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Targeting Cancer-Anorexia Cachexia Syndrome and Septic Inflammatory-Based Atrophy With R/S 1,3 Butanediol Acetoacetate Diester

Andrew P. Koutnik
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Targeting Cancer-Anorexia Cachexia Syndrome and Septic Inflammatory-Based Atrophy With R/S 1,3 Butanediol Acetoacetate Diester

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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Keywords: Cancer Cachexia, Skeletal Muscle, Atrophy, Ketone Bodies

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# TABLE OF CONTENTS

List of Tables ........................................................................................................ v
List of Figures ......................................................................................................... vi
List of Abbreviations ............................................................................................. ix
Abstract ................................................................................................................ xiii

Chapter 1: Cancer Cachexia ................................................................................ 1
1.1 Chapter Synopsis ............................................................................................ 1
1.2 Cancer Impact ................................................................................................ 1
1.3 Cancer Cachexia ............................................................................................. 3
1.3.1 History and Definitions ............................................................................. 3
1.3.2 Prevalence and Impact ............................................................................. 5
1.3.3 Skeletal Muscle Mass Across Health and Disease ................................. 9
1.3.4 Distinct Multifaceted Syndrome .............................................................. 14
   1.3.4.1 Metastasis ......................................................................................... 18
   1.3.4.2 Body Composition ........................................................................... 19
      1.3.4.2.1 Tumor Necrosis Factor-Alpha Signaling .................................. 20
      1.3.4.2.1.1 Skeletal Muscle ................................................................. 22
      1.3.4.2.1.2 Insulin and Insulin-Like Growth Factor 1 Signaling .......... 27
   1.3.4.2.2 Adipose Tissue ............................................................................. 32
   1.3.4.2.3 Ascites ......................................................................................... 35
   1.3.4.3 Inflammation ................................................................................... 36
   1.3.4.4 Anorexia .......................................................................................... 40
   1.3.4.5 Anemia ............................................................................................ 42
   1.3.4.6 Blood Urea Nitrogen ....................................................................... 44
   1.3.4.7 Total Protein ................................................................................... 45
   1.3.4.8 Hypoalbuminemia .......................................................................... 45
   1.3.4.9 Metabolic Derangement ................................................................. 46
1.4 Modeling Cancer-Anorexia Cachexia Syndrome ........................................ 50
   1.4.1 Xenograph ............................................................................................ 50
   1.4.2 Syngeneic ............................................................................................. 51
   1.4.3 Genetically Engineered ....................................................................... 52
   1.4.4 Modeling Gaps ..................................................................................... 55
      1.4.4.1 Metastasis ...................................................................................... 55
      1.4.4.2 Clinical Biomarkers ....................................................................... 56
Chapter 3: The VM-M3 Model of Systemic Metastasis Demonstrates the Full Progressive Cancer-Anorexia Syndrome

3.1 Chapter Synopsis

3.2 The VM-M3 Model of Systemic Metastasis Demonstrates the Full Progressive Cancer-Anorexia Cachexia Syndrome

3.2.1 Results and Discussion

3.2.1.1 Baseline Bodyweight, Age and Food Intake

3.2.1.2 Survival Analysis, Tumor Growth and Metastasis

3.2.1.3 Body Composition

3.2.1.4 Inflammation

3.2.1.5 Anorexia, Anemia, Protein Breakdown, Hypoalbuminemia, and Metabolic Derangement

3.3 References for Chapter 3

Chapter 4: The Ubiquitin-Proteasomal Pathway is Activated in VM-M3 Skeletal Muscle

4.1 Chapter Synopsis

4.2 The Ubiquitin-Proteasomal Pathway is Activated in VM-M3 Skeletal Muscle

4.2.1 Results and Discussion

4.3 References for Chapter 4

Chapter 5: Ketone Diester Attenuates Multifactorial Cancer-Anorexia Cachexia and Inflammatory Atrophy

5.1 Chapter Synopsis

5.2 Ketone Diester Attenuates Multifactorial Cancer-Anorexia Cachexia and Inflammatory Atrophy

5.2.1 Results and Discussion

5.2.1.1 Ketone Diester Mitigates Comorbidities, Tumor Burden, and Skeletal Muscle Atrophy in Cancer-Anorexia Cachexia Syndrome

5.2.1.2 Ketone Diester Mitigates Tissue Catabolism and Comorbidities in Inflammation-Induced Atrophy

5.3 References for Chapter 5

Chapter 6: Discussion – Implications for Cancer Cachexia Modeling and Therapy

6.1 Chapter Synopsis

6.2 Implications for Cancer Cachexia Modeling and Therapy

6.3 References for Chapter 6

Appendices
# LIST OF TABLES

Table 1.1. Characteristics of Skeletal Muscle Wasting……………………………………….. 18
Table 1.2. Characteristics of Cancer Cachexia Modeling Systems………………………….. 54
Table A.1 Experimental Design……………………………………………………………….. 218
LIST OF FIGURES

Figure 3.1. Baseline Sex, Bodyweight, and Age Controlled with Similar Survival in Males and Females .......................................................... 141

Figure 3.2. VM-M3 Have Progressive Tumor Growth and Spontaneous Systemic Metastases ................................................................. 142

Figure 3.3. VM-M3 Have Progressive Tumor Growth and Spontaneous Systemic Metastases ................................................................. 143

Figure 3.4. VM-M3 Males and Females Have Similar Survival ............................................. 144

Figure 3.5. VM-M3 Develop Skeletal Muscle and Adipose Tissue Wasting Not Represented in Bodyweight Measurements........................................ 145

Figure 3.6. VM-M3 Develop Prolonged Systemic Inflammation................................. 147

Figure 3.7. VM-M3 Alters Serum Cytokine Profile ..................................................... 149

Figure 3.8. VM-M3 Develop Anorexia, Anemia, Protein Breakdown, Hypoalbuminemia, and Metabolic Derangement ........................................ 151

Figure 3.9. VM-M3 Circulating Lipids ................................................................. 152

Figure 3.10. Alterations Seen in VM-M3 Cannot Be Explained By Altered Hydration Status ................................................................. 153

Figure 4.1. VM-M3 Skeletal Muscle Upregulates Ubiquitination, while 26S Proteasomal Quantity and Capacity Remains Stable ................................. 162

Figure 4.2. NF-κB is not upregulated in VM-M3 Skeletal Muscle .................................. 163
Figure 4.3. Lower Total RNA and Nuclear Fraction Protein Illustrate Wasting in VM-M3 Skeletal Muscle ................................................................. 164

Figure 4.4. Lower Circulating IGF-1/Insulin and Subsequent FOXO3a Nuclear Translocation is Upregulated in VM-M3 Skeletal Muscle ............................. 165

Figure 4.5. Reduced Insulin/IGF-1, increased FOXO3a Activation, and Increased Poly-Ubiquitination Observed in Atrophied VM-M3 Skeletal Muscle ............................... 166

Figure 5.1. Ketone Diester Administration into Food Altered Systemic Metabolism and Was Well-Tolerated ................................................................. 179

Figure 5.2. Baseline Sex, Bodyweight, Age, and Food Intake Were Controlled ................ 180

Figure 5.3. Ketone Diester Attenuated Comorbid Anorexia in VM-M3 Cancer-Anorexia Cachexia Syndrome ................................................................. 180

Figure 5.4. Ketone Diester Altered Tumor Burdon and Surrogate Markers of Metastatic Progression in VM-M3 Cancer-Anorexia Cachexia Syndrome ..................... 181

Figure 5.5. Ketone Diester Altered Circulating Metabolites in VM-M3 Cancer-Anorexia Cachexia Syndrome ................................................................. 182

Figure 5.6. Ketone Diester Altered Circulating Metabolites in VM-M3 Cancer-Anorexia Cachexia Syndrome ................................................................. 183

Figure 5.7. Ketone Diester Attenuates Cancer-Anorexia Cachexia Syndrome ................ 184

Figure 5.8. Ketone Diester Oral Gavage Rapidly Altered Circulating Metabolites ............. 185

Figure 5.9. Acute Ketone Diester Administration Attenuated Tissues Catabolism and Comorbid Anorexia During Sepsis ....................................................... 185

Figure 5.10. Acute Ketone Diester Administration Attenuated Tissues Catabolism and Time to Recovery During and Following Sepsis ........................................ 186
Figure 5.11. Acute Ketone Diester Administration Attenuated Tissues Catabolism During Sepsis When Controlling for Food Intake ................................................................. 187

Figure 5.12. Ketone Diester Attenuates Muscle Atrophy in Cancer-Independent Catabolic Environment ................................................................. 188

Figure 5.13. Ketone Diester Attenuates Muscle Atrophy in Cancer-Independent Catabolic Environment ................................................................. 189
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>4EB-P1</td>
<td>Eukaryotic translation initiation factor 4E-binding protein 1</td>
</tr>
<tr>
<td>AID</td>
<td>Auto Immune Deficiency Syndrome</td>
</tr>
<tr>
<td>Akt</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>AMPK</td>
<td>5' Adenosine Monophosphate-Activated Protein Kinase</td>
</tr>
</tbody>
</table>
| APCMin/+     | Adenomatous polyposis coli Multiple Intestinal Neoplasia/+
<p>| ATP          | Adenosine Triphosphate |
| BDNF         | Brain-Derived Neurotrophic Factor |
| BUN          | Blood Urea Nitrogen |
| C-26         | Colon-26 Mouse Model |
| Ca2+         | Calcium |
| CACS         | Cancer Anorexia Cachexia Syndrome |
| DHA          | Docosahexaenoic Acid |
| DXA          | Dual-Energy X-Ray Absorptiometry |
| eIF4E        | Eukaryotic Translation Initiation Factor 4E |
| EOL          | End of Life |
| EPA          | Eicosapentaenoic Acid |
| ERK          | Extracellular Signal-Regulated Kinase-1 |
| FFA          | Free Fatty Acid |
| FGF21        | Fibroblast Growth Factor 21 |</p>
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOXO3a</td>
<td>Forkhead Box O3a</td>
</tr>
<tr>
<td>GH</td>
<td>Growth Hormone</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon-Like Peptide-1</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose Transport Protein</td>
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<tr>
<td>GPR109a/HCAR2</td>
<td>G-Protein-Coupled Receptor 109a/ Hydroxycarboxylic Acid Receptor 2</td>
</tr>
<tr>
<td>Grb2/Sos</td>
<td>Growth Factor Receptor-Bound Protein 2/Son of Sevenless</td>
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<tr>
<td>GSK3</td>
<td>Glycogen Synthase Kinase 3</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone Deacetylases</td>
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<tr>
<td>HIF-1α</td>
<td>Hypoxia-Inducible Factor 1-Alpha</td>
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<tr>
<td>HMG CoA</td>
<td>3-Hydroxy-3-Methyl-Glutaryl-CoA</td>
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<tr>
<td>IGF-1</td>
<td>Insulin-Like Growth Factor 1</td>
</tr>
<tr>
<td>IGF-1R</td>
<td>Insulin-Like Growth Factor 1 Receptor</td>
</tr>
<tr>
<td>IGFBP-1</td>
<td>Insulin-Like Growth Factor Binding Protein 1</td>
</tr>
<tr>
<td>IkBa</td>
<td>Inhibitory Kappa Beta Alpha</td>
</tr>
<tr>
<td>IKKβ</td>
<td>Inhibitory Kappa Beta Kinase</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin Receptor</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin Receptor Substrate</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>K⁺</td>
<td>Potassium</td>
</tr>
<tr>
<td>KDE</td>
<td>R/S 1,3-Butanediol Acetoacetate Diester</td>
</tr>
<tr>
<td>Kras/Lkb1</td>
<td>KrasG12D/+;Lkb1f/f model</td>
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LLC ............................................................ Lewis Lung Carcinoma
LLYT ....................................................... Leucine-Leucine-Valine-Tyrosine
LPS .......................................................... Lipopolysaccharide
MAC16 ..................................................... Murine Adenocarcinoma 16 Model
MRI ........................................................ Magnetic Resonance Imaging
MAPK ...................................................... Mitogen-Activated Protein Kinase
MCFAs ....................................................... Medium Chain Fatty Acids
MCT ........................................................ Medium Chain Triglycerides
MEGACE ................................................... Megestrol Acetate
MEK ........................................................ MAPK/Extracellular Signal-Regulated Kinase
Mg2+ ........................................................ Magnesium
mRNA ........................................................ messenger Ribonucleic Acid
mTOR ........................................................ mammalian Target of Rapamycin
mTORC1 ...................................................... mammalian Target of Rapamycin Complex 1
MuRF1 ....................................................... Muscle RING Finger 1
Na+ .............................................................. Sodium
NAD .......................................................... Nicotinamide-Adenine Dinucleotide
NF-κB ...................................................... Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NH4+ ........................................................... Ammonium
NSAIDS ................................................. Non-Steroidal Anti-Inflammatory Drugs
NSCLC ..................................................... Non-Small Cell Lung Cancer
PCG-1α ........................................... Peroxisome Proliferator-activated Receptor Gamma Coactivator 1-Alpha
PDK1 ....................................................... Phosphoinositide-Dependent Kinase-1
PI3K ................................................................. Phosphoinositide 3-Kinase
PIP2 ............................................................... Phosphatidylinositol 4,5-Bisphosphate
PIP3 ............................................................... Phosphatidylinositol 3,4,5-Triphosphate
PPARγ ............................................................ Peroxisome Proliferator-Activated Receptor Gamma
ROS ................................................................. Reactive Oxygen Species
S6K ................................................................. Ribosomal Protein S6 kinase
SARMs ............................................................ Selective Androgen Receptor Modulators
SCOT ............................................................... Succinyl CoA Transferase
STAT3 ......................................................... Signal Transducer and Activator of Transcription 3
TCA ................................................................. Tricarboxylic Acid
TGF-β .............................................................. Transforming Growth Factor - Beta
TNF-α ............................................................... Tumor Necrosis Factor - Alpha
TRADD ........................ Tumor Necrosis Factor Receptor Type 1-Associated DEATH Domain
TRAF2 ........................ Tumor Necrosis Factor Receptor-Associated Factor 2
TSC ................................................................. Tuberous Sclerosis Complex
UCPs ............................................................... Uncoupling Proteins
VLCKD .......................................................... Very-Low Carbohydrate Ketogenic Diet
YAH-130 ......................................................... Yoshida Ascites Heptoma-130
Cancer anorexia cachexia syndrome (CACS) is a distinct atrophy disease negatively influencing multiple aspects of clinical care and patient quality of life. Although it directly causes 20% of all cancer-related deaths, there are currently no model systems that encompass the entire multifaceted syndrome, nor are there any effective therapeutic treatments. Here, we show that the VM-M3 mouse model of systemic metastasis demonstrates a novel, immunocompetent, logistically feasible, repeatable phenotype with progressive tumor growth, spontaneous metastatic spread, and the full multifaceted CACS with expected sex dimorphisms across tissue wasting. We also demonstrate that the ubiquitin proteasomal degradation pathway was significantly upregulated in association with reduced IGF-1/Insulin and increased FOXO3a activation, but not TNF-α-induced NF-κB activation, driving skeletal muscle atrophy. Additionally, we show that R/S 1,3-butanediol acetoacetate diester (Ketone Diester; KDE) administration shifted systemic metabolism, attenuated tumor burden, reduced tissue catabolism, and mitigated comorbid symptoms in both CACS and cancer-independent atrophy environments. Our findings suggest the ketone diester attenuates multifactorial CACS skeletal muscle atrophy and inflammation-induced tissue catabolism, demonstrating anti-catabolic effects of ketones bodies in multifactorial atrophy.
CHAPTER 1: CANCER CACHEXIA

1.1 Chapter Synopsis

Cancer remains a prevalent, devastating, and financially costly diagnosis. While “cures” are rare, early detection, improved understanding into cancer’s molecular pathology, and expanding treatment options will likely increase the number of patients “managing” this disease and its comorbidities. Cancer cachexia is a progressive, multifactorial, systemic syndrome negatively impacting patient health, outcomes, and survival. Cachexia pathology and treatment options largely result from animal model systems. However, all model systems demonstrate their own strengths and weaknesses, particularly when attempting to encapsulate the entire multifactorial syndrome. Additionally, cancer cachexia currently has no approved treatment option(s). In this review of literature, cancer cachexia’s history, definitions, prevalence, impact, distinct multifaceted components, modeling systems, and proposed treatment options are discussed.

1.2 Cancer Impact

Cancer is currently defined as a group of diseases characterized by the uncontrolled growth and spread of abnormal cells [1]. In 2011, Hanahan and Weinberg described the underlying hallmarks and/or characteristics of cancer cells which include sustaining proliferative signaling,
evading growth suppressors, activation of invasion and metastasis, enabling replicative immorality, inducing angiogenesis, resisting cell death, deregulating cellular genetics, avoiding immune destruction, tumor-promoting inflammation, and genome instability and mutation(s) [2]. These neoplastic, often heterogenous cells can directly or indirectly disrupt normal bodily functions and can result in host mortality if growth and invasion are left unbridled. It was estimated that approximately ½ of males and 1/3 of females will be diagnosed with cancer in their lifetime [3]. In 2019 alone, 1,762,450 diagnoses and 606,880 mortalities are estimated to result from cancer [1]. While incidence and mortality remains high, the 5-year relative survival rates continues to improve across all races and many cancer types [1]. While these improvement in patient outcomes can be tied to an ever growing body of research on the molecular pathology of cancer and expanding therapeutic options, the hallmark reduction in overall age-adjusted death rates starting in 1991 has largely been attributed to reductions in tobacco use and improved disease detection [4]. None the less, understanding cancer pathology and therapeutic development remains under extensive evaluation as cancer remains the second leading cause of death behind heart disease in the United States [4]. In fact, the Agency for Healthcare Research and Quality estimates the direct medical cost of cancer in the United States is $80.2 billion, demonstrating a tremendous economic burden [1]. Thus, while cancer prevention, detection, evaluation and therapeutic options are likely the cause of subtle, yet continuous, improvements in patient 5-year relative survival rates [1], “cures” are rare, likely leading to future increases in the number of cancer patients that fall into the category of disease and comorbid syndrome management. Cachexia illustrates a devasting cancer comorbidity present in 30% of all cancer patients and 50-80% of advanced cancer patients [5-7].
1.3 Cancer Cachexia

Cancer cachexia is a multifactorial atrophy syndrome characterized by skeletal muscle and often adipose tissue wasting that is typically accompanied by inflammation, anorexia, anemia, elevated protein breakdown, hypoalbuminemia and metabolic derangement [5, 6, 8]. Due to its multifaceted nature and complexity, cachexia is distinct from other forms of wasting and has been repeatedly shown to worsen prognosis, quality of life, and ability to receive, tolerate, and/or respond to standard of care [9-15]. Consequently, 20% of cancer-related deaths result directly from cachexia [8]. Unfortunately, the prevalence of cachexia-induced mortality in cancer patients remains unchanged from the initial mortality estimations in 1932, [16] as current modeling systems are limited [17-20] and therapeutic interventions ineffective [21-23].

1.3.1 History and Definitions

The word cachexia originates from the Greek words *kakos* and *hexis* meaning “bad” and “condition of appearance,” respectively [24, 25]. Attempts to define “cachexia” have evolved into present day [5, 8, 24-26]. Starting in 460-377 BC, Hippocrates described his observation of what can now be interpreted as cachexia in a patient, “the abdomen fills with water, the feet and legs swell, the shoulders, clavicles, chest and thighs melt away.” This observation describes both bodily wasting and what is likely ascites fluid, an indicator of potential metastasis [27]. Multiple other observations of what would be deemed cachexia were described between Hippocrates and the early 20th century [25]. Warren was one of the earliest attempts to give a clinical, yet testable description of cachexia in 1932 during an observational analysis [26]. Warren categorized cachexia as
progressive wasting, weakness, and anemia. Based on this definition, Warren went on to provide
the first statistical cancer cachexia mortality rates at 22%, which remain unchanged almost a
century later [8, 26]. In 2004, Dahele and Fearon proposed not defining the condition, but instead
describing it as a spectrum, as evidence had accumulated that cachexia was a complex multifaceted
syndrome with interpatient variability [24]. Dahele and Fearon described cachexia as having
variable degrees of weight loss, anorexia, anemia, fatigue, and ascites. Entering the 21st century,
little progress was being made in therapeutically addressing this syndrome. Many attributed this
to an absence of a unifying definition for cachexia. Thus, an attempt to provide a consensus
definition of the entire syndrome across chronic illnesses (cancer, chronic heart failure, chronic
obstructive pulmonary disease, chronic kidney disease, inflection/sepsis, etc.) was described by
Evans and colleagues in 2008 [5]. Evan et al. defined cachexia as 1) complex and multifaceted, 2)
distinct from other wasting/catabolic syndromes, and 3) encompassing most prominently skeletal
muscle loss, but also general weight loss (controlled for fluid retention/ascites), anorexia,
inflammation, metabolic derangement, altered hormonal profile, anemia, and hypoalbuminemia.
While Evan et al. described cachexia across chronic inflammatory disease, cachexia is most
prevalently observed in cancer [28]. Therefore, the definition was further refined in 2011 by Fearon
and colleagues [6]. Fearon et al. added “staging” characteristics. This three-stage progression was
broken into pre-cachexia, cachexia, and refractory cachexia based on body weight, food intake,
metabolic changes, inflammation, performance scores, and responsiveness to anti-neoplastic
treatments. Specifically, pre-cachexia was defined as initial weight loss ≤5%, anorexia, and
metabolic derangement. Cachexia was characterized as weight loss ≥5% (or based on metrics of
body mass index or sarcopenic diagnosis), reduced food intake, and systemic inflammation.
Finally, refractory cachexia was described as variable degrees of weight loss and an estimated
<3month patient survival. “Refractory” describes not only the patient’s inability to respond to anti-neoplastic treatment, but a general belief that patients cannot reverse or manage the progressive nature of this disease once it has reached this stage. While Warren described cachexia as wasting, weakness, anemia, Evans et al. as multifaceted and distinct, and Fearon et al. as a staged wasting process, all these definitions illustrate overlapping characteristics, but subtle discrepancies that helped evolve the definition into present day [5, 6, 26]. In 2014, Argiles et al. went on to provide an updated description of the disease which accounted for prior nuances, but also the array of cancer cachexia data across rodents and humans [8]. Argiles et al. described cancer cachexia as, 1) consensus hallmark biomarkers of weight loss- mainly from loss of skeletal muscle and body fat, along with inflammation; 2) primary symptoms of asthenia, anorexia, anemia, fatigue, and reduced quality of life; 3) multifactorial syndrome involving several metabolic pathways, in many tissues and organs. This definition, along with other’s pre-dating it, illustrate the complex, multifaceted, progressive, systemic, yet distinct nature of this disease. Unfortunately, this clinically impactful disease effecting cancer patient care, outcomes, and ultimately survival, remains without an approved treatment [7, 9-11, 13, 26, 29-31].

1.3.2 Prevalence and Impact

Cachexia is estimated to be present in 30% of all cancer patients [7]. However, 50-80% of patients with advanced/metastatic cancer experience cachexia, indicating that most metastatic patients will likely experience cachexia, regardless of tumor type [7, 8, 32]. Caillet et al. 2016 provided supportive evidence showing that 3% to 83% of aged (>65years of age) cancer patients experience malnutrition, a critical component of cachexia [33]. This wide range was attributed to
tumor type and stage heterogeneity. However, a consistently higher range (64-76.5%) of cancer patients with metastasis experienced comorbid symptoms, such as malnutrition [33]. Thus, cachexia appears to be a common cancer comorbidity that will affect most cancer patients as the cancer progresses.

Cachexia has repeatedly been shown to worsen the prognosis of cancer patients and be inversely correlated with quality of life [9-12, 34]. In one of the first observations of its kind, Dewys et al. in 1980 observed weight loss in 3,047 cancer patients across tumor type prior to receiving chemotherapy [34]. Dewy observed a common connection between weight loss and reduced survival in all tumor types. Additionally, weight loss was also higher (almost ubiquitously) across tumor type in the more advanced metastatic stage, illustrating both weight loss’ loss impact on survival and metastasis impact on cachexia incidence. In 2008, Bachman et al. observed 227 pancreatic ductal adenocarcinoma patients over 18 months and tracked for various cachexia biomarkers [weight loss, body mass index, inflammation (c-reactive protein), albumin, hemoglobin, anorexia], along with tumor size and metastasis [12]. Cachexic patients had reduced survival, higher metastatic load, increased inflammation, lower hemoglobin (anemia), lower protein, lower albumin (hypoalbuminemia), amongst other symptoms. Cachexic patients that had metastatic recurrence also had rapid cachexic onset. Bachman et al. was one of the first to directly speculate that metastasis may induce cachexia, stating, "metastatic dedifferentiation of the tumor might be a critical step in the development of tumor-associated cachexia." In 2014, Blum et al. went on to observe survival in 733 cancer patients of various tumor types using different cachexia models differentiated by how cachexia was defined (Models 1-3; [9]). Model 1 compared weight loss versus no-weight loss. Model 2 compared no cachexia vs pre-cachexia (weight loss 1%-5%)
vs cachexia (>5% weight loss or >2% with <20kg/m²) vs refractory cachexia (>15% weight loss and body mass index <23kg/m² or weight >20% last 6 month and body mass index <27kg/m²), loosely based on Fearon et al. 2011 definition [6]. Model 3 compared pre-cachexia to pre-cachexia with individual comorbid symptoms of cachexia [elevated inflammatory metrics (C-reactive protein) and anorexia] to determine if individual components of the syndrome could impact survival, independent of weight loss [9]. Across all models, those patients with either cachexia and/or cachexia components demonstrated reduced survival. In 2016, Vanhoutte et al. followed up on Blum’s work and also attempted to confirm Evans et al. 2008 and Fearon et al. 2011 diagnostic criteria in 167 cancer patients [5, 6, 11]. Vanhoutte et al. found that of the 167 cancer patients, 70% fit into the “looser” Fearon et al. 2011 definition, while 40% fit into the more multifaceted, encompassing definition by Evans et al. 2008. While patients categorized as cachexia by either definition had reduced survival, the more robust definition by Evans et al. resulted in greater reductions in survival, supporting the importance of encompassing comorbidities in defining cachexia. In 2017, Zhou et al. observed the prevalence and impact of comorbid symptoms in 306 advanced cachexia patients [29]. The presence and stage of cachexia had a progressive impact on the occurrence and severity of symptoms (appetite, sleep, fatigue, lack of energy, distress). However, in adjusted analysis, pain, fatigue, disturbed sleep, cognitive decline, anorexia, nausea, amongst several other symptoms, were observed in this cachexia cohort across stages of cancer. Additionally, Zhou et al. observed that more advance metastatic cancer patients had increased cachexia incidence. In 2018, Zhou et al. went on to observed 259 advanced cancer patients across cachexia stages [15]. Zhou et al. observed that patients with more severe cachexia had increase comorbid symptoms and reduced muscle mass, quality of life, and survival. In 2018, Judge et al. observed changes in muscle morphology, metrics of cachexia, metastasis, and survival in a
pancreatic ductal adenocarcinoma patient cohort [35]. They discovered patients with higher degrees of weight loss had higher observable collagen content. They also found that higher collagen content was associated with lymph node metastasis and reduced survival. Furthermore, in 2013, Wallengren et al. observed reduced survival across a heterogenous cohort of cancer patients with weight loss [10]. In support of Vanhoutte et al.’s findings [11], Wallengren et al. also observed that individual components of the multifaceted syndrome were consistently associated with decreased survival in a heterogeneous cohort of cancer patients [10]. In 2015, Martin et al. found that across 8,160 patients, that both weight loss and body mass index predicted survival, with differences in survival across the various body composition categories [31]. Taken together, these large clinical cachexia cohort analyses, across various cancer types, illustrates a clear and consistent prognostic role for cachexia in cancer patient survival [9-12, 15, 31, 34]. These data also illustrates that when cancer patients progress through the stages of cachexia, symptoms and survival worsens [6, 9, 11, 15, 29, 31]. Additionally, patients in the metastatic stage have a higher prevalence of cachexia diagnosis, regardless of tumor type and independent of treatment-induced cachexia [7, 12, 29, 33, 34]. This data also highlights the prognostic and defining importance of individual comorbid components of the cachexia symptoms in cancer patients, highlighting the complex and devastating nature of cancer cachexia. Ultimately, cachexia-induced mortality is currently estimated to be the direct cause of death in 20% of all cancer patients [8]. This mortality rate remains unchanged since its original estimations in 1932 [26], illustrating a pressing need for pathological understanding and therapeutic advancement to improve patient outcomes.
1.3.3 Skeletal Muscle Mass Across Health and Disease

While cachexia has consistently been associated with reduced survival and prognosis in patients [9-12, 34]. One such explanation on the cause(s) of this consistent association is that cachexia has been shown to reduce the ability to receive, tolerate, and/or respond to traditional forms of anti-neoplastic treatments [13, 14]. This was explained by altered chemotherapeutic kinetics and temporal drug exposure due to altered skeletal muscle mass. This work illustrated that muscle mass plays an important role in facilitating the efficacy of anti-neoplastic treatments. This critical observation, combined with evidence that reduced/altered anti-neoplastic treatment regimens may reduce anti-neoplastic anti-tumor efficacy [36], highlights how skeletal muscle mass may influence cancer, and then subsequently, cachexia outcomes. However, the role of muscle mass goes far beyond drug regulation. Skeletal muscle mass plays an integrated and multimodal role across health and disease [37]. In day-to-day health, muscle mass facilitates functional activity and mobility [38], regulates systemic glucose and amino acid metabolism [39, 40], provides an amino acid reservoir [37], and is a multi-organ and tissue molecular messenger [41]. Chronically, optimal muscle mass or methods and/or maintaining muscle mass are associated with improved physical wellbeing [42, 43], mental health [44-46], and survival [47-51] in those with and without disease.

With over 500 individual skeletal muscles are under direct, volitional, nervous system control, skeletal muscle is most commonly appreciated for its critical role in generating movement and maintaining functional activity [38]. Functional movement contributes to both physical autonomy and overall health [43-46]. This is best illustrated in analysis of those who “move” and
“don’t move.” Physical activity has been shown to increase insulin sensitivity, muscle protein synthesis, anti-oxidant enzyme activity, immune function, while reducing inflammation, fatigue, and incidence of obesity [52]. It has been observed that those who engage in more physical activity, have higher life-expectancies, extending up to 4.5 years for the most active [51]. Conversely, physical inactivity, often termed “sedentary,” is associated with elevated risk for disease and all-cause mortality [53, 54]. Thus, physical movement, both aerobic and anerobic, have been under investigation for their efficacy in cancer [44, 55] and cachexic patients [56, 57]. Aerobic exercise in rodents has been shown to both reduced tumor growth and improve skeletal muscle mass [58-60]. Resistance exercise (anerobic) in rodents has been shown to preserve muscle mass in the cachexia environment [61-64], even when the cachexia syndrome was unresolved [65]. Exercise has been demonstrated to be feasible, safe, improve physical performance, and attenuate psychological burden, cognitive decline, and cardiovascular events in cancer patients [43-45]. Consequently, maintenance of healthy muscle mass and function for physical activity may influence cancer, cachexia, and overall health outcomes.

