Eicosapentaenoic and Docosahexaenoic Fatty Acids Modify Skeletal Muscle Fat Infiltration in an Animal Model of Colorectal Cancer Receiving Chemotherapy

by

Alaa Almasud

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

Doctor of Philosophy

in

Nutrition and Metabolism

Department of Agricultural, Food and Nutritional Science
University of Alberta

© Alaa Almasud, 2017

ABSTRACT

Background**:* Myosteatosis is defined as low skeletal muscle radio-density with elevated amounts of intermuscular adipose tissue, assessed using computed tomography. Myosteatosis has been recently observed to be associated with mortality in people with cancer. Previous work from our lab reported that deposition of intermuscular fat occurs as patients progress through chemotherapy. However, when patients supplemented their daily intake with eicosapentaenoic acid (20:5n-3; EPA) and docosahexaenoic acid (22:6n-3; DHA), a reduction in intermuscular adipose tissue was observed. Mechanisms underlying myosteatosis in cancer have not been explored and no preclinical model of cancer associated with myosteatosis has been developed. Therefore, this study aimed to first establish a preclinical model of cancer and chemotherapy treatment associated with myosteatosis, and then assess if feeding a diet containing fish oil was efficacious in reducing tumor- and chemotherapy-associated fat accumulation within skeletal muscle. ***Methods: Fischer 344 rats were fed either a control diet for the entire study (control), or switched to a diet containing fish oil (2.3 g /100 g of diet) one week prior to tumor implantation (long term fish oil) or at the start of chemotherapy (adjuvant fish oil). Chemotherapy (irinotecan plus 5-fluorouracil) was initiated 2 weeks after tumor implantation (cycle-1) and 1 week thereafter (cycle-2). Reference animals received no tumor or treatment and consumed the control diet. Gastrocnemius and tibialis anterior muscles were frozen in melting isopentane cooled in liquid nitrogen (-156°C), and stored at -80°C until subsequent analyses. To assess myosteatosis, lipids were revealed histologically by Oil Red O staining and triglyceride fatty acids were quantified by gas chromatography. Expression of adipogenic transcription factors and mitochondrial density were assessed at the mRNA level by real-time RT-PCR. Mitochondrial enzymatic activities were assessed using spectrophotometry. Myosin Heavy Chain isoforms were identified by immunofluorescence. ***Results:*** Feeding a diet

containing fish oil reduced tumor- and subsequent chemotherapy-associated increases in muscle neutral lipid content, triglyceride fatty acid levels, and expression of adipogenic transcriptional factors that occurred in control diet fed animals. Lower neutral lipid content within the muscle following chemotherapy treatment in rats fed fish oil diet was associated with higher mitochondrial oxidative capacity. The adjuvant fish oil diet was as effective as the long term fish oil diet in mitigating chemotherapy-associated muscle fat content. ***Conclusion:*** Long term and adjuvant fish oil diets are both efficacious in reducing chemotherapy-associated myosteatosis by reducing expression of transcriptional factors involved in adipogenesis/lipogenesis, and improving mitochondrial oxidative capacity and density. The data we have assembled suggests that myosteatosis, an independent prognostic factor in cancer, is modifiable through dietary intake of EPA and DHA.

PREFACE

All the work presented in the present dissertation was conducted at University of Alberta. Experimental procedures presented in Chapter 3,4, and 5 were reviewed and approved by the University of Alberta Institutional Animal Care Committee and conducted in accordance with the Guidelines of the Canadian Council on Animal Care. This work was funded by the Canadian Institutes of Health Research (259704).

Part of the work presented in Chapter 1 was published by *Applied Physiology, Nutrition, and Metabolism* in 2014. The citation is: Julia B. Ewaschuk, Alaa Almasud, and Vera C. Mazurak. Role of n-3 fatty acids in muscle loss and myosteatosis. Appl. Physiol. Nutr. Metab. 39: 1-9 (2014) doi.org/10.1139/apnm-2013-0423. Chapter 3 is submitted to *Skeletal Muscle Journal* under title of Fish oil mitigates myosteatosis and improves chemotherapy efficacy in a preclinical model of colon cancer. Alaa. A. Almasud, Kaitlin. H. Giles, John. J. Miklavcic, Karen. J. B. Martins, Vickie. E. Baracos, Charles. T. Putman, Leluo. L. Guan, Vera. C. Mazurak. Chapter 4 will be submitted to *Lipids* after merging my results with the work conducted by Maryam Ebadi, a PhD student in our lab, working on adipose tissue alteration in the same animal model that is presented in this thesis.

The co-authors have given their permission for the work to be presented in this thesis. Contributions are detailed as follows: Dr. Vera Mazurak designed and wrote the grant that funded this study as well as supervised Alaa Almasud through the entire work. Alaa Almasud conducted the long-term fish oil and control experiments, analyzed all data, generated figures and tables and wrote the manuscript. Kaitlin Giles conducted the short term fish oil experiment. Dr. Karen Martins assisted in using Volocity software. Dr. Vickie Baracos and Dr. Charles Putman assisted in muscles dissection and provided feedback for Oil Red O analysis. All gene work was conducted

in Dr. Leluo Guan's lab. All named authors have read and helped in editing the manuscript and have agreed to submit the paper in its present form.

DEDICATION

To

My Mother

An angel on the earth who taught me to trust myself, never be a victim, never make excuses, and always face challenges to reach my dream. You are the reason behind my success; you are always my inspiration and I know that I am a strong woman because a strong woman raised me.

My Father

My first love, my hero, and my prince, who always adored and supported me unconditionally since I first opened my eyes, since my first smile, my first word, and my first step. I love you and I will always love you.

My Husband

My heart and soulmate, this work would not have been possible without you, as you, my love, shared with me the entire project and supported me throughout the whole process. You are the miracle who saved me from the darkness and makes my heart sing. Thank you for understanding how extremely important this work was to me and for supporting me at every step of the way, never allowing me to give up.

My Daughter

You are the light of my life; I never knew how much love my heart could hold until you called me mommy. I regret that this work has taken me away from you for a while, but I am sure that one day you will understand and take pride in your mother for her accomplishments.

My Aunt

I miss you so much and you will be always in my heart. Your battle with cancer was my inspiration in completing this project. I hate cancer so much because it took you away from us. I know that if you were here, you would be so proud of what I have reached.

My Brothers and Sister

Sana, Ahmed, Raya, Ali, Raad, and Mohammed, thanks for your continued love and support. I cannot imagine my life without all of you. I love you all so much.

ACKNOWLEDGEMENT

First and foremost, I would like to thank God for this accomplishment. A special thanks to the King of Saudi Arabia, King Salman bin Abdulaziz, who has given me the opportunity for education abroad. I owe a deep appreciation to all the people who are working at the Saudi Arabian Cultural Bureau in Canada, specifically Dr. Samia Osman, as they facilitated all of my educational arrangements from the first day I arrived to Canada until my graduation.

From my first day in the laboratory, the first letter of this thesis, to the final draft, I owe an immense debt of gratitude to my supervisor, Dr. Vera Mazurak. Her sound advice and careful guidance were invaluable through my entire PhD program at the University of Alberta. I have learned invaluable lessons from you, not only pertaining to scientific work, but also to teachings that extend far beyond into the real world. Thank you for imparting the skills that will shape my career and my professional life. I can never really thank you enough.

My deepest appreciation is extended to Dr. Vickie Baracos. There are no words to express my gratitude to you. Thank you for being my role model, for your guidance, and for your invaluable teaching experience. It was a great honor to be one of your students.

I am also very grateful to Dr. Ted Putman and Dr. Leluo Guan for providing valuable comments and suggestions to improve my research project. Thank you for your generosity and for welcoming me into your lab when I had no other place to go. I would not have been able to complete this thesis without your assistance.

I would also like to thank Dr. Carla Prado and Dr. Bodil Larsen for serving as an internal/external examiner during my candidacy exam. I extend my appreciation to Dr. Sambasivarao Damaraju and Dr. Simon Wing for providing feedback and working as an internal/external examiner during my final exam. Thank you, Dr. Diana Mager for serving as my examination chair.

Dalia, Abeer, and Aisha, thank you so much for always being around when I needed you. Dalia, thank you for spending hours of your time talking to me on the phone during my difficult moments. Aisha, I will never forget when you came to Edmonton just to support me when I had no one here. Abeer, we used to share all of the good and bad times together; one day, it will be all good times for you. Just hold on a little more and you will reach the light soon, so we can celebrate together.

Abha and Ian, I really appreciate your laboratory assistance. It was an absolute pleasure to work and learn from both of you throughout my entire project. Special thanks as well to my labmates Maryam, Kaitlin, Ana, Amrit, Sarah, Karen, and Magaly for all the times that we shared together; I will truly miss you all.

TABLE OF CONTENTS

CHAPTER 1: Introduction and Literature Review **1**

1.1 Introduction 1

1.2 Colorectal Cancer 3

1.3 First Line Chemotherapy Treatment Colorectal Cancer 4

1.4 Measuring Body Composition and muscle condition in Cancer 5

1.5 Myosteatosis in Cancer. 7

1.6 Lipid and Essential Fatty Acids 7

1.7 EPA and DHA Status in Cancer Patients 8

1.8 Mechanisms underlying myosteatosis and the potential role of EPA and DHA on each of these mechanisms 9

1.8.1 Adipogenesis Transcriptional factors 10

1.8.2 Skeletal Muscle Fatty Acids Composition 10

1.8.3 Skeletal Muscle Fiber Type Composition and Mitochondrial Oxidative Capacity 13

1.9 Animal Model of Myosteatosis and Diet 16

1.10 Conclusion 16

CHAPTER 2: Research Plan **22**

2.1 Rationale 22

2.2 Research Objectives and Hypotheses 24

CHAPTER 3: Fish Oil Mitigates Myosteatosis and Improves Chemotherapy Efficacy in a Preclinical Model of Colon Cancer **32**

3.1 Introduction 32

3.2 Materia and Methods 34

3.2.1 Animal Model and Experimental Design 35

3.2.2 Tumor Injection and Chemotherapy 36

3.2.3 Diet and Food Intake 36

3.2.4 Body Weight 37

3.2.5 Study Termination and Tissue Collection 37

3.2.6 Oil Red O and Hematoxylin Staining 37

3.2.7 Fatty Acid and Triglyceride Quantification and Composition 38

3.2.8 RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) 38
3.2.9 Statistical Analysis 39
3.3 Results 39
3.3.1 Food Intake and Body Weight 39
3.3.2 Identification of a Potential Model of Tumor- and Chemotherapy-Associated Myosteatosis 40
3.3.3 Effects of the Fish Oil Diets on Tumor- and Chemotherapy-Associated Myosteatosis 42
3.3.4 Effects of the Fish Oil Diets on Tumor Response to Chemotherapy 44
3.4 Discussion 44
3.4.1 Identification of a Potential Model of Tumor- and Chemotherapy-Associated Myosteatosis 45
3.4.2 Effects of the Fish Oil Diets on Tumor- and Chemotherapy-Associated Myosteatosis 47
3.4.3 Effects of the Fish Oil Diets on the Tumor Response to Chemotherapy 48
3.5 Conclusions 48
CHAPTER 4: Phospholipid and Triglyceride Compositions Reflect Dietary EPA and DHA Intervention in an Animal Model of Colorectal Cancer Receiving CPT-11 and 5-FU 59
4.1 Introduction 59
4.2 Material and Methods 61
4.2.1 Phospholipid and Triglyceride Quantification and Composition 61
4.2.2 Statistical Analysis 62
4.3 Results 62
4.3.1 Phospholipid Fatty Acid Profile 62
4.3.2 Triglyceride Fatty Acids Profile: 64
4.4 Discussion 66
4.4.1 PL and TG Fatty Acid Composition in Gastrocnemius Muscle of Model of Tumor- and Chemotherapy-Associated Myosteatosis 67
4.4.2 Effects of the Fish Oil Diets on PL and TG Fatty Acid Composition in Gastrocnemius Muscle of Model of Tumor- and Chemotherapy-Associated Myosteatosis 69
4.5 Conclusions 71
CHAPTER 5: Role of EPA and DHA on Skeletal Muscle Mitochondria in an Animal Model of Colorectal Cancer Associated Myosteatosis 77
5.1 Introduction 77

5.2 Material and Methods 80
5.2.1 Tissue Collection and Processing 80
5.2.2 Histology 80
5.2.3 Mitochondrial Enzymes Activity and Measures 83
5.2.4 RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) 83
5.2.5 Statistical Analysis 84
5.3 Results 84
5.3.1 Neutral Lipid Staining 84
5.3.2 Neutral Lipid Staining-Based Muscle Fiber Type 85
5.3.3 Tibialis Anterior Muscle Fiber Type Composition 86
5.3.4 Muscle Cross Sectional Area 88
5.3.5 Mitochondrial Oxidative Capacity and Density 88
5.4 Discussion 91
5.4.1 Potential Mechanisms Underlying Tumor- and Chemotherapy-Associated Myosteatosis in a Preclinical Model 92
5.4.2 Effects of the Fish Oil Diets on Mechanisms Underlying Tumor- and Chemotherapy-Associated Myosteatosis 94
5.5 Conclusion 95
CHAPTER 6: General Summary and Final Discussion 111
6.1 General Summary 111
6.2 General Discussion 117
6.2.1 Potential Model of Tumor- and Chemotherapy-Associated Myosteatosis 117
6.2.2 Role of the Fish Oil Diets on Tumor- and Chemotherapy-Associated Myosteatosis 122
6.3 Strength and Limitations 124
6.4 Considerations for Future Research 125
6.5 Conclusion 127
References 130
Appendix A 144
Appendix B 145
Appendix C 146

LIST OF TABLES

Table 1-1
Elongation and desaturation pathway of Arachidonic, Eicosapentaenoic, and Docosahexaenoic from their parent Linoleic and α-linolenic

Table 1-2
Skeletal muscle fiber type composition

Table 3-1
Composition of experimental diets.

Table 3-2
EPA and DHA content within the rat gastrocnemius muscle tryglyceride fraction.

Table 4-1
Fatty acids composition of muscle phospholipid of rats bearing tumor and receiving chemotherapy

Table 4-2
Fatty acids composition of muscle triglyceride of rats bearing tumor and receiving chemotherapy

Table 5-1
Immunofluorescence Oil Red O Antibodies Cocktail for Rat Muscle

Table 5-2
Immunofluorescence Myosin Heavy Chain Antibodies Cocktail for Rat Muscle

LIST OF FIGURES

Figure 1.1
Suggested mechanisms underlying myosteatosis associated with cancer and chemotherapy treatment from non-cancer conditions

Figure 1.2
Suggested mechanisms of the role of EPA and DHA on mitigating myosteatosis associated with cancer and chemotherapy treatment from non-cancer conditions

Figure 2.1
Features of the pre-clinical model that has been developed in our lab

Figure 2.2
A fish oil containing diet fed prior to tumor implantation prevented fat accumulation within gastrocnemius muscle

Figure 3.1
Experimental study design

Figure 3.2
Oil Red O staining and analysis of neutral lipid localization in rat gastrocnemius muscle

Figure 3.3
Total triglyceride fatty acid levels in the rat gastrocnemius muscle quantified using gas chromatography

Figure 3.4
Patterns of adipogenic transcriptional factor mRNA expression in the rat gastrocnemius muscle

Figure 3.5
Fiber cross-sectional area in the rat gastrocnemius muscle

Figure 3.5
Relative tumor volume of tumor-bearing rats who underwent one or two cycles of CPT-11/5-FU treatment

Figure 4.1
Total Phospholipid fatty acid levels in the rat gastrocnemius muscle quantified using gas chromatography

Figure 5.1
Examples of manual quantification of neutral lipid content-based muscle fiber type from healthy rats

Figure 5.2
Oil Red O staining in rat tibialis anterior muscle

Figure 5.3
Fibers associated with neutral lipid accumulation in rat tibialis anterior muscle

Figure 5.4
Fiber composition in tibialis anterior muscle of rats bearing a ward colon tumor and receiving CPT-11/5-FU

Figure 5.5
Hybrid fiber composition in tibialis anterior muscle of rats bearing a ward colon tumor and receiving CPT-11/5-FU

Figure 5.6
Succinate Dehydrogenase of rat tibialis anterior muscle

Figure 5.7
Activities of citrate synthase and hydroxyacyl-CoA dehydrogenase of rat tibialis anterior muscle

Figure 5.8
Patterns of mitochondrial biogenesis transcriptional factor mRNA expression in the rat tibialis anterior muscle

Figure 6.1
Summary of mechanisms underlying fat accumulation within skeletal muscle

LIST OF SYMBOLS AND ABBREVIATIONS

Δ	delta
5-FU	5-fluorouraci
AA	arachidonic acid
acetyl CoA	acetyl co-enzyme a
AIN-76	American Institute of Nutrition-76
ALA	alpha- linolenic acid
ANOVA	analysis of variance
CS	citrate synthase
CPT-11	irinotecan
CSA	cross sectional area
CT	computed tomography image
CO_2	carbon dioxide
C/EBPs CCAAT	enhancer binding proteins
C/EBPβ CCAAT	enhancer binding protein beta
C/EBPα CCAAT	enhancer binding protein alpha
C/EBPσ CCAAT	enhancer binding protein gamma
DHA	docosahexaenoic acid
EPA	eicosapentaenoic
GLUT4	glucose transporter 4
HADH	3-hydroxyacyl-CoA dehydrogenase
LA	linoleic acid
MHC	myosin heavy chain
MRI	magnetic resonance imaging

ND	not detected
NBF	neutral buffered formalin
PBS-T	phosphate buffered saline with Tween®
PCR	polymerase chain reaction
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator
PL	Phospholipid
PPAR-α	peroxisome proliferator-activated receptor alpha
PUFA	polyunsaturated fatty acids
SDH	succinate dehydrogenase
SFA	saturated fatty acid
SN38	7-ethyl-10-hydroxycamptothecin
SPSS	Statistical Package for the Social Sciences
SREBP1-c	sterol regulator element binding protein factor 1
TFAM	Transcription Factor A, Mitochondrial
TG	triglyceride
TMN	tumor; node; metastases
TNF-α	tumor necrosis factor-alpha
UCP3	uncoupling protein 3
mm	millimeter
µg	microgram
µm	micrometer

CHAPTER 1

Introduction and Literature Review

1.1 Introduction

The relationship between body composition and risk of disease has become more clearly understood in recent years, as the technology available to non-invasively quantify body components has improved. The advent of noninvasive radiological techniques such as magnetic resonance [MR] imaging and computed tomography [CT] has enabled new explorations of physiological and pathological variation in muscle. In particular, the widespread use of CT scans to assess tumors in the oncological setting enables accurate and precise determination of body composition over the cancer trajectory and has become the gold standard for body composition measurements in people with cancer. CT imaging used to assess body composition have been instrumental in revealing low muscle mass in people with cancer, affecting about half of patients at diagnosis (1-3). CT imaging has also revealed altered radiation attenuation characteristics of muscle in people with cancer [reviewed by (4)]. This is of particular interest as low muscle radiodensity, detected by CT imaging, has been identified as an independent predictor of poor outcomes and mortality in cancer patients (5-12). Low radiation attenuation of muscle has been well correlated with fat content of muscle biopsy specimens (13), therefore enabling categorization of muscle with pathological fat accumulation, or myosteatosis. Myosteatosis has been associated with insulin resistance and diabetes (14), detraining, and injury (15). Most recently, myosteatosis has also been described in cancer patients, and is associated with worsened outcomes, including poorer survival (6-11). These studies have prompted investigation into the causes and biological features of cancer-related muscle pathology in an animal model to derive mechanisms of change in body composition.

In oncological settings, one approach to improve the therapeutic index of chemotherapy is to combine these drugs with adjuvant factors that enhance chemotherapy efficacy and decrease toxicities. The n-3 fatty acids eicosapentaenoic [EPA, 20:5] and docosahexaenoic [DHA, 22:6] are bioactive lipids reported to improve tumor toxicity of chemotherapy drugs and with decreasing host toxicities associated with chemotherapy drugs such as cisplatin, anthracyclines and alkylating agents in a variety of animal models including breast, colorectal, prostate, and lung cancers [reviewed by (16-18)].

Few clinical studies have been conducted to demonstrate that inclusion of EPA and/or DHA in diet improves outcomes of patients actively undergoing chemotherapy treatment including enhanced tumor response to treatment, reduced toxicities associated with chemotherapy drugs, improved quality of life, and overall survival (2,19,20).Thus, the human studies investigating drug efficacy in combination with fish oil supplementation align with the wealth of experimental evidence demonstrating enhanced therapeutic index.

In addition to an enhanced therapeutic index of chemotherapy when EPA and DHA are provided in the diet, our previous work (1) established an important relationship between skeletal muscle mass and EPA and DHA. The amount of EPA and DHA in plasma of newly diagnosed patients with advanced colorectal and lung cancer is low (21-23) and relates to the amount of muscle the patient has as well as the intensity of loss the patient may experience (1). Non-small cell lung cancer patients undergoing platinum based doublet chemotherapy experienced muscle loss and exhibited an increase in intermuscular adipose tissue over the course of treatment, however, daily EPA and DHA supplementation [2.2g/day] during chemotherapy stabilized muscle mass and significantly reduced the amount of intermuscular fat, assessed by sequential CT imaging, compared to patients who received standard of care (24). Therefore, restoring EPA and

DHA through supplementation prevented muscle loss in the majority of patients and reduced fat accumulation in muscle. Collectively, these results suggest that EPA and DHA could potentially be used as adjuvant factors to enhance chemotherapy efficacy and to prevent or treat fat accumulation that occurs in the presence of tumor or during chemotherapy; however, this has not been directly studied.

While EPA and DHA appear to exhibit promising effects on body composition, the mechanisms by which these alterations occur are unknown in the neoplastic state. In order to investigate the biological features and causes of cancer-related myosteatosis, as well as the mechanisms through which EPA and DHA may be exerting protective effects in the tumor-bearing state, with and without chemotherapy; appropriate preclinical models need to be identified. To our knowledge, no neoplastic model of myosteatosis in cancer has been reported. Accordingly, a discussion of existing mechanistic literature pertaining to EPA and DHA in other conditions where myosteatosis is presented, including obesity and insulin resistance are discussed, and the applicability of these mechanisms to EPA and DHA activity on muscle condition in cancer are considered.

1.2 Colorectal Cancer

The number of new colorectal cancer cases and deaths continue to rise as the Canadian population ages. In Canada, there are approximately 25,100 diagnoses per year and 9,300 deaths reported from colorectal cancer (25). On average, 69 Canadians will be diagnosed with colorectal cancer and 25 Canadians will die from it every day (25). Colorectal cancer is diagnosed at one of five stages. This staging system ranges from 0 to IV, indicating the severity of the disease (26). At Stage 0, the cancer is in the earliest phase, and it has not progressed beyond the inner layer, or mucosa, of the colon or rectum. The treatment during this stage involves effectively removing the

polyp via colonoscopy. In Stages I and II, the tumor has grown through the wall of the colon and may extend into nearby tissue; however, at this point, the cancer has not yet reached the lymph nodes. The tumor becomes increasingly aggressive until Stage IV, which indicates that the cancer has spread to other organs within the body (26). In order to determine the size and location of the primary tumor, the involvement of the lymph nodes, and the extent of metastasis, specialists utilize TNM descriptors, where T indicates the primary tumor, N denotes regional lymph nodes and M signifies distant metastasis. If the tumor is detected in Stage I, the patient has a 90% chance of surviving for five years and surgical removal of the tumor may cure the cancer. However, in advanced stages of cancer, the median survival duration is 5–6 months (27) and combination treatment may be provided. This treatment includes options such as surgical resection, chemotherapy, radiation therapy and biotherapy or immunotherapy (27).

1.3 First Line Chemotherapy Treatment Colorectal Cancer

Chemotherapy remains an essential step in the treatment of advanced colorectal cancer. Most chemotherapy agents target cells that divide rapidly, regardless of whether or not these cells are cancerous. As a result, toxic side effects are associated with chemotherapy (28). The combination of 5-fluorouracil [5-FU] and irinotecan [CPT-11] represents an effective combination of drugs used to treat colorectal cancer patients (29).

5-FU, which was developed in 1957, has become part of the standard therapy for most malignancies arising in the gastrointestinal tract and breast as well as for head and neck cancers. 5-FU belongs to a family of anti-metabolite chemotherapy drugs. Anti-metabolite factors replace biological substances such as folic acid; when the cells incorporate these substances into cellular metabolism, cells are unable to divide. 5-FU is converted into fluorodeoxyuridine monophosphate and fluorodeoxyuridine triphosphate which both inhibit thymidylate synthase, an enzyme

responsible for DNA synthesis and repair. 5-FU could also convert to fluorouridine triphosphate which causes RNA damage and produces apoptosis in the tumor (30). Several toxicities have been associated with 5-FU, including vomiting and nausea, cytopenias secondary to bone marrow suppression, palmar-plantar erythrodysesthesia (hand-foot syndrome) and cardiotoxicity (31). The overall response rate for 5-FU as a single agent in metastatic colorectal cancer is <10% (32); however, when 5-FU is combined with other drugs, such as CPT-11, the rate of response is significantly increased to 40–50% (29).

Irinotecan is a water-soluble semi-synthetic that is isolated from the Chinese/Tibetan ornamental tree *Camptotheca Acuminata* (33). This drug was produced in Japan and has demonstrated antitumor activity against a wide range of tumors, including colorectal, esophageal, leukemia, gastric, non-small-cell and small-cell lung cancers as well as lymphomas (34). In vivo, irinotecan is converted in the liver to a metabolite, 7-ethyl-10-hydroxycamptothecin [SN38], which appears to contribute to the antitumor activity of CPT-11. Irinotecan possesses a novel mechanism of action that depends on the inhibition of the eukaryotic enzyme DNA replication and cell death [reviewed by (35)]. Irinotecan has emerged as one of the most effective antitumor drugs for a wide range of tumor types, especially when combined with 5-FU (29). However, diarrhea and myelosuppression remain common dose-limiting toxicities of this antitumor drug [reviewed by (35)].

1.4 Measuring Body Composition and Muscle Condition in Cancer

High resolution image-based techniques such as CT and MRI exhibit specificity and precision that have been extensively validated and applied in body composition research (36-38). Apart from the quantity of muscle and fat, image-based approaches also reveal additional features

such as excess inter- and intra-myocellular lipid accumulation within muscle tissue (36). The overall fat content of muscle can be indirectly and non-invasively assessed (39,40) by making use of the fact that adipose tissue attenuates the applied radiation in a characteristic manner. The value and sign of attenuation measurements are determined by the speed at which radiation passes through the tissue, and are measured most typically in Hounsfield Units [HU], where water and air have a value of 0 HU and 1000 HU, respectively. Radiation passes more slowly through lean tissue than through water, giving lean muscle tissue a mean radiation attenuation with a positive sign, most prominently around +50 HU. By contrast, radiation passes more quickly through lipid-containing tissue than it does through water, and therefore the mean attenuation of adipose tissue has a negative sign, usually -100 HU. The attenuation ranges of muscle [45-50 HU] and total adipose tissue [-190 to -30 HU] have been defined (13,36,40-42). Within the range of radiation attenuation values for muscle, the fat content of muscle can be evaluated, since the overall attenuation value of muscle is decreased when a high amount of intramuscular fat is present. Altered radiation attenuation of muscle has been well correlated with triglyceride content of muscle biopsy specimens (14). Thus, HU values defined using CT reflect the amount of intramuscular adipose tissue and are useful in categorizing muscle as normal or exhibiting a pathological triglyceride and/ or intramuscular adipose tissue accumulation, defined as myosteatosis. Image-based methods of assessing muscular density have been applied primarily in research settings, and are not yet in routine clinical use.

Very little data exists to define the biological and physiological features of low muscles attenuation. Muscle tissue normally contains only small amounts of fat not intended for long-term lipid storage, but to be used as a short term source of energy. There are two potential fat depots within skeletal muscle: fat within myocytes [intramyocellular fat] and visible fat within the fascia

surrounding skeletal muscle [intermuscular fat]. Further studies are required to determine the site of fat in myosteatosis [intermuscular, intramuscular or both], and the mechanisms by which muscle is apparently replaced by adipose tissue in conditions characterized by low radiation-attenuating muscle.

1.5 Myosteatosis in Cancer

Until recently, low attenuating muscle, myosteatosis, has only been described in conditions of aging, injury, insulin resistance and Type 2 diabetes (39,40,43-45). Recent studies applying CT imaging to understanding features of individuals with cancer have revealed a wide variation of muscle radiation attenuation (46-48). Loss of skeletal muscle mass in cancer appears to generally, but not always, be concurrent with myosteatosis. Strikingly, compared to normal values [45-50 HU], the median value for cancer patients [35 HU; (8)] comparable to results reported for obese or diabetic patients with some patients exhibiting values of muscle attenuation in the range of 20-25 HU. Low muscle radiation attenuation has now been identified as an independent risk factor for mortality in cancer patients (6-9). Other study reported that muscle of cancer patients contained more and larger intramuscular lipid droplets as severity of weight loss progressed (49). The nature and characteristics of these pathologies and why they confer greater risk are unresolved.

1.6 Lipid and Essential Fatty Acids

Fat is not only an important source of energy in the form of triglyceride, but it is also essential for the survival and functioning of human and animal organs. Lipid is essential for every cell membrane in the body. Phospholipid act as an interface between external environments and the cells, and also partition intracellular compartments. Fat also stimulates the release of hormones and cytokines as signaling molecules and it fulfills crucial functions as enzyme cofactors, electron

carriers and intracellular messengers. There are several different types of fatty acids, including saturated with no double bonds, monounsaturated with one double bond and polyunsaturated with two or more double bonds, which being further divided into omega-3 [n-3] and omega-6 [n-6] fatty acids (50).

Essential fatty acids are the fatty acids that cannot be synthesized in animal and human tissues, and therefore must be obtained through the diet. Humans and animals can synthesize saturated and monounsaturated acyl chains from acetyl co-enzyme-A [acetyl CoA]. However, mammals lack an enzyme that adds a double bond before the n-9 position, and, as a result, alpha-linolenic acid [18:3n-3] and linoleic acid [18:2n-6] must be obtained from diet. Plasma phospholipid composition reflects endogenous and dietary fatty acids, and has been used as an index of fatty acid status (17).

1.7 EPA and DHA Status in Cancer Patients

Several studies suggest the existence of abnormalities in the fatty acid metabolism of people with cancer (21,23,49). Wasting syndrome in advanced cancer patients can constitute a marker for not only adipose tissue wasting but also muscle wasting (8,46,47). Alterations in fatty acid metabolism may cause a reduction in the availability of n-3 fatty acids within the body, which may subsequently enhance the wasting condition. Pratt et al. showed that in comparison to healthy subjects, advanced cancer patients who lost five percent or more of their pre-illness body weight had depleted stores of plasma essential fatty acids within phospholipid fractions. Depletion became even more evident after high-dose chemotherapy, when DHA and EPA levels were reduced to approximately 7% of the control values (21). Clinical evidence suggests that newly diagnosed cancer patients exhibited low level of n-3 fatty acids. From this perspective, both cancer and the

therapeutic treatments for this disease may represent contributing factors in the decrease of n-3 fatty acids [reviewed by (22)]. Fatty acid patterns of gastrointestinal mucosa have been reported to be altered in human gastric cancer. Specifically, the researchers analyzed the total fatty acid contents and evaluated their relative composition among the total fatty acids in mucosa as well as in the phospholipid fractions contained in paired cancerous and non-cancerous gastric tissues. The results of this investigation showed that cancerous mucosa had a higher ratio of n-6 to n-3 fatty acids in phospholipid fraction. This finding occurred from high levels of arachidonic acid concurrent with low levels of DHA and EPA in both total fatty acids and phospholipid fractions (51). Similarly, a study by our group reported a reduction in plasma phospholipid n-3 fatty acids, including EPA and DHA, in non-small cell lung cancer patients. This loss was associated with a decrease in fat tissue (23) and muscle mass compared with patients of stable weight (1). However, the mechanisms controlling the alteration of essential fatty acids in cancer patients remain unknown.

1.8 Mechanisms Underlying Myosteatosis and The Potential Role of EPA and DHA on Each of These Mechanisms

Several mechanisms may contribute with the development of myosteatosis in non-cancer conditions such as obesity and insulin resistance. These mechanisms include: 1) increased expression of genes involving in the adipogenesis process and lipid synthesis in muscle such as CCAAT/enhancer binding protein [*C/EBPs*], peroxisome proliferator-activated receptor gamma [*PPARγ*], and sterol regulatory element binding protein 1c isoform [*SREBP-1c*]; 2) altered skeletal muscle triglyceride and phospholipid composition; 3) changing in skeletal muscle fiber composition and 4) modulated mitochondrial oxidative capacity [Figure 1.1].

1.8.1 Adipogenesis Transcriptional Factors

At the molecular level, the crucial roles of adipogenesis transcriptional factors have been studied in preclinical models of other conditions associated with myosteatosis such as obesity and diabetes (52). These genes are involving in activation of adipogenesis, lipid uptake, and lipogenesis by regulating downstream pathways of fatty acids synthesis and catabolism such as lipoprotein lipase, hormone sensitive lipase, acetyl CoA carboxylase, and fatty acid synthase, stearoyl coA desaturase (52-55). *PPARγ* is expressed in skeletal muscle in response to *C/EBPβ* and *C/EBPα* activation (56,57) and has been associated with ectopic fat accumulation into skeletal muscle in an animal model of high-fat-diet-induced insulin resistance (52,58).

EPA and DHA are known to have anti-adipogenic functions (59,60) and may have the ability to decrease mRNA expression of these genes in muscle cells. Increasing the EPA and DHA content of bovine muscle through diet resulted in a reduction in the transcription factor *SREBP-1c* and subsequent genes regulating downstream pathways of fatty acid synthesis in muscle tissue including acetyl CoA carboxylase, fatty acid synthase, stearoyl coA desaturase (61). Expression of *SREBP-1c* in muscle of cattle is reported to be 2-fold lower when fish oil versus soybean oil diets is provided (62). Collectively, these results suggested that adipogenesis transcriptional factors should be considered as one of the mechanisms underlying myosteatosis that occur in cancer, however, this has not been explored in cancer.

1.8.2 Skeletal Muscle Fatty Acids Composition

Alterations in the composition of plasma membrane phospholipid fatty acids modify the thickness and fluidity of the lipid bilayer, which can subsequently impact skeletal muscle function. Several cross sectional studies on obese and insulin resistance patients suggest that skeletal muscle

membrane phospholipid display a different fatty acid composition compared with lean subjects, with a higher proportion of the saturated fat palmitate [C16:0] and lower concentrations of linoleic acid n–6 fatty acid (63-65). Differences in membrane phospholipid fatty acids can impact skeletal muscle anabolism and increase intramyocellular triglyceride [Figure 1.1]. For example, a study evaluated the association between the level of muscle triglyceride and insulin resistance in nondiabetic normal-weight or obese people. Skeletal muscle triglyceride level was higher in obese patients compared to people with normal weight and it was negatively correlated with glucose uptake. In addition, the triglyceride fatty acid composition was significantly different between the two groups. Obese people exhibited higher concentration of saturated fatty acids compared to normal weight people and it was positively correlated with insulin resistance (66).

Antunes et al (67). studied the effect of cancer on mitochondrial phospholipid remodelling in wasted skeletal muscle and the consequences of this relationship for mitochondrial functionality. These authors used an animal model of urothelial carcinoma, which was induced through exposure to N-butyl-N-[4-hydroxybutyl]-nitrosamine [BBN]. The model experienced a significant loss of body weight due to a reduction in skeletal muscle mass. Additionally, histological evidence of muscle atrophy relates to reduced respiratory chain activity and enhanced expression of mitochondrial uncoupling protein 3 [UCP3, (67)]. In combination, these two factors decrease the ability of wasted muscle to produce ATP which could also result in fat accumulation within the muscle due to mitochondrial dysfunction. The implications of these observations suggest the regulation of phospholipid biosynthetic pathways as potential therapeutic targets for the management of muscle alteration in cancer.

Improvement of the insulin response is the most widely explored mechanism for the beneficial effects of EPA and DHA fatty acids on muscle and has been studied in many

experimental systems and in a number of non-cancer disease states (68-73). Dangardt et al (74). reported improved insulin sensitivity and reduced triglyceride accumulation in the muscle of obese children when EPA and DHA were provided. In a crossover design, adolescent children [14–17 y] were randomized to receive 1.2 g fatty acids [930 mg EPA, 290 mg DHA] or placebo for three months with a six-week washout period. Skeletal muscle biopsies revealed increased total n-3 fatty acids, EPA and DHA concentrations in muscle phospholipids during the supplemental period which was accompanied by improved glucose tolerance [by 39%] and restoration of insulin concentration [by 34%] as well as improved insulin sensitivity. While improvement in insulin response was observed only in female subjects, all subjects exhibited lower muscle triglyceride content after EPA and DHA supplementation compared to placebo (74). In a hyperinsulemic/hyperglycemic state, Smith et al. reported increases in mTOR signaling, muscle size and muscle protein synthesis after 8 weeks of supplementing with n-3 fatty acids in healthy adults (75).

In several experimental studies, EPA and DHA have been reported to support the anabolic potential of muscle (68,70,72,76). In rats, n-3 fatty acid incorporation into muscle increased membrane unsaturation concurrent with improved insulin sensitivity and decreased muscle triglyceride content (77). Fat-1 mice, which are capable of endogenous n-3 fatty acid synthesis, exhibit better glucose tolerance than control mice (68). Some, (70,76,78,79) but not all (80,81) studies have shown an effect of n-3 fatty acids on AKT activation, a regulator of muscle growth. Incorporation of EPA and DHA into the muscle membrane alters its composition, and may modulate key membrane substrates involved in the insulin signaling pathway and subsequent protein synthesis and improve muscle anabolism (82).

1.8.3 Skeletal Muscle Fiber Type Composition and Mitochondrial Oxidative Capacity

Lipids stored in muscle usually serve as a fuel for mitochondria oxidation (83), suggesting that a change in mitochondrial function and/or content within skeletal muscle may cause lipid accumulation inside of the muscle. Recent research has shown that an alteration in mitochondrial homeostasis in skeletal muscle has been associated with muscle wasting in cancer (84-86). Dysfunction of mitochondria has been linked to increased intramyocellular lipid content in non-cancer conditions, such as in elderly population [reviewed by (87)], insulin resistance and obesity [(88), Figure 1.2]. However, no existing studies have associated mitochondrial content and dysfunction to myosteatosis development in preclinical model of cancer. Thus, the potential association between mitochondria content or function and myosteatosis in cancer needs to be addressed in response to tumor and chemotherapy treatment.

