University of Alberta

Elevated Fatty Acid Content in Muscle is Prevented by EPA and DHA in an Animal Model of Colorectal Cancer Receiving CPT-11 / 5-FU

by

Alaa Almasud

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science
in
Nutrition and Metabolism

Department of Agricultural, Food and Nutritional Science

Fall 2012
Edmonton, Alberta

Abstract

This study aimed to assess the effect of irinotecan / 5-fluorouracil treatment on amount and types of fatty acids in skeletal muscles of tumor-bearing rats fed: a) control diet, b) fish oil diet. Rats bearing the Ward colorectal carcinoma were fed a semi-purified diet with or without fish oil prior to receiving chemotherapy. Gastrocnemius muscles were isolated 7 days later. Lipids were extracted and triglycerides (TG) and phospholipid (PL) separated by thin layer chromatography, and fatty acids (FAs) quantified by gas liquid chromatography. Rats receiving chemotherapy exhibited the most TG-FA in muscle tissue. Rats fed the fish oil diet exhibited lower total muscle TG compared with other groups. N-3 FA content in muscle TG and PL were higher in the fish oil group compared to other groups. This study suggests that fish oil modifies the fat accumulation in muscle that occurs during chemotherapy.

Acknowledgements

I would like to thank God for this accomplishment. This thesis is dedicated to the King of Saudi Arabia, king Abdullah bin Abdulaziz who has given me the opportunity for education abroad. A special thanks to the Saudi Arabian Cultural Bureau in Canada that facilitated everything from the first day I arrived to Canada until my graduation. I dedicate this achievement to my beloved country, Saudi Arabia.

From 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 master program at U of A.

This thesis would not have been possible unless my lovely husband shared with me the entire project and supported me throughout the whole process. Thank you sweetheart for offered me unconditional love and support since I met you in Jan, 2010. I really appreciate your caring and encouragement.

To my Mom, you are the reason behind my success as you are always my inspiration. To my brothers, Ahmed, Rayan, Ali, Ra'ad, and Mohammed thanks for your love, support, and everything you have provided to support me while I am here in Canada.

My deepest appreciation is extended to my committee members Dr Vickie Baracos and Dr Thomas Clandinin for providing valuable comments and suggestions to improve my research project. I would like to thank my external

examiner Dr Ted Putman, for providing feedbacks for my research, which will help me to improve.

Kelly and Abha I really appreciate your technical assistance without your help this research would not be possible. A special thanks as well to my friends Abeer, Maryam and Asha for all the nice time that we shared since I started my master program. Also, I would like to thank Taylor for answering all my questions while I was working in the lab.

Dalia and Aisha I will never forget our time together in Toronto. Thanks for all your support.

To each of the above, I extend my deepest appreciation.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

Δ	delta
5-FUl	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
BF_3	boron trifluoride
$CaCl_2$	calcium chloride
CMO	rats receiving CPT-11/5-FU and fed control diet
CPT-11	irinotecan
CT	computed tomography image
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
EFAs	essential fatty acids
EPA	eicosapentaenoic
FFA	free fatty acids
FO	rats receiving CPT-11/5-FU and fed fish oil diet
GLUT4	glucose transporter 4
HDL	high density lipoprotein

HSL	hormone sensitive lipase
IMCL	intramuscular lipid content
KOH	potassium hydroxide
LA	linoleic acid
LDL	low density lipoprotein
LPL	lipoprotein lipase
mL	milliliter
MUFA	monounsaturated fatty acids
ND	not detected
n-3 FA	n-3 fatty acid
PCR	polymerase chain reaction
PL	phospholipid
PPAR-α	peroxisome proliferator-activated receptor alpha
PUFA	polyunsaturated fatty acids
REF	healthy rats fed a control diet
SFA	saturated fatty acid
SN38	7-ethyl-10-hydroxycamptothecin
SPSS	Statistical Package for the Social Sciences
SREBF	sterol regulatory element binding transcription
SREBP1-c	sterol regulator element binding protein factor 1
TG	triglyceride
TMN	tumor; node; metastases
TNF-α	tumor necrosis factor-alpha
TUM	untreated rats with colon tumor fed a control diet

VLDL	very low density lipoprotein
μg	microgram
μL	microlitre

Chapter 1
Introduction and Literature Review

1.1 Colorectal Cancer

The number of new colorectal cancer cases and deaths continue to rise as the Canadian population ages. In Canada, there are approximately 22,200 diagnoses and 8,900 deaths reported from colorectal cancer each year (Canadian Cancer Statistics, 2011).Significant weight loss has been observed in patients with advanced colorectal cancers. Specifically, weight loss of greater than five percent of the normal body weight, known as cachexia, has been associated with poor quality of life, decreased response to treatment and mortality (Dewys et al., 1980). Colorectal cancer is diagnosed at one of five stages. This staging system ranges from 0 to IV, indicating the severity of the disease (Edge et al., 2010). 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 (Kinzler et al., 2002). 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 the advanced stages of cancer, the median survival duration is 5–6 months (Van Cutsem & Geboes, 2007) and combination treatment may be provided. This treatment includes options such as surgical resection, chemotherapy, radiation therapy and biotherapy or immunotherapy (Howlader et al., 2011; Kinzler et al., 2002).

1.2 Colorectal Cancer Chemotherapy

Chemotherapy remains an essential step in the treatment of metastatic 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 (Board et al., 2007). The combination of 5-fluorouracil (5-FU) and irinotecan (CPT-11) represents an effective combination of drugs used to treat colorectal cancer patients (Douillard et al., 2000; Saltz et al., 2000).

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. Furthermore, 5-FU is still the most common drug used to treat colorectal cancer (Malet-Martino et al., 2002). 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 as well as it could convert to fluorouridine triphosphate which causes RNA damage and produces apoptosis in the tumor (Longley et al., 2003). 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 (Tejwani et al., 2008). The overall response rate for 5-FU as a single agent in metastatic colorectal cancer is <10% (Bleiberg, 1997); however, when 5-FU is combined with other drugs, such as CPT-11, the rate of response is significantly increased to 40–50% (Saif & Cohenuram, 2006).

Irinotecan is a water-soluble semi-synthetic that is isolated from the Chinese/Tibetan ornamental tree *Camptotheca Acuminata* (Wall et al., 1966). In 1983, 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 (Xu et al., 2002). 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 (Rothenberg, 2001). Since its 1996 approval in the United States (Köhne et al., 2009), irinotecan has emerged as one of the most effective antitumor drugs for a wide range of tumor types, especially when combined with 5-FU (Douillard et al., 2000; Saltz et al., 2000). However, diarrhea and myelosuppression remain common dose-limiting toxicities of this antitumor drug (Rothenberg, 2001).

1.3 Cancer is Associated with Cachexia

Cachexia is a wasting syndrome involving the loss of skeletal muscle with or without the loss of adipose tissue (Evans et al., 2008; Fearon et al., 2011). This syndrome is characterized by anorexia and premature satiety, as well as by the dysfunction of lipid, protein and carbohydrate metabolism and hypercatabolism. Cachexia, which results either directly from various tumor factors or indirectly from an irregular host response to a tumor, is associated with poor quality of life, reduced response to chemotherapy and severe toxicity. Furthermore, cachexia may be the cause of death in a significant proportion of cancer patients (Tisdale, 2002).

1.4 Loss of Skeletal Muscle is a Key Feature of Cachexia

Severe skeletal muscle wasting is the hallmark feature of cachexia (Evans et al., 2008). Skeletal muscle mass that is reduced more than two standard deviations (SD) below that of healthy adults is defined as sarcopenia (Janssen et al., 2002), which was first described in the elderly (Baumgartner et al., 1998) and has recently been revealed in advanced lung (Murphy et al., 2010) and colorectal cancer patients (Lieffers, 2009). Sarcopenia is characterized by decreased muscle strength and function (Janssen et al., 2002), impaired mobility, reduced response to treatment, increased length of hospital stay and mortality (Lieffers et al., 2009).

The balance between protein synthesis and protein catabolism is important for muscle health. In healthy individuals, the process of fasting results in the catabolism of muscle proteins to provide amino acids for gluconeogenesis. During starvation, protein catabolism is reduced to preserve muscle mass by utilizing the

fat stores in the body. This essential process of conserving nitrogen during the reduction of food intake appears absent in cancer patients experiencing cachexia (Josep et al., 2005; Whitehouse et al., 2001). However, the cause of increased muscle catabolism and decreased muscle anabolism is not well understood. Several lines of evidence maintain that protein catabolism results from the presence of pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α). These cytokines play a role in enhancing muscle breakdown both in vivo and in vitro studies of cancer cachexia (Josep et al., 2005). In addition, the Cori cycle may contribute to increased muscle catabolism in cancer patients. This cycle consists of a metabolic pathway involving two inverse processes: the conversion of lactate to glucose and the transformation of glucose back to lactate. The synthesizing of lactate to glucose requires the input of energy. In tumor-bearing patients, the conversion rate of lactate to glucose increases, which may result in increased energy expenditure by these patients (Gold, 1974).

Chemotherapy is often used to treat cancer patients. However, a recent study reported that in comparison to non-sarcopenic patients, cancer patients with sarcopenia experience poor outcomes during chemotherapy treatment as well as a more rapid progression of tumor development (Prado et al., 2009). This phenomenon may result in the reduction of chemotherapy drugs or the termination of therapy.

1.5 Lipid Types and Functions

Lipid 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. Specifically, fat is crucial for brain development and functioning (Robert et al., 1994) as well as for cell membrane properties such as phospholipids (PL) and sterols (Hulbert et al., 2005). Lipid stimulates the release of hormones and cytokines as signaling molecules and it fulfill crucial functions as enzyme cofactors, electron carriers and intracellular messengers. There are several different types of fatty acids (FAs), including saturated with no double bonds (SFA), monounsaturated with one double bond (MUFA) and polyunsaturated with two or more double bonds (PUFA). In PUFAs, the number of carbon atoms and the position of the double bonds have a major effect on their physical properties (Hulbert et al., 2005; Nelson & Cox, 2008).

1.6 Essential Fatty Acids (EFAs)

Humans and animals can synthesize SFAs and MUFA 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 (ALA) 18:3n-3 and linoleic acid (LA) 18:2n-6 must be obtained from diet (Pawlosky et al., 1994).

Humans can synthesize eicosapentaenoic acid (EPA) 20:5n-3 and docosahexaenoic acid (DHA) 22:6-n-3 and AA from parent FAs ALA and LA respectively (see Figure 1-1). One recent study demonstrates that the conversion rate of ALA to EPA is approximately 5%, while the conversation rate from ALA

to DHA is less than 0.5% (Plourde et al., 2007). Thus, it has been suggested that EPA and DHA should be considered as essential FAs, especially in specific circumstances, such as the premature infants (Clandinin et al., 1997).

Rich sources of n-6 PUFA include nuts, seeds and vegetable oils such as corn, sunflower, soy and cottonseed. The n-3 FAs can be found in plant sources such as flaxseeds, walnuts and canola oils. The greatest amounts of DHA and EPA are found in fatty fish (Hulbert et al., 2005; Simopoulos et al., 1986), but sources of these FAs can also exist in egg yolk and meat, depending on the animals' diet (Simopoulos, 1989).

1.7 EPA and DHA Deficiency

Deficits of essential fatty acids are caused by a low dietary intake of ALA, EPA and DHA or by problems in the metabolic pathways, such as a fat digestion disorder, malabsorption or insufficient secretion of pancreatic lipase, which is essential in fat digestion. In the past, the recommended dietary ratio of n-6: n-3 was 4:1, whereas current dietary intake patterns are characterized by ratios that range from 10:1 to 20–25:1 (De Gomez & Brenner, 1975; Hulbert et al., 2005). For instance, DHA, which is one part of the n-3 family, is important for the human brain, so this ratio may influence the function of the brain (Haag, 2003; Robert et al., 1994).

The n-6 and n-3 acids utilize the same delta-6 (Δ-6) desaturase enzyme. Thus, a high intake of n-6 PUFAs indicates that fewer enzymes are available for n-3 PUFAs due to the saturation of enzyme complexes with high intakes of n-6.

Specifically, a high dietary intake of n-6 PUFAs can result in the decreased synthesis of long chain n-3 PUFAs in the body. Studies demonstrate that the ALA conversion rate in the body to EPA and DHA is low because of the small proportions of ALA in the diet. Moreover, high amounts of n-6 PUFAs from food intake would further reduce the conversion of ALA to EPA and DHA (Haag, 2003; Plourde & Cunnane, 2007). Researchers suggest that the levels of n-6 and n-3 as well as the balance between these acids may play a critical role in the prevention and treatment of chronic diseases, including immune disorders, inflammatory illnesses and cancers (Horrobin, 1993). These findings require consideration when making dietary recommendations for diabetes, cardiovascular and cancer patients.

