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Preliminary Study: Leucine Supplementation Exacerbates Muscle Wasting Independent of the Ubiquitin-Proteasome System

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Preliminary Study: Leucine Supplementation Exacerbates Muscle Wasting Independent of the Ubiquitin-Proteasome System

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ABSTRACT

Cancer cachexia is the rapid, drastic loss of muscle mass associated with cancer and is not reversible by conventional nutritional means (Brown et al., 2018; Brown et al., 2017). It occurs in ~80% of cancer patients and is responsible for 20-40% of cancer-related deaths (Brown et al., 2018; Brown et al., 2017). This condition leaves patients with fatigue, functional impairment, reduced quality of life, and a decrease in survival rates. Cachexia occurs through an imbalance between protein degradation and protein synthesis (Brown et al., 2018), which is associated with inflammation and altered metabolic processes.

Several studies have investigated the effects of leucine on cancer cachexia, as it is known for promoting muscle growth through the stimulation of anabolic signaling cascades while inhibiting catabolism. These studies have all been conducted for a short period of time and have shown promise for leucine as a treatment for cancer cachexia. However, one study found that long-term (6 weeks) leucine supplementation increased epididymal fat mass while decreasing muscle mass in rats fed a high-fat diet (Baum et al., 2016). In addition, Lee et al. (2019) demonstrated exacerbated atrophy in tumor-bearing mice after 28 days of low-dose leucine supplementation. Whether leucine is a viable long-term treatment for cancer cachexia requires further investigation.

10 C57BL/6 (WT) mice and 7 APCMin/+ (APC) mice were used in this study and given either tap water as a control or given 1.5% leucine-enriched water. Tissue harvest was conducted at ~24 weeks of age for genetic and western blot analysis. There was a main effect of genotype for decreased body and plantaris weight in APC mice compared to WT ($p < 0.05$), and a main effect of leucine for lower plantaris weight/tibia length in APC mice ($p < 0.05$), which appeared to be driven by the $APC^{Min/+}$ genotype ($p = 0.0841$). Cyclin D1 mRNA abundance was ~2-fold

greater in APC mice compared to WT, although no difference was found with treatment. No difference was found in MyoD or Myogenin mRNA abundance. Long-term leucine supplementation appears to exacerbate atrophy, which appeared to be independent of protein turnover or myogenesis disruption.

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Chapter 1: Introduction

INTRODUCTION

Skeletal muscle is a highly plastic tissue, capable of maintaining the mass needed to perform daily tasks. However, this ability is compromised when a body undergoes certain pathological conditions such as tumor growth, resulting in a condition known as cancer cachexia. Cancer cachexia occurs in about 50-85% of colorectal, pancreatic and lung cancer patients and is characterized by a dramatic loss in fat and muscle tissue, negatively affecting mortality and quality of life in cancer patients, and is inversely related to survival time (Aversa, et al., 2011; Cruz et al, 2017; VanderVeen et al., 2017; Assi, Derbre, Lefeuvre-Orfila & Rebillard, 2016). Consequently, it is one of the most devastating effects of cancer and has a direct correlation with tumor development (the degree of which varies with the type of cancer) (Salomao & Gomes-Marcondes, 2012). This drastic loss in muscle tissue can contribute to a decline in functional status and reduction in respiratory function (Aversa, et al., 2011). Not only does it affect the patient's mortality/survival time, it also impacts the patient's response to chemotherapy and increases susceptibility to chemotherapy-induced toxicity, as well as causing a higher incidence of postoperative complications (Van Norren, et al., 2009).

The effect of cancer cachexia on skeletal muscle has been extensively studied, due to its importance for health maintenance during aging, obesity, and many chronic diseases (Carson, Hardee & VanderVeen, 2016; VanderVeen et al., 2017). However, there are considerable gaps in our understanding of the roles of muscle properties that contribute to health and quality of life. In addition to mass, skeletal muscle has several properties that impart health benefits to cancer-ridden organisms, including metabolic capacity and substrate utilization flexibility (Carson et al., 2016). Skeletal muscle also has an endocrine function through the production and secretion of myokines (Carson et al., 2016). Muscle health can in part be controlled through the regulation of

protein turnover, which responds to a variety of stimuli. These stimuli work through intricate cellular signaling pathways involving several organelles and structures, all of which can become dysregulated in cancerous conditions.

Clearly, preventive measures are important and continue to be researched. However, nutritional therapies have yet to prove effective and there are currently no approved treatments for cancer cachexia due to the complexity and variability of the condition (Assi et al., 2016; Brown et al., 2018; Carson et al., 2016). Many recent studies have investigated the complex processes involved in cancer cachexia, as well as a myriad of potential treatments. One possible treatment is leucine supplementation. This branched chain amino acid has been shown to stimulate muscle protein synthesis through activation of the protein mTOR, as well as attenuating protein degradation by inhibiting ubiquitin protein ligases (Baum, 2005; Baptista et al, 2009; Junior et al., 2011).

The effects of leucine supplementation on cancer cachexia is not yet conclusive. Previous research has shown that leucine supplementation is efficacious in maintaining muscle mass in cancer cachexia, however these are short-term studies (12-21 days into treatment/cancer) (Cruz, Oliveira, & Gomes-Marcondes, 2017; Gomes-Marcondes, Ventrucci, Toledo, Cury, & Cooper, 2003). These studies also showed that leucine supplementation attenuated protein degradation and improved protein synthesis. In contrast, Viana et al (2016) showed leucine to have no effect on body weight. In addition, Lee et al. (2019) demonstrated that muscle mass loss was exacerbated in LLC tumor-bearing mice after 28 days of low-dose leucine supplementation.

The involvement of muscle mitochondria in wasting and metabolic quality is well recognized (Brown et al., 2017; Carson, Hardee & VanderVeen, 2016; VanderVeen, Fix & Carson, 2017). The role of mitochondria in cancer cachexia has therefore been investigated

intensively during the past few years (Brown et al., 2017; Carson, Hardee & VanderVeen, 2016; VanderVeen, Fix & Carson, 2017). The processes by which mitochondrial quality is maintained are regulated by a variety of stimuli, including frequency of use, inflammation, and hormonal signaling (Carson et al., 2016; Vanderveen et al., 2017). These processes include mitochondrial biogenesis, dynamics, and mitophagy (Brown et al., 2017). Mitochondria function has a role in ATP production, apoptosis, autophagy, and protein turnover (Carson et al., 2016). Many processes involved in mitochondrial regulation are altered in cachectic muscle (Brown et al., 2017). Brown et al. (2017) demonstrated that mitochondrial health is compromised before measurable muscle wasting in a Lewis Lung Carcinoma (LLC) model of cancer cachexia, beginning with increased reactive oxygen species (ROS) emission and followed by an impaired mitochondrial network and respiratory function.

The current knowledge regarding the effects of leucine supplementation on mitochondrial quality is limited. Mitochondrial content and biogenesis-related gene expression have both been shown to increase with leucine supplementation (Liang, Curry, Brown & Zemmel, 2014). In addition, D'Antona et al. (2010) demonstrated that antioxidant (SOD1, SOD2, and catalase) protein and gene expression increased with branched-chain amino acid (BCAA) supplementation as well, which could contribute to the modulation of ROS damage. Whether leucine supplementation influences mitochondrial dynamics and/or mitophagy has yet to be determined, and none of the processes related to mitochondrial quality and leucine supplementation has been studied in relation to cancer cachexia.

In addition to mitochondrial quality, the balance in muscle protein turnover affects muscle health, as well as mass. When protein synthesis is equal to protein degradation, mass stays the same (Aversa et al. 2011; Haegens et al., 2012). In catabolic conditions, such as when

an organism has developed cancer, this balance shifts to increased protein degradation and suppressed protein synthesis (Aversa et al., 2011; Brown et al., 2018). This state largely results from activation of the ubiquitin proteasome and autophagy systems (White et al., 2011). Two specific ubiquitin ligases have been identified as biomarkers of cachexia: MuRF1 and Atrogin-1 (Brown et al., 2018; Carson et al., 2010). Regarding anabolic pathways, cachexia-induced reduction of MAPK, Akt, peIF2**α** and mTOR activities decreases protein synthesis (Aversa et al., 2011, Viana & Gomes-Marcondes, 2015). As mentioned previously, leucine supplementation has been shown to improve muscle mass through the stimulation of mTOR and the suppression of some catabolic pathways, both in healthy and cachectic muscle (in short-term studies). Therefore, long-term supplementation of this BCAA may prove to be beneficial for protein turnover in tumor-bearing mice.

The purpose of this study is to investigate the effects of long-term leucine supplementation on cancer cachexia. Of interest are the signaling pathways and resultant outcomes of protein turnover and myogenesis. Based on the current literature, we hypothesize that leucine supplementation will increase protein anabolism and decrease catabolism while contributing to the impairment of myogenic regulation.

CHAPTER 2: Literature Review

There are two main model categories of cancer cachexia: 1) tumor injection and 2) genetic models. The focus of this literature review will be to discuss the models of cancer cachexia and the major components driving cancer cachexia: satellite cells, inflammation, dysregulated protein turnover, and altered oxidative metabolism. There will be a discussion of the pros and cons of the cachexia models and how each component muscle regeneration responds within a healthy cell, within the confines of cancer cachexia, and to leucine-promoted signals. Leucine will be discussed as an intervention for cancer cachexia as this topic is key to understanding the complex nature of prevention and/or treatment of cancer cachexia.

Satellite Cells

The ability of skeletal muscle to regenerate is due in large part to satellite cells. These cells are a population of heterogeneous, undifferentiated cells located between the basal lamina and sarcolemma of muscle cells and account for 2-7% of myofiber nuclei in adults (Fu, Wang & Hu, 2015). During hypertrophy or repair, satellite cells can fuse with existing muscle fibers for nuclei donation. They have a substantial nuclear-to-cytoplasmic ratio, little organelle content, and considerable amounts of heterochromatin.

In healthy, adult skeletal muscle, satellite cells remain in the G_O phase in which cells are inactive (Schultz, Gibson & Champion, 1978). Upon injury or mechanical stress satellite cells migrate from their niche and proliferate, after which a proportion of them differentiate. Once satellite cells enter the G_1 phase of the cells cycle, they are referred to as myoblasts.

Upon differentiation satellite cells contribute to muscle mass in 2 ways: fusion to pre-existing fibers and forming new fibers. When they fuse to existing fibers, they contribute to myonuclear accretion, which allows for growth and replacement of damaged nuclei. The

formation of new fibers is not common in skeletal muscle, but it does happen in smooth muscles, such as in the uterus.

Satellite Cell Quiescence

The ability of satellite cells to repair and maintain muscle mass is dependent on a variety of intrinsic and extrinsic signals. Myogenic regulatory factors (MRFs) have been shown to be necessary for formation of muscle and the presence of myogenic cells in adult skeletal muscle. These include Myogenin (also referred to as MRF4), MyoD (also known as myogenic determination factor 1), and Myf5 (myogenic factor 5). Paired box proteins 3 and 7 (Pax3 and Pax7) are also important in the regulation of myogenesis and are upstream of Myf5 and Myod in the myogenic signaling pathway.

While in the quiescent phase, satellite cells express Pax7, Pax3, and possibly Myf5 (Cornelison & Wold, 1997). Myf5 has been found in ~90% of quiescent cells, indicating myogenic commitment (Beauchamp et al., 2000). Pax 7 has a dual role of inducing myoblast proliferation and delaying differentiation through regulation of MyoD. Its involvement in expression of antiapoptotic factors has also been recognized. Pax3's role is not well understood, though what is known is that it is important in embryonic development. Myf 5 is thought to regulate proliferation rate and homeostasis and is expressed at high levels in isolated satellite cells; however, a small population of satellite cells do not express Myf5, for reasons that have yet to be elucidated.

