Skeletal muscle fat infiltration is reversed by dietary fish oil in an animal model of colorectal cancer receiving irinotecan and 5-fluorouracil

by

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Abstract

This study aimed to assess the effect of a Ward colorectal tumour, and irinotecan + 5-fluorouracil after 1- or 2-cycles in rats fed a diet with or without fish oil (FO), on the amount and types of fatty acids in rat skeletal muscle. Upon chemotherapy initiation, rats remained on control diet or began FO diet. Gastrocnemius muscles were isolated before tumour implantation, before chemotherapy, and after 1- and 2-cycles. Triglyceride (TG) and phospholipid (PL) fatty acids (FAs) were extracted, separated, and quantified. Compared to healthy rats, tumour-bearing rats exhibited higher TG-FA content. After 1-cycle, FO-fed rats exhibited lower TG-FA content compared to tumour-bearing rats. Compared to control-feeding, FO-feeding resulted in lower TG-FA after both 1- and 2-cycles. N-3 FA content in muscle TG-FA and PL-FA was higher in FO group compared to control-fed. This study suggests that FO fed during chemotherapy may attenuate tumour- and chemotherapy-associated skeletal muscle TG-FA infiltration.

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Δ	delta
5-FU	5-fluorouracil
AA	arachidonic acid
acetyl CoA	acetyl co-enzyme a
AIN-76	American Institute of Nutrition-76
AKT	protein kinase B
ALA	alpha-linoleic acid
ANOVA	analysis of variance
BF_3	boron trifluoride
$CaCl_2$	calcium chloride
CPT-11	irinotecan
CRC	colorectal cancer
CRP	C-reactive protein
CT	computed tomography
DHA	docosahexaenoic acid
DNA	deoxyribonucleic acid
EFA	essential fatty acid
EPA	eicosapentaenoic acid
FFA	free fatty acid
FO	fish oil
GLUT4	glucose transporter 4
HU	Hounsfield units
IFN- γ	interferon-γ
IGF	Insulin-like growth factor
IL-1	interleukin-1
IL-6	interleukin-6
IMAT	intermuscular adipose tissue
IRS	insulin receptor substrate
KOH	potassium hydroxide

LA	linoleic acid
mL	millilitre
mTOR	mammalian target of rapamyacin
MUFA	monounsaturated fatty acid
n-3 FA	n-3 fatty acid
n-6 FA	n-6 fatty acid
PI3K	phosphoinositide-3-kinase
PKB	protein kinase B
PL	phospholipid
PUFA	polyunsaturated fatty acid
SD	standard deviation
SFA	saturated fatty acid
SN-38	7-ethyl-10-hydroxycamptothecin
SPSS	Statistical Package for the Social Sciences
TG	triglyceride
TMN	tumour; node; metastases
TNF-α	tumour necrosis factor-alpha
UGT	uridine 5'-diphospho-glucuronosyltransferase
µg	microgram
µL	microlitre

CHAPTER 1

Introduction and Literature Review

1.1 Colorectal cancer

An estimated 2 in 5 Canadians will develop cancer in their lifetime, and 1 in 4 Canadians will die from cancer (Canadian Cancer Statistics, 2013). In Canada, colorectal cancer (CRC) is the second most common cause of cancer death for males and the third most common cause of cancer death for females, with 1 in 13 men and 1 in 15 women being diagnosed with CRC in their lifetime. CRC is linked to several modifiable risk factors including red and processed meat consumption, obesity, physical inactivity and smoking. CRC is most frequently diagnosed in people aged 65-74 (World Cancer Research Fund, 2011; Wolin et al., 2009). In patients with advanced CRC, significant weight loss has been reported (Lieffers et al., 2009; Fordy et al., 1999). Cachexia, defined as weight loss >5% of normal body weight over the past 6-months (Fearon et al., 2010) manifests in CRC patients, and has been associated with reduced physical function (Moses et al., 2004), reduced tolerance to anticancer treatments (Bachmann et al., 2008), and shorter survival (Dewys et al., 1980).

1.1.1 Treatment for colorectal cancer

Colorectal cancer is diagnosed according to the T (tumour) N (nodal) M (metastases) staging system (Edge et al., 2010), which is currently regarded as the strongest prognostic parameter for patients with colorectal cancer. CRC is diagnosed at

one of five stages, ranging from stage 0, where the carcinoma is localized *in situ* and requiring surgical removal, to stage IV which indicates that cancer cells have spread to other organs or sites within the body requiring advanced treatments (Oberoi et al., 2014; reviewed by Resch et al., 2013). Colorectal cancer progression can occur without any symptoms, and is commonly diagnosed in later stages when symptoms eventually appear (Oberoi et al., 2014). Early CRC detection is critical for treatment to be most effective. CRC diagnosed at Stage I or earlier has a 5-year relative survival rate of 93%, but if diagnosed in Stages II, III, and IV, the median 5-year relative survival rates are approximately 70-85%, 44-83%, and 7-8% respectively (Canadian Cancer Statistics, 2013).

The majority of CRC patients will receive chemotherapy treatment. Stages II, III, and IV will require chemotherapy treatment, with or without surgery. Treatment for CRC is chosen based on the TNM staging for an individual patient. The tumour stage, lymph node involvement, and distant metastases determine the specific treatment a patient will receive. CRC treatment may involve any combination of: surgical resection, radiotherapy, and/or chemotherapy. While routine colonoscopies have reduced the number of patients diagnosed at later stages of CRC (Canadian Cancer Statistics, 2013), most patients require chemotherapy as part of a multimodality treatment. First-line therapy in CRC involves a combination of the drugs, 5- fluorouracil (5-FU) and irinotecan (CPT-11; Douillard et al., 2000).

Irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin, CPT-11, Camptosar) is a water soluble, semi-synthetic derivative of camptothecin, an

alkaloid isolated from the *Camptotheca acuminata* plant. The main dose-limiting factor in CPT-11 administration is its primary toxicity, severe diarrhea (Douillard et al., 2000). Irinotecan can cause acute diarrhea (immediately after drug administration) or delayed diarrhea. Immediate-onset diarrhea is caused by acute cholinergic properties and is often accompanied by other symptoms of cholinergic excess, including abdominal cramping, rhinitis, lacrimation, and salivation (Stein et al., 2010). CPT-11 requires activation by tissue carboxylesterase to form the active metabolite SN-38 (7-ethyl-10-hydroxycamptothecin) that is responsible for both antitumour activities and gastrointestinal toxicity (Xue et al, 2007). SN-38 causes irreversible double-strand DNA breakage and apoptotic cell death by inhibiting DNA topoisomerase I by reversibly stabilizing the covalent enzyme-DNA intermediate (Hsiang et al., 1989). The eventual deactivation of SN-38 occurs by uridine 5'-diphospho-glucuronosyltransferase (UGTs) to form its nontoxic glucuronide (SN-38G) prior to biliary excretion (Xue et al., 2007), and is a proposed mechanism for the severe chronic diarrhea experienced in patients administered CPT-11 (Gupta et al., 1994). Research also suggests β-glucuronidases, produced by intestinal microflora, convert SN-38G back to SN-38 to be reabsorbed into the systemic circulation, localization and accumulation of the potent metabolite occurs causing direct local damage and diarrhea (reviewed by Ma and Macleod, 2003; Takasuna et al., 1996). Despite its dose-limiting toxicity, CPT-11 has emerged as one of the most effective antitumour drugs for CRC, in addition to other malignancies (Rothenburg, 2001) especially when administered in combination with 5-FU (Douillard et al., 2000).

5-FU is a potent anti-metabolite that is a well-established form of chemotherapy for CRC, and is used to treat a variety of malignancies such as breast, and head and neck

cancers (reviewed by Malet-Martino & Martino, 2002; Schmoll et al., 1999). Fluorouracil (FU) is the fluorinated analogue of uracil, originally synthesized in 1957. Heidelberger et al. (1957) synthesized 5-FU following the observation that rat hepatomas utilized radiolabeled uracil in higher concentrations than non-malignant tissues, indicating different enzymatic pathways for uracil utilization between malignant and non-malignant tissues (Rutman et al., 1954). The metabolite fluorodeoxyuridine triphosphate inhibits thymidylate synthase involved in DNA synthesis and repair. Additionally, 5-FU can convert to fluorouridine triphosphate, which incorporates into RNA, causing damage and apoptosis of tumour cells (Longley et al., 2003). Overall, 5-FU results in cell death in the absence of thymidine, essential for DNA synthesis (reviewed by Malet-Martino & Martino, 2002). As an anticancer agent 5-FU is effective, but similar to CPT-11, it is relatively toxic resulting in myelosuppression, cardiotoxicity, gastrointestinal disorders, vomiting, and nausea due to the phosphorylation of 5-FU in the digestive tract (reviewed by Wolko et al., 2005). In spite of the wealth of toxicities induced by 5-FU and CPT-11, these drugs endure as first-line therapy with the rate of response significantly enhanced when administered in combination.

1.2 Cancer cachexia

Body composition changes, particularly loss of skeletal muscle, are important components of the condition referred to as cachexia. A recent panel of experts aimed to define cachexia in a formal consensus, "as a multifactorial syndrome defined by an on-going loss of skeletal muscle mass, with or without loss of fat mass, that cannot be fully reversed by conventional nutritional support and leads to progressive functional

impairment" (Fearon et al., 2011). Cachexia, resulting from direct tumour responses or tumour-host interactions, is associated with anaemia, altered immune function, reduced caloric intake, poor quality of life, and reduced response to chemotherapy manifesting as severe toxicities. Furthermore, cachexia is associated with reduced survival (Bachmann et al., 2008; reviewed by Tisdale, 2002). The greatest frequency of weight loss in cancer patients is experienced in gastrointestinal, pancreatic, lung, and colorectal cancers (reviewed by MacDonald et al., 2003).

1.2.1 Skeletal muscle wasting in cancer

Loss of skeletal muscle mass, occurring with or without loss of body fat, is the pathological hallmark feature of cancer cachexia (Evans, 2010). Low muscle mass (sarcopenia) was originally characterized in the elderly; it is defined as skeletal muscle >2 standard deviations (SD) below that of healthy adults (Baumgartner et al., 1998). The characteristic feature of sarcopenia is decreased muscle strength and function (Janssen et al., 2002). Recently, skeletal muscle depletion in cancer patients has been shown to be independently associated with reduced survival time (Antoun et al., 2013; Martin et al., 2013). While muscle protein degradation may be increased, fat mass can be decreased or not changed (Evans, 2010). Inflammation is a major driver of cachexia, although a number of complex factors also underlie the condition. Anabolic processes are required for muscle synthesis to occur. An adequate supply of energy fuels and substrates, an anabolic endocrine environment, and physical movement must be present simultaneously for anabolism to occur. Insulin resistance in cancer patients (Dodesini et al., 2007) in addition to reductions in food intake, physical inactivity, and a catabolic endocrine

environment results in anabolic failure (Baracos, 2006). Loss of appetite (anorexia) is one of the most frequently reported symptoms in cancer, in addition to early satiety, hedonic and chemosensory alterations, gastrointestinal motility impairments, and multiple psychosocial factors (Baracos, 2006). Appetite is regulated by orexigenic and anorexigenic factors by peripheral metabolic signals to the brain. A large body of literature suggests that the signalling of metabolic sensors in the brainstem and hypothalamus are also altered in cachexia (Broberger, 2005; Laviano et al., 2002).

Muscle synthesis or breakdown relies on a balance between anabolic and catabolic factors. Pathological muscle depletion is characterized by a negative nitrogen balance from reduced protein synthesis, increased breakdown, or both (Costelli et al., 2000). Unlike starvation, the underlying metabolic abnormalities in cachexia are not corrected through increased caloric intake alone (Evans et al., 2008; Argilés et al., 2005). In healthy fasted individuals, muscle protein catabolism occurs to provide amino acids for gluconeogenesis in the liver, combined with a decreased metabolic rate, and a heavy reliance on fat for energy, all of which serve to preserve lean body mass (Giordano et al., 2003). On the other hand, the presence of a tumour elicits a systemic inflammatory response that triggers metabolic and anorexic alterations through direct tumour factors or tumour-host responses. Pro-inflammatory cytokines have been implicated to play a role in increased muscle catabolism and reduced muscle anabolism in both *in vivo* and *in vitro* models of cachexia (Argilés et al., 2005). The participation of proinflammatory cytokines including intereukin-1 (IL-1), interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) are thought to be the principal catabolic mediators in skeletal muscle. These proinflammatory cytokines have also been shown to act in synergy with one

another (Bedard et al., 1997), signalling activation of proteolysis in addition to inhibiting growth hormones and insulin-like growth factor (IGF)-1 signalling (Frost and Lang, 2004; 2005), further resulting in insulin resistance (de Alvaro et al., 2004; reviewed by Baracos, 2006). Muscle protein degradation has been shown to be directly related to IL-1 activity resulting in increased skeletal muscle catabolism (Baracos et al., 1983).

For several decades, whether decreased protein synthesis or enhanced proteolysis is the major determinant of muscle wasting has been a matter of debate. Recent studies have suggested that networks exist between anabolic and catabolic processes in skeletal muscle, with both systems being interdependently regulated (reviewed by Attaix et al., 2012). The most prominent mechanism in muscle wasting has been associated with the breakdown of myofibrillar proteins actin and myosin, in addition to reduced protein synthesis and inhibited amino acid uptake (Tisdale, 2002). In addition to the role of proinflammatory cytokines, activation of the adenosine triphosphate-dependant ubiquitin proteasome proteolytic system is implicated in cancer-associated muscle catabolism (Attaix et al., 1999). This system plays a major role in the breakdown of myofibrillar proteins. The ubiquitin proteasome system begins with the covalent attachment of polyubiquitin chains to a protein of target; these proteins are then recognized and subsequently degraded by the 26S proteasome complex (reviewed by Baracos, 2006). The activation of muscle specific E3 ubiquitin ligases has been reported to be increased seven- to tenfold in animal models of muscle atrophy (Dodson et al., 2011; Bodine et al., 2001).

1.3 Fatty muscle pathology

The majority of cancer patients receive computed tomography (CT) scans throughout their disease trajectory. CT images are one of the preferred methods for analyzing muscle mass in cancer patients (Fearon et al., 2010), however the use of CT images in determining muscular density has thus far been used primarily in research settings, and not applied in clinical practice (Ewaschuk et al., 2014). Analysis at the 3rd lumbar vertebra (L3) highly correlates with whole body volumes of muscle and adipose tissue (Shen et al., 2004; Heymsfield et al., 1997). Muscle radiation attenuation, also referred to as muscle density, is a radiologic characteristic of muscle related to a variety of outcomes (Esfandiari et al., 2014). Attenuation is measured in Hounsfield Units (HU), a linear scale centred on water (0 HU). Radiation attenuation values, which are determined by the speed at which radiation passes through a tissue, allow clinicians to quantify fat and muscle from the use of defined tissue-specific attenuation ranges (Milijkovic and Zmuda, 2010; reviewed by Aubrey et al., 2014). Reduced muscle attenuation within these defined ranges are believed to reflect the pathological infiltration of muscle with fat, as it has been shown to correspond closely to muscle lipid content and loss of muscle function (Esfandiari et al., 2014; Goodpaster et al., 2000). While fat is mainly stored in adipose tissue, there are two potential fat depots in skeletal muscle: (i) fat within myocytes (intramyocellular/intramuscular fat) or (ii) visible fat within the fascia surrounding skeletal muscle (intermuscular fat; Aubrey et al., 2014; Goodpaster et al., 2000). Since muscle depletion is characterized by both a reduction in muscle size and increased proportion of inter- and intramuscular fat (Delmonico et al., 2007), the use of CT images have been instrumental in advancing the understanding of this pathology.

Recent application of CT image analysis to cancer patients has revealed a high prevalence of low muscle mass along with a pathological accumulation of fat in the muscle. Reduced muscle attenuation in cancer patients, indicating an increased proportion of fat in muscle, has been associated with worsened outcomes and poorer survival (Sabel et al., 2011; Antoun et al., 2013; Martin et al., 2013). In considering the relationship between fatty muscle and overall survival, it has been suggested that muscle attenuation values be included in the prognostic scores for management of cancer patients (Antoun et al., 2013). Further studies are required to determine the location and mechanisms of fat infiltration into muscle. Since fatty muscle may be associated with survival in cancer patients, the nature of this pathology and its mechanisms necessitate resolution.

1.3.1 Insulin resistance in fatty muscle

Skeletal muscle is a crucial organ in the maintenance of glucose homeostasis. Skeletal muscle accounts for 80% of glucose disposal, and in the rested state, skeletal muscle fatty acid oxidation provides ~90% of energy requirements (Kelley et al., 1993). When myocytes become unresponsive to an insulin stimulus, glucose uptake into the cell is impaired (reviewed by Milijkovic & Zmuda, 2010). In healthy muscle cells, insulin stimulates the mobilization of the glucose transporter 4 (GLUT4) to the cell membrane surface, resulting in glucose uptake through a series of elaborate signalling cascade events. Insulin resistance is also characterized by hyperinsulinemia and increased hepatic gluconeogenesis.

A number of mediators and major signalling pathways controlling muscle hypertrophy rely on the activation of the insulin pathway, emphasizing the importance of insulin sensitivity in overall muscle maintenance and growth. There are two major signalling pathways that control protein synthesis: the insulin-like growth factor-1-phosphoinositide-3-kinase-AKT/protein kinase B-mammalian target of rapamyacin (IGF-1-PI3K-AKT/PKB-mTOR) pathway, acting as a positive regulator of muscle growth, and the myostatin-Smad2/3 pathway, acting as a negative regulator; additional pathways have also been identified (Schiaffino et al., 2013; see **Figure 1-1**). Activation of this pathway first begins with the binding of IGF-1 to its receptor, the IGF-1 receptor (IGF-1R). This binding initiates intracellular signalling activation of the AKT signalling pathway. AKT stimulates protein synthesis by activating mTOR and its downstream effects. mTOR interacts with several proteins to form two complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2; Schiaffino et al., 2013). mTOR is a master integrator of protein and lipid biosynthesis signals and growth factor–driven cell cycle progression (Wander et al., 2011). The crucial role of the IGF-1-PI3K-AKT/PKB-mTOR pathway in mediating muscle growth has been supported by a number of studies demonstrating that mTOR knockout results in severe myopathy. Bodine et al. (2001) investigated the role of the IGF-1-PI3K-AKT/PKB-mTOR signalling pathway in several in vivo models of skeletal muscle hypertrophy and atrophy. Results demonstrated that the IGF-1-PI3K-AKT/PKB-mTOR pathway was upregulated during hypertrophy and downregulated during muscle atrophy. Activation of the IGF-1-PI3K-AKT/PKB-mTOR pathway in vivo resulted in atrophy prevention and hypertrophy activation, while blocking the pathway resulted in the prevention of hypertrophy. Bodine et al. (2001) further concluded that activation of the IGF-1-PI3K-AKT/PKB-mTOR pathway and its downstream targets

results in the regulation of muscle fibre size, and disuse of the pathway results in muscle atrophy. Long et al. (2001) showed that IGF-1-PI3K-AKT/PKB-mTOR signalling is connected to other pathways for the regulation of protein turnover in muscle. Adapter proteins, insulin receptor substrate-1 and -2 (IRS-1; IRS-2), are essential in the preservation of skeletal muscle mass and inhibition of AMPK-associated proteolysis. IRS-1/2 both activate the IGF-1-PI3K-AKT/PKB-mTOR pathways and are involved in IGF1 and insulin signalling (Long et al., 2011). The activation of this entire pathway relies on insulin sensitivity, emphasizing its importance in muscle growth and maintenance.

Insulin resistance is a common feature of cancer cachexia and has been proposed as a causal factor of muscle wasting (Chevalier and Farsijani, 2013). Since cachexia is a metabolic syndrome affecting glucose, lipid, protein, and energy metabolism (Evans et al., 2008) alterations in glucose metabolism may specifically play a role in this pathology. Insulin resistance has been most commonly studied in type II diabetes. In contrast to type II diabetes, normal fasting serum levels of glucose and insulin are generally seen in cancer patients. Although endogenous glucose production is generally increased in cancer patients, hyperglycemia does not result (Leij-Halfwerk et al., 2000; Winter et al., 2012). In previous years, evidence has supported insulin resistance in a variety of cancer populations with or without weight loss (Cersosimo et al., 1991; Copeland et al., 1987; Yoshikawa et al., 1994). Compared to the diabetes literature, the use of the gold standard technique in measuring peripheral insulin sensitivity—the euglycemic hyperinsulinemic glucose clamp—has not been frequently utilized in cancer populations. A recent review by Chevalier and Farsijani (2013) revealed that only 9 studies in the cancer literature

measuring insulin resistance have utilized the gold standard euglycemic hyperinsulinemic glucose clamp. In one of these studies, Yoshikawa et al. (2001) examined peripheral insulin sensitivity in 32 cancer patients; this study aimed to determine the relationship between insulin resistance and tumour factors (type and stage), malnutrition, and inflammatory responses. The impetus for this investigation was from previous reports that insulin resistance in cancer patients was partly reversed immediately after tumour resection in cancer patients, suggesting a causal effect from the tumour itself or tumour-host interactions (Yoshikawa et al., 1994). Results suggested that insulin sensitivity was often reduced in patients with cancer, but the amount of glucose metabolized was not related to tumour site or stage. Further, reduced glucose uptake in cancer patients was not correlated with body-weight loss, serum albumin, or resting energy expenditure. Following their review of the literature, Chevalier and Farsijani (2013) concluded that with the exception of head and neck cancer, insulin resistance does not appear to be related to tumour site or burden, stage of disease, or degree of weight loss, but may weakly relate to the degree of inflammation in cancer patients.

Numerous reports have described a positive relationship between intramyocellular fat and insulin resistance; this relationship has been largely studied in aging, diabetic, and obese populations (Goodpaster et al., 2000; Perseghin et al., 1999). Cross-sectional studies in advanced aging reveal increases in both intramyocellular and intermuscular fat, possibly linked to activation, proliferation, and differentiation modifications in skeletal muscle precursor stem cells that instead become adipocytes in response to age-related alterations (reviewed by Milijkovic and Zmuda, 2010; Sinanan et al., 2006). In a large longitudinal study investigating skeletal muscle changes in elderly adults, Delmonico *et*

al. (2007) discovered deposition of intermuscular fat in both men and women increased with age independent of obesity, weight loss, or weight gain. While the majority of evidence supporting a relationship between fatty muscle and insulin resistance has been investigated in the diabetes population, evidence also supports this relationship in the cancer population. In cancer, there may be a potential lipid oversupply from increased lipolysis, lipid oxidation, and subsequent release of free fatty acids into the blood stream from adipose tissue stores, or impaired ability to store fat in adipose tissue, resulting in storage of fatty acids in skeletal muscle in a compensatory manner (Bing et al., 2000). The reduced insulin response in skeletal muscle may increase fat storage, reduce muscle strength, and result in overall muscle dysfunction (Wang et al., 2001). Inflammation is also believed to play a major role in myosteatosis development. Higher intermuscular fat content has been associated with increased levels of proinflammatory markers IL-6, C-reactive protein (CRP), and TNF-α (Beasley et al., 2009). While several different mechanisms have been proposed in the development of fatty muscle in cancer, insulin resistance remains a plausible explanation.

1.4 Lipids

Lipids play a variety of roles in metabolism, and are found in every cell membrane in the body. Lipids serve as important sources of energy in the form of triglycerides (TGs), signalling molecules, intracellular messengers, and hormone precursors. Membranes provide an interface between cells and the external environment, and also partition intracellular compartments (Hulbert et al., 2005). The lipid bilayer is composed of phospholipids (PL) containing differing fatty acyl chains. FAs are identified

according to the number of carbon atoms, number of double bonds, and position of the first double bond from the methyl end of the molecule (Bezard et al., 1994). The chain length and number of double bonds in fatty acids have a major effect on their physical properties; depending on the degree of unsaturation, the melting point of individual fatty acyl chains differs. The stereospecific numbering (sn) of carbons refers to the carbon atom with the polar head group attached. In membrane PLs, the sn-2 position is always an unsaturated acyl chain, while the sn-1 can be either a saturated or unsaturated acyl chain. Thus, the fatty acid chains in membranes have a significant effect on fluidity, and other dynamic properties of membranes (Hulbert et al., 2005). Fatty acids can be categorized as either saturated fatty acids (SFAs), monounsaturates (MUFAs), or polyunsaturates (PUFAs), with the PUFAs being further divided into omega-3 (n-3) and omega-6 (n-6) fatty acids.

1.4.1 Essential fatty acids

Essential fatty acids (EFAs) are fatty acids that cannot be synthesized in animal and human tissues, and therefore must be obtained through the diet. Animals can synthesize both SFA and MUFA acyl chains from acetyl-Coenzyme A and from non-lipid sources. Mammals do not have an enzyme that adds a double bond in the carbon chain before the n-9 position, rendering linoleic acid (LA; 18:2n-6) and α-linoleic acid (ALA; 18:3n-3) essential in the diet (see **Figure 1-2**). In 1929, the essentiality of fat in the diet was discovered when mice fed a fat-free diet developed pathologies, failed to grow, and eventually died (Burr & Burr, 1929). In following years, three PUFAs were deemed essential: LA, ALA, and arachidonic acid (AA; 20:4n-6), however the discovery

that AA could be synthesized from LA in animal models resulted in it no longer being considered essential in the diet. The majority of n-6 and n-3 essential fatty acids in the blood are contained in the plasma PLs. Plasma PL composition reflects endogenous and dietary fatty acids, and has been used as an index of fatty acid status (Murphy et al., 2012; Cvetkovic et al., 2010).

Humans have enzymes required to make the long-chain PUFAs, eicosopentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:5n-3) from the parent 18-carbon PUFAs, human evidence suggests that ~5% of ALA is converted to EPA, and <0.5% of ALA is converted to DHA making this process relatively inefficient (Plourde et al., 2007), leading to the suggestion that EPA and DHA be considered essential fatty acids. N-6 fatty acids are found in high concentrations in vegetable oils, n-3 fatty acids are found in nuts, flaxseeds, and in lesser quantities in canola oil; AA is found in products from terrestrian animals, while EPA and DHA are found in fatty fish, especially cold-water fish and their oils (Hulbert et al., 2005). Depending on the diets of animals, ALA can be found in egg yolks and meat (Simopolous, 1986; 1989).

Intakes of n-3 and n-6 fatty acids relative to one another is important to consider in the conversion of parent PUFAs to long chain PUFAs, specifically EPA and DHA. These two classes of EFA are not interconvertible, are metabolically and functionally distinct, and often have important opposing physiological functions (Simopolous, 2008). Through the desaturation-elongation pathways, n-6 and n-3 fatty acids utilize the same enzyme, Δ (delta)-6 desaturase enzyme. Since both fatty acids compete for the same enzyme, a high dietary intake of n-6 PUFAs can result in a decreased availability of the

long chain n-3 PUFAs. Research supports the estimate that human beings evolved on a diet with a ratio of n-6 to n-3 of ~1/1, whereas in Western diets the ratio is 15/1–16.7/1 (Simopoulos, 2002). Further, it is now recognized that Western diets are deficient in n-3 fatty acids, and have higher amounts of n-6 fatty acids compared with the diet on which human beings evolved and their genetic patterns were established (Simopolous, 2002).

1.4.2 Lipid metabolism and EFAs in cancer

Altered lipid metabolism in cancer has been documented in several animal and human studies. Abnormalities in lipid metabolism in cancer such as increased lipolysis (Taylor et al., 2002; Ryden et al., 2008), oxidation of free fatty acids (Douglas and Shaw, 1990), and hypertriglyceridemia (Tisdale, 1999) have been reported. Reduced insulin-mediated suppression of lipolysis in cancer results in elevated FFA content in plasma, and may be related to the storage of lipids in non-adipose tissue such as skeletal muscle (Gregoire et al., 1998). Moreover, the fatty acid status of patients with cancer has not been well characterized since one of the first investigations conducted over a decade ago (Pratt et al., 2002). Plasma lipid profiles reported in cancer patients reveal low concentrations of essential n-3 and n-6 fatty acids. Studies have reported cancer patients to have low plasma levels of n-3 PUFAs at diagnosis, with these levels worsening alongside therapeutic treatments for the disease (Murphy et al., 2012). Thus, it has been proposed that both the disease as well as therapeutic treatments may contribute to reduced n-3 PUFA status (Baracos et al., 2004).

A recent study has reported plasma PL fatty acids as 35% lower in colorectal and lung cancer patients closest to death versus those surviving >8 months (Murphy et al., 2010*a).* Pratt et al. (2002) showed that compared to healthy subjects, advanced cancer patients that lost >5% of their pre-illness weight had depleted PL in plasma. A depletion of EFA in PLs was amplified following high-dose chemotherapy, when DHA and EPA levels were reduced to ~7% of the control group values (Pratt et al., 2002). In addition to therapeutic treatments, progression and advancement of disease may play a role in EFA status. One other study reported that cytotoxic agents interfere with PUFA metabolism, and may limit the endogenous synthesis of C20:5n-3 and C22:6n-3 from C18:3n-3 and C20:4n-6 from C18:2n-6 (Marra et al., 1986). Murphy et al. (2012) reported that compared to patients with early stage disease, patients with advanced disease had abnormal fatty acid profiles including significantly lower amounts of total PL, saturated, and PUFAs including LA, AA, EPA, and DHA. Further analysis revealed that patients with advanced cancer who completed chemotherapy had stable fatty acid levels and maintained this stability 1-month post-treatment. In contrast, patients that did not complete chemotherapy for reasons of toxicity or disease progression had progressive losses of total PL, stearic, LA, and n-6 fatty acids. Findings from Murphy *et al.* (2010*a* and 2012) suggest that the loss of fatty acids may be influenced by the progression of the disease and chemotherapy treatment. Collectively, these findings suggest EFA alterations in cancer, while the underlying mechanism remains unknown.

1.5 Role of EPA and DHA in attenuating fatty muscle

Little is known about the role that nutrients may play in the attenuation of myosteatosis. Emerging evidence suggests that EPA and DHA may relate to the attenuation of fatty muscle. Supplementation with fish oil in advanced cancer patients has been shown to attenuate lean tissue wasting and weight loss (Barber et al., 1999; Fearon et al., 2003). In an attempt to determine the relationship between muscle mass and plasma n-3 fatty acids in the absence of supplementation, Murphy et al. (2010) analysed CT images to measure muscle mass and changes over the course of chemotherapy compared to plasma n-3 fatty acid status. Findings from this study demonstrated a relationship between n-3 fatty acids and rate of muscle change. Results demonstrated that compared to patients not losing muscle, patients experiencing muscle loss during chemotherapy had lower plasma EPA, DHA, and total n-3 fatty acid concentrations. Patients with the greatest loss of muscle mass had the lowest EPA, DHA, and total n-3 fatty acids. Furthermore, a portion of patients gained muscle during the study period; this group of patients also had the highest mean plasma EPA, DHA, and total n-3 fatty acids concentrations. These findings suggest an important relationship between EPA and DHA and muscle health, since depleted plasma n-3 fatty acids in cancer patients undergoing chemotherapy are associated with loss of muscle mass (Murphy et al., 2010).

