The Effect of Omega-3 Polyunsaturated Fatty Acids on Growth and microRNA-21 Expression in Estrogen Receptor-Positive Breast Cancer Cells (MCF-7)

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

Nutritional Sciences
University of Toronto

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Abstract

Targeted, effective and safe therapies for breast cancer are lacking and many are turning towards complementary therapies, such as FS oil (FSO) and fish oil (FO). Interest surrounding the role of microRNA (miR) in breast cancer has piqued over the last decade, and the interaction between miR and dietary factors is emerging.

This thesis examined the role of α-linolenic acid (ALA) alone or combined with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) at ratios seen in the serum post-FSO consumption on cell growth, miR-21 expression and molecular targets of miR-21 in estrogen receptor-positive breast cancer (MCF-7) cells. A reduction in cell growth and miR-21 expression was observed following fatty acid treatment of varying incubation times. Bcl-2 gene is one molecular target of miR-21 that was reduced by the fatty acid treatment. In conclusion, ALA alone or combined with EPA and DHA can reduce cell growth and miR-21 expression.
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Lastly, I would like to dedicate this thesis to my family. The completion of my MSc would not have been possible without their unconditional love and support. Thank you to my parents for their unwavering encouragement and belief in my abilities, especially when I was unable to see them in myself, and to my sister, for keeping me laughing throughout and reminding me to have fun and maintain balance in my life. I’m so grateful to have you all in my life and could not have made it through these two years without you by my side cheering me on.

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List of Abbreviations Used

4-HNE 4-hydroxynonenal
5-LOX 5-lipoxygenase
AA Arachidonic acid, 20:4n-6
AE Antiestrogen
AI Aromatase inhibitors
Akt Protein kinase B
ALA α-linolenic acid, 18:3n-3
ANOVA Analysis of variance
AP-1 Activator protein-1
ATCC American Type Cell Culture
BD Basal diet
BC Breast cancer
Bcl-2 B-cell lymphoma 2
COX Cyclooxygenase
CS-FBS Charcoal-stripped fetal bovine serum
DHA Docosahexaenoic acid
DMBA Dimethylbenz(a)anthrazene
E2 17β-estradiol
EGFR Epidermal growth factor receptor
EPA Eicosapentaenoic acid, 20:5n-3
ER Estrogen receptor
FBS Fetal bovine serum
FO Fish oil
FS Flaxseed
FSO Flaxseed oil
GAPDH Glyceraldehyde 3-phosphate dehydrogenase
GPCR G-protein coupled receptor
HER2 Human epidermal growth factor receptor 2
HPRD Human protein reference database
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-1R</td>
<td>Insulin-like growth factor-1 receptor</td>
</tr>
<tr>
<td>IL-6R</td>
<td>Interleukin-6 receptor</td>
</tr>
<tr>
<td>LA</td>
<td>Linoleic acid, 18:2n-6</td>
</tr>
<tr>
<td>LPO</td>
<td>Lipid peroxidation</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>miR-21</td>
<td>microRNA-21</td>
</tr>
<tr>
<td>MMTV</td>
<td>Mouse mammary tumour virus</td>
</tr>
<tr>
<td>n-3 PUFA</td>
<td>Omega-3 polyunsaturated fatty acid</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa B</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>OA</td>
<td>Oleic acid, 18:1n-9</td>
</tr>
<tr>
<td>pAkt</td>
<td>Phospho-protein kinase B</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PDCD4</td>
<td>Programmed cell death receptor 4</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide-3-kinase</td>
</tr>
<tr>
<td>PIN</td>
<td>Protein interaction network</td>
</tr>
<tr>
<td>PIP₂</td>
<td>Phosphatidylinositol (4,5)-bisphosphate</td>
</tr>
<tr>
<td>PIP₃</td>
<td>Phosphatidylinositol (3,4,5)-triphosphate</td>
</tr>
<tr>
<td>PPAR-γ</td>
<td>Peroxisome proliferator-activated receptor-γ</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homologue</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>SERM</td>
<td>Selective estrogen receptor modulator</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>TAM</td>
<td>Tamoxifen</td>
</tr>
<tr>
<td>TRBP</td>
<td>Transactivating response RNA-binding protein</td>
</tr>
</tbody>
</table>
UTR Untranslated region
Introduction

Breast cancer is one of the most commonly diagnosed cancers worldwide (DeSantis et al., 2014; Hutchison, 2010). Improvements in early detection and treatment have resulted in a significant reduction in breast cancer mortalities over the past two decades, however, over one million people are diagnosed with breast cancer each year (De Santis et al., 2014; Hutchison, 2010; Eccles et al., 2013). Its total economic burden surpasses all other cancers and thus scientific investigation into its cause and treatment is vital (Hutchison, 2010; Eccles et al., 2013). Both non-modifiable factors, such as age of menarche and menopause, and modifiable factors, such as diet and physical activity, can all influence risk (Ali et al., 2015).

Breast cancer is a diverse disease categorized by the presence or absence of hormone and growth factor receptors on the cell membrane (Criscitiello et al., 2012; Crown et al., 2012). The combination of the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor (HER2) all determine breast cancer subtype (Criscitiello et al., 2012). Overexpression or amplified activation of these receptors is commonly observed in breast cancer, and many of the novel pharmaceuticals developed to treat breast cancer target these receptors (Renoir et al., 2013; Sestak, 2014; Nahta and Esteva, 2007). However, cancer cells can adapt to overcome these therapies and many of the conventional treatment protocols involve detrimental side effects (Flower et al., 2013). Therefore, prevention and/or treatment through alteration of modifiable lifestyle factors, such as diet, are being sought (Howell et al., 2014).

Many breast cancer patients and physicians are interested in alternative and complementary medicine, such as functional foods and supplements, to aid in breast
cancer treatment (Flower et al., 2013; Howell et al., 2014). Two of the most commonly used complementary therapies, flaxseed (FS; *Linum usitatissimum*) and fish oil (FO), are both touted for their high concentration of omega-3 fatty acids (n-3 PUFAs) (Anderson and Taylor, 2012; Boucher et al., 2012). FS contains ~40% flaxseed oil (FSO), which is especially rich in the n-3 PUFA, alpha-linolenic acid (ALA; 18:3n-3) (Thompson and Mason, 2010; Truan et al., 2010). Exploring the effect of ALA on human health is of great interest as it accounts for ~90% of the total n-3 PUFAs in the North American diet (Simon et al., 2009). FO is rich in the n-3 PUFAs eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3).

The biological effects of n-3 PUFAs on breast cancer have been examined in observational and clinical studies, preclinical animal studies and in vitro studies. In general, ALA and FO have been shown to reduce the risk of breast cancer in observational studies and FS has displayed antitumourigenic effects in a randomized controlled trial of women with diagnosed breast cancer (Wakai et al., 2005; Chajes et al., 2012; Thompson et al., 2005). Preclinical animal studies have shown beneficial effects of FSO and FO, with mouse models of breast cancer consistently displaying reduced cell proliferation, tumour size and/or increased apoptosis following FSO/FO consumption (Truan et al., 2010; Rao et al., 2000; Thompson et al., 1996).

ALA is converted into the long chain metabolites EPA and DHA in vivo, and consumption of ALA or ALA-rich foods, such as FSO, results in a specific ratio of ALA:EPA:DHA in the serum and/or plasma. Controversy surrounding the role of each n-3 PUFA exists, and questions persist on whether the in vivo effects of ALA are the result of its long chain metabolites. In vitro studies have shown that ALA alone is effective at
reducing the growth of breast cancer cells and increasing apoptosis (Truan et al., 2010; Kim et al., 2009b; Wiggins et al., 2013). The specific effect of ALA can be confirmed in vitro as cells are not able to undergo the conversion process seen in vivo. EPA and DHA are also independently able to reduce the growth of breast cancer cell lines and/or increase apoptosis (Corsetto et al., 2011; Schley et al., 2007; Chamras et al., 2002).

The mechanisms of the n-3 PUFA effect on cell growth have not yet been fully elucidated. Recent findings have indicated that short, non-coding molecules known as microRNA (miR) are involved in breast cancer and their expression can be modulated by environmental factors such as diet (Farazi et al., 2013; Radojicic et al., 2011; Qiu et al., 2012), including n-3 PUFAs (Mandal et al., 2012; Davidson et al., 2009). miR control post-transcriptional gene expression, and a single miR can regulate numerous targets (Farazi et al., 2013; Zaleska et al., 2015). miR-21 is shown to be overexpressed in breast cancer and due to its widespread and complex effects within the cell has become a primary focus in breast cancer research (Fix et al., 2010; Jazbuytye and Thum, 2010). This study aims to investigate in vitro the effect of ALA, alone or in combination with its EPA and DHA metabolites, at levels seen in the serum of animals or humans fed FSO, on the growth of ER+ breast cancer cells (MCF-7), and its relationship to miR-21 expression and its molecular targets. Results should help elucidate another mechanism behind the growth reducing effects of n-3 PUFAs and may indicate the potential for a miR-based n-3 PUFA complementary therapy for individuals with breast cancer.
2.0 Literature Review

2.1 Breast Cancer

2.1.1 Breast Cancer Incidence and Risk Factors

Breast cancer is the second leading cause of cancer death in women in Canada and the United States after lung cancer, and is the second most commonly diagnosed cancer worldwide after skin cancers (DeSantis et al., 2014; Hutchison, 2010). The rate of diagnosis is continuing to rise, with an estimated 1.38 million new cases every year with the total economic burden of breast cancer surpassing all other cancers (Hutchison, 2010; Eccles et al., 2013). Although the current lifetime risk of breast cancer for a woman living in the United States is 1-in-8, an increase from the 1-in-11 seen in the 1970s, improvements in treatment and early detection have resulted in a 34% decrease in breast cancer deaths from 1990-2010 (DeSantis et al., 2014).

Breast cancer risk can be categorized into non-modifiable and modifiable factors (Ali et al., 2015). Non-modifiable risk factors include family history of breast cancer, benign breast disease, early age of menarche, late menopause and cause of menopause (Barnes et al., 2011). Modifiable risk factors include childbirth over the age of 30, nulliparity, duration of breastfeeding, use of hormone therapy, alcohol consumption, smoking and obesity (Ali et al., 2015; Barnes et al., 2011). Many of the factors, such as age of menarche/ menopause and hormone therapy, relate to the duration of time estrogen is circulating in body at high levels. Since estradiol (E2) is involved in the development and progression of breast cancer, the longer a women maintains high levels of this hormone, the greater her risk of developing breast cancer (Renoir et al., 2013).
2.1.2 Molecular Subtypes

Breast cancer is histopathologically categorized by the presence or absence of the ER, PR, and the overexpression of the HER2 (Criscitiello et al., 2012; Crown et al., 2012). Breast cancer can then be further classified as luminal A (ER+, PR+, Low/HER2), luminal B (ER+, PR+, HER2+), HER2-enriched groups (ER−, PR−, HER2+) and basal (ER−, PR−, Low/HER2−) based on its receptor composition (Zaleska, 2015). These subtypes are complicated by a woman’s age and corresponding E2 status, as E2 has been shown to modulate tumour growth (Renoir et al., 2013). It has been demonstrated clinically that each breast cancer subtype responds differently to therapies and carries diverse overall prognoses (Zaleska, 2015). Defining the molecular subtype is vital when establishing the most effective therapy regimen, which may include surgery, hormonal or biologic therapies, as well as chemotherapy and/or radiotherapy (Zaleska, 2015; Matsen and Neumayer, 2013). Breast cancer therapies often target growth factor receptors as activation of these receptors in breast cancer epithelial cells triggers proliferation, invasion and metastases (Nahta and Esteva, 2007). Overexpression and/or augmented activation of these receptors is commonly observed in human breast cancer and has been linked to poor prognoses (Nahta and Esteva, 2007).

2.1.3 Hormone Receptors and Effects

Both E2 and progesterone are involved in the growth of breast cancer cells and accomplish their role through binding and activating the ligand-dependent transcription factors, ER and PR (Tanos et al., 2012). The ER is very commonly expressed in breast cancer, and the steroid hormone, E2, plays a vital role in breast cancer progression.
through binding to either ERα or ERβ (Roy and Vadlamudi, 2012) (Figure 2.1). ERα is the primary ER located in the mammary epithelium, and following diffusion of E2 through the cell membrane and subsequent binding of E2 to ER, homo- or heterodimers of E2-ER translocate to the nucleus and bind to the estrogen response element (ERE). The binding of the E2-ER complex to the ERE triggers transcription of E2-sensitive genes, leading to increased cell proliferation and potential metastases in breast epithelial cells (Roy and Vadlamudi, 2012) (Figure 2.1). Non-ERE genomic signaling can also occur when the E2-ER complex interacts with transcription factors such as Jun and Fos, triggering transcription of E2-sensitive genes (Bjornstrom and Sjoberg, 2005). Lastly, ER can also be activated by growth factor receptors, as their downstream signaling can lead to the phosphorylation of transcription factors associated with the ERE (Bjornstrom and Sjoberg, 2005). ERβ also acts as a transcription factor; however, it appears to antagonize the effects of ERα as it has been shown to decrease cell proliferation and demonstrate anti-proliferative functions (Roy and Vadlamudi, 2012).

2.1.4 Growth Factor Receptors and Signaling

In addition to ER, additional receptors such as HER2, epidermal growth factor receptor (EGFR), and the insulin-like growth factor-1 receptor (IGF-1R) are also important signaling pathways in breast cancer (Nahta and Esteva, 2003).
Figure 2.1. Hormone signaling in breast cancer.
All of these tyrosine kinase receptors trigger downstream cellular signaling pathways, such as phosphosinositide-3-kinase (PI3K)/protein kinase B (Akt), which control cell proliferation and apoptosis in both normal and malignant breast cells (Zhang et al., 2008; Liu and Ma, 2014; Yerushalmi et al., 2011) (Figure 2.2). EGFR is overexpressed in 20%-80% of breast cancer cases and HER2 is seen to be overexpressed in 20%-30% of cases (Zhang et al., 2008). IGF-1R has been shown to be present in all subtypes of breast cancer, independent of hormone receptor status (Law et al., 2008). HER2 forms homodimers, as well as heterodimers with either EGFR or IGF-1R and this dimerization of receptors triggers downstream cell signal transduction pathways that control cell proliferation and cell death (Liu and Ma, 2014; Moasser, 2007; Bollig-Fischer et al., 2010). Overexpression of these receptors is advantageous for cancer cells as their downstream signaling triggers tumorigenesis (Zhang et al., 2008) (Figure 2.2).

2.1.5 Breast Cancer Cell Lines: MCF-7 Cells

MCF-7, a commonly used breast cancer cell line, was first established in 1970 when the cells from a 69-year-old woman with metastatic breast cancer were isolated from her pleural effusion (Comsa et al., 2015; Lee et al., 2015; Simstein et al., 2003). Over the past several decades, research conducted with MCF-7 cells has provided the medical field with more translational knowledge for patient care than any other breast cancer cell line (Comsa et al., 2015). MCF-7 cells are both ER+ and PR+ and for this reason are characterized as the luminal A molecular subtype.
Figure 2.2. Growth factor signaling in breast cancer
They are sensitive to the steroid hormone, E2, and require E2 in order to proliferate. Although often treated as equivalent, there is a large degree of variability seen within this cell type, and multiple phenotypes have been observed. These phenotypes have been shown to vary in gene expression profile, receptor expression and signaling pathway, and these differences result in distinct proliferation rates (Comsa et al., 2015). The variations in proliferation observed in different MCF-7 phenotypes appears to depend on the quantity or signaling of IGF-1R, and not E2.