Satellite Cells: From Activation to Proliferation

Upon activation of satellite cells, expression of Pax3, Pax7, and Myf5 is retained but MyoD is now expressed as well, which is responsible for proliferation (Brown et al., 2018). Most proliferating cells suppress Pax7 expression and up-regulate MyoD before differentiation; however, a small population maintains Pax7 expression, repress MyoD and return to quiescence. Proliferating satellite cells will begin to differentiate, during which all MRFs stop being expressed, except for Myogenin which is up regulated. In fact, Myogenin is known to play a role in the downregulation of Pax7 expression.

Satellite cells are not a homogenous population. Ten percent are stem cells and 90% are progenitor cells. The stem cells express Pax7+/Myf5- and can divide asynchronously, producing both stem and progenitor cells. This is due to miR-49, which is found in quiescent stem cells. MiR-49 inhibits Dek, keeping the stem cell from becoming a progenitor cell. Progenitor cells express Pax7+/Myf5+ and differentiate into muscle cells.

One of the key factors that releases the satellite cell from the G_O phase into the G_1 phase is Cyclin D1. Cyclin D1 is regulated in part by hepatic growth factor as well as IL-6 (Serrano, Baeza-Raja, Perduguero, Jardi & Munoz-Canoves, 2008). It is heavily expressed during proliferation of the satellite cell and suppresses the differentiation function of MyoD (Skapek, Rhee, Spicer & Lassar, 1995). Inflammatory cytokines and oxidative stress can initiate p38 activation which in turn activates MyoD transcription of Cyclin D1 inhibitors (Palacios et al, 2010).

Once the cells undergo multiple cycles of proliferation, terminal differentiation of the satellite cells is initiated by Myogenin and Myf6/Mrf4 (Brown et al., 2018; Smith, Janney & Allen, 1994).

Labeling and Identifying Satellite Cells

Fluorescence microscopy is often used traditionally by tagging Pax7. However, solely labeling Pax7 to confirm the state of the satellite cell is insufficient. By further labeling MyoD, the status of the cell can be observed as quiescent, proliferating or differentiating. If the satellite cell is quiescent or activated, it will not express MyoD.

Satellite Cells and Muscle Mass

Increases and decreases in mass are accompanied by alterations in muscle DNA content. This suggests that the ratio of muscle DNA-to-cytoplasmic volume remains constant, which is referred to as the myonuclear domain. Because myonuclei are post-mitotic, increases in muscle mass must be dependent on satellite cells to maintain constant myonuclear domain. By differentiating and fusing with muscle cells, satellite cells provide genetic machinery for increases in protein synthesis.

Satellite Cells and Cancer Cachexia

While less appreciated than the more common acute injuries to skeletal muscle, muscle atrophy is also related to muscle damage, a point that will be discussed in this section. Many conditions of atrophy, including cancer, have been associated with the impairment of muscle regeneration (Talbert & Guttridge, 2016). It has been shown that the sarcolemma of muscle fibers become damaged due to factors released by tumors (Brown et al., 2018). There appears to be a large increase in Pax7 protein expression in cachectic muscle, and He et al. (2016) showed that depletion of Pax7 was sufficient to attenuate muscle wasting by rescuing the muscle regeneration program. This demonstrated the contribution of Pax7 dysregulation to cancer cachexia; however, the mechanism by which this occurs is still unknown.

The ability of satellite cells to be activated does not seem to be the limiting factor in the impairment of muscle regeneration. For various atrophy conditions including cancer, there is an increase in muscle precursor cell number as muscle mass is lost. There are some who have suggested that this may be a result of a fusion defect, preventing myoblasts from repairing the damage (Talbert et al., 2016). He et al (2016) also showed that muscle precursor cells (MPCs) isolated from healthy mice fuse into injured muscle of cancerous mice at a reduced frequency compared to regenerating muscle in non-cancerous mice, suggesting that the environment of cachectic muscle is not conducive to myoblast fusion. Strengthening this hypothesis, upon removing the tumor these cells readily fused into muscle fibers. Another finding showed that tumor factors can induce satellite cell apoptosis in vitro, which may lead to a decrease in overall satellite cell number.

In addition to environmental factors, satellite cell protein expression seems to become dysfunctional and thus affect regeneration. Muscles from cancerous mice show large increases in Pax7 protein levels without increases in other myogenic transcription factor coding genes like MyoD and Myogenin, which in healthy cells would be induced to regulate the completion of proliferation and differentiation, respectively (Brown et al., 2018; He et al., 2016). An increase in the ratio of Pax7 to MyoD can prevent or delay the expression of Myogenin and differentiation. Thus, this dysregulation in Pax7/MyoD or Myogenin expression can lead to a block in muscle regeneration by keeping cells from eventually fusing into muscle fibers.

One possible reason for this alteration in Pax7+ expression is the NF-κB signaling pathway. This signaling is activated by TNF-α in cachectic muscle fibers via the degradation of NF-κB's inhibitor and has been shown to negatively regulate differentiation. He et al. (2016) showed that while NF-κB activity is absent in quiescent satellite cells, it is highly induced within

other Pax7+ cells in tumor-bearing mice. They also found that deletion of NF-κB signaling from Pax7+ cells is sufficient to rescue muscle wasting. This was associated with increased fusion of myoblasts into myofibers. Several inflammatory factors such as Tumor necrosis factor-α, angiotensin II, and myostatin have been linked to cancer cachexia and have been associated with NF-κB activity (Li, Schwartz, Waddell, Holloway & Reid, 1998; Sriram et al., 2011). Inflammation will be discussed in more detail later, including its potential role in cancer cachexia.

Inflammation

This section will discuss, in depth, the role of inflammation for skeletal muscle maintenance, the effect of leucine on inflammation, and current scientific observations of inflammation in response to cancer and its subsequent role in cancer cachexia.

As previously discussed, skeletal muscle is a highly plastic tissue, capable of remarkable adaptive changes in response to physiological stimuli. Inflammation plays an indirect role by cleaning damaged tissue and releasing factors that, among other factors, activate protein synthesis. In addition, proper inflammatory processes have a critical role in the guidance of satellite cell function and thus, muscle regeneration (Tidball, 2005). Therefore, healthy inflammation responses during skeletal muscle adaptations is vital for proper structural and functional maintenance.

Leukocytes, immune cells recruited to tissues via inflammation, are activated by damage to the tissue (Honda, Kimua & Rostami, 1990). Neutrophils will respond immediately to the site of damage due to chemoattractants released by the activated macrophages (Brigitte et al., 2010). These immune cells are vital for releasing pro-inflammatory cytokines such as TNFα, Il-6, IL-β and IFNγ and IL-10 (Puppa et al., 2014). During this time, an increase in activated, MyoD-

expressing satellite cells is also observed with the possibility of IFNγ as a candidate for key regulation of the inflammatory response (Cheng, Nguyen, Fantuzzi & Koh, 2008). Specifically, IFNγ has been shown to bind to myogenic precursor cells, which initiates the JAK/STAT pathway resulting in a proliferative, non-differentiating satellite cell. IL-6, a neutrophil- and muscle-released cytokine, also activates the JAK/STAT pathway (Carson et al., 2010). This leads to the subsequent activation and proliferation of satellite cells via regulation of cyclin D1 (Serrano et al., 2008).

In addition to IFNγ, TNFα has also been heavily implicated in its role for regulating myogenesis. Macrophages and monocytes release the biggest percentage of TNFα in the muscle microenvironment acutely following injury. TNFα plays a role in silencing Pax7 via MAPK signaling, resulting in more prominent differentiation of the satellite cells. TNFα has also been shown to increase differentiation of satellite cells by silencing the Notch-1 gene, which suppresses MyoD and Myogenin expression. It has been shown to suppress Notch-1 signaling, which suppresses MyoD and Myogenin expression thereby reducing differentiation and increasing proliferation of satellite cells (Acharyya et al., 2010).

In regenerating muscle, IL-6 is produced by infiltrating macrophages and neutrophils (Zhang et al., 2013), fibro-adipogenic progenitors (Joe et al., 2010), and satellite cells (Kami & Senba, 1998). It has also been shown that human myoblasts release IL-6 in response to other cytokines involved in the inflammatory process (Gallucci, Provenzano, Mazzarelli, Scuderi & Bartoccioni, 1998). The release of IL-6 might trigger and control the actions of satellite cells throughout the myogenic process, leading to proliferation, and possibly differentiation and fusion (Gao et al., 2017). Similarly, myoblasts derived from IL-6 null mice have displayed reduced differentiation and fusion capacities in vitro (Hoene, Runge, Haring, Schleicher & Weigert,

2013). Thus, IL-6 appears to have a crucial role in myogenesis. While IL-6 is mostly considered a pro-inflammatory cytokine, it also has been shown to have anti-inflammatory effects and to improve systemic insulin action (Wunderlich et al., 2010), which has a role in skeletal muscle protein synthesis.

Inflammation and Cancer

Clearly, inflammation is a key regulatory factor in skeletal muscle maintenance. Chronic inflammation, however, can disturb protein turnover and oxidative metabolism, both of which have been implicated in muscle wasting (Hardee et al., 2018; VanderVeen et al., 2017). Both systemic and muscle inflammation have been studied for their regulation of muscle wasting in cancer. The following section will discuss the current literature on the inflammatory factors in response to cancer and their role in cancer cachexia.

As mentioned previously, one of the hallmarks of cachexia is elevated, chronic inflammation (VanderVeen et al., 2017). Widely studied mediators of cancer-induced wasting include pro-inflammatory cytokines such as Tumor Necrosis Factor α (TNFα), Interleukin-6 (IL-6), transforming growth factor-β (TGF-β), IFNγ, and more (Cruz et al., 2017;VanderVeen, Fix & Carson, 2017; VanderVeen, Hardee, Fix & Carson, 2018). These have been shown to be up regulated in many cancer cachexia preclinical models (Cruz et al., 2017; Hardee et al., 2018; VanderVeen, Fix & Carson, 2017). While these all seem to be associated with cancer cachexia, IL-6 has been identified as a driver of cachexia through the interaction with cancer environments (Carson & Baltgalvis, 2010; Hardee et al., 2018). Indeed, disrupted protein turnover and metabolic homeostasis in the $APC^{Min/+}$ mouse, a well-studied cancer cachexia model, is dependent on elevated circulating IL-6 (Hardee et al., 2018; VanderVeen et al., 2018; Carson et al., 2010). The effects of IL-6 on protein turnover seem to, at least in part, be due to a dose-

dependent suppression of mTOR in $APC^{Min/+}$ mice, as well as its effect on the expression of lysosomal and ubiquitin-related mRNA and proteins (Hardee et al., 2018; White et al., 2011).

Muscle inflammatory signaling has also been investigated for the disruption of protein turnover and metabolism in cancer cachexia (Narsale et al., 2016). Chronic activation of signal transducer and activator of transcription 3 (STAT3), NF-***κ***B, p38, ERK1/2, and protein kinase B (Akt) signaling in muscle have all been implicated in muscle wasting (Brown et al., 2018; Hardee et al. 2018; Narsale et al., 2016; White et al., 2011).

Some interventions have been used to target inflammation specifically and have been shown to modulate the inflammatory response to cachexia. For example, Narsale et al. (2016) demonstrated that short-term administration of pyrrolidine dithiocarbamate, which has both anti-inflammatory and antioxidant properties, rescued signaling pathways involved in turnover regulation. In addition, Lundholm et al. (1994) showed that indomethacin, a cyclooxygenase inhibitor, prolonged survival in cancer patients when compared to a placebo.

Protein Metabolism

Exercise is a common model of muscle damage used in laboratory experiments. The current literature states that resistance exercise promotes muscle adaptation through gene expression and elevated myofibrillar protein synthesis (Bell, Seguin, Parise, Baker & Phillips, 2015). Once genes are transcribed, they then become translated using tRNA. The ribosomal complex is formed, and the peptide chain becomes elongated through the addition of amino acids corresponding to the genetic code. Once the addition of amino acids is terminated, the peptide chain is modified, during which the chain folds and side chains are added.

