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PRECLINICAL INVESTIGATIONS INTO THE ROLE OF OMEGA-3 FATTY ACIDS FOR BREAST CANCER PREVENTION

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by

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ABSTRACT

As discussed in **Chapter 1**, Breast cancer is the second leading cause of cancer death in women in the United States, with about 2 million women at high risk for developing the disease. A current strategy, approved by the FDA, for breast cancer prevention is the daily administration of selective estrogen receptor modulators(SERMS), tamoxifen and raloxifene. These SERMS have proven to be effective at reducing breast cancer incidence in women that are at high risk by 50% and 38%, respectively. However, these agents are poorly accepted as oral chemopreventives even by women at high risk for breast cancer because of concerns of side effects which include thromboembolic events and an increase in endometrial cancers. Furthermore, both agents are ineffective against the more aggressive estrogen receptor negative tumors. A series of experiments have been conducted in our laboratories to test the hypothesis that chemoprevention can be improved by combining SERMS with agents with different mechanisms of action. Such an approach can allow the use of low doses of SERMS and thus reduce their side effects. Literature data provide some support of the protective effects of omega-3 fatty acids against the development of several cancers, including breast cancer. However, the results remain inconsistent which could be due to confounding variables. These confounding variables which have been reported by our group include omega-3:omega-6(n-3:n-6) ratio and caloric intake. A previous study conducted in our laboratories showed that high ratios of omega-3:omega-6 fatty acids(25:1 n-3:n-6) are required to inhibit mammary carcinogenesis in the rat and such high ratios of omega-3:omega-6 fatty acids potentiated the chemopreventive efficacy of tamoxifen.

Studies conducted in **Chapter 2** were aimed to test the **hypothesis** that by using a proteomics approach, novel proteins can be identified that can provide insights into the molecular mechanism by which high ratios of omega-3:omega-6 fatty acids inhibit mammary

carcinogenesis. We further **hypothesize** that proteins identified in a minimally invasive fashion can be used for early detection and to monitor the efficacy of the chemopreventive agents.

We used an isobaric Tagging for Relative and Absolute Quantitation (iTRAQ) method to provide insights into the mechanism, at the protein level, responsible for the chemopreventive action of the high omega-3:omega-6 fatty acid ratios in the absence and presence of tamoxifen in the 1-methyl-1-nitrosourea(MNU)-induced mammary tumor model in the rat; selective proteins were further validated by western blotting. Compared to control (n-3:n-6, 1:1) diet, both 10:1 and 25:1 n-3:n-6 diets upregulated plasma vitamin D binding protein, gelsolin, and 14-3-3 sigma, reported to have tumor suppressive effects, whereas alpha-1B-glycoprotein which has been reported to be elevated in the serum of breast cancer patients was decreased. Compared to 25:1 n-3:n-6, the 25:1 n-3:n-6 plus tamoxifen diet downregulated apolipoprotein E, haptoglobin, and inter-alpha-inhibitor H4 heavy chain. Ingenuity Pathway Analysis (IPA) determined that the trends of specific proteins were related to lipid metabolism in the 25:1 n-3:n-6 group whereas the 25:1 n-3:n-6 plus tamoxifen group included proteins involved in cancer and inflammation. Collectively, our results show that several proteins were altered in a manner consistent with chemoprevention.

The Western diet is composed of a 15:1 n-6:n-3 ratio and would be impractical for women to consume high omega-3:omega-6 ratios on a daily basis. The chemopreventive efficacy of the high omega-3:omega-6 ratios suggests that a component of omega-3 fatty acids(eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA)) or one of its metabolites which is present in small amounts could account for the chemopreventive effects observed in the rat. Furthermore, considering the superior efficacy of DHA over EPA, in preclinical mammary carcinogenesis models as reported in the literature provided a strong rationale to focus our

studies on DHA. However, the highly unsaturated structure of DHA makes it susceptible to oxidation by aqueous medium, therefore a liposomal formulation of DHA would be ideal for oral administration, which is considered an appropriate route for chemoprevention studies.

In **Chapter 3**, we **hypothesize** that an ether lipid based nanoliposome delivery system of DHA would protect DHA from oxidation and fluctuation in pH of the gastrointestinal system, resulting in an ideal oral delivery system. Oral delivery is the appropriate route for chemoprevention studies. We further **hypothesize** that liposomal DHA will be more efficacious than free DHA at providing an anti-cancer effect *in vitro*, specifically in both estrogen receptor positive and estrogen receptor negative human breast cancer cell lines. Our results indicated that our liposomal formulation of DHA(size: 136nm, charge: -1.3mV) protected DHA from oxidation and acidic pH, as determined by dynamic light scattering. Our liposome also had a 60-80% encapsulation efficiency as determined by LC-MS/MS. Furthermore, liposomal DHA was found to be more efficacious at reducing cell viability, and increasing apoptosis in both MCF-7 and MDA-MB-231 cell lines. In addition, western blot analysis revealed that liposomal DHA was more efficacious at altering protein markers (P-AKT, P-S6, p21, cleaved PARP, BCL-2) of cell viability, the cell cycle, and apoptosis in human breast cancer cell lines in a manner consistent with chemoprevention.

We decided in **Chapter 4** to test our hypothesis that the oral administration of our acid stable liposome formulation of DHA would protect DHA from oxidation and acidic pH and thus increasing the bioavailability of DHA and its metabolites in the serum and at the mammary fat pad. To test our hypothesis, it was crucial to initially demonstrate whether our oral liposomal formulation was capable of delivering DHA and its metabolites to the circulation and mammary fat pad. Female rats (21-day old) were gavaged with liposomal DHA and sacrificed at 1, 3, and

6 hours and tissues and serum were collected at each time point. Fatty acids were extracted with a solution ethyl acetate/hexane and methylated using a solution of acetyl chloride, butylated hydroxytoluene, and methanol. Detection and quantification of DHA (expressed as % of administered dose) were achieved using Gas Chromatography Flame Ionization Detection(GC-FID), and liquid chromatography tandem mass spectroscopy(LC-MS/MS). The lipoxygenase (LOX) metabolites of DHA, known to play a role in anti-inflammation, were quantified using LC MS/MS. Following the oral administration of liposomal DHA, a significantly higher amount of DHA was found in the mammary fat pad (15-20%), 2-3 fold higher, than in the serum (4.5-6%). The LOX metabolites 4-hydroxy DHA, 14-hydroxy DHA, and 17-hydroxy DHA were also detected in both the serum (0.14%) and mammary fat pad (0.45%).

In summary, we demonstrated that high omega-3 fatty acids ratios(25:1) with and without tamoxifen were able to alter plasma proteins in a manner consistent with chemoprevention. Furthermore, we demonstrated for the first time the successful formulation of liposomal DHA for oral delivery of DHA. Our formulation protected DHA from acidic pH and oxidation and *in vitro* and *in vivo* results provided experimental data demonstrating the potential utility of liposomal DHA in the prevention of breast cancer initially in preclinical models and ultimately in the clinic. Future studies (detailed in **Chapter 5**) will be aimed at comparing the pharmacokinetics, pharmacodynamics and chemopreventive efficacy of liposomal DHA and free DHA in the rat.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

4E-BP1	4E Binding Protein 1
4-HDHA	4-Hydroxy docosahexaenoic acid
14-HDHA	14-Hydroxy docosahexaenoic acid
17-HDHA	17-Hydroxy docosahexaenoic acid
A-1B-G	Alpha-B-Glycoprotein
ADM	Active DHA Metabolites
ALA	Alpha-Linolenic Acid
AKT	Protein Kinase B
AIN	American Institute of Nutrition
ANG-1	Angiopoietin-1
ANOVA	Analysis of Variance
APO E	Apolipoprotein E
AR	Androgen Receptor
APP	Amyloid Precursor Protein
ATCC	American Type Culture Collection
AUC	Area Under the Curve
BAX	BCL-2 Association X Protein
BCL-2	B Cell Lymphoma Protein
BCPT	Breast Cancer Prevention Trial
BHT	Butylated Hydroxytoluene
BMI	Body Mass Index
BRCA1	BReast CAncer 1Protein
BRCA2	BReast CAncer 2 Protein
BSA	Bovine Serum Albumin
C15	Pentadecanoic Acid

C18	Nonpolar Silica Bead Outer Coating
CA 15-3	Cancer Antigen 15-3
CL-4B	Size Exclusion Beads
CPT1	Carnitine Palmitoyltransferase 1
CHCA	Alpha-cyano-hydroxycinnamic acid
CD8+	CD8 Molecular
CDK19	Cyclin Dependent Kinase 19
CE	Collision Energy
CL	Clearance
COX	Cyclooxygenase
CpG	Cytosine Guanine Nucleotide Repeats
Co	Concentration of Drug at Time 0
CYP	Cytochrome P450
CXP	Cell Exit Potential
D_5-DHA	Deuterated DHA
DC	Detergent Compatible Protein Assay
DCIS	Ductal Carcinoma In-situ
DE	Declustering Potential
DHA	Docosahexaenoic Acid
DLS	Dynamic Light Scattering
DMBA	7,12-Dimethylbenza(a)anthracene
DMEM	Dulbecco's Modified Eagle Medium
DMPC	Dimyristoyl phosphocholine
DOPE	1,2-dioleoyl-sn-glycero-3-3phosphoethanolamine
DOTAP	Dioleoxyloxy-3-trimethyl-ammonio-propane
DOTMA	Dioeoxy propyl trimethyl ammonium chloride

DPA	Docosapentanoic Acid
DPBS	Dulbecco's Phosphate Buffered Saline
EDP	Epoxydecosapentaenoic Acid
EDTA	Ethylenediaminetetraacetic acid
EET	Epoxyeicosatrienoic Acid
EF	Error Factor
EGCG	Epigallocatechin-3-gallate
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked Immunosorbent Assay
EMEM	Eagle's Minimum Essential Media
EP	Entrance Potential
EPA	Eicosapentanoic Acid
ER+	Estrogen Receptor Positive
ER-	Estrogen Receptor Negative
ERE	Estrogen Response Element
ERK	Extracellular Signal Regulated Kinases
EV	Electron Volt
FA	Fatty Acid
FADS1	Fatty Acid Desaturase 1
FADS2	Fatty Acid Desaturase 2
FASN	Fatty Acid Synthase
FATP	Fatty Acid Transporter Protein
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FDR	False Discovery Rate
FO	Fish Oil

FOXO1	Forkhead Box Protein O1
FXR	Farnesoid X Receptor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC-FID	Gas Chromatography Flame Ionization Detection
GcMAF	Macrophage Activating Factor
GPR120	G-Protein Coupled Receptor 120
HCL	Hydrochloric Acid
HDL	High Density Lipoprotein
Her2/neu	Receptor Tyrosine Protein Kinase erbB2
HIF-1α	Hypoxia Inducible Factor 1 Alpha
HPLC	High Performance Liquid Chromatography
HMEC	Human Mammary Epithelial Cells
HMGCR	3-hydroxy-3-methylglutaryl-Coa Reductase
IARC	International Agency for Research on Cancer
IC_{50}	Half Maximal Inhibitory Concentration
IGF-1R	Insulin Growth Factor 1R
IGg	Immunoglobulin G
IL-1	Interleukin 1
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-1B	Interleukin 1B
ITIH3	Inter-Alpha-Trypsin Inhibitor Heavy Chain 3
ITIH4	Inter-Alpha-Trypsin Inhibitor Heavy Chain 4
I.P.	Intraperitoneal
IPA	Ingenuity Pathway Analysis
iTRAQ	Isobaric Tagging for Relative and Absolute Quantitation

I.V.	Intravenous
JNK	c-Jun N-Terminal Kinase
KD	Knock down
KO	Knock out mice
Ki-67	Marker of Proliferation
LA	Linoleic Acid
LCC	Lewis Lung Carcinoma
LCIS	Lobelar Carcinoma In-situ
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrscopy
LDL	Low Density Lipoprotein
LGD1069	Rexinoid Agent
LPS	Lipopolysaccharide
LOX	Lipoxygease
MALDI TOF	Matrix Assisted Laser Desorption Ionization Time of Flight
MAPK	Mitogen Activated Protein Kinase
ME	Malate Dehydrogenase Enzymes
MFP	Mammary Fat Pad
MLV	Multilamellar Vesicles
MMTV	Mouse Mammary Tumor Virus
MPS	Mononuclear Phagocyte System
MRI	Magnetic Resonance Imaging
mTOR	Mammalian Target of Rapamycin
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium Bromide Salt
MNU	1-methyl-1-nitrosourea
N-3	Omega-3 Fatty Acid

N-6	Omega-6 Fatty Acid
NaOH	Sodium Hydroxide
NCBI	National Center for Biotechnology Information
NF-KB	Nuclear Factor Receptor Kappa Beta
NSABP	National Surgical Adjuvant Breast and Bowel Project
OIR	Oxygen Induced Retinopathy
OP	Obese Prone
P21	Cyclin Dependent Kinase Inhibitor 1
P27	Cyclin Dependent Kinase Inhibitor 1B
P70S6K	Ribosomal Protein S6 Kinase
P-ACC	Phosphorylated Acetyl Coa Carboxylase
PAI	Plasminogen Activator Inhibitor
PANTHER	Protein Analysis Through Evolutionary Relationships
PARP	Poly ADP Ribose Polymerse
P-AKT	Phosphorylated Protein Kinase B
P-AMK	Phosphorylated serine/threonine Protein Kinase
PBS	Phosphate Buffered Saline
PDGF	Platelet Derived Growth Factor
PI3K	Phosphoinositide 3 Kinase
PEG	Polyethylene Glycol
PGLA	Polylactic-co-glycolic acid
PK	Pharmacokinetics
PK	Plasma Kallikrein
PMSF	Phenylmethanesulfonylfluoride
PNM	Polymorphonuclear leukocytes
PPAR	Peroxisome Proliferator-Activated Receptor

PPRE	PPAR Response Element
PSEP	Proteomics System Performance Evaluation Pipeline Algorithm
PSI	Pounds Per Square Inch
PVDF	Polyvinylidene Fluoride
RES	Reticuloendothelial System
RPM	Revolutions Per Minute
RXR	Retinoid X Receptor
SCID Mice	Severe Combined Immunodeficiency Mice
SDS	Sodium Dodecyl Sulfate
sHE	Soluble Epoxide Hydroxylases
SIRT	Sirtuin Protein
SD	Sprague Dawley Rat
SERM	Selective Estrogen Receptor Modulator
SREBP-1	Sterol Regulatory Element Binding Protein
STAR	Study of Tamoxifen and Raloxifene
SCX	Strong Cation Exchange
$T_{1/2}$	Half-life
TAG	Triacylglycerol
TEAB	Triethylammonium Bicarbonate Buffer
TEB	Terminal End Buds
TEER	Trans epithelial electric resistance
TEM	Transmission electron microscopy
TF	Thomas-Friedenreich Antigen
TNF-α	Tumor Necrosis Factor Alpha
TSA	Trichostatin A

uPA	Urokinse-dependent plasminogen activator system
TP53	Tumor Protein P53
V_d	Volume of Distribution
VDBP	Vitamin D Binding Protein
VEGF	Vascular Endothelial Growth Factor
VLDL	Very Low Density Lipoprotein
ZP	Zeta Potential

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Chapter 1

Introduction

Etiology of Breast Cancer and Current and Alternative Approaches for Disease Management

1. Breast Cancer

1.1 Breast Cancer Statistics

Breast cancer is the second leading cause of cancer death in women [1]. In the United States, approximately 231,840 cases of invasive breast cancer are expected to be diagnosed in 2015, with 40,290 of these diagnosed women ultimately dying from the disease [1]. It is expected that 1 in every 8 women(12.5%) will be diagnosed in her lifetime with breast cancer in the United States, a country with one of the highest lifetime risk in the world attributed to lifestyle and reproductive factors[2]. African American women are less likely than white women to get breast cancer but more likely to die from it[3]. Furthermore, African American women are about three times more likely to develop the highly aggressive triple-negative breast cancer, which occurs more often in younger women[3]. Triple negative breast cancers count for 20% of the breast cancers diagnosed and are classified as lacking the estrogen receptor, progesterone receptor, and her2/neu receptors, making them impossible to target and very aggressive[4].

In the world, breast cancer is the most frequently diagnosed cancer in women [1]. The incidence of breast cancer has increased at a rate of 3.1% annually from 1980 to 2010 which are thought to be contributed to an increasing population, specifically an aging population [5]. In 2012 the International Agency for Research on Cancer(IARC) determined that breast cancer is common in both developed and less developed countries with 794,000 and 883,000 cases expected, respectively. Since 1985 the breast cancer mortality rate in developed countries has

been gradually increasing in middle income countries such as China, Japan, and Brazil due to these countries adopting a Western lifestyle[3]. However, with access to up to date healthcare women in developed countries can benefit from cutting edge treatments[3]. In poorer underdeveloped counties there has been a drastic increase in breast cancer which is attributed to minimal access to health care and one-size-fits-all regimen of chemotherapy and surgery options[3].

1.2 Etiology of Breast Cancer

A vast body of literature describes risk factors for breast cancer and proposes hypotheses for its etiology based on epidemiological and experimental studies[6]. It has become increasingly evident that endogenous natural estrogens and exogenously ingested estrogenic agents are associated with breast cancer risk. This view is supported by epidemiological studies relating prolonged estrogen exposure as a breast cancer risk, such as early menarche, late age at menopause, late age at first full-term pregnancy, nulliparity, absence of lactation, obesity, higher socioeconomic status, and post-menopausal hormone replacement therapy. The most compelling evidence for the role of estrogen induced breast cancer is derived from the protective role of the anti-estrogen tamoxifen in women considered at increased risk for breast cancer[7, 8]. Other risk factors including ionizing radiation, dietary fat, and alcohol intake, have been shown to be associated with breast cancer[6].

Although a woman has a higher chance of developing breast cancer at an older age(>40); the disease tends to be more aggressive in younger women(<40)[9]. The disease is more aggressive in younger women because they seem to have less hormone sensitive tumors that can metastasize to the lymph nodes[10]. Younger women(≤30) with breast cancer typically have an increased familial risk which can be associated with mutations in *BRCA1* and *BRCA2* genes. In

addition, genetic mutations including, *TP53* mutations, have been found in 50% of women with strong family histories, while only evident in 10% of women with no family history[11].

Lifestyle factors, such as obesity, influence a woman's chance of developing breast cancer. Obesity has been linked to postmenstrual women developing breast cancer, but overall the conclusions from numerous cohort and case studies have led to mixed results[12]. The difference in breast cancer incidence amongst some of these studies is thought to be contributed to the variation in the age and race of the women, method of Body Mass Index (BMI) collection, and hormone usage.

Obese women have more adipose tissue that can produce estrogen by the aromatase enzyme in localized regions such as the breast tissue.[13]. In addition, TNFα and IL-6 can be secreted by these adipocytes and increase the production of aromatase by promoting paracrine and endocrine pathways, thus increase estrogen and promote ER+ breast tumors[14].

2. Current Disease Management and Treatment Options

2.1 Screening for Breast Cancer

The American Cancer Society recommends that women over the age of 40 should participate in a mammography yearly, while self-examinations should be conducted beginning at the age of 20. Breast MRI and ultrasound are other ways to screen for breast cancer [15, 16]. Screening for breast cancer is crucial at a young age because aggressive subtypes of breast cancer are found more common in young women and this correlates with an increased risk of dying from the disease[17-19].

2.2 Treatment of Breast Cancer

Surgery

There are different types of surgery for different stages of breast cancer. These types include lumpectomy, partial, total, and radical mastectomy[20]. For a lumpectomy the tumor and surrounding breast tissue are removed, while a partial or total mastectomy requires the removal of one or both breasts[21]. For a radical mastectomy the removal of the breast and surrounding lymph nodes is conducted to prevent further spread of the disease[22].

Radiation Therapy

Radiation therapy consist of concentrating high energy x-rays(external beam radiation) or radioactive substances(brachytherapy) at the site of the cancer, usually at the tumor site[21]. Brachytherapy consist of implanting radioactive pellets, balloons, or tubes in the body cavity where the cancer is present and this is known as intracavitary radiation. The implant can also be administered near the cancer but not in a cavity in the body and this type of brachytherapy is known as interstitial radiation[21]. In most cases radiation therapy has been shown to reduce local recurrence of breast cancer when given after surgery by as high as 70%[23]. Advantages of external beam radiation include non-invasiveness of the therapy and homogenous dose distribution[24]. Multicatheter brachytherapy, the most popular radioactive invasive therapy, utilizes radioactive beads in a balloon at the site of tumor growth. A disadvantage is that infections from the placement of the beads occurs in about 10% of the population who uses them[25].

Chemotherapy

Chemotherapy is the use of cytotoxic anti-neoplastic drugs to inhibit cell growth, particularly in cancer cells. Neoadjuvant therapy, or the administration of chemotherapeutics

before surgery has been employed in clinical trials to determine its benefits over drug administration after surgery, known as adjuvant therapy[26, 27]. In the National Surgical Adjuvant Breast and Bowel Project, Protocols B-18 and B-27 (NSABP B-18 and B-27) studied the effects of chemotherapeutics doxorubicin and cyclophosphamide when given to women with breast cancer before and after surgery, respectively. The primary end points focused on disease free survival and overall survival, comparing both amongst patients who received adjuvant and neoadjuvant therapy. In both groups overall survival and disease free survival were found to be the same.[27].

3. Current Chemoprevention Strategies for Breast Cancer

3.1 Selective Estrogen Receptor Modulators and Breast Cancer Prevention

Chemoprevention is defined as the use of pharmacologic or natural agents that can be applied at any time in the multi-step carcinogenesis process before the detection of invasive disease in order to inhibit, delay, or reverse this process.[28]. There are currently 2 million women in the United States that are at high risk for breast cancer, thus prevention strategies are needed. Selective estrogen receptor modulators (SERMS) have been an option for chemoprevention in women that are high risk for breast cancer. The clinical trial initiated by the National Surgical Adjuvant Breast and Bowel Project known as the Breast Cancer Prevention Trial (BCPT P-1)[7] in women at high risk for breast cancer showed that the SERM, tamoxifen, prevented the development of invasive and non-invasive breast cancer by 49% and 50%, respectively, in comparison to placebo. In addition, there was a reduction in estrogen receptor positive cancer by 69%, but no effect on triple negative breast cancers. However, promising as the study may seem, tamoxifen did increase the risk of developing endometrial cancer. The Study of Tamoxifen and Raloxifene; (STAR P-2) [8] was an additional clinical trial where the

effects of tamoxifen were compared with raloxifene in high risk postmenopausal women, in an effort to determine if raloxifene would have similar effects as tamoxifen but with less endometrial cancers as a side effect.

The STAR Trial P-2 was a chemoprevention study based on having three decades of supportive data, both clinical and pre-clinical trials, on the effects of tamoxifen on early and advanced breast cancer [29, 30]. This study focused on the effects of tamoxifen(20mg/day) and raloxifene(60mg/day) in postmenopausal women with a high risk for breast cancer. Breast cancer risk was determined by the Gail Model Assessment that included a combination of risk factors such as age, hormone usage, personal history, breast cancer history, and cardiac health over a duration of five years. The end points of this study were invasive breast cancer, with secondary endpoints of endometrial cancer, and thromboembolic events, since it was determined in the NBCT P-1 that tamoxifen increased the occurrence of these undesirable secondary endpoints [7].

Following the five year trial, an 81-month follow-up was conducted to determine the effects tamoxifen and raloxifene on invasive and noninvasive breast cancer risk[31, 32]. Tamoxifen was more effective than raloxifene at preventing invasive breast cancer. Tamoxifen reduced the risk of developing invasive breast cancer by 50%, while raloxifene reduced the risk of invasive breast cancer by 39%. Tamoxifen was also more effective than raloxifene at preventing non-invasive breast cancer. Tamoxifen reduced the risk of non-invasive breast cancer by 50%, while raloxifene reduced the risk of non-invasive breast cancer by 38%[31]. As for secondary endpoints raloxifene was shown to have 45% less cases of endometrial cancers as compared to the tamoxifen group, while there was a significant decrease of 25% in thromboembolic events in the raloxifene group compared to the tamoxifen group[31, 32]. Thus, this study determined that raloxifene could be utilized as a chemopreventive agent for women

that were high risk for breast cancer, and result in similar protection against invasive breast cancer as tamoxifen with less side effects.

3.2 Omega-3 and Omega-6 Fatty Acid Chemistry and Metabolism

Omega-3 fatty acids have been studied in an effort to understand their ability to benefit human health. Specifically, their protective role in hormone-related cancers such as breast and prostate have been evaluated in epidemiological studies, and in pre-clinical systems [6].

The conventional chemical nomenclature is to systematically number the carbon atoms from the carboxyl-terminal end group **(Figure 1.1)**. The carbon atoms numbered two and three from the carboxyl group are referred to as the alpha(α) and beta(β) carbons, respectively. The last carbon is the omega(ω), or n-carbon(η), and the position of a double bond is indicated by the symbol Δ, followed by a number. For example, Δ^9 refers to a double bond between carbon atoms 9 and 10 from the carboxyl group. However, an accepted practice in describing the chemical

structure of fatty acid molecules is to start numbering at the methyl group (ω- or n-).

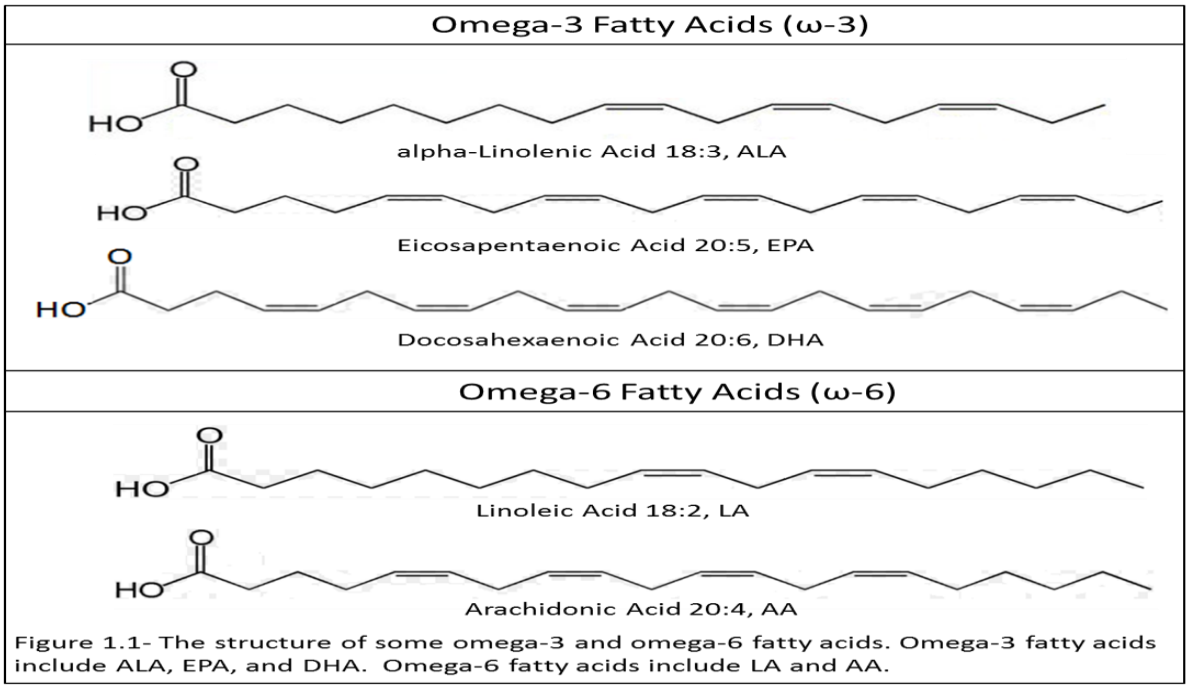


Figure 1.1- The structure of some omega-3 and omega-6 fatty acids. Omega-3 fatty acids include ALA, EPA, and DHA. Omega-6 fatty acids include LA and AA.

The omega-3, ω-3, (or n-3) and omega-6, ω-6, (or n-6) polyunsaturated fatty acids can not be synthesized by mammals, and because they must be obtained from exogenous sources, such as the diet, they are referred to as "essential fatty acids". The diet must provide them because in mammals the desaturase needed to synthesize a double bond in the Δ^{12} is lacking. Omega-3 fatty acids are represented by α-linolenic acid(C18:3 n-3, all-cis-9,12,15-octadecatrienoic acid, ALA), and omega-6 fatty acids are represented by linoleic acid(18:2 cis-9,cis-12-octadecadienoic acid, LA). Both ALA and LA are metabolized to longer chain fatty acids, largely in endoplasmic reticulum of the liver[33]. ALA is converted to eicosapentaenoic acid(C20:5, n-3, all-cis-eicosa-5,8,11,14,17-pentaenoic acid, EPA)and then to docosahexaenoic acid(C22:6, n-3, all cis-docosa-4,7,10,13,16,19-hexaenoic acid, DHA) in the peroxisomes, while LA is the metabolic precursor of arachidonic acid **(Figure1.2)**. The omega-6 fatty acids utilize

the same desaturases and elongases in the endoplasmic reticulum [6, 34], however such enzymes have a higher affinity for the omega-3 fatty acids in comparison to the omega-6 fatty acids. DHA can be further metabolized by lipoxygenases to form metabolites, such as 4-hydroxy-DHA, which has been shown to be involved in halting the promotion of angiogenesis[35]. The main sources to obtain omega-3 fatty acids are plants and aquatic life such as walnuts, chia seed, seaweed, and fish. Sources of omega-6 fatty acids include soy, corn, and safflower.

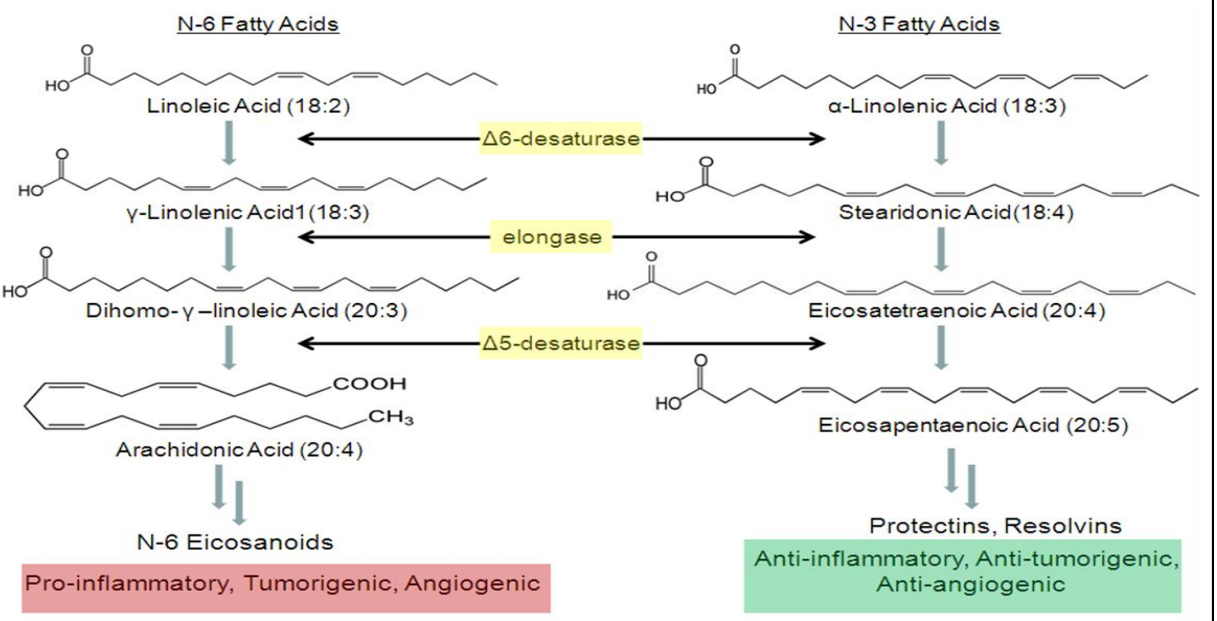


Figure 1.2- ω-3 and ω-6 fatty acid metabolism in mammals (selected molecules)

Currently, the Western diet contains omega-6:omega-3 ratio of 15:1[6]. The increase of omega-6 fatty acid in the diet is thought to contribute to the increase in development of chronic diseases [36]. It is hypothesized that increasing the omega-3 content not only allows for the competition of metabolic enzymes but also allows for omega-3 fatty acids to occupy the sn-2 position of phospholipids in cell membranes, making them more susceptible to cleavage by phospholipase A2. This cleavage results in the fatty acid to be metabolized further into

metabolites that can be protective against the development of certain cancers such as breast cancer[6].

Metabolic enzymes for omega-3 and omega-6 fatty acids include cycloxygenase(COX), lipoxygenase(LOX), and cytochrome P450 epoxygenases(CYP). The COX and LOX enzymes metabolize omega-3 and omega-6 fatty acids into metabolites with opposing effects on inflammation, cell proliferation, and apoptosis (anti-tumorigenic with omega-3, neutral to pro-tumorigenic with omega-6). Thus, the overall cellular abundance of omega-3 vs. omega-6 fatty acids can have a major impact on carcinogenesis [37-41]. The metabolism of omega-3 fatty acids by COX and LOX enzymes generates 3-series prostaglandins[41, 42] and leukotrienes[35] as well as mono-hydroxylated derivatives(e.g. active DHA metabolites, ADM) protectins, and resolvins[35, 43] which have anti-inflammatory and anti-angiogenic effects.

Omega-3 and omega-6 fatty acids are also substrates of cytochrome P450(CYP) epoxygenases which convert them to epoxy signaling lipids including epoxyeicosatrienoic(EET) acids derived from n-6 arachidonic acid and epoxydocosapentaenoic(EDP) acid from DHA[44-46]. DHA can compete with arachidonic acid metabolism by CYP epoxygenases leading to replacement of EETs with EDPs *in vivo*[45, 47, 48]. Previous reports have shown the contrasting role of EDPs and EETs in inflammation, angiogenesis, and tumorigenesis[48-56];the CYP epoxygenases and soluble epoxide hydrolases(sEH) pathway plays a critical role in mediating the anti-angiogenic and anti-tumorigenic effects of omega-3 fatty acids[57].

3.3 Omega-3 Fatty Acids and Breast Cancer Prevention: Epidemiological Studies

Case-control studies and cohort studies established a relationship between omega-3 fatty acids and the risk of developing breast cancer. Some case-control studies investigating the relationship between fish consumption and breast cancer risk have not shown a clear association

between fish consumption and breast cancer risk across many fish consuming countries[58, 59]. Fish is food source rich in omega-3 fatty acids making fish consumption an indicator of omega-3 intake. In case studies where biomarkers of fatty acid intake have been measured; the serum, adipose tissue, and erythrocyte membrane results have been weakly supportive of an inverse association between an increase in specific dietary omega-3s(DHA, EPA) and decreasing breast cancer risk [60-62]. Alpha-linolenic acid(ALA), a precursor of DHA and EPA has also been evaluated and has shown a weak association with decreasing breast cancer risk [63] [64].

Cohort studies in addition to case-control still have not made a strong inverse association between omega-3 fatty acids and breast cancer development[65, 66]. In addition, studies examining various omega-3:omega-6 ratios have been weakly supportive of an association with a decrease in breast cancer risk[67].

Overall, both case-control studies and cohort studies have not been able to identify a clear association with omega-3 fatty acids and risk of developing breast cancer. The many inconsistencies in omega-3 consumption across studies, and genetic variance in the populations studied could attribute to this inconclusiveness[6].

3.4 Omega-3 Fatty Acids and Breast Cancer Prevention: Pre-clinical Studies

In vivo

Most *in vivo* models to study the protective role of omega-3 fatty acids against the development of breast cancer have used the 1-methyl-1-nitrosourea(MNU) or 7,12-dimethylbenz(a)anthracene(DMBA) to induce breast cancer in the female Sprague Dawley rat[68, 69]. Typically, the carcinogen can be given at 21 or 50 days of age by intraperitoneal injection in the case of MNU, or by intragastric tube in the case of DMBA. It is hypothesized

that 60%-90% of the tumors that develop in these rat models are estrogen receptor positive (ER+)[70].

The results from most *in vivo* studies were in general supportive, indicating a protective role of omega-3 fatty acids against breast cancer development, but there were still many inconsistencies that made comparisons among various studies difficult. Signori, et al., did an extensive comprehensive review of studies involving fish oil [34]. This review revealed several confounding factors which may account for the inconsistencies reported *in vivo.* These factors include omega-3 fatty acid content of the diet, omega-3:omega-6 fatty acid ratio, type of omega-3 acids, calories from fat, timing of diet administration, and genetic variability in the fatty acid desaturase enzymes (FADS1 and FADS2)[34]. In an attempt to standardize some of the confounding variables in *in vivo* research and to clarify the role of omega-3 fatty acids as a chemopreventive Zhu et al., [71] utilized the 21-day old MNU Sprague Dawley rat model of breast carcinogenesis to evaluate various omega-3:omega-6 fatty acid ratios including 25:1, 10:1, 5:1, 1:1, 5:1, 10:1, 25:1 in the diet with and without tamoxifen (1.0 mg/kg diet) given ad libitum[71]. The fatty acid composition contains 10% saturated, 10% monounsaturated, and 10% polyunsaturated to meet the requirements of the recommended daily guidelines according to the Food and Drug Administration (FDA) [72]. The outcome of the study demonstrated that the high omega-3:omega-6 ratio, specifically 10:1 and 25:1 exerted a chemopreventive effect on breast cancer in the rat. The 10:1 omega-3:omega-6 ratio only resulted in a marginally significant decrease in cancer incidence, multiplicity, and burden with or without the addition of tamoxifen, while the 25:1 omega-3:omega-6 ratio with and without tamoxifen, resulted in a significant decrease in cancer incidence, multiplicity, and burden[71].

To elucidate the mechanisms that could account for the decrease in mammary cancer by a high omega-3:omega-6 fatty acid ratio Jiang et al, analyzed the protein profile by western blot analysis of the mammary tumors in the 25:1 in comparison to the 1:1, omega-3:omega-6 [73]. In the animals given 25:1 omega-3:omega-6 cell proliferation was reduced by 60% according to Ki-67 analysis. The reduction in cancer incidence by the 25:1 omega-3:omega-6 fatty acid ratio was further supported by a significant reduction in cyclin D1, and an increase in p21, and p27 proteins. On the basis of the results of protein expression, it was hypothesized that the tumor cells analyzed were arrested in G1/S transition of the cell cycle. The apoptotic index was increased significantly in the high omega-3:omega-6 tumors, and was further supported by a significant increase in Bax/BCL-2 ratio and cleaved PARP. Pathways involved in lipid metabolism, inflammation, and protein synthesis were also altered in the tumors of rats given the 25:1 omega-3;omega-6 ratio. Fatty acid synthetase, HMGCR, and SREBP-1 were all decreased indicative of a decrease in cholesterol synthesis and cell growth. Transcription factors HIF-1α, SIRT-1, and FOXO1(Thr24) were all decreased suggesting a decrease in inflammatory pathways. Proteins in the mTOR pathway, AKT(Ser473, P70S6K(thr389), and 4E-BP1(thr37) were all decreased indicating a decrease in mRNA translation and protein synthesis. Overall, these studies provided important insight on the cellular and molecular mechanism that can account for the protective effects of high omega-3:omega-6 fatty acid ratio on breast cancer.

Because of the significant protective effect of the high n-3:n-6 ratios, we hypothesized that a particular component(s) in fish oil(DHA, EPA) may account for the anticancer effects *in vivo.* Thus, in a 50-day old MNU Sprague Dawley rat model of mammary carcinogenesis Yuri et al., administered a diet consisting of 10% DHA, 10% EPA and 10% EPA+DHA ad libitum[68]. After 20 weeks 10% DHA was superior to 10% EPA and 10% EPA+EPA and significantly

reduced tumor incidence and multiplicity. Specifically, tumor incidence was reduced to 23%, 73%, and 65% in the DHA, EPA, and EPA+DHA groups, respectively. Tumor multiplicity was reduced to 0.23, 1.67, and 1.59 in the DHA, EPA, and EPA+DHA groups, respectively.

In a study by Noguchi et al., low amounts of DHA administered with a high fat diet still reduced tumor incidence in a mammary carcinogenesis model [69]. The study utilized a 50-day old Sprague Dawley rat and DMBA as the carcinogen to induce of mammary carcinogenesis. Thirty rats per group were given a high-fat(20% corn oil) or low-fat diet(0.5% corn oil) with 0.5mL DHA ethyl ester, EPA ethyl ester, or coconut oil twice a week via an intragastric tube, for 20 weeks. Tumor incidence in both the EPA and DHA groups were significantly decreased by 58% and 75%, respectively.

Finally, DHA metabolites have also been of interest, but up until this point only 4-hydroxy docosahexaenoic acid and 7-hydroxy docosahexaenoic acid have been investigated in CL57BL/6 knock-out mice for 5-LOX and wild-type controls[35]. A mouse model of oxygen-induced retinopathy(OIR) has indicated that the 5-LOX conversion of DHA to 4-hydroxy-docosahexaenoic acid attributed to a decrease in neovascularization in the retina, considering that 5-LOX$^{-/-}$ had lower amounts of the metabolite and more neovascularization. In addition, 4-hydroxy-docosahexaenoic acid was superior at activating PPARγ and decreasing angiogenesis in endothelial sprouting assays than at similar concentrations of 7-hydroxy docosahexaenoic acid. This particular study emphasized that DHA metabolites are responsible for the receptor activation and decreased angiogenesis, rather than the parent compound, DHA[35]. However, DHA metabolites have yet to be examined in cancer prevention.

