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Sept 26, 2018

"Investigating the anti-cancer effects of novel bioactive oils" (UA RES0017311)

Team Leader: Catherine Field
Co-Investigator: Randall Weselake, the Phytola team, Vera Mazurak
Organization: University of Alberta

Project Duration: 3 years
Project Start Date*: 04/01/2015
Project End Date*: 03/31/2018

*Note: Project was awarded April 2014 but officially began April 2015 due to a year delay in setting up agreements and the end date was extended until March 2018 due to a delay in obtaining a Canola source of DHA for the final objective.

EXECUTIVE SUMMARY

Breast cancer is the leading cause of cancer in Canadian women. It is estimated that 1 in 8 Canadian women will be develop breast cancer in their lifetime and 1 in 31 will die from the disease. Strategies to improve treatment of this disease are needed. Breast cancer is a heterogeneous disease that is stratified into histological and molecular subtypes. For a number of the forms of breast cancer, including triple-negative breast cancer, conventional therapies are limited in their successes and an improved understanding of disease progression is needed in conjunction with exploration of alternative therapies. It is well established that eicosapentaenoic (EPA) and docosahexaenoic (DHA) exert anti-cancer effects but dietary sources of these n-3 fatty acids (FA) are limited and the mechanisms to explain their anti-cancer effect is not known. Additionally, it is not known if other n-3 fatty acids such as punicic and stearidonic, that are found in small quantities in plant oils have the same biological effects as EPA and DHA. The overall aim of this grant was to establish the anti-cancer activity, the mechanisms and impact on chemotherapy treatment of: 1) high-DHA/EPA canola oil, an alternative to fish and single cell sources; 2) high-stearidonic flax oil, providing a FA that bypasses the rate limiting step in the synthesis of EPA/DHA; and 3) high-punicic acid canola oil, providing a FA that can be endogenously converted to c9t11 CLA.

The approach to addressing these objectives was to conduct a series of *in vitro* studies using human breast cancer cell lines. The two studied were the estrogen-receptor positive MCF-7 cell line that represents the majority of women diagnosed with breast cancer and the highly invasive triple-negative, MDA-MB-231 cell line that results in the poorest prognosis and is a candidate for neoadjuvant chemotherapy (chemotherapy prior to surgical resection and/or radiation). The *in vitro* studies were followed by a series of animal trials using two pre-clinical breast cancer

models and two different chemotherapy drugs used in standard care neoadjuvant breast cancer therapy.

In a series of peer reviewed publications we have provided evidence for the following:

- The fatty acids, punicic (1), stearidonic (2,3), eicosapentaenoic (2) and docosahexaenoic (2, 4) acid all have potent anti-cancer activities against both MDA-MB-231 and MCF-7 human breast cancer cells but not against non-tumorigenic MCF-12A (human breast cells).
- Using a well excepted pre-clinical mouse model of breast cancer (tumour-bearing *nu/nu* mice), we provided evidence that a diet supplemented with a high-stearidonic acid oil (3) or high DHA oil (4) reduced the growth of triple-negative breast cancer cells. We have confirmed the beneficial effects of DHA on tumour growth using a second, more robust pre-clinical model (NSG mice) with two different triple negative breast cancer tumours (4, unpublished results see report).
- We demonstrated that incubating MDA-MB-231 cells with DHA (4) or feeding DHA to MDA-MB-231 bearing *nu/nu* mice improved the action of the neoadjuvant chemotherapy, doxorubicin (4). We repeated this using 2 triple negative breast tumours in NSG mice and demonstrated that feeding DHA from either an algae source or from high DHA canola improved the anti-tumour activity of the other neoadjuvant chemotherapy, docetaxel (see report for unpublished results).
- Using genomics and molecular biological techniques, we have identified that the bioactive fatty acids exert their beneficial effect through their action on a number of genes and proteins that regulate apoptosis and growth (including cell cycle regulation). (see report for summary). Lipidomics suggests that changes in the fatty acid composition of cellular phospholipids may be the mediating factor (2-4).
- Together, our data from cell culture (1-4), pre-clinical models (3-4) and our critical reviews of the literature (5,6), suggests that consuming these fatty acids may be beneficial in the prevention and/or treatment of breast cancer and that the bioengeneering of oils could provide a dietary source of these fatty acids (7,8).

The data generated from this grant has provided the evidence needed to develop new engineered bioactive oils and to move to a clinical trial. Patent uptakes for new oils are pending and the DHA supplement will be used in a clinical trial, DHA-WIN (Docosahexaenoic acid (DHA) for Women with Early Breast Cancer in the Neoadjuvant Setting) that is expected to begin recruitment in Jan 2019.

