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UNIVERSITY OF NORTHERN COLORADO

Greeley, Colorado

The Graduate School

THE EFFECTS OF EXERCISE TRAINING ON CACHEXIA IN MICE
BEARING THE COLON-26 CARCINOMA

A Dissertation Submitted in Partial Fulfillment
Of the Requirements for the Degree of
Doctor of Philosophy

Nicole Wood

College of Natural and Health Sciences
School of Sport and Exercise Science

May 2019

This Dissertation by: Nicole Wood

Entitled: *The Effects of Exercise Training on Cachexia in Mice Bearing the Colon-26 Carcinoma.*

has been approved as meeting the requirement for the Degree of Doctor of Philosophy in the College of Natural and Health Sciences in the School of Sport and Exercise Science.

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ABSTRACT

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Colon cancer is third the most prevalent cancer in the United States in both men and women. However, it is also a largely preventable disease, as colorectal cancer (CRC) risk is increased by environmental factors such as poor diet and physical inactivity. Furthermore, CRC patients are highly susceptible to significant muscle wasting. Cachexia is classified as a multifactorial metabolic syndrome associated with impairments in immune function, fatigue, and overall weakness, that lead to increased morbidity and mortality. To date, multiple studies have shown that both aerobic and resistance training, individually, are highly effective in their ability to attenuate the deleterious effects of cachexia. However, little research has focused on the inclusion of a program that combines both modes of exercise, which may be more clinically relevant.

The purpose of the study was to examine the effects of multiple modes of exercise training on markers of cachexia to determine if treadmill training (TM), resistance training (RT), or a combination of the two (TM+RT) can effectively attenuate cachexia. A secondary purpose was to examine the involvement of interleukin-6 (IL-6) in the muscle wasting process and to determine if exercise training would reduce markers of systemic inflammation.

Six-week old male Balb/c mice were randomly selected to sedentary (SED; n = 24) or exercise (EX; n = 36) groups. Mice in the EX group were further randomized to

either a treadmill training (TM; n = 12), resistance training (RT; n = 12), or a combination (TM+RT; n = 12) group. At 11 weeks of age, half of the SED animals and all EX animals were inoculated with C26 cells; all EX mice continued their respective exercise protocols. Animals were sacrificed at 14 weeks of age. Cachexia was assessed via body mass, gastrocnemius mass and CSA, forelimb grip strength, and systemic inflammation.

Colon-26 carcinoma induced cachexia in SED+Tumor mice, as evident by significant decreases in body mass, gastrocnemius mass and CSA, declines in muscle function, and increases in systemic inflammation markers. Exercise, specifically RT, was able to provide significant improvements in all examined markers of cachexia, with the exception of relative body mass. Significant improvements also existed in TM+Tumor and TM+RT+Tumor in regard to gastrocnemius CSA and muscle function when compared to SED+Tumor.

These data suggest that exercise training, regardless of mode are able to provide significant benefit to cachectic mice and this may be due, in part, to decreases in systemic inflammation.

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CHAPTER I

INTRODUCTION

According to recent data from the American Cancer Society, more than 1.7 million people in America will be diagnosed with cancer, and nearly 600,000 people are estimated to die from the disease this year (Siegel, Miller, & Jemal, 2018). Though cancer-related deaths have decreased in recent years, due to advancements in detection and treatment, more people are living with cancer than ever before. Colorectal cancer (CRC) is among the highest in cancer death rates in the U.S. for both men and women (Mehl, Davis, Clements, et al., 2005a). Colorectal cancer risk is associated with environmental factors, such as diet and exercise. In fact, many studies have shown a decreased risk with increased physical activity (Aoi et al., 2010; Demarzo et al., 2008; Mehl, Davis, Clements, et al., 2005a; Puppa et al., 2012; Shin & Lee, 2011).

Cachexia, which is associated with an unintentional loss in body mass, is diagnosed in nearly 80% of cancer patients, and plays a role in 20% of cancer-related deaths (Baltgalvis, Berger, Pena, Davis, & Carson, 2008; Puppa et al., 2012). Cachexia contributes to not only muscle wasting, but also diminished adipose tissue and is associated with chronic inflammation, fatigue, and weakness, contributing to increased patient morbidity and mortality (Baltgalvis et al., 2008; Puppa et al., 2012). Cancer-related cachexia is especially prominent in gastrointestinal cancers, and patients can lose up to 30% of their original body weight (Baltgalvis et al., 2008). Though specific mechanisms behind cancer-induced cachexia remain unclear, evidence suggests that the

local release of pro-inflammatory cytokines may play a role, leading to changes in muscle protein metabolism. Alterations in muscles protein metabolism, in this case, result in an imbalance where protein degradation exceeds protein synthesis (Al-Majid & McCarthy, 2001).

Inflammatory mediators in the tumor microenvironment activate paracrine immune responses that can, in turn, promote tumor growth, proliferation, and angiogenesis, thus it is important to examine mechanisms that will help decrease chronic inflammation (Di Caro et al., 2016). Both systemic and intestinal inflammation contribute to the onset and severity of colon cancer (Mehl, Davis, Berger, & Carson, 2005b). Interleukin-6 (IL-6) is an inflammatory cytokine that plays a key role in mediating chronic inflammation and metabolic disturbances, therefore, an increase in circulating IL-6 can contribute to a reduction in overall body mass, muscle mass, and increase in protein degradation (Baltgalvis et al., 2008). Recent studies have shown that inhibiting IL-6 can attenuate muscle wasting (Baltgalvis et al., 2008). IL-6 is known to have both anti- and pro-inflammatory properties

Interventions that address muscle wasting are of particular interest to researchers. Evidence suggests that exercise may counteract some of the muscle metabolism imbalances caused by cancer-induced cachexia. Resistance training has a significant impact on muscle weight in tumor bearing mice. Al-Majid & McCarthy (2001) examined the effects of muscle stimulation to mimic resistance training in the Colon-26 (C-26) adenocarcinoma mouse model. Results indicated that tumor bearing animals had extensor digitorum longus (EDL) weights that were significantly smaller than their contralateral control muscles. Furthermore, EDL weights were significantly greater in the stimulated

EDL muscles of C-26 mice when compared to their contralateral control muscles, indicating that this repeated muscle contraction may, in fact, attenuate muscle wasting.

Many studies have shown that physical activity has an antitumor effect. Low to moderate-intensity exercise can trigger inhibition, or activation, of appropriate cellular signaling pathways that may slow carcinogenesis (Demarzo et al., 2008). The antitumor effects of physical activity appear to be the result from prevention during early stages of CRC, suggesting that individuals who exercise regularly are at a decreased risk (Aoi et al., 2010). Though exact mechanisms have not been completely clear, many potential mechanisms, such as immune system activation and metabolic improvements, have been identified in past research (Demarzo et al., 2008). In regard to CRC, it is thought that one potential mechanism behind decreased cancer risk may be due to exercise-induced gastrointestinal motility (Aoi et al., 2010). Another potential mechanism to examine is local inflammation. Inflammatory responses occur as a result of local tissue ischemia that is induced by exercise and can result in a stimulus to trigger long-term anti-inflammatory mechanisms (Demarzo et al., 2008). These mechanisms are mediated, in part, by muscle-derived IL-6, which can stimulate the release of anti-inflammatory cytokines, such as IL-10, and inhibit pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF- α). Furthermore, moderate physical activity has been shown to decrease macrophage IL-6, resulting in a decrease in colon cancer development (Mehl, Davis, Clements, et al., 2005a).

It is suggested that CRC is likely induced by environmental factors, such as physical inactivity, therefore, it is imperative to elucidate methods to prevent such a disease from occurring. For cancer patients who develop cachexia, it is crucial to identify

underlying mechanisms by which the disease occurs and increasing muscle mass is likely to offset the damaging effects, thus leading to decreases in morbidity and mortality.

Statement of Purpose

The purpose of this study was twofold: (a) to examine the effects of three different exercise training protocols on skeletal muscle wasting in male Balb/c mice bearing the C26 carcinoma and (b) to examine the role of circulating IL-6 in the severity of skeletal muscle wasting.

Research Questions and Hypotheses

- Q1 Will endurance exercise, resistance exercise, or a combination of both be associated with a reduced severity of skeletal muscle wasting in cachectic mice?
- H1 Chronic endurance exercise will attenuate cachexia-induced decreases in gastrocnemius muscle cross sectional area and wet muscle mass.
 - H2 Resistance training will attenuate cachexia-induced skeletal muscle wasting to a greater extent than endurance exercise.
 - H3 A combination of both resistance training and endurance training will attenuate skeletal muscle wasting to a greater extent than with either protocol alone.
- Q2 Does IL-6 play a significant role in cachexia-induced skeletal muscle wasting?
- H1 Circulating plasma IL-6 is associated with skeletal muscle wasting.
 - H2 Chronic exercise training will reduce circulating IL-6 in cachectic mice, regardless of training mode, thus reducing the severity of the atrophy.

Table 1

Abbreviations Used in This Study

Abbreviation	Explanation	Abbreviation	Explanation
AMP	adenosine monophosphate	mRNA	messenger ribonucleic acid
AMPK	AMP-activated protein kinase	O.C.T.	optimal cutting temperature
Apc	adenomatous polyposis coli	RNA	ribonucleic acid
ATP	adenosine triphosphate	SED	sedentary
C26	Colon-26 tumor cell	STAT	signal transducer and activator of transcription
CRC	colorectal cancer	TA	tibialis anterior
CSA	cross-sectional area	TM	treadmill
EDL	extensor digitorum longus	RT	resistance training
EDTA	ethylenediaminetetraacetic acid	TNF- α	tumor necrosis factor-alpha
EX	exercise training		
HFES	high frequency electrical stimulation		
IGF-1	insulin-like growth factor-1		
IGF-1	insulin-like growth factor-1 receptor		
IL-6	interleukin 6		
IL-10	interleukin 10		
ELISA	enzyme-linked immunosorbent assay		
Jak	janus kinase		
Kras	Kristen rat sarcoma virus		
MHC	myosin heavy chain		

Definitions of Terms

Atrophy - wasting, or degeneration, of cells that leads to a decrease in the cell size

Cachexia - unintentional loss in body mass that is associated with impaired immune function, chronic inflammation, fatigue, muscle wasting, and overall weakness.

Cell Culture - a process by which cells are grown under controlled conditions outside of their natural environment.

Cytokine - cell signaling molecules that aid in cell-to-cell communication in response to inflammation, infection, or trauma.

Electrical Stimulation - use of electrodes to induce a tetanic or fatiguing muscle contraction.

Hypertrophy - the enlargement of a tissue, such as skeletal muscle, that increases the size of its cells.

Myosin Heavy Chain - the motor protein of skeletal muscle thick filaments

Oncogene - a gene that has the potential to cause cancer.

Splenomegaly - abnormal enlargement of the spleen.

Tumor-Suppressor - an anti-oncogene that protects a cell from one step on the path to cancer.

Tumorigenesis - the formation of a tumor, or tumors.

Ubiquitination - the addition of a ubiquitin to a substrate protein that marks the protein for degradation via the proteasome.