In vitro

The omega-3 fatty acids, DHA and EPA, have been studied as potential inhibitors of cancer cell growth in breast cancer cell lines[74-79]. DHA has been shown to be superior agent to EPA at preventing cell growth in both estrogen-receptor positive(MCF-7) and estrogen receptor negative forms of breast cancer(MDA-MB-231,MDA-MB-435)[75-77]. The mechanism of cell growth inhibition has been attributed to a decrease in cell respiration in both cell lines[75-77], an increase in cell cycle arrest at G0/G1[78], and a decrease in cell cycle proteins cyclin A, B, and CDK19[78]. The triple negative MDA-MB-231 cells were shown to be more sensitive to DHA than the ER+ MCF-7 cells in some studies[77], but not in one study when cell proliferation was evaluated [75].

DHA has been shown to be a superior inducer of apoptosis in breast cancer cell lines, in comparison to EPA. A study by Kang et al[75], demonstrated that 25uM DHA after 72 hours of treatment induced apoptosis by reactive oxygen species formation that lead to activation of caspases-8 in MCF-7 cells. In MDA-MB-231 cells 100uM DHA after 24 hours lead to an increase of caspases-3 activation [76]. A study by Sun et al.[80], demonstrated that a glycoprotein on the surface of MCF-7 cells, specifically, glycoprotein syndecan-1(SDC-1) increased apoptosis through the interaction of a PPAR responsive element(PPRE) with the syndecan-1 gene promoter. The increased expression of syndecan-1 is thought to result in deactivation of pathways involving MAPKinase, MAPkinase kinase, and Bad, leading to apoptosis in the MCF-7 and SK-BR-3 cell lines [81].

Overall, the *in vitro* studies in various breast cancer cells showed a more protective role of a specific omega-3 fatty acid, docosahexaenoic acid(DHA). The most common pathways

studied to identify certain omega-3 fatty acids as chemopreventives were cell viability and apoptosis.

DHA has a protective role against breast cancer as determined by the *in vitro* and *in vivo* studies. To understand the protective role of DHA it is important to know the biodistribution of DHA when given orally. In a study by Yuri, et al[68] female Sprague Dawley rats were given 9.5g/100g diet of DHA or EPA, or both (4.75g DHA/EPA/100g diet) ad libitum for 20 weeks. To quantify the amount of DHA in the serum and mammary fat pad gas chromatography flame ionization detection (GC-FID) was used. At the end of 20 weeks approximately, 30% of the fatty acid composition of both the serum and mammary fat pad were composed of DHA in the rats fed only DHA. In rats given EPA approximately 2% and 0.5% of the fatty acid composition was DHA in the serum and mammary fat pad, respectively, illustrating that EPA was minimally converted to DHA. The rats given both EPA and DHA had 19% and 23% of their fatty acid composition as DHA, in the serum and mammary fat pad, respectively. Overall, it seems that DHA when given orally favors the breast tissue, possibly due to the breast tissue being mainly composed of fat, however percentage can vary[82].

4. Drug Delivery Systems of Chemopreventive Agents

4.1 Types of Liposomes: Conventional, Stealth, Cationic, and Immunosomes

A liposome is defined as a lipid bilayer of natural or synthetic phospholipids that forms a spherical microscopic vesicle. Liposomes contain a hydrophilic interior and a hydrophobic bilayer and are utilized in the delivery of molecules for the treatment or diagnosis of disease. These microscopic vesicles can be conventional or stealth liposomes, depending on the individual components of the formulation. Conventional liposomes can be composed of neutral phospholipids, such as phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin [83,

84]. Another hydrophilic component of most liposomes, cholesterol, allows for an increase in negative charge to avoid aggregation, an increase in encapsulation efficiency, and fusogenic behavior[85, 86].

Other types of liposomes include stealth liposomes, targeted liposomes such as immunosomes, and cationic liposomes. Stealth liposomes are unique in that they are utilized to have a longer circulation half-life in the vasculature than conventional liposomes. The longer circulation life is achieved by grafting polyethylene glycol (PEG) onto the phospholipid head group of the lipid membrane. The outer layer of this polymer on the liposome protects the liposomes from blood proteins such as oposins, lipases, and other lipoproteins, that have the ability to bind and destabilize the liposomes before it reaches its intended delivery site. In addition, the PEG inhibits the body's immune system in the form of the reticuloendothelial system, such as macrophages and Kupffer cells of the liver and spleen, from uptake and removing the liposomes from circulation. Differential scanning calorimetry studies [87] and dynamic light scattering have shown that the percentage and molecular weight of PEG can affect the phase transition and lead to the formation of a single lipid layer, known as a micelle. Greater than 40 mol% of PEG-350 caused an interdigitated gel phase bilayer instead of a liquid crystalline bilayer above the main phase transition temperature [87], while the same amount of PEG-2000 has shown to form micelles due to the steric hindrance of PEG [88]. The stealth activity of the liposome can be attributed to the interactions of the polymer chains on the outside and inner space of the membrane. The polymer on the outside of the membrane produces a steric hindrance and prevents large macromolecules from binding to the liposome, while polymer chains in the inner membrane produce an internal repulsive pressure that inhibits transport of small molecules across the liposome, preventing liposome rupture [87, 89] [88].

Targeted liposomes, are similar to stealth liposomes in that they typically contain PEG as a scaffold to bind targeting agents such as proteins or antibodies directly to a liposome. Liposomes can be effective at targeting cells of interest when targeting ligands are bound to PEG, rather than the lipids themselves [90]. Targeting ligands include antibodies, peptides, vitamins, and growth factors [91, 92]. For tumor delivery antibodies have been used to target receptors for internalization by endocytosis. Protein fragments such as transferrin have been bound to liposomes in an attempt to target the transferrin receptor on tumors [93, 94]. Folic acid, bound to liposomes has been successful at targeting tumor cells, resulting in folate mediated endocytosis as a method to delivery doxorubicin [95, 96]. Growth factors such as epidermal growth factor(EGRF) have been bound on the outside of liposomes for targeting cancer cells that have an over-expression of this receptor on their outer surface [97].

Cationic liposomes are vesicles that carry a positive charge [98] and are composed of positively charged surfactants as dioleoxy propyl trimethyl ammonium chloride(DOTMA) and dioleoxyloxy-3-trimethyl-ammonio-propane(DOTAP), however, neutral lipid such as 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine(DOPE) and the addition of cholesterol can lead to unilamellar liposomes [98]. These liposomes are utilized in the transfection of cells in-vitro with DNA. Liposomes have been employed in gene therapy with the delivery of antisense nucleic acids and DNA in various complexes [88].

4.2 Archaeosomes as Delivery Systems

Archaea is a type of prokaryote that can tolerate harsh environments [99]. Such types of archaea include the hyperthermophiles, thermoacidophiles, extreme halophiles, and methanogenic bacteria. Some, but not all archaea, coexist with other marine life in the deepest waters of the Arctic and Antarctic Oceans [100]. At such depths these microorganisms

encounter harsh conditions such as high temperature, acidity, and pressure. The survival of archaea in this unique environment can be attributed to their lipid structure in their cell membrane.

The lipid structure of archaeobacteria is like most lipids which consist of a hydrophobic nonpolar hydrocarbon chain attached to a polar head group, however, the nonpolar carbon chain is typically composed of branched phytanyl chains, 5-carbon repeating unit, linked to the sn-2,3 carbons of the glycerol backbone by ether bonds [101]. Diversity in these hydrocarbon chains can include cyclopentane rings and caldarchaeols [102].

In vitro studies focusing on archaeosome stability have determined that the unique lipids can be utilized into a liposome formulation that can have properties of thermal[103], serum[103], and pH stability[103-107]. These archaeosomes can have additional stability against biological processes as lipase attack[103]and bile salt emulsion[107]. Furthermore, archaeosome lipids have been attempted to be synthesized in the laboratory, making certain types of lipids commercially available and pure [108]. Recently, archaeosomes have been utilized in vaccine therapy, and drug delivery. Krishnan L. et al., have demonstrated that C57BL/6 mice could be immunized with a *Methanobrevibactor smithii* archaeosome formulation encapsulating antigenic melanoma peptides against B16 tumor cells injected subcutaneously [109]. The mice given archaeosome with peptides lived significantly longer with slower growing tumors and higher CD8+ T cell counts than control mice, indicating that archaeosomes could be utilized for adapting the immune system to inhibit certain cancers.

Archaeosome have also been applied to drug delivery to improve the therapeutic index of well-known anticancer agents for breast cancer[110, 111]. When MCF-7 cells were treated with archaeosomes containing Paclitaxel, the IC_{50} was 50% lower than with Paclitaxel alone,

illustrating that the liposome delivery system was more efficacious[110]. The unique properties of archaeosomes have made them attractive for the development of oral delivery systems. Considering their resistance to phospholipase attack and low pH [103-107], they have been employed *in vivo* models by the oral administration[112-114]; some formulations were found to increase their pay load in the blood significantly, making archaeosomes an attractive vehicle for the oral administration of anticancer and adjuvant therapies. Most formulations when given orally in mice were found not to be toxic[112].

4.3 Nanoparticles in Chemoprevention

Many nanomaterials and delivery systems have been utilized in pre-clinical and clinical chemotherapeutic and prevention such as liposomes as illustrated in **Figure 1.3**[115]. As compared to pharmaceutical agents natural bioactive compounds are more appealing in the arena of chemoprevention. Natural bioactive compounds include curcumin [116-118], resveratrol [119-121], epigallocatechin-3-gallate(EGCG) [122-124] and luteolin [125, 126]. Although, effective when given by gavage, most compounds need to be administered in high doses since their lipophilicity makes them poorly soluble in the aqueous environment [127], which decreases their bioavailability in pre-clinical models. Nanodelivery systems have provided a solution for the effective delivery of lipophilic compounds such as curcumin[128, 129], resveratrol[130, 131], EGCG[132, 133], and luteolin[134].

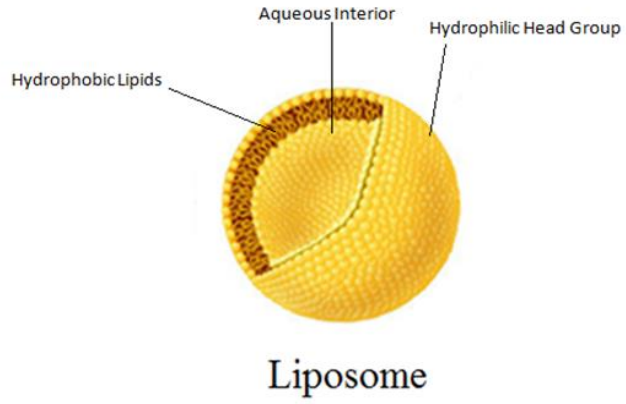


Figure 1.3- The Organization of a Liposome

Curcumin, a bioactive component found in *Curcuma longa*, has been classified as a potent anti-inflammatory agent due to its inhibition of the NF-kB pathway[135]. The compound is also a potent inducer of apoptosis[136] and has been shown to be effective at inhibiting benzo[a]pyrene-induced forestomach tumors in mice[137]. However, even though it has many roles in preventing carcinogenesis, curcumin has disadvantages such as poor solubility in an aqueous environment, and can be rapidly metabolized by the liver, limiting its bioavailability. To address these issues drug delivery systems have been developed to improve solubility and bioavailability of some chemopreventive agents.

Nanoparticles consisting of polylactic-co-glycolic acid(PLGA) have been developed for various routes of administration to deliver curcumin [138, 139]. Shaikh et al., determined that curcumin given by oral administration in a PLGA nanoparticle increased its bioavailability by 9-fold in the plasma of Sprague Dawley rats[138]. An increase in bioavailability was also seen the

plasma in a Balb/c mouse model when PLGA nanoparticles were given intravenously[139]. For parenteral administration, liposome formulations of curcumin consisting of conventional lipids have also been developed. In a xenograft model of head and neck squamous cell carcinoma, parenteral administration of liposome curcumin reduced the tumor mass, while decreased phosphorylation in the NF-kB and p-AKT pathways [140]. Intraductal administration of curcumin nanoparticles(Nanocurcumin) has also been successful at preventing tumor formation in the MNU-rat model of breast cancer[141]. When given intraductally into the mammary gland Nanocurcumin at doses of 2mg/12 mammary glands(per rat), there was a significant reduction in mammary tumors in comparison to rats administered free curcumin intraductally[141].

Resveratrol, a phytoalexin found in grapes, has been shown to inhibit proliferation and promote cell death in various types of cancer[142, 143]. However, its disadvantages as a chemopreventive, are poor solubility and bioavailability. PLGA nanoparticles containing resveratrol were shown to increase its solubility, leading to improved uptake as compared to the free compound[130]. In addition, liposomal resveratrol was determined to be a possible chemopreventive agent for prostate cancer in prostate specific PTEN-KO mice[144]. Also, in male B6C3F1/J mice, liposomal resveratrol was more efficacious than resveratrol alone at increasing the bioavailability of resveratrol in the serum and prostate tissue[144]. When liposomal resveratrol was administered together with liposomal curcumin, the combination was more effective at the increasing bioavailability of resveratrol and curcumin in the serum and prostate tissue than either liposome formulation alone[144].

Epigallocatechin-3-gallate(EGCG), a polyphenol component of green tea, is an inhibitor cell growth in many types of cancers[145-147]. In an attempt to eliminate toxicity and enhance bioavailability of EGCG Siddiqui et al., have developed a PLGA based nanoparticle for the

delivery of the compound to prostate cancer cells[148]; nano-EGCG produced the same significant decrease in cell proliferation of PC3 cells at a dose 10-fold lower than free EGCG. The nano-EGCG also significantly increased BAX, cleaved PARP, p21, and p27 markers of apoptosis and cell viability at a 10-fold lower dose than free EGCG. In a 22Rv1 xenograft nude mouse model an injected dose of 100µg nano-EGCG was more efficacious at reducing xenograft volume, decreasing prostate antigen protein, and increasing bioavailability of ECGC than 1mM of EGCG alone. In a nude mouse xenograft model of melanoma(Mel 928 xenograft) 100µg nano-EGCG given by oral intubation was more efficacious than 100 mg of free ECGC at reducing tumor volume and increasing BAX, p21, and p27, and cleaved PARP in the xenografts[149].

Luteolin, a glycosylated flavonoid that is found in green vegetables such as broccoli and celery, is known as a natural anti-inflammatory and anti-angiogenic agent [134]. In lung cancer luteolin displayed anticancer effects such as inducing cell cycle arrest [150] and increasing apoptosis [151, 152]. Luteolin also exerted similar effects in other cancer models[153, 154]. However, like many other chemopreventives, luteolin is poorly soluble in aqueous conditions making administration of the compound problematic, with the outcome of low bioavailability of the compound. To avoid these difficulties a nanoformulation of luteolin utilizing PGLA and Polyethylene glycol(PEG) has been developed for intraperitoneal administration in a Tu212 xenograft nude mouse model [134]. In-vitro antitumor efficacy experiments determined that nano-luteolin was more efficacious at decreasing cell proliferation in both head and neck as well as lung cancer cell lines, Tu212 and H292, respectively.

Collectively, the above mentioned studies indicate that improved efficacy of chemopreventive agents can be achieved by incorporating nanotechnology. Nanoparticles have

the ability to alter the physical properties of some chemopreventives making them more promising in future clinical trials.

4.4 Liposome Formulation Development and Classification Methods

Physical characteristics of liposomes can ultimately affect their biological activity *in vitro*, as well as, *in vivo*. Such characteristics that can define the stability of the liposome includes size distribution, lamellar structure, and outer leaflet surface charge(zeta potential)[88]. Typically, depending on the method of preparation solvents and lipid mechanics can determine many of these parameters. One method that can lead to liposomes with uniform size and encapsulation is the extrusion method.

The extrusion method**(Figure 1.4)** is a combination of the film drying method and the sonication method with the end product having an additional extrusion through a polycarbonate filter composed of pores of a known diameter[155]. Once a liposome is prepared the physical properties of the batch can be evaluated. One method of determining the size of a liposome is dynamic light scattering(DLS). DLS utilizes laser technology to measure the Brownian motions of liposomes in buffer solution and quantifies the velocity of these interactions, which is incorporated into the Stokes-Einstein equation to determine the average diameter of a particular liposome batch[156].

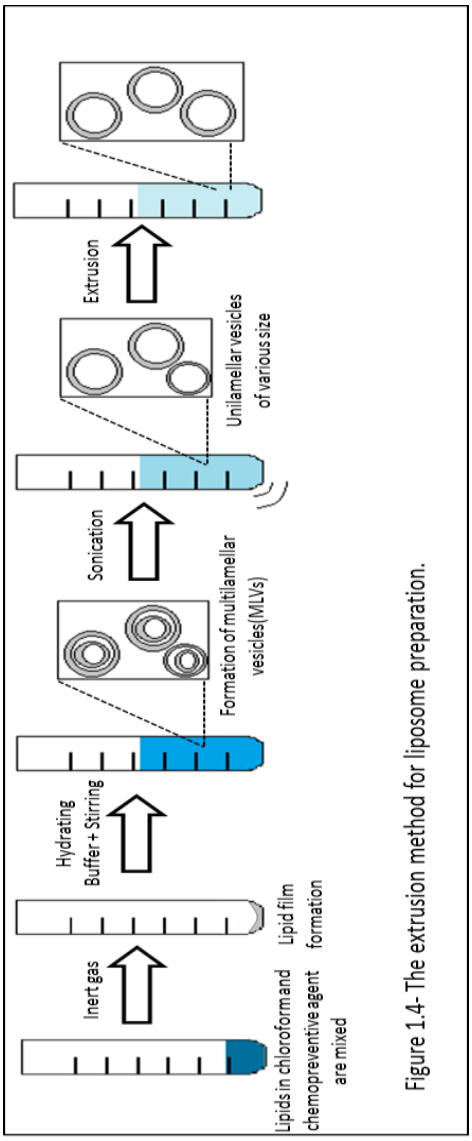


Figure 1.4- The extrusion method for liposome preparation.

Another method that can be applied to determine the size of liposomes is cryo-transmission electron microscopy(cryoTEM). CryoTEM is ideal for some liposomes, considering that DLS assumes all liposomes are spherical in shape which can lead to inconsistent results. CryoTEM allows for the shape of individual liposomes to be observed since they are

frozen and immobile allowing for the diameter of any individual liposome to be measured [157]. This technique also can be used for the determination of the lamellar structure, or number of bilayers of a particular liposome through observation [157].

The charge on the outer surface of the liposome can also determine its stability. This charge is influenced by the chemical groups protruding from the outer leaflet**(Figure 1.5)**, or outer layer of the liposome[158]. Typically the surface charge of a liposome is defined at pH 7 and is referred to as the zeta-potential. One method that can determine the surface charge of the liposome is Laser Doppler Velocimetry. This technique creates an electrical field in solution and monitors how this field affects the outer layer of ions that bind to the liposome due to static interactions[156]. These static interactions diffuse constantly at an interface known as the slipping plane. The velocity of these ions at the slipping plane is measured and from this the average surface charge of the liposome is determined in millivolts.

4.5 Pharmacokinetics and Biodistribution of Liposomes

The purpose of a liposome as a drug delivery system is to decrease both the dose and toxicity typically observed with the free drug, as well as increase selectivity of drug delivery to a certain organ of interest, and to provide a means of increasing solubility of drugs that are less readily absorbed by the oral delivery route. Liposomes have two distinct pharmacokinetic processes that include the pharmacokinetic behavior of the liposome carrier and the behavior of the free drug once it's released from the liposome carrier[88].

Conventional liposomes have pharmacokinetics that are dependent on the dose administered. The pharmacokinetics is based on dose due to the clearance of the drug by the reticuloendothelial system that is observed during first pass metabolism of the liver [159, 160].

In the liver Kupffer cells, hepatocytes, and macrophages can eliminate the liposome, however, upon saturation of these mechanisms the clearance of the liposome from circulation will decrease with increasing dose. In addition, factors such as size[161], charge[162], and lipid composition[163]can affect the clearance of liposomes.

Sterically stabilized liposomes are known to have polyethylene glycol(PEG) and other hydrophobic polymers linked to their lipid head groups **(Figure 1.5)**[158]. Their pharmacokinetics are similar to conventional liposomes(given intravenously) in that the liposome is also dependent on the dose administered, however, the clearance of these liposomes is less dependent on size and charge, especially below 300 nm. Due to the negatively charged hydrophobic polymers(3-10 mol% PEG in most cases)[164] shielding the liposome from being recognized by the reticuloendothelial system there is no saturation of the Kupffer cells of the liver at high doses after injection(intraperitoneally, subcatanously, or IV[161]), which allows for an increase in half-life($T_{1/2}$) in circulation.

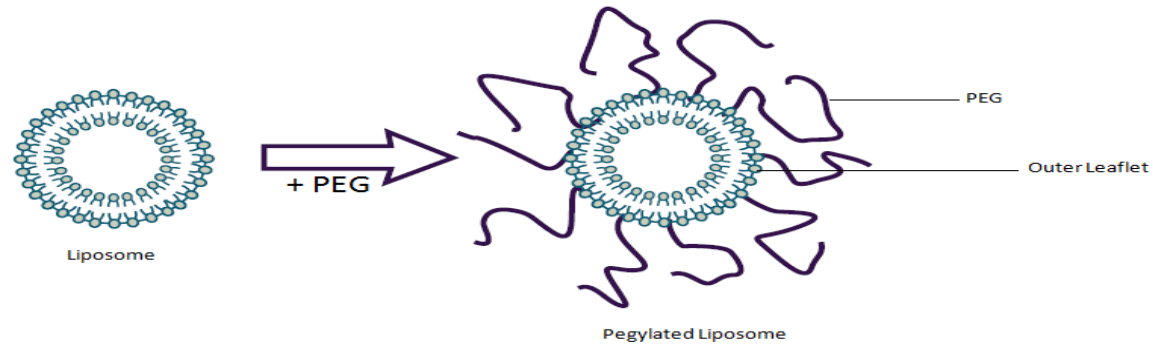


Figure 1.5- Schematic of a Poly Ethylene Glycol(PEG) Coated Liposome

Immunosomes, a type of targeted liposome, have shown to have higher half-lives than conventional liposomes when targeting antibodies were bound to hydrophilic polymers. Antibody density on the outer surface of the liposome also plays a role in clearance rate, with 25µg antibody/µmol phospholipid being optimum in improving the half-life of immunosomes in comparison to sterically stable liposome only [165].

Cationic liposomes, are used to deliver nucleic acids in an attempt to knock down the expression of genes *in vitro*[166] and *in vivo*[167]. Recently, it was shown that cationic liposomes of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine(DOPE), polyethylene glycol(PEG), and an antisense nucleotide were able to knock-down BCL-2 in ovarian xenografts *in-vivo*, while minimally distributed to the liver and spleen[167]. Other studies utilizing *in vivo* models have demonstrated the same efficacy of delivering siRNA to breast tumors[168].

4.6 Docosahexaenoic Acid (DHA) Liposomes

The omega-3 fatty acid DHA has been incorporated into conventional liposomes formulations[169-171]. The purpose of liposomal delivery of DHA in these studies was to increase its solubility when given intravenously and prevent colon cancer cell migration[170], increase permeability of tight junctions[169], and determine its ability to protect rat adrenal gland cells from cell death[171].

It is well known that omega-3 fatty acids are capable of preventing colon tumorigenesis[59, 172-174]. In the study by Ichihara et al.[170], the focus was to understand if DHA would be efficacious at preventing colon cancer metastasis to the liver, if given in a hybrid liposome formulation, by daily intravenous administration. The therapeutic effect of dimyristoyl phosphocholine and DHA liposome(100nm) at a molar ratio of 1:1 was observed in a HCT116 mouse xenograft model of colon cancer metathesis to the liver[170]. Overall, the formulation

prevented most HCT116 cells from migrating to the liver and increased apoptosis in the few HCT116 cells which reached the liver. The intravenous administration of the hybrid liposome formulation(136mg/kg/d DMPC, 65.7mg/kg/d DHA) was successful at preventing HCT116 migration in SCID mice after a period of 14 days[170].

DHA has also been proven to be involved in brain aging and neurodegeneration by affecting the fluidity of neuronal membranes. Specifically, DHA is the most abundant fatty acid in the brain and its role in neuroprotection includes gene expression [175], neurodevelopment [176] and membrane-protein mechanisms[177]. In the study by Eckert et al.[171], a conventional liposome formulation of DHA was applied every 48 hours for 5 days to an embryonic kidney cell line, HEK-293, that were transfected with human APP695 plasmid; the results indicated that their conventional liposome formulation(20uM) was able to increase membrane fluidity and levels of APP cleavage resulting in protection from calcium depletion of the endoplasmic reticulum leading to apoptosis. Furthermore, this protection was confirmed by modulation of the PI3K/Akt and JNK signaling pathways in PC12 cells, adrenal gland cells. The PC12 cells were treated with the cleaved APP protein, found in the used media of the HEK293 cells, treated with DHA. The sAPP component found in the used media had protective effects on the PC12 cells, possibly through inhibition of the JNK pathway and activation of the PI3K pathways.

In the laboratory of Hossain et al.[169], the permeability, transport, and uptake of omega-3 fatty acids on human colorectal cells, Caco-2 had been evaluated[169]. By utilizing natural sources(starfish and squid) of lipids that express DHA, liposomal DHA was synthesized by the thin film/sonication method. TEER(Trans Epithelial Electrical Resistance) measurements of Caco-2 monolayers treated with liposomal DHA ranging from 25-100uM in lipid concentration

were conducted to measure permeability. It was found that increasing amounts of liposomal DHA decreased TEER in a dose dependent fashion, indicating that liposomal DHA was able to increase permeability of tight junctions. Monitoring liposomal DHA transport with incorporation of luciferase yellow also provided further evidence for the increase permeability of the tight junctions on the Caco-2 monolayer. Overall, it was demonstrated that DHA was able to decrease tight junction adhesion and increased permeability between colorectal cells.

5. Research Objectives and Specific Aims

There are currently 2 million women in the United States that are at high risk for breast cancer. Current chemopreventive agents for breast cancer include the antiestrogens, tamoxifen and raloxifene, however, about 4% of women that are high risk would take such synthetic drugs on a daily basis to reduce their risk of developing breast cancer because of the fear of side effects such as thromboembolic events and increased risk of developing endometrial cancer [178-180]. A natural agent that has practically no side effects would be more appealing to women for chemoprevention.

Omega-3 fatty acids, a key component of fish oil, have been evaluated both in epidemiological studies and preclinical studies. Epidemiological studies have not been supportive in providing a clear relationship between omega-3 fatty acids ingested and a decrease in the risk of breast cancer. Preclinical studies provided more supportive evidence that omega-3 fatty acids may play a role in breast cancer prevention in *in vivo* models of breast cancer, but inconsistencies among various reports remain[34]. Recently, our laboratory demonstrated that a diet using high omega-3:omega-6 fatty acid ratio(25:1) prevented the development of tumors in chemically induced breast carcinogenesis in the rat[71].

Therefore, one of our goals is to better understand the underlying molecular mechanism of how the high omega-3 fatty acid ratio contributes to a decrease in breast cancer *in vivo.* Our **hypothesis** is that by utilizing a proteomics approach, novel proteins could be identified that could provide insights into the molecular mechanism of how omega-3 fatty acids have decreased mammary carcinogenesis in the rat. These proteins identified could be used as biomarkers for early detection and to monitor the efficacy of the omega-3 fatty acids as chemopreventive agents. We believe that it is important to take a minimally invasive approach which can provide important insights that could eventually be applied in the clinic. Thus in **Specific Aim 1** we propose to utilize a proteomics approach to gain insights into the mechanism at the protein level responsible for the chemopreventive action of high omega-3:omega-6 fatty acids(n-3:n-6) in the presence and absence of tamoxifen as observed in the MNU-mammary tumor model[181]. These proteins could be used to determine a potential mechanism through correlating their expression to the expression of proteins found in adenocarcinomas[182].

Feeding a diet consisting of a high omega-3:omega-6 fatty acid ratio(10:1 and 25:1) may seem promising in an attempt to prevent breast cancer. However, it is impossible to consume such high amounts of omega-3 fatty acids on a daily basis, considering that sources of omega-3s are nuts, fish, and certain seeds can provide only low and variable amounts of omega-3 fatty acids. Furthermore, the Western diet consist of an average omega-3:omega-6 fatty acid ratio of 1:15[6], making it difficult to achieve the 25:1 as seen in the preclinical experiment[71].

Previous studies evaluating breast cancer both, *in vitro* and *in vivo*, have supported a role of particular form of omega-3 fatty acids such as, DHA and EPA, as possible chemopreventive agents. DHA has been shown to be a superior to that of EPA in preventing breast cancer in

DMBA and MNU models of mammary carcinogenesis[68, 69] in the rat, and in MCF-7(ER+) and MDA-MB-231(triple negative) breast cancer cell lines[74-79].

Therefore, it is feasible to administer DHA supplements to women who are high risk on a daily basis, however, DHA is highly unsaturated with multiple double bonds, thus highly susceptible to oxidation and breakdown through free radical reactions. Furthermore, introduction to acidic aqueous conditions in the human digestive system will make it difficult to be absorbed. To address such a limitation, a drug delivery system that would protect DHA from the acidic and aqueous environment of the stomach would be ideal. Our **hypothesis** is that oral administration of liposomal DHA would enhance bioavailability by increasing the amount of DHA in circulation and that reaches the breast tissue, while protecting DHA from oxidation and pH fluctuations, making this delivery system a superior chemopreventive strategy.

Ether lipids similar in structure from the single-celled domain known as archaea are of interest for their thermal stability, pH stability and stability against phospholipase cleavage[103-107]. Therefore, in **Specific Aim 2** we propose to develop a liposomal formulation of DHA with ether lipids that can protect DHA from oxidation and fluctuations in pH for oral administration. Furthermore, we also propose to compare in **Specific Aim 2** its antitumor activity at the cellular and molecular levels, initially in breast cancer cell culture studies, *in vitro*. Oral administration is a plausible route for chemopreventive agents since women who are high risk for cancer are more willing to take an oral formulation than suffer through injections daily.

Following the demonstration of the anticancer activity of liposomal DHA *in vitro* the formulation will be evaluated in an animal model to observe its tissue distribution. Our **hypothesis** is that liposomal DHA will protect DHA from oxidation and fluctuation in pH which will increase DHA in circulation and at the breast tissue. Thus, in **Specific Aim 3,** the amount of

DHA delivered to the serum and mammary fat pad by liposomal DHA will be evaluated in a 21 day old female Sprague Dawley rat; the same animal used in chemoprevention studies[68, 69, 71]. Specifically we will measure DHA and its lipoxygenase (LOX) metabolites in the serum and in at the mammary fat pad by gas chromatography flame ionization detection(GC-FID)[183, 184] and liquid chromatography-tandem mass spectroscopy (LC-MS/MS), respectively. Furthermore, we will utilize LC-MS/MS for confirming the structure of DHA in the serum and in the mammary fat pad. Collectively, the work proposed in this thesis will provide the experimental data needed to make translationally-relevant effective chemopreventive dietary supplements, which can be employed successfully in the clinic for the management and prevention of breast cancer.

Chapter 2

A Proteomics Approach to Gain Insights into the Mechanisms of Chemoprevention by High Omega-3:Omega-6 Ratios in the Presence and Absence of Tamoxifen in the Rat

1. Objective

Worldwide, breast cancer is the most common and the most deadly cancer in women[3]. Prevention is a plausible approach to reduce breast cancer morbidity and mortality. The two selective estrogen receptor modulators (tamoxifen and raloxifene) have been shown to be effective chemopreventive agents by reducing the incidence of estrogen receptor positive breast cancer by 50% and 38%, respectively[7, 8, 31, 32]. However, the acceptance of these compounds for reducing breast cancer risk is extremely limited due to toxicity such as thromboembolic events which, although, rare, are significant when considering that these drugs are given to healthy women for prevention[185]. An additional limitation of tamoxifen and raloxifene is that neither drug reduces the incidence of estrogen receptor negative tumors[7, 8, 31]. The steroidal aromatase inhibitor exemestane has been shown to reduce the annual incidence of invasive breast cancer by 65% after a median follow-up period of three years[186]; whether this drug will be more acceptable to the general public remains to be determined. Clearly, there is an urgent need to develop highly effective chemopreventive agents with limited side effects.

Naturally-occurring agents are very appealing to the general public as compared to pharmacological agents. Omega-3 fatty acids have been shown to inhibit breast carcinogenesis in pre-clinical models[34, 68, 69, 71, 73]; however, inconsistencies remain among published reports. A comprehensive review by Signori et al.,[34] has identified several confounding

variables including the omega-3:omega-6 fatty acid ratio and caloric intake from fat that can explain the inconsistencies across these studies. Therefore, a study by Zhu et al. [71] has been conducted to better define the effective ratios of omega-3:omega-6 fatty acids as a chemopreventive regimen using a diet that provides 30% calories from fat as recommended by the FDA[72]. The results of this study[71] demonstrate that high omega-3:omega-6 fatty acid ratios(25:1), with and without tamoxifen(1.0mg tamoxifen citrate/kg diet) significantly inhibited MNU-induced mammary carcinogenesis in the female Sprague Dawley rat[71]. A follow-up study by our team illustrated that the high omega-3:omega-6 fatty acid ratio also altered breast tumor biology to favor an increase in apoptosis, and a decrease in cell proliferation, inflammation, and lipid metabolism in the same MNU Sprague Dawley rat model[73].

On the basis of results described above, studies conducted in **chapter 2** are designed to test our **hypothesis** that by utilizing a proteomic approach, novel proteins can be identified that can provide insights into the molecular mechanisms of the protective effect of omega-3 fatty acids. An additional goal is to identify, in a minimally-invasive fashion, proteins that are altered in response to treatment which can be used as a biomarker for early detection of the disease and to monitor the efficacy of the chemopreventive agents initially in preclinical animal models and ultimately in the clinic. The results of studies performed in this chapter have been published [181].

2. Background

2.1 Biomarkers of Breast Cancer

The ability to evaluate an individual's health based on the presence of proteins in serum and at the tissue of interest is a practice that has been used in clinical settings. The abundance of proteins ultimately determines the biological activity, specifically, if the protein levels are altered

or if there are post-translational modifications that may impact their functions. In breast cancer proteins can be altered at the genetic and post-translational level and this can alter their function. Such functions can include cell cycle, proliferation, apoptosis, and metastasis. Determining the protein expression in the serum and in the tissue of interest can provide insights on the molecular mechanisms of breast cancer development.

Even though research in the field of breast cancer biomarkers is expanding in an attempt to discover protein biomarkers for the early detection of disease, predict patient prognosis, and determine the likelihood of drug resistance in the clinical setting, currently a reliable biomarker for these purposes has not been identified[187]. Many studies have been conducted on breast cancer patient samples that include breast discharge, peripheral blood, bone marrow, and breast tissue[188-191]. Other studies have been conducted on breast cancer cell lines and pre-clinical animal models[192-196].

Many studies have focused on potential markers for metastasis in breast cancer patients. For example three particular proteins, urokinase-dependent plasminogen activator system (uPA), the plasminogen activator inhibitor (PAI) and the Thomsen-Friedenreich (TF) antigen have been assessed[187]. uPA is a tumor associated serine protease, while its inhibitor PAI, have been studied in patients with auxiliary node negative breast cancer[197]. However, early detection is still difficult to evaluate because finding markers that reflect carcinogenesis, the transition of a normal cell to a cancerous cell, instead of focusing on the end stage of the disease (cancer) has numerous challenges.

For early detection biological fluids, such as serum or nipple aspirate, have been the biological fluid of choice since it requires minimally invasive technique for collection. Proteins such as alpha-2S-glycoprotein and vitamin D binding protein were observed to be down-

regulated and up-regulated, respectively in the nipple aspirate of tumor-bearing breast cancer patients versus healthy volunteers[198], while Her-2/neu[199], CA 15-3[200, 201] have been observed in the serum of patients with breast cancer. In addition, the humoral response, or antibody abundance to certain proteins such as heat shock protein 90, HSP90[202, 203] and mutated p53[204-207] have been studied as possible responses to breast carcinogenesis.

In addition, as mentioned in **Chapter 1**, certain prognostic markers, such as the expression of certain genes, *BRCA1* and *BRCA2*, may be utilized for early detection and rare mutations with *PALB2*[208]. For pre-malignant lesions, such as DCIS, genes such as *ki-67, STK15, Survivin, CCNBI, MYBL2, PR, GSTM1, RB, and PTEN* have been reported[209].

3. Rationale

Our team has shown that a high omega-3:omega-6 fatty acid ratio(25:1, 10:1) inhibited MNU-induced mammary adenocarcinoma in female Sprague Dawley rats[71]. In a follow-up study, the molecular mechanism that can account for chemoprevention by high omega-3:omega-6 fatty acid ratios was investigated; the results showed a decrease in cell proliferation and increase in apoptosis in mammary tumors.[73]. In this chapter we used plasma, a minimally invasive fluid[210], from the same animals in an attempt to further understand the mechanism of chemoprevention at the protein level and identify biomarkers that could be used to monitor the effects of high amounts of omega-3 fatty acids during the process of the bioassay. We used iTRAQ(isobaric Tagging for Relative and Absolute Quantitation), a sensitive and robust proteomic technique and the results reported in this chapter show that several proteins were altered in a manner consistent with chemoprevention[181].

4. Experimental Design

4.1 4-Plex iTRAQ Analysis of Plasma Protein Expression in MNU-Induced Mammary Carcinogenesis in the Rat

4.1.1 Reagents

Albumin/IgG removal kits were purchased from EMD Millipore (Billerica, MA). iTRAQ reagents were obtained from Applied Biosystems Inc. (ABI, Foster City, CA). DC Protein Assay Reagents for protein quantification were obtained from Bio-Rad Laboratories (Hercules, CA). Reagents for Western blots were obtained from Bio-Rad Laboratories (Hercules, CA) and primary and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), Abcam (Cambridge, MA), and Cell Signaling (Davers, MA). Antibodies against vitamin D binding protein, VDBP (Santa Cruz Biotechnology, SC-32899), 14-3-3 Sigma (Abcam, ab14123), alpha-1-b-glycoprotein, A1BG, (Santa Cruz Biotechnology, sc-132613), gelsolin (Abcam, ab134183), apolipoprotein E (Abcam, ab20874), haptoglobin (Abcam, ab117316), and inter-alpha inhibitor H4 heavy chain, ITIH4 (Abcam, ab118283) were used. Chemilumnescent immunodetection reagents and autoradiography film were obtained from GE Healthcare (Piscataway, NJ).

4.1.2 Animals and Blood Collection

The development of breast cancer can be evaluated in rat models. There are two types of rat models of chemically induced breast carcinogenesis, dimethylbenz[α]anthracene (DMBA) and 1-methyl-1-nitrosourea (MNU).

MNU is a DNA alkylating agent and has an affinity for the terminal end buds(TEB) of the breast tissue in female Sprague Dawley rats. In addition, this alkylating activity is dependent

on the age of the rat, considering that sexual maturity in the female Sprague Dawley rat is around 50 days of age, where estrogen influences the morphogenesis at the breast tissue[211]. However, at 21 days of age the TEB is at a higher activity and thus rats at this age are more susceptible to MNU-induced mammary carcinogenesis[212].

The MNU-rat model of mammary carcinogenesis has been studied thoroughly[213-216]. The different types of benign and cancerous lesions seen in rat abdominal-inguinal mammary glands (ductal hyperplasia-mild, moderate, florid) and ductal carcinoma in-situ, DCIS (cribiform, comedo, papillary) are similar in morphology to human lesions[214, 217, 218]. Two weeks post-MNU administration benign lesions such as ductal hyperplasia can be observed. After five weeks these lesions can advance to mammary adenocarcinomas with about 60% being estrogen receptor positive[214, 215, 217, 218].

The plasma evaluated in this study was obtained from rats used in our published study [71] testing the chemopreventive effects of different omega-3:omega-6 fatty acid ratios, individually and in combination with tamoxifen. Briefly, female Sprague Dawley rats were injected i.p. at 21 days of age with 50 mg of MNU/kg body weight as previously described[71]. Seven days post-administration of the carcinogen all rats were randomized into treatment groups and were fed their respective experimental diets (30 rats/group): ad libitum consisting of 1:25, 1:10, 1:5, 1:1, 5:1, 10:1, 25:1 n-3:n-6 fatty acids with or without tamoxifen (1.0 mg tamoxifen citrate/kg diet). The ratio of polyunsaturated: saturated: monounsaturated fatty acids was 10%:10%:10%, according to FDA recommendation[72]. The rats were weighed and palpated for detection of mammary tumors twice a week. At the end of eight weeks the rats were sacrificed by cervical dislocation. Blood was collected through the retro-orbital-sinus and distributed into

heparinized capillary tubes and EDTA coated tubes. All samples were aliquoted and kept at -80°C.