One major regulator of cell growth is the protein known as mTOR. Two major signaling pathways lead to mTORC1 activation: Mapk/Erk as well as the PI3K/Akt pathways. mTOR has

been implicated in the phosphorylation of eukaryotic translation initiation factor 4E-binding protein 1 (4-EBP1) and ribosomal protein S6 kinase beta-1 (S6K1) (Haegens et al., 2012). There are two types of mTOR: mTORC1, which is involved in protein synthesis and autophagy repression, and mTORC2, which is involved in cell survival, metabolism, and cytoskeletal organization. Several factors regulate mTOR activity including growth factors, hormones, amino acids, energy availability, oxygen and stress.

Raptor and DEPTOR proteins are mTOR-associated binding proteins and seem to be negatively correlated with fractional synthetic rate (Nilsson et al., 2013). DEPTOR is a competitive inhibitor of mTOR and exhibits decreased expression in most cancer types. While this would seem like a positive occurrence, stimulation of mTOR by leucine leads to degradation of DEPTOR, which is necessary for activation of p70S6k, thus suppressing synthesis. REDD 1 also seems to negatively regulate rates of skeletal muscle protein synthesis by suppressing mTOR. In one study, deficiency of REDD1 was shown to enhance signaling through mTORC1 following electrically induced contractions in a mice study (Gordon, Steiner, Lang, Jefferson & Kimball, 2014).

For skeletal muscle to function and adapt, protein synthesis must be accompanied by autophagy. In fact, deficiency in basal autophagy has been shown to induce muscle damage (Lira et al., 2013). The process of autophagy facilitates clearance of dysfunctional, damaged, long-lived or misfolded proteins and/or organelles through the formation of double-membrane structures that form autophagosomes, which fuse with lysosomes to degrade materials (VanderVeen et al., 2017; Lira et al., 2013). There are two types of autophagy in healthy conditions. One is nonselective, which is induced by starvation, and selective, which involves the

specific removal of aggregated proteins and damaged organelles and is required for quality control.

The two major pathways that regulate autophagy are the ubiquitin proteasome and autophagy-lysosome pathways. The ubiquitin proteasome pathway results in the degradation of the greater part of proteins intracellularly and is responsible for regulated proteolysis, progression of the cell cycle, regulation of transcription, and the presentation of antigens (Reid & Li, 2001). Ubiquitin-activating enzyme (E1) first activates ubiquitin and is then shifted to the ubiquitin carrier protein (E2) active site. Ubiquitin conjugating enzymes (E3 or E3 protein ligase) are recognized by the bound E2, which allows the occurrence of conjugation reactions by the formation of ubiquitin chain by attaching with each other and the protein substrate. The proteasome can recognize a selected protein only after it is targeted by ubiquitin and then processed into smaller peptides (Glickman & Ceichanover, 2002). During proteolysis, there are three E3 protein ligases which are active in atrophy in muscle, namely E3α, MAFbx/atrogin-1 and MuRF1 (Bodine et al., 2001).

Protein Metabolism and Cancer Cachexia

Cancer cachexia has been shown to be partially mediated by altered protein metabolism. Part of this is due to reduced muscle protein synthesis (Hardee et al., 2018; Brown et al., 2018). For instance, IGF-1 expression and mTOR targets are suppressed in cancer cachexia (White et al., 2011), while AMPK and Raptor phosphorylation are increased as cachexia progresses (VanderVeen et al., 2017). However, Penna et al. (2010) demonstrated that muscle atrophy might be independent of IGF-1 activity, and IGF-1 and insulin treatment have not been shown to attenuate muscle wasting in cancer cachexia (Lazarus, Kambayashi, Lowry & Strassman, 1996).

One anabolic protein, mTOR, has conflicting evidence in the literature. mTOR activity has been shown to decrease with the development and progression of cachexia with $APC^{Min/+}$ mice as well as mice bearing Lewis cell carcinoma (White et al., 2011; Puppa, Gao, Narsale & Carson, 2014), mediated through activation of AMPK by IL-6 (Hardee et al., 2018). However, mTORC1 inhibition has been reported to prevent muscle loss in cancer cachexia (Pigna et al., 2016). In fact, pharmaceutical intervention with rapamycin restored autophagy in tumor-bearing mice and prevented cachexia (Pigna et al., 2016). The results of another study suggested that mTOR regulates the pro-cachectic factors IL-10 and IL-6 (Robert et al., 2012; Hatakeyama et al., 2016). On the other hand, Baselga et al. (2012) found that treatment of cancer patients with everolimus, an mTOR inhibitor, was associated with decreased weight and decreased appetite, showing that mTOR inhibitors may worsen cachexia. Given this evidence, it is unclear whether mTOR activation attenuates or contributes to cancer cachexia.

In addition to the dysregulation of anabolic pathways, autophagy has been found to be activated in muscles of cachectic animals (Brown et al., 2018). This activation might be a response to the inflammatory, nutritional and energy stresses induced by cancer growth to sustain cell survival. Whether this response contributes to cachexia is still unclear, however.

IL-6, a cytokine known to be up-regulated in cancer environments, is associated with both growth and atrophy (Gao et al., 2017). Under physiological conditions, IL-6 is an important regulator of hypertrophy. Gao et al. (2017) demonstrated that IL-6 administration to C2C12 myotubes induced protein synthesis in a dose-dependent manner, in which the positive effects seemed to diminish at higher concentrations of this cytokine. This protein synthesis was accompanied by STAT3 activation, Akt-mTORC1 activation, and increased SOCS3 expression. In another study, administration of IL-6 receptor antibodies has been shown to suppress the rate

of cachexia, however it was not enough to reverse the condition (White et al., 2011). There has also been some indication that IL-6 may have a role in mitophagy regulation (VanderVeen et al., 2017).

As mentioned previously, several pro-inflammatory cytokines are significantly up regulated in cancer patients. One such cytokine is TNF-α, which has multiple roles in muscle physiology. It is known that TNF-α can increase satellite cell differentiation, however it is also able to induce apoptotic cell death, cachectic condition and the inflammatory response. In addition to reducing food intake, it has been shown that TNF-α has an important role in cachectic muscle wasting through protein degradation (Li et al., 1998; Li & Reid, 2000). In fact, one study showed that mice lacking the TNF-α receptor protein type 1 had decreased muscle wasting when transplanted with Lewis lung carcinoma (Llovera et al., 1998). This effect is thought to be mediated through activation of the ubiquitin proteasome pathway through NF-κB signaling (Li & Reid, 2000; Reid & Li, 2001).

The ubiquitin proteasome pathway has been shown to be stimulated in cachectic patients (Khal, Wyke, Russell, Hine & Tisdale, 2005). Specifically, Atrogin-1/MAFbx and MuRF1 are strongly up-regulated in several models of cancer cachexia (Brown et al., 2018; Carson et al., 2010). Atrogin-1 promotes degradation of MyoD and eIF3-f, an activator of protein synthesis (Csibi et al., 2010; Tintignac et al., 2005), while MuRF1 ubiquitinates several structural proteins (Fielitz et al., 2007; Polge et al., 2011; Cohen et al., 2009).

One E3 ubiquitin ligase that found to play a role in cancer cachexia is TRAF6 (Narsale et al., 2016), which is found to be induced in gastric cancer patients and is a potent inducer of mitophagy (VanderVeen et al., 2017). TRAF6 knockout mice are resistant to muscle loss induced

by cancer (Paul et al., 2010) and have reduced Atrogin-1 and MuRF1 expression. TRAF6 is also necessary for optimal activation of JNK, AMPK, FoxO3 and NF-κB (Paul et al., 2012).

Mitochondria

Mitochondria are organelles that are essential for a variety of processes such as oxidative phosphorylation, ATP generation, autophagy, protein turnover, calcium signaling and apoptosis (Carson et al., 2018, 18). Oxidative metabolism is directly tied to mitochondrial content and is used in fiber type classification (Carson et al., 2016; VanderVeen et al., 2017).

The changes in mitochondrial health is partly achieved by the interplay between fusion and fission (Carson et al., 2016). Fusion of mitochondrial membranes facilitates optimal ATP production by enhancing mitochondrial networks and preventing mitochondrial DNA loss, while fission contributes to the removal of damaged mitochondria. Fusion is regulated by fusion proteins 1 and 2 (Mfn1/2) and optic atrophy protein 1 (OPA1) (Brown et al., 2017), the levels of which decrease with the increase of IL-6 (Carson et al., 2016). Fission is regulated through dynamin-related protein-1 (DRP1) and fission protein 1 (Fis1) expression (Carson et al., 2016).

Alongside the numerous mitochondrial processes, reactive oxygen species (ROS) are also produced by these organelles. ROS are involved in the maintenance of many signaling pathways (Bae, Oh, Rhee & Yoo, 2011). An overabundance of ROS can also lead to cellular and DNA damage, protein oxidation and apoptosis (Brown et al., 2017; VanderVeen et al., 2017). To avoid the buildup of damaged mitochondria, mitochondrial proteins damaged by ROS can be broken down by specialized mitochondrial enzymes (Smith, Hartley, Cocheme & Muphy, 2012). If the damage has not led to severe loss of function, mitochondria with damaged mitochondrial DNA can fuse with healthier mitochondria to borrow and/or transfer missing components (Youle & van der Bliek, 2012). Mitochondria that have accumulated greater damage and cannot maintain

function, are subject to fission, followed by mitophagy (VanderVeen et al., 2017). Following this process, mitochondria are replaced by active mitochondria produced in mitochondrial biogenesis.

Mitochondria in Cachectic Environments

Mitochondrial loss and dysfunction have been well characterized in many cachectic conditions and has been tied to muscle fatigability, weakness, and dysregulated protein turnover (Brown et al, 2017; Carson et al., 2016; VanderVeen et al., 2017). In fact, cachectic muscle wasting has been shown to primarily occur in glycolytic muscle when compared to more oxidative muscle (Brown et al., 2017; Carson et al., 2010).

Mitochondrial content and quality are regulated by mitochondrial biogenesis, fission/fusion, and mitophagy. Part of the dysregulation of mitochondrial processes associated with cachexia is the suppression of Mfn1/2 during the initial stages of this condition (VanderVeen et al., 2017). In addition, there is a decrease in Opa1 and Drp1 content, as well as an increase in Fis1 by the fourth week of tumor development (Brown et al., 2017). This suggests that cancer-induced disruption of muscle oxidative capacity begins early in the process of cachexia development and is accompanied by an increase in ROS production, along with an increase in fragmented mitochondria (Brown et al. 2017; VanderVeen et al., 2017).

Removal of damaged mitochondria through autophagy is essential to the maintenance of mitochondrial quality (Carson et al., 2016). This process is known as mitophagy and is linked to mitochondrial dynamics and health (Carson et al., 2016). Indeed, deletion of the autophagic gene Atg7 results in atrophy, mitochondrial abnormalities, and disorganization of sarcomeres (Carson et al., 2016). In contribution to the mitochondrial dysregulation seen with cachexia, it has been seen in $APC^{Min/+}$ mice that expression of autophagy proteins does not increase until severe

cachexia has developed (Carson et al., 2016); however, the mitophagy protein Bnip3 has been seen to increase in $APC^{Min/+}$ and LLC tumor-bearing mice (Brown et al, 2017).

The accumulation of dysfunctional mitochondria leads to the increase of ROS, which may instigate the muscle wasting observed in cachectic mice as it has been shown to promote catabolic functions such as the ubiquitin system (Li & Reid, 2000; Carson et al., 2016; VanderVeen et al., 2017). There are three mechanisms by which ROS may contribute to the increased rate of muscle wasting: (1) oxidative stress leads to calcium overload and activation of calcium-dependent proteases such as calpain; (2) oxidative stress stimulates the 20S proteasome system via the activation of caspase-3; (3) oxidative stress up-regulates expression of ubiquitin E3 ligases through NF-κB (Li & Reid, 2000). Further investigation is needed to determine through which ROS-induced mechanism cancer might instigate muscle wasting.

