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The Effect of Cancer Cachexia Severity and Eccentric Muscle Contractions on Selected Myofiber Metabolic Properties in Mouse Skeletal Muscle

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ABSTRACT

Cancer cachexia is a complex syndrome that induces skeletal muscle wasting and dysregulation of skeletal muscle metabolism. Alterations in area of myofibers and myofiber metabolic properties can significantly impact the function of skeletal muscle. The purpose of this study was to examine the effect of cachexia severity and resistance exercise training on frequency and area of selected myofiber metabolic characteristics during cachexia-induced myofiber atrophy. Male $Apc^{Min/+}$ (Min) mice were studied during the progression of cachexia (16-20 weeks of age) and stratified into groups based on the severity of cachexia. For the second study, male Min mice performed resistance exercise (RE) for 7 sessions over 2 weeks during the initiation of cachexia. Myofiber area and myofiber metabolic properties were examined through histological analysis (H&E, SDH, and PAS) in the tibialis anterior (TA) muscle. Cachexia severity progressively decreased TA cross-sectional area and frequency of high SDH activity (OX) fibers however there was also a progressive increased frequency of high glycogen content (HG) fibers. Regardless of SDH activity, myofiber area was decreased with cachexia. In cachectic mice, RE increased high SDH activity fiber frequency and selectively induced hypertrophy of low SDH activity (GL) fibers, however there were no changes in frequency of high glycogen content fiber with RE. These results demonstrate that during the progression of cancer cachexia, there is progressive myofiber atrophy regardless of SDH activity, a decrease in frequency of OX fibers and increase frequency

of HG fibers. RE is able to increase frequency of OX fibers and induce hypertrophy of GL fibers.

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CHAPTER 1

INTRODUCTION

According to recently published data from the CDC and NIH, cancer is ranked as the second-leading cause of death in the United States (1). NIH data indicates that the new case diagnosis incidence rate per year from 2004-2008 for cancer was 553.0/100,000 and in 2012 it is estimated that cancer deaths in the United States were approximately 577,190 (1). The NIH estimates that over-all costs of cancer in 2007 were 226.8 billion dollars with over half of that being direct medical costs and the other being related to indirect mortality costs (1). Prostate, lung and colon/rectum are the three most prevalent cancer types among men and breast, lung and colon/rectum are the three most prevalent cancer types for women (1). Cachexia is a condition often seen during the progression of cancer, which involves the unintentional loss of body weight from the wasting/atrophy of both skeletal muscle and adipose tissue (3). Cachexia is estimated to be present in 80% of advanced cancer patients and can be directly attributed to 20-40% of cancer related deaths (4). The causes of cancer cachexia are not fully understood however increased levels of inflammatory cytokines such as TNF-alpha, interferon-y and IL-6 are thought to have a role. Inflammation during cachexia has a significant negative effect on skeletal muscle (4, 5, 6), however, there are still questions relating to development of these changes in skeletal muscle that have not yet been answered. During the progression of cancer cachexia, there is a disruption in skeletal muscle structure, function and metabolic properties.

Skeletal muscle is composed of three different fiber types: Type I (slow-oxidative), Type IIa (fast-oxidative) and Type IIb (fast-glycolytic) fibers and all of these fibers are characterized by specific metabolic properties as reflected by their intracellular enzyme activity patterns (7). Type I and Type II can be classified independently of myofibrillar ATPase enzyme activity, as Type I are solely oxidative, while Type II can range from oxidative-glycolytic to primarily glycolytic. A critical component of each muscle fiber are the mitochondria, which have a role in energy production, apoptotic properties and overall the energy demands of the cell and are directly related to oxidative capacity (8). Type I fibers have very high mitochondrial density while Type II-a fibers have less and Type II-b have even less mitochondria. With higher mitochondrial density, Type I fibers have much greater oxidative capacity than that of Type II fibers. Muscle mitochondrial content is significantly decreased in wasting conditions associated with heart disease, COPD and cancer cachexia, additionally Type II fibers are more susceptible to cachexia and have greater losses in morphology and overall oxidative capacity than that of Type I (9, 10). More specifically, gastrocnemius and soleus muscle oxidative capacity, when measured through multiple techniques such as COX IV expression and SDH enzymatic activity staining, is negatively affected by cancer cachexia in $Apc^{Min/+}$ mice, a mouse model of colorectal cancer (8). While there are reductions in oxidative capacity during the progression of cancer cachexia, the mechanisms behind this change in oxidative capacity have yet to be examined. A potential relationship to this reduction in oxidative capacity and mitochondrial dysfunction could be linked to changes in intramuscular energy stores/metabolism and alterations in morphology.

Glycogen and lipid can both serve as energy stores in skeletal muscle and vary depending on muscle fiber type. Most endogenous fat is stored as triacylglycerol in both subcutaneous and deep adipose tissue, however smaller amounts are deposited in skeletal muscle fibers (intramuscular triacylglycerol – IMTG) and can be used as a substrate source during exercise (11). Electron microscopic analysis of skeletal muscle has shown that intramyocellular lipid droplets are located adjacent to the muscle mitochondria, implying that these IMTG stores function as a readily available source of fatty acids for oxidative purposes (12). Several studies have reported greater IMTG content in Type I fibers than Type II fibers, indicating that greater fat oxidative capacity in Type I fibers is associated with greater IMTG storage (12,13). These fatty acids are utilized in beta-oxidation in which the fatty acids are broken down in the mitochondria to generate acetyl-CoA for entry into the citric acid cycle for the production of ATP (14). Carbohydrates can also serve as an intramuscular energy store during rest and exercise, stored as intramuscular glycogen (15). Studies have demonstrated as exercise intensity increases, carbohydrate oxidation increases while the oxidation of lipids decreases due to factors such as: limited rate of mitochondrial fat oxidation, abundance of glycolytic enzymes and a shift to fast glycolytic muscle fibers at high exercise intensities (15). Intramuscular glycogen stores can be increased directly by dietary carbohydrate intake known as "glycogen loading" and also have been observed to be increased as an exercise training adaptation (16). Glycogen can be utilized through glycogenolysis producing glucose-1-phosphate which enters to the glycolytic pathways, converted to pyruvate which then can be utilized in the mitochondria (14). Several studies have also shown that while Type II fibers can utilize both oxidative and glycolytic properties, higher levels of

glycogen content are found in Type II fibers, even more in Type IIB (17). While the initial stage of glycolysis occurs in the cytosol, later stages with end products of glycolysis utilize the mitochondria for energy production from glycogen stores (14).

Resistance exercise training has been shown to have beneficial effects such as mitochondrial biogenesis, with increased oxidative capacity through increased activity of citrate synthase and succinate dehydrogenase (18). Resistance exercise is also known to induce hypertrophy of skeletal muscle, however less is known of the response of cachectic skeletal muscle to resistance exercise. Resistance exercise induces hypertrophy of myofibers, while glycolytic myofibers tend to have the greater hypertrophy as compared to oxidative myofibers with resistance exercise training. In regards to previous research, only one published study has examined the effect of cancer cachexia progression and changes in intramyocellular lipids and reported a significant increase with the progression of cachexia severity (19), however no studies have examined the effect of eccentric-induced hypertrophy in skeletal muscle on myofiber metabolic properties. Intramuscular glycogen and lipid stores have a significant role in the energy demands and oxidative capacity of skeletal muscle and while it has been established that oxidative capacity is significantly reduced during the progression of cancer cachexia, alterations in oxidative myofiber morphology and alterations in glycogen storage have yet to be examined. Also it is not known if resistance exercise training can alter these metabolic properties or morphology during cachexia.

Research has shown that cancer cachexia progression has numerous detrimental systemic effects that can disrupt skeletal muscle. Our lab has demonstrated that with cachexia there is a loss of oxidative capacity. While oxidative capacity has been shown

to be greatly reduced in cachectic skeletal muscle, this has not been further extended to examine the effect of cachexia severity . Our lab has established that in the $Apc^{Min/+}$ mouse there is myofiber degeneration/regeneration (20), a hyperactivation of Akt and pAMPK as well as a downregulation of p-mTOR (5). Risson et al. (21) found that in their mTOR muscle specific KO model there is a hyperactivation of Akt, GSK3*B* and downregulation of pmTOR and this was accompanied with myofiber atrophy and a loss of oxidative capacity (21), similar conditions observed in our $Apc^{Min/+}$ model. Interestingly, they found that in the presence of these conditions, intramuscular glycogen significantly increased. The conditions are very similar to our model and warrant the further investigation of these variables in our model. To our knowledge, no studies have examined any alterations in the metabolic properties such as frequency and size of SDH activity myofibers and high and low glycogen content myofibers during the progression of cancer cachexia. Also, while exercise through treadmill running has been utilized in the $Apc^{Min/+}$ mouse model suppressing IL-6 induced wasting, resistance exercise training using electrical stimulation bouts has not been examined as an intervention in cancer cachexia related to myofiber size, and frequency and size of SDH activity myofibers and high and low glycogen content myofibers. There are critical clinical implications that are being examined in this study in relation to how metabolic properties in skeletal muscle are affected during cancer cachexia and if changes can be made to selected myofiber metabolic properties with the intervention of resistance exercise. The proposed study's overall purpose is to examine the effect of cachexia severity and resistance exercise training on frequency and area of selected myofiber metabolic characteristics during cachexia-induced myofiber atrophy. The central hypothesis is that cachexia severity will

induce myofiber atrophy and there will be a reduction in frequency and area of high SDH activity myofibers and an increase in the frequency of high glycogen content fibers and resistance exercise will attenuate the loss of low SDH activity myofiber CSA and frequency of high SDH activity myofibers.

SPECIFIC AIMS

Aim 1. To determine the effect of cachexia severity on frequency and area of high and low SDH activity fibers and high and low glycogen content fibers during cachexia-induced myofiber atrophy

Aim 2. To determine the effect of resistance exercise training on frequency and size of high and low SDH activity fibers and high and low glycogen content fibers during the initiation of cancer cachexia.

With respect to expected outcomes, experiments in Aim 1 will establish if severity of cachexia affects overall myofiber area and frequency and size of both high and low SDH activity myofibers and high and low glycogen content myofibers. Aim 2 will establish if resistance exercise during the initiation of cachexia will induce hypertrophy and if there is a relationship in frequency and size of SDH activity myofibers and glycogen content myofibers in conjunction with this hypertrophy. Overall, we expect to find progressive decreases in frequency and area of high SDH activity myofibers and increases in high glycogen content myofibers related to the atrophy with severity of cachexia. Also, we expect to find that the intervention of resistance exercise will increase mean myofiber area and increase the frequency of high SDH activity myofibers, inducing hypertrophy in low SDH activity and high glycogen content myofibers.

CHAPTER 2

Review of Literature

Cancer is a disease that affects millions across the world each year and still ranks as the number two cause of death in America (1). While many underlying mechanisms are understood regarding cancer and its effects during the progression, there are still many unanswered questions as well. Cachexia is a condition often seen with cancer which can lead to wasting of both muscle and adipose tissue. Some of the effects associated with skeletal muscle mass loss during cachexia include a loss of contractile proteins, decreased myofiber area and overall reduction in muscle weight (3). One of the negative effects on skeletal muscle that has been examined is the loss of oxidative capacity which is directly related to mitochondria and energy production, particularly in slow oxidative fibers. Intramuscular energy stores, glycogen and lipids, have been shown to have significant links to the oxidative capacity in skeletal muscle. T readmill training has been established as a positive influence on oxidative capacity in skeletal muscle during the progression of cancer cachexia, there are still gaps in the role of resistance exercise on oxidative capacity and other metabolic changes during the progression of cancer cachexia. The literature review for this Master's Thesis is stratified into 3 sections: (1) Cachexia overview and systemic effects, (2) Skeletal muscle structure, metabolic properties and the effect of cancer cachexia, and (3) Exercise and effects on skeletal muscle and intervention in cancer cachexia. This literature

review will give a general overview of cancer cachexia and the overall systemic effects. The specific effects of the progression of cancer cachexia in skeletal muscle will also be discussed, particularly in the $Apc^{Min/+}$ model which is being used for this study. Since the progression of cancer cachexia negatively impacts skeletal muscle, this review will examine the changes in skeletal muscle. This review will focus on skeletal muscle structure and metabolic properties in different types of skeletal muscle and changes that occur during cancer cachexia. Finally this review will focus on exercise, both aerobic and resistance exercise on its influence on healthy and cachectic systemic variables and skeletal muscle specifically. Since resistance exercise is a specific aim of this study, more focus will be on resistance exercise than aerobic.

1. Cachexia and Mechanisms

While there are different types of atrophy that can affect skeletal muscle and adipose tissue, cachexia encompasses and can negatively affect both. The changes during the progression of cancer cachexia are a major part of this research so understanding what is known and not known during the progression is essential. The purpose of this section of the review of literature is to clearly define cachexia and describe the systemic changes and possible mechanisms of atrophy including changes in protein synthesis and degradation as well as the possible role of inflammatory cytokines.

Cachexia. Cachexia derives from the Greek word "kakos" which translates to bad and "hexis" which means condition (22). It affects nearly 80% of diagnosed cancer patients and patients of other diseases as well. Cachexia has been shown to occur in acquired immunodeficiency syndrome (AIDS), major trauma, severe sepsis and many others {4}. The condition of cachexia is usually defined as an unintentional weight loss which

derives from depletion of both adipose tissue and skeletal muscle mass as well. Cachexia should not be confused with starvation which also exhibits a decrease in body weight however these conditions differ in multiple facets. These conditions differ in the fact that during starvation there is increased glucose production from the liver through gluconeogenesis (production of glucose from alternative sources such as amino acids) and during long- term starvation most fuel is derived from free fatty acids released from adipose tissue which ultimately results in a conservation of muscle mass (19). However, in cachexia there is an equal loss of adipose tissue and skeletal muscle mass, often times with greater decreases found in skeletal muscle mass (4). The body composition changes are dramatically different during the progression of cancer cachexia in that body composition changes can often be reversed in simple starvation with the addition of extra calories where this is not seen in patients with cancer cachexia (23). A prospective randomized study examined the effects of frequent nutritional counseling in chemotherapy cancer patients with the significant addition of calories and protein into the diet with a control that had no counseling or change in diet. It was found that quality of life and response rate to the addition of calories was not different as compared to the control group (24).

Energy Metabolism. While resting energy expenditure (REE) can attribute to nearly of 70% of sedentary total energy expenditure, this can become altered during the progression of cancer cachexia (25). Resting energy expenditure is often increased in cancer patients, however studies have shown that this may vary depending on the type of tumor. While no increases were seen in resting energy expenditure in colorectal cancer patients, there were elevation in resting energy expenditure noted in lung and pancreatic

cancer patients (25, 26). One of the links from changes in resting energy expenditure to inflammation could arise from changes in protein synthesis in the liver. Elevated acute phase response (APR) are defined as changes in liver protein synthesis which changes the production of albumin to inflammatory markers such as CRP, fibrinogen and others (27). There seems to be an association with elevated REE and elevated APR in response which leads to increased levels of acute phase proteins which has been associated with shorter survival times in cancer patients. Increased REE during cancer cachexia could also be attributed to increased thermogenesis in skeletal muscle. Uncoupling protein 3 (UCP3), which is found in adipose and skeletal muscle (28), has been found to be significantly elevated in cancer patients, particular those experiencing great weight loss (29). There has been a suggestion that this increase in UCP3 mRNA can contribute to the increased resting energy expenditure and be related to the overall tissue catabolism, particularly skeletal muscle that is present during the progression of cancer cachexia.

Alterations in Protein Synthesis. Cachexia estimates for 20-35% of cancer related deaths (30) and the loss of skeletal muscle in cancer cachexia patients has been linked to reduced survival rates due to further complications in respiratory muscles and other skeletal muscle dysfunction (31, 32). A contributing factor to the loss in skeletal muscle mass is decreased levels of protein synthesis and increases in protein degradation. Lundholm et.al (33) showed in a larger study of cancer patients, when matched with controls, the cancer patients had significant decreases in protein synthesis rates and increased fractional degradation rate of proteins. During the progression of cancer cachexia while there is typically a reduction in protein synthesis rates and this has been suggested as directly related to decreased appetite and weight loss, some data has shown

otherwise. In an animal model where there were no significant changes in food consumption, a depressed protein synthesis rate and increased protein degradation rate was still present (34). This indicates that the protein synthesis rate is affected not only by energy intake and expenditure, however there is an alteration in underlying mechanisms. eIF2a is a translation initiating factor in protein synthesis (35) and has been suggested as a possible altered mechanism in the changes in protein synthesis. A linear relationship between the phosphorylation of PKR which led to a phosphorylation of eIF2a was observed in cancer patients as compared to controls which could be responsible for the loss in myofibrillar proteins (36). Eley et.al (37) also noted in a different study that there was hypophosphorylation of 4E-BP1, another translation factor with increases in eEF2 leading to decreased translation elongation negatively affecting protein synthesis rates. While the loss in muscle mass and loss of myofibrils during the progression of cancer cachexia can be due to reductions in protein synthesis rates, increases in protein degradation rates also are responsible.

Alterations in Protein Degradation. While alterations in protein synthesis have been attributed to cachexia during the progression of cancer, alterations in protein degradation also coincide with these which lead to detrimental effects. While there are multiple proteolytic pathways for the degradation of proteins in skeletal muscle, the ubiquitin-proteasome pathway has been the most researched. Khal et al (38) found in cachectic cancer patients with weight loss greater than 10% that the ubiquitin-proteasome pathway was most responsible for the degradation of proteins in skeletal muscle. One of the major contributing factors to the increase in ubiquitin-proteasome activity is an increase in TNF-alpha, an inflammatory cytokine (39). The increase in TNF-alpha has been

observed to lead to increased rates of proteolytic protein degradation via the ubiquitin-proteasome with formation of reactive oxygen species and upregulation of Nf- kB (40,41). This upregulation of NF-kB led to an increased production of other inflammatory cytokines and increased breakdown of myofibrillar proteins (42). Studies have suggested that these increased protein degradation rates through the ubiquitin-proteosome pathway are partly responsible for the increased REE due to the requirement of ATP in the proteolytic process (43). However, for lower weight loss patients, muscle biopsies in some studies have shown that the ubiquitin-proteosome pathway was unchanged and the loss was responsible from expression of mRNA for cathepsin B (44). While other myofibrillar proteins do not seem changed, myosin heavy chain (MHC) seem to be the greatest affected by the increase in the ubiquitin-proteosome activity during the progression of cancer cachexia, which with the decrease in protein synthesis leads to the skeletal muscle mass loss observed {16}.

The Role of IL-6. Interleukin-6 is a cytokine that has both pro-inflammatory and anti-inflammatory characteristics (45). IL-6 is associated with chronic inflammation as IL-6 is necessary for the induction of Th17 cells, which are a subset of T helper cells (46). Studies have shown elevated IL-6 levels in the blood and within the tumors in cancer patients with various types of cancer including breast, colorectal, pancreatic and many others (47, 48). Knupfer and Preiss (49) noted a distinct association between IL- 6 levels and tumor stage, size and survival rate. Becker et. al (50) found that IL-6 was critical in the development colitis-associated cancer and growth of intestinal tumors which could lead to activation of STAT3 as a downstream effect. IL-6 $^{-/-}$ mice in a colitis-induced model had reduced tumor development, in which Grivennikov et. al (51)

described a critical role of STAT3 activation though elevation of IL-6 for the proliferation and apoptosis observed. IL-6 has also been linked to the increased expression of VEGFR2, which contributes to vasculogenesis and angiogenesis, which then promotes proliferation of cancerous tumor cells in the colitis cancer model (52). IL-6 also can contribute to other types of cancer as Gao et. al (53) showed that there is possible involvement in an IL-6/STAT3 signaling cascade in the proliferation of adenocarcinomas and tumorigenesis in the lungs. Sansone et.al (54) also found a critical role of IL-6/Stat3 activation and interaction in the development of tumors in breast cancer. Downstream STAT3 activation via IL-6 expression has been shown to regulate tumorigenesis in multiple lines of cancer, demonstrating a critical dependent role for IL-6 during the development of cancer.

Proteolysis-inducing factor. Proteolysis-inducing factor (PIF) is a glycoprotein produced by tumors and leads to decreases in protein synthesis and increases in protein degradation (55). PIF has been detected in the urine of cachectic cancer patients of multiple cancer lines however was not observed in weight-stable cachectic patients (56). Some studies have shown specific PIF excretion in the urine related to the weight loss in cachectic patients with prostatic and gastrointestinal tumors (57) however other studies have disputed the significance of PIF in the weight loss of other cachectic patients (58). Williams et. al (59) performed a longer study of PIF excretion during weight loss, noting the majority of changes in PIF status were negative to positive. There seems to be a dispute in the literature regarding the role and importance of PIF during the development of cancer cachexia, however PIF does seem to have significance in the body weight loss, particularly skeletal muscle mass during the progression of cancer

cachexia. PIF is unlike cytokines as it is able to induce protein degradation in isolated skeletal muscle and also can lead to cachexia in non-tumor-bearing mice without changes in dietary intake (60). Interestingly, Todorov et. al (61) showed that PIF produced overall body weight loss in mice, specifically in non-fat mass without decrease in food intake. In this particular study, PIF was also observed to increase mRNA levels of ubiquitin and other proteasome subunits in skeletal muscle, suggesting a significant role in the previously mentioned ubiquitin-proteasome pathway of protein degradation. In isolated myotubes, muscle atrophy was observed via PIF as it was shown to decrease protein synthesis while increasing protein degradation. In this same study, the PIF was shown to have no significant effect on actin however there was a depletion of myosin (61). While an increase in proteasome activity is observed in cachectic patients particularly by PIF, PIF has been shown to also increase TPPII. TPPII is tripeptidyl peptidase II which is part of the downstream proteolytic cascade of the ubiquitin-proteasome pathway (62). Both the separate proteasome units and the increase in TPPII by PIF have been suggested to be related to a link in activation of NFkB.