The experimental procedures to collect plasma samples were previously described [181]. In this study we selected six plasma samples per group at random from rats fed a ratio of omega-3:omega-6 fatty acids of 1:1 (control)(Group 1), 10:1 (Group 2), 25:1 (Group 3), and 25:1 with tamoxifen(Group 4).

4.1.3 Protein Depletion and Quantitation

The abundant blood proteins, IgG and serum albumin, were mostly depleted from all plasma samples using an Albumin/IgG removal column (CalBiochem, Cat. No. 122642). To ensure proper removal of albumin an aliquot from each depleted sample was separated on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and stained with Coommasie Blue (Bio-Rad 161-0786). The samples were then precipitated according to manufacturer's procedure for the CalBiochem ProteoExtract Protein Precipitation Kit for volumes larger than 200µl. After the precipitation 150µl of MilliQ water, 33µl of 6M urea, and 10µl of 2% SDS solution was added to each precipitated pellet for solubilization. Following depletion, precipitation, and solubilization aliquots of all samples were examined by a DC Protein Assay to determine total protein concentration (Bio-Rad Laboratories).

4.1.4 iTRAQ Labeling of Protein Peptides

The plasma samples from each of the four groups (1:1 n-3:n-6, 10:1 n-3:n-6, 25:1 n-3:n-6, 25:1 n-3:n-6 plus tamoxifen) were assigned an isobaric tag for a 4-plex experiment as described in **Figure 2.1.** A total of six samples of Sprague Dawley rat plasma from each group was pooled (500 µg protein) together. From this pool 115 µg of protein was denatured, reduced, alkylated, and digested with 2% SDS, 5mM tris-(2-carboxyethyl) phosphine, 84mM

iodoacetamide (Sigma-Aldrich, St. Louis, MO), and trypsin (5:1, Promega, Madison, WI), respectively. The samples were dried under vacuum for two hours. To the contents of each tube an appropriate amount of 0.5M TEAB with ethanol was added until the pH of all samples was between 7.0-8.0. Each iTRAQ Tag (Applied Biosciences) was added to its designated pool of protein for a specific group and incubated in the dark, followed by drying by vacuum. Once samples were tagged all samples from each group were mixed and submitted to the Pennsylvania State University College of Medicine Proteomic/MS Core Facility where they were kept at -20°C until analysis.

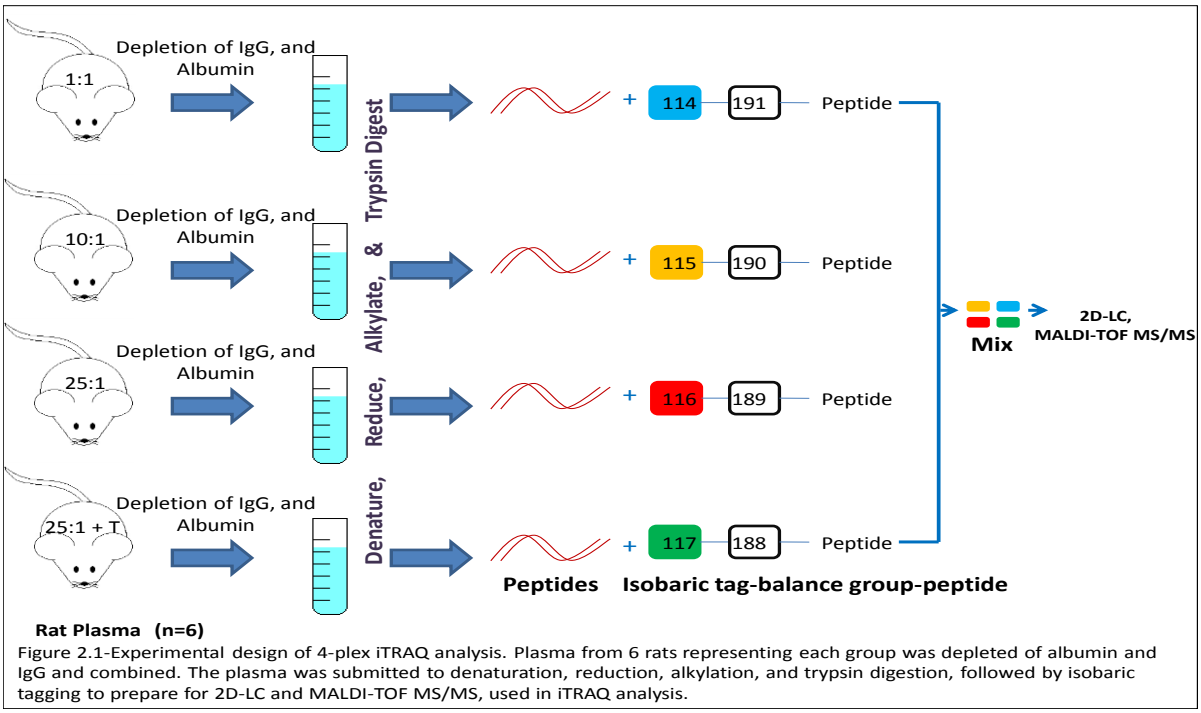


Figure 2.1-Experimental design of 4-plex iTRAQ analysis. Plasma from 6 rats representing each group was depleted of albumin and IgG and combined. The plasma was submitted to denaturation, reduction, alkylation, and trypsin digestion, followed by isobaric tagging to prepare for 2D-LC and MALDI-TOF MS/MS, used in iTRAQ analysis.

4.1.5 2D-LC Fractionation of iTRAQ Labeled Peptides

For better detection of proteins of lower abundance the labeled peptide mixtures were submitted to the Penn State Hershey Cancer Institute Proteomics/Mass Spectrometry Core for 2D-LC separation according to the following

website(http://www.pennstatehershey.org/web/core/proteinsmassspectometry/protocols/data-analysis). The first dimension separation of peptides by SCX was carried out as follows: The samples were dried and resuspended in loading buffer A(10 mM ammonium formate, pH 3.6, in 20% acetonitrile/80% water). The separations were conducted on a passivated Waters 600E HPLC system, using a 4.6 X 250 mm Polysulfoethyl Aspartamide column (PolyLC, Columbia, MD) at a flow rate of 1 mL/min. The HPLC separation was conducted according to the following procedure. Buffer A was used for a gradient separation at 100% (0- 22 minutes following sample injection), while buffer B(666 mM ammonium formate, pH 3.6, in 20% acetonitrile/80% water) was used for a gradient separation of 0%to 40% (22-48 min), 40% to 100% (48-49 min) 100% and isocratic (49-56 min), then at 56 min the gradient was switched back to 100% buffer A to re-equilibrate for the next injection. The first 28 mL of eluant (containing all flow-through fractions) were combined into one fraction, then 14 additional 2-mL fractions collected. The 15 SCX fractions were dried down to reduce volume and to remove the volatile ammonium formate salts, re-suspended in 9 µL of 2% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid and filtered prior to reverse phase C18 nanoflow-LC separation.

Next, second dimension separation was conducted by reverse phase nanoflow LC. Each fraction from SCX separation was auto-injected onto a Chromolith CapRod column (150 X 0.1 mm, Merck) using a 5 µL injector loop on a Tempo LC MALDI Spotting system (ABI-MDS/Sciex). The elution gradient was conducted with two buffers, buffer C (2% acetonitrile, 0.1% trifluoroacetic acid) and buffer D (98% acetonitrile, 0.1% trifluoroacetic acid). The elution gradient started at 95% buffer C/ 5% buffer D (2µL per minute flow rate from 0 to 3 min, switching to 2.5µL per minute at 3 min for the remainder of the gradient), changes from 5% buffer D to 38% buffer D (8.1-40 min), 38% buffer D to 80% buffer D (41-44 min), 80% buffer

D to 5% buffer D (44-49 min). A 3 µL per minute flow of MALDI matrix solution is added post-column (7 mg/mL recrystallized CHCA (a-cyano-hydroxycinnamic acid), 2 mg/mL ammonium phosphate, 0.1% trifluoroacetic acid, 80% acetonitrile). The combined eluted solution was automatically spotted onto a stainless steel MALDI target plate every 6 seconds (0.55 µL per spot), for a total of 370 spots per original SCX fraction.

4.1.6 Mass Spectroscopy(MS) Analysis of Peptides

The MS analysis of fractioned peptides was conducted in the Penn State Hershey Cancer Institute Proteomic/Mass Spectrometry Core. Following the drying of all spots derived from the SCX fractionation, thirteen calibrant spots (ABI 4700 Mix) were manually added to each silver plate. On the ABI 5800 MALDI TOF-TOF target plates (15 per experiment) were analyzed in a data-dependent manner. As each plate was placed into the instrument, a plate calibration/ MS Default calibration update was conducted, then the MS/MS default calibration was also updated. MS Spectra were acquired from each sample spot using the newly updated default calibration, with 500 laser shots per spot, laser intensity 3200 (this value can change somewhat with laser age and tuning). Automatically, a plate-wide interpretation was conducted, choosing the highest peak of each observed m/z value for subsequent MS/MS analysis. The mass spectra taken from 5,500 MALDI spots averaged 500 laser shots per spot at a Laser Power of 2,900. The most intense m/z as analyzed by the Paragon Algorithm were used for MS/MS. Approximately, 2,500 laser shots at laser power 4,000 were accumulated for the MS/MS spectrum. 5,500 MALDI spots were analyzed overall. The peptide fragmentation was induced by a collision gas (nitrogen) at 1.2 to 1.3 x 10^{-6} torr. A total of 14,349 MS/MS spectra were taken. The percent of MS/MS spectra identified with 95% confidence when compared to peptides in the NCBI database for *Rattus norvegicus* was 82.9%.

4.1.7 Database Search for Proteins Identified from MS and MS/MS Spectra

The combined MS and MS/MS data collected were analyzed by the Paragon Algorithm as implemented by Protein Pilot v4.0 software (ABI/MDS-Sciex)[219]. The Paragon Algorithm took into account the carboxymethylation of cysteine amino acids by iodoacetamide, any biological modifications, and any amino acid substitutions when assessing peptides for protein identification. The paragon algorithm compared each peptide sequence identified to peptide sequences for *Rattus norvegicus*. The peptides were each evaluated with regards to their Sequence Temperature Value (STV). MS/MS spectra were investigated using the *Rattus norvegicus* NCBI data sequences containing a total of 25,319 protein sequences. In addition, the spectra were also compared to 389 common lab contaminants (ABSciex_ContaminantDB_20070711) and a concatenated reverse decoy database, an additional 26,087 sequences. Palindromic sequences can not be identified by this method. Once peptides were identified a statistical analysis by the Pro Group Algorithm was conducted to determine the minimal set of protein identifications. This algorithm calculates the Unused Protein Score (Unused ProtScore) based only on peptide sequences that are only related to a particular protein. The more peptide sequences that are determined for a particular protein, the more confidence there is for that protein being identified. However, these peptides can not be used to identify another protein, if so, there is no confident match for that peptide and the peptide is eliminated from the overall analysis. Confident identifications of proteins were only accepted as having an unused protein score of 1.3 or greater, which illustrates that there is greater than 95% confidence that the protein is accurately identified in the analysis, by the Pro Group Algorithm. The equation to determine the unused protein score is the following: unused protscore=-log(1-(%

confidence/100)). For each peptide identified a percent confidence is determined. A stringent Local False Discovery Rate (FDR) estimation was conducted for all proteins. This is based on simultaneously searching a concatenated Decoy database which is the exact reverse of each protein sequence in the database to rid of any false positive results. The overall analysis identified 148 highly confident proteins. The lowest ranking protein ID on this list still has less than a 5% chance of being a False Positive ID as calculated by the PSEP (Proteomics System Performance Evaluation Pipeline Algorithm)[220].

4.1.8 Quantitation and Statistical Analysis of Proteins Identified from MS Results

The relative abundance of proteins identified was expressed as an average log ratio of intensity from all proteins identified. The log ratios of the proteins identified with a protein unused score of at least 1.3 in the groups 10:1 n-3:n-6, 25:1 n-3:n-6, 25:1 n-3:n-6 plus tamoxifen were compared to the log ratios of the 1:1 n-3:n-6 group (control-Tag 114) utilizing a Student's two-tailed t-test in Microsoft Excel. Only proteins that had a log ratio with a significant p-value($p \leq 0.05$) and an error factor less than or equal to 2 in the groups 10:1 n-3:n-6, and 25:1 n-3:n-6, and 25:1 n-3:n-6 fatty acids plus tamoxifen, when compared with the control group, were further investigated. An error factor less than 2 is equivalent to a standard deviation of 20% or less amongst the individual peptides measured by their intensity by MS/MS. To obtain a p-value for each protein, the individual peptides identifying each protein have a standard deviation of intensity, or error factor, less than 20%, and can not be false positive when compared to the decoy database. When comparing the intensity of these peptides to the intensity of the peptides found in the control group(1:1) by utilizing a Student t-test, the p value has to be less than 0.05. For the 148 proteins identified there were 444 t-tests(148 proteins x 3 treatment groups), one for each protein identified for each of the three experimental groups; the intensity of the peptides

determine by MS/MS for each treatment group were compared to the intensity of the peptides found in the 1:1 group(control).

4.1.9 Western Blot Analysis of Protein Expression Discovered in iTRAQ Analysis

Proteins from depleted plasma (depletion of IgG and serum albumin) were submitted to acrylamide gels that ranged from 10-20% acrylamide (Bio-Rad Laboratories) utilizing 14-35 µg protein per well. They were transferred to PVDF (Bio-Rad Laboratories), blocked with 5% milk, and probed over-night at 4°C with antibodies to validate the differences in protein levels discovered in the iTRAQ analysis. Proteins that were validated include VDBP, 14-3-3 sigma, A1BG, gelsolin, apolipoprotein E, haptoglobin, and ITIH4. The images were captured with Bio-Rad's GS800 Calibrated Densiometer and quantified with the Quantity One v4.5.0 1D Analysis Software (Bio-Rad Laboratories). The equal loading technique was utilized based on determining protein concentration using the Bio-Rad DC Protein Assay. Staining of the PVDF membrane with Ponceau S confirmed that the proteins were loaded equally. A Student t-test was utilized to determine significance of results which was considered a p-value of ≤ 0.05.

5. Results

5.1 Identification of Plasma Proteins by iTRAQ Analysis and Western Blotting

5.1.1 Plasma Proteins Identified by iTRAQ Analysis in MNU Rat Model of Mammary Carcinogenesis Given High Omega-3:Omega-6 Fatty Acids

As compared to control rats treated with MNU and fed a diet containing n-3:n-6 ratio of 1:1, tumor incidence was significantly decreased by 12%, 21%, and 72% in rats treated with MNU, but fed the 10:1 n-3:n-6, 25:1 n-3:n-6, and 25:1 n-3:n-6 plus tamoxifen, respectively [71,

73]. Tumor multiplicity was also decreased by 26%, 30%, and 80% in the rats fed 10:1 n-3:n-6, 25:1 n-3:n-6, and 25:1 n-3:n-6 plus tamoxifen, respectively.

Figure 2.2 illustrates a representative MS and peptide MS/MS spectrum of the corresponding amino acid sequence in y-ions, AGKEPGLQIWR, used in the identification and quantification of one of the plasma proteins identified in this study, gelsolin. The 4-plex iTRAQ analysis of the plasma samples from rats treated with set ratios of omega-3 and omega-6 fatty acids identified 148 proteins with high confidence (unused protein score with 95% confidence interval or greater).

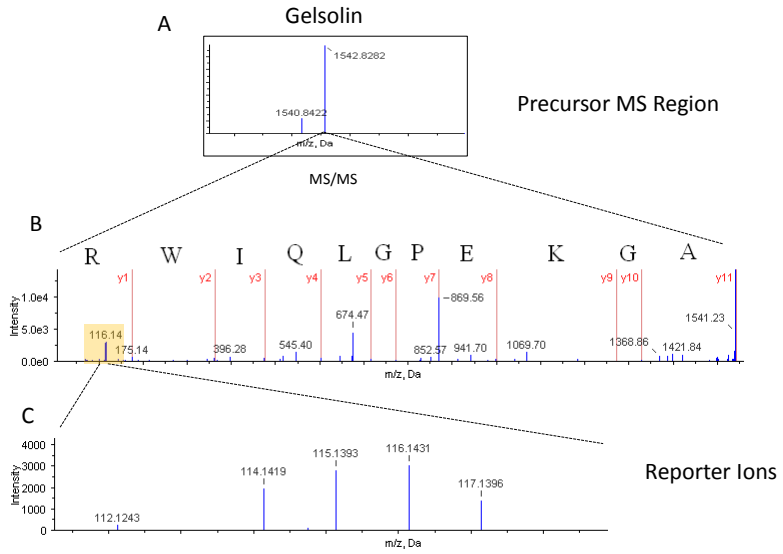


Figure 2.2-Identification of gelsolin. MALDI-TOF MS (A) for gelsolin and its tandem MS (B) showing the unique peptide AGKEPGLQIWR for the identification of gelsolin. The protein was quantitated by observing the intensities of the reporter ions (C) for groups 114, 115, 116, and 117.

From this list 10, 14, and 19 proteins were modulated significantly ($p \leq 0.05$) and with an error factor (EF) less than or equal to 2 (standard deviation ≤20%) in the groups given 10:1 n-3:n-6, 25:1 n-3:n-6, and 25:1 n-3:n-6 plus tamoxifen, respectively, when compared to the control

group given 1:1 n-3:n-6 ratio **(Table 2.1).**

Table 2.1- Proteins Modulated in Some Groups in Comparison to 1:1 n-3:n-6.

Protein	10:1 vs 1:1		25:1 vs 1:1		25:1 +Tamoxifen vs 1:1	
	Fold Change	p Value	Fold Change	p Value	Fold Change	p Value
inter-alpha-trypsin inhibitor heavy chain H3	**4.405549049**	**0.037132461**	7.65596581	0.001594627	7.943282127	0.000759842
fibrinogen gamma chain	**3.53183198**	**0.04742229**	2.46603894	0.079512857	1.659587026	0.083611593
fibrinogen alpha chain isoform 1	**3.162277937**	**0.003749724**	**3.31131101**	**0.002404082**	**2.728977919**	**0.004014052**
complement C3	**2.805433989**	**0.001147725**	**2.20800495**	**0.000559888**	1.318256974	0.278048187
coagulation factor XIII B chain	**2.511885881**	**0.004626506**	**2.67916799**	**0.0126862**	**2.443430901**	**0.01244645**
inter-alpha-inhibitor H4 heavy chain	**1.803017974**	**0.03057613**	**1.599558**	**0.0213216**	0.383707315	0.643387914
haptoglobin	**1.721868992**	**0.018680319**	0.51050502	0.060198981	0.087902263	2.23E-05
alpha-2-antiplasmin	**1.367728949**	**0.059626061**	1.31825697	0.070849828	**1.432188034**	**0.048097949**
alpha-1-macroglobulin	**1.318256974**	**0.005542278**	**1.44544005**	**0.002628269**	**0.839460015**	**0.047253091**
fibrinogen beta chain	**1.056818008**	**0.0104149**	**1.16949904**	**0.002058916**	**1.116863012**	**0.005659904**
fetuin-B	8.472273827	0.006295987	**10.0925303**	**0.003898517**	**5.861382008**	**0.030004229**
vitamin D-binding protein	3.250873089	0.169551998	**5.29663515**	**0.004626949**	**0.672976673**	**0.0167256**
apolipoprotein E	2.654606104	0.106729902	**4.9203949**	**0.002455108**	0.570164323	0.337399989
ceruloplasmin	1.28233099	0.331379294	**3.1915381**	**0.000434292**	1.08642602	0.565339804
serotransferrin	0.928966403	0.088144973	**2.46603894**	**0.003877083**	**1.202263951**	**0.01927135**
complement component C9	0.990831971	0.705974579	**1.04712903**	**0.03969308**	1.018591046	0.621905386
alpha-1B-glycoprotein	0.586138189	0.304905713	**0.1137627**	**0.000374327**	**0.01499685**	**3.62E-07**
Serine Protease Inhibitor A3M	2.051162004	0.098450407	2.22843504	0.089430854	**3.53183198**	**0.007889271**
kininogen-1 isoform 1	2.53512907	0.161166906	3.37287307	0.038619041	**2.884032011**	**0.01903403**
serine protease inhibitor A3N	1.51356101	0.819369674	1.247383	0.888150394	**2.728977919**	**0.037142001**
plasma kallikrein	1.527565956	0.210067406	1.65958703	0.178194895	**2.051162004**	**0.01194448**
gelsolin	1.614359021	0.169333905	2.88403201	0.000573623	**1.803017974**	**0.041453529**
leukemia inhibitory factor receptor	0.870963573	0.661621988	0.65463609	0.109953098	**0.416869402**	**0.02516003**
C4b-binding protein alpha chain	0.779830098	0.395195693	1.19124198	0.267823994	**0.416869402**	**0.02253277**
serum amyloid A-4 protein	0.170608193	0.01579879	0.26061529	0.090979457	**0.337287307**	**0.028261339**
fibronectin	1.330453992	0.793550193	0.68548822	0.223466396	**0.169044107**	**2.08E-06**
Hypothetical Protein	2.269865	0.098173	**0.519996**	**0.030653**	**0.316227794**	**0.01446376**

Table 2.1- All proteins identified with confidence (p≤0.05) by the iTRAQ analysis in all groups. The proteins in yellow are significantly modulated(p≤0.05) with an error factor less than 2 in comparison to the same proteins in the 1:1 n-3:n-6 group.

5.1.2 Proteins Modulated in Rats Administered a Diet Containing Different Ratios of Omega-3:Omega-6 Fatty Acids

Out of 148 proteins identified 10 proteins were significantly up-regulated in plasma of the Sprague Dawley rats (p≤0.05, EF≤2) given a dietary ratio of 10:1 n-3:n-6 fatty acids compared to the control group given the 1:1 ratio **(Table 2.2)**. Raw data can be found in excel folder: iTRAQ, file: CSU SAMPLES-115 116 117 to 114 SUMMARY.

The plasma from rats administered a diet of 25:1 n-3:n-6 fatty acids had 12 known proteins up-regulated and 2 proteins down-regulated according to our criteria (p≤0.05, EF≤2) as

illustrated in **Table 2.3**. Proteins such as VDBP and A1BG were differentially altered in a manner consistent with chemoprevention[221-224], thus protein expression was altered in the opposite manner that would be observed in breast cancer patients. VDBP was found to be up-regulated, while A1BG was down-regulated in response to increasing levels of n-3 fatty acids.

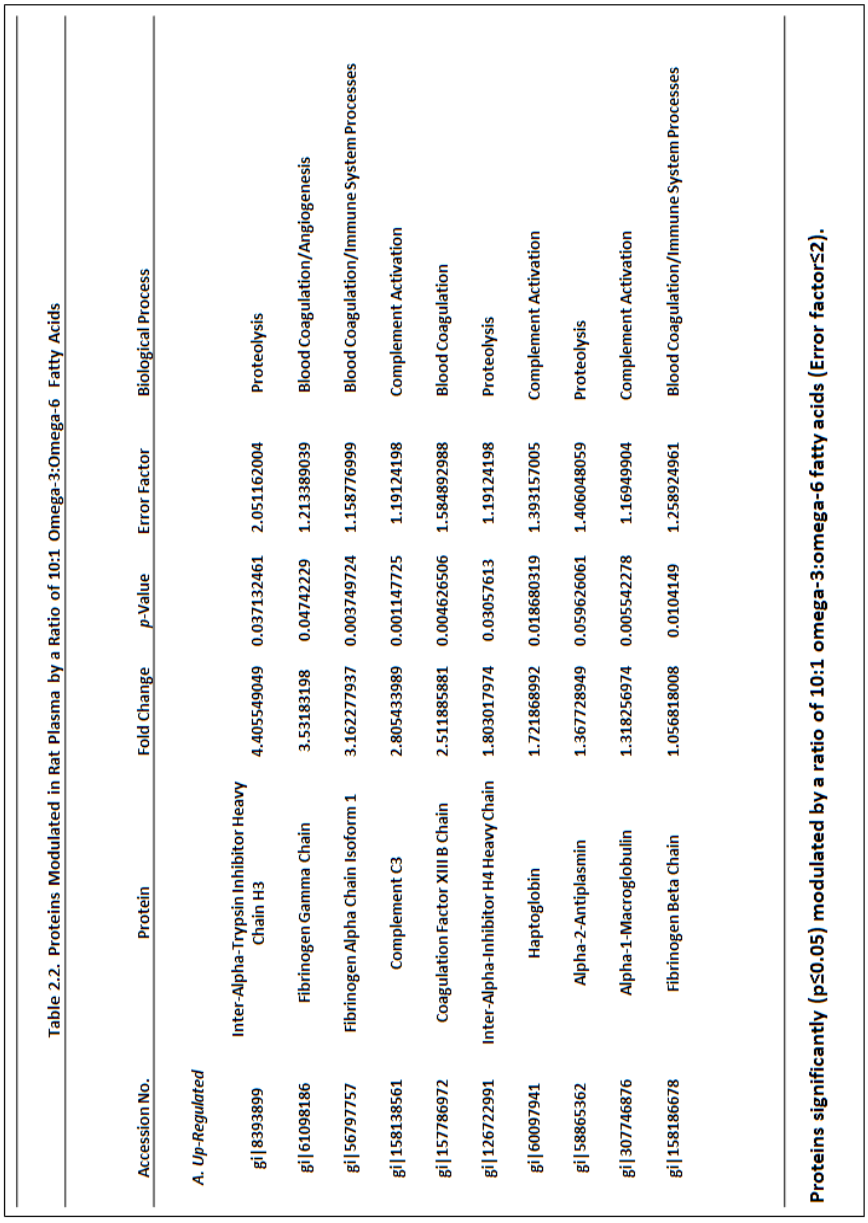


Table 2.2. Proteins Modulated in Rat Plasma by a Ratio of 10:1 Omega-3:Omega-6 Fatty Acids

Accession No.	Protein	Fold Change	*p*-Value	Error Factor	Biological Process
A. Up-Regulated					
gi|8393899	Inter-Alpha-Trypsin Inhibitor Heavy Chain H3	4.405549049	0.037132461	2.051162004	Proteolysis
gi|61098186	Fibrinogen Gamma Chain	3.53183198	0.04742229	1.213389039	Blood Coagulation/Angiogenesis
gi|56797757	Fibrinogen Alpha Chain Isoform 1	3.162277937	0.003749724	1.158776999	Blood Coagulation/Immune System Processes
gi|158138561	Complement C3	2.805433989	0.001147725	1.19124198	Complement Activation
gi|157786972	Coagulation Factor XIII B Chain	2.511885881	0.004626506	1.584892988	Blood Coagulation
gi|126722991	Inter-Alpha-Inhibitor H4 Heavy Chain	1.803017974	0.03057613	1.19124198	Proteolysis
gi|60097941	Haptoglobin	1.721868992	0.018680319	1.393157005	Complement Activation
gi|58865362	Alpha-2-Antiplasmin	1.367728949	0.059626061	1.406048059	Proteolysis
gi|307746876	Alpha-1-Macroglobulin	1.318256974	0.005542278	1.16949904	Complement Activation
gi|158186678	Fibrinogen Beta Chain	1.056818008	0.0104149	1.258924961	Blood Coagulation/Immune System Processes

Proteins significantly (p≤0.05) modulated by a ratio of 10:1 omega-3:omega-6 fatty acids (Error factor≤2).

Table 2.3. Proteins Modulated in Rat Plasma by a Ratio of 25:1 Omega-3:Omega-6 Fatty Acids

Accession No.	Protein	Fold Change	*p*-Value	Error Factor	Biological Process
A. Up-Regulated					
gi|17865327	Fetuin-B	10.09253025	0.003898517	1.721868992	N/A
gi|6978879	Vitamin D-Binding Protein	5.296635151	0.004626949	1.995262027	Transport
gi|162287337	Apolipoprotein E	4.920394897	0.002455108	1.976969957	Lipid Transport and Metabolism
gi|56797757	Fibrinogen Alpha Chain Isoform 1	3.311311007	0.002404082	1.202263951	Blood Coagulation/Immune system processes
gi|6978695	Ceruloplasmin	3.191538095	0.000434292	1.380383968	Transport
gi|157786972	Coagulation Factor XIII B Chain	2.679167986	0.0126862	1.721868992	Blood Coagulation
gi|61556986	Serotransferrin	2.466038942	0.003877083	1.116863012	Transport
gi|158138561	Complement C3	2.208004951	0.000559888	1.106624007	Complement Activation
gi|126722991	Inter-Alpha-Inhibitor H4 Heavy Chain	1.599557996	0.0213216	1.19124198	Proteolysis
gi|307746876	Alpha-1-Macroglobulin	1.445440054	0.002628269	1.202263951	Complement Activation
gi|158186678	Fibrinogen Beta Chain	1.16949904	0.002058916	1.330453992	Blood Coagulation/Immune System Processes
gi|16924006	Complement Component C9	1.047129035	0.03969308	1.106624007	Complement Activation
B. Down-Regulated					
gi|25453392	Alpha-1B-Glycoprotein	0.113762699	0.000374327	1.38038396	Response to Stimuli
gi|293348303	Hypothetical Protein	0.519995987	0.030652801	1.30617094	N/A

Proteins significantly modulated (p≤0.05) by a ratio of 25:1 omega-3:omega-6 fatty acids (Error factor≤2).

Table 2.4 illustrates 19 proteins were regulated by the dietary intervention of 25:1 n-3:n-6 fatty acids with tamoxifen according to our criteria ($p \leq 0.05$, $EF \leq 2$); 11 proteins were up-

regulated and 8 proteins were down-regulated. The statistically significant changes in observed iTRAQ ratios demonstrated that gelsolin was up-regulated, while A1BG was down-regulated further.

Table 2.4. Proteins Modulated in Rat Plasma by a Ratio of 25:1 Omega-3:Omega-6 Fatty Acids and Tamoxifen

Accession No.	Protein	Fold Change	*p*-Value	Error Factor	Biological Process
A. Up-Regulated					
gi|34867677	Serine Protease Inhibitor A3M	3.53183198	0.007889271	1.976969957	Proteolysis
gi|17865327	Fetuin-B	5.861382008	0.030004229	1.923092008	N/A
gi|7549773	Kininogen-1 Isoform 1	2.884032011	0.01903403	2.070141077	Blood Coagulation
gi|56797757	Fibrinogen Alpha Chain Isoform 1	2.728977919	0.004014052	1.127197981	Blood Coagulation/Immune System Processes
gi|13928716	Serine Protease Inhibitor A3N	2.728977919	0.037142001	1.887990952	Proteolysis
gi|157786972	Coagulation Factor XIII B Chain	2.443430901	0.01244645	1.659587026	Blood Coagulation
gi|162138905	Plasma Kallikrein	2.051162004	0.01194448	1.584892988	Proteolysis
gi|51854227	Gelsolin	1.803017974	0.041453529	1.690441012	Cell Component Morphogenesis
gi|58865362	Alpha-2-Antiplasmin	1.432188034	0.048097949	1.419057012	Proteolysis
gi|61556986	Serotransferrin	1.202263951	0.01927135	1.29419601	Transport
gi|158186678	Fibrinogen Beta Chain	1.116863012	0.005659904	1.16949904	Blood Coagulation/Immune System Processes
B. Down-Regulated					
gi|307746876	Alpha-1-Macroglobulin	0.839460015	0.047253091	1.106624007	Complement Activation
gi|6978879	Vitamin D-Binding Protein	0.672976673	0.0167256	1.330453992	Transport
gi|13591979	Leukemia Inhibitory Factor Receptor	0.416869402	0.02516003	1.67494297	Immune System Processes
gi|77539456	C4b-Binding Protein Alpha Chain	0.416869402	0.02253277	1.770109057	Complement Activation
gi|57164089	Serum Amyloid A-4 Protein	0.337287307	0.028261339	1.940886021	Immune system processes
gi|186972114	Fibronectin	0.169044107	2.08E-06	1.541700006	Cell Adhesion
gi|25453392	Alpha-1B-Glycoprotein	0.01499685	3.62E-07	1.30617094	Response to Stimuli
gi| 293348303	Hypothetical Protein	0.316227794	0.01446376	1.753880978	N/A

Proteins significantly modulated(p≤0.05) by a ratio of 25:1 omega-3:omega-6 fatty acids with tamoxifen (Error factor≤2)

5.1.3 Verification by Western Blot Analysis of Proteins Identified by iTRAQ

Next, we performed western blot analysis to verify the alterations in protein levels identified by iTRAQ. Several proteins were selected based on their known contributions to carcinogenesis and the availability of their antibodies. Gelsolin was found to be up-regulated in the 10:1 n-3:n-6, but significantly higher yet in the 25:1 n-3:n-6 group **(Figure 2.3A)**. A similar trend was seen with VDBP **(Figure 2.3B)**. However, the protein A1BG was found to be down-regulated in the 10:1 n-3:n-6, and was significantly lower in the 25:1 n-3:n-6 group **(Figure 2.3C)**. The protein 14-3-3 sigma, not identified by iTRAQ analysis but still of interest due to its well-known tumor suppressor activity in breast cancer[225-227] was observed to be increased significantly in the 10:1 and 25:1 n-3:n-6 groups **(Figure 2.3D)**.

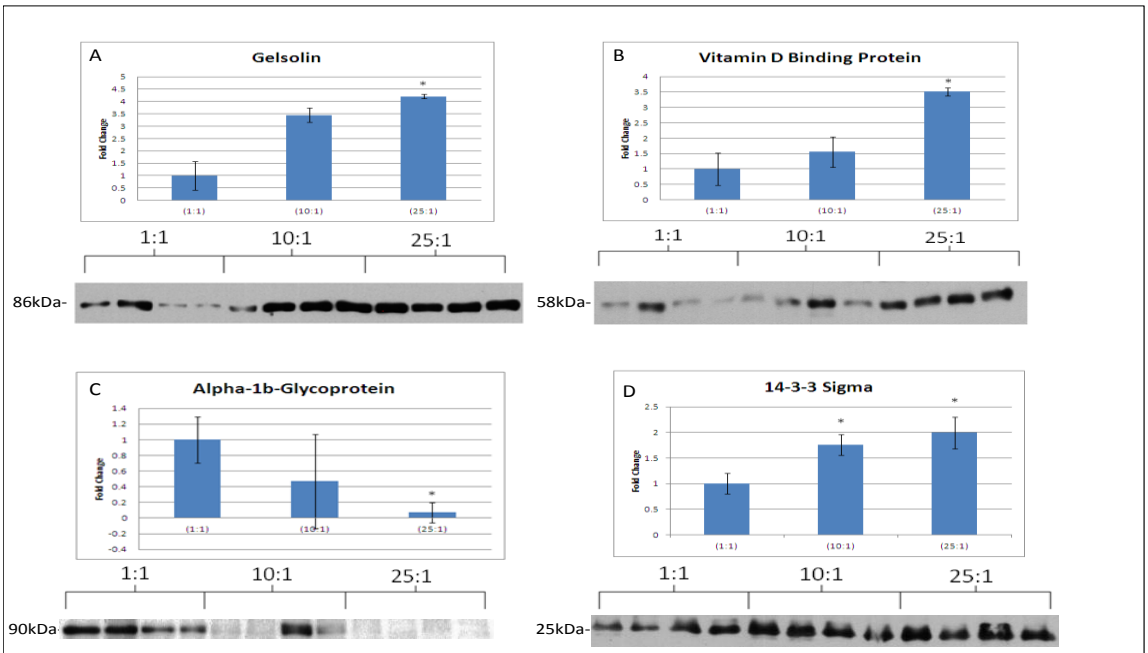


Figure 2.3-Western blot analysis of specific proteins for validation of iTRAQ analysis (comparison of 1:1, 10:1, and 25:1 n-3:n-6). A, gelsolin expression. B, vitamin D binding protein expression. C, alpha-1B-glycoprotein expression. D, 14-3-3 sigma expression; $p \leq 0.05$.

Proteins from depleted rat plasma representing the 25:1 and 25:1 n-3:n-6 plus tamoxifen groups were also analyzed by western blot analysis. Although the decrease in haptoglobin did

not reach significance, apolipoprotein E, and ITIH4 were significantly decreased in the plasma obtained from rats given a combination of 25:1 n-3:n-6 plus tamoxifen, in comparison to the 25:1 n-3:n-6 fatty acids alone, as shown in **Figures 2.4A**, **2.4B**, and **2.4C**.

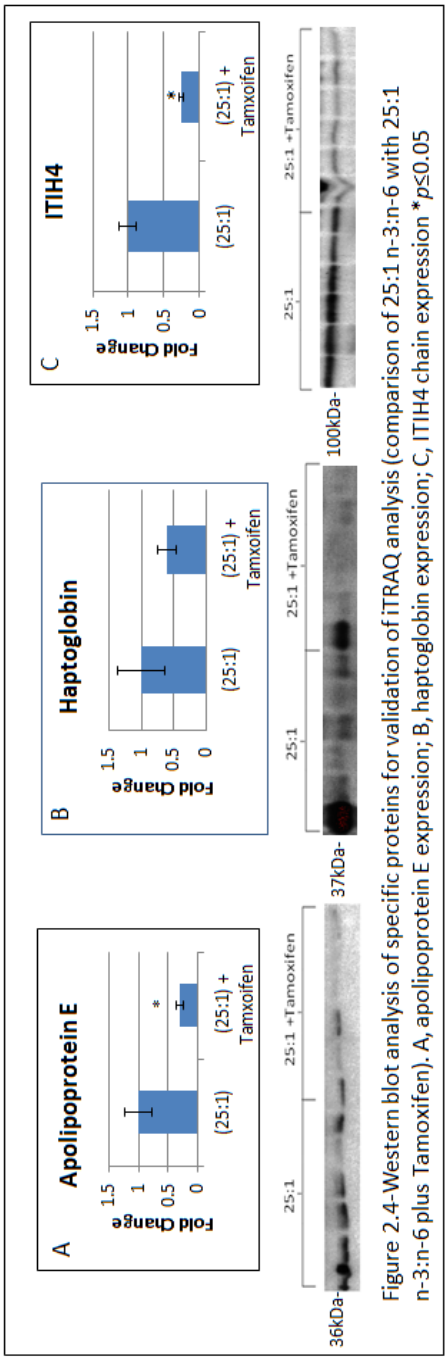


Figure 2.4-Western blot analysis of specific proteins for validation of iTRAQ analysis (comparison of 25:1 n-3:n-6 with 25:1 n-3:n-6 plus Tamoxifen). A, apolipoprotein E expression; B, haptoglobin expression; C, ITIH4 chain expression *$p \leq 0.05$

5.2 Biological Process Identification through Bioinformatics Techniques

5.2.1 Biological Significance of Proteins Modulated by n-3:n-6 Fatty Acid Ratios of 10:1, 25:1, and 25:1 plus Tamoxifen

All biological processes were defined through data according to PANTHER Classification System (Protein ANalysis THrough Evolutionary Relationships). Ten proteins identified in the 10:1 n-3:n-6 group are known to be involved in several biological processes that include complement activation (complement C3, haptoglobin, alpha-1-macroglobulin), proteolysis (complement C3, inter-alpha-trypsin inhibitor heavy chain H3, inter-alpha-inhibitor H4 heavy chain, alpha-2-antiplasmin, alpha-1-macroglobulin) and blood coagulation (fibrinogen gamma chain, fibrinogen alpha chain isoform 1, coagulation factor XIII B chain, fibrinogen beta chain). Additional biological processes that were determined by the PANTHER database are presented in **Figure 2.5A**.