Mitochondria and Leucine

Few studies have been done on the effect of leucine supplementation on mitochondrial biogenesis, dynamics, and mitophagy in the context of cancer cachexia. It has been shown to increase mitochondrial biogenesis through the increase in CoxIV mRNA (Liang 2014). Whether leucine increases PGC1α remains unclear. It has been shown by Liang et al. (2014) that leucine increases AMPK activity, which in turn activates PGC1α. In addition, several studies have demonstrated leucine's effect to increase PGC1α mRNA (Jiao, 2016; Junior, 2016; Liang 2014; Lee, 2019), however a 6-week study on the effects of leucine on rats fed a high-fat diet found that PGC1α decreased (Baum et al., 2016).

As far as we know there has not been any data regarding the effects of leucine on mitochondrial dynamics, however we can begin to speculate the effect of leucine on mitochondrial degradation, content, and quality. As most studies found that PGC1α increases

with leucine supplementation, which besides being a regulatory factor of mitochondrial biogenesis is also known to suppress the expression of atrogenes. Leucine is also known for its inhibitory effect on proteasome, calpain and cathepsin-dependent degradative pathways. As some of these processes are involved in mitophagy, it may be reasonable to hypothesize that leucine supplementation decreases the rate of mitophagy. In combination with the upregulation of mitochondrial biogenesis, this should lead to the increase of mitochondrial content. Indeed, Liang et al. (2014) found that leucine supplementation increased this content in C2C12 myotubes. As for whether this increase in content is accompanied by an improvement in mitochondrial quality is also a matter of speculation at this point. However, as leucine supplementation has been associated with a decrease in ROS production. To support this concept, it PCG1α is a known regulator of ROS defense systems, and PGC1α has been shown to increase with leucine supplementation.

Strategies for Attenuation of Cachexia

Several studies have been conducted to prevent and/or treat cancer cachexia. For instance, STAT3 and the myostatin pathway have been indicated as possible therapeutic targets for the preservation of skeletal muscle in cachexia (Bonetto et al., 2012; Busquets et al., 2012). ActRII inhibitors have shown to be a promising intervention for cancer cachexia (Hatakeyama et al., 2016). IGF-1 administration has been studied by Costelli et al. (2006) as a possible method of treatment, however it did not prevent cachexia.

Supplementation of leucine and HMB, a leucine metabolite, have also been studied. One study on the effects of HMB for 24 days attenuated muscle and body weight loss on an 8-day AH-130 injection model of cancer cachexia (Aversa et al., 2011). In studies performed by Gomes-Marcondes et al. (2003) and Cruz et al. (2017), 12 and 21 days, respectively, of leucine

supplementation in tumor-bearing rats showed improvements in muscle mass and protein content; however, Gomes-Marcondes also found that leucine supplementation accelerated tumor growth. Peters et al. (2011), Salomao et al. (2012), and Viana et al. (2015) all found a similar result. One of the few studies involving longer supplementation (6 weeks) with leucine showed that leucine decreased muscle mass in rats fed a high-fat diet (Baum et al., 2016). In addition, Lee et al. (2019) demonstrated that muscle mass loss was exacerbated in LLC tumor-bearing mice after 28 days of low-dose leucine supplementation. Further investigations of long-term leucine supplementation are needed to determine whether it is a viable treatment for cancer cachexia.

Leucine Supplementation

A plethora of data have shown the branched-chain amino acid leucine to initiate the greatest anabolic signaling response through the mTORC1-p70S6K1 pathway (Anthony, Anthony, Kimball & Jefforson, 2001; Haegens et al., 2012). However, this is not the only mechanism through which leucine has been investigated regarding its role in muscle mass regulation. Here we will discuss leucin supplementation as it pertains to satellite cells, inflammation, and protein metabolism.

Satellite Cells with Leucine Supplementation

The research done on the effects of leucine supplementation on satellite cells is promising. Wan et al. (2017) showed in a lipopolysaccharide model of muscle wasting that leucine supplementation was associated with a decrease in NF-κB activity, which shows promise for the attenuation of Pax7 dysregulation. Indeed, Pereira et al. (2015) showed that leucine supplementation in old rats is associated with an increase in satellite cell proliferation and a decrease in the ratio of Pax7 to MyoD.

Inflammation and Leucine

The investigation of the effect of leucine on inflammation is somewhat limited. One study showed leucine to attenuate muscle protein degradation in tumor-bearing rats (Cruz et al., 2017). However, this study only investigated the effects of leucine over a short time frame (21 days). This study found that leucine increased pro-inflammatory cytokines in tumor-bearing rats, including TNFα, IL-6, and IFNγ, as well as the TNFα-IL10 ratio by day 14, however they all returned to levels similar to day 7, with the exception of IFN-$\boldsymbol{\gamma}$. For a short period of time, this may help with the myogenic processes of satellite cells, as well as attenuate the progression of the cancer itself. It is not known, however, whether the elevation of IFN-$\boldsymbol{\gamma}$ would continue with prolonged leucine supplementation, or whether this would prove to be beneficial or detrimental.

Protein Metabolism and Leucine

There is a lot of conflicting evidence regarding the effects of leucine on protein metabolism. However, it is known to affect skeletal muscle anabolism through phosphorylation of mTOR and its downstream targets (Haegens et al., 2012; Deldicque et al., 2008). Increased leucine intake has also been shown to preserve lean mass and increase circulating levels of IGF-1 in calorie-restricted rats (Pedroso, Nishimura, de Matoso-Neto, Donato & Tirapequi, 2014). This does not directly link IGF-1 with lean mass preservation; however, this could be evidence that leucine activates mTOR through multiple pathways. This activation of mTOR may not be a positive attribute, however, as leucine-stimulated mTOR leads to the degradation of DEPTOR, which is necessary for the activation of the important synthetic factor p70S6k.

There is also conflicting evidence regarding the effects of leucine on glucose handling and insulin sensitivity. Junior et al. (2015) found that leucine supplementation worsened glucose homeostasis in sedentary and exercised mice. However, Baum et al. (2005) demonstrated that

leucine, both alone and in conjunction with carbohydrate, had minimal effect on glucose uptake in rats. The only difference both studies found in leucine's effect on insulin signaling was a blunted duration of insulin-induced PI 3-kinase activity when it was combined with carbohydrate (Baum et al., 2005) and a decrease in insulin-stimulated AMPK phosphorylation (Junior et al., 2011). Thus, while the effects of leucine on glucose sensitivity is yet under scrutiny, it does appear to lessen the effects of insulin on pathways that regulate protein metabolism.

Leucine supplementation has also been shown to alleviate dexamethasone-suppressed protein synthesis (Wang et al., 2016). In this study, the improvement in protein synthesis was accompanied by increased phosphorylation of mTOR and decreased phosphorylation of AMPK, a factor known to be up regulated with cancer cachexia.

A leucine-rich diet was shown to improve the cancer-induced down-regulation of the mTOR signaling pathway, rescuing mTOR and p70 activation in tumor-bearing mice (Viana & Gomes-Marcondes, 2015). The effects of leucine supplementation on SOCS3 has yet to be studied with respect to skeletal muscle, however expression was decreased in adipose tissues of rat fed a high-fat diet and didn't change in the hypothalamus of lactating rats (Lopez, Sanchez, Pico, Palou & Serra, 2010; Yuan, Han, Zhang, Xu & Qin, 2015).

In two separate studies, chronic leucine supplementation has been shown to decrease the expression of TNF-α in rats on a high-fat diet (Jiao et al., 2016; Li et al., 2013). In addition, in vitro incubation of muscle fibers showed a decrease in NF-$\boldsymbol{\kappa}$B activity, through which it is thought TNF-α activates the ubiquitin-proteasome pathway (Li et al, 2000). However, Torres-Leal et al. (2011) found that leucine supplementation had no effect on TNF-α in rats that have transitioned from a high-fat to low-fat diet.

Two studies have shown that leucine significantly attenuates Atrogin-1 and MuRF-1 gene expression in immobilized rats (Baptista et al., 2010; Baptista et al., 2013). Whether this is beneficial or not remains to be seen. This inhibition of the ubiquitin pathway could attenuate the imbalance between protein synthesis and degradation seen in cancer cachexia. However, it has been suggested that mitochondrial quality is partly regulated by the ubiquitin system (Lehmann, Udasin & Ciechanover, 2016). Impaired mitochondrial quality control has been found in cancer cachexia, in which the accumulation of reactive oxygen species may contribute to the weight loss found in cachexia (Brown et al., 2017). Thus, reducing the activation of the ubiquitin proteasome pathway may lead to exacerbating cancer cachexia.

Models of Cachexia

The diverse models of cancer cachexia can be divided into two categories: injection and genetic. Two injection models include Walker tumor injections and Lewis Lung Carcinoma (LLC) injections. Both cell lines can be cultured and expanded in any lab and can quickly cause the development of the cachectic phenotype (Pin et al., 2015). The LLC model is characterized by a loss of muscle mass and strength and develops the cachectic state by about four weeks (Brown et al., 2017). Using this model, mitochondrial health impairment has been demonstrated well before the onset of measurable atrophy in cancer cachexia (Brown et al., 2017). One drawback to consider is the ratio of tumor size to body weight. In implantation models, tumors can be up to 10% of an animal's body weight, which is a condition not normally found in human cancer or cachexia. This could place confounding inflammatory and metabolic challenges on the animal (Carson et al., 2010).

In contrast to injection cancer models, genetic models occur more gradually. One of the most well-studied genetic models of cancer cachexia is the $APC^{Min/+}$ mouse (Hardee et al., 2018;

Brown et al., 2017; Carson et al., 2010; Narsale et al., 2016). This strain has a genetic mutation in the adenomatous polyposis coli (APC) gene, which is a key tumor suppressor. Mutation of this gene results in the spontaneous development of intestinal and colon tumors by four weeks of age (Carson et al., 2010; Puppa et al., 2011). This model increases in weight similarly to the C57BL/ mouse until ~12 weeks of age, and then body weight decreases steadily between weeks ~12 and ~20 and leads to a 20-25% decrease in bodyweight (Puppa et al., 2011). This model requires more time than injection modalities for the animals to reach the cachectic state, however it does provide a valuable model that mimics human colorectal cancer and lacks anorexia (Baltgavis et al., 2008).

Conclusion

Cancer cachexia is characterized by a drastic, rapid loss in body weight, fat and muscle tissue, resulting in reduced quality of life and functionality (VanderVeen et al., 2017). This is partly due to increased inflammation, protein breakdown, and impaired mitochondria (Brown et al., 2017; VanderVeen et al., 2017). Interventions for treating/preventing cachexia have included, among other treatments, STAT3, myostatin pathway, and ActRII inhibition, and leucine supplementation. Thus far, full cachexia attenuation has not been achieved.

CHAPTER 3: Aims

AIMS

AIM 1: Examine how leucine supplementation affects protein turnover in cachectic mice.

Maintenance of muscle mass depends on the balance between protein synthesis and protein degradation. The first aim of this study will be to see how protein turnover responds to leucine supplementation in cachectic mice. Thus far, the response has yet to be completely elucidated. By performing qRT-PCR and Western Blot we will be able to analyze signaling pathways and mRNA expression related to protein synthesis and degradation.

AIM 2: Examine how leucine supplementation affects satellite cell dynamics.

The disruption of myogenesis during the progression of cancer cachexia is well-recognized (Brown et al., 2018). The effects of long-term leucine supplementation on satellite cell dynamics within the context of cancer cachexia has yet to be studied. To investigate the regulation of satellite cell dynamics in response to leucine supplementation in this cachectic model, we will use PCR to measure factors involved in activation and differentiation.

CHAPTER 4: Manuscript

Preliminary Study: Muscle Wasting is Exacerbated in APC$^{Min/+}$ Mice with Chronic Leucine Supplementation

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ABSTRACT

Preservation of muscle mass is important for the maintenance of quality of life during aging and a variety of diseases, including cancer. Cancer cachexia is characterized by rapid, drastic loss in fat and muscle mass, and is irreversible by conventional nutritional means. It occurs in ~80% of cancer patients and is responsible for 20-40% of cancer-related deaths and is especially prevalent in cancers of the gastrointestinal system (Brown et al., 2018; Brown et al., 2017; Puppa et al., 2011). This condition leaves patients with increased fatigue, loss of functional independence, reduced quality of life, and a decrease in overall survival rates. Cachexia occurs through an imbalance in skeletal muscle protein metabolism, with degradation rates overcoming that of protein synthesis (Brown et al., 2018; Brown et al., 2017). These changes are often in association with an increase in inflammation and an alteration in several metabolic processes.

