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The role of Protein Degradation in Cancer Cachexia in Female Tumor Bearing Mice

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The role of Protein Degradation in Cancer Cachexia in Female Tumor Bearing Mice.

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Abstract

Background: Cancer is a leading cause of death in the world in which half of the people affected by this disease die from its effects. Cancer-cachexia is a syndrome associated with the significant loss of skeletal muscle mass and function, which cannot be fully reversed by nutritional intervention alone and in turn, impairs the host. Cancer-cachexia affects 50-80% of cancer patients and is a primary cause of death accounting for 20-40% of cancer related deaths. Efforts to reverse the effects of cancer-related cachexia have been largely unsuccessful and have primarily focused on the late stages of CC. **Methods:** Lewis Lung Carcinoma (LLC) was injected into C57BL6/J mice into their hind flank at 8 weeks of age. The tumor was allowed to develop for a LT and HT group and was compared to a PBS control. **Results:** Tumor weight was significantly higher in HT mice in comparison to LT mice. Gastrocnemius and plantaris weight in LT and HT in comparison to PBS mice was lower than LT. The soleus was lower in weight in HT mice when compared to PBS mice. TA mass was significantly lower in HT mice when compared to PBS mice. Spleen mass was significantly higher. Fat weight was significantly lower in HT mice when compared to PBS. Atrogin-1 was significantly higher in HT mice when compared to PBS mice. MuRF-1 in HT mice was significantly greater than PBS mice. Ubiquitin HT was significantly greater when compared to PBS and greater than LT. IL-6 was significantly higher in HT mice when compared to PBS mice. Progesterone levels were significantly lower in HT and LT mice when compared to PBS mice. Estrogen levels were significantly lower in HT and LT mice when compared to PBS mice**. Conclusions:** There appears to be a protective nature in the role of estrogen and progesterone and a negative relationship between these hormones and muscle atrophy. Levels of the E3 ligases Atrogin-1 and MuRF-1 may be elevated in the absence and presence of atrophying muscle and are significantly elevated in atrophic female LLC mice.

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Dedication

I would like to dedicate this thesis to my loving parents Melody and Robert Thomas. Your belief in me my entire life has pushed me to soar at greater heights at each stage of my life and your unwavering love and support have been my source of strength. I would like to dedicate this to Madison Kennedy Lewis who is my rock. Without you, I wouldn't be able to function on a daily basis. Your support and love keep me going on days where I think I am at my wits end. This is dedicated as well to all of my family members that I lost to the effects of cancer and chronic disease that plagued my family at a young age. I hope that in the future, my work leads to a better understanding of these diseases in your honor. I would also like to dedicate this to my grandfather Leroy Goss. I wish you could be here to enjoy these moments with me today, but I know you are looking down on me as I work. My life's work has been and will always be dedicated to the memory of the man you were and always will be in my heart. May all my future endeavors be as passionately sought after in your memory and may my life be dedicated to continuing to serve others as unselfishly as you did.

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Chapter 1: Literature Review

Cancer-Cachexia Overview

Cancer is a leading cause of death in the world in which half of the people affected by this disease die from its effects [19]. In 2012 alone, a study sample of 20 large demographics of the world presented with 14.1 million new cases and 8.2 million deaths that were attributed to this disease [19]. Of these diagnoses, lung cancer had an incidence rate of 1.82 million new cases, consequently being the most commonly diagnosed [19]. Often in cancer, a common complication called cancer-cachexia is a prominent side effect. Cancer-cachexia is a syndrome associated with the significant loss of skeletal muscle mass and function, which cannot be fully reversed by nutritional intervention alone [2, 3, 45]. This in turn impairs the host [2, 3, 45]. This loss is dependent upon the type of tumor and the metabolic responses afforded per the tumor [12, 26]. Cancer-cachexia (CC) affects 50-80% of cancer patients and is a primary cause of death accounting for 20-40% of cancer related deaths [1, 2, 3, 26]. Identifying the mechanisms of cancer cachexia is crucial to possible future treatments and therapies to prevent or even reverse CC.

Unlike starvation, cachexia patients lose lean muscle as well as fat stores [48]. Some side effects of this disease include but are not limited to weight loss, anorexia, fatigue, anemia, edema, and aesthesia [48]. These side effects are associated with secondary side effects like anxiety, depression, nausea and vomiting, and sometimes depression [48]. Though anorexia is a hallmark symptom of this disease, it is not completely responsible for cachexia. Although anorexia (hypophagia) commonly occurs in cancer cachexia, cancer cachexia can and sometimes does develop in the absence of hypophagia [4, 17, 48, 51]. Cancer patients with cachexia have a

significantly decreased survival rate and lifespan compared to patients who are not cachectic [48, 51].

There are no current efficacious clinical therapies for CC. Efforts to reverse the effects of cancer-related cachexia have been largely unsuccessful and have primarily focused on the late stages of CC. There has been little effort to study the earlier stages of the disease and its development. Our laboratory revealed that mitochondrial health is compromised before the presence of cachectic phenotypes in male Lewis Lung Carcinoma (LLC) tumor-bearing mice [6]. This was viewed through a significant increase in mitochondrial ROS emission and declined respiratory function in muscles of tumor-bearing animals prior to onset of phenotypic markers of CC [6]. Interestingly, in other studies, it was found that lung cancer patients experienced a higher resting energy expenditure in comparison to healthy individuals and other forms of cancer [10, 51]. This suggests that the effects of lung cancer may elicit greater demands on the body when in comparison to other forms of cancer.