To determine whether restoring EPA and DHA status through supplementation could modify muscle mass and fatty muscle in lung cancer patients, patients received either standard of care (SOC) or were supplemented with 2.2 grams of fish oil per day (FO) (Murphy et al., 2011*b*). CT scans available between diagnosis and end of treatment

were analyzed. The SOC group experienced muscle loss during chemotherapy, with some patients losing up to 5.2 kg muscle. Concurrently, muscle loss in the SOC group was coupled with increases in intermuscular adipose tissue (IMAT). In contrast, the FO-group was able to maintain muscle mass during chemotherapy, and experienced a loss of IMAT over the course of treatment. Similar to the findings from Murphy et al. (2010*a*), patients with the greatest increases in plasma EPA concentrations also experienced the greatest gains in muscle mass during the intervention. These findings point to the potential role of FO not only in the maintenance of muscle mass during treatment, but also the ability for FO to modify IMAT. However, this has not been examined in a systematic way.

1.5.1 EPA and DHA in the insulin pathway

Insulin resistance is associated with defects in glucose transport (Bonadonna et al., 1996), translocation of the glucose transporter-4 (GLUT-4) to the sarcolemma (Kelley et al., 1996) and defects in insulin binding and signalling (Sesti et al., 2001). It is generally accepted that accumulation of lipid-derived intermediates in skeletal muscle are a major cause of insulin resistance (Bosma et al., 2012; Savage et al., 2008). Along with increases in IMAT in cancer patients (Murphy et al., 2011*b*) insensitivity to insulin has been observed in patients with cancer cachexia (Dodesini et al., 2007). In tumour-bearing mice, insulin resistance preceded weight loss and administration of Rosiglitazone, a drug used in the treatment of type II diabetes, improved insulin sensitivity and attenuated skeletal muscle proteolysis (Asp et al., 2010). The precise points of interaction and the relationship between n-3 fatty acids and the glucose-insulin signalling pathway have not been well explored in cancer cachexia (Asp et al., 2010). The majority of evidence

supporting n-3 fatty acids in insulin sensitivity is derived from the diabetes literature, and may contribute to understanding similar mechanisms in cancer cachexia.

In experimental models of diabetes, long chain n-3 fatty acids have been shown to improve insulin sensitivity and glucose uptake in skeletal muscle (Figueras et al., 2011). Results from human epidemiological studies indicate that n-3 PUFAs reduce the development of insulin resistance and diabetes (reviewed by Fedor and Kelley, 2009). A number of mechanisms have been proposed for the role of n-3 fatty acids in restoring insulin sensitivity. In type II diabetes, it is accepted that increased inflammation is one of the major factors that leads to the development of insulin resistance (reviewed by Fedor and Kelley, 2009). Moreover, it has been well established that changing the fatty acid composition of phospholipids in cellular membranes can alter insulin's ability to bind to its receptor (Gould et al., 1982). Feeding rats a high-fat diet results in skeletal muscle insulin resistance, even when n-6 polyunsaturated fatty acids are administered (Storlien et al., 1991). When a proportion of n-6 fatty acids were replaced by fish oil containing EPA and DHA, muscle insulin resistance induced by a high-fat diet was prevented (Storlein et al., 1991). Membranes enriched in unsaturated fatty acids have increased membrane fluidity and tend to bind more insulin than membranes enriched in saturated fatty acids (Field et al., 1988). A low-fat diet intervention with fish resulted in increased incorporation of EPA and DHA in skeletal muscle membrane phospholipids, and was significantly associated with improved insulin sensitivity in obese subjects (Haugaard et al., 2006). Improvements in glucose uptake after membrane enrichment with PUFA may be related to an increase in the time glucose transporters (GLUT4) remain in the plasma membrane, allowing an increased flux of glucose into the cell and expansion of the

intracellular glucose-6-phosphate pool (Nugent et al., 2001).

A reduction in skeletal muscle fatty acid oxidation through a reduction in mitochondrial content and/or fatty acid oxidation within mitochondria has been implicated in the development of insulin resistance. In the fat-1 mouse, Smith et al. (2010) examined the capacity for n-3 fatty acids to increase skeletal muscle fatty acid oxidation and improve insulin sensitivity. The fat-1 mouse is a transgenic model capable of synthesizing n-3 PUFA from n-6 PUFA, resulting in a reduced n-6/n-3 ratio in skeletal muscle. Results demonstrated that fat-1 mice had decreased skeletal muscle mitochondrial content, yet the intrinsic ability of mitochondria to oxidize fatty acids was unchanged. Overall, fat-1 mice demonstrated improved whole-body glucose tolerance despite lowered mitochondrial content; these findings demonstrate that skeletal muscle mitochondrial oxidative potential is not a prominent mediator in whole-body glucose tolerance (Smith et al., 2010). While mitochondrial oxidation changes may be involved in insulin resistance, other mechanisms also exist.

Similar to Smith et al. (2010), Stephens et al. (2014) aimed to determine if n-3 PUFAs play a role in restoring insulin sensitivity through mitochondrial pathways. Mitochondrial overload occurs when an excess entry of fatty acids into mitochondria causes an imbalance between β-oxidation and the demands of the tricarboxylic acid cycle, resulting in an accumulation of acetyl-CoA (reviewed by Schooneman et al., 2013). In a clinical trial, Stephens et al. (2014) aimed to determine if n-3 fatty acids restore insulin sensitivity in skeletal muscle by preventing mitochondrial overload. Six healthy young men received unsupplemented Intralipid® infusions, or Intralipid® infusions with 10% n-6

or 10% n-3 fatty acids (Omegaven®). The euglycemic-hyperinsulinaemic clamp technique was used to demonstrate improved insulin-stimulated whole body glucose infusion rate with n-3 supplementation. However, there was no difference in muscle acetyl-CoA accumulation, suggesting that the ability for n-3 fatty acids to improve insulin sensitivity is related to mechanisms other than mitochondrial overload.

In addition to insulin sensitizing effects, there is emerging evidence suggesting a muscle anabolic effect of long-chain n-3 fatty acids. In tumour-bearing mice, n-3 PUFA supplementation has been reported to maintain whole-body protein synthesis, whole-body protein net balance and muscle mass (van Norren et al., 2009). In young steers, EPA and DHA doubled insulin-stimulated whole-body protein synthesis and increased activation of the AKT/mTOR/p70S6K signalling pathway (Gingras et al., 2007). In both elderly and healthy young and middle-aged men and women, 8-weeks of 4 g long-chain n-3 supplementation resulted in increased anabolic response to insulin and amino acid infusion (Smith et al., 2011*a*, 2011*b*). The muscle protein fractional synthesis rate, phosphorylated mTOR levels, and muscle cell size significantly increased after n-3 supplementation during insulin and amino acid infusion. A post-hoc analysis revealed an increase in AKT and mTOR phosphorylation and activation (Smith et al., 2011*a*, 2011*b*). These results indicate that the ability for n-3 fatty acids to activate the mTOR pathway may require an anabolic stimulus, such as amino acids. A recent study reported that 1-week of supplementation with 5 grams of fish oil per day in healthy humans resulted in increased total mTOR content, with no further increases after 2- and 4-weeks (McGlory et al., 2014). In rodents, n-3 PUFA supplementation has been reported to alleviate soleus atrophy during a period of immobilization (Liu et al., 2013). In pigs, 21 days of fish oil

supplementation resulted in increases in muscle protein mass and mTOR protein content (Wei et al., 2013).

1.6 Animal models of cancer cachexia

Given the clinical need for effective treatments of cachexia, animal models serve a vital role in assessing the efficacy and safety of potential treatments and therapies prior to testing in humans (DeBoer, 2009). Animal models are generally used to study underlying biological mechanisms and provide evidence to support the development of future preclinical or clinical trials in cachexia therapy. However, there is a lack of standardization in the types of animal models used in these investigations, resulting in an impediment to current understandings (Baracos, 2006).

Animal models should ideally represent the human situation of cancer cachexia. Uniform monitoring of physical, physiological, metabolic, nutritional, and behavioral factors in various animal models must be established (Baracos, 2006). Human cancer is an extremely complex disease state, involving a variety of mechanisms, cytokines, inflammatory responses, and tumour-host interactions that in many cases do not translate to humans. Animal models of cancer may fall into several different categories: animals in which cancer occurs spontaneously through genetics, animals whose genes are altered to develop spontaneous tumours, animals that are exposed to environmental factors, or inoculated with tumour cells. Many different mechanisms may be involved simultaneously in humans, while animal models typically focus on one mechanism of cancer or cachexia. For example, cancer cachexia and/or anorexia can be induced in

animal models by giving inflammatory cytokines or prostaglandins. Inoculation with tumour drives tumour-host interactions, which may or may not result in cachexia, or administration of chemotherapy may or may not result in anorexia (Deans et al., 2005; DeBoer et al., 2009). All of these factors may concurrently be at play in humans. The limitations of each model need to be recognized to interpret the results within a human context.

To represent the human clinical situation of cancer/cachexia as closely as possible, several factors must be considered. Tumour cell type is often chosen in order to extrapolate the findings to the same population in humans. For example, the Lewis lung carcinoma is routinely used in animal models for lung cancer, and the Colon 26 (C26) adenocarcinoma cell line is used in animal models for colorectal cancer. Tumour cell lines are typically implanted subcutaneously into experimental animal models, and are left to grow until the tumour-burden induces cachexia symptoms (DeBoer et al., 2009). Although tumour cell implantation does induce symptoms of cachexia, these tumours rarely metastasize, a key difference from cachexia-causing neoplasms in humans (DeBoer et al., 2009). In the majority of animal models for cancer and cancer cachexia, murine rodents are used. The breed of animal is also an important factor to consider since genetic variation, gender effects, and breeding disparities exist between different laboratory animal classes. For example, the use of in-bred animals reduces genetic variability between each animal and allows researchers to duplicate experiments with little diversity between breeding batches (Alexander et al., 2005). However, some strains of in-bred animals are more susceptible to developing obesity and diabetes such as the C57BL/6J strain (Alexander et al., 2005), which can significantly affect study outcomes. Female

rodents are preferred over male rodents in studies with endpoints involving body weight changes since females have a slower growth curve and are less susceptible to rapid changes in response to dietary interventions. In contrast, studies attempting to limit hormonal fluctuations within an animal prefer male rodents (Graves et al., 2005).

1.6.1 Animal diets

Animal diets used in the laboratory are categorized into three main groups: cereal-based (non-purified) chow, purified or semi-purified, and chemically defined diets (AIN, 1977). Diets either have an open formula, indicating that the dietary composition has been published and is available to the scientific community, or diets can have a closed formula, indicating the dietary composition and ingredients are known only by the manufacturer (Reeves et al., 1997). Laboratory chow does not have a standard macronutrient composition (Xue et al., 2007), and varies from batch to batch and individual ingredients may also vary over time (Mead et al., 2006; Research Diets, 2014). Chows with closed formulas are incompletely characterized, making it nearly impossible to determine exactly what is in a particular batch (Xue et al., 2007). Although chow diets are formulated to meet minimum required intakes in murine models, they are not translatable to human dietary intakes (Xue et al., 2007). In comparison to laboratory chow, the ingredients included in purified or semi-purified diets are considered open formulas and are well characterized. There are several benefits to using semi-/purified diets over laboratory chow in murine animal designs. Diet modifications are required, in particular to study nutrient interventions. If the nutrient intervention involves a fat source, semi-purified diets enable the fat sources to be added by the researcher; for example, corn

oil replaced by olive oil, safflower oil or fish oil to determine the effects of changing the fat composition from saturated, to monounsaturated, to polyunsaturated fatty acids, respectively. Furthermore, semi-purified diets allow researchers to modify fat quantity and the proportion each type of fat contributes to the diet as a whole. Laboratory chow diets also contain micronutrients or compounds unbeknownst to researchers that may impact study endpoints and results. A number of studies have determined that the variability in commercial rodent chow diets interferes with the value of these animal models and experimental results (Jensen et al., 2007). The process of determining mechanisms and treatment effects of nutrient interventions may be masked or confounded by unknown compounds at varying levels present in many chow diets. Chow diets may significantly alter effects of experimental treatments, and in particular, affect gene and protein expression (Kozul et al., 2009).

In nutrient intervention studies, little attention is paid to background diets for their particular nutrient of interest. The phrase, 'a standard rodent chow was used' is written frequently throughout the literature, indicating that the content and composition of the animal diet is either unknown to researchers, or considered unimportant (Jensen et al., 2007). Knowing the composition and quantity of the diet fed is required to interpret findings relative to human disease states. The most basic formulation of an animal diet should at the very least be isocaloric and isonitrogenous between treatment and control diets, and contains proportions of macro- and micronutrients similar to human intakes (see **Figure 1-3)**. There is an abundance of evidence that suggests the fatty acid profile is of importance for the efficacy of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) *in vivo*, most animal models do not consider the fatty acid profile of the

background diet when designing nutrient intervention diets. The effectiveness of long-chain fatty acids EPA and DHA on enhancing drug antitumour activity has been demonstrated in several rodent models of cancer. Interestingly, a comparison of studies has shown that the effectiveness of EPA and DHA antineoplastic activities depends on the background diet, with effects greatest in studies where control diets contained fat sources of palm or corn oil, and subsequently low levels of total PUFA (Hardman et al., 2001; Shao et al., 1997). Control diets with fat sources from safflower or sunflower oil, therefore with higher levels of total PUFA, do not give the same results (Xue et al., 2007 and 2009; reviewed by Hajjaji and Bougnoux, 2013). Collectively, studies suggest that the amount of total PUFA in the diet may be an important factor in the ability for EPA and DHA to enhance tumour sensitivity to drugs. These findings emphasize the importance of a well-developed background diet in studies of nutrient interventions, particularly those with EPA and DHA.

Across nutrient intervention animal models there is a lack of standardization in dietary designs, content, source, and overall composition. While the ease and convenience of using standard laboratory chow may be clear, the effects a closed label dietary design may have on study endpoints needs to be acknowledged. Implementation of a standard dietary design will enable comparison of studies, continuity between research groups, and ensure that results obtained from animal models are applicable to humans, and translational across different studies. The potential for experimental endpoints to be affected by variations in food intake, macronutrient content, and diet composition is likely. Thus, matching diets within studies and meticulously designing diets that reflect typical human intakes is likely to result in reliable and interpretable

findings that have potential to be translated to the manifestation of cancer cachexia in humans.

1.7 Summary

Low muscle mass and fat infiltration into muscle are features that contribute to worsened outcomes in cancer patients, and have recently emerged as risk factors for death. However, these pathologies are incompletely understood. Fish oil, which is a concentrated source of EPA and DHA, has been observed to modify fatty muscle in human subjects, and evidence from our laboratory has shown that fatty muscle development during chemotherapy can be prevented in an animal model. Mechanisms surrounding the role of EPA and DHA in attenuating fatty muscle remain unknown, warranting further investigation to completely characterize muscle wasting and fat infiltration.

CHAPTER 1

Figures

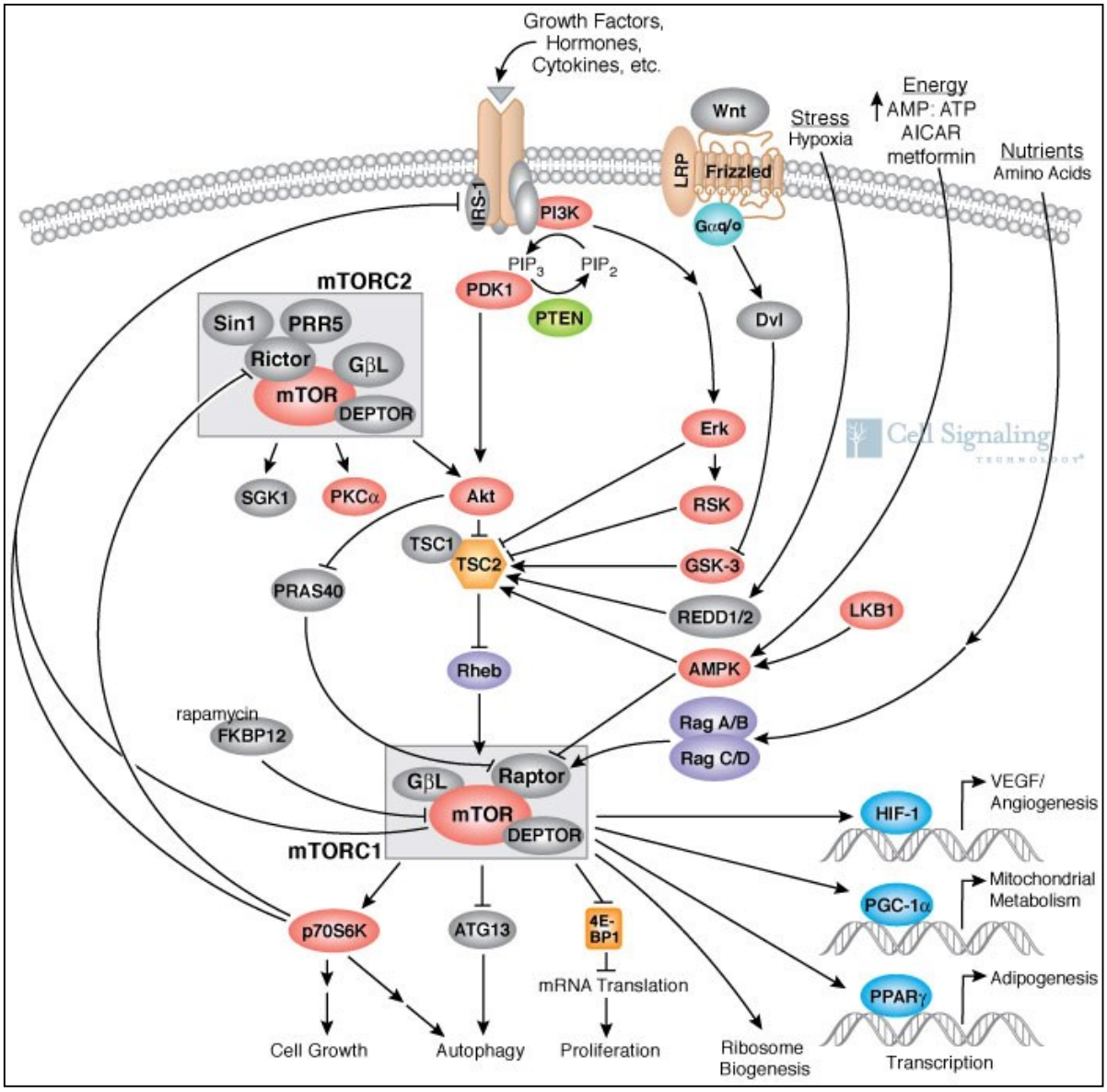

Figure 1-1. Overview of the insulin/AKT/mTOR pathway and downstream mediators. Illustration reproduced with permission, courtesy of Cell Signalling Technology, Inc. (www.cellsignal.com)

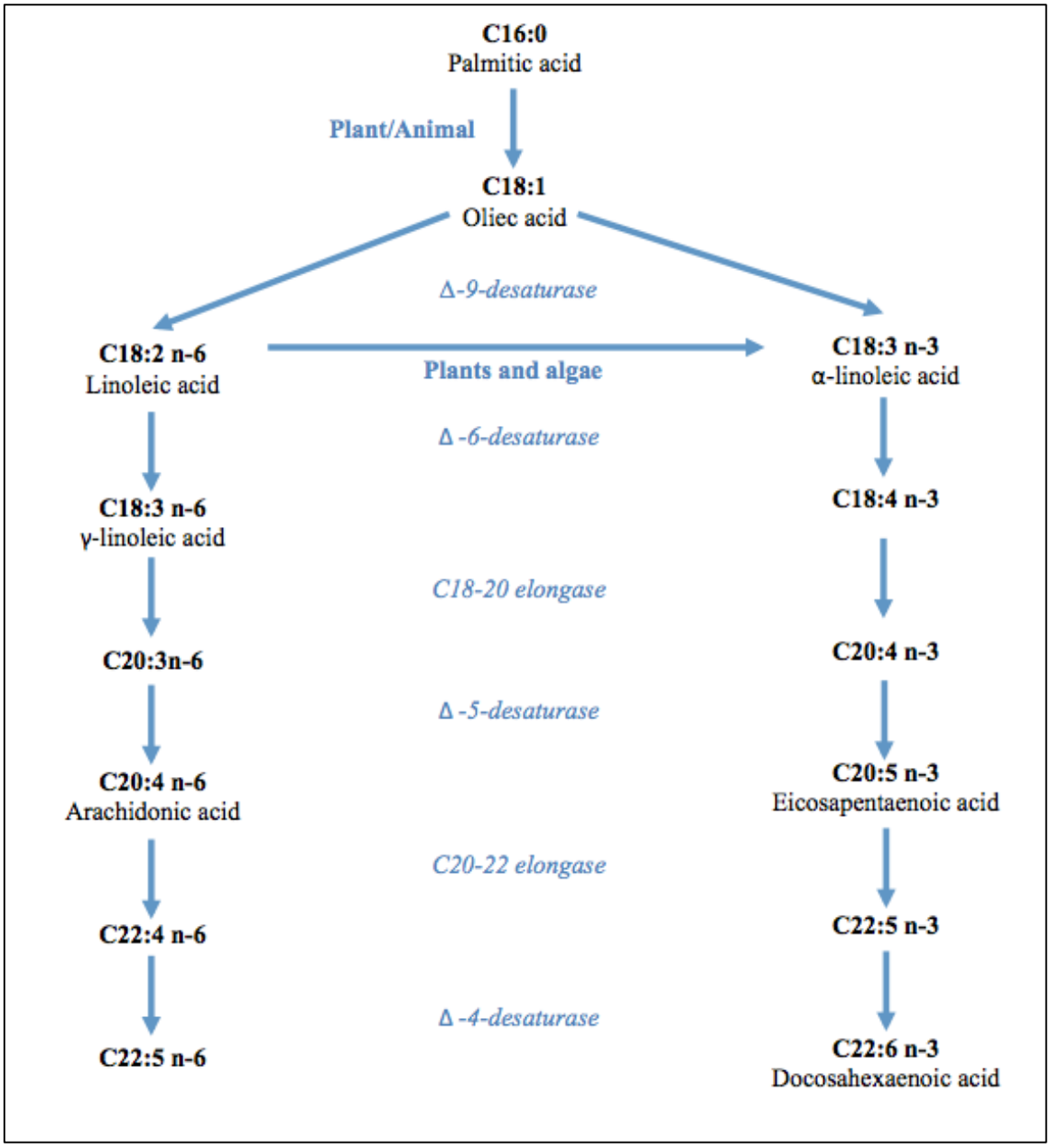

Figure 1-2. Schematic representation of essential fatty acids and their derivatives. Common fatty acid names are listed, and enzymes are italicized

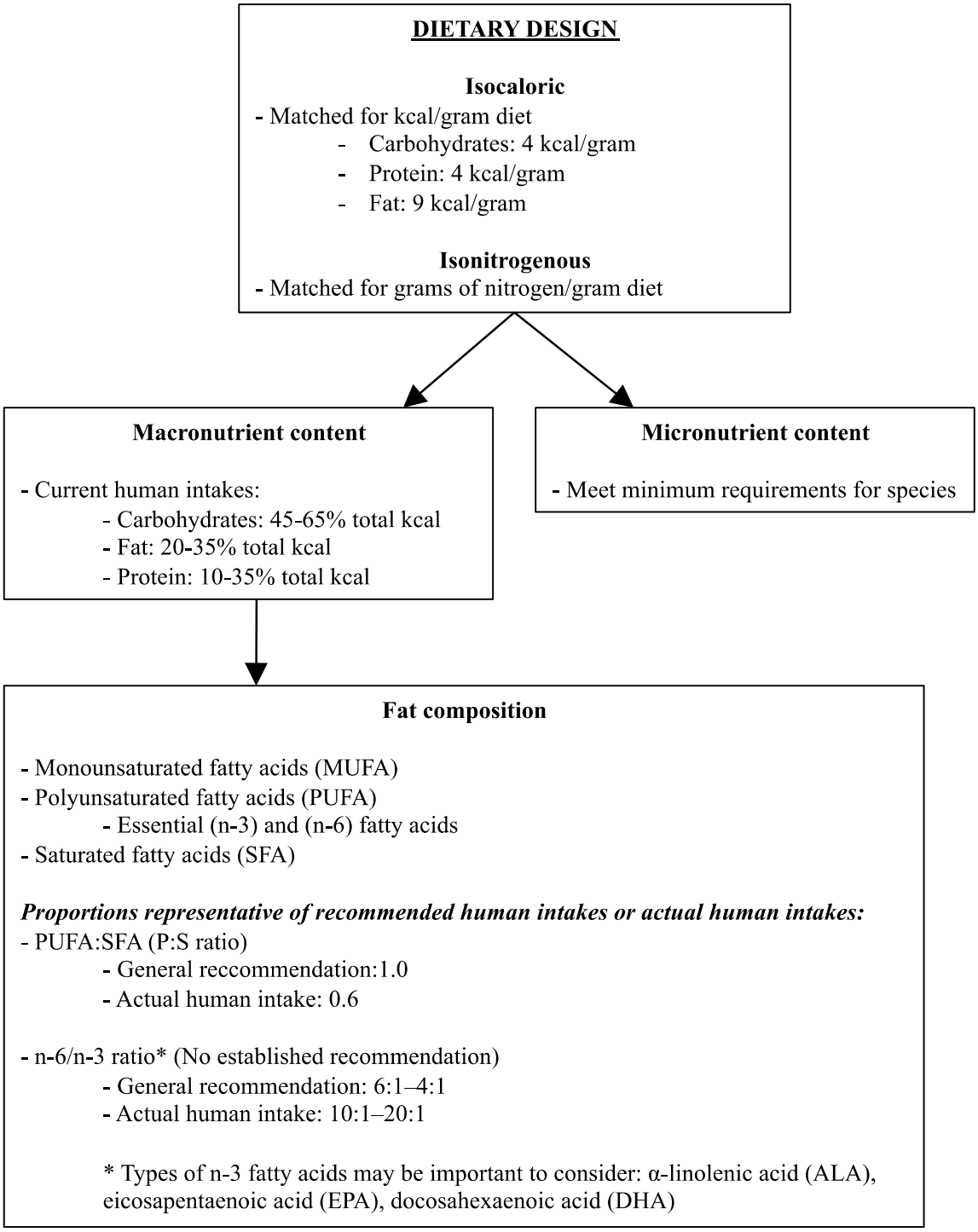

Figure 1-3. Schematic representation of proposed dietary design for use in animal models of cancer cachexia

CHAPTER 2

Research Plan

2.1 Rationale

Advanced cancer is associated with significant weight and muscle loss. High-resolution image-based techniques, such as CT, have revealed that in addition to low muscle mass, a pathological accumulation of fat in muscle tissue (myosteatosis) also occurs in cancer patients (Murphy et al., 2011*b*). Weight loss (>8%), low muscle mass, and myosteatosis are each independently associated with mortality (Martin et al., 2013; Prado et al., 2008). Further, since there is an association between loss of muscle (Lieffers et al., 2009), and more recently, skeletal muscle density, to proximity to death (Esfandiari et al., 2014) research suggests that skeletal muscle density be integrated into prognostic scores, thereby enhancing cancer patient management (Antoun et al., 2013). Although the relationship between myosteatosis and poor outcomes are recognized, the distinct mechanisms associated with myosteatosis have not been established.

First-line therapy for colorectal cancer is a combination of irinotecan (CPT-11) and 5-fluorouracil (5-FU). This treatment is currently the most effective treatment for the disease. However, chemotherapy may interfere with essential fatty acid metabolism and accelerate muscle wasting (Pratt et al., 2002). Previous clinical trials in our laboratory suggest a role for EPA and DHA in cancer-associated muscle changes. Subsequent studies reported that cancer patients undergoing chemotherapy had depleted levels of plasma EPA and DHA compared to healthy controls (Pratt et al., 2002). Cancer patients experiencing muscle gain during treatment exhibited the highest EPA and DHA plasma

concentrations, while patients with the greatest muscle loss exhibited the lowest EPA and DHA plasma concentrations (Murphy et al., 2010*a*). Lung cancer patients undergoing chemotherapy either received standard of care (SOC) or fish oil supplementation, a concentrated source of EPA and DHA, during cancer treatment (Murphy et al., 2011*b*). Patients in the SOC group experienced muscle loss during treatment concurrent with increases in intermuscular adipose tissue (IMAT), while those in the fish oil group experienced increases or stability in muscle mass during treatment, and concurrent reductions in IMAT. Results from these studies suggest an important relationship between muscle health and essential fatty acid status in cancer, and further suggest a potential role for EPA and DHA in modifying fatty muscle.

Findings from human clinical trials have been replicated in our laboratory in an animal model of colorectal cancer. Compared to healthy rats, tumour-bearing rats had higher concentrations of TG fatty acids in muscle, which progressively increased with treatment. Rats fed a fish oil diet for 3-weeks, prior to start of chemotherapy exhibited an increase in EPA and DHA in muscle TG and PL fatty acids concurrent with lower total TG fatty acid concentrations in muscle compared to the control group (Almasud, 2012). Results from this animal model suggest that EPA and DHA fed prior to chemotherapy initiation prevents fat infiltration into muscle; whether or not this intervention would be effective after fatty muscles develop is not known.

Several mechanisms have been proposed in the role EPA and DHA may play in ameliorating myosteatosis and muscle loss in conditions other than cancer. Cancer cachexia and diabetes share similarities in metabolic alterations; muscle wasting is a

hallmark of cachexia, while epidemiological evidence supports accelerated age-related muscle loss in type II diabetes (Chevalier & Farsijani, 2014). Further, insulin resistance, a pathway critical in muscle anabolism, manifests in both conditions, albeit has been studied much more extensively in the diabetes population (Winter et al., 2012; Copeland et al. 1987; Yoshikawa et al., 2001). N-3 fatty acids have been associated with improved insulin sensitivity through increasing the unsaturation of muscle membrane fatty acids and membrane fluidity (Martin de Santa Olalla et al., 2009), increasing the number of insulin receptors (Simopolous et al., 2002), and improving insulin action (Lichtenstein et al., 2000). These results suggest that EPA and DHA may restore insulin sensitivity in cancer, affecting downstream mediators of the insulin pathway, such as AKT and mTOR, involved in muscle anabolism, regeneration, and fat oxidation.

