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Molecular Mechanisms Governing Muscle Wasting in Cancer

Aneesha Dasgupta
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Molecular Mechanisms Governing Muscle Wasting in Cancer

By

Aneesha Dasgupta

A Dissertation
Presented to the Faculty of Nebraska Graduate College
in Partial Fulfillment for the Requirements of
the Degree of Doctor of Philosophy

Biochemistry and Molecular Biology Graduate Program
Under the Supervision of Dr. Pankaj K. Singh
April 2019

Supervisory Committee Members

Dr. Angie Rizzino, Ph.D.  Dr. Keith Johnson, Ph.D.
Dr. Melissa Teoh-Fitzgerald, Ph.D.  Dr. Pi-Wan Cheng, Ph.D.
Dedicated to my parents and brother who never laughed at my dreams.

And to my grandparents and forefathers without whose genes, I wouldn’t have started on this journey.

~
Acknowledgement

I am grateful to my Ph.D. mentor, Dr. Pankaj K. Singh. He has been responsible in inspiring, motivating and guiding me through my entire PhD journey. He has made me the best version of the graduate student that I could be. All the accolades and the awards that I have received in these last four years is due to his investment in me. He was able to instill the same amount of ambition, drive and motivation in me, which he has, for science and for making a difference. He has always asked me to look at grander scheme of events. We have not always agreed with each other, but he has always supported me when I needed him to. I have learnt a lot from him and I would like to thank him for contributing his time and effort in building my scientific acumen.

I would also like to thank all the past and current lab members. Each of them has contributed to my scientific journey. I would like to start by thanking Dr. Surendra K. Shukla. He has guided and taught me most of my skills that I attained in the study of cancer cachexia. He has always been available to answer all my questions and confusions and to oversee my experiments. I would like to thank Dr. Enza Vernucci, for being a constant inspiration to me in the lab and to teach me to keep working hard through all the frustrations and the failures. My time in the PhD program would not have been half as enjoyable and fulfilling without her beside me. I would also like to thank Dr. Jaime Abrego, Ryan King and Scott Mulder, who are not just my fellow graduate students, but have eventually become few of my best friends. These are the people with whom I have spent each day for the last 5 years. They have always helped me in dealing with large groups of mice surgeries,
late night mice necropsies and any other help that I have ever needed. They have been there through all the ups and downs in my PhD; we have celebrated the sorrows and the joys of all our experiments together. I would also like to thank Dr. Ravi Thakur with whom I have always had the most enlightening scientific arguments. He has never hesitated to help me or to answer my questions. This section is incomplete without mentioning Nina Chaika. I would like to thank her for being patient with me since the first year and to help me whenever I needed her. Somehow, she always had answers to all my technical struggles. My lab members would agree when I say that the lab would not be held together without her constant efforts.

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It took all these people and more for me to be able to finish this journey amid frustrations and anxiety.

Aneesha Dasgupta, 2019.
Abstract

Molecular Mechanisms Governing Muscle Wasting in Cancer

Aneesha Dasgupta, Ph.D.

University of Nebraska Medical Center, 2019

Supervisor: Pankaj K. Singh, Ph.D.

Pancreatic cancer is the third-leading cause of cancer-related deaths in the United States. About 80 percent of the pancreatic cancer patients suffer from cachexia and, about one-third die due to complexities related to the syndrome. Cachexia leads to a loss in body weight and cachectic patients are refractory to chemotherapy. Despite recent advances, the mechanisms of pancreatic cancer-cachexia and the potential therapeutic interventions remain poorly evaluated.

Sirtuins represent a class of proteins that are regulated by metabolic fluctuations in tissues. We observed a reduced expression of Sirt1 in spontaneous PDAC mice muscles, human pancreatic cancer muscles, and myotubes treated by cancer cell-conditioned media. We further observed that cancer cell-conditioned media upregulated the NF-κB and FoxO transcription factor pathways. NF-κB regulated the expression of NADPH Oxidase (Nox4) in cachectic muscles, thereby increasing reactive oxygen species (ROS). We also observed a negative correlation between Nox4 expression and muscle cross-sectional area in the cachectic muscles of PDAC patients. Inducible genetic knockout of Nox4 gene in muscles of tumor-bearing mice rescued the cachectic phenotype. Moreover, pharmacological blockade of Nox4 activity was successful in attenuating loss in body weight and muscle mass in tumor-bearing mice. Therefore, we concluded
that the Sirt1-Nox4 axis plays an important role in the manifestation of pancreatic cancer cachexia.

Studies have shown that chemotherapy in addition to tumor burden can induce body weight loss in cancer patients. We performed a single-center retrospective study of 162 patients at our institution by measuring muscle mass among patients with a diagnosis of pancreatic cancer at initial diagnosis and eight-week follow up post treatment. We found a significant improvement in overall survival and progression-free survival in patients with modest or no reduction in skeletal muscle index after eight weeks of chemotherapy. Furthermore, we treated mice with Gemcitabine and FOLFIRINOX, and observed a decrease in body weight, gastrocnemius muscle weight, grip-strength, and rotarod performance. We also observed an increase in chemotherapy-induced ROS in the mice. We observed that utilizing BMX-001, a SOD mimetic rescued the decrease in body weight, muscle weight, grip-strength, and rotarod performance in mice treated with chemotherapy. BMX-001 was also successful in rescuing chemotherapy-induced muscle wasting in tumor-bearing mice. We concluded that targeting ROS production in skeletal muscles could prevent chemotherapy-induced muscle wasting.
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AMPK</td>
<td>AMP-Activated Protein Kinase</td>
</tr>
<tr>
<td>APPR</td>
<td>Acute Protein Phase Response</td>
</tr>
<tr>
<td>ATGL</td>
<td>Adipose Triglyceride Lipases</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>CAMKII</td>
<td>Calmodulin-Dependent Kinase II</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin Immunoprecipitation</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese Hamster Ovary</td>
</tr>
<tr>
<td>CM</td>
<td>Conditioned Medium</td>
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<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CT</td>
<td>Computed Tomography</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacyl Glycerides</td>
</tr>
<tr>
<td>DEXA</td>
<td>Dual Energy X-ray Imaging</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>EPR</td>
<td>Electron Paramagnetic Resonance</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular Signal-Regulate Kinase</td>
</tr>
<tr>
<td>ESPEN</td>
<td>European Society for Clinical Nutrition and Metabolism</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>GHS-R</td>
<td>Growth Hormone Receptor Secretagogue Receptor</td>
</tr>
<tr>
<td>GKT</td>
<td>GKT 138731</td>
</tr>
<tr>
<td>GPX</td>
<td>Glutathione Peroxidase</td>
</tr>
<tr>
<td>GSEA</td>
<td>Gene Set Enrichment Analysis</td>
</tr>
</tbody>
</table>
GSK-3β  Glycogen synthase kinase 3β
HMGB-1  High-Motility Group Box 1
HSL  Hormone Sensitive Lipase
HU  Hounsfield Units
IACUC  Institutional Animal Care and Use Committee
IGF1  Insulin-like growth factor
IL-6  Interleukin 6
IR  Insulin Receptor
LMF  Lipid Mobilizing Factor
LVEF  Left Ventricular Ejection Fraction
MAFbx  Muscle Atrophy F box protein
MAG  Monoacylglycerols
MAPK  Mitogen-Activated Protein Kinase
Mfns  Mitofusins
MOI  Multiplicity of Infection
MURF1  Muscle ring finger containing protein-1
MyoD  Myosin Differentiation Factor
NADPH  Nicotinamide Adenine Dinucleotide Phosphate
NF-κB  Nuclear Factor κB
NOX  NADPH Oxidase
OS  Overall Survival
PBS  Phosphate Buffered Saline
PCR  Polymerase Chain Reaction
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIF</td>
<td>Proteolysis Inducing Factor</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for Advanced Glycation End-products</td>
</tr>
<tr>
<td>RAP</td>
<td>Rapid Autopsy Program</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radio-Immunoprecipitation Assay</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RVEF</td>
<td>Right Ventricular Ejection Fraction</td>
</tr>
<tr>
<td>shRNA</td>
<td>Stable Short Hairpin RNA</td>
</tr>
<tr>
<td>SIRT</td>
<td>Sirtuin</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide Dismutase</td>
</tr>
<tr>
<td>STACs</td>
<td>SIRT1-activating compounds</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal Transducer and Activator of Transcription</td>
</tr>
<tr>
<td>TAG</td>
<td>Triacylglycerides</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor beta</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor alpha</td>
</tr>
<tr>
<td>Trdxn</td>
<td>Thioredoxin</td>
</tr>
<tr>
<td>UCP-1</td>
<td>Uncoupling Protein-1</td>
</tr>
<tr>
<td>WAT</td>
<td>White Adipose Tissue</td>
</tr>
<tr>
<td>WL</td>
<td>Weight Loss</td>
</tr>
<tr>
<td>WLGS</td>
<td>Weight Loss Grading System</td>
</tr>
<tr>
<td>ZAG</td>
<td>Zinc alpha 2 glycoprotein</td>
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Chapter 1: Introduction