Cachexia and skeletal muscle atrophy present primarily due to the imbalance of protein turnover though the stimulus for this imbalance is not fully understood. This study will focus on the imbalance of protein turnover in LLC tumor bearing female mice with a focus on protein degradation [6, 33, 38]. Skeletal muscle mass is primarily dictated by muscle protein turnover, the ratio of protein synthesis to protein degradation. Therefore, the imbalance of protein turnover favoring protein degradation is a major feature of cachexia and other forms of skeletal muscle atrophy [33, 38]. The regulation of protein turnover involves diverse molecular mechanisms via upregulating and downregulating specific different cell signaling pathways, and gene expressions, resulting in the effects of muscle atrophy and protein catabolism. Understanding this is essential to comprehend its pathogenesis and possible therapies for the disease [6, 26, 33, 38].

Protein Catabolism

Muscles contain the highest concentration of protein in the entire body [4]. In cachexia, the largest loss of protein is therefore in skeletal muscle [30, 51]. These proteins are in a constant state of turnover in which there are two distinct functions: protein synthesis and protein degradation [8] and muscle health and mass depend on this turnover [4]. During CC, rates of protein degradation are elevated while rates of protein synthesis remain the same or are reduced, which in turn is associated with atrophy [5, 6, 12, 45]. Prior evidence displayed that 43 newly diagnosed cancer patients demonstrated a higher rate of protein degradation and lowered rates of protein synthesis [29,51]. Specifically, muscle protein synthetic rates of healthy patients account for 53% of total body synthesis while that of cachexia patients is only responsible for 8% [17,51]. This protein degradation is largely driven via the ubiquitin-proteasome system (UPS) [8]. This suggests that, due to CC, synthetic rates are largely downregulated, while degradation rates are largely upregulated. This could point toward the atrophy seen in CC.

Within the UPS, ubiquitin, a 76 amino acid polypeptide, tags old or damaged proteins for degradation through ligation and once it is ligated, it is shuttled to the proteasome and is committed to degradation or ingestion [8, 22, 23]. The proteasome is responsible for degrading the proteins tagged by Ubiquitin. This process is mediated by ligases E1, E2, and E3 [25]. Protein degradation is achieved by stimulation of the UPS system by specifically activating the E3 ligases Muscle RING finger 1 (MuRF-1) and muscle atrophy F-box protein-1 (Atrogin-1) [25, 54]. These E3 ligases have a vital role in ligating the selected proteins designated for degradation [8, 23, 25]. MuRF-1 encodes E3 ubiquitin ligases as well as acts as an E3 ligase to catalyze the ubiquitination of certain proteins [11, 25]. MuRF-1 has been shown to be

upregulated in atrophy in several pathologies including CC and deletion of MuRF-1 has been shown to lessen the muscle atrophy effects of CC [11, 12, 25].

Although both Atrogin-1 and MuRF-1 are E3 ubiquitin ligases, they play a similar role in the process of muscle atrophy, Atrogin-1 has been most dramatically upregulated prior to atrophy [45]. It has been reported that upregulated Atrogin-1 levels were 8 to 40-fold depending on the atrophic stimulus. This atrophy occurred prior to skeletal muscle loss [45]. Moreover, several proteins are activated along with these catabolic pathologies responsible for CC and the activation of these proteins determines the atrophy seen in CC [4]. Some of these proteins include Forkhead Box O proteins such as FoxO-1 and FoxO-3. These have been found to control the expression of these E3 ligases as an upstream stimulator [25, 54, 55]. Dephosphorylated FoxO-3 enter the nucleus to enhance expression of Atrogin-1 and MuRF-1 while FoxO-1 was found to initiate transcription for these Atrogin-1 and MURF-1 [54, 55]. FoxO family of proteins respond to different catabolic situations by promoting MuRF-1 and Atrogin-1 [54, 55]. FoxO proteins are involved in stress signaling, growth transcription, and are a downstream target of Protein Kinase B (Akt) [18]. While also known to control cell proliferation and death, it has also been known to control the pathways for tumor suppression [18].

In the functional form, FoxOs are located in the nucleus and the activated form is responsible for up-regulating MuRF-1 and Atrogin-1 [16, 18, 49]. The pathways for FoxO regulation can be activated by the Phosphoinositide 3-kinase (PI3K/Akt) pathway which is also responsible for proliferation and growth [18]. The PI3K/Akt in combination with the Insulin Growth-like Factor-1 pathway (IGF-1) has been shown to prevent the induction of muscle atrophy via UPS [49], via decreased activity of the FoxO pathway [18]. This inhibition has been shown to take place by inhibiting the induction of Atrogin-1 [18]. This thesis will focus on the

effects of the UPS in association with MuRF-1, Atrogin-1, FoxO-1, and FoxO-3 in relation to cancer-cachexia and muscle atrophy.

Development and Progression of Cancer-Cachexia

A distinguishing aspect of cancer-cachexia is the progressive nature in the deterioration of skeletal muscle [56]. Cachexia progresses in two distinct stages for the purpose of this study: pre-cachexia and cachexia [39]. Pre-cachexia is defined as the stage in which no significant reductions in skeletal muscle mass are detected, while cachexia will be defined as the state in which skeletal muscle masses are declined in the tumor-bearing state. Cachexia has the highest rate of body impairments due to the disease [39]. Studies have been dedicated to identifying progressive stages of this disease based on quantitative means which attribute certain levels of atrophy to different stages [29,]. The CASCO score is a tool used for staging human cachexia patients [2, 3, 29]. In a portion of the test, it states that more than 10% of body weight loss will be considered moderate, more than 15% as severe, and more than 20% as terminal [2, 3, 29]. As the disease progresses, muscle mass and function decline and in turn, exercise capacity, strength, and ability to perform daily tasks progressively decline as well [20]. With this decline in ability, the quality of life for patients also progressively declines until the patient has passed from the effects. Therefore, it is important to identify and study the early stages of CC to prevent the onset of cachexia and thereby avoid irreparable damage seen in the final stages of cachexia and to potentiate anti-cancer strategy [29].