NF-kB. nuclear factor kB, NF-kB, also seems have a significant role in the ubiquitin-proteasome pathway. NF-kB is suggested to be activated in response to PIF in which the ubiquitin-proteasome pathway is further induced (63), leading to greater protein degradation associated with the loss during cancer cachexia. Cai et. al (64), showed the link of IKKB/NF-kB to the induction of ubiquitin-proteasome pathway which resulted in very high levels of muscle wasting that were very similar to cachexia. Grannerman et. al (65) demonstrated that this activation of NF-kB also suppressed mRNA for MyoD, a myogenic transcription factor, which led to a reduction in synthesis of myosin.

Expression of MuRF1 and other subunits of the proteasome are also elevated due to activation of NF-kB leading to muscle loss as well (65). Resveratrol confirmed the activation of NF-kB to muscle wasting during the progression of cancer cachexia. Wyke et. al (66) showed that resveratrol, which inhibits the activation of NFkB through other targets, significantly attenuate the weight loss and protein degradation via the ubiquitin-proteasome pathway in MAC16 tumor bearing mice. The formation of reactive oxygen species via the activation of NF-kB by PIF has also been suggested as a link to the skeletal muscle atrophy observed during the progression of cancer cachexia (67). IL-6 production from the activation of NF-kB also results in the proliferation of the STAT3 pathway which then leads to the increase in survival genes which then leads to the production of other inflammatory cytokines (68). Thus, NF-kB in conjunction with other factors play a significant role in the chronic inflammation and skeletal muscle mass loss observed in cachectic cancer patients. While NF-kB and IL-6 seem to target skeletal muscle specifically, there are cytokines such as TNF-a, which target adipose tissue during the progression of cancer cachexia.

TNF-a. Tumor necrosis factor alpha (TNF-α) is another cytokine that has a critical role in systemic inflammation during an acute phase reaction (69). While the definitive role of TNF-a in the induction and progression of cancer cachexia still remains controversial, some studies have attempted to define a role for TNF-a in cachexia. It is known that individual variations in the TNF-a levels can attribute to polymorphisms, particular in the A allele in the promoter region of the TNF gene (70). This variation can lead to other downstream effects such as higher TNF-a transcription levels and an increased risk for many cancers (71, 72), while in other cancers these variations linked to the

development of cancer are not as consistent (73). Mader et. al (74) found that there was no correlation between TNF-a levels and weight loss in cancer patients. However, Karayiannakis et al.(75) observed that the levels of TNF-a in cancer patients was inversely correlated with body weight and body mass index. While evidence shows that TNF-a can induce lipid depletion in adipose tissue through various mechanisms (76, 77, 78), a true correlation linking TNF-a to the progression of cancer cachexia has yet to be established and needs to be examined further. While numerous factors may affect the inability to provide a direct link of TNF-a to degree of body weight loss, it seems that it is more likely that TNF-a levels coincide with tumor size and development (4). While individual alterations in cytokine levels and protein synthesis/degradation have a role in the progression of cancer cachexia, the culmination of all these factors can greatly affect multiple variables in cachectic patients.

Systemic Effects/ Quality of Life. While numerous specific effects from overexpression of cytokines and alterations in certain pathways have been suggested as causation for the reduction in skeletal muscle mass during cachexia, the culmination of all of these effects can lead to overall changes in systemic variables for cachectic cancer patients. Cachexia has been implicated as one of the most important factors in survival rates and quality of living for cancer patients (79). DeWys et. al (80) performed a retrospective evaluation of over 3000 cancer patients with differing types of cancer tumor types.

While moderate to severe weight loss was seen in 30-70% of patients, this wide range can be attributed to multiple variables such as tumor site, size, type etc. Interestingly, they found that there was a lower risk of weight loss in the breast and hematological patients and the patients with gastric, lung and pancreatic solid tumors were often likely

to lose 10% or more of their typical body weight. Buccheri and Ferrigo found that total weight loss was the best indicator of prognosis in 388 cases (81). There also seems to be a significant correlation of overall body weight change and survival. Hess et al (82) observed ovarian cancer patients and found that the risk of death increased by 7% for each 5% drop in body weight. It was also found that patients with the highest rate of weight loss had a median survival rate of 7.5 months while patients with low rates of weight loss had an average survival of 30.2 months (83). While changes in protein synthesis and degradation rates along with inflammatory cytokines have been linked to this weight loss, other variables also can contribute. Andreyev et al. (84) recorded data from over 1500 patients receiving surgical and chemotherapeutic treatments. This group observed that patients with greater weight loss had lower chemotherapy doses. This weight loss was correlated with other variables including overall survival rates and quality of life. Quality of life is a variable that is often affected by the progression of cancer cachexia. Unlike other measurements, quality of life is a subjective measurement assessed by every individual, however strong correlations are observed in quality of life in cachectic cancer patients. As the progression of cancer continues, measurements of quality of life have been shown to decrease in many patients (85). Since many of the measurements for quality of life are subjective, they include ratings of measurements such as fatigue, depression and other aspects of quality of life. Multiple studies have associated quality of life with management and survival rates during the progression of cancer cachexia. Definitions slightly vary from past studies, however current studies typically define quality of life as an individuals perceived physical, mental and social health status. Quality of life has shown to have a correlation to cancer progression in all

types of cancer. Coates et al. (86) found that overall quality of life and social functioning were significantly predictive of survival in cancer patients and Dancey et al (87) observed that overall quality of life was significantly associated with survival. Langendijk et al (88) found that in 198 lung cancer patients, overall quality of life was a strong prognostic factor in the survival rate. While quality of life is a subjective measurement in the progression of cancer cachexia, it is a strong indicator of the negative systemic effects of cachexia.

2. Skeletal Muscle (Structure, Metabolic Properties and Effect of Cachexia)

While the progression of cancer cachexia affects both adipose and skeletal muscle tissue, the aim of this particular research is its effect on skeletal muscle. There are different distinctions between types of skeletal muscle which will be examined in this particular part of the literature review. One of the metabolic properties of skeletal muscle that is being investigated is oxidative capacity. There is extensive research and literature regarding oxidative capacity in healthy skeletal muscle and changes during the progression of cancer cachexia. One of the variables of both of the aims is to examine any possible changes in intramuscular glycogen stores. While much is known of this and other intramuscular energy stores in healthy skeletal muscle, the research is more limited in diseased muscle.

Phenotypes. There are 3 muscle types in humans: skeletal, cardiac and smooth muscle. While these three types share some similarities, their differences vary depending upon multiple factors. In skeletal muscle there are 3 primary fiber types: Type I, Type IIA and Type IIB (89). These fiber types vary depending upon multiple differences in metabolic properties and other characteristics. Type I fibers are referred to as slow

oxidative, Type IIA fibers are fast oxidative and Type IIB are fast glycolytic. While Type I fibers only use oxidative capacity for generation of force production, Type II fibers can vary from oxidative to glycolytic depending upon sub-classification (90). Type I fibers typically have large amounts of mitochondria which are responsible for the increased oxidative capacity. Type IIA fibers also contain a significant number of mitochondria, however they are much less than that of Type I fibers. Type IIB store more glycogen than any other fiber type and have the lowest amount of mitochondria present (91). While Type I fibers have a slower contraction velocity than other fiber types, these are much more efficient in steady-state long time activities such as long distance running (91). Type IIA fibers have a much higher contraction velocity and are more useful in shorter activities than that of Type I fibers such as middle distance running. Type IIB fibers have the greatest contraction velocity as ATP are split very quickly however these fibers fatigue very fast and easily, therefore they are primary fiber types that are used in very short distance activities such as sprinting (90). While muscle fiber type shifts have been observed in sedentary and active individuals, phenotype shifts have been observed in diseased patients, specifically cachexia.

Muscle Phenotype Shift/Loss During Cancer Cachexia. Certain muscles, such as the gastrocnemius which is fast-twitch, seem to be more susceptible during muscle wasting (92). While certain muscles do appear to be more susceptible to the wasting observed during cancer cachexia, fiber type shifts have also been noted typically shifts towards a faster, more glycolytic phenotype. Diffee et al.(93) noted that while no type IIB MHC was detected in the control mice soleus muscles, Type IIB comprised of 19% of the total MHC in the experimental soleus muscle. Type I MHC was also decreased in the

C-26 mice as compared to the control mice. While reductions were seen in the Type I fibers, fiber loss and shifts were observed within the Type II fibers. Other studies have also reported that during the progression of cancer cachexia there is a decline in muscle mass that is also accompanied with a decrease in muscle protein concentration (94, 95). These studies and others have suggested that this loss in the muscle mass is related to the increased activity in the ubiquitin-proteasome pathway (96). Diffee et. al (93) also noted that the MHC isoform content changes were very similar to other interventions that had shown to decrease muscle mass such as spinal transection and decreased mechanical loading (97, 98). All of these results indicate changes in the Type II MHC isoforms and decrease in the amount of Type I MHC isoforms. While Type I muscle fibers are affected by cancer cachexia, often times the greater losses are noted in the Type II fiber types and shifts from Type IIB to Type IIA. One proposed mechanism for the decreased loss in the Type I fibers and Type II fiber shift is PCG-1a. PGFC-1a, peroxisome proliferator-activated receptor-y coactivator, is a transcriptional coactivator that has multiple roles including mitochondrial biogenesis and more importantly the fiber type conversion to a more oxidative phenotype in skeletal muscle (99). Over-expression of PGC- 1a in mice has resulted in an attenuation of muscle mass loss during denervation-induced atrophy, indicating an important mechanism in the regulation of muscle mass (99). PGC-1a is reduced during the progression of cancer cachexia which coincides with the loss in skeletal muscle mass (100) and it appears that PGC-1a plays a significant regulatory role. This overall loss in skeletal muscle mass has been validated in other studies demonstrating significant losses in mean cross sectional area of the soleus and shifts in fiber types. One study from our lab showed

that the overall body weight in the $Apc^{Min/+}$ mice had decreased 21% as compared to control and that the soleus mean fiber cross sectional area had decreased 24% and the gastrocnemius was reduced 45%, indicating and validating that Type II fibers, due to lower oxidative capacity appear to be more susceptible to losses associated with the progression of cancer cachexia (20).

Oxidative Capacity/Effect of Cachexia. The mitochondria are a critical regulator of skeletal muscle mass during the progression of cancer cachexia due to its multiple roles such as energy production, production of reactive oxygen species and others. The increased turnover of mitochondrial proteins and also a suppression of mitochondrial biogenesis have been suggested as a contributor to the loss observed in oxidative capacity (8). Predominantly fast-twtich muscles such as the gastrocnemius and the tibialis anterior seem to have the greatest reductions in muscle mass as compared to the soleus, which has a much greater oxidative capacity. This loss is observed in the progression of cancer cachexia, specifically colon cancer, as this has been observed in the $Apc^{Min/+}$ mouse (8,101) There is conflicting evidence as some have found that precachectic oxidative capacity appears to serve as a protective effect during the progression of cancer cachexia (102), however certain studies have shown otherwise (103). Wang et al. (103) used LLC (Lewis lung carcinoma implantation) model of mice and reported that there was increased mitochondrial biogenesis in the skeletal muscle of the tumor-implanted mice. Interestingly, it was reported that the increased mitochondrial biogenesis was not enough to rescue muscle loss and the over-expression of muscle PGC-1a led to increased tumor size. There appears to be debate on exactly how and to what degree cancer cachexia affects oxidative capacity in skeletal muscle and other factors that could influence this as well. Oxidative fibers such as the soleus appear to be

less susceptible to overall loss as compared to less oxidative fibers such as the EDL, however this has not been concluded with certainty.

Intramuscular Glycogen Stores. Carbohydrates can be utilized as a substrate during exercise and have been studied extensively (104,105,106). Carbohydrates can be stored as intramuscular glycogen and then utilized during exercise. Depletion of intramuscular glycogen has been noted as a limiting factor during prolonged exercise (104), while some data has tried to argue that exhaustive exercise does not always coincide with depleted intramuscular glycogen (107) however this is highly dependent upon training status. Training status and availability of intramuscular glycogen stores contributes to the degree that this becomes a limiting factor. One important factor that impacts intramuscular glycogen storage is the rate of hepatic glycogen mobilization and gluconeogenesis (108,109). Haff et al. (110) found that carbohydrate supplementation improved intramuscular glycogen stores thereby improving performance and duration during resistance exercise. While most literature indicates the critical role of intramuscular glycogen in the performance particularly in short intense bouts, there appears to be a few selected studies that dispute this dogma. Symons and Jacobs (111) found that a strict low carbohydrate diet resulted in significantly lower intramuscular glycogen stores versus the control diet. However, when both groups performed short-term high intensity exercise, performance was not impaired when compared across the groups. While certain diseases such as McArdles disease (112) can affect the ability of muscle to utilize stored intramuscular glycogen, the literature is very limited in the effect of diseases on intramuscular glycogen stores. Variations in diet and training status have been proven to affect intramuscular glycogen stores, however diseased states have not

been examined, particularly in cancer cachexia. Stephens et al.(113) examined intramyocellular lipid droplets and reported that these were increased in the presence of cancer and increased as weight loss increased. However, intramuscular glycogen stores have yet to be examined during the progression of cancer cachexia.

Cross Sectional Area Alterations. While metabolic changes have been reviewed, overall changes occur within skeletal muscle during the progression of cancer cachexia such as alterations in cross sectional area (CSA). Alterations in CSA are often associated with the body weight changes that are observed during the progression of cancer cachexia. CSA is a valid measurement of myofiber area and changes in CSA occur in many muscles, however Type II fibers tend to show the greatest amount of change in CSA (114, 115). The mass as well as the function are directly related to CSA in skeletal muscle, these alterations during the progression of cancer cachexia can explain the atrophy and loss of function that is often observed. Julienne et al (116) reported that significant changes occurred in cross-sectional area of skeletal muscle rather than changes in numbers when compared with controls. The more oxidative muscle such as the soleus did not show reduction in cross sectional area however the fast-twitch muscles such as the EDL and gastrocnemius were significantly reduced in cross sectional area. While some have reported that the soleus is not significantly affected, other studies have shown otherwise. Mehl et. al (20) reported that in $Apc^{Min/+}$ mice the soleus muscle in cachectic mice was significantly reduced as compared to B6 and was associated with a reduction in Type I percentage of fibers and an increase in Type IIa and TypeIIb. This indicated that while the soleus has been previously shown as less susceptible to the effects of cachexia due to its oxidative capacity, some have shown

reductions in cross-sectional area and fiber type changes. While the cross-sectional area of fibers has been analyzed in the cachectic model, there have been no studies that have examined the effect of the cachexia severity on CSA, oxidative myofiber morphology and cross- sectional area of glycogen dense/depleted fibers to notate any changes if any are present during the progression of cancer cachexia.

3. Exercise (Effects on Metabolic Properties and Cachexia)

Exercise has multiple health benefits that have been well established. Studies have provided data that supports the benefit of both aerobic and resistance exercise during the progression of wasting diseases. While the literature is quite extensive in the benefits of aerobic exercise during the progression of cancer cachexia, there is a lack of evidence from studies examining the effects of resistance training on skeletal muscle and other systemic variables.

Systemic Effects/Benefits of Exercise. There is a mass of literature that indicates the benefit of aerobic and resistance training exercise in both healthy and diseased persons. Some of the benefits include lowered LDL cholesterol, improved neural communication and stimulation, improved function of skeletal muscle and many others. Exercise has shown to have benefits in the immediate and long term effects. Altena et al. (117) reported significant beneficial changes in total cholesterol after only 4 weeks of aerobic exercise while in longer term studies, significant changes were reported in body mass, systolic/diastolic blood pressure, waist circumference and V02 max (118,119,120). In regards to short term responses, multiple studies reported an increase in HDL cholesterol (121) while other studies have reported that there were no

appreciable changes in HDL in the short term, however they were present in the long term. Beneficial changes in body composition were also reported. In regards to adiposity, multiple studies have reported significantly reduced body fat in the exercise groups versus non-exercise control (118,122). These changes in body fat were also associated with changes in waist and hip circumference. Murphy et al (119) found that in both continuous and accumulated exercise groups, there were reductions in the waist and hip circumference measurements. Studies have also reported improvements in VO2 max after exercise as compared to non-exercise controls (123,124). While multiple systemic effects are reported with exercise, there are beneficial effects in skeletal muscle and adaptations occur over time with training.

Resistance Exercise and Adipose Tissue/Fat Metabolism. Resistance exercise has been shown to have numerous beneficial effects, particularly on lean and fat tissue mass. There is evidence that repeated bouts of resistance exercise training can improve body composition by reducing fat mass and increasing lean body mass (125). Immediately post exercise it has been reported that intramuscular triglyceride stores are reduced as they were used for substrate fuel (126). This was further validated as resting respiratory exchange ratio was reduced immediately (127) and 15 hours following a bout of resistance exercise, suggesting fat oxidation post-exercise (128). Melanson et al. (129) reported that there was an increase of 5% in 24 hour fat oxidation following a bout of resistance exercise however it was not significant. Stich et al. (130) observed that after 60 minutes of exercise, lipolysis was at its highest rate which matched overall body fat oxidation, suggesting the use of fats of whole body for substrate use during resistance exercise. Orsmbee et al (128) reported that subcutaneous adipose tissue lipolysis and whole body fat oxidation were improved after an acute bout of resistance exercise. The

literature has shown that resistance exercise will improve fat oxidation and intramuscular triglyceride stores, however no work has been done with resistance exercise in the cancer cachectic model to note if these changes will still occur.

Resistance Exercise and Skeletal Muscle. Both resistance training and aerobic exercise has systemic beneficial effects as well as adaptations that occur in skeletal muscle through training. One of the adaptations that occurs in skeletal muscle over time with resistance training is known as hypertrophy. Hypertrophy refers to an increase in size the myofibrils, rather than an increase in fiber number which is known as hyperplasia (131). Most training studies have reported an increase in the cross-sectional area of muscle fibers ranging from 20-45% (132). Hather et al. (133) reported that glycolytic muscle fibers showed greater increases in cross-sectional area than that of oxidative muscle fibers. With the increase in cross-sectional area of muscle fibers are indicative of multiple changes that are occurring within the muscle fiber. Typically these are changes in actin and myosin protein filaments, more myofibrils, more sarcoplasm as well as increases in the connective tissue around the muscle fibers (131). Associated with changes in the cross-sectional area of skeletal muscle during are muscular strength gains. During the first few weeks of resistance training, much of the gain in strength is due to neural adaptations, rather than the cross-sectional area of the skeletal muscle as the motor unit recruitment increases and is more efficient. Longer term changes in muscular strength can be associated with the changes in cross- sectional area of the skeletal muscle. These changes in muscular strength can vary greatly as Kraemer reported ranges of +7% to +45% (134).

Resistance Exercise and Intramuscular Glycogen. Improvements in glucose

metabolism associated with skeletal muscle have been reported with resistance exercise training. An adaptation to exercise often noted is the increase in GLUT4 receptors. GLUT4 is a protein that is the insulin-regulated glucose transporter that is found in adipose and skeletal muscle (131). Improvements in glucose metabolism have been reported in clients engaging in regular resistance exercise training and when tested, improvements were noted in glucose tolerance and reduced insulin responses to oral glucose (135,136). While there are adaptations to skeletal muscle with resistance exercise, immediate effects have been noted and factors previous to the exercise can affect the short-term effects. Tesch et al. (137) reported that bodybuilders completing a circuit style weight lifting session had a muscle glycogen content that was 26% lower post-exercise than when compared to baseline measurements. While this decline was reported as modest, it still indicated that muscle glycogen was contributing to the resistance exercise session. Based upon this information Essen-Gustavsson and Tesch (138) repeated the same experiment however this time measuring muscle glycogen and intramuscular triglycerides. Muscle glycogen content decrease was similar as before at 28% and intramuscular triglycerides also were reduced by 30% immediately post-exercise. This data indicated that both muscle glycogen and triglycerides both contribute as energy substrates during weight training. Limiting the intake of muscle glycogen stores has also been shown to negatively affect performance. Leveritt and Abernathy (139) reported that depletion of glycogen negatively affected performance. The study was conducted with a control group and a group that was on a carbohydrate restricted group for 48 hours plus exhaustive exercise prior to testing. Both groups were tested in a resistance training protocol. While no differences were noted in the knee

extension trials, the squat performance had significantly decreased in the carbohydrate restricted group. One of the major factors suggested by the authors was that the knee extension trials were very short duration isokinetic bouts which energy production was mainly from creatine phosphate, while the longer isometric squat exercise utilized more glycogen, therefore the depletion of glycogen was more of an effect. While glycogen depletion has been shown to negatively affect performance, particularly in isometric exercise, glycogen loading has the opposite effect indicating the importance for intramuscular glycogen content in skeletal muscle for performance. Haff et al (140) while previously mentioned did a further study to examine the effect of muscle glycogen stores and glycogen loading on performance. Control subjects drank a placebo beverage while another group consumed a 250 gram carbohydrate supplement, then rested for 3 hours. After the rest period both groups performed squats at 55% of 1-RM until exhaustion. The group that has consumed the carbohydrate supplement were able to exercise significantly longer than the placebo drink group, the authors concluded that the ingestion of carbohydrates increased the muscle glycogen stores aided in improved performance in weight training activity.