This study focuses on the effect of an EPA and DHA enriched diet after tumour implantation and during chemotherapy (CPT-11 + 5-FU) on the TG and PL fatty acid content and composition in skeletal muscle of tumour-bearing rats. Further, this study aims to investigate phosphorylated mTOR and AKT concentrations, potential mediators of the insulin pathway, as a possible mechanism for a benefit of EPA and DHA.

2.2 Objectives and Hypotheses

Objective 1

To evaluate morphology, lipid content and fatty acid composition of gastrocnemius muscle of:

i) Healthy Fischer 344 rats fed a control diet

ii) Fischer 344 rats bearing the Ward colorectal carcinoma fed a control diet

iii) Fischer 344 rats bearing the Ward colorectal carcinoma fed a control diet receiving either 1- or 2-cycles of chemotherapy (CPT-11 + 5-FU)

iv) Fischer 344 rats bearing the Ward colorectal carcinoma fed a diet containing fish oil, receiving either 1- or 2-cycles of chemotherapy (CPT-11 + 5-FU)

Hypothesis 1

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

i) Lower muscle cross sectional area

ii) Higher content of TG fatty acids in skeletal muscle tissue

iii) Higher content of adipocytes between muscle fibres (intermyocellular) and/or lipid droplets within muscle fibres (intramyocellular)

Hypothesis 2

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

i) Lower body weight

ii) Higher content of TG fatty acids in skeletal muscle tissue

iii) Lower proportion of total n-3 fatty acids, EPA, and DHA in muscle triglyceride and phospholipid fractions

iv) Lower muscle cross sectional area

v) Higher content of adipocytes between muscle fibres (intermyocellular) and/or lipid droplets within muscle fibres (intramyocellular)

vi) The effects above will occur to a greater magnitude after 2-cycles of chemotherapy compared to 1-cycle.

Hypothesis 3

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

i) Higher body weight

ii) Lower content of TG fatty acids in skeletal muscle tissue

iii) Higher proportion of total n-3 fatty acids, EPA, and DHA in muscle triglyceride and phospholipid fractions

iv) Higher muscle cross sectional area

v) Lower content of adipocytes between muscle fibres (intermyocellular) and/or lipid droplets within muscle fibres (intramyocellular)

These hypotheses are investigated in Chapter 3. The aim of this work is to investigate if EPA and DHA diet could reverse the pathological fat accumulation that

occurs with growth of Ward colorectal tumour during chemotherapy and to determine if the accumulation of fat occurs in triglycerides between or inside of myocytes.

Objective 2

To evaluate protein concentrations of mTOR (pSer2448), total-AKT, and phosphorylated-AKT in gastrocnemius muscles of:

i) Healthy Fischer 344 rats fed a control diet

ii) Fischer 344 rats bearing the Ward colorectal carcinoma fed a control diet

iii) Fischer 344 rats bearing the Ward colorectal carcinoma fed a control diet receiving either 1- or 2-cycles of chemotherapy (CPT-11 + 5-FU)

iv) Fischer 344 rats bearing the Ward colorectal carcinoma fed a diet containing fish oil, receiving either 1- or 2-cycles of chemotherapy (CPT-11 + 5-FU)

Hypothesis 1

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

i) Lower total muscle protein

ii) Lower concentration of mTOR (pSer2448) protein

iii) Lower ratio of phosphorylated-AKT to total-AKT protein

Hypothesis 2

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

i) Lower total muscle protein

ii) Lower concentration of mTOR (pSer2448) protein

iii) Lower ratio of phosphorylated-AKT to total-AKT protein

iv) The effects above will occur to a greater magnitude after 2-cycles of chemotherapy compared to 1-cycle.

Hypothesis 3

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

i) Higher total muscle protein

ii) Higher concentration of mTOR (pSer2448) protein

iii) Higher ratio of phosphorylated-AKT to total-AKT protein

These hypotheses are investigated in Chapter 4. The aim of this work is to investigate a possible mechanism for the ability for EPA and DHA to reverse pathological fat accumulation in the muscle through altering mediators of the insulin pathway.

CHAPTER 3

Skeletal muscle fat infiltration is reversed by dietary fish oil in an animal model of colorectal cancer receiving irinotecan/5-fluorouracil

3.1 Introduction

The majority of fat in the body is stored in adipose tissue, however, fat can also be associated with skeletal muscle. Intramyocellular fat is located within the cytoplasm of myocytyes, and intermuscular adipocytes are located peripheral to myocytes (Wronska & Kmiec, 2012). Intramyocellular lipids/lipid droplets within skeletal muscle are usually in direct contact with mitochondria to allow for rapid utilization in times of exercise and act as fuel stores for mitochondrial fat oxidation (Schrauwen-Hinderling et al., 2006) but decrease with acute exercise (Decombaz et al., 2001; Krssak et al., 2000) and are no longer present after marathon running (Staron et al., 1989; Kayar et al., 1986). An increased presence of lipid droplets in skeletal muscle has been associated with pathological states such as type 2 diabetes, insulin resistance and ageing (Crane et al., 2010; Bostrom et al., 2009). Further, increases in lipid droplet numbers have been directly associated with mitochondrial dysfunction (Crane et al., 2010; Conley et al., 2000).

Abnormal accumulation of lipids within the skeletal muscle is considered to be pathological, and is referred to as myosteatosis. In contrast to hepatosteatosis, which is highly characterized in a number of disease states, myosteatosis is poorly characterized, especially in the cancer population. Myosteatosis has been observed in advanced

colorectal cancer (Lieffers et al., 2009) and lung cancer (Murphy et al., 2011*b*), and has been associated with significant weight loss (Murphy et al., 2011*a*; Stephens et al., 2011). Like sarcopenia, myosteatosis has been associated with worsened outcomes and poorer survival in cancer patients (Antoun et al., 2013; Martin et al., 2013; Sabel et al., 2011). This pathology in humans has not been completely characterized. In melanoma patients, Sabel et al. (2011) showed poor disease-free and distant disease-free survival in patients with the lowest muscle attenuation, which is a measurement correlating with fatty muscle (Goodpaster et al., 2000). Furthermore, patients with the lowest psoas muscle attenuation exhibited increased surgical complications and wound infection (Sabel et al., 2011). In patients with solid tumours of the lung and gastrointestinal tract, Martin et al. (2013) reported that reduced muscle attenuation was independently prognostic of poor survival. Antoun et al. (2013) reported significantly shorter overall survival in patients with metastatic renal cell carcinoma who exhibited skeletal muscle attenuation values below the median.

People with non-small cell lung cancer patients undergoing first-line chemotherapy either received standard of care (SOC) or were supplemented with fish oil (FO) containing EPA and DHA at a dose of 2.2 g/day. Skeletal muscle mass, assessed by CT, revealed that patients in the SOC group experienced muscle loss and increases in intermuscular adipose tissue (IMAT) over the course of chemotherapy, whereas patients in the FO group maintained or increased their muscle mass and lost IMAT. The capacity for fish oil supplementation to potentially attenuate fat deposition in muscle requires further investigation.

The objectives of this study were to determine the effect of a tumour (Ward colorectal carcinoma) on the lipid content and composition, in rat muscle tissue compared to healthy rats. The second objective was to compare the lipid content and composition in the muscle, as well as the morphological muscle characteristics in rats receiving 1- and 2-cycles of chemotherapy (CPT-11 + 5-FU) consuming a control diet, compared to rats consuming a diet containing fish oil.

It was hypothesized that the presence of a tumour increases the triglyceride fatty acids in muscle compared to a non-tumour bearing state, and that each cycle of chemotherapy treatment would further increase triglyceride content. A fish oil diet, containing EPA and DHA, fed during chemotherapy treatment to tumour-bearing rats will reduce the amount of triglyceride in muscle, and increase total n-3 fatty acids, specifically EPA and DHA content in phospholipid and triglyceride fatty acids compared to a control diet. It was hypothesized that the presence of a tumour would reduce the cross sectional muscle area and increase the content of lipids between or within myocytes. A fish oil diet, containing EPA and DHA, fed during chemotherapy treatment to tumour-bearing rats will reduce the lipid deposition between or inside of myoctyes compared to a control diet fed during chemotherapy.

3.2 Study Design

3.2.1 Animal model

Female Fischer 344 rats weighing 150-180 grams at 11-12 weeks old were

received from Charles River (St. Constant, Quebec, Canada). Rats were housed two per cage in a controlled, positive air pressure room kept at a constant temperature of 22°C. Cages contained bedding and filter tops. Rats received twelve hours of light per day. Water and food were provided *ad libitum* throughout the entire experiment (see **Figure 3-1**).

3.2.2 Tumour

One week after starting the control diet, rats were implanted with the Ward colorectal carcinoma (provided by: Dr. Y. Rustum, Department of Cancer Biology, Chair, Director of Institute Core Resources, Roswell Park Cancer Institute, Buffalo, NY; Cao & Rustum, 2000). The colorectal carcinoma cells were transplanted subcutaneously into the flank of the rats (0.05 g). Tumours were measured daily in centimetre (cm) units with callipers in three dimensions: length (L), width (W), and height (H). Tumour volume was calculated according to the following calculation: 0.5 x (L x W X H), and expressed as cm^3. Tumour volume was converted to mass using a density of: 1 g/cm^3. Tumours grew to approximately 2.3 cm^3 (or 1.2% body weight) before rats received chemotherapy. Tumour volume during chemotherapy was expressed as *relative tumour volume*. Relative tumour volume is a percentage change from the average tumour volume prior to chemotherapy (set at 100%).

3.2.3 Diet

Diets were based on the American Institute of Nutrition-76 (AIN-76) modified basal ingredients with fat-source omitted (Harlan Teklad). Diets contained 40% total kilocalories (kcal) from fat, 40% total kcal from carbohydrates, 20% total kcal from

protein (**Table 3-1**). All rats consumed a control diet beginning seven-days after the acclimation period until the first day of chemotherapy, when they either continued to consume the control diet or began a fish oil diet. The fish oil diet contained the same proportion of macronutrients as the control diet, differing only in fatty acid composition (2.0 g FO/100 g diet; **Table 3-2**). The added fish oil contained 51% fatty acids as EPA, and 21% fatty acids as DHA.

3.2.4 Body weight and food intake

Body weight was recorded every other day prior to chemotherapy, every day during chemotherapy, and at euthanization. Body weight was converted to tumour-free body weight for data interpretation and statistical analysis. Body weight during chemotherapy is expressed as *relative body weight*. Relative body weight is a percentage change from the average body weight prior to chemotherapy (set at 100%). Food intake was recorded every other day prior to chemotherapy, every day during chemotherapy, and at euthanization. Food intake during chemotherapy was expressed as *relative food intake.* Relative food intake is a percentage change from the average food intake prior to chemotherapy (set at 100%).

3.2.5 Chemotherapy (irinotecan + 5-fluorouracil)

Rats bearing a tumour, receiving no chemotherapy, served as a control group (TUM; n=8) and consumed control diet throughout the entire study. Rats serving as healthy controls (REF; n=8) did not receive tumour implantation or chemotherapy and

consumed the control diet throughout the entire study. For rats receiving chemotherapy, CPT-11 + 5-FU were administered one week apart. Atropine was administered at a dose of 1 mg/kg s.c. injection immediately before each CPT-11 injection. CPT-11 was administered at dose of 50 mg/kgBW/day s.c. injection on Day 0 (cycle-1), and Day 7 (cycle-2); 5-FU was administered at a dose of 50mg/kgBW/day s.c. injection on Day 1 (cycle-1), and Day 8 (cycle-2). On the first day of chemotherapy (Day 0), rats were assigned to one of two diet groups receiving either 1- or 2-cycles of chemotherapy: (1) control-fed rats receiving 1-cycle (CON1; n=8), 2-cycles (CON2; n=8) of chemotherapy (CON1n=16); or (2) fish oil-fed rats receiving 1-cycle (FO1; n=8), 2-cycles (FO2; n=8).

3.2.6 Study termination and tissue collection

Rats were euthanized by carbon dioxide (CO_2) asphyxiation on Day (-)14 (n=8); Day 0 (n=8); Day 7 (n=16); and Day 14 (n=16). Gastrocnemius muscles were isolated and rapidly frozen in liquid-nitrogen cooled isopentane (C_5H_{12}; 2-methylbutane) to achieve the melting point temperature of -161°C. Gastrocnemius muscles were then stored at -80°C until further analysis.

3.3 Methods and Fatty Acid Analysis

3.3.1 Muscle mass

Before lipid extraction began, the wet weight of frozen gastrocnemius muscles (stored at -80°C) was recorded. When both the left and right gastrocnemius muscles were available, the mean of the left and right dry muscle weights were recorded.

3.3.2 Modified Folch lipid extraction

Fat was extracted from gastrocnemius muscles (100 mg) using a modified Folch method (Folch et al., 1957). Glass homogenizing beads (0.5 mm diameter) were added to plastic homogenizing screw-top tubes in an amount to fill the bottom cone of the tube. Muscle samples were transferred into homogenizing tubes, and 0.8 mL of calcium chloride ($CaCl_2$; 0.025%) solution was subsequently added to each tube. Samples were homogenized in (FastPrep ®-24, MP Biomedicals, Santa Ana, California, USA) for 1 minute total, in 20-second intervals; samples were put on ice for 15-seconds between each homogenization interval to prevent the tissue temperature from increasing. After homogenizing, the tissue homogenate was transferred to glass methylation tubes and an additional 0.8 mL of $CaCl_2$ was added to each tube and vortexed. Subsequently, 8 mL of chloroform/methanol solution (C/Me; 2:1 v/v) was added to each tube and vortexed. Tubes were flushed with nitrogen gas before being stored overnight at 4°C.

The bottom layer containing lipids was transferred to a 5 mL methylation tube. The original tube was washed with 2 mL of chloroform/methanol/water (C/Me/H_2O; 86:14:1, v/v). Lipids were subsequently allowed to separate again, and the clean bottom layer was added to the 5 mL methylation tube. Samples were dried under nitrogen gas; 160 µL of C/Me was added to each tube and then vortexed.

3.3.3 Thin Layer Chromatography

Thin layer chromatography (TLC) plates (G plated, Silica Gel, 20 x 20 cm, 250 microns, Analtech Inc., Newark, DE) were placed into solvent tanks containing chloroform. Once the chloroform reached ~1.5 cm from the top of the plate, plates were removed from the solvent tank and left to dry. Plates were then heated for 1-hour at 160°C in an oven, and left to cool. Plates were labelled, and samples were spotted on plates in duplicate; the duplicate column for each sample was spotted on a second plate. Spotted plates were then placed into a solvent tank containing petroleum ether/ethyl ether/glacial acetic acid (PE/EE/AA; 80:20:1, v/v). Plates were left to run until the solvent mixture reached ~1.5 cm from the top of the plate. Plates were then removed from the solvent tank and left to air dry. Once dry, plates were sprayed with 0.1% ANSA (1-anilinonaphthalene-8-sulfonic acid ammonium salt) to visualize the phospholipid (PL) and triglyceride (TG) bands under ultraviolet light. PL and TG bands were identified and labelled, scraped, and added to individual methylation tubes containing internal standards, C17:0 (10.23 mg/100 mL hexane) and C15:0 (10.2 mg/100 mL hexane), respectively.

3.3.4 Phospholipid and Triglyceride methylation

To saponify TGs, 1 mL of potassium hydroxide (KOH) in methanol was added to TG tubes and heated for 1-hour in a dry bath at 110°C, and then allowed to cool. Two mL of hexane, and 1 mL of boron triflouride (BF_3) were added to tubes containing TG and PL scrapings. All tubes were heated on a dry bath for 1-hour at 110°C. Once tubes cooled, 1 mL of double distilled water (ddH_2O) was added. Tubes were vortexed and refrigerated at 4°C overnight to allow for separation. The clean top layer from each TG and PL tube was transferred to a gas liquid chromatography (GLC) glass vial and dried under nitrogen gas. Dried GLC vials were washed with 200 µL of hexane, and entire volume was pipetted into glass inserts, placed into GLC vials and capped. Samples were stored at -20°C until analysis with gas liquid chromatography.

3.3.5 Gas Liquid Chromatography

GLC was used to analyze samples. Methylated samples were subjected to GC-FID analysis on an Agilent 7890A instrument. Briefly, 1.5 µL of sample was injected onto a 30 m x 0.25 mm ID BP20 column with a 0.25 µm film thickness (SGE Analytical Science) using a split flow injection. The spilt flow to the GC column was set to 15 and the initial temperature of the inlet was held at 275˚C. The GC run parameters were as follows; initial temperature was set to 150 ˚C and held for one minute. The temperature was increased at a rate of 5˚C/min to a temperature of 180˚C. The temperature ramp rate was then slowed to 2˚C/min until a temperature of 225˚C was reached. After holding this temperature for five minutes, the temperature was quickly raised (80˚C/min) to 245˚C

and held for 3 minutes. Identification of the fatty acids present in the sample was achieved by comparing their retention times to that of a standard methylated fatty acid mixture (GLC461, Nu-chek Prep Inc., Elysian, USA).

3.3.6 Quantification of fatty acid composition

Peaks of saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids were separated between 6 and 24 carbon chain lengths. TG fatty acid amounts were calculated by using the area peak of the known concentration of the C15:0 internal standard peak, and PL fatty acid amount by comparison with the known concentration of the C17:0 internal standard peak. The proportion of individual fatty acids within each fraction was expressed as a percentage (%) of total fatty acids in sample. An acceptable level of variation between duplicates was set at 5%. Mean fatty acid amounts were calculated from duplicates; otherwise sample duplicates that varied >5% were re-analyzed using gas liquid chromatography.

3.3.7 Muscle sectioning

Frozen gastrocnemius muscles were embedded in optimal cutting temperature compound (Tissue-Tek® O.C.T™ Compound, Sakura Finetek Europe, Zoeterwoude, The Netherlands) and sectioned with a cryostat (Jung CM3000 Research Cryostat Microtome, Leica Microsystems, Solms, Germany) at -20˚C. Serial sections (10 µm) were thaw mounted on uncoated precleaned glass microscope slides (Fisherbrand Superfrost™ Plus

Microscope Slides, Fisher Scientific, Waltham, MA, USA). Muscle sections were left to air dry for approximately 1-hour and were stored at -80˚C until staining.

3.3.8 Hematoxylin and eosin staining and muscle cross sectional area quantification

Muscle sections were stained with hematoxylin and eosin (H&E) for preliminary morphological analysis. Sections were first air dried for several minutes to remove moisture. Slides were stained with filtered 0.1% Mayers Hematoxylin (Sigma-Aldrich, St. Louis, MO, USA) for ten minutes, and then rinsed in cool running deionized water for five minutes. Mayer's hematoxylin is free of alcohol or organic solvents to prevent removal of lipids during staining procedures. Slides were then dipped in 0.5% Eosin (1.5 g dissolved in 300 mL of 95% ethanol) 12 times, followed by dipping in deionized water. Slides were then dipped 10 times each in 50% ethanol and 70% ethanol, equilibrated in 95% ethanol for 30 seconds, and 100% ethanol for one minute before being dipped in xylene several times and mounted with Organo/Limonene mounting medium (Sigma-Aldrich, St. Louis, MO, USA) and a coverslip. Completed and stained sections were then photographed at 1X magnification (Gene Tools, Syngene, Synoptics Limited, Frederick, MD, USA) to ensure that the entire muscle was pictured. Photos of muscle sections were uploaded into a custom analysis program (original program developed by Dr. Dirk Pette, University of Konstanz, Konstanz, Germany and Dr. Ted Putman, University of Alberta, Edmonton, Alberta) for cross sectional area quantification. A haemocytometer scale bar was used for measurement, and whole muscle cross sectional area was determined in

triplicate. The mean of the triplicate measurements were used for quantitative comparisons.

3.3.9 Oil red O staining and qualitative analysis

Frozen muscle sections were air dried for several minutes to remove moisture before beginning staining. Prior to staining, oil red O was dissolved to a stock solution by adding 0.7 g oil red O to 100 mL propylene glycol (Fisher Scientific, Waltham, MA, USA) slowly, while stirring. The solution was heated to 100˚C but not over 110˚C for three minutes, while being stirred consistently and then filtered through Whatman paper #2 (Whatman, Maidstone, UK) to remove any crystallized oil red O. Frozen sections were fixed in 10% neutral buffered formalin for ten minutes, before being placed in distilled water (changed twice; soaked for two minutes each). Sections were placed in 50% propylene glycol, followed by absolute propylene glycol (changed twice; soaked for two minutes each). Sections were then soaked in oil red O solution for 20 minutes on an automatic shaker, and placed for three minutes each in: absolute propylene glycol, 85% propylene glycol, and 50% propylene glycol. Sections were then rinsed in distilled water, and counterstained in Mayer's Hematoxylin (Sigma-Aldrich, St. Louis, MO, USA) for five minutes before another rinse in distilled water. Sections were then ran under warm tap water, followed by a rinse in distilled water. Excess water was drained from sections before mounting with water-soluble mounting medium (GelTol Aqueous Mounting Medium, Sigma-Aldrich, St. Louis, MO, USA) and mounted with 24 x 50 mm glass coverslips. Qualitative analysis was performed blinded in a triplicate manner. Sections were visualized under a ZEISS AXIO Compound Light Microscope (AX10 Scope A.1,

Carl Zeiss Group, Toronto, ON, Canada) at magnifications of 2X, 10X, 20X, 40X and 63X. Colour images were taken with an Optronics MacroFire Digital Camera (Optronics, Goleta, CA, USA) using Leica TCS-SP2 spectral confocal and multiphoton system (Leica Camera, Solms, Germany). Sections were qualitatively described, followed by group comparisons.

3.4 Statistical Analysis

Data are reported as mean ± standard deviation (SD). Two-way independent sample *t*-tests were used to compare REF and TUM groups. One-way Analysis of variance (ANOVA) with Bonferroni post-hoc comparisons was used to compare fatty acids between TUM, CON1, CON2, FO1 and FO2 groups. Repeated measures-ANOVA with Bonferroni post-hoc comparisons was used to compare body weight and food intake changes during chemotherapy between groups. Significance for all statistical tests was reported at the level of $P<0.05$. Statistical analysis was completed using IBM Statistical Package for the Social Sciences (SPSS) for Windows (version 21.0, Chicago, IL).

3.5 Results

3.5.1 Body weight

During the first cycle of chemotherapy, the body weight of all groups decreased, and recovered by the end of the first cycle (Day 6; **Figure 3-2**). During the first cycle of chemotherapy, fish-oil fed rats (FO1, n=8; FO2, n=8) had a significantly higher body

weight overall compared to control-fed rats (CON1, n=8; CON2, n=8) (repeated-measures ANOVA, *P*=0.001). During the second cycle of chemotherapy, the body weight of all groups decreased again, and recovered by the end of the second cycle (Day 13). During the second cycle of chemotherapy, there was no significant difference in overall body weight between fish-oil fed rats (FO2, n=8) and control-fed rats (CON2, n=8).

3.5.2 Muscle mass

There were no significant differences between the left and right gastrocnemius muscles within the same animal, nor were there any significant differences in the gastrocnemius muscle weights (mean wet weight: 765 ± 61.2 mg) between any of the groups (REF, TUM, CON1, CON2, FO1, FO2).

3.5.3 Food intake

During the first cycle of chemotherapy, food intake decreased in all groups and was restored to baseline intakes by end of the first cycle (Day 6; **Figure 3-3**). Mean food intake during cycle-1 of chemotherapy was 6.8 ± 2.7 g/day in the control group (CON1 + CON2), and 6.6 ± 3.0 g/day in the fish oil group (FO1 + FO2) equivalent to 71.3 mg EPA + DHA/day (**Table 3-2**). During the first cycle of chemotherapy, there was no significant difference in food intake between fish-oil fed rats (FO1, n=8; FO2, n=8) and control-fed rats (CON1, n=8; CON2, n=8). During the second cycle of chemotherapy, food intake decreased again in both groups and was restored to baseline intakes by the end of the second cycle (Day 13). Mean food intake during cycle-2 in the control group (CON2)

was 7.5 ± 3.1 g/day, and in the fish oil group (FO2) was 6.0 ± 3.1 g/day, equivalent to 66.0 mg EPA + DHA/day). During the second cycle of chemotherapy, control-fed rats ate significantly more food (CO2, n=8) compared to fish oil fed rats (FO2, n=8; repeated measures-ANOVA, *P*=0.05).

3.5.4 Tumour volume

There were no significant differences between mean relative tumour volumes in fish oil-fed animals compared to control-fed animals after both 1- and 2-cycles of chemotherapy (**Figure 3-4**). Following the first cycle of chemotherapy, both fish oil and control-fed groups exhibited a significant decrease in tumour volume, with a slight increase in tumour volume by the end of cycle-1 (Day 6). The same pattern of reduced tumour volume was observed at the beginning of cycle-2 (Day 7 and Day 8) followed by a slight increase in tumour volume at the end of cycle-2 (Day 14; **Figure 3-4**). When relative tumour volume was expressed as a ratio of relative body weight, (relative TV/relative BW) control fed animals exhibited a higher ratio of relative TV/relative BW compared to fish oil-fed animals during the first cycle of chemotherapy (repeated measures-ANOVA; *P*=0.024). After the first cycle of chemotherapy began, both fish oil and control-fed groups exhibited a reduced ratio of relative TV/relative BW, with a slight increase in this ratio by the end of cycle-1 (Day 6; **Figure 3-5**), although the change occurred at a slower rate in the control group compared to the fish oil group. The same pattern of reduced relative TV/relative BW was observed at the beginning of cycle-2 (Day 7 and Day 8), which continued to decline until the end of cycle-2 (Day 14; **Figure 3-5**).

3.5.5 Triglyceride Fatty Acids

Total amount of triglyceride (TG) fatty acids in gastrocnemius muscle of rats was significantly higher in TUM compared to REF (P = 0.001; **Figure 3-6)** CPT-11 + 5-FU significantly increased TG fatty acid content in the muscle after each cycle of chemotherapy for both control and fish oil-fed animals (P=0.001; **Figure 3-7**, **Table 3-3**). Rats provided fish oil had significantly lower TG fatty acid content after 1-cycle of chemotherapy compared to TUM (P=0.001). Although the TG fatty acid content increased in the muscle after the second cycle of chemotherapy (FO2), the values were similar to rats that were bearing a tumour alone (P=0.001; **Figure 3-7**, **Table 3-3**). Total TG fatty acid content in TUM group compared to CON1 group was not significantly different. After both 1- and 2-cycles of chemotherapy, the fish oil group exhibited significantly less TG than rats consuming the control diet (P=0.001; **Figure 3-7**, **Table 3-3**). Overall, in rats receiving control diet, chemotherapy significantly increased TG fatty acids with each successive cycle, with CON2 having the highest total TG fatty acid content compared to all other groups (P=0.001).

Compared to REF, TUM had a higher proportion of C18:1 in TG fatty acids, and a lower total n-3 fatty acids in TG fatty acids. Compared to TUM group, FO1 and FO2 exhibited a significantly lower proportion of C16:0 (**Table 3-3**). After 1 cycle of chemotherapy, there was no significant difference in the proportion of C18:0 or total SFA between CON1 and FO1. Compared to control-fed rats (CON1, CON2), fish oil fed rats (FO1, FO2) exhibited a greater proportion of total n-3 fatty acids, EPA, and DHA in muscle TG fatty acids after both 1- and 2- cycles of chemotherapy (**Table 3-3**). After 2-

cycles of chemotherapy, CON2 also exhibited a greater proportion of total SFA in muscle TG fatty acids compared to FO2 (*P*=0.019; **Table 3-3**).

3.5.6 Phospholipid Fatty Acids

Total amount of phospholipid (PL) fatty acids in gastrocnemius muscle of rats was not different between TUM and REF (2990.9 ± 704.8 versus 4280.6 ± 1931.3). CPT-11 + 5-FU significantly reduced PL fatty acid content in control-fed animals after 1-cycle of chemotherapy (CON1; *P*=0.006) compared to TUM, and in fish-oil fed animals (FO1), PL fatty acid content was similar to TUM (**Figure 3-8**; **Table 3-4**). Total PL content in muscles of CON2, FO1, and FO2 groups were not significantly different from one another, but these groups exhibited significantly greater PL fatty acid content compared to CON1 (*P*=0.006).

Compared to REF, TUM had a higher proportion of C18:1 and lower proportion of C22:6 in PL fatty acids (*P*=0.004; P=0.001, respectively). After 1 cycle of chemotherapy, FO1 exhibited a lower proportion of C20:4n-6 in muscle PL compared to CON1 (*P*=0.001; **Table 3-4**). Between diet groups, control-fed animals exhibited a greater proportion of total SFA in muscle PL compared to fish oil-fed animals (FO1 + FO2) after both 1- and 2-cycles of chemotherapy (*P*=0.001). Compared to control-fed rats (CON1 + CON2), fish oil fed rats (FO1 + FO2) exhibited a greater proportion of total n-3 fatty acids, EPA, and DHA in muscle PL fatty acids, a lower proportion of total n-6 fatty acids in muscle PL fatty acids (CON1 versus FO1), and a reduced n-6/n-3 ratio after both 1- and 2- cycles of chemotherapy (**Table 3-4**).

3.5.7 Muscle cross sectional area

Muscle cross sectional area (CSA; mm^2) was significantly greater in REF compared to TUM (P=0.027; **Figure 3-9**). When TUM was compared to all other groups by one-way ANOVA, TUM was only smaller than FO2 (P=0.034; **Figure 3-10**). However, when the main-effect of diet was tested by an independent two-tailed *t*-test, comparing the effect of feeding a control diet without fish oil (CON1 + CON2) to feeding a fish oil diet (FO1 + FO2), fish-oil fed rats exhibited a significantly greater muscle CSA compared to control-fed rats (P=0.044; **Figure 3-11**).

3.5.8 Qualitative lipid analysis using red O staining

A qualitative analysis of muscle section images revealed that REF muscle sections contained no visible red staining of neutral lipids (**Figure 3-12**). Compared to REF, TUM exhibited a greater amount of neutral red staining in muscle sections. Red lipid stains appeared to be located between muscle fibres in TUM. There was a small amount of intramyocellular red staining, however the majority of staining was located in the periphery of myocytes (**Figure 3-13**). In contrast, CON1 contained a greater amount of intramyocellular red lipids staining compared to TUM, FO1, and FO2; qualitative analysis of CON1 muscle sections also revealed red lipid staining between myocytes, with the majority of red lipid staining uniformly distributed across individual muscle cells (**Figure 3-14**). In FO1 muscle sections, qualitative analysis revealed few visible red lipid stains. There was no visible red lipid staining located inside of myocytes or between muscle fibres at both lower and higher magnifications (**Figure 3-15**). CON2 muscle

sections exhibited the greatest density of red neutral lipid staining. Qualitative analysis revealed that CON2 muscle sections had several areas of densely stained areas with the majority of staining contained between muscle cells. There were few visible locations of intramyocellular red lipid staining at both lower and higher magnifications. Compared to CON1, CON2 contained a greater appearance of red staining however it did not appear uniformly distributed in CON2 compared to CON1; red lipid staining appeared to be concentrated in certain areas of the muscle sections in CON2 (**Figure 3-16**). Compared to FO1, FO2 contained a greater content of red neutral lipid staining in muscle sections, however the amount of visible stain was less than CON2. FO2 contained oil red O staining between muscle cells, with very little intramyocellular staining (**Figure 3-17**). Overall, qualitative analysis revealed that compared to control-fed animals, fish oil-fed animals appear to have less red neutral lipid staining.