Several studies have investigated the effects of leucine on cancer cachexia, as this branched-chain amino acid is known for promoting muscle growth through the stimulation of the anabolic signaling cascades while inhibiting catabolic processes. These studies have all been conducted for a short period of time, with the longest being 24 days, and have shown promise for leucine as a treatment for cancer cachexia. Whether leucine is viable as a long-term, chronic treatment requires further investigation. 10 C57BL/6 (WT) mice and 7 APCMin/+ (APC) mice were used in this study and given either tap water as a control or given 1.5% leucine-enriched water. Tissue harvest was conducted at 20 weeks of age for gene expression and western blot analysis. There was a main effect of genotype for lower body and plantaris weight in APC mice when compared to WT ($p < 0.05$), and a main effect of leucine for a lower plantaris/tibia length in APC mice ($p < 0.05$), which appeared to be driven by the $APC^{Min/+}$ genotype ($p = 0.0841$). Cyclin D1 mRNA abundance was ~2-fold greater in APC mice compared to WT, although no

difference was found with leucine supplementation. No difference was found in MyoD or Myogenin mRNA abundance. Long-term chronic leucine supplementation appears to exacerbate body weight and muscle mass loss. Our data suggest that this is independent of protein turnover or myogenesis disruption.

INTRODUCTION

In addition to mass, skeletal muscle has several properties that impart health benefits to cancer-ridden organisms. Skeletal muscle quality is aligned with metabolic capacity and substrate utilization flexibility (Carson, Hardee & VanderVeen, 2016). Skeletal muscle is heavily involved in metabolic capacity and substrate utilization flexibility and serves an endocrine function through the production and secretion of myokines (Carson et al., 2016). Muscle health can in part be controlled through the regulation of protein turnover, which responds to a variety of stimuli. These stimuli work through intricate cellular signaling pathways involving several organelles and structures, all of which can become dysregulated in cancerous conditions.

Many types of cancer are accompanied by a rapid, drastic loss in skeletal muscle tissue, which negatively affects mortality and quality of life in cancer patients. This condition, known as cancer cachexia, occurs in about 50-85% of colorectal, pancreatic and lung cancer patients and is inversely related to survival time (Aversa, et al., 2011; VanderVeen et al., 2017). Consequently, it is one of the most devastating effects of cancer and has a direct correlation with tumor development (the degree of which varies with the type of cancer) (Salomao & Gomes-Marcondes, 2012). This drastic loss in muscle tissue can contribute to a decline in functional status and reduction in respiratory function (Aversa, et al., 2011). Not only does atrophy affect the patient's survival time, it also impacts the patient's response to chemotherapy and increases susceptibility to chemotherapy-induced toxicity, as well as decreasing the patient's ability to withstand infection (Van Norren, et al., 2009).

Clearly, preventive measures are important and thus continue to be researched. However, nutritional therapies have yet to prove effective and there are currently no approved treatments for cancer cachexia due to the complexity and variability of the condition (Assi et al., 2016;

Brown et al., 2018). Many recent studies have investigated the complex processes involved in cancer cachexia, as well as a myriad of potential treatments. However, the main obstacle to treatment of this condition is that it is multifaceted. This condition is associated with anorexia, altered oxidative metabolism, insulin resistance, systemic inflammation, and disrupted protein turnover. Thus far no treatments have been found that address all contributing factors.

Some studies have shown promise, such as the study on ERK inhibition done by Penna et al. (2010), however this was a short-term experiment (4-13 days) and it is unknown how chronic inhibition of this protein would affect an organism with cancer. Studies targeting inflammation have been inconclusive (Maddedu et al., 2012; Wu, Fernandez & Criswell, 2013), and there are few studies involving oxidative metabolism. Narsale et al (2016) demonstrated that short-term antioxidant treatment rescued signaling for protein turnover and increased body weight and muscle mass in the $APC^{Min/+}$ mouse. However, Assi et al. (2016) showed that antioxidant treatment not only failed to attenuate the development of cachexia but also promoted tumor growth and premature death. Lastly, the role of protein turnover in the attenuation of cancer cachexia has also been studied. Specifically, the stimulation of skeletal muscle protein synthesis has been of great interest, through the major protein mTOR.

The cell signaling protein mTOR is known to be stimulated by the Branched Chain Amino Acid (BCAA) called leucine (Lee et al., 2019). Through inhibition of catabolism and the stimulation of mTOR, leucine is well known for its anabolic and mass-saving effects on skeletal muscle (Stipanuk, 2007). This BCAA has also been shown to prevent mitochondrial dysfunction in high-fat diet-induced obese mice (Li et al., 2012). While leucine has been shown to attenuate progression of cancer cachexia, these studies have only included short-term supplementation (12-

21 days) with this amino acid (Baptista et al., 2010; Cruz, Oliveira & Gomes-Marcondes, 2017; Peters et al., 2011).

To study cancer cachexia and its possible treatments, a variety of murine models are used. The diverse models of cancer cachexia can be divided into two categories: injection and genetic. One well-studied injection model is the LLC-induced cancer cachexia. This cell line can be easily cultured and expanded in any lab, and it takes four weeks for the cachectic phenotype to develop (Pin et al., 2015). The LLC model is characterized by a loss of muscle mass and strength and can develop the cachectic state by four weeks (Brown et al., 2017). Using this model, mitochondrial health impairment has been demonstrated well before the onset of measurable muscle wasting in cancer cachexia (Brown et al., 2017).

In contrast to injection cancer models, genetic models occur more gradually. One of the most well-studied genetic models of cancer cachexia is the $APC^{Min/+}$ mouse (Hardee et al., 2018). This strain has a genetic mutation in the adenomatous polyposis coli (APC) gene, which is a key tumor suppressor. Mutation of this gene results in the spontaneous development of intestinal and colon tumors (Carson et al., 2010; Puppa et al., 2011). This model requires more time than injection modalities for the animals to begin developing tumors, and even longer for them to develop the cachectic state. It does, however, mimic several characteristics found in human colorectal cancer and lacks anorexia (Baltgavis et al., 2008).

While the importance of treating cancer cachexia is well known, a viable treatment has yet to be found. Leucine supplementation has been particularly promising; however, administration of this amino acid has been up to three weeks at most. Therefore, the purpose of this study was to investigate the effect of long-term, chronic leucine supplementation on $APC^{Min/+}$ mice, which have a genetic mutation that leads to colorectal cancer and cachexia. We

hypothesized that leucine would attenuate the development of cancer cachexia through the stimulation of mTOR and the inhibition of catabolism. We demonstrate that chronic leucine administration not only exacerbated the cachectic condition but seemed to shorten the lifespan of tumor-bearing mice. These data suggest that leucine may not be a viable treatment option for cancer patients, although further studies are required to determine if other administration schedules have promise.

METHODS

Animals

10 male C57BL/6 (WT) and 10 $APC^{Min/+}$ (APC) mice were used for this study. APC and WT mice were randomly assigned to either no leucine (WTNL, n=5 and APCNL, n=5) or leucine supplemented (WTL, n=5 and APCL=5) groups. Mice were group housed in the University of Arkansas Central Laboratory Animal Facility. Animals were kept on a 12:12-hour light-dark cycle in a climate-controlled room with *ad libitum* access to normal chow and water throughout the study. Once weaned, animals in the NL group received normal water, while L groups were given 1.5% leucine-rich water (Perry et al., 2016). Food and water mass consumption were recorded weekly. Three APCL mice died before harvest, so n=2 was used for this group and n=7 was used for the APC genotype group in our data collection and statistical analysis. There was no difference in water consumption between NL and L (p=.9391) (Figure 1). At twenty weeks of age, tissue harvest was conducted. All methods used in this study were approved by the University of Arkansas Institutional Animal Care and Use Committee (IACUC).

Tissue Harvest

Mice were anesthetized with a subcutaneous injection of a cocktail containing ketamine hydrochloride (90 mg/kg body weight), xylaxine (3 mg/kg body weight), and acepromozine (1 mg/kg body weight). The plantaris was immediately flash frozen in liquid nitrogen and stored at -80°C for protein and gene expression analysis. Following removal of the muscles, tibias of both legs were extracted and measured via caliper. All collected tissue was stored in -80°C until processed for analyses.

RNA Isolation, cDNA synthesis, and Quantitative Real-Time PCR

RNA was removed from the plantaris muscle using a Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA). RNA was isolated using the Purelink mRNA minikit (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was reverse transcribed from 1 µg of total RNA using the Superscript Vilo cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA). Real-time PCR was performed, and results were analyzed using the StepOne Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was amplified in a 25 µL reaction containing appropriate probes and Taqman Gene Expression Mastermix (Thermo Fisher Scientific). Samples were incubated at 95°C for 4 minutes, followed by 40 cycles of denaturation, annealing and extension at 95°C, 55°C and 72°C, respectively, with fluorescence measured at the end of the extension step each cycle. Fluorescent probes for *18s, IGF-1, MyoD, Myogenin, Atrogin-1, MuRF-1, Cox IV and CCD1* were purchased from Applied Biosystems. RT-qPCR measured cycle threshold (Ct) and the ΔCt value was calculated as the difference between the Ct value and the 18s Ct value. Final quantification of gene expression was calculated using the ΔΔCT method Ct = [ΔCt(calibrator) – ΔCt(sample)]. Relative quantifications were then calculated as $2^{-\Delta\Delta Ct}$.

Western Blot

Protein quantification for protein metabolism was probed and analyzed via Western Blot. Plantaris muscle homogenate was fractioned in 6-15% SDS-polyacrylamide gels. Gels were then transferred to low-fluorescing polyvinylidene difluoride (PVDF) membranes. Membranes were stained with Ponceau S before blotting to verify equal loading of the gels. Membranes were blocked in a 1:1 solution of containing Tris-buffered saline (TBS) with 0.1% Tween®20 (TBST) for 2 hours. Primary antibodies for 4EBP-1 (pan) (#9644), p70S6K (pan) (#9202) and COX-IV (#11967) were purchased from Cell Signaling (MA, US) and were diluted 1:1000 in 5% milk, in TBST and incubated at 4°C overnight. Anti-mouse or anti-rabbit secondary antibodies (Cell Signaling) were diluted at 1:20000 in 5% milk, in TBST and incubated at room temperature for one hour. Fluorescence imaging was performed using the LI-COR Fc to visualize antibody-antigen interaction. Quantification of blotting images was performed through densitometry analysis using Image Studio Lite v. 5.2 software. Ponceau stained membranes were digitally scanned, and 45 kDa actin bands were quantified by densitometry to be used as a protein loading correction factor for each lane.

Statistical Analyses

Data were calculated using Statistical Package for the Social Sciences (SPSS version 23.0, Armonk, NY). All results were reported as mean ± SEM. A priori comparisons between WTNL and APCNL mice were conducted using Student's t-tests. A two-way ANOVA was performed to analyze main effects of genotype and treatment and any interactions between the dependent variables. Where significance was detected, a Fisher's LSD post-hoc analysis was conducted. Statistical significance was determined if $p \leq 0.05$.

RESULTS

Phenotypic Data

As shown in Table 1, at the end of ~20 weeks there was a main effect of genotype for $APC^{Min/+}$ mice to have lower body mass than WT mice ($p < 0.0001$). There was no difference between WT groups. Therefore, leucine treatment had no effect on WT mice body weights. There was a trend for body mass to be lower in APCL when compared to APCNL ($p = 0.0720$).

While no interaction was found, there was a main effect of genotype for lower plantaris weight/tibia length in $APC^{Min/+}$ mice when compared to WT (Table 1, $p < 0.0001$) and a main effect of leucine for lower plantaris weight/tibia length in APCL compared to APCNL ($p < 0.05$). The plantaris weight/tibia length was similar between WTNL and WTL.