Mitochondrial content of skeletal muscle could be affected by altering muscle fiber compositions [Table 1-2]. Skeletal muscle fibers are classified according to the type of myosin (slow or fast) and the degree of oxidative phosphorylation that the fiber undergoes. The type I and IIA fibers [slow acting] are rich in myoglobin [red fibers], and mitochondria compared with type IIB and IID/X fibers. Furthermore, type I and IIA have a slow contraction speed, low myosin ATPase activity, rely on oxidation phosphorylation ATP production, and are highly resistant to fatigue, thus they are called slow acting. Type IIB and IID/X fibers [white fibers] are fast acting, mitochondria poor, and rely on glycolytic ATP production. Muscle fibers vary in intramyocellular triglyceride content [Table 1-2]. Several researchers used electron microscopy and biochemical analysis to assess the concentration of intramyocellular triglyceride on single fibers in healthy male subjects. These studies have shown that intramyocellular triglyceride content was threefold higher in type I fibers than in type II fibers (89). Suggesting that intramyocellular triglyceride

concentration may be influenced by the amount of mitochondria inside the fibers as the greater fat oxidative capacity in the type I fiber is associated with greater intramyocellular triglyceride storage. Changes in skeletal muscle fiber composition and / or size could influence mitochondrial content within the muscle and interfere with fat metabolism. Experimental studies have reported the relationship between the oxidative capacity of muscles and depleted muscle mass during the development of cancer. In this study, the researchers collected gastrocnemius muscle from Apc [Min/+] mice at 20 weeks of age. Mitochondrial oxidative capacity was reduced in mice of severe loss of muscle mass. Specifically, muscle loss entailed a threefold reduction in the major regulation enzymes for oxidative phosphorylation including cytochrome-c oxidase complex subunit IV and a 50% reduction in the level of succinate dehydrogenase of gastrocnemius muscle of the Apc [Min/+] mouse compared to wild-type control mice. Specifically, these alterations occurred in the type IIA and IIB fibers of the gastrocnemius muscle which was distinguished by qRT-PCR (86). These findings suggest an association between the specific fiber type in cancer and the reduction of muscle oxidative capacity. However, this study neglected to assess the amount of lipids inside of the muscle. A recent study used Male C57Bl6/J mice to assess fiber type's specific lipid accumulation as well as muscle oxidative capacity by using tibialis anterior [highly glycolytic fiber, mainly type IIB] and soleus [highly oxidative muscle; mainly type I fiber] muscles in response to 12 weeks high fat diet intervention reported that triglycerides content in the soleus of the control group was ≈ two times higher than the tibialis anterior muscle (90). Triglyceride accumulation in muscle could be specific to fiber types. If the tumor-bearing state is decreasing the amount of specific fiber types, this may also affect which muscles accumulate intramyocellular triglyceride. However, fiber type's specific lipid accumulation has not been yet defined in cancer.

Many beneficial effects of EPA and DHA on mitochondrial content and oxidative capacity have been reported, including increasing mitochondria number [Figure 1.2], improved function of the enzyme complexes within the electron transport chain, and an improved capacity to appropriately use physiologically available fuels (91,92). Several experimental studies have shown that EPA and DHA enhance lipid oxidation in skeletal muscle via AMPK and PPAR-α activation. In addition, these two fatty acids increase mitochondrial biogenesis through the activation of PGC-1α in skeletal muscle cells (91,92). Another study used, male Wistar rats received either EPA or DHA for three months. In comparison to rats fed a DHA diet, rats fed EPA exhibited higher mitochondria biogenesis in type I fibres of soleus and diaphragm muscles as well as an increase in the size distributions of mitochondrial areas, which were associated with decreased lipid droplets in this specific type of fiber(93).

UCP3 is a mitochondrial membrane transporter expressed mainly in skeletal muscle where it plays an important role in energy expenditure and fat oxidation. EPA and DHA have been reported to up-regulate UCP3 mRNA and promotor activity in a dose-dependent manner in C2C12 muscle cells, potentially through an AMPK-mediated pathway (94). In the liver, lipogenesis is reduced and beta oxidation is elevated during n-3 fatty acid interventions [reviewed by (95)]. Thus, there is a growing body of evidence suggesting the presence of n-3 fatty acids are beneficial for energy homeostasis and body composition, with n-3 fatty acids reducing body fat in general, potentially through a greater partitioning of energy into lean versus fat tissue, combined with an improved capacity to appropriately use physiologically available fuels (78,96), processes which collectively support muscle anabolism and lower fat content of muscle.

1.9 Animal Model of Myosteatosis and Diet

An animal model has been developed and refined in our lab that corresponds to the delivery of therapy for colorectal cancer in humans (97,98). Fischer rats bearing the Ward colon 26 tumor receive two cycles of irinotecan plus 5-fluorouracil [CPT-11 plus 5-FU] therapy which carefully recapitulates first line therapy for colorectal cancer in humans (97,98). Diets resembling human diets in Westernized countries with respect to all macro- and micronutrients, including quantity and composition of dietary lipids (98) were provided. When this diet was supplemented with n-3 fatty acids [2.7% EPA + DHA], fatty acid enrichment of the muscle tissue occurred within 6 days at 2% and 4%, respectively (99). This enrichment thus represents a biologically relevant dose of n-3 with a resulting n-6: n-3 fatty acid ratio of 3.2, which is identical to the n-6: n-3 ratio reported for humans in muscle phospholipid after 3 weeks of supplementation with fish oil [2.4 g per day EPA + DHA; (75)]. This model has enabled us to demonstrate that tumor-bearing animals exhibit elevated muscle triglyceride content which is accelerated following chemotherapy, similar to what Murphy et al. reported in non-small cell lung cancer patients. Triglyceride accumulation in the muscle was prevented by dietary supplementation with EPA and DHA initiated prior to and continued during treatment (24). At present, no other reports exist regarding the effects of EPA and DHA on lipid infiltration of muscle in the neoplastic state, and these encouraging results will initiate further research in this area.

1.10 Conclusion

Low muscle mass and infiltration of muscle with fat are features of body composition which contribute to worsened outcomes in cancer and other diseases. The pathogenesis of muscle wasting and myosteatosis in cancer is incompletely understood. Low muscle mass and low attenuating muscle have recently emerged as independent risk factors for death and disability in

people with cancer (47,48). Low plasma concentrations of EPA and DHA are independently and strongly related to the presence of muscle loss over the treatment period (23), which suggests an important and potentially modifiable relationship between skeletal muscle metabolism and n-3 fatty acids. The ability to modify muscle wasting and intramuscular fat accumulation has a broad scope of application to aging, diabetes, obesity and various forms of muscle atrophy, which share these common features. Number of mechanisms may contribute to the ability of n-3 to alter body composition, including alterations in adipogenesis transcriptional factors, phospholipid composition, and fiber types and mitochondrial density as well as oxidative capacity. Before mechanisms can be more thoroughly defined for benefits of EPA and DHA, a more complete understanding of the features of the muscle characterized by fat infiltration is required and in order to investigate that, appropriate preclinical models of cancer associated-myosteatosis need to be identified.

Table 1-1 Elongation and desaturation pathway of Arachidonic, Eicosapentaenoic, and Docosahexaenoic from their parent Linoleic and α-linolenic

N-6	N-3
Linoleic (LA) (18:2 n-6)	α-linolenic (ALA) (18:3 n-6)
↓ **Desaturase Δ6**	↓ Desaturase Δ6
γ-linolenic (18:3 n-6)	Stearidonic (18:4 n-3)
↓ Elongase	↓ Elongase
Dihomo- γ-linolenic (20:3 n-6)	Eicostrienoic (20:4 n-3)
↓ Desaturase Δ5	↓ Desaturase Δ5
Arachidonic (AA) (20:4 n-6)	Eicosapentaenoic (EPA) (20:5 n-3)
↓ Elongase	↓ Elongase
Ardanic (22:4 n-6)	Docosapenraenoic (22:5 n-3)
↓ Desaturase Δ4	↓ Elongase
Docosapentaenoic (22:5 n-6)	Tetracosapentaenoic (24:5 n-3)
	↓ Desaturase Δ4
	Tetracosahexaenoic (24:6 n-3)
	↓ peroxisomal oxidation
	Docosahexaenoic (DHA) (22:6 n-3)

The red colors are the essential fatty acids from n-6 and n-3 families

Table 1-2 Skeletal muscle fiber type composition

	Type I	Type IIA	Type IIDX	Type IIB
Contracting Duration	Hours (Slow)	Less than 30 minutes (Moderately Fast)	Less than 5 minutes (Fast)	Less than 1 minutes (Very Fast)
Resistance to Fatigue	High	High	Moderate	Low
Mitochondrial Density and Oxidative Capacity	High	High	Moderate	Low
Main Fuel Storage	Triglyceride	Glycogen and Creatine Phosphate	Glycogen and Creatine Phosphate	Creatine Phosphate

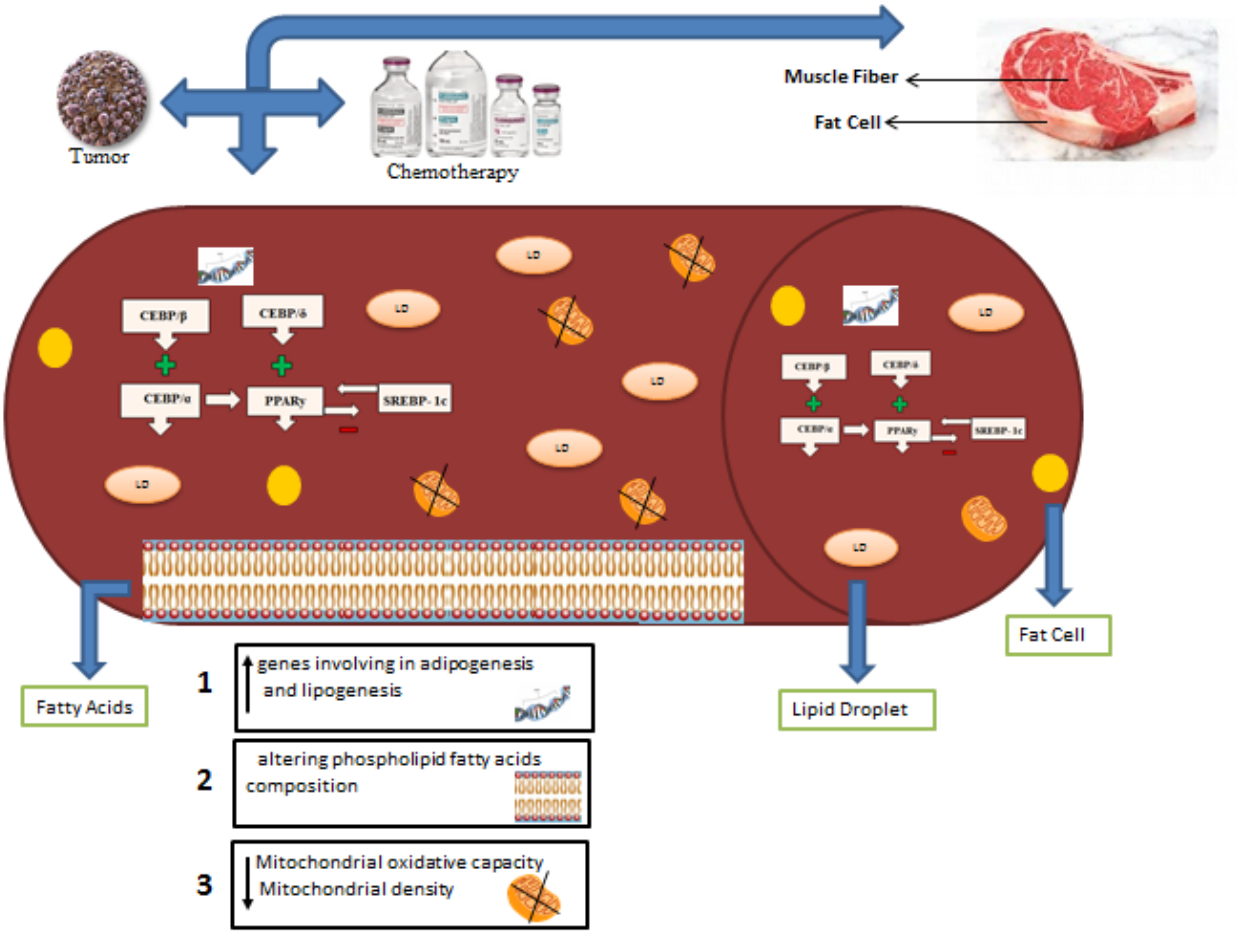


Figure 1.1 Suggested mechanisms underlying myosteatosis associated with cancer and chemotherapy treatment from non-cancer conditions

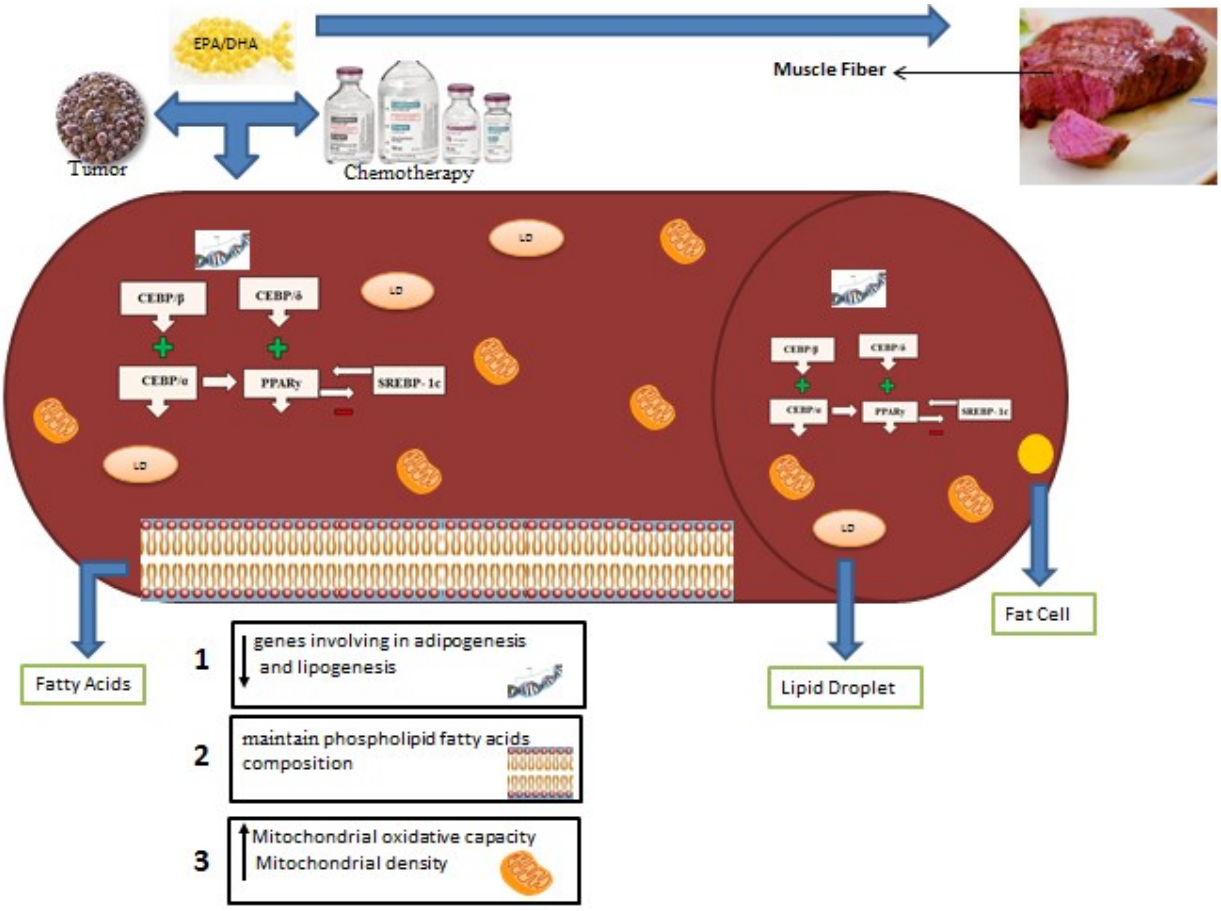


Figure 1.2 Suggested mechanisms of the role of EPA and DHA on mitigating myosteatosis associated with cancer and chemotherapy treatment from non-cancer conditions

CHAPTER 2

Research Plan

2.1 Rationale

The number of new colorectal cancer cases and deaths continue to rise as the Canadian population ages (25). Chemotherapy remains one of the essential treatments for advanced colorectal cancer patients (29). However, chemotherapy is associated with muscle wasting (1,3,6,24,48,100). Recently, non-invasive radiological image-based techniques, such as computed tomography [CT], have revealed that in addition to low muscle mass, a pathological accumulation of fat in skeletal muscle (myosteatosis) also occurs in cancer patients (6,7,9,24,46-48). The fat content of muscle can be evaluated by using CT, since the overall attenuation value of muscle is decreased as the triglyceride content of muscle increases [see Chapter 1 section 1.4]. Low muscle attenuation has been found to be associated with shorter progression- and disease-free survival (6,9), and overall survival (6-8) in cancer patients. Although the relationship between myosteatosis and poor outcomes is observed, the characteristics of increased fat content within skeletal muscle has not been resolved and no mechanisms associated with myosteatosis have been established in cancer.

Clinical practice aims to improve the effectiveness of chemotherapeutics to reduce tumor growth while mitigating associated side effects such as alterations in skeletal muscle condition. One approach to improve the therapeutic index of antineoplastic therapies is to combine cytotoxic drugs with adjuvant factors that enhance anti-tumor efficacy and reduce harmful side effects (101-105). Therefore, identification of an adjuvant factor to chemotherapy that can protect against myosteatosis would be of great benefit. The omega-3 polyunsaturated fatty acids eicosapentaenoic acid [EPA, 20:5n-3] and docosahexaenoic acid [DHA, 22:6n-3], which are highly abundant in fatty

fish and their oils, are emerging as promising nutritional adjuvants to chemotherapy as they have been reported to enhance anti-tumor effects and reduce drug-associated toxicities of antineoplastic agents including cisplatin, anthracyclines and alkylating agents in a variety of animal models including breast, colorectal, prostate, and lung cancers (7,16,97,106-112). A Previous clinical trial in our laboratory (1) established an important relationship between skeletal muscle mass and plasma level of EPA and DHA. Non-small cell lung cancer patients undergoing chemotherapy experienced muscle loss and exhibited an increase in intermuscular adipose tissue over chemotherapy treatment, however, daily EPA and DHA supplementation [2.2g/day] during chemotherapy stabilized muscle mass and significantly reduced the amount of intermuscular fat, assessed by CT imaging, compared to patients who received standard of care (24). Therefore, restoring n-3 fatty acids through supplementation prevented muscle loss in the majority of patients and reduced fat accumulation in muscle. Collectively, these results suggest that EPA and DHA are important for muscle health and could potentially mitigate fat accumulation that occurs in the presence of tumor or during chemotherapy. This has not been directly studied and no biological measure of fat in muscle was obtained in that study. Also, the mechanisms behind these observations are not defined.

Mechanisms associated with myosteatosis have been reported in non- cancer conditions such as obesity and insulin resistance. These mechanisms include: 1) increase of expression of genes involving in adipogenesis program and lipid synthesis in muscle such as CCAAT/enhancer binding protein [*C/EBPs*], peroxisome proliferator-activated receptor gamma [*PPARγ*], and sterol regulatory element binding protein 1c isoform [*SREBP-1c*]; 2) altering skeletal muscle phospholipid composition 3) changing in skeletal muscle oxidative fiber composition and 4)

mitochondrial dysfunction. The association between each of these mechanisms and myosteatosis has been discussed in details in the previous chapter [see Chapter 1 section 1.8].

To investigate the biological features and causes of cancer-related myosteatosis, as well as the mechanisms through which EPA and DHA may be exerting its protective effects in the tumor-bearing state, with and without chemotherapy treatment, appropriate preclinical models need to be identified. An animal model has been developed and refined in our laboratory that parallels the delivery of drug therapy for colorectal cancer in humans (98,99) is used. Specifically, Fischer rats bearing the Ward colon 26 tumor receive two cycles of irinotecan [CPT-11] and 5-fluorouracil [5-FU] therapy, which carefully recapitulates first line therapy for colorectal cancer in humans. This model closely features the same doses, cycles, and level of toxicity observed in humans [Figure 2.1]. Tumor bearing animals show considerable elevated triglyceride content, which is accelerated with chemotherapy [Figure 2.2 (97,99)], similar to what is observed clinically (24). To evaluate mechanisms associated with myosteatosis, this animal model enables interventions at different time points before and after myosteatosis has been developed to assess the ability of EPA and DHA to prevent and treat myosteatosis.

2.2 Research Objectives and Hypotheses

Objective 1

The objective of this work was to characterize a preclinical model of cancer-associated myosteatosis and then, use this model to investigate the effects of dietary EPA and DHA supplementation on tumor- and subsequent chemotherapy-associated fat content of muscle, as well as the tumor response to chemotherapy. Additionally, I wanted to determine whether EPA and

DHA supplementation beginning at the initiation of chemotherapy [adjuvant] has similar efficacy compared with starting EPA and DHA supplementation prior to tumor implantation [long term].

Hypothesis 1

It was hypothesized that compared to healthy rats not bearing a tumor, rats bearing the Ward colorectal tumor will exhibit:

i) Higher content of neutral lipid between muscle fiber and lipid droplets within muscle fiber
ii) Higher content of triglyceride fatty acid in skeletal muscle
iii) Higher mRNA expression of genes involved in adipogenesis program

Hypothesis 2

It was hypothesized that compared to tumor-bearing rats, rats receiving 1- or 2-cycles of chemotherapy will exhibit**:**

i) Higher content of neutral lipid between muscle fiber and lipid droplets within muscle fiber
ii) Higher content of triglyceride fatty acid in skeletal muscle
iii) Higher mRNA expression of genes involved in adipogenesis program

Hypothesis 3

It was hypothesized that compared to rats fed a control diet, rats receiving tumor alone or 1- or 2-cycles of chemotherapy fed a long term fish oil diet will exhibit:

i) Lower content of neutral lipid between muscle fibers and lipid droplets within muscle fibers
ii) Lower content of total triglyceride fatty acid in skeletal muscle

iii) Lower mRNA expression of genes involved in adipogenesis program

iv) Lower tumor volume following 1- and 2- cycles of chemotherapy

v) Adjuvant fish oil diet will be as effective as long term fish oil diet for each of the measures

These hypotheses are investigated in Chapter 3.

Objective 2

The objective of this work was to characterize the fatty acid composition of phospholipid and triglyceride of muscle tissue in a preclinical model of colon cancer with or without chemotherapy treatment fed a control diet or fish oil diet prior [long term] and after tumor implantation [adjuvant diet]. This will allow us to determine the association between the proportion of EPA and DHA in gastrocnemius muscle and total triglyceride content of muscle. In addition, we want to determine whether supplementation beginning at the initiation of chemotherapy [adjuvant] will result in similar fatty acids proportions in gastrocnemius muscle and similar effect on total triglyceride content of muscle as long term fish oil feeding.

Hypothesis 1

It was hypothesized that compared to healthy rats not bearing a tumor, rats bearing the Ward colorectal tumor will exhibit:

i) Lower total phospholipid content

ii) Lower proportion of EPA and DHA and total n-3 fatty acids

iii) Higher proportion of n6/n3 fatty acid ratio

iv) Higher proportion of saturated fatty acid

Hypothesis 2

It was hypothesized that compared to tumor-bearing rats, rats receiving 1- or 2-cycles of chemotherapy will exhibit**:**

i) Lower total phospholipid content

ii) Lower proportion of EPA and DHA

iii) Higher proportion of n6/n3 fatty acid ratio

iv) Higher proportion of saturated fatty acid

Hypothesis 3

It was hypothesized that compared to rats fed a control diet, rats received tumor alone or 1- or 2-cycles of chemotherapy fed a long term fish oil diet will exhibit:

i) Higher total phospholipid content

ii) Higher EPA and DHA

iii) Lower n6/n3 fatty acid ratio

iv) Lower saturated fatty acid

vi) Adjuvant fish oil diet will be as effective as long term fish oil diet for each of the measures

These hypotheses are investigated in Chapter 4.

Objective 3

The objective of this work was to evaluate skeletal muscle neutral lipid content and location, fiber composition, and if the accumulation is related to specific fiber type in a preclinical model of colon cancer fed a control diet or fish oil diet prior [long term] and after tumor implantation [adjuvant diet] and if there is a relationship between lipid accumulation within the tibialis anterior muscle and mitochondrial content and oxidative capacity. Additionally, we wanted to determine whether EPA and DHA supplementation beginning at the initiation of chemotherapy

[adjuvant] was able to elicit similar effects compared with beginning prior to tumor implantation [long term].

Hypothesis 1

It was hypothesized that compared to healthy rats not bearing a tumor, rats bearing the Ward colorectal tumor will exhibit:

i) Higher content of neutral lipid staining within muscle fiber
ii) Lower number of oxidative fiber
iii) Lower mitochondrial content and oxidative capacity

Hypothesis 2

It was hypothesized that compared to tumor-bearing rats, rats receiving 1- or 2-cycles of chemotherapy will exhibit**:**

i) Higher content of neutral lipid staining within muscle fiber
ii) Lower number of oxidative fiber
iii) Lower mitochondrial content and oxidative capacity

Hypothesis 3

It was hypothesized that compared to rats fed a control diet, rats received tumor alone or 1- or 2-cycles of chemotherapy fed a long term fish oil diet will exhibit:

i) Lower content of neutral lipid staining between muscle fiber
ii) Higher number of oxidative fiber
iii) Higher mitochondrial content and oxidative capacity
vii) Adjuvant fish oil diet will be as effective as long term fish oil diet for each of the measures

These hypotheses are investigated in Chapter 5.

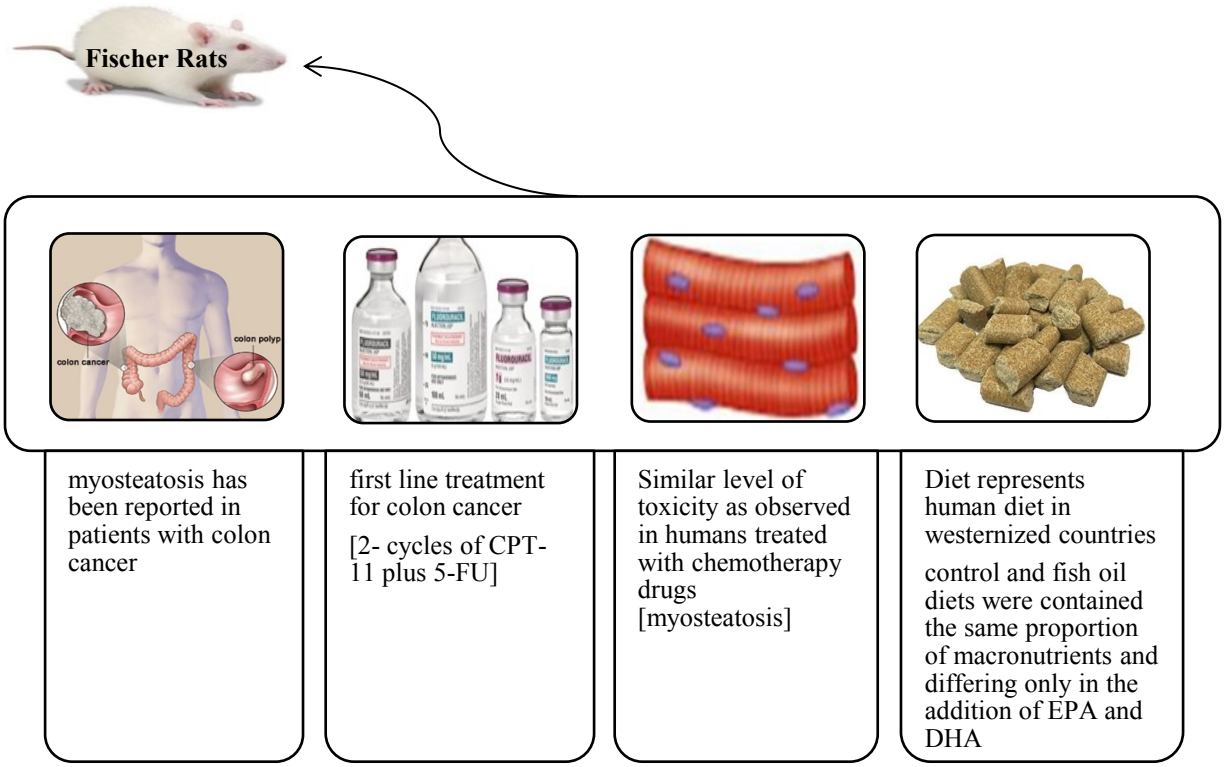


Figure 2.1 Features of the pre-clinical model that has been developed in our lab. CPT-11, irinotecan; 5-FU, 5-fluorouracil; EPA, eicosapentaenoic; DHA, docosahexaenoic

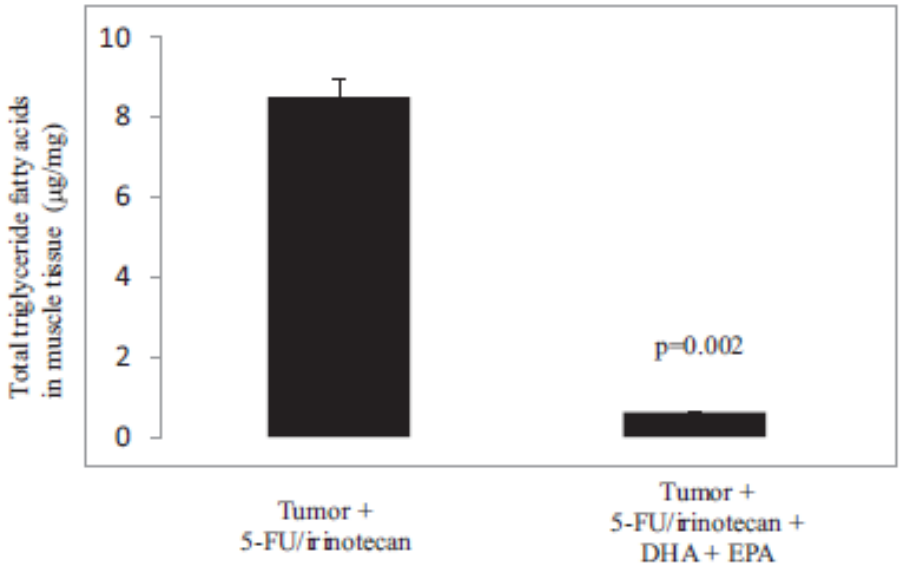


Figure 2.2 A fish oil containing diet fed prior to tumor implantation prevented fat accumulation within gastrocnemius muscle. Triglyceride fatty acid content of gastrocnemius muscle in a Ward colon tumor-bearing rat model, 7 days following treatment with CPT-11 and 5-FU and provided diets with or without fish oil that was fed prior to tumor implantation (2% *w*/*w*; $n = 8$ in each group). CPT-11, irinotecan; 5-FU, 5-fluorouracil; EPA, eicosapentaenoic; DHA, docosahexaenoic

CHAPTER 3

Fish Oil Mitigates Myosteatosis and Improves Chemotherapy Efficacy in a Preclinical Model of Colon Cancer

3.1 Introduction

Clinical practice aims to improve the effectiveness of chemotherapeutics to reduce tumor growth while mitigating harmful side effects. One approach to improve the therapeutic index of antineoplastic therapies is to combine cytotoxic drugs with adjuvant factors that enhance anti-tumor efficacy and reduce harmful side effects. Pathological alterations in skeletal muscle have been identified in cancer patients undergoing treatment, including muscle loss and fat accumulation (5-9,100,113). Specifically, myosteatosis, defined as the pathological accumulation of fat in skeletal muscle, is emerging as an important prognostic factor in the oncology setting (6-9). Low skeletal muscle density, which reflects high skeletal muscle fatty infiltration, is associated with shorter progression- and disease-free survival (6,9), and overall survival (6-8) in cancer patients treated with various therapies including chemotherapy. Therefore, identification of an adjuvant factor to chemotherapy that can protect against myosteatosis, in addition to enhancing tumor cytotoxicity, would be of great benefit.

The omega-3 polyunsaturated fatty acids, eicosapentaenoic acid [EPA, 20:5n-3] and docosahexaenoic acid [DHA, 22:6n-3], which are highly abundant in fish oil, are emerging as promising nutritional adjuvants to chemotherapy as they have been reported to reduce drug-associated toxicities and enhance anti-tumor effects in a variety of antineoplastic agents in a number of *in vitro* and *in vivo* preclinical models [reviewed by (16)]. There is also accumulating evidence from clinical trials that EPA and DHA supplementation improves patient outcomes during cancer chemotherapy, including improved muscle condition and a greater tumor response

rate [reviewed by (18)]. Specifically, we showed that advanced non-small cell lung cancer patients who supplemented with EPA and DHA during treatment had a preservation of muscle mass, less intermuscular adipose tissue, and better tumor responses compared to those not taking fish oil [standard of care] (2,24). Therefore, EPA and DHA may protect against myosteatosis while improving tumor response to antineoplastic agents. While EPA and DHA reduced myosteatosis in other human pathological conditions [reviewed by (97)], it has yet to be investigated in the oncology setting. Appropriate preclinical models are required to investigate the biological features and causes of cancer-associated myosteatosis, as well as the mechanisms through which EPA and DHA may be exerting their protective effects in the tumor-bearing state, with and without chemotherapy.

While conditions such as insulin resistance and obesity suggest that impaired skeletal muscle fatty acid metabolism may be responsible for pathological fat accumulation in muscle (114), cancer-associated myosteatosis may also involve mechanisms related to adipogenesis. Specifically, cancer has been shown to upregulate the expression of adipogenic genes in skeletal muscle, including CCAAT/enhancer-binding protein *[C/EBP]β*, a potent activator of adipogenesis (115). *C/EBPβ, δ,* and *α,* and peroxisome proliferator-activated receptor *[PPAR]γ*, are important transcriptional factors involved in robust adipocyte gene expression (116). For example, overexpression of *C/EBPα* and/or *PPARγ* have been shown to convert myoblasts into adipocytes by promoting adipogenesis and lipogenesis (117), and increase skeletal muscle triglyceride [TG] *in vivo* (58). Whereas, reducing the n-6/n-3 ratio decreased the expression of *PPARγ* and inhibited adipogenesis in the 3T3-L1 pre-adipocyte cell line (59). *In vivo,* feeding a diet rich in fish oil prevented myosteatosis [induced by a lard-based high fat diet)] with no changes in fatty acid oxidation, suggesting an alternative mechanism by which n-3 fatty acids modifies fat content of

muscle (118). Collectively, it appears that cancer-associated myosteatosis may involve mechanisms related to adipocyte gene expression, which can potentially be modified in the presence of n-3 fatty acids supplementation.

We have established an animal model to study interactions amongst tumor [rats bearing the Ward colon tumor] and chemotherapy, combined irinotecan [CPT-11] and 5-fluorouracil [5-FU], (98,110) that represents the first-line chemotherapy treatment regime for colorectal cancer, and elicits a similar level of toxicity as observed in humans treated with this drug combination. The current study aimed to identify a preclinical model of cancer-associated myosteatosis and then, use this model to investigate the effects of dietary EPA and DHA on tumor- and subsequent chemotherapy-associated fat content of muscle, as well as the tumor response to chemotherapy. Additionally, we wanted to determine whether feeding dietary EPA and DHA beginning at the initiation of chemotherapy [adjuvant] was able to elicit similar effects compared to a diet fed beginning prior to tumor implantation [long term]. We hypothesized that EPA and DHA supplementation would prevent tumor-associated myosteatosis before treatment, and that both the long term and adjuvant fish oil diets would similarly mitigate chemotherapy-associated fat accumulation in muscle through the inhibition of adipogenic/lipogenic transcriptional factor signaling, as well as enhance the tumor response to chemotherapy.

3.2 Materia and Methods

Experimental procedures were reviewed and approved by the University of Alberta Institutional Animal Care Committee and conducted in accordance with the Guidelines of the Canadian Council on Animal Care.

3.2.1 Animal Model and Experimental Design

Female Fischer 344 rats [n=72] weighing an average of 127 ± 18 g aged 11-12 weeks were received from Charles River [St. Constant, QC, Canada]. Rats were housed two per cage containing bedding and filter tops during the seven-day acclimation period and one per cage when initial diets were assigned. Rats received twelve hours of a light:dark cycle per day, and were kept in a positive air pressure room at a constant temperature [22°C]. Water and food was provided *ad libitum* throughout the entire experiment.

Experimental design is outlined in Figure 1. All rats were initially fed a control diet during the seven-day acclimation period and then, one week prior to tumor implantation, rats were randomly assigned to one of three diets: 1) control diet [n=24]; 2) long term fish oil diet [n=24]; 3) adjuvant fish oil diet (control diet until chemotherapy was initiated, then switched to the fish oil diet; n=16).

To examine a potential preclinical model of cancer-associated myosteatosis, two weeks after tumor implantation, rats on the control diet were either euthanized [n=8] or underwent one cycle [cycle-1; n=8] or two cycles [cycle-2; n=8] of chemotherapy. To investigate the effects of EPA and DHA supplementation before and during chemotherapy treatment, a group of rats on the long term fish oil diet were euthanized two weeks after tumor implantation [n=8], while the remaining rats on the long term and adjuvant fish oil diets underwent one cycle [n=8 each] or two cycles [n=8 each] of chemotherapy. Rats serving as a reference group [n=8] did not undergo tumor implantation or receive chemotherapy, consumed only the control diet throughout the entire study, and were otherwise handled in the same manner as the experimental groups.

3.2.2 Tumor Injection and Chemotherapy

The Ward colorectal carcinoma [0.05 g; provided by Dr. Y Rustum, Roswell Park Institute Buffalo, NY, USA] was transplanted subcutaneously into the flank of the rats under mild isoflurane anesthesia. Tumor size was calculated as described previously (98,110). Tumor volume was recorded every other day prior to initiation of chemotherapy, and every day during the two weeks that chemotherapy was administered. During chemotherapy, relative tumor volume for each animal is compared to the baseline volume [Day 0].

The day when chemotherapy was initiated was designated as Day 0. Cycle-1 consisted of CPT-11 [50 mg/kg body weight, *intraperitoneal*] administered on Day 0 and 5-FU [50 mg/kg body weight, *intraperitoneal*] administered on Day 1. Cycle-2 consisted of the same drug regime occurring one week after cycle-1 [Days 7 and 8]. Atropine [1 mg/kg body weight, *subcutaneous*] was administered immediately prior to each CPT-11 injection to alleviate early onset cholinergic symptoms (98).

