The role of the dietary long chain polyunsaturated fatty acid, docosahexaenoic acid, in prevention of breast cancer and its' efficacy during neoadjuvant breast cancer chemotherapy

by

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Abstract

Breast cancer (BC) is the most frequently diagnosed and the second leading cause of cancer related death in Canadian women. Docosahexaenoic acid (DHA) is an n-3 long chain polyunsaturated fatty acid (LCPUFA) that has shown efficacy in reducing BC cell growth, however its' role in prevention of BC or how it improves the efficacy of standard chemotherapy and the mechanisms involved have not been established. The overall objective of this thesis was to determine the efficacy of DHA in prevention and treatment of BC.

The relationship between plasma phospholipid fatty acid status and BC risk in a nested-case control study of women with BC (n=393) and age-matched controls (n=786) from Alberta's Tomorrow Project (ATP) and British Columbia Generations Project (BCGP) was determined. Women from BCGP had higher n-3 LCPUFA status compared to ATP (6.4±0.08% vs. 5.3±0.06%, $P<0.001$). Fatty acid status was not consistently associated with risk. In ATP among premenopausal women, total n-3 LCPUFA were positively associated with BC risk, while in BCGP, DHA and n-3 LCPUFA were associated with decreased cancer risk when the waist-to-hip ratio was <0.85. This study highlights the difficulty in using fatty acid status to predict BC risk in diverse populations.

In a series of *in vitro* and *in vivo* experiments with immortalized BC cells, we sought to establish the efficacy and mechanisms for how pre-treatment of BC cells with DHA improves the action of chemotherapy. First, we determined that DHA is differentially incorporated into whole cell and lipid raft membranes of BC cell lines, with higher incorporation occurring in MDA-MB-231 triple negative BC (TNBC) compared to estrogen receptor positive MCF-7 BC cells. Doxorubicin (DOX) chemotherapy treatment did not alter this incorporation. Microarray analysis

indicated that DHA+DOX treated MDA-MB-231 cells had upregulated expression of apoptosis genes (*RIPK1*, *Caspase-10*) and down regulated cell cycle gene expression (*Cyclin B1*, *WEE1*, *CDC25C,* all $P<0.05$). Mice fed a 2.8% w/w DHA diet and treated with 5 mg/kg DOX had 50% smaller MDA-MB-231 tumours compared to control (0% DHA) fed mice and increased expression of apoptotic proteins (Caspase-10 and Bid) combined with decreased cell cycle proteins (Cyclin B1 and Cdc25c, $P<0.05$).

We then employed a heterogeneous, drug resistant patient derived xenograft (PDX) model of TNBC. Mice bearing MAXF574 TNBC PDXs fed a 3.8% w/w DHA diet in combination with 5 mg/kg docetaxel (TXT) had a 57% reduction in tumour weight compared to mice fed a control diet ($P<0.004$) and a 64% reduction compared to control diet +TXT ($P<0.01$). DHA+TXT resulted in higher expression of proapoptotic proteins: RipK1 and Bid, lower expression of Ki67 proliferation marker, Bcl-2 and Parp and increased cell cycle arrest compared to control or Control+TXT mice ($P<0.05$). Next, to assess the efficacy of DHA at a lower dose, high DHA (HDHA 3.8% w/w) and low DHA (LDHA, 1.8% w/w) diets were fed to MAXF401 TNBC PDX bearing mice. Tumours from mice fed HDHA+TXT or LDHA+TXT were similar in size to each other, but 36% and 32% smaller than tumours from mice fed control+TXT, respectively ($P<0.05$). Both DHA doses resulted in increased necrotic tissue and decreased NFκB protein expression compared to control tumours, however only HDHA+TXT had increased expression of necroptosis related proteins: RIPK1, RIPK3 and MLKL ($P<0.05$). This work confirms the efficacy of DHA supplementation with TXT in two TNBC PDX models.

Our final translation was to determine the efficacy of supplementing 4.4 g/ day DHA in women undergoing neoadjuvant chemotherapy. A randomized, placebo-controlled trial was planned, received ethics and Health Canada approval and 49 women have been enrolled (80% of

total patients required). Women entering the study had an average age of 52 years with a BMI=28.5±1.0. Half the women are post-menopausal and at baseline, women had 2.3±0.1% DHA content in plasma phospholipids.

In summary, while we did not find that DHA phospholipid status reduced the risk of future BC, our data provided strong pre-clinical evidence of efficacy of DHA in combination with chemotherapeutics in reducing BC cell growth. The mechanisms of action through which DHA works include increased apoptosis, necroptosis and cell cycle arrest and decreased cellular proliferation. Collectively the evidence obtained from these studies details the role of DHA in a neoadjuvant setting that we hypothesize will be confirmed in the clinical trial.

Preface

This thesis is original work by Marnie Newell. The first research project involving humans, of which this thesis is a part, received research ethics approval from the Health Research Ethics Board of Alberta Cancer Committee, "N-3 long-chain polyunsaturated fatty acid status and breast cancer risk", HREBA.CC-17-0344, September 25, 2017. The research project involving animals, of which this thesis is a part, received research ethics approval from the Committee of Animal Policy and Welfare of the Faculty of Agriculture, Life and Environmental Sciences at the University of Alberta, Probing the anti-cancer properties of designer oils. Protocol #134, April 01, 2012. The second research project involving humans, of which this thesis is a part, received research ethics approval from the Health Research Ethics Board of Alberta Cancer Committee, "Docosahexaenoic acid (DHA) for Women with Breast Cancer in the Neoadjuvant Setting", HREBA.CC-18-0381, March 06, 2019. The contributions made by the candidate, Marnie Newell, and the co-authors to the completion of this work are described here.

A section in Chapter 1 has been published as M. Newell, K. Baker, L.M. Postovit and C.J. Field, "A Critical Review on the Effect of Docosahexaenoic Acid (DHA) on Cancer Cell Cycle Progression", *International Journal of Molecular Sciences* 18(8):1784. I was responsible for performing the literature search, disseminating and analyzing the data and preparing the manuscript. KB, LMP and CJF also analyzed the data and prepared the manuscript and CJF had primary responsibility for the final content. A second portion of Chapter 1 has been published as M. Newell, V. Mazurak, L.M. Postovit, C.J. Field, "N-3 Long-Chain Polyunsaturated Fatty Acids, Eicosapentaenoic and Docosahexaenoic Acid, and the Role of Supplementation during Cancer Treatment: A Scoping Review of Current Clinical Evidence", *cancers* 13: 1206. I was responsible for performing the literature search, disseminating and analyzing the data and

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Chapter 3 of this thesis has been accepted for publication as M. Newell, S. Ghosh[1], S. Goruk[2], M. Pakseresht, J.E. Vena, T.J.B. Dummer and C.J. Field, "A prospective analysis of plasma phospholipid fatty acids and breast cancer risk in two provinces in Canada", *Current Developments in Nutrition* DOI: 10.1093/cdn.nzab022. I was responsible for analyzing the data and preparing the manuscript. MP, JEV, TJBD and CJF designed the research study, obtained the specific funding for this specific analysis on this study; SG[2] conducted the research; SG[1] and MP performed the statistical analyses, SG[1], SG[2], MP, JEV, TJBD and CJF also analyzed the data and prepared the manuscript and CJF had primary responsibility for the final content.

Chapter 4 of this thesis has been published as M. Newell, D. Patel, S. Goruk and C.J. Field, "Docosahexaenoic Acid Incorporation Is Not Affected by Doxorubicin Chemotherapy in either Whole Cell or Lipid Raft Phospholipids of Breast Cancer Cells *in vitro* and Tumour Phospholipids *in vivo*", *Lipids* DOI 10.10002/lipd.12252. I was responsible for conducting the research, analyzing and performing statistical analyses of the data and preparing the manuscript. CJF designed the research study and obtained the specific funding for this study, DP, SG and CJF also analyzed the data and assisted in manuscript preparation and CJF had primary responsibility for the final content.

Chapter 5 of this thesis has been published as M. Newell, M. Brun and C.J. Field, "Treatment with DHA modifies the response of MDA-MB-231 breast cancer cells and tumours from *nu/nu* mice to doxorubicin through apoptosis and cell cycle arrest", *The Journal of Nutrition* DOI: https//doi.org/10.1093/jn/nxy224. I was responsible for conducting the research, analyzing and performing statistical analyses of the data and preparing the manuscript. CJF

designed the research study and obtained the specific funding for this study, MB and CJF also analyzed the data and assisted in manuscript preparation and CJF had primary responsibility for the final content.

Chapter 6 of this thesis has been published as M. Newell, S. Goruk, V. Mazurak, L.M. Postovit and C.J. Field, "Role of docosahexaenoic acid in enhancement of docetaxel action in patient derived breast cancer xenografts" *Breast Cancer Research and Treatment,* 177(2):357-367. I was responsible for conducting the research, analyzing and performing statistical analyses of the data and preparing the manuscript. LMP and CJF designed the research study and CJF obtained the funding for this study. SG, VM, LMP and CJF also analyzed the data and assisted in manuscript preparation and CJF had primary responsibility for the final content.

Chapter 7 of this thesis has been submitted for publication as M. Newell, S. Goruk, J. Schueler, V. Mazurak, L.M. Postovit and C.J. Field, "Docosahexaenoic acid enrichment of tumor phospholipid membranes increases tumor necroptosis in mice bearing triple negative breast cancer patient-derived xenografts". I was responsible for conducting the research, analyzing and performing statistical analyses of the data and preparing the manuscript. LMP and CJF designed the research study and CJF obtained the funding for this study. SG, JS, VM, LMP and CJF also analyzed the data and assisted in manuscript preparation and CJF had primary responsibility for the final content.

A section in Chapter 8 has been published M. Newell, J. Mackey, G. Bigras, M. Alvarez-Camacho, S. Goruk[1], S. Ghosh[2], A. Schmidt, D. Miede, A. Chisotti, LM. Postovit, K. Baker, V. Mazurak, K.S. Courneya, R. Berendt, W.F. Dong, G. Wood, S.K. Basi, A.A. Joy, K. King, J. Meza-Junco, X. Zhu and C.J. Field, "Protocol of a double blind, phase II randomized controlled trial to compare Docosahexaenoic acid (DHA) concomitant with neoadjuvant chemotherapy

versus neoadjuvant chemotherapy alone in the treatment of breast cancer: DHA WIN", *BMJ Open* 17;9(9):e030502. I was responsible for designing the protocol and writing the manuscript. JM, GB, MAC, SG[1], KB and CJF also designed the protocol. JM, GB, MAC, SG, SG, AS, DM, AC, LMP, KB, VM, KSC, RB, WFD, GW, SKB, AAJ, KK, JMJ, XZ and CJF assisted in manuscript preparation. JM, SG, KB, VM, LMP, and CJF obtained the specific funding for this study. CJF had primary responsibility for the final content. The results presented in Chapter 8 used baseline data collected for the DHA WIN trial. I was responsible for conducting the research, analyzing and performing statistical analyses of the data and writing the chapter.

Dedication

To Chloë and Chris.

You two are the very best.

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Table of Contents

List of Tables

List of Figures

List of Appendices

List of Abbreviations Used

5-FU: 5 fluorouracil

ALA: alpha linolenic acid

ARA: arachidonic acid

ASK1: apoptosis signal-regulating kinase 1

ATP: Alberta's Tomorrow Project

AURK: aurora kinase

BAD: Bcl-2-associated death promoter

BAX: Bcl-2-associated X

BC: breast cancer

BCGP: British Columbia Generations Project

BCL-2: B-cell CLL/lymphoma 2

BCLXL: B-cell CLL/lymphoma extra large

BID: BH3 interacting domain death agonist

BMI: body mass index

BSA: bovine serum albumin

CC: case-control

CDC2/CDK: cyclin dependent kinase

CDC25C: Cell division cycle 25 homolog C

CDHA: conjugated DHA

CRP: C reactive protein

DGLA: dihomo-gamma-linolenic acid

DHA: docosahexaenoic acid

DPA: docosapentaenoic acid

DR5: death receptor 5

DOX: doxorubicin

EGFR: epidermal growth factor receptor

EPA: eicosapentaenoic acid

EN / PN: enteral nutrition / parenteral nutrition

ER: estrogen receptor

FADD: Fas-Associated Via Death Domain

FAS: CD95 receptor

FCS: fetal calf serum

FO: fish oil

G1/G2: gap 1/ gap 2

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GPX4: glutathione peroxidase 4

HDHA: high docosahexaenoic acid

H&E: haematoxylin and eosin

HER2: human epidermal growth factor receptor 2

HRP: horseradish peroxidase

IFNγ: interferon gamma

LDHA: low docosahexaenoic acid

LA: linoleic acid

LCPUFA: long chain polyunsaturated fatty acid

LysoPC: lysophosphatidylcholine

MAPK: mitogen-activated protein kinase

MCM2: minichromosome maintenance complex component 2

MCT: medium chain triglycerides

METC: mitochondrial electron transfer chain

MLKL: mixed lineage kinase domain like protein

MMP/ MOMP: mitochondrial membrane potential

mRNA: messenger RNA

MUFA: monounsaturated fatty acid

N/A: not applicable

NC: no change

NCC: nested case-control

NSCLC: non-small cell lung cancer

NSG: NOD.Cg-Prkdc^{scid}Il2rg

OA: oleic acid

PAKT: phospho-akt

PARP: poly (ADP-ribose) polymerase

PBCL-2: phospho-bcl-2

PBMC: peripheral blood mononuclear cell

PC: phosphatidylcholine

PCNA: proliferating cell nuclear antigen

pCR: pathological complete response

PDX: patient derived xenograft

PE: phosphatidylethanolamine

PGE2: prostaglandin E2

PI: phosphatidylinositol

PI3K: phosphoinositide 3-kinase

PL: phospholipid

PLK1: polo-like kinase 1/serine/threonine-protein kinase

PR: progesterone receptor

PRB: retinoblastoma protein

PS: phosphatidylserine

PUFA: polyunsaturated fatty acid

RBC: red blood cell

RIP/RIPK: receptor-interacting protein

ROS: reactive oxygen species

RTPCR: real time quantitative polymerase chain reaction

SFA: saturated fatty acid

SM: sphingomyelin

SOC: standard of care

TBST: tris-buffered saline with Tween 20

TNBC: triple negative breast cancer

TG: triglyceride

TNFα: tumour necrosis factor alpha

TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling

TXT: docetaxel

WEE1: WEE1 homolog

WT: wild type

CHAPTER 1- Introduction[1]

1.1 Breast Cancer

Globally, breast cancer is the most frequently diagnosed cancer in women, accounting for approximately 2.1 million cases in 2018 or 11.6% of all newly diagnosed cancer cases (Bray et al. 2018). Despite ongoing advances in screening, prevention, diagnosis, and treatment, it remains the second leading cause of cancer-related death in women and one of the most expensive to treat (Luengo-Fernandez et al. 2013, Nadeem et al. 2016). Fifteen percent of all female cancer related deaths are attributable to breast cancer worldwide; accounting for greater than 620 000 deaths in 2018 (Bray et al. 2018). In Canada, an estimated 27 400 women will be diagnosed with breast cancer in 2020 and it is projected that 5 100 will die from the disease (Brenner et al. 2020). Breast cancer is not a single disease (Vargo-Gogola and Rosen 2007) rather a group of diseases with distinct genetic /genomic differences and clinical outcomes. It is routinely classified and treated based on histology, status of predictive markers (oestrogen receptor [ER], progesterone receptor [PR] and human epidermal growth factor receptor 2 [HER2]), lymph node status and tumour grade, resulting in stratification into four main subtypes (luminal A, luminal B, HER2 and basal-like). There are many commonalities and differences in features between subtypes and better understanding of these data will help shape future

[1] Section 1.5.3 was adapted from a review published by Newell M, Baker K, Postovit LM, Field CJ. (2017) A Critical Review on the Effect of Docosahexaenoic Acid (DHA) on Cancer Cell Cycle Progression. International Journal of Molecular Sciences 18(8):1784. Section 1.7 was adapted from a review published by Newell M, Mazurak V, Postovit LM, Field CJ. (2021) N-3 Long-Chain Polyunsaturated Fatty Acids, Eicosapentaenoic and Docosahexaenoic Acid, and the Role of Supplementation during Cancer Treatment: A Scoping Review of Current Clinical Evidence. Cancers 13: 1206.

diagnoses and treatments. **Table 1-1** provides a broad (but not exhaustive) summary of key classifying features of each subtype, highlighting the complexity and heterogeneity in breast cancer.

Table 1-1: Overview of Breast Cancer Subtypes[1]

Subtype	**Luminal A**	**Luminal B**	**Basal-like**	**HER2**
Prevalence	60-70%	10-20 %	10-15%	13-15 %
ER status	positive	Positive	negative	Negative
PR status	positive/ negative	positive/ negative	negative	Negative
HER2 status	negative	Positive	negative	Positive
Proliferation	low	High	high	High
DNA mutations	*TP53* (12%); *PIK3CA* (49%); *GATA* (14%); *MAP3K1* (14%)	*TP53* (32%); *PIK3CA* (32%); *MAP3K1* (5%)	*TP53* (84%); *PIK3CA* (7%)	TP53 (75%); *PIK3CA* (42%); *PIK3R1* (8%); *ERRB2* amplification (71%)
Sensitivity towards standard chemotherapy	endocrine and chemotherapy responsive	endocrine responsive and variably chemotherapy responsive; HER2+ are trastuzumab responsive	endocrine nonresponsive; chemotherapy responsive	chemotherapy responsive; trastuzumab responsive
Examples of immortalized cell lines	MCF-7, T47D, SUM185	BT474, ZR-75	MDA-MB-231, BT549, SUM190	SKBR3
Examples of patient derived xenografts	MAXF713	MAXF1398	MAXF401, MAXF574	MAXF2498, MAXF2500

[1](The Cancer Genome Atlas 2012, Holliday and Speirs 2011, Eskiler et al. 2018, Harbeck et al. 2019, Vincent et al. 2015)

To date, forty genes that have been implicated in breast cancer (Pereira et al. 2016). Basal-like and HER2 subtypes have a higher mutation rate while luminal A and B subtypes have

a more diverse spectrum of mutated genes (Goncalves et al. 2014). The extensive genetic / phenotypic variation has significant implications for the choice of biomarkers to guide clinical decisions (Burrell et al. 2013). Patients within these four 'groups' have different prognoses and respond differentially to treatment (Holliday and Speirs 2011). ER^+ (luminal A and B) breast cancer accounts for 75% of diagnoses, but therapeutic responses / outcomes vary (Aparicio and Caldas 2013). The primary focus of this thesis research has been on triple negative breast cancer (TNBC: ER-PR-HER2-) which falls into the basal-like subtype. This accounts for 10-15% of all breast cancer diagnoses (Harbeck et al. 2019); it is more difficult to treat and patients with TNBC have the poorest prognoses (Eskiler et al. 2018).

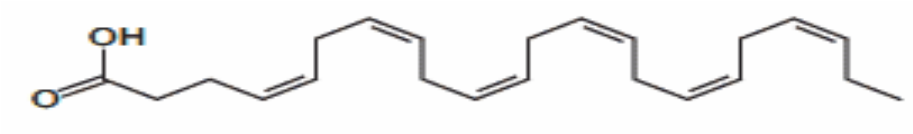
1.2 Modelling Breast Cancer

Breast cancer differs both in etiology among patients and also within the tumour microenvironment (inter- and intra-tumoural variation) (Turashvili and Brogi 2017). To address this heterogenicity, we have employed multiple models to study mechanisms of disease for this thesis research. While this thesis has primarily focused on TNBC breast cancer, we began our *in vitro* work with two immortalized cell lines: MDA-MB-231, representative of TNBC and MCF-7, representative of luminal A to assess the overall efficacy and generalizability of DHA treatment. These are two of the most widely used and best characterized human breast cancer cell lines (Lacroix and Leclercq 2004) and have also been extensively employed in animal studies (Gould et al. 2015). However, the dynamic, complex environments of breast cancer tumours are not adequately represented by immortalized breast cancer cell models. Patient derived xenograft (PDX) models, human tumour explants that are able to grown in an immune-deficient host (Gould et al. 2015), have emerged and better recapitulate this heterogenicity (Zhang and Lewis 2013, Dobrolecki et al. 2016). Two different TNBC PDXs have been employed in the studies

described in this thesis. MAXF574 is a poorly differentiated, well vascularized PDX and MAXF401 is a moderately differentiated, poorly vascularized PDX.

1.3 Docosahexaenoic Acid

Figure 1-1: Chemical Structure of docosahexaenoic acid C22:6 n-3 (Sigma Aldrich)



Docosahexaenoic acid (DHA) is a long chain polyunsaturated omega-3 (n-3) fatty acid (LCPUFA) with 22 carbons and 6 *cis*-double bonds (**Figure 1-1**). In humans there is a limited ability to elongate and desaturate the shorter chained n-3 fatty acids [including alpha-linolenic acid (ALA) and eicosapentaenoic acid (EPA)] to the longer chained DHA. Conversion of ALA to EPA has been reported to be between 0.3 and 8% in men or up to 21% in women and conversion to DHA is <1% in men and 9% in women (Calder 2016). Therefore, DHA is predominantly obtained by the body through dietary intake (Plourde et al. 2011). It is found in fatty fish including salmon, mackerel and whitefish (Salem et al. 1986), but is also available in the food supply as supplements and in fortified foods. In North America, the prevalence of the 'Western diet' leads to an estimated intake of only 100 mg / day, corresponding to ~280 µM DHA and ~100 µM EPA in fasted blood (Chapkin et al. 2008). Data obtained from 600 women in the Alberta Pregnancy Outcomes and Nutrition (APrON) cohort study found only 27% had adequate intake of n-3's during pregnancy and 25% postpartum (according to the EU recommendation for DHA) (Jia et al. 2015). Indeed, the Western diet typically provides only 0.1-0.2% of kcal is from n-3's whereas the Japanese obtain 1-2% DHA from their diets (Turk and

Chapkin 2013). It has been shown that supplementation can increase blood plasma levels by 7x for EPA and 2x for DHA (700 μM and 500 μM respectively), which can thereby lead to plasma membrane lipid enrichment (Chapkin et al. 2008). Stillwell et al. highlighted the effect of DHA incorporation and the various cell membrane property changes it induces: elastic compressibility, flexibility of the acyl chain and fluidity, fusion, rapid flip-flop, phase behaviour, protein function and ion permeability (Stillwell et al. 2003). The inherent bulkiness of DHA results in cell membranes that are not as tightly packed as a saturated membrane (Chapkin et al. 2008, Berquin et al. 2008). This was confirmed through a set of NMR experiments using labelled oxygen, where it was shown that bilayers composed of DHA are 2.7 times more permeable to water and 2.3 times more permeable to carboxyflourescein leakage than bilayers composed of oleic acid (Wassall et al. 2009).

Uptake of DHA may vary within and between individuals. Umhau et al. (2009) found that DHA supplementation increased plasma lipid content during the post-prandial period (4 hours after ingestion) 42% higher in an elderly population compared to young adults receiving the same supplementation. This four-fold increase was observed both in plasma triglycerides and in free fatty acids (Umhau et al. 2009). It appears important to maintain consumption of DHA in order to maintain the concentration in tissues as the reversal / loss from plasma occurs quite readily when dietary intake stops (Turk and Chapkin 2013). In studies of DHA content in the brain; DHA incorporation was reported at a rate of 3.8 ± 1.7 mg / day; with a half-life of 2.5 years, although the DHA concentration in the brain dropped by 5% within 49 days after it disappeared from plasma when preformed dietary DHA was removed (Umhau et al. 2009). Furthermore, retro-conversion of DHA to other fatty acids could reduce tissue concentrations. In

^{13}C tracer studies, retro-conversion of ^{13}C-DHA to ^{13}C-docosapentaenoic acid (DPA) and ^{13}C-EPA has been reported at 1.4% (Plourde et al. 2011).

DHA is found at concentrations of up to 50% in the acyl chains of synaptosomes, sperm and the retinal rod outer segment (Stillwell et al. 2005); yet in other tissues DHA incorporation is below 5 mol % of total phospholipid acyl chains. The concentrations of these other tissues can be increased several fold through provision of DHA in the diet (dietary enrichment) (Stillwell et al. 2005) although it is not taken up equally amongst different tissues. For example, in a dietary intervention mouse study with 5% fish oil diet, DHA was observed in tissues in the following order (based on the highest amount): breast (~700 μg DHA /g wet tissue) followed by an equal amount taken up in tumour, skin and uterus (~450 μg DHA /g wet tissue) (Kang et al. 2010). DHA uptake into phospholipids is rapid; it has been demonstrated that 25% of DHA conjugated to albumin incorporated into prostate cancer cells within 48 hours (Gu et al. 2015). Studies using tracers: ^{13}C-DHA, have shown supplementation of 250-280 mg ^{13}C-DHA resulted in peak enrichment of plasma triglycerides (TG) at 2 hours if the tracer was provided in TG form; or a peak at 6 hours post supplementation if the tracer was provided as phosphatidylcholine (Plourde et al. 2011). Because of this rapid uptake, there has been considerable research aimed at identifying the role of DHA in cell membrane-initiated events.

Exactly where DHA is taken up in the plasma membrane could be influential in membrane function. Interestingly, it is found in both the sn-1 and sn-2 position of phospholipids in synaptosomes, sperm and the retinal rod outer segment, yet in other tissues, it is primarily found in the sn-2 position (commonly with a saturated fatty acid such as palmitic or stearic acid in the sn-1 position) (Stillwell et al. 2005). Furthermore, it is primarily esterified to phosphatidylethanolamine (PE), found in the inner leaflet of the plasma membrane; with lesser

amounts in phosphatidylcholine (PC) (outer leaflet of the plasma membrane), and the other phospholipids (PLs) (Stillwell et al. 2005). It has been shown for example in T27A leukemia cells that DHA is 5.7 times more concentrated in PE vs. PC (Stillwell et al. 2005). In syanaptosomal membranes, DHA is highly concentrated in phosphatidylserine (PS, inner leaflet of the plasma membrane) (Salem et al.1986) and in Wistar rats the accumulation of DHA in the brain and heart was greatest in PE (Stillwell et al. 2005). Differences between uptake in breast tumours and non-tumourous breast tissue have been observed (Chajes et al. 1995). In PC, there was a greater proportion of LCPUFA (EPA and DHA) in tumour samples compared to non-tumourous tissue and conversely a lower proportion of n-6 fatty acids (linoleic and arachidonic). Interestingly there were no differences in the relative content of DHA or EPA in total phospholipids between tumourous and non-tumourous samples (Chajes et al. 1995). This suggests the bioactive capabilities of DHA / EPA with respect to reduced cell viability / decreased proliferation are only realized within the tumour microenvironment (discussed later in this chapter).

1.4 DHA and Lipid Rafts

Lipid rafts are ordered, sphingomyelin (SM)-rich/ cholesterol-rich micro domains that act as mobile platforms for protein activity (Lee et al. 2014). They are small, up to 14 nm in diameter, and enhanced signalling is due in part to their small size and increased frequency of protein-protein interactions (Turk and Chapkin 2013). They are integral docking platforms that initiate many cellular events such as T-cell activation, protein and lipid trafficking and signal transduction including ones for apoptosis or proliferation; signalling pathways that tend to be hyper-activated in cancer (Turk and Chapkin 2013). The highly unsaturated nature of DHA makes it sterically incompatible with the SM and cholesterol leading to disruption in raft and

protein signalling (Calder 2016). Mice fed an n-3 enriched diet were seen to have a reduction of cholesterol in colonocyte lipid rafts by 46% and splenic T-cells had 30% less sphingomyelin in their lipid rafts compared to mice fed an n-6 based diet (Turk and Chapkin 2013). Treatment with 50 μM DHA corresponded with a decrease in membrane cholesterol levels and an internalization of lipid rafts in MDA-MB-231 breast cancer cells; results that were attenuated when the cells were co-treated with cholesterol (Lee et al. 2014). DHA is reported to be twice as likely to be incorporated into rafts, compared to EPA (Berquin et al. 2008). While DHA (likely due to increased internalization) reduced cell surface levels of lipid rafts, EPA was shown to have little effect on the surface levels (Lee et al. 2014). In other studies, feeding, providing or increasing the content of n-3's including DHA, have been shown to increase raft clustering, creating large lipid raft domains (Turk and Chapkin 2013). These larger domains are believed to have diminished signalling capacity due to a reduction in protein-protein interactions (Turk and Chapkin 2013). DHA disrupts cell signalling initiated from lipid rafts by displacing key signalling molecules including EGFR, Src and Hsp90 from lipid rafts and down-regulates their activities in a time and dose-dependent manner (Lee et al. 2014). This disruption by DHA of lipid rafts could be a key factor in reducing cellular proliferation or increasing apoptosis (both discussed later this chapter).

1.5 DHA and Cancer –Epidemiological Evidence

Breast cancer incidence is not uniform across the world, with higher incidences occurring in Australia, Western and Northern Europe and North America and lower incidences in South Central / South Eastern Asia, Africa and Central America (Bray et al. 2018). This variance could be due in part to lifestyle factors that impact breast cancer risk, including diet and exercise (World Cancer Research Fund 2018). These preventable dietary and lifestyle factors and are

estimated to contribute to ~8% of all cancers (Brenner 2014) and as a result, there have been numerous epidemiological studies that have looked at the impact of diet, specifically the role of LCPUFA and dietary fat on breast cancer risk. In the Reykjavik Study, approximately 3000 women from Iceland were assessed and compared based on geographic location, fish intake and incidence of breast cancer (Haraldsdottir et al. 2017). The authors found that women who lived in coastal areas with high fish intake of 4 times per week had lower incidences of breast cancer compared to women who lived inland and had lower intake (HR 0.46, 95% CI, 0.22 -0.97) (Haraldsdottir et al. 2017). The Singapore Chinese Health Study found in a population of 35 298 women, a decreased risk of breast cancer with higher intake of n-3 fatty acids from marine sources (relative risk, RR 0.72, 95% CI, 0.53-0.98) (Gago-Dominguez et al. 2003). The Vitamin Lifestyle Cohort, comprised of 35 016 women in Washington, USA, found that fish oil supplementation could be inversely associated with breast cancer risk (HR, 0.68; 95% CI, 0.50-0.92) (Brasky et al. 2010). These examples suggest fish intake elicits a reduction in breast cancer risk yet several meta-analyses have concluded that the role of n-3 fatty acids in risk reduction remains unclear (MacLean et al. 2006, Yang et al. 2014, Zheng et al. 2013). It has been suggested that fatty acids in blood components (plasma, serum or red blood cells) could be used as biomarkers to predict breast cancer risk. To date, there have been several epidemiological studies that have explored fatty acids as biomarkers of breast cancer risk and the findings have been inconsistent. **Table 1-2** summarizes the current evidence. The data thus far suggests a decreased risk of breast cancer with increased fatty acid content of linoleic acid (Vatten et al. 1993, Rissanen et al. 2003, Shannon et al. 2007), stearic acid (Chajes et al. 1999), α-linolenic acid (Klein et al. 2000, Maillard et al. 2002), DHA (Maillard et al. 2002), EPA (Shannon et al. 2007, Witt et al. 2009), total n-6 (Vatten et al. 1993, Rissanen et al. 2003), total n-3 (Simonsen et

al. 1998), DI_{18} (Chajes et al. 1999) or PUFA (Rissanen et al. 2003); as well as increased risk of breast cancer with increased fatty acid content of palmitic acid (Shannon et al. 2007, Pala et al. 2001, Bassett et al. 2016), palmitoleic acid (Shannon et al. 2007, Chajes et al. 2008, Chajes et al. 2017), oleic acid (Pala et al. 2001), MUFA (Pala et al. 2001), SFA (Saadatian-Elahi et al. 2002, Bassett et al. 2016) and increased total n-3 (Simonsen et al. 1998). The inconsistencies could be due to a variety of factors: 1) these studies do not have a common source for the biomarker and instead come from: serum, plasma, red blood cells and breast adipose tissue (Brenna et al. 2018); 2) discrepancies dietary intake or dietary patterns based on geographic location (Dandamudi et al. 2018, Brennan et al. 2010, Xiao et al. 2019) and 3) failure of some studies to address known confounders.

Table 1-2: Characteristics of Epidemiological studies assessing risk of breast cancer and fatty acid status

Study design (Country)	Population (cases / control)	Measurement	Confounding Variables Included in analysis	Main Finding RR (95% CI) Highest vs. lowest category	Additional information	Ref.
Nested Case Control (Norway)	87/ 235	Serum PL (mg/l) Quartile analysis	Covariate information not collected and therefore not assessed	Decreased n-6 PUFA OR: 0.5 (0.2-1.0) and LA OR: 0.4 (0.2-1.0)	In women under 55; not seen in women over 55. No associations observed in n-3s.	(Vatten et al. 1993)
Nested Case Control (Europe: Germany, the Netherlands, Northern Ireland, Spain, Switzerland) (EURAMIC)	291/ 351	Buttocks adipose tissue (% total FA) Tertile analysis (split by country)	BMI, reproductive history, family history of BC, age at first birth if over 35. Confounders considered but not included: current/ former smoking, current alcohol, oral contraceptive used, HRT, age at menarche, socioeconomic status and region	Switzerland: decreased n-3 OR: 0.41 (0.17-1.0) Spain: increased n-3 OR: 4.56 (1.74-11.93); increased n-6 OR: 17.11 (5.58-52.47) All centers pooled: overall trend (P<0.055) of decreased n-3/n-6 OR: 0.65 (0.41-1.03)	Substantial variance in FA content from center to center Suggest ratio could be important	(Simonsen et al. 1998)
Nested Case Control (Sweden)	196/ 388	Serum PL (% total FA) Quartile analysis	Parity & age at first birth (split into 3), HRT (yes/no), menopausal status (above/ below).	Decreased stearic acid (18:0) OR: 0.49 (0.22-1.08); desaturation index	No association with n-3 and BC risk	(Chajes et al. 1999)

			BMI and Age at menarche kept as continuous data,	(18:0/18:1n9) OR: 0.5 (0.23-1.0) (P<0.06)		
Case Control (France)	123/ 59 benign breast disease	Breast adipose tissue PL (% total FA) Quartile analysis	Age and BMI -categorical with quartiles of distribution; menopausal status (yes/ no)	Decreased ALA OR: 0.36 (0.12-1.02)	No association observed with any other fatty acid	(Klein et al. 2000)
Nested Case Control (Italy) ORDET study	71/142	RBC PL (% total FA) Tertile analysis	Confounders considered: BMI, waist to hip ratio, age at menarche, age at first birth, age at menopause, months of lactation, parity, education -None used unadjusted OR presented	Increased oleic acid OR: 2.79 (1.24-6.28); increased MUFA OR: 5.21 (1.95-13.91); saturation index OR: 0.29 (0.13-0.64)	Suggest that these variables are dependent on Δ 9 desaturase activity	(Pala, Krogh, Muti, Chajes, et al. 2001)
Nested Case Control (United States)	73/74	Breast adipose tissue (µmol/g)	Confounders considered: Age, BMI, menopausal status, smoking status, family history of BC Only age adjusted for	LA+AA and total n-3 higher in cases vs. control		(Bagga et al. 2002)
Nested Case Control (United States)	197/ 197	Serum PL (% total FA) Quartile analysis	Included: Family history, age at first birth, cholesterol, history of treatment for benign breast conditions Not included: Age at menarche, BMI, Grouped by menopausal status	Post-menopausal: Increased SFA OR:1.96 (0.73-5.25); palmitic acid OR: 2.57 (0.99-6.61) Pre-menopausal: Myristic acid (14:0) OR: 2.22 (0.78-6.31)	No associations of LCPUFA to BC risk	(Saadatian-Elahi et al. 2004)

				Overall: total PUFAs OR:0.59 (0.31-1.09)		
Case Control (France)	241/88 benign breast disease	Breast adipose tissue (% total FA) Tertile analysis	Confounders considered: Age, height and BMI (assessed as continuous or tertiles), Menopausal status, Age, height and BMI as continuous variables used, menopausal status and BMI-menopause interaction	Decreased ALA OR: 0.39 (0.19-0.78); decreased DHA OR: 0.31 (0.13-0.75) and decreased LCn3/total n6 (0.33 (0.17-0.66); trend towards increase LA OR: 2.31 (1.15-4.67)	Suggest protective effect of n-3 on BC risk	(Maillard et al. 2002)
Nested Case Control (Finland)	127/ 242	Serum total lipids (% total FA) Tertile analysis	Confounders considered: BMI, serum cholesterol, smoking, alcohol consumption, number of pregnancies, parity, leisure-time exercise and education; mostly unadjusted results displayed	Decreased PUFA OR: 0.31 (0.12-0.77); decreased n-6 OR: 0.35 (0.14-0.84), decreased LA OR: 0.29 (0.12-0.73); higher trans-11 18:1 OR: 3.69 (1.35-10.06)	Stronger associations in post-menopausal vs premenopausal	(Rissanen et al. 2003)
Nested Case Control (Sweden) Malmo Cohort	12,803 total cohort; 237/673	RBC total lipids (% total FA) Quintile analysis	Multivariate analysis controlled for: height, waist circumference, BMI, HRT, age of first birth and alcohol	Associations between dietary FAs and RBC FA	No significant associations with BC risk and FA status	(Wirfalt et al. 2004)
Case Control (China)	322/1030	RBC (% total FA) Quartile analysis	Confounders considered: Family history of breast cancer, age at menarche, age at first full-term pregnancy,	Increased palmitic acid OR: 2.18 (1.14-4.15), palmitoleic acid		(Shannon et al. 2007)

			age at first live birth, total live births, number of prior benign breast lumps, duration of oral contraceptive/ IUD use, numbers of abortions, frequency of BSE practice, education, BMI, smoking, alcohol consumption, physical activity education; Employed: Duration of breast feeding, age at first birth, duration of IUD use, time since last abortion. Stratified by menopausal status	OR: 4.83 (2.58-9.06) and vaccenic acid OR: 2.21 (1.25-3.82); decreased total n-3 OR:0.55 (0.32-0.94), EPA OR: 0.45 (0.26-0.77) and LA OR: 0.67 (0.37-1.21)		
Nested Case Control (France) E3N -EPIC Study	19,934 total cohort; 363/702	Serum PL (% total FA and µmol/l) Quintile analysis	Multivariate analysis controlled for: Years of education (4 categories: <12, 12+14, 15-16, >17) BMI (continuous), adult height (continuous) HRT (ever/ never), alcohol use (continuous), age at first birth and parity combined, family history of BC in 1st degree relative, personal history of benign breast disease	Increased trans-palmitoleic and elaidic acids OR: 1.75 (1.08-2.83) corresponded to increased BC risk; Decreased 16:0/16:1n-7 saturation index OR: 0.66 (0.41-1.05)	Suggests high trans levels due to intake of industrialized foods	(Chajes et al. 2008)
Nested Case Control (Denmark)	463/ 635	Buttocks adipose PL (% total FA) Quintile analysis	Multivariate analysis controlled for: Years of education (<8, 8-10 and >10) BMI (<20, 20-25, >25), parity (0-6), age at first birth, age at	Total n-3 HR: 0.96 (0.64-1.43); EPA HR: 0.84 (0.58-1.23) and	No associations between n-3 PUFA and BC risk	(Witt et al. 2009)

			menarche (<12,12-14,>14, unknown), history of benign breast disease adult height (continuous) HRT (former/ never, current), moderate to vigorous PA, alcohol use (continuous), smoking (never, former, current x 3 cat)	DHA HR: 1.08 (0.73-1.58)		
Nested Case Control (United States) CARET Study	130/257	Serum PL (% total FA) Quartile analysis	Multivariate analysis controlled for: BMI, smoking (current / former), alcohol use	Decreased trans linoleic acid OR: 0.32 (0.17-2.78); Increase C16 trans fatty acids OR: 2.44 (1.02-5.82) in smokers; decreased C16 trans fatty acids OR: 0.67 (0.15-2.69) former smokers	No associations with former smokers FA and BC risk; No association between SFA, MUFA, PUFA and BC risk; Participants were post-menopausal and current or former smokers;	(Takata et al. 2009)
Case Control (United States)	248	RBC total lipids (% total FA) N/A	Age, BMI		Compared n-6 and n-3 status with breast density not BC risk per se, did not find any significant trends	(Hudson et al. 2013)
Nested Case Control (Denmark)	29,875 total cohort; 459 / 611	Buttocks adipose PL (% total FA) Used Treelets -7 levels/ groups	Multivariate analysis controlled for: BMI (<20, 20-25, 25-30, >30), smoking (never, former, current x 3 cat), alcohol use (continuous), HRT (never, former, current),		Determined no clear associations between patterns of FAs and BC risk, nor with hormone	(Schmidt et al. 2014)

			parity (0, 1, 2,3, >4), age at first birth (continuous), age at menarche (<12,12-14,>14, unknown), benign breast tumour surgery (yes, no) PA (<3.5 or >3.5 hrs/wk) Years of education (<7, 8-10 and >10) Ran both adjusted and unadjusted models	receptor status (ER+/-; PR+/-); Employed treelet transform for the statistical analysis	
United States	4 pregnancy-associated BC	breast milk PL - milk from each breast (mole% and concentration normalized to total breast milk protein) N/A	N/A	Trend of increased concentrations of AA, EPA, DPA in breast milk from cancer containing breast compared to non-cancer containing breast (P<0.10)	(Qin et al. 2014)
Prospective study (United States)	Women with elevated BC risk: 22 atypia; 40 no atypia;	RBC PL, Plasma PL and TG, Breast PL and TG (% total FA) Comparison between tissues	N/A	Women with atypia had lower total n-3 in RBC and plasma PL and lower n-3:n-6 ratios in plasma TAGs and breast TAGs; EPA+DHA:AA ratio in plasma TAGS lower in women with atypia	(Hidaka et al. 2015)
Case Control (Brazil)	38/ 75 benign breast disease,	Breast adipose tissue (% total FA)	Multivariate adjustments made but not specified	Decreased lauric, myristic, stearic acids and SFA in	(Conceicao et al. 2016)

		Comparison between groups			BC compared to BBD women; palmitoleic acid, erucic acid, MUFAs and saturation index increased in BC compared to BBD women	
Nested Case Control (Australia)	470 / 2,021 Melbourne Collaborative Cohort Study	Plasma PL (% total FA) Quintile analysis	Multivariate adjustments controlled for: country of birth, menopausal status (pre, post), age at menarche (<12, 12,13,>14); parity and lactation (nulliparous, parous and never lactated, parous and lactated); OC use (never, former, current), HRT (never, former, current), education (<primary, some high school, completed high school, degree/ diploma) alcohol (never, low, med, high) PA (4 cat), family history of cancer, BMI	Increased total SFA HR: 1.64 (1.17-2.30) and palmitic acid 1.86 (1.27-2.72)	ER+PR+ compared to ER-PR-: hormone positive group trended more closely to overall findings and the hormone negative group was less closely associated.	(Bassett et al. 2016)
Case Control (Japan)	3098 total cohort; 112 died of cancer	Serum PL (% total FA) Quartile analysis	Multivariate adjustments controlled for: age, sex, hypertension, diabetes, serum HDL and non-HDL cholesterol, BMI, CRP, smoking, alcohol, exercise	Decreased ratio EPA:AA HR: 1.94 (1.18-3.20);	Ratio of DHA:AA not found to be associated; not specifically BC cases (all cancers)	(Nagata et al. 2017)
Nested Case Control	2982/ 2982 controls	Plasma PL (% total FA)	Multivariate adjustments controlled for: BMI, education	Increased palmitoleic acid	No associations with n3 and BC risk	(Chajes et al. 2017)

(Europe) EPIC study		Quartile analysis	(low, med, high), height (continuous), HRT (never, ever), alcohol (continuous), age at first birth and parity combined (3 cat), energy intake	OR: 1.37 (1.14-1.64) and increased desaturation index (16:1n7/16:0) OR: 1.28 (1.07-1.54) Increased industrial trans fatty acids OR: 2.01 (1.03-3.90) in ER- BC patients only	overall or by hormone receptor	
Nested Case Control (United States) Nurses' Health Study II	794/ 794	RBC (% total FA) Quintile analysis	Multivariate included: age at menarche, age at first birth/ parity, breastfeeding, family history of BC, history of benign breast disease, BMI at age 18, weight change since 18, alcohol and PA Stratified by BMI, menopause and ER status	BMI ≥ 25 kg/m^2: Increased SFA OR: 1.85 (1.18-2.88), trans fatty acid OR: 2.33 (1.45-3.77), dairy derived FA (15:0,17:0 and 16:1 n-7t) OR: 1.83 (1.16-2.89); decreased N3 PUFA (ALA, EPA) OR:0.57 (0.36-0.89); BMI <25 kg/m^2: decreased SFA OR:0.68 (0.46-0.98)	No associations with BC risk and fatty acids; BMI could be a predictor	(Hirko et al. 2018)

Retrospective Study (United States)	19/ 23 benign/ high risk lesions; 19 with history of BC	Gradient-echo Spectroscopic Imaging N/A	Age, BMI	Postmenopausal women with BC had higher SFA and lower MUFA in mammary adipose tissue compared to other groups; women with cancer had significant correlation (r=0.57) with PUFA and BMI	(Lewin et al. 2019)

In contrast to the conflicting epidemiological evidence, there has been a vast amount of preclinical research that strongly suggests the efficacy of DHA as an anti-cancer therapeutic.

1.6 DHA and Cancer –Preclinical Evidence

Healthy cells maintain a balance between apoptosis, cell cycle progression and proliferation and dysregulation of these mechanisms are three of the hallmarks of cancer (Hanahan and Weinberg 2000, Hanahan and Weinberg 2011). Many cancers have defective apoptotic pathways that inhibit apoptotic events while others have defective cell proliferation / growth pathways that are highly constitutive and result in growth promotion and some have a combination of both (D'Eliseo and Velotti 2016). Enrichment of the membrane with DHA and subsequent modification of lipid rafts could therefore modulate these two key mechanisms in a cancer cell.

1.6.1 DHA, Cancer and Proliferation

Dysregulated signalling of growth factors has been implicated in cancer cell proliferation and its inhibition would convey a therapeutic advantage in the treatment of cancers. The ability of DHA to modulate / reduce the expression of key proliferation markers including epidermal growth factor receptor (EGFR), proliferating cell nuclear antigen (PCNA), and the P13K/Akt pathway is of great interest. An overview of current evidence is provided in **Table 1-3.** Decreased protein expression of EGFR was shown in MDA-MB-231 breast cancer cells treated with DHA, resulting in the inhibition of EGF-mediated signalling events. This is in part explained by a decrease in EGFR levels at the plasma membrane (Lee et al. 2014). The disruption of lipid rafts by DHA and exclusion of EGFR from the rafts is well documented in colonocytes, supressing cell proliferation in YAMC cells (Turk and Chapkin 2013) as well as breast (Schley et al. 2007, Lee et al. 2014) and lung cancers (Rogers et al. 2010).

Table 1-3: Overview of Growth and Apoptotic Studies in Representative Cancer cell lines *in vitro* and *in vivo*

Cancer Type	Cell Model	Concentration of DHA	Mechanism	Experimental Observations	Ref.
Colorectal	HT-29	25 µM	Apoptosis	↑ floating cells; ↑ sensitization to apoptosis	(Hofmanová et al. 2005)
Colorectal	YAMC / HCT116	0-200 µM	Apoptosis	↑ apoptosis measured by DNA fragmentation; ↑ MMP; ↑ p53 independent apoptosis	(Chapkin et al. 2008)
Colorectal	CaCo2	not given	Apoptosis	Activation of cytochrome c; ↑ caspase 10, 13, 8, 5 and 9	(Narayanan et al. 2001)
Colorectal	*in vivo* -rats	n-3 diet	Apoptosis	↑ apoptotic index assessed by TUNEL; ↓ BCL-2 levels	(Chapkin et al. 2008)
Pancreatic Bladder	PaCa-44 / EJ	150 µM	Apoptosis	↑ Caspase-8 activation	(Molinari et al. 2011)
Breast	MDA-MB-231 / MCF-7	25 µM	Apoptosis	↑ apoptosis in DHA treated TUNEL positive cells; caspase-8, -9, -6 inhibition ↑ cell viability	(Kang et al. 2010)
Breast	MDA-MB-231 / SKBR-3	50 µM	Apoptosis	↑ MMP; ↑ caspase -3 activation, PARP cleavage	(Lee et al. 2014)
Breast	MDA-MB-231 / MCF-7	60 µM	Apoptosis	↑CD95 protein expression	(Ewaschuk et al. 2012)
Breast	MDA-MB-231 / SKBR-3	50 µM	Apoptosis	Changes in mitochondrial membrane potential; ↑ in cleave caspase 3	(Stillwell and Wassall 2003)
Leukemia	EHEB/ MEC-2 / JVM-2	75, 50, 50 µM (respectively)	Apoptosis	↓ cell viability and ↑sensitization to apoptosis; higher lipid peroxidation; ↑ ROS; G2M arrest	(Fahrmann and Hardman 2013)
Breast	MDA-MB-231 / SKBR-3	50 µM	Growth	Downregulation of EGFR and Hsp90	(Lee et al. 2014)
Prostate	PC3 /LNCaP	60 µM	Growth	↑ due to modulation of P13K/AKT/ BAD pathway	(Berquin et al. 2008, Gu et al. 2015)
Colorectal	CaCo2	5-50 µM	Growth	↓ P13K/AKT; p38 MAPK	(Toit-Kohn et al. 2009)
Prostate	PC3 /LNCaP	60 µM	Growth	↑ due to modulation of P13K/AKT/ BAD pathway	(Berquin et al. 2008, Gu et al. 2015)
Breast	*in vivo* MCF-7 in mice	5% Fish oil diet	Growth	↓ PCNA staining in tumours from mice fed fish oil diet	(Kang et al. 2010)

Tumour tissue extracted from mice (MCF-7 xenografts) fed a 3% fish oil diet had decreased PCNA expression compared to tumour tissue from control mice. This suggests decreased proliferation of tumour cells in mice fed a diet

1.6.2 DHA, Cancer and Apoptosis

In cancer, inhibition of apoptosis has been associated with the evolution of normal epithelium to carcinoma (Chapkin et al. 2008). First, to determine if apoptosis is induced by n-3's or could be induced by other fatty acids, an *in vivo* study looked at rats with colonic carcinomas who were fed diets containing high amounts of either n-3's, n-6's, or n-9's and assessed for apoptosis by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). The apoptotic index was double in n-3 fed rats compared to n-6's, or n-9's (3.1 ± 0.5 vs. 1.3 ± 0.4 and 1.5 ± 0.3 respectively) (Chapkin et al. 2008). The ability of DHA to induce apoptosis *in vitro* has been shown in many tumour cell lines including: esophageal, gastric, breast, pancreatic, ovarian, prostate, bladder and colorectal cancers (D'Eliseo and Velotti 2016); **Table 1-3.** In MCF-7 breast cancer cells treated with 25 μM DHA, flow cytometric analysis showed an increase of apoptotic cells in dose and time dependent manner (annexin V/ propidium iodide staining); increased TUNEL positive staining in a time / exposure dependent manner (highest at 72 hours, the longest length of incubation) and an increase of TUNEL staining in MCF-7 tumours from mice fed a fish oil diet (Kang et al. 2010). An increase in apoptosis to 53.9% (annexin V positive staining) was also seen in MDA-MB-231 breast cancer cells in a time dependent manner after 16 hours of incubation with 50 μM DHA (Lee et al. 2014). At concentrations as low as 25 μM, DHA is able to sensitize HT-29 colon cancer cells to apoptosis (Hofmanová et al. 2005).

Apoptosis can occur via two main pathways: extrinsic or intrinsic. Execution of the extrinsic pathway occurs at the cell membrane whereas initiation of the intrinsic pathway

occurs at the mitochondria. Both pathways 'converge' in the cytosol in a common pathway hallmarked by executioner caspases- 3 -6 and -7 that results in apoptosis.

The extrinsic pathway is initiated by death receptors in the membrane, including: TNFα, CD95 and TRAIL followed by the clustering of death-inducing signalling complexes (DISCs), containing TRADD, FADD and RIPK1. Caspase-8 and -10 are activated by this complex and in turn activate the executioner caspases (Barnhart et al. 2003). Previous work from our lab has demonstrated that treatment with 60 μM DHA increased surface expression of the CD95 receptor and increased subsequent apoptosis in MDA-MB-231 breast cancer cells (Ewaschuk et al. 2012). In MCF-7 breast cancer cells, when caspase-8, -9 and -6 inhibitors are added in the presence of DHA, cell viability increased. Caspase-8 inhibitor completely abrogated the DHA effect whereas caspase-9 was only 80% abrogated. The pan caspase-1, -3, -4, -7 and caspase-6 had similar effect as caspase-9 inhibition. The importance of caspase-8 in induction of cell death was confirmed by siRNA (Kang et al. 2010). The fact that caspase-8 inhibition abolishes the effect of DHA shows the critical role that DHA has in the extrinsic apoptotic pathway. Increased expression of caspase-8 and caspase-9 was seen in DHA treated Caco-2 colon cancer cells as well as pancreatic and bladder cancer cells (Narayanan et al. 2001, Molinari et al. 2011). DHA incorporation into the plasma membrane, is critical in order for the modulation of death receptors and secondary messengers in the extrinsic pathway of apoptosis (D'Eliseo and Velotti 2016).

The intrinsic (mitochondrial) pathway is activated by endogenous stress signals, including reactive oxygen species (ROS), that increase mitochondrial membrane permeability, followed by the release of cytochrome C into the cytosol and subsequent caspase 9 activation of the executioner caspases (D'Eliseo and Velotti 2016). DHA is incorporated into the mitochondrial membrane phospholipids. It increases the susceptibility of the membrane to ROS which disrupts the mitochondrial permeability transition pore and

releases intermembrane proteins (Chapkin et al. 2008). In a comparison of DHA to linoleic acid (LA), incubation of mouse colonocytes (YAMC) with DHA resulted in a 120% increase (P<0.01) in mitochondrial membrane potential (MMP). Both the increase in lipid oxidation and MMP resulted in the induction of apoptosis (Chapkin et al. 2008). Confirmation of these findings was seen when cells were treated with Mito-Q (mitochondria-specific antioxidant) which blocks lipid oxidation and prevented apoptosis (Chapkin et al. 2008). Although the precise mechanism of mitochondrial ROS induced apoptosis is not known, it has been shown that DHA treatment in conjunction with butyrate of p53 +/+ and p53 deficient colon tumour cells (HCT116) resulted in mitochondrial accumulation of calcium and lipid peroxidation, thereby inducing p53 independent apoptosis (Chapkin et al. 2008). In breast cancer cells treated with increasing concentrations of DHA, there was an increase in mitochondrial membrane potential from 15.1% with no DHA to 84.9% with 50 μM DHA (Lee et al. 2014). Caspase-3 activation (indicated by a decrease in pro-caspase 3 and an increase in cleaved caspase 3) followed by PARP cleavage and apoptosis occurred in response to 50 μM DHA in MDA-MB-231 cells (Lee et al. 2014). Finally, in fish oil diet fed rats with colon cancer, assessment of anti-apoptotic protein BCL-2 and key regulator protein involved in the intrinsic apoptosis pathway was found to have decreased expression compared to control fed animals (Chapkin et al. 2008). These data are non-exhaustive as there has been considerable research into the ability of DHA to increase apoptosis in multiple cancer cell types and through both the extrinsic and intrinsic pathways. While the evidence is strong, it exists primarily in preclinical models and has to date relied on immortalized cell lines. There is a clear need for further research that reflects the heterogeneity of breast cancer.

1.6.3 DHA, Cancer and Cell Cycle Progression

Additionally, there is limited research into the effects of DHA on cell cycle regulation in cancer cells. The aim of this section is to provide a critical examination of studies investigating

Table 1-4: Overview of *in vitro* studies investigating cell cycle in cancer cells treated with docosahexaenoic acid

Cell Cycle	Cancer Model	Cancer Cell Line	Treatment	Cell Cycle Markers	Other Markers	Ref.
G1	Leukemia	KG1A	150 µM	↑ cells in G1 and ↓ in G2M	↑ apoptosis; ↑ DNA fragmentation; NC BCL2, ↑ Bax expression	(Yamagami et al. 2009)
G1	Neuroblastoma	LA-N-1; (HEK-293; WRL-68-control)	0–70 µM	↑ in cells in G1; ↓ expression of CDK2 and Cyclin E	↑ apoptosis; ↑ PS extern.; ↓ MMP; ↓ BCL-XL and ↑ Bax, Casp-3 and -9; Casp-8 NC	(So et al. 2015)
G1	Colorectal	HT-29	150 µM	↑ in cells in G1; ↓ Cyclin D1, E and A activation; ↓ expression of Cyclin A and pRb; ↓ E2F-1 DNA binding activity	NA	(Chen and Istfan 2001)
G1	Breast	4T1 (Mouse); MCF-7 (Human)	25–100 µM	↑ cells in G1; ↓ β-catenin, c-myc, Cyclin D1 in 4T1 cells	↑ apoptotic in 4T1 and MCF-7 cells	(Xue et al. 2014)
G1	Breast	MDA-MB-231, MCF-7, SK-BR-3, HCC1806	80 µM	↑ cells in G1; ↓ in p21 in MCF-7 and SK-BR-3, ↑ in HCC1086, NC in MDA-MB-231; NC in p27 or Cyclin D1	↑ apoptosis	(Lin et al. 2017)
G1	Breast	FM3A (Mouse)	10 µM	↑ cells in G1; ↑ p27; ↓ MAPK expression; NC p27 mRNA; ↓ Cyclin E, pCDK2 expression; NC Cyclin D; ↓ pRB	NA	(Khan et al. 2006)
G1	Breast	MCF-7, ZR-75-1, SK-BR-3, MCF-10A	100 or 300 µM	↑ in cells in G1; ↑ in sub G1; ↑ p21 (mRNA and protein) in MCF-10A; NC in G1; ↓ sub G1; ↓ p21 (mRNA)	↓ p-ERK ½ and STAT3 in SKBR3 and MCF-7 cells; ↑ p-ERK ½,	(Rescigno et al. 2016)

				in MCF-7; ↑ in G2M; ↑ in G1; ↓ p21 (mRNA) SKBR3	STAT3; ↑ p53 all cell lines	
G1	Gastric	AGS	7.5–45 μg/mL DHA; 1.5625–50 μg/mL 5-FU	↑ cells in G1 with DHA or 5-FU alone; ↑ cells in G1 more in combination; ↓ in S-phase	↓ in METC I, II, V expression	(Gao et al. 2016)
S	Leukemia	E6-1	0–30 μM	↑ cells in S; ↓ Cdk2, pRb and Cyclin A expression; ↑ p21	4-fold ↑ ceramide formation; ↓ Casp-3 expression	(Siddiqui et al. 2003)
S	Melanoma	SK-Mel-110 and SK-Mel-29 (control)	0.5–5 μg/mL	Two-fold ↑ SK-Mel-110 cells in S; ↓ pRb in SK-Mel-110; NC Cyclin D, E, p21, p27	↑ apoptosis in SK-Mel-110	(Albino et al. 2000)
S	Liver	MHCC97L	0–200 μM	↑ in cells in sub G1; prolonged S phase; ↓ in Cyclin A, E and CDK2	↓ *COX-2* mRNA; NC protein expression; ↓ Hsp27, GRP78, N-myc protein; ↑ SOD2	(Lee et al. 2010)
G2M	Pancreatic	MIA PaCa-2	10–100 μM *n*-3 emulsion	↑ in cells in G2; ↓ in G1, 13% ↑ in S-phase; large sub G1; ↓ Cdc2 (Cdk1) expression	↑ in apoptotic cells; ↓ BCL-2 expression; ↑ PARP cleavage product	(Dekoj et al. 2007)
G2M	Breast	MDA-MB-231	30–100 μM DHA	↑ cells in G2M; ↓ CDK1, Cyclin B1, Cyclin A, CDC25C, Cyclin B1p-Ser126 and NC Cyclin E	↑ apoptosis with ↑ concentrations DHA	(Barascu et al. 2006)
G2M	Leukemia	EHEB, JVM-2 and MEC-2	50 μM; 0.75 μM Dox	↑ in cells in G2M with DHA alone; ↑ in G2M with DHA + Dox (EHEB, JVM-2 and MEC-2); ↑ in G2M DHA + vincristine (JVM-2 and EPA) ↑ in G2M DHA + fludarabine (EHEB)	↑ cell death from Dox in EHEB, JVM-2 and MEC-2; ↑ cell death from vincristine in JVM-2 and MEC-2 and fludarabine in EHEB	(Fahrmann and Hardman 2013)
G2M	Prostate	LNCaP, DU145, PC3	25 μM; 0.6 nM TXT	↑ sub G1 cells; no diff between DHA, TXT, and combo; ↑ in G2M in LNCaP cells; >DHA + TXT than other treatments alone	↑ MMP collapse in DHA + TXT; ↑ MAP2K4, TNFRSF11A, RIPK1; ↓	(Shaikh et al. 2009)

					FADD, AKT1, MAX (microarray); RT-PCR opposite values	
G2M	Colorectal	CaCo2	FO (10–50 uM EPA 2:1 EPA:DHA); 0.25–1.0 μmol/L 5-FU	↑ cells in G2M with FO, ↑ in S with 5-FU and ↑ cells in S and ↓ in G2M with 5-FU and FO combined	↑ in apoptotic cells	(Jordan and Stein 2003)
G1 and G2M	Colorectal	COLO205 (wt p53) and WiDr (mutated p53)	125 μM	↑ in G1 in WiDr; ↑ G2M in COLO205	↓ proliferation in WiDr (NC in COLO205), ↑ apoptosis in COLO205, NC in WiDr	(Kato et al. 2007)
G1 and G2M	Colorectal	SW620 (chemotherapy resistant)	70 μM	↓ Cyclin D1, D3, A2, B2, F, CDK1, CDK2, CDK4, PCNA, CDC25B, CDC25C; ↑ p21, 14-3-3; ↓ mRNA transcript: G1/S: *CCND1, CCND3, CCNG2 CDC42, CDC45L, CDC7, CDK2, CDK2AP, CDK4, CIP1/P21, CDKN1A, E2F1, PCNA, UNG, G2M: AURKA, AURKB, BIRC5, BUB1, CCNA2, CCNA2, CCNB2, CNF, CDC2/CDK1, CDC20, CDC25B, CDC25C, CENPE, FOXO3A, PLK1*; ↑ p21, 14-3-3 protein	↑ Gadd-45A, Gadd45B and Gadd34, Casp-4, 7, TNFRSF10B mRNA; ↓ NFκB, p38-P, α, β-livin, ↑ t-livin (protein); NC total p38 or Survivin (protein)	(Slagsvold et al. 2010)
G1 and G2M	Breast	KLP-1	97 (CDHA) 270 (DHA) μmol/L	↑ cells in G2 with DHA; ↑ cells in G1 with CDHA; Cyclin D1; ↑ p21 expression	↑ apoptosis; ↑ p53; ↓ BCL-2; NC Bax	(Tsujita-Kyutoku et al. 2004)
G1 and G2M	Breast	MDA-MB-231 MCF-7	0–100 nmol/L Dox	↑ cells in G1 and G2M in MCF-7; ↑ G2M in MDA-MB-231; ↓ expression SKP2, p21, p27, Cyclin B, p53 in MCF-7; ↑ protein	NA	(Bar-On et al. 2007)

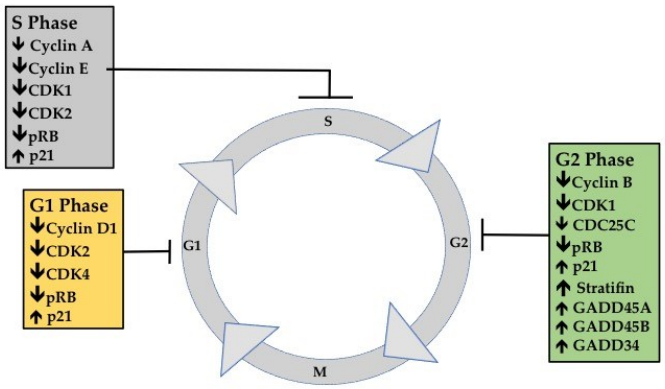
			expression SKP2, Cyclin B, p53 and ↓ p21 MDA-MB-231	

Table 1-5: Overview of *in vivo* studies investigating cell cycle in cancer cells treated with docosahexaenoic acid

Animal Model	Tumour Model	Treatment/Diet	Results	Ref.
BALB/c mice	KLP-1	0, 0.2%, 1.0% CDHA	NC body weight; ↓ in tumour volume and ↓ in metastases in 1.0% CDHA, but NC in tumour weight	(Tsujita-Kyutoku et al. 2004)
Rats	mammary tumours induced with 1M1N	high *n*-3 diet (3:1 EPA:DHA, 45 g/kg diet)	↓ in Cyclin D1, pRB ↑ p21, ↑ p27 protein expression; ↑ apoptotic markers	(Jiang et al. 2012)
BALB/c mice	4T1; mammary fat pad	5% fish oil	↓ tumour weight; ↓ in Cyclin D1, c-myc, B-catenin ↑ TUNEL + cells	(Xue et al. 2014)

the ability of DHA to stall progression during different cell cycle phases (**Table 1-4** and **1-5** and **Figure 1-2**) in cancer cells. This functionality of DHA in cancer could lead to growth inhibition, independently and in conjunction with chemotherapy.

Figure 1-2: Schematic illustrating the pleiotropic effects of docosahexaenoic acid (DHA) on genes and proteins throughout the cell cycle in various cancer cell types



(referenced in accompanying Table 1-4).

Progression through the cell cycle is tightly regulated and checkpoints at phase transitions during the cell cycle ensure that only healthy cells progress and proliferate. Loss of cell cycle control is one of the hallmarks of cancer (Hanahan and Weinberg 2000). Normal, non-cancerous cells monitor their environments and have the potential to either remain quiescent, proliferate, or become post-mitotic (Hanahan and Weinberg 2000). Once a cell is committed to entering the cell cycle, checkpoints regulated by cyclins and their associated cyclin dependent kinases are in place to monitor errors and avoid mutations (Hochegger et al. 2008). In a cancer cell with dysregulated growth, these genes and proteins are frequently overexpressed and checkpoint control is evaded. The key cell cycle checkpoint between G_2 and M phase was investigated in a retrospective cohort of breast tumour samples from 10 ER^+ (and/or) $PR^+/HER2^+$; 32 ER^+(and/or) $PR^+/HER2^-$; 1 $ER^-PR^-/HER2^+$ and 4 $ER^-PR^-/HER2^-$ patients. Seventy-six mitotic checkpoint genes were analyzed by RTPCR. It was

found *NDC80*, *BUB1*, *BUB1B*, *CCNB1*, *TACC3*, *TPX2*, *CCNA2*, *CDC2* and *CDC20* were significantly up-regulated in all tumour types compared to normal breast tissue and in addition to these genes, *NEK2*, *CENPE*, *BIRC5*, *CCNB2*, *AURKB*, *AURKA*, *TTK* and *PLK1* were found to be highly up-regulated in invasive breast tissues compared to normal breast tissues (Bieche et al. 2011). Some of these same mitotic genes are being investigated as emerging cancer therapy targets including ones (i.e. *AURKA* and *PLK1*) that are implicated in cancers with 'poorer prognosis'. To date the thirty AURK inhibitors tested have performed poorly in clinical trials, while PLK1 inhibitors have shown potential in solid tumours (Weiß and Efferth 2012). However, these results have not yet been reproduced in a clinical setting. Although many emerging cell cycle targets are still being investigated, currently there is no highly effective anti-mitotic drug that works in solid tumours and in patients (Dominguez-Brauer et al. 2015).

DHA has been shown to be cytotoxic to many cancer cell types and to have differential effects on a broad variety of cellular molecules and pathways; the mechanisms proposed to explain this may be phenotype-specific although this has not been clearly established (D'Eliseo and Velotti 2016). Furthermore, the ability of DHA to alter cancer cell progression through the cell cycle has not been extensively investigated. Cell cycle analysis was assessed in all studies by propidium iodide staining of cells and flow cytometric analysis. Of 21 studies, nine reported cell cycle stall at G1, three reported alterations in S phase, five reported cell cycle stall in G2M and another four studies reported that multi-phases were affected. **Figure 1-2** and **Table 1-4** provide a summary of cell cycle markers that have been reported to change with DHA treatment.

Treatment of cells with DHA stalled cell cycle progression in the G1 phase of the cell cycle in leukemia (Yamagami et al. 2009), colorectal (Chen and Istfan 2001), neuroblastoma (So et al.2015) and breast (Xue et al. 2014, Khan et al. 2006, Rescigno et al. 2016) cancer

cells. Acute myeloid leukemia cells (KG1A) treated with 100 μM DHA were measured at 0, 2, 4, 6, and 24 hours for cell cycle progression. At T=0, G1=57%, S=31% and G2M 11% for both control and DHA treated cells. Although there was a slight increase in G1=60%, no other changes were observed in the first 4 hours; by 24 hours the control and the DHA-treated cells in the G1 phase were, 56% and 68% respectively. Although, the trend did not reach statistical significance, the higher proportion of DHA treated cells stalled in G1 suggests the propensity for DHA treatments to induce cell cycle arrest. A similar trend was also observed in HL-60 leukemia cells where a 12-22% increase in G1 arrest was seen (Chiu et al. 2004). There were no changes in S phase and the proportion of cells in G2M decreased, but not significantly (Yamagami et al. 2009). Cell cycle analysis of breast (Xue et al. 2014) and neuroblastoma (So et al. 2015) cells showed a concentration dependent (25-150 μM in breast; 0-70 μM in neuroblastoma) increase in percentage of cells stalled at G1. However, in both studies, a concentration of 25 μM was sufficient to induce a significant effect. In LA-N-1 neuroblastoma cells it was also found that DHA treatment had a marginally increased yet non- significant efficacy over eicosapentaenoic acid (EPA) treatments at the same concentration (So et al.2015). Xue et al. compared human breast cells and mouse breast cells and also found a higher percentage of cells in G1 phase following a treatment with increasing concentrations of DHA in both cell types and a corresponding decrease in the S and G2M phases (Xue et al. 2014). A separate study looked at FM3A mouse breast cancer cells and found a significant increase in the proportion of cells in G1 and a corresponding decrease in S phase with 10 μM treatment for 12 hours (Khan et al. 2006). In another study, HT-29 colorectal cells were serum starved to synchronize their cell cycle to G1 and then stimulated with 150 μM DHA treatment. This resulted in an increase from 32% to 63% in the G1 phase of the cell cycle; a decrease from 47% to 30% in S phase and a reduction in G2 from 21% to 8% (Chen and Istfan 2001).

Rescigno et al., found that MCF10A (non-tumourigenic breast cells) and SKBR3 breast cancer cells treated with DHA (100 or 300 μM) had a greater proportion of cells in G1 after 24, 48 and 72 hours, while MCF-7 cells had a lower proportion of cells in G1 compared to control at the 48- and 72-hour time points (Rescigno et al.2016).

In conclusion a consistent propensity to stall during the G1 phase when treated with DHA was seen across four different cancer types with varied experimental conditions. In four of these studies (three on breast (Xue et al. 2014, Lin et al. 2017, Rescigno et al. 2016) and one leukemia (Yamagami et al. 2009)), cells were not synchronized prior to the flow cytometry experiments. Synchronization of the cell cycle by serum starvation moves the cells to the G1 phase before replacing the serum and commencing treatments. This limitation was overcome by other strengths in the studies: all three breast cancer cell studies assessed multiple cell lines and different DHA concentrations (Xue et al. 2014, Lin et al. 2017, Rescigno et al.2016). Yamagami et al. assessed time point variation so an increasing trend of cells accumulating in G1 could be seen over time (Yamagami et al. 2009). These studies used varying concentrations of DHA from 10 μM to 300 μM. It has been reported that, in serum starved cells, the concentration of DHA required to elicit a response can be four times lower than that required in the presence of serum (Siddiqui et al. 2003). For example, in the experiment where breast cells were treated with 10 μM they were grown with only 0.2% serum providing a possible explanation for the efficacy of DHA at this concentration (Khan et al. 2006). Only one study investigating DHA treatment in conjunction with chemotherapy reported cell cycle arrest in the G1 phase. In this study, gastric AGS cells were treated with 30 μg/mL DHA ± 12.5μg/mL 5-fluorouracil (5-FU) for 48 hours. The percentage of cells in the G1 phase was significantly increased compared to control in both DHA (19% higher), and 5-FU (33% higher); but no significant differences were observed between the two treatments. The effect of the DHA+ 5-FU combination was significantly higher with respect to individual

treatments (39%). The percentage of cells in S-phase decreased with treatment: 56% in the Control group, 34 % in the DHA, 25% in the 5-FU and 19% in the DHA+ 5-FU group. This study also assessed the effect of DHA +5-FU on the expression of mitochondrial electron transfer chain (METC) complexes and at 48 hours DHA+ 5-FU had decreased significantly their expression, different from control and individual treatments. These results suggest that DHA and/or 5-FU inhibit entry / exit to the METC, therefore disrupting energy metabolism within the cell (Gao et al. 2016). Interestingly two of the studies (focused on lung (Crnkovic et al. 2012) and neuroblastoma (So et al. 2015)) that reported G1 arrest with DHA and in the absence of chemotherapy also documented a decrease in mitochondrial membrane potential suggesting an effect of DHA on the mitochondrial function in a cancer cell.

Progression through G1 is regulated by Cyclin dependent kinases (CDK) 2, 4 and 6 and Cyclins D1, -2, -3 and Cyclin E (So et al. 2015) and it has been suggested that G1/S phase arrest is due in part to increased levels of p21, p27 and p53 and decreased levels of Cyclin D1 (Caldon et al. 2006). p21 and p27 are CDK inhibitors that inhibit Cyclin D/CDK4/6 and Cyclin E/CDK2 complexes (Slagsvold et al. 2010). Conflicting results have been reported for the effects of DHA on Cyclin D1. After DHA treatment, expression of Cyclin D1 was found to be reduced in lung (Crnkovic et al. 2012), MCF-7, 4T1 breast (Xue et al. 2014) and KLP-1 breast (Tsujita-Kyutoku et al. 2004) cells while other studies found no changes in Cyclin D1 expression in FM3A (Khan et al. 2006), MDA-MB-231, MCF-7 or SK-BR-3 breast cells (Lin et al. 2017).

Taken together, these results highlight the heterogenicity amongst different immortalized cell lines, not only in baseline differences in expression of cell cycle proteins but also in the response of cell cycle genes to DHA treatment. In HT-29 colorectal cells, treatment with DHA but not EPA, linoleic acid (LA), α-linolenic acid (ALA), nor arachidonic acid (ARA) reduced activation of Cyclin D1, E and A-dependent histone 1 kinases. The

authors suggest that DHA treatment resulting in reduced Cyclin A protein expression (confirmed by a Western blot) could be responsible for the effects seen on Cyclin A-dependent histone 1 kinase. Cyclin D1 or E protein expression was not measured (Chen and Istfan 2001). In a separate experiment, the antioxidant BHT was added with DHA and resulted in a reversal of the effects on Cyclin A. The authors suggest that cell cycle arrest could be due in part to increased oxidative stress with DHA treatment (Chen and Istfan 2001). It should be noted that, while there is considerable evidence that treatment with DHA increases the amount of cytotoxic lipid peroxidation products (Gonzalez et al. 1993, Germain et al. 1998) with subsequent induction of apoptosis (D'Eliseo and Velotti 2016), this study (Chen and Istfan 2001) was one of only two to link lipid peroxidation with cell cycle arrest. Decreased CDK2 protein expression was found in lung (Terano et al. 1999) and neuroblastoma cells (So et al. 2015); in fact, CDK2 relative expression of cells treated with 50 μM DHA was approximately 50% less than without DHA treatment (So et al. 2015). Similarly, Cyclin E expression was reduced to 30% of control with 50 μM DHA in these cells (So et al.2015). FM3A mouse breast cells also had reduced Cyclin E and pCDK2 expression after DHA treatment (Khan et al. 2006).

Regulation of cell cycle at the molecular level is maintained by the retinoblastoma protein (pRb), which sequesters E2F when in a hypophosphorylated state, and thereby inhibits proliferation (Hanahan and Weinberg 2000). Hypophosphorylated pRB was reported in lung (Terano et al. 1999), colorectal (Chen and Istfan 2001) and mouse breast cancer (Khan et al. 2006) cells after DHA treatment. In both the lung and colorectal studies pRb levels in DHA treated cells were compared to oleic acid or linoleic fatty acid-treated cells (Terano et al. 1999, Chen and Istfan 2001). Under normal circumstances, the phosphorylation of pRB results in its dissociation and release of E2F-1 which then induces entry to S-phase (Khan et al. 2006). Reduced activity of E2F-1 measured by a gel shift assay was seen in DHA

treated colorectal cells (Chen and Istfan 2001). In an *in vivo* model, Jiang et al. induced mammary tumours in rats and in addition to reduced tumours in rats fed a high n-3 diet, they also found lower cyclin D1, pRb and higher p21 and p27 protein expression with DHA treatment (Jiang et al. 2012). Levels of p53 and p21 increased in a time dependent manner in KLP-1 (Tsujita-Kyutoku et al. 2004), MCF-10A, MCF-7 and SKBR3 (Rescigno et al. 2016) breast cells treated with DHA. Although none of the studies to date have provided a complete analysis of all cell cycle markers, there appears to be consensus of a reduction of Cyclin D (Chen and Istfan 2001, Xue et al. 2014), CDK2 (So et al.2015), and pRb (Chen and Istfan 2001, Terano et al. 1999). The expression of p21 was found to be decreased in MCF-7 (Lin et al. 2017, Rescigno et al. 2016) and SKBR3 (Lin et al. 2017) breast cancer cells but was not changed in MDA-MB-231 breast cancer cells (Lin et al. 2017). p27 protein expression was not changed in MDA-MB-231, MCF-7 or SKBR3 cells (Lin et al. 2017), but was increased in mouse breast cells, although the mRNA expression was unchanged (Khan et al. 2006), suggesting a post-transcriptional effect of DHA on this protein. It is known that ERK1/2 and STAT3 phosphorylation leads to an increase in cell proliferation and survival as STAT3 up-regulates Cyclin D1 and p21 expression (Johnston and Grandis 2011). In SKBR3, and to lesser extent MCF-7 cells, there was decreased phosphorylation of ERK1/2 and STAT3 after DHA treatment (Rescigno et al. 2016), suggestive of a mechanism involving reduced STAT3 activation of p21 leading to G1 arrest. Together, these studies provide evidence that DHA treatment can stall cancer cells in G1 due in part to decreased Cyclin D, possibly via the associated kinase CDK2 and can prevent pRb phosphorylation and E2F-1 activation.

Only three studies were found reporting that DHA treatment affects transition through the S phase of the cell cycle. In the hepatocarcinoma cell line, MHCC97L, 50μM DHA was found to disrupt and prolong S phase transition (Lee et al. 2010). In this experiment, cells were BrdU-labelled and DNA synthesis time based on the relative movement of cells through

the cell cycle was measured. The time to progress through S phase increased from 18 to 21 hours after DHA treatment. In particular, the proportion of Jurkat leukemia cells in the S phase of the cell cycle (measured at 0 and 24 hours) increased from 30 % to 68 % with 30μM DHA treatment while G1 decreased (62% to 43%) (Lee et al. 2010). Control cells had a larger proportion of cells moving to G2 than the DHA treatment (11% vs. 2%), suggesting that cells were continuing to move through the cell cycle compared to the DHA cells that were accumulating in S phase (Siddiqui et al. 2003). Albino et al. compared two melanoma lines, one that was sensitive (SK-Mel-110) and one that was refractory (SK-Mel-29) to DHA and found the SK-Mel-110 cells accumulated in the S-phase (36% compared to 17%) with 2μg/ml DHA but no changes were seen in SK-Mel-29 with DHA treatment (Albino et al. 2000). The serum starvation, which induces cellular stress, in the leukemia and melanoma cells could explain the effectiveness of low concentrations of DHA used in these studies.

Cyclin E controls entry from late G1 to S phase and this is followed, as DNA synthesis begins with an increase in Cyclin A and associated CDK2 Cyclin A. CDK2 protein expression was found to be lower in metastatic MHCC97L liver cells (Lee et al. 2010) and leukemia (Siddiqui et al. 2003) cells treated with DHA. DHA also decreased Cyclin E protein expression and decreased COX-2 mRNA expression in MHCC97L cells (Lee et al. 2010). COX-2 is known to be overexpressed in many cancer types resulting in inhibition of apoptosis (D'Eliseo and Velotti 2016) and the authors suggest that further studies should be conducted in order to determine the relationship between reduced COX-2 and cell cycle arrest (Lee et al. 2010). Although the proliferation marker, pRb was hypophosphorylated in leukemia (Siddiqui et al. 2003) cells and SK-Mel-110 melanoma, no other cell markers (Cyclin D, E, p21 or p27) were different in this melanoma cell line after DHA treatment (Albino et al. 2000). Siddiqui et al. reported that a treatment of 10 μM DHA resulted in membrane incorporation, sphingomyelinase activation and a four-fold increase in ceramide

generation (Siddiqui et al. 2003). The authors proposed a pathway for DHA-mediated cell cycle arrest in S-phase in leukemia cells: initiation of p21 activation, which in turn leads to inhibition of the CDK2/Cyclin A complex, hypophosphorylation of pRb and subsequent arrest (Siddiqui et al. 2003).

The G2M checkpoint is a known target for cell cycle inhibition (Dominguez-Brauer et al. 2015) and the ability for DHA to arrest cells at this point has been studied in leukemia (Fahrmann and Hardman 2013), pancreatic (Dekoj et al. 2007), breast (Rescigno et al. 2016, Barascu et al. 2006, Tsujita-Kyutoku et al. 2004), and colorectal (Kato et al. 2007, Slagsvold et al. 2010) cancer cells. In a study comparing treatments with n-3 (mixture of EPA and DHA) or n-6 fatty acid emulsion (Omegaven), pancreatic cells (MIA PaCa-2) were found after 24h to accumulate in the G2M phase only when treated with 100 μM n-3 emulsion (Dekoj et al. 2007). However, at 48 hours there was only a small further increase suggesting that by 48 hours, cells had moved on to cell death / apoptosis (Dekoj et al. 2007). In a synchronized MDA-MB-231 breast cancer cell population, treatment with increasing concentrations of DHA resulted in cells stalled at 18 hours in the G2M phase of cell cycle in a dose-dependent manner (Barascu et al. 2006).

In the study by Rescigno et al., a higher proportion of SKBR3 cells were found in G2M with 100 and 300 μM treatment compared to control at 24, 48 and 72 hours (Rescigno et al. 2016). There is a growing number of studies demonstrating the synergistic efficacy of DHA in conjunction with chemotherapy (reviewed by D'Eliseo and Velotti (D'Eliseo and Velotti 2016)), however only three studies have reported cell cycle analysis. In CaCo2 cells treated with a (0.36 mL/L) fish oil (FO) emulsion it was found that there was a 2.2-fold increase in G2M with FO alone, but in combination with 5-FU, cells increased in the S phase from ~40% to 70% (Jordan and Stein 2003). This study did not look at any cell cycle markers to confirm the flow cytometric analysis of cell cycle and the FO was a mixture of EPA and

DHA, so some of these effects could be attributed to EPA, which has been proposed to have a different mechanism to explain anti-cancer effects on tumour cells (Jordan and Stein 2003). In a panel of 3 different leukemia cell lines and 3 different antineoplastic drugs it was found that treatment with 50 μM DHA elicited G2M arrest in JVM-2 and MEC-2 cells but not in EHEB cells. The addition of 1.5 μM doxorubicin or 42 μM fludarabine to 75 μM DHA elicited G2M arrest in EHEB cells. JVM-2 and MEC-2 leukemia cells treated with 50 μM DHA +1.5 μM doxorubicin treatment or 100nM of vincristine showed an increase in G2M arrest compared to DHA treatment alone. EPA and LA were also tested and LA did not induce cell cycle arrest and, although EPA did elicit a response it was less efficacious compared to DHA confirming, at the same dose, a differential ability of n-3 fatty acids to slow growth of malignant B-lymphocytes. The combination of DHA + doxorubicin (in EHEB, JVM-2 and MEC-2) or DHA + vincristine (in JVM-2 and MEC-2) showed increased chemo-sensitivity over chemotherapy alone highlighting the synergistic effects of DHA and chemotherapy (Fahrmann and Hardman 2013). In prostate LNCaP and PC3 cells enhanced effect was found with a dose of 25 μM DHA and 0.6 nM docetaxel (TXT). The synergism was found to be best at 48 hours and by 72 hours the beneficial results began to diminish. This could possibly be due to a limitation of available DHA in the media. LNCaP cells treated with DHA + TXT increased the percentage of cells in G2M phase of cell cycle compared to control, DHA or TXT alone. This study did not assess any other markers of cell cycle, but did investigate apoptosis and reported a depolarization/ collapse in MMP, which are an early sign of apoptosis as well as inhibition of the NFKB pathway with the combination of DHA +TXT (Shaikh et al. 2008). These results suggest that DHA treatments alone or in conjunction with chemotherapy target the cell cycle at the G2M phase in multiple cancer models.

In normal cell cycle progression, the transition from G2 to M phase is marked by an increase in Cyclin B and CDK1 expression as well as an increase in expression of mitotic genes including *CDC25C* and in the neoplastic cell cycle, CDK1 is thought to be necessary for tumourigenesis (Otto and Sicinski 2017). Protein expression of these three markers was found to be decreased in MDA-MB-231 breast cancer cells (Barascu et al. 2006) and CDK1 expression was lower in pancreatic cells after DHA treatment (Dekoj et al. 2007). The three studies that assessed DHA in combination with chemotherapy focused on enhanced chemo-sensitization with DHA treatment and did not assess G2M phase markers (Fahrmann and Hardman 2013, Shaikh et al. 2008, Jordan and Stein 2003), although Shaikh *et al.*, did assess changes in mitochondrial membrane potential and NFκB candidate genes (Shaikh et al. 2008).

A comparison of the response to DHA in cells harboring a wild type p53 versus a mutated p53 was made in a colorectal cancer model. An effect on the cell cycle after DHA treatment occurred in G1 in p53 mutated WiDr cells while an effect in G2M was seen in p53+ COLO205 cells. While COLO205 cells went on to programmed apoptotic death, WiDr cells were not stimulated by DHA to undergo apoptosis, but rather had reduced proliferation (Kato et al. 2007). This suggests that the fate of the cell and the phase of the cell cycle in which it gets arrested in response to DHA treatment may be dependent on the p53 status of the cell. In synchronized KLP-1 breast cancer cells treated with 200 μmol/L DHA or 97 μmol/L conjugated DHA (CDHA, a geometric isomer of DHA prepared by alkaline isomerization); differential effects were seen in cell cycle response. After 24 hours, in CDHA treated cells, the percentage of cells in G1 increased by 33% compared to control, whereas in DHA treated cells the percentage found in G2M increased by 22% compared to control (Tsujita-Kyutoku et al. 2004). This suggests that the formulation of DHA is also important in eliciting a cell cycle response. A comprehensive study of chemotherapy resistant colorectal

cancer SW620 cells reported that DHA treatment reduced the expression of many G1 and G2 genes both at transcript and protein level. In G1, *Cyclin D1*, *CDK1*, *CDK2* and *CDK4* had decreased expression (protein and mRNA) while p21 and 14-3-3 (stratifin) were decreased with 70μM DHA treatment. These factors are consistent with arrest at G1. Up-regulation of stratifin is an important event in cell cycle arrest as it anchors CDK1 in the cytoplasm and from there it is unable to form a complex with Cyclin B1 and induce mitosis (Slagsvold et al. 2010).

In G2M, there was a 2.5-fold increase of cells corresponding with a down regulation of mRNA in the following G2M checkpoint proteins: *AURKA*, *AURKB*, *BIRC5*, *BUB1*, *CCNA2*, *CCNA2*, *CCNB2*, *CNF*, *CDC2* /*CDK1*, *CDC20*, *CDC25B*, *CDC25C*, *CENPE*, *FOXO3A* and *PLK* and decreased protein expression in Cyclin B2, CDC5b and CDC25c (Slagsvold et al. 2010). These findings suggest that, in these cells after DHA treatment, p21 inhibits progression through the cell cycle resulting in arrest in either G1 or G2 depending on what phase of the cell cycle a cell is in upon treatment.

Treatment with DHA has been demonstrated in cell lines and preclinical models to inhibit cell proliferation or growth across a wide spectrum of cancers. There is considerable evidence that treatment with DHA is able to elicit arrest in the G1 phase, S phase and possibly G2M phase (particularly when co-treated with cytotoxic drugs) and decreases the expression of Cyclins and other cell cycle markers throughout the cell cycle (**Figure 1-2**). The efficacy, and the specificity of DHA likely depends on two main factors: (1) the molecular properties or type (invasiveness) of each cancer and (2) the variability in the experimental conditions, including time, concentration and synchronization of cells.

Emerging evidence highlights the complexities of treating human cancer cells due to extensive mutations including p53 (Leroy et al. 2014), KRAS (Lièvre et al. 2006), PIK3CA and PTEN (Jhawer et al. 2008) and indeed mutational status might contribute to the effect of

DHA. While mutational status is known for many immortalized cancer cell lines (COSMIC), cellular response to DHA on the basis of mutation hasn't been specifically addressed in these studies and there is not sufficient data available at this time to systematically look at this as a possible contributor.

Half of the studies reviewed synchronized the cells and this could be a key factor affecting not only the time point during which cells arrest in the cell cycle, but also what cell cycle markers are involved. While serum starvation does correct for synchronization, it may also induce additional stress to cells and make them more vulnerable to DHA treatment, possibly adding other variables that confound the ability to compare between cell types and experiments.

The ability of DHA to induce an effect at specific points in the cell cycle could be in part due to the broad ranges of doses (from 10 to 300 μM) and methods of exposing cells to fatty acids (free fatty acids or bound to albumin) used in reviewed studies. This highlights the need for a more biological marker of exposure such as membrane n-3 incorporation which might help explain the variability in responses reported. It may also be helpful to adjust for the different modes of administering: bovine serum albumin (BSA)-conjugated versus free fatty acids as it is likely that free fatty acids are taken up more readily than BSA-conjugated fatty acids (Tronstad et al. 2001).

The current evidence presented suggests G1 arrest occurs after a longer incubation compared to a shorter incubation time for G2 arrest. This could be due to the fact that, while G2M arrested cells become apoptotic and subsequently enter programmed cell death, G1 cells do not undergo apoptosis, but it could also be attributed to the limited data available. There are substantial gaps in the literature. Of the 20 *in vitro* studies only 5 specifically studied the effects of DHA on the cell cycle while the majority focused on apoptosis. The limited number of studies that have focused on cell cycle arrest and n-3 PUFA treatment results in the inability

to clearly elucidate the mechanisms that are affected by DHA treatment. While the role of lipid peroxidation on DHA action and apoptosis has been well established (Gonzalez et al. 1993, Germain et al. 1998), further studies are needed to provide evidence that changes in lipid peroxidation/oxidative stress may impact cell ability to proceed through the cell. Additionally, the effect of DHA on nuclear receptors (Sun et al. 2008) or transcription factors and subsequent implications on cell cycle progression will need to be explored. Furthermore, to really understand the effect of DHA on cell cycle arrest, future studies should include analysis of cell cycle markers throughout the entire cell cycle as only one study (with the chemotherapy resistant cells (Slagsvold et al. 2010)) provided evidence of markers throughout the entire cell cycle. In addition, the activation status of cyclins and cyclin dependent kinases are influenced by not only their expression levels but also their phosphorylation state (Hochegger et al. 2008, Blethrow et al. 2008) and it will be necessary to investigate whether the effect of DHA is due to changes in protein level versus changes in phosphorylation. Furthermore, understanding how DHA interferes with cell cycle will be important to determine if there is a role for DHA treatment in combination with chemotherapy for cell cycle inhibition.

1.7 DHA and Cancer –Clinical Evidence

Despite the extensive research into the efficacy of DHA in preclinical models of breast cancer (described earlier in this chapter), evidence in a patient population is limited. The difficulty in translating laboratory findings to a clinical setting arise from tremendous heterogeneities that exist within tumours (intra-tumoural) and between patients which cannot readily be replicated in immortalized cancer cell models. Moreover, the role of the tumour microenvironment, including cells of the immune system, is difficult to assess in immunocompromised animal models. Furthermore, side effects from cancer and the treatment for cancer have not been properly assessed in animal models. This includes pain, weight loss, quality of life (QOL) and peripheral neuropathy or other chemotherapy-specific

adverse effects. While this thesis is focused on DHA supplementation, currently there are only two clinical trials that report on supplementation with DHA alone. The aim of this scoping review is to examine the current clinical evidence on n-3 LCPUFA supplementation in cancer treatment and highlight areas where more clinical evidence is needed.

A spectrum of cancers including breast (Bougnoux et al. 2009, Darwito et al. 2019, Ghoreishi et al. 2012, Hershman et al. 2015, Shen et al. 2018, da Silva Paixão et al. 2017, Martínez et al. 2019, Gogos et al. 1998, Faber et al. 2013, Mansara et al. 2015), head and neck (Faber et al. 2013, Hanai et al. 2018, Solís-Martínez et al. 2018, Talvas et al. 2015), gastrointestinal (Bonatto et al. 2012, Gogos et al. 1998, Gómez-Candela et al. 2011, Swails et al. 1997, Gianotti et al. 1999, Wu et al. 2001, Jiang et al. 2010), gastric (Gómez-Candela et al. 2011, Chen et al. 2005, Wei et al. 2014), colorectal / rectal (Mocellin et al. 2013, de Aguiar et al. 2012, Camargo et al. 2016, Purasiri et al. 1994, Cockbain et al. 2014, Gómez-Candela et al. 2011, Trabal et al. 2010, Kemen et al. 1995, Persson et al. 2005, Pastore et al. 2014, Read et al. 2007, Braga et al. 2002, Liang et al. 2008, Zhu et al. 2012), esophageal (Faber et al. 2013, Gómez-Candela et al. 2011, Ryan et al. 2009, Talvas et al. 2015), leukemia/ lymphoma (Chagas et al. 2017), lung (Murphy et al. 2011, Finocchiaro et al. 2012, Lu et al. 2018, Gogos et al. 1998, Cerchietti et al. 2007, Faber et al. 2013, Gómez-Candela et al. 2011, Pastore et al. 2014, van der Meij et al. 2012, Sánchez-Lara et al. 2014, Bauer and Capra 2005), multiple myeloma (Maschio et al. 2018) and pancreas (Gogos et al. 1998, Wigmore et al. 2000, Werner et al. 2017, Gómez-Candela et al. 2011, Bauer and Capra 2005, Barber et al. 1999, Barber et al. 1999, Barber et al. 2001, Arshad et al. 2014) have been investigated for efficacy of n-3 supplementation pre -or post-treatment (da Silva Paixão et al. 2017, Martínez et al. 2019), concomitant with neoadjuvant chemotherapy or radiation (Bougnoux et al. 2009, Darwito et al. 2019, Ghoreishi et al. 2012, Hershman et al. 2015, Shen et al. 2018, Bonatto et al. 2012, Mocellin et al. 2013, de Aguiar et al. 2012, Camargo et al.

2016, Chagas et al. 2017, Murphy et al. 2011, Finocchiaro et al. 2012, Lu et al. 2018, Maschio et al. 2018, da Silva Paixão et al. 2017, Faber et al. 2013, Gómez-Candela et al. 2011, Solís-Martínez et al. 2018, Trabal et al. 2010, Shirai et al. 2017, van der Meij et al. 2012, Sánchez-Lara et al. 2014, Ryan et al. 2009, Talvas et al. 2015, Arshad et al. 2014, Mansara et al. 2015), in conjunction with surgery (Purasiri et al. 1994, Hanai et al. 2018, Solís-Martínez et al. 2018, Kemen et al. 1995, Chen et al. 2005, Ryan et al. 2009, Braga et al. 2002, Gianotti et al. 1999, Wu et al. 2001, Jiang et al. 2010, Swails et al. 1997, Zhu et al. 2012) or during palliative therapy (Gogos et al. 1998, Cockbain et al. 2014, Cerchietti et al. 2007, Wigmore et al. 2000, Werner et al. 2017, Persson et al. 2005, Shirai et al. 2017, Pastore et al. 2014, Barber et al. 1999, Barber et al. 1999, Barber et al. 2001, Read et al. 2007, Aiko et al. 2005). Furthermore, DHA and EPA treatment in a clinical setting has been delivered through multiple modalities including capsules: **Table 1-6** and **Table 1-7** (Darwito et al. 2019, Bougnoux et al. 2009, Ghoreishi et al. 2012, Hershman et al. 2015, Shen et al. 2018, Bonatto et al. 2012, Mocellin et al. 2013, de Aguiar et al. 2012, Camargo et al. 2016, Chagas et al. 2017, Murphy et al. 2011, Finocchiaro et al. 2012, Lu et al. 2018, Maschio et al. 2018, da Silva Paixão et al. 2017, Martínez et al. 2019, Gogos et al. 1998, Purasiri et al. 1994, Cockbain et al. 2014, Cerchietti et al. 2007, Wigmore et al. 2000, Werner et al. 2017, Mansara et al. 2015), oral: **Table 1-8** (Faber et al. 2013, Gómez-Candela et al. 2011, Hanai et al. 2018, Solís-Martínez et al. 2018, Trabal et al. 2010, Kemen et al. 1995, Persson et al. 2005, Pastore et al. 2014, van der Meij et al. 2012, Sánchez-Lara et al. 2014, Bauer and Capra 2005, Barber et al. 1999, Barber et al. 1999, Barber et al. 2001, Read et al. 2007, Shirai et al. 2017) and enteral / parenteral supplementation **Table 1-9** (Chen et al. 2005, Ryan et al. 2009, Braga et al. 2002, Liang et al. 2008, Talvas et al. 2015, Wu et al. 2001, Gianotti et al. 1999, Aiko et al. 2005, Swails et al. 1997, Jiang et al. 2010, Zhu et al. 2012, Wei et al. 2014, Arshad et al. 2014). Much of the current body of research has occurred in surgical or

palliative patients receiving oral or enteral / parenteral nutrition that is routinely employed in cancer patients to provide nutritional support, especially in instances of weight loss and cachexia or during surgical interventions. While this type of n-3 nutritional supplementation is beyond the scope of the current thesis work, the outcomes of these studies have been included as they provide evidence of efficacy with respect to beneficial immunomodulation, weight maintenance and improved quality of life in cancer therapy.

Table 1-6: Randomized controlled trials providing N-3 capsule supplementation concomitant with chemotherapy

Cancer type (Stage)	N (Int /CNT) Female /Male	Age, Body Weight & BMI (Int/CNT)	Chemotherapy	N-3 (total/ day= EPA/DHA) Control	Treatment duration	Experimental Findings	Reference
Breast (metastatic)	25 F	Age= 58 (32-71)	Cyclophosphamide, 5-FU, epirubicin	1.8 g DHA CNT= N/A	18 weeks	Stratified by amount DHA incorporated into plasma ↑ DHA group associated with longer time to progression (8.7 months vs. 3.5 months); ↑ OS (34 vs. 18 months; ↓ neutropenia, anaemia & thrombopenia	(Bougnoux et al. 2009)
Breast	57 F (30/27)	Age= 46.2±9.8/ 45.7±12.0 BMI=46.0±9.0/ 44.1±8.9	Paclitaxel	1.2 g =192 mg EPA/1.04 g DHA CNT =sunflower	4 cycles + 1 m post	N-3: 70% ↓ risk of peripheral neuropathy incidence	(Ghoreishi et al. 2012)
Breast (I-III)	209 F (102/107)	Age= 59.5/59.1 Body Weight=79.0 (77.3-79.8)	Anastrozole, Exemestane or Letrozole	3.3 g =2.24 g EPA /1.12 g DHA CNT = soybean & corn oil	24 weeks	Both groups: ↓ in pain symptoms in but no proof of n-3 efficacy; when stratified by BMI, n-3 significantly ↓ pain in obese patients	(Hershman et al. 2015, Shen et al. 2018)

Breast (IIIB)	48F (24/24)	Age= 46.5±8.1 /48.5±8.8	Cyclophosphamide, DOX, 5-FU	1.0 g N-3 CNT= unknown source	51 days	N-3: ↓ Ki67 (39.2±5.3 vs. 42.4±4.8, P=0.03), ↓ VEGF (29.5±5.4 vs. 32.7±5.2, P=0.04). ↑ OS (30.9±3.7 vs. 25.9±3.6 wks, P=0.05; HR=0.41, 95% CI: 0.20-0.84) and ↑ DFS (28.5±3.3 vs. 23.7±3.6, P=0.03; HR=0.44, 95% CI: 0.22-0.87)	(Darwito et al. 2019)
Breast (I-II)	5F	Age= 50 (34-60)	Cyclophosphamide+ 5-FU+ DOX/ Adriamycin or Paclitaxel	720 mg-1.08 g EPA+ 480-720 mg DHA	130-188 days	N-3: ↑ SOD, glutathione reductase & plasma antioxidant status; ↑ QOL	(Mansara et al. 2015)
Gastrointestinal	38 (19/19) 16F/22M	Age= 53.8±2.4 /54.9±3.2 Body Weight= 65.8±3.6 /69.5±3.6	5-FU and Leucovorin	700 mg =300 mg EPA /400 mg DHA CNT = N/A	8 weeks	N-3: ↑ in EPA & DHA in PBMCs, ↑ in phagocytosis, superoxide anion production & H_2O_2 productions, ↑ Weight, improved neutrophil function during chemo Control: ↓ Weight	(Bonatto et al. 2012)
Colorectal (III & IV)	11(6/5) 5F/6M	Age= 53.6±12.9 /55.2±7.7 Body Weight= 72.3±12.3 /68.1±12.1 BMI=28.6±6.3 /26.4±3.7	Xeloda, Oxaliplatin, 5-FU and/ or Leucovorin	600 mg =360 mg EPA/ 240 mg DHA CNT = N/A	9 weeks	N-3: Improved CRP, CRP/albumin and potentially prevented Weight loss	(Mocellin et al. 2013)

Colorectal & Rectal	23(11/12) 6F/17M	Age= 50.1±8.2 /54.3±9.3 Body Weight=73±16.8 /66.8±11.6 BMI=27.3±6.1 /25.0±3.4	Type not specified	600 mg =360 mg EPA/ 240 mg DHA CNT= N/A	9 weeks	N-3: ↓ CRP/albumin ratio	(de Aguiar et al. 2012)
Colorectal (II-IV)	30 (17/13) 10F/20M	Age= 52.1±7.6 /53.1±10.2	Chemotherapy type not stated	600 mg =360 mg EPA/ 240 mg DHA CNT=N/A	9 weeks	N-3: ↑ time to progression (20 vs. 11 months); ↓ carcinoembryonic antigen	(Camargo et al. 2016)
Leukemia Lymphoma	22 (9/13) 10F/12M	Age= 43.8/ 53.8 Body Weight= 68.1±10.3 / 72.4±11.6 BMI= 24.6±4.1 / 25.7±4.0	Type not specified	610 mg = 367 mg EPA/ 243 mg DHA CNT =N/A	9 weeks	N-3: ↓ CRP/albumin ratio from high to low; ↑ overall long-term survival (at 465 days) compared to control Control: ↓CRP/albumin ratio from high to medium	(Chagas et al. 2017)
NSCLC (III or IV)	46 (31/15) 22F/24M	Age= 64±1.7/ /63±2.1	Carboplatin and vinorelbine or Carboplatin and gemcitabine	2.2 g EPA +240-500 mg DHA CNT =SOC	6 weeks	N-3: ↑ chemo response rate, ↑ clinical benefit; ↑ 1-yr survival (trend)	(Murphy et al. 2011)
Lung (Advanced)	27 (13/14) 8F/19M	Age= 55.6±7.4 / 60.6±7.4 Body Weight= 75.1±16.1 / 68.0±12.8	Gemcitabine, Cisplatin	3.4 =2.04 g EPA/1.36 g DHA CNT =olive oil	66 days	N-3: ↑ in EPA+ DHA in plasma, ↑ in EPA in RBC; ↓ IL-6, PGE2 & ↑Body Weight; ↓ inflammatory indexes and oxidative status;	(Finocchiaro et al. 2012)

		BMI=26.2±7.0 / 25.2±3.9				Control: ↑ CRP, IL-6, TNF & ROS	
NSCLC (Advanced)	137 (77/60) 61F/76M	Age= 63.8±6.4 / 62.9±7.1 Body Weight= 67.2±11.5 / 70.1±12.3 BMI=23.5±2.1 / 23.9±2.4	Cisplatin, ±TXT, ± bevacizumab	710 mg =510 mg EPA / 200 mg DHA CNT =N/A	6 weeks	N-3 group ↓ CRP, IL-6 and PGE2; no change in QOL or nutritional status;	(Lu et al. 2018)
Multiple Myeloma	18 8F/11M	Age= 69 (57-76)	Bortezomib+ thalidomide +dexamethasone (84 days) or Bortezomib+ melphalan +prednisone	1.2 g ALA+ 800 mg DHA CNT =N/A	6 months	N-3: ↓ in onset or worsening of neuropathic pain, ↓ in chemo interruptions	(Maschio et al. 2018)

Abbreviations used: ALA, alpha linolenic acid; BMI, body mass index; CI, confidence interval; CNT, control; CRP, C-reactive protein; DFS, disease-free survival; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; F, female; g, gram(s); H_2O_2, hydrogen peroxide; HR, hazards ratio; IL, interleukin; Int, intervention group; M, male; MMP, matrix metalloproteinase; N, number; N/A, not applicable; NC, no change, NS, non-significant; NSCLC, non-small-cell lung cancer; OS, overall survival; PBMC, peripheral blood mononuclear cell, PGE2, prostaglandin E2; QOL, quality of life; RBC, red blood cell; Ref, reference; SOC, standard of care; SOD, super oxide dismutase; TNFα, tumor necrosis factor; VEGF, vascular endothelial growth factor.

Table 1-7: Randomized controlled trials providing N-3 capsule supplementation without chemotherapy

Cancer type (Stage)	N (Int/CNT) Female/ Male	Age, Body Weight & BMI (Int/CNT)	Chemotherapy	N-3 (EPA +DHA /day) Control (CNT)	Treatment duration	Experimental Findings	Reference
Breast (I-III)	37F (18/19)	Age= 48.6+9.0/ 53.4+7.5 BMI= 43% overweight, 30% obese	No chemo-N-3 supplementation prior to treatment	940 mg EPA+ 780 mg DHA CNT=2 g mineral oil	30 days	N-3: NC CD4+, CD8+, PGE, IL-6 Control: ↓ CD4+, NC PGE, IL-6, ↑ hsCRP	(da Silva Paixão et al. 2017)
Breast (I-III)	45	Age=57.3 (40-81) BMI= 28.9 (19.3-38.3)	Previous chemo (69.9%), previous radiotherapy (87%); currently on AI 67.3% or Tamoxifen 32.6%	1.38 g N-3 CNT =N/A	30 days	N-3: ↓ from baseline at D30 and D60 of CRP; 21.5% decrease in pain scale; ↓ in IFNγ at D30	(Martínez et al. 2019)
Breast, Gastrointestinal Lung, liver, pancreas (all metastasized)	64 (60 completed) 24F/36M	Age= 60±5 (F), 57±4 (M)/ 58±4 (F), 56±3 (M)	Previous surgery= 38, Previous chemo n=26, previous radiotherapy n=6, none=10	3.06 g EPA+ 2.07 g DHA CNT=sugar tablets	until death	Both groups: ↑ in survival in well-nourished vs. malnourished N-3: ↑ in survival, ↑ CD4/CD8	(Gogos et al. 1998)
Colorectal (local and advanced)	30	Age=63±2.3	Surgery	Group 1 (localized): 1.2 g GLA+ 1.06 g EPA+ 160 mg DHA	Group 1: until surgery Group 2 & 3: 6 months	Group 1: NC in immune parameters; Group 2= ↓ IL1B 3, 4, 5 & 6 months; ↓ IL-4	(Purasiri et al. 1994)

				Group 2 (advanced): T0-15 1.2 g GLA+ 1.06 g EPA+ 160 mg DHA, T16-30: 1.8 g GLA+ 1.6 g EPA+ 240 mg DHA; Months 2-6: 2.3 g GLA+ 2.1 g EPA+ 320 mg DHA Group 3= CNT (6 months)		at 2, 3, 4, 5 & 6; ↓ IL6 at 6 months; ↓ TNF at 2, 4, 5 & 6; ↓ IFNγ by month 4 Group 3: NC	
Colorectal (Dukes A-D) All with liver metastases	88 43 (17F/26M) /45 (10F/35M)	Age= 71 (35-87)/ 68 (44-82)	Previous chemotherapy	2 g EPA CNT =2 g MCT	12-65 days	N-3: ↑ EPA in tumour tissue, NC in Ki67, ↑ OS at 18 months (trend)	(Cockbain et al. 2014)
Advanced Lung (III-IV)	22 (10/12) 5F/17M	Age= 64 (44-90)/ 61 (44-83) Body Weight=60.1±8.2/62.8±9.7 BMI=24±6.2 /25.8±4.4	N/A	360 mg EPA+ 240 mg DHA +celecoxib CNT= 360 mg EPA+ 240 mg DHA	6 weeks	N-3+celecoxib: ↓ CRP; ↑ body weight and hand grip scores improved N-3 alone: ↓ CRP	(Cerchietti et al. 2007)

Pancreatic (II-IV)	26 14F/12M	Age= 56 (39-75) Body Weight =66.8 (56.0-75.1) BMI=23.2 (21.1-27.4)	N/A	EPA only Wk 1: 1 g Wk 2: 2 g Wk 3: 4 g Wk 4-12: 6 g CNT=N/A	12 weeks until death	Body weight stabilized and began to increase by week 4; CRP stabilized; median survival =6.8 months	(Wigmore et al. 2000)
Pancreatic	33 (18/15) 17F/16M	Age=70.3±8.2/ 71.3±7.5 Body Weight =62.9±6.5/ 71.4±15.3 BMI=21.3±1.7 /23.7±4.1	24 patients received chemo, 2 received radiotherapy (not all curative, most palliative)	Group 1: 103.5 mg EPA+ 204 mg DHA; Group 2: 127.5 mg EPA+ 184.5 mg DHA CNT=N/A	6 weeks	↑ in HDL in Group 1	(Werner et al. 2017)

Abbreviations used: BMI, body mass index; CD, cluster of differentiation; CNT, control; CRP, C-reactive protein; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; F, female; g, gram(s); GLA, gamma linolenic acid; HDL, high-density lipoprotein; hsCRP, high sensitivity CRP; IFNγ, interferon gamma; IL, interleukin; Int, intervention group; M, male; MCT, medium chain triglycerides; N, number; N/A, not applicable; NC, no change, OS, overall survival; PGE2, prostaglandin E2; Ref, reference; SOC, standard of care; TNFα, tumor necrosis factor.