(Note – The introduction is adapted from a review article which is under revision for submission as of April 2019)

The word cachexia is derived from the Greek words, “Kakos” meaning bad and “Hexis” meaning condition. It is a multifactorial syndrome leading to loss in body weight and is associated with many diseases, majorly cancer, AIDS, tuberculosis, and chronic obstructive pulmonary disorder. The diagnostic criteria for cachexia is defined as 5% loss in body weight over a duration of six months or 2% loss in body weight for those whose body mass index (BMI) is less than 20 kg/m². However, this diagnostic criterion did not include the prognostic significance of weight loss in patients who initially have a low, intermediate, and high BMI. In 2015, the diagnostic criteria for cachexia was revised by the consensus group and is now included in the international practice guidelines of the European Society for Clinical Nutrition and Metabolism (ESPEN). The group demonstrated differences in survival across five categories of BMI (< 20.0, 20.0 to 21.9, 22.0 to 24.9, 25.0 to 27.9, and 28.0 kg/m²) and five categories of percent weight loss (WL) (-2.5 to -5.9, -6.0 to -10.9, -11.0 to -14.9, ≥ 15.0%, and weight stable (±2.4%). They observed that weight-stable patients with BMI ≥ 25.0 kg/m² (grade 0) had the longest survival and percentage WL values associated with lower categories of BMI were related to shorter survival.

Cachexia differs from starvation in the way that the weight loss is due to depletion in adipose tissues and skeletal muscle mass but not non-muscle protein. Moreover, cachexia also differs from anorexia in the way that the loss in
muscle and adipose deposits cannot be reversed by conventional nutritional support and thus leads to progressive functional impairment\textsuperscript{3}.

Previous studies have shown that cachexia is responsible for about 20% of deaths of cancer patients\textsuperscript{4}, although this percentage varies with the tumor type, with an incidence of about 80\% in pancreatic cancer patients, 40 to 50\% in lung, prostate and colon cancer patients\textsuperscript{5,6}

The severity of cancer cachexia is inversely related to the survival of the patient and it almost always leads to a poor prognosis. The main characteristics of cancer cachexia is asthenia or lack of muscular strength. This entails the muscle wastage as well as physical and mental fatigue. Along with it, loss in mean body mass is the other trend in cachexia. It has also been observed that along with loss in skeletal muscle, loss in cardiac muscle also occurs in cancer cachectic patients. This might be a major reason of death in cachectic patients. The onset of cachexia significantly influences the course of the disease. Furthermore, most anti-tumor therapies also lead to muscle wasting and exacerbate the condition\textsuperscript{7}. Body weight loss negatively influences survival by prevention of completion of chemotherapy.

Cachexia affects the metabolism of the entire host body as it affects multiple organs. The severity of cachexia has been difficult to assess objectively and only recently a method to stage cachexia has been validated\textsuperscript{8}.

1. Diagnostic Techniques

Cachexia is typically diagnosed if a patient has lost 5\% of their body weight over a duration of 6 months or 2\% of their body weight for those with a BMI of less than
20 kg/m². Moreover, though none of them are in clinical use yet, there are several potential biomarkers which may be used to identify patients even before they show signs of cachexia or weight loss. Plasma levels of ghrelin, leptin and adiponectin have been found to be significantly higher in cachectic gastric cancer patients as compared to non-cachectic gastric cancer patients. Moreover, the plasma levels of c-reactive protein (CRP) has been shown to be increased in the plasma of cachectic cancer patients.

A computed topography (CT) scan at the lumbar vertebrae level 3-4 is also being utilized to measure skeletal muscle mass, which can detect small changes and can be used to diagnose cachexia. Skeletal muscle can be identified around the L3 and then demarcated and quantified based on preestablished Hounsfield units (HU). Patients can be compared directly based on the measurements around the lumbar region which can be correlated to whole body skeletal muscle. Magnetic resonance imaging, appendicular skeletal muscle index from dual energy x-ray imaging (DEXA), mid-upper arm muscle area by anthropometry and whole body fat-free mass index by bioimpedence analysis are other methods to assess cachexia in cancer patients.

2. Cachexia Targets

Cancer associated cachexia in patients affects more than just the skeletal muscles and the adipose tissues. It manifests effects on the whole body and alters the functioning and signaling in the organs/tissues (Figure 1).
a. Skeletal Muscle

In almost all cancers, muscle and weight loss is correlated with poor prognosis, increased risk of treatment-related toxicity, and poor quality of life\textsuperscript{15,16}. Additionally, preventing muscle wasting can directly improve survival in tumor-bearing mice\textsuperscript{17}. Skeletal muscles comprise about 40% of the body weight and represent major protein reservoir of the body. Skeletal muscles not only provide strength and movement for the body, but also aid in maintaining posture and regulating body temperature. The major cause of weakness and fatigue in cachectic patients is muscle wasting\textsuperscript{18}. The molecular basis of cancer-related muscle wasting is not fully understood. Majority of the evidence suggest an impairment in synthesis and degradation of protein and amino acid metabolism\textsuperscript{4}. Protein degradation in the muscles is mainly mediated by the activation of the ubiquitin-dependent proteasome pathway\textsuperscript{19}. However, calpain-dependent cleavage of myofibrils, which also leads to muscle wasting, acts upstream to the proteasome-dependent degradation\textsuperscript{20}. Furthermore, autophagy also plays a role in protein degradation\textsuperscript{21}. 
Figure 1: Cachexia is a multifactorial syndrome which affects the whole body.
b. Cardiac Muscle

Cardiac atrophy is characterized by decreased heart mass mainly due to the combination of decreased cell size and increased apoptosis. Cancer as well as, anti-cancer therapies lead to cardiac atrophy which leads to functional impairment of heart function. It leads to the increased burden of clinical symptoms such as increased breathlessness, lethargies and sometimes cardiac arrest, leading to mortality. A study conducted by Mamidanna et al. demonstrated that about 25.4% of colorectal patient died due to cardiac causes. Another study comprising of 500 cancer patients, including gastrointestinal cancer patients, has shown that cardiovascular peptides (N-terminal pro BNP-N-proBNP, mid-regional pro-atrial natriuretic peptide-MR-proANP, mid-regional pro-adrenomedullin-MRproADM, c-terminal pro-endothelin-1-CT-proET-1, copeptin) were upregulated in tumor-bearing patients. Cardiopeptides concentrations were significantly correlated with mortality independent of age, sex and tumor stage. Recently, a study including pancreatic cancer, lung cancer and gastrointestinal cancer patients along with healthy controls has demonstrated that body weight loss in these patients was accompanied with loss of heart weight.