3.6 Discussion

The pathological accumulation of fat into muscle (myosteatosis) has recently been described as an independent risk factor for mortality in cancer patients (Martin et al., 2013; Prado et al., 2008; Sabel et al., 2008), and may be an important prognostic factor to consider in cancer patient management (Antoun et al., 2013). This study is one of the first to quantify triglyceride fatty acids and measure the composition of fatty acids in an animal model of cancer receiving treatment (CPT-11 + 5-FU). Results from the present study revealed that compared to a control diet-fed group, a fish oil intervention started concurrent with and continuing throughout chemotherapy resulted in a two-fold decrease in TG fatty acid content compared to tumour-bearing animals. This finding of reversal of

fatty muscle by fish oil has not been previously reported in a model of cancer, nor in any other disease states to our knowledge. The findings from the present study support the results from a previous clinical trial conducted in our laboratory, that reported fish oil supplementation in advanced non-small cell lung cancer patients undergoing treatment to result in maintenance or increases in overall muscle mass, concurrent with a reduction in intermuscular adipose tissue, measured by CT, compared to patients receiving standard of care (Murphy et al., 2011*b*). Similar to this previous clinical trial, results from the present study also indicate that fish oil supplementation administered during chemotherapy results in reduced fat within the muscle compared to a control-fed group.

Findings from the present study parallel previous results from our laboratory (Almasud, 2012) where increased muscle TG content was observed in the muscle of tumour-bearing rats compared to a healthy reference group, confirming that 2-weeks of tumour growth results in fat deposition in the muscle. However, starting a fish oil-enriched diet at the time of chemotherapy reduced tumour-associated fatty muscle. Compared to healthy animals, control-fed animals receiving 2-cycles of chemotherapy had a 4-fold increase in muscle TG fatty acid content. Following the second cycle of chemotherapy, the muscle TG fatty acid content in the fish oil-fed group was similar to that observed in animals with tumours not receiving chemotherapy.

Muscle contains a variety of lipid species including triglycerides and diacylglycerols, phospholipids, free fatty acids, and cholesterol esters. The histological stain oil red O, which was used in the present study, allows for visualization of neutral lipids (mainly triglycerides) with an orange-red tint. This method has been applied to

visualize intramyocellular lipids and intermuscular adipocytes (Goodpaster et al., 2000; Koopman et al., 2001). There is one publication providing direct evidence that muscles of cancer patients exhibit an increased number of intramyocellular lipid droplets (Stephens et al., 2011). In the present study, qualitative observations reveal red neutral lipid staining both between and within muscle cells of tumour-bearing animals that progressively increased with each chemotherapy cycle. The increases observed in TG fatty acid content in control-fed animals after both 1- and 2-cycles of chemotherapy is in line with the higher content of red lipid staining observed in muscle sections compared to fish oil-fed animals. It was evident from qualitative analysis that with increased TG fatty acid content, there is a concurrent increase in the amount of red neutral lipid staining between myocytes, and to a lesser extent, within myocytes. The staining located between muscle cells may be attributed to adipocytes, however additional staining methods and histological analysis would be required in order to determine the nature of the lipid in these areas. This preliminary qualitative analysis supports other findings from cancer patients exhibiting an increased amount of intramyocellular lipid droplets (Stephens et al., 2011), however, successive cycles of chemotherapy, appears to contribute to increased content of lipids between muscle cells as well. Although myosteatosis is defined by an ectopic accumulation of lipids in muscle tissue, it is not known whether or not it is the absolute amount of fat, type of fat, or anatomical location of fat that may contribute to pathological effects. For example, studies have suggested that the accumulation of diacylglyercols, but not triglycerides, is independently associated with insulin resistance in non-adipose tissue (Chabowski et al., 2012). Future research is warranted in order to determine if the nature of the lipid species within muscle in a pathological state have differing biological effects.

Interestingly, after 1-cycle of chemotherapy, rats fed a control diet experienced a significant reduction in total muscle PL fatty acids compared to tumour-bearing rats receiving no chemotherapy, whereas rats fed a fish oil diet maintained their muscle PL fatty acid content throughout chemotherapy. Since the TG fatty acid content concurrently increased while PL fatty acid content decreased, the changes in PL content are likely not attributed to adipocyte or lipid droplets, and are more likely attributed to alterations in myocyte number or changes in organelles. This hypothesis is supported by the observation that animals fed a control diet had reduced muscle cross sectional area compared to fish oil-fed animals (**Figure 3-11**) however these proposed hypotheses would require further quantitative analysis of muscle fibre number and size. Following a second cycle of chemotherapy, total muscle PL fatty acids in the control-fed group significantly increased to comparable levels of tumour-bearing animals that received no chemotherapy. This change in PL content in cycle-2 may be a result of increased adipocyte or lipid droplet or changes in muscle fibres, since this was concurrently associated with a two-fold increase in TG fatty acid content. These findings are supported by the qualitative observations made from oil red O histology staining.

Remarkably, despite the significantly lower food intake in the fish oil group during cycle-2, fish oil-fed animals maintained their body weight (**Figures 3-2** and **3-3)**. Fish oil supplementation in combination with a reduced caloric diet promoted weight loss in obese individuals (Micallef et al., 2009), yet has been associated with weight maintenance in weight-losing cancer patients (Finocchiaro et al., 2012; Murphy et al., 2011*b*). EPA and DHA have been reported to increase the capacity for oxidative metabolism in muscle that may relate to changes in glycolytic enzymes and

improvements in metabolic flexibility (Hessvik et al., 2010). These findings may point to the ability for EPA and DHA to evoke varying effects depending on the biological background, or for the ability to operate differently depending on the tissue type. For example, in the muscle, EPA and DHA have been shown to promote activation of the mTOR pathway involved in muscle hypertrophy (reviewed by Gray and Da Boit, 2013; Smith et al., 2011) yet in tumours, EPA and DHA have been shown to inhibit activation of the mTOR pathway and reduce tumour cell growth (Friedrichs et al., 2011). Future investigations are warranted to determine the ability for EPA and DHA to differentially affect mechanisms in various tissues.

Fatty acid metabolism and fatty acid status during treatment for cancer patients is not well characterized. Depletion of long-chain PUFAs has been reported during high-dose chemotherapy (MacDonald et al., 2003; Pratt et al., 2002). These findings, as well as those from other clinical trials may suggest a conditional essentiality of long-chain PUFAs during treatment, since chemotherapy has been shown to alter the endogenous production of EPA and DHA from C18:3n-3 in rat muscle (Bordoni et al., 1999). The animal model used in the present study has previously been shown to increase n-3 fatty acid content in muscle tissue within 7-days of fish oil supplementation (unpublished data; see Xue et al., 2007). These results were confirmed in the present study, since fish oil-fed animals exhibited significantly higher proportions of n-3 fatty acids in muscle TG and PL fractions after 1-week of supplementation (FO1), and after 2-weeks of supplementation (FO2) while simultaneously receiving chemotherapy. In a recent study, humans were supplemented with 5 grams of fish oil per day for 4-weeks; biopsies revealed that in total muscle fatty acids, n-3 fatty acid composition increased after 2-weeks of supplementation

from 3.8% to 5.1% total fatty acids, and continued to rise after 4-weeks to ~6.8% total fatty acids (McGlory et al., 2014). In the present study, we observed similar increases in proportion of n-3 fatty acid content in both TG and PL fatty acids, even with one week of supplementation. Other studies have demonstrated that 8-weeks of fish oil supplementation results in a 2-fold increase in muscle PL n-3 fatty acid composition (Smith et al., 2011*a*, 2011*b*). We observed similar increases in both TG and PL fatty acids, achieved within only 1- and 2-weeks of supplementation. Furthermore, McGlory et al. (2014) found that unlike blood, there was no apparent saturation of n-3 PUFA composition in healthy human skeletal muscle within a 4-week time course. In cancer patients, a temporal assessment of n-3 fatty acid incorporation into tissue has not been conducted. Further work similar to McGlory et al. (2014) could be replicated in a cancer model to determine whether a more prolonged period of fish oil supplementation may be required in order to reach a saturation of n-3 fatty acids in skeletal muscle, especially during chemotherapy treatment (Pratt et al., 2002).

Results from the present study indicated that fish oil-fed rats exhibited significantly lower n-6/n-3 ratios (FO1, FO2) compared to control-fed rats (CON1, CON2). N-6 and n-3 fatty acids utilize the same Δ-6-desaturase enzyme, however the enzyme has a higher affinity for n-3 fatty acids compared to n-6. In the fat-1 mouse, which is capable of endogenous n-3 fatty acid synthesis, there was a significant reduction in n-6/n-3 fatty acid ratio in muscle tissue compared to control (Smith et al., 2010). In cancer patients, higher n-6/n-3 ratios have been associated with increased inflammatory markers, IL-6 and TNF-α and higher intermuscular fat content has been associated with increased levels of proinflammatory markers IL-6, C-reactive protein (CRP), and TNF-α

(Beasley et al., 2009). Inflammation has been shown to be a major driver of wasting in cancer (Fearon et al., 2012; Stephens et al., 2008), and thus, attenuating inflammatory markers via n-3 fatty acid may prove to a mechanism associated with improvements in muscle health in cancer. Future studies are warranted in order to determine additional mechanisms that EPA and DHA may be mediating in cancer-associated fatty muscle.

Presently, there are few other reports that demonstrate the effects of fish oil supplementation on lipid infiltration into muscle in a cancer state in experimental models nor in humans. The present results are novel and encouraging, warranting future research to determine the mechanisms by which EPA and DHA may attenuate fat deposition in muscle of cancer patients. When using animal models of cancer, measures of fatty muscle should be explored given the recent emergent value for prognostication.

CHAPTER 3

Tables

Table 3-1. Experimental diets

Ingredient (g/100 g of diet)		Control	Fish oil
Constant portion Modified AIN-76 basal mix (80g/100g)	Casein DL-Methionine Corn Starch Sucrose Vitamins (AIN-76) Minerals (AIN-76) Inositol Choline Bitartrate Cellulose	22.7 0.33 25.52 20.35 1.2 4.1 0.6 0.2 5.0	22.7 0.33 25.52 20.35 1.2 4.1 0.6 0.2 5.0
Variable portion Lipids (20g/100g)	Canola Stearine Olive oil Sunflower oil Canola oil Fish oil*	11.7 0.0 5.2 3.1 0.0	12.0 0.8 3.3 1.6 2.3
Total		**100**	**100**

Diets were isocaloric and isonitrogenous, both containing 230.3 g/kg diet of protein and 4,550.6 kcal/kg diet. Macronutrient proportions in both diets were 20% total kcal from protein, 40% total kcal from carbohydrates, and 40% total kcal from fat. The constant portion in both diets consisted of premixed modified American Institute of Nutrition-76 (AIN-76) basal ingredients, with fat source omitted (Harlan Teklad). The variable lipid portion was formulated to allow for the addition of fish oil in the treatment group. Other oils were supplied: canola stearine (ICN Biomedicals Inc.), olive oil (Safeway, Safeway brand, Alberta), sunflower oil (Safeway, Safflo brand, Alberta), canola oil (Safeway, Safeway brand, Alberta), and fish oil (DSM, formerly Ocean Nutrition Canada, Nova Scotia). 1 g of fish oil contains 360 mg EPA, and 180 mg DHA (determined by gas liquid chromatography); 1 g of diet supplied 12.4 mg of EPA + DHA.

Abbreviations: AIN-76, American Institute of Nutrition-76

Table 3-2. Fatty acid composition of experimental diets

% Fatty Acids	SFA	PUFA	MUFA	n-6	n-3	P/S	n-6/n-3
CONTROL DIET							
Canola oil	1.1	4.8	9.5	3.1	1.7	**0.35**	**9.29**
Canola stearine	54.8	0.0	0.8	0.0	0.0		
Sunflower oil	2.8	15.8	6.9	15.5	0.3		
Fish oil	0.0	0.0	0.0	0.0	0.0		
TOTAL	**58.7**	**20.6**	**17.3**	**18.6**	**2.0**		
FISH OIL DIET							
Canola oil	0.6	2.5	4.9	1.6	0.9	**0.37**	**1.52**
Canola stearine	56.2	0.0	0.9	0.0	0.0		
Sunflower oil	1.9	10.9	4.8	10.7	0.2		
Olive oil	0.7	0.5	2.7	0.5	0.1		
Fish oil	0.5	8.5	1.0	0.7	7.8		
TOTAL	**59.9**	**22.5**	**14.3**	**13.6**	**8.9**		

Proportion of total fatty acids (%) in control diet and fish oil diet. Experimental diets had equivalent proportions of SFA, PUFA, MUFA, n-6 fatty acids, and P/S ratio.

P/S ratio: Polyunsaturated fatty acid/saturated fatty acid ratio; n-6/n-3 ratio: n-6 fatty acid/n-3 fatty acid ratio

Abbreviations: SFA, saturated fatty acid; PUFA, polyunsaturated fatty acid; MUFA, monounsaturated fatty acid

Table 3-3. Fatty acid composition of muscle triglycerides in rats bearing the Ward colorectal carcinoma receiving chemotherapy

Fatty Acid %	NO CHEMO	CHEMO CYCLE 1		CHEMO CYCLE 2		ANOVA P value
	TUM n=7	CON1 n=8	FO1 n=8	CON2 n=7	FO2 n=7	
C16:0	20.9 ± 2.1^{a}	17.4 ± 2.7^{ab}	15.9 ± 2.8^{b}	20.8 ± 10.5^{ab}	16.2 ± 4.4^{b}	0.051
C16:1	4.5 ± 0.8	2.9 ± 0.9	2.7 ± 1.1	3.1 ± 1.3	4.5 ± 2.3	0.502
C18:0	7.9 ± 1.5	11.2 ± 2.3	11.2 ± 1.2	14.2 ± 8.9	8.6 ± 1.6	0.101
C18:1	41.6 ± 2.6	45.2 ± 2.9	41.9 ± 1.9	42.5 ± 7.6	44.6 ± 3.3	0.102
C18:2	20.0 ± 3.6^{a}	15.2 ± 1.5^{b}	19.5 ± 1.6^{b}	15.9 ± 5.9^{b}	19.5 ± 1.8^{b}	0.001
C18:3 n-3	0.6 ± 0.4^{a}	0.4 ± 0.2^{b}	1.3 ± 0.4^{a}	0.2 ± 0.1^{a}	1.2 ± 0.6^{a}	0.001
C20:4 (AA)	1.4 ± 0.2^{a}	3.0 ± 1.4^{b}	1.7 ± 0.4^{ab}	1.7 ± 0.7^{a}	1.4 ± 0.5^{ab}	0.014
C20:5 (EPA)	0.1 ± 0.2^{a}	0.4 ± 0.2^{a}	1.2 ± 0.2^{b}	0.0 ± 0.0^{a}	1.1 ± 0.5^{b}	0.001
C22:5	0.4 ± 0.2^{a}	0.7 ± 0.5^{a}	1.5 ± 0.3^{b}	0.2 ± 0.2^{a}	0.1 ± 0.2^{a}	0.002
C22:6 (DHA)	0.2 ± 0.4^{a}	0.1± 0.2^{a}	2.7 ± 0.8^{b}	0.0 ± 0.1^{a}	1.7 ± 0.5^{b}	0.001
∑SFA	29.0 ± 3.1^{ab}	30.1 ± 1.4^{ab}	27.2 ± 2.5^{ab}	35.0 ± 10.7^{a}	24.8 ± 3.8^{b}	0.019
∑MUFA	46.3 ± 3.1	48.1 ± 3.0	44.7 ± 2.5^{a}	45.6 ± 7.3	50.0 ± 1.7^{b}	0.015
∑n-6	22.9 ± 3.4	20.2 ± 2.0	22.6 ± 1.8	19.0 ± 5.0	22.2 ± 2.5	0.060
∑n-3	1.8 ± 0.9^{a}	1.7 ± 0.8^{a}	5.5 ± 1.4^{b}	0.5 ± 0.3^{c}	3.1 ± 0.7^{d}	0.001
n-6/n-3	12.7 ± 10.2^{a}	11.9 ± 9.3^{a}	4.1 ± 1.2^{b}	38.0 ± 8.8^{c}	7.2 ± 2.6^{b}	0.001
Total (µg/g)	2480.8 ± 568.3^{ab}	2159.7 ± 435.1^{b}	1253.6 ± 201.1^{c}	4320.0 ± 1621.9^{d}	3105.1 ± 798.7^{a}	0.001

Different superscripts indicate significant differences between groups ($P<0.05$). REF group not included in one-way ANOVA; TUM group used as reference in one-way ANOVA comparisons between CON1, CON2, FO1, FO2. Data is expressed as mean ± standard deviation. One-way ANOVA was used to determine significant differences between groups.

Triglyceride fatty acids in gastrocnemius muscles of Fischer 344 rats bearing the Ward colorectal carcinoma receiving 1- or 2- cycles of CPT-11 + 5-FU chemotherapy and fed either a fish oil (FO1, FO2) or control diet (CON1, CON2). Rats bearing only the Ward colorectal carcinoma, that did not receive chemotherapy (TUM) were used a reference group for comparison. A known volume of standard (C15:0) was used to determine the total amount of TG fatty acids (μg/g), and individual fatty acids were determined as proportion amounts of total TG, expressed as percentages (%).

Abbreviations: REF, Healthy; TUM, Tumour-bearing; CON1, Control diet + 1-cycle; CON2, Control diet + 2-cycles; FO1, Fish oil diet + 1-cycle; FO2, Fish oil diet + 2-cycles; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids

Table 3-4. Fatty acid composition of muscle phospholipids in rats bearing the Ward colorectal carcinoma receiving chemotherapy

Fatty Acid %	NO CHEMO	CHEMO CYCLE 1		CHEMO CYCLE 2		ANOVA P value
	TUM n=7	CON1 n=8	FO1 n=8	CON2 n=7	FO2 n=7	
C 16:0	20.8 ± 3. 7	21.7 ± 1.3	19.5 ± 1.6	19.1 ± 1.7	21.2 ± 1.3	0.056
C 16:1	0.6 ± 0.2 ^{ab}	0.7 ± 0.2 ^{ab}	0.4 ± 0.1^{a}	0.6 ± 0.3^{ab}	0.8 ± 0.4^{b}	0.011
C 18:0	19.2 ± 1.9	19.9 ± 1.7	20.9 ± 1.2	20.4 ± 3.9	18.8 ± 1.3	0.172
C 18:1	9.2 ± 1.5^{a}	11.8 ± 2.2^{b}	9.3 ± 0.9^{a}	10.0 ± 1.1^{b}	9.4 ± 1.2^{a}	0.004
C 18:2	11.8 ± 1.2^{a}	9.7 ± 0.8^{ab}	8.3 ± 1.0^{b}	9.3 ± 1.7^{b}	8.2 ± 0.8^{b}	0.001
C 18:3 n-3	0.2 ± 0.4	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.2	0.2 ± 0.2	0.257
C 20:4 (AA)	17.4 ± 2.6^{ab}	17.3 ± 1.2^{b}	14.8 ± 0.9^{a}	16.8 ± 1.0^{ab}	14.4 ± 1.3^{a}	0.001
C 20:5 (EPA)	0.3 ± 0.2^{a}	0.2 ± 0.1^{a}	1.7 ± 0.5^{b}	0.3 ± 0.4^{a}	1.7 ± 0.5^{b}	0.001
C 22:5	2.8 ± 0.3^{a}	2.6 ± 0.3^{a}	3.1 ± 0.2^{b}	2.3 ± 0.2^{a}	3.2 ± 0.4^{b}	0.001
C 22:6 (DHA)	0.0 ± 0.0^{a}	0.0 ± 0.1^{a}	20.2 ± 0.7^{b}	0.0 ± 0.1^{a}	20.3 ± 1.6^{b}	0.001
∑SFA	57.2 ± 1.1^{a}	55.7 ± 1.8^{ab}	41.3 ± 1.6^{c}	58.8 ± 3.0^{a}	41.3 ± 1.9^{c}	0.001
∑MUFA	9.8 ±1.5^{a}	12.7 ± 2.3^{b}	9.7 ± 1.0	10.6 ± 1.3	10.3 ± 1.8	0.001
∑n-6	29.5 ± 1.4^{a}	28.7 ± 1.7^{a}	24.0 ± 1.5^{b}	27.6 ± 3.3^{ab}	23.0 ± 1.8^{b}	0.001
∑n-3	3.4 ± 0.5^{a}	2.8 ± 0.4^{b}	25.0 ± 1.2^{c}	2.8 ± 1.1^{b}	25.4 ± 1.8^{c}	0.001
n-6/n-3	8.9 ± 0.8^{a}	10.7 ± 1.1^{a}	1.0 ± 0.1^{b}	10.0 ± 1.1^{a}	0.9 ± 0.1^{b}	0.001
Total (µg/g)	4280.6 ± 1931.3^{a}	1625.8 ± 325.5^{b}	5014.6 ± 1603.6^{c}	3249.5 ± 1795.9^{a}	4664.3 ± 1325.4^{a}	0.006

Different superscripts indicate significant differences between groups ($P<0.05$). REF group not included in one-way ANOVA; TUM group used as reference in one-way ANOVA comparisons between CON1, CON2, FO1, FO2. Data is expressed as mean ± standard deviation. One-way ANOVA was used to determine significant differences between groups.

Triglyceride fatty acids in gastrocnemius muscles of Fischer 344 rats bearing the Ward colorectal carcinoma receiving 1- or 2- cycles of CPT-11 + 5-FU chemotherapy and fed either a fish oil (FO1, FO2) or control diet (CON1, CON2). Rats bearing only the Ward colorectal carcinoma, that did not receive chemotherapy (TUM) were used a reference group for comparison. A known volume of standard (C17:0) was used to determine the total amount of PL fatty acids (μg/g), and individual fatty acids were determined as proportion amounts of total PL, expressed as percentages (%).

Abbreviations: REF, Healthy; TUM, Tumour-bearing; CON1, Control diet + 1-cycle; CON2, Control diet + 2-cycles; FO1, Fish oil diet + 1-cycle; FO2, Fish oil diet + 2-cycles; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids

CHAPTER 3

Figures

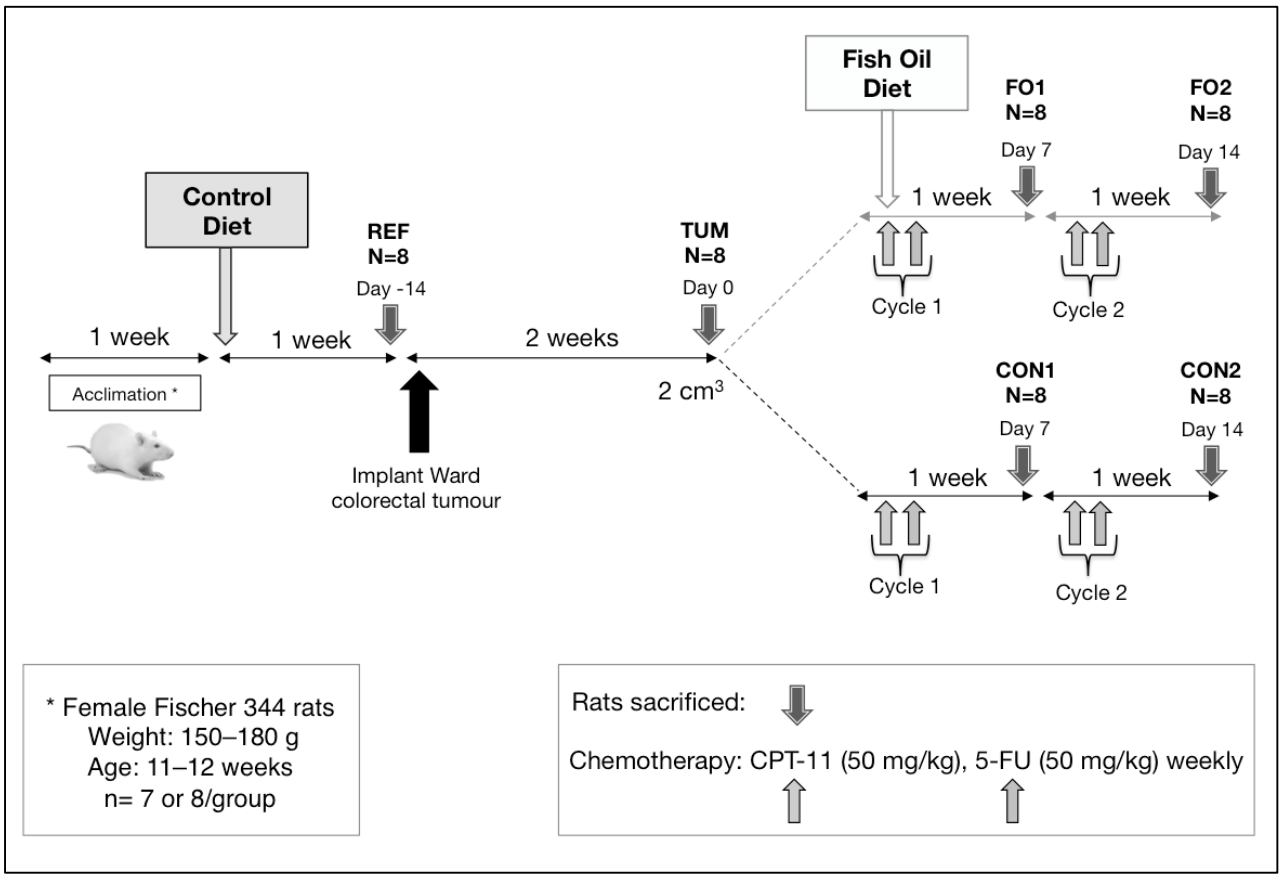

Figure 3-1. Experimental study design

The first day of chemotherapy cycle-1 designated as Day 0; the first day of chemotherapy cycle-2 designated as Day 7. Rats were sacrificed on Day 7 after the completion of cycle-1, and on Day 14 after the completion of cycle-2. CPT-11 was administered on Day 0 and Day 7; 5-FU was administered on Day 1 and Day 8.

Abbreviations: CPT-11, irinotecan; 5-FU, 5-fluorouracil; REF, Healthy; TUM, Tumour-bearing; CON1, Control diet + 1-cycle; CON2, Control diet + 2-cycles; FO1, Fish oil diet + 1-cycle; FO2, Fish oil diet + 2-cycles

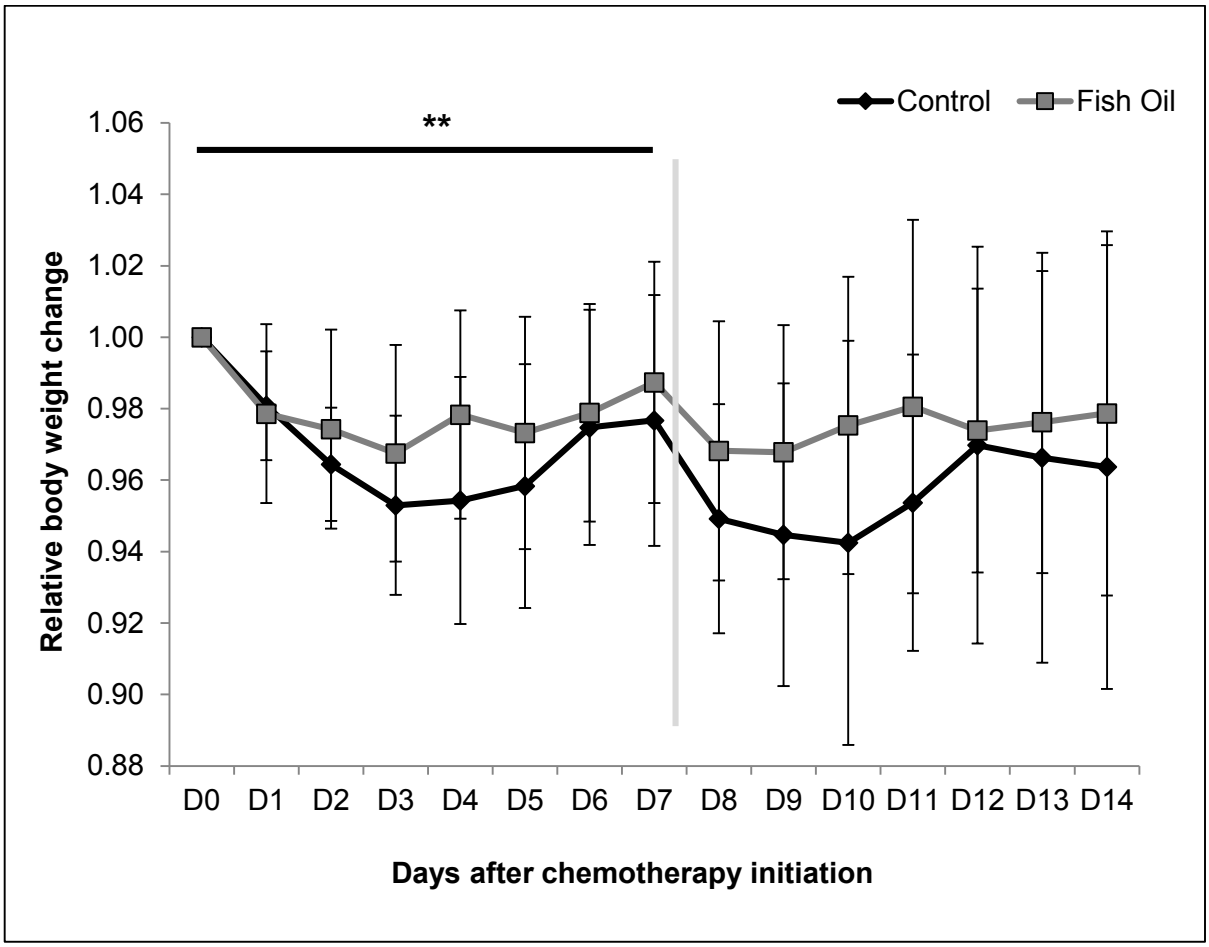

Figure 3-2. Relative body weight change in fish-oil fed and control-fed rats bearing the Ward colorectal carcinoma during chemotherapy (CPT-11 + 5-FU)

** Significant difference between fish oil-fed and control-fed animals during chemotherapy; determined by repeated measures-ANOVA, P=0.05

Body weight was measured every day during chemotherapy until euthanization. Relative body weight represents delta changes from baseline average body weight, prior to chemotherapy; average baseline body weight for each diet group set at 1.0. Data points represent mean ± standard deviation on each day. Chemotherapy cycle-1 began on Day 0 (D0); CPT-11 administered on D0, 5-FU administered on D2. Chemotherapy cycle-2 began on Day 7 (D7); CPT-11 administered on D7, 5-FU administered on D8. In cycle-1, n=16 rats per group (FO1 n=8, FO2 n=8; CO1 n=8, CO2=8). In cycle-2, n=8 rats/group (FO2 n=8; CO2 n=8).

