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# Role of Sex Differences on Cancer Cachexia Progression and Fibrosis during Cancer Cachexia Development

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Role of Sex Differences on Cancer Cachexia Progression and Fibrosis during Cancer Cachexia Development

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by

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## Abstract

Cancer cachexia is a multifactorial wasting syndrome characterized by losses in body mass >5% and occurs in approximately 80% of all cancer patients. Chronic inflammation and fibrosis play roles in cancer cachexia and a greater understanding of these contributing pathways to this pathology will pave the way for potential therapeutic avenues. While inflammation and fibrosis have been researched in various models of cancer cachexia, little to no studies have been performed in both sexes as most previous studies focus on males. **PURPOSE** The purpose of these studies is to investigate the role of fibrosis on cancer cachexia development as well as the effects of leucine supplementation on cancer cachexia in both sexes. **METHODS** Lewis Lung Carcinoma cells (LLC) or PBS (control) were injected into the hind-flank of C57Bl6/J mice at 8 wks age, and tumor allowed to develop for 1, 2, 3 or 4 wks. WT and $APC^{Min/+}$ mice were supplemented with regular or leucine-enriched water following weaning up until euthanasia at 20 weeks of age. Body and pertinent tissue and muscle weights were taken. Histology was used to measure CSA. RT-qPCR and immunoblotting were used for analysis of markers of fibrosis in the LLC study, and markers for inflammation, protein anabolism and catabolism, and myogenesis in the APC study. **RESULTS** Both male and female LLC mice exhibited increased fibrosis at the onset of cancer cachexia. Females, however, saw induction of pro-fibrotic markers such as TGF-β and Collagens 1 and 3 as early as 1wk post-injection when compared to males who saw no increases until 4wks when cachexia was developed. Male $APC^{Min/+}$ mice had greater reductions in body weight following leucine supplementation whereas female $APC^{Min/+}$ mice did not experience this effect of leucine. Higher elevation of IL-6 mRNA abundance accompanied the decrease in body weight in male $APC^{Min/+}$ mice. Myogenesis was altered in female mice, favoring satellite cell activation and differentiation with increases in MyoD and Myogenin over

quiescence. Myotube diameter was decreased following addition of blood plasma taken from male $APC^{Min/+}$ mice. **DISCUSSION** Fibrosis clearly plays an early role in the development of cancer cachexia through upregulation of the TGF-β pathway, but timing of TGF-β and collagen induction differed between sexes. However, the canonical effectors downstream of TGF-β were not altered in either sex in the LLC time course. Supplementation of leucine exacerbated cancer cachexia in male but not female $APC^{Min/+}$ mice, and elevation of inflammatory IL-6 accompanied this effect of leucine. Altered myogenesis in females suggests a protective effect from wasting in female mice and could explain the lack of reduction in myotube diameter observed in $APC^{Min/+}$ female plasma treated cells. These data provide novel insight into variations in fibrosis as well as responses to nutraceutical interventions for cancer cachexia between sexes.

## Acknowledgements

Special thanks to all members, past and present, of the Exercise Muscle Biology Lab and Cachexia Research Laboratory for all their assistance over the years. Also special thanks to my advisor Dr. Tyrone Washington for his guidance and collaboration over the course of this dissertation.

## Dedication

This dissertation is dedicated to my wife and two sons, Madison, Taylor, and Beaux.

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# Chapter 1

## Introduction

Cancer is a leading cause of death worldwide according to the World Health Organization (WHO), with estimations of upwards to 9.6 million deaths in 2018. In the U.S. alone, it is estimated ~600,000 people perished as a result of cancer in the year 2020, and according to the Center for Disease Control (CDC) ~39% of all men and women acquire some form of cancer in their lifetime [1, 2]. While the causes and severity of cancer has been well documented what is less understood is the concurrent wasting of muscle mass that a large portion of cancer patients can experience, otherwise known as cancer cachexia. Cancer cachexia is a multi-factorial wasting syndrome of skeletal muscle, which can be accompanied with or without losses in fat mass and worsens cancer patient health and ultimately outcomes [3]. Cancer cachexia is defined by losses of body mass >5% and occurs in ~80% of all cancer patients [4, 5]. Cancer cachexia is directly responsible for ~20% of cancer deaths, and therefore affects most cancer patients to at least some extent [4, 5, 6]. Of growing concern in cancer research is the apparent divergence in cancer severity and response to cancer treatments that occurs as it relates to differences in sex [7, 8, 9]. These sex differences create an added layer of difficulty to properly evaluate and subsequently create ubiquitously effective treatments, and thus require elucidation with regards to treating cancer cachexia and its associated complications. Creating a better understanding of cancer cachexia and treatment options that can help alleviate its severity in both sexes provides a substantial opportunity to improve and potentially save cancer patient lives on a global scale.

The multifactorial nature of cancer cachexia has resulted in a multifaceted approach to investigate and treat the underlying causes of lean tissue wasting. Of note, a dysregulation in

protein turnover observed in cachectic cancer patients has led to research investigating nutraceutical interventions to help improve protein anabolism and reduce the catabolic state of these patients [10, 11, 12, 13]. However, none to date have proven effective at alleviating the symptomatic wasting of muscle mass during cancer progression thus creating a need for further evaluation as to why this is the case. Also associated with wasting of skeletal muscle due to cancer is the development of increased fibrosis and replacement of healthy tissue with non-contractile fibrotic proteins [14, 15, 16]. Deposition of collagen and related extracellular matrix components in skeletal muscle further exacerbates muscle mass losses thereby worsening the cachectic phenotype in cancer patients. A thorough evaluation across both sexes of anabolic nutraceuticals and the role fibrosis plays on cancer cachexia could create potential therapeutic avenues for cancer cachexia treatments. This literature review will outline research that has shaped what we currently understand concerning cancer cachexia, nutraceutical interventions against cancer cachexia and muscle wasting, and the role fibrosis can play in cancer cachexia progression.

## Review of Literature

### *Cancer Cachexia Physiology*

Cancer cachexia affects a large portion of the cancer population and results in worsened patient health and mortality rates [17, 18]. Interventions targeting the root causes of cachexia progression have yet to yield effective improvements in skeletal muscle maintenance. Many of the key regulators of cancer cachexia are processes that influence the regulation of muscle mass, such as inflammation, protein turnover, atrophy, autophagy, and myogenesis [4, 13, 19, 20, 21]. Understanding these cellular processes within cancer cachexia and the role that sex differences can play on each is vital for future treatments.

#### *A. IL-6 and Inflammation*

Inflammation is a known instigator of cachexia progression when allowed to exceed healthy levels and possesses a profound systemic effect on skeletal muscle wasting [22]. Largely, the main contributors to this systemic induction of cachexia are pro-inflammatory cytokines such IL-6, IL-1. IL-8, TNF-α, and IFNγ [17]. Inhibition of these pro-inflammatory cytokines, namely IL-6, has shown improvements on skeletal muscle wasting [23, 24]. Due to strong link IL-6 has on inflammation regulation and ultimately cancer cachexia, evaluating IL-6 and how its elevation influences skeletal muscle wasting has been a great topic of research since its link to cancer cachexia was discovered [21, 23, 25-30]. Typically, IL-6 plasma concentration will increase following tumor formation and this increase can continue to extreme levels as cancers progress [25]. IL-6 has been shown to reduce protein synthesis via downstream inhibition of mTOR activity [31], potentially through the activation of AMPK [29]. JAK/STAT3 activation

has also been linked to cancer cachectic wasting in $C_2C_{12}$ myotubes, and inhibition of AMPK and STAT3 reversed the associated wasting in those same myotubes [29].

TNF-α is another potent cytokine shown to be elevated in cancer patients that can directly influence protein degradation through degradative processes such as the ubiquitin proteasome system (UPS), while simultaneously inhibiting protein synthesis similarly to IL-6 [27, 32]. TNF-α can activate NFκB to translocate to the cell nucleus and induce the transcription of E3 ligases and atrophy-related genes, also known as atrogenes [33, 34], such as Atrogin-1 and MuRF-1 [35]. Induction of the UPS and these related pathways will result in excessive tagging of proteins for degradation via the proteasome. Upregulation of E3 ligases and subsequent proteolytic degradation further exacerbates skeletal muscle wasting in cancer cachexia.

### *B. Protein Synthesis*

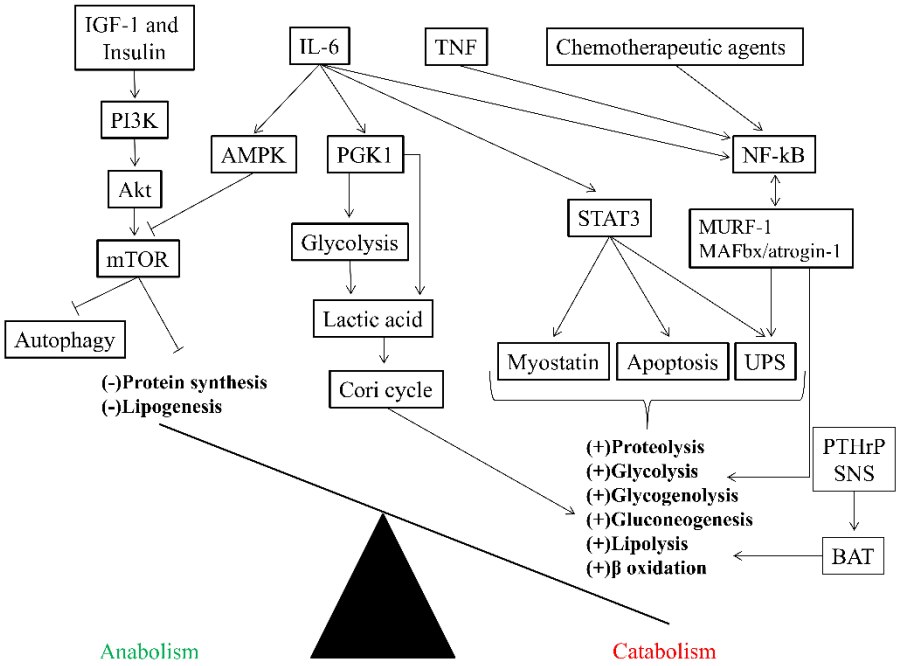


Fig 1-1. Flow chart depicting the role of protein turnover on cancer cachexia.

Imbalances in protein turnover in cancer cachexia patients is noted by dysregulation in protein anabolism compared to protein catabolism [36, 37, 38]. This imbalance leads to overall losses in muscle mass as proteins are being broken down at a greater rate than can be synthesized [39, 40] (Figure 1-1). The synthesis of proteins is a tightly regulated process that ultimately is controlled through successful formation of the ribosomal complex for translation of mRNA into proteins. Formation of the ribosomal complex begins with formation of the 43S pre-initiation complex by binding of the 40S ribosomal subunit to the initiator methionyl-tRNA in order to initiate translation [41]. Next, the eIF4F complex containing eIF4E (cap-binding protein), eIF4G (scaffold protein), and eIF4A (ATP-dependent mRNA helicase) can then combine with the 43S pre-initiation complex to form the 48S ribosomal complex [41, 42]. The formation of the eIF4F complex is the rate-limiting step for initiation and control of protein synthesis, where eIF4E cap-binding protein is sequestered by 4E-BPs, namely 4E-BP1, and only hyper-phosphorylation will release eIF4E from 4E-BP1[43, 44]. As such, analysis of the 4E-BP1 and its upstream signaling cascade have aided analyses aimed towards understanding fluctuations in protein synthesis.

Mechanistic target of rapamycin (mTOR) is the major regulator upstream of 4E-BP1, where mTOR aids in hyper-phosphorylation of 4E-BP1 and phosphorylation of ribosomal protein S6 kinase beta-1 (p70S6k) to promote translation initiation and greater production of proteins [45]. mTOR activity is known to be suppressed in cancer cachectic patients and mice, but the effects of various treatments targeting this protein synthesis pathway have not been completely elucidated [46, 47, 48]. 5' AMP-activated protein kinase also emerged a potent mediator of protein synthesis due to its capability to interact with and inhibit mTOR’s effect to activate its downstream effectors of protein synthesis p70S6k and 4E-BP1 [47]. Recent studies have supported this by reporting elevated AMPK levels in association with cancer cachexia, and

this has commonly been in conjunction with altered mTOR activity [47, 49]. Understanding the role of protein anabolism as it relates to cancer cachexia and potential treatments to improve protein anabolism likely is critical to alleviate muscle wasting.

### *C. Atrophy*

Muscle atrophy due to cancer cachexia progression relates to processes of protein degradation elevated to a greater extent than processes related to protein synthesis. The UPS is known to be upregulated in various models of muscle atrophy, especially in cancer cachexia [50, 51]. The proteasome plays a pivotal role in the breakdown of proteins tagged for degradation via ubiquitin tagging, and within the correct balance is necessary for the healthy removal of proteins [52, 53]. But at chronically elevated or even extreme levels as seen in cachectic patients the UPS results in excessive protein breakdown and ultimately muscle wasting.

The proteasome consists of two subunits, the catalytic core 20S subunit and the regulatory 19S subunit [53]. Combined these two subunits create a complete a functional 26S proteasome complex that will actively destroy proteins that have been labeled with ubiquitin tags

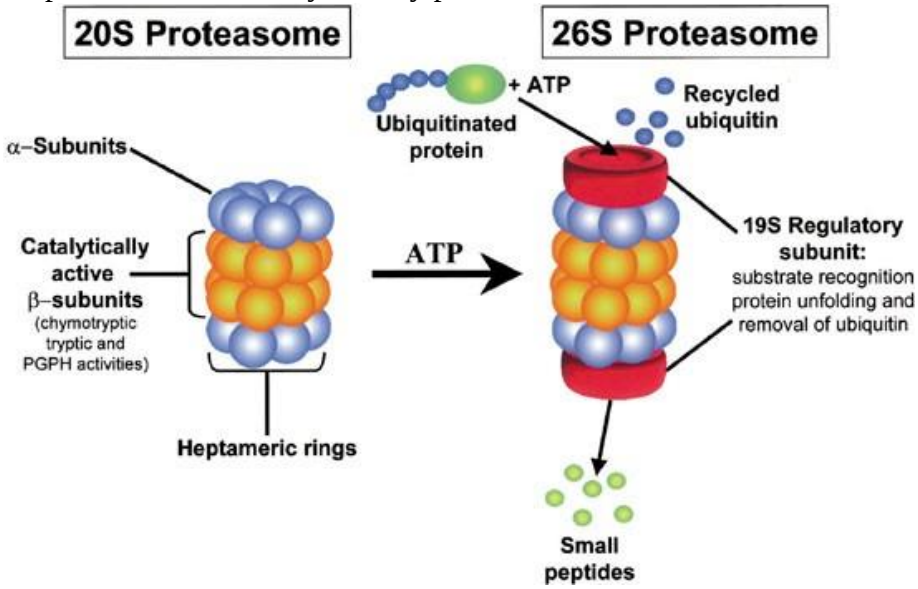


Fig 1-2. Ubiquitin Proteasome System (UPS).

[53, 54] (Fig 2). The process of ubiquitination revolves around three series of ubiquitin labeling enzymes: 1) ubiquitin-activating enzymes (E1), 2) ubiquitin-conjugating enzymes (E2), and 3) ubiquitin-protein ligases (E3) [53, 55]. The third step of ubiquitination involving the E3 ligases has become a valuable part of measuring relative levels of proteolysis in wasting diseases such as disuse, anorexia, and cancer cachexia [55, 56]. E3 ligases such as Muscle Atrophy F-box Protein-1(Atrogin-1) and Muscle Specific RING Finger-1 (MuRF-1) have been the focus of muscle wasting research, where increased transcription of these genes has been positively correlated with worsened muscle wasting [57, 58]. Atrogin-1 and MuRF-1 are primarily regulated by the FOXO-1 and FOXO-3 transcription factors, where upon their activation both FOXO-1/3 translocate to the nucleus of the cell and directly aid in transcription of Atrogin-1 and MuRF-1[53, 55, 59]. Therefore, evaluating the activation of FOXO-1 and FOXO-3 as well as their downstream transcriptional products in the form of Atrogin-1 and MuRF-1 can create a better understanding of relative proteolytic activity in wasting pathologies.

The FOXO1/3 family of transcription factors is regulated via the Protein Kinase B (from here on out known as AKT) pathway, where AKT phosphorylates FOXO1/3 and inactivates their transcriptional capability [60]. Due to AKT's prominent role in protein synthesis by its ability to activate mTOR, relative activity of these protein synthetic markers would then play a role in regulating proteolytic activity via Atrogin-1 and MuRF-1. Cytokines such as IL-6 also aid in upregulation of Atrogin-1 and MuRF-1 in cancer cachexia [61, 62]. Due to the intertwined relationship between inflammatory and protein synthetic pathways on proteolytic activity, evaluating all three in conjunction yields the greatest value in understanding the role of proteolysis on cancer cachexia.

### *D. Autophagy*

Autophagy, like the proteasome, is necessary for health destruction and removal of proteins but is also found to be dysregulated in cancer cachexia [63, 64]. Autophagy is induced in times of nutrient deficiency, oxidative stress, or reduced energy and is negatively impacted when protein synthesis levels are high as activation of mTOR inactivates autophagy mechanisms [65, 66]. Autophagy is the process of producing an autophagosome to induce the destruction of cellular proteins and even organelles for recycling of their components in times of cellular stress. Autophagy is initiated via the formation of a phagophore or nucleation membrane. The phagophore is elongated, resulting in a nascent autophagosome, which then can engulf proteins that are marked for degradation and is now considered mature. Finally, the mature autophagosome fuses with lysosomes, resulting in autolysosomes, and consumed contents are degraded via catalytic enzymes. Prominent markers associated with autophagosomal degradation of proteins are autophagosome membrane-associated LC3 I and LC3 II, and p62/sequestosome 1 (SQSTM1) [67, 68]. LC3 I is the cytosolic form which is then converted to LC3 II during lengthening of the phagophore to form the autophagosome. p62 interacts and sequesters ubiquitinated proteins to the autophagosome for degradation via autophagy as opposed to via the proteasome [69-71]. Impaired autophagy appears to occur in conjunction with cancer cachexia progression and may result in mitochondrial dysfunctions as well if damaged mitochondria and their cellular components are not successfully recycled [63, 72, 73]. Due to its related nature of breaking down ubiquitinated proteins similar to the proteasome, measuring autophagic markers in combination with proteasomal ones can create a comprehensive picture of protein degradation in cancer cachexia.

### *E. Myogenesis*

Myogenesis, or the development and regeneration of skeletal muscle tissue, is necessary for healthy skeletal muscle regeneration and maintenance. The myogenic process can be divided into three major stages of 1) Activation, 2) Proliferation and 3) Differentiation. Prior to activation a muscle damaging stimulus such as exercise or pathological conditions induces the myogenic process to begin in order to prevent muscle loss [74, 75]. Next muscle is infiltrated by neutrophils and then macrophages which breakdown damaged or necrotic tissue, as well as releasing potent growth factors such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF) and hepatocyte growth factor (HGF) [74-76]. These growth factors aid in activation of resident and quiescent satellite cells to begin the process of forming new myoblasts. While quiescent, satellite cells expressed the Paired box protein (Pax7) and no MyoD [76-79]. However, once activated by these growth factors, satellite cells switch towards greater MyoD expression in order to enter the cell cycle and multiply into more myoblasts known as the Proliferation stage [76-79]. Finally, Pax7 expression decreases and satellite cells express Myogenin where they then differentiate into myotubes and fuse at the site of injury to regenerate damaged myofibers [76-79]. The myogenic process has been shown to be impaired *in vitro* and *in vivo* [80, 81], but recently literature has aimed to understand what alterations in the myogenic process are involved with cancer cachexia progression. Most recent evidence ties the chronic elevation of inflammatory cytokines such as TNF-α, NFκB and IL-6 to alterations in key myogenic regulators such as Pax7 and MyoD [82-84]. Chronic overexpression of Pax7 in conjunction with NFκB has been observed in cachectic mice to the extent where satellite cell activation is impaired and therefore downstream proliferation and differentiation result in incomplete myogenesis and worsened muscle atrophy

[82-84]. These negative alterations and the role they play on myogenesis in cancer cachexia is necessary to determine to what extent impaired myogenesis correlates to cancer cachexia progression.