Cancer-associated cardiac cachexia may be the direct consequence of underlying heart disease, tumor burden itself or due to the chemotherapy, unfortunately, the detailed molecular mechanism is not well explored. Cancer-associated cardiac cachexia patients exhibit reduced cardiac functions such as left ventricular ejection fraction (LVEF), right ventricular ejection fraction (RVEF) and morphological alterations such as interventricular septum thickness and posterior
wall thickness. Increased level of several cardiac peptides has been reported in cancer patients. Increased level of BNP-NT-proBNP, MR-proANP, MRproADM, and CT-proET-1 were found to be correlated with survival of cancer patients. Altered organization of myofibrillar proteins, disrupted and degenerated sarcomere has been observed in preclinical models of cancer-associated cachexia. By utilizing C-26 cancer-associated cachexia model, Wysong et al. have shown that NF-κB inhibition exhibits cardio protective effect in tumor-bearing mice.

c. Adipose Tissue

Cancer cachexia also entails a significant loss of white adipose tissue, which is attributed to an increase in lipolysis rather than a decrease in lipogenesis. Inhibiting lipid mobilization rescued cancer-induced cachexia in mice models. Although according to the 2011 consensus, skeletal muscle loss was a necessity for the clinical diagnosis of cancer-associated cachexia, there has been evidence that lipid wasting may occur before muscle wasting. Therefore, the 2014 consensus which is based on initial BMI and percentage weight loss might be a more accurate method to grade cachexia.

Cachectic patients with gastrointestinal cancer have elevated levels of circulating free-fatty acids, triacylglycerol, and glycerol. These fatty acids can be taken up by skeletal muscles and high intramuscular fatty acids can induce the expression of the ubiquitin ligases (Atrogin-1 and MuRF-1), leading to skeletal muscle atrophy. This study was further validated by the correlation of lipid droplets in muscles of cancer patients with the extent of body weight loss. A
recent study also demonstrated architectural modification consisting of fibrosis and immune cell infiltration in the subcutaneous adipose tissues in gastrointestinal cancer patients\textsuperscript{37}.

Another interesting phenomenon of adipose metabolism in cancer associated cachexia is the “browning” of white adipose tissue (WAT), known as brown fat\textsuperscript{35,38}. These adipocytes have high mitochondrial content and increased expression of the uncoupling-1 protein (UCP-1) and function like brown adipocytes\textsuperscript{39}. UCP-1 is responsible for uncoupling the mitochondrial electron chain from ATP synthesis to thermogenesis\textsuperscript{40}. In 2014, Petruzzi et al demonstrated that WAT browning is an early event and increases systemic energy expenditure in preclinical models of cancer-induced cachexia\textsuperscript{41}. Another group demonstrated that upon using a genetically engineered mouse model that have reduced thermogenic potential and are resistant to browning, tumor-induced adipose tissue wasting was significantly inhibited\textsuperscript{38}. Interestingly, a recent study has demonstrated that succinate can be metabolized by adipocytes and can induce thermogenesis, implying a metabolic control of this process\textsuperscript{42}.

\textbf{d. Gut}

Studies have demonstrated that cachectic mice with colon cancer (APC min/+) undergo gut barrier disruption, which leads to increased systemic inflammation and plasma endotoxin concentrations\textsuperscript{43}. Moreover, gut microbiota affects the host metabolism and immunity, two characteristics of the pathophysiology of cachexia. \textit{Klebsiella oxytoca} is increased in cachectic C-26 bearing mice and alters the gut barrier function\textsuperscript{44}. Lipopolysaccharide-binding protein, mostly associated with
bacterial load, was increased in cachectic lung and colon cancer patients\textsuperscript{45}. These levels were also correlated with the serum IL-6 levels. Lipopolysaccharides, which are pro-inflammatory compounds, can induce muscle wasting through the toll-like receptor by activating the ubiquitin proteasome pathway and the autophagy lysosome pathway\textsuperscript{46}. Currently, the therapeutic potential of targeting gut microbiota is being examined by administering prebiotics and probiotics. For example, adding the bacteria \textit{L. reuturi} to drinking water of mice with colon cancer has been reported to lead to significantly increased gastrocnemius muscles and a statistically significant survival advantage compared to controls\textsuperscript{43}. This suggests that cachexia may be inhibited by the introduction of advantageous bacteria.

e. Liver

The liver plays a key role in whole-body metabolism. In both the liver and skeletal muscle, an opposing pattern of protein metabolism is observed. Muscle degradation leads to mobilization of amino acids like alanine and glutamine; while glutamine is taken up by the tumor as a nutrient source, alanine is taken up by the liver to produce glucose by gluconeogenesis\textsuperscript{47}. Cytokines have been shown to regulate liver metabolism by sustaining changes in the hepatic APPR (Acute Protein Phase Response). IL-6 is known to be the principal regulator of APPR in human hepatocytes\textsuperscript{48}. Activation of APPR in the liver leads to hypercatabolism because it reprioritizes whole-body protein metabolism from skeletal muscles to the generation of acute phase proteins\textsuperscript{49}. Production of acute phase proteins by the liver correlates with the mobilization of amino acids from the skeletal muscle,
which leads to loss of lean body mass. Further, Moses et al. found elevated APPR to correspond with decreased survival in pancreatic cancer patients.

3. Systemic Alterations in Cancer Cachexia

In this section, we will illustrate the systemic alterations in cancer cachexia, which includes the inflammatory, immune and metabolic pathways (Figure 2).

3.1 Cytokines

A loss in muscle weight is caused by a decrease in protein synthesis and an increase in protein degradation. The most studied cause of muscle degradation is the activation of the ubiquitination pathway by pro-inflammatory cytokines (Figure 3). A multitude of cytokines including TNF-α, IL-6, IL-1 have been implicated in inducing cachexia in gastrointestinal cancers. The hormone “cachectin” was discovered by Beutler et al. as a lipoprotein lipase suppressing hormone and was demonstrated to have detrimental effects on skeletal muscles plasma membrane potential. Cachectin was then recognized to correspond to the same protein as the human TNF-alpha. Following that, Oliff in 1987 demonstrated that injecting CHO cells producing cachectin/TNF-α in nude mice killed the mice faster as compared to those in which the CHO cells did not secrete cachectin. Moreover, it was also established that blockade of TNF-α with etanercept improved the tolerability of chemotherapy in patients with refractory gastric cancer. TNF-α demonstrated synergy with interferon-γ in decreasing the levels of myosin heavy chain in cultured myotubes and muscles of C-26.
Figure 2: Alterations in whole body metabolism. Tumor regulates whole body metabolism by releasing factors that affect the muscles, adipose deposits and the liver. Skeletal muscles and adipose tissues in turn secrete nutrients (amino acids and fatty acids) that feed the tumor. Liver associated CD68+ macrophages can also secrete cytokines in cachexia. Decrease in neuropeptide Y in the brain can lead to decrease in appetite which causes loss in body weight.
bearing mice. This study also established that myosin heavy chain was a target of the pro-cachectic factors\textsuperscript{59}.