Abbreviations: CPT-11, irinotecan; 5-FU, 5-fluorouracil; REF, Healthy; TUM, Tumour-bearing; CON1, Control diet + 1-cycle; CON2, Control diet + 2-cycles; FO1, Fish oil diet + 1-cycle; FO2, Fish oil diet + 2-cycles

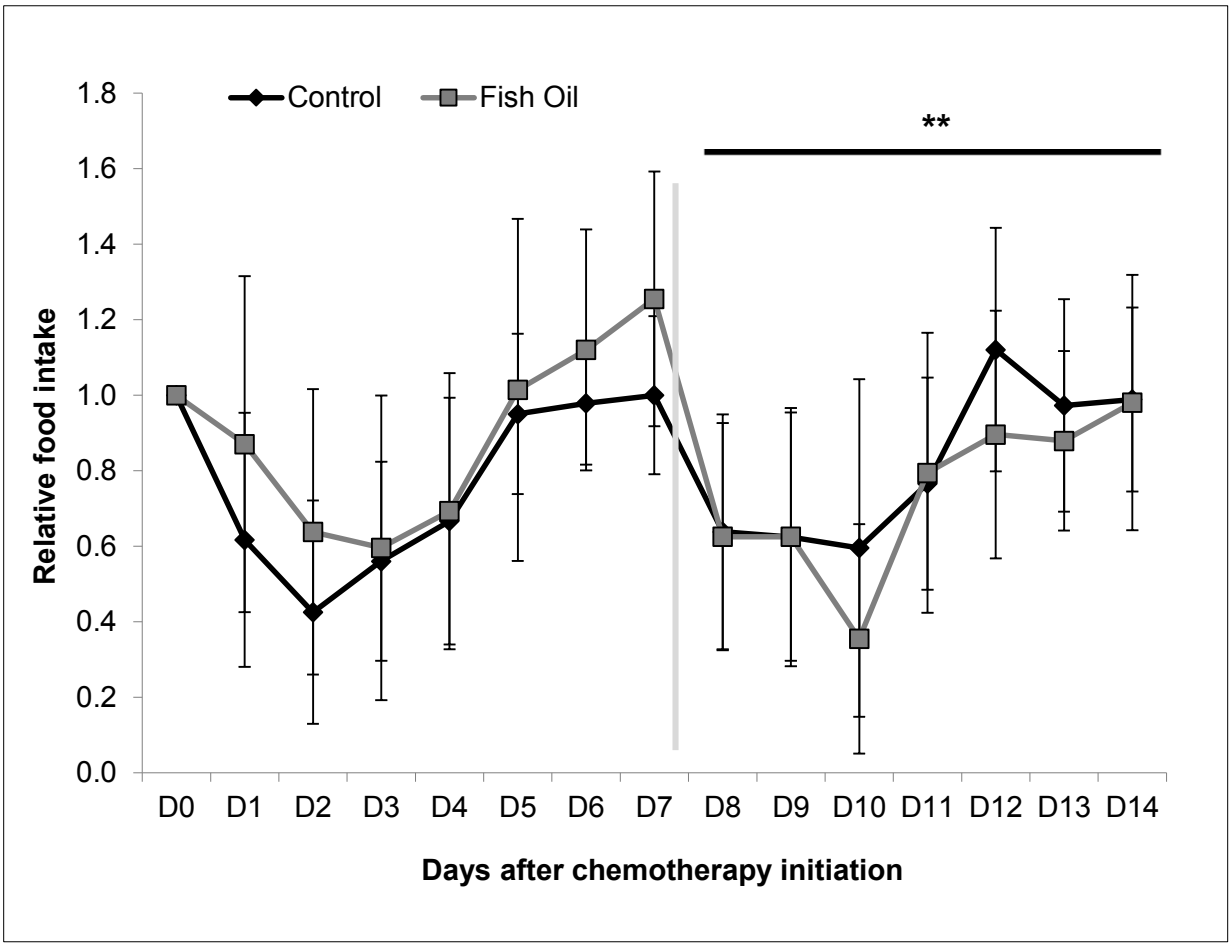

Figure 3-3. **Relative food intake change in fish-oil fed and control diet-fed rats bearing the Ward colorectal carcinoma during chemotherapy (CPT-11 + 5-FU)**

** Significant difference between fish oil-fed and control-fed animals during chemotherapy; determined by repeated measures-ANOVA, P=0.05

Food intake was measured every day during chemotherapy until euthanization. Relative food intake represents delta changes from baseline average food intake, prior to chemotherapy; average baseline food intake for each diet group set at 1.0. Data points represent mean ± standard deviation on each day. Chemotherapy cycle-1 began on Day 0 (D0); CPT-11 administered on D0, 5-FU administered on D2. Chemotherapy cycle-2 began on Day 7 (D7); CPT-11 administered on D7, 5-FU administered on D8. In cycle-1, n=16 rats per group (FO1 n=8, FO2 n=8; CO1 n=8, CO2=8). In cycle-2, n=8 rats/group (FO2 n=8; CO2 n=8)

Abbreviations: CPT-11, irinotecan; 5-FU, 5-fluorouracil; REF, Healthy; TUM, Tumour-bearing; CON1, Control diet + 1-cycle; CON2, Control diet + 2-cycles; FO1, Fish oil diet + 1-cycle; FO2, Fish oil diet + 2-cycles

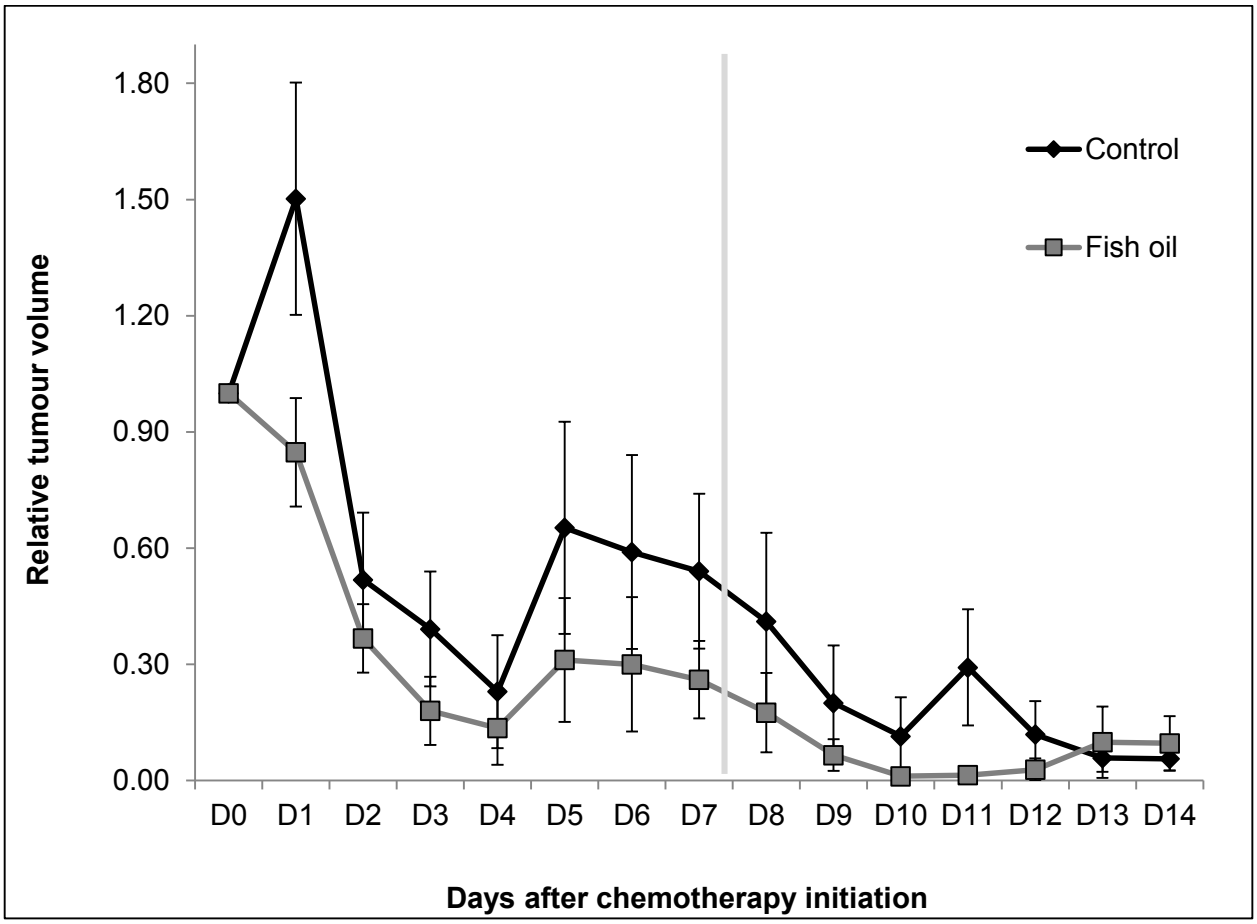

Figure 3-4. **Relative tumour size in fish oil-fed and control-fed rats bearing the Ward colorectal carcinoma during chemotherapy (CPT-11 + 5-FU)**

Fish oil and control diet groups were statistically compared by repeated measures-ANOVA (*P*=0.074).

Tumour volume was measured every day during chemotherapy until euthanization and expressed in cm^3. Relative tumour volume represents delta changes from baseline average tumour volume, prior to chemotherapy; average baseline tumour volume for each diet group set at 1.0. Data points represent mean ± standard deviation on each day. Chemotherapy cycle-1 began on Day 0 (D0); CPT-11 administered on D0, 5-FU administered on D2. Chemotherapy cycle-2 began on Day 7 (D7); CPT-11 administered on D7, 5-FU administered on D8. In cycle-1, n=16 rats per group (FO1 n=8, FO2 n=8; CO1 n=8, CO2=8). In cycle-2, n=8 rats/group (FO2 n=8; CO2 n=8).

Abbreviations: CPT-11, irinotecan; 5-FU, 5-fluorouracil; REF, Healthy; TUM, Tumour-bearing; CON1, Control diet + 1-cycle; CON2, Control diet + 2-cycles; FO1, Fish oil diet + 1-cycle; FO2, Fish oil diet + 2-cycles

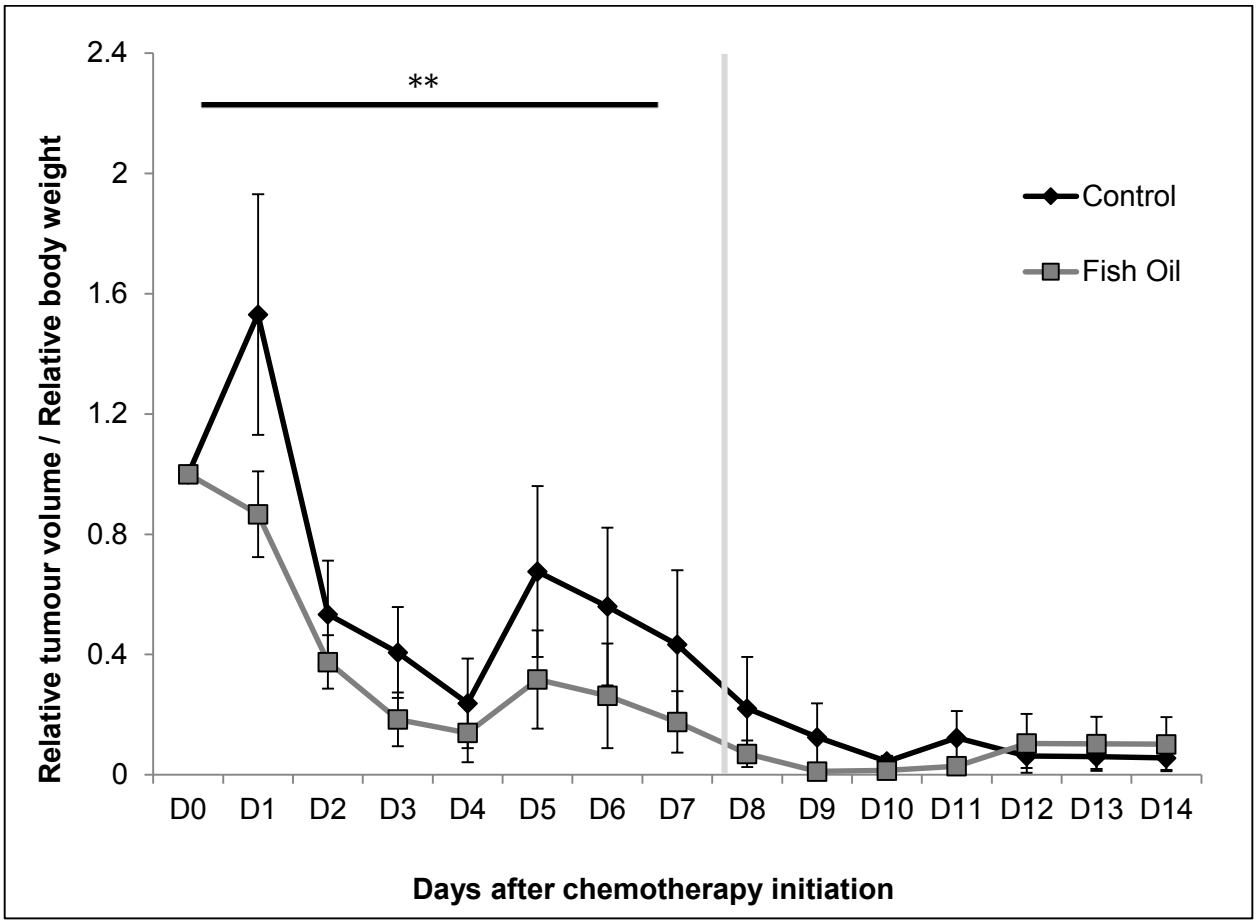

Figure 3-5. Relative tumour size expressed as a proportion of relative body weight in fish oil-fed and control-fed rats bearing the Ward colorectal carcinoma during chemotherapy (CPT-11 + 5-FU)

** Significant difference between fish oil-fed and control-fed animals during the first cycle of chemotherapy; determined by repeated measures-ANOVA (P=0.024)

Tumour volume was measured every day during chemotherapy until euthanization and expressed in cm^3. Relative tumour volume and relative body weight represent delta changes from baseline average tumour volume and average body weight, prior to chemotherapy; average baseline for each diet group set at 1.0. Data expressed as ratio of relative tumour volume/relative body weight. Data points represent mean ± standard deviation on each day. Chemotherapy cycle-1 began on Day 0 (D0); CPT-11 administered on D0, 5-FU administered on D2. Chemotherapy cycle-2 began on Day 7 (D7); CPT-11 administered on D7, 5-FU administered on D8. In cycle-1, n=16 rats per group (FO1 n=8, FO2 n=8; CO1 n=8, CO2=8). In cycle-2, n=8 rats/group (FO2 n=8; CO2 n=8).

Abbreviations: CPT-11, irinotecan; 5-FU, 5-fluorouracil; REF, Healthy; TUM, Tumour-bearing; CON1, Control diet + 1-cycle; CON2, Control diet + 2-cycles; FO1, Fish oil diet + 1-cycle; FO2, Fish oil diet + 2-cycles

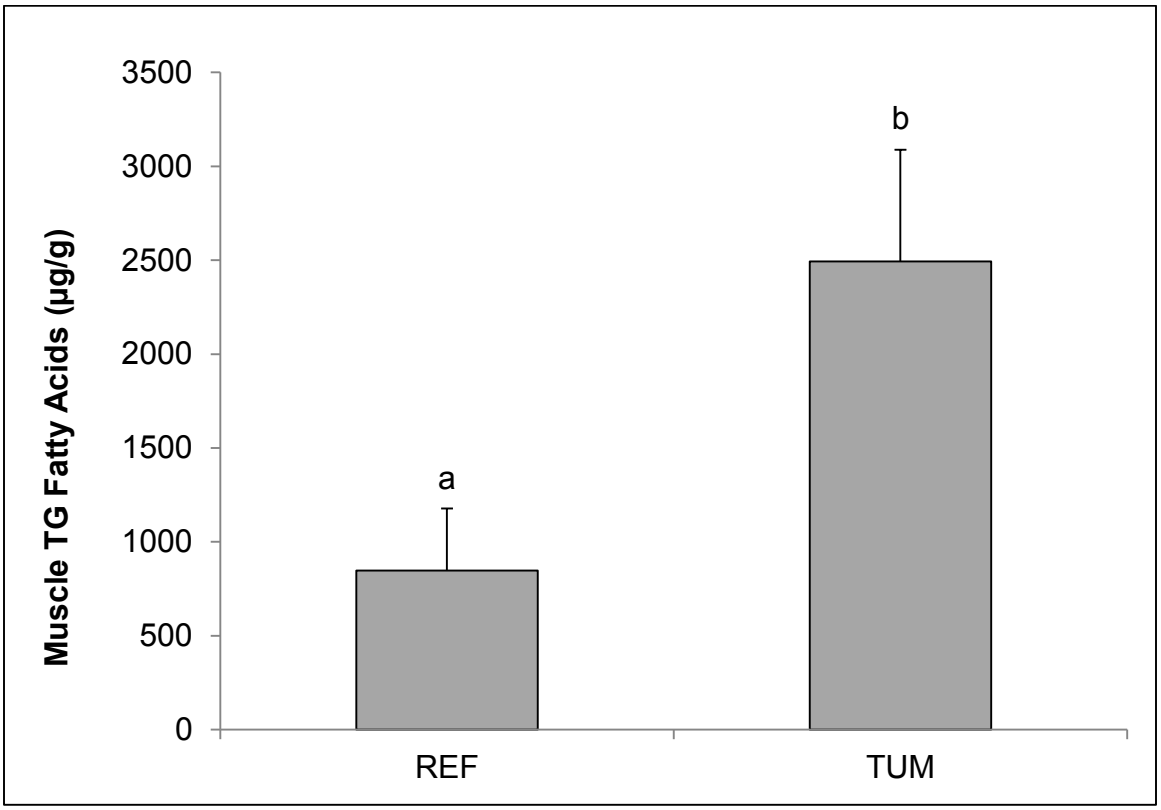

Figure 3-6. Effect of Ward colorectal carcinoma on total TG fatty acid content in gastrocnemius muscles

Different superscripts indicate significant differences between groups (P=0.001)

Significant difference between REF and TUM determined by two-sided t-test. Data is expressed as mean ± standard deviation.

Total muscle TG fatty acid content (µg/g) in healthy Fischer 344 rats (REF) compared to Fischer 344 rats bearing the Ward colorectal carcinoma (TUM), receiving no chemotherapy; N=8/group.

Abbreviations: CPT-11, irinotecan; 5-FU, 5-fluorouracil; REF, Healthy; TUM, Tumour-bearing

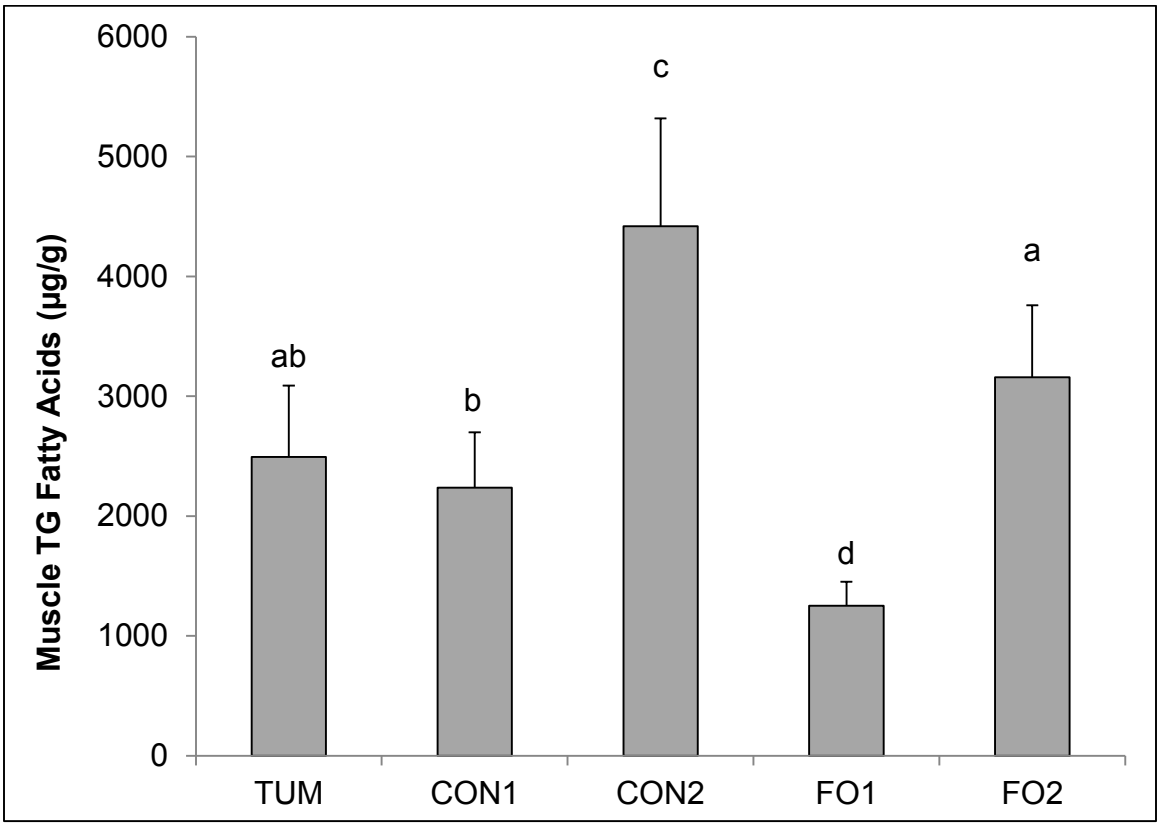

Figure 3-7. Comparison of total muscle TG content in fish oil-fed and control diet-fed animals receiving 1- or 2-cycles of chemotherapy (CPT-11 + 5-FU)

Different superscripts indicate significant differences between groups (*P*=0.001)

TUM group used as reference in one-way ANOVA comparisons between CON1, CON2, FO1, FO2. Data is expressed as mean ± standard deviation. One-way ANOVA was used to determine significant differences between groups. In cycle-1, n=16 rats per group (FO1 n=8, FO2 n=8; CO1 n=8, CO2=8). In cycle-2, n=8 rats/group (FO2 n=8; CO2 n=8)

Abbreviations: CPT-11, irinotecan; 5-FU, 5-fluorouracil; REF, Healthy; TUM, Tumour-bearing; CON1, Control diet + 1-cycle; CON2, Control diet + 2-cycles; FO1, Fish oil diet + 1-cycle; FO2, Fish oil diet + 2-cycles

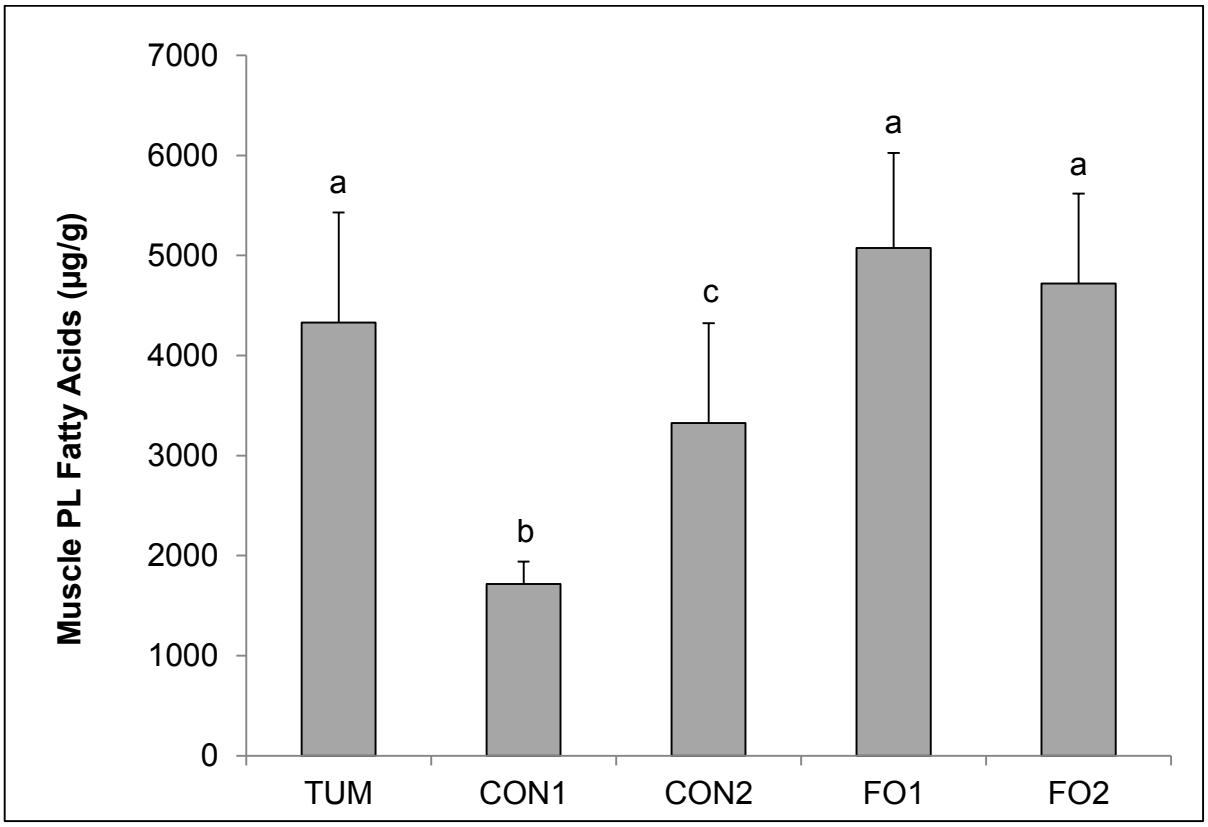

Figure 3-8. Comparison of total muscle PL content in fish oil-fed and control diet-fed animals receiving 1- or 2-cycles of chemotherapy (CPT-11 + 5-FU)

Different superscripts indicate significant differences between groups (*P*=0.006)

TUM group used as reference in one-way ANOVA comparisons between CON1, CON2, FO1, FO2. Data is expressed as mean ± standard deviation. One-way ANOVA was used to determine significant differences between groups. In cycle-1, n=16 rats per group (FO1 n=8, FO2 n=8; CO1 n=8, CO2=8). In cycle-2, n=8 rats/group (FO2 n=8; CO2 n=8)

Abbreviations: CPT-11, irinotecan; 5-FU, 5-fluorouracil; REF, Healthy; TUM, Tumour-bearing; CON1, Control diet + 1-cycle; CON2, Control diet + 2-cycles; FO1, Fish oil diet + 1-cycle; FO2, Fish oil diet + 2-cycles

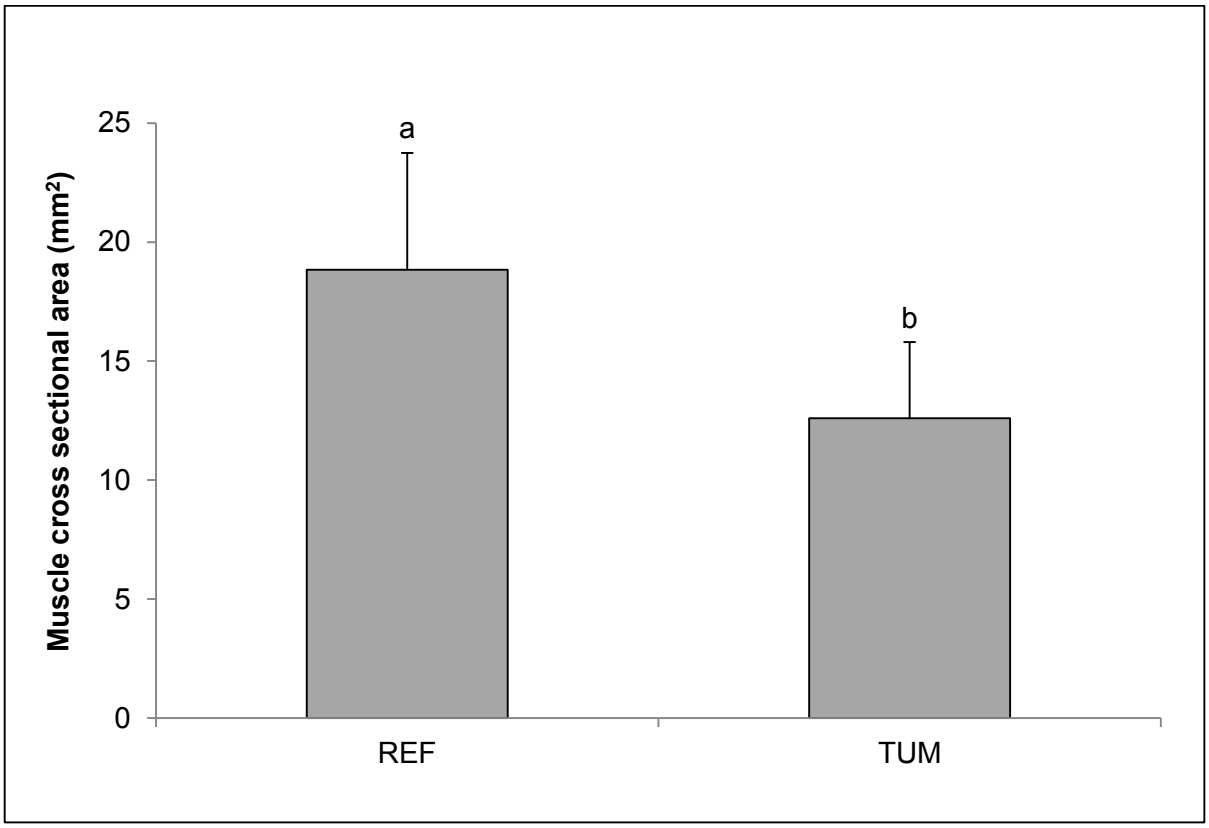

Figure 3-9. Effect of Ward colorectal carcinoma on muscle cross sectional area in gastrocnemius muscle of rats

Different superscripts indicate significant differences between groups (*P*=0.001)

Significant difference between REF and TUM determined by two-sided *t*-test Data is expressed as mean ± standard deviation. N=8/group

Abbreviations: CPT-11, irinotecan; 5-FU, 5-fluorouracil; REF, Healthy; TUM, Tumour-bearing