*Nutraceutical Interventions for Cancer Cachexia*

*A. Current literature*

Because cancer cachexia is marked by its irreversible losses in skeletal muscle mass a great deal of research has focused on alleviating this chronic wasting by use of nutritional and nutraceutical interventions. More specifically, attempts to improve caloric intake and evaluation of nutraceuticals aimed at alleviating cancer cachexia associated dysfunctions have been the main focus. Reduced appetite has been known to contribute to cancer cachexia progression as it results in reduced caloric intake [85, 86], however interventions aimed at solely improving calorie consumption have not effectively alleviated cancer cachexia associated wasting [85, 87, 88]. Nutraceuticals have the ability to target one or several cellular processes in such as protein synthesis or inflammation in an effort to improve each's effect on cancer cachexia progression [89-92]. Long-chain omega-3 fatty acid eicosapentaenoic acid (EPA) is one such nutraceutical that is known to improve inflammation by reducing NFκB expression and has been supported to improve muscle wasting in smaller human studies [93, 94, 95], but these findings were not supported in studies of larger sampling [96, 97]. Quercetin is another anti-inflammatory targeting nutraceutical that has been shown to improve and reduce IL-6 expression in $APC^{Min/+}$ mice [11, 98, 99]. Other nutraceuticals such as β-hydroxy-β-methylbutyrate (HMB) have been used in cachectic subjects for their ability to improve activation of protein synthesis markers such as mTOR and AKT with successful implications on cancer cachexia [100, 101]. In fact, HMB serves an example of a nutraceutical with the potential capability to improve markers of protein

synthesis and atrophic regulators such as FOXO1/3 and MuRF-1 [100-104]. Following years of research, no single nutraceutical has proven effective at alleviating cancer cachexia across all populations. In all likelihood a multi-targeted approach of a nutritional/nutraceutical along with additional interventions may be necessary to maximize anti-cachectic effects. In order to better understand what a multi-targeted approach for cancer cachexia should look like, a thorough evaluation of potential nutraceuticals on cancer cachexia is still required.

*B. Leucine*

Leucine is an essential amino acid and one of three branch chain amino acids (BCAA), along with iso-leucine and valine. Leucine is most notable of the BCAA for its capability to induce protein synthesis machinery in skeletal muscle via direct activation of mTOR [105-107]. Increases in protein synthesis have a protective effect on skeletal muscle from elevated protein catabolism which is observed across muscle wasting pathologies. Due to leucine's ability to stimulate mTOR it has been widely researched in skeletal muscle across different exercise and pathological conditions such as cancer cachexia [108-112]. Depending on the model of cancer cachexia being used and other experimental conditions leucine supplementation has shown either no improvements or marked improvements in protein synthesis and preservations in muscle mass [113]. Low doses of leucine supplementation in Lewis Lung Carcinoma (LLC) has shown no significant improvements in protein synthesis but did lead to greater expression of Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) a known regulator mitochondrial biogenesis [114]. Cruz et al. reported that a

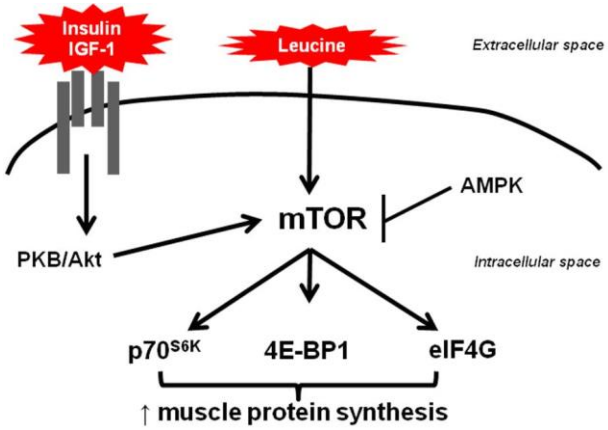


Fig 1-3. Leucine's role on protein synthesis in skeletal muscle.

leucine-rich diet resulted in improved muscle mass and protein content, but they contributed this effect to reduced pro-inflammatory and greater anti-inflammatory signaling [115]. Leucine supplementation has even shown improvements on proteasomal and lysosomal degradation in tumor-bearing rats, but the exact mechanism as to how was not elucidated [115]. Research by Baptista et al. suggests that leucine can reduce Atrogin-1 and MuRF-1 activity but is dependent on its ability to suppress FOXO 3 upstream of both atrogenes, as supported by their *in vitro* inhibition of leucine activity [116, 117]. Leucine dosage of 1.5% per volume has been reported in previous studies to stimulate markers of protein synthesis while having no impact on metabolic rates and has been widely utilized in leucine supplementation studies for that very reason [145, 146, 147]. These variations in effect and efficacy reveal the need for further evaluation of leucine supplementation and the underlying mechanisms that can contribute to any and all improvements on protein synthesis and cancer cachexia.

*Fibrosis in Cancer Cachexia*

*A. Extracellular matrix (ECM)*

The ECM was thought for many years to act as a scaffold within skeletal muscle to allow for proper anchoring and force production, but now we understand a great deal of the necessity and complex role the ECM plays in overall skeletal muscle health. The ECM has three major

layers within skeletal muscle starting with the epimysium surrounding the entire muscle, the perimysium surrounds muscle fiber bundles that comprise an entire muscle, and lastly the endomysium that surrounds individual muscle fibers [118]. The ECM itself accounts for ~5% of the dry weight of skeletal muscle [76]. One major key element of the ECM is that it surrounds the satellite cells responsible for skeletal muscle growth and regeneration. As such, the ECM maintains skeletal muscle structure and the crucial progenitor cells that manipulate myogenesis [76, 119]. The ECM is also the host to growth factors such as transforming growth factor-beta (TGF-β) that can aid in activation of satellite cells and downstream myogenic processes [76, 120]. Degradation or damage of the ECM results in release of these growth factors which can now exert their effects to satellite cells and aid in regeneration [76, 119]. This capability of the ECM to aid in repair and maintenance of skeletal muscle makes it critical for overall skeletal muscle health. However, the ECM can also negatively impact skeletal muscle if there is excess deposition of ECM components within skeletal muscle, a process known as fibrosis [14, 120]. Excessive fibrosis of skeletal muscle essentially replaces health skeletal muscle tissue with non-contractile and stiff ECM components. Fibrosis development can be traced back to persistent inflammation and as a result is prevalent in models of cancer cachexia [121]. TGF-β is controlled by inflammatory machinery such as IL-6 and is found to be upregulated in cancer cachexia, resulting in continuous accumulation of ECM components such as Collagens and worsening losses of skeletal muscle in cancer cachexia [15].

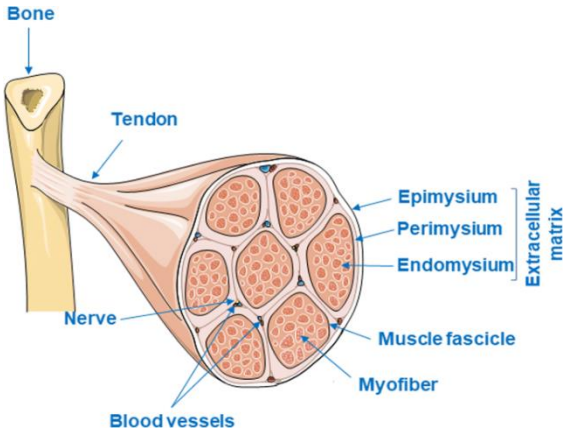


Fig 1-4. ECM structure in skeletal muscle

*B. Collagens, MMPs and TIMPs*

Collagen comprises the majority of the ECM. There are over 28 known types of Collagens all with varying characteristics and functions [122]. But within the skeletal muscle ECM, Collagens I and III are the most abundant components with polarizing characteristics. Of all the Collagens, Collagens I, II, and III make up over 90% of all Collagen in the body [123, 124]. All Collagens have a base structure revolving around a triple helix: two α1 chains and one α2 chain. Each chain consists of ~1050 amino acids, with the majority of those amino acids consisting of glycine, proline, and hydroxyproline. The repeating motif of this amino acid arrangement is Gly-Pro-X, where X can be any amino acid. This repeating but still customizable motif is what gives Collagen fibers similar and still unique forms and characteristics. Collagen 1 has more rigid and tensile characteristics, is pound-for-pound stronger than steel, and as such is a crucial component in tendons [76, 124]. Collagen III has received less attention in research than Collagen I as Collagen I is more readily isolated and extracted. Collagen III is a thinner, elastic collagen and as such is more malleable under stressful conditions [76, 124]. Due to the opposing characteristics of these collagens and their abundance within the skeletal muscle ECM recent research has sought to compare the relative expression of Collagen I and Collagen III [125-127]. Creation of this Collagen ratio would then give insights into the rigidity or elasticity of the ECM. The results of this ratio as it pertains to amounts of fibrotic material present together would aid in evaluation of the ECM as it relates to overall muscle health. ECM is known to be dysregulated in cancer cachexia as fibrosis continues to develop, however fluctuations in Collagen I and Collagen III in cancer cachexia as it relates to skeletal muscle lack scientific research.

As discussed above, the ECM is crucial for skeletal muscle health maintenance, but improper regulation of the ECM and its components leads to fibrosis and impaired muscle mass.

Matrix metalloproteinases (MMPs) are a family of proteases that breakdown collagens and therefore breakdown the ECM. MMPs are released by a variety of cells such as immune, myofiber, satellite, epithelial, and fibroblast which themselves are transcriptionally regulated by inflammatory cytokines such as TGF-β, IL-6, and TNF-α [76, 128]. Although there are many MMPs, MMP-2 and MMP-9 are consistently found to be linked to ECM degradation and fibrosis in skeletal muscle across a variety of pathologies [129-132]. Because MMPs are regulated in-part by inflammatory cytokines known to elevated in cancer cachexia they must play a part in fibrosis during cancer cachexia, but their exact role has yet to be elucidated.

MMPs regulate collagen and ECM breakdown in response to various stimuli such as muscle injury and damage. This breakdown of the ECM and its components can be useful such as when releasing growth factors following muscle damage to aid in the regeneration process, but chronic elevation of MMPs results in excess fibrosis and only worsens muscle health [121, 133]. Key regulators of MMPs are Tissue Inhibitor of Metalloproteinases (TIMPs) which inhibit the activity of MMPs [134]. TIMPs aid in maintaining the proper balance of MMP activity for proper myogenesis and muscle health while limiting fibrotic events to healthy levels [135]. When TIMPs are dysregulated MMPs will be left unchecked ultimately leading to ECM and skeletal muscle pathology [136, 137]. TIMP-1 and TIMP-2 inhibit MMP-9 and MMP-2, respectively, and the analysis of MMPs in association with their respective TIMPs is necessary for measuring degradation and over remodeling of the ECM.

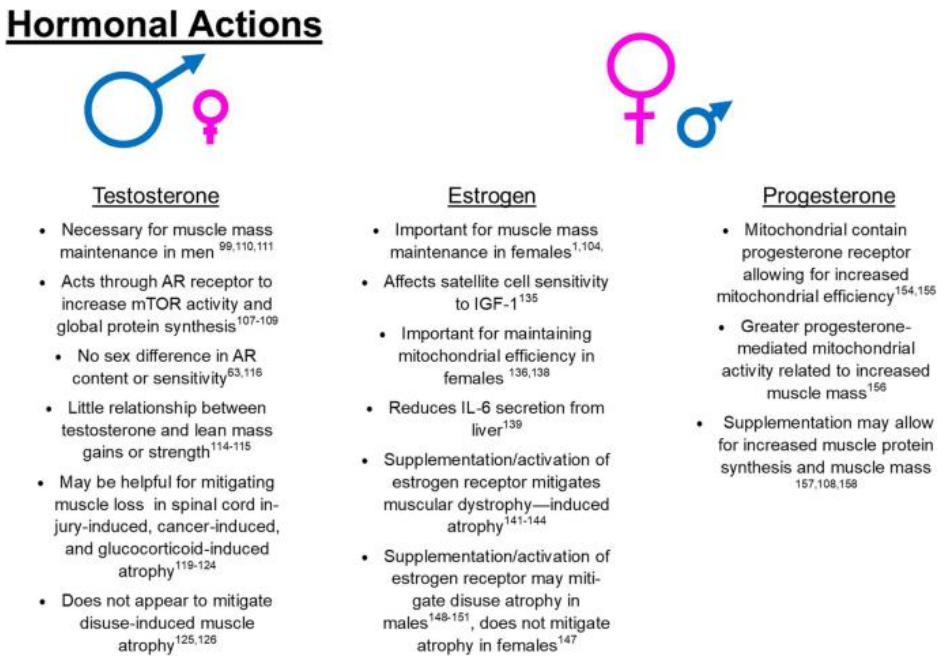


Fig 1-5. Hormonal effects on aspects of skeletal muscle health. Rosa-Caldwell & Greene, 2019.

Cancer cachexia is a multifaceted wasting syndrome characterized by chronic inflammation, dysregulated protein turnover, and excessive fibrosis [4, 13, 14, 15, 22, 36]. This inherent complexity of the condition has made finding effective therapies challenging. Adding to this challenge is the effect sex has on creating varied responses to treatments and even cancer cachexia itself [138]. Recent research has been aimed at evaluating cancer cachexia and various treatments in both sexes [8, 139, 140]. More specifically, research in females has been lacking where past studies tended to focus exclusively on male subjects to avoid complications that can arise due to hormonal variations from estrous cycling in females. Interestingly, current literature suggests that females are more susceptible to wasting from disuse and less susceptible to wasting from inflammatory conditions such as cancer cachexia [138]. The root causes of these sex

differences seem to center around muscle protein turnover, satellite cell dynamics, hormones, and mitochondria (Fig 5).

Hormonal estrogen has been observed as the main driver for female physiology to differ from males, and past studies of ovariectomized females resulted in increased adiposity, inflammatory expression, and lethargy [8, 143]. Vice versa, studies adding exogenous estrogen saw improvements in protection from skeletal muscle wasting [144]. With these improvements largely attributed to lessened inflammation in females. These recent studies all point to females displaying greater protection to inflammatory-based pathologies, namely cancer cachexia, due to the greater protective effects of estrogen on reducing inflammation.

Protein turnover has shown sexual dimorphism mainly in the presence of estrogen, where reductions in estrogen have been linked to reduced anabolism and increased inflammation and autophagy [138, 141]. This demonstrates protein turnover as possessing a sensitivity to relative amounts of circulating estrogen. Like estrogen's effect on protein turnover, higher testosterone in males and in subjects with exogenous supplementation of testosterone has been linked to greater content and proliferation capacity of satellite cells [142]. Recent research also suggests that males and females may favor or utilize different protein degradation pathways, whether with proteolysis or autophagy for example [34, 138]. In general, there is no longer a debate on whether sex can result in variations in muscle pathologies, the focus has now shifted on future research attempting to better understand the effects of sex on muscle pathologies and therapeutic interventions.

*Summary*

Cancer cachexia is an irreversible wasting condition that affects ~80% of all cancer patients and is accountable for ~20% of all cancer related mortality [4, 5, 6]. Causes for cancer cachexia are largely attributed to the chronic inflammation and protein imbalance observed as cancer progresses [10-13]. These dysregulations result in excessive protein degradation and fibrosis ultimately leading to skeletal muscle wasting and dysfunction [14-16]. Skeletal muscle comprises the majority of overall body mass and continuous muscle atrophy in cancer patients worsens patient outcomes by increasing morbidity and mortality [3].

The role sex plays on cancer cachexia has received little scientific attention until recently [138]. More recent efforts have been made to evaluate how male and female differences effect skeletal muscle physiology as it relates to cancer cachexia and even potential therapeutic interventions [8, 139, 140]. Variations in muscle physiology, hormone balance and protein turnover are known to occur between both sexes, but the role sex has on leucine supplementation and fibrosis as they relate to cancer cachexia has not been elucidated.

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# Chapter 2

## Doctoral Dissertation Proposal

Cancer is the second leading cause of death in the United States according to the Centers for Disease Control and Prevention (CDC), accounting for ~600,000 deaths yearly and trailing only heart disease in mortality [1, 2]. Concurrent with many cancers is the development of continual losses in skeletal muscle associated with cancer progression, otherwise known as cancer cachexia. This progressive wasting of skeletal muscle tissue is also associated with excess fibrosis of the extracellular matrix (ECM) and its components within skeletal muscle, and overall results in worsening of cancer patient health outcomes [3, 4, 5]. In fact, cancer cachexia is responsible for ~20% of all-cancer related deaths [6, 7, 8]. The difficulty in finding effective therapeutic approaches to cancer cachexia is due to the multi-factorial nature in which cachexia can develop, and to date no nutritional interventions have shown to be effective treatments. Along with current research into cachexia treatments in general is the rising need for evaluation of how sex has shown to create a divergent response to not only cancer cachexia development and progression [9], but to potential cachexia interventions as well [10]. Therefore, evaluation of cancer cachexia between sexes and how both respond to any possible intervention has become of crucial importance in recent cachexia research.

Cancer cachexia is driven by improper maintenance of protein flux, favoring catabolism over anabolism [11, 12]. No single nutritional intervention targeting this imbalance has been effective across all cancer types, but evaluation of cancer cachexia development and progression between sexes following treatment with a known stimulator of anabolism is lacking. The amino acid Leucine has been shown to drive anabolism through direct stimulation of mTOR, where an increase in mTOR activation could theoretically upregulate protein anabolism in order to

mitigate improper protein turnover [13, 14]. Increased fibrosis is also known to occur in cachectic patients further worsening losses in skeletal muscle mass [15, 16]. While fibrosis is known to be upregulated due to cancer progression, the fibrotic response during the early developmental stages of cachexia is not completely understood. Therefore, the central hypothesis of this proposal is two-fold: 1) leucine supplementation will induce proteins and related markers of anabolism, however this could exacerbate cancer cachexia due to the opportunistic nature of the tumor micro-environment; and 2) increases in fibrosis will be driven by dysregulation of extracellular matrix maintenance during the early, developmental stages of cancer cachexia. We also hypothesize there will be a dimorphic response to both leucine supplementation and fibrotic development between cachectic males and females.

**Aim 1.** Determine the effect of leucine supplementation on cancer cachexia in males and females. $APC^{Min/+}$ male and female mice will be given 1.5% leucine supplemented water (plain water control) to evaluate the effect of leucine on cancer cachexia. Tissue collection will occur at 20 weeks of age, or when cachexia becomes severe enough that mice become moribund. Analysis of leucine supplementation and its effect on the cachectic phenotype, inflammatory markers and protein turnover signaling will be used to elucidate leucine's specific role on cancer cachexia.

**Aim 2.** Analyze fibrosis during the development of cancer cachexia between males and females. Fibrosis during cancer cachexia development will be analyzed following injection of Lewis-lung carcinoma cells for 1, 2, 3, and 4wks (0wk PBS control) into the hind flank of male and female C57BL/6 mice. We will measure markers associated with ECM regulation and fibrosis at all timepoints to determine the role of the ECM on skeletal muscle mass during the development of cancer cachexia.

**Aim 3.** *in vitro* analysis of cancer cachexia in muscle cells following treatment with plasma from leucine supplemented males and females. Blood plasma collected from the $APC^{Min/+}$ mice in Aim 1 will be administered to differentiated myotube cells to evaluate leucine supplementation *in vitro* to go along with the *in vivo* analyses. Results obtained in Aim 3 will be portrayed alongside Aim in Chapter 4 in manuscript form.

The experiments outlined above will provide a thorough and novel analysis of the effectiveness of an anabolism-driven nutraceutical on cancer cachexia, as well as the role fibrosis plays on the developmental stages of cachexia between both sexes.

## Research Strategy

### A. Significance

**A1.** Cancer cachexia lacks an effective treatment and is shown to worsen cancer patient health and outcomes. Cancer cachexia worsens cancer severity and ultimately cancer patient outcomes. While skeletal muscle wasting is known to occur in cancer patients, there are no clear avenues for treatment due to the multi-factorial nature of the disease. This lack of an effective treatment avenue along with the prevalence and severity of cancer-related deaths has made cachexia a focal point of recent cancer research and a priority of the National Cancer Institute as its own grant initiative (PQ6). To date, the efficaciousness of nutraceutical interventions and the concurrent replacement of skeletal muscle with fibrotic tissue during cancer cachexia lack thorough evaluation. Due to the lack of effective treatments for cancer cachexia there is still an urgent need for investigation of novel therapeutics against and processes that contribute to the development and progression of cancer cachexia.