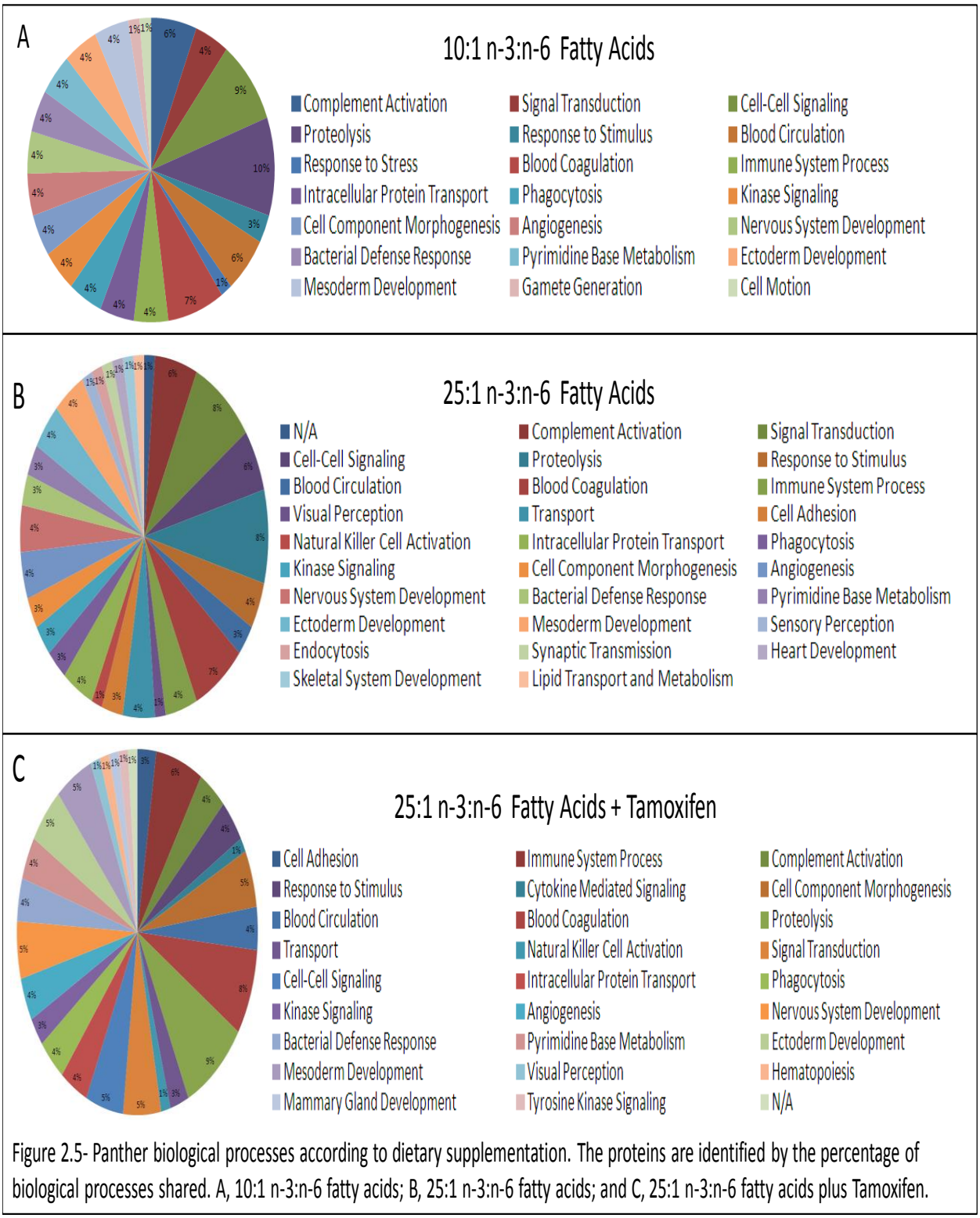


Figure 2.5- Panther biological processes according to dietary supplementation. The proteins are identified by the percentage of biological processes shared. A, 10:1 n-3:n-6 fatty acids; B, 25:1 n-3:n-6 fatty acids; and C, 25:1 n-3:n-6 fatty acids plus Tamoxifen.

In the 25:1 n-3:n-6 group, the biological processes of the 14 proteins identified include involvement with transport (VDBP, ceruloplasmin, serotransferrin), lipid transport and metabolism (apolipoprotein E), blood coagulation (fibrinogen alpha chain isoform 1, coagulation factor XIII B chain, fibrinogen beta chain), proteolysis (inter-alpha-inhibitor H4 heavy chain), complement activation (complement C3, complement component C9, alpha-1-macroglobulin), and responding to certain stimuli (A1BG). Fetuin B did not have a noted biological process according to PANTHER. A hypothetical protein with accession number 293348303 was also identified but its function still has to be determined. Additional biological processes that were determined by the PANTHER database are summarized in **Figure 2.5B**.

The plasma from rats administered a diet of 25:1 n-3:n-6 fatty acids plus tamoxifen had 19 proteins modulated. These proteins are known to be involved in immune system processes (fibrinogen beta chain, fibrinogen alpha chain isoform 1, leukemia inhibitory factor receptor, serum amyloid A-4), transport (VDBP, serotransferrin), proteolysis (plasma kallikrein, alpha-2-antiplasmin, serine protease inhibitors A3N and A3M), complement activation (C4b-binding protein alpha chain, alpha-1-macrogloblin), blood coagulation (kininogen-1 isoform 1, coagulation factor XIII B), cell adhesion (fibronectin), responding to stimuli (A1BG), and cell component morphogenesis (gelsolin). Fetuin B did not have a noted biological process according to PANTHER. A hypothetical protein with accession number 293348303 was also identified but its function still has to be determined. Additional biological processes that were determined by the PANTHER database are summarized in **Figure 2.5C**.

5.2.2 Ingenuity Pathway Analysis(IPA) of Proteins Quantitated via iTRAQ

Next, we were interested in determining a relationship amongst the proteins detected in our iTRAQ analysis. This would help us understand how their regulation is being controlled by

the various dietary interventions. Utilizing the program Ingenuity Pathway Analysis (IPA) allows for such relationships to be further investigated. Network functions that resulted in a Z-score higher than 3, was an indicator of high confidence when linking our proteins to that particular function. **Figure 2.6** illustrates the proteins that are unique and in common to 10:1, 25:1 n-3:n-6, and 25:1 n-3:n-6 plus tamoxifen groups identified by iTRAQ. These proteins have a p-value less than or equal to 0.05 and an error factor less than or equal to 2 when compared to 1:1 n-3:n-6.

IPA determined that the trend of regulation seen in some proteins in the 10:1 n-3:n-6 group were related to hematological system development and function. Specifically, the fold change seen in all proteins was related to this associated network function. IPA determined that the trends of specific proteins were related to lipid metabolism in the 25:1 n-3:n-6 group, while the 25:1 n-3:n-6 plus tamoxifen intervention included proteins involved in cancer and inflammation. Specifically, the increase in apolipoprotein E and VDBP were reported to be linked to lipid metabolism. In the 25:1 n-3:n-6 fatty acids plus tamoxifen group the decrease in A1BG and increase in gelsolin were linked to cancer.

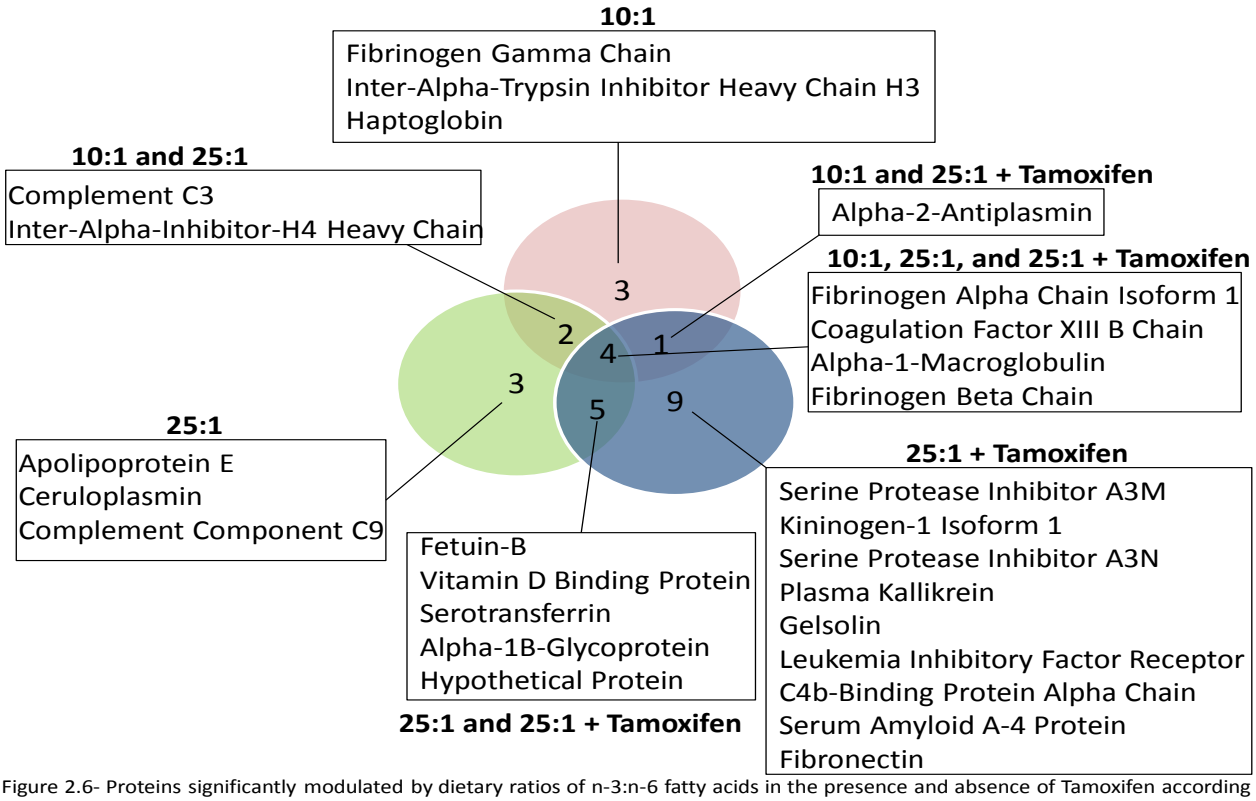


Figure 2.6- Proteins significantly modulated by dietary ratios of n-3:n-6 fatty acids in the presence and absence of Tamoxifen according to iTRAQ analysis ($p \leq 0.05$, ER≤2).

6. Discussion

To our knowledge this is the first study that examined the influence of effective chemopreventive amounts of omega-3 relative to omega-6 fatty acids on protein expression using iTRAQ method in a well-defined animal model of chemically-induced mammary carcinogenesis[71]. Our goal is to understand how ingestion of high amounts of omega-3 fatty acids in the absence and presence of tamoxifen influence the plasma proteome in a way that can provide insights into the mechanism of chemoprevention[71]. Furthermore, we focused on the plasma proteome, since it is a minimally-invasive, and consequently a practical method to measure biomarkers in clinical chemoprevention studies.

The results of the present study demonstrate that changes in plasma protein expression in rats that received high omega-3:omega-6 ratios with and without tamoxifen were consistent with chemoprevention[71]. In a previous study, we demonstrated[73] that the mechanism that can account for the chemopreventive activity of the high ratio of omega-3:omoega-6 (25:1) against the development of mammary cancer is due to the induction of apoptosis and more so the inhibition of cell proliferation in the rat mammary adenocarcinomas. In this previous report[73] we showed that in the target tissue (mammary gland) from the same animals from which plasma samples were collected using western blot analysis, changes in proteins involved in apoptosis, transcription regulation, growth factor regulation, and lipid metabolism were consistent with cancer prevention. Taken together the results of the present report (plasma proteins) and those of our previous study[73] (target organ proteins) provide a plausible justification of the potential utility of some of these proteins to monitor efficacy of omega-3 fatty acids as chemopreventive agents in clinical trials.

6.1 Proteins associated with Lipid Metabolism, Inflammation, and Cancer

We used the Ingenuity Pathway Analysis to investigate the biological role of the proteins altered by omega-3:omega-6 fatty acid ratios according to iTRAQ. We found that high ratios of omega-3:omega-6 fatty acids with and without tamoxifen affected plasma levels of proteins involved in lipid metabolism, inflammation, and cancer. It is proposed that omega-3:omega-6 fatty acid ratios influence the synthesis of lipids which is correlated with mammary tumor growth in MNU-treated rats[73].

i. Fetuin B

We showed that omega-3:omega-6 ratios of 10:1 and 25:1 up-regulated Fetuin B. Fetuin B, a glycoprotein of the fetuin family, has been shown to be involved in fatty acid metabolism in human liver cell lines as supported by the observation that pACC and pAMPK were down-regulated in fetuin B knockdown cells [228]. In knock-down studies with liver cell line, CCL-13, the decrease in fetuin B increased the expression of mRNA encoding malate dehydrogenase enzymes(*ME1, ME2, ME3*) as well as *ACC1* and *FASN*[228], known to be involved in fatty acid metabolism. It has also been shown to be decreased in liver during the acute phase of inflammation[229]. Therefore, the up-regulation of fetuin by high omega-3:omega-6 fatty acid ratios may provide a mechanism for the induction of pACC and pAMPK by high omega-3:omega-6 ratios as recently reported in mammary adenocarcinoma in the rat treated with MNU[73].

ii. Apolipoprotein E

Apolipoprotein E, responsible for fat and cholesterol transport, was down-regulated in the serum of rats given an omega-3:omega-6 dietary ratio of 25:1 in combination with tamoxifen, when compared to rats given only the 25:1 n-3:n-6 ratio, thus independently validating the iTRAQ results. There are four allelic variants of the apolipoprotein E gene (ε1, ε2, ε3, ε4,) with the ε4 gene being associated with breast cancer among Asian population[230]. High levels of APO E4 isoform are associated with high triglycerides(LDL, HDL, cholesterol) in breast cancer patients[231].

The apolipoprotein E genotypes are known to influence an individual's level of fatty acids, for example, there is a 4-12% variance in LDL-cholesterol concentrations depending on genotype[232]. This is further supported by the LDL-cholesterol concentration being 30% lower in people with the E2 genotype in comparison to the E4 genotype[233].

Currently, clinical studies have shown more support for certain apolipoprotein E genotypes effecting the lipid protein response to omega-3 fatty acids[234]. In patients with hypertriacylglycerolaemia, a supplement of 3g DHA/EPA day was found to decrease TAG in patients with the E2 genotype. Patients with the E3 genotype in response to supplementation showed a decrease in HDL-cholesterol and an increase in LDL-cholesterol. In another study with healthy subjects given 1.8g DHA/EPA daily the TAG levels were significantly decreased in the E4 genotype. It is hypothesized that the E2 and E3 genotypes have more affinity for HDL, lower in lipid content, while E4 genotype had an affinity for VLDL and TAG[235].

The phenotype of the APO E can influence the lipid profile of breast cancer patients, with the APO E4 isoform leading to increased triglyceride levels[231, 236]. The risk of developing breast cancer has been linked to higher triglyceride levels. Increased triglyceride levels have been attributed to a decreased in levels of sex hormone binding globulin, leading to high levels of estradiol and an increased risk of breast cancer [236, 237]. In addition, hypertriglyceridemia could lead to insulin resistance [236] linked to increased sex steroid levels in association with decreased sex hormone binding globulin levels, as well as with increased bioactive levels of insulin-like growth factor I, which may act synergistically with estrogen in promoting breast cancer[238]. However, APO E4 protein expression can influence tamoxifen's effect on the triglyceride levels in women with breast cancer, specifically plasma lipid levels that include a decrease in HDL cholesterol and triglycerides after 6 months of treatment[239]. In other studies

women given tamoxifen have shown the same increase in serum lipids independent of the APO E phenotype[240-242]. Overall, results are mixed with respect to tamoxifen's effect on TAG in individuals with the APOE4 genotype.

The PPARγ agonist, pioglitazone, and RXR agonist, 6-OH-11-O-Hydroxyphenanthrene, have shown to decrease expression of apolipoprotein E at the mRNA and protein level in MCF-7 cells[243]. APO E decrease was also shown with a decrease in Hif-1α in hypoxic MCF-7 exosomes(cancer stem cells) after treatments with the PPARγ and RXR agonists indicating that a decrease in APO E may also have an anti-inflammatory effect[243]. It is well known that omega-3 fatty acids are activators of the PPARγ pathways, thus a connection between APO E4 and PPARγ could mediate protective effects in the inflammatory and fatty acid synthesis pathways.

APO E and its relation to DHA homeostasis has also been evaluated in various mouse models expressing the different types of APO E genes given a diet with an omega-3:omega-6 fatty acid ratio of 7.69:1 for up to a year[244]. In homozygous APO E4 mice ALA and DHA were significantly lower in adipose tissue and in the liver than in the same organs of APO E3 mice. Also in the same APO E4 mice fatty acid transporter protein, FATP1, in the adipose tissue was 30% higher than in any other mouse genotype. In the liver the levels of FATP5 and FATP1 were 2-fold higher in APO E4 mice than in APO E2. However, this low amount of ALA and DHA may be resulting from an increase in carnitine palmitoyltransferase 1 (CPT1) which increases the β-oxidation of ALA in the liver.

iii. Haptoglobin

Haptoglobin, is an acute phase protein synthesized and dispersed by the liver, muscle, and adipose tissue and has a role in inflammatory states, such as obesity and cancer[245, 246]. Some studies[246] but not others[247, 248] have reported an increase in the level of this protein in the serum of breast cancer patients. Consistent with the iTRAQ analysis, haptoglobin was down-regulated in response to an increase in n-3 fatty acid administration, with a more pronounced effect in the group given high n-3 fatty acids with tamoxifen.

In Wistar rats administered a high fat diet with and without EPA ethyl ester by oral gavage(1g/kg body weight) for fifteen days to determine the effects of obesity on inflammatory markers at the mRNA level[249]. The rats only given the high fat diet were found to have high levels of haptoglobin in serum but not adipose tissue. In the rats given the high fat diet and EPA the protein expression of haptoglobin in the serum was the same as rats that were fed a normal chow diet. Levels of triglycerides, cholesterol, and insulin were reduced in the rats given EPA in comparison to rats given high fat diet alone, suggesting that insulin resistance could be alleviated.

Another study found that a more supportive role of haptoglobin mRNA levels in the adipose tissue with regards to inflammation[250]. In transgenic mice, expressing the TNF-α in the white adipose tissue, haptoglobin at the mRNA level were much higher than in non-transgenic animals, eluding that, tumor necrosis factor alpha, TNF-α, expression directly impacts the expression of haptoglobin. In 3T3-L1 adipocytes, the addition of lipopolysaccharide, (LPS), interlukin-6 (IL-6) or TNF-α, increased the expression of haptoglobin mRNA by 2.5-fold, 7-fold, and 2.5-fold over adipocytes that were not treated, indicating that inflammatory molecules

increase expression of haptoglobin, at least at the mRNA level[251]. In addition, the PPARγ agonist, rosiglitazone, was also able to reduce the haptoglobin mRNA levels in 3T3-L1 adipocytes, by 5-fold in comparison to control adipocytes. Thus, there seems to be a link between haptoglobin and inflammation, and this inflammation may be reduced upon PPARγ activation.

Tamoxifen, given as an adjuvant therapy, has also affected the levels of haptoglobin in postmenopausal breast cancer patients. Following a three month administration of the drug statistically significant decreases were observed in haptoglobin protein expression in the patient's serum[252].

iv. Vitamin D Binding Protein

Vitamin D binding protein, (VDBP), is involved in calcium homeostasis, immunity [253, 254], fatty acid transport and chemotaxis[255, 256] and anti-angiogenic[257] as well as anti-proliferation processes[258]. The protein is primarily synthesized in the liver and secreted into the plasma, where it binds to vitamin D and known to be taken up by human mammary cells via endocytosis[259]. With regards to immunity, VDBP is involved in macrophage activation by having its branched trisaccharide complex hydrolyzed by beta-glactosidase on B cells and salidase from T cells forming the macrophage activating factor (GcMAF). In patients with metastatic breast cancer it was found that administration of VDBP in the form of GcMAF decreased Nagalase activity, an indicator of macrophage activation that can lead to cell death[253]. *In vitro* GcMAF treated MCF-7 cells had macrophage-induced apoptosis after 72 hours of treatment [260].

A major role of VDBP is to transport vitamin D to peripheral tissues and to facilitate the tissue uptake of vitamin D[259]. This uptake occurs by 25-hydroxycholecalciferol(25D3) binding to VDBP in the blood stream, and then taken to the tissue of interest, where megalin and cublin and adapter protein disable-2(Dab2) bind the VDPB-25D3 complex and allow for cellular uptake in the form of receptor mediated endocytosis[261]. This protein has been noted as having a protective effect in breast cancer by facilitating transport of vitamin D metabolites[221] and has been shown to be down-regulated in the serum of breast cancer patients[262]. Some studies suggest that specific genetic variants of VDBP influence a woman's risk of developing breast cancer[263]. In the aggregate, our results show an increase in plasma levels of VDBP in rats fed a high omega-3:omega-6 fatty acid ratio which provides support for an additional possible mechanism of chemoprevention by omega-3 fatty acids.

VDBP has also been shown to be involved in lipid metabolism[264]. This protein has been shown to play a role in fatty acid transport and chemotaxis in diseases such as obesity-related diabetes[255]. It has been demonstrated *in vitro* that VDBP can bind to unsaturated fatty acids, possibly regulating their transport[265]. In elderly men VDBP concentrations have been shown to be directly correlated to body mass index, fat mass, and leptin level[266]. Elevated levels of VDBP have also been reported to be elevated in rats administered a high fat diet[267]. Certain mutations of the VDBP gene were significantly associated with fat content in humans[268]. In obese prone (OP) rats the levels of VDBP have been elevated in comparison to levels in normal rats making the argument that VDBP level can be used as a plausible biomarker for the etiology of obesity[264].

v. Gelsolin

In the present study we found gelsolin to be significantly up-regulated by high omega-3:omega-6 fatty acid ratios. The role of omega-3 fatty acids in the up-regulation of gelsolin is also supported by the observation that restriction of omega-3 fatty acids leads to gelsolin cleavage by caspases in the brains of a mouse model of Alzheimer's disease[269]. Its expression has been shown to be reduced in various human cancer cell lines and both human and chemically-induced rodent(i.e. rat and mouse) mammary tumors.[262, 270]. Studies utilizing MMTV-Her2/neu transgenic mice have shown down-regulation of gelsolin at the mRNA level in primary mammary tumors in comparison to the normal mammary gland[271]. Furthermore, it was demonstrated that gelsolin expression was reduced in human mammary lesions as the disease progressed; atypical ductal hyperplasia, ductal carcinoma in-situ, and invasive carcinoma[270, 272, 273]. In contrast, however, another report indicated that gelsolin levels were increased in the plasma of breast cancer patients[274], suggesting the role of gelsolin in carcinogenesis needs to be further investigated.

Gelsolin, a protein involved in actin polymerization in the nucleus, has been shown to be cleaved by caspases in the brain of Tg2576 mice after ingesting a diet of low DHA content[275]. Mechanistic studies have shown that gelsolin binds to the estrogen receptor after activation with 17-beta-estradiol in MCF-7 cells[276]. The interaction of ER-alpha with actin monomers is hypothesized to stabilize globular actin (g-actin) leading to filamentous actin (f-actin) formation in the process known as β-actin polymerization. Furthermore, it is hypothesized that ER-alpha and β-actin regulated transcription of ER-response genes influencing the pre-initiation complex formation and promoter activity, elongation, and ribnucleoprotein complex formation[277].

Gelsolin is known to take part in this process by severing and capping the end of actin filaments, controlling its polymerization, and apoptosis[278-280]. It is known that gelsolin is epigenetically down-regulated in MDA-MB-231, MCF-7, and T47D breast cancer cells, but not in normal human mammary epithelial cells, HMECS [279]. Upon treatment of the breast cancer cell lines with trichostatin A (TSA), a histone deacetylase inhibitor, the epigenetic pattern was reversed to what is seen in human mammary epithelial cells (HMECs) and caused an increase in gelsolin mRNA and protein expression. Following the treatment with the histone deacetylate inhibitor(TSA) the breast cancer cells had a nuclear morphology that was consistent with the process of apoptosis[279].

Gelsolin lacks a nuclear localization signal allowing it to co-translocate to the nucleus by binding to proteins[281]. It is known to interact with the androgen receptor and translocate to the nucleus, and in tumor cells it was determined that gelsolin aided in androgen receptor mediated growth by increasing its expression and interaction with AR[282].

vi. Alpha-1B-Glycoprotein

Our studies also confirmed that A1BG was down-regulated in a manner consistent with chemoprevention. A1BG is known to bind to CRISP-3[283] and is partially similar in amino acid sequence to the opossum protein oprin, a metalloproteinase[284]. In the serum of breast cancer patients A1BG was shown to be slightly increased in expression in comparison to healthy controls[223]. In pancreatic juice and pancreatic tissue of pancreatic cancer patients A1BG was up-regulated in comparison to cancer-free controls[285]. A1BG has also been shown to be elevated in the urine of bladder cancer patients compared to controls[226].

vii. Inter-Alpha-Tryspin-Inhibitor Heavy Chain 3/4

In this study, inter-alpha inhibitor H4 heavy chain (ITIH4); a protein secreted by the liver in times of trauma and involved in the acute phase response[286], was shown to have a downward trend as the amount of omega-3 fatty acids increased in the dietary groups. In serum from breast cancer patients it was shown that this protein as peptide fragments was up-regulated in comparison to healthy controls[287, 288]. ITIH4 has also proposed to be cleaved by plasma kallikrein in cancers such as ovarian and breast[288, 289] which could possibly explain the bands seen at 120kda and 100kda. However, it has been determined that ITIH4 is correlated with incidence of hypercholesteremia, specifically a particular homozygous point mutation[290]. It has also been shown to be up-regulated in plasma of Sprague Dawley rats that were prone to obesity[291]. Thus, the downward trend of the expression of ITIH4 is consistent with chemoprevention. Furthermore, the effects of tamoxifen on the expression of ITIH4 lead to the decrease in expression, potentially to tamoxifen having an anti-inflammatory effect.

ITIH3, inter-alpha-trypsin-inhibitor heavy chain 3, is known to bind to hyaluronic acid to form a component of the extracellular matrix. ITIH3 when over-expressed in human lung cancer cell line (H460M) was able to inhibit tumor growth and metastatic spreading[292]. Furthermore, ITIH3 is known to be down-regulated in cancers of the colon, lung, and breast[293, 294]. In our study we showed that this protein was increased in response to high n-3:n-6 ratio possibly playing a role in an inhibitory response against tumor growth.

viii. 14-3-3 sigma

Although not detected by iTRAQ, we showed by western blot analysis that a high omega-3:omega-6 ratio increased the plasma level of 14-3-3 sigma, a well-established tumor suppressor gene[227]. This protein has been shown to be down-regulated in many types of cancer including breast cancer[228, 295]. It is thought that hypermethylation at CpG islands at the gene promoter of the 14-3-3 sigma causes the down-regulation of the protein in breast cancer patients[296]. It was recently determined that DHA, a component of omega-3 fatty acids, increased the amount of 14-3-3 sigma protein expressed in colon cancer cells[297]. This increase in 14-3-3 sigma has led to an increase in the pro-apoptotic protein p38 MAPK leading to apoptosis in colon cancer cells[297]. Collectively, these data raise the possibility that omega-3 fatty acids may up-regulate 14-3-3 sigma through an epigenetic mechanism.

ix. Coagulations Factors (fibrinogen, Coagulation Factor XIII and Alpha-2-Antiplasmin

A relationship between coagulation and angiogenesis has been hypothesized[298, 299]. It has also been hypothesized that active coagulation can influence tumor stroma formation in breast cancer[300]. In one study an increase in fibrinogen and thrombin-antithrombin has been found to be indicative of hypercoagulation in breast cancer patients[301, 302]. The presence of procoagulatant molecules in the tumor and surrounding environment promote fibrinogen deposition and coagulation[303], all which is initiated by inflammatory pathways[304]. In a study by Tas et al., in peripheral blood samples of patients with both triple negative and ER+/PR+ tumors, fibrinogen was shown to be elevated in 52% and 64% of the patients,

respectively, but was not found to be significant[304]. Increases in fibrinogen were also seen in early and advanced staged breast cancer patients[305].

Coagulation factors of the X family have shown to be increased in expression as a result of tissue factor activator complexes excreted by tumor tissue[305]. The omega-3 fatty acid, EPA, has shown to decrease the amount of coagulation factors VII-X in male Wistar rats, indicating that it plays a role in blood coagulation[306]. However, other coagulation factors, such as fibrinogen, have not shown a correlation between omega-3 fatty acid intake and protein expression in the serum[306, 307] while one study has shown a correlation[308]. In addition, tamoxifen(10mg/day) and raloxifene(10mg/day) given to breast cancer patients for six weeks have shown to significantly reduce fibrinogen levels[309]. The same study also saw significant reductions in antithrombin III and cholesterol by the SERMs[309]. In other studies where fibrinogen was modulated negligibly by tamoxifen in breast cancer patients, there was a decrease in alpha-2-antiplasmin[310]. The decrease in alpha-2-antiplasmin was also seen in healthy patients administered the omega-3 fatty acid, MaxEPA[311]. The fibrin degrading enzyme PAi-1 has also shown to decrease in protein expression in the serum as a result of fish oil intake in patients with hypercholestremia, thus reducing inflammation. Considering that fibrinogen is the precursor to fibrin this downstream enzyme may also effect fibrinogen concentrations and the overall inflammatory response in the blood[312]. In our study increases in fibrinogen alpha, beta, and gamma chains, alpha-2-antiplasmin, and coagulation factor XIII B were seen in response to increasing omega-3:omega-6 ratio, suggesting a possible role in the coagulation and inflammatory responses.

x. *The Immune System (Complement C3 and C4, Plasma Kallikrein, and Kininogen-1)*

Complement C3, a protein involved in the complement pathway, is involved in both the innate and classical immune response. Cleavage of the C3 protein by C3-convertase results in the formation of C3a and C3b, which act as a precursor to some cytokines and an opsonizing agent, respectively. In breast cancer complement C3 has its expression at the mRNA level reduced by 4-hydroxytamoxifen in MDA-MB-231 cells. Via a luciferase reporter assay the decrease in expression, as a result of the AF-2 domain of the estrogen receptor alpha subunit being inhibited from binding to the promoter region of the complement C3 gene, was observed[313]. Thus, it was hypothesized that tamoxifen would have an inhibitory effect on the expression of the complement C3 protein. DHA was also shown to decrease the amount of complement C3 in healthy men administered the 15g/day docosahexaenoic acid triglyceride (DHASCO) for at least 90 days, however, this decrease in comparison to controls was not statistically significant[314]. DHA in these men, however, did decrease the amount of polymorphonuclear leukocytes (PNMs) in circulation. The decrease in PNMs in circulation could be an indicator of a decrease in the inflammatory response[314].

Another mechanism of complement C3 is lipid metabolism. In obese adults high levels of C3 have been correlated with high body mass index(BMI), abdominal fat distribution, blood pressure, triglycerides, blood glucose and metabolic syndrome[315]. Obesity is a chronic state of inflammation where high levels of C3 are regulated by IL-6 and IL-1B. It is thought that this increase of C3 is due to IL-6 and IL-1B response elements in the promoter of the C3 gene, particularly where the farnesoid X receptor, FXR, can bind once stimulated[316]. In addition, the cleavage products of C3, C3b and C3a-desArg, can alter adipocytes to increase triglyceride

metabolism, elevating triglyceride levels and increasing insulin resistance in obese individuals. Complement C3 can also activate C5 to form C5a which can influence adipose tissue to initiate an inflammatory response with macrophages and mast cells increasing cytokine production leading to tissue inflammation in obesity[317, 318]. Complement C4 has also been shown to be involved in lipid metabolism, particularly in the alternative pathway of complement activation, which can be triggered by adipose tissue[264]. Complement C3 and C4b were both increased in our study in response to high omega-3:omega-6 ratio suggesting their role in inflammation. However, the decrease in c4b binding protein expression, with the addition of tamoxifen to the high omega-3 diet, suggested that tamoxifen could be causing an anti-inflammatory response, leading to a significant decrease in expression of c4b binding protein.

Plasma kallikrein(PK), known as a kininogenin, are serine proteases found in the blood plasma that are involved in coagulation, inflammation, and the alternative complement pathway[319-321]. Specifically, PK can cleave bradykinin which can cause vascular leakage and can also initiate coagulation[320]. In addition, PK can substitute for factor D of the intrinsic complement pathway and aid C3 convertase to cleave C3 into C3a, C3b, and C5 leading to inflammation and possibly metastasis[320, 322]. PK is known to be involved in the prothrombin pathway, which is responsible for coagulation[322]. It's this coagulation that can lead to tumor angiogenesis and metastasis through the generation of fibrin by thrombin cleavage of fibrinogen. This leads to the attraction of endothelial cells and the up-regulation of VEGF on the surface of these cells, which activates HIF-1α to promote the production of several angiogenic factors such as platelet-derived growth factor(PDGF) and angiopoietin-1(ANG-1)[299]. Kininogen-1, a serine protease of the killikrein family, has been reported as being up-regulated in colorectal cancer[323] and urine of ovarian cancer patients[324]. It plays similar roles as plasma kallikrein

in thrombosis, fibrinolysis, inflammation[298], and oncogenesis[325]. Both proteins were increased in response to an increase in the omega-3:omega-6 ratio which could be indication of an inflammatory response.

xi. Serum Amyloid A

Serum amyloid A is an acute phase protein synthesized in hepatocytes and adipose tissue[326, 327] and stimulates the inflammatory response through initiating pathways involving IL-6, TNF-alpha, and IL-1[328, 329]. Levels of serum amyloid A have been found to be increased in women with obesity, specifically positively correlating with a larger waistline, very high BMI, and low HDL levels[330]. In addition, levels serum amyloid A were found to be increased in patients with ER- tumors in comparison to ER+ tumors. This increase in serum amyloid A has been seen in other studies investigating ER- and ER+ tumors[331]. Tamoxifen has shown to decrease serum amyloid A production in monocyte/macrophages by reducing CD36, a receptor that binds LDL and induces SAA production. This process is hypothesized to cause chronic inflammation in fat tissue, but it has yet to be observed in breast adipose tissue[332]. Omega-3 fatty acids are also known to decrease cytokine-induced lipolysis in adipocytes by reducing SAA concentrations, resulting in reduced inflammation[333]. This protein was down-regulated in response to both high omega-3:omega-6 with and without tamoxifen, which could explain an anti-inflammatory response.

xii. Fibronectin

Omega-3 fatty acids(2g/day) from marine and non-marine sources were able to decrease fibronectin expression which positively correlated with an increase in HDL in healthy

males[334]. *In vitro* studies with MDA-MB-231 cells have demonstrated that EPA in a dose dependent fashion can decrease cell adhesion to fibronectin, possibly through decreasing PKC expression, which has been demonstrated to decrease cell adhesion to the endothelium[335]. Possibly a decrease in fibronectin expression could also decrease cell adhesion and cell metastasis. Fibronectin is thought to promote tamoxifen resistance in M05 breast cancer tumors derived from BalB/C mice. After fibronectin treatment, cells that were resistant to tamoxifen had higher amounts of β1 integrin, a receptor for fibronectin, and there was an increased phosphorylation of the FAK protein and activation of MAPK/ERK 1/2 and PI3K/AKT[336]. In MCF-7 cells treated with fibronectin, tamoxifen resistance did occur which was found to be attributed to increased phosphorylation of ER-α which could be a result of the MAP/ERK 1/2 pathway [336]. Fibronectin expression was reduced significantly in the 25:1 n-3:n-6 plus tamoxifen group.

xiii. Ceruloplasmin

Ceruloplasmin is a known a copper-transport protein of the blood that can have ferroxidase activity. In female breast cancer patients the ceruloplasmin protein was found to be elevated following adjuvant therapy with tamoxifen[252, 337]. In pancreatic cancer patients given a fish oil supplement(2g EPA+ 1g DHA/day) for three weeks the ceruloplasmin protein was decreased in comparison to pancreatic cancer patients that did not receive the supplement[338]. Ceruloplasmin has been connected to LDL oxidation *ex vivo*[339]. Its Cu(II) metal ion is thought to interact with LDL result in oxidation of fatty acid chains[339], possibly an initiator of inflammation. In our study this protein was also increased in response only to the 10:1 n-3:n-6 ratio, possibly playing a role in the inflammatory response. With the addition of

tamoxifen to the high omega-3:omega-6 ratio the expression of ceruloplasmin was decreased indicating that tamoxifen could be having an anti-inflammatory effect.

xiv. Serotransferrin

Serotransferrin is a protein responsible for iron transport and homeostasis. In hemodialysis patients given 180mg EPA + 120mg DHA daily for four months, there was a minimal decrease in transferrin in comparison to patients given a placebo[340]. In oesophageal and breast cancer patients given an EPA supplement the level of serotransferrin did not change[341]. However, pancreatic cancer patients given an 2g EPA + 1g DHA/day for three weeks showed a significant increase in serum transferrin levels, in comparison to the control group given no supplementation[338]. Increased serum transferrin levels have been positively associated with insulin resistance, impaired glucose metabolism and hyperglycemia in older(60+) healthy humans[342]. Tamoxifen has not shown to alter the expression levels of serotransferrin in cancer patients[343]. Our study illustrated that the 25:1 n-3:n-6 diet increased serum transferrin, similar to literature data[338].

7. Summary and Concluding Remarks

The purpose of studies performed in **Chapter 2** is to gain insight into the mechanism that can account for chemoprevention at the protein level by a high omega-3:omega-6 ratio in the absence and presence of tamoxifen using an established proteomic approach (iTRAQ). The outcome of our iTRAQ analysis determined that proteins with diverse biological processes were being altered in a fashion that favored chemoprevention by the high omega-3:omega-6 fatty acid ratio. However, this was only true for some proteins, while others were being altered in a manner that was consistent with inflammation and coagulation, a response that can be mediated by

omega-6 fatty acids; a known tumor promoter. Thus our method, not being biased, allowed us to gain detailed information into the many mechanisms that fatty acids can have on the expression of blood plasma proteins in a well-defined rat model of mammary cancer.

As reported by our group[71, 73], the same high n-3:n-6 ratio (25:1) was found to have a protective effect on tumor incidence and multiplicity and further studies in the same rat model supported the high omega-3:omega-6 ratio as favoring a decrease in proliferation and an increase in apoptosis in mammary adenocarcinomas. Therefore seeking a minimally invasive approach that can be translated to humans we focused our efforts on elucidating the mechanisms that can account for chemoprevention based on expression levels of plasma proteins involved in cell proliferation, apoptosis, inflammation, cell cycle regulation, and lipid metabolism.

The expression level of several proteins in rat plasma in response to high omega-3:omega-6 ratios **(Figure 2.7)** included VDBP, haptoglobin, gelsolin, fetuin B, and 14-3-3 sigma. All proteins were altered by the high omega-3:omega-6 fatty acid ratio in a way that favored chemoprevention. Vitamin D binding protein, was found to be up-regulated in response to the high omega-3:omega-6 fatty acid ratio. VDBP is a protein of diverse biological function. The Gc-MAF structure is a macrophage-inducer of apoptosis in breast cancer cell lines, and VDBP can also transport fatty acids to various organs. It is known that the increase in vitamin D at the breast tissue can protect against breast cancer[253-255, 257, 258, 260, 263].

Haptoglobin, is known to play a role in the inflammatory response, which may explain how Jiang et al, [73] demonstrated the decrease in Hif-1α in mammary adenocarcinomas from the same animals. The literature has supported gelsolin decreasing in tissue and serum samples of breast cancer patients and attributes it decrease to a decrease in cytoskeletal organization which could lead to cancer development. Gelsolin was significantly increased in response to the

high omega-3:omega-6 ratio. Fetuin B was found to be up-regulated, which may explain the up-regulation of pAMK, and pACC in mammary adenocarcinomas[73], considering that fetuin B expression has been known to effect the expression of proteins involved in fatty acid metabolism[228]. 14-3-3 sigma, although not detected by ITRAQ, was found to be up-regulated by western blot. This protein is known to directly regulate pathways of the cell-cycle and apoptosis, which can explain how p21, p27 were up-regulated and cyclin D1 down-regulated in adenocarcinomas[73]. Furthermore, the increase in Bax, a pro-apoptotic protein found in adenocarcinomas[73], can be a result of 14-3-3 sigma up-regulating the p38 MAPK, which can directly up-regulate Bax protein.

To use a single protein as a biomarker would not explain the overall effects of omega-3 on the many pathways it can alter in the plasma and breast tissue. Therefore, a panel of proteins to explain the effects of high omega-3 fatty acids is needed. From the data in our study additional proteins that could be evaluated in addition to VDBP include, gelsolin, haptoglobin, 14-3-3 sigma, fetuin B, and A-1B-G. Therefore, VDBP, gelsolin, haptoglobin, 14-3-3 sigma, fetuin B, and A-1B-G proteins could be potentially used as a protein panel to evaluate the effects of high amounts of omega-3 fatty acids in both preclinical models and in the clinic.

However, even though VDBP, gelsolin, 14-3-3 sigma, fetuin B, and A-1B-G were increased in response to a high omega-3:omega-6 ratio it was decreased as a result of adding tamoxifen. This is not entirely unexpected because the addition of selective estrogen receptor modulator could influence other pathways that favor chemoprevention in a different manner. Considering that the effects of the high omega-3:omega-6 ratio with the addition of tamoxifen were superior to high omega-3:omega-6 ratios alone, an alternative mechanism that can account for prevention is depicted in **Figure 2.7**. With the addition of tamoxifen proteins involved in the

inflammatory process such as haptoglobin, serum amyloid A, c4b binding protein, and ceruloplasmin were all down-regulated. Furthermore, ITIH4, a protein linked to lipid metabolism, was down-regulated by the addition of tamoxifen to the high omega-3 diet.