2.2 Breast Cancer Therapy

2.2.1 Hormone and Growth Factor Receptor Therapies

Given the immense heterogeneity observed in solid breast tumours, sophisticated and targeted treatment options are a necessity (Hutchison, 2010). Hormone and growth factor receptors offer the potential for therapeutic interventions. In ER+ breast cancer, E2 demonstrates mitogenic activity when it binds to the two estrogen receptors, ERα and ERβ (Renoir et al., 2013). These receptors act as transcription factors and antiestrogens (AEs), selective estrogen receptor modulators (SERMs) and aromatase inhibitors (AIs) are used to inhibit the growth of ER+ breast cancer cells (Renoir et al., 2013). Tamoxifen (TAM) is a well-known SERM that is used for both the prevention and treatment of ER+ breast cancers (Sestak, 2014). In addition, the use of monoclonal antibodies to block individual receptors, as well as inhibit tyrosine kinase function, has also been used clinically (Nahta and Esteva, 2007). For example, Trastuzumab, a humanized monoclonal antibody directed against HER2, is approved for the treatment of patients with HER2 overexpressing metastatic breast cancer (Nahta and Esteva, 2007).
Unfortunately, many of the conventional treatment protocols involve powerful therapies which, although often life-saving, can result in detrimental side effects (Flower et al., 2013). This, along with the molecular complexity and potential adaptions of cancer cells to these pharmaceuticals, renders prevention through alteration of modifiable lifestyle factors optimal (Howell et al., 2014).

2.2.2 Diet, Lifestyle and Complementary Therapies

The American Institute for Cancer Research estimates that at least 40% of post-menopausal breast cancer cases could be prevented by a healthy lifestyle (Howell et al., 2014). Control of modifiable lifestyle factors, such as avoiding weight gain and obesity, engaging in regular physical activity and reducing alcohol intake may help reduce the risk of breast cancer (DeSantis et al., 2014; Howell et al., 2014). Recently, the incidence rate for ER- tumours has decreased, while the incidence of ER+ tumours has increased (DeSantis et al., 2014). This may be due in part to awareness surrounding healthy lifestyle habits, such as physical activity and fruit and vegetable consumption, which have been associated with a reduced risk of ER- breast cancers (DeSantis et al., 2014).

In addition to controlling modifiable lifestyle factors associated with breast cancer risk, many patients and clinicians alike are turning towards alternative and complementary medicine, such as specific dietary components, in an attempt to thwart the ramifications associated with current treatment methods (Flower et al., 2013; Howell et al., 2014). One of the most commonly used alternative therapies is flaxseed (FS; *Linum usitatissimum*), with a recent study reporting that 33% of women began consuming FS post-breast cancer diagnosis in Ontario, Canada alone (Boucher et al., 2012). FS is a rich source of the omega-3 polyunsaturated fatty acid (n-3 PUFA) α-linolenic acid (18:3n-3,
ALA) (Thompson and Mason, 2010). Flaxseed oil (FSO), which comprises approximately 40% of FS, is composed of nearly 60% ALA (Thompson and Mason, 2010; Truan et al., 2010). Examining the impact of ALA is of great interest in North America due to its readily accessible nature in our food supply, accounting for ~85-94% of the total n-3 PUFAs in the average diet (Simon et al., 2009). Furthermore, it has also been shown that dietary lipids, such as ALA, can be stored in adipose tissue and that one’s mammary adipose composition can be a direct reflection of their dietary intake (Klein et al., 2000; Anderson and Ma, 2009). Hence, dietary fat quality as opposed to quantity appears to be a key determinant in whether or not dietary lipids carry tumour-promoting or preventive properties (Klein et al., 2000). Numerous epidemiological studies, clinical trials, animal studies and in vitro studies have been carried out to determine both the safety and efficacy of FS and individual n-3 PUFAs on breast cancer development, risk, and treatment (Mason et al., 2013).

2.3 Omega-3 Polyunsaturated Fatty Acids (n-3 PUFAs)

n-3 PUFAs contain multiple double bonds, with the first double bond appearing at the third carbon within the hydrocarbon chain. Through a series of elongation and desaturation chemical reactions in vivo, ALA can be metabolized into the longer chain n-3 PUFAs, EPA and DHA (Cunnane, 2003; Hall et al., 2006). ALA is found primarily in plant sources, while EPA and DHA are found exclusively in marine sources such as fish and fish oil (FO) (Liu and Ma, 2014). The human body is unable to synthesize some fatty acids, which renders their ingestion essential from dietary sources or supplements (Visioli et al., 2012). Both ALA and the n-6 PUFA, linoleic acid (LA; 18:2n-6), are considered
essential fatty acids for humans (Mason et al., 2013). Since ALA can be converted into the longer chain n-3 PUFAs, EPA and DHA, consumption of ALA, or ALA-rich foods such as FS or FSO by animals or humans, results in a certain ratio of ALA:EPA:DHA in their blood serum or plasma. For example, consumption of 40g/kg FSO (levels found in a 10% FS diet; equivalent to 10-20g FSO in humans) in mice resulted in a significant increase in all n-3 PUFAs resulting in a ratio of 1.0:0.4:3.1 (ALA:EPA:DHA) (Table 2.1) (Truan et al., 2010). Consumption of 2g/day of FSO in humans resulted in a significant increase in ALA resulting in a ratio of 1.0:1.0:2.5 (Table 2.1) (Kaul et al., 2013). Due to this conversion, EPA and DHA are not considered essential. The difference observed between animal and human serum fatty acid ratios is likely the result of varying enzymatic capabilities; rodents are better equipped to convert ALA to the longer chain fatty acids, EPA and DHA, through alternating series of elongation and desaturation reactions as they display a more efficient ALA elongation process. Conversely, this conversion has been shown to be quite inefficient in humans due to the first rate limiting reaction catalyzed by Δ6-desaturase (Burdge and Calder, 2006) (Figure 2.3). In contrast, the conversion of ALA to EPA and DHA is not seen in vitro as cells lack the necessary enzymes required for this to occur. To date, no studies have been completed in vitro on breast cancer cell lines with the fatty acid serum ratios seen in vivo post-ALA consumption.

The dietary intake of ALA is approximately 10-fold greater than that of EPA and DHA in North America and therefore, learning about its effect on breast cancer risk and development is crucial (Arterburn et al., 2006).
2.4 Effect of n-3 PUFAs on Breast Cancer

2.4.1 Observational and Clinical studies

Over the past decade, prospective cohort studies and case-control studies have investigated the impact of n-3 PUFA (FS, FSO, fish and/or FO) consumption on breast cancer risk (Table 2.2). Out of 14 prospective cohort studies examined, 8 demonstrated a reduction in breast cancer risk associated with either total or marine n-3 PUFA, or ALA intake (Wakai et al., 2005; Gago-Dominguez et al., 2003; Murff et al., 2011; Brasky et al., 2010; Theibaut et al., 2009; Bougnoux et al., 1994; Patterson et al., 2011; Voorrips et al., 2002). No association between n-3 PUFAs and breast cancer risk was observed in 5/14 of the studies (Folsom and Demissie, 2004; Engeset et al., 2006; Cho et al., 2003; Park et al., 2012; Sczaniecka et al., 2012) and one study showed an increased risk of ER+ breast cancer associated with fish consumption (Stripp et al., 2003). The variation observed between the studies may be the result of subject selection, attrition bias, recall bias, inappropriate food frequency questionnaires and source of n-3 PUFA in the diet.

Case-control studies have generally displayed greater discrepancies in their results with 8/18 studies in Table 2.3 showing no association between total n-3 PUFA intake and breast cancer risk (Pala et al., 2001; Chajes et al., 2008; Takata et al., 2009; Wirfalt et al., 2002; Shannon et al., 2007; Nkondjock et al., 2003; Witt et al., 2009; Vatten et al., 1993).
Table 2.1. Serum concentration and ratios of ALA, EPA and DHA in animals and humans fed FSO

<table>
<thead>
<tr>
<th></th>
<th>ALA (µM)</th>
<th>EPA (µM)</th>
<th>DHA (µM)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Animals</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (µM)</td>
<td>4.5</td>
<td>~</td>
<td>50.4</td>
<td>1.0:~:11.2</td>
</tr>
<tr>
<td>FSO (µM)</td>
<td>61.0</td>
<td>22.2</td>
<td>193.2</td>
<td>1.0:0.4:3.1</td>
</tr>
<tr>
<td>Difference (FSO-control)</td>
<td>+56.5 ↑1255%</td>
<td>+22.2 ↑100%</td>
<td>+142.8 ↑283%</td>
<td></td>
</tr>
<tr>
<td><strong>Humans</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (µM)</td>
<td>53.5</td>
<td>74.7</td>
<td>172.9</td>
<td>1.0:1.5:3.8</td>
</tr>
<tr>
<td>FSO (µM)</td>
<td>90.1</td>
<td>84.6</td>
<td>195.4</td>
<td>1.0:1.0:2.5</td>
</tr>
<tr>
<td>Difference (FSO-control)</td>
<td>+ 36.6 ↑68%</td>
<td>+ 9.9 ↑13.2%</td>
<td>+ 22.5 ↑13%</td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.3. Conversion of $\alpha$-linolenic acid into eicosapentaenoic acid and docosahexaenoic acid in vivo.
Of the remaining studies, 5/18 showed a reduced risk of breast cancer associated with total n-3 PUFA consumption (Chajes et al., 2012; Kim et al., 2009a; Maillard et al., 2002; Goodstine et al., 2003; Saadatian-Elahi et al., 2002) and 3/18 showed a decreased risk associated with EPA and DHA intake (Pala et al., 2001; Kuriki et al., 2007; Bagga et al., 2002). The overall variability for all n-3 PUFA types and sources observed within observational studies may be reflective of the heterogeneity of the cohorts, the methods used to assess fatty acid consumption, PUFA dosage, timing of analyses following PUFA consumption and/or response endpoint.

ALA alone has been demonstrated to display protective effects against breast cancer in human studies. An inverse association between the ALA content of breast adipose tissue and breast cancer metastases, as well as an overall decreased risk of breast cancer in women consuming the greatest quantity of ALA was demonstrated in 2/18 of the case-control studies (Klein et al., 2000; Maillard et al., 2002). There is some controversy, however, as 1/18 of the case-control studies showed an increased risk of breast cancer associated with ALA intake (De Stefani et al., 1998). As these are observational studies, the source of ALA varies from location to location, and some of the negative or null findings with ALA and breast cancer risk may be attributed to red meat, which is a source of ALA, contrasted with plant-based sources such as FS (Bougnoux and Chajes, 2003). This hypothesis regarding different sources of ALA having different effects was supported by a cohort study, which showed beneficial effects of ALA from fruit, vegetables and vegetable oils, but an increased risk with ALA derived from nuts and other sources (Theibaut et al., 2009). Factors such as food source and
quantity of ALA, menopausal status and breast cancer subtype can all influence the effect of ALA.

Currently, only a small number of randomized controlled trials (RCTs) for n-3 PUFAs have also been conducted or are in the process of being completed. One study which examined the impact of FS on 32 post-menopausal breast cancer patients showed that 25g FS reduced cell proliferation and HER2 expression, and increased apoptosis, in tumours (Thompson et al., 2005). Based on the findings of observational studies, there is promise that additional RCTs will show favourable results for a role of n-3 PUFAs as a complementary therapy in breast cancer.

2.4.2 In Vivo Animal Studies

The impact of ALA, EPA and DHA, either alone or combined, on mammary tumour growth in rodents has been studied in xenograft, transgenic and chemically induced models of breast cancer (Table 2.3). Overall in preclinical animal studies an antitumourigenic effect was observed following consumption of FS, FSO, FO or pure n-3 PUFAs, EPA and DHA (Table 2.3).

Breast cancer cells derived from humans are administered into rodent mammary tissue, resulting in xenograft rodent models of breast cancer (Fantozzi and Christofori, 2006). Overall, studies involving xenograft models of breast cancer and consumption of FS and FSO, FO or pure EPA and DHA have shown reduced tumour growth rates and/or size of neoplastic masses (Truan et al., 2010; Saggar et al., 2010a,b; Chen et al., 2007, Chen et al., 2009, Chen et al., 2002; Wang et al., 2005; Fritsche and Johnston, 1990; Gonzalez et al., 1991; Hardman et al., 2001; Rose et al., 1995).
Table 2.2: Effect of n-3 PUFAs on breast cancer in humans: Observational and clinical studies

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Source of n-3/n-6 PUFA; Measure</th>
<th>Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Prospective Cohort</strong></td>
<td></td>
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</tr>
<tr>
<td>26,291 women, 129 BC patients</td>
<td>n-3 PUFA consumption FFQ</td>
<td>No association between total n-3 PUFA consumption and BC risk; ↓ risk BC with ↑ fish marine n-3 PUFA consumption</td>
<td>Wakai et al., 2005</td>
</tr>
<tr>
<td>35,298 women; 342 BC patients</td>
<td>n-3 PUFA FFQ: total vs marine vs ALA</td>
<td>↑ intake n-3 PUFAs= ↓ BC risk ↓ n-3 PUFA + ↑ n-6 PUFA= ↑ BC risk</td>
<td>Gago-Dominguez et al., 2003</td>
</tr>
<tr>
<td>72,571; 712 BC patients</td>
<td>Marine-derived n-3 PUFA; red meat (n-6 source); FFQ</td>
<td>↓ n-3 PUFA + ↑ n-6 PUFA= ↑ BC risk</td>
<td>Murff et al., 2012</td>
</tr>
<tr>
<td>35,016 women; 880 BC patients</td>
<td>Fish oil supplement; FFQ</td>
<td>↑ fish oil= ↓ BC risk (invasive ductal, but not lobular, carcinomas)</td>
<td>Brasky et al., 2010</td>
</tr>
<tr>
<td>41,836 postmenopausal women</td>
<td>FFQ regarding fish (marine n-3 PUFA) intake</td>
<td>No association between fish/marine n-3 PUFA consumption and BC risk</td>
<td>Folsom and Demissie, 2004</td>
</tr>
<tr>
<td>56,007 women; 1650 BC patients</td>
<td>FFQ for ALA, marine and total n-3 PUFA consumption</td>
<td>No association between BC risk and total or marine n-3 PUFA consumption; ↓ BC hazard ratio with ALA derived from fruit/vegetables/vegetable oils; ↑ BC hazard ratio with ALA derived from nuts</td>
<td>Thiebaut et al., 2009</td>
</tr>
<tr>
<td>23,693 women; 424 BC patients</td>
<td>Marine-derived n-3 PUFAs (fish); FFQ</td>
<td>↑ intake fish= ↑ incidence of (ER+) BC</td>
<td>Stripp et al., 2003</td>
</tr>
<tr>
<td>310,671 women; 4776 BC patients</td>
<td>Marine-derived n-3 PUFAs (fish); FFQ</td>
<td>No significant associations seen between fish intake and BC risk</td>
<td>Engeset et al., 2006</td>
</tr>
<tr>
<td>Study Cases</td>
<td>Description</td>
<td>Findings</td>
<td>Reference</td>
</tr>
<tr>
<td>-------------</td>
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</tr>
<tr>
<td>121 BC patients</td>
<td>Fatty acid methyl esters of breast adipose tissue</td>
<td>↓ BC metastases with higher ALA content (&gt; 0.38% total fatty acids)</td>
<td>Bougnoux et al., 1994</td>
</tr>
<tr>
<td>91, 369 women</td>
<td>n-3 PUFA consumption FFQ (both total and marine)</td>
<td>No association between n-3 PUFA consumption for either total or marine</td>
<td>Cho et al., 2003</td>
</tr>
<tr>
<td>3,598 women</td>
<td>24-hour recall assessing fish/marine n-3 PUFA consumption</td>
<td>↓ BC risk associated with greater intake of fish/marine-derived n-3 PUFAs</td>
<td>Patterson et al., 2011</td>
</tr>
<tr>
<td>88,974 postmenopausal women</td>
<td>FFQ for total n-3 PUFA + ALA, EPA and DHA consumption</td>
<td>No association between BC risk and total n-3 PUFA or ALA, EPA, DHA</td>
<td>Park et al., 2012</td>
</tr>
<tr>
<td>31, 024 women</td>
<td>FFQ of ALA, EPA and DHA consumption</td>
<td>No association observed between BC and ALA intake; ↓ BC risk associated with EPA and DHA intake</td>
<td>Sczaniecka et al., 2012</td>
</tr>
<tr>
<td>62,573 women</td>
<td>FFQ for ALA, EPA and DHA consumption</td>
<td>↓ BC risk associated with ALA consumption; no association between EPA and DHA intake</td>
<td>Voorips et al., 2002</td>
</tr>
</tbody>
</table>