**A2.** Supplementation with leucine may alleviate muscle wasting through activation of anabolism. Cancer cachexia occurs when rates of protein breakdown are greater than protein synthesis. This imbalance in protein turnover induces wasting of skeletal muscle and is known to affect ~50% of all cancer patients. The amino acid leucine has the unique capability to drive protein synthesis rates to increase via direct stimulation of mTOR. This induction of mTOR and its downstream effectors (e.g., 4E-BP1) results in translation progression, aiding in protein synthesis rates. Therefore, the addition of leucine could provide a potent nutraceutical-based treatment and correct the skeletal muscle wasting observed in cancer cachexia.

**A3.** Excess fibrosis is associated with cancer cachexia progression but the role of fibrosis in the developmental stages of early cancer cachexia is not fully understood. The replacement of healthy muscle mass with non-contractile and fibrotic tissue is a major contributor to cancer cachexia and its progression. What is less understood is the role of fibrosis towards development of cachexia in its early stages. The ECM that surrounds skeletal muscle is home to growth factors (TGF-β) and satellite cells that aid in skeletal muscle mass health and maintenance. Excessive upregulation of these growth factors ultimately leads to fibrotic infiltration into healthy skeletal muscle, thereby worsening cancer cachexia. Alterations in the ECM may begin prior to the development of cancer cachexia, but whether it is true, and the processes involved lack elucidation.

**A4.** *In vitro* analysis of leucine supplementation's effect on cancer cachexia will allow for a mechanistic evaluation at the cellular level alongside the *in vivo* approach used in A1. The dimorphism in response of males and females to cancer cachexia as well cancer cachexia treatments produces difficulty in evaluating potential therapies. These known sex differences require consideration when researching the capability of novel treatments of cancer and cancer

cachexia. Therefore, we propose to utilize an *in vitro* analysis of leucine supplementation on skeletal muscle cells. Addition of blood plasma taken from the non-cachectic and cachectic animals to skeletal muscle cells in culture will elucidate the role of circulating factors on cancer cachexia following supplementation with the amino acid leucine. Similarities in protein turnover and inflammatory signaling between mice in A1 and skeletal muscle cells in A4 will aid in pinpointing key pathways and processes contributing to cancer cachexia improvements or worsening severity between sexes. Therefore, results discovered in A4 will be included alongside those in A1 for a comprehensive analysis of cancer cachexia in conjunction with leucine supplementation.

My central hypothesis is two-fold: 1) leucine supplementation will induce proteins and related markers of anabolism, however this could exacerbate cancer cachexia due to the opportunistic nature of the tumor micro-environment; and 2) increases in fibrosis will be driven by dysregulation of extracellular matrix maintenance during the early, developmental stages of cancer cachexia. We also hypothesize there will be a dimorphic response to both leucine supplementation and fibrotic development between cachectic males and females.

**B. Innovation**

The proposed studies were innovative due to the targeting of a potent nutraceutical (leucine) for treatment of cancer cachexia, and the isolation of the early and developmental stages of cancer cachexia for fibrosis analysis. *In vitro* duplication of the experiments in Aim 1 also adds an innovative and robust comparison to strengthen any arguments for or against leucine supplementation as an appropriate nutraceutical intervention for the treatment of cancer cachexia. We believe we are the first research group to have such an experiment for *in vitro* analysis of leucine supplementation on cancer cachexia by plasma treatment of muscle cells from

leucine supplemented and cachectic mice. Inclusion of both male and female sexes in all proposed experiments also adds novelty to past research of leucine supplementation and fibrosis in cancer cachexia, where past studies are lacking in such considerations of sexual dimorphism.

## C. Approach

**Overall Approach.** In order to test the central hypothesis, we have performed two large *in vivo* experiments using male and female mice. $APC^{Min/+}$ (APC) mice, a well-established model of cancer cachexia due to colon cancer, were selected for analysis of leucine supplementation as a potential nutraceutical treatment. This model was chosen as this allows a more prolonged development of cancer cachexia compared to allograft models to allow an enhanced assessment of nutraceutical efficacy. LLC mice were created, and tissue harvested at 0, 1, 2, 3, and 4wk post-injection with 1million Lewis Lung Carcinoma (LLC) cells to isolate the developmental stages of cancer cachexia for fibrosis analysis. For these studies LLC was chosen as a model to allow for more careful control of the time course development of cancer cachexia. Plasma was extracted from the APC mice described above and used to treat muscle cells in culture for a novel *in vitro* evaluation of any differences in leucine supplementation on cancer cachexia prevalence due to sex and their inherent circulating factors.

**Specific Aim 1.** I hypothesize leucine supplementation will drive increases in markers of anabolism in both males and females, however this may or may not alleviate cancer cachexia severity. Preliminary weight data (Figure 1) suggest leucine supplementation worsened losses in body weight, at least in males, compared to those given normal water. This increased severity in wasting may be due to the opportunistic nature of cancer, thereby driving cancer growth and worsening pathways already associated with cancer cachexia progression. To test this hypothesis and understand why this exacerbated wasting was not observed in females, we evaluated the

inflammatory, protein turnover, and myogenic profiles of WT and APC mice following leucine supplementation.

*$APC^{Min/+}$ model of cancer cachexia:* APC mice were purchased from Jax Labs. APC-positive males were bred with C57BL/6 female mice. Mice were genotyped using PCR following weaning to determine genotype. APC-negative mice were used as a wild-type (WT) control. Mice were weaned at 4 weeks of age, where they were then supplemented with leucine-enriched water (1.5%) or normal water as a control. This created 4 experimental groups per sex based on

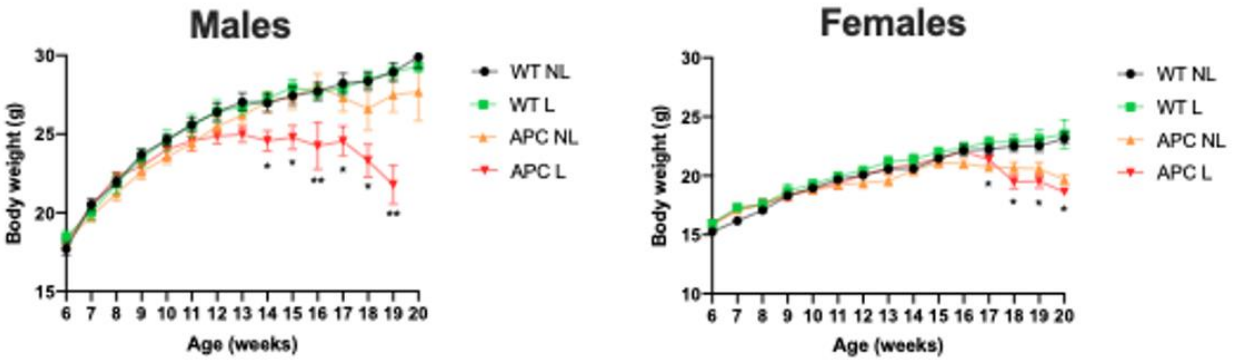


Figure 2-1. Preliminary body weight data from male and female APC mice supplemented with normal or leucine-enriched water collected by our lab group.

genotype and leucine supplementation described as WT No Leucine (WT NL), WT Leucine (WT L), APC No Leucine (APC NL), and APC Leucine (APC L). Tissue was collected at 20 weeks of age or if mice became moribund prior to harvest date.

*Confirmation of cancer cachexia:* Muscle wet weights, fat mass and measurements of muscle cross-sectional area (CSA) will be collected to confirm whether cachexia occurred, as well as whether there were any variations between groups. CSA measurements will be performed in conjunction with immunofluorescent fiber typing described below.

*Inflammatory Profile:* Classification of inflammatory markers associated with cancer cachexia in APC mice has been well established. Further analysis of known regulators of

inflammation following leucine supplementation will allow for comparison with past studies on how leucine exhibits its specific effects on cancer cachexia. This will be accomplished through measurements of spleen mass, and RT-PCR and Immunoblot inflammatory cytokines such as IL-6, TNF-α for a comprehensive analysis.

*Protein Turnover:* Analysis of markers involved in the synthesis and breakdown of proteins will confirm the role of leucine on cancer cachexia as it pertains to protein turnover. This will be completed through RT-PCR and Immunoblot of protein turnover such as p70S6k and 4E-BP1 similarly to classification of the inflammatory profile described above.

*Histological Analysis:* Tibialis anterior (TA) muscle was embedded in optimal cutting temperature (OCT) compound for sectioning of muscle at a thickness of 10μm. Immunofluorescent labeling of myosin heavy chains (MHCs) I, IIa, IIb in TA cross-sections will be performed for measurements of any shifts in fiber type composition. Labeling of these fibers will also allow for tracing of individual muscle fibers for CSA measurements for confirmation of muscle wasting due to cancer cachexia.

*Statistical Analyses:* Differences between groups will be determined via Two-way ANOVA for a global analysis, where a Tukey's post hoc test will then be performed to evaluate differences between means. All Two-way ANOVAs will be performed within sex and are not meant for direct comparisons between male and female data. Data was determined parametric via a Shapiro-Wilk test. Statistical significance will be set at $\alpha=0.05$ and $\beta=0.20$ (power = 0.80).

*Anticipated Results and Interpretation of Findings:* We expect leucine supplementation to alter markers of protein anabolism in WT and APC mice. Our preliminary data (Figure 1) show that leucine supplementation had a negative impact on skeletal muscle wasting at least in male mice. Analysis of the inflammatory profile and markers associated with protein turnover

will help to elucidate the major contributors towards the worsening of cancer cachexia induced by leucine supplementation.

**Specific Aim 2.** We hypothesized dysregulation of the ECM will coincide with cancer cachexia development in males and females, but there would be variations in severity and the signaling pathways contributing to the fibrotic response. To test this hypothesis, male and female mice were injected with either phosphate buffered saline (PBS) or LLC cells for 0 (PBS only), 1, 2, 3, and 4wks. Published data from our research group has demonstrated that cachexia developed at 4wks in these mice, therefore alterations in ECM signaling and composition in the weeks prior could identify key regulators of fibrosis during cancer cachexia development.

| Variable | PBS (n=24) | 1 Week (n=16) | 2 week (n=16) | 3 week (n=12) | 4 week (n=14) |
|---|---|---|---|---|---|
| Body Weight | 24.9±0.33a | 23.9±0.32a | 23.8±0.62a | 24.3±0.81a | 27.0±0.62b |
| Tumor Weight | N/A | 0.03±0.07a | 0.16±0.03a | 0.77±0.15b | 3.50±0.36c |
| BW - Tumor | 24.8±0.33 | 23.8±0.32 | 23.6±0.65 | 23.5±0.74 | 23.6±0.56 |
| TA | 45.14±0.85a | 43.80±1.16a | 44.54±1.15a | 42.88±1.64a | 38.34±1.31b |

Table 2-1. Weight data from male LLC model of cancer cachexia. Brown et al., 2017.

*Culturing of LLC Cells:* LLC cells were cultured in 10% Fetal Bovine Serum Growth Media with 1% Penicillin and Streptomycin added. Once confluent, cells will be trypsinized, counted and diluted in PBS for implantation.

*Induction of muscle atrophy by cancer-cachexia:* LLC cells will be implanted into the hind flank of anesthetized C57BL/6 mice at a concentration of $1X10^6$ cells in a 100 µL suspension of sterile PBS. The LLC tumor will be allowed to develop for up to 4 wks with cohorts harvested weekly. Sterile PBS was injected for a 0wk control group.

*Confirmation of model:* Tissue wet weights were measured for confirmation of muscle wasting due to cancer cachexia. *Fibrosis and Collagen Analysis:* Fibrosis will be quantified by histological staining of TA cross-sections via Picro Sirius Red. Picro Sirius Red stains collagen, the major component of the extracellular matrix, allowing for quantification of collagen deposition and overall fibrosis in skeletal muscle as cancer cachexia develops. We will also perform immunofluorescent labeling of Collagens 1 and 3 in TA cross-sections, where we can then create an analysis of the relative amount of Collagen 1 to Collagen 3. Collagen 1 is a more rigid collagen fiber, whereas collagen 3 has greater elasticity and is more tensile. Changes in the relative amounts of these collagen fibers can indicate alterations in the ECM surrounding skeletal muscle as it pertains to fibrosis due to cancer cachexia.

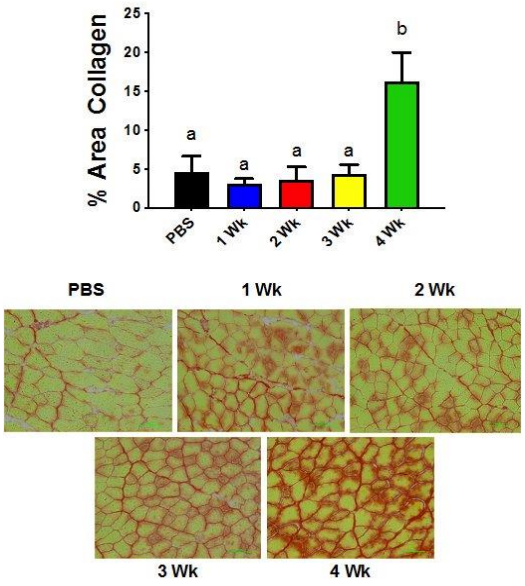


Figure 2-2. Preliminary data of collagen staining of skeletal muscle cross-sections via Picro Sirius Red.

*ECM dynamics:* RT-PCR and Immunoblot of collagens and regulating factors of the ECM within skeletal muscle will aid in identification of key molecules and processes that contribute to fibrosis during the development of cancer cachexia. RT-PCR and Immunoblot targets are as follows: Collagen 1, Collagen 3, MMP-2, MMP-9, Timp-1, TGF-β, β-catenin, SMAD 2, and SMAD 3.

*Muscle cell and Fibroblast cell Co-culture:* C2C12 myoblast cells and 3T3 fibroblast cells will be co-cultured *in vitro* for analysis of the same ECM-related pathways and markers described in the *in vivo* approach. C2C12 cells will be grown until they reach ~80% confluency,

where they will then be transferred to a 6-well plate and differentiated for 5 days. 3T3 fibroblast cells will then be added via a transwell-insert, which possesses a semi-permeable membrane, into the 6-well plate for creation of a skeletal muscle to ECM connection *in vitro*. LLC conditioned media (LCM) or control media will then be added to both cell types for 24hr, where we will then harvest both for the same RT-PCR and Immunoblot analyses described in the *in vivo* approach. Confirmation of cancer cachexia will be confirmed via measurements of myotube diameter prior to cell harvest.

*LLC conditioned media:* LLC cells were grown to 100% confluence. Media was then filtered and diluted to 25% total volume in serum free media. For the Control group, 10% fetal bovine serum growth media was diluted to 25% total volume with serum free media.

*Statistical Analyses:* Differences between groups will be determined via One-way ANOVA for a global analysis, where a Student's Newman Keuls post hoc test will then be performed to evaluate differences between means. Data was determined parametric via a Shapiro-Wilk test. Statistical significance will be set at $\alpha$=0.05 and $\beta$=0.20 (power = 0.80).

*Anticipated Results and Interpretation of Findings:* We expect ECM dynamics to be altered and fibrosis to increase as cancer cachexia begins to develop. This will be reflected in modifications to pathways and markers associated with ECM remodeling, and further reflected in changes to the ratio of Collagen 1 to Collagen 3 at the tissue level. However, these alterations will likely vary between males and females.

**Specific Aim 3.** We hypothesize addition of blood plasma from leucine supplemented and control mice to muscle cells in culture would aid in identification of the major pathways contributing to cancer cachexia between sexes. *in vitro* addition of plasma from male and female APC mice will aid in isolation of the dimorphic pathways responsible for the state of cancer

cachexia. This experiment gives a mechanistic boost in approach for analysis of leucine's capability, or potentially lack thereof, to alleviate cancer cachexia in both sexes.

*Plasma Treatments:* Plasma extracted from the female and male WT and APC mice used in Aim 1 will be added to cultured myotubes for *in vitro* analysis of cancer cachexia following leucine supplementation.

*Cachectic Plasma Analyses:* We will measure myotube diameter along with all RT-PCR and Immunoblot targets outlined in the *in vivo* approach in Aim 1. These analyses will ultimately provide a comprehensive comparison of leucine supplementation at the *in vivo* and *in vitro* level for both sexes. Any similarities or differences observed in RT-qPCR and Immunoblot targets at the *in vivo* or *in vitro* level will aid in elucidation of the major contributors of leucine's effect on cancer cachexia.

*Statistical Analyses:* Differences between groups will be determined via Two-way ANOVA for a global analysis, where a Tukey's post hoc test will then be performed to evaluate differences between means. All Two-way ANOVAs will be performed within sex and are not meant for direct comparisons between male and female data. Data was determined parametric via a Shapiro-Wilk test. Statistical significance will be set at $\alpha=0.05$ and $\beta=0.20$ (power = 0.80).

*Anticipated Results and Interpretation of Findings:* We expect the *in vitro* results to mirror those which are observed *in vivo* in Aim 1 on a sex-to-sex basis. Any variations will likely be minor, as plasma contains any circulating factors relative to each sex obtained from the WT and APC mice.

**Summary.** Cancer is a leading cause of death across the globe. Cancer cachexia is cancer-associated wasting of skeletal muscle that affects ~50% of all cancer patients, worsening patient outcomes. Due to the prevalence and severity of cancer and cancer cachexia there are

countless completed and ongoing studies aiming to better understand this multi-factorial wasting condition and how to properly treat it. Current nutritional interventions are ineffective at treating cancer cachexia, but not all nutraceutical avenues have been pursued. Fibrosis is known to coincide with cancer cachexia and its progression, but literature is lacking on how fibrosis and ECM dysregulation contribute to the initial development of cancer cachexia. Therefore, this proposal aims to evaluate a potent stimulator of anabolism, in the amino acid leucine, and fibrotic pathways in cancer cachexia and cancer cachexia development.

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# Chapter 3

## Leucine Supplementation Exacerbates Cancer Cachexia in a Male but not Female Model of Colon Cancer

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Running Title: Sexual Dimorphism of Leucine Supplementation on Cancer Cachexia

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**Abstract**

Cancer cachexia is a multi-factorial wasting syndrome that affects up to 80% of all cancer patients and is responsible for 20-40% of all cancer related deaths. To date, nutritional interventions have shown to be ineffective in alleviating cancer cachexia but there remains a lack of understanding as to why. More importantly, research is severely lacking in evaluation of nutraceutical interventions for cancer cachexia across both sexes. The purpose of this study was to evaluate the effects of leucine supplementation on cancer cachexia in both male (n=28) and female (n=32) $APC^{Min/+}$ (APC) and wild-type (WT) mice. Mice were supplemented with regular or leucine-enriched water following weaning up until euthanasia at 20 weeks of age. Male APC L mice exhibited exacerbated wasting with decreased body weight compared to male APC NL mice, and this effect of leucine was not observed in female APC mice. IL-6 mRNA was elevated in male APC L mice compared to male APC NL mice and this effect was also not observed in female mice. Female APC mice displayed greater MyoD mRNA abundance compared to WT and this was not observed in male mice. Myotube diameter was reduced following addition of blood plasma derived from male APC NL & L mice compared to WT but myotube diameter was unchanged following addition of female APC plasma. Our results indicate sexual dimorphism in markers of inflammation, protein anabolism and catabolism, and myogenesis during cancer cachexia following supplementation with leucine. We provide novel evidence for leucine supplementation to affect male and females differently during cancer cachexia and further highlights the need for inclusion of both sexes in future research.

## Introduction

Cancer is a leading cause of death worldwide according to the World Health Organization (WHO), with estimations of upwards to ~10 million deaths in 2019. In the U.S. alone, it is estimated ~600,000 people perished as a result of cancer in the year 2020 by the American Cancer Society (ACS), and according to the Center for Disease Control (CDC) ~39% of all men and women acquire some form of cancer in their lifetime. Cancer cachexia is a multifactorial wasting syndrome defined by losses of lean mass >5%, that can be accompanied with or without losses in fat mass and will affect ~80% of all cancer patients to varying extent (1, 2). Also of note, up to 40% of all cancer deaths can be directly attributed to cancer cachexia as it worsens patient health and overall outcomes (3). Currently no therapeutic interventions have shown to be effective in the treatment of cancer cachexia due to the pathology's multifactorial induction and regulation through a negative energy balance, systemic inflammation, and hormonal imbalances (4-6). Current scientific research has been aimed at understanding these cachexia-induced dysregulations in order to elucidate potential avenues for intervention but to date there is no current consensus of treatments with consistent efficacy (7).

