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Effects of a Leucine Supplementation on Mitochondrial Markers in Pre-Cachectic Female Mice

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TABLE OF CONTENTS

ABSTRACT

Cancer cachexia is a wasting syndrome characterized by extreme weight and skeletal muscle loss that results in death in many tumor bearing individuals. Because skeletal muscle requires a high amount of energy to function, skeletal muscle fiber cells need healthy mitochondria to function. When skeletal muscle loss atrophy occurs, the mitochondrial network is degraded. Different treatments for this muscle atrophy are being studied, one of which is a leucine supplementation since leucine has been found to stimulate skeletal muscle growth in other studies. However, current data suggests that a leucine supplementation in cachexic individuals exacerbates this atrophy. **PURPOSE:** This study will examine how leucine supplementation will affect the mitochondrial markers in pre-cachectic mice. **METHODS:** Female $APC^{Min/+}$ and their wild-type litter mates were assigned to either the leucine supplementation group, which will receive leucine in their water until tissue harvest, or no leucine supplementation group. The gastrocnemius was harvested at 13 weeks (pre-cachectic stage). Quantitative PCR was performed to determine mRNA abundance of OPA1 and BNIP3. A two-way ANOVA was used to analyze the data. **RESULTS:** There were no significant differences between in mitochondrial markers, OPA1 and BNIP3, between groups. There was a main effect of leucine and time, and there was an interaction between genotype and time. There were no significant differences in tissue weights, and the $APC^{Min/+}$ leucine group had the highest average polyp count. There was an interaction between genotype and treatment in peak torque averages, and the comparison between wild-type no leucine and $APC^{Min/+}$ no leucine was significant. **CONCLUSIONS:** Leucine does not have a significant effect on the mitochondrial quality or health of female mice in the pre-cachectic stage.

KEY WORDS: Leucine Supplementation, Mitochondrial Markers, Cancer Cachexia

CHAPTER I

INTRODUCTION

Cancer is the second leading cause of death in the world and is responsible for almost 10 million deaths each year [1]. One symptom associated with many cancer related deaths is a condition called cancer cachexia. Cancer cachexia, observed in approximately 80% of cancer cases, is the irreversible wasting syndrome that is characterized by skeletal muscle loss in an individual [2]. This skeletal muscle loss, also called skeletal muscle atrophy, leads to extreme loss of body weight and eventually death once 25-30% of the total body weight has been lost [2]. This makes cancer cachexia responsible for up to 40% of all cancer related deaths [3]. Various nutraceuticals are currently being studied as potential treatments for skeletal muscle atrophy. One promising nutraceutical is leucine.

Leucine is one of the three branched chain amino acid (BCAA) that make up almost one third of muscle protein. Leucine has been found to stimulate protein synthesis in skeletal muscle by acting as a mediator for the activities of the intracellular kinase mammalian target of rapamycin (mTOR) which influences protein translation [3,4]. A balance between protein synthesis and protein degradation within the skeletal muscle cells is what maintains skeletal muscle mass. When this balance is altered and protein degradation exceeds protein synthesis, skeletal muscle atrophy results. Finding a treatment to stimulate protein synthesis in cancer cachectic individuals where this balance is interrupted could be an attractive target in reducing skeletal muscle atrophy.

Skeletal muscle is a high demand tissue and accounts for most of one's daily energy consumption to support its growth and function [5]. To effectively take up energy, the skeletal muscle cells require good mitochondrial quality since mitochondria produce the ATP that drives these energy processes. Mitochondrial quality is the overall health and general function of the mitochondrial network in the muscle [6]. When a factor, such as cancer cachexia, negatively effects

this network, degeneration of the mitochondria occurs which can induce skeletal muscle atrophy as the cancer cachexia progresses [6]. This idea is supported by recent data showing that mitochondria quality does, indeed, decline with cancer cachexia [3]. While a leucine supplementation is being studied as a possible remedy, there have been a few drawbacks.

A recent study indicated a positive response in skeletal muscle regeneration to a leucine supplementation after exercise [3]. This led to the hypothesis that a leucine supplementation could reduce skeletal muscle atrophy. However, our lab has preliminary data suggesting leucine supplementation in cancer cachectic mice exacerbates skeletal muscle atrophy rather than suppresses it during tumor progression [7]. The cause of this exacerbation is unknown.

CHAPTER II

LITERATURE REVIEW

Cancer is the second leading cause of death in the world [1]. Cachexia, the wasting syndrome characterized by loss of weight and muscle mass over time, is a key indicator of cancer in individuals and is the leading cause of death in cancer patients [8]. Previous research has shown that cachexia induced by cancer is a multifactorial wasting syndrome. This indicates that the muscle wasting is a product of different factors associated with the growing tumor, rather than the result of only one element. Some of these other common factors that have been shown to aid inducing cachexia in cancer patients are asthenia, anorexia, and anemia. [2]

Asthenia is used to describe physical weakness or lack of energy. The Resting Energy Expenditure (REE) is used to predict the 24-hour energy expenditure of an individual by using indirect calorimetry. This helps calculate a percentage of total energy loss from the REE, which is correlated with levels of asthenia. Research has shown that REE in cancer patients varies depending on the malignancy type, but it typically higher in cancer patients compared to healthy individuals. One reason for this increase is attributed to increased thermogenesis in skeletal muscle and brown adipose tissue (BAT). Cachectic patients are shown to have more brown adipose tissue than healthy controls, providing a potential explanation for increased thermogenesis. [2]

Anorexia is another common factor in cachectic patients. Anorexia is the loss of appetite or desire to eat, which leads to extreme weight loss. In cancer induced anorexia, the patient has a decreased taste and smell of food, as well as having their metabolic rate altered so they experience early satiety. Researchers believe this is done by either a physical obstruction in the GI tract from the tumor, or the alteration of cholecystokinin (CCK) and their receptors, which are satiety inducing peptides, in cachectic patients. Cancer induced anorexia can also be traced to the

production of cytokines by the cancer cells. Cytokines support the growth of new tissue and blood vessels, creating a suitable environment for cancer cells, as well as transport substances that interact with normal endothelial cells in the body, mediating anorexia in the patient. Additionally, in cachectic patients, a substance called anemia inducing substance (AIS) is secreted by the cancer cells, which causes red blood cells to be osmotically fragile and reduce their deformability, as well as depresses immune functioning cells. AIS binds to the surface of RBCs and lowers its glucose influx and pyruvate kinase activity, leading to the dysfunction and lysis of the RBCs. The lysis of the RBCs leads to the onset of anemia in cachectic patients.[2]