1.8 Lipid Metabolism in Cancer Cachexia with Skeletal Muscle Loss

Human cancer cachexia is characterized by altered lipid metabolism. In comparison to non-cachectic patients, cachectic patients experience elevated lipolytic activity (Agustsson et al., 2007), which results in the elevated plasma levels of free fatty acids (FFA) and glycerol (Fiorenza et al., 2000; Tisdale, 2004). Furthermore, in advanced cancer patients, high-density lipoprotein cholesterol (HDL) and low-density lipoprotein (LDL) cholesterol concentrations are reportedly decreased (Fiorenza et al., 2000). This pattern may suggest a dysfunction of lipid absorption, chylomicron particles or very low density lipoprotein (VLDL) synthesis from the liver, which subsequently affects HDL and LDL levels. These changes may result from decreased lipoprotein lipase (LPL)

activity (Thompson et al., 1981) and increased hormone sensitive lipase (HSL) activity (Thompson et al., 1993).

In advanced cancer patients, a pathological fat accumulation in muscle, known as myosteatosis, has recently been observed (Murphy et al., 2010, 2011; Stephens et al., 2011). Myosteatosis has been discussed in conjunction with obesity (Van Loon et al., 2004), diabetes (Miljkovic et al., 2010), and some forms of muscle atrophy, such as aging (Baumgartner et al., 1998). However, myosteatosis has not been fully examined in cancer patients. A recent study of advanced cancer patients reported that weight loss is associated with an increase in the size and number of lipid droplets in muscle (Stephens et al., 2011). However, since this phenomenon has been also observed in obese patients (Van Loon et al., 2004), these findings suggest that muscle fat accumulation may not be related to positive or negative energy homeostasis; rather, this accumulation might relate to the underlying causes of an energy imbalance, including catabolic hormonal mediators such as pro-inflammatory cytokine and insulin resistance (Baracos et al., 2006).

The unresponsiveness of skeletal muscle to insulin is defined as insulin resistance. This condition is characterized by hyperinsulinemia, enhanced hepatic gluconeogenesis, and impaired insulin-stimulated glucose uptake into skeletal muscle. Specifically, insulin stimulates glucose uptake into skeletal muscle via mobilized vesicles containing glucose transporter 4 (GLUT4) through an elaborate signal transduction cascade to the muscle surface (Nelson & Cox, 2008).

In cancer, adipose tissue loses its sensitivity to the insulin-mediated suppression of lipolysis. Also, adipose tissue becomes activated to initiate pro-inflammatory cytokines such as TNF-α and IL-6 (Ryden et al., 2008), which cause an increase in plasma free fatty acids (FFA) (Bing et al., 2008). Increased levels of FFA in the blood may result in increased amounts of lipid stored in non-adipose tissue, such as skeletal muscle (Bertrand et al., 1978, 1980; Gregoire et al., 1998). However, the specific ways in which changes in lipid metabolism affect insulin signaling requires further examination.

White (2010) has examined the relationship between the oxidative capacity of muscles and depleted muscle mass, or sarcopenia, during the development of cancer cachexia. In this study, gastrocnemius and soleus muscle were collected from a cancer cachexia model ($Apc^{Min/+}$ mouse) at 20 weeks of age. Subsequently, the gastrocnemius muscle was separated into red and white fibers. Mice were classified as having mild, moderate or severe cachexia based on their body weight, gastrocnemius muscle mass and epididymal fat pad mass. When these variables were within 1 SD of the mean of matched WT mice, the mice were classified as having mild cachexia. If the variables were more than 2 SDs away from the mean, the mice were considered as having severe cachexia. Blood samples were collected to measure plasma IL-6. The mitochondria DNA of gastrocnemius muscle were assessed by using the quantitative real-time PCR. This study demonstrated that the amount of the mitochondria is reduced which may cause a decrease in muscles oxidative capacity in both red and white fibers because of a five-fold increase in IL-6, which was associated with insulin

resistance in severely cachectic mice, especially in comparison to mildly cachectic and healthy mice. Furthermore, during cancer cachexia, the gastrocnemius muscle mass was decreased by 41% and the soleus muscle mass was decreased by 34%, both of which were related to increasing IL-6 levels (White et al., 2010). The decreased muscle oxidative capacity in this study suggests that increased IL-6 may relate to mitochondria dysfunction. Lipids stored in muscle usually serve as a fuel for mitochondria oxidation (Hoppeler et al., 1973), suggesting that a decrease in the size or number of mitochondria may cause lipid accumulation inside of the muscle by insufficient oxidation of FFA. Thus, IL-6 may be associated with increased adipose tissue lipolysis as well as with enhanced IMCL and decreased muscle mass.

Several transcription factors, including peroxisome proliferator-activated receptor gamma (PPARγ), CCAAT/enhancer binding proteins (C/EBPs), such as C/EBPσ, C/EBPβ and C/EBPα, as well as sterol regulatory element binding protein 1c isoform (SREBP-1c), play essential roles in adipogenesis (Kawai et al., 2007). An understanding of adipocyte differentiation may help to gain insight into the elevated fat content of muscles observed in cancer. Bing et al. (2006) showed that the white adipose tissue of tumor-bearing mice experienced a major reduction in mRNA levels and in the protein content of adipogenic transcription factors, including C/EBPα, C/EBPβ and PPAR-γ, as well as GLUT-4 and leptin. The release of leptin from adipose tissue is responsible for stimulating adipocyte differentiation and B-oxidation outside adipose cells, especially in the skeletal muscle and liver (Bing et al, 2006). Decreased leptin levels may contribute to

lipid storage in non-adipose tissues, which has a detrimental effect on health. For instance, decreased insulin response in skeletal muscle increases fat storage and reduces muscle strength, hence resulting in muscle dysfunction and limited movement (Wang et al., 2001).

Furthermore, chemotherapy may play a role in increased IMCL, as recent findings using computed tomography (CT) images found that in non-small cell lung cancer patients receiving chemotherapy, the intramuscular adipose tissue, compared to the visceral and subcutaneous adipose tissues, is the only fat depot that increases at end of life (Murphy et al., 2010). However, the mechanism behind this increase in IMCL during chemotherapy is not defined.

1.9 EPA and DHA Fatty Acids Status in Cancer Patients

Several studies suggest that there are abnormalities in the FA metabolism for advanced cancer patients with wasting syndrome (Michael, 1999; Pratt, 2002). These alterations in FA metabolism may cause a reduction in the availability of n-3 and n-6 FAs within the body, which may enhance the cachectic condition. Pratt et al. (2002) showed that in comparison to healthy subjects, advanced cancer patients who lost five percent or more of their pre-illness weight had depleted stores of plasma EFA within PL fractions. In fact, this phenomenon was even more evident after high-dose chemotherapy, when DHA and EPA levels were reduced to about 7% of the control values (Pratt et al., 2002). Clinical evidence suggests that the n-3 PUFA status of newly-diagnosed cancer patients undergoing chemotherapy is low. Therefore, both the disease as well as the therapeutic treatments for it may be contributing factors in the decrease of n-3 PUFA status

(Baracos et al., 2004). Another study purported to investigate the way in which FA patterns of mucosa are altered in human gastric cancer. Specifically, researchers analyzed the total FAs and evaluated their relative composition among the total FAs in mucosa as well as in the PL FAs contained in paired cancerous and non-cancerous gastric tissues. As a result, this study showed that the n-6:n-3 FA ratio of PL FA was higher in cancerous mucosa mainly due to high levels of AA and low levels of DHA and EPA in both total FAs and in PL FAs (Ahn et al., 2001). A recent study by Murphy (2010) reported a clear relationship between muscle mass and plasma PL n-3 FAs in non-small cell lung cancer patients. In particular, this study found that in comparison with non-sarcopenic patients, patients with sarcopenia had the lowest concentration of n-3 FA, including EPA and DHA (Murphy et al., 2010). However, the alteration mechanism of essential FAs in cancer patients remains unknown.

1.10 The Effect of EPA and DHA n-3 PUFA on Cancer Patients

Evidence from epidemiological studies suggests that diets rich in n-3 PUFA might be associated with reduced cancer risk (Colomer et al., 2007). According to Cave et al. (1991), high n-3 FA inhibits tumor growth while diets rich in n-6 stimulate mammary metastases as well as tumor growth and development (Cave et al., 1991).

In addition, studies have reported that n-3 FAs have an anti-cachectic effect. Advanced cancer patients with weight loss received 14 days of fish oil supplementation, which caused increased levels of EPA and DHA in the plasma PL of these patients in comparison with the control group. Specifically, enhanced

plasma EPA was associated with increased body weight in those subjects (Pratt et al., 2002). Wigmore (2006) reported that dietary supplementation with EPA and DHA n-3 PUFAs reduced weight loss, increased appetite and improved quality of life in patients with cancer cachexia (Wigmore et al., 2006). In particular, EPA was associated with inhibited body weight loss and lipolysis in cachectic mice bearing a colon tumor as compared with a control group (Tisdale, 1996). However, in a different case, such as that of obese patients, fish oil is known to have an anti-adipogenic function. In 2007, a group of researchers investigated the effect of an EPA in 3T3-L1 fat cells on lipid metabolism and lipolysis. This study demonstrated that the amount of glycerol and FFA released into a medium from cells treated by EPA was higher than that in the control 3T3-L1 adipocytes. In addition, intracellular lipid accumulation significantly decreased in comparison to the control cells. This study also found that PPAR-γ, which is essential for adipocyte development, was downregulated by EPA. These results suggest that EPA may inhibit fat cell development and stimulate lipolysis (Lee et al., 2008). Perez-Matute (2007) assessed the effect of EPA on adipose tissue and weight gain in rats fed either control diets of chow or high-fat diets of cafeteria food. Accordingly, the animals were divided into four groups: control diet, high-fat diet, control diet with EPA, and high-fat diet with EPA. During the study, the WAT functions, including lipolysis, apoptosis, gene secretion, and insulin resistance, were measured. In comparison to the other groups, the rats that ate a high-fat diet experienced more weight gain and accumulated more fat mass. Moreover, this group had a higher TNF-α level. However, the rats that ate a high-fat diet with

EPA experienced less weight gain and decreased food intake, which was related to decreased leptin production from adipose tissues, especially in comparison to the rats without EPA (Perez-Matute et al., 2007).

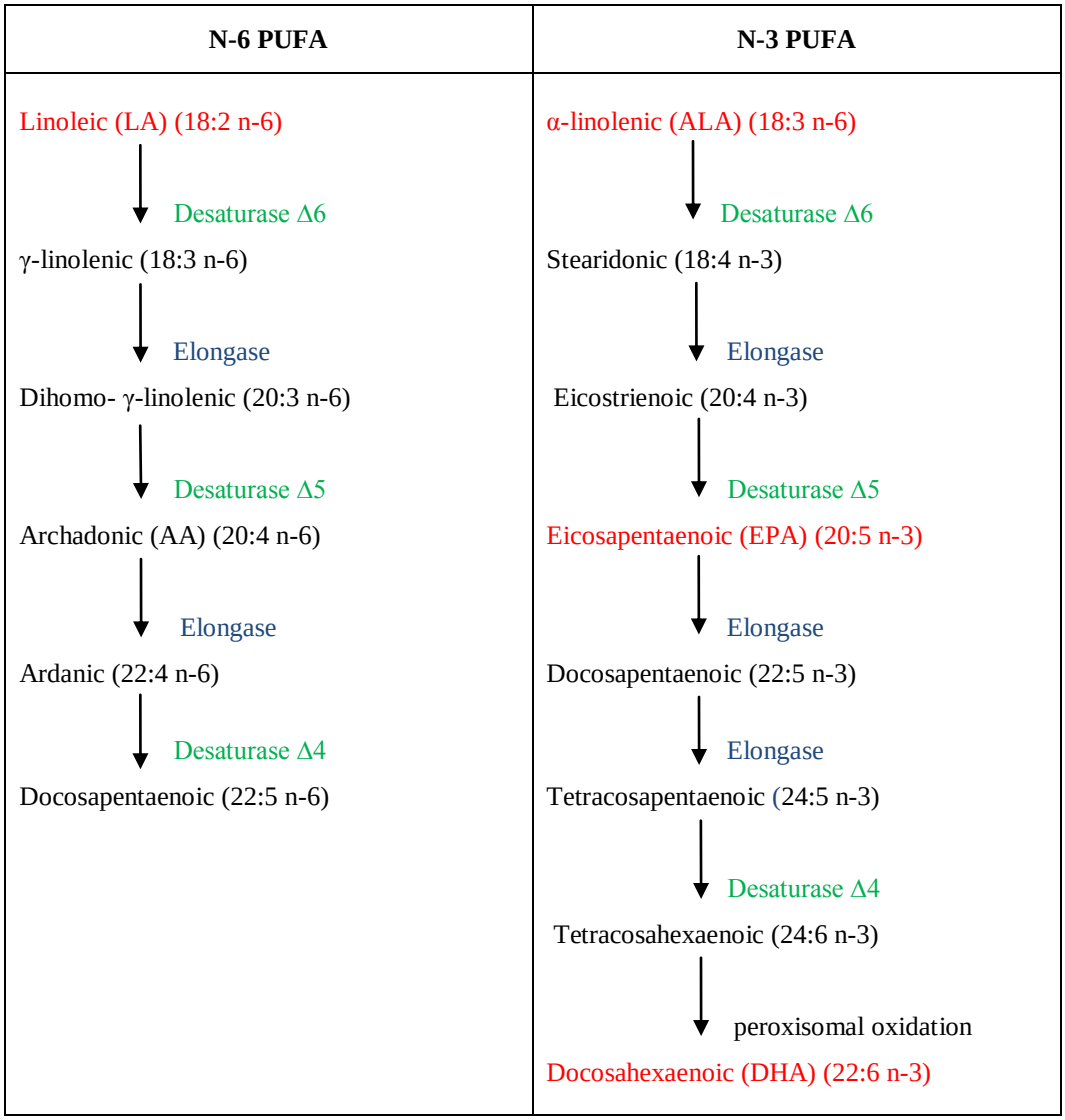
These studies suggest that EPA acts as anti-obesity and anti-cachectic factor. Therefore, EPA may have an essential role in not only regulating body weight but also in controlling energy balance. However, the operation of these mechanisms, including the effect of EPA on adipogenesis alteration, lipolysis and apoptosis, remains unclear.