References for Executive Summary

1. Gagnon AE, Newell M, Richard C, Weselake R, Field CJ. Growth inhibitory effects of conjugated linolenic and linoleic acid isomers on breast cancer cells in vitro. CRINA (Cancer Research Institute of Northern Alberta) Research Day (Nov 2014) Edmonton Alberta
2. Yu H-M, Newell M, Subedi K, Weselake RJ, Mazurak V, Field CJ. (2015) Bypassing the Delta 6-desaturase enzyme and directly providing n-3 and n-6 PUFA pathway intermediates reduces the survival of two human breast cancer cell lines. European Journal of Lipid Science and Technology Eur. J. Lipid Sci. Technol. 2015, 117, 1378–1390.
3. Subedi K, Yu H-M, Newell M, Weselake R, Meesapyodsuk D, Qiu X, Shah S and Field CJ. (2015) A stearidonic acid - enriched flax oil reduces the growth of human breast cancer in vitro and in vivo. Breast Cancer Research and Treatment 149:17-29 PMID: 25417173 DOI 10.1007/s10549-014-3212-3

4. Newell M, Brun M, Field CJ. Treatment with DHA modifies the response of MDA-MB-231 cells and tumors from nu/nu mice to doxorubicin through apoptosis and cell cycle arrest. Journal of Nutrition (accepted Aug 2018)
5. VanderSluis L, Mazurak VC, Damaraju S, Field CJ. (2017) Determination of the relative efficacy of long chain omega-3 polyunsaturated fatty acids for anti-cancer effects in human breast cancer models. International Journal of Molecular Science *18*(12), 2607; doi:10.3390/ijms18122607
6. Newell M, Baker K, Postovit L, Field CJ. (2017) A critical review on the effect of docosahexaenoic acid (DHA) on cancer cell cycle progression. International Journal of Molecular Science 18(8), 1784-1798; DOI:10.3390/ijms18081784
7. Weselake RJ, Woodfield H, Field CJ, Harwood JL (2017) Production of Edible Oils through Metabolic Engineering. In Food Lipids: Chemistry, Nutrition, and Biotechnology 4th edition, edited by Akoh CC, CRC Press, Taylor and Francis Group pages 973-996.
8. Holic R, Yang X, Mark K, Caldo KMP, Singer SD, Field CJ, Weselake RJ, Chen G. (2018) Bioactivity and biotechnological production of punicic acid, Applied Microbiology and Biotechnology 102(8):3537-3549.
9. Brun M, Field CJ. (2017) Enhancing Breast Cancer Treatment with Omega-3 Fatty Acids. FATS of Life. A Scientific Based Publication on healthy fats. Vol 22, Issue 1, 33-37.

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BACKGROUND

Original proposal:
Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), and c9t11 conjugated linoleic acid (CLA) have been demonstrated to have anti-cancer activity in human tumours and animals. Pre-clinical evidence suggests DHA/EPA can enhance chemotherapy action. As there are few sources of these fatty acids (FA), there is an opportunity for novel plant sources. This proposal aims to establish the anti-cancer activity, the mechanisms and impact on chemotherapy treatment of: 1) high-DHA/EPA canola oil, an alternative to fish and single cell sources; 2) high-stearidonic flax oil, providing a FA that bypasses the rate limited step in the synthesis of EPA/DHA; and 3) high-punicic acid canola oil, providing a FA that can be endogenously converted to c9t11 CLA. Efficacy and mechanisms will be assessed *in vitro* (+/- doxorubicin chemotherapy) using nutrigenomics and lipidomics in 3 human cancers that represent those afflicting women. These experiments will be followed by feeding the oils in a diet (with a fat composition representing that of the North American population) to *nu/nu* mice that have been implanted with human breast tumours. Establishing the efficacy and mechanism for these 3 bioactive oils on breast cancer will provide evidence to promote their use in humans and to move to clinical trials. This study will benefit the population at risk for and impacted by breast cancer, and the industries responsible for the development, production and refinement of novel oil products.

OBJECTIVES & DELIVERABLES (original proposal)

Overall aim: To clearly define the effects of 3 novel, designer oils on breast cancer growth and chemotherapy efficacy. We will assess the anti-breast cancer effects, the mechanisms of their action, and the impact on chemotherapy treatment of a high-DHA/EPA canola oil (HDO), a high-SDA flax oil (HSO), and a high-punicic acid canola oil (HPO).

Objective 1: To establish the impact of 2 novel designer oils (HDO and HPO) on breast cancer cell growth, *in vitro*, by measuring metabolic activity and markers of tumour cell proliferation and apoptosis (programed cell death)

Objective 2: To extend the findings of this cell culture work to an *in vivo* animal model of human breast cancer to verify the effectiveness of dietary intake of the 3 novel oils on breast cancer cell growth and determine optimal dosing and timing for maximal effects.

Objective 3: To identify the mechanisms of action of the 3 novel oils using nutri-genomic and lipidomic techniques, both *in vitro* and *in vivo*, and confirm findings by measuring protein expression, localization and activation of key receptors and proteins.