3.2.3 Diet and Food Intake

Diets were based on American Institute of Nutrition-76 modified basal ingredients with the fat-source omitted [Harlan Teklad, Indianapolis, IN, USA]. Control and fish oil diets contained 40% of total energy from fat, 40% from carbohydrates and 20% from protein [Table 3-1], representing the estimated average proportion of macronutrients typically consumed by humans. The fish oil diet contained the same proportion of macronutrients as the control diet, differing only in the addition of 2.3 g fish oil/100 g diet [Ocean Nutrition Canada, Dartmouth, NS, Canada]. Added fish oil replaced 2.3 g of other fat in the diet such that the total fat content [20 g/100g diet] and the polyunsaturated to saturated fat ratio did not differ between control and fish oil diets. Food intake was measured every other day prior to initiation of chemotherapy, and every day during the

two weeks that chemotherapy was administered. During chemotherapy, relative food intake for each animal is compared to the average relative food intake prior chemotherapy [Day -14 to Day 0].

3.2.4 Body Weight

Body weight was recorded on the same days as tumor volume. Body weight was converted to tumor-free body weight for data interpretation and statistical analysis. Body weight during chemotherapy was expressed relative to each animal's body weight at Day 0.

3.2.5 Study Termination and Tissue Collection

All rats were euthanized by carbon dioxide [CO_2] asphyxiation. At euthanization, gastrocnemius muscles were isolated, weighed, and frozen in melting isopentane cooled in liquid nitrogen [-156°C], and stored at -80°C until subsequent analyses.

3.2.6 Oil Red O and Hematoxylin Staining

Frozen gastrocnemius muscles were cryosectioned transversely [10 μm thick] and stained for neutral lipid content using Oil Red O as previously described (119). Sections were then rinsed in distilled water, and counterstained [5 min] in Mayer's Hematoxylin [Sigma-Aldrich, St. Louis, MO, USA], to delineate fibers for cross-sectional area [CSA] measurements, before another rinse in distilled water. Sections were visualized under a ZEISS AXIO Compound Light Microscope [AX10 Scope A.1, Carl Zeiss Group, Toronto, ON, Canada] at 200× magnification. Colour images were taken with an Optronics MacroFire Digital Camera [Optronics, Goleta, CA, USA] using a Leica TCS-SP2 spectral confocal and multiphoton system [Leica Camera, Solms, Germany]. Qualitative and quantitative analysis of Oil Red O staining was performed in blinded manner. Quantification of neutral lipid accumulation was analyzed by Volocity 6.3 Software [PerkinElmer,

Inc., Waltham, MA, USA]. An average of 800 ± 23 fibers was analyzed per muscle for the delineation of the proportion of fibers expressing Oil Red O staining. Muscle fiber CSA was measured using Image J software on 200 fibers per muscle.

3.2.7 Fatty Acid and Triglyceride Quantification and Composition

Gastrocnemius muscle [100 mg] was homogenized in a 1.6 ml calcium chloride [$CaCl_2$; 0.025%] solution with glass beads [0.5 mm diameter; FastPrep ®-24, MP Biomedicals, Santa Ana, CA, USA] in 20 sec intervals for 1 min total. Samples were placed on ice for at least 15 sec between each homogenization interval. Lipids were extracted using chloroform/methanol as previously described (120). The TG fraction was isolated on G-plates as previously described (1,21). The TG band was scraped from G-plates and the C15:0 internal standard [10.2 mg/100 ml hexane] was added, followed by saponification. TG was then methylated as previously described (1). Fatty acid composition was determined using gas chromatography-flame-ionisation detector analysis on a Varian 3900 [Varian Instruments, Georgetown, ON, Canada] as previously described (1). Peaks of saturated, monounsaturated and polyunsaturated fatty acids were separated between 6 and 24 carbon chain lengths and identified using a fatty acid standard of known composition [GLC461, Sigma-Aldrich]. Quantity of FA within the TG fractions was calculated by comparison with the known concentration of the C15:0 standard.

3.2.8 RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from gastrocnemius muscle [10 mg] using MagMax-96 total RNA isolation Kit [Ambion, Austin, TX, USA] following the manufacturer's protocol. For assessing RNA quantity and quality, a NanoDrop spectrophotometer [Thermo Scientific, Wilmington, DE] and Agilent 2100 Bioanalyzer [Agilent Technologies, Santa Clara, CA, USA] were used, respectively. Samples were then diluted with nuclease-free water to 7 ng/µl. High

Capacity cDNA Reverse Transcription kit [Applied Biosystems, Foster City, CA, USA] was used to reverse transcribe RNA to cDNA following the manufacturer's protocol. Pre-designed TaqMan® probes with a 6-carboxyfluorescein phosphoramidite [FAM™] label on the 5' end and primer sets [Applied Biosystem] were used to target the following genes: *C/EBPβ* [Rn01764319_m1], *C/EBPδ* [Rn02532096_s1], *C/EBPα* [Rn00560963_s1], *PPARγ* [Rn00440945_m1], and *SREBP*-1c [Mm00550339_g1]. *18S* rRNA [Rn03928990_g1] was stable among all samples, and therefore used as the endogenous control. qRT-PCR was performed on 1 *μl* cDNA samples, in triplicate, on an ABI 7900HT thermocycler [Applied Biosystems]. Relative changes in gene expression were determined using the $2^{-\Delta\Delta CT}$ method of analysis (121).

3.2.9 Statistical Analysis

Data are summarized as mean ± SD. One-way and a two-way repeated measures analysis of variance [ANOVA] were used to test differences in food intake, changes in body weight and tumor volume before and during chemotherapy treatment, respectively. A One-way ANOVA was used to test differences in Oil Red O staining, total TG, mRNA fold changes in gene expression, and fatty acids content. When a significant difference was observed, post-hoc analysis was completed using the Bonferroni model. A Pearson's correlation was used to test relationships between mRNA expression of various adipogenic/lipogenic transcriptional factors and total TG. Statistical significance was reported when p value <0.05. All statistical analyses were performed using SPSS 21.0 [Chicago, IL, USA] for Windows.

3.3 Results

3.3.1 Food Intake and Body Weight

After tumor implantation and prior to chemotherapy, the rats consuming fish oil had higher relative food intake compared to rats in control group [mean relative values: 1.0 ± 0.09 g/g food

intake prior tumor versus 0.9 ± 0.1 g/g food intake prior tumor, p<0.001; data not shown]. The fish oil group gained 13 ± 5 g of their body weight compared to 7 ± 3 g in the rats consuming the control diet [p< 0.03].

After each cycle of chemotherapy, food intake decreased in all group, and returned to baseline by the end of the cycle, however there were no significant differences between the groups [0.8 ± 0.1 g/day; data not shown]. Body weight decreased after each chemotherapy cycle and was significantly lower than baseline on the second, third and fourth day following chemotherapy injection [body weight decreased by 5%; p=0.04]. Average intake of EPA plus DHA during the chemotherapy period in the fish oil groups was 112 ± 25 mg/day.

3.3.2 Identification of a Potential Model of Tumor- and Chemotherapy-Associated Myosteatosis

Fat accumulation in muscle. First, to confirm that the Ward colon tumor-bearing rat and CPT-11/5-FU delivery models induced skeletal muscle fat accumulation, we quantified both the neutral lipid accumulation and TG content in the gastrocnemius muscle of rats on the control diet. Neutral lipids were quantified according to strong positive staining for Oil Red O within muscle fibers, and qualitatively for the presence of intermuscular fiber variations [Figure 3.2]. Two weeks following tumor implantation, the proportion of fibers expressing Oil Red O and the mean total TG content in the gastrocnemius muscle were 6-fold [P<0.001, Figure 3.2] and 3-fold [P<0.001, Figure 3.3] higher, respectively, in the control diet group compared with the reference group, reflecting tumor-associated fat accumulation in muscle. Subsequently, chemotherapy treatment further increased fat accumulation in the gastrocnemius muscle. Rats on the control diet who underwent cycle-2 displayed a large amount of intermuscular fiber Oil Red O staining [Figure 3.2], and significant increases in the proportion of fibers expressing Oil Red O [Figure 3.2] and mean

total TG content [Figure 3.3] compared with tumor-bearing only rats [P<0.001 and P<0.001, respectively] and rats who underwent cycle-1 [P<0.001 and P<0.001, respectively].

Adipogenic transcriptional factors in muscle. Next, we assessed expression of genes involved in the adipogenic transcriptional cascade including the upstream genes sterol regulatory element binding protein *[SREBP]-1c*, *C/EBPβ* and *C/EBPδ*, as well as the downstream genes *C/EBPα* and *PPARγ* in the gastrocnemius muscle to identify a potential mechanism for skeletal muscle fat accumulation in response to the tumor-bearing state before and during chemotherapy treatment. While expression of *SREBP-1c, C/EBPβ* and *C/EBPδ* were either not altered [i.e. *SREBP-1c* and *C/EBPβ*; Appendix A] or displayed a moderate increase in expression [i.e. *C/EBPδ*, P<0.002; Figure 3.4A], expression of *C/EBPα* and *PPARγ* showed remarkable increases in response to the tumor-bearing state and subsequent chemotherapy treatment in rats on the control diet [Figure 3.4A]. Specifically, in these animals, *C/EBPα* and *PPARγ* expression increased 26-fold [P<0.001] and 59-fold [P<0.001], respectively, in the tumor-bearing state before chemotherapy that significantly further increased in response to cycle-2 compared with reference animals [Figure 3.4A].

We further explored the relationships between *C/EBPδ, C/EBPα* and *PPARγ* expression and total TG content in the gastrocnemius muscle [Figure 3.4B]. The expression of *C/EBPδ, C/EBPα* and *PPARγ* were positively correlated with total TG content [Figure 3.4B], revealing that key transcription factors involved in adipogenesis are related to the TG content in muscle.

Muscle weight and fiber cross-sectional area. Further characterization of the gastrocnemius muscle showed that while muscle weight was not different between the groups [overall mean 760 ± 60 mg; data not shown], the tumor-bearing state [P<0.01] and subsequent

chemotherapy [P<0.001] resulted in lower mean muscle fiber CSA in rats on the control diet compared with reference animals [Figure 3.5]. This shows that moderate muscle atrophy occurred concurrent of fat accumulation in this animal model.

3.3.3 Effects of the Fish Oil Diets on Tumor- and Chemotherapy-Associated Myosteatosis

EPA and DHA content in muscle. Since chemotherapy treatment significantly reduced the EPA [i.e. cycle-2] and DHA [i.e. cycle-1 and cycle-2] content in the gastrocnemius muscle TG fraction of rats on the control diet compared with the reference animals [Table 3-2], we wanted to first confirm that the fish oil diets restored muscle EPA and DHA. Indeed, both fish oil diets were efficacious in maintaining or elevating EPA and DHA throughout both cycles of chemotherapy compared to reference [Table 3-2].

Fat accumulation in muscle. To test the hypothesis that in a Ward colon tumor-bearing rat model of myosteatosis, dietary EPA and DHA would mitigate tumor- and chemotherapy-associated fat accumulation in skeletal muscle, we measured neutral lipids and TG content in the gastrocnemius muscle of rats fed a diet containing fish oil beginning one week prior to tumor implantation [long term] and beginning when chemotherapy was initiated [adjuvant]. Before chemotherapy, tumor-bearing rats on the long term fish oil diet displayed proportions of fibers expressing Oil Red O [Figure 3.2] and total TG content [Figure 3.3] in the gastrocnemius muscle similar to levels observed in reference animals, which were significantly lower compared with tumor-bearing rats on the control diet [P<0.001 and P<0.003, respectively]. Subsequently, after both cycle-1 and cycle-2, tumor-bearing rats on the long term fish oil diet displayed minimal intermuscular Oil Red O staining [Figure 3.2], and exhibited over 40% less fibers expressing Oil Red O [Figure 3.2] and over 40% less total TG- muscle content [Figure 3.3] in the gastrocnemius muscle compared with their respective chemotherapy treated cycle, tumor-bearing control diet

groups. Additionally, cycle-1 and cycle-2 rats on the adjuvant fish oil diet displayed similar amounts of inter- [Figure 3.2] and intra-muscular [Figure 3.2] Oil Red O staining and total TG [Figure 3.3] in the gastrocnemius muscle compared with their respective long term fish oil diet group. Collectively, these results show that before chemotherapy, the long term fish oil diet prevented tumor-associated fat accumulation, and the adjuvant fish oil diet was equally efficacious as the long term fish oil diet in mitigating chemotherapy-associated fat accumulation in muscle.

Adipogenic transcription factors in muscle. In the gastrocnemius muscle, the long term fish oil diet prevented tumor-associated increases in *C/EBPδ, C/EBPα* and *PPARγ* expression, as the expression of these transcription factors were similar to reference animals and significantly lower compared to tumor-bearing rats on the control diet [*C/EBPδ*, $P<0.001$; *C/EBPα*, $P<0.02$; *PPARγ*, $P<0.02$; Figure 3.4A]. Subsequently, both fish oil diets greatly suppressed chemotherapy-associated expression of these transcriptional factors that occurred in rats on the control diet [Figure 3.4A]. Specifically, expression of *C/EBPδ, C/EBPα* and *PPARγ* remained at reference levels in the gastrocnemius muscle of rats on the long term fish oil diet who underwent chemotherapy, with the lone exception of cycle-2 *C/EBPα* expression that was 2-folder [$P<0.05$] greater compared with reference, but remained 20-fold [$P<0.03$] lower compared to cycle-2 rats fed control diet [Figure 3.4A]. Similarly, the adjuvant fish oil diet was able to fully reverse and significantly decrease expression of *C/EBPδ, C/EBPα* and *PPARγ* after cycle-1 and cycle-2, respectively [Figure 3.4A]. These results show that both fish oil diets greatly reduced tumor- and/or chemotherapy-associated increases in the expression of key adipogenic transcriptional factors.

Muscle weight and fiber cross-sectional area. All groups on the long term fish oil diet had similar muscle fiber CSA compared with reference animals, and were significantly greater than

their respective control diet groups following one and two cycles of chemotherapy [P<0.002 and P<0.005, respectively; Figure 3.5]. Additionally, muscle fiber CSA was not different between the long term fish oil and adjuvant fish oil groups after either cycle-1 or cycle-2 [Figure 3.5].

3.3.4 Effects of the Fish Oil Diets on Tumor Response to Chemotherapy

Tumors grew to 1.8 ± 0.4 cm^3 in size and were similar between all groups before starting chemotherapy treatment. Long term and adjuvant EPA and DHA supplementation similarly enhanced the anti-tumor activity of CPT-11/5-FU chemotherapy compared with the control diet [Figure 3.6]. In each of the 7 days following initiation of cycle-1, rats in both the long term and adjuvant fish oil groups had significantly smaller tumor volumes compared with tumor-bearing rats in the control diet group [P<0.01]. Notably, tumor volumes were not significantly different between the long term and adjuvant fish oil diet groups during either cycle-1 or cycle-2.

3.4 Discussion

To our knowledge, this study is the first to describe a preclinical model of tumor- and chemotherapy-associated myosteatosis. Using this model, we investigated the effects of a physiologically attainable level of EPA and DHA supplementation on the tumor-bearing state before and during chemotherapy-associated myosteatosis, as well as on the tumor response to chemotherapy. We show for the first time that feeding a diet containing EPA and DHA prevents tumor-associated myosteatosis, and that adjuvant- is similarly efficacious to long term- EPA and DHA feeding in greatly mitigating chemotherapy-associated myosteatosis, and in enhancing the tumor response to chemotherapy. Specifically, we show that in the gastrocnemius muscle of tumor-bearing animals before and during chemotherapy, neutral lipids and TG were elevated along with increased expression of key transcriptional factors involved in adipocyte gene expression, which were all greatly reduced in animals fed a diet containing fish oil either beginning before tumor-

implantation [long term] or at the initiation of chemotherapy [adjuvant]. Additionally, after cycle-1, tumor volume decreased in animals on the long term and adjuvant fish oil diets, to an equal extent, compared with those on a control diet. Collectively, our results suggest that EPA and DHA supplementation is a recognized effective adjuvant to chemotherapy to combat myosteatosis, in addition to its well-documented effect of enhancing tumor cytotoxicity [reviewed by (16)].

3.4.1 Identification of a Potential Model of Tumor- and Chemotherapy-Associated Myosteatosis

Myosteatosis is emerging as an important prognostic factor in the oncology setting, as it has been found to be associated with shorter survival in cancer patients treated with various therapies including chemotherapy (6-9). In this study, the Ward colon tumor model displayed robust fat accumulation within skeletal muscle that was exacerbated by successive chemotherapy cycles. Rollins *et al.* (7) has reported that the majority of cancer patients display myosteatosis in concert with muscle loss; two features that share a poor prognosis in advanced cachexic cancer patients (8). Similarly, the Ward colon tumor model also displayed decreased muscle fiber. These pathological changes in muscle have been identified during chemotherapy (1,5,7,8,113) and represent one of the toxicity effects of antineoplastic treatment. Collectively, this preclinical model displays features of human cancer with regards to skeletal muscle changes that occur in the majority of cancer patients undergoing chemotherapy.

Determining the anatomical location of pathological fat in cancer patients can be informative towards the identification of potential underlying signaling mechanisms. However, there is a dearth of information on this topic. Stephens *et al.* (49) examined intramuscular lipid droplets in weight-losing cancer patients, and their results suggest that the number and size of lipid droplets increase with cancer. Similarly, we observed an increase in neutral lipid content primarily

within muscle fibers, indicative of lipid droplets, in the tumor-bearing state that was exacerbated by successive chemotherapy cycles. Neutral lipids were also evident between muscle fibers, but only after the second cycle of chemotherapy, which may be attributed to the formation of intermuscular adipocytes. Collectively, it appears that signaling mechanisms involved in lipid droplet and adipocyte formation are involved in cancer-associated myosteatosis.

Lipid droplet formation occurs in various tissues in response to cellular stress (122), which can occur via *SREBP-1c* (123), an adipogenic transcriptional factor that plays a key role in fatty acid biosynthesis (124). Additionally, cellular stress responses occur in cancer and cancer therapies [reviewed by (125)]. Therefore, we examined skeletal muscle *SREBP-1c* expression, but found that it was not altered in response to the tumor-bearing state or subsequent chemotherapy. However, *C/EBPδ, C/EPBα* and *PPARγ* expression, which were all elevated in the skeletal muscles of tumor-bearing animals before and after chemotherapy in our study, are involved in both lipogenesis and adipogenesis in skeletal muscle (117,126,127). Specifically, increased *C/EBPδ* expression occurs in myopathic murine skeletal muscle that contains abnormally high amounts of lipid droplets within muscle fibers along with intermuscular adipocytes (126). Additionally, *C/EBPα* and/or *PPARγ* overexpression *in vitro* increases fatty acid uptake and incorporation into muscle lipids including TG, and augments lipid droplet accumulation (117,127). Likewise, these key transcriptional factors were related to skeletal muscle fat accumulation in this animal model that occurred mainly within, but also between muscle fibers. Collectively, it appears that the mechanisms responsible for cancer-associated myosteatosis involve key regulators of adipogenesis/lipogenesis.

3.4.2 Effects of the Fish Oil Diets on Tumor- and Chemotherapy-Associated Myosteatosis

Nutrient deficiencies have been observed in cancer patients undergoing chemotherapy including low levels of plasma EPA and DHA that appear to occur concurrently with muscle loss and fat deposition, which can be corrected when fish oil is provided to patients during treatment [10, 26]. We showed that patients receiving first-line chemotherapy for advanced non-small cell lung cancer lost muscle mass and gained skeletal muscle fat, while patients who supplemented with EPA and DHA [2.1 g/day] beginning on the first day of chemotherapy exhibited a maintenance or gain in muscle mass and reduction in intermuscular fat over the same time period (24). However, the mechanisms through which EPA and DHA supplementation exert this beneficial effect are unknown. Also, the extent to which adjuvant EPA and DHA supplementation during chemotherapy is as efficacious as beginning prior to a cancer diagnosis is important for application in the clinical setting.

EPA and DHA are known to have anti-adipogenic effects on adipose tissue and metabolism that occurs through multiple mechanisms (128), but little is known about this mechanistic regulation in cancer-associated fat accumulation in skeletal muscle. Here we demonstrate, for the first time, that EPA and DHA supplementation prevents tumor-associated increases in the transcriptional expression of *C/EBPδ, C/EBPα* and *PPARγ*, and greatly mitigates further increases during chemotherapy. These findings reveal that EPA and DHA exert their beneficial effects, at least in part, through the suppression of key adipogenic transcriptional factors that we show are related to total TG content in skeletal muscle. Additionally, findings from our study also demonstrate that adjuvant feeding of EPA and DHA [started on the same day as initiation of chemotherapy] is as effective as long term feeding [started prior to tumor implantation] in mitigating myosteatosis and inhibiting the expression of underlying transcriptional factors.

3.4.3 Effects of the Fish Oil Diets on the Tumor Response to Chemotherapy

The *in vivo* model used in the current study represents a prevalent chemotherapy regime for colorectal cancer patients, and mimics the human colon cancer response to CPT-11/5-FU treatment (98,110,129). Our results contribute to the large body of preclinical [reveiwed by (16)] and emerging clinical (19,24) evidence suggest that providing EPA and DHA concurrent with antineoplastic agents enhances anti-tumor effects. We additionally demonstrate that adjuvant is as effective as longer term EPA and DHA supplementation in enhancing the anti-tumor activity of CPT-11/5-FU chemotherapy. These effects are most likely due to the incorporation of these EPA and DHA in to cancer cell membrane phospholipids, thus modifying tumor properties. In doing so, a wide range of biological functions can be altered, such as eicosanoid production, signal transduction, membrane fluidity and cell interaction [reviewed by (22,130)].

3.5 Conclusions

The novel observations of pathological fat accumulation coupled with the expression of key adipogenic/lipogenic transcriptional factors in the skeletal muscles of an *in vivo* tumor-bearing model that underwent chemotherapy treatment, reveal a potential mechanism underlying fundamental alterations that may be involved in the development of myosteatosis in cancer patients. Long term and adjuvant dietary EPA and DHA feeding were equally efficacious in markedly improving the therapeutic index of CPT-11/5-FU chemotherapy by concurrently enhancing drug efficacy to the tumor while reducing the toxicity effect of fat accumulation within skeletal muscle. Our study highlights the therapeutic potential of EPA and DHA as promising nutritional adjuvants to chemotherapy, which is of particular importance given the prognostic value of myosteatosis as an independent predictive factor of short- and long-term outcomes in cancer. Findings from the present study are novel and encouraging, warranting future research to

determine further mechanisms by which dietary EPA and DHA supplementation attenuate pathological fat accumulation in the skeletal muscles of cancer patients.

Table 3-1 Composition of experimental diets.

Ingredient	Control diet	Fish oil diet
Constant portion (80% w/w of diet)		
Modified AIN-76 basal mix (g/100g of diet):		
Casein	22.7	22.7
DL-Methionine	0.3	0.3
Corn Starch	25.5	25.5
Sucrose	20.4	20.4
Vitamins (AIN-76)	1.2	1.2
Minerals (AIN-76)	4.1	4.1
Inositol	0.6	0.6
Choline Bitartrate	0.2	0.2
Cellulose	5.0	5.0
Variable portion (20% w/w of diet)		
Lipid source (g/100g of diet):		
Canola Stearine	11.7	12.0
Olive oil	0.0	0.8
Sunflower oil	5.2	3.3
Canola oil	3.1	1.6
Fish oil	0.0	2.3
Fatty acid composition (% of total fatty acids in the lipid source):		
Saturated fatty acids	58.7	59.9
Monounsaturated fatty acids	17.3	14.3
Polyunsaturated fatty acids	20.6	22.5
Total n-6	(18.6)	(13.6)
Total n-3	(2.0)	(8.9)
EPA	(0.0)	(5.1)
DHA	(0.0)	(2.1)
Other fatty acids	3.4	3.3

Diets were isocaloric and isonitrogenous. AIN, American Institute of Nutrition; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid. Fatty acid composition was measured by gas chromatography.

Table 3-2 EPA and DHA content within the rat gastrocnemius muscle tryglyceride fraction.

n3 fatty acids	Study Groups									P Value
		Control Diet			Long Term Fish Oil Diet			Adjuvant Fish Oil Diet		
	Reference	Tumor	Cycle1	Cycle2	Tumor	Cycle1	Cycle2	Cycle1	Cycle2	
EPA (µg/g)	0.9 ± 0.5a	0.4 ± 0.2ab	0.7 ± 0.5a	0.2 ± 0.2b	0.9 ± 0.4a	0.9 ± 0.1a	1.3 ± 0.7c	1.5 ± 0.3c	1.1 ± 0.5ac	P<0.001
DHA (µg/g)	0.7 ± 0.4a	0.6 ± 0.4a	0.1 ± 0.0b	NDb	1.7 ± 0.5c	2.6 ± 1.3d	2.6 ± 0.7d	2.7 ± 0.8d	1.7 ± 0.5d	P<0.001

Values are presented as means ± SD. Different letters indicate significant differences among groups. DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ND, not detectable.

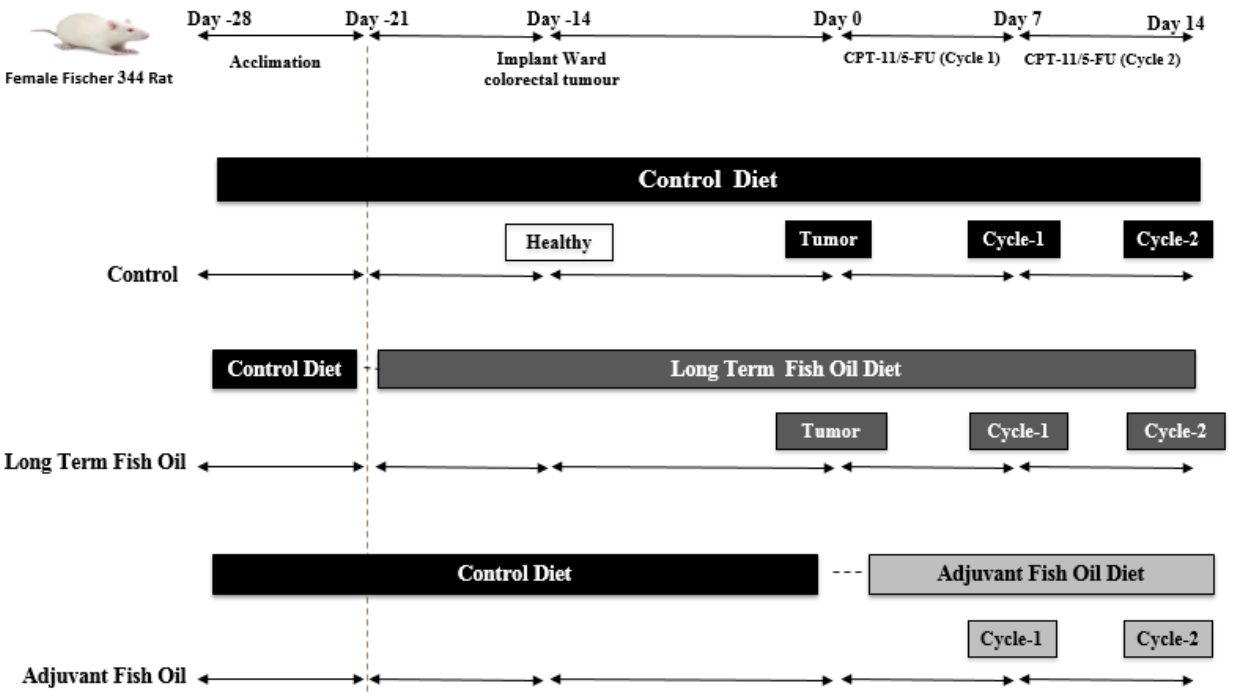


Figure 3.1 Experimental study design. The first day of chemotherapy cycle-1 designed as day 0 and the first day of chemotherapy cycle-2 designated as day 7. Rats were euthanized 7 days following the completion of each cycle. CPT-11 was administered on day 0 and day 7; 5-FU was administered on Day 1 and Day 8. Rats bearing tumor were euthanized two weeks following tumor implantation. Healthy rats received no tumor and treatment and were on control diet. Long term fish oil diet started one week prior to tumor implantation on Day -21. Adjuvant fish oil diet started at the same day of the first cycle of chemotherapy on Day 0. CPT-11, irinotecan; 5-FU, 5-fluorouracil.

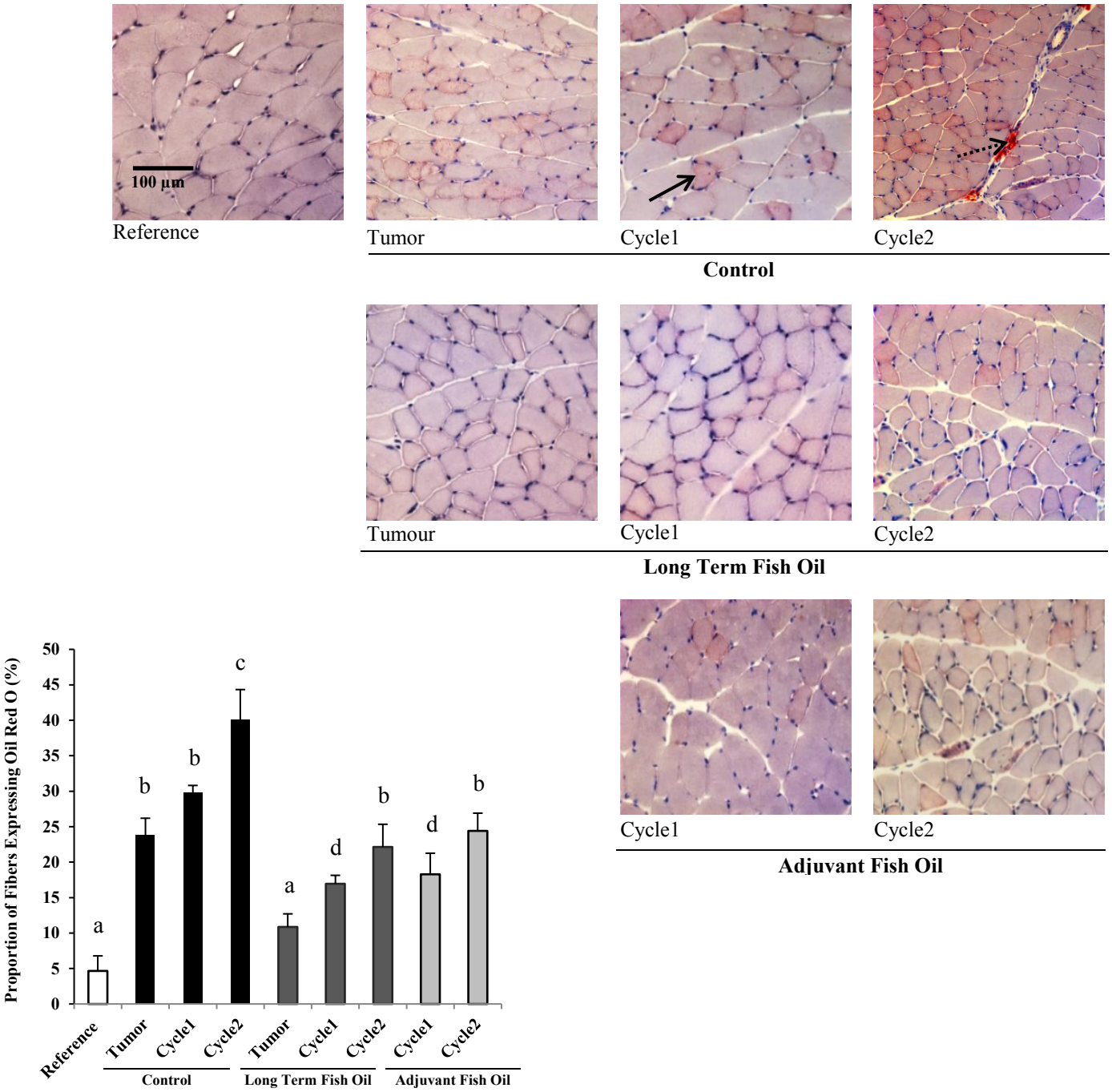


Figure 3.2 Oil Red O staining and analysis of neutral lipid localization in rat gastrocnemius muscle. In photomicrographs of Oil Red O stained muscle, solid arrow shows an example of a fiber stained positive for Oil Red O; dashed arrow shows an example of positive Oil Red O staining between muscle fibers. Scale bar represents 100 µm. Bars represent proportion of fibers stained positive for Oil Red O. Values are means ± SD. Different letters indicate significant differences among groups [$P<0.05$].

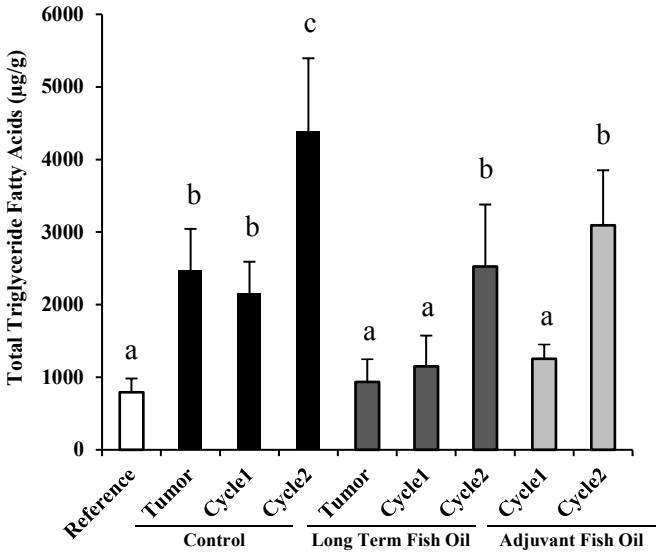
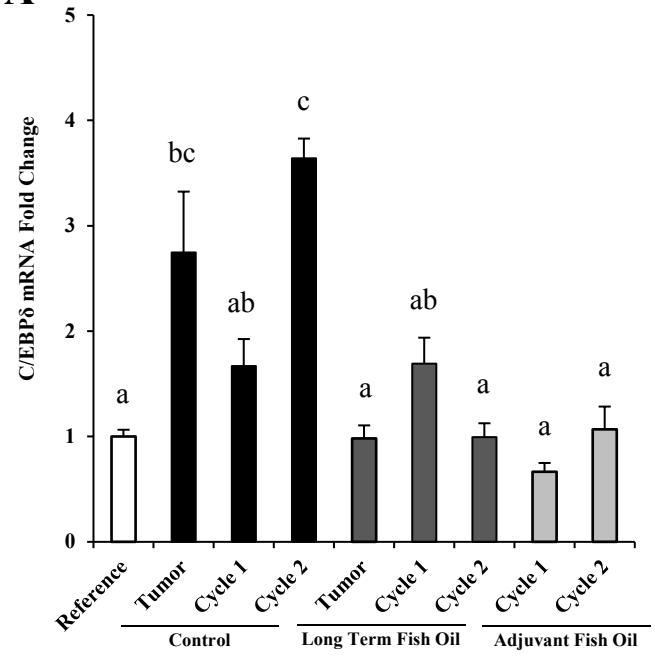
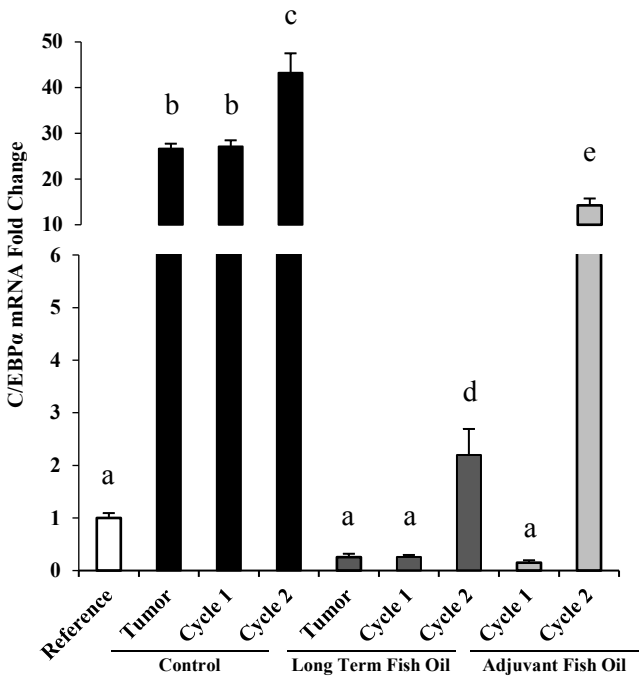
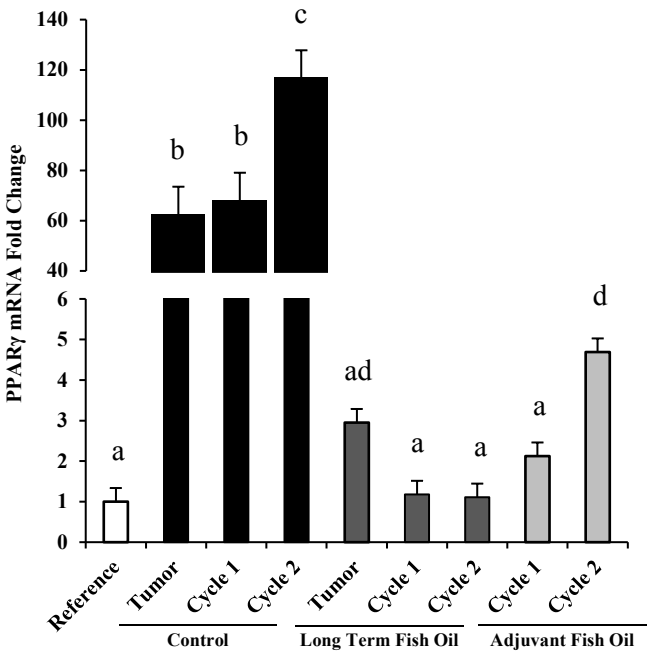


Figure 3.3 Total triglyceride fatty acid levels in the rat gastrocnemius muscle quantified using gas chromatography. Values are means ± SD. Different letters indicate significant differences among groups [$P<0.05$].