Case-Control

<table>
<thead>
<tr>
<th>Study Cases</th>
<th>Description</th>
<th>Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>71 BC patients; 212 controls</td>
<td>Erythrocyte fatty acids</td>
<td>No consistent findings between overall n-3 PUFAs and BC risk; highest levels of DHA were associated with reduced risk BC</td>
<td>Pala et al., 2001</td>
</tr>
<tr>
<td>197 BC patients; 394 controls</td>
<td>Serum phospholipid fatty acid composition</td>
<td>Total PUFAs (n-6 and n-3) showed slight protective effect against BC risk; no associations with EPA or DHA levels alone</td>
<td>Saadatian-Elahi et al., 2002</td>
</tr>
<tr>
<td>363 BC patients; 1131 controls</td>
<td>Serum phospholipid fatty acid composition</td>
<td>No significant associations between n-3 PUFA levels and BC risk</td>
<td>Chajes et al., 2008</td>
</tr>
<tr>
<td>130 BC patients; 387 controls</td>
<td>Serum phospholipid fatty acid composition</td>
<td>No significant associations between n-3 PUFA levels and BC risk</td>
<td>Takata et al., 2009</td>
</tr>
<tr>
<td>Study Size</td>
<td>Sample Type</td>
<td>Methodology</td>
<td>Findings</td>
</tr>
<tr>
<td>------------</td>
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</tr>
<tr>
<td>123 BC patients; 59 controls</td>
<td>Breast adipose fatty acid composition</td>
<td>Inverse association between ALA and BC risk</td>
<td>Klein et al., 2000</td>
</tr>
<tr>
<td>241 BC patients; 88 controls</td>
<td>Breast adipose fatty acid composition</td>
<td>↓ BC risk associated with greater ALA breast adipose tissue levels</td>
<td>Maillard et al., 2002</td>
</tr>
<tr>
<td>414 BC patients; 429 controls</td>
<td>FFQ assessing ALA intake</td>
<td>No significant associations between ALA intake and BC risk</td>
<td>Nkondjock et al., 2003</td>
</tr>
<tr>
<td>322 BC patients; 1030 controls</td>
<td>Erythrocyte fatty acid composition</td>
<td>No significant associations between n-3 PUFAs and BC risk</td>
<td>Shannon et al., 2007</td>
</tr>
<tr>
<td>103 BC patients; 309 controls</td>
<td>Erythrocyte fatty acid composition; dietary record</td>
<td>↓ risk of BC with highest erythrocyte concentrations of EPA and DHA; ↓ risk of BC with highest intakes of EPA and DHA</td>
<td>Kuriki et al., 2007</td>
</tr>
<tr>
<td>237 BC patients; 673 controls</td>
<td>Dietary history method assessing total n-3 PUFA intake</td>
<td>No significant associations between n-3 PUFA intake and BC risk</td>
<td>Wirfalt et al., 2002</td>
</tr>
<tr>
<td>73 BC patients; 74 controls</td>
<td>Breast adipose fatty acid composition</td>
<td>High EPA and DHA correlated with ↓ BC risk</td>
<td>Bagga et al., 2002</td>
</tr>
<tr>
<td>1000 BC patients; 1074 controls</td>
<td>FFQ</td>
<td>n-3 PUFA intake associated with ↓ BC risk in obese women</td>
<td>Chajes et al., 2012</td>
</tr>
<tr>
<td>358 BC patients; 360 controls</td>
<td>FFQ for fish consumption</td>
<td>High intake of fish was associated with ↓ BC risk</td>
<td>Kim et al., 2009a</td>
</tr>
<tr>
<td>565 BC patients; 554 controls</td>
<td>FFQ for n-3 and n-6 PUFA consumption</td>
<td>Higher n-3/n-6 PUFA ratio associated with ↓ risk of BC</td>
<td>Goodstine et al., 2003</td>
</tr>
<tr>
<td>463 BC patients; 1098 controls</td>
<td>Gluteal adipose fatty acid composition</td>
<td>No significant association observed between n-3 PUFA content and BC risk</td>
<td>Witt et al., 2009</td>
</tr>
<tr>
<td>65 BC patients, 260 controls</td>
<td>Serum phospholipid fatty acid composition composition</td>
<td>No association between ALA, EPA and DHA levels in serum phospholipids and BC risk</td>
<td>Vatten et al., 1993</td>
</tr>
<tr>
<td>365 BC patients, 397 controls</td>
<td>FFQ</td>
<td>↑ BC risk associated with ALA intake</td>
<td>De Stefani et al., 1998</td>
</tr>
<tr>
<td>Meta-analyses</td>
<td></td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>8 prospective cohort studies</td>
<td>Fish, ALA, EPA, DHA intake</td>
<td>No association between BC incidence and fish, total n-3 PUFA, EPA or DHA consumption; greatest ALA intake= ↓ BC risk</td>
<td>MaLean et al., 2006</td>
</tr>
<tr>
<td>21 prospective cohort studies</td>
<td>Tissue biomarkers or fish, ALA, EPA, DHA consumption</td>
<td>↓ BC risk associated with EPA and DHA consumption; no association between BC incidence and fish or ALA consumption</td>
<td>Zheng et al., 2013</td>
</tr>
<tr>
<td>7 case-control studies, 3 cohort studies</td>
<td>Fatty acid composition of both serum and adipose tissue</td>
<td>Case control: ↓ risk BC associated with high ALA consumption Cohort: no association between BC risk and ALA content; low E2 (postmenopausal) + high ALA content= ↑ BC risk</td>
<td>Saadatian-Elahi et al., 2004</td>
</tr>
<tr>
<td>11 prospective cohort studies</td>
<td>High n-3:n-6 intake ratio; BC risk</td>
<td>Higher n-3:n-6 ratio= ↓ risk BC</td>
<td>Yang et al., 2014</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Randomized Controlled Trials</th>
</tr>
</thead>
<tbody>
<tr>
<td>32 postmenopausal BC patients</td>
</tr>
<tr>
<td>28,000 men and women (women 55 years +)</td>
</tr>
<tr>
<td>60 women with diagnosed Stage I-III BC</td>
</tr>
<tr>
<td>25 women with ↑ Fish consumption</td>
</tr>
<tr>
<td><strong>risk BC</strong></td>
</tr>
<tr>
<td><strong>16 women with newly diagnosed BC</strong></td>
</tr>
</tbody>
</table>

Abbreviations: ALA= α-linolenic acid, BC= breast cancer, DHA= docosahexaenoic acid, E2= 17-β estradiol, EPA= eicosapentaenoic acid, FFQ= food frequency questionnaire, FO= fish oil, FS= flaxseed, FSO= flaxseed oil, n-3 PUFA= omega-3 polyunsaturated fatty acid, n-6 PUFA= omega-6 polyunsaturated fatty acid
Alternatively, gain of function mutations involving oncogenes or loss of function mutations involving cellular signaling apoptotic pathways, result in the generation of transgenic mouse models of breast cancer (Fantozzi and Christofori, 2006). Transgenic models fed either FSO or menhaden oil, a type of FO, displayed reduced tumour volume, reduced cell proliferation and/or extended tumour latency (Rao et al., 2000; MacLennan et al., 2013; Table 2.3).

The last type of rodent breast cancer model, chemically-induced models of breast cancer, trigger carcinogenesis via injection of carcinogens into rodents (Fantozzi and Christofori, 2006). On the whole, consumption of FSO, FO or isolated EPA and DHA resulted in reduced tumour incidence and multiplicity, reduced cell proliferation and increased apoptosis (Cognault et al., 2000; Thompson et al., 1996; Jiang et al., 2012; Manna et al., 2007; Table 2.3).

Animal consumption of ALA-rich FS and FSO has been correlated with a reduction in mammary tumour size via decreased cell proliferation, independent of its long chain metabolites, EPA and DHA (Truan et al., 2010; Saggar et al., 2010b; Chen et al., 2007; Chen et al., 2009; Chen et al., 2002; Fritsche et al., 1990; Wang et al., 2005; Gonzalez et al., 1991; Hardman et al., 2001; Rose et al., 1995). Looking at the individual effects of EPA and DHA, an 8% EPA supplemented diet was able to inhibit the growth of MDA-MB-434 mammary tumours in mice (Rose et al., 1995). DHA alone was also able to independently inhibit the growth of breast cancer cells in vitro and MDA-MB-435 mammary tumour development in vivo, in addition to inducing apoptosis (Rose et al., 1995).
2.4.3 In Vitro Studies

Cell culture experiments of both murine and human breast cancer cells have demonstrated that treatment with ALA, EPA and DHA, both alone and combined, can inhibit cell proliferation by limiting cell cycle progression and induce apoptosis (Table 2.4). In normal cells, through interactions between two cells or a cell and the extracellular matrix, growth homeostasis and cellular quiescence is maintained by the release of anti-proliferative signals by tumour suppressor genes (Stephenson et al., 2013). Cells communicate using these anti-proliferative signals via cell surface receptors and the ultimate effects include cells being either forced into G0 in the cell cycle, or triggered to enter a post-mitotic state where they remain dormant (Stephenson et al., 2013). In cancer cells, these signals become disturbed, and EPA and DHA in cell culture have been shown to alter the sensitivity of cancer cells to growth-inhibitory signaling (Stephenson et al., 2013). It has been repeatedly shown that ALA, EPA and DHA have a dose-dependent inhibitory effect on the growth of ER+ breast cancer cells, MCF-7 (Truan et al., 2010; Kim et al., 2009b; Vanden Heuvel, 2012; Grammatikos et al., 1994; Tran et al., 2010; Chajes et al., 1995; Wiggins et al., 2013; Gore et al., 1994; Corsetto et al., 2011; Schley et al., 2007; Chamras et al., 2002; Kang et al., 2010; Blanckaert et al., 2010; Rogers et al., 2010; See Table 2.5). The effects of EPA and DHA seem to be consistent across cell lines, with similar results seen for MCF-7 and MDA-MB-231 cells, while results with ALA appear to display some inconsistencies; some studies have shown only reductions in cell growth with ALA in MCF-7, while others have only shown reductions in MDA-MB-231 with ALA (Tran et al., 2010; Chajes et al., 1995) (Table 2.4).
### Table 2.3: Effect of n-3 PUFAs on breast cancer: Preclinical animal studies

<table>
<thead>
<tr>
<th>Model</th>
<th>Source of n-3 PUFA</th>
<th>Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVX athymic mice with MCF-7 xenografts</td>
<td>BD vs. 4% FSO diet; high E2; 8 weeks</td>
<td>↓ tumour growth and cell proliferation, ↑ apoptosis in FSO group vs. BD</td>
<td>Truan et al., 2010</td>
</tr>
<tr>
<td>OVX athymic mice; MCF-7 xenografts</td>
<td>TAM for both groups + BD or FSO (38.5g/kg) supplemented; low E2 mimicking post-menopausal state</td>
<td>FSO augmented TAM effect and ↓ cell proliferation by 35% and ↑ apoptosis by 76%</td>
<td>Saggar et al., 2010a</td>
</tr>
<tr>
<td>OVX athymic mice; MCF-7 xenografts</td>
<td>BD or FSO supplemented (38.5g/kg)</td>
<td>↓ tumour growth</td>
<td>Saggar et al., 2010b</td>
</tr>
<tr>
<td>OVX BALB/c nu/nu athymic mice; MCF-7 xenografts</td>
<td>BD or 5% and 10% supplemented FS diet</td>
<td>Significant ↓ initial tumour size</td>
<td>Chen et al., 2007</td>
</tr>
<tr>
<td>Ovarectomized BALB/c nu/nu athymic mice; MCF-7 xenograft</td>
<td>BD or 10% FS supplemented diet</td>
<td>FS diet significantly ↓ tumour size and cell proliferation; significantly ↑ apoptosis</td>
<td>Chen et al., 2009</td>
</tr>
<tr>
<td>Athymic nude mice (Ncr nu/nu); MDA-MB-435 xenograft</td>
<td>BD or 10% supplemented FS diet</td>
<td>Significant ↓ in tumour growth and cell proliferation</td>
<td>Chen et al., 2002</td>
</tr>
<tr>
<td>Athymic nude mice (Ncr nu/nu); MDA-MB-435 xenograft</td>
<td>BD or 10% supplemented FSO diet</td>
<td>Significant ↓ in tumour growth, cell proliferation and lymph node metastases; significant ↑ in apoptosis</td>
<td>Wang et al., 2005</td>
</tr>
<tr>
<td>Athymic BALB/c mice; 410 and 410.4 xenografts</td>
<td>BD; FSO or 4:1 FO:corn oil supplemented diets</td>
<td>Tumour weight, size and burden= CO &gt; FSO &gt; FO; tumours grew faster and had ↑ weight in CO vs. FSO mice; FSO&lt; FO &lt; CO for tumours tumour size and pulmonary metastases</td>
<td>Fritsche and Johnston, 1990</td>
</tr>
<tr>
<td>Athymic nude mice; MCF-7 xenografts</td>
<td>19% menhaden oil (contains ALA, EPA, DHA); 6-8 weeks</td>
<td>↓ tumour volume</td>
<td>Gonzalez et al., 1991</td>
</tr>
<tr>
<td>Athymic nu/nu mouse; MDA-MB-</td>
<td>3% fish oil concentration,</td>
<td>↓ tumour growth</td>
<td>Hardman et al., 2001</td>
</tr>
<tr>
<td>Study/Model/Induction</td>
<td>Design</td>
<td>Outcomes</td>
<td>Reference</td>
</tr>
<tr>
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</tr>
<tr>
<td>231 xenografts (contains ALA, EPA, DHA); 7 weeks</td>
<td>Athymic nu/nu mice (NCr-nu/nu); MDA-MB-435 xenograft</td>
<td>↓ overall tumour size, growth and weight</td>
<td>Rose et al., 1995</td>
</tr>
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<td></td>
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<tr>
<td>TG.NK mouse of FVB background with MMTV/c-neu transgene</td>
<td>Gavage of 0.05, 0.10 and 0.20ml/mouse FSO (topped up to 0.20ml/mouse with corn oil) or gavage of melatonin</td>
<td>Delayed onset of tumour with increased FSO dose; dose related negative trend in tumour incidence with higher FSO; ↓ tumour burden/mouse in highest FSO group</td>
<td>Rao et al., 2000</td>
</tr>
<tr>
<td>MMTV-neu(ndl)-YD5 fat-1 mice</td>
<td>3% menhaden oil diet</td>
<td>↓ total tumour volume and multiplicity</td>
<td>MacLennan et al., 2013</td>
</tr>
<tr>
<td>Sprague-Dawley rats with MNU-induced mammary tumours</td>
<td>15% FSO or 15% palm oil/sunflower oil supplemented diet; FSO ± vitamin E; FSO + vitamin E + oxidant (vitamin C + vitamin K)</td>
<td>↑ tumour growth FSO + vitamin E compared to – vitamin E; ↓ tumour area, multiplicity, incidence and number in FSO + vitamin E + oxidant compared to FSO + vitamin E + oxidant</td>
<td>Cognault et al., 2000</td>
</tr>
<tr>
<td>Sprague-Dawley rats with DMBA-induced tumours</td>
<td>BD; 2.5% or 5% FSO diet</td>
<td>↓ established tumour growth in 2.5% or 5% FSO diet vs. BD</td>
<td>Thompson et al., 1996</td>
</tr>
<tr>
<td>MNU chemically induced mammary carcinoma</td>
<td>Low n-3:n-6 diet= 0.7:1 High n-3:n-6 diet= 14.6:1</td>
<td>↓ cancer incidence, multiplicity, mass, cell proliferation and ↑ tumour latency and apoptotic index</td>
<td>Jiang et al., 2012</td>
</tr>
<tr>
<td>DMB A chemically induced mammary carcinoma</td>
<td>Maxepa fish oil (0.5ml/day= 90mg EPA, 60mg DHA)</td>
<td>Chemopreventive effect of Maxepa fish oil; ↓ cell proliferation and DNA protein breaks</td>
<td>Manna et al., 2007</td>
</tr>
</tbody>
</table>