Haptoglobin is found up-regulated in adipose tissue and plasma in state of chronic inflammation, known as obesity[245]. Specifically, cytokines such as IL-6, IL-1, and TNF-α are known to directly up-regulate haptoglobin at mRNA level. Haptoglobin also responds to PPARγ activation; PPARγ agonist decreased the expression of haptoglobin upon activation. Furthermore, tamoxifen is known to decrease its expression in the serum of breast cancer patients[252]. Thus, considering that haptoglobin was decreased further with the addition of tamoxifen to the high omega-3:omega-6 ratio in our study, it would be possible that this decrease reflects the reduction in the inflammatory state. Serum amyloid A, in patients given tamoxifen, has decreased in protein expression, possibly due to reducing macrophage activity[330]. C4b binding protein, involved in the complement pathway of inflammation was decreased by the combination approach, which is not unexpected since tamoxifen was able to reduce the expression of this protein in human breast cancer cell lines[313]. Ceruloplasmin, a copper-transport protein, was found to decrease in expression in the rats given tamoxifen, thus it seem that tamoxifen had a chemopreventive effect on this protein, which in literature has shown to be elevated in patients given tamoxifen as adjuvant therapy[252, 337]. ITIH4, a protein linked to lipid metabolism, has shown to be increased in patients with hypercholesteremia[290], however, the effects of tamoxifen have not been studied on the expression of this protein in the literature. The addition of tamoxifen led to a significant decrease in protein expression of ITIH4, potentially due to a decrease in lipid metabolism. Therefore, the protein panel including haptoglobin, serum amyloid A, c4b binding protein, ceruloplasmin, and ITIH4 could be utilized

as potential protein biomarkers in the preclinical models and in the clinic to monitor the effects high omega-3:omega-6 administration individually and in combination with tamoxifen.

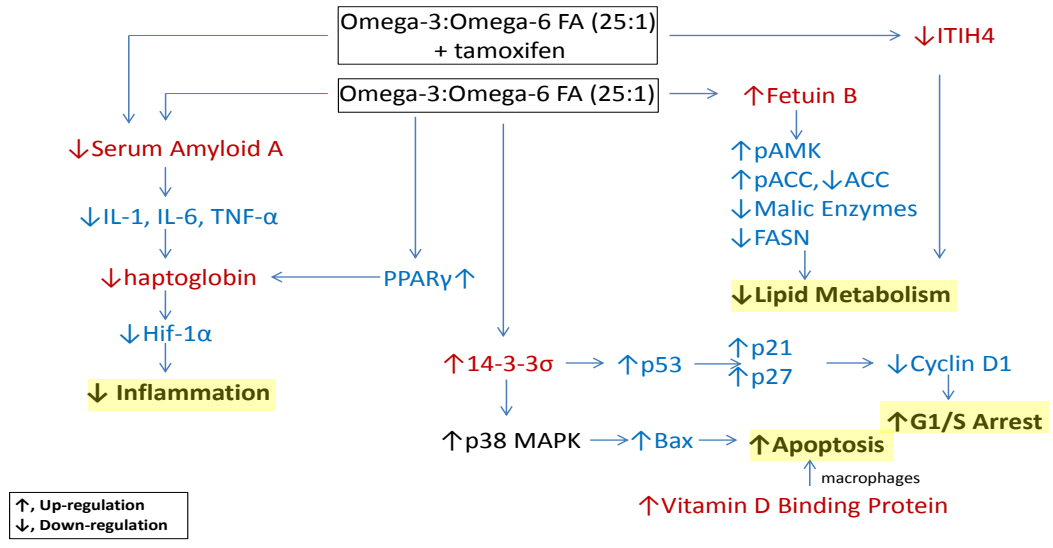


Figure 2.7- Possible mechanism of protein expression in plasma of Sprague Dawley rats as influenced by 25:1 n-3:n-6 ratio with and without tamoxifen. Proteins in blue were altered in mammary adenocarcinomas in rats treated with MNU, while proteins in red were altered in plasma obtained from the same animals. Proteins in black were found to be linked to apoptosis according to literature.

Overall, the expression of certain proteins in the plasma were altered in a manner consistent with a chemopreventive effect of the high omega-3:omega-6 ratio in the absence and presence of tamoxifen. In this study **(chapter 2)** we discussed the mechanism of how high omega-3 fatty acids with and without tamoxifen could lead to an alteration in the progression of carcinogenesis, since we evaluated the proteins in rats with adenocarcinomas. To determine if these proteins are biomarkers of early detection their expression could be evaluated at earlier time points in the same MNU rat model of mammary carcinogenesis. We would hypothesize that the proteins we identified in our study would have the same regulation in expression at these early time points if the omega-3 fatty acid with without tamoxifen could alter the protein expression in preneoplastic lesions. The panel of protein mentioned above could potentially be

ideal to utilize as biomarkers. The results reported in this chapter reveal that a single protein may not be realistic but multiple proteins can be used as potential biomarkers for disease progression and to monitor the efficacy of omega-3 fatty acids as chemopreventive agents in future clinical trials.

Chapter 3

A Novel Acid Stable Biologically Active Liposomal Formulation of Docosahexaenoic Acid for Breast Cancer Prevention

1. Objective

We have shown that ratios of omega-3 and omega-6 fatty acids that significantly inhibited chemically-induced mammary carcinogenesis in the rat were in excess of 10:1 suggesting that a particular omega-3 fatty acid may account for the chemoprevention of such high ratios. Docosahexaenoic acid (DHA; 22:6 n-3), a component in fish oil, has been shown to inhibit mammary cancer-induced by chemical carcinogenesis(7,12-dimethylbenz-[a]-anthracene and 1-methyl-1-nitrosourea) in the rat. The Western diet, with a ratio of 15:1 omega-6:omega-3 fatty acids, is not sufficient in providing the levels of DHA required for cancer prevention and since mammals can't synthesize this fatty acid its bioavailability is very low. Thus, a liposome that protects DHA from oxidation, low pH, and lipase attack would be an ideal biologically carrier for DHA and ultimately increase its bioavailability at the breast tissue. Although, a liposomal formulation for DHA for intravenous administration has been developed, this route of administration is not appropriate for chemoprevention for which oral delivery for long term use is the preferred approach. Therefore, we **hypothesized** that the oral delivery of a liposomal formulation of DHA which is resistant to oxidation and low gastric pH will enhance its bioavailability in the circulation and the breast tissues, and thus enhance the chemopreventive efficacy. Our **aims** are to develop an acid stable liposomal formulation of DHA and compare its biological activity, including apoptosis and cell viability, with free DHA *in vitro* using human breast cancer cell lines.

2. Background

2.1 Archaeosomes as Drug Delivery Vehicles

One source of lipids for the development of an acid stable liposome belong to the unique species, archaea, found in sea vents in the deep ocean, that have demonstrated a high tolerance to acidic conditions, high pressure, and heat[103, 105, 344, 345]. Their survival has been attributed to a particular set of lipids, highly saturated diphytanyl ether lipids, whose branching and ether bond structure make them resistant against harsh conditions[103, 105, 344, 345]. The ether linkages of these lipids are stable over a variety of temperatures and are resistant to attack by phospholipases[103], while the branched methyl groups provide a means of reducing membrane permeability and increase stability at low pHs[103, 344]. Specifically, natural archaeosomes, composed of such lipids, were stable from pH values ranging from 3-10 as observed by minimal leakage of the ether liposome in comparison to phosphatidylcholine ester-based liposomes[103]. Also, $C_{25,25}$-archaeol-based ether lipids in liposomes have been shown to resist changes in pH ranging from 5-10[345].

Archaea-based lipids have also shown to be well tolerated in high doses in a liposome formulation in *in vivo* studies[112]. In an *in vivo* study by Omri et al.[112], archaeosomes were prepared by the extrusion method with lipids extracted from various archaeobacteria(H. *salinarum*, N. *magadii*, M. *smithii*, M. *stadtmanae*). The archaeosomes at various doses (55mg, 275mg, and 550mg per kg body weight/day) were orally administered to balb/C mice for ten days. Clinical signs including lethargy, hyperactivity, and aggressiveness, body weight and temperature were recorded daily to monitor the side effects of the archaeosomes. Liver enzyme activity and organ weights were recorded following necropsy to observe differences in response

to dosage. The findings of this study in balb/C mice concluded that when given orally, archaeosomes caused moderate hyperactivity after one week of administration with no significant change in liver enzymes or organ weights when compared to controls. Overall, the study did not identify toxicity-related issues when the archaeosomes were given orally making archaeosomes an attractive oral delivery option.

3. Rationale

It has been postulated that the risk of breast cancer can be influenced by certain dietary components such as the amount and type of dietary fats ingested [6, 68, 69, 71, 73]. Specifically, omega-3 fatty acids, a component of fish oil, and omega-6 fatty acids have been suggested to decrease and increase breast cancer incidence, respectively[68, 69, 73]. Our laboratories have demonstrated that dietary administration of a high omega-3:omega-6 ratio, 25:1, reduced incidence and multiplicity of MNU induced rat mammary tumors by 21% and 30%, respectively[71, 73, 181]. An extensive analysis of the molecular signature underlying inhibition of mammary carcinoma by dietary omega-3 fatty acids revealed a dominant effect on suppression of proliferation over induction of apoptosis, although both processes were significantly affected[73]. Of interest, changes in cellular biomarkers related to proliferation and apoptosis were paralleled by concordant alterations in the plasma proteome of the same rats[73].

The high omega-3:omega-6 ratio needed to induce an antitumor effect suggests that the tumor protective effect of fish oil may be due to one of its components which may be present in small amounts. Since, administration of such high ratios of omega-3:omega-6 fatty acids in the diet is not feasible, it is necessary to develop a practical formulation of its active ingredient which can easily be administered to women at high risk for breast cancer. Based on evidence in

the literature and our own data we postulate that DHA is the active component of fish oil accounting for most of its antitumor action in breast cancer.

However, based on the chemical structure of DHA being highly unsaturated and vulnerable to oxidation and degradation under acidic conditions, as posed by the digestive track, the bioavailability of DHA would be limited[346-349]. Therefore, a delivery system that would allow for a concentrated amount of DHA to be taken orally on a daily basis would be an ideal chemopreventive strategy. An ideal system would be a nanoliposomal formulation that would encapsulate DHA utilizing non-toxic lipids that can protect DHA from pH fluctuations and oxidation. This protection from physiological stress would allow for the nanodelivery system to cross the enterocytes, enter the bloodstream and increase the bioavailability of DHA at the breast tissue. Hussain, et al, has demonstrated that particles under 200nm are ideal to cross the enterocytes of the intestine into circulation[344].

In this study we utilized the commercially available ether lipids: 1,2-di-O-hexadecyl-*sn*-glycero-3-phosphatidylcholine, and 1,2- di-O-phytanyl-*sn*-glycero-3-phosphatidylethanolamine considering their similarity in structure to $C_{25,25}$-archaeol-based ether lipids to construct an acid stable liposome formulation of DHA. We report here that this particular liposome formulation was superior at protecting DHA from oxidation and fluctuations in pH, making it a superior chemopreventive strategy for breast cancer. Furthermore, this unique liposome was more effective than free DHA at reducing cell viability, increasing apoptosis and altering certain proteins known to be involved in these cellular events in several human breast cancer cells lines with varied receptors status.

4. Experimental Design

4.1 Reagents

Detergent compatible protein assay reagents for protein quantification were obtained from Bio-Rad Laboratories, Hercules, CA. Reagents for Western blots were obtained from Bio-Rad Laboratories and primary and secondary antibodies were purchased from Santa Cruz Biotechnology and Cell Signaling. Primary antibodies against p21(#2947s), P-AKT(#4060p), AKT(#9272), P-S6(#4856s), S6(#2217s), PARP and Cleaved PARP(#9542s), and BCL-2(#2876s) were obtained from cell signaling. GAPDH(SC-32233) was obtained from Santa Cruz Biotechnology and used as a loading control. Chemilumnescent immunodetection reagents and autoradiography film were obtained from GE Healthcare. Ether lipids 1,2-dihexadecyl-*sn*-glycero-3-phosphocholine (999992) and 1,2-diphytanyl-*sn*-glycero-3-phosphoethanolamine (999985) were purchased from Avanti Polar Lipids, Alabaster, AL. Docosahexaenoic acid sodium salt (D8768) for the liposome formulation was purchased from Sigma Aldrich, St. Louis, MI. MTT reagent (M5655) for cell viability assays was purchased from Sigma Aldrich, St. Louis, MI. The Cell Death Detection Elisa Kit (11544675001) was purchased from Roche Applied Science, Indianapolis, IN.

4.2 Nanoformulation of Liposomal DHA and Ghost Liposome

Docosahexaenoic acid sodium salt(Sigma Aldrich, St. Louis, MI) was dissolved in absolute ethanol to yield a concentration of 10 mg/mL. 1,2-Diphytanyl-*sn*-glycero-3-phosphoethanolamine and 1,2-dihexadecyl-*sn*-glycero-3-phosphocholine were dissolved in chloroform to a concentration of 10 mg/mL. For the liposome formulation, 1,2-dihexadecyl-*sn*-glycero-3-phosphocholine, 1,2-diphytanyl-*sn*-glycero-3-phosphoenthanolamine and

docosahexaenoic acid were combined in a 6:3:1 molar ratio**(Table 3.1)**. For ghost liposomes 1,2-dihexadecyl-*sn*-glycero-3-phosphocholine and 1,2-diphytanyl-*sn*-glycero-3-phosphoenthanolamine were combined in a 6.67 and 3.33 molar ratio**(Table 3.2)**. The algorithm to determine the micromolar amount of DHA sodium salt to add to the formulation is the following: (total mg lipid in formulation *((1/(MW DHA/1000)+(MW PE Lipid/1000)*(Molar % PE Lipid)+(MW PC Lipid/1000)*(Molar % PC Lipid/Molar % DHA))). All other values can be added to the table as is. For the ghost formulation the algorithm to determine the micromolar amount of diphytanyl PE is the following: (total mg lipid in formulation *((1/(MW PE Lipid/1000)+(MW PC Lipid/1000)*(Molar % PE Lipid/ Molar % PC Lipids))). All other values can be added to the table as is. Lipids were dried to a film under a stream of nitrogen for four hours, then hydrated with 1mL of 0.9% degassed saline and heated (70°C) for ninety minutes with occasional vortexing. Next, the liposome solution was sonicated at 65°C for two and a half minutes, resulting in the liposome suspension to become less milky in appearance. The solution of lipids was extruded 13 times at 70°C through a 0.1um polycarbonate membrane with an Avanti-Mini Extruder(Avanti Polar Lipids, Alabaster, AL,USA). Liposomes were purified on a CL-4B column in 0.9% saline and stored at 4°C until further use. Liposome size(nanometers) and zeta potential(millivolts) for liposomal DHA and ghost were later measured, respectively, by dynamic light scattering and laser doppler velocrimetry(Zetasizer Nano ZS at 25°C). The amount of DHA in the liposome was determined by liquid chromatography tandem mass spectroscopy(LC-MS/MS)(Agilent 1100 Series capillary LC/MSD Ion Trap XCT equipped with an electrospray ion source).

Lipid	MW(mg/mmol)	Mg Lipid	µmol	Molar Ratio	Stock(mg/mL)	µL(1mL)
Dihexadecyl PC	706.08	6.1260	8.6761	6	10	612.60
Diphytanyl PE	776.205	3.36723	4.3381	3	10	336.723
Docosahexaenoic Acid Sodium Salt in Ethanol	350.47	0.5068	1.4460	1	10	50.68

Table 3.1- The formulation for liposomal DHA.

Lipid	MW(mg/mmol)	Mg Lipid	µmol	Molar Ratio	Stock(mg/mL)	µL(1mL)
Dihexadecyl PC	706.08	6.45644	9.1442	6.67	10	645.644
Diphytanyl PE	776.205	3.5436	4.5642	3.33	10	354.36

Table 3.2- The formulation for the ghost liposome.

4.3 pH Stability Studies (Dynamic Light Scattering, DLS)

First, solutions of dPBS were adjusted to pH 1-7.4 by adding an appropriate amount of 1.0 N HCl or 10 N NaOH and verified by a pH meter(Denver Instruments, Bohemia, NY). In a 1 mL cuvette 100 µL of liposomal DHA was added to 900 µL of pH adjusted dPBS and mixed. The pH of the mixture was further verified by the pH meter. Two batches of DHA nanoliposomes were run in triplicate for each pH point. This solution was submitted to dynamic light scattering analysis at 37°C for a duration of ten minutes to two hours. DLS Spectra were collected after ten minutes, one hour, and two hours as an indicator of stability by observing the size of the liposome.

4.4 Oxidation Studies (Liquid Chromatography Tandem Mass Spectrometry)

DHA and liposomal DHA , both in dPBS, were aliquoted into separated amber vials and stored at 37°C or 4°C for up to two weeks. An aliquot of each sample with D_5-DHA, an internal standard, were analyzed using an API 3200 LC MS/MS triple quadrupole mass spectrometer interfaced with an Agilent 1200 series HPLC using an Agilent extended C18 column (4.6 × 150 mm, 5µm.) The electrospray was in negative ion mode. The MS parameters were set as the following: electrospray source temperature and voltage were 230°C and -4.5kV, respectively; the declustering potential (DP) collision energy (CE), entrance potential (EP) and cell exit potential (CXP) were -40, -16, -3, and -12ev, respectively; the collision activated dissociation was set to 6 psi, while the curtain gas was set to 20 psi. The elution solvent program was 200uL/min gradient using solvent A (methanol) and solvent B (water). The gradient was 10% A to 100% A in five minutes, 100% A was held for another 28 minutes, and continued to 10% A in two minutes. DHA and D_5-DHA were detected after 31 minutes.

4.5 Cell Lines

Human breast cancer cell lines with varied receptor status were used in this study. MDA-MB-231(triple negative) and MCF-7(ER positive) cells lines were purchased from ATCC(American Type Culture Collection). MDA-MB-231 cells were cultured in DMEM with 10% Fetal Bovine Serum(FBS) and 1% Penicillin/Streptomycin. MCF-7 cells were cultured in EMEM with10% FBS, 0.01mg/mL human insulin and 1% Penicillin/Streptomycin. All cell lines were maintained in a humidified incubator at 37°C, 5% CO_2. For subculture, cells were submitted to 0.25% trypsin/EDTA detachment, followed centrifugation at 1200rpm for three minutes at 25°C and re-plated in growth media.

4.6 The Effects of Liposomal DHA, Ghost, DHA, and Ethanol on the Cell Viability of Human Breast Cancer Cell Lines

MDA-MB-231 cells were plated at $6.0X10^3$ cells per well in a 96-well tissue culture plate. MCF-7 were plated at $1.0X10^4$ cell/well. The cells were grown in 10% FBS,1% Pen/strep DMEM media for 24 hours prior to treatment. A concentration of 5µM-100µM of liposomal DHA, ghost(lipid equivalent of liposomal DHA) free DHA, and absolute ethanol(volume equivalent of DHA) were dissolved in full media and cells were exposed to treatment for 48 hours; this time point was selected since it was found to be optimum on the basis of literature data which examined DHA using various human breast cancer cell lines[75, 76, 79, 350-352] and our own data. Furthermore, our goal was to compare free DHA with liposomal DHA in these cell lines and determine the IC_{50}. Thus, an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue) assay was conducted to determine the effects of treatment on cell viability according to manufacturer's instructions. Cell viability was measured

at an absorbance wavelength of 570nm(SoftMax Pro 4.8) with a microplate reader(SpectraMax Plus 384, Molecular Devices, Sunnyvale, CA).

The IC_{50} was determined by analyzing the raw data with a four parameter log-logistic model of nonlinear regression[353]. Two experiments were conducted on each cell line with all treatments in quadruplicate. Based on this model the data was plotted as a log(dose) vs. percent viability.

4.7 The Effects of Liposomal DHA, Ghost, DHA, and Ethanol on the Apoptotic Activity of Human Breast Cancer Cell Lines

MDA-MB-231 and MCF-7 were plated at 50,000 cells/well respectively, in a 12-well tissue culture plate with full growth media (DMEM, 10% fetal bovine serum, 1% Penicillin/Streptomycin or EMEM, 10% fetal bovine serum, 1% Penicillin/Streptomycin, 0.1% insulin). After 24 hours the cells were treated with liposomal DHA, ghost liposomes, free DHA in ethanol, or ethanol in full media for 48 hours. Following the treatment, media was removed and cell lysis buffer was added for thirty minutes at 25°C. The buffer was collected in Eppendorf tubes and centrifuged for ten minutes at 20,000g. The supernatant (cytoplasmic fraction) was collected and diluted 1:10 with incubation buffer. Samples were placed at -20°C until needed. The cell lysates were then subjected to the protocol for a Cell Death Detection ELISA (Roche Diagnostics, Cat. No. 11544675001, v.07).

The Cell Death Detection ELISA was conducted as described below. To a 96-well plate 100uL of coating solution was added to each well. The plate was incubated for one hour at 25°C. The coating solution was removed and 200uL of incubation buffer was added following an incubation of thirty minutes at 25°C. The incubation buffer was removed and 200uL washing

buffer was added, removed and repeated three times. The cell lysates diluted 1:10 in incubation buffer were added to each well in the amount of 100uL and incubated for ninety minutes at 25°C. Next, 200uL of washing buffer was added, removed, and repeated three times. 100uL of conjugate solution was added to each well following incubation for ninety minutes at 25°C. Washing buffer was then added in the amount of 100uL, removed, and repeated three times. Finally, 100uL of substrate solution was added and the plate was incubated at room temperature on a plate shaker for ten minutes. Two experiments were conducted on each cell line with all treatments in quadruplicate.

4.8 Western Blot Analysis of Markers of Proliferation, Inflammation, and Apoptosis

MDA-MB-231 cells were in DMEM media supplemented with 10% FBS and 1% Penicillin/Streptomycin until at a confluence level of 60%. MCF-7 cells were plated EMEM with 10% FBS, 0.01% human insulin and 1% Penicillin/Streptomycin. These cells were then treated for 48 hours with 60 µM liposomal DHA, a lipid equivalent amount of ghost, 60 µM DHA in ethanol, and a volume equivalent of ethanol. After 48 hours the cells were collected and washed three times with dPBS. The cell pellet was mixed with 1X cell lysis buffer/1 mM PMSF and stored at 4°C overnight for lysing. Cell lysates were centrifuged at 1200 rpm for three minutes at 25°C separating the pellet and supernatant. All supernatants were collected and stored at -80°C until use.

Protein concentrations from cell lysates were measured by DC Protein Assay (Bio-Rad Laboratories). Proteins were submitted to 12% acrylamide gels(Bio-Rad Laboratories) using 30 µg of protein per well. They were transferred to PVDF(Bio-Rad Laboratories), blocked with 5% milk for one hour and probed over night at 4°C with antibodies to determine the effects liposomal DHA on protein expression of markers of cell proliferation, growth, and apoptosis.

Proteins examined include p21, AKT, P-AKT, S6, P-S6, Bax, BCL-2, PARP, and cleaved PARP. GAPDH was used as a loading control. The images were captured with Bio-Rad's GS800 Calibrated Densiometer and quantified with the Quantity One v4.5.0 1D Analysis Software (Bio-Rad Laboratories).

4.9 Statistical Analysis

The results of the MTT assay, cell death detection ELISA, and western blots are expressed as the mean ± S.E. (standard error). Differences among treatment groups for the MTT assay, and protein expression were considered significant at $p \leq 0.05$ and determined by a student *t*-test(2-tail paired). A four parameter log-logistic model using the R package dcr[353] was utilized to determine the IC_{50} value in both MCF-7 and MDA-MB-231 cell lines. In addition to a student T-Test, the results of the Cell Death Detection ELISA were analyzed by ANOVA, comparing liposomal DHA to free DHA on enrichment in breast cancer cell lines ($p \leq 0.05$).

5. Results

5.1 Formulation of Liposomal Docosahexaenoic Acid and Ghost Formulations

The liposomal DHA formulation was composed of a 6:3:1 molar ratio of 1,2-dihexadecyl-s*n*-glycero-3-phosphocholine, 1,2-diphytanyl-*sn*-glycero-phospho-3-ethanolamine, and DHA sodium salt**(Table 3.3)**.

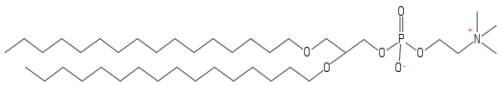
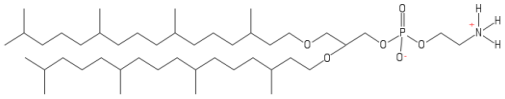
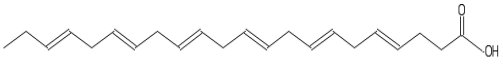
Lipid	Molecular Weight	Milligrams Lipid	µmol Lipid	Molar Ratio
1,2-dihexadecyl-sn-glycerophosphocholine 	706.08	6.1260	8.6761	6
1,2-diphytanyl-sn-glycerophosphoenatholamine 	776.205	3.36723	4.3381	3
Docosahexaenoic Acid Sodium Salt 	350.47	0.5068	1.4460	1

Table 3.3- The components for liposomal DHA include ether lipids and DHA in the molar quantities illustrated.

Dynamic light scattering characterized the liposomal DHA**(Figure 3.1A)** and ghost liposome**(Figure 3.1C)** to be unilamellar vesicles with a diameter of 136.8 ± 11.9nm and 148.9±4.11nm, respectively. The zeta-potential analysis determined that the DHA liposomes**(Figure 3.1B)** and ghost liposomes**(Figure 3.1D)** were slightly negative at -1.676 mV and -0.50967 mV, respectively. All measurements DLS and ZP measurements are an average of three individual liposome batches +/- S.E.M.

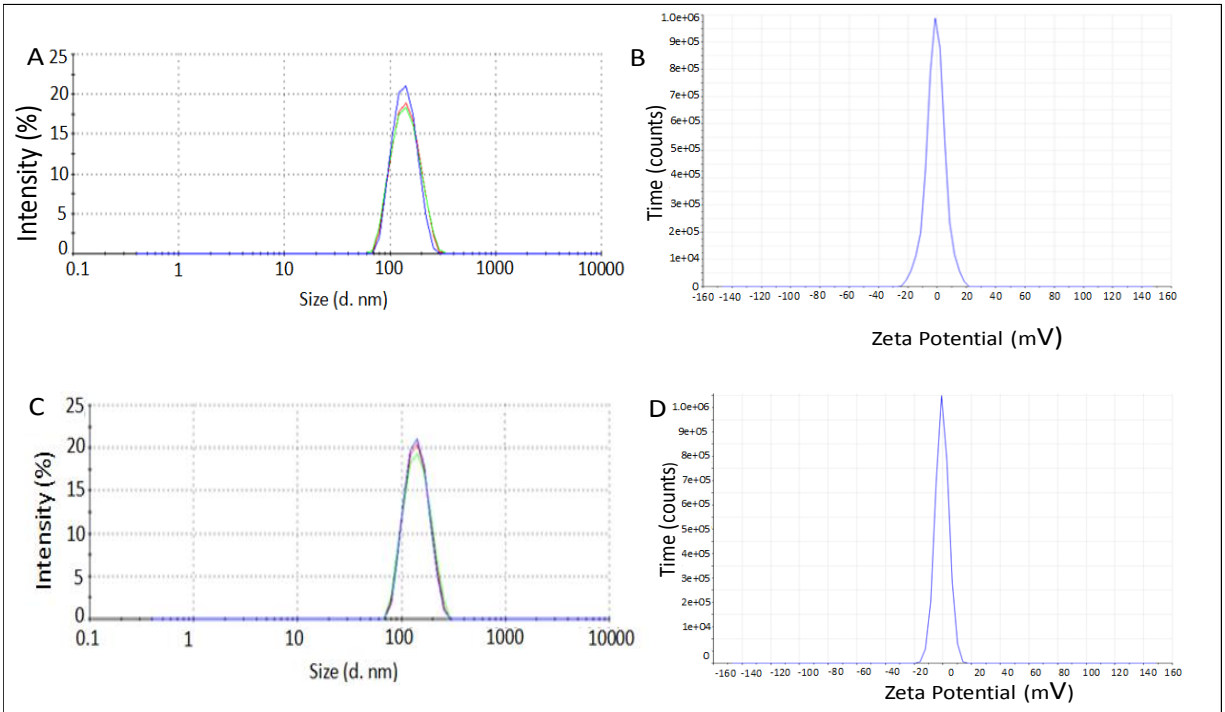


Figure 3.1- Characterization of liposomal DHA and ghost formulation size and zeta potential by dynamic light scattering (DLS) and doppler velocimetry (ZP), respectively. A. DLS of liposomal DHA , B. ZP of liposomal DHA, C. DLS of ghost, D. ZP of ghost.

By LC-MS/MS the encapsulation efficiency was determined. **Figure 3.2A** illustrates the standard curve of DHA at various concentrations. D_5-DHA was the internal standard. **Figure 3.2B** is the representation of the chromatogram that was observed following LC-MS/MS for liposomal DHA and D_5-DHA. Encapsulation efficiencies DHA in the liposomal formulations were determined by liquid chromatography tandem mass spectroscopy and ranged from 60% to 80%.

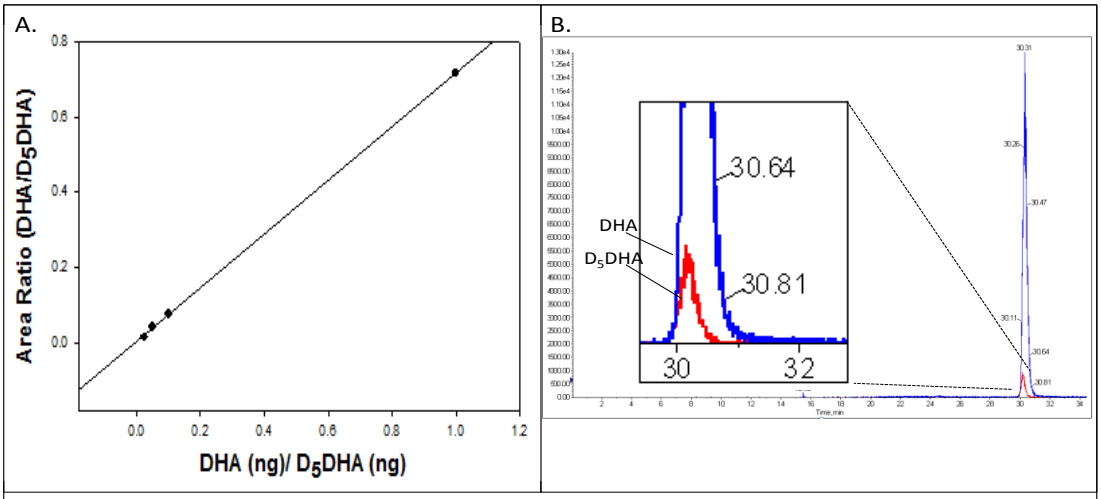


Figure 3.2- LC MS/MS analysis of DHA vs. D_5-DHA of a (A) standard curve for encapsulation efficiency determination. The LC-MS/MS chromatogram (B) of liposomal DHA and D_5-DHA to quantify encapsulation efficiency of DHA.

5.2 pH Stability Studies

We evaluated the stability of liposomal DHA under acidic conditions that mimic the stomach and small intestine (pH 1-7.4) by subjecting the liposomes to acidic Dulbecco's phosphate buffered saline (dPBS) and measuring the size, diameter in nanometers, by dynamic light scattering. The experiment was performed for two hours and at 37°C to mimic the time and temperature the liposome would be exposed to in the stomach and small intestine of a healthy subject. The size of liposomal DHA was measured at every pH point for the duration of two hours **(Figure 3.3).** At pH 7 and at ten minutes, one, and two hours the size of the liposome was 130.8 ± 0.3nm. Over the two hour duration as the pH decreased, the size of the liposome increased. Regardless of the increase in size of the liposome the DLS spectra did not show additional peaks above 1000 nm at any time point or pH, which is indicative of degradation. At pH 1, the most acidic pH tested, the liposome increased in size from 141.18 ± 3.38nm to 156.38

± 0.35nm, starting at ten minutes to two hours, possibly due to an increase in positive charge on the hydrophilic shell of the liposome leading to repulsion by the functional groups. However, this was not significant. The intact liposome was still below 200 nm in diameter at all pH points, making it ideal for the uptake and transfer into enterocytes of the intestine.

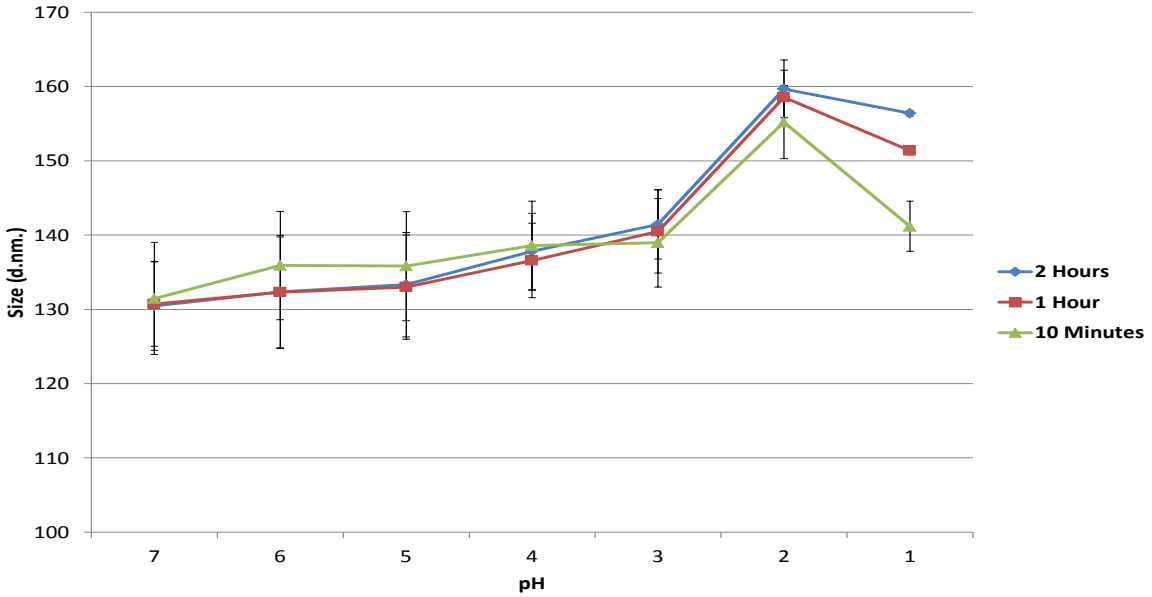


Figure 3.3- Stability of liposomal DHA as determined by DLS at pHs 1-7 for up to two hours. Data is presented as the average +/- S.E. of two individual samples.

5.3 Oxidation Studies

The ability of the liposome to protect DHA from oxidation was evaluated by LC-MS/MS. This was conducted over a two week period for liposomal DHA. Both liposomal DHA and free DHA were in an aqueous environment of dPBS, at either 4°C or 37°C, to mimic shelf life conditions and physiological conditions of the rat and human environment, respectively **(Figure 3.4)**. After one week the recovery of the liposomal DHA at 4°C was at 95.5% ± 2.25% of its original level at day 1, while the recovery of free DHA after one week was at 66.3% ± 15.3%. The results showed that the liposome had a protective effect on DHA shelf life. This protective

effect was also seen when comparing the liposomal DHA after two weeks to the free DHA after one week at 4°C. The liposomal formulation was also superior at protecting DHA from oxidation when compared to free DHA at 37°C. The liposomal DHA after one and two weeks contained 77.3% ± 4.56% and 64.13% ± 8.37% respectively of the original amount of DHA at day 1, while free DHA after one week in aqueous conditions contained 17.26% ± 8.39% of the original amount. Therefore, at physiological temperature, the liposome can protect DHA from degradation, making the delivery system ideal for *in vivo* oral administration.

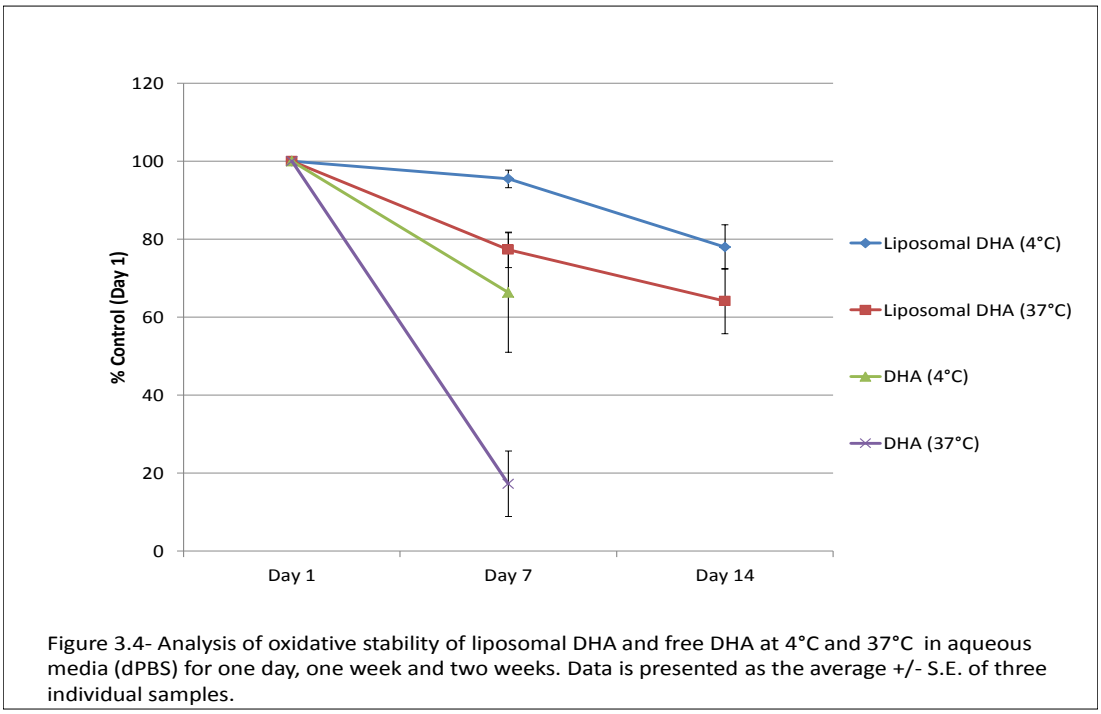


Figure 3.4- Analysis of oxidative stability of liposomal DHA and free DHA at 4°C and 37°C in aqueous media (dPBS) for one day, one week and two weeks. Data is presented as the average +/- S.E. of three individual samples.

5.4 The Biological Effects of Liposomal DHA on Human Breast Cancer Cell Lines-Cell Viability Studies

MTT assays were conducted for 48 hours with a single treatment of liposomal DHA utilizing diverse breast cancer cell lines, with regards to their receptor status, including MDA-MB-231(triple-negative), and MCF-7(ER+). Considering the rationale described in the

Experimental Design section we focused on comparing the effects of liposomal DHA and free DHA for 48 hours.

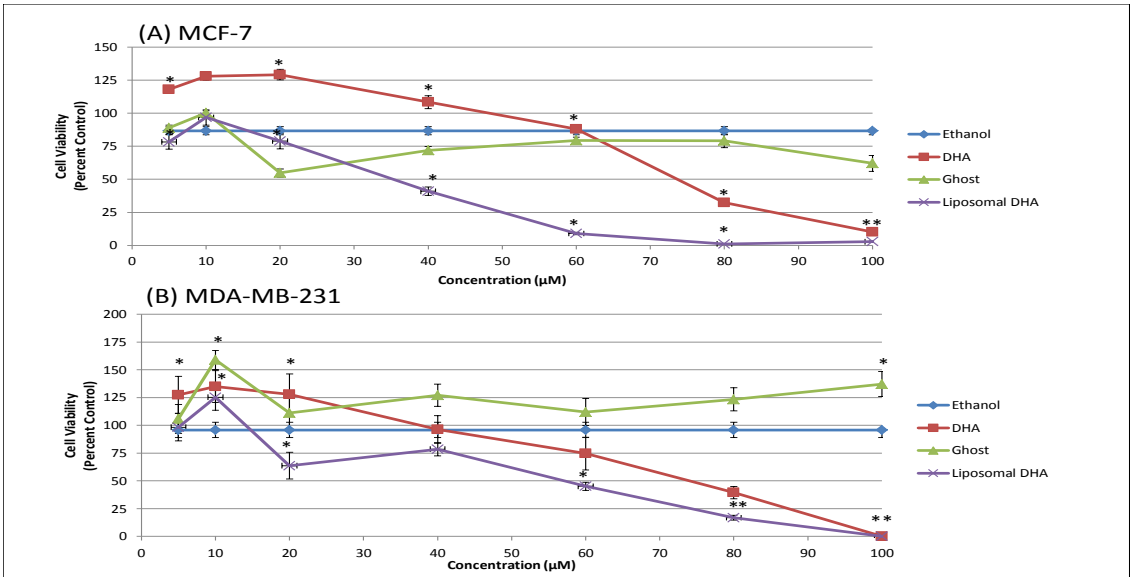


Figure 3.5- Cell Viability Studies of Liposomal DHA, Ghost, DHA, and Ethanol on MCF-7 and MDA-MB-231 Human Breast Cancer Cell Lines via an MTT Assay. A, Effects of ethanol (15μL), free DHA (5μM-100μM), ghost liposome (lipid equivalent), and liposomal DHA (5μM-100μM) on MCF-7 cells. B, Effects of ethanol (15μL), free DHA (5μM-100μM), ghost liposome (lipid equivalent), and liposomal DHA (5μM-100μM) on MDA-MB-231 cells. Data is presented as the average +/- S.E.M of 7-8 samples. *p≤0.05 in comparison to control.

