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The Effect of Leucine Supplementation on Mitochondrial Biogenesis within the LLC Model of Cancer Cachexia

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THE EFFECT OF LEUCINE SUPPLEMENTATION ON MITOCHONDRIAL BIOGENESIS WITHIN THE LLC MODEL OF CANCER CACHEXIA

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Abstract

The hallmarks of cachexia include the loss of leans mass due to disruption in muscle maintenance. Leucine has been shown to improve protein synthesis and mitochondrial biogenesis, key signaling pathways influenced by cachexia. This study aimed to elucidate the effects of leucine supplementation on mitochondrial biogenesis, within the Lewis Lung Carcinoma (LLC) implantation model of cancer cachexia. Twenty mice were divided into four equal groups (n = 5): Chow, leucine (Leu), LLC, LLC+Leu. At the age of 9-10 weeks, mice received a subcutaneous injection of $1x10^6$ LLC cells or phosphate buffered saline (PBS). Leu groups were then switched to diet supplemented with 5% leucine (w/w) for 4 weeks. Within the gastrocnemius, protein synthesis and mitochondrial content were decreased in LLC. Peroxisome proliferator-activated receptor Gamma Co-activator 1-α (PGC-1α) within LLC+Leu was increased relative to LLC. Leucine was unable to preserve protein synthesis, mitochondrial content, and reduce inflammation associated with LLC implantation.

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Key to Symbols and Abbreviations

ADP - Adenosine diphosphate

Akt - Protein Kinase B

AMP - Adenosine monophosphate

AMPK - AMP-activated protein kinase

ANOVA - Analysis of variance

APR - Acute Phase Response

ATP - Adenosine triphosphate

BCAA – Branched Chain Amino Acid

BCAT - Branched chain aminotransferase

BSA – Bovine Serum Albumin

Chow – Control diet

CRP - C-reactive protein

FOXO1 - Forkhead box protein O1

Glut4 - Glucose transporter type 4

HMB - β-hydroxy-β-methylbutyrate

HRP - Horseradish Peroxidase

IGF-1 - Insulin-like growth factor 1

IL-6 - Interleukin 6

IOD – Integrated optical density

JNK - Jun N-terminal kinase

Leu - Leucine supplemented

LLC - Lewis Lung Carcinoma

MAPK - Mitogen-Activated Protein Kinase

MCK - Muscle Creatine Kinase

mRNA - Messenger RNA

mTOR – Mammalian Target of Rapamycin

mtROS – Mitochondrial Reactive Oxygen Species

NAD - Nicotinamide Adenine Dinucleotide

NFκB - Nuclear Factor κ B

NRF - Nuclear Respiratory Factor

PBS – Phosphate Buffered Saline

PGC-1α - Peroxisome proliferator-activated receptor Gamma Co-activator 1-α

PIF - Proteolysis-Inducing Factor

PPAR - Peroxisome Proliferator-Activated Receptors

RNS - Reactive Nitrogen Species

ROS - Reactive oxygen species

RTF – Run to fatigue

SEM - Standard Error of Mean

SIRT1 – Sirtuin 1

STAT3 - Signal transducer and activator of transcription 3

TBST - Tris Buffered Saline with Tween® 20

TFAM - mitochondrial transcription factor A

TNFR1 - Tumor necrosis factor receptor 1

TNFα - Tumor necrosis factor α

TRAPS - TNFR1-associated periodic syndrome

UCP - Uncoupling proteins

UPS - Ubiquitin Proteasome System

Introduction

Cachexia refers to the loss of body mass in the presence of an underlying chronic illness. In cancer patients in particular, cachexia affects a large portion of the population and is thought to be responsible for more than 30% of all cancer-related deaths (von Haehling & Anker, 2010). In contrast to typical anorexic weight loss, there is increased loss of muscle mass relative to fat mass, potentially modulated by metabolic disruption induced by the chronic inflammation associated with cancer. In various models of disease, loss of lean mass is an indicator of increased mortality (Nanri et al., 2010; Pocock et al., 2008). The loss of lean mass induced by cancer is commonly attributed to the decreased skeletal muscle protein synthesis and stimulation of proteolytic pathways. In response, nutritional interventions have been utilized to increase food intake, preserve body mass, and improve quality of life, with varying degrees of success. However, due to different methods, results from these studies do not demonstrate consistent preservation of body mass or lowering mortality (Baldwin, Spiro, Ahern, & Emery, 2012; Balstad, Solheim, Strasser, Kaasa, & Bye, 2014). These findings suggest the need for additional strategies for management of weight loss induced by cancer cachexia.

Cancer Cachexia

Although many definitions exist, cachexia generally refers to the loss of body mass, both muscle and fat mass, in the presence of an underlying chronic disease. This loss of body mass is generally accompanied by inflammation and alterations in metabolic pathways. Cachexia affects a significant portion of cancer patients and it is associated with increased morbidity and mortality in cancer patients (von Haehling & Anker, 2010). In various states of disease, significant loss of lean body mass is correlated with increased

mortality (Nanri et al., 2010; Pocock et al., 2008). However, loss of body mass is not enough to identify cancer cachexia. Both systemic inflammation and reduced food intake can be factored in conjunction with loss of body mass to predict adverse functions of cachexia and poor prognosis (Fearon, Voss, Hustead, & Group, 2006).

Reduction in food intake and hypermetabolism. Many cancer patients experience anorexia, likely induced by the disease and its accompanying treatments including chemotherapy, surgery, and radiotherapy. In a study of sixty six cancer patients receiving end of life care, 61% had anorexia even though they were not receiving chemotherapy (Tranmer et al., 2003). Additionally, there is increasing evidence that weight loss due to pathologically induced anorexia, from cancer, HIV, or sepsis, is inherently different from anorexia nervosa. In the case of anorexia nervosa, fat mass is often lost first in a higher proportion than lean body mass due to prolonged starvation with ketone bodies being produced for energy supplementation to the brain. In the pathologically induced anorexia, there is a larger portion of lean body mass loss relative to fat mass (Tisdale, 2009). Fortunately, loss of body mass due to cachexia responds well to oral nutritional support, as a meta-analysis reviewing 13 studies totaling 1,414 patients, has shown that interventions were associated with an increased energy intake of 430 kcals per day and a weight gain of 1.9 kg over various durations (Baldwin et al., 2012). While nutritional intervention improved various quality of life factors, these studies were very heterogeneous and did not significantly affect mortality (Baldwin et al., 2012). When gastrointestinal function is impaired, oral nutrition can be supplemented with parenteral nutrition, providing caloric support to stabilize nutritional status (Pelzer et al., 2010). The fact that simply increasing caloric intake does not consistently preserve

muscle mass or improve mortality suggests that there is more to cachexia than overcoming anorexia.

In addition to anorexia, resting energy expenditure may be increased in various forms of cancer. Several factors may increase resting energy expenditure including increased acute phase response (APR), thermogenesis from skeletal muscle, or presence of brown adipose tissue (Tisdale, 2009). An increase in APR occurs when the liver changes protein synthesis from production of albumin to acute phase proteins such as C-Reactive protein (CRP). In addition to increasing resting energy expenditure, induction of APR in cachectic patients has been linked to inflammation (Scott, McMillan, Crilly, McArdle, & Milroy, 1996; Staal-van den Brekel, Dentener, Schols, Buurman, & Wouters, 1995), muscle wasting (Bonetto et al., 2011), reduced quality of life and shortened survival (Falconer et al., 1995; Vaughan, Martin, & Lewandowski, 2013). The increased thermogenesis from skeletal muscle and "browning" of adipose tissue is caused by increased expression of uncoupling proteins (UCPs) that mediate the proton gradient in the mitochondria. In the muscle, increased levels of UCP3 mRNA was associated with weight loss in adenocarcinoma patients (Collins, Bing, McCulloch, & Williams, 2002). In the MAC16 adenocarcinoma mouse model of cancer cachexia, UCP1 was elevated in adipose tissue while UCP2 and UCP3 were elevated in the muscle of tumor bearing groups (Bing et al., 2000). Both factors highlight cancer induced metabolic inefficiencies additively contribute to cachexia.

In some cancer patients, the total energy expenditure may be lower relative to that of a healthy individual, despite increased resting energy expenditure. Understandably, this is commonly attributed to the lack of physical activity in the cachectic population

(Moses, Slater, Preston, Barber, & Fearon, 2004). Inactivity is not only associated with increased systemic inflammation, but lower quality of life as well (Fischer, Berntsen, Perstrup, Eskildsen, & Pedersen, 2007; Tisdale, 2009). While physical activity provides many metabolic benefits, counteracting inflammation and improving mitochondrial function, exercise may not be possible for those in advanced stages of cancer. In addition to physical inactivity, there is also evidence that disturbances in mitochondrial function and elevations in APR are associated with chronic inflammation induced by cachexia.