Cancer cachexia depletes the nutritional resources throughout the body, specifically in the adipose tissue and skeletal muscle, because of the anemia, anorexia, and loss of energy expenditure. This compromises the body's ability to fight infections and eventually leads the individual to be too weak to withstand chemo and radiation therapy, exacerbating the loss of the body weight. Once 25-30% of the individual's total body weight is lost, the patient dies [2]. While cancer cachexia creates similar symptoms in patients affected, there are different models used when studying the effects of cancer cachexia.

With cachexia studies, generating clinical trials are difficult. The use of animal models has helped alleviate this difficulty and allowed researchers to study specific diseases, potential treatments, and new therapy regimens in more depth. Mice and rats specifically, have been shown to be popular models utilized in research involving cancer and its resulting symptoms. While there are many positives to using a mouse model in cancer cachexia studies, there are also some negative aspects that researchers must keep in mind. [9]

Animal models, specifically mice which are commonly used, provide effective ways to stimulate the muscle wasting that occurs in cancer cachexia, while controlling for different

variables, and monitoring the outcomes of treatments being studied. The mice models can be implanted with different tumor lines where the tumor(s) can grow to a certain burden before euthanizing the animal. For cachexia, the most common line used in models is Lewis Lung Carcinomas, but other lines such as C26 colorectal carcinomas, prostate tumors, and colon adenocarcinomas are also used depending on the project. The effects of these tumors in mice mimic the reactions to those tumors in humans, allowing researchers to observe the symptoms over the course of the experiment as well as test potential treatments. This allows for the generalizability of certain treatments and better understanding of the pathophysiology of different tumor cell lines. Different protocols are used for different tumor lines ensure that the experiments are ethical, and the mice aren't allowed to reach a point of suffering [9]. In this study, $APC^{min/+}$ mice will be used. The $APC^{min/+}$ mouse is an animal model with a mutation in the adenomatous polyposis coli (APC) gene, so the gene is eliminated in the mice. The APC gene prevents the formation of colon cancer, so this mutation causes the mice to develop tumors, also known as polyps, within their colon. In many cases of human colon cancer, the APC gene is mutated, so these mice models are good to use in studying this line of cancer. The $APC^{min/+}$ mice also display several of the same symptoms and characteristics as humans when the tumor burden increases, so results found in these animal studies can be generalized to human cases regarding colon cancer and muscle wasting. [10]

While there are many pros to using mice models when studying cancer cachexia, researchers must keep in mind a few potential drawbacks. One being that due to the complexity of cachexia and many factors that influence it, completely replicating it as it appears in humans is a challenge, and studies have shown where most tumor lines that are implanted in mice don't metastasize as they do in humans. Additionally, the differences between male and female growth responses in mice can make generalizability to humans difficult, and metabolic differences

between mice and humans can limit conclusions as many human cancer patients have other comorbidities over several years that can't be replicated with mice [11]. Despite these drawbacks, though, there are enough similarities between rodents and humans to justify the use of rodent models in studies regarding cancer cachexia.

Leucine is one of three branched-chain amino acids (BCAA), the other two being isoleucine and valine. Leucine has a predominating pH of 7.4, and contains an amino group, carboxyl group, and a side chain consisting of a branched, nonpolar hydrocarbon group. The BCAAs are considered an essential part of the human diet, as the body doesn't naturally produce them, and they make up roughly one third of muscle protein, with leucine being about 5-10% of the protein content. Metabolism of BCAAs is initiated in skeletal muscle, and leucine specifically has been shown to stimulate protein synthesis in the muscle. In combination with the other BCAAs, leucine supplementation was correlated with a decrease in skeletal muscle protein degradation as well, which is a key aspect of muscle wasting in cancer cachexia. [3]

A study done on the muscle protein mass in elderly men showed that an added leucine supplementation improved the muscle protein fractional synthesis rate and reduced the breakdown rate. The researchers found that the BCAAs, especially leucine, were most efficient at stimulating protein synthesis. Leucine, on its own, was able to stimulate muscle protein synthesis to the same extent as the other amino acids. However, researchers were unable to find an effect of leucine on whole body protein turnover. [4]

The amino acid, leucine, affects protein balance by targeting the protein synthetic pathways within the skeletal muscle. Leucine inhibits certain transcription factors like FOXO3a to prevent the degradation of proteins and can stimulate the synthesis by producing a higher p-mTOR content, which is a protein that controls skeletal muscle signaling. However, evidence suggests that leucine

supplementation affects the skeletal muscle regeneration protein levels differently in aged vs young mice. In aged mice, the added leucine resulted in an increase in relative and phosphorylated mTOR levels. But in young mice, higher mTOR levels were found in the injured mice, regardless of the status of an added leucine supplementation. Additionally, FOXO1 (a transcription factor for Atrogin-1 and MuRF-1) and Atrogin-1 levels, which are atrophy markers, were found to not be affected by leucine in young mice. The researchers concluded that while leucine has been shown to help reduce injury-induced inflammation, in terms of protein levels a leucine supplementation acts independently of the mTOR pathway in young mice, but dependently of the mTOR pathway in aged mice. In other words, in young mice, leucine had no significant effect on the stimulation of protein synthesis, and therefore the growth, of skeletal muscle. In aged mice however, leucine directly stimulated the pathway that controls muscle growth and regeneration. On the mRNA levels of FoxO, MuRF-1 (another atrophy marker protein), and Atrogin-1 specifically, leucine exhibited no effect in young mice, but appeared to stop the increase of FOXO3 after injury in aged mice [7]. This data suggests that aged muscles are more sensitive to a leucine supplementation during muscle regeneration.