In vitro and animal studies demonstrate a relationship between n-3 PUFA and cancer chemotherapy. Several experimental studies have reported that chemotherapy with fish oil enhances tumor cell death and reduces the toxic side effects to the host tissues (reviewed by Baracos et al., 2004). N-3 PUFA may interfere with the catabolic signal transduction pathways implicated in cancer cachexia and reduce the loss of lean body mass in advanced cancer patients. Murphy (2010) reported that in comparison to a control group, non-small cell lung cancer patients that supplemented chemotherapy with 2.2g/day EPA maintained their body weight, gained muscle mass and exhibited decreased IMCL (Murphy et al., 2010, 2011). However, the mechanisms regulating the way in which EPA and DHA affect the body weight, muscle mass, and IMCL of cancer patients during chemotherapy require further examination.

1.11 Summary

Cancer cachexia is a common syndrome in advanced cancer patients. Patients with cancer cachexia experience reduced body weight and muscle mass, increased IMCL, additional side effects from chemotherapy, higher metabolic rates, and poor quality of life with limited survival. Fish oil containing EPA and DHA has been reported to reduce many of these symptoms. However, the specific details of these mechanisms are not entirely clear, and therefore, further investigations need to be conducted in this area. EPA and DHA could utilize to reduce the cachexia syndrome and to offer an anticancer therapy for cachexia patients.

Figure 1-1. Desaturation and Elongation of n-6 and n-3



The red colors are the essential PUFA from n-6 and n-3 families. This figure presents the elongation and desaturation pathway of AA, EPA, and DHA from their parent LA and ALA.

References: Chapter 1

Agustsson, T., Ryden, M., Hoffstedt, J., van Harmelen, V., Dicker, A., Laurencikiene, J., . . . Arner, P. (2007). Mechanism of increased lipolysis in cancer cachexia. *Cancer Research, 67*(11), 5531–5537.

Ahn, J., Park, I. S., Lee, S. K., Kim, S. Y., Chung, E. J., Kim, J., . . . Lee-Kim, Y. C. (2001). Fatty acid patterns in gastric mucosa of stomach cancer patients. *Yonsei Medical Journal, 42*(2), 20–226.

Argilés, J. M., Sílvia, B., Antonio, F., & Francisco, J. L. (2005). Molecular mechanisms involved in muscle wasting in cancer and ageing: Cachexia versus sarcopenia. *International Journal of Biochemistry and Cell Biology, 37*, 1084–1104.

Baracos, V. E., Mazuak, V. C., & Ma, D. W. L. (2004). N-3 polyunsaturated fatty acids throughout the cancer trajectory: Influence on disease incidence, progression, response to therapy and cancer- associated cachexia. *Nutrition Research Reviews, 17*(2), 177–192.

Baracos, V. E. (2006). Cancer-associated cachexia and underlying biological mechanisms. *Annual Review of Nutrition, 26,* 435–461.

Barber, M. (2001). Cancer cachexia and its treatment with fish-oil-enriched nutritional supplementation. *Nutrition, 17*(9), 751–755.

Barber, M., Fearon, K., Tisdale, M., McMillan, D., & Ross, J. (2001). Effect of a fish oil-enriched nutritional supplement on metabolic mediators in patients with pancreatic cancer cachexia. *Nutrition & Cancer, 40*(2), 118–124.

Barber, M., Ross, J., Voss, A., Tisdale, M., & Fearon, K. (1999). The effect of an oral nutritional supplement enriched with fish oil on weight-loss in patients with pancreatic cancer. *British Journal of Cancer, 81*(1), 80.

Baumgartner, R. N., Koehler, K. M., Gallagher, D., Romero, L., Heymsfield, S. B., Ross, R. R., Garry, P. J., & Lindeman, R. D. (1998). Epidemiology of sarcopenia among the elderly in New Mexico. *American Journal of Epidemiology, 147*(8), 755–763.

Bertrand, H., Masoro, E., & Yu, B. (1978). Increasing adipocyte number as the basis for perirenaldepot growth in adult rats. *Science, 201*(4362), 1234–1235.

Bertrand, H. A., Lynd, F. T., Masoro, E. J., & Yu, B. P. (1980). Changes in adipose mass and cellularity through the adult life of rats fed ad libitum or a life-prolonging restricted diet. *Journal of Gerontology, 35,* 827–835.

Bing, C., & Trayhurn, P. (2008). Regulation of adipose tissue metabolism in cancer cachexia. *Current Opinion in Clinical Nutrition & Metabolic Care, 11*(3), 201–207.

Bleiberg, H. (1997). Colorectal cancer-is there an alternative to 5-FU? *European Journal of Cancer, 33*(4), 536–541.

Board, R. E., & Valle, J. W. (2007). Metastatic colorectal cancer: current systemic treatment options. *Drugs, 67*(13), 1851–1867.

Canadian Cancer Society's Steering Committee. (2011). *Canadian Cancer Statistics 2011.* Toronto, Ontario, Canada: Author.

Cave, W. (1991). Dietary n-3 (omega-3) polyunsaturated fatty acid effects on animal tumorigenesis. *FASEB Journal: Official Publication of The Federation of American Societies for Experimental Biology, 5*(8), 2160–2166.

Clandinin, M., Van Aerde, J., Parrott, A., Field, C., Euler, A., & Lien, E. (1997). Assessment of the efficacious dose of arachidonic and docosahexaenoic acids in preterm infant formulas: Fatty acid composition of erythrocyte membrane lipids. *Pediatric Research, 42*(6), 819–825.

Colomer, R., Moreno-Nogueira, J. M., Garcia-Luna, P. P., Garcia-Peris, P., Garcia-de-Lorenzo, A., Zarazaga, A., . . . Casimiro, C. (2007). N-3 fatty acids, cancer and cachexia: A systematic review of the literature. *British Journal of Nutrition, 97*(5), 823–831.

De Gomez, I. N. T., & Brenner, R. R. (1975). Oxidative desaturation of alpha-linolenic, linoleic, and stearic acid by human liver microsomes. *Lipids, 10(6),* 315–317.

Dewys, W. D., Begg, C., Lavin, P. T., Band, P. R., Bennett, J. M., Bertino, J. R., . . . Tormey, D. C. (1980). Prognostic effect of weight loss prior to chemotherapy in cancer patients. *The American Journal of Medicine, 69*(4), 491–497.

Douillard, J. Y., Cunningham, D., Roth, A. D., Navarro, M., James, R. D., Karasek, P., . . . Rougier, P. (2000). Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicentre randomized trial. *Lancet, 355*(9209), 1041–1047.

Edge, S., & Compton, C. (2010). The American Joint Committee on Cancer: The 7th edition of the AJCC cancer staging manual and the future of TNM. *Annals of Surgical Oncology, 17*(6), 1471–1474.

Erickson, K. L., & Hubbard, N. E. (1990). Dietary fat and tumor metastasis. *Nutrition Reviews, 48*(1), 6–14.

Evans, W. J., Morley, J. E., Argilés, J., Bales, C., Baracos, V., Guttridge, D., . . . Anker, S. D. (2008). Cachexia: A new definition. *Clinical Nutrition, 27*(6), 793–799.

Fearon, K., Strasser, F., Anker, S. D., Bosaeus, I., Bruera, E., Fainsinger, R. L., . . . Mantovani, G. (2011). Definition and classification of cancer cachexia: An international consensus. *Lancet Oncology, 12*(5), 489–495.

Fearon, K. C. H., von Meyenfeldt, M. F., Moses, A. G. W., van Geenen, R., Roy, A., Gouma, D. J., . . . Tisdale, M. J. (2003). Effect of a protein and energy dense n-3 fatty acid enriched oral supplement on loss of weight and lean tissue in cancer cachexia: A randomised double blind trial. *Gut, 52*(10), 1479–1486.

Fiorenza, A., Branchi, A., & Sommariva, D. (2000). Serum lipoprotein profile in patients with cancer: A comparison with non-cancer subjects. *International Journal of Clinical & Laboratory Research, 30*(3), 141–145.

Gogos, C. A., Ginopoulos, P., Salsa, B., Apostolidou, E., Zoumbos, N. C., & Kalfarentzos, F. (1998). Dietary omega-3 polyunsaturated fatty acids plus vitamin E restore immunodeficiency and prolong survival for severely ill patients with generalized malignancy: A randomised controlled trial. *Cancer,* 82 (2), 395–402.

Gold, J. (1974). Cancer cachexia and gluconeogenesis. *Annals of The New York Academy of Sciences, 230,* 103–110.

Gregoire, F. M., Smas, C. M., & Sul, H. S. (1998). Understanding adipocyte differentiation. *Physiological Reviews, 78(3),* 783–809.

Haag, M. (2003). Essential fatty acids and brain. *Canadian Journal of Psychiatry, 48*(3), 195–203.

Hoppeler, H., Luthi, P., Claassen, H., Weibel, E. R., & Howald, H. (1973). The ultrastructure of the normal human skeletal muscle: A morphometric analysis on untrained men, women and welltrained orienteers. *Pflugers Archiv: European Journal of Physiology, 344*(3), 217–232.

Horrobin, D. F. (1993). Omega-6 and omega-3 essential fatty acids in atherosclerosis. *Thieme E-Journals: Seminars in Thrombosis and Hemostatis, 19*(2), 129–137.

Howlader, N., Noone, A. M., Krapcho, M., Neyman, N., Aminou, R., Altekruse, S. F., . . . Cronin, K. A. (Eds.). (2011). Surveillance epidemiology and end result. *Cancer Statistics Review, 1975-2009 (Vintage 2009 Populations)* [based on November 2011]. Retrieved from http://seer.cancer.gov/csr/1975_2009_pops09/

Hulbert, A. J., Turner, N., Storlien, L. H., & Else, P. L. (2005). Dietary fats and membrane function, implications for metabolism and disease. *Biological Reviews of The Cambridge Philosophical Society, 80*(1), 155–169.

Janssen, I., Heymsfield, S., & Ross, R. (2002). Low relative skeletal muscle mass (sarcopenia) in older persons is associated with functional impairment and physical disability. *Journal of The American Geriatrics Society, 50*(5), 889–896.

Kawai, M., Namba, N., Mushiake, S., Etani, Y., Nishimura, R., Makishima, M., & Ozono, K. (2007). Growth hormone stimulates adipogenesis of 3T3-L1 cells through activation of the Stat5A/5B-PPARgamma pathway. *Journal of Molecular Endocrinology, 38*(1-2), 19–34.

Köhne, C., & Lenz, H. (2009). Chemotherapy with targeted agents for the treatment of metastatic colorectal cancer. *Oncologist, 14*(5), 478–488.

Lee, M., Kwun, I., & Kim, Y. (2008). Eicosapentaenoic acid increases lipolysis through up-regulation of the lipolytic gene expression and down-regulation of the adipogenic gene expression in 3T3-L1 adipocytes. *Genes & Nutrition, 2*(4), 327–330.