A key metabolic factor modulated by cancer cachexia is mitochondrial function. Due to increased inflammatory signaling and generation of reactive oxygen species (ROS) during cancer cachexia, mitochondrial biogenesis and function may be impaired. Various models of cancer cachexia exhibit decreased Peroxisome proliferator-activated receptor Gamma Co-activator 1-α (PGC-1α) expression and mitochondrial dysfunction in the presence of increased IL-6 and weight loss (Eley, Russell, & Tisdale, 2007; White et al., 2011). This impairment may affect the efficiency of metabolism and energy balance, consequently influencing weight loss. While these effects can be attenuated by exercise (Puppa et al., 2012), other strategies in conjunction with exercise could be developed to further preserve muscle mass and mitochondrial function.

One potential strategy may be the supplementation of the Branched Chain Amino Acid (BCAA) leucine, which has been established as a stimulator of protein synthesis through increased activity of the Mammalian Target of Rapamycin (mTOR) pathway. Leucine supplementation has also been shown to attenuate protein degradation, increase mitochondrial function, and modulate insulin signaling. In a tumor implantation model of cancer cachexia, Leucine supplementation mitigated the loss of

lean mass and increased protein synthesis, without increasing tumor mass (Eley et al., 2007). Thus, the therapeutic signaling produced from leucine supplementation may counter balance cachectic signaling induced by development of cancer. However, in pancreatic cancer cells leucine induced proliferation of tumor cells (K. A. Liu, Lashinger, Rasmussen, & Hursting, 2014). Additionally, Peters et al. demonstrated that leucine supplementation was unable to preserve body weight, but attenuated loss of muscle mass in glycolytic muscles (Peters et al., 2011). Although research has demonstrated the ability of leucine supplementation to attenuate muscle loss, little has been done to understand leucine's effect on maintaining skeletal muscle metabolism during cancer cachexia. More research needs to be done to understand the mechanisms by which leucine is preserving muscle mass and how it interacts with the tumor and both muscle and systemic metabolism.

Mitochondrial Function

Mitochondria are crucial for metabolic reactions necessary for energy production by processing products of lipid and carbohydrate metabolism. This dual membraned organelle hosts highly oxidative processes to produce the energy necessary for the cell, insulating these reactive components from the cytosol. Disruption in mitochondrial function has been shown in various disease states ranging from diabetes to heart failure, suggesting an important role in the progression of these pathological conditions (Kelley, He, Menshikova, & Ritov, 2002; Ventura-Clapier, Garnier, & Veksler, 2008). Various studies have linked disruption in mitochondrial function to increased oxidative stress through generation of reactive oxygen species (ROS) and vice-versa (Venditti, Di Stefano, & Di Meo, 2013). In models of diet-induced obesity and type II diabetes,

mitochondrial dysfunction, oxidative stress, chronic inflammation, and physical inactivity are often associated with each other (Bach et al., 2005; Fischer et al., 2007; Kelley et al., 2002). Mitochondrial dysfunction in muscle can lead to loss of muscle mass and function, impairing physical activity and overall health (Argilés, López-Soriano, & Busquets, 2015; Romanello & Sandri, 2015).

Mitochondrial function can be characterized through assessment of mitochondrial mass, metabolic efficiency, and maintenance. A main factor in mitochondrial mass is mitochondrial biogenesis, which can be defined as processes that increase mitochondrial mass and number. In the world of exercise, increases in muscle mitochondria are thought to be largely responsible for increased resistance to fatigue and is one of the proven benefits of training. On the molecular level, mitochondrial biogenesis is highly influenced by current energy balance and associated signaling molecules. Factors such as a low ATP/ADP*AMP or NADH/NAD balance lead to increased AMP-activated protein kinase (AMPK) and Sirtuin 1 (SIRT1) activity respectively, modulating the activity of Peroxisome proliferator-activated receptor gamma co-activator (PGC-1α). PGC-1α is a key regulator of mitochondrial biogenesis through its interactions with key transcription factors, such as increased activation of nuclear respiratory factor 1 (NRF1) and 2 (NRF2) that leads to increased activity of mitochondrial transcription factor A (TFAM). TFAM drives the transcription and replication of mitochondrial DNA, consequently driving mitochondrial biogenesis (Jornayvaz & Shulman, 2010).

In addition to exercise, other factors including inflammatory cytokines, caloric restriction, and various pharmacological agents have been shown to modulate mitochondrial biogenesis (Andreux, Houtkooper, & Auwerx, 2013; Jornayvaz &

Shulman, 2010). Furthermore, in some models of cancer cachexia, increased levels of interleukin-6 (IL-6) have been shown to decrease PGC-1α expression, resulting in disruption of mitochondrial biogenesis. To combat this disruption, both inhibition of IL-6 signaling and exercise have been shown to attenuate these effects (White et al., 2012). Due to the relationship between mitochondrial disruption and muscle loss, preservation of mitochondrial function may be a vital mechanism to prevent further disruption of metabolic pathways already affected by cancer cachexia and prevent muscle wasting.

Leucine Supplementation

Branched chain amino acids (BCAAs) Leucine, Valine, and Isoleucine are essential amino acids that are primarily metabolized in the skeletal muscle. The liver, which would normally metabolize the majority of dietary amino acids, lacks branched chain amino transferase (BCAT) allowing for the BCAAs to be spared from hepatic metabolism and be utilized by tissues containing BCATs such as the skeletal muscles and the brain (Pedroso, Zampieri, & Donato, 2015). Dietary Supplementation of BCAAs, specifically leucine, have been shown to modulate protein synthesis and degradation in skeletal muscle. In fact, dietary leucine has been shown to increase protein synthesis through increased mTOR signaling, improve mitochondrial function, improve glucose metabolism, and enhance insulin signaling in various models and tissues (Li, Xu, Lee, He, & Xie, 2012; Pedroso et al., 2015). While the stimulation of mTOR and subsequent protein synthesis is widely known, Leucine's effects on mitochondrial function, protein degradation, and glucose metabolism may prove to be just as valuable in metabolic disorders.

Attenuation of Protein Degradation. Leucine supplementation has been shown to attenuate protein degradation in models of muscle wasting and protein deficiency (Baptista et al., 2010; Sugawara, Ito, Nishizawa, & Nagasawa, 2009). Baptista et al. demonstrated that leucine supplementation in a model of hind limb immobilization attenuates soleus muscle mass loss. This attenuation was accompanied with decreased expression of E3 ligases, potentially down regulating protein degradation via the Ubiquitin Proteasome System (UPS) pathway (Baptista et al., 2010). In rats fed a protein free diet, leucine supplementation attenuated protein degradation without increasing protein synthesis. The active form of protein light chain 3, a marker of autophagy, was significantly decreased while proteinase activity and ubiquitin ligase mRNA expression were not. These findings suggest that leucine may attenuate muscle protein degradation due to protein deficit through inhibition of autophagy, rather than increasing protein synthesis (Sugawara et al., 2009). Mirza et al compared the attenuation of protein degradation from leucine supplementation and from Ca-β-hydroxy-β-methylbutyrate (HMB) supplementation, a leucine metabolite, in C2C12 myotubes. They found that both leucine and HMB attenuated decreases in protein synthesis induced by proteolysis-inducing factor (PIF), lipopolysaccharide, and angiotensin II, with HMB (50 μM) being more potent than Leucine (1 mM). Similar to the previously mentioned studies, this attenuation of protein degradation was measured as decreased activity of the UPS pathway through functional assessment of proteasome enzymatic activity and decreased western blot quantification proteasome subunits and ubiquitin ligases (Mirza, Pereira, Voss, & Tisdale, 2014). These findings show that leucine supplementation can attenuate

protein degradation independently of protein synthesis, further supporting its potential benefits for supplementation in highly catabolic states such as cachexia.

Influence on Mitochondrial Function. In addition to stimulation of protein synthesis and attenuation of protein degradation, leucine supplementation has been shown to modulate mitochondrial function, potentially through the activation of SIRT1 and phosphorylation of AMPK. Leucine supplementation in C2C12 myotubes significantly increase mitochondrial content, expression of genes related to mitochondrial biogenesis, fatty acid oxidation, SIRT1 activity and gene expression, and AMPK phosphorylation compared to the controls (Liang, Curry, Brown, & Zemel, 2014). In a similar study, 24 h leucine incubation with C2C12 myotubes increased peroxisome proliferator-activated receptors β/δ (PPAR) expression and markers of mitochondrial biogenesis when compared to valine incubation (control). Inhibition of PPARβ/δ resulted in decreased mitochondrial content and inhibition of mitochondrial oxygen consumption. In addition to mitochondrial effects, leucine incubation stimulated the phosphorylation of Akt and increased Glucose transporter type 4 (GLUT-4) content, subsequently increasing glucose uptake and resulted in increased PPARγ expression, fatty acid synthase expression, and lipid content suggesting improved lipid biogenesis and metabolism (Schnuck, Sunderland, Gannon, Kuennen, & Vaughan, 2016).