CHAPTER II

REVIEW OF LITERATURE

In 2018, it is estimated, by the American Cancer Society, that nearly 1.7 million new cancer cases will be diagnosed and greater than 600,000 cancer-related deaths will occur (Siegel et al., 2018). Colon cancer is among the highest in new cancer cases and mortality, among men and women, ranking third in the United States (Tan & Du, 2012). Nearly 80% of colon cancer cases are attributed to environmental factors, such as poor diet and physical inactivity, suggesting that colon cancer is also largely preventable (McClellan et al., 2014). Colorectal cancer (CRC) patients are highly susceptible to significant skeletal muscle wasting. Research suggests that nearly 80% of all cancer patients are diagnosed with cachexia, which plays a significant role in 20% of cancer-related deaths (Aulino et al., 2010; Bonetto et al., 2011; Bonetto et al., 2012; Coletti et al., 2016; Puppa et al., 2012). Low- to moderate-intensity exercise is effective in lowering the risk of cancer and may slow the progression of, or prevent, the development of cancer (Demarzo et al., 2008; Puppa et al., 2012). In addition, exercise is known to lower chronic inflammation, has been shown to be a significant factor in cancer prevention, and may play a role in attenuating the effects of cancer-induced cachexia (Puppa et al., 2012).

Colon Cancer

The colon, or large intestine, is a fundamental part of the gastrointestinal tract. It functions to absorb water, minerals, and nutrients, and to serve as a storage area for waste. The colon plays an important role in physiological economy of the host organism

and its biological nature increases susceptibility to development of diverse pathologies, such as cancer (Arvelo, Sojo, & Cotte, 2015). Generally speaking, colon cancer diagnoses occur late and detection is often difficult due to the rapid formation and rate of transfer through the bloodstream. Development of CRC can occur through the mutation of oncogenes, which affect cell proliferation and apoptosis, or through the inactivation of tumor suppressor genes.

The Colon-26 (C26) tumor was first developed in 1975 in an effort to establish a suitable murine colon cancer model to be used in biological and chemotherapy research (Corbett, Griswold, Roberts, Peckham, & Schabel, 1975). In their studies, 82 colon tumors were induced and transplanted in four different strains of inbred mice. Only four tumors, one of which was C26 (number 26 of 82), originating from female Balb/c mice, survived and were subject to serial passaging (Corbett et al., 1975). In regard to tumor formation, the C26 animal model provides similarities to human colon cancer, in that tumors are likely chemically induced, rather than spontaneously formed (Corbett et al., 1975). The C26 cell line is an N-nitroso-N-methylurethane induced, undifferentiated, grade IV colon carcinoma (Bhadury, López, Muralidharan, Nilsson, & Nilsson, 2013; Castle et al., 2014; Corbett et al., 1975). C26 is known to be highly tumorigenic, with a low tendency to metastasize, and exhibits high mortality in mice bearing the carcinoma (Aulino et al., 2010). This cell line expresses an oncogene mutation to the Kras, or p21, protein, which is observed in 35-45% of all CRC incidences (Castle et al., 2014; Tan & Du, 2012). This mutation increases activity of the epidermal growth factor receptor signaling pathway, leading to uncontrolled cell growth. Rodent models are widely used in the study of a variety of cancers, including CRC. The C26 cell line may be implanted

orthotopically or ectopically, as a solid tumor fragment (Aulino et al., 2010) or from cell culture (Khamoui, 2014). Furthermore, though C26 is a colon cancer, the cell line may be implanted in various locations, such as in the hind limbs (Hiroux et al., 2016), dorsally between the scapulae (Aulino et al., 2010; Khamoui, 2014), or into a skeletal muscle of interest (Hiroux et al., 2016). Additionally, mice bearing the C26 colon carcinoma are a well-characterized, extensively studied model of cancer-induced cachexia (Bonetto, Rupert, Barreto, & Zimmers, 2016). Mice implanted with the C26 carcinoma exhibit significant declines in body mass, with wasting of skeletal muscle and adipose tissue, without alterations in food intake (Al-Majid & McCarthy, 2001; Aulino et al., 2010; Bonetto et al., 2017).

Cachexia

Cachexia is identified as an unintentional loss of body mass, resulting from the progressive wasting of both skeletal muscle and adipose tissue (Baltgalvis et al., 2010; Bonetto et al., 2011; Khamoui, 2014; Puppa et al., 2012). The disorder occurs with many chronic diseases, including HIV and cancer, and is associated with poor prognosis and is implicated in at least 20% of cancer-related deaths (Aulino et al., 2010; Coletti et al., 2016). Cachexia can be defined as a metabolic syndrome that is associated with chronic inflammation, impaired immune function, hypermetabolism, fatigue, and overall weakness, leading to increased morbidity and mortality (Baltgalvis, et al., 2010; Hardee et al., 2016; Mehl, Davis, Berger, et al., 2005b; Puppa et al., 2012). One hallmark of this disorder is marked skeletal muscle atrophy that leads to significant declines in muscle strength and overall quality of life. Nearly 80% of all cancer patients are diagnosed with

cachexia, which is identified in those individuals exhibiting >5% loss of body weight (Mehl, Davis, Berger, et al., 2005b).

Skeletal muscle mass in humans is difficult to measure, as muscles cannot be fully excised, weighed, and examined. Though measurements of whole muscle volume, such as cross-sectional area (CSA), are often examined via imaging, the equipment required for such analysis is costly. This methodology is advantageous, as it allows researchers to investigate intact muscle which can then be complimented by examining skeletal muscle morphology via muscle biopsy. Muscle biopsies are, however, highly invasive. As a result, animal models have been widely used in cachexia research and have provided greater insight to the muscle wasting process.

Al-Majid and McCarthy (2001) found that C26 tumors induced significant wasting of the gastrocnemius and extensor digitorum longus (EDL) within 17-days post tumor inoculation in 7- to 8-week old female CD2F1 mice. It was reported that tumor-bearing mice experienced a significant loss in overall body mass by day 15 after inoculation, while control animals gained weight slightly. On day 17, tumor-bearing mice had a 10% lower body mass than controls (Al-Majid & McCarthy, 2001). Aulino et al. (2010) examined CSA in 7-week old Balb/c mice bearing the C26 carcinoma. Tumor-bearing mice displayed a marked decrease in muscle mass and CSA in the EDL and tibialis anterior (TA). It was also reported that tumor-bearing animals had a catastrophic loss in body weight (-30%) in the third week following tumor induction, and death ensued in 90% of mice within 32 days of transplantation.

**Cachexia & Systemic Inflammation:
Interleukin-6 & Signal Transducer
and Activator of Transcription
(STAT3) Signaling**

In both human and rodent models, nearly 80% of CRC cases are attributed to environmental factors, such as poor diet and physical inactivity (Baltgalvis et al., 2007; McClellan et al., 2014). The onset of cachexia is identified as multifactorial and research suggests it can be linked to increases in reactive oxygen species, metabolic abnormalities, changes in skeletal muscle protein turnover, and systemic inflammation. Multiple pro-inflammatory cytokines, such as IL-6, TNF- α , C-reactive protein, have been implicated in association with both the risk of CRC and cachexia.

IL-6 is a multifunctional cytokine, with pro- and anti-inflammatory properties, that is involved in a variety of host defenses and pathological processes. It can activate the transcription of genes involved in cellular proliferation, differentiation, and apoptosis. This is accomplished by binding to a plasma membrane receptor complex which contains the common signal transducing receptor chain glycoprotein 130 that induces signal transducer and activator of transcription 3 (STAT3) signaling with ligation (Baltgalvis et al., 2007). While transient increases in STAT3 activation provides anti-inflammatory effects, prolonged activation can induce pro-inflammatory effects. Under normal conditions, circulating levels of IL-6 are very low but can dramatically increase in response to tissue injury or other inflammatory conditions. In tumors, an inflammatory microenvironment is maintained. Heinrich, Behrmann, Haan, Hermanns, & Uller-newen (2003) identified mechanisms of IL-6 cytokine signaling and its regulation in a comprehensive review. Though IL-6 signaling is not completely understood due to the complexity of the cytokine and its signaling network, researchers continue to seek to

better understand the pathway. Circulating levels of IL-6 have been shown to increase significantly in both C26 and $Apc^{Min/+}$ mice.

Puppa et al. (2012) investigated the effects of IL-6 overexpression on the development of cachexia in $Apc^{Min/+}$ mice. At 12 weeks of age, animals were electroporated with either an empty vector or an IL-6 plasmid used to increase endogenous IL-6 production and were sacrificed two weeks later. Results of the study confirmed that systemic IL-6 overexpression leads to a significant decrease in both body weight and skeletal muscle mass in sedentary $Apc^{Min/+}$ mice. STAT3 and nuclear factor- κ B (NF κ B) signaling were also examined to understand how IL-6 overexpression might alter inflammatory signaling in muscle. STAT3 and NF κ B activation was induced in mice overexpressing IL-6, leading to increased inflammation in the quadriceps muscle. In another study, Baltgalvis et al. (2007) sought to determine the relationship between circulating IL-6 and tumor burden for the development of cachexia in $Apc^{Min/+}$ mice. In this study, $Apc^{Min/+}$ mice were compared to $Apc^{Min/+}/IL-6^{-/-}$ mice. Cachexia was scored based on body mass, gastrocnemius muscle weight, and epididymal fat weight. In $Apc^{Min/+}$ mice, there was a 61% decrease in gastrocnemius muscle weight and complete elimination of the epididymal fat pad in severely cachectic mice when compared to mice classified as mildly cachectic. Plasma IL-6 saw approximately a 10-fold increase in severely cachectic mice. Phosphorylated STAT3 increased 4.5-fold in severely cachectic mice when compared to mildly cachectic mice, showing there is, in fact, a significant increase in activation of the STAT3 signaling pathway. Data also revealed a significant inverse relationship between STAT3 activation and gastrocnemius muscle weight. Interestingly, IL-6 mRNA did not change with IL-6 overexpression, suggesting that the

STAT3 activation in skeletal muscle appears to be the result of changes in circulating IL-6 rather than muscle-derived IL-6. Together, these data suggest that elevated IL-6 significantly alters muscle weight and can induce cachexia in the mouse model.