Table 1-8: Randomized controlled trials providing oral N-3 supplementation

Cancer type (Stage)	N (Int /CNT) Female/ Male	Age Body Weight & BMI (Int/CNT)	Chemotherapy	N-3 (EPA +DHA /day) Control (CNT)	Treatment duration	Experimental Findings	Reference
Lung, Head & Neck, Gynecologic, Breast, Prostate, Urinary tract, Esophagus (I-IV)	38 20/18 14F/24M	Age=62.7±11.0 Body Weight= 70.8±12.6 BMI=24.8±3.5	Radiotherapy	2x 326 kcal: 2.4g EPA+1.2 g DHA +40 g protein CNT=N/A	7 days	N-3: ↓ serum PGE2 Control: ↑ serum PGE2 No differences in cytokine production	(Faber et al. 2013)
Stomach, Colon, Lung, Pancreas, Other	40	Age= 61.3±12.1/ 63.6±11.4 BMI= 20.9±3.7/ 22.2±3.8	Chemotherapy ±radiation or no treatment	600 kcal: 1.5 g EPA +19.5% protein CNT= Isocaloric supplement	1 month	Both groups: ↑ SF36 N-3: ↓ in IFNγ Control: ↑ in IFNγ	(Gómez-Candela et al. 2011)
Head & Neck (I-IV)	27 (13/14) 11F/16M	Age= 61.5(45-77)/ 66.1 (47-76) BMI=NS but cachexic	Surgery	600 kcal: 2.1 g EPA+32 g protein CNT= N/A	4 weeks	No differences between groups or from baseline	(Hanai et al. 2018)
Head & Neck (I-IV)	64 29F/ 35M	Age= 60±14/ 58±14 Body Weight= 58.8±1.4/ 61.1±11.5 BMI= 22.6±4.6/ 24±4.2 Weight loss= ~9kg in 3 months before entry	Surgery, radiotherapy, chemotherapy or combination	600 kcal: 2.2 g EPA + 1 g DHA +32 g protein	6 weeks	N-3: Weight maintenance, ↓CRP, TNFα & IFNγ Control: Weight loss (2.0±3.7 lbs),	(Solís-Martínez et al. 2018)

				CNT= Isocaloric supplement		↓ CRP, ↑ TNFα and IFNγ	
Colorectal (IV)	23 8F/13M	Age=61±11.6 Body Weight= 75.9±17.0 BMI=28±6.4	Chemotherapy 17 with previous chemotherapy	600 kcal: 2.2 g EPA + 0.92 g DHA +32 g protein CNT=N/A	9 weeks	N-3: ↓ in GM-CSF, ↑ RANTES, CRP (Wk 3) ↑ in GM-CSF & NC CRP (Wk 9), Correlations between: baseline IL-10 & survival, IL-6 & survival, IL-6 & CRP	(Read et al. 2007)
Colorectal (IV)	13 (5/6) 4F/9M	Age =61.5±15.8/ 68.2±15.6 Body Weight= 69.9±15.9/72.2±11.7 BMI= 25.8±4.3/ 26±3.3	5-FU+ oxaliplatin+ folinic acid or Capecitabine	600 kcal: 2 g EPA+ 0.9 g DHA +32 g protein CNT=N/A	12 weeks	N-3: ↑ Weight, NS improvement in QOL & appetite, NS ↓ in fatigue & pain	(Trabal et al. 2010)
Gastrointestinal	42 15F/27M	Age= 68.1/ 66.7 Body Weight= 69.1/67.8	Surgery	10.5% n-3 of 25% fat + 5.6 g protein in 100 ml (patients received 25kcal/kg body weight)	16 days post-operative	N-3: NC in albumin, transferrin, prealbumin, PHA; ↑ stimulated IFN, CD3+, CD3+HLADR, CD4+ & B lymphocytes Both groups: ↓ T lymphocytes (pre-op to post-op)	(Kemen et al. 1995)

Gastrointestinal (Advanced)	24 10F/14M	Age =66±9/ 69±10 Body Weight= 56.6(35-101)/ 61.8(33-80) BMI= 21.6±4.1/ 21.1±4.8 All had >10% Weight loss in past 6 months	Palliative (at least 2 rounds of chemo before study entry)	4.9 g EPA and 3.2 g DHA± melatonin CNT= Isocaloric supplement	4 weeks	N-3: 38% had Weight maintenance, No statistically significant changes in cytokines	(Persson et al. 2005)
Gastrointestinal (II-IV)	128 38F/90M	Age= 72.3±8.4/68.9±10.3 Body Weight= NS but 5% Weight loss before entry	44 adjuvant chemotherapy/ 84 palliative chemotherapy	600 kcal supplement: 2.2 g EPA + 0.92 g DHA +32 g protein CNT= N/A	6 months	N-3: ↓CRP Control: ↑ CRP	(Shirai et al. 2017)
Lung, Gastrointestinal (I-IV)	69 28F/21M	Age=63.5±11.8 BMI=NS but 87% moderate or severe malnutrition	Chemotherapy	600 kcal: 2.2 g EPA+ 33 g protein CNT= Isocaloric supplement	4 weeks	N-3: ↓ CRP (NS due to dropouts/ death only 18 in N-3 vs. 25 in control for final analysis)	(Pastore, et al. 2014)
NSCLC (III)	40 19 F/21M	Age= 58.4±12.0/ 57.2±8.1 Body Weight=77.1±14.6/ 64.7±7.4 BMI=24.8±4.1/ 23.0±2.4	Cisplatin±docetaxel or± bevacizumab +concurrent radiotherapy	600 kcal: 2.2 g EPA + 1 g DHA +32 g protein CNT= Isocaloric supplement	6 weeks	N-3: Weight maintenance, NC in CRP, IL-6, TNFp55, albumin and HLA-DR	(van der Meij et al. 2012)
NSCLC (III-IV)	84 (44 /40) 49F/43M	Age =58.8±14/ 61.1±12.4 Body Weight= 60.4±11/64.7±11; BMI= 24.2±3/ 25.2±4	Paclitaxel and cisplatinum	300 kcal: 1.1 g EPA + protein CNT= Isocaloric supplement	6 weeks supplement & up to 18 weeks chemo)	N-3: Weight maintenance; ↓ CRP, TNFα; ↑ protein intake improved global health status	(Sánchez-Lara et al. 2014)

		Weight loss before entry=8.8±8%/7.4±9%				(including fatigue & improved appetite); trend towards progressive free survival Control: Weight loss, ↑ neuropathy	
Pancreatic, NSCLC	7 2F/5M	Age= 55.1±5.0 =Body Weight= 77.5±11.5 (12% weight loss in previous 6 months) BMI=26.8±5.7	Gemcitabine± other	300 kcal: 1.1 g EPA+ 16 g protein CNT=N/A	8 weeks	N-3: ↑ in protein intake, total energy intake, body weight & QOL	(Bauer and Capra 2005)
Pancreatic (II-IV)	36 (18/18) (+6 no cancer controls)	Age= 64(56-66)/ 60(54-70) Body Weight= 55.0(46.5-60.5) / 58.5(47.8-70.7); pre-study weight loss =17.9% (15.9-20.7)/ 11.8% (5.6-23.5)	Palliative	2 x 610 kcal: 2.2 g EPA + 0.96 g DHA +32 g protein CNT=N/A	24 days	Baseline: Cancer patients: ↓ albumin, prealbumin & transferrin; ↑ CRP, fibrinogen, haptoglobin, ceruloplasmin; After intervention: N-3: ↑ albumin, prealbumin, transferrin; ↓ CRP; 1.0 kg Weight gain	(Barber et al. 1999)
Pancreatic (II-IV)	20 10F/10M	Age= 62 (51-75) Body Weight= 55.2 (48.8-61.2); 17.9% (15.9-22.8) Weight loss BMI= 19.8 (17.8-21.8)	Palliative surgical procedures	2 x 610 kcal: 2.2 g EPA, 0.96 g DHA +32 g protein CNT=N/A	3-7 weeks	N-3: Weight gain= 1.0 kg at 3wks, 2 kg at 7 wks; ↓ IL-6 in stim PBMCs & ↓ trend IL1β	(Barber et al. 1999, Barber et al. 2001)

						(p=0.07), NC in TNF, CRP, unstimulated production of cytokines, or serum concentrations of IL-6, sTNF-RI, sTNF-RII, or sIL-6R & NC leptin; ↑ in fasting insulin

Abbreviations used: BMI, body mass index; CD, cluster of differentiation; CNT, control; CRP, C-reactive protein; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; F, female; g, gram(s); GM-CSF, granulocyte macrophage colony-stimulating factor; HLADR, Human Leukocyte Antigen—DR; IFNγ, interferon gamma; IL, interleukin; Int, intervention group; kcal, kilocalorie; kg, kilogram; M, male; N, number; N/A, not applicable; NC, no change, NS, non-significant; NSCLC, non-small-cell lung cancer; PBMC, peripheral blood mononuclear cell, PGE2, prostaglandin E2; PHA, phytohaemagglutinin; QOL, quality of life; RANTES, regulated on activation, normal T cell expressed and secreted (CCL5); Ref, reference; SF36, short form (36) health survey; TNFα, tumor necrosis factor.

Table 1-9: Randomized controlled trials providing N-3 enteral or parenteral supplementation

Cancer type (Stage)	N (Int/CNT) Female/ Male	Age Body Weight & BMI (Int/CNT)	Chemotherapy	N-3 (EPA+DHA /day) Control (CNT)	Treatment duration	Experimental Findings	Reference
Esophageal (O-III)	27 4F/23M	Age= 67±3 / 64±2	N/A	150 mg n-3/100 ml (up to max 1.5L/day =2.25 g) + protein CNT= EN	day 0 & 8	N-3: NC IL-6 between grps, ↓ in IL-8 (day 1 and 3) and PGF1a (day 5)	(Aiko et al. 2005)
Esophageal (O-III)	53 (28/25) 5F/ 28M	Age= 62±11/ 65.7±9 Body Weight=73.6±14, /77.2±13 BMI= 24.6±3.4/ 27.1±4.1	Combined radiation+ chemotherapy: 5-FU & cisplatin + surgery or surgery alone	Pre-op: 2.2 g EPA enteral feed; Post-op: 0.45g EPA+ 0.19 g DHA/ 100 mL ~2.25 EPA/ day and 0.95g DHA / day oral CNT = EN	5+21 days	Both groups: ↑ CRP, IL-6 after surgery & ↓ after 21 d N-3: ↓ IL-10, IL-8	(Ryan et al. 2009)
Head & neck & Esophageal (II-IV)	28 (15/13) 5F/23M	Age= 57.7±9.9 / 3.3±10.4 Body Weight= 60.5±11.6/ 62.5±12.6 BMI= 22.0±3.6/ 22.3±4.6	Combined radiation+ chemotherapy: 5-FU & cisplatin	3.4g/L EPA+DHA CNT = EN	Chemo:5-7 weeks Int: 5 days before end of chemo	N-3: ↑ in CD62L, CD15 & NK cytotoxicity ↓ in CD4, CD8, CD45RA, CD19+,	(Talvas et al. 2015)

		All had ~10% Weight loss before the study				TCR α/β, TCRγ/δ, NK cells ↑ in PHA stimulated TNFα & PGE2 Control: similar to N-3, Genes for immune receptors, cytokines, inflammation markers & TFs were differentially expressed in n-3 vs. control	
Gastric	40 (20/ 20) 12F/28M	Age= 59.0±12.6	Surgery	Exact n-3 formulation not given+ 24% protein CNT = EN	9 days	N-3: ↑ prealbumin, transferrin, IgA, IgG, IgM, CD4, CD4/CD8 ratio & IL-2; ↓ IL-6 & TNF	(Chen et al. 2005)
Gastric (I-II)	46 (26/20) 20F/26M	Age= 59 (36-74)/ 50.5 (29-75) Body Weight= 65(45-89) /62 (42-88) BMI= 22.5(17.8-29.7)/ 22.2 (15.7-28.1)	Surgery	N-3: 0.2 g/kg body weight parenteral CNT = PN	6 days	Both groups: No difference in immunological parameters by flow, VEGF or IGF1, ↑ in CRP and IL1β N-3: ↓ in total protein, albumin, prealbumin, total cholesterol postop;	(Wei et al. 2014)

					Control: ↑ in IL-6 & TNF α	
Colorectal	200: 4 groups n=50 Control no supplement, Control+ supplement, N-3 before and after surgery & N-3 Preop only 82F/118M	Age= 62.2±10.4/ 61.8±9.9/ 60.5±11.5/ 63.0±8.1 20 Patients with Weight loss >10%	Surgery	3.3g N-3/L (patients received 25kcal/kg body weight) +protein (7+7 days) CNT = EN	N-3: ↑ Phagocytic ability of PMN compared to controls (did not drop post op), ↑ IL-6 post op, but lower compared to control; ↓ Delayed hypersensitivity & ↓ infection in supplemented groups; NC in IGs	(Braga et al. 2002)
Colorectal & Rectal	42 16F/25M	Age= 55.8±10.1/59.2±10.6 Body Weight=63.5±8.9, /65.4±9.2 BMI= 23.4±2.4 /23.9±2.8	Surgery	N-3: 0.2 g/kg body weight parenteral (7 days) CNT = EN	Both groups ↑ IL-6 on day of surgery N-3: ↑ CD4+ & ↓ IL-6 by day 8; NS ↓ TNF	(Liang et al. 2008)
Colorectal & Rectal (Duke B-C)	57 24F/33M	Age= 69.8±10.5 /70.8±6.4 BMI= 22.9±3.1 /23.2±3.6	Surgery	N-3: 0.2 g/kg body weight parenteral (7 days) CNT = EN	Both groups: ↓ CD4 on Day 8 vs day 1 N-3: ↓ CD8 day 1 & day 8; ↓ IL6 at day 8 compared to control	(Zhu et al. 2012)
Gastrointestinal	18 7F/11M	Age= 69.8±2.7 / 65.4±4.2 Body Weight= 67.5±4.5/ 59.6±3.0	Surgery	N-3: Avg =2.74g/d EPA, 1.24g/day DHA	N-3: ↓ in ALT, AST and Alk phos, ↓ in PGE2	(Swails et al. 1997)

		25% had moderate to severe protein calorie malnutrition		(7 days) CNT = EN		production in LPS stimulated cells	
Gastrointestinal	50 20F/30M	Age= 62.5±11.3 /60.9±12.5 11 patients with weight loss >10%	Surgery	N-3: 10.5% 28% fat in 100 ml (patients received 25kcal/kg body weight) + protein CNT = EN	7+7 days (Pre + Post)	N-3: ↑ Prealbumin and retinol binding protein & ↓ IL-6, IL-1RII & Delayed hypersensitivity at day 8, NC in IGs.	(Gianotti et al. 1999)
Gastrointestinal	48 17F/31M	Age= 55.2±12.1/ 52.6±9.8	Surgery	146 kj/kg/day: 100 ml =125kcal=79 mg EPA+ 30 mg DHA + 4 g protein CNT = EN	7+7 days (Pre + Post)	Both groups: ↑ PGE2 and CRP post operatively N-3: ↓ PGE2, CRP IL-6 & TNF by day8, NS ↓ in IL2, ↑ glutamine & arginine; ↓ in CD3+, CD4+ CD8+ & NK cells at day 1 & ↑ compared to baseline & compared to control at day 8	(Wu, et al. 2001)
Gastrointestinal (II-III)	204 73F/131M	Age= 56.3±10.1/ 58.2±11.0 Body Weight= 64.2±10.1/64.7±10.0 BMI= 22.8±2.6/ 23.1±3.1	Surgery	N-3: 0.2 g/kg body weight parenteral CNT = PN	8 days	N-3: ↓ in CD8 & NS ↓ in IL-6 & TNF compared to control at day 8	(Jiang et al. 2010)

Pancreatic (Advanced)	50 (20F/30M)	Age=68 (40-83)	Gemcitabine	N-3: up to 500 ml (4.3-8.6g of EPA+DHA) 1/wk CNT =N/A	Up to 6 cycles (24 weeks)	N-3: ↑ in perceived QOL; 10% ↑ in global health in 47% of patients	(Arshad et al. 2014)

Abbreviations used: ALT, alanine aminotransferase; AST, aspartate transaminase; BMI, body mass index; CD, cluster of differentiation; CNT, control; CRP, C-reactive protein; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; EN, standard enteral nutrition; F, female; g, gram(s); IG, immunoglobulin; IGF1, insulin-like growth factor 1; IL, interleukin; Int, intervention; kcal, kilocalorie; kg, kilogram; LPS, lipopolysaccharide; M, male; ml, millilitre; N, number; N/A, not applicable; NC, no change, NK, natural killer; NS, non-significant; PGE2, prostaglandin E2; PGF1a, prostaglandin F1a; PHA, phytohaemagglutinin; PMN, polymorphonuclear leukocytes; PN, standard parenteral nutrition; QOL, quality of life; Ref, reference; TCR, T-cell receptor; TNFα, tumor necrosis factor; VEGF, vascular endothelial growth factor.

1.7.1 DHA and Cancer –Clinical Evidence: N-3 type, amount and intervention length

Across the spectrum of studies, supplementation varied greatly in concentrations and intervention length. When the supplement was provided in an oral capsule form (**Tables 1-6** and **1-7**), daily supplementation ranged from 300 mg to 5 grams (g) of n-3 fatty acids with 72% of the studies providing 2 grams or less per day. DHA concentrations ranged from 0 to 2 grams with 91% of the studies providing 1 gram or less per day. When capsules were provided concomitant with chemotherapy the intervention length was 6-24 weeks and equivalent to the duration of chemotherapy (Bougnoux et al. 2009, Ghoreishi et al. 2012, Hershman et al. 2015, Shen et al. 2018, Darwito et al. 2019, Mansara et al. 2015, Bonatto et al. 2012, Mocellin et al. 2013, de Aguiar et al. 2012, Camargo et al. 2016, Chagas et al. 2017, Murphy et al. 2011, Finocchiaro et al. 2012, Lu et al. 2018, Maschio et al. 2018). In the absence of chemotherapy, supplementation was provided for 1- 6 months (da Silva Paixão et al. 2017, Martínez et al. 2019, Gogos et al. 1998, Purasiri et al. 1994, Cockbain et al. 2014, Cerchietti et al. 2007, Wigmore et al. 2000). Oral supplementation interventions were one week to 6 months in length, with over half of the studies ranging from 4-6 weeks in duration. Ten of the studies provided similar doses of EPA and DHA (2.2 g and 0.9 g per day respectively) with a range of 2 - 3.6 grams total n-3 per day (1.1 - 2.4 grams EPA± 0.9 - 1.2 g DHA) and in one study 4.9 grams of EPA + 3.2 grams of DHA per day (**Table 1-8**). The amount of n-3 fatty acids provided by enteral / parenteral nutrition was variably reported in the assessed studies; in some instances, 0.2 g n-3/ kg of body weight and in others from 2.2-3.3 g /per day (**Table 1-9**). In these studies, the interventions were acute, generally limited to before and after surgery and were approximately 5-9 days long.

1.7.2 DHA and Cancer –Clinical Evidence: Outcomes

Reported outcomes vary across the studies but are grouped below into the following categories: (1) weight gain or maintenance, (2) serious adverse events including neuropathy and length of hospital stay, (3) immunological measures, (4) quality of life, (5) overall survival or progression free survival and (6) additional parameters.

1.7.2.1 Weight

No breast cancer n-3 supplementation studies reported this metric, yet weight loss is a common side effect of other cancer sites and cancer therapies. Subsequently it has been reported on in many studies investigating the efficacy of n-3 supplementation particularly in advanced or palliative cancers and ones with defined pre-study weight loss. In general, oral liquid supplementation (which also included protein), was provided in instances where there was substantial weight loss prior to study entry and some evidence of malnutrition or cachexia. In the n-3 supplemented groups, there was weight gain in colorectal and pancreatic cancer studies (Trabal et al. 2010, Bauer and Capra 2005, Barber et al. 1999, Barber et al. 1999, Barber et al. 2001) or weight maintenance in head and neck, gastrointestinal and non-small cell lung cancer studies (Solís-Martínez et al. 2018, Persson et al. 2005, van der Meij et al. 2012).

Capsule supplementation has been employed in three studies with palliative patients. In advanced lung cancer patients with systemic immune metabolic syndrome (SIMS -defined by presence of cachexia, anorexia, ECOG > 2 and high CRP), the combination of 600 mg EPA+DHA and celecoxib (NSAID) increased body weight by approximately 1.2 kg compared to the n-3 capsules alone over a 6-week intervention (Cerchietti, et al. 2007). Pancreatic cancer patients who at study entry had lost approximately 13% body weight, stabilized their weight and began to increase it by week 4 with EPA supplementation and the authors suggest that EPA could be a safe, effective anti-cachectic agent that could result in weight gain (Wigmore et al.

2000). Finally, in a low dose EPA+DHA (300 mg total per day) study of palliative pancreatic cancer patients, where two different sources were provided (fish oil or marine phospholipid capsules), similar effects were observed between the 2 groups suggesting the efficacy of n-3s in weight stabilization (Werner et al. 2017).

Three studies reported weight gain when n-3 fatty acids capsules were provided with neoadjuvant chemotherapy. In advanced lung cancer patients supplemented with 3.4 grams of EPA+DHA concomitant with gemcitabine and cisplatin chemotherapy, body weight increased by 3.4 kg over the 66-day intervention (Finocchiaro et al. 2012). Gastrointestinal patients supplemented with 700 mg of EPA+DHA for 8 weeks with 5-fluorouracil and leucovorin chemotherapy gained an average of 1.7 kg over 8 weeks and although not significant to their baseline status, was significantly different from control patients who lost 2.5 kg average during this time frame (Bonatto et al. 2012). In colorectal cancer patients treated with xeloda, oxaliplatin, 5- fluorouracil and / or leucovorin therapy it was observed that 600 mg of EPA+DHA potentially prevented weight loss over 9 weeks when compared to standard of care control (Mocellin et al. 2013).

1.7.2.2 Serious adverse events

No studies reported serious adverse events attributable to n-3 supplementation, although in two instances with surgical patients for gastric and gastrointestinal cancers found improvement in overall postoperative recovery (Wei et al. 2014) and length of hospital stay (Jiang et al. 2010) respectively with parenteral nutrition. Lung cancer patients supplemented with up to 2.7 grams of EPA+DHA were observed to have a better chemotherapy response rate during neoadjuvant therapy (Murphy et al. 2011). Common side effects of cytotoxic therapies used in breast cancer treatment include febrile neutropenia and neuropathy (Ho and Mackey 2014) ad

two studies observed a beneficial effect of supplementation on their side effects during neoadjuvant chemotherapy. Metastatic patients supplemented with 1.8 grams of DHA per day had decreased neutropenia, anaemia & thrombopenia during cytotoxic chemotherapy (Bougnoux et al. 2009). Additionally, when women were supplemented with 1.2 grams of EPA+DHA, Ghoreishi et al. observed a 70% decreased risk of peripheral neuropathy incidence over 4 cycles of paclitaxel treatment (Ghoreishi et al. 2012). These beneficial effects were also observed in palliative esophageal cancer patients, where EPA + DHA supplementation resulted in decreased nausea, thromboembolism, leucopenia, and neutropenia (Chagas et al. 2017).

1.7.2.3 Immunological outcomes

Enteral or parenteral nutrition was commonly employed for surgical interventions, resulting in elevated inflammation. All studies assessed in this review reported immunological modulations resulting from n-3 fatty acid enrichment of the enteral / parenteral nutrition. This included improved immune cell response (Chen et al. 2005, Ryan et al. 2009, Braga et al. 2002, Liang et al. 2008), decreased interleukin (IL)-8 (Ryan et al. 2009, Aiko et al. 2005), IL-6 (Ryan et al. 2009), and IL-6 (at time points 8-21 days post-surgery compared to control) (Gianotti et al. 1999, Wu et al. 2001, Chen et al. 2005, Wei et al, 2014, Braga et al. 2002, Liang et al. 2008, Zhu et al. 2012, Ryan et al. 2009), modulation of functional capacity and gene expression of immune markers (Talvas et al. 2015), increase in T-lymphocytes, T helper and natural killer (NK) cells (Wu et al. 2001), modulated cytokine production (Gianotti et al. 1999, Aiko et al. 2005), decrease in prostaglandin E2 (PGE2) (Swails et al. 1997) and reduced incidence of systemic inflammatory response syndrome (Jiang et al. 2010, Zhu et al. 2012). C-reactive protein (CRP), a marker of inflammation that is often used as an indicator of poor prognosis, was routinely

elevated post-surgery but found to decrease during n-3 supplementation in the days following (Wu et al. 2001, Ryan et al. 2009).

Chemotherapy alters the immune response and inflammatory status yet evidence of beneficial immunomodulation with n-3 supplementation in non-surgical settings is limited. CRP was the most frequently assessed marker of inflammation, most often in advanced or palliative cancer patients, where it was observed that capsule/oral n-3 supplementation decreased CRP in head and neck (Solís-Martínez et al. 2018), lung (Pastore et al. 2014, Cerchietti et al. 2007, Van Der Meij et al. 2012), gastrointestinal (Pastore et al. 2014, Shirai et al. 2017), or pancreatic (Shirai et al. 2017) cancers or maintained CRP levels during n-3 supplementation in breast (da Silva Paixão et al. 2017, Martínez et al. 2019), lung (Finocchiaro et al. 2012) or pancreatic cancer (Wigmore et al. 2000) compared to either baseline levels or increased CRP in non-supplemented controls. Additionally, the CRP / albumin ratio believed to be a predictor of overall survival (Zhou et al. 2015) and was decreased during n-3 supplementation (Mocellin et al. 2013, de Aguiar et al. 2012, Chagas et al. 2017). Other markers reported to be beneficially decreased during n-3 supplementation include IL-6 (Finocchiaro et al. 2012), PGE2 (Faber et al. 2013), tumour necrosis factor alpha (TNFα) (Solís-Martínez et al. 2018, Sánchez-Lara et al. 2014) and interferon gamma (IFNγ) (Gómez-Candela et al. 2011, Solís-Martínez et al. 2018, Martínez et al. 2019). Purasiri et al., assessed both localized colorectal cancer patients (Purasiri et al. 1994). In the patients with localized cancer, supplementation with 1-gram EPA+ 160 mg DHA per day, short term until surgery, had no observed changes in immune parameters. However, in the advanced patients, where the amount of EPA+ DHA increased to 2.1 g EPA and 320 mg DHA daily for months 2 -6, a decrease in IL1β, IL-4, IL-6, TNFα, IFNγ were observed. Interestingly, no changes in cytokine levels occurred in the first 2 months, and the authors

suggest that long term supplementation results in significant decrease in circulating cytokines (Purasiri et al. 1994).

Additionally, neutrophil function during chemotherapy improved (Bonatto et al. 2012), cluster of differentiation (CD)4/CD8 ratio increased (Gogos et al. 1998) and CD4+, CD8+, PGE2 and IL-6 levels were maintained compared to elevation in controls (da Silva Paixão et al. 2017).

1.7.2.4 Quality of life

Changes in life quality have been frequently reported in clinical trial outcomes and often studies have employed the validated questionnaire from the European Organisation for Research and Treatment of Cancer-Quality of Life Questionnaire-C30 (Aaronson et al. 1993). In capsule or oral n-3 supplementation studies, improved perceived quality of life was reported in three instances (Trabal et al. 2010, Sánchez-Lara et al. 2014, Bauer and Capra 2005) and improved appetite reported twice; in colorectal and non-small cell lung cancer studies (Trabal et al. 2010, Sánchez-Lara et al. 2014). Musculoskeletal pain is a well-documented side effect of aromatase inhibitors (Henry et al. 2010). In women with previous chemotherapy or radiation and currently on aromatase inhibitors for breast cancer therapy, 1.4 g of n-3 per day resulted in a 21.5% decrease in pain scale after 30 days (Martínez et al. 2019). However, a second study in breast cancer patients on aromatase inhibitors found that supplementation with 3.3 grams of EPA+DHA for 24 weeks only decreased pain significantly in obese patients (Hershman et al. 2015, Shen et al. 2018).

1.7.2.5 Survival

Overall survival, progression free survival and disease-free survival are key metrics reported in clinical trials. In the current review of the literature, studies investigating the benefits

of n-3 supplementation via enteral / parenteral nutrition did not report on these metrics, likely due to the acute time frame of the interventions. However, a clinical benefit was observed in other studies. Stage III / IV lung cancer patients who received 1.1 g of EPA in a protein enriched oral supplement were reported to have a trend towards progression free survival (P<0.07) (Sánchez-Lara et al. 2014). In metastatic patients with a variety of cancers, high doses of n-3 (5 g EPA + DHA combo) increased survival (P < 0.02), which was further increased if stratified between well-nourished and malnourished patients (P<0.001) suggesting that malnutrition could be a predictor that affects n-3 supplementation prolonging survival (Gogos et al. 1998). Pancreatic patients generally have a median survival of 4.1 months, yet in a study by Wigmore et al. EPA supplementation (increasing doses up to a max 6 g per day for 12 weeks) increased the median survival =6.8 months (Wigmore et al. 2000). In a metastatic breast cancer study where all patients were DHA supplemented, stratification by amount of DHA incorporated into plasma showed that higher DHA incorporation was associated with longer time to progression (8.7 months vs. 3.5 months) and overall survival increased from 18 to 34 months (Bougnoux et al. 2009). One gram of n-3 per day for 51 days increased overall survival (30.9±3.7 versus 25.9±3.6 weeks, P=0.05; HR=0.41, 95% CI: 0.20-0.84) and disease-free survival increased (28.5±3.3 versus 23.7±3.6, P=0.03; HR=0.44, 95% CI: 0.22-0.87) in stage IIIB breast cancer patients (Darwito et al. 2019). Supplementation with 600 mg EPA+DHA increased time to progression in colorectal patients (20 months vs. 11 months for non-supplemented controls) (Camargo et al. 2016) and overall long-term survival (at 465 days) compared to control in leukemia / lymphoma patients (Chagas et al. 2017).

1.7.2.6 Additional parameters

Two of the included studies assessed the Ki67 proliferation index. Ki67 is a clinically relevant measure of efficacy in many clinical trials as it is expressed throughout the cell cycle (G_1, S, G_2 and M phases, but not in G_0) (Dowsett et al. 2011, Gerdes et al. 1984, Scholzen and Johannes 2000). Darwito et al. found decreased Ki67 expression in breast cancer patients receiving 1 g per day of n-3 supplement (39.2±5.3 versus 42.4±4.8, P=0.03) (Darwito et al. 2019), while there were no observed changes in the Ki67 proliferation index in patients with colorectal cancer and liver metastases when supplemented with 2 grams of EPA per day (Cockbain et al. 2014). Other experimentally relevant markers have been assessed in supplementation studies that suggest efficacy of n-3 fatty acids in modulating cancer outcomes including decreased oxidative status (Finocchiaro et al. 2012), increased glutathione reductase and plasma antioxidant status (Mansara et al. 2015), superoxide dismutase (Mansara et al. 2015, Bonatto et al. 2012) and phagocytosis and H_2O_2 (Bonatto et al. 2012) in plasma or peripheral mononuclear cells and vascular endothelial growth factor (VEGF) (29.5±5.4 versus 32.7±5.2, *P*=0.04) (Darwito et al. 2019), and carcinoembryonic antigen (Camargo et al. 2016) in tumours.

Collectively, these studies *in vitro*, *in vivo* and in humans suggest that n-3 fatty acids including EPA and DHA have anti-tumoural effects and this occurs through a variety of pathways including apoptosis, cell proliferation, cell cycle progression and immunomodulation. However, there are few studies that investigate the independent effects of DHA and the translation of work from animal models to humans is not clear. Further understanding of the role of DHA as a neoadjuvant therapy in breast cancer treatment is needed.

CHAPTER 2: Research plan

2.1 Rationale

Breast cancer is the most frequently diagnosed cancer and the second leading cause of cancer related death in Canadian women (Brenner et al. 2020). An estimated 27,400 women will be diagnosed with breast cancer in 2020 and it is projected that 5,100 will die (Brenner et al. 2020), yet targeted nutritional guidelines for prevention or treatment have not been established. Higher consumption of fish or fish oil has been associated with lower incidences of breast cancer (Albuquerque et al. 2014, Kuriki et al. 2007, Zheng et al. 2013). The relationship between individual fatty acids found in fish or fish oil, including docosahexaenoic acid (DHA), and breast cancer risk are unclear. An assessment of plasma fatty acid composition in women with or without breast cancer in a Canadian population has not been examined.

DHA has been shown to reduce growth and increase death of breast cancer cells in preclinical models (reviewed in (D'Eliseo and Velotti 2016, Liu and Ma 2014)) and enhance the anticancer actions of chemotherapy (Barascu et al. 2006, Ewaschuk et al. 2012, Germain et al. 1998, Kang et al. 2010). Many chemotherapeutics act by specifically targeting the tumour cell membrane (Hanahan and Weinberg 2000, Hanahan and Weinberg 2011). Uptake of DHA into cell membrane is well established (Plourde et al. 2011, Stillwell and Wassall 2003, Turk and Chapkin 2013, Wassall and Stillwell 2009), therefore it has been hypothesized that these membrane changes could play a critical role in increasing tumoural death (Escribá et al. 2008). Indeed, apoptosis and cellular proliferation are two key pathways that originate at the cell membrane and have been identified as contributing to the efficacy of DHA treatment (D'Eliseo and Velotti 2016, Liu et al. 2018). However, the membrane changes that occur within breast cancer cells treated with DHA in conjunction with chemotherapy have not been examined and

the mechanisms of action of DHA combined with chemotherapy have not been clearly elucidated. Additionally, breast cancer is a heterogeneous disease that differs both in etiology among patients and within the tumour microenvironment (inter- and intra-tumoural variation) (Turashvili and Brogi 2017). These dynamic, complex environments are not adequately represented by immortalized breast cancer cell models. Patient derived xenograft (PDX) models have recently emerged and better recapitulate the heterogenicity of breast cancer (Dobrolecki et al. 2016, Zhang and Lewis 2013). However, the role of n-3 fatty acids, specifically DHA, on PDXs has not been established.

Finally, the role of oral DHA in a clinical setting is unclear. It has been shown to improve outcomes in a small group of metastatic breast cancer patients (Bougnoux et al. 2009), yet the efficacy of DHA has not been determined in a randomized controlled trial of newly diagnosed breast cancer patents.

2.2 Objectives and hypotheses

The overall objective of this research was to determine the efficacy of DHA in prevention and treatment of breast cancer. To address this overarching objective, three specific and several sub-objectives and hypotheses were developed as follows:

1. The first objective of this research was to determine the relationship between fatty acid status in plasma phospholipids and breast cancer risk in a nested-case control study of Canadian women.
2. The second objective was to establish the efficacy and mechanisms for how pre-treatment of breast cancer cells with DHA improves the action of chemotherapy. This was further divided into sub-objectives and hypotheses:

a. The first sub-objective of objective 2 was to establish DHA incorporation into two phenotypically distinct immortalized breast cancer cell lines: MDA-MB-231 and MCF-7 cells, when provided prior to doxorubicin (DOX) chemotherapy in an *in vitro* model. We hypothesized that DHA would improve efficacy of DOX through incorporation into membrane phospholipids and lipid rafts and that this would occur in both cell lines. We further hypothesized that *in vivo* incorporation of DHA into MDA-MB-231 tumours would be representative of results observed *in vitro*.
b. The second sub-objective of objective 2 was to establish the efficacy and mechanisms for how pre-treatment with DHA improves the action of doxorubicin chemotherapy *in vitro* MDA-MB-231 cells. We hypothesized that treating MDA-MB-231 cells with DHA prior to DOX would enhance the anti-cancer actions of DOX through an increase in apoptosis and cell cycle arrest.
c. The third sub-objective of objective 2 was to confirm that feeding DHA improves the action of doxorubicin chemotherapy *in vivo nu/nu* mice implanted with MDA-MB-231 cells. We hypothesized that feeding a diet enriched with DHA to tumour bearing mice would reduce tumour growth and that this would occur through increased apoptosis and decreased cell cycle progression.
d. The fourth sub-objective of objective 2 was to examine how feeding DHA in a patient derived xenograft (PDX) model of triple negative breast cancer (TNBC) improves the efficacy of docetaxel (TXT) chemotherapy. We hypothesized that feeding a diet enriched with DHA to PDX tumour bearing mice would reduce tumour growth and that this would occur through increased apoptosis, decreased cell cycle progression and decreased proliferation.

e. The fifth sub-objective of objective 2 was to examine how different doses and sources of dietary DHA improve the efficacy of TXT chemotherapy in a PDX model of TNBC. We hypothesized that a dose effect would be observed, resulting in different levels of anti-tumoural response in PDX tumour bearing mice and that this would occur through changes in tumoural phospholipid DHA content.

3. The final objective of this thesis was to determine efficacy of supplemental DHA provided with neoadjuvant chemotherapy in women with breast cancer measured by changes in Ki67 index from biopsy to surgical excision. We hypothesized that supplementing DHA during chemotherapy would decrease tumour proliferation (Ki67) and improve patient outcomes as measured by improved immune response and a decrease in chemotherapy associated side-effects and progressive disease.

2.3 Chapter format

The above objectives and hypotheses were assessed in a series of different studies using cohort data, several pre-clinical animal models and a human randomized controlled trial. These studies are organized into thesis chapters, which have been submitted and / or accepted for publication as individual manuscripts.

Chapter 3 reports the risk of breast cancer in a nested case-control study within Alberta's Tomorrow Project (ATP) and British Columbia Generations Project (BCGP) and demonstrates regional variations in fatty acid status in a Canadian population. The relationships between fatty acid status and menopausal status or central adiposity were examined. Objective 1 was addressed in this chapter.

Chapter 4 reports the changes in phospholipid (PL) classes in MDA-MB-231 and MCF-7 breast cancer cells, when treated with DHA prior to DOX chemotherapy. We examined the

changes that occurred both in whole cell and lipid raft membrane PL classes and assessed these membrane changes in MDA-MB-231 tumours from *nu/nu* mice fed DHA in conjunction with DOX chemotherapy. Objective 2 and sub-objective 2(a) were addressed in this chapter.

Chapter 5 examines the efficacy and mechanisms for how pre-treatment with DHA improves the action of DOX chemotherapy in MDA-MB-231 cells *in vitro* and implanted into *nu/nu* mice. Two key pathways were examined: apoptosis and cell cycle. This is the first study to look at changes in gene expression resulting from DHA treatment in breast cancer cells. Objective 2 and sub-objectives 2(b) and 2(c) were addressed in this chapter.

Chapter 6 confirms the efficacy of DHA reported in Chapters 4 and 5 in an improved preclinical PDX model of TNBC. We examined apoptosis, cell cycle progression and proliferation as potential mechanisms that were involved in improved efficacy of DHA. This is the first study to report on the effects of dietary DHA in a PDX model. Objective 2 and sub-objective 2(d) were addressed in this chapter.

Chapter 7 examines the potential of lower doses of DHA from a plant oil source to improve chemotherapy efficacy and confirms phospholipid uptake of DHA reported in Chapter 4 in the preclinical PDX model of TNBC. We further examined the mechanism of necroptosis for its contribution to the anti-tumoural effects of DHA. This is the first study to report on phospholipid changes in a PDX model and necroptosis as a possible mechanism in DHA fed tumour bearing animals. Objective 2 and sub-objective 2(e) were addressed in this chapter.

Chapter 8 reports the protocol for the randomized controlled trial to determine efficacy of supplemental DHA provided with neoadjuvant chemotherapy in women with breast cancer. The baseline status of women enrolled in DHA WIN is presented. Objective 3 was addressed in this chapter.

Chapter 9 summarizes the results generated from this thesis and discusses the implications of DHA in breast cancer therapy and future research directions.

CHAPTER 3: A prospective analysis of plasma phospholipid fatty acids and breast cancer risk in two provinces in Canada[2]

3.1 Introduction

Globally, breast cancer is the most frequently diagnosed female cancer and the leading cause of cancer related death in women (Bray et al. 2018). Preventable dietary and lifestyle factors and are estimated to contribute to ~8% of all cancers (Brenner et al. 2012) yet epidemiological evidence linking dietary fat intake to breast cancer risk is inconsistent (MacLean et al. 2006, Murff et al. 2011, Goodstine et al. 2003, Brasky et al. 2010, Wakai et al. 2005, Gago-Dominguez et al. 2003, Tayyem et al. 2019, Thiebaut et al. 2009) and does not address complete fatty acid status. Thus, measurement of plasma phospholipids could be a more reliable biomarker and indicator of fatty acid status than dietary assessment (Patel et al. 2010). As phospholipids are primarily synthesized within the body, they provide a quantitative measurement of the composition of fats that are bioavailable and overcomes the potential limitations of food frequency and dietary assessment questionnaires. Plasma phospholipid analysis is a reasonable measure of long-chain polyunsaturated fatty acids (PUFA). They are reflective of the sum of processes including dietary intake, fatty acid synthesis and utilization. Dietary intake studies have suggested that long chain n-3 PUFA, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), could be protective, although the data are conflicting (MacLean et al. 2006, Zheng et al. 2013). Fatty acids are integral components of cellular processes involved in

[2] A version of this chapter has been accepted for publication: Newell M, Ghosh S, Goruk S, Pakseresht M, Vena JE, Dummer TJB, Field CJ. (2021) A prospective analysis of plasma phospholipid fatty acids and breast cancer risk in two provinces in Canada. Current Developments in Nutrition DOI: 10.1093/cdn/nzab022.

many cancer hallmarks including cellular proliferation, apoptosis, cell growth and metastasis (Wymann et al. 2008, Larsson et al. 2004). In *in vitro* and *in vivo* models, EPA and DHA have demonstrated an anticancer effect (D'Eliseo and Velotti 2016) therefore it is plausible that they could have a role in prevention as well.

To date, several epidemiological studies have explored circulating fatty acids as biomarkers of breast cancer risk with conflicting findings (Vatten et al. 1993, Simonsen et al. 1998, Chajes et al. 1999, Klein et al. 2000, Pala et al. 2001, Bagga et al. 2002, Saadatian-Elahi et al. 2002, Maillard et al. 2002, Rissanen et al. 2003, Wirfalt et al. 2004, Shannon et al. 2007, Chajes et al. 2008, Witt et al. 2009, Takata et al. 2009, Schmidt et al. 2014, Qin et al. 2014, Hidaka et al. 2015, Conceicao et al. 2016, Bassett et al. 2016, Nagata et al. 2017, Chajes et al. 2017, Hirko et al. 2018) but most have not considered geographic variations in dietary intake, body fatness or the influence of menopause on fatty acid composition. In this unique nested case-control study from a Canadian population, we studied two regionally distinct populations – the Alberta's Tomorrow Project (ATP) and British Columbia Generations Project (BCGP) cohorts – to examine the associations of fatty acid status with breast cancer risk including location, menopausal status and waist-to-hip ratio as key variables.

3.2 Methods

3.2.1 Study population

ATP and BCGP are prospective cohort studies and part of the larger CanPath (Canadian Partnership for Tomorrow's Health, country wide prospective cohort), created to investigate lifestyle, diet, environmental and genetic influences on risk of chronic diseases including cancer (Dummer et al. 2018). Detailed methodologies for study design and recruitment for both cohorts have been previously published (Dhalla et al. 2018, Robson et al. 2016). In brief, participants in

both cohorts were between the ages of 35-69, provided a health and lifestyle questionnaire, physical measurements, biosamples and consented to data linkage (including cancer registry). A total of 31 072 participants in ATP and 29 796 participants in BCGP were recruited between the years 2001-2015 (ATP) and 2009-2016 (BCGP). Non-fasted blood samples were collected pre-diagnosis therefore not subjected to any potential biases data collection following standard protocols, separated into blood components (red blood cells, serum, plasma and buffy coat) and stored either in liquid nitrogen or in a -80°C freezer (Ye et al. 2016, Dhalla et al. 2018). Ethical approval for the ATP for recruitment and data collection was obtained by the former Alberta Cancer Board Research Ethics Committee and the University of Calgary Conjoint Health Research Ethics Board. Ethical approval for the BCGP for data collection and recruitment was obtained by the University of British Columbia -British Columbia Cancer Agency Research Ethics Board.

3.2.2 Nested Cohort

Breast cancer cases in women that occurred from blood sample collection (respective study inclusion date) to December 31, 2019 were identified through linkage to the Alberta Cancer Registry and the British Columbia Cancer Registry. From these two cohorts, 393 females with a breast cancer diagnosis and age-matched control women (N=786, matched 2:1 with cancer cases) with no cancer (as of December 31, 2019) were identified. The current analysis includes 614 women from the ATP (203 cases and 411 controls) and 514 women from the BCGP (174 cases and 340 controls). Fifty-one samples were excluded from the analysis due to insufficient sample for phospholipid analysis or sample degradation prior to arrival in the lab. A flow diagram of final sample selection for fatty acid analysis is provided in **Appendix Figure 1**. Descriptive information on breast cancer subsets and hormone receptor status of the included

population is provided in **Appendix Tables 1** and **2**. Ethics approval for the current study was approved by the Health Research Ethics Board of Alberta Cancer Committee (HREBA.CC-17-0344).

3.2.3 Dietary Intake

In ATP, dietary intake over one year was estimated using the validated Canadian Diet History Questionnaire as described earlier (Robson et al. 2016). Briefly, this questionnaire was modified from the United States National Cancer Institute's Diet History Questionnaire that contains intake questions on 124 food items and portion sizes. Estimated nutrient intake was determined using the Canadian Nutrient File (Csizmadi et al. 2007). Dietary intake and supplement use from this food frequency questionnaire was available for 256 subjects from ATP (70 cases and 186 controls) and was used to determine if the plasma phospholipid n-3 status related to estimated dietary intake. Unfortunately, there was no dietary data collected for the BCGP.

3.2.4 Plasma phospholipid analysis

Cases and controls from both cohorts were processed within the same batch and laboratory personnel were blinded to participant information. To determine fatty acid concentration, 10 µg of C15 phosphatidylcholine internal standard (Nuchek Prep Inc) was added to 200 µl of plasma and phospholipids were extracted using a Folch method as previously described (Folch et al. 1957, Field et al. 1988). Briefly, lipids were extracted from the plasma sample and total phospholipids were separated by spotting the samples on a heat-activated silica gel 'G' thin layer chromatography (TLC) plate (Analtech, Newark, DE, USA) and developing plate in chamber with solvent containing 80:20:1 petroleum ether: diethyl ether: acetic acid. Methyl ester bands were prepared by a mixture of BF_3 and hexane at 100 ºC. Total

phospholipid fatty acids were separated by automated GLC 7890A (Agilent Technologies, Mississauga, ON, Canada) on a CP-Sil 88 column (100 m x 0.25 mm, Agilent) (Cruz-Hernandez et al. 2013). To control for variations between batches of samples, control measures were employed -in addition to the internal standard (concentration =20 μg/ml), individual GC peaks were identified and validated against phospholipid standards (GLC-502 and GLC-643) from NuChek Prep Inc. (Elysian, MN) which were run each batch to verify retention time and quantification for each individual fatty acid.

3.2.5 Statistical Analyses

Mean and standard deviations were reported for the continuous variables and frequency and proportions were used to describe the categorical variables. To assess characteristics of the study population between cases and controls, independent *t*-tests for continuous variables and chi squared tests for categorical variables were employed. Missing values were excluded from calculations. Odds ratios (OR) and their 95% confidence intervals (CI) were determined using binary logistic regression models to evaluate the association between the outcome variable (cases vs. controls) breast cancer risk and fatty acid status (both relative percent and concentration). Plasma fatty acids were categorized into quartiles together and for individual cohorts based on distribution of plasma levels in control women.

There are known associations between demographic and lifestyle factors with breast cancer and to account for this, multiple covariates, identified *a priori* were adjusted for in the effort to reduce potential confounding. Variables included in the adjusted models were: age, body mass index (BMI, kg/m^2, continuous variable), height (continuous variable), alcohol intake [never, infrequent (≤1-4 / month) and frequent (>2 / week)], combination of age at first birth and parity (nulliparous; first birth before 30 years with 1-2 children; first birth before 30 years with >

3 children; first birth after 30 years); age at menopause; menopausal hormone use (never and ever); family history of breast cancer; education (≤high school, some post-secondary, undergraduate degree or advanced degree); ethnicity (white or non-white) and age at menarche (<11 or >11). Means of fatty acids within each quartile were used to test for trends.

To determine if differences existed for fatty acid status in conjunction with other defined parameters, models were stratified according to BMI (healthy, overweight, obese) or menopausal status at baseline (pre or post), and sub-analyses were conducted. Statistical tests were two sided. SPSS version 25 (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.) was used for all statistical analyses.

3.3 Results

Overall, the majority of anthropometric and demographic characteristics at time of plasma ascertainment were similar between cases and controls when both ATP and BCGP cohorts were considered together with the following exceptions: cases had higher waist-to-hip ratios, higher alcohol consumption, had longer oral contraceptive use, more first-degree relatives with breast cancer and lower estimated mean total physical activity (**Table 3-1,** $P<0.04$). However, anthropometric and demographic characteristics were different between the ATP and BCGP cohorts, including living area, BMI distribution, waist-to-hip ratio, marital status, education, ethnicity, alcohol use, hysterectomy, gravidity, age at first pregnancy, oral contraceptive use, family history of cancer and number of first-degree relatives with breast cancer.

Table 3-1: Descriptive statistics for Alberta's Tomorrow Project and British Columbia Generations Project participants (n=1128)

	n	Breast Cancer (n=377)	Non-cancer (n=751)	Chi-square
Age	1128	(%)	(%)	0.87
35-50		63 (16.7)	131 (17.4)	
50-60		123 (32.6)	234 (31.2)	
60-80		191 (50.6)	386 (51.4)	
Living Area	976			0.63
Rural		58 (17.0)	100 (15.8)	
Urban		284 (83.0)	534 (84.2)	
BMI	1075	358	717	0.90
Underweight (<18.5 kg/m^2)		1 (0.3)	6 (0.8)	
Healthy weight (18.5 to <25 kg/m^2)		150 (41.9)	291 (40.6)	
Overweight (25 to <30 kg/m^2)		105 (29.3)	217 (30.3)	
Obese Class 1 (30 to <35 kg/m^2)		72 (20.1)	137 (19.1)	
Obese Class 2 (35 to <40 kg/m^2)		18 (5.0)	39 (5.4)	
Obese Class 3 (≥40 kg/m^2)		12 (3.4)	27 (3.8)	
Waist-to-hip ratio	1062	353	709	0.03
Below guidelines		151 (42.8)	354 (49.9)	
Above guidelines		202 (57.2)	355 (50.1)	
Marital Status	1124			0.41
Married or cohabitating		261 (69.8)	510 (68.0)	
Divorced, separated, widowed		83 (22.2)	161 (21.5)	
Single, never married		30 (8.2)	79 (10.5)	
Education	1124			0.33
Elementary school		7 (1.9)	15 (2.0)	
High school		87 (23.3)	150 (20.0)	
Trade or vocational school		35 (9.4)	82 (10.9)	
Diploma (community college, pre-university)		97 (25.9)	174 (23.2)	
University certificate below bachelor's level		14 (3.7)	51 (6.8)	
Bachelor's degree		83 (22.2)	176 (23.2)	
Graduate degree		51 (13.6)	104 (13.9)	
Income level	1089			0.94
<$50,000		102 (28.3)	214 (29.4)	
$50,000 to $99,999		136 (37.8)	273 (37.4)	
>$100,000		122(33.9)	242 (33.2)	
Ethnicity -Caucasian	1114			0.48
Yes		345(93.2)	685 (92.1)	
No		25 (6.8)	59 (7.9)	

Smoking status	1121			0.60
Never		202 (54.0)	388 (51.9)	
Former		152 (40.6)	325 (43.5)	
Current		20 (5.3)	34 (4.6)	
Alcohol	1121			0.04
Never		7 (1.9)	39 (5.2)	
≤1/month		119 (31.8)	245 (32.8)	
2-4 / month		82 (21.9)	177 (23.7)	
2-3 /week		80 (21.4)	130 (17.4)	
> 4 /week		86 (23.0)	156 (20.9)	
Menopausal Status	1119			0.71
Pre-menopausal		100 (26.9)	193 (25.8)	
Post-menopausal		272 (73.1)	554 (74.2)	
Age at Menopause	792			0.67
<44 y		60 (22.7)	126 (23.9)	
45 to 49 y		57 (21.6)	100 (18.9)	
≥50 y		147 (55.7)	302 (57.2)	
Mammogram	1121			0.32
Never		19 (5.1)	34 (4.5)	
<6 months		96 (25.8)	216 (28.8)	
6 months to 1 year		132 (35.5)	234 (31.2)	
1 to 2 years ago		88 (23.6)	185 (24.7)	
2 to 3 years ago		16 (4.3)	49 (6.5)	
>3 years ago		21 (5.6)	31 (4.1)	
Hysterectomy	1124			0.42
None		283 (75.9)	586 (78.0)	
Simple		90 (24.1)	165 (22.0)	
Total or bilateral oophorectomy	1115			0.38
Yes		56 (15.1)	98 (13.2)	
No		315 (84.9)	646 (86.8)	
Age at menarche	1090			0.92
<11 years		22 (6.1)	44 (6.0)	
11 years		41 (11.3)	70 (9.6)	
12 years		106 (29.2)	224 (30.8)	
13 years		109 (30.0)	214 (29.4)	
14 or greater		85 (23.4)	175 (24.1)	
Gravidity	1121			0.86
0		59 (15.8)	132 (17.7)	
1		42 (11.2)	80 (10.7)	
2 to 3		192 (51.3)	382 (51.1)	
>3		81 (21.6)	153 (20.5)	
Age at first pregnancy	1120			0.77
Not applicable		59 (15.6)	132 (17.6)	
<21 y		84 (22.4)	159 (21.3)	

21 to 25		86 (23.0)	188 (25.2)	
25 to>30		95 (25.4)	173 (23.2)	
>30 y		50 (13.4)	94 (12.6)	
Months of Lactation	1072			0.44
Not applicable		59 (16.2)	132 (18.7)	
0		67 (18.4)	118 (16.7)	
< 6 months		69 (18.9)	150 (21.2)	
7 to <12 months		52 (14.2)	76 (10.7)	
12 to <18 months		38 (10.4)	83 (11.7)	
>18 months		80 (21.9)	148 (20.9)	
Oral Contraceptive use	787			0.005
Never		37 (12.7)	97 (20.0)	
1-4		67 (23.0)	140 (28.2)	
5-9		75 (25.8)	116 (23.4)	
>10 years		112 (38.5)	143 (28.8)	
Hormone Fertility	1125			0.56
Yes		22 (5.9)	38 (5.1)	
Never		352 (94.1)	713 (94.9)	
Hormone Replacement	1119			0.82
Yes		160 (43.0)	316 (42.3)	
No		212 (57.0)	431 (57.7)	
Yearss of HRT	1085			0.10
Never		212 (58.6)	431 (59.6)	
0 to 4 years		55 (15.2)	138 (19.1)	
5 to 9 y		48 (13.2)	66 (9.1)	
>10 y		47 (13.0)	88 (12.2)	
Self-reported family history of cancer	1113			0.04
Yes		258 (69.9)	455 (65.2)	
No		111 (29.8)	259 (34.5)	
Number of first-degree relatives with BC	1124			0.0003
None		305 (81.6)	669 (89.2)	
≥1		69 (18.4)	81 (10.8)	
Physical Activity	728	282	447	*t* test
Mean total physical activity (MET-min/week)		2896.0±167.0	3526.7±159.4	0.008

(waist to hip ratio below guidelines: <0.85; above guidelines: ≧0.85)

The mean time between sample collection and breast cancer diagnoses for cases was 2.8 ± 0.10 years and there was no difference between cohorts (*P*=0.42). Eighty-five percent of the cases were estrogen receptor positive (ER+), 16% were human epidermal growth factor receptor

2 positive (HER2+) and 9% were triple negative [ER-PR- (progesterone receptor negative) HER2-] (Appendix Tables 1 and 2**)**. Two-sided students' *t*-tests were performed and no differences in fatty acid content based on receptor status of breast cancer cases were observed, therefore this was not used as a co-factor in analyses.

To confirm that plasma is reflective of dietary intake, we first assessed the Spearman's rank correlation coefficients of n-3 LCPUFA between reported dietary intake and plasma phospholipid fatty acid status in a sub-cohort of ATP (**Appendix Table 3**). Weak correlations were observed between plasma relative percent of long chain n-3 fatty acids (r=0.21, $P<0.01$), DHA (r=0.25, $P<0.001$), the combination of EPA+DHA (r=0.24, $P<0.001$) and energy adjusted n-3 fatty acid consumption (grams / 1000 kJ) in this sub-cohort of the study. These correlations remained consistent when assessed between plasma relative percent fatty acid and daily fish consumption, or unadjusted fatty acid consumption (Appendix Table 3).

The mean phospholipid fatty acid content (concentration and relative percent; **Table 3-2**) varied between cohorts, with BCGP participants having 20% higher n-3 polyunsaturated fatty acids (PUFA) fatty acids including eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) with lower saturated fatty acids (SFA) and a lower n-6:n-3 ratio compared to the ATP participants. Regardless of cancer status, we determined 84% of the fatty acids or fatty acid combinations were different between the two cohorts. For this reason, all subsequent analysis was blocked by cohort. A few differences were observed between cases and controls for individual fatty acids (Table 3-2). In ATP, arachidonic acid (ARA) was higher in cases vs. control ($P<0.02$, relative percent and concentration), and total concentration of monounsaturated fatty acids (MUFA), PUFA and total n-6 were also higher in cases versus

control (P<0.04). Cases in BCGP had lower 18:1 c11 (relative percent and concentration) and lower 18:1 n-9 (oleic acid) relative percent compared to controls.

We first assessed the data by running principal component analysis (PCA) to determine if there were clusters of fatty acids that grouped together to predict risk. However, this analysis did not elucidate any clusters that characterized risk. Multivariable odds ratios for breast cancer by quartiles of plasma phospholipid fatty acid were then assessed, with quartile one being the lowest relative percent (**Table 3-3**). Associations of fatty acids were similar in univariate and adjusted models; therefore, we have presented the adjusted results herein. In ATP positive associations between fatty acids and overall breast cancer risk were observed for total long chain n-6, ARA, DHA and the combination of EPA+DHA and a negative association was observed for total SFA and breast cancer risk. In BCGP, a positive association between fatty acids and overall breast cancer risk was observed for the ratio of n-6:n-3 with an observed positive trend in the ratio of ARA:DHA+EPA. Negative associations for 18:1 c11 and total long chain n-3 were also observed.

Breast cancer etiology differs depending on hormonal changes and menopausal status and this could be impacted by nutritional factors including fatty acid composition (World Cancer Research Fund 2018), therefore the data was stratified to examine differences in fatty acid status by menopausal status. Regardless of breast cancer status or cohort, fatty acid status was different between pre- and post-menopausal women (**Appendix Table 4**). While menopausal status did not affect the overall amount (relative percent) of SFA, MUFA and PUFA, the balance of n-6 and n-3 fatty acids within PUFA was different - premenopausal women had lower total n-3 and more total n-6 (yet lower total long chain n-6) than postmenopausal women.

Table 3-2: Plasma phospholipid fatty acid composition in relative percent (%) and concentration (µg/ml) among breast cancer cases and controls

Fatty Acids		Entire cohort			ATP			BCGP			ATP vs. BCGP*
		Cases	Control	*P*	Cases	Control	*P*	Cases	Control	*P*	
Saturates											
Total SFA[1]	%	46.6±0.1	46.8±0.1	0.18	47.3±0.1	47.6±0.1	**0.04**	45.8±0.2	45.8±0.1	0.83	*P*<0.001
	µg/ml	758.3±10.1	743.5±7.0	0.23	818.0±14.4	785.8±10.3	0.07	688.6±11.9	692.4±8.6	0.80	*P*<0.001
14:0 (Myristic acid)	%	0.3±0.0	0.4±0.1	0.57	0.3±0.0	0.3±0.0	0.12	0.4±0.0	0.4±0.0	0.36	*P*<0.001
	µg/ml	5.6±0.1	5.5± 0.1	0.46	5.6±0.2	5.5±0.1	0.70	5.5±0.2	5.4±0.1	0.48	
16:0 (Palmitic acid)	%	28.8±0.1	28.9±0.1	0.44	29.0±0.1	29.3±0.1	0.12	28.5±0.1	28.4±0.1	0.46	*P*<0.001
	µg/ml	467.6±6.1	458.5±4.3	0.22	500.9±8.6	482.6±6.2	0.09	428.6±7.6	429.3±5.5	0.94	*P*<0.001
17:0 (Margric acid)	%	0.4±0.0	0.4±0.0	0.32	0.4±0.0	0.4±0.0	0.24	0.4±0.0	0.4±0.0	0.94	*P*<0.001
	µg/ml	7.0±0.1	6.9±0.1	0.57	7.6±0.1	7.3±0.1	0.28	6.2±0.1	6.3±0.1	0.67	*P*<0.001
18:0 (Stearic acid)	%	15.4±0.1	15.5±0.0	0.62	15.9±0.1	15.8±0.1	0.67	14.9±0.1	15.1±0.1	0.22	*P*<0.001
	µg/ml	252.4±3.8	246.8±2.5	0.21	276.4±5.6	262.4±3.8	**0.04**	224.5±4.2	228.0±2.9	0.48	*P*<0.001
20:0 (Arachidic acid)	%	0.5±0.0	0.5±0.0	0.65	0.5±0.0	0.5±0.0	**0.04**	0.5±0.0	0.5±0.0	0.12	
	µg/ml	8.2±0.1	8.1±0.1	0.54	8.6±0.2	8.5±0.2	0.78	7.7±0.2	7.5±0.1	0.43	*P*<0.001
24:0 (Lignoceric acid)	%	1.1±0.0	1.1±0.0	0.10	1.1±0.0	1.2±0.0	**0.01**	1.1±0.0	1.0±0.0	0.36	*P*<0.001
	µg/ml	17.6±0.4	17.8±0.3	0.70	19.0±0.6	19.5±0.4	0.53	16.1±0.5	15.9±0.3	0.70	*P*<0.001
Monounsaturates											
Total MUFA[2]	%	13.8±0.1	13.9±0.0	0.23	13.9±0.1	13.9±0.1	0.86	13.6±0.1	13.9±0.1	**0.05**	
	µg/ml	224.9±3.3	221.1±2.2	0.33	241.7±4.8	230.1±3.3	**0.04**	205.3±4.0	210.3±2.8	0.31	*P*<0.001
16:1 n-7 (Palmitoleic acid)	%	0.7±0.0	0.7±0.0	0.70	0.7±0.0	0.7±0.0	0.88	0.8±0.0	08±0.0	0.51	*P*<0.001
	µg/ml	11.6±0.2	11.4±0.2	0.38	11.6±0.3	11.2±0.2	0.31	11.7±0.4	11.6±0.3	0.85	
18:1 c11 (Vaccenic acid)	%	0.2±0.0	0.2±0.0	**0.02**	0.2±0.0	0.2±0.0	0.24	0.2±0.0	0.2±0.0	**0.04**	
	µg/ml	3.2±0.1	3.5±0.1	0.06	3.5±0.2	3.7±0.1	0.53	2.8±0.2	3.3±0.2	**0.03**	*P*<0.001
18:1 n-9 (Oleic acid)	%	9.4±0.1	9.5±0.0	0.33	9.5±0.1	9.4±0.1	0.38	9.3±0.1	9.6±0.1	**0.01**	
	µg/ml	154.3±2.4	151.7±1.6	0.36	166.0±3.5	156.8±2.4	**0.03**	140.6±2.9	145.5±2.1	0.18	*P*<0.001
18:1 n-7 (Octadecenoic acid)	%	1.4±0.0	1.4±0.0	0.90	1.4±0.0	14±0.0	0.47	1.3±0.0	1.3±0.0	0.36	*P*<0.001
	µg/ml	22.1±0.4	21.5±0.2	0.19	24.3±0.5	22.9±0.4	**0.02**	19.5±0.4	19.8±0.3	0.42	*P*<0.001
24:1 n-9 (Nervonic acid)	%	2.1±0.0	2.1±0.0	0.94	2.1±0.0	2.1±0.0	0.25	2.0±0.0	2.0±0.0	0.15	*P*<0.001
	µg/ml	33.7±0.7	33.0±0.5	0.41	36.3±1.1	35.4±0.8	0.53	30.7±0.8	30.0±0.6	0.50	*P*<0.001
Polyunsaturates											

Total PUFA[3]	%	39.0±0.1	38.7±0.1	**0.05**	38.3±0.2	37.9±0.1	**0.05**	39.9±0.2	39.7±0.1	0.45	*P*<0.001
	µg/ml	635.5±8.6	618.5±6.3	0.12	664.7±12.5	630.4±9.3	**0.03**	601.3±11.1	604.1±8.2	0.84	*P*<0.001
Total n-6	%	33.2±0.1	32.9±0.1	0.07	32.9±0.2	32.6±0.1	0.07	33.5±0.2	33.3±0.1	0.44	*P*<0.001
	µg/ml	540.2±7.5	524.6±5.3	0.09	571.1±10.8	540.9±7.9	**0.03**	504.1±9.4	504.9±6.7	0.94	*P*<0.001
Total long chain n-6[4]	%	13.8±0.1	13.5±0.1	**0.01**	13.8±0.1	13.5±0.1	**0.03**	13.9±0.2	13.6±0.1	0.18	
	µg/ml	224.9±3.3	215.9±2.4	**0.03**	239.3±4.9	223.3±3.5	**0.01**	208.2±4.1	207.0±3.1	0.82	*P*<0.001
18:2 n-6 (Linoleic acid)	%	19.2±0.1	19.3±0.1	0.82	19.0±0.2	19.0±0.12	0.96	19.5±0.2	19.5±0.2	0.76	*P*<0.001
	µg/ml	313.7±4.9	307.3±3.4	0.28	330.2±7.1	316.2±5.0	0.11	294.4±6.5	296.4±4.4	0.79	*P*<0.001
18:3 n-6 (γ- Linolenic acid)	%	0.1±0.0	0.1±0.0	0.14	0.1±0.0	0.1±0.0	0.21	0.1±0.0	0.1±0.0	0.40	
	µg/ml	1.6±0.1	1.4±0.0	**0.05**	1.5±0.1	1.4±0.0	**0.04**	1.6±0.1	1.5±0.1	0.54	P<0.01
20:2 n-6 (Eicosadienoic acid)	%	0.3±0.0	0.3±0.0	0.20	0.3±0.0	0.3±0.0	0.53	0.3±0.0	0.30±0.0	0.28	*P*<0.001
	µg/ml	4.6±0.1	4.5±0.06	0.14	4.8±0.1	4.5±0.08	0.09	4.5±0.2	4.4±0.1	0.70	
20:3 n-6 (Dihomo-γ-Linolenic acid)	%	4.1±0.0	4.1±0.03	0.78	4.2±0.0	4.1±0.04	0.95	4.0±0.1	4.0±0.0	0.72	P<0.01
	µg/ml	66.7±1.1	65.1±0.8	0.24	72.0±1.6	68.7±1.2	0.11	60.6±1.4	60.8±1.0	0.92	*P*<0.001
20:4 n-6 (ARA)	%	9.0±0.1	8.8±0.1	**0.01**	9.0±0.1	8.6±0.1	**0.02**	9.1±0.1	8.9±0.1	0.18	P<0.01
	µg/ml	146.8±2.4	139.6±1.7	**0.01**	155.4±3.4	143.2±2.4	**0.004**	136.9±3.0	135.4±2.3	0.70	*P*<0.001
22:4 n-6 (Adrenic acid)	%	0.2±0.0	0.2±0.0	0.96	0.2±0.0	0.2±0.0	**0.03**	0.3±0.0	0.3±0.0	0.26	*P*<0.001
	µg/ml	4.0±0.1	3.9±0.1	0.74	4.2±0.1	3.8±0.1	**0.02**	3.7±0.2	4.1±0.1	0.11	
22:5 n-6 (Osbond acid)	%	0.2±0.0	0.2±0.0	0.93	0.2±0.0	0.2±0.0	0.16	0.2±0.0	0.2±0.0	0.14	*P*<0.001
	µg/ml	2.8±0.1	2.7±0.0	0.51	3.0±0.1	3.0±0.1	0.97	2.5±0.1	2.4±0.1	0.27	*P*<0.001
Total n-3	%	5.9±0.1	5.8±0.1	0.89	5.4±0.1	5.3±0.1	0.80	6.4±0.1	6.4±0.1	0.88	*P*<0.001
	µg/ml	95.3±1.8	93.9±1.5	0.56	93.6±2.3	89.5±1.9	0.20	97.2±2.8	99.2±2.2	0.60	*P*<0.001
Total long chain n-3[5]	%	5.4±0.1	5.4±0.1	0.97	5.0±0.1	5.0±0.1	0.80	6.0±0.1	6.0±0.1	0.72	*P*<0.001
	µg/ml	88.7±1.7	87.6±1.4	0.67	87.3±2.2	83.5±1.8	0.21	90.2±2.7	92.7±2.2	0.49	*P*<0.001
18:3 n-3 (α-Linolenic acid)	%	0.4±0.0	0.4±0.0	0.07	0.4±0.0	0.4±0.0	0.99	0.5±0.0	0.4±0.0	**0.03**	P<0.01
	µg/ml	6.6±0.2	6.2±0.1	**0.03**	6.3±0.2	6.1±0.1	0.21	7.0±0.3	6.5±0.2	0.09	P<0.01
20:4 n-3 (Eicosatetraenoic acid)	%	0.6±0.0	0.6±0.0	0.50	0.6±0.0	0.6±0.0	0.31	0.7±0.0	0.6±0.0	0.07	
	µg/ml	10.4±0.2	10.1±0.2	0.23	11.0±0.4	10.7±0.2	0.51	9.8±0.3	9.4±0.2	0.23	*P*<0.001
20:5 n-3 (EPA)	%	1.2±0.0	1.2±0.0	0.47	1.0±0.0	1.1±0.0	0.35	1.3±0.1	1.4±0.0	0.79	*P*<0.001
	µg/ml	18.9±0.6	19.4±0.5	0.58	17.9±0.7	18.1±0.6	0.86	20.1±1.1	20.9±0.9	0.54	*P*<0.001
22:5 n-3 (DPA)	%	0.8±0.0	0.8±0.0	0.57	0.8±0.0	0.7±0.0	0.24	0.8±0.0	0.8±0.0	0.64	*P*<0.001
	µg/ml	12.7±0.2	12.4±0.2	0.26	13.2±0.3	12.4±0.2	0.06	12.2±0.3	12.4±0.2	0.63	
22:6 n-3 (DHA)	%	2.9±0.0	2.8±0.0	0.78	2.6±0.0	2.5±0.0	0.26	3.2±0.1	3.2±0.0	0.46	*P*<0.001
	µg/ml	46.6±1.0	45.8±0.8	0.53	45.2±1.3	42.3±1.0	0.08	48.1±1.4	50.0±1.2	0.34	*P*<0.001
EPA+DHA	%	4.0±0.1	4.0±0.1	0.86	3.6±0.1	3.6±0.1	0.79	4.5±0.1	4.6±0.1	0.57	*P*<0.001

	μg/ml	65.5±1.4	65.1±1.2	0.87	63.2±1.8	60.4±1.5	0.26	68.2±2.3	70.9±1.9	0.39	*P*<0.001
Ratios											
Total n-6:Total n-3		6.1±0.1	6.1±0.1	0.74	6.5±0.1	6.5±0.1	0.63	5.6±0.1	5.6±1.7	0.89	*P*<0.001
ARA:DHA		3.5±0.1	3.4±0.0	0.79	3.7±0.1	3.8±0.1	0.64	3.1±0.1	3.0±0.1	0.25	*P*<0.001
ARA:EPA+DHA		2.5±0.0	2.4±0.0	0.66	2.7±0.1	2.7±0.0	0.79	2.3±0.1	2.2±0.0	**0.04**	*P*<0.001
DI_{16}		0.0±0.0	0.0±0.0	0.63	0.0±0.0	0.0±0.0	0.83	0.0±0.0	0.0±0.0	0.70	*P*<0.001
DI_{18}		0.6±0.0	0.6±0.0	0.81	0.6±0.0	0.6±0.0	0.56	0.6±0.0	0.6±0.0	0.28	*P*<0.001

[1]SFA= 14:0, 16:0, 17:0, 18:0, 20:0, 24:0. [2]MUFA= 16:1 n-7, 18:1 c11, 18:1 n-9, 18:1 n-7, 24:1 n-9. [3]PUFA= 18:2 n-6, 18:3n-6, 20:2 n-6, 20:3 n-6, 20:4 n-6, 22:4 n-6, 22:5 n-6, 18:3 n-3, 20:4 n-3, 20:5 n-3, 22:5 n-3, 22:6 n-3. [4]Total long chain n-6 =20:2 n-6, 20:3 n-6, 20:4 n-6, 22:4 n-6, 22:5 n-6. [5]Total long chain n-3 = 20:4 n-3, 20:5 n-3, 22:5 n-3, 22:6 n-3. Abbreviations: ARA, arachidonic acid; ATP, Alberta's Tomorrow Project; BCGP, British Columbia Generations Project; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; DI_{16}, desaturation index of 16:1:16:0; DI_{18}, desaturation index of 18:1:18:0.

*ATP vs. BCGP (Overall differences between pooled cases and controls from each cohort) as assessed by independent *t*-tests.

Interestingly, only in ATP did the association with breast cancer risk and several fatty acids vary by menopausal status (**Tables 3-4 -ATP** and **3-5-BCGP**). Specifically, positive associations between fatty acids and breast cancer risk were observed in premenopausal women for the desaturation index (ratio of oleic acid: stearic acid, 18:1 n-9:18:0) and total long chain n-3, driven by a positive association of EPA+DHA. A negative association was observed for total SFA, driven by the negative associations observed in 18:0 and 24:0.

In several instances, the second quartile conferred the highest level of risk and could be responsible for the overall positive association. This was observed for total n-3, total long chain n-3, DHA and the combination of EPA+DHA. In postmenopausal women, positive associations were observed for DHA and total long chain n-6 including ARA, while a negative association was observed for 16:0 and 18:3 n-3. Statistical interactions were observed for oleic acid (P_{int}=0.04) and total long chain n-6 fatty acids (P_{int}=0.05) suggesting positive associations in postmenopausal women versus premenopausal women. Conversely, statistical interactions observed for linoleic acid (P_{int}=0.05), ALA (P_{int}=0.03), total n-3 (P_{int}=0.09) and total long chain n-3 (P_{int}=0.07), suggested inverse associations in postmenopausal women compared to premenopausal women. In the BCGP cohort no clear associations or trends were observed in fatty acids when stratified by menopausal status.

Stratification by BMI (18 to <25, 25 to <30 and 30+) did not elucidate any clear patterns of risk (data not shown) however stratifying by waist-to-hip ratio produced associations between some fatty acids and breast cancer risk (**Table 3-6 -ATP** and **3-7-BCGP**). In the ATP cohort, positive associations with breast cancer risk were observed for 16:0 and DHA and negative associations for ALA and the ratio of ARA:DHA when the waist-to-hip ratio was <0.85.

Table 3-3: Multivariate* odds ratios (OR, 95% confidence intervals [CI]) of breast cancer according to quartiles of plasma phospholipid fatty acid relative percent in Alberta's Tomorrow Project British Columbia Generations Project

	OR (95% CI) Quartile							
Cohort	**ATP**				**BCGP**			
Quartile	**Q2vsQ1[a]**	**Q3vsQ1**	**Q4vsQ1**	***P trend***	**Q2vsQ1**	**Q3vsQ1**	**Q4vsQ1**	***P trend***
Saturates								
Fatty Acids Total SFA[1]	0.71 (0.43-1.17)	1.00 (0.61-1.64)	**0.54 (0.32-0.91)**	**0.05**	1.40 (0.78-2.48)	0.63 (0.34-1.18)	1.09 (0.60-1.96)	0.08
14:0 (Myristic acid)	0.71 (0.43-1.18)	0.71 (0.43-1.18)	0.79 (0.48-1.30)	0.49	1.09 (0.60-1.97)	1.55 (0.84-2.84)	1.38 (0.75-2.54)	0.44
16:0 (Palmitic acid)	1.26 (0.76-2.06)	0.86 (0.52-1.43)	0.75 (0.44-1.25)	0.23	1.24 (0.69-2.22)	0.87 (0.47-1.61)	1.37 (0.67-1.90)	0.44
18:0 (Stearic acid)	0.79 (0.47-1.33)	0.72 (0.43-1.21)	0.88 (0.52-1.50)	0.62	0.82 (0.47-1.46)	0.51 (0.28-0.94)	0.74 (0.41-1.34)	0.19
24:0 (Lignoceric acid)	1.11 (0.68-1.80)	0.65 (0.39-1.08)	0.63 (0.38-1.05)	0.06	0.80 (0.44-1.45)	1.45 (0.82-2.59)	0.81 (0.45-1.48)	0.15
Monounsaturates								
Total MUFA[2]	0.98 (0.60-1.62)	0.78 (0.47-1.29)	0.86 (0.52-1.42)	0.74	1.05 (0.59-1.85)	0.81 (0.46-1.43)	0.70 (0.38-1.30)	0.54
16:1 n-7 (Palmitoleic acid)	1.08 (0.66-1.78)	0.90 (0.54-1.51)	0.91 (0.54-1.51)	0.88	0.68 (0.38-1.22)	1.02 (0.56-1.84)	1.28 (0.71-2.30)	0.68
18:1 c11 (Vaccenic acid)	0.96 (0.58-1.57)	**0.54 (0.32-0.92)**	0.86 (0.52-1.41)	0.09	0.66 (0.40-1.10)	0.99 (0.56-1.76)	**0.45 (0.24-0.84)**	**0.04**
18:1 n-9 (Oleic acid)	0.83 (0.50-1.38)	0.97 (0.59-1.61)	0.98 (0.59-1.63)	0.88	1.40 (0.76-2.58)	1.48 (0.80-2.74)	1.11 (0.60-2.05)	0.53
18:1 n-7 (Octadecenoic acid)	0.94 (0.57-1.55)	0.95 (0.57-1.57)	1.23 (0.74-2.04)	0.69	1.40 (0.79-2.49)	1.11 (0.62-1.99)	0.88 (0.48-1.61)	0.46
24:1 n-9 (Nervonic acid)	0.90 (0.55-1.48)	0.76 (0.46-1.26)	0.73 (0.44-1.21)	0.59	1.00 (0.56-1.83)	1.27 (0.70-2.29)	1.23 (0.68-2.25)	0.78
Polyunsaturates								
Total PUFA[3]	0.99 (0.58-1.70)	**1.90 (1.15-3.13)**	1.15 (0.68-1.94)	**0.03**	1.50 (0.82-2.76)	1.29 (0.70-2.40)	1.60 (0.88-2.91)	0.43
Total n-6	1.11 (0.66-1.87)	1.38 (0.82-2.31)	1.46 (0.88-2.42)	0.42	0.74 (0.40-1.36)	1.24 (0.69-2.20)	1.08 (0.60-1.93)	0.40
Total long chain n-6[4]	0.66 (0.39-1.14)	1.17 (0.70-1.96)	1.60 (0.95-2.67)	**0.01**	1.34 (0.74-2.43)	0.92 (0.49-1.70)	1.46 (0.79-2.70)	0.35
18:2 n-6 (Linoleic acid)	0.75 (0.45-1.24)	**0.59 (0.35-0.99)**	0.87 (0.52-1.43)	0.22	1.25 (0.69-2.26)	0.91 (0.50-1.66)	1.42 (0.77-2.61)	0.44
18:3 n-6 (γ- Linolenic acid)	1.49 (0.90-2.48)	0.86 (0.51-1.47)	1.33 (0.79-2.24)	0.14	1.12 (0.61-2.05)	**1.81 (1.01-3.26)**	1.44 (0.78-2.63)	0.19
20:2 n-6 (Eicosadienoic acid)	1.32 (0.79-2.12)	1.00 (0.60-1.69)	1.47 (0.88-2.45)	0.33	1.12 (0.61-2.04)	1.16 (0.64-2.10)	1.07 (0.58-1.95)	0.96
20:3 n-6 (Dihomo-γ- Linolenic acid)	0.88 (0.53-1.47)	0.83 (0.49-1.41)	0.98 (0.58-1.68)	0.87	0.83 (0.45-1.51)	0.84 (0.46-1.54)	1.04 (0.56-1.94)	0.83
20:4 n-6 (ARA)	0.93 (0.55-1.59)	1.35 (0.80-2.26)	**1.67 (0.99-2.81)**	0.09	0.98 (0.54-1.79)	1.32 (0.62-2.06)	1.24 (0.67-2.27)	0.86
Total n-3	**1.94 (1.15-3.26)**	1.51 (0.89-2.57)	1.50 (0.88-2.56)	0.10	1.32 (0.74-2.34)	0.52 (0.28-0.99)	0.94 (0.51-1.75)	**0.03**
Total long chain n-3[5]	**1.92 (1.14-3.23)**	**1.67 (0.99-2.81)**	1.56 (0.92-2.67)	0.09	1.22 (0.69-2.16)	**0.50 (0.27-0.95)**	0.86 (0.46-1.59)	**0.03**
18:3 n-3 (α-Linolenic acid)	**0.52 (0.31-0.87)**	0.97 (0.59-1.59)	0.86 (0.52-1.43)	0.06	1.63 (0.91-2.93)	1.33 (0.72-2.45)	1.57 (0.87-2.86)	0.36
20:4 n-3 (Eicosatetraenoic acid)	0.82 (0.50-1.35)	0.76 (0.46-1.26)	0.80 (0.48-1.32)	0.72	1.08 (0.59-1.96)	1.11 (0.61-2.04)	1.33 (0.74-2.40)	0.80
20:5 n-3 (EPA)	1.09 (0.66-1.82)	1.14 (0.69-1.90)	1.05 (0.63-1.76)	0.96	0.87 (0.48-1.58)	0.89 (0.50-1.61)	0.81 (0.44-1.49)	0.92
22:5 n-3 (DPA)	1.21 (0.72-2.02)	1.04 (0.62-1.74)	1.47 (0.88-2.44)	0.44	0.83 (0.46-1.47)	0.72 (0.40-1.30)	0.66 (0.36-1.21)	0.56
22:6 n-3 (DHA)	**2.70 (1.59-4.58)**	**1.97 (1.15-3.37)**	**1.87 (1.08-3.23)**	**0.003**	1.39 (0.78-2.48)	0.94 (0.52-1.70)	0.76 (0.41-1.42)	0.26
EPA+DHA	**2.35 (1.39-3.98)**	**1.90 (1.12-3.23)**	**1.85 (1.08-3.18)**	**0.01**	1.30 (0.73-2.31)	0.69 (0.37-1.27)	0.88 (0.47-1.62)	0.20

Ratios								
Total n-6:Total n-3	0.94 (0.57-1.55)	0.92 (0.55-1.52)	0.72 (0.43-1.22)	0.64	0.62 (0.33-1.15)	1.45 (0.81-2.59)	1.26 (0.68-2.35)	**0.04**
ARA:DHA	1.25 (0.75-2.08)	1.53 (0.92-2.54)	0.82 (0.48-1.39)	0.09	0.92 (0.50-1.68)	1.13 (0.62-2.08)	1.55 (0.85-2.83)	0.33
ARA:EPA+DHA	1.04 (0.62-1.75)	**1.72 (1.04-2.85)**	0.73 (0.43-1.24)	**0.009**	1.18 (0.65-2.17)	1.25 (0.68-2.30)	**1.99 (1.08-3.69)**	0.14
DI_{16}	0.97 (0.58-1.61)	0.94 (0.57-1.56)	0.93 (0.57-1.52)	0.99	1.20 (0.66-2.20)	1.10 (0.61-1.96)	1.06 (0.58-1.93)	0.94
DI_{18}	1.02 (0.62-1.70)	0.92 (0.55-1.55)	1.21 (0.73-2.02)	0.75	0.96 (0.54-1.71)	0.59 (0.33-1.06)	0.93 (0.52-1.67)	0.28

*Adjusted for: smoking ever, alcohol consumption, hysterectomy ever, BMI, physical activity, age at first pregnancy, age at menopause, age at menarche.
[a]Quartile 1: lowest fatty acid content; reference quartile. Bold typeface indicates quartiles that are statistically significant compared to quartile 1 ($P<0.05$).
[1]SFA= 14:0, 16:0, 17:0, 18:0, 20:0, 24:0. [2]MUFA= 16:1 n-7, 18:1 c11, 18:1 n-9, 18:1 n-7, 24:1 n-9. [3]PUFA= 18:2 n-6, 18:3n-6, 20:2 n-6, 20:3 n-6, 20:4 n-6, 22:4 n-6, 22:5 n-6, 18:3 n-3, 20:4 n-3, 20:5 n-3, 22:5 n-3, 22:6 n-3.[4]Total long chain n-6 =20:2 n-6, 20:3 n-6, 20:4 n-6, 22:4 n-6, 22:5 n-6. [5]Total long chain n-3 = 20:4 n-3, 20:5 n-3, 22:5 n-3, 22:6 n-3. Abbreviations: ARA, arachidonic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; DI_{16}, desaturation index of 16:1:16:0; DI_{18}, desaturation index of 18:1:18:0.

Table 3-4: Multivariate* odds ratios (OR, 95% confidence intervals [CI]) of breast cancer according to quartiles of plasma phospholipid fatty acid relative percent by menopause status in Alberta's Tomorrow Project

Fatty Acids	Premenopausal				Postmenopausal				
	Quartile				Quartile				
	Q2vsQ1[a]	Q3vsQ1	Q4vsQ1	*P trend*	Q2vsQ1[a]	Q3vsQ1	Q4vsQ1	*P trend*	*P int*
	OR (95% CI)				OR (95% CI)				
Saturates									
Total SFA[1]	**0.27 (0.09-0.80)**	0.74 (0.28-1.94)	**0.22 (0.08-0.65)**	**0.02**	0.92 (0.51-1.67)	1.15 (0.64-2.06)	0.72 (0.39-1.33)	0.49	0.54
14:0 (Myristic acid)	0.41 (0.14-1.25)	1.17 (0.44-3.12)	0.73 (0.28-1.93)	0.26	0.79 (0.44-1.42)	**0.54 (0.29-1.00)**	0.81 (0.45-1.46)	0.27	0.14
16:0 (Palmitic acid)	0.72 (0.25-2.08)	0.51 (0.17-1.55)	0.98 (0.39-2.49)	0.57	1.52 (0.86-2.69)	0.99 (0.55-1.76)	0.57 (0.30-1.10)	**0.04**	0.11
18:0 (Stearic acid)	0.49 (0.18-1.33)	**0.33 (0.12-0.87)**	**0.27 (0.08-0.90)**	**0.06**	1.12 (0.59-2.12)	1.10 (0.57-2.13)	1.34 (0.71-2.52)	0.82	0.11
24:0 (Lignoceric acid)	0.57 (0.22-1.48)	0.66 (0.24-1.79)	**0.16 (0.05-0.50)**	**0.02**	1.38 (0.77-2.48)	0.65 (0.35-1.21)	0.95 (0.52-1.73)	0.11	**0.04**
Monounsaturates									
Total MUFA[2]	0.74 (0.24-2.21)	0.58 (0.18-1.84)	1.53 (0.50-4.65)	0.24	1.12 (0.63-1.98)	0.87 (0.49-1.55)	0.63 (0.34-1.14)	0.31	0.07
16:1 n-7 (Palmitoleic acid)	1.05 (0.41-2.68)	1.08 (0.40-2.98)	0.98 (0.36-2.67)	1.00	1.08 (0.59-1.95)	0.86 (0.47-1.56)	0.85 (0.46-1.56)	0.83	0.97
18:1 c11 (Vaccenic acid)	0.57 (0.21-1.54)	0.44 (0.16-1.23)	0.60 (0.22-1.61)	0.46	1.12 (0.62-2.00)	0.57 (0.30-1.06)	0.92 (0.50-1.67)	0.16	0.73
18:1 n-9 (Oleic acid)	0.53(0.18-1.58)	**0.30 (0.10-0.86)**	0.66 (0.25-1.72)	0.15	1.03 (0.57-1.86)	1.56 (0.86-2.84)	1.20 (0.64-2.24)	0.43	**0.04**
18:1 n-7 (Octadecenoic acid)	1.03 (0.38-2.84)	1.05 (0.38-2.92)	1.99 (0.71-5.56)	0.46	0.95 (0.53-1.70)	0.96 (0.53-1.72)	1.07 (0.59-1.93)	0.98	0.73
24:1 n-9 (Nervonic acid)	0.90 (0.34-2.41)	0.65 (0.25-1.68)	0.61 (0.22-1.67)	0.72	0.89 (0.50-1.60)	0.80 (0.44-1.45)	0.76 (0.42-1.39)	0.81	0.97
Polyunsaturates									
Total PUFA[3]	1.07 (0.37-3.11)	2.05 (0.77-5.45)	1.41 (0.48-4.15)	0.44	0.99 (0.53-1.86)	**1.98 (1.09-3.62)**	1.15 (0.63-2.11)	0.07	0.99
Total n-6	1.44 (0.47-4.41)	1.25 (0.44-3.52)	2.13 (0.75-6.05)	0.50	1.03 (0.56-1.88)	1.51 (0.83-2.74)	1.35 (0.75-2.44)	0.46	0.68
Total long chain n-6[4]	**0.38 (0.15-1.01)**	**0.41 (0.16-1.05)**	1.76 (0.51-6.11)	**0.03**	0.91 (0.46-1.80)	**1.95 (1.03-3.71)**	**2.03 (1.10-3.74)**	**0.01**	**0.05**
18:2 n-6 (Linoleic acid)	2.24 (0.73-6.86)	0.74 (0.22-2.51)	1.70 (0.53-5.45)	0.15	**0.50 (0.27-0.91)**	0.60 (0.34-1.06)	0.76 (0.43-1.36)	0.11	**0.05**
18:3 n-6 (γ- Linolenic acid)	1.32 (0.52-3.32)	0.73 (0.27-1.96)	0.48 (0.16-1.43)	0.33	1.66 (0.89-3.11)	0.92 (0.48-1.76)	1.75 (0.94-3.25)	0.08	0.35
20:2 n-6 (Eicosadienoic acid)	0.59 (0.19-1.85)	0.65 (0.23-1.87)	1.39 (0.50-3.82)	0.29	1.60 (0.90-2.87)	1.15 (0.62-2.11)	1.40 (0.76-2.57)	0.40	0.34
20:3 n-6 (Dihomo-γ-Linolenic acid)	0.48 (0.18-1.28)	0.46 (0.16-1.34)	0.49 (0.18-1.34)	0.36	1.14 (0.62-2.10)	1.04 (0.55-1.94)	1.30 (0.68-2.4)9	0.85	0.35
20:4 n-6 (ARA)	1.22 (0.48-3.10)	0.82 (0.31-2.22)	3.07 (0.89-10.60)	0.19	0.78 (0.40-1.52)	1.70 (0.92-3.16)	1.59 (0.88-2.89)	**0.04**	0.09
Total n-3	**5.39 (2.05-14.17)**	2.09 (0.77-5.68)	1.40 (0.47-4.16)	**0.007**	1.25 (0.66-2.37)	1.27 (0.67-2.39)	1.36 (0.73-2.53)	0.81	**0.09**
Total long chain n-3[5]	**5.42 (2.08-14.16)**	1.82 (0.66-5.02)	1.78 (0.58-5.43)	**0.007**	1.20 (0.63-2.28)	1.52 (0.82-2.84)	1.39 (0.74-2.58)	0.58	**0.07**
18:3 n-3 (α-Linolenic acid)	0.79 (0.24-2.55)	1.38 (0.48-3.91)	**3.05 (1.01-9.26)**	0.07	**0.47 (0.26-0.84)**	0.93 (0.52-1.66)	**0.56 (0.30-1.01)**	**0.03**	**0.03**
20:4 n-3 (Eicosatetraenoic acid)	0.78 (0.30-2.01)	0.48 (0.18-1.30)	0.44 (0.16-1.22)	0.33	0.84 (0.46-1.53)	0.88 (0.48-1.60)	0.98 (0.54-1.77)	0.93	0.50
20:5 n-3 (EPA)	1.71 (0.68-4.28)	1.07 (0.40-2.84)	0.98 (0.35-2.79)	0.65	0.92 (0.50-1.72)	1.09 (0.59-2.01)	1.02 (0.56-1.86)	0.96	0.78

22:5 n-3 (DPA)	1.71 (0.61-4.05)	0.82 (0.32-2.07)	1.30 (0.47-3.59)	0.60	1.13 (0.60-2.11)	1.20 (0.64-2.26)	1.59 (0.87-2.91)	0.46	0.66
22:6 n-3 (DHA)	**3.42 (1.26-9.27)**	**2.76 (1.05-7.28)**	2.44 (0.81-7.29)	0.07	**2.58 (1.36-4.89)**	1.75 (0.90-3.38)	1.75 (0.91-3.35)	**0.04**	0.90
EPA+DHA	**5.72 (2.09-15.70)**	2.60 (0.95-7.13)	2.06 (0.65-6.55)	**0.009**	1.74 (0.92-3.3)	1.68 (0.89-3.17)	1.70 (0.91-3.18)	0.28	0.37
Ratios									
Total n-6:Total n-3	1.25 (0.41-3.83)	1.36 (0.46-4.03)	0.69 (0.25-1.91)	0.48	0.88 (0.50-1.56)	0.83 (0.46-1.49)	0.79 (0.42-1.45)	0.88	0.76
ARA:DHA	2.38 (0.85-6.38)	1.17 (0.40-3.44)	0.68 (0.24-1.93)	0.10	1.01 (0.57-1.79)	**1.72 (0.99-2.97)**	0.88 (0.49-1.58)	0.13	0.14
ARA:EPA+DHA	1.12 (0.39-3.23)	1.66 (0.61-4.53)	0.57 (0.20-1.58)	0.18	1.04 (0.57-1.88)	**1.77 (0.98-3.18)**	0.82 (0.44-1.55)	0.07	0.86
DI_{16}	1.86 (0.71-4.89)	1.27 (0.47-3.41)	1.16 (0.44-3.10)	0.64	0.98 (0.53-1.79)	1.66 (0.93-2.99)	0.89 (0.47-1.67)	0.82	0.50
DI_{18}	0.63 (0.18-2.22)	1.04 (0.33-3.23)	**2.93 (0.98-8.81)**	**0.02**	1.20 (0.69-2.10)	0.95 (0.52-1.72)	0.79 (0.43-1.46)	0.61	**0.01**

[*]Adjusted for: smoking ever, alcohol consumption, hysterectomy ever, BMI, physical activity, age at first pregnancy, age at menopause, age at menarche.
[a]Quartile 1: lowest fatty acid content; reference quartile. Bold typeface indicates quartiles that are statistically significant compared to quartile 1 (P<0.05).
[1]SFA= 14:0, 16:0, 17:0, 18:0, 20:0, 24:0. [2]MUFA= 16:1 n-7, 18:1 c11, 18:1 n-9, 18:1 n-7, 24:1 n-9. [3]PUFA= 18:2 n-6, 18:3n-6, 20:2 n-6, 20:3 n-6, 20:4 n-6, 22:4 n-6, 22:5 n-6, 18:3 n-3, 20:4 n-3, 20:5 n-3, 22:5 n-3, 22:6 n-3.[4]Total long chain n-6 =20:2 n-6, 20:3 n-6, 20:4 n-6, 22:4 n-6, 22:5 n-6. [5]Total long chain n-3 = 20:4 n-3, 20:5 n-3, 22:5 n-3, 22:6 n-3. Abbreviations: ARA, arachidonic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; DI_{16}, desaturation index of 16:1:16:0; DI_{18}, desaturation index of 18:1:18:0.

Table 3-5: Multivariate* odds ratios (OR, 95% confidence intervals [CI]) of breast cancer according to quartiles of plasma phospholipid fatty acid relative percent by menopause status in British Columbia Generations Project

Fatty Acids	Premenopausal				Postmenopausal				
	Quartile				Quartile				
	Q2vsQ1[a]	Q3vsQ1	Q4vsQ1	*P trend*	Q2vsQ1[a]	Q3vsQ1	Q4vsQ1	*P trend*	*P int*
	OR (95% CI)				OR (95% CI)				
Saturates									
Total SFA[1]	1.72 (0.54-5.46)	0.42 (0.10-1.69)	1.56 (0.50-4.88)	0.20	1.22 (0.61-2.42)	0.65 (0.31-1.34)	0.88 (0.43-1.79)	0.36	0.48
14:0 (Myristic acid)	2.96 (0.93-9.40)	1.92 (0.56-6.64)	1.70 (0.46-6.22)	0.33	0.75 (0.36-1.56)	1.42 (0.69-2.94)	1.29 (0.63-2.64)	0.26	0.18
16:0 (Palmitic acid)	0.97 (0.30-3.20)	0.78 (0.21-2.94)	1.60 (0.48-5.32)	0.70	1.22 (0.61-2.43)	0.87 (0.43-1.77)	1.13 (0.57-2.24)	0.81	0.72
18:0 (Stearic acid)	1.04 (0.34-3.18)	**0.28 (0.09-0.91)**	0.60 (0.15-2.39)	0.13	0.79 (0.40-1.56)	0.64 (0.31-1.33)	0.86 (0.43-1.72)	0.70	0.68
24:0 (Lignoceric acid)	0.56 (0.16-1.96)	0.40 (0.11-1.38)	0.80 (0.25-2.56)	0.48	0.92 (0.44-1.90)	**2.40 (1.18-4.87)**	0.80 (0.38-1.67)	**0.007**	**0.05**
Monounsaturates									
Total MUFA[2]	0.69 (0.21-2.29)	0.76 (0.24-2.37)	0.68 (0.20-2.41)	0.92	1.23 (0.62-2.41)	0.83 (0.42-1.65)	0.80 (0.39-1.65)	0.61	0.86
16:1 n-7 (Palmitoleic acid)	0.74 (0.21-2.66)	1.01 (0.32-3.18)	1.94 (0.59-6.35)	0.52	0.97 (0.46-2.02)	1.03 (0.50-2.11)	1.21 (0.60-2.44)	0.92	0.69
18:1 c11 (Vaccenic acid)	0.37 (0.10-1.44)	0.94 (0.32-2.78)	0.31 (0.08-1.15)	0.16	0.78 (0.40-1.52)	0.98 (0.49-1.97)	0.50 (0.24-1.04)	0.23	0.85
18:1 n-9 (Oleic acid)	1.57 (0.44-5.56)	0.84 (0.26-2.69)	0.82 (0.25-2.70)	0.75	1.36 (0.66-2.82)	1.56 (0.74-3.28)	1.10 (0.53-2.29)	0.62	0.92
18:1 n-7 (Octadecenoic acid)	2.80 (0.84-9.33)	1.50 (0.40-5.66)	1.46 (0.40-5.29)	0.39	1.26 (0.64-2.48)	1.08 (0.54-2.13)	0.80 (0.39-1.64)	0.66	0.85
24:1 n-9 (Nervonic acid)	0.70 (0.24-2.02)	1.21 (0.39-3.71)	0.73 (0.19-2.77)	0.79	1.34 (0.63-2.84)	1.50 (0.73-3.10)	1.55 (0.76-3.18)	0.64	0.74
Polyunsaturates									
Total PUFA[3]	0.49 (0.14-1.78)	0.58 (0.16-2.07)	1.24 (0.42-3.63)	0.40	2.13 (1.04-4.39)	1.58 (0.76-3.27)	1.74 (0.83-3.62)	0.22	0.18
Total n-6	**0.12 (0.02-0.72)**	0.79 (0.24-2.59)	0.59 (0.20-1.77)	0.13	1.14 (0.57-2.29)	1.51 (0.77-2.98)	1.30(0.64-2.66)	0.67	0.18
Total long chain n-6[4]	1.16 (0.36-3.73)	2.01 (0.61-6.68)	1.43 (0.35-5.94)	0.67	1.33(0.65-2.72)	0.68 (0.32-1.44)	1.48 (0.73-3.00)	0.15	0.24
18:2 n-6 (Linoleic acid)	0.95 (0.23-3.85)	0.71 (0.20-2.57)	0.81 (0.24-2.69)	0.95	1.32 (0.66-2.63)	0.98 (0.49-1.96)	1.69 (0.82-3.51)	0.40	0.87
18:3 n-6 (γ- Linolenic acid)	2.52 (0.72-8.78)	2.95 0.86-10.08)	2.88 (0.84-9.88)	0.25	0.88 (0.46-1.94)	1.62 (0.80-3.26)	1.27 (0.62-2.61)	0.39	0.71
20:2 n-6 (Eicosadienoic acid)	1.62 (0.40-6.60)	2.41 (0.63-9.22)	2.36 (0.68-8.15)	0.51	1.08 (0.54-2.14)	0.97 (0.49-1.93)	0.87 (0.42-1.82)	0.95	0.66
20:3 n-6 (Dihomo-γ-Linolenic acid)	0.78 (0.22-2.74)	0.55 (0.16-1.88)	1.99 (0.546-7.32)	0.25	0.86 (0.43-1.75)	1.00 (0.49-2.03)	0.93 (0.44-1.94)	0.97	0.26
20:4 n-6 (ARA)	1.96 (0.60-6.37)	**3.72 (1.19-11.61)**	0.80 (0.17-3.69)	0.08	0.73 (0.35-1.52)	0.73 (0.35-1.53)	1.26 (0.62-2.58)	0.31	**0.02**
Total n-3	0.73 (0.25-2.13)	0.54 (0.15-2.01)	0.75 (0.21-2.68)	0.82	1.87 (0.90-3.89)	0.63 (0.29-1.35)	1.12 (0.53-2.38)	**0.02**	0.36
Total long chain n-3[5]	0.81 (0.28-2.31)	0.34(0.08-1.40)	0.70 (0.19-2.52)	0.52	1.52 (0.74-3.14)	0.65 (0.30-1.38)	0.98 (0.46-2.07)	0.11	0.55
18:3 n-3 (α-Linolenic acid)	2.35 (0.68-8.19)	1.47 (0.38-5.71)	3.46 (0.95-12.58)	0.25	1.48 (0.74-2.95)	1.50 (0.72-3.10)	1.25 (0.62-2.51)	0.66	0.25
20:4 n-3 (Eicosatetraenoic acid)	0.67 (0.22-2.06)	0.67 (0.18-2.54)	0.85 (0.27-2.68)	0.88	1.27 (0.61-2.65)	1.35 (0.66-2.75)	1.41 (0.68-2.92)	0.80	0.88
20:5 n-3 (EPA)	0.46 (0.13-1.62)	1.32 (0.43-4.02)	0.86 (0.24-2.99)	0.49	1.08 (0.52-2.23)	0.89 (0.42-1.86)	0.82 (0.38-1.74)	0.86	0.58

22:5 n-3 (DPA)	0.58 (0.19-1.74)	0.90 (0.28-2.92)	0.30 (0.06-1.37)	0.38	1.04 (0.51-2.11)	0.84 (0.41-1.74)	0.85 (0.42-1.72)	0.91	0.39
22:6 n-3 (DHA)	1.86 (0.62-5.54)	0.48 (0.12-1.91)	1.15 (0.34-3.90)	0.27	1.35 (0.66-2.76)	1.10 (0.55-2.21)	0.65 (0.31-1.39)	0.25	0.41
EPA+DHA	1.11 (0.39-3.15)	0.48 (0.12-1.90)	1.06 (0.30-3.72)	0.67	1.55 (0.75-3.17)	0.81 (0.39-1.67)	0.90 (0.43-1.89)	0.25	0.76
Ratios									
Total n-6:Total n-3	0.78 (0.16-3.79)	1.73 (0.48-6.29)	1.17 (0.33-4.17)	0.69	0.67 (0.33-1.36)	1.44 (0.74-2.82)	1.32 (0.62-2.80)	0.13	0.88
ARA:DHA	1.03 (0.28-3.76)	1.41 (0.42-4.78)	1.16 (0.31-4.42)	0.94	0.98 (0.49-1.97)	1.00 (0.48-2.10)	1.88 (0.93-3.80)	0.18	0.82
ARA:EPA+DHA	2.17 (0.55-8.54)	1.76 (0.45-6.94)	1.68 (0.42-6.68)	0.74	1.07 (0.53-2.17)	1.20 (0.59-2.44)	**2.40 (1.16-4.96)**	0.07	0.61
DI_{16}	1.87 (0.56-6.29)	1.66 (0.51-5.39)	1.49 (0.45-5.03)	0.75	1.05 (0.50-2.19)	1.07 (0.53-2.17)	1.06 (0.52-2.15)	1.00	0.98
DI_{18}	0.59 (016-2.19)	0.32 (0.09-1.13)	1.03 (0.29-3.60)	0.20	1.56 (0.54-2.04)	0.72 (0.36-1.44)	0.84 (0.42-1.67)	0.72	0.55

[*]Adjusted for: smoking ever, alcohol consumption, hysterectomy ever, BMI, physical activity, age at first pregnancy, age at menopause, age at menarche.
[a]Quartile 1: lowest fatty acid content; reference quartile. Bold typeface indicates quartiles that are statistically significant compared to quartile 1 ($P<0.05$).
[1]SFA= 14:0, 16:0, 17:0, 18:0, 20:0, 24:0. [2]MUFA= 16:1 n-7, 18:1 c11, 18:1 n-9, 18:1 n-7, 24:1 n-9. [3]PUFA= 18:2 n-6, 18:3n-6, 20:2 n-6, 20:3 n-6, 20:4 n-6, 22:4 n-6, 22:5 n-6, 18:3 n-3, 20:4 n-3, 20:5 n-3, 22:5 n-3, 22:6 n-3.[4]Total long chain n-6 =20:2 n-6, 20:3 n-6, 20:4 n-6, 22:4 n-6, 22:5 n-6. [5]Total long chain n-3 = 20:4 n-3, 20:5 n-3, 22:5 n-3, 22:6 n-3. Abbreviations: ARA, arachidonic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; DI_{16}, desaturation index of 16:1:16:0; DI_{18}, desaturation index of 18:1:18:0.