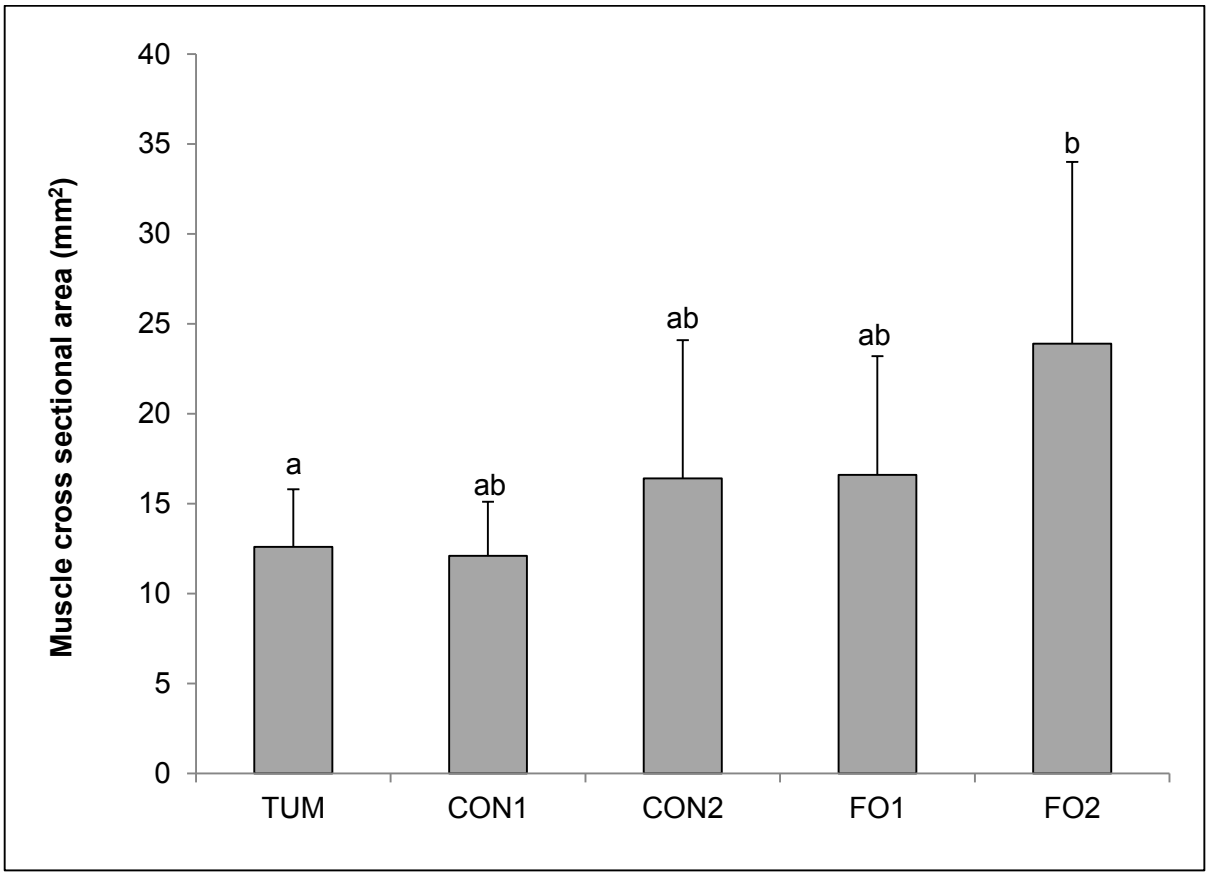

Figure 3-10. Comparison of muscle cross sectional area in fish oil-fed and control diet-fed animals receiving 1- or 2-cycles of chemotherapy (CPT-11 + 5-FU)

Different superscripts indicate significant differences between groups (P=0.05)

TUM group used as reference in one-way ANOVA comparisons between CON1, CON2, FO1, FO2. Data is expressed as mean ± standard deviation. One-way ANOVA was used to determine significant differences between groups. In cycle-1, n=16 rats per group (FO1 n=8, FO2 n=8; CO1 n=8, CO2=8). In cycle-2, n=8 rats/group (FO2 n=8; CO2 n=8)

Abbreviations: CPT-11, irinotecan; 5-FU, 5-fluorouracil; REF, Healthy; TUM, Tumour-bearing; CON1, Control diet + 1-cycle; CON2, Control diet + 2-cycles; FO1, Fish oil diet + 1-cycle; FO2, Fish oil diet + 2-cycles

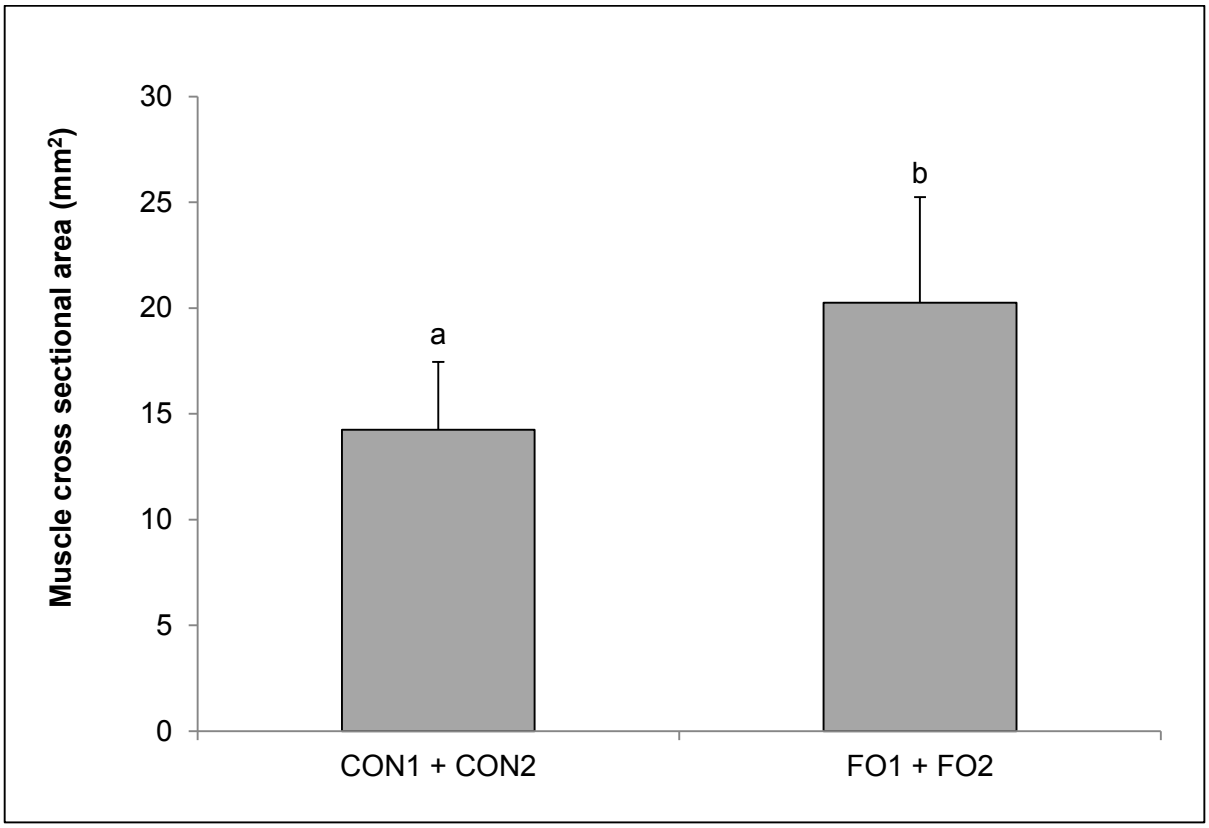

Figure 3-11. Comparison of muscle cross sectional area in fish oil-fed and control diet-fed animals receiving 1- and 2-cycles of chemotherapy (CPT-11 + 5-FU)

Different superscripts indicate significant differences between groups (*P*=0.05)

Significant difference between (CON1 + CON2) and (FO1 + FO2) groups determined by two-sided *t*-test. Data is expressed as mean ± standard deviation. In cycle-1, n=16 rats per group (FO1 n=8, FO2 n=8; CO1 n=8, CO2=8). In cycle-2, n=8 rats/group (FO2 n=8; CO2 n=8)

Abbreviations: CPT-11, irinotecan; 5-FU, 5-fluorouracil; CON1, Control diet + 1-cycle; CON2, Control diet + 2-cycles; FO1, Fish oil diet + 1-cycle; FO2, Fish oil diet + 2-cycles

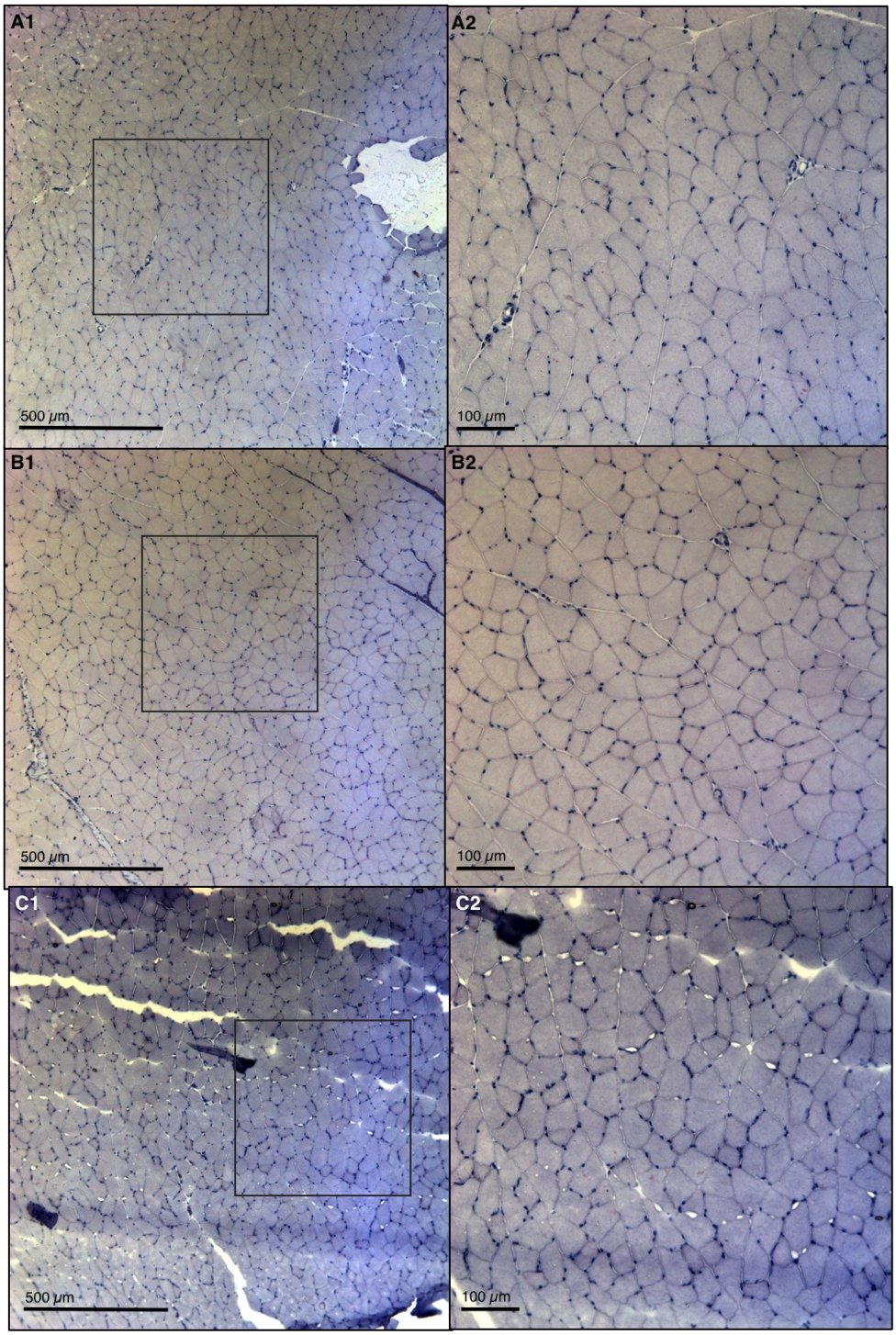

Figure 3-12. Representative panel of light microscope images of oil red O and Mayer's hematoxylin stained frozen gastrocnemius cross-sectional muscle sections from healthy (REF) animals

N=8 slides included in qualitative analysis. Images taken from a single muscle section contain the same letters at differing magnifications: A1, B1, C1 at x10 magnification; A2, B2, C2 at 20x magnification; Bars represent µm scales. Images in A2, B2, C2 taken at

higher magnifications from areas contained in black squares in images A1, B1, C1, respectively

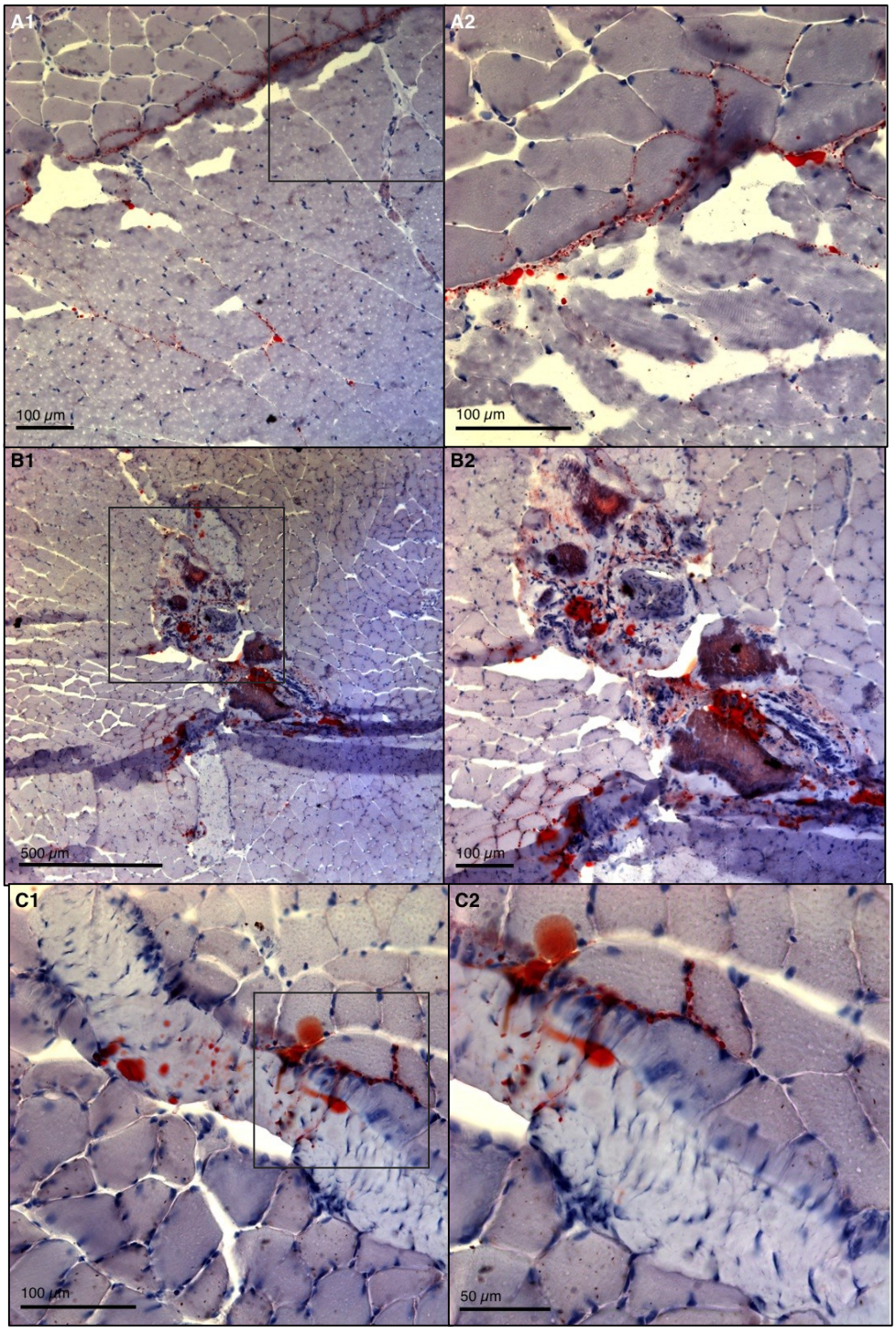

Figure 3-13. Representative panel of light microscope images of oil red O and Mayer's hematoxylin stained frozen gastrocnemius cross-sectional muscle sections from tumour-bearing (TUM) animals

N=7 slides included in qualitative analysis. Images taken from a single muscle section

contain the same letters at differing magnifications: B1 taken at 10x magnification; A1, B2 at 20x magnification; A2, C1 taken at 40x magnification; C2 taken at 63x magnification. Bars represent μm scales. Images in A2, B2, C2 taken at higher magnifications from areas contained in black squares in images A1, B1, C1, respectively

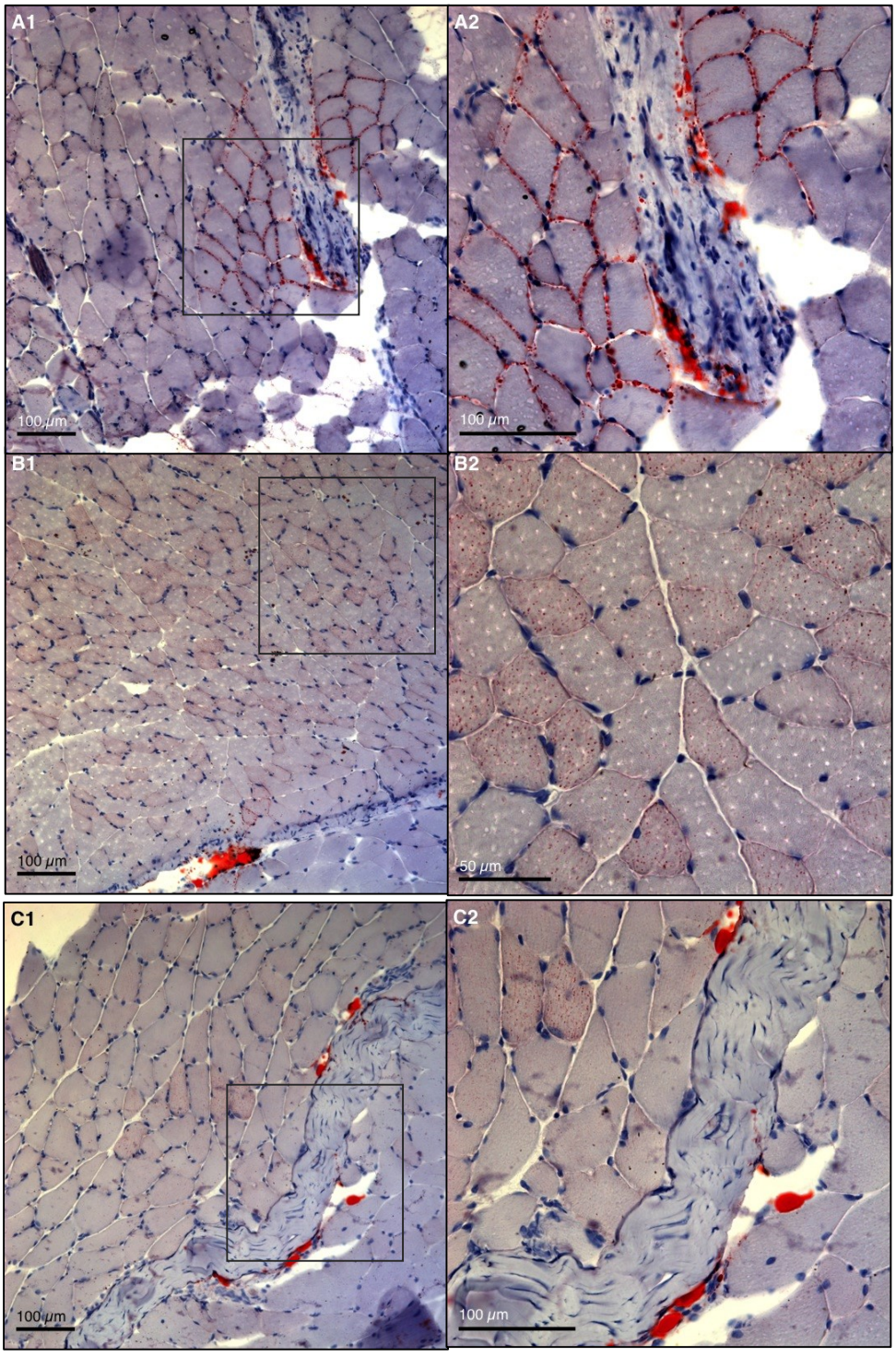

Figure 3-14. Representative panel of light microscope images of oil red O and Mayer's hematoxylin stained frozen gastrocnemius cross-sectional muscle sections from control-fed animals receiving 1-cycle of chemotherapy (CON1)

N=8 slides included in qualitative analysis. Images taken from a single muscle section contain the same letters at differing magnifications: A1, B1, C1 at 20x magnification; A2,

C2 taken at 40x magnification; B2 taken at 63x magnification. Bars represent μm scales. Images in A2, B2, C2 taken at higher magnifications from areas contained in black squares in images A1, B1, C1, respectively

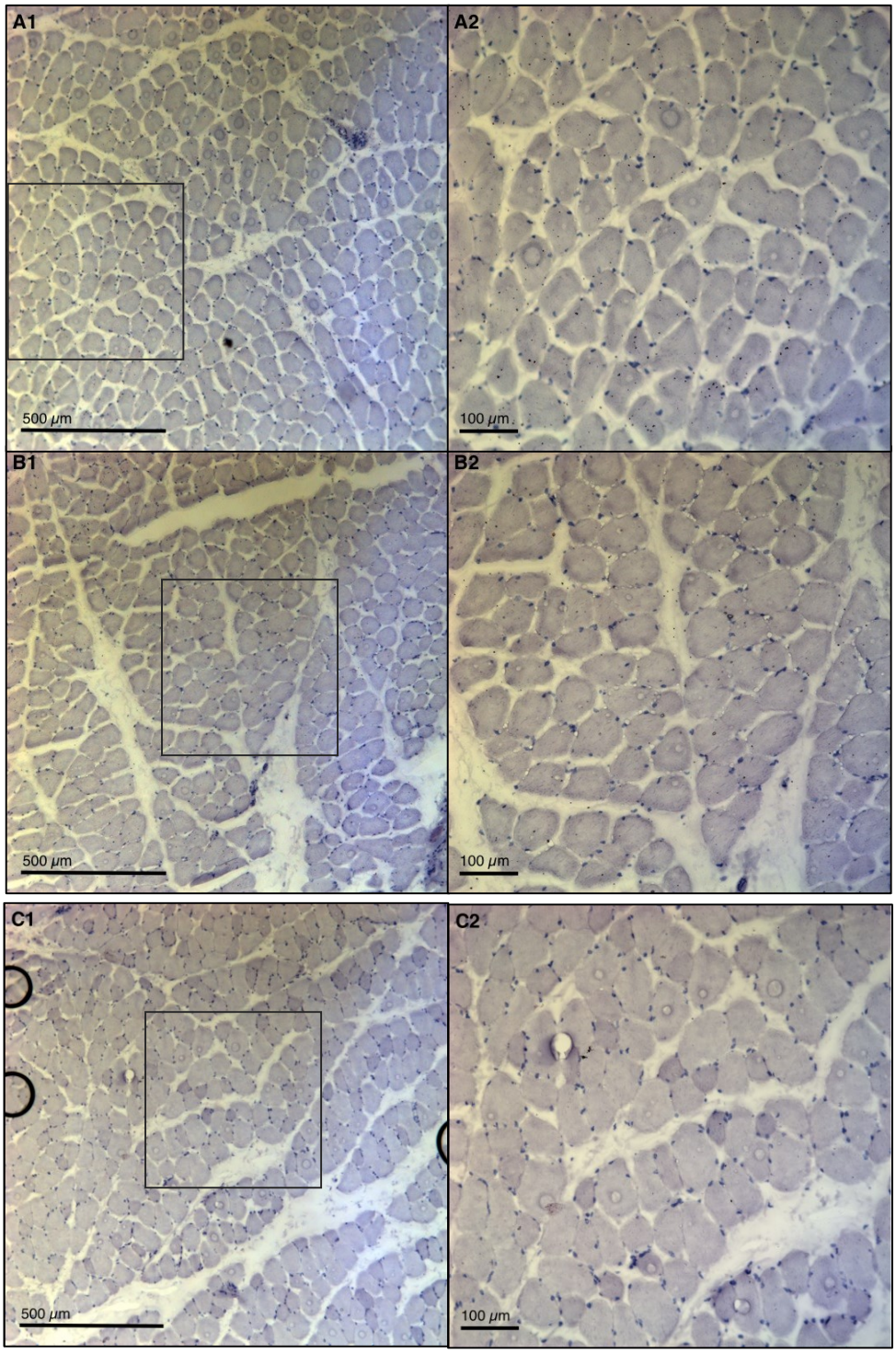

Figure 3-15. Representative panel of light microscope images of oil red O and Mayer's hematoxylin stained frozen gastrocnemius cross-sectional muscle sections from fish oil-fed animals receiving 1-cycle of chemotherapy (FO1)

N=8 slides included in qualitative analysis. Images taken from a single muscle section contain the same letters at differing magnifications: A1, B1, C1 at 10x magnification; A2,

B2, C2 taken at 20x magnification. Bars represent μm scales. Images in A2, B2, C2 taken at higher magnifications from areas contained in black squares in images A1, B1, C1, respectively

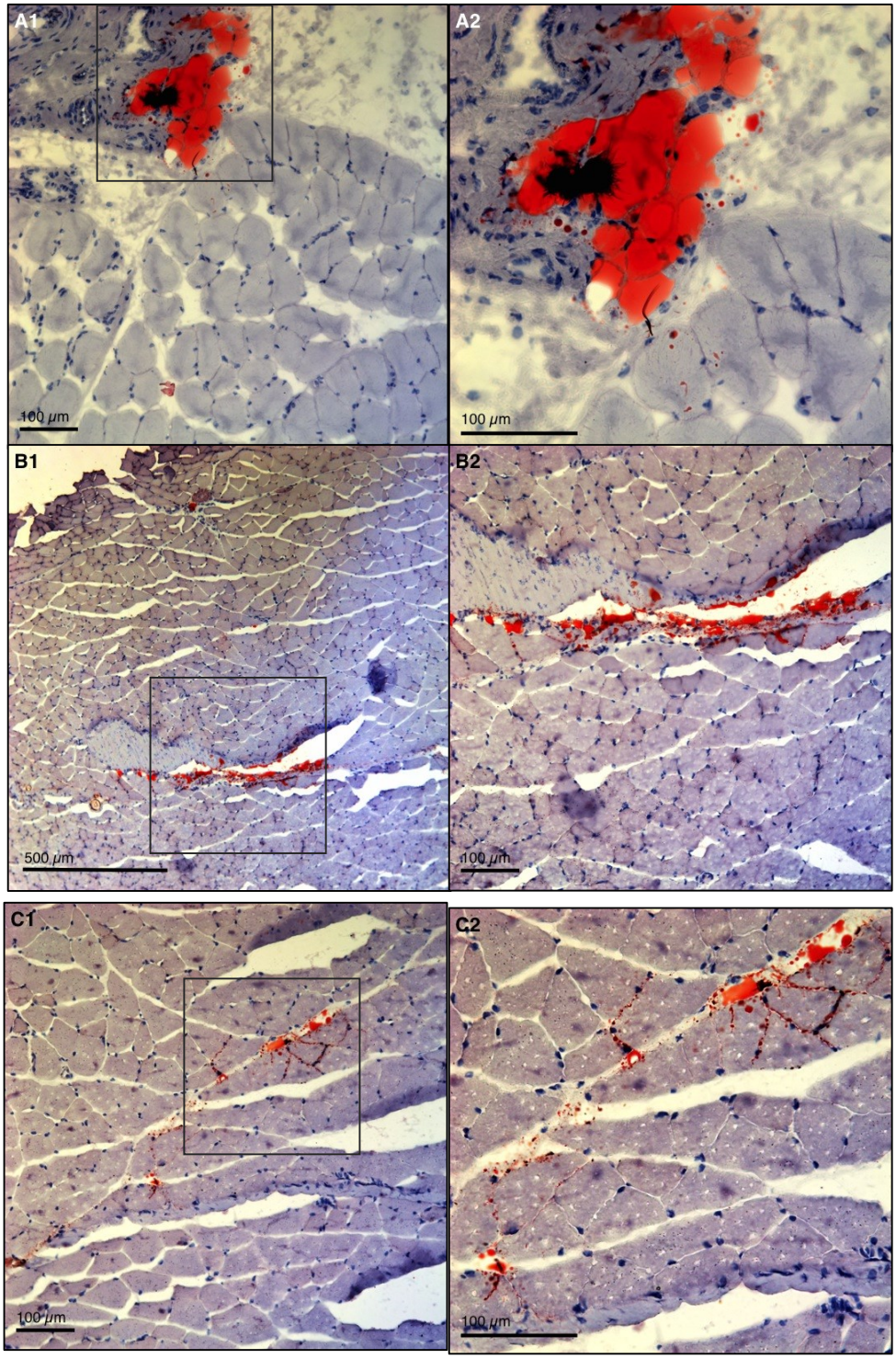

Figure 3-16. Representative panel of light microscope images of oil red O and Mayer's hematoxylin stained frozen gastrocnemius cross-sectional muscle sections from control-fed animals receiving 2-cycles of chemotherapy (CON2)

N=7 slides included in qualitative analysis. Images taken from a single muscle section contain the same letters at differing magnifications: B1 at 10x magnification; A1, B2, C1

taken at 20x magnification; A2, C2 taken at 40x magnification. Bars represent μm scales. Images in A2, B2, C2 taken at higher magnifications from areas contained in black squares in images A1, B1, C1, respectively