Objective 4: Based on the findings in objective 2 and 3, to assess the effectiveness of the oils alone and in combination in augmenting the anti-neoplastic effects with and without chemotherapy, *in vitro* and *in vivo*.

DELIVERABLES Proposed with ORIGNIAL PROPOSAL

1. Clear evidence of the efficacy of three novel, bioactive oils (from flax and canola) developed by Cargill/BASF or the Bioactive Oils Program and Alberta Innovates Phytola Centre, in the prevention and treatment of human breast cancer.
2. Evidence that the bioactive oils are not harmful (and possibly beneficial) to non-tumourigenic cells.
3. A clear understanding of the cellular fates of each fatty acid mixture, and the mechanisms responsible for the activity of the designer oils.
4. Establishment of the efficacy (adjuvant action) when combined with the first line treatment (chemotherapy) of human breast cancer.
5. An optimal mixture 'anti-cancer dietary oil' of these three oils that produces the best tumour treatment alone and with chemotherapy.

RESULTS AND DELIEVERABLES

Objective 1: To establish the impact of 2 novel designer oils High DHA/EPA Canola Oil (HDO) and High Punicic acid oil (HPO) on breast cancer cell growth, *in vitro*, by measuring metabolic activity and markers of tumour cell proliferation and apoptosis (already carried out for HSO).

HPO studies *in vitro*

A. The *in vitro* investigation establishing the anti-cancer activity of punicic acid were completed. Punicic Acid was found to have a high anti-cancer activity against human breast cancer cells, both triple negative (MBA-MD-231, Figure 1) and estrogen receptor positive (MCF-7, Figure 2) but did not harm 'non-cancer/malignant' breast cells lines (MCF-12A) (data not shown)

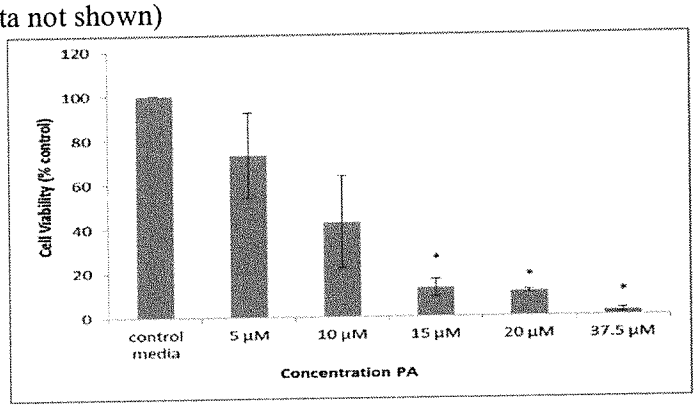

Figure 1: Effect of Punicic Acid on the viability (growth) of MDA-MB-231 Cells. Mean (±SEM) cell viability of MDA-MB-231 cells incubated with varying levels of PA expressed as a percent of control media treated cells. * Indicates viability of treated cells is significantly different from control treatment ($p<0.05$).

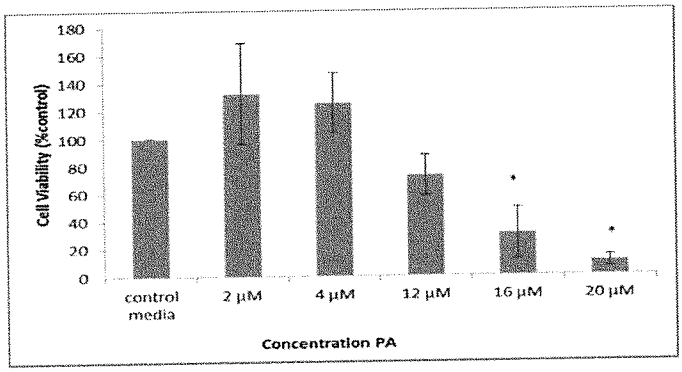

Figure 2: Effect of Punicic Acid on the viability (growth) of MCF-7 Cells. Mean (±SEM) cell viability of MCF-7 cells incubated with varying levels of PA expressed as a percent of control media treated cells. * Indicates viability of treated cells is significantly different from control treatment ($p<0.05$).

B. High Pucinic Acid Canola: The Phytola group successfully produced a T1 canola seed line over-expressing pomegranate conjugase (PgFADX)+ delta-12 desaturase (PgFAD2) to result in an oil containing 8% of the fatty acids as pucinic acid. Until the HPO patent is taken up by industry, there are no further plans to move to animal trials. The search for an industry partner was delayed (albeit not an objective of this grant) with the early retirement of the PI of Phytola, R. Weselake. To increase interest in the patent, we wrote the section on the health benefits of bioactive oils in a book on Food Lipids.