Beyond physical movement, skeletal muscle also plays a critical role in regulating systemic metabolism. This is particularly salient in glucose and amino acid metabolism in the presence and absence of nutrients [40, 66-69]. Upon ingestion of a mixed meal, carbohydrates are broken down into individual glucose molecules, while protein stimulates glucagon release and subsequent glycolysis, both directly and indirectly elevating circulating glucose levels, respectively [70-72]. In response to post-prandial glucose elevation, pancreatic beta cells facilitate proportional insulin release down stream of GLUT2 facilitated diffusion, oxidative phosphorylation-induced ATP generation, closure of ATP sensitive potassium channel, depolarization of voltage sensitive
calcium channels, and eventual exocytosis of insulin granules into system circulation [73]. Insulin binds insulin receptors and facilitate GLUT4-mediated glucose uptake and the return of circulating glucose back into homeostatic range. While exact percentage uptake varies, liver and skeletal muscle tissues absorb most post-prandial glucose. While the liver holds priority in healthy individuals (conversely to those administering exogenous insulin), insulin-mediated glucose uptake in the skeletal muscle is the largest scavenger of post-prandial glucose, primarily due to its sheer size, storage capabilities, and energetic needs [39]. Consequently, skeletal muscle plays a prominent role in regulating systemic glucose levels during glucose abundance. Alternatively, skeletal muscle, secondary to the liver, helps indirectly maintain glucose levels during periods of acute and/or chronic fasting [40, 66, 67, 74, 75]. During periods of energetic deficits, glucose levels lowers as endogenous energetic utilization exceeds exogenous glucose input. Following lower blood glucose levels, insulin release is reduced. This relative reduction in insulin drives the mobilization of amino acids from the skeletal muscle to systemic circulation [40]. Felig et al. demonstrated that during periods of fasting, following blood glucose and insulin reductions, skeletal muscle amino acid efflux is upregulated, primarily (>50%) in the form of alanine and glutamine [37, 40]. Subsequently, it was then shown that alanine was disproportionately consumed across the hepatic tissue to undergo gluconeogenesis via the Cahill cycle to help maintain circulating glucose levels and attenuate fatal hypoglycemia during periods of energy deficits [40]. Thus, the skeletal muscle plays an important, active, and plastic metabolic role in glucose homeostasis by consuming, storing, utilizing, and indirectly facilitating glucose production to maintain homeostatic glucose levels. The importance of this process is most commonly appreciated during its pathological dysfunction or absence in the case of type-2 or type-1 diabetes, respectively [71, 76]. Both are categorized as diseases of glucose dysfunction. The glycemic
mismanagement commonly observed in diabetic patients results in elevated risk for many of the leading causes of death and early mortality [77-80]. However, glucose also has well established roles in cancer and cachexic metabolism, as glucose uptake has been reported to be commonly elevated in a number of tumor types [81, 82] and altered glucose metabolism in cachexic skeletal muscle can contribute to energetic inefficiencies and tissue depletion [83-86]. While skeletal muscle has a critical role in glucose homeostasis and glucose metabolism has an established role in cancer and cachexia, a direct effect of muscle’s glucose homeostatic effect on cancer and/or cachexia remains unexplored.

Muscle mass make up an estimated 40% of total body mass and 40-60% of total body protein. Skeletal muscle is the primary source of amino acids which help sustain 1) tissue structure and function, 2) organ structure and function, 3) cellular protein concentrations, and 4) serum protein concentrations. During periods of amino acid demand, the skeletal muscle is the primary donator. A common example is during an acute inflammatory response. Upon inflammatory insult, proinflammatory cytokines, TNF-α, IL-6, and IL-1β are often elevated [87]. These cytokines help immune cells mount an optimal immune response. All three cytokines have been shown to play catabolic roles in the skeletal muscle. This amino acid efflux facilitates, “(1) the activation of T lymphocytes, B lymphocytes, natural killer cells and macrophages; (2) cellular redox state, gene expression and lymphocyte proliferation; and (3) the production of antibodies, cytokines and other cytotoxic substances” [88]. While liberating amino acid from the skeletal muscle for an optimal immune response is a critical component to combat acute inflammatory triggers (such as infection), this has pathological implications in cancer cachexia where prolonged inflammation is common, contributing to multiple component of the multifaceted syndrome including skeletal
muscle catabolism, adipose tissue wasting, anorexia, systemic metabolic alterations, etc. Conversely, the absence of sufficient muscle mass has been linked to increased risk for infection, reduced wound healing, and compromised immunity, amongst others [42].

An ever growing body of literature has started to show skeletal muscle autocrine, paracrine, and endocrine-like function via crosstalk with various other organs and tissues through molecules called myokines [41]. Examples of such include skeletal muscle IL-6 and BDNF release. Skeletal muscle IL-6 production has been demonstrated to increase GLP-1 secretion in pancreas and gut, increase lipolysis in adipose tissue, increase glucose uptake and fatty acid oxidation in skeletal muscle. Skeletal muscle BDNF production has been shown to increase brain learning and memory, along with muscle fatty acid oxidation. However, various other myokines have been linked to other tissues such as bone, breast, and liver, illustrating skeletal muscle as a critical tissue in multiorgan/tissue crosstalk.

Consequently, due to skeletal muscle mass’ multimodal role in health and disease it is no surprise that muscle mass predicts longevity in older adults [47] and negatively impact survival across various atrophy-based diseases including sarcopenia, infection, chronic obstructive pulmonary disease, chronic kidney disease, amongst others [47-50]. Additionally, skeletal muscle mass has implication on, and is implicated by, cancer, aging, infection, chronic obstructive pulmonary disease, chronic heart failure, chronic kidney disease, diabetes, disuse, muscular dystrophy, neuropathy, anorexia, etc. Unsurprisingly, skeletal muscle atrophy has a direct healthcare cost of 1.5% annually [42].
Collectively, skeletal muscle is one of the largest organ systems in the body making up almost ½ of total body mass and >½ of total body protein. This extremely dynamic, multifaceted, and plastic tissues plays critical roles in regulating systemic metabolism as well as engaging in multi-organ and tissue crosstalk to not only produce functional movement but also a maintain health, combat pathology, and prolong survival [42]. The importance of this tissue remains most salient in diseases of skeletal muscle atrophy, particularly cancer cachexia.

1.3.4 Distinct Multifaceted Syndrome

Cancer cachexia is distinct from other common atrophy-based diseases including starvation, caloric restriction, hyperthyroidism, sarcopenia, diabetes, disuse, muscular dystrophy, neuropathy, amongst others (Table 1.1). While skeletal muscle mass is a common component in atrophy diseases, the pathophysiology and multisystem consequences distinguish these various forms of atrophy.

Starvation and/or caloric restriction involve the reduction in energetic input relative to expenditure leading to the reliance of endogenous energy stores to maintain tissue energetic requirements [40, 66, 74, 75, 89]. Due to their abundance of stored energy and inherent plasticity (both anabolic and catabolic), adipose tissue and subsequently skeletal muscle are preferentially catabolized to supplement multiorgan and tissue energetic needs via their established roles in fatty acid, glucose, amino acid, and ketone metabolism. Anorexia also results in tissue catabolism via net reductions in energetic input versus output. However, anorexia can be distinguished from volitional reductions in calorie intake, especially in the context of cachexia [90]. Mounting
evidence suggests that anorexia in CACS may be the result of inflammation-induced appetite reduction via pro-inflammatory cytokines (TNF-α, IL-6, IL-1β; [87]). However, additional evidence suggests hormonal dysregulation (leptin, ghrelin, etc…) and neuropeptides may be contributing factors [90-92]. Conversely, starvation and caloric restriction often result in reductions in inflammatory biomarkers [93]. Additionally, while starvation, caloric restriction, and anorexia all result in net energetic deficits and catabolism of plastic energetic tissues, the ratio of skeletal muscle wasting to adipose tissue wasting is far greater in cachexia. In fact, during cachexia, skeletal muscle wasting has been reported to occur regardless of the degree of adipose tissue wasting. Additionally, while refeeding can reverse starvation and caloric restriction induced catabolism, this hasn’t proven effective in the cachexic atrophy environment [94-96].

Sarcopenia is a disease of aging that results in reduction in muscle mass and function [37, 97]. Anabolic resistance to insulin signaling, protein ingestion, and resistance exercise have been observed in aging populations [98-100]. Additionally, systemic inflammation has been suggested to contribute to sarcopenic wasting and may be contributory in the observed anabolic resistance. Hypogonadism is a common aspect of aging physiology which can contribute directly and indirectly to both reduced anabolism and subsequent muscle loss [101]. Lastly, while aging can lead to a subtle and progressive decline in muscle mass and function overtime, disuse or acute reductions in skeletal muscle loading (disuse atrophy), can lead to rapid robust declines in muscle size [102, 103]. This can be a common component to many disease pathologies as diagnosis and comorbid symptoms can alter patients’ ability and/or desire to engage in physical activity. Anabolic resistance, inflammation, hypogonadism and disuse atrophy are all common overlapping observation between sarcopenia and cachexia [5, 104-106]. The combination of all these factors
leads to loss of muscle across age that is then difficult to regain. Consequently, like cachexia, much attention has been placed on understanding and targeting catabolism in these disease states. Another interesting overlap is the reduction in muscle mass without the obligate adipose tissue wasting. While adipose tissue wasting is common in cachexia, it isn’t in sarcopenia. In fact, a common phenotype in sarcopenia is obesity, termed sarcopenic obesity [94, 100]. This has been observed as well in some cachexia trials where caloric intake was elevated, resulting in adipose tissue accumulation without meaningful changes in skeletal muscle mass [94-96]. However, critical differences between sarcopenic and cachexia are the atrophy timeline and robust comorbid nature of cachexia. Sarcopenia often occurs over many years, often decades [107], while cachexia occurs over weeks and months [6]. Additionally, cachexia accompanies anorexia, anemia, and hypoalbuminemia, which is not commonly characterized in sacrcopenic patients. Lastly, research has uncovered clinically effective options to combat sarcopenia in the form of resistance exercise, protein supplementation, and hormone replacement therapy, which are shown most effective when combined [37, 99]. However, there is not clinically effective standard of care treatment for cachexia. Thus, while cachexia and sarcopenia have many overlapping characteristic multiple key differences exist.

While diabetes is often seen as a disease of abundance typically accompanying obesity, underlying skeletal muscle atrophy is a common aspect of this disease [108]. While anorexia is not common in either type-1 or type-2 diabetes, reduced anabolic signaling is. In type-1 diabetes, reduced insulin signaling can occur due to insufficient endogenous insulin productions [109]. However, in type-2 diabetes reduced insulin signaling results from developed resistance [110]. Both forms of diabetes, like cachexia, have cited elevations in inflammation [108]. While this
appears to originate in cachexia from the underlying cancer pathology, diabetic inflammation appears to result from glycemic mismanagement [111]. While not common in type-1 diabetes, approximately a fourth of type-2 diabetic have been cited to develop anemia and hypogonadism [112]. Interestingly, a unique overlap with cachexia is altered glucose metabolism [113, 114]. Additionally, type-2 diabetics also have elevated circulating lactate at rest, which has been proposed to occur due to hyperinsulinemia [115, 116]. This is like the observed elevations in aerobic fermentation and lactate production seen at the skeletal muscle of cachexic patients [83, 117-119]. Additionally, atrophy in diabetes is a prolonged process. Thus, diabetic and cachexic patients can present with dysregulated metabolism and skeletal muscle atrophy, and a smaller percentage develop anemia and hypogonadism. However, weight, fat mass, and function loss, along with discrepant atrophy timelines are observed difference between diabetes and cachexia.

Lastly, hyperthyroidism, muscular dystrophy, neuropathology, like starvation, caloric restriction, sarcopenia, disuse, diabetes, all have overlapping and independent components, but none of these diseases encompass the multifaceted and dynamic nature of atrophy in cachexia. However, infection and sepsis illustrate a disease state with unique overlap to the cachexia phenotype. Like cachexia, sepsis causes rapid weight, muscle and fat loss, with anorexia, systemic inflammation, insulin resistance, reduced circulating anabolic hormones and signaling, anemia, and functional impairment [5]. In fact, evidence exists that overlapping skeletal muscle atrophy mechanisms contribute to reduced skeletal muscle mass across cachexia and septic atrophy. The key differences between sepsis and cachexia are atrophy the timeline and disease initiation. Sepsis, an often-fatal condition, results in very rapidly atrophy, on the order of days. Additionally, sepsis is often caused by a robust bacterial infection, while cachexia is most commonly tumor-driven.
Table 1.1. Characteristics of Skeletal Muscle Wasting

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1.3.4.1 Metastasis

An intriguing, and largely understudied area of inquiry revolves metastasis’ role in cancer cachexia. Presently, 30% of all cancer patients have cachexia [7]. However, incidence increases to 50-80% in metastatic cancer patients [7, 8, 29, 32]. Bachmann et al. demonstrated that pancreatic cancer patients with cachexia were more likely to have metastatic burden and argued that metastatic progression might be a critical step in the development of tumor-associated cachexia [12]. A year later, Bachmann et al. demonstrated that metastasis was diagnosed twice as frequently in pancreatic cancer patients who presented cachexia [120]. Shiono et al. reported that metastasis was associated with 2.4x more weight loss in lung cancer patients independent of treatment [121]. Judge et al. observed that skeletal muscle collagen content, a marker of skeletal fibrosis, was more prevalent in pancreatic ductal adenocarcinoma patients with lymph node metastasis [35]. Additionally, Caillet et al. 2016 provided supportive evidence for metastasis role in cachexia symptoms by revealing that a consistently higher range of aged (>65 years of age) cancer patients
experience malnutrition (64-76.5%) with metastasis [33]. Zhou et al. observed a higher prevalence of cachexia and refractory cachexia, along with cachexia symptoms, in patients with systemic metastasis compared to locally invasive tumors in a heterogeneous cohort of cancer patients [29]. In the two largest analysis on this topic to date, Dewy et al. observed approximately 3,000 patients and demonstrated that patients in the advanced metastatic state had higher incidence of cachexia in all tumor types, independent of treatment [34]. Muscaritoli also observed approximately 2,000 treatment naïve patients across 22 oncology treatment sites and found that patients in the advance metastatic stage had higher prevalence of weight loss, inflammation, and anorexia for all tumor types observed [122]. Furthermore, emergent evidence in rodent modeling indicates that the metastatic process may directly contribute to cachexia onset and progression [123]. Cumulatively, this evidence supports the notion that cancer progression and metastasis may be a common component of the cachexia syndrome, independent of cancer type or prior treatment [12, 29, 120-125].

1.3.4.2 Body Composition

The hallmark characteristic of cachexia is altered body composition [5, 6, 8]. Clinically, bodyweight is most commonly used as a surrogate marker for both skeletal muscle and often adipose tissue changes as changes in bodyweight commonly results in associated changes in skeletal muscle and adipose tissue. Additionally, bodyweight analysis is a low resource, rapid clinical evaluation of alteration in body composition. More refined, detailed measurements of body composition are bioelectrical impedance, hydrostatic weighing, dual-energy X-ray absorptiometry (DXA), magnetic resonance imaging (MRI; [126]). Typically, the more accurate and reliable the
measurement, the higher the analysis cost and resources investment. However, while loses in bodyweight often accompany loses in both skeletal muscle and adipose tissue, this association is particularly uncertain in the cancer cachexic environment and has led to the exclusion of adipose tissue wasting as an obligate characteristic in many cachexia definitions. Multiple confounding factors may lead to misinterpretation as to the nature of wasting and therapeutic outcomes within cachexic patients. This issue is highlighted in cancer patients with sarcopenia obesity and in cachexia trails using appetite stimulants where it has been reported that adipose tissue can increase while skeletal muscle progressively atrophies [94-96]. Additionally, alterations in fluid retention, namely ascites fluid, have been observed in cachexic patients [27, 127, 128]. Taken together, while changes in body composition are hallmark to the cachexia syndrome, differences in wasting across skeletal muscle and adipose tissue, along with comorbid alterations in fluid retention, illustrate confounding variables which may influence the accuracy of cachexia status based on superficial metrics of body composition alone.

1.3.4.2.1 Skeletal Muscle

Skeletal muscle is an extremely dynamic, multifaceted, and plastic tissues with critical roles in functional movement, metabolism, and multiorgan/tissue crosstalk which has an established role in promoting health and combating pathology. However, reduced skeletal muscle mass is a strong negative prognosis across various acute and chronic diseases [47-50]. In cancer, reduced skeletal muscle mass has been linked to worsen prognosis, quality of life, and ability to receive, tolerate, and/or respond to standard of care [9-15, 29, 35]. Thus, efforts have been placed into understanding the mechanisms driving skeletal muscle atrophy.
Fundamentally, skeletal muscle mass is the net balance between skeletal muscle breakdown and synthesis [129, 130]. Increased skeletal muscle breakdown and decrease synthesis has been observed in cancer cachexia, resulting in enhanced skeletal muscle protein turnover and subsequent loss of mass [131-134]. However, due to the catabolic rate of cachexia, much focus has been aligned on understanding and targeting the catabolic mechanisms driving the disease [8, 134].

Multiple driving mechanisms of skeletal muscle atrophy have been proposed and explored for cachexia [8], yet limited patient data leads to many unanswered questions related to the driving forces at the skeletal muscle level [17, 135, 136]. However, hierarchical assessment of molecular pathways can be used to determine potential translatable value. Proposed criteria for this could be assessed based on evidence in: 1) clinical cachexia, 2) experimental modeling, 3) conservation across multiple biological organisms, 4) role in anabolic and/or catabolism, 5) involvement across atrophy-based environments. The ubiquitin proteasomal degradation pathway has received considerable attention due to its established role in cellular protein turnover and catabolism across many atrophy-based diseases, with evidence across in vitro, rodent, and human cancer cachexia [130, 137-150]. Two conical activators of the ubiquitin proteasomal degradation pathway pathways with multifaceted roles across systemic atrophy that have undergone considerable investigation are TNF-α and IGF-1/Insulin signaling.
1.3.4.1.1 Tumor Necrosis Factor-Alpha Signaling

Pro-inflammatory cytokines TNF-α, IL-6, and IL-1β are believed to be important contributors to the cachexia phenotype in both human and/or rodent models of cachexia. These cytokines are produced and secreted from the tumor and/or produced by the tumor-host response in various tissues, including immune cells. TNF-α is one of the most studied pro-inflammatory cytokines. TNF-α originally known as cachectin, due to its believed role in cachexia pathogenesis, was originally derived from macrophage cell downstream of LPS-induced sepsis [151]. It was also observed that cachectin could potentially have an anti-tumor role in vitro, giving it the TNF-α name it retains today [152-154]. TNF-α’s role in facilitating wasting has undergone extensive evaluation across atrophy-based disease due to its common occurrence and numerous ways it contributes to the cachexia syndrome [87].

TNF-α travels through the blood as a homotrimer and binds to TNF-α-R1 receptor [155, 156]. TNF-R1 then signals through subdomains TRADD and TRAF2 to recruit IKKβ to enzymatically phosphorylate the IκBa which binds NF-κB, inhibiting NF-κB translocation into the nucleus. IKKβ phosphorylates IκBa at Ser32 and Ser36 which initiates a confirmations changes releasing NF-κB. Phosphorylated-IκBa is then polyubiquitinated by E3 ligases. IκBa is shuttled to the 26S proteasome and degraded into peptides. Peptides are further catabolized by intracellular peptidases into individual amino acids [137]. NF-κB is a dimer composed of Rel-family proteins. TNF-α stimulates the release of RelA(p50)/p65 heterodimer which quickly enters the nucleus and binds promoter regions for various proinflammatory (TNF-α, IL-6) [157] and catabolic (MuRF1 and Atrogin-1) genes [146, 158-161]. Within the skeletal muscle, MuRF1 and Atrogin-1 are
muscle (skeletal, smooth, and cardiac) specific E3 ligases which elevate as a result of various catabolic signaling and have been reported across various atrophying scenarios [162-165] including cachexia model systems [146, 161, 166-168]. These E3 ligases take myofibrillar proteins actin and myosin and polyubiquitinate their fragmented components to target them for 26S proteasomal degradation. TNF-α → IKKb → IkBa → NF-κB → nuclear translocation is a conical pathway [169] across various tissues resulting in the proteasomal degradation of myofibrillar components in skeletal muscle. TNF-α and NF-κB contributory role in the cachexia phenotype has also been reported in myofibrillar cell lines [146, 157, 159, 160, 170-175], cachexic rodent model systems [18, 158, 176-185], and most importantly, in cachexic patients [139-147], suggesting that these signals may represent potentially promising catabolic therapeutic targets.

TNF-α has also been shown to regulate skeletal muscle synthetic signaling [186, 187]. TNF-α has been shown to attenuate insulin/IGF-1-induced anabolic signaling at the skeletal muscle [186, 188] in a dose-dependent manner [187]. While TNF-α-induced insulin/IGF-1 resistance is not fully understood, multiple reports indicate this may be occurring at the receptor level at the skeletal muscle [186] and other tissues [189]. This anabolic resistance to insulin/IGF-1 signaling appears to be consistent with other pro-inflammatory cytokines IL-6 and IL-1β [186, 189], suggesting a inflammation-induced anabolic resistance that may be facilitated via pro-inflammatory cytokines at the receptor level [186-188, 190-192].

Inflammation appears to play an important role in energetic metabolism. TNF-α has been reported to directly shift skeletal muscle oxidative metabolism to aerobic fermentation in myocytes [193]. Mechanistically, TNF-α has also been demonstrated to reduce myocyte mitochondrial
respiration/oxidation in a NF-κB-dependent manner [118]. Increase aerobic fermentation and lactate production has also been observed in TNF-α stimulated myocytes via augmented NF-κB-induced HIF-1α signaling [119]. Evidence for this effect in cachexia was demonstrated in the gastrocnemius of C26 cancer cachexia model which demonstrated increased oxidative stress, impaired redox homeostasis, altered glucose metabolism, decreased TCA carbon cycling, and reduced amino acid abundance, all consistent with the “Warburg effect” [117]. However, in this study, the skeletal muscle “Warburg” phenotype was only partially modulated by inhibition of NF-κB signaling (Compound A inhibits NF-κB and altered redox balance and oxidative stress, but not glycolytic and amino acid phenotype), suggesting NF-κB may contribute to altered metabolism, but may not entirely explain the shift in skeletal muscle towards aerobic fermentation. Nonetheless, evidence for HIF-1α and redox balance contributing to aerobic fermentation in skeletal muscle was evaluated in aging muscle and illustrated a Warburg-like phenotype, even during sufficient O2, explained by age specific declines in nuclear NAD+ (altered redox balance) [194]. Evidence for inflammation-driven Warburg phenotype in skeletal muscle has also been observed in chronic obstructive pulmonary disease patients, a cachexic disease driven by inflammation [118, 119]. Remels et al. demonstrated that chronic obstructive pulmonary disease patients with elevated TNF-α mRNA had lower body mass index, markers of oxidative metabolism, and lower muscle mass (non-significant trends; [118]). Remels et al. went on to demonstrate that chronic obstructive pulmonary disease patients with higher levels of TNF-α in muscle biopsy samples, also had increased HIF-1α expression, further suggesting TNF-α induces enhanced aerobic fermentation in the skeletal muscle of cachexic patients [119]. Interestingly, direct analysis of skeletal muscle metabolite flux of cachexic patients compared to malnourished non-cachexic patients revealed a 7.5-fold higher lactate efflux from skeletal muscle [83]. These elevations in blood lactate can be
used to generate glucose via the Cori cycle, a process reported to be elevated in cachexic patients [84-86]. The Cori cycle is estimated to cause 250-300 kcal/day in fuel inefficiency in cachexic patients [195]. Interestingly, an estimated 250-400 kcal/day caloric deficit is reported in cachexic patients [196]. While the underlying mechanism of inflammation-induced alterations in energetic metabolism are not completely elucidated, evidence indicates that inflammation may be increasing aerobic fermentation (Warburg-like effect) in the skeletal muscle (potentially via NF-κB and/or HIF-1α signaling) and that lactate cycling may help explain nutritional deficiencies within cachexic patients, illustrating another way in which inflammation may contribute to the cachexic phenotype.

While skeletal muscle atrophy is a maladaptive response to inflammatory disease states, there is strong evolutionary rational for such a response. During acute inflammatory insult in the form of an infection, pathogen, or foreign entity (inflammatory drivers), such is the case with a tumor, the body mounts an immune-based inflammatory response which involves the upregulation of pro-inflammatory cytokines. As these inflammatory drivers threatens the host survival acutely, priority is taken to sequester/combat the/these inflammation initiator(s). While skeletal muscle mass has a clear role is health, and thus is preferentially persevered over adipose tissue during energetic deficits (absent pathology), it is sacrificed to preserve the host species acutely by driving pathways that attempt to clear the body of foreign/pathogen or foreign/pathogenic-like entities. Pro-inflammatory cytokines, central to this response, play a multifaceted role in facilitating an adequate immunologic response. They facilitate the systemic acute-phase response at the liver, mobilization of immune cells, catabolism of skeletal muscle protein and mobilization of amino acids, and inhibition of skeletal muscle anabolic signaling. Together, these molecules have
conserved redundancy to ensure that the immune system is mobilized and receives adequate energetic and anaplerotic substrates for optimal immune function, even at the expense of important host tissues, such as the skeletal muscle. While this response is essential for maintaining acute health when an inflammatory driver is not sequestered, tumors-driven inflammation can result in augmented catabolism and attenuated anabolism in the skeletal muscle chronically, resulting in progressive skeletal muscle loss. Unsurprisingly, skeletal muscle atrophy is seen in many chronic inflammatory environments beyond cancer cachexia, such as chronic obstructive pulmonary disease, chronic kidney disease, chronic heart failure, type-2 diabetes, amongst others. Unfortunately, this muscle loss further compromises the hosts ability to combat the underlying disease pathology, often leading to mortality in many patients. Thus, taken together, proinflammatory cytokines play a conserved role in the inflammatory response across tissues, facilitating catabolism and reducing synthesis in skeletal muscle as indicated from both in vitro and in vivo (mouse, rat, and human) analysis in atrophy-based diseases with underlying systemic inflammation. Consequently, proinflammatory inhibitors have been developed to combat inflammatory-driven pathology. A primary example are TNF-α inhibitors which have resulted in $25 billion dollars in sales, and are current standard of care for clinical use for rheumatoid arthritis, and are under exploratory investigation for other disease pathologies [197], including cancer cachexia [198]. However, preliminary clinical investigation into their efficacy in cancer cachexia are ongoing and current results are inconclusive on their effectiveness.
1.3.4.2.1.2 Insulin and Insulin-Like Growth Factor 1 Signaling

Insulin is a 51 amino acid hormone secreted by the pancreatic beta-cells with essential roles in regulating glucose metabolism and growth. It’s original discovery and isolation awarded Fredrick Banting and John Macleod the Nobel prize for its life saving capabilities in patients with absent or attenuated endogenous insulin production, type-1 diabetics [199]. Subsequently, insulin was directly and/or indirectly involved in additional Nobel prize winning discoveries [200-202]. Insulin is most known for its predominant role as a regulator of systemic glucose, lipid, and amino acid metabolism [110, 203]. Generally, relative insulin abundance leads to energy storage, cellular growth, and whole-body anabolism, while insulin shortage induces energetic mobilization and release, along with tissue atrophy. Thus, insulin has been described as a primary and central metabolic regulator between cellular and systemic anabolism/catabolism. IGF-1 is a 70 amino acid peptide primarily produced from the hepatic tissue due to growth hormone (GH) elevations (although most tissues can synthesize IGF-1) with overlapping amino acid structure, receptor binding, downstream signaling, and role in anabolism and catabolism to insulin [110, 203-206].

Insulin and/or IGF-1 bind two homologous receptors, insulin (IR) and/or IGF-1 receptors (IGF-1R), albeit at differing affinities [110, 203, 205, 207]. Upon binding, IR and/or IGF-1R undergo conformational changes, autophosphorylation of receptor tyrosine kinases, and recruitment of binding proteins that can facilitate PI3K/Akt and/or MAPK signaling. PI3K/Akt signaling is facilitated by binding and phosphorylation IRS [110]. IRS activates PI3K, which facilitates the phosphorylation of PIP2 to PIP3. PIP3 recruits PDK1 to the cellular membrane
which subsequently phosphorylates and activates Akt. Akt acts as a key regulator of insulin/IGF-1 signaling. Phosphorylated Akt:

1) negatively regulates TSC 2/1 to allow for mTORC1 activation. Activated mTORC1 inhibits eIF4E and activates S6K resulting in Cap-dependent translocation, translation elongation, mRNA biogenesis, and ribosomal biogenesis [208]. These processes are a key step in regulating skeletal muscle protein synthesis and cellular growth;

2) phosphorylates FOXO3a, a known catabolic regulator [209]. Phosphorylated FOXO3a is restricted to the cytosol cellular compartment, attenuating FOXO3a atrophy signaling;

3) inactivates GSK3 resulting in hepatic glycogen accumulation [210];

4) inhibits PCG-1α, a known promoter of gluconeogenesis and fatty acid oxidation [211, 212];

5) other regulatory effects [213, 214].

Alternatively, IR and IGF-1R also recruits Shc proteins to the tyrosine docking sites, which subsequently recruit Grb2/Sos and Ras/Raf [110]. Ras/Raf causes the phosphorylation of MEK, which phosphorylates ERK. This alternative phosphorylation cascade, termed MAPK, promotes cellular proliferation and gene transcription. Additionally, it was found that insulin and IGF-1 binding effect on IR and IGF-1R are concentration dependent, thus higher circulating insulin and/or IGF-1 results in greater signaling amplitude [205].

However, when insulin and IGF-1 levels are lowered, IR and IGF-1R binding is reduced and subsequently, Akt remains in a relatively inactive, unphosphorylated state. Inactivate Akt reduces FOXO3a phosphorylation [215]. In its unphosphorylated state FOXO3a translocates to the nucleus where it upregulates apoptotic, mitobiogenesis, antioxidant, but important to skeletal
muscle, atrophy gene expression. FOXO3a increases atrogin-1, a skeletal muscle specific E3 ligase sufficient to induce atrophy in low insulin/IGF-1 environment [209]. Atrogin-1 facilitates the ubiquitination of cellular proteins [162, 163]. Once cellular proteins become poly-ubiquitinated, they are shuttled to the 26S proteasome which breaks whole proteins into their smaller peptide components [137]. Cytosolic peptidases breakdown the inactive peptide fragments into individual amino acids. These amino acids can then be reused by the cell, converted to energetic substrates and/or ATP, undergo anaplerotic processes, and/or be utilized for synthesis of whole proteins. Additionally, while low insulin and IGF-1 have been shown to induce skeletal muscle tissue catabolism and amino acid efflux, reductions in these hormones also promotes increased glycogenolysis, gluconeogenesis, lipolysis, and fatty acid oxidation [110]. Taken together, insulin and IGF-1 regulate growth via dose-dependent regulation of metabolite (amino acids, glucose, and lipids) mobilization, storage, anaplerotic conversion, and energetic utilization.

Insulin/IGF-1’s essential role in muscle homeostasis was most convincingly shown via myocyte and rodent models with direct genetic alterations across nodes of the Insulin/IGF-1 pathway [130, 138]. Overexpression of IGF-1 has been demonstrated to increase contractile protein content and myogenesis in cultured muscle cells, while increasing skeletal muscle mass in rodent models [216-218]. Elevations of IGF-1 via viral transfection was sufficient to increase muscle mass and/or function in young, old sarcopenic, disuse, and dexamethasone treatment mice, demonstrating insulin/IGF-1 signaling can augment muscle mass and function across age, disease, and pharmacologically-induced atrophy [219-221]. Chronic elevations in insulin have been shown to increase weight in rodents [222]. Conversely, IGF-1 null rodents all present general growth retardation [223-225], while muscle specific IGF-1R knockout models present with reductions in
weight [226] and skeletal muscle [206, 227]. IR knockout rodent models have demonstrated reduction in skeletal muscle size and force production [228]. Additionally, reduction in production and circulating levels of insulin produces type-1 diabetes, a known catabolic condition without the supplementation of exogenous insulin [229, 230]. O’Neill et al. attempted to determine the relative contribution/importance of IR versus IGF-1 on skeletal muscle mass [206]. O’Neill et al. demonstrated that IR appears to provide a greater contribution to skeletal muscle homeostasis than IGF-1R as IR knockouts lost more muscle mass. However, while important, these findings may be explained by reports of IR/IGF-1R hybrid receptor formation in IGF-1R knockout models [226] potentially mitigating atrophy in IGF-1R knockouts. Additionally, overlapping insulin/IGF-1 ligand binding to alternative IR receptor may induce similar signaling pathways [206]. Together, regulation of circulating insulin and IGF-1, along with IR and IGF-1R, all strongly suggest a strong contributory role of upstream insulin/IGF-1 signaling component to tissue anabolism and catabolism.

In agreement with findings on circulating insulin/IGF-1 and receptor analysis in skeletal muscle homeostasis, constitutively active PI3K and/or Akt increase hypertrophy and attenuate atrophy [231-233]. Inducible skeletal muscle Akt causes rapid and pronounced increases in skeletal muscle mass and strength [234-236]. Akt knockout has been demonstrated to induced growth retardation and skeletal muscle atrophy [232, 237-239]. Additionally, knockout of muscle specific E3 Ligases MuRF-1 and atrogin-1 attenuated atrophy during denervation [162]. Additionally, while knocking out FOXO3a is insufficient to alter normal growth [240], constitutively active FOXO3a is sufficient to induce atrogin-1 and skeletal muscle atrophy [209]. Taken together, evidence in cell culture and rodent modeling across nodal downstream of IR and
IGF-1R indicate that elevations in insulin and/or IGF-1 signaling induce hypertrophy and attenuate atrophy, while reduction in this pathway are associated with E3 Ligase elevation, FOXO3a, and consistent skeletal muscle atrophy.

