Anti-cancer effects of n-3 long-chain polyunsaturated fatty acids (LCPUFA) on ovarian cancer growth *in vitro* and *in vivo*

by

Bahareh Hamedi

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science
in
Cancer Sciences

Department of Oncology
University of Alberta

Abstract

Objectives: Many ovarian cancer (OC) patients relapse with chemo-resistant disease, necessitating improved therapeutic approaches. Recent studies have determined that long chain polyunsaturated fatty acids (LCPUFA) may affect cancer cell growth and metastatic potential; however, few studies have tested these in combination with chemotherapy in OC models. Herein, we investigated the effects of n-3 LCPUFAs on OC cell growth, and sensitivity to carboplatin, both *in vitro* and *in vivo*.

Methods: *In vitro,* eight different OC cell lines were treated with different concentrations of fatty acids (0 to 320μM), alone and in combination with carboplatin, to assess the cytotoxicity. *In vivo,* mice transplanted with patient-derived xenograft (PDX) OC models were randomly assigned into four groups: basal diet (control), basal diet+carboplatin, docosahexaenoic acid diet (DHA, 3.8%), and DHA diet+carboplatin. Mice were fed with basal or DHA enriched diet for two weeks prior to weight-based treatment with carboplatin (40mg/kg every 3 days IP) and were kept on the same diet during chemotherapy.

Results: *In vitro*, DHA alone inhibited proliferation of ES2 and A2780cp OC cells. Linear regression analysis demonstrated that DHA enhanced the cytotoxic effects of carboplatin on ES2, A2780cp, SKOV3, and Kuramochi cells. DHA reduced cell proliferation and induced cell cycle alteration in ES2 cells. *In vivo*, DHA enriched diet significantly reduced the tumor growth and increased the sensitivity of PDX tumors to carboplatin. Histological evaluation showed that

the combination of DHA with carboplatin increased the area of necrosis and reduced the expression of Ki67 without induction of apoptosis.

Conclusions: *In vitro* and *in vivo* evidence showed that DHA might increase the sensitivity of OC cells to platinum-based chemotherapy. Further studies, as well as clinical trials, are necessary to provide a strong rationale for a targeted n-3 LCPUFA intervention, as an adjuvant to the antineoplastic drug.

Dedication

To Ali, Paria, Darya, Mom and Dad

Acknowledgments

I would like to express my deepest gratitude to my supervisor ***Dr. Lynne-Marie Postovit*** *for her academic guidance and support during my master research. I am forever grateful to Lynne for her kindness, understanding, and for all of the countless opportunities she has provided me with. It has been an absolute privilege working with such a force in oncology research and the welcoming and constructive environment she has created in her lab.*

I would like to extend my thanks to my supervisory committee members ***Dr. Catherine J. Field*** *and* ***Dr. Yangxin Fu*** *for their insightful suggestions and their guidance throughout the completion of this thesis.*

I would like to thank other defense committee members ***Dr. Roseline Godbout*** *and* ***Dr. Mary Hitt*** *for taking the time to read and critically discuss my thesis with me.*

I would like to thank ***Dr. Postovit's lab members****, for active collaboration to produce this work, for assistance and guiding me through several laboratory techniques, and for their insightful discussions throughout cancer research team weekly meetings. Specifically, I would like to thank* ***Dr. Olena Bilyk,*** *a postdoctoral fellow in Dr.Postovit's lab****,*** *for her assistance in every step of this project, for training me in all the lab basics and for her great job in the animal work. She was always available for help with her outstanding knowledge and great technical expertise.*

I also thank the PhD candidate in the Dr. Field's lab, ***Marnie Newell,*** *for assisting with the fatty acid preparation and phospholipid analysis, the staff of the Flow Cytometry Facility at the University of Alberta****, Aja Reiger*** *and* ***Sabina Baghirova****, for assistance in flow cytometry and FlowJo analysis, Microscope Unit at the Department of Pathology,* ***Shalawny Miller****, for helping with imaging of immunohistochemistry slides, and Imaging Facility at the Cross Caner,*

***Geraldine Barron**, for helping me in imaging with Confocal Microscope. This research was generously supported by a start-up funding donated by the Dr. Anthony Noujaim Legacy.*

I also owe a lot to my husband, Ali, and my daughters, Paria and Darya, for always being there by my side. Their love and support have given me the strength to be what I am today. Their endless motivation has fuelled in me the desire to work hard.

I am thankful to my parents for their constant words of encouragement and motivation from halfway across the world which has inspired me to do my best.

Table of Contents

List of Tables:

List of Figures:

Abbreviations

Acetyl CoA	acetyl coenzyme A
ADHD	attention deficit hyperactivity disorder
ALA	alpha-linolenic acid
ARID1A	AT-Rich Interaction Domain 1A
AUC	area under curve
BAK	BCL2 Antagonist/Killer 1
BAX	BCL2 Associated X
BCL	B cell lymphoma
BRAF	B-Raf proto-oncogen
BRCA1	breast cancer 1 gene
BRCA2	breast cancer 2 genes
CA125	cancer antigen 125
CCC	clear cell carcinoma
CDKN2A	cyclin-dependent kinase Inhibitor 2A
CDKs	cyclin-dependent kinases
COX	cyclooxygenase
CT	computerized tomography
CTNNB1	catenin Beta 1
Ctr	copper transporter
DHA	docosahexaenoic acid
EGFR	epidermal growth factor receptor
EOC	epithelial ovarian carcinomas
EPA	eicosapentaenoic acid
ERK	extracellular signal-regulated kinase
EZH2	zeste homologues 2
FAS	First apoptosis signal
FDA	Food and Drug Administration

FIGO	International Federation of Gynecology and Obstetrics
FNA	fine needle aspiration
H3K27me3	histone 3 lysine 27 trimethylation
HER-2	human epidermal growth factor receptor-2
HGSC	high-grade serous carcinoma
HNF-1β	Hepatocyte nuclear factor -1β
ICAM-1	intracellular adhesion molecule
IGF-1R	Insulin-like growth factor-1
IHC	immunohistochemistry
INK4a	inhibitors of CDK4
KRAS	Kirsten rat sarcoma gene
LA	Linoleic acid
LCPUFAs	long chain polyunsaturated fatty acids
LGALS4	lectin galactoside binding soluble 4
LGSC	low-grade serous ovarian carcinoma
LOX	lipoxygenase
LTs	leukotrienes
MAP	mitogen-activated protein
MAPKs	mitogen-activated protein kinases
MEK	MAPK ERK kinase
MMP-9	matrix metalloproteinase 9
MRI	magnetic resonance imaging MRI
m-TOR	mechanistic target of rapamycin
MTT	3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF1	Neurofibromatosis type 1
NFkB	nuclear factor kappa B
OC	ovarian cancer
OCT	organic cation transporters
OSE	varian surface epithelium

PARP	poly ADP ribose polymerase
PAX8	Paired box gene 8
PDX	patient-derived xenograft
PET	Positron Emission Tomography
PFI	platinum-free interval
PGs	prostaglandins
PIk3CA	phosphatidylinositol-4,5- bisphosphate 3-kinase catalytic subunit alpha
PIP3 ($PtdInsP_3$)	Phosphatidylinositol (3,4,5)-trisphosphate
PPAR-γ	peroxisome proliferator-activated receptor gamma
PPREs	PPAR response elements
PTEN	Phosphatase and tensin homolog
RB	retinoblastoma
RCT	randomized clinical trials
ROS	reactive oxygen species
SEER	Surveillance, Epidemiology, and End Results
STIC	serous tubal intraepithelial carcinoma
SWI/SNF	switch/sucrose non-fermentable
TIMP1	tissue inhibitor of metalloproteinases 1
TP53	Tumor Protein 53
TXs	thromboxane
VCAM	vascular cell adhesion protein 1 precursor
VEGF	vascular endothelial growth factor

Chapter 1

Introduction

Chapter One- Introduction

1. Ovarian cancer

1.1 Ovarian cancer epidemiology

Ovarian cancer is the second most common gynecological malignancy and the most lethal genital tract cancer in the USA and Canada (1, 2).

According to the Surveillance, Epidemiology, and End Results (SEER) Program 2011-2015, the number of new cases and deaths from ovarian cancer were 11.6 and 7.2 per 100,000 women per year, respectively (3). Based on the published data on SEER, about 15% of diagnosed ovarian cancers are localized and confined to the primary site with a 5-year relative survival rate of 92.3%. Regional disease, which spreads to regional lymph nodes, accounts for 20% of the tumors, while 59% of patients have distant metastasis at the time of diagnosis. The 5-year survival rates of the patients significantly decline to 74.5% and 29.2% in regional and metastatic diseases, respectively (3). Symptoms of the early-stage disease may be subtle, and this is the reason why most patients present with advanced stages of the disease. Even with the standard treatment of cytoreductive surgery in combination with taxanes-platinum-based chemotherapy, the majority of patients experience a recurrence with poor overall survival (4).

1.2 Etiology and risk factors of ovarian cancer

Most patients with ovarian cancer present in an advanced stage and consequently, the molecular or tissue biomarkers of the disease's early stages are not entirely determined (5). Moreover, even if tissue biomarkers for neoplastic transformation of the ovarian epithelium are known, it is difficult to apply this knowledge clinically because of the inaccessibility of ovarian tissue (6).

Therefore, the identification of the population at risk for ovarian cancer is primarily based on epidemiologic data.

Among all risk factors, reproductive and hormonal factors are the most established ones for ovarian cancer. Increased risk has consistently been associated with the following elements: higher lifelong numbers of menstrual cycles, nulliparity, low parity, hormonal replacement therapy, family history of breast and ovarian cancer, and the presence of mutations in the breast cancer 1 and breast cancer 2 genes (BRCA1 and BRCA2) (7 - 9). Breastfeeding, multiparity, tubal ligation, salpingectomy, combined oral contraceptive pills, late age at menarche, and early menopause have been inversely related to ovarian cancer in different studies (10, 11).

1.3 Histotypes and molecular characteristics of ovarian tumors

The majority of ovarian tumors are categorized into three different types, based on their anatomical structure and origin: epithelial ovarian tumors, sex-cord stromal tumors, and germ cell tumors.

Epithelial ovarian tumors

Epithelial ovarian carcinomas (EOC) are the most common malignant ovarian tumors. About 90% of malignant ovarian tumors are epithelial in origin but the exact origin of these epithelial cells is still obscure. The proposed origins include the ovary, fallopian tube, and peritoneum (12, 13). EOC is a genetically heterogeneous disease with distinct molecular and clinical characteristics. It is grouped into two general categories. Type I cancers account for a third of ovarian cancers and include low-grade serous ovarian carcinoma (LGSC), endometrioid adenocarcinoma, clear cell carcinoma, and mucinous adenocarcinoma. This group arises from well-established precursor lesions in the ovary such as low-grade lesions, Mullerian inclusion cysts, and endometriosis. The other two-thirds of ovarian cancers are serous carcinomas, most commonly high-grade serous

carcinoma (HGSC), which are classified as type II tumors and are responsible for the poor outcomes associated with this disease (14, 15).

Serous epithelial ovarian cancer

For many years, it was believed that the ovarian surface epithelium (OSE) was the origin of HGSC. Since the late 1990s, serous tubal intraepithelial carcinoma (STIC), which is a non-invasive tumor lesion formed in the distal fallopian tube, was proposed to be the origin of HGSC. Many clinical and pre-clinical study models support the fallopian tube STIC origin of HGSC. For example, HGSC cells resemble the secretory cells of the fallopian tube epithelium and express the transcription factor PAX8 that is a marker of fallopian tube secretory cells (16, 17). This histologic type is most common in the sixth decade of life with a mean age of 59.4. The tumors may contain multiple loculations, cystic and solid areas, as well as abundant papillae (18). Most HGSC tumors are disseminated at the time of diagnosis and prognosis is related to the stage of the disease at that time. Five-year survival rates vary from 76% to 9% in stages I to IV (12).

In HGSC of the ovary, the TP53 gene mutation is found in at least 95% of cases (19, 20). Several other mutations have been reported in HGSC of the ovary, such as RB1, NF1, CDK12 and amplification of MYC with lower frequency (20 - 22). Like HGSC, LGSC cells also express the transcription factor PAX8. The common mutations in LGSC of the ovary are KRAS and BRAF mutations. These two genes are regulators of the downstream RAS/RAF/MEK/ERK/MAP signal transduction pathway and have a significant role in the transmission of growth signals to the nucleus and the resulting transformation of neoplastic cells (23).

Endometrioid carcinoma

The origin of endometrioid carcinoma may be the malignant transformation of the endometriotic cysts or ovarian endometriosis (24, 25). Endometrioid carcinoma accounts for about 10 – 25% of

all EOC and is the second most common subtype after serous carcinomas. The malignant tumors are predominantly solid and unilateral but may be cystic or bilateral. The five-year survival rates vary according to the stage of cancer: 78% for stage I, 63% for stage II, 24% for stage III and 6% for stage IV (12).

Like endometrial cancers, endometrioid carcinoma of the ovary is more prevalent in patients with Lynch syndrome, and 13–20% of patients have microsatellite instability. PTEN, as well as PIK3CA mutations, are found in about 20% of cases, and as a result, activation of PI3K and inhibition of apoptosis happens (26, 27). Mutations in CTNNB1 (Catenin Beta 1) and tumor suppressor gene ARID1A (AT-Rich Interaction Domain 1A), are found in endometrioid carcinoma and involve the Wnt/ß-catenin signaling pathway and chromatin remodeling as a member of the SWI/SNF complex, respectively (26 - 28).

Clear cell carcinoma

The majority of patients with clear cell carcinoma (CCC) are in the fifth decade of life with a history of nulliparity and endometriosis. Most clear cell tumors are malignant and present with a solid or cystic mass forming clear, piglike or hobnail-like cells (29). CCC has a close association with endometriosis, similar to endometrioid carcinoma. Progression-free survival for CCC is poorer in the advanced stages compared to other types of ovarian cancer (30).

Hepatocyte nuclear factor -1β (HNF-1β) plays a role in glucose and glycogen metabolism by regulating several genes such as glutathione peroxidase 3, annexin A4, and dipeptidyl peptidase IV. It may be the reason for the typical morphological characteristics of this tumor which show cells with a clear appearance and a glycogen-rich cytoplasm (31 - 33). Other gene mutations are the same as endometrioid carcinoma such as PIK3CA, PTEN, ARID1A and KRAS mutations (34).

Mucinous carcinoma

Mucinous tumors of the ovary account for 10-15% of ovarian epithelial tumors and may be primary or metastatic. Primary lesions of the ovary are mostly unilateral and large with cystic and multilocular features, while the metastatic tumors of the gastrointestinal tract are smaller and bilateral (35). The cellular origin of mucinous tumors is not known, as they may resemble the cells of the gastric pylorus, intestine, endocervix, germ cell, or transitional cell nests (36, 37). KRAS mutation is prevalent in ovarian mucinous tumors and may play a role in ovarian tumorigenesis (38). Also, it has been shown that overexpression of LGALS4 is an early step in the molecular pathogenesis of ovarian mucinous tumors, and can be found in almost all tumors of this type (39). Other mutations such as BRAF, CDKN2A, TP53, and P16/INK4a may also present in these tumors (40).

Other histotypes of ovarian tumors

Sex cord-stromal tumors are less prevalent than EOC and account for about 3%-5% of all malignant ovarian tumors. These tumors originate from theca cells, granulosa cells, and other ovarian stromal cells. Granulosa cell tumors, thecomas, fibromas, Sertoli, and Sertoli-Leydig cell tumors are different histotypes of this family and are more common in the first menopausal decade (12, 42).

Germ cell tumors are believed to originate from primordial germ cells of the ovaries. They account for a relatively small proportion of all malignant ovarian tumors and, compared to EOC, are more prevalent in children and adolescents. Because of their sensitivity to chemotherapy, germ cell tumors are among the most curable cancers. Dysgerminoma, yolk sac tumors, embryonal carcinoma, choriocarcinoma, and teratomas are all different subtypes of germ cell tumors (12).

1.4 Staging of ovarian cancer

According to the updated International Federation of Gynecology and Obstetrics (FIGO) staging system, staging is conducted during and after surgical debulking and is based on the extent of tumor dissemination and on pathological criteria, assessed by analyzing tissue samples (41, 42). The revised version published in 2018, combines the classification for the ovarian, fallopian tube, and peritoneum cancer. The currently approved staging of ovarian cancer is shown in Table 1-1.

Stage I: Tumor confined to ovaries or fallopian tube(s)	
	T1-N0-M0
IA: Tumor limited to 1 ovary (capsule intact) or fallopian tube; no tumor on ovarian or fallopian tube surface; no malignant cells in the ascites or peritoneal washings	
	T1a-N0-M0
IB: Tumor limited to both ovaries (capsules intact) or fallopian tubes; no tumor on ovarian or fallopian tube surface; no malignant cells in the ascites or peritoneal washings	
	T1b-N0-M0
IC: Tumor limited to 1 or both ovaries or fallopian tubes, with any of the following:	
IC1: Surgical spill	
	T1c1-N0-M0
IC2: Capsule ruptured before surgery or tumor on ovarian or fallopian tube surface	
	T1c2-N0-M0
IC3: Malignant cells in the ascites or peritoneal washings	
	T1c3-N0-M0
Stage II: Tumor involves 1 or both ovaries or fallopian tubes with pelvic extension (below pelvic brim) or peritoneal cancer	
	T2-N0-M0
IIA: Extension and/or implants on uterus and/or fallopian tubes and/or ovaries	
	T2a-N0-M0
IIB: Extension to other pelvic intraperitoneal tissues	
	T2b-N0-M0
Stage III: Tumor involves 1 or both ovaries or fallopian tubes, or peritoneal cancer, with cytologically or histologically confirmed spread to the peritoneum outside the pelvis and/or metastasis to the retroperitoneal lymph nodes	
	T1/T2-N1-M0
IIIA1: Positive retroperitoneal lymph nodes only (cytologically or histologically proven):	
IIIA1(i) Metastasis up to 10 mm in greatest dimension	
IIIA1(ii) Metastasis more than 10 mm in greatest dimension	
IIIA2: Microscopic extrapelvic (above the pelvic brim) peritoneal involvement with or without positive retroperitoneal lymph nodes	
	T3a2-N0/N1-M0
IIIB: Macroscopic peritoneal metastasis beyond the pelvis up to 2 cm in greatest dimension, with or without metastasis to the retroperitoneal lymph nodes	
	T3b-N0/N1-M0
IIIC: Macroscopic peritoneal metastasis beyond the pelvis more than 2 cm in greatest dimension, with or without metastasis to the retroperitoneal lymph nodes (includes extension of tumor to capsule of liver and spleen without parenchymal involvement of either organ)	
	T3c-N0/N1-M0
Stage IV: Distant metastasis excluding peritoneal metastases	
Stage IVA: Pleural effusion with positive cytology	
Stage IVB: Parenchymal metastases and metastases to extra-abdominal organs (including inguinal lymph nodes and lymph nodes outside of the abdominal cavity)	
	Any T, any N, M1

Table 1-1: FIGO 2014 staging classification for the ovarian, fallopian tube, and peritoneal cancer (Revised in 2018, reproduced from Berek *et al,* 2018 (Ref. 42)).

1.5 Treatment approaches to ovarian cancer

1.5.1 Surgical treatment in ovarian cancer

Optimal staging and treatment can involve a total hysterectomy, bilateral salpingo-oophorectomy, omentectomy, peritoneal cytology, pelvic and para-aortic lymph node biopsy, biopsy of parietal peritoneum, Douglas pouch, intestinal tract, mesentery, and examination/sampling of surfaces of the diaphragm and any suspected lesions (42). Maximal debulking of the tumor with no residue is recommended even for advanced ovarian cancers because of the association with better patients' survival. It may include bowel resection, diaphragm resection, splenectomy and other procedures (41). Besides laparotomy, other approaches are also studied in the treatment of ovarian cancer, such as laparoscopic-assisted surgery, neoadjuvant chemotherapy, interval-debulking, intraperitoneal chemotherapy, and targeted molecular therapy.

1.5.2 Chemotherapy in ovarian cancer

Treatment with chemotherapy will depend on the staging and grading of the tumor from the pathologic report. It is currently recommended that all patients with ovarian cancer receive 3 to 6 cycles of combination chemotherapy as soon as 2-4 weeks after surgery, except for patients with stage IA/B and/ or Grade1 or 2. Standard first-line chemotherapy in ovarian cancer involves platinum and taxane combination therapy. (Table 1-2).

Drugs	*Dose*	*Administration (hr)*	*Interval*	*No. of Treatments*
Standard Regimens				
Paclitaxel Carboplatin	175 mg/m^2 AUC = 5-6	3	Every 3 weeks	6-8 cycles
Paclitaxel Cisplatin	135 mg/m^2 75 mg/m^2	3	Every 3 weeks	6-8 cycles
Alternative Drugs [a] (Can be given with platinum)				
Topotecan	1.0-1.25 mg/m^2 4.0 mg/m^2		Daily × 3-5 days Every 3 weeks, or weekly	
Gemcitabine	800-1,000 mg/m^2		Every 3 weeks	
Doxorubicin, liposomal	40-50 mg/m^2		Every 4 weeks	

AUC, area under the curve dose by Calvert formula.

[a] Drugs that can be substituted for *paclitaxel* if hypersensitivity to that drug occurs.

Table 1-2: Chemotherapy for advanced stages of ovarian cancer (AUC: area under the curve dose, reproduced from Berek et al., Ref. 42)

In the current study, we focused on platinum-based chemotherapy in ovarian cancer treatment.

1.6 Platinum-based chemotherapy

Introduction of cisplatin

40 years ago, alkylating agents such as melphalan, cyclophosphamide and chlorambucil were the main choices of treatment in women having advanced stages of ovarian cancer with the response rate of 20-60% (43). The discovery of cisplatin in the early 1980s was one of the most critical developments in the treatment of cancer. In ovarian cancer, cisplatin increased the response rate to 50-80%, and quickly became one of the components of first-line combination therapies; used with cyclophosphamide or doxorubicin (44, 45).

The introduction of paclitaxel in the 1990s changed the standard chemotherapy regimens in ovarian cancer, and paclitaxel plus cisplatin, become the standard of treatment (46, 47).

The taxanes were initially derived from the bark of the Pacific Yew tree, *Taxus brevifolia*.

Although the paclitaxel-cisplatin regimen showed a statistical improvement in progression-free survival and overall survival, it was accompanied by increased toxicity. Ototoxicity, nephrotoxicity, neurotoxicity, gastrointestinal toxicity and myelosuppression were associated with this treatment (43).

Carboplatin, introduced in 1985, had equal effectiveness but fewer side effects and tolerability advantages, and it has replaced cisplatin as the standard of treatment in ovarian cancer since 1998 (48). Carboplatin is a cisplatin derivative with the same mechanism of action but a different structure (Figure 1-1). The lower toxicity of carboplatin may be due to pharmacodynamics of the drug since 1,1-cyclobutanedicarboxylate is a poorer leaving group than chloride, then, carboplatin has lower reactivity rate compared to cisplatin and a lower rate of adduct formation (48).

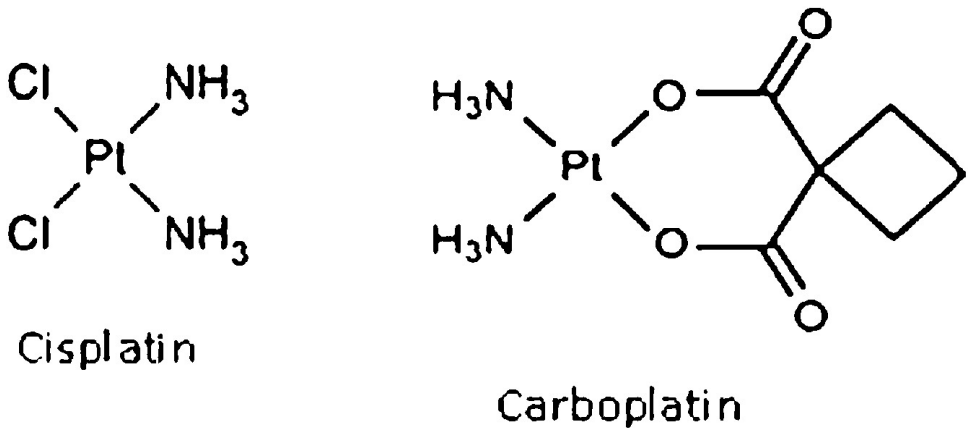


Figure 1-1: Chemical structure of Cisplatin and Carboplatin (Reproduced from Ref. 48).

Mechanism of action

DNA is the primary target of carboplatin. When carboplatin binds to DNA, it inhibits transcription, prevents DNA replication and induces cell death (49). Adducts associated with carboplatin can be mono-adducts or intra- and inter-chain adducts which affect DNA as well as some transduction pathways. Collectively, this induces apoptosis or necrosis in the tumor cells (50).

To exert its effects, carboplatin must cross the cell membrane to the cytosol and then to the nucleus. The cell can uptake carboplatin by passive diffusion, active transport by copper transporter Ctr1, or by organic cation transporters (OCTs) OCT1, OCT2, OCT3 (51, 52). Inside the cytosol, carboplatin undergoes hydrolysis of 1,1-cyclobutanedicarboxylate and becomes positively charged (Figure 1-2). This allows the drug to interact with nucleophilic molecules such as DNA, RNA, and proteins. A covalent binding occurs between carboplatin and the N7 site of purine bases which forms DNA-DNA or DNA-protein interactions (53, 54).

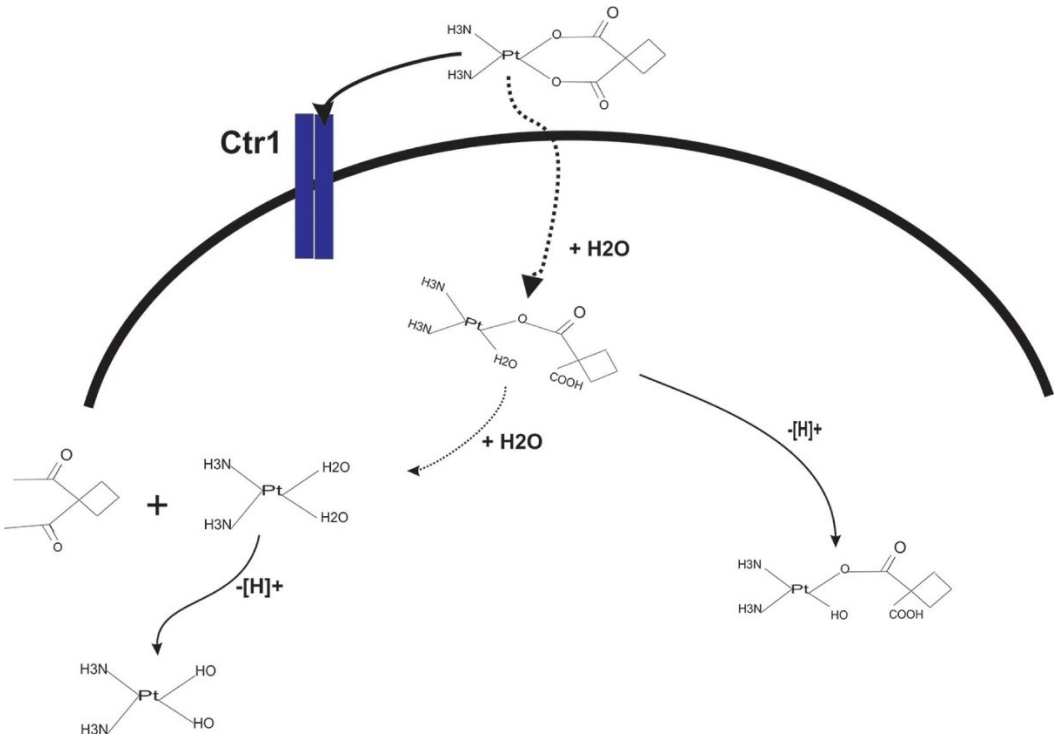


Figure 1-2: Carboplatin hydrolysis inside the cell (Ctr: copper transporter, adapted from Ref. 48).

1.7 Treatment assessment

After optimal cytoreductive surgery and subsequent combination chemotherapy, most patients have no evidence of disease. Because of the high recurrence rate, patients should have follow-up care to detect any recurrence as soon as possible, and the recommended strategies are tumor markers, radiologic assessments, and second-look surgery (42).

Tumor markers

CA125 is a surface glycoprotein associated with Mullerian epithelial tissue which is elevated at the time of diagnosis in about 80% of the patients with EOC. After optimal surgery and one or two cycles of chemotherapy, it often becomes undetectable. After an excellent response to treatment, elevated levels of CA125 almost always indicate disease relapse, with a positive predictive value

of 100%. Negative levels of CA125 are not conclusive, with a negative predictive value of only 56% (55).

Radiologic assessment

The imaging modalities are useful for assessing response in patients with measurable residual disease at the start of chemotherapy. If the patient has no or minimal residual disease after cytoreductive surgery, the value of the imaging is limited (56). Abdominopelvic ultrasound, CT-scan, MRI or FNA under the guide of CT, or MRI and PET scan are the available imaging modalities in patient follow up. Ascites, recurrent mass, liver, and omental metastasis may be detectable in the radiologic assessment (57).

Second-look surgery

As tumor markers and imaging assessment are too insensitive to exclude the subclinical disease, second-look surgery may be performed to evaluate the patients after successful cytoreductive surgery and chemotherapy. However, due to unproven benefits and possible side effects, this surgery is not recommended anymore (55, 58, and 59).

1.8 Recurrence and chemotherapy resistance in the EOC

In advanced stages of ovarian cancer, even in patients who completely respond to first-line chemotherapy, the recurrence rate is about 70% (60). At the time of recurrence, the choice of treatment is based on factors such as patient convenience, potential cytotoxicity, and possible benefits. The goals of treatment include improving or maintaining the quality of life, delaying progression, reducing disease-related symptoms, and prolonging survival. Secondary cytoreductive surgery, chemotherapy, hormonal therapy, targeted therapy, poly ADP ribose polymerase (PARP) inhibitors, and radiation all are available treatment modalities with different

indications and toxicities (61, 62). Although the second line of cytotoxic treatment offers a significant benefit in recurrent ovarian cancer, the response rate is generally lower, and the duration of remission is shorter compared to primary chemotherapy. It has been shown that the most reliable parameter is the platinum-free interval (PFI), which is the interval between the completion of the last platinum regimen and the start of new treatment. PFI shorter than six months is classified as platinum-resistant, and PFI longer than 12 months is considered to be platinum sensitive. In partially platinum-sensitive patients with the PFI between 6 and 12 months, the treatment result is controversial because of unsatisfactory results from treatment with platinum (63). If the patient is fully sensitive to platinum, then combination chemotherapy after recurrence includes a platinum agent. Patients with platinum-resistance and refractory diseases are also treated with chemotherapy but the response rates are less than 10%, and the median survival is lower than six months (64). Single-agent chemotherapy with active drugs is used to prevent unnecessary toxicity such as paclitaxel, docetaxel, gemcitabine, liposomal doxorubicin, oral etoposide, vinorelbine, and trabectedin (65).

Overall, chemotherapy efficacy in ovarian cancer is limited by the resistance of cancer cells, and as a result, chemoresistance may induce disease progression, reduce patient response to treatment, and decrease overall survival.

2. Polyunsaturated fatty acids

Omega-3 polyunsaturated fatty acids (PUFA) are widely recognized as dietary components which can improve human health. In this chapter, the chemical structure, dietary sources, absorption, metabolism, and health benefits of these fatty acids will be discussed.

2.1 Chemistry of PUFAs

Fatty acids are aliphatic monocarboxylic acids derived from an animal or vegetable, are naturally occurring, and have a chain of 4 – 28 carbons (66). The two classes of saturated and unsaturated fatty acids are defined based on the presence or absence of carbon double bonds. PUFAs are distinguished from monounsaturated and saturated fatty acids by the presence of at least two carbon double binds. A carboxyl group is present at one end, and a methyl group (the "n" or "ω" end) at the other end. In omega-3 or n-3 PUFAs, the first double bond is placed three carbons from the methyl end of the carbon chain, and in omega-6 or n-6 PUFAs, it is located six carbons away from the methyl end (67).

Several different omega-3 PUFAs exist, but the three main ones in the diet are:

1- α – Linolenic acid (C18:3 n-3, ALA)
2- Eicosapentaenoic acid (C20:5 n-3, EPA)
3- Docosahexaenoic acid (C22:6 n-3, DHA)

Two of the major omega-6s fatty acids that can be found in the diet are:

1- Linoleic acid (C18:2 n-6, LA)
2- Arachidonic acid (C 20: 4 n-6, AA)

In the human body, ALA and LA are considered the dietary essential fatty acids as our bodies cannot form carbon-carbon double bonds before the 9^{th} carbon from the methyl end. Also, consuming EPA and DHA from dietary supplements or marine sources are the only practical way

for their bioavailability to the body, as the conversion rate of ALA to EPA and DHA is very limited (68).

ALA is present in canola oil, walnuts, ground flaxseed, and soybeans. EPA and DHA are originally synthesized by microalga which is consumed by phytoplankton. When fish consume phytoplankton, the n-3 PUFAs accumulate in their tissue, and fish and seafood become the main food source of these two fatty acids (69).

2.2 Digestion and absorption of n-3 PUFAs

Lipids are hydrophobic, and their solubility is the key issue for digestion and absorption. After ingestion, short and medium-chain fatty acids are relatively soluble and can pass through the epithelial cells of the intestine and enter the circulatory system. However, long-chain fatty acids are water-insoluble, and their digestion is greatly aided by bile salts and the pancreatic lipases. Their final production, micelles, which is composed of fatty acids, phospholipids, monoglycerides, and bile salts, travel through the small intestinal epithelial cells mainly by passive transfusion for an overall efficiency of 95% fat absorption in human adults (68). Triglycerides, lipoproteins, cholesterols and other lipids are packed in the endoplasmic reticulum of epithelial cells to form chylomicrons, which release from enterocytes by exocytosis and enter the circulatory system via the thoracic duct (69).

2.3 Metabolism of PUFAs

After absorption, lipoprotein particles circulate in the bloodstream and deliver lipids to different organs for oxidation, metabolism, and storage in adipose tissue (69). Oxidation occurs in mitochondria, where long chain fatty acids are transported into mitochondria via a carnitine-

dependent pathway and undergo β-oxidation. The final products of this cycle are acetyl coenzyme A (CoA), carbon dioxide and large quantities of high energy phosphate bonds or ketone bodies (when there is limited carbohydrates available) (70).

***n-6 PUFAs**:* Mamalian cells cannot insert a double bond at the n-6 position of a fatty acid. Thus, n-6 PUFAs such as linoleic acids are essential in the diet. Under enzymatic activity of desaturases and elongases, linoleic acid forms arachidonic acid (Figure 2-1). n-6 PUFAs are found primarily in the cellular membrane phospholipids, and therefore, arachidonic acid is available in animal tissue. Arachidonic acid is the precursor to eicosanoids such as leukotrienes (LTs), thromboxane (TXs) and prostaglandins (PGs) that are the main components in hemodynamic, vascular tone and platelet aggregation (71).