Using MCF-7 cells(ER+) liposomal DHA was more efficacious at reducing cell viability than DHA alone from 5 μM to 100 μM**(Figure 3.5A).** In this cell line the ghost liposome and ethanol decreased cell viability but was not significant. In MCF-7 cells liposomal encapsulation significantly reduced the IC_{50} for DHA from 72.5 μM **(Figure 3.6C)**, to 38.8 μM (p=0.0017) **(Figure 3.6D).** The ghost liposome and ethanol control for both studies had no effect at either dose(data not shown). Of note, at 60 μM liposomal DHA, MCF-7 ER positive cells were far more sensitive than MDA-MB-231 triple negative cells.

Our results demonstrated that liposomal DHA and free DHA exerted a dose dependent decrease in cell viability from 5 μM to 100 μM in the MDA-MB-231 **(Figure 3.5B)** cell line. The ghost liposome had a slight increase in viability, however, was consistent, while ethanol had no effect. In the MDA-MB-231 cell line, liposomal DHA exerted a better effect than free DHA,

but the difference was not statistically significant (p=0.927); the IC_{50} is 65 µM for liposomal DHA and the IC_{50} is 69 µM for free DHA **(Figure 3.6B).**

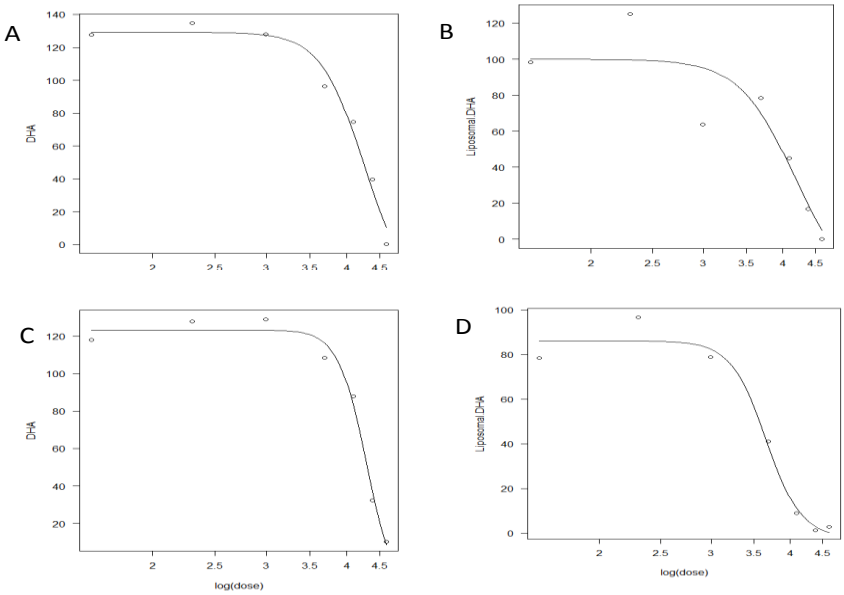


Figure 3.6- A 4-Parameter Log Logistic Model of Cell Viability Results A, Effects of DHA (5uM-100uM) on MDA-MB-231. B, Effects of liposomal DHA (5uM-100uM) on MDA-MB-231. C, Effects of DHA (5uM-100uM) on MCF-7. D, Effects of liposomal DHA on MCF-7. n=7.

5.5 The Biological Effects of Liposomal DHA on Human Breast Cancer Cell Lines-Apoptosis Studies

In this study we focused on the effect of liposomal DHA, ghost, and free DHA on apoptosis using one ER positive, MCF-7, and one triple negative, MDA-MB-231 cell line; treatments were conducted after the same duration employed in cell viability(48 hours). **Figures 3.7A and 3.7B** illustrate that the MCF-7 and MDA-MB-231 cell lines respond to liposomal DHA treatment. At 80 µM, 40 µM, 20 µM, and 10 µM the enrichment factor, or fold change observed as abundance of nucleosomes, in the MCF-7 cell line was 3.5, 2, 1.8, and 1.5 times higher than cell media controls($p \leq 0.05$), respectively**(Figure 3.7A)**. As evaluated by an ANOVA, liposomal DHA had a statistically significant effect on increasing apoptosis in

comparison to free DHA in the MCF-7 cell line(p=0.000173). The ghost liposome had a similar effect as 10 µM liposomal DHA on the MCF-7 cell line. **(Figure 3.7A)**.

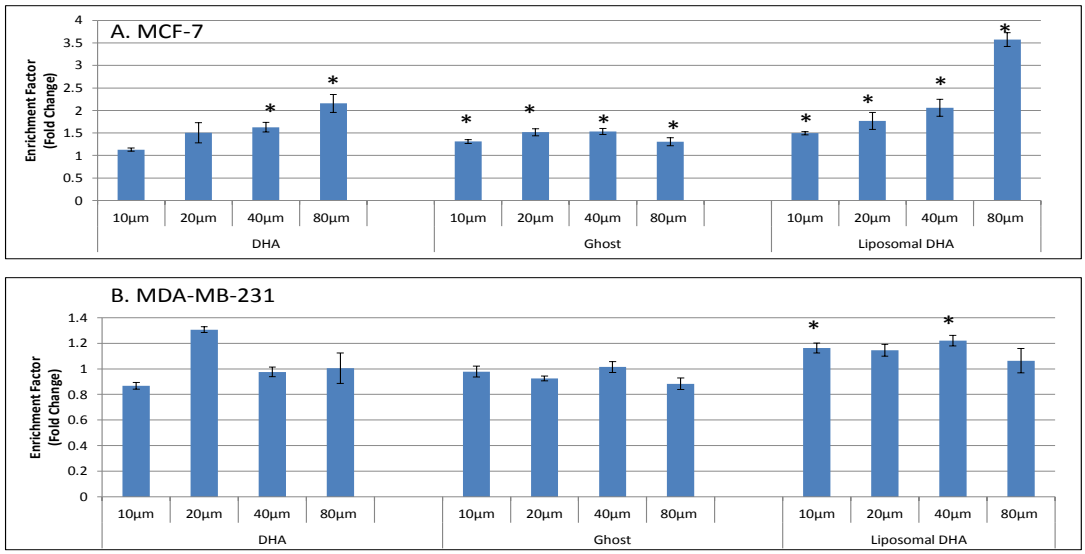


Figure 3.7-Apoptosis Analysis of Liposomal DHA, Ghost and DHA, on MCF-7 and MDA-MB-231 Human Breast Cancer Cell lines via a Cell Death Detection ELISA. A, Effects of DHA (10uM-80uM) ghost (10uM-80uM lipid equivalent), and liposomal DHA (10uM-80uM) on MDA-MB-231 cells. B, Effects of DHA (10uM-80uM) ghost (10uM-80uM lipid equivalent), and liposomal DHA (10uM-80uM) on MCF-7 cells. Data is presented as the % control cell viability +/- S.E.M of n=3. *p≤0.05 in comparison to control by a Student t-test.

Liposomal DHA and free DHA had no dose-dependent effect on the MDA-MB-231 cell line at any dose **(Figure 3.7B)**. The ghost liposome had no dose-dependent effect. As evaluated by an ANOVA test, liposomal DHA did induce more apoptosis than free DHA on the MDA-MB-231 cell line, however, it was not statistically significant(p=0.0738).

5.6 Protein Markers of Cell Viability, Cell Growth, and Apoptosis

Liposomal DHA has shown to be superior to DHA at decreasing cell viability and increasing apoptosis in breast cancer cell lines. We decided to investigate proteins involved in cell viability, cell growth, and apoptosis in the MCF-7**(Figure 3.8)** and MDA-MB-231**(Figure 3.9)** cell lines. Based on the IC_{50} values for MDA-MB-231 cell line**(Figure 3.5B)** we used a concentration of 60 µM for each treatment.

Specifically, P-AKT and P-S6 kinase, both mediators of the mTOR pathway, were found to be decreased significantly in the both the MCF-7**(Figure 3.8)** and MDA-MB-231**(Figure 3.9)** cell lines in comparison to free DHA. The cyclase inhibitor, p21was increased significantly in both breast cancer cell lines when treated with liposomal DHA, indicative of an arrest in the G1/S phase of the cell cycle.

Proteins directly involved in apoptosis were also modulated in the MCF-7 and MDA-MB-231 cell lines when treated with liposomal DHA. Cleaved PARP and Bax were significantly increased in the MCF-7 cell line**(Figure 3.8)** treated with 60 uM liposomal DHA, in comparison to 60 µM free DHA. Cleaved PARP and BCL-2 were significantly increased and decreased, respectively, in the MDA-MB-231 cell line**(Figure 3.9)** treated with the same amount of liposomal DHA, in comparison to free DHA. Taken together the acid-stable nanoliposomal delivery system of DHA was more efficacious at altering protein expression related to cell proliferation, cell growth, and apoptosis in a manner consistent with chemoprevention.

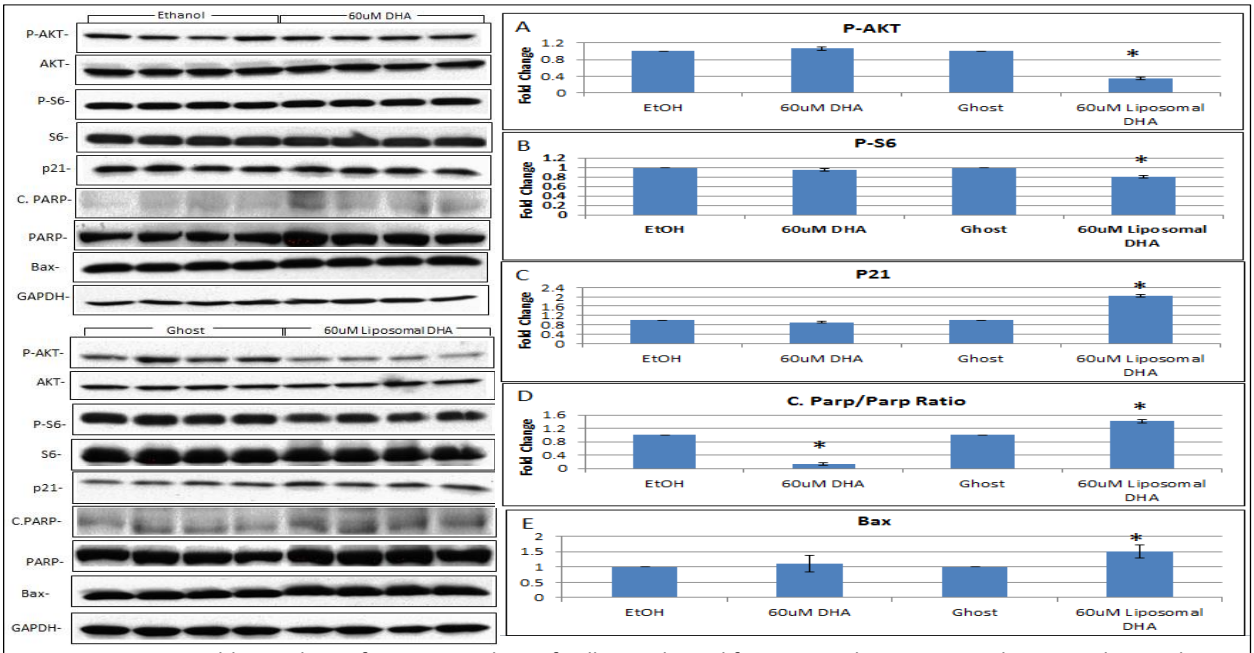


Figure 3.8- Western blot analysis of protein markers of cell growth, proliferation, and apoptosis in the MCF-7 human breast cancer cell line (comparison of 60 µM DHA to ethanol control, comparison of 60 µM liposomal DHA to ghost). (A) P-AKT, (B) P-S6, (C) p21, (D) Cleaved Parp/Parp ratio, (E) Bax. *$p \leq 0.05$ with respect to control.

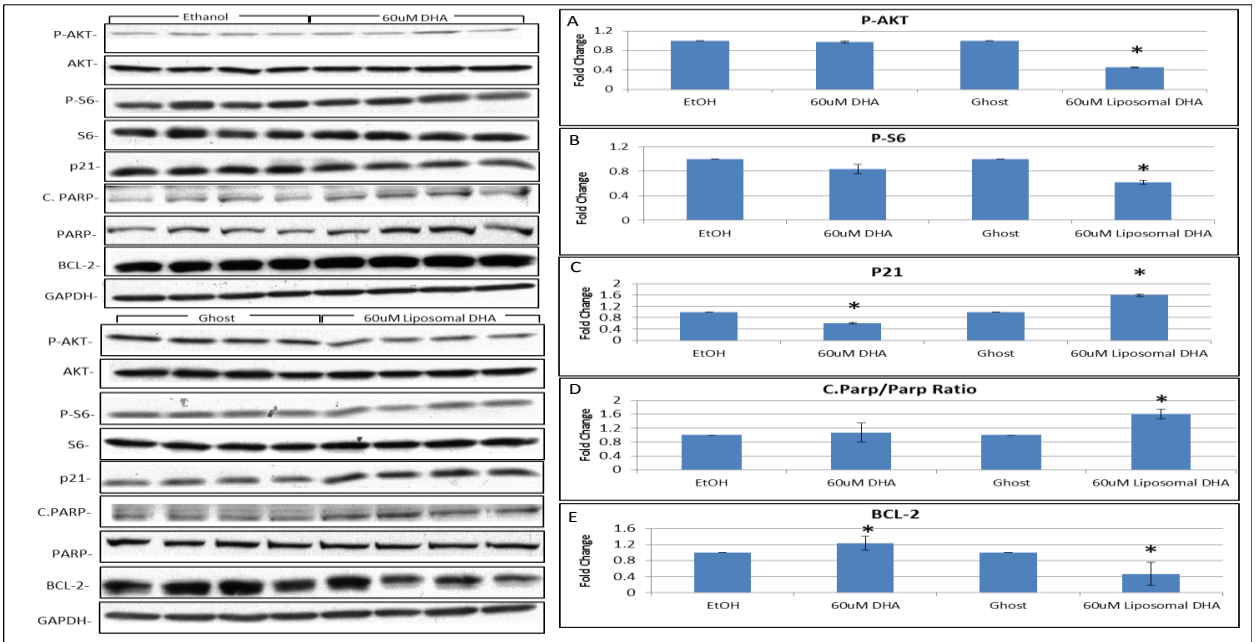


Figure 3.9- Western blot analysis of protein markers of cell growth, proliferation, and apoptosis in the MDA-MB-231 human breast cancer cell line (comparison of 60 µM DHA to ethanol control, comparison of 60 µM liposomal DHA to ghost). (A) P-AKT, (B) P-S6, (C) p21, (D) Cleaved PARP/PARP ratio, (E) BCL-2. *$p \leq 0.05$ with respect to control.

6. Discussion

This is the first study demonstrating the successful development of an acid stable liposomal formulation of docosahexaenoic acid as a potential chemopreventive for breast cancer. The goal of the study was to develop a bio-efficacious liposome that has the ability to protect DHA from oxidation and fluctuations in pH, specifically low pH, making it an ideal daily oral delivery system for chemoprevention trials in women who are high risk for breast cancer.

In our initial investigation we considered other liposomal formulations of DHA that have been reported previously[169-171] for reasons other than cancer prevention, including elucidating mechanisms of metathesis[170], Amyloid-Precursor Protein, APP, processing[171], and epithelial cell permeability[169]. When given for 14 days by intravenous injection a 1:1 DMPC:DHA formulation was able to prevent the metathesis of human colon cancer cells, HCT116 cells, to the lung and induced apoptosis in tumors in the liver in a xenograft mouse model[170]. Cleavage of APP, induced by a soy phosphatidylcholine/DHA liposome formulation, resulted in a decrease of apoptosis in HEK-293 APP-transfected cells [171]. A formulation utilizing star fish lipids(42% DHA), and squid lipids (8% DHA) was shown to increase permeability of tight junctions in human colon adenocarcinoma cells, Caco-2 cells, by reducing the polarity as measured by Trans Epithelial Electronic Resistance(TEER) as compared to a soy lipid control[169]. We did attempt to use conventional lipids, DPPC and DPPE, as described in literature[169-171] to make an acid stable liposome but the conventional formulation could not withstand acidic pH (**Figure 3.10**).

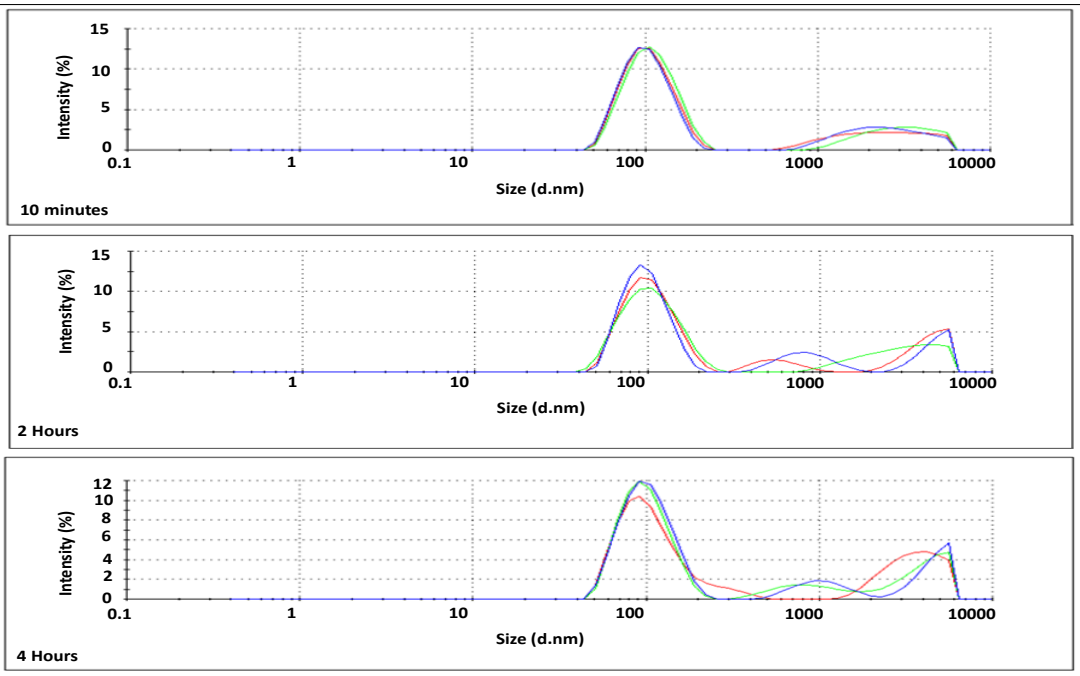


Figure 3.10- Dynamic light scattering of conventional liposome(DPPC, DPPE) DHA formulation. DLS was conducted on the conventional formulation at 10 minutes, 2 hours and 4 hours at pH 3. Additional peaks to the left of 100nm are an indication of liposome instability.

Overall, many different liposome formulations have been synthesized but a liposome formulation that could be given orally and protect DHA from oxidation and pH fluctuation for breast cancer prevention has never been reported. Since the convention lipids, DPPC and DPPE, composed of acyl chains bound to phosphodiester head groups by ester linkages, were proven to not be stable in acid, a unique blend of lipids had to be used to develop a liposome with characteristics that would make it feasible as an oral delivery system. By reviewing literature on the characteristics of microorganisms that can withstand harsh environments such as acidity, high temperature, and high pressure I was able to find a microorganism whose survival was attributed to its lipid composition in its plasma membrane. The microorganism known as Archaea was found to survive harsh conditions due to its plasma membrane being composed of ether lipids[103, 108]. To develop a liposomal delivery system feasible for oral administration in humans pure lipids similar in structure to the lipids found in Archaea needed to be utilized in a

liposomal formulation. The lipids in **Table 3.3** were pure since they were synthetic and commercially available, making them ideal for an oral liposomal formulation. Many DHA liposome formulations were developed, varying in lipid ratios, DHA composition, and protocol **(Table 3.4)**. These formulations were tested by dynamic light scattering to assess their size as a measure of stability. Many formulations were detected by DLS as a liposomal structure, however, would degrade shortly after in buffer solution (pH 7.4) making them unfeasible for an acid stable formulation. The successful formulation for liposomal DHA and the ghost liposome are highlighted in yellow in **Figure 3.4**. Both of these formulations remained stable for up to two weeks at 4°C in buffer. Liposome formulation 12 was further tested in acidic solutions **(Figure 3.4)** for pH stability and found to be our successful acid stable liposomal formulation of DHA.

Formulation	Lipids	Type of DHA	Molar Ratio (Lipid/DHA)	Detection by DLS
1	Diphytanyl PC	Oil	5.66/2.87	Yes
2	Dihexadecyl PC	Oil	5.66/2.87	Yes
3	Diphytanyl PC	Sodium Salt	5.66/2.87	Yes
4	Dihexadecyl PC	Sodium Salt	5.66/2.87	Yes
5	Dihexadecyl PC	Ethyl Ester	5.66/2.87	Yes
6	Diphytanyl PC	Ethyl Ester	5.66/2.87	Yes
7	Diphytanyl PC Diphytanyl PE	Oil	6/3/1	No
8	Dihexadecyl PC Diphytanyl PE	Oil	6/3/1	Yes
9	Dihexadecyl PC Diphytanyl PC	Salt	6/3/1	Yes
10	Dihexadecyl PC Diphytanyl PE	Oil	7/2/1	Yes
11	Dihexadecyl PC Diphytanyl PC	Oil	7/2/1	Yes
12	Dihexadecyl PC Diphytanyl PE	Salt	6/3/1	Yes
Conventional DHA Liposome	DPPC DPPE	Salt	6/3/1	Yes
Ghost 1	Dihexadecyl PC Dihexadecyl PC	--------	6.67/3.33	Yes
Ghost 2	Diphytanyl PC Diphytanyl PE	--------	6.67/3.33	No
Ghost 3	Dihexadecyl PC Dihexadecyl PE	--------	6.67/3.33	Yes
Conventional Ghost	DPPC DPPE	--------	6.67/3.33	Yes

Table 3.4- Formulations of Liposomal DHA and Ghost Liposomes. Successful formulations are highlighted in yellow.

Our formulation for liposomal DHA was first tested with regards to its stability at a pH that ranged from neutral to acidic(pHs 7.4-1) at 37°C, in an attempt to mimic the conditions of the stomach and small intestine[354]. We measured the diameter of liposomal DHA, via dynamic light scattering[156] , over a period of two hours to closely resemble the digestion time in a healthy human. We observed after two hours that the liposome did increase in size in a pH dependent fashion, as the liposome was exposed to more acidic conditions. The liposome after two hours at pH 1 was 156.38 ± 0.35 nm, with no degradation. Degradation would have been evident if any additional peaks of measurable intensity at a higher diameter would have been evident; however, this was not observed. The advantage of the liposome at pH 1 having this particular size is that literature supports that only particles 200 nm or less can cross the enterocytes of the intestine[170]. The polydispersity index, distribution of particle size, of liposomal DHA at all pHs was below 0.123 indicating size homogeneity with no aggregation. The polydispersity Index is calculated by a two parameter fit to the cumulants analysis[156]. For this oral delivery system to be successful *in vivo* it will have to cross the intestinal barrier to reach circulation, and have the ability to survive acidic pH, physiological temperature, with the diameter remaining below 200 nm.

The charge of liposomal DHA was evaluated by doppler effect velocimetry in the form of zeta-potential[156]. This is crucial because the electrostatic interactions of the liposomes with cell surface moieties can determine the fate of a liposome[355]. Our formulation of liposomal DHA was weakly negative with a zeta-potential of -1.676mV after five scans of the same sample by laser Doppler micro-electrophoresis. The weakly charged liposome has the advantage of not attracting components of the reticuloendothelial system[356]as readily as a highly charged

particle, allowing for it to remain in circulation. The ability of liposomal DHA to remain in circulation will improve its bioavailability.

Considering, the structure of DHA we focused on developing a formulation that can protect it from oxidation. The unique ether bonds of the lipids in our formulation render them resistant against nucleophilic attack, while the saturated acyl chains provide against oxidation in aqueous medium. This is ideal for liposomal DHA because the hydrophobic nature of the polyunsaturated fatty acid will lead to its encapsulation in the lipid bilayer[357]and protect DHA from oxidation and degradation. To evaluate the ability of the liposome to protect DHA we subjected liposomal DHA to aqueous conditions for two weeks at 4°C and 37°C, and free DHA under the same conditions for one week. Our results demonstrate that our liposome formulation had the ability to protect DHA under shelf-life conditions(4°C) and conditions that resembled physiological temperature(37°C).

The ability of liposomal DHA to affect cell viability(MDA-MB-231, MCF-7) and apoptosis(MDA-MB-231, MCF-7) in human breast cancer cell lines in comparison to free DHA, *in vitro*, was also evaluated in this study. Jiang, et al have shown that high amounts of dietary omega-3 fatty acids reduced cell viability and induced apoptosis in rat mammary adenocarcinoma-induced by 1-methyl-1-nitrosourea[73]. Specifically, protein targets assessed by western blot and proteomic approaches involved in the cell cycle, lipid metabolism, and inflammation were altered in a manner consistent with chemoprevention by the high ratio of omega-3:omega-6 fatty acids[73, 181].

The dose dependent response of DHA on the cell viability of the MDA-MB-231 cell line was consistent with literature[75, 76, 350]. The IC_{50} of liposomal DHA in this cell line was 65 µM, while the IC_{50} of free DHA was 69 µM after 48 hours of treatment. Previous reports have

supported the high sensitivity of MDA-MB-231 cells to DHA after 48 and 72 hours[75, 76, 350]; one study attributed the effect of DHA to the generation of reactive oxygen species[75]. MDA-MB-231 cells have been shown to be susceptible to a reduction in cell proliferation by 25% after being treated with DHA for 48 hours[350]. This reduction of cell viability by DHA treatment was further supported with a significant reduction seen after 72 hours, when MDA-MB-231 cells were treated with 20uM and 100uM DHA[76]. It is of particular significance that we showed that liposomal DHA is a superior anti-proliferating formulation than free DHA.

When we evaluated the effect of liposomal DHA and free DHA on receptor-positive breast cancer cell line, MCF-7, a significant dose dependent reduction in proliferation was observed. The sensitivity of MCF-7 cells to DHA is supported by the study by Kang et al.[75] that determined the IC_{50} of DHA alone to be 20.2uM after 72 hours of treatment. In the present study the IC_{50} of liposomal DHA was 38.8 µM, while the IC_{50} for free DHA was 72.5 µM after 48 hours of treatment, making it clear that liposomal DHA was more efficacious at reducing cell viability than free DHA.

Omega-3 fatty acids, including DHA, have also been shown to contribute to apoptosis in breast tumors induced by chemical carcinogenesis in the rat[71, 73] and in cell lines[75, 76]. Zhu et al., have shown that an increase apoptosis is favored in the rat model of mammary carcinogenesis given high omega-3:omega-6 fatty acid ratios, 25:1. Specifically, cell markers of apoptosis focusing on the intrinsic pathway have shown to be altered in these tumors[71]. Apoptosis induction by omega-3 fatty acid treatment is hypothesized to be caused by omega-3s being taken in by the cell and causing activation of certain transcription factors[71], dissociation of lipid rafts resulting in a decrease in oncogenic signaling[358] and formation of reactive oxygen species that can activate specific caspases pathways[75]. In *in vitro* systems, DHA has

also been shown to induce apoptosis in the MCF-7 and MDA-MB-231 cell lines by either the casepase-8[75]or caspase-3[76] pathways, respectively. Our results showed that after 48 hours there was a significant dose dependent increase in apoptosis in the MCF-7 cell line with liposomal DHA increasing from 10 μM to 80 μM, while a dose-dependent effect was not seen with the MDA-MB-231 cell line with liposomal DHA. The present investigation clearly demonstrates the pro-apoptotic activity of liposomal DHA exceeded that of free DHA.

Once the effects of cell viability and apoptosis were determined in response to free DHA, ghost liposome, and liposomal DHA, protein markers of cell proliferation and apoptosis were examined in an attempt to understand how liposomal DHA was more efficacious than free DHA in the MDA-MB-231 cell line. We decided to focus on markers of the cell proliferation and growth: p21, P-AKT, and P-S6 and markers of apoptosis: BCL-2, and cleaved PARP. It is known that omega-3 fatty acids can increase p21, and cleaved PARP, while decreasing BCL-2, P-AKT, and P-S6 in the mammary tumors of rats given high omega-3 fatty acids[71].

P21, a cyclase inhibitor of the G1/S phase of the cell cycle, has been shown to be increased in MDA-MB-231 cell lines when treated with 25 μM DHA[359]. In our study the significant increase of this protein after liposomal DHA treatment supported that the liposome was more efficacious at delivering DHA to the cell and reducing cell proliferation.

Literature data also support that certain DHA and EPA combinations have been able to reduce the expression of phosphorylated protein kinase B, P-AKT, in MDA-MB-231 cells[360]. In addition, P-AKT, a serine/threonine kinase known to aid in cell growth, proliferation, and protein translation in response to growth factors[361] could be responsible for up to 70% of breast cancer cases[362]. In our study it was shown to be significantly decreased in response to

liposomal DHA treatment. This further supports the dose dependent decrease seen in cell viability when MDA-MB-231 cells were treated with liposomal DHA.

P-S6 ribosomal protein was also decreased significantly in the MDA-MB-231 cell line when treated with liposomal DHA. In triple negative breast cancer cells this protein has been linked to an increase in cell proliferation, survival, and migration[363]. Specifically, the activation of the PI3K pathways is thought to increase mTORC1 activity and increase phosphorylation and activation of S6 kinase, and in some cases an activating mutation of the *PIK3A* gene can lead to an increase in P-S6[364].

Proteins indicative of apoptosis, BCL-2 and cleaved PARP, were also evaluated to determine if they contributed to the increase in apoptosis observed in the MDA-MB-231 cell line by the Cell Death Detection ELISA. BCL-2, an anti-apoptotic protein, inhibits caspase pathways and APAF-1 activation. One study illustrated that MDA-MB-231 cells treated with 50ng/mL DHA had a decrease in mRNA for BCL-2, indicative of apoptosis[79]. However, many other studies have focused on apoptosis in the MCF-7 cell line. BCL-2 protein expression has been shown to decrease in response to treatment with DHA in the ER+ cell line, MCF-7. In MCF-7 cells the BCL-2 was decreased after 48 hours of treatment with DHA which was indicative of apoptosis, but was not significant[365].

PARP, a nuclear poly (ADP-ribose) polymerase, is known to be involved in single strand break repair of DNA, aiding in cell survival and proliferation[366]. During apoptosis PARP is cleaved by caspases making it inactive and unable to repair damaged cells. BCL-2 and cleaved PARP protein expression was significantly decreased and increased, respectively, by our liposomal DHA formulation as compared to free DHA, in the MDA-MB-231 cell line. This

alteration in protein expression observed with cleaved BCL-2 and PARP indicated that our liposomal formulation was more efficacious at inducing apoptosis than free DHA.

7. Summary and Concluding Remarks

The purpose of the studies performed in **Chapter 3** was to develop a liposomal delivery system for DHA that would protect DHA from fluctuations in pH and oxidation from aqueous conditions. After many formulations testing various lipid blends, DHA compositions, and protocols we successfully developed the first oral liposome delivery system for DHA. Oral delivery was the route of interest in our study because oral delivery is the appropriate route for chemoprevention studies. Our results have demonstrated that a liposome formulation using lipids similar in structure to those found in archaeobacteria can provide a stable delivery system that contains the protective qualities needed to maintain the structural stability of DHA.

The potential for liposomal DHA to be utilized as a chemopreventive strategy for breast cancer was demonstrated by its ability to reduce cell viability and increase apoptosis in various cell lines of breast cancer with differing receptor status. Furthermore, this was supported by liposomal DHA being more efficacious than free DHA in altering protein markers of the cell cycle, cell growth, and apoptosis, which include p21, P-AKT, P-S6, cleaved PARP, and BCL-2. Overall, the ability of the nanoliposome to protect DHA while inducing anti-cancer effects makes it an ideal delivery system to test as a chemopreventive for breast cancer.

Chapter 4

Oral Administration of an Acid Stable Liposomal Formulation of Docosahexaenoic Acid (DHA) Delivers DHA and LOX-Metabolites to the Circulation and the Mammary Fat Pad of Sprague Dawley Rats

1. Objective

Although liposomal delivery systems of DHA for intravenous administration have been synthesized[169-171] our laboratory **(Chapter 3)** is the first to design an oral liposomal delivery system of DHA that is able to protect DHA from degradation due to oxidation and fluctuations in pH. We **hypothesize** that the oral administration would increase delivery of DHA in circulation and at the breast tissue as a result of protecting the encapsulated DHA from oxidation and fluctuations in pH, often found in the gastrointestinal tract. Therefore, our initial goal of **Aim 3** was to test the efficacy of our liposomal formulation to deliver DHA to the circulation and to the mammary fat pad, in the female Sprague Dawley rat. We further **hypothesize** that once in circulation and at the mammary fat pad, DHA would be metabolized into its hydroxylated derivatives catalyzed by lipoxygenase (LOX) enzymes.

2. Background

2.1 The 21 Day Old Sprague Dawley Rat Model of Mammary Carcinogenesis

Rodents have been employed to the study mammary carcinogenesis *in vivo* for more than 5 decades. One particular rodent, the Sprague Dawley rat, has been useful in studying mammary carcinogenesis and chemoprevention.

The chemical carcinogen 1-methyl-1-nitrosourea (MNU) is administered to a Sprague rat at 21 days of age via interperoteneal injection, and acts as a DNA alkylator with its primary site

of mutagenesis at the terminal end buds (TEB) of the mammary fat pad which undergoes branching morphogenesis[213]. This particular tissue experiences a high rate of mutations due to rapid proliferation which could be a result of a mutation in the ha-*ras* gene or microsattelite instability[70].

The mutagenesis from administering this chemical at 21 days results in lesions that over a period of five weeks progress into more aggressive forms of breast cancer, similar to the pattern seen in humans[213]. As illustrated in **Table 4.1** the lesions that develop over time progress from intraductal proliferation→ ductal carcinoma in-situ(DCIS)→ adenocarcinoma[213]. Clearly, this animal model is suitable not only to study mechanisms of chemical carcinogenesis but also tissue distribution of liposomal DHA; a potential chemopreventive agent.

Lesion Type	Time-post Carcinogen			
	14 days	21 days	28 days	35 days
Intraductal proliferation (%)	20	46.7	80	80
DCIS (%)	0	26.7	23.3	40
Adenocarcinoma(%)	0	26.7	86.7	93.3

Table 4.1- Histopathology of the 21 day old female Sprague Dawley rat model of mammary carcinogenesis.

2.2 Metabolites of Omega-3 Fatty Acids in Cancer Research

Metabolites of DHA have shown to be more effective than DHA alone at providing an anticancer effect, both *in vitro[367-369]* and *in vivo*[35, 57]. DHA can be metabolized by lipoxygenases[39, 370, 371], cyclooxygenases[368], and cytochrome-P450[46, 57, 372] in the breast tissue to form hydroxides, peroxides, and epoxides, respectively **(Figure 4.1)**.

Hydroxylated forms of DHA, including 4- and 17-hydroxy DHA, have shown to decrease inflammation and angiogenesis[35]. 4-Hydroxy DHA, a DHA metabolite of 5-lipooxygenase, is an inhibitor of angiogenesis in an oxygen induced diabetic retinopathy mouse model[35]. It was shown that 4-hydroxy DHA inhibited angiogenesis by activating PPARγ and decreasing expression of epidermal growth factor receptor(VEGF), decreasing neovascularization in the retina[35].

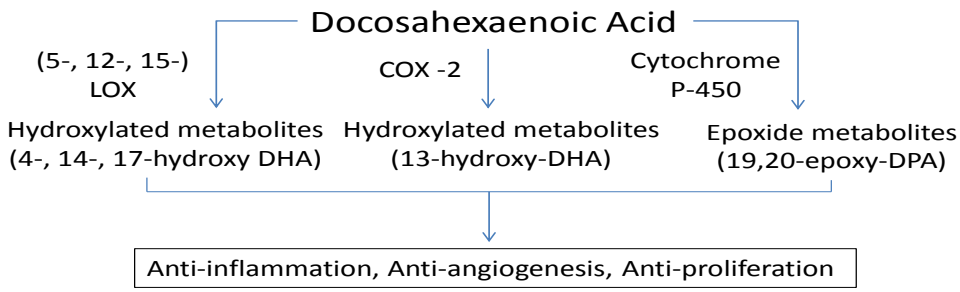


Figure 4.1- The pathways of DHA metabolism at the mammary fat pad. These pathways include lipoxygenases (5-, 12-, 15- LOX), cyclooxygenase-2(COX-2), and cytochrome P-450 enzymes.

17-Hydroxy DHA, a metabolite of 15-lipoxygenase, has shown to increase phagocytosis by macrophages *in vitro* promoting anti-inflammation. In addition, in dextran sodium sulfate induced colitis in a mouse model, 17-Hydroxy DHA dampened inflammation and increased resolution of the immune response.[371]. This metabolite has been linked to decreasing necroinflammation *in vivo* in mice hepatocytes and decreasing TNF-α in macrophages in the same model[39].

Cytochrome P450 is responsible for the conversion of DHA to epoxide and vicinal diol metabolites. DHA metabolites such as 19, 20-epoxydocosapentaenoic acid (19, 20-epoxy DPA) have shown to be highly potent antiarrhythmic properties in cardiomyocytes, thus have a potential cardioprotective behavior[372]. 19, 20- Epoxy-DPA acid, a lipid metabolite of DHA, has shown to decrease tumor growth and metastasize in a LLC(Lewis lung carcinoma) mouse model of lung cancer[57]. It is hypothesized that 19, 20-epoxy-DPA can block angiogenesis by inhibiting the VEGF-VEGF2 signaling pathway[57].

3. Rationale

Our laboratory is the first to synthesize a liposomal formulation of DHA for its potential utility for chemoprevention of breast cancer **(Chapter 3)**. This liposome has been characterized in both, acidic and neutral conditions (pHs 1-7.4) and at 4°C and 37°C in aqueous conditions. The result of these experiments illustrated that our liposome formulation was able to protect DHA from conditions of degradation due to acidity as well as protect DHA from oxidation at physiological temperature(37°C). In addition, we have shown that liposomal DHA when compared to free DHA was more efficacious at altering cell proliferation and apoptosis in both estrogen receptor positive and triple negative human breast cancer cell lines **(Chapter 3)**. We also showed that the expression of p21, P-AKT, P-S6, cleaved PARP, and BCL-2 was altered by

liposomal DHA in a manner that was consistent with our data demonstrating inhibition of cell proliferation and induction of apoptosis.

The initial goal of studies conducted in this chapter is to demonstrate that our liposomal formulation can deliver DHA to the circulation and the mammary fat pad prior to initiation of comparative pharmacokinetic and chemoprevention studies with free DHA. We chose the 21-day old female Sprague Dawley rat because this rodent model is well characterized to evaluate numerous chemopreventive agents[215, 216], including omega-3 fatty acids[68, 69, 73].

4. Experimental Design of Study

4.1 Reagents

Ether lipids 1,2-dihexadecyl-*sn*-glycero-3-phosphocholine (999992) and 1,2-diphytanyl-*sn*-glycero-3-phosphoethanolamine (999985) were purchased from Avanti Polar Lipids, Alabaster, AL. Docosahexaenoic acid sodium salt (D8768) for the liposome formulation was purchased from Sigma Aldrich, St. Louis, MI. D_5-DHA (10005057) was purchased from Cayman Chemical Co., Chicago, IL., for LC-MS/MS analysis. For a standard curve for GC-FID docosahexaenoic acid methyl ester(10006865) was purchased from Cayman Chemical Co. Chicago, IL., and methyl pentadecanoate (76560) was purchased from Sigma Aldrich, St. Louis, MI.

4.2 Nanoformulation of Liposomal DHA and Ghost Liposome

Docosahexaenoic acid sodium salt(Sigma Aldrich, St. Louis, MI) was dissolved in absolute ethanol to a concentration of 10 mg/mL. 1,2-diphytanyl-*sn*-glycero-3-phosphoethanolamine and 1,2-dihexadecyl-*sn*-glycero-3-phosphocholine were dissolved in chloroform to a concentration of 10 mg/mL. For the liposome formulation, 1,2-dihexadecyl-*sn*-glycero-3-phosphocholine, 1,2-diphytanyl-*sn*-glycero-3-phosphoenthanolamine and

docosahexaenoic acid were combined in a 6:3:1 molar ratio. For ghost liposomes 1,2-dihexadecyl-*sn*-glycero-3-phosphocholine and 1,2-diphytanyl-*sn*-glycero-3-phosphoenthanolamine were combined in a 6.67 and 3.33 molar ratio. Lipids were dried to a film under a stream of nitrogen for four hours, then hydrated with 1mL of 0.9% degassed saline and heated (70°C) for ninety minutes with occasional vortexing. Next, the liposome solution was sonicated at 65°C for two and a half minutes, resulting in the liposome suspension to become less milky in appearance. The solution of lipids was extruded 13 times at 70°C through a 0.1nm polycarbonate membrane with an Avanti-Mini Extruder(Avanti Polar Lipids, Alabaster, AL,USA). Each 1mL liposome batch were purified on a CL-4B column in 0.9% saline and stored at 4°C until further use. This process separated the free DHA from the liposomal DHA and conducted for each batch of liposomal DHA.