There was an increased level of circulating cytokines (IL-6, IL-\(\alpha\), TNF-\(\alpha\)) in the serum of colon and pancreatic cancer patients suggesting a network of cytokines collectively promoting cachexia\textsuperscript{60}. Elevated levels of IL-6 in serum of pancreatic cancer patients was associated with decreased muscle mass and severe fatigue\textsuperscript{51}. Circulating levels of IL-6 also have been demonstrated to be elevated in the plasma of spontaneous mouse models of colon cancer which led to loss in body weight\textsuperscript{53}. Another study by the same group also established that deleting IL-6 not only abrogated cachexia but also led to reduced tumor burden in the colon cancer mouse model\textsuperscript{61}.

The myosin differentiation factor, MyoD1, which binds to the promoter of myosin heavy chain is regulated by these cytokines. The cytokines TNF-\(\alpha\) and IL-6 activate the p38 MAPK and the NF\(\kappa\)B pathways which are needed to upregulate the E3 ligases MURF1 and MAFbx, which modulate breakdown of sarcomeres and inhibition of protein synthesis\textsuperscript{62}. MURF1 is responsible for modulating the ubiquitination of the myosin heavy chain and other thick filaments\textsuperscript{63,64}. The MURF1 and MAFbx family of proteins are regulated by the Forkhead box proteins, namely Foxo3, Foxo1 and Foxo4\textsuperscript{65}. Furthermore, it was also demonstrated that the injection of double negative foxo in the skeletal muscles of mice before implantation of tumor cells reduced the mRNA levels of MAFbx and MuRF1, further inhibiting muscle atrophy during cancer cachexia\textsuperscript{66}.  

Figure 3: Cytokines regulate muscle wasting factors. Immune cells and tumor cells can release cytokines which affects both the liver and the skeletal muscles. This can induce acute phase protein response in the liver. Cytokines can also induce the liver to release c-reactive protein (CRP) into the blood vessels. Cytokine released from the liver can also stimulate proteolysis in the skeletal muscles via the activation of NF-κB. Activation of NF-κB can induce the transcription of the muscle specific ubiquitin ligases- muscle ring finger containing protein-1 [MURF1] and muscle atrophy F box protein [MAFbx]. Amino acids like glutamine and alanine can be exported to the tumor to be used as nutrients.
3.2. Myostatin

A TGF-β family member, myostatin has been implicated in inducing cachexia. Genetic null mutations for myostatin in humans and animals demonstrated muscle hypertrophy\(^ {67,68} \). Myostatin is mainly synthesized and secreted from the skeletal muscle cells and function via the activin type IIb (ActRIIB) receptor. Although the exact mechanism is still unclear, studies suggest that there might be multiple pathways that act by inhibiting AKT and thus the downstream TORC1 pathways which promote protein synthesis\(^ {69,70} \). Inhibition of ActRIIB prevented muscle wasting and prolonged survival in C-26 colon tumor-bearing mice\(^ {17} \). A myostatin inhibitor, BYM338 is currently in the phase 2 trial for attenuating body weight loss in stage III/IV pancreatic cancer patients (ClinicalTrials.gov. Clinical study of BYM338 for the treatment of unintentional weight loss in patients with cancer of the lung or the pancreas). A humanized anti-myostatin antibody, LY2495655 is the phase 2 trial in combination with gemcitabine to evaluate its efficacy in improving survival and lean body mass in patients having advanced or metastatic pancreatic cancer. (ClinicalTrials.gov. A Phase 2 study of LY2495655 in participants with pancreatic cancer).

3.3. Proteolysis Inducing Factor

Proteolysis inducing factor (PIF) is produced by murine and human tumors. Immunohistochemistry has demonstrated the presence of PIF in the cytoplasm of gastrointestinal cancers and a correlation was established between its expression, detection in the urine and weight loss\(^ {71} \). In another study, PIF was detected in 80% of pancreatic cancer patients who suffered greater significant weight loss and had
increased rate of weight loss than patients who didn’t have PIF in their urine\textsuperscript{72}. It has been reported that this factor leads to increase in protein degradation and a decrease in protein synthesis\textsuperscript{73}. Interestingly, when PIF was isolated from the urine of cancer patients including pancreatic and colon, and injected into mice, it led to induction of cachexia, without reduction in food or water intake\textsuperscript{74}. Unlike the cytokines mentioned previously, PIF can act on isolated skeletal muscle and can generate a state of cachexia in non-tumor bearing model without a decrease in food intake\textsuperscript{75}. In addition, administration of PIF to normal mice upregulated the ubiquitin proteosomal pathway, indicating this as one of the mechanisms of action of PIF\textsuperscript{76}. These results were also validated in cell culture conditions with murine myotubes. In the murine myotubes, addition of PIF inhibited the inhibitor of NF-κB, which is IκBα. This resulted in more NF-κB entering the nucleus and an increased expression of the regulators of the ubiquitin- proteosomal proteolytic pathway\textsuperscript{77}. Moreover, activation of NADPH oxidases and reactive oxygen species (ROS) also play a role in PIF-induced expression of ubiquitin-proteosomal pathway which leads to muscle degradation\textsuperscript{78,79}. Increased ROS again leads to the degradation of IκBα, which releases NF-κB from its inactive state\textsuperscript{78}.

Although there has been a lot of evidence of inflammatory pathways being involved in cachexia in preclinical models, the clinical trials with anti-inflammatory agents have not shown promising results.
3.4. Insulin-like growth factor (IGF-1)

In recent years, the role played by insulin-like growth factor-1 (IGF-1) has been evaluated in the context of cancer-cachexia. It has been established that IGF-1 stimulates muscle protein synthesis, differentiation and proliferation of satellite cells. It mainly acts on the PI3K pathway which activates the downstream targets which are required for protein synthesis. It was observed that rats bearing Yoshida AH-130 ascites hepatoma suffered from muscle atrophy which was associated with decreased IGF-expression in the skeletal muscle. As a suggested adaptive mechanism, skeletal muscles also demonstrated an increase in the levels of the IGF-1 receptor. Moreover, muscle wasting was rescued by the administration of insulin but not IGF-1, possibly due to its low bioavailability. In a separate study, mouse models of colon adenocarcinoma and hepatoma demonstrated that the cachectic tumor-bearing mice had decreased mRNA levels of IGF-1 in the skeletal muscles. However, there was no difference in the activation of the PI3K pathway suggesting that some other pathways might be responsible for IGF-1-induced muscle wasting.