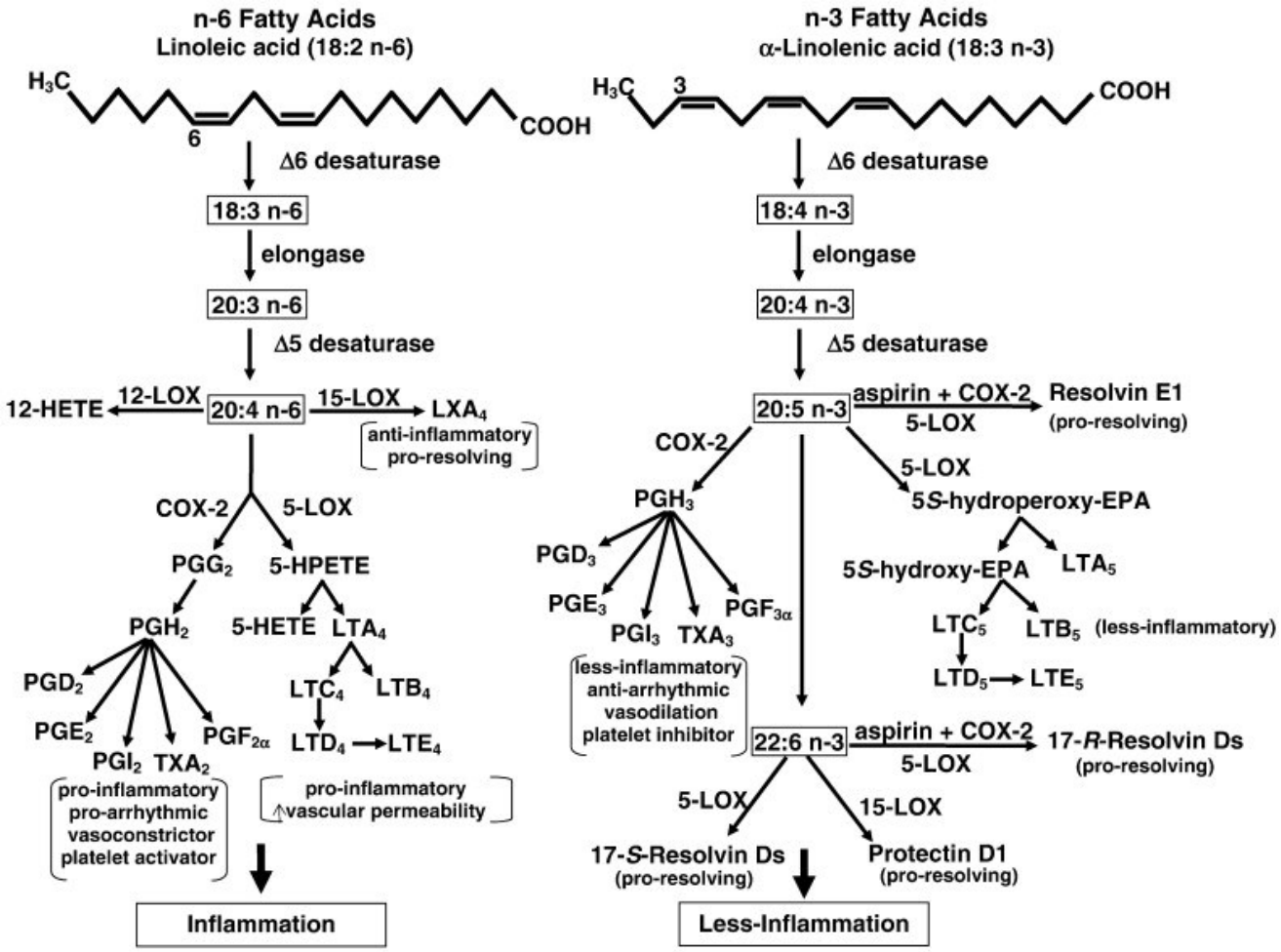


Figure 2-1: The metabolism of n-3 and n-6 PUFA and the biosynthesis of the eicosanoid and pro-resolving mediators. Abbreviations: PG, prostaglandin; HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; LOX, lipooxygenase; COX, cyclooxygenase; LT, leukotriene ; LX, lipoxin A (Adopted with permission from Ref. 67).

n-3 PUFAs*:* Like n-6 PUFAs, the human body is unable to insert a double bond at the n-3 position of a long-chain fatty acid, and thus requires a dietary source of these fatty acids. ALA is the main dietary source for n-3 PUFAs, and like linoleic acid, it can be further metabolized by elongation and desaturation enzymes to form EPA and DHA. EPA is the precursor for series 3 eicosanoids

and series 5 leukotrienes (Figure 2-1). DHA is a structural cellular membrane lipid and is enriched in some tissues such as the nervous system, retina, and sperm (72, 73).

Intracellular metabolic competition of n-3 PUFAs with arachidonic acid

In the AA pathway, following a series of stimulations, phospholipase A2 acts to release AA from the membrane. Free AA is then metabolized by cyclooxygenase (COX) and lipoxygenase (LOX) to eicosanoids (74). These eicosanoids (which are PGs, TXs, LTs and hydro fatty acids) are potent mediators of many physiological and pathological processes such as inflammation, survival, proliferation and cancer generation (75). Generally speaking, the eicosanoids made from n-6 PUFAs are more potent than those made from n-3 PUFAs. An increase in the dietary index of EPA and DHA results in a decrease in the eicosanoid production of AA, which reduces the response to oxidative metabolism of AA in different ways:

1. Both classes of fatty acids compete for the same desaturation enzymes, and as a result, ALA acts as an inhibitor of LA metabolism and vice versa. Additionally, EPA competes with AA for COX and 5-LOX with a shift toward the production of EPA-derived eicosanoids, of which many are biologically less potent (76, 77).
2. EPA and DHA can partially replace the AA in the cell membrane. They compete with AA for acylation in the sn-2 position of membrane phospholipids (77).
3. Both n-3 PUFAs (EPA, DHA) may be metabolized to some potent bioactive eicosanoids and docosanoids such as resolvins, docosatrienes and protectins with anti-inflammatory and protective characteristics (78, 79).
4. Both EPA and DHA down regulate COX2 expression, which is mainly expressed during inflammation and cancer (80).

2.4 n-3 PUFAs' benefits for human health

n-3 PUFAs have many health benefits. There is no clear set standard for the daily intake of n-3 PUFAs, but World Health Organization recommended a minimum daily dose of 250 – 300 mg combined EPA and DHA for healthy adults (81). Many observational studies have shown the potential health benefits of a higher intake of fish and other seafood (82); however, there is considerable controversy in scientific research about these positive effects. This section focuses on areas of health with possible involvement of n-3 PUFAs, as well as cancer prevention and treatment.

Cardiovascular diseases*:* The first epidemiological research from the 1970s found that there was a lower rate of mortality from cardiovascular diseases of Greenland Inuit, the Japanese and other fish-eating populations (82). Since then, many studies have evaluated the effects of n-3 PUFAs on cardiovascular risk factors and diseases such as high blood pressure, elevated plasma lipids, heart failure, coronary disease and fatal coronary heart disease (83, 84). In 2014, the FDA stated "supportive but not conclusive research shows that consumption of EPA and DHA omega-3 fatty acids may reduce the risk of coronary heart disease," and recommended dose of 2 g/day for both EPA and DHA. The dose of 2-4 g/day has also been recommended when patients need to decrease their triglycerides level (85).

Neural development and infant health: As mentioned before, high concentrations of DHA are present in the cellular membranes of the retina and brain. DHA is an essential factor in fetal growth and development. Many observational studies and randomized controlled trials have evaluated the effects of high maternal consumption or supplementation of n-3 PUFAs on different fetal growth and developmental factors (86 - 89). Although the results are not entirely conclusive, it has been shown that n-3 PUFAs supplementation could improve the pregnancy duration and mean birth

weight. The Dietary Guidelines for Americans and the American Academy of Pediatrics have therefore recommended intakes of 200 -300 mg/day of DHA during pregnancy (90).

Cognitive function and Alzheimer's disease*:* As DHA is an essential component of brain cell membrane phospholipids, it was hypothesized that n-3 PUFA might protect cognitive function by maintaining cell membrane integrity and neuronal function (91). Several meta-analyses and systematic reviews have assessed the effect of n-3 PUFAs on dementia and cognitive function in both healthy adults and Alzheimer patients. Overall, they concluded that n-3 PUFA might improve the attention, immediate recall, and processing speed in patients with mild cognitive impairment but not in the healthy elderly (92).

Cancer prevention: Many randomized clinical trials (RCTs), case-control studies, and prospective cohorts have found that higher intakes of n-3 PUFAs reduce the risk of cancer (mainly breast, colorectal and prostate). These hypotheses are based on the possible anti-inflammatory and antiproliferative capacity of n-3 PUFAs which has been shown in many *in vitro* and *in vivo* studies. However, the results are inconsistent. While some researchers have shown no association between higher intakes of n-3 PUFAs, some others found an association in negative or even in positive directions (93 – 100).

In breast cancer, evidence from several studies suggests that higher intakes of n-3 PUFAs may reduce the risk of cancer (93, 94). Some researchers also found a dose-dependent response between n-3 PUFAs intake and the risk of breast cancer (95).

In colorectal cancer, this association is unclear. A meta-analysis of 19 prospective cohort studies published in 2007 did not find any association between n-3 PUFAs intake and colorectal cancer, but they found a 12% lower risk of colorectal cancer in the highest fish consumption category compared to lowest fish consumption (96). Another cohort study showed that the associations vary

based on genetic characteristics and gender (97). More research is needed to clarify this additional association.

In prostate cancer, the relation between serum levels of n-3 PUFAs and the risk of cancer is even more inconsistent. Although some studies have shown the protective effects of PUFAs on prostate cancer risk (98, 99), some others showed a positive association (100 - 102).

In other cancers, the evidence is minimal. Overall, data from observational studies are inconsistent with the association between n-3 PUFAs and cancer risks, and additional RCTs are needed for clarification.

***Others*:** The benefits of n-3 PUFAs supplementation have been investigated in several conditions such as rheumatoid arthritis, depression, inflammatory bowel disease, ADHD, cystic fibrosis and childhood allergies. Findings to date suggest that n-3 PUFAs may be helpful in some of the above conditions, both alone and in combination with pharmacotherapy, but more research is needed to confirm this (81).

2.5 Mechanisms of n-3 PUFAs in cancer

Several *in vitro* and *in vivo* studies have shown the anti-cancer effects of n-3 PUFAs, and different mechanisms have been proposed. Overall, it has been hypothesized that the n-3 PUFAs may act through a few main cellular pathways and as a result influence the activity level of multiple other pathways. The main pathways are:

a. Incorporation of n-3 PUFAs in the cell membrane and changing the expression of multiple enzymes, receptors, proteins, and multiple downstream pathways
b. Alteration of the oxidative stress-dependent pathway which is related to apoptosis and proliferation

c. Metabolic conversion to other anti-inflammatory molecules
d. Alteration of the epigenetic control of genes involved in cell proliferation and survival
e. Anti-invasion and anti-metastatic effects on cancer cells

2.5.1 Incorporation of n-3 PUFAs into the tumor cell membrane

Dietary n-3 PUFAs influence fatty acid composition by integrating into the cell membrane phospholipids and affecting their fluidity and permeability (103, 104). These n-3 PUFAs-induced changes in the cell membrane may significantly affect the signaling process originating from the cell membrane.

Role of n-3 PUFAs on lipid raft and signal transduction

Lipid rafts are highly-ordered, glycolipid-enriched membrane domains which are less fluid than the surrounding membranes due to high levels of cholesterol, sphingolipids, and phospholipids with saturated fatty acids. Because of this unique environment, lipid rafts are essential domains for membrane receptors and key signaling proteins such as apoptotic signaling proteins (105 - 107). Cholesterol plays a significant role in maintaining the lipid raft microdomains as it is a spacer for the hydrocarbon chain of sphingolipids (108). Incorporation of EPA and DHA into the cell membrane alter the composition and disturb the organization of lipid rafts which suppress raft-associated cell signaling (109 - 111). It has been shown that n-3 PUFAs can modulate different intracellular signaling pathways by modulating the activity of some mediators such as epidermal growth factor receptor (EGFR), human epidermal growth factor receptor-2 (HER-2), peroxisome proliferator-activated receptor gamma (PPAR-γ), mitogen-activated protein kinases (MAPKs), and some others (112 - 114).

EGFR and HER-2

EGFR is a receptor tyrosine kinase which is involved in carcinogenesis, cell proliferation and survival (115). EGFR is generally activated by phosphorylation in response to extracellular ligands such as epidermal growth factor, and its dysregulation causes overexpression of EGFR which has been found in different cancers such as breast carcinoma (116 - 117). DHA induces apoptosis by inhibiting EGFR activation, which is mediated by caspase-3 or caspase-8 pathways (109, 118).

HER-2 is also a tyrosine kinase receptor which is overexpressed in 25-30% of invasive breast cancers with poor prognoses. HER-2 is a co-receptor for EGFR and Insulin-like growth factor-1 (IGF-1R) and activates downstream-signaling pathways such as MAPK and phosphatidylinositol 3-kinase (PI3K/AKT) resulting in cell proliferation and survival (119 - 122). Bcl-2 mediated apoptotic pathways also have been inhibited by HER-2, and research has demonstrated that the HER-2 receptor is down-regulated by n-3 PUFAs (119, 123).

Peroxisome proliferator-activated receptor Gamma (PPAR-γ)

PPAR-γ is a member of the PPAR family which is involved in cell proliferation and differentiation, and functions as a ligand-activated transcription factor. PPAR response elements (PPREs) are located in the regulatory regions of a variety of genes involved in the inflammatory response. It has been found that PUFAs and eicosanoids are PPAR ligands and may induce apoptosis by PPAR-γ activation in breast cancer cells (124 - 127). It has been shown that dietary supplementation with low n-6 / n-3 PUFA ratios could increase PPAR-γ protein content and reduce tumor burden in rat breast carcinogenesis models (128).

Bax / Bcl2

Apoptosis is a form of programmed cell death and is a tightly regulated and highly efficient process. Bcl2 is a well-known apoptosis-regulator protein, and much clinical and pre-clinical data demonstrate that in the majority of cancers, expression of Bcl2-family proteins --both pro- and anti-apoptotic-- are changed (129, 130). Treatment with DHA and EPA shifts the balance between pro- and anti-apoptotic Bcl2 proteins by downregulating anti-apoptotic Bcl2 and Bcl-xL proteins and upregulating pro-apoptotic Bak and Bcl-xS (131). Also, it has been shown that the Bax/Bcl-2 ratio predicts the cell susceptibility to apoptosis (132). Yip KM et al., in a colon cancer rat model, showed that feeding animals with high n-3 PUFAs such as fish oil resulted in down-regulation of Bcl2 and induction of apoptosis in cancer cells (133).

PI3K/Akt and nuclear factor kappa-B transcription factor (NF-κB)

Phosphatidylinositol 3-kinase (PI3K) is a heterodimeric lipid kinase which plays an essential role in cell apoptosis. Activation of the Akt pathway, downstream of PI3K, can function to promote anti-apoptotic signaling and to activate NF-κB (114). The primary result of PI3K activation is the generation of PIP3 as a second messenger in the cell membrane which activates a downstream serine/threonine kinase, Akt (134, 135). Akt acts as an anti-apoptotic signaling protein which should be translocated to the cell membrane for phosphorylation at Thr 308 and Ser 473. When the tumor cell membrane is enriched with n-3 PUFAs, it might affect the phosphorylation of Akt. Additionally, the PI3K/Akt pathway may promote cell survival via NF-κB, which is a critical regulator of the proliferation and migration of cancer cells (136, 137).

Alteration in the cell cycle and cell proliferation

Cell proliferation is a fundamental process in carcinogenesis. An unregulated cell cycle and the loss of dependency on external signals are characteristics of cancer cells. In a normal cell, the cell cycle is regulated by two important categories of regulatory molecules: the cyclins and the cyclin-dependent kinases (CDKs). In addition, retinoblastoma (RB) protein, p53 transcription factor, and CDK inhibitors are all negative regulators of the cell cycle (138, 139). Studies have shown that n-3 PUFAs could slow down the cell cycle or arrest malignant growth of cancer cells mostly in the S phase, or prevent G1/S progression. In cell lines that are sensitive to n-3 PUFAs, treatment changes the levels of cell cycle regulators such as cyclins, CDKs, and RB (140). For example, treatment with DHA has been shown to induce cell cycle arrest in G1 phase, S phase, and possibly G2/M phase and studies suggest that it also decreases the expression of cell cycle regulatory markers in a broad spectrum of cancers (141). Figure 2.2 illustrates the effects of DHA on regulators of cell cycle.

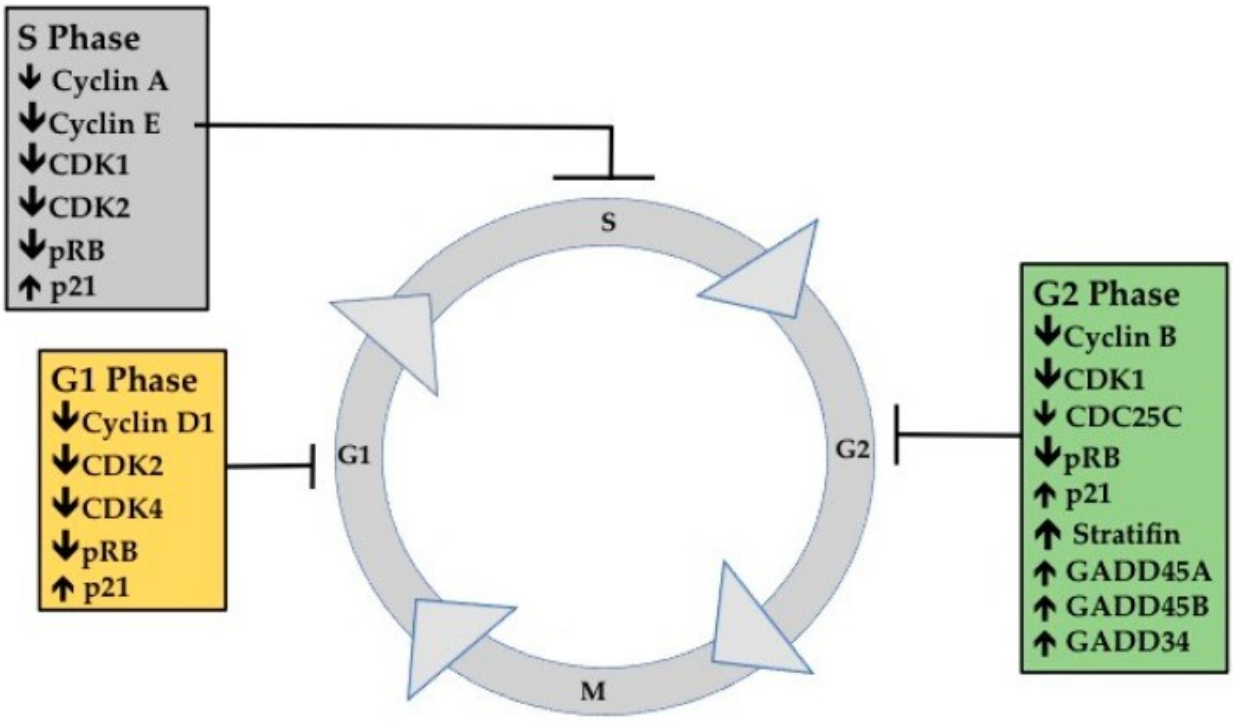


Figure 2-2: the schematic illustrates the possible mechanisms of effects of DHA on cell cycle genes and proteins expression in different cancer types (adopted from Ref. 141).

2.5.2 Effect of n-3 PUFAs on cellular lipid peroxidation and oxidative stress

Lipid peroxidation is a primary n-3 PUFAs anticancer mechanism (142, 143). When n-3 PUFAs incorporate into the tumor cell membranes, the cells get more susceptible to lipid peroxidation by reactive oxygen species. The outcome of this process is the formation of lipid radicals which react with oxygen to form free radicals which can propagate in the cell membrane by attacking the adjacent fatty acids (144). The exact mechanism of the fatty acid peroxidation on tumor growth inhibition is not clear, but n-3 PUFAs may induce cell death through ROS-induced apoptosis pathways (145). For example, in colon adenocarcinoma, DHA has been shown to induce lipid peroxidation and, as a consequence, to alter the molecular targets such as caspase-3, caspase-9 and MCL-1 anti-apoptotic protein (146). It has also been reported that incorporation of n-3 PUFAs in tumor lipids decreases cell survival and proliferation in breast cancer (116, 147 and 148).

2.5.3 Impact of n-3 PUFAs on inflammatory mediators

Eicosanoids are hormone-like lipids and key regulators and mediators of inflammation which typically derive from 20 carbon atom PUFAs [eicosa=20] (150). Eicosanoids include prostaglandins (PG), thromboxanes (TXs), and leukotrienes (LTs), which play a critical role in cellular growth and differentiation, platelet aggregation and modulation of inflammatory and immune responses. An important mechanism by which n-3 PUFAs may prevent cancer development is through inhibition of eicosanoid synthesis from AA (151). As mentioned before, LA and ALA are precursor fatty acids for the formation of eicosanoids and undergo the same desaturation/elongation reaction to form the long chain metabolites of AA and DHA, respectively. The affinity of delta 6-desaturase for ALA is more significant than for LA. As a result, higher intake of ALA and more incorporation of this fatty acid into the cell membrane phospholipids reduces the synthesis of AA from LA. Less AA will then be available to produce n-6 derived inflammatory eicosanoids (152 - 154).

To start the pathway (Figure 2.2), phospholipases liberate the PUFAs from membrane phospholipids, which supply the substrate for COX-1 and COX-2, LOX and cytochrome p450 monooxygenases (153). PGs and TXAs are the final products of COX while LOXs produce LTs, hydroxyl fatty acids and lipoxins. AA is the majority of PUFAs in the cell membrane, and its products have pro-inflammatory properties compared to EPA-derived eicosanoids which have mostly anti-inflammatory characteristics (152).

It has been shown that N-3 PUFA supplementation can reduce the production of LTB4 by 75% and PGE2 by 60% in human peripheral blood mononuclear cells (155). Also, Rose et al. have suggested that COX-2 and 5-LOX are down-regulated in mice fed with high n-3 PUFA diets (156).

Additionally, it has been shown that n-3 PUFAs act as the precursors of another group of lipid mediators (including resolvins, docosatrienes, and protectins) which exert potent anti-inflammatory actions and may be a potential target for some chronic inflammatory diseases as well as cancer (157, 158).

2.5.4 Role of n-3 PUFAs on epigenetic control of genes

N-3 PUFAs may also drive alterations in the epigenetic control of genes involved in apoptosis and proliferation pathways, by altering DNA cytosine methylation and precipitating changes in the covalent modification of histones. In 2012, Sadli et al. showed that DHA increases acetylation of histone 3 (H3) in the Bcl-2 locus, and decreases the acetylation, methylation, and phosphorylation of H3, leading to reduced caspase-3 levels in human neuronal M17 cells (159). In breast cancer, DHA may downregulate Zeste homologues 2 (EZH2) through posttranslational mechanisms. The downregulation of EZH2 was accompanied by a decrease in histone 3 lysine 27 trimethylation (H3K27me3) activity of EZH2, as well as upregulation of E-cadherin which is a known target of EZH2 (160).

2.5.5 Role of N-3 PUFAs in adhesion, angiogenesis and metastasis

Adhesion and angiogenesis are important processes involved in cancer metastasis (161, 162). DHA can inhibit adhesion by inhibiting Rho GTPase mediated cytoskeletal rearrangement and reducing intracellular adhesion molecule (ICAM-1) and vascular cell adhesion protein 1 precursor (VCAM-1) protein expression (163, 164). Also, n-3 PUFAs upregulate expression of E-cadherin ?in what type of cells which is vital in maintaining the integrity of intercellular adhesions (165).

DHA has been shown to reduce angiogenesis by decreasing the levels of vascular endothelial growth factor (VEGF) platelet-derived growth factor, and platelet-derived endothelial cell growth factor (161, 162).

Tumor metastasis and the subsequent growth in distant tissues is one of the major causes of failure in the treatment of cancer. AA-derived eicosanoids are involved in different stages of metastatic cascades, such as interactions between tumor cells and the formation of aggregates of tumor cells and platelets (166). As mentioned earlier, DHA and EPA-derived eicosanoids are less active and may inhibit this process (167). Additionally, incorporation of n-3 PUFAs into tumor cell membranes may alter the composition of fatty acids which may lead to some membrane alterations and reduce the metastatic potential of tumor cells (168 - 170).

2.6 n-3 PUFAs and ovarian cancer

The effect of n-3 PUFAs on prevention of EOC is not apparent. Based on a meta-analysis published in 2015 (171), there is no evidence of a relationship between dietary fat and fatty acid intakes and EOC risk. Additionally, Ibiebele et al. did not find any correlation between the intake of n-3 PUFAs and EOC prevention (172). However, other studies have demonstrated that n-3 PUFAs show protective effects against ovarian cancer. High levels of n-3 PUFAs in the diet may reduce the risk and severity of ovarian cancer (ref). Hens fed with a flaxseed diet, for example, showed a reduction in ovarian cancer severity and incidence and an increase in survival (173, 174).

It has been illustrated that n-3 PUFAs can induce apoptosis and reduce cell growth and viability in ovarian cancer cells (refs). Han et al.(ref) reported that incubation with EPA could induce apoptosis in SKOV3 cells. They showed that EPA inhibited phosphorylation of ERK 1/2, down-regulate ERK1/2, mTOR/ NF-κB pathway, and induce S-phase cell cycle arrest (ref). Also, n-3

PUFAs and especially DHA was shown to suppress the growth of TOV-21G cells by activation of PPARγ and P53 (175). Additionally, Zajdel et al. *(ref)* showed that in SKOV3 and OVCAR3 cell lines, DHA suppressed the cell viability and proliferation and induced cell death by activation of caspases and apoptosis pathways. They also compared DHA with AA but did not see any significant effects with AA (176). Furthermore, evaluation of the effects of n-3 PUFAs on three different cell lines -- SKOV3, TOV-21G, and OVCAR3-- showed the induction of apoptosis and/or down-regulation of VEGF which causes growth suppression in these cell lines (177).

Another possible mechanism of n-3 PUFAs on ovarian cancer cells may be through ROS-dependent MAP kinase activation. Tanaka et al. (ref) examined the effects of n-6 and n-3 PUFAs on cell death in two ovarian cell lines: KF28 and HAC2. They showed that DHA and γ-LA induce cell death in KF28 but not in HAC2. This effect was correlated with the activation of JNK and p38 Map kinase, which are stimulated by ROS (178). They also showed that vitamin E, an antioxidant, attenuated the response.

Additionally, the effects of n-3 PUFAs on ovarian cancer growth may be due to their role in invasion and metastasis. Researchers investigated the effects of DHA on invasion and metastasis of ovarian cancer cell lines A2780, HO8910 and SKOV3 (ref). They showed that DHA inhibited the invasion and metastasis of ovarian cancer by up-regulation of WAVE3, VEGF and MMP-9 expression and down-regulation of KISS, TIMP-1, and PPAR-γ expression, and by modulation of NF-κB signaling pathway (179).

3. Rationale and hypotheses:

3.1 Rationale

Ovarian cancer (OC) is the most lethal gynecological malignancy in the USA and Europe (1, 2).

Due to subtle symptoms of the early-stage disease, and unavailable accepted screening methods, most of the patients present with advanced stages of the disease which significantly reduces the response rate to the treatments and the overall survival of the patients (3). In advanced stages of ovarian cancer, even in patients who completely respond to first-line chemotherapy, the recurrence rate is about 70% (60). Even with the standard treatment of cytoreductive surgery in combination with taxanes-platinum-based chemotherapy, the majority of patients experience a recurrence with poor overall survival, necessitating improved therapeutic approaches (4).

There is a strong rationale for studying nutritional interventions in cancer prevention and therapy. Recent studies have determined that LCPUFA may affect cancer cell growth and metastatic potential; however few studies have tested these in combination with chemotherapy in OC models.

Dietary n-3 PUFAs influence fatty acid composition by integrating into the cell membrane phospholipids and affecting their fluidity and permeability (103, 104). These n-3 PUFAs-induced changes in the cell membrane may significantly affect the signaling process originating from the cell membrane. Overall, it has been hypothesized that the n-3 PUFAs may act through some cellular pathways and influence the activity level of multiple other pathways, such as incorporation into the cell membranes (109 – 111) and changing the expression of multiple enzymes, receptors, and downstream pathways (115 - 133), induction of apoptosis by alteration of the oxidative stress-dependent pathway (142 – 154), metabolic conversion to other anti-

inflammatory molecules (151 – 154), alteration of the epigenetic control of genes (159, 160) as well as anti-invasion and anti-metastatic effects on cancer cells (161 – 170).

Herein, we will investigate the effects of n-3 LCPUFAs on OC cell growth, and sensitivity to carboplatin, both *in vitro* and *in vivo*.

3.2 Hypothesis and objectives

Hypothesis: Omega-3 long chain polyunsaturated fatty acids increase the efficacy of carboplatin in ovarian cancer cells.

The overall aim of this study is to determine if DHA enhances the cytotoxic effects of carboplatin in ovarian cancer cell models, both *in vitro* and *in vivo*. This aim is achieved by addressing the following objectives:

Objective 1: To determine if DHA can enhance the effects of carboplatin on ovarian cancer cells, in vitro.

The study has included two immortalized ovarian surface epithelium (IOSE), and Fallopian tube (FT194) cell lines as well as eight ovarian cancer cell lines from different subtypes of epithelial ovarian cancer consists of three clear cell carcinoma cell lines (ES2, OCI-C1P, and OCI-C4P), three high grade serous ovarian cancer cell lines (OV90, JHOS2, and Kuramochi), one endometrioid carcinoma with resistance to cisplatin (A2780cp), and one serous epithelial ovarian cancer cell line (SKOV3). The cell viability and metabolic activity of the different cell lines have been analyzed after treatment with DHA, OA, and LA, both alone and in combination with carboplatin.

Objective 2: To identify potential mechanisms that could explain the effects of n-3 LCPUFA on human ovarian cancer cells.

Incorporation of fatty acids into the whole cell phospholipids has been analyzed by Gas Chromatograph. To show the effects of fatty acids on proliferation, cell cycle analysis and expression of proteins related to proliferation have been analyzed. Analysis of apoptosis by flow cytometery and expression of apoptosis related genes and proteins are performed to realize the mechanism of effects of fatty acids.

Objective 3: To determine if DHA can enhance the effects of carboplatin on human ovarian HGSC-PDX, in vivo.

To achieve this aim, Nude mice have been engrafted with ovarian HGSC-PDX tumor and grouped to four, based on the treatment. The first group receives basal diet, the second group receives basal diet plus carboplatin, the third group is on DHA-enriched diet, and the last group is treated with DHA-enriched diet plus carboplatin. Incorporation of DHA into the cell membrane of liver and tumor cells were analyzed by Gas chromatograph. We have evaluated the effects and the mechanism of effects of DHA treatment on the PDX samples.

Chapter 2

Materials and Methods

Chapter Two- Materials and Methods:

1. Cell lines and reagents:

Human ovarian cancer cell lines (ES2, SKOV3, and OV90) were obtained from ATCC (Manassas, VA, USA) and maintained in culture media according to ATCC recommendations. Immortalized ovarian surface epithelial (IOSE) cells and A2780cp OC cells were kindly provided by Dr. Fu's Lab (Department of Oncology, UofA). IOSE cells were cultured in 1:1 MCBD 105:199 (Sigma; Oakville, Ontario, Canada), 2.2 g/L $NaHCO_3$ plus 10% fetal bovine serum (FBS) (Sigma), and A2780cp OC cells were cultured in RPMI/1640 supplemented with 10% FBS and 2 mM Glutamine (Gibco). Immortalized fallopian tube epithelial cells (FT194) were kindly provided by Ronny Drapkin (Department of Obstetrics & Gynecology, University of Pennsylvania) and maintained in DMEM/ Ham F12 (Gibco; Grand Island, NY, USA), and 2% Ultroser Serum Substitute (Pall Corporation, France). Both IOSE and FT194 cells were transformed to immortalized cell lines by telomerase reverse transcriptase and SV40 T-Antigen.

Kuramochi ovarian cancer cell line was purchased from JCRB cell bank (Sekisui, XenoTech, LCC; Kansas City, KS, USA) and maintained in RPMI/1640 and 10% FBS. The JHOS-2 ovarian cancer cell line was purchased from RIKEN BRC cell bank (Tokyo, Japan) and cultured in DMEM/F12, 10% FBS and 0.1mM non-essential amino acids (Gibco). OCI-C1p and OCI-C4P ovarian cancer cells were purchased from Sylvester Comprehensive Cancer Center (Miller School of Medicine, University of Miami, USA) and maintained in OCMI media (University of Miami, USA).

Among of the ovarian cancer cell lines, OV90, JHOS-2 (BRCA1 mutation), and Kuramochi (*BRCA2* mutation) are all High-Grade Serous Ovarian Adenocarcinoma cells (HGSOC). ES2, OCI-C1p, and OCI-C4p cells are Clear Cell Carcinoma (CCC) histologic subtype. A2780cp is

ovarian Endometrioid Carcinoma (EC) (resistant to cisplatin) and SKOV3 is an epithelial ovarian carcinoma cell line, unlikely to be HGSOC (180, 181). Based on the previous published data (180, 181), A2780cp, JHOS-2, and OCI-C1p ovarian cancer cell lines have wild type P53 genes but ES2, OV90, SKOV3, Kuramochi, and OCI-C4p cell lines have mutation in P53 genes.

Carboplatin (Millipore Sigma; Germany) was used as a chemotherapeutic drug at concentrations ranging from 0 to 400 µM.

2. Cytotoxicity assay:

In the present study, cytotoxicity was determined by the 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Roche Diagnostics GmbH; Mannheim Germany). A trypan blue exclusion assay was also used as additional cytotoxicity assay.

For the MTT assay, the cells were cultured at 5 x 10^3 cells/well in 96-well flat-bottom plates (VWR; Radnor, PA, USA) with 100 µl of media for 24 hours. After 24 hours, the media was replaced with 100 µl of fresh media containing DHA, OA or LA at concentrations ranging from 0 to 320 µM. Each treatment was done in triplicate and in three biological replicates. After 72 hours, 10 µl/well MTT labeling reagent (final concentration 0.5 mg/ml) was added in each well. 4 hours later, 100 µl/well solubilization solution was added and plates were incubated for 24 hours in a humidified atmosphere (+37°C, 5 % CO2). Optical density (OD) was read at 570 nm by using Fluostar Omega micro-plate reader (BMG LABTECH; Offenburg, Germany) and the absorbance of each treatment was converted to relative cell viability.

For the trypan blue exclusion assay, cells were cultured in three replicates at a density of 5 x 10^4 cells/well in a 24 well flat-bottom cell culture plate (VWR) for 24 hours in 2 ml of culture media. After 24 hours, media was replaced, and the cells were treated with different fatty acids with

concentrations of 0 µM to 320 µM for 72 hours. Following this incubation period, cells were detached with 300 µl of 0.05% trypsin and the reaction was stopped with appropriate media. The cells were centrifuged for 5 min at 1500 rpm and suspended in culture media. A 10 µl aliquot of cells was mixed with 10 µl of 0.04% trypan blue solution (Invitrogen; OR, USA) and viable cells were counted using both automated cell counter (Invitrogen; OR, USA) and a hemocytometer under a light microscope.