In a mouse model of diet-induced obesity, leucine supplementation has been shown to increase mitochondrial biogenesis through increases in SIRT1 activity, resulting in decreases in PGC-1α acetylation, increasing mitochondrial mRNA (PGC-1α, NRF1, TFAM), and increased citrate synthase activity in both the liver and adipose tissue. Additionally, leucine supplementation also improved similar measures of oxidative

capacity in the skeletal muscle, decreased FOXO1 acetylation, and improved glucose metabolism (Li et al., 2012). These findings build a strong case for the improvement of mitochondrial function through Leucine supplementation, potentially improving outcomes of metabolic disorders like type 2 diabetes. Moreover, the ability of leucine to stimulate protein synthesis, attenuate degradation, improve mitochondrial function, and improve insulin sensitivity highlight its potential to improve outcomes of cancer cachexia.

Combating Cachexia with anabolic stimulation or improvement of mitochondrial function. In a few models of cancer cachexia, stimulation of the mTOR pathway has been shown to mitigate muscle loss (Chen et al., 2016; Eley et al., 2007). Chen et al supplemented Salidroside, a commonly consumed plant derivative, in LLC and CT-26 models of cancer cachexia and in C2C12 myotubes. They found that supplementation of Salidroside preserved muscle mass, improved food intake, and improved mTOR signaling without increasing tumor mass. Salidroside also improved outcomes in conjunction with a chemotherapeutic agent and improved mTOR signaling in C2C12 myotubes incubated with TNF-α (Chen et al., 2016). Utilizing a similar strategy, Eley et al. showed that supplementation of Leucine in a MAC16 implantation model of murine cancer cachexia improved protein synthesis, attenuated degradation, and preserved muscle mass, without increasing tumor mass. Western blot analysis of the skeletal muscle confirmed that these improvements were accompanied with improved mTOR signaling. Leucine supplementation in C2C12 also confirmed resistance to suppression of protein synthesis by PIF and angiotensin II (Eley et al., 2007). Together,

these studies support the use of nutritional anabolic stimulators in select cancer models to preserve muscle mass, potentially without increasing tumor burden.

Other labs have considered increasing mitochondrial biogenesis or function to combat cachectic effects. In a LLC implantation model of murine cancer cachexia, PGC-1α overexpression in the skeletal muscle increased mitochondrial biogenesis, content, and enzymatic activity. However, this increase in muscle mitochondrial function alone did not preserve muscle mass and may have increased tumor growth as tumor mass was significantly increased in the PGC-1α over expression group (Wang, Pickrell, Zimmers, & Moraes, 2012). In other models of muscle wasting, supplementation of resveratrol, an anti-oxidant and stimulator of mitochondrial function, has been shown to preserve mitochondrial function and attenuate muscle wasting (J. Liu et al., 2015). Resveratrol supplementation in a C26 adenocarcinoma model was reported to preserve lean, fat, and skeletal muscle mass potentially through attenuation of UPS degradation and Nuclear factor κ B (NFκB) inflammatory signaling (Shadfar et al., 2011). These studies support the use of nutritional supplements to improve protein synthesis, preserve mitochondrial function, and attenuate protein degradation to combat the catabolic effects of cachexia. Moreover, the supplementation of leucine has largely been beneficial in numerous disease models, with few or no associated side effects (Pedroso et al., 2015). However, one last connection remains to be confirmed: the influence of leucine on mitochondrial function under cancer cachexia.

Therefore, this experiment aimed to determine the effects of dietary leucine supplementation on mitochondrial function within the LLC implantation model of cancer cachexia. A secondary aim was to analyze the effect of leucine on skeletal muscle mass

and function within cancer cachexia, allowing us to correlate these factors with mitochondrial function. Based on the current literature, we hypothesized that leucine should improve mitochondrial biogenesis within the LLC implantation model of cancer cachexia, with improved measures of protein synthesis and glucose metabolism. Furthermore, if those measures were improved, we expected preservation of skeletal muscle mass relative to the LLC group.

The results from this experiment should further characterize the relationship between leucine supplementation, mitochondrial function, protein synthesis, maintenance of skeletal muscle, and LLC induced cachexia. Should the dietary supplementation of leucine prove beneficial under the influence of cancer, these effects may support therapeutic use to combat cachexia.

Materials and methods

Cell Culture

The Lewis Lung Carcinoma (LLC) cell line utilized for this experiment was provided as a gift from the Carson Lab (University of South Carolina). The LLC cells were grown in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and penicillin/streptomycin, refreshing media every 48h. Just prior to tumor implantation, LLC cells were rinsed with phosphate buffered saline (PBS) and incubated with trypsin to release the cells from the dish. The cells were then suspended in PBS at a concentration of $1x10^7$ cells per mL, such that each mouse received a 100 µl, subcutaneous injection of one million cells in their right flank.

Animals

All experimental and housing protocols were approved by the Institutional Animal Care and Use Committee of the University of Memphis.

C57BL/6 male mice (n = 20), aged 7-8 weeks, were individually housed in a climate controlled room on a 12:12-h light-dark cycle. Upon arrival, all mice were allowed two weeks to acclimate to the new housing facilities before being divided into 4 groups: Chow n = 5, Leucine Enriched (Leu) n = 5, Lewis Lung Carcinoma Implanted (LLC) n = 5 and LLC+Leu n = 5. The mice were allocated such that each group had equivalent average weight. In the week preceding tumor implantation, the mice underwent pre-conditional testing including a run to fatigue, and fasting blood glucose measure. After two weeks, at approximately 9-10 weeks of age, LLC groups received the subcutaneous 100 μL injection of $1x10^6$ LLC cells suspended in PBS while control groups received an equivalent volume of PBS alone. At this point, food was switched from standard chow to their respective experimental diets s, with Leu group diets being supplemented with Leucine 5% w/w. For details on dietary composition of each diet, consult Table 1.

Subsequently, the mice were monitored over the course of 28 days with an endpoint run to fatigue, blood draw, and glucose tolerance test conducted within a week of euthanasia. Food intake and body weight were measured every 48 h and the mice were checked daily for overall health. If the mice failed to eat over the course of 48 h, had severely impaired movement, lost >20% of body weight over 48 h, had a tumor larger than 2.5 cm in diameter, lacked responsiveness, or showed other severe signs of ill health, the mice were euthanized immediately. Mice were fasted 5 h prior to euthanasia, fasting

blood glucose measures, and blood draws. Mice were injected with puromycin (0.04 µM/g BW) 30 minutes prior to tissue collection for assays measuring protein synthesis. Immediately before euthanasia, mice were anesthetized with isoflurane and tissues were harvested. For a list of collected tissues and respective weights, reference Table 2.

For further biochemical analysis, the gastrocnemius of the hind limb was removed, weighed, snap frozen in liquid nitrogen, and stored at -80 °C. A visual representation of how these methods were conducted throughout the experiment can be seen in Figure 1.

Run to Fatigue

During run to fatigue testing, mice were placed on the treadmill briefly to allow acclimation prior to testing. The acclimation, warmup, and run to fatigue was conducted on an incline of 5%. The pre and post run to fatigue starts with warmup period consisting of a 5 m/min walk, a 10 m/min walk, and 15 m/min run at 5 minutes each. After the warm up, the run to fatigue began and the timer was started. The mice ran at 20 m/min for 30 min before the speed was increased to 25 m/min for the remainder of the trial. If mice stopped running or slipped off the end of the treadmill, the mice were encouraged to return to running with a slight push with a gloved hand. Mice that continuously fell off or refused to run were removed and their time was taken. After two hours, the run to fatigue was ended.

Fasting blood glucose

Within the week preceding tumor implantation and within the last week of experimental conditions, mice were fasted 5 h before blood glucose levels were measured with a glucometer (Bayer Contour) using blood from a tail snip.

Western Blot

A portion of the excised gastrocnemius muscle was homogenized using a Kontes glass homogenizer in a 10:1 v/w ratio in ice cold Muller buffer containing protease and phosphatase inhibitors. The samples were centrifuged for 10 minutes at 10,000 g before the supernatant was removed into a separate microtube. The total protein concentration of this lysate was quantified using the Bradford protein assay. Samples were diluted to 3 μg protein/μL in appropriate amounts of diluent buffer and 5X Lane Marking Reducing Sample Buffer and were heated at 95° C for 10 minutes. Samples were then resolved via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 4-15% gradient gel and subsequently transferred on to a PVDF membrane. The blot was then blocked with 5% BSA for 1 h before being incubated in primary antibody according to manufacture specification. After multiple washes (3 washes at 5 min each) in Tris-buffered saline-0.1% Tween 20 (TBST) blots were incubated in appropriate HRP-conjugated secondary antibodies (1:5,000 in 5% BSA for 2 h). For more details on western blot conditions and source of antibodies, reference Table 2. The blots were then washed again in TBST before being visualized with a chemiluminescent agent and imaged using a Fotodyne® 60-7020 bench top imager. All bands were quantified via densitometry via ImageJ.

Protein synthesis was quantified with a western blot adaptation of the SUrface SEnsing of Translation (SUnSET) technique (Schmidt, Clavarino, Ceppi, & Pierre, 2009). The puromycin injected (0.04μMol/ g BW) 30 minutes prior to sacrifice is incorporated into elongating peptide chains, preventing further peptides from binding. Consequently, the peptides with incorporated puromycin are released from the ribosome,

accumulating in the cell. At low levels, quantification of these puromycin incorporated peptide chains directly correlates with protein synthesis. Utilizing the western blot methodology, immunoblotting with a puromycin antibody allow quantification of protein synthesis.