While genetic modeling provides considerable evidence for insulin/IGF-1’s role in muscle homeostasis, studies modulating nutritional intake further suggest a centralized role of insulin/IGF-1 in skeletal muscle. Reducing caloric intake has consistently been shown to induce reductions in bodyweight, from both skeletal muscle and adipose tissue compartments, albeit at differing rates. Clinically, patients undergoing caloric restriction and/or fast have reduced insulin [74], IGF-1 [241-243], bodyweight and muscle mass. Alternatively, during periods of feeding, carbohydrate and protein intake increase insulin and IGF-1 levels [71, 72, 241, 244]. Elevated carbohydrate intake increases circulating glucose levels which stimulate the release of insulin from pancreatic beta-cells. Elevated amino acids elevated glucagon and facilitate glycogenolysis, further elevating glucose over a prolonged period [71]. Additionally, amino acid intake regulates IGFBP-1 expression, an inhibitor molecule that is dynamically regulated by food intake and inhibits IGF-1 binding to its respective receptor [245]. Reduced insulin is associated with increased IGFBP-1 [244]. However, increase insulin decreases IGFBP-1 production and increases IGF-1 bioactivity. Thus, regulating nutritional intake alters circulating insulin and IGF-1 concentrations and/or bioactivity and this is suggested to explain alteration in tissue mass that accompany nutritional alterations.
Insulin/IGF-1’s role in tissue homeostasis and mass has also been observed in patients. Clear examples of insulin’s influence on growth is present upon diagnosis of type-1 diabetes. Type-1 diabetes is a disease of endogenous insulin deficiencies which can result in whole body wasting and skeletal muscle atrophy until administration of exogenous insulin [229, 230]. It has also been demonstrated that type-1 diabetic patients during periods of acute insulin deficiency upregulate FOXO3a signaling in skeletal muscle [109]. Type-2 diabetic patients with insulin resistance and dysregulated insulin/IGF-1 signaling, demonstrate 60% higher FOXO expression [108]. An inverse association has also been observed between skeletal muscle mass and insulin resistance [246]. Additionally, patients with IGF-1R mutations and GH deficiency and/or insensitivity have been demonstrated to present with retarded growth [247-249]. However, exogenous administration of GH has been shown to stimulates hepatic release of IGF-1 resulting in restoration of normal growth in patients with GH deficiency [250]. Support for insulin/IGF-1’s role in muscle homeostasis have been suggested in patients across multiple atrophy environments including cachexia [148-150, 251-258], nutrient deprivation/anorexia [241-243], sepsis [259-262], diabetes [109], sarcopenia [263], amongst others. Insulin/IGF-1 signaling appears to play a central role in muscle homeostasis in myocytes, rodents, and humans, along with evidence across atrophy-based conditions, including cachexia.

1.3.4.2.2 Adipose Tissue

Adipose tissue is best known for its plastic role in energy storage/release in response to alterations in systemic energetic balance. Like skeletal muscle, adipose tissue plays important functions across physiological systems, including immune, endocrine, metabolic, amongst others.
Adipose tissue also facilitates multiorgan crosstalk via secretory adipokines, which include cytokines (TNF-α, IL-6) and hormones (leptin, adiponectin). Alternatively, adipose tissue contains receptors for and receive signals from these same cytokines and endocrine molecules, including insulin and IGF-1, which play a regulatory role in adipose tissue size. Both surplus and deficiencies in adipose tissue can lead to adverse health and disease outcomes. Excess adipose tissue increases the risk for cardiovascular disease, obesity, type-2 diabetes, certain cancers, amongst others [266]. Alternatively, adipose tissue wasting is a common component to cancer cachexia wasting [8]. Cachexia definitions often exclude adipose tissue wasting as an obligate component to the multifaceted disease [5, 6] due to prior evidence on discrepant tissue wasting across body tissue compartments during overfeeding studies [94-96]. Simultaneous skeletal muscle atrophy and adipose tissue hypertrophy have also been observed in sarcopenic [267] and type-2 diabetic patients [108]. Excess adipose tissue can be problematic in the cachexic environment as it may hamper clinical diagnosis, drive systemic metabolic dysfunction, and contribute to a pro-tumor environment.

Cachexic adipose tissue wasting has been attributed to several mechanisms including inflammatory, hormonal, and altered mitochondrial energetic efficiency [8]. Like skeletal muscle, TNF-α plays a regulatory role in adipose tissue. TNF-α has been demonstrated to induce lipolysis via NF-κB signaling in human adipocytes [268]. Additionally, TNF-α-induced lipolysis and glycerol release in 3T3-L1 mouse adipocytes [269]. Stephens and Pekala found that TNF-α-induced insulin resistance and inhibited insulin receptor gene expression in 3T3-L1 adipocytes [270]. Torti et al. 1985 found that TNF-α inhibited gene expression of lipogenesis enzymes [271].
Thus, TNF-α induces catabolism, disrupts glucose metabolism, and inhibits anabolism in adipose tissue.

Hormonally, IR and IGF-1R are both present on adipocytes, while IR appears to play a more prominent role due to higher IR:IGF-1R ratio in differentiated adipocytes [205, 272, 273]. Interestingly, IR/IGF-1R knockout studies have revealed a 95% reduction in white adipose tissue with IR knockout, but only 25% reduction with IGF-1R knockout [274], demonstrating the importance of both insulin and IGF-1 on adipose tissue mass. Insulin has an undisputed role in adipose tissue homoeostasis. Insulin is known to facilitate increased lipogenesis and lipid synthesis, and reduced lipolysis and fatty acid oxidation [275]. Together, insulin and IGF-1 both play important roles in adipose tissue homeostasis and metabolism, with insulin playing the primary role, and thus help regulate adipose tissue size.

Adipose tissue can be broken into categories based their color and metabolic characteristics [276]. White adipose tissue is commonly characterized for its stored energy. Conversely, brown adipose tissue is known for thermogenic heat generation resulting from uncoupling protein (UCPs) which utilize energy to dissipate heat but results in inefficient utilization of energetic resources. During cachexia, adipose tissue can transition from white to brown and result in adipose tissue wasting [8, 277]. Thus, inflammation, hormones, and inefficiency energy expenditure all contribute to alterations in adipose tissue metabolism and have been demonstrated to facilitate wasting in the cachexic environment [8].
Adipose tissue catabolism has been recently described to potentially precede and contribute to skeletal muscle wasting in cachexia. Das et al. in 2011 demonstrate that enzymatic breakdown of adipose tissue can occur prior to skeletal muscle wasting and contribute to the cachexic phenotype. Das et al. also demonstrated that genetically inhibiting lipolysis can attenuate skeletal muscle wasting and cachexic phenotype [278]. In support of this theory, Fukawa et al. also recently found that adipose tissue wasting and fatty acid oxidation precede skeletal muscle atrophy, and inhibition of fatty acid oxidation can rescue skeletal muscle wasting across a number of cancer cachexia modeling systems [279]. Consequently, both findings reinvigorated interest in adipose tissues contributory and potentially facilitatory roles in cancer cachexia. Taken together, adipose tissue is a multifaceted and plastic tissue which undergoes cachexic dysregulation and catabolism and may play a contributory role in skeletal muscle wasting.

1.3.4.2.3 Ascites

Ascites fluid is the accumulation of fluid in the peritoneal cavity [27, 127]. While liver cirrhosis makes up the overwhelming percentage of ascites diagnoses (81%), malignant ascites (ascites in the presence of cancer) accounts for approximately 10% of all ascites diagnoses and is most commonly seen in advance cancer [27]. While estimated prevalence across cancer patient populations varies, a range of 3.0-37.7% of cancer patients will develop ascites fluid [128]. Due to malignant ascites’ association with the advanced metastatic stage and comorbid symptoms, it has been cited to worsen quality of life and prognosis [127, 128]. Malignant ascites is believed to be a result of disruption to hepatic function, lymphatic drainage, and/or damage to the abdominal lining [127]. Altered hepatic function is believed to result from direct tumor blockage of the hepatic
portal vein, inflammation (TNF-α [280, 281], IL-6 [282-284], IL-1β [285]), and/or altered hormone levels (insulin [286]) which may drive a shift in hepatic resources away from “negative” acute phase response proteins (albumin, transferrin, etc..) towards “positive” acute phase response protein (C-reactive protein, hepcidin, serum amyloid A, etc…) production. This reduction in albumin, also termed hypoalbuminemia, is commonly observed in cachexic patients and can result in shifted osmotic gradients, reducing blood volume and increasing extravascular fluid accumulation (additional discussion in sections 1.3.4.8 Albumin). Additionally, the lymphatic system facilitates a constant and critical role in uptake and redistribution of fluids throughout the body to retain homeostatic intra- extravascular fluid. However, disruption to this system may cause inefficient extravascular fluid reuptake and redistribution resulting in ascites accumulation [287]. Cancer may also directly disrupt the abdominal wall via cancer cell accumulation or indirectly induce inflammation-driven abdominal leakage [127]. While the cause and consequences of ascites remains an understudied aspect of cachexia, when present, malignant ascites can be indicative of metastatic progression, can be a confounding influence on body composition, and is associated with adverse outcomes.

1.3.4.3 Inflammation

Inflammation has received considerable interest due to its influence on various components of the cachexia phenotype [8, 21, 87]. The origins of inflammation in cachexia are not completely understood but have largely been attributed to both tumor secretion and/or host-tissue immune/acute phase response which can result in sustained systemic inflammation. Growing evidence indicates that tumors can produce and secrete proinflammatory mediators. In 2011,
Hanahan and Weinberg updated the hallmarks of cancer to include “tumor-promoting inflammation” as an enabling characteristic of tumor cells [2], as inflammation can contribute to proliferation and survival of malignant cells by increasing angiogenesis, metastasis, adaptive immune evasion, and anti-neoplastic futility [288]. Central to cancer’s inflammatory response are TNF-α, IL-6, and IL-1β cytokines and downstream NF-κB and STAT3 signaling, which also regulate skeletal muscle and adipose tissue homeostasis in cachexia (as discussed in section 1.3.4.2.1.1 Tumor Necrosis Factor-Alpha Signaling). Additionally, the host-response to tumor-burden can induce systemic inflammation, a process originally termed “immune surveillance” [289]. Immune surveillance described a hypothesis that immune cells constantly survey the host to remove pathogens and/or invading tissue including malignant cells. However, emergent evidence illustrates a spectrum of host-immune system and tumor immune evasion responses that can result in acute and robust immune invasion and deletion of a selective malignant subpopulations [290]. Consequently, this leaves immune resistant malignant cells and tumor microenvironments which can result in chronic host-immune reactivity and tumor adaptive host-immune evasion. Therefore, both the tumor and host-immune system can induce a chronic systemic inflammatory response which can influence tumor growth and metastatic progression. However, in non-malignant tissue such as skeletal muscle and adipose tissue, proinflammatory cytokines play an integral role in catabolism [21, 291-293]. Proinflammatory mediator’s TNF-α, IL-6, and IL-1β can directly induce tissue catabolism, inhibit synthesis, and/or attenuate therapeutic responsiveness to anabolic agents by inducing anabolic resistance (additional discussion in sections 1.3.4.2.1.1 Tumor Necrosis Factor-Alpha Signaling). Elevated levels of proinflammatory cytokines such a TNF-α, IL-6, and IL-1β have been reported to reduce appetite levels, promoting anorexia via appetite regulation, taste distortion, and malabsorption [294].
Anorexia confounds energetic balance, exacerbates wasting, and impact morbidity and mortality, influencing the entire cachexia syndrome (additional discussion in sections 1.3.4.4 Anorexia). Inflammation has been shown to disrupt systemic and cellular metabolism in the cachexic environment. Proinflammatory cytokines disrupt insulin/IGF-1 signaling, induce insulin resistance, inhibit oxidative metabolism, increase aerobic fermentation, induce amino acid anabolic resistance (which can exacerbate catabolic signals), and attenuate anabolic therapeutic strategies (additional discussion in sections 1.3.4.2.1.1 Tumor Necrosis Factor-Alpha Signaling). Beyond inflammation’s role in cancer growth and progression, tissue composition/atrophy, and anorexia, inflammation can contribute to alterations in common clinical assessment tools. Common clinical biomarkers evaluated in cachexic patients are anemic status, systemic surrogate metrics of protein breakdown, and albumin as these are a part of routine clinical chemistry and blood count assessment (additional discussion in sections 1.3.4.5 Anemia, 1.3.4.6 Blood Urea Nitrogen, 1.3.4.7 Total Protein, and 1.3.4.8 Hypoalbuminemia). Anemia is the reduction in red blood cell count, hemoglobin, and/or hematocrit resulting in weakness, fatigue, reduced quality of life, and increased mortality risk in cancer patients. Inflammation has been shown to play a multifaceted role in facilitating anemia by augmenting acute response protein and attenuating red blood cell production and functionality. Resolution of inflammation has been shown to improve and/or resolve anemia (additional discussion in section 1.3.4.5 Anemia). Blood urea nitrogen (BUN) and total protein are surrogate markers of systemic protein breakdown [295]. BUN is the biproduct of protein catabolism via urea cycle resulting in nitrogen exchange to urea which is retained in the blood until excreted via the kidneys. Total protein quantifies all circulating protein molecules and has been used as a surrogate marker for systemic protein breakdown (and nutritional status). Inflammation can lead to elevations in BUN and reductions in total protein via catabolic
signaling in muscle [295], altered hepatic protein dynamics [296-298], and indirect regulation of nutritional intake (additional discussion in sections 1.3.4.6 Blood Urea Nitrogen and 1.3.4.7 Total Protein). Finally, albumin is the most abundant protein in systemic circulation and plays a critical role in osmotic regulation, lipophilic molecule transport, amongst other roles [299-301]. However, hypoalbuminemia is also observed in cachexic patient as inflammatory cytokines can directly signaling hepatic tissue to decrease albumin production in preference for positive acute phase protein (additional discussion in section 1.3.4.8 Hypoalbuminemia). It has also been speculated that inflammation plays a contributing role in depression and reduced quality of life that can accompany the cachexic condition [302-304]. Taken together, inflammation plays a multifaceted role across many components of the cachexia syndrome.

Anti-inflammatory agents have been administered in cachexia patients with positive [305-308] and neutral effects [309] on body composition [310]. While more clinical trials will determine the efficacy of these agent, the current mixed clinical results suggest that sequestering inflammation will likely be helpful, but insufficient to attenuate the entire CACS. None the less, as inflammation is a common characteristic of cachexia, influencing various aspects of the disease phenotype, disrupting therapeutic potential, and inducing a self-perpetuating cycle often resulting in chronic systemic elevation, attenuating inflammation in cancer cachexia appears critical to the efficacy of any future multimodal anti-cachexia therapeutic strategy.
1.3.4.4 Anorexia

Food intake has a clear role in regulating energy balance and thus muscle mass. Anorexia, or the pathological reductions in food intake, has been commonly reported in the cachexic environment [5, 6, 15, 33, 311]. Anorexia is believed to result from a number of potential contributors that primarily alter hypothalamic appetite regulation and intestinal absorption [90]. While the terminology used to describe cancer cachexia has varied, cancer cachexia in the presence of comorbid anorexia is associated with higher morbidity and mortality, and is termed cancer anorexia cachexia syndrome (CACS) [25]. Thus, exploration into the mechanisms driving reduced food intake have been explored to assist patients in overcoming this seemingly correctable problem.

Evidence for inflammations involvement in the anorexic process is seen across numerous inflammatory diseases. Consequently, pro-inflammatory cytokines’ role in regulatory food intake have received considerable attention. Evaluation into direct anorexic effects of TNF-α, IL-6 and IL-1β have demonstrate cytokine-induced reductions in food intake which can be corrected by antibody sequestration, suggesting pro-inflammatory cytokines may play a contributing mechanistic role in anorexia in CACS. Neuropeptide γ and pro-opiomelanocortin have gained interest due to their central role in regulating appetite in the hypothalamus. It has been demonstrated that elevations in proinflammatory cytokine gene expression (IL-1) is elevated in anorexic models. Additionally, cerebrospinal IL-1 levels were inversely correlated with food intake. Lastly, antibody sequestration of IL-1 abolished anorexia in the same model system.
Hormones known to have regulatory roles in appetite [312], have been explored in cachexia particular, including leptin and ghrelin [91, 150, 313]. Leptin is a hormone expressed and secreted from adipose tissue [264, 265]. It has gained considerable interest in the field of obesity as leptin levels have been shown to be linked to hunger and bodyweight. Normally, as bodyweight reduces, leptin levels increase and drive appetite as a counter regulatory signal to maintain bodyweight homeostasis [312]. However, leptin resistance has been reported in cachexia, disrupting leptin’s anti-anorexic role even when adipose tissue is deficient [91]. Ghrelin is a temporally regulated hormone that appears to acutely drive hunger from meal to meal [312]. However, interestingly, ghrelin analogues have been shown to help assist cachectic patients regulate appetite [314, 315].

While pro-inflammatory cytokines and potentially hormonal signaling appear to be mechanistic drivers of altered food intake, increasing caloric intake alone in cachexic patients has not been proven efficacious [95, 96]. In fact, while patients in these trials appear to be able to gain weight, the weight appears to be in adipose, not muscle. Similar body composition changes accompany elevation in caloric intake in non-diseased environments during post-diet refeeding, where adipose tissue has great anabolic plasticity than muscle in response to caloric surplus [312, 316]. However, since caloric surplus can drive systemic anabolism, this may present a unique contraindication for the underlying cause of cachexia, cancer. Thus, optimizing nutrient composition appears to be a more logical approach than just applying nutrient surplus when considering both cancer and cachexia together. A high-protein diet is a primary example of such an approach as high protein diets have been shown in healthy and sarcopenic patients to contribute to improve skeletal muscle mass independent of fat mass changes [316, 317]. While data does
illustrate that increasing amino acid in cancer/cachexia patients may assist with overcoming damped immune system, and higher protein diets may help overcome reported anabolic resistance, data to date has remained unclear on its efficacy the cachetic environment [318]. This is likely because elevating protein intake alone is not sufficient to address the entire CACS, along with known amino acid anabolic resistance in inflammatory environments [8, 21]. However, protein intake appears to still be important as having sufficient amino acid intake is a critical nutrient required to mount a sufficient anabolic response in the muscle and to retain adequate immunologic function. Taken together, due to food intake inherent link to muscle mass, optimizing nutritional intake will likely be a logical contributor to any successful anti-cachexia multi-model therapeutic approach.

1.3.4.5 Anemia

Red blood cell count, hemoglobin, and hematocrit are metrics of absolute and relative red blood cell concentration in the blood. However, reductions in these biomarkers is termed anemia and is often seen in cachetic patients [5, 10, 12, 148, 251, 311, 319-321]. Across chronic diseases, cancer is second only to infection in estimated anemia prevalence at 30-70% [322], even independent of anti-neoplastic treatments [323]. Red blood cells contain hemoglobin which transport oxygen throughout the circulation to various tissues to participate in cellular respiration, redox balance, amongst others. Due to oxygens role in mitochondrial respiration, reduced red blood cells or oxygen-carrying hemoglobin molecules can result in fatigue, weakness, and reduced functional capabilities [322]. Confounding the anemic prevalence in cachetic patients is that
anemia is a common consequence of anti-neoplastic standard of care therapeutic interventions including surgery, chemotherapy, and radiation therapy [324].

Mechanisms driving anemia in cachexia patients are multifactorial but nutritional status and pro-inflammatory cytokines have been demonstrated to play direct roles. Iron is a critical component of hemoglobin and insufficient iron intake can result in altered production of functional red blood cells [325]. However, folate, vitamin B12 and vitamin C appear to also be important to attenuate nutritionally-induced deficiencies. As anorexia is a common component of CACS, nutritionally-induced anemia introduces a potential risk factor. However, inflammation is another common and critical component to CACS and proinflammatory cytokines have been central in the investigation of inflammation driven anemia. TNF-α administration over a 7-10 day period, twice a day, resulted in anorexia, tissue wasting, and significant anemia [326]. The alteration in anemia were a result of reduced red blood cell count. Inflammatory cytokines, including TNF-α and IL-6 and acute phase proteins (which inflammatory cytokines can facilitates the production of [327]) have been demonstrated to disrupt or be associated with altered iron homeostasis [322, 323, 328] via inhibited release [329] and altered gene expression [330]. TNF-α and IL-6 both inhibit erythropoiesis [322]. Rodent experiments have also demonstrated that TNF-α facilitated immune mediated erythrocyte phagocytosis. TNF-α transgenic mice with chronic elevations in TNF-α developed alterations in red blood cell populations, functional iron deficiency, apoptosis in bone marrow cell populations, and reductions in hemoglobin and hematocrit, all manifesting in multiple facilitating characteristics of anemia independent of any of cytokines influence [331]. In 2015, Maccio et al. found that anemia was found in 63% of patients and that cytokine levels could predict anemia status when analyzing 888 cancer patients in a large prospective observational study [332].
Further evidence for inflammations impact on anemia can be observed with anti-inflammatory treatments. Anti-TNF-α and anti-IL-6 therapy decreased acute phase response proteins and increased hemoglobin across various inflammatory disease states, including cachexia [333-337].

However, beyond function impairment, anemic cancer patients frequently reported reduced quality of life [338]. Resolution of anemia in cancer patients has resulted in improvements in fatigue and quality of life, supporting anemia as a proposed cause of altered fatigue and quality of life in cancer patients. Together, anemia is common components of CACS, perpetuated by standard of care, malnutrition, and inflammation and plays a multifaceted role in patient’s functionality and quality of life. Addressing anemia via anti-inflammatory and/or nutritional optimization strategies have demonstrated efficacy in attenuating anemia and augmenting quality of life in patients.

1.3.4.6 Blood Urea Nitrogen

BUN is a surrogate marker of protein turnover [295]. During protein degradation, amino acids are fluxed from the skeletal muscle. Owens et al. found that in fasted subject, alanine is the predominant amino acid fluxed from the skeletal muscle and taken up for gluconeogenesis in hepatic tissue [40]. The Cahill cycle, also known as alanine-glucose cycle, was name after this discovery. In the hepatic tissue alanine is converted to pyruvate, producing glutamate and NH₄⁺ [339]. Glutamate and NH₄⁺ are converted to Glutamine for transport to the urea cycle. Most urea produced is sent to the circulation as BUN and eventually excreted via the urine [295]. The remaining urea is disbursed throughout the body or excreted via the large intestine. Consequently,
elevated BUN can result from increased protein turnover. However, BUN has also been used to assess kidney function as reduced urinary excretion due to reduced glomerular filtration rate could result in elevated BUN. However, in systemic wasting syndromes such as cachexia, protein breakdown is elevated [340]. Inflammation’ contributes to this by elevating systemic protein catabolism. TNF-α, not IL-6, has also been suggested to increase urea synthesis, but it remains unclear whether this is via direct or indirect regulation [296-298].

1.3.4.7 Total Protein

Total protein is a global marker of total circulating protein levels and is indicative of general protein balance. Malnutrition, catabolism, liver or kidney dysfunction, amongst others can all influence this metric. Reduction in adequate nutrition, reduced protein synthesis, and/or accelerated protein breakdown can result in reduced circulating protein levels. Cachexia patients commonly observe malnutrition and reduced protein synthesis [12, 311, 319].

1.3.4.8 Hypoalbuminemia

Albumin makes up approximately 50% of total serum protein content and is synthesized by the hepatic tissue [299, 300]. Albumin’s primary functions are to maintain osmotic pressure and hydrophilic molecular transport, but also has antioxidant functionality. Albumin production is optimized via adequate nutrient intake and subsequent elevations in anabolic hormones such as insulin [286, 301]. However, albumin production is conversely reduced via inadequate protein balance [18] and pro-inflammatory cytokines TNF-α [280, 281], IL-6 [282-284], IL-1β [285],
and/or altered hormone levels (insulin [286]). Inadequate protein intake can reduce synthesis via reduced anaplerotic substrates. Inflammatory cytokines appear to reduce hepatic synthesis of albumin at hepatic gene expression level. However, while decreased synthesis is believed to be the primary cause of reduced albumin, also termed hypoalbuminemia, albumin can also be degraded and leak into extravascular compartments which can cause edema and/or exacerbate ascites accumulation in metastatic patients (for more detailed discussion see 1.3.4.2.3 Ascites). Reductions in serum albumin are observed in infection, liver cirrhosis, and gastrointestinal disorders/malabsorption. Importantly, hypoalbuminemia is part of the diagnostic criteria for cachexia [5]. While hypoalbuminemia is commonly observed in CACS [9, 10, 12, 148, 251, 311, 320, 321], it is also associated with reduced survival in cachetic patients [10]. Taken together, hypoalbuminemia is a critical diagnostic component of CACS and is driven by multifactorial causes including nutritional, altered hepatic function, and extravascular relocation, with inverse relationship with survival.

1.3.4.9 Metabolic Derangement

Alterations in metabolism have been observed in CACS which promote the cachexia phenotype and skeletal muscle atrophy. These include alterations in glucose, amino acid, lactate, and fatty acid metabolism.

Glucose is a critical six-carbon metabolite with important roles in tumor and extra-tumoral tissues. Interest into glucose’s role in cancer came front and center following Warburg’s findings that cancer cells underwent fermentation even in the presence of oxygen, termed aerobic
fermentation [341]. These findings were later termed the “Warburg Effect” and have been a central point of discussion and investigation in the growing field of cancer metabolism as “The warburg effect” confer multiple growth advantages to rapidly proliferating cancer cells [81, 82]. However, this increase metabolic demand for glucose (and glutamine) seen across many cancer cells may have consequences for extra-tumoral tissues.

Skeletal muscle is a very plastic tissue with important roles in amino acid and glucose metabolism (for more detailed discussion see 1.3.3 Skeletal Muscle Mass Across Health and Disease and 2.1 Fasting). During period of increased metabolic demand and/or catabolism, skeletal muscle proteins are catabolized and fluxed into circulation. This is observed in the fasted state when blood glucose levels drop resulting in the eventual reduction in circulating insulin and IGF-1 [209]. This reduced insulin and IGF-1, lowers insulin/IGF-1 signaling in the skeletal muscle, driving ubiquitin proteosomal degradation, resulting in amino acid mobilization. However, half-century old data in humans demonstrated that during these catabolic states, alanine and glutamine made up over 50% of amino acids released from the skeletal muscle into circulation, even though these two amino acids only make up 15% of total skeletal muscle amino acid composition [37, 40, 68, 69, 342]. It was also found that alanine, the most abundant amino acid released from the skeletal muscle, was shuttled to the hepatic tissue where it underwent robust uptake and gluconeogenesis to sustain homeostatic circulating glucose levels [40]. It was also found that skeletal muscle appeared to be the primary site of glutamine synthesis and release when compared to other tissues [68]. Importantly, Vander Heiden et al. described that glucose and glutamine are the primary source of carbon, nitrogen, energy, and reduce equivalents for cell growth and division, particularly cancer cells [82]. These historical findings in skeletal muscle in concert with
emergent understanding into cancer cell metabolism illustrate a synergistic metabolic relationship between proliferating tumor cells and catabolic skeletal muscle [8].

Tumor cells can also indirectly influence systemic glucose by promoting inflammatory signaling. As described earlier, one of the enabling characteristics in cancer cells are there “tumor-promoting inflammation.” [2]. While this inflammation can promote proliferation and survival of malignant cells [288], it can also induce insulin resistance at the skeletal muscle levels as proinflammatory cytokines have been shown to disrupt insulin/IGF-1 signaling at the receptor level [186-188, 190-192]. This altered signaling promotes skeletal muscle catabolism and amino acid release, but also increasing circulating glucose by inhibiting insulin/IGF-1-induced skeletal muscle glucose uptake (for more detailed discussion see 1.3.4.2.1.2 Insulin and Insulin-Like Growth Factor 1 Signaling and 2.1 Fasting). Additionally, inflammation directly induces catabolic signaling in the skeletal muscle via TNF-α, IL-6, and IL-1β, all contributing to amino acid efflux (for more detailed discussion see 1.3.4.2.1.1 Tumor Necrosis Factor-Alpha Signaling). Together, inflammation can induced catabolic signaling and attenuate skeletal muscle glucose uptake, conferring metabolic changes in skeletal muscle which promote glucose availability for tumor proliferation and growth [8].

While the Warburg effect has also been observed in cancer cells, it has also been observed in the skeletal muscle downstream of pro-inflammatory signaling in cachexic patients (for more detailed discussion see 1.3.4.2.1.1 Tumor Necrosis Factor-Alpha Signaling). While this metabolic alteration has been described to confer advantages to cancer and immune cells, it appears to be detrimental to skeletal muscle mass, driving inefficient metabolic pathways and catabolism.
Additionally, emergent evidence in cancer patients illustrates that some tumors may increase lactate uptake [343], demonstrating yet another potential advantage given to tumor cells at the expense of skeletal muscle mass.

Beyond skeletal muscle, adipose tissue also undergoes metabolic alterations which promote catabolism and transformation during cachexia progression which may facilitate advantages to tumor cells (for more detailed discussion see 1.3.4.2.2 Adipose Tissue). Adipose tissue is the richest store of triglycerides found in the body. During periods of catabolism, triglycerides can be broken down into glycerol and free-fatty acids. Glycerol can be converted at the liver into glucose. Glucose and free-fatty acids can be utilized by tumoral tissue to meet energetic and growth demands [8]. While inflammation can drive skeletal muscle insulin/IGF-1 signaling resistance, it also does so in adipose tissue, promoting adipose tissue catabolism. Indirectly, inflammation also promote anorexia which can promote the mobilization of tissue from both skeletal muscle and adipose tissue. Additionally, the most abundant form of adipose tissue, white adipose tissue, can undergo transformation into energetically inefficient brown adipose tissue, termed “browning.” Brown adipose tissue undergo increased uncoupling and consequently increase catabolism via energetic inefficiency.

Taken together, cancer patients can undergo metabolic alterations across various tissues, specifically skeletal muscle, hepatic, and adipose tissue, which facilitate the mobilization of metabolic substrates which support cancer cell growth and proliferation, while simultaneously promoting the development of CACS.
1.4 Modeling Cancer-Anorexia Cachexia Syndrome

Advances in defining the pathology of, and therapeutic response to cachexia have largely resulted from animal model systems [17]. Updated definitions of CACS describe it as a distinct, multifaceted, progressive, atrophy syndrome most commonly observed in the advanced metastatic environment characterized by skeletal muscle wasting, often accompanied by adipose tissue wasting and comorbid symptoms of inflammation, anorexia, anemia, elevated protein breakdown, hypoalbuminemia, and metabolic derangement [5, 6, 8]. Modeling the entire CACS creates a considerable barrier due to its multiple components [17-20]. Thus, all cachexic modeling systems present limitations. Bennani-Baiti and Walsh argued that an ideal model should replicate as much of the human condition as possible while being reliable and reproducible across multiple labs [19]. Modeling systems have historically been categorized by method of cancer induction/formation. These include xenograph, syngeneic (orthotopic and ectopic), and genetically engineered.

1.4.1 Xenograph

Xenograph animal models are defined by their ability to harbor foreign tissue without immunologic rejection [344]. These models allow for implantation of foreign tumors (mouse and human) without immune rejection as xenograph mice have compromised immune systems. The most common use of xenograph model is implantation of human primary tumors to evaluate drug efficacy. Newer xenograph modeling has also incorporated human immune cells into immunocompromised mice to attempt to better reconstitute the tumor-host interaction.

- **Advantages:**
Rapid evaluation of drug-tumor response
Allow for logistically feasible and predictable experimental designs

- Disadvantages:
  - Immunocompromised
    - Inflammatory response critical to cachexia phenotype [8, 30, 345]
  - Host-tumor microenvironment response often does not replicate host-tumor interaction
  - Do not often metastasize

1.4.2 Syngeneic

Syngeneic mouse models are the most abundant form of cancer modeling [346]. They involve the implantation of mouse cancer cells at the origin (orthotopic) or distant sites (ectopic). Implanted cells often originated from spontaneously develop, resected and cultured mouse tumor cells.