Recent pre-clinical evidence from our laboratory demonstrates marked weight loss accompanied with a decreased cross-sectional area (CSA) and fractional synthetic rate (FSR) of muscle protein in male LLC tumor-bearing cachectic mice [5,6]. However, several cell cycling

and myogenic factors including cyclin D, Pax 7, MyoD, and Myogenin were reduced during pre-cachexia without changes in CSA and FSR [5,6], indicating molecular degenerations occur during the early stages of CC and these factors may initiate the mechanisms involved in weight loss seen in CC. Despite this, the majority of both clinical and pre-clinical cancer studies have largely focused on the late stages of cachexia. In addition, most pre-clinical studies of CC mainly focused on the male animals.

Biological Sex

Prior pre-clinical studies of CC have been committed primarily toward the male sex, thereby lacking the information regarding that of the female sex during the development and progression of CC although dichotomous pathological patterns between sexes have been reported in different forms of cancer such as gastric cancer [46] and lung cancer [44]. Moreover, recent research points towards distinct differences between male and female atrophic response in association with CC [43]. Therefore, understanding of the development of CC in both male and female cohorts is critical to develop required knowledge for appropriate therapeutic intervention across cancer patients.

A distinct difference between males and females is that females tend to have a higher concentration of type-1 oxidative muscles than males do in the same muscle [9, 21]. This may play an important role in CC because it has a greater recorded influence on glycolytic fibers according to previous studies [41, 43, 50]. Additionally, female rodents have shown to exhibit lower basal ubiquitin-proteasomal activity compared to male rodents while highly increased inflammation-mediated muscle atrophy has been seen in male rodents compared to female counterparts [25, 36, 43]. Recall that the ubiquitin-proteasomal pathway is a primary pathway

responsible for protein degradation and cachexia [8, 23, 25, 54]. This pathway includes the effects of the FoxO family of proteins. FoxO-3 activity is consequently also lowered when compared to males due to this difference in pathways [23, 25].

Though females display lower FoxO-3 content, the ubiquitin marked protein levels are curiously much higher than in their male counterparts [30, 55]. It seems that there is a distinct difference in cachexia between females and males exist with males utilizing the ubiquitin-protease pathway and females having more inclination toward the autophagy pathway and model of atrophy and cachexia [37, 42, 43].

Purpose

The purpose of this study is to examine the ubiquitin-ligase degradative pathway in the development and progression of cancer cachexia in female LLC tumor-bearing mice. This is in an effort to develop preventive measures to combat the effects of early stage cachexia and understanding the early development of the condition in LLC female mice throughout the progression of cancer cachexia. I hypothesize that during cancer cachexia, there will be significantly elevated levels of activity in the ubiquitin-ligase degradative pathway with higher levels seen on Atrogin, MuRF, and FoxOs.

Objectives for Data Analysis

In order to further investigate the progression of cancer-cachexia through the role of the ubiquitin-proteasome pathway, this study will: 1) describe the phenotype of the LLC tumor-bearing female mice used in this study, including tissue weight and body weight, 2) assess differential content of proteins related to muscle protein catabolism (Ubiquitin, Atrogin-1, and

MuRF-1) in development of cachexia in female mice, 3) to assess differential content of mRNA of genes related to the ubiquitin-proteasome pathway (Foxo-1, Foxo-3, p-Foxo-1, p-Foxo-3) via real-time qPCR.

II. **Methods**

For this proposal, animal experiments were previously completed by Dr. Greene's laboratory group. My work consists of analysis of previously collected tissue sample only and does not involve direct use of the research animals.

Animal Intervention

Animal experiments for this project were performed at the University of Arkansas in Fayetteville, Arkansas. The mice used for this study were a cohort of C57BL/6J female mice (stock 000664) purchased from Jackson Laboratories (Bar Harbor, ME, USA). These animals were housed in a temperature-controlled environment in a 12:12 h light-dark cycle with *ad libitum* access to normal rodent chow and water. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Arkansas.

In this present study, LLC implantation was utilized to induce cancer-cachexia. The cohort of mice used was 8 weeks of age at the time of implantation in the hind flank. The tissues used in this study were completely on sample analysis.

Lewis Lung Carcinoma Cell Culture and Implantation

Lewis lung carcinoma cells (ATCC CRL-164) were used in this project. These cells were plated in culture flasks in DMEM with 1% penicillin and streptomycin, and with 10% fetal

bovine serum. Upon confluence, cells were then be trypsinized and counted. These cells were later diluted in PBS for implantation and plated at passage 2. The mice described earlier were then implanted with the LLC cells subcutaneously in their hind flank at the age of 8 weeks. The tumor was allowed to develop for 1-4 weeks. A control cohort was developed by administering an equal volume injection of PBS at 8 weeks of age and the control group will be age-matched with 4 weeks of tumor-bearing (the most cachectic) mice during the harvest (12 weeks of age at tissue collection). At the end of the appointed period of tumor growth mice were euthanized and skeletal muscles collected. Muscles were weighed and immediately snap-frozen in liquid nitrogen and later stored at -80°C for further biological analysis.

Tissue Collection and Sample Preparation for Immunoblotting

Mice were 12 weeks of age at the time of harvest. Neither cohort of mice was fasting at the time of harvesting of tissue. Muscle tissue samples were collected with mice under isoflurane anesthesia and mice euthanized while under the plane of anesthesia. Gastrocnemius muscles were collected during harvesting and weighed after harvesting and then snap-frozen in liquid nitrogen and later stored at -80°C for further biological analysis.