ALA= α-linolenic acid, BD= basal diet, DHA= docosahexaenoic acid, DMB A= dimethylbenz(α)anthracene, E2= 17-β estradiol, EPA= eicosapentaenoic acid, = fish oil, FS= flaxseed, FSO= flaxseed oil, MMTV= mouse mammary tumour virus, n-3 PUFA= omega-3 polyunsaturated fatty acid, NMU= N-nitrosomethyl-urea, OVX= ovariectomized, TAM= tamoxifen
Breast cancer cells ex vivo lack the enzymes required to convert ALA to EPA and DHA, therefore, any effects seen with ALA can be assumed to be the result of ALA alone, and not the longer chain metabolites. The individual effects of EPA and DHA have been studied in vitro in breast cancer as well (Table 2.4). Studies have shown that EPA alone is able to induce apoptosis in human breast cancer cells in vitro and DHA alone is able to independently inhibit the growth of breast cancer cells (Rose et al., 1995; Kang et al., 2010).

Overall, it appears that n-3 PUFAs exert beneficial effects in reducing breast cancer risk in humans and actively triggering cell death in in vivo and in vitro studies. The promising results seen in preclinical animal models regarding the antitumorigenic effects of n-3 PUFAs support the need for additional RCTs to further evaluate the role of FSO, FS or FO as potential complementary therapies in breast cancer treatment. The mechanisms of the effect of ALA alone or in combination with its metabolites, EPA and DHA, has not been fully elucidated.

2.5 Potential Mechanisms of n-3 PUFA Effect

n-3 PUFAs may possess anti-cancer effects and may accomplish these through several mechanisms including (a) alteration of cell membrane composition, (b) inhibition of arachidonic acid metabolism and its derivatives, (c) lipid peroxidation, as well as (d) modulation of cell membrane receptors, transcription factors and various signaling molecules derived from lipids (Corsetto et al., 2011; Mansara et al., 2011; Deshpande et al., 2013).
Table 2.4: Effect of n-3 PUFAs on breast cancer in vitro studies

<table>
<thead>
<tr>
<th>Model</th>
<th>n-3 PUFA treatment</th>
<th>Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7 cells</td>
<td>50µM ALA + 1nM E2 for 5 days</td>
<td>↓ cell proliferation</td>
<td>Truan et al., 2010</td>
</tr>
<tr>
<td>MCF-7</td>
<td>100µM ALA for 24, 48 and 72 hours</td>
<td>↓ cell growth (dose- and time-dependent); ↑ apoptosis (dose-dependent)</td>
<td>Kim et al., 2009b</td>
</tr>
<tr>
<td>MCF-7 cells</td>
<td>25-100µM ALA; time</td>
<td>↓ cell proliferation following 100µM ALA treatment</td>
<td>Vanden Heuvel, 2012</td>
</tr>
<tr>
<td>MCF-7</td>
<td>0-30µM ALA; 7 days</td>
<td>↓ cell growth at 18-30uM</td>
<td>Grammatikos et al., 1994</td>
</tr>
<tr>
<td>MCF-7; MDA-MB-231</td>
<td>50 and 100µM ALA; 5 days</td>
<td>↓ cell growth with 100uM ALA in MCF-7; no significant changes seen in MDA-MB-231</td>
<td>Tran et al., 2010</td>
</tr>
<tr>
<td>MCF-7; MDA-MB-231</td>
<td>71.83µM ALA; 5 days</td>
<td>↓ cell growth of MDA-MB-231 only</td>
<td>Chajes et al., 1995</td>
</tr>
<tr>
<td>MCF-7; MDA-MB-231; MDA-MB-468; BT-474</td>
<td>75µM ALA; 96 hours</td>
<td>Significant ↓ in cell growth seen in all cell lines</td>
<td>Wiggins et al., 2013</td>
</tr>
<tr>
<td>MCF-7 cells</td>
<td>102.7µM ALA; up to 7 days</td>
<td>↓ cell growth</td>
<td>Gore et al., 1994</td>
</tr>
<tr>
<td>MDA-MB-231 cells</td>
<td>150µM ALA + 40µM OA + 40µM LA; 48 hours</td>
<td>No change in cell growth</td>
<td>Yu et al., 2011</td>
</tr>
<tr>
<td>MDA-MB-231 cells</td>
<td>10-200µM ALA, 24 hours</td>
<td>↓ cell number</td>
<td>Horia and Watkins, 2005</td>
</tr>
<tr>
<td>SKBr3 cells; BT-474 cells</td>
<td>10-20µM ALA ± TRAS; 48 hours</td>
<td>↓ HER2 expression; ALA dose-dependent ↓ in cell proliferation in + TRAS condition</td>
<td>Menendez et al., 2006</td>
</tr>
<tr>
<td>MCF-7; MDA-MB-231</td>
<td>50-300µM EPA; DHA; 72 hours</td>
<td>Significant ↓ in cell growth (starting at 220µM for DHA and 240µM for EPA)</td>
<td>Corsetto et al., 2011</td>
</tr>
<tr>
<td>Cell Line</td>
<td>Treatment</td>
<td>Effect</td>
<td>Reference</td>
</tr>
<tr>
<td>-------------------</td>
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<td>---------------------------------------------</td>
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<tr>
<td>MDA-MB-231</td>
<td>60µM EPA + 40µM DHA +/− 75µM LA; 45µM EPA + 30µM DHA +/− 75µM LA</td>
<td>Both doses significantly ↓ cell growth</td>
<td>Schley et al., 2007</td>
</tr>
<tr>
<td>MCF-7</td>
<td>100µM DHA or EPA</td>
<td>↓ cell growth</td>
<td>Chamras et al., 2002</td>
</tr>
<tr>
<td>MCF-7, MDA-MB-231, MDA-MB-435 cells</td>
<td>20.2µM DHA, 57.4µM EPA (MCF-7), 70-100µM DHA, &gt; 200µM EPA (MDA-MB-231/435)</td>
<td>Significant ↓ cell viability for all cell lines; MCF-7 cells were more sensitive to DHA and EPA vs MDA-MB-231/435</td>
<td>Kang et al., 2010</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>20-100µM DHA; 24-72 hours</td>
<td>↓ cell proliferation and ↑ apoptosis</td>
<td>Blanckaert et al., 2010</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>100µM DHA; 48 hours</td>
<td>↓ cell growth and proliferation</td>
<td>Rogers et al., 2010</td>
</tr>
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</table>

ALA= α-linolenic acid, DHA= docosahexaenoic acid, E2= 17-β estradiol, EPA= eicosapentaenoic acid
2.5.1 Alteration of Cell Membrane Composition

A major class of lipids found in the cell membrane, glycerophospholipids, are altered by dietary fatty acid intake (Menendez and Lupu, 2007; Gu et al., 2013). Consumption of ALA, EPA and/or DHA modifies the membrane composition so that more of these n-3 PUFA’s are found within the glycerophospholipid than n-6 PUFAs (Gu et al., 2013). This then alters membrane fluidity and permeability, as well as disrupts cholesterol-rich regions in the cell membrane known as lipid rafts (Gu et al., 2013; Chenais and Blanckaert, 2012). Various cellular events such as apoptosis, protein signaling and trafficking, and cellular migration are then seen to be impaired in breast cancer cells (Babina et al., 2011). Literature points to the role of the cell membrane, with specific focus on lipid rafts, as a key player in these aberrant mechanisms (Babina et al., 2011). Proteins involved in cell signal transduction such as receptor tyrosine kinases (RTKs), G-protein coupled receptors (GPCRs), G-proteins, kinases and phosphatases are found concentrated within cholesterol and glycosphingolipid-rich regions in the plasma membrane known as lipid rafts (Chini and Parenti, 2004; Turk and Chapkin, 2013; Babina et al., 2011). The signaling that originates from rafts is seen to be hyperactive in cancer and can trigger numerous tumour-promoting events, such as increased cell proliferation (Turk and Chapkin, 2004).

Cancer cell membranes typically contain a greater concentration of saturated fatty acids and cholesterol than non-malignant cells (Babina et al., 2011). Consumption of ALA, EPA and/or DHA is thought to be capable of altering the composition of these diverse raft domains, and in turn modulate cancer cell biology (Turk and Chapkin, 2004). Disruption of raft proteins, such as lateral movement of proteins from raft to non-raft
domains, or changes in the fatty acid composition of rafts by n-3 PUFAs, can result in altered oncogenic signaling in breast cancer cells (Babina et al., 2011). Signaling proteins critical in breast cancer pathogenesis found in lipid rafts include EGFR, HER2, interleukin-6 receptor (IL-6R), and GPCR’s (Dethlefsen et al., 2013; Knupfer and Preiss, 2007; Masuda et al., 2012; Feigin et al, 2014). EPA and DHA have been shown to block EGFR signaling, and DHA alone has been shown to induce apoptosis in MCF-7 and MDA-MB-231 cells via downregulation of EGFR (Corsetto et al., 2011). ALA has been shown to significantly downregulate HER-2 protein expression in vivo in MCF-7 xenograft mice following consumption of FSO (Truan et al., 2010). EPA and DHA rich FO has also been shown to result in the formation of larger raft domains, reducing oncogenic signaling from raft domains in T-cells from fat-1 mice, which express the n-3 desaturase allowing them to endogenously produce n-3 PUFAs (Kang et al., 2004). It is proposed that n-3 PUFAs accomplish these alterations by either 1) modifying palmitoylation status of lipid raft proteins, or 2) reducing cholesterol levels within the raft, which promotes the lateral movement of proteins from raft to non-raft domains (Babina et al., 2011). Localization of these proteins to lipid rafts is crucial for the functionality of these proteins and their downstream signaling pathways (Babina et al., 2011) (Figure 2.5).

2.5.2 Inhibition of Arachidonic Acid Metabolism and its Derivatives

Both n-3 and n-6 PUFAs can be converted into signaling molecules known as eicosanoids, which are important hormone-like molecules that control platelet aggregation, cell growth and differentiation (Larsson et al., 2004). n-6 PUFAs ultimately
lead to the conversion of inflammatory eicosanoids through the production of arachidonic acid (AA), while n-3 PUFAs, like EPA, lead to the synthesis of anti-inflammatory eicosanoids through EPA (Young et al., 2011; Calder, 2006) (Figure 2.4). EPA competes with AA for eicosanoid synthesis and can also block AA metabolism thereby reducing inflammatory by-products (Calder, 2006). This is critical due to the role of inflammation in cancer initiation and progression (Calder, 2006) (Figure 2.4).

2.5.3 Lipid Peroxidation

ALA, EPA and DHA can also decrease cell growth and increase cell death or apoptosis due to their unsaturated nature, which readily becomes oxidized in a process known as lipid peroxidation (Mansara et al., 2011; Deshpande et al., 2013). Lipid peroxidation results in the production of free radicals, leading to the induction of oxidative stress in cells, which has the potential to damage cellular proteins involved in tumour suppression (Mansara et al., 2011). In addition to acting directly, fatty acids can also be converted into lipid mediators, such as eicosanoids, which are capable of triggering a variety of effects in the cell (Liu and Ma, 2014) (Figure 2.5).
Figure 2.4. Metabolism of n-6 and n-3 polyunsaturated fatty acids into lipid mediators.
2.5.4 Modulation of Cell Membrane Receptors, Transcription Factors and Various Signaling Molecules Derived from Lipids

In addition to impacting proteins found on the cellular membrane, n-3 PUFAs are also able to modulate the activity of nuclear proteins and transcription factors found within the cell (Liu and Ma, 2014). Peroxisome proliferator-activated receptor-gamma (PPARγ), a member of the nuclear receptor family PPAR, is found in breast tissue and is a prominent transcription factor in breast cells (Georgiadi and Kersten, 2012; Gurnell et al., 2013). It binds to DNA sequence elements and controls lipid metabolism, cell proliferation and differentiation and inflammation (Desvergne and Wahli, 1999; Flachs et al., 2009). EPA and DHA have been shown to be ligands or agonists for PPARγ and both cell treatments with EPA and DHA and supplementation with FO in rats has been shown to increase PPARγ protein content (Flachs et al., 2009); this is advantageous as greater PPARγ activity correlates with reduced tumour burden (Jiang et al., 2012). A second nuclear protein, Ki-67, used as a marker of tumour growth and aids in determining prognosis, has been shown to be downregulated by ALA-rich flaxseed oil in rodents, paired with an overall reduction in tumour size (Truan et al., 2010; Saggar et al., 2010b). A third nuclear protein similar to Ki-67, Proliferating Cell Nuclear Antigen (PCNA), is involved in DNA replication and repair and can be used as a marker of cell proliferation (Taftachi et al., 2005; Malkas et al., 2006; Strzalka and Ziemienowicz., 2011). Menhaden oil or FO supplementation in rats has been shown to decrease the expression of PCNA, which then led to a reduction in actively dividing tumour cells (Olivo and Hilakivi, 2005) (Figure 2.5).
Cell proliferation is only one aspect of modulating tumour growth; initiation of apoptosis or cell death is especially critical in terms of controlling neoplastic progression. B cell lymphoma-2 (Bcl-2) is a crucial regulator of apoptosis and its overexpression promotes survival of breast cancer cells (Van Slooten et al., 1996). Bax, a pro-apoptotic member of the Bcl-2 family of proteins may be upregulated in the presence of elevated n-3 PUFAs in the diet (either in the form of FO or canola oil), while anti-apoptotic Bcl-2 may be downregulated (Manna et al., 2008; Van Slooten et al., 1996; Oltavi et al., 1993; Ion et al., 2010). In addition to the Bcl-2 family proteins, PI3K/Akt are also crucial regulators of apoptosis and wild-type signaling is anti-apoptotic in nature (Ravacci et al., 2013, Jiang et al., 2012, Schley et al., 2005). FO supplementation in rodents has been shown to downregulate Akt or inhibit its phosphorylation and subsequent activation (Ravacci et al., 2013; DeGraffenried et al., 2003). Downstream signaling from Akt leads to the activation of nuclear factor kappa-B (NF-κB), an important transcription factor which controls several genes involved in cell proliferation, metastases and angiogenesis (Dolcet et al., 2005; Wu et al., 2005). Therefore, downregulation of Akt by EPA and DHA would subsequently lead to a reduction in NF-κB and in turn modulate genes controlled by this transcription factor (Figure 2.5).