Regarding muscle loss and muscle regeneration, most of this activity happens within the skeletal muscle. Skeletal muscle is made up of three types of muscle fibers: type 1, type 2a, and type 2b. Type 1 fibers are slow twitch fibers. They are the smallest of the muscle fiber types, contract with low force, produce low power, and have a very high mitochondrial density. They are the most resistant to fatigue and are mainly used in aerobic activity such as distance running. Type 2a and type 2b are fast twitch fibers. Type 2a fibers are larger than type 1 fibers, but smaller than type 2 fibers. They contract with intermediate force, produce intermediate power, and have a high mitochondrial density. Type 2a fibers are quick to fatigue and are mainly used in long term

anaerobic activity such as weightlifting. Type 2b fibers are the largest fiber type. They contract with high force, have the higher power, and a low mitochondrial density. These fibers are used in short term anaerobic activity. Type 2b muscle fibers are the quickest to fatigue and are found in most animal models used in research, but they are not found in humans. However, humans have a type 2x fiber that performs similarly to type 2b. [12]

Typical muscles used for observing skeletal muscle atrophy in cancer cachexia studies are the gastrocnemius, the soleus, the tibialis anterior, the extensor digitorum longus (EDL) and the plantaris. Cross sectional area of these muscles is used to compare cachectic patients to healthy patients, and each muscle has different metabolic and contractile properties, allowing the researchers to have a variety of data from different factors. Some studies suggest that a shift from slow to fast fiber types, and therefore a higher force production, may occur during cancer cachexia to compensate for the decrease in muscle mass from skeletal muscle atrophy. Previous animal studies show that the type 2 muscle fibers may be more prone to atrophy indicating that type 2 fibers are targeted and are more sensitive in states of cachexia. In addition, it has been found that this cachexia and subsequent skeletal muscle mass reduction is due to a decrease in muscle fiber size, rather than a decrease in the number of muscle fibers [8].

Skeletal muscle generates force for movement and posture maintenance. When the muscle atrophies, it prevents the muscle from being able to generate force to its full capacity. In cancer cachectic patients, the maximum force contraction is decreased in several muscles compared to health patients, as well as the cachectic patients having a weakened grip strength. While this reduction in force and increase in muscle fatigue is mainly attributed to a decrease in fiber size, some evidence suggests that contractile dysfunction and an impairment in calcium handling within the fibers are also responsible for the change in patients with cachexia. Muscle contraction

produces the muscle force, and it relies on calcium to activate the cross-bridge cycle and generate the contraction. In cases of cachexia, the fibers may have a change in calcium sensitivity, causing the ability of the muscle to contract to be dysfunctional, leading to changes in the generated force[8]. A major factor contributing to the correct functionality of skeletal muscle is the mitochondria and its ability to produce ATP via oxidative phosphorylation during muscle contraction.

The mitochondria is an organelle within cells that is responsible for oxidative metabolism. Oxidative phosphorylation is the process that generates ATP from the consumption of oxygen and movement of electrons down the mitochondrial electron transport chain. Mitochondrial degeneration occurring within muscle cells can impair energy production, ultimately leading to muscle atrophy. Mitochondria degeneration is a common occurrence in cachectic individuals, and many researchers believe that mitochondrial degeneration is a critical step in the development of the cachexia. [6]

Mitochondrial degeneration results from poor mitochondrial quality. Mitochondrial quality is the general health and function of the mitochondrial network within cells. This quality can be measured by testing the mitochondrial oxygen consumption (VO_2) from sampled fiber bundles. Since mitochondria rely on oxygen consumption, the amount of oxygen being consumed and used can tell the health of the mitochondria within the muscle samples. When the quality of the mitochondria is poor, it can lead to impaired ATP production as well as excessive production of mitochondrial reactive oxygen species (ROS), which damage the cell's health.[13] These reactive oxygen species can allow the development of cancer cachexia. The amount of ROS produced can also be measured to determine if the mitochondrial function is poor by sampling the fibers and using a method that converts the ROS into a red fluorescent oxidation product. [6]

In skeletal muscle specifically, there are several processes utilized by the mitochondria to regulate the production of ROS and manage damaged mitochondria, such as biogenesis of new mitochondrial components and fusion or fission of mitochondrial regions within the network. In cancer cachexia, however, these regulatory processes are impaired, which allows the mitochondrial network to be degraded and the muscle to atrophy with the progression of the cancer [6]. These various mitochondrial processes can be indicated by mitochondrial markers such as BNIP3, OPA1, COX4, MFN2, TFAM, and CRMP1. The levels of these markers can indicate the health of the mitochondria.

In a recent study looking at the mitochondrial degeneration before cancer cachexia, researchers found that the mitochondrial biogenesis was dysregulated. This was indicated by almost a 50% decrease in mRNA contents that serve as biogenesis regulators of mitochondria, before the cachexia occurred. They observed that the mitochondria content 4 weeks post tumor implantation, was lower. Additionally, the mitochondrial ROS production 1 week post tumor implantation, was 2-fold greater and remained elevated. [6]

They found there was increased mitochondrial oxidative stress followed by mitochondrial degeneration and loss of function in the muscle cells prior to the onset of muscle atrophy in the tumor bearing mice. Amounts of biogenesis (marked by levels of Pgc-1a and Ppara), fusion (marked by levels of Mfn2), and mitophagy (marked by levels of Bnip3) markers indicated that the mitochondrial quality impairment begins with downregulated biogenesis and fusion regulators. This was followed by an upregulation of mitophagy regulators. Mitophagy is the self-degradation of mitochondria. Fis1, a marker for the number of fragmented mitochondria, was also upregulated[6].