Intramuscular Triglycerides and Resistance Exercise. While intramuscular triglyceride stores can be utilized as a substrate during exercise, high accumulations of intramuscular lipids have been linked to health problems. Multiple studies have noted significant correlations between high intramuscular lipid contents and skeletal muscle insulin resistance (141, 142) in obesity and Type 2 diabetes. High levels of intramuscular lipids are also found in athletes as well which has been referenced as the "athletes' paradox." Studies have shown that athletes, particularly endurance trained

athletes, have similar intramuscular lipid content as that of insulin resistance obese and type 2 diabetes patients (143). Changes in intramuscular lipid content have also been noted in older individuals after beginning an exercise program associated with improved overall fitness and oxidative capacity. Dubé et al. (144) found that just moderate improvements in physical activity increased use of intramuscular triglyceride stores, associated with improvements in intramuscular glycogen stores and increased oxidative capacity. This study indicated that exercise has beneficial effects on oxidative capacity associated with improvements on intramuscular glycogen and triglyceride stores in healthy muscle. While many studies have examined the effects of intramuscular energy stores and oxidative capacity in response to resistance exercise, none have examined in wasting conditions such as cancer cachexia.

CHAPTER 3

THE EFFECT OF CACHEXIA SEVERITY ON SELECTED MYOFIBER METABOLIC PROPERTIES IN $APC^{MIN/+}$ MICE

ABSTRACT

Cancer cachexia disrupts skeletal muscle metabolism in conjunction with muscle mass loss. Alterations in myofiber area and metabolic properties can significantly impact the function of skeletal muscle. The purpose of this study was to determine the effect of cachexia severity on frequency and area of high and low SDH activity fibers and high and low glycogen content fibers during cachexia-induced myofiber atrophy. Male $Apc^{Min/+}$ mice were studied during the progression of cachexia (16-20 weeks of age) and stratified into groups based on the severity of cachexia [weight stable WS (no body weight (BW) change), moderate (5-19% BW change) and severe (≥20% BW change)]. Myofiber area and myofiber metabolic properties were examined through histological analysis (H&E, SDH, and PAS) in the tibialis anterior (TA) muscle. Cachexia severity progressively decreased TA mass and TA mean myofiber cross-sectional area (CSA). The frequency of high SDH activity fibers (OX) was reduced in mice with moderate and severe cachexia (23% and 45%, $p<0.01$). Additionally, CSA was reduced in both OX and low SDH activity (GL) myofibers. However, there was an increase in frequency of high glycogen content fibers (HG) in both moderate and severe cachexia (24% and 55%, $p<0.05$). There were no differences found in any of the variables between wild-type

controls and $Apc^{Min/+}$ WS. These results demonstrate there is progressive myofiber atrophy regardless of SDH activity with cachexia severity. Additionally, there is a progressive reduction in the frequency of OX myofibers and an increase in the freqeuncy HG fibers with cachexia severity.

INTRODUCTION

According to recently published data from the CDC and NIH, cancer is ranked as the second-leading cause of death in the United States (1,2). The NIH estimates that over-all costs of cancers in 2007 were 227 billion dollars with over half of that being direct medical costs and the other being related to indirect mortality costs (1). Cachexia is condition which involves a wasting of both adipose and skeletal muscle tissue and is associated with over 50% of cancer patients and attributed to 40% of cancer deaths (3). All of the mechanisms are not clear in regards to the progression of cachexia and multiple inflammatory cytokines have been identified as possible contributors to the progression (4). While much research has been done on skeletal muscle during the progression of cancer cachexia (5, 8, 20), there are still numerous gaps in the research regarding specific metabolic properties. Investigating and identifying the alterations in skeletal muscle metabolic properties during the progression of cancer cachexia is essential in moving towards treatment and further understanding the debilitating condition.

A hallmark characteristic of cachexia is a loss of both adipose and skeletal muscle tissue (4). During the progression of cancer cachexia severity, there is an imbalance between protein synthesis and degradation leading to the loss in skeletal muscle mass. This imbalance is due to a decrease in myofibrillar protein synthesis rates and increase in

multiple proteolytic genes such as polyubiquitins, Ub ligases atrogin-1/MAFbx and MuRF-1 which contribute to the increased protein degradation (3,6). Skeletal muscle is composed of three different primary fiber types, and these have been shown to be differentially affected during the progression of cachexia (20). These fibers are characterized by specific metabolic properties as reflected by their intracellular enzyme activity patterns; type I fibers are highly oxidative fibers and Type II can range from oxidative-glycolytic (Type IIa) to solely glycolytic (Type IIb) (20). While there are numerous metabolic properties that are specific to each muscle based upon their enzyme activity pattern, certain specific metabolic properties in $Apc^{Min/+}$ mice have yet to be further investigated.

Oxidative capacity is defined as the maximal capacity of a tissue to utilize oxygen for energy production, which is directly related to the amount of mitochondria in the skeletal muscle fiber (8). While there are numerous methods that can be utilized to measure oxidative capacity, a common histological technique is succinate dehydrogenase (SDH) staining. The SDH enzyme is located in the inner membrane of the mitochondria and oxidizes succinate to fumarate in Kreb's cycle. The staining technique will stain the SDH enzyme and a greater intensity of the staining indicates greater more mitochondria and enzymatic activity, and this activity is indicative of oxidative capacity (9). Another differential factor in skeletal muscle fiber types is substrate utilization. Type I fibers are more oxidative utilizing free fatty acids (FFA) for energy production while Type II fibers are more glycolytic using glycogen for energy production (145). Glucose is stored as glycogen in skeletal muscle via glycogen synthase (GYS1 for skeletal muscle) and is utilized via glycogen phosphorylase (16). One validated method of determining skeletal

muscle glycogen content is periodic acid Schiff (PAS) staining. PAS staining is used to detect polysaccharides such as glycogen. The greater the numbers of fibers with dark intense staining has shown to strongly positively correlate with total skeletal muscle glycogen content (46). Metabolism is known to be dysregulated with cachexia, however specific myofibers metabolic properties related to skeletal muscle metabolism are less understood. Elucidating any changes in myofiber metabolic properties during cachexia-induced myofiber atrophy is essential for further examining the effects of cachexia on skeletal muscle to move towards further understanding the mechanisms of change and treatment of cachexia.

While human models of cachexia patients have been used in numerous studies, the use of the $Apc^{Min/+}$ mouse is a widely used method of examining the effects of the progression of cancer cachexia as it has been shown to correlate and prove as an effective model similar to humans (20). The $Apc^{Min/+}$ model is from the alteration of the tumor suppressor gene (Apc) and these mice are highly susceptible to spontaneous intestinal adenoma formation which is very similar to the human model of colon cancer. Much data has been shown using the $Apc^{Min/+}$ model with increases in protein degradation, reductions in protein synthesis, myofiber degeneration and other characteristics with the progression of cancer cachexia (5, 8, 20). Oxidative capacity has been shown to be reduced with a selected degree of cachexia in selected muscle however alterations in high and low SDH activity myofibers has not been examined neither has high and low glycogen content myofibers. Therefore, the purpose of this study is to examine the effect of cancer cachexia severity on frequency and size of high and low SDH activity myofibers and high and low glycogen content fibers during cachexia-induced myofiber

atrophy. The hypothesis of this study is that cachexia severity will progressively induce myofiber atrophy and will reduce the frequency and size of high SDH activity myofibers however will increase the frequency of high glycogen content myofibers.

MATERIALS AND METHODS

Animals. All WT and $Apc^{Min/+}$ mice used in this study were on a C57BL/6 background and were originally purchased from Jackson Laboratories (Bar Harbor, ME). All mice were bred at the University of South Carolina's Colon Cancer Research Center Mouse Core breeding facility that is housed in the University's Animal Resource facility, as previously described. For all mice in the study, the room was maintained on a 12:12-h light-dark cycle with the light period starting at 0700. Mice were provided standard rodent chow (cat. no. 8604 Rodent Diet; Harlan Teklad, Madison, WI) and water ad libitum. Body weights and food intake were measured weekly. All animal experimentation was approved by the University of South Carolina's Institutional Animal Care and Use Committee.

Determination of cachexia symptom severity. The inherent variability in cachexia development between mice is a strength of the $Apc^{Min/+}$ mouse model, and $Apc^{Min/+}$ mice. Cachexia severity was determined to classify the groups for the $Apc^{Min/+}$ mice. Mice were classified as having moderate or severe cachexia based on their body mass loss change from peak body weight (16 weeks) to sacrifice (20 weeks). Mice were categorized as having moderate cachexia if body weight at sacrifice was 5-19% change from peak body weight. Mice were categorized as having severe cachexia if body weight

at sacrifice was equal or greater to 20% change from peak body weight. This categorization is based upon published studies from our lab (5, 8)

Tissue collection. Mice were given a subcutaneous injection of ketamine-xylazine-acepromazine cocktail (1.4 ml/kg body wt). Skeletal muscles, spleens, livers, and tibias were excised. The right tibialis anterior (TA) was placed in optimal cutting temperature (OCT) solution and frozen in isopentane cooled in liquid nitrogen. All issues were rinsed in PBS, snap frozen in liquid nitrogen, and stored at -80°C until further analysis.

Tissue morphological analysis. Cross-sectional area was performed as previously described (20). Briefly, transverse sections (10 um) were cut from OCT mounted distal TA muscle on a cryostat at -20°C. Hematoxylin and eosin (H&E) staining was performed on sections for all muscle samples for cross-sectional area (CSA). Digital photographs were taken from each H&E section at a 40x magnification with a Nikon spot camera, and approx. 125 fibers/animal were traced with imaging software (ImageJ - NIH) in a blinded fashion.

Succinate Dehydrogenase Staining. Succinate dehydrogenase (SDH) staining was performed as previously described to characterize mitochondrial enzyme function/oxidative capacity in the TA muscle (5). Sectioning of muscle was performed the same as previously described. The frozen sections were dried at room temperature for 10 min. Sections were incubated in a solution made up of 0.2 M phosphate buffer (pH 7.4), 0.1 M MgCl2, 0.2 M succinic acid, and 2.4 mM nitroblue tetrazolium at 37°C for 45 min. The sections were then washed in deionized water for 3 min, dehydrated in 50% ethanol for 2 min, and mounted for viewing with mounting media. Digital photographs were

taken from each section at a 10x and 40x magnification with a Nikon spot camera, and fibers were traced with imaging software (ImageJ NIH). The percentage of SDH positive fibers was then determined at 10x. Images were converted post-hoc to grayscale 8 bit and the background of each image was subtracted to then create an integrated optical density (IOD) value and then categorized as positively stained (high) or nonstained (low). The percentage of each stain was quantified and expressed as percent per total muscle fibers. For SDH myofiber area analysis, high and low SDH activity fibers were traced at a 40x magnification in a blinded fashion. Approx 100/fibers per animal were traced for myofiber area analysis.

Intramuscular Glycogen Content. Periodic acid Schiff (PAS) staining was performed to quantify intramuscular glycogen content. The frozen sections were dried at room temperature for 5 minutes. The slides were then incubated in Carnoy's fixative (60% ethanol, 30% chloroform and 10% glacial acetic acid) for 30 minutes at room temperature. The slides were then placed in 0.5% periodic acid solution for 15 minutes and then rinsed in dH_20. The slides were then placed in Schiff reagent for 15 minutes and then washed in luke-warm tap water for 5 minutes for color to fully develop. Slides were counter-stained in hematoxylin for one minute, washed in dH_20, dehydrated and coverslipped. Digital photographs were taken from each section at 10x and 40x magnification with a Nikon spot camera, and fibers were counted and traced with imaging software (ImageJ NIH). The percentage of PAS positive and negative fibers was then determined at 10x. The PAS bright-field images were converted-post hoc into eight-bit grayscale values and the background of each image was subtracted to then create an integrated optical density (IOD) value and then categorized as positively stained (high

glycogen) or nonstained (low glycogen). The percentage of each stain was quantified and expressed as percent per total muscle fibers. Fibers with high glycogen content were quantified as an integrated optical density as 2 standard deviations from empty fibers, light stained fibers with no stain present. Percent counts of high and low glycogen content were then quantified as a percent total of muscle fibers. For PAS myofiber area analysis, high and low glycogen content fibers were traced at a 40x magnification in a blinded fashion. Approx 100/fibers per animal were traced for myofiber area analysis.

Statistical Analysis. Results are reported as the means ± SE. Variables were analyzed with ANOVA to determine differences between groups. Post-hoc analyses were performed with Student-Newman-Keuls methods. Chi-square analysis was utilized for all frequency histograms. Pearson correlation analysis was used for correlations. The accepted level of significance was set at $p < 0.05$.

RESULTS

Body weight and tibialis anterior (TA) muscle mass.

At 20 weeks of age, wild-type and $Apc^{Min/+}$ mice were sacrificed and $Apc^{Min/+}$ mice were assigned to one of three groups dependent on weight loss from peak body weight. These stratifications have been detailed in previous studies from our lab (5, 8, 20) in regards to the $Apc^{Min/+}$ model to represent moderate and severe degrees of cachexia. In the current investigation $Apc^{Min/+}$ WS had no change in body weight however, both $Apc^{Min/+}$ moderate and severe mice had decreased body weight from peak to sacrifice. This decrease represented ~12.3% body weight loss from peak body weight in the $Apc^{Min/+}$ moderate group and ~22.6% body weight loss from peak body weight in

the $Apc^{Min/+}$ severe group ($p<0.05$, Table 3.1). Tibia length was measured as a marker of body size and was not significantly different between groups. TA mass at sacrifice for wild-type was ~53 mg and ~51 mg for $Apc^{Min/+}$ WS. TA mass was reduced 28% in the $Apc^{Min/+}$ moderate group and 47% in the $Apc^{Min/+}$ severe group. ($p<0.05$, Table 3.1)

Tibialis anterior (TA) myofiber area in wild-type and $Apc^{Min/+}$ mice

To determine the effect of cachexia severity on myofiber morphology, H&E staining was utilized to measure mean myofiber cross-sectional area (CSA) (Figure 3.1A). There were no significant differences in CSA between Wt and $Apc^{Min/+}$ WS (Figure 3.1B). There was a 31% decrease in mean CSA of myofibers in $Apc^{Min/+}$ moderate and 60% decrease in the $Apc^{Min/+}$ severe group ($p<0.05$, Figure 3.1B). There were no significant differences in the fiber area frequency distribution between Wt and $Apc^{Min/+}$ WS (Figure 3.1C). When fiber size was compared, cachexia severity progressively increased the percentage of smaller fibers and decreased the percentage of larger fibers. ($p<0.01$, Figure 3.1D)

Frequency and size of high and low SDH activity myofibers in the TA of wild-type and $Apc^{Min/+}$ mice

Previously, our lab has shown that oxidative capacity is reduced with cachexia. SDH staining was performed to examine the effect of cachexia severity on frequency and size of high and low SDH activity myofibers (Figure 3.2A). There were no significant differences in the frequency of fibers between Wt and $Apc^{Min/+}$ WS, indicating cancer alone does not alter frequency of either high of low SDH activity myofibers (Figure 3.2B). There was a 23% reduction in the frequency of high SDH activity fibers in

$Apc^{Min/+}$ moderate and a 45% decrease in the $Apc^{Min/+}$ severe group, however cachexia induced an increase in the frequency of low SDH activity fibers in $Apc^{Min/+}$ moderate and severe, 15% and 24% respectively ($p<0.05$, Figure 3.2B). Mean CSA of high SDH activity fibers and low SDH activity fibers were not significantly different between Wt and $Apc^{Min/+}$ WS (Figure 3.2C, Figure 3.2D). Decreases were observed in both SDH dark and light fibers in $Apc^{Min/+}$ moderate and severe. There was an 18% decrease in CSA of SDH dark fibers in the $Apc^{Min/+}$ moderate group and 39% decrease in the severe group ($p<0.05$, Figure 3.2C). Cachexia also reduced the CSA of SDH light fibers in $Apc^{Min/+}$ moderate and severe groups, 31% and 52% respecitively ($P<0.05$, Figure 3.2 C). This indicates there is myofiber atrophy despite high or low SDH activity ($p<0.05$, Figure 3.2C). When fiber size distribution was compared, cachexia severity progressively increased the percentage of smaller myofibers and reduced the percentage of larger myofibers in both high and low SDH activity ($p<0.05$, Figure 3.2E).

Relationship between frequency and size of high and low SDH activity myofibers with cachexia

To determine the relationship between frequency of SDH activity fibers and the area of these myofibers, correlations were generated using these variables. A positive correlation ($r^2 = 0.66$, $p = .002$) was observed between SDH dark fiber frequency (%) and SDH dark fiber CSA (um^2), indicating a higher frequency of high SDH activity fibers corresponds with greater CSA of high SDH activity fibers (Figure 3.3C). indicating that cachexia induces an increase in frequency of low SDH activity fibers, however these fibers still atrophy with cachexia. Therefore, cachexia will reduce the CSA despite high or low SDH activity of myofibers.

Frequency and size of high and low glycogen content myofibers in the TA of wild-type and Apc$^{Min/+}$ mice

To determine the effect of cachexia severity on the frequency and size of high and low glycogen content myofibers, Periodic Acid Schiff staining (PAS) was utilized. The greater amount of glycogen within the fibers results in a greater intensity of the stain (Figure 3.3A). There were no significant differences in the percentage of high glycogen content or low glycogen content fibers between Wt and Apc$^{Min/+}$ WS, indicating cancer alone has no effect on high and low glycogen content myofibers (Figure 3.3B, 3.3C). Cachexia severity induced a 24% increase in the frequency of high glycogen content fibers in ApcMin/+ moderate and 55% in Apc$^{Min/+}$ severe ($p<0.05$, Figure 3.3B). Cachexia severity also decreased the frequency of low glycogen content fibers, % in ApcMin/+ moderate and % in ApcMin severe. While the frequency of high glycogen content fibers was increased, cachexia decreased the CSA of high glycogen content fibers 35% in Apc$^{Min/+}$ moderate and 54% in the Apc$^{Min/+}$ severe group. Additionally, cachexia reduced the CSA of low glycogen content fibers in ApcMin/+ moderate and severe mice, 20% and 45% respectively ($p<0.05$, Figure 3.3C). When fiber size distribution was compared, cachexia severity progressively increased the percentage of smaller PAS dark and light fibers ($p<0.05$, Figure 3.3E).

Relationship between frequency and size of myofiber metabolic properties with moderate and severe cachexia

To determine the relationship between the frequency of high SDH activity myofibers and CSA of high SDH myofibers a correlation was generated using these variables. A strong

positive correlation ($r^2 = 0.63$, $p = 0.003$) was observed, indicating as frequency of high SDH activity fibers is decreased with cachexia there is reduced CSA of these fibers (Figure 3.4 A). Also, to determine the relationship between the frequency of low SDH myofibers and the CSA of low SDH myofibers a correlation was generated using these varaibles. A strong negative correlation ($r^2 = -0.61$, $p = 0.007$) was observed, indicating as cachexia increases the frequency of low SDH activity myofibers, atrophy of these fibers progresses (Figure 3.4 B). Additionally, a correlation was generated with the frequency of high SDH activity myofiber and frequency of high glycogen content myofibers (Figure 3.4 C). A negative correlation ($r^2 = -0.63$, $p = 0.004$) was observed indicating that with the progression of cachexia there is a reduction of high SDH activity myofibers yet an increase in high glycogen content myofibers.

DISCUSSION

Cancer cachexia is a catabolic condition that leads to death in 1/3 of cancer patients and negatively affects both muscle and adipose tissue (4). While much research has been published in regards to the multiple skeletal muscle alterations with cachexia (5, 6, 8, 16) the morphological and metabolic changes during the progression still need to be further defined for treatment purposes. For our study we utilized the $Apc^{Min/+}$ model with (moderate, severe) and without (weight stable) cachexia to examine the alterations in myofiber area and selected myofiber metabolic properties. A strength of using the $Apc^{Min/+}$ weight stable (WS) with $Apc^{Min/+}$ cachectic model allows the extrapolation of changes with cachexia and cancer alone. To this end, this study demonstrates that there is progressive myofiber atrophy during the progression of cancer cachexia. Severity of cachexia also progressively reduces the frequency of high SDH activity myofibers

however increases the frequency of high glycogen content myofibers. Despite a myofiber having high or low SDH activity or high or low glycogen content, there is wasting in all of these myofibers. These data suggest that myofiber area and selected myofiber metabolic characteristics are related and highly influenced by the severity of cachexia.