Effects of Cachexia on Skeletal Muscle Structure and Function

Though cachexia is marked by a loss in both skeletal muscle and adipose tissue, skeletal muscle degradation will be the topic of focus here. Cachectic patients can lose up to 30% of their total body mass, of which muscle mass accounts for a large portion (Mehl, Davis, Clements, et al., 2005a). As skeletal muscle degrades, muscle function and overall strength decline, leading to a decreased quality of life and increased mortality. Increases in protein degradation are often accompanied by decreases in protein synthesis, thus altering overall protein turnover. In addition to IL-6 and STAT3 signaling, the ubiquitin-proteasome pathway is also implicated in protein degradation and its constituents are often upregulated in cachectic muscles (Mehl, Davis, Clements, et al., 2005a). The ubiquitin-proteasome pathway is an ATP-dependent pathway that plays a predominant role in degradation of myofibrillar proteins. In this pathway, myosin heavy chain (MHC) is selectively targeted, leading to the degradation of thick myofilaments and a change in myosin isoform expression. In this pathway, E3 ligases atrogen-1/MAFbx and MuRF-1 regulate muscle atrophy during cachectic conditions (Mehl, Davis, Clements, et al., 2005a). Conversely, depletion of these E3 ligases results in hypertrophy (Hanai et al., 2007)

Myosin Heavy Chain Expression

Muscle fibers can be classified based on contractile speed, MHC, and metabolic capacity. MHC isoforms are critical in determining functional variations among skeletal muscles (Wells, Edwards, & Bernstein, 1996). In rodent skeletal muscles, four MHC types have been identified, including MHCI (slow twitch), and three isoforms of MHCII (fast twitch), including IIa, IIx, and IIb. MHC is a major target of protein degradation, and in cachectic muscles, type II fibers are compromised, while type I fibers are largely unaffected (Mehl, Davis, Clements, et al., 2005a). White, Baynes, et al. (2011b) showed that, at the initiation of cachexia (< 5% loss in body weight), there was a 19% reduction in the rate of myofibrillar protein synthesis that continued to decline further with the progression of cachexia. Furthermore, the gastrocnemius, specifically, displayed a 17% decrease in mass during this same period that also continued to decline with the progression of cachexia. White, Baynes, et al., (2011b) also found that total protein degradation was increased by 45% in $Apc^{Min/+}$ mice at initial weight loss (mild cachexia), while $Apc^{Min/+}$ mice who were identified as severely cachectic, with extreme weight loss, had a total protein degradation increase of 188%. In addition, Acharyya et al. (2004) found significant alterations in MHCIIb in the TA and gastrocnemius muscles of C26 tumor-bearing mice when compared to non tumor-bearing controls, while MHCI dominant muscles, such as the soleus, saw very little change. It was also reported that no other myofibrillar proteins were observed to be altered in C26 tumor-bearing mice (Acharyya et al., 2004).

Diffie, Kalfas, Al-Majid, & McCarthy (2002) examined changes to myosin isoform expression with cachectic condition in CD2F1 mice bearing the C26 carcinoma.

In tumor-bearing mice, there was a shift in MHC expression in the soleus, with an increase in the expression of MHCIIb and a decrease in the expression of MHCI. In control animals, soleus muscles had no traceable expression of MHCIIb. Furthermore, no shifts in MHC expression in the gastrocnemius, an already highly glycolytic fiber, occurred. Similar to findings from several other studies (Baltgalvis et al., 2010; Hardee et al., 2016; Khamoui, 2014; Mehl, Davis, Berger, et al., 2005b), the atrophy seen in cachectic muscles preferentially occurs in glycolytic fibers rather than oxidative. This is supported by the significant loss of mass observed in muscles containing mostly MHCII fibers, such as the EDL and gastrocnemius, and shift in fiber type found in highly oxidative muscles, such as the soleus (Al-Majid & McCarthy, 2001; Diffie et al., 2002).

Protein Degradation Mechanisms and Pathways

As discussed previously, many cellular signaling pathways are involved in protein degradation and many have been specifically implicated in cancer cachexia. The ubiquitin-proteasome pathway has been identified as one of the major mechanisms involved in the regulation in skeletal muscle wasting (Acharyya et al., 2004; Diffie et al., 2002; Khal, Wyke, Russell, Hine, & Tisdale, 2005). In C26 tumor-bearing mice, the expression of ubiquitin and muscle-specific E3 ligases, MuRF1 and atrogin-1/MAFbx are markedly increased in cachectic muscles when compared to controls (Acharyya et al., 2004; Mota et al., 2017). Similarly, Aulino et al. (2010) noted significant up-regulation of atrogin-1 expression in tumor-bearing mice. Western blot analysis also revealed protein ubiquitination was both qualitatively and quantitatively affected by C26 tumors. In addition, protein ubiquitination led to impairments in muscle function. EDL and soleus muscles of C26 mice demonstrated a lower maximal force and a diminished resistance to

fatigue than their control counterparts. Together, these findings support the involvement of the ubiquitin-proteasome pathway in cancer-induced muscle wasting and muscle dysfunction.

In addition to E3 ligases, the ubiquitin-activating enzyme (E1) and ubiquitin-conjugating enzyme (E2) can be quantified to demonstrate activation of the ubiquitin-proteasome pathway. Khal et al. (2005) investigated the effects of cachexia on E2 and found that its mRNA expression was increased two-fold in mice exhibiting a 12% weight loss and remained elevated up to a 20% weight loss. C2 and C5 (α and β proteasome regulatory subunits, respectively), also demonstrated significant increases in mRNA expression with the same weight loss range. Interestingly, E2, C2, and C5 were not significantly increased with weight losses greater than 20%. Furthermore, in agreement with other cancer cachexia studies, Khal et al. (2005) reported a direct relationship between loss in body weight and decreases in gastrocnemius mass, signifying that type II fibers are, in fact, the most affected by cachexia and that the ubiquitin-proteasome pathway is primarily responsible for protein degradation in tumor bearing mice.

Exercise Training

To date, numerous studies have reported the anti-tumor effects of physical activity, as low- to moderate-intensity exercise can trigger inhibition, or activation, of appropriate cellular signaling pathways that may slow carcinogenesis (Demarzo et al., 2008). In CRC, the anti-tumor effect appears to result from prevention during early stages of the disease, suggesting that individuals who exercise regularly have a reduced risk (Aoi et al., 2010). Although exact mechanisms for the anti-tumor effects of exercise are not completely understood, several potential mechanisms have been identified, including

immune system activation and metabolic improvements that occur with physical activity. In regard to CRC, it has been suggested that exercise-induced improvements in gastrointestinal motility may play a role in the decreased risk of the disease (Aoi et al., 2010). Chronic systemic inflammation has been widely regarded as major cause of CRC (Baltgalvis et al., 2007; Mehl, Davis, Clements, et al., 2005a, 2010). In addition, transient inflammation that results from exercise-induced local tissue ischemia can trigger long-term anti-inflammatory responses (Demarzo et al., 2008). These mechanisms are mediated, in part, by muscle-derived IL-6, which can stimulate the release of anti-inflammatory cytokines, such as IL-10, and inhibit pro-inflammatory cytokines, such as tumor necrosis factor- α (TNF- α). Furthermore, moderate physical activity has been shown to decrease macrophage IL-6, resulting in a decrease in colon cancer development (Mehl, Davis, Clements, et al., 2005a). These aforementioned mechanisms, in addition to skeletal muscle hypertrophy that occurs with exercise training, may also help to offset the effects of cancer-induced cachexia, thus reducing morbidity and mortality in individuals with cancer.

Effects of Endurance Exercise on Tumorigenesis

It has been suggested that physical inactivity is one of the leading environmental factors increasing the risk for CRC (McClellan et al., 2014). As such, endurance exercise has been used in numerous studies to assess the effects of exercise on tumorigenesis and the development of cancer-induced cachexia and it has been shown that exercise can prevent colon tumor formation and progression (Baltgalvis et al., 2007). Though tumors arise from various tissue types, all have similar characteristics in that they produce

growth factors, escape apoptosis, and have uncontrolled cell division and growth and exercise can offer protection through various mechanisms.

In $Apc^{Min/+}$ mice, several studies have demonstrated the anti-tumor effects of exercise. Baltgalvis et al. (2008) proposed that exercise may play a critical role in regulating pathways that lead to the development and growth of intestinal polyps. Nine weeks of treadmill running reduced macrophage infiltration, as identified by a decrease in F4/80-positive cells, a macrophage marker, in intestinal polyps. Using a similar experimental design, McClellan et al. (2014) reported 48% fewer larger intestinal polyps in $Apc^{Min/+}$ mice. However, no differences were detected in the number of small- or medium-sized polyps, and no difference were seen in the overall number of polyps. These results indicate that while exercise may not completely attenuate colon cancer, it may serve to reduce the number of large polyps, which could aid in improving gastrointestinal motility. Furthermore, investigators also found that F4/80 mRNA expression decreased. Researchers from this laboratory found similar results in $Apc^{Min/+}$ mice performing wheel running activity (Baltgalvis et al., 2010). Similarly, Hiroux et al. (2016) investigated the ability of exercise to counteract tumor cell growth in C26 tumor-bearing Balb/c mice. In this experimental design, C26 cells were injected into the TA of the animals. There were no differences in wheel running activity between tumor-bearing animals and controls, suggesting that tumor induction did not affect physical activity. The wheel run activity was determined to be of low-intensity, which still lead to a significant reduction of tumor growth when compared to sedentary animals.

Effects of Exercise on Cachexia

In addition to the investigations on tumorigenesis with exercise, researchers have examined the effects of cachexia with exercise. As previously discussed, mechanisms for both incidence of CRC development and skeletal muscle wasting largely occur as a result of increases in local and systemic inflammation, so it is likely that using exercise to reduce inflammation would be an effective modality for both. By examining muscle mass and skeletal muscle morphology, researchers can identify adaptations that occur with exercise. Resistance and endurance training have both been shown to be effective in attenuating the muscle wasting process that occurs with cachexia (Al-Majid & McCarthy, 2001; Coletti et al., 2016; Hardee et al., 2016; Pigna et al., 2016).

In 2013, investigators examined the effects of resistance training on C26 tumor-bearing mice (Al-Majid & McCarthy, 2001). Though morphology was not assessed in this study, researchers did report significant increases in muscle weights when comparing sedentary versus exercised, tumor-bearing animals. This suggests that cachexia was attenuated via the use of electrical stimulation to mimic resistance training. To investigate muscle morphology alterations with exercise, Hardee et al. (2016) examined CSA in the TA of tumor-bearing mice. Animals in this study were also subjected to high-frequency electrical stimulation (HFES) as a form of resistance training. Over a two-week exercise training period, $Apc^{Min/+}$ mice experienced multiple bouts of HFES that generated tetanic contractions. Myofiber CSA was significantly increased in the TA of tumor-bearing mice who underwent HFES, when compared to mice who did not receive HFES, suggesting that repeated bouts of tetanic contractions are sufficient to induce hypertrophy and reduce the severity of muscle wasting.

Endurance exercise has been shown to exhibit similar effects. In 2016, Pigna et al. examined the effects of voluntary wheel running on cancer cachexia in C26 tumor-bearing mice. Groups performed 19 days of wheel running. C26 mice in wheel running cages had significantly greater TA CSA, when compared to C26 mice housed in cages without wheels. Similar to other studies, mean body mass was also significantly greater in tumor-bearing mice when subjected to endurance exercise.

Exercise-Induced Skeletal Muscle Hypertrophy

Since skeletal muscle atrophy is a central hallmark of cachexia, it is essential that researchers investigate interventions that may help attenuate the wasting process. By mitigating muscle atrophy, individuals with cachexia have a significantly increased quality of life and reduced rate of mortality (White, Baltgalvis, et al., 2011a). By better understanding the mechanisms responsible for skeletal muscle wasting, researchers and clinicians can develop appropriate exercise training programs to induce hypertrophy. Resistance exercise is one such approach to address the increase in protein degradation and reduction in protein synthesis that accompanies cancer-induced cachexia (Al-Majid & McCarthy, 2001; Hardee et al., 2016; Khamoui, 2014; Wong & Booth, 1988). In 1988, Wong & Booth examined a weight training intervention to produce skeletal muscle hypertrophy. Electrical stimulation was used to mimic near maximal muscle contractions, without inducing fatigue, which would be similar to that seen in humans during a bout of resistance training. In this protocol, rats underwent low repetitions and high training loads in 20 second intervals, with 5-minutes rest between sets. Each training bout consisted of a progression in which load increased by 200 g per set. In resistance-trained rats, wet muscle weight increased in plantar flexors by 13-18%. The gastrocnemius of resistance

trained rats experienced significant increases in wet weight, protein content, and RNA content following 16 weeks of training (Wong & Booth, 1988).