3.5. Oxidative Stress

Reactive oxygen species are highly unstable and short-lived molecules that play a major role in signaling and physiological pathways. Increase in oxidative stress is another characteristic of cachexia/anorexia. In rats bearing Yoshida AH-130 hepatoma, there was an increased levels of total protein carbonylation in the cachectic skeletal muscles. This increase in oxidative modification was not compensated by an increase in the antioxidants, which might be a reason for
increased oxidative stress in cancer-induced cachexia\textsuperscript{84,85}. It has also been reported that mice implanted with Chinese hamster ovary cells transfected with TNF-α become cachectic and lead to an increase in oxidative stress and nitric oxide synthase in the skeletal muscles. This lead to a decrease in the myosin creatine kinase which is needed for muscle differentiation\textsuperscript{86}. Oxidative stress has also been reported to activate NF-κB which regulates the ubiquitin proteasome pathway\textsuperscript{87}. Moreover, it has been demonstrated that hydrogen peroxide induces ubiquitin conjugation to proteins through the transcriptional regulation of the E2 and E3 enzymes in mice C2C12 myotubes\textsuperscript{88}. In 2007, Russell \textit{et al} suggested that PIF and angiotensin can upregulate the ubiquitin proteasome pathway by ROS produced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (Nox) in murine myotubes\textsuperscript{79}. In the skeletal muscles of cachectic tumor-bearing mice (MAC-16), there was an increase in Nox4 which generates hydrogen peroxide and a decrease in antioxidants such as glutathione peroxidases, catalases and superoxide dismutases\textsuperscript{85}.

\textbf{3.6. Impaired regeneration of muscle fibers}

Muscle atrophy can not only be associated with increased muscle proteolysis, but also decreased muscle regeneration or differentiation. Expression of Pax7, a marker of muscle satellite cells was demonstrated to be increased in the skeletal muscles of C-26 bearing mice. Sustained expression of Pax7 results in muscle progenitors being in a state of self-renewal which results in impaired differentiation and inhibition of fusion. This is also indicated by decreased levels of MyoD, which is a downstream target of Pax7\textsuperscript{89}. Moreover, adenovirus overexpression of MyoD
in skeletal muscles of tumor-bearing mice rescued muscle wasting. Pax7 was also reported to be increased in the muscles of cachectic gastric cancer patients. MyoD is also demonstrated to be inhibited by the pro-inflammatory cytokines such as TNF-α via NF-κB signaling.

3.7. Appetite and Adipose Tissue Loss

A major aspect of cancer patients is the loss in appetite, which in turn may lead to anorexia. Studies have demonstrated that about 50% of patients confirm irregularities in their food habits at diagnosis. Anorexia is differentiated from cachexia, in the way that in anorexia there is a decreased interest to eat which may lead to decreased food intake. Cachexia is the loss of adipose and muscle mass which leads to hypoalbuminemia and asthenia that limits patient's physical activity and inhibits protein synthesis. Cachexia is dissimilar from starvation as there is preservation of lean body mass in starvation. Cancer patients suffer from both anorexia and cachexia, though the loss in body weight cannot be attributed to decreased food intake. This was further validated by studies demonstrating muscle wasting even in the presence of normal food intake. Moreover, it has also been observed that administration of food and nutrients are unable to reverse the muscle wasting.

Along with skeletal muscle wasting, cancer cachexia entails a significant loss of white adipose tissues. This loss has been attributed to an increase in lipolysis rather than a decrease in lipogenesis. Cachectic patients with gastrointestinal cancer have elevated levels of circulating free fatty acids, triacyl
glycerol and glycerol\textsuperscript{34}. Some studies have also implied the loss in WAT precedes the loss in muscle mass\textsuperscript{33}.

3.8. Adipokines

Leptin is one of the major hormones associated with appetite. It decreases food intake and increases energy expenditure. It was the first hormone to be discovered which is secreted from adipocytes. It can cross the blood-brain barrier\textsuperscript{96} and has its receptors at the hypothalamus. Its receptors are also present peripherally at the skeletal muscles, pancreatic islet cells, kidneys and some other tissues\textsuperscript{97}. Along with adipocytes, leptin has also been observed to be secreted by the gastric and epithelial tissues\textsuperscript{98}. There have been conflicting reports on the serum level of leptin in cachectic cancer patients. One study reported that serum leptin levels are observed to be decreased in patients with gastric cancer as compared to the normal healthy control but there was no significant difference in cachectic and non-cachectic patients\textsuperscript{99}. Another study demonstrated that cachectic gastric cancer patients had significantly higher level of serum leptin than the non-cachectic patients; and a negative correlation was found between leptin levels and BMI\textsuperscript{9}.

There is also a definite crosstalk between leptin signaling and inflammation. During fasting, leptin levels decrease and then again increase during the postprandial phase. When fasted hamsters were treated with TNF-\(\alpha\) and IL-1, they showed an increased levels of leptin mRNA in the adipose tissues\textsuperscript{100}. This increase in leptin was correlated with a decline in food intake. The cross talk between the leptin and the inflammation pathways have also been demonstrated in studies wherein peripheral leptin administration caused hypothalamic inflammation and
central injection of IL-1 receptor (IL-1r) antagonist inhibited the suppression of food intake caused by central or peripheral injection of leptin\textsuperscript{101,102}.

Another important hormone associated with cachexia is the adipokine called adiponectin. Adiponectin is specifically released by adipose tissues. There is an inverse correlation between adiponectin and obesity and levels of adiponectin increases with weight loss\textsuperscript{103}. Serum levels of adiponectin is increased in cachectic colon cancer patients\textsuperscript{104}. In contradiction, reports have also shown association of low levels of adiponectin to increased risk of gastric cancer\textsuperscript{105}. An explanation for this might be that TNF-\textalpha inhibits the secretion of adiponectin from adipocytes\textsuperscript{106,107}. It has also been demonstrated that adiponectin is increased in the cases of cardiac cachexia irrespective of the body mass index. Adiponectin regulates the activity of ceramidase which removes acyl chains from ceramides. Ceramides are related to inflammation and insulin resistance\textsuperscript{108}. In other studies, administration of adiponectin, increased the molecules which are involved in fatty acid transport and energy consumption such as acyl-coA oxidase and uncoupling protein-2\textsuperscript{92}. These molecular processes decreases the tissue triglyceride content in skeletal muscles.

3.9. Ghrelin

Ghrelin, a natural ligand for the growth hormone receptor secretagogue receptor (GHS-R) that is produced in the fundus of the stomach, is another important that plays a key role in cancer cachexia\textsuperscript{109}. However, ghrelin is shown to stimulate food intake and adiposity by a GH-independent mechanism\textsuperscript{110}. Counterintuitively, patients with cancer and cardiac cachexia have increased circulating concentrations of ghrelin, perhaps due to negative energy balance\textsuperscript{104,111}. Even in
such cases, ghrelin administration resulted in an increase in food intake\textsuperscript{112,113}. Studies have also demonstrated that Ghrelin decreases serum levels of IL-6. Considering the role of inflammation in cachexia, this might be another mechanism by which ghrelin can improve cachexia. However, it is not clear if all these hormones play critical roles in cachexia in tumors of different origins.

3.10. Neuronal Regulation

Cachexia is mostly also associated with decreased food intake and change in taste perception\textsuperscript{4}. These are characteristics of anorexia which worsens the manifestation of cachexia. It has been reported that both orexigenic (appetite-stimulating) and anorexigenic (appetite-suppressing) pathways are altered in cancer patients\textsuperscript{114}. Increase in cytokines can activate anorexigenic pathways and inhibit orexigenic pathways leading to the decrease of neuropeptide Y and thus decrease in appetite\textsuperscript{114}. The melanocortin system can also participate in the process of muscle wasting. Thus, in a mouse model of colon cancer, melanocortin receptor antagonist abrogated muscle depletion\textsuperscript{115}.