Immunoblotting

The frozen gastrocnemius muscles were powdered and that of 20mg will be collected into a 1.5mL centrifuge tube of liquid nitrogen. The powdered protein was transferred into an ice-cold glass homogenizing tube with 250μL of buffer containing phosphatase inhibitor cocktails (Sigma-Aldrich, St. Louis, MO USA), 80 mM dithiothreitol, 0.23 M Tris–HCL, pH 6.8, 0.57 mM 2-mercaptoethanol, 4.5% w/v SDS, 45% glycerol, 0.04% w/v Bromophenol Blue complete,

mini protease inhibitor cocktail (Roche, Indianapolis, IN, USA), was homogenized using a glass homogenizer. This was later denatured at 95°C followed by centrifugation at 2,000 rpm. The supernatant was aspirated and transferred into a new 1.5 centrifuge tube. An RC/DC assay (500–0119, Bio-Rad, Hercules, CA, USA) was used in order to accurately determine concentrations. 40μg total protein was loaded into 10% gel and resolved by SDS-Page at 100V for 2 hours.

After resolving, this was transferred to a Polyvinylidene difluoride (PVDF) membrane and blocked. The blocking was accomplished with 5% powdered milk in Tris-buffered saline with 0.2% Tween 20 (TBST) at room temperature for an hour. The membrane was then incubated with the primary antibodies diluted in Odyssey blocking buffer (LICOR Biosciences, Lincoln, NE) with 0.1% Tween 20 overnight at 4°C. The primary antibodies were specific to Ubiquitin (Cell Signaling 3933S), FOXO1 (Cell Signaling 2880S), pFOXO1 (Cell Signaling 9461S), pFOXO3 (Cell Signaling 9466S), and FOXO3 (Cell Signaling 12829S). Secondary antibodies from LICOR will be conjugated with Infrared (IR) Dye. Manufacturer protocols was abided by. Membranes was later be imaged using LICOR Odyssey FC through IR detection. Bands will be normalized at 45 kDa. Ponceau S stain was used as a loading control.

RNA isolation, cDNA synthesis, and qualitative real-time qPCR

During the time of harvesting, different skeletal muscles was collected and later frozen. 20mg of samples were pulverized completely before suspension in TRIzol reagent. This was homogenized using Polytron for ~5 s and then transferred into 1.5 ml microtube on ice. 200μl of chloroform was added to the samples after 15 min. The samples were then centrifuged for 25 min. The supernatant liquid was removed and placed in a new tube. 70% of DEPC treated ethanol was then added in equal amount, while the sample is loaded into a RNeasy column.

Using an RNA isolation kit (K145002; Invitrogen, Carlsbad, CA, USA), RNA isolation was then processed. 30ul of DNase treated RNA was collected. The purity of this RNA was verified by fluorometry using 260/280nm ratios read on a Bio-Tek Power Wave XC microplate reader (BioTek Instruments Inc., Winooski, VT, USA) with Take3 microvolume plate and Gen5 software. 1 μg of RNA was reverse-transcribed into cDNA. This was accomplished using VILO Superscript reagent (11755050; Invitrogen, Carlsbad, CA, USA). cDNA was diluted to 1:100 (10ng/μL) during this process.

Using TaqMan probe reagent and Step-One real-time RT-PCR instrumentation (Applied BioSystems, Foster City, CA, USA), Real-time polymerase chain reaction (PCR) was performed on the sample. The manufacturer's protocol was followed for analyzing Ct values. The PCR protocol was followed in this order: 4 minutes of incubations, 45 cycles of denaturation, annealing and extension at 95 °C, 60 °C, and 72°C. Target TaqMan probes for Atrogin-1 (Mm00499523) and MuRF-1 (Mm01185221) purchased from Applied Biosystems (Life Technologies). Final gene expression was calculated using the ΔΔCT method. Relative quantification was calculated as $2^{-\Delta\Delta CT}$.

Statistical Analysis

Data was analyzed by a one-way ANOVA with the independent factor of tumor burden and factor levels of PBS, LT and HT. When significant F ratios were found, pairwise differences between means were analyzed by a Tukey post-hoc. Significance was denoted at $p<0.05$. All data was analyzed with SAS statistical software (SAS Institute, Cary, NC).

III. Results:

COVID-19

Due to the circumstances and the CDC recommendations for social distancing/closing of facilities, this project was interrupted during the vital time of the data collection. These circumstances are beyond our control and have, in turn, affected the ability to compile all the data that was sought after in this project. This project will, however, proceed with the data that was able to be collected. Due to this change, measures of IL-6, progesterone, and estrogen were included as data in this project to assist in the analysis of the results.

Characterization of the progression of Lewis Lung carcinoma-induced cancer-cachexia in female mice (Table 1 and Figure 1).

During data analysis, we observed dichotomous phenotypes in tumor mass were in 3- and 4-week animals. Due to this, three- and four-week animals were re-divided into low tumor bearing (LT) and high tumor bearing (HT). Table 1 and Figure 1 display phenotypic descriptors for body and tissues weights. When values from body weight and tumor weight were normalized, it was seen that there was no significant difference in body weight. Tumor weight was significantly higher (290.54%) in HT mice in comparison to LT mice ($p<0.05$) (Table 1). There was no significant difference in gastrocnemius and plantaris weight in LT or HT in comparison to PBS mice, but that of HT was lower than LT by 6.3% ($P<0.05$). The soleus was 13.4% lower in weight in HT mice when compared to PBS mice ($p<0.05$) (Figure 1a). The EDL mass was not significantly lower in HT mice when compared to PBS mice (Table 1). TA mass was significantly lower (10.74%) in HT mice when compared to PBS mice ($p<0.05$) (Figure 1b). Spleen mass was significantly higher (295.12%) in HT in comparison to the PBS group (Table

1). Increased spleen size was used to measure inflammation. Fat weight was significantly lower (36.92%) in HT mice when compared to PBS ($p<0.05$) (Table 1).