Skeletal muscle mass loss and alterations in morphology are associated with reduced mobility and quality of life, reduced function and shorter life span (4, 17). Skeletal muscle wasting is a hallmark characteristic of cancer cachexia (16, 18) and myofiber degeneration has been previously demonstrated in our lab (7). Muscle atrophy associated with cancer cachexia can range from 10% to 50% (19), typically it has been observed that fast-glycolytic fibers are more susceptible to atrophy as compared to slow-oxidative fibers. While degeneration was shown in our lab with use of the ApcMin/+ model, there was no further investigation if cancer alone is enough to induce alterations in skeletal muscle myofiber area or any differences in degrees of cachexia severity. We found that there were no alterations in myofiber are with cancer alone, however there were significant decreases in mean myofiber area of the TA muscle in $Apc^{Min/+}$ moderately cachectic mice and these decreases were further exacerbated in $Apc^{Min/+}$ severely cachectic mice. There was a decrease in mean CSA in the $Apc^{Min/+}$ moderate group, this was decrease was even greater in $Apc^{Min/+}$ severe group which suggest there is no "plateau" in myofiber atrophy and it progresses with cachexia severity. The frequency distributions of CSA suggest a leftward shift indicating of a greater percentage of smaller myofibers in both degrees of cachexia severity, suggesting progressive myofiber atrophy. This indicates a continued loss of myofibrils during the progression of cachexia. While

these shifts were observed, there were no investigation into fiber type alterations with cancer alone and cachexia severity. Future work should examine alterations in fiber type expression and morphology with the progression of cachexia to also investigate if changes also occur.

Oxidative metabolism is a characteristic of skeletal muscle and is highly influenced by fiber phenotype (9). Skeletal muscle that is comprised of primarily oxidative Type I fibers have a higher capacity for oxidative metabolism than skeletal muscle that is primarily glycolytic Type II fibers (20). Studies have investigated how oxidative metabolism of muscle can influence its catabolic susceptibility (21,22). Another line of investigation from our lab has examined mitochondrial function and oxidative capacity in the $Apc^{Min/+}$ mouse. It was shown that in $Apc^{Min/+}$ mice with cachexia there was a reduction in mitochondrial function and oxidative capacity (5). While this work utilized the $Apc^{Min/+}$ severe, our line of investigation utilized the weight stable model and two degrees of body weight loss to elucidate the changes with cancer alone and progression of cachexia. Also, our study was novel in that it examined the alterations of freqnecy and size of high SDH activity and low SDH activity myofibers with different degrees of cachexia severity. We report here that there is no change in frequency or size of both high and low SDH activity myofiber between wild-type and $Apc^{Min/+}$ weight stable indicating no effect of cancer alone. However cachexia induced a 23% decrease in high SDH acvity myofibers in the $Apc^{Min/+}$ moderate group and 45% decrease in the $Apc^{Min/+}$ severe group. SDH activity can be a measurement of oxidative capacity of a myofiber, therefore high SDH activity myofibers are typically considered highly oxidative. This loss in highly oxidative fibers coincides and extends upon

previously published data from our lab that there is a loss in oxidative capacity in cachexia. This loss is not observed in cancer alone and is progressive with cachexia severity. We also observed a decrease in oxidative fiber morphology, as SDH dark fibers were reduced by 18% in the $Apc^{Min/+}$ moderate group and 43% in the $Apc^{Min/+}$ severe group. Both high and low SDH activity myofibers were succeptible cachexia-induce myofiber atrophy. This is interesting as it suggests neither oxidative nor glycolytic potential serves as a protective mechanism of cachectic muscle wasting. While it has been suggested that oxidative potential can serve as a protective effect from the atrophic effects of cachexia (19), our data demonstrates significant reductions in oxidative fiber morphology, particularly in the severely cachectic stage. Further histological work should utilize enzymatic techniques (such as NADH stain) to further delineate the effects of cachexia severity on oxidative potential of skeletal muscle fibers.

While previously mentioned variables have been measured in different aspects in the $Apc^{Min/+}$ model, this is the first to examine intramuscular glycogen content during the progression of cancer cachexia. Metabolic dysregulation affects multiple systemic variables during the progression of cancer cachexia and while glucose metabolism has been observed to be one of these, the storage of glycogen in skeletal muscle has yet to be measured. Our study found there were no alterations in intramuscular glycogen with cancer alone however intramuscular glycogen content was increased in both groups of cachexia severity, and that in the severely cachectic group there was a 103% increase in glycogen content. This is of great interest because it was expected for glycogen content to decrease due to the decreased oxidative capacity and impaired skeletal muscle function with progression of cachexia. Possible explanations could be related to AMPK signaling

and accumulation of glucose-6-phosphate (G6P) which provides allosteric regulation that overrides the effect of AMPK (23). Acutely, AMPK down regulates the storage of glycogen however when chronically elevated, glycogen content has been observed to increase (23,24). The proposed mechanism of this observation is that while AMPK is inhibiting glycogen synthase and glycogen storage, the increase of glucose from GLUT-4 results in an over-accumulation of G6P which allosterically regulates glycogen synthase, overriding the effects of AMPK and results in increased skeletal muscle glycogen (23). Previous literature from our lab has indicated that AMPK is chronically elevated in the $Apc^{Min/+}$ model (8) however no work has looked at downstream targets such as G6P and glycogen synthase, so this could be a possible mechanism to explain the increase in high glycogen myofibers with the progression of cachexia.

Several strengths and limitations to the current study exist. This is the first study to examine the effects of cancer cachexia severity on SDH activity myofibers and glycogen content myofibers. While other studies in our lab have examined myofiber degeneration and oxidative capacity, this is the first to examine these particular variables with the use of $Apc^{Min/+}$ WS and in differing degrees of cachexia and utilize the TA muscle. The $Apc^{Min/+}$ WS is a strength as it allows the examination of the effects of cancer alone as opposed to cachexia since there is no associated weight loss. Another strength to the study is the use of the $Apc^{Min/+}$ model. The development and progression of cachexia is slower in the $Apc^{Min/+}$ model and is similar to what is seen in humans (25). Both the technique for measuring SDH activity of myofibers and glycogen content myofibers have been validated in other studies so our measurement of our particular variables is appropriate. Both SDH staining and PAS staining are semi-quantitative

measurements so care must be taken in the interpretation of results. Staining technique and quantification of digital images were repeated and validated, however error can still occur. Further work should examine direct measurements of oxidative capacity (Seahorse Biosciences) and intramuscular glycogen content (total glycogen content assay). Previous studies have employed these methods for direct measurements of these variables however this has not been done in conjunction with each other in the $Apc^{Min/+}$ model.

In summary, our data demonstrates cachectic skeletal muscle undergoes multiple changes in myofiber area and myofiber metabolic properties. Specifically, oxidative high SDH activity myofibers are progressively decreased with the severity of cancer cachexia while there is an increase in high glycogen content myofibers. Also it was found despite SDH activity there was a decrease in myofiber area in both high and low SDH activity myofibers. Similar to previous reports, the decrease in overall myofiber area and high SDH activity myofibers was expected results however the increase in high glycogen content myofibers quite interesting and intriguing. Taken collectively, the data suggest that there are multiple morphological and metabolic disruptions in skeletal muscle with the progression of cancer cachexia that can ultimately lead to reduced function. Future research should continue to examine the changes in intramuscular glycogen content with the utilization of total content assays and possible pathways that are responsible for these metabolic changes. Understanding the changes observed in this study and further examining the molecular signaling pathways associated with the changes during the progression could provide better understanding of the alterations in skeletal muscle with cancer cachexia to move towards better treatment.

Table 3.1. *Body weight change, tibia length, muscle and epididymal fat pad weights in 20-week-old male wild-type and ApcMin/+ mice.*

		Body Weight					
Group	**n**	**Peak (g)**	**Sac (g)**	**Change (%)**	**Tibia (mm)**	**TA (mg)**	**Epi Fat (mg)**
WT	5	26.6 ± 0.6	26.5 ± 0.6	0.1 ± 0.3	17.5 ± 0.3	53 ± 6	532 ± 31
ApcMin^{+}							
WS	5	25.6 ± 0.4	25.5 ± 0.3	0.0 ± 0.0	17.4 ± 0.1	51 ± 3	430 ± 25
Mod	6	24.3 ± 0.4*	21.8 ± 0.9*†	-10.3 ±1.8*	17.3 ± 0.2	38 ± 4*	59 ± 12
Severe	5	23.5 ± 0.5*†	18.5 ± 0.4$^{\#}$	-20.6 ± 1.8$^{\#}$	17.2 ± 0.1	28 ± 23$^{\#}$	0 ± 0$^{\#}$

At 20 weeks of age, wild-type and Apc$^{Min/+}$ were categorized based upon weight loss from peak to sacrifice. Moderate, Apc$^{Min/+}$ having 5-19% body weight loss from peak to sacrifice; Severe, Apc$^{Min/+}$ having $\geq$ 20% body weight loss from peak to sacrifice. All muscles, organs and fat were excised at time of sacrifice. Sac, sacrifice; g, gram; mg, milligram; TA, tibialis anterior muscle. Values are means ± SE. Sac, sacrifice; g, gram; mg, milligram; TA, tibialis anterior muscle. * Different from wild-type group, † different from weight stable group, # different from all groups. Significance was set at $P < 0.05$.

Table 3.2. *Summary table of alterations in specific myofiber characteristics in fiber area (um^2) and frequency (%) of weight stable and cachectic Apc$^{Min/+}$ mice.*

	Frequency (%)		CSA (um2)		CSA Frequency	
Myofiber Characteristic	***Cancer***	***Cachexia***	***Cancer***	***Cachexia***	***Cancer***	***Cachexia***
High SDH Activity	⟷	↓	⟷	↓	⟷	←
Low SDH Activity	⟷	↓	⟷	↑	⟷	←
High Glycogen Content	⟷	↓	⟷	↑	⟷	←
Low Glycogen Content	⟷	↓	⟷	↓	⟷	←

Cancer is defined as Apc$^{Min/+}$ weight stable with no body weight change, cachexia are Apc$^{Min/+}$ moderate and severe with body weight loss. Arrows indicate significance in changes and direction of changes with frequency and CSA. Arrows in CSA frequency indicate shift. No change ⟷ Increase ↑ decrease ↓ leftward shift to smaller myofibers ←

Figure Legends

Figure 3.1. Tibialis Anterior (TA) myofiber area in wild-type and Apc$^{Min/+}$ mice. ***A:*** *Top left*, representative image of wild-type at 40x; *top right*, Apc$^{Min/+}$ weight stable (WS); *bottom left*, Apc$^{Min/+}$ moderate; *bottom right*, Apc$^{Min/+}$ severe. ***B:*** Mean CSA (um^2) of TA in wild-type and Apc$^{Min/+}$. ***C-D:*** Mean CSA distributions of wild-type, Apc$^{Min/+}$ WS, Apc$^{Min/+}$ moderate and Apc$^{Min/+}$ severe. Data were analyzed using ANOVA with Student-Newman-Keuls post-hoc and Chi-Square where appropriate. * significantly different from wild-type group, † significantly different from weight stable group, # significantly different from all groups. Significance was set at $P < 0.05$

Figure 3.2. Frequency and size of high and low SDH activity myofibers in the TA of wild-type and Apc$^{Min/+}$ mice mice. SDH staining was utilized to quantify frequency and size of high and low SDH activity myofibers in the tibialis anterior (TA) muscle during the progression of cachexia ***A:*** *Top left*, representative image of wild-type at 10x; *top right*, Apc$^{Min/+}$ weight stable (WS); *bottom left*, Apc$^{Min/+}$ moderate; *bottom right*, Apc$^{Min/+}$ severe. Arrows indicate high SDH activity fibers *B:* High and Low SDH activity fiber frequency (%) in wild-type and Apc$^{Min/+}$. ***C:*** Mean cross-sectional area (CSA) of high SDH activity fibers and low SDH activity fibers (um^2) in the TA of wild-type and Apc$^{Min/+}$. ***D.*** Frequency histogram of CSA (um^2) in high SDH activity fibers of Wt and Apc$^{Min/+}$ WS. ***E.*** Frequency histogram of CSA (um^2) in high SDH activity fibers of Apc$^{Min/+}$ WS, Moderate, and Severe. ***F.*** Frequency histogram of CSA (um^2) in low SDH activity fibers of Wt and Apc$^{Min/+}$ WS. ***G.*** Frequency histogram of CSA (um^2) in low SDH activity fibers of Apc$^{Min/+}$ WS, Apc$^{Min/+}$ Moderate and Apc$^{Min/+}$ Severe. Data were analyzed using ANOVA with Student-Newman-Keuls post-hoc analysis and Chi-

Square where appropriate. * significantly different from wild-type group, † significantly different from weight stable group, # significantly different from all groups. Significance was set at $p < 0.05$

Figure 3.3. Frequency and size of high and low glycogen content myofibers in the TA of wild-type and Apc$^{Min/+}$ mice. PAS staining was utilized to quantify frequency and size of high and low glycogen content myofibers in the tibialis anterior (TA) muscle during the progression of cachexia ***A*****:** *Top left*, representative image of wild-type at 10x; *top right*, Apc$^{Min/+}$ weight stable (WS); *bottom left*, Apc$^{Min/+}$ moderate; *bottom right*, Apc$^{Min/+}$ severe. ***B:*** High and low glycogen content fiber frequency (%) in wild-type and Apc$^{Min/+}$. ***C:*** Mean cross-sectional area (CSA) of high and low glycogen content myofibers (um^2) in the TA in wild-type and Apc$^{Min/+}$. ***D.*** Frequency histogram of CSA (um2) in high glycogen content fibers of Wt and Apc$^{Min/+}$ WS. ***E.*** Frequency histogram of CSA (um2) in high glycogen content fibers of Apc$^{Min/+}$ WS, Moderate, Severe. ***F***. Frequency histogram of CSA (um2) in low glycogen content fibers of Wt and Apc$^{Min/+}$ WS. ***G***. Frequency histogram of CSA (um2) in low glycogen content fibers of Apc$^{Min/+}$ WS, Moderate and Severe. Arrows indicate high glycogen fibers. Data were analyzed using ANOVA with Student-Newman-Keuls post-hoc analysis and Chi-Square where appropriate . * significantly different from wild-type group, † significantly different from weight stable group, # significantly different from all groups. Significance was set at $P < 0.05$

Figure 3.4. Relationship between frequency and size of selected myofiber metabolic characteristics in mice with moderate and severe cachexia. A: Correlation of High SDH activity fiber frequency (%) and high SDH activity fiber CSA (um^2). B:

Correlation of low SDH activity fiber frequency (%) and low SDH activity activity fiber CSA (um^2). ***C:*** Correlation of high SDH activity fiber frequency (%) and high glycogen content fiber frequency (%). Data were analyzed using Pearson correlation analysis

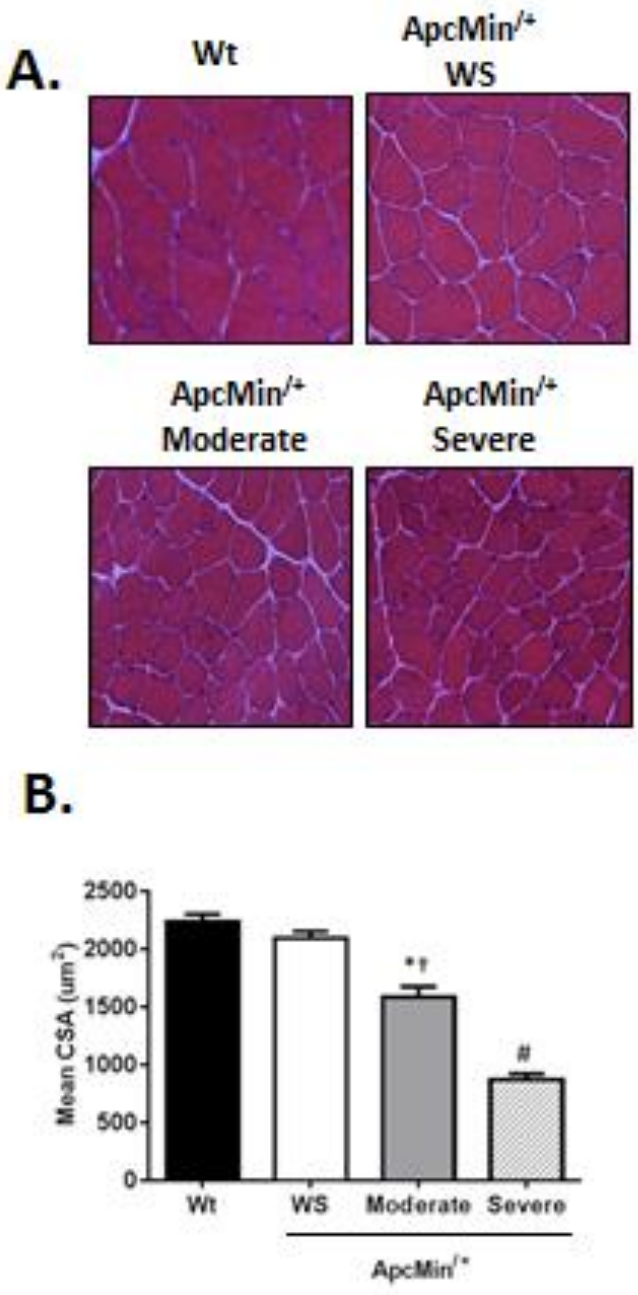


Figure 3.1 Tibialis Anterior (TA) myofiber area in wild-type and $Apc^{Min/+}$ mice

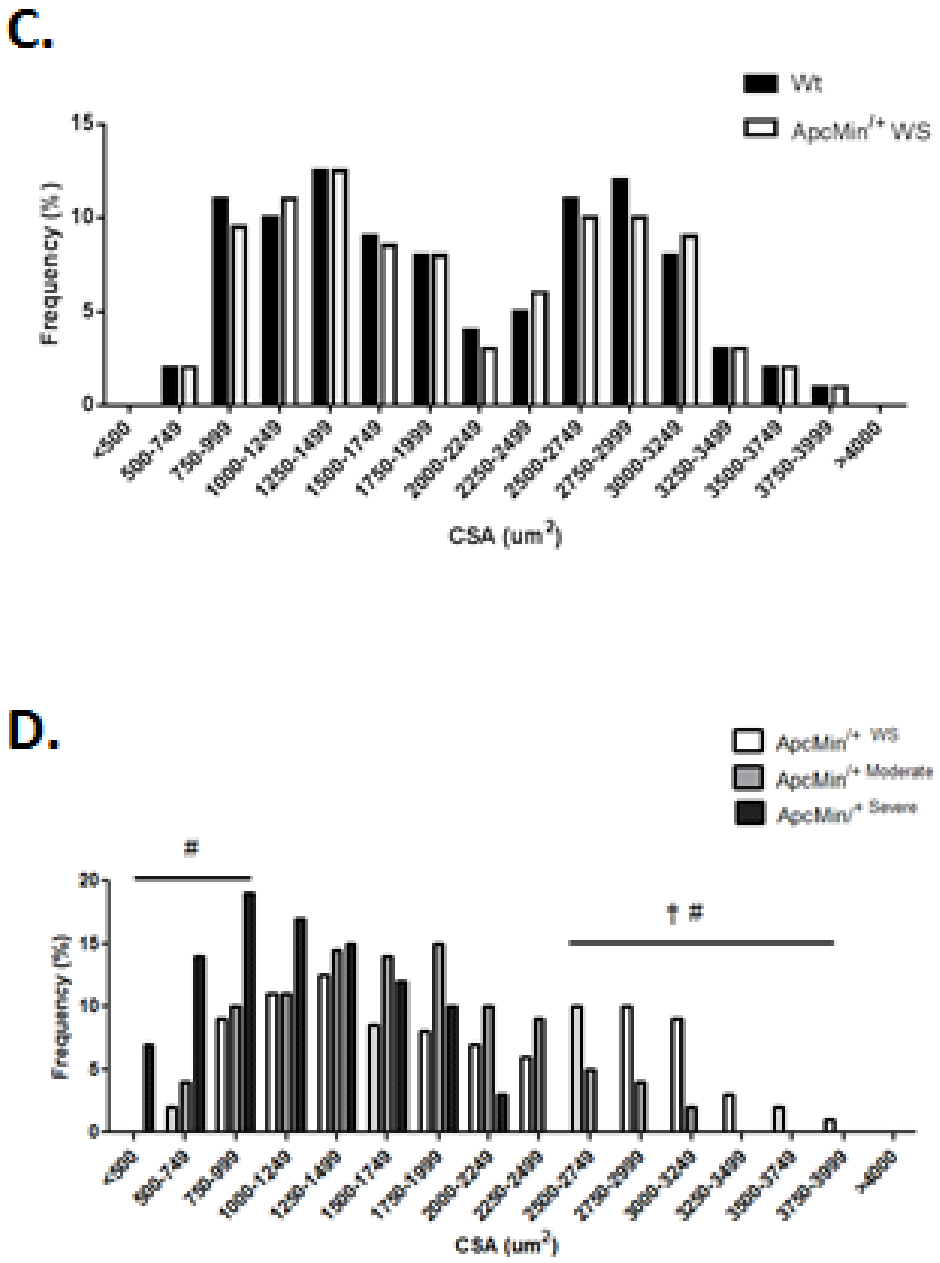


Figure 3.1 Tibialis Anterior (TA) myofiber area in wild-type and $Apc^{Min/+}$ mice