Next, two batches of liposome were combined in an Amicon Ultra-4 Centrifugal Filter Unit with Ultracel-100 membrane(Millipore, Billerica, MA, USA). The tubes were centrifuged for 4 hours at 3500rpm and 4°C to a volume of 1mL. Following, two tubes were then combined for a total of 4 liposome DHA batches per tube(4mL). This was centrifuged for a total of 6 hours to a final volume ranging from 300µL-500µL. Each of the centrifuged batches was combined to a total volume of 8 mL.

Liposome size(nanometers) and zeta potential(millivolts) for DHA liposome and ghost were later confirmed, respectively, by dynamic light scattering and laser Doppler velocrimetry(Zetasizer Nano ZS at 25°C). The level of DHA content in the liposome was determined by liquid chromatography tandem mass spectroscopy(Agilent 1100 Series capillary LC/MSD Ion Trap XCT equipped with an electrospray ion source). To prepare liposomes for the above analysis the final concentrated liposomal DHA solution was split into two 4mL

solutions(36x concentration each). From each 30uL was aliquoted and placed into 525uL of Dulbecco's phosphate buffered saline(dpbs) for final concentration of 1x liposomal DHA. Twenty microliters of this solution was placed into 1980uL dPBS for a 1:100 dilution. From this 230uL of the 1:100 dilution was placed in a vial with 20uL of the internal standard, D_5-DHA, for MS/MS analysis. The remainder of 1x liposomal DHA was utilized in DLS and analysis and zeta potential determination.

4.3 *In Vivo* Experimental Setup

Female Sprague Rat pups (n=9) (14 days old) were purchased from Charles River Laboratories to determine the tissue distribution of DHA given in a liposome formulation; we initially focused on assessing levels of DHA mainly in the serum and the target organ, the mammary fat pad.

Sprague Dawley Rats were housed in poly-carbonated cages in a ventilated rack caging system (3 rats/cage) under controlled conditions with a 12 hour light/dark cycle at 50% humidity and 21°C temperature. All rats were given AIN-76A diet ad libitum starting one day before treatment. Diets were stored at 4°C until needed.

Following quarantine for one week rat pups were distributed by weight into three groups (3 rats/group). Rats in group one were orally administered liposomal DHA and sacrificed one hour after administration. Rats in group two were orally administer liposomal DHA and sacrificed three hours after administration. Rats in group three were orally administered liposomal DHA and sacrificed six hours after administration. To perform intragastric administration, 21 day old female Sprague Dawley rats were positioned on their back and a gavage needle (a 3.8 cm long needle with a stainless steel ball affixed to the tip to prevent accidental rupture of esophagus) was inserted into the mouth, through the esophagus, and finally

to the stomach where 400uL of liposomal DHA in 0.9% saline was introduced (1.43mg/rat). Body weights were recorded before gavage. At the time of sacrifice rats were euthanized via inhalation of gaseous carbon dioxide. Both blood and mammary fat pad(MFP) were collected. Blood was obtained by cardiac puncture into BD vacutainer serum blood collection tubes (BD Diagnostics, Franklin Lakes, NJ, USA) and centrifuged at 4^{o}C for 15 min at 3,000 rpm to recover serum. All serum was aliquoted into three Eppendorf tubes and stored at -80°C. The mammary fat pad was wrapped in tin foil and flash frozen in liquid nitrogen and stored at -80°C.

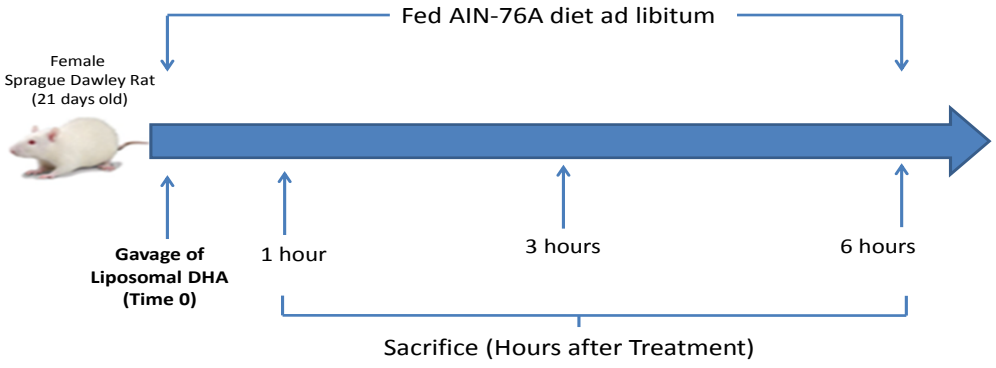


Figure 4.2- Experiment design to determine tissue distribution of liposomal DHA.
Female Sprague Dawley rats(21 days old) were orally administered a dose of liposomal DHA. Following gavage after 1, 3, and 6 hours three rats per group were sacrificed by gaseous CO_2. The blood and mammary fat pad was collected.

4.4 Analysis of Total Fatty Acids

4.4.1 Serum

Serum(100uL) was placed in a 13X100 glass tube. Two milliliters of butylated hydroxytoluene, BHT, that had been dissolved in methanol (20mg/1L) and pentadecanoic acid (0.075mg/mL) were added to the serum and placed on ice. Two hundred microliters of ice cold acetyl chloride was added to each serum sample. The samples were stirred at room temperature

for 24 hours. After 24 hours 5mL of ice cold 6% K_2CO_3 was added to each sample tube followed by 600µL of BHT in hexane solution. This solution was vortexed (speed 8) at room temperature for one minute and centrifuged for 10 minutes at room temperature. The top layer (organic layer) was collected and stored at -20°C. This solution was diluted 1:10 in hexane and submitted to fatty acid analysis by gas chromatography flame ionization detection(GC-FID).

To construct a standard curve methyl pentadecanoate (0.238mg/mL) was dissolved in hexane and evaporated to near dryness. Docosahexaenoic acid methyl ester(250,000 ng/uL) was diluted to a concentration of 2500ng/uL, 1000ng/uL, 600ng/uL, 400ng/uL, 200ng/uL, 100ng/uL, 50ng/uL and added to separate vials of the methyl pentadecanoate. These solutions were further diluted 1:10 to a final concentration of 250ng/uL, 100ng/uL, 60ng/uL, 40ng/uL, 20ng/uL, 10ng/uL, 5ng/uL with a methyl pentadecanoate concentration of 0.0238mg/mL.

4.4.2 Mammary Fat Pad

Whole organs were weighed and recorded. These organs include the mammary fat pad, liver, spleen, stomach, lung, kidney, and brain. From each organ approximately 100mg was used for analysis; it was minced, and placed in a homogenizing tube. To the tube 1mL of hexane and 1mL of ethyl acetate was added. The sample was vortexed (speed 8) for five minutes at room temperature. Twenty microliters of acetic acid (1% of total solution) was added to the tube. The tube was sonicated at room temperature for fifteen minutes, with the tissue becoming noticeably smaller. The sample was centrifuged for five minutes and the remaining liquid was removed from the remaining tissue. This solution was added to a vial and speed vacuumed on low for thirty minutes resulting in a semi-dry white layer. To this layer a 2 mL of butylated hydroxytoluene, BHT, that had been dissolved in methanol (20mg/1L) and pentadecanoic acid (0.225 mg/mL) were added to the serum and placed on ice. Two hundred microliters of ice cold

acetyl chloride was added to each serum sample. The samples were stirred at room temperature for 24 hours. After 24 hours 5mL of ice cold 6% K_2CO_3 was added to each sample tube followed by 600µL of a 20mg/L BHT in hexane solution. This solution was vortexed (speed 8) at room temperature for one minute and centrifuged for 10 minutes at room temperature. The top layer (organic layer) was collected and stored at -20°C. This solution was diluted 1:10 in hexane and submitted to fatty acid analysis gas chromatography- flame ionization detection.

4.5 Gas chromatography-Flame Ionization Detection (GC-FID) Analysis

One microliter of sample(in hexane) was injected into the GC oven (temperature of 100°C). The inject sample mixed with a carrier gas (no splitting) of helium and flowed into an Agilent CP-wax 52 CB column (30m, 0.25mm, 0.25µM) at a rate of 1.2mL/min helium. The temperature increased from 100°C to 265°C, after 23 minutes (7.17°C increase/min). The temperature increases causing the fatty acids to become volatile, dissociate from the column, and combust in a hydrogen flame. A detector (280°C) recognizes the combustion as a change in electrical resistance. The internal standard, the methyl ester(ME) of pentadecanoic acid was detected after 12.14 minutes, while DHA methyl ester was detected after 21.3 minutes for both serum**(Figure 4.3)** and mammary fat pad.

4.6 LC-MS/MS Analysis to Confirm DHA in the Mammary Fat Pad and Serum

The DHA and D_5DHA were analyzed using an API 3200 LC MS/MS triple quadrupole mass spectrometer interfaced with an Agilent 1200 series HPLC using an Agilent extended C18 column (4.6 × 150 mm, 5µm.) The electrospray was in negative ion mode. The MS parameters were set as the following: electrospray source temperature and voltage were 230°C and -4.5kV, respectively; the declustering potential (DP) collision energy (CE), entrance potential (EP) and cell exit potential (CXP) were -40, -16, -3, and -12ev, respectively; the collision activated

dissociation was set to 6 psi, while the curtain gas was set to 20 psi. The elution solvent program was 200uL/min gradient using solvent A (methanol) and solvent B (water). The gradient was 10% A to 100% A in five minutes, 100% A was held for another twenty eight minutes, and continued to 10% A in two minutes. DHA and D_5DHA were detected after 31 minutes.

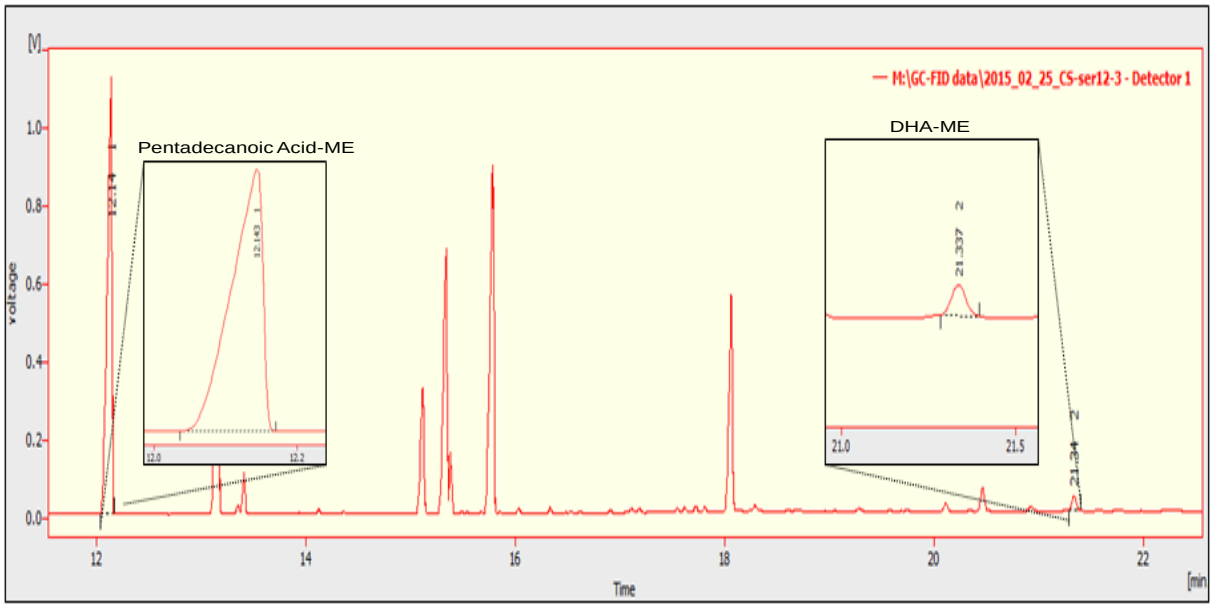


Figure 4.3- GC-FID chromatograph of DHA-ME and pentadecanoic acid-ME internal standard in the serum of a Sprague Dawley rat given liposomal DHA.

4.7 Data analysis

Clarity Software(v. 3.0.02.244, DataApex) was used to integrate peaks for both methyl esters derived from pentadecanoic acid and DHA to determine the area under the curve (AUC) following GC-FID analysis. The values were integrated by dividing AUC docosahexaenoic acid methyl ester by methyl pentadecanoate. This integrated value was placed into the slope equation ($Y=0.5016+0.009$, $R^2=0.9996$) from the standard curve **(Figure 4.4)** to yield a ratio: nanomols docosahexaenoic acid methyl ester/ nanomols methyl pentadecanoate. The ratio was converted to nanograms docosahexaenoic acid methyl ester/ milliliter serum. The following equation was

used to determine the percent of dose of liposomal DHA administered found in the serum: Percent of Dose=(nanomol DHA methyl ester/nanomol pentadecanoic acid)*586nanomols pentadecanoic acid standard*molecular weight of DHA)/100uL serum)*total serum)/1430000 nanograms DHA orally administered in liposome)*100). The following equation was used to determine the percent of dose of liposomal DHA administered found in the mammary fat pad: Percent of Dose=(nanomol DHA methyl ester/nanomol pentadecanoic acid)*586nanomols pentadecanoic acid standard*molecular weight of DHA)/mg MFP used in extraction)*total mg MFP collected per rat)/1430000 nanograms DHA orally administered in liposome)*100).All calculations were conducted in Microsoft Excel. A student t-test was applied for statistical analysis.

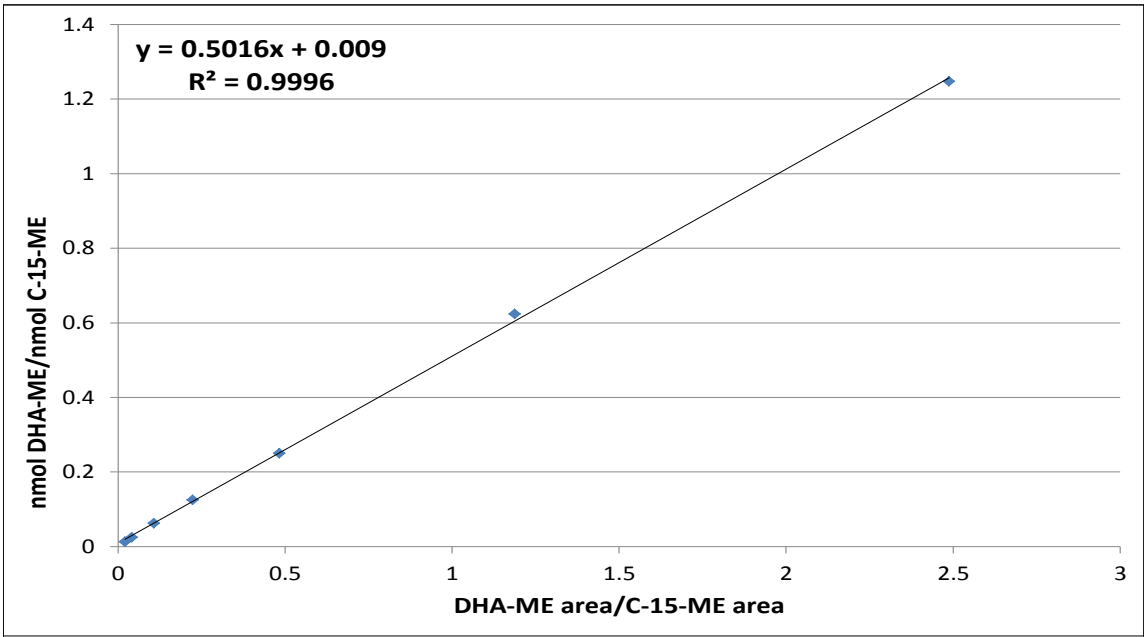


Figure 4.4- The standard curve for quantification of methylated DHA(DHA-ME) using methylated pentadecanoic acid (C-15-ME)as an internal standard for GC-FID.

4.8 Analysis of DHA Metabolites

4.8.1 Serum

Five hundred microliters of serum was placed in an Eppendorf tube with 1mL extracting solution (25% hexane, 75% ethyl acetate, 1% acetic acid), 350uL buffer (1M NaOAC, 5% MeOH, pH 6) and five microliters of 20mg/L BHT in methanol. The mixture was vortexed for five minutes, then centrifuged at 10,000 rpm at 4°C for five minutes, resulting in an aqueous and organic layer. The organic layer was removed and stored in a vial. To the aqueous layer an addition of 1mL of extracting solution was added. This mixture was vortexed and centrifuged as mentioned above. This extraction was conducted on the aqueous layer for a total of three times, resulting in a total of 3mL of organic solution for the analysis of DHA metabolites by LC-MS/MS. The following equation was used to determine the percent of dose of liposomal DHA administered found as DHA metabolites in the serum: Percent of Dose=(AUC DHA methyl ester/AUC metabolites standard)*500 nanograms metabolite standard/10)/100µL serum)*total uL serum collected per rat)/1430000 nanograms DHA orally administered in liposome)*100)

4.8.2 Mammary Fat Pad

Approximately 100 mg of mammary fat pad was removed, minced, and placed in a homogenizing tube. To the tube 1mL of extracting solution (25% hexane, 75% ethyl acetate, 1% acetic acid), 350uL buffer (1M NaOAC, 5% MeOH, pH 6) and five microliters of 20mg/L BHT in methanol. The tube was sonicated at room temperature for fifteen minutes, with the tissue becoming noticeably smaller. The sample was centrifuged for five minutes and the remaining liquid was removed from the remaining tissue. This liquid was submitted to LC-MS/MS for DHA metabolites analysis. The following equation was used to determine the percent of dose of liposomal DHA administered found as DHA metabolites in the MFP: Percent of Dose=(AUC

DHA methyl ester/AUC metabolites standard)*500 nanograms metabolite standard/10)/mg MFP used for extraction)*total mg MFP collected per rat)/1430000 nanograms DHA orally administered in liposome)*100)

4.9 LC-MS/MS Analysis of DHA Metabolites

The analysis was carried out on an AB Sciex QTRAP 4500 mass spectrometer interfaced with an Agilent 1100 series HPLC using a Zorbax SBC18 column (2.1 x 150mm, 3.5µm). The electrospray ionization was done in negative ion mode. The MS parameters were the following: electrospray source temperature and voltage were 300°C and -4.5kv, respectively; the declustering potential (DP), collision energy (CE), entrance potential (EP) and cell exit potential (CXP) were -85, -16, -10, and -13ev, respectively; the collision activated dissociation was set to medium, while the curtain gas was set to 35 psi. The elution solvent program was 250µL/min gradient using solvent A (water) and solvent B (acetonitrile). The gradient was 90% A to 10% B in ten minutes, followed by 10% A and 90% B in ten minutes, and 90% A to 10% B for eight minutes. Metabolites and standards were eluted after 14 minutes. Metabolites analyzed include 4-hydroxy DHA, 14-hydroxy DHA, and 17-hydroxy DHA. Standards for 4-hydroxy DHA(#33200), 14-hydroxy DHA(#15253), and 17-hydroxy DHA(#33650) were purchased from Cayman Chemical Company.

5. Results

5.1 Liposomal DHA Can Deliver DHA to the Circulation of the Sprague Dawley Rat

We orally administered a single dose of liposomal DHA (1.43mg DHA in 400µL saline) to Sprague Dawley rats at 21 days of age. Blood was collected 1, 3, and 6 hours after treatment. The serum from these rats was analyzed by GC-FID to determine the fatty acid concentration, specifically focusing on DHA content. It is expected that there would be a miniscule amount of

DHA in the rats at the time of oral administration because the AIN-76A diet contains no DHA, but contains alpha linolenic acid. Literature has shown when alpha-linolenic acid is given in a diet with 5% calories from fat only 1% of the alpha-linolenic acid will convert to DHA[373].

It was determined that 4.5-6% of the DHA dose administered in the form of a liposome was found in the serum at the different time points **(Figure 4.5)**. Overall, there was little variation in the amount of DHA at either time point.

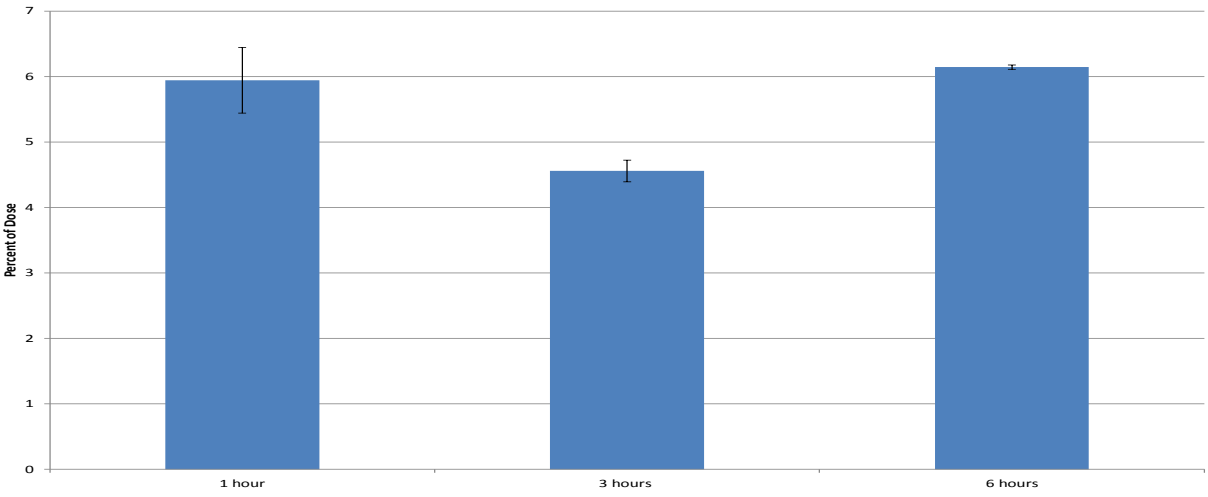


Figure 4.5- Percent of dose of DHA in the serum following the oral administration of liposomal DHA to Sprague Dawley rats. Three rats per group were sacrificed after 1, 3, and 6 hours.

5.2 Liposomal DHA Can Deliver DHA to the Mammary Fat Pad of the Sprague Dawley Rat

A single dose of liposomal DHA was orally administered to Sprague Dawley rats at 21 days of age. Following 1, 3, and 6 hours all rats were sacrificed and the mammary fat pad was collected. The fatty acids, specifically focusing on the content in the mammary fat pad, was analyzed by GC-FID.

We found that 15-20% of the DHA dose administered in the form of a liposome was detected in the mammary fat pad **(Figure 4.6)**. The amount of DHA detected at all time points was similar. However, the overall amounts of DHA in the mammary fat pad were significantly

higher than in the serum by 2-3 times**(Figure 4.5)**, thus the liposomal formulation delivered DHA to the mammary fat pad in high abundance in comparison to the serum.

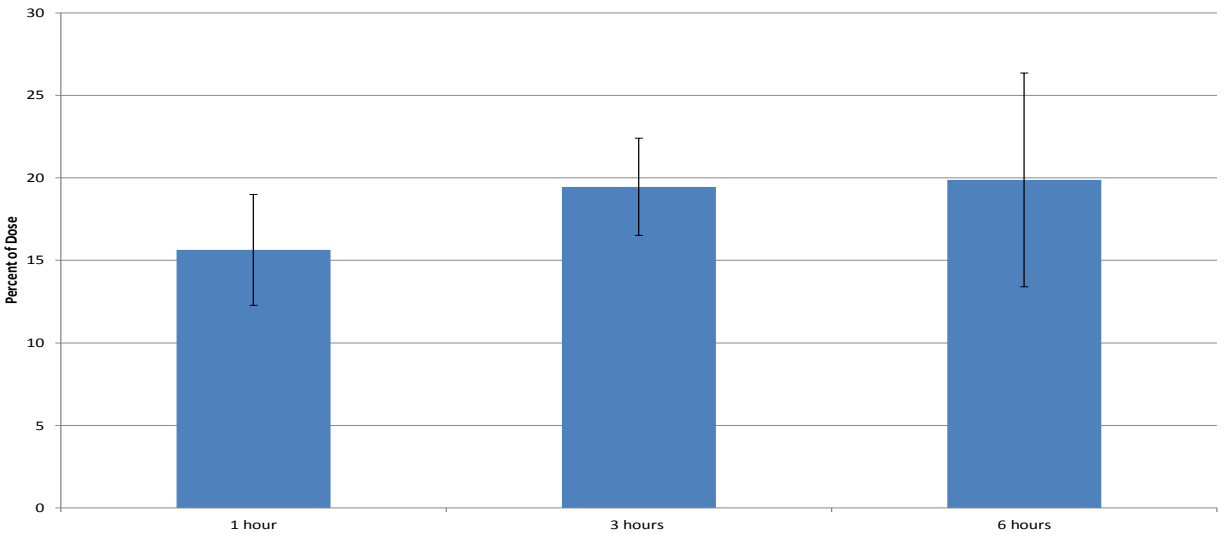


Figure 4.6- Percent of dose of DHA in the mammary fat pad following the oral administration of liposomal DHA to Sprague Dawley rats. Three rats per group were sacrificed 1, 3, and 6 hours after oral administration of liposomal DHA.

5.3 LC-MS/MS Analysis Confirmed the Presence of DHA in the Serum

Docosahexaenoic acid was found in the serum and mammary fat pad of the Sprague Dawley rats that were given liposomal DHA by oral administration. We used the LC-MS/MS method to validate the structural identity of DHA detected by GC-FID. **Figure 4.7** illustrates that LC-MS/MS chromatogram seen using a standard of DHA **(Figure 4.7A);** we also confirmed the presence of DHA in the serum **(Figure 4.7B)**.

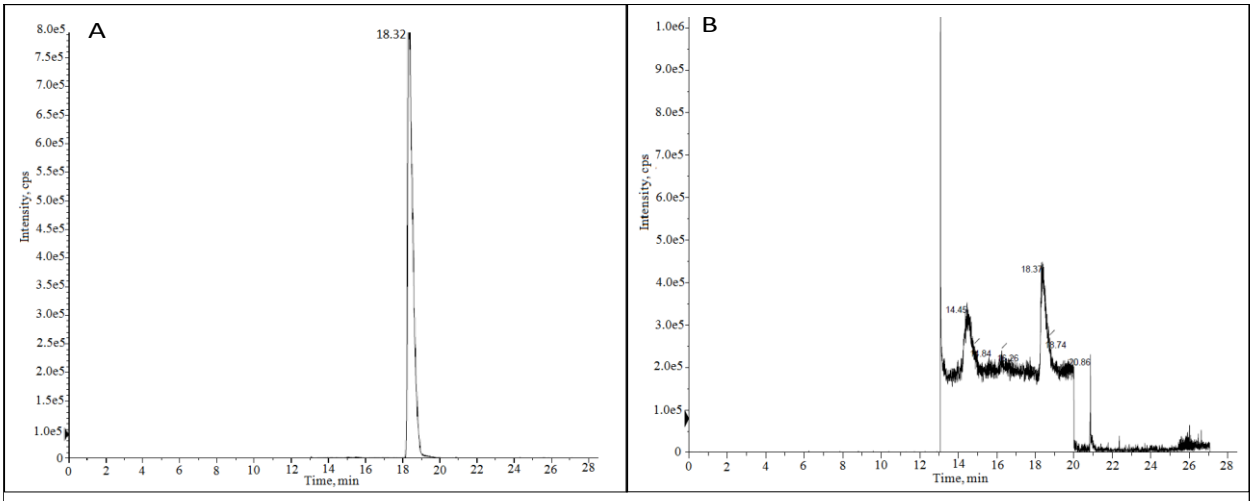


Figure 4.7- Validation of DHA in a serum sample by LC-MS/MS. LC-MS/MS chromatogram of A.) DHA standard and B.) DHA in rat serum. We confirmed the structure of DHA in the serum of rats treated with liposomal DHA; retention time 18.32 minutes.

5.4 Liposomal DHA Can Deliver LOX Metabolites of DHA to the Serum of the Sprague Dawley Rat

The DHA metabolites, 4-hydroxy DHA, 14-hydroxy DHA, and 17-hydroxy DHA are known metabolites of the lipoxygenase pathway **(Figure 4.8)**. Following the liposomal delivery of DHA we decided to focus on LOX metabolites using LC-MS/MS and we identified 4-hydroxy DHA, 14-hydroxy DHA, and 17-hydroxy DHA in the serum**(Figure 4.9)**. **Figures 4.9 A, B and C** represent the synthetic standards for 4-hydroxy DHA, 14-hydroxy DHA, and 17-hydroxy DHA, respectively. **Figures D, E, and F** show the detection of 4-hydroxy DHA, 14-hydroxy DHA, and 17-hydroxy DHA metabolites in the serum, respectively. The synthetic standards and the serum metabolites were detected by LC-MS/MS after 13-14 minutes.

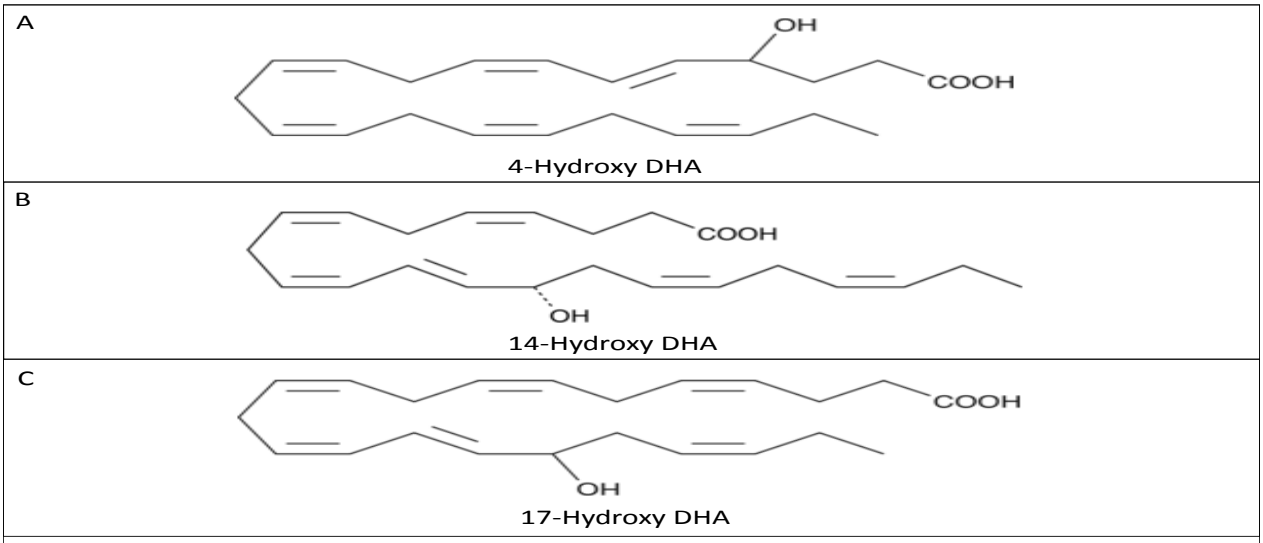


Figure 4.8- The lipoxygenase metabolites of DHA: A) 4-Hydroxy DHA, B)14-Hydroxy DHA, and C)17- Hydroxy DHA)

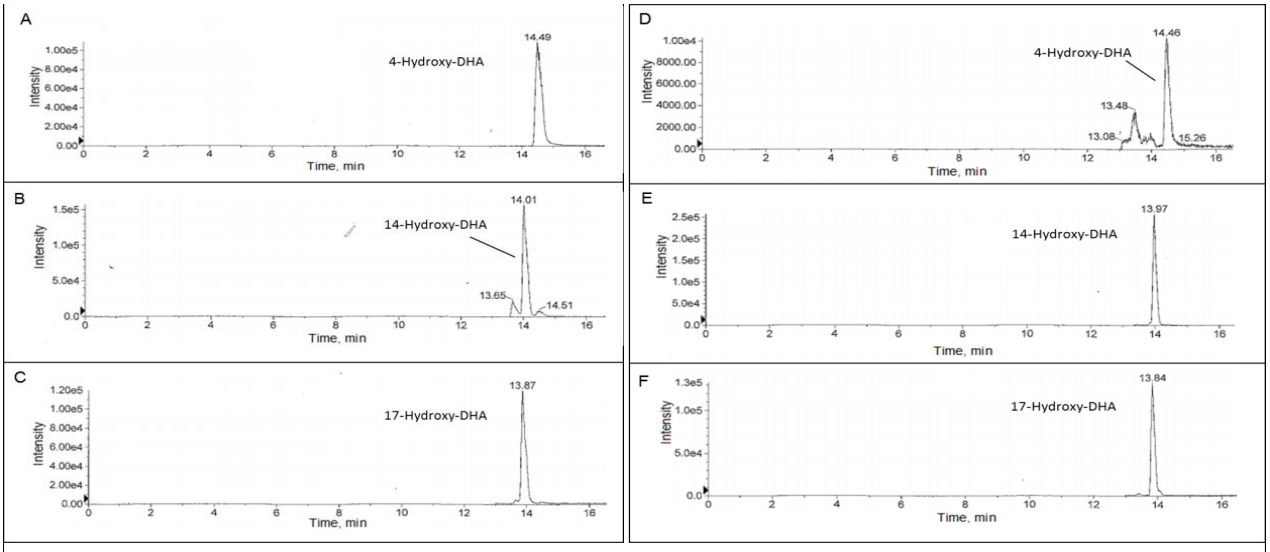


Figure 4.9- LC-MS/MS analysis of Metabolites of DHA; standards and serum samples. Standards include A) 4-hydroxy DHA, B) 14-hydroxy DHA, C) 17-hydroxy DHA, while serum samples include D) 4-hydroxy DHA, E) 14-hydroxy DHA, F) 17-hydroxy DHA.

Once we identified the metabolites in our samples we were able to quantify them by comparing the area under the curve for each metabolite to our standards. By this method we observed that a small percentage of our original dose of DHA was metabolized and found in the

serum. Expressed as percent of dose approximately, 0.001-0.005% of 4-hydroxy DHA, 0.1-0.26% of 14-hydroxy DHA, and 0.05-0.14% of 17-hydroxy DHA were found in the serum **(Figure 4.10)**. Overall, there was little difference in the amount of 14-hyroxy DHA and 17-hydroxy DHA across all time points in the serum. It appears that the amounts of the metabolites are higher at the earliest time point, specifically 4-hydroxy DHA, and decrease over time in the serum.

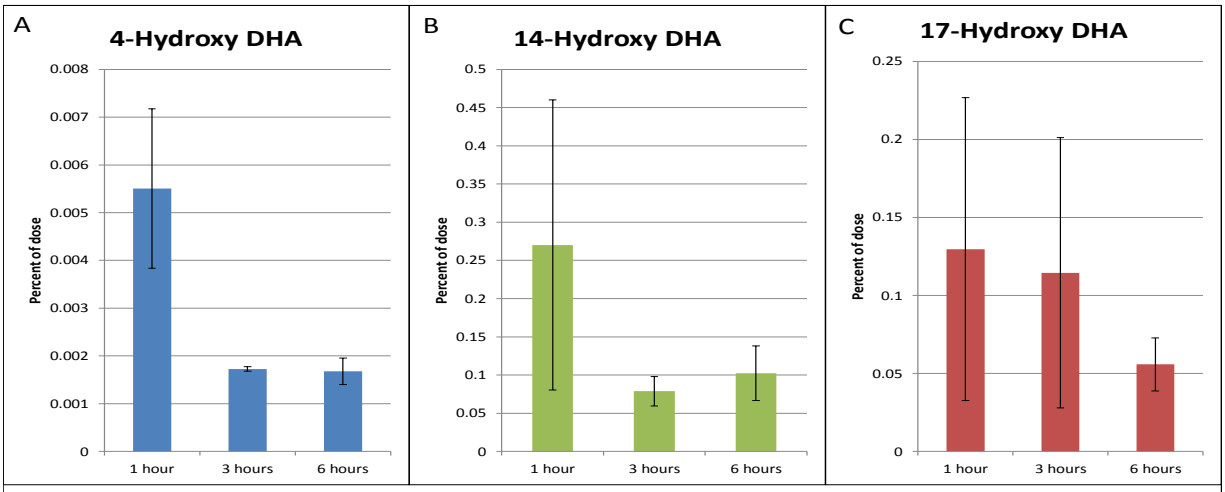


Figure 4.10- LOX metabolites in the serum expressed as percent of the dose of liposomal DHA administered. These metabolites include A) 4-hydroxy DHA, B) 14-hydroxy DHA, and C) 17-hydroxy DHA.

5.5 Liposomal DHA Can Deliver LOX Metabolites of DHA to the Mammary Fat Pad of the Sprague Dawley Rat

In addition to the serum we also quantified the amount of LOX metabolites of DHA in the mammary fat pad by LC-MS/MS. As a percentage of the dose of liposomal DHA administered, approximately 0.00005-0.00064% of 4-hydroxy DHA, 0.18-0.45% of 14-hydroxy DHA, and 0.1-0.28% of 17-hydroxy DHA are found in the mammary fat pad **(Figure 4.11)**. It

appears that the levels of all three metabolites increased over time. Overall, the mammary fat pad had an increased amount of 14-hyroxy DHA and 17-hydroxy DHA in comparison to the serum; this is expected since the mammary fat pad contained a higher dose of DHA delivered from the liposome.

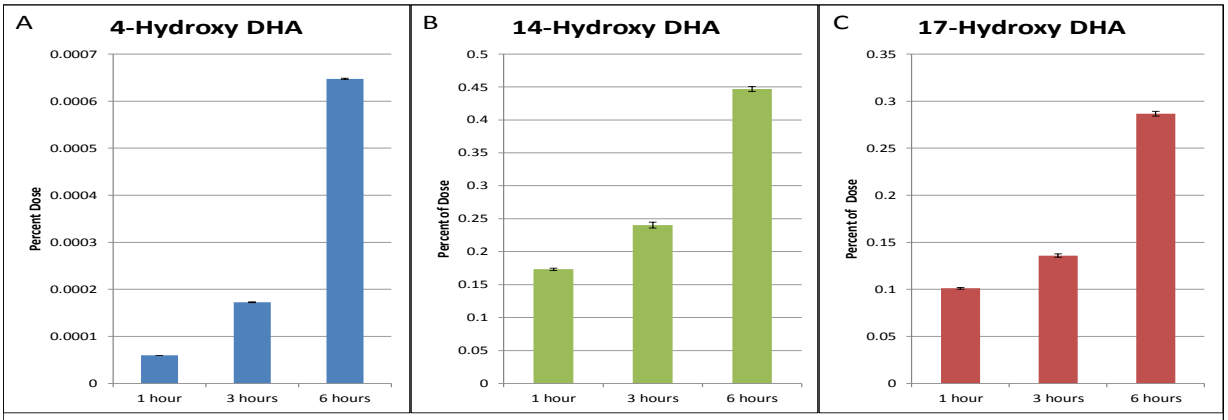


Figure 4.11- LOX metabolites in the mammary fat pad expressed as percent of the dose of liposomal DHA administered. These metabolites include A) 4-hydroxy DHA, B) 14-hydroxy DHA, and C) 17-hydroxy DHA.

6. Discussion

Our preliminary data demonstrates the first preclinical study to successfully administer a liposomal formulation of DHA by oral administration in an established rat model extensively used for assessing efficacies of numerous chemopreventive agents. The goal of our study was to evaluate if liposomal DHA could deliver DHA to the circulation and to the target organ, the mammary fat pad. In addition, we also evaluated the amount of LOX DHA metabolites known to have anti-inflammatory effects in the serum and at the mammary fat pad.

Tissue distribution studies with omega-3 fatty acid consumption have evaluated omega-3 fatty acids in organs, plasma, and red blood cells in preclinical models[374, 375] and clinical

studies[376, 377]. In a study where Sprague Dawley rats were given DHA and EPA in phospholipid and triglyceride forms, with a corn oil diet as a control, it was shown that DHA in the form of phospholipids was incorporated into muscle, liver, and surrounding adipose tissue[375]. Overall, the DHA phospholipids had a high amount of incorporation into all three organ systems. This could potentially explain how our liposome formulation of DHA composed of archaea phospholipids could facilitate such a high amount of DHA into tissues such as the mammary fat pad **(Figure 4.6)**. Another study illustrated that female Wistar rats fed a diet high in DHA(5% energy from fat) for five weeks had a high amount of DHA incorporated into fatty tissue[374]. Considering the mammary fat pad is composed of mostly adipose tissue[378], it would be reasonable to expect that our liposome formulation would deliver the majority of its DHA to the mammary fat pad.

Clinical studies have also focused on evaluating the omega-3 fatty acids content in the blood, focusing on the incorporation of fatty acids into the membranes of erythrocytes. In one clinical study healthy adults were supplemented with 510mg DHA/day(microalgal oil) for 29 days, and their blood was evaluated for DHA glycerophospholipids daily by LC-MS/MS[376]. The results showed that certain lipid varieties of DHA, phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine species, increased by 12-25% in the plasma. Our results**(Figure 4.5)** illustrated that liposomal DHA treatment resulted in a maximum of 6% of the liposomal dose in the serum, similar to results seen in literature[376]. Another study analyzed omega-3 fatty acid erythrocyte content in women to observe a potential link between omega-3 content and inflammation[377]. The study provided evidence that women with high amount of erythrocyte omega-3 fatty acids also had a high amount of omega-3 fatty acids in the breast tissue.