Cancer cachexia is a wasting syndrome partially characterized by the favoring of catabolism over anabolism of proteins, as a result there has been numerous studies evaluating nutritional interventions on correcting this imbalance (8-10). To date these nutritional interventions have largely been proven ineffective across multiple models of cancer cachexia, however there is still a considerable lack of understanding as to the mechanisms that contribute to the inefficiency of nutritional interventions. L-leucine is a branched chain amino acid known to stimulate activation of mammalian target of rapamycin (mTOR) in the protein synthesis pathway. Studies utilizing leucine as a potential therapeutic have observed improvements in

mitochondrial quantity (11), reductions in protein inhibition and improved protein synthesis (12), improvements in inflammatory markers IL-6 and TNF-α (13), as well as increasing mTOR activation in various models of cancer cachexia (14). Therefore, utilization of a potent nutraceutical such as leucine could provide therapeutic benefits to cachectic cancer patients.

Adding into the overall difficulty and complexity of evaluating cancer cachexia is the emerging role that males and females display a sexual dimorphism to cancer, cancer cachexia, and even cancer treatments (15-20). For example, females in general tend to benefit from the anti-inflammatory effects of estrogen and therefore can experience lesser losses in body weight, muscle mass, and muscle function compared to males during cancer cachexia (17). Skeletal muscle of females also exhibits greater mitochondrial quality and fatigue resistance (17, 21). Recent studies overall indicate greater susceptibility of male skeletal muscle to cancer cachexia (17, 22), but very few studies have directly compared this sexual dimorphism simultaneously and most that do exist focused on phenotypic observations (23, 24). Further evaluation of cancer cachexia and potential therapeutics is critical to a holistic understanding of this pathology as cancer and cancer cachexia affects both sexes indiscriminately.

To our knowledge, there has not been a study on leucine supplementation as a nutritional intervention across both sexes in a common model of cancer cachexia. Therefore, the purpose of this study was to utilize the well-known model of cancer cachexia in the $APC^{Min/+}$ strain of male and female mice in conjunction with leucine supplementation. By supplementing their normal diet with leucine, we hypothesized that male and female mice would exhibit reductions in muscle wasting as cancer cachexia progressed. We tested this hypothesis by taking phenotypic measurements as well as a comprehensive analysis of key signaling pathways surrounding protein turnover and inflammation that are known to be particularly dysregulated in $APC^{Min/+}$

(APC) mice. As shown in the results of this study, leucine supplementation exacerbated cancer cachexia in male but not female mice. However, the direct link to this difference remains unclear and requires further evaluation in future research.

## Methods

### Animals and Interventions

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Arkansas, Fayetteville. APC-positive male mice and APC-negative female mice were initially purchased from Jackson Laboratories for APC colony production. APC-positive males were bred with C57BL/6 female mice as colon cancer is a contraindication for pregnancy. Mice were genotyped using semi-quantitative PCR following weaning to determine genotype. Males and females determined to be APC-negative following genotyping were used as a wild-type (WT) control. Mice were weaned at 4 weeks of age, where they were then supplemented with leucine-enriched water (1.5%) or normal water as a control until tissue collection and euthanasia at 20 weeks of age. This created 4 experimental groups per sex based on genotype and leucine supplementation described as: WT No Leucine (WT NL; Male n=7, Female n=8), WT Leucine (WT L; Male n=7, Female n=8), APC No Leucine (APC NL; Male n=7; Female n=8), and APC Leucine (APC L; Male n=7; Female n=8). Tissue was collected at 20 weeks of age or if mice became moribund prior to harvest date. Animal tissues, organs, and blood plasma were collected under isoflurane anesthesia prior to euthanasia. Tissues were weighed and snap-frozen in liquid nitrogen for further processing and stored at −80°C. The tibialis anterior (TA) muscle was submerged in optimum cutting temperature compound (OCT) and then placed in liquid nitrogen cooled isopentane. OCT mounted tissue was then stored at -80°C for future histological and microscopic analysis.

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

RNA isolation, cDNA synthesis, and quantitative real-time PCR were performed as we have previously described (25, 26). All targets were assayed using Taqman probes for the following targets: 18S (Mm03928990_g1), IL-6 (Mm00446190_m1), IL-1β (Mm00434228_m1), MyoD (Mm00440387_m1), MyoG (Mm00446194_m1), Pax7 (Mm01354484_m1), NFκB (Mm00476361_m1), TNF-α (Mm00476361_m1), UBC (Mm01198158_m1), Deptor (Mm01195339_m1), IGF-1 (Mm00439560_m1), Foxo1 (Mm00490671_m1), Foxo3 (Mm01185722_m1), Atrogin-1 (Mm00499523_m1), MuRF-1 (Mm01185221_m1). Taqman probes were purchased from Applied Biosystems. RT-qPCR measured cycle threshold (Ct) and the ΔCt value was calculated as the difference between the Ct value and the 18S Ct value. Final quantification of mRNA abundance was calculated using the ΔΔCT method Ct = [ΔCt(calibrator) – ΔCt(sample)]. Relative quantifications were then calculated as $2^{-}$ΔΔCt. 18S Ct values were confirmed to not differ between experimental conditions for any comparison.

**Immunoblotting**

Immunoblotting was performed as we have previously described (25, 26). Protein concentration and equal loading of SDS-page gels was determined via RC DC assay™ (Bio-Rad), and target protein content was normalized to Ponceau S staining of 45kDa band. Membranes were probed overnight for primary antibodies. Protein targets were purchased from Cell Signaling Technology (CST) and selected based upon prior literature targeting key components of inflammation and protein turnover (27-28). Antibodies were diluted in Tris-buffered saline, 0.1% Tween 20 with 5% milk. Membranes were imaged on LiCor Odyssey FC using IR detection and images analyzed by LiCor Image Studio™.

**Histology**

TA muscle stored in OCT were cryosectioned into 10μm thick sections on polarized microscope slides. Muscle sections were then histologically stained with Succinate Dehydrogenase (SDH) for quantification and analysis of oxidative muscle phenotype by counting relative presence of SDH+ (purple) and SDH- (white) fibers within the TA muscle. Following SDH staining, cross-sectional area (CSA) of muscle was determined by manually tracing of SDH+ and SDH- fibers by a blinded researcher. Images were analyzed using Nikon NIS Elements BR software package.

***in vitro APC$^{Min/+}$ Plasma Treatment***

C2C12 myoblast cells (ATCC CRL-1772) were cultured for an *in vitro* analysis of the effect of blood plasma derived from all male and female groups on myotubes. C2C12 cells were grown until they reached ~80% confluency at passage 2, where they were then transferred to a 6-well plate and differentiated for 5 days. Plasma-infused media was created by taking blood plasma from a single representative mouse from all treatment groups and adding to serum free DMEM at a concentration of 20μL/mL of media. Cells were treated with the plasma-infused media for over 24hr after differentiation, where they were then harvested for subsequent RT-qPCR and Western Blot analysis.

**Statistical Analysis**

All data was determined parametric via a Shapiro-Wilk test prior to any parametric analysis. Differences between groups was determined via Two-way ANOVA for a global analysis, where a Tukey's post hoc test was then performed to evaluate differences between means following a significant F-test. All Two-way ANOVAs were performed within sex and are not meant for direct comparisons between male and female data. Statistical

significance was set at $\alpha=0.05$. All data were analyzed using the Statistical Analysis System (SAS) and figures were compiled using GraphPad Prism and data expressed as mean ± standard error of the mean (SEM).

## Results

### Confirmation of Cancer Cachexia and Effect of Leucine Supplementation

Cancer cachexia was confirmed in males and females via body, tissue, and organ weights in Figure 1. Male APC NL mice had decreased body weight ~17% compared to WT mice and APC L mice exhibited further losses in body weight of ~12% compared to APC NL mice ($p<0.05$; Figure 1A). There was a main effect of the APC genotype to reduce tibialis anterior (M = ~34%; F = ~13%), gastrocnemius (M = ~40%; F = ~32%), plantaris (M = ~16%; F = ~27%), and soleus weight (M = ~12%; F = ~12%) ($p<0.05$; Figure 1B-E). There was a main effect of the APC genotype to increase spleen weight ~550% in male mice and ~400% in female mice ($p<0.05$; Figure 1F). There was a main effect of genotype to reduce fat weight ~91% in male mice and ~98% in females, although there was no detectable fat in the APC L females ($p<0.05$; Figure 1G). Male APC L appeared to develop severe cachexia to the extent where all male APC L mice became moribund and did not survive to the 20-week end point of this study (Figure 2A). This effect was not observed in female APC L mice and male APC L mice exhibited significant losses in body weight of ~11% by 14 weeks of age, also not observed in female mice, and this significant loss in body weight was never recovered prior to euthanasia ($p<0.05$; Figure 2A & 2B). Survival curves in Figure 2C and 2D show drops in survival rate by 10% by 14 weeks, 40% by 19 weeks, and 100% by 20 weeks in male APC L mice, whereas female APC NL were the only group to lose subjects prior to the 20-week euthanasia. We also note that water and food intake did not differ across either sex or treatment group.

**Sexual Dimorphism of Inflammation**

mRNA abundance of the classical markers of cancer cachexia associated inflammation were analyzed *in vivo* and *in vitro*. IL-6 mRNA abundance was ~3.5-fold greater in both APC groups compared to WT in male mice, and IL-6 mRNA was ~2-fold greater in APC L compared to APC NL ($p<0.05$; Figure 3A). In female mice there was a main effect of APC to have ~4-fold greater IL-6 mRNA compared to WT mice ($p<0.05$; Figure 3E). There were no differences in TNF-α and NFκB mRNA in male mice ($p>0.05$; Figure 3B & 3C), but there was a main effect of APC to have ~1.5-fold and ~0.5-fold greater mRNA abundance of TNF-α and NFκB in female mice compared to WT, respectively ($p<0.05$; Figure 3F & 3G). IL-1β mRNA was elevated ~3-fold and ~7-fold in both male and female APC mice compared to WT ($p<0.05$; Figure 3D & 3H). There was a main effect of the APC genotype to increase IL-6 mRNA abundance ~1 and ~3-fold in male and female plasma-treated myotubes compared to WT ($p<0.05$: Figure 3I & 3M). TFN-α mRNA was increased ~1-fold in WT L compared to WT NL treated myotubes from male plasma, but there was a ~1-fold reduction in TNF-α mRNA in APC L compared to APC NL treated myotubes ($p<0.05$; Figure 3J). In female plasma treated myotubes there was a main effect of APC genotype to increase TNF-α mRNA abundance ~1-fold ($p<0.05$; Figure 3N). There were no differences in NFκB mRNA in male plasma treated myotubes ($p>0.05$; Figure 3K), however NFκB mRNA was elevated ~1-fold in APC plasma treated myotubes ($p<0.05$; Figure 3O). There was a main effect of APC to increase IL-1β mRNA ~1 and ~0.5-fold in male and female plasma treated myotubes, respectively ($p<0.05$; Figure 3L & 3P).

**Markers of Atrophy, Proteolysis and Protein Anabolism Inhibition**

There was a main effect of APC mice to have increased FOXO1 and FOXO3 mRNA abundance in male and female mice ~2-fold and ~3-fold, respectively ($p<0.05$; Figure 4A, B, E

& F). There were no differences in Deptor mRNA in either male or female mice ($p>0.05$; Figure 4C, D, G & H), but there was a main effect of APC mice to have ~2-fold higher UBC mRNA abundance compared to WT ($p<0.05$; Figure 4D & H). FOXO1 mRNA was ~1-fold greater in APC female plasma treated myotubes compared to WT ($p<0.05$; Figure 4M) and was not different in male plasma treated myotubes ($p>0.05$; Figure 4I). There were main effects of FOXO3 to be altered in male and female APC plasma treated myotubes, but alterations differed in male APC myotubes to have ~0.5-fold less FOXO3 mRNA ($p<0.05$; Figure 4J) and female APC myotubes to have ~2-fold greater mRNA abundance when both were compared to WT ($p<0.05$; Figure 4N). No alterations were detected in Deptor mRNA either male or female plasma treated myotubes ($p>0.05$; Figure 4K & 4O). UBC mRNA was reduced ~0.3-fold in male APC plasma treated myotubes ($p<0.05$; Figure 4L) but no differences in female plasma treated myotubes were detected ($p<0.05$; Figure 4P).

**Muscle Myogenesis**

No differences in Pax7 mRNA abundance were detected in either male or female mice ($p>0.05$; Figure 5A & E). MyoD was increased ~2-fold in female APC mice compared to WT ($p<0.05$; Figure 5F), but no differences were observed in male mice ($p>0.05$; Figure 5B). There was a main effect for Myogenin mRNA to be increased in male and female APC mice ~1 and ~2-fold, respectively ($p<0.05$; Figure 5C &G). IGF-1 mRNA did not change across WT and APC groups in either sex ($p>0.05$; Figure 5D & 5H).

There were main effects for genotype to decrease Pax7 mRNA and for leucine to increase Pax7 in male plasma treated myotubes ($p<0.05$; Figure 5I). Pax7 mRNA was elevated ~0.5-fold in WT L compared to WT NL plasma treated myotubes, but APC L myotubes exhibited ~0.5-fold less mRNA compared to APC NL myotubes ($p<0.05$; Figure 5M). MyoD mRNA abundance

did not differ in any groups for male and female plasma treated myotubes ($p>0.05$; Figure 5J & 5N). There was a main effect for leucine to increase IGF-1 mRNA abundance ~0.3-fold in male plasma treated myotubes ($p<0.05$; Figure 5L). IGF-1 mRNA was increased in WT L treated myotubes ~0.3-fold, but APC L treated myotubes saw a ~0.3-fold decrease in IGF-1 mRNA abundance ($p<0.05$; Figure 5P).

**Protein Anabolism**

Male mice had no changes in phospho:total AKT protein content ($p>0.05$; Figure 6A), but female mice saw ~0.5-fold more phospho:total AKT in WT L compared to WT NL and ~0.3-fold less phospho:total AKT in APC L compared to APC NL mice ($p<0.05$; Figure 6D). There was a main effect of leucine supplementation to increase phoshpo:total p70 protein content in both male and female WT and APC mice ~2-fold ($p<0.05$; Figure 6B & 6E). There was a main effect of the APC genotype and leucine supplementation to increase phospho:total 4E-BP1 protein content in male mice ~2-fold ($p<0.05$; Figure 6C) and a main effect of leucine to increase phospho:total 4E-BP1 protein content ~2-fold in female mice ($p<0.05$; Figure 6F). There was a main effect of blood plasma from leucine supplemented male mice to have ~0.5-fold phospho:total AKT protein in myotubes ($p<0.05$; Figure 6G), however plasma from leucine supplemented female mice resulted in a ~0.5-fold increase in phospho:total AKT protein ($p<0.05$; Figure 6J). No differences in phospho:total p70 and 4E-BP1 protein were detected in male plasma ($p>0.05$; Figure 6H & I). Phospho:total p70 protein was ~0.3-fold lower in WT L plasma treated myotubes compared to WT NL, but APC L plasma treated myotubes saw ~0.4-fold increase phospho:total p70 protein compared to APC N ($p<0.05$; Figure 6K). There was a main effect for female APC plasma to increase phospho:total 4E-BP1 content compared to WT ($p<0.05$; Figure 6L).

**Deeper Dive into Protein Breakdown**

There was a main effect for APC mice to have ~7-fold greater Atrogin-1 mRNA abundance in male mice and ~3-fold greater Atrogin-1 mRNA in female mice compared to WT ($p<0.05$; Figure 7A & D). MuRF-1 mRNA was elevated ~5-fold in male and female APC mice compared to WT ($p<0.05$; Figure 7B & E). There were no changes in ubiquitin protein content in either male or female mice ($p>0.05$; Figure 7C & F). Atrogin-1 mRNA abundance was decreased ~0.4-fold in APC compared to WT mice in male plasma treated myotubes ($p<0.05$; Figure 7G). There was a main effect of the APC genotype and Leucine supplementation to increase Atrogin-1 mRNA in ~2-fold in APC plasma treated myotubes compared to WT treated ($p<0.05$; Figure 7J). MuRF-1 mRNA was increased ~0.3-fold in WT and APC male plasma treated myotubes due a main effect of leucine, and APC had a main effect of decreasing MuRF-1 mRNA ~0.3-fold compared to WT ($p<0.05$; Figure 7H). There was a main effect of APC to elevate MuRF-1 mRNA abundance ~2.2-fold in female plasma treated myotubes ($p<0.05$; Figure 7K). Ubiquitin protein content was decreased ~0.7 and ~0.5-fold in WT NL male plasma treated myotubes compared to WT L and both APC groups ($p<0.05$; Figure 7I). No differences in ubiquitin protein content were detected between any groups in female plasma treated myotubes ($p>0.05$; Figure 7L).

**Skeletal Muscle CSA and Myotube Diameter**

There was a main effect of the APC genotype to reduce CSA of skeletal muscle in both male and female mice ~27% and ~30%, respectively ($p<0.05$; Figure 8A-D). These decrease in muscle CSA were the same regardless of leucine supplementation or being identified as SHD (+) or SDH (-) fibers ($p>0.05$; Figure 8A-D). Myotube diameter was decreased following addition of male APC plasma ~13% when compared to WT plasma treated myotubes ($p<0.05$; Figure 8E).

No changes in myotube diameter following addition of female plasma were detected across any groups ($p>0.05$; Figure 8F).

**Discussion**

Cancer cachexia is a thoroughly researched model of muscle wasting that is notably characterized by chronically elevated and systemic inflammation (29-31). Nutritional interventions have also been thoroughly researched in treating cancer cachexia with little to no success (8-10). Yet there remains little research of nutritional interventions on cancer cachexia across both sexes, and therefore, in this study we aimed to investigate the effects of leucine supplementation on cancer cachexia and how sex can influence the outcome. The simplest and yet major finding observed in this study was the effect of leucine supplementation to reduce body weight in male APC mice ~12% compared to APC NL mice. This effect of leucine supplementation to decrease overall body weight was not observed in female mice, even though both sexes displayed similar decreases in muscle, tissue and organ weights due to cancer cachexia. In both male and female APC mice, skeletal muscle CSA was reduced regardless of oxidative phenotype (+ or -) and leucine supplementation had no added effect. Of note, myotube diameter decreased significantly following addition of either male APC NL or APC L blood plasma whereas female plasma created no differences in myotube diameter across group. To our knowledge, this study is the first to report this effect of leucine to potentially exacerbate cancer cachexia but in a sex-dependent fashion *in vivo*.

Elevated levels of IL-6 are well documented in APC mice (32-35) and our findings further support this observation, especially in male APC mice where their decreased body weight following leucine supplementation corresponded with an increase IL-6 mRNA abundance. While female mice displayed no increases in IL-6 mRNA following leucine supplementation, female APC mice did have higher TNF-α and NFκB mRNA abundance compared to WT which was not

observed in males. These variations in inflammatory markers suggests potential differences in downstream effectors and pathways influencing cachectic wasting between sexes. It is noteworthy to mention that the rise in TNF-α and NFκB mRNA translated to female plasma treated myotubes as well, although male plasma treated myotubes interestingly saw increases and decreases in TNFα due to leucine in WT and APC plasma, respectively. TNF-α and NFκB have previously been linked to the E3 ligases Atrogin-1 and MuRF-1 in models of colorectal cancer (36-38) and following these observations we evaluated the E3 ligases and total ubiquitin protein content. However, we saw no clear trends pinpointing the observed differences in IL-6, TNF-α, and NFκB to impact rates of proteolysis and support the dimorphic impact of leucine on body weight between sexes. This contrasts previous findings by Baltgavis et al. and White et al. in 2009 and 2011 (39, 40), where elevations in IL-6 were tied to increased Atrogin-1 and ubiquitination of proteins in APC mice. So, while leucine supplementation appeared to impact cancer cachexia negatively and selectively in males, the exact cellular process responsible beyond elevated IL-6 remains unclear.