2.6 microRNA

microRNAs (miRs) are small, non-coding endogenous RNAs approximately 18-25 nucleotides long (Farazi et al., 2013). They govern post-transcriptional gene expression, reducing their abundance and/or translational efficiency thereby regulating protein expression and modulating numerous cellular processes (Farazi et al., 2013; Krol
et al., 2010; Lee et al., 2013; Cascione et al., 2013; Radojicic et al., 2011; Parasramka et al., 2011). There are 2,588 unique mature human miRs (miRBase, 2014) and over one-third of human genes are postulated to be directly targeted by miRs (Kim et al., 2009c). The combination of miRs in each cell type determines the outcome of thousands of mRNAs (Kim et al., 2009c). miR biogenesis begins in the nucleus and is regulated by transcription factors in a tissue-specific manner (Krol et al., 2010). Primary transcripts (pri-miRs) are generated by RNA polymerase II and the first step of miR maturation involves cleavage at the stem of the hairpin structure by the nuclear RNase III-type protein Drosha, with the help of DiGeorge Syndrome Critical Region 8 Protein (DGCR8); this releases a small hairpin called the pre-miR (Kim et al., 2009c).

The pre-miR is then exported from the nucleus into the cytoplasm, a process mediated by exportin-5. The pre-miR is then cleaved near the terminal loop by the enzyme Dicer, along with the transactivating response RNA-binding protein (TRBP). Once cleaved, the RNA duplex associates with Argonaute (AGO) proteins forming a complex known as the RNA-induced silencing complex or RISC (Krol et al., 2010). Once in RISC, the miR guides the complex to target recognition sites in its mRNA (Kim et al., 2009c) (Figure 2.6). Target genes are downregulated via complementary base-pairing between the 5’ seed region of the miR and the 3’ untranslated region (UTR) of the mRNA, resulting in destabilization of the transcript, or by improper base pairing, which represses translation (Farazi et al., 2013; Parasramka et al., 2011).
Figure 2.5. Potential mechanisms of n-3 polyunsaturated fatty acids effect
The degree of complementarity between the miR and mRNA dictates the outcome of the target mRNA; perfect complementarity between the miR and mRNA results in cleavage and degradation of the transcript, while imperfect base pairing results in repression of translation (Parasramka et al., 2011). This repression has been shown to have significant time-, cell- and tissue-specificity (Vera et al., 2013). A single miR can target hundreds of mRNAs and it is postulated that approximately 50% of protein-coding genes are regulated by miRs (Farazi et al., 2013; Krol et al., 2010). One mRNA may be targeted by several miRs at the 3’-UTR and a single miR can bind multiple targets (Zaleska et al., 2015). Complementary or cooperative actions of several miRs on the same gene may also occur, increasing the already complex and diverse interactions observed between miR and mRNA targets (Zaleska et al., 2015).

Regulation of miR is a dynamic process and multiple factors control their expression. Time course experiments are crucial for elucidating the temporal changes observed in not only miR, but also their gene targets (Li et al., 2010; Nazarov et al., 2013). Transcription factors can promote the expression of many miRs, which may target the same genes or several genes involved in the same signaling pathways (Zaleska et al., 2015; Vera et al., 2013). In terms of miR regulation, biological networks of miRs are controlled by positive and negative feedback loops, which display both steady state and transient patterns; regulatory mechanisms such as active degradation of mature miRs are also vital for miR homeostasis (Vera et al., 2013; Ruegger and Grobhans, 2012). In a positive feedback loop, the expression of the miR of interest is inhibited by one of its target proteins, while in a negative feedback loop, the transcription factor targeted by the miR goes on to activate miR expression.
Figure 2.6. Biogenesis of microRNA. Modified from Winters et al., 2009.
Positive feedback loops can result in stable expression of both the miR and transcription factor in question, while negative feedback loops can result in waxing and waning of the miR and transcription factor being studied (Vera et al., 2013). A positive feedback loop may be seen in cancer where chronically high expression levels of a miR is often observed, whereas a negative feedback loop can maintain homeostasis and prevent uninhibited cell proliferation seen in cancer (Vera et al., 2013).

2.6.1 miR and Breast Cancer

miRs have been shown to modulate the expression of several tumorigenesis-associated genes, including those involved in cell development, proliferation, inflammation, cell cycle regulation, differentiation, apoptosis evasion, genomic instability, chromatin structure, angiogenesis, replicative immortalization, metabolism, morphogenesis, and invasion (Farazi et al., 2013; Radojicic et al., 2011; Becker Buscaglia and Li, 2011). Within cancer, miRs are known as either tumour suppressor miRs or oncogenic miRs (“oncomiRs”). A reduction in tumour suppressor miRs results in an increase of detrimental miR-target oncoproteins, while an increase in oncomiRs results in a reduction of essential tumour suppressor proteins (Parasramka et al., 2011). In general, oncomiRs exhibit anti-apoptotic activity, whereas tumour suppressor miRNAs display anti-proliferative and pro-apoptotic function (Radojicic et al., 2011). In either case, augmented cell proliferation can result in a neoplastic formation (Parasramka et al., 2011). Due to their widespread and complex effects within the cell, miRs are an intriguing candidate in the study of many of the pathologies observed in humans at present (Krol et al., 2010).
Aberrant expression of miRs observed in breast cancer patients compared to control patients indicates a high probability that specific miRs are key players in breast cancer pathogenesis (Lee et al., 2013). These changes in miR expression observed when comparing healthy controls and cancer patients may be attributed to the location of the miRs within the genome (Farazi et al., 2013). miRs often shown to be dysregulated in cancer are disproportionately found to be located in regions of chromosomal instability or near chromosomal breakpoints or fragile sites (Farazi et al., 2013). Since miRs have many gene targets, impaired miR expression has many detrimental effects within the cell. miR-target pairs have been determined by combining data from miR target prediction databases and expression profiles of miR and mRNA (Lee et al., 2013). From here, miR-regulated protein interaction networks (PINs) can then be used to ascertain pairings with the help of the human protein reference database (HPRD) (Lee et al., 2013). Lastly, the functions of these pairings can be revealed through functional enrichment analysis (Lee et al., 2013). Through this methodology, breast cancer-related miRs and the associated functions of the PINs have been elucidated (Lee et al., 2013). Since one miR may target multiple genes, and a single gene can be targeted by multiple miRs, the complexity is immense. Figure 2.7 represents an example of a PIN showcasing a subset of miRs most commonly known to target PTEN and Bcl-2 (Dweep and Gretz, 2015). This shows that one gene can be targeted by several miRs and also that one miR can target more than one gene, as is the case with miR-21.
miR-21 was found to be a prominent breast cancer-related miR (Lee et al., 2013). Interestingly, the miR-21 gene is found within a fragile site in the human genome, which may help to explain why it is overexpressed in several types of cancer, including breast cancer (Krichevsky and Gabriely, 2009). Several studies have shown that circulating extracellular miRs, such as miR-21, are easily detectable tumour biomarkers and may provide the potential to act as novel, non-invasive, diagnostic, prognostic, treatment response predictors and therapeutic aids in breast cancer (Zaleska et al., 2015; Christodoulatos and Dalamaga, 2014).
2.6.2 miR-21 in Breast Cancer

miR-21 has been shown to be significantly overexpressed in ER+ breast cancer patients compared to healthy controls, and is known as an oncomiR (Radojicic et al., 2011). miRs can be found in circulating extracellular plasma in breast cancer patients and a positive correlation between elevated miR-21 expression levels and poor patient outcomes has been observed (Jazbutyte and Thum, 2010; Radojicic et al., 2011). For this reason, miR-21 may be a promising clinical biomarker in breast cancer. High levels of miR-21 have been shown to play a prominent role in uncontrolled cell proliferation; this is supported by studies showing that anti-miR-21 inhibits MCF-7 cell growth both in vitro as well as tumour growth in a MCF-7 xenograft mouse model of breast cancer in vivo (Si et al., 2007). MCF-7 cells express many miRs often seen to be dysregulated in breast cancer and therefore may be a strong model for studying miR regulatory networks (Farazi et al., 2013). A microarray study in MCF-7 cells demonstrated that miR-21 was one of the most highly expressed miRs in MCF-7 cells (Fix et al., 2010).

Further investigations have examined the correlation between environmental factors, such as diet, smoking and alcohol, and miRs (Qiu et al., 2012). Qiu et al. (2012) found that the most highly expressed miRs interacted with the greatest number of environmental factors. In addition, they also found that the disease spectrum width (DSW) (the number of diseases associated with a single miR) of a miR was significantly associated with the number of environmental factors that interacted with it. Interestingly, miR-21 was found to display the widest DSW in their study, and demonstrated the most interacting environmental factors (Qiu et al. 2012). Research highlights the potential for dietary interventions, in particular n-3 PUFAs, in altering miR expression (Visioli et al.,
Recent investigations have suggested a modulatory effect of the n-3 PUFAs EPA and DHA on miR activity, and in turn neoplastic progression, in breast cancer (Nassipour et al., 2012; Shah et al., 2012). DHA, the long chain metabolic product of ALA derived from marine organisms, has been shown to significantly attenuate miR-21 expression in vitro in both MCF-7 and MDA-MB-231 cells (Mandal et al., 2012). FO has also been shown to reduce miR-21 in vivo in xenograft mice with implanted MDA-MB-231 mammary tumours (Mandal et al., 2012). The mechanism leading to this modulation of miR remains unclear, and it is undetermined whether the bioactive effects of n-3 PUFAs on miRs are due directly to the fatty acids themselves, or to metabolites/lipid mediators such as resolvins, lipoxins, protectins, and maresins (Visioli et al., 2012).

### 2.7 Proposed Mechanisms of n-3 PUFAs on miR-21 in Breast Cancer

DHA and FO have been shown to significantly reduce miR-21 expression and activity, both in vitro and in vivo, as well as impacting miR-21 cellular targets involved in cell growth and apoptosis (Mandal et al., 2012). The mechanism(s) by which this occurs remains unclear, however, pathways involving its direct targets and other associated proteins such as PTEN, Bcl-2, PDCD4 and NF-kB have been suggested (Mandal et al., 2012; Shah et al., 2012; Parasramka et al., 2011; Tables 2.5 and 2.6).

#### 2.7.1 miR-21 Targets Involved in Cell Growth and Apoptosis

miR-21 has been shown to directly target phosphatase and tensin homolog (PTEN), a phosphatidylinositol-3,4,5-trisphosphate 3 (PIP3)-phosphatase that inhibits the
PI3K pathway while dephosphorylating PIP3, and subsequently preventing activation of Akt (Jazbutyte and Thum, 2010). The PI3K pathway has been shown to be constitutively active in a variety of cancers as it plays an important role in controlling cell survival (Jazbutyte and Thum, 2010). miR-21 has been shown to target and modulate PTEN expression in numerous cancers, including breast cancer (Mandal et al., 2012; Jazbutyte and Thum, 2010). Investigations carried out in MCF-7 and triple-negative, MDA-MB-231, cell lines demonstrated that 152nM DHA treatment for 24 hours significantly reduced miR-21 expression (Mandal et al., 2012).

Transfection experiments in the same cell lines further confirmed that miR-21 regulates PTEN protein expression and that inhibition of miR-21 resulted in an upregulation of PTEN. Although cell viability was not investigated, this would be expected to result in an attenuation of aberrant cell growth. PTEN is a negative regulator of PI3K/Akt signaling, therefore, a greater quantity of PTEN would inhibit the PI3K/Akt pathway and reduce cell growth (Mandal et al., 2012) (Figure 2.8).
Figure 2.8. Potential role of dietary factors on miR-21 activity. Modified from Qiu et al., 2012.
Table 2.5: Selected experimentally verified miR-21 targets in breast cancer cell lines

<table>
<thead>
<tr>
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<th>Cell line</th>
<th>Function</th>
<th>References</th>
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<tbody>
<tr>
<td>PTEN</td>
<td>MCF-7; MDA-MB-231</td>
<td>Tumour suppressor, reduces cell proliferation</td>
<td>Mandal et al., 2012; Wickramasinghe et al., 2009</td>
</tr>
<tr>
<td>PDCD4</td>
<td>MCF-7</td>
<td>Tumour suppressor, decreases cell invasion</td>
<td>Frankel et al., 2008; Wickramasinghe et al., 2009</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>MCF-7</td>
<td>Apoptosis inhibitor</td>
<td>Wickramasinghe et al., 2009; Si et al., 2007</td>
</tr>
</tbody>
</table>

Table 2.6: Transcription factors involved in miR-21 gene regulation

<table>
<thead>
<tr>
<th>Factor</th>
<th>Cell line</th>
<th>Effect on miR-21</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP-1</td>
<td>MCF-7</td>
<td>Activates miR-21 transcription</td>
<td>Wickramasinghe et al., 2009</td>
</tr>
<tr>
<td>STAT3</td>
<td>HCT116; HT29; PC3; A5549; Hep3B</td>
<td>Induces miR-21 synthesis</td>
<td>Iliopoulos et al., 2010</td>
</tr>
<tr>
<td>NF-κB</td>
<td>AGS</td>
<td>Induces miR-21 synthesis</td>
<td>Shin et al., 2010</td>
</tr>
</tbody>
</table>
Additional known targets of miR-21 include the apoptotic regulator, Bcl-2 and the tumour suppressor, PDCD4 (Si et al., 2007; Zaleska, 2015). Notably, anti-miR-21 oligonucleotides have been shown to suppress cell growth, as well as tumour growth, and these findings are associated with an increase in apoptosis and a reduction in Bcl-2 in MCF-7 cells (Si et al., 2007). In addition, studies in MCF-7 cells have also shown that inhibition of miR-21 results in an increase in PDCD4 and PTEN (Wickramasinghe et al., 2009). Therefore, it is postulated that ALA, alone or combined with EPA and DHA, may downregulate miR-21, leading to a reduction in cell growth and increase in apoptosis via secondary modulation of its targets, PTEN, Bcl-2 or PDCD4 (Figure 2.8).