3. Preparation of conjugated fatty acids:

DHA, OA, and LA were prepared based on published protocol (182). Briefly, fatty acids were purchased from Matreya LLC (JMS Biolynx, Brockville, ON), dissolved in hexane to obtain stock fatty acid solutions (100mg/10ml) and stored in -20°C. Fatty acids were conjugated to bovine serum albumin (BSA, Fisher Scientific, Edmonton, Alberta) as follows: 1.2 mL (12mg) of the stock solution of fatty acid was dried under Nitrogen gas and re-suspended in 1 mL of 0.1 M potassium hydroxide (KOH) and incubated at 50°C for 10 min. Conjugation to BSA was achieved by drop-wise addition of 9 mL of 7.5% BSA (prepared in sterile double distilled water and filtered through a 0.2-micron filter (Fisher Scientific, Edmonton, Alberta) and incubated at room temperature for three hours followed by overnight incubation at 4°C. FAs were aliquated into sterile 1.5 mL Eppendorf microcentrifuge tubes (Fisher Scientific, Edmonton, Alberta) and stored at -20°C until further use.

4. Whole cell phospholipid analysis:

Whole cell phospholipids (PL) were extracted by a modified Folch procedure as previously described (110, 183). Total PLs were separated on Silica “G” thin layer chromatography (TLC) plates (Analtech Inc., Newark, DE) in an 80:20:1 v/v ratio of petroleum ether: diethyl ether: glacial acetic acid for 20 minutes (184). 8-anilino-1-naphthalene-sulfonic acid (ANSA) was used

to visualize the PL band under ultraviolet light. PLs were methylated at 110°C with 1.5 mL boron trifluoride and 2 mL hexane for 1 hour followed by 1 mL of ddH2O for overnight incubation. The hexane layer from the methylation step was removed, dried down under nitrogen gas and reconstituted in 75 µl hexane. Then, fatty acid methyl esters (FAME) were separated and quantified with an Agilent 7890A Gas Chromatograph system with a 100m CP-Sil 88 fused capillary column (Agilent Technologies, Mississauga, ON); heated to 250°C to volatilize fatty acids in order to be carried through the capillary column (highly polar stationary phase) by using hydrogen gas (mobile phase). Fatty acids elute through the column based on the boiling point, polarity and degree of saturation; smaller, non-polar compounds elute first. The retention time and proportion relative to the concentration of different fatty acids in the sample is recorded as a chromatogram where the areas of the individual fatty acid peaks can be quantified and expressed as a percentage.

(Both fatty acid preparations and whole cell PL analysis were kindly done at Dr. Field's laboratory).

5. Flow cytometry:

5.1 Apoptosis assay: ES2 and Kuramochi cell lines were plated in 75cm^2 flasks and after 24 hours, cells were treated with DHA IC_{25} (10 µM), 10 µM of OA, carboplatin IC_{25} (10 µM) or combination of DHA + carboplatin or OA + carboplatin. After 72 hours, cells were detached by 0.05% trypsin and stained for apoptosis. For that, cells were first stained with Zombie Aqua viability dye (BioLegend) for 15 – 20 min following the manufacturer's protocol, washed with Annexin-V binding buffer (BioLegend), and stained with Annexin-V FITC (BioLegend) for 15 min in dark place on ice. Cells were washed and re-suspended in Annexin-V binding buffer and immediately subjected to flow cytometry analysis using FACS Canto II (Becton Dickinson,

Mississauga, ON, Canada) calibrated and gated with cytometer set-up, unstained and single stained samples. FlowJo software (Tree Star, Ashland, OR, USA, version 10.0.8) was used for data analysis.

5.2 Cell cycle analysis: ES2 cells were plated in 75cm^2 flasks and treated with 23 μM (DHA IC_{50}) of DHA, OA, and LA for 0, 6, 12 and 24 hours. Then, the cells were collected, fixed in 70% ethanol and stored at -20°C before cell cycle analysis.

After removal of ethanol, cells were washed with phosphate buffered saline (PBS) twice and stained with a solution containing Propidium Iodide (PI, Sigma-Aldrich), Triton X-100 (Fisher Scientific; New Jersey, USA) and DNase-free RNase A (Sigma; St. Louis, USA; to 10 ml of 0.1% Triton X-100 in PBS added 2mg DNase-free RNase A and 200 μl of 1 mg/ml PI) and incubated for 30 min at room temperature. Flow cytometry was done on a FACS Canto II (Becton Dickinson, Mississauga, ON, Canada), and analyzed by FlowJo software (Tree Star, Ashland, OR, USA, version 10.0.8).

6. Protein extraction:

ES2 cell lines were cultured in 75 cm^2 flasks and treated with 23 μM of DHA and OA for 24, 48 and 72 hours. After the incubation time, cells were washed with cold PBS twice and lysed with cold RIPA buffer (1ml of buffer per 75 cm2 flask, Thermo Scientific; Rockford, IL, USA) mixed 1:100 with Halt protease and phosphatase inhibitor cocktail (Thermo Scientific). Cells were kept on ice for 5 min, scraped, gathered and transferred to a microcentrifuge tube. Samples were centrifuged at ~14,000 x g for 15 min to pellet the cell debris and the supernatant transferred to a new tube and stored in -80°C.

The protein concentration was determined by Micro BCA Protein™ Assay Kit (Thermo Fisher) according to the manufacturer's instruction and measured on a Fluostar Omega plate reader (BMG LABTECH; Offenburg, Germany).

7. Western Blot analysis:

Samples containing 50µg protein, 4x Laemmli sample buffer (Bio-Rad ; Hercules, California, USA) and 5% 2-mercaptoethanol (Sigma-Aldrich ; St. Louis, Missouri, USA) were boiled for 10 min and loaded onto 10% or 12% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE) based on the protein of interest molecular weight. After electrophoresis, the protein was transferred to polyvinylidene difluoride (PVDF, Bio-Rad) membranes using the Trans-Blot Turbo (setting: 25v and 1.5 A for 10 min, Bio-Rad). The membranes were blocked by Odyssey blocking buffer (LI-COR; Lincoln, Nebraska, USA) for 1 hour and then incubated with primary antibody diluted in 3% bovine serum albumin (BSA) in TBS-T (Tris-buffered saline, 0.1% Tween 20) at 4°C overnight. For a list of primary antibodies and dilutions refer to Table 2-1.

Table 2-1: List of antibodies used in Western Blot.

	Antibody	Dilution	Molecular weight	Company	Catalog #
1	Caspase-3	1/1000	35 KDa	Cell signaling technology; Danvers, Massachusetts, USA	9662
2	Cleaved Caspase-3	1/1000	17, 19 KDa	Cell signaling technology; Danvers, Massachusetts, USA	9664
3	Caspase-9	1/1000	47 KDa	Cell signaling technology; Danvers, Massachusetts, USA	9502
4	Cleaved Caspase-9	1/1000	17, 37 KDa	Cell signaling technology; Danvers, Massachusetts, USA	9501
5	PARP	1/1000	116 KDa	Cell signaling technology; Danvers, Massachusetts, USA	9532
6	Cleaved PARP	1/1000	89 KDa	Cell signaling technology; Danvers, Massachusetts, USA	9541
7	Anti-FADD	1/1000	28 KDa	Abcam Inc; Toronto, ON, Canada	GR84671-23
8	Histone H-3	1/1000	~17 KDa	Cell signaling technology; Danvers, Massachusetts, USA	4499
10	p-Histone 3 (S10)	1/1000	~17 KDa	Cell signaling technology; Danvers, Massachusetts, USA	3377
11	p-Histone 3 (S28)	1/1000	~17 KDa	Cell signaling technology; Danvers, Massachusetts, USA	9713
13	Tubulin	1/10000	55 KDa	LI-COR Biosciences; Lincoln, NE, USA	926-42213

Next, the membranes were washed with TBS-T and probed with horseradish peroxide (HRP)-conjugated secondary antibodies (LI-COR anti-mouse or anti-rabbit) for one hour at room temperature. Then, the membranes were washed to remove excess secondary antibody and LI-COR Odyssey CLx infra-red imaging system was used to detect the immunofluorescent intensity.

8. RNA extraction and RT-PCR analysis of apoptotic genes:

ES2 cells were cultured in 75 cm^2 flasks and treated with 23 µM of DHA for 0 and16 hours. Then, the RNeasy Plus Mini Kit (Qiagen; Hilden, Germany) was used to extract total RNA following the manufacturer's protocol. 3µl of purified RNA was quantified by Epoch plate reader (Biotek; Winooski, Vermont, USA).

2µg of total RNA was used to make cDNA by using high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, California, USA) following the manufacturer's instruction.

15µl of cDNA was mixed with 1080µl of TaqMan gene expression master mix (Applied Biosystems) and 1065 µl of water based on the manufacturer's instruction and Real-time PCR analysis was performed by Applied Biosystems 7300 Real-Time PCR System as follow:

1- Activation: 95°C 10 min
2- Denaturation 95°C 15 seconds
3- Annealing/extension 60°C 1 min and returned to step 2 for total cycles of 40.

Data was collected and analyzed by the Applied Biosystems 7300 software.

For the list of the gene see Table 2-2.

Table 2-2: TaqMan human apoptosis plate layout with gene symbols. A1 – A4 are the control genes

Gene Symbol	1	2	3	4	5	6	7	8	9	10	11	12
A	18S	GAPDH	HPRT1	GUSB	BIRC2	APAF1	BAD	BAK1	BAX	BBC3	BCAP31	BCL10
B	BCL2	BCL2A1	BCL2L1	BCL2L10	BCL2L11	BCL2L13	BCL2L14	BCL2L2	BCL3	BID	BIK	NAIP
C	BIRC3	XIAP	BIRC5,EPR1	BIRC6	BIRC7	BIRC8	BNIP3	BNIP3L	BOK	NOD2	NOD1	CARD6
D	CARD9	CASP1	CASP10	CASP14	CASP2	CASP3	CASP4	CASP5	CASP6	CASP7	CASP8	CASP8AP2
E	CASP9	CFLAR	CHUK	CRADD	DAPK1	DEDD	DEDD2	DIABLO	IFT57	FADD	FAS	FASLG
F	HIP1	HRK	HTRA2	CARD18	IKBKB	IKBKE	IKBKG	LRDD	LTA	LTB	MCL1	NLRP1
G	NFKB1	NFKB2	NFKBIA	NFKBIB	NFKBIE	NFKBIZ	PEA15	PMAIP1	PYCARD	REL	RELA	RELB
H	RIPK1	RIPK2	TBK1	TNF	TNFRSF10A	TNFRSF10B	TNFRSF1A	TNFRSF1B	TNFRSF21	TNFRSF25	TNFSF10	TRADD

9. Immunofluorescence staining:

ES2 cells were grown overnight on sterile glass slides in the appropriate medium. Cells were treated with DHA IC_{25} (10 μM), 10 μM of OA, carboplatin IC_{25} (10 μM) for ES2 cells, or combination of drugs for 72 hours. After the incubation period, slides were washed in PBS 3 times and fixed in 4% paraformaldehyde for 10 minutes. Then, slides were washed 3 more times, permeabilized with 0.2% Triton X-100 in PBS for 5 minutes and incubated with FAS antibody (1:200 dilution, Santa Cruz Biotechnology; Dallas, Texas, USA) for 3 hours. After washing with PBS, slides were incubated with secondary antibody (1:500, Alexa Flour; Cell signaling technology) for 1 hour. Slides were counterstained with DAPI nuclear stain (1:5000 dilution), washed twice and mounted with Dako Fluorescent Mounting Medium (Dako North America; Carpinteria, CA, USA) and analyzed by confocal microscope.

10. *In vivo* tumor implantation:

All steps of animal works and experiments were approved by the Animal Use Subcommittee at the University of Alberta. (AUP2496).

Patient-derived xenografts (PDX) model:

Our PDX-550 model was obtained through a collaboration with Oncotest (Charles River, Freiburg, Germany) and is a high grade serous ovarian serous carcinoma (HGSC PDX550). Viable pieces of the tumor (~1mm in diameter) were engrafted subcutaneously into the flank area of 7-8-week-old female NSG mice. A total of 26 mice (two tumors per mice) was used for DHA study plus chemotherapy intervention. Mice were divided into 4 random groups and treated as follow:

Group 1: Control diet - 5 mice

Group 2: Control diet plus carboplatin - 7 mice

Group 3: DHA enriched diet - 7 mice

Group 4: DHA enriched diet plus carboplatin - 7 mice

The nutritional intervention started 3 weeks after tumor implantation. Mice were fed with control or DHA enriched diet two weeks prior to chemotherapy (intraperitoneal carboplatin 40 mg/kg every 3 days) intervention and were kept on control or DHA diet during chemotherapy. See Table 2-3 for the fatty acid composition of control and DHA diets.

Table 2-3: Control vs DHA-enriched mice diet formulation (W/W %)

Oil	Control diet	DHA diet
C12:00	0.00	0.34
C14:0	1.13	2.19
C16:0	22.87	21.08
C16:1n9	1.76	1.80
C18:0	13.75	12.39
C18:1n9	36.37	38.66
C18:1c11	0.23	0.42
C18:2n6	21.01	15.15
C20:0	0.01	0.02
C18:3n3	2.38	3.37
C20:2n6	0.01	0.01
C20:3n6	0.03	0.04
C20:4n6	0.42	0.42
C20:5n3	0.00	0.00
C22:0	0.01	0.04
C22:5n3	0.00	0.04
C22:6n3	0.00	3.83
TOTAL	99.97	99.78
SFA	37.77	36.05
PUFA	23.85	22.85
MUFA	38.35	40.88
total	99.97	99.78
n-6	21.47	15.61
n-3	2.38	7.24
n6/n3	9.02	2.16
P/S	0.63	0.63

Tumor size was measured twice weekly with a digital caliper and mice were sacrificed when tumors reached 10 mm in diameter (2-3 weeks after chemotherapy). Tumors were collected, weighed and one-half of the tumors was fixed with 4% paraformaldehyde, embedded in paraffin,

and sent for histology to be sectioned and stained with H & E or used for immunohistochemistry (IHC). A small piece of the tumor and liver were sent for fatty acid analysis.

11. Immunohistochemistry staining:

IHC for Ki67 and FAS: All tumor samples were IHC stained for Ki67 (a marker of proliferation) and FAS (as indicator of apoptosis pathway activation), both antibodies were from Santa Cruz Biotechnology (Dallas, Texas, USA).

For Ki67 and FAS staining, formalin-fixed paraffin-embedded tumor tissue sections were placed on a slide warmer at 60°C for a minimum of 30 min. De-paraffinization using fresh xylenes (Thermo Fisher scientific, Burlington, ON, Canada) and hydration using 100, 95, 70 and 60% graded ethanol was done followed by brief incubation in water.

Antigen retrieval was done at 100°C for 20 min in DAKO target retrieval solution PH 6 (DAKO North American Carpinteria, CA, and the USA) and cooled to room temperature. Sections were rinsed with water and tissue was circled with an ImmEdge pen (Vector Laboratories; Burlingame, CA USA), blocked with one drop of peroxidase block (DAKO) for 5 min and washed with Tris-buffered saline and 0.1% Tween (TBS-T) for 5 min.

After that, sections were blocked with DAKO protein Serum Free for 15 min. Protein block was removed and replaced with antibodies, Ki67 or FAS both at 1:200 dilutions, and all made in DAKO antibody diluent and kept in a humidified chamber at 4°C overnight.

Slides were then washed in TBS-T for 5 times and incubated with 100µl of secondary antibody DAKO labeled Polymer-HRP anti-mouse for 60 min at room temperature in the humidified

chamber. Next, washing was done in TBS-T for 5 times and each section was covered by 75-100µl 3,3-diaminobenzidine chromogen solution (DAKO) for a maximum of 8 min. Then, the reaction was stopped by rinsing the sections with Milli-Q water several times to remove the background staining. Hematoxylin (DAKO) was used to counterstain the nucleus followed by slide wash in water. Sections were dehydrated using graded ethanol (60, 70, 95, and 100%) and cleared with xylenesSlides were dried and coverslips mounted with 15µl of Vecta Mount permanent mounting medium (Vector Laboratories).

TUNEL assay: Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed to detect DNA fragmentation as a characteristic of late-stage apoptosis. TUNEL staining was done on formalin-fixed paraffin-embedded sections using ApopTag Peroxidase *In situ* Apoptosis Detection Kit (Millipore Corporation; Temecula, CA, USA) following the manufacturer's protocol. As it was optional in the instruction, we used proteinase K (20µg/ml, Millipore Corporation) in order to pre-treat the tissue.

12. Analysis of H&E and IHC slides:

After staining, the entire cross-sectional area of all the slides was scanned by Aperio Digital Pathology slide scanner (Leica Biosystems; Concord, ON, Canada).

Analysis of H&E tumor sections for necrosis: Histological features were identified manually and quantified using image analysis software (Image Scope; Leica Biosystems). To quantitate the abundance of the necrotic features (e.g. tumor necrosis) per tumor cross-sectional area (185), the whole slide image was opened in Image Scope analysis software and measured (draw around) the entire cross-section area of the tumor. Second, identified and measured (draw around) all histological necrotic features. When the area of necrosis was determined, a percentage of

necrosis and dead cells was calculated (total area of necrosis/ total section area) by Image Scope. The data were then subjected to the appropriate statistical analysis.

Analysis of IHC sections: For Ki67, the number of positive and negative cells were counted in 10 high power (40x) fields of each section (necrotic areas were excluded). Then, the percentage of positive immunolabeled cells over the total number of the cells and a relative percentage in each selected field was calculated following the recommended protocol (186). For CD95, we analyzed at least 10 fields/section for the translocation of CD95 antigen to the cell membrane.

13. Data analysis:

Data are shown as mean ± SD of three to five independent experiments. Statistical analysis was performed using GraphPad Prism 8. Fatty acid-carboplatin interaction analysis was performed by Dr. Maryna Yaskina, Women and Children's Health Research Institute (ECHA, 5-083), University of Alberta, using SAS ver. 9.4. Statistical significance between every two independent groups was determined by the unpaired t-test and between multiple groups by the one-way or two-way ANOVA test, and the $P < 0.05$ defined as significant.

Chapter 3

Results

Chapter Three- Results

1. n-3 PUFAs have no cytotoxic effects on immortalized epithelial surface ovarian (OSE) and fallopian tube cell lines (FT194)

Chemotherapy treatment kills cancer cells but can also damage healthy cells. Damage to normal cells is one of the major side effects of anti-cancer drugs; hence a therapeutic window, in which a compound kills cancer, but not normal cells, must be ascertained in order to consider it as a therapy. Accordingly, we first determined the effects of n-3 PUFAs on normal cells, shown to contribute as the cells of origin (193) to ovarian cancer. For these studies, we used immortalized human ovarian surface epithelial and fallopian tube cells (IOSE and FT194, respectively). In both IOSE and FT194 cells, treatment with different doses of DHA caused no significant differences in cell viability when compared to control groups (OA and LA) as estimated with the MTT assays, $p > 0.05$ (Figure 3-1).

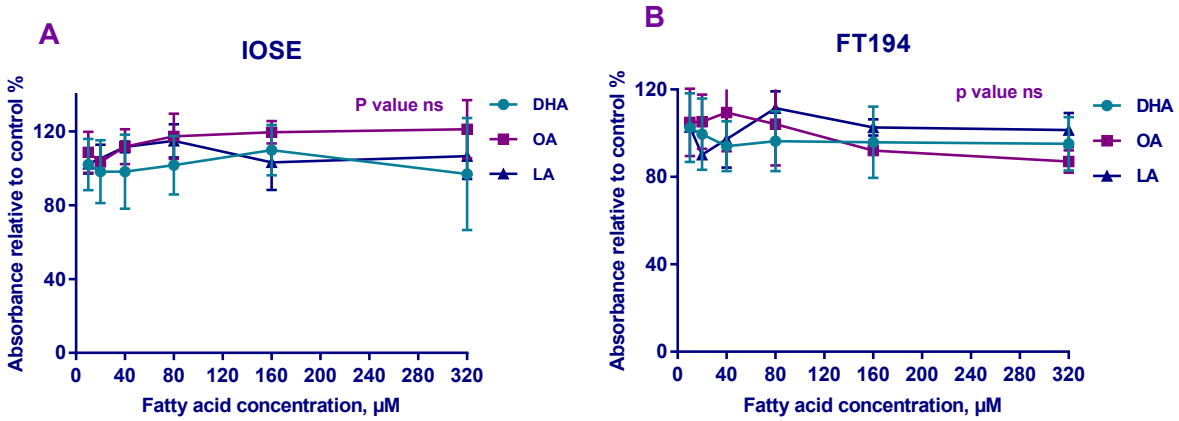


Figure 3-1: DHA, OA, and LA do not reduce the viability of immortalized ovarian surface epithelium (A) and fallopian tube cells (B). Cells were treated with 0µM to 320µM of fatty acids for 72 hours, and the cell viability was measured with MTT assay (as described in materials and methods). Cells were cultured in triplicates and for 3 separate passages. Each point represents the mean ± SD. P values were not statistically significant in both cell lines (calculated by one-way ANOVA).

2. Effects of DHA on metabolic activity of ovarian cancer cell lines.

We next measured the effects of DHA treatment on ovarian cancer cell lines. All the cell lines were treated with different concentrations of DHA from 0 µM to 320 µM for 72 hours. As shown in Figure 3-2, DHA significantly decreased ES2 and A2780cp cell metabolic activity (as determined by MTT, which was used as a surrogate for cell number) in a concentration-dependent manner. The other cell lines (OV90, SKOV3, Kuramochi, JHOS2, OCI-C1p, and OCI-C4p) were all resistant to DHA even in high concentrations.

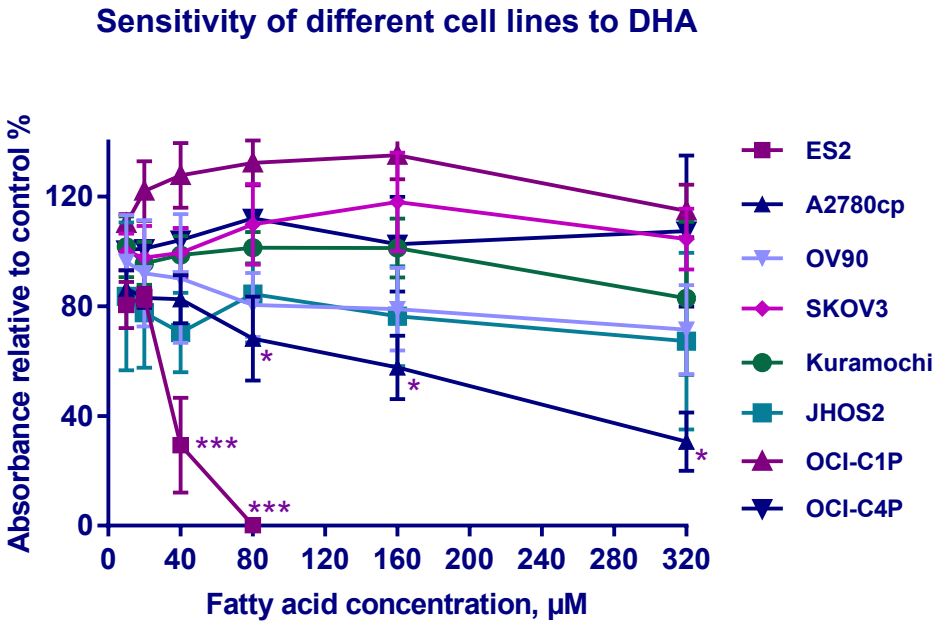


Figure 3-2: Concentration-dependent effect of DHA on cell growth and metabolic activity of ES2, A2780cp, OV90, SKOV3, Kuramochi, JHOS-2, OCI-C1p, and OCI-C4p. Cells were treated with 0µM to 320µM of fatty acids for 72 hours, and the cell viability was measured with MTT assay (as described in materials and methods). Cells were cultured in triplicates and for 3 separate passages. Each point represents the mean ± SD. P value was calculated by unpaired t-test. "*" = p value<0.05; "**" = p value<0.01; and "***" = p value<0.001.

3. **DHA significantly reduces the cell viability and metabolic activity of ES2 and A2780cp ovarian cell lines (compared to OA and LA), but the other cell lines were relatively resistant to fatty acids.**

The proliferation of ES2 and A2780cp cells were significantly inhibited by DHA in a concentration-dependent manner (Figure 3-3). ES2 cell line, which is a clear cell subtype of ovarian cancer was sensitive to DHA at very low concentrations (p value= 0.001). Moreover, the inhibitory effects of DHA were more pronounced than those of OA and LA.

When compared to untreated control, 100 µM DHA inhibited cell proliferation by approximately 100% and 35% in ES2 and A2780cp cells, respectively ($P < 0.05$).

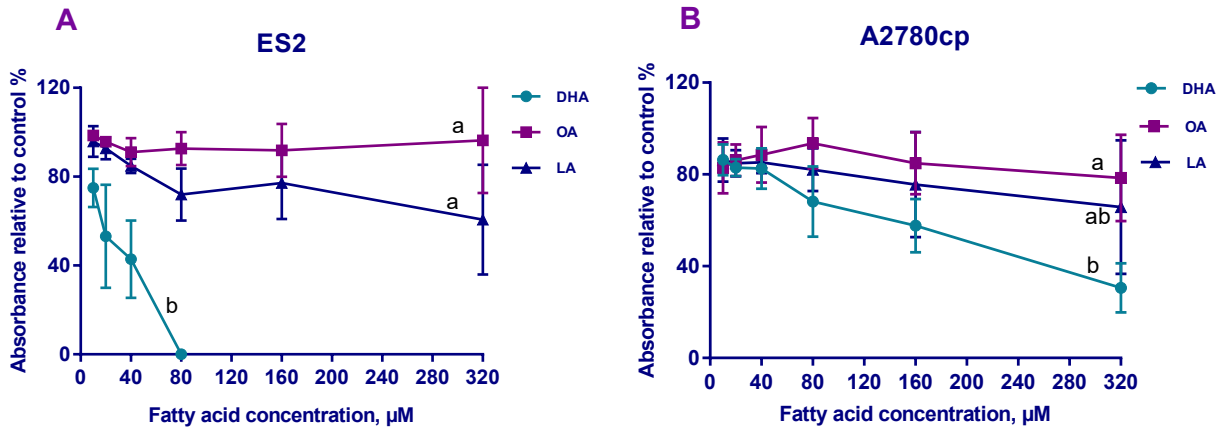


Figure 3-3: DHA reduces the viability of ES2 (A) and A2780cp (B) human ovarian cancer cells. Cells were treated with 0µM to 320µM of fatty acids for 72 hours, and the cell viability was measured with MTT assay (as described in materials and methods). Cells were cultured in triplicates and for 3 separate passages. Each point represents the mean ± SD. P value was calculated by one-way ANOVA, followed by Turkey's multiple comparison tests. Bars that do not share a common letter are significantly different ($p<0.05$).

We also evaluated the cytotoxicity of the fatty acids on the other ovarian cancer cell lines (OV90, Kuramochi, JHOS-2, OCI-C1P, OCI-C4P, and SKOV3). As it is shown in Figure 3-4, all other cells were resistant to the cytotoxic effects of DHA.

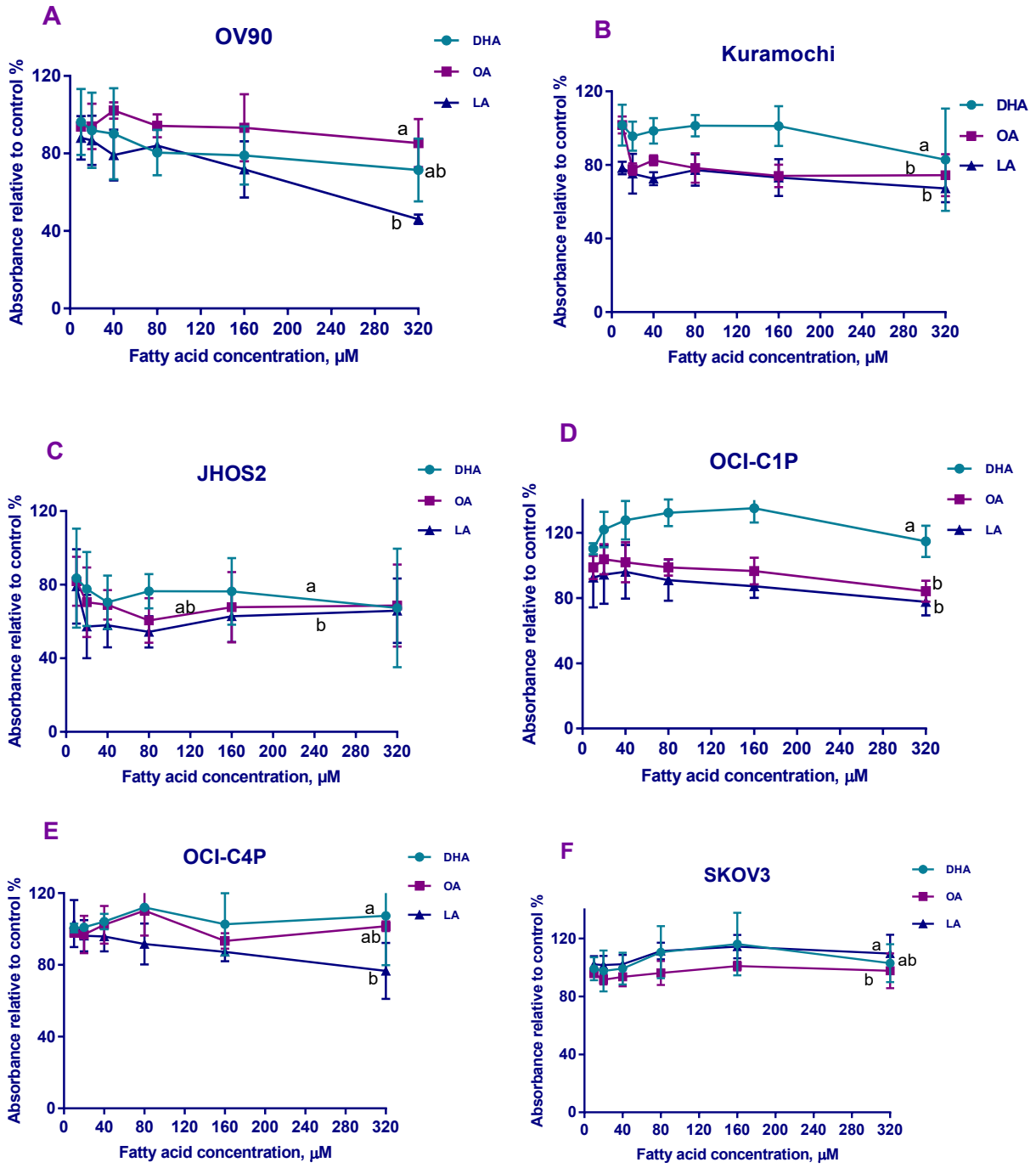


Figure 3-4: DHA does not reduce the viability of OV90, Kuramochi, JHOS2, OCI-C1p, OCI-C4P, and SKOV3 (A-F). Cells were treated with 0µM to 320µM of fatty acids for 72 hours, and the cell viability was measured with MTT assay (as described in materials and methods). Cells were cultured in triplicates and for 3 separate passages. Each point represents the mean ± SD. P value was calculated by one-way ANOVA, followed by Turkey's multiple comparison tests. Bars that do not share a common letter are significantly different ($p<0.05$).

Interestingly, we observed that LA induced more cell cytotoxicity than DHA in OV90 and JHOS2. Another finding was that DHA increased the viability of OCI-C1P and OCI-C4P cells, which all justify the inconsistency between the previous studies.

To validate the results of MTT assay, Trypan Blue exclusion assay was performed on ES2 and Kuramochi cell lines (ES2 as a sensitive and Kuramochi as a resistant one) after treatment with different concentrations of fatty acids for 72 hours (Figure 3-5). The results of Trypan Blue assay were almost the same as the MTT assay.

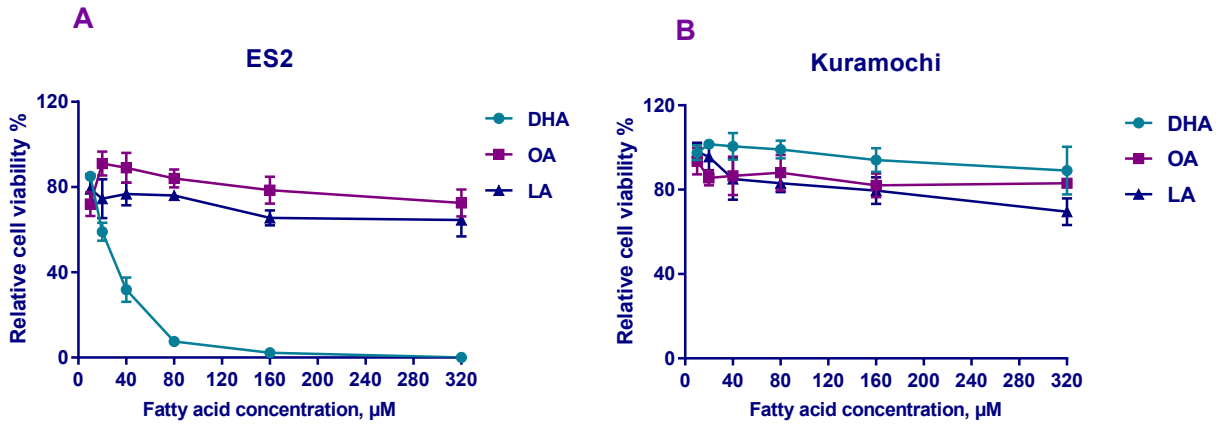


Figure 3-5: DHA reduces the viability of ES2 (A) human ovarian cancer cells but does not reduce the viability of Kuramochi cells (B). Cells were treated with 0µM to 320µM of fatty acids for 72 hours, and the cell viability was measured with Trypan Blue exclusion assay. (as described in materials and methods). Cells were cultured in triplicates and for 2 separate passages. Each point represents the mean ± SD. P value was not calculated due to an inadequate number of replications.

4. DHA increases the efficacy of carboplatin

4.1 The sensitivity of different ovarian cancer cell lines to carboplatin

Prior to testing the effects of combination therapy, a cytotoxicity assay was performed to determine the IC_{50} concentration for carboplatin in each ovarian cancer cell line. For that, the cells were plated for 24h and treated with different concentrations of carboplatin (0µM–400µM)

for 72h. After the incubation period, the metabolic activity of the cells was evaluated by MTT assay. Table 3-1 shows the IC_{50} of carboplatin in different cell lines. IC_{50} values ranged from 29-120 μM.