Al-Majid & McCarthy (2001) investigated the effects of electrical stimulation, to mimic resistance exercise, as an intervention for cachexia by way of induction of skeletal muscle hypertrophy. C26 tumor-bearing mice were subject to electrical stimulation of the sciatic (motor) nerve that would induce simultaneous eccentric contraction of dorsiflexor muscles, such as the EDL and TA, and concentric contraction of plantar flexor muscles, such as the soleus and gastrocnemius. Results of this study showed significant increases in the weight of the TA and gastrocnemius. In the EDL, tumor-bearing mice displayed significantly lower muscle weights, but exercise training did not attenuate that wasting. It was suggested that this may be due to dorsiflexor muscles contracting against a higher load than plantar flexors, since the plantar flexor muscles are larger.

The use of electrical stimulation has often been used by researchers in an attempt to mimic resistance training (Al-Majid & McCarthy, 2001; Aulino et al., 2010; Khamoui, 2014; Wong & Booth, 1988). However, in 2001, Yao, Jee, Chen, Li, & Frost developed a novel method to mimic such exercise training. It was speculated that this new methodology would provide a less stressful environment for rodents, while still producing resistance training adaptations seen with more invasive and stressful methods such as electrical stimulation. This novel methodology can be considered a raised cage method, by which rats are initially housed in normal height cages and subsequently subject to progressive increases in the height of food and water by placing spacers between the standard cage and cage lid. By raising the height of food and water, rats would be forced to assume an erect bipedal stance in order to feed. Yao et al. (2001)

suggested this method would mimic the upright stance seen in humans, and thus may be translatable to resistance training methods used in human subjects. Results of this study demonstrated significant increases in both whole body and hind limb muscle weights by an average of 6% over a period of four weeks and 12% at 12-weeks.

Khamoui (2014) described the use of high tension exercise, where rats were subjected to ladder climbing. In this study, exercise training was conducted on a 100 cm long ladder with grips that were spaced 1 cm apart. Weight loading was progressively increased by attaching weights to the animal's tail. Muscle function and CSA were then measured. In non-tumor bearing animals, high tension exercise increased grip strength when compared to both control animals and animals performing low tension exercise, while tumor-bearing animals experienced significant decreases in grip strength, regardless of exercise training. Tumor bearing animals exhibited significantly lower gastrocnemius weights, when compared to controls and myofiber CSA was significantly lower in tumor-bearing mice relative to controls. Though investigators did not see any significant changes in myofiber CSA with low- or high-tension exercise, there was a trend towards increasing CSA. Collectively, results of these studies demonstrate the effectiveness in using various resistance training modalities to improve both function and structure of skeletal muscle.

Mechanisms Responsible for Exercise-Induced Cachexia Protection

As discussed, many studies have reported that exercise, both resistance and endurance, can provide protection from the skeletal muscle wasting that occurs with cancer cachexia. Many mechanisms have been suggested for this attenuation, but most

revolve around the idea of reducing chronic inflammation and activation of cellular signaling pathways that promote muscle hypertrophy, by either activating protein synthesis pathways or inactivating protein degradation pathways. Muscle contraction results in a rapid and transient, localized inflammatory response (Trenerry, Carey, Ward, Farnfield, & Cameron-Smith, 2008). During recovery, pro-inflammatory cytokines that have been released in response to tissue ischemia, are mobilized and activated. This response can trigger mechanisms that elicit a long-term anti-inflammatory response and induce myogenesis.

Signal transducer and activator of transcription, through the Janus kinase (Jak)/STAT signaling cascade, has been implicated in the mediation of cellular proliferation, differentiation, and may play a key role in the muscle regeneration process through activation of satellite cell proliferation during damaging stimuli. IL-6 levels play a significant role in the progression of cachexia and muscle-derived IL-6 causes a transient increase in circulating IL-6 that has been linked to metabolic signaling with exercise (Puppa et al., 2012). This differs from chronically elevated IL-6, which has been associated with inflammation. Using an IL-6 overexpression mouse model, Puppa et al. (2012) evaluated the effects of regular treadmill exercise on cachexia. In prior studies, it has been shown that IL-6 overexpression can accelerate muscle wasting in $Apc^{Min/+}$ mice, but does not induce cachexia in wild-type mice (Baltgalvis et al., 2007). Here, it was reported that IL-6 overexpression significantly reduced the body mass in $Apc^{Min/+}$ mice and that exercise was able to effectively prevent this loss in body mass. AMP-activated protein kinase (AMPK) has been shown to exhibit catabolic effects as it can inhibit protein synthesis. Puppa et al. (2012) found that AMPK activation was also increased

threefold in IL-6 overexpressed $Apc^{Min/+}$ mice, while exercise was able to mitigate this increase.

Insulin-like growth factor-1 (IGF-1) has also been suggested as a mechanism responsible for exercise-induced skeletal muscle growth in cachexia. When IGF-1 binds to its receptor, it induces a signaling cascade involving the activation of protein kinase B and mammalian target of rapamycin. In addition, IGF-1 stimulates cellular proliferation and differentiation of satellite cells and can act to inhibit the ubiquitin-proteasome pathway that is responsible for protein degradation (Costelli et al., 2006). In cancer cachexia, IGF-1 is shown to be downregulated. The gastrocnemius of male Wistar rats inoculated with Yoshida AH-130 ascites hepatoma cells displayed significant loss of body mass and muscle mass of the gastrocnemius, EDL, TA, and soleus. In addition, IGF-1 and IGF1-R mRNA expression exhibits a marked decrease of 80% in the gastrocnemius of cachectic rats, when compared to controls (Costelli et al., 2006). In a study conducted by Kido et al. (2016), resistance training was shown to increase IGF-1 expression in skeletal muscle by 63%. Interestingly, IGF-1 expression was shown to be decreased with treadmill running. Conversely, Khamoui (2014) reported increases in IGF-1 expression in the gastrocnemius and plantaris in C26 cachectic mice. Though these results differ, it demonstrates the ability of physical activity, resistance and endurance, to increase IGF-1, thus inducing myofiber growth.

Human Models of Cancer Cachexia

In addition to rodent models, researchers have also examined cachexia in humans (Blauwhoff-Buskermolen, Langius, Becker, Verheul, & de van der Schueren, 2017). Researchers often induce cachexia in rodents, via tumor inoculation, as it provides a

quick and efficient way to study the disease. However, translation of this model into humans is the ultimate goal, as cachexia leads to a reduced quality of life and reduced survival (Blauwhoff-Buskermolen et al., 2017). In addition, it is often difficult to assess and identify cachexia in humans. Measurement of muscle mass in humans can be both expensive and invasive. A framework of measurement options has been provided by Fearon et al. (2011), including dual energy X-ray imaging, computed tomography, bioelectrical impedance analysis, and mid-upper arm muscle area. Muscle biopsies can also be performed in an effort to quantify CSA, but this methodology is highly invasive.

The rodent model displays mechanisms of cancer cachexia and adaptations to exercise that mimic those seen in humans, such as alterations in IL-6 and STAT3. Trenergy, Carey, Ward, & Cameron-Smith (2007) investigated the impact of resistance training on protein levels of Jak/STAT3 and IL-6. Thirteen active, but untrained, males participated in an acute bout of resistance training, followed by a 12-week resistance training program. The acute bout assessed maximal voluntary contraction and peak torque. Investigators collected muscle biopsies from participants at rest and again three hours following completion of the exercise test. The 12-week training program consisted of progressive resistance training on 3 days each week, with a minimum of a 48-hour rest period in between sessions. Participants started with a relatively light load, at 50%, and then progressively increased to training at 80% of their 1-repetition maximum (1-RM). All participants showed significant increases in their 1-RM for leg press, bench press, seated row, and leg extension at the end of the program. IL-6 protein expression significantly increased following the acute bout of exercise performed on an isokinetic dynamometer. STAT3 phosphorylation was examined in the vastus lateralis and

significant increases were reported in both trained and untrained muscle biopsy samples. Trenerry, Della Gatta, Larsen, Garnham, & Cameron-Smith (2011) also investigated STAT3 responsive genes to assess the potential for resistance training to affect downstream targets. C-FOS, c-MYC, and SOCS3 were all significantly increased 3 hours following an acute bout of resistance exercise. Similarly, in 2008, Trenerry et al. found that exercise-induced STAT3 signaling increases with age. In this study, healthy young men (age 20.4 +/- 0.8) and healthy older men (age 67.4 +/- 1.3) performed an acute bout of resistance exercise using an isokinetic dynamometer. Muscle biopsies were performed on each participant at rest and two hours following the bout of exercise. STAT3 phosphorylation was significantly increased in both younger and older males. However, older males had significantly greater increases in JUNB (16-fold increase) and c-MYC (44-fold increase), which are downstream targets of STAT3. The Jak/STAT3 signaling pathway has been identified as a mediator of skeletal muscle adaptation and these data support that idea. These data also support the application of exercise training of offset muscle wasting conditions, such as cancer cachexia.

Summary

CRC is one of the most common cancers and it is responsible for the third most cancer-related deaths among men and women in the U.S. Environmental factors, such as lack of physical activity and diet, are highly associated with an increased risk of developing CRC. Unfortunately, CRC patients are at a high risk for developing cachexia, a metabolic disease linked to increased morbidity and mortality. Cachexia contributes to skeletal muscle and adipose tissue wasting and is associated with chronic inflammation. The unintentional loss in body mass that occurs with cachexia increases overall fatigue

and weakness. Fortunately, exercise training can lead to activation of cellular signaling pathways that ultimately induce an anti-inflammatory response. In tumor-bearing rodent models, chronic exercise training is shown to decrease systemic inflammation and decrease tumor growth. Additionally, both resistance and endurance exercise have been shown to increase protein synthesis and decrease protein degradation via mechanisms that inhibit the ubiquitin-proteasome pathway. In cachectic patients, physical activity may increase skeletal muscle CSA, thus increasing function and overall quality of life.

CHAPTER III
MATERIALS AND METHODS
Experimental Design

The primary purpose of this study was to examine both endurance and resistance training modalities to identify the effects of exercise on varying skeletal muscle morphological properties in mice with cancer-induced cachexia. A secondary purpose of this study was to determine the relationship between inflammation and the severity of cachexia. These exercise effects were examined using an 8-week exercise training protocol that consisted of endurance training, resistance training, or a combination of both. At six weeks of age, Balb/c mice were randomly selected to sedentary or exercise training groups. At 11 weeks of age, mice selected to tumor bearing groups (n = 48) were inoculated with 1×10^6 C26 tumor cells. Control animals (n = 12) remained sedentary, with no tumor cell inoculation. Upon sacrifice of control and experimental animals, the gastrocnemius was harvested, weighed, and prepared for histology, while the spleen was harvested and weighed to determine splenomegaly. Blood was collected via left ventricular puncture, following sacrifice, and centrifuged for plasma collection. Table 2 outlines the experimental design of this study.