Book Chapter: Weselake RJ, Woodfield H, Field CJ, Harwood JL (2017) Production of Edible Oils through Metabolic Engineering. In Food Lipids: Chemistry, Nutrition, and Biotechnology 4th edition, edited by Akoh CC, CRC Press, Taylor and Francis Group pages 973-996. (see accompanying zip file for copy)

Patent: Weselake RJ, Mietkiewska E. Gene combinations for producing punicic acid in transgenic plants. U.S. Patent Application No. 14/224,582 filed March 25, 2014 (U.S. Provisional Patent Application No. 61/804,877; filed March 25, 2013). Note this patent was filed prior to this grant but some of the data was later added to it to support it.

C. Upon the retirement of Dr. Weselake and the dissolving of Phytola, we began a new collaborations with Dr. Gavin Chan We added some of the data from our literature review on punicic acid to a comprehensive literature review on the potential role of punicic acid on health (albeit not supported directly from this grant).

Holic R, Yang X, Mark K, Caldo KMP, Singer SD, Field CJ, Weselake RJ, Chen G. (2018) Bioactivity and biotechnological production of punicic acid, Applied Microbiology and Biotechnology 102(8):3537-3549.

Abstract: Punicic acid (PuA; 18: $3\Delta^{9cis,11trans,13cis}$) is an unusual 18-carbon fatty acid bearing three conjugated double bonds. It has been shown to exhibit a myriad of beneficial bioactivities including anti-cancer, anti-diabetes, anti-obesity, antioxidant, and anti-inflammatory properties. Pomegranate (*Punica granatum*) seed oil contains approximately 80% PuA and is currently the major natural source of this remarkable fatty acid. While both PuA and pomegranate seed oil have been used as functional ingredients in foods and cosmetics for some time, their value in pharmaceutical/medical and industrial applications are presently under further exploration. Unfortunately, the availability of PuA is severely limited by the low yield and unstable supply of pomegranate seeds. In addition, efforts to produce PuA in transgenic crops have been limited by a relatively low content of PuA in the resulting seed oil. The production of PuA in engineered microorganisms with modern fermentation technology is therefore a promising and emerging method with the potential to resolve this predicament. In this paper, we provide a comprehensive review of this unusual fatty acid, covering topics ranging from its natural sources, biosynthesis, extraction and analysis, bioactivity, health benefits, and industrial applications, to recent efforts and future perspectives on the production of PuA in engineered plants and microorganisms.

High DHA Canola

In 2015 BASF (who had originally indicted they would provide the n-3 LCPUFA Canola) decided to discontinue the production of the n-3 LCPUFA canola and the patent was transferred

to Monsanto. We then discussed this with Monsanto about obtaining oil from the high DHA/EPA Canola (for the animal feeding trials) but the company made the decision to not to commercialize the product. We began discussions (James Petrie, CSIRO Australia and Malcolm Devine, NUSEED Australia), wrote a proposal to the Board and received approval (Dec 2016) to obtain HPO from Nuseed Australia. The oil arrived in Dec 2017 and the feeding trials began Jan 2018. (see later objectives for update)

i) Critical literature review of the anti-cancer activity of EPA and DHA. In anticipation of obtaining NUSEED canola (composition not yet released to us), we performed extensive experiments with human tumour cells to identify the effects of different ratios of DHA and EPA on breast cancer cells (markers of proliferation and apoptosis).

We published a critical review of what was known about the relative anti-cancer efficacy of DHA and EPA. It was published in 2017. (copy in the zip folder accompanying this report):

VanderSluis L, Mazurak VC, Damaraju S, Field CJ. (2017) Determination of the relative efficacy of long chain omega-3 polyunsaturated fatty acids for anti-cancer effects in human breast cancer models. International Journal of Molecular Science *18*(12), 2607; doi:10.3390/ijms18122607

Abstract: Epidemiological studies have associated high fish oil consumption with decreased risk of breast cancer (BC). n-3 long chain polyunsaturated fatty acids (n-3 LCPUFA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) found in fish and fish oils exert anti-cancer effects. However, few studies have examined the relative efficacy of EPA and DHA alone and in mixtures on BC subtypes. This was the objective of the present review, as this research is a necessity for the translation of findings to human health and disease. The literature suggests that DHA has a greater anti-cancer effect in triple negative BC (TNBC). In estrogen positive (ER+) BC, DHA has a greater effect on cell viability, while both fatty acids have similar effects on apoptosis and proliferation. These effects are associated with preferential uptake of DHA into TNBC lipid rafts and EPA in ER+ BC. EPA:DHA mixtures have anti-cancer activity; however, the ratio of EPA:DHA does not predict the relative incorporation of these two fatty acids into membrane lipids as EPA appears to be preferentially incorporated. In summary, DHA and EPA should be considered separately in the context of BC prevention. The elucidation of optimal EPA:DHA ratios will be important for designing targeted n-3 LCPUFA treatments.