Table 3-1: Sensitivity of different cell lines to carboplatin (IC50 after 72h of treatment)

Carboplatin IC50 (μM) in different ovarian cancer cell lines			
Cell line	**IC50**	**Cell line**	**IC50**
ES2	29μM	Kuramochi	39μM
A2780cp	120μM	JHOS-2	75μM
OV90	40μM	OCI-C1P	60μM
SKOV3	42μM	OCI-C4P	34μM

4.2 Combination therapy with fatty acids and carboplatin decreases survival of ovarian cancer cells in vitro.

We assessed cell viability of ovarian cancer cell lines when treating the cells with a combination of fatty acids (DHA vs OA) and carboplatin. First, cells were plated in 96 well plates at 5 x 10^3 cells/well for 24h and then treated with IC_{50} of carboplatin in the presence or absence of different concentrations of DHA or OA (0 to 320 μM) for 72h. As shown in Figure 3-6, in ES2, A2780cp, SKOV3, and Kuramochi cell lines, cell viability was reduced by combination therapy of DHA and carboplatin as compared to either treatment alone. In ES2, A2780cp, SKOV3, and Kuramochi cells, DHA enhanced the cytotoxic effects of carboplatin (p value<0.05).

As an interesting finding, in ES2 and A2780cp cells, cell viability was suppressed more by the co-administration of DHA and carboplatin than by a combination of OA and carboplatin, but in SKOV3 and Kuramochi cell lines, OA was as effective as DHA when combined with carboplatin. In the other cell lines (OV90, JHOS-2, OCI-C1P, and OCI-C4P), fatty acids did not enhance the cytotoxic effects of carboplatin (Figure 3-7). We did not find any relationship

between the response to fatty acids (alone and in combination with carboplatin) and p53 mutation status in the different ovarian cancer cell lines.

P-values were calculated by one-way ANOVA, followed by Turkey's comparison test. Statistical analysis to ascertain the correlation between DHA and carboplatin was done by biostatistician, Dr. Maryna Yaskina, Women and Children's Health Research Institute (ECHA, 5-083), University of Alberta.

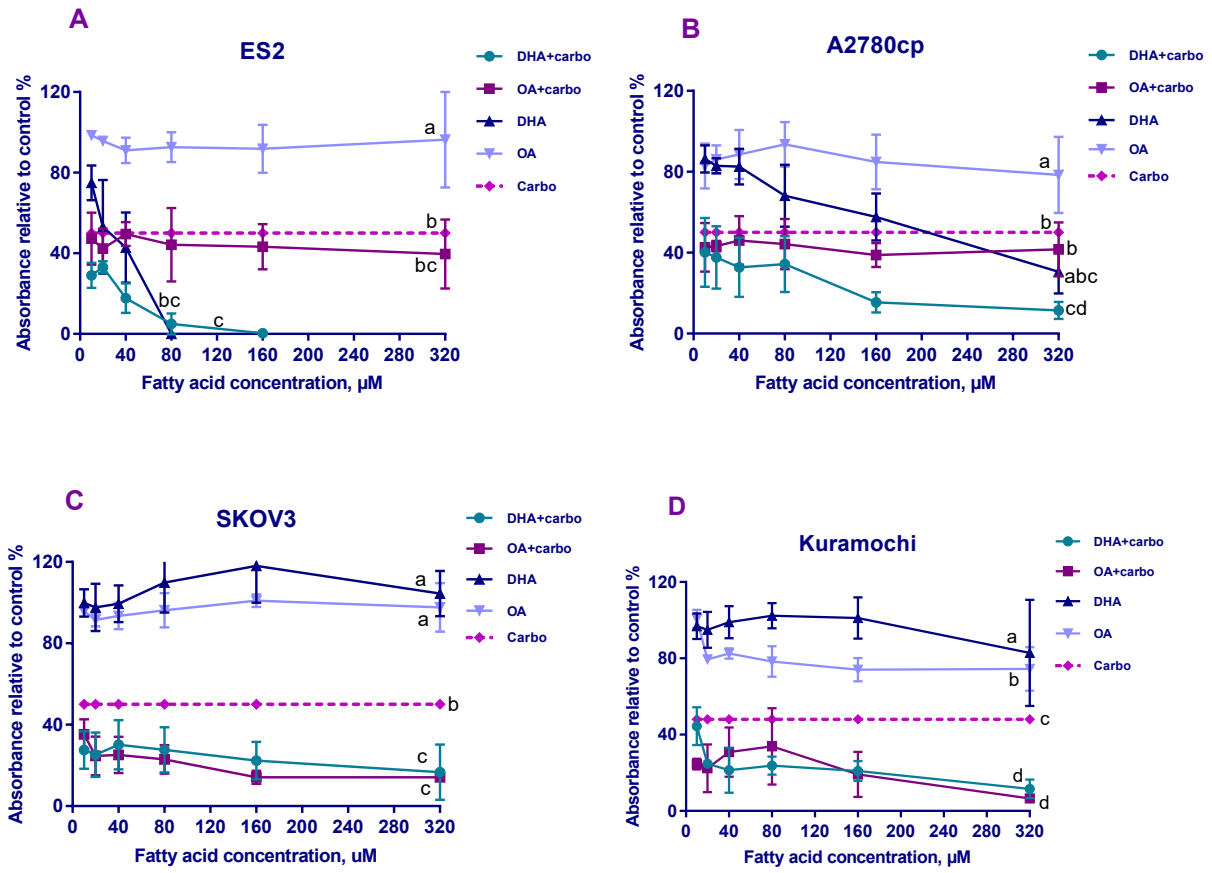


Figure 3-6. DHA in combination with carboplatin reduces the viability of ES2 (A) and A2780cp (B), SKOV3 (C), and Kuramochi (D) human ovarian cancer cells. Cells were treated with 0μM to 320μM of fatty acids and IC_{50} of carboplatin (Table 3-1) for 72 hours, and the cell viability was measured with MTT assay. Cells were cultured in triplicates and for 3 separate passages. Each point represents the mean ± SD. P value was calculated by one-way ANOVA, followed by Turkey's multiple comparison tests. Bars that do not share a common letter are significantly different ($p<0.05$).

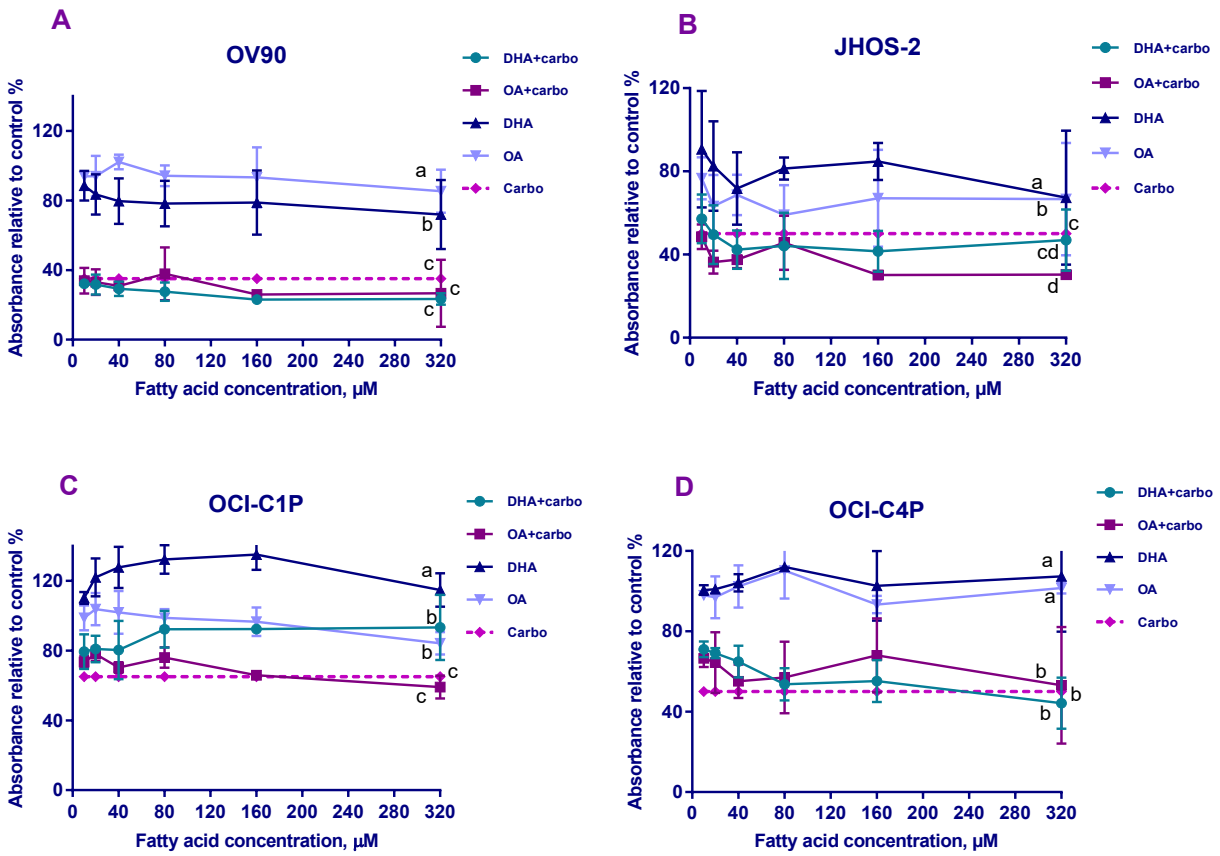


Figure 3-7. DHA in combination with carboplatin does not reduce the viability of OV90 (A), JHOS2 (B), OCI-C1P (C) and OCI-C4P (D) human ovarian cancer cells. Cells were treated with 0µM to 320µM of fatty acids and IC_{50} of carboplatin (Table 3-1) for 72 hours, and the cell viability was measured with MTT assay. Cells were cultured in triplicates and for 3 separate passages. Each point represents the mean ± SD. P value was calculated by one-way ANOVA, followed by Turkey's multiple comparison tests. Bars that do not share a common letter are significantly different ($p<0.05$).

We next sought to determine whether fatty acids could alter the IC_{50} of carboplatin. We selected two cell lines, SKOV3 and Kuramochi, that are resistant to fatty acids and treated them with escalating doses of carboplatin (from 0 to 200 µM) in the presence or absence of 160 µM of DHA, OA or LA for 72h. After the incubation time, cell proliferation was evaluated by Trypan Blue exclusion assay to check the cell viability (and to confirm the accuracy of MTT assay). As shown in Figure 3-8, combination therapy with fatty acids reduced the IC_{50} of carboplatin. In the

SKOV3 cell line, IC_{50} of carboplatin alone is 41.9 μM and in combination with DHA, it is 4.35 μM. Similarly, in the Kuramochi cell line, the IC_{50} of carboplatin alone and in combination with DHA is 39.11 μM and 5.17 μM, respectively. These data confirm that DHA enhances the cytotoxic effect of carboplatin, *in vitro* (p-value was significant in the concentrations of 100 μM and lower of carboplatin). Interestingly, in the SKOV3 and Kuramochi cell lines, treatment with OA and LA in combination with carboplatin, reduced the IC_{50} of carboplatin. Further studies should be performed before making any conclusions.

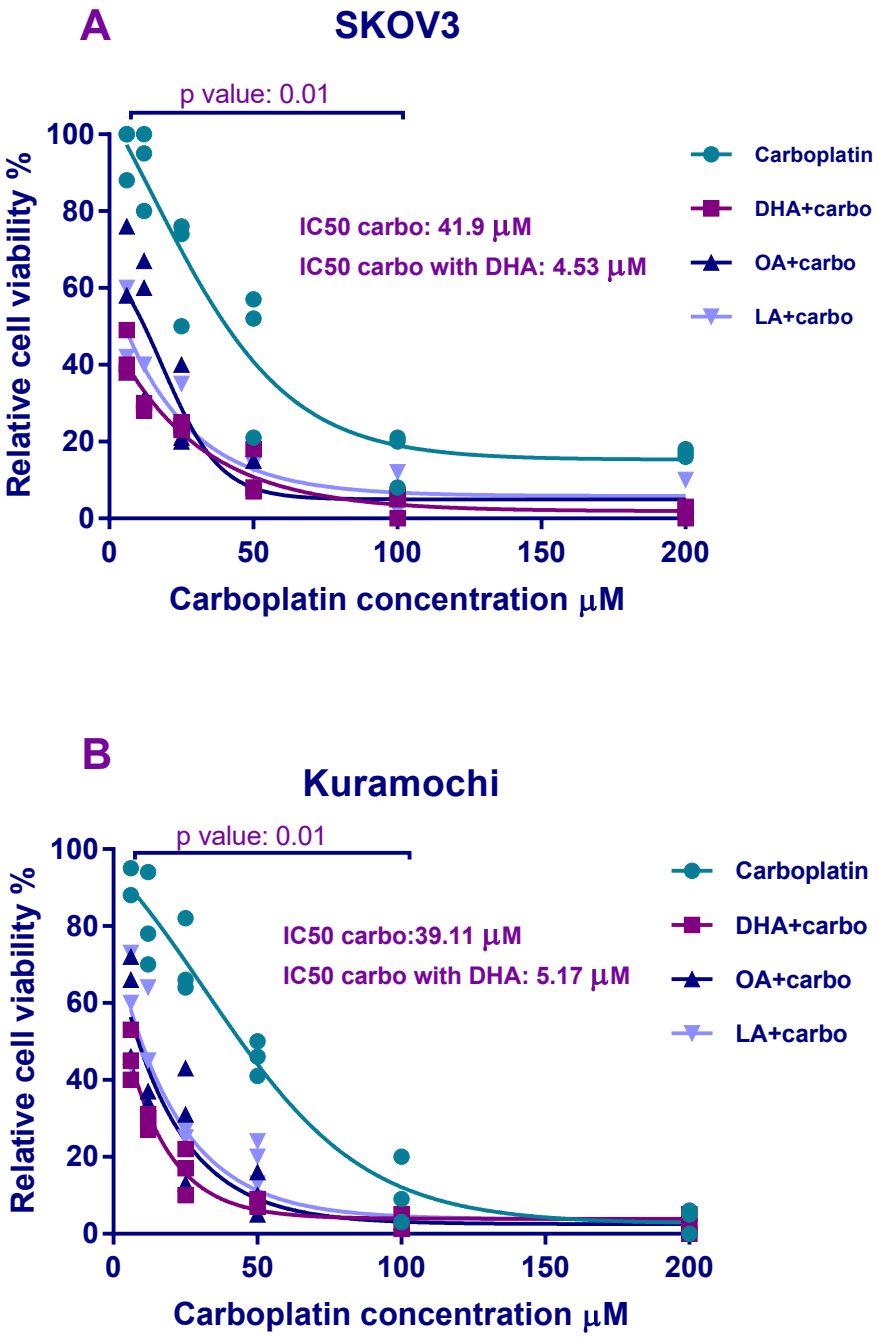


Figure 3-8. The cytotoxic dose-dependent effect of carboplatin alone or in combination with DHA, OA, and LA. SKOV3 (A), and Kuramochi (B) cell lines were treated with escalating doses of carboplatin and 160 µM of DHA, OA or LA for 72 hours, and the cell viability was measured with Trypan Blue exclusion assay (as described in materials and methods). Cells were cultured in triplicates and for 3 separate passages. Each point represents the mean of the separate experiment. P value was calculated by one-way ANOVA and $p<0.05$ considered to be significant. For both cell lines in the carboplatin concentrations of 100 µM and lower, the p-value was significant.

5. Lipid Analysis

5.1 Effect of DHA and OA treatments on fatty acid content of whole cell phospholipids in ES2, A2780cp, and SKOV3 ovarian cancer cells

Selective fatty acid profiles for whole cell PL are shown in Tables 5-1 to 5-3 for ES2, A2780cp, and SKOV3 ovarian cancer cells, respectively. Cells were treated with 23 μM of DHA or OA for 3 days and then sent for analysis of whole cell PL.

The fatty acid profiles for whole cell PL were also analyzed to compare the following:

1) Saturated fatty acid content (∑SFA): C14:0, C16:0, C17:0, C18:0, C20:0, C24:0
2) Mono-unsaturated fatty acids (∑MUFA): C16:1, C18:1
3) Poly-unsaturated fatty acids (∑PUFA): C18:2 n-6, C18:3 n-3, C20:2 n-6, C20:3 n-6, C20:4 n-6, C20:5 n-3, C20:4 n-6, C20:5 n-3, and C20:6 n-3.
4) ∑N-3 PUFAs: C18:3 n-3, C20:5 n-3, C20:5 n-3, and C20:6 n-3.
5) ∑N-6 PUFAs: C18:2 n-6, C20:2 n-6, C20:3 n-6, C20:4 n-6, and C20:4 n-6.

The effect of fatty acid treatments on changes in whole cell PLs is summarized in Figures 3-9, 3-10 and 3-11.

Table 3-2: Change in selected fatty acids of whole cell PLs in ES2 ovarian cancer cells incubated with control (without treatment), DHA or OA for 72h (% w/w of total fatty acids)

ES2			
Fatty acids	**Control**	**DHA 3 days**	**OA 3 days**
C18:1 n9 (OA)	26.19±7.22	22.30±4.61	29.49±4.45
C18:2 n6 (LA)	2.94±0.96	2.95±0.69	2.89±0.74
C20:4 n6 (AA)	6.40±2.30	3.63±0.99	5.15±0.31
C20:5 n3 (EPA)	0.24±0.10	0.35±0.15	0.22±0.10
C22:5 n3 (DPA)	1.35±0.25	1.01±0.28	1.18±0.16
C22:6 n3 (DHA)	2.81±0.57	**5.45±0.96****	2.19±0.46
∑SFA	47.02±3.33	51.86±3.29	46.34±2.74
∑MUFA	27.57±6.85	23.55±4.23	30.80±4.23
∑PUFA	16.19±3.96	15.87±1.51	14.51±0.78
∑N3	4.98±0.83	**7.41±1.36***	4.35±0.78
∑N6	11.20±3.34	8.45±1.09	8.45±1.06

Values are mean percent composition ± SD (n=4); "OA" = oleic acid; "LA" = linoleic acid; "AA" = arachidonic acid; "EPA"=eicosapentaenoic acid; "DPA"= docosapentanoic acid; "DHA"= docosahexanoic acid; "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each FA identified, **bold** indicate significant difference compared to control (without treatment). "*" = p value<0.05; "**" = p value<0.01; and "***" = p value<0.001.

Table 3-3: Change in selected fatty acids of whole cell PLs in A2780cp ovarian cancer cells incubated with control (without treatment), DHA or OA for 72h (% w/w of total fatty acids).

A2780cp			
Fatty acids	**Control**	**DHA 3 days**	**OA 3 days**
C18:1 n9 (OA)	28.03±1.95	**13.86±0.87*** **	32.36±5.02
C18:2 n6 (LA)	1.76±0.32	3.40±0.57	1.93±0.39
C20:4 n6 (AA)	8.02±0.51	**5.53±0.73***	7.42±1.01
C20:5 n3 (EPA)	0.21±0.03	0.41±0.03	0.24±0.10
C22:5 n3 (DPA)	1.35±0.24	1.12±0.15	1.16±0.24
C22:6 n3 (DHA)	2.76±0.22	**14.60±2.44** **	2.57±0.19
∑SFA	43.19±0.45	48.28±2.39	39.93±2.92
∑MUFA	30.06±1.92	**15.62±0.88*** **	34.16±5.08
∑PUFA	17.06±1.21	**27.82±3.83** **	16.71±1.75
∑N3	4.88±0.37	**16.43±2.49** **	4.66±0.28
∑N6	12.17±0.85	11.39±1.33	12.04±1.63

Values are mean percent composition ± SD (n=4); "OA" = oleic acid; "LA" = linoleic acid; "AA" = arachidonic acid; "EPA"=eicosapentaenoic acid; "DPA"= docosapentanoic acid; "DHA"= docosahexanoic acid; "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each FA identified, **bold*** indicate significant difference compared to control (without treatment). "*" = p value<0.05; "**" = p value<0.01; and "***" = p value<0.001.

Table 3-4: Change in selected fatty acids of whole cell PLs in SKOV3 ovarian cancer cells incubated with control (without treatment), DHA or OA for 72h (% w/w of total fatty acids).

SKOV3			
Fatty acids	**Control**	**DHA 3 days**	**OA 3 days**
C18:1 n9 (OA)	18.81±1.29	17.77±1.19	26.38±6.05
C18:2 n6 (LA)	4.15±0.08	4.53±0.18	3.73±0.84
C20:4 n6 (AA)	10.17±1.20	8.33±0.41	9.59±1.89
C20:5 n3 (EPA)	0.26±0.04	0.45±0.17	0.20±0.06
C22:5 n3 (DPA)	1.56±0.35	1.30±0.36	1.36±0.12
C22:6 n3 (DHA)	1.92±0.12	5.84±2.84	1.60±0.04
∑SFA	36.69±21.26	36.74±21.21	33.30±19.37
∑MUFA	15.16±8.82	14.39±8.36	21.09±13.22
∑PUFA	15.22±8.84	17.01±10.15	14.31±8.60
∑N3	2.97±1.75	5.84±4.05	2.65±1.53
∑N6	12.24±7.16	11.17±6.48	11.66±7.16

Values are mean percent composition ± SD (n=4); "OA" = oleic acid; "LA" = linoleic acid; "AA" = arachidonic acid; "EPA"=eicosapentaenoic acid; "DPA"= docosapentanoic acid; "DHA"= docosahexanoic acid; "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each FA identified, **bold*** indicate significant difference compared to control (without treatment). "*" = p value<0.05; "**" = p value<0.01; and "***" = p value<0.001.

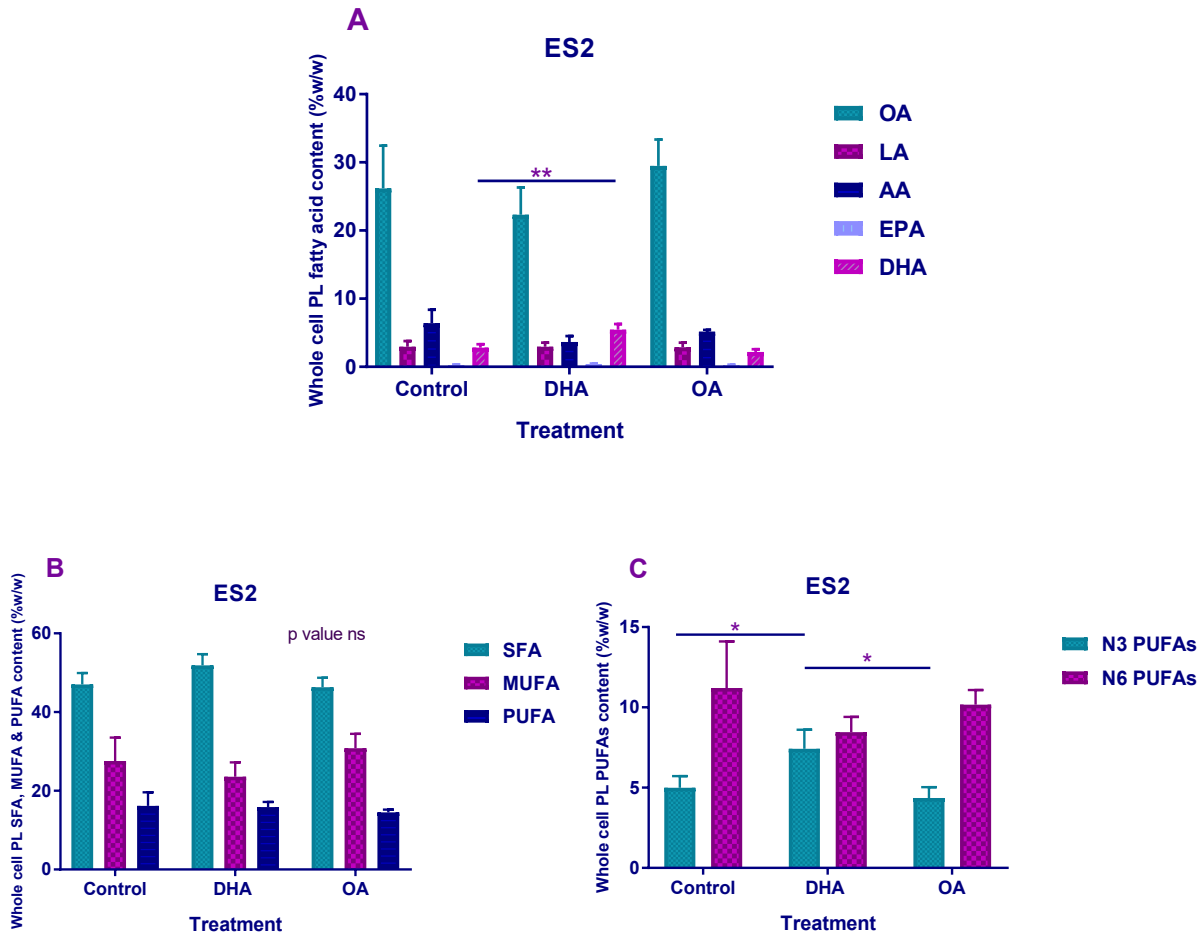


Figure 3-9: Effect of DHA and OA treatments in ES2 ovarian cancer cells on whole cell total PL content (%w/w) in (A) single fatty acids; (B) SFA, MUFA, and PUFA; and (C) total n3 and n6 PUFAs. Bars represent the mean ± SD for ES2 ovarian cancer cells (n=4 separate experiments and passages). Differences in fatty acid content with each treatment relative to control were tested using a t-test. "OA" = oleic acid; "LA" = linoleic acid; "AA" = arachidonic acid; "EPA" =eicosapentaenoic acid; "DHA"= docosahexaenoic acid; "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. "*" = p value<0.05; "**" = p value<0.01; and "***" = p value<0.001.

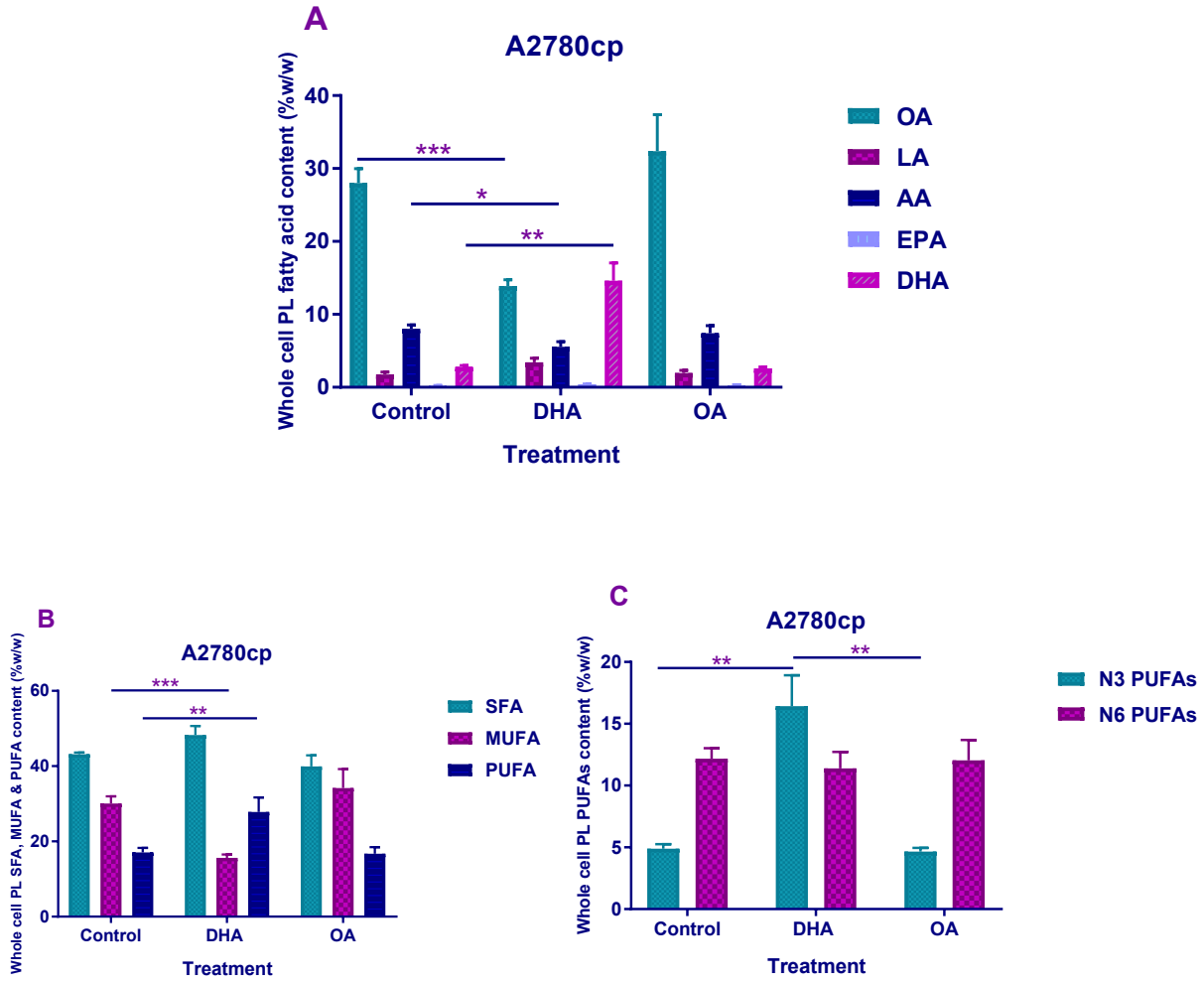


Figure 3-10: Effect of DHA and OA treatments in A2780cp ovarian cancer cells on whole cell total PL content (%w/w) in (A) single fatty acids; (B) SFA, MUFA, and PUFA; and (C) total n3 and n6 PUFAs. Bars represent the mean ± SD for ES2 ovarian cancer cells (n=3 separate experiments and passages). Differences in fatty acid content with each treatment relative to control were tested using a t-test. "OA"= oleic acid; "LA"= linoleic acid; "AA"= arachidonic acid; "EPA"=eicosapentaenoic acid; "DHA"= docosahexaenoic acid; "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. "*" = p value<0.05; "**" = p value<0.01; and "***" = p value<0.001.

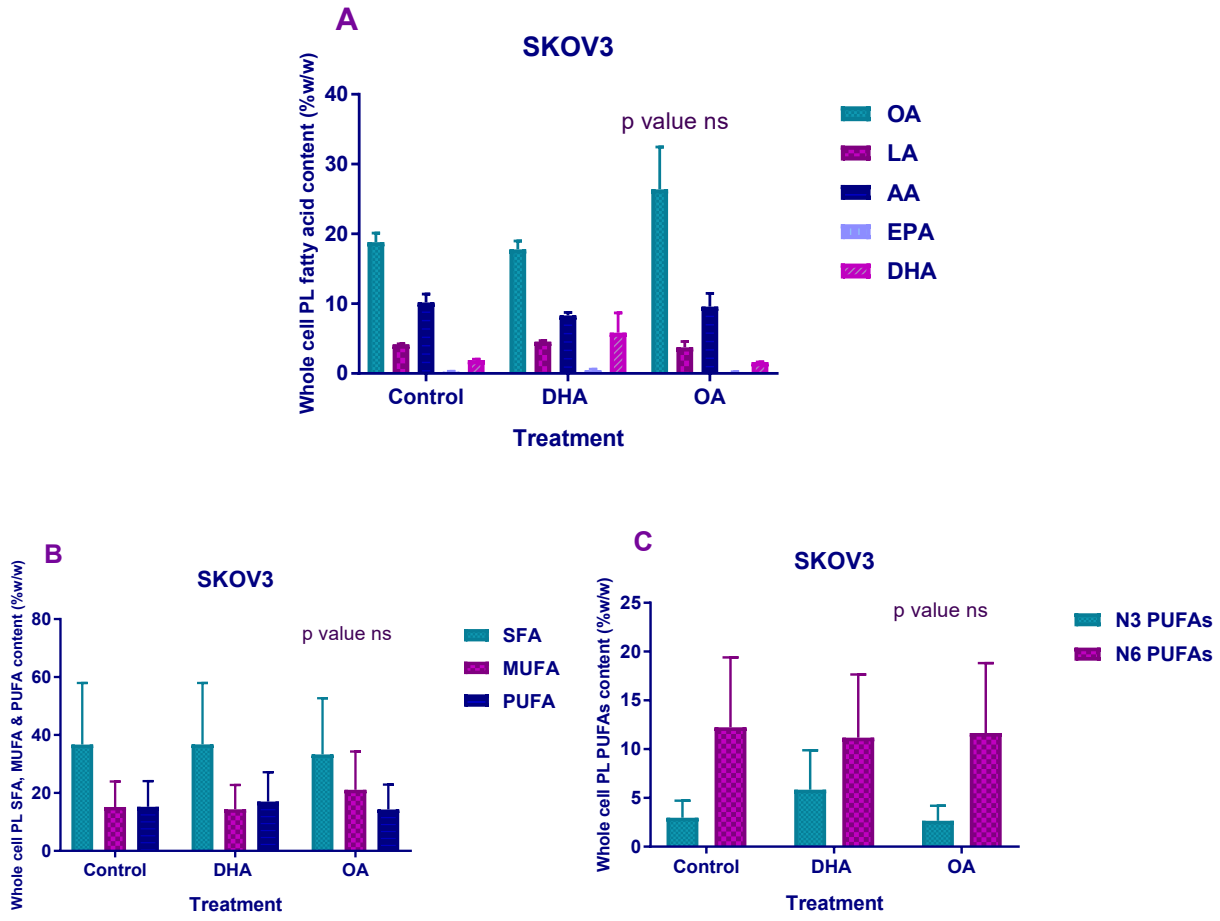


Figure 3-11: Effect of DHA and OA treatments in SKOV3 ovarian cancer cells on whole cell total PL content (%w/w) in (A) single fatty acids; (B) SFA, MUFA, and PUFA; and (C) total n3 and n6 PUFAs. Bars represent the mean ± SD for ES2 ovarian cancer cells (n=3 separate experiments and passages). Differences in fatty acid content with each treatment relative to control were tested using a t-test. "OA"= oleic acid; "LA"= linoleic acid; "AA"= arachidonic acid; "EPA"=eicosapentaenoic acid; "DHA"= docosahexaenoic acid; "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. "*" = p value<0.05; "**" = p value<0.01; and "***" = p value<0.001.

When DHA was provided to ES2 ovarian cancer cells (Table 3-2 and Fig. 3-9), there was a significant increase in both DHA and n-3 PUFAs content in total cell PL relative to control. In this

cell line, treatment with DHA decreased the inflammatory mediator of AA, but the difference was not statistically significant.

In the A2780cp ovarian cancer cell line, the effect of treatment with fatty acids on whole cell PL content was more prominent. As shown in Table 3-3 and Figure 3-10, treatment with DHA increased the cell content of DHA, n-3 PUFAs and total PUFAs. In addition to the incorporation of DHA in the cell membrane, the levels of OA, AA, and MUFA were reduced in the A2780cp cells.

In SKOV3 which was resistant to DHA treatment (Figure 3-4), the presence of DHA did not appear to affect the composition of the cellular PLs (Table 3-4 and Figure 3-11). Although after treatment with DHA, the cell content of DHA and n-3 PUFAs were increased, the p-value was not statistically significant. Additionally, OA had no significant effect on the whole cell PL content of this resistant cell line.