After imaging, blots were stained with Ponceau to visualize bound protein and were imaged using the bright field setting on the Fotodyne® imager. Immunoblotted proteins were then normalized to the inverted densitometry quantification of the Ponceau stain. Data is presented as relative integrated optical density (IOD) normalized to inverted densitometric quantification of ponceau staining with the control group average set to 1.

Statistical Analysis

All statistical analyses were conducted using GraphPad Prism 7® software. All data are presented as means $\pm$ Standard Error of Mean (SEM). Statistical significance was set at $\alpha = 0.05$. Western blot data were analyzed via two-way analysis of variance (ANOVA). Pre-planned t-tests were used to examine the effect of LLC, the effect of leucine in the control condition, and the effect of leucine in the LLC condition.

Results

All mice in the Chow, Leu, and LLC groups completed the study with no complications. Within the LLC+Leu group, one mouse experienced tumor growth that impaired mobility, requiring euthanasia three days before the rest. Another mouse within the LLC+Leu group did not develop a tumor and resulting data was excluded from analysis ($n = 4$).

Experimental effects on food intake and body composition. Neither LLC implantation nor leucine supplementation had significant effects on body weight (Figure

2A) or daily food intake (Figure 2B) over the course of this experiment. Leucine had no effect on tumor growth (Table 3). There were no independent effects of LLC implantation or leucine supplementation on tumor free body mass, epididymal fat mass, gastrocnemius mass, or hind limb mass, calculated as the sum of the hind limb muscles taken, between groups (Figure 2C-E). Within the LLC implantation groups preplanned t-tests revealed a significant decrease in gastrocnemius and hind limb mass of the leucine supplemented group when compared to the control chow group.

Experimental effects on skeletal muscle function and fasting glucose. A run to fatigue test was conducted the week preceding euthanasia and tissue collection. There was a main effect of LLC implantation ($p>0.05$) to decrease endurance running performance measured by the run to fatigue (Figure 3A). Within the same week, fasting blood glucose was measured and recorded via glucometer. An interaction between LLC implantation and leucine supplementation was present within the final fasting glucose measures, with a T-test revealing a significant decrease in the LLC+Leu group compared to the LLC group (Figure 3B). Considering individual changes in response to experimental conditions, a change in fasting glucose was calculated for each mouse, as a percent difference of pre-experimental measures to post-experimental measures of fasting glucose (Figure 3C). Considering % change from pre-experimental measures, a T-test revealed that leucine supplementation in the LLC implanted mice induced a significant decrease in fasting glucose ($P<0.05$) compared to the control chow group.

Experimental effects on markers of mitochondrial function within skeletal muscle. Western blot analysis in the gastrocnemius muscle revealed effects of LLC implantation and leucine supplementation on protein expression levels of various

mitochondrial markers. An interaction between LLC implantation and leucine supplementation was present within PGC-1α expression levels (Figure 4A), a marker of mitochondrial biogenesis. A significant increase of PGC-1α protein levels in the LLC+Leu group was seen relative to LLC. Cytochrome C (Figure 4B), a marker of mitochondrial content, showed a main effect LLC implantation decreasing expression levels, with a significant decrease ($p<0.05$) in the LLC group compared to the Chow. Analysis of CV-ATPA5 (Figure 4C), commonly known as ATP synthase or mitochondrial complex V, showed a main effect of LLC implantation reducing expression levels. Levels of CIII-UQCRC2 (Figure 4D), a component of complex mitochondrial complex III or ubiquinol-cytochrome c reductase complex, showed a main effect of LLC implantation, resulting in significant decreases ($p<0.05$) in both LLC and LLC+Leu groups, when compared to the control. For both CII-SDHB (Figure 4E), a subunit of succinate dehydrogenase or complex II, and CI-NDUFB8 (Figure 4F), subunit of mitochondrial complex I or NADH dehydrogenase, expression levels were not significantly different between groups.

Experimental effects on protein synthesis. Protein synthesis was quantified by western blot adaptation of the SUnSET technique, measuring incorporation of injected puromycin into elongating peptides. Within the gastrocnemius, there was a main effect of LLC implantation for decreased puromycin levels (Figure 5A), Additionally, a significant decrease ($p<0.05$) was detected in the LLC+Leu group when compared to the control. Interestingly, there was no effect of Leucine or LLC tumor implantation on the phosphorylation of mTOR (Figure 5B).

Experimental effects on phosphorylation of STAT3. The proportion of STAT3 phosphorylation can indicate state of inflammation. There was a main effect of LLC implantation ($P<0.05$) to increase STAT3 phosphorylation (Figure 5C). In the non-tumor groups, leucine significantly decreased STAT3 phosphorylation ($p<0.05$), but this was not maintained in the tumor implantation groups.

Discussion

Preservation of lean mass is often the goal when treating cachexia, as loss of lean mass is associated with increased mortality within cancer and many other pathological populations including: heart failure, liver cirrhosis, sarcopenia, and more (Kalafateli, Konstantakis, Thomopoulos, & Triantos, 2015; Nanri et al., 2010; Pocock et al., 2008; Visser & Schaap, 2011). Cachectic signaling secreted by the tumor induces a chronic state of inflammation, which has been linked with muscle wasting and disruption of energy metabolism. Consequently, strategies utilizing nutritional intervention and exercise can support maintenance of skeletal muscle and improve metabolic measures, with reduced interactions with pharmacological therapies for cancer treatment (Argilés, Busquets, López-Soriano, Costelli, & Penna, 2012; Baldwin et al., 2012). In addition to improving caloric and micronutrient content, there is evidence that utilization of nutraceuticals, food based nutrients at pharmacological doses, can provide benefits through improvements in metabolic signaling in skeletal muscle.

This research explores whether the Branched Chain Amino Acid (BCAA) leucine can improve metabolic measures impaired by cachexia. Within the models of exercise, diet, and disease, leucine has been shown to improve protein synthesis and preserve muscle mass. In non-pathological models, leucine supplementation increases

mitochondrial biogenesis and content. The focus of this experiment was mitochondrial biogenesis, as loss of mitochondria can be associated with cachexia and muscle wasting. Therefore, our experiment aimed to elucidate how leucine signaling influences mitochondrial biogenesis within the LLC model of cancer cachexia and whether that interaction affects muscle mass and function.

Both leucine supplementation and LLC implantation had limited effects on food intake and body composition. Neither 5% leucine supplementation nor LLC implantation affected food intake over the course of the experiment, suggesting the absence of anorexia. Even minor anorexic tendencies can result in large differences in body weight. Since food intake did not differ between groups, we can infer that the influences of LLC implantation on body composition are independent of anorexia. However, neither LLC implantation nor leucine supplementation independently affected tumor free body weight, fat mass, or muscle mass (Figure 2C-D). This is in contrast to previous studies which have shown LLC-induced decreases in tumor free body mass and gastrocnemius weight when compared to controls (Puppa, Gao, Narsale, & Carson, 2014). Interestingly, in the current study leucine supplementation in the LLC condition resulted in a decrease of muscle mass in the gastrocnemius. This contrasts with other reports that supplementing leucine within models of cachexia preserved muscle mass (Eley et al., 2007; Peters et al., 2011).

Similar to our study, Peters et al. utilized a tumor implantation model of cachexia to reveal dose dependent effects of dietary leucine on preservation of muscle mass. The C-26 adenocarcinoma model induced significant decreases in body and muscle mass over the course of 21 days, a more acute window of cachexia (Peters et al., 2011). The authors

noted that this model progressively develops anorexia, as noted by a significant decrease in food intake on the final day. Providing 14.6% leucine preserved the gastrocnemius and tibialis anterior under tumor burden, but lower leucine content of 9.6% did not result in a significant preservation of body or muscle mass, similar to our study. While both diets initially appear to have higher leucine content than our 5% supplementation (w/w), the 9.6%, and 14.6%, also accounts for leucine from dietary protein. The shortened experimental period, leucine dosage, and progression into anorexia may influence rates of muscle loss within their model, which may contribute to different results for the present study.

In a study analyzing the effect of branched chain amino acids in a MAC16 model of cancer cachexia, Eley et al. demonstrated that leucine supplementation preserves soleus muscle mass and increases protein synthesis in the gastrocnemius when compared to the control tumor group (Eley et al., 2007). While these results are, again, in contrast to ours, methodology differed as leucine supplementation was administered daily in a PBS solution (1g Leucine/kg of bodyweight) by gavage, only after tumor groups had lost 5% of their starting body weight. Additionally, the mice were sacrificed after 20% of their starting body weight had been lost, a period of 5 days, ensuring that the main outcome of cachexia, significant loss of body mass, was present (van Hall et al., 2008). Despite measuring similar outcomes in a similar disease state, Eley's acute delivery of leucine supplementation into mice progressing into cachexia is in stark contrast to the study where the diet provided 5% w/w of leucine, immediately after LLC implantation.