- Advantages:
  - Immunocompetent
  - Synergistic growth and development with host
    - Improved with ectopic implantation
  - Logistically feasible experiment designs

- Disadvantages:
  - Primary tumor implanted, not spontaneously developed
• Ectopic surgical implantation may introduce logistical and reproducibility complications across groups
  o Often do not metastasize
  • Those that do often bypass steps in metastatic progression (i.e. i.v. implantation, i.p. implantation, surgical removal, etc.)
  o Often rapid tumor progression and rapid cachexic decline.

1.4.3 Genetically Engineered

Genetically engineered modelling systems present numerous advances in recapitulating human tumor development [344, 347]. Genetic mutations (often at tumor promotor and/or suppressor regions) are placed at specific location throughout the genome to engineer tumor onset in animals. Consequently, models can be developed to recapitulate the most common genetic mutations found in human cancers and recreate “spontaneous” tumor formation. Due to overlapping mutations, variability in tumor onset and metastatic progress, these models’ systems are believed to present many overlaps to human model environment compared to other current modeling systems.

• **Advantages:**
  o Immunocompetent
  o Spontaneous tissue specific tumor development
  o Extended tumor and metastatic progression
  o Multistep carcinogenesis

• **Disadvantages:**
  o Variable tumor onset, tumor burden, metastatic progression, cachexia onset
Logistically difficult and expensive to design appropriately powered experimental designs

Argued to not mimic human tumor complexity

Xenograph models are currently not recommended for cachexic modeling due to the inability to model the immune aspect critical to the cachexic phenotype [17, 19]. Syngeneic modeling has been the most common form of cachexia modeling due to its ease of implementation, reproducibility, and evaluations. However, cachexic wasting is often rapid, dissimilar to the more prolonged and progressive process seen in the human condition. Additionally, historically many syngeneic cachexic models do not metastasize. However, one critical advantage to these models besides their ease of use is that they model the tumor-host interaction better than xenograph modeling. Genetically engineered modeling systems have started to become the “preferred” modeling system (over xenograph) for evaluating carcinogenesis due to their ability to “spontaneously” recapitulate multiple steps of this process via clinically overlapping and inducible genetic mutations. These model system present considerable advancement for cachexic modeling due to the extended catabolic timeline and intact host-tumor interaction. The primary limitations to genetically engineered modeling systems is logistical. Variability is reported with cancer onset, tumor burden, metastasis, and cachexia progression. Thus, appropriately powered experiments can be logistically cumbersome, limiting their widespread use. The most cited and utilized cancer cachexia models include C-26, Walker 256, MAC16, YAH-130, and LLC [17]. The nuances of these models and notable genetically engineering models are summarized in Table 1.2 below.
<table>
<thead>
<tr>
<th>Model</th>
<th>Muscle Wasting</th>
<th>Adipose Wasting</th>
<th>Anorexia</th>
<th>Inflammation</th>
<th>Metabolic Derangement</th>
<th>Hypo-Gonadism</th>
<th>Anemia</th>
<th>Hypo-albuminemia</th>
<th>Elevated BUN</th>
<th>Metastasis</th>
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1.4.4 Modeling Gaps

While all modeling systems present strengths and weakness, one common limitation is the inability to model the full CACS. While modeling systems that do not present all facets of the disease may help answer research question specific to the nuances presented in each model system (i.e. anorexia’s influence on cachexia develop; inflammations role in muscle wasting; etc), it also may limit translatability of findings to the full CACS presented in the clinical environment. Commonly observed limitations include lack of metastasis modeling and validation of clinical biomarkers.

1.4.4.1 Metastasis

An intriguing, and largely understudied area of inquiry revolves metastasis’ role in cancer cachexia. Metastases role in CACS is best illustrated by observing the incidence of CACS with and without metastases present [7, 8, 12, 29, 32, 120-125]. Additionally, a consistently high rate of cachexia is observed in the limited modeling systems that do develop cachexia and metastasis, including KrasG12D/+;Lkb1f/f, APCMin/+ , and some versions of LLC model. Recent reports of KrasG12D/+;Lkb1f/f model of NSCLC demonstrated CACS in some animals, as this model demonstrates variable degrees of tumor formation, metastatic burden, and cachexia onset [369]. Interestingly, the KrasG12D/+;Lkb1f/f animals that developed lymph metastases also reported to consistently develop CACS. APCMin/+ is a genetically engineered colon cancer model which develops intestinal polys and variable degrees of tumor progression, invasion, metastasis in a minority of animals [368, 370], and cachexia onset [371]. However, the animals with metastatic
burden outside the intestinal tract also present with a consistent cachexic phenotype (personal communication). LLC is one of the most commonly utilized models, but methods of tumor inoculation have led to different phenotypes. However, the metastatic version of the LLC model does consistently develop cachexia. However, this form of the LLC model requires ectopic implantation and surgical resection of the primary tumor to allow for metastasis to occur back to tumor origin (lung). While current cachexic modeling systemic that do metastasis present with more consistent cachexic phenotype, metastasis and cachexia onset can be variable, demonstrating logistical limitations of these model systems. Thus, many model systems do not metastasize significantly despite consistent clinical reports demonstrating a higher incidence of cachexia and its comorbidities in the advanced metastatic stage. However, in the modeling systems that do demonstrate metastasis, it is inconsistent, and often results in variable degrees of cachexia, presenting logistical limitations.

1.4.4.2 Clinical Biomarkers

Red blood cell count, hemoglobin, hematocrit, blood urea nitrogen, total protein, and albumin are all commonly measured in CACS patients to determine anemia [5, 10, 12, 148, 251, 311, 319-321], elevated protein breakdown [12, 311, 319, 340], and hypoalbuminemia [9, 10, 12, 148, 251, 311, 320, 321] status. However, very few modeling systems evaluate these biomarkers to determine modeling overlap leaving many unanswered questions revolving the clinical translatability.
1.4.5 VM-M3 Model of Systemic Metastasis

The VM-M3 model of systemic metastasis is a syngeneic mouse model derived from a spontaneous glioma tumor, which, when implanted subcutaneously, results in systemic metastasis throughout the animal, including to the liver, lungs, brain, kidneys, and spleen. Of particular interest, the VM-M3 model reliably recreates the spontaneous metastatic process (primary tumor formation, followed by establishment of secondary tumors), is syngeneic with VM/Dk background strain, and permits bioluminescent-based monitoring of disease progression [372], and has a reliable and consistent metastatic phenotype [372-377]. This convenient and consistent model system exhibits many of the hallmarks of the metastatic condition, mirroring important aspects observed in clinical cachexia, but has not yet been explored for the CACS phenotype.

1.5 Treating Cancer-Anorexia Cachexia Syndrome

With a century of research, cancer cachexia remains a disease without a clinically approved treatment option. This has been attributed to [17]:

1) Complexity of syndrome
2) Difficulty recruiting patients into clinical trials
3) Difficulty following patients
4) Underlying pathology not completely understood

Many mono-therapeutic approaches have shown mixed success at small clinical trial scale but these benefits have not yet translated up to larger scale clinical trials [310]. Consequently, much discussion has emerged whether a single therapy will ever be sufficient to treat a multifactorial
problem [17, 21, 310, 345]. Thus, multimodal approaches have been suggested to move towards findings a clinically effective approach to altering the course and/or burden of cancer cachexia. An ideal therapeutic option would likely need to satisfy the following criteria:

1. Acceptable toxicity (ideally none)
2. Neutral or anti-tumor response
3. Attenuate muscle catabolism
4. Normalize system inflammation
5. Normalize dietary intake once ideal bodyweight is achieved
6. Improves quality of life

Current therapeutic options that have been, or are under investigation include nutritional, appetite stimulants, anti-inflammatories, and anabolic agents.

1.5.1 Nutritional

1.5.1.1 High Calorie Diet

Cancer cachexia patients have commonly been cited to be malnourished [5, 6, 15, 33, 196, 311]. Kumar et al. proposed that cancer cachexia patients are often in a 250-400kcal/day caloric deficit. Combined with the known anorexic effect see in CACS, initial attempt to combat the disease involve increasing caloric intake. However, high calorie diets alone have not been shown to combat the disease, but to instead increase adipose tissue accumulation [95, 96]. Thus, it has been generally accepted within the cachexia field that increasing caloric intake alone will not sufficiently combat the disease. None the less, it has been shown that oncological nutritional
recommendations amongst many oncological treatment institutes usually involves a high carbohydrate and high calorie diet [378]. However, nutrition plays a clear role in energy balance and not all dietary compositions have the same effect on body composition [316] and/or tumor response [81]. Consequently, specific changes in macronutrient composition have been attempted.

1.5.1.2 High Protein Diet

High protein diets have been considered to combat cancer cachexia as [316, 317]:

1) Protein is a fundamental building block in skeletal muscle

2) Protein ingestion absent pathology facilitates both an anabolic and anti-catabolic response

3) Protein appears to improve skeletal muscle body composition, compared to fat or carbohydrate during overfeeding studies

4) High protein diet has been shown to attenuate muscle wasting in age-related muscle atrophy

While this nutritional approach appears logical, data determining the efficacy of high protein diets in cachexic patients is lacking [318]. Another confounding factor in the success of high protein nutrition in cancer cachexia is the known anabolic resistance reported in cancer cachexia patients believed to be caused by inflammation [8, 21].
1.5.2 Appetite Stimulants

1.5.2.1 Progestins

Regardless of the known inadequacy of simply increasing caloric intake in combating cancer cachexia, appetite stimulants are the most studied of all proposed anti-cachexia drugs with approximately 100 clinical trials thus far. However, while correcting caloric intake has not been successful in cancer cachexia, the clear role of normalizing caloric intake is essential in preventing energetic deficits which are known confounder in cachexic wasting. Progestins are now known as appetite stimulants. However, they were first synthesized and isolated in 1931 and termed progesterone [379]. One of the most studied forms of this drug is megestrol acetate (MEGACE), which was originally used as an oral contraceptive [380]. In 1994, McCarthy believed that MEGACE could stimulate the synthesis, transport, and release of neuropeptide γ [381], a key regulator of appetite in the hypothalamus. It was later approved for anorexia, cachexia, or unexpected weight loss in AIDS patients. It is now appreciated MEGACE can improve appetite and food intake, which could influence weight and quality of life. However, it is currently not approved for cancer cachexia, has been shown to induce hyperglycemia, and when cancer cachexia patients are overfed, they predominantly gain adipose tissue [96, 382].

1.5.2.2 Ghrelin Mimetics

Ghrelin has been known as an appetite stimulating gastric peptide hormone that is transiently regulated by food intake [312]. Ghrelin has also been shown to increase IGF-1, GH,
serotonin, alters autonomic regulation to increase gastric emptying, attenuates anorexigenic mediators, and augments orexigenic mediators [383]. In 2004, Neary et al. showed that ghrelin could improve appetite in anorexic cancer patients [384]. In 2010, Lundholm et al. found that high dose ghrelin improved appetite, attenuate fat-loss, did not cause adverse effects, but fat-free mass was not reported [385]. In 2013, Garcia et al. found that anamorelin, a ghrelin mimetic, increased bodyweight, GH, IGF-1, and appetite in 16 heterogeneous patients with cancer cachexia [314]. While positive results have been reported and multiple groups are currently investigating ghrelin as an anti-cachexia therapy, in 2018 the Cochrane database published an evaluation of ghrelin and found that there is currently insufficient evidence for its recommendation in cancer cachexia [92]. Additionally, there has been caution raised for ghrelin’s use due to its effect on elevation IGF-1 and a report in rodents indicating that cancer cachexia may induce ghrelin resistance potentially confounding the success of these molecules [310, 386].

1.5.2.3 Cannabinoids

Cannabinoids are a diverse category of chemical compounds derived from the marijuana plant that stimulate cannabinoid receptors. Interest in cannabinoids for cancer cachexia arose due to their ability to promote appetite, weight gain, alter mood, potential anti-inflammatory effects, amongst others [387]. However, separate analysis of clinical trials in cancer patients came to separate conclusions on cannabinoid effect on appetite [387, 388]. However, both analyses indicated that no other effects have been demonstrated in cancer cachexia patients.
1.5.3 Anti-Inflammatories

1.5.3.1 Corticosteroids

Corticosteroids are both appetite stimulants and anti-inflammatory agents. “Adrenal cortical hormone” was originally extract from adrenal corticol animal extracts [389]. First clinical use of these molecules was for inflammatory disorders such as rheumatoid arthritis. However, subsequent evaluation discovered a long-list of side effects, one of which was increased appetite. However, of interest, corticosteroids have known atrophy effects at the skeletal muscle [209]. In fact, dexamethasone is the most common technique to attenuate IGF-1 signaling and thus result in atrophy in cultured myocytes. Additionally, corticosteroids are known to induce insulin resistance, worsen infection risk, amongst others [21]. Thus, while corticosteroids have orexogenic and anti-inflammatory effects, their pro-cachexia side effects results have resulted in cautious use of these compounds for anti-cachexic therapeutic strategies.

1.5.3.2 Non-Steroidal Anti-Inflammatory Drugs

Non-steroidal anti-inflammatory drugs (NSAIDS) are commonly used to treat pain, fever, and inflammation and can be purchased with and without a prescription. Common examples include ibuprofen and Celebrex. They have also been assessed in cancer cachexia and have shown improvements across various cachexia parameters including weight, muscular function, appetite, inflammatory markers, etc. [390-393]. However, they are often not evaluated in isolation so
isolated efficacy is hard to determine [390-392]. Two separate reviews determined that NSAIDs did not have enough scientific support for their use in treating cancer cachexia [394, 395].

1.5.3.3 Omega-3 Fish Oils

Omega-3 fatty acids, predominantly EPA and DHA, have been under widespread investigations when it was speculated that omega-3 fatty acid deficiency in the western diet may be contributing to adverse health outcomes [396]. Since this point, omega-3 fatty acids have been shown to have a plethora of health benefits [397]. Additionally, these non-toxic food supplements have also been shown to have anti-tumor effects [398]. In 2003, Fearon et al. found that omega-3 fatty acid and protein supplementation could improve cancer cachexia lean mass and quality of life [307]. However, from 2007-2012, three separate reviews indicated that there was not enough evidence to recommend their use [310]. However, recent reports on improvement to cancer standard of care illustrates that while these non-toxic supplements do not have enough evidence for their use, they seem to have no adverse effects and having the potential to improve various aspects of cancer patient outcomes [308, 399].

1.5.3.4 Anti-Tumor Necrosis Factor-Alpha & Interleukin-6

TNF-α and IL-6 have contributory roles in various aspects of the cachexia phenotype. Consequently, direct anti-body sequestration of them cytokines have been proposed. Initial results have been mixed with recent indications of potential subject drop out due to speculated side effects. None the less, additional clinical trials are underway and will likely help evaluate the efficacy of
these therapies for future clinical use (for more detailed discussion see 1.3.4.2.1.2 Tumor Necrosis Factor-Alpha Signaling and 1.3.4.3 Inflammation).

1.5.4 Anabolics

1.5.4.1 Testosterone

Anabolic agents have been proposed to induce hypertrophy to combat the elevated catabolism in skeletal muscle. Testosterone is one of the most well-known androgenic hormones in the body playing well-established roles in regulating skeletal muscle homeostasis and function [400]. However, cancer cachexia patients have cited hypogonadism which is indicated to perpetuate the cachexic phenotype and influence survival [5]. Thus, testosterone replacement therapy has been proposed for cancer cachexia patients [401]. While thorough investigation into the efficacy of hormone replacement therapy is lacking, small scale pilot evaluation indicates improvements, albeit not significant, in fatigue [402], skeletal muscle, quality of life, but not survival [401]. Testosterone is currently only in preliminary analysis and future trials will reveal whether this hormonal replacement therapy may modulate skeletal muscle and function in this multifaceted atrophy disease.

1.5.4.2 Selective Androgen Receptor Modulators

Selective Androgen Receptor Modulators (SARMs) were developed to regulate androgen receptors activity while reducing off-target effects of traditional hormone replacement therapy.
Ostarine/enobosarm are the most well-known SARM therapies. Previous trails have revealed improvement in muscle mass and physical activity [403]. A 2018 review indicated that of the potential pharmacological therapeutics, anobosarm and anamorelin (ghrelin mimetic) were the only two promising therapies.

1.5.4.3 Myostatin Inhibitors

Myostatin is a molecule apart of the TGF-β family with role in regulating skeletal muscle hypertrophy. Myostatin has been proposed to have a role in inducing atrophy across several atrophy-related diseases [404]. Myostatin effect on skeletal muscle regulation is best appreciated when animals with exceptionally large muscle mass also lacked myostatin, termed “double muscled” [405]. Myostatin has been found to decrease PI3K signaling, while upregulating Smad cascade, promoting skeletal muscle breakdown [404]. To date, data in cachexic patients in limited [406]. However, it seemingly direct role on skeletal muscle may make it an ideal therapy in a multimodal approach.

1.5.4.4 Insulin

Insulin therapy has been proposed due to insulin’s multifaceted role in anabolism and inhibiting catabolism (for more detailed discussion see 1.3.4.2.1.2 Insulin and Insulin-Like Growth Factor 1 Signaling). Yet, only one trials has evaluate insulins use in cancer cachexia patients. In 2007, Lundholm et al. evaluated insulin with “best available palliative care” (indomethacin/anti-inflammatory, erythropoietin/anti-anemia, oral supplements, parental nutrition) or just best
palliative care alone in advanced gastrointestinal malignancies [407]. The insulin treated group increase carbohydrate intake, increased fat mass, decrease serum-FFAs, but did not influence muscle. This is in line with the effect with other studies increasing caloric intake via appetite stimulants. However, patients did see an improvement in survival, likely indicating these patients were at greater risk for cachexia-induced mortality than cancer-induce mortality. None the less, insulin therapy has not sense been evaluated. This is likely due to the clear tumor promoting role of insulin across many tumor types. Thus, insulin is unlikely to be recommended in cachexia therapy unless in treating patients at end of life where cachexia burden outweighs risk for tumor growth.

1.5.5 Exercise

The widespread benefits of physical activity on both disease and non-diseased individuals alike are wide-spread and well supported (for more detailed discussion see 1.3.3 Skeletal Muscle Mass Across Health and Disease). These effects, as well as exercise’s direct role in retaining muscle mass, make it an ideal therapy to combat cachexia [57]. As a result, exercise is generally recommended to cachexia patients [310] However, exercise may not be feasible for many cachexia patients as fatigue, reduced functionality, and attenuate psychological status is commonly cited in patients. Thus, upfront therapeutic improvement of patient’s functionality and comorbid syndromes will likely be required to improve patient adherence.
1.6 Closing Remarks

A comprehensive review of the literature indicates that CACS is a distinct catabolic disease negatively influencing multiple aspects of clinical care and patient quality of life. Although it directly causes 1/5 of all cancer-related deaths, there are currently no model systems that encompass the entire multifaceted syndrome, nor are there any effective therapeutic treatments. The following chapter will discuss exogenous ketones therapy as a potential anti-cachexia therapeutic option.

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86
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CHAPTER 2 NUTRITIONAL KETOSIS

2.1 Chapter Synopsis:

Nutritional ketosis describes a physiological state defined by the elevation of circulating ketone bodies in the blood via altered nutritional status. The typical physiological range of ketones bodies for the average human in the fed state is 0-0.2mM and primarily remains <0.5mM [1]. Thus, ≥0.5mM has been described as the physiological “cut-off” for nutritional ketosis. There are multiple way to achieve nutritional ketosis and predominantly include prolonged fasting [2-5], very-low carbohydrate ketogenic diet (VLCKD; [6-8]), and exogenous ketone supplements [9, 10]. While there are other methods of elevating ketone bodies via exercise [1], pharmacological [11, 12], and pathological [13], etc., this review will focus on a historical perspective of nutritional ketosis via fasting, VLCKD, and exogenous ketones bodies and the potential therapeutic application of ketone bodies in skeletal muscle atrophy.

2.2 Fasting

Fasting describes the abstinence of exogenous dietary calories for an allotted period. When exogenous nutrients are stopped, the body begin to rely universally on endogenous energetic storage to retain vital tissue functionality. Maintaining homeostatic circulating blood glucose levels is the predominant driver of physiological alterations/adaptation that occurs upon the
initiation and prolongation of fasting [2, 3]. Initially, within the first 4-6 hours post-prandial, exogenous nutrients from carbohydrates (glucose) and protein (amino acids) meet peripheral and central tissue glycolytic demands and sustain circulating blood glucose levels. However, as the fast prolongs and central and peripheral glucose demands are unchanged, circulating blood glucose concentrations drop. Reduced blood glucose is the initiating step facilitating fasted metabolism driven predominantly by reduced insulin, and increase glucagon and catecholamines which [2, 4, 5, 14-19]:

1) increases hepatic gluconeogenesis and glycogenolysis;
2) removes the blockade on lipolysis and fatty acid oxidation and decreases lipogenesis;
3) increases the mobilization of amino acids from skeletal muscle;
4) increase in ketogenesis.

This multi-step process of reducing glucose and increasing fatty acid and ketone bodies utilization has recently been termed “keto-adaptation” [20].

2.2.1 Elevated Hepatic Gluconeogenesis and Glycolysis

Hepatic gluconeogenesis and glycolysis are involved in the five differential steps of glucose homeostasis [2].

Step 1) In the 4-6 hours following a mixed meal, carbohydrates are broken down into individual molecules of glucose which directly raise blood glucose [21-23]. Protein is metabolized into amino acid which increase glucagon levels and subsequent hepatic glucose release. Fat intake has an inconsequential influence on blood glucose immediately post-prandial. However, as these
post-prandial circulating glucose molecules are metabolized to meet central and peripheral tissue glucose demands, glycogen stores are catabolized and mobilized to maintain circulating glucose homeostasis [2, 24]. Within the first 4-6 hours post-prandial, central and peripheral tissue glycolytic demands remain unchanged.

Step 2) Reduced blood glucose causes reduced insulin release from pancreatic beta-cells due to reduced GLUT2 glucose uptake and ATP generation [15-18]. The reduced circulating insulin decreases GLUT4 translocations and glucose uptake in both skeletal muscle and adipose tissue, reducing glucose utilization. Additionally, the reduced blood glucose also promotes glucagon release from pancreatic alpha cells and increased catecholamines (potentially via autonomic regulation). Both catecholamine and glucagon trigger the enzymatic breakdown of glycogen stores from the liver (glycolysis), while lowered insulin remove the inhibition of this process [15, 16, 25], allowing for the normalization of circulating glucose levels for up 16 hours post-prandial [2]. However, the liver only stores approximately 100g of carbohydrates, limiting its capacity to sustain extra-hepatic tissue glycolytic demands [26]. Thus, reduced insulin, increased glucagon, and increased catecholamines facilitate elevated lipolysis, free-fatty acid mobilization, and subsequent ketone-body synthesis [2, 18]. Within this first 16 hours post-prandial, the liver, muscle and adipose tissue all lower their reliance on glycolytic metabolism and shift towards fatty acids oxidation. The increased fatty acid oxidation is accompanied by subtle elevations in circulating ketone bodies β-hydroxybutyrate and acetoacetate which also supplement both central and peripheral tissue energetic requirements [5].
Step 3) As glycogen stores diminish 16 to 48 hours post-prandial, hepatic gluconeogenesis becomes the predominant metabolic pathway sustaining extra-hepatic tissue glycolytic demands [2]. During this period, the liver, muscle and adipose tissues further reduce reliance on glycolytic metabolism and upregulate fatty acid metabolism.

Step 4) 48 hours to 24 days post-prandial, glycogen stores are commonly diminished, and the primary form of endogenous glucose is now gluconeogenesis from both the liver and renal tissues [2]. Fatty acids become the predominant fuel for most tissue with the exclusion of the brain, red blood cells, renal medulla, and a small portion in the muscle, which still rely on glucose. Circulating ketone levels become progressively higher and contribute proportionately to energetic needs.

Step 5) An estimated 21-24 days into the fast, gluconeogenesis remains the only endogenous source of glucose with almost all tissues primarily relying on fatty acid metabolism [2]. However, this final step in maintaining glucose homeostasis is characterized by maximally elevated and plateaued circulating ketone bodies (5.0-6.0 mM). At this final stage of glucose homeostasis, fatty acid and ketone metabolism make up the vast majority of systemic energetic metabolism. However, amino acid, glycerol, and lactate gluconeogenesis generate glucose to sustain circulating glucose needs and energy metabolism for obligate glucose users (i.e. red blood cells).
2.2.2 Lipolysis, Fatty Acid Oxidation, and Lipogenesis

Insulin is well known for its ability to augment adipogenesis, increase adipose tissue stores in a caloric surplus, and inhibit lipolysis [19, 27]. Insulin facilitates this effect via IRs. IRs are abundant on adipocytes and insulin binding facilitates both glucose and FFA uptake and lipogenesis [28]. Additionally, insulin is a potent inhibitor of lipolysis via inhibition of hormone-sensitive lipase. Insulin and IRs importance to these processes are best demonstrated in adipose tissue IR and IGF-1R knockout models demonstrating marked reductions in adipose tissue size and reduced ability to increase adipose tissue when challenged with a high fat diet [27].

While reduced insulin removes the blockade on lipolysis and FFA mobilization, catecholamines and glucagon stimulate lipolysis and catabolism of triglycerides into FFA and glycerol components [16, 25]. Together, these processes result in increased serum FFA and glycerol levels as observed during a fast [2, 24, 29, 30]. Of note, FFA and glycerol levels rapidly elevate within the first three days of a fast followed by subtle elevations until day 17, suggesting rapid elevations in fatty acid mobilization and subsequent fatty acid utilization. Evidence for the systemic shift to fat oxidation has been indicated by respiratory calorimetry [5, 31-33]. At the hepatic tissue the increased FFA availability drives fatty acid oxidation and subsequent ketogenesis in the lower insulin environment [34]. While elevations and oxidation of FFAs appears to be a very acute change, ketone production appears to be a progressive as it takes multiple days before ketone bodies reach maximal and plateaued blood concentrations [5]. Thus, during prolonged starvation as insulin lowers, glucagon and catecholamines elevate, glucose availability
lowers, and FFA and ketone body availability increases, endogenous adipose tissue stores progressively make up the predominant endogenous fuel source for systemic energetic demands.

### 2.2.3 Skeletal Muscle and Hepatic Amino Acid Metabolism

Insulin/IGF-1 are known regulators of skeletal muscle mass [35]. Elevations in insulin and IGF-1 trig...
conserved role in maintaining extra-muscular tissue function. However, interestingly, catabolism of skeletal muscle to maintain gluconeogenesis is slowed upon ketone body elevation (for more detailed discussion see 2.4.4 Amino Acids and 2.5 Anti-Catabolic Effects of Ketone Bodies). While this clearly seems to be in part indirectly driven in part by ketone supplementing energetic needs of various tissues [2], the exact mechanisms of ketone-induced altered skeletal muscle catabolism during prolonged fasting, lowered insulin/IGF-1 environment, has not been determined.

2.2.4 Ketogenesis

When insulin is sufficiently attenuated, adipose tissue lipolysis causes peripheral tissue mobilization and hepatic FFA uptake. When FFAs reach the liver in a lower insulin environment, they undergo oxidation to acetyl CoA. HMG CoA regulates the irreversible step of acetyl CoA’s entry into the ketogenesis pathway (Newman 2014). HMG CoA is regulated via several molecules that are modulated by insulin/IGF-1 signaling (mTOR, PPARγ, FGF21, etc…), resulting in the synthesis of both β-hydroxybutyrate and acetoacetate (interconverted via β-hydroxybutyrate dehydrogenase). However, hepatic tissue lacks succinyl CoA transferase (SCOT), a critical ketolytic enzyme facilitating the conversion of acetoacetate to acetylacetyl CoA [48]. The level of SCOT activity has been demonstrated to be proportional to tissue ketolytic capacity [49]. Thus, the lack of SCOT in the hepatic tissue inhibits its ketone oxidative capacity. However, reduced SCOT in the liver may be explained in part or whole by extra-hepatic tissue energy demands during low glucose/insulin environments. While the brain readily utilizes glucose for energy, it cannot readily transport and/or efficiently utilize long-chain fatty acids [50]. However, in 1967 Owen et
al. made a seminal discovery that ketone bodies are consumed across the cerebral tissue, supplementing 60% of brain energy demands, making hepatic ketogenesis the predominant cerebral nutrient during prolonged fasting [5]. Recent discoveries have demonstrated that ketone bodies may be the preferred cerebral fuel as higher ketone bodies result in proportionally high brain ketone utilization [51-53], while glucose utilization is limited by cerebral energetic demands [51]. Uniquely, ketone bodies also have a physiological range that is approximately 4-fold larger than glucose, lactate, pyruvate, alanine, FFAs, triglycerides, or glycerol [54]. Thus, the maximally elevated ketone levels during fasting, ketone-induced cerebral ATP production, attenuated ketolysis in hepatic tissue, and the unique physiological concentration range of ketone bodies suggests that these metabolites are evolutionarily conserved to, in part, maintain cerebral energy metabolism during lower glucose/insulin environments. However, β-hydroxybutyrate and acetoacetate are not just metabolized by cerebral tissue [52], they are readily transported to peripheral and central tissues through MCT transporters where they play a plethora of roles across metabolism, inflammation, oxidation stress, epigenetic, amongst others [55, 56]. These metabolic and signaling targets of ketone bodies appear to be relevant in regulating skeletal muscle mass during disease and non-disease atrophy [57].

2.3 Very-Low Carbohydrate Ketogenic Diet

A VLCKD is a high fat, low to moderate protein, very low carbohydrate, typically in the form of fibrous vegetables, diet. VLCKD has undergone extensive investigation in recent years due to emergent relevance across various disease pathologies [7, 58-64] and data on the signaling effects on ketone bodies [55, 56, 65-71]. Interestingly, a VLCKD has been demonstrated to
produce an overlapping metabolic environment to fasting. When exogenous carbohydrates are removed from the diet and protein is kept at low enough levels, systemic metabolism begins to shift towards fat-based metabolism [6-8, 20, 72]. VLCKD also replicates many signaling effects observed in fasting/caloric restriction environments [69].

Differences exist between a VLCKD and fasting. One clear difference is in calories and energy homeostasis. Since VLCKD does not require obligate caloric restriction, one can maintain nutritional ketosis without reducing calories [8]. One final difference between VLCKD and fasting are circulating IGF-1 levels. While both fasting and VLCKD lower IGF-1 levels, VLCKD results in ~20% reduction in circulating IGF-1 [73, 74], while fasting can result in a 80% reduction [42] compared to standard diet ad libitum control groups. The reason for this alteration in IGF-1 levels may be differences in calories and amino acid precursors, such as methionine, for IGF-1 synthesis [75, 76]. In support of this hypothesis is the growth impairment seen in a percentage of children on a very carbohydrate and low protein diet [77], normal growth observed in children on a very low carbohydrate and high protein diet [61], and growth retardation correlated with both low protein and calorie intake [76].

One critical issue with the utilization of a VLCKD is that all dietary regimens, regardless of composition, come with a common issue of compliance [78, 79]. This may be further complicated and potentially compromised in the disease state where multiple other factors influence dietary and lifestyle change adherence (nutritional education, family support, etc…; [80]). Consequently, attempts have been made to replicate various aspects of a nutritional ketosis without introducing the potential confounding variable of dietary adherence.
2.4 Exogenous Ketone Bodies

Exogenous ketone bodies have been developed to allow for the rapid elevation of ketone bodies, and associated metabolic changes, without obligate changes in dietary/lifestyle [9, 10]. Consequently, this may result in improved patient compliance and achievement of a state of nutritional ketosis [57]. There are currently multiple forms of exogenous ketones bodies that can be placed into three categories: fats, salts, and esters.