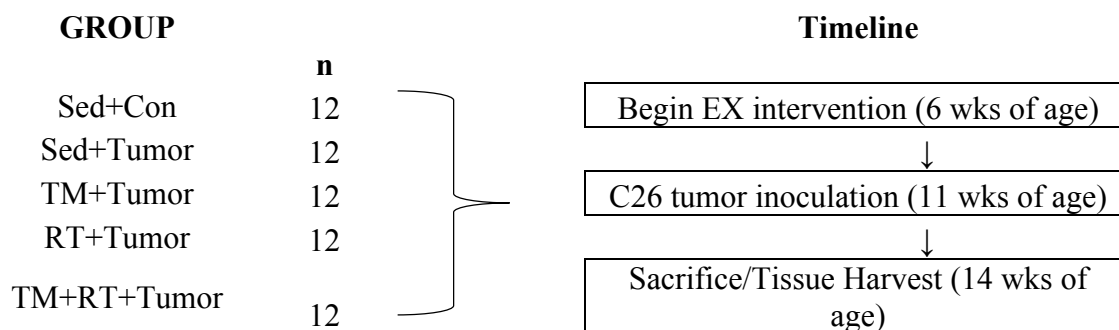


Figure 1. Experimental Design. Note. Sed, sedentary; TM, treadmill; RT, resistance trained

Animals and Animal Care

Male Balb/c mice (The Jackson Laboratory; Bar Harbor, ME; stock #000651; n = 60) were housed one per cage in a temperature-controlled facility with a 12:12-hour light-dark cycle at the University of Northern Colorado Animal Research Facility. Mice were provided standard chow and water ad libitum. All procedures for the study were approved by the University of Northern Colorado Institutional Animal Care and Use Committee (IACUC) and were in compliance with the Animal Welfare Act guidelines.

Exercise Training Protocol

Resistance Training

Animals randomly selected to the RT group were initially housed in standard, 12.5 cm, cages. Cage height was progressively increased, forcing mice to raise up onto their hind limbs to reach food and water. On day one of the exercise protocol, mice were moved to cages with a height of 15.5 cm, where they remained for one week. Following the first week of the protocol, the cage height was increased by 2.5 cm, resulting in a total cage height of 18 cm. This final cage height was maintained for the remainder of the eight-week intervention. During the first three weeks of the exercise protocol, food and

body mass were measured and water monitored daily to ensure animals were able reach both food and water. Thereafter, food and water were monitored three days per week and body mass measured weekly.

Endurance Training

Animals randomly selected to the TM group were housed in cages that were of the standard 12.5 cm height for the entirety of the eight-week intervention. All TM animals were acclimated to the treadmill for 3 days prior to the beginning of the intervention. Acclimatization consisted of running on the treadmill at gradually increased speeds (10-15 m/min) for 20 minutes. The running protocol for the intervention consisted of a brief 5-minute warm-up at a speed of 10 m/min and 5% grade, followed by 55-minutes of running at 15 m/min and 5% grade. Mice ran during their dark cycle, under red lights, 5 days/wk, for a total of 8 weeks.

Combination Training

Animals randomly selected to the combination group (TM+RT) followed both the RT and TM protocols described above. Mice in the combination group were continuously housed in cages with the progressive height increase, to mimic resistance training, and removed for treadmill running 5 days/wk for 8 weeks.

Colon-26 Cell Culture and Inoculation

Colon-26 cells (DCTD Tumor Repository, National Cancer Institute; Frederick, MD) were suspended in Roswell Park Memorial Institute (RPMI) 1640 complete growth medium containing 10% FB Essence, 1% L-glutamine, and 1% streptomycin/penicillin and grown in an incubator at 37°C in a humidified atmosphere of 5% CO₂ in air. Cells were counted using a commercially available cell counter (Invitrogen; Carlsbad, CA).

On the day of implantation, adherent cells were dissociated with a trypsin-EDTA solution and cell concentration in cells/ m^2 were determined. 1×10^6 cells were resuspended in sterile PBS for tumor cell inoculation. Prior to injection, mice were anesthetized using isoflurane by inhalation, and maintained under anesthesia during the time necessary to perform the tumor inoculation. Cells were injected subcutaneously, between the scapulae, using a sterile 1-mL insulin syringe with a 25-gauge needle.

Following implantation of the C26 cells, until time of sacrifice, animals and tumors were monitored daily for end point criteria, including relative tumor size, significant loss in body mass, body score condition (Figure 2), and tumor ulceration. Body weight measurements were taken daily. Once palpable, tumors were also measured daily. At the time of sacrifice, tumors were removed and weighed.

**BC 1**

Mouse is emaciated.

- *Skeletal structure extremely prominent; little or no flesh cover.*
- *Vertebrae distinctly segmented.*

**BC 2**

Mouse is underconditioned.

- *Segmentation of vertebral column evident.*
- *Dorsal pelvic bones are readily palpable.*

**BC 3**

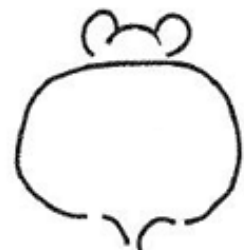
Mouse is well-conditioned.

- *Vertebrae and dorsal pelvis not prominent; palpable with slight pressure.*

**BC 4**

Mouse is overconditioned.

- *Spine is a continuous column.*
- *Vertebrae palpable only with firm pressure.*

**BC 5**

Mouse is obese.

- *Mouse is smooth and bulky.*
- *Bone structure disappears under flesh and subcutaneous fat.*

A "+" or a "-" can be added to the body condition score if additional increments are necessary (i.e. ...2+, 2, 2-...)

Figure 2. Body condition and assessment score (Ullman-Cullere & Foltz, 1999)

***In-Vivo* Skeletal Muscle Function**

As weakness is one common hallmark of cachexia, *in-vivo* skeletal muscle function was measured via force production prior to sacrifice. Force production was measured using a commercially available grip strength meter designed for rodents (Columbus Instruments, Columbus, OH). Forelimb grip strength was measured as mice grasped on to a metal grid attached to a force transducer with both front paws. Mice were placed onto the metal grid and once the mouse had a firm, and secure, grasp on the grid, the mouse was gently pulled away, by the distal end of the tail, in a slow and steady manner until the mouse released its grip on the metal grid (Tan et al., 2013; Wang, Sorenson, Spinner, & Windebank, 2008). The force produced at the point of release was recorded as a single trial. After each trial, mice were returned to their cage for a 5-minute recovery period. Each mouse performed this procedure the exact same way for a total of five trials. Grip strength for each animal was calculated as the mean score from the five trials. The mean value for each animal was then converted to a relative strength value, taking into account body mass.

Sacrifice and Tissue Collection

Upon completion of the exercise intervention, mice were weighed to obtain final body mass, sedated using isoflurane, and euthanized by cervical crush. The gastrocnemius, spleen, and tumor were excised and blood collected via left ventricular puncture. All excised tissues were weighed prior to preparation and storage. The gastrocnemius was placed in optimal cutting temperature (O.C.T) and frozen in isopentane cooled in liquid nitrogen. Blood was centrifuged to collect plasma and then flash frozen in liquid nitrogen and stored at -80°C until further biochemical analysis.

Histochemical Analyses

Transverse sections from the midbelly region of the gastrocnemius were sectioned at 10 μm on a cryostat (Leica; CM1950) at -20°C . Hematoxylin & Eosin (H&E) staining was done on muscle sections to measure cross-sectional area (CSA). Digital images were taken (Olympus DP21) and imaging software (ImageJ, NIH; Bethesda, MD) was used to trace fibers using a handheld computer mouse. The number of pixels traced was calibrated to a defined area in square micrometers. Immunofluorescence was used to determine skeletal muscle fiber type via identification of myosin heavy chain IIA (SC-71) and IIB (BF-F3) expression. Myosin heavy chain (MHC) primary antibodies were purchased from the Developmental Studies Hybridoma Bank (University of Iowa; Ames, IA). Muscle sections were air dried and placed in a blocking solution containing 1% bovine serum albumin (BSA), 1% dry milk, 1% Triton-x, and 10% natural goat serum for one hour at room temperature and then incubated in MHC primary antibodies (IIa, 1:600; IIB, 1:100) for two hours at room temperature. Muscle sections were rinsed in phosphate buffered solution (PBS) 3 x 5 minutes and then incubated in a secondary antibody containing AlexaFluor 488 (ThermoFisher Scientific; Waltham, MA; 1:500) for one hour at room temperature. Digital images (Leica Microsystems DM1RB) at 40X magnification were taken and MHC types IIA and IIB were visualized and measured using imaging software (Image J; NIH; Bethesda, MD).

Biochemical Analysis

Upon sacrifice, blood samples were collected from all subjects via left ventricular puncture. Samples were kept on ice and centrifuged at 1,000 g for 10 minutes and 4°C . Plasma was aliquotted and stored at -80°C until further analysis. Plasma IL-6 was

analyzed via enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions (Abcam, ab100712; Cambridge, MA).

Statistical Analyses

Statistical analyses were completed using GraphPad Prism statistical software. All data are presented as means \pm standard deviation ($M \pm SD$). For body mass and gastrocnemius wet mass, a one-way analysis of variation (ANOVA) was performed to identify differences between SED, SED+Tumor, TM+Tumor, RT+Tumor, and TM+RT+Tumor groups. For CSA, a one-way ANOVA was performed to identify differences in gastrocnemius myofiber size between groups. Additionally, a one-way ANOVA was performed to identify any differences in IL-6 concentrations between SED and EX groups. For ANOVA analyses detecting significant differences ($P < 0.05$), a Tukey's *post hoc* test was performed to identify where significant differences existed. The alpha level was set at 0.05.

CHAPTER IV

RESULTS

The purpose of this study was to examine the effects of endurance and resistance training on skeletal muscle wasting in C26 tumor-bearing mice. A secondary purpose was to examine what, role, if any, IL-6 has on the severity of skeletal muscle wasting in tumor-bearing mice.

General Observations

All animals were acclimated to treadmill exercise prior to onset of the endurance training protocol and no animals were removed from the study for non-compliance. Chow-consumption was measured for resistance-trained animals to ensure their ability to reach the raised food and water and to ensure that body or muscle wasting did not occur due to lack of food intake. All RT animals were able to reach food and water throughout the study, and intake did not decline with tumor condition (Table 2). Furthermore, all animals inoculated with the C26 carcinoma cells developed measureable tumors, though relatively small and TM running did not decline with tumor growth. No significant differences were observed in tumor mass between any groups ($P>0.05$) and exercise did not significantly reduce tumor mass when compared to SED+Tumor (Figure 3). However, TM+Tumor had a -21% difference in tumor mass when compared to SED+Tumor. No animals were euthanized according to tumor end-point criteria or body condition score as outlined in the Methods section. There were no significant differences in animal body mass at the time of tumor inoculation. However, at the time of sacrifice,

SED+Tumor body mass was significantly lower than SED+Con body mass ($P<0.05$).

General body mass observations are presented in Table 3. SED+Con relative gastrocnemius wet mass was significantly lower than SED+Tumor ($P<0.05$), while no other significant differences between groups were observed in relative gastrocnemius wet mass.