6. Mechanism of the effect of DHA is by inhibition of proliferation in ES2 ovarian cancer cells

6.1 DHA induces cell cycle arrest in ES2 ovarian cancer cells

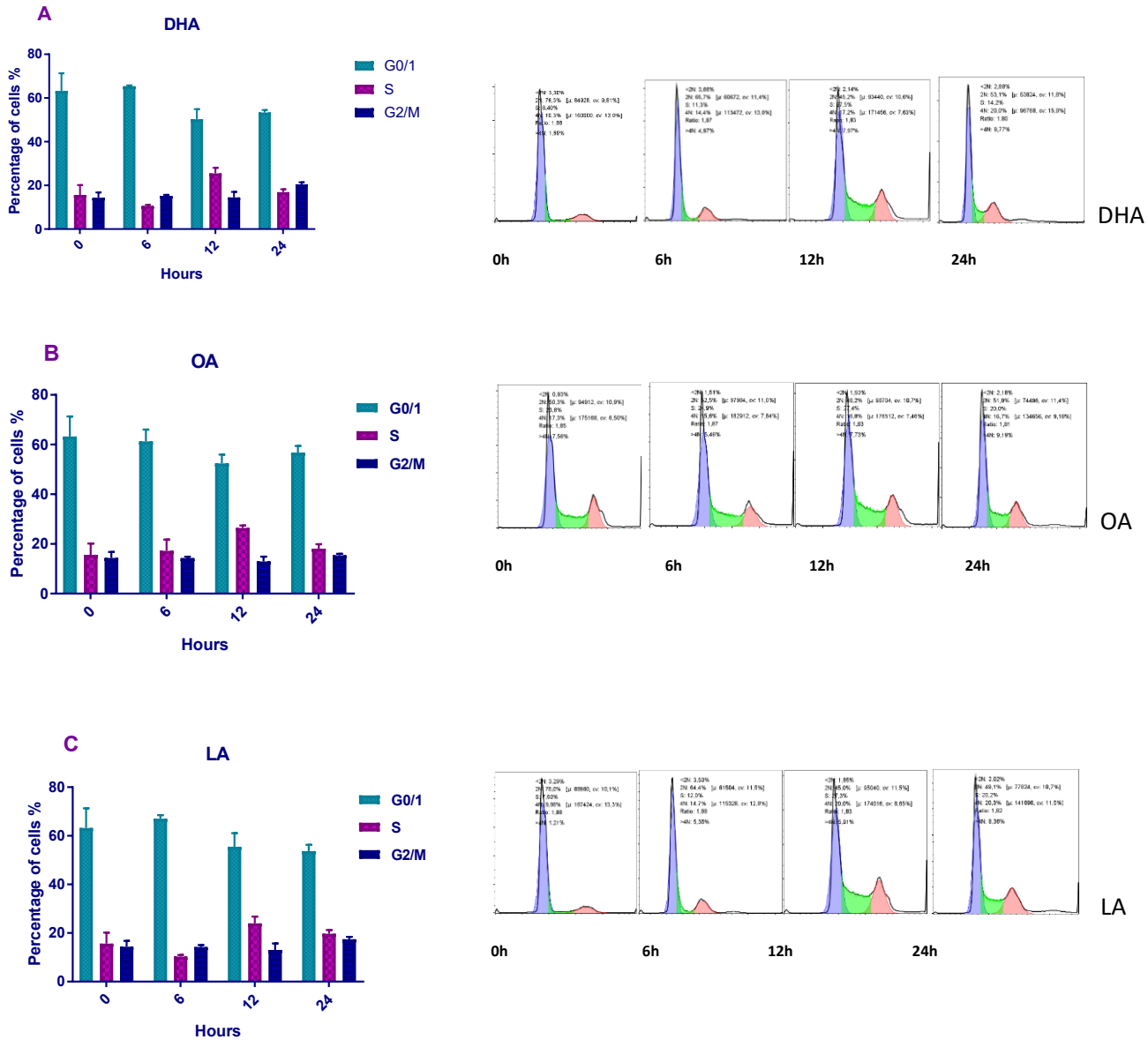
Cell cycle analysis:

To uncover the function of DHA, OA, and LA on ES2 ovarian cancer cell growth, cell cycle distribution was monitored by flow cytometry. As shown in Figure 3-12, Panel (A), after 12h of treatment with 23 μM (IC_{50}) of DHA, 50.2% of ES2 cells were in the G0/G1 phase, 25.5% were in S phase and about 14.4% were in G2/M phase, which was significantly different in G0/G1 and S phase compared to 6h of treatment (65.3% in G0/G1 phase, 10.5% in S phase and 15.2% in G2/M phase). Additionally, there is a significant difference in the G0/G1 phase after 24h of treatment with DHA compared to 6h, 53.3%, and 65.3%, respectively.

Figure 3-12, Panel B shows the same pattern of the cell cycle in ES2 cells after treatment with 23 μM of OA. Although we can see a reduction in the percentage of the cells in G0/G1 phase (63% at 0h, 61.2% at 6h and 52.4% at 12h) and an increase in the number of cells in S phase (15.6% at 0h, 17.2% at 6h and 26.4% at 12h) up to 12h of treatment with OA, the differences were not statistically significant.

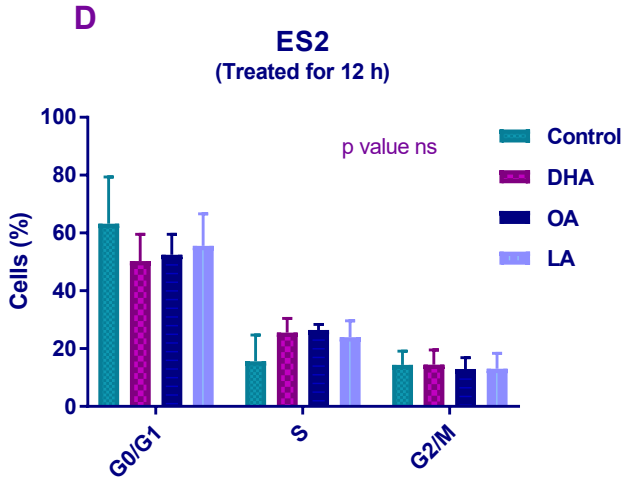
Similar to DHA, the cell cycle distribution of LA treated ES2 cells (23 μM of LA) was significantly different at different time points. As shown in Figure 3-12 panel C, there was a significant reduction of the percentage of the cells at G0/G1 phase from 6h to 24 h (67% at 6h, 55.5% at 12h and 53.7% at 24 phase) and a significant increase in the percentage of the cells in S phase after 12h of treatment compared to 6h (10.2% compared to 23.9%).

In Figure 3-12 (panel D and Table E), the distribution of cells in the cell cycle phases were evaluated after 12 hours of treatment by different fatty acids. As it is shown, fatty acids and especially DHA, increased the percentage of the cells in S phase and reduced the cell population in the G0/G1 phase, but the p-value was not significant. These results suggest that fatty acids may be involved in the cell cycle distribution of ovarian cancer cells.



Continued

D



E

cell cycle phase ratio	Control	DHA, 12h	OA, 12h	LA, 12h
G1/S	6.0±1.96	2.09±0.39	2.0±0.17	3.36±1.26
G1/G2	5.07±1.20	3.92±0.78	4.48±0.82	5.02±1.14
G1/(S+G2)	2.71±0.77	**1.33±0.24***	**1.37±0.16***	1.92±0.57

Figure 3-12: DHA and LA change the cell cycle distribution in ES2 ovarian cancer cells.
The cell cycle in fatty acid treated cells was monitored with fluorescence-activated cell sorting (FACS) analysis (A, B, and C). Panel (D) illustrates the percentage of the cells in each phase of the cell cycle after 12 hours of treatment with 23 μM of different fatty acids. Although the percentage of the cells in the S phase increased by fatty acid treatment, the p-value was > 0.05 (calculated by unpaired t-test between each treatment compared to control). To further analyze the pattern of the cell cycle, G1 ratio to other cell cycle phases were calculated (Table E). After 12 hours of treatment with DHA, the majority of the cells arrested in S and G2 phases (although the p-value was not significant for each phase separately). "DHA" = docosahexaenoic acid; "OA" = oleic acid; "LA" = linoleic acid. "*" = p value<0.05.

***Western blot analysis of proliferation protein expression**:*

To determine the effects of fatty acids (DHA and OA) on protein expression associated with proliferation, we treated ES2 cancer cells with DHA (for 24, 48 and 72 hours of treatment) and OA (for 24, 48 and 72 hours). As shown in Figure 3-13, compared with the control without treatment, the level of phosphorylated histone H3 Ser10 was reduced after 48h of treatment with

DHA, and after 72 hours of treatment with OA. Phosphorylated histone H3 Ser28 was reduced by both treatments after only 24 hours.

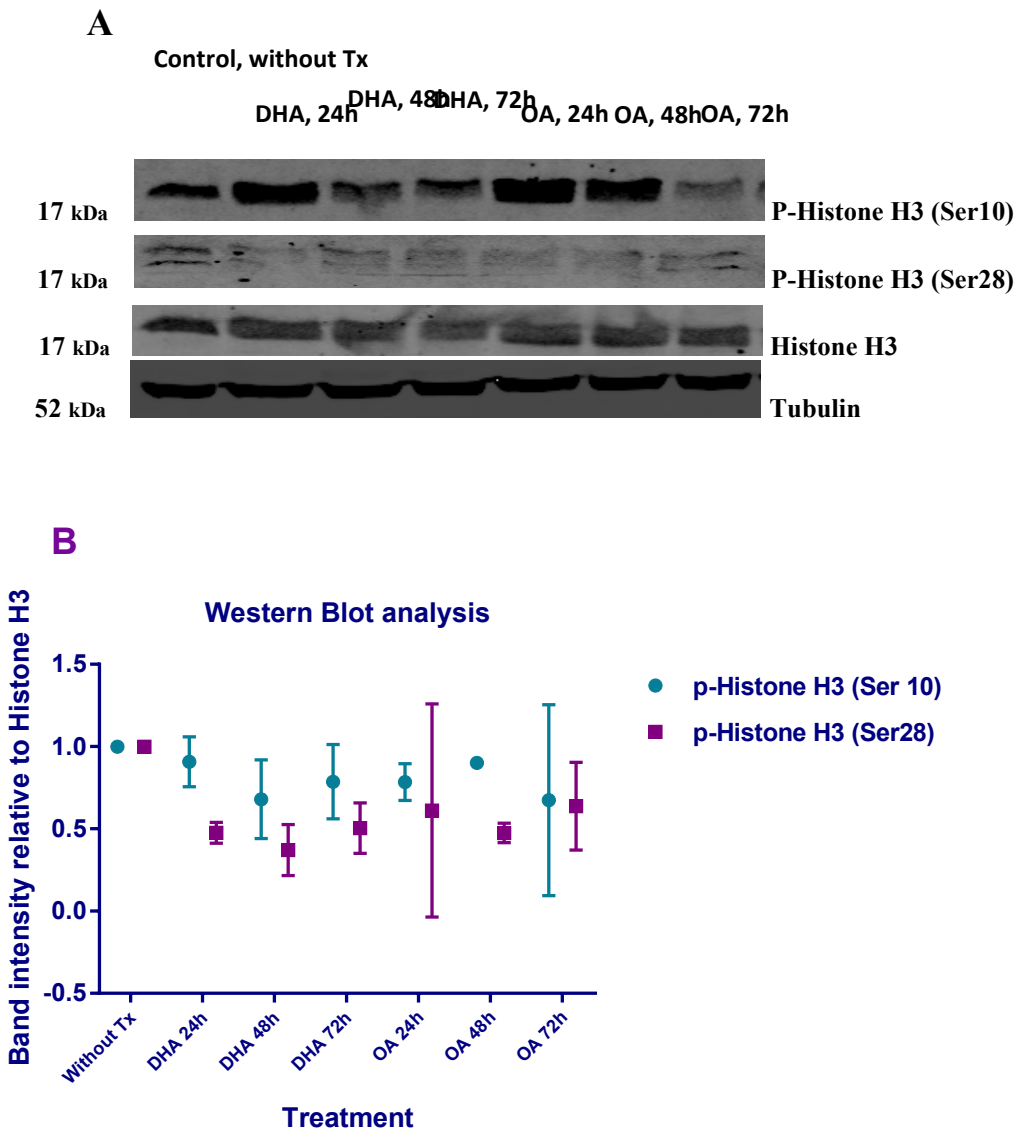


Figure 3-13: The effect of DHA and OA on the expression of proteins associated with proliferation. The cells were treated with 27 µM of DHA and OA for 0, 24, 48 and 72 hours. Western blot analysis was performed using antibodies against Histone H-3, P-Histone H3 (Ser10), P-Histone H3 (Ser28). Tubulin and Histone H3 were used as loading controls. **Panel (B)** illustrates the relative p-histone H3 ser10 and ser28 band intensities relative to histone H3. The band intensities are calculated by ImageJ software and normalized to Tubulin. n=2 separate independent experiments.

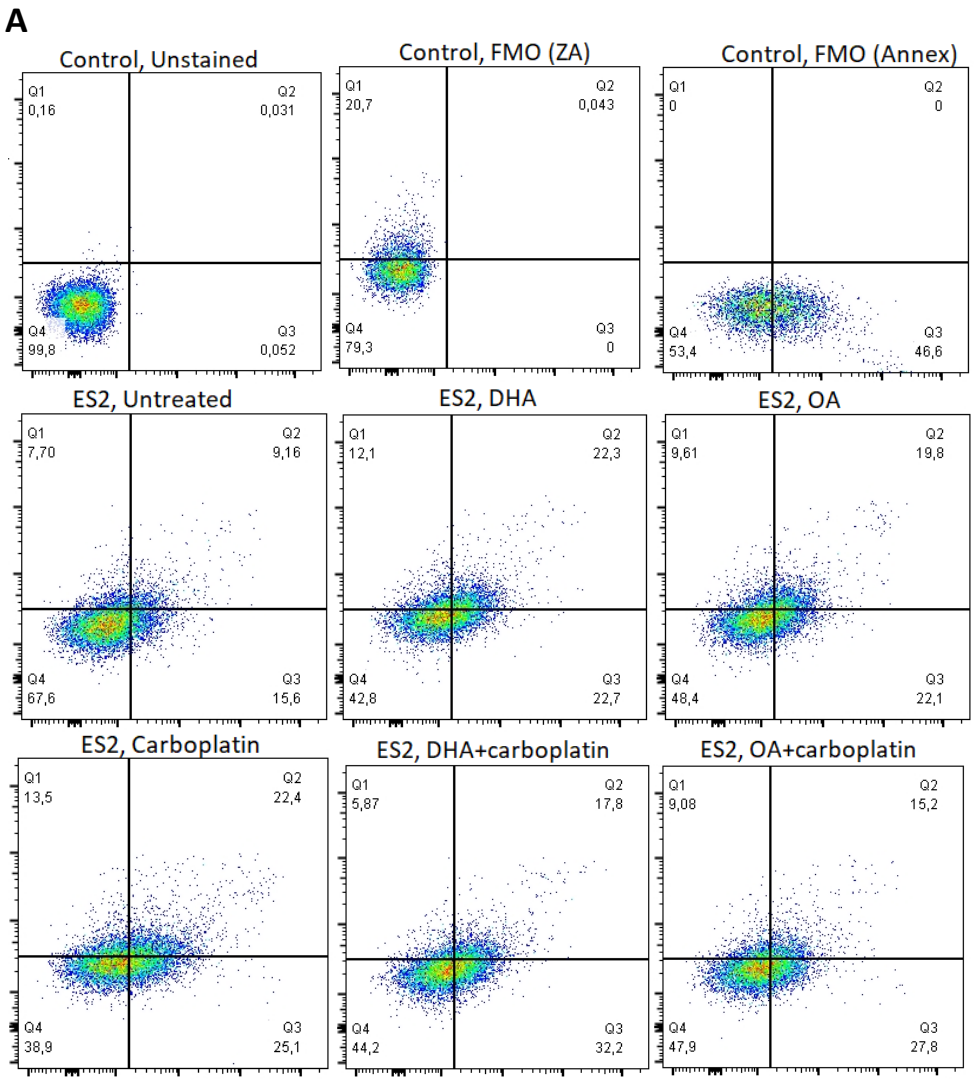
6.2 DHA does not affect apoptosis in ES2 ovarian cancer cells

Annexin V/ZA apoptosis assay:

To further define the mechanism of cell death in ovarian cancer cells, we extended our experiment to examine apoptosis by flow cytometry using Annexin V / Zombie Aqua (ZA) staining. Es2 cells were treated with 10 μM of fatty acids (DHA and OA) alone and in combination with 10 μM of carboplatin for 72 hours. Early apoptosis which is represented by the Ann V+/ ZA– cells was evaluated. Figure 3-14, Panel (A), shows a representative example of flow cytometry analysis. As it is shown in panel (B), we found that the average percentage of ES2 cells in early apoptosis was 20.9% in control group, 27.4% in DHA, 27.2% in OA, 29.4% in carboplatin, 33.7% in DHA+carboplatin and 29.5% in OA+carboplatin treatment. The differences were not statistically significant. The 20% of early apoptosis in the untreated ES2 cells may be due to the short doubling time of the cells which was about 19 hours.

Western blot analysis of apoptosis protein expression:

To detect the effects of fatty acids (DHA and OA) on protein expression associated with apoptosis, we evaluated protein expression of markers of apoptosis using ES2 cancer cells treated with 23 μM of DHA (for 24, 48 and 72 hours of treatment) and 23 μM of OA (for 24, 48 and 72 hours). As shown in Figure 3-15, the level of expression of caspase-9, cleaved caspase-9, caspase-3, cleaved caspase-3, caspase-7, PARP, and anti-FADD was not significantly different between our control and treatment groups (the expression of cleaved caspase-7 and cleaved PARP was not detected).



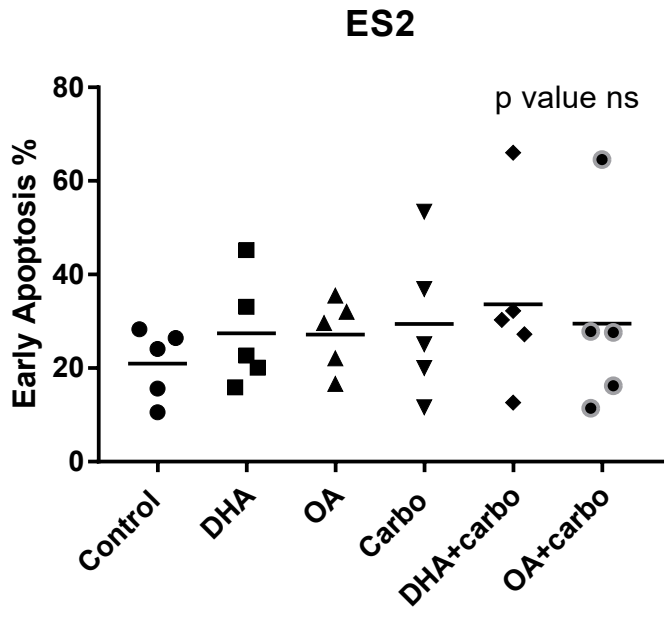


Figure 3-14: Effect of fatty acid treatment on induction of apoptosis both alone and in combination with carboplatin. For the experiment shown in **panel (A)**, the cells were treated with IC25 of DHA and the matched dose of OA as well as IC25 of carboplatin. Cells were treated for 72h and stained with Zombie Aqua (ZA) and Annexin V (Ann V). Early apoptotic cells are annexin V positive/ZA negative, which are the cells on the bottom right quadrant. Positive cells for ZA demonstrate dead cells**. Panel (B)** compiled results for five independent experiments focusing on early apoptosis. P value was not significant.

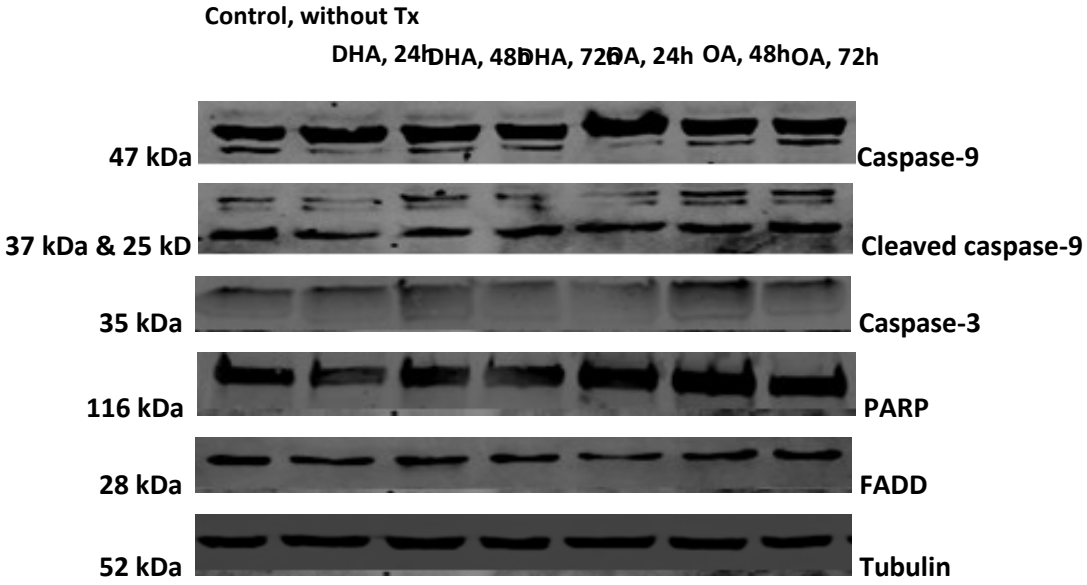


Figure 3-15: The effect of DHA and OA on the expression of proteins associated with apoptosis. The cells were treated with 27 μM of DHA and OA for 0, 24, 48 and 72 hours. Western blot analysis was performed using antibodies against caspase-9, cleaved caspase-9, caspase-3, cleaved caspase-3, PARP, cleaved PARP, and FADD. Tubulin was used as the reference gene. No significant band was detected for cleaved caspase-3 and cleaved PARP.

RT-PCR analysis of gene expression of the apoptosis pathway:

An RT-PCR array was then used in order to determine whether the expression of genes related to apoptosis may be affected by treatment with fatty acids. The gene expression array kit evaluated the expression of 92 different genes in the apoptosis and related pathways and the results are shown in the Figure 3-16 (A). In ES2 ovarian cancer cells, DHA, did not induce a conclusive pattern of gene expression alterations. Figure 3-16 panel (B) illustrates the simplified apoptosis pathway and the effects of DHA on that pathway based on the RT-PCR results.

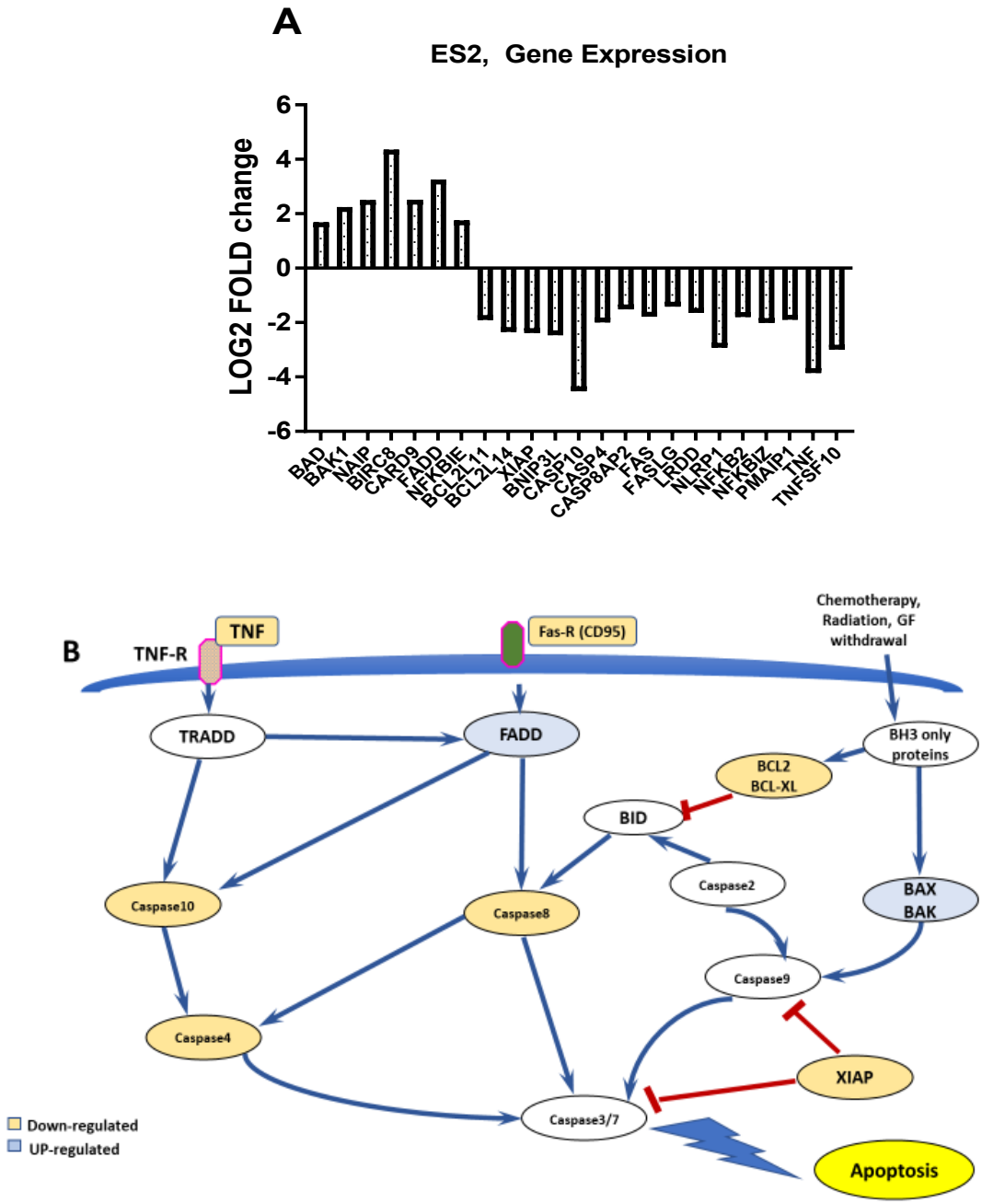


Figure 3-16: The effect of DHA on the expression of genes associated with apoptosis (A). ES2 cells were treated without and with DHA for 16 hours. RT-PCR analysis was performed to assess the relative expression of 92 genes which have a role in the apoptosis pathway, by Human apoptosis gene array kit. The fold change of gene expression was calculated by LOG 2 fold change and the results of >1.5 fold change, is shown in the graph A. **Panel (B)** shows a simplified apoptosis pathway diagram and our results in panel (A) are applied in order to find a relation between the results.

7. DHA-rich environment suppresses in vivo tumor growth

To conduct a more precise preclinical assessment of the efficacy of DHA in treating ovarian tumors, we took advantage of a human HGSC-PDX model (PDX-550, untreated tumor with 70% sensitivity to carboplatin), and evaluated the effect of DHA treatment on the *in vivo* growth of a PDX-ovarian cancer model engrafted subcutaneously in immunocompromised mice. Engrafted PDX tumors were grown for two weeks before treatment was initiated and then divided into four groups and treated with regular vs DHA-enriched diet without and with intraperitoneal carboplatin, 40mg/kg every 3 days (n=6 in control diet, n=7 in control diet+carboplatin, n=6 in DHA-enriched diet and n=7 DHA diet+carboplatin). Mice were monitored for body weight twice weekly (Figure 3-17). There was not a significant difference in the body weight of mice between the four treatment groups.

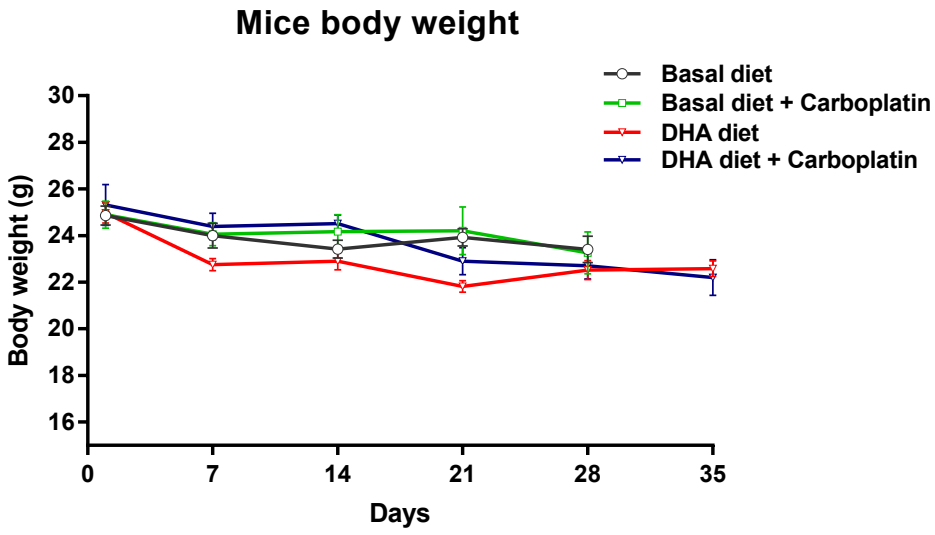


Figure 3-17: DHA, alone and in combination with carboplatin has no effect on the PDX-engrafted mice body weight. DHA enriched diet alone and in combination with carboplatin had no effect on HGSOC PDX-engrafted mice body weight compared to basal diet alone or in combination with the drug. Mice were fed with basal or DHA enriched diet for two weeks prior to weight-based treatment with carboplatin (40mg/kg every 3 days IP) and were kept on the same diet during chemotherapy. Body weight was not significantly altered by DHA treatment (by ANOVA). Error bars are mean ± SEM.

Figure 3-18 illustrates the growth curve of HGSC PDX engrafted tumors. In about 35 days of follow up, DHA enriched diet and DHA diet+carboplatin significantly reduced the growth of tumors compared to control diet and control diet+carboplatin, respectively. The differences between the slopes of tumor growth lines were statistically significant (DHA alone vs. basal diet F=10.46, p=0.02; DHA plus carboplatin vs. basal diet plus carboplatin F=4.48, p=0.03).

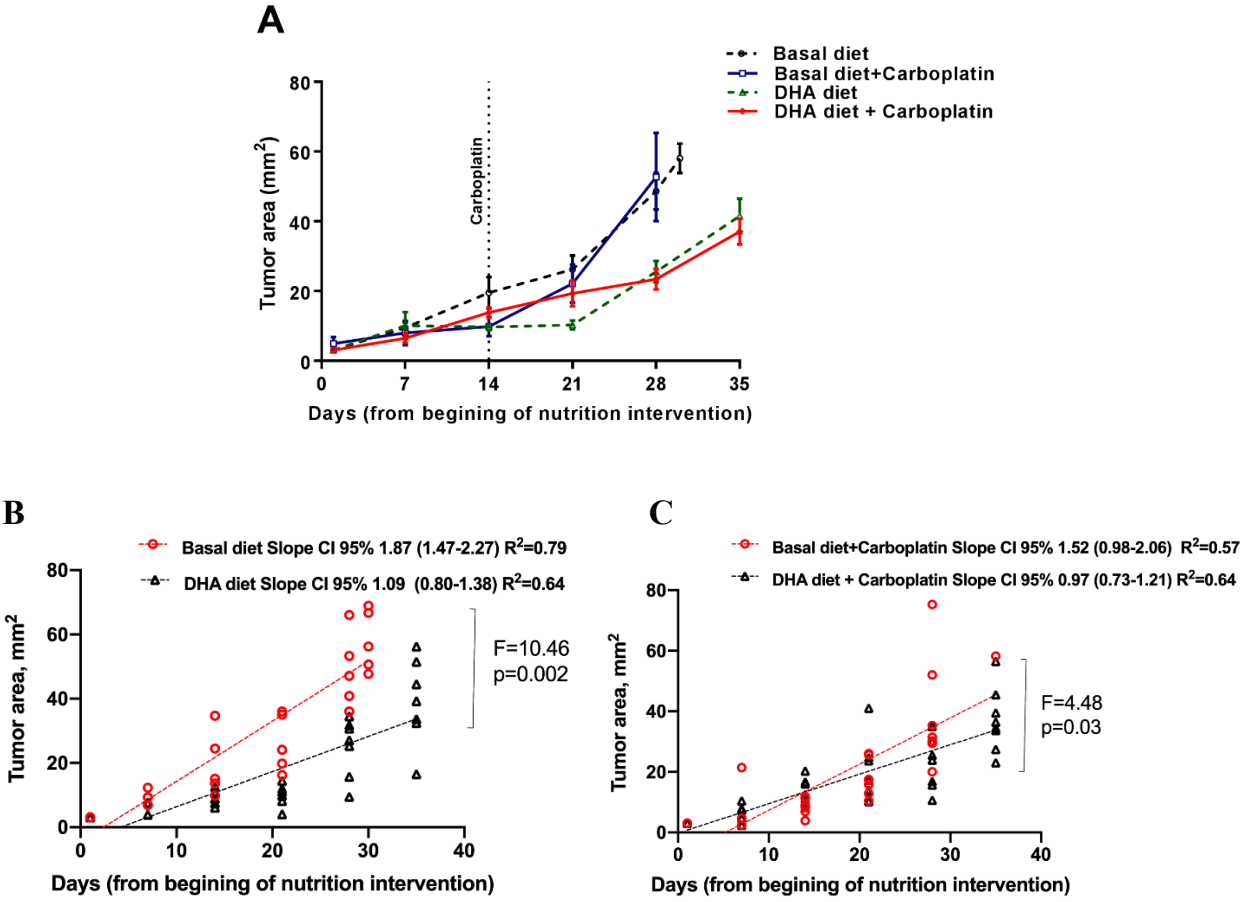


Figure 3-18. The growth of HGSOC PDX model engrafted subcutaneously during dietary supplementation in combination with standard-of-care carboplatin therapy (A). DHA enriched diet alone and in combination with carboplatin (B & C) significantly reduced the tumor growth of HGSOC PDX model compared to basal diet alone or basal diet in combination with the drug.
Mice were fed with basal or DHA enriched diet for two weeks prior to treatment with carboplatin (40mg/kg every 3 days IP) and were kept on the same diet during chemotherapy. The tumor area was calculated as an area of an ellipse. Data represent the linear regression model with each replicate.

8. DHA induced necrosis and reduced proliferation within the HGSOC PDX model:

To further evaluate the effect of DHA on HGSC tumors in PDX models, resected tumors from euthanized animals were formalin fixed and paraffin embedded and sectioned for analysis. H&E staining, IHC analysis, and TUNEL assays were performed.

Examination of the H&E sections confirmed that HGSC tumors had formed in all cases. Interestingly, we noted that the area of necrosis relative to the whole tumor area was higher in DHA combination therapy with carboplatin compared to carboplatin treatment alone (Figure 3-19). The IHC analysis with specific antibodies showed that the levels of the proliferation marker, Ki67, was markedly decreased in tumors treated with DHA and DHA+carboplatin compared to control diet and control diet+carboplatin, respectively (Figure 3-20). On average, about 30% of tumor cells stained positive for Ki67 when treated with DHA or the combination of DHA and carboplatin compared to 40% and 35% for control diet and control diet plus carboplatin, respectively.