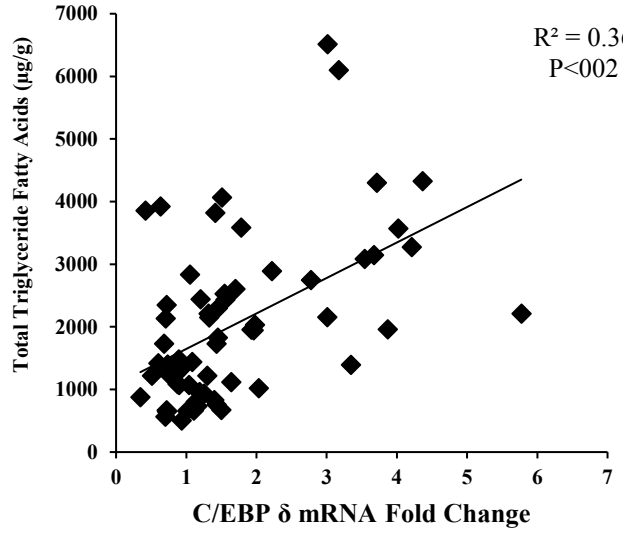
A

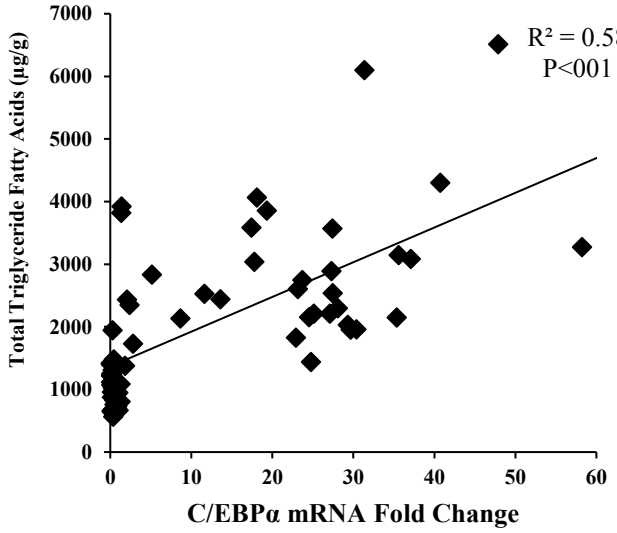

C/EBPδ mRNA Fold Change
a
bc
ab
c
a
ab
a
a
a
Reference
Tumor
Cycle 1
Cycle 2
Tumor
Cycle 1
Cycle 2
Cycle 1
Cycle 2
Control
Long Term Fish Oil
Adjuvant Fish Oil


C/EBPα mRNA Fold Change
a
b
b
c
a
a
d
a
e
Reference
Tumor
Cycle 1
Cycle 2
Tumor
Cycle 1
Cycle 2
Cycle 1
Cycle 2
Control
Long Term Fish Oil
Adjuvant Fish Oil


PPARγ mRNA Fold Change
a
b
b
c
ad
a
a
a
d
Reference
Tumor
Cycle 1
Cycle 2
Tumor
Cycle 1
Cycle 2
Cycle 1
Cycle 2
Control
Long Term Fish Oil
Adjuvant Fish Oil

B


Total Triglyceride Fatty Acids (µg/g)
R² = 0.36
P<002
C/EBP δ mRNA Fold Change


Total Triglyceride Fatty Acids (µg/g)
R² = 0.58
P<001
C/EBPα mRNA Fold Change

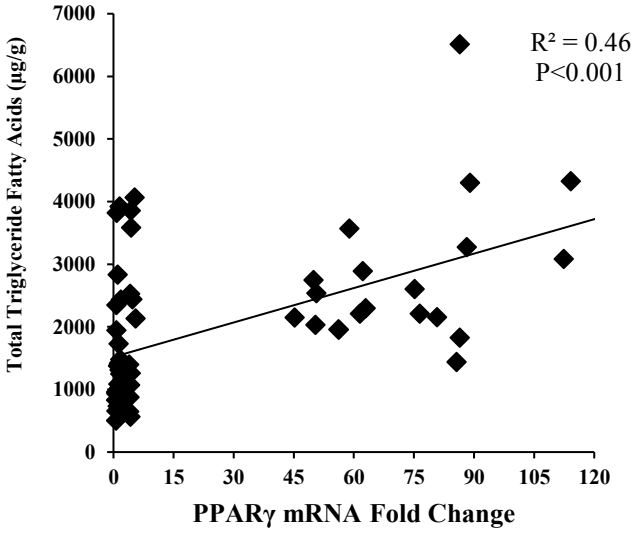

Total Triglyceride Fatty Acids (µg/g)
R² = 0.46
P<0.001
PPARγ mRNA Fold Change

Figure 3.4 Patterns of adipogenic transcriptional factor mRNA expression in the rat gastrocnemius muscle. **A)** Fold changes in *C/EPBδ, C/EBPα* and *PPARγ* expression levels as determined by the $2^{-\Delta\Delta CT}$ method of analysis. Values are means ± SD. Different letters indicate significant differences among groups [$P<0.05$]. **B)** The correlation between each gene and the total triglyceride fatty acid level.

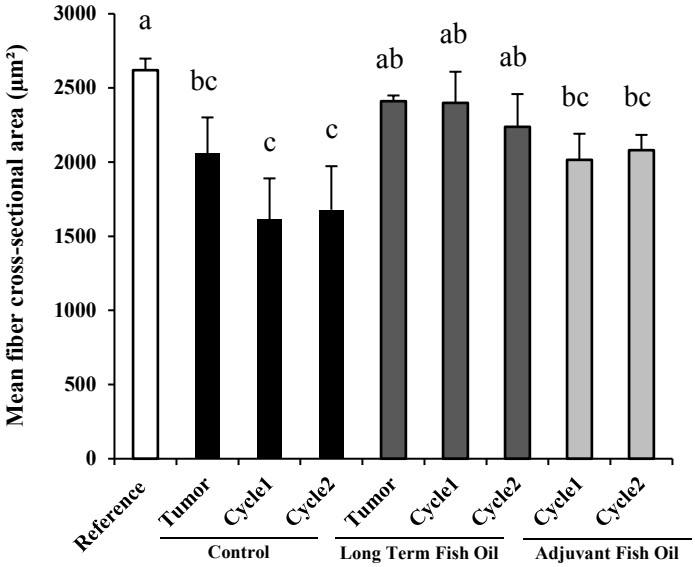


Figure 3.5 Fiber cross-sectional area in the rat gastrocnemius muscle. Values are means ± SD. Different letters indicate significant differences among groups [P<0.05].

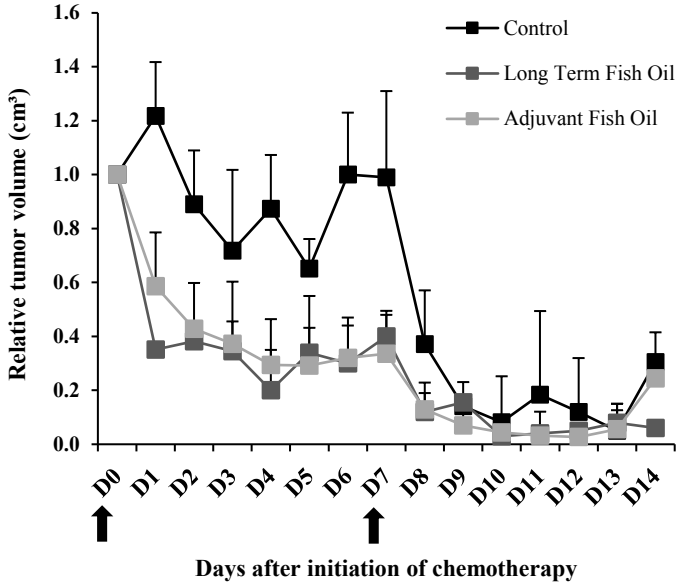


Figure 3.5 Relative tumor volume of tumor-bearing rats who underwent one or two cycles of CPT-11/5-FU treatment. Relative tumor volume is compared to the baseline volume when chemotherapy was initiated [Day 0]. Black arrow shows the days when single CPT-11 injections [50mg/kg] occurred [Days 0, 7]; white arrow shows the days when single 5-FU injections [50mg/kg] occurred [Days 1, 8]. Values are means ± SD.

CHAPTER 4

Phospholipid and Triglyceride Compositions Reflect Dietary EPA and DHA Intervention in an Animal Model of Colorectal Cancer Receiving CPT-11 and 5-FU

4.1 Introduction

Application of computed tomography [CT] scans obtained in an oncological setting to evaluate body composition has revealed that muscle with low radiodensity and elevated amounts of intermuscular adipose tissue, defined as myosteatosis, occurs in cancer patients (6,7,9,24,46-48). It has been reported recently that presence of myosteatosis reduces the length of survival in cancer patients (6-9). We have reported that intermuscular fat infiltration occurs as patients progress through chemotherapy. However, when cancer patients supplemented their daily intake with eicosapentaenoic acid [20:5n-3; EPA] and docosahexaenoic acid [22:6n-3; DHA], reductions in intermuscular adipose tissue were observed (24). This clinical result is supported by a pre-clinical study where we observed marked triglyceride accumulation to occur within muscle during tumor growth. Feeding dietary EPA and DHA lowered the triglyceride content of muscle to that of healthy animals after only 6 days of feeding [see Chapter 3, Figure 3.3]. Therefore, these data suggest that myosteatosis, an independent prognostic factor for mortality in cancer, is modifiable through dietary intervention with EPA and DHA.

Clinical evidence suggests that the n-3 polyunsaturated fatty acid status of newly-diagnosed cancer patients undergoing chemotherapy is low (1,21). In comparison to healthy subjects, advanced cancer patients had depleted stores of plasma n-3 fatty acids within plasma phospholipid [PL] fraction after high-dose chemotherapy, when EPA and DHA levels were reduced to about 7% of the control values (21). Our group has reported a reduction in EPA and DHA and total n-3 fatty acid, in non-small cell lung cancer patients (1). Another study aimed to

investigate the way in which fatty acids patterns of gastrointestinal mucosa are altered in human gastric cancer. Specifically, researchers assessed the fatty acid composition of PL fraction of mucosa in paired cancerous and non-cancerous gastric tissues. This study showed that the n-6/n-3 ratio was higher in PL of cancerous mucosa mainly due to high levels of arachidonic acid [20:4n-6] coupled by low levels of EPA and DHA (51). Therefore, both the disease as well as the therapeutic treatments may be contributing factors in reducing n-3 fatty acids (7,9,24,47,48,100). Collectively, these results suggest that EPA and DHA are important for muscle health and could potentially mitigate fat accumulation that occurs in the presence of tumor or during chemotherapy [discussed in details in Chapter 3]. However, fatty acid composition of skeletal muscle from cancer patients has not been characterized and no biological measure of fat in muscle was reported in cancer associated with myosteatosis or neither during EPA and DHA intervention.

In order to investigate the skeletal muscle fatty acids profile in cancer-related myosteatosis, as well as during EPA and DHA intervention, Fischer rats bearing the Ward colon 26 tumor and receiving two cycles of CPT-11 plus 5-FU therapy were used. Tumor-bearing animals show elevated triglyceride [TG] content, which is accelerated with chemotherapy [see Chapter 3, Figure 3.2], similar to what is observed clinically (24). Additionally, findings from our study also demonstrate both adjuvant feeding of EPA and DHA [started on the same day as initiation of chemotherapy] as well as long term feeding [started prior to tumor implantation] were effective in reducing TG content of gastrocnemius muscle that known to occur in the presence of tumor and during chemotherapy [see Chapter 3, Figure 3.3]. The objectives of this study were 1) to determine the fatty acid profile of TG and PL fractions of gastrocnemius muscle tissue in a preclinical model of cancer-associated myosteatosis either prior or after rats received 1- or 2-cycles of CPT-11/5-FU, and 2) to determine the fatty acids profile of TG and PL fractions in

gastrocnemius muscle when dietary EPA and DHA is provided either beginning at the initiation of chemotherapy [adjuvant] or beginning prior to tumor implantation [long term]. It was hypothesized that compared to healthy rats not bearing a tumor, rats bearing the Ward colorectal tumor alone or receiving 1- and -2 cycles of chemotherapy and associated with myosteatosis will exhibit lower PL content, higher saturated fatty acids and n6/n3 fatty acid ratio, and lower EPA and DHA and total n-3 fatty acid. However, providing a diet containing EPA and DHA fed prior [long term] to, and following [adjuvant] tumor implantation would increase total PL content, decrease saturated fatty acids and n6/n3, and increase EPA and DHA and total n-3 in both PL and TG fractions compared to rats fed a control diet. Also, adjuvant fish oil diet will be as effective as fish oil diet in all measures.

4.2 Material and Methods

Experimental design including, animal model, dietary design, tumor and chemotherapy description, body weight and food intake measurements, and total muscle weight are reported in Chapter 3 section 3.2.

4.2.1 Phospholipid and Triglyceride Quantification and Composition

Gastrocnemius muscle [100 mg] homogenization and preparation for lipid extraction has been described in Chapter 3 section 3.2.6. Lipids were extracted using chloroform/methanol as previously described (120). The TG and PL fraction was isolated on G-plates as previously described (1,21). The TG and PL bands were scraped from G-plates and C15:0 [10.2 mg/100 ml hexane] and C17:0 [10 mg/100 ml hexane] standards were added respectively, followed by saponification for TG only. TG and PL were then methylated as previously described (1). Fatty acid compositions were determined using gas chromatography-flame-ionisation detector analysis on a Varian 3900 [Varian Instruments, Georgetown, ON, Canada] as previously described (1).

Peaks of saturated, monounsaturated and polyunsaturated fatty acids were separated between 6 and 24 carbon chain lengths and identified using a fatty acids standard of known composition [GLC461, Sigma-Aldrich]. Quantities of fatty acids within the TG and PL fractions were calculated by comparison with the known concentration of the C15:0 and C17:0 standards.

4.2.2 Statistical Analysis

Data are summarized as mean ± SD. A One-way ANOVA was used to test differences in total PL and TG as well as the fatty acid composition in PL and TG fractions. When a significant difference was observed, post-hoc analysis was completed using the Bonferroni model. Statistical significance was reported when p value <0.05. All statistical analyses were performed using SPSS 21.0 [Chicago, IL, USA] for Windows.

4.3 Results

4.3.1 Phospholipid Fatty Acid Profile

Fatty Acid Composition of PL in a Preclinical Model of Tumor- and Chemotherapy-Associated Myosteatosis: First, to confirm that the Ward colon tumor-bearing rat and CPT-11/5-FU delivery models induced an alteration in fatty acid composition within the PL fraction, we quantified total PL fatty acids as well as PL fatty acid composition in the gastrocnemius muscle of rats bearing-tumor with or without receiving 1- or 2- cycles of chemotherapy and were on control diet. The mean total PL fatty acid content in the gastrocnemius muscles of rats bearing-tumor only was not different from reference rats [Figure 4.1]. On the other hand, rats receiving 1- cycle chemotherapy exhibited a significant reduction of mean PL fatty acid compared to rats bearing-tumor only [P<0.02, Figure 4.1]. After 2- cycle of chemotherapy, amount of PL was returned to baseline levels.

Tumor-bearing rats exhibited similar proportion of total n-3 and n-6 fatty acids compared to reference group [Table 4-1]. However, the proportions of total saturated and monounsaturated fatty acid, driven by higher proportion of C18:1 n-9, were significantly higher in tumor-bearing rats compared to reference animals [P<0.001 and P<0.001 and P<0.001, respectively; Table 4-1]. After both 1- and 2-cycles of chemotherapy, proportions of C18:1 n-9 and total monounsaturated fatty acids were significantly higher than tumor-bearing animals [P<0.001 and P<0.001, respectively; Table 4-1], whereas total proportion of saturated fatty acid significantly increased after the second cycle only [P<0.001]. Proportion of n-6 fatty acids were significantly decreased following cycle-1 and cycle-2 chemotherapy [P<0.04 and P<0.02, respectively] driven by lower proportion of C18:2 [P<0.001 and P<0.001, respectively]. Total proportions of n-3 fatty acids were comparable between all groups on control diet.

Effects of Fish Oil Diets on PL Fatty Acid Composition in a Preclinical Model of Tumor- and Chemotherapy-Associated Myosteatosis*:* We next wanted to confirm the effect of a long term compared to the adjuvant fish oil diets in total PL in the gastrocnemius muscles. Tumor-bearing rats and those went under 1- and 2- cycles of chemotherapy and fed long term fish oil diet exhibited > 2 fold higher total PL content [P<0.001 and P<0.001 and P<0.001, respectively] in the gastrocnemius muscles compared to their respective control groups [Figure 4.1]. Rats fed adjuvant fish oil diet and went under 1- and 2- cycles of chemotherapy exhibited similar level of total PL compared to reference animal [Figure 4.1].

Before chemotherapy, tumor-bearing rats on the long term fish oil diet displayed lower proportion of total saturated fatty acids [P<0.001], driven by lower proportion of C16:0 [P<0.04], as well as lower proportion of n-6 fatty acids [P<0.001], driven by lower proportion of C18:2 [P<0.005] and C20:4 [P<0.05], compared to tumor-bearing rats on control diet [Table 4-1]. On the

other hand, proportion of n-3 fatty acids [P<0.001] including EPA [P<0.005] and DHA [P<0.001] were significantly higher in rats fed long term fish oil diet compared to their respective rats on the control diet [Table 4-1]. In addition, long term fish oil diet induced a significant reduction in the ratio of n-6/n-3 fatty acids [P<0.001] in tumor-bearing rats compared to animals fed a control diet. After both cycle-1 and cycle-2, proportion of all fatty acids remained unchanged compared with rats bearing only tumor and fed a long term fish oil diet [Table 4-1]. Additionally, cycle-1 and cycle-2 rats on the adjuvant fish oil diet displayed similar proportions of total saturated fatty acid, n-6, and n-3 including EPA and DHA, and n-6/n-3 ratio in the gastrocnemius muscle compared with their respective long term fish oil diet group [Table 4-1].

4.3.2 Triglyceride Fatty Acids Profile:

Fatty Acids Compositions of TG in a Preclinical Model of Tumor- and Chemotherapy-Associated Myosteatosis: We next quantified total TG fatty acids as well as TG fatty acid composition in the gastrocnemius muscle of rats on the control diet. The total TG fatty acid content in the gastrocnemius muscle has been presented in chapter 3 [Figure 3.3]. Total saturated fatty acids including C16:0 and C18:0 were comparable between tumor bearing animals and reference group. However, tumor-bearing rats exhibited higher total monounsaturated fatty acids [P<0.001], driven by higher C18:1 [P<0.001], and lower proportion of total n-6 fatty acid [P<0.03] and n-3 fatty acids [P<0.05], driven by lower C18:3 [P<0.001, Table 4-2]. Rats on the control diet that underwent 2-cycles of chemotherapy displayed a higher proportion of saturated fatty acids compared to tumor-bearing rat only [P<0.04, Table 4-2], driven by higher proportion of C18:0 [P<0.005]. Rats receiving 2-cycles of chemotherapy exhibited significant decreases in total n-3 fatty acids [P<0.002] and DHA was not detected at any time after chemotherapy treatment. Two

cycles of chemotherapy further cause a significant increase in n-6/n-3 ratio in TG fraction of gastrocnemius muscle compared to rats bearing only tumor [P<0.001, Table 4-2].

Effects of the Fish Oil Diets on TG fatty acids composition in a Preclinical Model of Tumor- and Chemotherapy-Associated Myosteatosis: Since both tumor and chemotherapy treatment significantly decreased n-3 fatty acids [Table 4-2], we wanted confirm that the fish oil diets restored muscle essential fatty acids including EPA and DHA. We also wanted to confirm the effect of a long term compared to the adjuvant fish oil diets in total TG in the gastrocnemius muscles [Table 4-2]. Total TG fatty acids in these rats were reported in Chapter 3 [Figure 3.3].

Tumor-bearing rats on the long term fish oil diet displayed lower proportion of n-6 fatty acids [P<0.05], driven by lower proportion C18:2 [P<0.001], and higher proportion of n-3 fatty acids [P<0.001], driven by higher EPA [P<0.05] and DHA [P<0.001], compared to tumor-bearing rats on control diet [Table 4-2]. In addition, long term fish oil diet induced a significant reduction in the proportion of n-6/n-3 fatty acids [P<0.001] in tumor-bearing rats compared to animals fed a control diet. Subsequently, after both cycle-1 and cycle-2, rats on the long term fish oil diet exhibited lower proportion of n-6 [P<0.001 and P<0.001, respectively] and n-6/n-3 ratio [P<0.001 and P<0.001, respectively] driven by higher proportion of total n-3 fatty acids [P<0.001 and P<0.001, respectively], driven by higher proportion of DHA [P<0.001 and P<0.001, respectively] compared to their respective control animals [Table 4-2]. Additionally, cycle-1 and cycle-2 rats on the adjuvant fish oil diet displayed similar effect on fatty acid composition in the gastrocnemius muscle compared with their respective long term fish oil diet group [Table 4-2].

4.4 Discussion

This study is the first to use a preclinical model of tumor- and chemotherapy-associated myosteatosis [see Chapter 3] to investigate the effects of tumor and chemotherapy on PL and TG content and composition in skeletal muscle. Additionally, we investigated the effect of dietary EPA and DHA fed prior to tumor implantation [Long term] and concurrent with the first cycle of chemotherapy [adjuvant] on PL and TG content and compositions in skeletal muscle. We show in the previous study [see Chapter 3] for the first time that feeding a diet containing EPA and DHA prevents tumor-associated myosteatosis, and that adjuvant feeding fish oil- is similarly efficacious to long term- EPA and DHA feeding in greatly mitigating chemotherapy-associated myosteatosis. While in the previous study we show that total TG content in the gastrocnemius muscle of tumor-bearing animals before and during chemotherapy that fed control diet increased [see Chapter 3, Figure 3.3], in this study we show that total PL decreases [Figure 4.1] following the first cycle of chemotherapy in control fed animals but is markedly increased in the gastrocnemius muscle of fish oil fed animals.

Rats bearing-tumor alone or undergoing one or two cycles of chemotherapy and fed a control diet exhibited higher saturated fatty acids, while n-3 fatty acids including EPA were significantly reduced. DHA was not detected in either PL and TG compared to reference animals. However, animals fed a diet containing fish oil either beginning before tumor-implantation [long term] or at the initiation of chemotherapy [adjuvant] exhibited lower saturated fatty acids, n-6, while n-3 fatty acids including EPA and DHA were significantly increased in gastrocnemius muscle compared to their respective control groups. Collectively, our results suggest that PL and TG composition of gastrocnemius muscle reflect EPA and DHA intervention which may associate with preventing and treating myosteatosis in these animal models [see Chapter 3]. Feeding EPA

and DHA for short term [a week] was as effective as feeding EPA and DHA for long term [4 weeks] to be incorporated into gastrocnemius muscle tissue.

4.4.1 PL and TG Fatty Acid Composition in Gastrocnemius Muscle of Model of Tumor- and Chemotherapy-Associated Myosteatosis

Myosteatosis is considered as an important prognostic factor in the oncology setting, as it has been found to be associated with mortality in cancer patients treated with chemotherapy (6-9). In this study, the Ward colon tumor model displayed robust fat accumulation within skeletal muscle that was exacerbated by successive chemotherapy cycles [see Chapter 3]. Biological measure of fat in muscle of cancer associated with myosteatosis is not explored. Muscle contains different lipid species such as TG and PL. In the present study, the mean total PL fatty acids content in the gastrocnemius muscles of rats bearing-tumor and received 1- cycle of chemotherapy treatment is lower than reference animals. The reduction in total PL that was observed following the first cycle of chemotherapy could be attributed to a reduction in either number or size of muscle fiber. In the previous study [see Chapter 3] we have reported that chemotherapy resulted in lower mean muscle fiber cross sectional area in rats on the control diet compared with reference animals [see Chapter 3, Figure 3.5]. Total muscle PL fatty acids in the control-fed rats receiving 2- cycles of chemotherapy significantly increased to similar levels of tumor-bearing rats that received no treatment. The increase in PL content of gastrocnemius muscle following the second cycle of chemotherapy could be a result of increased adipocytes and/or lipid droplets membranes since it was concurrently associated with increase in TG fatty acid content. These findings are supported by Oil Red O staining as it showed an increase in neutral lipid content primarily within muscle fibers, indicative of lipid droplets, and also evident between muscle fibers, after the second cycle

of chemotherapy, which may be attributed to the formation of intermuscular adipocytes [see Chapter 3, Figure 3.2].

Not only the total amount of PL fatty acids is important but also the fatty acid composition of PL within the muscle can contribute to muscle metabolism. Alterations in the composition of membrane fatty acids modifies the thickness and fluidity of the lipid bilayer, which can subsequently impact skeletal muscle lipid content. In non-cancer conditions associated with myosteatosis such as obesity and insulin resistance, it has been reported that skeletal muscle membrane PL display a different fatty acid composition compared with lean subjects, with a higher proportion of the saturated fat and lower concentrations of polyunsaturated fatty acid (63-65). In this study, we found that tumor bearing rats and those receiving 1- and 2-cycles of chemotherapy exhibited higher proportion of total saturated fatty acid compared to reference animal in PL fraction [Table 4-1]. Preclinical models of insulin resistance reported that higher saturated fat–laden membranes are associated with insulin resistance, which is similarly observed in humans (66).

Bordoni et al reported that tumour as well as chemotherapy treatment induces alterations in the desaturation and elongation of alpha-linolenic acid [C18:3 n-3] to EPA and DHA in heart muscle (131). Similarly, in this study we observed that tumor bearing only rats and those received 1- and 2-cycles of chemotherapy exhibited low proportion of total n-3 fatty acids and DHA was not detected following chemotherapy treatment in PL and TG fractions of gastrocnemius muscle. These results suggested a conditional essentiality of EPA and DHA in cancer and during treatment.

This study found that the proportion of n-6/n-3 ratio was significantly increased in the presence of the tumor and following 1- and 2- cycles of chemotherapy in TG fraction which driven

by lower n-3 fatty acids compared to reference rats. Increase the ratio of n-6/n-3 derives eicosanoids that modulate the production of pro-inflammatory cytokines (132), such as interleukin-6 [IL-6] and tumor necrosis factor alpha [TNF-α] that may cause an increase in muscle catabolism and mitochondrial dysfunction (86,133,134). Fats stored in muscle usually serve as a fuel for mitochondria oxidation (83), suggesting that a decrease in the size or number of mitochondria may cause lipid accumulation inside of the muscle. Collectively, these results suggest that fatty acid composition in both PL and TG are altered in the presence of Ward colon tumor and following chemotherapy treatment with higher saturated fatty acid, n-6/n-3 ratio and lower n-3 fatty acids including EPA and DHA compared to reference rats. Further investigation is required to assess pathways that may affected due to the changes in fatty acid composition and contribute to increase TG content within muscle tissue.

4.4.2 Effects of the Fish Oil Diets on PL and TG Fatty Acid Composition in Gastrocnemius Muscle of Model of Tumor- and Chemotherapy-Associated Myosteatosis

Nutrient deficiencies have been observed in cancer patients following chemotherapy treatment including low levels of plasma EPA and DHA that appear to occur concurrently with muscle loss and fat deposition within the muscle, which can be corrected by providing fish oil supplementation to cancer patients during treatment (1,24).

The animal model used in the present study demonstrated that incorporation of EPA and DHA into both TG and PL fatty acids was similar between rats fed fish oil for either long term [3 weeks minimum] or short term [one week minimum]. On the other hand, saturated fatty acids as well as n6/n-3 proportion were significantly decreased in PL fraction following fish oil supplementation compared to rats fed a control diet. Healthy people supplemented with 5 grams of fish oil per day had an increase in n-3 fatty acids [3.8% to 5.1% total fatty acids] after 2-weeks,

and it further increased after 4-weeks to ~6.8% total fatty acids (135). Other studies have demonstrated that 8-weeks of fish oil supplementation results in a 2-fold increase in muscle PL n-3 fatty acid composition in healthy subjects [5.04% to 9.03% total fatty acids; (75,76). In the present study, we observed similar increases in proportion of n-3 fatty acid content in both TG and PL fatty acids, even with one week of supplementation [adjuvant-cycle-1 chemotherapy]. In cancer patients, a temporal assessment of n-3 fatty acid incorporation into tissue has not been conducted Further work similar to McGlory et al. (132) could be replicated in a cancer model to determine whether a prolonged period of fish oil supplementation may be required to reach a saturation of n-3 fatty acids in skeletal muscle, especially during chemotherapy treatment (21). Improvement of the insulin response is the most widely explored mechanism for the beneficial effects of EPA and DHA fatty acids on muscle and has been studied in many experimental systems and in a number of non-cancer disease states (68-73). In several experimental studies, EPA and DHA have been reported to support the anabolic potential of muscle (68,70,72,76). In rats, n-3 fatty acid incorporation into muscle increased membrane unsaturation concurrent with improved insulin sensitivity and decreased muscle triglyceride content (77). Fat-1 mice, which are capable of endogenous n-3 fatty acid synthesis, exhibit better glucose tolerance than control mice (68). Some, (70,76,78,79) but not all (80,81) studies have shown an effect of n-3 polyunsaturated fatty acids on AKT activation, a regulator of muscle growth. Incorporation of EPA and DHA into the muscle membrane alters its composition, and may modulate key membrane substrates involved in the insulin signaling pathway and subsequent protein synthesis and improve muscle anabolism (82).

We have also observed that PL content in gastrocnemius muscles was significantly higher in rats fed dietary fish oil compared to their respective control groups. The increase in total PL could be attributed to increase in either number or size of muscle fiber. In the previous study [see

Chapter 3] we have reported that rats fed a fish oil diet exhibited higher mean muscle fiber cross sectional area following chemotherapy treatment compared to their respective control diet groups [see Chapter 3, Figure 3.5], however this observation would require further quantitative analysis of the number of muscle fiber. The increase in PL content of gastrocnemius muscle in these groups could be also a result of increased cells within the muscle such as mitochondria. Higher number of mitochondria in skeletal muscle has been reported during EPA and DHA intervention through the activation of PGC-1α in skeletal muscle [reviewed by (91)]. However, further research is needed to investigate the mechanisms underlying the beneficial effect of EPA and DHA provided in the diet on skeletal muscle in cancer.

4.5 Conclusions

The novel observations of pathological fat accumulation [see chapter 3] coupled with lower total PL, lower n-3, driven by undetectable DHA following chemotherapy treatment, and higher saturated fatty acids and n-6/n-3 ratio in the skeletal muscles of an *in vivo* tumor-bearing model that underwent chemotherapy treatment, reveal a potential alteration in fatty acid composition within skeletal muscle that may be involved in the development of myosteatosis in cancer patients. Long term and adjuvant dietary EPA and DHA feeding were equally efficacious in markedly reducing TG fatty acids content within the muscle [see Chapter 3, Figure 3.3] and improving the incorporation of EPA and DHA fatty acids in both PL and TG fraction within the muscle which was also coupled by reducing proportion saturated fatty acids and n-6/n-3 ratio. These results are novel future research to determine further mechanisms by which dietary EPA and DHA supplementation mitigate myosteatosis in the skeletal muscles of cancer patients.

Table 4-1 Fatty acids composition of muscle phospholipid of rats bearing tumor and receiving chemotherapy

Fatty Acids	**Study Groups**									**P Value**
(%)		Control Diet			Long Term Fish Oil Diet			Adjuvant Fish Oil Diet		
	Reference	Tumor	Cycle1	Cycle2	Tumor	Cycle1	Cycle2	Cycle1	Cycle2	
C 16:0	20.9 ± 2.6ac	21.1 ± 1.1ac	22.0 ± 1.4ab	20.5 ± 4.5ac	18.3 ± 1.4b	21.1 ± 2.0ac	20.2 ± 1.6ac	19.5 ± 1.6c	21.2 ± 1.1ab	P<0.001
C 18:0	21.9 ± 2.6a	21.4 ± 2.5a	20.2 ± 1.7a	22.5 ± 3.5a	20.6 ± 1.0a	22.2 ± 2.1a	22.0 ± 1.6a	21.2 ± 1.2a	18.8 ± 1.3b	P<0.003
C 18:1 n-9	4.1 ± 1.7a	10.3 ± 1.8b	12.0 ± 2.2c	12.3 ± 3.9c	7.3 ± 1.9d	9.5 ± 1.4bd	10.5 ± 2.4b	9.3 ± 0.9b	9.4 ± 1.1b	P<0.001
C 18:2 n-6	11.9 ± 0.9a	12.0 ± 1.2a	9.9 ± 0.8b	9.6 ± 2.1b	7.4 ± 1.0c	8.0 ± 1.1bc	8.5 ± 0.9bc	8.4 ± 0.9bc	8.2 ± 0.8bc	P<0.001
C 18:3 n-3	0.1 ± 0.1a	0.1 ± 0.1a	NDa	NDa	0.1 ± 0.1a	0.1 ± 0.1a	0.2 ± 0.1b	0.1 ± 0.1a	0.2 ± 0.1b	P<0.021
C 20:4 n-6	17.6 ± 2.6a	16.9 ± 1.2a	17.3 ± 1.2a	16.2 ± 2.0a	13.3 ± 2.3bc	10.2 ± 1.9b	9.4 ± 2.1b	15.0 ± 0.9ca	14.4 ± 1.3c	P<0.001
C 20:5 n-3	0.1 ± 0.1a	0.2 ± 0.1a	0.1 ± 0.1a	0.1 ± 0.1a	1.7 ± 0.3b	2.2 ± 0.3c	2.5 ± 0.2c	1.7 ± 0.5b	1.7 ± 0.5b	P<0.001
C 22:6 n-3	0.2 ± 0.1a	NDb	NDb	NDb	20.2 ± 2.0c	20.5 ± 1.1c	20.6 ± 1.5c	20.2 ± 0.7c	20.3 ± 1.6c	P<0.001
∑SFA	50.7 ± 1.7a	55.8 ± 1.6b	55.7 ± 1.8b	60.2 ± 2.6c	45.3 ± 1.7d	45.1 ± 1.3d	43.8 ± 1.1de	41.3 ± 1.6e	41.3 ± 1.9e	P<0.001
∑MUFA	4.6 ± 1.7a	10.9 ± 1.9b	12.7 ± 2.3c	13.1 ± 3.9c	7.8 ± 2.0d	10.1 ± 1.5b	11.0 ± 2.5b	9.7 ± 1.0b	10.3 ± 1.4b	P<0.001
∑n-6	31.1 ± 2.7a	30.3 ± 2.0a	28.7 ± 1.7b	26.7 ± 2.0c	21.1 ± 1.6de	18.7 ± 1.3d	18.5 ± 1.9d	23.9 ± 1.4e	23.0 ± 1.7e	P<0.001
∑n-3	3.1 ± 0.3a	3.2 ± 0.3a	2.7 ± 0.3a	2.3 ± 0.2a	25.7 ± 2.3b	26.1 ± 1.2b	26.5 ± 1.6b	25.0 ± 1.2b	25.4 ± 1.8b	P<0.001
n-6/n-3	10.5 ± 1.2a	9.5 ± 0.5a	10.5 ± 1.2a	11.3 ± 1.0a	0.8 ± 0.1b	0.7 ± 0.1b	0.7 ± 0.1b	1.0 ± 0.1b	0.9 ± 0.1b	P<0.001

Values are presented as means ± SD. Different letters indicate significant differences among groups. Fatty acids composition of muscle phospholipid was quantified using gas chromatography SFA, saturated fatty acid; MUFA, Monounsaturated fatty acid; ND, not detectable; C 20:5 n-3, eicosapentaenoic acid (EPA); C 22:6, docosahexaenoic acid (DHA).

Table 4-2 Fatty acids composition of muscle triglyceride of rats bearing tumor and receiving chemotherapy

Fatty Acids	**Study Groups**									**P Value**
(%)		Control Diet			Long Term Fish Oil Diet			Adjuvant Fish Oil Diet		
	Reference	Tumor	Cycle1	Cycle2	Tumor	Cycle1	Cycle2	Cycle1	Cycle2	
C 16:0	19.0 ± 4.3ab	20.9 ± 2.1a	17.4 ± 2.7ab	17.5 ± 2.3ab	20.0 ± 1.7a	17.9 ± 2.1ab	18.2 ± 1.6ab	15.9 ± 2.7b	16.2 ± 4.4ab	P<0.02
C 18:0	10.0 ± 2.9ac	7.9 ± 1.5a	11.2 ± 2.3bc	15.2 ± 4.3c	10.5 ± 2.2ac	12.0 ± 3.8bc	12.1 ± 2.9bc	11.2 ± 1.2bc	8.6 ± 1.6a	P<0.02
C 18:1 n-9	33.9 ± 6.5a	41.6 ± 2.6b	45.2 ± 2.9c	42.5 ± 5.6bc	37.9 ± 2.7a	42.3 ± 1.2bc	42.7 ± 2.2bc	42.3 ± 1.2bc	44.6 ± 3.3bc	P<0.001
C 18:2 n-6	20.8 ± 3.8a	20.0 ± 3.6a	15.2 ± 1.5b	15.9 ± 2.9b	15.8 ± 3.0b	13.8 ± 2.7bc	12.2 ± 3.3c	19.5 ± 1.6a	19.5 ± 1.8a	P<0.001
C 18:3 n-3	1.4 ± 0.3a	0.6 ± 0.4b	0.4 ± 0.2bc	0.2 ± 0.1bc	0.6 ± 0.3b	0.8 ± 0.2b	0.6 ± 0.3b	0.5 ± 0.4bc	0.2 ± 0.1c	P<0.001
C 20:4 n-6	1.8 ± 0.4a	1.4 ± 0.2ac	3.0 ± 1.2b	1.7 ± 0.7a	1.2 ± 0.3ac	0.9 ± 0.2c	0.9 ± 0.3c	1.7 ± 0.4a	1.5 ± 0.6ac	P<0.001
C 20:5 n-3	0.9 ± 0.3ac	0.4 ± 0.2ab	0.7 ± 0.2a	0.2 ± 0.2b	0.9 ± 0.4ac	0.9 ± 0.1a	1.3 ± 0.7c	1.5 ± 0.3c	1.1 ± 0.5c	P<0.001
C 22:6 n-3	0.7 ± 0.4a	0.6 ± 0.4a	NDb	NDb	1.7 ± 0.5c	2.6 ± 1.1c	2.6 ± 0.7c	2.7 ± 0.8c	1.7 ± 0.5c	P<0.001
∑SFA	29.3 ± 2.9a	28.9 ± 1.1a	30.1 ± 1.4ab	32.7 ± 2.0b	31.8 ± 3.4a	30.5 ± 2.8ab	30.8 ± 3.3ab	27.2 ± 2.5ac	24.8 ± 3.8c	P<0.005
∑MUFA	39.8 ± 2.2a	46.3 ± 3.1bc	48.1 ± 3.0bd	45.6 ± 3.3bc	40.8 ± 3.3a	45.8 ± 2.1bc	45.9 ± 3.1bc	44.7 ± 2.5c	50.0 ± 1.7d	P<0.001
∑n-6	26.9 ± 2.3a	22.9 ± 1.4b	20.2 ± 2.0bc	19.0 ± 2.0c	19.4 ± 1.8c	16.3 ± 2.1d	14.8 ± 2.6d	22.6 ± 1.8b	22.2 ± 2.5b	P<0.001
∑n-3	3.3 ± 0.6a	1.8 ± 0.3b	1.7 ± 0.4b	0.5 ± 0.3c	5.5 ± 1.5d	6.1 ± 2.0d	6.8 ± 2.0d	5.5 ± 1.4d	5.1 ± 0.7d	P<0.001
n-6/n-3	10.9 ± 2.9a	17.2 ± 5.2b	15.3 ± 3.1b	49.2 ± 13.2c	3.8 ± 1.2d	2.9 ± 1.1d	2.4 ± 1.0d	4.4 ± 1.2d	7.8 ± 2.6a	P<0.001

Values are presented as means ± SD. Different letters indicate significant differences among groups. Fatty acids composition of muscle triglyceride was quantified using gas chromatography. SFA, saturated fatty acid; MUFA, Monounsaturated fatty acid; ND, not detectable; C 20:5 n-3, eicosapentaenoic acid (EPA); C 22:6, docosahexaenoic acid (DHA).