FOXO1 and FOXO3 are the key effectors involved in ubiquitination of proteins targeted for degradation by the ubiquitin proteasome system (UPS) downstream of E3 ligase induction, both of which have been shown to be upregulated in cancer cachexia (41, 42). Here we report APC mice having increased FOXO1, FOXO3, and UBC mRNA regardless of sex and all of these increases translated *in vitro* except for FOXO1 in male plasma and UBC in female plasma. While not completely consistent *in vivo* and *in vitro* these findings support increased rates of proteolysis during cancer cachexia in both sexes, with leucine having no added effect. In female APC mice there was an interesting finding for ~2-fold increased MyoD mRNA abundance not observed in males. While both sexes had increases in Myogenin mRNA in APC mice, the

elevation of both MyoD and Myogenin could indicate enhanced satellite cell activation and differentiation to upregulate myogenesis and combat cachexia associated muscle wasting. That MyoD was not elevated in male APC mice similar to females could be of importance as well in this regard. Elevation of Myogenin translated to female APC plasma treated myotubes, and interestingly Pax7 mRNA was decreased in female APC L treated myotubes compared to APC NL. Taking that data in conjunction with male APC L treated myotubes having a ~0.3-fold reduction in Myogenin compared to APC NL supports the possibility for female-specific muscle protection in cancer cachexia via a shift in myogenesis to strongly favor activation and differentiation of satellite cells. A recent study by Lim et al., 2021 (43) also reported reductions in Pax7 and MyoD in a Lewis Lung Carcinoma (LLC) model of cancer cachexia. The conservation of this observation in myogenic regulatory factors suggests a potentially large role for myogenesis in protection of skeletal muscle in females affected by cancer cachexia.

Leucine supplementation increased protein content of mTOR's downstream effectors p70s6K and 4E-BP1 in mice, regardless of genotype or sex. We observed no changes in p70s6k or 4E-BP1 protein in male plasma treated myotubes and increases in 4E-BP1 in both female APC plasma treated myotube groups. Overall, the anticipated effects of leucine supplementation on markers of protein anabolism translated to both sexes *in vivo* but not *in vitro*. IGF-1 mRNA was unchanged *in vivo* but leucine supplementation increased IGF-1 in male WT and APC plasma treated myotubes, whereas female APC L plasma treated myotubes saw decreased IGF-1 mRNA. The lack of consistency *in vivo* to *in vitro* points to the added complexity of comparing males and females, as we note vast differences in protein anabolism likely contributable to sex specific circulating factors in these mice.

In conclusion, we aimed to investigate the effects of leucine supplementation on cancer cachexia in both sexes. The divergence in markers of inflammation, protein anabolism, and myogenesis between males and females during cachexia reported here highlights the need for inclusion of both sexes in future studies, as interventions in pathologies can have sex-based effects on patient outcomes. This need for inclusion of both sexes is even enhanced when considering leucine had such a potent effect to exacerbate cancer cachexia in males, as noted by increased losses in body weight and increased mortality. While leucine supplementation exacerbated cancer cachexia in males, the exact mechanisms involved downstream of IL-6, TNF-α and NFκB remain unclear. Further research is required but potential therapies that target these inflammatory pathways may prove beneficial to those suffering from cancer cachexia more so than those targeting protein anabolism.

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**Disclosures**

No conflict of Interest, financial or otherwise, are declared by the authors.

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## Figure Legends

### Figure 1. Descriptive Measurements

Body (A), muscle (B-E), and tissue weights (F-G) of male and female WT and APC mice. All values are represented as means ± SEM. Main effects of genotype and leucine are labeled as such, and different letters signify differences in means between groups following an interaction with significance set at $p<0.05$.

### Figure 2. Body Weights and Survival Rate

Body weight (A-B) and percent survival (C-D) of male and female WT and APC mice. Body weights are represented as means ± SEM and percent survival as relative percentage. Single asterisks signify significance from WT only and double asterisks signify significance compared to APC NL with $p<0.05$.

### Figure 3. Inflammation

mRNA analysis of inflammatory markers in male and female mice as well as plasma treated myotubes (A-P). Values are represented as means ± SEM. Main effects of genotype and leucine are labeled as such, and asterisks denote differences between means following an interaction with alpha set at $p<0.05$.

### Figure 4. Protein Degradation

mRNA analysis of protein breakdown in male and female mice as well as plasma treated myotubes (A-P). Values are represented as means ± SEM. Main effects of genotype and leucine are labeled as such, and asterisks denote differences between means following an interaction with alpha set at $p<0.05$.

### Figure 5. Myogenesis

mRNA analysis of markers of myogenesis in male and female mice as well as plasma treated myotubes (A-P). Values are represented as means ± SEM. Main effects of genotype and leucine are labeled as such, and asterisks denote differences between means following an interaction with alpha set at $p<0.05$.

### Figure 6. Protein Anabolism

Protein content of markers associated with protein anabolism in male and female mice as well as plasma treated myotubes (A-P). Values are represented as means ± SEM. Main effects of genotype and leucine are labeled as such, and asterisks denote differences between means following an interaction with alpha set at $p<0.05$.

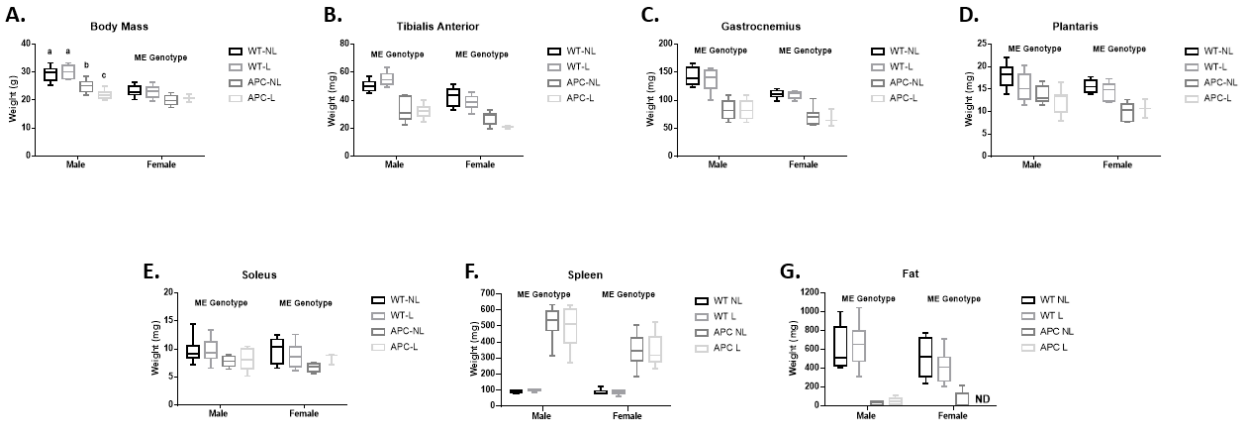
**Figure 7. Deeper Dive into Protein Degradation**

mRNA abundance of markers of protein breakdown in male (A-B) and female mice (D-E) as well as male (G-H) and female (J-K) plasma treated myotubes. Protein content of ubiquitin in male and female mice (C & F) and male and female plasma treated myotubes (I & L). Values are represented as means ± SEM. Main effects of genotype and leucine are labeled as such, and asterisks denote differences between means following an interaction with alpha set at $p<0.05$.

**Figure 8. Microscopy *in vivo* and *in vitro***

Cross-sectional area of TA muscle in male and female mice (A-D). Myotube diameter following addition of blood plasma from male and female mice (E-F). Values are represented as means ± SEM. Main effects of genotype and leucine are labeled as such, and asterisks denote differences between means following an interaction with alpha set at $p<0.05$.

## Figures

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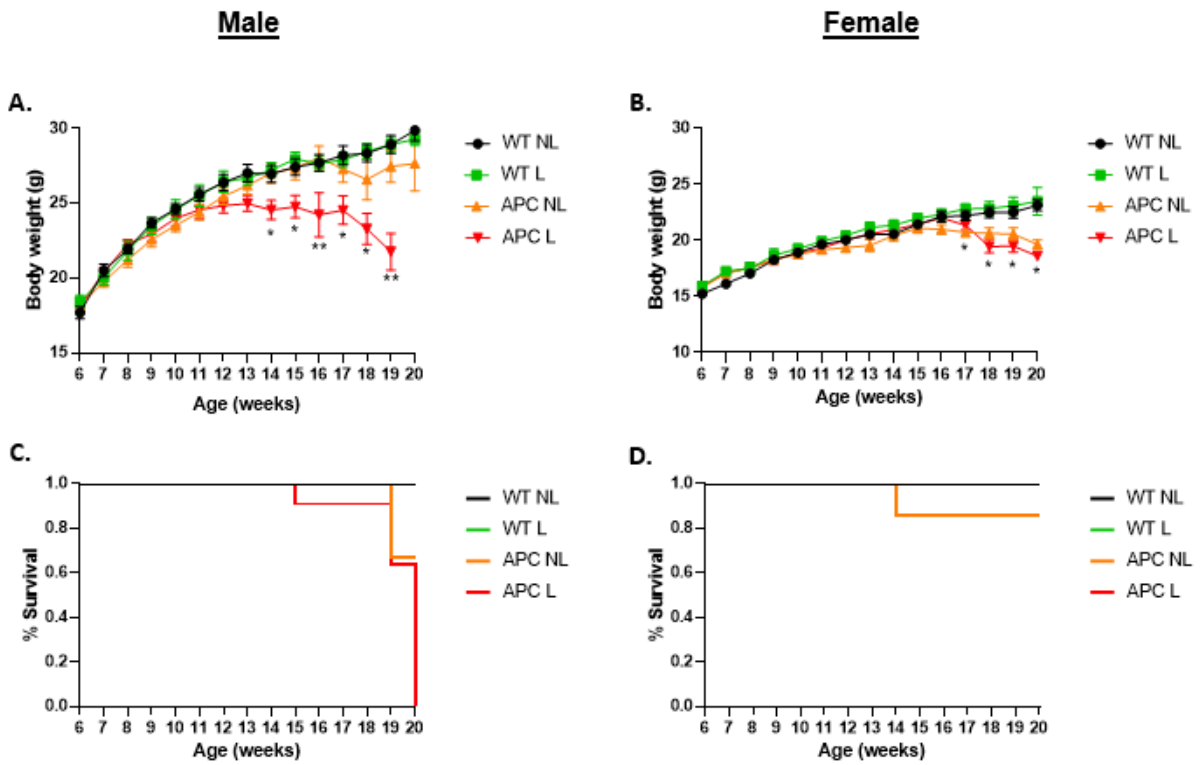


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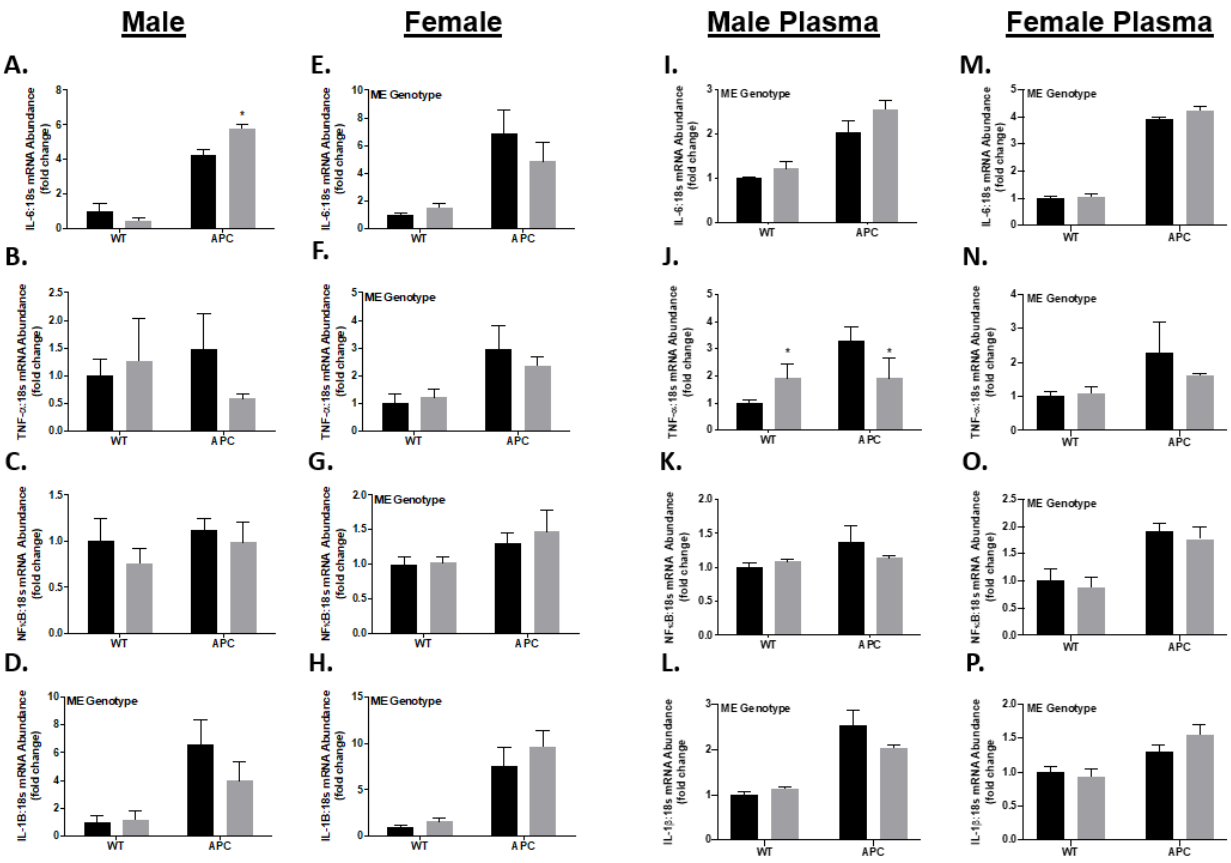


**Figure 3.** mRNA analysis of inflammatory markers in male and female mice as well as plasma treated myotubes (A-P). Values are represented as means ± SEM. Main effects of genotype and leucine are labeled as such, and asterisks denote differences between means following an interaction with alpha set at $p<0.05$.

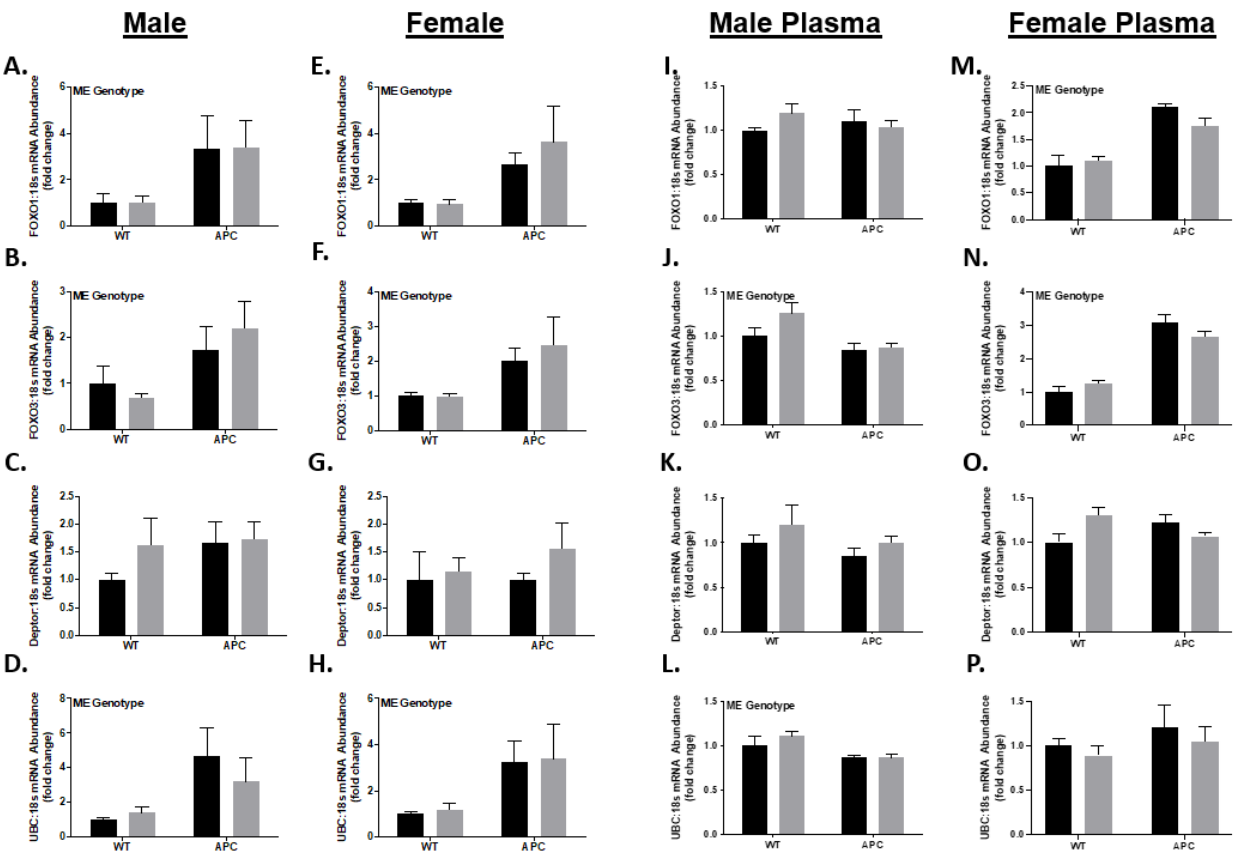


**Figure 4.** mRNA analysis of protein breakdown in male and female mice as well as plasma treated myotubes (A-P). Values are represented as means ± SEM. Main effects of genotype and leucine are labeled as such, and asterisks denote differences between means following an interaction with alpha set at $p<0.05$.

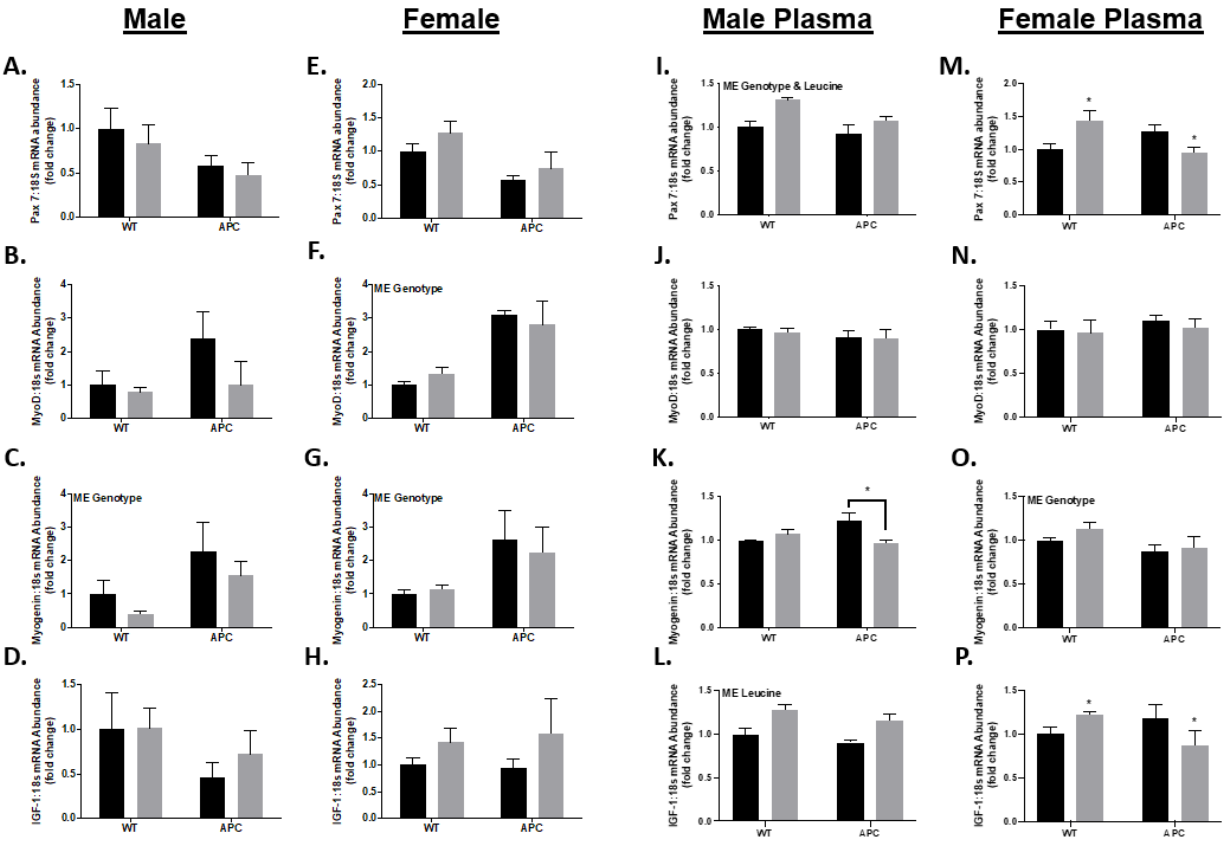


**Figure 5.** mRNA analysis of markers of myogenesis in male and female mice as well as plasma treated myotubes (A-P). Values are represented as means ± SEM. Main effects of genotype and leucine are labeled as such, and asterisks denote differences between means following an interaction with alpha set at $p<0.05$.