Lieffers, J., Mourtzakis, M., Hall, K., McCargar, L., Prado, C., & Baracos, V. (2009). A viscerally driven cachexia syndrome in patients with advanced colorectal cancer: Contributions of organ and tumor mass to whole-body energy demands. *American Journal of Clinical Nutrition, 89*(4), 1173–1179.

Longley, D. B., & Harkin, D. P. (2003). 5-Fluorouracil: mechanisms of action and clinical strategies. *Nature Reviews Cancer, 3*(5), 330–338.

Malet-Martino, M., & Martino, R. (2002). Clinical studies of three oral prodrugs of 5-fluorouracil. *The Oncologist, 7*(4), 288–323.

Miljkovic, I., & Zmuda, J. (2010). Epidemiology of myosteatosis. *Current Opinion in Clinical Nutrition and Metabolic Care, 13*(3), 260–264.

Murphy, R., Mourtzakis, M., Chu, Q., Reiman, T., & Mazurak, V. (2010). Skeletal muscle depletion is associated with reduced plasma (n-3) fatty acids in non-small cell lung cancer patients. *Journal of Nutrition, 140*(9), 1602–1606.

Murphy, R., Mourtzakis, M., Chu, Q., Baracos, V., Reiman, T., & Mazurak, V. (2011). Nutritional intervention with fish oil provides a benefit over standard of care on weight, and skeletal muscle mass in non-small cell lung cancer patients receiving chemotherapy. *Cancer, 117(8),* 1775–1782.

Nelson, D. L., Lehninger, A. L., & Cox, M. M. (2008). *Lehninger principles of biochemistry.* New York, NY: W. H. Freeman.

Pawlosky, R., Barnes, A., & Salem, N., Jr. (1994). Essential fatty acid metabolism in the feline: Relationship between liver and brain production of long-chain polyunsaturated fatty acids. *Journal of Lipid Research, 35*(11), 2032–2040.

Perez-Matute, P., Perez-Echarri, N., Martinez, J., Marti, A., & Moreno-Aliaga, M. (2007). Eicosapentaenoic acid actions on adiposity and insulin resistance in control and high-fat-fed rats: Role of apoptosis, adiponectin and tumour necrosis factor-alpha. *British Journal of Nutrition, 97*(2), 389–398.

Plourde, M., & Cunnane, S. (2007). Extremely limited synthesis of long chain polyunsaturates in adults: Implications for their dietary essentiality and use as supplements. *Applied Physiology, Nutrition & Metabolism, 32*(4), 619–634.

Prado, C. M., Baracos, V. E., McCargar, L. J., Reiman, T., Mourtzakis, M., Tonkin, K., . . . Sawyer, M. B. (2009). Sarcopenia as a determinant of chemotherapy toxicity and time to tumor progression in metastatic breast cancer patients receiving capecitabine treatment. *Clinical Cancer Research, 15*(8), 2920–2926.

Pratt, V. C., Watanabe, S., Bruera, E., Mackey, J., Clandinin, M. T., Baracos, V. E., & Field, C. J. (2002). Plasma and neutrophil fatty acid composition in advanced cancer patients and response to fish oil supplementation. *British Journal of Cancer, 87,* 1370–1378.

Rothenberg, M. L. (2001). Irinotecan (CPT-11): Recent developments and future directions colorectal cancer and beyond. *Oncologist, 6*(1), 66–80.

Rydén, M., Agustsson, T., Laurencikiene, J., Britton, T., Sjölin, E., Isaksson, B., Permert, J., & Arner, P. (2008). Lipolysis–not inflammation, cell death, or lipogenesis–is involved in adipose tissue loss in cancer cachexia. *Cancer, 113*(7), 1695–1704.

Saif, M., & Cohenuram, M. (2006). Role of panitumumab in the management of metastatic colorectal cancer. *Clinical Colorectal Cancer, 6*(2), 118–124.

Saltz, L. B., Cox, J. V., Blanke, C., Rosen, L. S., Fehrenbacher, L., Moore, M. J., . . . Miller, L. M. (2000). Irinotecan plus fluorouracil and leucovorin for metastatic colorectal cancer. Irinotecan Study Group. *New England Journal of Medicine, 343*(13), 905–914.

Simopoulos, A., & Salem, N. (1986). Purslane: A terrestrial source of omega-3 fatty acids. *The New England Journal of Medicine, 315*(13), 833.

Simopoulos, A. P., & Salem, N. (1989). N-3 fatty acids in eggs from range-fed Greek chickens. *New England Journal of Medicine, 321*(20), 1412.

Stephens, N., Skipworth, R., MacDonald, A., Greig, C., Ross, J., & Fearon, K. (2011). Intramyocellular lipid droplets increase with progression of cachexia in cancer patients. *Journal of Cachexia, Sarcopenia & Muscle, 2*(2), 111–117.

Tejwani, A., Yates, T., & Tejwani, S. (2008). Cardiotoxicity with fluorouracil and capecitabine revisited. *Community Oncology, 5*(10), 566–568.

Thompson, M., Cooper, S., Parry, B., & Tuckey, J. (1993). Increased expression of the mRNA for hormone-sensitive lipase in adipose tissue of cancer patients. *Biochimica Et Biophysica Acta, 1180*(3), 236–242.

Thompson, M. P., Koons, J. E., Tan, E. T., & Grigor, M. R. (1981). Modified lipoprotein lipase activities, rates of lipogenesis, and lipolysis as factors leading to lipid depletion in C57BL mice bearing the preputial gland tumor. *Cancer Research, 41*(8), 3228–3232.

Tisdale, M. (1996). Inhibition of lipolysis and muscle protein degradation by EPA in cancer cachexia [Supplement]. *Nutrition, 12*(1), S31–S33.

Tisdale, M. J. (1999). Wasting in cancer. *American Society for Nutritional Sciences, 129*(1), 243–246.

Tisdale, M. J. (2002). Cachexia in cancer patients. *Nature Reviews Cancer, 2*, 862–871.

Tisdale, M. J. (2004). Cancer cachexia. *Langenbeck's Archives of Surgery, 389*, 299–305.

Van Cutsem, E., & Geboes, K. (2007). The multidisciplinary management of gastrointestinal cancer: The integration of cytotoxics and biologicals in the treatment of metastatic colorectal cancer. *Clinical Gastroenterology, 21*(6), 1089–1108.

Van Loon, L., Koopman, R., Manders, R., van der Weegen, W., van Kranenburg, G., & Keizer, H. (2004). Intramyocellular lipid content in type 2 diabetes patients compared with overweight sedentary men and highly trained endurance athletes. *American Journal of Physiology-Endocrinology and Metabolism, 287*(3), E558–E565.

Vogelstein, B., & Kinzler, K. W. (Eds.). (2002). *The genetic basis of human cancer.* New York, NY: McGraw-Hill.

Wang, Z., Pan, W., Lee, Y., Kakuma, T., Zhou, Y., & Unger, R. (2001). The role of leptin resistance in the lipid abnormalities of aging. *FASEB Journal: Official Publication of The Federation of American Societies for Experimental Biology, 15*(1), 108–114.

Wall, M. E., Wani, M. C., Cook, C. E., Palmer, K., McPhail, A., & Sim, G. (1966). Plant antitumor agents: i. isolation and structure of camptothecin a novel alkaloidal leukemia and tumor inhibitor from camptotheca acuminata. *Journal of The American Chemical Society, 88*(16), 3888–3890.

White, J., Baltgalvis, K., Puppa, M., Sato, S., Baynes, J., & Carson, J. (2010). Muscle oxidative capacity during IL-6-dependent cancer cachexia. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology, 300*(2), R201–R211.

Whitehouse, A., Smith, H., Drake, J., & Tisdale, M. (2001). Mechanism of attenuation of skeletal muscle protein catabolism in cancer cachexia by eicosapentaenoic acid. *Cancer Research, 61*(9), 3604–3609.

Wigmore, S. J., Ross, J. A., Falconer, J. S., Plester, C. E., Tisdale, M. J., Carter, D. C., & Fearon, K. C. (2006). The effect of polyunsaturated fatty acids on the progress of cachexia in patients with pancreatic cancer [Supplement]. *Nutrition, 12*(1), S27–S30.

Xu, Y., & Villalona-Calero, M. (2002). Irinotecan: Mechanisms of tumor resistance and novel strategies for modulating its activity. *Annals of Oncology, 13*(12), 1841–1851.

Chapter 2
Research Plan

2.1 Rationale

Significant weight and muscle loss has been observed in advanced cancer. Sarcopenia (low muscle mass) and pathological fat accumulation in muscle (myosteatosis) were first recognized in aging and obesity and more recently in cancer patients (Murphy et al., 2010; Stephens et al., 2011). Myosteatosis and sarcopenia are independent risk factors for mortality in cancer patients (Martin et al., 2011). However, the characteristics of these features have not been resolved Intramuscular lipid content (IMCL) has been observed to be increased during chemotherapy and was reported to be decreased when eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3) is provided in the diet (Pratt et al., 2002; Murphy et al., 2010). IMCL has been estimated by using computed tomography (CT) (Murphy et al., 2010; Prado et al., 2008); however triglyceride (TG) content and fatty acids types within muscle tissue have not been assessed.

In previous studies from our lab have reported that cancer patients with low muscle mass exhibit lower plasma EPA and DHA than patients without sarcopenia (Murphy et al., 2010; Pratt et al., 2002). These studies suggest a relationship between the concentration of phospholipid (PL) n-3 and muscularity.

Chemotherapy drugs such as irinotecan (CPT-11) and 5-fluorouracil (5-FU) represent an effective treatment for colorectal cancer patients (Douillard et al., 2000). However, therapeutic treatments for cancer have been reported to accelerate muscle wasting, increase IMCL, and may interfere with n-3 fatty acid

metabolism (Murphy et al., 2009, 2011). Patients receiving fish oil during chemotherapy treatment had increased body weight and muscle mass and lower IMCL as well as improved response to chemotherapy (Murphy et al., 2011). Therefore, EPA and DHA may provide several benefits during chemotherapy. This study is focusing on the effect of CPT-11/5-FU as well as a fish oil diet during chemotherapy on the TG and PL content and fatty acids composition within muscle tissue of rats bearing a colon tumor.

2.2 Research Objectives and Hypotheses

Objective 1: To evaluate lipid content and composition of gastrocnemius muscle tissue of Ward colorectal tumor bearing rats one week after receiving CPT-11 + 5-FU compared to tumor bearing rats not receiving chemotherapy.

Hypothesis: It is hypothesized that: Compared to rats not receiving CPT-11/5-FU, rats undergoing CPT-11/5-FU treatment will exhibit:

i. Lower body weight.

ii. Higher content of TG fatty acid in muscle tissue.

iii. Greater amounts and proportions of saturated fatty acids in muscle TG.

iv. Lower proportions of EPA and DHA fatty acids in muscle TG and PL.

Objective 2: To evaluate lipid content and composition of gastrocnemius muscle tissue in Ward colorectal tumor bearing rats one week after receiving

CPT-11/ 5-FU and fed a diet containing fish oil compared to tumor bearing rats undergoing CPT-11/5-FU consuming a diet without fish oil.

Hypothesis: It is hypothesized that: Compared to rats fed a control diet and receiving CPT-11/5-FU, rats undergoing CPT-11/5-FU treatment and fed fish oil diet will exhibit:

i. Higher body weight.

ii. Lower content of TG fatty acid in muscle tissue.

iii. Lower amounts and proportions of saturated fatty acids

iv. Higher proportions of n-3 fatty acids in muscle triglyceride.

v. Higher proportions of EPA and DHA in muscle phospholipid.

These hypotheses will be investigated in chapter 3. This work represents a first step in measuring fatty acid composition of TG and PL of muscle tissue of colon cancer model fed a control diet or fish oil diet prior and during CPT-11/5-FU treatment. The aim of this work is to investigate if EPA and DHA diet could prevent the pathological fat accumulation in colorectal cancer undergoing chemotherapy.

References: Chapter 2

Douillard, J. Y.; Cunningham, D.; Roth, A. D.; Navarro, M.; James, R. D.; Karasek, P.; Jandik, P.; Iveson, T.; Carmichael, J.; Alakl, M.; Gruia, G.; Awad, L.; Rougier, P. (2000). Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicentre randomized trial. *Lancet, 355*(9209), 1041–1047.

Martin, L., Birdsell, L., MacDonald, N., Reiman, A., Clandinin, T., McCargar, L., Baracos, V. (2011). *Cancer cachexia in the age of obesity: Skeletal muscle wasting is a powerful prognostic factor, independent of body mass index.* Manuscript submitted for publication.

Murphy, R., Mourtzakis, M., Chu, Q., Baracos, V., Reiman, T., & Mazurak, V. (2011). Nutritional intervention with fish oil provides a benefit over standard of care on weight, and skeletal muscle mass in non-small cell lung cancer patients receiving chemotherapy. *Cancer, 117(8),* 1775–1782.