3.11. Lipid Mobilizing Factor (LMF)/Zinc alpha 2 glycoprotein (ZAG)

The search for a tumor-secreting product having lipid mobilizing activity led to the purification of a 43 kDa glycoprotein from the cachexia-inducing MAC16 tumor\textsuperscript{116}. Zinc α2 Glycoprotein (ZAG) acts as a lipid mobilizing factor and is overexpressed in certain malignant tumors\textsuperscript{117}. ZAG has been reported to be present in the urine of cachectic pancreatic cancer patients but not non-cachectic cancer patients\textsuperscript{116}. Moreover, a study also established the possibility of utilizing serum levels of
ZAG/LMF as a biomarker for pancreatic cancer cachexia. Expression of ZAG was also observed more in pancreatic cancer tumors of cachectic patients than non-cachectic patients upon immunohistochemical analysis\textsuperscript{118}. LMF/ZAG can directly activate adenylyl cyclase in a GTP dependent process; thereby inducing lipolysis\textsuperscript{119}. It has been shown that administration of ZAG to mice reduces body fat and induces hormone sensitive lipase (HSL)\textsuperscript{120}. In 2004, Trayhurn and colleagues demonstrated that ZAG is also expressed in white adipose tissues (WAT) and is observed to be overexpressed in adipocytes of MAC16-bearing cachectic mice\textsuperscript{121}. ZAG is also expressed in brown adipose tissue (BAT) in cachectic mice, which is required for lipid utilization\textsuperscript{121}. Thus, BAT increases energy utilization by generating heat instead of ATP in cachectic mice by upregulating BAT UCP1 mRNA, via ZAG.

3.12. Triglyceride Lipases

White adipose tissue (WAT) is the reservoir for triacylglycerides (TAG). The TAGs in the WATs are broken down by adipose triglyceride lipase (ATGL), in a rate limiting step to form diacyl glycerides (DAGs). The DAGs are broken down by hormone sensitive lipase (HSL) to form monoacylglycerols (MAGs), which is further broken down into free fatty acids and glycerol by monoglyceride lipases (MGL). Many factors regulate these lipases which lead to increase or decrease of lipolysis. In cancer cachexia, there is evidence of decrease in TAGs in WAT rather than complete cell death. The levels of HSL were reported to be two-fold higher in the WAT of cancer patients as compared to the control group\textsuperscript{122}. HSL is activated by its phosphorylation which is modulated by protein kinase A, extracellular signal-
regulated kinase (ERK), glycogen synthase kinase 3β (GSK-3β; previously known as GSK-4), calmodulin-dependent kinase II (CAMKII), as well as AMP-activated protein kinase (AMPK)\textsuperscript{123,124}. It was demonstrated that the ATGL activity is increased in murine cachexia models with increase in tumor burden and WAT mobilization\textsuperscript{32}. ATGL knockout mice showed protection from tumor induced lipolysis. Moreover, cachectic cancer patients showed significantly increased ATGL activity in their WAT tissues\textsuperscript{32}. In addition, ATGL is also observed to be expressed in skeletal muscles and reported to play an important role in lipid metabolism in muscles\textsuperscript{125}. HSL activity and expression in skeletal muscles is also reported to lead to insulin resistance\textsuperscript{126}.

3.13. Insulin Resistance

Glucose intolerance was the first metabolic abnormality that was recorded in cancer patients\textsuperscript{127}. The main hormone to control blood glucose levels is insulin, which plays a major role in whole body metabolism including fat, amino acid and protein metabolism. Consumption of a meal triggers the release of insulin from pancreatic beta cells which decreases circulating blood glucose levels. This happens due to the action of insulin on muscles and fat deposits which makes these tissues utilize glucose thereby decreasing its concentration in the blood.

In 1965, one of the earliest large scale studies to report metabolic effects of cancer, including cancer of the GI tract, in patients demonstrated a significant percentage of cancer patients suffering from glucose intolerance or diabetes\textsuperscript{128}. It has been reported that there is a high incidence of glucose intolerance in pancreatic cancer patients, which cannot be explained by impaired secretion of
insulin\textsuperscript{129}. Furthermore, it has also been demonstrated that insulin infusions into skeletal muscle increases amino acid uptake and reduces proteolysis\textsuperscript{130}. Higher doses of insulin infusions decrease the blood glucose levels but have no effect on protein mass\textsuperscript{130}. Therefore, there is evidence that insulin affects skeletal muscle mass and alterations in the levels of insulin can perturb proteolysis.

There has also been evidence regarding the development of glucose intolerance before the manifestation of cancer. Norton and his colleagues demonstrated that in 27 otherwise healthy patients with localized sarcoma, glucose intolerance occurred before other signs of cachexia appeared\textsuperscript{131}. Furthermore, in the said cohort, those patients who had less than ideal body weight showed lower glucose tolerance than the other patients. These studies indicate the role of glucose tolerance and insulin sensitivity in muscle wasting in cancer patients. Mice bearing colon-C26 adenocarcinoma demonstrated a blunted blood glucose response to insulin in an insulin tolerance test before a difference in body weight was observed. This suggested that insulin resistance is an early event in the development of cancer cachexia\textsuperscript{132}. One of the factors identified for impaired insulin signaling is TNF-\(\alpha\), which is observed to decrease the autophosphorylation of the insulin receptor(IR) and the IR substrate\textsuperscript{1}\textsuperscript{133}.

The insulin signaling pathway overlaps with the ubiquitin proteolytic pathway. Insulin binding to its receptor activates the PI3K and AKT. AKT suppresses FOXO3 and caspase-3 activation, thus decreasing the expression of the muscle ubiquitin ligases atrogin-1 and murf-1. However, in cancer cachexia, when patients suffer from insulin resistance, the suppression of PI3K and AKT is
decreased and there is an increased expression of the components of the ubiquitin proteolytic pathway. This is one mechanism through which insulin resistance can control muscle degradation\textsuperscript{134}. Insulin resistance might fuel tumor growth in two possible ways – first, by promoting muscle wasting and thus mobilizing amino acids into circulation; second by promoting liver gluconeogenesis\textsuperscript{135} which would increase REE, tissue wasting and would fuel cancer aerobic glycolysis.

3.14. Fatty Acid Oxidation

Cancer cell-secreted inflammatory factors can lead to increased fatty acid metabolism and p38 stress signature in the muscles\textsuperscript{136}. Moreover, pharmacological blockade of fatty acid oxidation improves muscle mass and body weight \textit{in vivo}\textsuperscript{136}.

The metabolic capacity of skeletal muscles is a substantial characteristic in the pathophysiology of cancer cachexia. Muscle fiber subtypes are traditionally delineated by the isoform of myosin heavy chain (MHC) expressed (eg. slow twitch- type I; fast twitch- type IIA, IIB, and IIX) that impacts the metabolic phenotypes and mitochondrial capacity\textsuperscript{137}. Myogenesis tends to alter the metabolic profile from a glycolytic phenotype toward a greater dependency on oxidative phosphorylation\textsuperscript{138}. MHC subtype I and IIA containing isoforms most heavily rely on oxidative metabolism, and MHC type IIB and IIX are highly glycolytic\textsuperscript{139}. The rate of protein synthesis is greater in the oxidative muscle fibers as compared to the glycolytic ones\textsuperscript{140}. Of note, multiple mice models of cancer cachexia display the loss of the oxidative phenotype\textsuperscript{141}. Extrapolating this evidence, there are also studies which demonstrate decreased protein synthesis in mouse models of
cancer cachexia. While it is not known if muscle wasting leads to changes in MHC subtypes, metabolic profiling studies indicate decreased overall glycolytic capacity.