Polyp data

After ~20 weeks, the number of polyps of the $APC^{Min/+}$ and WT mice was 46.57 ± 2.44 and 0.00 ± 0.00, respectively. The total number of polyps did not change between APCNL and APCL (Figure 1A). There was no difference in the number of polyps larger than one millimeter. However, there were more polyps under 1mm in the APCL group when compared to APCNL.

Markers of Myogenesis

There was a main effect of genotype for a lower Cyclin D1 mRNA abundance in $APC^{Min/+}$ mice compared to WT (Fig. 2A, $p < 0.05$). No difference was found in MyoD mRNA abundance (Fig 2C). No difference was found in Myogenin mRNA abundance (Fig 2E, $p < 0.05$).

Markers of Protein Turnover

IGF1 mRNA abundance had a trend to be lower in the APCNL group compared to WTNL (Fig3F, $p = 0.0616$), although no difference in IGF-1 mRNA abundance was found with leucine treatment. There was a trend toward higher p70 protein content in APCNL compared to WTNL (Figure 3B, $p = 0.0559$), although no difference in protein content was found in P70 with leucine treatment. There was a main effect of genotype for lower 4EBP1 protein abundance in $APC^{Min/+}$ mice compared to WT (Figure 3C, $p < 0.05$). An eight-fold increase of Atrogin-1 abundance in the APCNL group was found compared to WTNL, WTL and APCL groups (Fig 3D, $p < 0.05$). There was a main effect of genotype for there to be a greater MuRF- 1 mRNA abundance in $APC^{Min/+}$ mice compared to WT. There was a main effect of treatment for there to be less MuRF-1 mRNA abundance in the APCL group compared to APCNL (Fig 3E, $p < 0.05$).

DISCUSSION

The purpose of this study was to investigate whether long-term supplementation of leucine to $APC^{Min/+}$ mice would attenuate the cachectic process associated with the cancer that they develop. Not only did leucine fail to attenuate cancer cachexia, to our knowledge we are among the first to provide evidence suggesting that chronic, long-term leucine treatment seems to exacerbate the loss of body weight and muscle mass. This is in accordance with a finding by Baum et al. (2016) in which rats fed a high-fat diet had lower muscle masses after being fed leucine for 6 weeks, which is longer than any cachexia-related study has been performed to date. It also confirms the findings of Lee et al. (2019), in which muscle mass loss was exacerbated in LLC tumor-bearing mice after 28 days of low-dose leucine supplementation. Based on the results, this effect does not seem to occur due to alterations in myogenesis or protein turnover.

The process of myogenesis is normally triggered by damage to the muscle and then leads to a series of processes involving satellite cell dynamics and overall skeletal muscle repair and, in many cases, hypertrophy. Under cancerous conditions, however, there is some dysregulation in satellite cell dynamics that impedes myogenesis and contributes to muscle wasting (Brown et al., 2018). In this study, we found that genotype had a main effect to decrease Cyclin D1 mRNA abundance, however there was no difference in Myogenin or MyoD. These findings are in agreement with Brown et al. (2018), who also found in their 4-week time course study that Cyclin D1 decreased with the progression of cachexia, and that while Myogenin and MyoD decreased initially, they rebounded to basal levels by week 4.

Evidence regarding the effect of leucine on myogenesis has yet to be confirmed. Haegens et al. (2012) found that leucine supplementation did nothing to affect myogenesis in C2C12 myotubes, and Perry et al. (2016) found no effect of leucine on Myogenin or MyoD in aged or young mice following injury. It has been shown, however, to preserve Cyclin D1 protein abundance in hindlimb suspension models (Bajotto, Sato, Kitaura & Shimomura, 2011), increase MyoD in young and old rats (Pereira et al., 2015), and increase Myogenin in preterm rats (Dai, Yu, Shen, Guo & Qiu, 2015). In contrast, our study showed no change in Cyclin D1, MyoD or Myogenin mRNA abundance in WT or $APC^{Min/+}$ mice. Han et al. (2007) suggested that the stimulating effect of leucine on myogenesis may be through mTOR and its downstream targets.

Protein turnover has been demonstrated to be dysregulated during cancer cachexia. In cachectic environments, IGF-1 expression has been shown to decrease (White et al., 2011). With the increase in inflammation, which is common in cachectic environments, there is evidence to support that mTOR signaling also decreases, along with mTOR targets p70 and 4EBP-1 (White, Puppa, Gao, Sato, Welle & Carson, 2013; Hardee et al., 2018). In contrast, our data show no

change in IGF-1 expression and a trend for an increase in p70 protein abundance in cachectic mice; however, there was a decrease in 4EBP-1 abundance. Further investigation is needed to determine whether there is a change in phosphorylation of the latter two markers, however Baum et al. (2016) found in one of the longest leucine studies to date that there was no effect of leucine on 4EBP-1 or p70 after 6 weeks of supplementation in rats fed a high-fat diet.

In cancerous conditions, a leucine-rich diet was shown to increase protein synthesis by improving the downregulation of the mTOR signaling pathway, rescuing mTOR and p70 activation in tumor-bearing mice (Viana & Gomes-Marcondes, 2014). A mixture of leucine and valine has also been shown to increase the phosphorylation of 4EBP-1 in tumor-bearing mice (Eley, Russell and Tisdale, 2007). Our data showed no change in IGF-1, p70 or 4EBP-1 with leucine supplementation, which further corroborates the findings of Baum et al. (2016). That said, further investigation is required to determine if these conditions would affect the phosphorylation of p70 and/or 4EBP-1.

While markers for protein synthesis decrease in cachectic conditions, ubiquitin ligases Atrogin-1 and MuRF-1 have been shown to increase (Brown et al., 2018). Our data presented a confirmatory 8-fold increase of Atrogin in cachectic mice and a main effect for genotype to increase MuRF-1, adding further support to these findings in the current literature.

In addition to stimulating protein synthesis pathways, leucine has also been shown to attenuate Atrogin and MuRF-1 abundance in immobilized rats (Baptista et al., 2010; Baptista et al., 2013). Confirmatory evidence showed that leucine supplementation attenuated Atrogin in $APC^{Min/+}$ mice and had a main effect for lowered MuRF-1 in $APC^{Min/+}$ mice.

There are a couple of possible explanations for this anomaly. As Lee et al. (2019) speculates, the combination of the stimulating effect both cancer and leucine independently have

on energy expenditure may be exacerbating the overall energy imbalance. Nilsson et al. (2016) point out that DEPTOR, a competitive inhibitor of mTOR, decreases in protein expression in most cancer types. In addition, leucine-induced stimulation of mTOR leads to the degradation of DEPTOR, which is necessary for the activation of p70. As p70 is important for the process of protein synthesis, leucine may exacerbate the cancer-induced suppression of DEPTOR, resulting in a decrease in p70 activity. One limitation to this study is the muscle used in this project. While leucine has been shown to have a greater effect on the cross-sectional area of Type 1 fiber types, the muscle used in this project was the plantaris, which is predominantly made up of fast-twitch fibers (Perry et al., 2016).

While we did not investigate this process, another possible avenue to consider is autophagy dysregulation. As Lee et al. (2019) demonstrated, STAT3, which is an inflammatory marker, is suppressed with leucine supplementation in healthy mice. According to Puppa et al. (2014), inhibition of STAT3 can decrease FOXO3a phosphorylation, which is a key regulator of autophagy. This is further evidenced by the study conducted by Lee et al. (2019). Additionally, mTOR, which is stimulated by leucine, can regulate the expression of autophagy genes (White et al., 2011). Autophagy is necessary to remove dysfunctional mitochondria to prevent an increase in ROS, which can induce atrophy if left unchecked (Assi et al., 2016; Carson et al., 2016). Mitochondrial content and function are disrupted in cachectic environments, as evidenced by Brown et al. (2017). Although leucine has been shown to increase mitochondrial biogenesis (Lee et al., 2019), it is not yet known whether leucine improves overall quality and function. Bnip3, a mitophagy marker, increases dramatically in cachectic mice due the increase in inflammation (Brown et al., 2017; VanderVeen et al., 2017), while Zheng et al. (2019) demonstrated that

leucine inhibits expression of this autophagic marker. This could lead to a buildup of degenerated mitochondria, resulting in excessive ROS production (VanderVeen et al., 2017).

The purpose of this study was to investigate whether long-term supplementation of leucine to $APC^{Min/+}$ mice would attenuate the development of cachexia. Not only did it fail to attenuate this disease, it seemed to exacerbate it. Further study is required to determine whether this has anything to do with phosphorylation of mTOR's downstream targets, and whether any other physiological functions are negatively affected by long-term leucine supplementation. That said, the present evidence would indicate that caution should be exercised when considering prescribing leucine as a treatment for cancer patients.

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CONFLICT OF INTEREST

The authors do not have any conflicts of interest to declare.

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CHAPTER 5: Conclusion

Conclusion

Based on the present study, it appears that long-term leucine supplementation not only fails to attenuate cancer cachexia, but it seems to exacerbate skeletal muscle mass loss. This would suggest that caution should be employed when considering leucine as a prescription for cancer patients, as the result may be opposite of that intended. Further research is required to determine the cause of this anomaly. Leucine seems to have some positive effect in short-term studies, so it may also be beneficial to test different administration models.

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APPENDIX

Table Legends

Table 1. Bodyweight, plantaris weight, tibia length, and plantaris weight normalized to tibia length at ~24 weeks. Values are means ± SEM. * Indicates a main effect of genotype. ** indicates a main effect of leucine. Statistical significance we set at an alpha of $p \leq 0.05$.

Figure Legends

Figure 1. Animal Data. (A) Average amount of water drunk per day. (B) Total number of polyps in APCMin/+ mice treatment comparison. (c) Relative frequency of polyps by size at ~24 weeks. * Signifies a trend ($p = 0.10$) for an increase in relative number of <1mm tumors with leucine administration.

Figure 2. mRNA abundance of satellite cell dynamics-associated factors at ~24weeks. (A) mRNA abundance of Cyclin D1. (B) T-test comparison of mRNA abundance of Cyclin D1. (C) mRNA abundance of MyoD. (D) T-test comparison of mRNA abundance of MyoD. (E) mRNA abundance of Myogenin. (F) T-test comparison of Myogenin mRNA abundance in APCNL and WTNL. Main effects (ME) of genotype and/or injury appear on each graph when relevant. $p \leq 0.05$.

Figure 3. mRNA abundance and protein content of protein synthetic factors p70 and 4E-BP-1 at ~24 weeks. (A) p70 protein content. (B) T-test comparison of p70 protein content in APCNL and WTNL. (C) 4E-BP1 protein content. (D) T-test comparison of 4E-BP1 protein content in APCNL and WTNL.

Figure 4. mRNA abundance of degradative factors Atrogin-1 and MuRF-1 at ~24wks. (A) Atrogin-1 mRNA abundance. (B) T-test comparison of Atrogin-1 mRNA abundance in APCNL and WTNL. (C) MuRF-1 mRNA abundance. (D) T-test comparison of MuRF-1 mRNA abundance in APCNL and WTNL

Figure 5. mRNA abundance of anabolic signal IGF-1 at ~24 weeks. (A) IGF-1 mRNA abundance. (B) T-test comparison of IGF-1 mRNA abundance in APCNL and WTNL. Main effects (ME) of genotype and/or injury appear on each graph when relevant. $p \leq 0.05$.

Figure 6. mRNA abundance and protein content of Cox4. (A) Cox4 mRNA abundance. (B) T-test comparison of Cox4 mRNA abundance in APCNL and WTNL. (C) Cox4 mRNA abundance. Main effects (ME) of genotype and/or injury appear on each graph when relevant. $p \leq 0.05$.

	Group	Body Weight (g)	Plantaris (mg)	Tibia Length (mg)	Plantaris mass/ TL (mg/mm)
Wild Type	No Leu	29.0 ± 1.0	17.2 ± 0.7	16.7 ± 0.1	1.03 ± 0.3
	Leu	29.1 ± 0.8	16.7 ± 1.2	16.8 ± 0.1	1.00 ± 0.45
$APC^{Min/+}$	No Leu	25.0 ± 0.6*	13.8 ± 0.9	15.9 ± 0.2	0.83 ± 0.05*
	Leu	21.1 ± 0.8	9.3 ± 0.7	16.5 ± 0.2	0.59 ± 0.04**

Table 1.