Murphy, R. A., Mourtzakis, M., Chu, Q., Reiman, T., & Mazurak, V. (2010). Skeletal muscle depletion is associated with reduced plasma (n-3) fatty acids in non-small cell lung cancer patients. *Journal of Nutrition, 140*(9), 1602–1606.

Murphy, R. A., Wilke, M. S., Perrine, M., Pawlowicz, M., Mourtzakis, M., Lieffers, J. R., . . . Mazurak, V. C. (2010). Loss of adipose tissue and plasma phospholipid: Relationship to survival in advanced cancer patients. *Clinical Nutrition*, *29,* 482–487.

Prado, CM; Baracos, VE; McCargar, LJ; Reiman, T; Mourtzakis, M; Tonkin, K; Mackey, JR; Koski, S; Pituskin, E; Sawyer, MB. (2009). Sarcopenia as a determinant of chemotherapy toxicity and time to tumor progression in metastatic breast cancer patients receiving capecitabine treatment. *Clinical Cancer Research, 15*(8), 2920–2926.

Pratt, V., Watanabe, S., Bruera, E., Mackey, J., Clandinin, M., Baracos, V., & Field, C. (2002). Plasma and neutrophil fatty acid composition in advanced cancer patients and response to fish oil supplementation. *British Journal of Cancer, 87*(12), 1370–1378.

Stephens, N., Skipworth, R., MacDonald, A., Greig, C., Ross, J., & Fearon, K. (2011). Intramyocellular lipid droplets increase with progression of cachexia in cancer patients. *Journal of Cachexia, Sarcopenia & Muscle, 2*(2), 111–117.

Tisdale, M. J. (2004). Cancer cachexia. *Langenbeck's Archives of Surgery*, *389*, 299–305.

Chapter 3
Elevated Fatty Acid Content in Muscle is Prevented by EPA and DHA in an Animal Model of Colorectal Cancer Receiving Irinotecan / 5FU

3.1 Introduction

Muscle tissue is not designed for long-term lipid storage, and usually contains a very small amount of usable fat (reviewed by Miljkovic & Zmuda, 2010). Myosteatosis, the pathological accumulation of fat in muscle, first observed in aging (Baumgartner et al., 1998), has recently been observed in advanced colorectal (Lieffers et al., 2009) and lung cancer (Murphy et al., 2010). In advanced cancer, myosteatosis is associated with significant weight loss (Murphy et al., 2011; Stephens et al., 2011). Weight loss greater than 5% of usual body weight has been characterized by dysfunction of lipid, protein, and carbohydrate metabolism, hypercatabolism, poor quality of life, decreased response to treatment, hospitalization, and mortality (Martin et al., 2011; reviewed by Tisdale, 1999, 2004). However, myosteatosis in cancer patients is not characterized.

Irinotecan (CPT-11) and 5-fluorouracil (5-FU) represent an effective combination of drugs used to treat colorectal cancer patients (Douillard et al., 2000). However, these drugs may contribute to alterations in lipid metabolism (Murphy et al., 2009; Pant, 2011). Clinical evidence suggests that the n-3 polyunsaturated fatty acids (PUFA) status of newly diagnosed patients with advanced lung and colorectal cancer undergoing chemotherapy is low

(Pawlowicz, 2008; Vicie et al., 2004). Daily eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3) supplementation (2.4g/day) during platinum based doublet chemotherapy increased muscle mass and decreased intramuscular adipose tissue, assessed by computed tomography imaging (CT) in non-small cell lung cancer patients as compared to patients who received no supplement (Murphy et al., 2011). However, fatty acid amount and composition in the skeletal muscle was not assessed. The effect of EPA and DHA supplementation potentially reducing IMCL accumulation requires further investigation.

The objectives of this study were:

1. to compare the lipid content and fatty acid composition in the muscle tissue of Ward colorectal tumor-bearing rats receiving CPT-11 and 5-FU to those in rats not receiving chemotherapy, and
2. to compare the lipid content and fatty acid composition of muscle tissue in Ward colorectal tumor-bearing rats consuming a diet containing fish oil to those of rats consuming a diet without fish oil prior and during chemotherapy treatment.

It is hypothesized that chemotherapy treatment will increase the total amount of triglycerides in muscle TG and PL will contain lower amounts of n-3 fatty acids compared to rats not receiving chemotherapy. A diet containing EPA and DHA during chemotherapy treatment will reduce the amount of triglyceride in muscle while increasing the n-3 fatty acids, especially the EPA and DHA content in both

phospholipids and triglycerides. Providing fish oil fatty acids will be associated with higher body weight after the chemotherapy treatment.

3.2 Study Design

3.2.1 Rats

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. Female fisher rats weighing 150–180g at 11–12 weeks old were received from Charles River (St. Constant, QC, Canada). The rats were housed two per cage in a controlled positive air pressured room and provided with cages containing bedding and filter tops. The temperature was 22°C and the rats received twelve hours of light per day. Water and food were available for ad libitum consumption throughout the experiment.

3.2.2 Diet

The diets were based on the American Institute of Nutrition-76 (AIN-76). They contained 40% of energy from fat, with a polyunsaturated to saturated fat ratio of 0.35 (see Table 3-1). All rats were on a control diet during the seven-day acclimation period. Subsequently, rats were assigned to one of two diet groups: the first group remained on the control diet (n = 25) while the second group (n = 9) was fed a diet similar in composition but containing fish oil (2.3 g FO/100 g containing 0.64% EPA and 0.16% DHA).

3.2.3 Tumor and CPT-11/5-FU

Two weeks after starting the experimental diets, the Ward colorectal carcinoma (provided by Dr Y Rustum, Roswell Park Institute Buffalo, NY, USA) (0.05g) was transplanted subcutaneously into the flank of the rats under slight isoflurane anaesthesia. Calipers were used to measure the tumors in three dimensions as length (L), width (W), and height (H). Tumor volume was estimated as (cm^3) = 0.5 x L x W x H. When the tumor reached approximately 2.3 cm^3 or 1.2% of body weight, rats were randomly assigned to either receive CPT-11/5-FU (CMO, control diet; n = 8 and FO, fish oil diet; n = 9) or no treatment (TUM, control diet; n = 9). CPT-11(50 mg/kg/day) was initiated on Day 0, and 5-FU (50 mg/ kg/day) was administered on Day 1. Rats that served as a healthy group (REF, n = 8) for comparison did not receive tumor implantation or chemotherapy and received the control diet throughout the study (see Figure 3-1). Rats were euthanized by CO_2 asphyxiation on Day 8. Gastrocnemius muscles were isolated and immediately frozen at –80°C until fatty acid analysis was conducted. During the study, body weight was reported twice: on the day rats were received and on the day rats were euthanized.

3.3 Methods and Fatty Acid Analysis

3.3.1 Lipid Extraction

Muscle tissues (50 mg pieces) were kept frozen at –80°C until lipid analysis. One mL of chloroform-methanol mixture (2:1) was added to the samples in methylation tubes and sonicated; subsequently, 3 mL of the same mixture was

added to each tube and vortexed. Finally, calcium chloride (800 µL) was added to the tubes and vortexed. Samples were stored at 4°C overnight.

The clear bottom layer containing the lipid was transferred to a methylation tube. The original tube was washed with 1mL of chloroform-methanol-water (86:14:1); subsequently, allowed to separate and then bottom layer transferred. Samples were dried under nitrogen gas and 160 µL chloroform was added and then vortexed.

3.3.2 Thin Layer Chromatography

Thin layer chromatography plates (G plated, Silica Gel G, 20 × 20cm, 250 microns, Analtech Inc., Newark, DE) were heated for one hour at 160°C in an oven. Samples were spotted in duplicate columns on the G plates. Plates containing the spotted samples were placed into solvent mixture tank (80: 20: 1 petroleum ether-ethyl ether- glacial acetic acid). Once the solvent reached the top of the plate, plates were removed from the solvent tanks and left to dry. Plates were sprayed with 0.1% ANSA (8-anilino-1-naphthalene-sulfonic acid) to visualize the phospholipid (PL) and triglyceride (TG) bands under ultraviolet light. PL and TG bands were identified, scraped and added to methylation tubes.

3.3.3 Phospholipid and Triglyceride Methylation

C17:0 (50 µg/mL) was added to tubes containing PL. Two mL of hexane and one mL of boron trifluoride (BF_3) were added to each tube. Samples were heated in a dry bath for one hour at 110 °C and then left to cool at room temperature. The standard C15:0 (100 µg/mL) was added to each TG tube and dried under nitrogen gas. Once the solution was dry, 1 mL of potassium

hydroxide (KOH) in methanol was added and heated for one hour in the dry bath at 110 °C. Subsequently, hexane (2mL) and BF_3 (1mL) were added to TG tubes and heated again for one hour in the dry bath at 110°C. Once cooled, distilled water (1mL) was added to both TG and PL samples and vortexed. Tubes were refrigerated for 15 minutes to allow for separation. The top layer from each of the TG and PL tubes were transferred to a GC vial and dried under nitrogen gas. Hexane (200 µL) was added to the dried samples and was pipetted into a glass insert and placed into GC vials. Samples were stored at –20°C until analysis with gas liquid chromatography (GLC).

3.3.4 Gas Liquid Chromatography

GLC was used to analyze the samples (Varian 3900, Varian Instruments, Georgetown, ON) on a fused silica BP20 capillary column (30m x 0.22 mm internal diameter, SGE Instruments). Automated GLC was used to separate the fatty acid methyl esters by means of a Galaxie Chromatography Data System and a Varian CP-8400 Autosampler equipped with Varian model 3900 (Varian Instruments, Georgetown, ON). In particular, the abounded phase fused silica capillary column (BP20: 30 m x 0.25um OD SGE product) was used. The carrier helium gas was used at a flow rate of 28ml/min with a split injector (20:1). During the process, the GLC oven temperature was increased from 150°C to 190°C at 20˚C /min and then increased from 190˚C to 220˚C at 2˚C/min. Subsequently, the temperature was held constant for 8 minutes and then increased to 240°C at 5˚C/min for a total analysis time of 46 minutes. Saturated, monounsaturated, and polyunsaturated fatty acids were separated between 6 and 24 carbon chain

lengths. The peaks were compared to a known standard, and the amount (µg) and proportion (%) of each fatty acid were determined.

3.4 Statistical Analysis

The data is presented as a mean with ± SD. Independent samples and *t*-tests were used for comparison between the two control groups. A one-way analysis of variance (ANOVA) was used to determine significant differences between groups. Statistical significance was reported when the *p* value was <0.05. When a significant overall difference was observed, post-hoc analysis was completed using the Bonferroni model. All statistical analyses were performed using SPSS 17.0 (Chicago, IL) for Windows.

3.5 Results

3.5.1 Body Weight

TUM group exhibited lower body weight than REF group (161±5.8 g versus 170.9 ± 9.2; P = 0.03) (see Appendix A). Body weight of rats on CMO group was significantly lower than those on FO group (155±6.9 g versus 167.8±10.0 g; P = 0.011) but was not differ than rats in TUM group (155±6.9 g versus 161±5.8 g; P = 0.412). Body weight of FO group was similar to TUM (167.8±10.0 g versus 161±5.8 g; P = 0.260) (see Figure 3-2).

3.5.2 Triglyceride

Total amount of TG fatty acid within the gastrocnemius muscle was comparable between TUM and FO. CMO contained the greatest amount of TG fatty acids compared all other groups. The proportion of SFA was significantly

higher in the FO group compared to other groups, due to the higher proportion of C16:0 and C18:0, however on a quantitative basis amount of SFA was lowest in the FO compared to TUM and CMO (0.3 ± 0.1µg/mg versus 1.0±2.0 µg/mg versus 3.7±5.0 µg/mg; P = 0.028) as this group contained the lowest amount of TG fatty acids. The proportion of MUFA was lowest in the FO group largely due to lower C18:1n-9. Total n-6 fatty acids were significantly lower in FO compared to the other groups, mainly due to a smaller proportion of C18:2n-6. FO exhibited the highest proportion of C20:5 n-3, C22:6 n-3, and total n-3 fatty acids in skeletal muscle TG. On the other hand, C20:5 n-3 was not detected in TUM and CMO groups (see Table 3-2). There were few differences in fatty acid composition of muscle TG between the REF and TUM groups. C16:0 was significantly higher in REF but the proportions of C18:0 and total SFA were comparable between groups. TUM exhibited a higher proportion of 18:1 and total MUFA compared to REF. There were no significant differences in C20:5 n-3 and C22:6 n-3 between REF and TUM groups (see Appendix B).