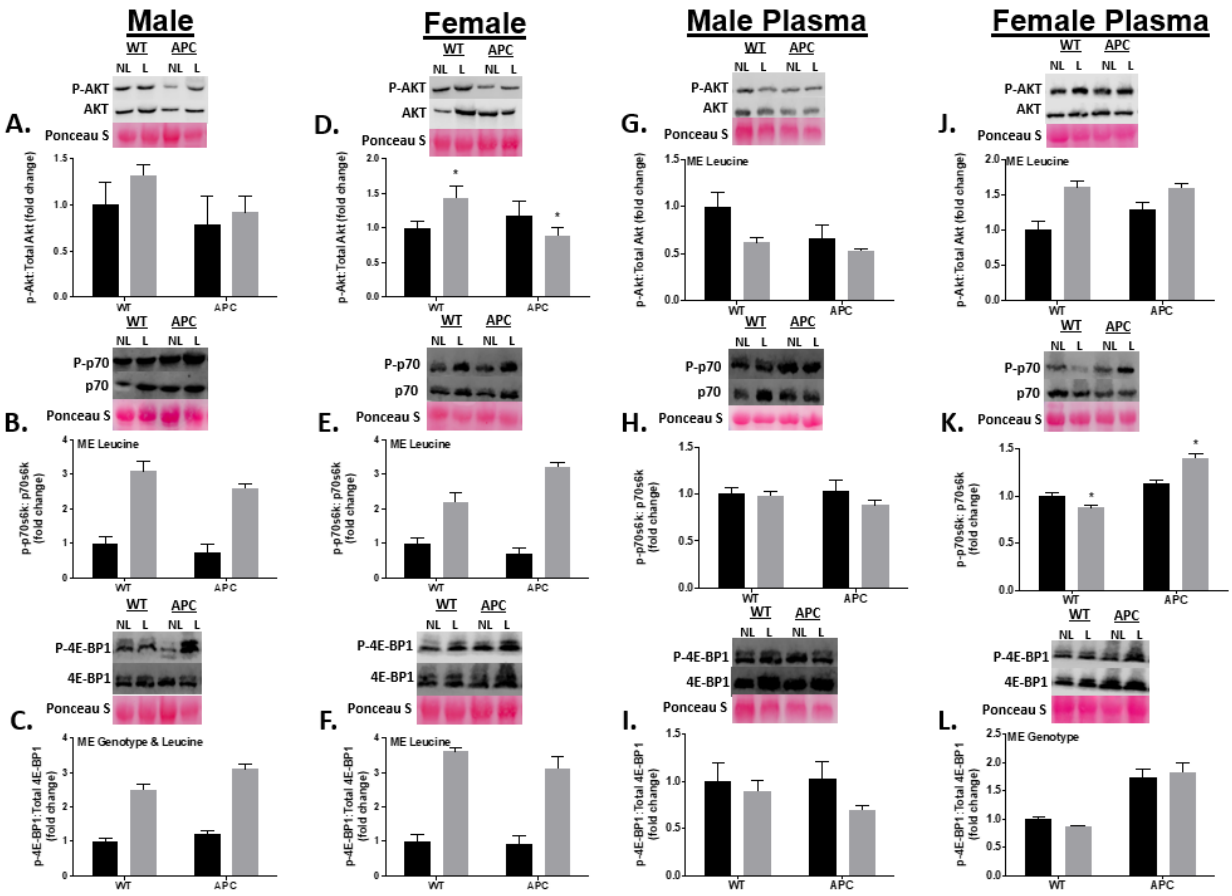


**Figure 6.** Protein content of markers associated with protein anabolism in male and female mice as well as plasma treated myotubes (A-P). Values are represented as means ± SEM. Main effects of genotype and leucine are labeled as such, and asterisks denote differences between means following an interaction with alpha set at $p<0.05$.

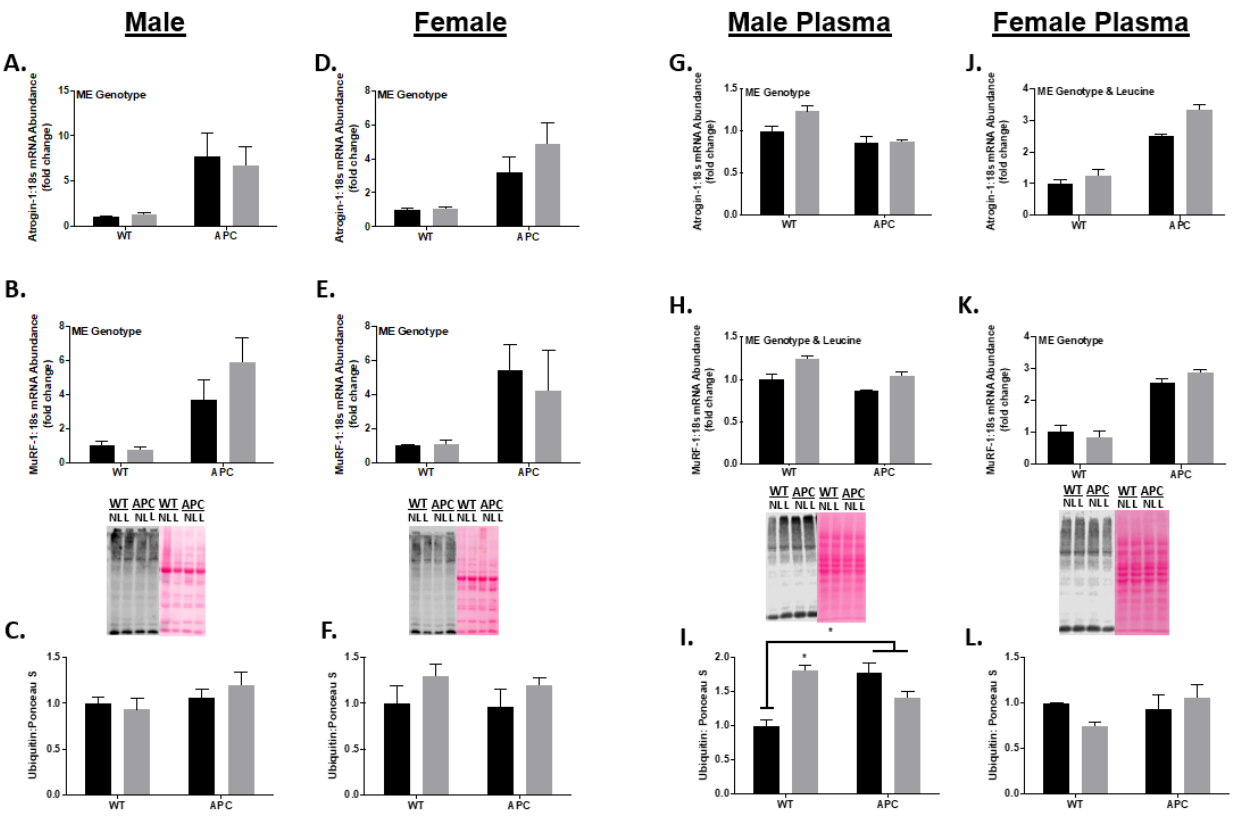


**Figure 7.** mRNA abundance of markers of protein breakdown in male (A-B) and female mice (D-E) as well as male (G-H) and female (J-K) plasma treated myotubes. Protein content of ubiquitin in male and female mice (C & F) and male and female plasma treated myotubes (I & L). Values are represented as means ± SEM. Main effects of genotype and leucine are labeled as such, and asterisks denote differences between means following an interaction with alpha set at $p<0.05$.

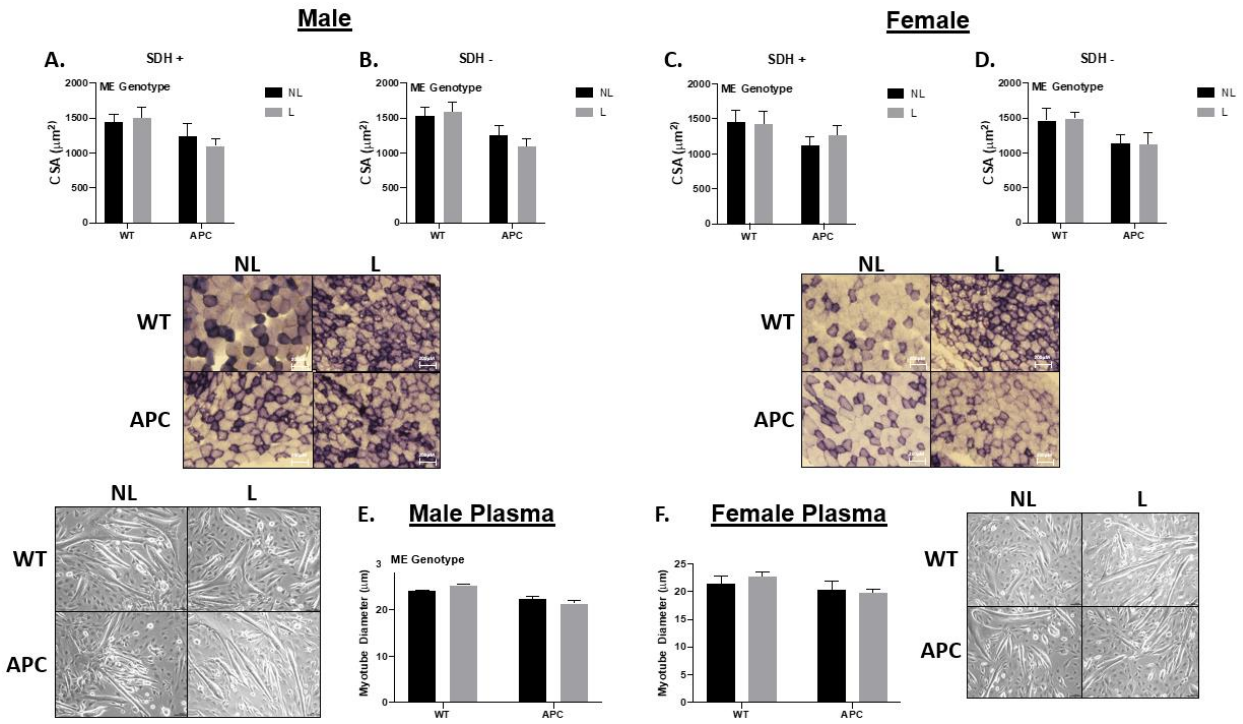


**Figure 8.** Cross-sectional area of TA muscle in male and female mice (A-D). Myotube diameter following addition of blood plasma from male and female mice (E-F). Values are represented as means ± SEM. Main effects of genotype and leucine are labeled as such, and asterisks denote differences between means following an interaction with alpha set at $p<0.05$. alpha set at $p<0.05$.

# Chapter 4

## Sexual Differences of Fibrosis During the Development of Cancer Cachexia

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**Abstract**

Cancer cachexia is characterized by losses in lean body mass >5% and its progression results in worsened quality of life and even outcomes in cancer patients. The chronic inflammation and suppression of protein synthetic pathways have been researched extensively. We reported mitochondrial degeneration preceded cancer cachexia development in male mice, however no research to date has investigated the role and impact of fibrosis during the early stages and development of cancer cachexia. The purpose of this study was to determine if fibrosis occurs during cancer cachexia development, and to evaluate this in both sexes. 60 female and 82 male C57BL/6J mice were injected with PBS or Lewis Lung Carcinoma at 8-week age, and tumors developed for 1, 2, 3, or 4 weeks to assess the time course of cancer cachexia development. 3wk and 4wk female tumor-bearing mice displayed a dichotomy in tumor growth and were reassigned to high Tumor (HT) and low Tumor (LT) groups. i*n vivo* analyses were also performed on co-cultured C2C12 and 3T3 cells for skeletal muscle-ECM interactions in a cachectic environment *in vtiro*. 4wk male and HT female mice displayed reduced tibialis anterior and fat weight compared to PBS. Collagen deposition in skeletal muscle was increased at 1wk, LT and HT in female and 4wk in male mice. MMP-9 was increased in HT and 4wk mice and MMP-9 inhibitor, TIMP-1, was only decreased in female mice at 1wk, LT and HT. TGF-β was elevated at 1wk and 4wk for female and male mice, respectively. 3T3 fibroblasts expressed ~350-500 more MMP-9 mRNA than C2C12 myotubes *in vitro,* and LCM treatment reduce the relative amounts of Collagen 3 to 1 mRNA. These data present novel insights into fibrosis during cancer cachexia development across both sexes.

## Introduction

Cancer cachexia is a progressive and involuntary wasting condition of lean body mass that afflicts approximately 80% of all cancer patients and can be attributed to 20% of cancer-associated deaths (1, 2). Cancer cachexia is defined as losses in body mass >5% and this can be accompanied with or without losses in adipose tissue (3, 4). To date, no singular method of treatment has shown effective at eliminating cancer cachexia development due to the systemic and complex nature of its underlying mechanisms (5-7). Further complicating research into effective treatments for cancer cachexia is the emerging role of sexual dimorphism on cancer cachexia progression, as recent studies have shown that males and females can differentially respond to cancer cachexia (8-10). Metabolic and inflammatory instabilities are known contributors to cancer cachexia progression and have been greatly researched (11, 12), but little to none is known of the early role of fibrosis potentially plays on the development of cancer cachexia.

Excessive fibrosis of healthy skeletal muscle has been shown to occur and worsen overall outcomes in cancer patients (13). More so, unlike metabolic and inflammatory abnormalities that can improve following cancer cessation, this fibrosis of vital and functional lean tissue is irreversible and can affect patients throughout their lifespan (14). Transforming growth factor-β (TGF-β) is a massive transcriptional activator of many cellular processes including the activation of satellite cells as well as degradation and rearrangement of the extracellular matrix (ECM) that surrounds skeletal muscle (15, 16). However, upregulation of TGF-β can result in excessive ECM dysregulation and deposition of ECM components ultimately resulting in the replacement of healthy skeletal muscle with non-contractile proteins such as collagens (17, 18). This upregulation of TGF-β can be induced via its upstream regulator in Interleukin (IL)-6, an

inflammatory marker which is commonly overexpressed in cachectic cancer patients (19). Taken together, these data suggest that fibrosis in cancer patients is altered via TGF-β, and evaluation of TGF-β and its downstream effectors could provide insights on fibrosis as it pertains to cancer cachexia progression.

Males and females exhibit sexual dimorphism in skeletal muscle properties such as fiber type composition within the same muscle, mitochondrial content, and number of satellite cells innately, and this dimorphism is even documented in some muscle pathologies such as disuse atrophy (10, 20). Differences in the relative amounts of circulating hormones like testosterone and estrogen only adds to the complexity of the male and female phenotypic makeup within skeletal muscle. For example, testosterone is typically higher in males and promotes muscle protein synthesis and regeneration, while estrogen is typically higher in females and has a greater impact on reducing inflammation (21, 22). Therefore, female mice could possess a greater protective effect from inflammatory-based muscle wasting conditions such as cancer cachexia when compared to males (10). Innate differences between sexes can therefore greatly affect overall skeletal muscle health and even skeletal muscle health in pathologies such as cancer cachexia (8, 10).

This study is the first to evaluate the role of fibrosis during the development of cancer cachexia across both sexes. Implantation of Lewis Lung Carcinoma (LLC) cells allows for the controlled development of cancer cachexia, and therefore a mechanistic analysis of fibrotic pathways during this initial stage of cachexia. Evaluation of these fibrotic pathways could elucidate potential avenues for therapeutic interventions aimed towards the prevention of fibrosis and its contributions to cancer cachexia development. Therefore, the purpose of this study was to utilize the implantation of LLC cells to analyze key regulators of the ECM in a controlled

development of cancer cachexia. We hypothesized that the ECM and signaling pathways involved in its modulation would be altered prior to cachexia. Herein this study we provide novel evidence of altered fibrotic signaling coinciding with cancer cachexia development, however this varied between sexes. These data highlight the emerging role of fibrosis on cancer cachexia even in the earliest stages, and further strengthens the growing need for research in both males and females as sex continues to create diverging responses to cancer cachexia and its underlying mechanisms.

## Methods

### Animals and Interventions

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Arkansas, Fayetteville. Male and female C57BL/6J mice were purchased from Jackson Laboratories. The mice were kept on a 12:12 h light–dark cycle with *ad libitum* access to normal rodent chow and water. LLC cells ($1 \times 10^6$) suspended in 100 μL sterile PBS were implanted to the hind flank of mice at 8 weeks of age as previously described (23). The tumor was then allowed to develop for 1, 2, 3, or 4 weeks in separate cohorts. For sham control, one group of mice received a bolus injection of 100 μL sterile PBS. PBS controls were age-matched to the most cachectic group (4 weeks post-implantation, 12 weeks of age at tissue collection). In the female mice, there was a clear dichotomization between the 3- and 4-weeks groups to cluster into smaller or larger tumor sizes. These 2 groups were then isolated and split into low tumor (LT; ≤1.2g) and high tumor (HT; ≥2g) groups based on this dichotomization of tumor size as denoted in Table 1 below. Animal tissues, organs, and blood plasma were quickly collected under isoflurane anesthesia prior to euthanasia. Tissues were weighed and snap-frozen in liquid nitrogen for further processing and stored at −80°C. The

tibialis anterior muscle from males (TA) and plantaris muscle from females were submerged in optimum cutting temperature compound (OCT) and then placed in liquid nitrogen cooled isopentane. OCT mounted tissue was then stored at -80°C for histological analysis.

**Lewis Lung Carcinoma and Implantation**

LLC cells were prepared as described previously (23). LLC cells (ATCC CRL-1642) were plated at passage 2. Cells were cultured in 250 ml culture flasks in DMEM supplemented with 10% fetal bovine serum supplemented with 1% penicillin and streptomycin. Once cells reached confluence, they were counted, trypsinized and diluted in PBS for implantation. Mice were anesthetized with isoflurane and hair was removed from the right hind flank. LLC cells ($1x10^6$) suspended in 100 µl sterile PBS and injected subcutaneously into the hind flank of mice at 8 weeks of age as previously described (23). Tumors developed for up to 4 weeks. Experimental endpoints were adjusted for signs of distress and veterinary recommendation for humane care.

**Myoblast and Fibroblast Co-Culture**

C2C12 myoblast cells (ATCC CRL-1772) and 3T3 fibroblast cells (ATCC CRL-1658) were co-cultured for an *in vitro* analysis of skeletal muscle to ECM interaction. C2C12 cells were grown until they reached ~80% confluency at passage 2, where they were then transferred to a 6-well plate and differentiated for 5 days. 3T3 fibroblast cells were then added via a transwell-insert, which possesses a semi-permeable membrane, into the 6-well plate for creation of a skeletal muscle to ECM connection *in vitro*. LLC conditioned media (LCM) or control media were added to both cell types for 24hr, where they were then harvested for subsequent RT-qPCR and Western Blot analysis.

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

RNA isolation, cDNA synthesis, and quantitative real-time PCR were performed as we have previously described (24-26). All targets were assayed using Taqman probes including: 18S (Mm03928990_g1), Collagen 1 (Mm00801666_g1), Collagen 3 (Mm00802305_g1), MMP-2 (Mm00439498_m1), MMP-9 (Mm00439498_m1), Timp-1 (Mm01341361_m1), TGF-β1 (Mm01178820_m1), SMAD 2 (Mm00487530_m1), SMAD 3 (Mm01170760_m1). Taqman probes were purchased from Applied Biosystems. RT-qPCR measured cycle threshold (Ct) and the ΔCt value was calculated as the difference between the Ct value and the 18S Ct value. Final quantification of mRNA abundance was calculated using the ΔΔCT method Ct = [ΔCt(calibrator) – ΔCt(sample)]. Relative quantifications were then calculated as $2^{-}$ΔΔCt. 18S Ct values were confirmed to not differ between experimental conditions for any comparison.