In addition to modulating miR-21 targets, it has been shown previously that DHA inhibits activation of NF-κB in breast cancer cells (Ghosh-Choudhury et al., 2009; Ghosh-Choudhury et al., 2010). Incubation of MCF-7 and MDA-MB-231 cells with DHA resulted in a reduction in pri-miR-21, suggesting that the regulation of miR-21 is transcriptional in nature (Mandal et al., 2012). It has been shown that the promoter of miR-21 contains an NF-κB binding element and DHA treatment has been shown to inhibit miR-21 expression by inhibiting NF-κB activity in both MCF-7 and MDA-MB-231 breast cancer cells (Zhou et al., 2010; Mandal et al., 2012).
Figure 2.9. Proposed mechanisms of n-3 PUFAs on miR-21 in breast cancer. Modified from Mandal et al., 2012.
2.7.2 miR, n-3 PUFAs and Lipid Rafts

Downstream signaling from lipid raft proteins can result in either activation or repression of various transcription factors which control the biogenesis of miR (Krol et al., 2010). Since n-3 PUFAs alter the composition of the cell membrane, including lipid raft domains, it is possible that they could alter downstream signaling of proteins and transcription factors, which control the synthesis of miR.

In vitro findings in MDA-MB-231 cells have indicated that DHA treatment triggers the lateral movement of EGFR from raft to non-raft domains and it appears that EGFR signaling originating from within rafts is oncogenic in nature (induces cell proliferation), while EGFR signaling outside of raft domains appears to induce apoptosis via phosphorylation and eventual activation of pro-apoptotic p38 mitogen activated protein kinase (MAPK) (Schley et al., 2007; Babina et al., 2011). Activation of p38 MAPK results in further downstream activation of transcription factors involved in the control of miR. The alterations in downstream signaling following disruption of EGFR within lipid rafts is hypothesized to influence miR biogenesis. Proteins such as Ras, MAPK/Erk, and Akt all lead to either the activation or repression of transcription factors, such as NF-κB and activator protein-1 (AP-1), which regulate the synthesis of miR-21. A reduction in these proteins would point to a concomitant reduction in transcription factor activity, and hence, miR biogenesis. Furthermore, Dicer and TRBP are two crucial proteins of the “microRNA-generating complex,” involved in the initiation of miR biogenesis (Paroo et al., 2009). Upstream, MAPK/Erk is responsible for the phosphorylation and activation of TRBP (Paroo et al., 2009). Consequently, a reduction
in MAPK/Erk activity, as seen when EGFR translocates to non-raft regions, is postulated to result in a reduction in miR biogenesis. In addition, MAPK/Erk is an upstream activator of the transcription factor, c-Myc, which also controls biogenesis of certain miRs (Krol et al., 2010). Therefore, it is suggested that reduced MAPK/Erk activity will lead to a suppression of c-Myc activation, and ultimately, reduced synthesis of oncogenic miRs, such as miR-21.

2.7.3 miR, n-3 PUFAs and Lipid Peroxidation

Lipid peroxidation can result following treatment of cells with ALA, EPA and/or DHA and the by-products of lipid peroxidation including 4-hydroxynonenal (4-HNE), have been shown to alter miR expression in a leukemia cell line (Deshpande et al., 2013; Pizzimenti et al., 2009). Reactive oxygen species (ROS), such as hydrogen peroxide (H₂O₂), superoxide anion and hydroxyl radicals also result following treatment of cells with ALA, EPA and/or DHA, leading to a suppression in cell growth and an increase in cell death (Kang et al., 2010; Xu et al., 2012; Deshpande et al., 2013). DHA treatment of MCF-7 cells as well as consumption of DHA in a xenograft mouse model of breast cancer (MCF-7 or MDA-MB-231 cells injected) resulted in an increase of ROS species, which lead to an elevation of apoptotic cell death (Kang et al., 2010). In addition, treatment of MCF-7 cells in vitro with ALA resulted in a concomitant increase in LPO paired with a reduction in nitric oxide (NO) and cell viability (Deshpande et al., 2013). The effect of n-3 PUFAs on miR in breast cancer remains unknown, although, they have been shown to modulate the expression of specific miRs in other, non-breast cancer cell lines. Many of the studies on miR were conducted with DHA, none with EPA and ALA,
or their combination. Since ALA is the major n-3 PUFA in the North American diet and it is converted to EPA and DHA, although to a limited extent, it is of interest to determine the effect of serum levels of ALA alone or combined with its metabolites EPA and DHA seen when ALA rich diets are consumed by animal or humans on miR-21 expression.

2.8 Summary and Questions

Despite advances in breast cancer, many issues still remain. Current treatments often result in negative side effects and drug resistance, in addition to the immense costs associated with developing and distributing novel breast cancer pharmaceuticals. For this reason, the attention surrounding lifestyle changes and complementary medicine has increased, and foods rich in n-3 PUFAs, such as FS and fish, have been of great interest.

In humans, consumption of n-3 PUFAs, as well as a high n-3:n-6 PUFA ratio in the diet is preventive and reduces the risk of breast cancer. n-3 PUFA consumption in animal models has been shown to reduce tumour size, decrease cell proliferation and increase apoptosis. Although in vivo exploration is the optimal method for studying the bioactive effects of n-3 PUFAs, one difficulty is the inability to differentiate the effects of individual fatty acids, such as ALA, as conversion to the longer chain metabolites, EPA and DHA, occurs. In this regard, studies in vitro are advantageous as breast cancer cell lines are unable to convert ALA into EPA and DHA.

The effects of EPA and DHA alone and combined as well as the effect of ALA alone have been studied in breast cancer cells lines. However, the combination of the three fatty acids at physiological concentrations and ratios seen in animal or human serum after the intake of FSO has not yet been studied in vitro in breast cancer cell lines. Mimicking the ratio of ALA:EPA:DHA observed post-ALA consumption in cell culture
treatments may help to provide the most physiologically-relevant results. Simultaneous treatment with multiple fatty acids may lead to more effective investigations of mechanisms surrounding the n-3 PUFA effect in vitro.

In addition, the role of n-3 PUFAs on miR, specifically miR-21, expression and mechanisms involving miR-21 regulation in breast cancer remains unknown. The effect of DHA alone on miR-21 and its targets has been examined, however, the combination of ALA, EPA and DHA on miR-21 and its gene targets has not yet been investigated. The issue of time is a pertinent factor when studying miRs, as their dynamic nature makes it challenging to identify an overall mechanism of effect, as both expression of miRs themselves, as well as their gene targets change rapidly and repeatedly over time. Moreover, the ability of multiple miRs to act on a single gene, or one miR to act on multiple genes makes this an even more arduous exploration. Varying n-3 PUFA treatment time points in vitro will each likely result in different miR-21 and mRNA target profiles, confirming this added complexity. Defining all mechanisms involved and teasing out miR-21-related ones from non-miR-21 ones will be challenging, however, patterns may be elucidated from the temporal changes observed in both miR-21 and its targets.
3.0 Objectives, Hypotheses, Experimental Approach and Rationale

3.1 Objectives

Overall: To further understand using in vitro studies the mechanisms of the n-3 PUFA effect on growth of breast cancer cells (MCF-7) with specific focus on the role of miR-21 expression.

Specific: 1. To investigate the effect of ALA alone and combined with EPA and DHA and background fatty acids, oleic acid (OA) and linoleic acid (LA), on breast cancer cell (MCF-7) growth and its relationship to miR-21 expression (Study 1 and 2).
2. To determine whether molecular target genes or related proteins of miR-21 such as PTEN, EGFR and Bcl-2 and Akt are affected by n-3 PUFAs (Study 3).

3.2 Hypothesis

ALA alone or combined with EPA and DHA, will reduce the growth of breast cancer cells (MCF-7) which is related to attenuation of miR-21 expression and consequent upregulation of PTEN and downregulation of Bcl-2, EGFR and Akt (Figure 3.1).
Figure 3.1. Hypothesis
3.3 Experimental Approach and Rationale

Figure 3.2 provides an overview of the experimental approach. **Study 1** was initially conducted to determine the effect of ALA alone or ALA combined with background fatty acids, OA and LA, to determine whether (a) background fatty acids would influence cell growth or miR expression and (b) the effect observed was due exclusively to ALA, and not simply due to the presence of any fatty acid in the treatment medium. Hence the growth of MCF-7 cells and miR-21 expression were measured following treatment with ALA (100µM), either alone or with background fatty acids, OA (40µM) and LA (40µM) initially for 96 and 48 hours. The 100µM dose of ALA was selected based on previous dose response experiments conducted in our lab, demonstrating it to be the most effective in reducing cell growth, yet still physiological dose, in MCF-7 cells (Wiggins et al., 2013). The background fatty acids dosages of 40µM were the same as that used by Ewaschuk et al. (2012). A trypan blue exclusion assay was then carried out to determine the impact of the fatty acids on cell growth and qPCR assay to determine miR-21 expression. When it was established that cell growth was not influenced by the background fatty acids, ALA treatment for 24 hours and all subsequent treatments in Studies 2 and 3 were conducted only with added background fatty acids.

**Study 2** examined the effect on cell growth and miR-21 expression of ALA alone versus combined with EPA and DHA at levels and ratios seen in the serum of animals and humans following FSO consumption. The serum fatty acid ratios described earlier (Table 2.1) were used to determine fatty acid concentrations for treatments, ensuring the concentrations used were physiological. As shown in Table 3.1, all treatments delivered a total dose of 112µM of n-3 PUFA (ALA+EPA+DHA), regardless of fatty acid
combination, and all treatments also contained background fatty acids at the same 
concentrations in Study 1. MCF-7 cells were incubated for 1, 3 and 24 hour(s). Due to 
the potency of the animal and human ratio fatty acid treatments (AnR and HuR, 
respectively), shorter time points were chosen in Study 2 to ensure an adequate quantity 
of cells remained following incubation to conduct analyses. A trypan blue exclusion 
assay determined cell growth and qPCR assay examined changes in miR-21 expression 
(Figure 3.2).

**Study 3** was conducted to determine the effect of n-3 PUFAs on molecular targets 
of miR-21 such as PTEN, EGFR and Bcl-2 genes and PTEN and Akt protein to 
determine a possible mechanistic explanation for the changes in growth and miR-21 
observed in **Study 1 and 2**. mRNA was analyzed via qPCR and protein was examined 
through Western blot at both 12 and 24 hours (Figure 3.2). These time points were 
chosen to accommodate for the lag time seen in gene and protein expression following 
changes in miR-21 expression.

Both technical and biological replicates were carried out in triplicate for both cell 
culture and qPCR experiments.
Figure 3.2. Experimental Approach
X= background fatty acids, OA and LA
Table 3.1. Fatty acid dose used for treatment

<table>
<thead>
<tr>
<th>Ratios</th>
<th>ALA (µM)</th>
<th>EPA (µM)</th>
<th>DHA (µM)</th>
<th>OA (µM)</th>
<th>LA (µM)</th>
<th>Total FA dose (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal FA Ratio (AnR)</td>
<td>25</td>
<td>9</td>
<td>78</td>
<td>40</td>
<td>40</td>
<td>112+80</td>
</tr>
<tr>
<td>Human FA Ratio (HuR)</td>
<td>25</td>
<td>25</td>
<td>62</td>
<td>40</td>
<td>40</td>
<td>112+80</td>
</tr>
</tbody>
</table>

X= background fatty acids, OA and LA
4.0 Materials and Methods

4.1 Cell Line and Culture

The ER+, PR+ breast cancer cell line, MCF-7, was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in DMEM medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA) and 1% antibiotic-antimycotic solution containing penicillin, streptomycin and amphotericin B (Gibco). MCF-7 cells were maintained in a humidified 37°C, 5% CO2 atmosphere incubator.

4.2 Treatment Media

ALA, EPA, OA and LA (all >99% pure, Sigma-Aldrich) and DHA (>98% pure, Sigma-Aldrich), stock solutions were prepared in 100% ethanol and stored at -20°C following flushing with nitrogen gas to reduce oxidation. To prepare the fatty acid treatment of choice, a volume of stock solution was pipetted into a 15mL centrifuge tube and the ethanol was evaporated with nitrogen gas. An appropriate volume of charcoal stripped FBS (CS-FBS; Sigma-Aldrich) was then added to the tube creating a 4mM fatty acid solution. This was placed in the 37°C, 5% CO2 atmosphere incubator for 1 hour to allow the fatty acids to complex with the albumin in the CS-FBS. The fatty acid-CS-FBS solution was then added to phenol red free DMEM-F12 (Gibco), which was supplemented with 1% antibiotic-antimycotic, additional CS-FBS to reach a 5% FBS treatment solution, and 1nM E2 (Sigma-Aldrich) dissolved in ethanol. Appropriate volumes of fatty acid-CS-FBS solutions were then added to create desired treatment concentrations and fatty acid ratios. The control treatment medium was equivalent to the
treatment medium with the exception of the fatty acid addition; background fatty acids were added to the control media when also contained in the treatment. The total treatment incubation time varied depending on the study and experiment. For 96 hours treatment time, treatment media was refreshed at 48 hours.

4.3 Cell Culture and Trypan Blue Assay for Cell Growth

Cells in the log phase of growth were plated in 6-well tissue culture plates (Sarstedt, Numbrecht, Germany) in complete DMEM medium at a density of 9.6x10^4 to 1.92x10^5 cells/well (greater density required in Study 2 and 3 for treatments containing EPA and DHA) and allowed to adhere for 72 hours. Complete DMEM medium was then removed and treatment media was added containing the appropriate concentration and/or ratio of fatty acids along with 1nM E2 to three wells per treatment condition. Treatment media was replaced at 48 hours for 96 hour treatment time. After the desired elapsed time (1, 3, 24, 48 or 96 hours), cells were collected from each well with 0.25% trypsin-EDTA (Sigma-Aldrich), centrifuged and resuspended in 200µl of DMEM medium. A 10µl aliquot was then promptly added to 10µl 0.4% trypan blue stain (Gibco) and total and viable cells were counted using a TC20 automated cell counter (Bio-Rad, Hercules, CA, USA). The total live cell number for each well was recorded and the average viable cell counts of the three wells for each treatment condition (three technical replicates) was divided by the mean of the control wells to present the data as a percentage of the untreated control cell number.
4.4 Quantitative Polymerase Chain Reaction (qPCR) Assay for miR-21 and Gene Expression

Total RNA was extracted from 9.6x10^4 to 1.92x10^5 cells/well after treatment for 1, 3, 24, 48 or 96 hours (for miR analyses) or for 12 and 24 hours (for mRNA analyses) as described in section 4.2 using the mirVana™ miRNA isolation kit, with phenol. RNA concentration and quality was measured using the NanoDrop 2000 Spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA). For miR-21 analysis, 10ng of total RNA per 15µl reverse transcription reaction was then used to synthesize cDNA using the TaqMan® microRNA Reverse Transcription kit. TaqMan® Small RNA assay (20X) along with TaqMan® Universal PCR Master Mix II (2X), no UNG was added to the cDNA and 10µl of this was placed into each well in triplicate into a 384-well qPCR plate. qPCR set-up was the following: 95°C for 10 minutes, and then 40 cycles of 1) 95°C for 15 seconds and 2) 60°C for 60 seconds.