Fragmented mitochondria cannot generate ATP. Without the generation of ATP, the cell is put under significant oxidative stress. This could be contributed to the observed decrease in muscle

mass in tumor bearing mice. The researchers explained that this data supports the notion that mitochondrial network degeneration and dysfunction occurs before cancer cachexia. [6]

In the same study, the researchers found that damaged mitochondria directly promote atrophy signaling in skeletal muscle and is a result of the tumor environment. Inflammation, reduced protein synthesis, increased protein catabolism, and altered myogenesis lead to increased ROS production and the loss of normal mitochondrial function in cancer individuals. Oxidative stress from the damaged mitochondria leads to skeletal muscle wasting, further supporting that mitochondrial dysregulation occurs before the onset of cancer cachexia, and that muscle mitochondria are a key target of the cancer resulting in cancer-induced muscle wasting. [6]

CHAPTER III

PRELIMINARY DATA AND SPECIFIC AIMS

Body mass data is presented in ***Figure 1.*** Body mass increased in all groups from 6 to 20 weeks except for the $APC^{Min/+}$ leucine group. $APC^{Min/+}$ group increased body mass up to week 12. We did note a lower body mass in the $APC^{Min/+}$ leucine group compared to the other groups starting at week 14. At 20 weeks there was no effect of leucine supplementation on body mass of wild-type mice. At 20 weeks the $APC^{Min/+}$ leucine group had a lower body mass than all other groups. In conclusion, leucine supplementation exacerbated body mass wasting in male $APC^{Min/+}$ mice.

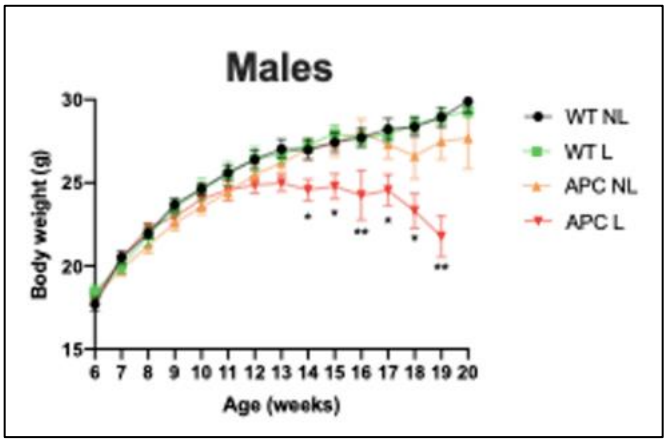

Figure 1- Body mass changes in APCmin/+ leucine male mice.

Purpose and Hypothesis

The purpose of this study was to evaluate the effects of a leucine supplementation on mitochondrial markers in pre-cachectic, female mice.

We hypothesized that a leucine supplementation would exacerbate the decline of mitochondrial quality in the female mice prior to the onset of cancer cachexia.

CHAPTER IV

METHODS

Animals

We used female $APC^{Min/+}$ mice bred on a C56BL/6 background that we purchased from Jackson Laboratory (Bar Harbor, ME) and bred at the University of Arkansas. Approval was obtained from the University of Arkansas Institutional Animal Care and Use Committee. These mice were genetically predisposed for the development of colorectal adenomas starting at 4 weeks of age, and therefore make for an excellent model of colon cancer and associated wasting throughout their lifespan. All mice were given standard rodent chow and access to water *ad libitum* and kept on a 12h:12h light/dark cycle. Following weaning, mice were split into 4 groups based on their genotype (Wild-type (WT) or $APC^{Min/+}$), condition, and assignment to a leucine (L) or no-leucine (NL) supplementation as shown in ***Figure 2***. Food and water consumption were weighed weekly in conjunction with body weight measurements across all groups.

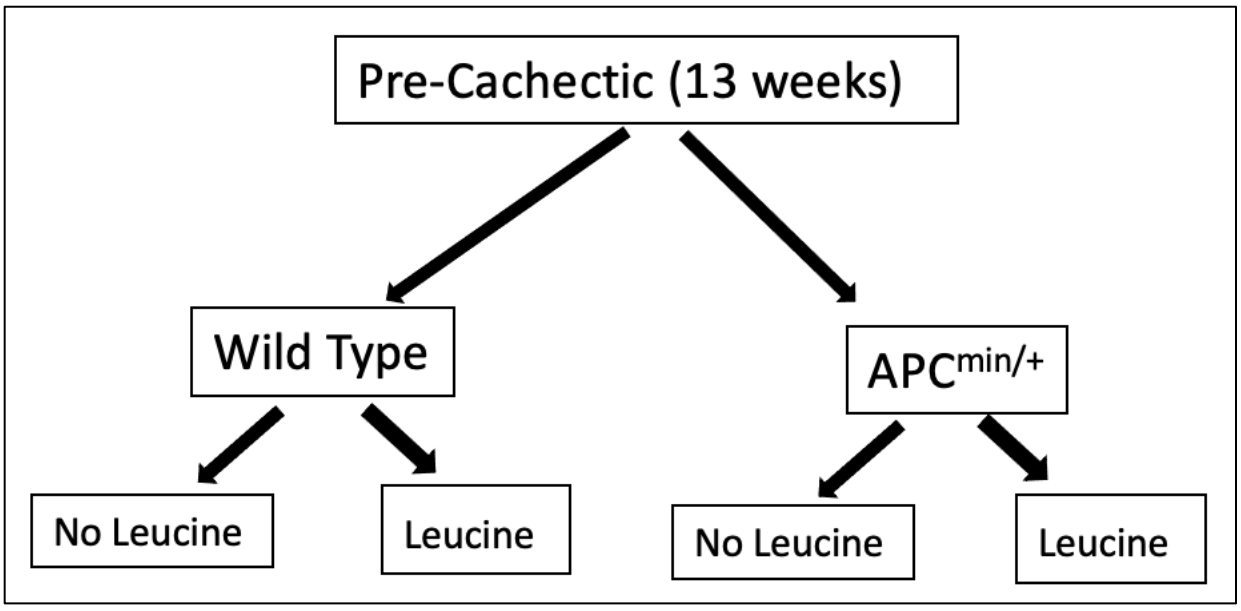

Figure 2- Schematic of group breakdown for experiment.

Leucine Supplementation

Following weaning, mice in the leucine supplementation groups were given leucine in their water at a dose of 1.5g/100 mL as previously described [7,14], with NL counterparts receiving the same water source but without leucine enrichment.