To extend our *in vitro* findings, TUNEL staining was done to evaluate early apoptosis in the tumor cells. The nuclei percentage of nuclei positive for TUNEL was almost the same in all treatment groups with a range of 1.7% to 2% (Figure 3-21). These data indicated that DHA alone and in combination to carboplatin inhibited PDX tumor growth through inhibition of proliferation signaling and induction of necrosis and that apoptosis was not different at the time of sacrifice.

In order to evaluate the expression of CD95 death receptor after treatment with DHA and carboplatin, formalin fixed and paraffin embedded tumor sections were IHC stained with anti-CD95 antibody (Figure 3-22, Panel A). There were no significant differences in the expression of the death receptor in the four treatment groups. In order to show the effects of DHA treatment on CD95 antigen expression *in vitro,* the ES2 cells were treated with 10 μM of DHA alone and in

combination with 10 μM of carboplatin for 72h and the expression of CD95 antigen was evaluated by immunofluorescent staining (Figure 3-22, Panel B). Like *in vivo* results, treatment with DHA did not alter the expression of CD95 in ES2 cells

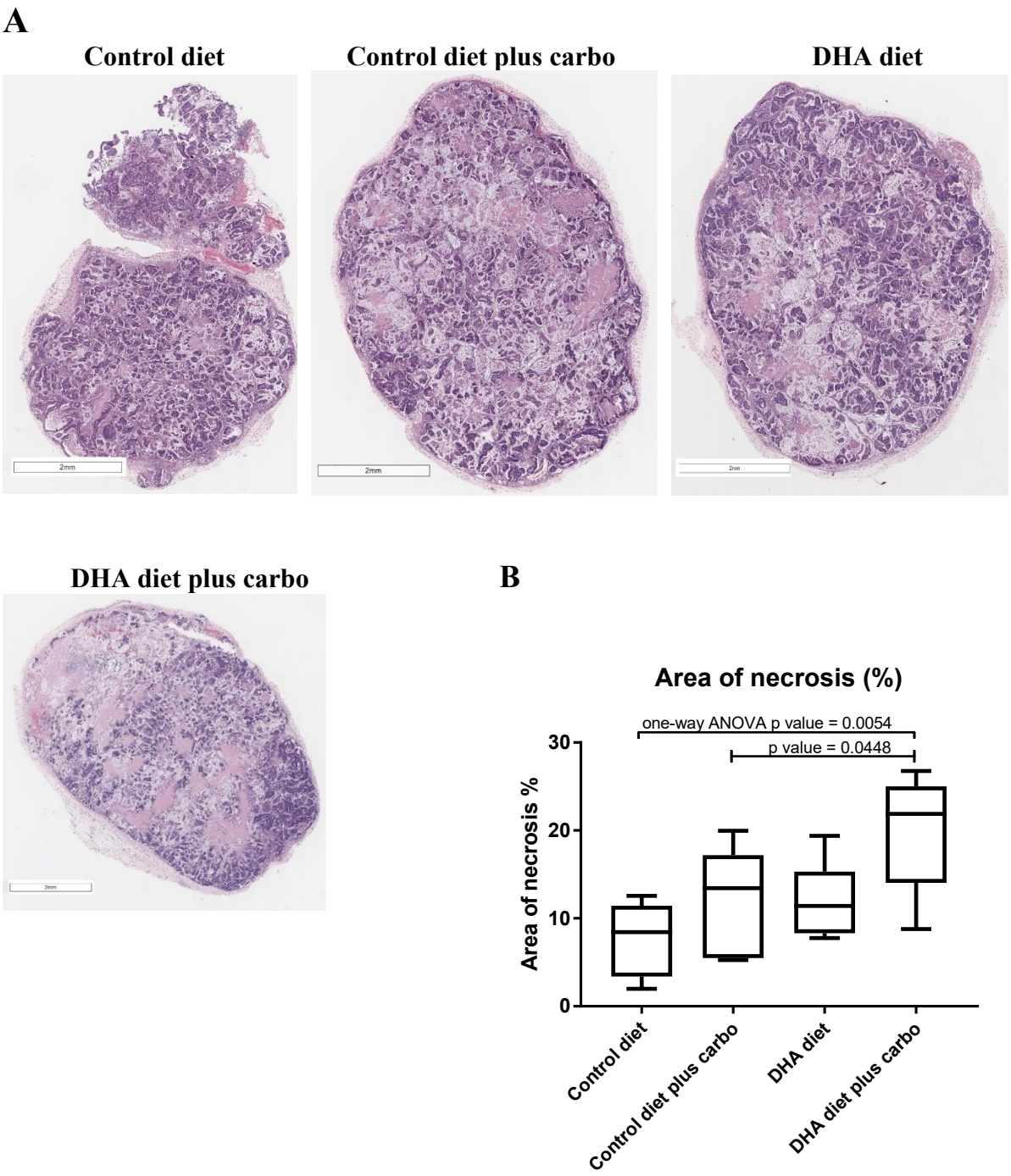
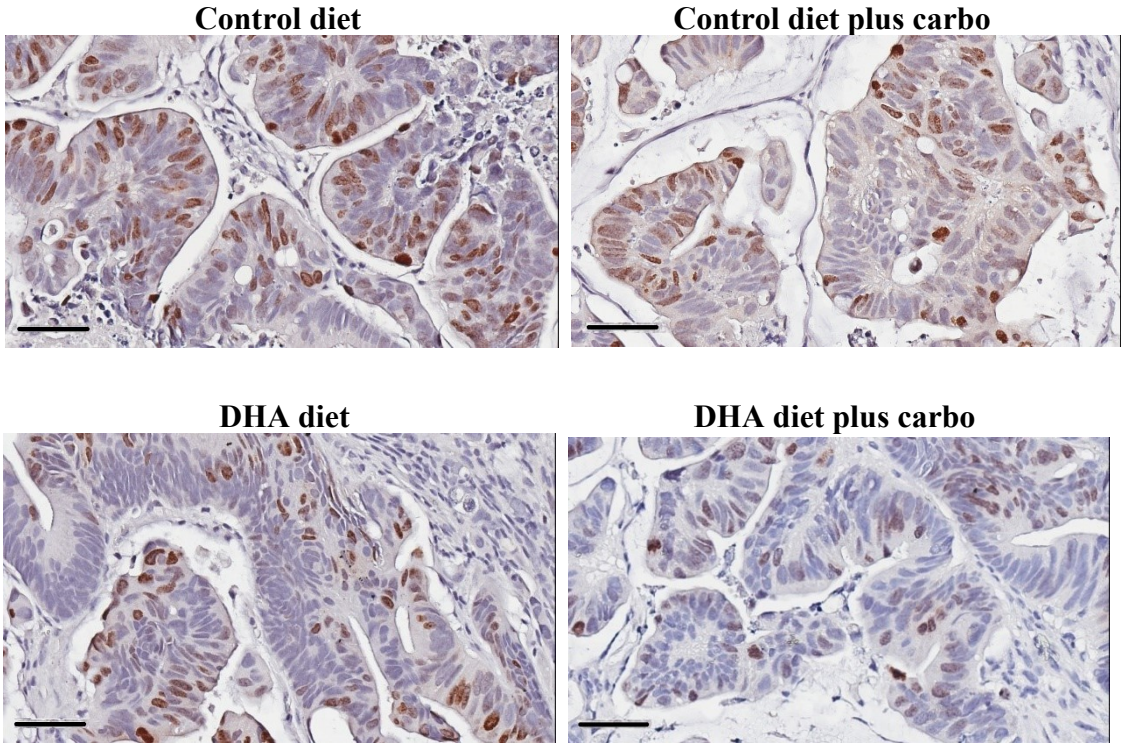


Figure 3-19: DHA induces tumor necrosis in Human HGSC-PDX model.
Mice were fed with basal or DHA enriched diet for two weeks prior to weight-based treatment with carboplatin (40mg/kg every 3 days IP) and were kept on the same diet during chemotherapy.
Panel (A)displays the representative H&E staining whole slide images of the four treatment groups. The size bars indicate 2mm. **Panel (B):** quantitative analysis of the necrosis area in all treatment groups. (p-value between every 2 groups was calculated by unpaired t-test). $p<0.05$ is significant.

A



B

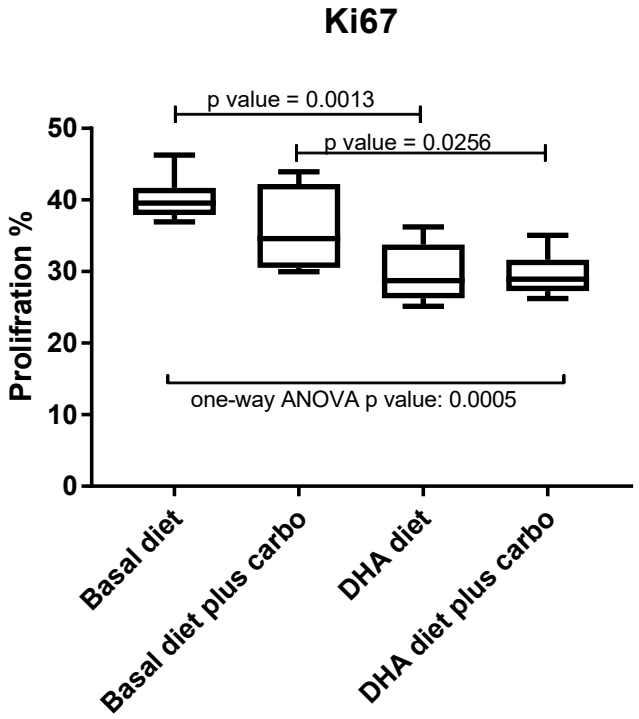
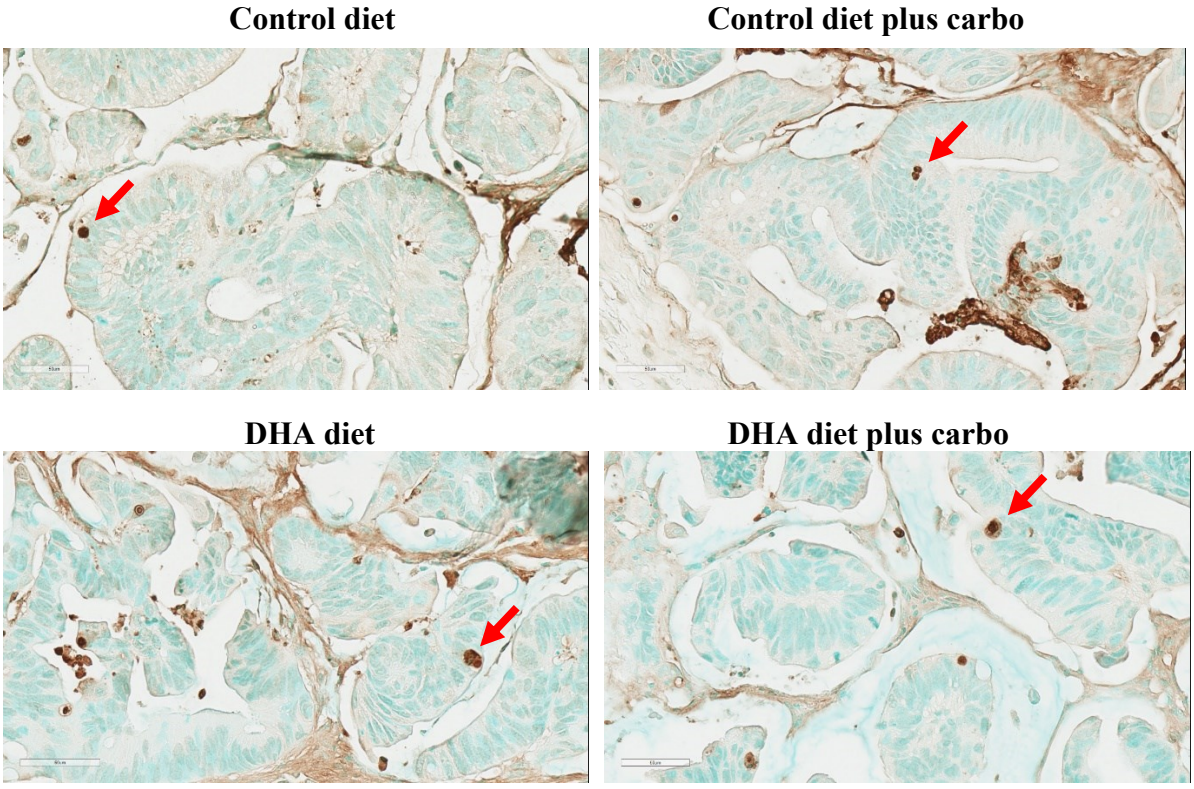


Figure 3-20: DHA reduces expression of Ki67 in Human HGSC-PDX model. Mice were fed with basal or DHA enriched diet for two weeks prior to weight-based treatment with carboplatin (40mg/kg every 3 days IP) and were kept on the same diet during chemotherapy.
Panel (A) displays the representative Ki67 IHC staining images of the four treatment groups. The size bars indicate 50 µm. **Panel (B):** quantitative analysis of the positive nuclei for Ki67 in all treatment groups (p-value between each 2 groups was calculated by unpaired t-test).

A



B

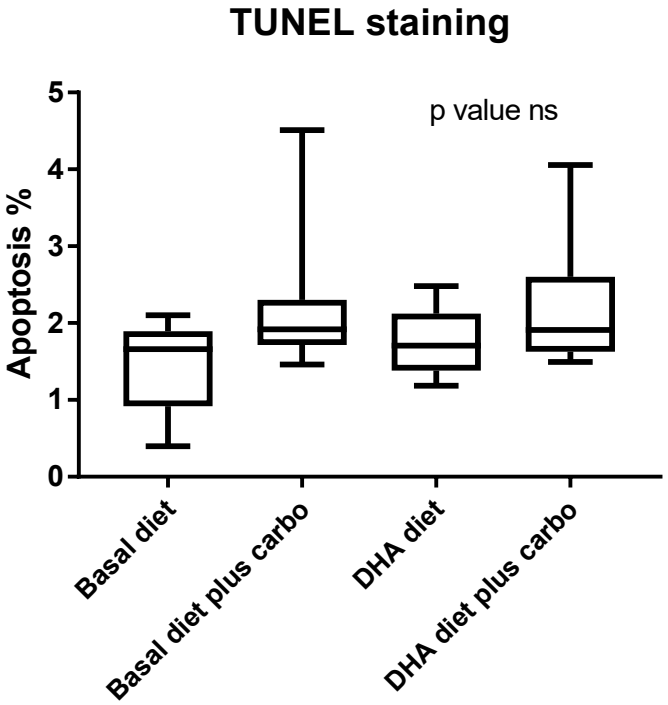
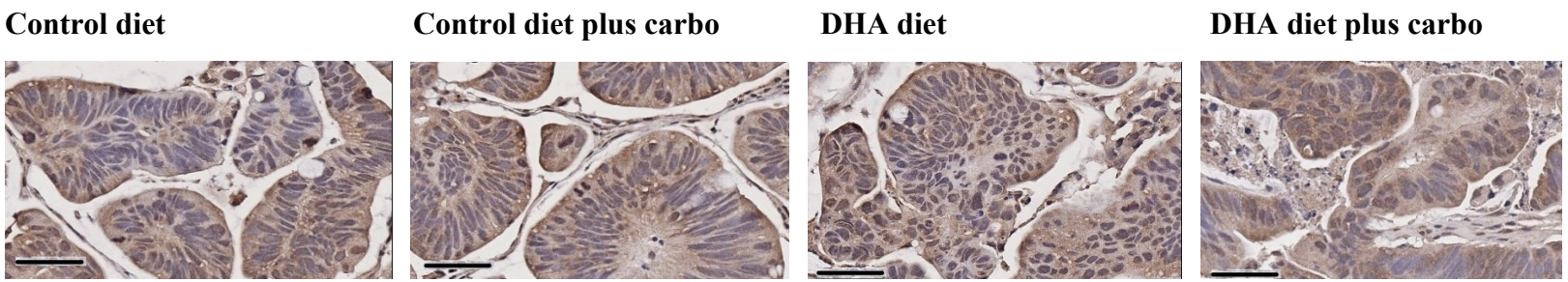


Figure 3-21: DHA does not affect tumor apoptosis in Human HGSC-PDX model.
Mice were fed with basal or DHA enriched diet for two weeks prior to weight-based treatment with carboplatin (40mg/kg every 3 days IP) and were kept on the same diet during chemotherapy.
Panel (A) displays the representative TUNEL staining images of the four treatment groups and the red arrows show the positive cells. The size bars on the lower left side of images indicate 50 µm.
Panel (B): quantitative analysis of the positive nuclei for TUNEL in all treatment groups.

A



B

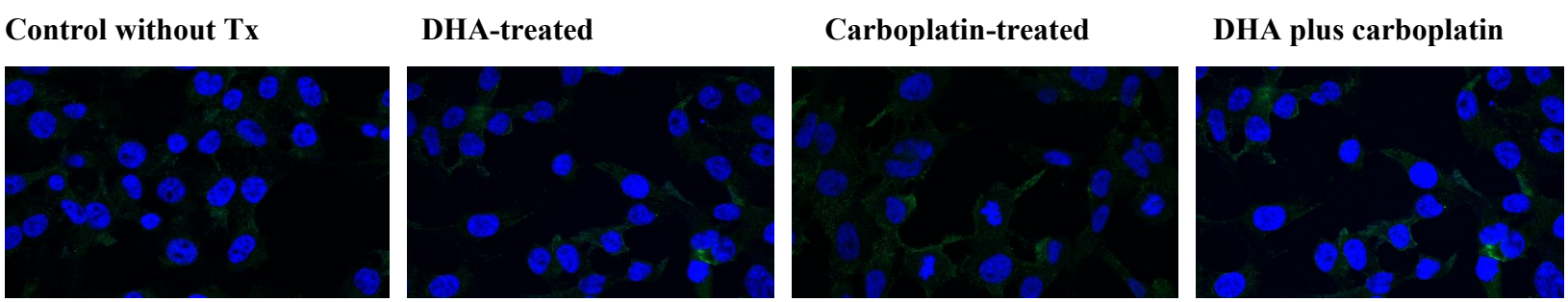


Figure 3-22 DHA does not affect the expression of CD95 antigen both *in vivo* and *in vitro*.
In **panel A** (*in vivo*), the treatment schedule consisted of control vs DHA-enriched diet without and with a combination of carboplatin (Mice were fed with basal or DHA enriched diet for two weeks prior to weight-based treatment with carboplatin (40mg/kg every 3 days IP) and were kept on the same diet during chemotherapy. The expression of CD95 antigen was evaluated in the four treatment groups. There was no gross significant difference in the expression of CD95 in the treatment vs the control groups. The size bars indicate 50 µm. In **panel B** (*in vitro*), ES2 ovarian cancer cells were treated with DHA and carboplatin, alone and in combination, for 72 hours to evaluate the expression of CD95 by immunofluorescent staining and confocal microscope. There was no significant difference in the expression of CD95 antigen in the treatment groups compared to control.

9. Effect of DHA treatment alone and in combination with carboplatin on fatty acid contents of whole cell phospholipids within the HGSOC PDX tumors and mice liver tissue:

To further evaluate the incorporation of DHA in HGSC tumors in PDX models as well as in livers, a small piece of the resected tumors and livers from euthanized animals were sent for fatty acids profile analysis and the results are shown in Tables 3-5 and 3-6.

The fatty acid profiles for whole cell PL were also analyzed to compare the following:

Saturated fatty acid content (∑SFA), mono-unsaturated fatty acids (∑MUFA), polyunsaturated fatty acids (∑PUFA), ∑N-3 PUFAs and ∑N-6 PUFAs.

The effect of fatty acid treatments on the alteration of cellular fatty acid components is summarized in Figures 3-23 and 3-24.

.

Table 3-5: Change in selected fatty acids of whole cell PLs in HGSC PDX-550 ovarian cancer model treated with control vs DHA diet ± carboplatin (% w/w of total fatty acids).

PDX-550 tumor fatty acid composition				
Fatty acids	**Control diet**	**Control diet + carbo**	**DHA enriched diet**	**DHA diet + carbo**
C18:1 n9 (OA)	11.15±8.44	9.62±8.12	8.14±7.37	8.32±8.35
C18:2 n6 (LA)	14.92±1.65	14.82±1.37	14.65±1.59	15.53±1.16
C20:4 n6 (AA)	12.48±1.14	12.80±1.78	**8.13±1.25*** **	**9.33±0.52*** **
C20:5 n3 (EPA)	0.15±0.00	0.19±0.03	0.82±0.20	0.99±0.15
C22:5 n3 (DPA)	0.20±0.07	0.19±0.06	0.25±0.11	0.23±0.06
C22:6 n3 (DHA)	2.96±0.54	3.07±0.94	**4.62±1.38***	**4.80±1.18****
∑SFA	49.63±7.62	51.72±8.49	54.36±7.96	53.11±8.45
∑MUFA	14.73±8.00	12.51±7.59	12.28±6.68	11.27±8.24
∑PUFA	34.46±2.66	34.51±3.39	32.06±3.79	34.50±2.05
∑N3	4.15±0.42	4.28±0.83	**6.64±1.55****	**6.94±1.22*** **
∑N6	30.30±2.43	30.22±2.74	**25.41±2.42****	**27.55±1.47***

Values are mean percent composition ± SD (n=6 in control diet, n=7 in control diet+carboplatin, n=6 in DHA-enriched diet and n=7 DHA diet+carboplatin); "OA"= oleic acid; "LA"= linoleic acid; "AA"= arachidonic acid; "EPA"=eicosapentaenoic acid; "DPA"= docosapentanoic acid; "DHA"= docosahexanoic acid; "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each FA identified, **bold*** indicate significant difference compared to control (without treatment). "*" = p value<0.05; "**" = p value<0.01; and "***" = p value<0.001.

Table 3-6: Change in selected fatty acids of whole cell PLs in the immunocompromised mice liver treated with control vs DHA diet ± carboplatin (% w/w of total fatty acids).

Mice liver fatty acid composition				
Fatty acids	**Control diet**	**Control diet + carbo**	**DHA enriched diet**	**DHA diet + carbo**
C18:1 n9 (OA)	11.62±3.76	11.83±2.88	8.99±0.73	9.66±0.73
C18:2 n6 (LA)	12.98±0.90	13.62±0.65	13.49±0.22	12.18±0.59
C20:4 n6 (AA)	19.10±1.17	17.90±1.81	**11.99±0.27*** **	**13.35±2.81***
C20:5 n3 (EPA)	0.09±0.03	0.12±0.01	**1.48±0.08*** **	**1.32±0.17*** **
C22:5 n3 (DPA)	0.39±0.03	0.39±0.02	0.39±0.05	0.43±0.09
C22:6 n3 (DHA)	11.42±1.64	12.27±0.92	**17.31±0.55** **	**17.07±2.88***
∑SFA	41.20±1.37	40.55±1.25	43.32±0.61	43.12±0.62
∑MUFA	12.05±3.73	12.28±2.84	9.52±0.75	10.09±0.73
∑PUFA	46.50±2.32	46.91±1.84	47.06±0.30	46.66±0.52
∑N3	12.26±1.73	13.12±0.92	**19.48±0.57*** **	**19.11±2.79***
∑N6	34.23±1.58	33.79±2.74	**27.57±0.46*** **	**27.54±2.82***

Values are mean percent composition ± SD (n=4); "OA"= oleic acid; "LA"= linoleic acid; "AA"= arachidonic acid; "EPA"=eicosapentaenoic acid; "DPA"= docosapentanoic acid; "DHA"= docosahexaenoic acid; "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each FA identified, **bold*** indicate significant difference compared to control (without treatment). "*" = p value<0.05; "**" = p value<0.01; and "***" = p value<0.001.

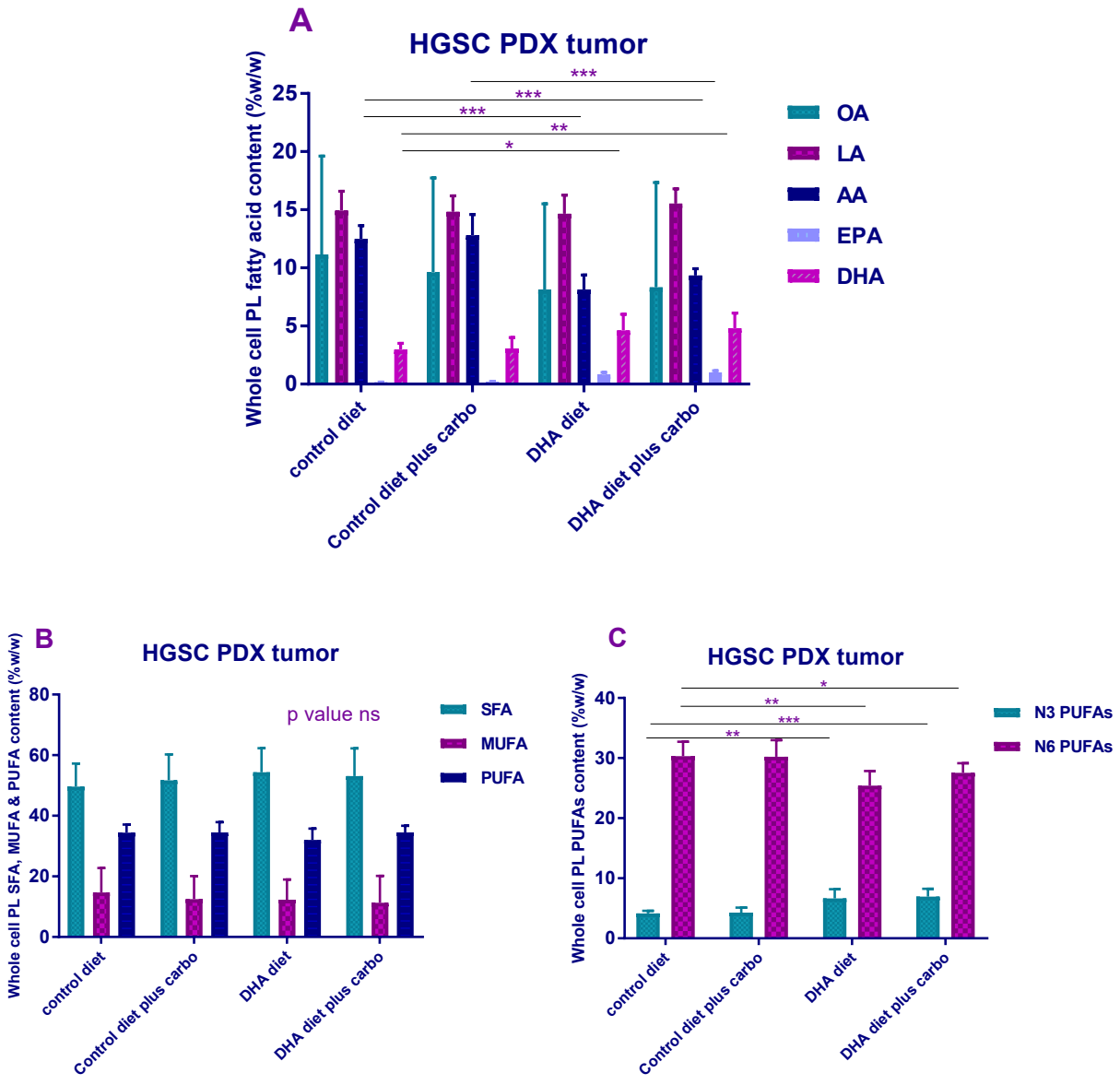


Figure 3-23: Effect of DHA diet with and without carboplatin in HGSC-PDX tumor model on whole cell total PL content (%w/w); (A) single fatty acids; (B) SFA, MUFA and PUFA; and (C) total n3 and n6 PUFAs. Bars represent the mean ± SD (n=6 in control diet, n=7 in control diet+carboplatin, n=6 in DHA-enriched diet and n=7 DHA diet+carboplatin). Differences in fatty acid content with each treatment relative to control were tested using a t-test. "OA"= oleic acid; "LA"= linoleic acid; "AA"= arachidonic acid; "EPA"=eicosapentaenoic acid; "DHA"= docosahexaenoic acid; "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. "*" = p value<0.05; "**" = p value<0.01; and "***" = p value<0.001.

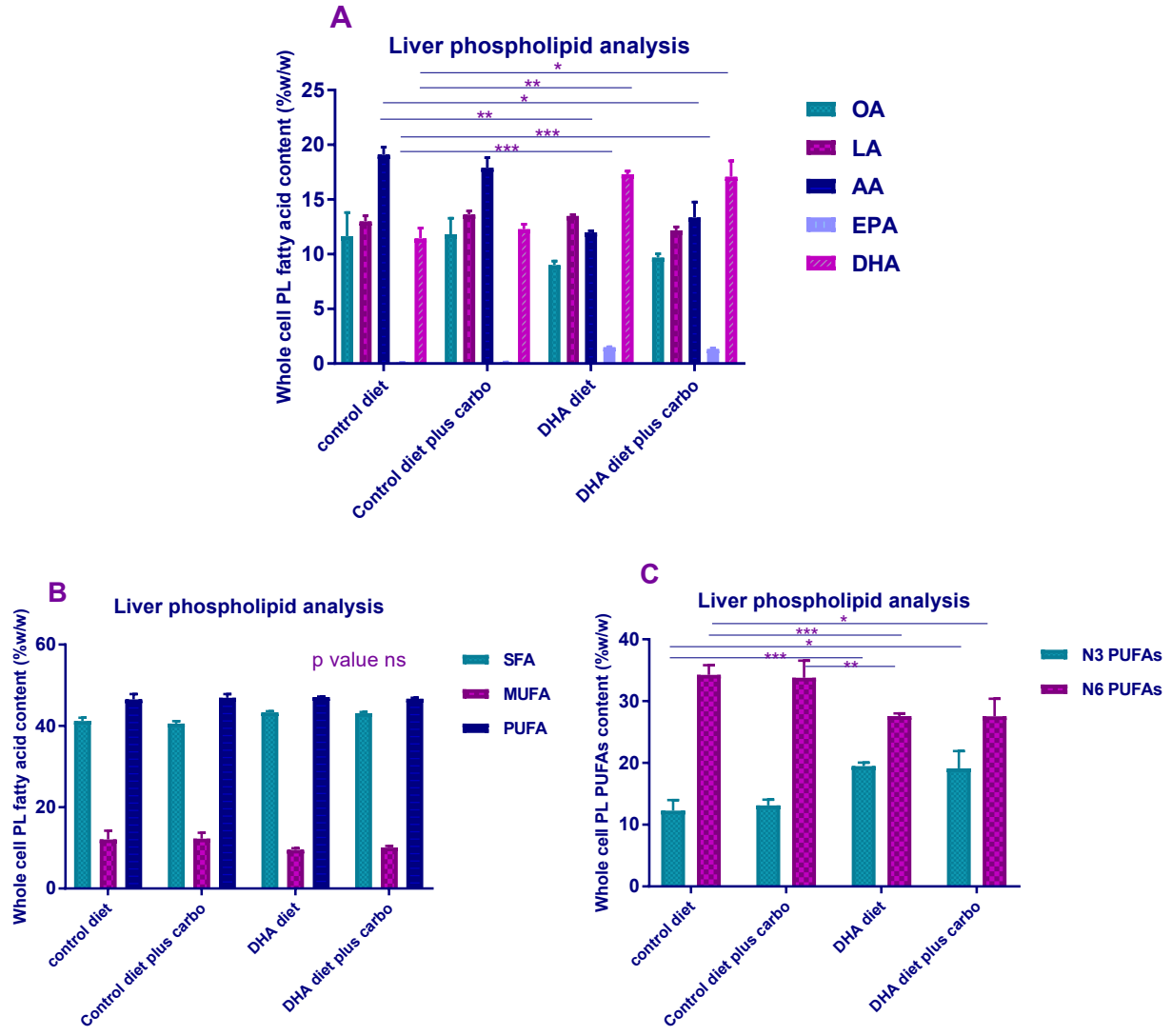


Figure 3-24: Effect of DHA diet with and without carboplatin on whole cell total PL content of mice liver (%w/w); (A) single fatty acids; (B) SFA, MUFA, and PUFA; and (C) total n3 and n6 PUFAs. Bars represent the mean ± SD (n=6 in control diet, n=7 in control diet+carboplatin, n=6 in DHA-enriched diet and n=7 DHA diet+carboplatin). Differences in fatty acid content with each treatment relative to control were tested using a t-test. "OA"= oleic acid; "LA"= linoleic acid; "AA"= arachidonic acid; "EPA"=eicosapentaenoic acid; "DHA"= docosahexaenoic acid; "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. "*" = p value<0.05; "**" = p value<0.01; and "***" = p value<0.001.

When the PDX tumors were treated with DHA alone and in combination with carboplatin, there was a significant decrease in AA level in the whole tumor phospholipid analysis. At the same time,

DHA is effectively incorporated in the tumor cell membrane as the level of DHA significantly increased after treatment (Table 3-5 and Figure 3-23). We also showed that treatment with DHA increased the whole level of n-3 PUFAs and decreased the level of n-6 PUFAs in the tumor tissue. All these changes are in favor of reduction of inflammation as well as DHA incorporation into the tumor cell membrane.

In order to compare the incorporation of DHA in tumor tissue with the normal tissue, and to ensure uptake through the digestive system, we also evaluated the whole cell phospholipid composition of the mice livers. As it is shown in Table 3-6 and Figure 3-24, liver tissue followed the same pattern of phospholipid alterations as in tumor tissue.

Treatment with DHA increased the level of DHA and n-3 PUFAs and decreased the level of AA and n-6 PUFAs in the liver tissue in comparison to without DHA. It may be concluded that tumor tissue uptakes DHA at the same rate and efficacy as other normal tissues such as the liver. Additionally, DHA can effectively reduce inflammatory mediators such as AA in the tumor and liver cells.

Chapter 4

Discussion

Chapter four- Discussion

As most ovarian cancer patients relapse with the chemo-resistant disease, further advances in cancer research are needed to improve treatment outcome. There is a strong rationale for studying nutritional interventions, such as PUFAs, in cancer prevention and therapy. Although several studies have shown the correlation of PUFAs with cancer progression and treatment, the exact patho-chemical interactions of n-3 PUFAs and n-6 PUFAs with the tumor tissues are not clear. The primary purpose of this study was to investigate the potential anti-cancer effects of fatty acids on ovarian cancer cells *in vitro*. Rather than testing single therapy, we illustrated that DHA treatment increased the sensitivity of the cells to carboplatin. Few studies have tested the anti-cancer effects of DHA in combination with chemotherapy in ovarian cancer models, and this is the significance of our study. As the next objective, we attempted to elucidate potential mechanisms by which n-3 PUFAs achieve their effects, and as the last goal, we conducted an *in vivo* study with a human PDX ovarian tumor model transplanted into immune-compromised mice to investigate if the *in vitro* findings were applicable *in vivo* as well.

1. DHA exerts cytotoxic effects on ovarian cancer cell lines but is not toxic in immortalized ovarian and fallopian tube cells.

As the first step, to evaluate the anti-proliferative effects of fatty acids on non-malignant cells, we selected two immortalized ovarian surface epithelium (IOSE) and fallopian tube (FT194) cell lines. As shown in Figure 3-1, fatty acids did not reduce the cellular growth and viability of the normal cells. This was a favorable finding because a therapeutic intervention that could enhance the effects of chemotherapy and as a result, improve the clinical outcome of the patients without causing additional side effects on non-tumoral cells, would be clinically advantageous.