**Immunoblotting**

Immunoblotting was performed as we have previously described (27, 28). Protein concentration and equal loading of SDS-page gels was determined via RC DC assay™ (Bio-Rad), and target protein content was normalized to Ponceau S staining of 45kDa band. Membranes were probed overnight for primary antibodies. Protein targets were purchased from Cell Signaling Technology (CST) and selected based upon prior literature targeting key components of ECM and fibrotic signaling pathways (29-33): β-Catenin (D10A8), Phospho-SMAD 2 (Ser465/467)/SMAD 3 (SerSMAD423/425) (D27F4), SMAD 2/3 (D7G7). Antibodies were diluted in Tris-buffered saline, 0.1% Tween 20 with 5% milk. Membranes were imaged on LiCor Odyssey FC using IR detection and images analyzed by LiCor Image Studio™.

**Histology**

TA and plantaris skeletal muscle stored in OCT were cryosectioned into 10μm thick sections on polarized microscope slides. Muscle sections were then histologically stained with Picro Sirius Red for quantification and analysis of overall collagen content within the muscle. Cross-sections were also labeled with immunofluorescent antibodies for both Collagen 1 and Collagen 3 for quantification and analysis of their relative amounts. Images were analyzed using Nikon NIS Elements BR software package.

**Statistical Analysis**

Normality of data was established via a Shapiro Wilk test prior to any parametric analysis. A one-way ANOVA was employed as the global analysis for each dependent variable in both experiments. Where significance was detected, differences among means were determined by a Student–Newman–Keuls post hoc test. For all experiments, $\alpha$ error rate was set at 0.05 for all statistical tests. All data were analyzed using the Statistical Analysis System (SAS) and figures were compiled using GraphPad Prism and data expressed as mean ± standard error of the mean (SEM).

**Results**

**Confirmation of Cachexia Development.**

Measurements of body, skeletal muscle, organ and tissue weights are represented in Table 1. Neither male nor female mice saw significant reductions in tumor-free body weight compared to PBS controls ($p>0.05$), however both experienced reductions of tibialis anterior muscle weight of ~11% and 15% in the HT and 4wk groups, respectively ($p<0.05$). Only males saw a significant reduction in gastrocnemius weight of ~12% compared to PBS ($p<0.05$). Both females and males had increases of ~33% and ~28% in liver weight and splenomegaly of ~395%

and ~451% in the HT and 4wk groups compared to PBS, respectively ($p<0.05$). Fat weight also decreased ~37% and ~35% for the HT and 4wk groups compared to PBS, respectively ($p<0.05$). This data suggests cachectic losses in tibialis anterior muscle and fat mass, as well as cancer associated increases in liver and spleen mass compared to control mice.

**Excessive Deposition of Collagen in Skeletal Muscle**

Picro Sirius Red staining of collagen within the plantaris and tibialis anterior muscle was performed to determine overall fibrosis of both female and male mice skeletal muscle during the development of cancer cachexia. 1wk and LT and HT female mice saw ~1.5-2% increases ($p<0.05$) in the area of muscle that can be attributed as collagen compared to the PBS and 2wk groups (Figure 1A). Male mice saw no significant increases in the relative area of collagen within the muscle until 4 weeks post-injection with LLC where there was a ~3-fold increase ($p<0.05$) in the percentage of collagen in the muscle compared to all other groups (Figure 1B).

**ECM Components and Modifiers are Altered during Cancer Cachexia Development**

1wk female mice displayed significant increases in Collagen 3 and 1 mRNA abundance of ~0.7 and ~1.8-fold compared to all other groups, respectively ($p<0.05$; Figure 2A & 2B), however the relative ratio of Collagen 3:1 mRNA abundance was decreased ~0.5-fold in 1wk female mice compared to PBS ($p<0.05$; Figure 2C). Male mice saw no differences in Collagen 3 and 1 mRNA abundance ($p>0.05$ Figure 2D-F). MMP-2 mRNA abundance increased ~1-fold in female mice at 1wk compared to the LT and HT groups ($p<0.05$; Figure 2G), and MMP-9 mRNA abundance increased ~2.5-fold in the HT group compared to PBS ($p<0.05$; Figure 2H). TIMP-1, which binds and inhibits MMP-9 in its latent state, exhibited decreased mRNA abundance ~0.7-fold in the 1wk, LT and HT groups compared to PBS in female mice ($p<0.05$; Figure 2I). Male mice saw no changes in MM-2 and TIMP-1 mRNA abundance ($p>0.05$; 2J &

2L) but saw a similar increase of ~2-fold in mRNA abundance of MMP-9 in the most cachectic 4wk group when compared to PBS ($p<0.05$; Figure 2K).

**TGF-β-regulated Fibrosis during Cancer Cachexia Development**

TGF-β1 mRNA abundance was increased ~1.2-fold at 1wk in female mice compared to PBS ($p<0.05$; Figure 3A), and while there was a trend for SMAD 2 and 3 to exhibit similar increased mRNA abundance there was ultimately no significance ($p>0.05$; Figure 3B & 3C). There were no alterations in protein content for β-catenin or total SMAD 2/3 in female mice ($p>0.05$; Figure 3D & 3E), and protein content for phosphorylate levels of SMAD 2/3 were not detectable (ND). TGF-β1 mRNA abundance increased ~0.5-fold in 4wk male mice compared to PBS ($p<0.05$; Figure 3F), but there were no differences or trends detected in mRNA abundance of SMAD 2 and 3 ($p>0.05$; Figure 3G & 3H). Male mice also saw no differences in protein content of β-catenin and SMAD 2 and 3 ($p>0.05$; Figure 3I & 3J) and phosphorylated SMAD 2 and 3 were ND similar to female mice.

**Major Collagens within Skeletal Muscle Fluctuate during Cancer Cachexia Development**

The relative ratios of collagens 3 and 1 were measured via immunofluorescent labeling to observe variations of the major proteins that constitute the ECM during cancer cachexia development. The amount of collagen 3 to collagen 1 present in the TA muscle of male mice decreased ~1-fold at 1wk and 3wk compared to PBS ($p<0.05$; Figure 4A).

***in vitro* Analysis of Skeletal Muscle-ECM Dynamics in a Cachectic Environment**

There was a main effect of C2C12 myotubes cells to have ~0.5-fold greater mRNA abundance of TGF-β1 compared to 3T3 fibroblasts ($p<0.05$; Figure 5A). There was a main effect of myotubes to have ~4-fold less Collagen 1 mRNA abundance, and a main effect of LCM treatment to reduce Collagen 1 mRNA abundance across both cell types ($p<0.05$; Figure 5B).

There were main effects for myotubes to have ~0.8-fold greater Collagen 3:1 ratio of mRNA abundance and for LCM treatment to increase this ratio as well ($p<0.05$; Figure 3C). There were no differences in mRNA abundance of MMP-2 in either myotubes or 3T3 cells ($p>0.05$; Figure 3D), however mRNA abundance of MMP-9 was ~450-fold greater in 3T3 cells than in myotubes and LCM treatment reduced MMP-9 mRNA ~200-fold in 3T3 cells compared to Vehicle ($p<0.05$; Figure 3E). There was a main effect for 3T3 cells to have decreases mRNA abundance of TIMP-1 by ~0.6-fold compared to C2C12 cells ($p<0.05$; Figure 3F). No differences were observed in C2C12 or 3T3 cells for protein content of phosphorylated-total SMAD 2/3 and β-catenin ($p>0.05$; Figure 3G & 3H). Myotube diameter was reduced ~4μM following treatment with LCM compared to Vehicle ($p<0.05$; Figure 3I).

**Discussion**

In this study, we are the first to evaluate and report variations in fibrosis and associated markers during the development of cancer cachexia in both sexes. Our analysis consisted of major ECM components such as collagens, MMPs and TIMPs involved with breakdown and remodeling of the ECM, and a key signaling pathway associated with fibrosis across pathologies in TGF-β and its downstream effectors. These key markers of fibrosis were analyzed *in vivo* and *in vitro* to create a comprehensive approach to understanding if the ECM is altered during the development of cancer cachexia, and if so, what are the likely contributors to the result. While no direct comparisons can be made between males and females, there appears to be differences in the timing of increased fibrosis of the skeletal muscle as cancer cachexia begins to develop. Female mice saw significant alterations in collagen content, collagen mRNA, TIMP-1 mRNA, and TGF-β mRNA as early as 1wk post-injection with LLC cells (Figures 1-3). Male mice on the other hand displayed no significance in fibrosis or fibrosis-related markers until 4wk almost

across the board, with the sole exception of Collagen 3:1 being decreased ~1-fold following immunofluorescent labeling (Figure 4). *in vitro* co-culturing of myotubes and fibroblast cells in a cachectic environment yielded interesting findings, albeit in the absence of sex distinctions, but with the added distinction of isolating muscle and ECM associated cell lines (Figure 5). Of note, both C2C12 and 3T3 cells had higher/lower relative mRNA abundance in certain collagen, MMPs, and TIMP-1 relative to one another. Addition of LCM did indeed reduce myotube diameter significantly as well as reduce Collagen 1 mRNA and increase Collagen 3:1 mRNA in both cell types. These findings support the growing theme across literature for divergent responses to myopathologies between male and female sexes as well as reinforce the need for these sex-based comparisons in future studies (8, 35, 36).

As reported by Lim et al. (34), the dichotomization of tumor burden observed in the female mice supports the correlation with overall tumor burden to development of cancer cachexia. However, with regards to incidence of fibrosis, we observed increased collagen content and alterations in ECM components and regulators as early as 1wk in female mice. This observation indicates that while tumor burden still is tightly correlated to cancer cachexia in both sexes, the early stages of fibrotic development as it pertains to cancer cachexia is not as tightly correlated to overall tumor burden. Male mice didn't observe the same early stages alterations outside of a decrease in Collagen 3:1 at 1wk in male (Figure 4). These data suggest that both males and females experience alterations in their ECM as early as 1wk post-injection with LLC cells, but not with complete consistency across the associated regulators in MMPs, TIMP-1, and TGF-β. Recent studies report reductions in TGF-β mRNA in cardiac muscle in a Colon adenocarcinoma cells 26 (CT26) model of cancer cachexia (37) and increased TGF-β mRNA in adipose and skeletal muscle tissue in models of pancreatic cancer cachexia (13, 38), but all

studies exhibit increased fibrosis. TGF-β then appears to be a clear and consistent regulator of fibrosis in cancer cachexia, and our findings suggest its role can even precede development, at least in female mice.

The role of TGF-β in fibrosis across pathologies has been well documented (39-41). The canonical pathway of TGF-β is to activate its downstream regulators in SMAD 2 and 3, which in turn can induce expression of collagens such as Collagen 1 and 3 (40). Dysregulations in TGF-β leads to improper deposition of ECM components via SMAD 2 and 3, ultimately resulting in fibrosis. However, here we report no significant alterations in SMAD 2 and 3 mRNA or protein content in either male or female mice or even *in vitro* following addition of LCM (Figure 3). We also measured β-catenin content, which resulted in no differences as well, as cross-talk between TGF-β and the Wnt/β-catenin pathway has been noted in previous literature (42-45). The lack of SMAD 2 and 3 or β-catenin induction appears to suggest a non-canonical pathway is involved in regulation of the ECM and its components in this model of cancer cachexia. However, what pathway(s) is/are involved remains unclear and requires further research.

Our final experiment aimed to isolate the mechanistic interaction between skeletal muscle and the ECM at a cellular level. To do so, we co-cultured C2C12 myotubes with 3T3 fibroblasts *in vitro* and then created a cachectic environment via addition of LCM. We then analyzed the same key components and regulators of the ECM and fibrotic pathways that were targeted in the *in vivo* approach (Figure 5). One major overall finding was the discrepancies in relative mRNA abundance between the myotube and fibroblast cells across the various markers. Of particular interest was the stark contrast observed in MMP-9 abundance, where fibroblast cells exhibited upwards of ~500-fold greater amounts of MMP-9 mRNA. Several recent studies have linked MMP-9 secretion not only to increased fibrosis, but more specifically to enhanced tumor growth

and formation (46, 47). LCM addition increased Collagen 3:1 mRNA abundance in both cell types as well as decreased MMP-9 mRNA in fibroblast cells by ~200-fold, however LCM addition had no effect on any other markers of fibrosis.

In summary, this study set out to obtain a better understanding of fibrosis during the very early stages and development of cancer cachexia in both sexes. The findings we report support the growing trend for females and males to display divergent responses to pathologies. While both sexes displayed increased fibrosis at their heaviest tumor burden compared to controls, females uniquely exhibited alterations to markers associated with fibrosis and ECM regulation as early as 1wk post-injection with LLC. Due to both sexes possessing increases in collagen deposition at the onset of cancer cachexia, it appears that fibrosis plays a role in the development of cancer cachexia as well as its well-noted progression (13,). Overall, increased fibrosis is involved with the development of cancer cachexia and males and females seemingly differ in the timing of alterations in the relevant fibrosis-related markers. However, more research is necessary to ascertain the players involved in the elevated collagen deposition and fibrosis shown in our results as TGF-β was elevated but the traditional downstream effectors in SMADs 2 and 3 and β-catenin were unchanged. A greater understanding of these pathways involved with fibrosis development during the pre- and early-stage development of cancer cachexia could point to potential therapeutic avenues and improve overall patient outcomes.

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**Disclosures**

No conflict of Interest, financial or otherwise, are declared by the authors.

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## Figure Legends

### Table 1. Descriptive Measurements

Body weight, tumor weight, hindlimb skeletal muscle weight, organ and tissue weights of PBS and LLC-injected C57BL6/J mice. Values are represented as means ± SEM with significance set at $p<0.05$. Different letters signify differences in group means following a significant F-test and Tukey's post-hoc analysis. Female data reported in Lim et al. 2021 (34), and male data as reported in Brown et al. 2017 (23).

### Figure 1. Collagen Content in Muscle

Collagen deposition within skeletal muscle measured via Picro Sirius Red staining of overall collagen in the plantaris (female) and tibialis anterior muscle (male). Values are represented as means ± SEM with significance set at $p<0.05$. Different letters signify differences in group means following a significant F-test and Tukey's post-hoc analysis.

### Figure 2. ECM Remodeling Regulators

Relative mRNA abundance of major collagen types, MMPs and TIMPs within the tibialis anterior muscle. Values are represented as means ± SEM with significance set at $p<0.05$. Different letters signify differences in group means following a significant F-test and Tukey's post-hoc analysis.

### Figure 3. Fibrotic Pathways

Relative mRNA abundance of TGF-β1, SMAD 2 and 3 as well as protein levels of β-catenin and SMAD 2 and 3 within the tibialis anterior muscle. Values are represented as means ± SEM with significance set at $p<0.05$. Different letters signify differences in group means following a significant F-test and Tukey's post-hoc analysis.

### Figure 4. Immunofluorescence of Collagens 3 and 1

Ratio of collagen 3 to 1 in the tibialis anterior muscle following immunofluorescent labeling. Values are represented as means ± SEM with significance set at $p<0.05$. Different letters signify differences in group means following a significant F-test and Tukey's post-hoc analysis.

### Figure 5. *in vitro* Analysis of Fibrosis

Relative mRNA abundance of collagens, MMPs, and TIMP-1 as well as protein levels of SMAD 2 and 3 and β-catenin from Vehicle and LCM treated C2C12 myotubes and 3T3 fibroblasts. Myotube diameter of differentiated C2C12 cells was also calculated following addition of LCM. Values are represented as means ± SEM with significance set at $p<0.05$.

## Figures

**Table 1**. Body weight, tumor weight, hindlimb skeletal muscle weight, organ and tissue weights of PBS and LLC-injected C57BL6/J mice. Values are represented as means ± SEM with significance set at $p<0.05$. Different letters signify differences in group means following a significant F-test and Tukey's post-hoc analysis. Female data reported in Lim et al. 2021 (34), and male data as reported in Brown et al. 2017 (23).

**Female**

| | PBS (n = 10) | 1 Week (n = 11) | 2 Weeks (n = 12) | LT (n= 13) | HT (n = 14) |
|---|---|---|---|---|---|
| **Body Weight (g)** | 19.03 ± 0.17 a | 17.36 ± 0.21 b | 18.35 ± 0.26 ab | 19.90 ± 0.30 ac | 21.41 ± 0.43 d |
| **Tumor Weight (g)** | N/A | 0.04 ± 0.01 a | 0.24 ± 0.04 a | 0.73 ± 0.11 b | 2.85 ± 0.15 c |
| **Body Weight –Tumor (g)** | 19.03 ± 0.17 a | 17.33 ± 0.21 b | 18.10 ± 0.26 ab | 19.17 ± 0.29 a | 18.55 ± 0.42ab |
| **Gastrocnemius (mg)** | 91.39 ± 1.16 a | 84.17 ± 1.43 b | 87.18 ± 1.27 ab | 93.12 ± 1.31 a | 88.09 ± 1.76 ab |
| **Tibialis Anterior (mg)** | 37.60 ± 0.85 a | 35.15 ± 0.74 ab | 35.09 ± 0.74 ab | 36.78 ± 0.70 a | 33.56 ± 0.73 b |
| **Liver (mg)** | 776.48 ± 31.47 a | 754.45 ± 31.00 a | 796.31 ± 19.83 ab | 910.48 ± 31.30 bc | 1036.33 ± 38.95 d |
| **Spleen (mg)** | 81.87 ± 3.52 a | 69.15 ± 3.06 a | 89.31 ± 6.25 a | 134.67 ± 8.77 b | 323.47 ± 18.44 c |
| **Fat (mg)** | 316.42 ± 30.09 a | 284.70 ± 24.58 ab | 274.04 ± 19.90 ab | 301.6 ± 25.52 a | 199.59 ± 22.60 b |
| **Tibia Length (mm)** | 16.57 ± 0.05 a | 16.15 ± 0.04 b | 16.30 ± 0.08 bc | 16.62 ± 0.06 ad | 16.64 ± 0.05 ad |

**Male**

| | PBS (n=24) | 1 Week (n=16) | 2 Weeks (n=16) | 3 Weeks (n=12) | 4 Weeks (n=14) |
|---|---|---|---|---|---|
| **Body Weight (g)** | 24.9 ± 0.33 a | 23.9 ± 0.32 a | 23.8 ± 0.62 a | 24.3 ± 0.81 a | 27.0 ± 0.62 b |
| **Tumor Weight (g)** | N/A | 0.03 ± 0.07 a | 0.16 ± 0.03 a | 0.77 ± 0.15 b | 3.50 ± 0.36 c |
| **Body Weight – Tumor (g)** | 24.8 ± 0.33 | 23.8 ± 0.32 | 23.6 ± 0.65 | 23.5 ± 0.74 | 23.6 ± 0.56 |
| **Gastrocnemius (mg)** | 134.6 ± 2.07 a | 129.44 ± 2.49 a | 130.00 ± 4.08 a | 125.16 ± 3.47 ab | 118.39 ± 4.05 b |
| **Tibialis Anterior (mg)** | 45.14 ± 0.85 a | 43.80 ± 1.16 a | 44.54 ± 1.15 a | 42.88 ± 1.64 a | 38.34 ± 1.31 b |
| **Liver (mg)** | 1059.55 ± 32.20 a | 1038.85 ± 42.62 a | 981.30 ± 39.47 a | 965.35 ± 53.33 a | 1354.15 ± 53.15 b |
| **Spleen (mg)** | 81.14 ± 3.24 a | 81.86 ± 2.77 a | 85.73 ± 2.59 a | 222.88 ± 31.07 b | 366.54 ± 31.80 c |
| **Fat (mg)** | 367.58 ± 12.87 a | 332.59 ± 22.53 a | 348.29 ± 12.78 a | 316.75 ± 33.64 a | 240.47 ± 19.17 b |
| **Tibia Length (mm)** | 17.41 ± 0.09 | 17.36 ± 0.05 | 17.29 ± 0.117 | 17.43 ± 0.093 | 17.33 ± 0.04 |

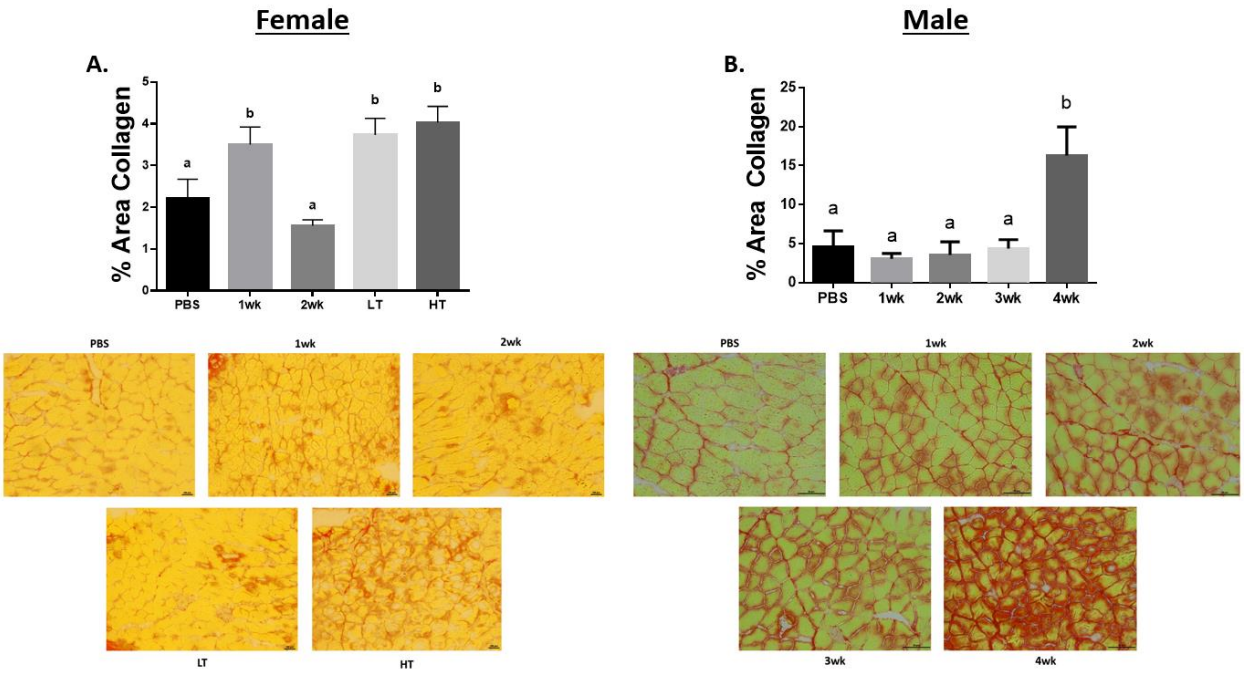


**Figure 1**. Collagen deposition within skeletal muscle measured via Picro Sirius Red staining of overall collagen in the plantaris (female) and tibialis anterior muscle (male). Values are represented as means ± SEM with significance set at $p<0.05$. Different letters signify differences in group means following a significant F-test and Tukey's post-hoc analysis.