3.5.3 Phospholipid

The total amount of PL fatty acids in gastrocnemius muscle was comparable among all of the groups. The proportion of SFA including C16:0 and C18:0 and MUFA including C18:1 n-9 were comparable between the groups. FO exhibited significantly less 20:4 n-6 than the other two groups (see Table 3-3). FO exhibited the highest proportion of n-3 fatty acids due to higher C20:5n-3 and C22:6n-3 compared with the other two groups. In comparing the TUM and REF groups, total amount of PL fatty acids was higher in the TUM group. There were

no significant differences in the proportion of C16:0 and C18:0 between the groups. However, REF rats exhibited higher total SFA than TUM. Proportions of n-6 and n-3 were higher in the TUM group, largely due to a higher proportion of C18:2 n-6 and C 22:6 n-3 respectively (see Appendix C).

3.6 Discussion

This is the first study to measure the amount and types of fatty acids within skeletal muscle obtained from a Ward colon tumor bearing rats following one cycle of CPT-11/5-FU treatment. The data presented in this study supports observations of a clinical study in our lab (Murphy et al., 2011). The present study revealed that the combination of CPT-11 and 5-FU significantly decreased body weight and altered fatty acid TG and PL amount and composition inside the gastrocnemius muscle of rats bearing the Ward colon tumor. EPA and DHA in diet prevented body weight loss during chemotherapy treatment. Although intramuscular triglyceride appears to increase after one cycle of CPT-11/5-FU, EPA and DHA supplementation prevented triglyceride accumulation in muscle during chemotherapy treatment.

Myosteatosis and loss of muscle mass and body weight are important predictors of treatment response and mortality in cancer patients (Prado et al., 2009; Martin et al., 2011). However, the fatty acid profile of skeletal muscle of cancer patients is not well characterized. In a previous study, it was reported that patients with advanced non small cell lung cancer experienced a significant loss of muscle mass with gained in intramuscular adipose tissue during cisplatin-based chemotherapy. These observations were recognized by using CT images to

quantify body compositions. Interestingly, EPA and DHA supplementation increased muscle mass and decreased intramuscular adipose tissue compared to patients who received no intervention and exhibited muscle loss and myosteatosis (Murphy et al., 2011). No analysis of the fat in muscle was performed in that study.

In the present study we found a negative association between intramyocellular fat and body weight. Rats received CPT-11/5FU and fed a control diet exhibited more TG within the muscle and lower body weight compared to other groups. One study has shown that in non-small cell lung cancer patients receiving chemotherapy, the intramuscular adipose tissue, compared to visceral and subcutaneous adipose tissues, is the only fat depot that increases at the end of life (Murphy et al., 2010). Another study reported that in the presence of cancer, weight loss is associated with an increase in the size and number of lipid droplets in muscle (Stephens et al., 2011). While intramyocellular lipid droplets have also been observed in obese patients (Van Loon et al., 2004), these findings suggest that muscle fat infiltration may not be associated with a positive or negative energy homeostasis; rather, it might relate to the underlying causes of this imbalance, including catabolic hormonal mediators such as pro-inflammatory cytokine and insulin resistance (discussed in chapter 1, under title of Lipid Metabolism in Cancer Cachexia with Skeletal Muscle Loss)

Not surprisingly that rats consuming the FO diet had the highest proportion of EPA and DHA and total n-3 fatty acids as these were presented in the diet. However, it was interesting that the fish oil diet prevented body weight

loss and TG accumulation prior and during CPT-11/5-FU treatment compared to rats had no intervention with EPA and DHA. These data support evidence from clinical studies which suggest that supplementation of EPA and DHA may maintain body weight and prevent fat accumulation inside of the muscle. In advanced cancer patients with weight loss, supplementation with EPA and DHA increased the amount of EPA and DHA in the plasma, which was associated with higher body weights compared to subjects not receiving fish oil (Pratt et al., 2002). In another study skeletal mass and adipose tissue mass of 40 non-small cell lung cancer patients were measured using CT images. Patients received either fish oil (2.2g EPA/day) during chemotherapy (n=16) or standard of care; no intervention (n=24). Blood was collected and weight was recorded at baseline and throughout chemotherapy. Daily EPA and DHA supplementation during platinum based doublet chemotherapy increased muscle mass and decreased intramuscular adipose tissue in non small cell lung cancer patients compared to control group (Murphy et al., 2011). A previous study reported the same findings in advanced pancreatic cancer patients with weight loss, where fish oil normalized the metabolic environment including reduced the production IL-6 and increased serum insulin concentration. These changes occurred with association with weight gain (Barber et al., 2001). This evidence from clinical studies and our experiment may suggest that EPA and DHA are essential components for muscle health and may help to maintain body weight and reduce the pathological accumulation of fat in muscle. However, the mechanism of how EPA and DHA affect muscle mass needs further studies.

Linoleic acid (C18:2 n-6) is the dietary precursor for synthesizing arachidonic acid (AA) (C20:4 n-6) and other fatty acids within the n-6 series, including C22:4 n-6 and C22:5 n-6. This study found that the proportion of LA is significantly higher in CMO in comparison to other groups, which accounted for increase of n-6 in CMO group. Increase in the amount of n-6 derived eicosanoids modulates the production of pro-inflammatory cytokines (Nielsen et al., 2005, Calder, 1997), such as IL-6, which may lead to an increase in muscle catabolism and mitochondrial dysfunction (White et al., 2010). Lipids stored in muscle usually serve as a fuel for mitochondria oxidation (Hoppeler et al., 1973), suggesting that a decrease in the size or number of mitochondria may cause lipid accumulation inside of the muscle. In this study increased EPA, DHA and total n-3 fatty acids in the muscle triglyceride and phospholipid of rats consumed fish oil diet caused a significant reduction in n-6/n-3 fatty acids ratio in both TG and PL of muscle tissue, especially compared to CMO group. Furthermore, N-6 and n-3 utilize the same Δ-6 desaturases enzyme; however, it appears that Δ-6 desaturases activity prefers n-3 to n-6 PUFAs because n-3 fatty acids have higher affinity for Δ-6 than for n-6 fatty acids (Plourde & Cunnane, 2007). In 2010, Brennan used the fat-1 mouse which has the ability to convert n-6 fatty acids to longer chain n-3 fatty acids. These mice exhibited a significant reduction in n-6/n-3 fatty acids ratio due to increase n-3 in several tissues including skeletal muscle compared to wild type mice (Brennan et al., 2010). Increase n-6/n-3 ratio has been observed to be associated with increase IL-6 and TNF-alpha in cancer patients (Tashiro et al., 1998).

In conclusion, this study indicated that rats receiving one cycle of CPT-11/5-FU have a lower body weight and a higher amount of triglyceride inside the gastrocnemius muscle in comparison to rats that did not receive chemotherapy. Also, chemotherapy treatment seems to change the fatty acid composition in both triglycerides and phospholipids. However, a fish oil diet prevented body weight loss and resulted in a lower amount of triglycerides during chemotherapy treatmentCPT-11/5-FU treated rats that were fed a fish oil diet exhibited lower amounts of SFA and n-6 fatty acids as well as higher amounts of n-3 fatty acids.

Assessing the effect of EPA and DHA on skeletal muscle fatty acid composition after several cycle of chemotherapy would also report if EPA and DHA supplementation could prevent body weight loss and fatty acids infiltration of skeletal muscle following several cycle of treatment or not. In the present study we assessed the effect of fish oil on IMCL after only one cycle of CPT-11 /5-FU, however, clinically cancer patients receive no less than three cycle of chemotherapy. In addition, the mechanisms of altered fatty acids composition in muscle during chemotherapy as well as the effect of EPA and DHA on IMCL during chemotherapy need further studies.

Table 3-1. Experimental Diets

Ingredient (g/100 g of diet)		Control	Fish oil
Constant portion Modified AIN-76 basal mix (46 g/100 g)	Casein Methionine Mize starch Glucose Vitamins (AIN-76) Minerals (AIN-76) Inositol Choline	25.2 0.25 23.7 13.95 1 5 0.6 0.3	25.2 0.25 23.7 13.95 1 5 0.6 0.3
Variable portion Lipids (20 g/100 g)	Soybean stearine Linseed oil Sunflower oil Safflower oil Fish oil*	15.22 0.4 0 4.38 0	13.84 0.4 3.46 0 2.3
Fibers (10 g/100 g)	Cellulose	10	10
Total		100	100

All diets contained 262 g of protein and 15.48 MJ of energy per kilogram. The constant portion consisted of the premixed modified American Institute of Nutrition-76 (AIN-76) basal ingredients (Harlan Teklad); the variable portion was formulated to allow the addition of selected fat/fiber/amino acid elements. Other ingredients were supplied: soybean stearine (ICN Biomedicals, Inc.), safflower oil (Canadian Superstore, President's Choice, AB), linseed oil (Planet Organic, Gold Top, AB), fish oil (Ocean Nutrition Canada). Fish oil containing diets contained C18:3(3), C 20:5(3), C 22:5(3), and C 22:6(3), respectively, at 0.2%, 3.2%, 0.2%, and 0.8% of total fatty acids, and had an n-6:n-3 ratio of 3.8. Control diets contained 1.1% of total fatty acids as C18:3(3) and had an n-6: n-3 ratio of 21.0 (Adapted from H. Xue et al., 2007).

Table 3-2. Fatty Acid Composition of Muscle Triglycerides in Rats Bearing a Ward Colorectal Carcinoma

Fatty Acid (%)	TUM (n = 9)	CMO (n = 8)	FO (n = 9)	P-Value
C 16:0	21.6 ± 2.4	21.3 ± 1.9	24.8 ± 3.4≠	0.03
C 18:0	12.1 ± 4.6	11.3 ± 3.1	23.9 ± 5.0*≠	P<0.001
C 18:1 n-9	32.2 ± 2.8	28.6 ± 4.1	24.2 ± 2.3*≠	P<0.001
C 18:2 n-6	26.8 ± 3.6	29.9 ± 3.1	21.9 ± 3.8*≠	P<0.001
C 20:4 n-6	1.2 ± 0.2	1.7 ± 1.0	2.0 ± 1.5	0.32
C 20:5 n-3	ND	ND	1.8 ± 1.5*≠	P<0.001
C 22:6 n-3	0.4 ± 0.7	0.1 ± 0.4	4.2 ± 1.7*≠	P<0.001
∑SFA	34.1 ± 3.6	33.0 ± 4.5	48.9 ± 7.9*≠	P<0.001
∑MUFA	34.6 ± 2.5	32.5 ± 3.9	25.1 ± 2.4*≠	P<0.001
∑n-6	28.9 ± 4.1	32.1 ± 4.0	23.9 ± 4.4*≠	P<0.001
∑n-3	1.2 ± 0.7	0.6 ± 0.7	6.9 ± 3.7*≠	P<0.001
n-6/n-3	32.5 ± 21.0	20.4 ± 26.2	3.2 ± 1.6*	0.01
Total (µg/mg)	4.7 ± 4.0	8.5 ± 9.2	0.6 ± 0.4≠	0.03

*Significantly different from Tumor (P< 0.05)

≠ Significantly different from irinotecan/5-FU (P< 0.05)

Fatty acid within gastrocnemius muscle of rats bearing a Ward colon cancer received CPT-11/5-FU either fed a fish oil (FO) or control diet (CMO). Not treated rats (TUM) used as reference to compare. A hundred µL of C15:0 (100 µg/mL) was used to determine the total amount of fatty acids (µg/mg). Individual fatty acids were determined as proportionate amount (%) of total TG. Data is expressed as means ± standard deviation. One way ANOVA was used to determine significant differences between groups. Abbreviations: SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; ND, not detected.