Table 3-6: Multivariate* odds ratios (OR, 95% confidence intervals [CI]) of breast cancer according to quartiles of plasma phospholipid fatty acid relative percent by waist-to-hip ratio in Alberta's Tomorrow Project

Fatty Acids	Waist-to-hip ratio <0.85[1]				Waist-to-hip ratio ≥0.85[1]				
	Quartile				Quartile				
	Q2vsQ1[a]	Q3vsQ1	Q4vsQ1	*P trend*	Q2vsQ1[a]	Q3vsQ1	Q4vsQ1	*P trend*	*P int*
	OR (95% CI)				OR (95% CI)				
Saturates									
Total SFA[1]	0.48 (0.22-1.05)	0.58 (0.27-1.26)	0.71 (0.32-1.59)	0.28	1.08 (0.54-2.20)	1.40 (0.71-2.76)	0.52 (0.26-1.07)	**0.04**	0.06
14:0 (Myristic acid)	0.69 (0.32-1.53)	0.64 (0.28-1.45)	1.48 (0.68-3.26)	0.18	0.79 (0.39-1.57)	0.66 (0.33-1.32)	0.52 (0.26-1.04)	0.30	0.07
16:0 (Palmitic acid)	**2.72 (1.21-6.11)**	0.89 (0.37-2.13)	1.48 (0.62-3.56)	**0.03**	0.72 (0.36-1.41)	0.84 (0.44-1.62)	0.53 (0.27-1.05)	0.32	**0.05**
18:0 (Stearic acid)	0.58 (0.27-1.22)	0.48 (0.21-1.14)	0.71 (0.30-1.66)	0.32	1.12 (0.53-2.37)	0.95 (0.47-1.92)	1.01 (0.49-2.07)	0.97	0.73
24:0 (Lignoceric acid)	0.59 (0.27-1.27)	**0.43 (0.18-1.00)**	**0.42 (0.19-0.94)**	0.13	1.75 (0.91-3.39)	0.78 (0.39-1.54)	0.85 (0.43-1.70)	0.08	0.30
Monounsaturates									
Total MUFA[2]	0.77 (0.34-1.76)	0.69 (0.30-1.57)	0.61 (0.27-1.40)	0.69	1.03 (0.53-2.00)	0.86 (0.44-1.69)	0.99 (0.51-1.93)	0.96	0.84
16:1 n-7 (Palmitoleic acid)	1.54 (0.70-3.41)	1.54 (0.69-3.43)	1.30 (0.55-3.07)	0.68	0.78 (0.40-1.52)	0.56 (0.28-1.15)	0.67 (0.35-1.30)	0.43	0.23
18:1 c11 (Vaccenic acid)	1.47 (0.67-3.22)	0.56 (0.23-1.37)	1.27 (0.57-2.85)	0.15	0.76 (0.39-1.50)	0.57 (0.29-1.12)	0.65 (0.33-1.28)	0.39	0.40
18:1 n-9 (Oleic acid)	1.38 (0.62-3.03)	0.82 (0.36-1.87)	1.31 (0.59-2.92)	0.57	0.60 (0.30-1.20)	1.20 (0.61-2.37)	0.82 (0.41-1.66)	0.24	0.20
18:1 n-7 (Octadecenoic acid)	**0.35 (0.14-0.89)**	0.63 (0.28-1.41)	1.13 (0.51-2.51)	**0.05**	1.56 (0.82-2.95)	1.39 (0.70-2.76)	1.40 (0.70-2.81)	0.57	**0.04**
24:1 n-9 (Nervonic acid)	0.87 (0.39-1.92)	0.62 (0.28-1.38)	0.75 (0.34-1.67)	0.68	1.07 (0.54-2.10)	0.98 (0.50-1.92)	0.76 (0.39-1.49)	0.78	0.73
Polyunsaturates									
Total PUFA[3]	1.30 (0.54-3.13)	1.57 (0.68-3.62)	1.38 (0.60-3.20)	0.76	0.72 (0.35-1.47)	**2.11 (1.10-4.07)**	0.95 (0.47-1.90)	**0.01**	0.48
Total n-6	1.48 (0.64-3.44)	0.93 (0.40-2.18)	1.31 (0.58-2.96)	0.65	0.92 (0.46-1.84)	1.72 (0.87-3.40)	1.57 (0.81-3.06)	0.18	0.30
Total long chain n-6[4]	1.27 (0.56-2.87)	1.33 (0.58-3.05)	1.41 (0.62-3.18)	0.85	0.47 (0.21-1.02)	1.27 (0.63-2.56)	1.81 (0.88-3.72)	**0.003**	0.32
18:2 n-6 (Linoleic acid)	0.66 (0.29-1.53)	**0.42 (0.18-1.00)**	0.74 (0.34-1.62)	0.27	0.81 (0.42-1.55)	0.73 (0.38-1.42)	0.88 (0.44-1.76)	0.82	0.74
18:3 n-6 (γ- Linolenic acid)	**2.22 (1.05-4.67)**	0.70 (0.29-1.69)	1.11 (0.48-2.58)	**0.05**	1.07 (0.52-2.21)	0.85 (0.42-1.74)	1.24 (0.61-2.50)	0.73	0.26
20:2 n-6 (Eicosadienoic acid)	1.08 (0.47-2.49)	0.80 (0.34-1.90)	1.69 (0.76-3.78)	0.30	1.57 (0.80-3.09)	1.15 (0.56-2.20)	1.25 (0.62-2.52)	0.58	0.62
20:3 n-6 (Dihomo-γ-Linolenic acid)	1.47 (0.70-3.10)	1.04 (0.45-2.40)	1.31 (0.54-3.18)	0.72	0.54 (0.26-1.14)	0.61 (0.29-1.29)	0.76 (0.37-1.55)	0.39	0.35
20:4 n-6 (ARA)	0.67 (0.28-1.59)	1.58 (0.69-3.59)	1.21 (0.54-2.69)	0.26	1.43 (0.69-2.96)	1.46 (0.70-3.04)	**2.54 (1.22-5.32)**	0.08	0.18
Total n-3	1.76 (0.76-4.10)	1.68 (0.71-3.94)	1.87 (0.78-4.46)	0.48	**2.02 (1.02-4.02)**	1.44 (0.71-2.90)	1.32 (0.65-2.68)	0.24	0.95
Total long chain n-3[5]	2.02 (0.85-4.77)	2.22 (0.94-5.20)	2.17 (0.90-5.24)	0.24	1.80 (0.91-2.57)	1.48 (0.74-2.96)	1.30 (0.64-2.64)	0.09	0.93

18:3 n-3 (α-Linolenic acid)	**0.29 (0.12-0.69)**	0.66 (0.29-1.49)	0.60 (0.27-1.30)	**0.05**	0.67 (0.34-1.32)	1.03 (0.53-1.99)	1.00 (0.50-2.01)	0.56	0.47
20:4 n-3 (Eicosatetraenoic acid)	0.53 (0.24-1.16)	0.66 (0.28-1.53)	0.65 (0.29-1.43)	0.45	1.22 (0.62-2.40)	0.78 (0.40-1.54)	0.89 (0.45-1.76)	0.62	0.49
20:5 n-3 (EPA)	0.96 (0.43-2.14)	1.14 (0.52-2.52)	1.00 (0.45-2.26)	0.98	1.25 (0.62-2.50)	1.19 (0.59-2.39)	1.15 (0.57-2.31)	0.94	0.96
22:5 n-3 (DPA)	1.61 (0.70-3.72)	1.69 (0.72-4.01)	2.11 (0.92-4.87)	0.37	1.07 (0.54-2.12)	0.79 (0.40-1.55)	1.26 (0.63-2.51)	0.60	0.51
22:6 n-3 (DHA)	**2.65 (1.06-6.60)**	**2.95 (1.20-7.28)**	**3.40 (1.37-8.40)**	**0.05**	**2.92 (1.46-5.82)**	1.52 (0.75-3.11)	1.32 (0.63-2.77)	**0.02**	0.38
EPA+DHA	**2.34 (1.00-5.53)**	2.15 (0.90-5.14)	**2.79 (1.16-6.72)**	0.12	**2.51 (1.25-5.02)**	1.86 (0.92-3.77)	1.46 (0.71-3.01)	0.06	0.71
Ratios									
Total n-6:Total n-3	0.77 (0.34-1.70)	0.64 (0.28-1.47)	0.48 (0.21-1.12)	0.38	1.00 (0.50-2.00)	1.11 (0.57-2.17)	0.90 (0.44-1.80)	0.94	0.84
ARA:DHA	1.87 (0.85-4.10)	0.84 (0.38-1.87)	**0.42 (0.17-1.02)**	**0.01**	0.80 (0.39-1.66)	**2.31 (1.15-4.65)**	1.12 (0.55-2.30)	**0.01**	**0.002**
ARA:EPA+DHA	1.40 (0.64-3.07)	1.14 (0.51-2.56)	0.48 (0.20-1.15)	0.09	0.72 (0.35-1.49)	**2.14 (1.09-4.18)**	0.96 (0.47-1.95)	**0.01**	**0.07**
DI_{16}	0.97 (0.44-2.17)	1.41 (0.64-3.08)	1.04 (0.46-2.35)	0.79	0.88 (0.44-1.73)	0.64 (0.32-1.27)	0.79 (0.42-1.48)	0.63	0.34
DI_{18}	0.59 (0.25-1.38)	0.44 (0.17-1.11)	0.85 (0.38-1.91)	0.26	1.48 (0.77-2.85)	1.44 (0.75-2.76)	1.38 (0.68-2.79)	0.62	0.11

*Adjusted for: smoking ever, alcohol consumption, hysterectomy ever, BMI, physical activity, age at first pregnancy, age at menopause, age at menarche.
[a]Quartile 1: lowest fatty acid content; reference quartile. Bold typeface indicates quartiles that are statistically significant compared to quartile 1 ($P<0.05$).
[1]Waist-to-hip ratio below guidelines <0.85 and above guidelines ≥0.85 (for women).[2]SFA= 14:0, 16:0, 17:0, 18:0, 20:0, 24:0. [3]MUFA= 16:1 n-7, 18:1 c11, 18:1 n-9, 18:1 n-7, 24:1 n-9. [4]PUFA= 18:2 n-6, 18:3n-6, 20:2 n-6, 20:3 n-6, 20:4 n-6, 22:4 n-6, 22:5 n-6, 18:3 n-3, 20:4 n-3, 20:5 n-3, 22:5 n-3, 22:6 n-3. [5]Total long chain n-6 =20:2 n-6, 20:3 n-6, 20:4 n-6, 22:4 n-6, 22:5 n-6. [6]Total long chain n-3 = 20:4 n-3, 20:5 n-3, 22:5 n-3, 22:6 n-3. Abbreviations: ARA, arachidonic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; DI_{16}, desaturation index of 16:1:16:0; DI_{18}, desaturation index of 18:1:18:0.

Table 3-7: Multivariate* odds ratios (OR, 95% confidence intervals [CI]) of breast cancer according to quartiles of plasma phospholipid fatty acid relative percent by waist-to-hip ratio in British Columbia Generations Project

Fatty Acids	Waist-to-hip ratio <0.85[1]				Waist-to-hip ratio ≥0.85[1]				
	Quartile				Quartile				
	Q2vsQ1[a]	Q3vsQ1	Q4vsQ1	*P trend*	Q2vsQ1[a]	Q3vsQ1	Q4vsQ1	*P trend*	*P int*
	OR (95% CI)				OR (95% CI)				
Saturates									
Total SFA[1]	1.22 (0.58-2.55)	0.68 (0.30-1.56)	0.72 (0.31-1.69)	0.50	1.54 (0.57-4.16)	0.61 (0.22-1.65)	1.44 (0.55-3.72)	0.13	0.64
14:0 (Myristic acid)	1.08 (0.49-2.38)	1.60 (0.69-3.71)	1.07 (0.46-2.53)	0.68	1.10 (0.43-2.83)	1.41 (0.56-3.52)	1.64 (0.65-4.11)	0.68	0.78
16:0 (Palmitic acid)	1.02 (0.47-2.21)	0.74 (0.33-1.63)	1.12 (0.48-2.60)	0.78	1.60 (0.64-4.01)	1.22 (0.45-3.30)	1.63 (0.70-3.81)	0.66	0.80
18:0 (Stearic acid)	0.64 (0.31-1.34)	**0.31 (0.13-0.74)**	0.62 (0.26-1.49)	0.07	1.31 (0.50-3.44)	0.91 (0.36-2.33)	1.01 (0.42-2.44)	0.88	0.48
24:0 (Lignoceric acid)	0.68 (0.28-1.65)	1.80 (0.83-3.88)	0.53 (0.22-1.29)	**0.02**	0.86 (0.36-2.06)	0.94 (0.38-2.33)	1.02 (0.42-2.44)	0.98	0.22
Monounsaturates									
Total MUFA[2]	0.81 (0.36-1.82)	0.78 (0.34-1.79)	0.77 (0.34-1.78)	0.92	1.39 (0.58-3.30)	0.74 (0.33-1.66)	0.57 (0.22-1.44)	0.28	0.51
16:1 n-7 (Palmitoleic acid)	1.30 (0.55-3.08)	0.93 (0.38-2.27)	1.76 (0.73-4.24)	0.43	0.56 (0.21-1.49)	1.23 (0.53-2.89)	0.98 (0.43-2.24)	0.46	0.35
18:1 c11 (Vaccenic acid)	0.73 (0.32-1.68)	0.91 (0.41-2.04)	**0.30 (0.12-0.72)**	**0.03**	0.67 (0.29-1.57)	1.18 (0.51-2.71)	0.75 (0.30-1.88)	0.57	0.51
18:1 n-9 (Oleic acid)	1.03 (0.46-2.31)	0.84 (0.36-1.97)	0.88 (0.39-1.99)	0.96	2.52 (0.94-6.75)	**2.85 (1.11-7.34)**	1.43 (0.55-3.72)	0.10	0.36
18:1 n-7 (Octadecenoic acid)	1.42 (0.61-3.33)	1.79 (0.75-4.28)	1.23 (0.52-2.92)	0.61	1.73 (0.74-4.02)	0.73 (0.32-1.65)	0.75 (0.31-1.83)	0.21	0.38
24:1 n-9 (Nervonic acid)	1.17 (0.49-2.80)	1.64 (0.70-3.89)	1.64 (0.67-4.06)	0.59	0.95 (0.40-2.26)	1.07 (0.45-2.51)	1.02 (0.44-2.34)	1.00	0.91
Polyunsaturates									
Total PUFA[3]	2.04 (0.78-5.35)	1.50 (0.57-3.97)	2.16 (0.85-5.49)	0.36	1.06 (0.46-2.48)	1.30 (0.56-3.06)	1.32 (0.57-3.05)	0.89	0.71
Total n-6	0.83 (0.33-2.05)	1.42 (0.62-3.28)	1.51 (0.67-3.41)	0.46	0.71 (0.30-1.68)	1.25 (0.54-2.89)	0.76 (0.32-1.81)	0.57	0.66
Total long chain n-6[4]	1.44 (0.66-3.14)	0.88 (0.37-2.13)	1.91 (0.84-4.36)	0.28	1.33 (0.51-3.51)	0.93 (0.36-2.38)	1.22 (0.47-3.20)	0.83	0.89
18:2 n-6 (Linoleic acid)	1.16 (0.46-2.88)	0.83 (0.33-2.05)	1.55 (0.66-3.64)	0.44	1.33 (0.59-2.99)	0.94 (0.42-2.13)	1.26 (0.50-3.19)	0.83	0.90
18:3 n-6 (γ- Linolenic acid)	1.30 (0.58-2.90)	**2.98 (1.29-6.87)**	1.38 (0.56-3.37)	0.06	1.07 (0.40-2.87)	1.16 (0.49-2.73)	1.38 (0.58-3.28)	0.90	0.27
20:2 n-6 (Eicosadienoic acid)	0.96 (0.41-2.25)	1.46 (0.66-3.22)	1.23 (0.52-2.91)	0.71	1.26 (0.52-3.08)	0.82 (0.33-2.04)	0.99 (0.41-2.39)	0.81	0.52
20:3 n-6 (Dihomo-γ-Linolenic acid)	0.70 (0.32-1.52)	0.67 (0.30-1.47)	1.27 (0.54-2.99)	0.41	1.06 (0.39-2.88)	1.03 (0.38-2.78)	0.94 (0.36-2.45)	0.99	0.83
20:4 n-6 (ARA)	1.53 (0.68-3.46)	1.41 (0.60-3.32)	1.60 (0.69-3.70)	0.68	0.56 (0.22-1.43)	0.86 (0.35-2.09)	0.98 (0.38-2.53)	0.55	0.52
Total n-3	1.14 (0.50-2.59)	**0.30 (0.12-0.77)**	0.76 (0.32-1.77)	**0.03**	1.66 (0.72-3.85)	0.94 (0.38-2.34)	1.21 (0.47-3.08)	0.53	0.58
Total long chain n-3[5]	1.05 (0.46-2.36)	**0.29 (0.12-075)**	0.66 (0.28-1.54)	**0.03**	1.51 (0.66-3.48)	0.87 (0.35-2.16)	1.14 (0.44-2.91)	0.60	0.62
18:3 n-3 (α-Linolenic acid)	**2.56 (1.09-6.02)**	1.87 (0.76-4.62)	1.84 (0.75-4.50)	0.20	1.06 (0.46-2.48)	0.97 (0.39-2.40)	1.33 (0.56-3.12)	0.89	0.47
20:4 n-3 (Eicosatetraenoic acid)	1.47 (0.61-3.53)	1.43 (0.60-3.42)	1.36 (0.58-3.22)	0.83	0.84 (0.36-1.99)	0.90 (0.37-2.17)	1.38 (0.58-3.26)	0.69	0.74

20:5 n-3 (EPA)	1.64 (0.73-3.87)	0.48 (0.20-1.20)	0.97 (0.42-2.24)	0.06	0.42 (0.16-1.04)	1.37 (0.57-3.28)	0.64 (0.25-1.65)	**0.04**	**0.002**
22:5 n-3 (DPA)	0.71 (0.32-1.58)	0.65 (0.28-1.49)	0.55 (0.23-1.31)	0.57	0.96 (0.40-2.32)	0.76 (0.31-1.84)	0.71 (0.31-1.66)	0.83	0.99
22:6 n-3 (DHA)	1.33 (0.58-3.02)	**0.38 (0.14-0.97)**	0.66 (0.28-1.53)	**0.03**	1.31 (0.57-3.05)	2.09 (0.91-4.84)	0.86 (0.33-2.26)	0.21	**0.05**
EPA+DHA	1.02 (0.46-2.26)	**0.27 (0.10-0.71)**	0.66 (0.29-1.50)	**0.03**	1.75 (0.75-407)	1.57 (0.65-3.78)	1.20 (0.46-3.17)	0.57	0.13
Ratios									
Total n-6:Total n-3	**0.32 (0.12-0.87)**	1.74 (0.78-3.84)	1.65 (0.72-3.82)	**0.005**	0.91 (0.36-2.26)	1.21 (0.51-2.89)	0.89 (0.33-2.37)	0.87	0.16
ARA:DHA	**0.44 (0.18-1.04)**	1.13 (0.48-2.68)	1.88 (0.84-4.17)	**0.02**	2.02 (0.77-5.26)	1.33 (0.53-3.31)	1.44 (0.56-3.70)	0.54	**0.05**
ARA:EPA+DHA	0.64 (0.28-1.50)	1.26 (0.54-2.95)	2.05 (0.92-4.58)	0.06	2.34 (0.90-6.08)	1.47 (0.58-3.69)	2.28 (0.85-6.13)	0.25	0.27
DI_{16}	2.18 (0.92-5.15)	1.34 (0.56-3.20)	1.38 (0.56-3.40)	0.32	0.65 (0.26-1.66)	0.97 (0.42-2.25)	0.93 (0.40-2.16)	0.80	0.31
DI_{18}	0.70 (0.29-1.65)	0.59 (0.26-1.35)	1.09 (0.48-2.48)	0.41	1.23 (0.54-2.80)	0.49 (0.20-1.20)	0.71 (0.30-1.70)	0.22	0.59

[*]Adjusted for: smoking ever, alcohol consumption, hysterectomy ever, BMI, physical activity, age at first pregnancy, age at menopause, age at menarche.
[a]Quartile 1: lowest fatty acid content; reference quartile. Bold typeface indicates quartiles that are statistically significant compared to quartile 1 ($P<0.05$).
[1]Waist-to-hip ratio below guidelines <0.85 and above guidelines ≥0.85 (for women).[2]SFA= 14:0, 16:0, 17:0, 18:0, 20:0, 24:0. [3]MUFA= 16:1 n-7, 18:1 c11, 18:1 n-9, 18:1 n-7, 24:1 n-9. [4]PUFA= 18:2 n-6, 18:3n-6, 20:2 n-6, 20:3 n-6, 20:4 n-6, 22:4 n-6, 22:5 n-6, 18:3 n-3, 20:4 n-3, 20:5 n-3, 22:5 n-3, 22:6 n-3. [5]Total long chain n-6 =20:2 n-6, 20:3 n-6, 20:4 n-6, 22:4 n-6, 22:5 n-6. [6]Total long chain n-3 = 20:4 n-3, 20:5 n-3, 22:5 n-3, 22:6 n-3. Abbreviations: ARA, arachidonic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; DI_{16}, desaturation index of 16:1:16:0; DI_{18}, desaturation index of 18:1:18:0.

When the waist-to-hip ratio was ≥0.85, positive associations with breast cancer risk were observed for DHA and total long chain n-6 (largely influenced by ARA) and a negative association was observed for SFA. In ATP interactions were observed in three instances, suggesting decreased risk with increased 16:0 (P_{int}=0.05), and increased risk with increased 18:1 n-7 (P_{int}=0.04), or the ratio of ARA:DHA (P_{int}=0.002) if waist-to-hip was ≥0.85. In the BCGP cohort, when stratified by waist-to-hip ratio, several fatty acids were associated with decreased risk of breast cancer when waist-to-hip ratio was <0.85, including 18:1 c11, DHA, EPA+DHA, total n-3 and total long chain n-3. The ratios of n-6:n-3 and ARA:DHA were associated with increased breast cancer risk when the waist-to-hip ratio was <0.85. EPA was associated with decreased risk when waist-to-hip ratio was ≥0.85. There was a statistical interaction for DHA (P_{int}=0.05), suggesting a negative association among women with a waist-to-hip ratio of <0.85 compared to women with a waist-to-hip was ≥0.85. In two other instances statistical interactions were observed: EPA (P_{int}=0.002), and the ratio of ARA:DHA (P_{int}=0.05) however they are not linear interactions and suggest there may be an optimal range for reduced risk.

3.4 Discussion

In this large, prospective study of two geographically distinct Canadian cohorts, we found regional heterogenicity in fatty acid status, wherein women in the BCGP cohort had higher levels of plasma n-3 PUFA, specifically EPA and DHA compared to women in the ATP cohort. Considered in isolation, ATP and BCGP had inconsistent associations of plasma phospholipid fatty acid status with breast cancer risk. When adjusted for possible confounders, in ATP SFA were associated with reduced risk while ARA, DHA, the combination of EPA+DHA, and long chain n-6 fatty acids, were associated with increased breast cancer risk; whereas in the BCGP, 18:1 c11 and long chain n-3 were inversely associated and the ratio of total n-6:total n-3 was

positively associated with breast cancer risk. *A priori* stratification revealed that these associations were driven by waist-to-hip ratio in BCGP and both waist-to-hip ratio and menopausal status in ATP. Our study is unique as it has been conducted for the first time in a Canadian population, highlighting provincial variations in fatty acid status. To our knowledge it is the first study to assess associations between breast cancer risk and fatty acids stratified by waist-to-hip ratio (a more accurate measurement of central adiposity compared to BMI).

The use of plasma for phospholipid fatty acid analysis provides an easily accessible, minimally invasive sample that has longer fatty acid stability compared to red blood cells (Brenna et al. 2018). Extraction of plasma phospholipids versus total plasma lipids avoids the pool of postprandial triacylgycerols and is believed to be a reliable estimation of a persons' usual fatty acid status (Brenna et al 2018.). Furthermore, the EPA and DHA plasma phospholipid content observed in this study are similar to a previous study in pre and post-menopausal Canadian women (Stark et al. 2003). While both ATP and BCGP are large, robust longitudinal studies, dietary intake data were not collected for BCGP therefore it is not possible to comment specifically on the status of EPA and DHA relative to dietary intake in this cohort. However, using the limited dietary intake available for ATP, we observed moderate but consistent correlations between recalled intake (diet and supplements) and plasma phospholipid DHA and EPA composition. Additionally, a 2016 study that assessed dietary patterns in women at high risk for breast cancer reported plasma phospholipid ranges similar to the current study in women consuming a 'modern diet' versus a 'traditional diet'. Dietary information for the dietary pattern study was obtained from a cohort of Canadian women in Ontario and British Columbia (Hidaka et al. 2017, Martin et al. 2011). Taken together, this information infers the reliability of the plasma phospholipid data we obtained in the absence of dietary intake data.

The regional differences observed in our study are consistent with the discrepant findings previously reported in the literature (Vatten et al. 1993, Simonsen et al. 1998, Chajes et al. 1999, Klein et al. 2000, Pala et al. 2001, Bagga et al. 2002, Saadatian-Elahi et al. 2002, Maillard et al. 2002, Rissanen et al. 2003, Wirfalt et al. 2004, Shannon et al. 2007, Chajes et al. 2008, Witt et al. 2009, Takata et al. 2009, Schmidt et al. 2014, Qin et al. 2014, Hidaka et al. 2015, Conceicao et al. 2016, Bassett et al. 2016, Nagata et al. 2017, Chajes et al. 2017, Hirko et al. 2018). Current evidence, from studies using varied tissue sources of fatty acids—breast adipose tissue, erythrocytes, serum and plasma—suggests a lower risk of breast cancer with higher fatty acid content of linoleic acid (Vatten et al. 1993, Rissanen et al. 2003, Shannon et al. 2007), stearic acid (Chajes et al. 1999), α-linolenic acid (Klein et al. 2000, Maillard et al. 2002), DHA (Maillard et al. 2002), EPA (Shannon et al. 2007, Witt et al. 2009), total n-6 (Vatten et al. 1993, Rissanen et al. 2003), total n-3 (Simonsen et al. 1998), DI_{18} (Chajes et al. 1999) or PUFA (Rissanen et al. 2003); as well as elevated risk of breast cancer with higher fatty acid content of palmitic acid (Shannon et al. 2007, Pala et al. 2001, Bassett et al. 2016), palmitoleic acid (Shannon et al. 2007, Chajes et al. 2008, Chajes et al. 2017), oleic acid (Pala et al. 2001), MUFA (Pala et al. 2001), SFA (Saadatian-Elahi et al. 2002, Bassett et al. 2016) and total n-3 (Simonsen et al. 1998). The difficulty in forming a strong conclusion from these studies could be due in part to a variety of factors: 1) differences in dietary intake or dietary patterns based on geographic location (Dandamudi et al. 2018, Brennan et al. 2010, Xiao et al. 2019), 2) heterogeneity in tissue sources (Brenna et al. 2018); 3) controls in two instances were women with breast benign disease (Klein et al. 2000, Maillard et al. 2002), 4) several studies only assessed post-menopausal women (Simonsen et al. 1998, Pala et al. 2001, Wirfalt et al. 2004, Witt et al. 2009, Takata et al. 2009), while others had groups primarily containing pre-menopausal women (Klein et al. 2000,

Shannon et al. 2007, Hirko et al. 2018) and 5) failure of some studies to address (consider or list) in their analysis known risk factors such as BMI or age. Many studies that have used fatty acids as a biomarker are based on European or Asian populations (Chajes et al. 1999, Pala et al. 2001, Wirfalt et al. 2004, Shannon et al. 2007, Chajes et al. 2008); regions that have higher levels of EPA+DHA in blood fractions compared to North American populations (Stark et al. 2016). Two studies from the United States had DHA content similar to ATP (Takata et al. 2009, Hirko et al. 2018) and an Australian study had DHA content similar to our BCGP cohort (Bassett et al. 2016), yet no clear associations between DHA and breast cancer risk were established in these studies. Furthermore, it is possible that changes in dietary intake after sample collection could influence future cancer incidence. The regional variations in fatty acid content including differences in levels of long chain PUFA underscore the complexity of employing plasma phospholipid fatty acid status as a biomarker for breast cancer risk. The availability of two cohorts with distinctly different fatty acid profiles provided a means of assessing the impact of n-3 LCPUFA on breast cancer risk and offers evidence that suggests demographic or geographic influences on dietary intake and resultant fatty acid content impact future breast cancer risk. Indeed, the quartiles for relative percent DHA in BCGP were 25-30% higher than the levels in ATP and the results suggest that this variance in fatty acid content could influence risk when the data is stratified by menopause or body composition.

In isolation, both menopausal status and body composition are both known to influence breast cancer risk (World Cancer Research Fund 2018). Breast cancer is a hormone-related, heterogeneous disease, whose etiology differs based on menopausal status (Harbeck et al. 2019, Davis et al. 2015). In premenopausal women, the risk of breast cancer is inversely associated with higher body fatness, while the opposite is true for postmenopausal women (positively

associated) (World Cancer Research Fund 2018). This is believed to be related to the fact that the major source of estrogen post-menopause comes from adipose tissue, resulting in obese women having higher serum concentrations of estradiol and a resultant increased risk (Key 2011). It is therefore plausible that in order to establish clear associations between fatty acid content and breast cancer risk, one must not only consider geographic location and the dietary influences on fatty acid status, but also the menopausal state and measures of body fat distribution of the women. The *a priori* stratification employed in this study allowed us to further delineate the observations observed in the original multivariate analysis and determine that risk is dependent on the multifactorial combination of fatty acid status, menopause status and body fatness. While no associations were observed in BCGP when stratified by menopausal status, in premenopausal women in ATP, SFA (driven by 18:0 and 24:0) were associated with decreased risk, which is contrary to previously reported findings suggesting higher levels of SFA were associated with an increased risk (Saadatian-Elahi et al. 2002, Bassett et al. 2016, Hirko et al. 2018). Interestingly, samples in these studies had lower amounts of total SFA (40 – 42%), compared to the ATP cohort (approximately 47%). How a higher content could be protective or how the balance or mixture of saturated fatty acids could influence risk should be explored further. From a mechanistic perspective, stearic acid (18:0) has been suggested to induce apoptosis in breast cancer cells (Evans et al. 2009) and availability to tumours could therefore be protective against tumoural development. Dietary components including carbohydrates influence the status of fatty acids and could affect the fat / carbohydrate ratio as excess carbohydrates are converted to medium chain fatty acids and MUFA through de novo lipogenesis (Hellerstein et al. 1996). Therefore, we examined key fatty acids that could be reflective of a higher carbohydrate intake and at the desaturation ratios (ratio of product to substrate, 18:1 n-9:18:0). The desaturation

index can be used as a surrogate marker of stearoyl-CoA desaturase (SCD, Δ9 desaturase) activity as it is a possible indirect marker of *de novo* lipid synthesis. Increased SCD activity has been implicated in increased tumoural growth (Peck et al. 2019, Igal 2010, Hodson et al. 2013) and while it varies with breast cancer subtype, it is believed to influence breast cancer survival (Holder et al. 2013). It is important to use caution when interpreting associations derived from the calculated ratio as it is a surrogate marker of SCD as it does not take into consideration other factors that could influence SCD activity, nor is it solely representative of endogenous synthesis; however, DI_{18} was associated with increased risk in premenopausal ATP women. This combination of decreased OR with stearic acid and increased OR with DI_{18} has been previously observed in two European studies (Chajes et al. 1999, Chajes et al. 2017), but was not observed in the BCGP cohort.

Essential fatty acids, including linoleic and long chain n-3 fatty acids, must be obtained from the diet as they are not endogenously synthesized. The relationship between the status of these fatty acids and breast cancer risk continues to be unclear (Hanson et al. 2020). Statistical interactions for linoleic acid, ALA and total long chain n-3 suggested inverse associations in postmenopausal women compared to premenopausal women in ATP. This is in accordance with two prior studies that have suggested inverse associations with breast cancer for linoleic acid (Vatten et al. 1993, Rissanen et al. 2003). Contrary to positive epidemiological associations of EPA and DHA with risk reduction, in ATP long chain n-3 fatty acids were associated with increased risk of breast cancer in premenopausal women. This association of long chain n-3 fatty acids with risk has been previously observed in two prospective studies of prostate cancer (Brasky et al. 2011, Brasky et al. 2013) and these cohorts had a similar DHA status in phospholipids compared to our ATP cohort. However, it is important to note that in ATP, women

in the second quartile were associated with the highest risk and while risk increased in the fourth quartile it was trending downwards. This could suggest an optimal range for reduced risk. Furthermore, in concordance with a meta-analysis (Zheng et al. 2013), we observed a trend towards risk reduction in post-menopausal women based on n-3 status, driven by a decrease in risk due associated with ALA. It has been suggested that the beneficial effects of these long chain n-3 PUFAs occurs after long term exposure. This is an interesting hypothesis and merits further investigation.

There have been several studies that have established an association between body fatness (primarily using BMI) and breast cancer risk, particularly in North American populations (World Cancer Research Fund 2018). In Alberta, it is estimated that 8% of breast cancer cases are attributable to being overweight/ obese, as measured by BMI (Brenner et al. 2017). Weight distribution and central adiposity, determined by the waist-to-hip ratio, is thought to be a better modality to assess regional adiposity compared to BMI alone as it has been shown to better predict morbidity and is considered a stronger predictor of all cancer risk (Barberio et al. 2019). To our knowledge, this is the first nested case-control study using a prospective longitudinal cohort to examine associations between fatty acid status and waist-to-hip ratio. While there is a strong relationship between obesity and dietary intake, associations of fatty acid status and breast cancer risk amongst healthy, overweight or obese women have not been thoroughly explored. The differences between the two cohorts, ATP and BCGP, provide striking contrast and suggest the influence of specific fatty acids obtained from the diet on cancer risk. In the ATP cohort, in women with a waist-to-hip ratio <0.85, DHA was positively associated and the ratio of ARA:DHA negatively associated with breast cancer. Yet in the BCGP cohort, women with a waist-to-hip ratio <0.85 had a decreased risk of breast cancer with increased DHA, EPA+DHA,

total n-3, total long chain n-3 and an increased risk of breast cancer with higher total n-6:total n-3 or ARA:DHA ratios. These associations were attenuated in women with waist-to-hip ratios that were above guidelines, suggesting that the protective effect of these fatty acids on breast cancer risk may be decreased in overweight or obese women.

Strengths of our study include: 1) availability of cases and controls nested within two robust longitudinal population-based cohorts, with a large number of breast cancer incidences and extensive harmonized epidemiological data (Fortier et al. 2019); 2) biosamples that were obtained pre-diagnosis therefore not subjected to any potential biases in collection of data; 3) all biosamples were processed at the same facility and time frame to avoid any discrepancies in sample processing and 4) biolinkage to provincial cancer registries in Alberta and British Columbia to confirm cancer cases.

Limitations of the study include that both the ATP and BCGP cohort do not encompass the same sociodemographic diversity observed in the Canadian population and could limit generalizability. For example, the two cohorts combined identified predominantly as Caucasian (92.4%) and there were a slightly higher proportion of women who were overweight or obese compared to the overall Canadian population (58.3% versus 53.4% according to the Canadian Community Health Survey cycle 2.2) (Robson et al. 2016, Dhalla et al. 2018). In addition, although the biological values obtained are reflective of net metabolic processes and are of scientific merit, dietary intake data was only available for a sub-cohort from the ATP and therefore we cannot confirm to what degree fatty acid status from a single non-fasting plasma sample represents dietary intake for the combined cohorts. Future longitudinal studies would benefit from including this metric in their data collection. Furthermore, it is possible that changes in dietary intake after sample collection could influence future cancer incidence. Finally,

multiple associations were assessed in this study based on biologically justified *a priori* hypotheses. However, accounting for these comparisons with Bonferroni and Holm-Bonferroni corrections for statistical significance would reduce the number of associations observed to reach statistical significance and we interpret these results with caution. The available number of breast cancer cases in each cohort is a limitation of this study and it is possible that a larger cohort is needed to identify small differences in fatty acids associated with breast cancer risk.

3.5 Final Conclusions

Our study is the first to demonstrate regional variations in fatty acid status and subsequent breast cancer risk in a Canadian population. Dietary intake affects long chain n-3 fatty acid status and researchers need to consider that an optimal range and balance or mixture of other fatty acids could influence the protective effect of these long chain PUFAs. Furthermore, that these associations were observed with *a priori* stratification by menopausal status and central adiposity suggests the importance of modifiable dietary intake in addition to other metabolic or endocrine factors that potentially mediate fatty acid status. It highlights the complexity and difficulty in using a single biomarker to predict breast cancer risk. Further investigation into these associations could identify strategies for breast cancer prevention.

CHAPTER 4: DHA incorporation is not affected by doxorubicin chemotherapy in either whole cell or lipid raft phospholipids of breast cancer cells in vitro and tumour phospholipids *in vivo*[3]

4.1 Introduction

Breast cancer is a heterogeneous disease that can be broadly categorized into four main subtypes, Luminal A (estrogen receptor (ER) positive, progesterone receptor (PR) positive/negative, human epidermal growth factor receptor 2 (HER2) negative; ER+PR+/-HER2-), Luminal B (ER+PR+/-HER2+), HER2+ (ER-PR-HER2+) and basal-like which includes triple negative breast cancer (TNBC; ER-PR-HER2-). There are several cell lines frequently used for *in vitro* exploratory studies of breast cancer including MCF-7 and MDA-MB-231 cells. MCF-7 cells are representative of Luminal A; have slower growth and are responsive to both endocrine therapy as well as systemic chemotherapy. MDA-MB-231 cells, representative of TNBC, have more aggressive growth and an intermediate response to chemotherapy (Holliday et al. 2011).

The anti-tumourigenic properties of n-3 long chain polyunsaturated fatty acids (LCPUFA), specifically docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), have been shown in both cell culture and animal studies (Chajes et al. 1995, Schley et al. 2007, Rose et al. 1995, D'Eliseo et al. 2016, Ewaschuk et al. 2012, Newell et al. 2019, Newell et al. 2019). DHA has displayed greater cytotoxicity than EPA at the same concentration in MDA-MB-231 and MCF-7 cells (Barascu et al. 2006, Ewaschuk et al. 2012). Additionally, combinatory effects

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of DHA and chemotherapy drugs have been described in MDA-MB-231 (Ewaschuk et al. 2012, Maheo et al. 2005, Menendez et al. 2005, Germain et al. 1998), MCF-7 (Kang et al. 2010, Maheo et al. 2005), SKBR3 and BT-474 (Menendez et al. 2005) cell lines; in rodent feeding studies (Kang et al. 2010, Newell et al. 2019) and in one human metastatic breast cancer open trial (Bougnoux et al. 2006, Bougnoux et al. 2009).

Enriching DHA in cell membranes could enhance the ability of DHA to elicit an anti-cancer response as increased phospholipid (PL) membrane content of DHA has been shown to change elastic compressibility, flexibility of the acyl chain, fluidity, fusion, rapid flip-flop and phase behavior of the membrane as well as changes to protein function (transporters, receptors, ion channels), ion permeability, lipid raft function, membrane-mediated signaling pathways, production of free oxygen radicals and lipid peroxidation and modulation of gene expression (Stillwell et al. 2005, Stillwell et al. 2003, He et al. 2015, Das 1999). Thus, the n-3 LCPUFA content of membrane PL appear to be key components of a dynamic and asymmetric cellular membrane that can directly impact the cellular function and could thereby influence the cells' ability to respond to chemotherapy.

Lipid rafts are ordered micro domains (10-200 nm) within the cellular membrane that are enriched with sphingolipids, cholesterol, saturated fatty acids and glycosylphosphatidylinositol-linked proteins (Shaw 2006, Simons et al. 2000, Foster et al. 2003, Schley et al. 2007). Incorporation of EPA and DHA into the lipid rafts attained by incubation of cancer cells with DHA is well documented (Schley et al. 2007, Rogers et al. 2010, Biondo et al. 2008) and has been shown to enhance clustering to form large raft domains (Kim et al. 2008, Chapkin et al. 2008, Ma et al. 2004). These domains act as mobile docking platforms, home to cell signaling proteins (including Fas, epidermal growth factor receptor (EGFR), Src, Akt, Ras, Hsp90 and

MLKL), and initiate many cellular events such as protein / lipid trafficking and signal transduction including ones for proliferation, apoptosis or necrosis (Schley et al. 2007, Ewaschuk et al. 2012, Lee et al. 2014, Turk and Chapkin 2013, Chen et al. 2013). DHA is known to disrupt lipid raft signaling (Chapkin et al. 2008, Turk and Chapkin 2013, Lee et al. 2014), which can lead to changes in cell proliferation, apoptosis and survival (Lee et al. 2014).

We have previously determined the efficacy of treating MDA-MB-231 and MCF-7 cells with DHA in conjunction with the chemotoxic drug, doxorubicin (DOX) and the modulation of CD95 protein apoptotic activity within lipid rafts (Ewaschuk et al. 2012). Recently, we have also established the efficacy of DHA in conjunction with DOX on reducing tumour size in *nu/nu* mice implanted with MDA-MB-231 breast cancer cells (Newell et al. 2019). DOX is an anthracycline commonly used in the treatment of breast cancer. Its mode of action is through the inhibition of DNA topoisomerase II, the initiation of reactive oxygen species (ROS) production (Sinha et al. 1989, Conklin 2004) and induction of apoptosis and necrosis (Wei et al. 2015, Gaba et al. 2016, Tacar et al. 2013). For the current study, MDA-MB-231 and MCF-7 cell lines were chosen because they have different growth characteristics, yet both have been demonstrated to respond to n-3 LCPUFA treatment and to chemotherapeutics, albeit differently. The purpose of the current study was two-fold. First, we compared the effects of DHA supplementation and co-treatment with DOX on the membrane lipid composition of MDA-MB-231 and MCF-7 cells. Specifically, we looked at the change in the relative composition of PL classes, DHA incorporation in whole cell versus lipid raft membranes. Second, we explored whether the relative changes in PL DHA content of MDA-MB-231 cells could be extended to the PL from MDA-MB-231 tumours grown in mice fed a DHA supplemented diet and treated with DOX that we have previously demonstrated reduced tumour growth (Newell et al. 2019).

4.2 Methods

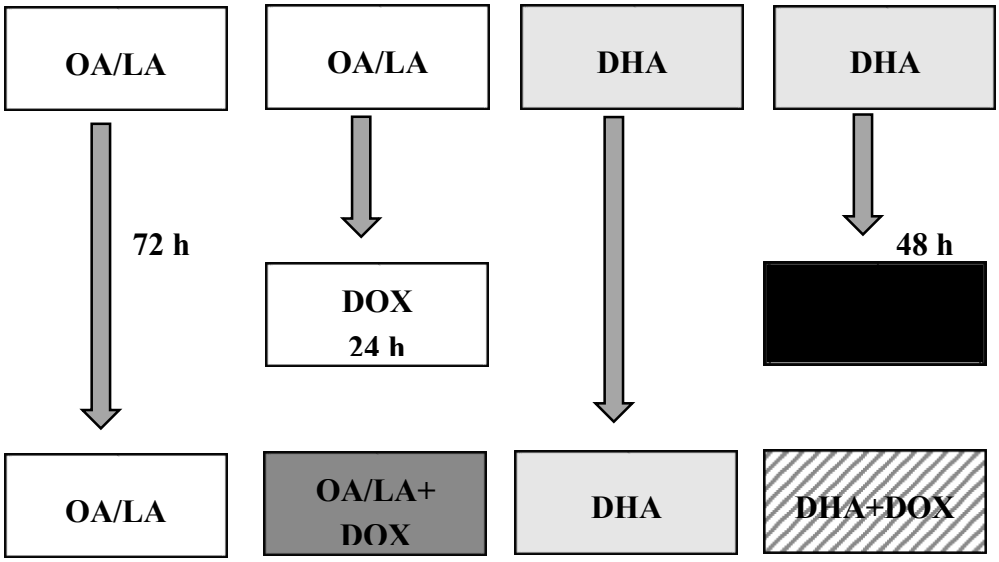
4.2.1 Cell Culture Conditions

MDA-MB-231 and MCF-7 cells were obtained from American Type Culture Collection (Rockville, MD, USA). MDA-MB-231 cells were maintained in Iscove's Modified Dulbecco's medium, and MCF-7 cells in Minimal Essential medium (MEM) with 10 mg/ml insulin and 1% sodium pyruvate. All media was supplemented with 5% vol:vol fetal calf serum (FCS) and 1% vol:vol penicillin and streptomycin (all media components from Fisher Scientific, Edmonton, AB, Canada). Cells were grown at 37° C in 5% carbon dioxide at 98% relative humidity. Bovine serum albumin (BSA, Fisher Scientific, Edmonton, AB, Canada) conjugated DHA, oleic acid (OA) and linoleic acid (LA) (Matreya, MJS Biolynx, Brockville, ON, Canada), were prepared as previously described (Ewaschuk et al. 2012). The concentrations of DOX and fatty acids were determined from previously published cell viability experiments that resulted in decreased viability. Briefly, all cells were supplemented with a control fatty acid background of OA/LA (40 μmol/L OA and 40 μmol/L LA). First, to confirm incorporation necessary for our experimental treatment paradigms, cells were incubated with 60 μmol/L DHA in OA/LA background for 2, 4, 6, 12, 24, 48 and 72 hours and assessed for fatty acid composition (**Appendix Table 5 and Appendix Figure 2**). Subsequently, cells were subjected to 4 conditions: OA/LA (control), DHA (60 μmol/L on OA/LA background), OA/LA+DOX (0.2233 μmol/L for MDA-MB-231 and 0.8427 μmol/L for MCF-7 cells) and DHA+DOX (on OA/LA background) (Ewaschuk et al. 2012).

Cells were seeded at 1 x 10^6 cells per flask (75cm^2) in 15 mL of fatty acid-free medium and allowed to adhere. After 48 hours the medium was replaced with 15 mL of fresh medium containing experimental and background fatty acids and incubated for either 72 hours

(reapplication of media every 24 hours) or 48 hours of fatty acid pre-treatment, followed by DOX for 24 hours. After 72 hours the cells were then harvested using trypsin–EDTA (Fisher Scientific, Edmonton, AB, Canada). (Experimental Layout in **Figure 4-1**).

Figure 4-1: Experimental design of DHA treatment with or without DOX on MDA-MB-231 or MCF-7 breast cancer cells *in vitro*[1]



[1]MDA-MB-231 cells were pre-treated with 40 μM/ 40 μM OA/LA control medium with or without DHA (60 μM) for 72 hours or 48 hours followed by treatment with DOX (0.22 μmol/L for MDA-MB-231 and 0.84 μmol/L for MCF-7 cells) for 24 hours.

4.2.2 Lipid Raft Isolation

Lipid rafts were isolated from the tumour cells as previously described (Schley et al. 2007), with the following modification: samples were centrifuged at 50,000 rpm for 8 h at 4° C. Fraction 3 was identified as the fraction containing the lipid raft based on the presence of raft marker $G_{\alpha i}$. This fraction was diluted to 5 mL with ice-cold TNE (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA), and centrifuged at 50,000 rpm for 38 minutes to pellet the raft.

4.2.3 Experimental Diets and Animals

Animal experiments were reviewed and approved (AUP00000134) by the University of Alberta Animal Policy and Welfare Committee and were in accordance with the Canadian Council on Animal Care guidelines. A basal mix diet formulated based on the AIN-76 diet was obtained from Teklad (TD84172; Harlan Laboratories). The complete macronutrient and micronutrient composition has been previously published by our group (Robinson et al. 1998). To this diet we supplemented 20%wt:wt fat (equivalent to ~40% of total energy from fat, **Table 4-1**). This is on the higher range of fat intake for humans, but is employed to promote tumour growth. The fatty acid composition of the diets was achieved by blending oils to obtain a DHA content in the DHA diet of 2.8% w/w fat. The amount of DHA in the diet was selected to obtain a ~4% plasma PL concentration (Newell et al. 2019) as this concentration has been associated with improved outcomes in metastatic breast cancer patients receiving chemotherapy (Bougnoux et al. 2009). It is a requirement of our animal care facility that diets supplied to immunocompromised animals, including the nude mice in this study, be irradiated to prevent exposure to foodborne pathogens (DeRuiter et al. 2002). Therefore, diets were irradiated for 72 hours at 8 kGy and stored at -20° C until used. Fatty acid analysis by gas liquid chromatography pre- and post-irradiation confirmed that the fat composition was not altered by irradiation (data not shown).

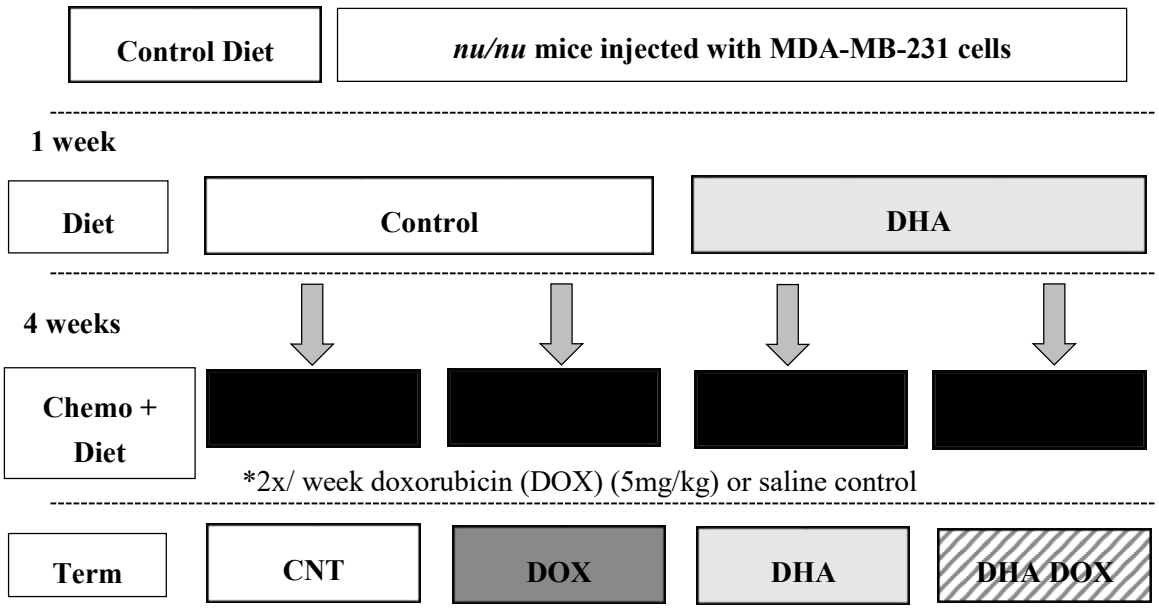
Table 4-1: Major fatty acids in the control and DHA-enriched diet

Fatty acids	Control diet	DHA diet
	g/100 g fat	
16:0	21.5±0.1	21.3±0.8
18:0	11.1±0.3	10.7±0.3
18:1 n-9	47.9±0.3	45.2±2.5
18:2 n-6	13.9±0.0	13.1±1.1
18:3 n-3	ND	0.6±0.0
18:3 n-6	1.13±0.0	0.3±0.0
20:4 n-6	0.5±0.1	0.5±0.0
22:6 n-3	ND	2.8±0.1
Total SFA	33.7±0.4	34.5±1.3
Total MUFA	49.7±0.2	47.2±2.4
Total PUFA	15.0±0.1	17.2±1.2
Total n-3	0	3.4±0.0
Total n-6	15.0±0.1	13.8±1.1
n-6/n-3 ratio	ND	4.1±0.3
P/S ratio	0.5±0.0	0.5±0.0

Values are the mean percentage of 3 batches of diet as determined by gas liquid chromatography after irradiation (Cruz-Hernandez et al. 2013). Diets contained 200 g/kg of fat that was a blend of sunflower oil, fully hydrogenated canola, olive oil, canola and Arasco oil (DSM Nutritional Products USA). The DHA in the DHA diet was provided by adding diet DHAsco (DSM Nutritional Products, USA). Minor fatty acids are not reported; therefore, totals do not add up to 100 %. Abbreviations used: saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), polyunsaturated to saturated fatty acid ratio (P/S), not detected (ND; < 0.05%).

Immune-deficient 6-week old female *nu/nu* mice (Charles River Laboratories International, Inc.) were housed under aseptic conditions with autoclaved bedding and water in biocontainment. Mice were fed the control diet for 3 days and then injected one time subcutaneously below the upper right scapula with MDA-MB-231 cells (2x 10^6 cells/ 100µL in 5% FCS Iscove's medium). Mice were fed control diet *ad libitum* until the tumour reached 50 mm^3 and then randomized into diet groups (control or DHA). Mice were fed *ad libitum* for one week and then further randomized to receive 5 mg/kg DOX chemotherapy or 0.9% saline injections twice weekly for 4 weeks. Body weights and food intake were monitored three times per week throughout the experiment. Mice were euthanized, tumours carefully excised and weighed, formalin fixed for immunohistochemistry or flash frozen for lipid analysis. Individuals performing the excision and weighing of the tumour and all subsequent assays were blinded to the diet treatments (Experimental Layout **Figure 4-2**).

Figure 4-2: Experimental Design of DHA dietary supplementation with or without DOX in *nu/nu* mice injected with MDA-MB-231 breast cancer cells



4.2.4 Phospholipid Class Composition

Major lipid classes were quantified by hydrophilic interaction liquid chromatography-tandem mass spectrometry (HILIC LC-MS/MS) with an Agilent 1200 series HPLC system in tandem with a 3200 QTRAP mass spectrometer (AB SCIEX; Concord, ON, Canada) as previously described (Xiong et al. 2012).

4.2.5 Fatty Acid Composition Analysis

Lipids from whole cells, pelleted rafts and tumours were extracted by modified Folch as previously described (Field et al. 1988, Folch et al. 1957). Total PL and PL classes: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI) and sphingomyelin (SM) were further separated by thin layer chromatography and fatty acid content measured by gas-liquid chromatography on automated GLC 7890A (Agilent Technologies, Mississauga, ON, Canada) on a CP-Sil 88 column (100 m x 0.25 mm, Agilent) as previously described (Cruz-Hernandez et al. 2013).

4.2.6 Immunohistochemistry

Paraffin-embedded tumour sections were deparaffinized as previously described (Newell et al. 2019) and stained with Harris Modified Hematoxylin and Eosin Y (H&E, Fisher Scientific, Edmonton, AB, Canada) to assess morphology and regions of necrosis. Slides were scanned with an Aperio Scanner and relative percent of necrosis was determined with Image Scope software.

4.2.7 Statistical Analyses

All statistical analyses were conducted using the SAS, version 9.4 (SAS Institute, Cary, NC, USA). Data are reported as means ±SEM. Data were tested for normal distribution and, once confirmed, analyzed by two-way analysis of variance (ANOVA), to assess drug and diet interactions, using a PROC GLM method. For distribution of PL classes, a three-way ANOVA

using a PROC MIXED procedure. While differences of $P \geq 0.05$ were considered significant, in some instances, *P* values are provided where there is a trend towards significance. Independent 2-tailed t-tests were performed for fold change comparisons of cell lines.

4.3 Results

4.3.1 Phospholipid Class Relative Composition of Whole Cell Membrane and Lipid Rafts of MDA-MB-231 cells and MCF-7 cells

We first sought to determine the relative composition of PL classes of both whole cell and lipid raft PL of MDA-MB-231 and MCF-7 cells treated with DHA with or without DOX chemotherapy. In both whole cell and plasma membrane lipid rafts, phosphatidylcholine (PC, 46.8- 58.1%) and phosphatidylethanolamine (PE, 20.2- 32.0%) were found to be the most abundant lipid classes in the two cell lines (**Table 4-2**). In MDA-MB-231 cells, the relative amount of PE, phosphatidylinositol (PI) and PC was higher, and the relative amount of phosphatidylserine (PS) and sphingomyelin (SM) was lower in whole cell compared to rafts ($P<0.04$). In MCF-7 cells, the relative amount of PE, PI, and PS was higher, and the relative amount of PC and SM was lower in whole cell compared to rafts ($P<0.002$). Most notably, the SM content of rafts was 4% higher in MDA-MB-231 cells and 7% higher in MCF-7 cells compared to whole cell membranes, confirming lipid raft isolation (Sezgin et al. 2017). There were no fatty acid treatment effects on the relative composition of PL classes but a chemotherapy treatment effect was observed in three instances. In MDA-MB-231 cells, the relative amount of PE and LysoPC in DOX treated cells, was lower than non-DOX treated cells ($P<0.04$) and the relative amount of PS in MCF-7 DOX treated cells, was higher than non-DOX treated cells ($P<0.0001$). A media treatment and membrane type interaction was observed in MDA-MB-231 cells, resulting in higher relative amount of PI in cells that received DHA ($P<0.05$). In both cell

lines there was a significant interaction between DOX and membrane type regarding the relative amount of PC and PS ($P<0.05$). Specifically, cells that received DOX had higher PC content in whole cell membranes and lower in raft membranes in both cell lines. MDA-MB-231 cells that received DOX had lower relative amounts in whole cell membranes cells and higher relative amounts of PS in lipid rafts ($P<0.05$).

4.3.2 Characterization of Whole Cell and Lipid Raft Membrane fatty acid composition of the PL classes in MDA-MB-231 and MCF-7 cells

We then sought to determine the fatty acid composition of the five main PL classes in whole cell and lipid raft membranes of both MDA-MB-231 and MCF-7 breast cancer cells treated with DHA and in the presence or absence of DOX. As expected, DHA treatment compared to OA/LA control, with or without DOX significantly increased the DHA content in both cell lines and in all PL classes except for SM, likely due to its' high level of saturation (**Figure 4-3**). Overall, there was a higher relative percent incorporation of DHA into the four PL moieties in MDA-MB-231 cells (**Figure 4-3 A & C**, $P<0.0001$) compared to MCF-7 cells (**Figure 4-3 B & D**, $P<0.001$), in both whole cell and raft lipids. DOX treatment affected the amount of DHA present, resulting in lower DHA accumulation compared to DHA alone in PI of MDA-MB-231whole cell membranes and PE, PC and PI in lipid rafts in both cell lines (**Figure 4-3**, $P<0.05$).

Table 4-2: Phospholipid class composition (relative % of total PL) of whole cell and lipid raft membranes for MDA-MB-231 and MCF-7 breast cancer cells

	WHOLE CELL MEMBRANE				LIPID RAFT				P^{Mem}	P^{Drug}	P^{Media}	P^{Int}
MDA-MB-231	OA/ LA	OA/LA+ DOX	DHA	DHA+ DOX	OA/ LA	OA/LA+ DOX	DHA	DHA+ DOX				
PE	22.8±0.1	22.5±0.2	24.2±1.3	21.6±0.4	21.8±0.0	21.3±0.9	21.3±0.3	20.2±0.5	***P*≤0.01**	***P*≤0.05**	NS	
PI	6.4±0.1	6.0±0.4	7.5±0.2	6.8±0.1	6.4±0.1	6.1±0.2	5.7±0.6	6.2±0.4	***P*≤0.05**	NS	NS	**Med*Mem *P*≤0.05**
PS	6.6±0.9	4.9±0.1	7.9±0.3	6.1±0.3	8.4±0.5	9.8±0.9	8.3±1.4	8.9±1.0	***P*≤0.001**	NS	NS	**Drug*Mem *P*≤0.05**
PC	54.6±1.1	58.1±0.8	52.0±1.2	57.4±0.6	51.5±0.8	50.3±1.8	52.4±2.1	52.5±1.0	***P*≤0.001**	NS	NS	**Drug *Mem *P*≤0.05**
SM	8.8±0.2	8.0±0.4	7.9±0.2	7.7±0.2	11.6±0.4	12.3±0.3	12.0±0.4	11.9±1.0	***P*<0.001**	NS	NS	
LysoPC	0.7±0.0	0.4±0.0	0.5±0.0	0.5±0.0	0.3±0.1	0.3±0.0	0.3±0.0	0.3±0.0	***P*<0.001**	***P*=0.05**	*P*=0.09	**Drug *Mem *P*≤0.01**
MCF-7												
PE	32.0±0.4	29.3±0.7	31.2±0.2	28.8±0.4	23.2±1.0	27.4±2.9	25.3±1.6	24.0±0.6	***P*<0.001**	NS	NS	Drug *Mem *P*=0.06
PI	9.0±0.8	8.9±0.1	8.9±0.4	8.9±0.3	8.1±0.2	7.7±0.3	7.3±0.4	8.1±0.4	***P*≤0.005**	NS	NS	
PS	7.1±0.2	7.8±0.3	7.2±0.3	7.5±0.2	2.6±0.2	5.2±0.1	2.8±0.4	4.9±0.2	***P*<0.001**	***P*<0.001**	NS	**Drug*Mem *P*≤0.001**
PC	46.8±1.2	48.9±1.2	47.8±0.8	50.4±0.4	53.1±1.1	48.0±1.4	53.0±1.3	51.5±0.5	***P*≤0.005**	NS	NS	**Drug*Mem *P*≤0.005**
SM	5.1±0.3	5.0±0.2	5.0±0.2	4.3±0.5	12.5±1.0	11.0±1.2	11.1±0.6	11.1±0.5	***P*<0.001**	NS	NS	
LysoPC	ND	ND	ND	ND	0.5±0.1	0.6±0.1	0.5±0.1	0.5±0.1	NA	NS	NS	

OA/LA: 40 μM OA/ 40 μM LA; DHA: 60 μM DHA 40 μM OA/ 40 μM LA; DOX: 0.22 μmol/L for MDA-MB-231 and 0.84 μmol/L for MCF-7 cells; Values represent the mean ± SEM (n=4 independent experiments). Data were analyzed using a 3-factor ANOVA to assess fatty acid supplemented media, drug and membrane type interactions within each cell type. P^{Mem} is the *P* value for the main effect of membrane type in the MIXED model; P^{Drug} is the *P* value for the main effect of the drug in the MIXED model; P^{Media} is the *P* value for the main effect of the fatty acid supplementation in the MIXED model; P^{Int} is the *P* value for the main effect of an interaction between media, drug and /or membrane in the MIXED model and is only indicated if significance was observed.

Figure 4-3. DHA incorporation into PL classes of MDA-MB-231 and MCF-7 whole cell and lipid raft membranes

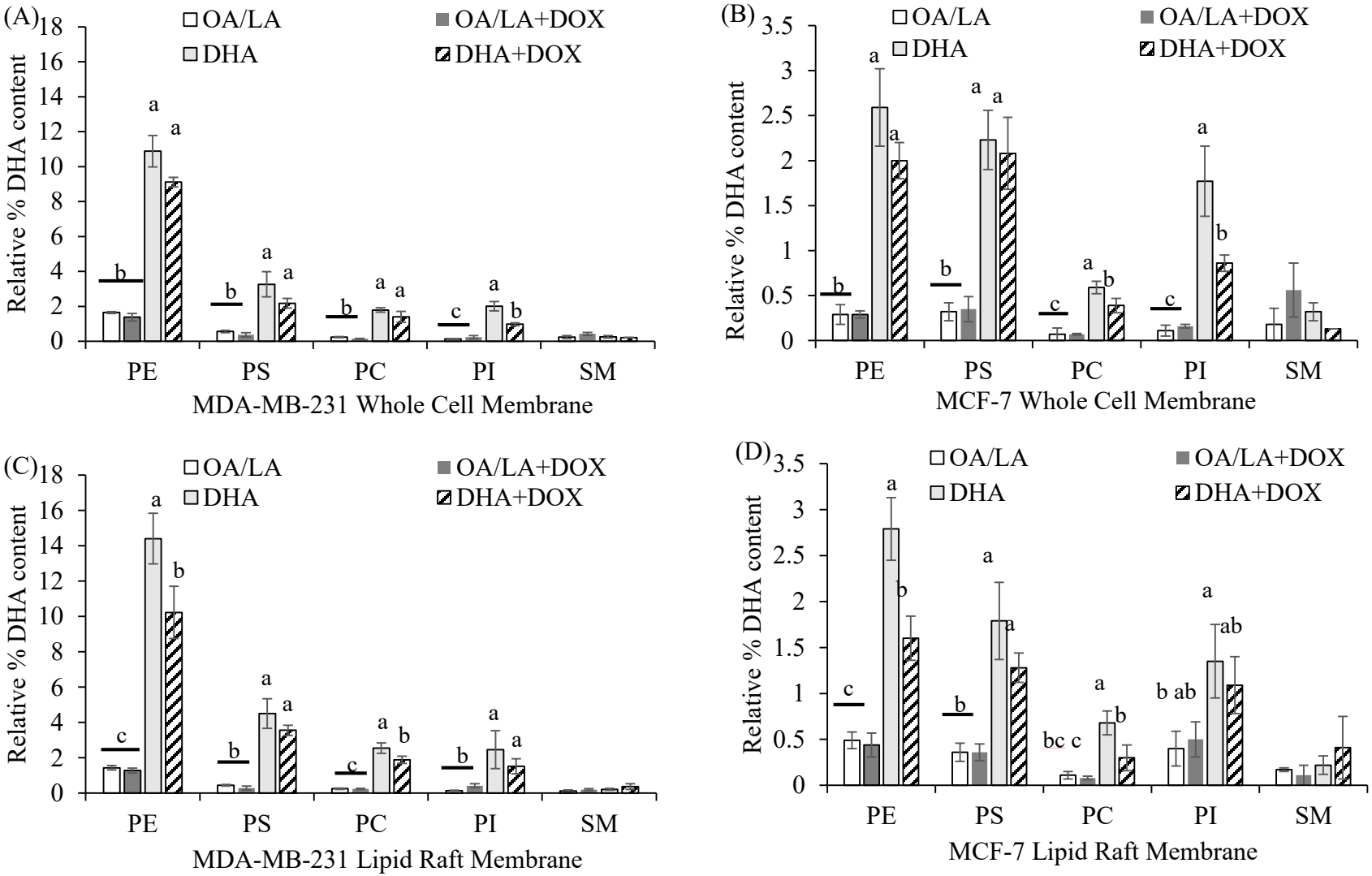
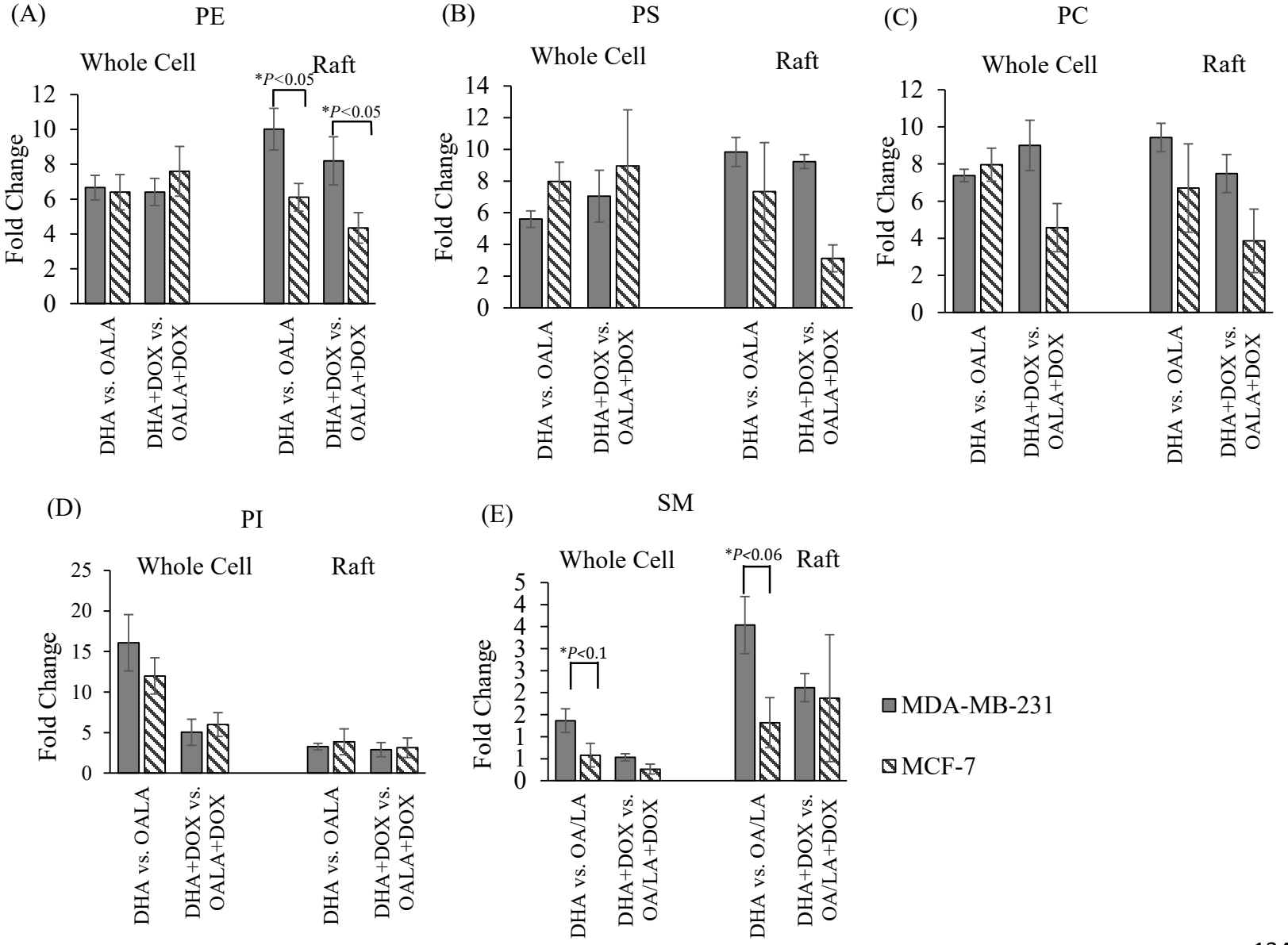


Figure 4-4. Fold change of DHA incorporation into MDA-MB-231 and MCF-7 cells, whole cell membrane and lipid raft membrane in Phosphatidylethanolamine, Phosphatidylserine, Phosphatidylcholine, Phosphatidylinositol and Sphingomyelin



To account for the faster rate of growth by MDA-MB-231 cells, we compared the fold change differences of DHA in each cell line and membrane type: DHA compared to OA/LA and DHA+DOX compared to OA/LA+DOX (**Figure 4-4 A-E**). No significant differences between cell lines were observed in the relative fold change in DHA incorporation in the whole cell yet significantly higher fold change incorporation in PE (**Figure 4-4 A**, $P<0.05$) and PS (**Figure 4-4 B**, $P<0.005$) were observed in lipid raft membranes.

Differences in the composition of other fatty acids were also measured (**Tables 4-3 to 4-12**; $P<0.05$). In the whole cell membranes of MDA-MB-231 cells, treatment with DHA in the presence or absence DOX, resulted in lower proportion of arachidonic acid (ARA) in PE, PC and PI; lower C24:1 n-9 in PE, PC, PS, PI and SM, lower OA in PS and PC; higher eicosapentaenoic acid (EPA) in PE, PI and SM (DHA+DOX only) and higher docosapentaenoic acid (DPA) n-3 in PC and PI compared to OA/LA and OA/LA+DOX. Additionally, whole cell membrane SM composition of MDA-MB-231 cells treated with DHA+DOX had higher alpha-linolenic acid than cells treated with DHA alone (**Tables 4-3, 4-5, 4-7, 4-9** and **4-11**; $P<0.05$). In lipid rafts of MDA-MB-231 cells, treatment with DHA in the presence or absence DOX, resulted in lower proportion of ARA in PE and PC; lower C24:1 n-9 in PE and PC; lower OA in PS and PC; higher EPA in PE and higher (DPA)n-3 in PE and PC compared to OA/LA and OA/LA+DOX (**Tables 4-3, 4-5, 4-7, 4-9** and **4-11**; $P<0.05$). The membrane fatty acid composition of MCF-7 cells was not altered to the same extent as observed in MDA-MB-231 cells. In the MCF-7 whole cell membrane moieties, there was lower C24:1 n-9 observed in PE, lower DPA(n-3) in PE, PC and PI and lower OA in PS and SM in cells treated with DHA or DHA+DOX compared to OA/LA or OA/LA+DOX (**Tables 4-4, 4-6, 4-8, 4-10** and **4-12**; $P<0.05$).

Table 4-3: Fatty Acid composition (relative percent) of PE phospholipids from whole cell and lipid raft membranes of MDA-MB-231 breast cancer cells

	Whole Cell				*P* values			Lipid Raft				*P* values		
Fatty Acids	OA/LA	OA/LA+ DOX	DHA	DHA+ DOX	P^{Media}	P^{Drug}	P^{Int}	OA/LA	OA/LA+ DOX	DHA	DHA+ DOX	P^{Media}	P^{Drug}	P^{Int}
14:0	0.8±0.4	0.3±0.1	0.7±0.2	1.8±1.2	ns	ns	ns	0.5±0.1	0.5±0.1	0.6±0.1	0.6±0.1	*P*<0.07	ns	ns
16:0	7.0±0.21	7.6±0.6	7.1±0.2	6.4±0.2	ns	ns	*P*<0.04	8.3±0.7	8.3±0.9	7.4±0.7	8.1±0.2	ns	ns	ns
16:1 n-9	0.5±0.0	0.4±0.1	0.4±0.1	0.5±0.0	ns	ns	ns	0.5±0.0	0.5±0.0	0.5±0.1	0.5±0.0	ns	ns	ns
17:0	0.7±0.1	0.6±0.1	0.6±0.1	0.6±0.2	ns	ns	ns	0.6±0.2	0.5±0.1	0.5±0.0	0.5±0.1	ns	ns	ns
18:0	23.8±0.6	25.5±0.7	27.7±1.2	25.4±2.1	*P*<0.10	ns	ns	25.9±1.3	26.2±0.9	27.4±1.5	27.7±1.2	ns	ns	ns
18:1 n-9	30.0±0.3	29.2±0.7	24.9±0.7	24.0±1.3	*P*<0.0001	ns	ns	27.±0.7	27.5±2.4	22.6±0.6	24.5±1.6	*P*<0.02	ns	ns
18:2 n-6	11.0±1.4	11.2±1.6	12.0±1.0	13.2±1.8	ns	ns	ns	6.0±0.5	8.1±1.2	8.4±0.7	9.6±1.1	*P*<0.10	*P*<0.13	ns
18:3 n-6	0.6±0.2	0.8±0.2	0.4±0.1	0.6±0.1	ns	ns	ns	0.7±0.0	0.6±0.1	0.5±0.0	0.5±0.1	*P*<0.06	ns	ns
20:2 n-6	1.4±0.3	1.7±0.0	1.5±0.2	1.6±0.2	ns	ns	ns	0.8±0.1	0.9±0.3	0.9±0.1	0.9±0.3	ns	ns	ns
20:3 n-6	1.8±0.1	1.9±0.1	1.9±0.3	2.0±0.4	ns	ns	ns	2.2±0.2	2.0±0.2	2.5±0.5	2.4±0.4	ns	ns	ns
20:4 n-6	12.6±1.1	11.6±1.0	7.9±1.0	8.8±1.4	*P*<0.008	ns	ns	14.6±1.9	14.5±2.9	7.9±1.4	8.9±1.2	*P*<0.009	ns	ns
20:5 n-3	0.2±0.0	0.2±0.0	1.0±0.1	0.8±0.1	*P*<0.0001	ns	ns	0.4±0.1	0.4±0.1	1.4±0.1	1.4±0.2	*P*<0.0001	ns	ns
24:0	0.3±0.1	0.4±0.2	0.2±0.1	0.3±0.1	ns	ns	ns	0.6±0.0	0.5±0.0	1.0±0.3	0.6±0.2	ns	ns	ns
24:1 n-9	5.6±0.3	5.7±0.4	1.2±0.3	1.5±0.2	*P*<0.0001	ns	ns	7.8±0.6	6.6±0.9	1.8±0.2	1.8±0.1	*P*<0.0001	ns	ns
22:5 n-6	0.2±0.0	0.1±0.0	0.1±0.0	0.2±0.0	ns	ns	*P*<0.13	0.3±0.1	0.1±0.0	0.2±0.0	0.1±0.0	ns	*P*<0.10	ns
22:5 n-3	1.3±0.0	1.2±0.3	1.2±0.1	1.3±0.1	ns	ns	ns	1.3±0.1	1.2±0.1	1.6±0.1	1.7±0.2	*P*<0.009	ns	ns
22:6 n-3	1.6±0.0	1.4±0.2	10.9±0.9	9.1±0.3	*P*<0.0001	*P*<0.10	ns	1.4±0.1	1.3±0.1	14.4±1.4	10.2±1.5	*P*<0.0001	*P*<0.07	*P*<0.11
SFA	32.7±0.3	34.3±0.4	36.4±0.8	35.8±0.4	*P*<0.0009	ns	*P*<0.10	36.6±1.9	38.6±3.4	37.0±2.2	37.4±1.3	ns	ns	ns
MUFA	36.1±0.4	35.3±0.7	26.6±0.6	25.9±1.1	*P*<0.0001	ns	ns	35.2±0.1	32.2±3.3	24.9±0.5	26.8±1.7	*P*<0.004	ns	ns
n-6 PUFA	27.4±0.3	26.8±1.1	23.6±0.6	26.4±0.6	*P*<0.0006	*P*<0.03	*P*<0.008	24.5±1.9	25.2±1.3	20.1±1.4	22.0±0.3	*P*<0.02	ns	ns
n-3 PUFA	3.7±0.3	3.5±0.7	13.5±0.8	11.9±0.3	*P*<0.0001	ns	ns	3.8±0.2	4.1±0.6	18.0±1.6	13.8±1.9	*P*<0.0001	*P*<0.16	*P*<0.11

OA/LA: 40 μM OA/ 40 μM LA; DHA: 60 μM DHA 40 μM OA/ 40 μM LA; DOX: 0.22 μmol/L. Values are % relative to the total fatty acid content ± SE. P^{Media}: *P* value from the main effect of the fatty acid supplementation in the PROC GLM procedure, P^{Drug}: *P* value from the main effect of DOX in the PROC GLM procedure. P^{Int}: *P* interaction between media and DOX in the PROC GLM procedure.

Table 4-4: Fatty Acid composition (relative percent) of PE phospholipids from whole cell and lipid raft membranes of MCF-7 breast cancer cells

	Whole Cell				*P* values			Lipid Raft				*P* values		
Fatty Acids	OA/LA	OA/LA+ DOX	DHA	DHA+ DOX	P^{Media}	P^{Drug}	P^{Int}	OA/LA	OA/LA+ DOX	DHA	DHA+ DOX	P^{Media}	P^{Drug}	P^{Int}
14:0	0.3±0.0	0.3±0.0	0.8±0.4	0.3±0.0	ns	ns	ns	1.4±0.4	1.8±0.4	1.5±0.4	1.4±0.1	ns	ns	ns
16:0	6.1±0.1	6.0±0.3	7.3±0.6	5.8±0.2	ns	*P*<0.03	*P*<0.08	10.5±1.6	12.3±1.0	12.2±0.9	11.8±0.9	ns	ns	ns
16:1 n-9	0.8±0.3	0.7±0.2	1.0±0.1	1.1±0.4	ns	ns	ns	0.9±0.2	1.2±0.2	1.0±0.0	1.0±0.2	ns	ns	ns
17:0	0.9±0.3	0.6±0.1	0.8±0.1	0.5±0.1	ns	ns	ns	0.7±0.1	1.1±0.4	1.0±0.3	0.8±0.2	ns	ns	ns
18:0	20.8±1.3	24.7±2.2	24.4±1.5	21.9±1.8	ns	ns	*P*<0.12	25.5±0.81	25.0±0.8	28.2±1.0	26.6±1.2	*P*<0.05	ns	ns
18:1 n-9	43.8±0.5	42.0±1.0	37.4±0.7	42.5±0.8	*P*<0.01	*P*<0.03	*P*<0.002	31.5±2.5	30.2±0.7	27.2±1.2	29.4±0.8	*P*<0.11	ns	ns
18:2 n-6	18.6±0.5	18.2±1.4	19.4±0.6	18.9±0.4	ns	ns	ns	19.6±1.0	18.8±1.1	18.1±1.0	19.3±0.8	ns	ns	ns
18:3 n-6	1.2±0.2	0.8±0.1	0.6±0.2	0.9±0.0	*P*<0.06	ns	*P*<0.03	1.4±0.1	1.2±0.0	1.0±0.1	0.9±0.2	*P*<0.01	ns	ns
20:2 n-6	1.0±0.1	1.1±0.2	0.7±0.1	0.8±0.0	*P*<0.03	ns	ns	1.7±0.2	1.6±0.1	1.2±0.2	1.5±0.2	ns	ns	ns
20:3 n-6	0.6±0.1	0.6±0.1	0.7±0.1	0.6±0.1	ns	ns	ns	0.8±0.2	0.4±0.1	0.7±0.1	0.7±0.2	ns	ns	ns
20:4 n-6	3.9±0.5	2.7±0.5	3.2±0.4	3.±0.4	ns	ns	ns	3.9±1.0	3.5±0.2	3.2±0.6	3.2±0.2	ns	ns	ns
20:5 n-3	0.3±0.1	0.2±0.0	0.2±0.1	0.2±0.0	ns	ns	ns	0.1±0.0	0.1±0.0	0.3±0.1	0.4±0.2	ns	ns	ns
24:0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	ns	ns	ns	0.1±0.1	0.2±0.0	0.2±0.0	0.4±0.3	ns	ns	ns
24:1 n-9	0.2±0.1	0.3±0.0	0.1±0.0	0.1±0.0	*P*<0.0007	ns	ns	0.2±0.0	0.2±0.1	0.1±0.0	0.2±0.1	ns	ns	ns
22:5 n-6	0.1±0.0	0.1±0.0	0.2±0.1	0.1±0.0	ns	ns	ns	0.2±0.1	0.2±0.1	0.1±0.0	0.1±0.0	ns	ns	ns
22:5 n-3	0.8±0.1	0.7±0.1	0.2±0.0	0.4±0.0	*P*<0.0005	ns	ns	0.6±0.1	0.5±0.1	0.3±0.0	0.3±0.1	*P*<0.003	ns	ns
22:6 n-3	0.3±0.1	0.3±0.0	2.6±0.4	2.0±0.2	*P*<0.0001	*P*<0.04	ns	0.5±0.1	0.4±0.1	2.8±0.3	1.6±0.2	*P*<0.0001	*P*<0.02	*P*<0.02
Σ SFA	28.3±1.6	32.0±2.3	33.5±0.8	29.4±1.2	ns	ns	*P*<0.08	38.3±2.6	40.5±1.0	43.0±2.1	41.1±1.6	ns	ns	ns
Σ MUFA	44.8±0.8	43.0±1.2	38.6±0.8	43.7±0.7	*P*<0.03	*P*<0.04	*P*<0.004	32.6±2.4	31.7±0.8	28.4±1.2	30.5±0.8	ns	ns	ns
Σ n-6 PUFA	24.5±0.9	23.0±2.1	24.3±1.1	23.5±0.9	ns	ns	ns	26.6±1.6	25.1±0.8	24.2±1.3	25.1±0.8	ns	ns	ns
Σ n-3 PUFA	2.5±0.3	2.0±0.3	3.7±0.5	3.4±0.2	*P*<0.003	*P*<0.13	ns	2.6±0.2	2.7±0.4	4.4±0.4	3.2±0.4	*P*<0.001	*P*<0.02	*P*<0.10

OA/LA: 40 μM OA/ 40 μM LA; DHA: 60 μM DHA 40 μM OA/ 40 μM LA; DOX: 0.84 μmol/L for MCF-7 cells. Values are percentages relative to the total fatty acid content ± SE. Data were analyzed using a 2-factor ANOVA to assess media and drug interactions within each cell type. P^{Media}: *P* value from the main effect of the fatty acid supplementation in the PROC GLM procedure, P^{Drug}: *P* value from the main effect of DOX in the PROC GLM procedure. P^{Int}: *P* interaction between media and DOX in the PROC GLM procedure.

Table 4-5: Fatty acid composition (relative percent) of PS phospholipids from whole cell and lipid raft membranes of MDA-MB-231 breast cancer cells

	Whole Cell				*P* values			Lipid Raft				*P* values		
Fatty Acids	OA/LA	OA/LA+ DOX	DHA	DHA+ DOX	P^{Media}	P^{Drug}	P^{Int}	OA/LA	OA/LA+ DOX	DHA	DHA+ DOX	P^{Media}	P^{Drug}	P^{Int}
14:0	0.5±0.1	2.2±1.1	0.4±0.0	1.4±0.3	ns	*P*<0.06	ns	0.8±0.1	1.2±0.3	1.2±0.4	0.7±0.2	ns	ns	ns
16:0	6.5±0.4	5.8±0.9	8.0±0.8	6.7±0.6	*P*<0.10	ns	ns	9.4±1.3	9.2±2.6	8.6±0.2	8.0±0.5	ns	ns	ns
16:1 n-9	0.6±0.1	0.5±0.0	0.6±0.1	0.6±0.2	ns	ns	ns	0.5±0.0	0.9±0.2	0.7±0.1	0.6±0.1	ns	ns	*P*<0.08
17:0	0.7±0.0	0.6±0.1	0.6±0.2	0.7±0.0	ns	ns	ns	0.±0.0	0.5±0.0	0.6±0.0	0.5±0.1	ns	ns	ns
18:0	39.4±1.3	36.8±1.0	42.6±3.4	44.8±1.6	*P*<0.04	ns	ns	37.0±1.8	37.4±1.4	41.3±0.8	38.3±1.0	*P*<0.09	ns	ns
18:1 n-9	23.6±1.3	21.0±1.3	19.9±0.9	19.0±0.8	*P*<0.02	ns	ns	25.3±0.8	26.3±2.2	18.6±1.8	21.9±2.0	*P*<0.02	ns	ns
18:2 n-6	8.1±1.0	10.8±0.1	9.6±0.7	10.4±1.6	ns	ns	ns	5.6±0.8	10.0±0.6	7.0±1.8	9.0±1.8	ns	ns	ns
18:3 n-6	0.6±0.1	0.5±0.2	0.3±0.0	0.5±0.1	ns	ns	ns	0.6±0.1	0.5±0.1	0.4±0.0	0.4±0.1	ns	ns	ns
20:2 n-6	0.8±0.1	1.1±0.2	0.9±0.1	0.9±0.2	ns	ns	ns	0.5±0.0	0.3±0.1	0.6±0.1	0.5±0.1	*P*<0.05	*P*<0.06	ns
20:3 n-6	2.6±0.5	2.5±0.5	2.6±0.5	2.3±0.7	ns	ns	ns	3.0±0.1	2.6±0.4	2.8±0.2	3.0±0.2	ns	ns	ns
20:4 n-6	2.3±0.1	2.1±0.2	1.5±0.4	1.4±0.0	*P*<0.03	ns	ns	1.7±0.4	1.7±0.3	0.9±0.1	1.1±0.2	*P*<0.03	ns	ns
20:5 n-3	0.3±0.0	0.2±0.1	0.1±0.01	0.3±0.1	ns	ns	*P*<0.04	0.2±0.1	0.2±0.1	0.1±0.0	0.1±0.0	ns	ns	ns
24:0	3.6±0.7	3.7±0.5	2.7±0.4	3.7±0.1	ns	ns	ns	4.5±1.9	2.5±0.8	3.1±0.9	2.7±1.4	ns	ns	ns
24:1 n-9	6.9±0.9	6.0±1.3	3.0±0.3	3.5±0.3	*P*<0.002	ns	ns	6.2±1.0	5.8±1.3	4.1±1.2	3.7±1.2	*P*<0.12	ns	ns
22:4 n-6	0.3±0.1	0.3±0.0	0.2±0.1	0.2±0.0	ns	ns	ns	0.3±0.0	0.3±0.1	0.2±0.1	0.2±0.1	ns	ns	ns
22:5 n-6	1.7±0.3	1.4±0.0	0.7±0.1	1.2±0.1	ns	ns	ns	0.4±0.0	0.3±0.0	0.4±0.1	0.5±0.1	ns	ns	ns
22:5 n-3	0.4±0.0	0.4±0.1	0.3±0.1	0.2±0.1	ns	ns	ns	0.4±0.0	0.3±0.1	4.5±0.8	3.6±0.3	*P*<0.08	ns	ns
22:6 n-3	0.6±0.1	0.4±0.1	3.3±0.7	2.2±0.3	*P*<0.0004	ns	ns	0.4±0.0	0.3±0.1	4.5±0.8	3.6±0.3	*P*<0.0001	ns	ns
Σ SFA	51.1±0.7	49.4±2.5	55.7±2.5	57.5±0.6	*P*<0.008	ns	ns	54.6±1.2	52.2±2.3	58.9±2.1	54.6±3.4	ns	ns	ns
Σ MUFA	31.0±0.9	27.9±0.8	23.4±1.0	23.0±0.7	*P*<0.0001	*P*<0.09	ns	32.2±0.3	33.0±1.3	23.3±0.8	26.3±1.2	*P*<0.0001	*P*<0.12	ns
Σ n-6 PUFA	16.0±1.3	21.1±2.4	16.9±1.6	16.2±1.0	ns	ns	*P*<0.11	11.6±0.8	13.5±1.4	12.1±1.6	14.4±1.8	ns	ns	ns
Σ n-3 PUFA	1.9±0.1	1.6±0.2	4.0±0.8	3.2±0.3	*P*<0.003	ns	ns	1.6±0.2	1.3±0.2	5.7±0.8	4.6±0.4	*P*<0.0001	ns	ns

OA/LA: 40 μM OA/ 40 μM LA; DHA: 60 μM DHA 40 μM OA/ 40 μM LA; DOX: 0.22 μmol/L for MDA-MB-231 cells. Values are percentages relative to the total fatty acid content ± SE. Data were analyzed using a 2-factor ANOVA to assess media and drug interactions within each cell type. P^{Media}: *P* value from the main effect of the fatty acid supplementation in the PROC GLM procedure, P^{Drug}: *P* value from the main effect of DOX in the PROC GLM procedure. P^{Int}: *P* interaction between media and DOX in the PROC GLM procedure.

Table 4-6: Fatty acid composition (relative percent) of PS phospholipids from whole cell and lipid raft membranes of MCF-7 breast cancer cells

	Whole Cell				*P* values			Lipid Raft				*P* values		
Fatty Acids	OA/LA	OA/LA+ DOX	DHA	DHA+ DOX	P^{Media}	P^{Drug}	P^{Int}	OA/LA	OA/LA+ DOX	DHA	DHA+ DOX	P^{Media}	P^{Drug}	P^{Int}
14:0	1.2±0.4	2.0±0.2	2.1±0.4	1.5±0.3	ns	ns	*P*<0.08	1.7±0.3	3.8±0.6	1.9±0.3	3.4±0.3	ns	*P*<0.006	ns
16:0	6.8±0.2	7.1±0.4	12.0±2.6	6.6±0.7	ns	ns	ns	13.8±1.1	13.9±3.4	21.0±3.1	19.1±2.6	*P*<0.06	ns	ns
16:1 n-9	0.8±0.2	1.4±0.3	1.2±0.2	1.0±0.2	ns	ns	*P*<0.08	1.5±0.2	1.6±0.3	0.9±0.2	1.4±0.4	*P*<0.10	ns	ns
17:0	1.2±0.2	1.2±0.3	1.3±0.2	1.1±0.2	ns	ns	ns	0.6±0.0	0.5±0.1	3.2±1.7	1.4±0.4	ns	ns	ns
18:0	45.0±3.0	39.3±0.8	35.2±5.5	40.8±1.8	ns	ns	ns	33.5±3.5	33.1±2.5	31.8±2.9	36.8±0.3	ns	ns	ns
18:1 n-9	25.4±0.9	28.2±14.0	26.4±2.1	26.8±2.0	ns	ns	ns	21.6±3.5	22.0±4.0	19.9±3.9	17.0±1.5	ns	ns	ns
18:2 n-6	10.5±1.7	12.7±1.6	12.8±1.9	12.6±0.6	ns	ns	ns	12.5±3.1	13.8±3.5	7.9±2.4	9.0±0.2	ns	ns	ns
18:3 n-6	0.6±0.0	0.8±0.1	0.6±0.1	0.6±0.0	ns	ns	ns	0.8±0.2	0.7±0.1	1.4±0.4	0.5±0.1	ns	ns	ns
20:2 n-6	0.7±0.2	0.7±0.2	0.8±0.2	0.6±0.1	ns	ns	ns	0.5±0.1	0.4±0.1	0.5±0.2	0.8±0.4	ns	ns	ns
20:3 n-6	1.2±0.0	0.9±0.1	1.1±0.2	1.2±0.3	ns	ns	ns	0.8±0.3	0.9±0.2	0.8±0.1	1.3±0.2	ns	ns	ns
20:4 n-6	2.1±0.6	2.1±0.5	1.9±0.2	2.1±0.4	ns	ns	ns	2.2±1.0	2.4±0.8	1.4±0.3	2.3±0.6	ns	ns	ns
20:5 n-3	0.5±0.1	0.7±0.3	0.4±0.2	0.5±0.2	ns	ns	ns	0.2±0.0	0.4±0.2	0.1±0.0	0.2±0.2	ns	ns	ns
24:0	0.5±0.2	0.5±0.1	0.4±0.1	0.6±0.1	ns	ns	ns	0.7±0.2	0.9±0.3	0.9±0.4	1.3±0.4	ns	ns	ns
24:1 n-9	1.2±0.3	0.8±0.0	0.7±0.1	0.9±0.2	ns	ns	ns	1.4±0.4	2.0±0.7	0.8±0.1	2.2±0.9	ns	*P*<0.12	ns
22:4 n-6	0.37±0.1	0.3±0.1	0.2±0.1	0.2±0.0	ns	ns	ns	0.3±0.1	0.4±0.2	0.1±0.0	0.5±0.4	ns	ns	ns
22:5 n-6	0.4±0.1	0.6±0.3	0.5±0.1	0.5±0.2	ns	ns	ns	0.4±0.2	0.8±0.3	0.2±0.1	0.7±0.3	ns	*P*<0.10	ns
22:5 n-3	0.7±0.2	0.5±0.2	0.3±0.0	0.4±0.1	*P*<0.03	ns	ns	0.5±0.1	0.4±0.2	0.2±0.1	0.5±0.2	ns	ns	ns
22:6 n-3	0.3±0.1	0.4±0.1	2.2±0.3	2.1±0.4	*P*<0.0001	ns	ns	0.4±0.1	0.1±0.1	1.8±0.4	1.3±0.2	*P*<0.0008	ns	ns
Σ SFA	55.2±3.4	50.1±1.1	50.943.2	50.6±1.1	ns	ns	ns	56.9±7.2	59.5±8.7	60.2±7.4	61.9±1.	ns	ns	ns
Σ MUFA	27.4±0.7	30.5±1.2	28.3±2.2	28.7±1.9	ns	ns	ns	24.5±3.7	22.0±4.6	21.6±4.0	21.1±0.12	ns	ns	ns
Σ n-6 PUFA	15.3±2.7	17.2±1.0	17.3±1.7	17.1±0.8	ns	ns	ns	16.7±3.3	16.6±3.9	14.5±3.5	14.5±1.7	ns	ns	ns
Σ n-3 PUFA	2.1±0.5	2.3±0.4	3.4±0.4	3.6±0.6	*P*<0.02	ns	ns	1.8±0.4	1.8±0.4	3.7±0.4	2.6±0.3	*P*<0.002	ns	ns

OA/LA: 40 μM OA/ 40 μM LA; DHA: 60 μM DHA 40 μM OA/ 40 μM LA; DOX: 0.84 μmol/L for MCF-7 cells. Values are percentages relative to the total fatty acid content ± SE. Data were analyzed using a 2-factor ANOVA to assess media and drug interactions within each cell type. P^{Media}: *P* value from the main effect of the fatty acid supplementation in the PROC GLM procedure, P^{Drug}: *P* value from the main effect of DOX in the PROC GLM procedure. P^{Int}: *P* interaction between media and DOX in the PROC GLM procedure.

Table 4-7: Fatty acid composition (relative percent) of PC phospholipids from whole cell and lipid raft membranes of MDA-MB-231 breast cancer cells

	Whole Cell				*P* values			Lipid Raft				*P* values		
Fatty Acids	OA/LA	OA/LA+ DOX	DHA	DHA+ DOX	P^{Media}	P^{Drug}	P^{Int}	OA/LA	OA/LA+ DOX	DHA	DHA+ DOX	P^{Media}	P^{Drug}	P^{Int}
14:0	1.2±0.1	1.4±0.2	1.5±0.1	1.2±0.2	ns	ns	ns	1.9±0.2	2.0±0.2	1.5±0.2	1.7±0.1	*P*<0.06	ns	ns
16:0	21.5±1.5	23.8±1.7	23.0±1.0	23.1±1.2	ns	ns	ns	27.0±1.4	27.2±1.3	26.2±0.2	27.6±1.2	ns	ns	ns
16:1 n-9	2.2±0.2	1.9±0.2	1.7±0.2	1.7±0.2	ns	ns	ns	2.8±0.2	1.8±0.3	1.8±0.2	1.9±0.2	ns	ns	*P*<0.06
17:0	0.6±0.0	0.8±0.2	1.0±0.2	0.8±0.2	ns	ns	ns	0.8±0.0	0.8±0.1	0.6±0.0	0.7±0.1	ns	ns	ns
18:0	9.5±0.5	10.4±1.2	13.2±0.8	13.0±1.2	*P*<0.005	ns	ns	11.5±0.2	14.0±1.2	15.5±1.1	14.1±0.3	*P*<0.06	ns	*P*<0.05
18:1 n-9	35.0±2.0	27.5±1.3	29.0±2.1	27.8±2.2	ns	ns	ns	34.5±0.8	29.9±2.4	28.3±0.1	25.0±0.3	*P*<0.01	*P*<0.03	ns
18:2 n-6	19.7±2.4	21.7±2.1	20.4±0.4	23.6±2.3	ns	ns	ns	11.3±0.7	11.8±1.4	13.9±0.9	15.8±1.3	*P*<0.02	ns	ns
18:3 n-6	1.1±0.2	1.0±0.2	0.6±0.1	0.8±0.1	*P*<0.02	ns	ns	0.8±0.1	0.7±0.2	0.6±0.1	0.5±0.1	ns	ns	ns
20:2 n-6	2.2±0.5	2.9±0.6	1.9±0.3	2.5±0.6	ns	ns	ns	1.0±0.0	1.0±0.1	0.9±0.1	1.0±0.1	ns	ns	ns
20:3 n-6	1.2±0.1	1.0±0.2	1.0±0.2	1.0±0.3	ns	ns	ns	1.4±0.1	1.0±0.2	1.4±0.1	1.2±0.2	ns	*P*<0.08	ns
20:4 n-6	1.9±0.1	2.0±0.4	1.1±0.2	1.3±0.3	*P*<0.01	ns	ns	3.6±0.6	2.9±0.6	1.9±0.1	1.3±0.2	*P*<0.01	ns	ns
20:5 n-3	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	ns	ns	ns	0.1±0.	0.2±01	0.3±0.1	0.3±0.0	*P*<0.005	ns	ns
24:0	0.3±0.1	0.3±0.1	0.2±0.0	0.3±0.1	ns	ns	ns	0.6±0.0	0.6±0.1	0.7±0.1	0.8±0.2	ns	ns	ns
24:1 n-9	1.3±0.2	1.2±0.2	0.5±0.0	0.5±0.2	*P*<0.0005	ns	ns	1.9±0.3	1.4±0.2	0.6±0.1	1.0±0.3	*P*<0.008	ns	ns
22:4 n-6	0.2±0.0	0.1±0.0	0.4±0.1	0.0±0.0	ns	ns	ns	0.1±0.0	0.2±0.1	0.1±0.0	0.1±0.0	ns	ns	ns
22:5 n-6	0.1±0.0	0.2±0.1	0.2±0.0	0.2±0.1	ns	ns	ns	0.1±0.0	0.2±0.0	0.1±0.0	0.2±0.1	ns	ns	ns
22:5 n-3	0.2±0.0	0.2±0.0	0.4±0.0	0.4±0.1	*P*<0.0002	ns	ns	0.2±0.0	0.4±0.2	0.9±0.2	0.5±0.0	*P*<0.02	ns	*P*<0.07
22:6 n-3	0.2±0.0	0.2±0.0	1.8±0.1	1.4±0.3	*P*<0.0001	ns	ns	0.2±0.0	0.2±0.0	2.6±0.3	1.9±0.2	*P*<0.0001	*P*<0.09	*P*<0.13
Σ SFA	33.5±1.8	36.8±1.4	38.9±1.0	38.5±0.6	*P*<0.02	ns	ns	41.9±1.2	47.2±3.2	46.7±2.5	47.2±2.8	ns	ns	ns
Σ MUFA	38.5±2.1	33.7±3.3	31.5±2.3	30.0±2.0	*P*<0.06	ns	ns	37.4±1.0	31.9±2.5	30.2±1.2	28.8±1.8	*P*<0.02	*P*<0.10	ns
Σ n-6 PUFA	26.2±2.6	27.9±2.4	26.6±1.6	28.6±2.4	ns	ns	ns	19.3±0.6	19.3±1.0	18.8±1.2	20.8±1.5	ns	ns	ns
Σ n-3 PUFA	1.8±0.2	1.6±0.2	3.0±0.2	2.8±0.3	*P*<0.0002	ns	ns	1.4±0.1	1.5±0.1	4.4±0.2	3.2±0.4	*P*<0.0001	*P*<0.06	*P*<0.02

OA/LA: 40 μM OA/ 40 μM LA; DHA: 60 μM DHA 40 μM OA/ 40 μM LA; DOX: 0.22 μmol/L for MDA-MB-231. Values are percentages relative to the total fatty acid content ± SE. Data were analyzed using a 2-factor ANOVA to assess media and drug interactions within each cell type. P^{Media}: *P* value from the main effect of the fatty acid supplementation in the PROC GLM procedure, P^{Drug}: *P* value from the main effect of DOX in the PROC GLM procedure. P^{Int}: *P* interaction between media and DOX in the PROC GLM procedure.

Table 4-8: Fatty acid composition (relative percent) of PC phospholipids from whole cell and lipid raft membranes of MCF-7 breast cancer cells

	Whole Cell				*P* values			Lipid Raft				*P* values		
Fatty Acids	OA/LA	OA/LA+ DOX	DHA	DHA+ DOX	P^{Media}	P^{Drug}	P^{Int}	OA/LA	OA/LA+ DOX	DHA	DHA+ DOX	P^{Media}	P^{Drug}	P^{Int}
14:0	3.1±0.1	3.±0.3	2.7±0.1	2.3±0.2	*P*<0.003	ns	ns	4.8±0.6	4.4±0.5	2.9±0.2	3.0±0.2	*P*<0.003	ns	ns
16:0	27.9±0.8	27.7±1.6	27.1±1.5	25.9±0.7	ns	ns	ns	35.9±2.2	34.0±0.6	33.8±1.5	30.4±1.8	ns	ns	ns
16:1 n-9	2.4±0.4	2.1±1.0	3.0±0.5	2.3±0.3	ns	ns	ns	2.8±0.3	2.4±0.3	3.0±0.2	2.8±0.2	ns	ns	ns
17:0	0.9±0.3	2.2±0.6	1.4±0.5	1.7±0.0	ns	ns	ns	0.4±0.0	1.2±0.7	0.9±0.4	0.6±0.1	ns	ns	ns
18:0	5.0±0.3	4.8±0.5	6.4±0.4	6.0±0.2	*P*<0.04	ns	ns	7.0±0.9	9.1±1.6	8.9±0.6	9.4±1.4	ns	ns	ns
18:1 n-9	34.4±0.5	33.4±1.4	32.7±0.9	35.5±0.5	ns	ns	*P*<0.05	24.1±0.5	22.3±2.0	25.2±0.6	24.6±1.7	ns	ns	ns
18:2 n-6	20.5±1.4	21.4±2.7	22.7±0.8	20.0±3.1	ns	ns	ns	18.6±0.8	18.5±0.7	20.1±1.4	21.3±0.8	*P*<0.07	ns	ns
18:3 n-6	0.9±0.1	0.9±0.1	0.7±0.0	0.7±0.2	*P*<0.04	ns	ns	1.1±0.1	1.0±0.1	0.7±0.1	0.9±0.1	*P*<0.06	ns	ns
20:2 n-6	1.2±0.1	1.2±0.2	1.0±0.1	0.9±0.2	*P*<0.08	ns	ns	1.5±0.1	1.7±0.4	1.0±0.1	0.9±0.2	*P*<0.02	ns	ns
20:3 n-6	0.2±0.8	0.3±0.1	0.2±0.0	0.2±0.1	ns	ns	ns	0.3±0.1	0.3±0.0	0.2±0.0	0.4±0.1	ns	ns	ns
20:4 n-6	0.8±0.1	0.7±0.1	0.6±0.1	0.6±0.2	ns	ns	ns	1.1±0.2	1.0±0.2	1.0±0.1	1.0±0.0	ns	ns	ns
20:5 n-3	0.1±0.1	0.1±0.0	0.1±0.0	0.1±0.1	ns	ns	ns	0.2±0.0	0.1±0.0	0.1±0.0	0.2±0.1	ns	ns	ns
24:0	0.1±0.4	0.2±0.1	0.1±0.0	0.2±0.0	ns	ns	ns	0.3±0.0	0.2±0.0	0.3±0.1	0.4±0.1	ns	ns	ns
24:1 n-9	0.4±0.1	0.4±0.2	0.3±0.0	0.4±0.2	ns	ns	ns	1.2±0.2	0.8±0.2	0.8±0.2	1.2±0.6	ns	ns	ns
22:4 n-6	0.1±0.0	0.1±0.0	0.0±0.0	0.0±0.0	*P*<0.005	ns	*P*<0.06	0.1±0.0	0.0±0.0	0.1±0.0	0.1±0.0	ns	ns	ns
22:5 n-6	0.1±0.0	0.2±0.0	0.1±0.0	0.2±0.1	ns	ns	ns	0.3±0.1	0.3±0.1	0.2±0.0	0.3±0.2	ns	ns	ns
22:5 n-3	0.1±0.0	0.1±0.0	0.0±0.0	0.1±0.0	*P*<0.005	ns	ns	0.2±0.0	0.1±0.0	0.1±0.0	0.1±0.0	ns	ns	ns
22:6 n-3	0.1±0.1	0.1±0.0	0.6±0.1	0.3±0.1	*P*<0.0001	*P*<0.03	*P*<0.02	0.1±0.0	0.1±0.0	0.7±0.1	0.3±0.1	*P*<0.001	ns	*P*<0.1
Σ SFA	38.7±2.1	39.0±1.8	37.8±1.1	38.6±1.8	ns	ns	ns	48.5±1.9	50.6±1.4	46.8±2.0	50.6±5.6	ns	ns	ns
Σ MUFA	37.2±0.5	35.9±1.4	36.0±1.2	38.2±0.6	ns	ns	*P*<0.11	28.1±0.7	25.6±2.1	29.0±0.7	26.7±2.7	ns	ns	ns
Σ n-6 PUFA	22.9±1.6	24.0±3.0	24.7±1.0	22.0±3.5	ns	ns	ns	21.9±1.0	22.5±0.8	22.6±1.3	21.4±2.8	ns	ns	ns
Σ n-3 PUFA	1.2±0.1	1.2±0.2	1.4±0.1	1.2±0.3	ns	ns	ns	1.5±0.2	1.3±0.1	1.6±0.2	1.4±0.3	ns	ns	ns

OA/LA: 40 μM OA/ 40 μM LA; DHA: 60 μM DHA 40 μM OA/ 40 μM LA; DOX: 0.84 μmol/L for MCF-7 cells. Values are percentages relative to the total fatty acid content ± SE. Data were analyzed using a 2-factor ANOVA to assess media and drug interactions within each cell type. P^{Media}: *P* value from the main effect of the fatty acid supplementation in the PROC GLM procedure, P^{Drug}: *P* value from the main effect of DOX in the PROC GLM procedure. P^{Int}: *P* interaction between media and DOX in the PROC GLM procedure.