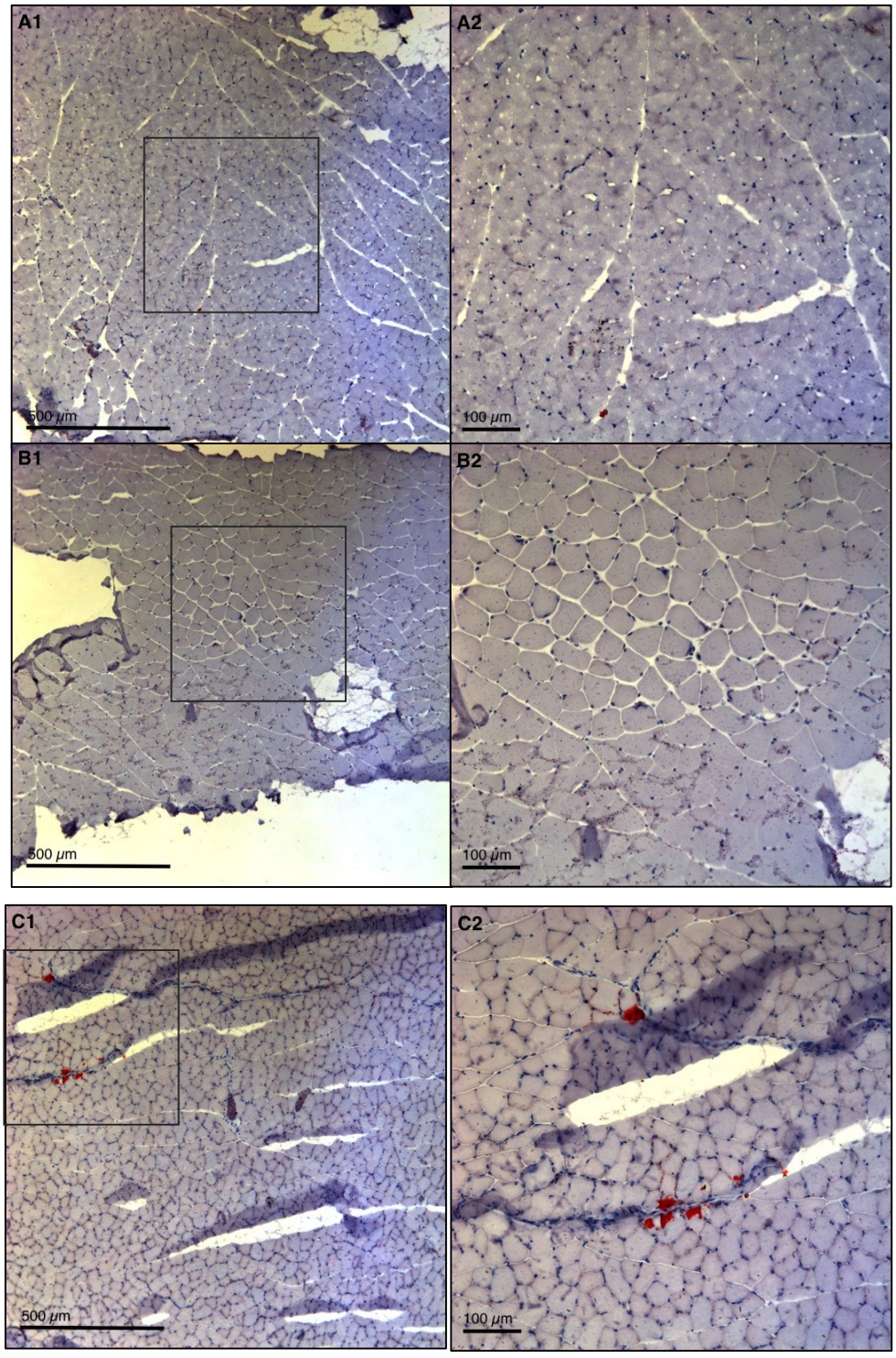

Figure 3-17. Representative panel of light microscope images of oil red O and Mayer's hematoxylin stained frozen gastrocnemius cross-sectional muscle sections from fish oil-fed animals receiving 2-cycles of chemotherapy (FO2)

N=7 slides included in qualitative analysis. Images taken from a single muscle section contain the same letters at differing magnifications: A1, B1, C1 at 10x magnification; A2,

B2, C2 taken at 20x magnification. Bars represent μm scales. Images in A2, B2, C2 taken at higher magnifications from areas contained in black squares in images A1, B1, C1, respectively

CHAPTER 4

Effects of fish oil feeding on mTOR, phosphorylated-AKT, and total AKT protein levels in rat gastrocnemius muscles

4.1 Introduction

The anti-inflammatory properties of EPA and DHA have been one of the main mechanisms proposed for their ability to improve muscle mass and function. However, studies indicate that EPA and DHA may operate through other mechanisms. Long chain n-3 fatty acids have been shown to exhibit anabolic effects in the absence of inflammation, suggesting a possible activation of protein synthesis through insulin signalling pathways (Smith et al., 2011*a*). Insulin resistance has been implicated in the development of fatty muscle in people with diabetes (Goodpaster et al., 2000; Perseghin et al., 1999), and is thought to play an important role in the loss of muscle mass in disease states (Gray and Da Boit, 2013). There is a large body of evidence from experimental and clinical studies in other disease states to support the role of insulin resistance in muscle loss (see Chapter 1, Section 1.3.1). Animal studies have demonstrated that EPA and DHA can improve insulin sensitivity and increase muscle glucose uptake in rats (Tishinsky et al., 2012), and EPA and DHA have also been reported to support the anabolic potential of muscle through improved insulin sensitivity (Flachs et al., 2009; Martin de Santa Olalla et al., 2009; Smith et al., 2010). In humans, Smith *et al.* (2011*b*) reported increases in mTOR signalling, overall muscle size, and muscle protein synthesis in healthy adults following 8-weeks of supplementation with n-3 fatty acids. In rodents, n-3 PUFA supplementation has been reported to alleviate soleus atrophy during a period of

immobilization (Liu et al., 2013). In pigs, 21 days of fish oil supplementation resulted in increases in muscle protein mass and mTOR protein content (Wei et al., 2013). One school of thought suggests that since insulin sensitivity is required for fat oxidation, EPA and DHA may play a role in the attenuation of fatty muscle seen in a tumour-bearing state by restoring insulin sensitivity and subsequently, restoring fat oxidation in fatty muscle. However, other mechanisms have also been proposed.

The role of the protein kinase B and mammalian target of rapamyacin (AKT/mTOR) signalling pathway in muscle anabolism has been investigated in several models of skeletal muscle hypertrophy and atrophy (Molkentin et al., 1998; Semarian et al., 1999; Rommel et al., 2001). Studies have shown that the AKT/mTOR pathway upregulation occurs during hypertrophy and is downregulated in atrophic muscle; further, activation of the pathway can oppose muscle atrophy induced by disease (Bodine et al., 2001). mTOR is a mediator of the insulin pathway signalling, and is a critical factor in the regulation of tissue metabolism in adipose tissue, skeletal muscle, liver, and pancreas. mTOR is a serine/threonine protein kinase that is part of two distinct signalling complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Huang and Houghton, 2003). mTORC1 coordinates signals from energy status, stress, oxygen levels, growth factors, and amino acid levels in the body. This coordination subsequently regulates processes involved in lipid synthesis, protein synthesis, and autophagy (Schiaffino et al., 2013). mTORC2 is specifically sensitive to insulin, while it does not respond to amino acids or rapamyacin. mTORC2 controls ion transport and cellular shape by mediating protein kinases. mTORC1 is regulated through signals from AKT, AMPK, and IκKβ, which activate the complex (Bodine et al., 2001). Direct phosphorylation of

mTOR occurs at Ser2448 by AKT kinase and p70S6 kinase. Phosphorylation at the Ser2448 site is a biomarker for the activation state of the phosphotidyl-inositol kinase (PI3K) pathway, in addition to the activation of mTOR (Jacinto and Hall, 2003; see Chapter 1, **Figure 1-1**).

Protein kinase B, also known as AKT, is a serine/threonine- specific protein kinase that is involved in cellular proliferation, protein synthesis, cell survival and apoptosis inhibition, glucose metabolism, and transcription of a number of genes. AKT is activated by insulin and other agonists, resulting in transport from the cytosol to the plasma membrane. At the plasma membrane, the carboxy terminus of AKT is phosphorylated at Ser473 by mTORC and is transformed into its active form. Phosphorylated-AKT can then go on to activate or inhibit its targets, one of them being mTOR. AKT plays a critical role in the insulin-signalling pathway, and is crucial for glucose transport. AKT contains a protein domain that binds with high affinity to phosphoinositides. AKT specifically binds either PIP_2 or PIP_3. EPA and DHA alter the fatty acid composition of the skeletal muscle membrane, and may influence anabolic signalling that occur via phospholipids in the membrane. Desaturation of the membrane has been associated with increased potency of the conversion of PIP_2 to PIP_3 in the plasma membrane via PI3K, which subsequently activates PDK1 and phosphorylates mTOR into its active form (Alessi et al., 1997). Furthermore, reduced insulin sensitivity has been associated with decreased concentrations of polyunsaturated fatty acids in skeletal-muscle phospholipids, suggesting that changes in the fatty-acid composition of muscles may modulate the action of insulin (Borkman et al., 1993).

The objectives of this study were to compare the levels of mTOR pSer2448, phosphorylated-AKT (pSer473), and total-AKT (1/2/3) in gastrocnemius muscles of rats fed a fish oil diet to those fed a diet without fish oil. It is hypothesized that the presence of a tumour will reduce the total amount of protein in muscles compared to a non-tumour bearing state, and reduce the amount of total mTOR, pAKT, and total-AKT. Treatment with CPT-11 + 5-FU will result in further reductions in all protein types, specifically protein levels of mTOR and phosphorylated-AKT, resulting in a reduced ratio of pAKT/AKT. It is hypothesized that a fish oil diet containing EPA and DHA fed during chemotherapy treatment will maintain total protein content in the muscle, and further maintain proteins involved in the insulin pathway: the phosphorylated active form of mTOR and AKT proteins, resulting in an increased ratio of pAKT/AKT.

4.2 Study Design

Animal model, dietary design, tumour and chemotherapy descriptions can be found in Chapter 3, Section 3.2.

4.3 Methods

4.3.1 Muscle total protein quantification

Total amount of protein in gastrocnemius muscle samples was determined by conducting a commercially available protein assay using bicinchoninic acid (BCA) (Pierce BCA Protein Assay Kit, Thermo Scientific, Rockford, IL, USA). The protein

assay combined the reduction of copper (Cu) ions by protein in an alkaline medium and used calorimetric detection and quantification of total protein in the samples. A bovine serum albumin (BSA) standard of known concentrations was diluted and run alongside unknown sample concentrations; the concentration of each unknown sample was determined using a spectrophotometer (Spectra Max 190, Molecular Devices, Sunnyvale, CA, USA) based on the standard curve at 450nm. The individual concentrations of each sample were determined from spectrophotometer duplicate readings (mean values of duplicate readings) and adjusted according to the dilution factor.

4.3.2 Preparation of cell extracts from tissue homogenates

Frozen gastrocnemius muscles (-80°C) were homogenized with a frozen mortar and pestle. Samples were then homogenized in cell extraction buffer centrifuged, and supernatants were removed for analysis; pellets were discarded. The sample protein concentration was quantified by the previously described BCA protein assay (see section 4.3.1). Samples were then diluted to desired concentrations for analysis on ELISA plates.

4.3.3 Mammalian target of rapamyacin phosphorylated protein enzyme-linked immunosorbent assay

mTOR pSer2448 *in vitro* enzyme-linked immunosorbent assay (ELISA) kit was performed for the quantitative measurement of Ser2448 of mTOR protein in muscle tissue lysates. The ELISA kit utilized TMB substrate and during incubation was catalyzed

by HRP. The ELISA kit was performed according to the protocol of the commercially available mTOR pSer2448 ELISA kit (Abcam, Cambridge, United Kingdom).

4.3.4 Total and phosphorylated AKT protein enzyme-linked immunosorbent assay

The three isoforms of AKT (1/2/3) phosphorylated at pS473 and AKT1 (Total) *in vitro* SimpleStep™ ELISA Kit was performed for the semi-quantitative measurement of p-AKT and total-AKT in muscle tissue lysates. The ELISA kit utilized a combined capture antibody/analyte detector antibody, which was immobilized via immunoaffinity of an anti-tag antibody coating the wells. The ELISA kit utilized TMB substrate and during incubation wass catalyzed by HRP. The ELISA kit was performed according to the protocol in the commercially available AKT 1/2/3 (pS473) + AKT1 (Total) SimpleStep™ Kit (Abcam, Cambridge, United Kingdom).

4.3.5 Quantification of mTOR pSer2448 protein

A positive control sample extract was prepared in our laboratory from a sample tissue homogenate. Tissue lysate was prepared, according to the method described in section 4.3.1. The sample protein concentration in the extract was quantified using a protein assay described in section 4.3.1. The known protein concentration of the tissue lysate was used as the positive control standard for the mTOR pSer2448 ELISA kit. The tissue lysate was serially diluted and run alongside unknown sample concentrations. The concentration of each unknown sample was determined using a spectrophotometer

(Spectra Max 190, Molecular Devices, Sunnyvale, CA, USA) based on the standard curve at 450nm. The individual concentrations of mTOR pSer2448 protein in each sample was determined from the spectrophotometer duplicate readings (mean values of duplicate readings) and adjusted according to the dilution factor.

4.3.6 Quantification of total- and phosphorylated-AKT

A lyophilized AKT 1/2/3 control lysate standard (Abcam, Cambridge, United Kingdom) with known concentrations was serially diluted and run alongside unknown sample concentrations. The concentration of each unknown sample was determined using a spectrophotometer (Spectra Max 190, Molecular Devices, Sunnyvale, CA, USA) based on the standard curve at 450nm. The individual concentrations of total- and phosphorylated-AKT protein in each sample was determined from the spectrophotometer duplicate readings (mean values of duplicate readings) and adjusted according to the dilution factor.

4.4 Statistical analysis

Data are reported as mean ± standard deviation (SD). Two-way independent sample *t*-tests were used to compare REF and TUM groups. One-way Analysis of variance (ANOVA) with Bonferroni post-hoc comparisons was used to compare total protein, mTOR pSer2448, phospho-AKT, and total-AKT concentrations between TUM, CON1, CON2, FO1 and FO2 groups. Significance for all statistical tests was reported at

the level of $P<0.05$. Statistical analysis was completed using IBM Statistical Package for the Social Sciences (SPSS) for Windows (version 21.0, Chicago, IL).

4.5 Results

4.5.1 Total muscle protein

The amount of total soluble protein/g muscle tissue in REF was significantly higher than total soluble protein/g muscle tissue in TUM (**Figure 4-1**). The amount of total protein/g muscle tissue in TUM was not significantly different from any other group: CON1, CON2, FO1, or FO2, nor were there any significant differences between these groups (**Figure 4-2**). The lack of significance between groups (CON1, CON2, FO1, FO2) may be explained by large standard deviations between group means. When a significant main-effect was tested between control-fed and fish-oil fed animals, fish-oil fed animals (FO1 + FO2) exhibited a greater amount of total soluble protein/gram tissue compared to control-fed animals (CON1 + CON2) (25,690 ± 6,780 μg protein/g muscle versus 14,384 ± 10,170 μg protein/g muscle; $P=0.03$).

4.5.2 Proportion of mTOR pSer2448 protein in muscle

The proportion of mTOR pSer2448 in muscle (expressed as arbitrary units) was significantly higher in REF compared to TUM (**Figure 4-3**). The amount of mTOR pSer2448 in TUM was not significantly different than any other group: CON1, CON2, FO1, or FO2, nor were there any significant differences between these groups when they

were statistically compared to one another (**Figure 4-4**). CON2 appears to have the lowest proportion of mTOR pSer2448 in muscle compared to all other groups, however when individually compared to all other groups, statistical significance was not achieved (P>0.05). When a significant main-effect was tested between control-fed animals (CON1 + CON2) and fish-oil fed animals (FO1 + FO2), there was no significant difference between groups.

4.5.3 Proportion of phosphorylated-AKT and total-AKT protein in muscle

The amount of total-AKT and phospho-AKT (expressed as arbitrary units) in REF was not significantly different from TUM; however, the ratio of pAKT/AKT was significantly higher in REF compared to TUM (**Figure 4-5**). The ratio of pAKT/AKT in TUM was not significantly different from any other group: CON1, CON2, FO1, or FO2, nor were there any significant differences between these groups when they were statistically compared to one another (**Figure 4-6**). When a significant main-effect was tested between control-fed animals (CON1 + CON2) and fish-oil fed animals (FO1 + FO2), there was a trend for fish-oil fed animals to have an increased ratio of pAKT/AKT (P=0.10).

4.6 Discussion

This preliminary study aimed to determine if fish oil-feeding had an effect on mediators of the insulin pathway in skeletal muscle, namely AKT and mTOR proteins. Results from this study indicated that a tumour-bearing state decreases total protein

content of muscle compared to healthy rats. Muscle wasting and proteolysis are commonly reported in a tumour-bearing state (reviewed by Tisdale, 2002), potentially driven by inflammatory mediators and/or tumour-derived factors. It has been well established that in states of catabolic disease or inadequate caloric intake, cell protein breakdown, especially in skeletal muscle, increases to provide the organism with amino acids essential for gluconeogenesis, protein synthesis, and energy production (Mitch and Goldberg, 1996). When muscle experiences an assault, such as chemotherapy, it attempts to maintain homeostasis and may upregulate the genes and subsequent production of proteins that are associated with muscle protein synthesis. Results from the present study indicate that fish oil-fed animals receiving 1- and 2- cycles of chemotherapy exhibited a significantly higher content of muscle protein compared to animals fed a diet without fish oil, also receiving 1- and 2- cycles of chemotherapy. These findings may indicate that EPA and DHA were able to maintain muscle protein content, or overall, reduce muscle atrophy during chemotherapy relative to the control-fed groups. Since the protein assay in this study measured all soluble protein within the sample, the values reflect a variety of different proteins within the muscle, and further analysis would be required to determine degradation or synthesis of specific proteins. The mechanisms of protein breakdown can be further measured by isotopic amino acid tracing techniques or by measuring rate of protein degradation via accumulation of a specific amino acid (Lecker et al., 1999).

Compared to healthy rats, tumour-bearing rats exhibited a reduced proportion of phosphorylated mTOR pSer2448 in muscle protein, as well as a reduced ratio of pAKT/AKT. The phosphorylation of a protein dramatically alters its half-life and either enhances or inhibits its susceptibility to degradation, depending on the function of the

protein enabling homeostasis in the muscle (Lecker et al., 1999). Although there was no significant differences between fish oil and control-fed animals receiving 1- or 2-cycles of chemotherapy in mTOR pSer2448, phospho-AKT, or total-AKT, there was an overall main-effect of fish oil on the pAKT/AKT ratio. Fish oil-fed animals overall exhibited a higher ratio of pAKT/AKT compared to control-fed animals. These findings indicate that EPA and DHA may enhance the phosphorylation of AKT. A variety of mechanisms have been proposed in the ability of n-3 fatty acids to activate AKT; IL-2 mediated activation and phosphorylation of AKT, or through increased desaturation of the membrane and subsequent increased phosphorylation of AKT (Hulbert et al., 2005).

Results from the present study were surprising, since several other studies have demonstrated an enhanced activation of AKT (Flachs et al., 2009; Smith et al., 2010) and mTOR (Smith et al., 2011*a*, 2011*b*; Wei et al., 2013, McGlory et al., 2014) with n-3 fatty acid supplementation. Although these mediators have been proposed mechanisms for EPA and DHA in the attenuation of fatty muscle, it is possible that EPA and DHA may be affecting other mediators in the insulin pathway, or operating through different mechanisms altogether. A recent study reported that 1-week of supplementation with 5 grams of fish oil per day in healthy humans resulted in increased total mTOR protein content, although no further increases were observed after 2- and 4-weeks of continued supplementation (McGlory et al., 2014). These findings suggest that changes in mTOR protein content may be acute with fish oil supplementation. Although studies have demonstrated an ability for dietary fish oil to promote muscle protein synthesis through promoted AKT signalling in rats (You et al., 2010), other studies have shown conflicting results for the ability of EPA and DHA to promote AKT phosphorylation (Finlin et al.,

2012). Moreover, there is evidence for effects of EPA and DHA on other mediators of the insulin pathway (Hessvik et al., 2010). Mikami *et al.* (2012) reported that in diabetic and obese mice fed a fish oil diet with taurine, GLUT-4 expression was significantly greater compared to control diet group. EPA and DHA may also be attenuating fatty muscle through energy utilization and enhanced capacity for fat oxidation. Myotubes cultured with EPA exhibited an enhanced capacity for fatty acid oxidation, whereas culturing with linoleic and oleic acids did not enhance fat oxidation (Hessvik et al., 2010). Furthermore, n-3 fatty acids have been shown to increase the number of mitochondria (Granci et al., 2013) and improved the function of enzymes in the electron transport chain (Vaughan et al., 2012). Moreover, in an attempt to characterize the mitochondrial membrane phospholipid composition, one study found that 12-weeks of supplementation with 2 grams of EPA and 1 gram of DHA resulted in improved respiration kinetics in human skeletal muscle mitochondria through alterations in membrane structure. Omega- 3 supplementation also increased the capacity for mitochondrial reactive oxygen species emission without altering the content of oxidative products, suggesting the absence of oxidative damage (Herbst et al., 2014). These are other potential roles for EPA and DHA evoking a reduction in TG fatty acids that were not explored in this study.

Another possible explanation for the surprising results in the present study may be attributed to the method used. The ELISA kits are more sensitive to cell lysate samples as opposed to tissue lysates given the variability that can result from the homogenization of tissue sections. In the literature, western blot analysis is the most common method to measure AKT and mTOR protein content in tissue, and serves to be a feasible method to validate these results in future studies. Moreover, given the complexity of the insulin-

signalling pathway, (see Chapter 1, **Figure 1-1**) there are additional proteins that can be examined in this pathway, such as phosphotidol-inositol-3-kinase (PI3K), an upstream activator of AKT. Eight-weeks of fish oil supplementation in aging rats resulted in enhanced PI3K activity and content in muscle, with no changes in mTOR or AKT phosphorylation (Kamolrat et al., 2013).

Since low muscle mass and myosteatosis appear to occur concurrently to low levels of EPA and DHA in cancer patients, EPA and DHA may be nutrients essential for muscle anabolism (Murphy et al., 2011). Several different mechanisms have been proposed in the ability for EPA and DHA to attenuate muscle mass and fat infiltration in cancer and other disease states, further research is warranted in this area since it is possible that multiple mechanisms may be concurrently at play.

CHAPTER 4

Figures

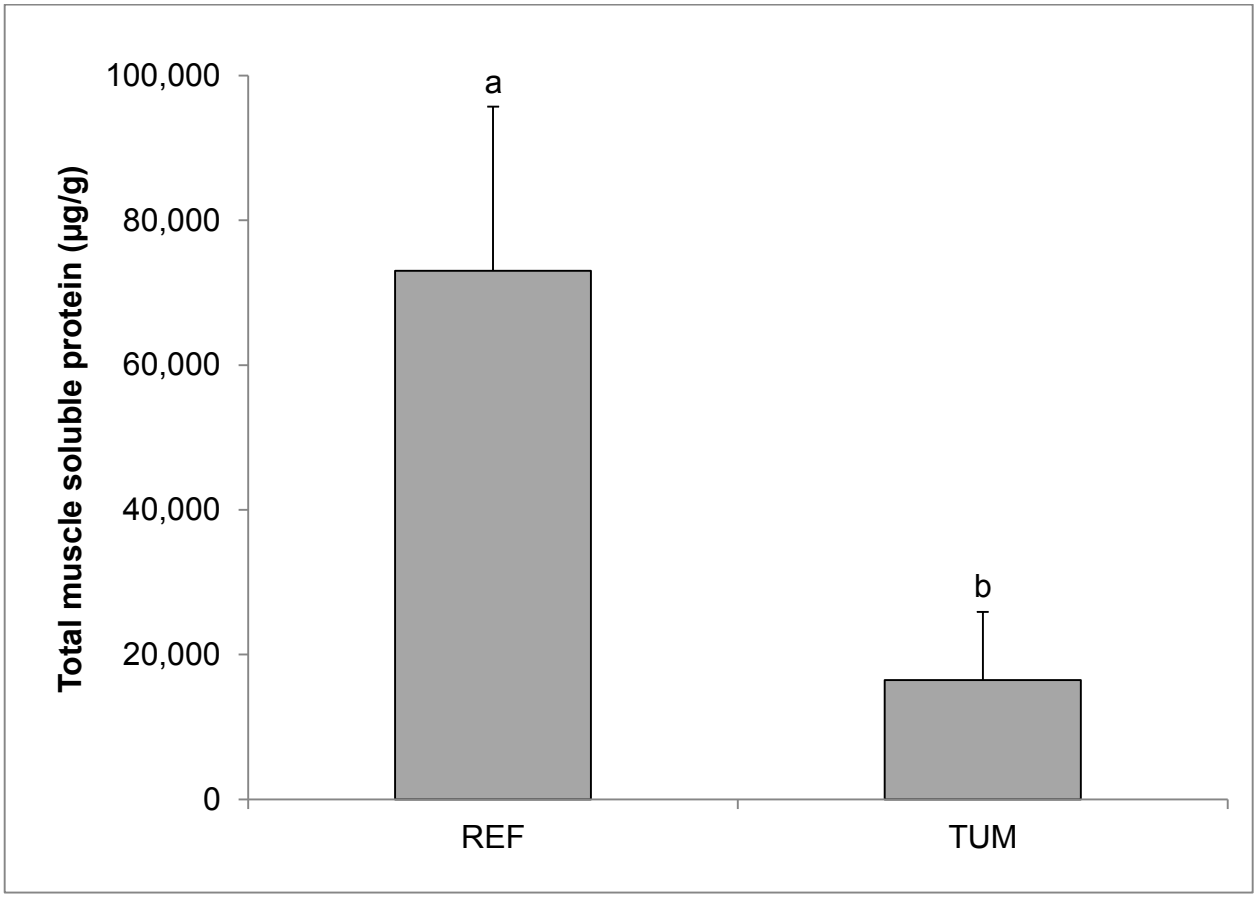

Figure 4-1. Effect of Ward colorectal carcinoma on total protein in gastrocnemius muscle of rats

Different superscripts indicate significant differences between groups (*P*=0.05).

Significant difference between REF and TUM groups determined by two-sided *t*-test Data is expressed as mean ± standard deviation. N=7 or 8/group. Total muscle protein (µg soluble protein/gram muscle tissue) in healthy Fischer 344 rats (REF) compared to Fischer 344 rats bearing the Ward colorectal carcinoma (TUM), receiving no chemotherapy.

Abbreviations: REF, Healthy; TUM, Tumour-bearing; CON1, Control diet + 1-cycle; CON2, Control diet + 2-cycles; FO1, Fish oil diet + 1-cycle; FO2, Fish oil diet + 2-cycles

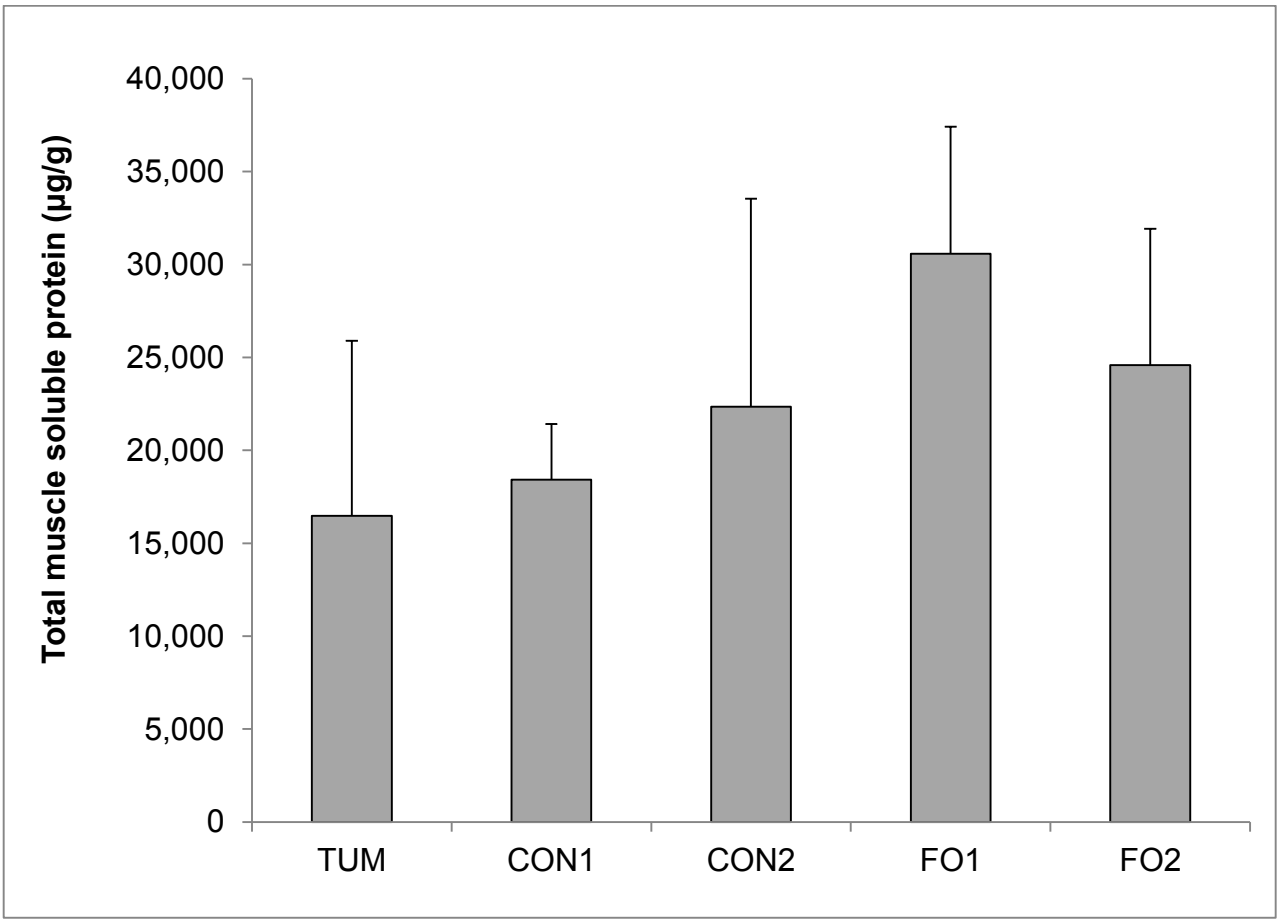

Figure 4-2. Comparison of total muscle protein in fish-oil fed and control-fed animals receiving 1- or 2-cycles of chemotherapy (CPT-11 + 5-FU)

No significant differences in total muscle protein (µg soluble protein/gram muscle tissue) between groups (P=0.10). TUM group used as reference in one-way ANOVA comparisons between CON1, CON2, FO1, FO2. Data is expressed as mean ± standard deviation. N=7 or 8/group. One-way ANOVA was used to determine significant differences between groups.