ii) Completion of a M.Sc. thesis: M.Sc. Student (Laura VanderSluis) completed her M.Sc. addressing this objective and successfully defended in Sept 2018. *At least 1 manuscript is in progress and will be submitted over the next year.*

Thesis Title: Determination of the Relative Efficacy of Docosahexaenoic Acid and Eicosapentaenoic Acid in Two-Dimensional and Three-Dimensional Cell Culture Models of Human Breast Cancer (completed Sept 2018)

Abstract: Omega-3 long chain polyunsaturated fatty acids (n-3 LCPUFA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), decrease breast cancer cell viability. DHA, EPA, and dietary relevant DHA:EPA mixtures have not been systematically investigated in two-dimensional (2D) cell culture models of human breast cancer and compared to three-dimensional (3D) cell culture models, which recapitulate the tumour microenvironment. The overall objectives of this thesis were to use MDA-MB-231 (triple negative (estrogen receptor-, progesterone receptor-, human epidermal growth factor receptor (Her2)-) and SK-BR-3 (Her2+) human breast cancer cells to: 1) determine if differences exist between DHA, EPA, and DHA:EPA mixtures on cell viability, tumour fatty acid composition, and proteins related to cell death and growth pathways in 2D culture and 2) determine if the effects are

maintained in 3D culture. In 2D culture, cells were incubated with 100, 150, or 200 μM DHA, EPA, or DHA:EPA mixtures (1:1 or 2:1) with a background fatty acid mixture (oleic/linoleic acid). In MDA-MB-231 cells all treatments decreased cell viability to the same extent at 100 and 150 μM (25-29% and 19-26%, respectively, $p<0.05$). DHA was more efficacious than other treatments at 200 μM (59% vs. 36-44%, $p<0.05$). Relative EPA +docosapentaenoic acid (DPA) and DHA content (%w/w) in total phospholipids (PL) and PL classes differed between DHA, EPA, and 2:1 treatments and the 1:1 mixture (EPA+DPA≈DHA vs. EPA+DPA>DHA, $p<0.05$). Similar decreases in cell content of apoptotic proteins RIPK1 (16%-28%, $p<0.05$), FADD (14%-31%, $p<0.05$), and increases in phosphorylated epidermal growth factor receptor (84%-96%, $p<0.05$) with all treatments may account for the similar effects on cell viability at 100 and 150 μM. In SK-BR-3 cells, EPA decreased cell viability to the greatest extent at each dose tested (35-47% vs. 17-39%, $p<0.05$). The relative EPA+DPA content in total PL and PL classes differed with EPA, DHA, and 1:1 treatments compared to the 2:1 mixture (EPA+DPA≈DHA vs. DHA>EPA+DPA, $p<0.05$). Increases in CD95 death receptor and decreased FADD content (14 and 22%, $p<0.05$) may explain the effect of EPA. In 3D culture, changes in EPA+DPA and DHA content with n-3 LCPUFA treatments in whole cell fatty acids were consistent with 2D culture. However, there were increases in MDA-MB-231 spheroid growth (26%, $p<0.05$) and SK-BR-3 aggregate formation (38-62%, $p<0.05$), suggesting these indices are not appropriate for studying the anti-cancer effects of n-3 LCPUFA established in 2D culture and animal feeding trials. Collectively, this research is important for using n-3 LCPUFA mixtures to target breast cancer tumours.

Objectives 2-4

The remaining objectives will be discussed together as they were met through a number of cell culture and animal feeding trials

Objective 2: To extend the findings of this cell culture work to an *in vivo* animal model of human breast cancer to verify the effectiveness of dietary intake of the 3 novel oils on breast cancer cell growth and determine optimal dosing and timing for maximal effects.

Objective 3: To identify the mechanisms of action of the 3 novel oils using nutri-genomic and lipidomic techniques, both *in vitro* and *in vivo*, and confirm findings by measuring protein expression, localization and activation of key receptors and proteins.

Objective 4: Based on the findings in objective 2 and 3, to assess the effectiveness of the oils alone and in combination in augmenting the anti-neoplastic effects with and without chemotherapy, *in vitro* and *in vivo*.