Table 4-9: Fatty acid composition (relative percent) of PI phospholipids from whole cell and lipid raft membranes of MDA-MB-231 breast cancer cells

	Whole Cell				*P* values			Lipid Raft				*P* values		
Fatty Acids	OA/LA	OA/LA+ DOX	DHA	DHA+ DOX	P^{Media}	P^{Drug}	P^{Int}	OA/LA	OA/LA+ DOX	DHA	DHA+ DOX	P^{Media}	P^{Drug}	P^{Int}
14:0	1.1±0.5	2.2±1.1	0.3±0.0	1.2±0.7	ns	ns	ns	2.1±0.5	0.9±0.2	2.7±0.1	2.2±0.8	*P*<0.02	*P*<0.09	ns
16:0	4.5±0.6	4.4±0.3	4.3±0.8	4.3±0.0	ns	ns	ns	21.2±2.5	17.6±3.9	13.8±4.0	26.4±0.4	Ns	ns	*P*<0.07
16:1 n-9	0.5±0.1	0.3±0.1	0.3±0.1	0.5±0.0	ns	ns	ns	0.6±0.2	0.7±0.1	1.2±0.3	0.8±0.1	*P*<0.04	ns	ns
17:0	0.4±0.0	0.5±0.2	0.7±0.2	0.5±0.1	ns	ns	ns	0.6±0.2	2.8±1.0	1.5±0.3	0.6±0.1	*P*<0.11	ns	*P*<0.02
18:0	49.5±4.3	48.8±2.1	48.5±2.3	48.0±4.0	ns	ns	ns	37.0±6.4	23.4±1.4	28.1±0.8	25.8±0.9	Ns	*P*<0.05	ns
18:1 n-9	12.4±1.1	10.5±1.1	12.4±0.6	14.4±3.0	ns	ns	ns	14.4±1.2	18.8±3.3	16.8±3.3	11.3±0.1	Ns	ns	*P*<0.05
18:2 n-6	7.4±1.4	9.0±0.5	9.9±0.7	9.1±0.4	ns	ns	ns	5.0±0.8	8.5±1.2	8.2±1.3	7.1±1.0	Ns	ns	*P*<0.07
18:3 n-6	0.4±0.0	0.3±0.0	0.1±0.0	0.2±0.1	*P*<0.002	ns	*P*<0.07	0.3±0.2	1.2±0.1	1.1±0.29	0.4±0.1	Ns	ns	*P*<0.001
20:2 n-6	0.5±0.1	1.3±0.4	0.5±0.2	1.1±0.2	ns	ns	ns	0.8±0.6	0.5±0.1	0.4±0.1	0.7±0.2	Ns	ns	ns
20:3 n-6	3.2±0.2	3.0±0.1	5.1±0.7	5.5±0.2	*P*<0.001	ns	ns	2.3±0.6	2.9±0.2	4.5±0.8	4.7±0.7	*P*<0.01	ns	ns
20:4 n-6	16.8±1.5	16.6±0.2	13.2±1.2	12.2±0.7	*P*<0.01	ns	ns	3.1±1.6	3.5±1.1	1.6±0.7	4.7±1.7	Ns	ns	ns
20:5 n-3	0.1±0.0	0.2±0.1	0.3±0.0	0.4±0.2	ns	ns	ns	1.4±0.0	0.2±0.1	1.4±0.3	0.3±0.1	Ns	*P*<0.0001	ns
24:0	0.2±0.1	0.4±0.2	0.2±0.1	0.2±0.0	ns	ns	ns	3.0±1.4	9.9±1.0	5.5±2.1	7.0±2.6	Ns	*P*<0.06	ns
24:1 n-9	1.5±0.1	1.4±0.2	0.7±0.0	0.6±0.2	ns	ns	ns	4.4±1.2	11.2±0.5	4.8±1.9	4.3±1.7	*P*<0.06	*P*<0.09	*P*<0.04
22:4 n-6	0.1±0.0	0.4±0.2	0.1±0.1	0.3±0.2	ns	*P*<0.10	ns	0.2±0.1	0.4±0.1	0.5±0.2	0.3±0.2	Ns	ns	ns
22:5 n-6	0.1±0.0	0.3±0.2	0.3±0.2	0.2±0.1	ns	ns	ns	0.3±0.2	0.6±0.2	0.6±0.1	0.4±0.2	Ns	ns	ns
22:5 n-3	0.2±0.0	0.2±0.0	0.5±0.1	0.4±0.0	*P*<0.002	ns	ns	0.3±0.0	0.3±0.0	0.5±0.0	0.5±0.1	*P*<0.05	ns	ns
22:6 n-3	0.1±0.0	0.2±0.1	2.0±0.3	1.0±0.1	*P*<0.0001	*P*<0.06	*P*<0.01	0.1±0.0	0.4±0.1	2.5±1.1	1.5±0.4	*P*<0.02	ns	ns
Σ SFA	56.0±3.4	56.2±1.1	54.8±2.2	54.2±3.2	ns	ns	ns	58.5±2.9	53.4±3.7	60.5±6.7	61.3±2.6	Ns	ns	ns
Σ MUFA	14.6±1.4	12.2±1.0	12.6±1.1	15.5±2.7	ns	ns	*P*<0.10	19.5±1.9	25.5±3.5	22.6±3.0	16.2±1.8	Ns	ns	*P*<0.13
Σ n-6 PUFA	28.5±2.4	30.7±1.0	29.6±1.4	28.4±0.3	ns	ns	ns	19.3±4.2	19.1±2.8	13.2±2.9	19.8±3.8	Ns	ns	ns
Σ n-3 PUFA	$0.9±0.2^{b}$	$0.9±0.1^{b}$	$3.0±0.4^{a}$	$1.9±0.2^{b}$	*P*<0.0003	*P*<0.12	*P*<0.10	2.8±1.2	2.0±0.4	3.6±1.4	2.7±0.5	Ns	ns	ns

OA/LA: 40 μM OA/ 40 μM LA; DHA: 60 μM DHA 40 μM OA/ 40 μM LA; DOX: 0.22 μmol/L for MDA-MB-231. Values are percentages relative to the total fatty acid content ± SE. Data were analyzed using a 2-factor ANOVA to assess media and drug interactions within each cell type. P^{Media}: *P* value from the main effect of the fatty acid supplementation in the PROC GLM procedure, P^{Drug}: *P* value from the main effect of DOX in the PROC GLM procedure. P^{Int}: *P* interaction between media and DOX in the PROC GLM procedure.

Table 4-10: Fatty Acid composition (relative percent) of PI phospholipids from whole cell and lipid raft membranes of MCF-7 breast cancer cells

	Whole Cell				*P* values			Lipid Raft				*P* values		
Fatty Acids	OA/LA	OA/LA+ DOX	DHA	DHA+ DOX	P^{Media}	P^{Drug}	P^{Int}	OA/LA	OA/LA+ DOX	DHA	DHA+ DOX	P^{Media}	P^{Drug}	P^{Int}
14:0	2.1±0.4	1.1±0.1	1.9±0.7	1.4±0.2	ns	ns	ns	1.1±0.2	2.2±0.4	4.27±1.32	2.6±0.9	ns	ns	ns
16:0	7.5±0.8	10.6±1.3	8.9±1.4	8.7±0.8	ns	*P*<0.05	*P*<0.08	10.0±1.0	14.7±0.9	16.68±0.50	14.0±1.4	P<0.02	ns	*P*<0.008
16:1 n-9	1.4±0.4	1.2±0.4	0.9±0.1	0.9±0.2	ns	ns	ns	1.1±0.1	1.0±0.2	1.28±0.14	1.6±0.5	ns	ns	ns
17:0	1.1±0.3	0.6±0.1	1.1±0.0	0.9±0.2	ns	ns	ns	0.6±0.1	0.5±0.0	0.95±0.25	0.6±0.2	ns	ns	ns
18:0	46.2±1.6	43.6±1.7	42.2±3.5	42.8±2.0	ns	ns	*P*<0.12	39.8±5.4	40.3±0.2	35.33±2.42	27.2±1.3	*P*<0.05	ns	ns
18:1 n-9	17.8±2.7	18.2±3.2	21.2±1.8	22.4±1.6	*P*<0.008	*P*<0.06	ns	18.6±6.1	14.8±1.1	14.26±3.36	10.9±3.6	ns	ns	ns
18:2 n-6	13.7±1.4	13.4±2.2	15.4±1.1	15.0±1.2	ns	ns	ns	17.1±0.5	14.8±1.6	11.99±1.82	16.2±2.6	ns	ns	ns
18:3 n-6	0.6±0.4	0.2±0.0	0.3±0.1	0.3±0.0	*P*<0.06	ns	*P*<0.03	0.6±0.3	0.9±0.3	0.78±0.26	0.8±0.4	ns	ns	ns
20:2 n-6	1.4±0.1	1.3±0.2	0.8±0.2	1.2±0.0	*P*<0.03	ns	ns	1.4±0.5	1.7±0.1	1.17±0.50	0.4±0.2	ns	ns	ns
20:3 n-6	1.3±0.4	0.8±0.1	1.0±0.2	1.2±0.2	ns	ns	ns	0.4±0.1	0.7±0.1	0.81±0.27	0.6±0.0	ns	ns	ns
20:4 n-6	3.2±0.5	3.4±1.3	2.2±0.3	4.0±0.3	ns	ns	ns	2.3±0.6	3.2±0.9	2.34±0.86	2.1±0.8	ns	ns	ns
20:5 n-3	0.7±0.4	0.5±0.2	0.3±0.1	0.5±0.2	ns	ns	ns	0.2±0.1	0.3±0.1	0.33±0.0.09	0.2±0.1	ns	ns	*P*<0.13
24:0	0.5±0.4	0.1±0.0	0.2±0.1	0.1±0.0	ns	ns	ns	0.4±0.1	0.7±0.2	0.51±0.10	0.8±0.4	ns	ns	ns
24:1 n-9	0.2±0.1	0.3±0.1	0.3±0.1	0.3±0.2	ns	ns	ns	0.6±0.2	0.6±0.2	0.48±0.20	0.4±0.0	ns	ns	ns
22:4 n-6	0.5±0.4	0.1±0.1	0.2±0.0	0.2±0.1	ns	ns	ns	0.2±0.1	0.2±0.0	0.48±0.16	0.3±0.3	ns	ns	ns
22:5 n-6	0.9±0.6	0.3±0.1	0.6±0.1	0.3±0.1	ns	ns	ns	0.3±0.0	0.2±0.0	0.61±0.27	0.5±0.2	ns	ns	ns
22:5 n-3	1.0±0.6	0.3±0.1	0.2±0.0	0.1±0.0	*P*<0.0004	ns	ns	0.4±0.1	0.4±0.0	0.44±0.16	0.3±0.2	ns	ns	ns
22:6 n-3	0.1±0.1	0.2±0.0	1.8±0.4	0.9±0.1	*P*<0.0001	ns	ns	0.4±0.2	0.5±0.2	1.35±0.40	1.1±0.3	*P*<0.03	ns	ns
Σ SFA	57.3±1.7	60.5±5.2	54.2±3.7	53.5±2.1	ns	ns	*P*<0.08	59.8±8.9	55.6±4.7	63.7±5.4	61.0±10.1	ns	ns	ns
Σ MUFA	19.4±2.9	19.7±3.0	22.4±1.8	23.5±1.7	*P*<0.01	*P*<0.10	*P*<0.004	20.3±6.0	21.0±4.8	16.0±3.1	20.0±6.1	ns	ns	ns
Σ n-6 PUFA	21.0±0.5	18.6±3.5	20.8±2.0	21.3±0.5	ns	ns	ns	18.3±3.7	21.1±0.3	17.4±2.6	16.6±4.0	ns	ns	ns
Σ n-3 PUFA	2.3±1.3	1.2±0.2	2.6±0.4	1.7±0.3	*P*<0.002	ns	ns	1.6±0.5	2.3±0.3	2.9±0.4	2.4±0.7	ns	ns	ns

P^{Media}: *P* value from the main effect of the fatty acid supplementation in the PROC GLM procedure, P^{Drug}: *P* value from the main effect of DOX in the PROC GLM procedure. P^{Int}: *P* interaction between media and DOX in the PROC GLM procedure.

Table 4-11: Fatty Acid composition (relative percent) of SM phospholipids from whole cell and lipid raft membranes of MDA-MB-231 breast cancer cells

	Whole Cell				*P* values			Lipid Raft				*P* values		
Fatty Acids	OA/LA	OA/LA+ DOX	DHA	DHA+ DOX	P^{Media}	P^{Drug}	P^{Int}	OA/LA	OA/LA+ DOX	DHA	DHA+ DOX	P^{Media}	P^{Drug}	P^{Int}
14:0	3.0±0.6	4.2±2.3	3.1±0.9	3.8±1.0	ns	ns	ns	4.1±0.9	3.3±0.8	3.0±0.1	2.0±0.4	*P*<0.11	ns	*P*<0.008
16:0	39.0±2.9	36.8±3.9	39.6±3.1	35.6±2.5	ns	ns	ns	38.3±3.8	31.6±3.5	38.6±0.2	31.9±3.8	ns	*P*<0.11	ns
16:1 n-9	0.6±0.2	0.5±0.2	0.7±0.2	1.0±0.1	ns	ns	ns	0.7±0.0	0.9±0.0	1.1±0.1	0.8±0.2	ns	ns	ns
17:0	1.7±0.0	1.4±0.2	1.6±0.1	1.3±0.1	ns	ns	ns	0.8±0.0	2.1±0.4	1.0±0.0[b]	0.9±0.1	*P*<0.04	*P*<0.02	ns
18:0	7.4±0.3	9.1±1.1	8.0±0.5	9.4±0.8	ns	*P*<0.04	ns	11.6±1.0	13.6±0.4	10.6±1.4	16.6±0.9	ns	*P*<0.004	ns
18:1 n-9	5.2±1.3	3.8±0.3	2.7±0.2	3.8±0.3	ns	ns	ns	14.2±0.2	11.5±0.6	9.9±1.1	12.4±3.1	ns	ns	ns
18:2 n-6	1.8±0.6	4.5±0.2	1.8±0.4	2.2±0.4	ns	ns	ns	5.8±0.8	5.8±0.3	4.4±0.4	7.4±1.4	ns	ns	ns
18:3 n-6	0.2±0.0	0.1±0.1	0.1±0.0	1.5±0.1	*P*<0.0001	*P*<0.0001	*P*<0.0001	0.4±0.0	0.4±0.1	0.4±0.2	0.6±0.1	ns	ns	ns
20:2 n-6	0.4±0.1	0.5±0.2	0.4±0.1	1.7±0.2	*P*<0.007	*P*<0.002	*P*<0.006	0.1±0.0	0.8±0.4	0.4±0.2	0.1±0.0	ns	ns	ns
20:3 n-6	3.8±0.8	3.3±1.0	3.8±0.5	2.9±0.6	ns	ns	ns	2.3±0.1	2.3±0.6	2.8±0.4	2.1±0.2	ns	ns	ns
20:4 n-6	1.3±0.5	0.6±0.2	0.8±0.3	2.5±0.4	ns	ns	ns	0.7±0.3	0.7±0.3	0.9±0.4	0.6±0.2	ns	ns	ns
20:5 n-3	0.1±0.0	0.2±0.1	0.1±0.0	1.3±0.7	*P*<0.10	*P*<0.04	*P*<0.08	0.1±0.0	0.1±0.0	0.1±0.0	0.2±0.0	ns	*P*<0.09	ns
24:0	4.7±2.0	6.8±0.2	6.6±3.1	10.1±1.8	ns	ns	ns	6.8±0.3	5.5±0.8	11.4±0.6	6.4±1.3	*P*<0.11	*P*<0.03	ns
24:1 n-9	22.2±1.3	18.8±2.2	20.0±1.2	15.8±0.8	*P*<0.10	*P*<0.03	ns	14.4±0.7	15.6±2.2	12.9±2.0	11.0±1.4	ns	ns	ns
22:4 n-6	0.2±0.1	0.6±0.0	0.3±0.1	0.7±0.3	ns	*P*<0.05	ns	0.14±0.0	0.4±0.2	0.3±0.1	0.4±0.1	ns	ns	ns
22:5 n-6	6.5±1.0	6.9±1.0	6.4±1.0	4.7±1.2	ns	ns	ns	2.2±0.6	2.6±1.2	3.7±0.3	2.7±0.6	ns	ns	ns
22:5 n-3	0.2±0.1	0.2±0.1	0.2±0.0	0.7±0.4	ns	ns	ns	0.2±0.0	0.4±0.1	0.4±0.1	0.4±0.0	ns	ns	ns
22:6 n-3	0.2±0.1	0.4±0.1	0.3±0.1	0.2±0.0	ns	ns	ns	0.1±0.1	0.2±0.0	0.2±0.0	0.4±0.2	ns	ns	ns
Σ SFA	55.6±2.4	60.4±1.8	59.0±1.9	61.6±1.4	ns	*P*<0.10	ns	60.4±3.4	60.9±6.0	65.0±1.8	61.6±4.8	ns	ns	ns
Σ MUFA	28.0±0.9	23.5±1.8	25.4±1.4	20.5±0.7	*P*<0.05	*P*<0.004	ns	27.4±2.3	24.9±3.9	22.3±0.7	22.9±3.7	ns	ns	ns
Σ n-6 PUFA	15.6±1.6	15.1±1.4	14.9±0.6	13.5±2.2	ns	ns	ns	11.4±1.1	13.0±1.9	11.8±1.2	14.0±1.2	ns	ns	ns
Σ n-3 PUFA	0.7±0.1	1.0±0.1	0.8±0.1	4.4±0.8	*P*<0.002	*P*<0.0002	*P*<0.0007	0.8±0.1	1.2±0.2	1.0±0.3	1.6±0.2	ns	*P*<0.05	ns

OA/LA: 40 μM OA/ 40 μM LA; DHA: 60 μM DHA 40 μM OA/ 40 μM LA; DOX: 0.22 μmol/L for MDA-MB-231. Values are percentages relative to the total fatty acid content ± SE. Data were analyzed using a 2-factor ANOVA to assess media and drug interactions within each cell type. P^{Media}: *P* value from the main effect of the fatty acid supplementation in the PROC GLM procedure, P^{Drug}: *P* value from the main effect of DOX in the PROC GLM procedure. P^{Int}: *P* interaction between media and DOX in the PROC GLM procedure.

Table 4-12: Fatty acid composition (relative percent) of SM phospholipids from whole cell and lipid raft membranes of MCF-7 breast cancer cells

	Whole Cell				*P* values			Lipid Raft				*P* values		
Fatty Acids	OA/LA	OA/LA+ DOX	DHA	DHA+ DOX	P^{Media}	P^{Drug}	P^{Int}	OA/LA	OA/LA+ DOX	DHA	DHA+ DOX	P^{Media}	P^{Drug}	P^{Int}
14:0	4.1±0.5	4.6±1.0	4.7±0.7	4.6±0.6	ns	ns	ns	6.3±1.6	10.4±1.6	8.8±0.7[a]	4.3±0.7	ns	ns	ns
16:0	36.1±2.1	34.3±2.3	37.2±5.8	38.7±3.6	ns	ns	ns	34.6±3.4	26.0±2.9	36.7±4.5	30.1±3.9	ns	*P*<0.11	ns
16:1 n-9	1.7±0.3	1.6±0.8	1.4±0.2	1.5±0.6	ns	ns	ns	1.7±0.1	1.3±0.1	2.0±0.2	1.0±0.3	ns	*P*<0.008	*P*<0.10
17:0	1.6±0.5	1.7±0.4	2.1±0.4	1.6±0.7	ns	ns	ns	0.6±0.1	0.5±0.0	1.3±0.6	0.5±0.2	ns	ns	*P*<0.007
18:0	14.8±1.6	15.6±3.9	15.2±1.8	11.2±0.8	ns	ns	ns	14.8±0.5	16.2±3.5	15.0±0.9	15.9±1.2	ns	ns	ns
18:1 n-9	4.5±1.5	12.8±3.7	7.8±0.3	12.4±1.4	ns	*P*<0.04	ns	3.4±0.2	8.2±3.6	7.3±1.5	8.0±1.9	ns	ns	ns
18:2 n-6	2.7±0.4	6.4±2.2	6.5±2.7	6.8±2.2	ns	ns	ns	2.3±0.6	9.8±4.4	4.9±0.5	3.5±2.2	ns	ns	ns
18:3 n-6	0.3±0.1	0.4±0.1	0.3±0.2	0.2±0.1	ns	ns	ns	0.2±0.0	0.6±0.4	0.5±0.1	0.7±0.2	ns	ns	ns
20:2 n-6	1.2±0.6	1.6±0.4	1.4±0.5	1.4±0.6	ns	ns	ns	0.2±0.0	0.2±0.2	0.1±0.0	0.2±0.1	ns	ns	ns
20:3 n-6	2.3±0.4	2.7±0.2	2.3±0.5	2.8±0.3	ns	ns	ns	1.9±0.6	1.1±0.5	2.4±0.4	2.1±0.4	ns	ns	ns
20:4 n-6	0.5±0.0	1.6±0.4	0.8±0.5	0.9±0.3	ns	ns	ns	1.6±0.7	0.7±0.0	1.6±0.8	1.1±0.5	ns	ns	ns
20:5 n-3	3.4±0.7	2.8±0.7	3.4±1.2	3.2±0.5	ns	ns	ns	0.4±0.2	0.4±0.1	0.4±0.2	0.4±0.2	ns	ns	ns
24:0	3.4±0.7	2.8±0.7	3.4±1.2	3.2±0.5	ns	ns	ns	2.6±0.6	1.3±0.5	3.4±0.7	2.8±0.4	*P*<0.07	ns	ns
24:1 n-9	15.8±2.9	11.8±3.6	11.8±2.3	11.6±1.3	ns	ns	ns	14.9±4.5	7.8±3.7	12.0±1.9	10.7±2.8	ns	ns	ns
22:4 n-6	0.5±0.2	0.2±0.1	0.2±0.1	0.2±0.1	ns	ns	*P*<0.08	0.2±0.0	0.1±0.1	0.3±0.2	0.2±0.1	ns	ns	ns
22:5 n-6	3.8±1.6	3.9±1.6	3.1±1.6	2.6±0.5	ns	ns	ns	0.3±0.2	0.8±0.2	0.3±0.3	0.4±0.3	ns	ns	ns
22:5 n-3	0.2±0.2	0.5±0.3	0.4±0.1	0.0±0.0	ns	ns	ns	4.6±1.6	2.2±1.2	2.8±0.4	1.8±0.1	ns	ns	ns
22:6 n-3	0.2±0.2	0.6±0.3	0.3±0.1	0.1±0.0	ns	ns	ns	0.2±0.0	0.1±0.1	0.2±0.1	0.4±0.3	ns	ns	ns
Σ SFA	59.9±2.9	55.1±1.4	55.4±7.7	59.3±3.2	ns	ns	ns	56.5±4.3	63.5±7.4	65.2±2.2	60.8±7.7	ns	ns	ns
Σ MUFA	23.7±1.7	23.8±0.8	26.7±5.6	23.0±1.5	ns	ns	ns	26.3±2.5	21.3±4.1	21.3±1.1	23.9±3.6	ns	ns	ns
Σ n-6 PUFA	14.6±1.8	18.1±1.0	15.8±2.5	16.4±2.0	ns	ns	ns	11.5±3.3	12.7±3.8	9.6±0.8	11.1±3.8	ns	ns	ns
Σ n-3 PUFA	1.8±0.7	3.0±0.2	2.1±0.4	1.3±0.4	*P*<0.11	ns	*P*<0.02	5.7±1.4	2.5±1.1	3.9±0.7	4.3±1.7	ns	ns	ns

OA/LA: 40 μM OA/ 40 μM LA; DHA: 60 μM DHA 40 μM OA/ 40 μM LA; DOX: 0.84 μmol/L for MCF-7 cells. Values are percentages relative to the total fatty acid content ± SE. P^{Media}: *P* value from the main effect of the fatty acid supplementation in the PROC GLM procedure, P^{Drug}: *P* value from the main effect of DOX in the PROC GLM procedure. P^{Int}: *P* interaction between media and DOX in the PROC GLM procedure.

4.3.3 Fatty Acid Composition of MDA-MB-231 Tumour Total Phospholipids and Phospholipid classes from nu/nu mice

Next, we sought to examine if changes in fatty acid composition of membrane PL classes observed *in vitro* also occurred *in vivo* from mice fed a DHA supplemented diet. MCF-7 cells grow slowly and are weakly invasive *in vivo* compared to MDA-MB-231 (Seibert et al. 1983), therefore MDA-MB-231 cells were chosen for the *in vivo* model. First, we determined the total PL composition of the MDA-MB-231 tumours excised from *nu/nu* mice (**Table 4-13**). In tumours from mice fed the DHA diet (with or without DOX), there was significantly lower in ARA and C24:1 n-9 and higher in EPA, DPA and DHA. A negative correlation between tumour ARA content and tumour DHA content was observed (r=-0.42; P<0.01; **Figure 4-5 A**). The higher n-3 PUFA was accompanied by a lower n-6 PUFA content (P<0.0001).

Figure 4-5. (A) Correlation between tumour ARA content and DHA content (B) DHA incorporation (relative %) into PL classes of MDA-MB-231 tumours excised from *nu/nu* mice

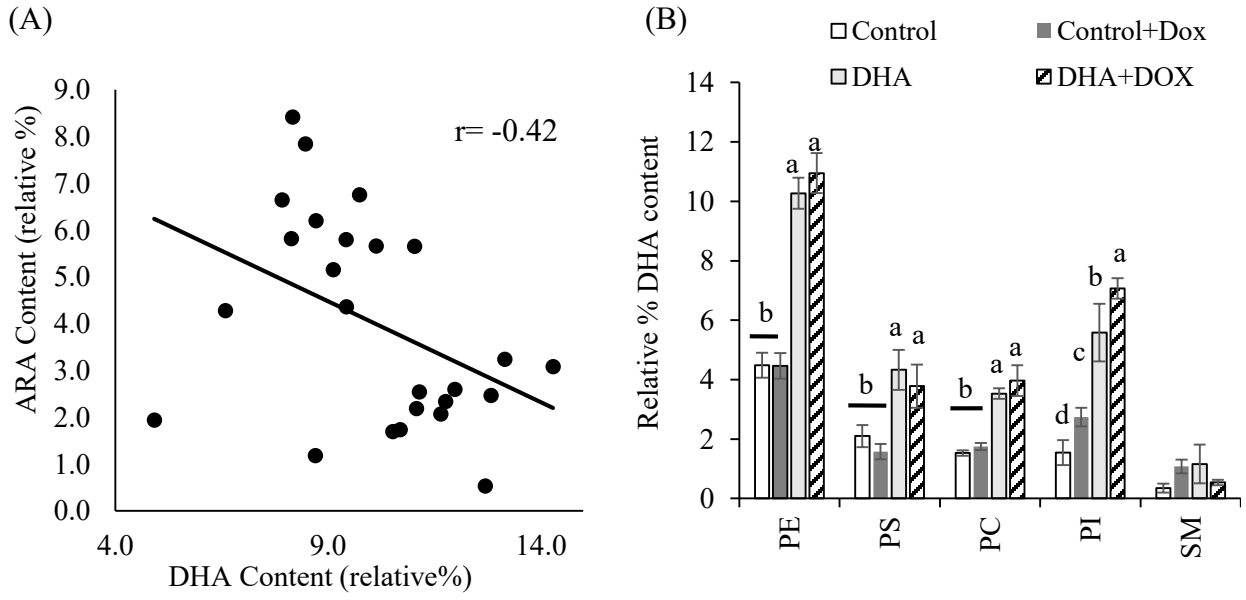


Table 4-13: Effect of the control and DHA diets with or without DOX on fatty acid composition (relative %) of MDA-MB-231 tumour total phospholipids from *nu/nu* mice

	Control Diet		DHA Diet		*P* values		
Fatty Acids	**Control**	**Control + DOX**	**DHA**	**DHA +DOX**	P^{Diet}	P^{Drug}	P^{Int}
14:0	0.8±0.1	1.1±0.4	1.1±0.1	0.8±0.1	ns	ns	ns
16:0	17.2±0.5	17.5±0.5	18.6±0.9	16.7±0.8	ns	ns	ns
16:1 n-9	1.0±0.0	1.1±0.1	1.3±0.2	1.2±0.1	ns	ns	ns
17:0	1.2±0.2	2.9±0.8	1.9±0.3	2.0±0.3	ns	*P*<0.07	*P*<0.11
18:0	26.6±0.9	23.5±0.6	24.6±1.5	23.5±0.1	ns	*P*<0.06	
18:1 n-9	18.5±0.2	18.8±0.4	19.2±0.4	18.6±0.5	ns	ns	ns
18:2 n-6	8.9±0.3	9.7±0.6	9.7±0.4	10.2±0.4	*P*<0.10	ns	ns
20:0	0.4±0.0	0.4±0.0	0.4±0.1	0.4±0.0	ns	ns	ns
18:3 n-6	0.5±0.1	0.4±0.0	0.4±0.1	0.5±0.0	ns	ns	ns
18:3 n-3	0.2±0.1	0.1±0.0	0.1±0.0	0.0±0.0	*P*<0.05	ns	ns
20:2 n-6	1.3±0.1	1.2±0.2	1.2±0.0	1.2±0.1	ns	ns	ns
20:3 n-6	2.0±0.1	2.0±0.2	2.5±0.2	2.5±0.1	*P*<0.0008	ns	ns
20:4 n-6	11.1±0.5	12.6±0.5	7.6±0.7	9.4±0.4	*P*<0.0001	*P*<0.004	ns
20:5 n-3	0.1±0.0	0.1±0.0	0.3±0.0	0.4±0.0	*P*<0.0001	*P*<0.002	*P*<0.09
24:0	1.1±0.0	1.1±0.2	1.1±0.0	1.0±0.1	ns	ns	ns
24:1 n-9	4.2±0.2	3.7±0.4	2.3±0.2	2.4±0.1	*P*<0.0001	ns	ns
22:4 n-6	0.7±0.1	0.7±0.0	0.4±0.2	0.2±0.0	*P*<0.001	ns	ns
22:5 n-6	0.1±0.0	0.2±0.0	0.1±0.0	0.1±0.0	*P*<0.02	*P*<0.04	ns
22:5 n-3	0.5±0.0	0.5±0.0	0.7±0.1	0.9±0.0	*P*<0.0001	ns	*P*<0.06
22:6 n-3	2.1±0.2	2.4±0.4	5.1±0.7	6.3±0.6	*P*<0.0001	ns	ns
Σ SFA	48.0±1.3	45.8±1.2	47.6±2.3	44.7±0.6	ns	*P*<0.10	ns
Σ MUFA	23.7±0.3	24.0±0.4	22.7±0.5	22.2±0.6	*P*<0.008	ns	ns
Σ n-6 PUFA	24.6±0.7	27.6±0.3	22.0±1.2	24.2±0.4	*P*<0.002	*P*<0.003	ns
Σ n-3 PUFA	2.8±0.3	3.0±0.4	6.2±0.8	7.6±0.6	*P*<0.0001	*P*<0.14	ns

Values are percentages relative to the total fatty acid content ± SE (n=6 mice per group). Data were analyzed using a 2-factor ANOVA to assess diet and drug interactions within each cell type. P^{Diet}: *P* value from the main effect of the diet in the PROC GLM procedure, P^{Drug}: *P* value from the main effect of DOX in the PROC GLM procedure. P^{Int}: *P* interaction between Diet and DOX in the PROC GLM procedure.

Tumours from mice fed a DHA diet with or without DOX treatment had significantly higher DHA content in PE, PS, PC and PI in the excised tumours compared to tumours from Control or Control+DOX fed mice (**Tables 4-14 to 4-18** and **Figure 4-5 B**; *P*<0.005).

Fatty acids other than DHA were also observed to be altered in the tumours from mice fed the DHA diet compared to control diet. In MDA-MB-231 tumours excised from mice fed DHA with or without DOX, there was lower ARA in PE, PC and PI (P<0.0001); lower C24:1(9) in PE, PC, PS and PI (P<0.006); higher LA in PS and PC (P<0.01), higher EPA in PE and PC (P<0.0001) and higher DPA n-3 in PE, PC and PI (P<0.0001) compared to tumours from mice fed the control diet (**Tables 4-14 to 4-18**).

Table 4-14: Phosphatidylethanolamine phospholipid fatty acid composition (relative %) from MDA-MB-231 tumours excised from *nu/nu* mice

	Control Diet		DHA Diet		*P* values		
Fatty Acids	**Control**	**Control + DOX**	**DHA**	**DHA +DOX**	P^{Diet}	P^{Drug}	P^{Int}
14:0	0.2±0.1	0.3±0.1	0.2±0.1	0.3±0.1	ns	ns	ns
16:0	6.1±0.6	5.3±0.2	6.8±0.6	5.9±0.2	ns	P<0.13	ns
16:1 n-9	0.3±0.0	0.3±0.1	0.4±0.0	0.3±0.0	ns	P<0.06	ns
17:0	0.8±0.2	0.4±0.0	0.7±0.2	0.5±0.0	ns	ns	ns
18:0	31.4±1.3	33.1±0.2	32.0±0.6	33.5±0.6	ns	P<0.10	ns
18:1 n-9	15.1±0.8	13.0±0.4	15.3±0.8	12.6±0.1	ns	P<0.003	ns
18:2 n-6	5.3±0.4	5.6±0.3	6.0±0.2	5.5±0.3	ns	ns	ns
20:0	0.3±0.03	0.2±0.0	0.2±0.0	0.2±0.0	P<0.11	ns	ns
18:3 n-6	0.2±0.03	0.1±0.1	0.1±0.0	0.1±0.0	P<0.10	P<0.09	ns
18:3 n-3	0.5±0.0	0.4±0.0	0.5±0.0	0.4±0.0	ns	P<0.005	ns
20:2 n-6	0.9±0.0	0.9±0.1	0.8±0.1	0.6±0.1	P<0.03	ns	ns
20:3 n-6	2.3±0.2	2.8±0.3	3.3±0.2	3.0±0.2	P<0.007	ns	P<0.11
20:4 n-6	23.1±0.9	23.9±1.4	16.7±0.8	19.9±1.3	P<0.0001	P<0.06	ns
20:5 n-3	0.1±0.01	0.2±0.0	0.6±0.0	0.8±0.1	P<0.0001	P<0.07	ns
24:0	0.2±0.0	0.2±0.0	0.3±0.1	0.2±0.0	ns	ns	ns
24:1 n-9	8.2±0.4	6.5±0. 3	3.8±0.2	3.4±0.2	P<0.0001	P<0.007	P<0.05
22:4 n-6	1.3±0.1	1.2±0.0	0.4±0.0	0.4±0.0	P<0.0001	ns	ns
22:5 n-6	0.1±0.0	0.0±0.0	0.0±0.0	0.1±0.0	ns	ns	ns
22:5 n-3	1.1±0.1	1.0±0.0	1.6±0.1	1.6±0.0	P<0.0001	ns	ns
22:6 n-3	4.5±0.4	4.5±0.4	10.3±0.5	11.0±0.7	P<0.0001	ns	ns
Σ SFA	37.7±1.4	39.6±0.4	40.3±0.6	40.6±0.7	P<0.05	ns	ns
Σ MUFA	22.8±2.9	19.8±0.6	19.5±0.8	16.3±0.2	P<0.0005	P<0.003	ns
Σ n-6 PUFA	33.2±1.9	34.6±0.6	27.2±0.9	29.5±0.9	P<0.0001	P<0.06	ns
Σ n-3 PUFA	6.2±1.1	6.0±0.4	12.9±0.6	13.6±0.7	P<0.0001	ns	ns

P^{Diet}: P value from the main effect of the diet in the PROC GLM procedure, P^{Drug}: P value from the main effect of DOX in the PROC GLM procedure. P^{Int}: P interaction between Diet and DOX.

Table 4-15: Phosphatidylserine phospholipid fatty acid composition (relative %) from MDA-MB-231 tumours excised from *nu/nu* mice

	Control Diet		DHA Diet		P values		
Fatty Acids	**Control**	**Control + DOX**	**DHA**	**DHA +DOX**	P^{Diet}	P^{Drug}	P^{Int}
14:0	0.4±0.2	1.9±0.5	1.2±0.3	1.8±0.4	ns	*P*<0.04	ns
16:0	3.9±1.0	10.8±1.4	12.0±2.7	15.0±1.0	*P*<0.02	*P*<0.07	ns
16:1 n-9	0.5±0.1	0.8±0.1	1.0±0.2	0.8±0.0	*P*<0.11	ns	*P*<0.09
17:0	0.7±0.3	1.1±0.1	0.9±0.1	1.0±0.1	ns	ns	ns
18:0	42.2±1.9	37.0±1.3	35.3±3.0	34.0±1.6	*P*<0.05	ns	ns
18:1 n-9	22.8±0.7	20.2±1.7	21.5±0.5	19.6±0.7	ns	*P*<0.02	ns
18:2 n-6	4.6±0.5	6.1±0.3	6.7±0.7	7.6±0.4	*P*<0.01	*P*<0.10	ns
20:0	1.2±0.2	1.0±0.1	0.9±0.2	0.7±0.0	*P*<0.08	ns	ns
18:3 n-6	0.3±0.0	0.4±0.1	0.3±0.0	0.3±0.1	ns	ns	ns
18:3 n-3	0.6±0.1	0.5±0.2	0.7±0.1	0.6±0.0	ns	ns	ns
20:2 n-6	0.8±0.1	1.0±0.1	1.0±0.1	1.0±0.2	ns	ns	ns
20:3 n-6	3.4±0.4	2.6±0.2	3.2±0.4	2.5±0.2	ns	*P*<0.07	ns
20:4 n-6	4.8±0.3	7.1±0.8	5.1±0.6	5.4±0.4	ns	*P*<0.08	*P*<0.10
20:5 n-3	0.2±0.1	0.5±0.2	0.2±0.0	0.3±0.0	ns	*P*<0.05	ns
24:0	1.6±0.1	2.4±0.2	1.8±0.07	1.8±0.2	ns	*P*<0.06	*P*<0.03
24:1 n-9	4.7±0.8	2.9±0.4	2.1±0.2	1.8±0.2	*P*<0.0002	*P*<0.04	*P*<0.08
22:4 n-6	1.3±0.1	0.9±0.1	0.7±0.1	0.9±0.1	*P*<0.01	ns	*P*<0.04
22:5 n-6	0.1±0.0	0.3±0.0	0.1±0.0	0.2±0.0	ns	*P*<0.03	ns
22:5 n-3	0.6±0.0	0.5±0.1	0.7±0.1	0.7±0.1	*P*<0.07	ns	ns
22:6 n-3	2.1±0.4	1.6±0.3	4.3±0.7	3.8±0.7	*P*<0.005	ns	ns
Σ SFA	52.8±1.2	54.2±1.7	52.0±0.6	54.2±1.1	ns	*P*<0.11	ns
Σ MUFA	27.9±1.2	23.9±1.6	24.6±0.5	22.2±0.7	*P*<0.01	*P*<0.004	ns
Σ n-6 PUFA	15.3±0.2	18.4±1.3	17.1±1.0	17.8±1.0	ns	*P*<0.12	ns
Σ n-3 PUFA	3.5±0.4	3.0±0.5	5.9±0.6	5.4±0.8	*P*<0.04	*P*<0.11	ns

Values are percentages relative to the total fatty acid content ± SE (n=6 mice per group). P^{Diet}: *P* value from the main effect of the diet in the PROC GLM procedure, P^{Drug}: *P* value from the main effect of DOX in the PROC GLM procedure. P^{Int}: *P* interaction between Diet and DOX in the PROC GLM procedure.

Table 4-16: Phosphatidylcholine phospholipid fatty acid composition (relative %) from MDA-MB-231 tumours excised from *nu/nu* mice

	Control Diet		DHA Diet		*P* values		
Fatty Acids	**Control**	**Control + DOX**	**DHA**	**DHA +DOX**	P^{Diet}	P^{Drug}	P^{Int}
14:0	1.0±0.1	1.1±0.2	1.2±0.1	1.0±0.1	ns	ns	ns
16:0	24.0±0.4	25.7±0.6	25.6±0.4	26.7±0.3	*P*<0.002	*P*<0.003	ns
16:1 n-9	1.8±0.2	1.7±0.3	1.6±0.1	2.1±0.2	ns	ns	ns
17:0	0.5±0.2	0.5±0.1	0.6±0.0	0.6±0.0	ns	ns	ns
18:0	20.5±0.3	18.9±1.0	20.2±0.5	18.7±0.5	ns	*P*<0.009	ns
18:1 n-9	22.1±0.4	23.1±0.8	22.6±0.4	22.3±0.6	ns	ns	ns
18:2 n-6	11.8±0.1	11.6±0.7	12.6±0.3	13.2±0.6	*P*<0.01	ns	ns
20:0	0.3±0.0	0.3±0.0	0.4±0.0	0.3±0.0	ns	ns	ns
18:3 n-6	0.8±0.1	0.3±0.2	0.7±0.0	0.6±0.0	ns	*P*<0.002	*P*<0.04
18:3 n-3	0.1±0.0	0.3±0.1	0.1±0.0	0.1±0.0	*P*<0.04	*P*<0.07	*P*<0.02
20:2 n-6	1.5±0.0	1.3±0.3	1.4±0.0	1.1±0.3	ns	ns	ns
20:3 n-6	1.6±0.0	1.6±0.0	2.0±0.1	1.7±0.1	*P*<0.0009	*P*<0.009	*P*<0.03
20:4 n-6	8.8±0.3	8.4±0.6	5.2±0.2	5.5±0.4	*P*<0.0001	ns	ns
20:5 n-3	0.1±0.0	0.1±0.0	0.2±0.0	0.3±0.0	*P*<0.0001	*P*<0.05	ns
24:0	0.3±0.0	0.4±0.0	0.3±0.03	0.3±0.0	ns	ns	*P*<0.06
24:1 n-9	2.5±0.1	2.0±0.2	1.2±0.0	1.0±0.0	*P*<0.0001	*P*<0.001	*P*<0.10
22:4 n-6	0.4±0.0	0.4±0.0	0.1±0.0	0.1±0.0	*P*<0.0001	ns	ns
22:5 n-6	0.0±0.0	0.1±0.0	0.0±0.0	0.0±0.0	*P*<0.05	*P*<0.08	*P*<0.04
22:5 n-3	0.4±0.0	0.3±0.0	0.5±0.0	0.6±0.0	*P*<0.0001	ns	ns
22:6 n-3	1.5±0.1	1.8±0.1	3.5±0.2	4.0±0.5	*P*<0.0001	ns	ns
Σ SFA	46.6±0.6	49.7±3.0	48.1±0.4	47.5±0.4	ns	ns	*P*<0.08
Σ MUFA	26.4±0.5	27.2±0.8	25.4±0.4	25.4±0.8	*P*<0.05	ns	ns
Σ n-6 PUFA	25.0±0.2	23.7±0.3	22.1±0.4	22.2±0.4	*P*<0.0001	*P*<0.09	*P*<0.04
Σ n-3 PUFA	2.0±0.1	2.5±0.3	4.4±0.2	4.9±0.5	*P*<0.0001	*P*<0.14	ns

Values are percentages relative to the total fatty acid content ± SE (n=6 mice per group). P^{Diet}: *P* value from the main effect of the diet in the PROC GLM procedure, P^{Drug}: *P* value from the main effect of DOX in the PROC GLM procedure. P^{Int}: *P* interaction between Diet and DOX in the PROC GLM procedure.

Table 4-17: Phosphatidylinositol phospholipid fatty acid composition (relative %) from MDA-MB-231 tumours excised from *nu/nu* mice

	Control Diet		DHA Diet		*P* values		
Fatty Acids	**Control**	**Control + DOX**	**DHA**	**DHA +DOX**	P^{Diet}	P^{Drug}	P^{Int}
14:0	0.7±0.3	1.6±0.8	0.6±0.2	0.9±0.4	ns	ns	ns
16:0	3.4±0.4	2.2±0.2	3.5±0.7	2.3±0.1	ns	*P*<0.03	ns
16:1 n-9	0.3±0.0	0.6±0.3	0.4±0.0	0.4±0.0	ns	ns	ns
17:0	0.3±0.0	0.3±0.0	0.3±0.0	0.2±0.0	ns	ns	ns
18:0	46.4±0.6	42.7±1.9	45.4±0.5	45.6±0.9	ns	*P*<0.10	*P*<0.05
18:1 n-9	15.6±2.9	20.8±2.5	17.8±3.1	21.5±0.5	ns	*P*<0.12	ns
18:2 n-6	4.3±0.2	4.4±0.2	4.8±0.2	4.4±0.2	ns	ns	ns
20:0	0.8±0.2	1.2±0.2	0.9±0.2	1.4±0.1	ns	*P*<0.03	ns
18:3 n-6	0.1±0.0	0.2±0.1	0.2±0.0	0.2±0.0	ns	ns	ns
18:3 n-3	0.4±0.1	0.5±0.1	0.5±0.1	0.6±0.0	ns	ns	ns
20:2 n-6	0.9±0.1	0.7±0.1	0.8±0.1	0.5±0.0		*P*<0.005	ns
20:3 n-6	4.0±0.1	3.9±0.5	5.0±0.2	4.8±0.1	*P*<0.001	ns	ns
20:4 n-6	14.1±4.2	7.2±2.8	3.2±0.2	3.3±0.3	*P*<0.0001	ns	ns
20:5 n-3	0.1±0.0	0.1±0.0	0.2±0.0	0.2±0.0	ns	ns	ns
24:0	0.9±0.4	1.6±0.4	1.2±0.3	2.0±0.1	ns	*P*<0.04	ns
24:1 n-9	4.5±0.6	4.6±1.0	2.8±0.3	3.1±0.2	*P*<0.006	ns	ns
22:4 n-6	0.9±0.1	1.2±0.1	0.4±0.0	0.4±0.0	*P*<0.0001	*P*<0.07	ns
22:5 n-6	0.1±0.0	0.2±0.1	0.1±0.0	0.1±0.0	ns	ns	ns
22:5 n-3	0.5±0.0	0.7±0.0	0.9±0.1	1.0±0.0	*P*<0.0001	*P*<0.007	ns
22:6 n-3	1.6±0.4	2.7±0.3	5.6±1.0	7.1±0.3	*P*<0.0001	*P*<0.05	ns
Σ SFA	52.6±0.6	52.0±0.9	51.9±0.7	52.4±1.1	ns	ns	ns
Σ MUFA	20.4±3.4	26.1±3.2	21.0±3.3	25.0±0.7	ns	ns	ns
Σ n-6 PUFA	24.3±4.2	17.7±2.4	13.9±0.2	13.7±0.4	ns	*P*<0.08	ns
Σ n-3 PUFA	2.6±0.6	4.0±0.3	7.2±1.0	8.8±0.3	*P*<0.0001	*P*<0.04	ns

Values are percentages relative to the total fatty acid content ± SE (n=6 mice per group). Data were analyzed using a 2-factor ANOVA to assess diet and drug interactions within each cell type. P^{Diet}: *P* value from the main effect of the diet in the PROC GLM procedure, P^{Drug}: *P* value from the main effect of DOX in the PROC GLM procedure. P^{Int}: *P* interaction between Diet and DOX in the PROC GLM procedure. DHA, docosahexaenoic acid; DOX, doxorubicin; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

Table 4-18: Sphingomyelin phospholipid fatty acid composition (relative %) from MDA-MB-231 tumours excised from *nu/nu* mice

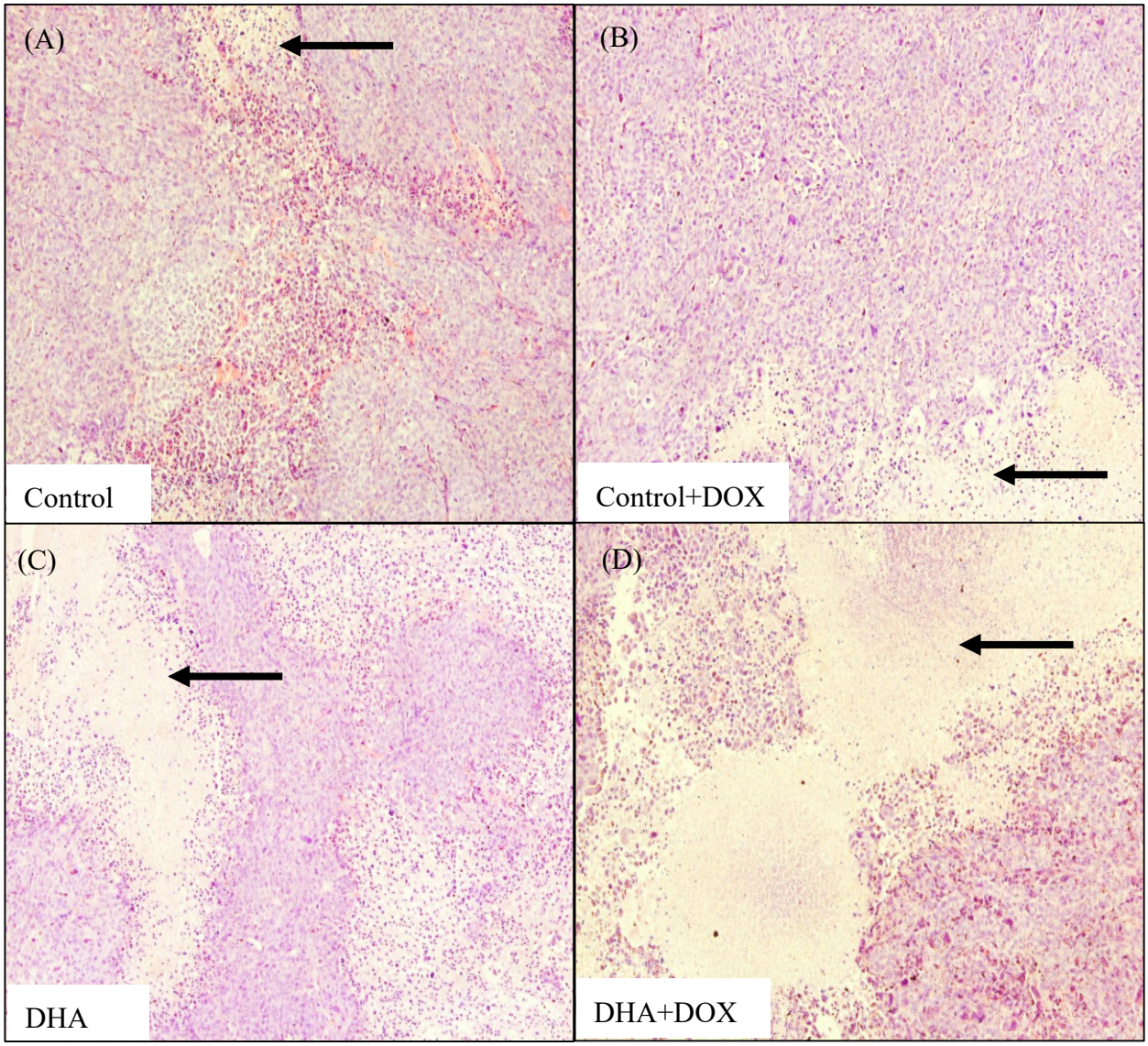
	Control Diet		DHA Diet		*P* values		
Fatty Acids	**Control**	**Control + DOX**	**DHA**	**DHA +DOX**	P^{Diet}	P^{Drug}	P^{Int}
14:0	2.3±0.6	3.6±1.2	3.3±0.6	1.4±0.1	ns	ns	*P*<0.03
16:0	40.3±1.4	41.9±2.0	38.8±1.9	40.0±3.3	ns	ns	ns
16:1 n-9	0.9±0.2	0.9±0.4	1.5±0.8	1.4±0.8	ns	ns	ns
17:0	0.8±0.1	0.9±0.1	0.9±0.1	1.9±1.2	ns	ns	ns
18:0	15.0±0.9	11.7±0.7	9.3±0.8	9.4±0.8	*P*<0.002	*P*<0.10	*P*<0.05
18:1 n-9	12.8±0.9	5.8±0.9	6.1±1.2	5.6±0.8	*P*<0.004	*P*<0.004	*P*<0.006
18:2 n-6	3.5±1.0	3.4±1.1	2.2±0.9	2.2±0.6	ns	ns	ns
20:0	1.2±0.5	0.8±0.0	0.8±0.1	2.6±1.8	ns	ns	ns
18:3 n-6	0.2±0.1	0.2±0.1	0.2±0.0	0.2±0.1	ns	ns	ns
18:3 n-3	0.3±0.1	0.2±0.1	0.2±0.0	0.4±0.3	ns	ns	ns
20:2 n-6	0.6±0.2	0.4±0.1	0.8±0.3	0.6±0.4	ns	ns	ns
20:3 n-6	4.1±0.5	4.3±0.8	5.0±0.5	6.0±0.2	*P*<0.02	ns	ns
20:4 n-6	0.9±0.2	1.2±0.4	0.5±0.2	0.7±0.3	*P*<0.13	ns	ns
20:5 n-3	0.6±0.2	0.7±0.2	0.6±0.3	1.7±1.0	ns	ns	ns
24:0	10.7±2.01	12.5±3.6	15.0±1.4	14.4±1.7	ns	ns	ns
24:1 n-9	7.0±1.1	7.5±1.8	8.6±1.0	8.5±1.2	ns	ns	ns
22:4 n-6	0.2±0.1	0.5±0.2	0.3±0.1	0.3±0.1	ns	ns	ns
22:5 n-6	1.2±0.2	1.6±0.3	1.3±0.1	1.6±0.1	ns	*P*<0.12	ns
22:5 n-3	0.1±0.0	0.2±0.1	0.2±0.1	0.1±0.0	ns	ns	ns
22:6 n-3	0.4±0.2	1.1±0.2	1.2±0.6	0.5±0.1	ns	ns	*P*<0.09
Σ SFA	65.2±2.6	71.5±1.0	66.8±2.6	69.7±2.4	ns	*P*<0.08	ns
Σ MUFA	20.7±3.4	14.2±1.9	18.2±2.1	15.4±0.5	ns	*P*<0.08	ns
Σ n-6 PUFA	10.8±0.7	11.6±0.7	12.0±1.4	11.6±0.7	ns	ns	ns
Σ n-3 PUFA	1.3±0.3	2.2±0.6	2.2±0.9	2.7±1.3	ns	ns	ns

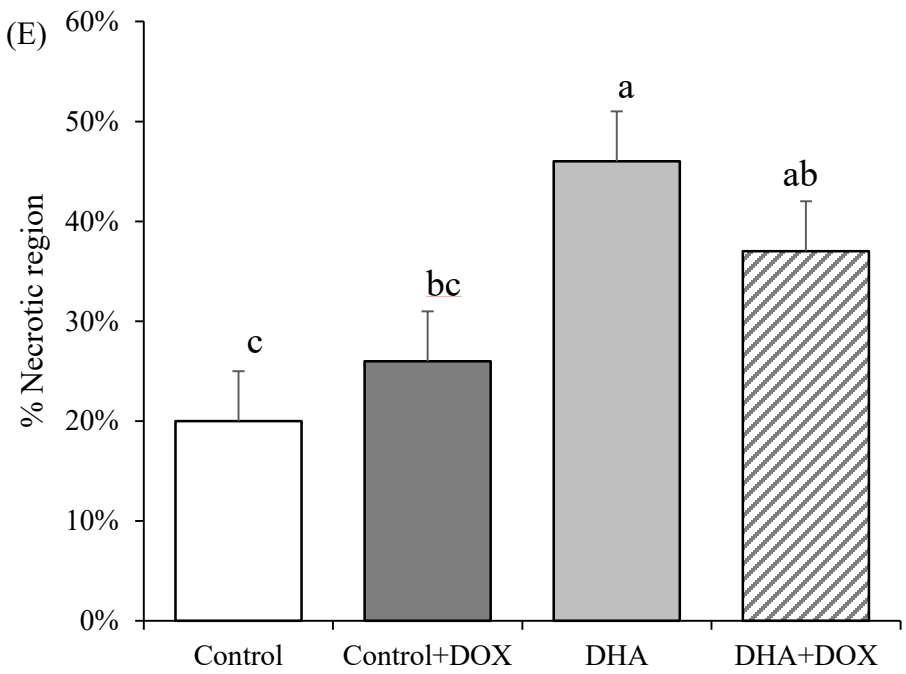
Values are percentages relative to the total fatty acid content ± SE (n=6 mice per group). P^{Diet}: *P* value from the main effect of the diet in the PROC GLM procedure, P^{Drug}: *P* value from the main effect of DOX in the PROC GLM procedure. P^{Int}: *P* interaction between Diet and DOX in the PROC GLM procedure.

4.3.4 MDA-MB-231 Tumour Morphology

Tumours from mice fed a DHA diet (with or without DOX) were found to have greater necrotic areas compared to mice in the groups fed the control diet (with or without DOX) **(Figure 4-6 A-D and E**; $P<0.05$). DOX treatment did not have an additional effect on the measured necrotic area in either diet group.

Figure 4-6. MDA-MB-231 tumour tissue H&E staining. Representative (A) Control (B) Control DOX (C) DHA and (D) DHA DOX tumours with regions of necrotic tissue highlighted with arrows. (E) Relative percent of necrotic regions in tumours compared to control.





4.4 Discussion

The therapeutic effects of n-3 LCPUFA in breast cancer have been widely investigated and our group has previously established that treating MDA-MB-231 (ER-) and MCF-7 (ER+) cells with n-3 LCPUFA including DHA results in decreased viability and increased anti-cancer effect of the chemotherapeutic DOX (Ewaschuk et al. 2012, Newell et al. 2019, Subedi et al. 2015). DHA treatment has been shown to induce a beneficial anti-cancer effect through multiple mechanisms, including increased apoptosis, oxidative stress and cell cycle arrest and decreased cell proliferation (reviewed by (Newell et al. 2017, D'Eliseo et al. 2016, Liu et al. 2014)). Many of these mechanisms are initiated at the cell membrane, including the CD95 death receptor pathway (Barnhart et al. 2003) and EGFR signaling (Oda et al. 2005, Schley et al. 2007), making it important to understand how treatment with DHA alters the membrane in the cancer cell. Other groups have previously assessed the membrane composition of MDA-MB-231 and MCF-7 breast cancer cells incubated with n-3 LCPUFA (Yu et al. 2015, Corsetto et al. 2011, Barascu et

al. 2006); yet there has not been a comprehensive assessment of how DHA treatment modulates the membrane during neoadjuvant chemotherapy and how fatty acid compositional changes in the cell membrane how this might contribute to the previously documented anti-cancer effects. Herein we have described for the first time the compositional differences between these two distinct breast cancer cell lines, supplemented with DHA alone or in conjunction with DOX chemotherapy; both in the whole cell and lipid raft membranes *in vitro* and in MDA-MB-231 tumours *in vivo* in an attempt to better understand the anti-cancer effects of DHA in a heterogeneous model.

Previously*, in situ* mass spectrometry and hierarchical cluster analysis of untreated breast cancer cell lines reported distinct cell membrane lipid phenotype differences. MDA-MB-231 cells have comparatively more PC than other breast cancer cell lines and MCF-7 cells have comparatively more PE, PI and SM (He et al. 2015). While these cells were not treated with fatty acids and therefore a direct comparison is not possible, some similarities to the current study were observed. The whole cell membrane PL class composition of the MDA-MB-231 cells (regardless of treatment) had higher amounts of PC and SM which are preferentially enriched on the external leaflet of the plasma membrane (Escribá et al. 2008, Verkleij et al. 1973), while the whole cell membranes of MCF-7 cells were observed to have higher amounts of PL moieties that are primarily located on the internal leaflet of the membrane: PE and PI (regardless of treatment) and PS (in OA/LA treated cells only). The distribution of these classes plays an important role in cell functioning. PC is known to contribute to cancer cell proliferation and apoptosis (Ridgway 2013) and its higher concentration could play a role in the prolific nature of MDA-MB-231 cells.

Fatty acid treatment did not result in changes in membrane class distribution within each PL moiety, however, a significant DOX effect was observed in MCF-7 cells in the lipid raft

component where the proportion of PS increased by two-fold and in both cell lines a significant DOX and membrane type interaction was observed. PS plays a role in apoptosis and therefore this could attribute to the increase observed in the raft component of both cell lines with DOX treatment. DHA incorporation into glycerophospholipids occurs primarily through its esterification to PE and lesser amounts to the other PL (Stillwell et al. 2003, Stillwell et al. 2005, Gu et al. 2013). In T27A leukemia cells it has been shown in that DHA is 5.7 times more concentrated in PE vs. PC (Zerouga et al. 1996) and this difference was also observed in the current study. In DHA treated MDA-MB-231 cells there were 6 times more DHA in PE vs. PC and in DHA treated MCF-7 cells there was 4.4 times more DHA in PE vs. PC. DHA has been reported to be highly incorporated into rafts, (Berquin et al. 2008) and in the current study upon treatment with DHA (in the presence or absence of DOX), we observed significantly more DHA in lipid rafts in both cell lines in PE, PS, PC and PI.

Compared to MCF-7 cells, MDA-MB-231 cells have a faster population doubling time. This necessitates an increase in plasma membrane production and therefore explains the higher incorporation of DHA in a set time period. To account for differences in growth rate, we compared the amount of DHA in the treated cells to the OA/LA control and determined that MDA-MB-231 and MCF-7 cells had similar fold change in DHA into each of the PL moieties of the whole cell membrane. However, we observed a consistent trend towards higher DHA in the lipid raft portion of MDA-MB-231 cells compared to MCF-7 cells in all PL moieties except for PI. Furthermore, a stark contrast between the two cell lines was observed when assessing the presence of EPA and DPA. In whole body ^{13}C tracer studies, retro-conversion of ^{13}C-DHA to ^{13}C-DPA and ^{13}C-EPA has been reported at 1.4% (Plourde et al. 2011). In the present study, retro-conversion of DHA to DPA and EPA occurred only in MDA-MB-231 cells, with an

increase in EPA in PE, PI and an increase in DPA in PI, PC in the whole cell membrane and an increase in EPA in PE, and PC and an increase in DPA PE, PI and PC in the rafts. Conversely, in MCF-7 cells, a lower relative content of DPA was seen in PE, PI, PS and PC in whole cell and in PE in the rafts. This difference was previously observed in a study measuring DHA effect on total PL composition by our group (Yu et al. 2015). These findings highlight the differences between the two cell lines and reinforce the importance of using multiple cell lines in a study, due to the heterogeneity of breast cancer.

The translatability of *in vitro* lipid incorporation to *in vivo* tumours was tested as we sought to determine if changes observed *in vitro* could be extended to the MDA-MB-231 tumour grown in mice when fed a DHA diet with DOX chemotherapy. While a direct comparison is not feasible, given the nature of the additional fatty acids provided in the fat mix of the diet, we did observe similar patterns in incorporation. Consistent with *in vitro* observations, there was similar percent incorporation of DHA into PE in MDA-MB-231 tumours, and interestingly, there was a higher relative percent DHA in all other PL moieties in the tumour compared to *in vitro* data. This could be due to the reported high fatty acid uptake by tumours (Sauer et al. 1990, Das 1999). Changes in tumour membrane due to DHA uptake with a reciprocal displacement of ARA has been cited as a possible mechanism for its anti-cancer efficacy (Merendino et al. 2013). Indeed, we observed both increased DHA and decreased ARA in MDA-MB-231 whole cell membranes *in vitro* and in tumours from mice fed a DHA diet. Furthermore, we have established that feeding a DHA enriched diet in conjunction with DOX significantly reduces the size of MDA-MB-231 tumours in mice (Newell et al. 2019).

We previously found that membrane initiated signaling resulted in decreased cellular proliferation and increased apoptosis in tumours from DHA fed, DOX treated mice (Newell et al.

2019). Recently Pizato et al. determined that treating MDA-MB-231 cells with DHA increased necrotic conditions including ruptured membranes (Pizato et al. 2018). Indeed, it is possible for both apoptosis and necrosis within the same tissue (Ozben 2007, Higuchi 2003) and in the current study we found that regardless of DOX, tumours from animals fed DHA had significantly higher regions of necrotic tissue. While one result of DOX metabolism is known to be an increase in necrosis (Tacar et al. 2013) and a correlation between DOX treatment and necrosis has been observed in an *in vivo* model of liver cancer (Gaba et al. 2016), we found that the increase in necrotic tissues occurred independently of DOX. Indeed, lipids are known to be important modulators of many forms of regulated cell death including apoptosis, necrosis, ferroptosis and pyroptosis (Magtanong et al. 2016).

4.4.1 Conclusions

This comprehensive study followed DHA incorporation into whole cell and lipid raft membrane PL in two phenotypically different cell lines in conjunction with chemotherapy. Differential membrane enrichment of DHA occurred between cell lines; in PL moieties and in membrane types (whole cell and lipid raft), highlighting the heterogeneity of breast cancer and the necessity of using multiple cell lines in preliminary analyses. However, we established in both cell lines and *in vivo* that incorporation of DHA into PL resulted in a decrease in ARA and was not hindered by DOX chemotherapy. Membrane changes produced in the feeding trial were similar to what we observed *in vitro* confirming the translatability and provides a good justification for using *in vitro* tissue culture models for preliminary dietary supplementation research. Researchers could then be confident that their findings would translate to the next level of pre-clinical investigations.

Chapter 5: Treatment with DHA modifies the response of MDA-MB-231 breast cancer cells and tumours from *nu/nu* mice to doxorubicin through apoptosis and cell cycle arrest[4]

5.1 Introduction

Triple negative (ER^- PR^- $HER2^-$) breast cancer accounts for 15%-20% of diagnosed breast cancers in women in North America and is reported to be more aggressive with poorer prognosis (Hurvitz et al. 2016, Haffty et al. 2006). More than 80% of women with triple negative breast cancer receive treatment with chemotherapy regimens that include anthracyclines such as doxorubicin (DOX) (Hurvitz et al. 2016). Although many women will benefit from this therapy, they will be exposed to the well-documented early and late toxicity of anthracyclines, including cardiotoxicity and leukemia (Ky et al. 2014, Petrelli et al. 2012). Thus, any new intervention that has the potential to improve the action of this drug without exposing the women to additional toxicities would be beneficial.

Studies conducted both *in vitro* and *in vivo* feeding trials have consistently shown that omega-3 long chain polyunsaturated fatty acids (n-3 LCPUFA), particularly docosahexaenoic acid (DHA), have anti-cancer properties in a number of different cancers, including breast cancer (Newell et al. 2017, Liu et al. 2014, D'Eliseo and Velotti 2016) [reviewed by (Siddiqui et al. 2011, Berquin et al. 2008, Sawyer et al. 2010)]. The majority of studies demonstrating anti-cancer effects of DHA on breast tumours have examined tumour development while only a few have focused on using DHA in treatment with the goal of reducing the growth of established mammary tumours (Connolly et al. 1999, Rose et al. 1996, Sun et al. 2011, Wu et al. 2005, Xue

[4] A version of this chapter has been published: Newell M, Brun M, Field CJ. (2019) Treatment with DHA modifies the response of MDA-MB-231 breast cancer cells and tumours from *nu/nu* mice to doxorubicin through apoptosis and cell cycle arrest. The Journal of Nutrition doi: https//doi.org/10.1093/jn/nxy224.

et al. 2014). Of considerable interest, DHA also improved response to anthracycline-based therapy in a phase II clinical trial of metastatic breast cancer patients. Bougnoux et al. (Bougnoux et al. 2006, Bougnoux et al. 2009) published compelling evidence that in an open-label trial, DHA supplementation given during DOX chemotherapy improved survival in these patients. This is exciting pilot data, but was studied only in advanced cancer. To be considered a candidate as adjuvant for current drug therapies, it is essential to have a mechanistic understanding of, and evidence supporting, the benefits of DHA in pre-clinical models. In support of this, there is a growing body of evidence that DHA treatment can improve the efficacy of chemotherapy drugs for cancers at other sites, including lung and colorectal cancer (reviewed by (Merendino et al. 2013, D'Eliseo and Velotti 2016, Calviello et al. 2009, Hardman 2004, Bougnoux et al. 2010)). There is also evidence in triple negative MDA-MB-231 cells that incubation with DHA can improve the efficacy of anthracyclines (Ewaschuk et al. 2012, Germain et al. 1998, Maheo et al. 2005). Although multiple molecular mechanisms have been proposed to explain the beneficial effects of DHA on anti-cancer chemotherapeutic agents (D'Eliseo and Velotti 2016, Sawyer et al. 2010, Merendino et al. 2013) these mechanisms are still not clearly elucidated. It is known that n-3 LCPUFA are rapidly incorporated into cell membranes, altering the structure and function of membrane receptors and signals (reviewed by (D'Eliseo and Velotti 2016, Bougnoux et al. 2010, Merendino et al. 2013, Biondo et al. 2008)). However, the effects of DHA on apoptosis and cell cycle progression when drugs such as DOX are administered are not clear. The purposes of the current study were 1) to identify the effects of pretreating MDA-MB-231 cells with DHA on the effects of DOX on the gene expression so as to elucidate key pathways that may explain the benefits of DHA and 2) to confirm these findings in a feeding trial using a well-characterized pre-clinical animal model.

5.2 Methods

5.2.1 Cell culture conditions

MDA-MB-231 cells, (American Type Culture Collection, Manassas, VA), were maintained in Iscove's Modified Dulbecco's medium supplemented with 5 % v/v fetal calf serum (FCS) and 1% v/v penicillin and streptomycin (Fisher Scientific, Edmonton AB) and grown at 37° C in 5 % CO_2 at 98 % relative humidity. Cells were seeded in 12-well plates at 1.5 x 10^5 cells/mL and grown to 80% confluence. For fatty acid treatments, oleic acid (OA; 18:1 n-9), linoleic acid (LA; 18:2 n-6) and docosahexaenoic acid (DHA; 22:6 n-3) (Matreya, MJS Biolynx, Brockville, ON) were dissolved in ethanol and conjugated to bovine serum albumin (BSA, Fisher Scientific, Edmonton, AB), as previously described (Ewaschuk et al. 2012). For experimental conditions, all cells were supplemented with a control fatty acid background of OALA (40 μM OA and 40 μM LA). The control lipid mixture was used to ensure that the effects of DHA were not due to fatty acid toxicity or to limiting the supply of essential fatty acids. Cells were subjected to four conditions: OALA control, DHA (60 μM on a background of OALA), OALA + doxorubicin (DOX), and DHA DOX (on a background of OALA). Control OALA and DHA treated cells were incubated with fatty acids for 72 hours, with media refreshed every 24 hours. For DOX alone, cells were incubated for 48 hours on the background of OALA and then treated with DOX 0.41 μM in the presence of OALA for 24 hours. For the DHA DOX group, cells were incubated with DHA for 48 hours, and then DOX was applied in the presence of DHA for the final 24 hours. The concentrations of DOX and fatty acids used and length of exposure were based on dose response curves previously published so as to achieve a 40% decrease in cell viability with DHA and a 20% decrease with DOX (Ewaschuk et al. 2012). All *in vitro* experiments were repeated four times on cells from independent cell passages.

5.2.2 Cell viability: WST-1

Cellular viability of MDA-MB-231 cells under experimental conditions was determined with cell proliferation reagent WST-1 (Roche Applied Science, Laval PQ) as per manufacturer's instructions.

5.2.3 Microarray analysis

After treatment, cells were rinsed twice with PBS and RNA was isolated using Trizol (Life Technologies, Burlington, ON), as per manufacturer's instructions. RNA concentration and purity were assessed using a NanoDrop spectrophotometer (Thermo Scientific, Nepean, ON). Microarray processing of purified RNA was performed at The Centre for Applied Genomics, The Hospital for Sick Children, Toronto, Canada. Affymetrix GeneChip Human Gene 2.0 ST arrays were used. Gene expression data were imported into Partek Genomics Suite 6.5 (Partek, St Louis, Mo) as CEL files, using default parameters. Raw data were pre-processed, including background correction, normalization, and summarization using robust multiarray average analysis, and expression data were Log2 transformed. Principal-component analysis (PCA) was carried out to identify outliers and evaluate whether batch effects, cell type or treatment significantly impacted the data. Differential expression analysis was performed using ANOVA with the Restricted Maximum Likelihood procedure applied. Gene lists of significantly altered genes (compared with control) were created using a cutoff of $P < 0.05$, 1.2-fold change. This cutoff was selected to determine a larger number of potential genes differentially affected by the treatments. Comparisons were determined *a posteriori* to identify the effects of DHA vs. OALA, DHA DOX vs. OALA, DOX vs. OALA and DHA DOX vs DOX. Lists of significantly altered genes were then imported into Ingenuity Pathway Analysis. Gene networks and canonical pathways representing key genes were generated using the curated Ingenuity Pathways Analysis

(IPA) database (IPA®, QIAGEN Redwood City, CA). The functional analysis identified the biological functions that were most significant to the data sets. Fisher's exact test was performed to calculate a *P*-value determining the probability that each biological function assigned to the data set was due to chance alone. In the graphical representations, the color of genes in the networks indicate the degree of significant down-regulation (blue with DOX alone or green with DHA DOX) or up-regulation (pink with DOX alone or yellow with DHA DOX) of gene expression. Additionally, we have provided all the data in an open format. The dataset supporting the conclusions of this article is included within the article and its additional files. A supplemental dataset is in an open data base at the University of Alberta (doi:10.7939/DVN/TNLMAN) and the data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al. 2002) and are accessible through GEO Series accession number GSE113427 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE113427).

5.2.4 Western blot analysis

MDA-MB-231 whole cell lysates and tumour samples were prepared for western blot analysis as previously described (Schley et al. 2005) and Western blots were performed as previously described (Subedi et al. 2015). Primary antibodies to BCL2, Bid, Cyclin B1, Cdc25C, (Cell Signaling Technology, New England Biolabs, Whitby, ON, Canada), and Wee1, Caspase 10 and Ripk1 (Abcam, Cedarlane, Burlington, ON, Canada) were diluted 1:1000 in 5% w/v BSA- Tris-buffered saline with Tween 20 (TBST). Membranes were cut to probe for both the primary antibody of interest and GAPDH from the same replicate. GAPDH was used as a loading control at a concentration of 1:5000 in 5% w/v BSA-TBST. Primary antibodies were detected with anti-rabbit IgG Horseradish Peroxidase (HRP)-linked secondary (Cell Signaling

Technology, Whitby ON) or anti-mouse IgG HRP-linked secondary (Abcam, Cedarlane, Burlington, ON, Canada) using Pierce ECL 2 Western Blotting Substrate (Fisher Scientific, Edmonton AB) and visualized on a Typhoon^{TM} Trio+ variable mode imager (GE Life Sciences, Baie d'Urfe, PQ). The relative intensities of bands were quantified using ImageQuant TL software. Images are a representative experiment from 4 separate experiments.

5.2.5 TUNEL Assay and Apoptosis

Treated MDA-MB-231 cells were washed once in phosphate-buffered saline (PBS), fixed in 1% paraformaldehyde in PBS and then assayed for cell cycle with APO-BrdU™ TUNEL Assay Kit (Life Technologies, ON) as per manufacturers' instructions. Propidium iodide stained cells were visualized by flow cytometry with a FACSCanto (BD Biosciences, Mississauga, ON, Canada) and histogram distribution analyzed using Kaluza Software (Beckman Coulter, Mississauga, ON, Canada). Apoptosis was assessed with Annexin V-FITC Apoptosis Detection Kit (Abcam, ON) as per manufacturers' instructions. Fixed cells were visualized by flow cytometry with a FACSCanto (BD Biosciences, Mississauga, ON, Canada) and analyzed according to the fluorescence intensity relative to control using Kaluza Software (Beckman Coulter, Mississauga, ON, Canada).

5.2.6 Experimental diets and animals

A nutritionally complete basal mix diet (fat omitted) was obtained from Teklad (TD.84172, Harlan Laboratories, Madison, WI). This formula is based on the AIN-76 diet. The completed composition of the basal diet has been described in detail in a previously published Journal of Nutrition manuscript (Robinson et al. 1998). For this study we supplemented the control and experimental diets at 20% w/w fat which equates to ~40% of total energy from fat. The fatty acid composition of the diets (**Appendix Table 6)** were achieved by blending oils so as

to obtain a polyunsaturated to saturated fatty acid ratio of 0.5 and a DHA content in the DHA diet of 2.8% w/w fat. This level of DHA in the diet was selected to achieve a plasma phospholipid concentration of approximately 4% DHA (see **Appendix Table 7**), the concentration reported to be associated with improved survival in women undergoing chemotherapy for metastatic breast cancer (Bougnoux et al. 2009). All diets were irradiated for 72h at 8 kGy and stored at -20°C until used. Fatty acid analysis by gas liquid chromatography pre- and post-irradiation confirmed that the fat composition was not altered by irradiation (data not shown). Animal experiments were reviewed and approved (AUP00000134) by the University of Alberta Animal Policy and Welfare Committee and were in accordance with the Canadian Council on Animal Care guidelines. Immune-deficient 6-week old female *nu/nu* mice (Charles River Laboratories International, Inc) were housed in bio-containment under aseptic conditions with autoclaved bedding and water. After 3 days feeding the control diet, MDA-MB-231 cells (2 x 10^6 cells/100µl in 5% FCS Iscove's media) were injected one time subcutaneously below the upper right scapula (Subedi et al. 2015). Mice were fed control diet *ad libitum* until the tumour reached approximately 50 mm^3 (Marlind et al. 2008, North et al. 2011) then randomized (n=6 for each experimental group) to one of two experimental diets. Tumours were measured twice weekly with calipers and estimated volume (volume= (length x width x width)/2) was calculated. After one week of DHA or control diet, the animals were further randomized to receive 5mg/kg doxorubicin chemotherapy as previously established in MDA-MB-231 cells (Hardman et al. 2001) or 0.9% saline injections twice weekly for four weeks. After the diet treatment began, body weight and food intake were regularly monitored. Mice were euthanized, tumours carefully excised and weighed, and formalin fixed for immunohistochemistry or homogenized for protein analysis. Plasma phospholipids were extracted using a modified Folch procedure (Field et al.

1988, Folch et al. 1957) and phospholipid fatty acid composition was determined by gas-liquid chromatography as previously described (Schonberg et al. 1995). The individuals performing the excision and weighing of the tumour and all subsequent assays were blinded to the diet treatments.

5.2.7 Immunohistochemistry

Paraffin embedded tumour sections were deparaffinized in xylene and rehydrated in alcohol. Antigen retrieval was performed by immersing slides in 10 mM sodium citrate buffer (pH 6.0) for 15 minutes in a pressure boiler, followed by 10 minutes quenching in 3% hydrogen peroxide and 30 minutes of 5% goat serum-TBST. Slides were incubated overnight at 4° with antibodies (1:50 in 5% goat serum-TBST) for Plk1 (Cell Signaling Technology, New England Biolabs, Whitby, ON, Canada), Cyclin B1, Cyclin B2, Wee 1, Birc5, CD95 and cdc25C (Abcam, Cedarlane, Burlington, ON, Canada). Slides were incubated with SignalStain Boost IHC Detection Reagent (HRP, rabbit; Cell Signaling Technology, New England Biolabs, Whitby, ON, Canada) at room temperature for 30 minutes and positively stained cells were visualized using ImmunoDetector liquid DAB (Bio SB Inc., Santa Barbara, CA, USA). Apoptosis in tumour sections was assessed using the ApopTag® Peroxidase *In Situ* Apoptosis Detection Kit (EMD Millipore, Temecula, CA) according to manufacturer's instructions. Sections were imaged using AxioCam (Carl Zeiss microscopy) and the proportion of positive cells was determined.

5.2.8 Statistical analysis

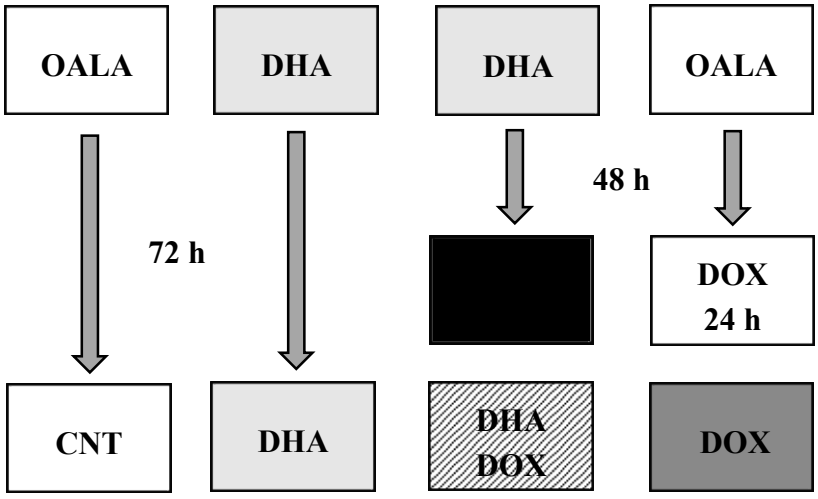
All statistical analyses (except the microarrays, described above) were conducted using SAS, (version 9.4, Cary, NC, USA). Data is reported as mean ± standard error of the mean (SEM) unless otherwise indicated. For the cell culture study, data were analyzed using a 2-way ANOVA to assess diet and drug interactions, followed by post hoc analysis using DUNCAN to

identify significant differences between treatments (P<0.05). For the *in vivo* study, data were tested for normal distribution and if not normally distributed the Kruskal-Wallis test for non-parametric analysis was used and indicated in the results. Normally distributed results were analyzed using a 2-way ANOVA to assess diet and drug interactions, followed by post hoc analysis using DUNCAN to identify significant differences between treatments (P<0.05).

5.3 Results

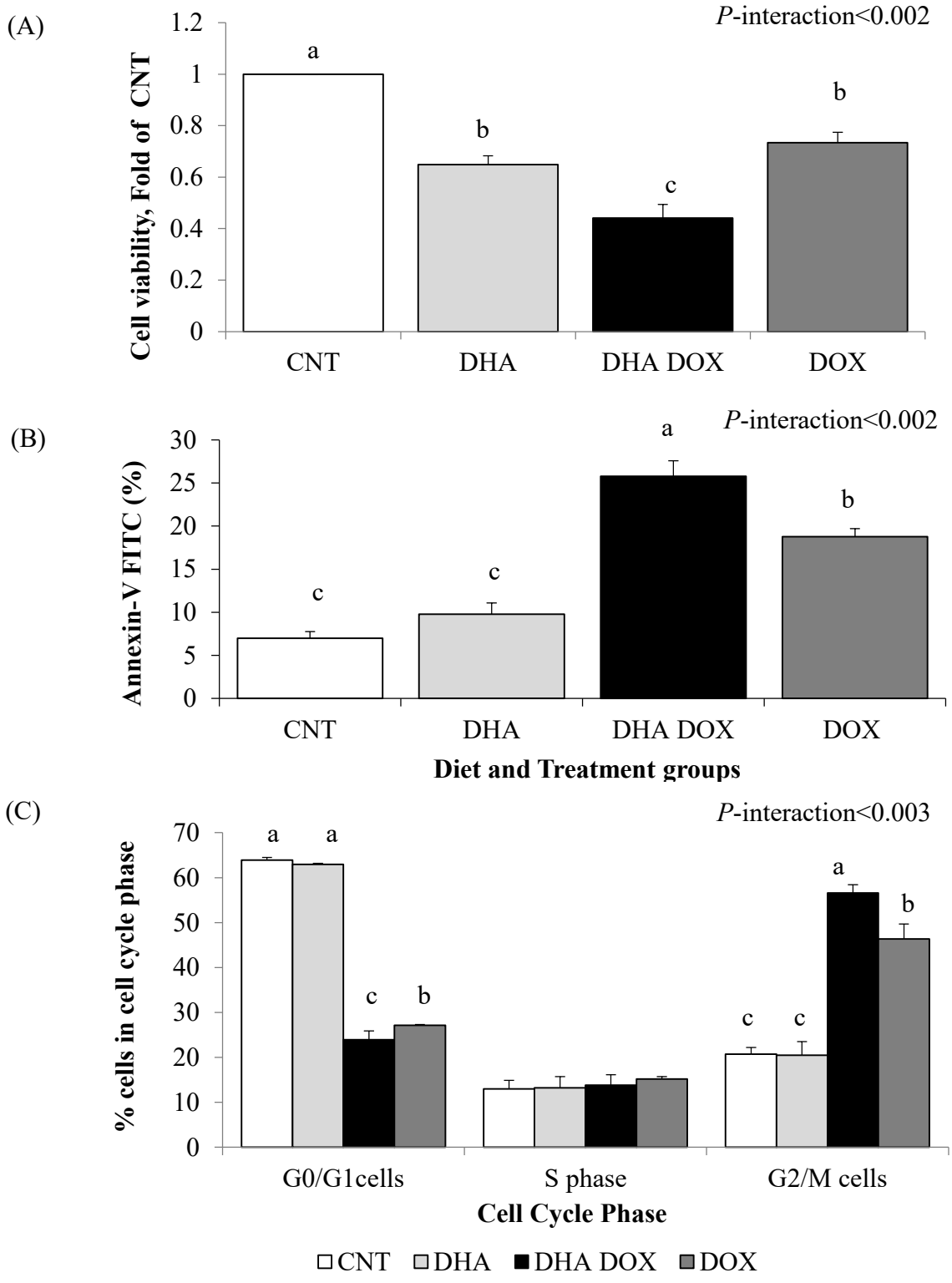
5.3.1 Effect of treatments on cell growth and cell cycle progression

Figure 5-1: Experimental design for determining efficacy of DHA with or without Doxorubicin *in vitro*



Compared to the control condition (Experimental layout **Figure 5-1**), treatment with DOX or with DHA significantly reduced cell viability of MDA-MB-231 cells in culture (**Figure 5-2 A**). Incubation with DHA prior to DOX further reduced cell viability of MDA-MB-231 cells (P<0.05).

Figure 5-2: Efficacy of DHA with or without DOX on MDA-MB-231 cells in terms of cell viability, apoptosis and cell cycle

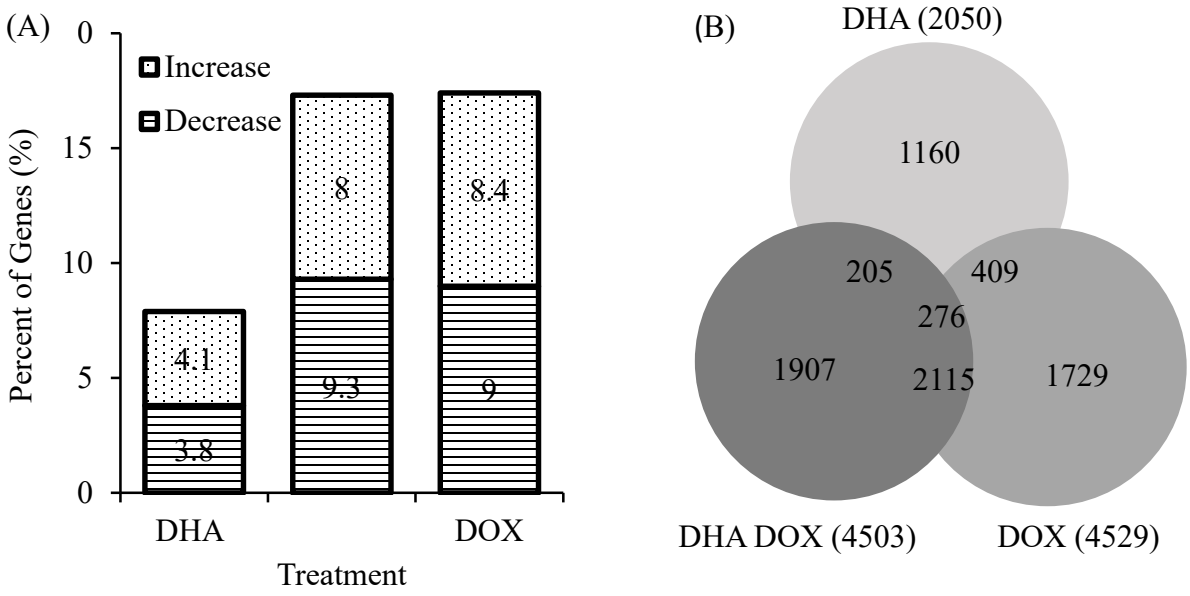


While incubation with DHA reduced cell viability, there was no increase in apoptosis. However, pre-incubation with DHA prior to DOX increased DOX mediated apoptosis in these cells compared to DOX alone (**Figure 5-2 B**). DHA DOX resulted in a lower proportion of cells in G_0/G_1 and a higher proportion in G_2/M phase compared to DOX (**Figure 5-2 C**). Values represent the mean ± SEM (n=4 independent experiments). Means that do not share a common letter are significantly different (P<0.05) based on post hoc DUNCAN analysis. The P values for the main effect of the diet are: Cell viability: P = <0.0001; Apoptosis: P = 0.002; G0/1 phase: P = 0.28; S phase: P =0.23; G2/M phase: P =0.23; and the main effect of the treatment: Cell viability: P = <0.0001; Apoptosis: P = <0.0001; G0/1 phase: P <0.001; S phase: P =0.07; G2/M phase: P =0.0001. P-interaction is the interaction between DHA diet and DOX chemotherapy treatment; P interaction: G0/1 phase: P = 0.40; S phase: P =0.14; G2/M phase: P =0.13.

5.3.2 Effect of treatments on gene expression

Out of 26054 genes, 2050 (7.9%) in DHA, 4529 (17.4%) in DOX and 4503 (17.3%) in DHA DOX group were significantly altered (fold change > 1.2 and P≤0.05) compared to the control fatty acid treatment (**Figure 5-3 A**). Out of these, 982 (3.8%), 2344 (9%) and 2414 (9.3%) were decreased and 1068 (4.1%), 2185 (8.4%) and 2089 (8.0%) were increased in DHA, DOX and DHA DOX group respectively, compared to control. As illustrated in a Venn diagram, showing the overlap and discrepancies between genes expressed (≥ 1.2-fold change, $P \leq 0.05$) in the 3 cell treatments, 1160 (4.5%), 1729 (6.6%) and 1907 (7.3%) genes were unique to DHA, DOX and DHA DOX group respectively, compared to control. The number in parentheses indicates the total number of genes expressed in each treatment type (**Figure 5-3 B**).

Figure 5-3: Genome wide gene expression in DHA, DOX and DHA DOX treated MDA-MB-231 cells compared to control



To explore how DHA DOX altered gene expression compared to DOX alone, we examined the predicted functions of differentially expressed genes. Both DOX, and DHA DOX decreased the expression of genes involved in cell cycle regulation, proliferation, tumour growth and transformation, survival, and viability and increased the expression of genes involved in apoptosis and cell death. Gene data analysis suggested top networks and cellular functions affected by DHA DOX treatment were Cyclins and Cell Cycle Regulation and Death Receptor Signaling pathways ($P<0.05$, **Appendix Figure 3**). Using these networks and cellular functions we then selected key genes in this pathway that were altered by the DHA DOX treatment for further analysis. Interestingly, DHA DOX consistently increased the number of genes altered in each category compared to DOX.

5.3.3 Effect of treatments on proteins involved in apoptosis

Key genes involved with apoptotic signaling with altered expression following treatment are listed in **Table 5-1**. Treatment of cells with DOX resulted in significant increase in expression of the pro-apoptotic genes *CASP9*, *CD95*, and *RIPK1*, and decreased expression of the anti-apoptotic genes *BCL2, BIRC5, BIRC6, BCL2A1.* When MDA-MB-231 breast cancer cells were incubated with DHA prior to DOX treatment, there was a consistent increase in the change in expression, and an increase in the number of both pro- and anti-apoptotic genes altered. Additional changes in DHA DOX treated cells include reduced expression of the anti-apoptotic gene *BCL-XL,* and increased expression of the pro-apoptotic genes *BID*, *BIK,* and *CASP10* compared to DOX alone, as well as altered expression of *CXCL10, ICAM-1, IKBKE, TNFAIP3*, and *TNFSF15*, which modulate apoptotic signaling.

Table 5-1: Genes associated with apoptosis including death receptor signaling pathway altered by DHA with or without DOX or DOX alone in MDA-MB-231 cells

Gene Symbol	Entrez gene name	DHA vs OALA	DHA DOX vs OALA	DOX vs OALA	DHA DOX vs DOX
		Fold change	Fold change	Fold change	Fold change
ACTA1	Actin, alpha 1	1.19	-1.20	1.18	-1.41*
APO2L	TNF (ligand) superfamily, member 10 (TNFSF10)	-1.26*	1.18	-1.05	1.23*
BAG1	BCL2-associated anthanogene	1.00	-1.26*	-1.17	-1.07
BCL-2	B-cell CLL/lymphoma 2	1.01	-1.33*	-1.32*	-1.01
BCL2A1	BCL2 related A1	-1.10	1.38*	-1.19*	1.64*
BCL-XL	BCL2-like 1	1.01	-1.21*	-1.11	-1.08
BID	BH3 interacting domain death agonist	-1.05	1.07	-1.14	1.22*
BIK	BCL2 interacting killer	1.07	1.22*	1.08	1.13
BIRC5	Baculoviral IAP repeat containing 5	1.05	-1.44*	-1.21*	-1.2*
CASP2	Caspase 2	1.09	-1.09	1.16	-1.26*
CASP9	Caspase 9	-1.09	1.35*	1.16*	1.17*

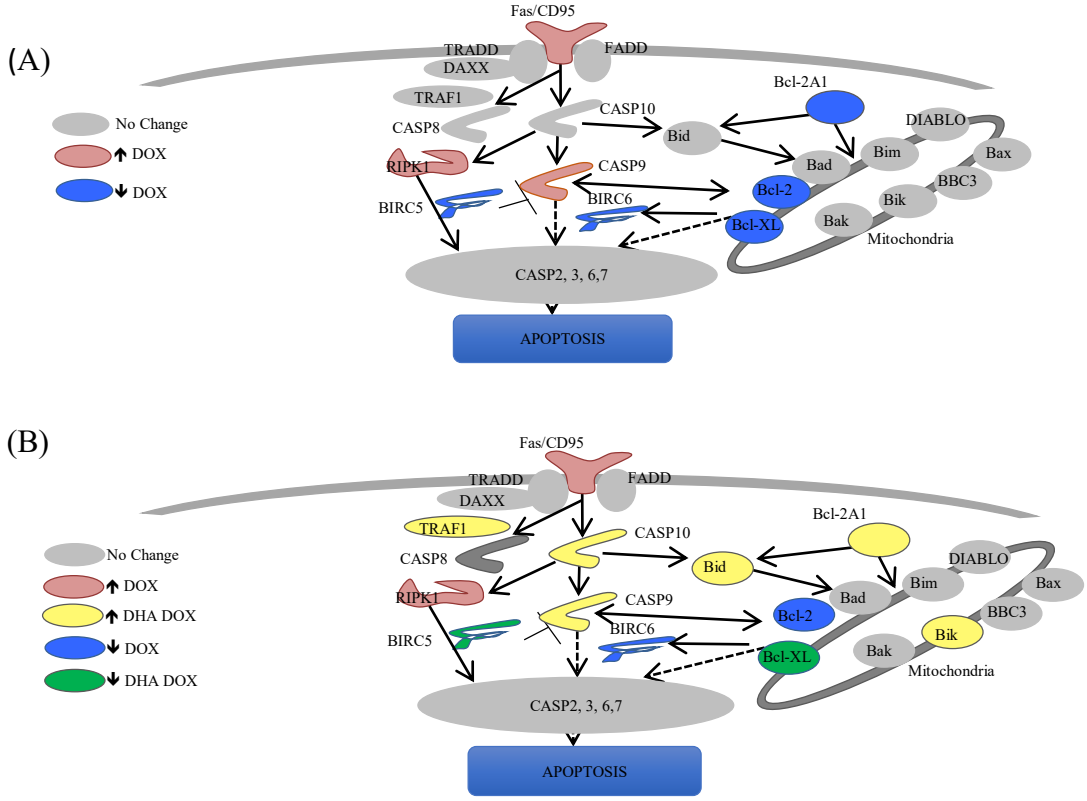
CASP10	Caspase 10	-1.16*	1.27*	1.09	1.16*
CXCL10	Chemokine (C-X-C motif) ligand 10	-1.15	2.69*	1.44	1.87*
EGR1	Early growth response 3	-1.01	1.78*	1.20	1.48*
FAS	CD95	-1.11	1.23*	1.15*	1.10
PARP15	Poly(ADA-ribose)polymerase family, member 15	-1.03	1.31	-1.06	1.39*
RIPK1	Receptor (TNFRSF)-interacting serine-threonine kinase 1	1.00	1.21*	1.19*	1.02
TRAF1	TNF receptor-associated factor 1	-1.14	1.43*	1.10	1.30*
TNFAIP3	Tumour necrosis factor, alpha-induced protein 3	-1.12	1.75*	1.33	1.32*
TNFSF15	Tumour necrosis factor (ligand) superfamily member 15	-1.21	2.79*	1.65	1.69*

* Indicates significantly different. Genes are considered significantly increased (positive value) or decreased (negative value) based on the selection criteria ($P \leq 0.05$ and fold change ≥ 1.2). However important genes in death receptor pathway only significantly changed at 1.1-fold are also shown.

A complete list of the genes involved in cell death, apoptosis and cell survival that were modified by any of the 3 treatments (not present in Table 5-1) can be found in **Appendix Tables 8-12**.

A schematic representation of apoptosis genes differentially expressed in MDA-MB-231 cells pre-treated with DHA prior to DOX compared to OALA control is shown in **Figure 5-4 A-B**. Genes differentially expressed with DOX treatment alone are shown in **Figure 5-4 A** and **Table 5-1**. Genes associated with apoptosis including death receptor signaling pathway altered by DHA with or without DOX or DOX alone in MDA-MB-231 cells differentially expressed in cells pre-treated with DHA prior to DOX are shown in **Figure 5-4 B** (where there is differential expression compared to OALA but not DOX the gene color is the same as for the DOX treatment).

Figure 5-4: Apoptotic Gene Expression in DHA treated MDA-MB-231 cells treated with or without DOX

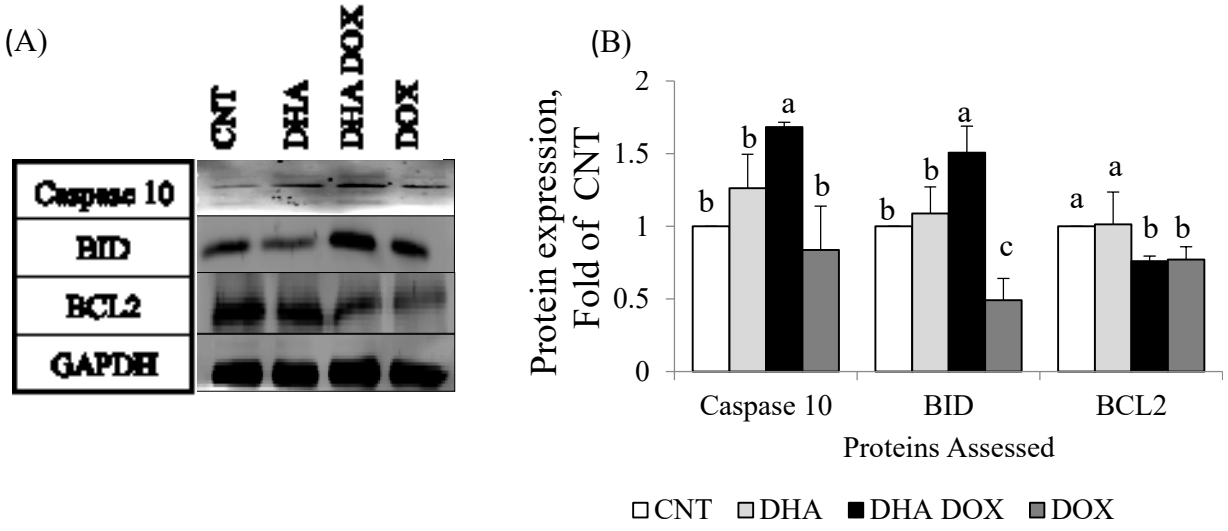


Grey colored genes indicate no change in expression compared to OALA control, pink colored genes indicate increased expression in DOX compared to OALA control, blue colored genes indicate decreased expression in DOX compared to OALA control, yellow colored genes indicate increased expression compared to DOX alone or OALA control, green colored genes indicate decreased expression compared to DOX alone or OALA control. (A) DOX treated cells (B) DHA DOX treated cells. Significantly altered genes (compared with CNT) were created using a cut off of $P < 0.05$, 1.2-fold change.

Changes in expression of selective apoptosis genes identified by microarray were verified by immunoblot analysis of treated MDA-MB-231 cells (**Figure 5-5 A** & **B**). Consistent with the microarray data, treatment with DHA prior to DOX increased the levels of Caspase-10

and BID and decreased the levels of BCL2 (P<0.05). Values represent the mean ± SEM (n=4 independent experiments). Labeled means without a common letter differ (P<0.05) based on post hoc DUNCAN analysis. The P values for the main effect of the diet are: Caspase 10: P = 0.04; BID: P =0.07; BCL2: P =0.80; the main effect of the treatment: Caspase 10: P = 0.67; BID: P =0.80; BCL2: P =0.12; and P interaction: Caspase 10: P = 0.2; BID: P =0.13; BCL2: P =0.79.

Figure 5-5: Apoptotic Protein Expression in DHA treated MDA-MB-231 cells treated with or without D



5.3.4 Effect of treatments on proteins involved in cell cycle transition

This analysis revealed significant changes in expression of genes associated with cell cycle transition (G2/M transition) following treatment with DOX in the presence and absence of DHA. Key genes associated with cell cycle transition (G2/M checkpoint) are listed in **Table 5-2**. The change in expression following treatment with DOX was augmented by exposure to DHA for a number of genes, including *CCNB2*, *CDC25C*, *CKS2*, and *MDM2*. A complete list of the genes involved in cell cycle regulation that were modified by any of the 3 treatments (not present in Table 5-2) can be found in Appendix Tables 8-12.

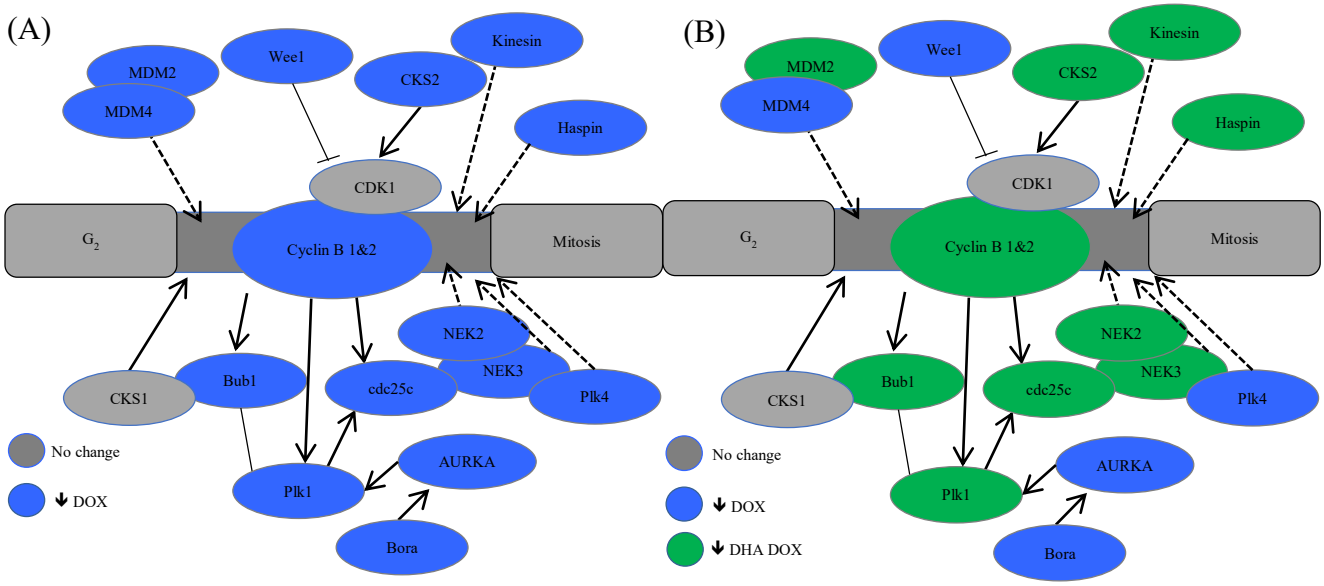
Table 5-2: Genes associated with G2/M DNA checkpoint regulation pathways that differ significantly between DHA DOX and the OALA Control in MDA-MB-231 cells

		DHA vs OALA	DHA DOX vs OALA	DOX vs OALA	DHA DOX vs DOX
Gene Symbol	**Entrez gene name**	Fold change	Fold change	Fold change	Fold change
14-3-3σ	14-3-3 Sigma	1.0	1.29*	1.34*	-1.04
AURKA	Aurora kinase A	1.05	-1.80*	-1.59*	-1.13
BORA	Bora, aurora kinase A activator	1.09*	-1.74*	-1.62*	-1.07
BRCA1	Breast cancer 1, early onset	1.01	1.36*	1.60*	-1.18*
CCNB1	Cyclin B1	1.05	-2.08*	-1.90*	-1.09*
CCNB2	Cyclin B2	1.02	-1.58*	-1.36*	-1.16*
CCNB3	Cyclin B3	1.11	-1.01	1.15	-1.16
CDK1/CDC2	Cyclin-dependent kinase 1	1.06	-1.10	1.05	-1.15*
CDC20	Cell division cycle 20 homolog	1.17	-1.88*	-1.68*	-1.12*
CDC25C	Cell division cycle 25 homolog C	-1.08	-1.75*	-1.38*	-1.27*
CKS2	CDC28 protein kinase regulatory subunit 2	1.13*	-1.68*	-1.33*	-1.27*
HIPK2	Homeodomain interacting protein kinase 2	1.17	-1.40	-1.10	-1.27*
MDM2	E3 ubiquitin protein ligase	1.04	-1.20*	-1.20	-1.0
MDM4	p53 binding protein homolog	-1.09	-1.20	-1.20	1.0
NDC80	NDC80 kinetochore complex component homolog	1.02	-1.56*	-1.33*	-1.17*
NEK2	NIMA (never in mitosis gene a)-related kinase 2	-1.13	-1.96*	-1.71*	-1.15*
NEK3	NIMA (never in mitosis gene a)-related kinase 3	-1.07	-1.80*	-1.40*	-1.28*
p21Cip1	Cyclin-dependent kinase inhibitor 1A	1.07	1.33*	1.26*	1.05
PLK1	Polo-like kinase 1	1.03	-2.98*	-2.60*	-1.14*
PLK4	Polo-like kinase 4	-1.00	-1.33*	-1.19*	-1.12
TOP2	Topoisomerase (DNA) II alpha	-1.07	-1.26*	-1.14	-1.1
WEE1	WEE1 homolog	-1.01	-1.55*	-1.41*	-1.10*

* Indicates significantly different. Genes are considered significantly increased (positive value) or decreased (negative value) based on the selection criteria ($P \leq 0.05$ and fold change ≥ 1.2). However important genes in cell cycle only significantly changed at 1.1-fold are also shown.

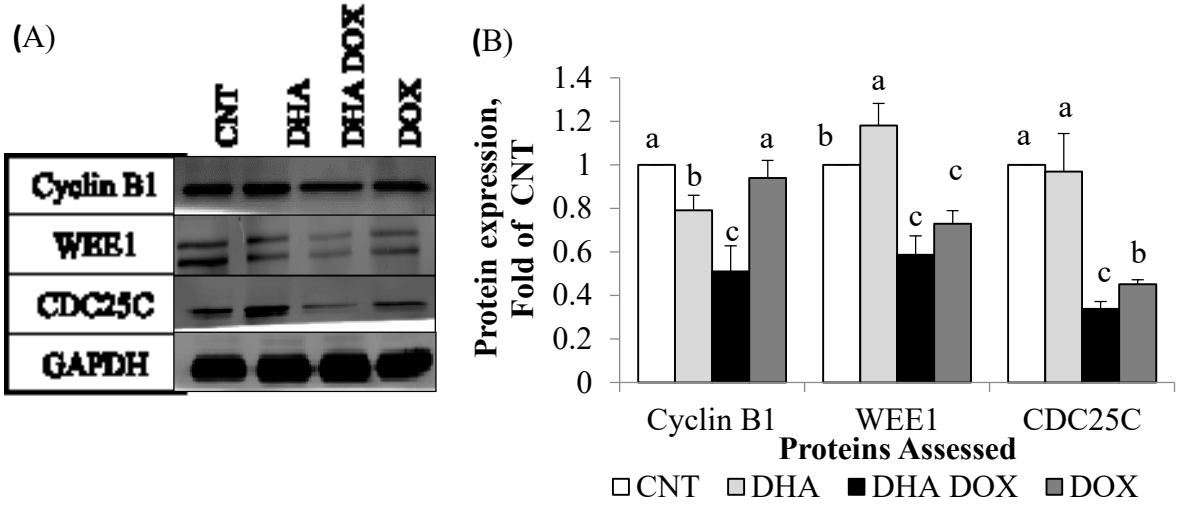
A schematic representation of cell cycle genes differentially expressed in MDA-MB-231 cells pre-treated with DHA prior to DOX compared to OALA control is shown in **Figure 5-4 A-B**. Many of these changes in expression of genes involved in the G2/M transition were verified by immunoblot analysis of treated MDA-MB-231 cells (**Figure 5-5 A & B**). Consistent with the microarray data, DOX treatment reduced the levels of WEE1 and CDC25C (P<0.05). Treatment with DHA prior to DOX further reduced the levels of CDC25C and Cyclin B1 in MDA-MB-231 cells (P<0.05).

Figure 5-4: Cell Cycle Gene Expression in DHA treated MDA-MB-231 cells treated with or without DOX



Grey colored genes indicate no change in expression compared to OALA control, blue colored genes indicate decreased expression in DOX compared to OALA control and green colored genes indicate decreased expression compared to DOX alone or OALA control. (A) DOX treated cells (B) DHA DOX treated cells. Significantly altered genes (compared with OALA) were created using a cut-off of P <0.05, 1.2-fold change.

Figure 5-5: Cell Cycle Protein Expression in DHA treated MDA-MB-231 cells treated with or without DO

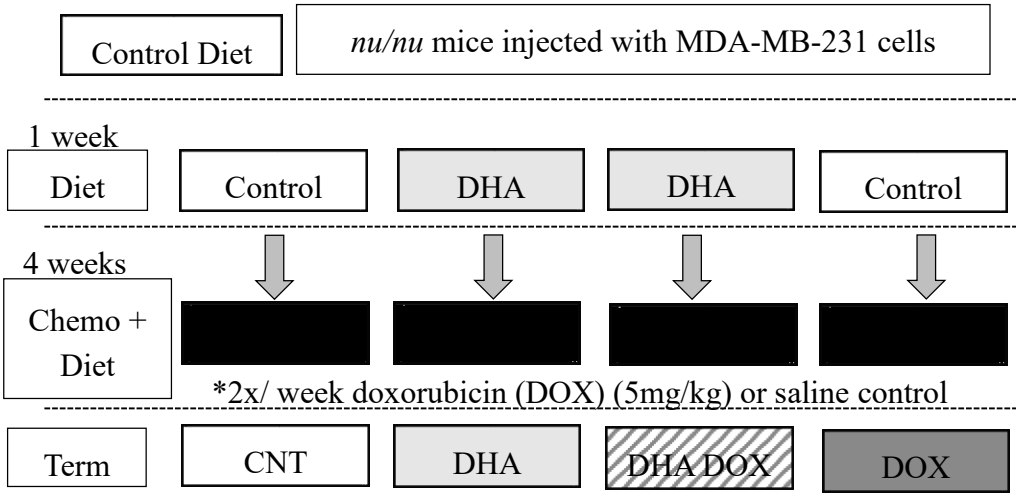


(A) Representative Western blot of cell cycle proteins and (B) densitometric quantification of blots for Cyclin B1, WEE1 and CDC25C. Values represent the mean ± SEM (n=4 independent experiments). Labeled means without a common letter differ (P<0.05) based on post hoc DUNCAN analysis. The P values for the main effect of the diet are: Cyclin B1: P =0.01; WEE1: P =0.56; CDC25C: P =0.55; the main effect of the treatment: Cyclin B1: P =0.05; WEE1: P = 0.001; CDC25C: P =0.006; and P interaction: Cyclin B1: P =0.19; WEE1: P =0.05; CDC25C: P =0.96.