Table 3-3. Fatty Acid Composition of Muscle Phospholipid in Rats Bearing a Ward Colorectal Carcinoma

Fatty Acid (%)	TUM (n = 9)	CMO (n = 8)	FO (n = 9)	P-Value
C 16:0	19.9 ± 7.6	24.3 ± 2.6	22.6 ± 3.4	0.21
C 18:0	27.4 ± 3.3	26.5 ± 0.9	27.5 ± 2.9	0.74
C 18:1 n-9	3.7 ± 1.0	3.6 ± 0.8	3.7 ± 2.5	0.96
C 18:2 n-6	12.7 ± 1.7	11.5 ± 2.0	13.2 ± 3.2	0.34
C 20:4 n-6	16.7 ± 3.2	15.1 ± 1.5	8.4 ± 2.2*≠	P<0.001
C 20:5 n-3	0.1 ± 0.1	ND	2.0 ± 0.9*≠	P<0.001
C 22:6 n-3	12.8 ± 2.1	10.0 ± 2.0*	15.8 ± 1.3*≠	P<0.001
∑SFA	48.5 ± 4.9	53.2 ± 3.8	51.1 ± 3.3	0.08
∑MUFA	5.6 ± 1.2	6.2 ± 1.0	6.1 ± 2.8	0.81
∑n-6	29.8 ± 3.8	26.8 ± 1.0	21.7 ± 2.6*≠	P<0.001
∑n-3	15.6 ± 2.2	12.8 ± 2.6	20.2 ± 1.9*≠	P<0.001
n-6/n-3	1.9 ± 0.3	2.2 ± 0.5	1.1 ± 0.2*≠	P<0.001
Total (µg/mg)	5.8 ± 1.3	4.0 ± 2.3	4.2 ± 0.9	0.08

*Significantly different from Tumor (P< 0.05)

≠ Significantly different from irinotecan/5-FU (P< 0.05)

Fatty acid within gastrocnemius muscle of rats bearing a Ward colon cancer received CPT-11/5-FU either fed a fish oil (FO) or control diet (CMO). Not treated rats (TUM) used as reference to compare. A hundred µL of C17:0 standard (50 µg/mL) was used to determine the total amount of fatty acids (µg/mg). Individual fatty acids were determined as proportionate amount (%) of total TG. Data is expressed as means ± standard deviation. One way ANOVA was used to determine significant differences between groups. Abbreviations: SFA, saturated fatty acids; MUFA, monounsaturated fatty acids ; ND, not detected.

Figure 3-1. Study Design

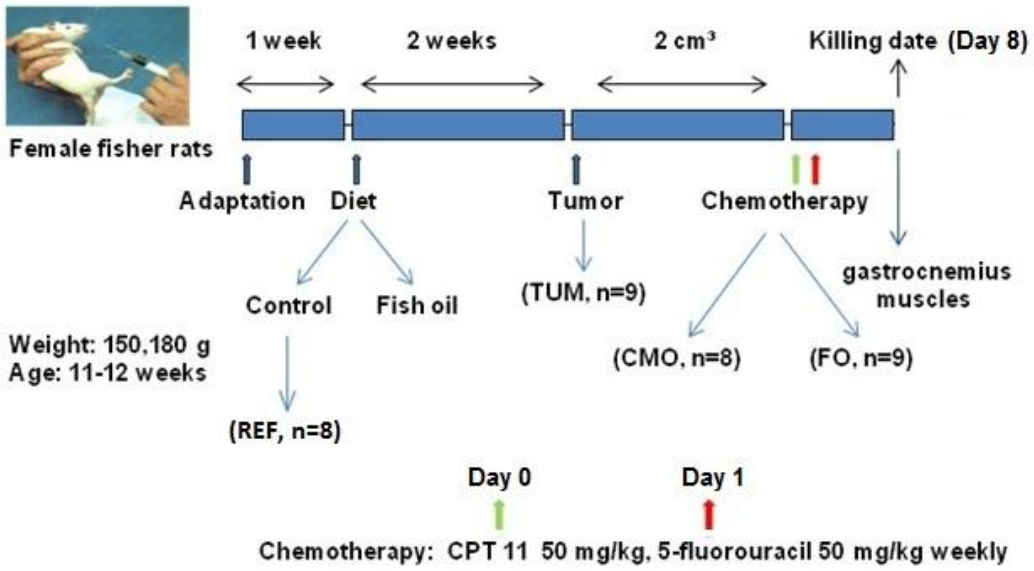
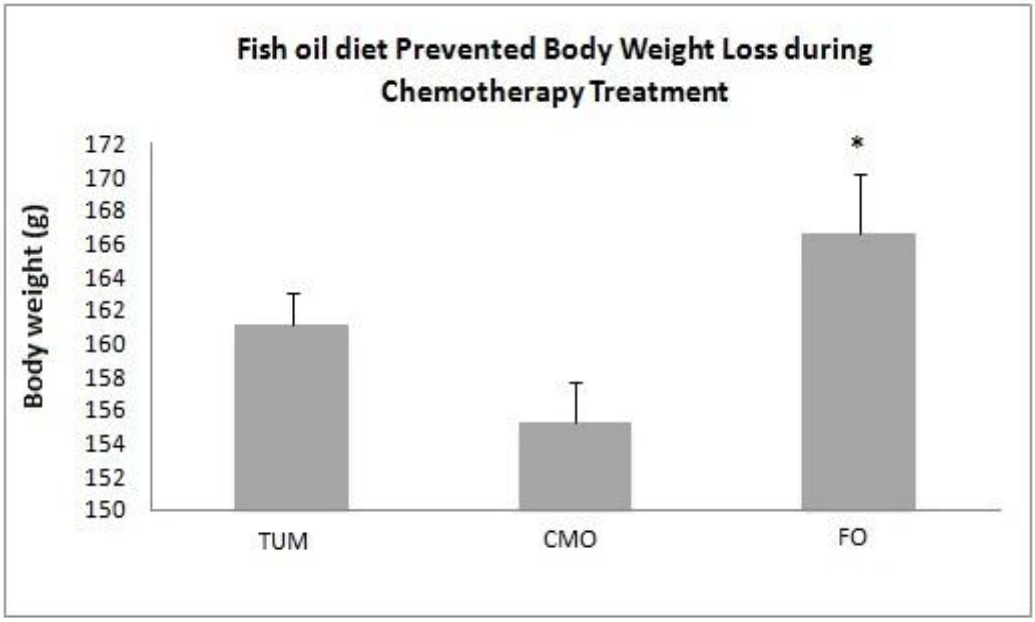


Figure 3-2. Effect of Fish Oil Diet During Chemotherapy



*Significant between fish oil vs control diet during CPT-11/5-FU treatments.

Body weight of rats bearing a Ward colon cancer received CPT-11/5-FU either fed a fish oil (FO) or control diet (CMO). Not treated rats (TUM) used as reference to compare. Body weight between groups was measured on the euthanized day. Data is expressed as means ± standard error. Significant differences ($P<0.05$) were determined using oneway ANOVA.

References: Chapter 3

Baracos, V. E., Mazuak, V. C., & Ma, D. W. L. (2004). N-3 polyunsaturated fatty acids throughout the cancer trajectory: Influence on disease incidence, progression, response to therapy and cancer- associated cachexia. *Nutrition Research Reviews, 17*(2), 177–192.

Baracos, V. E. (2006). Cancer-associated cachexia and underlying biological mechanisms. *Annual Review of Nutrition, 26,* 435–461.

Barber, M., Fearon, K., Tisdale, M., McMillan, D., & Ross, J. (2001). Effect of a fish oil-enriched nutritional supplement on metabolic mediators in patients with pancreatic cancer cachexia. *Nutrition & Cancer, 40*(2), 118–124.

Baumgartner, R. N., Koehler, K. M., Gallagher, D., Romero, L., Heymsfield, S. B., Ross, R. R., Garry, P. J., & Lindeman, R. D. (1998). Epidemiology of sarcopenia among the elderly in New Mexico. *American Journal of Epidemiology, 147,* 755–763.

Smith, B., Holloway, G., Reza-Lopez, S., Jeram, S., Kang, J., & Ma, D. (2010). A decreased n-6/n-3 ratio in the fat-1 mouse is associated with improved glucose tolerance. *Applied Physiology Nutrition and Metabolism-Physiologie (Appliquee Nutrition Et Metabolisme), 35*(5), 699–706.

Calder, P. (1997). N-3 Polyunsaturated fatty acids and cytokine production in health and disease. *Annals of Nutrition & Metabolism, 41*(4), 203–234.

Douillard, J. Y.; Cunningham, D.; Roth, A. D.; Navarro, M.; James, R. D.; Karasek, P.; Jandik, P.; Iveson, T.; Carmichael, J.; Alakl, M.; Gruia, G.; Awad, L.; Rougier, P. (2000). Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicentre randomized trial. *Lancet, 355*(9209), 1041–1047.

Evans, W. J., Morley, J. E., Argilés, J., Bales, C., Baracos, V., Guttridge, D., . . . Anker, S. D. (2008). Cachexia: A new definition. *Clinical Nutrition, 27*(6), 793–799.

Fearon, K. (2011). Cancer cachexia and fat-muscle physiology. *New England Journal of Medicine, 365*(6), 565–567.

Hoppeler, H., Luthi, P., Claassen, H., Weibel, E. R., & Howald, H. (1973). The ultrastructure of the normal human skeletal muscle: A morphometric analysis on untrained men, women and welltrained orienteers. *Pflugers Archiv European Journal of Physiology, 344,* 217–232.

Johannsen, D. L., Conley, K. E., Bajpeyi, S., Punyanitya, M., Gallagher, D., Zhang, Z., . . . Ravussin, E. (2012). Ectopic lipid accumulation and reduced glucose tolerance in elderly adults are accompanied by altered skeletal muscle mitochondrial activity. *The Journal of Clinical Endocrinology and Metabolism, 97*(1), 242–250.

Kuk, J., Saunders, T., Davidson, L., & Ross, R. (2009). Age-related changes in total and regional fat distribution. *Ageing Research Reviews, 8*(4), 339–348.

Lieffers, J., Mourtzakis, M., Hall, K., McCargar, L., Prado, C., & Baracos, V. (2009). A viscerally driven cachexia syndrome in patients with advanced colorectal cancer: Contributions of organ and tumor mass to whole-body energy demands. *American Journal of Clinical Nutrition, 89*(4), 1173–1179.

Martin, L., Birdsell, L., MacDonald, N., Reiman, A., Clandinin, T., McCargar, L., . . . Baracos, V. (2011). *Cancer cachexia in the age of obesity: Skeletal muscle wasting is a powerful prognostic factor, independent of body mass index.* Manuscript submitted for publication.

Miljkovic, I., & Zmuda, J. (2010). Epidemiology of myosteatosis. *Current Opinion in Clinical Nutrition & Metabolic Care, 13*(3), 260–264.

Murphy, R., Mourtzakis, M., Chu, Q., Baracos, V., Reiman, T., & Mazurak, V. (2011). Nutritional intervention with fish oil provides a benefit over standard of care on weight, and skeletal muscle mass in non-small cell lung cancer patients receiving chemotherapy. *Cancer, 117(8),* 1775–1782.

Murphy, R., Mourtzakis, M., Chu, Q., Reiman, T., & Mazurak, V. (2010). Skeletal muscle depletion is associated with reduced plasma (n-3) fatty acids in non-small cell lung cancer patients. *Journal of Nutrition, 140*(9), 1602–1606.

Nielsen, A. A., Jørgensen, L. G., Nielsen, J. N., Eivindson, M., Grønbaek, H., Vind, I., . . . Hey, H. (2005). Omega-3 fatty acids inhibit an increase of proinflammatory cytokines in patients with active Crohn's disease compared with omega-6 fatty acids. *Alimentary Pharmacology & Therapeutics, 2*2(11-12), 1121–1128.

Pant, A. (2011). *Alteration of liver fat metabolism following irinotecan plus5-fluorouracil treatment* (Master's thesis, University of Alberta, Edmonton, Alberta, Canada). Retrieved from http://hdl.handle.net/10048/2410

Pawlowicz, M. C. (2008). *What happens to essential fatty acids in cancer and during chemotherapy treatment?* (Master's thesis, University of Alberta [Book and Record Depository, QP 752 E84 P39 2008], Edmonton, Alberta, Canada).

Plourde, M., & Cunnane, S. (2007). Extremely limited synthesis of long chain polyunsaturates in adults: Implications for their dietary essentiality and use as supplements. *Applied Physiology, Nutrition & Metabolism, 32*(4), 619–634.

Prado, C. M., Baracos, V. E., McCargar, L. J., Reiman, T., Mourtzakis, M., Tonkin, K., . . . Sawyer, M. B. (2009). Sarcopenia as a determinant of chemotherapy toxicity and time to tumor progression in metastatic breast cancer patients receiving capecitabine treatment. *Clinical Cancer Research, 15*(8), 2920–2926.

Pratt, V. C., Watanabe, S., Bruera, E., Mackey, J., Clandinin, M. T., Baracos, V. E., & Field, C. J. (2002). Plasma and neutrophil fatty acid composition in advanced cancer patients and response to fish oil supplementation. *British Journal of Cancer, 87,* 1370–1378.

Smith, B., Holloway, G., Reza-Lopez, S., Jeram, S., Kang, J., & Ma, D. (2010). A decreased n-6/n-3 ratio in the fat-1 mouse is associated with improved glucose tolerance. *Applied Physiology, Nutrition & Metabolism, 35*(5), 699–706.

Stephens, N., Skipworth, R., MacDonald, A., Greig, C., Ross, J., & Fearon, K. (2011). Intramyocellular lipid droplets increase with progression of cachexia in cancer patients. *Journal Of Cachexia, Sarcopenia & Muscle*, *2*(2),111–117.