2.4.1 Fats

Fats typically fall into three categories based on carbon chain length, short, medium, and long chain fatty acids [81]. Octanoic (8C), decanoic acid (10C), and dodecanoic acid (12C) make up medium chain fatty acids (MCFAs) and are naturally occurring in a variety in animal and vegetable fats. MCFA appear to present unique metabolic characteristics over shorter or longer chain fatty acids. MCFAs more readily reach hepatic tissue (GI absorption and do not require chylomicron transport), readily enter mitochondria without obligate carnitine transport, and rapidly undergo beta-oxidation to acetyl CoA and ketogenesis. These effects are observed when MCFAs are exogenously administered as they induce a larger thermogenic effect and rapidly elevate circulating ketone bodies, unlike short chain or long-chain fatty acids [9, 51, 81, 82]. Thus, exogenous MCFAs can be readily converted to elevated ketone bodies, inducing nutritional ketosis. While MCFAs can induce nutritional ketosis, MCFAs ketogenic potential, appears to be limited by GI absorption of these fats (and potentially to a lesser degree hepatic metabolic capacity).
2.4.2 Salts

Ketone salts are a synthetic form of exogenous ketone bodies composed of β-hydroxybutyrate ionically bound to monovalent or divalent cations (Na+, K+, Mg2+, Ca2+) to create a salt. Thus, β-hydroxybutyrate-salts accompany a mineral load depending on the choice and proportion of cation used to synthesize the salt. β-hydroxybutyrate-salts are typically in a racemic mixture of approximately 50% R-β-hydroxybutyrate and 50% S-β-hydroxybutyrate [10]. This has metabolic, signaling and analytic consequences. R-β-hydroxybutyrate is the physiological form of β-hydroxybutyrate synthesized during fasting, VLCKD, and upon MCFA ingestion. S-β-hydroxybutyrate appears to undergo differential metabolism than R-β-hydroxybutyrate [10, 83, 84]. 47-75% of S-β-hydroxybutyrate is converted to acetyl CoA and metabolism back to R-β-hydroxybutyrate, while remaining portion of S-β-hydroxybutyrate is metabolized via non-ketolytic pathways [84].

Upon ingestion, β-hydroxybutyrate-salts are absorbed through intestinal MCT transporters and enter systemic circulation where they can be readily metabolized in extra-hepatic tissue. Stubbs et al. demonstrated that when ingestion of 3.2 mmol/kg of racemic β-hydroxybutyrate-salt induced 1mM R-β-hydroxybutyrate 1.5 hours post-ingestion [10]. While, S-β-hydroxybutyrate also achieved maximal elevation at 1.5 hours, circulating S-β-hydroxybutyrate levels remained elevated at approximately 2mM for atleast 4hours, whereas R-β-hydroxybutyrate was back to baseline at 2hour post-ingestion. Interesting combining β-hydroxybutyrate+MCFAs has been demonstrated to prolong R β-hydroxybutyrate elevation in rats longer than MCFA or β-
hydroxybutyrate alone [9], demonstrate unique exogenous ketone formulations with altered pharmacokinetics.

2.4.3 Esters

Ketone Esters are synthetic molecules composed of an alcohol esterified to an individual or multiple ketone bodies [9, 10, 85, 86]. Two primary examples that have been explored in the literature are $R$-1,3-Butanediol β-hydroxybutyrate Monoester and $R/S$-1,3 Butanediol Acetoacetate Diester. These molecules can more potently elevated β-hydroxybutyrate and Acetoacetate in a dose-dependent manner than either MCFAs and/or ketone salts [9, 10]. Upon ingestion, 1,3-Butanediol is cleaved from the attached ketone bodies by gastric esterase’s [87]. Ketone bodies readily cross the small intestine via MCT transporters [55]. However, 1,3-Butanediol is transported to the liver where it is metabolized by alcohol dehydrogenase and aldehyde dehydrogenase into β-hydroxybutyrate. The key factors that distinguishes these two esters is the ketone body composition. The monoester is composed of 1:1 1,3-Butanediol:β-hydroxybutyrate and results in $R$-β-hydroxybutyrate:Acetoacetate elevations in a 4:1 manner [10]. The diester is composed of $R/S$-1,3-Butanediol:Acetoacetate in a 1:2 ratio and elevates $R$-β-hydroxybutyrate:$S$-β-hydroxybutyrate:Acetoacetate in 0.5:0.5:1 ratio with a 1:1 ratio of $R/S$-β-hydroxybutyrate to Acetoacetate observed in circulation [86]. These molecules have been shown to effectively raise ketones in a dose-dependent manner and appears to currently be the most potent way to induced nutritional ketosis via exogenous ketone supplementation.
2.4.4 Others

While MCFAs, ketone salts, and ketone esters represent the primary form of exogenous ketone bodies explored in the literature, other exogenous ketones exist with various ketone compositions and elevations including monoacetoacetin [88-92], lithium-acetoacetate [93-95], tris-glycerol-β-hydroxybutyrate ester [96], amongst others. Monoacetoacetin is a glycerol attached to acetoacetate and primarily elevates acetoacetate and has been explored as alternative to traditional parental nutrition in rodent modeling. However, monoacetoacetin often requires glycerol control to elucidate ketone specific effects. Lithium-acetoacetate is a lithium bound to acetoacetate to stabilize acetoacetate, as acetoacetate is extremely unstable and rapidly degrades in isolation [97]. Lithium-acetoacetate has primary been used in cell-culture experiment to determine acetoacetate effect [93-95], but is confounded by lithium potential neurologic effects [98]. Tris-Glycerol-β-hydroxybutyrate ester is a glycerol molecule attached to three β-hydroxybutyrate molecules has been only undergone preliminary analysis in Parkinson rodent modeling [96].

2.5 Ketone Body Metabolism

Exogenous ketone bodies induce robust metabolic alterations and present many similarities and differences between other forms of nutritional ketosis. Like fasting and VLCKD, exogenous ketone bodies, regardless of formulation and without dietary restriction, rapidly raise circulating ketone levels [9, 10]. However, ketone esters have been demonstrated to be much more potent than ketone salts when exogenously administered [9, 10], demonstrating differences in potencies of
various exogenous ketone bodies. Additionally, exogenous ketone bodies are able to rapidly raise ketone levels within minutes of oral administration [9, 10], demonstrating a clear temporal difference between exogenous ketones and both fasting [2, 41] and VLCKD [6, 8, 72] which can take hours to days to observe endogenous R-β-hydroxybutyrate ≥0.5mM in adults. Additionally, while fasting and VLCKD increase endogenous production of ketone bodies, exogenous ketone administration can temporarily reduce endogenous ketone production [52]. Together, exogenous ketones rapidly induce nutritional ketosis without dietary restriction and present both similarities and difference to fasting and VLCKD.

2.5.1 Glucose

Acute exogenous ketones bodies have been shown to consistently lower circulating blood glucose levels across populations [9, 10, 44, 99-105]. However, several factors have been cited to be contributing to this glycemic response. Ketone bodies have been observed to induce insulin release from pancreatic islet cell extract [106]. Serum insulin elevations have also been observed along with exogenous ketone administration [10, 99]. However, exogenous ketones have also been demonstrated to lower blood glucose independent of insulin elevations [44, 52, 99, 103, 104]. Combined, this data seems to indicate that ketones may elevate insulin, but that elevations in insulin concentrations are not required to induce blood glucose lowering effect. Alterations in insulin sensitivity may also explain blood glucose lowering effects in studies where insulin levels remain unchanged. Myette-Cote demonstrated that exogenous ketone administration with oral-glucose load decrease glucose exposure, while insulin levels remained the same, demonstrating elevation in insulin sensitivity markers [107]. Mikkelsen et al. demonstrated that as Na+-β-
hydroxybutyrate was exogenously infused into patient, glucose lowered, but insulin also lowered, suggesting potential insulin sensitivity changes [52]. Beylot et al. demonstrated that ketone infused during euglycemic clamp reduced endogenous glucose production indicating that ketones reduced glucose production via reducing availability of gluconeogenic precursors alanine [108]. Miles et al. showed similar findings in reduced glucose production driven by reduced alanine availability [103]. Beylot et al. demonstrated the ability to lower glucose production in septic patients [109]. Analysis in muscle preparations demonstrated that ketones reduced glucose oxidation rates but increase glycogen synthesis [110-112]. Recent analysis using ketone monoester co-administered with glucose in humans demonstrated augmented glycogen synthesis when compared to glucose administration alone [113]. Additionally, we demonstrated that chronic exogenous ketone supplementation via gavage or food significantly elevated ketones bodies and lowered blood glucose [9, 114]. Together, these results indicate that exogenous ketone bodies reduce blood glucose 1) with or without alterations in insulin concentration or sensitivity, 2) via attenuated glucose production via reductions in anaplerotic precursors, and 3) via lowered glucose oxidation in preferences for glycogen storage.

2.5.2 Lipids

Exogenous ketone bodies directly and radily modulate lipid metabolism. Taggart et al. demonstrated that β-hydroxybutyrate binds HCAR2/GPR109a and inhibits lipolysis by inhibiting NF-κB activity in adipose tissue [115]. A number of studies where exogenous ketone administration reduced FFAs and glycerol with or without changes in insulin [52, 99-101, 104, 107-109, 116]. This effect also appeared to be dose-dependent [52]. This mechanism and direct
effect of ketone bodies help explain a potential self-regulating system on lipolysis during low
insulin environment of fasting and VLCKD. However, this also demonstrates a contrast to fasting
and VLCKD as both these nutritional approaches upregulate lipolysis and FFA mobilization, while
exogenous ketone inhibits it via HCAR2/GPR109a.

2.5.3 Lactate

Exogenous ketones bodies have shown mixed results in regulating lactate metabolism. Both administration of either monoester or diester has reduced lactate productions during exercise [100, 101, 117]. However, lactate production has been demonstrated to potentially be elevated on VLCKD during exercise [7, 118]. Exogenous ketones administered in a diet in cancer cachexia models and patients reduced lactate production [105, 116]. However, some studies have indicated that ketones do not alter lactate level [44]. Conversely to both these findings, are reports of lactate elevations with exogenous ketone administration [103, 108, 119]. Together, these results indicate that ketone bodies have a mixed response on lactate metabolism that may be explained by ketone body concentration, composition, and therapeutic environment.

2.5.4 Amino Acids

Exogenous ketone bodies have been shown to directly regulate amino acid metabolism and protein kinetics [44, 92, 103-105, 108, 119]. β-hydroxybutyrate infusion has been demonstrated to lower BUN and urinary excretion [44, 92] similar to what is observed during fasting upon ketone body elevation [2, 4, 24] and during VLCKD [8]. Interestingly, pathological elevations in ketone
bodies up to approximately 10 mM in uncontrolled type-1 diabetic patient with insufficient insulin present with a 48% reduction in BUN compared to uncontrolled type-1 diabetic patient on insulin [13]. Exogenously administered ketone bodies have been shown to directly inhibit alanine flux from skeletal muscle [103, 105, 108] in a dose-dependent manner regardless of fed or fasted status [44], similar to what has been demonstrated during fasting when ketone bodies elevate [4].

Exogenous ketone bodies have been shown to attenuate markers of leucine oxidation [92, 104] and enhance leucine induced mTORC1 response in skeletal muscle [120] similar to systemic reduction to leucine oxidation [8] and increased skeletal muscle mTORC1 activation observed in VLCKD [121]. However, two studies determined that exogenous ketone administration did not demonstrate superior leucine kinetics when directly compared to either a glucose [88, 89] or amino acid formulation [109]. Conversely, in further analysis of ketone-induced modulation of leucine kinetics, Nair et al. intravenously infused β-hydroxybutyrate post-prandial and observed decreased leucine oxidation, increased muscle leucine incorporation, increased fractional whole body protein synthesis, and no changes in insulin when compared to saline-only infusion [104]. Maiz et al. infused burned rats with monoacetoacetin which reduced weight loss, urinary nitrogen excretion, and leucine release, and increased in skeletal muscle protein synthesis rates, without changes in glucose or insulin levels, compared to group receiving amino acids-only via parental nutrition [92]. Maiz et al also found that ketones and dextrose infusion with amino acids had similar effects on improving protein kinetics. Birkhahn et al. demonstrated similar leucine kinetics for both glucose and ketone infusion in two separate rat studies [88, 89]. In 1986, Birkhahn et al. conducted a pilot evaluation in rats with five different monoacetoacetin and/or glucose+glycerol formulations [88]. While the authors argued that monoacetoacetate+glucose at 45 kcal/day induced more optimal
bodyweight, behavior, and nitrogen excretion compared to non-ketone groups, authors also did not observe statistical differences across these groups in nitrogen and leucine. However, this analysis is hard to interpret as authors experienced a host of experimental complications confounding interpretation of work. However, in 1988, the same group found that urinary nitrogen losses, leucine oxidation, and leucine turnover were similar between intravenous infusion of monoacetoacetin and glucose versus glucose-only in traumatized rats (induce leg fracture), but monoacetoacetin had the benefit of not resulting in hyperglycemia [89]. In 1994, Beylot infused 12 intensive care unit septic patients with either β-hydroxybutyrate or amino acids and found no differences across groups in leucine oxidation [109]. Taken together, the body of evidence indicates that exogenous ketone bodies reduce leucine oxidation but may not be superior to other macronutrients in modulating leucine kinetics.

Lastly, Thomsen et al. in 2018 found that intravenously infusion of β-hydroxybutyrate in endotoxin infused patients resulted in significantly reduced muscle catabolism and improved muscle protein turnover. Exogenous ketone bodies also demonstrated superior muscle protein turnover calculations when compared to elevated glucose infusion and hyperinsulinemia, even when controlling for β-hydroxybutyrate’s confounding anti-lipolytic and anti-inflammatory effect via HCAR2/GPR109a on NF-κB [119]. These results demonstrate a robust and superior anti-catabolic effect of ketone bodies in patients using gold-standard muscle turnover analysis in an inflammation-based atrophy environment.

Together these results strongly suggest that exogenous ketone bodies alter skeletal muscle amino acid kinetics as indicated by reduced BUN/urinary nitrogen, reduced alanine flux, reduced
leucine oxidation (which may not be superior to other macronutrients on leucine kinetics), and increased anabolic signaling (when co-administered with amino acid).

2.6 Anticatabolic Effects of Ketones Bodies

2.6.1 History

Fasting, VLCKD, and exogenous ketone bodies all induce metabolic alterations that demonstrate the potential to induce anti-catabolic effects on skeletal muscle. However, initial evaluation into this question was spawned by studies over a century old. In 1915, Benedict conducted one the initial analysis into the physiological consequences of prolonged fasting [122]. While this 31-day observation was not the first to observe the effect of extreme caloric deprivation, it was unique in its robust demonstration of the resilience/adaptive capabilities of humans in prolonged extreme caloric deficits. Interestingly, Benedict did analysis of systemic protein kinetics using nitrogen excretion. Benedict observed that in both his subjects and in prior analyses, that as fasting prolongs, an unexpected and unexplained reductions in nitrogen extraction was commonly observed. These observations led to a sequence of seminal research studies into the metabolic adaption that occur during prolonged energy deficits [2-5, 24, 41, 123-125]. In 1966, George Cahill confirmed a reduced nitrogen excretion in prolonged fasting using more reliable techniques. He calculated that as glycogen becomes depleted and gluconeogenic precursors become the only source of glucose production, amino acid catabolism, as calculated based on nitrogen excretion (3.73g/day), could only explain 10% (or 13.25g glucose/day) of the glucose needed to maintain brain energy glucose demands (110-145g/day). Cahill calculated that 10% of adipose tissue
breakdown would allow glycerol to contribute 19.2g glucose/day. From both glycerol and amino acid catabolism, only 33g glucose were being produced from gluconeogenic precursors, leaving a 77-122g glucose deficit in the brain. However, into two follow up analyses Cahill directly evaluated tissue metabolite utilization and production using arterial to venous metabolite concentration analysis [4, 5]. Cahill’s team discovered that approximately 300g ketones/day were being produced by hepatic ketogenesis and ketone bodies made up 66% of brain energy metabolism. Notably, Cahill also discovered that as ketone bodies elevated, alanine flux from the skeletal muscle reduced 3-fold along with reductions in nitrogen excretion [4, 5].

From 1973-1975, multiple analyses confirmed associations and/or direct effect between elevations in ketones bodies and reduced metrics of protein breakdown. Blackburn et al. 1973 found that infusion of protein instead of glucose in septic and obese patients caused high ketone elevations and improved nitrogen balance [126]. However, when these same patients were infused with glucose-only, the anti-catabolic benefits on nitrogen balance disappeared. That same year, Kies et al. found that 1,3-butanediol ingestion in humans, a exogenous ketogenic precursor, improved nitrogen balance and decreased blood glucose (through decrease gluconeogenic enzymes) compared to starches even when calories were controlled between groups [127]. In 1975, Hoover made the same observation in post-operative patients demonstrating that higher ketones improved nitrogen balance, while high glucose and insulin cause reduced nitrogen balance [128]. That same year, Smith et al. observed that in a cohort of severe accident patients, higher ketone levels were associated with lower urinary nitrogen excretion. Additionally, Smith et al. also observed that in the patients with higher circulating ketone bodies, alanine, lactate, and pyruvate were all reduced [129]. In 1975, Sherwin et al. was the first observation that exogenous ketone
administration directly modulated alterations in skeletal muscle homeostasis [44]. Sherwin et al. observed that during prolonged fasts, patients with elevated ketone bodies present reductions in alanine. However, Sherwin et al. also demonstrated that when fasted and septic subjects were infused with ketone bodies, alanine were further reduced (6-fold reduction) in a dose-dependent manner without changes in insulin. Sherwin went on to discover that ketones still induced reductions in alanine in subjects who were in the fed state, illustrating a consistent effect of ketone bodies on alanine regardless of energetic status.

From 1978-1988 Birkhahn did several trials ion rodents looking at monoacetoacetin, one the original exogenous ketone formulations [88, 89, 92]. In 1978, Birkhahn infused monoacetoacetin at various dosages into rats and found that it was well tolerated and induced elevations in ketone that could surpass fasting levels [90]. Due to this success, in 1985 Maiz et al. found that infusion of monoacetoacetin into burned rats reduced weight loss, urinary nitrogen, and leucine release excretion without changes in glucose or insulin levels [92]. Conversely, glucose infusion increased insulin and decreased ketone bodies, but had similar effect on decreasing weight loss, nitrogen excretion, and leucine release to ketone bodies (calorie-controlled environment). Maiz et al. argued that ketone bodies, as a none nitrogen source, can replace glucose in this burned environment as an important regulator of muscle protein breakdown biomarkers when calories are equivalent. In 1986, Birkhahn et al. did a pilot evaluation comparing various formulations of glucose, glycerol, and monoacetoacetin [88]. They found that 45kcal/day infusion of 50:50 mixture of glucose+glycerol cause hyperglycemia, while a 45kcal/day infusion of monoacetoacetin cause normoglycemia but elevated ketones. However, both rats lost weight. It was determined that a 67:33 mixture of monoacetoacetin:glucose results in normoglycemia, elevated ketones, weight
gain, and nitrogen retention in rats. However, authors described a host of complications due to technique difficult of IV infusion illustrating a confounding factor across groups. None the less, Birkhahn et al. argued that a majority monoacetoacetin formulation with low enough glucose prevent hyperglycemia, but improved muscle turnover biomarkers. In a follow up study, Kripke et al. evaluated this monoacetoacetin:glucose formulation in rodent models of intestinal tissue atrophy model [91]. Kripke et al. found that monoacetoacetin induced a positive nitrogen balance and reductions in mucosal atrophy (commonly seen in total parenteral nutrition). Interestingly, atrophy of jejunum and colon were inhibited in acetoacetate group compared to glycerol and standard rodent chow control groups. These results illustrated calorie superiority of ketone as anticatabolic fuel compared to glucose in peripheral tissue outside of skeletal muscle. These results also introduce the possibility that amino acid flux from skeletal muscle may be partially reduced due to reduced peripheral tissue amino acid demand.

In 1987 and 1988, the first evaluation of exogenous ketones bodies (MCFAs and $\beta$-hydroxybutyrate) in cancer cachexia model and patients, respectively [105, 116]. Tisdale et al. found that exogenous ketone diets (68%MCFAs; 60%MCFAs+$\beta$-hydroxybutyrate; 80%MCFAs; 80%MCFAs+$\beta$-hydroxybutyrate) all reduced tumor weight in inverse ratio to percentage of exogenous ketones in the diet (higher MCT+$\beta$-hydroxybutyrate:lower tumor weight). Tisdale et al. also found that higher exogenous ketones attenuated bodyweight and muscle loss in both tumor bearing and non-tumor bearing models. Additionally, all ketone diets reduced lactate levels in cachexia models. Lastly, all exogenous ketone diets reduced FFA and increased $\beta$-hydroxybutyrate and acetoacetate, without altering blood glucose. The success of this preclinical analysis spurred a small clinical trial with five extremely malnourished malignant cancer patient (~32% bodyweight
lost) who underwent intragastric feeding of standard diet or exogenous ketone diet with 70%MCFAs and 4mmol/kg/d of arginine β-hydroxybutyrate for 6 days [105]. Exogenous ketone diet group produced improvement in >2kg in bodyweight in just 6 days. The standard diet group observed a doubling in ALT, reduced albumin, elevated urea, but didn’t have any difference from exogenous ketone diet group on overall nitrogen balance. This initial evaluations into the efficacy of exogenous ketone bodies into cancer cachexia demonstrated improvement across various metrics of skeletal muscle homeostasis and clinical confounding biomarkers. In 1988, Nair et al. did an analysis into intravenous infusion of β-hydroxybutyrate during the post-absorptive stage [104]. β-hydroxybutyrate infusion increased whole body protein synthesis, reduced leucine oxidation and increase leucine muscle incorporation.

2.6.2 Nutritional Ketosis Limitations

An absence of literature on ketone bodies influence on skeletal muscle homoeostasis was observed from 1989-2013. Waning interest may be due to logistical limitation revolving ketogenic therapies. While fasting/severe caloric restriction induce nutritional ketosis, both results in atrophy due to catabolism of endogenous energy stores to sustain energy needs. Thus, while unique insights into ketone physiology could be gleaned from evaluation of fasting physiology, fasting/caloric restriction is contraindicated in atrophy-based disease. While a VLCKD circumvents the caloric reduction of fasting and induces nutritional ketosis, many within the clinical community remain hesitant about implementing a high fat diet [130] even in disease states where therapeutic evidence is robust [64]. Furthermore, dietary change is difficult for some patients [78, 79] and may be further complicated in the disease state [80]. Additionally, the predominant other method conducted to
upregulated circulating ketones bodies was via intravenous infusion which is limited to the clinical and/or research setting. However, the emergence of orally administered exogenous ketone bodies accompanied a rapid proliferation of studies evaluating nutritional ketosis for an array of health, disease, and performance applications including interest in skeletal muscle regulation.

### 2.6.3 Emergent Evidence

In 2014, Shukla et al. evaluated the effects of ketone bodies in a model of pancreatic cancer cachexia [131]. Treatment with β-hydroxybutyrate and lithium acetoacetate (or lithum) caused no harm to normalized pancreatic cells (and no change in pH). β-hydroxybutyrate and lithium acetoacetate reduced cell survival in pancreatic cancer cell line in dose-dependent manner. β-hydroxybutyrate and lithium acetoacetate also reduced glucose uptake, lactate release, glutamine uptake, cellular ATP content, and ROS levels. While, pancreatic cell line media induced wasting in C2C12 and 3T3L1 (adipocyte) cell lines, treatment with ketone bodies attenuated cancer cell media induced atrophy in C2C12 and 3T3-L1 and reduced gene expression in C2C12 myotubes of atrophying genes (mRNA Atrogin-1, MuRF-1, etc.). This work demonstrated an ability of ketone to both play an anti-tumor and anti-cachexia role, with direct effects on both myocyte and adipocyte function.

In 2016, Zou et al. assessed acetoacetate’s effect on muscle in both in vitro myocytes, in vivo healthy muscle, and mdx muscular dystrophy rodent modeling [70]. They demonstrated that acetoacetate promoted dose and time-dependent proliferation in C2C12 myoblast, an effect that was replicated with acetoacetate administration in vivo via satellite cell activation and
proliferation. Zou et al. also demonstrated an ability of acetoacetate to potentiate IGF-1 signaling in C2C12 cells resulting in proliferation and partial inhibition of myostatin. Additionally, in a model of muscular dystrophy, acetoacetate improved muscle architecture and function. Mechanistically, Zou et al. indicated that the proliferative capacity of acetoacetate in myoblasts was via MEK-ERK signaling. Interestingly, acetoacetate has been demonstrated to stimulate this proliferative pathways in hepatocytes [132] and BRAFV600E cells [133], suggesting a consistent, metabolically independent signaling effect of acetoacetate downstream of insulin/IGF-1 signaling cascade.

In 2017, Vandoorne et al. evaluated the effect of oral exogenous ketone administration on insulin/IGF-1/mTORC1 signaling in human skeletal muscle post-exercise [120]. Ketone ester enhanced mTORC1, S6K, and 4EB-P1, while lowering AMPK in skeletal muscle biopsy samples. In C2C12, β-hydroxybutyrate or acetoacetate alone did not stimulate mTORC1, S6K, 4EB-P1. However, β-hydroxybutyrate (4mM) and lithium acetoacetate (1.4mM) combined did stimulate mTORC1 biomarkers. β-hydroxybutyrate+acetoacetate combined with 1.5mM or 5mM Leucine enhanced Leucine-induced mTORC1 signaling, even when 1.5mM Leucine did not stimulate mTORC1 response alone. These results reveal that oral exogenous ketone administration may augment anabolic signaling in the skeletal muscle when co-administered with amino acids.

In 2018, Roberts et al. conducted a large analysis on the effects of various diets formulations (standard diet, low carbohydrate diet, and VLCKD) on aging mice, a model of sarcopenia [121]. Mice on a VLCKD, but not low carbohydrate diet, had increased lean body mass, strength, skeletal muscle mTOR signaling, suggesting potential skeletal muscle specific effects of
ketone bodies. Interestingly, mice on VLCKD also demonstrated decreased mTOR signaling within the liver, demonstrating tissue specific effects on VLCKD on cellular signaling. Additionally, mice on a VLCKD also demonstrated increase longevity and reduced tumor incidence demonstrating benefits to not only the muscle, but on metric of healthspan and lifespan.

In 2018, Thomsen et al. evaluated the skeletal muscle kinetics of 10 young healthy patients who were infused with endotoxin/LPS to induce septic atrophy phenotype [119]. Interestingly, this group controlled for the know anti-lipolytic and anti-inflammatory signal of β-hydroxybutyrate on HCAR2/GPR109a receptor by drugging the receptor with a niacin derivative. These researchers found that infusion of β-hydroxybutyrate produced a profound anti-catabolic response and an improvement in net protein turnover without increasing muscle synthesis, when compared to saline- or FFA-infused groups. Additionally, the anti-catabolic response of β-hydroxybutyrate was more profound than hyperinsulinemia+euglycemic clamp environment, indicating that β-hydroxybutyrate may be a more potent anti-catabolic stimulus than elevated insulin and glucose combined. Together, these results demonstrated a profound anti-catabolic response during disease induced atrophy in human patients which was better than hyperinsulinemia + elevated glucose, FFA, and saline exposure.

In 2019, it was proposed by our group that emergent evidence, historical anti-atrophy literature, and emergent evidence for metabolic, inflammatory, oxidative stress, and epigenetic signaling roles of ketone bodies illustrates that ketone bodies may be an ideal therapy for multifactorial diseases of skeletal muscle atrophy [57]. While evidence supports this hypothesis, direct chronic effects of exogenous ketone bodies on the skeletal muscle has been undetermined.
2.7 Closing Remarks

Evaluation into nutritional ketosis’ role in skeletal muscle homeostasis dates back over a century. Both historical and emergent evidence indicate ketone bodies may modulate glucose, lipids, amino acids, and lactate, and have anti-catabolic properties. However, limitations on administering nutritional ketosis has attenuated clinical translatability and research interest. Interestingly, emergent development of exogenous ketone bodies and discovery of ketone body signaling across metabolism, inflammation, oxidative stress, epigenetic, amongst others, has reinvigorated interested. However, direct evaluation into the therapeutic efficacy of exogenous ketone bodies in multifactorial diseases of atrophy, remains undetermined.

2.8 References for Chapter 2


93. Bartmann, C., et al., *Beta-hydroxybutyrate (3-OHB) can influence the energetic phenotype of breast cancer cells, but does not impact their proliferation and the response to chemotherapy or radiation*. Cancer Metab, 2018. 6: p. 8.


CHAPTER 3: THE VM-M3 MODEL OF SYSTEMIC METASTASIS DEMONSTRATES THE FULL PROGRESSIVE CANCER-ANOРЕXIA CACHEXIA SNYDROME

3.1 Chapter Synopsis

In this chapter we present data demonstrating that the VM-M3 model of systemic metastasis develops progressive tumor growth and subsequent systemic metastasis spread. The VM-M3 model also demonstrates progressive skeletal muscle wasting with temporal difference across skeletal muscle compartments. VM-M3 Males demonstrate progressive adipose tissue wasting, while VM-M3 Females retain fat mass until after week 3. Additionally, both Males and Females develop systemic inflammation, anorexia, anemia, elevated protein breakdown, hypoalbuminemia, and metabolic derangement. Taken together, data demonstrates that the VM-M3 model of systemic metastasis demonstrates the full progressive cancer-anorexia cachexia syndrome.

3.2 The VM-M3 Model of Systemic Metastasis Demonstrates the Full Progressive Cancer-Anorexia Cachexia Syndrome

As described, cancer-anorexia cachexia syndrome (CACS) is a distinct catabolic disease negatively influencing multiple aspects of clinical care, patient quality of life, and directly causing 1/5 of all cancer-related deaths. This multifaceted syndrome is most common in advanced
metastatic disease and clinically manifests with numerous systemic changes including tissue wasting, inflammation, anorexia, anemia, protein breakdown, hypoalbuminemia, and metabolic derangement. However, no current model reflects the entire multifaceted syndrome, especially the spontaneous metastatic progression or accounts for sex-specific characteristics. However, the VM-M3 model exhibits many of the hallmarks of the metastatic condition, mirroring important aspects observed in clinical cachexia. As such, we hypothesized that the VM-M3 syngeneic model of systemic metastatic would more accurately recapitulate the diagnostic hallmarks of CACS. We suggest that the addition of a multifactorial progressive CACS model that develops advanced metastatic disease would help address a major gap in CACS modeling.

**Results & Discussion:**

An inability to model the clinically reported physiologic environment of progressive metastatic CACS has impeded clinical advancements in the most commonly effected patient population, advanced metastatic cancer patients [134-137]. Our results demonstrate that the VM-M3 model of systemic metastasis replicates the progressive and spontaneous nature of metastatic disease which facilitates the development of the full clinical CACS via progressive wasting of skeletal muscle and adipose tissue compartments, systemic inflammation, anorexia, anemia, elevated protein breakdown, and metabolic derangement in both males and females with sex-specific discrepancies.

**Baseline Bodyweight, Age and Food Intake:**
Bodyweight, age, and food intake can influence various aspects of health and disease progression, especially the cachexic phenotype. Thus, to ensure adequate comparison across groups, animals were separated into groups based on equivalent bodyweight, age, and food intake. VM-M3 animals (CA-M and CA-F) were paired to an animal of equivalent sham-matched control (SH-M and SH-F). Baseline bodyweight did not significantly differ across groups of equivalent sex but did differ across sex (Figure 3.1A). Baseline age and food intake did not differ within or across sex. Following confirmation of cachexia phenotype at EOL, follow up time course analysis was conducted to evaluate cachexic progression at specified timepoints (week 1, week 2, week 3) prior to EOL. Differences were not seen in bodyweight, age and food intake within sex (Figure 3.1B). While age and food intake could be controlled within and across sex, bodyweight could only be controlled within as males are consistently higher in bodyweight at equivalent ages the across lifespan, regardless of rodent species.

**Survival Analysis, Tumor Growth and Metastasis:**

Clinical reports consistently indicate that cachexia is most prevalent during metastatic disease [134-143]. To determine tumor growth, metastatic progression and survival specific to cancer and sex, cancer male (CA-M) and females (CA-F) were matched to sham males (SH-M) and females (SH-F). Following implantation of either 1 million VM-M3 cells carrying the reporter *Luciferase* gene or PBS vehicle-only into VM/Dk background strain, tumor growth and bioluminescent spread were tracked weekly. CA-M and CA-F developed a primary tumor at the implantation site week 1, followed by visible metastatic spread from tumor origin to various tissues, verified during *in vivo* whole animal and *ex vivo* organ and tissue bioluminescent imaging
(Figure 3.2A-D). Primary tumor weight increased progressively from week 1 to end of life (EOL; Figure 3.3A), with similar metastatic invasion into the liver, spleen, adipose tissue, and ascites fluid (Figure 3.3C) between CA-M and CA-F. This is consistent with the progressive nature of systemic metastatic disease [144], where cachexia and comorbidities are most commonly reported in clinical cancer cachexia [134-143]. Additionally, mean survival did not differ between CA-M and CA-F (31.3±1.6 and 32.3±2.4 days, respectively; Figure 3.4A-B) illustrating similar tumor burden, metastatic spread, and survival across sexes.