5.3.5 Effect of feeding a DHA diet to tumour bearing mice on tumour growth

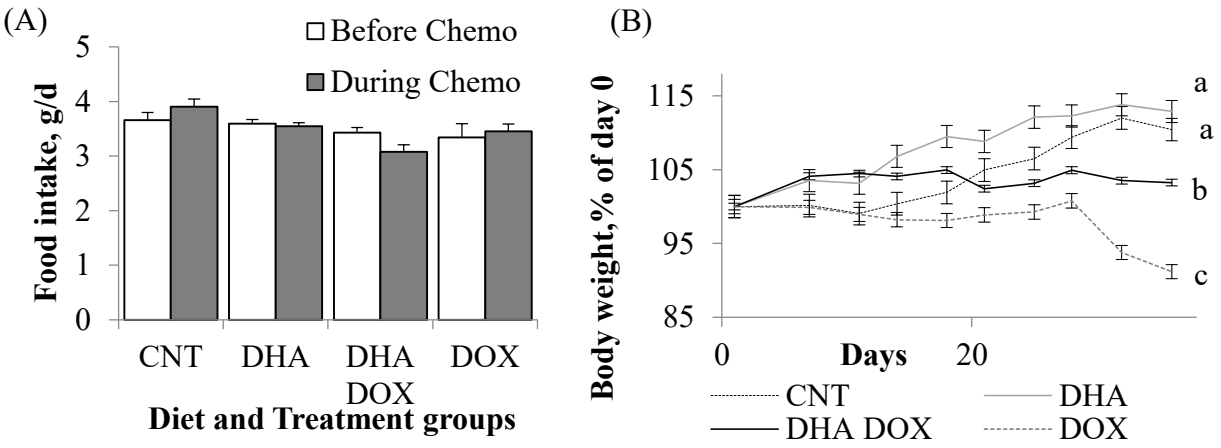
There was no significant difference in food intake among treatments (**Figure 5-6 A**), however, mice treated with DOX (consuming either of the experimental diets) weighed less at the end of the experiment compared to those not receiving DOX (**Figure 5-6 B**, P<0.05). Mice fed control diet and treated with DOX lost weight, while mice in DHA DOX treatment group did not (**Figure 5-6 B**, P<0.05).

Figure 5-6: Experimental Design of DHA dietary supplementation with or without DOX on MDA-MB-231 tumour growth in *nu/nu* mice[1]



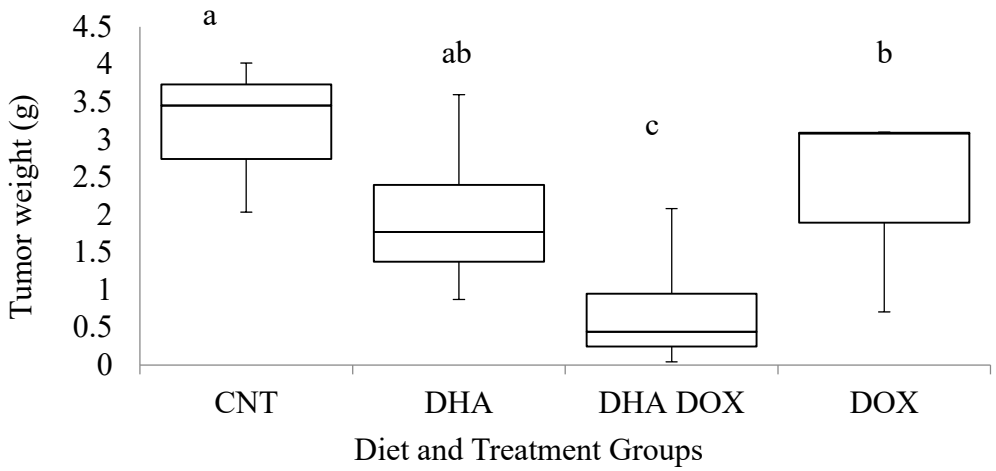
[1]Mice were injected with 2 x 10^6 MDA-MB-231 cells and maintained on control diet for 4 weeks. One week prior to commencing chemotherapy the mice were randomized into control or DHA diet groups and subsequently into chemotherapy (twice weekly) or control groups for an additional 4 weeks.

Figure 5-6: Effect of DHA dietary supplementation with or without DOX on (A) average daily food intakes and (B) body weights on MDA-MB-231 tumour bearing *nu/nu* mice[1]



[1]Normalized to 100%, letters signify differences in body weights at termination.

Figure 5-7: Effect of DHA dietary supplementation with or without DOX on on MDA-MB-231 tumour growth in *nu/nu* mice[1]



[1]Excised tumour weights of *nu/nu* mice fed control or DHA diet with or without DOX. Values represent the mean ± SEM (n=6 mice per group). Kruskal-Wallis test for non-parametric analysis was employed for tumour weights (P =0.04). Labeled means without a common letter differ (P<0.05) based on post hoc DUNCAN analysis.

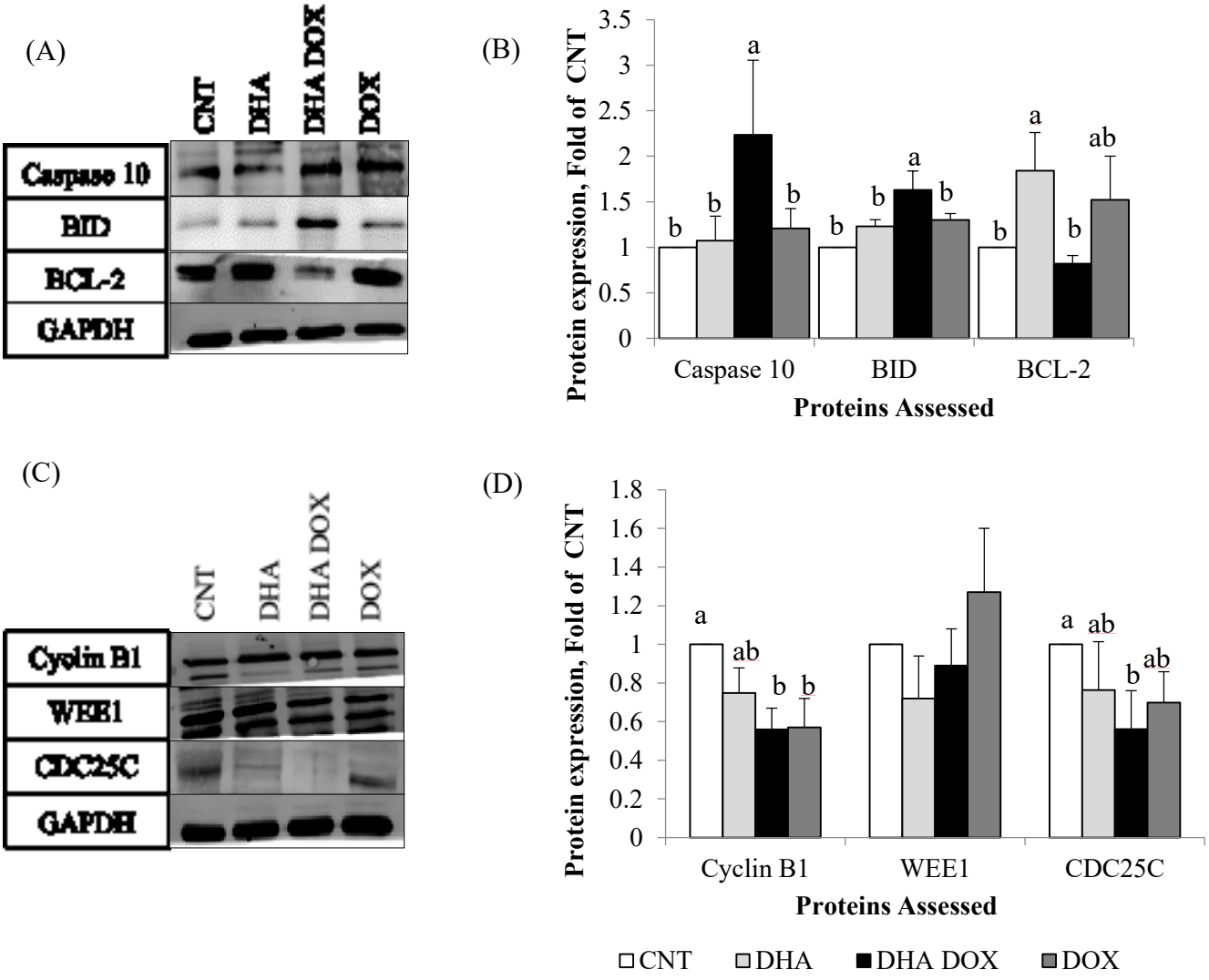
Treatment with DOX lowered the tumour burden and feeding DHA with DOX treatment further reduced tumour weight (**Figure 5-7**, P<0.05). Percent fatty acid in plasma phospholipid levels of mice were quantified in the four treatment groups and relative percent of DHA in the plasma phospholipids was significantly higher in the DHA DOX group compared to control (4.38±0.15 vs. 1.30±0.40, P<0.05. **Appendix Table 7**).

5.3.6 Effect of feeding a DHA diet on proteins involved in apoptosis and cell cycle transition *in vivo*

Proteins involved in apoptosis and the G2/M transition that were identified as differentially expressed between DHA DOX and OALA or DOX in the *in vitro* microarrays using MDA-MB-231 cells were confirmed by immunoblot analysis of *in vivo* tumour extracts

(**Figure 5-8 A-D)**. Caspase-10 and BID were increased while BCL2, Cyclin B1 and CDC25C were decreased. WEE1 was not different in tumour protein extracts.

Figure 5-8. Effect of DHA dietary supplementation with or without DOX on protein expression of (A) apoptosis and (C) cell cycle molecules in MDA-MB-231 tumours extracted from *nu/nu* mice, with (B) (D) with densitometric quantification of blots from (A) (C).

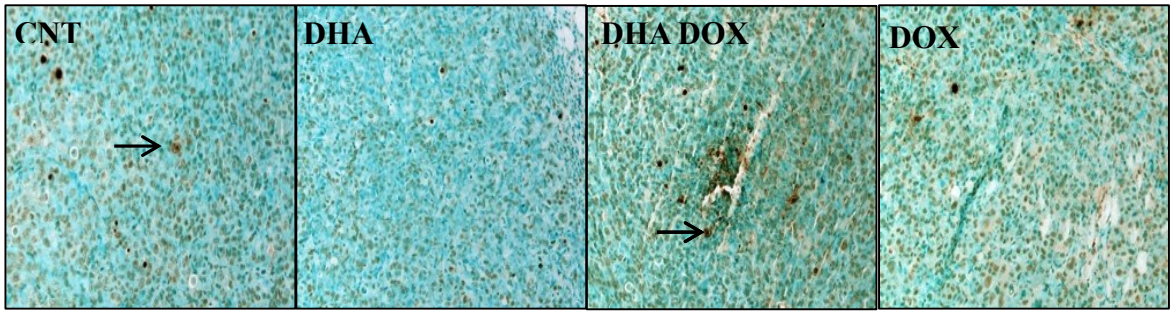


The *P* values for the main effect of the diet are: Caspase 10: *P* =0.19; BID: *P* =0.09; BCL2: *P* =0.84; Cyclin B1: *P* =0.47; WEE1: *P* =0.17; CDC25C: *P* =0.31; the main effect of the treatment:

Caspase 10: P =0.11; BID: P =0.04; BCL2: P =0.49; Cyclin B1: P =0.10; WEE1: P =0.33; CDC25C: P =0.18; and P interaction: Caspase 10: P =0.25; BID: P =0.77; BCL2: P =0.04; Cyclin B1: P =0.52; WEE1: P =0.83; CDC25C: P =0.78.

TUNEL staining of the tumours from the DHA DOX animals further confirmed increased apoptosis (**Figure 5-9**) and CD95 expression in addition to reduced expression of BIRC5, Cyclin B1, PLK1, WEE1 and CDC25C (**Appendix Figure 4**).

Figure 5-9: Effect of DHA dietary supplementation with or without DOX on immunohistochemical analysis of apoptosis by TUNEL assay in MDA-MB-231 tumours extracted from *nu/nu* mice[1]



[1]Positive staining is dark brown color and nuclei are stained green (methyl green). Values represent the mean ± SEM (n=6). Labeled means without a common letter differ (P<0.05) based on post hoc DUNCAN analysis.

5.4 Discussion

MDA-MB-231 cells readily form tumours in mice but only have an intermediate response to chemotherapy (Holliday et al. 2011). We have previously reported that that pre-treating MDA-MB-231 with DHA prior to DOX enhances the efficacy of DOX (Ewaschuk et al. 2012). The current study extends these findings by identifying two mechanisms by which DHA mediates these effects. Specifically, we show that DHA increases expression of pro-apoptotic genes and decreases expression of cell cycle genes *in vitro* and *in vivo*, thereby enhancing tumour cell

apoptosis and slowing tumour growth. Whereas DOX treatment alone led to significantly reduced body weight, we found that this side-effect was mitigated by DHA feeding during treatment. These findings are consistent with those of other studies (Lu et al. 2011, Zombeck et al. 2013), including one showing that feeding fish oil to mice implanted with the MCF-7 tumours (Kang et al. 2010) decreased tumour growth without altering body weight or food intake. In a clinical study of metastatic breast cancer patients with DHA supplementation by Bougnoux et. al (Bougnoux et al. 2009), it was observed that women with highest incorporation of DHA into their plasma (up to 4.3% increase from baseline DHA content) had the most improved outcomes We extend these findings by achieving this plasma phospholipid DHA concentrations in our animal study.

The ability of tumour cells to evade apoptosis is a well-established hallmark of cancer (Hanahan et al. 2000, Hanahan et al. 2011) and it can occur via extrinsic or intrinsic pathways. The molecular mechanisms involved in these cell death pathways overlap and can be co-activated during apoptotic events. DHA has been previously shown to induce apoptosis (reviewed in (D'Eliseo et al. 2016, Blanckaert et al. 2010)) in breast tumour cells (Kang et al. 2010, Chamras et al. 2002), including MDA-MB-231 cells (Ewaschuk et al. 2012, Rose et al. 1994). In the current study, gene, protein and immunohistochemical analysis suggests that the effects of DHA on DOX occurred through facilitating apoptosis via effects on multiple molecules in both pathways. In the extrinsic pathway, membrane receptor CD95 (FAS) was increased with DOX treatment. We have previously demonstrated that DHA increases the movement of CD95 receptors into membrane rafts, which act as signalling platforms within the cell (Ewaschuk et al. 2012) and in the current study, using an antibody to block CD95 prevented the beneficial effects of DHA on DOX-induced apoptosis. CD95 receptors cluster in a death

complex, which then activates downstream initiator caspases: Caspase-8 and Caspase-10. Interestingly, there was no change in Caspase-8 while Caspase-10 was up regulated by DHA DOX compared to DOX alone. The intrinsic signalling pathway that initiates apoptosis is regulated by mitochondrial outer membrane potential (MOMP), involving a diverse array of non-receptor-initiated stimuli that produce intracellular signals leading to release of cytochrome *c* and activation of Caspase-9 (up regulated in DHA DOX). *BIRC5* (Survivin), an inhibitor of the intrinsic pathway, is highly expressed in most human tumours (Mita et al. 2008) and in the present study this gene was down regulated with DHA treatment prior to DOX, suggesting a mechanism to explain the higher expression of Caspase-9. Consistent with our findings in breast cancer cells, down regulation of BIRC5 by DHA has been reported in colon tumours (Calviello et al. 2009, Sam et al. 2016, Slagsvold et al. 2010), and chemical induced murine mammary tumours (Siddiqui et al. 2013).

BCL-2 expression, both *in vitro* and *in vivo*, was reduced by DOX (with and without DHA treatment), while *BCL-$_X$L* gene expression was significantly down regulated with DHA DOX, but not DOX alone, suggesting enhanced efficacy of DOX with DHA treatment. Consistent with this, BCL2A1 was decreased with treatment, but unexpectedly in cells treated with DHA DOX, gene expression was 1.4-fold higher. While BCL2, BCL-$_X$L and BCL2A1 are pro-survival molecules and are amplified in cancer cells (Vogler 2012) (including breast tumours (Beverly et al. 2009)), BCL2A1 is unique among the other anti-apoptotic BCL2 members since it does not have a motif that allows for insertion into the mitochondrial membrane (Beverly et al. 2009). It has been shown that cleaved BCL2A1 is able to exert independent cytotoxic effects (Valero et al. 2012). This could, in part, explain findings that up regulation of this gene does not induce tumourigenesis (Chuang et al. 2002). Additionally, in CD95 mediated apoptosis,

increased expression of anti-apoptotic BCL2 family members that cannot inhibit apoptosis once it has begun (Barnhart et al. 2003). Our findings in MDA-MB-231 cells suggest that the increased membrane expression of CD95 is key in the beneficial effects of DHA on DOX. Gene expression of the pro-apoptotic members of the *BCL2* family, *BIK* and *BID*, were up regulated with DHA prior to DOX compared to DOX alone, although the expression of *BID* did not differ significantly from the control treatment. Interestingly an increase in BID protein expression was confirmed both *in vitro* and *in vivo*. BID, long established to be the link between the extrinsic and intrinsic apoptotic pathways (Luo et al. 1998), is a direct substrate for Caspase-10 and can activate the pro-apoptotic protein BAX (Correia et al. 2015). We have previously shown that treating/feeding stearidonic acid, another n-3 LCPUFA increased BID expression in MDA-MB-231 cells/tumours (Subedi et al. 2015), but to our knowledge, we are the first to demonstrate the up regulation of this apoptotic gene with DHA treatment administered prior to DOX.

In addition to apoptosis, deregulation of the cell cycle is a second, well- established hallmark of cancer (Okada et al. 2004, Hanahan et al. 2000, Hanahan et al. 2011). Incubation with DHA prior to DOX arrested a greater proportion of cells at the G_2M checkpoint than did the control fatty acid mixture. This is consistent with findings of a synergy or chemo-sensitization effect of DHA on DOX that has also been observed in B-Cell chronic lymphocytic leukemia cells incubated with DHA (Fahrmann 2013). In a retrospective analysis of breast tumours, genes involved in the G_2M transition were found to be overexpressed and predicted evasion of checkpoint control (Bieche et al. 2011). While the independent effect of DHA on cell cycle progression during G_2M phase is not well documented in the literature (Newell et al. 2017), the effect of DOX on the disruption of the cell cycle is well established (Ling 1996, Siu Wai 1999, Bilim 2000, Tyagi 2002). The mechanisms by which DOX induces cell cycle arrest are not

entirely clear (Imreh 2011, Park 2012). Although DHA alone did not alter cell cycle progression or expression of major cell cycle genes, our findings support the conclusion that DHA's enhancement of DOX efficacy occurs at the level of cell cycle progression.

Cell cycle progression is regulated by cyclins and their cyclin dependent kinases (Otto et al. 2017) DOX treatment reduced the gene expression of Cyclin B1 (levels of which are often elevated in tumours, leading to cells entering the M phase prematurely and unregulated proliferation (Aaltonen et al. 2009) but the amount of protein was not reduced significantly in cells or tumours until DHA was provided. Barascu et al. studied effects of EPA and DHA on MDA-MB-231 cells and found that providing either of these fatty acids led to a higher proportion of cells in the G2/M phase, and similar to the current study, also observed that DHA treatment of MDA-MB-231 cells reduced the expression of Cyclin B1 (Barascu et al. 2006). DHA pre-treatment with DOX also amplified the DOX mediated down-regulation of a number of other genes involved in G_2M phase transition. DHA pre-treatment prior to DOX administration resulted in a 1.3-fold further decrease in expression of CKS2, a protein that binds to CDK1 and CDK2. Up regulation of CKS2 is associated with larger tumour size, poor tumour differentiation and reduced survival (Wang et al. 2014). Additionally, the gene and protein for the tyrosine phosphatase, CDC25C was further down regulated in DHA DOX. This enzyme dephosphorylates Cyclin B-bound CDC2(CDK1) and this triggers mitotic entry into mitosis. These findings suggest that feeding DHA in combination with DOX treatment not only amplifies the effect of DOX on some key proteins critical for cell cycle but may have effects on additional proteins involved in cell cycle arrest.

Because of the critical importance of the G_2M transition of the cell cycle, many of the genes involved have been investigated as targets for cancer therapy. Inhibition of PLK1 resulted

in improved survival in acute myeloid lymphoma (Gjertsen et al. 2015). Increased BUB1 (Sotiriou et al. 2003) (required at the spindle assembly checkpoint of mitosis) and NEK2 (Cappello et al. 2014) (involved in centrosome separation) correlates with poor clinical prognosis in basal (ER^-) tumours (Dominguez-Brauer et al. 2015). PLK4, NEK2, and BUB1 have all emerged as potential anti-mitotic checkpoint inhibitors (Dominguez-Brauer et al. 2015) and all were significantly down-regulated in DHA DOX treated cells compared to DOX alone.

5.5 Conclusions

In the current study we examined the effect of pre-treatment with DHA on anti-tumour action of a breast cancer drug, DOX, on a triple negative human breast cancer cell line. We have demonstrated that DHA treatment alone, although cytotoxic to MDA-MB-231 cells, had a minimal effect on the expression of genes that regulate tumour cell apoptosis or cell cycle progression. This is consistent with previous studies that have identified that the main effects of DHA (in the absence of cytotoxic drugs) is via modifications in receptors and signals in the plasma membrane (reviewed by (Newell et al. 2017)). Although the cellular processes that lead to increased death of MDA-MB-231 cells after combined DHA and DOX treatment are complex and multifaceted, our work suggests that this occurred, in a multi-pronged manner, through amplifying the DOX-mediated increase in apoptosis (most probably via CD95) and blockage of cell cycle progression at the G_2M check point. These findings, together with the evidence that DHA mitigates weight loss during chemotherapy and reduces overall tumour size provides strong evidence to justify the need for clinical evaluation of this well tolerated dietary fatty acid on patient clinical outcomes and survival.

CHAPTER 6: Role of docosahexaenoic acid in enhancement of docetaxel action in patient derived breast cancer xenografts[5]

6.1 Introduction

Despite advances in prevention, screening, diagnoses and treatment, breast cancer is expected to account for more than 627,000 deaths worldwide annually (World Health Organization 2017). One of the difficulties that arises in successfully treating breast cancer is that it manifests as a heterogeneous group of diseases, rather than a single disease (Turashvili et al. 2017, Dobrolecki et al. 2016). Moreover, tumours employ multiple methods to survive and proliferate, creating a complex, continuously evolving environment which contributes to therapy resistance (Hanahan and Weinberg 2000, Hanahan and Weinberg 2011). This is particularly true for triple negative breast cancers (TNBC: estrogen, progesterone and human epidermal growth factor receptor negative, $ER^-PR^-HER2^-$). TNBC accounts for 15-20% of all breast cancer diagnoses in North America and is characterized by an aggressive trajectory that results in poorer patient prognoses (Pal et al. 2011, Hurvitz et al. 2016, Haffty et al. 2006). Targeted drug therapy is not yet an option for most women diagnosed with TNBC. Rather, most patients are treated with adjuvant or neoadjuvant systemic chemotherapy such as docetaxel (TXT) (Ho et al. 2014). TXT is known to be ineffective against certain breast cancers including metastatic cancers where the response rate is 30-50% (Noguchi 2006). However, it remains difficult to predict in the TNBC population who will respond favorably to docetaxel.

[5] A version of this chapter has been published: Newell M, Goruk S, Mazurak V, Postovit L, Field CJ. (2019) Role of docosahexaenoic acid in enhancement of docetaxel action in patient derived breast cancer xenografts. Breast Cancer Research and Treatment https://doi.org/10.1007/s10549-019-05331-8

The pleiotropic anti-cancer effects of the n-3 long chain polyunsaturated fatty acid (LCPUFA), docosahexaenoic acid (DHA), have been previously established in breast cancer cells *in vitro* and *in vivo* (reviewed by D'Eliseo and Velotti 2016, Newell et al. 2017, Liu et al. 2014). There is a growing evidence suggesting that DHA might also be beneficial when provided in conjunction with chemotherapy in a number of cancers *in vitro* (Calviello et al. 2005, Ewaschuk et al. 2012, Granci et al. 2013, Lindskog et al. 2006, Shaikh et al. 2008, Vibet et al. 2008) and in a few animal models of breast cancer (Chauvin et al. 2016, Newell et al. 2019, Vibet et al. 2008). While many of the multidimensional hallmarks of cancer (Hanahan and Weinberg 2000, Hanahan and Weinberg 2011) appear to be targeted by LCPUFA (reviewed in D'Eliseo and Velotti 2016, Newell et al. 2017, Liu et al. 2014), much of this work has relied on the use of immortalized cell lines (*in vitro* and implanted in immunodeficient animals *in vivo*) that do not represent the intratumoural heterogeneity of patient tumours (Turashvili et al. 2017, Whittle et al. 2015).

A major challenge in translating laboratory findings to a clinical setting is the inability of cell line derived preclinical models to recapitulate genomic and microenvironmental heterogeneities (Dobrolecki et al. 2016, Ellis et al. 2010, Gillet et al. 2011, Hait 2010, Gould et al. 2015). Patient derived xenografts (PDXs) bridge this gap and more closely recapitulate the heterogenicity and gene expression of primary tumours (Zhang et al. 2014, Whittle et al. 2015). Herein, we employed two TNBC PDX models, one well differentiated and the other poorly differentiated, in order to determine if feeding DHA enhances the anticancer actions of docetaxel. We further sought to explore potential mechanisms involved in the anticancer effects of DHA.

6.2 Methods

6.2.1 Experimental diets

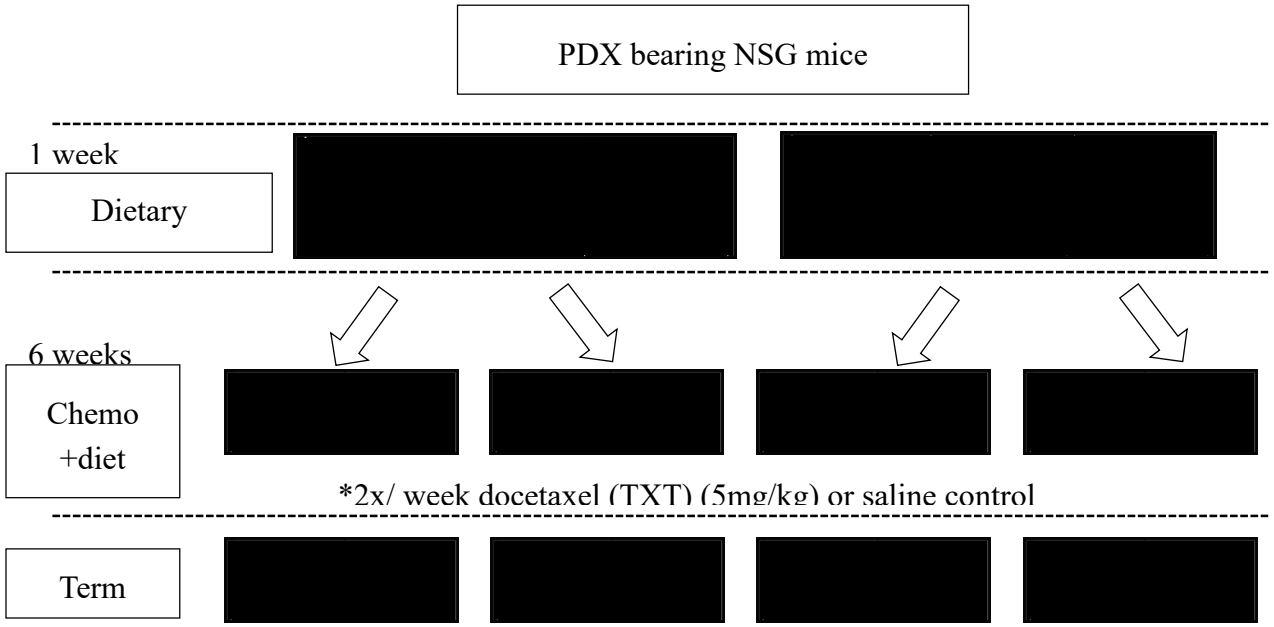
Nutritionally complete diets were composed of a basal mix diet from Teklad, (TD.84172; Harlan Laboratories, Madison, WI), with macronutrient composition as previously described (Robinson et al. 1998)) and contained 20% w/w fat. The fatty acid composition of the diets (**Appendix Table 13**) was achieved by blending oils to obtain a DHA content in the DHA diets of 3.9% w/w of total fat (DHASCO™, DSM, Columbia, MD). The amount of DHA in the diet was selected to achieve a plasma phospholipid DHA concentration of >5% w/w of total fatty acids. This concentration is believed to be clinically relevant as it has been associated with prolonged survival in women with metastatic breast cancer undergoing chemotherapy (Bougnoux et al. 2009). Because the animals in this study are immunocompromised, it is a requirement of our animal care facility that diets fed to these animals are irradiated to prevent potential exposure to foodborne pathogens (DeRuiter et al. 2002). Diets were irradiated for 108h at 8 kGy and stored at -20° C until used. Fatty acid analysis by gas liquid chromatography pre- and post-irradiation confirmed that the fat composition was not altered by irradiation (data not shown). While DHA in combination with EPA has been shown to exert-anticancer effects (VanderSluis et al. 2017), DHA alone has been shown to enhance the actions of chemotherapy (**Appendix Table 14**) and therefore the diet was designed to contain only DHA.

6.2.2 Experimental animals

Animal experiments were reviewed and approved (AUP00000134) by the University of Alberta Animal Policy and Welfare Committee and were in accordance with the Canadian Council on Animal Care guidelines. Immune-deficient 6-week old female NOD.Cg-Prkdc^{scid}Il2rg (NSG) mice were housed in bio-containment under aseptic conditions with autoclaved bedding and water. In separate experiments, PDX tumour sections approximately

30mm^3, representative of basal-like triple negative breast cancers (MAXF574 and MAXF401, Charles River Oncotest™ PDX models) were implanted into the left flank of each NSG mouse. MAXF 574 is a poorly differentiated, well-vascularized PDX, whereas MAXF 401 is moderately differentiated and poorly vascularized (**Appendix Table 15** for description of PDXs). Once tumours were approximately 50 mm^3 (measured by calipers and the equation: Volume (mm^3) = length (mm) x width^2 (mm^2) x 0.5), mice were randomized into diet groups: 0% DHA (control) or 3.9% w/w DHA (DHA) (**Figure 6-1**).

Figure 6-1: Experimental design of dietary DHA with or without TXT on MAXF574 TNBC PDX growth in NSG mice[1]



[1]Mice were implanted with TNBC MAXF574 tumour sections approximately 30 mm^3.

One week prior to commencing chemotherapy the mice were randomized into control or DHA diet groups and subsequently into chemotherapy (twice weekly) or control groups for an additional 6 weeks. Experimental groups are defined as: control, control+TXT, DHA and DHA+TXT (n=8 mice per group). Mice were fed ad libitum for one week and then further

randomized for chemotherapy treatments. As six rounds of neoadjuvant chemotherapy is standard of care for breast cancer patients prescribed neoadjuvant therapy, the mice received 5mg/kg docetaxel chemotherapy or 0.9% saline (placebo) injection twice weekly for 6 weeks and then the experiment was ended. Body weights and food intake were monitored three times per week throughout the experiment. Mice were euthanized, tumours carefully excised and weighed; one piece was formalin fixed for immunohistochemistry and another was homogenized for protein analysis. Individuals performing the excision and weighing of the tumour and all subsequent assays were blinded to the diet / chemotherapy treatments.

6.2.3 Western blot analysis

Tumours were minced and protein lysates were prepared and Western blots were performed as previously described (Schley et al. 2005, Subedi et al. 2015). Primary antibodies to β Catenin, BCL-2, BCLXL, Bad, Bax, Bid, Caspase 3, Caspase 7, Caspase 8, CD95, Cyclin B1, Cdc25C, CDC2, DR5, EGFR, FADD, MAPK, MCM2, pAKT, PARP, pBCL-2, PI3K, PCNA, PLK1, Ras (Cell Signaling Technology, New England Biolabs, Whitby, ON), Caspase 10, GPX4, Ripk1, Survivin and Wee1 (Abcam, Cedarlane, Burlington, ON) were diluted 1:1000 in 5% w/v BSA-TBST. GAPDH (Cell Signaling Technology, New England Biolabs, Whitby, ON) was used as a loading control at a concentration of 1:5000 in 5% w/v BSA-TBST and the secondary antibody, Anti-rabbit IgG HRP (Cell Signaling Technology, Whitby ON) or Goat Anti-Mouse IgG (Abcam, Cedarlane, Burlington, ON). Subsequently, membranes were developed using Pierce ECL 2 Western Blotting Substrate (Fisher Scientific, Edmonton AB) and visualized on a Typhoon^{TM} Trio+ variable mode imager (GE Life Sciences, Baie d'Urfe, PQ). The relative intensities of band signals were quantified using ImageQuant TL software.

6.2.4 Immunohistochemistry

Tumour sections were incubated with antibodies for CD95, Cyclin B1 and Ki67 (Cell Signaling Technology, Whitby ON), and positively stained cells were visualized using ImmunoDetector liquid 3,3'-Diaminobenzidine (DAB, Bio SB Inc., Santa Barbara, CA, USA). Apoptosis in tumour sections was assessed using the ApopTag® Peroxidase In Situ Apoptosis Detection Kit (EMD Millipore, Temecula, CA) according to manufacturer's instructions. Sections were imaged using AxioCam and the proportion of positive cells was determined using MetaMorph software (Carl Ziess Canada Ltd, North York ON).

6.2.5 Statistical analysis

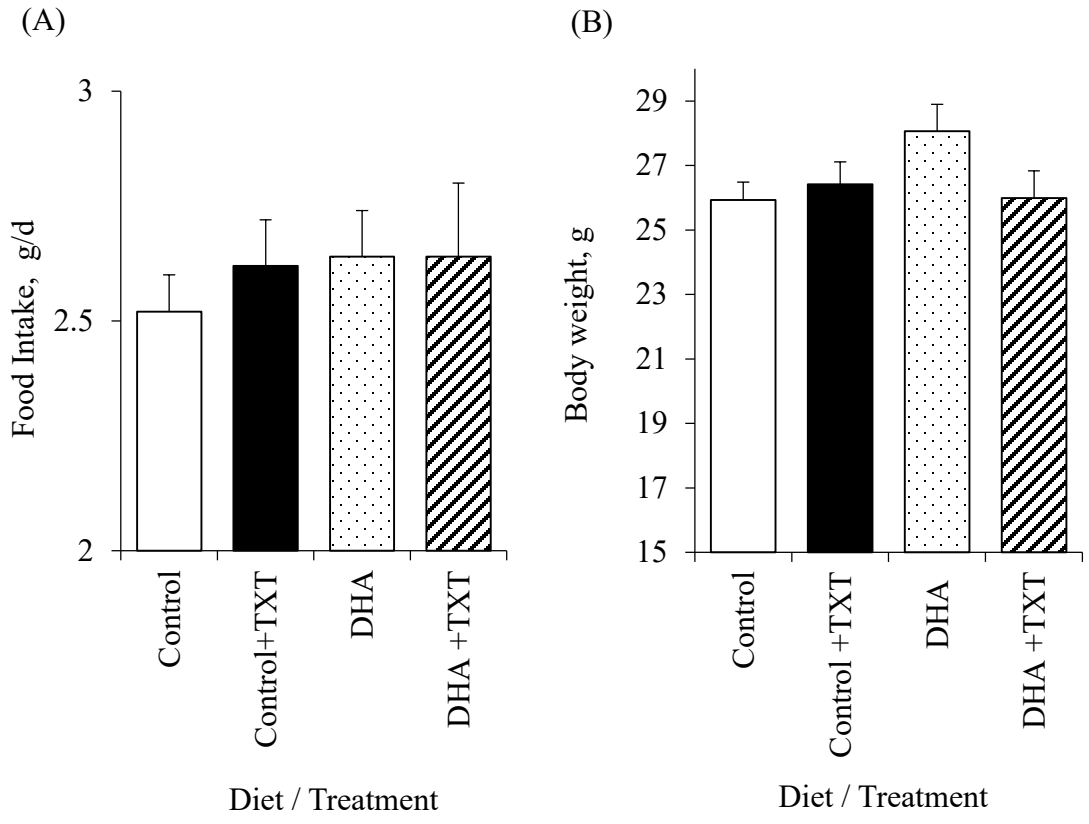
Statistical analyses were carried out using SAS, version 9.4 (SAS Institute Inc. Cary, NC). Data were analyzed for normal distribution and if normally distributed analyzed by 2-way ANOVA followed by post hoc Duncan analysis using to identify significant differences between treatments ($P<0.05$). Non-parametric data (tumour weights and volume) were analyzed using the Kruskal-Wallis to identify differences followed by post hoc analysis using the Mann-Witney test to identify differences between treatments. All statistical tests were two sided and *P* values less than 0.05 were considered statistically significant.

6.3 Results

6.3.1 Effect of DHA dietary supplementation on MAXF574 TNBC PDX tumour growth

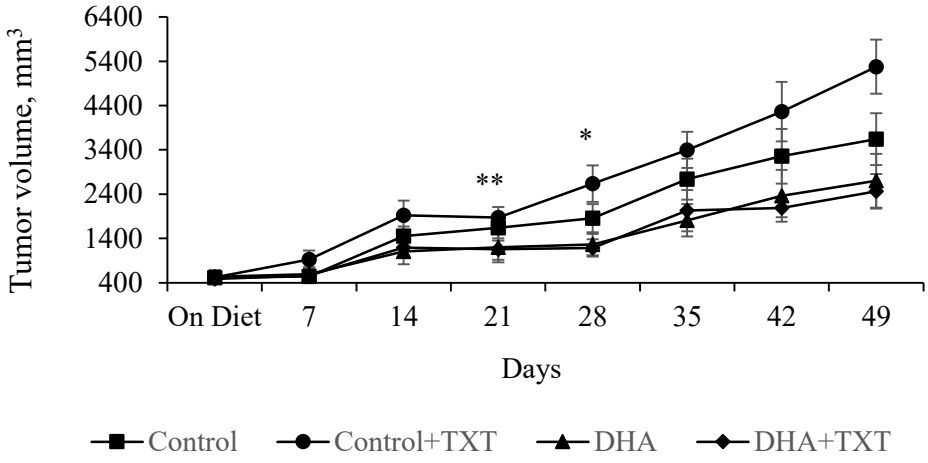
We first assessed whether DHA in conjunction with TXT chemotherapy could reduce tumour growth in a TNBC PDX model compared to control+TXT. Mice bearing MAXF574 PDX tumours did not differ in food intake and final body weight did not differ amongst treatments (**Figure 6-2 A** & **B**).

Figure 6-2: Effect of dietary DHA with or without TXT on (A) Average daily food intake of MAXF574 tumour bearing mice and (B) Final body weight of MAXF574 tumour bearing mice

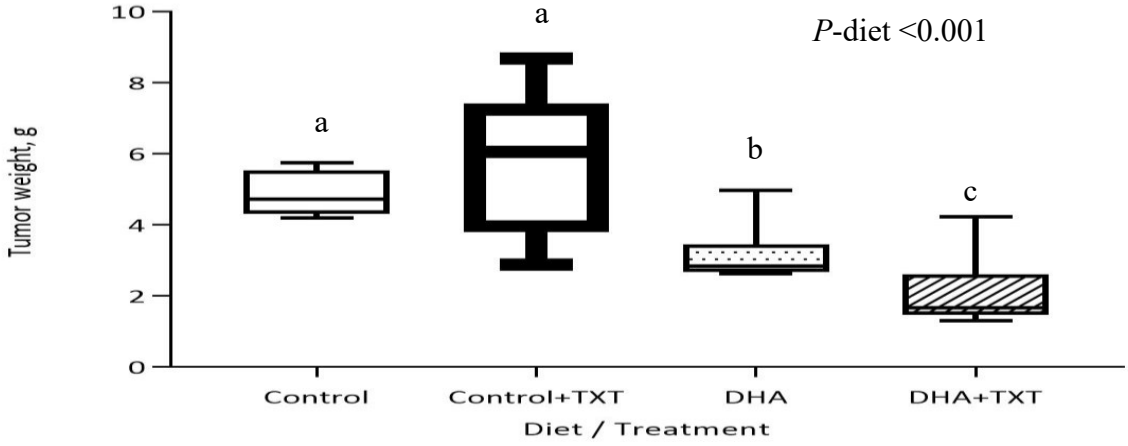


Final tumour volumes after 6 weeks of chemotherapy were lower in the DHA (P <0.02) and DHA+TXT (P <0.002) groups compared to control+TXT (**Figure 6-3 A**). Excised tumour weight was significantly lower in the DHA+TXT group compared to control+TXT (P <0.01), as well as control (P<0.004) and DHA alone (P <0.04; **Figure 6-3 B**). There was no significant difference in final tumour weight in control compared to control+TXT (P <0.5; **Figure 6-3 B**).

Figure 6-3: Effect of dietary DHA with or without TXT on MAXF574 TNBC PDX growth in NSG mice



(A) Average tumour volume of MAXF574 tumour bearing mice, * denotes statistical difference from day 28 (P<0.05) of DHA compared to Control+TXT, ** denotes statistical difference from day 21 (P<0.05) of DHA+TXT compared to Control+TXT.



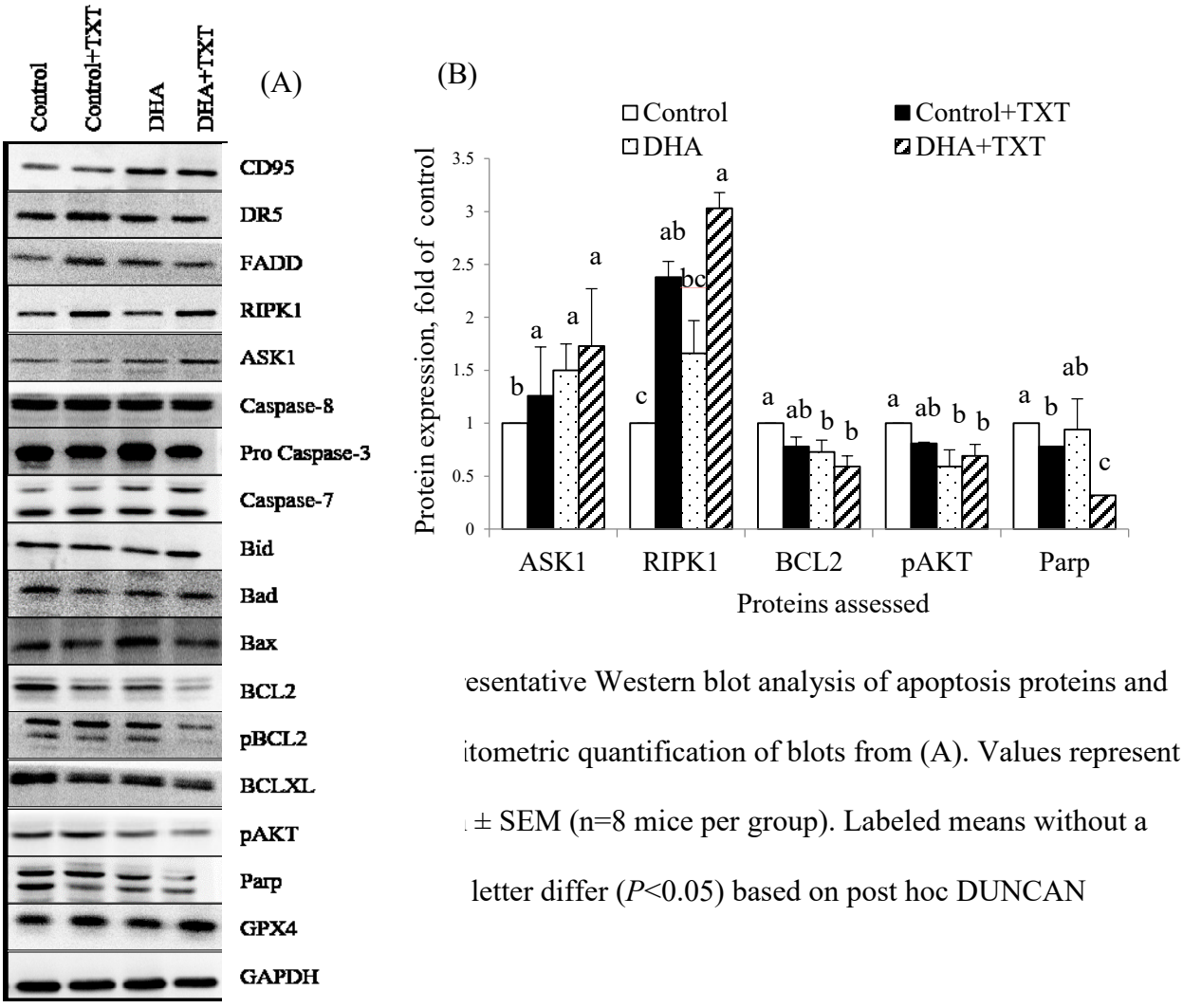
(B) excised tumour weight of MAXF574 tumour bearing mice. Kruskal-Wallis test for non-parametric analysis was employed to compare tumour weight between groups. The P value for the main effect of the diet= P<0.001; comparison of Control to DHA+TXT =P <0.004;

comparison of Control to DHA =P <0.02; comparison of Control+TXT to DHA =P <0.03 and comparison of Control to Control+TXT =P <0.48.

6.3.2 Effect of DHA dietary supplementation on apoptotic signalling

To assess the ability of DHA dietary supplementation to enhance apoptotic signalling, MAXF574 tumours were assessed for markers of apoptosis by Western blot analysis and immunohistochemistry. While there were no changes in whole cell protein expression of apoptotic markers CD95, DR5, FADD, Caspase 8, Caspase 7, Bad, Bax or GPX4 (**Figure 6-4 A**), DHA+TXT tumour extracts were found to have differential expression of TXT-mediated apoptotic pathway proteins, specifically a lower expression of Bcl-2, pro-Caspase 3, pAKT and Parp as well as a higher expression of ASK1, Bid and Ripk1 compared to control (P<0.05) but not control+TXT (**Figure 6-4 A & B**).

Figure 6-4: Apoptotic protein expression in MAXF574 PDX tumour extracts



esentative Western blot analysis of apoptosis proteins and

itometric quantification of blots from (A). Values represent

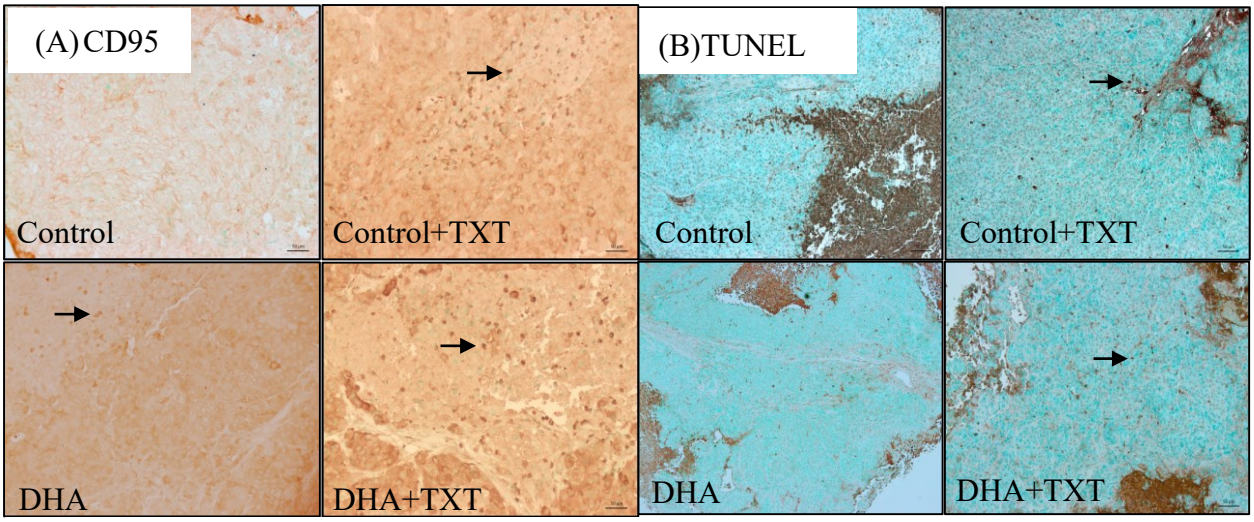
. ± SEM (n=8 mice per group). Labeled means without a

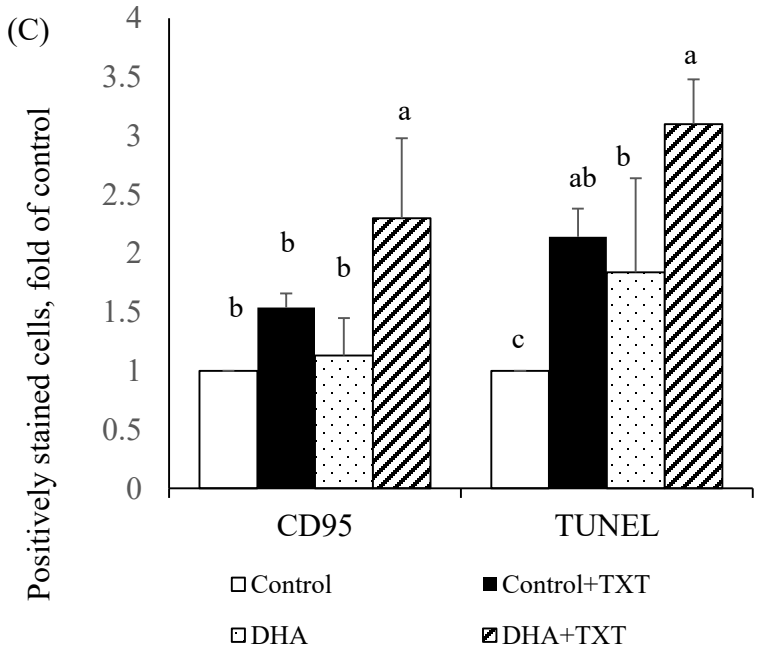
letter differ (P<0.05) based on post hoc DUNCAN

The P values for the main effect of the diet are: ASK1: P=0.05; RIPK1: P=0.1; BCL2: P=0.08; pAKT: P=0.04; Parp: P=0.13; the main effect of the treatment: ASK1: P=0.29; RIPK1: P=0.002; BCL2: P=0.19; pAKT: P=0.71; Parp: P=0.03 and P interaction: ASK1: P=0.93; RIPK1: P=0.98; BCL2: P=0.75; pAKT: P=0.21; Parp: P=0.24.

Immunohistochemical analysis of tumours from DHA+TXT mice determined CD95 expression (**Figure 6-5 A**) to be higher than control and control+TXT tumours (P<0.05). DHA+TXT tumours had higher TUNEL expression **(Figure 6-5 B & C**) compared to control but not significantly different control+TXT tumour groups (P<0.05).

Figure 6-5: Immunohistochemical staining in MAXF574 PDX tumour extracts



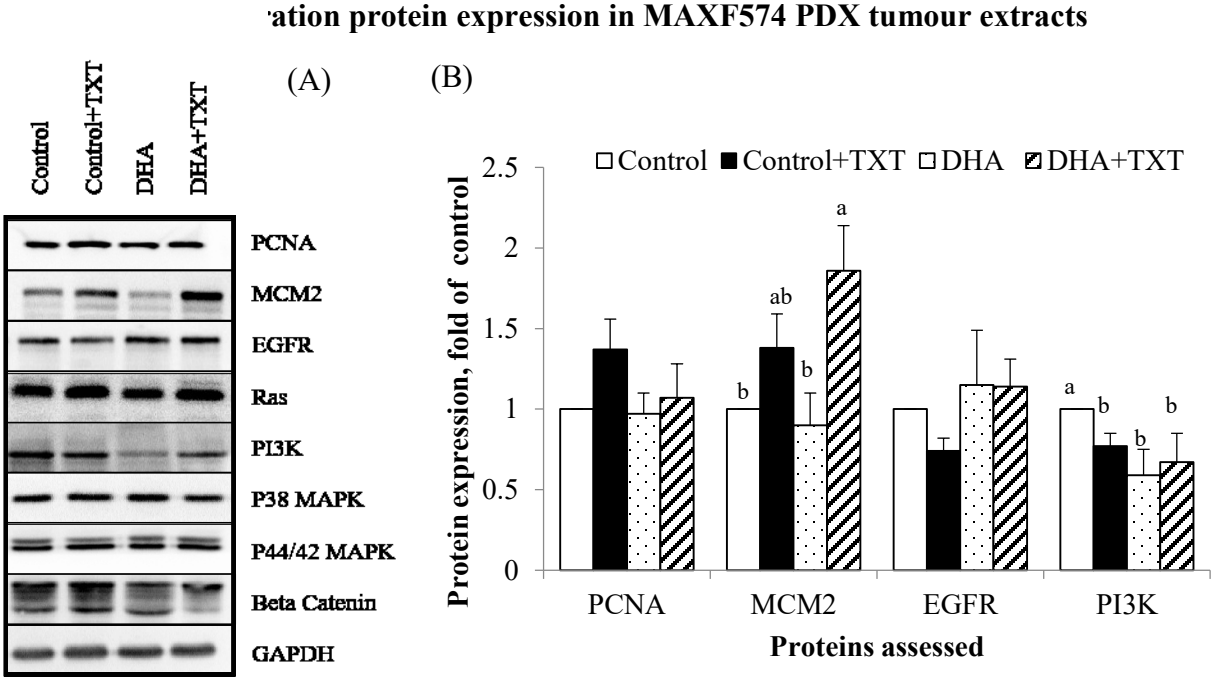


(A) Immunohistochemical staining of CD95 (B) relative quantification of CD95 and TUNEL staining and (C) apoptosis by TUNEL assay. Positive staining is dark brown color and indicated by arrows. Values represent the mean ± SEM (n=8 mice per group).

Labeled means without a common letter differ ($P<0.05$) based on post hoc DUNCAN analysis.

6.3.3 Effect of DHA supplementation on markers of proliferation

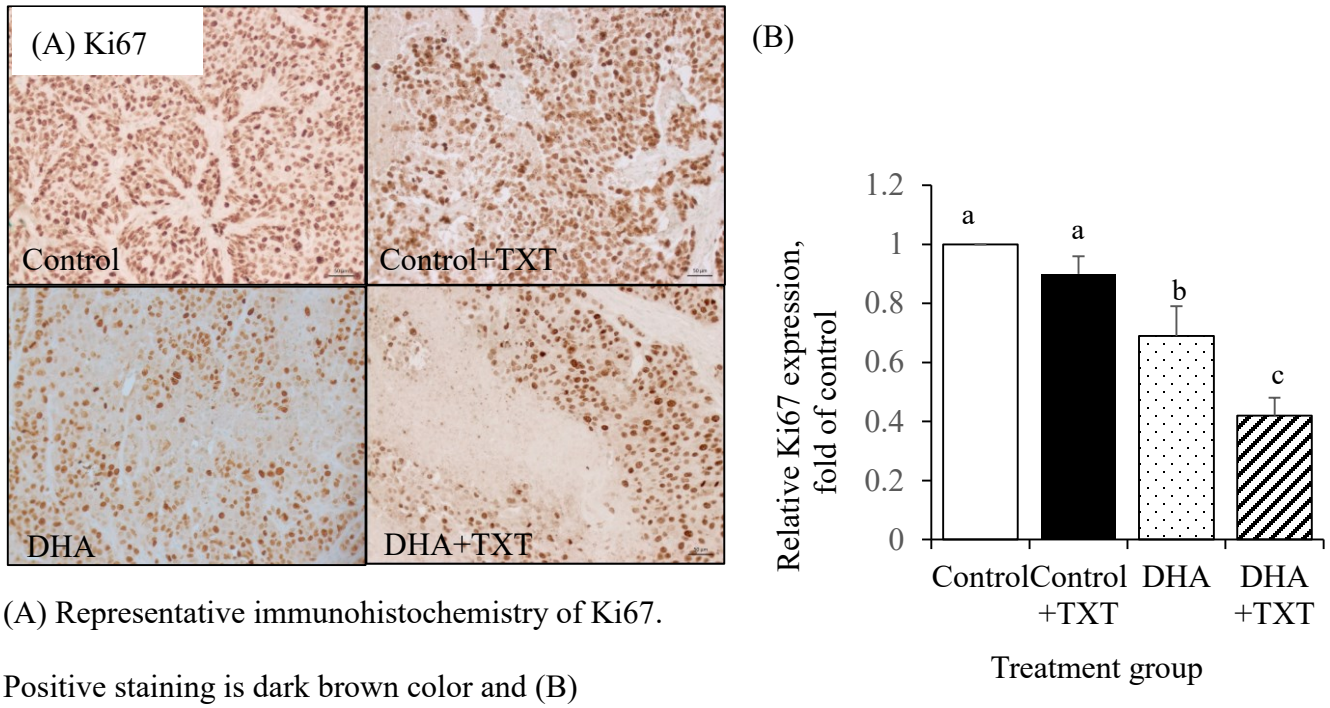
Changes in proliferation of MAXF574 tumours from mice fed DHA in combination with TXT compared to tumours from control fed mice with or without TXT were assessed by Western blot analysis and immunohistochemistry. There were no differences in proliferation or growth associated markers PCNA, EGFR, Ras, p38 MAPK, p44/42 MAPK or β Catenin amongst treatments. MCM2 was higher in both control+TXT and DHA+TXT groups and PI3K was lower in all experimental groups compared to control (**Figure 6-6 A & B**, P <0.05).



(A) Representative Western blot analysis of proliferation proteins and (B) densitometric quantification of blots from (A). Values represent the mean ± SEM (n=8). Labeled means without a common letter differ (P<0.05) based on post hoc DUNCAN analysis. The P values for the main effect of the diet are: PCNA: P=0.31; MCM2: P=0.36; EGFR: P=0.19; PI3K: P=0.05; the main effect of the treatment: PCNA: P=0.1; MCM2: P=0.003; EGFR: P=0.51; PI3K: P=0.56 and P interaction: PCNA: P=0.4; MCM2: P=0.17; EGFR: P=0.54; PI3K: P=0.25.

Tumours from mice fed DHA were found to have lower Ki67 staining compared to control and control+TXT groups and tumours from mice in the DHA+TXT had lower Ki67 staining compared to all groups (**Figure 6-7 A & B**, P <0.05).

Figure 6-7: Proliferation protein expression in MAXF574 PDX tumour extracts



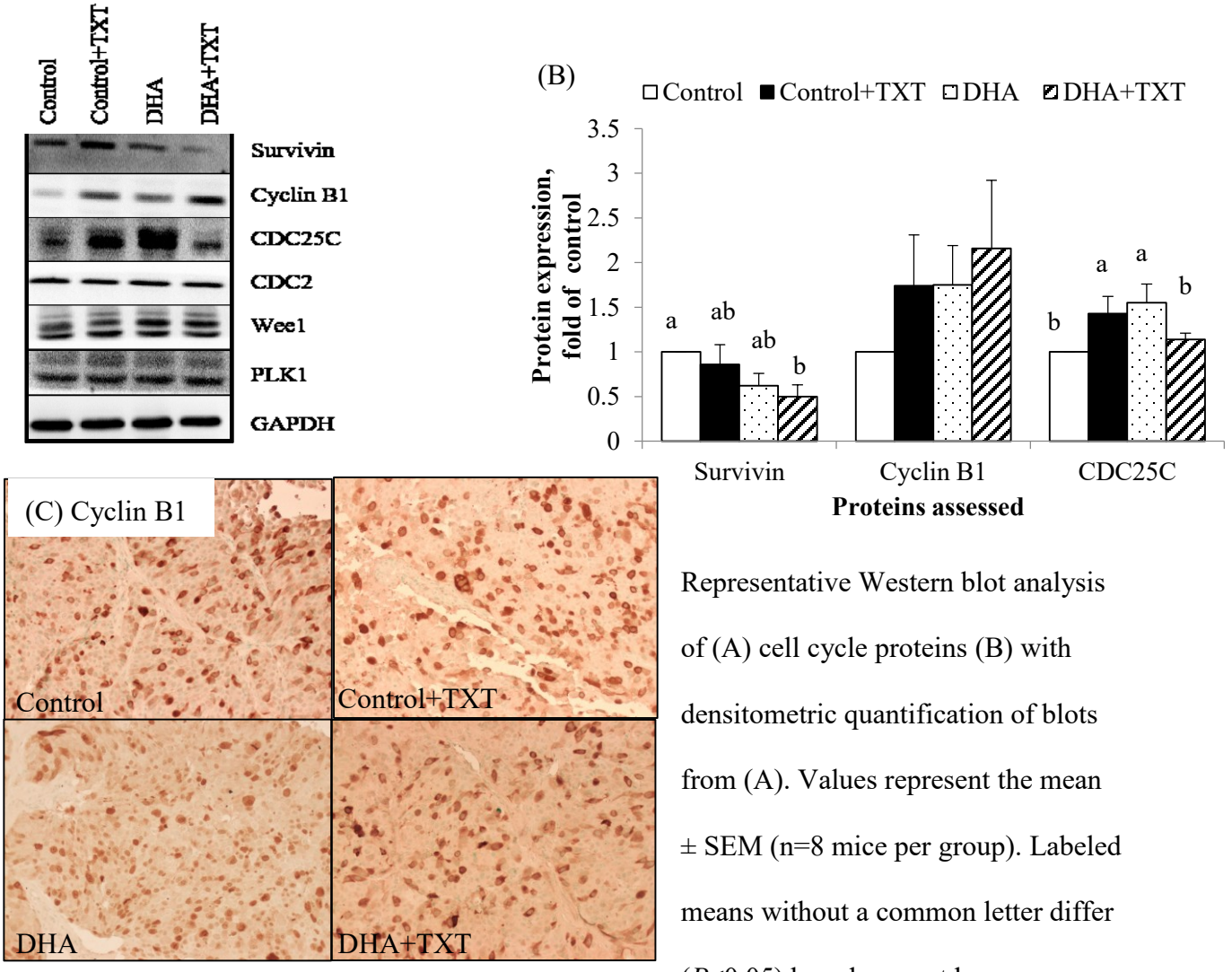
(A) Representative immunohistochemistry of Ki67. Positive staining is dark brown color and (B) quantification of Ki67 (P<0.05). Values represent the mean ± SEM (n=8). Labeled means without a common letter differ (P<0.05) based on post hoc DUNCAN analysis.

6.3.4 Effect of DHA dietary supplementation on markers of cell cycle progression

To assess the ability of DHA dietary supplementation to change signals associated with the cell cycle, MAXF574 tumours were assessed for markers of cell cycle progression by Western blot analysis (**Figure 6-8 A** & **B**) and immunohistochemistry (**Figure 6-8 C**). No differences were observed in CDC2, PLK1 or Wee1. However, Survivin expression was lower in DHA+TXT compared to control and control+TXT. In addition, cdc25c expression was lower in DHA+TXT compared to control+TXT but did not differ from the control (P <0.05). While

Cyclin B1 protein expression trended higher in DHA+TXT samples, (P <0.1), immunohistochemical staining was not significantly different amongst samples.

Figure 6-8: Cell cycle protein expression in MAXF574 PDX tumour extracts

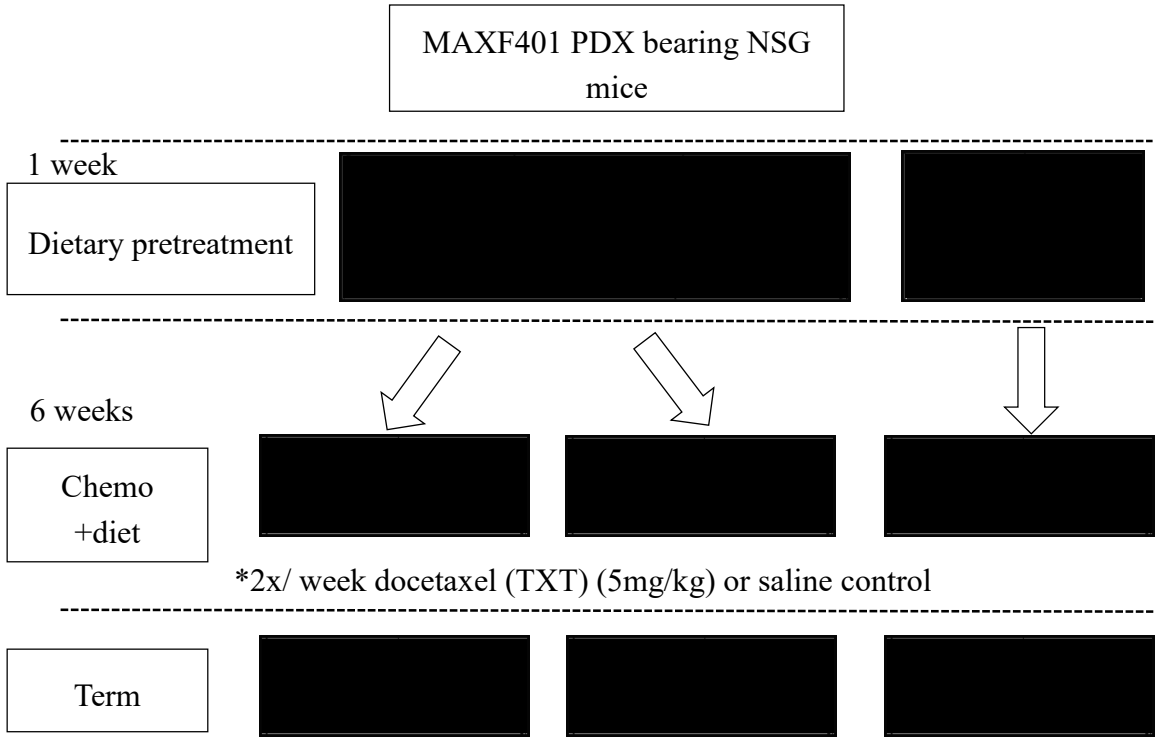


Representative Western blot analysis of (A) cell cycle proteins (B) with densitometric quantification of blots from (A). Values represent the mean ± SEM (n=8 mice per group). Labeled means without a common letter differ (P<0.05) based on post hoc DUNCAN analysis. The P values for the main effect of the diet are: Survivin: P=0.0.02; Cyclin B1: P=0.28; CDC25C: P=0.38; the main effect of the treatment: Survivin: P=0.4; Cyclin B1: P=0.29; CDC25C: P=0.96 and P interaction: Survivin: P=0.95; Cyclin B1: P=0.76; CDC25C: P=0.01. (C) Representative immunohistochemistry of Cyclin B1. Positive staining is dark brown color and nuclei are stained green (methyl green).

6.3.5 Effect of DHA dietary supplementation on MAXF401 tumour growth

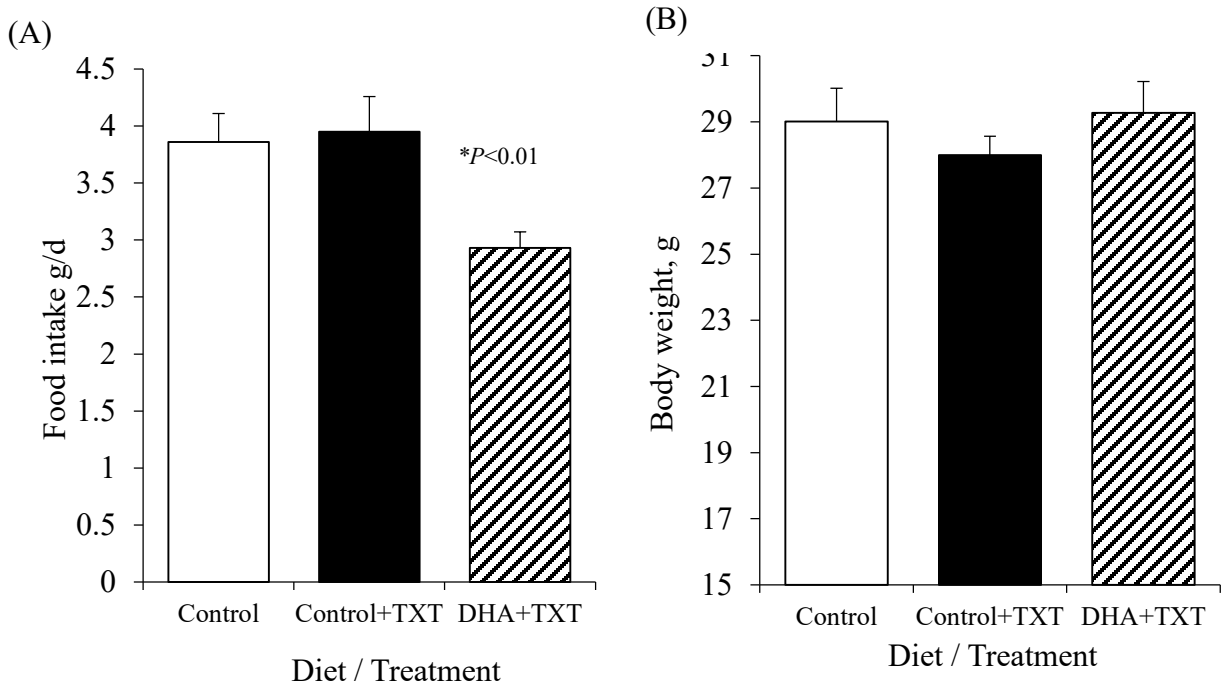
To confirm if the effects observed were applicable to other triple negative human tumours, a second TNBC PDX experiment was conducted. We sought to answer two key questions with this experiment: 1) Reproducibility: can we confirm efficacy in a second model and 2) Does DHA enhance the anticancer actions of docetaxel? Therefore, we employed a clinically relevant experimental design where our comparison was between control+TXT and DHA+TXT and there was no DHA alone group (**Figure 6-9**).

Figure 6-9: Experimental design of dietary DHA with or without TXT on MAXF401 TNBC PDX growth in NSG mice[1]



[1]Mice were implanted with TNBC MAXF401 PDX tumor sections approximately 30 mm^3. One week prior to commencing chemotherapy the mice were randomize into control or DHA diet groups and subsequently into chemotherapy (twice weekly) or control groups for an additional 6 weeks. Experimental groups are defined as control, control+TXT and DHA+TXT.

Figure 6-10: Effect of dietary DHA with TXT on (A) Average daily food intake of MAXF401 tumour bearing mice and (B) Final body weight of MAXF401 tumour bearing mice

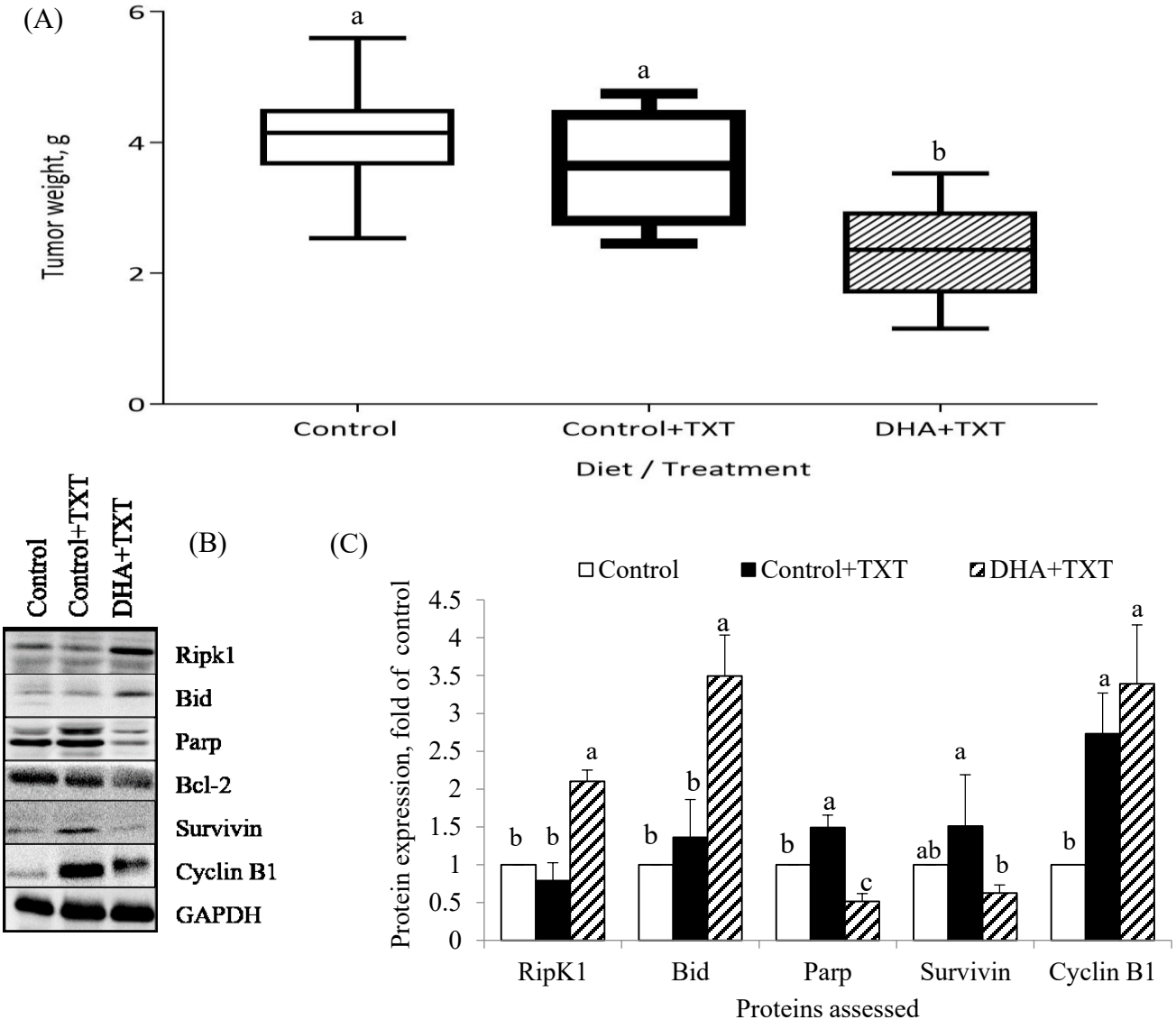


Mice bearing MAXF401 xenografts fed DHA had reduced average daily food intake compared to those fed the control diet (**Figure 6-10 A**), but there were no differences in body weight before (data not shown) or after chemotherapy amongst groups (**Figure 6-10 B**).

Excised tumour weight was lower for the DHA+TXT group compared to control+TXT (P <0.05) and control alone (P <0.004). For mice fed the control diet, TXT treatment did not significantly reduce tumour weight (P <0.44) (**Figure 6-11 A**).

Excised tumours were then assessed by Western blot analysis for select markers that were found to be differentially expressed in the first PDX experiment (**Figure 6-11 A & B**). DHA+TXT tumour protein extracts were found to have a higher expression of Ripk1, Bid and Cyclin B1 compared to control and control+TXT (P <0.05); lower expression of Bcl-2 and Parp compared to control and control+TXT (P <0.05); and lower expression of Survivin compared to control (P <0.05) but not significantly different from control+TXT.

Figure 6-11: Effect of dietary DHA with or without TXT on MAXF401 TNBC PDX growth and protein expression in NSG mice



(A) Excised tumour weight of MAXF401 tumour bearing mice (B) Representative Western blot analysis of proteins and (C) densitometric quantification of blots from (B). Kruskal-Wallis test for non-parametric analysis was employed for tumour weights. The *P* value for the main effect of the diet= $P<0.002$; comparison of Control to DHA+TXT =$P<0.004$; comparison of Control+TXT to DHA+TXT =$P<0.049$; and comparison of Control to Control+TXT =$P<0.44$. For the Western blots, values represent the mean ± SEM (n=8). Labeled means without a common letter differ ($P<0.05$) based on post hoc DUNCAN analysis. The *P* values for the main effect of the diet are: RIPK1: $P=0.004$; Bid: $P=0.002$; Parp: $P=0.0004$; Survivin: $P=0.1$; Cyclin B1: $P=0.48$ the main effect of the treatment: RIPK1: $P=0.001$; Bid: $P=0.35$; Parp: $P=0.16$; Survivin: $P=0.55$; Cyclin B1: $P=0.04$ and *P* interaction RIPK1: $P=0.01$; Bid: $P=0.09$; Parp: $P=0.05$; Survivin: $P=0.45$; Cyclin B1: $P=0.78$.

6.4 Discussion

To our knowledge, the current study is the first to report the anticancer effects of DHA supplementation concomitant with chemotherapy in a PDX model of BC. Previous studies by our group and others have determined the efficacy and potential mechanisms of action of DHA in conjunction with chemotherapy *in vitro* and *in vivo* models of breast cancer (**Appendix Table 14**) (Bougnoux et al. 2009, Darwito et al. 2019, Chauvin et al. 2016, Ewaschuk et al. 2012, Newell et al. 2019, Germain et al. 1998, Maheo et al. 2005, Vibet et al. 2008, Mason et al. 2015, deGraffenried et al. 2003). While these studies have elucidated mechanisms of action for DHA in conjunction with chemotherapy, they have relied on immortalized cell lines that do not reflect the heterogeneity of a primary patient tumour. For this reason, we have moved to the more clinically translatable heterogeneous PDX model to determine if DHA had efficacy in this model. We observed, in two different PDX models of TNBC, that feeding a diet supplemented

with DHA results in a significant reduction in tumour growth, and a greater response to first line docetaxel therapy. In fact, for control fed mice, docetaxel alone did not result in smaller tumours in either PDX model, rather resulted in greater variation in tumour sizes in the control+TXT group (although not significantly different than control alone). Interestingly, MAXF574 tumours excised from mice fed a DHA diet without chemotherapy were significantly smaller than tumours from mice fed control diet with or without chemotherapy. This has been observed in other pre-clinical models of breast cancer (Xue et al. 2014, Ghosh-Choudhury et al. 2009, Kang et al. 2010, Rose et al. 1997) and these results suggest that future studies exploring the effect of DHA alone in the PDX model would be warranted. However, this will not be translatable to medical treatment as women with TNBC are prescribed chemotherapy in clinic. For this reason, we focused on the comparison of tumours from mice fed DHA in conjunction with TXT to control+TXT that was assessed in this study. MAXF574 tumours excised from mice fed a DHA diet in conjunction with TXT were 64% smaller than tumours from mice fed a control diet concomitant with chemotherapy. Assessment of the second TNBC PDX, MAXF401, confirmed the positive synergism between DHA and TXT with a 34% reduction of tumour growth. The beneficial effects of DHA on docetaxel, in two different PDXs occurred without altering body weight; an important measure for improved clinical outcomes in some cancers (Murphy et al. 2011, Mocellin et al. 2014, Bonatto et al. 2012).

TNBC is known to be aggressive. It has a less favourable prognosis (Hurvitz et al. 2016, Haffty et al. 2006) and does not respond well to targeted therapies. The ability of neoplasms to employ multiple hallmarks of cancer as a means to survive and proliferate, creates a complex, continuously evolving environment that proves difficult to treat with standard or targeted chemotherapeutics (Hanahan et al. 2000, Hanahan et al. 2011). The efficacy of a treatment may

rely on its ability to elicit a response from numerous pathways within the tumour that are involved in apoptosis and proliferation, a phenomenon that was observed in the present study. We found that DHA improved the action of TXT in a multi-faceted way by simultaneously increasing apoptotic cell death through changes in ASK1, Ripk1, BCL2, pAKT and Parp; disrupting proliferation as indicated by changes in Ki67, and suppressing the progression of the cell cycle through changes in Survivin. Collectively, these alterations could contribute to substantially reduced tumour burden. These specific pathways are key hallmarks known to be implicated in cancer progression (Hanahan et al. 2000, Hanahan et al. 2011) and although they were not completely abrogated by the combined effects of DHA and docetaxel, the cumulative effects of differentially expressed proteins, in overlapping pathways, likely contributed to a striking reduction in tumour weight with combined therapies.

The apoptotic response, known to be triggered by both docetaxel (Ganansia-Leymarie et al. 2003) and DHA (Ewaschuk et al. 2012, Schley et al. 2005, Schley et al. 2007, Mansara et al. 2015, Grammatikos et al. 1994, Kang et al. 2010, Pogash et al. 2015) is consistent with the higher CD95 and TUNEL staining in DHA+TXT tumour sections and with the observed upregulation of pro-apoptotic and decreased anti-apoptotic protein expression in DHA+TXT tumours compared to control+TXT tumours. In DHA+TXT protein extracts there was a reduction in pro-caspase 3 suggesting cleavage and activation for initiation of apoptosis (McIlwain et al. 2013). Parp, one of the earliest substrates to be cleaved by caspase-3 during apoptosis (Duriez et al. 1997), was found to be substantially diminished in DHA+TXT protein extracts in both PDX models. Also, of note was the lower expression of Bcl-2, a potent inhibitor of apoptosis. This protein has been identified as a candidate biomarker of clinical response to docetaxel (Noguchi 2006). Additionally, a systematic review concluded that the negative

expression of Bcl-2 predicted favourable response to chemotherapy and predicted remission post neoadjuvant chemotherapy (Yang et al. 2013). Taken together, these results suggest that feeding DHA facilitates apoptosis in PDX tumours treated with TXT compared to mice fed a diet with a fat composition similar to that of the North American diet. We also tested whether feeding DHA could improve the anti-proliferative effects of TXT. The nuclear antigen Ki67, is considered a marker of cellular proliferation/cellular mitosis in breast tumour biopsies (Urruticoechea et al. 2005, Dowsett et al. 2011), and is used in clinic to assess efficacy of neoadjuvant chemotherapy prior to surgical removal (pre- and post- measurements of Ki67) (Matsubara et al. 2013). In the present study, we found a that TXT significantly lowered Ki67 expression in tumours from animals fed DHA, the combined effects of TXT and DHA resulted in an even lower Ki67 content.

Within the cell, TXT binds to β tubulin preventing depolymerization of microtubules thereby leading to cell cycle arrest (McGrogan et al. 2008) with an increase in Cyclin B1 expression (Motwani et al. 2003, Burrell et al. 2013) as cells become paused in the G2M phase. In both PDX experiments, DHA+TXT tumour extracts trended (P=0.1) towards increased Cyclin B1 protein expression compared to control although the localization and distribution of cyclin B1 was not found to be different in immunohistochemical analysis. Survivin reaches its peak expression in G2M (Mita et al. 2008) and is overexpressed in many cancers (McGrogan et al. 2008). Positive expression of Survivin correlates with poorer patient prognosis (Hinnis et al. 2007) and in the current study, feeding DHA with TXT treatment was found to reduce Survivin expression. This is consistent with facilitating the effects of TXT on cell cycle arrest.

There are limitations to our study. Other n-3 LCPUFA have been shown to exert anti-cancer effects, including EPA alone *in vitro* (Yu et al. 2015, VanderSluis et al. 2017) and EPA in

combination with DHA (VanderSluis et al. 2017) but more studies are needed to investigate the efficacy of these combinations in conjunction with chemotherapy. Additionally, as the response to docetaxel alone did not result in a reduction in tumour size, the mode of action through which DHA overcomes chemotherapy resistance in the PDX model should be investigated in future studies. Finally, the translational applicability of the immunocompromised mouse model used in this study should be considered. It is well established that DHA is a beneficial modulator on the immune system (Calder 2015) and it has been shown in other mammary cancer models that it can reduce inflammation and improve anti-tumour immune function (Robinson et al. 2002, Paixao et al. 2017). A clinical trial to determine the efficacy of DHA concomitant with chemotherapy is currently undergoing the approval process with the objective to assess the impact of supplementing women with DHA during neoadjuvant chemotherapy. This study will specifically quantify the effects of DHA on the Ki67 index in the tumour and will determine whether it can counteract the negative effects of chemotherapy on immunity (ClinicalTrials.gov Identifier: NCT03831178).

6.4.1 Conclusions

In summary, our findings provide clear evidence that supplementing the diet with DHA improves the efficacy of the neoadjuvant cytotoxic drug, TXT, in two chemo-resistant preclinical PDX models. A single mechanism could not be identified but analysis of cellular proteins suggest that this occurs by effects on apoptosis and cell cycle regulation concomitant with proliferation.

CHAPTER 7: Docosahexaenoic acid enrichment of tumour phospholipid membranes increases tumour necroptosis in mice bearing triple negative breast cancer patient-derived xenografts[6]

7.1 Introduction

Triple negative breast cancer (TNBC; estrogen negative, progesterone negative, human epidermal growth factor receptor 2 negative; ER-PR-HER2-) is an aggressive subtype that accounts for up to 20% of all breast cancer diagnoses and results in grave outcomes (Pal et al. 2011, Hurvitz et al. 2016, Haffty et al. 2006). TNBC can rarely be treated by targeted therapies (Pal et al. 2011, Hurvitz et al. 2016, Haffty et al. 2006), therefore systemic chemotherapy including docetaxel (TXT) is commonly employed in clinic (Ho et al. 2014). TXT is ineffective against certain breast cancers, and it is unclear which patients will respond favorably to treatment (Noguchi 2006). The difficulty in improving treatment options is due in part to the heterogeneous nature of the disease (Turashvili et al. 2017, Dobrolecki et al. 2016) and the dynamic tumour microenvironment that is able to evade systemic chemotherapeutics.

There is a growing body of evidence, in preclinical *in vitro* and *in vivo* models, that suggests supplementation with the long chain polyunsaturated fatty acid (LCPUFA), docosahexaenoic acid (DHA), alone (reviewed in (Newell et al. 2017, D'Eliseo et al. 2016, Liu et al. 2014)) or in combination with chemotherapeutics (Ewaschuk et al. 2012, Calviello et al. 2005, Shaikh et al. 2008, Vibet et al. 2008, Chauvin et al. 2016, Newell et al. 2019) results in a

[6] A version of this chapter has been submitted for publication and is under review: Newell M, Goruk S, Schueler J, Mazurak V, Postovit LM, Field CJ (2021) Docosahexaenoic acid enrichment of tumour phospholipid membranes increases tumour necroptosis in mice bearing triple negative breast cancer patient-derived xenografts

strong antineoplastic response in breast and other cancers. The incorporation of DHA into phospholipid membranes could be a key factor in the anticancer response as it influences membrane initiated signaling and subsequent downstream events. We have previously demonstrated incorporation of DHA into the lipid raft microdomains of immortalized breast cancer cell membranes (Newell et al. 2020). Lipid rafts are ordered, mobile platforms that initiate many cellular events key to cancer progression, including apoptosis and proliferation (Lee et al. 2014, Turk and Chapkin 2013). Indeed, membrane associated mechanisms including increased apoptosis (Newell et al. 2019, Ewaschuk et al. 2012, Calviello et al. 2005, Chiu et al. 2004, Kang et al. 2010, Siddiqui et al. 2013, Sun et al. 2011), decreased proliferation (Schley et al. 2007, Li et al. 2015, Rogers et al. 2010) and cell cycle arrest (Albino et al. 2000, Chiu et al. 2004, Khan et al. 2006, Moustaka et al. 2019, Siddiqui et al. 2003, Slagsvold et al. 2010, Xue et al. 2014) have been identified as routes of DHA action. However other mechanisms including necroptosis have not been explored extensively.

Necroptosis is the process of regulated necrotic death (Degterev et al. 2005) that involves formation of a necrosome – a complex that consists of receptor-interacting protein kinase 1 (RIPK1), RIPK3 and Mixed lineage kinase domain like protein (MLKL) (Vandenabeele et al. 2010, Dondelinger et al. 2016) and is a potential target for tumour cells that are resistant to apoptotic cell death. The ability of DHA to induce necroptosis has been investigated *in vitro* in L929 mouse fibrosarcoma cells (Pacheco et al. 2014) and Ou et al, described necrotic human hepatocellular carcinoma xenografts (Ou et al. 2017) but there is currently no evidence to describe the mechanisms by which DHA may affect cell viability in a model of breast cancer.

The dose of DHA required to elicit an anti-tumoural response or how different doses affect the relative distribution of DHA into phospholipid classes of tumoural membranes is not

known. Our group has previously established that DHA dietary supplementation reduces tumour growth by increasing apoptosis, cell cycle arrest and decreasing proliferation in patient derived xenografts (Newell et al. 2019). PDXs are heterogeneous, containing both stromal and cancer cell components, and more closely recapitulate the primary tumour microenvironment thereby increasing the translatability to a clinical setting (Zhang et al. 2013, Whittle et al. 2015). In the current study, two dietary doses and sources of DHA were employed to determine if the anticancer effects of DHA in a TNBC PDX model are dose dependent. The extent to which dosing may affect the relative distribution of DHA into tumour membrane phospholipid classes was also considered. We then explored alterations in the NFκB pathway, necroptosis and lipid rafts. Finally, the involvement of DHA in necroptosis in was confirmed in a second TNBC PDX model.

7.2 Materials and Methods

7.2.1 Experimental diets and animals

Animal experiments were reviewed and approved (AUP00000134) by the University of Alberta Animal Policy and Welfare Committee and were in accordance with the Canadian Council on Animal Care guidelines. Diets were nutritionally complete and contained 20% w/w fat (**Table 7-1**), the macronutrient composition as previously described (Robinson et al. 1998). The fatty acid composition of the diets was achieved by blending oils and fats to obtain a DHA content in the DHA diet of 3.8% w/w fat. The diets containing DHA were balanced for total n-3 content due to the higher 18:3 n-3 content in the low DHA diet. Diets were irradiated for 108h at 8 kGy and stored at -20° C until used. Fatty acid analysis by gas liquid chromatography pre and post-irradiation confirmed that the fat composition was not altered by irradiation (data not shown).

Table 7-1: Major Fatty Acids in the Control, HDHA and LDHA diets

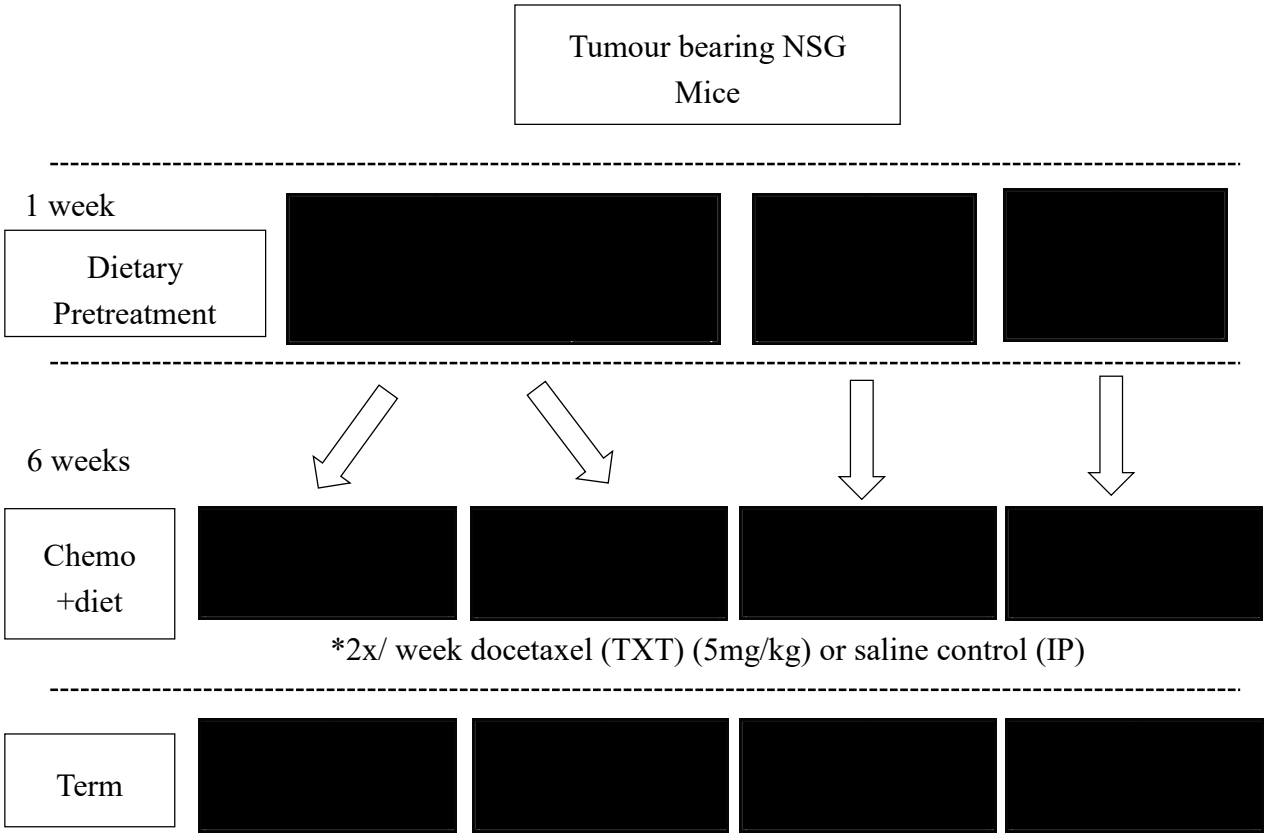
	Control	HDHA	LDHA
	% Total fatty acids		
16:0	22.9	21.1	20.7
18:0	13.8	12.4	12.8
18:1 n-9	36.4	38.7	40.8
18:2 n-6	21.0	15.2	13.1
18:3 n-3	2.4	3.4	5.2
18:4 n-3	0.00	0.0	0.4
20:4 n-6	0.42	0.4	0.4
22:6 n-3 (DHA)	**0.0**	**3.8**	**1.6**
Total SFA	37.8	36.0	34.8
Total PUFA	23.8	22.8	21.1
Total MUFA	38.4	40.9	43.1
Total n-6	21.5	15.6	13.7
Total n-3	2.4	7.2	7.4
P/S	0.6	0.6	0.6

Values are the mean percentage of 3 batches of diet as determined by gas liquid chromatography (Cruz-Hernandez et al.). Diets contained 200 g/kg of fat that was a blend of lard, vegetable oil, canola oil, olive oil, flax oil, vegetable shortening and Arasco oil (DSM Nutritional Products, USA). The DHA in the HDHA diet was provided by adding diet DHAsco (DSM Nutritional Products, USA) and for the LDHA diet Nuseed Canola Oil (generously donated by Nuseed, Canada). Minor fatty acids are not reported; therefore, totals do not add up to 100 %. Abbreviations used: SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; P/S, polyunsaturated to saturated fatty acid ratio.

Immune-deficient 6-week old female NOD.Cb-Prkdc^{scid}Il2rg (NSG) mice were obtained from a University of Alberta breeding colony and housed in bio-containment under aseptic conditions with autoclaved bedding and water. MAXF401 patient derived xenograft (PDX) tumour sections were implanted into the left flank of NSG mice. Once tumours were approximately 50 mm^3 (measured by calipers and the equation: Volume (mm^3) = length (mm) x width^2 (mm^2) x 0.5), mice were randomized into diet groups (control, HDHA or LDHA; 20% w/w total fat). Mice were fed *ad libitum* for one week and then further randomized to receive intraperitoneal injections of 5mg/kg docetaxel (TXT) chemotherapy or 0.9% saline injections twice weekly for six weeks. Experimental groups are defined as: control, control+TXT,

HDHA+TXT [3.8% wt:wt high DHA(HDHA) diet] and LDHA+TXT [1.6% wt:wt low DHA diet (LDHA)] (Experimental Layout **Figure 7-1**).

Figure 7-1: Effect of two dietary doses of DHA with TXT on MAXF401 TNBC PDX growth in NSG mice



For confirmation of DHA incorporation into lipids and necrosis, a second experiment with MAXF574 PDXs was conducted with experimental paradigms as previously described (Newell et al. 2019). Body weights and food intake were monitored three times/ week throughout the experiment. Mice were euthanized, tumours carefully excised and weighed, formalin fixed for immunohistochemistry, homogenized for protein analysis or flash frozen for lipid analysis. Individuals performing the excision and weighing of the tumour and all subsequent assays were blinded to the diet treatments.

7.2.2 Fatty Acid Composition Analysis

Lipids from tumours, plasma, liver and gastrocnemius muscle were extracted by Folch as previously described (Field et al. 1988, Folch et al. 1957). Total phospholipids and phospholipid classes: phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and sphingomyelin (SM) were separated thin layer chromatography and fatty acids separated by automated GLC 7890A (Agilent Technologies, ON, Canada) on a CP-Sil 88 column (100 m x 0.25 mm, Agilent) as previously described (Cruz-Hernandez et al. 2013).

7.2.3 Lipid Raft extraction

Lipid rafts were isolated as previously described (Schley et al. 2007, Newell et al. 2020) with the following modification: tumour sections were finely minced in ice-cold TNE (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA) and passed sequentially 10 times each through a 16 gauge, 18 gauge and 21-gauge needle to ensure adequate mincing of tumour for lysing and raft separation.

7.2.4 Western Blot analysis

Protein lysates were prepared from minced tumours and Western blots performed as previously described (Schley et al. 2005, Subedi et al. 2015). Primary antibodies to CD95, EGFR, IKKB, MLKL, NFκB, RIPK1, RIPK3, TNFR1 and TRAF2 (Cell Signaling Technology, New England Biolabs, ON, Canada) were diluted 1:1000 in 5%w/v BSA-TBST. Gα1 (1:1000) and GAPDH (1:10 000) (Cell Signaling Technology, New England Biolabs, ON, Canada) were used as loading controls for lipid rafts and whole cell protein respectively and the secondary antibody anti-rabbit IgG HRP (Cell Signaling Technology, New England Biolabs, ON, Canada) was employed. Membranes were developed using Pierce ECL 2 Western Blotting Substrate (Fisher Scientific, AB, Canada) and visualized on a TyphoonTM Trio+variable mode imager

(GE Life Sciences, PQ, Canada). ImageQuant TL software was used to quantify band signal relative intensities.

7.2.5 Immunohistochemistry

Paraffin-embedded tumour sections were deparaffinized as previously described (Newell et al. 2019) and stained with Harris Modified Hematoxylin and Eosin Y (H&E, Fisher Scientific, AB, Canada) to assess tumour morphology and necrotic regions. The entire cross-sectional area of the slides was scanned with an Aperio Digital Pathology Scanner (Leica Biosystems, Canada) and relative percent of necrosis (area of necrosis over total tumour area) determined with Image Scope software.

7.2.6 Statistical analysis

Statistical analyses were carried out using SAS, version 9.4. Data were tested for normal distribution and if not normally distributed, log transformed before analyzing by 2-way ANOVA followed by post hoc analysis using Tukey's test to identify significant differences between treatments ($P<0.05$). Graphs were generated using GraphPad Prism (GraphPad Software, Inc., CA, USA). Significance (P-values <0.05). Spearman's rank correlation coefficient was used to determine correlation between variables.

7.3 Results

7.3.1 Effect on tumour growth of feeding different doses of DHA diet to tumour bearing mice

Our group has previously established the efficacy of DHA in conjunction with TXT on reducing tumour size in two PDX models of breast cancer (MAXF574 and MAXF401) (Newell et al. 2019). We further sought to determine if a lower dietary dose of DHA would elicit the same response. Mice in both DHA dietary groups had reduced average daily food intake

compared to those fed the control diet, but there were no differences in body weight before (not illustrated) or after chemotherapy amongst groups (**Table 7-2**).

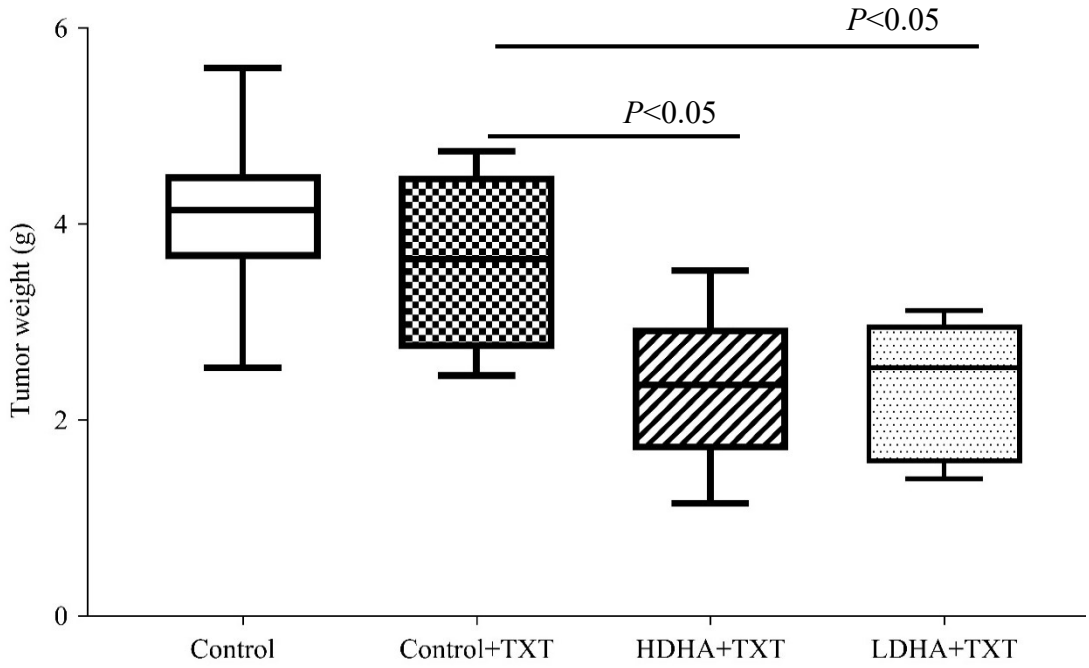
Table 7-2: Body Weight and Food intake of NSG mice bearing MAXF401 PDXs fed control or DHA diet with or without chemotherapy

Parameter	Control	Control+TXT	HDHA+TXT	LDHA+TXT
Final Body Weight (g)	29.0±1.0	28.0±0.6	29.3±1.0	28.5±0.9
Daily Food Intake	3.7±0.2[a]	3.7±0.3[a]	3.0±0.2[b]	3.0±0.2[b]

Values are presented as means ±SEM (n=7). Labeled means without a common letter differ (P<0.05) based on post hoc DUNCAN analysis. Abbreviations used: HDHA, High Docosahexaenoic acid; LDHA, Low Docosahexaenoic acid; TXT, Docetaxel.

Excised tumour weight was lower for both the HDHA+TXT and LDHA+TXT groups compared to control and control+TXT (P <0.05). For mice fed the control diet, TXT treatment did not significantly reduce tumour weight (P <0.44, **Figure 7-2**).

Figure 7-2: Effect of two dietary doses of DHA with TXT on excised tumour weight from MAXF401 tumour bearing mice



Kruskal-Wallis test for non-parametric analysis was employed to compare tumour weights between groups. The *P* value for the main effect of the diet =$P<0.05$.

7.3.2 Effect of DHA on Complete Blood Count Parameters

To assess if chemotherapy and / or DHA diet affected the blood profile of the mice, a CBC with differential was performed. No significant differences were observed in the CBC parameters between any of the diet/ treatment groups and all were within the reference ranges (**Appendix Table 16**).

7.3.3 Effect of feeding a DHA diet on incorporation of long chain polyunsaturated fatty acids in plasma, tumour, liver and muscle phospholipids

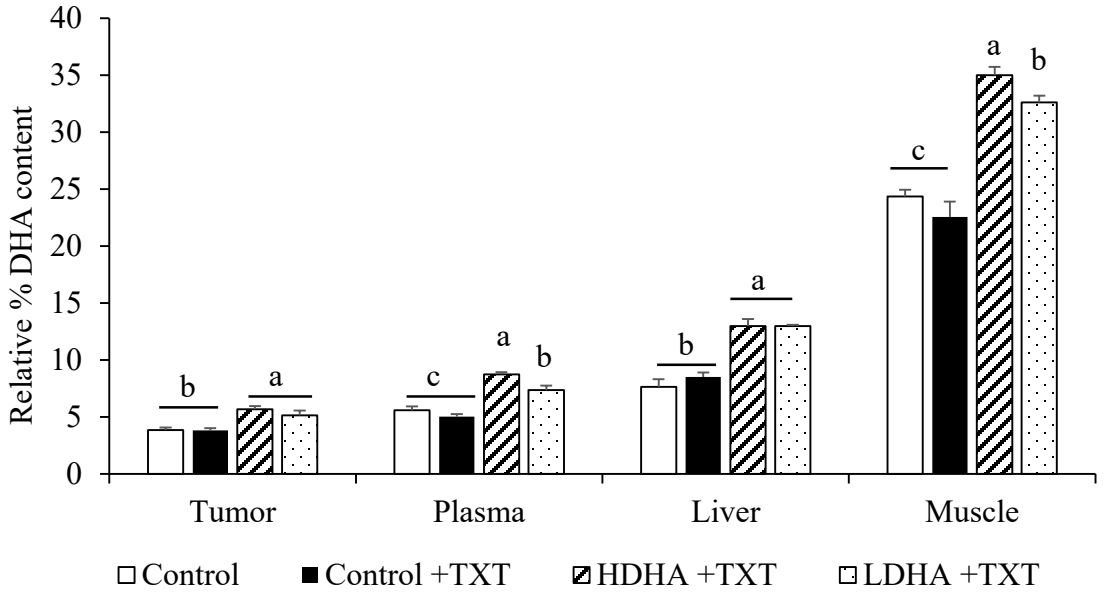
The content of LCPUFA: arachidonic (ARA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and DHA in tumour, plasma, liver and gastrocnemius muscle phospholipids are shown in **Table 7-3** and **Appendix Tables 17 and 18**. As predicted, ARA was higher in control fed animals in all tissues assessed. A dose effect of higher DHA incorporation in the HDHA+TXT group was observed only in muscle and plasma (**Figure 7-3**, $P<0.05$). EPA was significantly higher in LDHA+TXT compared to HDHA+TXT and control groups in plasma and muscle and DPA was significantly higher in the LDHA+TXT group compared to HDHA+TXT and control groups in the tumour, liver and muscle ($P<0.05$).

Table 7-3: LCPUFA composition of total phospholipids in tumour, plasma, liver and gastrocnemius muscle excised from NSG mice implanted with MAXF401 tumours

	Control	Control+TXT	HDHA+TXT	LDHA+TXT
Tumour				
20:4 n-6 (ARA)	15.6±0.4^{b}	17.1±0.7^{a}	12.7±0.2^{c}	12.3±0.6^{c}
20:5 n-3 (EPA)	0.4±0.0^{b}	0.3±0.0^{b}	0.8±0.1^{a}	1.0±0.1^{a}
22:5 n-3 (DPA)	0.7±0.0^{c}	0.6±0.0^{c}	0.9±0.0^{b}	1.2±0.1^{a}
22:6 n-3 (DHA)	3.8±0.2^{b}	3.8±0.2^{b}	5.7±0.3^{a}	5.1±0.4^{a}
Plasma				
20:4 n-6 (ARA)	13.8±0.5^{a}	13.0±0.4^{a}	7.5±0.4^{b}	7.9±0.4^{b}
20:5 n-3 (EPA)	0.1±0.0^{c}	0.1±0.0^{c}	0.4±0.1^{b}	0.6±0.0^{a}
22:5 n-3 (DPA)	0.2±0.0	0.2±0.0	0.3±0.0	0.3±0.0
22:6 n-3 (DHA)	5.6±0.4^{c}	5.0±0.2^{c}	8.7±0.2^{a}	7.4±0.4^{b}
Liver				
20:4 n-6 (ARA)	16.8±0.4^{a}	17.6±0.2^{a}	10.9±0.4^{c}	12.6±0.3^{b}
20:5 n-3 (EPA)	0.1±0.0^{b}	0.1±0.0^{b}	0.9±0.1^{a}	0.9±0.1^{a}
22:5 n-3 (DPA)	0.1±0.0^{c}	0.1±0.0^{c}	0.2±0.0^{b}	0.2±0.0^{a}
22:6 n-3 (DHA)	7.6±0.7^{b}	8.5±0.4^{b}	13.0±0.6^{a}	13.0±0.1^{a}
Gastrocnemius muscle				
20:4 n-6 (ARA)	10.0±0.3^{a}	10.0±0.2^{a}	4.6±0.2^{b}	5.0±0.2^{b}
20:5 n-3 (EPA)	0.1±0.0^{c}	0.1±0.0^{c}	0.1±0.0^{b}	0.3±0.0^{a}
22:5 n-3 (DPA)	2.0±0.1^{a}	1.8±0.2^{a}	1.1±0.1^{b}	2.0±0.1^{a}
22:6 n-3 (DHA)	24.4±0.6^{c}	22.6±1.3^{c}	35.0±0.7^{a}	32.6±0.6^{b}

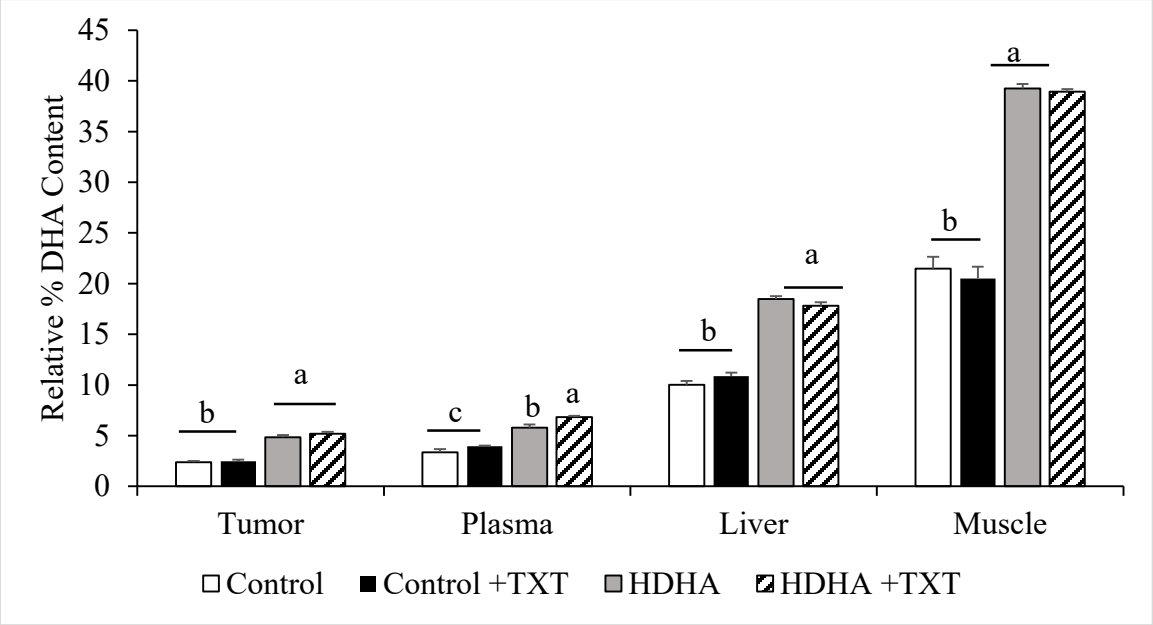
Values are percentages relative to the total fatty acid content ± SE (n=7). Within the rows labeled means without a common letter differ (P<0.05) based on post hoc DUNCAN analysis. Abbreviations used: ARA, arachidonic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; HDHA, high DHA; LDHA, low DHA; TXT, docetaxel.