For mRNA analysis, 2µg of total RNA per 20µl reverse transcription reaction were used to synthesize cDNA using the High Capacity cDNA Reverse Transcription Kit. qPCR was conducted with the TaqMan® Gene Expression Master Mix (2X) along with TaqMan® Gene Expression Assay primers including PTEN (ID: Hs02621230), EGFR (ID: Hs01076078) and Bcl-2 (ID: Hs00608023) in a 10µl reaction in triplicate using a 384-well qPCR plate. qPCR set-up was the following: 50°C for 2 minutes, 95°C for 10 minutes, and then 40 cycles of 1) 95°C or 15 seconds and 2) 60°C for 60 seconds. Data were normalized to the endogenous control U6 (ID: 001973) (for miR-21) or beta-2 microglobulin (B2M) (ID: Hs00984230) (for mRNA) and fold change was calculated
with the $2^{\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). All reagents used were from Life Technologies, Foster City, MA, USA.

4.5 Protein Biomarker Expression

Cells were plated and treated as in Section 4.3. After 12 or 24 hours cell treatment as described in section 4.2, protein was extracted on ice using RIPA lysis buffer (1X) (Cell Signaling Technology, Beverly, MA, USA) containing protease inhibitors (Complete Mini, EDTA-free Protease Inhibitor Cocktail Tablets, Roche) and phosphate inhibitors (PhosSTOP Phosphatase Inhibitor Cocktail Tablets, Roche). Briefly, each well of the 6-well plate was washed with ice cold PBS, and then 40-60µl RIPA buffer was added to each well for 5 minutes. Cell scrapers were used to collect cells, and this lysate solution was then placed on a shaker for 30 minutes at 4°C and centrifuged at 12,000 RPM for 20 minutes at 4°C. Supernatant was aliquoted and immediately stored at -80°C. Equivalent quantities of total protein were separated by electrophoresis on 7.5% polyacrylamide gels (200V for 40 minutes) and then transferred to polyvinylidene difluoride (PVDF) membranes. After blocking with 5% non-fat dry milk in blocking buffer for one hour, membranes were incubated overnight at 4°C with primary antibody in a 1% blocking buffer. Primary antibodies included: PTEN (1:1000), Bcl-2 (1:1000), EGF (1:1000), GAPDH (1:4000), pAkt (1:2000) and total Akt (1:4000) (Cell Signaling Technology (CST), Beverley, MA, USA. Membranes were then incubated with a 1% blocking buffer along with anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Beverley, MA, USA, 1:4000) for 1 hour and proteins were detected using LuminataTM Crescendo Western HRP Substrate chemiluminescent reagent (EMD
Millipore, WBLUR0100, Billerica, MA, USA). Chemiluminescence was then detected on X-ray film (Clonex Corporation, Markham, ON, Canada) using a Konica Minolta™ SRX-101A Film Processor. Densitometric analysis was performed using ImageJ software (National Institutes of Health (NIH)) and the relative intensity unit of each protein biomarker was determined by dividing each sample’s band intensity by its GAPDH band intensity.

4.6 Statistics

Statistical analysis of data was completed using Graph Pad Prism 5 (Graphpad) and Sigma Stat 3.5 (Jandel Scientific). All data are presented as mean ± standard error of mean. For all experiments, significance was set at p<0.05.

In Study 1 each treatment group was calculated as % of control viable cell number when examining cell viability, and control was either no treatment or + background fatty acids. A two-way ANOVA was conducted to determine independent effects of ALA, background fatty acids and their interaction on cell viability at each of 48 and 96 hour treatments. A one-way ANOVA with post-hoc Tukey test was conducted to analyze differences in +/- background fatty acid treatments on miR-21 expression at each of 48 and 96 hour treatments. At 24 hours, a paired t-test was conducted to analyze cell viability and a Mann-Whitney rank sum test was used to analyze differences in miR-21 expression.

In Study 2, as in Study 1, each treatment group was calculated as % of control viable cell number when examining cell viability. Paired t-tests were conducted when
two groups were being compared, and one-way ANOVA with post-hoc Tukey test was conducted when comparing three or more groups.

In Study 3 a one-way ANOVA with post-hoc Tukey test was conducted to determine differences in mRNA and protein expression between treatment groups.
5.0 Results

5.1 Study 1a: Effect of ALA alone or in combination with background fatty acids on cell viability and miR-21 expression at 48 and 96 hours treatment

ALA (100µM) alone and combined with background fatty acids significantly reduced the growth of MCF-7 cells after 48 hours or 96 hours treatment in a 24-well plate (Figure 5.1). A significant ALA effect (48 hours p= 0.045; 96 hours p= <0.001), but no background fatty acid effect (48 hours p= 0.768; 96 hours p= 0.806) were observed. There was no interaction between ALA and background fatty acids at either time point (48 hours p=0.769; 96 hours p= 0.844), so the presence of background fatty acids did not influence the ALA effect. There appears to be a limit for the reduction in growth that is attainable, as both 48 hours and 96 hours displayed similar reductions in growth in response to ALA alone treatment (48 hours= 42.6% ± 6.90 reduction; 96 hours= 39.33% ± 3.71 reduction).

ALA alone and combined with background fatty acids significantly increased the expression of miR-21 at both 48 hours (p= 0.0012) and 96 hours (p=0.0003) when compared to the control (Figure 5.1). Fold-changes in miR-21 expression were: 48 hours: ALA alone or ALA + background fatty acids= 1.2-1.3; 96 hours: ALA alone or ALA + background fatty acids = 1.3-1.4. Background fatty acids did not influence ALA effect.
Figure 5.1. Effect of ALA alone or in combination with background fatty acids on cell viability and miR-21 expression after 48 and 96 hours treatment. Bars with different letters are significantly different (p<0.05). ‘X’ denotes background fatty acids in all figures.
5.2 Study 1b: Effect of ALA combined with background fatty acids on cell viability and miR-21 after 24 hours treatment

Since there was a reduction in cell growth following 48 and 96 hours treatment, yet no reduction in miR-21 expression, it was thought that changes in miR-21 expression may have occurred at an earlier time point, initiating these reductions in cell growth observed at the later time points. The effect of a shorter ALA treatment time was therefore tested. In addition, only the ALA treatment combined with background fatty acids was tested as it was previously determined in the 48/96 hour studies that background fatty acids did not alter the ALA effect (See section 5.1).

Following 24 hours treatment with 100µM ALA combined with background fatty acids, no significant change in cell growth was observed (8.82% ± 4.98 reduction; p=0.3306) (Figure 5.2).

Similar to 48/96 hour findings, miR-21 expression was significantly increased following 24 hours treatment with ALA combined with background fatty acids (p=0.026) (Figure 5.2).
Figure 5.2. Effect of ALA combined with background fatty acids on cell viability and miR-21 expression after 24 hours treatment. Bars with different letters are significantly different (p<0.05).
5.3 Study 2a: Effect of ALA combined with EPA and DHA on cell viability and miR-21 expression after 24 hours treatment

MCF-7 cells were treated with ALA, combined with EPA and DHA, at ratios observed post-FSO consumption. The AnR (25µM ALA:9µM EPA:78µM DHA) significantly reduced cell growth (82.2% ± 10.23 reduction; p= 0.0019), while the HuR (25µM ALA:25µM EPA:62µM DHA) non-significantly reduced growth (33.5% ± 5.84 reduction; p= 0.0621) (Figure 5.3).

miR-21 expression was significantly downregulated following 24 hours treatment with the AnR (p= <0.0001; fold-change= 0.5 ± 0.07) when compared to the control, similar to the results seen in cell growth. No significant changes in miR-21 expression were observed after 24 hours treatment with the HuR (fold-change= 1.3 ± 0.13) (Figure 5.3).

5.4 Study 2b: Effect of ALA alone or combined with EPA and DHA miR-21 expression after 1 and 3 hours treatment

miR expression levels are known to change quite rapidly, therefore, the effect of ALA combined with EPA and DHA on miR-21 expression following 1 and 3 hour incubation periods was examined. miR-21 expression was significantly reduced (p= <0.0001 after both 1 and 3 hours) following treatment with 112µM total n-3 PUFAs combined with 80µM total background fatty acids (Figure 5.4). The 1 and 3 hour fold-changes in miR-21 expression were: ALA= 0.77- 0.79; AnR= 0.67- 0.66; HuR= 0.68-0.74. It appears that the reduction in miR-21 expression was sustained from 1 to 3 hours, as there is no change in expression between the two time points.
Figure 5.3. Effect of ALA combined with EPA and DHA on cell viability and miR-21 expression after 24 hours treatment. Bars with different letters are statistically significant (p<0.05). AnR= animal ratio fatty acids; HuR= human ratio fatty acids; X= background fatty acids.
5.5 Study 3a: Effect of ALA alone or combined with EPA and DHA on gene expression after 12 and 24 hours treatment

PTEN gene expression was reduced significantly following 12 hour treatment with ALA and AnR when compared to the control (p= 0.0017) and both did not differ significantly from each other (Figure 5.5). No significant difference between HuR and the control was observed. PTEN gene expression remained significantly reduced following 24 hour treatment with AnR and HuR when compared to the control (p= 0.0006). No significant difference between ALA treatment and the control was observed (Figure 5.5).

After 12 hours treatment, EGFR gene expression showed no significant differences between treatments and the control (Figure 5.5). After 24 hours treatment,
only HuR resulted in significantly greater EGFR mRNA expression when compared to the control, ALA and AnR (p= <0.0001).

At 12 hours, all fatty acid treatments significantly reduced Bcl-2 gene expression when compared to the control, but AnR displayed a significantly greater reducing effect when compared to both ALA and HuR (p= <0.0001) (Figure 5.5). At 24 hours, there was a significant reduction (p= <0.0001) in Bcl-2 expression following all three treatments and no differences were observed between the treatments (Figure 5.5).

5.6 Study 3b: Effect of ALA alone or combined with EPA and DHA on protein biomarker expression after 12 and 24 hours treatment

Following both 12 and 24 hours treatment, no difference in PTEN protein expression was observed among the treatment groups and the control (Figure 5.6).

After 12 hours treatment, pAkt protein expression was significantly greater after AnR and HuR when compared to the control (p= 0.0053; Figure 5.6). There was no significant difference between the control and ALA treatment and no significant difference between the AnR and HuR. Following 24 hours treatment, a significant reduction was seen after both ALA and AnR when compared to the control (p= 0.0173; Figure 5.6) and there was no difference in reduction between ALA and AnR. Although the reduction caused by HuR (33.99%) was close to that caused by ALA (46.33%), it did not differ significantly from the control due to its larger variability.
Figure 5.5. Effect of ALA combined with EPA and DHA on PTEN, EGFR and Bcl-2 gene expression. Bars with different letters are significantly different (p<0.05). AnR= animal ratio fatty acids; HuR= human ratio fatty acids; X= background fatty acids.
Figure 5.6. Effect of ALA alone or combined with EPA and DHA on PTEN and pAkt protein expression. Bars with different letters are significantly different (p<0.05). AnR= animal ratio fatty acids; HuR= human ratio fatty acids; X= background fatty acids.
6.0 Discussion

This study has shown that ALA alone or in combination with EPA and DHA can reduce breast cancer cell growth and miR-21 expression in a time-dependent fashion, as summarized in Table 6.1, with limited changes in the analyzed molecular targets of miR-21.

Table 6.1. Summary of cell viability and miR-21 expression results

<table>
<thead>
<tr>
<th>Treatment (all +X)</th>
<th>Cell viability (% reduction)</th>
<th>miR-21 expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALA 96 hours</td>
<td>42.3</td>
<td>↑ 1.3</td>
</tr>
<tr>
<td>ALA 48 hours</td>
<td>32.5</td>
<td>↑ 1.2</td>
</tr>
<tr>
<td>ALA 24 hours</td>
<td>8.8</td>
<td>↑ 1.3</td>
</tr>
<tr>
<td>AnR 24 hours</td>
<td>82.2</td>
<td>↓ 0.5</td>
</tr>
<tr>
<td>HuR 24 hours</td>
<td>33.5</td>
<td>↔ 1.3</td>
</tr>
<tr>
<td>ALA; 1, 3 hours</td>
<td>↔</td>
<td>↓ 0.8, 0.8</td>
</tr>
<tr>
<td>AnR; 1, 3 hours</td>
<td>↔</td>
<td>↓ 0.7, 0.7</td>
</tr>
<tr>
<td>HuR; 1, 3 hours</td>
<td>↔</td>
<td>↓ 0.7, 0.7</td>
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</tbody>
</table>
Both 48 and 96 hours of 100µM ALA treatment with and without background fatty acids, OA (40µM) and LA (40µM), significantly reduced the growth of MCF-7 cells (Figure 5.1). No effect of background fatty acids was seen, and no interaction between ALA and background fatty acids was observed. The combination of OA and LA did not influence the ALA effect. This shows that the growth reducing effect seen is due exclusively to ALA, and not simply to the addition of any fatty acid into the treatment media. For this reason, experiments at subsequent time points for both ALA and ALA combined with EPA and DHA were only conducted in the presence of background fatty acids to produce more physiologically relevant results.

Although no significant reduction in cell growth was seen following 24 hours treatment with ALA or HuR, ALA combined with DHA (AnR) at ratios seen in the serum of animals fed ALA significantly reduced the growth of MCF-7 cells at 24 hours (Figure 5.2 and 5.3). These findings are likely due to the higher quantity of DHA found in the AnR treatment versus the HuR (78µM versus 62µM). Kang et al. (2010) showed that in MCF-7 cells, the IC_{50} values for DHA and EPA were 20.2 and 57.4µM, respectively. Previous preliminary work in our lab has shown that the IC_{50} for ALA is 67.2µM. Additional work by Chamras et al. (2002) also demonstrated that when MCF-7 cells were treated with equivalent doses of EPA and DHA, DHA resulted in nearly a 25% greater reduction in cell growth than EPA. This demonstrates that MCF-7 cells are 1) more sensitive to DHA and/or 2) that DHA possesses a greater bioactive capacity. It is postulated that the greater effect of DHA is due to its ability to trigger an increased production of ROS in breast cancer cells; this subsequently leads to cell death via apoptosis (Kang et al., 2010).
In contrast with cell growth, 100μM ALA with and without background fatty acids significantly increased miR-21 expression at 24, 48 and 96 hours (Figure 5.1 and 5.2). It was hypothesized that miR-21 expression would be reduced in response to ALA treatment; however, there are several factors which may explain the increase. miR are dynamic molecules, which have been shown to display continuous and rapidly changing expression levels (Vera et al., 2013). Moreover, a single miR, such as miR-21, does not work in isolation of other miRs and its genes targets. Many miRs either work together cooperatively or antagonize one another to dictate certain gene expression, leading to a specific phenotype (Qiu et al., 2012). Since only miR-21 was examined, it is possible that at these time points miR-21 is not the dominant oncogenic miR, and overexpression of other oncogenic miRs govern the cellular changes that occur at these time points. Thus, it is possible that ALA downregulated other more powerful oncogenic miRs than miR-21 at these time points (24, 48 and 96 hours) that were not examined in this study. Elevated miR-21 would be expected to result in an increase in cell proliferation, however, this does not correlate with the cell viability findings observed following 48 and 96 hours treatment. Therefore, it could be postulated that ALA is reducing oncomirs other than miR-21 and resulting in a reduction in cell growth. It may also be that miR-21 expression is very persistent at these later time points, and a greater dose of ALA would be required to downregulate its expression. This dosage theory is supported by the findings seen following treatment with the AnR and HuR, which deliver a higher total dose of fatty acid (112μM versus 100μM at 24 hours), including EPA and DHA.