Electrophysiology

Electrophysiology protocol was performed as previously described [6]. Mice were anaesthetized and the peroneal nerve innervating the TA was stimulated at 10, 20, 40, 60, 80 100, 125, 150, 200, 250, and 300Hz with 400 ms pulse to induce peak tension. 11 measurements were taken, and 60 seconds of rest were allowed between each measurement.

Tissue Harvest

The gastrocnemius, EDL, soleus, plantaris, tibialis anterior muscles were extracted along with the heart, liver, spleen, fat, and tibia as previously described [15]. The tissues were immediately flash frozen in liquid nitrogen and stored in -80°C for mRNA abundance analysis. Mice were euthanized via exsanguination by removal of the heart.

RNA isolation, cDNA Synthesis, and quantitative Real Time Polymerase Chain Reaction

RNA isolation, cDNA Synthesis, and quantitative real time polymerase chain reaction were performed as previously described [6]. RNA was extracted isolated using the Purelink RNA mini kit (Thermo Fisher Scientific, Waltham, MA, USA). Using Superscript Vilo cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA), cDNA was reverse transcribed from 1 μg of total

RNA. Real-Time PCR was performed using the following probes: OPA1 (Mm01349709_m1), BNIP3 (Mm01275600_g1), and 18S (Mm03928990_g1) [16]. Final quantification of the relative quantification of mRNA abundance was calculated using the ΔΔCt method.

Statistical Analysis

All data was analyzed by Two-way ANOVA (genotype X leucine supplementation) to determine significant main effects and interactions. A Two-way Repeated Measures ANOVA was performed on the longitudinal body weight data. A non-paired T-test was used to determine differences in polyp counts in the $APC^{Min/+}$ groups. Post-hoc analysis via the Newman-Keuls test was performed. Significance was established at an alpha level of 0.05.

CHAPTER V

RESULTS

Body Mass and Polyp Counts

There was a main effect ($p = 0.0077$) of leucine to decrease body weight independent of genotype, a main effect ($p = 0.0001$) of time to increase body weight, and an interaction between genotype and time ($p = 0.0004$) (***Figure 3).*** There were no significant differences in the final body weights. However, there was a trend in the decreased body weight of the $APC^{Min/+}$ group ($p = 0.067$) and a trend in the interaction between genotype and leucine treatment ($p = 0.065$) in the final body weight data (***Figure 4)***. Polyps were counted in the colons of each mouse to confirm the genotype of either wild-type or $APC^{Min/+}$. As expected, the wild type mice had an average of 0 polyps, while the $APC^{Min/+}$ groups averaged between 20-30 polyps (***Figure 5).***

Tissue Weights

There were no statistically significant differences in tissue weights between groups. ***Table 1*** displays this data. The spleen was enlarged, on average, in the $APC^{Min/+}$ groups ($p = 0.082$) but this difference was not significant (***Figure 6).*** The heart in both leucine treatment groups was, on average, smaller in weight ($p = 0.095$), but this difference was also not significant.

Mitochondrial Markers

The gastrocnemius muscle was used in analysis of mRNA abundance of mitochondrial markers (***Figure 7)***. There were no significant differences between groups of mRNA abundances. Both $APC^{Min/+}$ groups had lower average levels of OPA1 compared to the wild-type groups ($p = 0.076$). For BNIP3 mRNA abundance, the $APC^{Min/+}$ no leucine group had the lowest abundance of the 4 groups. The BNIP3 abundance in the $APC^{Min/+}$ leucine group, however, was similar to those of

both wild-type groups. There was a trend towards an interaction between genotype and treatment for BNIP3 abundance ($p = 0.055$) (***Figure 8, Figure 9)***.

Force Data

There was no statistically significant difference between torque frequencies when analyzing the electrophysiology data for the mice in each group (***Figure 10)***. The average peak torque for each group was calculated and compared to the other groups. There was a significant interaction between genotype and treatment ($p = 0.04$) in peak torque, and the comparison between the wild-type no leucine group, and $APC^{Min/+}$ no leucine group was significant. There was a trend of leucine increasing force production in the wild-type groups ($p = 0.06$) (***Figure 11)***.

CHAPTER VI

DISCUSSION

The primary purpose of this study was to see how a leucine supplementation effected the mitochondrial quality of female $APC^{Min/+}$ mice in the pre-cachectic state compared to healthy, female mice. Data on tissue sizes, body weight, and muscle strength were also collected to provide further insight into how leucine effected the mice. Leucine is commonly used in protein supplements and has been shown to help stimulate skeletal muscle synthesis in healthy individuals[3,4]. So, it was assumed it could help slow muscle atrophy in cancer patients. However, preliminary data from our laboratory has suggested that a leucine supplementation may exacerbate muscle atrophy in male cancer individuals rather than help them. This study was done to investigate if this trend continued in females and how early this may start. We showed that leucine did not have a significant effect on mitochondrial markers between pre-cachectic wild type and $APC^{Min/+}$ mice. The mitochondria are critical organelles that provide the power, via ATP, for a cell to live and function. When the mitochondria are damaged, the cell is not able to properly function[6]. In this study, two mitochondria markers were used to determine the mitochondria health in the mice. The two markers were OPA1 and BNIP3. OPA1 is a marker for mitochondrial fusion, and BNIP3 is a marker for mitophagy [6]. While there were no significant differences in the levels of each marker between groups, there was a trend of OPA1 ($p = 0.076$) being downregulated in both $APC^{Min/+}$ groups, with the $APC^{Min/+}$ leucine group being downregulated the most. This indicates that the $APC^{Min/+}$ mice have less mitochondrial fusion [6], and while it wasn't a significant difference, leucine may play a role in downregulating that process even further. BNIP3 marker abundance didn't have any significant differences but trended towards a possible interaction

between genotype and leucine treatment ($p = 0.055$). The lack of significant differences in the results could be because these mice were pre-cachectic, so muscle atrophy hadn't begun yet.