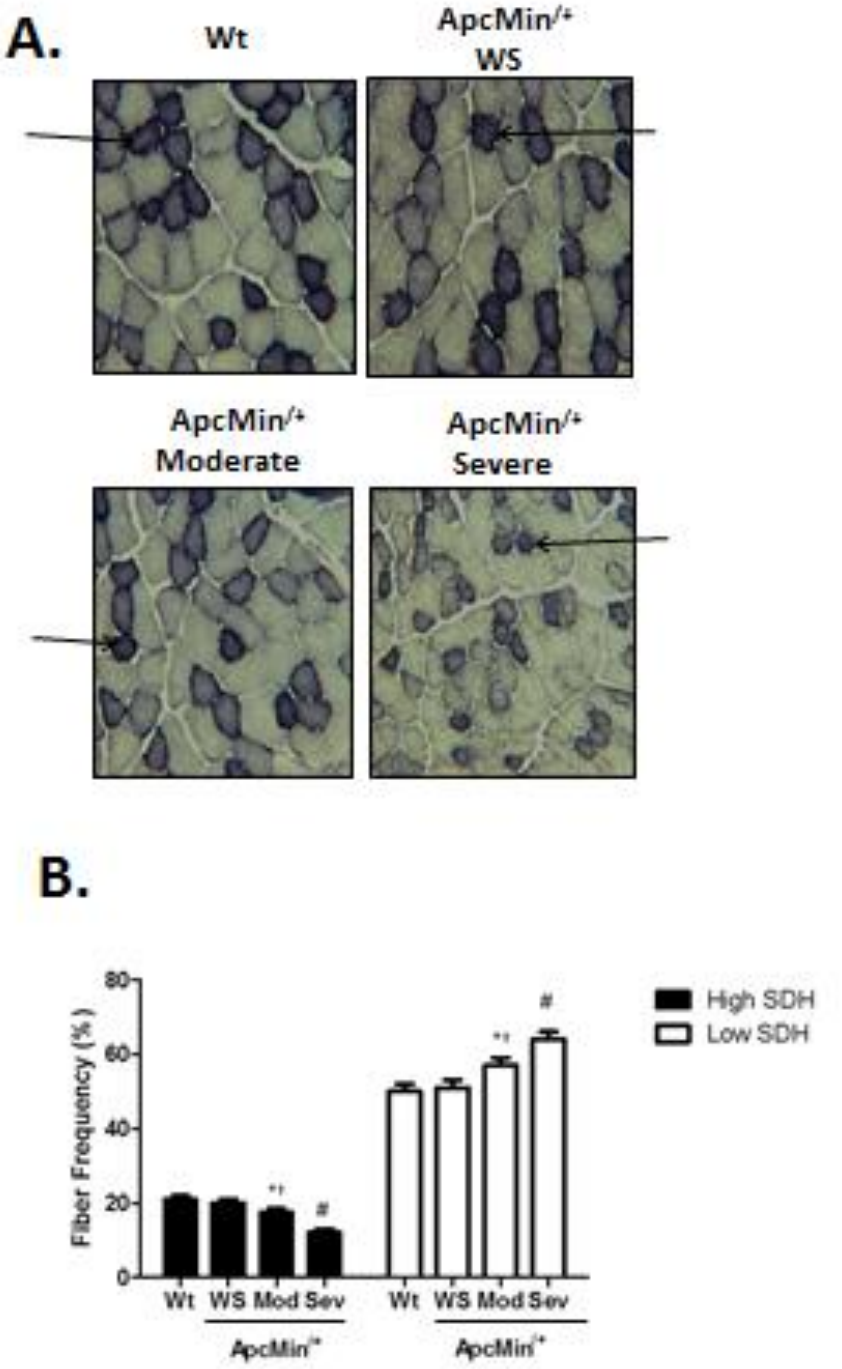


Figure 3.2 Frequency and size of high and low SDH activity myofibers in the TA of wild-type and $Apc^{Min/+}$ mice

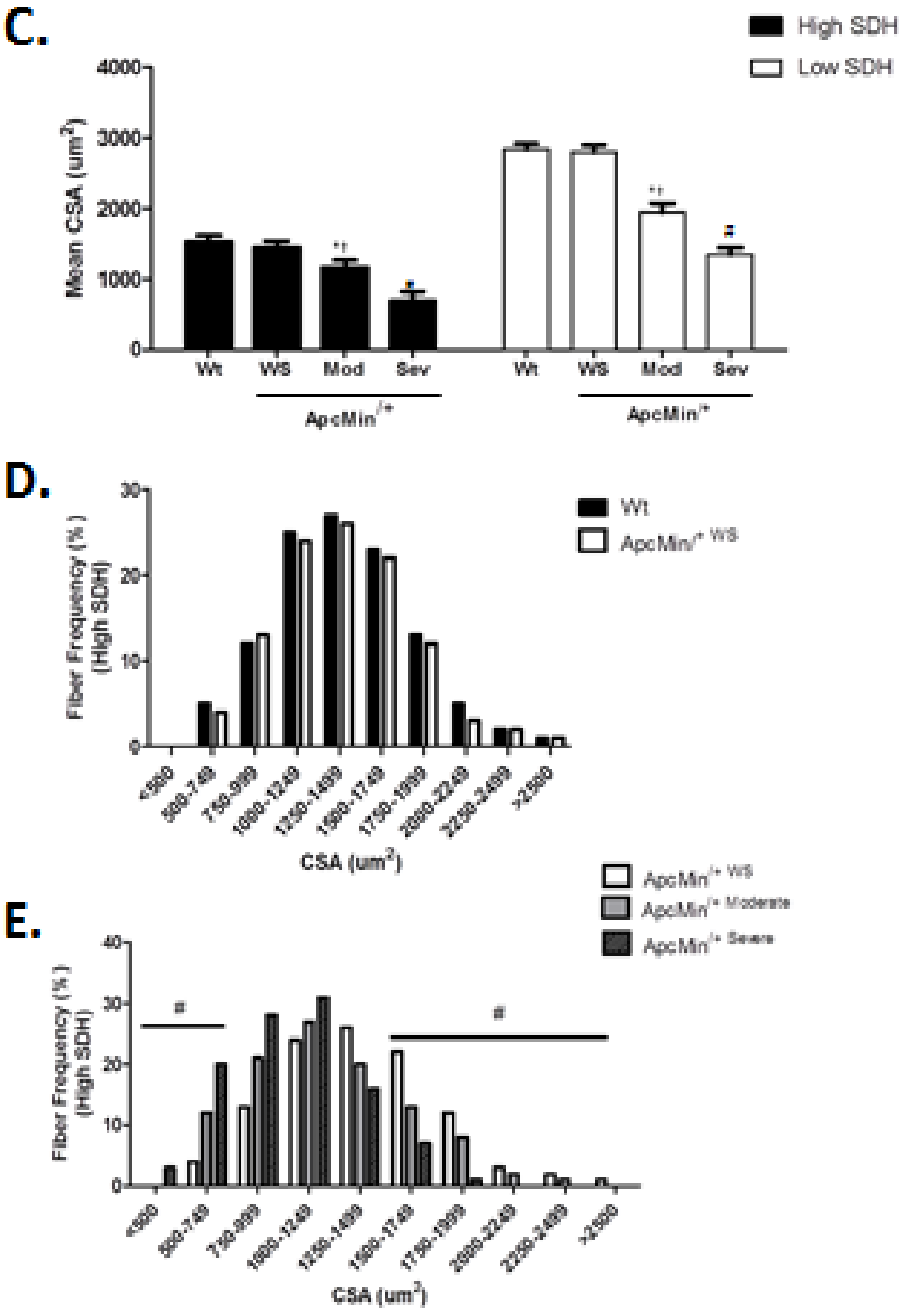


Figure 3.2 Frequency and size of high and low SDH activity myofibers in the TA of wild-type and $Apc^{Min/+}$ mice

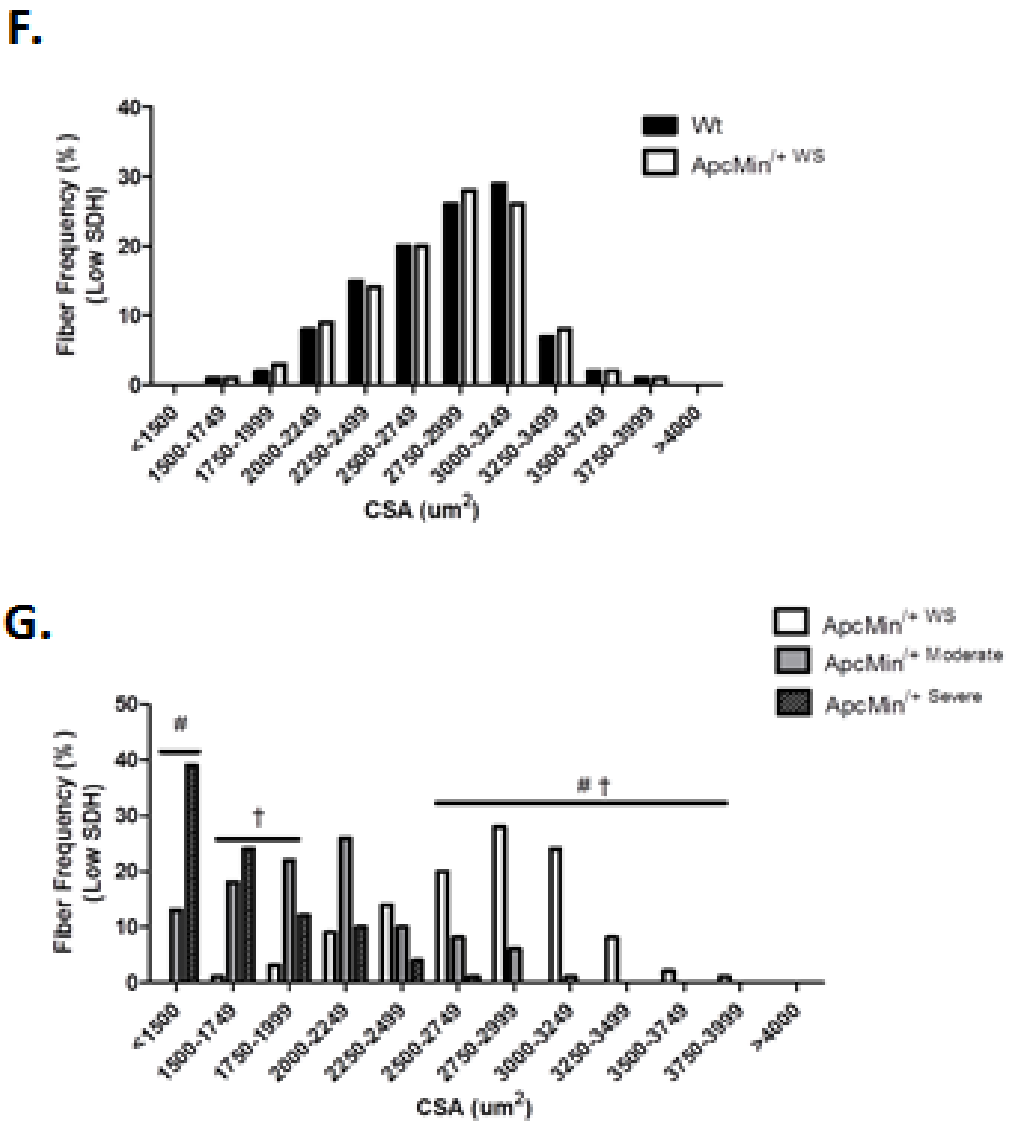


Figure 3.2 Frequency and size of high and low SDH activity myofibers in the TA of wild-type and $Apc^{Min/+}$ mice

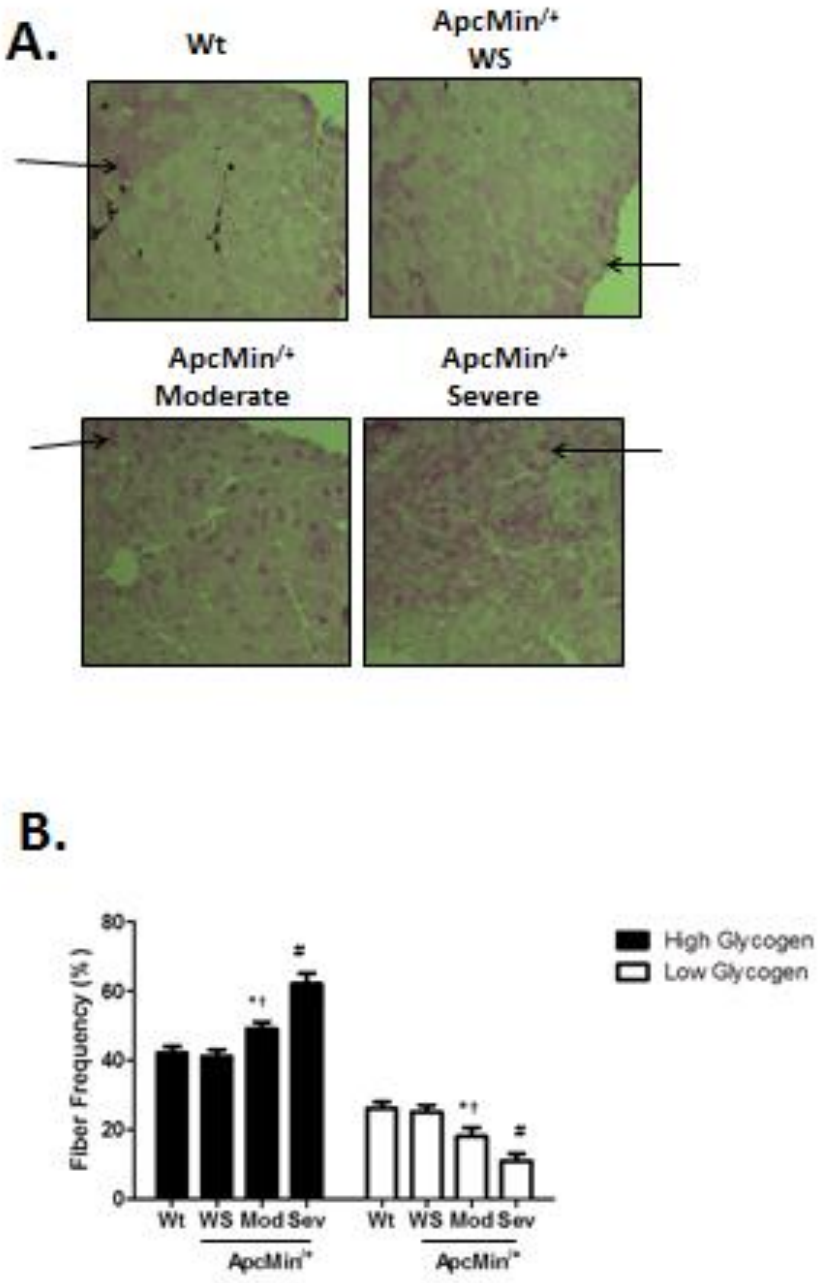


Figure 3.4 Frequency and size of high and low glycogen content myofibers in the TA ofwild-type and $Apc^{Min/+}$ mice

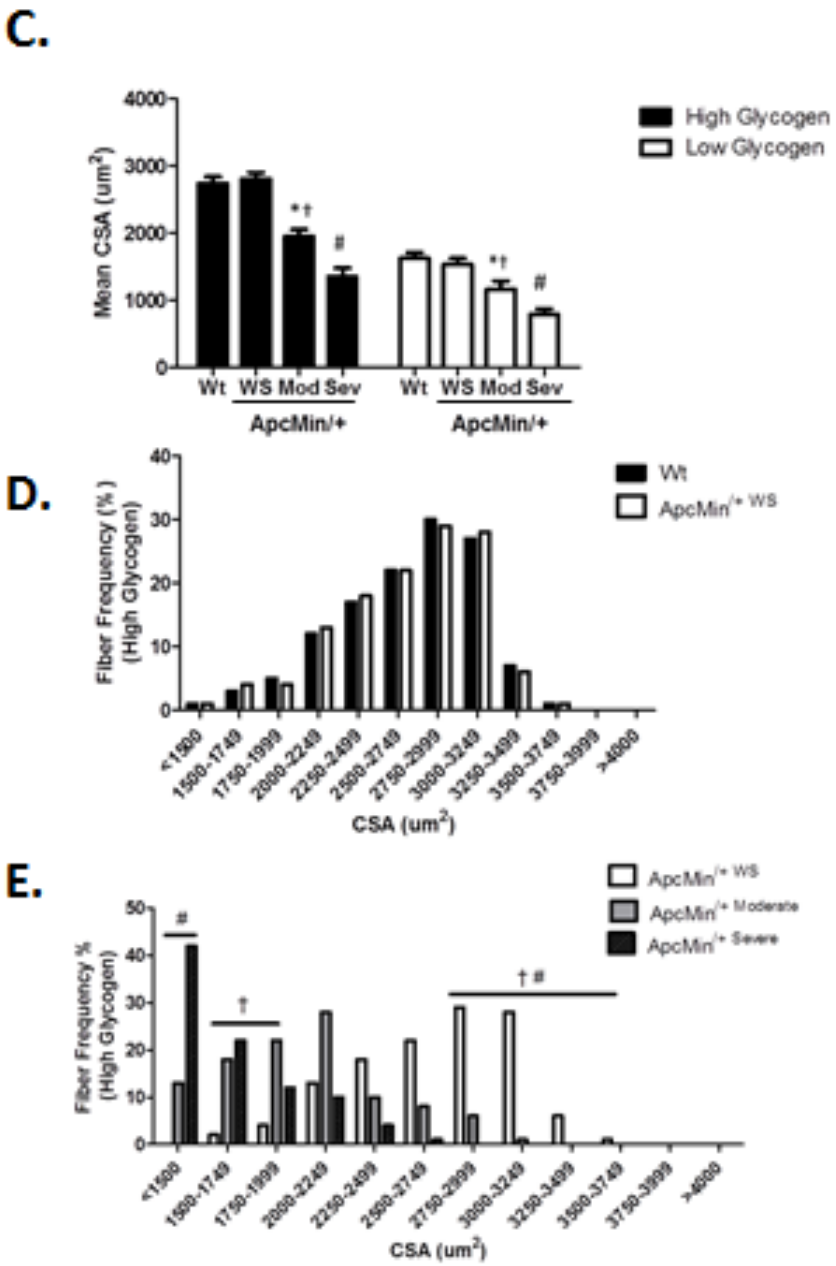
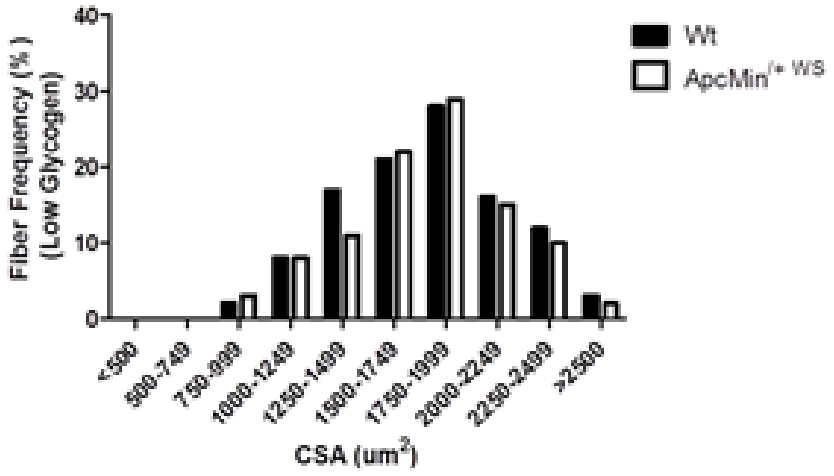


Figure 3.4 Frequency and size of high and low glycogen content myofibers in the TA of wild-type and $Apc^{Min/+}$ mice

F.



G.