The fact that our mice did not experience the predicted decrease in body or muscle mass under LLC implantation, as seen in previous studies or other models,

suggest that they did not develop a severe state of cachexia. Moreover, the decrease in muscle mass found with leucine supplementation in conjunction with LLC implantation was not expected, suggesting a different state of disease progression than other studies. Despite, not experiencing pathological wasting, our analysis of protein synthesis and mitochondrial function suggest that some cachectic signaling may be occurring, perhaps in a state of pre-cachexia.

Leucine supplementation and LLC implantation had limited effects muscle protein synthesis. In the current study, LLC implantation significantly impaired protein synthesis, as demonstrated by decreases in puromycin labeling in both LLC and LLC+Leu groups (Figure 4A). Unexpectedly, the administration of leucine supplemented chow was unable to induce protein synthesis in the skeletal muscle in both the control group and the LLC inoculated mice. Similarly, skeletal muscle mTOR activity was not increased with Leucine supplementation (Figure 5B). Interestingly, leucine supplementation in the LLC model resulted in a decrease puromycin incorporation, and unaltered mTOR phosphorylation. This discrepancy between puromycin, a functional measure of protein synthesis, and mTOR phosphorylation, an indicator of anabolic signaling, suggests an effect of cachectic signaling within the LLC implantation groups that is occurring downstream of mTOR activation.

The disconnect between measurements of protein synthesis and mTOR activation may result from influences of inflammation. A main effect of LLC implantation is increased phosphorylation of STAT3, a marker of inflammation, as seen in the skeletal muscle. This contrasts with the decreased proportions of STAT3 phosphorylation in Leucine supplemented group without LLC implantation (Figure 5C). These findings

show that inflammatory signaling is increased by LLC implantation and decreased by leucine supplementation, but leucine supplementation is not sufficient to decrease muscle inflammatory signaling in the pre-cachectic condition.

While the inflammatory signaling induced by LLC implantation indicates the presence of cachectic signaling, chronic inflammation may also explain the reduced efficacy of leucine supplementation within our study. In a state of induced sepsis, stimulation of mTOR by leucine supplementation was blocked. To demonstrate that not all anabolic signaling was blocked, exogenous insulin like growth factor-1 (IGF1) activated the same pathways that were not responsive to leucine with sepsis (Lang & Frost, 2004). These findings suggest that under inflammatory conditions, skeletal muscle may not respond to anabolic signaling by leucine supplementation. In a model of age induced muscle loss, antioxidants restored anti-catabolic blunting by leucine supplementation (Marzani et al., 2008). These findings suggest that anabolic signaling is heavily influenced by the state of inflammation.

While inflammation can be attributed to infection and tumor secretion, the generation of reactive oxygen species (ROS) can also contribute. Interestingly, mitochondrial dysfunction can lead to increased ROS generation if the highly oxidative reactions utilized to produce energy are exposed to the cytosol. There is ample evidence that mitochondrial dysfunction can lead to inflammation. Within the model of TNFR1-associated periodic syndrome (TRAPS), a disorder characterized by fever and severe localized inflammation, there are higher levels of mitochondrial ROS (mtROS). The increased mtROS was correlated with increased activation of Jun N-terminal kinase (JNK) and p38, as characterized by persistent Mitogen-Activated Protein Kinase

(MAPK) activation. Inhibition of mtROS inhibited MAPK activation and the production of inflammatory cytokines (Bulua et al., 2011).

These findings coincide with Barreiro et al. who revealed increases in protein carbonylation, a marker of protein oxidation, and 3-nitrotyrosine, a marker of reactive nitrogen species production, in the gastrocnemius of cachectic mice. In addition to evidence of increased ROS and reactive nitrogen species (RNS) induced oxidation, antioxidant enzymes were not significantly increased, suggesting potential inefficiencies in mitigating oxidative damage (Barreiro et al., 2005).

However, there is also evidence that inflammation can induce mitochondrial dysfunction. Within the $Apc^{Min/+}$ model of cancer cachexia, the severity of the cachectic condition is related with decreases in mitochondrial biogenesis and content, as well as disruption in mitochondrial maintenance. Administration of an IL-6 receptor antibody improved mitochondrial function while IL-6 over expression accelerated muscle wasting and suppressed PGC-1α expression (White et al., 2012). These findings reinforce the relationship between mitochondrial dysfunction and inflammation, providing evidence that aggravation of each can induce aggravation of the other.

In addition to inflammation, mTOR has been established as a modulator of mitochondria biogenesis. Cunningham et al. revealed that inhibition of mTOR by rapamycin in skeletal muscle cells decreases transcription of PGC-1α and NRFs, thereby decreasing mitochondrial gene expression and oxidative capacity. Using computational genomics, the transcription factor Yin-Yang 1 (YY1) was identified as a common target of mTOR and PGC-1α. The rapamycin dependent inhibition relies on YY1, resulting in failure to interact and coactivate with PGC-1α. Additionally, YY1 knockdown decreased

mitochondrial gene expression and respiration (Cunningham et al., 2007). This relationship between mTOR and PGC-1α effectively link protein synthesis to mitochondrial biogenesis, as inhibition of one may also inhibit the other.

Leucine supplementation improves mitochondrial biogenesis under LLC implantation. Despite the unexpected lack of effect on protein synthesis, leucine did improve mitochondrial biogenesis as PGC-1α was increased in the LLC+Leu group relative to the LLC group (Figure 4A). This is in agreement with in vitro studies demonstrating that leucine supplementation in the media significantly increased PGC-1α expression in C2C12 myotubes (Liang et al., 2014). Considering mitochondrial content, there were no significant differences between the LLC and LLC+Leu groups, suggesting no functional increase in mitochondrial biogenesis by leucine in to the LLC implantation model. This was corroborated by the decreases in Cytochrome C and CIII in the LLC implanted mice relative to the control (Figure 4B and 4D). The increase in PGC-1α suggests signaling for mitochondrial biogenesis; however, without a functional protein synthesis pathway mitochondrial biogenesis cannot occur as the majority of mitochondrial proteins are synthesized in the cytosol and transported into the mitochondria.

Simply increasing mitochondrial biogenesis or content may not be enough to impart protection from tumor-induced muscle wasting. Wang et al. utilized a transgenic Muscle Creatine Kinase (MCK)-PGC-1α mouse strain overexpressing PGC-1α skeletal muscle to explore influences of mitochondrial content in muscle preservation under cachexia. Like this current study, they utilized an LLC implantation model into their MCK-PGC-1α strain with C57BL/6 wild-type as the control. The MCK-PGC-1α strain

maintained upregulation of PGC-1α under tumor burden, resulting in increased mitochondrial biogenesis and content within the skeletal muscle. This coincided with increased PGC-1α, associated mRNA signaling, and mitochondrial content. Despite increased mitochondrial function, the MCK-PGC-1α groups experienced significantly decreased tumor free body weight, no preservation of skeletal muscle, and an unexpected increase in tumor mass. These changes may be attributed to increased secretion of skeletal muscle myokines as the MCK-PGC-1α groups experienced elevated IL-6 relative to their respective wild-type control (Wang et al., 2012). These results resonate with ours, as leucine increased PGC-1α without preserving muscle mass or increasing protein synthesis under tumor burden. However, leucine supplementation did not increase mitochondrial content or increase inflammation as seen in the MCK-PGC-1α mice suggesting a more complex signal by leucine.

While increases in skeletal muscle PGC-1α alone may not be sufficient to protect from tumor-induced muscle atrophy, increasing mitochondrial content through pharmacologic or nutraceutical means may impart some benefit. The use of resveratrol, an established anti-oxidant and stimulator of mitochondrial function, has been used to preserve body mass in cachectic and glucocorticoid induced models of muscle wasting(J. Liu et al., 2015; Shadfar et al., 2011). In a model of dexamethasone induced muscle atrophy, resveratrol reversed loss of mitochondrial content and preserved muscle mass, highlighting mitochondrial dysfunction as a key factor in muscle loss (J. Liu et al., 2015). While Shadfar et al did not analyze mitochondrial function, they demonstrated that resveratrol can inhibit skeletal and cardiac muscle atrophy under a C26 adenocarcinoma implantation model of cachexia, highlighting how the antioxidant capabilities inhibits

NFκB (Shadfar et al., 2011). These papers in conjunction suggest that nutraceutical inhibition of inflammatory signaling and restoration of mitochondrial function can preserve muscle mass without targeting mTOR.

BCAAs, glucose, and insulin. In addition to impairing mitochondrial function and inducing systemic inflammation, insulin resistance is a common symptom among patients with cachexia. There is even some evidence that insulin resistance precedes the onset of cachectic symptoms, and continues to worsen as cachexia progresses (Honors & Kinzig, 2012). Interestingly, leucine has been shown to have a complex interaction with insulin signaling and glucose metabolism. In cases of obesity and type II diabetes, endogenous elevation of circulating BCAAs has commonly been associated with the development of insulin resistance (Lynch & Adams, 2014; McCormack et al., 2013). This contrasts with various studies reporting increases in insulin sensitivity and increased glucose tolerance with leucine supplementation.