### 3.15. Mitochondrial biogenesis and dynamics

Dynamic mitochondrial networks fulfill the energy demands of different cells and play a very important role in cellular physiology. Due to the high energy demands in muscles, mitochondria, the cellular energy factories, play a significant role in muscle physiology and pathologies. Dysregulation of mitochondrial biogenesis and dynamics regulates muscle mass and physiology by regulating different molecular and signaling events. Recently, Brown et al., have shown that mitochondrial degeneration precedes the muscle atrophy in murine models of cancer-associated cachexia. Several published studies and genome-wide dataset analysis from different animal models of cancer-associated cachexia have revealed altered expression of genes involved mitochondrial biogenesis and dynamics.

Skeletal muscles from cancer patients display significant alterations in the expression of genes involved in mitochondrial dynamics. A study involving 18 patients of gastric adenocarcinoma (nine cachetic and nine non-cachetic) along with 9 healthy controls, showed altered expression of mitochondrial dynamics genes. Cachetic cancer patient muscles displayed reduced expression of FIS1 fission protein, and lipidated and non-lipidated forms of LC3B. ApcMin/+ mouse model of colon cancer-associated cachexia also displays altered expression of proteins regulating mitochondrial dynamics, including PGC1-α and mitofusins.
(Mfn1, Mfn2), even before changes in mitochondrial mass are apparent at the early stages of muscle wasting\textsuperscript{149}. However, with the progression of the cachectic phenotype these mice exhibit the loss of mitochondrial content with decreased expression of PGC1-\(\alpha\) and mitofusins, and increased expression of Fis1\textsuperscript{149}. C-26 tumor-bearing mice, another model of colon cancer-associated cachexia, present reduced expression of PGC1-\(\alpha\), TFAM, and TOM20 in skeletal muscles, which correlates well with the reduced cross-sectional area of gastrocnemius muscles and increased expression of cachectic markers\textsuperscript{150}. C-26 tumor-bearing mice skeletal muscles also exhibit distorted mitochondria, impaired oxidative capacity, and reduced ATP regeneration that may significantly impact the contractile function and predispose the mice to muscle wasting\textsuperscript{151}. Altered mitochondrial biogenesis and dynamics leads to bioenergetic insufficiency and increased oxidative stress due to generation of reactive oxygen species (ROS). Reduced oxidative capacity, altered protein synthesis and membrane fluidity, and post-translationally-modified mitochondrial proteins altogether hampers the mitochondrial function in cachectic muscles\textsuperscript{4}. While a few preclinical cachexia models have evaluated alterations in mitochondrial homeostasis, the broad scope of mitochondrial biogenesis and dynamics in gastrointestinal cancer-associated cachexia in patients is still underexplored. More clinical and preclinical studies under relevant physiological conditions can significantly improve our understanding of the dynamic mitochondrial networks in cancer-associated cachexia.
3.16. Zinc Metabolism

Cachectic muscles in patients and animal models display significant accumulation of zinc\textsuperscript{152}. Animal models with fibrosarcoma, lewis lung carcinoma, and murine-adenocarcinoma demonstrate accumulation of zinc in the cachectic skeletal muscles\textsuperscript{153}. Siren \textit{et al.} hypothesized that though zinc homeostasis is normally tightly regulated, during inflammation, infection, or cancer zinc dyshomeostasis can occur via an acute phase protein response (APPR)\textsuperscript{152}. Regarding the potential mechanisms of zinc accumulation in cachectic muscles, zinc transporters are induced in cachectic muscles. The cachectic cytokine IL-6 regulates the zinc transporter (Zip14) in the liver which contributes to the hypozincemia during APPR\textsuperscript{154}. Furthermore, due to the critical role of zinc in immune regulation, altered zinc metabolism can also contribute to systemic inflammation as observed in cachexia\textsuperscript{155}. Recently, Wang \textit{et al.} also demonstrated an upregulation of ZIP14 in the cachectic muscles of metastatic cancer models\textsuperscript{156}. Transforming growth factor beta (TGF-β) and TNF-α mediated the induction of ZIP14 expression in the muscles of these models. ZIP14-mediated Zn accumulation also blocked muscle differentiation, along with decreased levels of myosin heavy chain\textsuperscript{156}.

4. Immunological aspects of GI cancer-induced cachexia

The immunological status of the host body is a major determinant in progression of cancer and cachexia. Studies have demonstrated a strong decline in contact hypersensitivity, a parameter for cell-mediated immunity, in C26 tumor-bearing
cachectic mice\textsuperscript{157}. Interestingly, this decline was observed even before the mice underwent weight-loss, therefore implicating the importance of immunity in the pre-cachectic state. Cachectic pancreatic cancer patients also had an increased infiltration of activated macrophages in the liver as indicated by CD68 immunoreactivity\textsuperscript{158}. The amount of IL-6 staining in hepatocytes surrounding the CD68-positive macrophages was significantly increased in cachectic pancreatic cancer patients as compared to non-cachectic patients, indicating that liver parenchymal cells may be triggered by macrophages to produce pro-inflammatory cytokines like IL-6\textsuperscript{158}.

5. Chemotherapy-induced cachexia in gastrointestinal cancers

Anti-tumor cytotoxic agents have several deleterious impacts on normal tissues and physiology\textsuperscript{159} (Table 1). Chemotherapeutic agents have been shown to exhibit pro-inflammatory properties\textsuperscript{160,161} and deregulate nitrogen metabolism of the host body\textsuperscript{162}. Chemotherapeutic agents such as cisplatin, FOLFOX (5-FU, leucovorin, oxaliplatin), FOLFIRINOX (5-FU, leucovorin, irinotecan), doxorubicin and sorafenib contribute to myopathy independent of their anti-tumor effect in preclinical models of cancer-induced cachexia\textsuperscript{163,164,165,166,167}. Recently it has also been shown that muscle wasting is associated with long term use of mTOR inhibitors\textsuperscript{168}. In a longitudinal study of colorectal cancer patients with metastasis, it was observed that muscle area decreased significantly during combination of capecitabine plus oxaliplatin with or without bevacizumab treatment and it was independently associated with survival in patients\textsuperscript{169}. Similarly, Eriksson et al., have reported that muscle mass decreases during neoadjuvant chemotherapy in
patients with resectable colorectal liver metastases. Gemcitabine, the first line standard of care for pancreatic cancer has been shown to induce muscle atrophy in a mouse model of pancreatic cancer.