Adipose tissue plays an important role in lipid homeostasis by storing fatty acids as triglycerides[379]. Fish oil has shown to modify the storage and secretions from adipose tissue when taken in the diet. Omega-3 fatty acids, especially DHA, have shown to interact with PPARγ[380] leading to adipocyte differentiation[381]. In addition to fish oil, phytanic acid, a component of our ether lipid liposome formulation has shown to play a role in adipocyte differentiation[382]. Considering the roles of these particular fatty acids in adipose tissue biochemistry it does explain that the components of our liposomal formulation of DHA are ideal for adipocyte uptake at the mammary fat pad.

Furthermore, the characteristics of our liposome also provide an explanation for the increase in DHA in circulation and at the mammary fat pad. There are two potential mechanisms for our liposome formulation to be taken up into circulation. The first mechanism considers the size of the liposome. Literature has illustrated that nanoparticles at least 200nm in diameter are capable of being taken up by the enterocytes of the intestine, specifically the M cells of the Peyer's Patch via endocytosis[344]. Our liposome was approximately 136nm; an ideal size for endothelial cell uptake. It consists of ether lipids, similar to lipids found in archaea, has shown a specificity of being taken up by the M cells of the intestine[114].

Another proposed mechanism was illustrated in a study by Hossain etal. [169]; these authors applied DHA liposomes, composed of lecithin-based lipids, to Caco-2 cells grown on polycarbonate transwell filters. These transwell filters consisted of apical and basolateral chambers that would allow for the evaluation of liposomal DHA to increase the permeability of the tight junctions in between Caco-2 cells. The study revealed that DHA liposomes could increase the permeability between Caco-2 cells, potentially by reducing levels of occludin[383].

This suggested that DHA liposomes could make endothelial cells of the intestine more permeable, allowing DHA to be further taken into circulation in an *in vivo* model.

In addition to DHA, LOX metabolites of DHA found in the serum and mammary fat pad were also analyzed. Studies have shown DHA metabolites formed from the LOX pathway such as 4-hydroxy DHA, 14-hydroxy DHA, and 17-hydroxy DHA have been analyzed in serum samples of healthy humans by LC-MS/MS[384]. The metabolites 14-hydroxy DHA and 17-hydroxy DHA have been found in higher abundance in human serum samples in comparison to 4-hydroxy DHA. In mouse serum samples a similar trend was seen DHA metabolites[384]. This trend seen in literature is also consistent with our data in the rat for both the mammary fat pad and serum, where 14-hydroxy DHA and 17-hydroxy DHA are in higher abundance than 4-hydroxy DHA.

4-Hydroxy DHA is known to further oxidize to 4-oxo-DHA[385] by the LOX pathway. 4-Oxo-DHA was shown to be more potent at decreasing cell viability in the MDA-MB-231 cell line[367], thus seems to play a more protective role than 4-hydroxy DHA *in vitro*. In our study 4-hydroxy DHA was in lower abundance than the other two LOX metabolites evaluated in both serum and the mammary fat pad. Previous studies have shown that the stability of both 4-hydroxy DHA and 17-hydroxy DHA are equivalent in various aqueous mediums[386]. Thus, our data indicates that 4-hydroxy DHA is in less abundance in the serum and mammary fat pad due to being further metabolized to 4-oxo-DHA, and not due to degradation.

7. Summary and Concluding Remarks

The purpose of the studies performed in **Chapter 4** was to evaluate, *in vivo,* a liposomal delivery system for DHA that we determined in **Chapter 3** would protect DHA from acidic pH and oxidation from aqueous conditions. Our results have demonstrated that a liposomal

formulation of DHA given by oral administration to a 21 day old rat delivered DHA to the circulation and the mammary fat pad. Approximately, 4.5-6% of the liposomal dose of DHA was found in the serum, while 15%-20% was found in the mammary fat pad. This 2-3 fold increase in the mammary fat pad was significant, indicating that our liposomal formulation was capable of delivering DHA to the mammary fat pad.

In addition to DHA, we also identified DHA metabolites derived from the LOX pathway in the serum and mammary fat pad. A small percentage 4-hydroxy DHA, 14-hydroxy DHA, and 17-hydroxy DHA was found in the serum and mammary fat pad. The identification of these metabolites proved that liposomal DHA was able to deliver DHA to the tissues where it could be metabolized. Overall, our study demonstrated that liposomal DHA could deliver both DHA and its more effective anti-proliferating metabolites in both the circulation and the mammary fat pad; the results of this study suggest the potential utilization of this formulation as a chemopreventive strategy for breast cancer. Nevertheless, a comparative pharmacokinetic study of liposomal DHA and free DHA should be explored in the future.

Chapter 5

Summary and Future Directions

1. Chemopreventive Strategies for Breast Cancer

In 2015 it is expected that 231,840 cases of breast cancer will be diagnosed in women, with 40,290 resulting in death[1]. Risk factors for developing breast cancer include age, family history, inherited mutations (*BRCA1, BRCA2*), radiation exposure, estrogen levels, and diet. Current chemopreventive strategies for breast cancer include the use of selective estrogen receptor modulators, tamoxifen and raloxifene, daily for women who have a high risk of developing breast cancer[31, 32]. These agents, even though effective, are not without side effects that include an increase in endometrial cancers and thromboembolic events. In the United States there are currently 2 million women that are high risk for breast cancer, but less than 4% are interested in taking such agents on a daily basis because of the fear of side effects[387]. In addition, these FDA approved chemopreventive strategies are only preventive against estrogen receptor positive(ER+) breast cancers and will not prevent estrogen receptor negative(ER-) breast cancers.

There are potential preventive strategies for ER- breast cancers that have been investigated in ER- preclinical models, specifically focusing on Her-2/neu and triple negative breast cancers. The combination of rexinoids, and selective estrogen receptor modulators Arzoxifene and Acolbifene have been effective at preventing tumors for developing in a MMTV-neu mouse model of mammary cancer[388]. The COX-2 inhibitor, Celecoxib, in combination with the rexinoid, LGD1069, has also been a potential combination for the delay of Her-2/neu breast tumors[389]. Other potential preventive strategies include using agents such as metformin,

statins, poly(ADP-ribose) polymerase inhibitors[390], however, these agents for breast cancer prevention have only been validated in preclinical studies and still need to be tested in humans.

Natural agents such as EGCG[124], a component of green tea, resveratrol[391], a component in red grapes, and omega-3s[68, 69], components of fish oil have been investigated as potential chemopreventives in breast cancer. Epidemiological studies have provided some evidence that omega-3 consumption has an inverse relationship with the risk of breast cancer, but overall the results are mixed[392-394]. Preclinical studies have also been very supportive of omega-3 fatty acids as a breast cancer preventive[68, 69], but some inconsistencies remain[395]. These inconsistencies have been linked to several confounding variables that were not accounted for in the preclinical studies such as fish oil source, omega-3:omega-6 fatty acid ratio, and fat caloric intake[34].

To better identify the role of the omega-3:omega-6 ratio and the standardization of calorie intake in chemoprevention these variables were investigated in an MNU rodent model of mammary carcinogenesis[71]. The study determined that female rats(21 days of age) fed a high ratio of omega-3 fatty acids ad libitum with and without tamoxifen for 8 weeks had a decrease in tumor incidence, multiplicity and burden. However, only rats given a 25:1 n-3:n-6 ratio with and without tamoxifen had a significant reduction in tumor incidence when compared to the control(1:1 n-3:n-6). To understand this reduction in tumor incidence by high omega-3:omega-6 ratio I decided to study the effects of these high omega-3 fatty acids with and without Tamoxifen at the protein level using plasma as a minimally invasive approach to elucidate the mechanisms that can account for mammary cancer prevention[181]. The proteins could provide mechanistic insights and possibly be potential biomarkers to monitor disease development and efficacy of omega-3 as a potential chemopreventive.

2. Summary of Results from this Study

2.1 Elucidating a Mechanism to Explain the Preventive Effect of High Omega-3:Omega-6 Ratios in an MNU- Rat Model of Mammary Carcinogenesis

The mechanism that can explain the decrease in cancer incidence observed as a result of high omega-3 intake(25:1 n-3:n-6 ratio) has been determined by our collaborators in adenocarcinomas of rats at the protein level by western blot analysis**(Figure 5.1)**[73]. Pathways involved in cell proliferation and growth, inflammation, and lipid metabolism were altered in a manner that favored a decrease in cell proliferation more so than an increase in apoptotic cell death. This mechanism explained the decrease in adenocarcinomas seen in the rats fed a high omega-3:omega-6 ratio. However, a minimally invasive approach that can provide insights into the mechanism of action and potential biomarkers for disease progression and monitor the efficacy of chemopreventive agents is highly preferred.

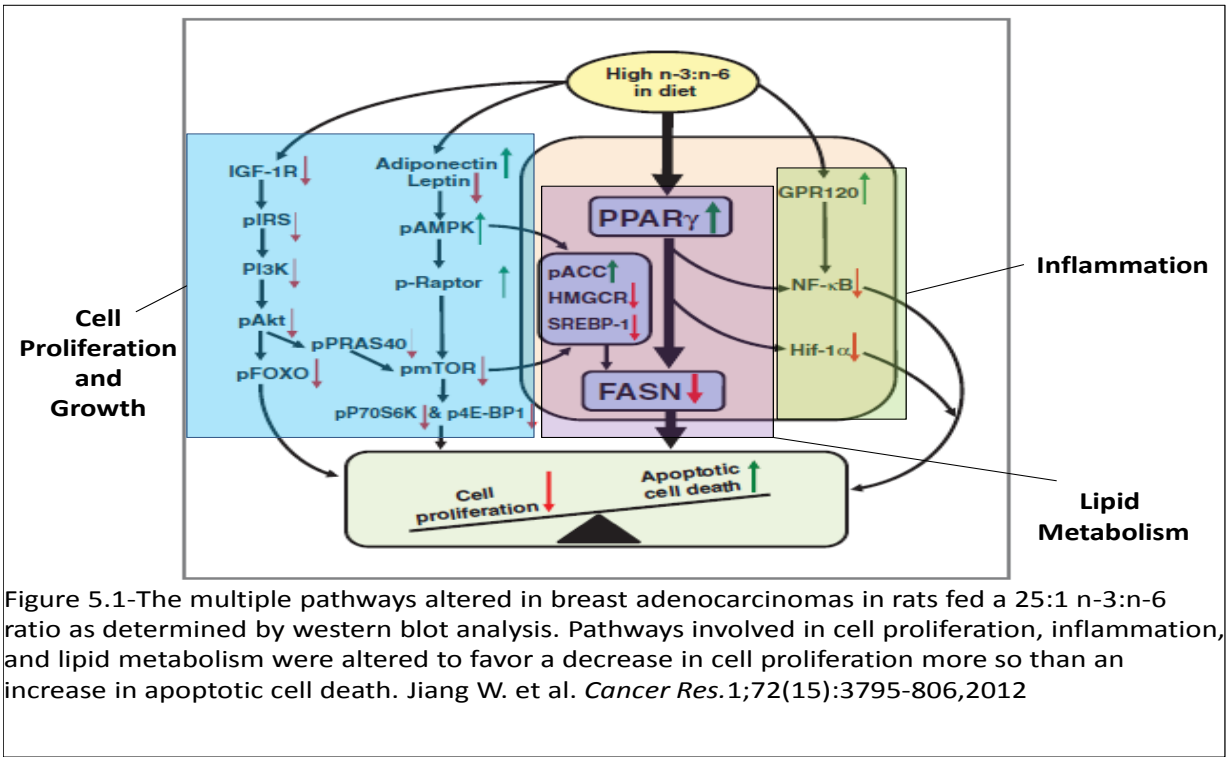


Figure 5.1-The multiple pathways altered in breast adenocarcinomas in rats fed a 25:1 n-3:n-6 ratio as determined by western blot analysis. Pathways involved in cell proliferation, inflammation, and lipid metabolism were altered to favor a decrease in cell proliferation more so than an increase in apoptotic cell death. Jiang W. et al. *Cancer Res.*1;72(15):3795-806,2012

I utilized **(Chapter 2)** the iTRAQ method(isobaric Tagging for Relative and Absolute Quantitation) to measure protein expression in the rat plasma proteome in response to the high omega-3:omega-6 fatty acid diet in the presence and absence of tamoxifen[181]. Groups included the 1:1 n-3:n-6(control), 10:1 n-3:n-6, 25:1 n-3:n-6, 25:1 n-3:n-6 with tamoxifen (1.0mg/kg diet). Our iTRAQ experiment revealed that out of 148 high confident proteins identified, 10, 14, and 19 proteins were modified significantly($p \leq 0.05$) in the 10:1, 25:1 and 25:1 plus tamoxifen group in comparison to our control(1:1). These proteins were evaluated by western blot in individual rats to validate our iTRAQ experiment but to also focus on proteins that were involved in breast cancer. We found that the high omega-3:omega-6 ratio alone, was able to increase the expression of gelsolin, vitamin D binding protein, and 14-3-3 sigma, while decreasing the expression of alpha-1B-glycoprotein. The high omega-3:omega-6 ratio with tamoxifen decreased apolipoprotein E, haptoglobin, and inter alpha inhibitor heavy chain 4, all three involved in breast cancer.

Once some of our proteins were validated by western blot analysis we decided to investigate a potential mechanism based on protein expression in the plasma[223, 249, 287, 288]. We conducted a thorough literature search and determined that our proteins were able to be paired with proteins found in the adenocarcinoma study[73]. Therefore, we determined that the plasma protein expression in conjunction with the protein expression observed in adenocarcinomas in the same animals could explain the alterations in pathways involving cell proliferation and growth, lipid metabolism, and inflammation. In particular, we determined that vitamin D binding protein could be utilized as a potential biomarker for 25:1 n-3:n-6, while haptoglobin could be utilized as a potential biomarker for the 25:1 n-3:n-6 plus tamoxifen group. However, to validate such proteins as reliable biomarkers, their detection and quantification in

women given a diet high in omega-3 fatty acids with and without tamoxifen needs to be investigated.

2.2 Development and Evaluation of the Biological Activity of Liposomal DHA

Our previous study[71] determined that high omega-3:omega-6 ratios were preventive in an MNU model of mammary carcinogenesis. However, for women at high risk for breast cancer to consume such high amounts of omega-3 fatty acids on a daily basis would be impossible, especially due to the Western diet consisting of 15:1 n-6:n-3 ratio. Literature data support a role of DHA or EPA in having an anticancer effect, *in vitro* and *in vivo*, with DHA being more efficacious than EPA in these preclinical studies[68, 69]. Therefore, we hypothesized that an abundance of DHA or its metabolites could be influencing the decrease that we saw on cancer incidence[73].

It might be assumed that if DHA were to be consumed as a supplement form on a daily basis that a high amount of DHA would be absorbed into circulation, but the susceptibility of DHA to oxidation has been illustrated in literature[346]. Specifically, the unsaturation of DHA can make it susceptible to oxidation in aqueous medium and in acidic environments. Therefore to develop a delivery system that could protect DHA from oxidation and acidic pH would be ideal for daily oral administration.

I successfully developed a liposomal formulation using ether lipids as a means of protecting DHA from oxidation and acidic pH **(Chapter 3)**. Our liposomal formulation was approximately 136nm, weakly negative, and had a high encapsulation efficiency of DHA ranging from 60-80%. Ether lipids were preferred since they mimicked the same chemical structure of ether lipids found in the microorganism archaea, a species known to tolerate such environments

as high pressure, high heat, and acidity. By measuring the diameter our liposomes by dynamic light scattering in response to acidic conditions we were able to validate that our liposome was stable at acidic and neutral pHs at physiological temperature(37°C). In addition we determined that our liposome could protect DHA from oxidation in aqueous medium at 37°C.

Once our liposomal formulation of DHA was characterized we evaluated its biological activity in comparison to free DHA *in vitro* using human breast cancer cell lines. Specifically, we focused on cell viability and apoptosis pathways to evaluate the efficacy of our liposome because these pathways were found to be effected by high omega-3:omega-6 fatty acids in a previous study[73]. We found that our liposomal formulation was more efficacious at decreasing cell viability than free DHA in both MCF-7(ER+) and MDA-MB-231(ER-,PR-,Her2/neu-). The IC_{50} of liposomal DHA was 38.8uM and 65uM in MCF-7 and MDA-MB-231, respectively. For free DHA in the MCF-7 and MDA-MB-231 cell lines the IC_{50}s were 72.5uM and 69uM, respectively.

Apoptosis assays determined that our liposomal formulation was significantly more efficacious at increasing apoptosis in comparison to free DHA in the MCF-7 cell line. The ghost liposome at all doses had a similar effect to the lowest dose of liposomal DHA administered. In the MDA-MB-231 cell line liposomal DHA was more efficacious than free DHA at increasing apoptosis, however, this efficacy was not significant. The ghost had no effect on this cell line at any doses used in this study.

Once it was concluded that our liposome could alter both cell proliferation and apoptosis pathways in human breast cancer cell lines, protein markers known to be involved in both pathways were evaluated initially in the MDA-MB-231 cell line. Specifically, 60 μM of liposomal DHA and free DHA were administered to this cell line because it was approximately

the IC_{50} for liposomal DHA. We found that markers of cell growth(P-AKT, P-S6), the cell cycle(p21), and apoptosis(cleaved PARP, BCL-2) were altered more efficaciously by liposomal DHA in a manner that was consisted with chemoprevention.

2.3 Evaluation of Liposomal DHA on Tissue Distribution in a Rat Model

Once our liposomal formulation of DHA was characterized as being acid stable we decided to administer our formulation by oral administration to 21 day old female Sprague Dawley rats. Following the administration of liposomal DHA, rats were sacrificed at several time points. We were interested in evaluating the amount of DHA that could be delivered to the serum and to the mammary fat pad.

By GC-FID we found that up to 20% of our liposomal dose could be found in the mammary fat pad after 6 hours. In addition, we found that up to 6% of the liposomal dose could be found in the serum after 6 hours. This approximated to about a 2-3 fold significant increase in DHA at the mammary fat pad in comparison to the serum. This proved that our liposome formulation was effective at delivering DHA to the circulation and the mammary fat pad.

However, we were also interested in determining if the DHA that reached these sites can be metabolized to hydroxylated metabolites catalyzed by the lipoxygenases. These metabolites are known to have more potent anticancer activity than DHA. By LC-MS/MS we determined that low levels of 4-hydroxy DHA, 14-hydroxy DHA, and 17-hydroxy DHA were found in the mammary fat pad. Even though these were a fraction of a percent of the dose of liposome administered they were still higher than the amount of metabolites found in the serum. Thus, our liposome formulation was capable at delivering DHA and its metabolites to the mammary fat pad and the serum.

3. Future Directions

3.1 Validation of Protein Biomarkers in High Risk Women

In phase 2 clinical trials, studies focusing on fatty acid intake in women who are at high risk for developing breast cancer are typically focused on specific plasma proteins involved in inflammation (COX-2, IL-6, IL-8, TNF-α)[396-398]. Our iTRAQ study found proteins such as serum amyloid A, haptoglobin, and ITIH4, proteins known to be involved in inflammation, altered in response to high omega-3 fatty acids. Considering that omega-3 fatty acids modify alternative pathways of inflammation, besides those involving interleukin proteins, serum analysis in patients given high omega-3 fatty acids should not be limited to IL-6, IL-8, and TNF-α. In addition, proteins involved in pathways such as lipid metabolism and cell proliferation, besides ki-67, should be considered.

We currently have some preliminary data focusing on the expression of some proteins found in our iTRAQ experiment in women at high risk based on breast density. These women were given Lovaza, an omega-3 supplement, with and without raloxifene for 12 months**(Figure 5.2)**. These preliminary results demonstrate that some of the proteins we found in our iTRAQ study were present in the plasma proteome of women given omega-3 supplementation with and without raloxifene. However, these proteins were not modulated significantly in response to treatment in these women after one year which may be attributed to the fact that these women are healthy and thus the treatment had no effect on the basal level of these proteins. Further analysis of these proteins needs to be done at later time points to observe if these proteins can be altered after prolonged treatment (2-years). Also, the effect of Lovaza and raloxifene intake on protein expression should be examined in obese women(BMI ≥25), since its known that there are higher

levels of inflammation at the breast tissue due to an increase in adipose tissues. In addition, other proteins discovered in our iTRAQ study should be evaluated.

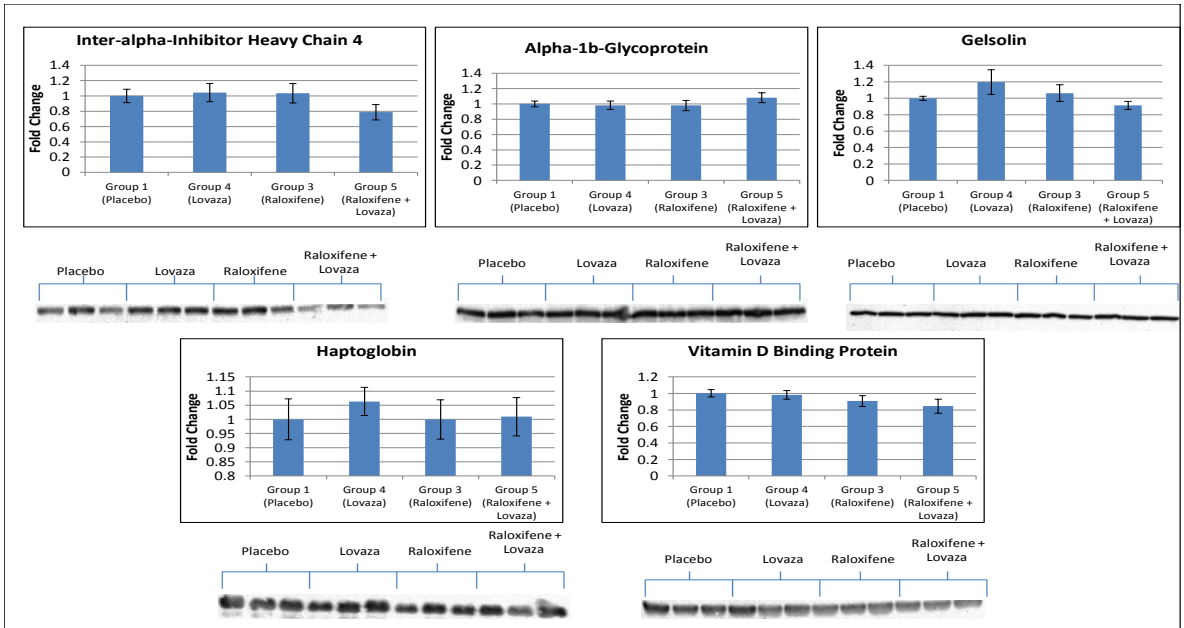


Figure 5.2- Women at high risk for breast cancer were administered Lovaza with and without Raloxifene. To evaluate the response to treatment plasma proteins involved in cell proliferation, inflammation, and lipid metabolism were measured by western blot analysis. (n=3/group)

3.2 Liposomal DHA

3.2.1 Modifications for Increased Circulation

Liposomes have been applied to the field of drug delivery for decades. In particular liposomes are an ideal delivery system since their components typically mimic lipids found in human cellular membranes, resulting in a delivery system that can interact with human cells and have minimum toxicity. However, components of the human immune system can recognize these delivery systems, resulting in their clearance and destruction. Specifically, the reticuloendothelial system, also known as mononuclear phagocyte system(MNS), has posed as a regulating factor when it comes to liposome circulation in the blood. The spleen and liver are both known to be involved in this process, with their macrophages and Kupffer cells,

respectively, removing liposomes from circulation. This can be an obstacle when it comes to achieving a long circulation life for a delivery system in an attempt to deliver an agent to a particular organ of interest.

There are ways to avoid this clearance mechanism. One way to circumvent this process is by the addition of polyethylene glycol polymers(PEG), known as pegylation, having a molecular weight of 1000-3000, to the outer leaflet of the liposome[399, 400]. Pegylation of a liposome prevents albumin, a blood protein known to bind and transport fatty acids, from binding and disrupting the lipid bilayer. In addition, PEG also protects the liposome from being coated with opsonizing proteins in the blood, which can direct the liposome to being taken up by Kupffer cells in the liver[401]. Finally, pegylation can also affect the packing of a liposome's lipids making the interaction tighter and the liposome more stable[401]. It has been shown that lipids with PEG covalently bound to phosphatidylethanolamine in amounts ranging from 5-7% can increase circulatory life of the liposome[399]. The addition of cholesterol to these pegylated liposomes may also act as a spacer and reduce PEG chain-chain interactions[399].

Our current liposome formulation is composed of ether lipids and DHA. It has the ability to deliver up to 20% of its dose to the mammary fat pad and about 6% of its dose to the serum. Thus, our liposome formulation is successful at delivering a high amount of DHA to the organ of interest. However, if our 1,2-di-phytanyl-sn-glycero-ethanolamine lipid had PEG covalently attached to its ethanolamine group it may increase the circulation of our liposome in the blood, resulting in a higher amount of the liposome to come in contact with the mammary fat pad. At the present time a PEG version of our ether lipids is not commercially available but this polymer might be added to our lipids if a chemical synthesis is designed to do so. Furthermore, if synthesized and applied to our current formulation, the pegylated liposome will have to be

submitted to acidic conditions and analyzed via dynamic light scattering to evaluate if this additional polymer is stable in acidic conditions, and at similar pHs seen in the gastrointestinal track (pHs 1-7).

3.2.2 Improvement of Liposome Biological Activity for the Prevention of Triple Negative Breast Cancers

Omega-3 fatty acids have been proven to have a preventive effect on the development of estrogen receptor positive tumors[68, 69, 71] and Her-2/neu positive tumors[402] in preclinical models of breast cancer. However, the effects of omega-3 fatty acids on certain triple negative tumor models of breast cancer, such as the polyoma middle T model, have not been as promising[402]. This difference in the effects of omega-3 fatty acids as a result of receptor status is due to differences in the tumor biology. For example COX-2, an estrogen receptor target gene that is downstream the Her-2/neu pathway was shown to be down regulated in estrogen receptor positive tumors in response to fish oil[403]. Also, DHA has shown to be incorporated into the plasma membrane of MCF-7 cells more readily than MDA-MB-231 cells leading to a decrease in lipid rafts and oncogenic signaling in the MCF-7 cell line[404]. Furthermore, DHA has shown to effect p53 signaling in the MCF-7 cell line leading to a decrease in cell proliferation, while p53 in the MDA-MB-231 cell line is mutated and its protein expression has little effect on oncogenic signaling.

Our collaborators[367] have determined that the lipoxygenase metabolites of DHA are more effective than DHA at significantly decreasing cell proliferation in the MDA-MB-231 cell line. Therefore, we hypothesize that a liposomal delivery system that could protect 4-hydroxy DHA or 4-oxo DHA from oxidation and pH fluctuations would be more effective than liposomal DHA at preventing triple negative breast cancers *in vivo*. Furthermore, this liposomal delivery

system of metabolites of DHA would be expected to be more effective than liposomal DHA at decreasing cell proliferation in triple negative breast cancer cell lines, since these cell lines are more sensitive to DHA metabolites than receptor positive cell lines.

3.2.3 *In Vivo* Evaluation of Liposome Uptake

Our liposome formulation has shown to effectively deliver DHA into the circulation and at the breast tissue. This is due to our formulation protecting DHA from oxidation and acidity in the gastrointestinal track of the rat, allowing for optimal uptake of DHA. The mode of uptake in the intestine still needs to be determined. There are two theories on the uptake of DHA that include being taken up by M Cells of the Peyer's Patch of the intestine[344] and increasing the permeability of tight junctions between intestinal cells to allow for the liposome to pass into the lymphatic system[169].

One method to determine if our liposome is intact when it gets into circulation would be to orally administer it to the 21 day old Sprague Dawley rat. Approximately 1, 3, and 6 hours later lymphatic fluid could be collected from each rat and submitted to dynamic light scattering to observe if a liposome could be identified. Alternatively, a portion of this lymphatic fluid could be submitted to cryo-transmission microscopy(cryoTEM) to observe any liposomal structures.

3.2.4. Pharmacokinetic(PK) Studies of Liposomal DHA and Free DHA

PK studies of liposomes have been conducted to determine if the liposomal formulation delivering a particular agent is more efficacious than the agent alone in a preclinical model. In a previous PK study where liposomes delivering short chain ceramide were evaluated; the liposome formulation, ghost, and free fatty acid was administered to a Sprague Dawley rat[405]. Pharamcokinetic parameters including C_o, AUC, CL, $t_{1/2}$, and V_d were determined by analyzing the plasma over many time points following liposome administration. The exact parameters

would have to be determined for liposomal DHA and free DHA to determine if the liposomal formulation is more efficacious at delivering DHA to the circulation *in vivo*. In this case the same dose of DHA would have to be administered by oral gavage for both liposomal DHA and free DHA. Also, the amount of ghost liposome given by oral administration would have to be the lipid equivalent of liposomal DHA. Utilizing a deuterated form of DHA (D_5-DHA) may also facilitate the detection of both liposomal and free DHA.

3.3 Chemoprevention Studies

3.3.1 Chemoprevention Studies in ER+ Preclinical Models of Breast Cancer

In our *in vivo* study we were able to determine the percent of the dose of our liposome that reached the serum and mammary fat pad. Since our liposomal formulation was effective at delivering up to 20% of the dose to the mammary fat pad we are confident that it would make for an ideal delivery system for DHA in chemoprevention studies. Chemoprevention studies using fatty acids have been conducted with success as reported in literature for both ER+[68, 69, 71, 183, 406] and ER-[403, 407] breast cancers. Specifically, ER+ models include chemically-induced mammary carcinogenesis with MNU[68, 71, 183, 406] and DMBA[69] in the rat, while ER- models include the MMTV-Her2/neu mouse model[403] and a polyoma middle T transgenic mouse model for triple negative breast cancer[407].

Our liposome formulation was orally administered to a 21 day old Sprague Dawley rat as described in **Chapter 4**. We initially chose to give this liposome to the rat at this particular age because in prior chemoprevention studies where omega-3 fatty acids were evaluated they were also administered in the diet at this age. This is the ideal age to administer chemopreventive agents in a chemically induced rat model of mammary carcinogenesis because the agent will have the ability to start reversing the damage done by prior carcinogen administration on the

proliferative terminal end buds of the mammary fat pad[215]. In addition, the continuation of the omega-3 fatty acids ad libitum[71] on a daily basis or twice a week by intragastric administration[69] will enhance its ability to counteract the effects of the carcinogen, resulting in a preventive effect on tumor formation. However, the progression of carcinogenesis has been well defined in the 21 day old rat model of MNU-induced mammary[215] carcinogenesis and the effect of our liposome formulation on the early development of lesions such as atypia hyperplasia and ductal carcinoma in-situ could be evaluated.

In addition, the chemoprevention study in an ER+ rat model of chemically induced mammary carcinogenesis could be conducted in a 50 day old rat [68, 69]. The administration of liposomal DHA to a 50 day old female Sprague Dawley rat would prevent any confounding variables that could be related to pre-pubertal effects. However, in a 50 day old model the duration is much longer and takes up to 20 weeks post-carcinogen administration to develop palpable tumors, requiring an additional 12 weeks of liposomal DHA administration in comparison to the 21 day old model that develops the same tumors only 8 weeks following carcinogen administration.

Furthermore, the effects of liposomal DHA could be assessed on preneoplastic lesions, such as intraductal proliferations(atypia ductal hyperplasia) and ductal carcinoma in-situ**(Table 4.1)** in the MNU Sprague Dawley rat model of mammary carcinogenesis. With this experimental set-up one week following carcinogen administration(i.p.), liposomal DHA could be given by oral administration to Sprague Dawley rats and the rats could be sacrificed 1 week, 2 weeks, and 3 weeks after oral administration. Depending on the time of sacrifice the percentage of intraductal proliferations and DCIS will differ. Since these lesions can not be palpated the use of laser microdissection will allow for the proper removal of lesions[408]. These lesions could then

be tested by immunohistochemistry techniques for markers of proliferation such as ki-67. These preneoplastic lesions could also be homogenized and the protein expression could be evaluated by western blot analysis for protein markers of cell growth, the cell cycle, and apoptosis to assess the preventive effects of liposomal DHA on protein expression. The proteins found to be altered in our iTRAQ experiment **(Chapter 2)** in a manner consistent with chemoprevention by the high omega-3 ratios with and without tamoxifen could be evaluated in these preneoplastic lesions. By evaluating the alteration in protein expression in these lesions it would provide more information on their expression at earlier time points in the MNU model. We could then determine if the proteins found in our study **(Chapter 2)** would be appropriate for further testing in the clinic as potential biomarkers in high risk women administered omega-3 fatty acids with and without antiestrogens as a form of chemoprevention. Obviously, more than one protein would have to be evaluated in preneoplastic lesions in response to high omega-3 fatty acids with and without tamoxifen because we know from our iTRAQ experiment and literature[73, 409] that omega-3 fatty acids and antiestrogens can alter multiple pathways. Proteins that could be evaluated in preneoplastic lesions in response to high omega-3 fatty acids include VDBP, haptoglobin, gelsolin, fetuin B, and 14-3-3 sigma. Proteins that could be evaluated in preneoplastic lesions in response to the high omega-3 fatty acids with tamoxifen include haptoglobin, serum amyloid A, c4b binding protein, ceruloplasmin, and ITIH4.

3.3.2 Chemoprevention Studies in ER- Preclinical Models of Breast Cancer

Omega-3 fatty acids have also been studied in ER- mouse models of mammary cancer, that include Her2/neu[403] and triple negative[407] types of breast cancer. The polyoma middle T transgeneic mouse, a model that forms ER+ tumors that progress to ER- tumors over time, has been used to observe the effects of fish oil with and without tamoxifen. Similar to models

previously mentioned administration of a chemopreventive is started at 3 weeks of age. It is thought that by antagonizing the estrogen receptor in this model, tumors can't progress to the triple negative stage. Overall, studies have been supportive with fish oil and tamoxifen, in combination, reducing palpable lesions. This model would be ideal for the evaluation of liposomal DHA as a preventive agent for triple negative breast cancer.

MMTV-Her2/neu transgenic models[403] have been applied to evaluating the effects of omega-3 fatty acids on pre-invasive mammary lesions. When fish oil was administered to the Her-2/neu transgenic mice for up to 35 weeks a dose dependent decrease in early developing lesions, including atypia hyperplasia, and cellular proliferation(ki67) was observed. Our liposome formulation could also be administered to the same mouse model for a period of 35 weeks to observe its effects of pre-invasive lesion development.

Other mouse models of breast cancer, include the MMTV-Wnt-1[410] and MMTV-M-Wnt mouse models. These have been utilized to investigate the effects of the omega-3 fatty acids on the inflammatory profile in these mice that were fed a diet to induced obesity(DIO)(60% kcal % fat diet)[411]. These mice provide a means to study basal-like and claudin-low breast cancer subtypes, known to be more aggressive in states of obesity[412]. In the DIO mice given 0.025% DHA plus EPA daily the tumor burden was significantly less than mice given no omega-3 fatty acids for both MMTV-Wnt-1 and MMTV-M-Wnt tumors [411]. Furthermore, 0.025% DHA plus EPA decreased inflammatory protein expression including the expression of COX-2 and phospho-p65 in both MMTV-Wnt-1 and MMTV-M-Wnt tumors. Clearly, the administration of omega-3 fatty acids can offset the pro-tumor effects of obesity in some preclinical models.

3.3.3 Additional Mechanistic Studies Involving Chemopreventive Models

In addition to tumor palpation and screening for pre-invasive lesions genomic and proteomic studies could be conducted on the tumors and lesions to elucidate the mechanism of action for the liposomal DHA formulation. Similar to the work conducted in other *in vivo* studies evaluating omega-3 fatty acids and breast cancer, we could observe the effects of liposomal DHA in comparison to free DHA on altering pathways of inflammation, lipid metabolism, and cell proliferation in plasma[181] and tumor development[73]. We could also observe changes in expression of ki-67, a marker of cell proliferation, in pre-neoplastic lesions by immunohistochemistry to observe if liposomal DHA is more effective than free DHA at decreasing cell proliferation. Her-2/neu is known to be an up-stream mediator of COX-2 and FASN RNA expression. By RNA isolation and qRT-PCR the expression of these transcriptions could be assessed in mammary tumors in rodents treated with both liposomal DHA and free DHA to evaluate efficacy of the liposomal delivery system.

4. Concluding Remarks

Plasma protein biomarkers have the potential to provide insights into the mechanism of cancer development, and to evaluate the efficacies of chemopreventive agents for breast cancer. By utilizing a chemically-induced mammary carcinogenesis rat model, we identified plasma proteins that could explain the reduction in mammary cancer incidence, multiplicity and burden, seen in response to a high omega-3:omega-6 ratio with and without tamoxifen. These proteins can be evaluated in plasma samples from our current clinical trial in women at high risk for breast cancer given omega-3 fatty acids or raloxifene and in combination as daily supplements. Some of these proteins have been detected in women given the omega-3 supplement Lovaza with

and without raloxifene after 1 year **(Figure 5.1)**, but more work needs to be done on evaluating these plasma proteins after longer durations of omega-3 supplementation(2-years). In addition, plasma protein expression may be used as a complement to breast density to assess risk for breast cancer development. Other methods, such as evaluating markers of inflammation (IL-6,IL-8, COX-2) in addition to the plasma proteins we found may also provide a means to understand the chemopreventive effects of omega-3 fatty acids on breast cancer.

For breast cancer prevention, effective ways of absorbing a large amount of omega-3 fatty acids should be considered. Commercially available supplements have been a potential way to consume high amounts of omega-3 fatty acids on a daily basis but there are some caveats with this practice. First, the bioavailability of these supplements can vary between supplements because of the variability in the composition and chemical structure of the fatty acids[34]. For example, fish oil may provide a mixture of fatty acids that can affect the diet differently than a particular fatty acid(DHA, EPA). Second, the chemical structure of fatty acids is not equal amongst all supplements. Most fatty acid supplements have the omega-3 in a triglyceride form. The location of a fatty acid on either the *sn-1, sn-2,* or *sn-3* position on a glycerol backbone can affect fatty acid absorption[413]. Depending on the source, the omega-3 fatty can vary in position on the glycerol backbone which can influence its interactions with pancreatic lipase and effect overall absorption. Also, omega-3 fatty acids that have been converted into ethyl esters may not be as readily absorbed as fatty acid triglycerols[414]. Finally, more importantly, the highly unsaturated structure of DHA can be readily oxidized in aqueous mediums which can result in little DHA being absorbed[346, 347].

Considering the above caveats of fatty acid supplements, there is an urgent need for a liposomal delivery system of DHA for increasing its bioavailability in circulation and at the

mammary fat pad. The liposome delivery system was proven by our work to protect DHA from oxidation and acidic pH, decrease cell viability and increase apoptosis in human breast cancer cell lines, making it a potential delivery system for DHA. Furthermore, we proved that when administered to a preclinical model, such as a rat by oral administration, approximately, 20% of our liposomal formulation was delivered to the mammary fat pad, while about 6% was found in the serum. Thus, our oral liposomal delivery system was able to increase the bioavailability of DHA. To further validate our liposomal formulation as a chemopreventive the liposome will need to be applied to a preclinical chemoprevention model and be compared with free DHA to observe if it will be more efficacious *in vivo*, and to evaluate its side effects over a longer period of oral administration. In summary our results provided experimental data demonstrating the potential utility of liposomal DHA in the prevention of breast cancer initially in preclinical models and ultimately in the clinic.

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VITA

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Peer Reviewed Publications:

1. **Skibinski CG**, Thompson HJ, Das A, Manni A, Bortner JD, Stanley A, Stanley BA, El-Bayoumy K. Proteomic Changes Induced by Effective Chemopreventive Ratios of n-3:n-6 Fatty Acids and Tamoxifen against MNU-Induced Mammary Cancer in the Rat. Cancer Prev Res (Phila). 2013 Sep;6(9):979-88. PMID: 23880232.
2. Andrea Manni, Karam El Bayoumy, **Christine G. Skibinski**, Henry J. Thompson, Julia Santucci-Pereira, Lucas Tadeu Bidinotto and Jose Russo. Combination of Antiestrogens and Omega-3 Fatty Acids for Breast Cancer Prevention. BioMed Research International, vol. 2015, Article ID 638645, 10 pages, 2015
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4. **Skibinski CG**, Das A, Kester M, Chen KM, Manni A, El-Bayoumy K. A Biologically Active Acid Stable Liposome Formulation of Docosahexaenoic Acid (DHA) for Breast Cancer Prevention. *Cancer Prevention Research.* (Submitted).

Intellectual Property

March 2013 Development of an Acid Stable Liposome Formulation
Provisional Patent Application Serial Number: 61/788,564

National Conferences

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2014 **Skibinski CG**, Das A., Kester M., Chen KM., Manni A, El-Bayoumy K. A Novel Biologically Active Stable Liposome of Docosahexaenoic Acid (DHA) for Breast Cancer Prevention. Poster Session presented at American Association for Cancer Research Annual Meeting. 2014 April 5-9. San Diego, California.

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