Upregulated Ubiquitin Proteasome System activity in high tumor-bearing mice

Atrogin-1 was significantly higher in HT mice with a 3.4-fold increase when compared to PBS mice ($p<0.05$) (Figure 2). MuRF-1 in HT mice was significantly greater than PBS mice with a 4.3-fold increase ($p<0.05$) (Figure 2a). Due to CDC restrictions and COVID-19 social distancing, I was unable to obtain a complete dataset for ubiquitin. Two immunoblotting experiments were successfully completed with ubiquitin and will be used as preliminary data. Ubiquitin HT was significantly greater, 13.52%, when compared to PBS, 94% greater than 1week, 138.20% greater than 2week, and 128.65% greater than LT ($p<0.05$).

Induction of Inflammation markers in muscles of high tumor-bearing mice

IL-6 was measured in order to mark inflammation. IL-6 was significantly higher (1.95-fold higher) in HT mice when compared to PBS mice ($p<0.05$) (Figure 3).

Hormonal changes in Lewis Lung carcinoma tumor-bearing female mice:

Progesterone levels were significantly lower in HT and LT mice (62.50% and 42.00%, respectively) when compared to PBS mice ($p<0.05$). Estrogen levels were significantly lower in HT and LT mice (41.61% and 67.8%, respectively) when compared to PBS mice ($p<0.05$)

IV. Discussion:

Cancer-cachexia is associated with the loss of muscle mass and this is primarily driven by protein imbalance favoring protein degradation. This has been studied in relatively great detail in late stage CC, however, we are among the first to study CC in this time course manner during the development and progression of LLC-induced CC in female mice. Our laboratory data demonstrated that mitochondrial health, such as mitochondrial oxidative capacity and ROS emission, is negatively impacted well before the onset of muscle atrophy in LLC-induced tumor-bearing male mice [7]. These data suggest that significant changes in cellular health and signaling occur prior to onset of CC. This pathway was the primary catabolic pathway upregulated in CC suggesting it may be a key driver in the protein imbalance in CC [6]. As a result of the current study, we have concluded that the ubiquitin-proteasome system is at least partially responsible for the muscle wasting seen in our cohort of female mice. Upstream signaling of MuRF-1 and Atrogin-1 suggest that these E3 ligases play a role in the mediation of the signaling cascades seen in the UPS degradative pathway in this model of CC. Interestingly, the higher levels of IL-6, and lower levels of estrogen, and progesterone seen in high tumor cachectic mice point toward possible therapeutic implications associated with the female seen in this and previous studies. The search for therapeutic interventions regarding muscle wasting is ongoing and mostly ineffective. The data in this project examines the overview of a complex pathophysiology in the early stages of CC in female LLC tumor-bearing mice.

Cancer-Cachexia induced muscle atrophy in Lewis Lung Carcinoma female mice

In the current study, cachectic muscle mass loss was marked by phenotypic descriptors for tissue and body weight. Dichotomous phenotypes in tumor mass in 3- and 4-week tumor-

bearing animals were observed, therefore, we re-divided mice into low tumor-bearing (LT) and high tumor-bearing (HT) groups. We did not see a significant difference in gastrocnemius and plantaris weight in LT or HT in comparison to PBS mice as we expected. When we compared LT and HT groups in gastrocnemius and soleus, the LT group in the gastrocnemius was slightly higher than the PBS in weight, while in the soleus, is was significantly lower in LT when compared to PBS. A distinct difference between males and females is that females tend to have a higher concentration of type-1 oxidative muscles than males do into the same muscle [9, 21]. This plays an important due to CC having a greater recorded influence on glycolytic fibers according to previous studies [43, 41, 50]. This may explain the results we recorded in regard to the lack of atrophy seen in the LT and HT mice in the gastrocnemius due to its mixed fiber nature, however, it does not explain the lack of atrophy we saw in the plantaris and EDL, which are fast-glycolytic fiber types. The EDL may be atrophied but difficult to detect due to difficulty in dissection.

Interestingly, the soleus (an oxidative muscle fiber type) showed a significant decrease in weight, also contradicting previous studies where oxidative muscle fiber types showed less effects of atrophy when on comparison with glycolytic [6]. Soleus decrease was shown up to 24% in previous studies [24]. This may be due to the difference in sex mitigating the levels of atrophy seen in the soleus. Glycolytic TA showed atrophic responses in regard to the recorded weight, following the statement above in regard to atrophy targeting more glycolytic fiber. The atrophy seen in the fat tissue (36.92% decrease) is the highest level of atrophy seen in this study. The muscle examined in this study did not atrophy to the same level as the fat on these female mice. This suggests that these mice display mild to moderate cachexia during our 4-week time course.