Modification to objectives: Due to the HPO oil commercialization being on hold until patent uptake, we focused this objective on the two bioactive oils of the original proposal; the high steradonic (SDA) flax oil, and the high DHA Canola (HDO).

c. A critical review of the literature was completed to identify the mechanisms by which DHA influences cell cycle arrest in tumour cells (copy in the zip folder accompanying this report):

Manuscript: Newell M, Baker K, Postovit L, Field CJ. (2017) A critical review on the effect of docosahexaenoic acid (DHA) on cancer cell cycle progression. International Journal of Molecular Science 18(8), 1784-1798; DOI:10.3390/ijms18081784

Abstract: Globally, there were 14.1 million new cancer diagnoses and 8.2 million cancer deaths in 2012. For many cancers, conventional therapies are limited in their successes and an improved understanding of disease progression is needed in conjunction with exploration of alternative therapies. The long chain polyunsaturated fatty acid, docosahexaenoic acid (DHA), has been shown to enhance many cellular responses that reduce cancer cell viability

and decrease proliferation both *in vitro* and *in vivo*. A small number of studies suggest that DHA improves chemotherapy outcomes in cancer patients. It is readily incorporated into cancer cell membranes and, as a result there has been considerable research regarding cell membrane-initiated events. For example, DHA has been shown to mediate the induction of apoptosis/reduction of proliferation *in vitro* and *in vivo*. However, there is limited research into the effect of DHA on cell cycle regulation in cancer cells and the mechanism(s) by which DHA acts are not fully understood. The purpose of the current review is to provide a critical examination of the literature investigating the ability of DHA to stall progression during different cell cycle phases in cancer cells, as well as the consequences that these changes may have on tumour growth, independently and in conjunction with chemotherapy.

ii) Genomic analysis was performed on human breast cancer cells (MDA-MB-231 and MCF-7) incubated with

   a. EPA or DHA compared to a control fatty acid
   b. SDA compared to a control fatty acid

Summary of the Results:

a. The objective of this study was to determine alterations in gene expression in breast cancer cells treated with docosahexaenoic acid (DHA) or eicosapentaenoic acid (EPA) compared to linoleic acid. MDA-MB-231 (estrogen receptor, ER^-) and MCF-7 (ER^+) human breast cancer cell lines were treated with culture media (control) or 100 μM linoleic acid (LA; n-6 fatty acid control), DHA or EPA for 48 h. Cellular viability was assessed by WST-1 assay and gene expression was measured using Affymetrix Gene Chip Human Gene ST 1.0 microarray chips. Data were analyzed for differential gene expression and network analysis was conducted using Ingenuity Pathway Analysis software. EPA and DHA both altered genes associated with cancer and anti-proliferation. Genes altered only by DHA impacted networks of genes related to cancer, cell death, cell cycle and tumor morphology, compared with all other treatments ($P<0.05$), while EPA uniquely impacted an array of non-cancer related networks, including tissue development and organismal injury ($P<0.05$). This study provided a transcriptome-level mechanistic explanation for the differences in viability induced by EPA and DHA, in two genetically and phenotypically different breast cancer cell lines and it is expected that manuscripts will be completed by the end of 2019.

b. In a second experiment, the difference in genome wide gene expression level in MDA-MB-231 breast cancer cells treated with SDA compared to a control fatty acid mixture was determined. Microarray data showed that out of 26,057 expressed genes in MDA-MB-231, 2239 (8.6%) were significantly changed ($p \leq 0.05$). Out of 8.6% genes, 42% were up-regulated and 58% down-regulated. We used two metrics (activation of Z-score and p-value) in IPA to identify the most important downstream biological functions that are expected to be decreased in SDA compared to control according to our microarray data. These functions connected to several cell processes such as cell survival and viability, inflammatory response, cellular movement, cell to cell signalling, proliferation and cancer characteristics (adhesion, angiogenesis and metastasis). The function of cell death tended to be increased however did not reach significant (Z-score- 0.379 and p-value -7.9E-12). We further analyzed these functions in more depth for the difference in the increase and decrease gene expression. The genes increased or decreased by 1.2-fold associated with are shown in Table 1.

Table 1. Genes significantly up or down regulated in different groups in MDA-MB-231 cells in response to SDA compared to the control fatty acids

Gene symbol	Entrez gene name	Fold change
Apoptosis		
CASP10	Caspase 10, apoptosis-related cysteine peptidase	-1.2
BCL2A1	BCL2-related protein A1	-1.8
BIRC3	Baculoviral IAP repeat containing 3	-1.4
DUSP1	Dual specificity phosphatase 1	-1.2
CAPN11	Calpain 11	-1.3
MCL1	Myeloid cell leukemia sequence 1	-1.2
APAF1	Apoptotic peptidase activating factor 1	-1.2
BAK1	BCL2-antagonist/killer 1	-1.2
CAPN10	Calpain 10	1.2
Growth factors		
VEGFA	Vascular endothelial growth factor A	-1.2
GDF-15	Growth differentiation factor 15	-1.4
COX and prostaglandin		
PTGES	Prostaglandin E synthase	-1.4
PTGS2/COX2	Prostaglandin-endoperoxide synthase 2	-2.3
TNF family		
TRAF1	TNF receptor-associated factor 1	-1.6
TNF	Tumor necrosis factor	-1.3
TNIP1	TNFAIP3 interacting protein 1	-1.4
TNFRSF10B	tumor necrosis factor receptor superfamily, member 10b	-1.2
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	-1.5
TNIP3	TNFAIP3 interacting protein 3	-1.6
TNFSF15	Tumor necrosis factor (ligand) superfamily, member 15	-1.6
LTBR	Lymphotoxin beta receptor	-1.2
TRADD	TNFRSF1A-associated via death domain	-1.3
Cell cycle		
CCNE2	Cyclin E2	1.2
CKS2	CDC28 protein kinase regulatory subunit 2	1.2
Cell adhesion and organization		
ICAM1	Intercellular adhesion molecule 1	-1.7
MMP14	Matrix metallopeptidase 14	-1.2
SDC4	Syndecan 4	-1.3
Transcription factors		
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer	-1.5
JUNB	Jun B proto-oncogene	-1.4
STAT5A	Signal transducer and activator of transcription 5A	-1.3
IKBKE	Inhibitor of kappa light polypeptide gene enhancer	-1.2
XBP1	X-box binding protein 1	-1.2