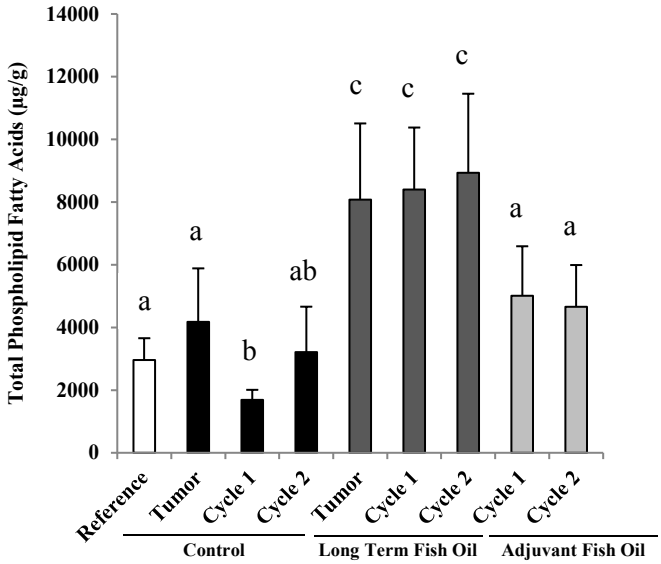


Figure 4.1 Total Phospholipid fatty acid levels in the rat gastrocnemius muscle quantified using gas chromatography. Values are means ± SD. Different letters indicate significant differences among groups ($P<0.05$).

CHAPTER 5

Role of EPA and DHA on Skeletal Muscle Mitochondria in an Animal Model of Colorectal Cancer Associated Myosteatosis

5.1 Introduction

In skeletal muscles, fat consists of intra-myocellular lipid droplets within the cytoplasm of myocytes and intermuscular adipocytes (136). These fat stores constitute the main substrate that fuels skeletal muscle contraction (137); however, excessive deposition of triglyceride within skeletal muscle, which should contain only small amounts of fat, represents a pathological condition. Specifically, this state represents aberrant of deposition, synthesis and/ or elimination of triglycerides. The pathological accumulation of fat within muscle, termed myosteatosis, is a serious consequence of the tumor bearing state and chemotherapy treatments that is considered as a predictor of time-to-death (6-9) . Methods used to measure myosteatosis in humans have been reported [reviewed by (97,138)], however, the mechanistic underpinnings of this condition still require characterization.

Recent human and animal studies have investigated the manner in which fat infiltrates muscle in the tumor-bearing state. The size and number of intramyocellular lipid droplets has been shown to increase in the presence of cancer (49). In our previous study (reported in chapter 3), we revealed that presence of the Ward colon tumor in an animal model significantly increased triglyceride fatty acid content, measured biochemically and visually using Oil Red O, in the gastrocnemius muscles compared to healthy rats and it was further increased following the second cycle of chemotherapy compared to untreated tumor-bearing rats and it was associated with an increase in gene expression involved in adipogenesis and lipogenesis. Collectively, these results

showed that both tumor and chemotherapy treatment could increase lipid synthesis within the muscle.

Increased lipid synthesis within the muscle along with lower fat utilization would result in fat accumulation within the muscle as lipids stored in muscle usually are used as a fuel for mitochondria oxidation (83). Skeletal muscle mitochondrial content may be modified if there is a changing in muscle fiber composition. Normal rat gastrocnemius and tibialis anterior muscles, for example, are comprised of mixed fiber types including type I and II (139). Type I fibers rely on mitochondrial oxidative phosphorylation for ATP production, are high in oxidative enzymes, low in glycolytic markers, and have high capacity to store and utilize fat, while type II fibers primarily use glycolysis [type IIB] or both glycolysis and mitochondrial oxidative phosphorylation [type IIA and type IIDX] for ATP production, store glycogen, and have less mitochondria. Therefore, biochemical properties of muscles with different fiber type composition may underlie differential metabolic response in the tumor-bearing state. An experimental study showed a significant reduction in the major regulation enzymes for oxidative phosphorylation including cytochrome-c oxidase complex subunit IV and a 50% reduction in the level of succinate dehydrogenase [SDH] as well as the main transcriptional factor for mitochondrial biogenesis, Peroxisome Proliferator-Activated Receptor-Gamma Coactivator *[PGC-1α]* in gastrocnemius muscle of the Apc [Min/+] mouse compared to wild-type control mice (86). However, fat content within the muscle has not been assessed.

In non-cancer conditions, the dysfunction of mitochondria has been linked to myosteatosis in elderly population (reviewed by (87), insulin resistance and obesity (88). An experimental study used tibialis anterior [highly glycolytic fiber, mainly type IIB] and soleus [highly oxidative muscle; mainly type I fiber] muscles of male C57Bl6/J mice to assess lipid accumulation in specific fibers

type as well as muscle oxidative capacity in response to 12 weeks of high fat diet intervention reported that triglyceride content in the oxidative soleus muscle of the control group was ≈ two-fold higher compared with the glycolytic tibialis anterior muscle (90). This study showed that the accumulation in muscle triglyceride content is higher in oxidative fiber types. However, in the tumor-bearing state of fiber type specific lipid accumulation has not been yet characterized.

Many beneficial effects of eicosapentaenoic [EPA, 20:5n-3] and docosahexaenoic [DHA, 22:6n-3] fatty acids on mitochondrial content and oxidative capacity have been reported including the stimulation of mitochondrial biogenesis through activation of *PGC-1α* in skeletal muscle cells and increased function of the major enzymes in the electron transport chain [reviewed by (91)]. However, the role of EPA and DHA on mitochondrial density as well as oxidative capacity has not been investigated in relation to myosteatosis in any neoplastic condition.

The objective of this work was first evaluate total neutral lipid content within tibialis anterior muscle to confirm our previous findings observed in gastrocnemius muscles [see Chapter 3, Figure 3.3], and then assess if the lipid accumulation is related to a specific fiber type in a preclinical model of colon cancer fed a control diet or fish oil diet prior to [long term] and after tumor implantation [adjuvant diet]. We also wanted to explore if these observations could be related to mitochondrial content and oxidative capacity. Additionally, we wanted to determine whether EPA and DHA supplementation beginning at the initiation of chemotherapy[adjuvant] had similar outcomes compared with starting prior to tumor implantation [long term]. It was hypothesized that compared to healthy rats not bearing a tumor, both rats bearing the Ward colorectal tumor alone or received 1- and -2 cycles of chemotherapy will exhibit higher content of neutral lipid staining within muscle fiber, and neutral lipid accumulation would be associated with lower mitochondrial density and oxidative capacity. However, rats fed a diet containing EPA and

DHA prior [long term] to, and following [adjuvant] tumor implantation will exhibit lower neutral lipid staining within muscle fiber and higher mitochondrial density and oxidative capacity compared to their respective control groups.

5.2 Material and Methods

Experimental design including, animal model, dietary design, tumor and chemotherapy description, body weight and food intake measurements are reported in Chapter 3 section 3.2.

5.2.1 Tissue Collection and Processing

All rats were euthanized by carbon dioxide [CO_2] asphyxiation. At euthanization, tibialis anterior muscles were isolated, weighed, and frozen in melting isopentane cooled in liquid nitrogen [-156°C], and stored at -80°C until subsequent analyses. For histology staining frozen tibialis anterior muscles were serially cryosectioned transversely [3 serial sections per sample, 10 μm thick] using a refrigerated cryostat [−20 °C, CTI Cryostat, International Equipment Cryostat, Needham Heights, MA] for Oil Red O stain, immunofluorescence Myosin Heavy Chain [MHC] stain, and SDH stain. One hundred mg portions of muscles were kept frozen at -80 for mRNA and protein analyses.

5.2.2 Histology

5.2.2.1 Oil Red O, Laminin and Dystrophin Staining

Slides were air dried for 30 min at room temperature and then fixed in 10% neutral buffered formalin [NBF, Sigma-Aldrich Canada Co., Oakville, Ontario] for one hour at room temperature. Slides were washed in phosphate buffered saline with Tween® 20 [PBS-T; Polysorbate, Abcam Inc, Toronto, ON] for 5 min followed by 2 x 5 min in PBS. Primary antibodies [500 μl; diluted in blocking solution, Table 5-1] were applied then slides were incubated in a humidity chamber for

two hours at room temperature. All slides were washed 3 x 5min with PBS. Secondary antibodies [500 µl; diluted in blocking solution, Table 5-1] were applied, then slides were incubated in a humidity chamber for 2 hours at room temperature. Slides were then washed 3 x 5min in PBS and stained for neutral lipid content using Oil Red O as previously described (119). Sections were then rinsed with deionized water 3 x 30 sec, then with running tap water for 5 min, and mounted with ProLong ™ [Molecular Probes™, Eugene, OR] mounting medium and 1.5-thickness coverslips and laid flat for 24 hours at room temperature. Fluorescent images were taken within 2-3 days.

5.2.2.2 Immunofluorescence Detection of Myosin Heavy Chain-Based Muscle Fiber Types

Fiber types were identified in tibialis anterior muscles by using Immunofluorescence MHC staining technique. Slides were air dry for 30 min at room temperature and then fixed with pre-chilled 100% acetone at -20ºC for 10 min. Muscles were permeabilized for 5 min in PBS-T followed by 3 x 5 min wash in PBS. Samples were blocked with blocking solution containing 10% normal goat serum and 1% albumin in PBS-T and kept overnight in a humidity chamber at 4 ºC. On the second day, samples were washed with PBS followed by incubation with primary antibodies [500 µl, diluted in blocking solution, Table 5-2] for 2 hours at room temperature. Slides were then washed 3 x 5 min in PBS and incubated in a humidity chamber for 2 hours with secondary antibodies [500 µl, diluted in blocking solution, Table 5-2] at room temperature. Slides were then washed 3 x 5min in PBS. Slides were mounted with ProLong ™ [Molecular Probes™, Eugene, OR] mounting medium and 1.5-thickness coverslips, and were laid flat for 24 hours at room temperatures and fluorescent images were taken within 2-3 days.

5.2.2.3 Myosin Heavy Chain and Oil Red O Confocal Imaging and Analyses

Muscle sections were visualized with a spinning disk confocal microscope [Olympus IX-81 motorised microscope base, Yokagawa CSUX1 spinning disk confocal scan-head] equipped

with pumped diode lasers [blue: 44mW 405 nm, green: 50mW 491 nm, red 50mW 561nm, far-red: 45mW 642nm] and an EM-CCD cooled camera [Hamamatsu; Quorum Technologies, Guelph, ON, Canada]. Individual z-stacked images were taken across the entire muscle cross section with a 20x oil lens, and then stitched and merged together into composite images using Volocity 6.3 software [PerkinElmer, Waltham, MA, USA]. Each muscle fiber type [I, IIA, IIB, IIDX, I/IIA, and IIA/IIDX] was presented as proportion within an average of 7691 ± 1074 fibers per sample. Entire muscle as well as muscle fiber cross sectional area [CSA] was measured using Volocity 6.3 software. Qualitative and quantitative analysis of Oil Red O staining was performed in a blinded manner. Minimum pixel intensity for positive Oil Red O staining was set manually. Neutral lipid content within muscle fibers, indicative of lipid droplets, was presented as proportion of fibers expressing Oil Red O within an average of 4390 ± 810 fibers per sample. Neutral lipid content between muscle fibers, which may be indicative to the formation of intermuscular adipocytes, was presented as proportion of area [μm^2] expressing Oil Red O between muscle fibers to whole muscle cross sectional area. Neutral lipid content-based muscle fiber type was performed manually. The two serial section slides, stained with either Oil Red O or MHC, were matched manually to identify fibers associated with neutral lipid accumulation. The analysis was conducted on 200 fibers per sample [50 fibers/ type, see example Figure 5.1]. Data are presented positive Oil Red O mean pixel intensity of each fiber/ minimum pixel intensity**.**

5.2.2.4 Succinate Dehydrogenase Histochemical Stain

Muscle oxidative capacity was identified in tibialis anterior muscles by using SDH staining as previously described (140). SDH mean pixel intensity of whole tibialis anterior muscle CSA was measured using Image J software. Data are presented as mean SDH intensity of whole muscle CSA.

5.2.3 Mitochondrial Enzymes Activity and Measures

Frozen muscle [≈ 30mg] was pulverized on Dry ice and under liquid N2. Samples then were homogenized [1:10, wt/vol] in an ice-cold 100 mM KH_2PO_4-Na_2HPO_4 buffer [pH 7.2] containing 5 mM EDTA. Homogenized samples were processed on ice with ≈15 turns of the blue Teflon pestle following by stirring with a micro-stair bar for 20 min. Samples went through three freeze [dry ice] and thaw [on ice] cycles and then centrifuged for 30 min at 20,000 ×g at 4°C. Supernatants were transferred to new 1.5 mL tubes on ice. Muscle total protein was determined according to Bradford (141). Activities of citrate synthase [CS] and 3-hydroxyacyl-CoA dehydrogenase [HADH] were determined as previously described (142). Samples were evaluated in duplicate. Results were presented as Unit/gram [U/g] of total protein.

5.2.4 RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA extraction and RNA quantity and quality measurements were reported in Chapter 3 section 3.2.7. High Capacity cDNA Reverse Transcription kit [Applied Biosystems, Foster City, CA, USA] was used to reverse transcribe RNA to cDNA following the manufacturer's protocol. Pre-designed TaqMan® probes with a 6-carboxyfluorescein phosphoramidite [FAM™] label on the 5' end and primer sets Applied Biosystem] were used to target the following genes: Peroxisome Proliferator-Activated Receptor-Gamma Coactivator [*PGC-1α;* Rn 00580241_m1] and Transcription Factor A, Mitochondrial [*TFAM;* Rn 00580051_m1]. *18S* rRNA [Rn03928990_g1] was unaffected, and thus used as the endogenous control. qRT-PCR was performed on 1 μl cDNA samples, in triplicate, on an ABI 7900HT thermocycler [Applied Biosystems]. Relative changes in gene expression were determined using the $2^{-\Delta\Delta CT}$ method.

5.2.5 Statistical Analysis

Data are summarized as mean ± SD. One-way analysis of variance [ANOVA] were used to test differences in muscle fiber type composition, muscle cross sectional area, Oil Red O staining, SDH, mitochondrial enzymes activities and mRNA fold changes in gene expression. When a significant difference was observed, post-hoc analysis was completed using the Bonferroni model. A Pearson's correlation test was used to test relationships between mRNA expression of various genes as well as mitochondrial enzyme activities and neutral lipid content. Statistical significance was reported when p value <0.05. All statistical analyses were performed using SPSS 21.0 [Chicago, IL, USA] for Windows.

5.3 Results

5.3.1 Neutral Lipid Staining

To test the hypothesis that presence of Ward colon tumor as well as chemotherapy treatment will increase neutral lipid content within muscle fibers, we measured neutral lipids content in the tibialis anterior muscle of rats fed a control diet. Neutral lipids were semi-quantified according to strong positive staining for Oil Red O within myocytes. Two weeks following tumor implantation, the proportion of fibers expressing Oil Red O in the tibialis anterior muscle was 23 % higher in the control diet group compared with the reference group [P<0.001, Figure 5.2], reflecting tumor-associated fat accumulation in muscle and these results are similar to our previous findings in gastrocnemius muscle [see Chapter 3, Figure 3.2]. Subsequently, rats that received chemotherapy treatment exhibited higher neutral lipid content following cycle-1 [P<0.04] and cycle-2 [P<0.001] compared to reference animals [Figure 5.2].

To test the hypothesis that in a Ward colon tumor-bearing rat model of myosteatosis, dietary EPA and DHA would mitigate tumor- and chemotherapy-associated fat accumulation in

skeletal muscle, we measured neutral lipids content in the tibialis anterior muscle of rats fed a diet containing fish oil starting one week prior to tumor implantation [long term] and beginning when chemotherapy was initiated [adjuvant]. Before chemotherapy, tumor-bearing rats on the long term fish oil diet displayed proportions of fibers expressing Oil Red O similar to levels observed in reference animals, which were significantly lower compared with tumor-bearing rats on the control diet [$P<0.001$, Figure 5.2]. Subsequently, after both cycle-1 and cycle-2, fibers expressing Oil Red O in the tibialis anterior muscle were significantly lower in tumor-bearing rats on the long term fish oil diet compared with their respective control groups. However, rats on the adjuvant fish oil diet displayed similar amounts of fibers expressing Oil Red O staining compared with their respective control diet group after each cycle of chemotherapy [Figure 5.2]. We further quantified neutral lipids staining between muscle fibers. Neutral lipid between muscle fibers was quantified as proportional area of Oil Red O staining between muscle fibers to whole muscle CSA. Following cycle-1 chemotherapy, tumor-bearing rats on the control diet exhibited the highest proportion of neutral lipids content between muscle fibers compared with all groups including rats on control and fish oil diets [$P<0.04$, Figure 5.2]. All other groups on control or fish diets exhibited similar proportion of neutral lipids content between muscle fibers compared with reference rats. Collectively, these results show that both Ward colon tumor and chemotherapy treatment induced myosteatosis in tibialis anterior muscle and the long term fish oil diet mitigated chemotherapy-associated fat accumulation in muscle similar to what was observed for gastrocnemius muscle [see chapter 3, Figure 3.2]

5.3.2 Neutral Lipid Staining-Based Muscle Fiber Type

Fibers associated with neutral lipid accumulation were also assessed. Positive Oil Red O staining was observed in Type I, IIA and IIDX and there was no positive Oil Red O staining in

type IIB for all groups including rats on control diet, long term fish oil diet, and adjuvant fish oil diet [see example Figure 5.1]. There was no significant difference in fiber-associated neutral lipid accumulation between all groups. However, the distribution of neutral lipid staining was shifted from IIDX<I<IIA in healthy and long term fish oil fed groups to IIDX=I=IIA in control and adjuvant fish oil fed groups [Figure 5.3].

5.3.3 Tibialis Anterior Muscle Fiber Type Composition

Type I: Prior to chemotherapy treatment, tumor-bearing rats on control diet exhibited similar proportion of type I compared to reference animals. However, following chemotherapy treatments, rats receiving cycle-1 and cycle-2 exhibited fewer type I fibers compared to tumor-bearing only rats [$P<0.04$ and $P<0.03$, respectively; Figure 5.4A]. Before chemotherapy, tumor-bearing rats on the long term fish oil diet displayed similar proportion of type I compared with reference animals and was significantly higher following cycle-1 and cycle-2 chemotherapy compared to their respective group on control diet [$P<0.03$ and $P<0.05$, respectively; Figure 5.4A]. Rats on the adjuvant fish oil diet exhibited similar proportion of type I compared to their respective long term fish oil fed groups [Figure 5.4A].

Type IIA: In tumor-bearing only rats, proportion of type IIA fibers was significantly higher compared to reference animals [$P<0.05$] and it restored to baseline following cycle-1 chemotherapy treatment. The proportion of type IIA was not significantly different following cycle-2 chemotherapy treatment compared with tumor-bearing only rats and rats that received cycle-1 chemotherapy on the control diet [Figure 5.4B]. The Proportion of type IIA fibers in long term fish oil fed rats was similar to reference animals in tibialis anterior of rats bearing only tumor and following cycle-1 chemotherapy. However, cycle-2 chemotherapy treatment significantly decreased the proportion of type IIA in rats fed long term fish oil diet compared to its respective

control group [P<0.001, Figure 5.4B]. Following cycle-1 chemotherapy treatment, rats on the adjuvant fish oil diet exhibited similar proportion of type IIA compared to their respective long term fish oil fed groups and was significantly higher following cycle-2 chemotherapy treatment [Figure 5.4 B].

Type IIDX and IIB: The proportions of type IIXD and type IIB fibers were not significantly different between groups on the control and long term fish oil diet [Figure 5.4C and D]. Rats on the adjuvant fish oil diet exhibited similar proportion of type IIB compared to their respective long term fish oil fed groups [Figure 5.4D] and the proportion of type IIDX fibers was only higher after cycle-1 chemotherapy in rats on the adjuvant fish oil diet [P<0.05, Figure 5.4C].

Hybrid fibers I/IIA and IIA/IIDX: The proportion of hybrid I/IIA fibers was significantly higher in the presence of the tumor and following cycle-1 and cycle-2 chemotherapy compared with reference animals [P<0.01, P<0.01, P<0.01, respectively; Figure 5.5]. The proportion of type IIA/IIDX hybrid fibers was only higher following cycle-1 chemotherapy treatment compared to reference animals [P<0.05, Figure 5.5]. The proportion of type IIA/IIDX was comparable to reference animals in the presence of the tumor and following cycle-1 chemotherapy in rats fed long term fish oil diet and higher following cycle-2 chemotherapy compared to reference animals [P<0.04]. The proportion of hybrid I/IIA fibers in rats fed adjuvant fish oil diet was not different from their respective gropes on long term fish oil diet following cycle-1 and cycle-2 chemotherapy, and was significantly lower compared to their respective control groups [P<0.01, P<0.01, respectively; Figure 5.5]. The proportion of type IIA/IIDX was not detectable after cycle-1 chemotherapy in rats fed adjuvant fish oil diet and was comparable to its respective rats on long term fish oil diet following cycle-2 chemotherapy [Figure 5.5].

5.3.4 Muscle Cross Sectional Area

There were no significant differences in total muscle cross sectional [CSA; mm^2] of tibialis anterior muscles between all groups [mean muscle CSA: 16 ± 1.8 mm^2, Appendix B] and neither fiber cross sectional area for each fiber type [mean fiber cross sectional area: 1622.8 ± 91.8 μm^2, Appendix C].

5.3.5 Mitochondrial Oxidative Capacity and Density

5.3.5.1 Succinate Dehydrogenase Staining

Two weeks following tumor implantation and prior to chemotherapy treatment, intensity of SDH staining was significantly higher in tibialis anterior muscle of rats bearing only tumor compared to reference rats [$P<0.001$, Figure 5.6]. Following both cycle-1 and cycle-2 chemotherapy treatment SDH staining was significantly reduced compared to rats bearing only tumor with no treatment [$P<0.001$, $P<0.001$; respectively].

We next assessed SDH staining intensity in rats fed long term or adjuvant fish oil diets. Rats bearing only tumor and fed long term fish oil diet exhibited similar level of SDH staining compared with reference animals and was significantly lower compared with its respective control group [$P<0.001$, Figure 5.6]. Following cycle-1 and cycle-2 chemotherapy, rats fed long term fish oil diet displayed higher SDH staining compared with their respective control groups [cycle-1: $P<0.001$ and cycle-2: $P<0.001$]. Rats fed adjuvant fish oil diet and receiving cycle-1 chemotherapy treatment displayed higher SDH staining [Figure 5.6] in tibialis anterior muscle compared with their respective group on control diet [$P<0.001$]. Following cycle-2 chemotherapy, SDH staining was similar to their respective long term fish group.

5.3.5.2 Citrate Synthase, and Hydroxyacyl-CoA Dehydrogenase

Since the presence of the tumor and chemotherapy treatment significantly increased neutral lipid content in the tibialis anterior muscle of rats on the control diet compared with the reference animals [Figure 5.2], we assessed CS and HADH to identify associations between mitochondrial enzyme activities and lipid content within the muscle. Two weeks following tumor implantation and prior to chemotherapy treatment, the activity of CS was not affected compared with reference rats [Figure 5.7A]. The activity of HADH on the other hand was significantly reduced in rats bearing only tumor compared with reference rats [$P<0.001$, Figure 5.7A]. The activity of both CS and HADH were similar with rats bearing only tumor following cycle-1 and cycle-2 chemotherapy treatment and were significantly lower than reference rats [cycle-1: $P<0.001$and $P<0.001$, cycle-2: $P<0.04$ and $P<0.001$; respectively].

We next assessed CS and HADH in rats fed long term or adjuvant fish oil diets. The activities of CS and HADH [Figure 5.7A] were significantly higher in tibialis anterior muscle of rats bearing only tumor and fed long term fish oil diet compared with tumor-bearing rats fed the control diet [$P<0.002$ and $P<0.002$; respectively]. Following cycle-1 and cycle-2 chemotherapy, rats fed long term fish oil diet displayed higher CS and HADH activities compared with their respective control groups [cycle-1: $P<0.001$ and $P<0.001$, cycle-2: $P<0.001$ and $P<0.001$; respectively].

Similarly, rats fed adjuvant fish oil diet and receiving cycle-1 chemotherapy treatment displayed higher CS and HADH [Figure 5.7A] activities in tibialis anterior muscle compared with their respective control groups [$P<0.001$ and $P<0.001$; respectively]. Following cycle-2

chemotherapy, HADH activity was similar to their respective long term fish oil groups. However, CS activity was significantly lower than rats fed long term fish oil diet [P<0.001].

We further explored the relationships between SC and HADH activities and neutral lipid content in the tibialis anterior muscle. The proportion of fibers expressing neutral lipid content was negatively correlated with SC and HADH [Figure 5.7B].

5.3.5.3 Peroxisome Proliferator-Activated Receptor-Gamma Coactivator 1-Alpha and Transcription Factor A Mitochondrial

Next, we assessed expression of genes involved in the mitochondrial biogenesis including the *PGC-1α* and *TFAM* in the tibialis anterior muscle. Expression of *PGC-1α* and *TFAM* showed remarkable increases in response to the tumor-bearing state [P<0.001 and P<0.001, respectively] compared to reference animals [Figure 5.8]. Following cycle-1 and cycle-2 chemotherapy treatment in rats on the control diet, both *PGC-1α* and *TFAM* were significantly decreased compared to rats bearing-only tumor [cycle-1, P<0.001 and P<0.001; cycle-2, P<0.001 and P<0.001; respectively].

We further assessed the expression of *PGC-1α* and *TFAM* in rats fed a diet containing fish oil either for long term or as an adjuvant. All groups on long term fish oil diet, including rats-bearing only tumor or those received one or two cycles of chemotherapy, exhibited similar level of the expression of *PGC-1α* compared with reference rats [Figure 5.8] and was significantly higher after cycle-2 compared with its respective control group [P<0.001]. The expression of *TFAM* in rats fed a long term fish oil diet was comparable to reference animals in the presence of the tumor and following cycle-1 chemotherapy. After cycle-2 chemotherapy, *TFAM* was

significantly higher in rats fed long term fish oil diet compared with its respective control group [P<0.001, Figure 5.8].

Similarly, the rats on adjuvant fish oil diet exhibited similar level of the expression of *PGC-1α* compared with rats fed a long term fish oil diet after cycle-1 and cycle-2 chemotherapy treatment. The expression of *TFAM* was similar to reference animals after cycle-1 and was significantly higher compared with its respective control rats after cycle-2 chemotherapy [P<0.001, Figure 5.8].

We further explored the relationships between *PGC-1α* and *TFAM* expression and neutral lipid content in the tibialis anterior muscle and there were no correlations observed [data not shown].

5.4 Discussion

This study is the first to describe mechanisms underlying fat accumulation within skeletal muscle using a preclinical model of tumor- and chemotherapy-associated myosteatosis. Using this animal model, we investigated the relationship between neutral lipid content within tibialis anterior muscle and genes involved in mitochondrial biogenesis as well as mitochondrial enzymes activates. We also examined whether fat accumulation occurs in a fiber-type-specific manner. Furthermore, feeding a diet containing EPA and DHA mitigates tumor- and chemotherapy associated myosteatosis through increasing markers of mitochondrial oxidative capacity as well as mitochondrial density that was observed to be reduced following chemotherapy treatments in tumor bearing rats. Specifically, we show that in the tibialis anterior muscle of tumor-bearing animals following chemotherapy, neutral lipids were elevated along with decreased oxidative type I fibers as well as SC and HADH activities, the major enzymes involving in mitochondrial

oxidation pathway, which were all greatly increased in animals fed a diet containing fish oil either beginning before tumor-implantation [long term] or at the initiation of chemotherapy [adjuvant]. Collectively, our results suggest that EPA and DHA supplementation is a recognized effective adjuvant to chemotherapy to combat myosteatosis through improving mitochondrial oxidative capacity as well as density which in further may improve muscle health in the presence of tumor and following chemotherapy treatments.

5.4.1 Potential Mechanisms Underlying Tumor- and Chemotherapy-Associated Myosteatosis in a Preclinical Model

In the oncology setting, myosteatosis detected by CT scan has been identified as an important prognostic factor associated with shorter survival in cancer patients treated with chemotherapy (6-9). However, mechanisms underlying myosteatosis have not been established yet. In order to investigate the biological features and causes of cancer-related myosteatosis, appropriate preclinical models need to be identified. In this study, the Ward colon tumor model displayed robust neutral lipid accumulation within tibialis anterior muscle that was exacerbated by the 2- cycle of chemotherapy similar to what was observed in gastrocnemius muscle [see chapter 3, Figure 3.2]. There is no neoplastic model of myosteatosis in cancer has been reported. This preclinical model displays features of human cancer with regards to fat accumulation within skeletal muscle that occur in some cancer patients undergoing chemotherapy. Using this animal model enables us to investigate mechanisms associated with myosteatosis in tumor-bearing rats and following chemotherapy treatments.

In the present study, while untreated tumor-bearing rats exhibited a significant increase in neutral lipid accumulation compared to reference rats, CS activity was not altered, and HADH was significantly decreased. On the other hand, the expression of the genes involving in mitochondrial

biogenesis including *PGC-1α* and *TFAM* were significantly increased in untreated tumor bearing rats compared with reference animals combined by the increase in the proportion of oxidative fiber type IIA. A reduction in mitochondrial oxidative capacity as well as mitochondrial content has been linked to increased intramyocellular lipid content in non-cancer conditions, such as elderly population [reviewed by (87)] insulin resistance and obesity (88). However, no existing studies have associated mitochondrial content and dysfunction to myosteatosis development in preclinical model of cancer. Similar to our findings, a reduction in the major regulation enzymes for oxidative phosphorylation including cytochrome-c oxidase complex subunit IV and SDH has been reported in gastrocnemius muscle of an Apc [Min/+] mice during the development of cancer compared to wild-type control mice (86). However, these authors did not measure fat in muscle.

Following chemotherapy treatment, rats bearing-tumor displayed a significant reduction in type I fibers, all proteins and genes involving in mitochondrial oxidation pathway as well as mitochondrial biogenesis. Additionally, we found a negative correlation between mitochondrial oxidative enzymes, CS and HADH and neutral lipid content. We have also observed a significant increase in hybrid fibers, fibers co-expressing two or more MHC isoforms [I/IIA] following each cycle of chemotherapy compared to reference animals. Fiber type composition has been reported in one recent study in cancer patients (143). This study reported no significant differences in I/IIA fibers between cancer patients and control. However, in this study authors excluded fibers <40 diameters which can contribute to their findings because the size of I/IIA hybrid fibers is usually small. These findings are novel in the cancer research and further investigations are required to understand the association between the shifting between fibers and myosteatosis. Collectively, it appears that the mechanisms responsible for treated cancer-associated myosteatosis involve key regulators of mitochondrial oxidative capacity and mitochondrial density. CPT-11 plus 5-FU is

first line- treatment for advanced colorectal cancer patients. Grivicich et al reported a reduction in mitochondrial membrane content of human colon carcinoma cell lines after CPT-11 plus 5-FU treatment (144). Both drugs could target mitochondrial DNA and RNA and cause mitochondrial damage in skeletal muscle (145). Thus, measuring the availability of these drugs within skeletal muscle as well as mitochondrial DNA in the present animal model would be an area of interest in future work.

5.4.2 Effects of the Fish Oil Diets on Mechanisms Underlying Tumor- and Chemotherapy-Associated Myosteatosis

Nutrient deficiencies have been observed in cancer patients undergoing chemotherapy including low levels of plasma EPA and DHA that appear to occur with fat deposition, which can be corrected when fish oil is provided to patients during treatment (1,24). Similar results have been observed in the present animal model [presented in chapter 3]. However, the mechanisms through which EPA and DHA supplementation exert this beneficial effect are unknown. Also, the extent to which adjuvant EPA and DHA supplementation during chemotherapy is as efficacious as beginning prior to a cancer diagnosis is important for application in the clinical setting.

Some researchers have reported the beneficial effects of EPA and DHA on mitochondrial content and oxidative capacity including higher mitochondrial content, improved function of the enzyme complexes within the electron transport chain, and an improved capacity to appropriately use physiologically available fuels in skeletal muscle [reviewed by (91)], but little is known about this mechanistic regulation in cancer-associated fat accumulation in skeletal muscle. Here we demonstrate, for the first time, that EPA and DHA supplementation prevents tumor- and treatment associated increases in neutral lipid through increase mitochondrial enzymes activities SC and HADH. Collectively, these results suggested that fish oil enhanced mitochondrial oxidative

capacity decreased in the presence of the tumor and during chemotherapy confirming that mitochondrial oxidation capacity is associated with decreased lipid content within the muscle in long term fish oil fed rats.

Adjuvant fish oil fed rats receiving cycle-1 chemotherapy treatment exhibited a similar amount of neutral lipid staining within muscle fibers compared with their respective long term fish oil diet group but, it was significantly higher following 2-cycle of chemotherapy treatment. Following cycle-2 chemotherapy CS activity *TFAM* were significantly lower than rats fed long term fish oil diet and received two cycles chemotherapy treatment, suggesting that both mitochondrial density and oxidative capacity could be decreased in adjuvant fish oil fed rats and received 2- cycles of chemotherapy. These results suggested that longer term of fish oil supplementation may be required or either increasing the dose of the supplement during chemotherapy treatment to maintain lipid content within the muscle similar to the level of healthy.

5.5 Conclusion

The novel observations of myosteatosis coupled with lower mitochondrial enzymes activities, oxidative capacity, and expression of genes involving in mitochondrial biogenesis in the skeletal muscles of an *in vivo* tumor-bearing model that underwent chemotherapy treatment, reveal potential mechanisms underlying fundamental alterations in skeletal muscle fat metabolism that may be involved in the development of myosteatosis in cancer patients. Long term and adjuvant dietary EPA and DHA feeding were both efficacious in markedly improving muscle health by reducing the toxicity effect, fat accumulation within skeletal muscle caused by CPT-11 and 5-FU. However, for the short term intervention, a higher dose of EPA and DHA may be required to treat myosteatosis. This study highlights the therapeutic potential of EPA and DHA as promising nutritional adjuvants to chemotherapy. The results from the present study are novel in the cancer

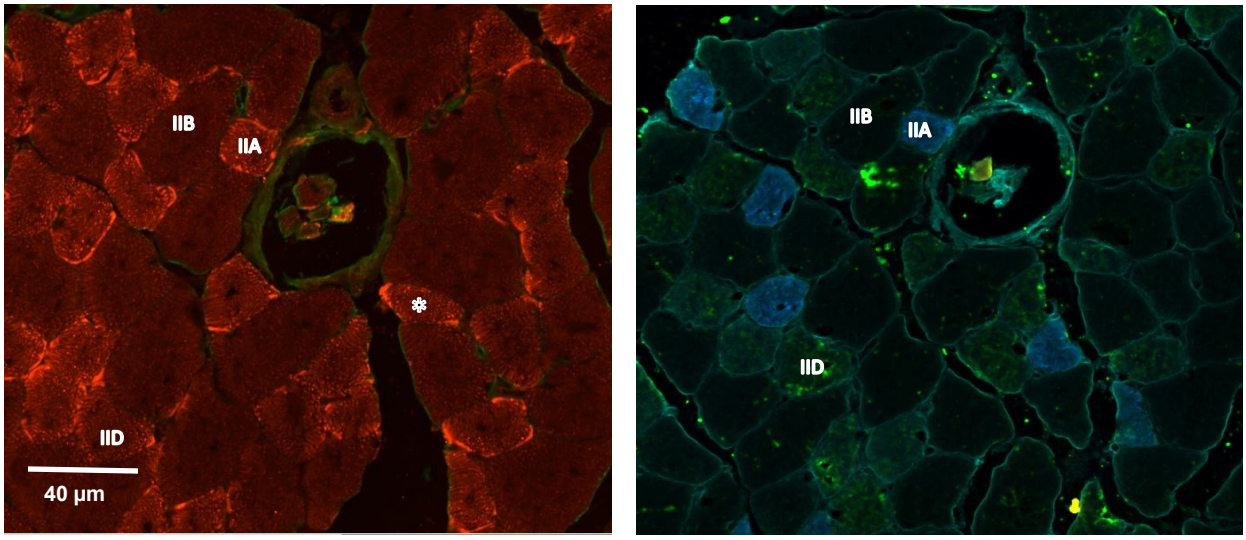
research and encourages further investigation to determine additional mechanisms by which dietary EPA and DHA supplementation attenuate myosteatosis in the skeletal muscles that known to be associated with higher mortality in cancer patients.