Induction of the ubiquitin-proteasome pathway

The data we collected in this study displayed increased protein degradation during the 4-week time course after LLC implantation. This was marked by the upregulation in the E3 ligases Atrogin-1 and MuRF-1 which in turn upregulated ubiquitination. The ubiquitin marked protein levels were much higher than in their male counterparts in previous literature [43, 55] which is consistent with the results of this study as well, though data are currently only descriptive in nature. The expression of atrogenes, Atrogin-1 and MuRF-1, have a role in this upregulation in ubiquitination and this was likely caused by enhanced Foxo-1 protein content. These results in the female model are consistent with our previous studies in male mice, however, the level of muscle atrophy was not consistent in the female, as seen in the male when it comes to fiber type and the significance if atrophy [6]. This being said, these raised levels of Atrogin-1 and MuRF-1 were, however, displayed in the HT and LT groups, but significant data was not displayed in the early stages of these female mice. In males, there was observed degradative signaling for ubiquitin, Atrogin-1, and MuRF-1, in the early stages, however, raised signaling was only seen in the female model in the later stages [6]. Female rodents have been shown to exhibit lowered basal ubiquitin-proteasomal activity compared to male rodents while highly increased inflammation-mediated muscle atrophy has been seen in male rodents compared to female counterparts in previous studies. [24, 36, 43]. This could account for the difference in early levels of E3 ligase expression when compared to the male study. Females show a greater inclination toward the autophagy pathway and models of atrophy including cachexia [43]. Our study displays similar results from our prior male study in which protein degradative signaling proceeds in the presence or absence of muscle atrophy.

Hormone analysis within the female tumor-bearing mice.

Due to the reproductive nature of the female sex there have been several findings that have led researchers to believe that the ovarian hormones have a significant potential to affect cachexia progression [13, 34, 35, 53]. IL-6 mRNA was measured in order to mark inflammation in this experiment and was found to be significantly higher (1.95-fold higher) in HT mice when compared to PBS mice. Increased spleen size was used as a surrogate measure of inflammation and that is what was displayed in our cohort of mice. Spleen mass was significantly higher (295.12%) in HT in comparison to the PBS group. This inflammation may be in regard to several upregulated inflammation markers and pathologies such as IL-6. The results of our experiments displayed a 41.61% decrease in estrogen levels when compared to PBS mice in this experiment as well. This data suggests that female tumor-bearing mice have upregulated inflammatory pathways and downregulated estrogen. The upregulation in IL-6 may be associated with the lack of estrogen and its inability to protect skeletal muscle from IL-6. In previous studies however, when IL-6 was overexpressed in female $Apc^{Min/+}$, it has no effect on CC progression [24]. Estrogen and progesterone are hormones specific to muscle health in the female sex [43]. Estrogen and progesterone have been shown to have hypertrophic effects in muscle metabolism as well as protective effects for muscle for females through diminishing the effects of Interlukin-6 (IL-6) which is partially responsible for inflammation-induced atrophy [40, 43]. Estrogen has been shown to increase mitochondrial health in muscle with increased rates of oxygen consumption in comparison to the removal of estrogen [27, 43, 52]. Progesterone has not been studied as closely as estrogen in relation to muscle quality, however, it is known that progesterone spikes drastically during the luteal phase of the menstrual cycle for females and has been shown to increase mitochondrial Reactive Oxygen Species emission (ROS), which assist in

muscle adaptation during exercise, and mediate mitochondrial efficiency which is a quality of healthy muscle [5, 28, 43]. Removal of these sex specific hormones, via ovariectomy, resulted in muscle atrophy when studied in other literature [15, 43, 47]. With this in consideration, estrogen supplementation alone has not been shown to remedy the effects of cancer-cachexia or atrophy [43].

In addition to the female's difference in hormones, the female also experiences a cycle in which several discoveries have stemmed [13, 24, 34, 35, 53]. Acyclicity along with enhanced inflammatory gene expression in female mice was shown to also mark severe cachectic conditions [24]. This experiment did not control for cyclicity. Our data from this study show that, as a result of CC, estrogen (an upregulated hormone in cyclic female mice) was significantly lower in HT and LT mice when compared to the healthy control. It may be possible that if estrogen was supplemented in excess, that there may be therapeutic implications for the female mouse. Progesterone also showed a 67.8% decrease in HT when compared to the healthy control, however, it showed ~30-fold decrease in LT and HT when compared to PBS. This suggests that progesterone's role in CC could possibly be more than estrogen in the HT group when compared to the PBS group, only being decreased by 41.61%, but progesterone may not have as big of an effect when the LT/HT group is compared to PBS. The exact role of progesterone and estrogen together is unknown when it comes to CC and warrants further investigation in future studies.

Limitations

Limitations of this study start with those enforced by the CDC in response to COVID-19. The data in this study will be used as preliminary data up until the date that the lab is reopened, and we are allowed to complete our data set. Though we have a glimpse of what is happening in

this model, further investigation into the role of Foxo-1 and Foxo-3 via western blot must be accomplished to get a closer look into this issue. Additionally, the lack of control for the estrus cycle in the female mouse was another limitation of this study.

V.Conclusion

In this study, we measured atrophy-related RNA content, analyzed muscle atrophy, and hormonal imbalance throughout the progression of CC. Based on our data, increased protein degradation and muscle atrophy induced by the UPS likely major contributors to the onset and progression of CC and the muscle atrophy seen in this model. There appears to be a protective nature in the role of estrogen and progesterone and a negative relationship between these hormones and muscle atrophy. Levels of the E3 ligases Atrogin-1 and MuRF-1 may be elevated in the absence and presence of atrophying muscle and are significantly elevated in atrophic female LLC mice. The search for successful clinical therapies continues for CC and muscle atrophy. The inhibition of protein degradation may assist in the regulation of CC and muscle atrophy. This data provides information to help solve the problem of muscle atrophy seen in CC and future clinical therapies for CC.

Tables and Figures

Table 1. Tissue weights in PBS, LT, and HT mice. Different lettering denotes statistical significance between groups, alpha set at p<0 .05. Data ± SEM.