The miR-21 expression results mirrored those of the cell growth results following treatment with the AnR and HuR (Figure 5.3). The AnR caused significant reductions in
miR-21, similar to what was seen for cell growth, and the HuR displayed no change in miR-21 expression when compared to the control; this is similar to the null result observed in terms of cell growth following HuR treatment. This differential expression of miR-21 in response to varying fatty acid treatment ratios is postulated to be related to the DHA level. DHA has been shown to alter lipid raft composition, translocating growth factor receptors from raft to non-raft domains (Corsetto et al., 2011; Babina et al., 2011). This then alters their downstream signaling pathways, rendering them less oncogenic in nature. Transcription factors, which typically terminate growth factor receptor signaling pathways are responsible for the promotion of miR-21 synthesis (Vera et al., 2013). If DHA altered growth factor signaling via lipid raft disruption, it likely also altered downstream transcription factor activity. This could potentially lead to reduced miR-21 synthesis and therefore decreased expression of mature miR-21. Alternatively, others have hypothesized that DHA may be able to act as a “natural” antagomiR, acting similar to an antisense oligonucleotide and directly inhibiting miR-21 expression and function (Mandal et al., 2012). Furthermore, since the AnR and HuR were not tested at later (48 and 96 hour) time points, it is unknown how the expression would change then, if at all, or if it would mimic the results of Study 1 with ALA. It is possible that DHA sustains this reduction in miR-21 expression throughout all time points, however, it is also possible that n-3 PUFAs work on other oncogenic miRs other than miR-21 within its same regulatory network at later time points. Investigation into these later time points was not possible as insufficient cells remained for analyses following 48 and 96 hours treatment.

Although cell viability was not examined at 1 and 3 hours as no effect is yet seen at these early time points, miR-21 expression was found to be significantly
downregulated at both time points in response to all three treatments (Figure 5.4). This downregulation was sustained from 1 to 3 hours, as there was no change in expression between the two time points. miR-21 may be more sensitive to n-3 PUFAs at earlier time points, or may be the dominant oncogenic miR within its signaling network at these earlier time points. This may leave it more susceptible to environmental factors, such as n-3 PUFAs, at the earlier time points. It is suspected that these earlier reductions in miR-21 preceded the reductions in cell growth that were observed at the later (24, 48, 96 hour) time points. The AnR is the only treatment that resulted in reduced miR-21 expression at all time points (1, 3 and 24 hours). It is postulated that this is due to the bioactivity of DHA on miR-21 in MCF-7 cells. Potentially greater doses of ALA alone and/or ALA combined with EPA, only at the earlier time points, would have elicited similar results, especially since their IC₅₀ is greater than that of DHA.

Taken together these results suggest that n-3 PUFAs are able to modulate miR-21 expression. The susceptibility of miR-21 to n-3 PUFAs appears to be temporal in nature, and also specific to the type of n-3 PUFA and dosage (Table 6.1).

In study 3, the biomarker targets did not all change according to the original hypothesis (Figure 6.1). PTEN mRNA was significantly reduced when compared to the control following ALA and AnR treatments; no change was observed when comparing HuR to the control. At 24 hours, a significant reduction in expression was seen for both AnR and HuR, while no significant change with ALA was observed when compared to the control (Figure 5.5). It was originally hypothesized that PTEN mRNA would increase in response to reduced miR-21, as PTEN is a tumour suppressor and a direct target of miR-21. However, PTEN mRNA at both 12 and 24 hours was reduced following AnR,
which juxtaposes the reduction in miR-21 seen at 1, 3 and 24 hours after AnR. This may be due to the lag time, which is typically seen between miR and mRNA expression (Jayaswal et al., 2009). Alteration in a single miR may require up to 48 hours before any change in its mRNA target is observed (Jayaswal et al., 2009). This time lag for the protein form of the target would then be even longer (Jayaswal et al., 2009). Therefore, one of three events may have occurred which could explain these results: 1) PTEN mRNA expression does not change until 48 hours, or simply any time later than 24 hours; 2) miR-21 does not target PTEN in response to n-3 PUFA-induced reduction, and other regulatory mechanisms or miRs within the miR-21 regulatory network are preventing this interaction from occurring; 3) miR-21 has to be a specific fold-change reduction for a certain period of time in order to possess the power necessary to alter gene expression.

The overall outcome of a miR-mRNA interaction can be altered by both the binding strength of the target site and the quantity of miR present (Orang et al., 2014). If the miR reduction is not great enough to exert its expected effect, an instantaneous reduction in its target gene can be observed, known as a pulse (Leung and Sharp, 2010). Once the miR reaches its required quantity, it can then exert its intended effect, ultimately leading to upregulation of its target due to lack of inhibition via reduced miR; in this case, we would expect to observe these dynamics in PTEN (Leung and Sharp, 2010). miR-21 was seen to have the greatest reduction in fold-change expression at 24 hours, and perhaps it was after this time point or “pulse,” that it was then able to initiate a rise in PTEN mRNA.

A second mRNA and target of miR-21, Bcl-2, was reduced across all treatments at both 12 and 24 hour time points (Figure 5.5). At the 12 hour time point, the reduction was greatest for AnR, which is hypothesized to be the result of the greater DHA content
of the AnR. In contrast to PTEN, Bcl-2 was hypothesized to decrease in response to reduced miR-21 expression, therefore, these findings support the initial hypothesis. Since all treatments reduced miR-21 expression at 1 and 3 hours, it appears that Bcl-2 has an initial lag time of approximately 9 hours, with a full time lag of 21 hours, where the greatest reduction in Bcl-2 was revealed. Bcl-2 is oncogenic and anti-apoptotic in nature, and so its reduced expression would lead to a reduction in cell growth and an increase in apoptosis. We observed a significant reduction in cell growth at 24 hours for AnR and 48 hours for ALA and HuR. Therefore, one mechanism of the growth reduction observed may have been mitigated by Bcl-2, with earlier reductions in miR-21 ultimately triggering the observed decrease in cell growth.

The last mRNA investigated, EGFR, did not display any significant changes in expression when compared to the control for all treatments at both time points, with the exception of HuR at 24 hours, which showed a significant increase in EGFR mRNA when compared to the control (Figure 5.5). EGFR is not a target of miR-21, but has been shown to be targeted by DHA, triggering its movement from raft to non-raft domains (Corsetto et al., 2011; Schley et al., 2007; Rogers et al., 2010). However, this displacement from lipid rafts typically does not lead to any alterations in EGFR mRNA quantity (Corsetto et al., 2011; Schley et al., 2011; Rogers et al., 2010). Since no other treatments displayed upregulation of EGFR, and a reduction in overall cell growth at later time points was observed, which contrasts with increased EGFR expression, it is likely this was a transient alteration in expression which was caught at this specific time point. Further investigations would be required into this finding in order to determine if a mechanism regarding elevated EGFR mRNA dose exist.
No significant changes in PTEN protein expression were observed with any of the treatments when compared to the control at both 12 and 24 hours (Figure 5.6). Although PTEN protein was hypothesized to increase in response to depression of miR-21, the finding is not surprising due to the recurring issue of time. Literature examining the correlation between mRNA and protein is lacking in mammalian cells, however, the correlation between the two at a single time point is found to be poor (Maier et al., 2009). Depending on the model system, correlations between protein and mRNA can be as little as 40% (Vogel and Marcotte, 2013). Multiple factors relating to post-transcriptional regulation can affect mRNA-protein correlation including translational efficiency, protein half-lives, and mRNA distribution within the cell (Maier et al., 2009). Any of these factors, in addition to timing, may be responsible for these null PTEN protein results. Investigation into PTEN protein expression at later time points, such as 48, 72 or 96 hours, would provide more information regarding its relationship with miR-21.

The second protein examined, pAkt, as part of the PI3K/Akt pathway, was postulated to decrease to help to explain the reduction in cell growth. At 12 hours, pAkt protein expression increased in response to AnR and HuR when compared to the control, and decreased at 24 hours following ALA and AnR when compared to the control (Figure 5.6). At 24 hours, HuR also resulted in a reduction that did not reach statistical significance. The discrepancy observed at the 12 hour time point may be related to any of the aforementioned factors regarding protein translation. At 24 hours, although all treatments were expected to reduce pAkt protein expression, only ALA and AnR did so as a larger variability was observed with the HuR treatment. This variation may be related to the lower quantity of DHA found within the HuR treatment.
A reduction in pAkt protein at 24 hours may be related to the reduction in Bcl-2 mRNA that was observed at 24 hours. Bratton et al. (2010) has shown that Akt is able to regulate Bcl-2 expression through ERα in MCF-7 cells. Acting together, these reductions in pAkt and Bcl-2 may help to explain a potential mechanism of the growth reduction that was observed.

In summary, ALA alone or combined with EPA and DHA did reduce cell growth. No change in EGFR was observed, however, a reduction in pAkt, along with subsequent reductions in miR-21 and Bcl-2 were seen. This was paired with no detectable changes in PTEN (Figure 6.1).

miR are complex molecules able to affect numerous genes within the cell. Although n-3 PUFAs reduced miR-21 expression at earlier time points (1 and 3 hours) among all treatments, the precise effect that this had on all of its target mRNAs remains unknown. Only a select few targets were investigated, and the issue of time appears to be pivotal when examining miR-mRNA-protein interactions. Ultimately, proteins are the true causal forces within the cell and their quantity and interactions with other biomolecules are the most important aspect to study (Greenbaum et al., 2003). Analyses at additional time points, as well as further exploration of miR gene targets would provide a more comprehensive view of the mechanisms at play which are triggering the resultant reduction in cell growth that was ultimately observed across all treatments.
Figure 6.1. Observed changes in biomarker expression following n-3 PUFA treatment
7.0 Conclusion

1) ALA alone or combined with EPA and DHA at ratios seen in the serum post-ALA consumption reduces the growth of ER+ breast cancer cells (MCF-7) at 48-96 hours, as well as the expression of miR-21 at 1-3 hours treatment time. An exception is AnR, which additionally reduced cell growth and miR-21 expression at 24 hours. The miR-21 findings were seen to be temporal in nature, as the fatty acid treatment incubation time significantly impacted miR-21 expression.

2) Of the expected change in molecular targets of miR-21 and associated biomarkers investigated at the gene and protein levels (upregulation of PTEN, downregulation of Bcl-2, EGFR, pAkt), only the Bcl-2 gene was reduced, which provides a possible explanation for the reduction of MCF-7 cell growth. Further analyses need to be conducted to determine whether or not this is a defined mechanism.

8.0 Study Strengths

This study has many strengths including 1) treatment at multiple time points, 2) in vitro fatty acid treatment with physiological ratios of n-3 PUFAs, 3) testing specific fatty acids as no conversion takes place in an in vitro system, and 4) providing further evidence displaying that miR are susceptible to modulation by dietary factors. Within the miR literature, very few studies have been conducted using multiple time points, and this study revealed the pivotal role of time on miR expression, and how this also translates to gene and protein biomarker expression. Treating cells in vitro with the concentration of n-3 PUFAs seen in vivo following FSO consumption helps to provide the most
physiologically relevant results. The emerging field of miR is rapidly expanding, and the role of dietary factors on miR remains unclear. Further investigations into the role of bioactive food components on miR are advantageous as they may be a low risk, inexpensive alternative to miR-modulating pharmaceuticals.

9.0 Limitations and Future Directions

Although this study helped to elucidate the effect of n-3 PUFAs on cell growth and miR-21 expression, there are some limitations that can be considered for future work.

1) Only a single miR, miR-21, was examined. Many miRs are seen to be dysregulated in breast cancer, and investigating many miRs will help provide a more complete picture of the mechanisms at play.

2) The molecular targets of miR-21 examined were chosen based on the results seen in previous literature. Additional targets can be studied, which based on bioinformatics analysis, may include tropomyosin, sprouty 1 and 2 and Cdc25A (Jazbutyte and Thum, 2010).

3) The experiments looked exclusively at a cancerous cell line. Running all experiments alongside a non-tumourigenic breast cancer cell line will help further to further validate our findings and support that they are specific to malignant cells.

4) Transfection experiments in which miR-21 is specifically inhibited and subsequent examination of its targets are carried out will help confirm the role of miR-21.
5) Little to no cells were left for analysis following 48 hours incubation with AnR and HuR, however, it would have been interesting to examine miR-21 expression, as well as molecular targets of miR-21, at 48 and 96 hours treatment with AnR and HuR.

6) Only one breast cancer cell line (MCF-7) was examined. It would be interesting to investigate how non-ER+ breast cancer cell lines respond to fatty acid ratio treatments, and if an equivalent effect on miR-21 would be observed.

7) In vitro studies were conducted as it allowed studying the effect of individual n-3 PUFAs, particularly of ALA, as cells cannot convert ALA into EPA and DHA due to lack of necessary enzymes. In vitro studies are also a necessary initial stage in scientific research. However, the results may not necessarily translate in vivo as the cells are treated outside of their normal environment and lack what would be seen in vivo—namely blood supply, surrounding tissues, an immune system, organ systems, and many metabolic processes.

Overall, future research to address the above limitations is necessary. First of all, a more comprehensive analysis of the role of miR in breast cancer and the n-3 PUFA effect are required. A miR expression profiling assay of MCF-7 cells would help define miRs that are either up- or downregulated in ER+ breast cancer cells. Interactions between prominent oncomiRs or tumour suppressor miRs, as well as miR regulatory networks could then be investigated. From here, further analyses may explore the impact of n-3 PUFA on miR expression and dynamics.
Subsequent investigations into the molecular targets of dysregulated miRs would then be warranted. A more comprehensive approach could involve a global gene expression analysis to define miR gene targets and/or a proteomics approach to further investigate the mechanism of the n-3 PUFA effect. Following this, exploration of these effects in additional cell lines, especially the triple-negative subtype, would help to determine whether or not the effects observed are seen across different breast cancer cell lines. After this, preclinical animal research would be needed to determine if the results seen in vitro could be translated in vivo in animal studies. Ideally, clinical investigations in humans are the ultimate objective, as the potential for a miR-based n-3 PUFA complementary therapy would be both low risk and cost effective. Targeting miR would be an advantageous approach to take as the origin of many of the cellular dysfunctions observed in breast cancer is being targeted as miR goes on to alter gene and protein expression. This may help to avoid the development of drug resistance, a common issue seen with many of the current targeted therapies.

10.0 Implications

The findings in this thesis provide preliminary support for the use of ALA alone or combined with EPA and DHA as a complementary therapy for patients with ER+ breast cancer. Both clinicians and patients are increasingly turning towards functional foods, such as FS, FSO and FO, in order to treat or prevent diseases such as breast cancer. The potential to target miR-21 provides a possible therapy that can effectively alter the expression of numerous genes and proteins involved in breast cancer.
11.0 References


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