There was a main effect of leucine on body weight ($p = 0.0077$), as the mice on leucine were smaller on average. This difference is interesting because leucine is seen as a supplement that is taken to stimulate skeletal muscle synthesis, and subsequently increase body weight. However, this data indicates that a leucine supplementation depresses the body weight in female mice. The potential cause of this is unknown though and further research would be needed. A leucine treatment did tend to increase force production in the wild-type mice ($p = 0.06$) when looking at the peak torque data, supporting the idea that leucine stimulates skeletal muscle growth in healthy individuals[6]. This trend was lost in the $APC^{Min/+}$ group, though, and it is not known why the $APC^{Min/+}$ no leucine group had a significantly higher peak torque than the wild-type no leucine group. Further research would be needed to understand this and see if this significance continued in female mice in the cachectic stage. The main effect of time ($p = 0.0001$) was expected because the mice were growing to reach their final weight. There was also a significant interaction between genotype and timepoint ($p = 0.0004$), which was also expected as the APC mice should not grow as the wild type mice do as they near the cachectic state. There was a trend towards an interaction between genotype and treatment ($p = 0.065$), which could indicate the potential effects of leucine in healthy versus non-healthy individuals. Data from female mice in the cachectic stage would be needed to confirm this trend though. The lack of significant interaction, in this study, between leucine and genotype is most likely because these mice had not reached the cachectic stage yet, so muscle atrophy had not begun and the conclusion of a significant effect of leucine in pre-cachexia cannot be drawn. There were also no significant findings in the tissue weights between the four groups, which is most likely the result of the mice being in the pre-cachectic stage as well. The

trends of the enlarged spleen in the $APC^{Min/+}$ groups and smaller hearts in the leucine treatment groups, could be of interest if more longitudinal data was available to confirm these trends.

The average polyp count between the two $APC^{Min/+}$ groups is something to note. Though it was not a significant difference ($p = 0.75$), the average polyp count for the $APC^{Min/+}$ leucine group was about 10 higher than the average polyp count for the $APC^{Min/+}$ no leucine group. This finding could eventually support the preliminary data that a leucine supplementation exacerbates the cancer in individuals. However, given that there was no significant difference between groups in the pre-cachectic stage, this claim cannot be supported and further data looking at the longitudinal effects would be needed to confirm this. Because there were no significant differences between the wild-type and $APC^{Min/+}$ groups, despite this higher polyp count, this also brings forth the idea of females potentially being protected from the physical effects of leucine. The more polyps a mouse has, the more the mouse should exhibit physical symptoms of the illness such as lower body weight or higher mitophagy (BNIP3) markers. Since neither of these were found to be significant in the $APC^{Min/+}$ leucine group, despite having the highest average polyp count, this could indicate that females are more protected than their male counterparts, but further investigation would need to be done to confirm this and understand what could be causing it.

In conclusion, a leucine supplementation does not have any significant effects on female mice in the pre-cachectic stage. While the data doesn't support a leucine supplementation helping slow or prevent muscle atrophy, the data also doesn't indicate that a leucine supplementation exacerbates muscle atrophy as was seen in male mice in preliminary data. The mitochondrial quality between the wild-type and $APC^{Min/+}$ groups did not differ significantly, indicating that leucine has no significant effect on mitochondrial health on females before cachexia. Because there were some trends observed in the data, though, future studies should focus on female mice in the

cachectic stage to see if these trends become significant. The lack of significant differences in this pre-cachectic data also introduces the idea that females may be sheltered against the significant effects of leucine, unlike their male counterparts. This would be a topic that future studies could focus on to better understand if this idea is true and begin to understand why this difference exists between males and females.

While this study yielded interesting results, there were some limitations. One being that our "n" for each females group was low, and slightly uneven. This could have potentially skewed data or made trends appear that may not otherwise be there with a higher "n" for each group. Another limitation is that male data was not included because we were not able to achieve an adequate "n" for each group of males in time. This lack of opposite sex data limits generalizability of the results. A third limitation is that this study only focused on the pre-cachectic stage and did not include data on females in the cachectic stage, which is needed to draw more accurate conclusions about the effects of leucine. Despite these limitations, this study provides a solid footing for future studies to build off and better understand how leucine effects individuals with cancer cachexia. This information is one step closer to potentially helping patients find relief who are experiencing cancer cachexia and continue moving towards a treatment for this devastating condition.

CHAPTER VII

FIGURES AND TABLES

Tissue	Soleus	Plantaris	Gastrocnemius	EDL	TA	Heart	Liver	Spleen	Fat	Tibia	Body Weight	Avg. Polyps
Group												
F- WTNL	7.82 ± 0.54	14.23 ± 0.64	111.19 ± 5.18	9.47 ± 1.19	38.01 ± 1.69	98.47 ± 2.25	942.7 ± 50.34	88.25 ± 3.55	305.63 ± 28.47	16.97 ± 0.22	21.5 ± 0.39	0
F- WTL	6.42 ± 0.39	12.86 ± 1.48	105.58 ± 3.95	6.79 ± 0.83	37.18 ± 1.66	93.68 ± 2.99	899.38 ± 35.30	80.13 ± 15.15	218.53 ± 37.46	16.60 ± 0.22	19.51 ± 0.54	0
F- APCNL	6.73 ± 0.33	13.98 ± 1.96	113.65 ± 6.41	8.28 ± 1.61	36.58 ± 3.14	97.33 ± 6.80	917.57 ± 65.87	107.67 ± 9.74	231.2 ± 38.59	16.73 ± 0.07	19.35 ± 0.18	22.5 ± 0.5
F- APCL	6.40 ± 1.05	11.91 ± 2.01	101.30 ± 12.44	9.09 ± 1.79	36.67 ± 2.53	89.35 ± 3.4	915 ± 88.43	111.33 ± 18.71	240.12 ± 50.53	16.50 ± 0.09	19.53 ± 0.85	32.5 ± 27.5

Table 1- Compilation of average tissue weights with standard error.