Group	***PBS***	***LT***	***HT***
Body Weight by Tumor(g)	*19.03* ± 0.59 a	*19.9* ± 1.10 a	*21.4* ± 1.63 b
Tumor Weight (mg)	*N/A a*	*728.55* ± 380.92 b	*2845.25* ± 559.95 c
Gastrocnemius (mg)	*91.39* ± 4.01 ab	*93.12* ± 4.73 a	*87.21* ± 5.93 b
Soleus (mg)	*7.41* ± 0.56 a	*7.23* ± 0.54 a	*6.40* ± 0.91 b
Plantaris (mg)	*13.01* ± 0.82 ab	*13.25* ± 1.28 a	*12.24* ± 1.01 b
EDL (mg)	*8.40* ± 0.81	*8.11* ± 0.83	*7.90* ± 1.0
TA (mg)	*37.60* ± 2.94 a	*36.78* ± 2.53 a	*33.56* ± 2.72 b
Spleen (mg)	*81.87* ± 12.18 a	*143.16* ± 31.62 b	*323.47* ± 69.02 c
Fat Weight (mg)	*316.42* ± 104.24 a	*301.60* ± 92.0 a	*199.59* ± 84.54 b

Figures

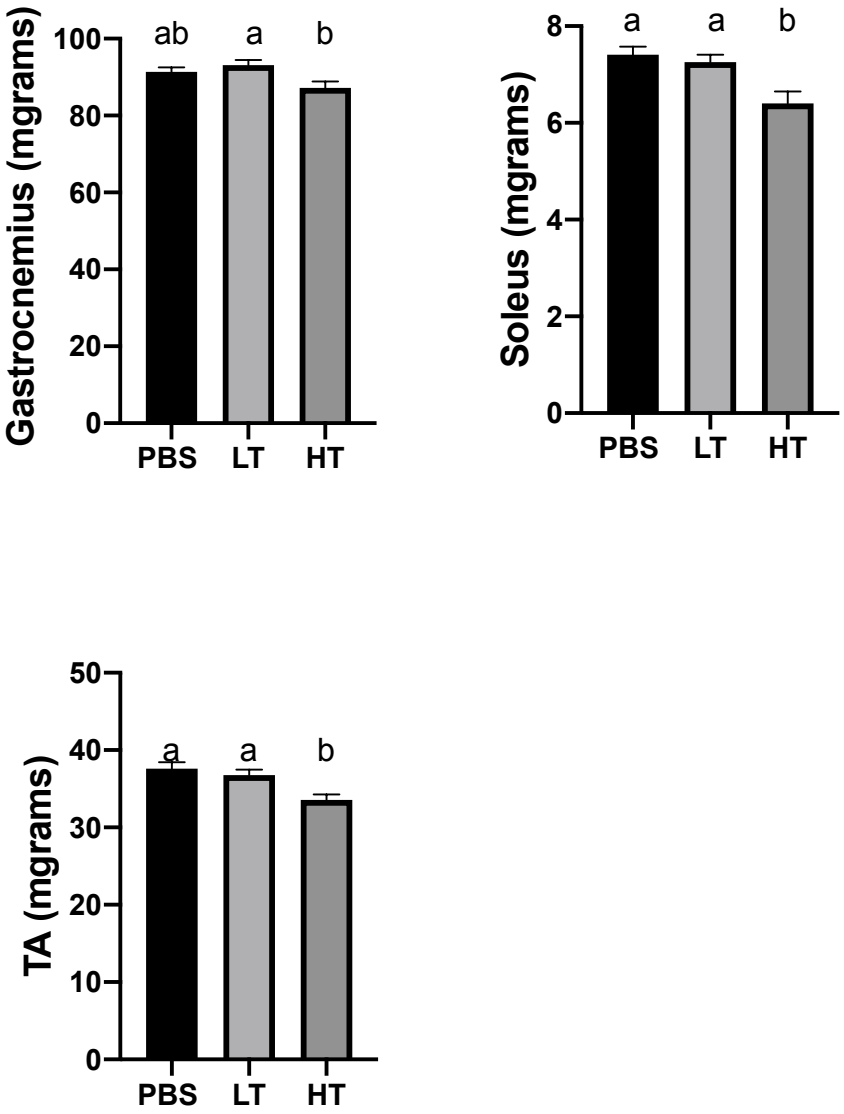

Figure 1. Comparison of tissue weight of different muscle types in Lewis Lung Carcinoma tumor-bearing female mice. (A) Gastrocnemius muscle (mixed muscle fiber), (B) soleus muscle (slow twitch-oxidative muscle fibers), and (C) tibialis anterior muscle (fast twitch-glycolytic muscle fiber). Different lettering denotes statistical significance between groups, alpha set at $p<0.05$. Data ± SEM.