Table 2

Food Consumption During a 21-day Period After C26 Inoculation

SED+ Con (g)	SED+ Tumor (g)	TM+ Tumor (g)	RT+ Tumor (g)	TM+RT+ Tumor (g)
81.3 ± 4.7	81 ± 2.9	84.3 ± 5.5	85.9 ± 4.5	83.3 ± 9.2

Note: Data are represented as $M \pm SD$. Con = control; TM = treadmill trained; RT = resistance trained

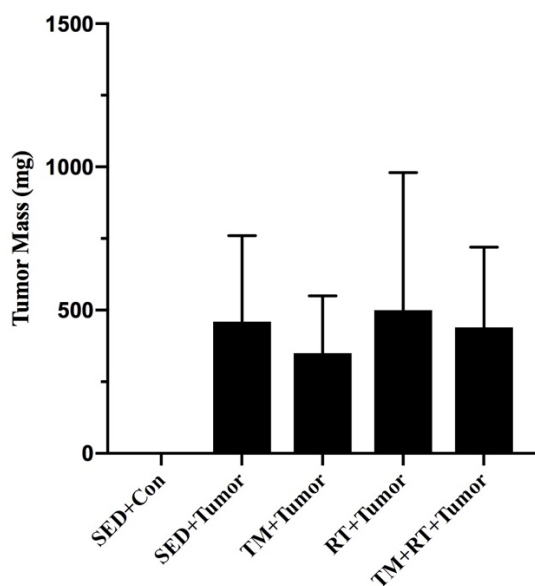


Figure 3. Tumor Mass. $p > 0.05$.

Table 3

Animal Characteristics

	<i>N</i>	Initial body mass (g) <i>M ± SD</i>	Injection day body mass (g) <i>M ± SD</i>	Final body mass with tumor (g) <i>M ± SD</i>	Final body mass (g) <i>M ± SD</i>
Sed+ Con	12	20.5 ± 1.7	26.5 ± 3.2	-	29.75 ± 3.6
Sed+ Tumor	12	20.3 ± 1.4	27.2 ± 2.4	26.2 ± 2.4	25.8 ± 2.5*
TM+ Tumor	12	21.3 ± 1.4	25.8 ± 2.3	29.0 ± 2.3	28.6 ± 2.4
RT+ Tumor	12	21.6 ± 1.9	25.4 ± 2.0	29.2 ± 1.8	28.7 ± 2.0†
TM+RT+ Tumor	12	22.3 ± 1.3	25.7 ± 0.8	28.9 ± 0.7	28.3 ± 1.1

Note. All EX groups are tumor-bearing. Final body mass obtained after subtracting tumor mass. Data are represented as *M ± SD*. SED = sedentary; Con = control; TM = treadmill trained; RT = resistance trained. $p < 0.05$.

* $p < 0.05$ versus SED+Con.

† $p < 0.05$ versus SED+Tumor.

Skeletal Muscle Function

Forelimb muscular strength was measured via a grip strength meter (Columbus Instruments; Columbus, OH). Grip strength was measured in all animals prior to sacrifice in order to assess any loss in muscle function that may be attributable to cachexia. At the time of sacrifice, SED+Tumor grip strength was significantly lower ($P < 0.05$) when compared to SED+Con. Additionally, handgrip for TM+Tumor and TM+RT+Tumor were also significantly lower ($P < 0.05$) when compared to SED+Con. Furthermore,

RT+Tumor had a significantly greater grip strength ($P < 0.05$) when compared to SED+Tumor. Additionally, RT+Tumor was not significantly different ($P > 0.05$) when compared to SED+Con. Absolute and relative forelimb grip strength data are presented in Figures 4 and 5, respectively.

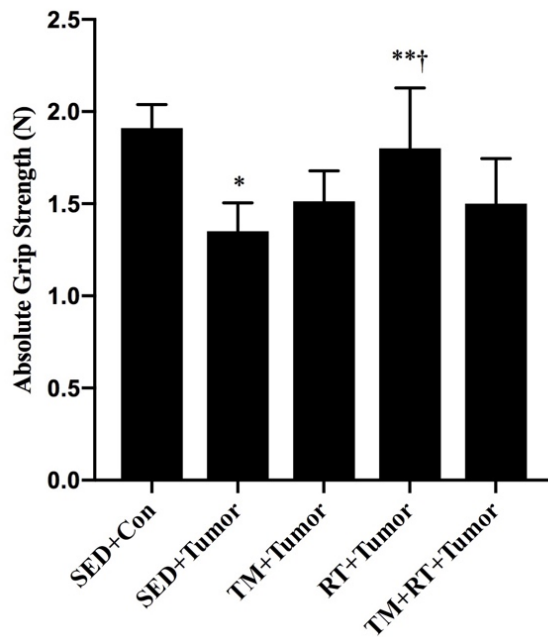


Figure 4. Absolute Grip Strength. Con = control; TM = treadmill trained; RT = resistance trained. $p < 0.05$.

* $p < 0.01$ versus SED+Con; ** $p < 0.01$ versus SED+Tumor; † $p < 0.05$ versus TM+Tumor, TM+RT+Tumor

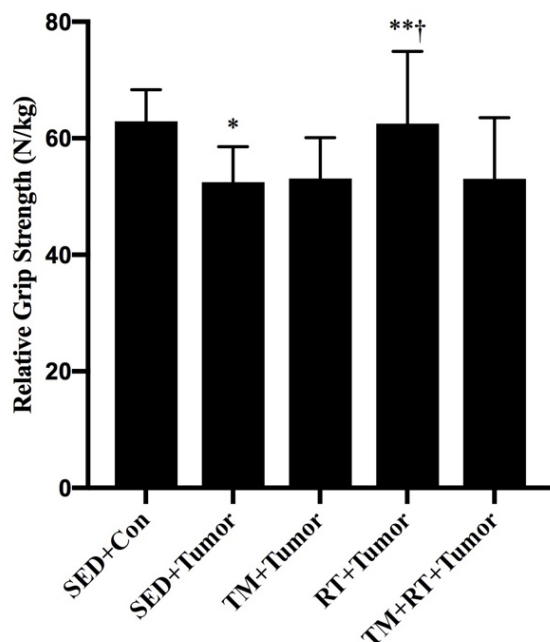


Figure 5. Relative Grip Strength. Con = control; TM = treadmill trained; RT = resistance trained. $p < 0.05$.

* $p < 0.05$ versus SED+Con; ** $p < 0.05$ versus SED+Tumor; † $p < 0.05$ versus TM+Tumor, TM+RT+Tumor

Gastrocnemius Mass and Cross-Sectional Area

Relative gastrocnemius mass and CSA data are summarized in Figure 6. Relative gastrocnemius mass was significantly lower ($P < 0.05$) in SED+Tumor when compared to SED+Con. No other groups displayed significant differences in wet mass of the gastrocnemius. Though relative gastrocnemius mass was not significantly different between SED and EX tumor groups, increases were seen in all EX groups, when compared to SED+Tumor. A 4% increase in gastrocnemius mass was observed in TM+Tumor, while there were 5.6% and 15% increases in gastrocnemius mass for RT+Tumor and TM+RT+Tumor, respectively. Histological analysis revealed that gastrocnemius CSA was also significantly lower ($P < 0.01$) in SED+Tumor when compared to SED+Con. All EX groups (TM+Tumor, RT+Tumor, and TM+RT+Tumor)

had significantly greater CSA ($P < 0.01$) than SED+Tumor and none of the EX groups (TM+Tumor, RT+Tumor, and TM+RT+Tumor) were significantly different ($P > 0.05$) than SED+Con. Additionally, immunofluorescence revealed no significant differences between MHCIIa and MHCIIb fiber CSA, within each group. Myosin heavy chain fiber type CSA data are presented in Table 4.

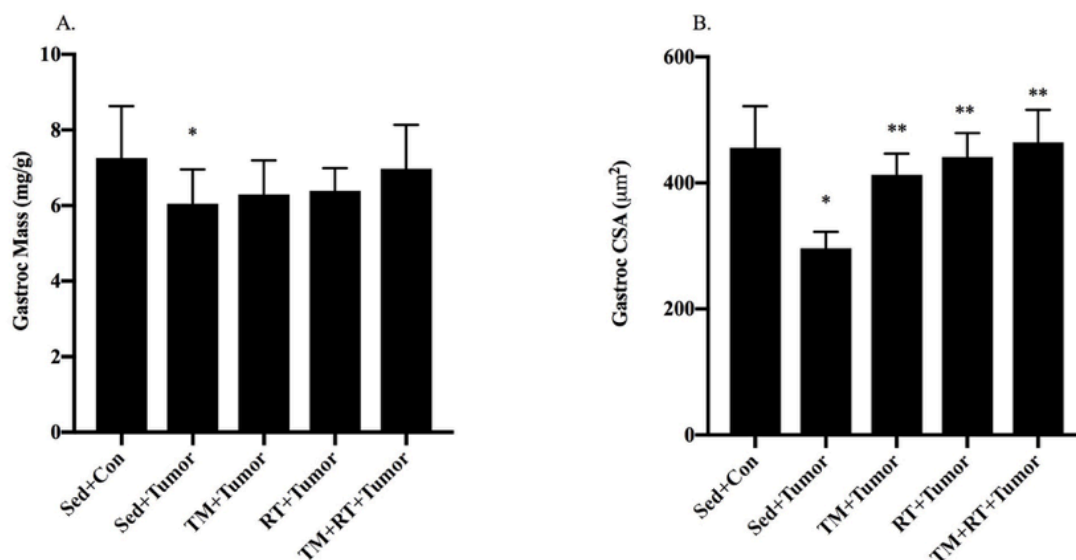


Figure 6. Gastrocnemius Mass and CSA. (A) Relative gastrocnemius mass; * $p < 0.05$ versus SED/Con, (B) Gastrocnemius cross-sectional area; * $p < 0.01$ versus SED+Con, ** $p < 0.01$ versus SED+Tumor. Con = control; TM = treadmill trained; RT = resistance trained. Values are $M \pm SD$. $p < 0.05$.

Table 4

CSA of Myosin Heavy Chain IIa and IIb Fibers

	SED+Con (μm^2)	SED+Tumor (μm^2)	TM+Tumor (μm^2)	RT+Tumor (μm^2)	TM+RT+Tumor (μm^2)
Fiber Type	$M \pm SD$	$M \pm SD$	$M \pm SD$	$M \pm SD$	$M \pm SD$
MHCIIa	435 \pm 41	307 \pm 48*	420 \pm 31†	437 \pm 38†	470 \pm 44†
MHCIIb	428 \pm 51	290 \pm 27*	410 \pm 31†	455 \pm 38†	463 \pm 50†

Note. Data are represented as $M \pm SD$. SED = sedentary; TM = treadmill trained; RT = resistance trained

* $p < 0.05$ vs SED+Con

† $p < 0.05$ vs SED+Tumor

Systemic Inflammation

During infection and other inflammatory challenges, the spleen initiates an immune response directed towards the tissue in need of repair (Mehl, Davis, Clements, et al., 2005a). The spleen is known to increase in size during this response. Splenomegaly is a marker of increased systemic inflammation and was thus measured in all groups. A significant increase in spleen weight was observed in SED+Tumor ($P < 0.01$) when compared to SED+Con. Exercise (TM+Tumor, RT+Tumor, and TM+RT+Tumor) was able to significantly decrease splenomegaly ($P < 0.01$) in tumor-bearing mice when compared to their sedentary counterparts. Spleen mass data are summarized in Figure 7.