The results from these studies are being written up for a manuscript that will be submitted in 2019.

iii) Animal trials with and/or without chemotherapy (doxorubicin or docetaxael) were conducted feeding the different bioactive oils (SDA-enriched oil, DHA-algae source, DHA-enriched Canola) to mice (*nu/nu* or NSG) compared to a control fatty acid diet on the growth of different models of human breast cancer (MDA-MB-231 triple negative breast cancer cells, patient-derived xenografts)

Results of the animal trials:

i. SDA-enriched oil

A. We identified the anti-cancer activity of SDA and the other long chain n-3 polyunsaturated fatty acids to halt the growth of human breast cancer cells. This was a component of a M.Sc. thesis (H-M Yu) defended in 2015. (manuscript in the accompanying zip folder)

Yu H-M, Newell M, Subedi K, Weselake RJ, Mazurak V, **Field CJ**. (2015) Bypassing the Delta 6-desaturase enzyme and directly providing n-3 and n-6 PUFA pathway intermediates reduces the survival of two human breast cancer cell lines. Eur. J. Lipid Sci. Technol. 2015, 117, 1378–1390.

Abstract: The n-3 long chain polyunsaturated fatty acids (PUFA), eicosapentaenoic (EPA), and docosahexaenoic (DHA) acid, and in some studies, the n-6 long chain PUFA arachidonic acid (AA), inhibit survival of breast cancer cells in vitro and in vivo. Less is known about the intermediates synthesized from the dietary 18 carbon fatty acids. The objective of this study was to determine the survival and phospholipid (PL) fatty acid composition of tumorigenic (MDA-MB-231, MCF-7) and nontumorigenic (MCF-12A) breast cells treated with intermediates in the n-3 and n-6 pathway. N-3 intermediates, stearidonic acid (SDA) and eicosatetraenoic acid (ETA), and n-6 intermediates, g-linolenic acid (GLA) and dihomo g-linolenic acid (DGLA), reduced ($P<0.05$) the growth of tumorigenic but not MCF-12A cells. All treatments resulted in a higher PUFA and saturated content and a lower monounsaturated content in tumorigenic cells ($P<0.05$). For MDA-MB-231 PL, treatment with SDA and ETA increased ETA, EPA, and docosapentaenoic acid (DPA) and lowered AA, while treatment with GLA and DGLA primarily increased DGLA with only a small decrease in the DPA and DHA ($P<0.05$). For MCF-7 PL, due to low desaturase activity, the n-3 intermediates increased ETA and the n-6 increased DGLA ($P<0.05$). Our findings suggest that the less studied n-6 (GLA and DGLA) and n-3 (SDA and ETA) fatty acids could serve as alternative dietary sources of bioactive lipids targeted at breast cancer.

B. A diet containing the high SDA oil compared to a control fatty acid mixture (that did not contain any SDA) was fed to *nu/nu* mice implanted with the human MD-MBA-231(triple negative highly invasive) breast cancer tumour. The accompanying cell culture studies were done to identify the mechanism for the beneficial effects. Lipidomics analysis was also done on the cells and the tumours and reported in the published manuscript (manuscript in the zip folder with this report).