Table 5-1 Immunofluorescence Oil Red O Antibodies Cocktail for Rat Muscle

Primary Antibodies	Secondary Antibodies	Antigen	Isotype	Species/Fluorescent Tag:	Dilution Factor
Laminin	-	Laminin	IgG	rabbit	1:100
Dystrophin		Dystrophin	IgG	rabbit	1:25
-	Laminin/ Dystrophin	Laminin/ Dystrophin	IgG	Goat Anti-Rabbit 488 (green)	1:400

Table 5-2 Immunofluorescence Myosin Heavy Chain Antibodies Cocktail for Rat Muscle

Primary Antibodies	Secondary Antibodies	Antigen	Isotype	Species/Fluorescent Tag:	Dilution Factor
Laminin (Lam)	-	Laminin	IgG	rabbit	1:100
Dystrophin (Dys)	-	Dystrophin	IgG	rabbit	1:25
BAD5	-	MHC I	IgG2b	mouse	1:50
6H1	-	MHC IID	IgM	mouse	1:1
SC71	-	MHC IIA	IgG1	mouse	1:50
-	Lam/Dys	Lam/Dys	IgG	Goat Anti-Rabbit 647(far red)	1:400
-	BAD5	MHC I	IgG2b (γ2b)	Goat Anti-Mouse 568 (red)	1:200
-	6H1	MHC IID	IgM	Goat Anti-Mouse 488 (green)	1:400
-	SC71	MHC IIA	IgG1 (γ1)	Goat Anti-Mouse 405 (blue)	1:200



Oil Red O Staining

Myosin Heavy Chain Staining

Figure 5.1 Examples of manual quantification of neutral lipid content-based muscle fiber type from healthy rats. Fiber types were identified in tibialis anterior muscles by using immunofluorescence myosin heavy chain staining method. * represents fiber stained positive for Oil Red O. Each fiber type is identified using different laser color; Type IIA: blue, Type IIDX: green, Type IIB no stain.

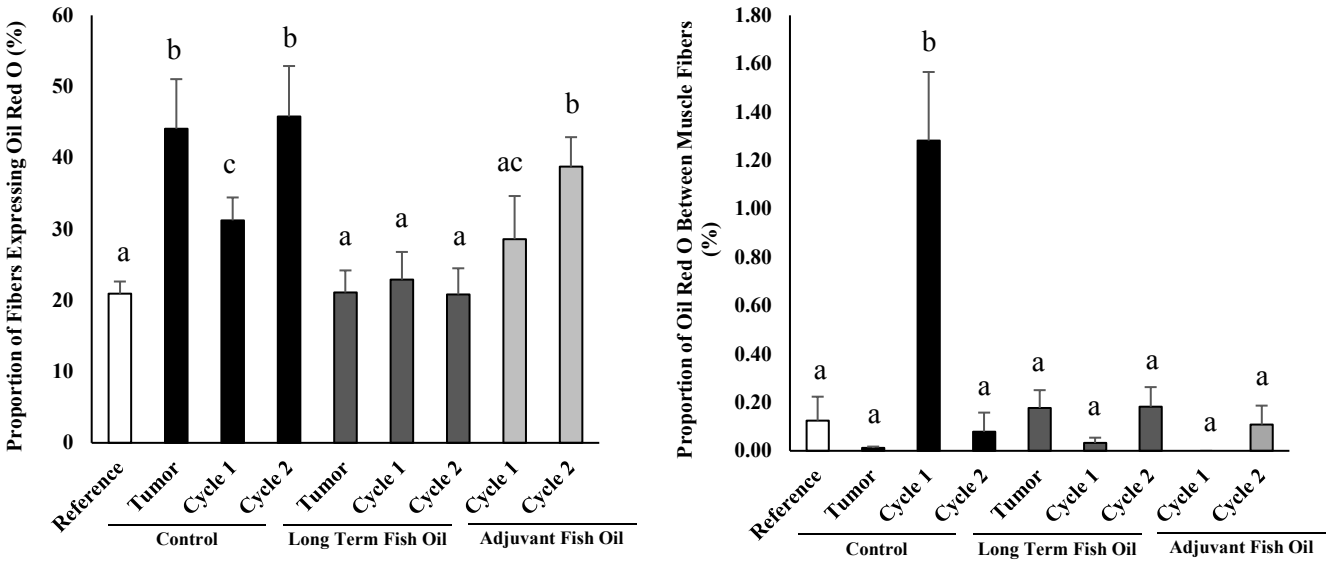


Figure 5.2 Oil Red O staining in rat tibialis anterior muscle. **A)** represents proportion of positive fibers expressing Oil Red O staining. **B)** represents area expressing positive Oil Red O staining between muscle fibers. Values are means ± SD. Different letters indicate significant differences among groups ($P<0.05$). n=7 or 6/group

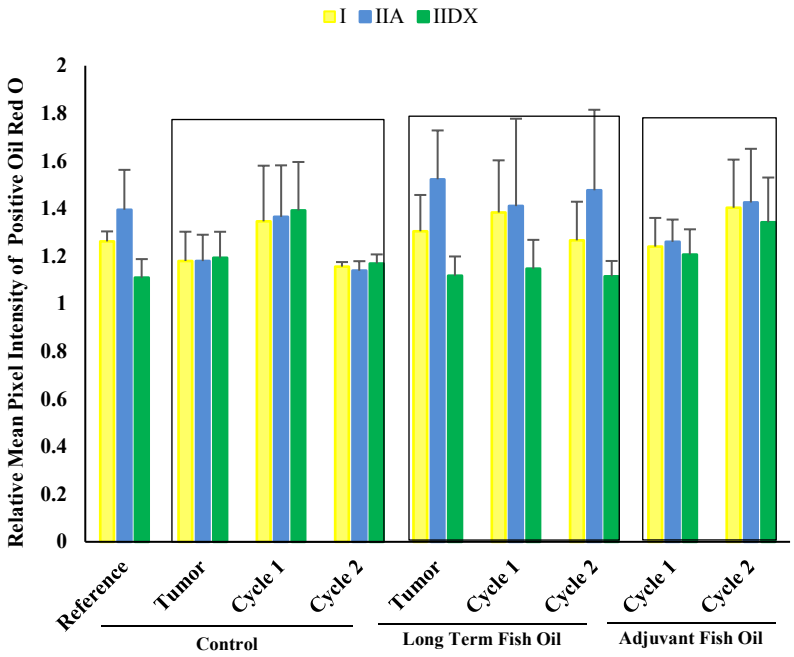
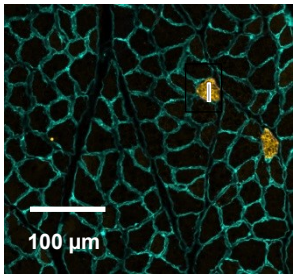
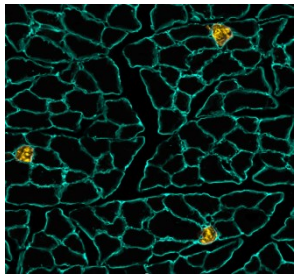


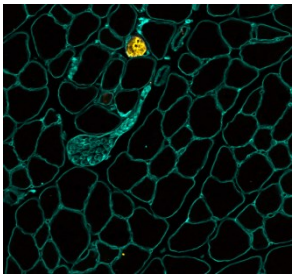
Figure 5.3 Fibers associated with neutral lipid accumulation in rat tibialis anterior muscle. Yellow bars represent type I, Blue bars represent type IIA, and green bars represent type IIDX. Data are presented as mean pixel intensity of positive Oil Red O staining /minimum positive Oil Red O pixel intensity. Values are means ± SD. n=5/group

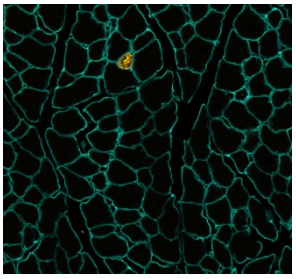
A



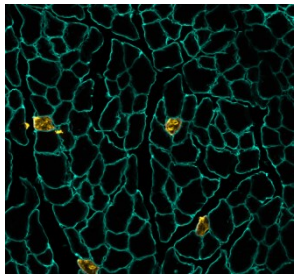
Reference

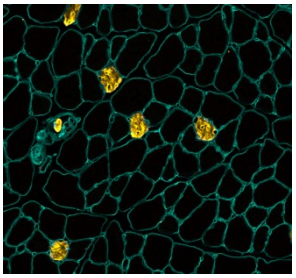

Tumor

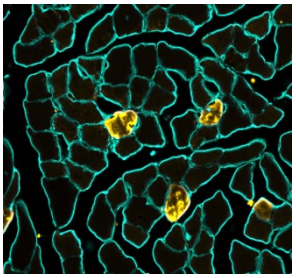

Cycle1


Cycle2

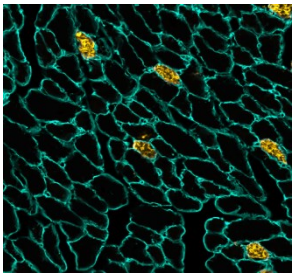
Control

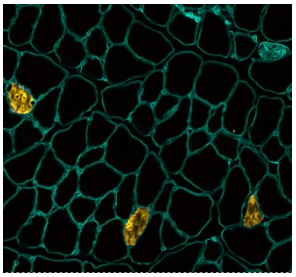

Tumour


Cycle1

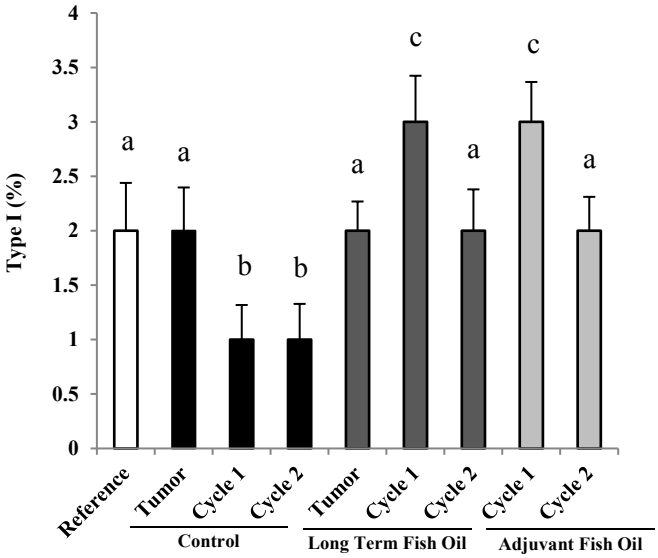

Cycle2

Long Term Fish Oil

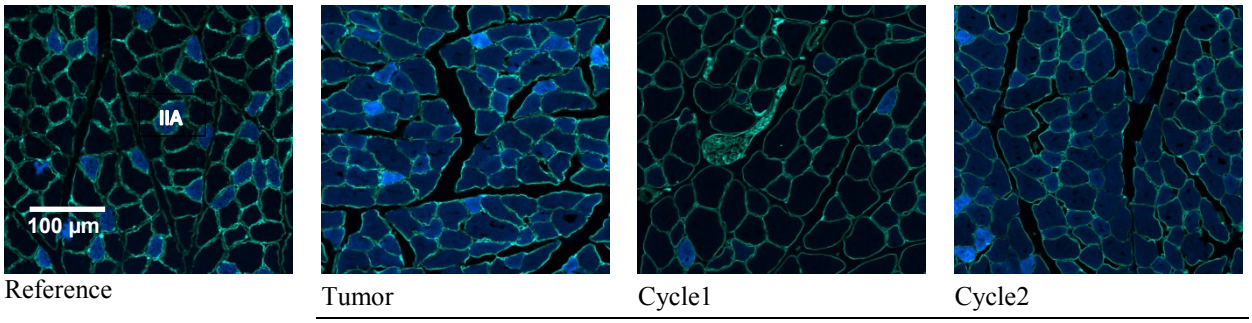

Cycle1

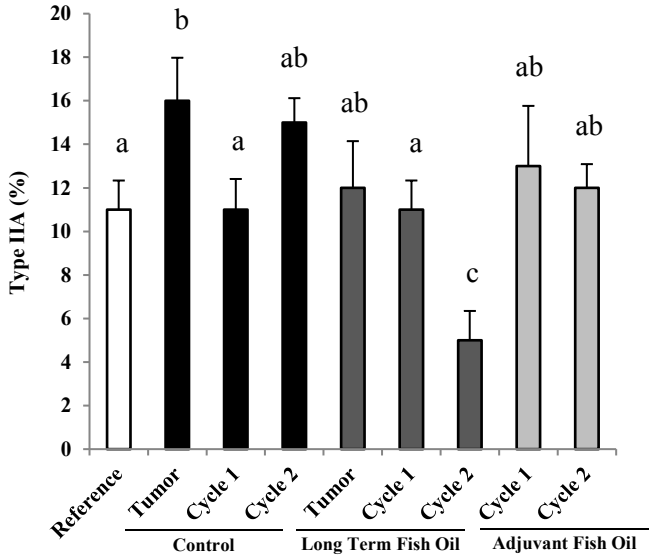

Cycle2

Adjuvant Fish Oil

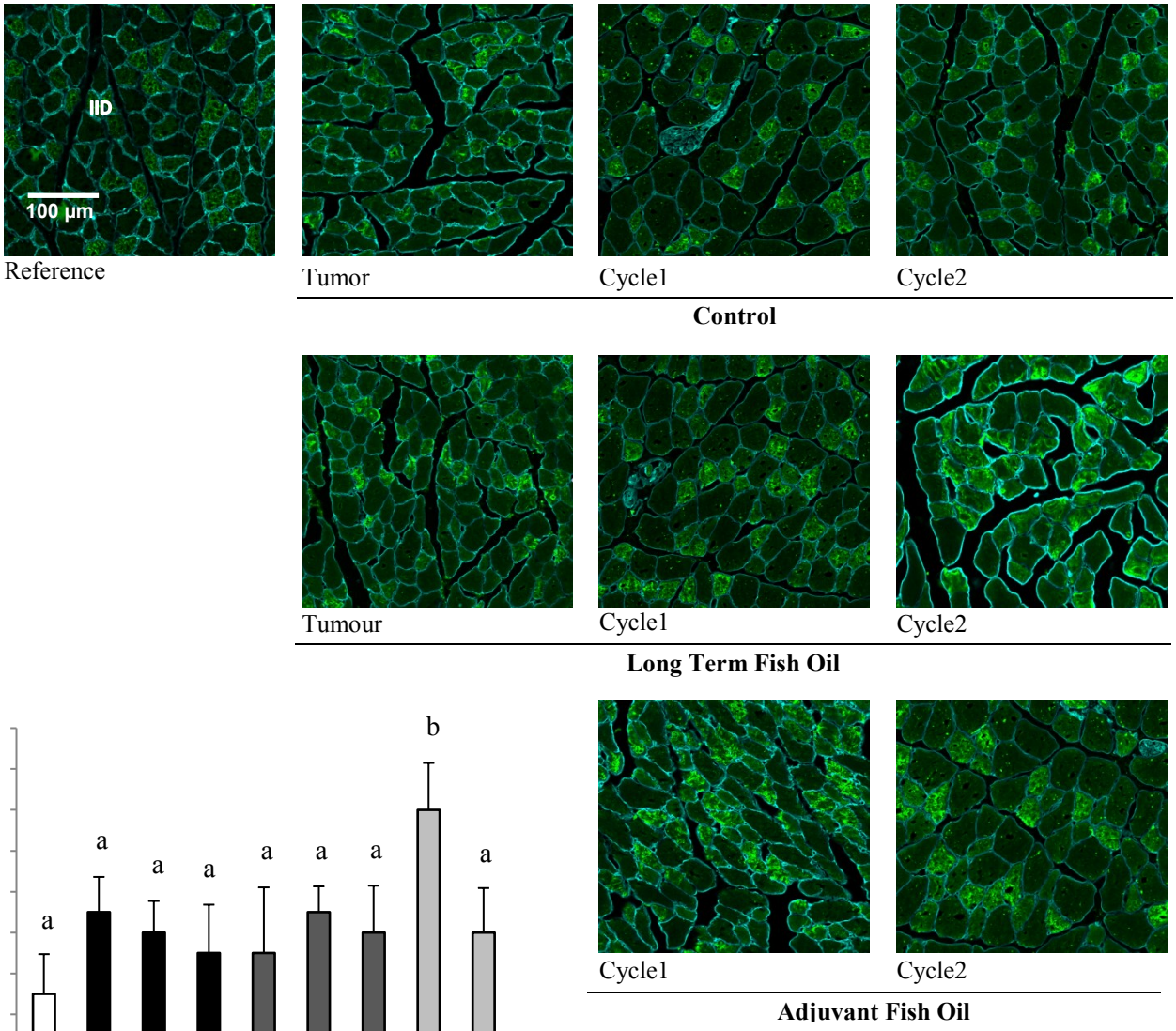


B


IIA
100 µm
Reference
Tumor
Cycle1
Cycle2
Control
Tumour
Cycle1
Cycle2
Long Term Fish Oil
Cycle1
Cycle2
Adjuvant Fish Oil


Type IIA (%)
20
18
16
14
12
10
8
6
4
2
0
a
b
a
ab
ab
a
c
ab
ab
Reference
Tumor
Cycle 1
Cycle 2
Tumor
Cycle 1
Cycle 2
Cycle 1
Cycle 2
Control
Long Term Fish Oil
Adjuvant Fish Oil

C


IID
100 µm
Reference
Tumor
Cycle1
Cycle2
Control
Tumour
Cycle1
Cycle2
Long Term Fish Oil
Cycle1
Cycle2
Adjuvant Fish Oil
Type IIDX (%)
0
2
4
6
8
10
12
14
16
18
20
a
a
a
a
a
a
a
b
a
Reference
Tumor
Cycle 1
Cycle 2
Tumor
Cycle 1
Cycle 2
Cycle 1
Cycle 2
Control
Long Term Fish Oil
Adjuvant Fish Oil

D

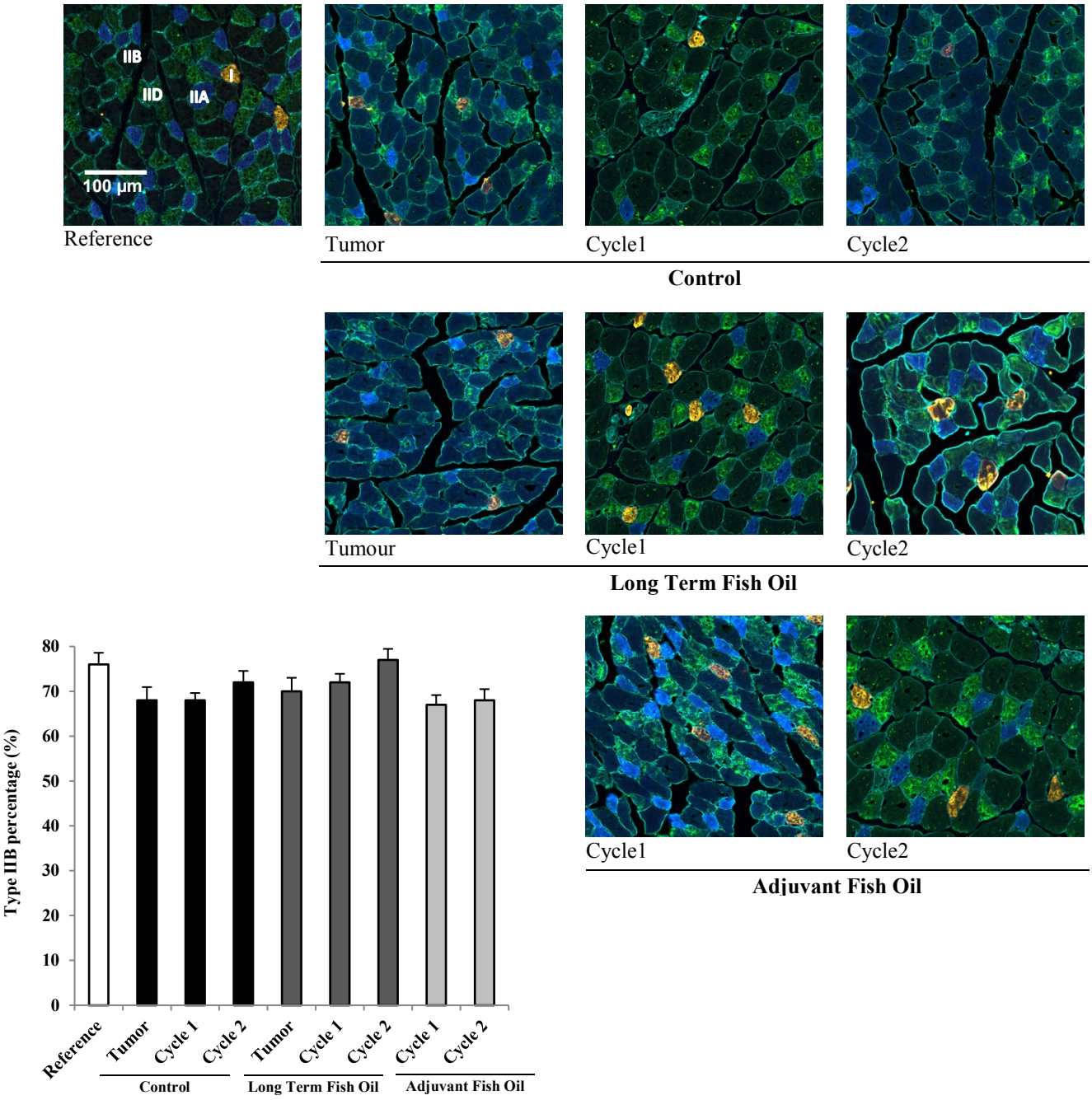


Figure 5.4 Fiber composition in tibialis anterior muscle of rats bearing a ward colon tumor and receiving CPT-11/5-FU. Rats were fed control diet or fish oil diet either prior or following to tumor implantation. Results were quantified using immunofluorescence myosin heavy chain method. Each fiber type is identified using different laser color; **A)** Type I: yellow, **B)** Type IIA: blue, **C)**

Type IIDX: green, **D)** Type IIB no stain. Data are presented as proportion of each fiber type. Values are means ± SD. Different letters indicate significant differences among groups ($P<0.05$). n=6/group.

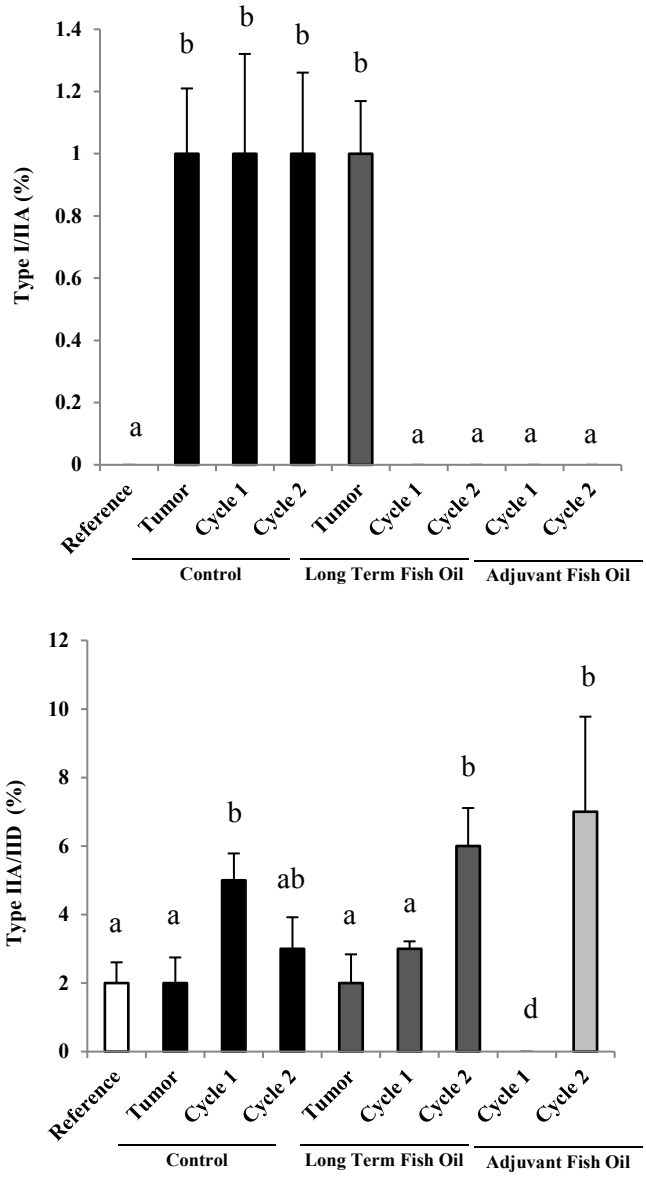


Figure 5.5 Hybrid fiber composition in tibialis anterior muscle of rats bearing a ward colon tumor and receiving CPT-11/5-FU. Rats were fed control diet or fish oil diet either prior or following to tumor implantation. Data were quantified using immunofluorescence myosin heavy chain method. Data are presented as proportion of each fiber type. Values are means ± SD. Different letters indicate significant differences among groups ($P<0.05$). n=6/group.

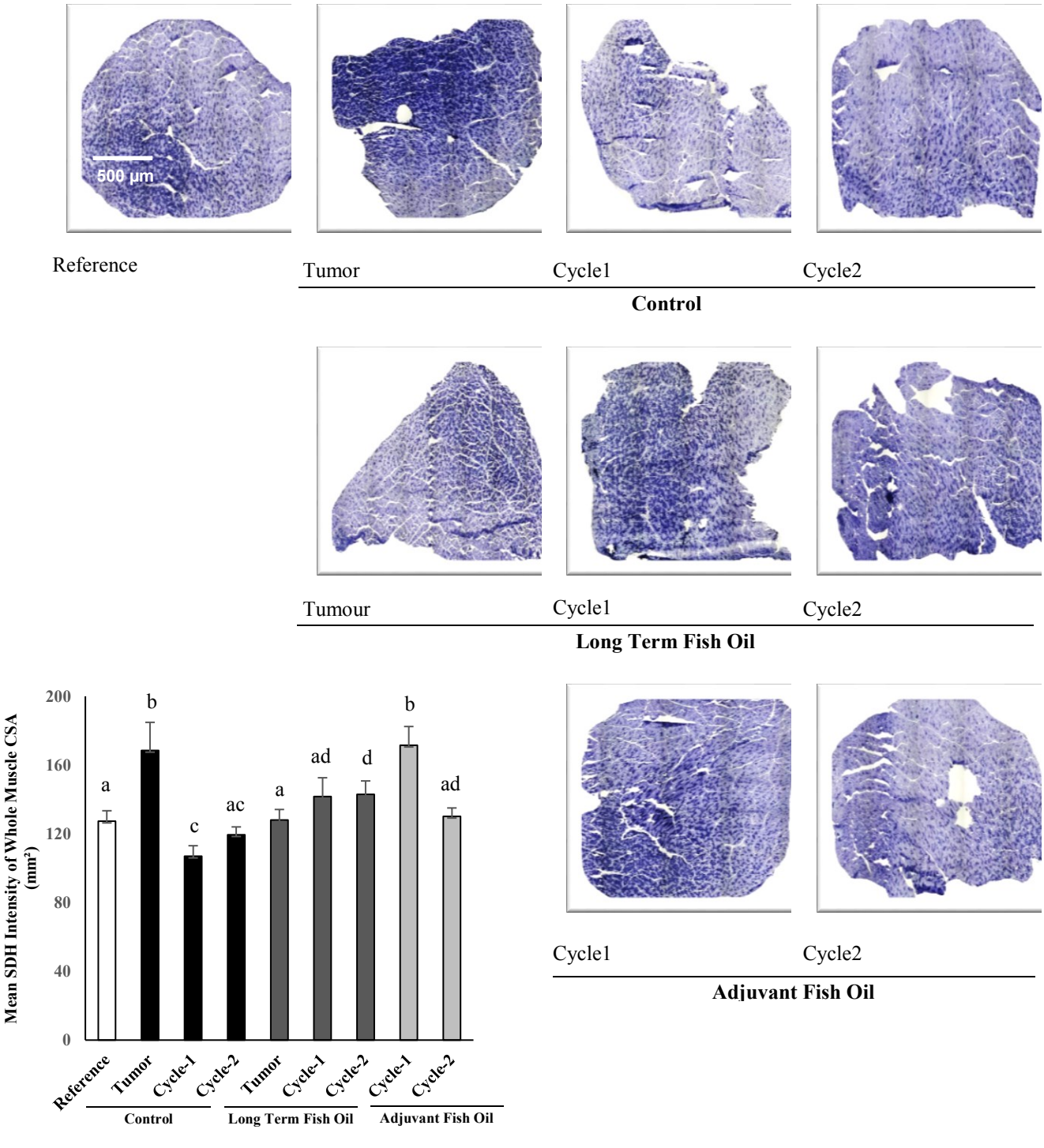


Figure 5.6 Succinate Dehydrogenase (SDH) of rat tibialis anterior muscle. Data are presented as mean SDH intensity of whole muscle CSA. Values are means ± SD. Different letters indicate significant differences among groups ($P<0.05$). n=8 or 7/ group.

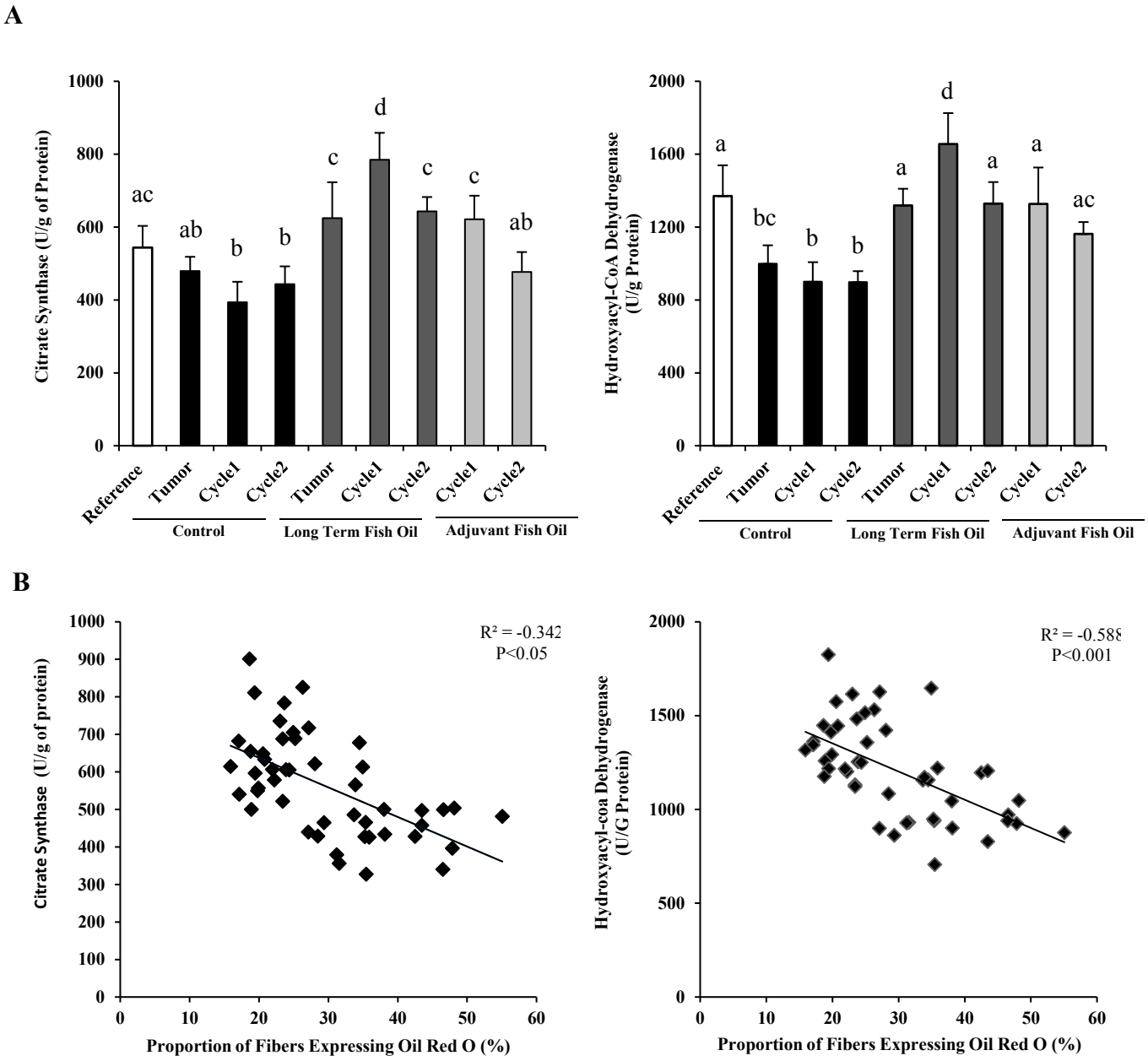


Figure 5.7 A) Activities of citrate synthase (SC) and 3-hydroxyacyl-CoA dehydrogenase (HADH) of rat tibialis anterior muscle. Results were quantified using spectrophotometry. Samples were evaluated in duplicate. Results were presented as U/g of total protein. Values are means ± SD. Different letters indicate significant differences among groups (P<0.05). n=8 or 7/ group. **B)** represents the correlation between each protein and the proportion of fiber expressing neutral lipid content.

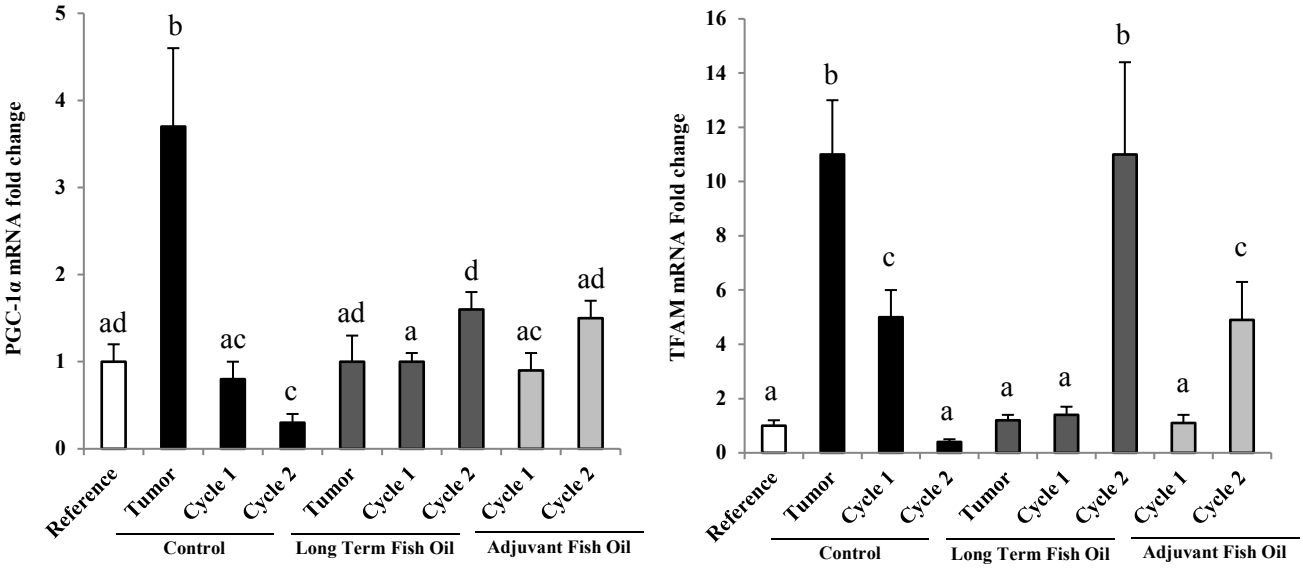


Figure 5.8 Patterns of mitochondrial biogenesis transcriptional factor mRNA expression in the rat tibialis anterior muscle. Fold changes in peroxisome proliferator-activated receptor-gamma coactivator 1-alpha (*PGC-1α*) and transcription factor A, mitochondrial (*TFAM*) expression levels as determined by the $2^{-\Delta\Delta CT}$ method of analysis. Values are means ± SD. Different letters indicate significant differences among groups ($P<0.05$). n=8 or 7/ group.

CHAPTER 6

General Summary and Final Discussion

6.1 General Summary

The first objective of this thesis [presented in Chapter 3] was to characterize a preclinical model of cancer-associated myosteatosis and then, use this model to investigate the effects of dietary omega-3 polyunsaturated fatty acids eicosapentaenoic acid [EPA, 20:5n-3] and docosahexaenoic acid [DHA, 22:6n-3] on tumor- and subsequent chemotherapy-associated fat content of muscle, as well as the tumor response to chemotherapy. Additionally, we wanted to determine whether EPA and DHA supplementation beginning at the initiation of chemotherapy [adjuvant] evokes similar efficacy compared with starting EPA and DHA supplementation prior to tumor implantation [long term].

Effect of tumor and chemotherapy: Two weeks following tumor implantation, the proportion of fibers staining positive for Oil Red O and the mean total TG content in the gastrocnemius muscle were 6-fold and 3-fold higher, respectively, in tumor-bearing rats compared with the reference group, reflecting that our preclinical tumor model is associated fat accumulation in muscle. 2-cycles of chemotherapy treatment further increased fat accumulation in the gastrocnemius muscle compared to tumor-bearing rats.

To determine mechanisms associated with the deposition of fat into gastrocnemius muscle, we assessed expression of genes involved in the adipogenic transcriptional cascade and we report that downstream genes including CCAAT/enhancer-binding protein *[C/EBP]α* and peroxisome proliferator-activated receptor *[PPAR]γ* expression increased 26-fold and 59-fold, respectively, in the tumor-bearing state compared with reference animals and the expression of these genes were further increased following 2- cycles of chemotherapy. We have also reported that total TG content

in the gastrocnemius muscle was positively correlated with increased the expression of *C/EBPδ, C/EBPα* and *PPARγ* revealing that key transcription factors involved in adipogenesis are related to the TG content in muscle. Collectively, it appears that these results support our hypotheses [see Chapter 2]. We have established a preclinical model of cancer- and chemotherapy associated myosteatosis and we have also reported that cancer- and chemotherapy associated myosteatosis maybe involve mechanisms related to increase the expression adipogenesis and lipogenesis factors in skeletal muscle.

Effect of fish oil diets: Before chemotherapy, tumor-bearing rats on the long term fish oil diet displayed proportions of fibers expressing Oil Red O and total TG content in the gastrocnemius muscle similar to levels observed in reference animals, which were significantly lower compared with tumor-bearing rats on the control diet. Subsequently, after both cycle-1 and cycle-2, tumor-bearing rats on the long term fish oil diet exhibited fewer fibers stinging positive for Oil Red O and lower total TG muscle content in the gastrocnemius muscle compared with their respective chemotherapy treated cycle on control diet groups. Additionally, cycle-1 and cycle-2 rats on the adjuvant fish oil diet displayed similar amounts of Oil Red O staining and total TG in the gastrocnemius muscle compared with their respective long term fish oil diet group. Collectively, these results show that before chemotherapy, the long term fish oil diet prevented tumor-associated fat accumulation, and the adjuvant fish oil diet was equally efficacious as the long term fish oil diet in mitigating chemotherapy-associated fat accumulation in muscle and restored muscle condition to that of reference animals.