Within this study, leucine supplementation decreased fasting glucose under tumor burden, resulting in a significant decrease from pre-experimental measures when compared to the chow group. These findings align with reports of leucine increasing insulin sensitivity as signaling from exogenous supplementation may be inherently different than endogenous increases.

This discrepancy may involve reasons why circulating BCAAs could increase without exogenous supplementation. A common instance of increased circulating BCAAs is during the process of muscle breakdown. As proteins within the muscle are broken down, the amino acid components are released into circulation to be recycled. As mentioned previously, the liver has a decreased ability to metabolize BCAAs due to the

lack of BCAT, resulting in an increase of BCAAs in circulation. Alternatively, BCAAs have been shown to be elevated in obese individuals and may predict the onset of type II diabetes (McCormack et al., 2013). BCAAs may be elevated by the body to enhance insulin signaling and glucose metabolism, only to experience desensitization of therapeutic signaling effects.

Within the realm of cachexia, increased circulating levels of BCAAs have been correlated with increased rates of protein turnover (Argiles, Costelli, Carbo, & LopezSoriano, 1996). While the cause is not certain, it is thought that the muscle wasting and overall catabolic condition induce the release of amino acids into circulation for increased energy demands of both the muscle and tumor. A slim possibility is that the cachectic processes/signals that induce insulin resistance and inflammatory signaling, also induce release of BCAAs. Considering these factors, future research should explore differences between chronic and acute leucine supplementation.

In some agreement with our hypothesis, our experiment demonstrates that leucine supplementation increased signaling of mitochondrial biogenesis under cachexia. However, despite increased PGC-1α within the LLC+LEU group, this did not result in significant improvements in mitochondrial content, as defined by cytochrome C and mitochondrial complexes. Unexpectedly, leucine supplementation did not preserve muscle mass or increase protein synthesis, contrary to other studies utilizing leucine within cachexia (Eley et al., 2007; Peters et al., 2011). While our results show improvement in fasting blood glucose and attenuation of inflammatory signaling, it is uncertain whether the 5% leucine supplementation provided overall benefit, as LLC implantation and leucine supplementation in conjunction, resulted in significantly

decreased gastrocnemius and hind limb muscle mass. Based on the current results of this study, it would be difficult to recommend leucine supplementation in the manner it was applied within our experiment. Major aspects of leucine signaling are still unknown, as it has not been revealed how a single amino acid induces such an array of signaling. As with many signaling agents, desensitization or downregulation of associated signaling may occur, suggesting more acute durations of supplementation may be more beneficial. These findings answered our main question, but left us with more. Stimulation of anabolic signaling may provide support to many models of disease. However, more research is needed to elucidate how leucine signaling is induced and how aspects of cachexia block leucine induced mitochondrial biogenesis.

Limitations and Future Direction

This study is limited by two main factors: low sample size and lack of cachectic characteristics. The current data represents only half of the planned mice, as the 2nd cohort of mice is currently undergoing experimental conditions. Additionally, the absence of tumor generation for one mouse compromised statistical analysis of LLC+Leu group, reducing sample size (n = 4). In the remaining mice, decreased cachectic characteristics can be attributed to shortened period under tumor burden. Within the LLC+Leu group, one mouse (001) required premature euthanasia due to health complications. Another mouse experienced ulceration of the tumor, prompting euthanasia the next day. Despite the enlarged tumor mass, limited effects of cachexia were seen within these mice. However, with the completion of the next cohort, differences between conditions may become more pronounced, allowing us improved comparison of experimental conditions.

Considering future experimentation, metabolic aspects should be measured within glycolytic muscle or the liver, as cachexia and leucine may affect these tissues differently. Additional markers of mitochondrial function and protein synthesis can be assessed via western blot analysis to define where disruptions occur along the signaling pathways. While phosphorylation of STAT3 indicates state of inflammatory signaling, direct measures of inflammatory cytokines in the blood can confirm the presence of cachectic signaling. If the cachectic signaling is confirmed in circulation, we could potentially elucidate interaction between Leucine supplementation and inflammatory signaling. Of particular interest are upstream promoters of PGC-1α (AMPK and SIRT1), markers of mitochondrial maintenance (mitochondrial fusion and fission proteins), and aspects of protein degradation pathways. Future studies should examine the effect of leucine supplementation in combination with anti-oxidants to further enhance mitochondrial biogenesis and content.

Tables

Table 1: Composition of Diets

The composition of each diet is listed, with diets being identical save for the addition of 5% Leucine w/w and red dye.

	Chow		Leu	
Product #	**D10001**		**D16121405**	
	gm%	*kcal%*	gm%	*kcal%*
Protein	20	*21*	24	*25*
Carbohydrate	66	*68*	63	*64*
Fat	5	*12*	5	*11*
Total		*100.0*		*100.0*
kcal/gm	3.90		3.91	
Ingredient	**gm**	***kcal***	**gm**	***kcal***
Casein	200	*800*	200	*800*
DL-Methionine	3	*12*	3	*12*
L-Leucine	0	*0*	53	*212*
Corn Starch	150	*600*	150	*600*
Sucrose	500	*2000*	500	*2000*
Cellulose, BW200	50	*0*	50	*0*
Corn Oil	50	*450*	50	*450*
Mineral Mix S10001	35	*0*	35	*0*
Vitamin Mix V10001	10	*40*	10	*40*
Choline Bitartrate	2	*0*	2	*0*
Dye FD&C Red#40	0	*0*	0.05	*0*
Dye FD&C Blue#1	0	*0*	0	*0*
Total	**1000**	***3902***	**1053.05**	***4114***
% Added Leucine	0.0		5.0	

Table 2: Antibody information

The antibodies used throughout this experiment, source of antibody, dilution, and incubation time. All antibodies were diluted in 5% BSA in TBST.

Primary Antibody	Source	Dilution	Incubation Time	Secondary Antibody	Source	Dilution	Incubation Time
OXPHOS Cocktail	abcam	1:2000	Overnight	Mouse	Cell Signaling	1:5000	2 h
Puromycin	Millipore	1:5000	Overnight	Mouse	Cell Signaling	1:5000	2 h
PGC-1α	Abcam	1:2000	Overnight	Rabbit	Cell Signaling	1:5000	2 h
P-STAT3	Cell Signaling	1:2000	Overnight	Rabbit	Cell Signaling	1:5000	2 h
STAT3	Cell Signaling	1:5000	Overnight	Rabbit	Cell Signaling	1:5000	2 h
P-MTOR	Cell Signaling	1:2000	Overnight	Rabbit	Cell Signaling	1:5000	2 h
MTOR	Cell Signaling	1:5000	Overnight	Rabbit	Cell Signaling	1:5000	2 h

Table 3: Body composition

Upon euthanasia, mice were weighed and tissues were collected. Body, muscle, tumor, fat, and organ mass are presented as means ± standard deviation.

	Control		LLC	
	Chow (n=5)	Leu (n=5)	LLC (n=5)	LLC + Leu (n=4)
BW Pre (g)	22.1 ± 1.6	22.7 ± 1.8	22.0 ± 1.0	21.9 ± 0.9
BW Sac (-Tumor) (g)	23.0 ± 1.3	23.9 ± 2.0	22.9 ± 1.4	21.5 ± 1.6
Tumor (g)	N/A	N/A	1.7 ± 1.1	1.5 ± 1.0
Sol (mg)	9.5 ± 0.6	8.8 ± 1.4	9.3 ± 0.9	7.8 ± 2.2
Plant (mg)	17.1 ± 0.6	16.9 ± 1.5	17.8 ± 1.4	14.6 ± 2.3
Gastrocnemius (mg)	119.3 ± 11.3	119.2 ± 11.2	121.2 ± 7.0	105.2 ± 11.1
TA (mg)	42.1 ± 3.9	42.9 ± 5.2	39.4 ± 6.3	36.0 ± 6.5
EDL (mg)	10.8 ± 1.9	10.3 ± 1.2	10.7 ± 1.4	8.5 ± 1.9
Quad (mg)	87.9 ± 9.2	90.1 ± 8.9	90.3 ± 3.1	73.5 ± 9.0
Total Hindlimb (mg)	198.7 ± 17.3	198.1 ± 20.0	198.4 ± 13.7	172.0 ± 23.3
Liver (mg)	1055.2 ± 78.5	1019.1 ± 236.1	1198.1 ± 86.5	1142.4 ± 143.4
Spleen (mg)	63.5 ± 4.0	74.6 ± 25.8	140.4 ± 52.3	151.9 ± 69.5
Epi Fat (mg)	363.4 ± 48.9	424.7 ± 128.8	352.1 ± 60.2	290.3 ± 113.4
Heart (mg)	150.8 ± 12.9	165.7 ± 45.9	121.9 ± 5.0	135.7 ± 37.8