Figure 7-3: Effect of two dietary doses of DHA with TXT on DHA incorporation (relative percent of total fatty acid) into tumour, plasma, liver and muscle



To confirm DHA incorporation in tissue, total phospholipids from a second PDX model (MAXF574) was assessed. In this model, tumours from mice fed HDHA without chemotherapy were also assessed. A similar trend was observed in this model, with lower ARA, significantly higher EPA, DPA and DHA in the HDHA fed animals with or without TXT compared control groups in all plasma, tumour, liver and gastrocnemius muscle (**Figure 7-4**; **Appendix Tables 18** and **19**; $P < 0.05$).

Figure 7-4: Incorporation of dietary DHA into tumour, plasma, liver and muscle of NSG mice implanted with MAXF574 TNBC PDX and treated with or without TXT



7.3.4 Effect on feeding a DHA diet on composition of phospholipid classes in PDX tumours

Fatty acid composition of the five main phospholipid classes in MAXF401 PDX tumours excised from mice fed the experimental diets were determined (**Table 7-4** and **Appendix Tables 19-25**). Tumours from mice fed either DHA diet had significantly higher EPA, DPA and DHA content in PE, PC and PI compared to control fed mice (**Figure 7-5 A**) and a dose effect for DHA was observed in PE ($P<0.05$) and PI ($P<004$). A corresponding decrease in ARA was observed in PE, PS, PC and PI (**Figure 7-5 B**, $P<0.05$). Phospholipid class incorporation of DHA into PE, PS, PC and PI with a corresponding decrease in ARA was confirmed in the second PDX MAXF574 model (**Figure 7-6 A and B**, Appendix Tables 19-25).

Table 7-4: Fatty acid composition (relative % of total fatty acids) of phospholipid classes from MAXF401 PDX tumours excised from NSG mice

Fatty Acids				
PE	**Control**	**Control+TXT**	**HDHA+TXT**	**LDHA+TXT**
18:1 n-9 (OA)	13.7±0.9^{a}	9.4±0.7^{b}	14.4±0.2^{a}	14.2±0.4^{a}
18:2 n-6 (LA)	3.2±0.2^{a}	2.3±0.1^{b}	3.0±0.1^{a}	3.0±0.1^{a}
20:4 n-6 (ARA)	26.0±0.5^{a}	26.4±0.1^{a}	21.0±0.7^{b}	20.1±0.8^{b}
20:5 n-3 (EPA)	0.3±0.0^{b}	0.4±0.1^{b}	1.4±0.1^{a}	1.6±0.2^{a}
22:5 n-3 (DPA)	1.2±0.0^{bc}	1.0±0.1^{c}	1.5±0.1^{b}	1.9±0.1^{a}
22:6 n-3 (DHA)	7.8±0.4^{b}	7.3±0.7^{b}	10.8±0.7^{a}	9.1±0.6^{b}
PS				
18:1 n-9 (OA)	26.1±0.7^{b}	23.3±0.9^{c}	27.7±0.5^{ab}	29.2±0.9^{a}
18:2 n-6 (LA)	2.8±0.2	2.6±0.2	2.7±0.3	2.9±0.2
20:4 n-6 (ARA)	5.4±0.3^{a}	5.4±0.4^{a}	3.4±0.4^{b}	3.6±0.5^{b}
20:5 n-3 (EPA)	0.2±0.0	0.2±0.0	0.4±0.1	0.2±0.1
22:5 n-3 (DPA)	0.1±0.0^{c}	0.2±0.0^{bc}	0.4±0.1^{ab}	0.7±0.2^{a}
22:6 n-3 (DHA)	2.6±0.2	2.3±0.1	2.2±0.3	2.1±0.3
PC				
18:1 n-9 (OA)	20.5±0.6^{b}	19.4±0.6^{b}	23.1±0.7^{a}	22.5±0.5^{a}
18:2 n-6 (LA)	6.9±0.2	6.8±0.4	6.5±0.4	6.5±0.2
20:4 n-6 (ARA)	11.5±0.5^{a}	12.6±0.9^{a}	8.2±0.4^{b}	8.7±0.3^{b}
20:5 n-3 (EPA)	0.2±0.0^{c}	0.3±0.0^{c}	0.4±0.1^{b}	0.6±0.1^{a}
22:5 n-3 (DPA)	0.4±0.0^{b}	0.4±0.0^{b}	0.4±0.0^{b}	0.6±0.1^{a}
22:6 n-3 (DHA)	1.6±0.1^{b}	1.9±0.1^{b}	2.6±0.2^{a}	2.5±0.2^{a}
PI				
18:1 n-9 (OA)	8.8±0.7	9.5±0.4	9.7±0.5	9.9±0.7
18:2 n-6 (LA)	3.4±0.2	3.0±0.5	3.1±0.2	3.0±0.2
20:4 n-6 (ARA)	25.5±1.3^{a}	25.8±1.2^{a}	22.0±1.2^{b}	22.2±1.6^{b}
20:5 n-3 (EPA)	0.1±0.0^{b}	0.1±0.0^{b}	0.3±0.1^{a}	0.3±0.1^{a}
22:5 n-3 (DPA)	0.5±0.0^{b}	0.5±0.0^{b}	0.8±0.0^{a}	0.8±0.1^{a}
22:6 n-3 (DHA)	2.3±0.1^{c}	2.3±0.1^{c}	3.6±0.2^{a}	2.8±0.3^{b}
SM				
18:1 n-9 (OA)	1.9±0.4^{b}	4.4±1.8^{a}	4.5±0.3^{a}	4.6±0.6^{a}
18:2 n-6 (LA)	4.3±0.2^{a}	3.6±0.2 ^{b}	2.7±0.1^{c}	2.5±0.1^{c}
20:4 n-6 (ARA)	0.9±0.1^{b}	1.0±0.1^{b}	0.6±0.2^{b}	1.5±0.4^{a}
20:5 n-3 (EPA)	2.7±0.2^{a}	2.7±0.2^{a}	2.2±0.1^{b}	2.1±0.1^{b}
22:5 n-3 (DPA)	0.3±0.0	0.3±0.0	0.2±0.0	0.2±0.0
22:6 n-3 (DHA)	0.5±0.2	0.4±0.2	0.5±0.0	0.4±0.1

Values are percentages relative to the total fatty acid content ± SE (n=7). Within the rows labeled means without a common letter differ (P<0.05) based on post hoc DUNCAN analysis.

Figure 7-5: Lipid incorporation (relative % of total fatty acids) into PL classes of (A) DHA in MAXF401 and (B) ARA in MAXF401

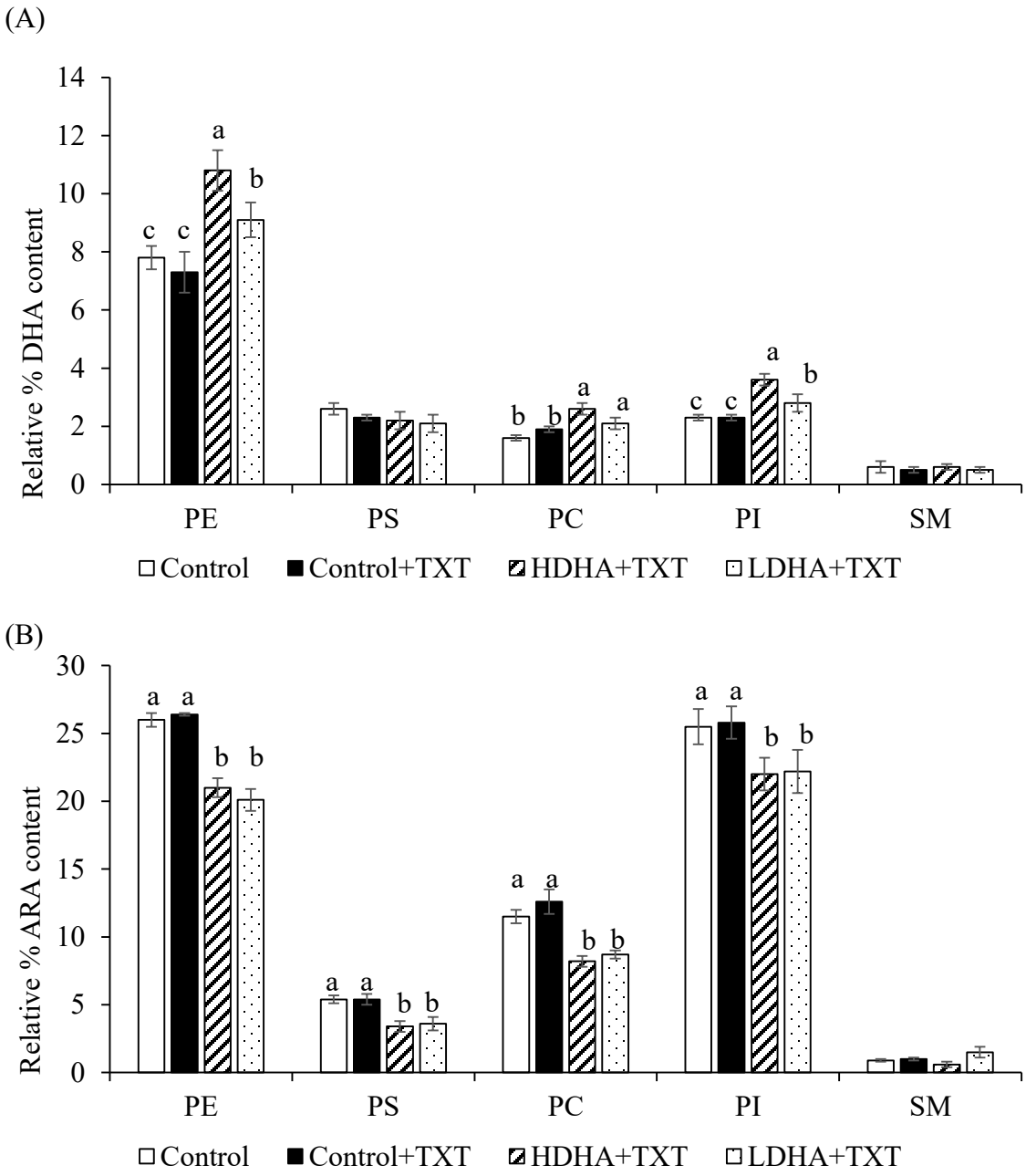
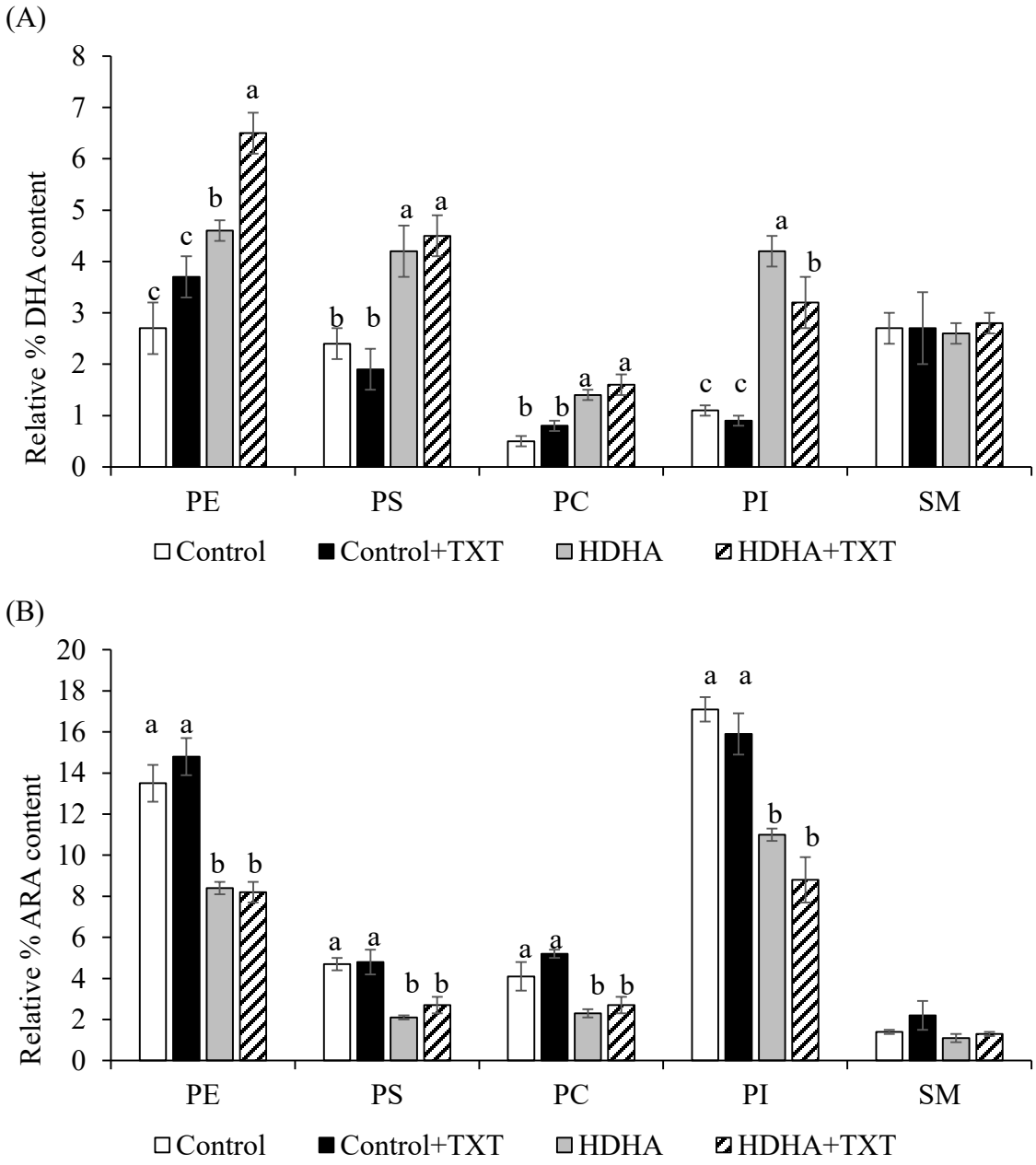


Figure 7-6: Lipid incorporation (relative % of total fatty acids) into PL classes of (A) DHA in MAXF574 and (B) ARA in MAXF574



7.3.5 Effect of DHA on markers of necroptosis

Our group has previously determined that smaller tumours observed in mice fed DHA in combination with chemotherapy treatment was in part due to increased apoptosis and cell cycle arrest in conjunction with decreased proliferation (Newell et al. 2019). We further sought to

determine if necroptosis was similarly increased in tumours from mice fed a DHA diet and treated with TXT. Necrotic regions were assessed through H&E staining and quantified (**Figure 7-6A & B**).

Figure 7-6 A: Necroptosis in MAXF401 tumours: Representative H&E staining of control, control + TXT, HDHA + TXT and LDHA + TXT tumours with regions of necrotic tissue highlighted with arrows

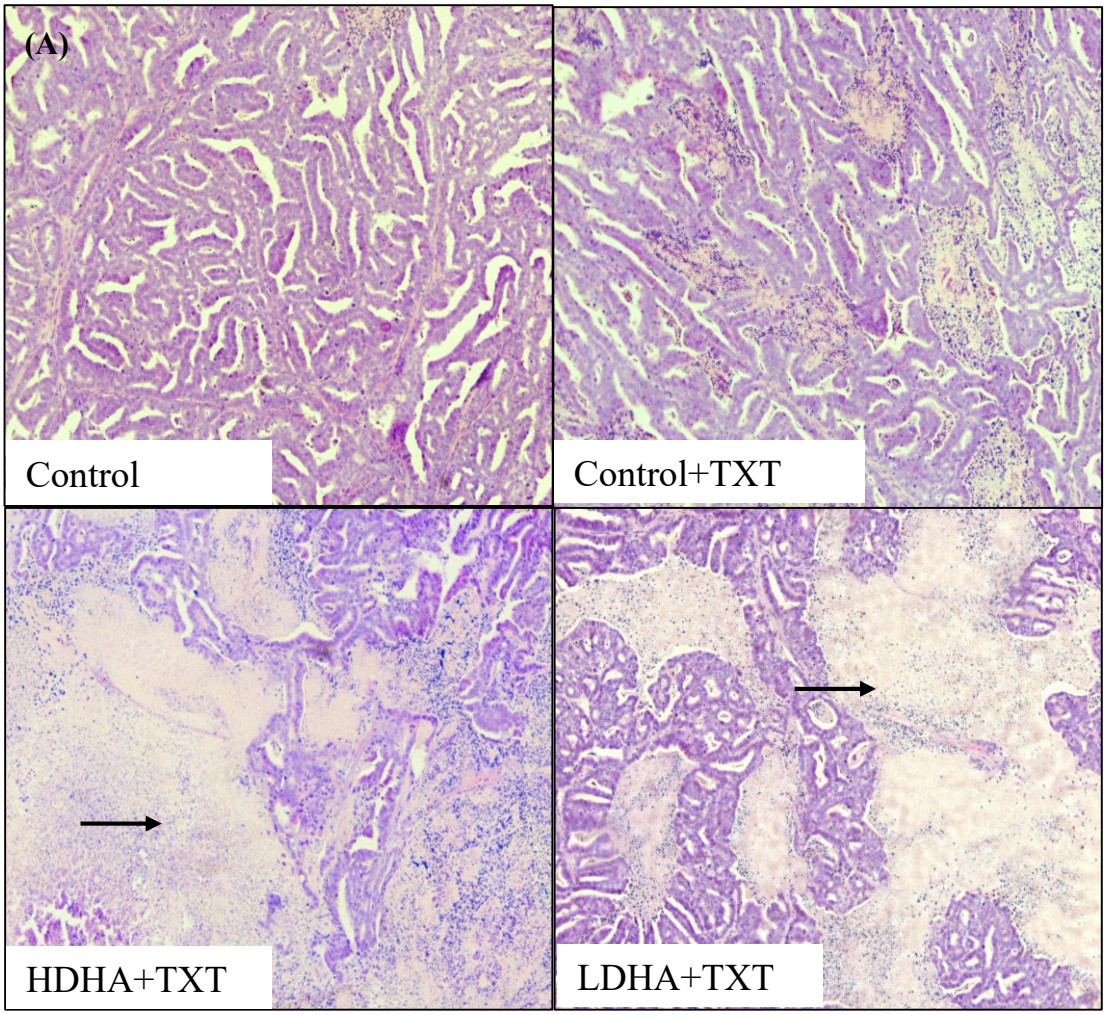
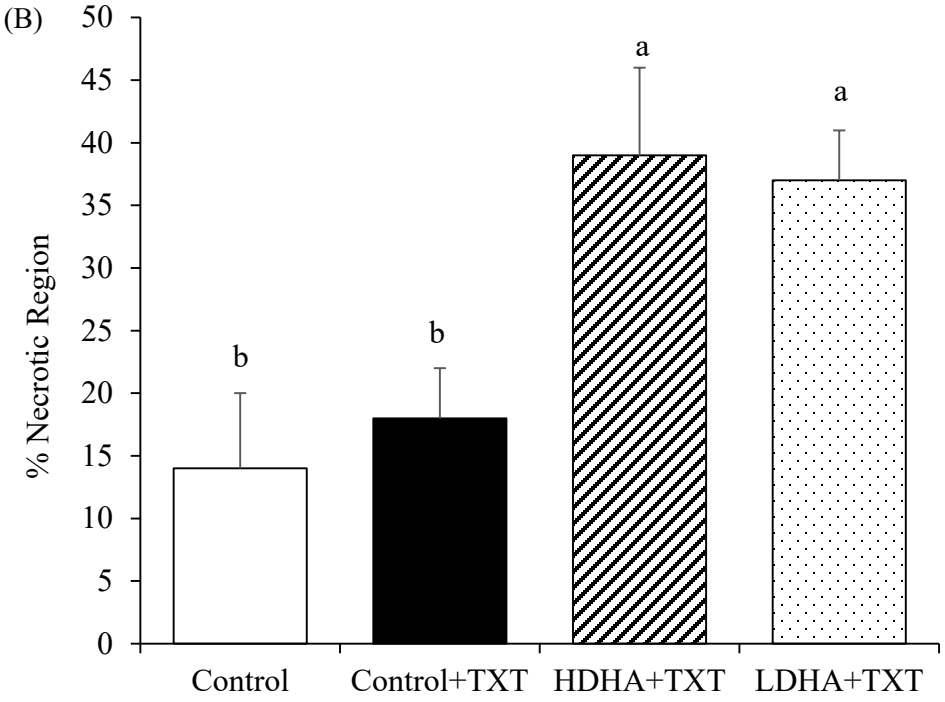


Figure 7-6 B: Necroptosis in MAXF401 tumours: Relative percent of necrotic regions in analyzed tissues



An increase in necrotic areas was observed in both the HDHA+TXT and LDHA+TXT groups ($P<0.05$). The increase in necrosis was confirmed by analysis of tumours from the MAXF574 PDX experiment (**Figure 7-7 A & B**) where there was significantly more necrosis in HDHA+TXT compared to HDHA alone or control tumours ($P<0.05$). Necroptosis related proteins were then assessed in excised tumours. HDHA+TXT tumour protein extracts were found to have increased expression of TNFR1, RIPK1, RIPK3 and MLKL compared to control and control+TXT tumours. The expression of these proteins in LDHA+TXT extracts compared to control or control+TXT were not significantly changed. NFκB was significantly downregulated in HDHA+TXT and LDHA+TXT compared to control and control+TXT and IKKB was significantly downregulated in HDHA+TXT compared to control and control+TXT

(**Figure 7-8 A & B**, $P<0.05$). A proposed pathway with arrows indicating proteins changed by HDHA+TXT is shown in **Figure 7-9**.

Figure 7-7 A: Necroptosis in MAXF574 tumours: Representative H&E staining of control, control + TXT, HDHA and HDHA + TXT tumours with regions of necrotic tissue highlighted with arrows

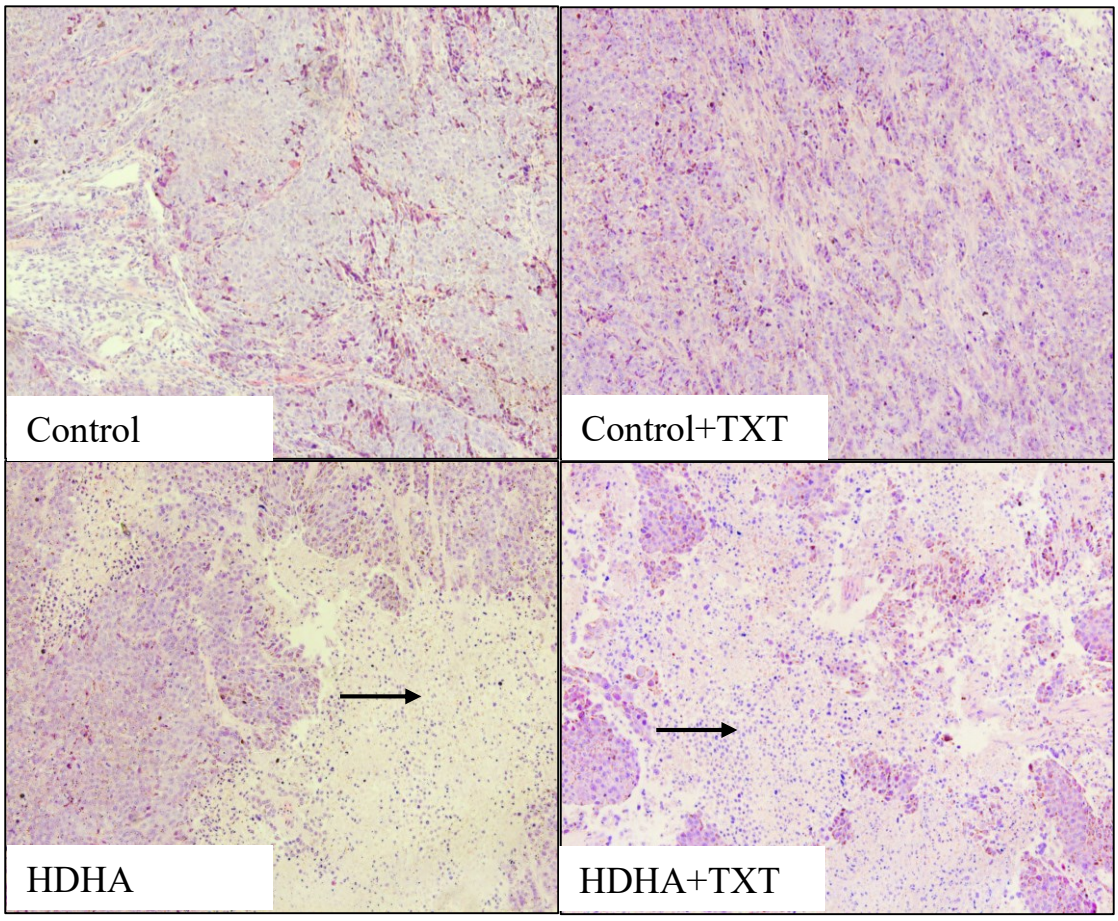


Figure 7-7 B: Necroptosis in MAXF574 tumours: Relative percent of necrotic regions in analyzed tissues

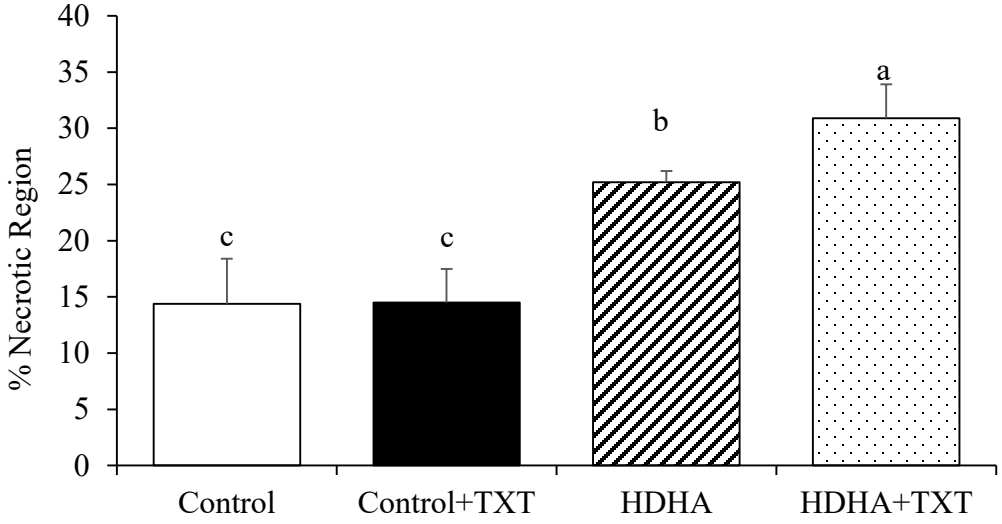
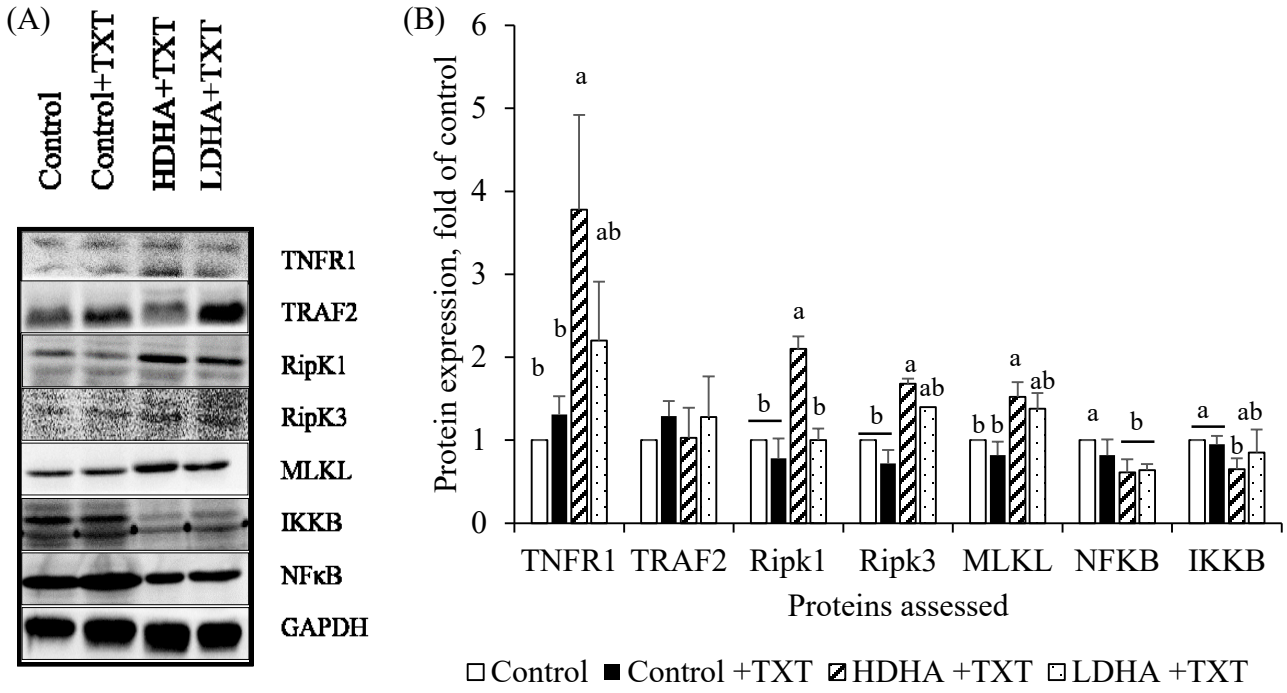
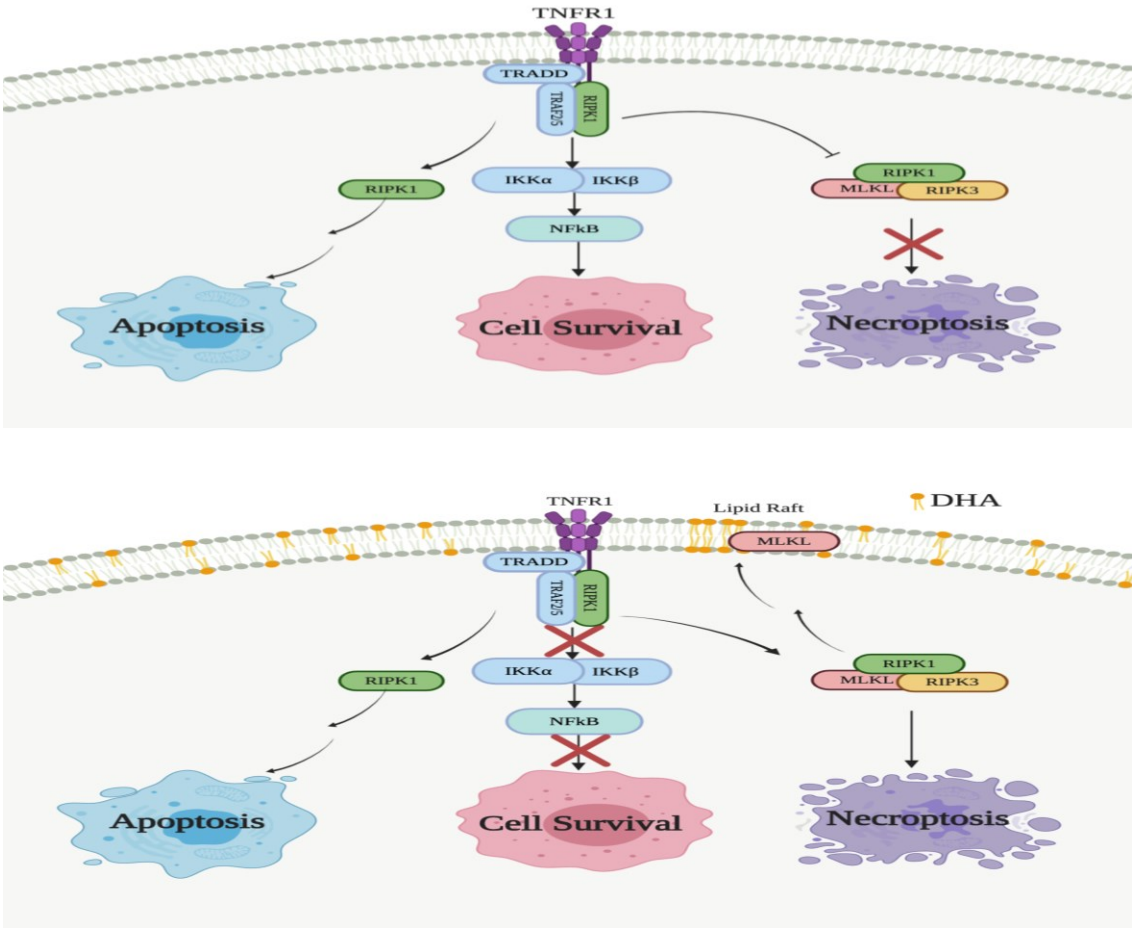


Figure 7-8: Protein analysis of necroptosis in MAXF401 tumours



(A) Representative Western blot analysis of proteins involved in necroptosis and (B) quantification of blots (normalized to loading control, GAPDH) from (A). Values represent the mean ±SEM (n=8 mice per group). Labeled means without a common letter differ ($P<0.05$) based on post hoc DUNCAN analysis.

Figure 7-9: Schematic illustration of proteins differentially expressed in necroptosis pathway of HDHA + TXT treated tumours.

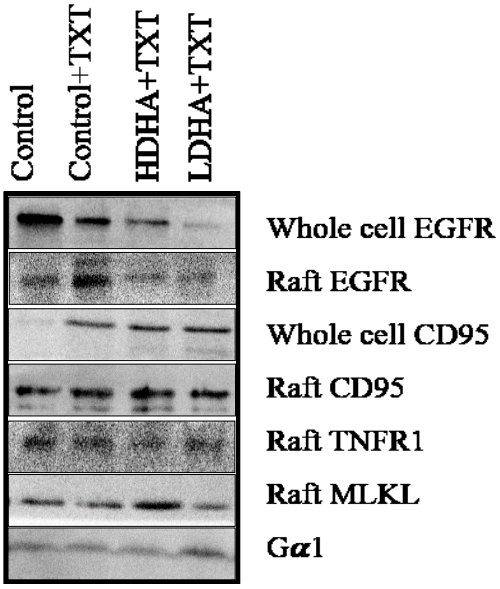


7.3.6 Effect of DHA on markers in the lipid raft

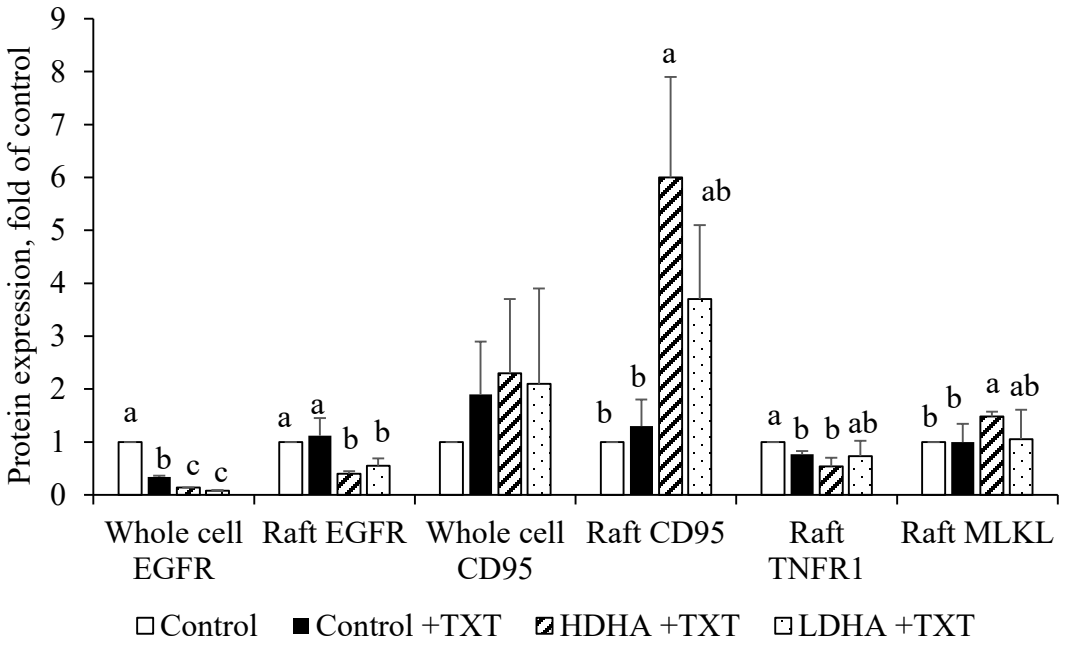
We next sought to determine if the DHA dietary enrichment resulted in differential expression of receptors found in the lipid raft component. First, we confirmed differential expression (whole cell versus raft) of two membrane proteins involved in proliferation and apoptosis, EGFR and CD95 respectively. HDHA+TXT and LDHA+TXT tumour protein extracts had lower expression of EGFR in both whole cell and rafts membranes, whereas with CD95 there were no changes observed in whole cell protein extracts and only HDHA+TXT raft extracts were found to have increased CD95 expression (**Figure 7-10 A & B**). Next, to confirm the effect

of DHA dietary supplementation on lipid raft markers of necroptosis, TNFR1 and MLKL presence in the rafts was assessed. There was a decrease in expression of raft TNFR1 in HDHA+TXT compared to control (but not different from control+TXT or LDHA+TXT) and an increase in expression of raft MLKL in HDHA+TXT compared to control or control+TXT (P<0.05). We further confirmed changes in necroptotic protein expression in lipid raft protein extracts from excised MAXF574 xenografts. Protein extracts from HDHA and HDHA+TXT had decreased expression of TNFR1 and increased expression of MLKL compared to control or control +TXT (**Figure 7-11A & B**, P<0.05).

Figure 7-10: Protein Expression in Lipid Rafts from excised MAXF401 Tumours

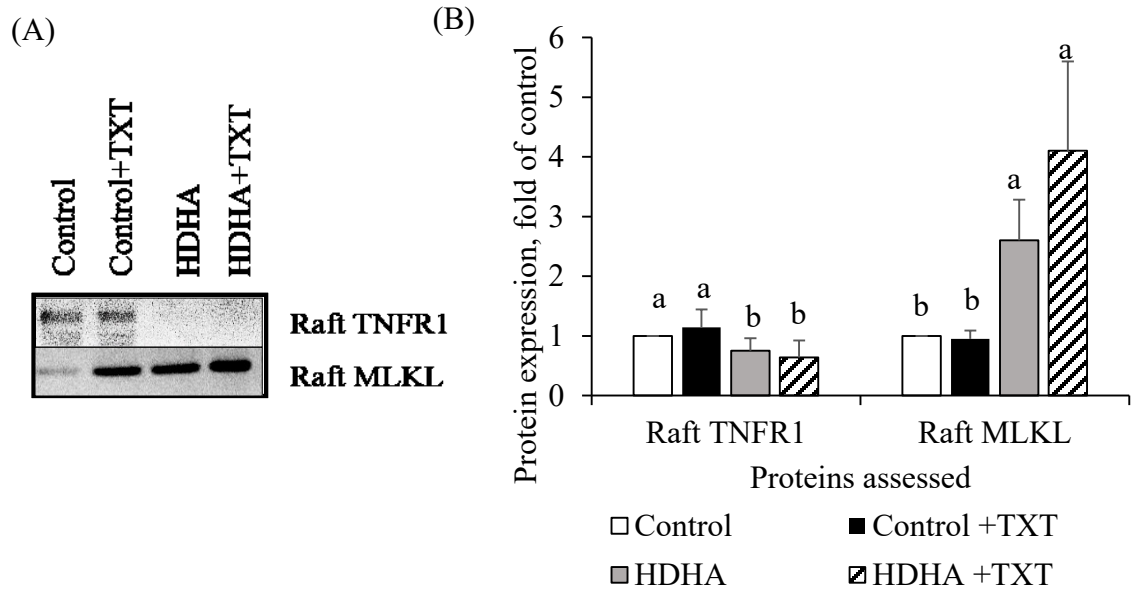


(A) Representative Western blot analysis of lipid raft proteins involved in proliferation, apoptosis and necroptosis in MAXF401 tumours (n=4 per group).



(B) quantification of blots (normalized to loading control, Gαi) from (A). Values represent the mean ±SEM (n=4 per group). Labeled means without a common letter differ (P<0.05) based on post hoc DUNCAN analysis.

Figure 7-11: Protein Expression in Tumour Lipid Rafts from excised MAXF574 Tumours



(B) quantification of blots from (A). Values represent the mean ±SEM (n=4 per group). Labeled means without a common letter differ (P<0.05) based on post hoc DUNCAN analysis.

7.4 Discussion

Patient derived xenografts (PDX) are clinically translatable preclinical models that better recapitulate primary human tumours (compared to immortalized cell models) both with respect to tumoural heterogeneity and biology (Dobrolecki et al. 2016). Our group has previously described the anti-tumoural effects of DHA in a PDX model of breast cancer (Newell et al. 2019). In this study, we extend these findings by defining the uptake of DHA into multiple tissues and identifying two additional mechanisms for DHA action. Specifically, we investigated the ability of dietary DHA at 1.6% or 3.8% w/w of total fat to reduce tumoural growth through decreased activation of the NFκB pathway, increased necroptosis and modulation of lipid raft proteins in tumours.

We had predicted that a higher dietary dose of DHA would result in smaller tumours compared to all other groups. However, tumours were reduced in both the HDHA+TXT and the LDHA+TXT groups to the same extent. A significant dose effect was observed only in the amount of DHA in plasma phospholipids of the HDHA+TXT animals suggesting a maximal incorporation of DHA into tumour phospholipids could be responsible for similar tumour sizes in these groups. It is important to note that although the diets in this study differed in DHA content, both had an equivalent amount of total n-3 fatty acid content due to higher alpha linolenic acid (ALA) and stearidonic acid (SDA) content in the LDHA diet. It is very possible that these n-3's contributed to the anti-cancer effects observed in this study. Indeed, the ability of ALA to reduce cancer cell growth has been demonstrated in *in vitro* breast cancer cell models (Mason et al. 2015, Kim et al. 2009, Yu et al. 2015) and our lab group has previously demonstrated the anti-tumoural efficacy of SDA in an *in vivo* model of breast cancer (Subedi et al. 2015). While there were no differences in amounts of ALA or SDA observed in total phospholipids of tumour,

plasma, liver or muscle among any of the diet groups (Supplemental Tables 2 and 3), elongation of these fatty acids is well documented in the literature ((Yu et al. 2015, Patel et al. 2019) and reviewed by Brenna et al (Brenna et al. 2009)). There was a higher amount of eicosapentaenoic acid (EPA, in plasma and muscle) and docosapentaenoic acid (DPA, in tumour and muscle) in the LDHA+TXT animals compared to all other groups suggesting the elongation of fatty acids from ALA and SDA occurred as these are the LCPUFA end products of these fatty acids, not DHA (Brenna et al. 2009).

In DHA fed animals, all tissues assessed had increased amounts of phospholipid DHA and while there was variance in the relative proportion of DHA amongst the tissues, in each instance the relative increase of DHA was approximately 1.5-fold compared to control. Elevated endogenous levels of DHA in livers and muscle of mice have been previously established (Couture et al. 1995) and was observed in this study. Furthermore, despite ubiquitous distribution of DHA, its' toxic effects have been reported to be tumour tissue specific in both preclinical (Hajjaji et al. 2012) and human trials (Bougnoux et al. 2009, Murphy et al. 2011). An augmented fatty acid uptake in tumours is well established (Sauer et al. 1990, Das 1999) and changes in tumour membranes due to DHA uptake with a reciprocal displacement of arachidonic acid (ARA) has been suggested as a possible mechanism for its' anti-cancer efficacy (Merendino et al. 2013). Our group has previously described this occurrence in immortalized breast cancer cell tumours from nude mice fed DHA in conjunction with chemotherapy (Newell et al. 2020) and similarly, both increased DHA and decreased ARA in PDX tumours from mice fed either DHA diet was observed in this study.

Many key mechanisms influencing cancer progression, such as proliferation and apoptosis, originate at the cell membrane (Hanahan and Weinberg 2000, Hanahan and Weinberg

2011) and because of this, the modulation and subsequent composition of membrane phospholipids are important features that influence cell survival or death (Magtanong et al. 2016). Membrane associated tumour necrosis factor receptor 1 (TNFR1) promotes cell survival via the cell signaling pathway that leads to IKK and subsequent NFκB activation (Hayden et al. 2012). Constitutively active NFκB occurs in many cancers (Ghosh et al. 2002) promoting cell proliferation, angiogenesis, invasion and inflammation (Nakanishi et al. 2005). Camandola et al. demonstrated that fatty acid supplementation of U937 histiocytic lymphoma cells with ARA, but not EPA, resulted in upregulation of NFκB activity (Camandola et al. 1996). Conversely, mice fed a diet enriched with n-3 fatty acids had decreased activation of NFκB in liver extracts (Hardman 2002). We observed decreased expression of both IKKB and NFκB in tumour proteins from mice fed either DHA diet. While this has been previously observed in an *in vitro* model of DHA+TXT treated prostate cancer cells (Shaikh et al. 2008), we believe we are the first to report this in a PDX model of breast cancer. Decreased NFκB activity could enable TNFR1 activated cells to switch from pro-survival to pro-death. TNFR1 engagement on its' own is generally insufficient to kill cells, therefore it has been suggested that the inhibition of NFκB makes it possible (Dondelinger et al. 2016). Furthermore, TNFR1 translocation to lipid rafts is necessary for NFκB signaling (Legler et al. 2003) and we observed a decrease in TNFR1 in lipid rafts isolated from tumour tissue of mice fed DHA diets in both MAXF401 and MAXF574 PDX studies.

DHA incorporation into phospholipid classes occurred to the greatest extent in phosphatidylethanolamine (PE) and in lesser amounts in other phospholipid classes as has been previously described by our group and others (Newell et al. 2020, Gu et al. 2013, Stillwell et al. 2005, Stillwell et al. 2003). Interestingly, a dose effect- with more DHA incorporated into the PE

and phosphatidylinositol (PI) membranes of HDHA group compared to the LDHA group occurred. PE has a range of functions including promoting oxidative phosphorylation and autophagy (Calzada et al. 2016), as well as having a role in both paraptosis (Bury et al. 2013, Chidley et al. 2016) and ferroptosis (D'Herde et al. 2017), but it's possible role in necroptosis has not been elucidated. However, a role for PI in necroptosis has been established (Zhang et al. 2020, Magtanong et al. 2016). DHA increases membrane fluidity and permeability (Stillwell et al. 2003) which can result in a loss of membrane integrity and contribute to necroptotic cell death (Zhang et al. 2018).

Our group has recently described increased necrotic tissue in tumours excised from mice fed a DHA diet (compared to mice fed a control diet without DHA) (Newell et al. 2020). In the current study, immunohistochemical analysis of both DHA groups showed similar increases in necrotic tissue compared to tumour tissue from control fed mice, yet protein analysis showed a significant increase in markers of necroptosis only in the HDHA group with non-significant changes in the LDHA group. We observed increased expression of necrosome components: RIPK1, RIPK3 and MLKL in the HDHA+TXT group compared to all other groups. It could be that while a lower dose of DHA is effective in reducing the size of tumours, the higher dose resulted in a quantifiable metabolic changes within the tumour. Indeed, it is plausible that the increased DHA content observed in the PI class of the HDHA group could have played a role in differential protein expression that resulted in increased expression of MLKL combined with decreased expression of TNFR1 in lipid rafts of the HDHA+TXT group. Phosphorylation of PI generates phosphatidylinositol 4,5-bisphosphate (Katan et al. 2020), a derivative of PI that is important in membrane trafficking, cell signaling and promotes necroptosis through translocation of MLKL to the plasma membrane (Dondelinger et al. 2014, Galluzzi et al. 2014, Dondelinger et

al. 2016). Finally, while the ability of taxanes to induce necroptosis has been shown in immortalized breast cancer cells treated with docetaxel (Mann et al. 2020) and in paclitaxel treated lung adenocarcinomas (Diao et al. 2016), we found no differences in IHC or protein expression in tumours from control fed plus docetaxel animals compared to control alone. This could be in part due to the fact that the PDX's employed in this study were known to be chemotherapy resistant.

Our lab group has previously demonstrated incorporation of DHA into lipid rafts in MDA-MB-231 and MCF-7 immortalized breast cancer cells (Newell et al. 2020). While we did not measure the fatty acid content of rafts in the current study, we did observe changes in raft protein expression from DHA fed animals. Lipid raft signaling pathways for apoptosis or proliferation tend to be hyper-activated in cancer (Turk and Chapkin 2013) and we observed, in conjunction with the differential expression of markers of necroptosis in lipid rafts, the reduced expression of a proliferation marker (EGFR) and increased expression of an apoptosis marker (CD95), highlighting the extensive effects of DHA that originate at the membrane. As observed herein, apoptosis and necroptosis can occur at the same time as they are not mutually exclusive (Ozben 2007, Higuchi 2003) and it is believed that the necrosome can form in parallel with a RIPK1, FADD and caspase 8 apoptosis complex (Dondelinger et al. 2016).

In summary, the current study examined the effect of two doses of dietary DHA on the growth of PDX tumours in NSG mice. We have demonstrated that both doses are effective at reducing tumoural growth and while a dose effect was observed in plasma phospholipids, it did not translate to tumours. However, the ability of DHA to elicit an anti-tumoural response at a lower dose in a PDX model of breast cancer could be important when translated to a clinical setting where breast cancer patients could consume varied amounts of DHA. A clinical trial is

currently ongoing to test this hypothesis (ClinicalTrials.gov Identifier: NCT03831178) (Newell et al. 2019). Taken together, the results from the current study and our previous PDX study (Newell et al. 2019) suggest the multifactorial actions of DHA occur through increased apoptosis and decreased cell proliferation. Additionally, we report for the first time the actions of DHA on increasing necroptosis and decreasing NFκB activity to result in a profound anticancer effect.

Chapter 8: Baseline assessment of women diagnosed with breast cancer enrolled in a double blind, phase II randomized controlled trial to compare Docosahexaenoic acid (DHA) concomitant with neoadjuvant chemotherapy versus neoadjuvant chemotherapy: DHA WIN[7]

8.1 Introduction

Despite improvements in early diagnosis and treatment, breast cancer remains the second leading cause of cancer related death in women (World Health Organization 2017). While neoadjuvant chemotherapy aims to improve surgical resection outcomes and reduce/eliminate micrometastases (Mamounas et al. 2001, Teshome et al. 2014), pathological complete response (pCR) is not achieved by all patients (Teshome et al. 2014). Increasing the efficacy of neoadjuvant treatment without adding additional side-effects would benefit this population.

DHA is an omega-3 long chain polyunsaturated fatty acid (n-3 LCPUFA). The majority of n-3 fatty acids are in the form of the 18-carbon fatty acid alpha-linolenic acid (ALA). While DHA can be synthesized from ALA and other n-3 LCPUFA in the body, endogenous synthesis is low (Burdge et al. 2003, Calder 2016). Consequently, the direct consumption of this fatty acid is the only way to significantly increase levels of DHA in tissues (Plourde et al. 2011). Supplementation can increase blood plasma DHA concentration by 2-fold (500 μM), which can lead to plasma membrane lipid enrichment (Chapkin et al. 2008). Incorporation of DHA into

[7] Sections 8.1-8.14 of this chapter have been adapted from the published protocol: Newell M, Mackey J, Bigras G, Alvarez-Camacho M, Goruk S, Ghosh S, Schmidt A, Miede D, Chisotti A, Postovit L, Baker K, Mazurak V, Courneya KS, Berendt R, Dong WF, Wood G, Basi SK, Joy AA, King K, Meza-Junco J, Zhu X, Field CJ. (2019) Protocol of a double blind, phase II randomized controlled trial to compare Docosahexaenoic acid (DHA) concomitant with neoadjuvant chemotherapy versus neoadjuvant chemotherapy alone in the treatment of breast cancer: DHA WIN. *BMJ Open*. 9:e030502. Doi: 10.1136/bmjopen-2019-030502

tumour membrane phospholipids has been shown to reduce breast cancer cell proliferation (Schley et al. 2007, Rogers et al. 2010) and increase apoptosis (Lee et al. 2014, Ewaschuk et al. 2012, Newell et al. 2019, Kang et al. 2010, Schley et al. 2005, Ghosh-Choudhury et al. 2009) *in vitro* and decreases tumour growth in animal models (Manni et al. 2014, Mason et al. 2015, Ghosh-Choudhury et al. 2009, Kang et al. 2010). Additionally, providing/feeding DHA has been shown to increase the efficacy of different chemotherapeutic drugs in animal models of breast cancer (Newell et al. 2019, Chauvin et al. 2016, Barascu et al. 2006, Ewaschuk et al. 2012). While there is limited clinical evidence, it has been shown that increased dietary intake of n-3 LCPUFA, including DHA, results in increased DHA incorporation in breast adipose tissue (Yee et al. 2010) and this correlates with improved response to chemotherapy (Bougnoux et al. 1999). In an open label trial with advanced metastatic breast cancer patients, DHA supplementation and enrichment into plasma phospholipids was associated with improved outcomes (Bougnoux et al. 2009). Other clinical trials have reported that supplementation with n-3 LCPUFA at a wide range of doses (0.6 g-8.6 g/day) increased tolerability of chemotherapeutic drugs in a range of malignancies at other sites, include lung, pancreatic and colorectal (reviewed in (Morland et al. 2016)). Consequently, we hypothesize that the therapeutic index (efficacy: toxicity ratio) of neoadjuvant breast cancer chemotherapy will be improved with the addition of DHA to the treatment.

Breast cancer proliferation can be assessed by immunohistochemical (IHC) analysis of cells staining positive for the nuclear antigen Ki67 (Dowsett et al. 2011), as it is expressed in all phases of the cell cycle, G_1, S, G_2, and M, but not in G_0 (Gerdes et al. 1984, Scholzen et al. 2000). The proportion of cells staining for Ki67 is frequently used as a primary endpoint to measure efficacy of neoadjuvant therapy in clinical trials. The Ki67 index, defining the change

between pre- and post-treatment Ki67, has been reported to be an independent prognostic factor in luminal A, luminal B, triple-negative, and HER2+ breast cancer, and has been reported to be a useful surrogate marker of relapse free survival in luminal B, triple-negative, and HER2+ breast cancer (Jones et al. 2009, Matsubara et al. 2013). The objective of this RCT is to assess the efficacy of supplemental DHA combined with neoadjuvant chemotherapy in treatment naïve women with breast cancer measured by changes in Ki67 index from biopsy to surgical excision. We hypothesize that DHA supplementation will increase plasma phospholipid DHA and improve response to neoadjuvant chemotherapy assessed by a decrease in the Ki67 index. This protocol follows the Standard Protocol Items for Randomized Trials (SPIRIT) guideline (Spirit Checklist: **Appendix Table 26**, WHO Checklist: **Appendix Table 27**) (Chan et al. 2013).

8.2 Methods

8.2.1 Study Design

The DHA-WIN trial will be a two-arm, double blind phase II randomized controlled trial comparing DHA supplementation and placebo (vegetable oil). The proposed study design with outcomes depicted is shown in **Figure 8-1** and SPIRIT participant flow chart is shown in **Figure 8-2**.

Figure 8-1: Flowchart of Trial Design with Endpoints and Proposed Experimental Analyses

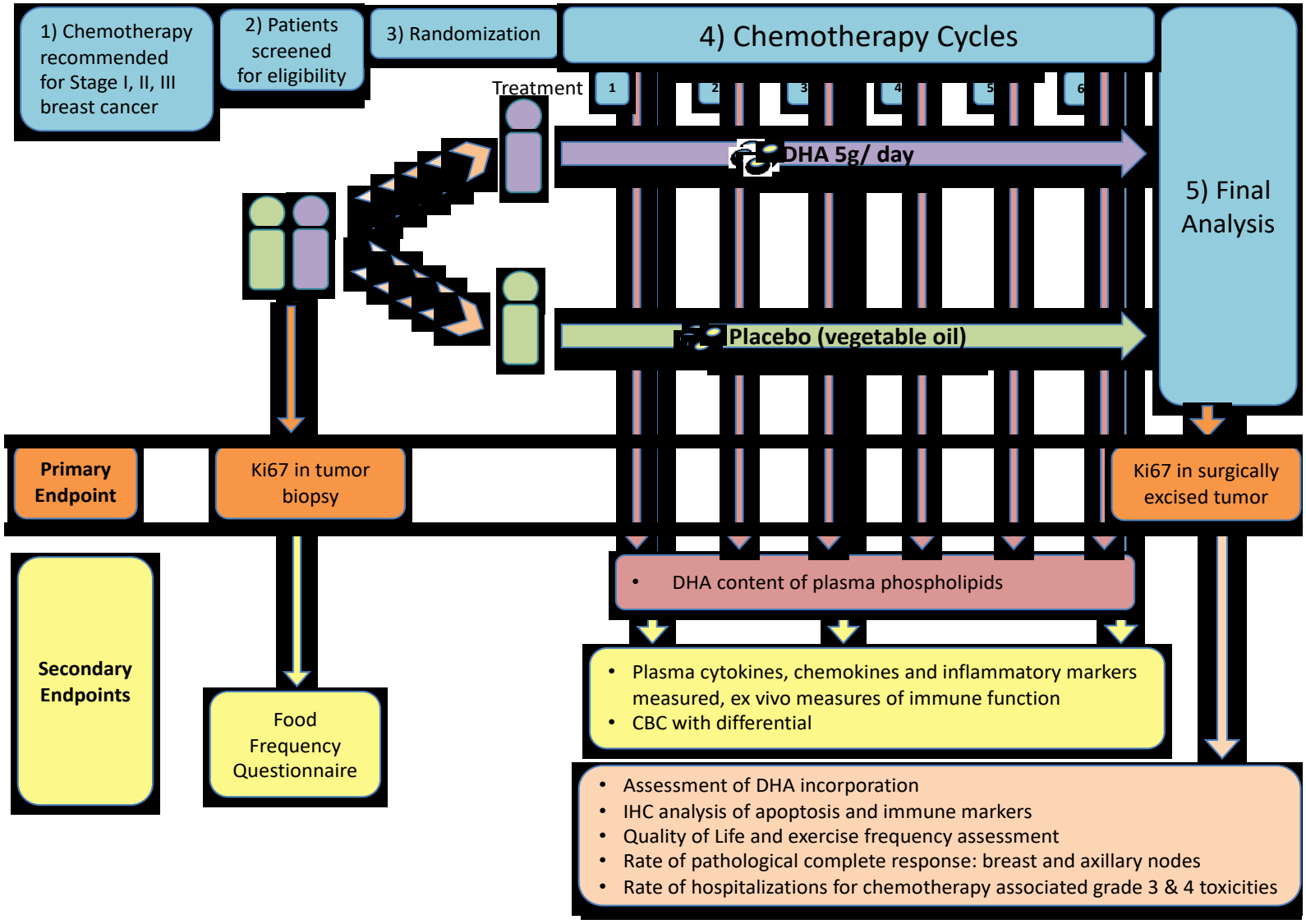
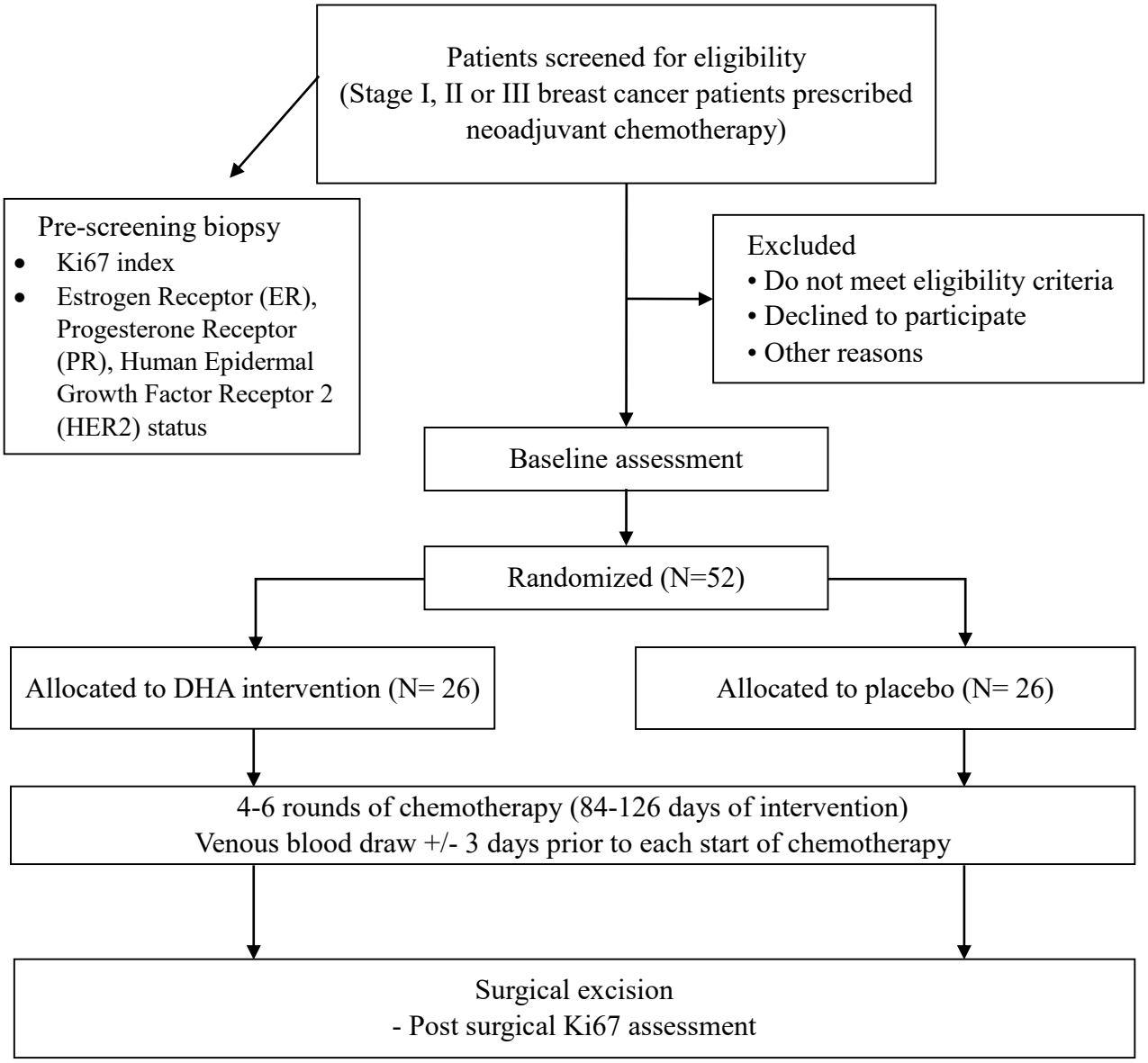


Figure 8-2: SPIRIT patient flow diagram of the DHA WIN trial



8.2.2 Study Population

Eligible women with invasive breast cancer (clinical stage I, II or III) for whom systemic chemotherapy (Arnaout et al. 2018) is recommended prior to surgery. The study will occur at the Cross Cancer Institute, with central laboratory and clinical analyses occurring the University of Alberta, both in Edmonton, Alberta, Canada. Inclusion and exclusion criteria are listed in **Table 8-1**.

Table 8-1: Inclusion and Exclusion Criteria for DHAWIN

Inclusion Criteria
1) ECOG Performance status of 0 or 1
2) Hematology and biochemistry assessments [CBC and differential, partial thromboplastin time (PTT), prothrombin time/ international normalized ratio (PT/INR), aspartate aminotransferase (AST), alkaline phosphatase (ALP), bilirubin, and creatinine] within normal range unless determined not clinically significant by the qualified investigator
3) Ability to take oral medications
4) Adequate tissue specimen for diagnosis, biomarkers, and endpoint Ki67 assays
Exclusion Criteria
1) Patients undergoing surgery prior to chemotherapy
2) Current or previous (within 2 months) daily use (>1 day/week) use of omega-3, fish oil, or other supplements or foods containing DHA (at daily doses > 200 mg)
3) Known allergy to soy or corn
4) Continued intake of supplements containing Vitamin C, Vitamin E or β-carotene exceeding the DRI, or other anti-oxidant supplements
5) Symptomatic but untreated cholelithiasis
6) History of deep venous thrombosis, active thrombophlebitis, pulmonary embolism, stroke, acute myocardial infarction, congestive cardiac failure, untreated hypertension, known inherited hypercoagulable disorder
7) Diagnosis of any other malignancy within the previous year except for adequately treated basal cell or squamous cell skin cancer
8) Medically documented history of a psychiatric disorder that would preclude consent
9) Partial or complete loss of vision or diplopia, from ophthalmic vascular disease
10) Hypersensitivity to any component of the container

8.2.3 Intervention

All women in this trial will receive standard of care chemotherapy throughout the duration of the trial. Breast cancer chemotherapy is developed in a guideline-coordinated system by a single team residing at the Cross Cancer Institute. Consequently, there are only two chemotherapy regimens that are used for neoadjuvant chemotherapy in this population. Each of the two regimens are six cycles in length and given at three-week intervals with a resultant chemotherapy regimen duration of 18 weeks. Both regimens are docetaxel based. For HER2

negative disease, patients universally receive the FEC-D (fluorouracil, epirubicin, cyclophosphamide; docetaxel) (Roche et al. 2006) regimen as neoadjuvant therapy, while HER2 positive patients receive the DCH regimen (docetaxel, carboplatinum, trastuzumab) (Slamon et al. 2011).

Patients will be prescribed either 5 g/day DHA (in 11- 1g capsules), in the form of DHA enriched algae-sourced triglyceride oil capsules (life'sDHA™ S40-O400) or 11g placebo (corn/soy oil blend) per day (capsules from DSM Nutritional Products, Columbia, MD, **Appendix Table 28** for the main fatty acid content of DHA and the placebo). The placebo is balanced for PUFA content with linoleic acid to match the DHA treatment. The amount of additional linoleic acid in the diet of this group is not expected to increase inflammation (Johnson et al. 2012) and has not been shown to elicit a tumoural response (Yu et al. 2015). The capsules are to be taken orally throughout the day as tolerated (at any time, with or without food). Capsules are identical in appearance and composition (other than the oils) to maintain blinding of participants and study staff. As the DHA source is an algae-synthesized triglyceride, there are no differences in texture or taste.

All patients will begin a cytotoxic chemotherapy regimen intended to require 18 weeks for delivery. The intervention (DHA or placebo) will commence at the start of the first cycle of chemotherapy and continue through 4-6 cycles of chemotherapy (3 weeks/cycle). Should a patient not be able to complete the full six cycles of therapy, the timing of surgery remains 3-5 weeks after the last cycle of chemotherapy is delivered. As local guidelines mandate surgery between 3 and 5 weeks from the last round of chemotherapy, DHA/placebo will be continued until this time (21-35 days after the last administration of cytotoxic chemotherapy).

All patients will be dispensed an additional bottle of DHA/placebo capsules at the beginning of the study to account for circumstances where their treatment is delayed due to treatment associated toxicities (including but not limited to vomiting, diarrhea, abnormalities in blood work, fatigue or severe mouth sores). The patients will be requested to continue taking the DHA or placebo as tolerated and will be dispensed additional capsules as necessary. The extra capsules will remain with the patient until the end of the study.

Patients will be encouraged to take the supplements as tolerated (throughout the day at any time, with or without food). Treatment adherence will be monitored by a review of the patient dosing diary and recording the number of any remaining capsules returned at the end of study visit following the last dose of DHA/placebo. Non-compliance will be assessed as consuming less than 50% of the weekly dose for 2 consecutive cycles. No additional natural health product is permitted beyond a daily multi-vitamin.

8.3 Outcome Measurements

Study outcome timelines are summarized in **Table 8-2**. Briefly, outcomes will be measured at baseline, within ± 3 days of chemotherapy and/or post-intervention (surgical excision). Electronic medical record and/or paper chart review of local control, relapse free survival and overall survival will occur at 3, 5, and 10 years to explore possible effects on long-term outcome.

Table 8-2: DHA WIN assessment schedule based on the Standard Protocol Items: Recommendations for Interventional Trials

	STUDY PERIOD												
		Chemotherapy											
	Enrolment	Cycle 1		Cycle 2		Cycle 3		Cycle 4		Cycle 5		Cycle 6		Surgery
TIMEPOINT**	$-t_1$	*Day 1*[(1)]	*Day 20*	*Day 1*	*Day 20*	*Day 1*	*Day 20*	*Day 1*	*Day 20*	*Day 1*	*Day 20*	*Day 1*	*Day 20*	t_x
ENROLMENT:														
Eligibility screen	X													
Informed consent	X													
Randomization	X													
INTERVENTIONS: [(2)]														
DHA 5 grams/day (11 - 1g capsules)		←	—	—	—	—	—	—	—	—	—	—	→	
Vegetable oil placebo (11 -1g capsules/day)		←	—	—	—	—	—	—	—	—	—	—	→	
Medication Diary		←	—	—	—	—	—	—	—	—	—	—	→	
Assessment of Compliance				X		X		X		X		X	X	
ASSESSMENTS:														
BASELINE / ONGOING														
Demographic data collection	X													
Tumour analysis for Grade/ER/PR/HER2[(3)]	X													

Physical Exam / anthropometric measurements	X	X		X		X		X		X		X	X
Relevant medical history /current medical conditions	X			X		X		X		X		X	X
ESAS questionnaire	X	X		X		X		X		X		X	X
Blood Chemistry	X											X [4]	
CBC and differential	X							X				X [4]	
Adverse Events		X		X		X		X		X		X	X
Assessment of Relevant Toxicities		X		X		X		X		X		X	
Primary Outcome													
Tumour analysis of Ki67	X												X
Secondary Outcome													
Assessment of immune function:	X							X				X[4]	
Assessment of DHA incorporation	X			X		X		X		X		X	
Tumour analysis of apoptosis and TILs	X												X
Exploratory Outcomes													
Grade 1, 2 neuropathy assessment		X		X		X		X		X		X	
Pathological complete response													X
Breast conservation													X

Assessment of surgical blood loss													X
Study Associated Questionnaires													
Food frequency questionnaire[5]	X												
Quality of life questionnaire	X											X	
Godin Exercise Questionnaire	X			X		X		X		X		X	X	

ESAS: Edmonton Symptom Assessment System

(1) Day 1 is the day 1 of chemotherapy cycle.

(2) If patients' chemotherapy is delayed due to associated toxicities, they will be encouraged to continue taking the DHA/placebo capsules as tolerated.

(3) From previously collected biopsy.

(4) Tests required at the end of the last round of chemotherapy (i.e., end of cycle 4, 5 or 6 as per patients' individual treatment plan).

(5) Food frequency questionnaire can be completed anytime within the first cycle (21 days) of chemotherapy.

8.3.1 Primary Outcome

The primary outcome of this study is the change in Ki67 from pre-treatment core needle biopsy to surgical excision. It will be calculated by image analysis and will follow analytical and pre-analytical recommendations of Dowsett et al. (Dowsett et al. 2011). The percent change in Ki67 index at experimental end (surgical excision) from baseline will be determined on a log scale and the mean percent change in Ki67 level from baseline will be calculated. Ki67 assays will be performed and reported as part of the routine diagnostic services. A semi-automated computer algorithm scoring system will be employed as previously described (Acs et al. 2019) using the platform QuPath (Bankhead et al. 2017). It is expected that 5g DHA/day will result in a clinically relevant decrease in Ki67.

8.3.2 Secondary Outcomes

8.3.2.1 DHA incorporation into phospholipids: The changes in level of DHA incorporation in plasma phospholipids will be assessed at baseline and at day 1 (±3 days) of each cycle of chemotherapy (2-6) and end of cycle 6 to identify the range of DHA incorporation in this patient population. The use of plasma rather than red blood cells or whole blood for this study is supported by the recent recommendations for best practices for fatty acids described by Brenna et al (Brenna et al. 2018). Analysis of the plasma phospholipid rather than plasma total lipids avoids the postprandial fluctuation of the triacylglycerol pool and is believed to adequately represent the cell membrane composition (Brenna et al. 2018). From our hypothesis and previously published data (Bougnoux et al. 2009), it is expected that supplementing with DHA will result in a significant increase in DHA incorporation. If possible, with the small study size, we will also assess differences in DHA incorporation in patients with different breast cancer subtypes and if subtype or disease stage affects DHA incorporation into plasma, controlling for

the reported dose taken by the patient. The goal is to determine if plasma phospholipid DHA content can be used to predict treatment outcomes. We will also assess incorporation of other essential fatty acids (linoleic, linolenic, arachidonic, eicosapentaenoic, docosapentaenoic) to determine if there are differences between or within treatment groups.

8.3.2.2 Systemic immune function: Systemic immune function will be assessed on blood samples obtained at baseline, beginning of chemotherapy cycle 4 (day 1± 3 days) and at the end of chemotherapy treatment. Changes in markers of systemic immune cell type and function will be assessed following supplementation compared to baseline and the change from baseline compared to patients receiving the placebo. We will also examine the relationship between changes in activation markers and the level of DHA incorporation, changes in systemic inflammation (CRP, IL-6, TNFα) and immune function (ability to produce IL-2 after stimulation *in vitro*) following DHA supplementation.

8.3.2.3 Identify factors that may affect DHA incorporation into plasma phospholipids: If incorporation of DHA into plasma phospholipids is variable within the DHA treatment arm, possible factors that may influence incorporation will be assessed between high and low incorporators. These parameters will be assessed at the end of the study from data collected throughout the study.

8.3.2.4 Examine changes in markers for apoptosis: Caspase-3 presence in the excised tumour, as percent positive cells, will be calculated by image analysis and a comparison of expression levels at experimental end (surgical excision) to baseline will be determined in patients receiving DHA supplementation and compared to patients receiving placebo. Proportions of negative cells, weakly positive cells and strongly positive cells will be scored by two pathologists and the staining intensity, assessed by QuPath, (Bankhead et al. 2017) will be

recorded independently. Increased apoptosis measured by Caspase-3 is a clinically relevant marker of cell death.

8.3.2.5 Examine changes in tumour infiltrating lymphocytes (TILs): CD4+ and CD8+ in the excised tumour, as a number of positive cells for a given area, will be calculated by image analysis. A comparison post-treatment of expression levels at experimental end (surgical excision) to baseline will be determined in patients receiving DHA supplementation and compared to patients receiving placebo. The differences will be compared between treatments and within the treatment group, related to plasma DHA concentrations. Increased infiltration of TILs is potential marker that could be used to predict treatment patient outcomes.

8.3.2.6 Pathological complete response (pCR) rate: pCR in resected breast tissue and all sampled axillary nodes will be assessed as absence of invasive cancer by haematoxylin and eosin evaluation as per standard of care. Pathologic complete response will be classified as ypT0/is ypN0 and will be determined at the end of study after surgical resection as part of standard of care assessment.

8.3.2.7 Comparison of rate of chemotherapy associated grade 3 and 4 toxicities: Rate of chemotherapy associated grade 3 and 4 toxicities, and chemotherapy-associated hospitalizations will be compared between DHA and placebo arms. Any changes will then be examined in regards to level of supplementation and DHA incorporation. These analyses will be completed at the end of study after surgical resection.

8.3.3 Exploratory outcomes

8.3.3.1 Food frequency questionnaire (FFQ): Assessment of the FFQ to compare the estimated (pre-diagnosis) usual intake of macronutrients on an energy basis (including fat content and

composition) between the two groups at baseline. In the future, the overall medians/means of the subjects in this study will be compared to age-matched women in the Alberta Tomorrow Project.

8.3.3.2 Quality of life: Assessment in changes in quality of life will be determined by questionnaire employed at baseline and end of treatment. Comparisons will be assessed from end of treatment to baseline within and between treatment groups.

8.3.3.3 Exercise behavior: Assessment of exercise behavior will be determined by questionnaire employed at baseline, each cycle of chemotherapy and end of treatment. Comparisons will be assessed from end of treatment to baseline within and between treatment groups.

8.3.3.4 Breast conservation: The rate of breast conservation, specifically the rate of lumpectomy and mastectomy, will be determined by review of surgical and pathological reports at the end of study after surgical resection.

8.3.3.5 Volume of surgical blood loss: High intakes of n-3 LCPUFA (that contain some DHA) have been studied to determine if they increase bleeding time (Watson et al. 2009, Eritsland et al. 1995). We will review surgical report estimates of blood loss to see if there is a qualitative or quantitative difference between placebo and treatment arms, once adjusted for the magnitude of surgery (lumpectomy vs. mastectomy vs. mastectomy + immediate reconstruction; sentinel node dissection vs. full axillary dissection). It is not expected that we will see a difference as it is eicosapentaenoic acid (EPA, the precursor to DHA) that has antithrombotic and antiplatelet properties (Knapp et al. 1986).

8.3.3.6 Local control, relapse free survival and overall survival: Local control, relapse free survival and overall survival will be analyzed by review of electronic medical records, registry reports, and/or paper medical charts at 3, 5, and 10 years to explore possible effects on long-term outcome.

8.4 Participant timeline

Breast cancer patients receiving neoadjuvant chemotherapy account for approximately 20% of newly diagnosed breast cancer patients, approximately 10-12/month at the Cross Cancer Institute. Assuming a conservative accrual rate of 30%, accrual is estimated to be completed in 14-18 months with 3-4 patients recruited per month. Each patient will be enrolled for the duration of their individual chemotherapy regimen, an estimated 12-18 weeks (84-126 days) beginning at the start of the first cycle of chemotherapy and continued through 4-6 cycles of chemotherapy (3 weeks/cycle). The intervention will be discontinued 21-35 days after the last administration of cytotoxic chemotherapy when surgery to remove the tumour occurs. See **Figure 8-1** for a schematic of the participant timeline.

8.5 Sample Size

Fifty-two women prescribed neoadjuvant breast cancer chemotherapy will be enrolled in a 2-arm trial with 26 participants/arm. The sample size calculation is based on the primary objective, which is to determine the efficacy of supplemental DHA provided with standard neoadjuvant as measured by change in the Ki67 index from biopsy to surgical excision. Group sample sizes of 23 patients in each group are required to achieve 81% power to detect a difference between the group proportions of 0.4. The proportion in group one is assumed to be 0.3 under the null hypothesis and 0.7 under the alternate hypothesis. The proportion in group two which is the control group is 0.3. The test statistic used is the two-sided t-test. The significance level of the test was targeted at 0.05 and the significance level actually achieved by this design is about 0.0497. Assuming a dropout rate estimated at approximately 10% for this patient population, which is approximately 5 patients, a total of 52 patients (26 patients in the DHA supplementation group, and 26 in the placebo group) is required for the study.

8.6 Recruitment

Oncologists and clinical trial nurses at the Cross Cancer Institute in Edmonton, Canada will recruit newly diagnosed breast cancer patients. Patients will be screened for eligibility by the clinical trial nurses and eligible, interested patients will receive a detailed explanation of the study by the study coordinators and written informed consent will be obtained (**Appendix Files 1 & 2**).

8.7 Randomization and Blinding

A biostatistician will generate a patient randomization list and randomized bottle numbers by covariate-adaptive randomization (block randomization). The randomized bottle numbers will be provided to DSM for labeling for both the DHA and placebo groups and the randomized bottle list will also be provided to the unblinded Clinical Trials Coordinator (CTC, Clinical Trials Unit) and the unblinded pharmacist. Patients will be stratified by histological subtype and then randomized. The allocation of the study arm (as the study is blinded, hence, the study arm A and B will be used as this will not identify the placebo or intervention arm) and a unique study identifier will be conducted using the REDCap database. The key to the study arm A and B will only be provided to the unblinded CTC, statistician and the pharmacist. The study coordinator will enter the new patient information in REDCap and assign the unique ID and arm. This information will be shared with the unblinded CTC and the unblinded pharmacy staff. The pharmacy staff will assign the correct bottle numbers based on the study arm at day 1 of each chemotherapy cycle. Following the allocation of the bottle numbers, this information will then be shared with the study coordinator and the unblinded CTC. The bottle ID will be entered in the REDCap database by the study co-ordinator. All future bottle allocations with the unique bottle ID will be entered into the REDCap database. The key to the study arm will be kept in password

protected computers and will only be shared in an urgent need for breaking of the blind. When a blinding code is broken, the date and reason for unblinding must be fully documented in source documents and entered on the case report form. Every effort should be made by site staff to ensure that the treatment arm in which the unblinded patient is assigned is communicated only to those site staff that require the information for treatment purposes. To assist in maintaining the blind of the patients, supplements and placebo are identical in size, shape, color and texture, in addition to identical bottles for dispensing. Patients, pathologists, physicians, and researchers will be blinded to patient enrolment in the study and throughout trial. Blinding will only be dropped after analysis of fatty acids, systemic immune function and Ki67 is complete.

8.8 Data Collection, Management and Analysis

Study methods are summarized in **Table 8-3**. Briefly, data will be collected and measured at baseline, within ± 3 days of chemotherapy and/or post-intervention (surgical excision). Electronic medical record and/or paper chart review of local control, relapse free survival and overall survival will occur at 3, 5, and 10 years. All data will be entered and maintained in the REDCap trial database. Baseline measurements will be analyzed once all participants have been enrolled and all other analyses will occur at completion of trial.

8.8.1 Primary Outcome

Ki67 will be tested by immunohistochemistry (IHC) by the diagnostic biomarker laboratory at the Cross Cancer Institute using the MIB1 antibody on 4 μm sections from formalin fixed paraffin embedded (FFPE) needle core biopsy surgical specimens. At final analyses, Ki67 staining will be repeated as a single IHC stain and interpreted by image analysis. At the time of Ki67 interpretation, slides will be de-identified and coded to ensure the pathologist is blinded to the experimental group. In addition, the original single stained slides

Table 8-3: Variables, Measures and methods of analysis

VARIABLE / OUTCOME	OUTCOME MEASURE	METHOD	STATISTICAL ANALYSIS
Primary:			
Efficacy of supplemental DHA provided with standard neoadjuvant chemotherapy as measured by change in Ki67	Ki67 labelling index	Immunohistochemistry	95% t-confidence interval for mean percent change in Ki67. Independent t-test to compare change between the study groups (Acs et al. 2019)
Secondary:			
1. DHA incorporation into plasma phospholipids	Fatty acid composition of plasma phospholipids	Gas chromatography	Paired t-test will be used to compare the mean percent change in the DHA level of patients after each cycle with their baseline values. If the data is not normally distributed, the Wilcoxon signed rank test will be employed for this comparison. A 95% t-confidence interval for the mean percent change in the DHA from baseline will be compared to patients receiving placebo
2. Systemic immune function	a) Immune cell subset identification b) Plasma cytokines c) Ex vivo stimulated immune cell response	a) Flow cytometry b and c) ELISA and MesoScale	Repeated Measures ANOVA with post-hoc analysis
3. Identify factors that may affect DHA incorporation into	Factors assessed after calculating high and low DHA incorporators:		Independent t-test will be conducted to compare the mean values between the two study groups. Chi-square test will be conducted to determine correlation between

tumour tissue and plasma phospholipids.	a) Weight (BMI) b) Age c) The usual diet estimated from the FFQ d) Composition of dietary fat estimated from the FFQ e) Histology of the tumour (provided from the biopsy) f) Amount of DHA consumed (adherence to the supplement) g) % incorporation of other fatty acids		two categorical variables for outcome measures listed
4. Examine changes in markers for apoptosis	Caspase-3	Immunohistochemistry	Within subject and between subject variability between the two groups will be tested using generalized estimating equation (GEE) method
5. Examine changes in markers for tumour infiltrating lymphocytes	CD4+/CD8+	Immunohistochemistry	Within subject and between subject variability between the two groups will be tested using generalized estimating equation (GEE) method
6. Describe the rate of pathological complete response in breast and in axillary nodes	Absence of invasive cancer on haematoxylin and eosin evaluation	Immunohistochemistry	pCR= ypT0/is ypN0 95% t-confidence interval using independent t-test for mean percent change between treatment groups

7. Describe the rate of grade 3 and 4 chemotherapy associated toxicities.	Rate of grade 3 /4 toxicities and chemotherapy associated hospitalizations	Chart review	95% t-confidence interval using independent t-test for mean percent change in events between treatment groups
Exploratory Outcomes			
1. Food Frequency Questionnaire	DHQ II questionnaire	Questionnaire	Independent t-test of macronutrient and fat content / composition between groups
2. Quality of Life	Baseline and Endpoint questionnaires	Questionnaire	Paired t-test for continuous variables and McNemar's for categorical variables for mean percent change in events between treatment groups
3. Exercise	Godin Exercise questionnaire	Questionnaire	Paired t-test for continuous variables and McNemar's for categorical variables for mean percent change in events between treatment groups
4. Assess the rate of breast conservation	Rate of lumpectomy and mastectomy.	Chart review	Chi-square tests
5. Assess the volume of surgical blood loss.	Review surgical reports for quantitative / qualitative loss of blood	Chart review	Independent t-test
6. Analyze local control, relapse free survival and overall survival	Electronic medical record and / or paper medical chart review at.3, 5, and 10 years to explore possible effects on long-term outcome	Chart review	Kaplan-Meier estimates along with the survival curves, log-rank test will be used for statistical comparison between groups

will be interpreted visually by research staff. All Ki67 values (routine and image analysis) will be recorded as absolute percentage and H-score in the REDCap trial database and the participants' case report form. The Ki67 index is validated and used in clinic as a marker of proliferation. The Ki67 index (absolute % and H-score (Ishibashi et al. 2003) of biopsy and surgical resection (after chemotherapy) will be compared on each participant and between participants receiving DHA compared to placebo.

8.8.2 Secondary Outcomes

DHA incorporation into plasma phospholipids will be measured in venous blood from patients at baseline (time of enrolment in trial), and at day 1 (± 3 days) of each chemotherapy cycle by a technician blinded to the treatment group. Venous blood will be collected in coated EDTA tubes and centrifuged at 750x g for 10 min to obtain plasma. Red blood cells will be immediately frozen and banked at -80°C for storage for future secondary analysis. Plasma will be separated into 6 aliquots and immediately frozen at -80°C for storage. Plasma will be extracted by the Folch procedure (Folch et al. 1957, Field et al. 1988), phospholipids separated by thin layer chromatography and fatty acid content (concentration and relative percent) measured by gas-liquid chromatography as previously described (Schonberg et al. 1995). The percentage change in DHA from baseline will be compared in each patient and a 95% t-confidence interval for the mean percent change in DHA from baseline will be compared to patients receiving placebo. An internal standard is used to identify and quantify the fatty acids. This standard measure for fatty acid status has coefficient of variation <5% and individual GC peaks are validated against phospholipid standards (GLC-502 and GLC-643) from NuChek (Elysian, MN).

Phenotyping of immune cell subsets will be measured using whole blood (collected in EDTA tubes). The various cell types will be identified using specific fluorescently labelled monoclonal antibodies (mAb) to surface receptors (See **Appendix Table 29** for list of antibodies). These will be quantified by flow cytometry, as previously described (Field et al. 2008). With the remaining blood, peripheral mononuclear cells will be isolated and purified on a Ficoll density gradient of Histopaque 1077 as previously described (Field et al. 2008, Field et al. 2000). To measure cytokine production in isolated lymphocytes, cells will be cultured in media with or without the mitogens, Phytohemagglutinin (PHA) or Lipopolysaccharide (LPS), for 48 h as previously described (Richard et al. 2017). Supernatant will be collected and stored at -80°C for *ex vivo* measures of immune function (ability and pattern of cytokines produced after stimulation). IL-1 β, IL-2, IL-6, IL-10, TNF α, and IFN-γ (pg/ml) cytokines will be measured using electrochemiluminescent multiplex assays (MesoScale Discovery) or by individual ELISA assays. Cytokines listed above and inflammatory markers including C-reactive protein (CRP) in plasma will be measured by electrochemiluminescent multiplex assays (MesoScale Discovery) as previously described (Lewis et al. 2016). Cytokines and inflammatory markers in plasma and cytokines from cultured lymphocytes will be analyzed when all samples have been collected. Changes in systemic immune function will be assessed in patients compared to baseline and compared between groups. The data analysis will occur at completion of trial. Cytokines are done in duplicate and the coefficient of variance is <15%. Phenotypes will be collected as a relative percent of total cells and the change compared between treatments. Additionally, white blood cells that are not used for the immune assays will be assessed for fatty acid composition.

If DHA incorporation into plasma phospholipids is significantly different within the DHA supplementation arm, factors that may influence incorporation will be compared in low vs. high incorporators, to identify possible factors that predict incorporation including BMI, age, the estimated macronutrient intake and composition of dietary fat of the women (estimated from the FFQ), histology of the tumour (provided from the biopsy), the amount of DHA consumed (adherence to the supplement) and length of time DHA consumed (if treatment is ended early) . We will also assess incorporation of other fatty acids (palmitic, oleic, linoleic, linolenic, arachidonic, eicosapentaenoic, docosapentaenoic) to determine if there are differences between or within treatment groups.

Caspase-3 changes and changes in CD4 and CD8 will be tested by IHC by the diagnostic biomarker laboratory at the Cross Cancer Institute on 4 μm sections from FFPE surgical specimens. At final analyses, IHC staining will be interpreted by image analysis. At time of interpretation, slides will be de-identified and coded to maintain the blind. All values (routine and image analysis) will be recorded as absolute percentage. Caspase-3 is a validated marker of apoptosis and CD4 and CD8 are validated markers for lymphocytes. The calculated indices (absolute % and H-score) of biopsy and surgical resection will be compared on each participant and between participants receiving DHA compared to placebo.

Pathological complete response in resected breast tissue and axillary nodes will be assessed in hematoxylin and eosin stained tissue for evidence of invasive disease according to standard of care and recorded in patients' case report form. The rate of pathological complete response in breast tissue and axillary nodes after surgical resection will be compared between participants receiving DHA supplementation compared to placebo.

Grade 3 and 4 toxicities will be assessed and recorded by the clinical trial nurse. Toxicities will be assessed on day 1 (± 3 days) of each chemotherapy cycle. Dates of hospitalization will be recorded in patients' case report form. Rates of chemotherapy-associated grade 3/4 toxicities, all grade neuropathy and hospitalizations will be compared between DHA supplementation and placebo arms as scored by a medical oncologist in a standardized toxicity/ neuropathy form for each cycle of chemotherapy.

8.8.3 Exploratory outcomes

The FFQ is a validated questionnaire for macronutrient intake (Thompson et al. 2002, Subar et al. 2001, Kipnis et al. 2003). The quality of life questionnaire is a validated questionnaire from European Organization for Research and Treatment of Cancer- Quality of Life Questionnaire-C30 (EORTC-QLQ-C30) (Aaronson et al. 1993). Exercise behavior will be assessed using the modified Godin Leisure-Time Exercise Questionnaire (GLTEQ) (Godin et al. 1997, Courneya et al. 1999). Assessment of changes in quality of life and exercise behavior will be compared from time points collected to baseline within and between treatment groups. We do not expect the supplement/placebo to influence this variable but since exercise alters immune function, quality of life and tumour growth we have included it herein to determine if it changes during therapy.

The rate of breast conservation, specifically the rate of lumpectomy and modified radical mastectomy, will be determined by surgical and pathological reports at time of surgical resection. Volume estimates of blood loss will be assessed by review of surgical reports to see if there is a qualitative or quantitative difference between placebo and treatment arms, once adjusted for the type of surgery (lumpectomy vs. mastectomy vs. mastectomy + immediate reconstruction; sentinel node dissection vs. full axillary dissection). Local control, relapse free

survival and overall survival will be analyzed by electronic medical record and/or paper medical chart review at 3, 5, and 10 years. Data will be validated by a medical oncologist.

8.9 Data Management

All data will be entered and maintained in the REDCap trial database. Direct access to clinical and laboratory information on enrolled trial patients will be limited to the principal investigator, co-investigators, trainees/staff who have had the appropriate training and approval and study nurses and study coordinators who will have access to the source documents through the electronic medical record and laboratory information system at the Cross Cancer Institute. All patients will have biopsy and tumour samples for analysis and we do not expect any missing data for the primary endpoint (Ki67). If supplement compliance is below 50% for more than two consecutive cycles, or if participants do not complete chemotherapy (to a minimum of 4 cycles), they will be excluded from final analysis of the primary endpoint. If patients do not have sufficient blood samples for the secondary analyses (DHA incorporation, systemic immune function), analysis will be performed using data from the remaining patients.

8.10 Statistical Methods

8.10.1 Primary Outcome

The percent change in Ki67 will be determined as an absolute percentage and H-score. The number of patients showing a decrease and the 95% confidence interval for the mean percent change in the Ki67 level from baseline in patients receiving DHA supplementation will be compared to patients receiving placebo. The mean change will be measured using an independent t-test between the two groups.

8.10.2 Secondary Outcomes

Paired t-tests will be used to compare the mean percent change in the plasma DHA level of the patients after each cycle of chemotherapy with their baseline values. If the data is not normally distributed, the Wilcoxon signed rank test will be used to compare the plasma DHA level after each cycle of chemotherapy with baseline. The difference in plasma phospholipid DHA from baseline and between DHA supplementation and placebo arms will be calculated, and the 95% confidence interval for the mean percent change in DHA level from baseline and groups will be assessed.

If systemic immune function data is not normally distributed, it will be log transformed prior to analysis and the normality assumptions will be tested again. Repeated measures ANOVA with post hoc analysis will be used to determine if there is an effect of treatment on immune function.

Factors affecting DHA incorporation will be examined by independent t-tests to compare the mean values between the DHA and placebo groups. Chi-square tests will be conducted to determine correlation between two categorical variables for the outcome measures listed.
The within subject and between subject variability between the two groups for the mean percent change in apoptosis and tumour infiltrating lymphocyte markers will be tested using the generalized estimating equation (GEE) method.

The 95% confidence interval using independent t-tests will be conducted for the mean percent change in pathological complete response and rates of grade 3 and 4 chemotherapy associated toxicities and hospitalization in patients receiving DHA supplementation compared to patients receiving placebo.

8.10.3 Exploratory outcomes

Independent t-tests for macronutrient and fat content obtained from the food frequency questionnaire will be examined between groups. Paired t-tests for continuous variables and McNemar's test for categorical variables will be assessed for mean percent change in events between treatment arms for the quality of life and exercise questionnaires. Chi-square tests will be used to compare the degree of breast conservation and the volume of surgical blood loss will employ an independent t-test between the two study arms. Rate of local control will be compared between treatment arms using t-test of proportions. Recurrence-free survival and survival will be analyzed using the log rank test on Kaplan-Meier survival curves.

SAS software, version 9.4 (SAS Institute Inc., Cary, NC), will be used for statistical analysis. A p-value <0.05 level will be used for all statistical significance. Two-sided tests will be used for all statistical tests.

8.11 Data Monitoring

The trial activities performed at the Cross Cancer Institute will be monitored by the Cross Cancer Institute, Investigator Initiated Trials Data Safety Monitoring Board (DSMB). The DSMB is independent of the investigator and is composed of representatives from both medical and radiation oncology.

The investigator will assess the relationship between protocol treatment and the occurrence of adverse events (AEs) and this assessment will be recorded in the database for adverse events. This study will use the International Common Terminology Criteria for Adverse Events (CTCAE), version 5.0, for adverse event reporting. The reporting period for adverse events will start at the time the patient takes the first dose of DHA/placebo through and including 28 calendar days after last administration of study agent. If serious adverse reaction

to treatment occurs, the Natural and Non-prescription Health Products Directorate (NNHPD), Clinical Trial Unit, Health Ethics Research Board of Alberta, Cancer Committee (HREBA.CC) and DSM will be notified as per guidelines. After 25 evaluable patients, all data and results will be submitted to the DSMB for review.

8.12 Auditing

As per the SPIRIT guidelines, the investigators, Cross Cancer Institute and the University of Alberta will permit trial-related monitoring, audits, REB, DSMB review, and regulatory inspection(s), providing direct access to paper and/or electronic documentation pertaining to the clinical study (e.g. CRFs, source documents such as hospital patient charts and investigator study files). All site facilities related to the study conduct could be visited during an audit (e.g. pharmacy, laboratory, outpatient department) and are agreed to co-operate and provide assistance at reasonable times and places with respect to any auditing activity.

8.13 Patient and Public Involvement

Patients were not involved in the protocol development or study design. However, oncologists and clinical trial nurses who work in the breast tumour group are involved in patient screening to assess eligibility for the study. The HREBA-CC approved informed consent will be obtained from patients prior to their involvement in the study and it informs patients of their right to withdraw at any time. At the end of the trial, results will be disseminated to the public through seminars, public talks and in peer-reviewed journals.

8.14 Ethics and dissemination

DHA WIN has received Health Canada approval (#HC6-24-c220167), full ethical approval from the Health Research Ethics Board of Alberta – Cancer Committee (Protocol #: HREBA.CC- 18-0381) and is registered at clinicaltrials.gov (Identifier: NCT03831178).

Protocol amendments will be submitted to HREBA.CC, Health Canada and the clinical trial registry prior to study implementation according to regulatory requirements. The formal consent of a participant, using the HREBA-CC-approved consent form (**Appendix File 1**), will be obtained by a clinical trial nurse before the participant is enrolled in the study and will be signed by the patient, and the principle investigator. A voluntary optional consent form for use of participant data and biological specimens (**Appendix File 2**), will be offered at time of enrollment. Patient confidentiality and anonymity will be maintained and identities protected from unauthorized parties.

Access to data will be restricted to the primary investigators and statistician. They will grant access to other team members as governed and approved by ethics. Ancillary care post-trial will occur as routine standard of care for all participants. Our objective is to determine the efficacy of using DHA supplementation concomitant with chemotherapy and as such our results will be disseminated to clinicians for implementation in future treatment paradigms. The results will be submitted to peer-reviewed journals and presented at national and international conferences.

8.15 Results

Original calculations for the DHA WIN trial determined that 52 women would be required to sufficiently statistically power this study with an estimated 10% dropout rate. Unfortunately, 26% of the patients have withdrawn to date. Reasons for withdrawal will be assessed at trial completion, however to ensure statistical power, the target recruitment has been increased to 61 participants. To date there has been 80% of patients recruited for the study and some baseline data has been evaluated.

8.15.1 Patient Characteristics

Baseline characteristics of women enrolled in DHA WIN are listed in **Table 8-4** and the clinical-pathological characteristics of the study population are listed in **Table 8-5** with the data stratified by body mass index (BMI). Overall, women entering the study had an average age of 52 years with a BMI=28.5±1.0. Obese women trended towards significantly higher C reactive protein (CRP) levels compared to healthy weight women (4.6±1.8 vs. 2.0±1.2, P=0.15). Eighty-four percent of the women identified as Caucasian and 51% are post-menopausal. Twenty-two women presented with a HER2+ histological subtype (ER±PR±HER2+), 15 were TNBC (ER-PR-HER2-), 9 women were luminal A (ER+PR±HER2-), and 3 were luminal B (ER+PR±HER2±). Women were stage IIA (n=12), IIB (n=16), IIIA (n=16) or IIIB (n=2). Three women were at enrolled at an unknown stage.

8.15.2 Composition of Fatty Acids in Plasma and Red Blood Cells (RBC) at Baseline

Baseline fatty acid composition of plasma membrane phospholipids and red blood cell total lipids were established for 49 women (**Table 8-6)**. There was 8.3±0.3% arachidonic acid and 2.3±0.1% DHA in the plasma phospholipids, similar to the RBC total lipid content of 8.5±0.3% arachidonic acid and 1.9±0.1% DHA. Spearman's rank correlation coefficient for DHA was calculated to confirm that plasma fatty acid composition is reflective of RBC total lipids and a strong correlation was observed (r=0.58, P<0.0001). When stratified by BMI, there were no differences in fatty acid status (data not shown).

Table 8-4: Baseline population information of DHA WIN Participants stratified by BMI (n=49)

	Healthy (n=15)	Overweight (n=17)	Obese (n=17)
Age (years)	50±3	54±2	52±3
Weight (kg)	56±2	72±1	96±4
BMI[1] (kg/m^2)	22±0	27±0	36±1
CRP at screening (mg/ml)	2±1	3±1	5±2
Ethnicity			
Indigenous	0	0	4
Asian	4	1	2
Black	1	0	1
Caucasian	10	17	14
Diabetes		1	3
Smoking status			
Never	12	13	12
Current	3	4	4
Number of Cigarettes per day for smokers	9±1	8±3	10±4
Use of Recreational Drugs (CBD or THC oils and gummies)	1	1	2
Menopausal Status			
Pre-menopausal	8	6	10
Post-menopausal	7	11	7
Age at Menopause (years)	49±2	51±1	55±2
Age at Menarche (years)	14±0	12±0	12±0
Unknown	2	1	2

[1]BMI status: Healthy 18 to <25 kg/m^2, Overweight 25 to <30 kg/m^2, Obese 30 or higher kg/m^2
Abbreviations used: body mass index, BMI; C reactive protein, CRP.

Table 8-5: Clinical pathological characteristics of DHA WIN Participants stratified by BMI (n=49)

	Healthy (n=15)	Overweight (n=17)	Obese (n=17)
Tumour grade			
1	0	1 (6%)	0
2	13 (87%)	8 (47%)	9 (53%)
3	2 (14%)	7 (41%)	4 (24%)
4	0	1 (6%)	1 (6%)
Unknown	0	0	3(18%)
Nodal Status			
0	3 (20%)	6 (35%)	3 (18%)
1	10 (67%)	7 (41%)	9 (53%)
2	1 (7%)	2 (12%)	2 (12%)
3	1 (7%)	0	0
Unknown	0	2 (12%)	3 (18%)
Disease Stage			
IIA	3 (20%)	6 (35%)	3 (18%)
IIB	8 (53%)	2 (12%)	6 (35%)
IIIA	4 (27%)	7 (41%)	5 (29%)
IIIB	0	1 (6%)	1 (6%)
Unknown	0	1 (6%)	2 (12%)
ER Status			
Positive	10 (67%)	7 (41%)	11 (65%)
Negative	5 (33%)	10 (59%)	6 (35%)
PR Status			
Positive	6 (40%)	5 (30%)	6 (35%)
Negative	9 (60%)	12 (70%)	11 (65%)
HER2 Status			
Positive	5 (33%)	10 (59%)	7 (41%)
Negative	10 (67%)	7 (41%)	10 (59%)
Histological Subtype			
Luminal A	5 (33%)	1 (6%)	3 (18%)
Luminal B	0	0	3 (18%)
HER2+	5 (33%)	10 (59%)	7 (41%)
TNBC	5 (33%)	6 (35%)	4 (24%)
ECOG Status			
0	15 (100%)	14 (82%)	13 (76%)
1	0	2 (12%)	3 (19%)

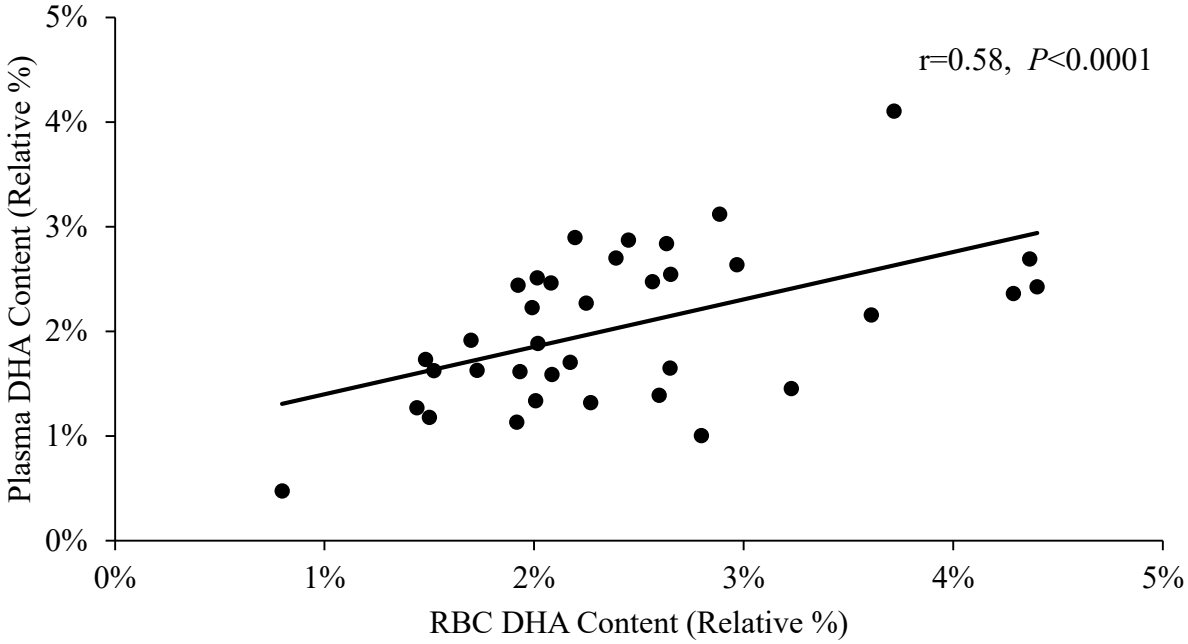
Abbreviations used: Estrogen receptor, ER; progesterone receptor; PR, human epidermal growth factor receptor 2; HER2; triple negative breast cancer; TNBC, Eastern Cooperative Oncology Group; ECOG.

Table 8-6: Baseline plasma phospholipid and red blood cell (RBC) total lipid fatty acid composition (relative percent of total fatty acids) among DHA WIN breast cancer patients (n=49)

Fatty Acid	Plasma PL	RBC total lipids
	% of total fatty acids	
14:0	0.3±0.0	0.8±0.1
16:0	31.2±0.3	26.6±0.5
16:1 n-9	0.5±0.0	0.3±0.0
17:0	0.5±0.0	0.5±0.0
18:0	16.0±0.2	16.9±0.6
18:1 n-9	1.2±0.1	0.5±0.0
18:1 n-9	9.7±0.2	16.8±0.6
18:1 n-9	1.5±0.0	1.3±0.1
18:2 n-6	18.4±0.4	8.4±0.2
20:0	0.5±0.1	0.3±0.0
18:3 n-6	0.3±0.0	0.5±0.0
18:3 n-3	0.3±0.0	0.4±0.0
20:2 n-6	0.3±0.0	0.3±0.0
20:3 n-6	3.3±0.1	1.9±0.1
20:4 n-6	8.3±0.3	8.5±0.3
20:4 n-3	0.3±0.0	0.2±0.0
20:5 n-3	0.7±0.1	0.5±0.1
24:0	0.9±0.0	3.5±0.3
24:1 n-9	1.5±0.1	5.0±0.1
22:4 n-6	0.8±0.1	2.1±0.1
22:5 n-6	0.2±0.0	0.6±0.0
22:5 n-3	0.7±0.0	1.3±0.1
22:6 n-3	**2.3±0.1**	**1.9±0.1**
Σ SFA	49.6±0.4	48.7±1.0
Σ PUFA	36.0±0.5	26.6±0.5
Σ MUFA	14.4±0.2	23.9±0.6
Σ N-6	31.6±0.5	22.3±0.4
Σ N-3	4.4±0.2	4.3 ±0.2

Abbreviations used: Abbreviations used: saturated fatty acids, SFA; monounsaturated fatty acids, MUFA; polyunsaturated fatty acids, PUFA.