We also showed that long term fish oil diet prevented tumor-associated increases in *C/EBPδ, C/EBPα* and *PPARγ* expression, as the expression of these transcription factors were similar to reference animals and significantly lower than tumor-bearing rats on the control diet,

Subsequently, both fish oil diets greatly suppressed chemotherapy-associated expression of these transcriptional factors that occurred in rats on the control diet. Similarly, the adjuvant fish oil diet was able to fully restore and significantly decrease expression of *C/EBPδ, C/EBPα* and *PPARγ* after cycle-1 and cycle-2, respectively. These results show that both fish oil diets greatly reduced tumor- and/or chemotherapy-associated increases in the expression of key adipogenic transcriptional factors.

We also reported that long term and adjuvant EPA and DHA supplementation similarly enhanced the anti-tumor activity of chemotherapy compared with the control diet. In each of the 6 days following initiation of cycle-1, rats in both the long term and adjuvant fish oil groups had significantly smaller tumor volumes compared with tumor-bearing rats in the control diet group. Notably, tumor volumes were not significantly different between the long term and adjuvant fish oil diet groups during either cycle-1 or cycle-2.

The second objective of this thesis [presented in chapter 4] was to characterize the fatty acid composition of phospholipid [PL] and TG of muscle tissue in a preclinical model of colon cancer with or without chemotherapy treatment fed a control diet or fish oil diet prior [long term] and after tumor implantation [adjuvant diet]. This enabled a determination of the impact of tumor and chemotherapy treatment on fatty acids content and whether EPA and DHA supplementation beginning at the initiation of chemotherapy [adjuvant] will result in similar fatty acids proportions in gastrocnemius muscle as long term fish oil feeding.

Effect of tumor and chemotherapy: This study reported that the mean total PL fatty acid content in the gastrocnemius muscles of rats bearing-tumor was not different from reference rats, as opposed to our hypothesis [see Chapter 2]. While presence of the tumor did not alter total PL

in muscle, we found that total n-3 fatty acids were significantly lower than reference animals in rats bearing-tumor in both PL and TG fractions, driven by lower C18:3 and EPA, in TG fraction and undetectable DHA in PL fraction. Ratio of n-6/n-3 was significantly higher in rats bearing tumor compared to reference animals in TG fraction.

Following chemotherapy treatments, rats receiving 1- cycle chemotherapy had lower total PL fatty acid compared with tumor-bearing animals. After 2- cycles of chemotherapy, the amount of PL returned to baseline levels. DHA was not detected after either cycle of chemotherapy in PL and TG fractions in control fed animals. Two cycles of chemotherapy further cause a significant increase in the n-6/n-3 fatty acid ratio in TG fraction of gastrocnemius muscle compared to rats bearing only tumor. Rats on the control diet that underwent 2-cycles of chemotherapy had higher proportion of saturated fatty acids compared to tumor-bearing rat only which was driven by higher proportion of C18:0 in PL fraction. Collectively, these results suggest that fatty acid composition in both PL and TG are altered with chemotherapy treatment treating rats with higher saturated fatty acid, n-6/n-3 ratio and lower n-3 fatty acids including DHA compared to tumor bearing-rats.

Effect of fish oil diets: All animals fed long term fish oil diet exhibited > 2 fold higher total PL content in the gastrocnemius muscles compared to their respective groups on control diet. Rats fed adjuvant fish oil diet and underwent 1- and 2- cycles of chemotherapy exhibited similar level of total PL compared to reference animal. Total proportion of n-3 fatty acids including EPA and DHA were significantly higher in groups fed long term fish oil diet compared to their respective groups on the control diet in both PL and TG fractions. As a result, long term fish oil diet induced a significant reduction in the ratio of n-6/n-3 compared with animals fed a control diet. A similar effect of increasing n-3 fatty acid content in the gastrocnemius muscle compared with their

respective long term fish oil diet group was observed in the rats on the adjuvant fish oil diet after 1- and 2- cycles of chemotherapy. These results showed that both fish oil diets were efficacious in maintaining or elevating n-3 fatty acids including EPA and DHA, that known to be reduced or not detected during chemotherapy treatment.

The final objective of this thesis [presented in chapter 5] was to evaluate skeletal muscle fiber composition, neutral lipid content and location and determine if the accumulation is related to specific fiber type, as well as mitochondrial density and oxidative capacity in a preclinical model of colon cancer. Additionally, we wanted to determine whether EPA and DHA supplementation beginning at the initiation of chemotherapy [adjuvant] was able to elicit similar effects compared with beginning prior to tumor implantation [long term]. Because this objective was achieved using the tibialis anterior muscle, a muscle comprised of mixed fiber types, we first confirmed that the tibialis anterior muscle responds in a similar manner to the gastrocnemius muscle in response to the treatment and the three diet interventions: control diet, long term fish oil diet, and adjuvant fish oil diet.

Effect of tumor and chemotherapy: Two weeks following tumor implantation, the proportion of fibers expressing neutral lipid, measured by Oil Red O, in the tibialis anterior muscle were 23 fold higher in the control diet group compared with the reference group, reflecting tumor-associated fat accumulation in muscle similar to gastrocnemius muscle. The activity of Hydroxyacyl-CoA Dehydrogenase [HADH] in tibialis anterior muscle of rats bearing only tumor was significantly reduced compared with reference rats. Expression of genes involving in mitochondrial biogenesis such as Peroxisome Proliferator-Activated Receptor-Gamma Coactivator 1-Alpha *[PGC-1α]* and Transcription Factor A Mitochondrial *[TFAM]* showed remarkable increases in response to the tumor-bearing state compared with reference rats.

Collectively, these results showed that while oxidative fibers [IIA] and genes involving in mitochondrial biogenesis increased within the tibialis anterior muscle in the presence of the tumor, as opposed to our hypothesis, the activity of mitochondrial enzymes decreased.

Rats receiving chemotherapy treatment and fed a control diet exhibited higher neutral lipid content, specifically, following cycle-2 compared to untreated tumor-bearing rats. Proportions of oxidative fibers, type I, were significantly lower in tibialis anterior muscles of rats-bearing tumor following both cycles of chemotherapy treatment and proportion of type IIA was lower only following cycle-1 of chemotherapy compared to untreated tumor bearing rats. Following cycle-1 and cycle-2 chemotherapy treatment, both CS and HADH activities were similar with rats bearing only tumor and were significantly lower than reference rats. Both *PGC-1α* and *TFAM* were significantly decreased compared to rats bearing-only tumor following both cycles of chemotherapy in tibialis anterior muscle. Collectively, these results support our hypothesis and showed that chemotherapy treatment increased neutral lipid content and decreased the expression of genes involve in mitochondrial biogenesis as well as the activity of mitochondrial enzymes in tibialis anterior muscle.

Effect of fish oil diets: Before chemotherapy, tumor-bearing rats on the long term fish oil diet displayed proportions of fibers expressing Oil Red O in the tibialis anterior muscle similar to levels observed in reference animals, which were significantly lower compared with tumor-bearing rats on the control diet. Subsequently, after both cycle-1 and cycle-2, tumor-bearing rats on the long term fish oil diet exhibited less fibers expressing Oil Red O in the tibialis anterior muscle compared with their respective control chemotherapy treated cycle groups.

Before chemotherapy, tumor-bearing rats on the long term fish oil diet displayed similar proportion of type I, oxidative fiber, compared with reference animals which was significantly higher following cycle-1 and cycle-2 chemotherapy compared to their respective group on control diet. The activity of CS and HADH were significantly higher in tibialis anterior muscle of rats bearing only tumor and fed long term fish oil diet compared with tumor-bearing rats fed control diet. Following cycle-1 and cycle-2 chemotherapy, rats fed long term fish oil diet displayed higher CS and HADH activities compared with their respective control groups. Collectively, these suggested that fish oil enhanced the activity of mitochondrial enzymes that known to be decreased in the presence of the tumor and during chemotherapy confirming that mitochondrial oxidative capacity is associated with decreased lipids content within the muscle in long term fish oil fed rats.

Rats received cycle-1 chemotherapy treatment on the adjuvant fish oil diet displayed similar amounts of fibers expressing Oil Red O staining compared with their respective long term fish oil diet group but, on the other hand, it was significantly higher following cycle-2 chemotherapy treatment. Following cycle-2 chemotherapy CS activity and expression of *TFAM* were significantly lower than rats fed long term fish oil diet and received 2- cycles chemotherapy treatment which could be related to increase neutral lipid content following cycle-2 chemotherapy in adjuvant fish oil group.

6.2 General Discussion

6.2.1 Potential Model of Tumor- and Chemotherapy-Associated Myosteatosis

Recently, non-invasive radiological image-based techniques, such as computed tomography (CT), have revealed that low muscle radiation attenuation is associated with shorter survival in cancer patients (5-12). Studies evaluating muscle fat infiltration in preclinical models of cancer have not yet appeared in the literature because the observation that myosteatosis shortens

survival in cancer patients is very recent (5-11). Although the relationship between myosteatosis and poor outcomes is observed, the characteristics of increased fat content within skeletal muscle has not been resolved and no mechanisms associated with myosteatosis have been established in cancer. Appropriate preclinical model of myosteatosis is required to investigate the biological features and causes of cancer-associated myosteatosis, as well as the mechanisms underlying this feature and potential treatments. This is the first work to report fat accumulation in muscle of a pre-clinical model of cancer and treatment. In the present animal model, we have reported that fat, in the form of TG, accumulates in the gastrocnemius muscle [see Chapter 3] as well as tibialis anterior muscle [see Chapter 5] in the tumor-bearing state and is increased further during chemotherapy similar to what has been observed in humans (24). Chemotherapy is often used to treat cancer patients. However, toxicities may result in a reduction of chemotherapy dose or the termination of therapy. One other study has examined human muscle from a biological prospective. Authors have assessed intramuscular lipid droplets in weight-losing cancer patients, and their results suggest that the number and size of lipid droplets increase with cancer (49). Similarly, we observed an increase in neutral lipid content primarily within muscle fibers, indicative of lipid droplets, in the tumor-bearing state that was exacerbated by successive chemotherapy cycles.

The muscles that we used in the present work are mixed fiber types with higher proportion of glycolytic fibers, IIB, compared with oxidative fibers, I and IIA. In humans, myosteatosis was also observed in a mixed fiber type muscle, such as *rectus abdominus,*(8). Findings from the present animal model enable the study of mechanisms that could be underlying myosteatosis in cancer in two different muscles, gastrocnemius and tibialis anterior which could be difficult to be obtained from humans due to either small sample size (~1 g) or the variations between the patients

because of cancer stage, age, gender, and or treatments each of which may contribute to deleterious effects on muscle. Additional variation may be introduced by the sampling site of the biopsy in humans; fiber composition is not similar between deep region or superficial region (146). These factors introduce variation that may limit the scope of investigations that can be performed in humans. The model used in this work demonstrates similar muscle pathology as observed muscle from humans with cancer treatment therefore in ongoing work by others in my lab this model can be used to understand mechanisms underlying myosteatosis and interventions that can be applied to treat myosteatosis.

In the present work, we have reported two major pathways that could be associated with myosteatosis that occurs in the presence of the tumor and during chemotherapy in skeletal muscle. In Chapter 3, we have showed that increase TG content of gastrocnemius muscle was associated elevated expression of genes involved in lipid synthesis. In chapter 5, we have reported that while the proportion of fibers expressing Oil Red O increased in tibialis anterior muscle in the presence of the tumor and following 1- and 2- cycles of chemotherapy compared with reference animals, mitochondrial enzymes activities were decreased. These results suggest that cancer-associated myosteatosis may involve mechanisms related to adipogenesis and mitochondrial oxidative capacity. However, these findings are novel and we still lack the mechanisms behind the impact of tumor and chemotherapy on these pathways. Evaluation of adipose tissue from the same animals has revealed that mitochondrial dysfunction occurs following CPT-11 plus 5-FU in peri-uterine adipose tissue. One of the mechanisms by which this drug combination causes tumor death is through mitochondrial toxicities (142) and it appears that they evoke similar effects in peripheral tissues as well. A reduction in mitochondrial content of human colon carcinoma cell lines after CPT-11 plus 5-FU treatment was reported (144). These findings suggest that both drugs could

target mitochondrial and cause mitochondrial damage in tissues other than tumor such as adipose tissue and skeletal muscle. In addition, one mechanism for elevated fat in muscle is if adipose tissue is unable to store excess circulating fats. Smaller adipocytes and a reduction in the expression of proteins involved in lipid accumulation in adipose tissue have been observed in the present animal model following chemotherapy treatment (147). These results could contribute to increase the expression of adipogenesis genes in skeletal muscle following chemotherapy treatment in this animal model. Further investigations are required to support this hypothesis such as measuring the downstream target genes for adipogenesis transcriptional factors including lipoprotein lipase and hormone sensitive lipase, in addition to fatty acids transporters and fatty acids binding proteins as increase the expression of these proteins can be related to increase lipid uptake by muscle tissue.

In Chapter 4, we have reported a reduction in total PL following cycle-1 chemotherapy treatment. The reduction in total PL could be attributed to a reduction in either number or size of muscle fiber. In chapter 3, we have reported that chemotherapy resulted in lower mean muscle fiber cross sectional area in rats on the control diet compared with reference animals [Figure 3-5]. Clinically, a reduction in skeletal muscle fiber cross sectional area have been reported in cancer patients who either weight stable or who had a history of weight loss compared to non-diseased control (143,148). Loss of skeletal muscle mass in cancer appears to generally, but not always, be concurrent with elevated intramuscular adipose tissue [reviewed by (4,97)]. In the present work, we mainly focused on studying pathways related to myosteatosis; however, fiber atrophy and muscle wasting could be an area of interest for further investigation. Measuring body composition by applying imaging techniques for example in the present animal model could reveal an interesting result.

Following the second cycle of chemotherapy, we observed an increase in PL content of gastrocnemius muscle which could be a result of increased adipocyte and lipid droplets since it was concurrently associated with increase in TG fatty acid content. These findings are supported by Oil Red O staining as it showed an increase in neutral lipid content primarily within muscle fibers, indicative of lipid droplets, and also evident between muscle fibers, after the second cycle of chemotherapy, which may be attributed to the formation of intermuscular adipocytes [see Chapter 3, Figure 3.2].

In the present study, we have also reported a higher ratio of n-6/n-3 in TG, driven by lower total n-3 fatty acids, EPA and DHA, following chemotherapy treatment. A reduction in n-3, including EPA and DHA levels in plasma PL has been observed during high-dose of chemotherapy and appear to occur concurrently with muscle loss and fat deposition within skeletal muscle (1,21,24). Increase the ratio of n-6/n-3 could derive eicosanoids that modulate the production of pro-inflammatory cytokines (132), such as interleukin-6 [IL-6] and tumor necrosis factor alpha [TNF-α]. Inflammation is a central driver of muscle wasting and mitochondrial dysfunction in the neoplastic state (86,133,134,149,150). In the present study, we have reported a significant decrease in mitochondrial enzymes activities which may contribute to decrease mitochondrial oxidative capacity [see chapter 5, Figure 5.6] as well as a decrease in muscle fiber cross sectional area following chemotherapy treatment [see Chapter 3, Figure 3.5]. Thus, measuring factors related to inflammation in the present animal model could be an area of interest for further investigations. Our method of fatty acid quantification does not enable fatty acid composition of mitochondrial membrane to be determined but rather isolates all the PL fatty acids in the cell and therefore would also include the PL membranes of the organelles such as mitochondria.

6.2.2 Role of the Fish Oil Diets on Tumor- and Chemotherapy-Associated Myosteatosis

In the present study, we investigated the effects of a physiologically attainable level of EPA and DHA supplementation on the tumor-bearing state before and during chemotherapy-associated myosteatosis, as well mechanisms that were associated with myosteatosis in the present animal model. We show for the first time that feeding a diet containing EPA and DHA prevents tumor-associated myosteatosis in both gastrocnemius [see Chapter 3] and tibialis anterior muscles [see Chapter 5], and that adjuvant- is similarly efficacious to long term- EPA and DHA feeding in mitigating chemotherapy-associated myosteatosis in skeletal muscle following both cycles of chemotherapy. These findings support a previous clinical trial from our lab showed that advanced non-small cell lung cancer patients receiving chemotherapy gained skeletal muscle fat, while patients who supplemented with EPA and DHA [2.1 g/day] after diagnosis and during chemotherapy exhibited a reduction in intermuscular fat over the same time period (24).

We show that animals fed a diet containing fish oil either beginning before tumor-implantation [long term] or at the initiation of chemotherapy [adjuvant], neutral lipids and TG were decreased along with decreased expression of transcriptional factors involved adipogenesis/lipogenesis in the gastrocnemius muscle [see chapter 3, Figure 3.4] and increased mitochondrial enzymes activities in tibialis anterior muscle compared with their respective control groups [see chapter 5, Figure 5.6]. Many beneficial effects of n-3 fatty acid on mitochondrial functions have been reported, including higher number of mitochondria, improved function of the enzyme complexes within the electron transport chain, and an improved capacity to appropriately use physiologically available fuels (78,91,96). In the present study, we measured mRNA of genes involved in mitochondrial biogenesis, however, not all mRNA translate to proteins. Thus, further work is required to measure mitochondrial proteins or mitochondrial DNA to confirm our results.

In chapter 4, we have reported that while total TG decreased in the gastrocnemius muscle of animal model during fish oil intervention, the significant increase in PL is not likely attributed to adipocytes or lipid droplets, and it seems more likely to attributed to either increase myocytes number, cross sectional area, and making more cells like mitochondria. We have reported in Chapter 5, that genes involving in mitochondrial biogenesis were increased in tibialis anterior muscle of rats fed long term fish oil diet following chemotherapy treatment compared with their respective control groups. These findings could contribute to increase total PL during fish oil intervention. Similar to our findings in this model of cancer, some experimental studies have reported the beneficial effects of EPA and DHA on mitochondrial content including higher number of mitochondria in skeletal muscle [reviewed by (91)]. However, the mechanisms through which dietary EPA and DHA supplementation improves muscle health during chemotherapy require further investigation. The requirement of EPA and DHA for muscle integrity has not been directly studied; however, evidence from studies by our group and others' suggest improvement in muscle health occurs when a supply of EPA and DHA is maintained (1,24,61,75,103,151,152). A number of mechanisms of action have been associated with the effects of EPA and DHA on muscle mass and myosteatosis, in a variety-of non-cancer conditions [reviewed by (97)] including their association with anti-inflammatory properties (152,153) and improvement of the insulin response in many experimental systems and in a number of non-cancer disease states (68-73).

The animal model used in the present study demonstrated that incorporation of EPA and DHA into both TG and PL fatty acids was similar between rats fed fish oil for either long term [3 weeks minimum] or short term [one week minimum]. Healthy people supplemented with 5 grams of fish oil per day had an increase in n-3 fatty acids [3.8% to 5.1% total fatty acids] after 2-weeks, and it further increased after 4-weeks to [~6.8%] total fatty acids (135). Other studies have

demonstrated that 8-weeks of fish oil supplementation results in a 2-fold increase in muscle PL n-3 fatty acid composition in healthy subjects [5.04% to 9.03%] total fatty acids; (75,76). In the present study, we observed similar increases in proportion of n-3 fatty acid content in both TG and PL fatty acids, even with one week of supplementation [adjuvant-cycle-1 chemotherapy].

6.3 Strength and Limitations

The first novel aspect of our study is that it is the first to report fat accumulation in muscle of a pre-clinical model of cancer and treatment. Mechanisms and therapies have not yet been explored using any experimental system. The animal model used in this study represents the delivery of drug therapy for colorectal cancer in humans with respect to drug dosing and toxicities using a combination of CPT-11 and 5-FU which is first-line treatment for colorectal cancer. The second novel aspect of our study is the design of our diets as both control and fish oil diets have equal amount of energy and protein per gram of diet. Diets are within the range of macronutrient intake by some humans in Westernized countries. In addition, both diets contained similar proportion of fat and were matched for polyunsaturated fatty acids/ saturated fatty acids and were only differ in the composition of fatty acids. Most of the studies used animal models utilized standard laboratory chow diet. Chow diet is usually provided with undefined of nutrient source and do not reflect typical human dietary intakes [reviewed by (154)]. The third novel aspect of this study is that presence of myosteatosis was confirmed in two different muscles including gastrocnemius and tibialis anterior. The fourth novel aspect of our study is using long and adjuvant [short term] fish oil diet. In humans, the long-term fish oil group translates to consumption of fish oil prior to cancer diagnosis whereas the adjuvant fish oil feeding group is most similar to the design of trials investigating fish oil supplementation. These timelines of the fish oil diets enable us to study the ability of fish oil to prevent [long term] and treat [adjuvant] tumor and treatment-

associated fat accumulation into muscle.

This study has several limitations, in this study maximum 2-cycles of chemotherapy were used while clinically, cancer patients would receive multiple cycles of chemotherapy. Thus, it is going to be interesting to study the effect of fish oil with additional cycles of chemotherapy that will better represents what cancer patients receive clinically. Additionally, these animals were not fasted at the termination time. Fasting animals before the termination would allow for additional analyses such as insulin sensitivity and glucose homeostasis. The animal models that we used are young and healthy, while clinically, cancer patients usually present with different comorbidities such as obesity and diabetes. Myosteatosis was first observed in these conditions before cancer, thus applying models of obesity or diabetes to the protocol of the current animal model presented in this study will reveal more findings regarding the ability of fish oil intervention to attenuate fatty muscle.

6.4 Considerations for Future Research

Further research is required to investigate muscle conditions in the presence of the tumor and during treatments in response to control and fish oil diets. For example, neutral lipid content in muscle increased after the 2- cycle in adjuvant fish oil rats. Since cancer patients usually receive multiple cycles of chemotherapy, thus, including more cycles of chemotherapy treatment to the present protocol will be more relevant clinically. Measuring the effect of higher doses of fish oil intervention in the present animal model could reveal better findings. There is no study evaluating the effect of different doses of fish oil on myosteatosis and muscle health in cancer. Furthermore, fasting all animals before the termination time would enable measures of myosteatosis that involve the insulin pathway. However, a previous study from our lab conducted on the present animal model showed no significant differences in mTOR (pSer2448) protein concentration in

gastrocnemius muscle between rats fed control diet and rats fed an adjuvant fish oil diet following both cycles of chemotherapy or the ratio of phosphorylated-AKT (pSer473) to total AKT. These preliminary findings could be related to the method that was used to measure the protein concentration, ELISA kits, as this method is not commonly used in rat tissue compared to Western Blot analysis(155). Other method such as electron microscopy to investigate mitochondrial number and shape can be used in future studies. In addition, assessing fatty acids binding and transporting genes and proteins such as fatty acids binding proteins and CD36, as well as inflammation markers, such as IL-6, TNF-α, CRP, in the present animal model would be areas that require further investigation. Poor outcomes of myosteatosis has been reported by using CT scan and the correlation between CT scan radiation attenuation of skeletal muscle and fat content of muscle biopsy specimens has been reported in only one study (14). More investigation in this area is required to establish a cutoff of myosteatosis based on both CT scan radiation attenuation as well as TG content within the muscle to enable understand the mechanisms underlying the association between myosteatosis and poor outcomes and higher mortality in cancer. Also, we have to acknowledge that skeletal muscle TG content has been reported to be higher in healthy people after endurance exercise align with higher mitochondrial enzymes activities and oxidation capacity (156,157). Increase the amount of TG content in this case is not pathological but instead is required to serve as essential fuel for muscle contraction. On the other hand, fatty muscle in cancer represents a pathological condition because of it is association with poor outcomes and mortality. In chapter 5, we investigated the expression of *PGC-1α* in the present animal model as it plays an essential role in mitochondrial biogenesis, but in addition to that *PGC-1α* seems to have a role also on neuromuscular junction [NMJ, (158)], chemical area where the cross talk between motor neurons and muscle fibers occur. Fiber type switching is controlled by neuronal input, such

as exercise adaptations of the NMJ causes fiber type switch towards oxidative fibers [type I], Thus studying NMJ in the present animal model and it is association with fiber type switching and myosteatosis could be an area of interest for further direction.

6.5 Conclusion

Figure 6-1 represents the novel observations of fat accumulation that was associated with increased the expression of key adipogenic/lipogenic transcriptional factors, altering fatty acids composition with lower EPA, DHA, and total n-3 fatty acids which drive an increase in the n-6/n-3 ratio, and lower mitochondrial oxidative capacity in the skeletal muscles of an *in vivo* tumor-bearing model that underwent chemotherapy treatment. These findings reveal potential mechanisms underlying fundamental alterations that may be involved in the development of myosteatosis in cancer patients. Long term and adjuvant dietary EPA and DHA feeding were both efficacious in markedly improving muscle health by reducing the toxicity effect of CPT-11 and 5-FU on fat accumulation within skeletal muscle. Our study highlights the therapeutic potential of EPA and DHA as promising nutritional adjuvants to chemotherapy to reduce muscle toxicities associated with drug delivery. Findings from the present study are encouraging, warranting future research to determine further mechanisms by which dietary EPA and DHA supplementation attenuate pathological fat accumulation in the skeletal muscles that known to be associated with higher mortality in cancer patients.

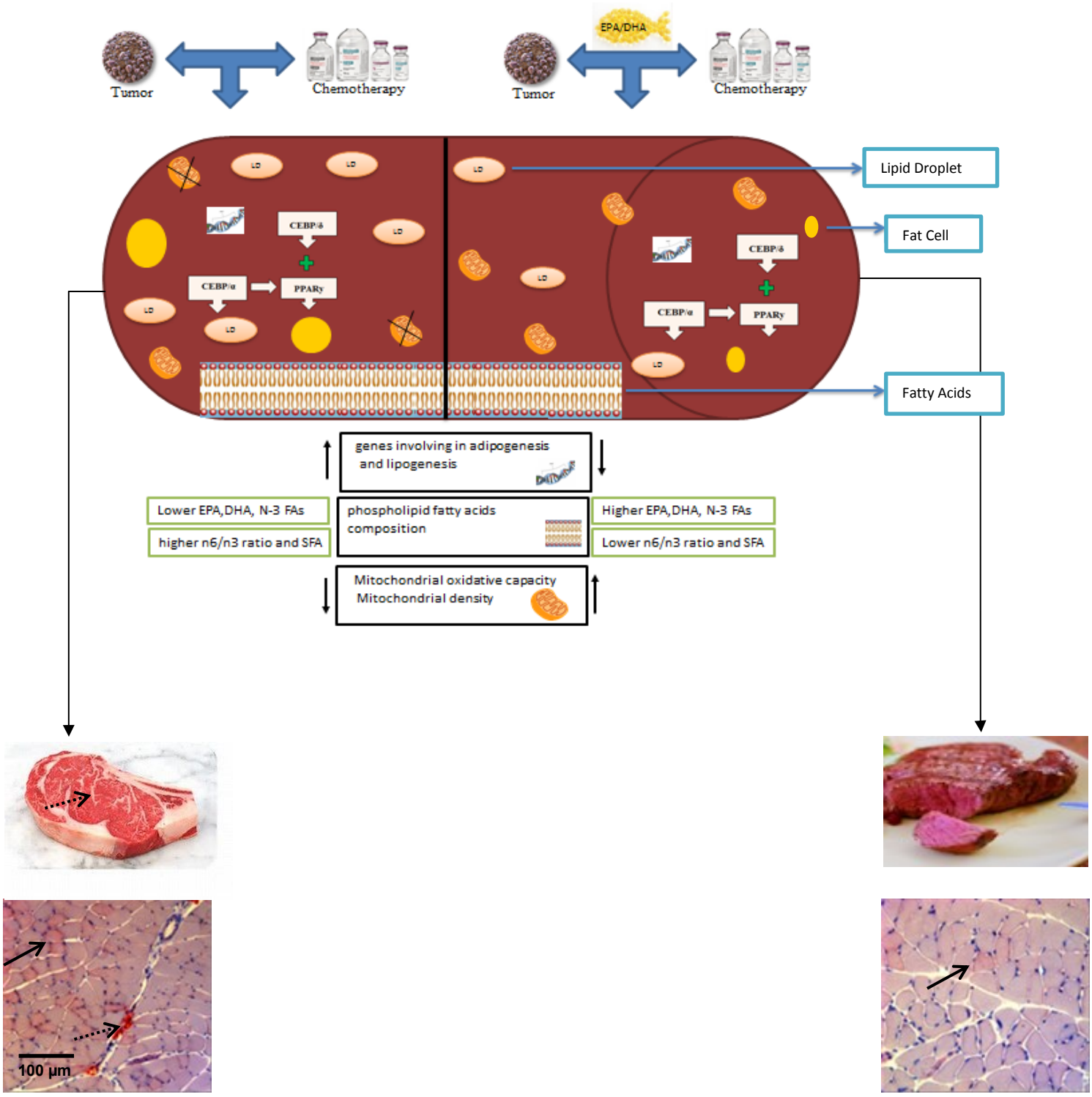


Figure 6.1 Summary of mechanisms underlying fat accumulation within skeletal muscle. A Ward colon tumor-bearing rat model underwent 1- and 2- cycles of irinotecan plus 5-fluorouracil and

provided diets with or without fish oil that was fed either prior to tumor implantation (long term) or at the same day of the first cycle of (adjuvant) to tumor implantation**.** Both fish oil containing diets were able to prevent and treat, respectively, fat accumulation within skeletal muscle that known to occur in the presence of the tumor and following chemotherapy treatment. Histology figures represent gastrocnemius muscles obtained from rats fed either control diet or long term fish oil diet and both received 2- cycles of chemotherapy (presented in Chapter 3, Figure 3-2). Muscles stained with Oil Red O to identify neutral lipid content within the muscle. Solid arrow shows an example of a positive fiber expressing Oil Red O; dashed arrow shows an example of Oil Red O staining between muscle fibers. Scale bar represents 100 μm.

References:

(1) Murphy RA, Mourtzakis M, Chu QS, Reiman T, Mazurak VC. Skeletal muscle depletion is associated with reduced plasma (n-3) fatty acids in non-small cell lung cancer patients. J Nutr 2010 Sep;140(9):1602-1606.

(2) Murphy RA, Mourtzakis M, Chu QS, Baracos VE, Reiman T, Mazurak VC. Supplementation with fish oil increases first-line chemotherapy efficacy in patients with advanced nonsmall cell lung cancer. Cancer 2011 Aug 15;117(16):3774-3780.

(3) Prado CM, Baracos VE, McCargar LJ, Reiman T, Mourtzakis M, Tonkin K, et al. Sarcopenia as a determinant of chemotherapy toxicity and time to tumor progression in metastatic breast cancer patients receiving capecitabine treatment. Clin Cancer Res 2009 Apr 15;15(8):2920-2926.

(4) Aubrey J, Esfandiari N, Baracos VE, Buteau FA, Frenette J, Putman CT, et al. Measurement of skeletal muscle radiation attenuation and basis of its biological variation. Acta Physiol (Oxf) 2014 Mar;210(3):489-497.

(5) Miyamoto Y, Baba Y, Sakamoto Y, Ohuchi M. Negative Impact of Skeletal Muscle Loss after Systemic Chemotherapy in Patients with Unresectable Colorectal Cancer. 2015;10(6):e0129742.

(6) Antoun S, Lanoy E, Iacovelli R, Albiges-Sauvin L, Loriot Y, Merad-Taoufik M, et al. Skeletal muscle density predicts prognosis in patients with metastatic renal cell carcinoma treated with targeted therapies. Cancer 2013 Jun 25.

(7) Rollins KE, Tewari N, Ackner A, Awwad A, Madhusudan S, Macdonald IA, et al. The impact of sarcopenia and myosteatosis on outcomes of unresectable pancreatic cancer or distal cholangiocarcinoma. Clin Nutr 2015 Sep 1.

(8) Martin L, Birdsell L, Macdonald N, Reiman T, Clandinin MT, McCargar LJ, et al. Cancer cachexia in the age of obesity: skeletal muscle depletion is a powerful prognostic factor, independent of body mass index. J Clin Oncol 2013 Apr 20;31(12):1539-1547.

(9) Sabel MS, Lee J, Cai S, Englesbe MJ, Holcombe S, Wang S. Sarcopenia as a prognostic factor among patients with stage III melanoma. Ann Surg Oncol 2011 Dec;18(13):3579-3585.

(10) Chu MP, Lieffers J, Ghosh S, Belch AR, Chua NS, Fontaine A, et al. Skeletal Muscle Radio-Density Is an Independent Predictor of Response and Outcomes in Follicular Lymphoma Treated with Chemoimmunotherapy. PLoS ONE 2015 06;10(6):1-11.

(11) van Dijk D,P.J., Bakens MJAM, Coolsen MME, Rensen SS, van Dam R,M., Bours MJL, et al. Low skeletal muscle radiation attenuation and visceral adiposity are associated with overall survival and surgical site infections in patients with pancreatic cancer. J Cachexia Sarcopenia Muscle 2016 10/26.

(12) Sjøblom B, Grønberg BH, Wentzel-Larsen T, Baracos VE, Hjermstad MJ, Aass N, et al. Skeletal muscle radiodensity is prognostic for survival in patients with advanced non-small cell lung cancer. Clin Nutr 2016 12;35(6):1386-1393.

(13) Goodpaster BH, Kelley DE, Thaete FL, He J, Ross R. Skeletal muscle attenuation determined by computed tomography is associated with skeletal muscle lipid content. J Appl Physiol 2000 Jul;89(1):104-110.

(14) Miljkovic I, Zmuda JM. Epidemiology of myosteatosis. Curr Opin Clin Nutr Metab Care 2010 May;13(3):260-264.

(15) Lang T, Cauley JA, Tylavsky F, Bauer D, Cummings S, Harris TB, et al. Computed tomographic measurements of thigh muscle cross-sectional area and attenuation coefficient predict hip fracture: the health, aging, and body composition study. J Bone Miner Res 2010 Mar;25(3):513-519.

(16) Hajjaji N, Bougnoux P. Selective sensitization of tumors to chemotherapy by marine-derived lipids: a review. Cancer Treat Rev 2013 Aug;39(5):473-488.

(17) Murphy RA, Mourtzakis M, Mazurak VC. N-3 Polyunsaturated Fatty Acids: the Potential Role for Supplementation in Cancer. CURR OPIN CLIN NUTR METABOLIC CARE 2012 2012;15(3):246-251.

(18) Morland SL, Martins KJB, Mazurak VC. n-3 polyunsaturated fatty acid supplementation during cancer chemotherapy. Journal of Nutrition & Intermediary Metabolism 2015.

(19) Bougnoux P, Hajjaji N, Ferrasson MN, Giraudeau B, Couet C, Le Floch O. Improving outcome of chemotherapy of metastatic breast cancer by docosahexaenoic acid: a phase II trial. Br J Cancer 2009 Dec 15;101(12):1978-1985.

(20) Arshad A, Isherwood J, Mann C, Cooke J, Pollard C. Intravenous omega-3 Fatty Acids Plus Gemcitabine: Potential to Improve Response and Quality of Life in Advanced Pancreatic Cancer. 2015 Jul 28.

(21) Pratt VC, Watanabe S, Bruera E, Mackey J, Clandinin MT, Baracos VE, et al. Plasma and neutrophil fatty acid composition in advanced cancer patients and response to fish oil supplementation. Br J Cancer 2002 Dec 2;87(12):1370-1378.

(22) Baracos VE, Mazurak VC, Ma DW. 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 Dec;17(2):177-192.

(23) Murphy RA, Wilke MS, Perrine M, Pawlowicz M, Mourtzakis M, Lieffers JR, et al. Loss of adipose tissue and plasma phospholipids: relationship to survival in advanced cancer patients. Clin Nutr 2010 Aug;29(4):482-487.

(24) Murphy RA, Mourtzakis M, Chu QS, Baracos VE, Reiman T, Mazurak VC. Nutritional intervention with fish oil provides a benefit over standard of care for weight and skeletal muscle mass in patients with nonsmall cell lung cancer receiving chemotherapy. Cancer 2011 Apr 15;117(8):1775-1782.

(25) Canadian Cancer Society's Advisory Committee on Cancer Statistics. Canadian Cancer Statistics. Toronto, ON: Canadian Cancer Society 2015.

(26) Edge SB, Compton CC. The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM. Ann Surg Oncol 2010 06;17(6):1471-1474.

(27) Van Cutsem E, Geboes K. The multidisciplinary management of gastrointestinal cancer. The integration of cytotoxics and biologicals in the treatment of metastatic colorectal cancer. Best Pract Res Clin Gastroenterol 2007;21(6):1089-1108.

(28) Board RE, Valle JW. Metastatic colorectal cancer: current systemic treatment options. Drugs 2007 08;67(13):1851-1867 17p.

(29) Douillard JY, Cunningham D, Roth AD, Navarro M, James RD, Karasek P, et al. Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicentre randomised trial. Lancet 2000 Mar 25;355(9209):1041-1047.

(30) Longley DB, Harkin DP, Johnston PG. 5-Fluorouracil: mechanisms of action and clinical strategies. Nature Reviews Cancer 2003 05;3(5):330.

(31) Tejwani A, Yates T, Tejwani S. Cardiotoxicity with fluorouracil and capecitabine revisited. COMMUNITY ONCOL 2008 2008;5(10):566-568 3p.

(32) Bleiberg H. Colorectal cancer--is there an alternative to 5-FU? European journal of cancer (Oxford, England : 1990) JID - 9005373 1997.

(33) Wall ME, Wani MC, Natschke SM, Nicholas AW. Plant antitumor agents. 22. Isolation of 11-hydroxycamptothecin from Camptotheca acuminata Decne: total synthesis and biological activity. J Med Chem 1986 08;29(8):1553-1555.

(34) Xu Y, Villalona-Calero M. Irinotecan: mechanisms of tumor resistance and novel strategies for modulating its activity. Ann Oncol 2002 12;13(12):1841-1851.

(35) Rothenberg ML. Irinotecan (CPT-11): recent developments and future directions--colorectal cancer and beyond. Oncologist 2001;6(1):66-80.

(36) Heymsfield SB, Wang Z, Baumgartner RN, Ross R. Human body composition: advances in models and methods. Annu Rev Nutr 1997;17:527-558.

(37) Mitsiopoulos N, Baumgartner RN, Heymsfield SB, Lyons W, Gallagher D, Ross R. Cadaver validation of skeletal muscle measurement by magnetic resonance imaging and computerized tomography. J Appl Physiol 1998 Jul;85(1):115-122.

(38) Shen W, Punyanitya M, Wang Z, Gallagher D, St-Onge MP, Albu J, et al. Total body skeletal muscle and adipose tissue volumes: estimation from a single abdominal cross-sectional image. J Appl Physiol 2004 Dec;97(6):2333-2338.

(39) Evans W. Functional and metabolic consequences of sarcopenia. J Nutr 1997 May;127(5 Suppl):998S-1003S.