Although ovarian cancer is designated as a single disease, it is characterized by clinical, pathological and biological heterogeneity. For this reason, we decided to test if the effects of DHA on ovarian cancer cells is limited to a specific subtype. Thus, we selected cell lines from different pathologic subtypes of ovarian cancer and evaluated the cytotoxic effects of fatty acids on the cells. We found out that between all selected cell lines, just ES2 (clear cell carcinoma) and A2780cp (endometrioid carcinoma) cells were susceptible to the cytotoxic effects of DHA (Figure 3-3). The ES2 line was the most sensitive to DHA. Therefore, we questioned if the effect of DHA is histotype-dependent. To examine that, we evaluated two more clear cell carcinoma cell lines (OCI-C1P and OCI-C4P), but those cell lines were completely resistant to the cytotoxic effects of DHA, and DHA increased the cell viability in both cells (Figure 3-4). Amongst the other cell lines, OV90 (HGSC), SKOV3 (serous carcinoma), and Kuramochi (HGSC) were all resistant to DHA as shown in Figure 3-4. It seems that the cytotoxic effect of DHA is not histotype-specific and there are some other determinants which should be considered in the prediction of sensitivity but are not yet known.

Previous studies have demonstrated the effects of n-3 PUFAs on ovarian cancer cells both *in vitro* and *in vivo*. Tanaka *et al.* (178) examined the effects of n-3 and n-6 PUFAs on cell death in KF28 and HAC2 ovarian cancer cell lines. They found that KF28 cell line was susceptible to both n-3 and n-6 PUFAs but that HAC2 was resistant. Some other cell lines such as TOV-21G, SKOV3, OVCAR3, A2780, and HO8910 were found to be sensitive to different n-3 PUFAs in a dose and time-dependent manner (175, 176, and 179).

This effect is not limited to ovarian cancer cells. Indeed, n-3 PUFAs anti-proliferative effects are highlighted in hormone-dependent malignancies such as breast cancer (116, 117). Ewaschuk *et al.*

(182), showed that both DHA and EPA inhibited the cell growth of MDA-MB-231, MCF-7, and SKBr-3 breast cancer cell lines. They also concluded that pre-treatment of the estrogen receptor negative MDA-MB-231 cells with DHA could increase the effect of anti-cancer drugs. In many types of cancers such as prostate cancer (188), colorectal cancer (146), esophageal cancer (189), gastric carcinoma (190), and hepatocellular carcinoma (191), n-3 PUFAs showed anti-cancer effects. The common point of all these studies is that n-3 PUFAs cause growth inhibition in a dose-, time-, and cell line-dependent manner.

2. DHA enhances the carboplatin cytotoxicity

In spite of the new modalities in the ovarian cancer treatment, the overall survival rate and the quality of life of the patients are still poor (4). Carboplatin as an anti-cancer agent is the first-line chemotherapy which has broad activity in ovarian cancer; however, the major disadvantage of carboplatin is resistance and its low therapeutic response in advanced cancers (63). Improving the efficacy of chemotherapy in ovarian cancer is an urgent need for increasing the overall survival rate and quality of life for the patients. Therefore, the development of new treatment modalities for ovarian cancer is urgently needed.

n-3 PUFAs have consistently been shown to improve the sensitivity of various tumors to chemotherapy both *in vitro* and *in vivo*. The present study investigated the therapeutic potential of DHA as an adjuvant for carboplatin in ovarian cancer. We showed that in some ovarian cancer cell lines, n-3 PUFAs increased the cytotoxic effects of chemotherapy. As illustrated in Figure 3-5, in ES2 and A2780cp cell lines which were sensitive to DHA alone, combination therapy with DHA and the IC_{50} of carboplatin reduces the cell viability significantly compared to carboplatin alone.

Interestingly, in SKOV3, and Kuramochi cell lines, which both were resistant to fatty acids alone, a combination of DHA and carboplatin reduced the cell viability significantly (Figure 3-6).

We did not find any relationship between the response to fatty acids (alone and in combination with carboplatin) and p53 mutation status in the different ovarian cancer cell lines.

Additionally, in OCI-C1P cells, DHA reduced the cytotoxic effects of carboplatin significantly. These findings may explain the reason for inconsistency between different studies.

In the two last cell lines, we evaluated the effects of combination therapy in two different ways:

1- As shown in Figure 3-6, cells were treated with different concentrations of fatty acids in combination with the IC_{50} of carboplatin for 72 hours and after the incubation, the metabolic activity of the cells was checked by MTT assay. By this method, we showed a significant decline in the metabolic activity of the cells in combination therapy compared to monotherapy.

2- To validate our MTT results, we treated SKOV3 and Kuramochi cell lines with 160μM of DHA, OA or LA and escalating concentrations of carboplatin (Figure 3-8) for 72 hours and evaluated the cell viability by Trypan Blue assay. We observed a significant decrease in the IC_{50} of carboplatin when combined with DHA. In SKOV3 cells, IC_{50} was 41.9 μM for carboplatin alone, but when combined with DHA, the IC_{50} was reduced to 4.53 μM. The same pattern was observed for Kuramochi cells, as the IC_{50} reduced from 39.11 μM to 5.17 μM in carboplatin and combination therapy, respectively.

In both SKOV3 and Kuramochi cell lines (Figure 3-8), fatty acids enhanced the cytotoxic effects of carboplatin and this difference was significant at the lower concentrations of carboplatin (less than 100 μM).

Collectively, our results indicate that combination therapy with DHA and carboplatin enhance cell cytotoxicity to carboplatin in some ovarian cancer cell lines.

Several studies have shown an association between n-3 PUFAs and improvement in the efficacy of chemotherapy against cancer. For example, by n-3 PUFAs administration, the efficacy of doxorubicin in lung cancer (192), mitomycin-C in colorectal cancer (193), doxorubicin, vincristine and fludarabine in chronic lymphoid leukemia (194), and 5-fluorouracil in gastric cancer (190), as well as many other anti-cancer drugs, may be enhanced. This effect may be clinically beneficial to the patients, as n-3 PUFAs may provide a better outcome for patients treated with standard therapy. There is an interesting finding to be considered. As shown in Figure 3-6, in SKOV3 and Kuramochi cell lines which were sensitive to the combination of DHA and carboplatin, treatment with OA (a monounsaturated omega-9 fatty acid) similarly reduced cell viability. This suggests that just an unsaturated fatty acid-enriched environment (and not particularly n-3 PUFAs) might enhance the efficacy of anti-cancer drugs in some cell lines, but further investigations are needed before any conclusion can be made.

3. Fatty acids incorporated into the tumor cells and changed the composition of whole cell phospholipids *in vitro*

Incorporation of fatty acids into the cell membrane is an important step in their function as they are able to alter the composition of the cell membrane, disturb the organization of lipid rafts, and modulate different intracellular signaling pathways (103, 104, and 109). In the present study, whole cell PL was examined in ES2, A2780cp, and SKOV3 cell lines after treatment with DHA or OA for 72 hours. As the plasma membrane contains most of the cell PLs, we assumed that whole cell PL is an appropriate representative of plasma membrane composition of fatty acids. If

DHA and OA were successfully incorporated into the plasma cell membrane, it would be expected that a significant change in the level of DHA, OA, ∑N3, and ∑N6 fatty acids would occur following treatment with these fatty acids.

As shown in Table 3-2 and Figure 3-9, in the ES2 cell line, DHA significantly increased the proportion of DHA and ∑N3 PUFAs in the whole cell PLs. Additionally, it increased the concentration of EPA and decreased the total n-6 PUFA levels but the differences were not statistically significant.

In A2780cp cells (Table 3-3 and Figure 3-10), the effect of treatment was even more robust. Treatment with DHA increased the percentage of DHA and ∑N3 PUFAs and decreased the cellular level of OA and MUFAs. However, in the OA-treated cells, the differences in fatty acid composition were not significant.

In the SKOV3 cell line (which was resistant to fatty acids), treatment with DHA and OA did not induce significant changes in the level of whole cell PLs (Table 3-4, and Figure 3-11). Although treatment with DHA increased the level of EPA and DHA in SKOV3 cells, the difference was not statistically significant.

Overall, it seems that DHA incorporated more efficiently in the sensitive cell lines (ES2 and A2780cp).

Effects of fatty acid treatment on AA: In both sensitive cell lines, A2780cp and ES2 cells, AA concentration decreased with DHA treatment. There was a larger decrease in AA level in A2780cp ovarian cancer cells than in ES2. Although in ES2 cells the level of AA decreased by about 50%, the p-value was not significant (p-value ~ 0.06). In the resistant SKOV3 cells, the level of AA did not change (Tables 3-1, 3-2, and 3-3). It is likely that DHA was better able to compete with AA

for incorporation into the cell membranes in the sensitive cell lines and this intrinsic characteristic of the cells might play a role in the growth inhibition and cell death induced by DHA.

The AA pathway has been shown to play a key role in carcinogenesis. AA and its metabolites are essential modulators of signaling pathways which contribute to proliferation, invasion, and angiogenesis (75). DHA can replace AA in the cell membrane and compete for COX and LOX enzymes. As a result, DHA plays an anti-inflammatory role in the cancer cells which may counteract carcinogenesis (77).

Taken together, this finding suggests that the decrease observed in total PL AA may have contributed to a decrease in cell proliferation and an increase in cell death.

4. DHA plays an anti-proliferative role in ES2 cells

As mentioned before, many investigators have demonstrated that n-3 PUFAs have protective effects against breast, prostate, and colon cancer. It is generally accepted that n-3 PUFAs integrate into the cell membranes, produce lipid mediators, induce oxidative stress, and alter membrane conformation and signal transduction. Inhibition of cell proliferation and induction of apoptosis are two major accepted mechanisms of these fatty acids that have been investigated in many studies. In some cancers such as leukemia (194), colon carcinoma (195), and melanoma (196), cell cycle arrest has been shown to be the major mechanism by which n-3 PUFAs impede cancer cell growth. However, in some other types such as pancreatic cancer (197), colon cancer (198), leukemia (199), and breast cancer (109), induction of apoptosis played a more significant role. In our study, we investigated the effects of DHA treatment on both cell proliferation and apoptosis induction.

5. DHA alters the cell cycle distribution of ES2 cells

We performed cell cycle analysis to investigate if treatment with DHA, OA, and LA was associated with cell proliferation inhibition. The influence of DHA, OA and LA treatment on G0/G1, S and G2/M fractions of the cell cycle were assessed by PI staining and flow cytometry. The cell cycle phases were evaluated at 0, 6, 12 and 24 hours of treatment with 28μM of DHA, and the matched doses of OA and LA compared to control without treatment. Figure 3-12 represents the results of the analysis. In DHA-treated cells, the cell cycle changes started after 6 hours of treatment. We observed a significant reduction in the percentage of the cells in G0/G1 phase from 65.3% at 6 hours to 50.2% at 12 hours of treatment. In addition, the population of cells in S-phase significantly increased at 12 hours of treatment, from 10.5% to 25.5% at 6 and 12 hours, respectively. We observed the same pattern of cell cycle changes in LA-treated ES2 cells which was a reduction in G0/G1 phase population from 67.0% at 6 hours to about 55.5% in 24 hours as well as a significant increase in the cell population at S phase after 12 hours of treatment. OA had no effect on the cell cycle phases. We concluded that DHA and OA might induce cell cycle arrest in ES2 cells line.

To evaluate if this finding may be translated to a specific phase arrest, we analyzed the cell cycle distribution at 12 hours of treatment with fatty acids as well as the ratio of G1/S, G1/G2, and G1/(S+G2) in control (without treatment) compared to 12 hours of treatment (the time at which the differences in phases were the greatest compared to control). As shown in Figure 3-12 (Panel D and Table E), although DHA treatment reduced the cell population in the G0/G1 phase and increased the cells in the S-phase, the p-value was not significant. Further studies are needed to show the cell cycle alteration by fatty acid treatment.

Cancer cells are characterized by cell cycle abnormalities which as a result, lead to unregulated growth and proliferation (138). The ability of DHA to alter the cell cycle progression in cancer

cells is still obscure. Many investigators have shown in cell lines and preclinical models that DHA can inhibit proliferation and cell cycle progression in many different types of cancer. In a review done by Newell et al. (141), 21 studies were analyzed to evaluate the cell cycle alterations with DHA treatment in a broad range of cancers. They reported G1 phase arrest in leukemia, colorectal, neuroblastoma and breast cancer, S phase arrest in hepatocellular carcinoma, leukemia, and melanoma, and G2/M phase stall in leukemia, pancreatic, breast, and colorectal cancer as well as a multi-phase stall in four other studies. They also concluded that treatment with DHA inhibits the expression of cyclins and other cell cycle markers. To explain the differences between the studies, they concluded that DHA effects may be due to the molecular properties of each cancer, variability in the conditions of the different experiments such as time, concentration of fatty acids, methods of cell exposure to the fatty acids (free or conjugated with albumin), and synchronization of the cells at the beginning of the experiment. All the mentioned points may be applied to our study as well. Therefore, as a future direction, we plan to understand how DHA interferes with cell cycle and for that, we will analyze the cell cycle progression in synchronized cells to see and compare the progression and duration of each phase after treatment with DHA alone and in combination with chemotherapy. The cell cycle markers such as cyclins and cyclin-dependent kinases will also be analyzed as another step.

DHA treatment reduces markers of proliferation in Western blot: Histone H3 is a nuclear core protein of chromatin. It has an important role in chromosome condensation, and after phosphorylation of serine-10 and serine-28 residues, induces cell cycle progression during mitosis and meiosis (200). Phosphorylation of histone H3 occurs during late G2 phase to early prophase. It is shown that phosphorylated histone H3 is a very sensitive and specific marker of mitosis and

its expression has a direct correlation with outcome in various types of cancer such as meningioma, astrocytoma, and melanoma (201 - 203).

In accordance with the results of viability assays and cell cycle analyses, Western blot analyses revealed that histone H3 phosphorylation was lower in ES2 cells treated with DHA and OA for 24, 48 and 72 hours compared to control without treatment (Figure 3-13) which indicated reduction of mitosis.

Collectively, our results suggest that DHA treatment decreases proliferation; however, this should be evaluated in other cell lines as well in combination with chemotherapy.

6. DHA does not induce apoptosis in ES2 cells

Many studies have shown the apoptotic effect of n-3 PUFAs on cancer cells both *in vitro* and *in vivo*. Incorporation of n-3 PUFAs into the cancer cell membranes is assumed to be the primary step in the induction of apoptosis (116, 147). It is known that many cell signaling pathways are regulated by DHA and its metabolites, and this effect may be through induction of lipid peroxidation (146), inhibition of PI3K/Akt pathway (114), downregulation of BCL2 and BCL-xL, and other mechanisms. To further investigate the mechanism of DHA on ovarian cancer cells, we evaluated apoptosis by Annexin V/ZA flow cytometry assays, Western blotting, and apoptosis gene pathway analyses.

Annexin V/ZA assay by flow cytometry: We treated the ES2 cells with IC_{25} (10 μM) of DHA and the matched dose of OA, without and with IC_{25} (10 μM) of carboplatin for 72 hours and then performed Annexin V/ZA assay to distinguish apoptosis from live and dead cells. As shown in Figure 3-14 although combination therapy induces more apoptosis than single drug treatment, the differences were not significant.

Western blot analysis: To evaluate the effect of DHA on the expression of proteins associated with apoptosis, we treated the ES2 cells with DHA and OA for 0, 24, 48 and 72 hours and assessed the expression of caspase-3, cleaved caspase-3, caspase-9, cleaved caspase-9, PARP, cleaved PARP, and FADD. As it is shown in Figure 3-15 treatment with fatty acids did not change the expression of apoptotic pathway proteins up to 72 hours of treatment. This was further evidence that apoptosis is not a mechanism of cell death in ES2 cells induced by DHA.

Apoptotic gene expression: Apoptosis pathway gene expression was performed by TaqMan human apoptosis array after 16 hours of treatment of ES2 cells with IC_{50} (28μM) of DHA. The array evaluated 92 different genes in the apoptosis pathway. As shown in Figure 3-16 A, we observed multiple alterations in the expression of apoptotic and anti-apoptotic genes. For example, treatment with DHA upregulated the expression of BAD, BAK1, BIRC8 and FADD and downregulated the expression of BCL2L11, BCL2L14, caspase-4, caspase-8, FAS and FAS ligand, TNF and TNFSF10. Some of these changes (such as downregulation of BCL2 and upregulation of FADD, BAD, and BAK1) are in favor of apoptosis and some are against apoptosis (such as the alteration in FAS, FASL, and caspases). Figure 3-16 B illustrates the results of the gene expression array in a diagram to visualize the relation of important genes and their alterations in response to DHA treatment in our study.

Based on the current data, it seems that both categories of apoptosis and anti-apoptosis pathways work to compensate each other, and the net final effect is not a significant induction of apoptosis in ES2 cell line after DHA treatment. Overall, mechanistically, DHA affected the cells by an alteration in the cell cycle phases and did not affect apoptosis in ES2 ovarian cancer cells (in the current study).

7. DHA decreases the tumor growth rate of human PDX ovarian tumor model without any effects on the body weight of mice

To extend our *in vitro* findings, we conducted an animal study with human HGSC-PDX tumors engrafted in immunocompromised mice. As Figure 3-17 illustrates the mice body weight was comparable between control diet, DHA diet, Control+carbo, and DHA+carbo treatment groups.

In addition, we showed that the tumor growth rate was lower in the DHA group compared to the control diet and DHA+carboplatin compared to carboplatin alone (Figure 3-18). We might conclude that DHA when combined with chemotherapy did not have negative impacts on body weight and food intake.

8. DHA induces necrosis and reduces the proliferation rate but does not affect apoptosis and CD95 expression, *in vivo*

We evaluated the H&E stained tumor sections to see the area of cell death. As Figure 3-19 shows, DHA increased the area of necrosis in the PDX ovarian tumors when combined with carboplatin, compared to carboplatin alone. As the dead cells may be the result of apoptosis, necrosis, autophagy, or necroptosis, it is not practical to distinguish the mechanism of cell death and therefore, we called the area of dead cells as necrosis.

To assess the anti-proliferation effect of DHA in PDX ovarian tumor tissue, Ki67 (a cell proliferation marker) was examined using IHC. As shown in Figure 3-20, tumors from the DHA-enriched diet group, had fewer Ki67 positive cells than those from the control diet group. In addition, DHA+carboplatin reduced the Ki67 expression more effectively compared to carboplatin alone.

To evaluate the apoptotic effects of DHA on tumor tissue, we performed TUNEL and CD95 staining by IHC. We determined that DHA might not affect apoptosis and the number of positive nuclei in TUNEL assay were comparable in all four treatment groups (Figure 3-21). It should be considered that TUNEL staining detects the late stages of apoptosis. For the detection of early apoptosis, some other markers such as PARP or cleaved caspases may be more accurate.

Similarly, CD95 staining did not show any differences between treatment and control groups. CD95 is a cytoplasmic marker and it is difficult to quantify and compare its expression in different sections. Accordingly, we evaluated multiple fields of different slide sections and did not see any significant differences in the intensity of staining (Figure 3-22 (A)). To confirm the equality of the expression of CD95 after treatment with DHA as we found in IHC, we performed an *in vitro* experiment. We treated the ES2 cells with DHA, OA as well as combination with chemotherapy and evaluated the expression of CD95 by IF using confocal microscopy. As it is shown in Figure 3-22 (B), expression of CD95 was not significantly different amongst all treatment groups compared to control.

9. Fatty acids incorporated into the tumor and liver cells and change the composition of whole cell phospholipids*, in vivo*

Whole tumor and liver cell phospholipid extraction were analyzed for fatty acid composition. As shown in Table 3-5 and Figure 3-23, whole tumor cell PL analysis revealed successful incorporation of DHA into the cell membranes. High DHA intake increased the percentage of DHA from 2.96% to 4.62% (W/W%) and total n-3 PUFAs from 4.15% to 6.64% in control vs DHA diet group but decreased the concentration of n-6 PUFAs in the tumor cells. Figure 3-22

illustrates the differences between the amount of n-6 and n-3 PUFAs in the DHA group which was inversely correlated, indicating a partial replacement of n-3 by n-6 fatty acids.

Similar to the *in vitro* experiment, enrichment of DHA in the diet leads to the partial replacement of AA in whole cell PLs. As we mentioned before, a higher concentration of n-3 PUFAs compared to AA in the PLs of tumor cells reduces the production of inflammatory mediators from AA and increases the production of anti-inflammatory mediators from n-3 PUFAs. Knowing that AA has a role in the pathogenesis of cancers, cellular growth, and proliferation, this replacement may inhibit carcinogenesis and reduce proliferation in tumor cells.

In addition, we showed the same pattern of whole cell PLs in the livers of mice treated with DHA (Table 3-6 and Figure 3-24). Similar to tumor tissue, treatment with DHA increased the percentage of EPA, DHA, and total n-3 PUFAs and decreased the AA and n-6 PUFAs concentration.

Comparison between tumor and liver PL analysis demonstrate two important facts; first, dietary DHA successfully incorporated into the cell membranes, and second, tumors uptake DHA as effective as normal tissues such as the liver.

10. Strengths and limitations of the study

The current study has several strengths as well as limitations. One of the points of power is that we analyzed multiple ovarian cancer cell lines from different subtypes of epithelial ovarian cancer to reduce the selection bias. We also treated the cells with three different fatty acids DHA, OA, and LA (n-3, n-9, and n-6 PUFAs, respectively) in six different concentrations from 0 μM to 320 μM and with two methods of cell survival analysis (MTT vs trypan blue assay). To evaluate the mechanism of DHA *in vitro,* apoptosis and cell cycle were analyzed by different complementary methods (flow cytometery, western blot, and RT-PCR). In the in vivo section of the study, the

animal model was well organized by the enough number of mice and appropriate monitoring. The PDX tumor might mimic the real tumor condition and response to the treatments as in the humans. However, the current study had some limitations which may interfere with the real results. Our ovarian cancer cell lines were different based on the specific culture media and the cell proliferation rate. These may affect the availability and the uptake of the fatty acids by different cell lines. We also treated the cells with single fatty acids which may cause some essential fatty acid deficiency in the cells and change the cellular response to the treatments. Combination of LA and OA as a control baseline treatment and then adding DHA to this combination, may be a better protocol to evaluate the effects of DHA on the ovarian cancer cells.

Our study did not have enough power to analyze the synergistic effects of DHA and carboplatin combination therapy. To evaluate synergy, multiple replications of cells treated with different concentrations of DHA and carboplatin at the same time in needed which will be performed soon. As another point, in the *in vivo* experiments, PDX tumors were engrafted in the mice flank area subcutaneously and carboplatin was injected intraperitoneally. Flank area may not be the ideal place for ovarian cancer growth and tissue availability of the intraperitoneal carboplatin is not clear and all these may interfere with the results of the study.

11. Conclusions and future directions

In conclusion, DHA might have some promising effects on inhibiting ovarian tumor growth and increasing the efficacy of chemotherapy, both *in vitro* and *in vivo*. Although these findings provide meaningful evidence, they cannot accurately show the real nature of DHA in cancer patients. Further investigations should be carried out to demonstrate the anti-cancer activity of DHA in preclinical and clinical settings. Based on that, DHA might be used as an adjunct treatment to the

standard chemotherapy treatment of ovarian cancer to enhance treatment efficacy and patient outcome.

As a future direction, synergistic effects of DHA on carboplatin in the ovarian cancer cell lines will be analyzed by appropriate experiments. Furthermore, cell cycle analysis will be repeated and the cells will be synchronized before the experiment. Although synchronization by serum starvation may make the cells more vulnerable to cytotoxic effects of DHA, it may provide more accurate results on cell cycle progression. To clarify the mechanism of anti-proliferation and cell cycle arrest, we will analyze the checkpoints of cell cycle such as cyclins and cyclin-dependent kinases at the level of gene expression and protein expression in the ovarian cancer cell lines.

As another future step, we will establish a new set of animal experiments to evaluate the anti-tumor effects of DHA. For that, we will transplant another human high grade serous ovarian PDX into mice and the animals will be treated with DHA-enriched diet and IV injections of carboplatin, in order to better mimic the clinical application of this drug. Finally, taking together all the results, a clinical trial may be justified wherein we shall determine the effects of DHA on ovarian cancer patients.

References:

1. Canadian Cancer Statistics, A 2018 special report on cancer incidence by stage. Government of Canada. 2018.
2. Mallen AR, Townsend MK, Tworoger SS. Risk factors for ovarian carcinoma. *Hematology/Oncology Clinics of North America*. 2018;32(6):891-902.
3. Cancer Statistics. National Cancer Institute, Surveillance, Epidemiology, and End Results Program. http://seer.cancer.gov/. Accessed 20 Aug 2016.
4. Bristow RE, Puri I, Chi DS. Cytoreductive surgery for recurrent ovarian cancer: a meta-analysis. *Gynecol Oncol*. 2009; 112:265–74.
5. Hunn J., Rodriguez G.C. Ovarian Cancer: Etiology, Risk Factors, and Epidemiology. *Clinical Obstetrics and Gynecology.* 2012; 55(1):3–23.
6. Cramer DW, BastRC Jr, Berg CD, et al. Ovarian cancer biomarker performance in prostate, lung, colorectal, and ovarian cancer screening trial specimens. *Cancer Prev Res.* 2011; 4:365–374.
7. Beral V, Million Women Study, Collaborators, Bull D, et al. Ovarian cancer and hormone replacement therapy in the Million Women Study. *Lancet* 2007; 369: 1703–10.
8. Danforth KN, Tworoger SS, Hecht JL, et al. A prospective study of postmenopausal hormone uses and ovarian cancer risk. *Br J Cancer* 2007; 96:151–6.
9. Pearce CL, Chung K, Pike MC, et al. Increased ovarian cancer risk associated with menopausal estrogen therapy is reduced by adding a progestin. *Cancer* 2009; 115:531–9.
10. Trabert B, Wentzensen N, Yang HP, et al. Ovarian cancer and menopausal hormone therapy in the NIH-AARP diet and health study. *Br J Cancer* 2012; 107: 1181–7.
11. Wentzensen N, Poole EM, Trabert B, et al. Ovarian cancer risk factors by histologic subtype: an analysis from the ovarian cancer cohort consortium. *J Clin Oncol* 2016; 34:2888–98.
12. Chen, V. W., Ruiz, B., Killeen, J. L., et al. Pathology and classification of ovarian tumors. *Cancer* 2003; 97: 2631-2642.
13. Berek, J. S., Kehoe, S. T., Kumar, L. and Friedlander, M. Cancer of the ovary, fallopian tube, and peritoneum. *Int J Gynecol Obstet*. 2018:143: 59-78.
14. Rojas V, Hirshfield KM, Ganesan S, Rodriguez-Rodriguez L. Molecular Characterization of Epithelial Ovarian Cancer: Implications for Diagnosis and Treatment. *Int J Mol Sci*. 2016; 17(12):2113.
15. Rosen DG, Yang G, Liu G, et al. Ovarian cancer: pathology, biology, and disease models. *Front Biosci (Landmark Ed).* 2009; 14:2089-102.
16. Lee Y, Miron A, Drapkin R, Nucci MR, Medeiros F, Saleemuddin A, et al. A candidate precursor to serous carcinoma that originates in the distal fallopian tube. J Pathol. 2007; 211:26–35.
17. Paik DY, Janzen DM, Schafenacker AM, Velasco VS, Shung MS, Cheng D, et al. Stem-like epithelial cells are concentrated in the distal end of the fallopian tube: a site for injury and serous cancer initiation. *Stem Cells.* 2012; 30:2487–97.
18. Scully R, Sobin L. Histological typing of ovarian tumors, volume 9. New York: Springer Berlin, 1999.
19. Salani R, Kurman RJ, Giuntoli R 2nd, Gardner G, Bristow R, Wang TL, et al. Assessment of TP53 mutation using purified tissue samples of ovarian serous carcinomas reveals a higher mutation rate than previously reported and does not correlate with drug resistance. *Int J Gynecol Cancer*. 2008; 18:487–91.

20. Network CGAR. Integrated genomic analyses of ovarian carcinoma. *Nature.* 2011; 474:609–15.
21. Merajver SD, Pham TM, Caduff RF, Chen M, Poy EL, Cooney KA, et al. Somatic mutations in the BRCA1 gene in sporadic ovarian tumors. *Nat Genet*. 1995; 9:439–43.
22. Sangha N, Wu R, Kuick R, Powers S, Mu D, Fiander D, et al. Neurofibromin 1 (NF1) defects are common in human ovarian serous carcinomas and co-occur with TP53 mutations. *Neoplasia.* 2008; 10:1362–72.
23. Peyssonnaux C, Eychene A. The Raf/MEK/ERK pathway: new concepts of activation. *Biol Cell.* 2001; 93:53–62.
24. Scully RE, Richardson GS, Barlow JF. The development of malignancy in endometriosis. *Clin Obstet Gynecol*. 1966; 9:384–411.
25. Sainz de la Cuesta R, Eichhorn JH, Rice LW, Fuller AF Jr, Nikrui N, Goff BA. Histologic transformation of benign endometriosis to early epithelial ovarian cancer. *Gynecol Oncol*. 1996; 60:238–44.
26. Obata K, Morland SJ, Watson RH, Hitchcock A, Chenevix-Trench G, Thomas EJ, et al. Frequent PTEN/MMAC mutations in endometrioid but not serous or mucinous epithelial ovarian tumors. *Cancer Res*. 1998; 58:2095–7.
27. Catasus L, Bussaglia E, Rodrguez I, Gallardo A, Pons C, Irving JA, et al. Molecular genetic alterations in endometrioid carcinomas of the ovary: similar frequency of beta-catenin abnormalities but lower rate of microsatellite instability and PTEN alterations than in uterine endometrioid carcinomas. *Hum Pathol*. 2004; 35:1360–8.
28. Wright K, Wilson P, Morland S, Campbell I, Walsh M, Hurst T, et al. Beta-catenin mutation and expression analysis in ovarian cancer: exon 3 mutations and nuclear translocation in 16% of endometrioid tumors. *Int J Cancer*. 1999; 82:625–9.
29. Carmen MG., Birrer M., Schorge JO. Clear cell carcinoma of the ovary: a review of the literature. *Gynecol Oncol*. 2012 S; 126(3):481-90.
30. Oliver KE., Brady WE., Birrer M., et al. An evaluation of progression free survival and overall survival of ovarian cancer patients with clear cell carcinoma versus serous carcinoma treated with platinum therapy: An NRG Oncology/Gynecologic Oncology Group experience. *Gynecol Oncol*. 2017; 147(2): 243–249.
31. Senkel S, Lucas B, Klein-Hitpass L, Ryffel GU. Identification of target genes of the transcription factor HNF1beta and HNF1alpha in a human embryonic kidney cell line. *Biochim Biophys Acta*. 1731; 2005:179–90.
32. Kobayashi H, Yamada Y, Kanayama S, Furukawa N, Noguchi T, Haruta S, et al. The role of hepatocyte nuclear factor-1beta in the pathogenesis of clear cell carcinoma of the ovary. *Int J Gynecol Cancer*. 2009; 19:471–9.
33. Tanaka T, Tomaru Y, Nomura Y, Miura H, Suzuki M, Hayashizaki Y. Comprehensive search for HNF-1beta-regulated genes in mouse hepatoma cells perturbed by transcription regulatory factor-targeted RNAi. *Nucleic Acids Res*. 2004; 32:2740–50.
34. Kim SL., Lee JW. Lee M., et al. Genomic landscape of ovarian clear cell carcinoma via whole exome sequencing. *Gynecol Oncol*. 2018; 148 (2):375-382.
35. Tashiro H., Imamura Y., Motohara T., et al. Morphological and molecular pathogenesis of epithelial ovarian tumors. In: Frontiers in ovarian cancer science. *Springer nature* 2017; 3:48-9.
36. Vang R, Gown AM, Zhao C, Barry TS, Isacson C, Richardson MS, et al. Ovarian mucinous tumors associated with mature cystic teratomas: morphologic and immunohistochemical

analysis identifies a subset of potential teratomatous origin that shares features of lower gastrointestinal tract mucinous tumors more commonly encountered as secondary tumors in the ovary. *Am J Surg Pathol.* 2007; 31:854–69.
37. Seidman JD, Khedmati F. Exploring the histogenesis of ovarian mucinous and transitional cell (Brenner) neoplasms and their relationship with Walthard cell nests: a study of 120 tumors. Arch *Pathol Lab Med*. 2008; 132:1753–60.
38. Cuatrecasas M, Villanueva A, Matias-Guiu X, Prat J. K-ras mutations in mucinous ovarian tumors: a clinicopathologic and molecular study of 95 cases. *Cancer*. 1997; 79:1581–6.
39. Heinzelmann-Schwarz VA, Gardiner-Garden M, Henshall SM, Scurry JP, Scolyer RA, Smith AN, et al. A distinct molecular profile associated with mucinous epithelial ovarian cancer. *Br J Cancer*. 2006; 94:904–13.
40. Ryland GL, Hunter SM, Doyle MA, Caramia F, Li J, Rowley SM, et al. Mutational landscape of mucinous ovarian carcinoma and its neoplastic precursors. *Genome Med.* 2015; 7:87.
41. Garcia-Soto AE, Boren T, Wingo SN, Heffernen T, Miller DS. Is comprehensive surgical staging needed for thorough evaluation of early-stage ovarian carcinoma? *Am J Obstet Gynecol.* 2012; 206:242.e241–5.
42. Berek JS, Kehoe ST, Kumar L, Friedlander M. FIGO cancer report 2018: Cancer of the ovary, fallopian tube, and peritoneum. *Obstet Gynecol.* 2018; 59-78.
43. McGuire WP, Markman M. Primary ovarian cancer chemotherapy: current standards of care. *Br J Cancer*. 2003; 89 Suppl. 3: S3-8.
44. Omura G, Blessing JA, Ehrlich CE, Miller A, Yordan E, Creasman WT, Homesley HD. A randomized trial of cyclophosphamide and doxorubicin with or without cisplatin in advanced ovarian carcinoma. *Cancer*. 1986; 57:1725–30.
45. Omura GA, Bundy BN, Berek JS, Curry S, Delgado G, Mortel R. Randomized trial of cyclophosphamide plus cisplatin with or without doxorubicin in ovarian carcinoma: a Gynecologicb Oncology Group Study. *J Clin Oncol*. 1989; 7:457–65.
46. McGuire WP, Hoskins WJ, Brady MF, Kucera PR, Partridge EE, Look KY, et al. Cyclophosphamide and cisplatin versus paclitaxel and cisplatin: a phase III randomized trial in patients with suboptimal stage III/IV ovarian cancer (from the Gynecologic Oncology Group). *Semin Oncol.* 1996;23:40–7.
47. Piccart MJ, Bertelsen K, James K, Cassidy J, Mangioni C, Simonsen E, et al. Randomized intergroup trial of cisplatin-paclitaxel versus cisplatin-cyclophosphamide in women with advanced epithelial ovarian cancer: three-year results. *J Natl Cancer Inst*. 2000; 92:699–708.
48. De Sousa GF., De Graziele F., Wlodarczyk SR., et al. Carboplatin: molecular mechanisms of action associated with chemoresistance. *Brazilian Journal of Pharmaceutical Sciences* 2014; 50(4):693-701
49. Brabec V., Kasparkova J. Modifications of DNA by platinum complexes. Relation to resistance of tumors to platinum antitumor drugs. *Drug Resist. Updat*. 2005; 8:131- 46.
50. Hah SS., Stivers KM., De Vere White RW., et al. Kinetics of carboplatin-DNA binding in genomic DNA and bladder cancer cells as determined by accelerator mass spectrometry. *Chem Res Toxicol.* 2006; 19: 622-626.
51. Wang D, Lippard SJ. Cellular processing of platinum anticancer drugs. *Nature reviews. Drug discovery.* Apr 2005; 4(4):307-320.