Figure 8-3. Correlation between Plasma phospholipid and RBC DHA content



8.15.3 Complete blood count (CBC) and differential and immune cell phenotypes

All women had CBC counts within the normal range at baseline. (**Table 8-7**). The use of five-colour flow cytometry allowed for identification of surface molecules and immune cell phenotyping: CD3/CD25/CD8/CD4, CD3/CD16/CD56, CD3/CD152/CD28/CD86, CD3/CD196/CD4/ CD183, CD45RO/CD45RA/CD8/CD4, CD20/CD25/HLADR/CD14/CD27, CD1a/CD11b/CD11c/CD1c/CD141, CD107a/CD8/CD279/CD95/CD103 and CD3/CD127/CD26/CD4.

Table 8-7: Baseline complete blood count (CBC) analysis of women with breast cancer in DHA WIN (n=49)

Parameter	Normal Range	Baseline
WBC ($x10^9$ /L)	3.5-10.5	7.01±0.23
RBC ($x10^{12}$ /L)	3.50-5.00	4.52±0.05
Lymphocytes ($x10^9$ /L)	0.8-3.3	1.85±0.10
Neutrophils ($x10^9$ /L)	2.0-7.5	4.52±0.19
Basophils ($x10^9$ /L)	0-0.1	0.02±0.01
Eosinophils ($x10^9$ /L)	0-0.5	0.14±0.02
Monocytes ($x10^9$ /L)	0.1-1.0	0.45±0.02

Only some preliminary baseline data is presented in **Table 8-8**. At baseline, the lymphocyte population of women enrolled in DHA WIN had 72.9±1.2% T cells (CD3+), 50.7±1.6% helper T cells (CD4+) and 24.7±1.2% cytotoxic T cells (CD8+). Additionally, 7.6±0.5% of the helper T cells expressed CD25 (IL-2 receptor) and 9.6±0.6 of cytotoxic T cells expressed CD103 (**Table 8-8)**.

Table 8-8: Immune cell phenotypes of women with breast cancer in DHA WIN (n=44)

Cell Phenotype	
	% of total lymphocytes
Total CD3+ (T cells)	72.9±1.2
CD4+ (Helper T cells)	50.7±1.6
CD8+ (Cytotoxic T cells)	24.7±1.2
CD4+/CD8+ Ratio	2.4±0.2
	% of CD4+ cells
CD25+	7.6±0.5
	% of CD8+ cells
CD103+	9.6±0.6
CD107a+	8.0±0.7
CD279 (PD-1)	10.2±0.8
	PBMC (% of total lymphocytes and monocytes)
Total CD20+ (B cells)	21.3±1.0
Total CD14 (monocytes)	12.3±0.7

8.16 Discussion

Luminal A is the most predominant histological subtype diagnosed in breast cancer, however HER2+ and TNBC patients are more often prescribed neoadjuvant chemotherapy (Harbeck et al. 2019) and as a result are the predominant participants enrolled in DHA WIN. The average age of 52 is consistent with what is observed in the broader Canadian population wherein 83% of women diagnosed with breast cancer are over 50 years of age (Statistics 2016).

Only two patients currently enrolled were below the age of 35, as breast cancer diagnoses below this age are rare (Harbeck et al. 2019). There is an even distribution of participants amongst healthy, overweight and obese BMI categories which is consistent with the Albertan population as previously reported in 1358 women with cancer in Alberta's Tomorrow Project (Barberio et al. 2019). Obese women present with a heightened inflammatory state, indicated through elevated levels of CRP (Allin et al. 2011), and while not significant, a trend was observed for higher CRP in obese women compared to women of healthy weight in the current study. Levels of CRP above 2 mg/ml are predictors of increased risk of cancer (Allin et al. 2011) and levels in this range were observed in all groups in the current study. Supplementation with DHA beneficially reduces systemic markers of inflammation (summarized in (Richard et al. 2016)) and we predict that women in the DHA arm of this study will have reduced levels of CRP or maintain their current levels compared to women in the placebo.

Women enrolled in DHA WIN had lower amounts of DHA in their plasma phospholipids and RBC total lipids compared to other studies of Canadian adults where the reported DHA content in plasma phospholipids ranged from 3-5% (Stark et al. 2016). However, these values were similar to the plasma DHA content assessed from a large cohort of 614 female participants in Alberta's Tomorrow Project (paper currently under review). Bougnoux et al. found that patients who incorporated higher levels of DHA into their plasma phospholipids had both longer time to progression and increased overall survival in a population of metastatic breast cancer patients who were prescribed 1.8 grams of DHA per day (Bougnoux et al. 2009). Therefore, it could be that regardless of the baseline status, how much DHA is incorporated throughout the trial in each patient from the DHA arm could be a predictor of overall outcomes. Additionally, the fatty acid content of plasma phospholipids will be assessed at the beginning of each cycle of

chemotherapy and will provide a confirmation of compliance (in addition to clinical trial nurses counting leftover capsules) throughout the study.

Chemotherapy reduces levels of circulating lymphocytes and modulates the phenotypes of these lymphocytes as they repopulate in breast cancer patients (Verma et al. 2016). This changed phenotype is typified by an increase in naïve B cells, decreased number of memory B cells, and a corresponding increase in memory T cells (Verma et al. 2016). Conversely, supplementation with omega-3 fatty acids has been shown to maintain CD4+ T cells throughout chemotherapy in head and neck cancer patients (Talvas et al. 2015) and in breast cancer patients prior to treatment (da Silva Paixão et al. 2017) but the effects of DHA supplementation on breast cancer patients during neoadjuvant chemotherapy are currently unknown. Additionally, compared to normal breast tissue, breast cancer tissue is observed to have an increased number of adaptive and innate immune infiltrates, identified by flow cytometry (Gil Del Alcazar et al. 2017). Obtaining breast cancer tissue for analysis was not practical for the current study. However, whole cell blood immunophenotyping has emerged as a method with high specificity that allows for assessment of immune function in cancer patients and requires a minimal sample of whole blood (Rühle et al. 2016). The proportion of white blood cells, neutrophils, lymphocytes and monocytes were all within normal reference ranges at baseline for DHA WIN participants. The extensive immune panel designed for this study will provide, for the first time, a comprehensive assessment of immune changes in breast cancer patients before neoadjuvant therapy and throughout their treatment. Mounting an effective anticancer immune response requires the combination of cytotoxic T cells (CD8+), helper T cells (CD4+), natural killer (NK) cells and dendritic cells (DC), while a suppression of the immune response sees the involvement of regulatory T cells (Treg) and M2 tumour associated macrophages (TAMs) (Luengo-Fernandez

et al. 2013). We predict that women in the DHA arm of the trial will have a modulated immune response observed through maintenance of the CD4+/CD8+ ratio, and decreased activation of Tregs. Immune suppressive PD-1 (CD279+) is expressed on activated T cells and other immune cells (Keir et al. 2008). It's ligand (PD-L1) has increased expression in breast tumour types that have a high proliferation rates (as measured by Ki67) (Janakiram et al. 2012) such as the HER2+ and TNBC subtypes (Harbeck et al. 2019) Upon binding to PD-L1, PD-1 reduces T cell activation and as such, the PD-L1/PD-1 pathway is important in the immune response in breast cancers (Janakiram et al. 2012). The DHA WIN population is comprised of 76% HER2+ and TNBC subtypes therefore we predict that supplementation with DHA will modulate this immune response in our population.

8.16.1 Conclusions

The DHA WIN trial is expected to complete recruitment in the spring of 2021. This trial will provide, for the first time, a comprehensive assessment of the role of DHA in breast cancer therapy. The ability of DHA to reduce proliferation (as measured by Ki67 status) and its' role in immune system modulation will be determined. The outcomes of this trial will provide valuable information to clinicians and could be beneficial to future cancer patients.

CHAPTER 9: Final Discussion

9.1 General objectives

The overall objectives of this thesis were to determine the efficacy of DHA in prevention and treatment of breast cancer through 1) determining the relationship between fatty acid status in plasma phospholipids and breast cancer risk; 2) establishing the efficacy and mechanisms for how pre-treatment of breast cancer cells with DHA improves the action of chemotherapy and 3) determining the efficacy of supplemental DHA provided with neoadjuvant chemotherapy in women with breast cancer.

9.2 Summary of Results

9.2.1 Objective 1: Relationship between fatty acid status and breast cancer risk

The first objective of this research was to determine the relationship between fatty acid status in plasma phospholipids and breast cancer risk in a nested-case control study of Canadian women. In Chapter 3, the fatty acid composition of plasma phospholipids from women with breast cancer (*n*=393) and age-matched controls (*n*=786) from Alberta's Tomorrow Project (ATP) and British Columbia Generations Project (BCGP) cohorts were quantified. Association between fatty acid content and breast cancer risk were evaluated. Differences in fatty acid status based on geographical location were observed as women in BCGP had a higher n-3 fatty acid status compared to ATP women. Overall, fatty acid status had inconsistent associations with risk. However, our findings suggested positive associations of total long chain n-3 fatty acids in premenopausal ATP women and negative associations of these fatty acids in BCGP women with a waist-to-hip ratio below guidelines. This chapter has been accepted for publication in Current Developments in Nutrition (Newell et al. 2021).

9.2.2 Objective 2: Establish the efficacy and mechanisms for how DHA improves the action of chemotherapy.

a) *The first sub-objective of objective 2 was to establish DHA incorporation into two phenotypically distinct immortalized breast cancer cell lines: MDA-MB-231 and MCF-7 cells, when provided prior to doxorubicin (DOX) chemotherapy in an in vitro model. We hypothesized that DHA would improve efficacy of DOX through the DHA incorporation into membrane phospholipids and lipid rafts and that this would occur in both cell lines. We further hypothesized that in vivo incorporation of DHA into MDA-MB-231 tumours would be representative of results observed in vitro.*

This hypothesis was partly supported by results presented in Chapter 4. Interestingly, regardless of DOX, the relative percent incorporation of DHA was higher in MDA-MB-231 cells compared to MCF-7 cells in phosphatidylserine, phosphatidylethanolamine, and phosphatidylcholine (both in whole cell and lipid rafts). Furthermore, DHA treatment increased EPA content in MDA-MB-231 cells but not MCF-7 cells. In mice bearing MDA-MB-231 tumours, feeding DHA resulted in increased DHA in all phospholipid moieties except sphingomyelin and increased tumour necrotic regions, confirming the translatability of our *in vitro* work. This chapter has been published in Lipids (Newell et al. 2020).

b) *The second sub-objective of objective 2 was to establish the efficacy and mechanisms for how pre-treatment with DHA improves the action of doxorubicin chemotherapy in vitro MDA-MB-231 cells. We hypothesized that treating MDA-MB-231 cells with DHA prior to DOX would enhance the anti-cancer actions of DOX through an increase in apoptosis and cell cycle arrest.*

This hypothesis was supported by results presented in Chapter 5. Microarray analysis indicated that MDA-MB-231 cells treated with DHA in conjunction with DOX, compared with cells treated with oleic and linoleic acid as a control and treated with DOX, had upregulated expression of apoptosis genes and downregulated expression of cell cycle genes. Gene expression was confirmed by analysis of protein expression. This data is contained in a published manuscript (Newell et al. 2019).

c) *The third sub-objective of objective 2 was to confirm that feeding DHA improves the action of doxorubicin chemotherapy in in vivo nu/nu mice implanted with MDA-MB-231 cells. We hypothesized that feeding a diet enriched with DHA to tumour bearing mice would reduce tumour growth and that this would occur through increased apoptosis and decreased cell cycle progression.*

This hypothesis was supported by results presented in Chapter 5. DHA DOX–treated mice had tumours that were 50% smaller than control mice. Analysis of tumours from DHA DOX mice showed increased pro-apoptotic proteins and decreased cell cycle proteins compared with control mice. These results in conjunction with the results from the previous sub-objective suggest that DHA supplementation facilitates the action of DOX through amplification of the effect on apoptosis and cell cycle genes. This data has been published in Journal of Nutrition (Newell et al. 2019).

d) *The fourth sub-objective of objective 2 was to examine how feeding DHA in a patient derived xenograft (PDX) model of triple negative breast cancer (TNBC) improves the efficacy of docetaxel (TXT) chemotherapy. We hypothesized that feeding a diet enriched with DHA to PDX tumour bearing mice would reduce tumour growth and that this would occur through increased apoptosis, decreased cell cycle progression and decreased proliferation.*

This hypothesis was supported by results presented in Chapter 6. In two different TNBC PDX models we observed a reduction of tumour growth in mice fed a DHA diet and treated with TXT compared to mice fed a control diet with TXT. Tumours from DHA fed mice had increased expression of pro-apoptotic proteins, decreased cell cycle progression proteins and decreased cellular proliferation. This chapter has been published in Breast Cancer Research and Treatment (Newell et al. 2019).

e) *The fifth sub-objective of objective 2 was to examine how different doses and sources of dietary DHA improve the efficacy of TXT chemotherapy in a PDX model of TNBC. We hypothesized that a dose effect would be observed, resulting in different levels of anti-tumour response in PDX tumour bearing mice and that this would occur through changes in tumour phospholipid DHA content.*

This hypothesis was partly supported by results presented in Chapter 7. Tumours from mice fed either a high dose of DHA+TXT or a low dose DHA+TXT were similar in size to each other, but were smaller than tumours from mice control fed +TXT. A dose effect of DHA incorporation, related to dietary intake, was observed in plasma total phospholipids and in phosphatidylethanolamine and phosphatidylinositol. Both doses of DHA resulted in similarly increased necrotic tissue and decreased NFκB protein expression compared to control tumours, however only the high dose DHA+TXT had higher expression of necroptosis related proteins and changed expression of necroptosis related proteins in the lipid raft portion of tumour extracts. This chapter has been submitted for publication and is currently under review.

9.2.3 Objective 3: To determine efficacy of supplemental DHA provided with neoadjuvant chemotherapy in women with breast cancer.

We hypothesized that supplementing DHA during chemotherapy would decrease tumour proliferation (Ki67) and improve patient outcomes as measured by improved immune response and a decrease in chemotherapy associated side-effects and progressive disease. This objective has not been completed for this thesis submission however considerable progress has been made and the hypotheses remain. The trial was designed and received full ethical approval from the Health Research Ethics Board of Alberta – Cancer Committee (Protocol #: HREBA.CC- 18-0381) and Health Canada approval (#HC6-24-c220167), and began in Sept 2019. The trial protocol was published in the British Medical Journal Open (Newell et al. 2019). The results presented in Chapter 8 detail the baseline status of 49 women currently enrolled in the trial. Baseline assessment of these women found the population was evenly distributed between pre and post-menopausal, had slightly elevated CRP and a DHA fatty acid content similar to the status of Albertan women described in Chapter 3 of this thesis. The recruitment for this trial is expected to be completed in spring of 2021 with final outcomes to be disseminated by late fall 2021.

9.3 General discussion and future directions

9.3.1 The role of DHA in breast cancer prevention

Studies have consistently associated high fish intake with reduced incidence of breast cancer (Haraldsdottir et al. 2017, Gago-Dominguez et al. 2003), yet to date the association between the amount of DHA in blood components (RBC, plasma or serum) and risk of breast cancer is unclear. A decreased risk of breast cancer with higher fatty acid content of DHA (Maillard et al. 2002), EPA (Shannon et al. 2007, Witt et al. 2009) or total n-3 (Simonsen et al. 1998) in blood components (serum, plasma or erythrocytes) has been observed, however several other studies did not observe mitigation of risk with higher DHA content in blood components

(Vatten et al. 1993, Simonsen et al. 1998, Chajes et al. 1999, Klein et al. 2000, Pala et al. 2001, Bagga et al. 2002, Saadatian-Elahi et al. 2002, Maillard et al. 2002, Rissanen et al. 2003, Wirfalt et al. 2004, Shannon et al. 2007, Chajes et al. 2008, Witt et al. 2009, Takata et al. 2009, Schmidt et al. 2014, Qin et al. 2014, Hidaka et al. 2015, Conceicao et al. 2016, Bassett et al. 2016, Nagata et al. 2017, Chajes et al. 2017, Hirko et al. 2018). Differences in dietary intake or dietary patterns based on geographic location and resultant differences in fatty acid status, play a role in the inconsistent conclusions from these studies (Dandamudi et al. 2018, Brennan et al. 2010, Xiao et al. 2019). Our study assessed for the first time the associations between breast cancer risk and fatty acid status in a Canadian population and our results are consistent with the discrepant reporting in the literature. While our research suggested that regional variations in fatty acid status influenced breast cancer risk, we expanded on this evidence, by assessing two key metrics that have been identified as influencers of breast cancer risk: menopausal status and body composition (World Cancer Research Fund 2018). In premenopausal Albertan women, higher saturated fatty acid content conferred a protective effect contrary to previous studies that suggested an increased risk (Saadatian-Elahi et al. 2002, Bassett et al. 2016, Hirko et al. 2018). Additionally, positive associations with breast cancer of total n-3 LCPUFA in premenopausal Albertan women who had lower n-3 LCPUFA content (specifically EPA and DHA) in their blood. This was contrary to observations in British Columbian women where negative associations of these fatty acids were observed in women with a waist-to-hip ratio below guidelines. This study reinforces the difficulty in using a single measure of fatty acid status to predict breast cancer risk in different groups, yet it does not eliminate the possibility that DHA could play a role in breast cancer prevention. It could be that the intake in the Canadian population is too low to confer a protective effect. Future longitudinal studies should include

multiple measures of both dietary intake and plasma in the collected data to more accurately determine how fatty acid status reflects dietary intake. There have been no other studies examining associations between risk of breast cancer and fatty acid status in Canada. A future expansion of the current study to the larger country wide prospective cohort (Canadian Partnership for Tomorrow's Health), could help delineate fatty acid relationships that are unclear in this cohort that contained only 351 breast cancer patients and provide an assessment of regional variation across Canada and how these differences might influence the risk of breast cancer. However, considering that globally there is a current lack of clear evidence in studies thus far, it is possible that even a larger cohort would not support the hypothesis of a preventative benefit of DHA or n-3 LCPUFA. Future researchers could instead pursue a systems approach to a cohort study wherein bioinformatics and high-throughput technology (DNA sequencing) could be employed to create an unbiased assessment of multiple parameters (Hartwell et al. 2006) within these populations and how n-3 fatty acid status is related.

9.3.2 Mechanisms of DHA action

In a series of *in vitro* and *in vivo* experiments, we have increased the evidence supporting the multifaceted actions of DHA in combination with chemotherapy. First, in two different breast cancer cell lines, one representative of Luminal A and one of TNBC, we confirmed that incorporation of DHA is not altered by chemotherapy. Modulation of lipid membranes by DHA intake occurred as it was incorporated into phosphatidylcholine, phosphatidylinositol, phosphatidylserine and phosphatidylethanolamine in both whole cell and lipid raft membranes. There was differential incorporation between the two cell lines which could account for the differential effects of DHA (and other n-3 LCPUFA including EPA) observed in other cancer types and cell lines (reviewed by (D'Eliseo and Velotti 2016)). TNBC accounts for 10-15% of all

diagnoses, yet it is more aggressive and has less favourable responses to current treatments (Harbeck et al. 2019). While this thesis has sought to better describe the mechanisms of action of DHA in TNBC, future researchers should investigate the mechanisms involved with DHA action in other breast cancer subtypes. Indeed, there have been over 100 different breast cancer cell lines established, yet only a small number account for more than two-thirds of published studies (Lacroix et al. 2004) (including MCF-7 and MDA-MB-231 that we have reported on in the current thesis). Furthermore, breast cancer subtypes differ in the degrees to which their *in vitro* cell line is translatable to their respective tumour type (Vincent et al. 2015). To properly account for the heterogeneity of breast cancer and how DHA affects cellular mechanisms, future studies will need to incorporate a more diverse range of cell lines into their *in vitro* experimental plans. This includes not only investigating all the histological subtypes (and potentially multiple cell types within each subtype), but also an assessment of emerging drug resistant models. How DHA could improve the actions of chemotherapy in a breast cancer model that is resistant to chemotherapy is unknown yet of major clinical importance. Furthermore, while pursuing these questions in an *in vitro* model, future researchers should consider employing a three-dimensional (3D) model that more accurately reflects a tumour microenvironment (Bissell et al. 2002, Vidi et al. 2013). There have been no studies published to date assessing the role of DHA in a 3D model, yet this could provide a means to explore multiple cell lines, doses and combinations of fatty acids (discussed below) in a more clinically relevant *in vitro* model (Vinci et al. 2012).

We have described how DHA, when employed in combination with chemotherapy, modulated gene expression related to apoptosis, cell death, cell proliferation, cell cycle progression and affected expression of protein in these pathways in a TNBC model. It is clear that the actions of DHA are multifaceted, and future studies should build on the current evidence

to explore other mechanisms of action. This includes the role of DHA in epigenetics. Epigenetic changes, such as modulating transcription through DNA methylation, are known to result in initiation and progression of breast cancer (Lacroix et al. 2004). While the ability of DHA to induce epigenetic modifications has been explored in hematological (Moloudizargari et al. 2018) and colorectal cancers (Moradi Sarabi et al. 2019, Triff et al. 2015), there has not been a link established in breast cancer.

In this thesis our models focused on DHA as the n-3 LCPUFA provided as the supplementation and while we have established a broad range of anticancer actions for DHA, we have not assessed the role of other n-3 LCPUFAs: EPA, DPA or SDA. To that end, many questions remain and future researchers should consider the following questions when designing their studies: is there an optimal combination of EPA, DPA, SDA and / or DHA? Furthermore, does the combination of EPA, DPA, SDA and / or DHA depend on the histological subtype of breast cancer? For example, could one component be more efficacious against a more aggressive subtype? Indeed, we have shown that DHA had differential incorporation into whole cell and lipid raft membranes of luminal A and TNBC cell lines, therefore it is plausible that combinations of these fatty acids would have differential effects. A comprehensive assessment of how these fatty acids could be used in combination would be beneficial in establishing future guidelines for use as an adjuvant to therapy.

Our *in vivo* experiments confirmed the mechanisms elucidated in the *in vitro* studies. We first assessed changes including decreased tumour size, increased necrosis, apoptosis, and decreased proliferation in the well-established immortalized tumour model. We then described for the first time, mechanisms of DHA action in a PDX model of breast cancer. The PDX model is a ‘gold-standard’ of preclinical model, with high heterogeneity that establishes a bridge

between previous work and future clinical work. To confirm the effects of DHA that resulted in increased apoptosis, necroptosis, decreased cell proliferation and modulation of lipid rafts, we employed 2 different models of TNBC. Researchers should consider expanding on this PDX work to include investigating HER2+ breast cancer. There is a poor prognosis for HER2+ breast cancer patients if targeted Trastuzamab (Herceptin) therapy is unsuccessful (Chung et al. 2013), yet currently there is limited evidence of the effects of DHA on Her2+ cells *in vitro* (Altenburg et al. 2011, Sun et al. 2011, Rescigno et al. 2016). Employing a HER2+ PDX model with DHA dietary supplementation would clarify the current *in vitro* evidence and provide rationale to explore DHA supplementation in HER2+ breast cancer patients in clinic. Additionally, an important next step for researchers would be to expand on the PDX model presented in this thesis, where PDXs were implanted subcutaneously, and assess DHA dietary supplementation when PDXs are implanted orthotopically. An orthotopic implantation into mammary tissue is a better representation of a true tumour microenvironment and therefore researchers could more accurately investigate metastatic events (Lwin et al. 2018). There is an increasing body of evidence that supports the role of DHA and inhibition of metastases (reviewed by (Merendino et al. 2013)), yet much of this research has been done in immortalized cell lines that can not accurately recapitulate metastases in a heterogenic model.

9.3.3 DHA supplementation in clinic

This thesis work has not yet confirmed the role of DHA supplementation in a neoadjuvant setting for breast cancer patients, yet the upcoming results of the DHA WIN trial will provide evidence for new recommendations. Therefore, while we have convincing pre-clinical data, we are not yet able to comment on the efficacy of DHA in the ongoing DHA WIN neoadjuvant trial,

there are considerations to propose with respect to design of future studies that could increase our understanding of DHA as a concurrent therapy in clinic.

First, building on the hypothesis that while this thesis has explored the role of DHA, there is a plausible role for EPA or the combination of EPA and DHA and a future trial should strive to determine the role of EPA in the neoadjuvant setting. There is limited data on breast cancer clinical trials involving n-3 LCPUFA. Indeed, there have only been four trials that have supplemented with n-3 LCPUFA in combination with chemotherapy treatment (Bougnoux et al. 2009, Ghoreishi et al. 2012, Darwito et al. 2019, Mansara et al. 2015). Of these, Bougnoux et al. employed DHA alone and the other three used varied combinations of EPA and DHA. It is well established that EPA and DHA have similar anti-tumour activity but this appears to be medicated through unique mechanisms of action in cancer and other chronic diseases (Gorjão et al. 2009, Serini et al. 2011, VanderSluis et al. 2017, Dyall 2015, Asztalos et al. 2016) and may affect the response to cytotoxic drugs differently (Ewaschuk et al. 2012). In an antineoplastic setting, while EPA more strongly inhibits arachidonic-derived prostaglandin production, DHA, among other mechanisms of action, is known to modulate membrane lipid rafts, increase production of oxidative products and beneficially bind / activate nuclear receptors to a greater extent than EPA (Serini et al. 2011). Therefore, future consideration should be given to determining the optimal combination of fatty acids prescribed to increase efficacy in clinic, both to reduce tumour growth and improve the efficacy of chemotherapies used.

This thesis has detailed several mechanisms of action for DHA, including apoptosis, necroptosis, cellular proliferation, and cell cycle progression. However, the scope of the current DHA WIN trial does not include a comprehensive assessment of the mechanisms of DHA action. A logical step for future researchers would be to integrate the preclinical evidence into

assessment in clinic. It is difficult to obtain fresh tumour tissue during a clinical trial, however many of these hallmarks of cancer (Hanahan et al. 2000, Hanahan et al. 2011) can be investigated through immunohistochemical analysis of preserved tissue. Furthermore, well established protocols would enable researchers to extract DNA from paraffin embedded tissue (Pikor et al. 2011) to detail genetic and epigenetic changes that have occurred with DHA treatment concomitant with chemotherapy.

It is difficult to properly assess certain hallmarks of cancer in a preclinical setting. The DHA WIN trial will offer a comprehensive analysis of immune function, however, induction of angiogenesis and activation of metastases are two hallmarks (Hanahan et al. 2000, Hanahan et al. 2011) that should be assessed in a future clinical setting. There is preclinical evidence from other groups that suggest efficacy of n-3 LCPUFA supplementation on key markers of angiogenesis, vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs), in breast (Blanckaert et al. 2010, Mandal et al. 2010), colorectal (Zou et al. 2015) and ovarian cancers (Wang et al. 2016), as well as limited evidence from two clinical trials. Darwito et al. reported a decrease in VEGF in tumour samples of breast cancer patients supplemented with 1 gram of n-3 throughout chemotherapy (Darwito et al. 2019) while Khojastehfard et al. reported decreased MMP-1 and MMP-9 expression in gastric cancer patients supplemented with 3.6 grams of a n-3 (amount of DHA not specified), n-6 and n-9 combination (Khojastehfard et al. 2019). A future trial with more clearly defined amounts of EPA and or DHA would help further delineate the role of DHA in reducing the induction of angiogenesis. The epithelial-mesenchymal transition (EMT) is an important process that occurs during breast cancer cell invasion / metastasis (Fedele et al. 2017). Currently, limited preclinical evidence supports the role for DHA in EMT inhibition in prostate cells (Bianchini et al. 2012), and reduction in EMT related proteins including

Gremlin-1 expression in breast cancer cells (Sung et al. 2020) and Twist suppression in cholangiocarcinoma cells (Lin et al. 2019). However, movement through the EMT transition is complex sequence of events, requiring the coordination of many proteins in addition to those mentioned here. Building on this current evidence through assessment of these markers in clinic could be an interesting direction for future studies.

9.4 Conclusions

There are limitations to my thesis work. As detailed in future directions, this thesis solely explored the role of DHA in the prevention and treatment of breast cancer. While the rationale in using one fatty acid was based on previous work, we can not conclude that DHA should be the only n-3 LCPUFA used in the treatment of breast cancer. Additionally, this thesis did not explore optimal doses or ratios and this could limit the application of our results. Future researchers should consider these two key factors (dose and type of fatty acid) in experimental design. Experimental limitations include 1) the use of a single chemotherapeutic agent whereas women in clinic would receive a combination of chemotherapy agents during their treatment, 2) the use of an immunocompromised model that is therefore not reflective of humans and 3) the use of subcutaneous versus orthotopic implanted tumours as described above. Additionally, while this thesis assessed a variety of mechanisms, it will be important to explore additional cellular mechanisms and pathways, including hypoxia, PI3K and Wnt/β-catenin signalling to further improve our understanding of how DHA targets breast cancer. Finally, this thesis primarily focused on TNBC. Breast cancer exists as a heterogeneous disease and confining this work to one subtype that accounts for 10-15% of all diagnoses limits the translatability of this body of work.

However, overall the current thesis has increased our understanding of the role of DHA as an adjuvant to breast cancer therapy. The strength of this body of work is the comprehensive assessment of the role of DHA from *in vitro*, to two different models of *in vivo* and finally a clinical trial. We have presented evidence of using oral DHA in reducing tumoural growth and cell cycle progression, in combination with increasing cell death (via apoptosis and necroptosis), in multiple *in vitro* and *in vivo* models of breast cancer. Future integration of additional parameters as suggested in the above discussion would increase our understanding of the anticancer functions of this unique fatty acid and could ultimately improve outcomes for breast cancer patients.

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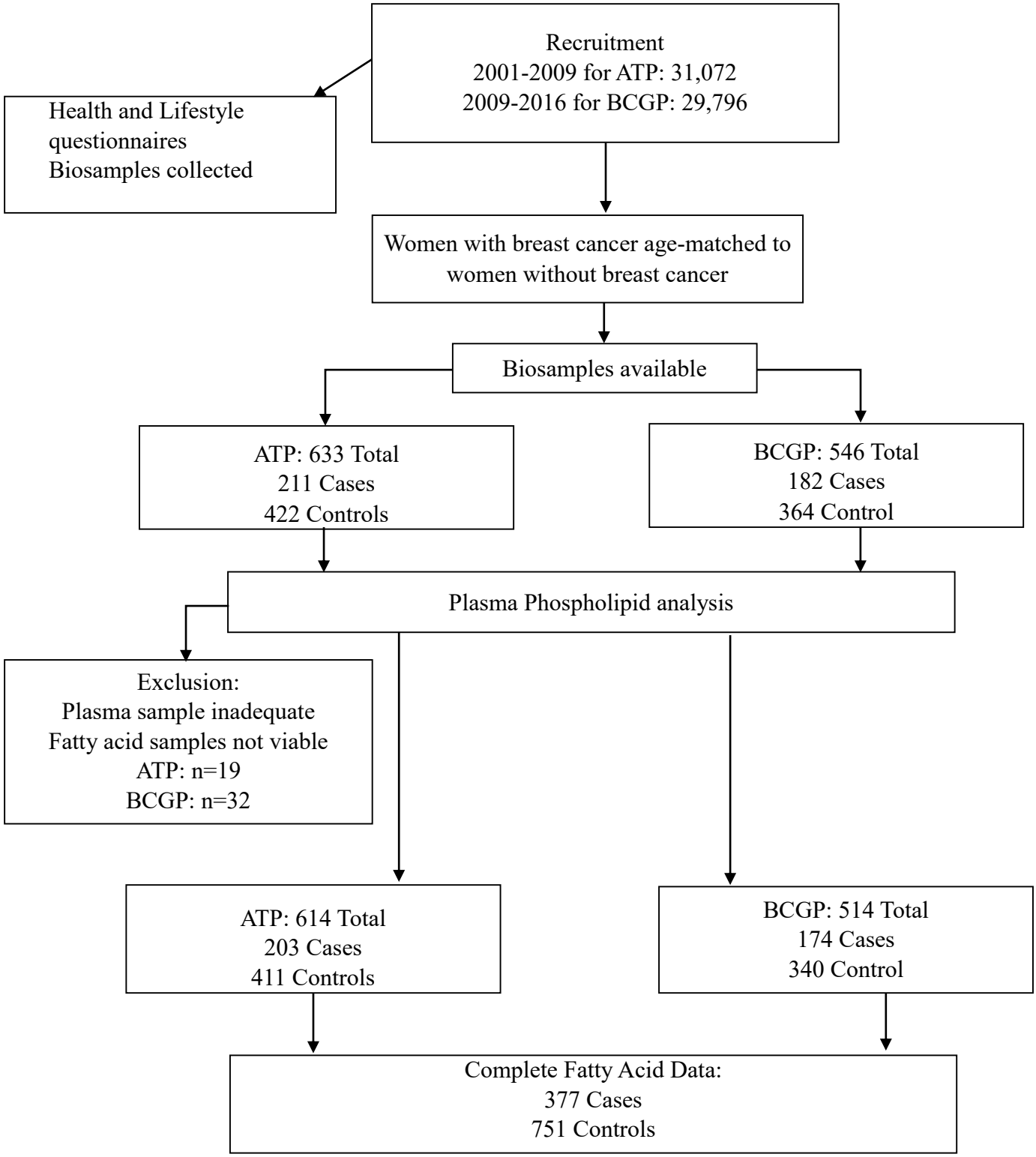
Appendix Figure 1: Flow diagram of final sample selection for fatty acid analysis from ATP and BCGP



Appendix Table 1: Breast Cancer subsets among Alberta's Tomorrow Project and British Columbia Generations Project participants (n=377)

Neoplasm	Cases	% total	Mean age at diagnosis
Carcinoma, NOS, Epithelial tumour, malignant	3	0.80	48.3±7.3
Spindle cell carcinoma, NOS	1	0.26	67
Tubular adenocarcinoma	6	1.59	62±3.6
Apocrine adenocarcinoma	5	1.32	68±2.8
Mucinous adenocarcinoma	3	0.79	63.33±9.4
Infiltrating duct carcinoma, NOS	284	75.3	60.9±0.5
Intraductal micropapillary carcinoma	3	0.80	57±2.3
Lobular carcinoma	26	6.90	60.8±1.5
Infiltrating duct and lobular carcinoma	20	5.30	60.6±2.0
Infiltrating duct mixed with other types of carcinoma	20	5.30	62.6±1.8
Paget disease and infiltrating duct carcinoma of breast	1	0.26	53
Paget disease and intraductal carcinoma of breast	1	0.26	65
Metaplastic carcinoma, NOS	3	0.80	50.7±2.0
Adenomyoepithelioma	1	0.26	62

NOS, not otherwise specified; Based on the International Classification of Diseases for Oncology

Appendix Table 2: Hormone Receptor Status of Breast Cancer cases in Alberta's Tomorrow Project and British Columbia Generations Project participants (n=377)

Hormone receptor status	Cases / total cases (%)
ER^+	323/377 (85.7)
ER^-	47/377 (12.5)
PR^+	272/377 (72.1)
PR^-	98/377 (26.0)
HER^+	60/377 (15.9)
HER^-	305/377 (9.3)

Appendix Table 3: Spearman's rank correlation coefficients between plasma fatty acid content and estimated dietary and supplement intake of n-3 from the FFQ

	Overall (n=256)
Between plasma fatty acid content (relative percent) and unadjusted fatty acid consumption (g/day)	
PUFA	0.048
Long chain n-3 PUFA	0.180*
EPA	0.125
DHA	0.228**
DPA	-0.079
EPA+DHA	0.204**
Between plasma fatty acid content (relative percent) and energy adjusted n-3 fatty acid consumption (g/1000 kJ)	
PUFA	0.065
Long chain n-3 PUFA	0.209**
EPA	0.175*
DHA	0.247***
DPA	-0.067
EPA+DHA	0.245***
Between plasma fatty acid content (relative percent) and fish consumption (oz/day)	
PUFA	0.053
Long chain n-3 PUFA	0.165*
EPA	0.130
DHA	0.189**
DPA	0.053
EPA+DHA	0.186**

*P<0.05, **P<0.01, ***P<0.001

Appendix Table 4: Plasma phospholipid fatty acid composition (relative percent, %) by menopausal status

Fatty acid	Entire Cohort			ATP			BCGP		
	Pre-menopausal	Post-menopausal	*P*-value	Pre-menopausal	Post-menopausal	*P*-value	Pre-menopausal	Post-menopausal	*P*-value
					Saturates				
Total SFA[1]	46.7±0.1	46.8±0.1	0.70	47.6±0.2	47.5±0.1	0.58	45.7±0.2	45.8±0.1	0.41
14:0 (Myristic acid)	0.4±0.0	0.3±0.0	0.49	0.4±0.0	0.3±0.0	0.06	0.4±0.0	0.4±0.0	0.18
16:0 (Palmitic acid)	29.2±0.1	28.8±0.1	**0.00**	29.7±0.2	29.1±0.1	**0.00**	28.6±0.2	28.4±0.1	0.36
17:0 (Margric acid)	0.4±0.0	0.4±0.0	0.79	0.4±0.0	0.4±0.0	0.64	0.4±0.0	0.4±0.0	0.39
18:0 (Stearic acid)	15.2±0.1	15.6±0.0	**0.00**	15.4±0.1	16.0±0.1	**0.00**	14.8±0.1	15.1±0.1	**0.02**
20:0 (Arachidic acid)	0.5±0.0	0.5±0.0	0.31	0.5±0.0	0.5±0.0	0.75	0.5±0.0	0.5±0.0	0.08
24:0 (Lignoceric acid)	1.1±0.0	1.1±0.0	0.60	1.1±0.0	1.1±0.0	0.89	1.0±0.0	1.1±0.0	0.37
					Monounsaturates				
Total MUFA[2]	13.9±0.1	13.8±0.0	0.20	14.1±0.1	13.8±0.1	0.07	13.8±0.1	13.8±0.1	0.99
16:1 n-7 (Palmitoleic acid)	0.7±0.0	0.7±0.0	0.14	0.7±0.0	0.7±0.0	0.53	0.7±0.0	0.8±0.0	0.09
18:1 c11 (Vaccenic acid)	0.2±0.0	0.2±0.0	**0.01**	0.2±0.0	0.2±0.0	0.07	0.2±0.0	0.2±0.0	0.10
18:1 n-9 (Oleic acid)	9.6±0.1	9.4±0.0	**0.02**	9.6±0.1	9.4±0.0	0.09	9.6±0.1	9.4±0.1	0.09
18:1 n-7 (Octadecenoic acid)	1.4±0.0	1.4±0.0	0.90	1.4±0.0	1.4±0.0	0.56	1.3±0.0	1.3±0.0	0.53
24:1 n-9 (Nervonic acid)	2.0±0.0	2.1±0.0	**0.04**	2.1±0.0	2.1±0.0	0.86	1.9±0.0	2.0±0.0	**0.00**
					Polyunsaturates				
Total PUFA[3]	38.8±0.2	38.8±0.1	0.72	37.8±0.2	38.1±0.1	0.11	39.9±0.2	39.7±0.1	0.45
Total n-6	33.3±0.2	32.9±0.1	**0.01**	32.8±0.2	32.6±0.1	0.43	33.8±0.2	33.1±0.1	**0.01**
Total long chain n-6[4]	13.2±0.1	13.8±0.1	**0.00**	13.1±0.1	13.8±0.0	**0.00**	13.3±0.2	13.9±0.1	**0.01**
18:2 n-6 (Linoleic acid)	20.0±0.2	19.0±0.1	**0.00**	19.6±0.2	18.8±0.1	**0.00**	20.4±0.3	19.2±0.1	**0.00**
18:3 n-6 (γ- Linolenic acid)	0.1±0.0	0.1±0.0	0.67	0.1±0.0	0.1±0.0	0.44	0.1±0.0	0.1±0.0	1.00
20:2 n-6 (Eicosadienoic acid)	0.3±0.0	0.3±0.0	**0.00**	0.3±0.0	0.3±0.0	**0.00**	0.3±0.0	0.3±0.0	**0.02**
20:3 n-6 (Dihomo-γ-Linolenic acid)	4.1±0.0	4.1±0.0	0.49	4.1±0.1	4.2±0.0	0.63	4.0±0.1	4.0±0.0	0.66
20:4 n-6 (ARA)	8.4±0.1	9.0±0.1	**0.00**	8.3±0.1	8.9±0.1	**0.00**	8.6±0.1	9.1±0.1	**0.00**
22:4 n-6 (Adrenic acid)	0.3±0.0	0.2±0.0	**0.01**	0.2±0.0	0.2±0.0	0.77	0.3±0.0	0.3±0.0	**0.01**
22:5 n-6 (Osbond acid)	0.2±0.0	0.2±0.0	0.49	0.2±0.0	0.2±0.0	0.60	0.2±0.0	0.2±0.0	0.54
Total n-3	5.5±0.1	6.0±0.1	**0.00**	5.0±0.1	5.5±0.1	**0.00**	6.1±0.2	6.6±0.1	**0.00**
Total long chain n-3[5]	5.1±0.1	5.6±0.1	**0.00**	4.6±0.1	5.1±0.0	**0.00**	5.6±0.2	6.2±0.1	**0.00**

18:3 n-3 (α-Linolenic acid)	0.4±0.0	0.4±0.0	0.95	0.4±0.0	0.4±0.0	0.08	0.4±0.0	0.4±0.0	0.16
20:4 n-3 (Eicosatetraenoic acid)	0.6±0.0	0.6±0.0	**0.06**	0.6±0.0	0.6±0.0	0.80	0.6±0.0	0.6±0.0	**0.02**
20:5 n-3 (EPA)	1.0±0.0	1.2±0.0	**0.00**	0.9±0.0	1.1±0.0	**0.00**	1.2±0.1	1.4±0.0	**0.01**
22:5 n-3 (DPA)	0.7±0.0	0.8±0.0	**0.00**	0.7±0.0	0.8±0.0	**0.01**	0.8±0.0	0.8±0.0	**0.00**
22:6 n-3 (DHA)	2.7±0.1	2.9±0.0	**0.00**	2.4±0.1	2.6±0.0	**0.01**	3.1±0.1	3.3±0.0	0.09
Ratios									
Total n-6:Total n-3	6.6±0.1	5.9±0.1	**0.00**	7.0±0.1	6.4±0.1	**0.00**	6.1±0.2	5.4±0.1	**0.00**
ARA:DHA	3.5±0.1	3.4±0.0	0.35	3.9±0.1	3.7±0.1	0.17	3.1±0.1	3.0±0.1	0.91
ARA:EPA+DHA	2.6±0.0	2.4±0.0	0.06	2.8±0.1	2.6±0.0	0.07	2.3±0.1	2.2±0.0	0.26
DI_{16}	0.0±0.0	0.0±0.0	**0.02**	0.0±0.0	0.0±0.0	0.15	0.0±0.0	0.0±0.0	**0.03**
DI_{18}	0.6±0.0	0.6±0.0	**0.00**	0.6±0.0	0.6±0.0	**0.00**	0.7±0.0	0.6±0.0	**0.01**

[1]SFA= 14:0, 16:0, 17:0, 18:0, 20:0, 24:0.

[2]MUFA= 16:1 n-7, 18:1 c11, 18:1 n-9, 18:1 n-7, 24:1 n-9.

[3]PUFA= 18:2 n-6, 18:3n-6, 20:2 n-6, 20:3 n-6, 20:4 n-6, 22:4 n-6, 22:5 n-6, 18:3 n-3, 20:4 n-3, 20:5 n-3, 22:5 n-3, 22:6 n-3.

[4]Total long chain n-6 =20:2 n-6, 20:3 n-6, 20:4 n-6, 22:4 n-6, 22:5 n-6.

[5]Total long chain n-3 = 20:4 n-3, 20:5 n-3, 22:5 n-3, 22:6 n-3.

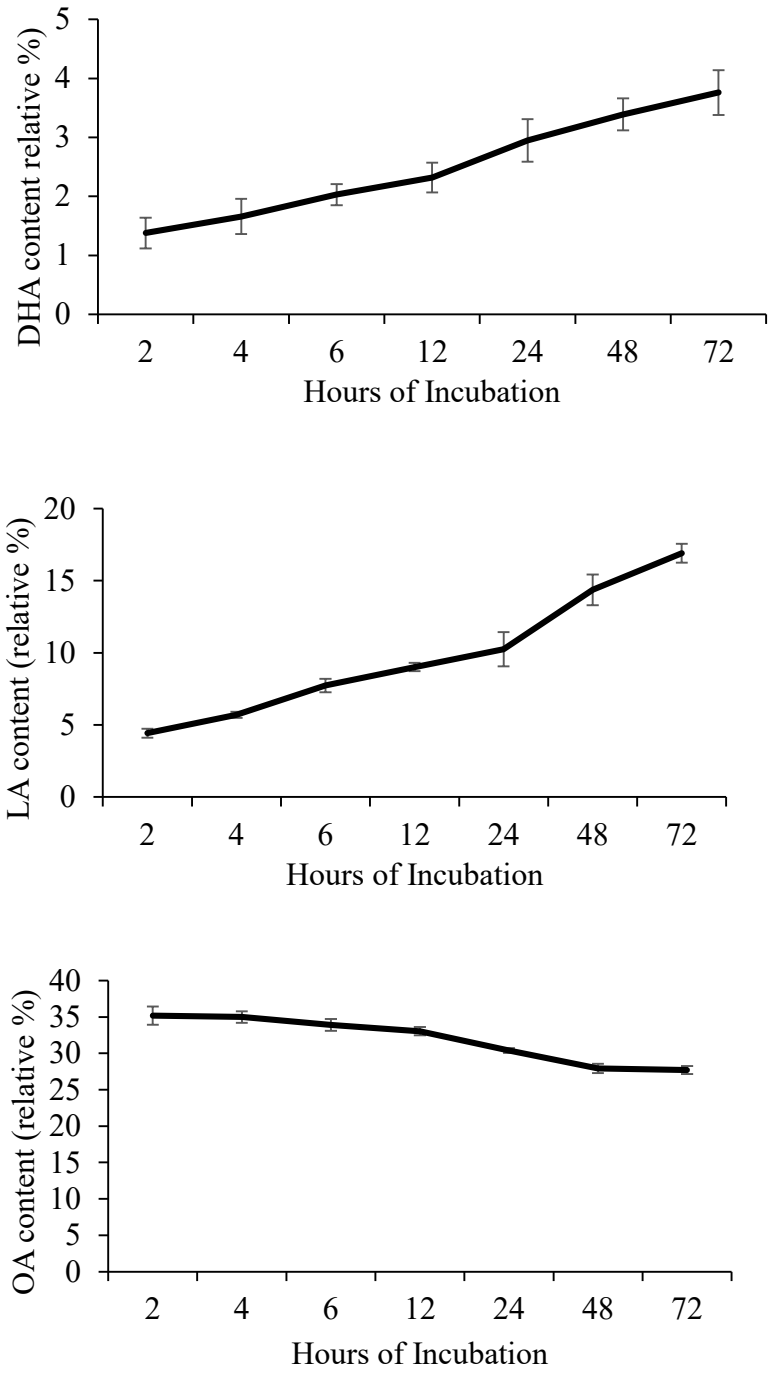
Abbreviations: ARA, arachidonic acid; ATP, Alberta's Tomorrow Project; BCGP, British Columbia Generations Project; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; DI_{16}, desaturation index of 16:1:16:0; DI_{18}, desaturation index of 18:1:18:0.

Appendix Table 5: Whole Cell Total Phospholipid Composition (relative %) of MDA-MB-231 breast cancer cells incubated with 60 μM DHA for up to 72 hours

Fatty Acid	2 hours	4 hours	6 hours	12 hours	24 hours	48 hours	72 hours
14:0	1.51±0.03^{a}	1.34±0.07^{b}	1.37±0.10^{ab}	1.24±0.07^{bc}	1.29±0.09^{bc}	1.12±0.04^{c}	0.97±0.04^{d}
16:0	18.87±0.96	18.06±0.61	17.85±0.33	17.77±0.10	19.10±0.36	18.88±0.13	18.04±0.21
16:1 n-9	5.18±0.18^{a}	4.34±0.20^{b}	4.07±0.28^{b}	3.26±0.34^{c}	2.34±0.34^{d}	1.45±0.13^{e}	1.19±0.07^{e}
17:0	0.40±0.05	0.72±0.28	0.45±0.03	0.44±0.07	0.58±0.08	0.55±0.01	0.63±0.03
18:0	21.72±1.30	21.24±0.88	20.53±0.94	20.45±0.67	20.66±0.80	20.61±0.29	19.54±0.42
18:1 n-9	35.18±1.25^{a}	35.00±0.79^{a}	33.91±0.83^{a}	33.04±0.57^{a}	30.39±0.33^{b}	27.94±0.63^{c}	27.71±0.56^{c}
18:2 n-6	4.43±0.31^{f}	5.70±0.21^{ef}	7.73±0.46^{de}	9.01±0.29^{cd}	10.25±1.19^{c}	14.37±1.07^{b}	16.90±0.66^{a}
18:3 n-3	1.05±0.15^{a}	1.06±0.21^{a}	0.87±0.16^{ab}	0.78±0.13^{ab}	0.64±0.07^{ab}	0.46±0.03^{b}	0.49±0.03^{b}
20:2 n-6	0.86±0.31^{b}	0.75±0.34^{b}	0.71±0.26^{b}	0.66±0.12^{b}	0.60±0.11^{b}	1.04±0.34^{ab}	1.65±0.13^{a}
20:3 n-6	1.61±0.17	1.80±0.13	1.81±0.20	2.03±0.33	2.17±0.44	1.93±0.22	1.69±0.17
20:4 n-6	2.43±0.28^{b}	2.93±0.38^{ab}	3.13±0.27^{ab}	3.66±0.31^{a}	3.81±0.54^{a}	3.32±0.18^{ab}	2.53±0.17^{b}
20:5 n-3	0.20±0.04	0.28±0.02	0.24±0.03	0.18±0.03	0.22±0.04	0.21±0.03	0.23±0.04
24:0	2.24±0.10^{a}	2.21±0.19^{a}	2.14±0.13^{a}	1.93±0.12^{ab}	1.69±0.07^{bc}	1.43±0.07^{cd}	1.31±0.11^{d}
24:1 n-9	1.33±0.15^{c}	1.44±0.05^{c}	1.60±0.09^{ab}	1.76±0.04^{ab}	1.91±0.11^{a}	1.88±0.07^{a}	1.98±0.06^{a}
22:4 n-6	0.14±0.03^{ab}	0.12±0.03^{ab}	0.16±0.04^{a}	0.13±0.03^{ab}	0.14±0.04^{ab}	0.07±0.01^{ab}	0.06±0.01^{b}
22:5 n-6	0.05±0.01^{d}	0.05±0.01^{d}	0.08±0.01^{cd}	0.15±0.04^{c}	0.13±0.02^{c}	0.23±0.02^{b}	0.33±0.03^{a}
22:5 n-3	0.41±0.09	0.43±0.06	0.39±0.07	0.41±0.04	0.46±0.08	0.44±0.07	0.45±0.06
22:6 n-3	1.38±0.26^{d}	1.66±0.30^{cd}	2.03±0.18^{cd}	2.32±0.25^{bc}	2.95±0.36^{ab}	3.39±0.27^{a}	3.76±0.38^{a}
SFA	45.25±2.29^{a}	43.99±1.44^{ab}	42.89±1.25^{ab}	42.17±0.60^{ab}	43.66±0.44^{ab}	42.99±0.15^{ab}	40.77±0.55^{b}
MUFA	41.70±1.52^{a}	40.78±0.66^{a}	39.58±1.09^{ab}	38.07±0.38^{b}	34.64±0.20^{c}	31.27±0.81^{d}	30.88±0.59^{d}
n-6 PUFA	9.52±0.79^{f}	11.34±0.57^{e}	13.61±0.31^{d}	15.64±0.38^{c}	17.10±0.37^{c}	20.98±1.02^{b}	23.16±0.56^{a}
n-3 PUFA	3.53±0.83^{b}	3.89±0.49^{ab}	3.92±0.38^{ab}	4.13±0.43^{ab}	4.59±0.54^{ab}	4.77±0.36^{ab}	5.19±0.50^{a}

MDA-MB-231 breast cancer cells were exposed to fatty acids for 2 hours, 4 hours, 6 hours, 12 hours, 24 hours, 48 hours or 72 hours. Composition of fatty acids from total phospholipids was determined from 4 experiments. Values are percentages relative to the total fatty acid content ± SE. Within rows the values with different superscripts are significantly different (P<0.05). Values without superscripts are not significantly different.

Appendix Figure 2: Temporal increase of DHA in whole cell total PL (relative %) of MDA-MB-231 breast cancer cells incubated with 60 µM DHA for up to 72 hours.



Values represent the mean ± SEM (n=4 per time point). Letters indicate time points significant differences between time points (P<0.05) based one-way ANOVA with post hoc Duncan analysis.

Appendix Table 6: Major fatty acids in the control and DHA-enriched diet

Fatty acids	Control diet	DHA diet
	g/100 g fat	
16:0	21.5±0.06	21.3±0.82
18:0	11.1±0.32	10.7±0.28
18:1 n-9	47.9±0.3	45.2±2.5
18:2 n-6	13.9±0.03	13.1±1.1
18:3 n-3	ND	0.56±0.04
18:3 n-6	1.13±0.01	0.26±0.00
20:4 n-6	0.46±0.06	0.46±0.0
22:6 n-3	ND	2.82±0.09
Total SFA	33.7±0.41	34.5±1.32
Total MUFA	49.7±0.22	47.2±2.42
Total PUFA	15.0±0.07	17.2±1.16
Total n-3	0	3.38±0.05
Total n-6	15.0±0.07	13.8±1.10
n-6/n-3 ratio	ND	4.09±0.27
P/S ratio	0.46±0.00	0.5±0.01

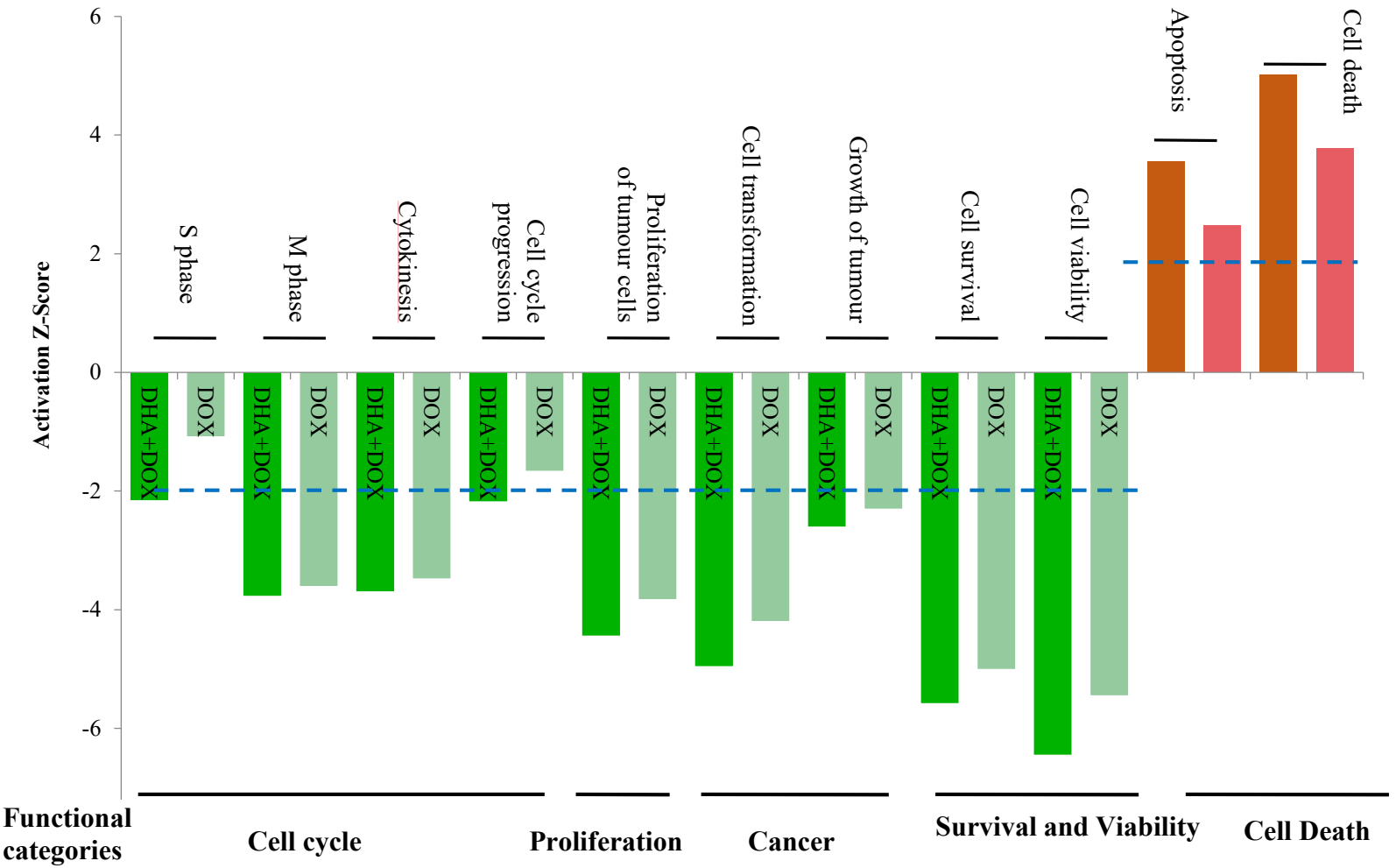
Values are the mean percentage of 3 batches of diet as determined by gas liquid chromatography (Cruz-Hernandez et al.). Diets contained 200 g/kg of fat that was a blend of sunflower oil, fully hydrogenated canola, olive oil, canola and Arasco oil (DSM Nutritional Products USA). The DHA in the DHA diet was provided by adding diet DHAsco (DSM Nutritional Products, USA). Minor fatty acids are not reported; therefore totals do not add up to 100 %. Abbreviations used: saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), polyunsaturated to saturated fatty acid ratio (P/S), not detected (ND; < 0.05%).

Appendix Table 7: Fatty acid composition of plasma phospholipids from *nu/nu* mice following dietary and chemotherapy interventions

	Control	DHA	DHA DOX	CONTROL DOX	Diet Interaction	Drug Interaction
14:0	0.43±0.1	0.54±0.11	0.45±0.04	0.37±0.08	NS	NS
16:0	21.3±1.32	21.2±2.04	21.3±1.32	18.9±0.89	NS	NS
16:1 n-9	0.64±0.08	0.82±0.17	0.68±0.07	0.84±0.12	NS	NS
18:0	30.4±1.02[a]	26.80±2.39[b]	27.8±0.97[b]	24.6±2.42[b]	NS	NS
18:1 n-9	18.0±2.46	20.3±2.53	16.5±1.96	23.0±4.35	NS	NS
18:2 n-6	19.5±2.53	21.9±0.45	21.7±1.79	23.4±1.17	NS	NS
20:0	0.12±0.03	0.07±0.01	0.21±0.13	0.08±0.01	NS	NS
18:3 n-6	0.32±0.06	0.56±0.22	0.22±0.06	0.64±0.19	NS	NS
18:3 n-3	0.31±0.12	0.37±0.01	0.18±0.07	0.25±0.07	NS	NS
20:2 n-6	0.23±0.13	0.06±0.01	0.18±0.08	0.09±0.04	NS	NS
20:3 n-6	0.52±0.14[b]	0.93±0.14[a]	1.29±0.09[a]	0.46±0.14[b]	+	NS
20:4 n-6	6.21±1.75	2.60±0.34	3.96±0.14	5.60±2.11	NS	NS
20:5 n-3	0.08±0.01[b]	0.44±0.08[a]	0.68±0.12[a]	0.06±0.02[b]	+	NS
24:0	0.13±0.03	0.07±0.01	0.08±0.04	0.09±0.02	NS	NS
24:1 n-9	0.15±0.06	0.10±0.04	0.12±0.01	0.14±0.01	NS	NS
22:4 n-6	0.08±0.02	0.03±0.01	0.03±0.01	0.07±0.02	+	NS
22:5 n-6	0.14±0.04	0.08±0.04	0.05±0.02	0.08±0.03	NS	NS
22:5 n-3	0.11±0.02	0.19±0.06	0.22±0.05	0.15±0.05	NS	NS
22:6 n-3	1.30±0.40[c]	2.94±0.24[b]	4.38±0.15[a]	1.09±0.43[c]	+	NS

Nu/nu mice were injected with 2 x 10^6 MDA-MB-231 breast cancer cells and maintained on control diet for 4 weeks. One week prior to commencing chemotherapy the mice were randomized into control or DHA diet groups and subsequently into chemotherapy (twice weekly) or control groups for an additional 4 weeks. Values represent the mean± SEM (n=6 mice per group). Plasma phospholipids were extracted using a modified Folch procedure (Field et al. , Folch et al.) and phospholipid fatty acid composition was determined by gas-liquid chromatography as previously described (Cruz-Hernandez et al.).

Appendix Figure 3: IPA regulation z-score algorithm to identify biological functions that are expected to be more active (positive z-score) or less active (negative z- score) according to our microarray data. In order to enhance the stringency of our analysis, we considered only functions with a z-score ≥ ± 2.0 (indicated by blue dotted line). Biological function is significant *P*-value ≤ 0.05.



Appendix Table 8: Genes associated with cell death (CD), necrosis (NE) and apoptosis (AP); cell survival (CS) and viability (CV), proliferation of cancer cell (PC), growth of tumour (GT) and cell transformation (CT) and cell cycle (CC) increased or decreased in DHA DOX vs DOX

		DHA vs OA		DHA DOX vs OA		DOX vs OA		DHA DOX vs DOX					
GENE	**Entrez Gene Name**	P-value	fold change	P-value	fold change	P-value	fold change	P-value	fold change				
BIRC5	Baculoviral IAP repeat containing 5	0.27	1.05	0.00	-1.44	0.01	-1.21	0.00	-1.18	CD AP	PC GT CT	CS CV	C C
BAG1	BCL2-associated anthanogene	0.93	1.00	0.00	-1.30	0.00	-1.20	0.15	-1.10	CD NE AP	PC GT	CS CV	
KITLG	KIT ligand	0.51	-1.04	0.02	-1.22	0.32	-1.07	0.09	-1.14	CD NE AP	PC GT	CS CV	
mir-181	microRNA 181	0.45	1.07	0.00	1.57	0.04	1.23	0.03	1.28	CD NE AP	PC GT	CS CV	
mir-221	microRNA 221	0.73	1.02	0.00	-1.49	0.00	-1.38	0.16	-1.08	CD NE AP	PC GT	CS	
MIR17HG	miR-17-92 cluster host gene	0.43	-1.10	0.03	-1.29	0.06	-1.20	0.58	-1.10	CD NE AP	PC GT	CS CV	
PBK	PDZ binding kinase	0.53	1.02	0.00	-1.36	0.02	-1.09	0.00	-1.25	CD NE AP	PC CT	CS CV	
PBX3	Pre-B-Cell leukemia homeobox 3	0.28	1.04	0.00	-1.21	0.00	-1.13	0.09	-1.07	CD NE AP	PC CT	CS CV	
PLK1	Polo-like kinase 1	0.38	1.03	0.00	-2.98	0.00	-2.60	0.00	-1.14	CD NE AP	PC GT	CS CV	
TNFSF13	TNFSF12-TNFSF13 readthrough	0.72	-1.00	0.00	-1.24	0.05	-1.10	0.03	-1.10	AP	PC GT	CS CV	
AURKA	Aurora kinase A	0.37	1.00	0.00	-1.80	0.00	-1.60	0.06	-1.10	CD NE AP	PC GT CT		C C
BRCA1	breast cancer 1, early onset	0.82	1.01	0.00	1.36	0.01	1.60	0.82	-1.18	CD AP	GT CT		C C
RARB	Retinoic acid receptor, beta	0.45	-1.11	0.02	1.55	0.21	1.20	0.12	1.29	CD NE AP	PC GT		C C
SKP2	S-phase kinase-associated protein 2	0.05	1.16	0.00	-1.45	0.41	-1.06	0.00	-1.37	CD NE AP	PC GT CT		C C
UBE2C	Ubiquitin-conjugating enzyme E2C	0.20	1.05	0.00	-1.51	0.00	-1.33	0.02	-1.13	CD NE	PC GT		C C

		DHA vs OA		DHA DOX vs OA		DOX vs OA		DHA DOX vs DOX				
GENE	**Entrez Gene Name**	P-value	fold change	P-value	fold change	P-value	fold change	P-value	fold change			
IGFBP3	Insulin-like growth factor binding protein 3	0.03	-1.48	0.01	1.73	0.87	1.03	0.01	1.69	CD NE AP	CS CV	C C
WEE1	WEE1 homolog (S. pombe)	0.66	-1.01	0.00	-1.55	0.00	-1.41	0.02	-1.10	CD NE AP	CS CV	C C
CD24	Cluster of Differentiation 24	0.00	-1.50	0.01	1.54	0.35	1.10	0.03	1.37	CD NE AP		C C
CENPF	Centromere protein F	0.81	-1.00	0.00	-1.61	0.00	-1.30	0.00	-1.20	CD AP		C C
INHBA	Inhibin, beta A	0.36	-1.24	0.01	2.21	0.47	1.18	0.03	1.87	CD NE AP		C C
KIF14	kinesin family member 14	0.86	-1.01	0.00	-2.40	0.00	-2.09	0.05	-1.15	CD NE AP		C C
MAD2L1	MAD2 mitotic arrest deficient-like 1	0.13	1.06	0.00	-1.21	0.84	1.01	0.00	-1.22	AP		C C
MKI67	Ki-67	0.21	1.05	0.00	-1.32	0.00	-1.15	0.01	-1.15	CD NE AP		C C
NEK2	NIMA -related kinase 2	0.02	-1.13	0.00	-1.97	0.00	-1.71	0.02	-1.15	CD NE		C C
RACGAP1	Rac GTPase activating protein 1	0.45	-1.06	0.00	-1.44	0.02	-1.21	0.05	-1.20	CD NE AP		C C
ANGPT1	angiopoietin 1	0.98	1.00	0.01	-1.58	0.02	-1.36	0.27	-1.16	CD NE AP	CS CV	
BCL2	B-cell lymphoma 2	0.82	1.00	0.00	-1.30	0.00	-1.30	0.84	-1.00	CD NE AP	CS CV	
BCL2L1	BCL2-like 1	0.69	1.00	0.00	-1.20	0.00	-1.10	0.05	-1.10	CD NE AP	CS CV	
CAMK4	calcium/calmodulin-dependent protein kinase IV	0.02	1.16	0.01	-1.24	0.07	-1.11	0.10	-1.11	AP	CS CV	
CASP9	Caspase 9	0.03	-1.09	0.00	1.35	0.00	1.16	0.00	1.17	CD NE AP	CS CV	
CKAP5	cytoskeleton associated protein 5	0.58	-1.02	0.00	-1.44	0.00	-1.27	0.02	-1.13	CD NE AP	CS CV	
CXCL10	chemokine (C-X-C motif) ligand 10	0.44	-1.15	0.00	2.69	0.07	1.44	0.01	1.87	CD NE AP	CS CV	

		DHA vs OA		DHA DOX vs OA		DOX vs OA		DHA DOX vs DOX			
GENE	**Entrez Gene Name**	P-value	fold change	P-value	fold change	P-value	fold change	P-value	fold change		
EGF	Epidermal Growth Factor	0.29	1.05	0.00	-1.28	0.00	-1.27	0.97	-1.00	CD AP	CS CV
FBXO32	F-box protein 32	0.15	-1.13	0.00	1.39	0.20	1.11	0.03	1.25	CD NE AP	CS CV
IL1B	Interleukin 1, beta	0.07	-1.42	0.01	1.92	0.58	-1.10	0.00	2.12	CD NE AP	CS CV
IL24	Interleukin 24	0.46	1.12	0.00	2.31	0.00	2.42	0.79	-1.04	CD NE AP	CS CV
KIF11	kinesin family member 11	0.57	1.02	0.00	-1.31	0.00	-1.15	0.00	-1.14	CD NE AP	CS CV
LCN2	lipocalin 2	0.27	-1.13	0.01	1.47	0.04	1.28	0.26	1.15	CD NE AP	CS CV
LMNA	lamin A/C	0.69	1.01	0.00	-1.30	0.00	-1.19	0.04	-1.10	AP	CS CV
MAP3K12	mitogen-activated protein kinase kinase kinase 12	0.04	-1.08	0.00	1.20	0.33	1.04	0.00	1.15	CD NE AP	CS CV
MAP3K4	mitogen-activated protein kinase kinase kinase 4	0.72	-1.01	0.00	-1.26	0.00	-1.14	0.02	-1.11	CD NE AP	CS CV
NDC80	NDC80 kinetochore complex component homolog	0.72	1.02	0.00	-1.56	0.00	-1.33	0.01	-1.17	CD NE AP	CS CV
NKX3-1	NK3 homeobox 1	0.47	1.04	0.00	1.33	0.01	1.18	0.04	1.13	CD	CS CV
NUF2	NDC80 kinetochore complex component, homolog	0.64	1.02	0.00	-1.54	0.00	-1.34	0.01	-1.15	CD NE AP	CS CV
OLR1	oxidized low density lipoprotein (lectin-like) receptor 1	0.78	1.05	0.00	2.46	0.02	1.64	0.08	1.50	CD NE AP	CS CV
PDPK1	3-phosphoinositide dependent protein kinase-1	0.09	1.09	0.00	-1.20	0.18	1.06	0.00	-1.27	CD NE AP	CS CV
XDH	xanthine dehydrogenase	0.26	-1.11	0.03	1.30	0.22	-1.12	0.00	1.46	CD NE AP	CS CV
TPX2	TPX2, microtubule-associated, homolog	0.18	1.04	0.00	-1.40	0.00	-1.29	0.02	-1.09	CD NE AP	PC GT

		DHA vs OA		DHA DOX vs OA		DOX vs OA		DHA DOX vs DOX				
GENE	**Entrez Gene Name**	P-value	fold change	P-value	fold change	P-value	fold change	P-value	fold change			
ANLN	anillin, actin binding protein	0.47	-1.02	0.00	-1.41	0.00	-1.32	0.03	-1.07		CS CV	C C
CCNB1	cyclin B1	0.17	1.05	0.00	-2.08	0.00	-1.90	0.03	-1.09		CS CV	C C
FOSL1	FOS-like antigen 1	0.11	1.10	0.00	-1.45	0.00	-1.20	0.01	-1.20		CS CV	C C
TOP2A	topoisomerase II	0.72	-1.00	0.00	-1.26	0.00	-1.10	0.03	-1.10		CS	C C
PBX1	Pre-B-Cell leukemia homeobox 1	0.04	-1.20	0.02	-1.35	0.08	-1.20	0.31	-1.10	PC CT	CS CV	
SHFM1	Split hand/foot malformation (ectrodactyly) type 1	0.60	-1.10	0.01	-1.49	0.02	-1.40	0.54	-1.10	PC CT	CS CV	
HMGB1	High mobility group box 1	0.64	-1.00	0.00	-1.23	0.01	-1.20	0.24	-1.10	PC GT	CS CV	
PPP2R2B	Protein phosphatase 2, regulatory subunit B, beta	0.73	-1.02	0.01	-1.24	0.33	-1.06	0.03	-1.17	PC GT	CS CV	
RNF126	Ring finger protein 126	0.12	1.10	0.00	-1.38	0.02	-1.10	0.01	-1.20	PC GT	CS CV	
PTTG1	Pituitary tumour-transforming 1	0.95	-1.00	0.00	-1.75	0.00	-1.61	0.17	-1.10	PC CT		C C
CCNA1	Cyclin A1	0.54	-1.05	0.00	-1.36	0.00	-1.46	0.40	1.10	PC GT		C C
DUSP1	Dual specificity phosphatase 1	0.05	-1.23	0.02	1.37	0.90	-1.01	0.01	1.38	PC GT		C C

P-values and fold change are shown. Genes are considered significantly increased (positive value) or decreased (negative value) based on the selection criteria ($P \leq 0.05$, fold change ≥ 1.2). However important genes only significantly changed at 1.1 fold are also shown.

Appendix Table 9: Genes associated in S phase, M phase, cytokinesis and cell cycle progression; increased or decreased in DHA DOX compared to DOX

		DHA vs OA		DHA DOX vs OA		DOX vs OA		DHA DOX vs DOX	
Gene Symbol	**Entrez Gene Name**	P-value	fold change	P-value	fold change	P-value	fold change	P-value	fold change
	S phase								
c-RAF	Raf-1 proto-oncogene, serine/threonine kinase	0.58	-1.01	0.01	-1.09	0.12	-1.04	0.17	-1.04
CDC25A	cell division cycle 25A	0.09	1.14	0.06	1.18	0.00	1.34	0.12	-1.14
CDC25C	Cell division cycle 25 homolog C	0.30	-1.1	0.00	-1.7	0.00	-1.4	0.02	-1.3
CDK2	cyclin-dependent kinase 2	0.15	1.06	0.96	-1.00	0.06	1.08	0.08	-1.08
CDK7	cyclin-dependent kinase 7	0.15	-1.05	0.03	-1.10	0.05	-1.08	0.60	-1.02
CCNA1	cyclin A1	0.54	-1.05	0.00	-1.36	0.00	-1.46	0.40	1.10
CCNE1	cyclin E1	0.11	1.14	0.20	1.12	0.00	1.35	0.05	-1.20
CCNH	cyclin H	0.97	1.00	0.93	1.00	0.23	-1.04	0.25	1.04
E2F8	E2F transcription factor 8	0.15	1.1	0.03	1.3	0.00	1.6	0.04	-1.3
MXD1	MAX dimerization protein 1	0.08	-1.1	0.00	1.6	0.00	1.3	0.00	1.3
p21CIP1	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	0.04	1.07	0.00	1.33	0.00	1.26	0.15	1.05
p27KIP1	cyclin-dependent kinase inhibitor 1B (p27, Kip1)	0.15	-1.07	0.38	-1.04	0.10	-1.08	0.48	1.03
RB	retinoblastoma 1	0.44	1.01	0.03	-1.06	0.00	-1.08	0.27	1.02
SKP2	S-phase kinase-associated protein 2	0.05	1.16	0.00	-1.45	0.41	-1.06	0.02	-1.37
	M phase								
AKAP12	A kinase (PRKA) anchor protein 12	0.61	-1.0	0.00	-1.4	0.04	-1.2	0.03	-1.2
CCNB1	Cyclin B1	0.17	1.05	0.00	-2.08	0.00	-1.90	0.03	-1.10
CDCA8	Cell division cycle associated 8	0.07	1.10	0.00	-2.05	0.00	-1.74	0.01	-1.18
CDC20	cell division cycle 20	0.01	1.17	0.00	-1.88	0.00	-1.68	0.06	-1.10
CDC7	cell division cycle 7	0.66	1.02	0.05	1.10	0.01	1.14	0.42	-1.00
CENPF	Centromere protein F	0.81	-1.01	0.00	-1.61	0.00	-1.32	0.00	-1.22

		DHA vs OA		DHA DOX vs OA		DOX vs OA		DHA DOX vs DOX	
Gene Symbol	**Entrez Gene Name**	P-value	fold change	P-value	fold change	P-value	fold change	P-value	fold change
CKS2	CDC28 protein kinase regulatory subunit 2	0.02	1.13	0.00	-1.68	0.00	-1.33	0.00	-1.27
EG5	kinesin family member 11	0.57	1.02	0.00	-1.31	0.00	-1.15	0.00	-1.10
Emi1	F-box protein 5	0.13	1.07	0.05	1.11	0.00	1.19	0.16	-1.10
Esp1	extra spindle pole bodies homolog 1 (S. cerevisiae)	0.62	-1.03	0.00	-1.29	0.01	-1.18	0.17	-1.10
FZR1	fizzy/cell division cycle 20 related 1 (Drosophila)	0.76	-1.01	0.00	-1.22	0.00	-1.16	0.23	-1.10
HIPK2	Homeodomain interacting protein kinase 2	0.09	1.2	0.01	-1.4	0.29	-1.1	0.03	-1.3
INCENP	Inner centromere protein antigens	0.04	1.08	0.00	-1.45	0.00	-1.21	0.00	-1.20
KIF23	kinesin family member 23	0.62	1.03	0.00	-1.62	0.00	-1.36	0.02	-1.20
KIF20A	Kinesin family member 20A	0.69	-1.01	0.00	-2.84	0.00	-2.43	0.00	-1.17
LIMK1	LIM domain kinase 1	0.62	1.0	0.00	-1.3	0.14	-1.1	0.00	-1.2
MAD2L1	MAD2 mitotic arrest deficient-like 1	0.13	1.06	0.00	-1.21	0.84	1.01	0.00	-1.22
NDC80	NDC80 kinetochore complex component homolog	0.72	1.0	0.00	-1.6	0.00	-1.3	0.01	-1.2
NUF2	NDC80 kinetochore complex component, homolog	0.64	1.0	0.00	-1.5	0.00	-1.3	0.01	-1.2
PRC1	protein regulator of cytokinesis 1	0.97	1.00	0.00	-1.42	0.00	-1.25	0.04	-1.10
PTTG1	pituitary tumour-transforming 1	0.95	-1.00	0.00	-1.75	0.00	-1.61	0.17	-1.10
TGF-β1	transforming growth factor, beta 1	0.08	-1.13	0.06	1.16	0.94	1.00	0.07	1.20
WEE1	WEE1 homolog (S. pombe)	0.66	-1.01	0.00	-1.55	0.00	-1.41	0.02	-1.10
	Cytokinesis								
ANLN	Anillin, actin binding protein	0.47	-1.00	0.00	-1.41	0.00	-1.30	0.03	-1.10
AURKA	Aurora kinase A	0.37	1.00	0.00	-1.80	0.00	-1.60	0.06	-1.10
CDC20	cell division cycle 20	0.01	1.20	0.00	-1.88	0.00	-1.70	0.06	-1.10
CDC25B	cell division cycle 25B	0.84	-1.00	0.04	-1.10	0.01	-1.10	0.58	1.00
CENPV	Centromere protein V	0.08	1.10	0.01	-1.12	0.28	-1.00	0.05	-1.10

		DHA vs OA		DHA DOX vs OA		DOX vs OA		DHA DOX vs DOX	
Gene Symbol	**Entrez Gene Name**	P-value	fold change	P-value	fold change	P-value	fold change	P-value	fold change
CKAP2	Cytoskeleton associated protein 2	0.66	1.00	0.00	-1.58	0.00	-1.50	0.18	-1.10
ECT2	Epithelial cell transforming 2	0.90	1.00	0.00	-1.26	0.00	-1.20	0.11	-1.10
HIPK2	Homeodomain-interacting protein kinase 2	0.09	1.20	0.01	-1.40	0.29	-1.10	0.03	-1.30
KIF14	kinesin family member 14	0.86	-1.00	0.00	-2.40	0.00	-2.10	0.05	-1.10
KIF20A	kinesin family member 20A	0.69	-1.00	0.00	-2.84	0.00	-2.40	0.00	-1.20
KIF20B	kinesin family member 20B	0.93	-1.00	0.00	-1.66	0.00	-1.50	0.04	-1.10
KIF23	kinesin family member 23	0.62	1.00	0.00	-1.62	0.00	-1.40	0.02	-1.20
KIF4A	kinesin family member 4A	0.53	1.00	0.00	-1.48	0.00	-1.40	0.17	-1.10
KIFC1	kinesin family member C1	0.96	1.00	0.00	-1.26	0.01	-1.10	0.03	-1.10
mir125b1	micro-RNA 125B1	0.07	-1.40	0.00	-2.14	0.00	-2.10	0.96	-1.00
NEK2	NIMA -related kinase 2	0.02	-1.10	0.00	-1.97	0.00	-1.70	0.02	-1.10
PRC1	Protein regulator of cytokinesis	0.97	1.00	0.00	-1.43	0.00	-1.20	0.04	-1.10
RACGAP1	Rac GTPase activating protein 1	0.45	-1.10	0.00	-1.44	0.02	-1.20	0.05	-1.20
TOP2A	topoisomerase II	0.72	-1.00	0.00	-1.26	0.00	-1.10	0.03	-1.10
TRRAP	transformation / transcription domain associated protein	0.70	-1.00	0.00	-1.26	0.03	-1.10	0.05	-1.10
	Cell Cycle Progression								
BIRC5	Baculoviral IAP repeat containing 5	0.27	1.10	0.00	-1.44	0.00	-1.20	0.01	-1.20
BRCA1	Breast cancer 1, early onset	0.82	1.00	0.00	1.36	0.00	1.60	0.01	-1.20
CCNB1	Cyclin B1	0.17	1.00	0.00	-2.08	0.00	-1.90	0.03	-1.10
CCNF	Cyclin F	0.96	-1.00	0.00	-1.63	0.00	-1.50	0.18	-1.10
CD24	Cluster of Differentiation 24	0.00	-1.50	0.01	1.54	0.35	1.10	0.03	1.40
CDKN3	Cyclin-dependent kinase inhibitor 3	0.22	1.10	0.00	-1.32	0.02	-1.10	0.01	-1.20
DBF4	DBF4 Zinc finger	0.05	1.10	0.00	-1.69	0.00	-1.30	0.00	-1.30
DUSP1	Dual specificity phosphatase 1	0.05	-1.20	0.02	1.37	0.90	-1.00	0.01	1.40
EGR1	Early growth response 1	0.82	-1.0	0.00	1.8	0.01	1.2	0.00	1.5
FAM72A	family with sequence similarity 72	0.21	1.20	0.02	-1.47	0.09	-1.30	0.32	-1.20

		DHA vs OA		DHA DOX vs OA		DOX vs OA		DHA DOX vs DOX	
Gene Symbol	**Entrez Gene Name**	P-value	fold change	P-value	fold change	P-value	fold change	P-value	fold change
FOSL1	FOS-like antigen 1	0.11	1.10	0.00	-1.45	0.00	-1.20	0.01	-1.20
IGFBP3	Insulin-like growth factor binding protein 3	0.03	-1.50	0.01	1.70	0.87	1.00	0.01	1.70
IL7	Interleukin 7	0.85	-1.00	0.00	-1.22	0.00	-1.20	0.00	1.00
INHBA	Inhibin, beta A	0.36	-1.20	0.01	2.21	0.47	1.20	0.03	1.90
MKI67	Ki-67	0.21	1.10	0.00	-1.32	0.00	-1.10	0.01	-1.20
PCNA	Proliferating cell nuclear antigen	0.06	1.1	0.01	1.2	0.00	1.4	0.02	-1.2
RARB	Retinoic acid receptor, beta	0.45	-1.10	0.02	1.55	0.21	1.20	0.12	1.30
RHOB	Ras homolog family member B	0.27	-1.10	0.03	1.18	0.78	1.00	0.05	1.20
TTC5	Tetratricopeptide repeat domain 5	0.46	-1.10	0.00	-1.71	0.00	-1.60	0.31	-1.10
UBE2C	Ubiquitin-conjugating enzyme E2C	0.20	1.10	0.00	-1.51	0.00	-1.30	0.02	-1.10

P-values and fold change are shown. Genes are considered significantly increased (positive value) or decreased (negative value) based on the selection criteria ($P \leq 0.05$, fold change ≥ 1.2). However important genes required for cell cycle only significantly changed at 1.1-fold are also shown.

Appendix 10: Genes associated with proliferation of cancer cells (PC), growth of tumour (GT) and cell transformation (CT) increased or decreased in DHA DOX vs DOX

		DHA vs OA		DHA DOX vs OA		DOX vs OA		DHA DOX vs DOX		
Gene Symbol	**Entrez Gene Name**	P-value	fold change	P-value	fold change	P-value	fold change	P-value	fold change	
ACVR1C	Activin A receptor, type IC	0.55	-1.00	0.02	1.25	0.26	1.10	0.13	1.10	PC GT
ALCAM	Activated leukocyte cell adhesion molecule	0.26	-1.10	0.00	-1.28	0.00	-1.00	0.15	1.10	PC GT
ARRDC3	Arrestin domain containing 3	0.04	-1.20	0.01	1.40	0.51	1.10	0.02	1.30	PC GT
AURKA	Aurora kinase A	0.37	1.00	0.00	-1.80	0.00	-1.60	0.06	-1.10	PC GT CT
BAG1	BCL2-associated anthanogene	0.93	1.00	0.00	-1.30	0.00	-1.20	0.15	-1.10	PC GT
BIRC5	Baculoviral IAP repeat containing 5	0.27	1.05	0.00	-1.44	0.00	-1.21	0.01	-1.18	PC GT CT
BRCA1	Breast cancer 1, early onset	0.82	1.01	0.00	1.36	0.00	1.60	0.01	-1.18	GT CT
EFNA1	Ephrin-A1	0.08	-1.20	0.01	1.43	0.01	1.30	0.55	1.10	PC GT
FOSL1	FOS-like antigen 1	0.11	1.09	0.00	-1.45	0.00	-1.21	0.01	-1.20	PC GT CT
HBEGF	Heparin-binding EGF-like growth factor	0.73	1.00	0.00	-1.29	0.00	-1.40	0.07	1.10	PC GT
HMGA1	High mobility group AT-hook 1	0.06	1.10	0.00	-1.37	0.00	-1.30	0.16	-1.10	PC GT CT
HMMR	Hyaluronan-mediated motility receptor (RHAMM)	0.81	1.00	0.00	-1.80	0.00	-1.60	0.13	-1.10	PC GT
ID1	Inhibitor of DNA binding 1	0.00	-1.40	1.00	-1.28	0.00	-1.40	0.21	1.10	PC GT
IFNAR1	Interferon (alpha, beta and omega) receptor 1	0.38	-1.00	0.00	1.30	0.00	1.20	0.14	1.10	PC GT
IGFBP1	Insulin-like growth factor 2 mRNA binding protein 1	0.14	-1.30	0.00	2.09	0.08	1.30	0.02	1.60	PC GT
KITLG	KIT ligand	0.51	-1.00	0.02	-1.20	0.32	-1.10	0.09	-1.10	PC GT
mir-181	microRNA 181	0.45	1.10	0.00	1.57	0.04	1.20	0.03	1.30	PC GT
mir-221	microRNA 221	0.73	1.00	0.00	-1.52	0.00	-1.40	0.16	-1.10	PC GT
MIR17HG	miR-17-92 cluster host gene	0.43	-1.10	0.03	-1.29	0.06	-1.20	0.58	-1.10	PC GT
NCAPG	Non-SMC condensin I complex, subunit G	0.22	1.10	0.01	-1.20	0.06	-1.10	0.16	-1.10	PC GT

		DHA vs OA		DHA DOX vs OA		DOX vs OA		DHA DOX vs DOX		
Gene Symbol	**Entrez Gene Name**	P-value	fold change	P-value	fold change	P-value	fold change	P-value	fold change	
NOV	Nephroblastoma overexpressed	0.54	-1.10	0.00	1.90	0.03	1.40	0.12	1.30	PC CT
PBK	PDZ binding kinase	0.53	1.02	0.00	-1.36	0.02	-1.09	0.00	-1.25	PC CT
PBX3	Pre-B-Cell leukemia homeobox 3	0.28	1.00	0.00	-1.20	0.00	-1.10	0.09	-1.10	PC CT
PLK1	Polo-like kinase 1	0.38	-1.00	0.00	-2.98	0.00	-2.60	0.00	-1.10	PC GT
PROX1	Prospero homeobox 1	0.79	-1.00	0.00	1.24	0.23	1.00	0.00	1.20	PC GT
S100A7	S100 calcium binding protein A7	0.84	1.00	0.04	-1.40	0.10	-1.20	0.48	-1.10	PC GT
SERPINA3	Serpin peptidase inhibitor, clade A	0.14	-1.20	0.00	1.62	0.30	1.10	0.00	1.50	PC CT
SKP2	S-phase kinase-associated protein 2	0.05	1.16	0.00	-1.45	0.41	-1.06	0.00	-1.37	PC GT CT
SOD2	Superoxide dismutase 2	0.12	-1.30	0.01	1.71	0.32	1.10	0.02	1.50	PC GT
TACSTD2	Tumour-associated calcium signal transducer 2	0.23	-1.10	0.00	-1.39	0.00	-1.30	0.37	-1.10	PC GT
TFDP1	Transcription factor Dp-1	0.83.	1.00	0.00	-1.21	0.01	-1.10	0.17	-1.10	PC CT
TGFB2	Transforming growth factor, beta-induced	0.00	-1.20	0.00	-1.32	0.00	-1.40	0.30	1.10	GT CT
TNFSF13	TNFSF12-TNFSF13 readthrough	0.72	-1.00	0.00	-1.24	0.05	-1.10	0.03	-1.10	PC GT
TRRAP	Transformation / transcription domain associated protein	0.70	-1.00	0.00	-1.26	0.03	-1.10	0.05	-1.10	PC CT
UBE2C	Ubiquitin-conjugating enzyme E2C	0.20	1.10	0.00	-1.51	0.00	-1.30	0.02	-1.10	PC GT

P-values and fold change are shown. Genes are considered significantly increased (positive value) or decreased (negative value) based on the selection criteria ($P \leq 0.05$, fold change ≥ 1.2). However important genes required for cell cycle only significantly changed at 1.1-fold are also shown.

Appendix Table 11: Genes associated with cell survival (CS) and viability (CV) increased or decreased in DHA DOX vs DOX

		DHA vs OA		DHA DOX vs OA		DOX vs OA		DHA DOX vs DOX		
Gene Symbol	**Entrez Gene Name**	P-value	fold change	P-value	fold change	P-value	fold change	P-value	fold change	
ALB	albumin	0.14	-1.14	0.03	1.27	0.16	1.13	0.23	1.12	CS CV
AQP3	aquaporin 3 (Gill blood group)	0.05	1.17	0.00	1.70	0.00	1.46	0.08	1.16	CS CV
BCKDK	branched chain ketoacid dehydrogenase kinase	0.00	1.14	0.00	-1.26	0.01	-1.12	0.01	-1.12	CS CV
BDNF	brain-derived neurotrophic factor	0.01	-1.10	0.00	-1.24	0.00	-1.12	0.01	-1.11	CS CV
BIK	BCL2-interacting killer (apoptosis-inducing)	0.16	1.07	0.00	1.22	0.09	1.08	0.02	1.13	CS CV
BMP4	bone morphogenetic protein 4	0.33	1.06	0.00	-1.28	0.42	-1.04	0.01	-1.22	CS CV
BRD2	bromodomain containing 2	0.10	-1.10	0.01	1.20	0.73	-1.02	0.01	1.23	CS CV
CCNG1	cyclin G1	0.51	1.03	0.00	-1.29	0.01	-1.13	0.01	-1.14	CS CV
CD38	CD38 molecule	0.46	-1.12	0.01	1.78	0.04	1.43	0.23	1.24	CS CV
CHCHD6	coiled-coil-helix-coiled-coil-helix domain containing 6	0.55	1.02	0.00	-1.20	0.58	-1.02	0.01	-1.17	CS CV
CLDN4	claudin 4	0.24	-1.12	0.02	-1.32	0.08	-1.19	0.33	-1.11	CS CV
CTLA4	cytotoxic T-lymphocyte-associated protein 4	0.44	-1.04	0.00	-1.26	0.87	-1.01	0.00	-1.25	CS CV
CXCL11	chemokine (C-X-C motif) ligand 11	0.52	-1.10	0.00	2.64	0.00	1.75	0.03	1.51	CS
DCLRE1A	DNA cross-link repair 1A	0.27	-1.07	0.01	-1.24	0.07	-1.13	0.19	-1.10	CS CV
H2AFX	H2A histone family, member X	0.85	1.01	0.00	-1.40	0.00	-1.19	0.00	-1.18	CS CV
HBEGF	Heparin-binding EGF-like growth factor	0.73	1.00	0.00	-1.29	0.00	-1.40	0.07	1.10	CS CV

		DHA vs OA		DHA DOX vs OA		DOX vs OA		DHA DOX vs DOX		
Gene Symbol	**Entrez Gene Name**	P-value	fold change	P-value	fold change	P-value	fold change	P-value	fold change	
HMGB2	high mobility group box 2	0.23	1.05	0.00	-1.23	0.03	-1.09	0.02	-1.12	CS CV
ID4	inhibitor of DNA binding 4, dominant negative helix-loop-helix prot	0.09	-1.15	0.01	1.31	0.31	-1.08	0.00	1.42	CS CV
KAT5	K(lysine) acetyltransferase 5	0.94	-1.00	0.01	-1.28	0.04	-1.16	0.20	-1.10	CS CV
KHK	ketohexokinase (fructokinase	0.21	-1.07	0.00	-1.26	0.10	-1.09	0.03	-1.15	CS CV
KIF18A	Kinesin family member 18A	0.43	1.03	0.00	-1.81	0.00	-1.50	0.00	-1.21	CS CV
MGAT5	mannosyl (alpha-1,6-)-glycoprotein beta-1,6-N-acetyl-glucosaminyl	0.33	-1.05	0.00	-1.54	0.00	-1.42	0.02	-1.09	CS CV
mir-210	microRNA 210	0.87	1.01	0.01	1.22	0.15	1.10	0.15	1.11	CS CV
mir-515	microRNA 520e	0.86	-1.03	0.03	-1.50	0.69	-1.06	0.05	-1.42	CS CV
MMP13	matrix metallopeptidase 13 (collagenase 3)	0.84	1.04	0.02	1.73	0.70	1.07	0.04	1.62	CS CV
NEK3	NIMA (never in mitosis gene a)-related kinase 3	0.65	-1.07	0.01	-1.80	0.04	-1.40	0.15	-1.28	CS CV
SERPINE1	serpin peptidase inhibitor, clade E (nexin, plasminogen activa	0.11	1.12	0.00	1.53	0.01	1.25	0.02	1.22	CS CV
THOC1	THO complex 1	0.24	1.04	0.00	-1.24	0.12	-1.06	0.00	-1.16	CS CV

Genes are considered significantly increased (positive value) or decreased (negative value) based on the selection criteria ($P \leq 0.05$, fold change ≥ 1.2). However important genes required for cell cycle only significantly changed at 1.1-fold are also shown.

Appendix Table 12: Genes associated with cell death (CD), necrosis (NE) and apoptosis (AP) increased or decreased in DHA DOX compared to DOX

		DHA vs OA		DHA DOX vs OA		DOX vs OA		DHA DOX vs DOX		
Gene Symbol	**Entrez Gene Name**	P-value	fold change	P-value	fold change	P-value	fold change	P-value	fold change	
AGR2	anterior gradient 2 homolog (Xenopus laevis)	0.04	-1.60	0.03	1.75	0.68	-1.10	0.02	1.90	CD NE
AKAP12	A kinase (PRKA) anchor protein 12	0.61	-1.03	0.00	-1.35	0.04	-1.15	0.03	-1.18	CD NE AP
APOL1	apolipoprotein L, 1	0.55	-1.10	0.01	1.55	0.07	1.30	0.12	1.20	CD NE
ASNS	Asparagine synthetase	0.08	1.10	0.00	-1.40	0.00	-1.30	0.38	-1.10	CD NE AP
BNIP1	BCL2/adenovirus E1B 19kDa interacting protein 1	0.58	1.00	0.00	-1.28	0.24	-1.10	0.01	-1.20	CD AP
BUB1	budding uninhibited by benzimidazoles 1 homolog (yeast)	0.13	1.10	0.00	-1.87	0.00	-1.46	0.00	-1.23	NE
C3	complement component 3	0.17	-1.30	0.00	2.35	0.35	1.20	0.02	1.90	CD NE AP
C9	complement component 9	0.09	1.10	0.00	1.30	0.74	1.00	0.00	1.20	CD NE
CASP10	Caspase 10	0.02	-1.16	0.00	1.27	0.12	1.09	0.03	1.16	CD NE AP
CAV1	Caveolin 1	0.33	1.00	0.00	-1.30	0.00	-1.20	0.01	-1.10	CD NE AP
CCBE1	collagen and calcium binding EGF domains 1	0.21	-1.10	0.00	-1.54	0.00	-1.50	0.94	-1.00	CD AP
CDC6	Cell division cycle 6 homolog	0.01	1.16	0.00	1.24	0.01	1.44	0.00	-1.16	CD NE AP
CDCA2	Cell division cycle associated 2	0.16	1.10	0.00	-1.50	0.00	-1.30	0.08	-1.20	CD NE AP
CDH3	cadherin 3, type 1, P-cadherin (placental)	0.15	-1.10	0.00	1.53	0.00	1.45	0.41	1.10	CD NE
CDH4	cadherin 4, type 1, R-cadherin (retinal)	0.74	1.00	0.00	-1.43	0.00	-1.50	0.74	1.00	CD AP
CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	0.33	-1.10	0.01	1.35	0.04	1.20	0.31	1.10	CD NE AP
CENPA	centromere protein A	0.14	1.10	0.00	-1.73	0.00	-1.40	0.00	-1.20	CD AP
CENPE	centromere protein E	0.62	1.00	0.00	-2.19	0.00	-1.90	0.06	-1.10	CD NE AP
CSK	c-src tyrosine kinase	0.32	1.04	0.00	-1.23	0.01	-1.12	0.04	-1.10	CD NE AP

		DHA vs OA		DHA DOX vs OA		DOX vs OA		DHA DOX vs DOX		
Gene Symbol	**Entrez Gene Name**	P-value	fold change	P-value	fold change	P-value	fold change	P-value	fold change	
CTNNBIP1	catenin, beta interacting protein 1	0.55	1.03	0.00	-1.30	0.07	-1.11	0.02	-1.17	CD AP
CTSS	cathepsin S	0.29	-1.13	0.00	1.65	0.03	1.32	0.09	1.25	CD NE AP
CUL9	cullin 9	0.01	-1.10	0.00	1.20	0.10	1.05	0.00	1.14	CD NE AP
CYBB	cytochrome b-245, beta polypeptide	0.06	-1.08	0.00	-1.34	0.00	-1.21	0.03	-1.11	NE
DAPK1	death-associated protein kinase 1	0.07	-1.24	0.05	1.31	0.93	1.01	0.05	1.30	CD NE AP
DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	0.16	-1.09	0.00	1.28	0.78	1.02	0.01	1.26	CD NE AP
E2F4	E2F transcription factor 4, p107	0.30	1.05	0.00	-1.29	0.01	-1.17	0.06	-1.11	CD NE AP
EGR1	early growth response 1	0.82	-1.01	0.00	1.78	0.01	1.20	0.00	1.48	CD NE
EPHB6	EPH receptor B6	0.48	1.04	0.02	1.19	0.93	1.01	0.02	1.19	CD NE AP
ERN1	endoplasmic reticulum to nucleus signaling 1	0.49	-1.06	0.00	1.77	0.01	1.30	0.01	1.36	CD NE AP
F3	coagulation factor III (thromboplastin, tissue factor)	0.04	-1.08	0.00	1.35	0.00	1.18	0.01	1.14	AP
FAS	Fas (TNF receptor superfamily, member 6)	0.01	-1.11	0.00	1.23	0.00	1.15	0.10	1.07	CD NE AP
FOS	FBJ murine osteosarcoma viral oncogene homolog	0.90	-1.01	0.00	1.24	0.47	1.03	0.00	1.20	CD NE AP
FSTL3	follistatin-like 3 (secreted glycoprotein)	0.00	-1.16	0.00	1.24	0.61	1.02	0.00	1.22	CD NE AP
G0S2	G0-Switch gene 2	0.25	1.06	0.00	1.35	0.01	1.19	0.04	1.14	CD NE AP
G2E3	G2/M-phase specific E3 ubiquitin protein ligase	0.33	-1.03	0.00	-1.98	0.00	-1.81	0.01	-1.09	CD AP
GCLM	glutamate-cysteine ligase, modifier subunit	0.01	1.18	0.00	-1.40	0.00	-1.22	0.02	-1.15	CD NE AP
HIST1H2BO	histone cluster 1, H2bo	0.84	-1.02	0.00	-1.42	0.06	-1.16	0.03	-1.22	CD NE

		DHA vs OA		DHA DOX vs OA		DOX vs OA		DHA DOX vs DOX		
Gene Symbol	**Entrez Gene Name**	P-value	fold change	P-value	fold change	P-value	fold change	P-value	fold change	
HSPB8	heat shock 22kDa protein 8	0.45	-1.09	0.02	1.42	0.07	1.24	0.30	1.14	CD NE AP
ICAM1	intercellular adhesion molecule 1	0.04	-1.22	0.00	1.54	0.11	1.12	0.01	1.37	CD NE AP
ID4	Inhibitor of DNA binding 4	0.09	-1.15	0.01	1.31	0.31	-1.08	0.00	1.42	CD NE AP
IDO1	indoleamine 2,3-dioxygenase 1	0.54	-1.13	0.01	2.08	0.04	1.56	0.20	1.33	CD AP
IGFBP6	Insulin-like growth factor binding protein 6	0.62	-1.03	0.00	1.54	0.00	1.25	0.01	1.23	CD NE AP
IL23A	interleukin 23, alpha subunit p19	0.05	1.08	0.00	1.53	0.00	1.31	0.00	1.17	CD
ISG15	ISG15 ubiquitin-like modifier	0.50	1.04	0.00	1.30	0.04	1.15	0.11	1.12	CD NE AP
ITPR1	inositol 1,4,5-trisphosphate receptor, type 1	0.02	-1.18	0.00	1.32	0.97	-1.00	0.00	1.32	CD NE AP
JAK3	Janus kinase 3	1.00	-1.00	0.00	1.40	0.01	1.26	0.23	1.11	CD NE AP
JUN	jun proto-oncogene	0.30	-1.07	0.01	1.24	0.18	1.10	0.12	1.13	CD NE AP
JUNB	Jun B proto-oncogene	0.00	-1.29	0.00	1.56	0.03	1.18	0.00	1.31	CD NE AP
JUND	Jun D proto-oncogene	0.02	-1.10	0.00	1.37	0.01	1.11	0.00	1.24	CD NE AP
KIF18A	Kinesin family member 18A	0.43	1.03	0.00	-1.81	0.00	-1.50	0.00	-1.21	CD NE AP
KLF4	Kruppel-like factor 4 (gut)	0.50	-1.03	0.00	1.26	0.01	1.15	0.08	1.10	CD NE AP
KSR1	kinase suppressor of ras 1	0.08	-1.11	0.00	1.26	0.07	1.11	0.05	1.14	CD NE AP
LTB	lymphotoxin beta (TNF superfamily, member 3)	0.45	-1.07	0.03	1.29	0.50	1.06	0.08	1.21	CD AP
MAPK13	mitogen-activated protein kinase 13	0.03	-1.09	0.00	1.36	0.00	1.25	0.05	1.09	CD NE AP
MXD1	MAX dimerization protein 1	0.08	-1.11	0.00	1.59	0.00	1.27	0.00	1.25	CD NE AP

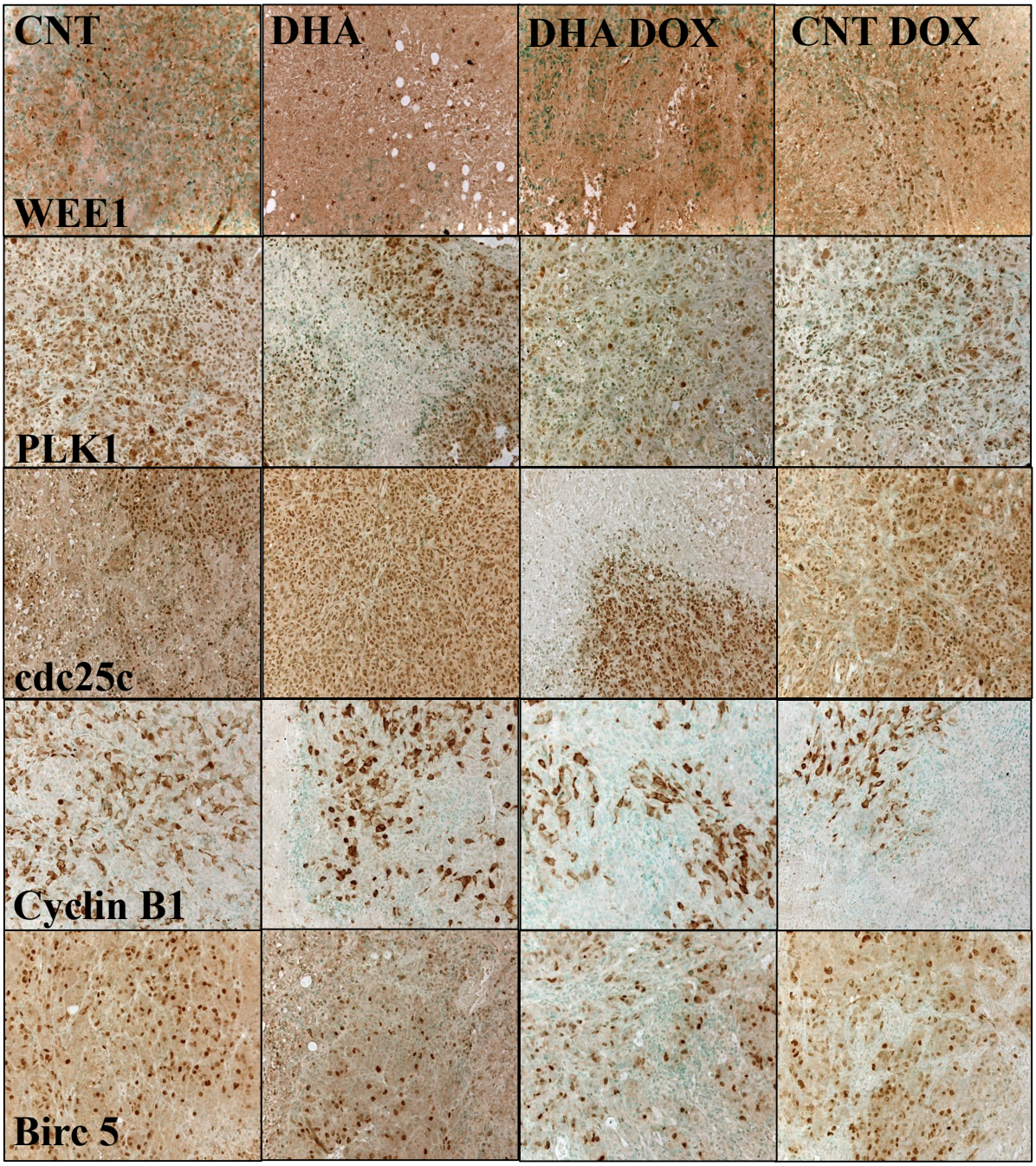
		DHA vs OA		DHA DOX vs OA		DOX vs OA		DHA DOX vs DOX		
Gene Symbol	**Entrez Gene Name**	P-value	fold change	P-value	fold change	P-value	fold change	P-value	fold change	
MDC1	mediator of DNA-damage checkpoint 1	0.95	-1.01	0.04	-1.71	0.38	-1.20	0.14	-1.43	CD NE AP
mir-296	microRNA 296	0.06	-1.20	0.00	-2.15	0.23	1.12	0.00	-2.40	CD NE AP
MLKL	mixed lineage kinase domain-like	0.04	-1.16	0.02	1.24	0.96	1.00	0.02	1.24	CD NE
MLLT11	myeloid/lymphoid or mixed-lineage leukemia	0.24	1.08	0.00	1.50	0.00	1.33	0.12	1.13	CD AP
MMP1	matrix metallopeptidase 1 (interstitial collagenase)	0.34	1.12	0.00	1.75	0.15	1.20	0.02	1.46	CD NE AP
MX1	myxovirus (influenza virus) resistance 1, interferon-inducible p	0.16	-1.20	0.03	1.42	0.24	1.16	0.16	1.22	CD NE
MYCT1	myc target 1	0.49	-1.11	0.03	1.51	0.40	1.14	0.11	1.33	CD AP
NANOG	Nanog homeobox	0.40	-1.12	0.04	-1.43	0.06	-1.32	0.60	-1.08	CD NE AP
NCF2	neutrophil cytosolic factor 2	0.65	-1.07	0.00	1.81	0.09	1.31	0.07	1.39	CD NE AP
NDUFAF 1	NADH dehydrogenase (ubiquinone) complex I, assembly factor 1	0.92	-1.01	0.03	1.20	0.96	-1.00	0.03	1.21	CD NE AP
NEU3	Membrane sialidase 3	0.59	1.03	0.00	-1.33	0.01	-1.21	0.16	-1.10	CD NE AP
NF2	neurofibromin 2 (merlin	0.34	1.06	0.02	-1.20	0.13	-1.10	0.24	-1.09	CD NE AP
NQO2	NAD(P)H dehydrogenase, quinone 2	0.19	1.06	0.00	1.53	0.00	1.34	0.01	1.14	CD NE AP
NRG1	neuregulin 1	0.76	1.02	0.03	1.20	0.89	-1.01	0.03	1.21	CD NE AP
NTN4	netrin 4	0.30	-1.16	0.01	1.70	0.30	1.17	0.04	1.46	CD AP
OAS1	2'-5'-oligoadenylate synthetase 1	0.39	1.10	0.00	1.91	0.01	1.36	0.01	1.41	CD AP
OPTN	optineurin	0.00	-1.16	0.00	1.20	0.01	1.08	0.00	1.11	CD AP
PARP14	poly (ADP-ribose) polymerase family, member 14	0.11	-1.11	0.00	1.35	0.01	1.21	0.11	1.12	CD NE
PI3	Peptidase Inhibitor 3	0.37	1.18	0.00	2.95	0.00	1.97	0.07	1.50	CD NE AP

		DHA vs OA		DHA DOX vs OA		DOX vs OA		DHA DOX vs DOX		
Gene Symbol	**Entrez Gene Name**	P-value	fold change	P-value	fold change	P-value	fold change	P-value	fold change	
PIGA	phosphatidylinositol glycan anchor biosynthesis, class A	0.05	-1.14	0.00	-1.55	0.00	-1.38	0.10	-1.12	CD
PLK4	Polo-like kinase 4	0.99	-1.00	0.00	-1.33	0.01	-1.19	0.08	-1.12	CD NE AP
PON2	paraoxonase 2	0.51	1.06	0.02	-1.31	0.63	-1.04	0.04	-1.25	CD AP
PPP1R15A	protein phosphatase 1, regulatory subunit 15A	0.55	1.03	0.00	1.24	0.02	1.13	0.07	1.10	CD NE AP
RASD1	RAS, dexamethasone-induced 1	0.10	-1.23	0.01	1.47	0.66	1.05	0.03	1.39	CD NE AP
RGS4	regulator of G-protein signaling 4	0.01	1.19	0.00	-1.28	0.14	-1.09	0.03	-1.17	CD NE AP
RNASE3	ribonuclease, RNase A family, 3	0.01	1.35	0.01	1.37	0.22	-1.12	0.00	1.54	CD NE
RNF144B	ring finger protein 144B	0.33	-1.10	0.01	1.40	0.94	1.01	0.01	1.39	CD NE AP
S1PR2	sphingosine-1-phosphate receptor 2	0.18	-1.05	0.00	1.45	0.00	1.30	0.01	1.12	NE AP
SAT1	Spermidine N1-acetyltransferase	0.08	-1.29	0.00	1.80	0.30	1.15	0.01	1.56	CD NE AP
SDC4	syndecan 4	0.06	-1.07	0.00	1.24	0.00	1.14	0.04	1.09	CD NE AP
SIGIRR	single immunoglobulin and toll-interleukin 1 receptor (TIR) d	0.53	-1.03	0.00	-1.29	0.02	-1.13	0.03	-1.14	CD NE
SKIL	SKI-like oncogene	0.10	-1.12	0.00	1.41	0.15	1.10	0.01	1.28	CD NE AP
SLAMF7	SLAM family member 7	0.24	-1.13	0.00	1.51	0.14	1.17	0.04	1.29	CD
SLC12A7	solute carrier family 12	0.38	-1.06	0.03	-1.20	0.40	-1.06	0.09	-1.14	CD
SOCS2	suppressor of cytokine signaling 2	0.61	1.04	0.04	-1.21	0.31	-1.08	0.17	-1.12	CD AP
STAT2	signal transducer and activator of transcription 2, 113kDa	0.01	-1.23	0.00	1.42	0.01	1.25	0.14	1.13	CD NE AP
STC1	stanniocalcin 1	0.01	-1.16	0.00	1.28	0.09	1.09	0.01	1.18	CD NE
SUN2	Sad1 and UNC84 domain containing 2	0.22	-1.06	0.00	-1.24	0.02	-1.13	0.07	-1.10	CD NE AP
TACC3	transforming, acidic coiled-coil containing protein 3	1.00	1.00	0.00	-1.51	0.00	-1.24	0.01	-1.22	CD NE AP

		DHA vs OA		DHA DOX vs OA		DOX vs OA		DHA DOX vs DOX		
Gene Symbol	**Entrez Gene Name**	P-value	fold change	P-value	fold change	P-value	fold change	P-value	fold change	
TFAM	transcription factor A, mitochondria	0.40	-1.06	0.04	-1.20	0.33	-1.07	0.17	-1.12	CD NE AP
TFPI2	tissue factor pathway inhibitor 2	0.20	-1.08	0.00	1.91	0.00	1.68	0.06	1.13	CD NE AP
TGFBI	transforming growth factor, beta 1	0.03	-1.22	0.00	1.50	0.03	1.21	0.03	1.25	CD AP
TLR2	toll-like receptor 2	0.37	-1.07	0.02	1.25	0.20	1.10	0.13	1.14	CD NE AP
TLR4	toll-like receptor 4	0.46	-1.08	0.00	1.60	0.56	1.06	0.01	1.50	CD NE AP
TMEM173	transmembrane protein 173	0.78	-1.01	0.00	1.35	0.00	1.17	0.01	1.15	CD NE
TNFRSF9	tumour necrosis factor receptor superfamily, member 9	0.00	-1.24	0.00	1.83	0.00	1.54	0.02	1.19	CD NE AP
TNFSF15	tumour necrosis factor (ligand) superfamily, member 15	0.22	-1.21	0.00	2.79	0.01	1.65	0.01	1.69	CD NE AP
TRAP1	TNF receptor-associated protein 1	0.05	1.14	0.00	-1.34	0.01	-1.20	0.11	-1.12	CD NE AP
TRPV1	transient receptor potential cation channel, subfamily V, member	0.05	-1.14	0.00	1.73	0.00	1.55	0.14	1.12	CD NE AP
VEGFB	vascular endothelial growth factor B	0.40	-1.05	0.01	-1.23	0.81	1.02	0.01	-1.25	CD NE AP
XAF1	XIAP associated factor 1	0.66	-1.04	0.00	1.47	0.02	1.26	0.13	1.16	CD NE AP
ZYX	zyxin	0.21	1.06	0.00	-1.41	0.00	-1.22	0.02	-1.16	CD NE

P-values and fold change are shown. Genes are considered significantly increased (positive value) or decreased (negative value) based on the selection criteria ($P \leq 0.05$, fold change ≥ 1.2)

Appendix Figure 4: Effect of control or DHA diet with or without chemotherapy on the expression of apoptosis and cell cycle molecules in MDA-MB-231 tumours extracted from *nu/nu* mice.