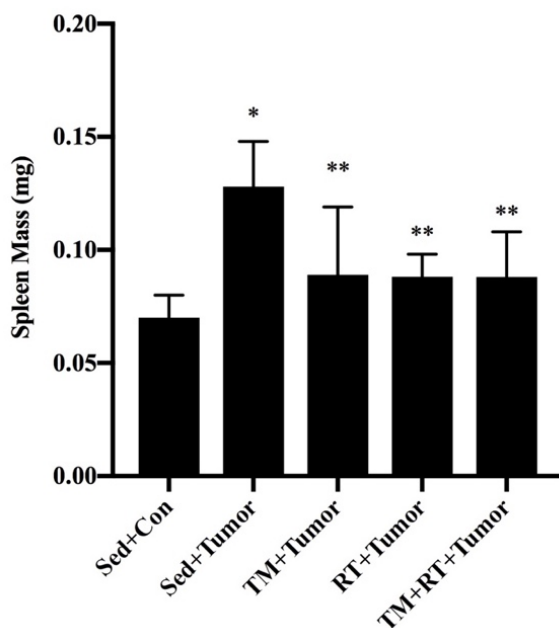


Figure 7. Splenomegaly. Con = control; TM = treadmill trained; RT = resistance trained. $p < 0.05$.

* $p < 0.01$ versus SED+Con

** $p < 0.01$ versus SED+Tumor

Systemic inflammation is also marked by increases in plasma IL-6. There was a significant increase in circulating IL-6 in SED+Tumor ($P < 0.01$) when compared to SED+Con. As shown in Figure 8, exercise was able to attenuate this increase in plasma IL-6. TM+Tumor, RT+Tumor, and TM+RT+Tumor had significantly lower plasma IL-6 levels ($P < 0.01$) than SED+Tumor and the plasma IL-6 levels in the exercised groups were not significantly different than SED+Con

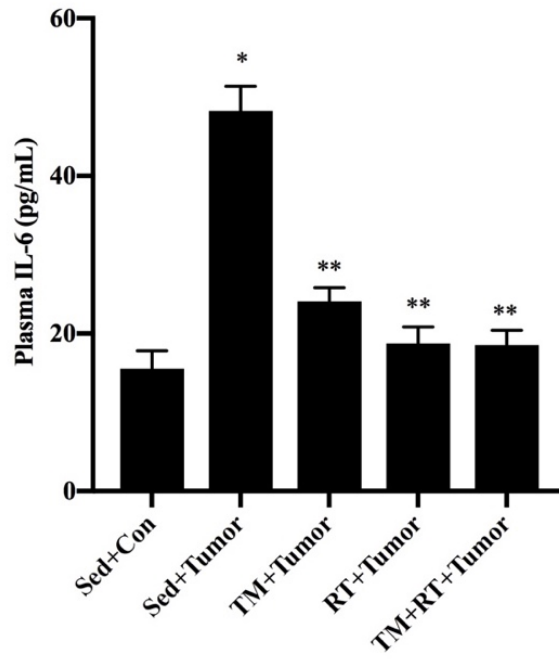


Figure 8. Plasma IL-6 Concentration; * $p < 0.01$ versus SED+Con, ** $p < 0.01$ versus SED+Tumor. Values are $M \pm SD$. $p < 0.05$. Note. $n=8$.

CHAPTER V

DISCUSSION

Colorectal cancer is the third most commonly diagnosed cancer and the second leading cause of cancer death in the U.S. for both men and women. Furthermore, CRC is largely preventable as the increase in risk can be attributed to lifestyle factors, including physical inactivity. Additionally, individuals with gastrointestinal cancers are more susceptible to developing cachexia, leading to significant loss in body weight, reducing overall quality of life, and increasing morbidity (Hardee et al., 2014; Puppa et al., 2012). It is imperative that all healthcare professionals promote strategies that prevent such disease. Numerous animal studies have examined not only the effects of exercise on cancer itself, but also its effects on muscle characteristics associated with cachexia (Demarzo et al., 2008; Puppa et al., 2012). In fact, research has demonstrated that both endurance and resistance training can attenuate skeletal muscle wasting, citing that both modes of exercise are effective in reducing cachectic symptoms (Al-Majid & McCarthy, 2001; Hardee et al., 2016; Ranjbar, Ballarò, Bover, & Pin, 2019). Though considerable research has been conducted in this area, to date, there has been negligible exploration on the use of a combined exercise training protocol, in which rodents in a single group perform both endurance and resistance training. Therefore, the primary purpose of the present study was to examine the effects of two modes of exercise training, each independently and a combination of the two, on skeletal muscle wasting in cachectic mice.

Colon-26 carcinoma is a well-characterized and extensively cited model for cancer cachexia in mice (Bonetto et al., 2016). In the present study, inoculation of mice with the C26 cells did in fact induce cachexia in the sedentary tumor-bearing group (SED+Tumor), with a nearly 14% difference in body mass between SED+Con and Sed+Tumor. This loss in body mass was not due to decreases in food consumption during the tumor-bearing period (Table 2). Previous works have defined cachexia as a loss of lean tissue mass that is associated with a decrease in body mass greater than 5% in the presence of chronic illness (Diffie et al., 2002; Martin et al., 2013; Mehl, Davis, Berger, et al., 2005b). In the present study, SED+Tumor mice lost 5.2% of their injection-day body mass. Though the loss in body mass in the present study is consistent with cachexia classifications, the degree of loss is considerably more mild than previous findings. Diffie et al. (2002) observed a 30% decrease in non-tumor body mass in C26 tumor-bearing mice when compared to controls. Similarly, Al-Majid & McCarthy (2001) observed a 20% decline in carcass weight of tumor-bearing mice when compared to non tumor-bearing controls. Conversely, Khamoui (2014) discovered a mere (though still cachectic) 6% decline in body mass, using a similar model. These findings are consistent with the present study. Skeletal muscle mass is another key hallmark in identifying cachexia. Previous research has shown significant differences in muscle mass, with muscle loss ranging from to 20-40%, when comparing tumor-bearing mice to control mice. In the present study, relative gastrocnemius mass was more than 16% less in SED+Tumor, when compared to SED+Con. So, while the C26 tumor did produce significant wasting of both body mass and gastrocnemius mass, the degree of wasting was less than in previous studies. Together, these findings suggest that the degree of loss

is highly variable and likely multifactorial. In the above mentioned studies, though similar models and methodologies were used, all had slight variations in the animal strain, preparation of the C26 cells and number of cells injected. Additionally, each study used cells obtained from different laboratories, which can lead to considerable variability in the degree of cachexia. Murphy, Chee, Trieu, Naim, & Lynch (2012), reported mild to severe forms of cachexia when using different C26 adenocarcinoma cells from various laboratories, indicating that the cachectic phenotype can vary significantly even in the same cell line. Cachexia, while characterized as a weight loss >5%, may also appear in patients exhibiting a small tumor burden that is less than 0.01% of their total body mass (Yasumoto et al., 1995). In the present study, tumor burden was 0.02% of total body weight in the SED+Tumor group. Nevertheless, it is clear in the present study that mice inoculated with C26 cells exhibited overt signs of cachexia.

Changes in skeletal muscle structure have also been noted in cachectic models. As has been observed in several cachexia studies, mice in the present study underwent significant atrophy of the gastrocnemius muscle, as evidenced by decreases in overall myofiber CSA (-35%). The mouse gastrocnemius muscle has a mixed phenotype, with nearly 80% being classified as type IIb (MHCIIb), exhibiting overt glycolytic properties. Additionally, while shifts in MHC expression (increases in type IIb and decreases in type I) have been noted in muscles known to be rich in slow-twitch fibers, such as the soleus, no shifts in fiber type have been noted in muscles rich in fast-twitch fibers, such as the gastrocnemius, tibialis anterior, and plantaris. However, in another mouse model of cachexia, the *Apc*^{min/+} C57BLJ6 mouse, Baltgalvis et al. (2010) observed that only mice who were classified as severely cachectic had reductions in both MHCIIa and MHCIIb

myofiber CSA, while those classified as moderately cachectic only had decreases in MHCIIb in the gastrocnemius. These data suggest that preferential fiber type wasting, or shifting, may be based on the severity of the disease. The current study demonstrated that no significant differences in MHCIIa and MHCIIb within each group existed, and the CSA of both fiber types were similar to total fiber CSA, suggesting that there was no preferential wasting of fiber type, as each atrophied similarly. These findings are in agreement with other studies (Diffie et al., 2002; Hardee et al., 2016). Mechanisms responsible for the muscle wasting associated with cancer cachexia are highly complex and multifactorial. Induction and severity of cachexia are also dependent on tumor phenotype and host genotype. In addition, apoptotic pathways are activated in cachectic conditions and proapoptotic B cell leukemia/lymphoma 2 (BCL2)-associated X protein (BAX) protein expression may be a key regulator in cachexia. Baltgalvis et al., (2010) examined BAX as a molecular marker of muscle wasting and demonstrated that gene and protein expression increased with increasing severity of cachexia. Additionally, it was demonstrated in the same study that BCL2, which inhibits BAX, was either weakly expressed, or not expressed at all, thus allowing the BAX pathway to induce apoptosis. It was concluded that apoptosis occurs regardless of the muscle's oxidative phenotype, as BAX was increased in both type I and type II fibers of the gastrocnemius. In addition, it was demonstrated that, while mean MHCIIa fiber CSA did not change, there was a shift in MHCIIa distribution, with an increase in small type IIa fibers and a decrease in large type IIa fibers (Baltgalvis et al., 2010). These data suggest that cachexia may preferentially target highly glycolytic fibers for wasting, but cause fiber type shifting in fibers with greater oxidative properties. Myosin heavy chain IIx fibers were analyzed, but

not observed through immunofluorescence, thus, it is possible that, in the present study, fiber type shifting may have occurred (MHCIIx \rightarrow MHCIIb). Additionally, though not analyzed in the present study, it is possible that MHCI, a highly oxidative fiber type, may have demonstrated shifting towards a MHCIIa fiber.