Subedi K, Yu H-M, Newell M, Weselake R, Meesapyodsuk D, Qiu X, Shah S and Field CJ. (2015) A stearidonic acid - enriched flax oil reduces the growth of human breast cancer *in vitro* and *in vivo*. Breast Cancer Research and Treatment 149:17-29 PMID: 25417173 DOI 10.1007/s10549-014-3212-3

Abstract: The 20 and 22 carbon n-3 long-chain polyunsaturated fatty acids (LCPUFA) inhibit the growth of tumors in vitro and in animal models, but less is known about the 18 carbon n-3, stearidonic acid (SDA). This study aimed to establish and determine a mechanism for the anticancer activity of SDA-enriched oil (SO). SO (26 % of lipid) was produced by genetically engineering flax and used to treat human tumourigenic (MDA-MB-231, MCF-7) and non-tumorigenic (MCF-12A) breast cells. *Nu/nu* mice bearing MDA-MB-231 tumor were fed SO (SDA, 4 % of fat). Cell/tumour growth, phospholipid (PL) composition, apoptosis, CD95, and pro-apoptotic molecules were determined in SO-treated cells/tumors. Compared to a control lipid mixture, SO reduced ($P<0.05$) the number of tumourigenic, but not MCF-12A cells, and resulted in higher concentration of most of the n-3 fatty acids in PL of all cells ($P <0.05$). However, docosapentaenoic acid increased only in tumourigenic cells ($P <0.05$). SO diet decreased tumour growth and resulted in more n-3 LCPUFA, including DPA and less arachidonic acid (AA) levels in major tumor PL ($P <0.05$). Treatment of MDA-MB-231 cells/tumours with SO resulted in more apoptotic cells (in tumours) and *in vivo* and *in vitro*, more CD95 positive cells and a higher expression of apoptotic molecules -

Caspase-10, Bad, or Bid (P <0.05). Supplementing SO alters total PL and PL classes by increasing membrane content of n-3 LCPUFA and lowering AA (*in vivo*), which is associated with increased CD95-mediated apoptosis, thereby suggesting a possible mechanism for reduce tumour survival.

ii. DHA-Algae Source

Nu/nu mice implanted with the human MDA-MB-231(triple negative highly invasive) breast cancer cells treated with or without the chemotherapy drug doxorubicin. The effects on the mice and tumours were compared to mice fed the control fatty acid mixture, that did not contain DHA and was more similar to the fat mixture of the North American Diet. (manuscript in the zip folder with this report). Lipidomics analysis was performed and the results will be reported in a future manuscript

Newell M, Brun M, Field CJ. Treatment with DHA modifies the response of MDA-MB-231 cells and tumors from *nu/nu* mice to doxorubicin through apoptosis and cell cycle arrest. Journal of Nutrition (accepted Aug 2018, copy of the accepted manuscript in zip folder accompanying this report)

Abstract: Background: Docosahexaenoic acid (DHA) has been shown to reduce growth of breast cancer cells *in vitro, in vivo* and it may benefit the action of cytotoxic cancer drugs. The mechanisms for these observations are not completely understood.
Objectives: We sought to explore how pre-treatment of MDA-MB-231 breast cancer cells with DHA alters gene expression with doxorubicin (DOX) treatment and confirm that feeding DHA to tumor bearing *nu/nu* mice improves the efficacy of DOX.
Methods: MDA-MB-231 cells were subjected to 4 conditions: a control mixture of 40 μM linoleic and 40 μM oleic acid (OALA), DHA (60 μM plus OALA), OALA doxorubicin (DOX, 0.41 μM), or, DHA DOX (plus OALA) and assessed for effects on viability and function. Female *nu/nu* mice (6-week old) bearing MDA-MB-231 tumors were randomized to a nutritionally complete diet (20 g ± 2.8 g w/w DHA /100 g diet) containing a polyunsaturated/saturated fat ratio of 0.5, with or without a twice weekly injection of 5 mg/kg twice weekly injection of DOX for 4 weeks.
Results: Microarray and protein analysis indicated that DHA DOX cells, compared to OA/LA DOX, had up-regulated expression of apoptosis genes, Caspase-10 (1.3 fold), Caspase-9 (1.4 fold), and *RIPK1* (1.2 fold), while down-regulating cell cycle genes, Cyclin B1 (-2.1 fold), *WEE1* (-1.6 fold), and *CDC25C* (-1.8 fold) (P<0.05). DHA DOX treated mice had 50% smaller tumors compared to control mice (P<0.05). Analysis of pro-apoptotic proteins from DHA DOX mice tumors showed increased Caspase-10 (2.2 fold), and Bid (1.6 fold), decreased BCL2 (0.8 fold) and decreased cell cycle proteins Cyclin B1 and Cdc25c, (both 0.5 fold) compared to control mice (P<0.05).
Conclusions: Supplementation with DHA facilitated the action of DOX in MDA-MB-231 cells and in *nu/nu* mice and this may occur via amplification of the effect of DOX on apoptosis and cell cycle genes.

iii. DHA dose study using both the **DHA-Canola** (low dose) and DHA-Algae (high dose) sources

Animal experiments are complete. The results have not yet been submitted for publication as there are still analyses pending. Below is a brief description of the study and the main results.

Objectives:

To determine the anti-cancer effects of supplementing diets with two doses and sources of DHA on the response to neoadjuvant chemotherapy (docetaxel) in mouse model of a patient-derived breast cancer xenograft (PDX).