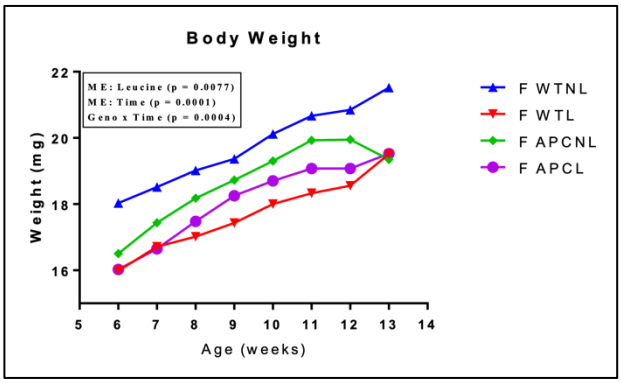

Figure 3- Average body weight over time per group.

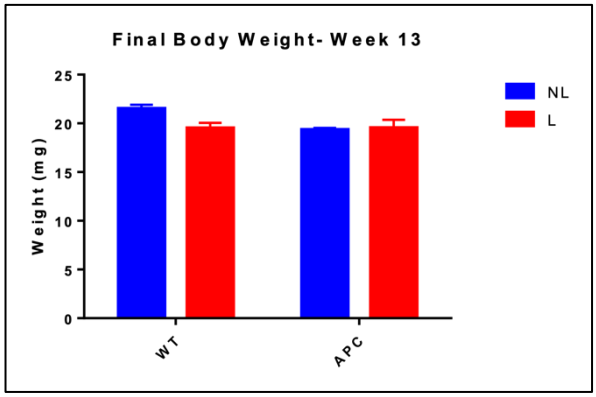

Figure 4- Final body weight comparison with standard error.

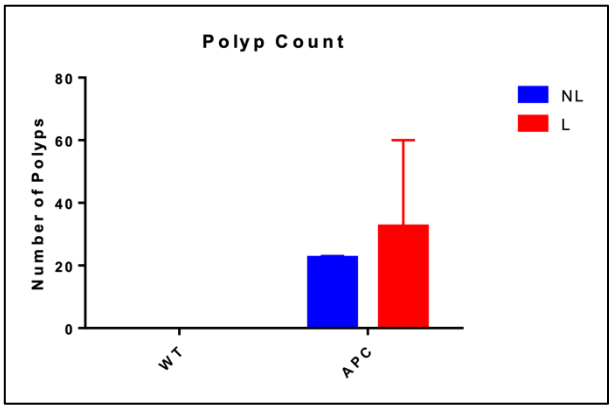

Figure 5- Average polyp count per group with standard error.

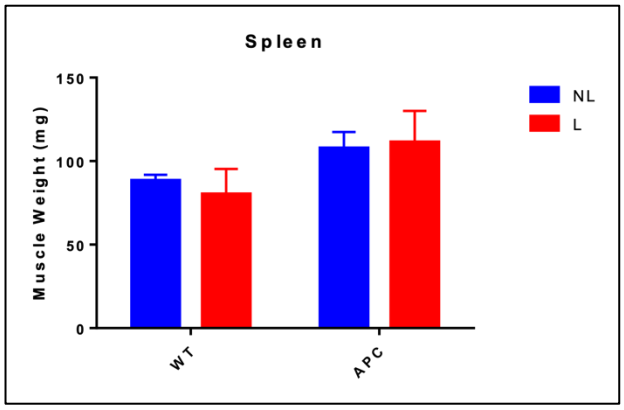

Figure 6- Comparison of average spleen weight with standard error.

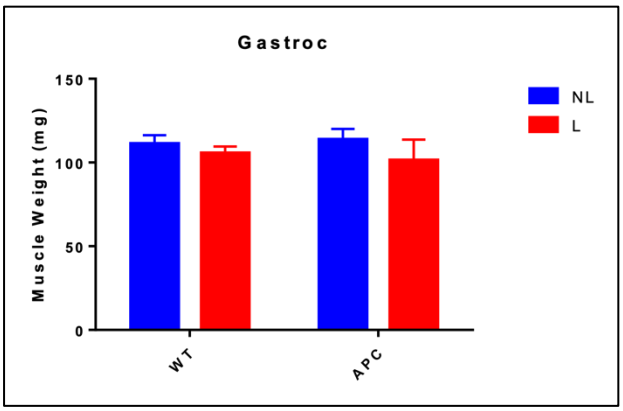

Figure 7- Comparison of average gastrocnemius weight with standard error.

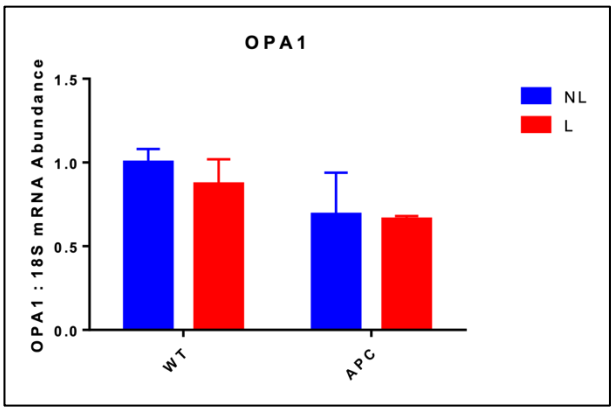

Figure 8- Average OPA1 abundance per group normalized to 18s with standard error.

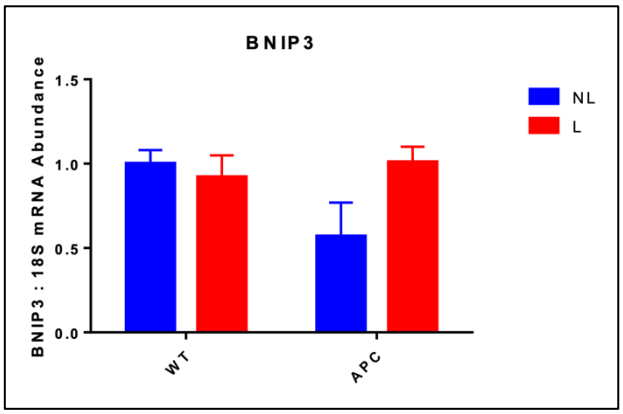

Figure 9- Average BNIP3 abundance per group normalized to 18s with standard error.