52. Hall MD, Okabe M, Shen DW, Liang XJ, Gottesman MM. The role of cellular accumulation in determining sensitivity to platinum-based chemotherapy. *Annual review of pharmacology and toxicology.* 2008; 48:495-535.
53. McWhinney S.R., Goldberg R.M., Mcleod H.L. Platinum neurotoxicity pharmacogenetics. Mol. *Cancer Ther*. 2009; 8:10-16.
54. Burger H, Loos WJ, Eechoute K, et al. Drug transporters of platinum-based anticancer agents and their clinical significance. *Drug Resist Update.* 2011; 14(1):22-34
55. Berek JS, Longacre TA, Friedlander M. Ovarian, fallopian tube, and peritoneal cancer. In"Berek & Novak's Gynecology". 15th edition. Wolters Kluwer/ Lippincott Williams & Wilkins. 2012:233-242.
56. Fishman EK1, Mendelson E, Böhm-Vélez M, Bree R, Finberg H, et al. Staging and follow-up of ovarian cancer. American colledge of radiology. ACR appropriateness criteria. *Radiology*. 2000; 215: Supp 889-902.
57. Mitchell DG, Javitt MC, Glanc P, Bennett GL, et al. ACR appropriateness criteria staging and follow-up of ovarian cancer. *JACR.* 2013;10 (11):822-7.
58. Chambers SK1, Chambers JT, Kohorn EI, Lawrence R, Schwartz PE. Evaluation of the role of second-look surgery in ovarian cancer. *Obstet Gynecol*. 1988; 72(3 Pt 1):404-8.
59. Eisenkop SM, Friedman RL, Spirtos NM. The role of secondary cytoreductive surgery in the treatment of patients with recurrent epithelial ovarian carcinoma. *Cancer.* 2000; 88:144–153.
60. Heintz AP, Odicino F, Maisonneuve P, Quinn MA, Benedet JL, Creasman WT, et al. Carcinoma of the ovary. FIGO 26th Annual Report on the results of treatment in gynecological cancer. *Int J Gynaecol Obstet*. 2006; 95(Suppl 1):S161–92.
61. Mullen MM, Kuroki LM, Thaker PH. Novel treatment options in platinum-sensitive recurrent ovarian cancer: A review. *Gynecologic Oncology*. 2019; 152(2):416-425.
62. Pignata S., Cecere SC., Du Bois A., et al. Treatment of recurrent ovarian cancer. *Annals of Oncology*. 2017; 28(suppl 8): viii51–viii56.
63. Pignata S, Scambia G, Bologna A, et al. Randomized controlled trial testing the efficacy of platinum-free interval prolongation in advanced ovarian cancer: The MITO-8, MaNGO, BGOG-Ov1, AGO-Ovar2.16, ENGOT-Ov1, GCIG study. *JCO*. 2017;35(29):3347-3353.
64. Parmar MK, Ledermann JA, Colombo N, du Bois A, Delaloye JF, Kristensen GB, et al. Paclitaxel plus platinum-based chemotherapy versus conventional platinum-based chemotherapy in women with relapsed ovarian cancer: the ICON4/AGO-OVAR-2.2 trial. *Lancet.* 2003; 361:2099–106.
65. (Guidelines) National Comprehensive Cancer Network (NCCN). Ovarian cancer including fallopian tube cancer and primary peritoneal cancer. 2016.
66. Moss G.P, Smith P.A.S, Tavernier D. IUPAC Compendium of Chemical Terminology. Pure and Applied Chemistry. 67(2nd ed.). International Union of Pure and Applied Chemistry. 1997;1307–1375.
67. Adkins Y, Kelley DS. Mechanisms underlying the cardioprotective effects of omega-3 polyunsaturated fatty acids. *J Nutr Biochem.* 2010; 21(9):781–92.
68. Jones PJH, Rideout T. Lipids, sterols, and their metabolites. In: Ross AC, Caballero B, Cousins RJ, Tucker KL, Ziegler TR, eds. Modern Nutrition in Health and Disease. 11th ed. Baltimore, MD: Lippincott Williams & Wilkins; 2014.

69. Lichtenstein AH, Jones PJH. Lipids: absorption and transport. In: Erdman JW, Macdonald IA, Zeisel SH, eds. Present Knowledge in Nutrition. 10th ed. Washington, DC: Wiley-Blackwell; 2012:118-31.
70. Emken EA., Adlof RO., Duval SM., et al. Effect of dietary arachidonic acid on metabolism of deuterated linoleic acid by adult male subjects. *Lipids* 1998; 33(5):471-480.
71. Gabbs M, Leng S, Devassy JG, Monirujjaman M, Aukema HM. Advances in our understanding of oxylipins derived from dietary PUFAs. *Adv Nutr* 2015; 6:513-40.
72. Institute of Medicine, Food and Nutrition Board. Dietary reference intakes for energy, carbohydrate, fiber, fat, fatty acids, cholesterol, protein, and amino acids (macronutrients). Washington, DC: National Academy Press; 2005.
73. SanGiovanni JP, Chew EY. The role of omega-3 long-chain polyunsaturated fatty acids in health and disease of the retina. *Prog Retin Eye Res* 2005;24:87-138
74. Samuelsson B, Dahlen SE, Lindgren JA, Rouzer CA, Serhan CN. Leukotrienes and lipoxins: Structures, biosynthesis, and biological effects. *Science* 1987; 237(4819):1171–6.
75. Funk CD. Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science* 2001; 294(5548):1871–5.
76. Karmali RA. Eicosanoids in neoplasia. *Prev Med* 1987; 16(4) 493–502.
77. Lands WE. Biochemistry and physiology of n-3 fatty acids. *FASEB J* 1992; 6(8):2530–6.
78. Serhan CN. Novel eicosanoid and docosanoid mediators: Resolvins, docosatrienes, and neuroprotectins. *Curr Opin Clin Nutr Metab Care* 2005; 8(2): 115–21.
79. Swamy MV, Cooma I, Patlolla JM, Simi B, Reddy BS, Rao CV. Modulation of cyclooxygenase-2 activities by the combined action of celecoxib and decosahexaenoic acid: Novel strategies for colon cancer prevention and treatment. *Mol Cancer Ther* 2004; 3(2):215–21.
80. Lee SA, Kim HJ, Chang KC, et al. DHA and EPA Down-regulate COX-2 Expression through Suppression of NF-kappaB Activity in LPS-treated Human Umbilical Vein Endothelial Cells. *Korean J Physiol Pharmacol*. 2009;13(4):301–307.
81. World Health Organization. Interim Summary of Conclusions and Dietary Recommendations on Total Fat & Fatty Acids. 2010.
82. National institutes of Health, Office of Dietary Supplements. Omega-3 fatty acids, fact sheet for health professionals. 2018.
83. Djousse L, Akinkuolie AO, Wu JH, Ding EL, Gaziano JM. Fish consumption, omega-3 fatty acids and risk of heart failure: a meta-analysis. *Clin Nutr* 2012; 31:846-53. [PubMed abstract]
84. Del Gobbo LC, Imamura F, Aslibekyan S, Marklund M, Virtanen JK, Wennberg M, et al. Omega-3 polyunsaturated fatty acid biomarkers and coronary heart disease: pooling project of 19 cohort studies. *JAMA Intern Med* 2016; 176:1155-66.
85. U.S. Food and Drug Administration. Summary of qualified health claims subject to enforcement discretion. 2014.
86. U.S. Department of Health and Human Services, U.S. Department of Agriculture. 2015–2020 dietary guidelines for Americans. 2015.

87. Hibbeln JR, Davis JM, Steer C, Emmett P, Rogers I, Williams C, et al. Maternal seafood consumption in pregnancy and neurodevelopmental outcomes in childhood (ALSPAC study): an observational cohort study. *Lancet*. 2007; 369:578-85.
88. Makrides M, Gibson RA, McPhee AJ, Yelland L, Quinlivan J, Ryan P, et al. Effect of DHA supplementation during pregnancy on maternal depression and neurodevelopment of young children: a randomized controlled trial. *JAMA* 2010; 304:1675-83.
89. Makrides M, Gould JF, Gawlik NR, Yelland LN, Smithers LG, Anderson PJ, et al. Four-year follow-up of children born to women in a randomized trial of prenatal DHA supplementation. *JAMA* 2014; 311:1802-4.
90. Section on Breastfeeding. Breastfeeding and the use of human milk. *Pediatrics* 2012; 129:2011-3552.
91. Sydenham E, Dangour AD, Lim WS. Omega 3 fatty acid for the prevention of cognitive decline and dementia. *Cochrane Database Syst Rev* 2012; 6:CD005379.
92. Mazereeuw G, Lanctot KL, Chau SA, Swardfager W, Herrmann N. Effects of omega-3 fatty acids on cognitive performance: a meta-analysis. *Neurobiol Aging* 2012; 33:1482 e17-29.
93. Gago-Dominguez M, Yuan JM, Sun CL, Lee HP, Yu MC. Opposing effects of dietary n-3 and n-6 fatty acids on mammary carcinogenesis: The Singapore Chinese Health Study. *Br J Cancer* 2003;89:1686-92
94. Brasky TM, Lampe JW, Potter JD, Patterson RE, White E. Specialty supplements and breast cancer risk in the VITamins And Lifestyle (VITAL) Cohort. *Cancer Epidemiol Biomarkers Prev* 2010; 19:1696-708.
95. Zheng JS, Hu XJ, Zhao YM, Yang J, Li D. Intake of fish and marine n-3 polyunsaturated fatty acids and risk of breast cancer: meta-analysis of data from 21 independent prospective cohort studies. *BMJ* 2013; 346:f3706.
96. Geelen A, Schouten JM, Kamphuis C, Stam BE, Burema J, Renkema JM, et al. Fish consumption, n-3 fatty acids, and colorectal cancer: a meta-analysis of prospective cohort studies. *Am J Epidemiol* 2007;166:1116-25
97. Kantor ED, Lampe JW, Peters U, Vaughan TL, White E. Long-chain omega-3 polyunsaturated fatty acid intake and risk of colorectal cancer. *Nutr Cancer* 2014; 66:716-27.
98. Bosire C, Stampfer MJ, Subar AF, Park Y, Kirkpatrick SI, Chiuve SE, et al. Index-based dietary patterns and the risk of prostate cancer in the NIH-AARP diet and health study. *Am J Epidemiol* 2013; 177:504-13?
99. Augustsson K, Michaud DS, Rimm EB, Leitzmann MF, Stampfer MJ, Willett WC, et al. A prospective study of intake of fish and marine fatty acids and prostate cancer. *Cancer Epidemiol Biomarkers Prev.* 2003; 12:64-7.
100. Brasky TM, Till C, White E, Neuhouser ML, Song X, Goodman P, et al. Serum phospholipid fatty acids and prostate cancer risk: results from the prostate cancer prevention trial. *Am J Epidemiol*. 2011; 173:1429-39

101. Brasky TM, Darke AK, Song X, Tangen CM, Goodman PJ, Thompson IM, et al. Plasma phospholipid fatty acids and prostate cancer risk in the SELECT trial. *J Natl Cancer Inst*. 2013; 105:1132-41.
102. Alexander W. Prostate cancer risk and omega-3 Fatty Acid intake from fish oil: a closer look at media messages versus research findings. *P T* 2013; 38:561-4.
103. Stillwell W, Jenski LJ, Crump FT, Ehringer W. Effect of docosahexaenoic acid on mouse mitochondrial membrane properties. *Lipids*. 1997; 32:497–506.
104. Wassall SR, Stillwell W. Docosahexaenoic acid domains: The ultimate non-raft membrane domain. *Chem Phys Lipids*. 2008; 153:57–63.
105. Ma DW, Seo J, Switzer KC, Fan YY, McMurray DN, Lupton JR et al. N-3 PUFA and membrane microdomains: a new frontier in bioactive lipid research. *J Nutr Biochem*. 2004; 15:700–706.
106. Brown DA, London E. Functions of lipid rafts in biological membranes. *Annu Rev Cell Dev Biol.* 1998; 14:111–136.
107. Calder PC, Yaqoob P. Lipid rafts—composition, characterization, and controversies. *J Nutr.* 2007; 137(3):545–7
108. Murai T. The role of lipid rafts in cancer cell adhesion and migration. *Int. J. Cell Biol*. 2012;
109. Blanckaert V, Ulmann L, Mimouni V, Antol J, Brancquart L, Chénais B. Docosahexaenoic acid intake decreases proliferation, increases apoptosis and decreases the invasive potential of the human breast carcinoma cell line MDA-MB-231. *Int J Oncol.* 2010; 36:737–42.
110. Schley P.D., Brindley D.N., Field C.J. (*n*-3) PUFA alter raft lipid composition and decrease epidermal growth factor receptor levels in lipid rafts of human breast cancer cells. *J. Nutr.* 2007; 137:548–553.
111. Rogers K.R., Kikawa K.D., Mouradian M., Hernandez K., McKinnon K.M., Ahwah S.M., Pardini R.S. Docosahexaenoic acid alters epidermal growth factor receptor-related signaling by disrupting its lipid raft association. *Carcinogenesis*. 2010; 31:1523–1530.
112. Nathalie V, Lajoie-Mazenc I, Auge N, Suc I, Frisach MF, et al. Activation of epithelial growth factor receptor pathway by unsaturated fatty acids. *Circ Res*. 1999; 85:892–9.
113. Flock MR, Harris WS, Kris-Etherton PM. Long-chain omega-3 fatty acids: time to establish a dietary reference intake. *Nutr Rev.* 2013; 71(10):692–707.
114. Liu J, Ma DW. The role of n-3 polyunsaturated fatty acids in the prevention and treatment of breast cancer. *Nutrients*. 2014; 6(11):5184-223.
115. Zandi R., Larsen A.B., Andersen P., Stockhausen M.T., Poulsen H.S. Mechanisms for oncogenic activation of the epidermal growth factor receptor. *Cell Signal*. 2007; 19:2013–2023.
116. Corsetto P.A., Montorfano G., Zava S., Jovenitti I.E., Cremona A., Berra B., Rizzo A.M. Effects of *n*-3 PUFAs on breast cancer cells through their incorporation in plasma membrane. *Lipids Health Dis.* 2011; 10:73.
117. Bhargava R., Gerald W.L., Li A.R., Pan Q., Lal P., Ladanyi M., Chen B. EGFR gene amplification in breast cancer: Correlation with epidermal growth factor receptor mRNA

and protein expression and HER-2 status and absence of EGFR-activating mutations. *Mod. Pathol.* 2005; 18:1027–1033.

118. Kang KS, Wang P, Yamabe N, Fukui M, Jay T, Zhu BT. Docosahexaenoic acid induces apoptosis in MCF-7 cells in vitro and in vivo via reactive oxygen species formation and caspase 8 activation. *PLoS One*. 2010; 5(4):e10296.
119. Zou Z, Bellenger S, Massey KA, Nicolaou A, Geissler A, Bidu C, Bonnotte B, Pierre AS, Minville-Walz M, Rialland M, et al. Inhibition of the HER2 pathway by n-3 polyunsaturated fatty acids prevents breast cancer in fat-1 transgenic mice. *J Lipid Res*. 2013; 54:3453–3463.
120. Tovey S.M., Brown S., Doughty J.C., Mallon E.A., Cooke T.G., Edwards J. Poor survival outcomes in HER2-positive breast cancer patients with low-grade, node-negative tumors. *Br. J. Cancer.* 2009; 100:680–683.
121. Bollig-Fischer A., Dziubinski M., Boyer A., Haddad R., Giroux C.N., Ethier S.P. HER-2 signaling, acquisition of growth factor independence, and regulation of biological networks associated with cell transformation. *Cancer Res*. 2010; 70:7862–7873.
122. Moasser M.M. The oncogene HER2: Its' signaling and transforming functions and its role in human cancer pathogenesis. *Oncogene*. 2007; 26:6469–6487.
123. Menéndez JA, Vázquez-Martín A, Ropero S, Colomer R, Lupu R. HER2 (erbB-2)-targeted effects of the omega-3 polyunsaturated fatty acid, alpha-linolenic acid (ALA; 18:3n-3) in breast cancer cells: The 'fat features' of the 'Mediterranean diet' as an 'anti-HER2 cocktail'. *Clin Transl Oncol.* 2006; 8:812–820.
124. Mobraten K, Haug TM, Kleiveland CR, Lea T. Omega-3 and omega-6 PUFAs induce the same GPR120-mediated signalling events, but with different kinetics and intensity in Caco-2 cells. *Lipids Health Dis.* 2013; 12:101.
125. Desvergne B., Wahli W. Peroxisome proliferator-activated receptors: Nuclear control of metabolism. *Endocr. Rev.* 1999; 20:649–688.
126. Rahman MM, Veigas M, Williams PJ, Fernandes G. DHA is a more potent inhibitor of breast cancer metastasis to bone and related osteolysis than EPA. *Breast Cancer Res Treat.* 2013; 141(3).
127. Flachs P., Rossmeisl M., Bryhn M., Kopecky J. Cellular and molecular effects of *n*-3 polyunsaturated fatty acids on adipose tissue biology and metabolism. *Clin. Sci*. 2009; 116:1–16.
128. Jiang W., Zhu Z., McGinley J.N., El B.K., Manni A., Thompson H.J. Identification of a molecular signature underlying inhibition of mammary carcinoma growth by dietary *n*-3 fatty acids. *Cancer Res.* 2012; 72:3795–3806.
129. Oltvai Z.N., Milliman C.L., Korsmeyer S.J. Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. *Cell.* 1993; 74:609–619.
130. Van Slooten H.J., Clahsen P.C., van Dierendonck J.H., Duval C., Pallud C., Mandard A.M., obelle-Deroide A., van de Velde C.J., van de Vijver M.J. Expression of Bcl-2 in node-negative breast cancer is associated with various prognostic factors, but does not predict response to one course of perioperative chemotherapy. *Br. J. Cancer*. 1996; 74:78–85.

131. Stephenson JA, Al-Taan O, Arshad A, Morgan B, Metcalfe MS, Dennison AR. The multifaceted effects of omega-3 polyunsaturated fatty acids on the hallmarks of cancer. *J Lipids*. 2013; 2013:261247.
132. Raisova M., Hossini A.M., Eberle J., Riebeling C., Wieder T., Sturm I., Daniel P.T., Orfanos C.E., Geilen C.C. The Bax/Bcl-2 ratio determines the susceptibility of human melanoma cells to CD95/Fas-mediated apoptosis. *J. Investig. Dermatol.* 2001; 117:333–340.
133. Baracos VE, Mazurak VC, Ma DWL. n-3 polyunsaturated fatty acids throughout the cancer trajectory: influence on disease incidence, progression, response to therapy and cancer-associated cachexia. *Nutr Res Rev.* 2004; 17:177–92.
134. Vivanco I., Sawyers C.L. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat. Rev. Cancer.* 2002; 2:489–501.
135. Ion G., Akinsete J.A., Hardman W.E. Maternal consumption of canola oil suppressed mammary gland tumorigenesis in C3 (1) TAg mice offspring. *BMC Cancer.* 2010; 10:81.
136. Wu J.T., Kral J.G. The NF-κB/IκB signaling system: A molecular target in breast cancer therapy. *J. Surg. Res.* 2005; 123:158–169.
137. Dolcet X., Llobet D., Pallares J., Matias-Guiu X. NF-κB in development and progression of human cancer. *Virchows Arch.* 2005; 446:475–482.
138. Dictor M, Ehinger M, Mertens F, Akervall J, Wennerberg J. Abnormal cell cycle regulation in malignancy. *Am J Clin Path*. 1999; 112:S40–S52.
139. Barnum KJ, O'Connell MJ. Cell cycle regulation by checkpoints. *Methods Mol Biol.* 2014; 1170:29-40.
140. Siddiqui RA, Shaikh SR, Sech LA, Yount HR, StillwellW, Zaloga GP. Omega 3-fatty acids: health benefits and cellular mechanisms of action. *Mini Rev Med Chem*. 2004; 4:859–871.
141. Newell M, Baker K, Postovit LM, Field CJ. A Critical Review on the Effect of Docosahexaenoic Acid (DHA) on Cancer Cell Cycle Progression. *Int J Mol Sci*. 2017; 18(8):1784.
142. Pauwels EK, Kairemo K. Fatty acid facts, part II: role in the prevention of carcinogenesis, or, more fish on the dish? *Drug News Perspect.* 2008; 21:504–10.
143. Tapiero H, Ba GN, Couvreur P, Tew KD. Polyunsaturated fatty acids (PUFA) and eicosanoids in human health and pathologies. *Biomed Pharmacother*. 2002; 56(5):215–22.
144. Halliwell B, Chirico S. Lipid peroxidation: its mechanism, measurement, and significance. *Am J Clin Nutr.* 1993; 57(suppl):715S–725S.
145. Fukui M, Kang KS, Okada K, Zhu BT. EPA, an omega-3 fatty acid, induces apoptosis in human pancreatic cancer cells: role of ros accumulation, caspase-8 activation, and autophagy induction. *J Cell Biochem*. 2012; 114(1):192–203.
146. Hofmanova J, Vaculova A, Kozubik A. Polyunsaturated fatty acids sensitize human colon adenocarcinoma HT-29 cells to death receptor-mediated apoptosis. *Cancer Lett.* 2005; 218:33–41.
147. Cao W., Ma Z., Rasenick M.M., Yeh S., Yu J. N-3 poly-unsaturated fatty acids shift estrogen signaling to inhibit human breast cancer cell growth. *PLoS ONE*. 2012;7:e52838

148. Yu H.M. Bypassing the D6-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.
149. Barascu A., Besson P., Le Floch O., Bougnoux P., Jourdan M.L. CDK1-cyclin B1 mediates the inhibition of proliferation induced by omega-3 fatty acids in MDA-MB-231 breast cancer cells. *Int. J. Biochem. Cell Biol*. 2006; 38:196–208.
150. Calder PC. Omega-3 fatty acids and inflammatory processes. *Nutrients*. 2010; 2(3):355-74.
151. Larsson S.C., Kumlin M., Ingelman-Sundberg M., Wolk A. Dietary long-chain *n*-3 fatty acids for the prevention of cancer: A review of potential mechanisms. *Am. J. Clin. Nutr*. 2004; 79:935–945.
152. Burdge G.C., Calder P.C. Conversion of α-linolenic acid to longer-chain polyunsaturated fatty acids in human adults. *Reprod. Nutr. Dev.* 2005; 45:581–597.
153. Healy D.A., Wallace F.A., Miles E.A., Calder P.C., Newsholm P. Effect of low-to-moderate amounts of dietary fish oil on neutrophil lipid composition and function. *Lipids*. 2000; 35:763–768.
154. Young L.R., Kurzer M.S., Thomas W., Redmon J.B., Raatz S.K. Effect of dietary fat and omega-3 fatty acids on urinary eicosanoids and sex hormone concentrations in postmenopausal women: A randomized controlled feeding trial. *Nutr. Cancer*. 2011; 63:930–939.
155. Wei N., Wang B., Zhang Q.Y., Mi M.T., Zhu J.D., Yu X.P., Yuan J.L., Chen K., Wang J., Chang H. Effects of different dietary fatty acids on the fatty acid compositions and the expression of lipid metabolic-related genes in mammary tumor tissues of rats. *Nutr. Cancer*. 2008; 60:810–825.
156. Rose D.P., Connolly J.M., Rayburn J., Coleman M. Influence of diets containing eicosapentaenoic or docosahexaenoic acid on growth and metastasis of breast cancer cells in nude mice. *J. Natl. Cancer Inst*. 1995; 87:587–592.
157. Kang J.X., Weylandt K.H. Modulation of inflammatory cytokines by omega-3 fatty acids. *Subcell. Biochem.* 2008; 49:133–143.
158. Weylandt K.H., Kang J.X., Wiedenmann B., Baumgart D.C. Lipoxins and resolvins in inflammatory bowel disease. *Inflamm. Bowel. Dis.* 2007; 13:797–799.
159. Sadli N., Ackland ML, de Mel D., Sinclair AJ., and Suphioglu C. Effects of zinc and DHA on the epigenetic regulation of human neuronal cells. *Cellular Physiology and Biochemistry.* 2012; 29(1-2):87–98.
160. Dimri M., Bommi PV., Sahasrabuddhe AA., et al. Dietary omega-3 polyunsaturated fatty acids suppress expression of EZH2 in breast cancer cells. *Carcinogenesis*. 2010; 31(3):489–495.
161. Victory R, Saed GM, Diamond MP. Antiadhesion effects of docosahexaenoic acid on normal human peritoneal and adhesion fibroblasts. *Fertil Steril.* 2007 ;88 :1657-1662.
162. Spencer L, Mann C, Metcalfe M, Webb M, et al. The effect of omega-3 FAs on tumor angiogenesis and their therapeutic potential. *Eur J Cancer.* 2009; 45:2077-2086.
163. Gleissman H., Johnsen JI., Konger P. Omega-3 fatty acids in cancer, the protectors of good and the killers of evil. *Experi cell research*. 2010; 316(8):1365-1373.

164. Goua M, Mulgrew S, Frank J, Rees D, A.A, et al. Regulation of adhesion molecule expression in human endothelial and smooth muscle cells by omega-3 fatty acids and conjugated linoleic acids: involvement of the transcription factor NF-kappaB? *Prostaglandins Leukot Essent Fatty Acids.* 2008(78):33–43.
165. Mareel M, Leroy A. Clinical, cellular, and molecular aspects of cancer invasion. *Physiol Rev.* 2003; 83:337–376.
166. Rose DP, Connolly JM, Coleman M. Effect of omega-3 fatty acids on the progression of metastases after the surgical excision of human breast cancer cell solid tumors growing in nude mice. *Clin Cancer Res.* 1996; 2:1751–6.
167. Zhang G, Panigrahy D, Mahakiane LM, Yang J, Liu J-Y, Leea KSS, et al. Epoxy metabolites of docosahexaenoic acid (DHA) inhibit angiogenesis, tumor growth, and metastasis. *PNAS.* 2013; 110(16):6530–35.
168. Rose DP, Connolly JM, Liu XH. Effects of linoleic acid and gamma-linolenic acid on the growth and metastasis of a human breast cancer cell line in nude mice and on its growth and invasive capacity *in vitro*. *Nutr Cancer*. 1995; 24:33–45.
169. Kinoshita K, Noguchi M, Tanaka M. Effects of linoleic acid, eicosapentaenoic acid, and docosahexaenoic acid on the growth and metastasis of MM48 mammary tumor transplants in mice. *Int J Oncol.* 1996; 8:575–581.
170. Kimura Y, Sumiyoshi M. Antitumor and antimetastatic actions of eicosapentaenoic acid ethylester and its by-products formed during accelerated stability testing. *Cancer Sci.* 2005; 96:441–450.
171. Hou R, Wu QJ, Gong TT, Jiang L. Dietary fat and fatty acid intake and epithelial ovarian cancer risk: evidence from epidemiological studies. *Oncotarget*. 2015; 6(40):43099-119.
172. Ibiebele TI., Nagle CM., Bain CJ., Webb PM. Intake of omega-3 and omega-6 fatty acids and risk of ovarian cancer. *Cancer Causes & Controls*. 2012; 23(11):1775-1783.
173. Ansenberger K, Richards C, Zhuge Y, et al. Decreased severity of ovarian cancer and increased survival in hens fed a flaxseed-enriched diet for 1 year. *Gynecol Oncol*. 2010; 117(2):341-7.
174. Eilati E, Bahr JM, Hales DB. Long term consumption of flaxseed enriched diet decreased ovarian cancer incidence and prostaglandin E_2in hens. *Gynecol Oncol*. 2013; 130(3):620-8.
175. Han L., Zhang Y., Meng M., Cheng D., WangC. Eicosapentaenoic acid induced SKOV-3 cell apoptosis through ERK1/2–mTOR–NF-[kappa] B pathways. *Anti-Cance Drugs*. 2016; 27(7):635-642.
176. Zajdel A., Kałucka M., Chodurek E., Wilczok A. DHA but not AA Enhances Cisplatin Cytotoxicity in Ovarian Cancer Cells. *Nutr Cancer*. 2018; 11:1-8.
177. Sharma A., Belna J., Logan J., et al. The effects of omega-3 fatty acids on growth regulation of epithelial ovarian cancer cell lines. *Gynecol Oncol*. 2005; 99(1):58-64.
178. Tanaka A., Yamamoto A., Murota K., et al. Polyunsaturated fatty acids induce ovarian cancer cell death through ROS-dependent MAP kinase activation. *Biochem Biophys Res commun.* 2017; 493:468-473.
179. Wang YC., Wu YN., Wang SL., et al. Docosahexanoic acis modulates invasion and metastasis of human ovarian cancer via multiple molecular pathways. *Int J Gynecol Cancer*. 2016; 26(6):994-1003.

180. Ince T.A, Sousa A.D, Jones M.A, et al. Characterization of twenty-five ovarian tumor cell lines that phenocopy primary tumors. *Nature communications*. 2015; 6:7419.
181. Papp E, Hallberg D, Konecny GE, et al. Integrated Genomic, Epigenomic, and Expression Analyses of Ovarian Cancer Cell Lines. *Cell Rep*. 2018;25(9):2617–2633.
182. Ewaschuk JB, Newell M, Field CJ. Docosahexaenoic acid improves chemotherapy efficacy by inducing CD95 translocation to lipid rafts in ER (-) breast cancer cells. *Lipids*. 2012; 47(11):1019-30.
183. Robinson, L.E., Clandinin M.T, and Field C.J. The role of dietary long-chain n-3 fatty acids in anti-cancer immune defense and R3230AC mammary tumor growth in rats: influence of diet fat composition. *Breast Cancer Res Treat.* 2002. 73(2): 145-60.
184. Cruz-Hernandez, C., et al. Direct quantification of fatty acids in human milk by gas chromatography. J Chromatogr A. 2013. 1284: 174-9.
185. Gurcan MN, Boucheron LE, Can A, et al. Histopathological image analysis: a review. *IEEE Rev Biomed Eng*. 2009; 2:147-71.
186. Taylor C.R, Levenson R.M. Quantification of immunohistochemistry-issues concerning methods, utility and semiquantitative assessment II. *Histopathology*. 2006; 49:411-424.
187. Bilyk O, Coatham M, Jewer M, Postovit LM. Epithelial-to-Mesenchymal Transition in the Female Reproductive Tract: From Normal Functioning to Disease Pathology. *Front Oncol*. 2017; 7:145.
188. Oono K, Takahashi K, Sukehara S, et al. Inhibition of PC3 human prostate cancer cell proliferation, invasion and migration by eicosapentaenoic acid and docosahexaenoic acid. *Mol Clin Oncol*. 2017; 7(2):217-220.
189. Eltweri AM, Howells LM, Thomas AL, Dennison AR, Bowrey DJ. Effects of Omegaven®, EPA, DHA and oxaliplatin on oesophageal adenocarcinoma cell lines growth, cytokine and cell signal biomarkers expression. *Lipids Health Dis*. 2018; 17(1):19.
190. Gao K, Liang Q, Zhao ZH, Li YF, Wang SF. Synergistic anticancer properties of docosahexaenoic acid and 5-fluorouracil through interference with energy metabolism and cell cycle arrest in human gastric cancer cell line AGS cells. *World J Gastroenterol*. 2016; 22(10):2971-80.
191. Sun SN, Jia WD, Chen H, et al. Docosahexaenoic acid (DHA) induces apoptosis in human hepatocellular carcinoma cells. *Int J Clin Exp Pathol*. 2013; 6(2):281-9.
192. Hardman WE, Moyer MP, Cameron IL. Dietary fish oil sensitizes A549 lung xenografts to doxorubicin chemotherapy Cancer Letters. 2000; 151(2): 145-151.
193. Yang T, Fang S, ZhangHX, et al. N-3 PUFAs have antiproliferative and apoptotic effects on human colorectal cancer stem-like cells in vitro. The Journal of Nutritional Biochemistry.2013;24(5):744-753.
194. Fahrmann JF, Hardman WE. Omega 3 fatty acids increase the chemo-sensitivity of B-CLL-derived cell lines EHEB and MEC-2 and of B-PLL-derived cell line JVM-2 to anti-cancer drugs doxorubicin, vincristine, and fludarabine. *Lipids Health Dis*. 2013; 12:36.
195. Chen ZY and Istfan NW: Docosahexaenoic acid, a major constituent of fish oil diets, prevents activation of cyclin-dependent kinases and S-phase entry by serum stimulation in HT-29 cells. *Prostaglandins Leukot Essent Fatty Acids*.2001;(64):67–73.

196. Albino AP, et al.: Cell cycle arrest and apoptosis of melanoma cells by docosahexaenoic acid: association with decreased pRb phosphorylation. Cancer Res. 2000;(60):4139–4155.
197. Hawkins RA, Sangster K, and Arends MJ. Apoptotic death of pancreatic cancer cells induced by polyunsaturated fatty acids varies with double bond number and involves an oxidative mechanism. *J Pathol* 185, 61–70, 1998.
198. Chen ZY and Istfan NW: Docosahexaenoic acid is a potent inducer of apoptosis in HT-29 colon cancer cells. *Prostaglandins Leukot Essent Fatty Acids*.2000;(63):301–308.
199. Arita K, et al.: Mechanism of apoptosis in HL-60 cells induced by n-3 and n-6 polyunsaturated fatty acids. *Biochem Pharmacol*. 2001;(62)821–828.
200. Kim JY, Jeong HS, Chung T, et al. The value of phosphohistone H3 as a proliferation marker for evaluating invasive breast cancers: A comparative study with Ki67. *Oncotarget.* 2017;8(39):65064-65076.
201. Ribalta T, McCutcheon IE, Aldape KD, Bruner JM, Fuller GN. The mitosis-specific antibody anti-phosphohistone-H3 (PHH3) facilitates rapid reliable grading of meningiomas according to WHO 2000 criteria. *Am J Surg Pathol*. 2004; 28:1532–1536.
202. Colman H, Giannini C, Huang L, Gonzalez J, Hess K, Bruner J, Fuller G, Langford L, Pelloski C, Aaron J, Burger P, Aldape K. Assessment and prognostic significance of mitotic index using the mitosis marker phospho-histone H3 in low and intermediate-grade infiltrating astrocytomas. *Am J Surg Pathol*. 2006; 30:657–664.
203. Tetzlaff MT, Curry JL, Ivan D, Wang WL, Torres-Cabala CA, Bassett RL, Valencia KM, McLemore MS, Ross MI, Prieto VG. Immunodetection of phosphohistone H3 as a surrogate of mitotic figure count and clinical outcome in cutaneous melanoma. *Mod Pathol*. 2013; 26:1153–1160.