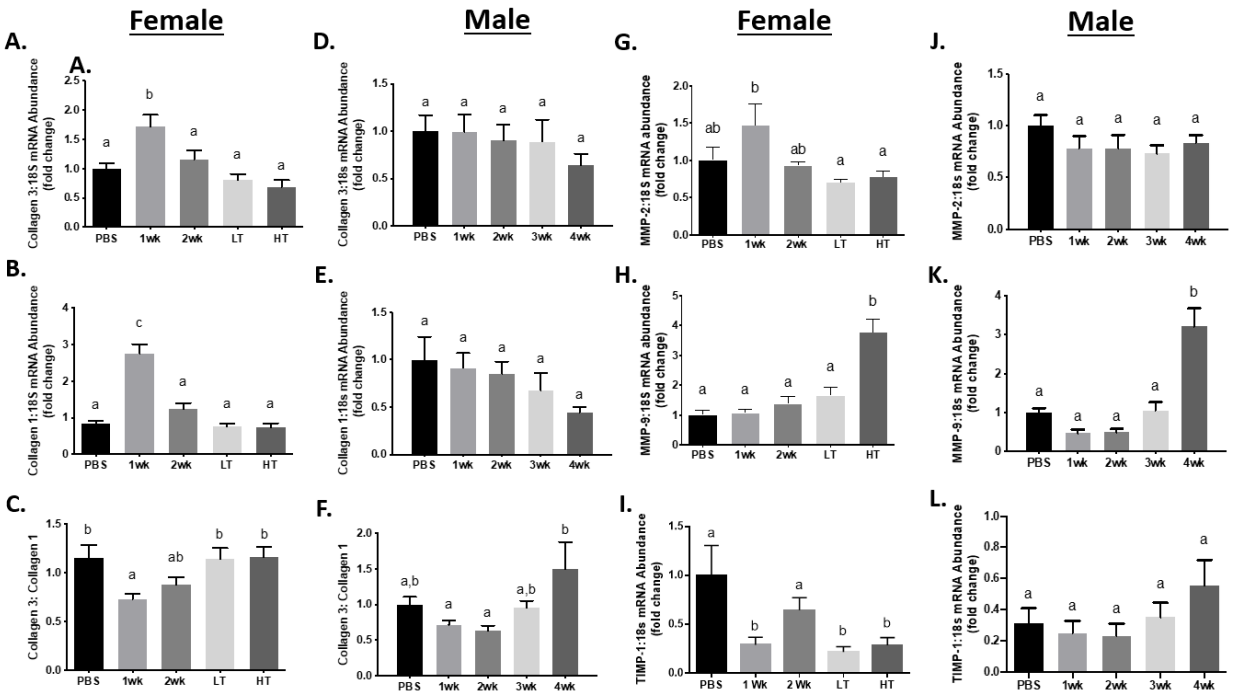


**Figure 2**. Relative mRNA abundance of major collagen types, MMPs and TIMPs within the tibialis anterior muscle. Values are represented as means ± SEM with significance set at $p<0.05$. Different letters signify differences in group means following a significant F-test and Tukey's post-hoc analysis.

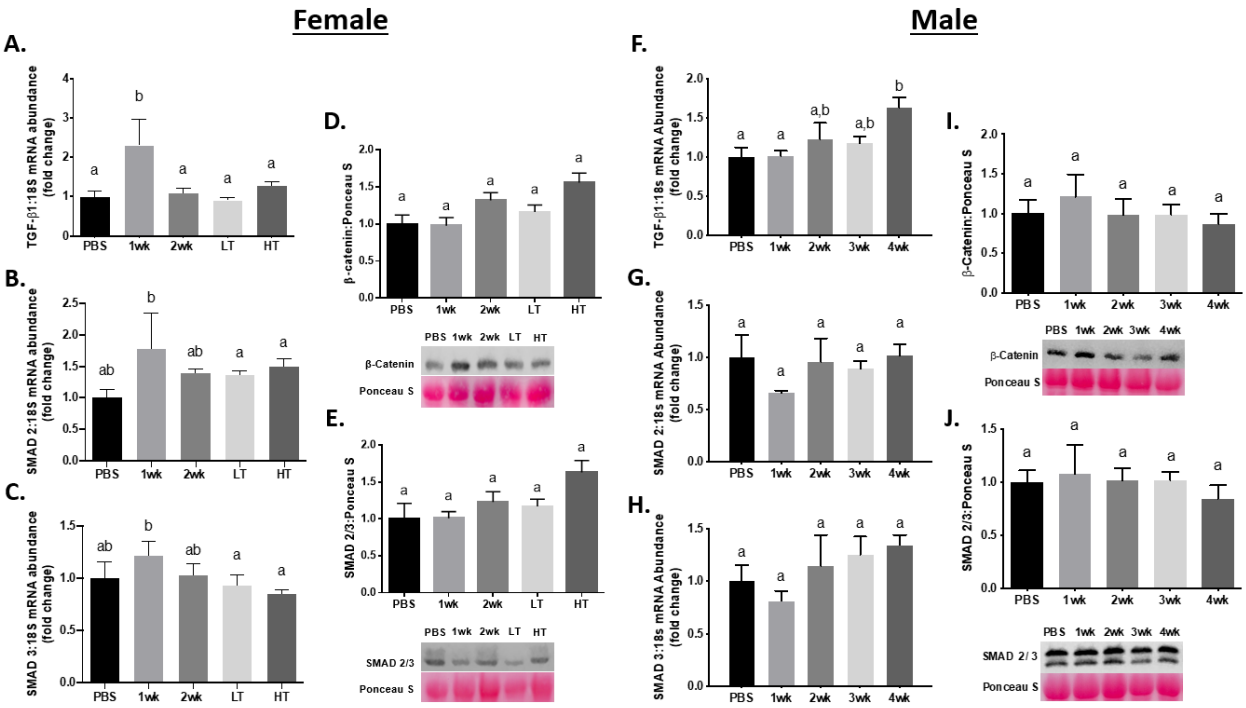


**Figure 3**. Relative mRNA abundance of TGF-β1, SMAD 2 and 3 as well as protein levels of β-catenin and SMAD 2 and 3 within the tibialis anterior muscle. Values are represented as means ± SEM with significance set at $p<0.05$. Different letters signify differences in group means following a significant F-test and Tukey's post-hoc analysis.

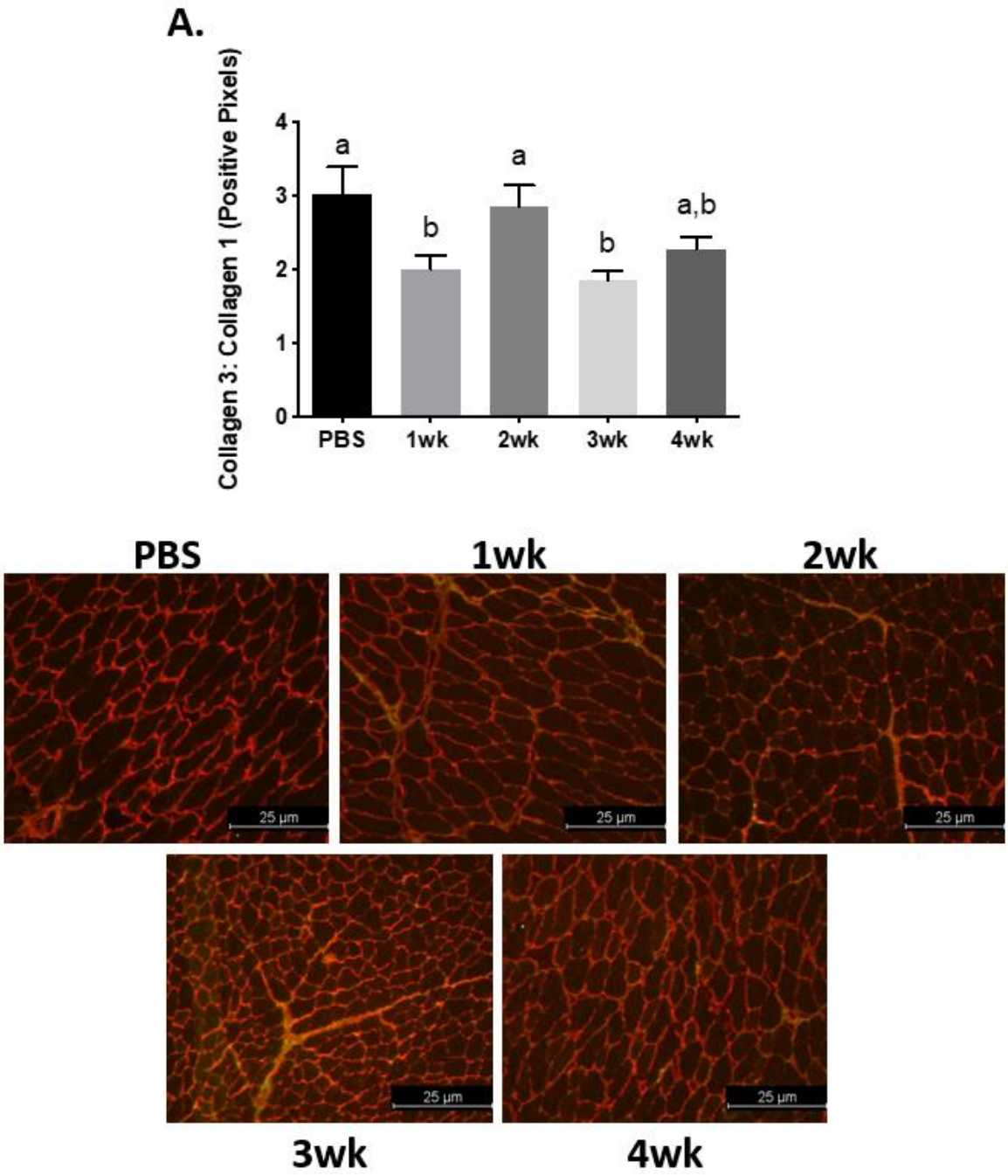


**Figure 4**. Ratio of collagen 3 to 1 in the tibialis anterior muscle following immunofluorescent labeling. Values are represented as means ± SEM with significance set at $p<0.05$. Different letters signify differences in group means following a significant F-test and Tukey's post-hoc analysis.

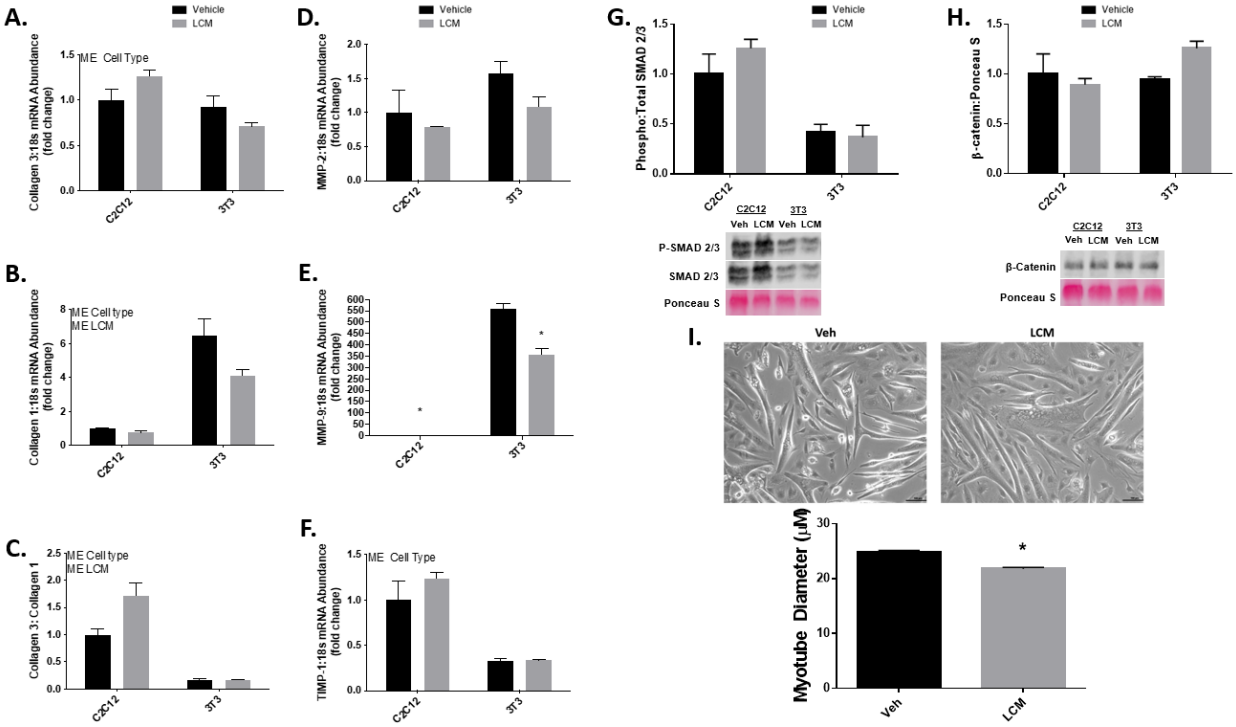


**Figure 5**. Relative mRNA abundance of collagens, MMPs, and TIMP-1 as well as protein levels of SMAD 2 and 3 and β-catenin from Vehicle and LCM treated C2C12 myotubes and 3T3 fibroblasts. Myotube diameter of differentiated C2C12 cells was also calculated following addition of LCM. Values are represented as means ± SEM with significance set at $p<0.05$.

# Chapter 5

## Overall Discussion

In this dissertation I examined sexual dimorphism of cancer cachexia in two different models of cancer cachexia in LLC and $APC^{Min/+}$ mice. One model focused on the role of fibrosis during the stages before and up to the development of cancer cachexia, and the second model focused on the effects of leucine supplementation on cancer cachexia progression. The data presented strongly support the emerging trend for sexual dimorphism in cancer cachexia across various models, and therefore the growing need for inclusion of both sexes in future studies. Here I show increased fibrosis in both sexes as cachexia develops at 4wks post-injection with LLC but with female mice exhibiting earlier inductions in TGF-β in males. I also show exacerbated losses in body weight in male $APC^{Min/+}$ mice following supplementation with leucine and this effect was not observed in female $APC^{Min/+}$ mice.

Future studies in both models should focus on elucidating the underlying pathways involved in the dimorphic sexual response. LLC males and females saw initial increases in TGF-β at different timepoints, and the typically understood downstream effectors in SMAD 2 and 3 as well β-catenin were unaffected in both sexes. The losses in body weight observed in male $APC^{Min/+}$ mice was not accompanied by greater reductions in any muscle weights, and more importantly not uniquely accompanied by upregulation of any markers of proteolysis or protein anabolism inhibition.

### Fibrosis Occurs during the Development of Cancer Cachexia in Both Male and Females

In this study I analyzed collagen content and markers associated with fibrosis in both sexes following cancer implantation up to the development of cancer cachexia. Based on our

data both males and females exhibit fibrosis as cancer cachexia develops. However, females saw alterations in TGF-β as early as 1wk post-injection with LLC whereas males saw no increase until 4wks. As SMAD 2 and 3 as well as β-catenin did not alter as cancer cachexia developed it appears that a non-canonical pathway could be involved in these early stages of fibrosis. Although, it is important to note the early increases in TGF-β were accompanied with increases in collagen 3 and 1 mRNA and overall collagen content with actual muscle tissue. These data suggest females experience earlier alterations in fibrosis during cancer cachexia development compared to males.

**Leucine Supplementation Exacerbates Cancer Cachexia in Males but Not Females**

To my knowledge I am the first to report greater sex-dependent losses in body weight in cancer cachexia following leucine supplementation. These losses in body weight observed in male $APC^{Min/+}$ mice was accompanied with higher IL-6 mRNA abundance, and interestingly not also with higher NFκB or TNF-α. In fact, myotubes treated with male APC L plasma saw decreases in TNF-α specifically. Female mice uniquely displayed elevation of MyoD mRNA in $APC^{Min/+}$ mice as well as decreases in Pax7 mRNA in APC L plasma treated myotubes. These shifts in markers of satellite cell dynamics to favor proliferation and differentiation could point to enhanced muscle regeneration and an overall protective effect of skeletal muscle not observed in male mice. The potentiality of this female protective effect is further supported when myotube diameter was measured, as only the male $APC^{Min/+}$ plasma treated myotubes experienced reductions in diameter.

## Concluding Statements

These studies show novel evidence for sex differences in two separate models of cancer cachexia. Males and females can possess inherent variations in responses to both interventions and pathologies, and a lack of evaluation of both sexes in future studies does not set up future clinical treatments up for success.

# Appendix

UNIVERSITY OF ARKANSAS

*Office of Research Compliance*

To: Tyrone Washington
Fr: Jeff Wolchok
Date: November 13, 2020
Subject: IACUC Approval
Exp. Date: August 2, 2021

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your modification to administer deuterium oxide heavy water in AUP #18137, *Cancer Cachexia and Leucine Supplementation*.

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 the above expiration date, 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 three years at a time.

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

JCW/rek

UNIVERSITY OF ARKANSAS

Office of Research Compliance

| | |
|---|---|
| To: | Tyrone Washington |
| Fr: | Craig Coon |
| Date: | August 15th, 2018 |
| Subject: | IACUC Approval |
| Expiration Date: | August 3rd, 2019 |

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol # **18137:** *Cancer Cachexia and Leucine Supplementation*.

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 August 3rd, 2019 you can submit a modification to extend project up to 3 years, or submit a new protocol. 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: Tyrone Washington, Wesley Haynie, Megan Caldwell, Katarina Bejarano, and Nicholas Greene. 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

UNIVERSITY OF ARKANSAS

Office of Research Compliance

MEMORANDUM

TO: Nicholas Greene

FROM: Craig N. Coon, Chairman

DATE: 7/13/15

SUBJECT: IACUC Approval

Expiration Date: Jan 1, 2018

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol 15065: "Mitochondrial Degeneration in the Onset of Cancer-Cachexia Induced Muscle Atrophy" you may begin work immediately

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 Jan 1, 2018 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 IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/aem

cc: Animal Welfare Veterinarian