Tashiro, T., Yamamori, H., Takagi, K., Hayashi, N., Furukawa, K., Nakajima, N. (1998), N-3 versus n-6 polyunsaturated fatty acids in critical illness. *Nutrition*, *14*(6), 551–553.

Tisdale, M. J. (1999). Wasting in cancer. *American Society for Nutritional Sciences, 129*(1), 243–246.

Tisdale, M. J. (2004). Cancer cachexia. *Langenbeck's Archives of Surgery, 389,* 299–305.

Van Loon, L., Koopman, R., Manders, R., van der Weegen, W., van Kranenburg, G., & Keizer, H. (2004). Intramyocellular lipid content in type 2 diabetes patients compared with overweight sedentary men and highly trained endurance athletes. *American Journal of Physiology-Endocrinology and Metabolism, 287*(3), E558–E565.

White, J., Baltgalvis, K., Puppa, M., Sato, S., Baynes, J., & Carson, J. (2010). Muscle oxidative capacity during IL-6-dependent cancer cachexia. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology, 300*(2), R201–R211.

Xue, H., Sawyer, M., Field, C., Dieleman, L., & Baracos, V. (2007). Nutritional modulation of antitumor efficacy and diarrhea toxicity related to irinotecan chemotherapy in rats bearing the ward colon tumor. *Clinical Cancer Research, 13*(23), 7146–7154.

Chapter 4
Conclusion

The first objective of the present study was to evaluate lipid content and composition of gastrocnemius muscle tissue of Ward colorectal tumor bearing rats one week after receiving CPT-11 + 5-FU compared to tumor bearing rats not receiving chemotherapy. It was hypothesized that rats undergoing CPT-11/5-FU treatment would exhibit,

1. lower body weight,
2. higher content of triglyceride fatty acid in muscle tissue,
3. greater amounts and proportions of saturated fatty acids in muscle triglyceride , and
4. lower proportions of EPA and DHA fatty acids in muscle triglyceride and phospholipid.

This study reported that rats receiving CPT-11/5-FU had lower body weight and exhibited the higher amount of fatty acid triglyceride inside the gastrocnemius muscle, especially in comparison to those fed a fish oil diet prior and during the chemotherapy treatment. The proportion of saturated fatty acids within the muscle triglycerides was significantly higher in FO compared to TUM and CMO groups. However, the proportion of SFA in FO was similar to REF group, suggesting that the proportion of SFA within muscle triglycerides in FO was not pathological. Furthermore, the proportion of linoleic acid was significantly higher in CMO in comparison to the rats in the other two groups,

which may account for increase n-6 in CMO group. The proportion of n-3 was lower in CMO due to lower EPA and DHA comparing to other groups in both triglyceride and phospholipid.

The second objective was to evaluate lipid content and composition of gastrocnemius muscle tissue in Ward colorectal tumor bearing rats one week after receiving CPT-11/ 5-FU and fed a diet containing fish oil compared to tumor bearing rats undergoing CPT-11/5-FU consuming a diet without fish oil. It was hypothesized that rats undergoing CPT-11/5-FU treatment and feeding fish oil diet would exhibit,

1. higher body weight,
2. lower content of triglyceride fatty acid in muscle tissue,
3. lower amounts and proportions of saturated fatty acids,
4. higher proportions of n-3 fatty acids in muscle triglyceride,
5. higher proportions of EPA and DHA in muscle phospholipid compared to rats underwent CPT-11/5-FU and fed a control diet.

This experiment reported that EPA and DHA diet maintains body weight and prevented triglyceride accumulation in muscle during chemotherapy treatment. Furthermore, rats that consumed a fish oil diet during chemotherapy exhibited less total SFA, largely due to lower C16:0 and C18:0 as well as higher EPA, DHA and total n-3. These findings support the hypothesis in this study and also suggest that EPA and DHA may be essential nutrients for maintains of healthy body weight and may prevent TG accumulation within skeletal muscle.

This is the first study to demonstrate elevated lipid content in muscle during CPT-11 treatment within the muscle of rats bearing a Ward colorectal carcinoma. This study reported that the effect of CPT-11/5-FU on intramuscular adipose tissue is eliminated when fish oil is provided in the diet. This parallels human studies where there was higher IMAT, assessed by CT imaging, following cisplatin based doublet chemotherapy that was ameliorated when patients were provided fish oil during their therapy (Murphy et al., 2011).

The strength of this study is we used two control groups; one did not receive a tumor or chemotherapy and fed a control diet (Healthy Rats). The other group has a tumor but no chemotherapy provided and fed a control diet. We used a tumor group to compare the effect of the chemotherapy and fish oil diet during chemotherapy on fatty acids composition because chemotherapy is provided to people with tumor, not to healthy people. However, tumor produces a different lipid homeostasis compared with healthy individuals. Therefore, we compared rats bearing a colon tumor with healthy rats to assess the effect of the tumor on fatty acids of both triglyceride and phospholipid and also on the body weight.

This study has several limitations. We did assess total amount of fat and fatty acids composition after only one cycle of chemotherapy but clinically patients typically receive a minimum of two cycles of chemotherapy. In advanced cancer patients, low level of n-3 fatty acids in plasma PL has been observed following chemotherapy treatment, with further declines following high dose chemotherapy (Murphy et al., 2010; Pratt et al., 2002). Measuring skeletal muscle fatty acid composition after several cycle of chemotherapy would also report if

EPA and DHA supplementation could prevent body weight loss and fatty acids infiltration of skeletal muscle following several cycle of treatment or not. Furthermore, we did present that chemotherapy has an effect on body weight and fatty acids composition of muscle tissue in cancer model but the mechanism of altered fatty acids composition in muscle during chemotherapy is not fully addressed. Assessing genes and proteins related to fatty acids metabolism including the adipogenesis transcriptional factors (PPARs, C/EBPs, SHREBP-1c) in addition to the proinflammatory-cytokines (IL-6 and TNF-alpha) and the function of mitochondria in colorectal cancer would be areas that require further investigation. EPA and DHA presented to have a positive effect on body weight and also it prevented the accumulation of triglyceride inside the muscle of rats bearing a Ward colorectal carcinoma undergoing chemotherapy treatment. However, we do not know how EPA and DHA interfere with chemotherapy. Thus, addressing the metabolism of these fatty acids during chemotherapy needs further studies.

This study enables us to report that feeding a 2.3g of fish oil per 100g of the diet prior and during receiving CPT-11/ 5-FU able to prevent body weight loss and triglyceride accumulation inside of skeletal muscle of rats bearing a Ward colorectal carcinoma and it could be used clinically to prevent myosteatosis during chemotherapy.

References: Chapter 4

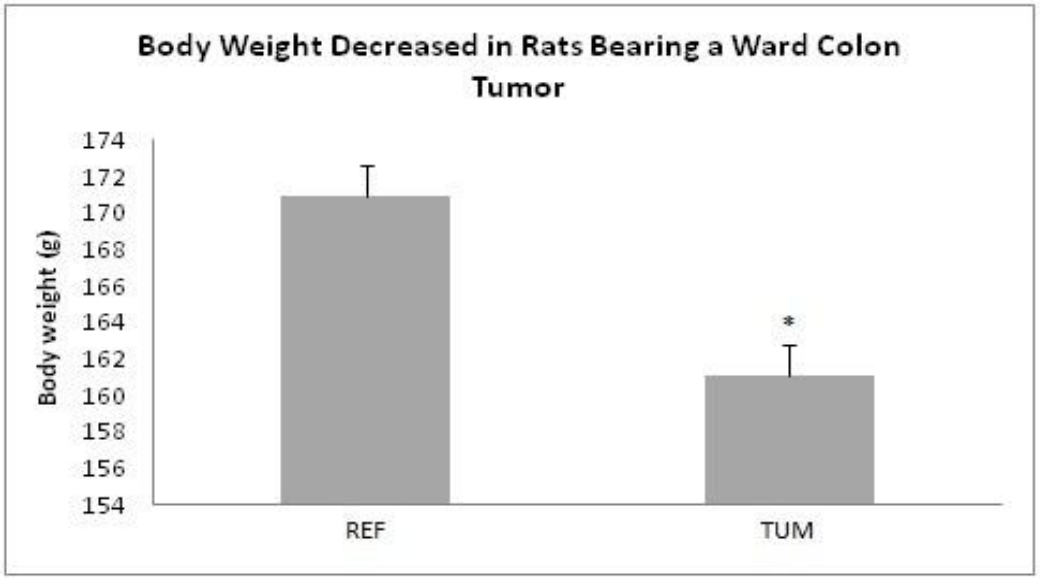
Murphy, R., Mourtzakis, M., Chu, Q., Baracos, V., Reiman, T., & Mazurak, V. (2011). Nutritional intervention with fish oil provides a benefit over standard of care on weight, and skeletal muscle mass in non-small cell lung cancer patients receiving chemotherapy. *Cancer, 117*(8), 1775–1782.

Murphy, R., Mourtzakis, M., Chu, Q., Reiman, T., & Mazurak, V. (2010). Skeletal muscle depletion is associated with reduced plasma (n-3) fatty acids in non-small cell lung cancer patients. *Journal of Nutrition, 140*(9), 1602–1606.

Pratt, V. C., Watanabe, S., Bruera, E., Mackey, J., Clandinin, M. T., Baracos, V. E., & Field, C. J. (2002). Plasma and neutrophil fatty acid composition in advanced cancer patients and response to fish oil supplementation. *British Journal of Cancer, 87,* 1370–1378.

Appendix A

Body Weight in Rats with Ward Colon Tumor



* P = 0.03

Body weight between Healthy (REF) and rats had a Ward colon tumor with no treatment (TUM) groups was measured on the euthanized day. Data is expressed as means ± standard error. Significant differences (P‹0.05) were determined using independent-sample *t*-test.

Appendix B
Fatty Acid Composition in Healthy Rats and Rats Bearing a Ward Colorectal Carcinoma Muscle Triglycerides

Fatty Acid (%)	REF (n = 8)			TUM (n = 9)			P-Value
C 16:0	29.9	±	9.6	21.6	±	2.4	0.03
C 18:0	20.0	±	16.8	12.1	±	4.6	0.20
C 18:1 n-9	19.5	±	11.6	32.2	±	2.8	0.01
C 18:2 n-6	20.8	±	11.5	26.8	±	3.6	0.16
C 20:4 n-6	2.7	±	3.1	1.2	±	0.2	0.17
C 20:5 n-3		ND			ND		0.14
C 22:6 n-3		ND		0.4	±	0.7	0.18
∑SFA	50.7	±	25.6	34.1	±	3.6	0.07
∑MUFA	22.8	±	12.8	34.6	±	2.5	0.01
∑n-6	24.0	±	12.9	28.9	±	4.1	0.31
∑n-3	0.3	±	0.3	1.2	±	0.7	0.01
n-6/n-3	32.5	±	21.0	27.6	±	35.2	0.74
Total(µg/mg)	1.6	±	1.6	4.7	±	4.0	0.10

C15:0 (100 µg/mL) was used to determine the total amount of fatty acids (µg/mg). Individual fatty acids were determined as proportionate amount (%) of total TG. Data is expressed as means ± standard deviation. Independent sample T-Test was used to determine significant between groups. Abbreviations: SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; ND, not detected.

Appendix C

Fatty Acid Composition in Healthy Rats and Rats Bearing a Ward Colorectal Carcinoma Muscle Phospholipid

Fatty Acid (%)	REF (n = 8)			TUM (n = 9)			P-Value
C 16:0	27.9	±	4.0	19.9	±	7.6	0.02
C 18:0	26.5	±	2.1	27.4	±	3.3	0.53
C 18:1 n-9	4.0	±	1.7	3.7	±	1.0	0.64
C 18:2 n-6	10.6	±	1.5	12.7	±	1.7	0.01
C 20:4 n-6	14.3	±	1.9	16.6	±	3.2	0.95
C 20:5 n-3		ND		0.1	±	0.1	0.25
C 22:6 n-3	10.5	±	0.7	12.8	±	2.1	0.01
∑SFA	56.4	±	4.0	48.5	±	4.9	P<0.001
∑MUFA	5.9	±	1.8	5.6	±	1.2	0.74
∑n-6	25.0	±	2.4	29.8	±	3.8	P<0.001
∑n-3	12.4	±	1.0	15.6	±	2.2	P<0.001
n-6/n-3	2.0	±	0.2	2.9	±	3.0	0.52
Total (µg/mg)	3.4	±	1.0	5.8	±	1.3	P<0.001

C17:0 (50 µg/mL) was used to determine the total amount of fatty acids (µg/mg). Individual fatty acids were determined as proportionate amount (%) of total TG. Data is expressed as means ± standard deviation. Independent sample T-Test was used to determine significant between groups. Abbreviations: SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; ND, not detected.