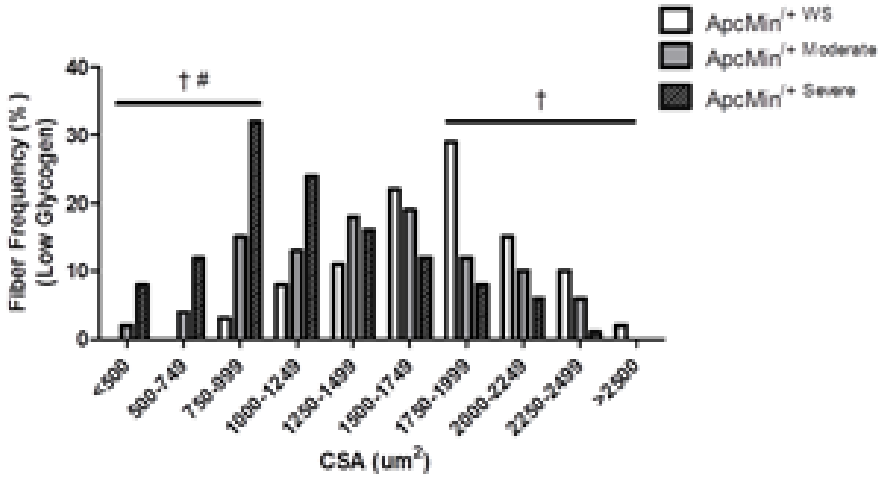


Figure 3.4 Frequency and size of high and low glycogen content myofibers in the TA of wild-type and $Apc^{Min/+}$ mice

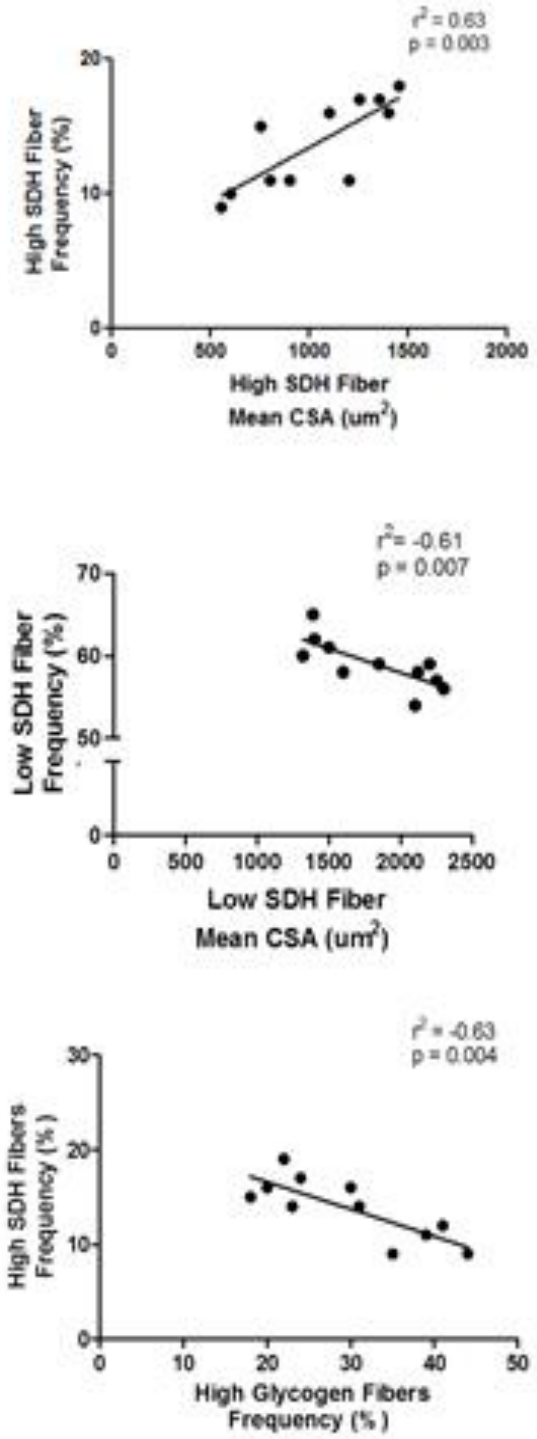


Figure 3.4 Relationship between frequency and size of myofiber metabolic properties with moderate and severe cachexia

CHAPTER 4

THE EFFECT OF ECCENTRIC CONTRACTIONS ON SELECTED MYOFIBER METABOLIC PROPERTIES IN APC$^{MIN/+}$ MICE

ABSTRACT

Cancer cachexia is a complex wasting syndrome which induces a loss in skeletal muscle mass and dysregulation of skeletal muscle metabolism. There are currently no approved treatment modalities for cachexia. The purpose of this study was to determine the effect of resistance exercise training on frequency and size of high and low SDH activity fibers and high and low glycogen content fibers during the initiation of cancer cachexia.. 16-week male C57BL/6 and Apc$^{Min/+}$ (Min) mice performed resistance exercise (RE) by stimulating the sciatic nerve in the left leg (Ex) for 7 sessions over 2 weeks, the right leg served as a control (Con). Histological analysis (H&E, SDH, and PAS) was utilized to examine myofiber area, myofiber SDH activity and glycogen content of myofibers in the tibialis anterior (TA) muscle. Cachexia reduced TA mass and myofiber CSA, RE was able to increase both TA mass and myofiber CSA (Ex>Con). RE induced a 26% ($p<0.05$) increase in the frequency of high SDH activity myofibers, which were reduced with cachexia. Cachexia reduced CSA of both high and low SDH activity myofibers, however RE selectively induced hypertrophy in low SDH activity myofibers. In regards to myofiber glycogen, cachexia induced a 38% ($p<0.01$) increase in high glycogen content fibers. RE did not increase the frequency of high glycogen content fibers,

however induced hypertrophy of high glycogen content fibers. In summary, cachexia-induced myofiber atrophy is independent of myofiber SDH activity. Additionally, RE during cancer cachexia can increase high SDH activity myofiber frequency and reverse low SDH activity myofiber wasting.

INTRODUCTION

Cachexia is a complex wasting syndrome that is associated with such symptoms inflammation and metabolic abnormalities, accompanied by a loss of lean body mass and fat mass (1,2). Approximately 50% of cancer patients experience progressive wasting of adipose tissue and/or skeletal muscle mass (3), and cachexia accounts for about 30% of cancer deaths (4). Prostate, lung and colon/rectum are the three most prevalent cancer types among men and breast, lung and colon/rectum are the three most prevalent cancer types for women (1). Multiple models have been utilized to examine the effects of cancer cachexia, our lab utilizes the $Apc^{Min/+}$ mouse which is an established model of colorectal cancer and cachexia (5, 8, 20). This model closely relates to the colorectal cancer observed in humans, with similar levels of IL-6 and polyp burden, so this model is advantageous in examining the alterations during the progression of cancer cachexia. The mechanisms underlying the wasting of skeletal muscle are still being examined, and there are no currently approved treatments modalities for cachexia. Resistance exercise has been shown to be beneficial to muscle mass and total protein content in colon-26 adenocarcinoma mice (147), however alterations in morphology and metabolic properties in skeletal muscle with resistance exercise during cachexia have yet to be examined.

While multiple alternative interventions have been utilized to reduce the wasting in cachectic patients, most are without any positive results. Appetite stimulants and

nutritional interventions (24) have been utilized, however there was no attenuation in the muscle weight loss in these patients. Nutritional interventions that provide excess calories have shown to increase body weight, however this was due to water retention rather than an increase in skeletal muscle mass (148). As well, the use of an appetite stimulant (megestrol acetate) has been utilized with observed increases in body weight, however similar to the previous study this was due to an increase in water retention and increase in fatty tissue (24). Therefore, the use of resistance exercise may be more beneficial in targeting skeletal muscle to the attenuate the loss in body weight observed with cachexia.

Resistance exercise training has been shown to increase muscle mass in both human and animal models (132, 133). Al-Majid and McCarthy reported increases in both EDL muscle weight and total protein content with resistance exercise in C-26 mice (147). While this study suggested the benefit of resistance exercise on skeletal muscle with cachexia, there was no measurement of dry weight of muscle or morphology examined, so it is not clear if any morphological changes in the EDL or if edema was responsible for the increase observed. Resistance training has been shown to coincide with adaptations in metabolic properties, such as an increase in intramuscular glycogen and oxidative capacity. Oxidative capacity is defined as the maximal capacity of a tissue to utilize oxygen for energy production, which is directly related to the amount of mitochondria in the skeletal muscle fiber (8). Succinate dehydrogenase (SDH) staining is a semi-quantitative measurement of the oxidative capacity of a measured tissue as SDH is an enzyme located in the mitochondrial membrane. In histological uses, a particular tissue such as skeletal muscle can be stained and the darker the SDH stain of the fibers,

generally the more oxidative the fibers are thought the be. Fibers that do not stain for the SDH are typically regarded as more glycolytic. The utilization of a resistance exercise intervention has been observed to increase oxidative capacity in patients with chronic kidney disease (149) however no published studies have examined the changes in the $Apc^{Min/+}$ model. While oxidative metabolism is a critical component in skeletal muscle, energy substrate utilization also serves a critical purpose in proper function and less is known about glycogen in cachectic skeletal muscle. Glucose is stored in skeletal muscle via glycogen synthase (GYS1 for skeletal muscle) as glycogen and is utilized via glycogen phosphorylase (16) Intramuscular glycogen can be utilized as a substrate during exercise (140) and increases in resting intramuscular glycogen levels have been noted in those who have undergone resistance exercise programs (150). However no studies have examined how glycogen or glucose metabolism is altered with resistance exercise in cachectic skeletal muscle.

Resistance exercise has been shown to attenuate wasting in skeletal muscle in multiple diseases and alter metabolic properties, however the effects of resistance exercise on cachectic skeletal muscle myofiber area and myofiber metabolic properties has not been investigated in the $Apc^{Min/+}$ model. Therefore, the purpose of this study was to examine whether resistance exercise would induce hypertrophy in cachectic skeletal muscle and affect the frequency of high and low SDH activity fibers and high and low glycogen content fibers.The hypothesis is that resistance exercise training will attenuate cachexia-induced muscle wasting and the loss of high SDH activity myofibers and induce hypertrophy in low SDH activity myofibers and high glycogen content myofibers in the $Apc^{Min/+}$ mouse.

MATERIALS AND METHODS

Animals. All WT and $Apc^{Min/+}$ mice used in this study were on a C57BL/6 background and were originally purchased from Jackson Laboratories (Bar Harbor, ME). All mice were bred at the University of South Carolina's Colon Cancer Research Center Mouse Core breeding facility that is housed in the University's Animal Resource facility, as previously described. For all mice in the study, the room was maintained on a 12:12-h light-dark cycle with the light period starting at 0700. Mice were provided standard rodent chow (cat. No. 8604 Rodent Diet; Harlan Teklad, Madison, WI) and water ad libitum. Body weights and food intake were measured weekly. All animal experimentation was approved by the University of South Carolina's Institutional Animal Care and Use Committee.

Resistance Exercise Protocol. To determine the effects of resistance exercise on cachectic skeletal muscle myofiber area and myofiber metabolic properties, a modified hindlimb electrical stimulation protocol was used (150). Two 28 gauge needle electrodes were placed in the left leg posterior to the knee and femur to allow stimulation of the sciatic nerve. Sham treatment was also performed in the right leg with no stimulation. Tetanic muscle contractions were generated using a Grass Stimulator (Grass Instruments, Quincy, MA) at a frequency of 100 Hz, 6-12V, 1ms duration, 9 ms delay for 10 sets of 6 repetitions, with each repetition lasting 3 s. Ten seconds recovery was given between repetitions and 50 seconds rest was allowed between sets. This protocol has been shown to recruit all motor units (fast and slow) and results in net plantar flexion of the ankle (151). Therefore, the plantar flexors (gastrocnemius, soleus, and plantaris) undergo

concentric contractions and the dorsiflexors [tibialis anterior (TA) and extensor digitorum longus (EDL)] undergo eccentric contractions. Electrical stimulated resistance exercise was initiated in week 16 and for a total of 7 sessions, with each session separated by 48 h for 2 weeks. Mice were sacrificed 48 hours after the last training session, in week 18 of the study.

Tissue collection. Mice were given a subcutaneous injection of ketamine-xylazine-acepromazine cocktail (1.4 ml/kg body wt). Skeletal muscles, spleens, livers, and tibias were excised. Both the right and left tibialis anterior were placed in optimal cutting temperature (OCT) solution and frozen in isopentane cooled in liquid nitrogen. All issues were rinsed in PBS, snap frozen in liquid nitrogen, and stored at -80°C until further analysis.

Tissue morphological analysis. Cross-sectional area (CSA) was performed as previously described (14). Briefly, transverse sections (10 um) were cut from OCT mounted distal TA muscle on a cryostat at -20°C. Hematoxylin and eosin (H&E) staining was performed on sections for all muscle samples for cross-sectional area. Digital photographs were taken from each H&E section at a 40x magnification with a Nikon spot camera, and approx. 125 fibers/animal were traced with imaging software (ImageJ - NIH) in a blinded fashion.

Succinate Dehydrogenase Staining. Succinate dehydrogenase (SDH) staining was performed as previously described to characterize mitochondrial enzyme function/oxidative capacity in the TA muscle (5). Sectioning of muscle was performed the same as previously described. The frozen sections were dried at room temperature for 10

min. Sections were incubated in a solution made up of 0.2 M phosphate buffer (pH 7.4), 0.1 M MgCl2, 0.2 M succinic acid, and 2.4 mM nitroblue tetrazolium at 37°C for 45 min. The sections were then washed in deionized water for 3 min, dehydrated in 50% ethanol for 2 min, and mounted for viewing with mounting media. Digital photographs were taken from each section at a 10x and 40x magnification with a Nikon spot camera, and fibers were traced with imaging software (ImageJ NIH). The percentage of SDH positive fibers was then determined at 10x. The background of each image was subtracted to then create an integrated optical density (IOD) value and then categorized as positively stained (high SDH activity) or nonstained (low SDH activity). The percentage of each stain was quantified and expressed as percent per total muscle fibers. For SDH myofiber area analysis, high and low SDH activity fibers were traced at a 40x magnification in a blinded fashion. Approx 100/fibers per animal were traced for myofiber area analysis.

Intramuscular Glycogen Content. Periodic acid Schiff (PAS) staining was performed to quantify intramuscular glycogen content. Periodic acid oxidizes the vicinal diols in sugars creating pairs of aldehydes at ends of a monosaccharide ring. The aldehydes then react with the Schiff reagent to give the purple-magenta color observed. The frozen sections were dried at room temperature for 5 minutes. The slides were then incubated in Carnoy's fixative (60% ethanol, 30% chloroform and 10% glacial acetic acid) for 30 minutes at room temperature. The slides were then placed in 0.5% periodic acid solution for 15 minutes and then rinsed in dH20. The slides were then placed in Schiff reagent for 15 minutes and then washed in luke-warm tap water for 5 minutes for color to fully develop. Slides were counter-stained in hematoxylin for one minute, washed in dH_2O, dehydrated and coverslipped. Digital photographs were taken from each section at 10x

and 40x magnification with a Nikon spot camera, and fibers were traced with imaging software (ImageJ NIH). The percentage of PAS positive and negative fibers was then determined at 10x. The PAS bright-field images were converted-post hoc into eight-bit grayscale values to quantify glycogen. Fibers with high glycogen content were quantified as an integrated optical density as 2 standard deviations from empty fibers, light stained fibers with no stain present. Percent counts of high and low glycogen content fibers were then quantified as a percent total of muscle fibers. For PAS myofiber area analysis, high and low glycogen content fibers were traced at a 40x magnification in a blinded fashion. Approx 100/fibers per animal were traced for myofiber area analysis.

Statistical Analysis. Results are reported as the means ± SE. Variables were analyzed with two ANOVA with repeated measures to determine differences and effect between genotype and exercise. Chi-square analysis was utilized for all frequency histograms. The accepted level of significance was set at $p < 0.05$.

RESULTS

Body weight change and tibialis anterior (TA) muscle mass

At 16 weeks of age, wild-type and $Apc^{Min/+}$ mice began a resistance exercise training protocol (detailed in Figure 4.1). The left leg was stimulated via percutaneous electrical stimulation for resistance exercise training and the right leg served as an inter-animal control. Overall $Apc^{Min/+}$ mice had ~4.4% BW loss from peak body weight to body weight at sacrifice ($p<0.05$, Table 4.1). Tibia length was not significantly different between groups. TA mass was significantly different in both exercise and control leg in $Apc^{Min/+}$ mice as compared to WT ($p<0.05$, Table 4.1). There was an increase in TA mass

in the exercise TA versus control in both groups (5.3% WT and 3.6% in $Apc^{Min/+}$), however these were not significant (Figure 4.2).

Tibialis Anterior (TA) myofiber area in wild-type and $Apc^{Min/+}$ mice with resistance exercise training

To determine the effect of the intervention of a resistance exercise training protocol on myofiber morphology with cachexia, H&E staining was utilized to measure mean myofiber cross-sectional area (CSA) (Figure 4.2 A). Mean CSA of the control $Apc^{Min/+}$ TA was decreased 24.2% ($p<0.05$) as compared to control WT (Figure 4.2 B). Mean CSA increased 10.4% in WT exercise as compared to control and 28.9% ($p<0.05$) in $Apc^{Min/+}$ exercise as compared to control (Figure 4.2 B). There was a rightward shift with exercise in both WT and $Apc^{Min/+}$ indicating an increase in larger myofibers (Figure 4.1 C,D).

Frequency and size of high and low SDH activity myofibers in wild-type and $Apc^{Min/+}$ mice with resistance exercise training

Previously, our lab has shown that oxidative capacity is reduced in the $Apc^{Min/+}$ and resistance exercise has been shown to improve oxidative capacity. To this end SDH staining was performed to examine the effect of cachexia and resistance exercise training on oxidative capacity and oxidative fiber morphology (Figure 4.3 A). There was a 28% reduction in the frequency of high SDH activity fibers in $Apc^{Min/+}$ control compared to WT control ($p<0.05$, Figure 4.2 B). There was a 13.2% increase in the frequency of high SDH activity fibers in the WT exercise compared to control and 29.2% ($p<0.05$) increase in $Apc^{Min/+}$ (Figure 4.3 B). Both exercise and control CSA of high SDH activity fibers

were significantly lower in $Apc^{Min/+}$ mice ($p<0.05$, Figure 4.3 C). There was a 5.1% increase in CSA of high SDH activity fibers in the WT exercise as compared to control and a 5.4% increase in $Apc^{Min/+}$ exercise as compared to control (Figure 4.3 C). Only CSA of control low SDH activity fibers were significantly lower in $Apc^{Min/+}$ mice ($p<0.05$, Figure 4.3 C). There was an 10.3% increase in CSA of low SDH activity fibers in the WT exercise as compared to control and a 22.3% increase ($p <0.05$) in $Apc^{Min/+}$ exercise as compared to $Apc^{Min/+}$ control (Figure 4.3 D).

Frequency and size of high and low glycogen myofibers in the Tibialis anterior of WT and ApcMin/+ mice with resistance exercise trainng

Periodic Acid Schiff staining (PAS) was utilized to determine the effect of cachexia and resistance exercise on high and low glycogen myofibersy, intramuscular glycogen content was quantified with Periodic Acid Schiff staining (PAS). The greater amount of glycogen within the fibers results in a greater intensity of the stain (Figure 4.4A). There was a 38% increase in the frequency of high glycogen fibers in the $Apc^{Min/+}$ control as compared to WT control, suggesting an increase in intramuscular glycogen with cachexia (Figure 4.4B). There was no significant effect of exercise in either WT or $Apc^{Min/+}$ to increase the frequency of high glycogen myofibers (Figure 4.4 B). Genotype had a significant effect on the CSA of high glycogen content fibers as both exercise and control were significantly lowered however resistance exercise training increased the CSA of high glycogen content fibers of $Apc^{Min/+}$ mice ($p<0.05$, Figure 4.4 C). Genotype also had a significant effect on the on the CSA of low glycogen content fibers as both exercise and control were lower however there was no significant effect of exercise in either genotype ($p<0.05$, Figure 4.4 D).

DISCUSSION

Cancer cachexia is a catabolic condition that leads to death in 1/3 of cancer patients and negatively affects both muscle and adipose tissue (4). For our study we utilized the $Apc^{Min/+}$ model, a mouse model of colorectal cancer. While much research has been published in regards to the multiple skeletal muscle alterations with cachexia (5, 6, 8, 16) the intervention of resistance exercise training has been less investigated. Al-Majid and McCarthy utilized a similar resistance exercise protocol in C-26 mice and found beneficial effects, however there was no morphological analysis of the muscle or examinations of metabolic properties (147). Our study aimed to examine the effects of resistance exercise on skeletal muscle myofiber area and specific myofiber metabolic properties in $Apc^{Min/+}$ mice. To this end, this study demonstrates that there are significant beneficial effects of resistance exercise during the initiation of cancer cachexia. In $Apc^{Min/+}$ mice there were significant increases in mean cross-sectional area in the exercise leg as compared to control. Also, in $Apc^{Min/+}$ mice there was an increase in oxidative myofibers and increases in cross-sectional area of glycolytic myofibers. Frequency of high glycogen myofibers were significantly higher in the control leg of $Apc^{Min/+}$ mice as compared to wild-type, however there was no significant increase in high glycogen fibers with resistance exercise in WT or $Apc^{Min/+}$ mice. This suggests possible metabolic dysfunction with uptake of glycogen however oxidative metabolism was able to improve with resistance exercise. This data suggests that resistance exercise can be very beneficial for the maintenance and improvement of skeletal muscle mass and oxidative capacity during the initiation of cancer cachexia.