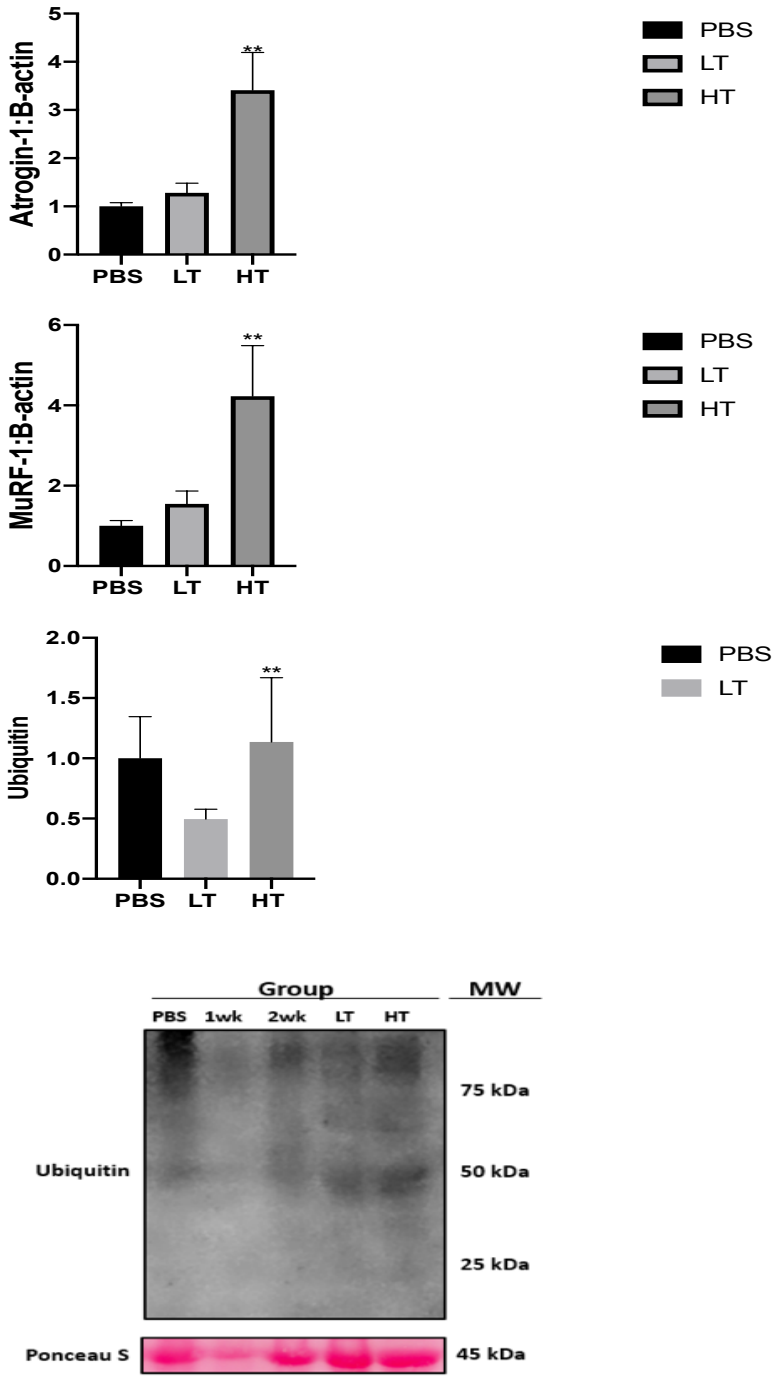

Figure 2.
Comparison of degradative signaling in Lewis Lung Carcinoma tumor-bearing female mice. (A) Atrogin-1 in PBS, LT and HT (B) MuRF-1 in PBS, LT and HT (C) Ubiquitin in in PBS, LT and HT and (D) Ponceau stain and WB for Ubiquitin. Different lettering denotes statistical significance between groups, alpha set at p<0 .05. Data ± SEM.

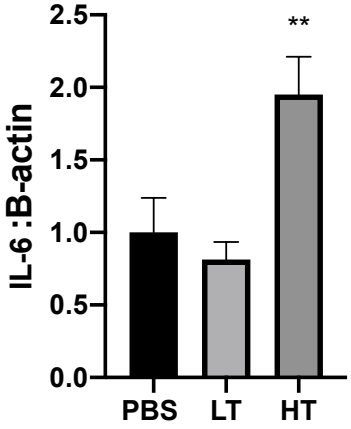

Figure 3.
Comparison of synthesis signaling in Lewis Lung Carcinoma tumor-bearing female mice. (3) IL-6 in PBS, 1week, 2week, LT and HT. Different lettering denotes statistical significance between groups, alpha set at p<0 .05. Data ± SEM.

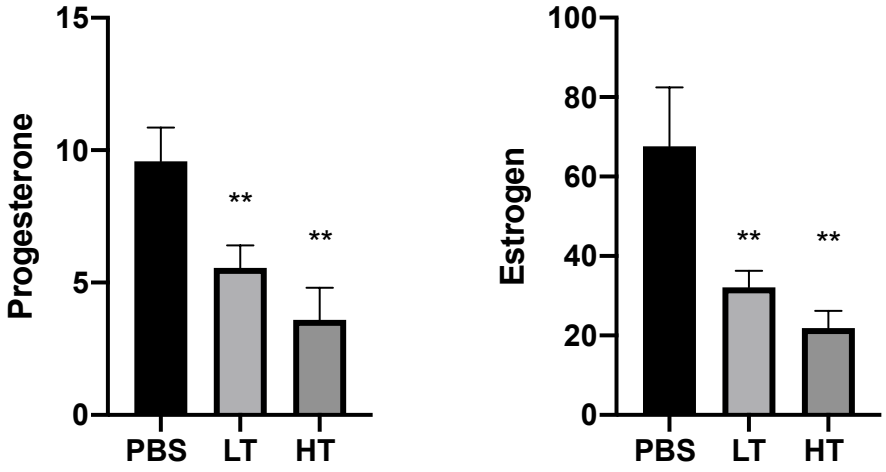

Figure 4.
Comparison of hormone levels in Lewis Lung Carcinoma tumor-bearing female mice. (A) Progesterone in PBS, LT and HT (B) Estrogen in PBS, LT and HT and Different lettering denotes statistical significance between groups, alpha set at p<0 .05. Data ± SEM.

VI. References

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VI. Appendix

UNIVERSITY OF ARKANSAS
Office of Research Compliance

To:	Nicholas Greene
Fr:	Craig Coon
Date:	April 12th, 2018
Subject:	IACUC Approval
Expiration Date:	April 5th, 2021

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol # **18111:** *Mitochondrial degeneration in the development of cancer-induced muscle loss in female mice.*

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond April 5th, 2021 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

The following individuals are approved to work on this study: Nicholas Greene, Tyrone Washington, Megan Rosa, and Wesley Haynie. Please submit personnel additions to this protocol via the modification form prior to their start of work.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/tmp

18111