Representative Immunohistochemical analysis of cell cycle markers: Wee1, Plk1, cdc25c, Cyclin B1 and Birc5. Positive staining is dark brown color and nuclei are stained green (methyl green).

Appendix Table 13: Major fatty acids in the control and DHA-enriched diet

Fatty acids	Control diet	DHA diet
	g/100 g fat	
16:0	21.9±0.04	22.7±0.79
18:0	12.4±0.43	12.2±0.12
18:1 n-9	34.9±1.53	33.6±2.1
18:2 n-6	23.9±1.7	18.5±1.7
18:3 n-6	1.9±0.38	2.6±0.86
20:4 n-6	0.43±0.01	0.43±0.01
22:6 n-3	ND	3.9±0.01
Total SFA	37.5±0.58	39.0±1.41
Total MUFA	26.3±1.37	25.3±0.82
Total PUFA	36.0±1.99	34.9±2.53
Total n-3	0.3±0.13	3.9±0.00
Total n-6	26.1±1.24	23.6±0.27
P/S ratio	0.70±0.03	0.65±0.00

Values are the mean percentage of 3 batches of diet as determined by gas liquid chromatography(Cruz-Hernandez et al.). Diets contained 200 g/kg of fat that was a blend of lard, vegetable oil, canola oil, vegetable shortening and Arasco oil (DSM Nutritional Products USA). The DHA in the DHA diet was provided by adding diet DHAsco (DSM Nutritional Products, USA). Minor fatty acids are not reported; therefore totals do not add up to 100 %. Abbreviations used: saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), polyunsaturated to saturated fatty acid ratio (P/S), not detected (ND; < 0.05%).

Appendix Table 14: Overview of studies investigating Omega-3 supplementation in conjunction with chemotherapy in breast cancer

Model	Study Type	Omega-3 treatment	Chemotherapy Treatment	Main Outcomes	Reference
Human	Open label Phase II	1.8 g DHA	5-FU, epirubicin, cyclophosphamide	Women with higher plasma DHA concentrations associated with longer time to progression and OS compared to women with lower plasma DHA levels	(Bougnoux et al.)
Human	Randomized double blind controlled trial	1 g Omega-3 (51 days)	Cyclophosphamide, doxorubicin, 5-FU	Decreased Ki67 and VEGF expression and increased DFS in intervention group compared to control group	(Darwito et al.)
In vitro	MDA-MB-231 TNBC	30 μM DHA (5 days)	0.5 nM Docetaxel	↓pERK, pAKT; increased docetaxel sensitivity	(Chauvin et al.)
In vitro	MDA-MB-231 TNBC	60 μM DHA (3 days)	0.41 μM Doxorubicin	↑CD95 translocation to lipid rafts	(Ewaschuk et al.)
In vitro	MDA-MB-231 TNBC	60 μM DHA (3 days)	0.41 μM Doxorubicin	↑ apoptosis, ↓ cell cycle progression	(Newell, Brun, et al.)
In vitro	MDA-MB-231 TNBC	29 μM DHA (6 days)	10^{-7} M Doxorubicin	Increased doxorubicin cytotoxicity in combination with DHA	(Germain et al.)
In vitro	MDA-MB-231 TNBC; MCF-7 DOX (doxorubicin resistant)	30 μM DHA (7 days)	10^{-10} M to 10^{-4} M Doxorubicin	Increased doxorubicin cytotoxicity in combination with DHA in both cell lines	(Maheo et al.)
In vitro	MDA-MB-231 TNBC MCF-7 (ER+/PR+)	30 μM DHA (7 days)	5 nM Doxorubicin (MDA's) 20 nM Doxorubicin (MCF-7)	Increased doxorubicin cytotoxicity in combination with DHA with ↓ GPX1 response	(Vibet et al.)

Appendix Table 15: Characteristics of MAXF574 and MAXF401 Patient Derived Xenografts

Characteristics	MAXF574	MAXF401
Differentiation	Poorly differentiated	Moderately differentiated
Vascularization	Well vascularized	Poorly vascularized
Patient age at surgery	Unknown	51
Patient histology	Invasive ductal carcinoma	Adeno carcinoma highly metastatic
Stage at surgery	Not available	Metastasis to lung
Previous Chemotherapy / Radiotherapy	Not available	Radiation
Origin of xenograft	Unknown	Metastasis

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Appendix Table 16: Complete Blood Count analysis of MAXF401 PDX bearing NSG mice fed control or DHA diets with or without docetaxel chemotherapy

Parameter	Concentration	Normal Range	Control	Control+TXT	HDHA+TXT	LDHA+TXT
WBC	$x10^3$ cells/µl	3.2-12.7	5.2±0.7	5.2±0.7	6.1±0.7	6.0±0.7
RBC	$x10^6$ cells/µl	7.0-10.1	8.4±0.3	9.1±0.5	8.8±0.2	8.8±0.3
HGB	g/L	118-149	135.6±4.4	132.0±10.7	135.6±1.7	126.5±6.2
HCT		0.37-0.47	0.5±0.0	0.5±0.0	0.5±0.0	0.5±0.0
MCV	fL	42.2-59.2	57.9±0.4	56.3±0.5	55.2±0.2	54.6±0.4
MCH	pg	13.8-18.4	16.1±0.3	16.0±0.5	15.1±0.2	14.4±0.5
MCHC	g/dL	31.0-34.7	279.2±2.3	275.0±2.6	272.1±2.5	273.0±2.7
PLT	$x10^3$ cells/µl	766-1657	1055.5±116.7	1102.0±99.6	1213.0±88.2	1192.7±41.4
RDW	$x10^3$ cells/µl	11.7-15.1	16.2±0.3	15.8±0.6	16.0±0.2	15.6±0.1
NEUT	%	6.8-31.1	59.9±1.3	60.7±5.0	67.9±2.9	64.4±4.3
LYMPH	%	60.2-95.0	23.0±1.1	22.0±4.4	17.7±1.5	19.8±2.7
MONO	%	0.0-4.3	9.6±1.6	10.9±3.2	7.9±1.3	10.6±1.7
EOS	%	0.2-5.9	4.6±1.9	4.0±1.6	2.5±0.4	2.6±0.3
BASO	%	0>1-0.3	1.2±0.4	1.5±0.1	1.6±0.3	2.6±0.8
LUC	%	0.0-3.2	0.8±0.2	0.4±0.2	1.8±0.4	1.3±0.2
RETIC	%	1.4-5.7	7.0±0.8	5.0±1.5	4.8±0.3	4.7±0.5

NSG Mice were implanted with Patient derived xenografts and randomized to control or DHA diets once tumours were established for one week prior to commencing chemotherapy. Mice received docetaxel chemotherapy (5mg/kg) or 0.9% saline control twice weekly for 6 weeks. Values represent the mean ± SEM (n=7). Abbreviations used: BASO, basophils; EOS, eosinophils; HDHA, high docosahexaenoic acid; HCT, hematocrit; HGB, hemoglobin; LDHA, low docosahexaenoic acid; LYMPH, lymphocytes; LUC, large unstained cells; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MONO, monocytes; NEUT, neutrophils; PDX, patient derived xenograft; PLT, platelets; RBC, red blood cells; RDW, red cell distribution; RETIC, reticulocytes; TXT, docetaxel; WBC, white blood cells.

Appendix Table 17: Total phospholipid composition (relative % total fatty acids) of tumours and plasma from NSG mice implanted with MAXF401 PDX tumours

	Tumour				Plasma			
	Control	**Control+TXT**	**HDHA+TXT**	**LDHA+TXT**	**Control**	**Control+TXT**	**HDHA+TXT**	**LDHA+TXT**
14:0	0.9±0.0	1.0±0.1	1.0±0.0	1.0±0.1	0.1±0.04	0.1±0.0	0.1±0.0	0.1±0.0
16:0	22.8±0.4	23.3±0.3	22.9±0.4	23.3±0.5	26.3±0.4	25.9±0.5	27.2±0.5	26.5±0.7
16:1 n-9	4.1±0.2^{ab}	3.8±0.4^{b}	4.8±0.2^{a}	4.1±0.2^{ab}	0.4±0.0	0.4±0.0	0.4±0.02	0.4±0.0
18:0	20.0±0.3^{ab}	21.0±0.5^{a}	19.0±0.4^{b}	20.2±0.4^{ab}	22.4±0.5^{b}	23.2±0.6^{ab}	23.4±0.4^{ab}	24.7±0.7^{a}
18:1 n-9	17.6±0.6^{b}	16.8±0.6^{b}	19.6±0.3^{a}	19.3±0.6^{a}	8.2±0.4^{c}	8.6±0.2^{bc}	9.3±0.2^{ab}	10.0±0.3^{a}
18:2 n-6	5.4±0.2	5.8±0.3	5.6±0.1	5.2±0.2	19.1±0.8	19.8±0.5	18.7±0.5	18.4±0.7
20:0	0.6±0.0	0.4±0.0	0.5±0.0	0.5±0.0	0.1±0.0^{a}	0.1±0.0^{b}	0.1±0.0^{ab}	0.1±0.0^{ab}
18:3 n-6	0.3±0.0	0.3±0.0	0.3±0.0	0.3±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.2±0.0
18:3 n-3	0.3±0.0	0.2±0.0	0.3±0.0	0.3±0.0	0.1±0.0^{b}	0.1±0.0^{b}	0.1±0.0^{a}	0.1±0.0^{a}
20:2 n-6	0.4±0.0	0.3±0.1	0.6±0.0	0.4±0.1	0.3±0.0	0.3±0.0	0.4±0.0	0.4±0.0
20:3 n-6	1.4±0.2	1.2±0.1	1.2±0.1	1.2±0.1	1.2±0.0^{b}	1.2±0.0^{b}	1.8±0.1^{a}	1.6±0.1^{a}
20:4 n-6	15.6±0.4^{b}	17.1±0.7^{a}	12.7±0.2^{c}	12.3±0.6^{c}	13.8±0.5^{a}	13.0±0.4^{a}	7.5±0.4^{b}	7.9±0.4^{b}
20:5 n-3	0.4±0.0^{b}	0.3±0.0^{b}	0.8±0.1^{a}	1.0±0.1^{a}	0.1±0.0^{c}	0.1±0.0^{c}	0.4±0.1^{b}	0.6±0.0^{a}
24:0	1.3±0.0	1.3±0.1	1.3±0.0	1.5±0.2	0.2±0.0	0.1±0.0	0.2±0.1	0.1±0.0
24:1 n-9	1.6±0.1^{a}	1.3±0.1^{b}	1.4±0.1^{ab}	1.6±0.1^{ab}	0.2±0.0^{a}	0.3±0.0^{a}	0.2±0.0^{b}	0.2±0.0^{b}
22:4 n-6	0.1±0.0^{a}	0.1±0.0^{b}	0.1±0.0^{b}	0.1±0.0^{b}	0.6±0.1	0.5±0.0	0.4±0.1	0.6±0.1
22:5 n-6	0.3±0.0	0.3±0.0	0.2±0.0	0.4±0.1	0.4±0.0^{a}	0.5±0.0^{a}	0.4±0.1^{a}	0.1±0.0^{b}
22:5 n-3	0.7±0.0^{c}	0.6±0.0^{c}	0.9±0.0^{b}	1.2±0.1^{a}	0.2±0.0	0.2±0.0	0.3±0.0	0.3±0.0
22:6 n-3	3.8±0.2^{b}	3.8±0.2^{b}	5.7±0.3^{a}	5.1±0.4^{a}	5.6±0.4^{c}	5.0±0.2^{c}	8.7±0.2^{a}	7.4±0.4^{b}
Total SFA	47.7±0.3^{a}	47.9±0.7^{a}	45.7±0.6^{b}	46.1±0.8^{ab}	49.5±0.4^{c}	49.8±0.6^{bc}	51.4±0.6^{ab}	51.8±0.6^{a}
Total PUFA	29.0±0.6	30.3±1.0	28.6±0.5	27.8±1.2	41.7±0.3^{a}	41.0±0.7^{a}	38.9±0.6^{b}	37.6±0.6^{b}
Total MUFA	23.3±0.7^{bc}	21.8±1.0^{c}	25.7±0.4^{a}	20.3±0.6^{ab}	8.8±0.4^{c}	9.3±0.2^{bc}	9.8±0.2^{ab}	10.5±0.3^{a}
Total N-6	23.5±0.4^{a}	25.0±0.8^{a}	20.6±0.2^{b}	21.9±0.8^{b}	35.6±0.4^{a}	35.4±0.6^{a}	29.3±0.5^{b}	29.2±0.5^{b}
Total N-3	5.5±0.2^{b}	5.3±0.2^{b}	8.0±0.4^{a}	7.7±0.5^{a}	6.0±0.4^{c}	5.5±0.2^{c}	9.6±0.2^{a}	8.5±0.4^{b}

NSG mice implanted with MAXF401 PDX tumours were maintained on a 20± (± 3.8± DHA) w/w diet for 7 weeks (1-week diet alone followed by 6 weeks with or without TXT treatment). Values are percentages relative to the total fatty acid content ± SE (n=7). Within the rows labeled means without a common letter differ (P<0.05) based on post hoc DUNCAN analysis. Abbreviations used: HDHA, high docosahexaenoic acid; LDHA, low docosahexaenoic acid; MUFA, monounsaturated fatty acids; PDX, patient derived xenograft; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TXT, docetaxel.

Appendix Table 18: Total phospholipid composition (relative % total fatty acids) of liver and gastrocnemius muscle from NSG mice implanted with MAXF401 PDX tumours

	Liver				Gastrocnemius muscle			
	Control	**Control+TXT**	**HDHA+TXT**	**LDHA+TXT**	**Control**	**Control+TXT**	**HDHA+TXT**	**LDHA+TXT**
14:0	0.1±0.0^{a}	0.0±0.0^{ab}	0.0±0.0 ^{a}	0.0±0.0^{b}	0.1±0.0^{b}	0.3±0.1^{ab}	0.3±0.0^{ab}	0.4±0.1^{a}
16:0	23.5±0.4^{a}	22.0±0.4^{c}	23.4±0.6^{ab}	22.3±0.3^{bc}	28.0±0.4	26.6±0.9	27.7±0.4	27.2±1.0
16:1 n-9	0.5±0.0^{a}	0.4±0.0^{ab}	0.4±0.0^{ab}	0.4±0.0^{b}	1.3±0.1	1.3±0.2	1.2±0.1	1.2±0.1
18:0	25.6±0.6	24.5±0.6	24.7±0.6	24.8±0.4	15.6±0.3	16.4±0.6	15.9±0.3	16.5±0.6
18:1 n-9	8.8±0.4	8.8±0.2	9.2±0.3	9.0±0.3	7.8±0.3	9.2±1.0	8.0±0.4	8.2±0.6
18:2 n-6	12.2±0.3^{a}	12.0±0.2^{ab}	12.4±0.3^{a}	11.4±0.2^{b}	5.4±0.2^{a}	6.3±0.7^{a}	3.7±0.2^{b}	3.9±0.4^{b}
20:0	0.4±0.0^{bc}	0.5±0.0^{a}	0.3±0.0^{c}	0.4±0.0^{b}	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0
18:3 n-6	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.0±0.0^{b}	0.0±0.0^{b}	0.0±0.0^{ab}	0.0±0.0^{a}
18:3 n-3	0.3±0.0	0.4±0.0	0.3±0.0	0.3±0.0	0.3±0.0^{b}	0.2±0.0^{b}	0.3±0.0^{b}	0.4±0.0^{a}
20:2 n-6	0.4±0.0	0.4±0.0	0.4±0.0	0.3±0.0	0.5±0.0^{a}	0.5±0.0^{a}	0.4±0.0^{b}	0.4±0.0^{b}
20:3 n-6	1.4±0.1^{c}	1.7±0.0^{b}	2.0±0.1^{a}	2.2±0.1^{a}	0.6±0.0^{ab}	0.6±0.1^{a}	0.5±0.0^{ab}	0.5±0.0^{b}
20:4 n-6	16.8±0.4^{a}	17.6±0.2^{a}	10.9±0.4^{c}	12.6±0.3^{b}	10.0±0.3^{a}	10.0±0.2^{a}	4.6±0.2^{b}	5.0±0.2^{b}
20:5 n-3	0.1±0.0^{b}	0.1±0.0^{b}	0.9±0.1^{a}	0.9±0.1^{a}	0.1±0.0^{c}	0.1±0.0^{c}	0.1±0.0^{b}	0.3±0.0^{a}
24:0	0.2±0.0^{b}	0.3±0.0^{a}	0.2±0.0^{ab}	0.3±0.0^{a}	0.2±0.0	0.2±0.1	0.2±0.0	0.1±0.0
24:1 n-9	0.6±0.1^{b}	1.0±0.0^{a}	0.6±0.1^{b}	0.7±0.0^{b}	0.2±0.0	0.2±0.1	0.2±0.0	0.2±0.0
22:4 n-6	0.5±0.1^{a}	0.5±0.1^{a}	0.1±0.0^{b}	0.1±0.0^{b}	1.2±0.0^{a}	1.2±0.0^{a}	0.3±0.0^{b}	0.3±0.0^{b}
22:5 n-6	0.2±0.0^{b}	0.2±0.0^{b}	0.2±0.0^{b}	0.4±0.0^{a}	2.0±0.1^{a}	1.8±0.2^{a}	0.1±0.0^{b}	0.3±0.0^{b}
22:5 n-3	0.1±0.0^{c}	0.1±0.0^{c}	0.2±0.0^{b}	0.2±0.0^{a}	2.0±0.1^{a}	1.8±0.2^{a}	1.1±0.0^{b}	2.0±0.1^{a}
22:6 n-3	7.6±0.7^{b}	8.5±0.4^{b}	13.0±0.6^{a}	13.0±0.1^{a}	24.4±0.6^{c}	22.6±1.3^{c}	35.0±0.7^{a}	32.6±0.6^{b}
Total SFA	50.2±0.8^{a}	47.8±0.6^{b}	49.2±0.8^{ab}	48.2±0.4^{ab}	44.4±0.3	44.1±0.6	44.5±0.4	44.7±0.6
Total PUFA	40.0±0.9	42.0±0.6	40.7±0.7	41.7±0.4	46.3±0.3	45.1±1.0	46.2±0.5	45.7±0.4
Total MUFA	9.8±0.4	10.2±0.2	10.1±0.3	10.1±0.3	9.3±0.4	10.8±1.3	9.4±0.4	9.6±0.7
Total N-6	31.6±0.4^{a}	32.7±0.4^{a}	26.1±0.4^{b}	27.0±0.3^{b}	19.6±0.6^{a}	20.4±0.7^{a}	9.6±0.43^{b}	10.4±0.4^{b}
Total N-3	8.4±0.7^{b}	9.3±0.4^{b}	14.6±0.6^{a}	14.7±0.1^{a}	26.7±0.7^{b}	24.7±1.4^{b}	36.5±0.8^{a}	35.3±0.6^{a}

NSG mice implanted with MAXF401 PDX tumours were maintained on a 20± (± 3.8± DHA) w/w diet for 7 weeks (1-week diet alone followed by 6 weeks with or without TXT treatment). Values are percentages relative to the total fatty acid content ± SE (n=7). Within the rows labeled means without a common letter differ (P<0.05) based on post hoc DUNCAN analysis. Abbreviations used: HDHA, high docosahexaenoic acid; LDHA, low docosahexaenoic acid; MUFA, monounsaturated fatty acids; PDX, patient derived xenograft; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TXT, docetaxel.

Appendix Table 19: Total phospholipid composition (relative % total fatty acids) of tumours and plasma from NSG mice implanted with MAXF574 PDX tumours

	Tumour				Plasma			
	Control	**Control+TXT**	**HDHA**	**HDHA+TXT**	**Control**	**Control+TXT**	**HDHA**	**HDHA+TXT**
14:0	1.9±0.1	1.8±0.1	2.0±0.2	2.0±0.1	0.4±0.0^{b}	0.3±0.1^{bc*}	0.6±0.1^{a}	0.1±0.0^{c*}
16:0	21.9±0.3	21.8±0.4	22.1±0.3	21.9±0.2	23.5±0.6^{a}	21.7±0.8^{b*}	22.4±0.3^{ab}	21.6±0.3^{b*}
16:1 n-9	4.2±0.1	4.6±0.3	4.6±0.4	4.5±0.1	0.4±0.0^{b}	0.3±0.0^{b*}	0.6±0.1^{a}	0.3±0.0^{b*}
18:0	12.9±0.4	13.4±0.4	13.8±0.8	14.0±0.2	27.1±0.9^{b}	29.6±0.7^{ab}	30.6±0.6^{a}	29.4±0.8^{ab}
18:1 n-9	27.9±0.6^{a}	27.1±1.19^{ab}	25.2±0.7^{b}	25.6±0.5^{b}	10.6±0.3	10.3±0.34	8.9±0.1	9.1±0.3
18:2 n-6	10.0±0.2^{b}	10.3±0.4^{b*}	12.3±0.4^{a}	12.9±0.2^{a*}	16.1±0.8^{b}	15.0±0.5^{b}	20.4±0.4^{a}	21.0±0.5^{a}
20:0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0^{b}	0.2±0.0^{ab}	0.2±0.0^{ab}	0.2±0.0^{a}
18:3 n-6	0.9±0.1^{a}	1.0±0.0^{a}	0.7±0.0^{b}	0.7±0.0^{b}	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
18:3 n-3	0.8±0.1^{a}	0.8±0.0^{a}	0.6±0.0^{b}	0.7±0.0^{b}	0.3±0.0^{ab}	0.3±0.0^{a*}	0.2±0.0^{b}	0.2±0.0^{b*}
20:2 n-6	1.3±0.1	1.2±0.0	1.2±0.0	1.3±0.0	0.3±0.0^{a}	0.4±0.0^{a*}	0.2±0.0^{b}	0.3±0.0^{a*}
20:3 n-6	1.4±0.0^{c}	1.4±0.0^{bc*}	1.8±0.1^{a}	1.5±0.0^{b*}	1.0±0.1^{c}	1.2±0.0^{c*}	1.7±0.1^{b}	1.9±0.0^{a*}
20:4 n-6	9.7±0.3^{a}	9.6±0.2^{a}	5.8±0.3^{b}	5.5±0.2^{b}	12.7±0.7^{a}	14.1±0.5^{a}	6.0±0.3^{b}	6.4±0.2^{b}
20:5 n-3	0.1±0.0^{b}	0.1±0.0^{b*}	0.4±0.0 ^{a}	0.4±0.0^{a*}	0.1±0.0^{c}	0.1±0.0^{c}	0.3±0.0^{b}	0.4±0.0^{a}
24:0	0.4±0.1^{b}	0.3±0.0^{b}	0.6±0.0^{a}	0.4±0.0^{b}	0.1±0.1^{b}	0.2±0.0^{b}	0.3±0.0^{a}	0.3±0.0^{a}
24:1 n-9	1.4±0.1^{a}	1.4±0.1^{a}	0.6±0.0^{b}	0.5±0.0^{b}	0.9±0.1	1.0±0.1	0.8±0.01	1.0±0.0
22:4 n-6	1.4±0.2^{b}	1.2±0.1^{b*}	1.7±0.1^{a}	1.2±0.1^{b*}	0.2±0.0^{a}	0.2±0.0^{b}	0.1±0.0^{bc}	0.1±0.0^{c}
22:5 n-6	0.5±0.0^{a}	0.5±0.0^{a}	0.3±0.0^{b}	0.2±0.0^{b}	0.7±0.0^{a}	0.7±0.1^{a}	0.2±0.0^{b}	0.2±0.0^{b}
22:5 n-3	0.2±0.0^{b}	0.2±0.0^{b}	0.7±0.0^{a}	0.7±0.0^{a}	0.1±0.0^{b}	0.1±0.0^{b}	0.2±0.0^{a}	0.1±0.0^{ab}
22:6 n-3	2.4±0.2^{b}	2.5±0.2^{b}	4.8±0.2^{a}	5.2±0.2^{a}	3.3±0.3^{c}	4.0±0.0^{c*}	5.8±0.3^{b}	6.8±0.1^{a*}
Total SFA	37.9±0.6	38.1±0.7	39.3±0.4	39.1±0.5	51.8±0.9	52.3±0.4	54.5±0.5	51.9±0.6
Total PUFA	28.6±0.2^{b}	28.8±0.4^{b}	30.3±0.6^{a}	30.3±0.4^{a}	35.1±0.9^{b}	36.0±0.4^{ab*}	35.3±0.4^{b}	37.7±0.5^{a*}
Total MUFA	33.5±0.6^{a}	33.1±1.0^{a}	30.4±0.9^{b}	30.6±0.5 ^{b}	11.8±0.3^{a}	11.7±0.4^{a}	10.3±0.2^{b}	10.4±0.4^{b}
Total N-6	25.2±0.2^{a}	25.3±0.5^{a}	23.5±0.5^{b}	23.3±0.2^{b}	31.1±0.8^{a}	31.4±0.4^{a}	28.7±0.2^{b}	29.9±0.4^{ab}
Total N-3	3.4±0.2^{b}	3.5±0.2^{b*}	6.5±0.2^{a}	7.0±0.2^{a*}	4.0±0.3^{c}	4.6±0.0^{c*}	6.6±0.3^{b}	7.8±0.2^{a*}

NSG mice implanted with MAXF574 PDX tumours were maintained on a 20% (± 3.8% DHA) w/w diet for 7 weeks (1-week diet alone followed by 6 weeks with or without TXT treatment). Values are percentages relative to the total fatty acid content ± SE (n=7). Within the rows labeled means without a common letter differ and * indicates a chemotherapy effect (P<0.05) based on post hoc DUNCAN analysis. Abbreviations used: HDHA, high docosahexaenoic acid; MUFA, monounsaturated fatty acids; PDX, patient derived xenograft; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TXT, docetaxel.

Appendix Table 20: Total phospholipid composition (relative % total fatty acids) of liver and gastrocnemius muscle from NSG mice implanted with MAXF574 PDX tumours

	Liver				Gastrocnemius muscle			
	Control	**Control+TXT**	**HDHA**	**HDHA+TXT**	**Control**	**Control + TXT**	**HDHA**	**HDHA+TXT**
14:0	ND	ND	ND	ND	0.4±0.1	0.4±0.1	0.2±0.1	0.2±0.0
16:0	19.0±0.3^{a}	17.6±0.3^{b}	17.2±0.3^{b}	17.6±0.3^{b}	27.1±0.3	29.2±1.5	25.0±0.3	24.7±1.1
16:1 n-9	0.4±0.0^{ab}	0.4±0.0^{b}	0.4±0.02^{ab}	0.4±0.0^{a}	1.0±0.0	1.4±0.1	1.2±0.1	1.2±0.1
18:0	22.6±0.5^{bc}	23.8±0.3^{b}	24.5±0.4^{ab}	24.3±0.6^{ab}	15.8±0.6	15.1±0.6	17.2±0.1	16.6±1.2
18:1 n-9	9.7±0.2	9.2±0.2	8.2±0.1	8.6±0.3	8.4±0.3	8.7±0.2	6.8±0.4	7.1±0.3
18:2 n-6	10.9±0.3^{b}	10.9±0.4^{b}	14.2±0.3^{a}	14.3±0.3^{a}	5.4±0.2	4.9±0.2	4.0±0.1	4.5±0.6
20:0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0
18:3 n-6	0.1±0.0^{b}	0.1±0.0^{b}	0.1±0.0^{a}	0.1±0.0^{a}	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
18:3 n-3	0.2±0.0	0.2±0.0	0.1±0.0	0.2±0.0	0.1±0.0	0.2±0.1	0.1±0.0	0.1±0.0
20:2 n-6	0.3±0.0	0.3±0.0	0.3±0.0	0.3±0.0	0.4±0.0	0.5±0.0	0.2±0.0	0.2±0.0
20:3 n-6	1.0±0.0^{b}	1.0±0.1^{b}	2.0±0.0^{a}	2.1±0.1^{a}	0.7±0.0	0.7±0.0	0.4±0.0	0.4±0.0
20:4 n-6	22.0±0.3^{a}	22.0±0.2^{a}	11.9±0.3^{b}	11.4±0.4^{b}	11.2±0.2	10.8±0.4	3.2±0.1	3.6±0.1
20:5 n-3	0.1±0.0^{c}	0.1±0.0^{c*}	0.8±0.0^{b}	1.1±0.1^{a*}	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0
24:0	0.6±0.0^{a}	0.6±0.0^{a}	0.1±0.0^{b}	0.2±0.0^{b}	0.3±0.1	0.1±0.1	0.2±0.0	0.2±0.1
24:1 n-9	0.7±0.0^{b}	0.8±0.0^{ab}	0.6±0.0^{c}	0.7±0.0^{ab}	0.1±0.0	0.2±0.1	0.2±0.1	0.3±0.0
22:4 n-6	1.6±0.2^{a}	1.4±0.1^{a}	0.0±0.0^{b}	0.0±0.0^{b}	1.6±0.1	1.7±0.0	0.1±0.0	0.1±0.0
22:5 n-6	0.1±0.0^{b}	0.1±0.1^{a}	0.1±0.0^{ab}	0.1±0.0^{b}	3.6±0.2	3.3±0.2	0.1±0.0	0.1±0.0
22:5 n-3	0.3±0.0^{b}	0.3±0.0^{ab}	0.3±0.0^{ab}	0.4±0.0^{a}	1.8±0.1	1.8±0.2	1.3±0.1	1.2±0.0
22:6 n-3	10.0±0.4^{b}	10.9±0.4^{b}	18.5±0.3^{a}	17.8±0.4^{a}	21.5±1.2	20.5±1.2	39.3±0.4	38.9±0.3
Total SFA	42.6±0.3	42.3±0.2	42.2±0.4	42.4±0.4	44.1±0.8	45.2±2.0	43.1±0.2	42.1±0.0
Total PUFA	46.5±0.3^{c}	47.3±0.2^{bc}	48.5±0.4^{a}	47.8±0.2^{ab}	46.4±1.0	44.4±1.8	48.8±0.3	49.3±0.4
Total MUFA	10.9±0.2^{a}	10.4±0.2^{ab}	9.3±0.1^{c}	9.8±0.3^{bc}	9.5±0.2	10.3±0.1	8.1±0.3	8.6±0.4
Total N-6	35.9±0.2^{a}	35.8±0.4^{a}	28.6±0.3^{b}	28.3±0.3^{b}	22.9±0.4	21.9±0.4	8.0±0.1	9.0±0.7
Total N-3	10.6±0.4^{b}	11.5±0.3^{b}	19.9±0.3^{a}	19.6±0.4^{a}	23.5±1.3	22.5±1.4	40.8±0.4	40.3±0.3

NSG mice implanted with MAXF574 PDX tumours were maintained on a 20% (± 3.8% DHA) w/w diet for 7 weeks (1-week diet alone followed by 6 weeks with or without TXT treatment). Values are percentages relative to the total fatty acid content ± SE (n=7). Within the rows labeled means without a common letter differ and * indicates a chemotherapy effect (P<0.05) based on post hoc DUNCAN analysis. Abbreviations used: HDHA, high docosahexaenoic acid; MUFA, monounsaturated fatty acids; PDX, patient derived xenograft; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TXT, docetaxel.

Appendix Table 21: Phosphatidylethanolamine phospholipid fatty acid composition (relative %) from NSG mice implanted with MAXF401 or MAXF574 PDX tumours

	MAXF401				MAXF574			
	Control	**Control + TXT**	**HDHA + TXT**	**LDHA+TXT**	**Control**	**Control + TXT**	**HDHA**	**HDHA + TXT**
14:0	0.2±0.0	0.2±0.0	0.2±0.0	0.4±0.1	0.4±0.1^{b}	0.7±0.2^{b}	0.4±0.0^{b}	1.4±0.3^{a}
16:0	12.9±0.8	14.1±0.3	13.6±1.0	14.3±1.0	11.4±0.7	10.8±0.6	11.6±0.4	11.4±0.4
16:1 n-9	1.6±0.3^{a}	1.0±0.1^{b}	1.5±0.2^{a}	1.5±0.1^{a}	3.2±0.3^{b}	3.2±0.3^{b}	4.2±0.2^{a}	3.7±0.2^{ab}
18:0	23.5±0.6^{c}	27.7±0.7^{a}	25.1±0.6^{b}	26.5±0.7^{ab}	13.4±0.8^{b}	14.0±0.4^{ab}	14.4±0.8^{ab}	15.1±0.3^{a}
18:1 n-9	13.7±0.9^{a}	9.4±0.7^{b}	14.4±0.2^{a}	14.2±0.4^{a}	35.5±2.6	35.2±0.5	36.2±1.3	32.9±0.2
18:2 n-6	3.2±0.2^{a}	2.3±0.1^{b}	3.0±0.1^{a}	3.0±0.1^{a}	9.2±0.6^{c}	9.3±0.2^{c}	13.3±0.3^{a}	12.0±0.4^{b}
20:0	0.2±0.0	0.2±0.0	0.2±0.1	0.3±0.0	0.1±0.0	0.1±0.0	0.2±0.0	0.2±0.1
18:3 n-6	0.5±0.1^{b}	0.5±0.1^{b}	0.4±0.1^{b}	0.8±0.1^{a}	1.2±0.2^{a}	1.3±0.1^{a}	0.9±0.1^{b}	0.9±0.0^{b}
18:3 n-3	0.3±0.0^{b}	0.6±0.1^{a}	0.7±0.1^{a}	0.4±0.0^{b}	0.8±0.1^{a}	0.8±0.1^{a}	0.6±0.0^{b}	0.8±0.0^{a}
20:2 n-6	0.2±0.0	0.3±0.1	0.4±0.1	0.3±0.0	1.1±0.1^{a}	1.1±0.1^{a}	0.9±0.0^{b}	1.0±0.0^{ab}
20:3 n-6	1.2±0.1^{b}	1.0±0.0^{c}	1.4±0.0^{a}	1.2±0.0^{b}	2.3±0.9	1.4±0.0	1.6±0.1	1.4±0.1
20:4 n-6	26.0±0.5^{a}	26.4±0.1^{a}	21.0±0.7^{b}	20.1±0.8^{b}	13.5±0.9^{a}	14.8±0.9^{a}	8.4±0.3^{b}	8.2±0.5^{b}
20:5 n-3	0.3±0.0^{b}	0.4±0.1^{b}	1.4±0.1^{a}	1.6±0.2^{a}	0.1±0.0^{b}	0.1±0.0^{b}	0.2±0.0^{b}	0.7±0.0^{a}
24:0	0.3±0.1^{b}	0.4±0.0^{a}	0.3±0.0^{b}	0.4±0.0^{a}	1.7±0.4^{a}	0.4±0.1^{c}	0.8±0.1^{b}	0.4±0.1^{c}
24:1 n-9	0.3±0.0^{a}	0.4±0.0^{a}	0.2±0.0^{b}	0.3±0.0^{a}	0.6±0.4^{a}	0.1±0.0^{b}	0.5±0.1^{a}	0.6±0.0^{a}
22:4 n-6	5.2±0.3^{a}	4.8±0.2^{a}	2.3±0.1^{b}	2.4±0.1^{b}	1.0±0.3^{b}	1.8±0.1^{s}	0.2±0.1^{c}	0.2±0.0^{c}
22:5 n-6	1.1±0.1^{a}	1.1±0.1^{a}	0.3±0.0^{b}	0.3±0.0^{b}	0.4±0.3^{ab}	0.5±0.1^{a}	0.1±0.0^{b}	0.2±0.0^{b}
22:5 n-3	1.2±0.0^{bc}	1.0±0.1^{c}	1.5±0.1^{b}	1.9±0.1^{a}	0.3±0.0^{b}	0.4±0.0^{b}	0.6±0.1^{a}	0.8±0.0^{a}
22:6 n-3	7.8±0.4^{b}	7.3±0.7^{b}	10.8±0.7^{a}	9.1±0.6^{b}	2.7±0.5^{c}	3.7±0.4^{c}	4.6±0.2^{b}	6.5±0.4^{a}
Total SFA	37.6±1.4^{b}	44.57±1.8^{a}	40.6±1.3^{ab}	42.9±1.6^{a}	27.9±2.9	26.3±0.7	27.6±1.0	29.8±0.5
Total PUFA	46.8±0.9^{a}	44.8±1.9^{ab}	43.2±1.3^{ab}	41.1±1.7^{b}	32.7±0.9^{ab}	35.2±1.3^{a}	31.5±0.7^{b}	32.9±0.7^{b}
Total MUFA	15.6±1.0^{a}	10.7±0.8^{b}	16.2±0.3^{a}	16.0±0.5^{a}	39.4±2.6	38.5±0.8	40.9±1.3	37.2±0.3
Total N-6	37.2±0.7^{a}	35.3±1.2^{a}	28.7±0.8^{b}	28.0±0.9^{b}	28.8±0.6^{a}	30.2±0.9^{a}	25.3±0.5^{b}	23.9±0.4^{b}
Total N-3	9.6±0.4^{b}	9.5±0.8^{b}	14.5±0.8^{a}	13.1±0.8^{a}	4.0±0.5^{c}	5.0±0.4^{c}	6.2±0.3^{b}	9.0±0.4^{a}

NSG mice implanted with MAXF401 or MAXF574 PDX tumours were maintained on a 20% (± 3.8% or 1.6 % DHA) w/w diet for 7 weeks (1-week diet alone followed by 6 weeks with or without TXT treatment). Values are percentages relative to the total fatty acid content ± SE (n=7). Within the rows and tumour type, labeled means without a common letter differ (P<0.05) based on post hoc DUNCAN analysis. Abbreviations used: HDHA, high docosahexaenoic acid; LDHA, low docosahexaenoic acid; MUFA, monounsaturated fatty acids; PDX, patient derived xenograft; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TXT, docetaxel.

Appendix Table 22: Phosphatidylserine phospholipid fatty acid composition (relative %) from NSG mice implanted with MAXF401 or MAXF574 PDX tumours

	MAXF401				MAXF574			
	Control	**Control + TXT**	**HDHA + TXT**	**LDHA+TXT**	**Control**	**Control + TXT**	**HDHA**	**HDHA+ TXT**
14:0	0.4±0.1^{ab}	0.7±0.1^{a}	0.2±0.1^{b}	0.3±0.1^{b}	0.3±0.0	0.3±0.0	0.5±0.1	0.5±0.2
16:0	4.2±0.5	5.9±0.9	4.6±0.9	4.2±0.5	6.0±0.4	5.2±0.0	4.9±0.7	5.9±0.4
16:1 n-9	1.9±0.1^{bc}	1.4±0.3^{c}	2.4±0.1^{a}	2.2±0.1^{ab}	2.3±0.1^{a}	2.4±0.4^{a}	1.8±0.2^{b}	1.5±0.1^{b}
18:0	46.8±0.2^{b}	49.5±0.4^{a}	49.5±0.6^{a}	49.2±0.8^{a}	36.7±1.0^{b}	35.7±0.4^{b}	40.3±1.7^{a}	40.5±0.7^{a}
18:1 n-9	26.1±0.7^{b}	23.3±0.9^{c}	27.7±0.5^{ab}	29.2±0.9^{a}	24.9±0.6	25.8±0.7	23.0±1.4	22.8±0.2
18:2 n-6	2.8±0.2	2.6±0.2	2.7±0.3	2.9±0.2	7.2±0.3^{b}	7.0±0.3^{b}	9.1±0.4^{a}	9.0±0.2^{a}
20:0	1.2±0.0	1.3±0.0	1.3±0.1	1.3±0.2	0.1±0.0^{c}	0.4±0.1^{b}	0.6±0.0^{a}	0.6±0.0^{a}
18:3 n-6	0.3±0.0	0.4±0.1	0.3±0.0	0.2±0.0	0.6±0.0^{b}	1.0±0.2^{a}	1.1±0.2^{a}	0.8±0.1^{b}
18:3 n-3	0.5±0.0	0.5±0.1	0.5±0.0	0.3±0.0	1.1±0.2	1.1±0.0	1.0±0.1	0.9±0.0
20:2 n-6	0.4±0.0	0.4±0.1	0.5±0.1	0.4±0.1	1.0±0.1	0.9±0.1	1.0±0.1	0.8±0.0
20:3 n-6	2.0±0.1^{a}	1.8±0.1^{ab}	1.8±0.2^{ab}	1.7±0.1^{b}	1.8±0.1	2.5±0.6	2.1±0.3	1.8±0.1
20:4 n-6	5.4±0.3^{a}	5.4±0.4^{a}	3.4±0.4^{b}	3.6±0.5^{b}	4.7±0.3^{a}	4.8±0.6^{a}	2.1±0.1^{b}	2.7±0.4^{b}
20:5 n-3	0.2±0.0	0.2±0.0	0.4±0.1	0.2±0.1	0.3±0.2	0.3±0.1	0.4±0.2	0.4±0.1
24:0	2.1±0.4^{a}	1.5±0.5^{ab}	0.7±0.4^{b}	0.2±0.0^{b}	2.2±0.3	2.5±0.1	1.7±0.8	1.9±0.2
24:1 n-9	0.8±0.3	1.3±0.4	0.8±0.1	0.0±0.0	4.6±1.2	4.1±0.6	3.7±0.4	2.8±0.2
22:4 n-6	0.2±0.1	0.5±0.2	0.2±0.1	0.6±0.1	3.0±0.1^{a}	2.5±0.1^{a}	1.1±0.2^{b}	1.2±0.0^{b}
22:5 n-6	0.5±0.0	0.6±0.0	0.5±0.1	0.3±0.2	0.5±0.0	0.7±0.1	0.6±0.1	0.5±0.1
22:5 n-3	0.1±0.0^{c}	0.2±0.0^{bc}	0.4±0.1^{ab}	0.7±0.2^{a}	0.3±0.0^{b}	0.4±0.0^{ab}	0.5±0.1^{a}	0.5±0.1^{a}
22:6 n-3	2.6±0.2	2.3±0.1	2.2±0.3	2.1±0.3	2.4±0.3^{b}	1.9±0.4^{b}	4.2±0.5^{a}	4.5±0.4^{a}
Total SFA	56.2±1.0^{ab}	59.1±1.4^{a}	56.6±1.4^{ab}	55.6±0.6^{b}	46.0±1.8	44.4±0.6	48.4±2.6	49.6±0.7
Total PUFA	15.0±0.3^{a}	14.8±0.6^{a}	12.7±1.1^{b}	13.0±1.2^{ab}	22.1±1.3	23.3±1.0	23.1±1.3	23.2±0.8
Total MUFA	28.8±0.8^{b}	26.0±1.2^{c}	30.7±0.6^{ab}	31.4±0.9^{a}	31.9±1.7^{ab}	32.3±0.4^{a}	28.4±1.4^{bc}	27.2±0.3^{c}
Total N-6	11.5±0.3^{a}	11.6±0.5^{a}	9.2±0.7^{b}	9.6±0.8^{b}	21.1±0.8^{a}	19.5±0.9^{a}	17.6±0.7^{b}	16.8±0.5^{b}
Total N-3	3.5±0.2	3.3±0.2	3.4±0.4	3.4±0.4	4.6±0.6^{b}	3.8±0.1^{b}	6.0±0.8^{a}	6.5±0.4^{a}

NSG mice implanted with MAXF401 or MAXF574 PDX tumours were maintained on a 20% (± 3.8% or 1.6 % DHA) w/w diet for 7 weeks (1-week diet alone followed by 6 weeks with or without TXT treatment). Values are percentages relative to the total fatty acid content ± SE (n=7). Within the rows and tumour type, labeled means without a common letter differ (P<0.05) based on post hoc DUNCAN analysis. Abbreviations used: HDHA, high docosahexaenoic acid; LDHA, low docosahexaenoic acid; MUFA, monounsaturated fatty acids; PDX, patient derived xenograft; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TXT, docetaxel.

Appendix Table 23: Phosphatidylcholine phospholipid fatty acid composition (relative %) from NSG mice implanted with MAXF401 or MAXF574 PDX tumours

	MAXF401				MAXF574			
	Control	**Control + TXT**	**HDHA + TXT**	**LDHA+TXT**	**Control**	**Control + TXT**	**DHA**	**DHA + TXT**
14:0	1.5±0.1^{a}	1.2±0.1^{b}	1.5±0.1^{a}	1.6±0.1^{a}	3.0±0.2^{b}	3.4±0.1^{a}	3.6±0.1^{a}	3.3±0.1^{ab}
16:0	34.3±0.5^{a}	33.7±0.7^{b}	35.6±0.6^{a}	34.9±0.7^{a}	38.0±1.4^{ab}	35.3±0.5^{b}	40.3±1.0^{a}	36.5±1.7^{ab}
16:1 n-9	6.1±0.3^{ab}	5.5±0.4^{b}	6.5±0.4^{a}	6.2±0.2^{ab}	5.7±0.4	5.8±0.4	5.0±0.5	4.8±0.6
18:0	11.6±0.5^{b}	13.1±0.6^{a}	11.3±0.6^{a}	12.0±0.5^{ab}	8.0±0.2	6.8±0.2	7.6±0.7	8.3±0.5
18:1 n-9	20.5±0.6^{b}	19.4±0.6^{b}	23.1±0.7^{a}	22.5±0.5^{a}	25.9±0.2^{a}	26.6±0.4^{a}	24.0±1.0^{b}	24.3±0.5^{b}
18:2 n-6	6.9±0.2	6.8±0.4	6.5±0.4	6.5±0.2	10.7±0.9	10.8±0.0	10.6±1.8	13.4±0.9
20:0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.1±0.0	0.2±0.0	0.1±0.0
18:3 n-6	0.4±0.0	0.4±0.0	0.4±0.0	0.4±0.0	0.5±0.1^{b}	0.8±0.0^{a}	0.3±0.0^{c}	0.5±0.0^{b}
18:3 n-3	0.3±0.0	0.3±0.0	0.4±0.0	0.4±0.0	0.5±0.1^{ab}	0.6±0.0^{a}	0.4±0.0^{b}	0.6±0.0^{ab}
20:2 n-6	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.7±0.1^{b}	0.9±0.0^{a}	0.7±0.0^{b}	0.8±0.0^{a}
20:3 n-6	1.2±0.0^{b}	1.1±0.0^{b}	1.3±0.0^{a}	1.3±0.0^{a}	0.6±0.1	0.8±0.0	0.7±0.0	0.8±0.1
20:4 n-6	11.5±0.5^{a}	12.6±0.9^{a}	8.2±0.4^{b}	8.7±0.3^{b}	4.1±0.7^{a}	5.2±0.2^{a}	2.3±0.2^{b}	2.7±0.4^{b}
20:5 n-3	0.2±0.0^{c}	0.3±0.0^{c}	0.4±0.1^{b}	0.6±0.1^{a}	0.1±0.0	0.1±0.1	0.1±0.0	0.1±0.0
24:0	0.1±0.0	0.1±0.0	0.1±0.0	0.2±0.1	0.1±0.0	0.1±0.0	0.4±0.2	0.2±0.0
24:1 n-9	0.1±0.0^{b}	0.5±0.2^{a}	0.5±0.1^{a}	0.6±0.0^{a}	0.3±0.1^{ab}	0.5±0.1^{a}	0.3±0.1^{b}	0.4±0.0^{ab}
22:4 n-6	1.3±0.1^{a}	1.0±0.2^{a}	0.0±0.0^{b}	0.1±0.0^{b}	0.2±0.1^{ab}	0.2±0.0^{ab}	0.2±0.0^{a}	0.1±0.0^{b}
22:5 n-6	0.3±0.0^{a}	0.2±0.0^{a}	0.1±0.0^{b}	0.1±0.0^{b}	0.1±0.0	0.1±0.0	0.2±0.1	0.0±0.0
22:5 n-3	0.4±0.0^{b}	0.4±0.0^{b}	0.4±0.0^{b}	0.6±0.1^{a}	0.1±0.0	0.1±0.0	0.3±0.1	0.2±0.0
22:6 n-3	1.6±0.1^{b}	1.9±0.1^{b}	2.6±0.2^{a}	2.5±0.2^{a}	0.5±0.1^{b}	0.8±0.1^{b}	1.4±0.1^{a}	1.6±0.2^{a}
Total SFA	48.4±0.5	49.2±1.2	49.5±1.0	49.6±1.1	50.0±1.8^{b}	46.7±0.7^{b}	54.0±1.6^{a}	49.5±2.0^{b}
Total PUFA	24.8±0.8^{a}	25.3±1.5^{a}	20.4±0.9^{b}	21.1±0.9^{b}	18.1±1.6	20.4±0.5	17.3±2.0	21.0±1.7
Total MUFA	26.7±0.8^{b}	25.5±1.1^{b}	30.1±0.9^{a}	29.3±0.7^{a}	31.9±0.2^{a}	32.9±0.8^{a}	28.6±0.8^{b}	29.6±0.5^{ab}
Total N-6	21.8±0.7^{a}	22.2±1.4^{a}	16.7±0.7^{b}	17.1±0.5^{b}	17.0±1.5^{ab}	18.7±0.4^{a}	14.5±1.8^{b}	18.5±1.4^{a}
Total N-3	2.7±0.2^{b}	2.9±0.2^{b}	3.8±0.3^{a}	4.3±0.2^{a}	1.2±0.2^{c}	1.7±0.1^{b}	2.1±0.4^{ab}	2.5±0.3^{a}

NSG mice implanted with MAXF401 or MAXF574 PDX tumours were maintained on a 20% (± 3.8% or 1.6 % DHA) w/w diet for 7 weeks (1-week diet alone followed by 6 weeks with or without TXT treatment). Values are percentages relative to the total fatty acid content ± SE (n=7). Within the rows and tumour type, labeled means without a common letter differ (P<0.05) based on post hoc DUNCAN analysis. Abbreviations used: HDHA, high docosahexaenoic acid; LDHA, low docosahexaenoic acid; MUFA, monounsaturated fatty acids; PDX, patient derived xenograft; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TXT, docetaxel.

Appendix Table 24: Phosphatidylinositol phospholipid fatty acid composition (relative %) from NSG mice implanted with MAXF401 or MAXF574 PDX tumours

	MAXF401				MAXF574			
	Control	**Control + TXT**	**HDHA + TXT**	**LDHA+TXT**	**Control**	**Control + TXT**	**DHA**	**DHA + TXT**
14:0	0.3±0.0^{b}	0.4±0.1^{b}	0.3±0.0^{b}	0.6±0.2^{a}	0.4±0.0^{b}	0.5±0.1^{b}	0.5±0.1^{b}	0.9±0.2^{a}
16:0	11.1±1.1	11.1±1.7	10.9±1.0	11.5±1.5	8.5±0.6^{b}	9.4±1.2^{ab}	9.3±0.5^{ab}	11.8±1.4^{a}
16:1 n-9	1.6±0.1	1.7±0.2	2.1±0.4	1.7±0.1	1.5±0.2	2.3±1.0	1.4±0.0	1.4±0.2
18:0	39.3±0.6^{b}	38.2±0.7^{b}	40.0±0.5^{b}	42.3±1.4^{a}	33.1±0.8	33.3±1.6	34.4±1.0	35.6±0.9
18:1 n-9	8.8±0.7	9.5±0.4	9.7±0.5	9.9±0.7	24.0±1.0^{ab}	25.1±1.0^{a}	22.6±0.8^{b}	23.5±0.4^{ab}
18:2 n-6	3.4±0.2	3.0±0.5	3.1±0.2	3.0±0.2	2.9±0.1^{b}	2.9±0.1^{b}	4.1±0.1^{a}	3.9±0.3^{a}
20:0	0.3±0.0	0.2±0.0	0.2±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.2±0.0
18:3 n-6	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.6±0.1^{a}	0.6±0.1^{a}	0.4±0.0^{b}	0.4±0.0^{b}
18:3 n-3	0.4±0.1	0.2±0.1	0.2±0.0	0.2±0.0	0.8±0.1^{a}	0.6±0.2^{b}	0.6±0.0^{b}	0.7±0.0^{ab}
20:2 n-6	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	2.6±0.2^{ab}	2.1±0.2^{b}	3.3±0.1^{a}	3.1±0.2^{a}
20:3 n-6	2.3±0.2^{b}	2.0±0.1^{bc}	3.3±0.3^{a}	1.5±0.2^{c}	3.3±0.2^{b}	3.0±0.2^{b}	4.9±0.2^{a}	3.6±0.4^{b}
20:4 n-6	25.5±1.3^{a}	25.8±1.2^{a}	22.0±1.2^{b}	22.2±1.6^{b}	17.1±0.6^{a}	15.9±1.0^{a}	11.0±0.3^{b}	8.8±1.1^{b}
20:5 n-3	0.1±0.0^{b}	0.1±0.0^{b}	0.3±0.1^{a}	0.3±0.1^{a}	0.1±0.0	0.1±0.0	0.1±0.0	0.2±0.0
24:0	0.2±0.0	0.3±0.0	0.5±0.1	0.3±0.0	2.2±0.1^{a}	0.1±0.0^{b}	0.1±0.0^{b}	0.2±0.0^{b}
24:1 n-9	2.0±0.1^{a}	1.8±0.0^{a}	1.2±0.1^{b}	0.3±0.0^{c}	1.4±0.4^{a}	1.5±0.2^{a}	1.1±0.1^{ab}	0.9±0.1^{b}
22:4 n-6	0.4±0.0^{b}	0.4±0.0^{b}	0.3±0.1^{b}	1.0±0.2^{a}	0.6±0.1^{a}	0.8±0.3^{a}	0.2±0.0^{b}	0.3±0.1^{b}
22:5 n-6	0.3±0.2	0.2±0.0	0.1±0.0	0.3±0.0	0.4±0.2	0.4±0.2	0.2±0.0	0.2±0.0
22:5 n-3	0.5±0.0^{b}	0.5±0.0^{b}	0.8±0.0^{a}	0.8±0.1^{a}	0.2±0.0^{b}	0.1±0.0^{b}	1.0±0.1^{a}	0.8±0.1^{a}
22:6 n-3	2.3±0.1^{c}	2.3±0.1^{c}	3.6±0.2^{a}	2.8±0.3^{b}	1.1±0.1^{c}	0.9±0.1^{c}	4.2±0.3^{a}	3.2±0.5^{b}
Total SFA	51.8±1.0	52.0±1.1	52.6±1.4	55.7±2.9	43.4±0.8^{b}	43.8±1.8^{b}	44.7±1.3^{ab}	48.9±2.4^{a}
Total PUFA	35.8±1.2	35.0±1.1	34.4±1.5	32.4±2.4	29.8±0.8^{a}	27.4±1.2^{ab}	30.2±0.5^{a}	25.3±2.4^{b}
Total MUFA	12.4±0.7	13.0±0.5	13.0±0.7	11.9±0.8	26.8±0.7^{ab}	28.8±1.7^{ab}	25.1±0.9^{b}	25.8±0.4^{b}
Total N-6	32.3±1.3	31.7±1.1	29.3±1.5	28.4±2.1	27.4±0.7^{a}	25.6±1.1^{a}	24.2±0.2^{b}	20.3±1.8^{c}
Total N-3	3.4±0.2^{c}	3.2±0.1^{c}	5.1±0.1^{a}	4.1±0.4^{b}	2.4±0.1^{b}	1.8±0.1^{b}	6.0±0.4^{a}	5.0±0.6^{a}

NSG mice implanted with MAXF401 or MAXF574 PDX tumours were maintained on a 20% (± 3.8% or 1.6 % DHA) w/w diet for 7 weeks (1-week diet alone followed by 6 weeks with or without TXT treatment). Values are percentages relative to the total fatty acid content ± SE (n=7). Within the rows and tumour type, labeled means without a common letter differ (P<0.05) based on post hoc DUNCAN analysis. Abbreviations used: HDHA, high docosahexaenoic acid; LDHA, low docosahexaenoic acid; MUFA, monounsaturated fatty acids; PDX, patient derived xenograft; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TXT, docetaxel.

Appendix Table 25: Sphingomyelin phospholipid fatty acid composition (relative %) from NSG mice implanted with MAXF401 or MAXF574 PDX tumours

	MAXF401				MAXF574			
	Control	**Control + TXT**	**HDHA + TXT**	**LDHA+TXT**	**Control**	**Control + TXT**	**DHA**	**DHA + TXT**
14:0	1.0±0.1^{b}	0.7±0.1^{b}	1.8±0.1^{a}	1.0±0.1^{b}	3.6±1.0^{b}	3.4±0.1^{b}	4.8±0.4^{b}	9.2±1.1^{a}
16:0	31.8±1.8^{b}	33.9±0.9^{ab}	35.1±1.1^{a}	34.9±1.2^{ab}	45.4±0.6	43.0±0.8	46.2±2.5	42.0±1.8
16:1 n-9	0.7±0.2^{b}	0.8±0.2^{b}	1.6±0.3^{a}	1.4±0.2^{a}	0.8±0.3^{a}	0.8±0.7^{ab}	0.2±0.0^{b}	0.2±0.0^{b}
18:0	13.3±0.6	16.5±1.5	13.7±2.1	13.8±1.3	6.3±0.6^{a}	4.2±0.5^{b}	5.4±0.5^{ab}	6.4±0.5^{a}
18:1 n-9	1.9±0.4^{b}	4.4±1.8^{a}	4.5±0.3^{a}	4.6±0.6^{a}	2.3±0.5	2.3±1.0	0.8±0.1	1.4±0.2
18:2 n-6	4.3±0.2^{a}	3.6±0.2 ^{b}	2.7±0.1^{c}	2.5±0.1^{c}	0.4±0.1^{b}	1.7±1.4^{a}	0.2±0.0^{b}	0.5±0.2^{b}
20:0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.4±0.1	0.6±0.2	0.2±0.0	0.4±0.1
18:3 n-6	0.0±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.7±0.0^{a}	0.2±0.2^{b}	0.8±0.1^{a}	0.8±0.0^{a}
18:3 n-3	0.5±0.0^{a}	0.4±0.0^{b}	0.3±0.1^{c}	0.3±0.1^{c}	0.5±0.1	0.2±0.0	0.1±0.0	0.4±0.2
20:2 n-6	0.1±0.0^{b}	0.3±0.1^{a}	0.3±0.1^{ab}	0.3±0.1^{ab}	0.2±0.1	0.2±0.1	0.1±0.0	0.2±0.1
20:3 n-6	14.9 ±0.6^{a}	13.2±0.8^{ab}	11.5±0.7^{bc}	10.4±0.6^{c}	3.2±0.2^{ab}	2.5±0.4^{b}	3.8±0.3^{a}	3.7±0.2^{a}
20:4 n-6	0.9±0.1^{b}	1.0±0.1^{b}	0.6±0.2^{b}	1.5±0.4^{a}	1.4±0.1^{b}	2.2±0.7^{a}	1.1±0.2^{b}	1.3±0.1^{b}
20:5 n-3	2.7±0.2^{a}	2.7±0.2^{a}	2.2±0.1^{b}	2.1±0.1^{b}	0.6±0.1^{ab}	0.4±0.0^{b}	0.7±0.0^{a}	0.7±0.0^{a}
24:0	11.4±0.8	9.7±1.0	11.4±0.7	11.7±1.3	6.0±0.6^{ab}	5.1±0.5^{b}	7.4±0.4^{a}	7.5±0.5^{a}
24:1 n-9	14.3±0.8^{a}	10.4±1.4^{b}	11.8±1.3^{b}	12.0±1.2^{ab}	20.7±1.3	24.5±5.9	20.9±2.3	18.6±0.8
22:4 n-6	0.3±0.1^{ab}	0.4±0.0^{a}	0.2±0.0^{b}	0.2±0.0^{b}	1.9±0.2	2.1±0.3	2.3±0.2	1.8±0.3
22:5 n-6	0.3±0.0^{a}	0.2±0.0^{b}	0.5±0.2^{a}	0.2±0.0^{b}	0.3±0.0	0.2±0.0	0.3±0.1	0.7±0.3
22:5 n-3	0.3±0.0	0.3±0.0	0.2±0.0	0.3±0.1	0.4±0.0^{a}	0.4±0.0^{ab}	0.3±0.0^{b}	0.4±0.0^{a}
22:6 n-3	0.5±0.2	0.4±0.2	0.5±0.0	0.4±0.1	2.7±0.3	2.7±0.7	2.6±0.2	2.8±0.2
Total SFA	58.0±1.5^{b}	61.8±1.6^{ab}	63.0±2.3^{a}	63.4±1.7^{a}	64.7±1.9^{a}	56.8±0.7^{b}	65.8±2.4^{a}	66.3±0.8^{a}
Total PUFA	25.1±1.1^{a}	22.6±1.2^{ab}	19.2±0.9^{b}	18.5±0.7^{b}	11.6±0.6^{b}	12.9±0.9^{ab}	12.4±0.2^{ab}	13.5±0.5^{a}
Total MUFA	16.8±0.6	15.6±1.7	17.9±1.7	18.0±1.4	23.7±1.8^{b}	30.3±1.6^{a}	21.9±2.3^{b}	20.2±0.7^{b}
Total N-6	21.0±0.7^{a}	18.8±0.9 ^{a}	15.8±0.8^{b}	15.5±0.6^{b}	7.6±0.4^{b}	9.2±1.6^{ab}	8.7±0.1^{a}	9.0±0.3^{a}
Total N-3	4.1±0.4^{a}	3.8±0.3^{a}	3.4±0.1^{ab}	3.1±0.2^{b}	4.0±0.3	3.7±0.7	3.7±0.1	4.5±0.4

NSG mice implanted with MAXF401 or MAXF574 PDX tumours were maintained on a 20% (± 3.8% or 1.6 % DHA) w/w diet for 7 weeks (1-week diet alone followed by 6 weeks with or without TXT treatment). Values are percentages relative to the total fatty acid content ± SE (n=7). Within the rows and tumour type, labeled means without a common letter differ (P<0.05) based on post hoc DUNCAN analysis. Abbreviations used: HDHA, high docosahexaenoic acid; LDHA, low docosahexaenoic acid; MUFA, monounsaturated fatty acids; PDX, patient derived xenograft; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TXT, docetaxel.

Appendix Table 26: SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Reported on Page No
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	286
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	314
	2b	All items from the World Health Organization Trial Registration Data Set	403
Protocol version	3	Date and version identifier	N/A
Funding	4	Sources and types of financial, material, and other support	N/A
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	N/A
	5b	Name and contact information for the trial sponsor	N/A
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	N/A
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	313-314
Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	286-288
	6b	Explanation for choice of comparators	292
Objectives	7	Specific objectives or hypotheses	289
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	Fig 8-1, Fig 8-2
Methods: Participants, interventions, and outcomes			

Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	290
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	Table 8-1 291
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	291-292
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	301
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	293
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	-
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	297-300 Table 8-2
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	302
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	301
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	302
Methods: Assignment of interventions (for controlled trials)			
Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable	302

		to those who enrol participants or assign interventions	
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	302
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	302
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	302
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	302
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	303-310 Table 8-3
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	-
Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	311
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	311-313 Table 8-3
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	311-313
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	-

Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	313
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	314
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	314
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	314
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	314-315
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	314-315
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	405
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	405
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	315
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	N/A
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	315

Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	315
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	315
	31b	Authorship eligibility guidelines and any intended use of professional writers	N/A
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	-
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	405
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	405

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.

Appendix Table 27: World Health Organization Trial Registration Data Set DHA WIN Summary

Data Category	Information
Primary registry and trial identifying number	ClinicalTrials.gov: NCT03831178
Date of registration in primary registry	February 5, 2019
Secondary identifying numbers	IIT-0005
Sources of monetary or material support	Canadian Institutes of Health Research (CIHR), AHS Cancer Control Alberta, Butler Family Foundation
Primary sponsor	AHS Cancer Control Alberta
Secondary sponsors	University of Alberta
Contact for public queries	Deborah Miede: Deborah.Miede@albertahealthservices.ca
Contact for scientific queries	Catherine Field: Catherine.field@ualberta.ca
Public title	DHA WIN
Scientific title	Docosahexaenoic acid (DHA) for Women with breast cancer in the neoadjuvant setting
Country of recruitment	Canada
Health condition or problems studied	Breast cancer
Interventions	DHA supplementation (5 g/ day) or equal amount of vegetable oil placebo for the duration of the participants chemotherapy treatment
Key inclusion and exclusion criteria	Inclusion: ECOG Performance status of 0 or 1; Hematology and biochemistry assessments within normal range; ability to take oral medication; adequate tissue specimen for diagnosis, biomarkers and endpoint Ki67 assays Exclusion: Patients undergoing surgery prior to chemotherapy; Current or previous (within 2 months) daily use (>1 day/week) use of omega-3, fish oil, or other supplements or foods containing DHA (at daily doses > 200 mg); Known allergy to soy or corn; Continued intake of supplements containing Vitamin C, Vitamin E or β-carotene exceeding the DRI, or other anti-oxidant supplements; History of deep venous thrombosis, active thrombophlebitis, pulmonary embolism, stroke, acute myocardial infarction, congestive cardiac failure, untreated hypertension, known inherited hypercoagulable disorder; Diagnosis of any

	other malignancy within the previous year except for adequately treated basal cell or squamous cell skin cancer
Study type	Randomized controlled trial
Date of first enrolment	Expected August 2019
Target sample size	52
Recruitment status	Not yet recruiting
Primary outcomes	Percent change in Ki67 index from baseline to surgical excision
Key secondary outcomes	Percent of DHA in plasma phospholipids; systemic immune function; Identify factors that may affect DHA incorporation into plasma phospholipids; Examine changes in markers for apoptosis and tumour infiltrating lymphocytes; pathological complete response; Comparison of rate of chemotherapy associated grade 3 and 4 toxicities

Appendix File 1: Informed Consent Form for Participation in a Research Study

DHA for Women with Breast Cancer in the Neoadjuvant Setting

DHA to improve effectiveness of Chemotherapy in Breast Cancer

Protocol ID: *IIT-0005*

Study Doctor: *Dr. John Mackey*
Department of Medical Oncology
Cross Cancer Institute
780-432-8221

Sponsor/Funder(s): *Alberta Health Services- Cross Cancer Institute*

Emergency Contact Number (24 hours / 7 days a week): *780-965-8824*

Non-Emergency contact numbers are noted at the end of this document under the section heading "WHO DO I CONTACT FOR QUESTIONS?".

For assistance with terminology within this consent form, please refer to the Canadian Cancer Society Glossary of Terms at http://info.cancer.ca/e/glossary/glossary.html.

You are being invited to participate in a research study because you have stage I, II or III breast cancer which has not spread to distant parts of the body and will be receiving chemotherapy prior to surgery. This consent form provides detailed information about the study to assist you with making an informed decision. Please read this document carefully and ask any questions you may have. All questions should be answered to your satisfaction before you decide whether to participate.

The study staff will tell you about timelines for making your decision. You may find it helpful to discuss the study with family and friends so that you can make the best possible decision within the given timelines.

Taking part in this study is voluntary. You may choose not to take part or, if you choose to participate, you may leave the study at any time without giving a reason. Deciding not to take part or deciding to leave the study will not result in any penalty or any loss of medical or health-related benefits to which you are entitled.

The study doctor, who is one of the researchers, will discuss this study with you and will answer any questions you may have. If you do consent to participate in this study, you will need to sign and date this consent form. You will receive a copy of the signed form.

WHAT IS THE BACKGROUND INFORMATION FOR THIS STUDY?

Docosahexaenoic acid (DHA) is an omega-3 fatty acid commonly found in fish and fish oil. In the body, DHA is found in the membranes of cells. DHA is important for brain development, and in the immune system. DHA is also beneficial in heart disease. A diet high in DHA can reduce the incidence of breast cancer.

Incubating breast cancer cells with DHA in cell culture (cells in a dish in a laboratory) decreases the growth of the breast cancer cells, and increases the death of these cells. This is specific to cancer cells, since DHA has no effect on normal breast cells. When breast cancer cells are treated with chemotherapy drugs and DHA, DHA increases the effectiveness of chemotherapy resulting in increased death of the cancer cells.

When mice with breast tumours are fed DHA and treated with chemotherapy their tumours are much smaller than mice who are not fed DHA. In a previous clinical trial, women with metastatic breast cancer were given DHA supplements and treated with chemotherapy. DHA supplements appeared to improve the response to chemotherapy for some women.

Taking DHA may also reduce some side effects of chemotherapy in women with breast cancer. In these previous trials, no side-effects of taking DHA supplements were found.

Health Canada, the regulatory body that oversees the use of natural health products, drugs and devices in Canada, has not approved the sale or use of this DHA supplement to treat this kind of cancer, although they have allowed its use in this study.

The Health Research Ethics Board of Alberta – Cancer Committee (HREBA-CC), which oversees the ethical acceptability of research involving humans, has reviewed and granted ethics approval for this study.

WHY IS THIS STUDY BEING DONE?

This study will test if taking a DHA supplement during chemotherapy for breast cancer increases the effectiveness of the chemotherapy. The purpose of this study is to find out what effects a new agent, DHA supplementation, has on you and your breast cancer.

The investigators of this study are also interested in exploring the factors that may affect DHA incorporation in your blood, such as your weight and height, usual food intake (including amount and type of fat eaten), tumour type and the amount of DHA supplement consumed in the study.

WHAT ARE OTHER OPTIONS IF I DECIDE NOT TO PARTICIPATE IN THIS STUDY?

You do not have to take part in this study, in order to receive continued medical care. Other alternatives in addition to standard care may include:

- Other experimental studies may be available if you decide not take part in this study.

- Continuing regular observation and routine follow-up care e.g., symptom management

Please talk to the study doctor or your care doctor about the known benefits and risks of these other options before you decide to take part in this study. Your study or care doctor can also discuss with you what will happen if you decide not to undertake any treatment at this time.

HOW MANY PEOPLE WILL TAKE PART IN THIS STUDY?

Up to 52 people will take part in this study.

WHAT WILL HAPPEN DURING THIS STUDY?

ASSIGNMENT TO A GROUP

If you decide to participate then you will be "randomized" into one of the groups described below. Randomization means that you are put into a group by chance (like flipping a coin). There is no way to predict which group you will be assigned to. You will have an equal chance of being placed in either DHA supplementation or placebo group. Neither you, the study staff, nor the study doctor can choose what group you will be in.

This is a double-blinded study, which means that neither you nor the study doctor or study staff will know which group you are in. This is done so that you and the study doctor will not be influenced by expectations of the effects of the study agent. Your treatment will be identified if medically necessary by a process referred to as unblinding. Requests to reveal your assignment for your information or participation in other research studies will not be considered until the study has been completed and the results are known.

STUDY INTERVENTION

Group 1 (Experimental intervention): standard intervention of neoadjuvant chemotherapy plus experimental intervention of DHA supplementation.
If you are randomized into this group, you will take DHA capsules by mouth every day during chemotherapy treatment (4-6 cycles of chemotherapy, which would last approximately12-18 weeks)

Group 2 (Non-experimental intervention): standard intervention of neoadjuvant chemotherapy

If you are randomized into this group you will take placebo capsules containing corn/soy oil by mouth every day during chemotherapy treatment (4-6 cycles of chemotherapy, which would last approximately *12-18 weeks).*

Other important information on study intervention:
If you have side effects while you are on this study, the study doctor may make changes to the intervention.

STUDY PROCEDURES

Established Procedures

The following established procedures will be done as part of this study. Some of these procedures may be done as part of your standard care, in which case the results may be used. Some may be done more frequently than if you were not taking part in this study. Some of these procedures may be done solely for the purpose of the study. If the results show that you are not able to continue participating in the study, the study doctor will let you know.

Screening:

- Signed Informed Consent
- Review of inclusion / exclusion criteria
- Confirmation of no known allergies to soybean or corn oil (participants with allergies to soy or corn will be excluded from the study).
- Demographic data
- Physical examination
- You will be asked about your ability to carry out daily activities
- Body height and weight
- Vital signs
- Documentation of the diagnosis and disease stage
- Confirmation of no previous or concomitant treatment
- Complete medical / oncological history and consultation
- Questionnaire about your symptoms and well-being (ESAS questionnaire)
- Quality of Life questionnaire
- Exercise questionnaire
- Food frequency questionnaire (to be completed before the end of the first cycle of chemotherapy)
- Blood sample
- Your biopsy sample will be analyzed for standard tumour analysis: Grade; ER/PR/HER2; Ki67 to be requested if not already performed and other disease-related biomarkers.
- Adverse events before start of treatment

Chemotherapy Cycles (will take place prior to each chemotherapy administration):

- Physical exam
- You will be asked about your ability to carry out daily activities (cycle 1 and upon completion of your chemotherapy).
- Weight
- Vital signs
- Adverse events
- Blood sample
- You will take the DHA/placebo capsules by mouth every day during chemotherapy treatment (4-6 cycles of chemotherapy, which would last approximately 12-18 weeks)
- Quality of Life questionnaire (only at end of 6^{th} cycle)
- Exercise questionnaire

Upon completion of chemotherapy:
If you undergo a surgical procedure to remove the tumour after chemotherapy, we will collect information from your records regarding the extent of the surgical procedure and amount of blood loss. In addition, your tumour sample will be reassessed for Ki67 and other disease-related biomarkers.

Questionnaires
You will be provided with a questionnaire about food intake by research staff during cycle 1 of this study. The purpose of the questionnaire is to determine the amount of DHA in your diet, and other foods that can affect DHA in the body. The questionnaire will take about 1 hr. to complete.

You will also be asked to complete questionnaires about your symptoms and well-being (ESAS questionnaire and exercise questionnaire) at the beginning of each chemotherapy cycle. It may take you 15-20 minutes to complete both questionnaires.

The information you provide is for research purposes only and will remain strictly confidential. Some of the questions are personal; you may choose not to answer them.

Participant Diaries
You will be asked to keep a diary to record *your study supplement capsules intake.* Please record *the times and number of capsules when you take the capsules each day*. You will be asked to return the diary to *the Cross Cancer Institute at the end of each cycle*.

MANDATORY SAMPLE COLLECTION
The researchers doing this study need to do tests on samples as described below. *The biopsy sample will be examined to make sure you have the type of cancer that is being studied in the research study. The surgical resection will be examined and compared to the biopsy sample to see how the cancer cells respond to DHA supplementation. Blood samples will be examined to see how DHA supplementation affects the amount of DHA in these samples, and if DHA alters immune cells.*

The collection of these samples is a necessary part of this study and will be used only for this purpose. The samples will not be sold.

Once these tests have been completed, any leftover samples will be returned to the facility from which they were obtained if needed or destroyed, unless you wish to give permission for other future research purposes, in which case you will be given a separate optional consent form to sign.

Hereditary genetic testing (to look at whether cancer runs in your family) will not be done on these samples.

Reports about research tests done with your samples will be given to the study doctor(s). If you would like to learn the results of this research, please let them know.

Tissue Collection (Mandatory)
A small sample of your tissue that has already been removed by a previous surgery or biopsy will be obtained by the researchers doing this study. No further surgeries or biopsies are required of you for this purpose.

As part of your standard of care and necessary for this study, you will have had a tissue biopsy. Upon completion of your chemotherapy treatment and as part of your standard of care, you may undergo a surgical procedure to remove the tumour from your breast. The amount of tissue to be removed will depend on the size and location of the tumour. Your doctor will give you more details regarding this procedure.

A sample of the tissues obtained from the initial biopsy and from the subsequent breast surgery will be sent to a laboratory at the *Cross Cancer Institute, and at the University of Alberta in Edmonton, Alberta, Canada,* where they will be examined to confirm your diagnosis *and examine how DHA alters tumour growth, and the amount of DHA in tumour cells*.

Blood Collection (Mandatory)
Blood samples will be taken by inserting a needle into a vein in your arm. These will be taken at the same time as your study related tests whenever possible upon *entry to the study, at the beginning of every cycle of chemotherapy (every three weeks), on day 20 of cycle 3 and before surgery. One tablespoon of blood will be collected for this study at those times*. These blood samples will be sent to a laboratory at the *Cross Cancer Institute and the University of Alberta in Edmonton, Alberta, Canada* where they will be examined to *measure the different cells in your blood, and the amount of DHA in these cells.*

Identification of Samples
To protect your identity, the information that will be on your samples will be limited to the *pathology identification number, and an identification number for the study.*
Despite protections being in place, there is a risk of unintentional release of information that could lead to loss of privacy. Due to technological advances in genetics, there is also a risk of unintentional release of genetic information from the samples. This information can be linked back to you and can lead to possible future discrimination in employment or insurance, against you or your biological relatives.

Withdrawal of Samples
If you no longer want your samples to be used in this research, you should tell the study doctor. The study doctor will ensure the samples are returned to the hospital from which they were obtained, if needed, or destroyed.

You can request withdrawal of your sample(s) until *you have received your blinded capsules* when the samples will be made anonymous. It won't be possible to return samples after this because the researchers will not know which samples are yours.

You will not be able to continue to participate in this study if required samples are withdrawn.

Assessments	Screening (within 21 days before chemotherapy)	Chemotherapy Cycle 1		Chemotherapy Cycle 2		Chemotherapy Cycle 3		Chemotherapy Cycle 4		Chemotherapy Cycle 5		Chemotherapy Cycle 6		End of Treatment	Surgery
		Day 1[2]	Day 20 (+/- 3 days)	Day 1	Day 20 (+/- 3 days)	Day 1	Day 20 (+/- 3 days)	Day 1	Day 20 (+/- 3 days)	Day 1	Day 20 (+/- 3 days	Day 1	Day 20 (+/- 3 days	Within 30 days after last dose	
Informed Consent	X														
Confirmation of previous or current medications	X	X		X		X		X		X		X		X	
Demographic data collection	X														
Physical Exam	X	X		X		X		X		X		X		X	
You will be asked about your ability to carry out daily activities	X	X												X	
Height	X														
Weight	X	X												X	
Vital Signs	X	X		X		X		X		X		X		X	
You will be asked about your medical history or current medical conditions	X	X		X		X		X		X		X		X	
You will be asked to complete questionnaires about your symptoms and well-being (ESAS questionnaire)	X	X		X		X		X		X		X		X	
You will be asked to complete questionnaire about your quality of life	X													X	
Exercise questionnaire	X	X		X		X		X		X		X		X	

Food frequency questionnaire		X (anytime within the first cycle)									
Blood will be taken for routine tests to monitor your health	X	X	X	X	X	X	X	X	X		
A sample of your tumour will be analyzed for disease-related biomarkers (signs related to your disease)	X										X
Blood will be collected to measure signs of immune function	X					X			X		
Blood will be collected to measure the level of study treatment in your blood lipids	X		X	X		X	X	X	X		
Treatment: DHA/Placebo		Days 1-21	Days 1-21	Days 1-21		Days 1-21	Days 1-21	Days 1-21			
You will complete a diary with your capsule intake		Days 1 -21	Days 1 -21	Days 1 -21		Days 1 -21	Days 1 -21	Days 1 -21			
You will be asked about any side effects which may or not be related to the study treatment	X	X	X	X		X	X	X		X	
We will collect results from your surgery report											X

OPTIONAL RESEARCH

The researchers doing this study are interested in doing additional optional research. You will be given a separate optional study consent form(s) to read and sign if you wish to give permission to this. You may decide not to participate in the "optional" study and still participate in this main study.

WHAT ARE THE POTENTIAL SIDE EFFECTS FROM PARTICIPATING IN THIS STUDY?

You may experience side effects from participating in this study. Some side effects are known and are listed below, but there may be side effects that are not expected. You should discuss these with the study doctor.

There are no known side effects of this omega 3 (DHA) supplement. A non-medicinal ingredient in this nutritional supplement that may cause an allergic reaction includes gelatin.

The risks and side-effects of the standard or usual treatment will be explained to you as part of your standard care. These risks are not included in this consent form.

A Data and Safety Monitoring Board (DSMB), an independent group of experts, will be reviewing the data throughout the conduct of the study to ensure continuing participant safety as well as scientific validity and quality of the research.

WHAT ARE THE REPRODUCTIVE RISKS?
There appears to be no effect of the nutritional product on the human reproductive system.

WHAT ARE THE BENEFITS OF PARTICIPATING IN THIS STUDY?
Participation in this study may or may not be of personal benefit to you. However, based on the results of this study, it is hoped that in the long-term, patient care can be improved.

WHAT ARE MY RESPONSIBILITIES AS A STUDY PARTICIPANT?

If you choose to participate in this study, you will be expected to:

- Tell the study doctor about your current medical conditions;
- Tell the study doctor about all prescription and non-prescription medications and supplements, including vitamins and herbals, that you may be taking and check with the study doctor before starting, stopping or changing any of these. This is for your safety as these may interact with the intervention you receive on this study;
- Tell the study doctor if you are thinking about participating on another research study;
- Attend all scheduled study visits and undergo all of the procedures described above;
- Return any unused DHA / placebo products;

- Return any *diaries and food frequency questionnaires* taken home to complete;
- Tell the study doctor if you become pregnant while participating on this study;
- Avoid taking fish oil supplements, or any supplements containing DHA.
- Stop taking other *supplements of vitamin C, vitamin E, or β-carotene exceeding the DRI (daily recommended intake), or other anti-oxidant supplements. A multivitamin with vitamin C, E, and β-carotene below the DRI are permitted (75 mg/day vitamin C, 15 mg/day vitamin E, and 700 µg/day β-carotene). A member of the research staff will go through the details of multivitamin intake to ensure it is within the guidelines.*
- *DHA supplement/ placebo capsules are* meant for you alone, and must not be shared with others. If someone accidently takes the capsules, the *intake should be recorded in medication diary, and the study staff should be informed.*

HOW LONG WILL I BE PARTICIPATING IN THIS STUDY?

The study intervention will last as long as it takes for you to receive your chemotherapy (about *12-18 weeks)*.
You may be seen more often if the study doctor determines that this is necessary or if your cancer *gets worse*.

WILL THERE BE ANY LONG-TERM FOLLOW-UP INVOLVED WITH THIS STUDY?

No matter which group you are randomized to, and even if you stop receiving the study intervention early, we would like to keep track of your health for *10 years to look at the long-term effects of your participation on the study*. We would do this *by accessing electronic or paper medical chart review at 3, 5 and 10 years after treatment.*

In the event it is necessary to further evaluate the safety or efficacy of the *DHA supplement*, it may be necessary to have access to additional information about your health status. The study team may attempt to obtain study-related information about your health from you or from other private sources, including your care physician and *electronic or paper medical chart review*. This may include contacting you again by phone or letter, but only if you have not withdrawn your consent for future contact. However, contacting you, your care physician or using other private sources of information, is optional, please indicate your decision using the check boxes below.

You give permission to the study doctor or member of the study team to attempt to obtain study-related information about your health status to further evaluate the safety or efficacy of *DHA supplementation*. This may include contacting your care physician, or by contacting you by phone or letter (i.e., future contact).

☐ Yes ☐ No Participant's Initials: _____

Name/phone number of care physician: _____
In addition, the study team may also attempt to obtain study-relevant information about your health information from public sources such as national patient registries (e.g., cancer registries)

If the study doctor needs to follow up with you but cannot locate you, either because you have moved and not updated your contact information or if, for some reason, your contact information is no longer accurate, the study doctor would like to obtain your new contact information (e.g., address, telephone number) by calling or writing to the persons you've named as your secondary contacts. This is optional, please indicate your decision using the check boxes below.

You give permission to the study doctor or member of the study team to contact your secondary contacts if the study doctor or study team no longer have accurate contact information for you.

☐ Yes ☐ No Participant's Initials: _____

Name/phone number of secondary contacts: _____

If the study doctor cannot obtain information through your secondary contacts, he/she would like to ask for assistance of a third party that specializes in locating persons. The study doctor may only share limited information about you (name and last known address) with a third party locator. None of your personal health or study-related information will be shared with the third party locator. The third party locator will consult public sources and databases to obtain your current contact information but will not contact you. The third party locator will only share this information with the study doctor or study team to help complete the follow-up stage of the study. Only the study doctor or a member of the study team will attempt to contact you directly. This is optional, please indicate your decision using the check boxes below.

If the study doctor is not able to obtain your contact information from your secondary contacts, you give permission for the study doctor to provide your name and last location to a third party that specializes in locating persons.

☐ Yes ☐ No Participant's Initials: _____

CAN I CHOOSE TO LEAVE THIS STUDY EARLY?

You can choose to end your participation in this research (called early withdrawal) at any time without having to provide a reason. If you choose to withdraw early from the study without finishing the intervention, procedure or follow-up, you are encouraged to contact the study doctor or study staff.

You may be asked questions about your experience with the study intervention, and to have laboratory tests and physical examinations considered necessary to safely stop your study involvement.

You may withdraw your permission to use information that was collected about you for this study at any time by letting the study doctor know. However, this would also mean that you withdraw from the study.

Information that was recorded before you withdrew will be used by the researchers for the purposes of the study, but no additional information will be collected or sent to the sponsor after you withdraw your permission.

CAN MY PARTICIPATION IN THIS STUDY END EARLY?

The study doctor may stop your participation in the study early, and without your consent, for reasons
such as:

- The intervention does not work for you;
- You are unable to tolerate the study intervention;
- You are unable to complete all required study procedures;
- New information shows that the study intervention is no longer in your best interest;
- The study doctor no longer feels this is the best treatment for you;
- A regulatory authority (for example, Health Canada) or the research ethics board withdraws permission for the study to continue;
- Your treatment assignment becomes known to others (the study doctor or study staff);

If you are removed from the study, the study doctor will discuss the reasons with you and plans will be made for your continued care outside of the study.

HOW WILL MY PERSONAL INFORMATION BE KEPT CONFIDENTIAL?

If you decide to participate in this study, the study doctor and study staff will only collect the information they need for this study.

Records identifying you, including information collect from your medical files/records, such as your Electronic Medical Records (EMR), Netcare, charts, etc., will be kept confidential to the extent permitted by the applicable laws, will not be disclosed or made publicly available, except as described in this consent document.

Authorized representatives of the following organizations may look at your identifiable medical/clinical study records at the site where these records are held for quality assurance purposes and/or to verify that the information collected for the study is correct and follows proper laws and guidelines:

- Members of the Regulatory/Audit team at *Cross Cancer Institute,* for quality assurance purposes;
- The Health Research Ethics Board of Alberta – Cancer Committee, which oversees the ethical conduct of this study;
- Health Canada, which oversees the use of natural health products/drugs/devices in Canada and the conduct of clinical trials;

Authorized representatives of the above organizations and of the University of Alberta may **receive** information related to the study from your medical/clinical study records that will be kept confidential in a secure location and may be used in current or future relevant health research. Your name or other information that may identify you will not be provided (i.e., the information will be de-identified). The records received by these organizations will be coded with a number. The key that indicates what number you have been assigned will be kept secure by the researchers directly involved with your study and will not be released.

Any disclosure of your identifiable health information will be done in accordance with federal and provincial laws including the Alberta Health Information Act (HIA). The organizations listed above are required to have organizational policies and procedures to protect the information they see or receive about you, except where disclosure may be required by law. The study doctor will ensure that any personal health information collected for this study is kept in a secure and confidential location at the *Cross Cancer Institute, Edmonton Alberta* as also required by law.

If the results of this study are published, your identity will remain confidential. It is expected that the information collected during the study will be *used in analyses and will be published/presented to the scientific community at meetings and in journals*. This information may also be used as part of a submission to regulatory authorities around the world to support the approval of this intervention.

Even though the likelihood that someone may identify you from the study data is very small, it can never be completely eliminated. Every effort will be made to keep your identifiable information confidential, and to follow the ethical and legal rules about collecting, using and disclosing this information.

WILL MY HEALTHCARE PROVIDER(S) BE INFORMED OF MY PARTICIPATION IN THIS STUDY?

Your family doctor/health care provider will not be informed by the study team that you are taking part in the study. You can choose to let your family doctor/health care provider know, if you like. If you are undecided, the study doctor can discuss this with you.

WILL THERE BE ANY COSTS INVOLVED WITH PARTICIPATING IN THIS STUDY?

The DHA supplement/ placeb*o* will be given to you free of charge while you take part in this study.

Taking part in this study may result in added costs to you. For example:

- There may be costs associated with hospital visits. For instance, parking, transportation, or snacks/meals during the study.

Possible Costs After the Study is Complete

You may not be able to receive the study intervention after your participation in the study is completed. There are several possible reasons for this, some of which are:

- The intervention may not turn out to be effective or safe;

- The intervention may not be approved for use in Canada;
- Your caregivers may not feel it is the best option for you;
- You may decide it is too expensive and insurance coverage may not be available;
- The intervention, even if approved in Canada, may not be available free of charge.

The study doctor will discuss these options with you.

WILL I BE COMPENSATED FOR PARTICIPATING IN THIS STUDY?
You will not be paid for taking part in this study.

It is possible that the research conducted using your samples and/or study data may eventually lead to the development of new diagnostic tests, new drugs or devices, or other commercial products. There are no plans to provide payment to you if this happens.

In the case of research-related side effects or injury, as a direct result of participating in this research, you will receive all medical treatments or services recommended by your doctors.

Although no funds have been set aside to compensate you in the event of injury or illness related to the study treatment or procedures, you do not give up any of your legal rights for compensation by signing this form.

WHAT ARE MY RIGHTS AS A PARTICIPANT IN THIS STUDY?
You will be told, in a timely manner, about new information that may be relevant to your willingness to stay in this study.

You have the right to be informed of the results of this study once the entire study is complete. If you would like to be informed of these results, please contact the study doctor.

The results of this study will be available on a clinical registry; refer to the section titled “Where can I find online information about this study?”.

Your rights to privacy are legally protected by federal and provincial laws that require safeguards to ensure that your privacy is respected.

By signing this form you do not give up any of your legal rights against the hospital, investigators, sponsor, involved institutions for compensation or their agents, nor does this form relieve these parties from their legal and professional responsibilities.

IS THERE CONFLICT OF INTEREST RELATED TO THIS STUDY?

There are no conflicts of interest declared between the study doctor and sponsor of this study.

WHAT IF RESEARCHERS DISCOVER SOMETHING ABOUT ME AS A RESEARCH PARTICIPANT?

During the study, the researchers may learn something about you that they didn't expect. For example, the researchers may *find out that you have another medical condition*.

If any clinically important information about your health is obtained as a result of your participation in this study, you will be given the opportunity at that time to decide whether you wish to be made aware of that information.

WHERE CAN I FIND ONLINE INFORMATION ABOUT THIS STUDY?

A description of this clinical trial will be available on *http://www.ClinicalTrials.gov*.

The study registration number to use this website is: NCT03831178

This website will not include information that can identify you. You can search for this website at any time.

WHO DO I CONTACT FOR QUESTIONS?

If you have questions about taking part in this study, or if you suffer a research-related injury, you should talk to the study doctor, co-investigator or study nurse. These person(s) are:

Dr. John Mackey	780-432-8221
Name	Telephone
Name	Telephone
Name	Telephone

He can also be paged through the Cross Cancer Institute switchboard at (780) 432-8771.

If you have questions about your rights as a participant or about ethical issues related to this study and you would like to talk to someone who is not involved in the conduct of the study, please contact the Office of the Health Research Ethics Board of Alberta – Cancer Committee at:

Telephone: 780-423-5727 Toll Free: 1-877-423-5727

You will be given a copy of this signed and dated consent form prior to participating in this study.

SIGNATURES

Part 1 - to be completed by the potential participant.

	Yes	No
Do you understand that you have been asked to take part in a research study?	☐	☐
Do you understand why this study is being done?	☐	☐
Do you understand the potential benefits of taking part in this study?	☐	☐
Do you understand the risks of taking part in this study and the risks of becoming pregnant or fathering a child during this study?	☐	☐
Do you understand what you will be asked to do should you decide to take part in this study?	☐	☐
Do you understand the alternatives to participating in this study?	☐	☐
Do you understand that you are free to leave the study at any time, without out having to give reason and without affecting your future health care?	☐	☐
Do you understand who will see your records, including health information that identifies you?	☐	☐
Do you understand that by signing this consent form you are giving us permission to access your health information and specimens if applicable?	☐	☐
Do you understand that by signing this consent form that you do not give up any of your legal rights?	☐	☐
Have you had enough opportunity to ask questions and discuss this study?	☐	☐

By signing this form I agree, to participate in this study.

Signature of Participant	PRINTED NAME	Date

Part 2 - to be completed by the study doctor or designee who conducted the informed consent discussion. Only compete this section if the potential participant has **agreed** to participate.
I believe that the person signing this form understands what is involved in the study and has freely decided to participate.

Signature of Person Conducting the Consent Discussion	PRINTED NAME	Date

Part 3 - to be completed only if the participant is unable to read or requires assistance of an oral translator/interpreter.

- The informed consent form was accurately explained to, and apparently understood by the participant.
- Informed consent was freely given by *or on behalf of* the participant.

Signature of Impartial Witness/Interpreter	PRINTED NAME	Date

Appendix File 2: Informed Consent Form for Participation in Optional Research

DHA for Women with Breast Cancer in the Neoadjuvant Setting (DHA WIN)

DHA to improve effectiveness of Chemotherapy in Breast Cancer

Protocol ID:	IIT-0005
Researcher:	Dr. John Mackey Department of Medical Oncology Cross Cancer Institute 780-432-8221
Funder(s)/Sponsor:	Alberta Health Services- Cross Cancer Institute

INTRODUCTION

In addition to the main study, you also are being invited to take part in optional research. Although it is optional, the study of human samples and data focusing on the prevention, diagnosis and treatment of cancer and other diseases is an important part of research. Taking part in this optional research is voluntary. You still can take part in the main study, and will continue to receive treatment and care even if you say "no" to any or all of this optional research now or later. This form and your discussion with the researcher/study staff will give you the information you need to make your decision.

WHY IS THIS OPTIONAL RESEARCH BEING DONE?

The researchers conducting this research are interested in doing the following:

- Biomarker research for the main study using tumour tissue / blood already collected
- Bio-banking for use in future research using tumour tissue / blood already collected

As part of this optional research, the researchers would like to examine your tumour tissue/blood samples to look for any **biomarkers** (small "signature" molecules or indicators) in your cancer cells or circulating in your blood. These biomarkers might help predict which patients are most likely to be affected by the study drug. This is called biomarker research.

Bio-banking is the collection, storage, and use of human body samples and related health information for future research. It provides an important resource for health research locally, across Canada, and around the world. The researchers doing the main study are also interested in storing your tissue/blood samples for future research. The research that may be done on your samples in the future is unknown at this time. It may be related to your condition or it may be used to address research questions that are unrelated.

Some of this research may be about genes. Genes carry information about features, such as hair or eye colour. This research may include looking at changes in genes found in you and in people who are related to you. These changes may be inherited (passed on in families). This is called hereditary genetic testing. Researchers also may be interested in the way that genes affect health and disease, or how your body responds to treatment.

WHAT WILL HAPPEN DURING THIS OPTIONAL RESEARCH?
You may take part in all or some of the optional research described here, it is your choice. If you agree to take part:

- the samples used for this optional research have already been collected as part of your standard of care. No further biopsies or surgeries are needed for this purpose.
- the blood samples used for this optional research will be those left over or remaining from your participation in the main study. No further biopsies or surgeries are needed for this purpose.

HOW WILL MY SAMPLES BE HANDLED?
Your sample(s) and some related health information already collected from your participation in the main study will be sent to the Nutritional Immunology laboratory at the University of Alberta, Edmonton, AB, for analysis. The samples and data will be kept indefinitely or until they are used up, destroyed or returned to the hospital where you had your surgery or biopsy.

Qualified researchers can submit a request to use the materials stored at the University of Alberta. Your samples and related health information will be used only by researchers whose requests have been accepted by the sponsor and who have met regulatory requirements and secured ethics approval for their research. The samples and data may be sent to other countries. Your name or any other information that could directly identify you will not be given to these researchers.

The results of research done on your samples *will not* be added to your personal health records and you or the researcher *will not* know the results.

WHAT ARE THE RISKS OF PARTICIPATING IN THIS OPTIONAL RESEARCH?
Risks related to sample collection:

- Since the tissue sample(s) already have been collected for the main study or as part of your standard of care, no additional physical risks are expected.

Risks related to the disclosure of personal health information:

- There is a risk that someone could get access to the personal information in your personal health records or other information researchers have stored about you.
- There is a risk that someone could trace the information in a central or public database back to you. Even without your name or other identifiers, your genetic information is unique to you. The researchers believe the chance that someone will identify you is very small, but the risk may change in the future as people come up with new ways of tracing information.
- New health information about inherited traits that might affect you or your blood relatives could be found during a study. The researchers believe the chance these things will happen is very small, but cannot promise that they will not occur.
- Due to the rapid pace of technological advances, the potential future use of genetic information is unknown and therefore the potential future risks also are unknown.
- There may be risks to eligibility for employment or insurance if the results of genetic testing were inadvertently disclosed to certain parties.
- Genetic information cannot be protected from court-ordered disclosure.

WHAT ARE THE POTENTIAL BENEFITS OF PARTICIPATING IN THIS OPTIONAL RESEARCH?

You will not benefit directly from taking part in this optional research. However, research done with your donated samples or health information may benefit other patients with your condition or other similar or related condition(s).

HOW WILL MY PERSONAL INFORMATION BE KEPT PRIVATE?

Your privacy is very important to the researchers and they will make every effort to protect it. Here are the steps they will take:

- When your sample(s) are sent to the laboratory, no information identifying you (such as your name, date of birth, health insurance number) will be provided or shared. Samples may be identified by your study code.
- The samples that are provided to researchers by the Cross Cancer Institute are identified only by that biobank code; researchers will not know who you are.
- The list that links the samples to your personal identifiers (i.e., name) will be kept separate from your sample(s) *and health information* in a secure and confidential location at the main study site. If you change your mind about participating in this optional research, this list will be used to locate and return or destroy your samples. Decoding can only be done by the researcher or an individual authorized by the researcher.
- Study records will be kept for 25 years.
- A record of your participation in this optional study will be kept with your main study records and may be monitored for quality assurance.

Information that identifies you, will be kept confidential and, to the extent permitted by the applicable laws, will not be disclosed or made publicly available except as described in this document. If research results are published, your name and other personal information will not be used.

Qualified representatives of the sponsor will make sure the study has been done properly by checking your records at the researcher's site. Regulatory authorities, such as Health Canada and the applicable Research Ethics Board also may wish to check that the study has been done properly, and may also have direct access to your personal health information. Except as expressly stated in this section, all of the information provided in the main study consent form about confidentiality and direct access to your personal health information applies to this optional research consent form.

WHAT IF RESEARCHERS DISCOVER SOMETHING ABOUT ME DURING THE STUDY?

During the study, the researchers may learn something about you that they didn't expect. For example, the researchers may find out that you have another medical condition.

If any new clinically important information about your health is obtained as a result of your participation in this optional research, you will be given the opportunity to decide whether you wish to be made aware of that information.

WILL THERE BE ANY COSTS OR COMPENSATION INVOLVED WITH THS

RESEARCH?

There are no costs to you. You will not be paid for taking part. No samples or information/data will be sold.
It is possible that the research conducted using your samples and/or my data may eventually lead to the development of new diagnostic tests, new drugs or other commercial products. There are no plans to provide payment to you if this happens.

WHAT ARE MY RIGHTS AS A PARTICIPANT IN THIS OPTIONAL RESEARCH?

You will be told, in a timely manner, about new information that may be relevant to your willingness to stay in this study.

If you decide you no longer want your samples or related health information to be used, you should tell the researcher. Any sample(s) that remain(s) in the laboratory will be destroyed (if blood) or returned to the hospital where you had your original biopsy or surgery (if tumour block). If tests have already been done on your sample and included in an analysis or publication, it will not be possible to withdraw these results.

You will be given a copy of this signed and dated consent form prior to participating in this study.

IS THERE ANY CONFLICT OF INTEREST RELATED TO THIS OPTIONAL RESEARCH?

There are no current or potential conflicts of interest concerning the optional research study.

WHO DO I CONTACT FOR QUESTIONS RELATED TO THIS OPTIONAL RESEARCH?

If you have questions about the use of your samples/data for optional research, or if you suffer a research-related injury, contact the researcher of this optional study:

Catherine J Field	780-492-5297
Name	Telephone Number

If you have questions about your rights as a participant or about ethical issues related to this optional research and you would like to speak to someone not involved in its conduct, please contact the Office of the Health Research Ethics Board of Alberta – Cancer Committee at: 780-423-5727 or toll-free 1-877-423-5727.

UNDERSTANDING AND SIGNATURES PAGE

Please circle your answer to show whether or not you would like to take part in the optional research:

I agree that samples which were already collected and related health information may be used for the optional research described above.

YES NO

I agree that my samples and related health information may be kept in a biobank for use in future health research related to my condition or may be used to address research questions that are unrelated.

YES NO

I agree that the researcher, or their representative, may contact me or my physician to see if I wish to learn about results from this research.

YES NO

You will be given a copy of this signed and dated consent form prior to participating in this optional research.

SIGNATURES

PARTICIPANT ACKNOWLEDGEMENT

- I understand the information within this optional consent form.
- All of my questions have been answered to my satisfaction.
- I am aware of the risks and potential benefits to me of participating in this optional research.
- I allow access to my personal health information and samples as explained in this form.
- I understand that I do not give up any of my legal rights by signing this consent form.
- I agree to take part in this optional research as described and where "YES" above has been circled.

_____	_____	_____
Signature of Participant	Printed Name	Date

STUDY TEAM ACKNOWLEDGEMENT

I believe that the person signing this form understands what is involved in this optional research and has freely decided to participate.

_____	_____	_____
Signature of Person Conducting the Consent Discussion	Printed Name	Date

PARTICIPANT ASSISTANCE (IMPARTIAL WITNESS)

This section is to be completed only if the participant is unable to read the consent document. The individual assisting the participant must be impartial.

- The informed consent form was accurately explained to, and apparently understood by the research participant.
- Informed consent was freely given by the participant.

_____	_____	_____
Signature of Impartial Witness	Printed Name	Date

TRANSLATOR/INTERPRETER ACKNOWLEDGEMENT

This section is to be completed only if the participant requires the assistance of a qualified oral translator/interpreter. The interpreter must be impartial.

- The informed consent discussion was accurately explained to, and apparently understood by the research participant.
- A sight translation of the consent document was provided by the interpreter as directed by the research staff conducting the consent process.

_____	_____	_____
Signature of Interpreter	Printed Name	Date

Appendix Table 28: Fatty Acid Composition of DHA supplement and Placebo

Fatty Acid	DHA capsule	Placebo
16:0	16.9	10.9
18:0	0.1	2.7
18:1n-9	4.8	23.2
18:2n6	0.5	53.5
18:3n-3	<0.1	4.7
20:5n-3	1.0	<0.1
22:5n-3	0.5	<0.1
22:5n-6	18.1	<0.1
22:6n-3	43.4	<0.1

Appendix Table 29: List of Antibodies used for immune cell phenotyping

CD1a	**FITC**	**300104**
CD1c	BV421	331526
CD3	FITC	300306
CD4	APC	357408
CD8	PerCP/Cy5.5	344710
CD11b	PE	301306
CD11c	APC	301614
CD14	APC	367118
CD16	PE	302008
CD20	FITC	302304
CD25	PE	302606
CD27	PECy7	356412
CD28	APC	302912
CD45RA	PE	304108
CD45RO	FITC	304204
CD56	APC	362504
CD86	PCP	374210
CD95	BV421	305624
CD103	PECy7	350212
CD107	PE	328608
CD141	PECy7	344110
CD152	PE	369604
CD183	PerCP/Cy5.5	353720
CD196	PE	353410
CD279	APC	329908
FOXP3	FITC	320106
HLADR	PerCP/Cy5.5	307630