The VM-M3 model of systemic metastasis presents a unique tumor growth and metastatic progression which is distinct from other models which require intravenous implantation or the assistance of surgical resection of the primary tumor [145-150]. The reliable, reproducible, and logistically feasible nature of this model [151-156] may present numerous experimental advantages over emergent genetically engineered modeling systems which often require lengthy and costly experimental designs due to heterogenous tumor onset and growth, metastatic progression, and cachexia occurrence [12, 144, 157]. Additionally, while metastases can directly disrupt cancer-burdened tissue and indirectly disrupt non-cancerous tissue through secretory factors or alterations in host-tissue response [134, 145, 158, 159], emerging evidence demonstrates that the metastatic process may directly contribute to the cachexia phenotype [145], further highlighting the need to model the complex metastatic CACS.

**Body Composition:**
Skeletal muscle and adipose tissue atrophy are common hallmarks of cancer cachexia [134, 160, 161]. To determine whether the VM-M3 model had progressive tissue atrophy, bodyweight was tracked daily during initial analysis and weekly during subsequent analysis as a superficial marker of body recomposition (Figure 3.5A). CA-M and CA-F gained significantly more bodyweight than sham groups (Figure 3.5B); however, a characteristic feature of the VM-M3 model upon presentation of widespread metastatic burden is the accumulation of ascites fluid in the intraperitoneal cavity, which contributes to the elevation in bodyweight. This characteristic has been observed in metastatic patients [162]. To address this concern, ascites fluid was weighed at EOL to determine its contribution to bodyweight gain. Ascites fluid accounted for 5.5±1.0g and 6.3±1.4g in CA-M and CA-F bodyweights, respectively (Figure 3.5C), explaining in part, along with tumor burden, the elevations in bodyweight seen in VM-M3 animals. Bodyweight being most commonly used clinically as a surrogate marker for both due to ease of assessment. It is important to note, however, that tissues can atrophy and/or hypertrophy in an asymmetric manner, highlighted in cancer patients with sarcopenia obesity and in cachexia trials using appetite stimulants, where it has been reported that adipose tissue can increase while skeletal muscle progressively atrophies [163-165]. Clinically, attributing bodyweight changes to skeletal muscle and/or adipose tissue loss can inaccurately represent the nature of wasting and misguide treatment strategies and “successes” [163-165]. Similarly, we demonstrate the non-specificity of bodyweight as a determinate for cachexia status.

To directly assess cachexic tissue recomposition, skeletal muscle, adipose, and tissue weights were gathered at EOL. Upon confirmation of tissue wasting at EOL, follow up week-by-week cohort analyses were conducted to determine the temporal/progressive nature of atrophy.
across tissues and groups. All individual tissues were set to ratio with baseline bodyweight (not influenced by cachexic progression) to allow for appropriate comparison across groups. CA-M saw a significant decrease in gastrocnemius and soleus weights starting at week 2 (-12.0%) and extending to EOL (-19.5%), compared to SH-M (Figure 3.5D), consistent with the progressive nature of cachexic wasting [161]. Similar trends were observed in CA-F, with decreased gastrocnemius and soleus weights at week 2 (-10.7%; p=0.09), compared to SH-F, which were significantly decreased within and across groups at EOL (-20.8%). Quadriceps weight was retained in both CA-M and CA-F until EOL (Figure 3.5E), indicating temporal and tissue-specific differences in skeletal muscle wasting which have been observed previously in cachexic models [166] and other clinical atrophy conditions [167-169]. Interestingly, while calf weight atrophied in a progressive manner, we did not observe decreases in quadriceps tissue until after week 3. This preferential retention in quadriceps muscle mass over gastrocnemius and soleus has also been reported in rodent C26 cachexia modeling [166] and in patients with disuse atrophy [167-169], and may be explained by gene expression differences across muscle groups [170] as epigenetic modulation of gene expression has been shown to regulate wasting in cachexia, disuse atrophy, and nutrient deprivation [171, 172].

While skeletal muscle wasting is central to the disease, adipose tissue wasting has gained considerable attention as recent reports have demonstrated that adipose tissue wasting may regulate skeletal muscle wasting in several cachexic models [173]. CA-M also presented with progressive decreases in intraperitoneal adipose tissue, with significant decreases at week 3 and EOL within and across groups (Figure 3.5F). Discrepantly, CA-F retained adipose tissue mass up to week 3, followed by rapid and complete wasting of adipose tissue between these timepoints.
This altered skeletal muscle and adipose tissue recomposition observed in CA-F is consistent with previous reports in other female cachexic preclinical models [174] and patients [175, 176], and might be explained by inherent hormonal differences influencing skeletal muscle [177] and adipose tissue [178]. The data here serves to confirm sex-specific cachexic discrepancies accompanies progressive tissue wasting in the VM-M3 model.

Inflammation:

Inflammation has been reported to drive multiple facets of the cachexia phenotype, including tissue wasting, anorexia, metabolic abnormalities, and tumor progression, among others [179, 180]. To determine if VM-M3 animals developed systemic inflammation, spleen weight, white blood cell count, and cytokines were measured. Both CA-M and CA-F developed splenomegaly (Figure 3.6A); however, increased spleen weight could also be attributed to tumor burden and/or other immunologic initiators [181]. Thus, to determine whether enlarged spleen weight could be directly attributed to tumor burden alone, a ratio of tumor burden (bioluminescence in photon/sec) to cancer-induced tissue weight changes (cancer to sham tissue weight differences) was calculated for the primary tumor, liver, and spleen, as the primary tumor bioluminescence would be directly proportional to increased tumor size and serve as a control ratio. The ratio of tumor burden to cancer-induced tissue weight differences (Figure 3.6C) was similar between the primary tumor and liver, but dissimilar to spleen, indicating that the change in weight within the liver could be primarily and/or completely attributed to within organ tumor burden. The spleen weight changes, along with G-CSF elevations (Figure 3.7A) demonstrated spleen enlargement was not due to tumor burden but was indicative of a prominent immunologic
response [182-185]. Additionally, white blood cell counts, along with cellular subpopulations (monocytes and granulocytes), were significantly elevated in CA-M (Figure 3.6D). In CA-F, however, white blood cell counts were not significantly elevated, although they did show similar trends to CA-M. TNF-α and IL-6, proinflammatory cytokines commonly reported in the cachexic phenotype, were elevated in CA-M and CA-F across and within groups (Fig 3E-F) [134, 179, 186]. However, IL-1β, another proinflammatory cytokine reportedly associated with some cachexic phenotypes, was not significantly altered within or across groups (Fig 3G). These inflammatory biomarkers at the organ, cellular, and molecular levels within VM-M3 animals are indicative of a prolonged systemic inflammatory response and consistent with the cancer cachexia phenotype [134, 179, 186].

Anti-cachexic cytokine IL-13 showed significant elevations at EOL in both groups indicating potential anti-inflammatory response at late stage (Figure S7A-D; [186]). IL-5 was elevated at week 2 and regressed to baseline at EOL (Figure 3.7B; [187, 188]). IL-5, which has been associated with adipose tissue fibrosis in cachexic patients and shown to contribute to lung metastasis in various rodent models, was elevated at week 2 and came back to baseline at EOL (Figure 3.7C; [187, 188]). IP-10 and KC (murine analogue for IL-8), both previously reported to be elevated in preclinical and clinical cachexic analysis [189], were elevated at week 2 and persisted into EOL in the VM-M3 modeling system (Figure 3.7D-E). Taken together, CA-M and CA-F show indications of a prolonged systemic inflammatory environment at the organ, cellular, and molecular levels. These progressive elevations in pro-CACS inflammatory markers and associated CACS cytokines indicate a robust inflammatory environment which could not be
Anorexia, Anemia, Protein Breakdown, Hypoalbuminemia, and Metabolic Derangement

Anorexia, anemia, elevated markers of protein breakdown, hypoalbuminemia, and metabolic derangement remain prominent clinical comorbidities of the full CACS [190], but are often not evaluated in pre-clinical modeling to determine the potential clinical relevance of these model systems [160]. To determine whether the VM-M3 model developed the full CACS as clinically presented, all aforementioned clinical comorbidities were evaluated. Food intake was monitored daily to determine the presence of anorexia, a common, contributory, and clinically impactful characteristic of CACS [134, 138, 160, 161, 190, 191]. Both CA-M and CA-F developed anorexia, compared to the elevation in food intake seen in both SH-M and SH-F (Figure 3.8A). Anemia can be a result of inflammation and/or shifted metabolic demands away from red blood cell production [181, 192], which can contribute to fatigue and might explain the noticeable lethargy/functional decline in cachexic patients [160, 193] and VM-M3 animals. CA-M developed anemia as indicated by significantly reduced hemoglobin (Figure 3.8B), hematocrit, and red blood cell count. CA-F had significant reductions in red blood cell count and trends for decreases in hemoglobin and hematocrit. BUN and total protein, clinical markers of whole-body protein kinetics, are commonly elevated and decreased, respectively, in CACS patients [139, 194-196]. CA-M and CA-F had significantly elevated BUN levels and decreases in total protein levels (Figure 3.8B), illustrating increased systemic protein breakdown. Albumin, another critical biomarker commonly reduced in CACS patients, was decreased in VM-M3 animals (Figure 3.8B).

overcome by EOL elevations in anti-cachexic cytokines within both male and female cancer-burdened animals, consistent with the CACS phenotype [134, 179, 186].
This decrease is hypothesized to be a consequence of shunting of hepatic resources towards acute response proteins during the inflammatory response in CACS [160, 193] and/or nutritional status [197], and has been linked to higher mortality in cancer, cachexia, and other atrophy diseases [193, 197, 198].

Unsurprisingly, metabolic abnormalities are seen in cachexic patients [134, 179]. The serum metabolites of blood glucose, $R$-$\beta$-hydroxybutyrate, and lactate were each measured weekly as metrics of metabolic alterations. Blood glucose did not change, except for a significant decrease from baseline to week 1 in CA-M (Figure 3.8C); however this did not exclude the possibility that metabolic changes were occurring while the serum metabolites remained within the homeostatic range, as elevated glucose turnover has been reported in cachexic patients [199-201]. Blood $R$-$\beta$-hydroxybutyrate did not differ significantly between sham and cancer groups for male and female mice, except for week 3 for males and week 1 for females. Follow up analysis indicated significant elevations in blood lactate (Figure 3.8C). Interestingly, direct analysis of skeletal muscle metabolite flux of cachexic patients compared to malnourished non-cachexic patients revealed a 7.5x higher lactate efflux from skeletal muscle [201]. A potential mechanistic explanation for elevated glucose turnover and lactate production at the skeletal muscle level is through the pro-inflammatory cytokine TNF-$\alpha$, which has been reported to directly shift skeletal muscle oxidative metabolism to inefficient aerobic fermentation in myocytes [202]. This is consistent with the elevations in both TNF-$\alpha$ (Figure 3.6E) and blood lactate (Figure 3.8C) that we observed. Alternatively, elevated lactate could be, in part, explained by increased VM-M3 tumor burden as these cells are highly glycolytic and undergo aerobic fermentation [153]. These elevations in blood lactate can be used to generate glucose via the Cori cycle, a process reported to be elevated in cachexic patients [200, 203, 204]. The Cori cycle is estimated to cause 250-300 kcal/day in fuel
inefficiency in cachexic patients [199]. Interestingly, an estimated 250-400 kcal/day caloric deficit is reported in cachexic patients [205], indicating that lactate cycling may help explain nutritional deficiencies within cachexic patients and be contributing to cachexia in our model. Additionally, total cholesterol was significantly reduced, and triglycerides had trends for reductions in VM-M3 animals, without changes in lipase, suggesting alterations in circulating lipids without changes in enzymatic capacity (Figure 3.9A). Reductions in cholesterol and triglycerides have been reported in cachexic patients [196, 206], however, not universally [207], which may be explained by differences in lipase activity or differential timepoint assessment. Additionally, metrics of hydrations were largely unchanged across groups demonstrating that changes in circulating biomarkers could not be explained by altered hydration status (Figure 3.10A).

**Conclusion**

Taken together, we demonstrated that the VM-M3 had the comprehensive clinical biomarkers observed in cachexic patients [160], but often unevaluated in rodent model systems, including anemia, metrics of protein breakdown, hypoalbuminemia, and metabolic derangement. This is salient as many mechanisms and therapeutic strategies proposed for CACS are largely driven from modeling systems known to not recapitulate or remain unevaluated for the full CACS. In conclusion, our findings suggest that the VM-M3 model of systemic metastasis provides a novel, immunocompetent, logistically feasible, repeatable phenotype with progressive tumor growth, spontaneous metastatic spread, and the full multifaceted CACS with expected sex dimorphisms across tissue wasting. This novel model addressed three current gaps in CACS modeling 1) the progressive spontaneous metastatic environment, 2) the full clinical syndrome of
progressive wasting of skeletal muscle and adipose tissue compartments, anorexia, systemic inflammation, metabolic derangement, anemia, hypoalbuminemia, and elevated protein breakdown, and 3) sex-specific temporal cachexic discrepancies.

Figure 3.1. Baseline Sex, Bodyweight, and Age Controlled with Similar Survival in Males and Females. (A) Sham Males (SH-M), Cancer Males (CA-M), Sham Females (SH-F), Cancer Females (CA-F) bodyweight (SH-M, n=20; CA-M, n=20; SH-F, n=18; CA-F, n=18), age (SH-M, n=20; CA-M, n=20; SH-F, n=18; CA-F, n=18), and food intake (SH-M, n=12; CA-M, n=12; SH-F, n=11; CA-F, n=12) were matched at baseline. (B) Sham Males (SH-M) week 1 (n=4), SH-M week 2 (n=4), SH-M week 3 (n=5), Cancer Males (CA-M) week 1 (n=4), CA-M week 2 (n=4), CA-M week 3 (n=5), SH-F week 1 (n=5), SH-F week 2 (n=5), and SH-F week 3 (n=6), Cancer
Females (CA-F) week 1 (n=5), CA-F week 2 (n=5), CA-F week 3 (n=6) were similar in bodyweight and age, although females tended to smaller. No significant differences in food intake were observed between CA-F or SH-F except between SH-F week 3 and CA-F week 3. Differences were observed across male groups and CA-F week 2 & 3. Differences across groups at each timepoint were analyzed with One-Way ANOVA with Tukey’s post-hoc (A,B). Data are mean ± SEM. *P>0.05, *P<0.05, **P<0.01, ****P<0.0001.

Figure 3.2. VM-M3 Have Progressive Tumor Growth and Spontaneous Systemic Metastases.

(A-B) Weekly in vivo IVIS luciferin imaging for representative animal Cancer-Female #3 (CA-F3) in the prone and supine positions, respectively. Primary tumor is visible week 1, subsequent progressive metastases starting week 2 till end of life (EOL). Red line indicates region of interest (ROI). Color Scale/Radiance: 3.62e6-1.21e7 photons/sec/cm²/sr. (C) Ex vivo anterior and posterior view of CA-F3’s organ tissues (liver, spleen, adipose tissue, and primary tumor) via IVIS luciferin imaging at EOL indicates metastatic spread. Red line indicates region of interest (ROI).
Figure 3.3. VM-M3 Have Progressive Tumor Growth and Spontaneous Systemic Metastases.

(A) Primary tumor weight progressively increased in both CA-M (Week 1, \(n=4\); Week 2, \(n=4\); Week 3, \(n=5\); EOL, \(n=11\)) and CA-F (Week 1, \(n=4\); Week 2, \(n=5\); Week 3, \(n=6\); EOL, \(n=10\)) week 1 to EOL. Primary tumor weight at EOL was significantly larger than week 2 and 3 for both CA-M and CA-F. (B) CA-M (\(n=12\)) and CA-F (\(n=12\)) data points represent the sum of both prone and supine in vivo bioluminescence for each individual animal. Tumor burden was progressive. (C) Whole animal in vivo (\(n=12/\text{group}\)) and ex-vivo IVIS luciferin imaging (Primary Tumor, CA-
M $n=11$, CA-F $n=9$; Liver, Spleen, CA-M $n=12$, CA-F $n=12$; Intraperitoneal Adipose Tissue, CA-M $n=12$, CA-F $n=10$; Ascites Fluid, CA-M $n=7$, CA-F $n=5$) indicate similar tumor burden. Data information: Within group differences across time were analyzed with One-Way ANOVA with Tukey’s post-hoc (A). Differences across groups were analyzed with unpaired t-test (A,C). Data are mean ± SEM. $^{*}P<0.05$.

Figure 3.4. VM-M3 Males and Females Have Similar Survival (A) Survival Curve for Cancer Males (CA-M, $n=20$) and Females (CA-F, $n=18$) shows no significant differences. (B) Mean Survival for CA-M ($n=20, 31.3$ days) and CA-F ($n=18, 32.3$ days). Data information: Kaplan–Meier analysis and log-rank test (A). Differences across groups were analyzed with unpaired t-test (B). Data are mean ± SEM. $^{*}P<0.05$. 
Figure 3.5. VM-M3 Develop Skeletal Muscle and Adipose Tissue Wasting Not Represented in Bodyweight Measurements. (A) Sham Males (SH-M, \(n=20\)), Cancer Males (CA-M, \(n=20\)), Sham Females (SH-F, \(n=17\)), and (D) Cancer Females (CA-F, \(n=18\)) bodyweight. (B) Change in bodyweight across groups (SH-M, \(n=20\)), (CA-M, \(n=20\)), (SH-F, \(n=17\)), and (CA-F, \(n=18\)). (C) Ascites fluid weight at end of life in CA-M (5.5g, \(n=8\)) and CA-F (6.2g, \(n=6\)). (D) End of life (EOL) gastrocnemius & soleus weights as a ratio baseline bodyweight for SH-M, CA-M (M week 1, \(n=4\); week 2, \(n=4\); week 3, \(n=5\); EOL, \(n=20\)), SH-F, CA-F (F week 1, \(n=5\); week 2, \(n=5\); week 3, \(n=6\); EOL, \(n=17\)). (E) EOL quadricep weights as a ratio baseline bodyweight for SH-M, CA-M (M week 1, \(n=4\); week 2, \(n=4\); week 3, \(n=5\); EOL, \(n=3\)), SH-F, CA-F (F week 1, \(n=5\); week 2, \(n=5\); week 3, \(n=6\); EOL, \(n=4\)). (F) EOL Intraperitoneal adipose tissue weights as a ratio baseline bodyweight for SH-M, CA-M (M week 1, \(n=4\); week 2, \(n=4\); week 3, \(n=5\); EOL, \(n=8\)), SH-F, CA-F (F week 1, \(n=5\); week 2, \(n=5\); week 3, \(n=6\); EOL, \(n=6\)). Data information: Within group differences across time were analyzed with One-Way ANOVA with Tukey’s post-hoc (D-F). Differences across groups at each timepoint were analyzed with unpaired t-test (B-F). Data are mean ± SEM. \(^{##}P>0.05\), \(^{*}P<0.05\), \(^{**}P<0.01\), \(^{***}P<0.001\), \(^{****}P<0.0001\).
Figure 3.6. VM-M3 Develop Prolonged Systemic Inflammation. (A) End of life (EOL) spleen weights as a ratio baseline bodyweight for Sham Males (SH-M), Cancer Males (CA-M) (M week 1, \( n=4 \); week 2, \( n=4 \); week 3, \( n=5 \); end of life, EOL, \( n=20 \)), Sham Females (SH-F), and Cancer Females (CA-F) (F week 1, \( n=5 \); week 2, \( n=5 \); week 3, \( n=6 \); EOL, \( n=17 \)). (B) End of Life (EOL) liver weights as a ratio baseline bodyweight for Sham Males (SH-M), Cancer Males (CA-M) (M week 1, \( n=4 \); week 2, \( n=4 \); week 3, \( n=5 \); end of life, EOL, \( n=20 \)), Sham Females (SH-F), and Cancer Females (CA-F) (F week 1, \( n=5 \); week 2, \( n=5 \); week 3, \( n=6 \); EOL, \( n=17 \)). (C) Primary tumor (\( n=20 \)), liver (\( n=24 \)), and spleen (\( n=24 \)) weight change to bioluminescence ratio via IVIS luciferin imaging. (D) White blood cell counts analyzed via impedance analysis (SH-M, \( n=9 \); SH-F, \( n=10 \); CA-M, \( n=9 \); CA-F, \( n=10 \)). (E-G) Cytokines quantification via Luminex fluorophore intensity analysis (\( n=9-12/group/timepoint \)). Data information: Within and across group differences were analyzed with One-Way ANOVA with Tukey’s post-hoc with \( >3 \) comparisons (A,B) and Fischer LSD post-hoc for \( \leq 3 \) comparisons (C, E-G). Differences across groups (B,E-G) or within sexes (D) at each timepoint were analyzed with unpaired t-test. Prior to ANOVA cytokine analysis, robust regression and outlier removal (ROUT) with coefficient \( Q=1\% \) was used as non-physiologic/error values were detected (E-G). Data are mean ± SEM. *\( P<0.05 \), **\( P<0.01 \), ***\( P<0.001 \), ****\( P<0.0001 \).
Figure 3.7. VM-M3 Alters Serum Cytokine Profile. (A-F) Cytokines quantification via Luminex fluorophore intensity analysis (n=8-12/group/timepoint). Data information: Within group differences across time were analyzed with One-Way ANOVA with Fischer LSD post-hoc for ≤3 comparisons (A-F). Differences across groups at each timepoint were analyzed with unpaired t-test. (A-F) Prior to ANOVA cytokine analysis, robust regression and outlier removal (ROUT) with coefficient Q=1% was used as non-physiologic/error values were detected (A-F). Data are mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
Figure 3.8. VM-M3 Develop Anorexia, Anemia, Protein Breakdown, Hypoalbuminemia, and Metabolic Derangement. (A) Food Intake was tracked with 4-day average analyzed at end of life
to baseline \(n=12\)/group). (B) Hemoglobin \(n=10\)/group), Hematocrit (males \(n=10\)/group; females \(n=9\)/group), and Red Blood Cell Count \(n=10\)/group) were quantified with impedance analysis. Blood Urea Nitrogen \(n=5\)/group), Total Protein (males \(n=9\)/group; females \(n=5-6\)/group), and Albumin (males \(n=5\)/group; females \(n=6\)/group) were analyzed via colorimetry analysis. (C) Serum metabolites were quantified via enzyme interaction \(n=8-12\)/group). Data information:

Within group differences across time were analyzed with One-Way ANOVA with Tukey’s post-hoc with \(>3\) comparisons (C). Differences across groups (A,C) or within sexes (B) at each timepoint were analyzed with unpaired t-test. Abbreviations: SH-M, Sham Males; CA-M, Cancer Males; SH-F, Sham Females; CA-F, Cancer Females. Data are mean ± SEM. *\(P<0.05\), **\(P<0.01\), ***\(P<0.001\), ****\(P<0.0001\).

**Figure 3.9. VM-M3 Circulating Lipids.** (A) Total Cholesterol \(n=5\)/group), Triglycerides \(n=4\)/group) and Lipase \(n=5\)/group) were analyzed via colorimetry analysis. Data information:

Across sex differences were analyzed with unpaired t-test. Abbreviations: SH-M, Sham Males; CA-M, Cancer Males; SH-F, Sham Females; CA-F, Cancer Females. Data are mean ± SEM. *\(P<0.05\).
Figure 3.10. Alterations Seen in VM-M3 Cannot Be Explained By Altered Hydration Status.

(A) Organic Phosphorous (males $n=5$/group; female $n=4$/group), Calcium (males $n=5$/group; female $n=4$/group), Sodium ($n=3$/group), Potassium (males $n=3$/group; female $n=4$/group), Chloride (males $n=2-3$/group; female $n=4$/group), Magnesium (males $n=3$/group; female $n=4$/group) were quantified via potentiometrics. Data information: Across sex differences were analyzed with unpaired t-test. Abbreviations: SH-M, Sham Males; CA-M, Cancer Males; SH-F, Sham Females; CA-F, Cancer Females. Data are mean ± SEM. **$P<0.01$.

References for Chapter 3


CHAPTER 4: THE UBIQUITIN-PROTEASOMAL PATHWAY IS ACTIVATED IN VM-M3 SKELETAL MUSCLE

4.1 Chapter Synopsis

In this chapter we present data demonstrating that the ubiquitin-proteasomal pathway is upregulated in VM-M3 skeletal muscle. While TNF-α was upregulated in VM-M3 serum, non-significant alterations in NF-κB/p65 nuclear localization suggested potential pathway inactivation. Further confirmation via E3 ligase expression and potentially likely explained by IL-10 upregulation. However, further evaluation of the other primary upstream modulator/activator of the ubiquitin-proteasomal pathway, reduced IGF-1/insulin signaling, revealed significantly lowered IGF-1/Insulin and subsequent FOXO3a nuclear translocation, indicating reduced IGF-1/Insulin signaling appears to drive ubiquitin-proteasomal degradation in atrophied VM-M3 skeletal muscle.

4.2 The Ubiquitin-Proteasomal Pathway is Activated in VM-M3 Skeletal Muscle

As described, multiple catabolic mechanisms have been proposed to drive skeletal muscle atrophy in cancer-anorexia cachexia syndrome (CACS; [134]). However, mechanisms of skeletal muscle atrophy in CACS have largely been confined to rodent modeling, with little confirmation in clinical setting [134, 208]. Yet, clinical evidence does illustrate that ubiquitin-proteasomal degradation may be a contributory atrophy mechanism in the skeletal muscle of cachexic patients.
[193, 209-219]. This ubiquitin-proteasomal degradation pathway has been shown to be conically activated via two independent upstream pathways: 1) TNF-α induced NF-κB nuclear translocation, upregulated E3 ligation, and increase protein ubiquitination, and 2) reduced IGF-1/Insulin-induced FOXO3a nuclear translocation, upregulated atrogin-1, and increase protein ubiquitination [134, 220]. Mechanistic evidence across atrophy modeling indicates that both mechanisms are sufficient to drive skeletal muscle atrophy [40, 221]. As prior analysis indicated TNF-α was upregulated in VM-M3 modeling (Chapter 3), we hypothesized that TNF-α induced NF-κB signaling would drive ubiquitin-proteasomal degradation in VM-M3 skeletal muscle. We suggest that understanding the mechanism of skeletal muscle atrophy in a metastatic, progressive, and comprehensive CACS model may help to determine potential translatability of these rodent mechanisms to the clinical setting of atrophy-based disease, and thus expand mechanistic in vivo atrophy modeling in the multifaceted CACS environment.

**Results & Discussion:**

Multiple cachexic atrophy mechanisms have been identified in rodent modeling, though many lack clinical confirmation in patients [134]. Emergent clinical data in cachexic patients has indicated a potential catabolic role for the ubiquitin-proteasomal degradation pathway [193, 209-218]. To determine the mechanism of skeletal muscle atrophy in the VM-M3 model of CACS, activation status of the ubiquitin-proteasomal pathway was analyzed. Results confirmed poly-ubiquitination activation (Figure 4.1A) [43]. To determine downstream regulation of proteasomal activity, 26S Proteasomal quantity and capacity were quantified via 20S proteasomal core western and LLYT fluorescent capacity, respectively. Neither quantity (Figure 4.1B) nor capacity (Figure 4.1C) of the
26S proteasome was altered, indicating atrophy occurred via upregulation of ubiquitination, but without altering 26S proteasomal quantity or capacity.

TNF-α has also been shown to play a direct role in skeletal muscle atrophy by upregulating NF-κB and subsequently ubiquitin-proteasomal activity [222, 223]. Following prior confirmation of TNF-α elevation (Figure 3.6E), NF-κB activation was explored in VM-M3 skeletal muscle to determine which upstream pathway augmented ubiquitination. Nuclear fraction versus total NF-κB protein analysis revealed no significant differences between CA-M and SH-M (Figure 4.2A), indicating inactivation; however, NF-κB is temporally regulated, and analysis may have missed the activation timepoint [224, 225]. Thus, mRNA MyoD, MuRF-1, Atrogin-1 and GRP109a/HCAR2, and protein levels of MuRF-1 and Atrogin-1 were analyzed to confirm NF-κB activation/inactivation status. MyoD expression drives skeletal muscle synthesis, but activation of NF-κB is known to directly induce degradation of mRNA MyoD [226]. Results indicated mRNA MyoD was unchanged in CA-M (Figure 4.2B). Additionally, skeletal muscle-specific E3 ligase gene expression, which can be upregulated downstream NF-κB activation, showed no changes in mRNA MuRF-1 (Figure 4.2C). However, nonsignificant elevations in mRNA Atrogin-1 were observed (Figure 4.2D). These findings were confirmed by E3 ligase protein levels (Figure 4.2C-D). Additionally, GPR109a/HCAR2, a receptor protein known to be upregulated by NF-κB activation and subsequently induce negative feedback upon NF-κB pathway [227, 228], revealed no changes (Figure 4.2E). Nonetheless, total RNA and nuclear fraction protein levels were significantly reduced in the VM-M3 skeletal muscle (Figure 4.3A-B), confirming a catabolic state. Since TNF-α is known to conically activate NF-κB and all markers indicated NF-κB pathway inactivation, a reported counter-regulator of TNF-α-induced NF-κB activation in muscle, IL-10,
was explored [229]. IL-10 was elevated in CA-M potentially explaining NF-κB inactivation status (Figure 4.2F) even in the presence of TNF-α (Figure 3.6E) and poly-ubiquitination upregulation (Figure 4.1A). Together, these results indicate NF-κB inactivation via direct and indirect analysis even in the presence of elevated TNF-α, poly-ubiquitination, and muscle wasting, and reduced skeletal muscle total RNA and protein levels, likely due to know counter-regulation of NF-κB signaling in muscle by IL-10.

Another upstream activator of the ubiquitin-proteasomal pathway is IGF-1/insulin, FOXO3a, and Atrogin-1 signaling [40]. Since Atrogin-1, poly-ubiquitination, and in vivo and ex vivo catabolism were confirmed, serum IGF-1 and insulin levels were analyzed in VM-M3 animals to determine if this was the upstream mechanism activating ubiquitin-proteasomal degradation. Quantification of serum IGF-1 and insulin revealed 26-fold and 4.6-fold downregulation of both anabolic hormones, respectively (Figure 4.4A-B). IGF-1 and insulin both bind to similar receptors which activate insulin signaling and a reduction in both can reduce insulin signaling, decrease FOXO3a phosphorylation, and increase FOXO3a nuclear translocation, sufficient to initiate skeletal muscle atrophy through the ubiquitin-proteasomal pathway [40]. Quantification of nuclear fraction FOXO3a revealed a 2.3-fold increase (Figure 4.4C), indicating pathway activation. Thus, the ubiquitin-proteasomal degradation pathway appeared to be significantly upregulated via reduced IGF-1/insulin and increased FOXO3a activation, but not through TNF-α-induced NF-κB activation (Figure 4.5). Importantly, IGF-1/insulin signaling has an established role in muscle homeostasis and alterations in this pathway have been observed in patients across multiple atrophy environments including cachexia [193, 218, 219, 230-237], nutrient deprivation/anorexia [42, 238, 239], sepsis [240-243], diabetes [244], sarcopenia [245], amongst others, demonstrating a
potentially clinically relevant mechanism in atrophy-based disease. These results reveal a conserved, targetable, and translatable pathway driving VM-M3 skeletal muscle atrophy. Additionally, while STAT3- and autophagy-induced atrophy signaling remain unexplored in the current analysis, the observed elevations in serum IL-6 and skeletal muscle FOXO3a nuclear localization indicates that we cannot exclude these potentially contributory pathways.