(40) Roubenoff R. Sarcopenic obesity: does muscle loss cause fat gain? Lessons from rheumatoid arthritis and osteoarthritis. Ann N Y Acad Sci 2000 May;904:553-557.

(41) Janssen I, Heymsfield SB, Ross R. Low relative skeletal muscle mass (sarcopenia) in older persons is associated with functional impairment and physical disability. J Am Geriatr Soc 2002 May;50(5):889-896.

(42) Mourtzakis M, Prado CM, Lieffers JR, Reiman T, McCargar LJ, Baracos VE. A practical and precise approach to quantification of body composition in cancer patients using computed tomography images acquired during routine care. Appl Physiol Nutr Metab 2008 Oct;33(5):997-1006.

(43) Baumgartner RN, Koehler KM, Gallagher D, Romero L, Heymsfield SB, Ross RR, et al. Epidemiology of sarcopenia among the elderly in New Mexico. Am J Epidemiol 1998 Apr 15;147(8):755-763.

(44) Bouchard DR, Dionne IJ, Brochu M. Sarcopenic/obesity and physical capacity in older men and women: data from the Nutrition as a Determinant of Successful Aging (NuAge)-the Quebec longitudinal Study. Obesity (Silver Spring) 2009 Nov;17(11):2082-2088.

(45) Rolland Y, Lauwers-Cances V, Cristini C, Abellan van Kan G, Janssen I, Morley JE, et al. Difficulties with physical function associated with obesity, sarcopenia, and sarcopenic-obesity in community-dwelling elderly women: the EPIDOS (EPIDemiologie de l'OSteoporose) Study. Am J Clin Nutr 2009 Jun;89(6):1895-1900.

(46) Baracos VE, Reiman T, Mourtzakis M, Gioulbasanis I, Antoun S. Body composition in patients with non-small cell lung cancer: a contemporary view of cancer cachexia with the use of computed tomography image analysis. Am J Clin Nutr 2010 Apr;91(4):1133S-1137S.

(47) Prado CM, Lieffers JR, McCargar LJ, Reiman T, Sawyer MB, Martin L, et al. Prevalence and clinical implications of sarcopenic obesity in patients with solid tumours of the respiratory and gastrointestinal tracts: a population-based study. Lancet Oncol 2008 Jul;9(7):629-635.

(48) Tan BH, Birdsell LA, Martin L, Baracos VE, Fearon KC. Sarcopenia in an overweight or obese patient is an adverse prognostic factor in pancreatic cancer. Clin Cancer Res 2009 Nov 15;15(22):6973-6979.

(49) Stephens NA, Skipworth RJ, Macdonald AJ, Greig CA, Ross JA, Fearon KC. Intramyocellular lipid droplets increase with progression of cachexia in cancer patients. J Cachexia Sarcopenia Muscle 2011 Jun;2(2):111-117.

(50) Sampath H, Ntambi JM. Polyunsaturated Fatty Acid Regulation of Genes of Lipid Metabolism. Annu Rev Nutr 2005 08;25(1):317-C-4.

(51) Ahn J, Park IS, Lee KS, Kim SY, Chung EJ, Kim J, et al. Fatty acid patterns in gastric mucosa of stomach cancer patients. Yonsei Med J 2001 Apr;42(2):220-226.

(52) Ji-Ming Ye, Doyle PJ, Iglesias MA, Watson DG, Cooney GJ, Kraegen EW. Peroxisome Proliferator-Activated Receptor (PPAR)-α Activation Lowers Muscle Lipids and Improves Insulin Sensitivity in High Fat-Fed Rats. Diabetes 2001 02;50(2):411-417.

(53) Muoio DM, MacLean PS, Lang DB, Li S, Houmard JA, Way JM, et al. Fatty acid homeostasis and induction of lipid regulatory genes in skeletal muscles of peroxisome proliferator-activated receptor (PPAR) alpha knock-out mice. Evidence for compensatory regulation by PPAR delta. J Biol Chem 2002 Jul 19;277(29):26089-26097.

(54) Gilde AJ, Van Bilsen M. Peroxisome proliferator-activated receptors (PPARS): regulators of gene expression in heart and skeletal muscle. Acta Physiol Scand 2003 Aug;178(4):425-434.

(55) Pandey P, Okuda H, Watabe M, Pai S, Liu W, Kobayashi A, et al. Resveratrol suppresses growth of cancer stem-like cells by inhibiting fatty acid synthase. Breast Cancer Research & Treatment 2011 11/15;130(2):387-398.

(56) Taniguchi M, Guan LL, Zhang B, Dodson MV, Okine E, Moore SS. Adipogenesis of bovine perimuscular preadipocytes. Biochem Biophys Res Commun 2008 Feb 1;366(1):54-59.

(57) Hausman GJ, Dodson MV, Ajuwon K, Azain M, Barnes KM, Guan LL, et al. Board-invited review: the biology and regulation of preadipocytes and adipocytes in meat animals. J Anim Sci 2009 Apr;87(4):1218-1246.

(58) Chabowski A, Żendzian-Piotrowska M, Nawrocki A, Górski J. Not only accumulation, but also saturation status of intramuscular lipids is significantly affected by PPARγ activation. Acta Physiologica 2012 05;205(1):145-158.

(59) An L, Pang Y, Gao H, Tao L, Miao K, Wu Z, et al. Heterologous expression of C. elegans fat-1 decreases the n-6/n-3 fatty acid ratio and inhibits adipogenesis in 3T3-L1 cells. Biochemical & Biophysical Research Communications 2012 11/23;428(3):405-410.

(60) Wójcik C, Lohe K, Kuang C, Xiao Y, Jouni Z, Poels E. Modulation of adipocyte differentiation by omega-3 polyunsaturated fatty acids involves the ubiquitin-proteasome system. J Cell Mol Med 2014 04;18(4):590-599.

(61) Hiller B, Herdmann A, Nuernberg K. Dietary n-3 fatty acids significantly suppress lipogenesis in bovine muscle and adipose tissue: a functional genomics approach. Lipids 2011 Jul;46(7):557-567.

(62) Waters SM, Kelly JP, O'Boyle P, Moloney AP, Kenny DA. Effect of level and duration of dietary n-3 polyunsaturated fatty acid supplementation on the transcriptional regulation of Delta9-desaturase in muscle of beef cattle. J Anim Sci 2009 Jan;87(1):244-252.

(63) Borkman M, Storlien LH, Pan DA, Jenkins AB, Chisholm DJ, Campbell LV. The relation between insulin sensitivity and the fatty-acid composition of skeletal-muscle phospholipids. N Engl J Med 1993 01/28;328(4):238-244.

(64) Pan DA, Lillioja S, Milner MR, Kriketos AD, Baur LA, Bogardus C, et al. Skeletal muscle membrane lipid composition is related to adiposity and insulin action. J Clin Invest 1995 12;96(6):2802-2808.

(65) Storlien LH, FAU PD, FAU KA, O'Connor J FAU - Caterson,,I.D., FAU CI, FAU CG, et al. Skeletal muscle membrane lipids and insulin resistance. Lipids JID - 0060450 1021.

(66) Manco M, Mingrone G, Greco AV, Capristo E, Gniuli D, De Gaetano A, et al. Insulin resistance directly correlates with increased saturated fatty acids in skeletal muscle triglycerides. Metabolism 2000 02;49(2):220-224.

(67) Antunes D, Padrao AI, Maciel E, Santinha D, Oliveira P, Vitorino R, et al. Molecular insights into mitochondrial dysfunction in cancer-related muscle wasting. Biochim Biophys Acta 2014 Jun;1841(6):896-905.

(68) Smith BK, Holloway GP, Reza-Lopez S, Jeram SM, Kang JX, Ma DW. A decreased n-6/n-3 ratio in the fat-1 mouse is associated with improved glucose tolerance. Appl Physiol Nutr Metab 2010 Oct;35(5):699-706.

(69) Moreno-Aliaga MJ, Lorente-Cebrian S, Martinez JA. Regulation of adipokine secretion by n-3 fatty acids. Proc Nutr Soc 2010 Aug;69(3):324-332.

(70) 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 (Lond) 2009 Jan;116(1):1-16.

(71) Kopecky J, Rossmeisl M, Flachs P, Kuda O, Brauner P, Jilkova Z, et al. n-3 PUFA: bioavailability and modulation of adipose tissue function. Proc Nutr Soc 2009 Nov;68(4):361-369.

(72) Martin de Santa Olalla L, Sanchez Muniz FJ, Vaquero MP. N-3 fatty acids in glucose metabolism and insulin sensitivity. Nutr Hosp 2009 Mar-Apr;24(2):113-127.

(73) Browning LM. n-3 Polyunsaturated fatty acids, inflammation and obesity-related disease. Proc Nutr Soc 2003 May;62(2):447-453.

(74) Dangardt F, Chen Y, Gronowitz E, Dahlgren J, Friberg P, Strandvik B. High physiological omega-3 Fatty Acid supplementation affects muscle Fatty Acid composition and glucose and insulin homeostasis in obese adolescents. J Nutr Metab 2012;2012:395757.

(75) Smith GI, Atherton P, Reeds DN, Mohammed BS, Rankin D, Rennie MJ, et al. Dietary omega-3 fatty acid supplementation increases the rate of muscle protein synthesis in older adults: a randomized controlled trial. Am J Clin Nutr 2011 Feb;93(2):402-412.

(76) Smith GI, Atherton P, Reeds DN, Mohammed BS, Rankin D, Rennie MJ, et al. Omega-3 polyunsaturated fatty acids augment the muscle protein anabolic response to hyperinsulinaemia-hyperaminoacidaemia in healthy young and middle-aged men and women. Clin Sci (Lond) 2011 Sep;121(6):267-278.

(77) Kraegen EW, Cooney GJ. Free fatty acids and skeletal muscle insulin resistance. Curr Opin Lipidol 2008 Jun;19(3):235-241.

(78) Vaughan RA, Garcia-Smith R, Bisoffi M, Conn CA, Trujillo KA. Conjugated linoleic acid or omega 3 fatty acids increase mitochondrial biosynthesis and metabolism in skeletal muscle cells. Lipids Health Dis 2012 Oct 30;11:142-511X-11-142.

(79) Mikami N, Hosokawa M, Miyashita K. Dietary combination of fish oil and taurine decreases fat accumulation and ameliorates blood glucose levels in type 2 diabetic/obese KK-A(y) mice. J Food Sci 2012 Jun;77(6):H114-20.

(80) Hessvik NP, Bakke SS, Fredriksson K, Boekschoten MV, Fjorkenstad A, Koster G, et al. Metabolic switching of human myotubes is improved by n-3 fatty acids. J Lipid Res 2010 Aug;51(8):2090-2104.

(81) Finlin BS, Varma V, Nolen GT, Dube J, Starnes CP, Rasouli N, et al. DHA reduces the atrophy-associated Fn14 protein in differentiated myotubes during coculture with macrophages. J Nutr Biochem 2012 Aug;23(8):885-891.

(82) Mansilla F, Dominguez CA, Yeadon JE, Corydon TJ, Burden SJ, Knudsen CR. Translation elongation factor eEF1A binds to a novel myosin binding protein-C-like protein. J Cell Biochem 2008 Oct 15;105(3):847-858.

(83) Hoppeler H, Luthi P, Claassen H, Weibel ER, Howald H. The ultrastructure of the normal human skeletal muscle. A morphometric analysis on untrained men, women and well-trained orienteers. Pflugers Arch 1973 Nov 28;344(3):217-232.

(84) Wang X, Pickrell AM, Zimmers TA, Moraes CT. Increase in muscle mitochondrial biogenesis does not prevent muscle loss but increased tumor size in a mouse model of acute cancer-induced cachexia. PLoS One 2012;7(3):e33426.

(85) Julienne CM, Dumas JF, Goupille C, Pinault M, Berri C, Collin A, et al. Cancer cachexia is associated with a decrease in skeletal muscle mitochondrial oxidative capacities without alteration of ATP production efficiency. J Cachexia Sarcopenia Muscle 2012 Dec;3(4):265-275.

(86) White JP, Baltgalvis KA, Puppa MJ, Sato S, Baynes JW, Carson JA. Muscle oxidative capacity during IL-6-dependent cancer cachexia. Am J Physiol Regul Integr Comp Physiol 2011 Feb;300(2):R201-11.

(87) Peterson CM, Johannsen DL, Ravussin E. Skeletal Muscle Mitochondria and Aging: A Review. Journal of Aging Research 2012 01:1-20.

(88) Montgomery MK, Turner N. Mitochondrial dysfunction and insulin resistance: an update. Endocr Connect 2015 03;4(1):R1-R15.

(89) Kiens B. Skeletal muscle lipid metabolism in exercise and insulin resistance. Physiol Rev 2006 Jan;86(1):205-243.

(90) Collino M, Mastrocola R, Nigro D, Chiazza F, Aragno M, D'Antona G, et al. Variability in myosteatosis and insulin resistance induced by high-fat diet in mouse skeletal muscles. Biomed Res Int 2014;2014:569623-569623.

(91) Vaughan RA, Mermier CM, Bisoffi M, Trujillo KA, Conn CA. Dietary stimulators of the PGC-1 superfamily and mitochondrial biosynthesis in skeletal muscle. A mini-review. J Physiol Biochem 2014 Mar;70(1):271-284.

(92) Herbst EAF, Paglialunga S, Gerling C, Whitfield J, Mukai K, Chabowski A, et al. Omega-3 supplementation alters mitochondrial membrane composition and respiration kinetics in human skeletal muscle. J Physiol (Lond) 2014 03/15;592(6):1341-1352.

(93) Totland GK, Madsen L, Klementsen B, Vaagenes H, Kryvi H, Froyland L, et al. Proliferation of mitochondria and gene expression of carnitine palmitoyltransferase and fatty acyl-CoA oxidase in rat skeletal muscle, heart and liver by hypolipidemic fatty acids. Biol Cell 2000 Aug;92(5):317-329.

(94) Lee MS, Kim IH, Kim Y. Effects of eicosapentaenoic acid and docosahexaenoic acid on uncoupling protein 3 gene expression in C(2)C(12) muscle cells. Nutrients 2013 May 21;5(5):1660-1671.

(95) Harris WS, Bulchandani D. Why do omega-3 fatty acids lower serum triglycerides? Curr Opin Lipidol 2006 Aug;17(4):387-393.

(96) Horakova O, Medrikova D, van Schothorst EM, Bunschoten A, Flachs P, Kus V, et al. Preservation of metabolic flexibility in skeletal muscle by a combined use of n-3 PUFA and rosiglitazone in dietary obese mice. PLoS One 2012;7(8):e43764.

(97) Ewaschuk JB, Almasud A, Mazurak VC. Role of n-3 fatty acids in muscle loss and myosteatosis. Appl Physiol Nutr Metab 2014 Jun;39(6):654-662.

(98) Xue H, Sawyer MB, Field CJ, Dieleman LA, Baracos VE. Nutritional modulation of antitumor efficacy and diarrhea toxicity related to irinotecan chemotherapy in rats bearing the ward colon tumor. Clin Cancer Res 2007 Dec 1;13(23):7146-7154.

(99) Almasud, A. Elevated Fatty Acid Content in Muscle is Prevented by EPA and DHA in an Animal Model of Colorectal Cancer Receiving CPT-11 / 5-FUuniversity of Alberta; 2012.

(100) Antoun S, Birdsell L, Sawyer MB, Venner P. Association of skeletal muscle wasting with treatment with sorafenib in patients with advanced renal cell carcinoma: results from a placebo-controlled study. 2010 Feb 20;28(6):1054-1060.

(101) Osterlund P, Ruotsalainen T, Korpela R, Saxelin M, Ollus A, Valta P, et al. Lactobacillus supplementation for diarrhoea related to chemotherapy of colorectal cancer: a randomised study. Br J Cancer 2007 Oct 22;97(8):1028-1034.

(102) Sun X, Zhang J, Gupta R, Macgibbon AK, Kuhn-Sherlock B, Krissansen GW. Dairy milk fat augments paclitaxel therapy to suppress tumour metastasis in mice, and protects against the side-effects of chemotherapy. Clin Exp Metastasis 2011 Oct;28(7):675-688.

(103) Castillero E, Martin AI, Lopez-Menduina M, Villanua MA, Lopez-Calderon A. Eicosapentaenoic acid attenuates arthritis-induced muscle wasting acting on atrogin-1 and on myogenic regulatory factors. Am J Physiol Regul Integr Comp Physiol 2009 Nov;297(5):R1322-31.

(104) Wang H, Li TL, Hsia S, Su IL, Chan YL, Wu CJ. Skeletal muscle atrophy is attenuated in tumor-bearing mice under chemotherapy by treatment with fish oil and selenium. 2015 Apr 10;6(10):7758-7773.

(105) Xue H, Sawyer MB, Wischmeyer PE, Baracos VE. Nutrition modulation of gastrointestinal toxicity related to cancer chemotherapy: from preclinical findings to clinical strategy. JPEN J Parenter Enteral Nutr 2011 Jan;35(1):74-90.

(106) Hardman WE, Avula CP, Fernandes G, Cameron IL. Three percent dietary fish oil concentrate increased efficacy of doxorubicin against MDA-MB 231 breast cancer xenografts. Clin Cancer Res 2001 Jul;7(7):2041-2049.

(107) Yam D, Peled A, Shinitzky M. Suppression of tumor growth and metastasis by dietary fish oil combined with vitamins E and C and cisplatin. Cancer Chemother Pharmacol 2001;47(1):34-40.

(108) Hardman WE, Moyer MP, Cameron IL. Fish oil supplementation enhanced CPT-11 (irinotecan) efficacy against MCF7 breast carcinoma xenografts and ameliorated intestinal side-effects. Br J Cancer 1999 Oct;81(3):440-448.

(109) Jordan A, Stein J. Effect of an omega-3 fatty acid containing lipid emulsion alone and in combination with 5-fluorouracil (5-FU) on growth of the colon cancer cell line Caco-2. Eur J Nutr 2003 Dec;42(6):324-331.

(110) Xue H, Le Roy S, Sawyer MB, Field CJ, Dieleman LA, Baracos VE. Single and combined supplementation of glutamine and n-3 polyunsaturated fatty acids on host tolerance and tumour response to 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin (CPT-11)/5-fluorouracil chemotherapy in rats bearing Ward colon tumour. Br J Nutr 2009 Aug;102(3):434-442.

(111) Germain E, Bonnet P, Aubourg L, Grangeponte MC, Chajes V, Bougnoux P. Anthracycline-induced cardiac toxicity is not increased by dietary omega-3 fatty acids. Pharmacol Res 2003 Feb;47(2):111-117.

(112) Shaikh IA, Brown I, Schofield AC, Wahle KW, Heys SD. Docosahexaenoic acid enhances the efficacy of docetaxel in prostate cancer cells by modulation of apoptosis: the role of genes associated with the NF-kappaB pathway. Prostate 2008 Nov 1;68(15):1635-1646.

(113) Blauwhoff-Buskermolen S, Versteeg KS, de van dS, den Braver NR, Berkhof J, Langius JAE, et al. Loss of Muscle Mass During Chemotherapy Is Predictive for Poor Survival of Patients With Metastatic Colorectal Cancer. J Clin Oncol 2016 04/20;34(12):1339-1344.

(114) Moro C, Bajpeyi S, Smith SR. Determinants of intramyocellular triglyceride turnover: implications for insulin sensitivity. Am J Physiol Endocrinol Metab 2008 02;294(2):E203-E213.

(115) Marchildon F, Lamarche É, Lala-Tabbert N, St-Louis C, Wiper-Bergeron N. Expression of CCAAT/Enhancer Binding Protein Beta in Muscle Satellite Cells Inhibits Myogenesis in Cancer Cachexia. PLoS ONE 2015 12/28;10(12):1-16.

(116) Lefterova MI, Zhang Y, Steger DJ, Schupp M, Schug J, Cristancho A, et al. RESEARCH PAPER:PPARγ and C/EBP factors orchestrate adipocyte biology via adjacent binding on a genome-wide scale. Genes Dev 2008 11;22(21):6-6.

(117) Hu E, Tontonoz P, Spiegelman BM. Transdifferentiation of Myoblasts by the Adipogenic Transcription Factors PPARγ and C/EBPα. Proc Natl Acad Sci U S A 1995(21):9856.

(118) Liu M, Montgomery MK, Fiveash CE, Osborne B, Cooney GJ, Bell-Anderson K, et al. PPARa-independent actions of omega-3 PUFAs contribute to their beneficial effects on adiposity and glucose homeostasis. Sci Rep 2014 07/02;4:5538-5538.

(119) Koopman R, Schaart G, Hesselink MK. Optimisation of oil red O staining permits combination with immunofluorescence and automated quantification of lipids. Histochem Cell Biol 2001 Jul;116(1):63-68.

(120) Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 1957 May;226(1):497-509.

(121) Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001 12;25(4):402-408.

(122) Lee S, Zhang J, Choi AMK, Kim HP. Mitochondrial Dysfunction Induces Formation of Lipid Droplets as a Generalized Response to Stress. doi:10 1155/2013/327167 2013.

(123) Sakamoto K. Oxidative stress induced lipid accumulation via SREBP1c activation in HepG2 cells. Biochem Biophys Res Commun 2008(4).

(124) Brey CW, Nelder MP, Hailemariam T, Gaugler R, Hashmi S. Krüppel-like family of transcription factors: an emerging new frontier in fat biology. Int J Biol Sci 2009 10/01;5(6):622-636.

(125) Chircop M, Speidel D. Cellular stress responses in cancer and cancer therapy. Front Oncol 2014 10/29;4:304-304.

(126) Grounds MD, Terrill JR, Radley-Crabb H, Robertson T, Papadimitriou J, Spuler S, et al. Lipid accumulation in dysferlin-deficient muscles. Am J Pathol 2014 06;184(6):1668-1676.

(127) Hu S, Yao J, Howe AA, Menke BM, Sivitz WI, Spector AA, et al. Peroxisome proliferator-activated receptor γ decouples fatty acid uptake from lipid inhibition of insulin signaling in skeletal muscle. Mol Endocrinol 2012 06;26(6):977-988.

(128) Todorcevic M, Hodson L. The Effect of Marine Derived n-3 Fatty Acids on Adipose Tissue Metabolism and Function. J Clin Med 2015 12/31;5(1).

(129) Cao S, Rustum YM. Synergistic antitumor activity of irinotecan in combination with 5-fluorouracil in rats bearing advanced colorectal cancer: role of drug sequence and dose. Cancer Res 2000 Jul 15;60(14):3717-3721.

(130) Conklin KA. Dietary polyunsaturated fatty acids: impact on cancer chemotherapy and radiation. Altern Med Rev 2002 02;7(1):4-21.

(131) Bordoni A, Biagi P, Hrelia S. The impairment of essential fatty acid metabolism as a key factor in doxorubicin-induced damage in cultured rat cardiomyocytes. BBA - Molecular and Cell Biology of Lipids 1999;1440:100-106.

(132) Calder PC. N-3 Polyunsaturated Fatty Acids, Inflammation, and Inflammatory Diseases. Am J Clin Nutr 2006 Jun;83(6 Suppl):1505S-1519S.

(133) White JP, Puppa MJ, Sato S, Gao S, Price RL, Baynes JW, et al. IL-6 regulation on skeletal muscle mitochondrial remodeling during cancer cachexia in the ApcMin/+ mouse. Skelet Muscle 2012 Jul 6;2:14-5040-2-14.

(134) White PJ, Arita M, Taguchi R, Kang JX, Marette A. Transgenic restoration of long-chain n-3 fatty acids in insulin target tissues improves resolution capacity and alleviates obesity-linked inflammation and insulin resistance in high-fat-fed mice. Diabetes 2010 Dec;59(12):3066-3073.

(135) McGlory C, Galloway SDR, Hamilton DL, McClintock C, Breen L, Dick JR, et al. Temporal changes in human skeletal muscle and blood lipid composition with fish oil supplementation. Prostaglandins, Leukotrienes and Essential Fatty Acids (PLEFA) 2014;90:199-206.

(136) Wronska A, Kmiec Z. Structural and biochemical characteristics of various white adipose tissue depots. Acta Physiologica 2012 06;205(2):194-208.

(137) Rouffet D, Villars C, Fissoune R, Sappey-Marinier D, Laville M, Ibarrola D, et al. Intramyocellular lipid variations in active older men: relationship with aerobic fitness. Acta Physiologica 2013 03;207(3):516-523.

(138) Aubrey J, Esfandiari N, Baracos VE, Buteau FA, Frenette J, Putman CT, Mazurak VC. Measurement of skeletal muscle radiation attenuation and basis of biological variation. 2013.

(139) Bloemberg D, Quadrilatero J. Rapid Determination of Myosin Heavy Chain Expression in Rat, Mouse, and Human Skeletal Muscle Using Multicolor Immunofluorescence Analysis. PLoS ONE 2012 04;7(4):1-11.

(140) Blanco CE, Sieck GC, Edgerton VR. Quantitative histochemical determination of succinic dehydrogenase activity in skeletal muscle fibres. Histochem J 1988 04;20(4):230-243.

(141) Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976 05/07;72:248-254.

(142) Bass A, Brdiczka D, Eyer P, Hofer S, Pette D. Metabolic Differentiation of Distinct Muscle Types at the Level of Enzymatic Organization. European Journal of Biochemistry 1969 11;10(2):198-206.

(143) Toth MJ, Callahan DM, Miller MS, Tourville TW, Hackett SB, Couch ME, et al. Skeletal muscle fiber size and fiber type distribution in human cancer: Effects of weight loss and relationship to physical function. Clin Nutr 2016 03/08.

(144) Grivicich I, Regner A, da Rocha A,B., Grass LB, Alves PAG, Kayser GB, et al. Irinotecan/5-fluorouracil combination induces alterations in mitochondrial membrane potential and caspases on colon cancer cell lines. Oncol Res 2005;15(7-8):385-392.

(145) Barreto R, Mandili G, Witzmann FA, Novelli F, Zimmers TA, Bonetto A. Cancer and Chemotherapy Contribute to Muscle Loss by Activating Common Signaling Pathways. Front Physiol 2016 10/19;7:472-472.

(146) Häggmark T, Thorstensson A. Fibre types in human abdominal muscles. Acta Physiol Scand 1979 12;107(4):319-325.

(147) Ebadi M. Adipose tissue alterations in an animal model of colorectal cancer. 2017.

(148) Weber M, Krakowski-Roosen H, Schroder L, Kinscherf R, Krix M, Kopp-Schneider A, et al. Morphology, metabolism, microcirculation, and strength of skeletal muscles in cancer-related cachexia. Acta Oncol 2009 01;48(1):116-124.

(149) Fearon K, Arends J, Baracos V. Understanding the mechanisms and treatment options in cancer cachexia. NATURE REV CLIN ONCOL 2013 2013;10(2):90-99 10p.

(150) Fearon KCH, Glass DJ, Guttridge DC. Cancer cachexia: mediators, signaling, and metabolic pathways. Cell Metab 2012 08/08;16(2):153-166.

(151) Penna F, Busquets S, Pin F, Toledo M, Baccino FM, Lopez-Soriano FJ, et al. Combined approach to counteract experimental cancer cachexia: eicosapentaenoic acid and training exercise. J Cachexia Sarcopenia Muscle 2011 Jun;2(2):95-104.

(152) Read JA, Beale PJ, Volker DH, Smith N, Childs A, Clarke SJ. Nutrition intervention using an eicosapentaenoic acid (EPA)-containing supplement in patients with advanced colorectal cancer. Effects on nutritional and inflammatory status: a phase II trial. Support Care Cancer 2007 Mar;15(3):301-307.

(153) Wigmore SJ, Fearon KC, Maingay JP, Ross JA. Down-regulation of the acute-phase response in patients with pancreatic cancer cachexia receiving oral eicosapentaenoic acid is mediated via suppression of interleukin-6. Clin Sci (Lond) 1997 Feb;92(2):215-221.

(154) Giles K, Guan C, Jagoe TR, Mazurak V. Diet composition as a source of variation in experimental animal models of cancer cachexia. J Cachexia Sarcopenia Muscle 2016 05;7(2):110-125.

(155) Giles K. Skeletal muscle fat infiltration is reversed by dietary fish oil in an animal model of colorectal cancer receiving irinotecan and 5-fluorouracilUniversity of Alberta; 2014.

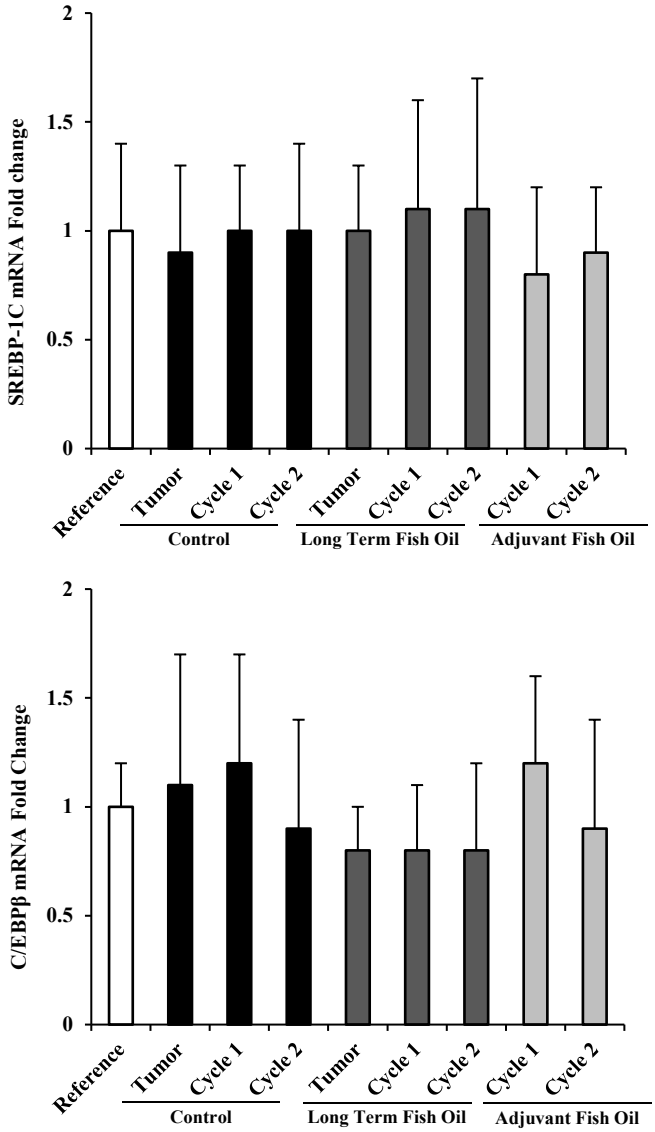
(156) Schrauwen-Hinderling VB, Hesselink MK, Schrauwen P, Kooi ME. Intramyocellular lipid content in human skeletal muscle. Obesity (Silver Spring) 2006 Mar;14(3):357-367.

(157) Corcoran MP, Lamon-Fava S, Fielding RA. Skeletal muscle lipid deposition and insulin resistance: effect of dietary fatty acids and exercise. Am J Clin Nutr 2007 03;85(3):662-677.

(158) Arnold A, Gill J, Christe M, Ruiz R, McGuirk S, St-Pierre J, et al. Morphological and functional remodelling of the neuromuscular junction by skeletal muscle PGC-1α. Nat Commun 2014 04/01;5:3569-3569.

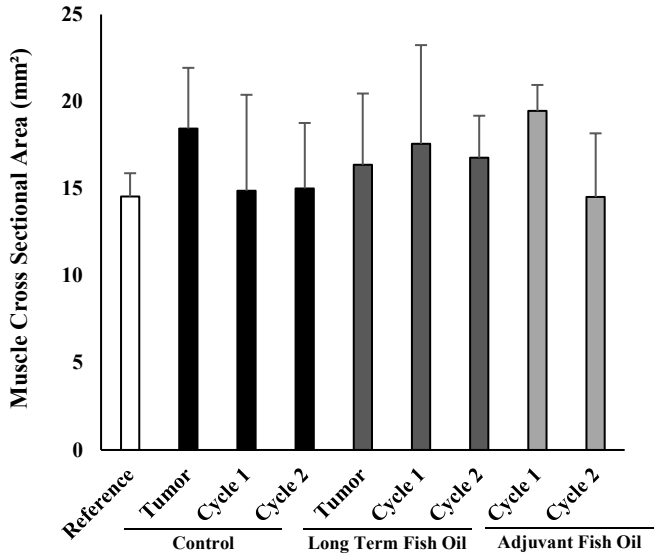
Appendix A

SREBP-1c and *C/EBPβ* expression in the rat gastrocnemius muscle



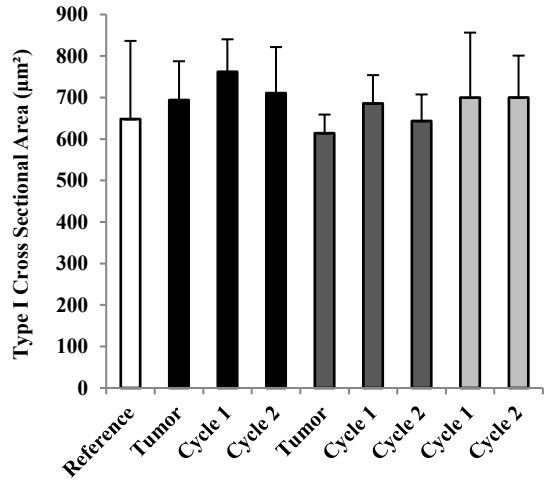
Appendix B

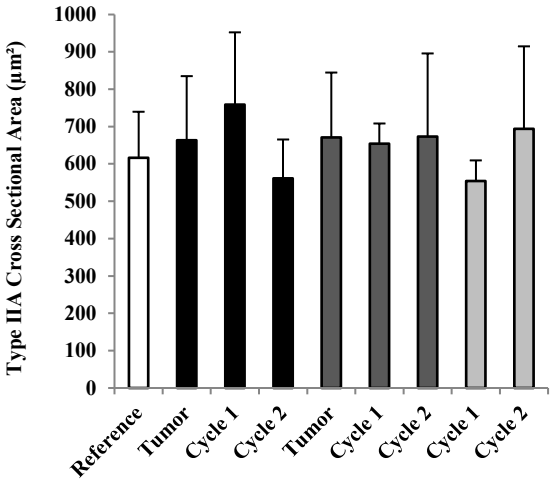
Tibialis anterior whole muscle cross sectional area of rats bearing a ward colon tumor and receiving CPT-11/5-FU fed a fish Oil diet either prior or following to tumor implantation

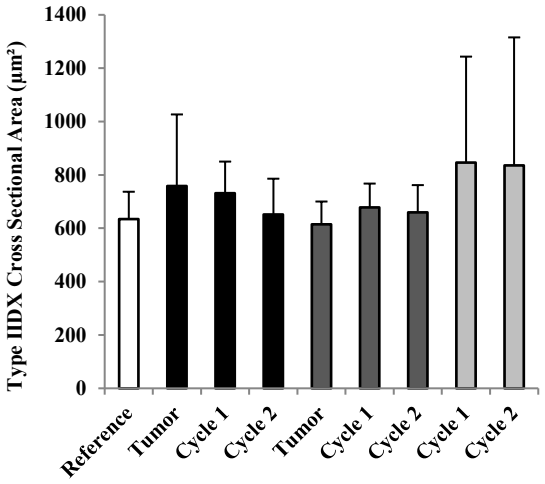


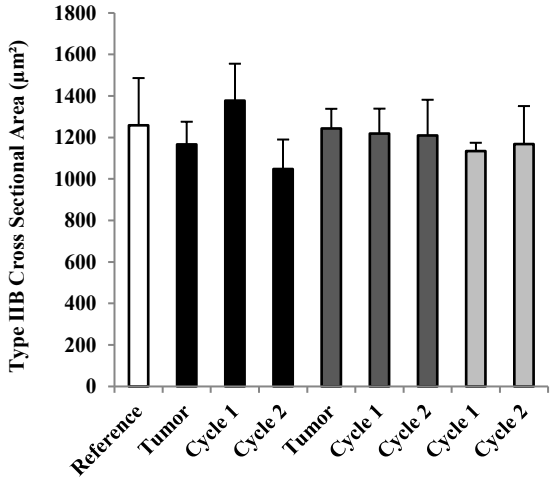
Appendix C

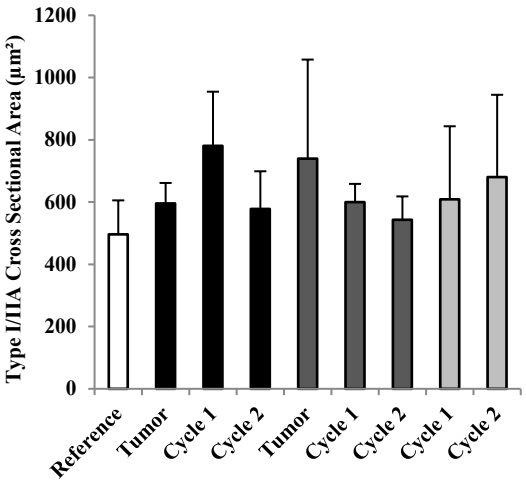
Tibialis anterior fiber types cross sectional area of rats bearing a ward colon tumor and receiving CPT-11/5-FU fed a fish oil diet either prior or following to tumor implantation

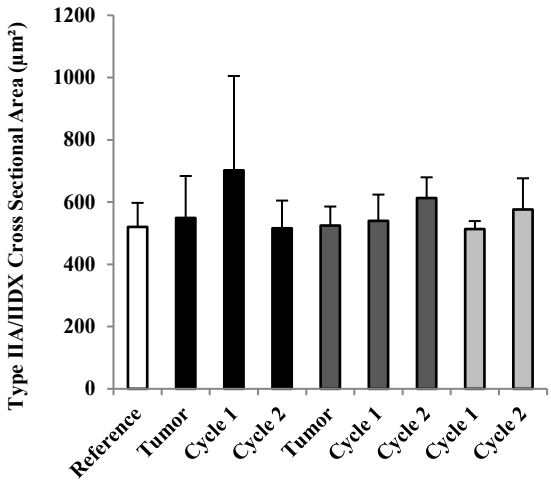









Type I/IIA Cross Sectional Area (µm²)
1200
1000
800
600
400
200
0
Reference
Tumor
Cycle 1
Cycle 2
Tumor
Cycle 1
Cycle 2
Cycle 1
Cycle 2


Type IIA/IIDX Cross Sectional Area (µm²)
1200
1000
800
600
400
200
0
Reference
Tumor
Cycle 1
Cycle 2
Tumor
Cycle 1
Cycle 2
Cycle 1
Cycle 2