Cachexia also produces significant declines in muscle function, which can ultimately lead to a substantial reduction in overall quality of life. Handgrip strength has been shown to be reduced by up to 25% in cancer patients with cachexia (Guo, Zhang, Ma, Zhang, & Huang, 1996). Severe reductions in handgrip strength can significantly alter a patient's ability to perform seemingly simple tasks. In the current study, forelimb grip strength was measured to assess muscle function. SED+Tumor demonstrated a -18% difference in strength, when compared to SED+Con. Other studies have reported similar declines in grip strength performance in cachectic mice (Khamoui, 2014; Murphy et al., 2012; Ranjbar, et al., 2019). A number of different factors have been shown to contribute to this muscle dysfunction. Ubiquitin-proteasome pathway constituents, for example, have been implicated in both structural and functional declines, as this pathway plays a predominant role in protein degradation. Though not evaluated in the present study, previous works have shown significant declines in total protein concentration (Al-Majid & McCarthy, 2001) and significant increases in MuRF1 and atrogen-1 (Baltgalvis et al., 2010; Khamoui, 2014; Mota et al., 2017; Tatebayashi et al., 2018). Additionally, Aulino et al., (2010) concluded that the observed up-regulation of atrogen-1 in tumor-bearing mice was accompanied by protein ubiquitination and ultimately resulted in muscle function impairments. Forkhead box proteins (FoxO) are additional ubiquitin-proteasome constituents that have been identified as key regulators of gene expression in cancer

cachexia, affecting both structural and functional integrity of the extracellular matrix and muscle sarcomere and regulating genes involved in atrophy-related transcriptional pathways, including the IL-6 pathway (Judge et al., 2014). FoxO-1 and -3 appear to have the most involvement of the FoxO family in cachexia, as they are activated in multiple types of skeletal muscle atrophy, including cancer cachexia (Bouchè, Lozanoska-Ochser, Proietti, & Madaro, 2018; Judge et al., 2014). To further demonstrate the involvement of the ubiquitin-proteasome pathway in muscle atrophy, researchers have used models which showed that blocking FoxO is able to prevent C26-induced muscle fiber atrophy (Judge et al., 2014; Milan et al., 2015).

Aerobic and resistance training have been demonstrated to be effective in combatting the muscle wasting and declines in muscle function observed in cachectic mice. In the present study, exercise training was able to improve detriments in skeletal muscle mass, muscle function, and gastrocnemius CSA. Specifically, resistance training provided the most benefit to the improvement of forelimb handgrip strength, and, though not significant, improved relative gastrocnemius mass by greater than 15% when compared to SED+Tumor. Additionally, gastrocnemius CSA was significantly greater in all exercised tumor-bearing groups (TM+Tumor, RT+Tumor, TM+RT+Tumor) when compared to SED+Tumor, suggesting that both aerobic and resistance exercise were able to significantly attenuate declines in myofiber size. Interestingly, the significant improvements in gastrocnemius CSA were not accompanied by significant increases in strength (+2%) or relative gastrocnemius mass (+5%) in TM+Tumor or RT+TM+Tumor. These findings are not in agreement with other literature, in which aerobic exercise was able to attenuate these symptoms of cachexia (Hardee et al., 2016; Khamoui, 2014).

While other researchers have used HFES or fatigue protocols, the current study used treadmill running as the modality for aerobic exercise. Though HFES and treadmill running both likely target hindlimb muscles, muscle function was assessed via forelimb grip strength. Furthermore, the current study used a novel, and modified, version of resistance training for rodents as described by Yao et al., (2001) where food and water were progressively raised and mice were required to raise up onto hind limbs to access food. In this model, functional improvement in hind limb muscular strength may be expected. However, it was observed that mice in the RT groups (RT+Tumor and TM+RT+Tumor) also spent much time hanging onto the wire cage tops with forelimbs, thus potentially producing strength improvements. This observation suggests that, while all EX groups had greater hind limb CSA, the RT protocol alone was better able to specifically improve forelimb strength when compared to the TM groups. It could be concluded that the novel RT protocol used for the present study was able to produce positive adaptation through specificity of exercise training.

Though not examined in the current study, the mechanisms responsible for the improvements in strength and CSA are likely those responsible for stimulating exercise-induced hypertrophy in skeletal muscle. For example, increases in IGF-1 and the downstream target, mechanistic target of rapamycin (mTOR), have been observed in exercising mice (Costelli et al., 2006). Mechanistic target of rapamycin functions as a serine/threonine protein kinase that regulates multiple cellular processes including cell growth and protein synthesis. Resistance training is known to maximize the hypertrophic response since heavy loading preferentially recruits fast-twitch fibers, such as MHCIIa and MHCIIb. Tatebayashi et al., (2018) demonstrated that, in CD2F1 mice inoculated

with the C26 tumor cells, HFES was able to elicit mTOR activation and significantly decrease E3 ubiquitin ligase mRNA of MuRF-1, atrogin-1, and FoxO. In addition, although chronically elevated IL-6 has been shown to exacerbate cachexia, exercise triggers a local, transient, increase in circulating IL-6 via the JAK/STAT signaling cascade that may play a key role in muscle regeneration through the stimulation of satellite cell proliferation (Baltgalvis et al., 2007; Puppa et al., 2012).

A secondary purpose of this study was to examine the effects of systemic inflammation, specifically IL-6, on cachexia. Systemic inflammation was marked by significant increases in both spleen mass (splenomegaly) and plasma IL-6 concentration in SED+Tumor mice, when compared to SED+Con. Chronic inflammation is associated with increased risk of both CRC and cachexia. Splenomegaly is also seen in cachectic mouse models and is noted as a marker of increased systemic inflammation and tumor progression (Aulino et al., 2010). It has been speculated that splenomegaly may indirectly enhance muscle wasting by competing for amino acids and other substrates, in essence starving the muscle or building blocks necessary to maintain or increase mass (Zimmers, Fishel, & Bonetto, 2016). Exercise training, regardless of mode, was able to attenuate the significant increases in both inflammatory markers (IL-6 and splenomegaly). The skeletal muscle inflammatory response, energy status, and protein homeostasis are known to be disrupted in cachexia and are implicated in the regulation of muscle atrophy (Hardee et al., 2018). Interestingly, many of the pathways involved in muscle wasting during cachexia, such as IL-6 and STAT3 signaling, are also upregulated by exercise (Hardee et al., 2018). In the tumor microenvironment, NF κ B stimulates activation of STAT3 through IL-6, leading to increased systemic inflammation. Hardee et al. (2018)

demonstrated that short-term treatment with pyrrolidine dithiocarbamate (an NF κ B inhibitor) was able to attenuate the suppression of mTORC1 signaling while concurrently reducing STAT3 activation in cachectic mice. Together, these data further support the involvement of inflammation and suppression of protein synthesis pathways in cachexia.

Interleukin-6, in addition to acting as a pro-inflammatory cytokine, also exerts endocrine and metabolic functions on various organs, including skeletal muscle. As a myokine, IL-6 acts to promote the proliferation of satellite cells to stimulate skeletal muscle repair and renewal (Belizário, Fontes-Oliveira, Borges, Kashiabara, & Vannier, 2016; Pedersen et al., 2004). During exercise, cytokines and myokines are produced to help maintain the metabolic homeostasis of proteins (Febbraio & Pedersen, 2002). It has also been demonstrated that IL-6 can induce muscle hypertrophy via the STAT3 signaling pathway (Serrano, Baeza-Raja, Perdiguero, Jardí, & Muñoz-Cánoves, 2008) and downstream targets such as the myogenic regulatory factor, MyoD1, which mediates the transcription of genes involved in myogenesis (Tierney et al., 2014). With physical activity, IL-6 is typically elevated during, and immediately following the exercise bout. In the present study, it was demonstrated that IL-6 remained elevated even 24-hours following the cessation of physical activity, suggesting that chronically elevated levels of circulating plasma IL-6 existed. To further address the role of IL-6 in tumor-bearing, cachectic, mice, Baltgalvis et al., (2010) have shown that electroporation with IL-6 directly into the muscle does not affect the size or function of skeletal muscle in non-tumor bearing mice, even with a 4-fold increase in circulating IL-6. So, although exercise does increase circulating IL-6, that alone may not be sufficient to induce skeletal muscle wasting or muscle dysfunction. Additionally, IL-6^{-/-} mice display no wasting in MHCIIa

or IIb fibers (Baltgalvis et al., 2010). Together, these data suggest that, while IL-6 is not the sole cause of skeletal muscle wasting, its presence is likely involved. It is concluded that IL-6 is a complicated, multifunctional, cytokine that can play a role in increased systemic inflammation, cachexia-associated atrophy, as well as hypertrophy in response to muscle damage, as with exercise.

Conclusions and Clinical Implications

Cachexia affects nearly 80% of all cancer patients by impairing immune function, increasing fatigue and overall weakness, and decreasing overall quality of life. Chemotherapy effectiveness is also decreased by the presence of cachexia (Manole, Ceafalan, Popescu, Dumitru, & Bastian, 2018). Thus, there is a need for research elucidating mechanisms that can decrease the incidence and severity of cachexia. Furthermore, research done in cell culture and rodent models should be translatable to the clinical population. In humans, cachexia is more difficult to detect, and significant loss in overall body mass is likely the only indication of the disease, without performing invasive procedures. Additionally, cachexia is not typically detected until late-stage or terminal cancer is evident. Results from this investigation indicate that C26 cells were able to produce tumors and thus, induce cachexia. Cachexia symptoms were marked by significant declines in body mass, muscle function, muscle mass, and increased systemic inflammation. Exercise, regardless of mode was able to attenuate the reduced muscle CSA and associated loss in strength and improve systemic inflammation. Together, these data give further insight into the benefits of both endurance and resistance training. It is also imperative to highlight the usage of moderate-intensity activity in the current protocol. Cachexia is characterized, in part, by chronic fatigue, thus making it difficult

for cancer survivors to exercise at high intensities. Additionally, the RT protocol adapted for the current study utilized body weight activity, in which mice essentially climb on the wire cage tops. Thus, it is possible that moderate-intensity exercises or body weight exercises, are sufficient to induce positive adaptation by reducing the severity of muscle wasting, improving muscular strength, and thereby improving overall quality of life. Additionally, because high-intensity activity may further compromise immune function, these data suggest that moderate-intensity activity is appropriate for cancer survivors and may confer significant benefits. Because the present study began with a period of pre-C26 inoculation exercise, it could be concluded that prior chronic physical activity may provide additional benefit in counteracting the severity of the cachexia and its symptoms. Moreover, obesity appears to be a key factor involved with increased incidence and severity of cachexia, cancer recurrence, and poor prognosis (Martin et al., 2013; Schwartz & Winters-Stone, 2009). It is possible that, because cancer survivors tend to become more sedentary during, and after treatment, a positive energy balance is created, thus worsening body composition, consequently providing further evidence that physical activity could be of great benefit to cancer survivors (Schwartz & Winters-Stone, 2009). Data from the current study supports the implementation of both aerobic and resistance exercise in rehabilitation programs for cancer survivors.

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APPENDIX

**UNIVERSITY OF NORTHERN COLORADO
INSTITUTIONAL ANIMAL CARE
AND USE COMMITTEE**



IACUC Memorandum

To: Dr. Reid Hayward
From: Laura Martin, Director of Compliance and Operations
CC: IACUC Files
Date: 07/09/17
Re: IACUC Protocol 1511CE-RH-R-18 Annual Renewal Approval

The UNC IACUC has reviewed your annual renewal request for animal use protocol 1511CE-RH-R-18.

The committee's review was based on the requirements of the Government Principles, the Public Health Policy, the USDA Animal Welfare Act and Regulations, and the Guide for the Care and Use of Laboratory Animals, as well as university policies and procedures related to the care and use of live vertebrate animals at the University of Northern Colorado.

Based on the review, the IACUC has determined that all review criteria have been adequately addressed. The PI/PD is approved to perform the experiments or procedures as described in the identified protocol for an additional year.