Skeletal muscle mass loss and alterations in morphology are associated with reduced mobility and quality of life, reduced function and shorter life span (4, 17). Skeletal muscle wasting is a hallmark characteristic of cancer cachexia (16, 18) and myofiber degeneration has been previously demonstrated in our lab (7). Muscle atrophy associated with cancer cachexia can range from 10% to 50% (19), typically it has been observed that fast-glycolytic fibers are more susceptible to atrophy as compared to slow-oxidative fibers. While previous studies have shown that resistance exercise improves TA mass and protein content with cachexia (147) there has been no investigation into the changes in skeletal muscle morphology. First, our work showed that there was a significant decrease in TA CSA in the control leg of Min mice as compared to the control leg of WT mice. This has been shown in our lab but this further validates this finding of myofiber atrophy associated with overall muscle mass loss. However, our novel finding is that resistance exercise increased CSA in the TA of Min mice during the initiation of cachexia. Interestingly there was no significant increase in TA mass however there were increases in TA CSA. There were increases in larger myofibers and a decrease in smaller myofibers so possibly this increase in CSA but no change in mass can be accounted to less but bigger myofibers. This needs to be investigated further if a reliable method is available to accurately quantify the number of myofibers present. While myofiber atrophy has been shown in the Min model it has also been shown that there are decreases in oxidative capacity (5). Increases in oxidative capacity have been observed with resistance exercise training so we decided to examine the effect of resistance exercise training on oxidative capacity in the Min model.

Oxidative metabolism is a characteristic of skeletal muscle and is highly influenced by fiber phenotype (9). Skeletal muscle that is comprised of primarily oxidative Type I fibers have a higher capacity for oxidative metabolism than skeletal muscle that is primarily glycolytic Type II fibers (20). Studies have investigated how oxidative metabolism of muscle can influence its catabolic susceptibility (21, 22). Another line of investigation from our lab has examined mitochondrial function and oxidative capacity in the $Apc^{Min/+}$ mouse. It was shown that in $Apc^{Min/+}$ mice with cachexia there was a reduction in mitochondrial function and oxidative capacity (5). Our work here further validates this finding, as the Min mice in this study had significantly lower frequency of SDH dark fibers in the control leg as compared to WT. Oxidative myofibers were also significantly lower which has not been previously published. Our novel finding is that while the frequency of high SDH activity fibers is not significantly increased in WT, it is significantly increased in Min mice. The frequency of high SDH activity fibers in the exercise TA of the Min mice is almost to the control value of WT mice, suggesting there is a possible restoration of oxidative capacity with the intervention of resistance exercise. Also there were increases in the CSA of both oxidative myofibers and glycolytic myofibers in Min mice demonstrating that Min mice are able to respond to resistance exercise. This data is intriguing in that it suggests oxidative capacity and oxidative/glycolytic myofibers can respond to resistance exercise training during the initiation of cachexia and further biochemical work should be done to further examine the influence of resistance exercise on oxidative capacity during cachexia.

While oxidative capacity and oxidative metabolism have been shown to be dysregulated in the $Apc^{Min/+}$ model, there have been basic investigations into glucose

metabolism however none in glycogen metabolism and glycogen storage. Particularly there have been no investigations on the influence of resistance exercise training on glycogen content in cachectic skeletal muscle. Our study found there was a significant increase in high glycogen content fibers in the control leg of Min mice, suggesting an increase in intramuscular glycogen content with cachexia. Typically there is an increase in intramuscular glycogen content with resistance exercise due to an increase in the translocation of GLUT-4, a transmembrane protein responsible for the entry of glucose into muscle (131). Our data found that there was no increase in intramuscular glycogen content in WT with resistance exercise, possibly due to the limited amount of training sessions. Some literature has suggested that this increase in GLUT-4 leading to increased intramuscular glycogen stores requires longer training than 7 sessions as performed here. There was a significant increase in the glycogen content in the Min control TA as compared to WT. However while intramuscular glycogen content trended to increase with exercise in Min mice, this was not significant. It was of great interest that intramuscular glycogen content had increased in the Min control and exercise leg as compared to WT. Possible explanations could be related to AMPK signaling and accumulation of glucose-6-phosphate (G6P) which provides allosteric regulation that overrides the effect of AMPK (23). Acutely, AMPK down regulates the storage of glycogen however when chronically elevated, glycogen content has been observed to increase (23, 24). The proposed mechanism of this observation is that while AMPK is inhibiting glycogen synthase and glycogen storage, the increase of glucose from GLUT-4 results in an over-accumulation of G6P which allosterically regulates glycogen synthase, overriding the effects of AMPK and results in increased skeletal muscle glycogen (23).

Previous literature from our lab has indicated that AMPK is chronically elevated in the $Apc^{Min/+}$ model (8) however no work has looked at downstream targets such as G6P and glycogen synthase, so this could be a possible mechanism to explain the increase in intramuscular glycogen with the progression of cachexia.

Several strengths and limitations to the current study exist. This is the first study to examine the effects of resistance exercise training on skeletal muscle morphology and metabolic properties during the initiation of cancer cachexia. However this resistance exercise protocol only lasted for 2 weeks with 7 sessions. While benefits were observed, a longer training model might be more reliably extrapolated for repeated exercise in the human model. Both the technique for measuring oxidative capacity and intramuscular glycogen have been validated in other studies so our measurement of our particular variables is appropriate. However, SDH staining and PAS staining are semi-quantitative measurements so care must be taken in the interpretation of results. Staining technique and quantification of digital images were repeated and validated, however error can still occur. Further work should examine direct measurements of oxidative capacity (Seahorse Biosciences) and intramuscular glycogen content (total glycogen content assay). Previous studies have employed these methods for direct measurements of these variables however this has not been done in conjunction with each other in the $Apc^{Min/+}$ model.

In summary, our data demonstrates cachectic skeletal muscle undergoes multiple changes in morphology and metabolic properties. Specifically, frequency of high SDH activity myofibers is decreased with cachexia however there is an increase in high glycogen content myofibers. It was also found that the intervention of a resistance

exercise training protocol had beneficial results in the cachectic Min mice. The intervention of a resistance exercise training is able to improve myofiber area and also increase the frequency of high SDH activity myofibers, suggesting an increase in oxidative capacity. Future research should continue to examine the changes in intramuscular glycogen content with the utilization of total content assays and possible pathways that are responsible for these metabolic changes. Understanding the changes observed in this study and further examining the molecular signaling pathways associated with these changes due to cachexia and resistance exercise training need to be furthered for better treatment modalities for cachectic patients.

Table 4.1. *Body weight change, tibia length, TA mass in control and exercise in male wild-type and Apc$^{Min/+}$ mice*

		Body Weight				TA		
Group	n	16 wk BW (%)	18 wk BW (%)	Change (%)	Tibia Length (mm)	Cntrl TA (mg)	Ex TA (mg)	Diff (%)
WT	5	26.0 ± 1.7	26.2 ± 1.5	0.7 ± 1.2	17.1 ± 0.1	48 ± 1.5	50.5 ± 1.2	5.3 ± 2.1
Apc$^{Min/}$	5	24.8 ± 0.7	23.7 ± 0.8	-4.4 ± 1.4*	17.0 ± 0.2	41 ± 0.9*	42.5 ± 0.8*	3.6 ± 1.5

All muscles, organs and fat were excised at time of sacrifice. BW, body weight; g, gram; mm, millimeter; mg, milligram; TA, tibialis anterior muscle. Values are means ± SE. Sac, sacrifice; g, gram; mg, milligram; TA, tibialis anterior muscle. Two-way ANOVA with repeated measures used for statistical analysis * Main effect of genotype, different from wild-type group. Significance was set at $P < 0.05$.

Table 4.2. *Summary table of alterations in morphology and metabolic properties with resistance exercise training in wild-type and Apc^{Min+} mice.*

	WT		$Apc^{Min/+}$	
Myofiber Characteristic	***Control***	***Exercise***	***Control***	***Exercise***
High SDH Activity %	--	↔	↓	↑
High SDH Activity CSA	--	↔	↓	↑
High Glycogen Content %	--	↔	↑	↔
High Glycogen Content CSA	--	↔	↓	↑

Figure Legends

Figure 4.1. Experimental design for resistance exercise training protocol. At 16 weeks of age both WT and $Apc^{Min/+}$ began a resistance exercise training protocol. The left leg was exercised via percutaneous electrical stimulation resulting in net plantar flexion of the ankle. The right leg served as an inter-animal control. Each session consisted of 10 sets of 6 reps, with each repetition lasting 3 seconds. Total of 7 sessions over two weeks. Mice were sacrificed 48 hours following the last training session.

Figure 4.2. TA mass difference with exercise in wild-type and $ApcMin^{/+}$ mice

The difference in TA mass between the exercise and control leg of wild-type and ApcMin/+ with resistance exercise training.

Figure 4.3. Tibialis Anterior (TA) myofiber area in wild-type and $Apc^{Min/+}$ mice with resistance exercise training. ***A:*** *Top left*, representative image of wild-type control at 40x; *top right*, wild-type with exercise; *bottom left*, $Apc^{Min/+}$ control; *bottom right*, $Apc^{Min/+}$ exercise. **B*:*** Mean CSA (um^2) of TA in wild-type and $Apc^{Min/+}$ control and exercise. ***C-D:*** Mean CSA distributions of wild-type and $Apc^{Min/+}$ control and exercise. Data were analyzed using repeated measures two-way ANOVA. * indicates significance of exercise within genotype. Significance was set at $P < 0.05$

Figure 4.4 High and low SDH activity myofiber frequency and area in wild-type and $Apc^{Min/+}$ mice with resistance exercise training. SDH staining was utilized to quantify frequency and size of high and low SDH activity myofibers in the tibialis anterior (TA) muscle during the progression of cachexia **A:** *Top left*, representative image of wild-type control; *top right*, wild-type exercise; *bottom left*, $Apc^{Min/+}$ control;

bottom right, $Apc^{Min/+}$ exercise. ***B:*** SDH dark (frequency %) in wild-type and $Apc^{Min/+}$ control and exercise. ***C-D:*** Mean cross-sectional area (CSA) of SDH dark fibers and SDH light fibers (um^2) in the TA of wild-type and $Apc^{Min/+}$ control and exercise. Data were analyzed using repeated measures two-way ANOVA. * indicates significance of exercise within genotype. Significance was set at $p < 0.05$

Figure 4.5 Frequency and size of high and low glycogen content myofibers in wild-type and $Apc^{Min/+}$ mice with resistance exercise training. PAS staining was utilized to quantify frequency and size of high and low glycogen content myofibers in the tibialis anterior (TA) ***A:*** *Top left*, representative image of wild-type control at 10x; *top right*, wild-type exercise; *bottom left*, $Apc^{Min/+}$ control; *bottom right*, $Apc^{Min/+}$ exercise. ***B:*** PAS dark fiber fiber (frequency %) in wild-type and $Apc^{Min/+}$ control and exercise. ***C-D:*** Mean cross-sectional area (CSA) of high and low glycogen content myofibers (um^2) of TA in wild-type and $Apc^{Min/+}$ control and exercise. Data were analyzed using repeated measures two-way ANOVA . * indicates significance of exercise within genotype. Significance was set at $P < 0.05$

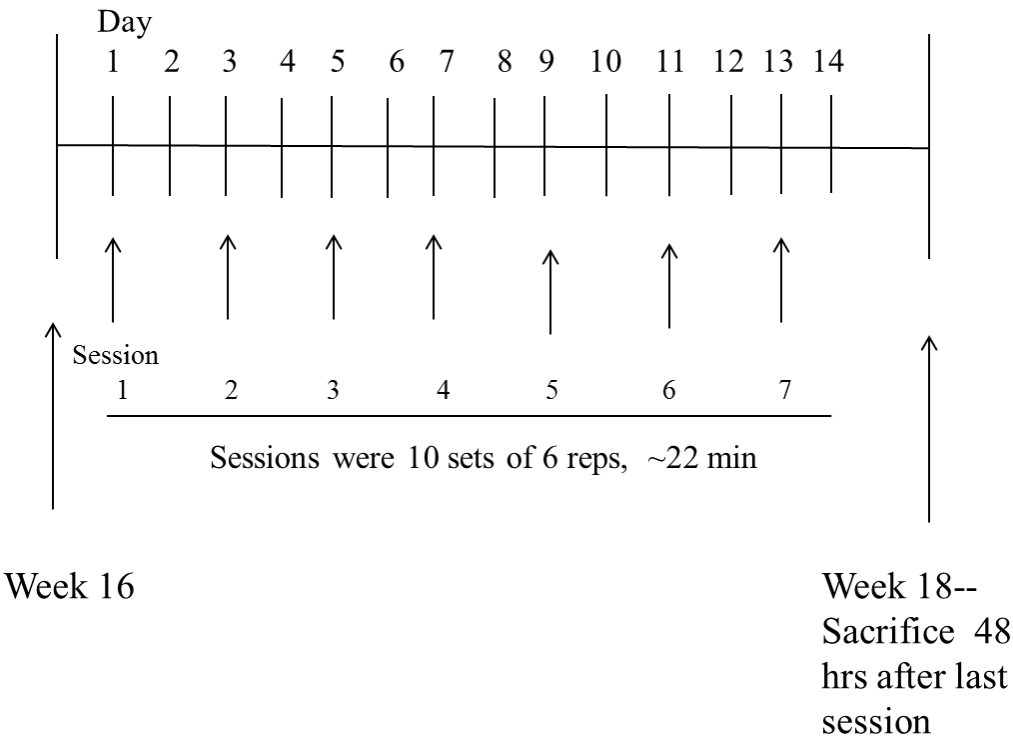


Figure 4.1. Experimental design for resistance exercise training protocol

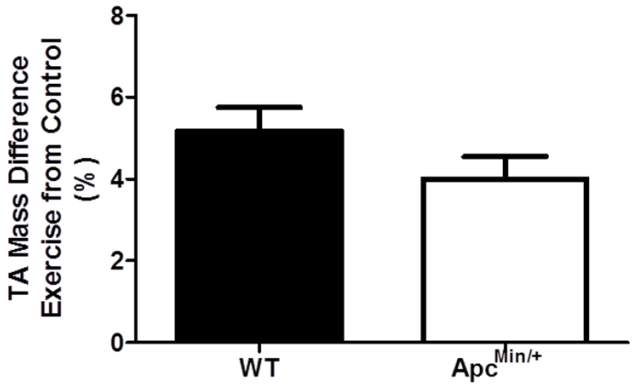


Figure 4.2 Tibialis Anterior (TA) mass difference between exercise and control in wild-type and ApcMin/+ mice

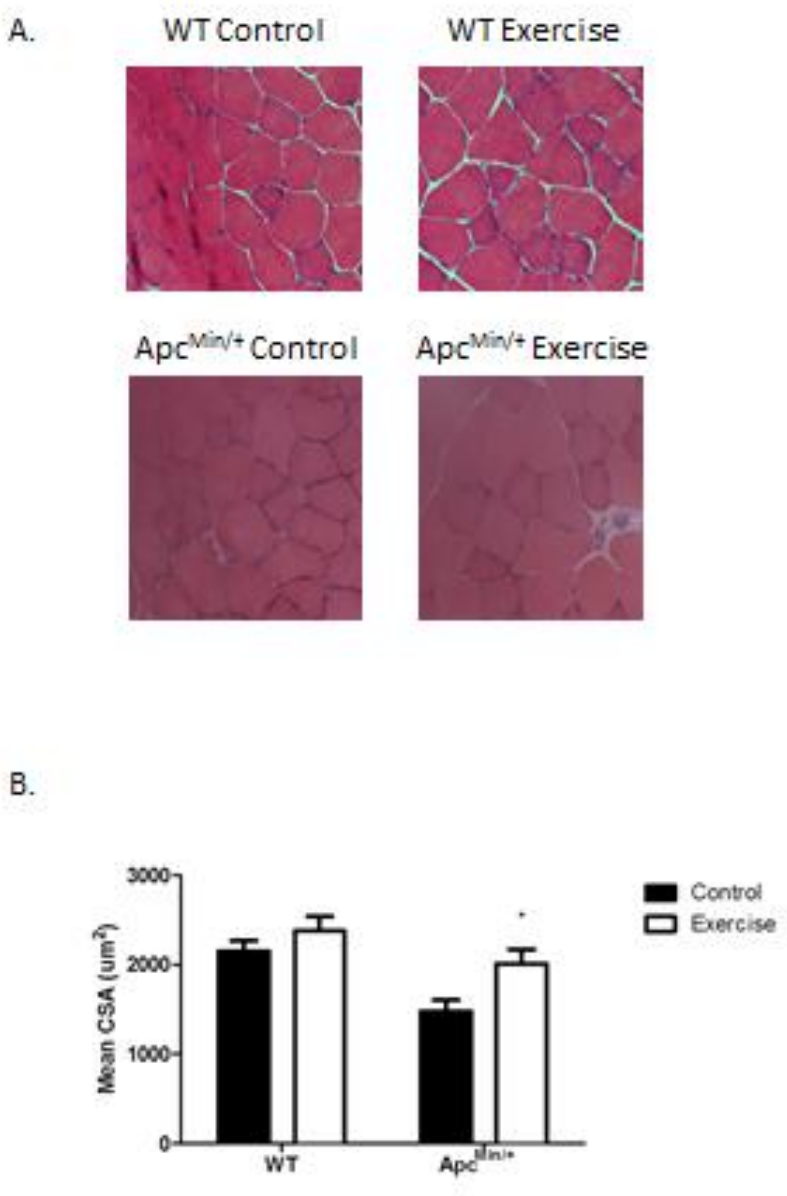


Figure 4.3 Tibialis Anterior (TA) myofiber area in wild-type and $Apc^{Min/+}$ mice with resistance exercise training

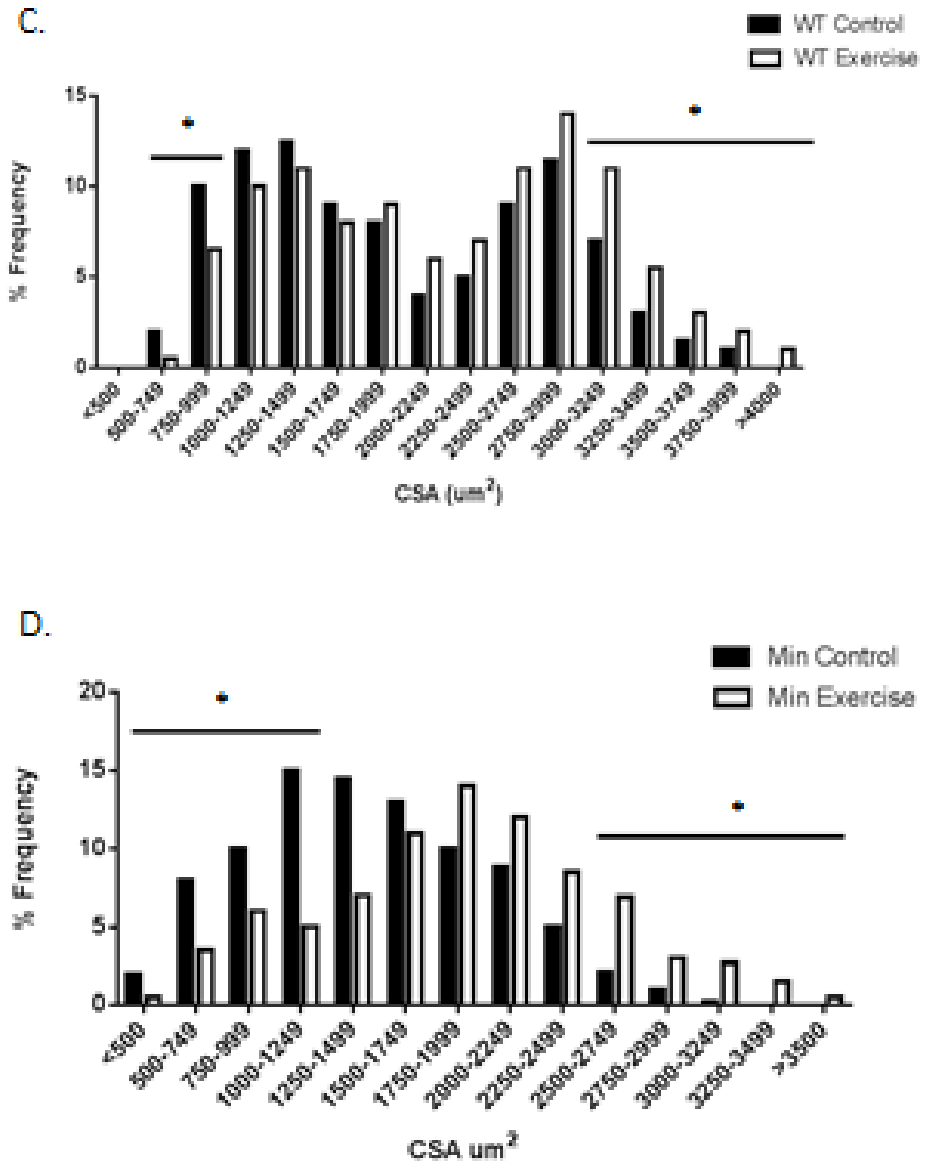


Figure 4.3 Tibialis Anterior (TA) myofiber area in wild-type and $Apc^{Min/+}$ mice with resistance exercise training