Figures

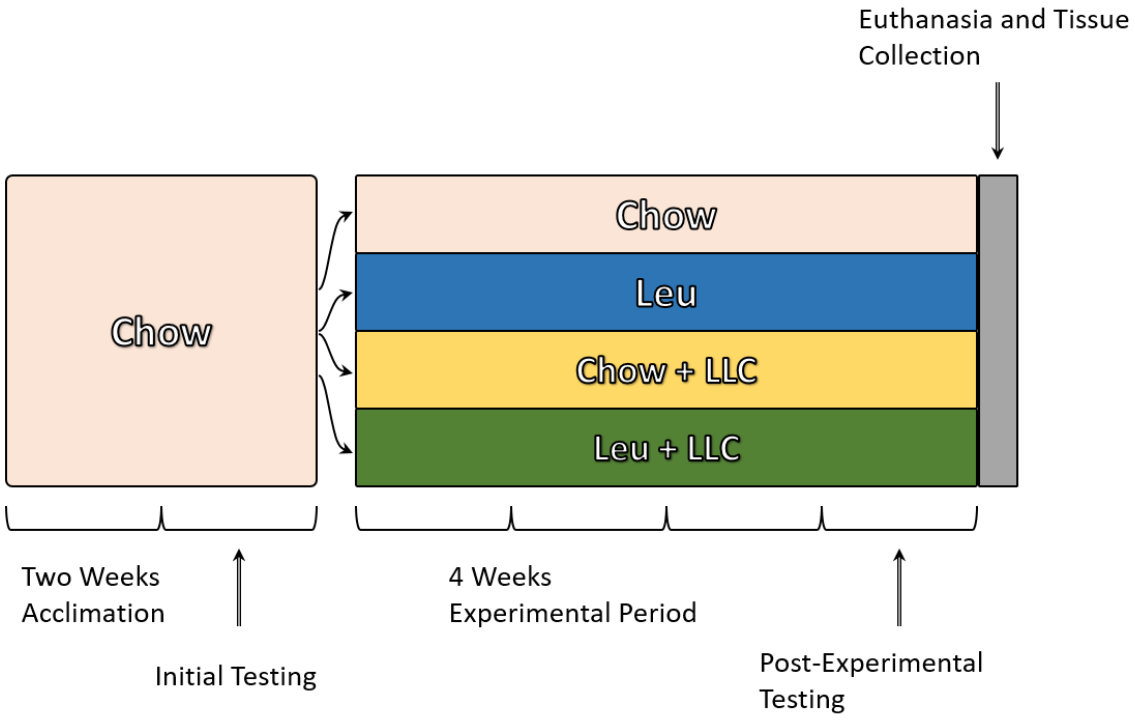


Figure 1 – **Experimental Design:**

Male, wild type C57BL/6 mice (n = 20), aged 7-8 weeks old were individually housed and allowed *ad libitum* food intake of standard chow to acclimate to the facility for two weeks. In the week, preceding tumor implantation, mice underwent pre-experimental testing including a fasting glucose, blood draw, and run to fatigue exercise test. At the end of their acclimation period, the mice were divided into 4 equal groups: Chow, Leucine (Leu), Chow + Lewis Lung Carcinoma (LLC), and Leu + LLC (n = 5). LLC groups received an injection of $1x10^6$ cells suspended in PBS subcutaneously while Non-LLC groups received a PBS injection of equivalent volume. Leu groups received a diet consisting of 5% Leu (w/w). The mice were monitored for 4 weeks post-injection and post experimental testing was completed in the final week preceding euthanasia and tissue collection.

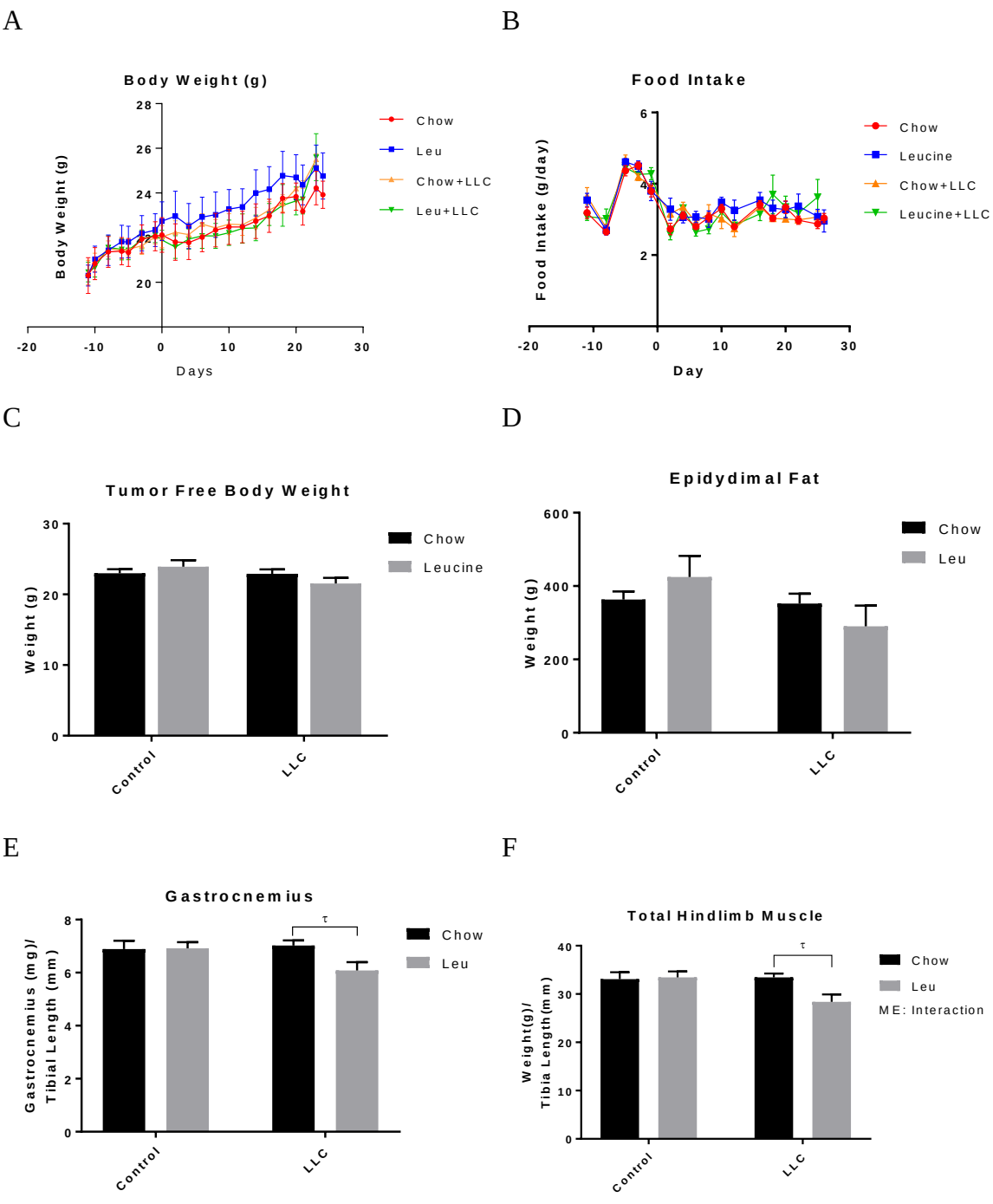
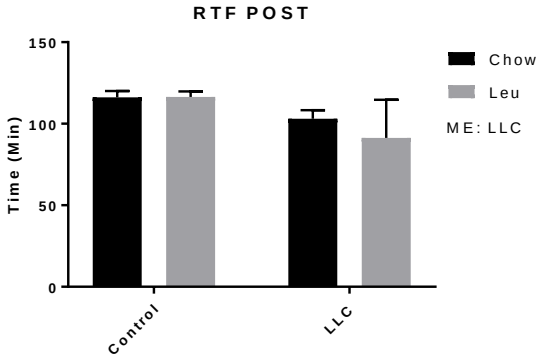


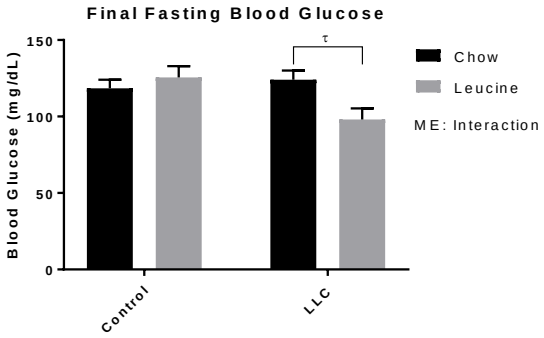
Figure 2 – Body mass and composition:

A) Body weights (g) and B) daily food intake (g/day) of the mice were recorded over time, with the "0" time point representing tumor injection. At the end of the experimental period, C) tumor free body weight, D) tumor mass, E) epidydimal fat mass normalized to tumor free body weight, and F) average gastric weight normalized to tibia length were calculated. Data are represented as mean ± SEM. Significance was set at $p>0.05$. τ significant base on t-test.