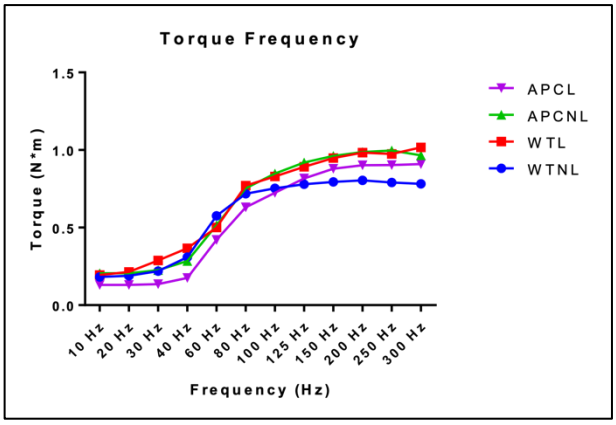

Figure 10- Average torque frequency per group.

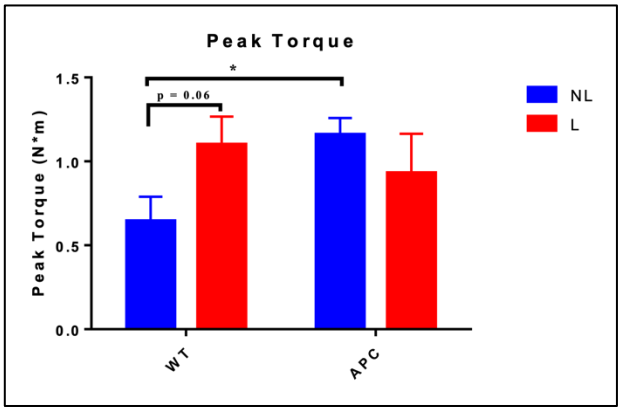

Figure 11- Average peak torque per group with standard error.

CHAPTER VIII

REFERENCES

1. Roser MR, Hannah. Cancer. Our World In Data. https://ourworldindata.org/cancer. 2015.
2. Dhanapal R, Saraswathi T, Govind RN. Cancer cachexia. *J Oral Maxillofac Pathol*. 2011;15:257-260. doi: 10.4103/0973-029X.86670
3. Mero A. Leucine supplementation and intensive training. *Sports Med*. 1999;27:347-358. doi: 10.2165/00007256-199927060-00001
4. Rieu I, Balage M, Sornet C, Giraudet C, Pujos E, Grizard J, Mosoni L, Dardevet D. Leucine supplementation improves muscle protein synthesis in elderly men independently of hyperaminoacidaemia. *J Physiol*. 2006;575:305-315. doi: 10.1113/jphysiol.2006.110742
5. Giordano A, Calvani M, Petillo O, Carteni' M, Melone MR, Peluso G. Skeletal muscle metabolism in physiology and in cancer disease. *J Cell Biochem*. 2003;90:170-186. doi: 10.1002/jcb.10601
6. Brown JL, Rosa-Caldwell ME, Lee DE, Blackwell TA, Brown LA, Perry RA, Haynie WS, Hardee JP, Carson JA, Wiggs MP, et al. Mitochondrial degeneration precedes the development of muscle atrophy in progression of cancer cachexia in tumour-bearing mice. *J Cachexia Sarcopenia Muscle*. 2017;8:926-938. doi: 10.1002/jcsm.12232
7. Perry RA, Brown LA, Lee DE, Brown JL, Baum JI, Greene NP, Washington TA. Differential effects of leucine supplementation in young and aged mice at the onset of skeletal muscle regeneration. *Mech Ageing Dev*. 2016;157:7-16. doi: 10.1016/j.mad.2016.05.007
8. Martin A, Freyssenet D. Phenotypic features of cancer cachexia-related loss of skeletal muscle mass and function: lessons from human and animal studies. *J Cachexia Sarcopenia Muscle*. 2021;12:252-273. doi: 10.1002/jcsm.12678
9. Deboer MD. Animal models of anorexia and cachexia. *Expert Opin Drug Discov*. 2009;4:1145-1155. doi: 10.1517/17460440903300842
10. Baltgalvis KA, Berger FG, Peña MM, Mark Davis J, White JP, Carson JA. Activity level, apoptosis, and development of cachexia in Apc(Min/+) mice. *J Appl Physiol (1985)*. 2010;109:1155-1161. doi: 10.1152/japplphysiol.00442.2010
11. Giles K, Guan C, Jagoe TR, Mazurak V. Diet composition as a source of variation in experimental animal models of cancer cachexia. *J Cachexia Sarcopenia Muscle*. 2016;7:110-125. doi: 10.1002/jcsm.12058
12. Karp J. Characteristics of the Three Muscle Fiber Types. In; 2004.
13. Laker RC, Xu P, Ryall KA, Sujkowski A, Kenwood BM, Chain KH, Zhang M, Royal MA, Hoehn KL, Driscoll M, et al. A novel MitoTimer reporter gene for mitochondrial content, structure, stress, and damage in vivo. *J Biol Chem*. 2014;289:12005-12015. doi: 10.1074/jbc.M113.530527
14. Costa Júnior JM, Rosa MR, Protzek AO, de Paula FM, Ferreira SM, Rezende LF, Vanzela EC, Zoppi CC, Silveira LR, Kettelhut IC, et al. Leucine supplementation does not affect protein turnover and impairs the beneficial effects of endurance training on glucose homeostasis in healthy mice. *Amino Acids*. 2015;47:745-755. doi: 10.1007/s00726-014-1903-z

15. Washington TA, Brown L, Smith DA, Davis G, Baum J, Bottje W. Monocarboxylate transporter expression at the onset of skeletal muscle regeneration. *Physiol Rep*. 2013;1:e00075. doi: 10.1002/phy2.75
16. Greene NP, Lee DE, Brown JL, Rosa ME, Brown LA, Perry RA, Henry JN, Washington TA. Mitochondrial quality control, promoted by PGC-1α, is dysregulated by Western diet-induced obesity and partially restored by moderate physical activity in mice. *Physiol Rep*. 2015;3. doi: 10.14814/phy2.12470