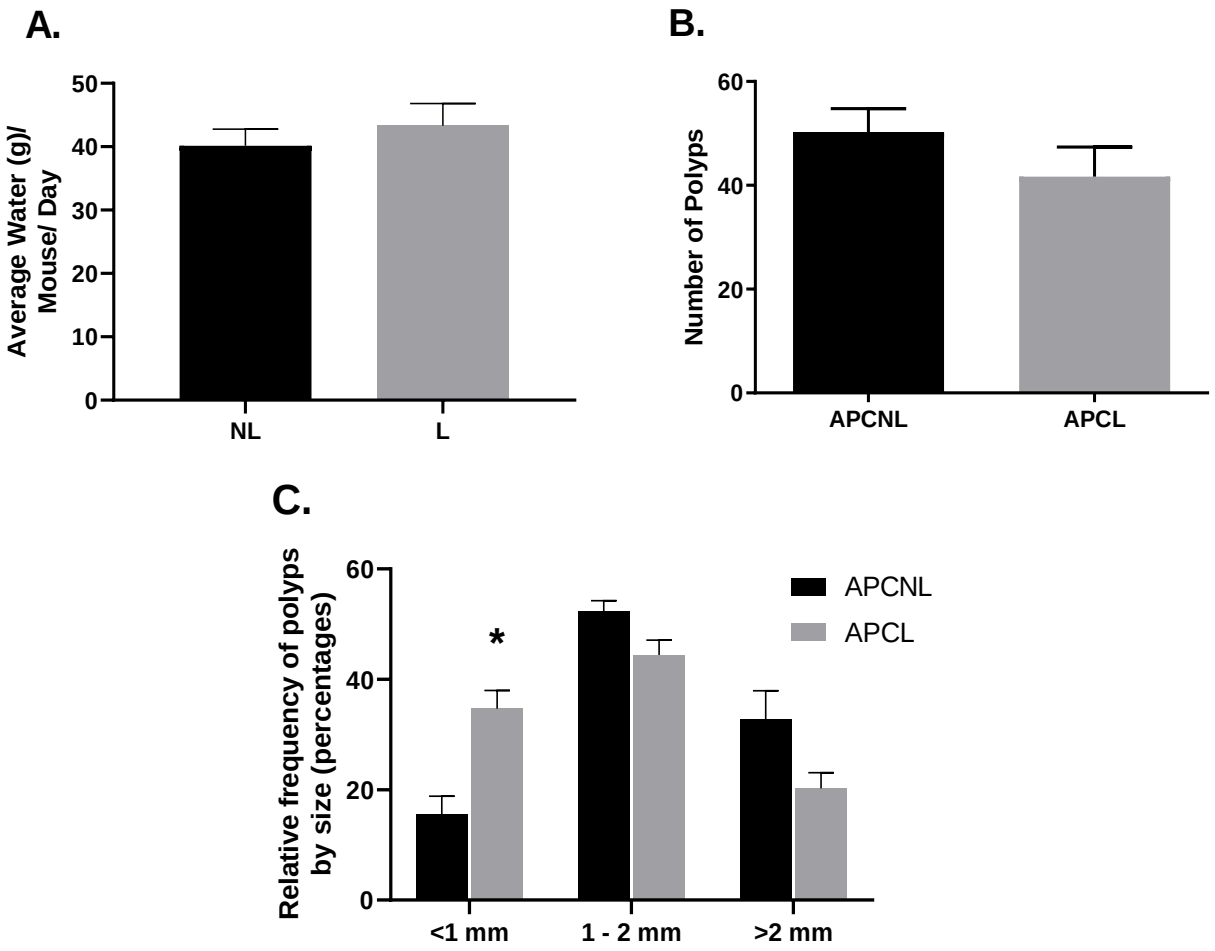
A.
Average Water (g)/ Mouse/ Day
50
40
30
20
10
0
NL
L
B.
Number of Polyps
60
40
20
0
APCNL
APCL
C.
Relative frequency of polyps by size (percentages)
60
40
20
0
*
APCNL
APCL
<1 mm
1 - 2 mm
>2 mm

Figure 1.

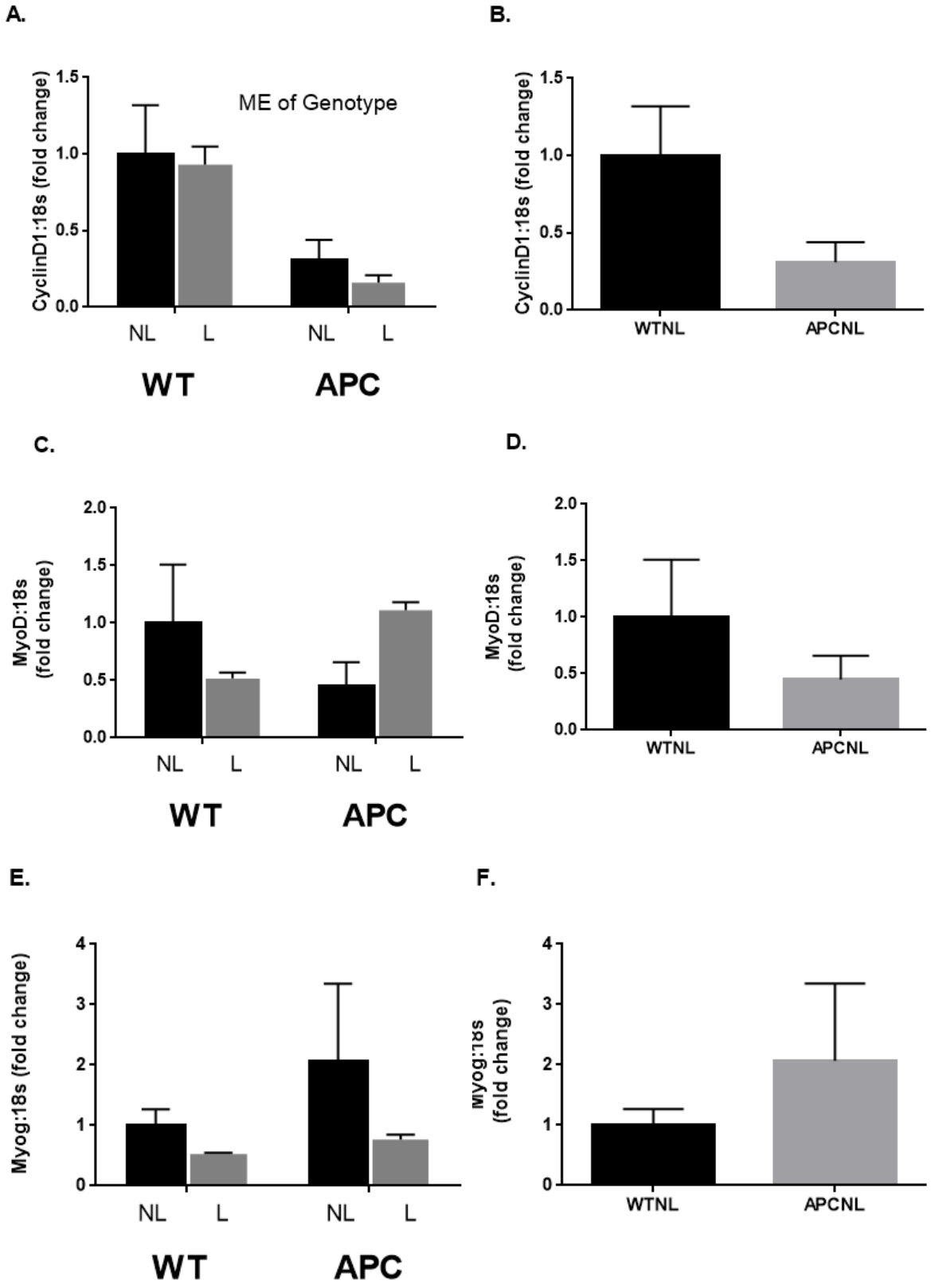
A.
CyclinD1:18s (fold change)
1.5
1.0
0.5
0.0
ME of Genotype
NL
L
NL
L
WT
APC
B.
CyclinD1:18s (fold change)
1.5
1.0
0.5
0.0
WTNL
APCNL
C.
MyoD:18s (fold change)
2.0
1.5
1.0
0.5
0.0
NL
L
NL
L
WT
APC
D.
MyoD:18s (fold change)
2.0
1.5
1.0
0.5
0.0
WTNL
APCNL
E.
Myog:18s (fold change)
4
3
2
1
0
NL
L
NL
L
WT
APC
F.
Myog:18s (fold change)
4
3
2
1
0
WTNL
APCNL

Figure 2.

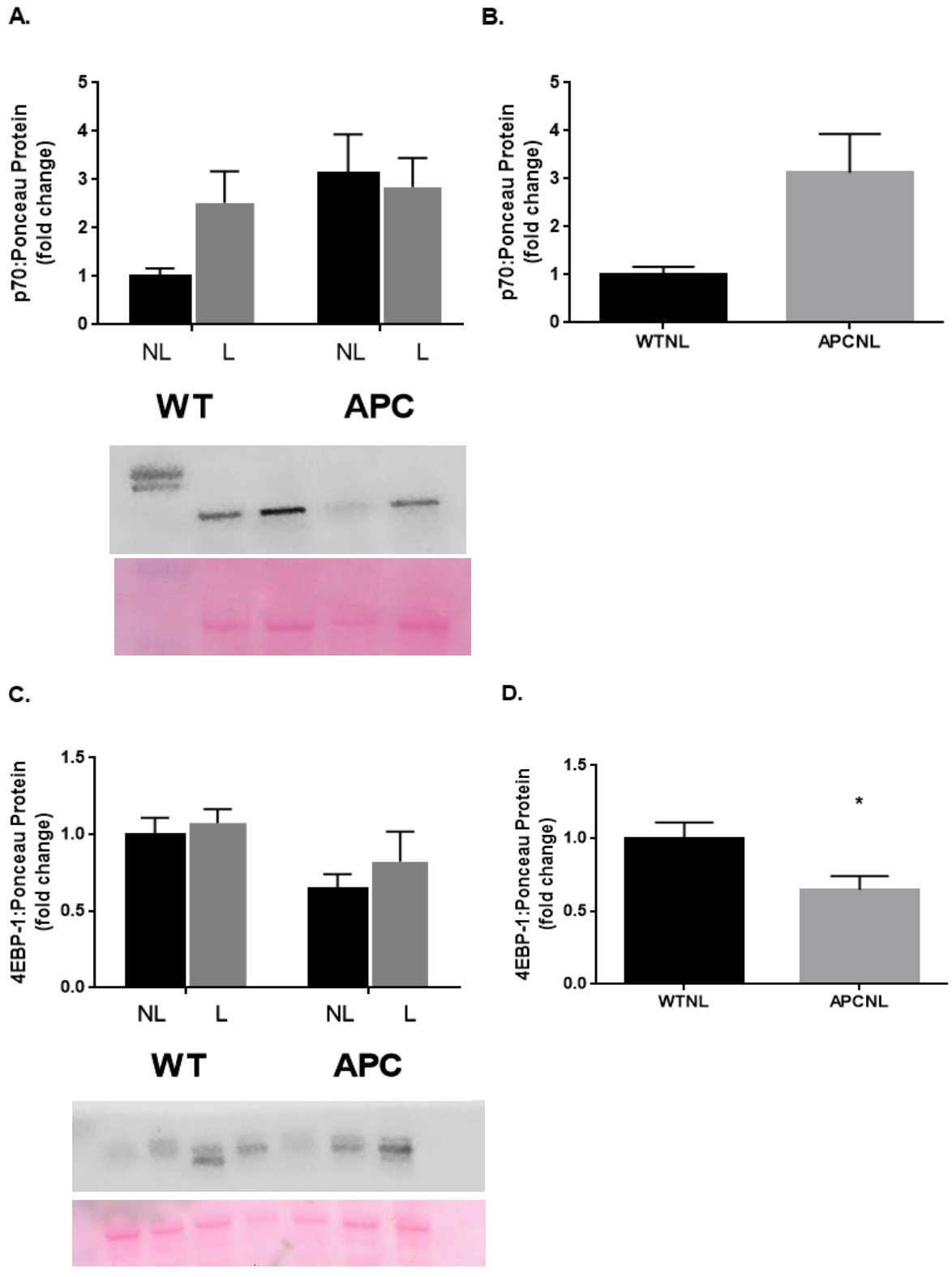
A.
p70:Ponceau Protein (fold change)
5
4
3
2
1
0
NL
L
NL
L
WT
APC
B.
p70:Ponceau Protein (fold change)
WTNL
APCNL
C.
4EBP-1:Ponceau Protein (fold change)
1.5
1.0
0.5
0.0
NL
L
NL
L
WT
APC
D.
4EBP-1:Ponceau Protein (fold change)
*
WTNL
APCNL

Figure 3.