A



B



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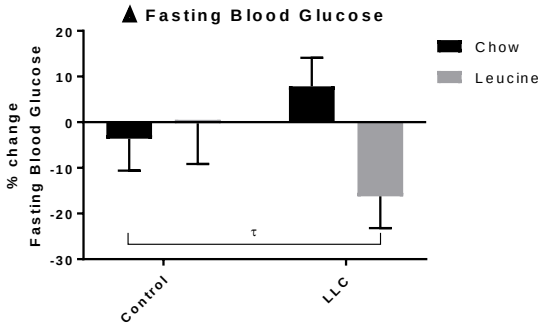


Figure 3 – **Endurance performance and fasting glucose:**

A) The post-experimental run to fatigue is presented in minutes with a maximum time of 120. B) Post experimental fasting blood glucose levels measured via glucometer were recorded after a 5h fasting period, C) Change in fasting blood glucose was calculated as a percent change from pre-implantation measures within the last week of tumor implantation. Data are represented as mean $\pm$ SEM. Significance was set at $p>0.05$. τ significant base on t-test. ME indicates main effect.

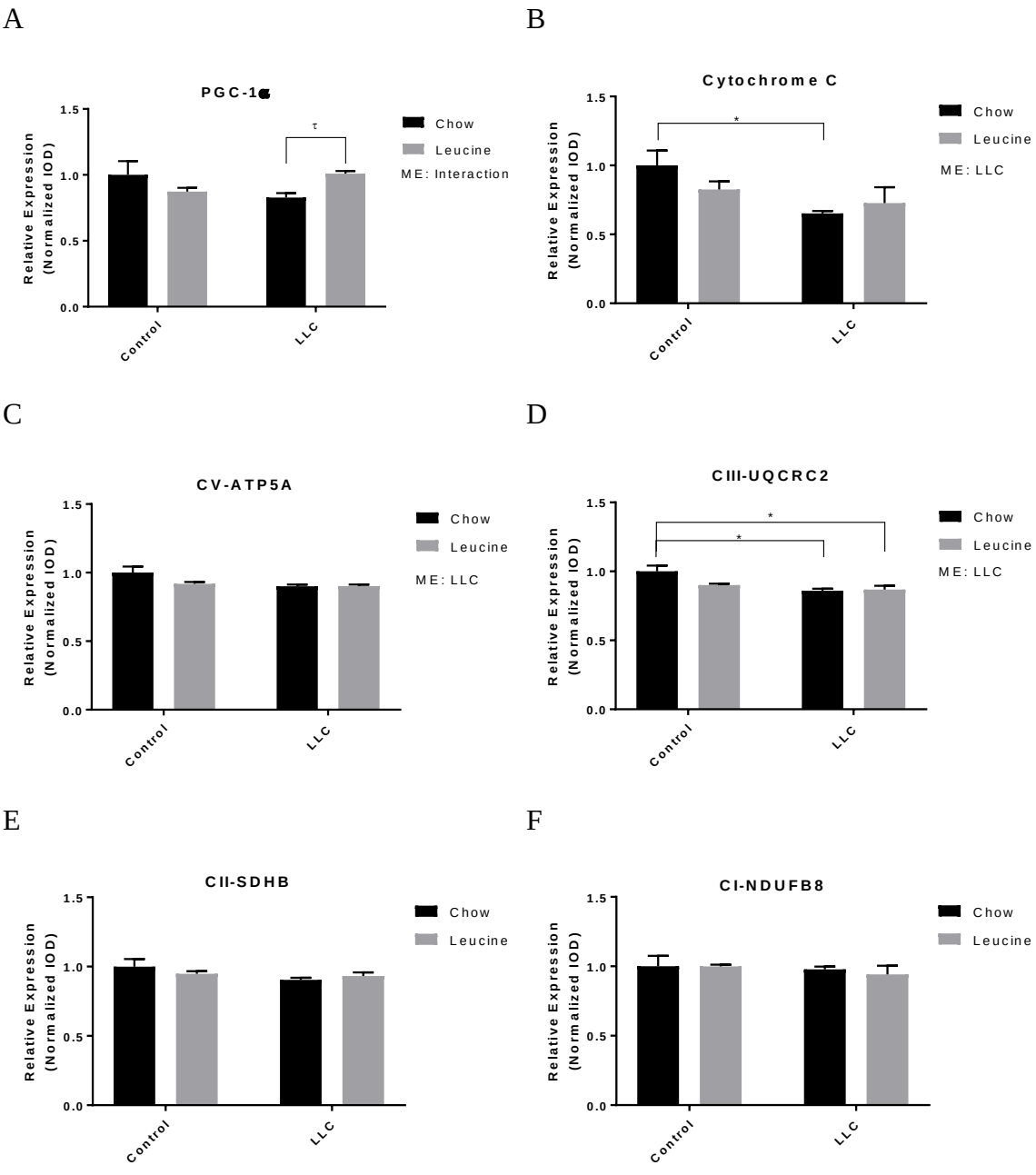
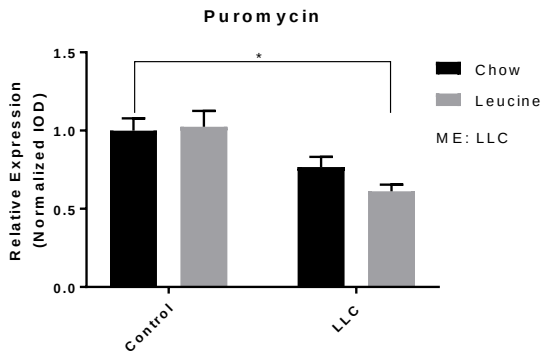


Figure 4 – **Measures of mitochondrial biogenesis in the gastrocnemius:**

Utilizing western blot analysis of the gastrocnemius tissue, expression of proteins key to mitochondrial biogenesis and content were quantified. Values for A) PGC-1α, B) Cytochrome C, C) CV-ATP5A, D) CIII-UQCRC2, E) CII-SDHB, and F) CI-NDUFB8 are presented as IOD normalized to Ponceau staining. * indicates significant difference calculated by repeated measures from a Two-Way ANOVA; τ indicates significant difference calculated by pre-planned T-test ($p>0.05$). ME indicates main effect.

A



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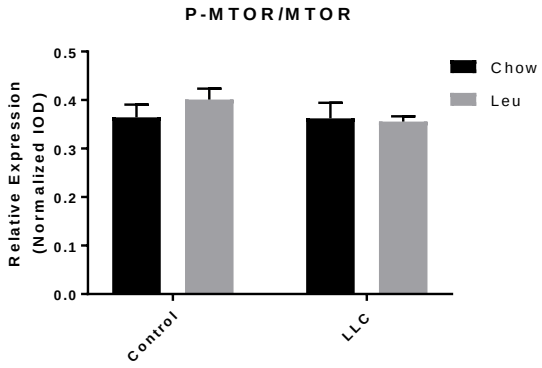

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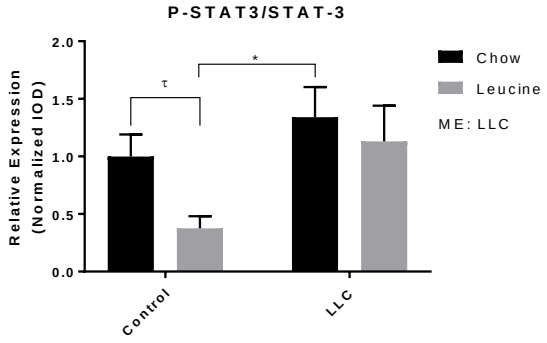

Figure 5- Measures of Protein synthesis and other metabolic signaling:

Utilizing the Western Blot adaptation of the SUnSET technique, protein synthesis was quantified within the gastrocnemius through the measurement of A) puromycin, presented as IOD normalized to inverted densitometry of Ponceau staining. The phosphorylated forms of B) MTOR (P-MTOR) and C) STAT3 (P-STAT) are presented as a ratio over its non-phosphorylated form. Data are represented as mean $\pm$ SEM. Significance was set at $p>0.05$. * indicates significant difference calculated by repeated measures from a Two-Way ANOVA; τ indicates significant difference calculated by t-test. ME indicates main effect.

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Appendix

IACUC Approval

IACUC PROTOCOL ACTION FORM

To:	[redacted]
From	Institutional Animal Care and Use Committee
Subject	Animal Research Protocol
Date	October 16, 2016

The institutional Animal Care and Use Committee (IACUC) has taken the following action concerning your Animal Research Protocol No. 0789 Effect of Leucine Supplementation on Cancer-induced Cachexia Progression

☒ Your protocol is approved for the following period:

From: October 14, 2016 To: October 13, 2019

☐ Your protocol is not approved for the following reasons (see attached memo).

☐ Your protocol is renewed without changes for the following period:

From: _____ To: _____

☐ Your protocol is renewed with the changes described in your IACUC Animal Research Protocol Update/Amendment Memorandum dated _____ for the following period:

From: _____ To: _____

☐ Your protocol is not renewed and the animals have been properly disposed of as described in your IACUC Animal Research Protocol Update/Amendment Memorandum dated _____

[redacted] Interim Chair of the IACUC

[redacted] Veterinarian
And Director of the Animal Care Facilities