Figure 4.1. VM-M3 Skeletal Muscle Upregulates Ubiquitination, while 26S Proteasomal Quantity and Capacity Remains Stable. (A) Immunoblotting and quantitative densitometry analysis indicated Poly-Ubiquitination was significantly reduced in Cancer Males (CA-M) compared to Sham Males (SH-M; n=12). (B) Immunoblotting and quantitative densitometry analysis indicated 20S Proteasomal Core was significantly reduced in CA-M compared to SH-M (n=12/group). (C) Fluorometric enzymatic assay indicated no change in 20S proteasomal capacity (n=12/group). Data information: Differences across groups were analyzed with unpaired t-test (A-C). Data are mean ± SEM. ns P>0.05, ***P<0.001.
Figure 4.2. NF-κB is not upregulated in VM-M3 Skeletal Muscle. (A) Immunoblotting Total and Nuclear Fraction NF-κB (65 kDa), and Ponceau Staining. Densitometry quantification did not indicate altered NF-κB activation between Sham Males (SH-M; n=11) and Cancer Males (CA-M;
(B) RT-qPCR analysis of mRNA MyoD in SH-M and CA-M revealed no differences ($n=12$/group). (C) RT-qPCR analysis of mRNA MuRF-1, immunoblotting and quantitative densitometry analysis of MuRF-1 in SH-M and CA-M revealed no differences ($n=12$/group). (D) RT-qPCR analysis of mRNA Atrogin-1, immunoblotting and quantitative densitometry analysis of Atrogin-1 in SH-M and CA-M revealed non-significant elevations ($n=12$/group). (E) RT-qPCR analysis of mRNA GPR109a/HCAR2 in SH-M and CA-M revealed no differences ($n=12$/group). (F) Cytokines quantification via Luminex fluorophore intensity analysis ($n=7-11$/group) indicate elevations in IL-10. Data information: Within group differences across time were analyzed with One-Way ANOVA with Fischer LSD post-hoc for ≤3 comparisons (F). Differences across groups at each timepoint were analyzed with unpaired t-test (A-K). Prior to ANOVA cytokine analysis, robust regression and outlier removal (ROUT) with coefficient $Q=1\%$ was used as non-physiologic/error values were detected (G). Data are mean ± SEM. $^{ns}P>0.05$, $^{*}P<0.01$, $^{****}P<0.0001$.

Figure 4.3. Lower Total RNA and Nuclear Fraction Protein Illustrate Wasting in VM-M3 Skeletal Muscle. (A) Total RNA was quantified via UV Spectroscopy and was significantly reduced in CA-M compared to SH-M ($n=12$). (B) BCA colorimetric assay indicated Nuclear Fraction Total Protein was significantly reduced in CA-M ($n=10$) compared to SH-M ($n=12$).
information. Differences across groups were analyzed with unpaired t-test (A-B). Data are mean ± SEM. *$P<0.05$, ***$P<0.001$.

Figure 4.4. Lower Circulating IGF-1/Insulin and Subsequent FOXO3a Nuclear Translocation is Upregulated in VM-M3 Skeletal Muscle. (A-B) Serum hormone levels quantified via Luminex xMAP fluorophore intensity analysis demonstrated 26-fold reduction in Insulin-like Growth Factor-1 (IGF-1) and 4.5-fold reduction in Insulin in Cancer Males (CA-M) compared to Sham Males (SH-M; $n=11$/group). (C) Immunoblotting and quantitative densitometry analysis indicated Nuclear Fraction FOXO3a was significantly increased (2.4-fold) in CA-M ($n=12$) compared to SH-M ($n=11$). Data information: Differences across groups were analyzed with unpaired t-test (A-C). Data are mean ± SEM. **$P<0.01$, ****$P<0.0001$. 
Figure 4.5. Reduced Insulin/IGF-1, increased FOXO3a Activation, and Increased Poly-Ubiquitination Observed in Atrophied VM-M3 Skeletal Muscle. The ubiquitin proteasomal degradation pathway was significantly upregulated via reduced Insulin/IGF-1 and increased FOXO3a activation, but not through TNF-α-induced NF-κB activation, explaining skeletal muscle atrophy. Data information: Faded images illustrates no change/reduction in protein quantify and/or activation status in cancer animals compared to sham animals. Non-faded images represent elevated protein quantify or activation status in cancer animals compared to sham animals.

References for Chapter 4


CHAPTER 5: KETONE DIESTER ATTENUATES MULTIFACTORIAL CANCER-ANOREXIA CACHEXIA AND INFLAMMATORY ATROPHY

5.1 Chapter Synopsis

In this chapter we present data demonstrating that chronic administration of R/S 1,3 Butanediol Acetoacetate Diester (Ketone Diester; KDE) into VM/Dk alters circulating metabolites and is well tolerated. We also demonstrate that chronic administration of KDE attenuated anorexia, altered systemic metabolism, reduced tumor burden and metastatic spread, and attenuated skeletal muscle atrophy, while not altering systemic anabolic hormone levels. Additionally, we demonstrate that a single acute administration of KDE rapidly altered systemic metabolism, and reduced tissue catabolism, anorexia, and time to recovery in lipopolysaccharide (LPS)/septic animals. KDE attenuated tissue catabolism even when LPS animals were pairfed. Taken together, KDE attenuates multifactorial wasting across multiple atrophy environments even when controlling for cancer and comorbid symptoms.

5.2 Ketone Diester Attenuates Multifactorial Cancer-Anorexia Cachexia and Inflammatory Atrophy

As described, while current models are a critical limitation to clinical advancement, a lack of effective and/or supportive treatments remains a century old problem [246, 247]. However, work dating back a half-century illustrated that patients who underwent extreme nutrient
deprivation produced endogenous metabolites called ketone bodies that progressively altered systemic metabolism and skeletal muscle protein kinetics which was hypothesized to prolong life during catabolic states by attenuating muscle atrophy [3-5]. Additionally, dietary or infusion-induced elevations of ketone bodies have been linked to altered metrics of protein breakdown across various atrophy [44, 119, 126, 128, 129] and post-absorptive [104, 120] conditions. These ketone bodies, which are the end-product of fatty acid oxidation and subsequent ketogenesis in the hepatic tissue, are shown to be upregulated during fasting, reduced carbohydrate metabolism, and/or direct IV infusion. Yet, dietary restriction may be contraindicated in atrophy-based disease/environments and IV infusion is limited to the clinical/research environment [80]. Recent development of orally administrated exogenous ketone molecules has circumventing previous barriers to ketone body elevation and has led to the rapid expansion of research unveiling direct metabolite effects on metabolism, inflammation, oxidative stress, and epigenetic regulation, among others [1, 10, 56, 57, 60, 248, 249]. While our team hypothesized that exogenous ketone bodies may mitigate inflammatory-based atrophy disease [57], their chronic anti-catabolic effects in multifactorial wasting diseases have remained untested until now. We hypothesized that KDE administration would attenuate atrophy in multifactorial inflammatory wasting syndromes. We suggest that a novel, non-toxic, pluripotent, anti-catabolic agent may provide translatable therapeutic support in dynamic and progressive inflammatory wasting environments.
5.2.1 Results and Discussion

5.2.1.1 Ketone Diester Mitigates Comorbidities, Tumor Burden, and Skeletal Muscle Atrophy in Cancer-Anorexia Cachexia Syndrome

Evidence from human, preclinical, and *in vitro* data has implicated IGF-1/Insulin, FOXO3a, and the ubiquitin-proteasomal pathway in muscle homeostasis and numerous atrophy conditions [245, 250]. Interestingly, patients undergoing extreme nutrient deprivation (prolonged fasting/anorexia) upregulated endogenous ketone body production, which was hypothesized to allow for prolonged survival via a protective and progressive metabolic adaptation and attenuated muscle atrophy [2-5, 44, 123]. Previously, dietary restrictions and/or infusion-induced elevations of ketone bodies limited exploration and clinical translation [80], however a novel, orally ingestible exogenous KDE offers a solution. KDE is a non-toxic synthetic exogenous ketone compound composed of a *R/S* 1,3-butanediol backbone esterified to two acetoacetates. Upon oral administration, KDE increases circulating ketone bodies β-hydroxybutyrate and acetoacetate, while decreasing blood glucose in a dose-dependent manner without the barriers of whole lifestyle/dietary changes or IV infusion [9, 86]. Thus, the KDE was assessed to determine its effect on anorexia, systemic metabolism, tumor and metastatic burden, and skeletal muscle catabolism in the full progressive CACS.

To determine whether KDE could alter systemic metabolism, preliminary analysis of blood metabolites was measured at baseline following a standard diet and after a 4-day transition to standard diet supplemented with either 20%KDE, 25%KDE, or 30%KDE. Results indicated
20%KDE and 30%KDE caused significant elevations in blood ketone levels, with 30%KDE inducing the highest ketone elevations (Figure 5.1B). All KDE groups resulted in significant reductions in blood glucose levels, with 30%KDE inducing the greatest reduction in glucose (Figure 5.1A). A second study was initiated to expand upon these findings, as prior work revealed that exogenous administration of ketone bodies may attenuate ad libitum food intake [153, 251, 252] and confound evaluation of KDE effect on tissue catabolism. To control for this effect, food palatability was increased, and the KDE was incrementally integrated (5%/day) over a 7-day period to achieve 30%KDE. The 30%KDE was well-tolerated as indicated by bodyweight maintenance (Figure 5.1C) and since 30%KDE caused the greatest alteration in systemic metabolism, mirroring metabolite alteration seen during extreme nutrient deprivation [3-5, 24, 41, 123], 30%KDE was administered in VM-M3 CACS (KDE+VM-M3) to determine whether KDE would alter the course of CACS-induced atrophy compared to VM-M3 CACS-alone (VM-M3) and PBS-only (Sham). Baseline sex, age, and food intake were controlled across groups (Figure 5.2A-C). Food intake was tracked daily to determine if KDE administration altered the anorexic phenotype. VM-M3 experienced a reduction in food intake (Figure 5.3A); however, animals in the KDE+VM-M3 demonstrated attenuation of the predicted anorexic phenotype in this CACS model. Seemingly contrary to the KDE anti-anorexic effects presented here are the KDE-induced reduction in food intake previously reported [153, 251, 252]. However, altered standard diet palatability and step-wise integration of KDE mitigated this confounding variable in non-diseased animals. While unexplored, due to inflammation’s established role in anorexia [179] and ketone bodies’ reported anti-inflammatory effects [56], it is tempting to speculate that ketones may be mitigating anorexia via alterations in systemic inflammation in CACS.
To determine whether KDE could alter metabolism in CACS, blood ketones, glucose, and lactate were measured weekly. KDE induced sustained elevations in circulating ketones and reductions in blood glucose in KDE+VM-M3 compared to both VM-M3 and Sham (Figure 5.5BA-B), illustrating KDE-induced alterations in CACS systemic metabolism. This presented a major step toward the clinical advancement of ketone therapeutics as fasting-induced ketone elevations and glucose reductions in cachexic patients are contraindicated, as nutrient deprivations will exacerbate tissue atrophy. While the VLCKD has overlapping metabolic changes and circumvents nutrient deprivation, major dietary modifications are difficult for some to sustain [78, 79], and compliance can be further complicated by disease [80]. Additionally, direct IV infusion of metabolites into circulation allows for dose-dependent elevations in circulating ketone bodies without altering diet, but this is limited to the clinical or research environment. Thus, KDE provides an orally consumable and well tolerated method for modulating systemic metabolism without dietary restriction or direct circulatory infusion.

No difference was found between KDE+VM-M3 and VM-M3 for blood lactate (Figure 5.5C), potentially indicating similar tumor or inflammation-induced aerobic fermentation [47, 202]. Thus, to determine if KDE altered tumor burden, in vivo bioluminescence imaging was analyzed weekly. Interestingly, KDE+VM-M3 demonstrated non-significant reductions in whole animal tumor burden (Figure 5.4A). Additionally, ascites fluid, a surrogate marker of metastatic spread [162], was also significantly reduced in KDE+VM-M3 compared to VM-M3 (Figure 5.4B), suggesting KDE-induced reductions in tumor burden and metastatic spread. Tumor burden and metastatic spread directly impact cachexia risk and progression. Prior work has demonstrated that a VLCKD can attenuate cancer burden across various preclinical cancer models [60, 253]. Ketone
bodies have also been demonstrated to induce direct anti-cancer effects which have been proposed to occur via alterations in energetic metabolism, oxidative stress, inflammation, and/or epigenetic regulation. Here we demonstrate that chronic KDE administration on top of a standard diet mitigated tumor and metastatic burden. Interestingly, this effect was present even though comorbid anorexia was mitigated in the KDE group.

Tissue catabolism/atrophy is the hallmark characteristic of CACS and ketone bodies have been shown to be associated with or directly reduce metrics of protein breakdown across various populations [4, 126, 128, 129] while demonstrating direct ketone-induced effects via acute IV infusion [44, 104, 119] and/or in vitro analysis [70, 131]. However, direct chronic effects of ketone bodies on the principle outcomes, tissue catabolism, have not been determined, nor has efficacy of orally administered exogenous ketone bodies been tested across various multifaceted atrophy environments. To determine if KDE altered body composition and whether this was through alteration in model specific catabolic drivers, bodyweight was tracked daily, while IGF-1, insulin, cardiac tissue, adipose tissue, and skeletal muscle tissue were evaluated 3 weeks post-implantation, prior to EOL. KDE+VM-M3 had a lower bodyweight compared to VM-M3 and Sham (Figure 5.6A); however, as both ascites fluid and tumor burden confound bodyweight in VM-M3 animals, bodyweight alone could not reliably indicate tissue atrophy in the VM-M3 model of CACS. To determine if KDE altered IGF-1 or insulin, serum hormone levels were evaluated. KDE+VM-M3 and VM-M3 both demonstrated significantly reduced serum IGF-1 and insulin, demonstrating that KDE did not alter circulating anabolic hormone levels (Figure 5.5D-E). Cardiac tissue was found to be unaltered by CACS or across group (Figure 5.6B). Additionally, adipose tissue atrophied to equivalent levels in KDE+VM-M3 and VM-M3, indicating KDE was unable to alter adipose tissue
catabolism (Figure 5.6C). However, the KDE did attenuate muscle atrophy across numerous skeletal muscle tissues (Figure 5.6D) without altering circulating anabolic hormone levels (Figure 5.5D-E). Taken together, the KDE was well tolerated, attenuated anorexia, altered systemic metabolism, attenuated tumor burden and metastatic spread, and reduced skeletal muscle atrophy without changing circulating IGF-1 and insulin levels, illustrating a unique and multifaceted anti-CACS therapy (Figure 5.7).

5.2.1.2 Ketone Diester Mitigates Tissue Catabolism and Comorbidities in Inflammation-Induced Atrophy

Reductions in anorexia, tumor burden and metastatic spread, and skeletal muscle atrophy all present ideal outcomes in the clinical environment of CACS. It should be noted, however, that altered anorexia and tumor burden and metastatic spread can confound interpretation of the direct effects of the KDE on skeletal muscle atrophy. To determine direct effects of KDE on tissue catabolism when controlling for confounding variables of cancer and anorexia, KDE was evaluated in a cancer-independent inflammation-induced atrophy environment of lipopolysaccharide (LPS)-induced sepsis, which has been shown to produce an overlapping multifactorial atrophy environment of low IGF-1/insulin, systemic inflammation, anorexia, anemia, hypoalbuminemia, metabolic derangement, and ubiquitin proteasomal signaling [160]. Due to the rapid nature of LPS-induced atrophy, various dosages of KDE were gavaged to determine their ability to rapidly shift systemic metabolism. Both 4mL/kg and 5mL/kg KDE dosages resulted in significant and rapid reductions in blood glucose and elevations in blood ketones (Figure 5.8A-B). We observed elevations in circulating ketone bodies and decreases in blood glucose in a dose-dependent manner.
when administered acutely via oral gavage on top of a standard diet, similar to what was observed via chronic feeding in VM-M3 CACS. This demonstrates that the KDE can acutely and chronically alter systemic metabolism.

To determine KDE effect on cancer-independent atrophy, a maximal non-fatal LPS dose (10mg/kg) was administered, followed by a single 4mL/kg water (LPS) or KDE (KDE+LPS) gavage in bodyweight matched animals (Figure 5.9A). KDE reduced tissue catabolism 47% within the first 24 hours post-LPS administration (Figure 5.9B). Consistent with what was found in CACS, KDE also significantly reduced anorexic symptoms (Figure 5.9C) indicating that the KDE attenuates anorexia in two multifaceted inflammatory atrophy environments, again suggesting potential anti-inflammatory role of KDE in both.

Animals were followed over a 13-day period to determine chronic effects of KDE. KDE significantly attenuated chronic LPS-induced catabolism (Figure 5.10A). To determine whether KDE would alter length of recovery time from a maximal non-fatal LPS dose, bodyweight was assessed over 13-day time-period to determine length of time before animals returned to baseline bodyweight. KDE+LPS recovered within 10 days, while LPS did not recover within the 13-day time-period (Figure 5.10B-C). While this illustrates that a single KDE administration could rapidly alter systemic metabolism and attenuate catabolism and comorbid symptoms, reducing anorexia can potentially confound catabolism. To determine if KDE attenuated catabolism in post-LPS administration, bodyweight matched animals were pair-fed and administered a single 4mL/kg gavage (Figure 5.11A). Pair-fed KDE+LPS significantly reduced tissue catabolism 28% compared to LPS group (Figure 5.11B), indicating KDE attenuated catabolism independent of food intake.
Taken together, the KDE attenuated anorexia and tissue catabolism in a cancer-independent inflammatory-atrophy environment when comorbidities were controlled, suggesting a direct anti-catabolic effect of KDE in these multifactorial catabolic environments (Figure 5.12).

Here we demonstrate that chronic administration of oral exogenous ketone bodies mitigated catabolism in two multifaceted, yet overlapping, inflammatory environments (Figure 5.13). First, KDE attenuated ubiquitin proteasomal-driven atrophy in CACS without altering upstream anabolic hormones. Second, KDE mitigated LPS/sepsis catabolism when controlling for anorexia. Mechanistically, patients have reduced IGF-1/insulin, decreased and stable glucose, and increased ketone bodies during prolonged fasting-induced atrophy [4, 5, 41, 44]. These studies also uncovered that as ketone bodies elevated, metrics of protein breakdown progressively decreased along with a 3-6-fold reduction in amino acid efflux from skeletal muscle, suggesting a direct ketone-induced effect on protein turnover [4, 44]. Additionally, recent work in patients with LPS/sepsis demonstrated that direct IV infusion of ketone bodies induced a potent acute anticatabolic response in the skeletal muscle, even when controlling for GPR109a/HCAR2 potential confounding signaling effects on NF-κB [57, 119]. Mechanistically, as fasting [40-42], LPS/sepsis [254] and CACS [134, 255] have all been shown to reduce IGF-1/insulin signaling, combined with our work and others indicating that ketone bodies attenuate catabolism in all three [4, 44, 119], even when controlling for confounding HCAR2/NF-κB signaling, ketone-induced histone acetylation’s through inhibition of Class I and II HDAC inhibitors is a possible anticatabolic mechanism. Supportive of this hypothesis is evidence that 1) class I and II HDAC induced atrophy effects across multiple catabolic environments [172, 256]; 2) inhibited Class I and II HDAC and subsequent histone acetylation in skeletal muscle is sufficient to inhibit skeletal
muscle atrophy via disruption of FOXO3a-induce atrophy [172]; 3) ketone bodies inhibit class I and II HDACs and subsequently increase histone acetylation across tissues [65], including skeletal muscle [121]; 4) ketone bodies’ anti-atrophy effect across multiple catabolic environments [4, 44, 57, 70, 119, 131]. However, contrary to this hypothesis is evidence that FOXO3a levels increase with diet-induced ketone elevations across tissues [55, 121]. Yet, neither analysis indicated FOXO3a nuclear activation state or FOXO3a gene expression. Additionally, FOXO3a has consistently been shown to induce atrophy in skeletal muscle, which is contrasting with work demonstrating ketone bodies’ anti-atrophy effect in environments where IGF-1/insulin signaling is commonly suppressed [4, 44, 119]. Thus, it appears that ketone-induced inhibition of class I and II HDAC, subsequent histone acetylation, and disruption of FOXO3a-induce atrophy, is a plausible mechanism attenuating atrophy. Notably, various reports attempting to inhibit FOXO3a and ubiquitin-proteasomal degradation in the inflammatory environment via augmenting upstream pathway inhibitors, IGF-1/insulin, have not proven efficacious [254, 257, 258]. This is likely due to skeletal muscle resistance to IGF-1/insulin signaling in these inflammatory environments [259-264]. While the location of inflammation-induced IGF-1/insulin resistance is not fully understood, multiple reports indicate this may be occurring at the receptor level [263-265]. Interestingly, our work demonstrating ketone bodies’ anticatabolic effect in both a low IGF-1/insulin signaling, and pro-inflammatory environments further supports the proposed downstream anti-catabolic mechanism and demonstrates that ketones present a mitigative tool for this catabolic pathway by circumventing previous therapeutic resistance. Additionally, discussion has emerged around the proposed dichotomy of IGF-1 on skeletal muscle mass, cancer growth and metastasis [266], and longevity [245]. Here we have shown a unique ability to attenuate skeletal muscle atrophy without elevating IGF-1 or insulin, demonstrating a therapeutic strategy that attenuates muscle mass
atrophy while mitigating perceived risk for adverse health or disease outcomes. Beyond tissue atrophy/catabolism, ketone bodies have been demonstrated to directly regulate metabolism, reduce oxidative stress, attenuate inflammation, regulate epigenetics, among others [56]. Consequently, ketone bodies may help support improved patient outcomes across various multifaceted diseases including cancer, CACS, sepsis, and others, where tissue catabolism is only one facet compromising patient outcomes.

Figure 5.1. Ketone Diester Administration into Food Altered Systemic Metabolism and Was Well-Tolerated. (A-B) Serum metabolites were quantified via enzyme interaction for animals fed 20% (n=5), 25% (n=3), 30% (n=5) Ketone Diester (KDE) on top of standard diet. 30%KDE induced largest alterations in systemic metabolism. (C) Bodyweight tracked daily with step-wise increase in KDE indicating tolerability of 30%KDE incorporated with highly palatable standard diet (HPSD) (n=4). Data information: Across group differences were analyzed with One-Way ANOVA with Fischer LSD post-hoc for ≤3 comparisons (A-B). Differences within groups (A) at
each timepoint were analyzed with unpaired t-test. Within group differences across time were analyzed with One-Way ANOVA with Tukey’s post-hoc with >3 comparisons (C). Data are mean ± SEM. *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$.

Figure 5.2. Baseline Sex, Bodyweight, Age, and Food Intake Were Controlled. (A) Baseline bodyweight ($n=12$/group). (B) Baseline age ($n=12$/group). (C) Baseline food intake ($n=12$/group). Data information: Across group differences were analyzed with One-Way ANOVA with Fischer LSD post-hoc for ≤3 comparisons (A-C). Data are mean ± SEM. Abbreviations: KDE+VM-M3, 30% R/S 1,3-Butanediol Acetoacetate Diester in VM-M3 Cancer Anorexia Cachexia Syndrome Model; VM-M3, VM-M3 Cancer Anorexia Cachexia Syndrome Model; Sham, sham injected VM/Dk.

Figure 5.3. Ketone Diester Attenuated Comorbid Anorexia in VM-M3 Cancer-Anorexia Cachexia Syndrome. (A) Food Intake was tracked daily and compared across groups ($n=3$/group). (B) Total calories consumed per animal over study duration ($n=3$/group). Data
information: Group = Cage = 3-4 animals. Differences across groups at each timepoint were analyzed with One-Way ANOVA with Fischer LSD post-hoc for ≤3 comparisons (A). Across group differences were analyzed with One-Way ANOVA with Fischer LSD post-hoc for ≤3 comparisons (B). Data are mean ± SEM. KDE-VM-M3 vs VM-M3 *P<0.05, KDE-VM-M3 vs Sham §P<0.05, VM-M3 vs. Sham ¥P<0.05 (A). Abbreviations: KDE+VM-M3, 30% R/S 1,3-Butanediol Acetoacetate Diester in VM-M3 Cancer Anorexia Cachexia Syndrome Model; VM-M3, VM-M3 Cancer Anorexia Cachexia Syndrome Model; Sham, sham injected VM/Dk.

Figure 5.4. Ketone Diester Altered Tumor Burdon and Surrogate Markers of Metastatic Progression in VM-M3 Cancer-Anorexia Cachexia Syndrome. (A) KDE+VM-M3 (n=12) and VM-M3 (n=12) data points represent the average of both prone and supine in vivo bioluminescence for each individual animal. KDE+VM-M3 has non-significant reductions in tumor burden. (B) Ascites fluid weight, surrogate marker of metastatic progression, at week 3 in KDE+VM-M3 (1.9g, n=11), VM-M3 (3.0g, n=11), and Sham (0g, n=11). Data information: Differences across groups at each timepoint were analyzed with unpaired t-test for individual comparisons (A, B). Data are mean ± SEM. *P<0.05 (B). Abbreviations: KDE+VM-M3, 30% R/S 1,3-Butanediol Acetoacetate Diester in VM-M3 Cancer Anorexia Cachexia Syndrome Model; VM-M3, VM-M3 Cancer Anorexia Cachexia Syndrome Model; Sham, sham injected VM/Dk.
Figure 5.5. Ketone Diester Altered Circulating Metabolites in VM-M3 Cancer-Anorexia Cachexia Syndrome. (A-C) Serum metabolites were quantified for Ketone Diester + VM-M3 Cancer Anorexia Cachexia Syndrome (KDE+VM-M3, n=12), VM-M3 (n=12), and Sham (n=11) via enzyme interaction. KDE+VM-M3 demonstrated shifts in system metabolism. (D-E) Serum hormone levels quantified via Luminex fluorophore intensity analysis demonstrated significant reductions in Insulin-like Growth Factor-1 (IGF-1) and Insulin in KDE-VM-M3 (n=12) and VM-M3 (n=11) compared to SH-M (n=11). Data information: Differences across groups at each timepoint were analyzed with One-Way ANOVA with Fischer LSD post-hoc for ≤3 comparisons (A-E). Data are mean ± SEM. KDE-VM-M3 vs VM-M3 *P<0.05, KDE-VM-M3 vs Sham §P<0.05, VM-M3 vs Sham ¥P<0.05 (A-C). nsP>0.05, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 (D-E). Abbreviations: KDE+VM-M3, 30% R/S 1,3-Butanediol Acetoacetate Diester in VM-M3 Cancer Anorexia Cachexia Syndrome Model; VM-M3, VM-M3 Cancer Anorexia Cachexia Syndrome Model; Sham, sham injected VM/Dk.
Figure 5.6. Ketone Diester Altered Circulating Metabolites in VM-M3 Cancer-Anorexia Cachexia Syndrome. (A) Daily bodyweight for KDE+VM-M3 (n=12), VM-M3 (n=12), and Sham (n=11). VM-M3 and Sham saw an increase in bodyweight compared to KDE-VM-M3, starting day 14. (B) Cardiac tissue weight set to baseline bodyweight (n=10/group) with no significant difference across groups. (C) Intraperitoneal adipose tissue weight set to baseline
bodyweight with significant reductions in KDE+VM-M3 and VM-M3 compared Sham (n=10/group). (D) Hind limb skeletal muscle weights set to baseline bodyweight showed KDE+VM-M3 attenuated skeletal muscle atrophy across a number of tissues compared to VM-M3 (n=10-11/group). Data information: Differences across groups at each timepoint were analyzed with One-Way ANOVA with Fischer LSD post-hoc for ≤3 comparisons (A-D). Data are mean ± SEM. KDE-VM-M3 vs VM-M3 *P<0.05, KDE-VM-M3 vs Sham §P<0.05, VM-M3 vs. Sham ¥P<0.05 (A). ns P>0.05, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 (B-D). Abbreviations: KDE+VM-M3, 30% R/S 1,3-Butanediol Acetoacetate Diester in VM-M3 Cancer Anorexia Cachexia Syndrome Model; VM-M3, VM-M3 Cancer Anorexia Cachexia Syndrome Model; Sham, sham injected VM/Dk.

Figure 5.7. Ketone Diester Attenuates Cancer-Anorexia Cachexia Syndrome. R/S 1,3-Butanediol Acetoacetate Diester (Ketone Diester) chronic administration on top of standard diet altered systemic metabolism and produced an anti-anorexic, anti-tumor, and anti-catabolic effect without altering anabolic hormones levels, illustrating a multifaceted anti-CACS therapeutic strategy. Abbreviations: VM-M3, VM-M3 Cancer Anorexia Cachexia Syndrome Model.
Figure 5.8. Ketone Diester Oral Gavage Rapidly Altered Circulating Metabolites. (A-B) Serum metabolites were quantified for Ketone Diester (KDE) at 2mL/kg \( (n=3) \), 3mL/kg \( (n=2) \), 4mL/kg \( (n=3) \), 5mL/kg \( (n=3) \) via enzyme interaction. 4mL/kg KDE gavage shifted systemic metabolism. Data information: Within group differences compared to timepoint “0” were analyzed with One-Way ANOVA with Fischer LSD post-hoc for \( \leq 3 \) comparisons (A). Data are mean ± SEM. 4mL/kg different from timepoint “0” \( \frac{\$}{P}<0.05 \), 5mL/kg different from timepoint “0” \( \frac{¥}{P}<0.05 \) (A). Abbreviations: R/S 1,3-Butanediol Acetoacetate Diester (Ketone Diester).

Figure 5.9. Acute Ketone Diester Administration Attenuated Tissues Catabolism and Comorbid Anorexia During Sepsis. (A) Baseline bodyweight for animals prior to 10mg/kg lipopolysaccharide administration with either 4mL water gavage (LPS, \( n=4 \)) or 4mL/kg ketone diester (KDE+LPS, \( n=5 \)) gavage administration. (B) Percentage bodyweight lost 1-day post
10mg/kg lipopolysaccharide administration with either 4mL water gavage (LPS, \( n=4 \)) or 4mL/kg KDE (KDE+LPS, \( n=5 \)) gavage administration following. (C) Percentage caloric restriction following LPS (\( n=4 \)) or KDE+LPS (\( n=5 \)). Data information: Difference across groups were analyzed with unpaired t-test for individual comparisons (A-C). Data are mean ± SEM. \( ^{ns}P>0.05 \).

Abbreviations: R/S 1,3-Butanediol Acetoacetate Diester (Ketone Diester, KDE).

Figure 5.10. Acute Ketone Diester Administration Attenuated Tissues Catabolism and Time to Recovery During and Following Sepsis. (A) Percentage bodyweight lost tracked for 13-days following LPS (\( n=4 \)) or KDE+LPS (\( n=5 \)). (B-C) Time to recovery as time taken to return to baseline bodyweight following LPS (\( n=4 \)) or KDE+LPS (\( n=5 \)). Data information: Difference across groups were analyzed unpaired (A). Differences across timepoint were analyzed with paired
t-test (B-C). Data are mean ± SEM. \( ^{\text{ns}}P>0.05, *P<0.05, **P<0.01, ***P<0.001 \) (A-C).

Abbreviations:

\( R/S \) 1,3-Butanediol Acetoacetate Diester (Ketone Diester, KDE); 10mg/kg lipopolysaccharide administration with 4mL water gavage (LPS); 10mg/kg lipopolysaccharide administration with 4mL/kg \( R/S \) 1,3-Butanediol Acetoacetate Diester (KDE+LPS).