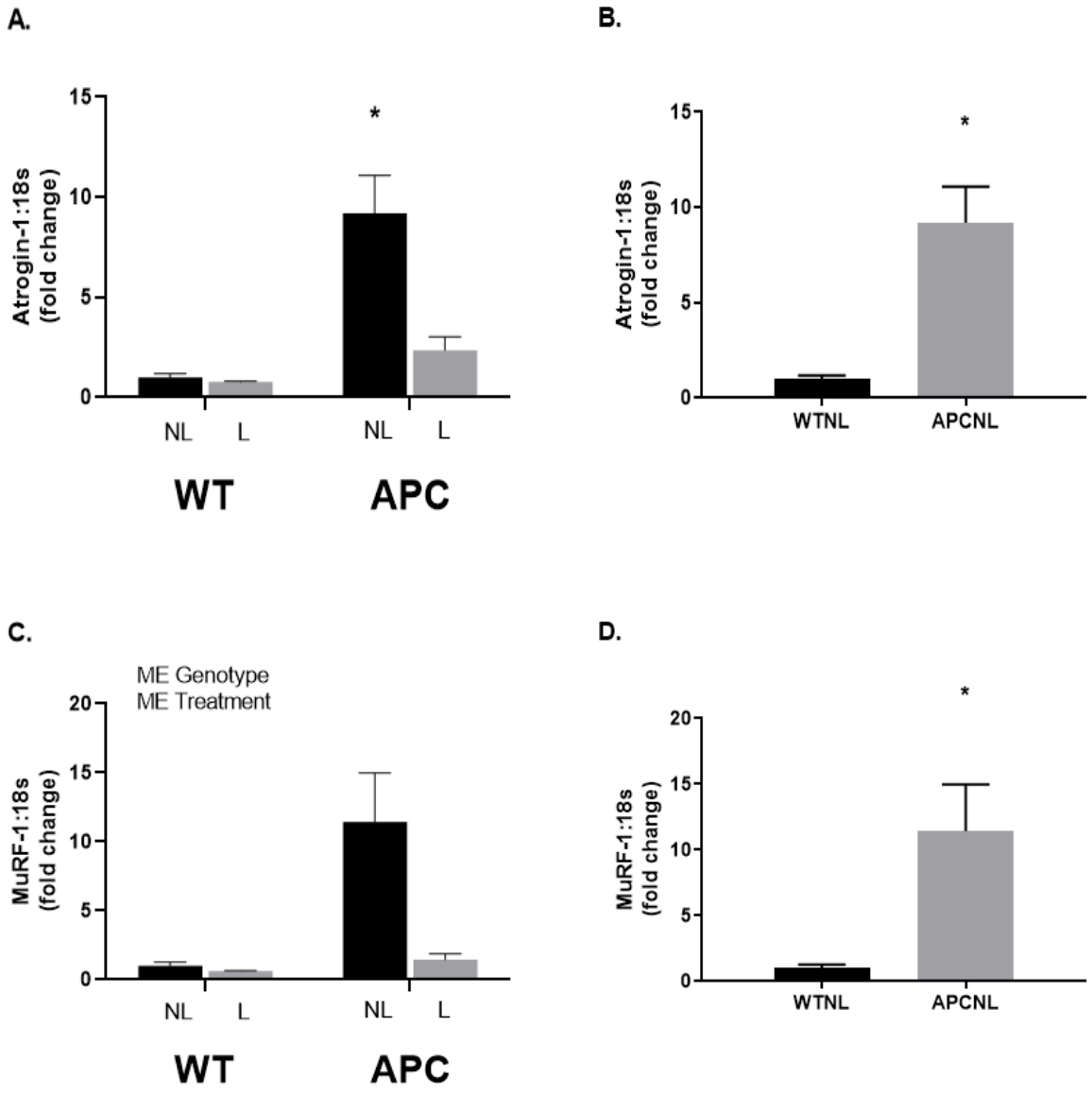
A.
Atrogin-1:18s (fold change)
15
10
5
0
*
NL
L
NL
L
WT
APC
B.
Atrogin-1:18s (fold change)
15
10
5
0
*
WTNL
APCNL
C.
ME Genotype
ME Treatment
MuRF-1:18s (fold change)
20
15
10
5
0
NL
L
NL
L
WT
APC
D.
MuRF-1:18s (fold change)
20
15
10
5
0
*
WTNL
APCNL

Figure 4.

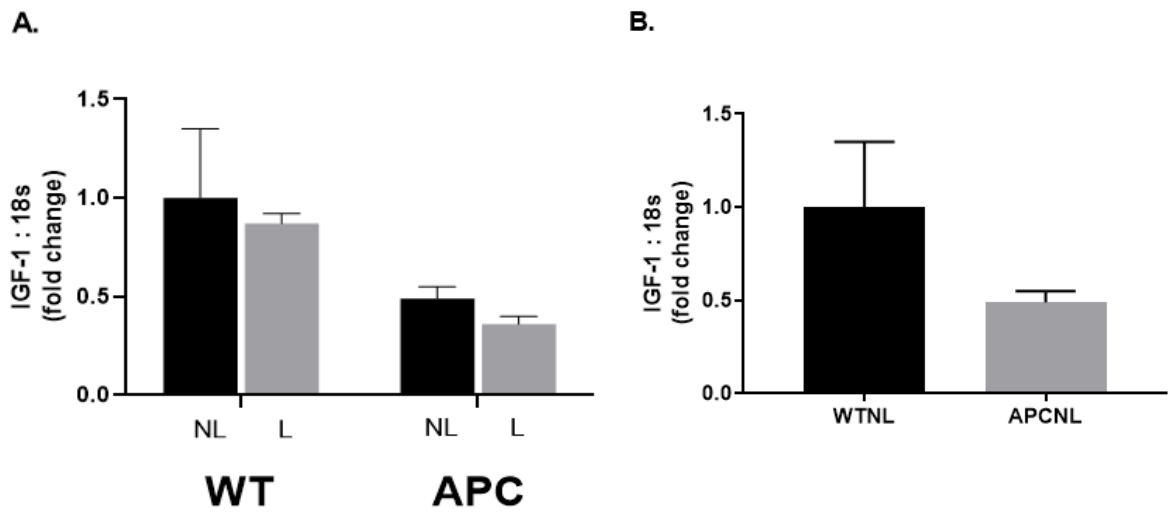
A.
IGF-1 : 18s
(fold change)
1.5
1.0
0.5
0.0
NL
L
NL
L
WT
APC
B.
IGF-1 : 18s
(fold change)
1.5
1.0
0.5
0.0
WTNL
APCNL

Figure 5.

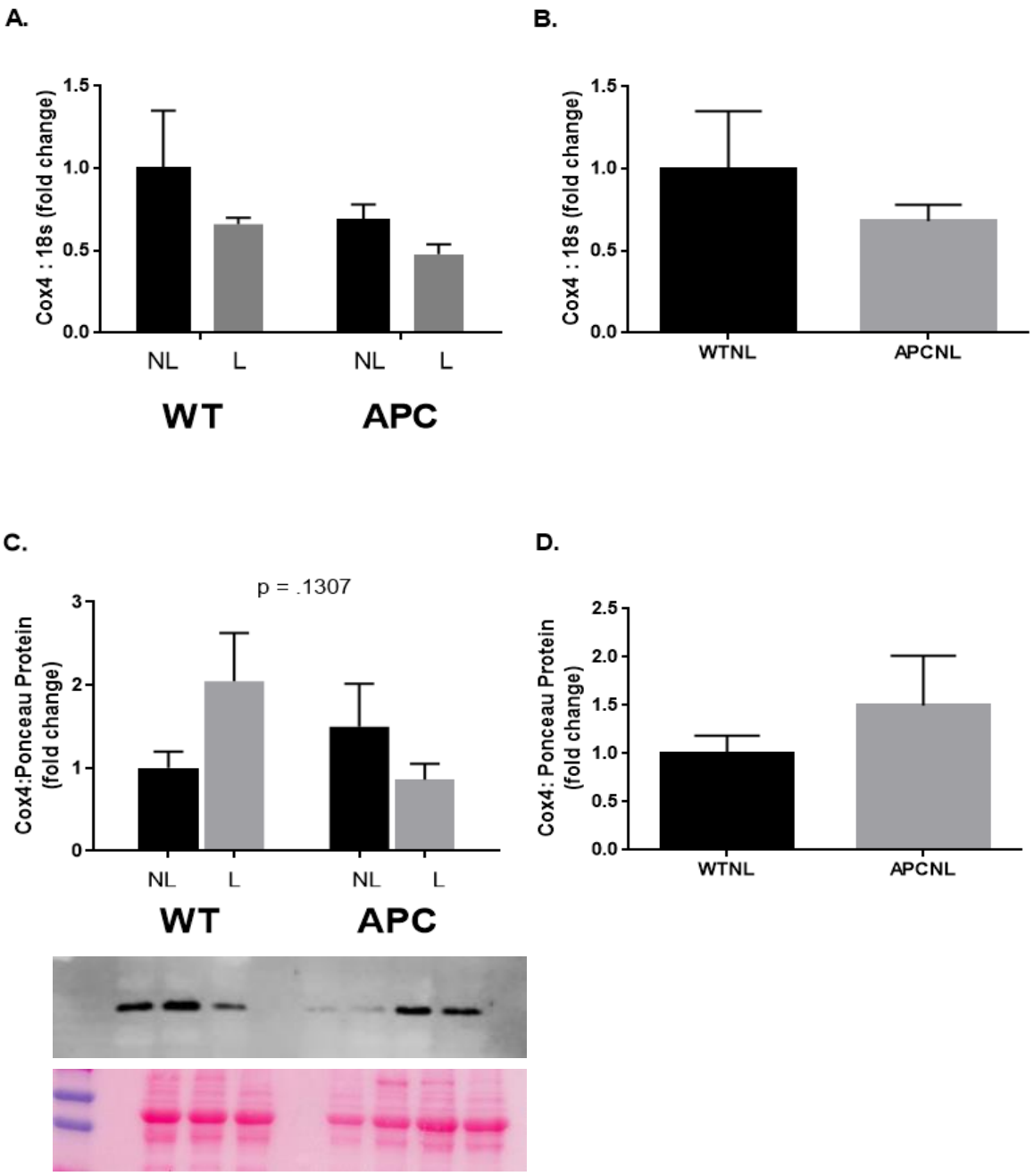
A.
Cox4 : 18s (fold change)
1.5
1.0
0.5
0.0
NL
L
NL
L
WT
APC
B.
Cox4 : 18s (fold change)
1.5
1.0
0.5
0.0
WTNL
APCNL
C.
p = .1307
Cox4:Ponceau Protein (fold change)
3
2
1
0
NL
L
NL
L
WT
APC
D.
Cox4: Ponceau Protein (fold change)
2.5
2.0
1.5
1.0
0.5
0.0
WTNL
APCNL

Figure 6.