Abbreviations: CPT-11, irinotecan; 5-FU, 5-fluorouracil; REF, Healthy; TUM, Tumour-bearing; CON1, Control diet + 1-cycle; CON2, Control diet + 2-cycles; FO1, Fish oil diet + 1-cycle; FO2, Fish oil diet + 2-cycles

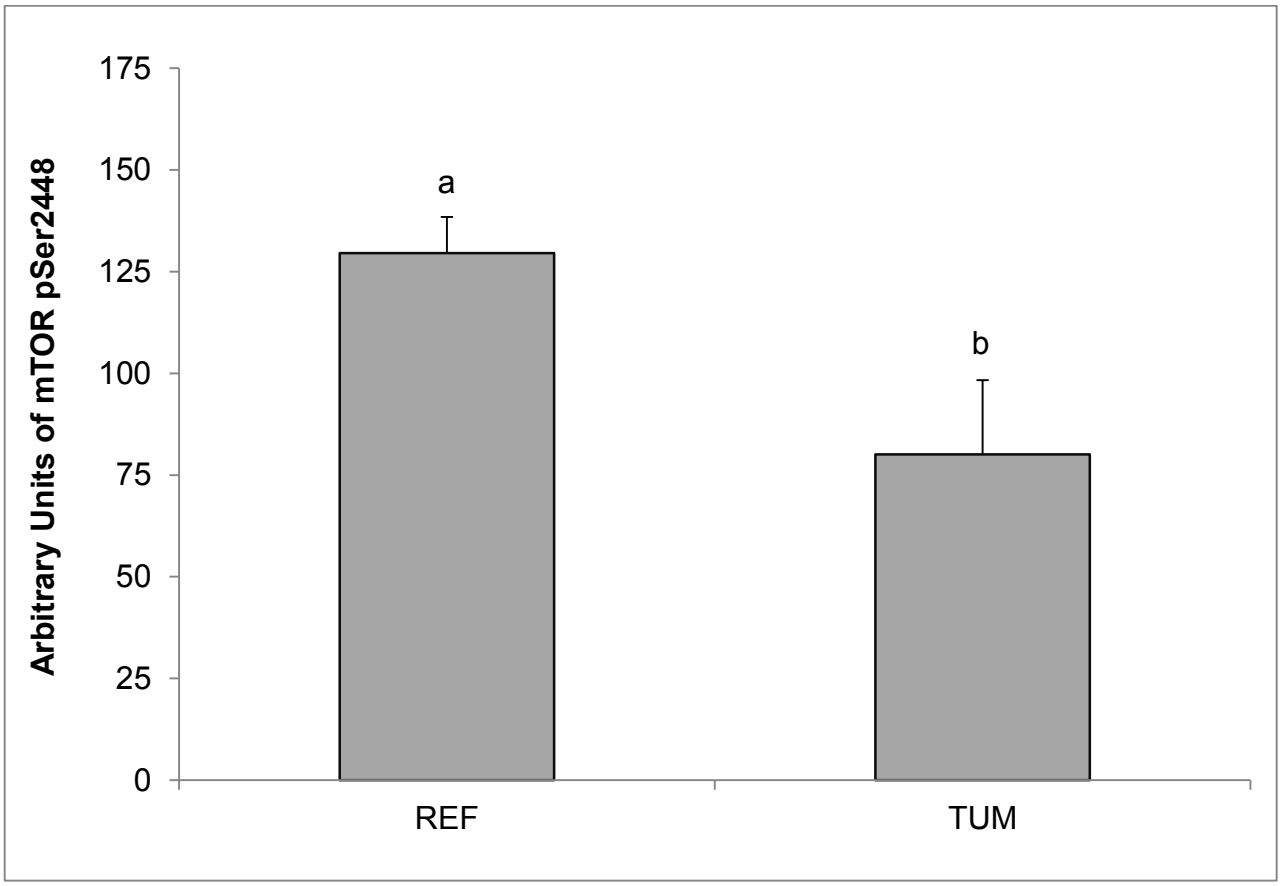

Figure 4-3. Effect of Ward colorectal carcinoma on proportion of mTOR protein in total protein in gastrocnemius muscle of rats

Different superscripts indicate significant differences between groups (P=0.05). Significant difference between REF and TUM groups determined by two-sided t-test Data is expressed as mean ± standard deviation. N=7 or 8/group.

Arbitrary units (AU) of mTOR pSer2448 in healthy Fischer 344 rats (REF) compared to Fischer 344 rats bearing the Ward colorectal carcinoma (TUM), receiving no chemotherapy. mTOR pSer2448 arbitrary units adjusted for total protein/gram muscle tissue.

Abbreviations: REF, Healthy; TUM, Tumour-bearing; CON1, Control diet + 1-cycle; CON2, Control diet + 2-cycles; FO1, Fish oil diet + 1-cycle; FO2, Fish oil diet + 2-cycles

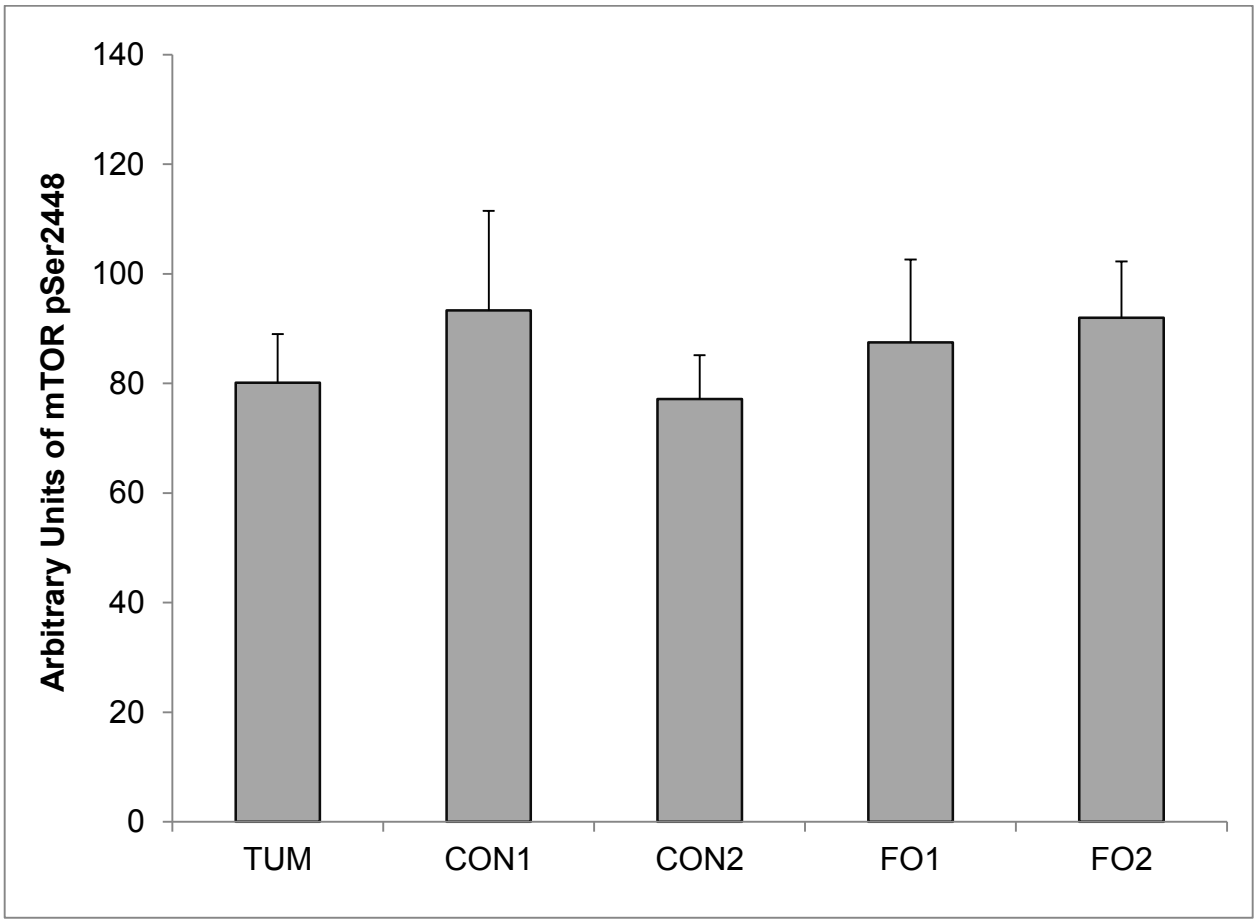

Figure 4-4. Comparison of proportion mTOR protein in total protein fish-oil fed and control-fed animals receiving 1- or 2-cycles of chemotherapy (CPT-11 + 5-FU)

No significant differences in AU of mTOR pSer2448 between groups (*P*=0.14). TUM group used as reference in one-way ANOVA comparisons between CON1, CON2, FO1, FO2. Data is expressed as mean ± standard deviation. N=7 or 8/group. One-way ANOVA was used to determine significant differences between groups.

Arbitrary units (AU) of mTOR pSer2448 adjusted for total protein/gram muscle tissue.

Abbreviations: CPT-11, irinotecan; 5-FU, 5-fluorouracil; REF, Healthy; TUM, Tumour-bearing; CON1, Control diet + 1-cycle; CON2, Control diet + 2-cycles; FO1, Fish oil diet + 1-cycle; FO2, Fish oil diet + 2-cycles

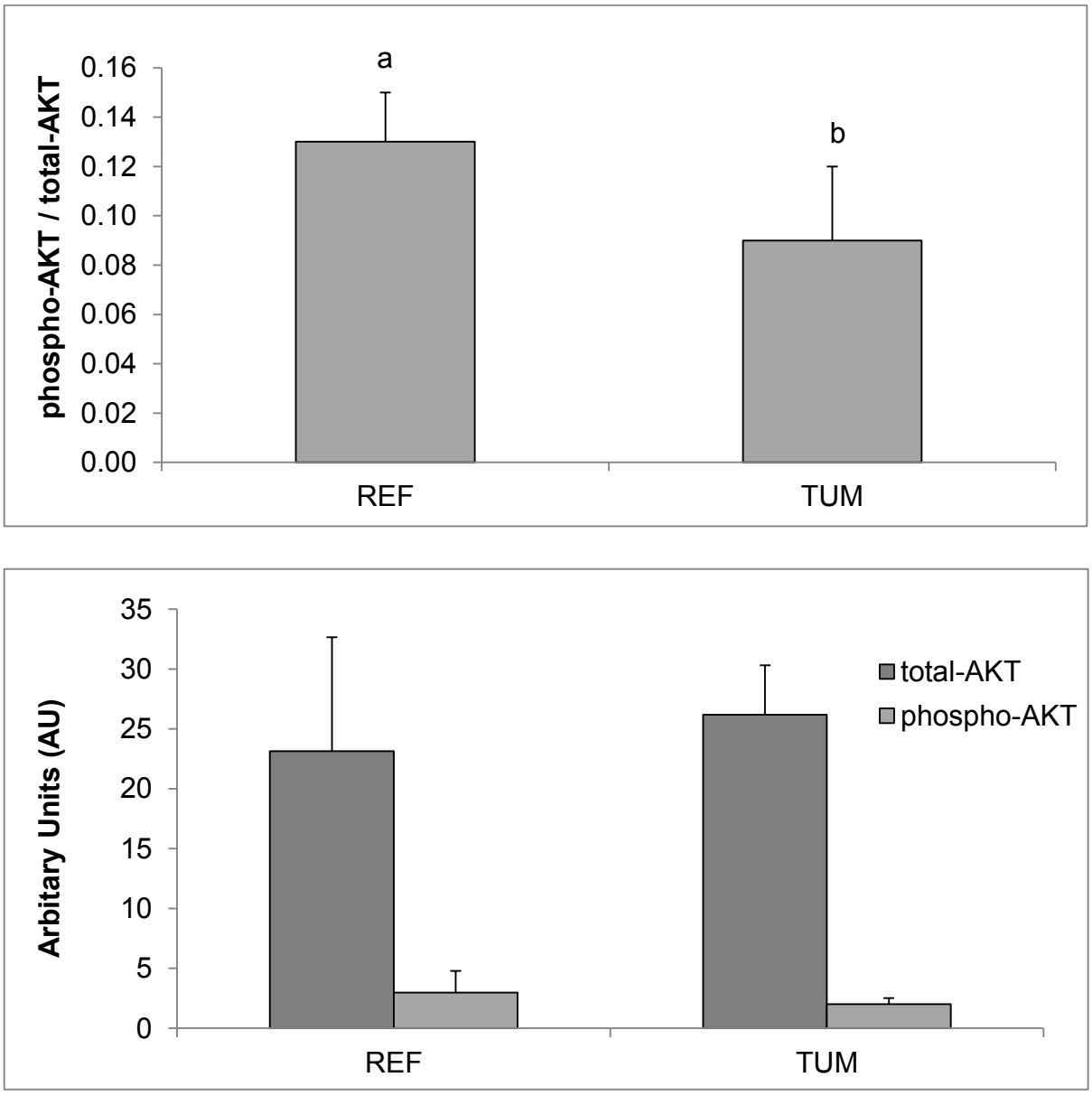

Figure 4-5. Effect of Ward colorectal carcinoma on ratio of phospho-AKT to total-AKT protein in gastrocnemius muscles of rats

Different superscripts indicate significant differences between groups (P=0.05). Significant difference between REF and TUM groups determined by two-sided t-test Data is expressed as mean ± standard deviation. N=7 or 8/group. Phosphorylated-AKT and total-AKT expressed as ratio and arbitrary units (AU) in healthy Fischer 344 rats (REF) compared to Fischer 344 rats bearing the Ward colorectal carcinoma (TUM), receiving no chemotherapy. Phosphorylated-AKT and total-AKT arbitrary units adjusted for total protein/gram muscle tissue.

Abbreviations: REF, Healthy; TUM, Tumour-bearing; CON1, Control diet + 1-cycle; CON2, Control diet + 2-cycles; FO1, Fish oil diet + 1-cycle; FO2, Fish oil diet + 2-cycles

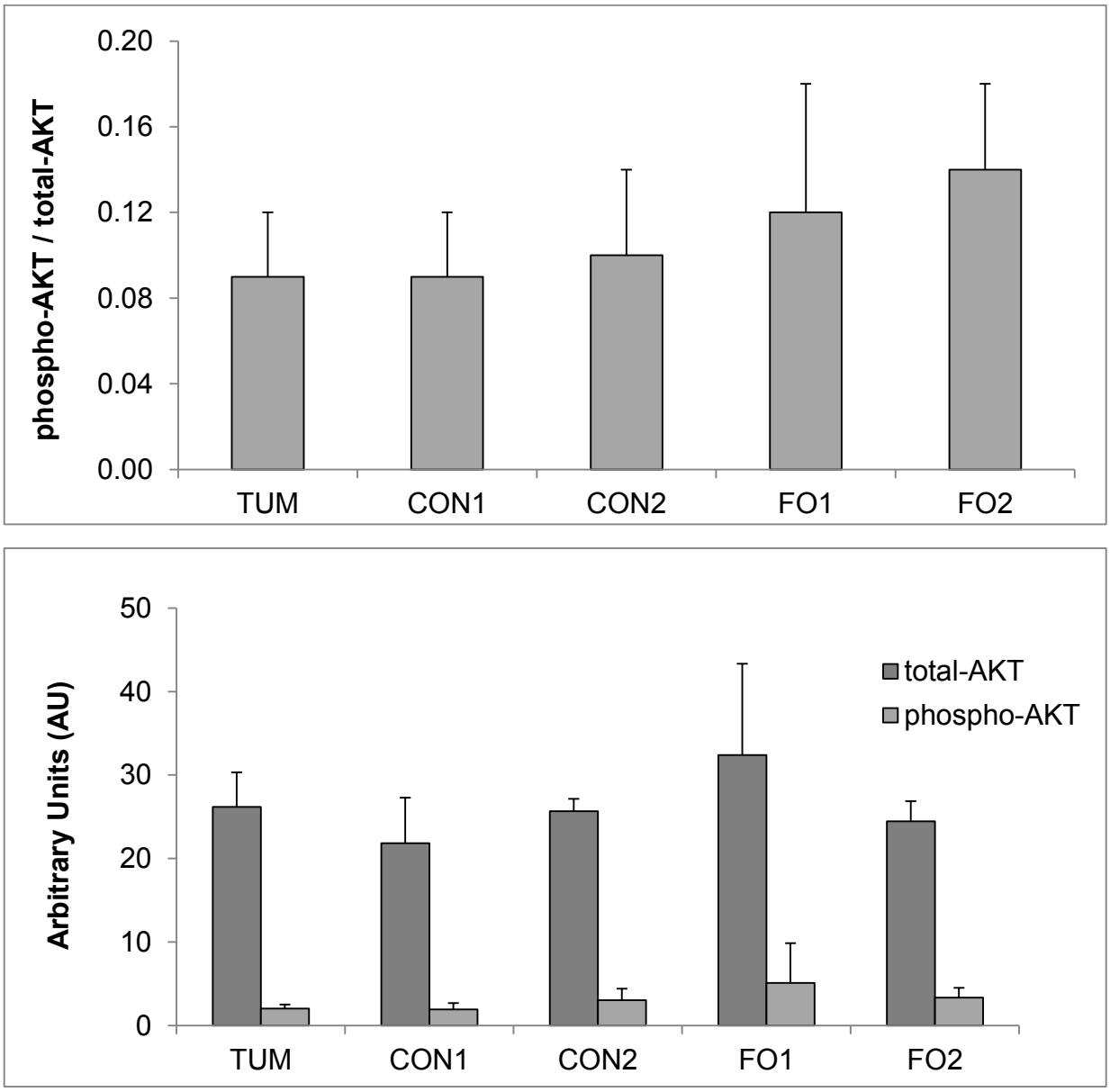

Figure 4-6. Comparison of the ratio of phospho-AKT to total-AKT protein in fish-oil fed and control-fed animals receiving 1- or 2-cycles of chemotherapy (CPT-11 + 5-FU)

No significant differences between groups (P=0.10). TUM group used as reference in one-way ANOVA comparisons between CON1, CON2, FO1, FO2. Data is expressed as mean ± standard deviation. One-way ANOVA was used to determine significant differences between groups. N=7 or 8/group. Phosphorylated-AKT and total-AKT expressed as ratio, and arbitrary units (AU). Arbitrary units of Phosphorylated-AKT and total-AKT adjusted for total protein/gram muscle tissue.

Abbreviations: CPT-11, irinotecan; 5-FU, 5-fluorouracil; REF, Healthy; TUM, Tumour-bearing; CON1, Control diet + 1-cycle; CON2, Control diet + 2-cycles; FO1, Fish oil diet + 1-cycle; FO2, Fish oil diet + 2-cycles

CHAPTER 5

General Summary and Future Research

5.1 General Summary

The first objective of the present study was to evaluate the morphology, lipid content and composition of gastrocnemius muscles of healthy Fischer 344 rats, and tumour-bearing rats fed a control diet with or without fish oil receiving 1- or 2-cycles of chemotherapy (CPT-11 + 5-FU).

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

i) Lower muscle cross sectional area

ii) Higher content of TG fatty acids in skeletal muscle tissue

iii) Higher content of adipocytes between muscle fibres (intermyocellular) and/or lipid droplets within muscle fibres (intramyocellular)

This study reported that rats bearing the Ward colorectal carcinoma for 2-weeks exhibited a significantly higher amount of triglyceride fatty acids within the gastrocnemius muscle tissue compared to healthy rats. TG fatty acid content was further characterized by reduced proportions and amounts of n-3 fatty acids compared to healthy rats. Increases in TG fatty acids were apparent in the qualitative histology analysis, which revealed increased red neutral lipid staining in the muscles of tumour-bearing animals

compared to healthy animals. Total phospholipid fatty acids were also significantly increased after 2-weeks of tumour growth compared to healthy rats. Since there was greater content of red lipid staining in tumour-bearing animals, the increase in phospholipids may be attributed to increased adipocyte content, and thus more membrane contributing to total phospholipid content. However, this hypothesis would require quantitative analysis to determine adipocyte number and size.

It was hypothesized that compared to tumour-bearing rats, rats receiving 1-cycle or 2-cycles of chemotherapy would exhibit:

i) Lower body weight
ii) Higher content of TG fatty acids in skeletal muscle tissue
iii) Lower proportion of total n-3 fatty acids, EPA, and DHA in muscle triglyceride and phospholipid fractions
iv) Lower muscle cross sectional area
v) Higher content of adipocytes between muscle fibres (intermyocellular) and/or lipid droplets within muscle fibres (intramyocellular)
vi) The effects above will occur to a greater magnitude after 2-cycles of chemotherapy compared to 1-cycle.

Chemotherapy resulted in significantly higher TG fatty acid content after 1-cycle and further after 2-cycles in control fed animals. This study reported that in control-fed and fish oil groups, the TG fatty acids content significantly increased after the second

cycle of chemotherapy compared to the first cycle. Interestingly, the amount of TG fatty acids in fish oil-fed animals after 2-cycles was similar to animals bearing tumour alone. These findings indicate a possible ability for fish oil to attenuate chemotherapy associated fat deposition in the muscle. Compared to cycle-1, there were no significant changes in EPA and DHA within groups, however between groups, the fish oil group exhibited significantly greater proportion of EPA, DHA, and total n-3 fatty acids compared to the control group in both TG and PL fatty acids. There was no significant difference in muscle cross sectional area after 1-cycle compared to 2-cycles in any of the groups. In both control and fish oil-fed groups, body weight and food intake both declined during each chemotherapy cycle, but recovered to baseline levels by the end of cycle-1 and cycle-2.

It was hypothesized that compared to rats receiving 1- or 2-cycles of chemotherapy and fed a control diet, rats receiving 1- or 2- cycles of chemotherapy and fed a fish oil diet would exhibit:

i) Higher body weight

ii) Lower content of TG fatty acids in skeletal muscle tissue

iii) Higher proportion of total n-3 fatty acids, EPA, and DHA in muscle triglyceride and phospholipid fractions

iv) Higher muscle cross sectional area

v) Lower content of adipocytes between muscle fibres (intermyocellular) and/or lipid droplets within muscle fibres (intramyocellular)

After 1-cycle, control-fed animals had significantly lower total phospholipid fatty acids. As mentioned, there was a 3-fold increase in total TG fatty acid content with 2-weeks of tumour growth. Interestingly, after 1-cycle of chemotherapy there was no significant difference in TG fatty acid compared to tumour-bearing animals. This finding indicates that the reduction in phospholipid content seen after 1-cycle in control fed animals may be due to loss of membrane from myocytes or organelles. Although there was no significant difference in muscle cross sectional areas between tumour-bearing rats and rats receiving 1-cycle, this reduction in total phospholipid fatty acids after chemotherapy may be a result of muscle fibre atrophy. However, this hypothesis would require validation with myosin heavy chain immunohistochemical staining to determine the proportion of various fibre types and potential fibre-type specific atrophy.

Compared to control fed rats receiving chemotherapy, fish oil fed rats exhibited lower triglyceride fatty acid content after both 1- and 2-cycles and a greater proportion of n-3 fatty acids in both phospholipid and triglyceride fatty acids. After 2-cycles, control-fed animals had the highest ratio of n-6/n-3 fatty acids compared to all other groups, due to a significant reduction in total n-3 fatty acids after 2-cycles of chemotherapy. Total phospholipid fatty acid content was highest in fish-oil fed animals, with this difference most markedly expressed after 1-cycle of chemotherapy. After 1-cycle of chemotherapy, muscle fat content of fish oil-fed rats was similar to rats bearing tumour alone suggesting that the lipid content was not pathological in the fish oil group. These findings indicate that increases in PL fatty acid content observed after 1-cycle in fish-oil fed rats is likely not a result of increases in adipocyte or lipid droplet content, but rather, changes in organelle number or size changes, or hypertrophy of the muscle fibres. The main effect of

fish oil feeding resulted in increased muscle cross sectional area compared to control-fed animals, which may be a result of muscle hypertrophy. To test this hypothesis, further research is warranted to quantify muscle fibre type, numbers and size beyond qualitative observations.

The second objective of the present study was to evaluate the protein concentration of mTOR (pSer2448), total-AKT (1/2/3), and phosphorylated-AKT (pSer473) in gastrocnemius muscles of healthy Fischer 344 rats, rats bearing the Ward colorectal carcinoma, tumour-bearing rats fed a control diet with and without fish oil receiving 1-cycle of chemotherapy (CPT-11 + 5-FU) and 2-cycles of chemotherapy.

It was hypothesized that compared to healthy rats not bearing a tumour, rats bearing the Ward colorectal carcinoma would exhibit:

i) Lower total muscle protein

ii) Lower concentration of mTOR (pSer2448) protein

iii) Lower ratio of phosphorylated-AKT to total-AKT protein

This study reported a significantly higher total protein content in healthy rats compared to tumour-bearing rats. However, compared to healthy rats, there were no significant differences in mTOR, phosphorylated-AKT or total-AKT protein in tumour-bearing rats.

It was hypothesized that compared to rats receiving 1- and 2-cycles of chemotherapy and fed a fish oil diet, rats receiving 1- and 2-cycles of chemotherapy and fed a control diet without fish oil will exhibit:

i) Lower total muscle protein

ii) Lower concentration of mTOR (pSer2448) protein

iii) Lower ratio of phosphorylated-AKT to total-AKT protein

This study reported a significantly higher total protein content in healthy rats relative to all other groups, but no significant differences between any other groups. There were no significant differences in mTOR (pSer2448) protein concentration between any of the groups or the ratio of phosphorylated-AKT (pSer473) to total AKT (1/2/3). These findings were surprising, since EPA and DHA have been previously shown to maintain whole-body protein synthesis, whole-body protein net balance and muscle mass in tumour-bearing mice (van Norren et al., 2009). In young steers provided EPA and DHA in the diet, insulin-stimulated whole-body protein synthesis was increased by 2-fold, along with increased activation of the AKT/mTOR/p70S6K signalling pathway (Gingras et al., 2007). In humans, young, middle-aged, and elderly men and women receiving 8-weeks of 4 g long-chain n-3 supplementation experienced an increased anabolic response to insulin and amino acid infusion (Smith et al., 2009, 2011*a*, 2011*b*). The muscle protein fractional synthesis rate, phosphorylated mTOR levels, and muscle cell size significantly increased after n-3 supplementation during insulin and amino acid infusion. A post-hoc analysis revealed an increase in AKT and mTOR phosphorylation and activation (Smith et al., 2009, 2011*a*, 2011*b*). Possible explanations for this finding

may be due to the method that was used to measure the tissue protein concentrations. Since this research was preliminary, ELISA kits were used as the first step in determining any possible changes of mTOR or AKT proteins with fish oil feeding. The ELISA kit methods are not as commonly used in rat tissue compared to Western Blot analysis. A large majority of the literature uses Western Blot analysis to measure mTOR and AKT protein concentrations. Future investigation into mTOR and AKT protein concentrations with Western Blot analysis is warranted.

5.2 Study strengths, limitations and future research

Strengths of this study include the use of two control groups; healthy rats that did not receive a tumour or chemotherapy fed a control diet (REF), and tumour-bearing rats that did not receive chemotherapy and fed a control diet (TUM). We determined the effect of a tumour by comparing REF and TUM groups, and determined the effect of chemotherapy by comparing TUM with CON1 and CON2. We were further able to determine the effect of fish oil feeding by comparing FO1 and FO2 to TUM, and CON1 and CON2. A tumour-bearing group receiving no chemotherapy (TUM) was chosen as the reference group for all comparisons, since chemotherapy is administered only to people with tumours. Furthermore, an additional strength of this study is the clinical relevance of the fish oil intervention. Patients will most likely receive a positive tumour diagnosis before beginning a dietary or clinical trial intervention of fish oil. Since rats began a fish oil intervention after tumour implantation, at the same time chemotherapy was initiated, the timeline of the present study closely models the clinical situation.

A significant strength of this study was the dietary design. Control and fish oil diets contained the same amount of energy and protein per gram of diet. Both diets were designed to represent the proportion of macronutrients typically consumed by humans, in addition to the ratios of polyunsaturated/saturated fat and n-6/n-3 fatty acids (see Chapter 1, **Figure 1-3**; Chapter 3, **Table 3-1** and **3-2**). Furthermore, control and fish oil diets contained the same proportion of fat, were matched for PUFA/SFA ratios, and differed only in the composition of fatty acids. The majority of animal models utilize standard laboratory chow, derived from undefined nutrient sources with unknown macronutrient, micronutrient, and fatty acid composition that do not reflect typical human dietary intakes.

This study has several limitations. Although groups were compared after both 1- and 2-cycles of chemotherapy, cancer patients would most likely receive multiple cycles of chemotherapy. Investigating changes that occur with additional cycles of chemotherapy in future models will better represent the treatment regime cancer patients receive clinically. Furthermore, the use of healthy and young animals may not allow study findings to be translational to humans, since the majority of cancer patients present with concurrent comorbidities. The use of animal models that represent for example, diabetes or obesity may produce results that are more relevant to what is seen presently in humans with cancer. Since diabetes, aging, and obesity themselves are associated with myosteatosis, applying these models to the protocol of the current animal model will contribute further findings for the ability of a fish oil intervention to attenuate fatty muscle.

Further research is warranted for the investigation of muscle morphological and quantitative differences between control and fish oil-fed groups. The lipid analysis used in the present study via gas chromatography determines total triglyceride fatty acid content, which would include lipid droplets and adipocytes. The stain used in the histological analysis, oil red O, stains all neutral lipids including lipid droplets. It is possible to stain for nuclei to determine if the lipids stained are in fact adipocytes, containing a nucleus, or lipid droplets with no nuclei present. Another method that can be explored in future studies to determine the location of lipids within the muscle is electron microscopy. In muscles of weight-losing cancer patients, one study demonstrated increased numbers of intramyocellular lipid droplets with the use of electron microscopy (Stephens et al., 2011). Similarly, this method can be applied in rat gastrocnemius muscles to determine the type and location of lipids present in the tissue.

Future studies should attempt to fast all animals upon study termination. Fasted animals will allow for additional analyses related to insulin sensitivity and glucose homeostasis measurements such as calculating HOMA-IR values. Additionally, future research is warranted to determine the effects of EPA or DHA supplementation independently, collectively with fish oil supplementation. Each of these fatty acids may be evoking different effects, which collectively reduce tumour size, increase muscle mass and reduce muscle TG content. Since fish oil contains other fatty acids in addition to EPA and DHA, it may be interesting to compare the results from the present study to a similar model with two additional study arms: EPA supplementation alone, and DHA supplementation alone.

5.3 Conclusion

Findings from the present study allow us to report that feeding fish oil at a physiological level (2.3 grams of fish oil (828 mg EPA, 414 mg DHA) per 100 grams of diet) was able to both prevent and reverse tumour-associated fatty muscle development, and further attenuate fat deposition into skeletal muscle during 1- and 2-cycles of chemotherapy treatment (CPT-11 + 5-FU) in Fischer 344 rats. Since myosteatosis has been shown to be independently associated with mortality in human cancer patients (Sabel et al., 2011, Antoun et al., 2013, Martin et al., 2013), fish oil feeding may clinically have the potential to reverse tumour-associated myosteatosis, and may further prevent myosteatosis development during chemotherapy treatment.

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