**Figure 5.11. Acute Ketone Diester Administration Attenuated Tissues Catabolism During Sepsis When Controlling for Food Intake.** (A) Baseline bodyweight for animals prior to 10mg/kg lipopolysaccharide administration with either 4mL water gavage (LPS, \( n=4 \)) or 4mL/kg KDE (KDE+LPS, \( n=4 \)) gavage administration, and pair feeding. (B) Percentage bodyweight lost in pair fed animals following (LPS, \( n=4 \)) or KDE+LPS (\( n=4 \)). Data information: Difference across groups were analyzed with unpaired t-test (A-B). Data are mean ± SEM. \( ^{\text{ns}}P>0.05, *P<0.05 \) (A-B). Abbreviations: \( R/S \) 1,3-Butanediol Acetoacetate Diester (Ketone Diester, KDE); 10mg/kg lipopolysaccharide administration with 4mL water gavage (LPS); 10mg/kg lipopolysaccharide administration with 4mL/kg \( R/S \) 1,3-Butanediol Acetoacetate Diester (KDE+LPS).
Figure 5.12. Ketone Diester Attenuates Muscle Atrophy in Cancer-Independent Catabolic Environment. Gavage administration of KDE alters systemic metabolism and attenuates tissue catabolism and anorexia post-LPS. This effect is maintained when controlling for comorbid anorexic behavior. Abbreviations: R/S 1,3-Butanediol Acetoacetate Diester (Ketone Diester, KDE).
Figure 5.13. Ketone Diester Attenuates Muscle Atrophy in Cancer-Independent Catabolic Environment. VM-M3 model of systemic metastasis demonstrated the comprehensive CACS with progressive tumor burden and metastatic disease, progressive skeletal muscle and adipose tissue wasting, prolonged systemic inflammation, anorexia, anemia, elevated protein breakdown, hypoalbuminemia, and metabolic derangement with expected sex-specific cachectic discrepancies. VM-M3 skeletal muscle atrophy via reduced IGF-1/insulin, increased FOXO3a, and subsequent ubiquitin proteasomal degradation. Chronic KDE administration on top of standard diet in CACS was well tolerated, altered systemic metabolism, and attenuated tumor burden, anorexia, and skeletal muscle atrophy. Acute KDE administration in LPS/sepsis altered systemic metabolism, reduced anorexia, reduced time to recovery, and attenuated tissue catabolism even when food intake was controlled. Abbreviations: R/S 1,3-Butanediol Acetoacetate Diester (Ketone Diester).

5.3 References for Chapter 5:


CHAPTER 6 DISCUSSION – IMPLICATIONS FOR CANCER CACHEXIA MODELING AND THERAPY

6.1 Chapter Synopsis

Here we summarize the potential implications and future considerations of exogenous ketone bodies in atrophy-based disease, specifically CACS, presented in this dissertation. A more comprehension discussion of the data can be found in previous chapters.

6.2 Implications for Cancer Cachexia Modeling and Therapy

The goal of this dissertation work was to discover a comprehensive multifactorial CACS model, determine relevant skeletal muscle signaling, and evaluate the potential therapeutic efficacy of exogenous ketone bodies in the context of multifactorial CACS and inflammatory atrophy. First, we demonstrated that the VM-M3 model demonstrates a novel, immunocompetent, logistically feasible, repeatable phenotype with progressive tumor growth and metastasis, skeletal muscle and adipose tissue wasting, proinflammatory environment, and anorexia, along with observed metrics of anemia, elevated BUN, reduced total protein, hypoalbuminemia, and metabolic derangement in both male and females with expected sex-specific discrepancies. We also demonstrate that the ubiquitin proteasomal degradation pathway was significantly upregulated in association with reduced IGF-1/insulin and increased FOXO3a activation, but not TNF-α-induced NF-κB activation, driving skeletal muscle atrophy. Finally, we show that $R/S$ 1,3-butanediol acetoacetate
diester (Ketone Diester; KDE) administration shifted systemic metabolism, attenuated tumor burden, reduced tissue catabolism independent of food intake, and mitigated comorbid symptoms in both CACS and cancer-independent atrophy environments.

These findings address several gaps present in current modeling systems and therapeutic treatment. First, very few of the most common cachexia model systems spontaneously metastasize presenting a considerable limitation as incidence of both cachexia and cachexia comorbidities have been reported to be much higher across all tumor types and independent of treatment in cancer cachexia patients [267, 268]. Additionally, emergent evidence has indicated the metastatic process may contribute to skeletal muscle wasting [145]. Here we demonstrate that the VM-M3 is a repeatable and logistically feasible model that produces a primary tumor with subsequent metastatic spread, recreating characteristics of the spontaneous metastatic process [144], overcoming gaps in current metastatic CACS modeling [208, 269, 270]. Second, many of the most commonly used models only partially recreate or have not been confirmed to have the full CACS, notably anemia, BUN, total protein, and albumin. Consequently, it is hard to determine whether the pathophysiology, mechanisms, and treatment discovered in these model systems will translate to the comprehensive CACS clinical setting. However, the VM-M3 model fills a considerable gap in recreating the comprehensive CACS as clinical described, illustrating a model with translatable potential. Third, while sex discrepancies in CACS have not been extensively investigated, limited data suggests potential differences in the degree of wasting and cachexia burden, with males experiencing greater cachexia [174-176]. We discovered that while both VM-M3 male and females presents with a similar CACS phenotype, greater tissue wasting and cachexia burden was observed in male, potentially introducing a model system which may allow for elucidation of sex-specific
cachexic discrepancies. Finally, limited CACS patient skeletal muscle atrophy data presents a considerable barrier to determining translatable mechanisms from models to humans. However, IGF/insulin signaling has a conserved, conical, centralized and regulatory capacity in skeletal muscle across in vitro, preclinical, cachexic patients, and other atrophy-based diseases (Section 1.3.4.2.1.2 Insulin and Insulin-Like Growth Factor 1 Signaling). We reveal that the VM-M3 model has upregulation of ubiquitin proteasomal degradation pathway via low IGF-1/insulin and increased FOXO3a signaling, revealing a targetable and potentially translatable atrophy target.

Novel and effective CACS therapies are urgently needed as patients currently have no standard of care with over a century worth of research into this disease. Nutritional ketosis has been demonstrated to have systemic and pluripotent signaling effects [9, 56, 69] which have been hypothesized by our group to potentially mitigate multifactorial atrophy disease [57]. However, logistical limitations have impeded translatable potential [78, 79, 271]. We reveal that acute oral administration and chronic food incorporation of exogenous ketone bodies is well tolerated and rapidly induces metabolite changes similar to chronic fasting, demonstrating a more translatable approach to therapeutically inducing nutritional ketosis.

Ketone bodies have been demonstrated to alter systemic physiology and induce a plethora of signaling effects [56, 69] which have anticipated relevance for CACS [57], cancer [60, 271], other therapeutic approaches [104, 120], and overall patient health [59, 248, 249, 272]. We demonstrate that ketone bodies inhibit skeletal muscle atrophy and tissue catabolism in multifactorial CACS and septic/inflammatory atrophy. While there are potentially multiple ways in which ketone bodies regulate skeletal muscle catabolism, historical data has demonstrated that
nutritional ketosis consistently reduces BUN [2, 44, 92], attenuates alanine flux [4, 44, 103, 105, 108], reduces circulating glucose and glycolysis without obligate insulin [9, 10, 44, 99-105], augments ERK/MEK proliferative signaling [70, 132, 133], and reduces HDAC activity and elevates acetylation status [65, 121], with demonstrated abilities to also reduce leucine oxidation in the fed state [92, 104], augment protein synthesis [104, 120], attenuate inflammation [66, 273], increase antioxidant production in extra-muscular tissues [65, 67], and reduce lactate [100, 101, 105, 116, 117]. Thus, due to the pluripotent nature of these metabolites in skeletal muscle atrophy, combined with our data in multifactorial tissue atrophy, it seems plausible that exogenous ketone bodies may mitigate atrophy in both CACS and other catabolic, albeit less complex, atrophy-based diseases.

While regulating skeletal muscle is central to CACS disease, nutritional ketosis has been demonstrated to be a promising therapy in several cancers [60, 253, 271]. Across 57 preclinical trials in VLCKD and cancer, 60%, 17%, and 10% have reported anti-tumor, neutral, or pro-tumor effects, respectively [271]. Most clinical reports have been limited to case reports (10) or small trials assessing safety and feasibility (8). None the less, these reports revealed feasibility, tolerability, and safety for those who adhered to the dietary regimen. These reported anti-cancer responses have been attributed to altered systemic and mitochondrial metabolism, inflammation, oxidative stress, tumor microenvironment, amongst others [60, 271]. Here we also report anti-tumor and metastatic effects of exogenous ketone bodies in CACS. While nutritional ketosis reveals anti-cancer potential, many of the prescribed effect of nutritional ketosis have been illustrated to result directly from ketone-bodies themselves, demonstrating the promising potential for exogenous ketone to modulate both CACS and cancer.
While our studies reveal the benefit of administration of exogenous ketone alone, additive and/or synergistic multi-modal approaches are likely to induce more potent anti-CACS responses. Currently, a high-protein diet is recommended for CACS patients due to protein’s inherent role in skeletal muscle but has not yet been determined to be efficacious in isolation [274]. However, exogenous ketone bodies have been shown to augment the muscle synthesis response when co-administered with protein [104, 120]. While concern may arise due to mTOR role in growth of cancer cells, recent longevity studies have suggested that nutritional ketosis may augment skeletal muscle mTOR while reducing mTOR in extra-muscular tissues, inducing tissue specific effects [121]. Inflammation influence numerous aspects of CACS including cancer, skeletal muscle and adipose tissue wasting, anorexia, metabolic derangement, anabolic resistance, well-being, amongst others (Chapter1; [134, 179, 275]). Prior work has indicated that exogenous administration of the KDE reduced LPS-induced inflammation [66, 273]. Combined with our results indicating the ability of exogenous ketone bodies to reduced anorexia and skeletal muscle catabolism in inflammatory atrophy environments, it seems likely that exogenous ketone administration may mitigate inflammation and its downstream consequences in CACS. Anabolic agents have been proposed to augment muscle synthesis. As ketone bodies have been demonstrated to induce a more potential anti-catabolic than pro-synthetic response [119], it seem likely that coadministration of anabolic agents with exogenous ketone bodies may further augment muscle protein turnover in favor of muscle retention. Lastly, most cancer patients will likely be receiving some form of standard of care treatment to manage their neoplastic disease. Importantly, nutritional ketosis has been demonstrated to potentially synergize with current standard of care (radiation, chemotherapy, and others) while mitigating damage to surrounding tissues [271]. Together, exogenous ketone
bodies may augment many of the currently proposed CACS and cancer therapies and be an integral part of augmenting patient outcomes.

Beyond cancer and CACS, nutritional ketosis has demonstrated efficacy in a number of diseases including, but not limited to seizures [86, 276], obesity [59], diabetes [61, 64], poly cystic ovarian disease [59], cardiovascular [248], with current investigation into longevity [121, 277-279], performance [101, 280-285], psychological status [286, 287]. Thus, it is tempting to speculate that exogenous ketone bodies may have benefits across patient overall health and well-being which are independent of cancer and muscle wasting.

The finding presented in this dissertation along with historical and emergent data into the possible effects of exogenous ketones reveal considerable potential for this therapeutic strategy. However, further evaluation into both VM-M3 modeling and exogenous ketone therapies will help advance understanding into these research and therapeutic tools. First, while it was shown that the VM-M3 model demonstrates increased ubiquitin proteasomal degradation via low IGF-1/insulin signaling, other unexplored mechanisms, notably IL-6-induced STAT3, FOXO3a regulation of autophagy, and mTORC1/eIF4e/S6K synthetic signaling all present potential mechanism regulating VM-M3 skeletal muscle signaling. Second, while this work reveals that ketone bodies’ hold to potential to improve multiple component of CACS, future work should determine how KDE are regulating skeletal muscle signaling in VM-M3 and other translatable model systems. Third, optimal human pharmacokinetic dosing along with metrics of safety with chronic use will be a critical next step in translating this therapy into the clinical setting. As both oral gavage and food incorporation were successful in inducing nutritional ketosis in our model system, both
options may be considered when implemented clinically. Lastly, as it is likely that exogenous ketones will have varying degrees of efficacy across cancer type and cachexia patients [57, 253, 271], determining the optimal therapeutic populations will be critical as more evidence emergences on the effects of nutritional ketosis’ across cancer and cachexia.

In conclusion, since the VM-M3 model comprehensively recapitulate the diagnostic metrics of clinical CACS, we expect that it may serve as an ideal modeling system for evaluating translatable findings on cachexia pathology and progression, multi-organ and tissue crosstalk, translatable mechanisms of atrophy, and therapeutic efficacy of future treatments. Additionally, KDE treatment attenuated cancer, atrophy, and comorbid symptoms, KDE appears to present a multimodal therapeutic option that could assist across multiple facets of multifaceted CACS, inflammatory/septic atrophy, and other atrophy-based diseases. Future studies could expand our understanding on the VM-M3 model, ketone-induced mechanism in CACS, and optimal methods and populations for KDE administration.

References for Chapter 6:

48. Fukao, T., et al., Enzymes of ketone body utilization in human tissues: protein and messenger RNA levels of succinyl-coenzyme A (CoA):3-ketoacid CoA transferase and


93. Bartmann, C., et al., *Beta-hydroxybutyrate (3-OHB) can influence the energetic phenotype of breast cancer cells, but does not impact their proliferation and the response to chemotherapy or radiation*. Cancer Metab, 2018. 6: p. 8.


APPENDIX A: MATERIALS AND METHODS

A.1 Ethics Statement

All procedures were approved by the USF Institutional Animal Care and Use Committee (USF IACUC; Protocol Number R1900 & R5829) and performed under strict adherence to the NIH Guide for the Care and Use of Laboratory Animals.

A.2 Cell Culture

VM-M3 cells were obtained as a gift from Dr. Thomas Seyfried (Boston College, Chestnut Hill, MA). VM-M3 cells were derived from a spontaneous tumor in a VM/Dk inbred mouse and adapted to cell culture [156]. VM-M3 cells were transduced with a lentivirus vector containing the firefly luciferase gene under control of the cytomegalovirus promoter as previously described [156]. VM-M3 cells were cultured in D-glucose, L-glutamine, and sodium pyruvate-free Dulbecco’s Modified Eagle Medium (Gibco, Life Technologies) supplemented with 10% fetal bovine serum (Invitrogen), 25mM D-glucose (Fisher Scientific), 2mM L-glutamine (Gibco, Life Technologies), 1% penicillin-streptomycin (Invitrogen), and 10mM HEPES buffer (Gibco, Life Technologies). Cells were maintained at 37°C in 95% air, 5% CO2 in a humidified incubator.
A.3 Animals

Five breeding pairs of the VM/Dk strain of mice were used to establish and propagate a VM/Dk mouse colony in the University of South Florida (USF) Morsani College of Medicine Vivarium according to standard husbandry protocol. Male and female VM/Dk mice between 10-23 weeks of age were singly housed to accurately assess individual animal food intake. Bodyweight and food intake were tracked daily prior to and post-social isolation to ensure bodyweight and food intake normalized prior to cell inoculation (Table A.1). Animals were distributed into one of four groups: SH-M, SH-F, CA-M, and CA-F. Each cancer animal was matched with a sham animal of equivalent sex, bodyweight, age, and food intake (Fig 3.1) to ensure appropriate cancer and sex-specific comparisons. To determine KDE-induced effects in CACS, VM/Dk animals were distributed into one of three groups: KDE+VM-M3, VM-M3, and Sham with equivalent sex, bodyweight, age and food intake at baseline (Fig 5.2A-C). To determine KDE-induced effects post-LPS administration, VM/Dk animals were distributed into two groups (Fig 5.9A & 5.11A): KDE+LPS and LPS of equivalent bodyweights. All procedures were approved by the USF Institutional Animal Care and Use Committee (USF IACUC; Protocol Number R1900 & R5829) and performed under strict adherence to the NIH Guide for the Care and Use of Laboratory Animals.
Table A.1 Experiment Design

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A.4 VM-M3 Cell and Lipopolysaccharide Implantation

For VM-M3 implantation in the model characterization experiments, ~1x10⁷ VM-M3 cells in 300μL sterile PBS (CA-M and CA-F) or 300μL sterile PBS-only (SH-M and SH-F) were injected subcutaneously into the left abdominal flank resulting in primary tumor formation at the injection site, and subsequently systemically metastasizes to most major organs, namely the liver, kidneys, spleen, lungs, and brain as previously described [151-153, 156, 288, 289]. Additional model analysis revealed metastatic disease and CACS temporal progression could be replicated with intraperitoneal administration of VM-M3 cells. This method of implantation further minimized variability in markers of tumor progression, and therefore provides a useful optional method technique in the VM-M3 model for efficient analysis of potential therapeutic agents. Therefore, to determine KDE-induced effects on CACS, 1x10⁷ VM-M3 cells in 300μL sterile PBS
(KDE+VM-M3 and VM-M3) or 300μL sterile PBS-only (Sham) were administered intraperitoneal. For LPS administration in the septic atrophy model, LPS (Escherichia coli O55:B5; L2880; Sigma-Aldrich, St. Louis, MO) was diluted in sterile PBS and administered intraperitoneal at 10mg/kg as pilot work indicated a maximal, yet non-fatal, ~15% bodyweight reduction at 10mg/kg dose.

A.5 Survival Analysis

Animal health and behavior were assessed daily. VM-M3 animals were humanely euthanized together with their sham-matched controls (sex, bodyweight, age, and food intake matched at baseline) by exsanguination under isoflurane (Henry Schein Animal Health, Dublin, OH) according to IACUC guidelines upon presentation of EOL defined criteria (decreased response to stimuli, failure to thrive, labored breathing and/or locomotion, and/or complete cessation of food intake). Survival time was recorded.

A.6 Tumor Growth and Metastasis

Tumor growth was monitored weekly as a measure of bioluminescence using the Xenogen IVIS Spectrum system (Caliper LS, Hopkinton, MA). Data acquisition and analysis was performed using the Living Image® software (Caliper LS). Approximately 15 minutes prior to in vivo imaging, VM/Dk mice received an intraperitoneal injection of D-Luciferin (50 mg/kg; 88293, Thermo Fisher Scientific; Waltham, MA)). Bioluminescent signal was obtained using the IVIS Lumina cooled CCD camera system in both prone and supine positions. As only the cancer cells
were transfected with the luciferase gene, bioluminescent signal (photons/sec) of the whole animal was measured and tracked over time as an indicator of tumor burden and metastatic spread. At EOL, primary tumor, spleen, liver, and adipose tissue were gathered and saturated with D-Luciferin (10µL D-Luciferin+PBS/g tissue at 5mg D-Luciferin/mL PBS dilution) for 5 mins and imaged to determine tumor burden. Ascites fluid was imaged 15 minutes after resuspension with D-Luciferin (20µL D-Luciferin+PBS/mL ascites fluid at 5mg D-Luciferin/mL PBS dilution) to assess presence of circulating tumor cells.

A.7 Body Composition

Bodyweight was assessed daily at the same time (7:00-9:00). At EOL, bodyweight and weights of ascites fluid, primary tumor, calf (combined gastrocnemius & soleus), anterior thigh (quadriiceps), intraperitoneal fat pads, liver and spleen tissue were measured. A follow-up time course experiment was conducted to weigh tissues at weeks 1, 2, and 3 to assess weekly changes in bodyweight and aforementioned tissues for both cancer and sham-matched control animals. All tissue weights were normalized to baseline bodyweight (not influenced by cachexic progression) to allow for appropriate comparison between animals. For follow up evaluation of KDE, bodyweight was tracked daily with tissue weight determined at 21-days, prior to EOL.

A.8 Food Intake and Ketone Diester Administration

Standard diet dry food (2018 Teklad Global 18% Protein Rodent Diet, Harlan) was mixed with deionized water (1g dry food/mL deionized water) into a consistent paste and placed on a 100
x 15mm dish. Food intake was tracked daily at the same time (7:00-9:00) and replaced every other day to ensure fresh food. Due to sinusoidal changes in food intake observed every other day, a 4-day average was taken at EOL and baseline to calculate changes in anorexic symptoms. For evaluation of KDE effect on anorexia, food intake was tracked daily in at the same time (7:00-9:00). As pilot work indicated reductions in ad libitum food intake with 20-30% by weight KDE incorporation standard diet, 1%/weight saccharine (Sigma-Aldrich) and 5%/weight peanut butter (Natural Jif Creamy, J.M. Smucker Company, Orrville, OH) were added to paste to increase palatability of standard diet across groups (HPSD). Additional pilot work revealed that incremental incorporation of KDE from 0-30%KDE/weight at 5%KDE/weight/day was better tolerated and did not result in changes in bodyweight across time (Fig 5.1C). Consequently, upon VM-M3 inoculation, KDE+VM-M3 received ad libitum 0%KDE/weight day 1, 5%KDE day 2, 10%KDE day 3, 15%KDE day 4, 20%KDE day 5, 25%KDE day 6, 30%KDE day 7 through 21 on top of HPSD. VM-M3 and Sham received HPSD ad libitum. As pilot work indicated potential water evaporation in food paste in ventilated cages, dehydration standard curve was calculated across various volumes of plated food (5, 10, 25.0, 50.0, 100g/food) with and without KDE over a two-day period. Standard curve equation [water evaporated = 0.2472 (original grams of water) + 1.364] was used to correct for amount of dehydration prior to calculating caloric intake. For LPS experiments, 1x4mL/kg KDE gavage or 1x4mL/kg water gavage were administered for KDE+LPS and LPS-only, respectively. Ab libitum food intake was tracked daily during initial analysis and pair-fed during subsequent analysis.
A.9 Inflammation

Whole blood was gathered at baseline, week 2, and EOL prior to anesthetics exposure via submandibular puncture to prevent anesthetic influence on inflammatory biomarkers [290] and to avoid potential ascites contamination from cardiac puncture. 60uL of whole blood was placed into a K2EDTA tube (BD Microtainer, Franklin, NJ), relabeled for blinded analysis, and analyzed via HemoTrue (Heska, Loveland, Colorado) to assess white blood cell counts. Remaining whole blood was placed into serum separator tubes (MiniCollect 0.8mL, Kremsunster, Austria) and centrifuged (13,000rpm, 4°C, 15min) to isolate serum. 25μL of serum was mixed with 25μL of saline, relabeled for blinded analysis, and analyzed using Bio-Plex (Bio-Rad, Hercules, California) fluorescent bead technology to generate cytokine concentrations via a standard curve (EVE Technology Mouse Cytokine/Chemokine Array 31-Plex). To determine whether spleen or liver enlargement in VM-M3 animals was explained by tumor burden alone or other immunologic factors, tissue bioluminescence was divided by the difference in tissue weight between VM-M3 and sham-matched controls. This bioluminescence to tissue weight ratio for both the liver and spleen was compared to primary tumor to determine if the organ weight differences could be explained by tumor burden alone.

- **Primary Tumor:** \( \text{Tumor Burden/Organ (mg)} = \frac{\text{Primary Tumor Bioluminescence}}{\text{Primary Tumor Weight}} \)

- **Liver:** \( \text{Tumor Burden/ΔOrgan (mg)} = \frac{\text{Liver Bioluminescence}}{\text{(Cancer Burdened Liver Weight – Sham Liver Weight)}} \)

- **Spleen:** \( \text{Tumor Burden/ΔOrgan (mg)} = \frac{\text{Spleen Bioluminescence}}{\text{(Cancer Burdened Spleen Weight – Sham Spleen Weight)}} \)
A.10 Metabolic Biomarkers

Blood glucose and \( R \) \( \beta \)-hydroxybutyrate were measured using Precision Xtra™ Blood Glucose & Ketone Monitoring System (Abbott Laboratories, Abbott Park, IL). Blood lactate concentration was measured using LACTATE PLUS Lactate Meter (Nova Biomedical). IGF-1 and Insulin were relabeled for blinded analysis and subsequently analyzed using Luminex 100 system (Luminex, Austin, TX) with R&D Systems Mouse 1-Plex Luminex Assay (R&D Systems, Minneapolis, MN) and Milliplex Mouse Multiplex Kit (Millipore, St. Charles, MO), respectively, using manufacturer’s protocol.

A.11 Clinical Cachexia Biomarker

At EOL, blood was gathered as previously described and red blood cell count relabeled for blinded analysis via HemoTrue (Heska, Loveland, Colorado). Remaining whole blood was centrifuged (13,000rpm, 4°C, 15min) down in serum separator tubes, relabeled for blinded analysis (MiniCollect 0.8mL, Kremsunster, Austria), and analyzed via DRI-CHEM 7000 (Heska) to determine clinical chemistry concentrations.

A.12 Muscle Tissue Collection and Processing

Muscle tissue was immediately gathered, separated and flash frozen in liquid nitrogen and stored at -80°C at harvest. Prior to tissue processing, all tissues were deidentified and relabeled for blinded analysis. For protein and RNA analyses tissues were removed from ~80°C and crushed.
using a liquid nitrogen-cooled mortar and pestle. For protein analysis, ~30mg of powdered tissue was placed in 1.7mL microcentrifuge tubes containing 500μL of ice-cold cell lysis buffer [20mM Tris-HCl (pH 7.5), 150mM NaCl, 1mM Na2EDTA, 1mM EGTA, 1% Triton (Cell Signaling, Danvers, MA)] pre-stocked with protease and Tyr/Ser/Thr phosphatase inhibitors (2.5mM sodium pyrophosphate, 1mM β-glycerophosphate, 1mM Na3VO4, 1µg/mL leupeptin). Samples were then homogenized by hand using tight micropetstles, insoluble proteins were removed with centrifugation at 500g for 5 minutes and obtained sample lysates were stored at −80°C prior to Western blotting and other biochemical assays (described below). For total RNA analysis, ~15–30mg of powdered tissue was weighed using an analytical scale with a sensitivity of 0.001g (Mettler-Toledo; Columbus, OH). Tissue was then homogenized in 1.7mL microcentrifuge tubes containing 500μL of Ribozol (Ameresco; Solon, OH) via micropetcle manipulation and RNA isolation was performed per manufacturer recommendations. Total RNA concentrations were then determined in duplicate using a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific), and total RNA per unit muscle weight was used as a surrogate for ribosome density as in past publications [291, 292].

**A.13 Western Blot Analysis**

Whole-tissue sample lysates obtained through cell lysis buffer processing (described above) were batch process-assayed for total protein content using a BCA Protein Assay Kit (Thermo Fisher Scientific). Lysates were then prepared for Western blotting using 4x Laemmli buffer at 1μg/μL. Following sample preparation, 18μL samples were loaded onto 4–15% SDS polyacrylamide gels (Bio-Rad; Hercules, CA) and subjected to electrophoresis (180V for 45–60
minutes) using premade 1x SDS-PAGE running buffer (Ameresco; Framingham, MA) in order of Sham, then Cancer, from animal number 1 to 12. Proteins were then transferred (200mA for 2 hours) to polyvinylidene difluoride membranes (Bio-Rad), Ponceau stained and imaged to ensure equal protein loading between lanes. Membranes were then blocked for 1 hour at room temperature with 5% non-fat milk powder in Tris-buffered saline with 0.1% Tween-20 (TBST; Ameresco). Rabbit anti-mouse pan NF-kB/p65 (1:1000; Cell Signaling, catalog #: 8242), rabbit anti-mouse MuRF-1 (1:500; Abcam, Cambridge, MA; catalog #: ab172479), rabbit anti-mouse Atrogin-1 (1:500; Abcam, catalog #: ab74023), rabbit anti-mouse FOXO3a (1:500; Cell Signaling, catalog #: 2497), rabbit anti-mouse ubiquitin (1:1,000; catalog #: 3933; Cell Signaling) and rabbit anti-mouse 20S proteasome core (1:500; Millipore Sigma, Burlingame, MA; catalog #: ST1053) were incubated with membranes overnight at 4°C in TBST with 5% bovine serum albumin (BSA). The following day, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:2000; Cell Signaling; catalog #: 7074; Danvers, MA) in TBST with 5%BSA at room temperature for 1 hour (1:2,000). Membrane development was performed using an enhanced chemiluminescent reagent (Luminata Forte HRP substrate; EMD Millipore, Billerica, MA), and band densitometry was performed using a gel documentation system and associated densitometry software (UVP, Upland, CA). Densitometry values for all targets were divided by whole-lane Ponceau densities. Density/Ponceau were divided by the Sham mean and expressed as relative fold change.
A.14 PCR

Two µg of RNA was reverse transcribed into cDNA for RT-PCR analysis with cDNA synthesis reagents (Quanta Biosciences, Gaithersburg, MD) per the manufacturer’s recommendations. RT-PCR was performed using gene-specific primers and SYBR green chemistry (Quanta Biosciences). Primer sequences used were as follows: beta-glucuronidase (housekeeping gene, HKG): forward primer 5′-TCAGCTCTGTGACCAGATACG-3′, reverse primer 5′-GCCACAGACCACATCAACAAC-3′; MyoD: forward primer 5′-CCTGCCCTCCACATCCTTTT-3′, reverse primer 5′-GAAGGGGGAGAGTGGGGTAT-3′; Atrogin-1/MAFbx: forward primer 5′-CCATCCTCTTTTTTTTCCGTC-3′, reverse primer 5′-ATCACGTCCTCCACCTGGCTG-3′; MuRF-1: forward primer 5′-TGACGATGAGGAGGG-3′, reverse primer 5′-TTTACCCTCTGTGACACGC-3′; GPR109a: forward primer 5′-GATGAAAACATCGCCAAGGT-3′, reverse primer 5′-CCTCCAGTCCCAGTTATGGA-3′; IGF-1: forward primer 5′-ACCACCTGTGACCTCAGTC-3′, reverse primer 5′-CTCCTGGAAAACCAGAACA-3′. Melt curve analyses confirmed that only one RT-PCR product was obtained with each primer set. PCR calculations were performed as previously described by our laboratory [293]. Briefly, $2^{ΔCq}$ values for each gene of interest at each time point were calculated whereby $ΔCq = Cq$ – geometric mean housekeeping gene $Cq$ values. All values for a given mRNA target were then normalized to the Sham mean and expressed as relative fold-change.
A.15 20S Proteasome Capacity Assay

Skeletal muscle protein from whole-tissue sample lysates (40 µg) obtained through cell lysis buffer processing (described above) were batch processed for 20S proteasome activity using commercially available fluorometric kits (catalog #: APT280; Millipore Sigma; Burlington, MA, United States) as previously described by our laboratory [293]. Assay readings are presented as relative fluorometric units. The average coefficient of variation for all duplicates was 10.7%.

A.16 Statistics

GraphPad Prism 7 software was used for all statistical analysis. Parametric tests were performed for all data sets as all groups were considered normally distributed. Unpaired or paired student’s t tests were performed for the comparison of two groups. One-Way ANOVA with Tukey’s multiple comparison post-hoc test was performed for more than three comparisons while Fischer LSD post-hoc was used for three comparisons or less. Results were considered significant when p<0.05. Robust regression and outlier removal (ROUT) with coefficient Q=1% was only used prior to cytokine analysis as non-physiologic/error values were independently indicated [294].

A.17 References for Appendix A


ABOUT THE AUTHOR

Andrew Koutnik was born and raised in Tallahassee, FL. He is a researcher studying the influence of nutrition and metabolism on health, disease, and performance at University of South Florida Morsani College of Medicine. He originally began his research path at Florida State University in the Exercise Science studying the influence of nutrition, exercise, supplementation, and environmental extremes on health-based outcomes in normal and clinical populations. During his time at Florida State University, Andrew graduated *Suma Cum Lade with Honors*, contributed to 10 published manuscripts, was awarded the Honors in the Major Medallion, Bess Honor These Award, and ACC Meeting of the Minds Travel & Presentation Award. From Florida State University, he was awarded the USF Presidential Fellowship and transitioned into Biomedical Research with the Metabolic Medicine Lab at University of South Florida Morsani College of Medicine with a focus on studying metabolism and metabolic
therapies for health, disease, and performance outcomes. During his time at University of South
Florida, Andrew has contributed to and/or authored 10 publications (additional in review),
conducted two researcher projects on NASA NEEMO 22, gave a TEDx Talk titled “Rethinking
Nutrition for Type-1 Diabetics,” gave numerous oral and poster domestic and internal
presentations, was awarded NASA NEEMO 22 Topside Support Crew Award, NASE NEEMO
22 USF/IHMC Research Team Award, and 2017 and 2019 USF Student Research Award
Recipient: “Alternative energy substrates as metabolic therapies” and “Targeting Cancer
Cachexia with Ketone Supplementation” respectively, and has author two separated pending
patents revolving the use of metabolic tools for disease management. Personally, Andrew
Koutnik also has Type-1 Diabetes which has and continues to give an incredible in-depth
personal journey into the world of our metabolism, how it works, how day-to-day life
(sometimes moment-by-moment choices) influences it, and how these changes on metabolism
can have far reaching effects over other aspects of our physiology. This combination of
researching nutrition and exercise, but also dealing with its benefits and consequences daily as
a Type-1 Diabetic, created a strong appreciation for the metabolic role nutrition and lifestyle
can play into disease prevention and management. Andrew is the lucky husband to Kelly Hope
Koutnik and father to Stellan Oakes Koutnik.