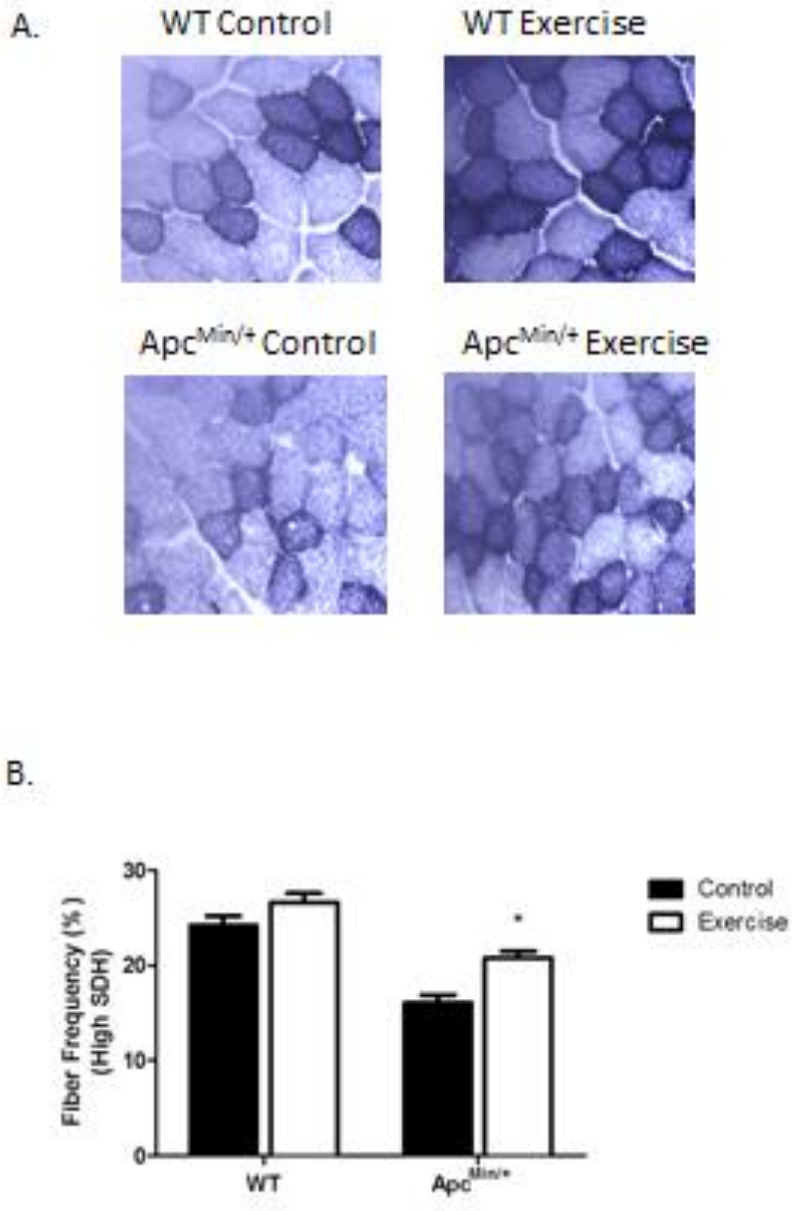
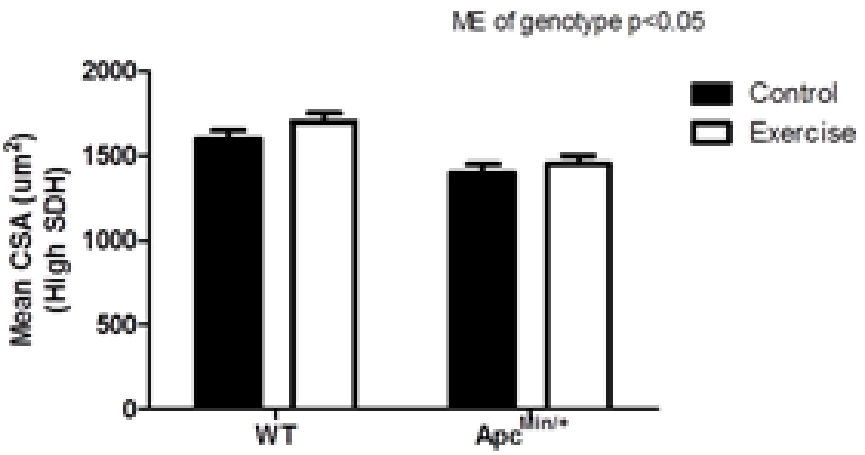


Figure 4.4 Frequency and size of high and low SDH activity myofibers in wild-type and $Apc^{Min/+}$ mice with resistance exercise training

C.



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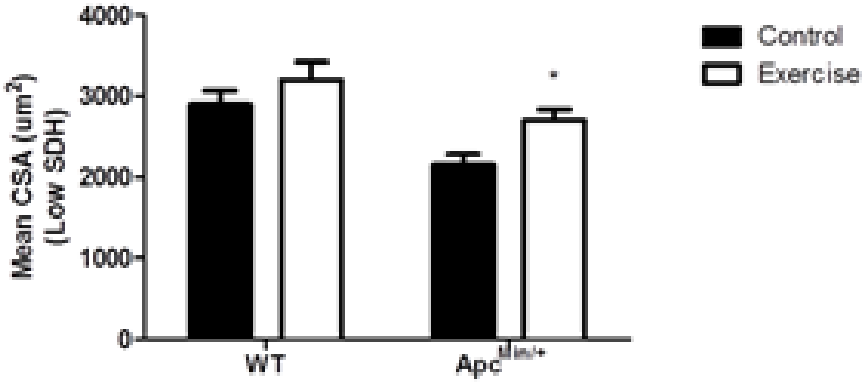


Figure 4.4 Frequency and size of high and low SDH activity myofibers in wild-type and $Apc^{Min/+}$ mice with resistance exercise training

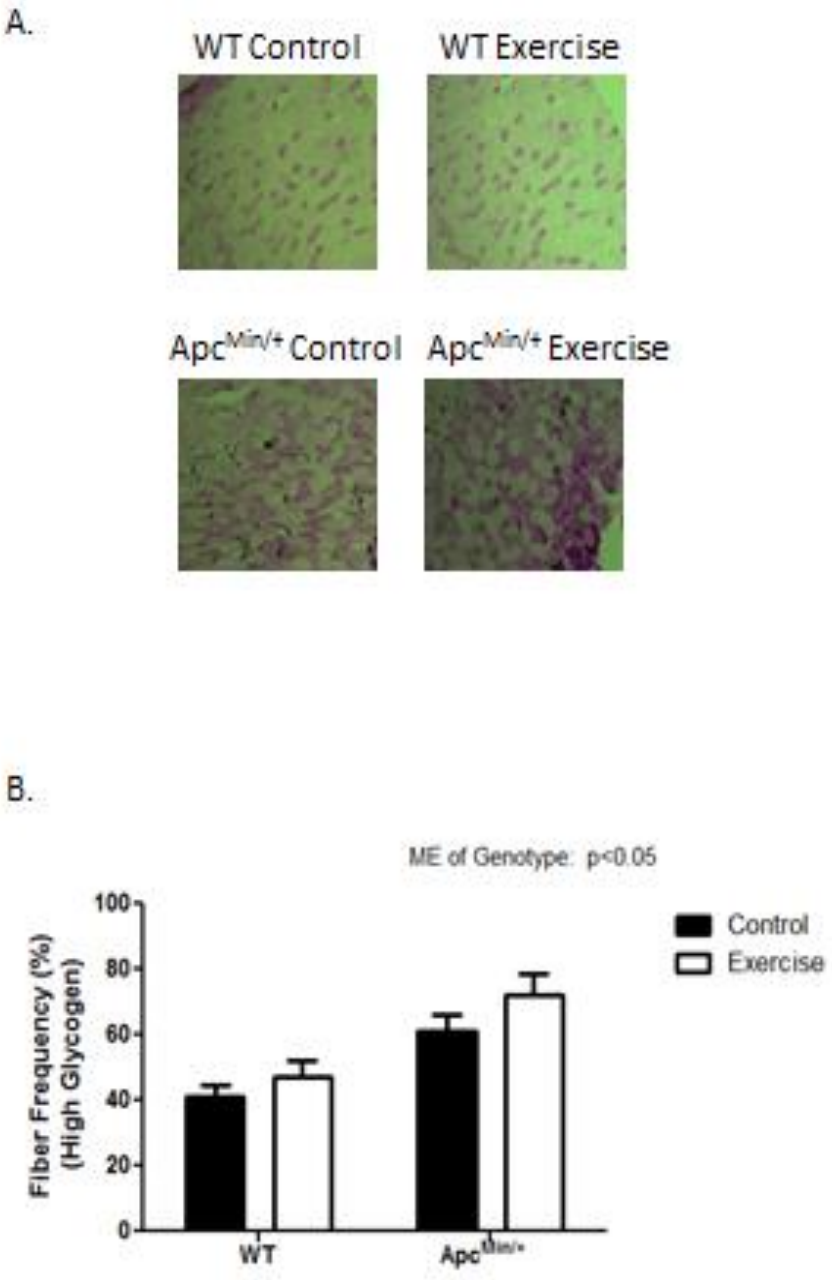
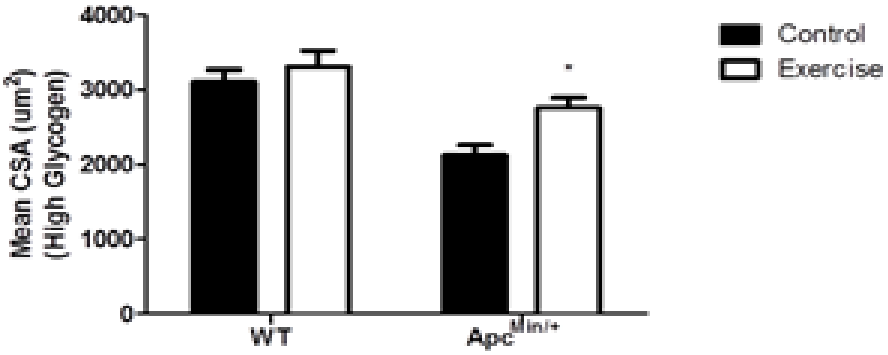


Figure 4.5 Frequency and size of high and low glycogen content myofibers in wild-type and $Apc^{Min/+}$ mice with resistance exercise training

C.



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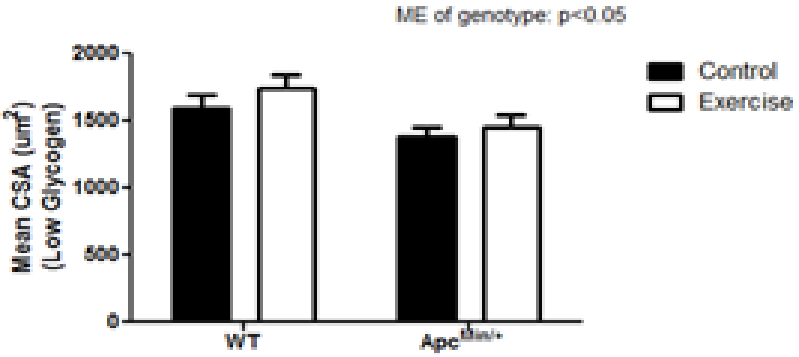

Figure 4.5 Frequency and size of high and low glycogen content myofibers in wild-type and $Apc^{Min/+}$ mice with resistance exercise training

CHAPTER 5

OVERALL DISCUSSION

Cachexia is a condition often seen during the progression of cancer, which involves the unintentional loss of body weight from the wasting/atrophy of both skeletal muscle and adipose tissue (3, 4). While much research has been done to further understood the mechanisms underlying the causes of cachexia and the treatment, there are still many unanswered questions. This study aimed to examine some of the lesser understood effects and to also examine the effect of resistance exercise in cachexia. There were two specific aims for this study. The first was to examine the effect of cachexia severity on skeletal muscle myofiber area and myofiber metabolic properties during cachexia-induced myofiber area. The second aim was to examine the effect of resistance exercise training on selected myofiber metabolic properties in the initiation of cachexia.

Overall this study had many intriguing findings that have led to the necessity of further investigation. In the first study the aim was to the examine the effect of cachexia severity on skeletal muscle myofiber area and specific myofiber metabolic properties. It is known that cachexia reduces skeletal muscle mass and area of myofibers, however it is not known if certain myofiber metabolic properties are related to the myofiber atrophy observed with cachexia. In the first study we found that cachexia severity highly

influences the mean cross-sectional area of myofiber as progressive atrophy was observed with cachexia severity progression. Moderate cachexia induced a higher percentage of smaller myofibers and this was further exacerbated with severe cachexia. Oxidative capacity has been shown to be reduced with cachexia and we decided to investigate this further with SDH staining to investigate the relationship of SDH activity and myofiber atrophy during cancer cachexia.

In this study we report that cachexia reduces the frequency of high SDH activity myofibers and increases the frequency of low SDH activity myofibers. This effect is progressive with cachexia severity. Despite SDH activity of myofibers, there was atrophy in both high and low SDH activity myofibers. Some literature has suggested that oxidative capacity of myofibers can possibly serve as a protective effect from wasting, however we demonstrate that high SDH activity myofibers (oxidative) are reduced in both frequency and area with cachexia. Also low SDH activity myofibers (glycolytic) are succeptible to wasting, even more so than those of high SDH activity myofibers when examining the percentage loss of mean CSA. This coincides with previously published from our lab reporting a decrease in oxidative capacity in skeletal muscle with cachexia. oxidative capacity and severe cachexia reduced oxidative capacity further. Interestingly, while cachexia induced an increase in the frequency of low SDH activity myofibers, cachexia reduced the area of these myofibers. . Disuse usually results in a reduction in the area of both slow-oxidative and fast-glycolytic myofibers. Some literature has suggested that oxidative fibers are more succeptible, some that glycolytic fibers are more succeptible. ApcMin/+ mice have dramatically reduced physical activity, due to tumor burden and wasting from cachexia. It is quite possible that this lack of activity is one

mechanism leading to further signaling mechanisms leading to the loss of oxidative myofibers and an increase in glycolytic myofibers. Loss of mitochondrial proteins and alterations in fusion/fission are also leading to this loss in oxidative myofiber frequency and area with cachexia. Further investigation needs to be conducted to further understand the mechanisms that are responsible for the reduction in frequency of oxidative myofibers and increase in the frequency of glycolytic myofibers.

In addition to the reduction in high SDH activity myofibers, there was a progressive increase in high glycogen content myofibers with cachexia severity progression. This was an interesting finding that has not been previously shown. Our lab has shown that in the $Apc^{Min/+}$ mouse there is myofiber degeneration/regeneration (20), a hyperactivation of Akt and pAMPK as well as a downregulation of p-mTOR (5). Risson et al. (21) found that in their mTOR muscle specific KO model there is a hyperactivation of Akt, GSK3B and downregulation of p-mTOR and this was accompanied with myofiber atrophy and a loss of oxidative capacity (21). Phosphorylation of GSK3B leads to an inactivation of this protein, leading to an increase of glycogen synthase and a reduction in glycogen phosphorylase which can lead to an increase in intramuscular glycogen. In this study we found that there was an increase in the frequency of high glycogen content myofibers despite the myofiber atrophy. The increase in high glycogen content myofibers suggests that there is possibly increased intramuscular glycogen levels, however this needs to be further investigated. The hyperphosphorylation of Akt and GSK3B could be the mechanism through which our $Apc^{Min/+}$ model is increasing high glycogen content myofibers with cachexia progression.

Further work needs to be done in conjunction with our findings here to further elucidate the mechanisms behind these observations.

Furthermore in line with glycogen metabolism and storage, the ApcMin/+ mice are insulin resistant with elevated resting blood glucose. Insulin plays a critical role in glycogen metabolism and glycogen storage in skeletal muscle. Type 2 diabetes patients have elevated blood glucose, reduced oxidative enzymes and reduced intramuscular glycogen. While ApcMin/+ mice have been found to have elevated blood glucose and reduced oxidative enzymes, we found in this study that there was an increase in frequency of high glycogen content fibers suggesting an increase in intramuscular glycogen. While ApcMin/+ mice are insulin resistant and possible downregualtion of GLUT-4, it is possible that other mechanisms are overriding the insulin response. These mechanisms could include the previously discussed hyperphosphorylated Akt and GSK3B which could induce the increases in intramuscular glycogen content. He and Kelly (152) reported an increase of glycolytic enzymes and decrease in oxidative enzymes with insulin resistance in type-2 diabetes patients . While it has been found that oxidative enzymes are reduced in the ApcMin/+, less is known about glycolytic enzymes. With the increase in high glycogen content fibers, these warrants further investigation of glycogen metabolism. The relationship between insulin and glycogen metabolism should be further examined in the ApcMin/+ model to further elucidate the mechanisms behind the observed changes in metabolic properties.

Resistance exercise training was utilized in aim 2 to investigate its effect on overall morphology and specific metabolic properties in $Apc^{Min/+}$ mice. The use of a resistance exercise protocol has been utilized in a mouse model of cancer (147) however

there were shortcomings in this study as no work was done to investigate any morphological changes in the skeletal muscle or any alterations in myofiber metabolic properties. The increases in muscle mass that were observed in this study could have simply been due to edema rather than any morphological changes. Therefore we chose to pursue this further and examine these particular variables in the $Apc^{Min/+}$ model with a resistance exercise training program. For our model we utilized a modified protocol from Baar and Esser (151) in which the left leg was stimulated via percutaneous electrical stimulation for 7 sessions over 14 days. These sessions consisted of 6 sets, 10 reps (at 3ms each) in which these sessions lasted approximately 22 minutes each. The right leg served as an inter-animal control. Our findings first demonstrate similar findings (20) that overall TA mass and CSA is reduced in the Min mouse. However, with the intervention of a resistance exercise protocol TA CSA significantly increased. Examining a frequency distribution it is clear there is an increase in larger fibers, suggesting hypertrophy is possible during the initiation of cachexia. The initiation of cachexia is a critical time in which skeletal muscle mass and function begins to decline. This work illustrates that at this critical time skeletal muscle is able to respond to the resistance exercise and hypertrophy, which can prove beneficial to the cachectic patient.

Our lab has also previously shown that with myofiber atrophy there is a loss in oxidative capacity in the Min mouse (8), we decided next to examine the influence of resistance exercise on oxidative capacity. It was found that the frequency of SDH dark fibers significantly increased and these fibers increased in CSA as well. This suggests that there is an improvement in oxidative capacity and integrity of oxidative myofibers with resistance exercise in the Min mouse. Oxidative metabolism is critical for energy

expenditure and losses in this can lead to fatigue and loss of endurance capacity. Our data suggests that resistance exercise training can be beneficial in restoring this however further work should be done to examine signaling and other markers of oxidative metabolism (ex. COX IV, cytochrome C, mitochondrial DNA). Since oxidaitive metabolism is compromised in the Min mouse (8) and improved with resistance exercise, we next decided to examine myofiber glycogen content to observe if any changes occur with the alterations in myofiber SDH activity.

Typically a training adaptation with resistance exercise training observed is an improvement in the translocation of GLUT-4, carrying glucose into the cell for conversion to glycogen. Interestingly we found that there was an increase in intramuscular glycogen in the control leg of the Min mouse. However, resistance exercise training did not significantly high glycogen content myofibers in either WT or Min mice. Our finding of elevated glycogen is interesting and needs to be pursued further. There appears to be either an alteration in glycogen synthase (conversion of glucose to glycogen) or an alteration in glycogen phosphorylase (breakdown of glycogen to glucose) or possibly both. Typically the Min mouse has issues with the "ramp up" stage of exercise and this could be due to the increased glycogen stores that it is not able to utilize. Therefore if resistance exercise is increasing glycogen levels even further and not able to utilize that glycogen, it could be detrimental. Therefore further work should be done to investigate the effects of resistance exercise training on intramuscular glycogen stores during cachexia.

Overall out study had many interesting findings that need to pursued further to understand the mechanisms that are responsible for these changes. Our data shows that

during the progression of cachexia there is progressive atrophy in myofibers that is not observed in cancer alone. Along with this progressive atrophy in myofibers there is a significant decrease in the frequency of high SDH activity myofibers. Despite SDH activity, there is myofiber atrophy in both high and low SDH activity myofibers suggesting both oxidative and glycolytic fibers are succeptible to cachectic wasting. Along with this decrease in oxidative capacity, there is a signficiant increase in the frequency of high glycogen fibers even those these fibers are atrophying. The mechanisms behind these changes are occurring need to be further investigated to understand the signaling pathways leading to these alterations. Additionally, resistance exercise proved to be beneficial in the imrpovement of myofiber area and improvement of the frequency of high SDH activity myofibers, suggesting an improvement in oxidative capacity. However since the intramuscular glycogen appears to be elevated in the control Min mouse with an increase in high glycogen content fiber frequency, care must be taken with an intense resistance exercise program that might further increase intramuscular glycogen levels. In conclusion we suggest that with the progression of cancer cachexia there is progressive myofiber atrophy with significant alterations in myofiber metabolic properties in skeletal muscle, however resistance exercise appears to be beneficial during the initiation of cachexia.

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