Molecular mechanism of chemotherapy-induced muscle wasting varies widely. Chemotherapy-induced alteration of ROS homeostasis plays important role in muscle weakness and fatigue. FOLFOX has been shown to induce muscle atrophy by activating ERK1/2 and p38MAPKs signaling pathways in addition to modulating mitochondrial biogenesis. Widely used anti-cancer agent doxorubicin induces muscle atrophy by altering calcium homeostasis of muscle and inducing autophagy. Doxorubicin also modulates TNF1R activity in skeletal muscles. Furthermore, it has been shown that doxorubicin treatment results in hyperglycemia and insulin resistance which is mainly mediated by negative regulation of AMPK signaling pathway in mice muscles. Baretto et al. have demonstrated that chemotherapy-induced muscle wasting and cancer-induced muscle atrophy share common alterations in signaling pathways. Modulation of glucocorticoid receptor mediated signaling has been reported to play an important role in 5-FU mediated muscle atrophy in murine skeletal muscles. Only a few pre-clinical studies have explored the therapeutic options to manage chemotherapy-induced cachexia. It has been shown that ACVR2B/Fc prevents FOLFRI mediated muscle loss. Mirtazapine, an antidepressant drug has shown to prevent gemcitabine-induced mild cachexia in mouse model of pancreatic cancer.
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6. Regulation of muscle atrophy by microRNAs

MicroRNAs (miRNAs) are short noncoding RNAs which play an important role in post-transcriptional gene regulation, thus affecting several key biological processes including muscle atrophy\textsuperscript{179}. Recently Narsimnhan \textit{et al.} have shown that several miRNAs are differentially expressed in skeletal muscles of cachectic versus non-cachectic pancreatic and colorectal cancer patients\textsuperscript{180}. They observed that miR-3184-3p, miR-1296-5p and let-7d were significantly upregulated in the muscles of cachectic patients\textsuperscript{180}. Moreover, it has been shown that miR-378 regulates lipolysis in gastrointestinal cancer patients\textsuperscript{181}. Pancreatic tumor-derived microvesicles containing miR-21 induces myotube atrophy by inducing apoptosis through TLR activation\textsuperscript{182}. miR-21 has been also shown to be upregulated in denervation induced muscle atrophy and it regulates translational initiation process\textsuperscript{183}. Overall, these evidences suggest that several miRNAs regulate muscle atrophy and lipolysis in gastrointestinal cancer-induced cachexia.

7. Serum Metabolic Profile

Recent studies have demonstrated distinct metabolic profiles in cachectic cancer patients compared to non-cachectic cancer patients\textsuperscript{184}. Cancer patients with cachexia presented decreased glucose and elevated lactate in the serum, compared to the non-cachectic ones. Metabolomic profiling identified 45 distinguishing metabolites and 18 metabolic pathways that were associated with cachexia. Out of the 45 metabolites, logistic regression analysis revealed diagnostic potential of a three-metabolite signature, consisting of carnosine, phenylacetate, and leucine. Similar metabolic alterations have been observed in
mice sera\textsuperscript{185}. In addition to the metabolic changes observed in the serum specimens, gastrocnemius muscle specimens from murine cancer cachexia models also reflect metabolic changes associated with wasting\textsuperscript{185}. Of note, that the common metabolic changes between human and the murine model included leucine, phenylacetate, and carnosine.

Another study utilizing a cohort of 390 cancer patients demonstrated that 49\% of the patients had hypermetabolism which was associated with clinical and biological features of precachexia\textsuperscript{186}. Hypermetabolism also correlated with decreased survival in metastatic cancer patients\textsuperscript{187}.

8. Metabolic Alterations in the Tumor

Altered metabolism is a known hallmark of cancer cells (Figure 4). Under normal conditions, metabolic pathways are highly regulated, but once the cells undergo malignant transformation due to the induction of oncogenes or suppression of tumor suppressors, tumor cells reprogram the metabolic pathways to need their growing cellular requirements\textsuperscript{188}. The metabolic pathways that majorly get reprogrammed is glycolysis, glutaminolysis and fatty acid synthesis. Recent studies by our laboratory have demonstrated that pancreatic cancer patients on chemotherapy who had a higher PET signal had poor prognosis, suggesting that patients having higher glucose uptake, had poor survival\textsuperscript{189}. 

**Figure 4 : Metabolic alterations in tumor and serum.** Metabolic alterations in tumor, distinct uptake and release of metabolites from the tumor and its uptake in the muscles regulate muscle wasting.
Extrapolating this evidence, it can be hypothesized that different tumors can have different metabolic alterations which can affect patient prognosis and survival. Several reports have demonstrated that specific metabolite uptakes are required for tumor progression, for example glutamine\textsuperscript{190}, serine\textsuperscript{191}, branched amino acids and lipids\textsuperscript{192} in cancers including gastrointestinal cancers. Interestingly, in 2014 Mayers \textit{et al} profiled the prediagnostic plasma metabolite levels of patients with pancreatic cancer and observed an increased level of branched chain amino acids as compared to the plasma of control cohorts\textsuperscript{193}. This suggests that whole-body protein break down occurs even before the cancer is diagnosed in patients. Furthermore, Luo \textit{et al} identified metabolic cross talk occurring between colorectal cancer and skeletal muscles. It was demonstrated that colorectal tumors release high-motility group box 1 (HMGB1) which leads to the metabolic reprogramming of skeletal muscle through RAGE (Receptor for Advanced Glycation End-products) by inducing autophagy and release of free amino acids in the plasma (Figure 5).\textsuperscript{13}C tracing experiments confirmed the transfer of carbon skeleton from the muscles to the tumor.

Cachexia has also been shown to be associated with hypoalbuminemia\textsuperscript{194}. But this condition was shown to be independent of functional capacity of the liver and, in turn, was said to be related to increased protein breakdown or increased utilization of protein by the tumor. It was also demonstrated that insulin releasing compounds, like glucose, failed to release insulin in cachectic rats\textsuperscript{195}. This study, along with their own, led Jasani and colleagues to conclude that in order to combat cachexia it is not only essential to improve dietary protein intake but also reduce
the metabolic activity of the tumor tissue\(^{194}\). Since only protein replenishment has served to be effective in non-malignant cachexia, combating the metabolic activities of the tumor tissue is crucial.

9. Advances Made in Altering Metabolic Pathways

Cachexia, till date has no FDA approved therapies. In the clinic, cachexia is managed by nutritional supplements or hormones to increase appetite. In research labs, attempts are being made to evaluate compounds which can target the tumor and combat muscle wasting (Figure 6).

a. Ketone Bodies and Fenofibrate

It has been demonstrated by Shukla et al in 2014 that a ketogenic diet leads to diminished glucose flux in the pancreatic cancer cell lines\(^{196}\). This also reduced glutamine uptake, ATP content and reduced survival in pancreatic cancer cell lines while inducing apoptosis. The metabolic reprogramming of pancreatic cancer cell lines reduced cachexia in the cell line model. This was further confirmed in mouse xenograft models. Hence, following a ketogenic diet might be a successful strategy to combat cachexia.

A unique metabolic gene signature in a murine model of cancer cachexia, which involved losing the peroxisome proliferator-activated receptor-\(\alpha\) (PPAR\(\alpha\))-dependent ketone production in the liver, was also reported\(^{197}\). There was a rise of glucocorticoid levels which correlated with degeneration of muscle fibers and increased hepatic gluconeogenesis. This loss in skeletal mass could be prevented by restoring the ketone production by a PPAR\(\alpha\) agonist, fenofibrate\(^{197}\).
Figure 5: Tumor cells secrete factors which degrade skeletal muscles. HMGB1 is released from the tumor cells and is shown to inhibit the conversion of phosphoenolpyruvate (PEP) to pyruvate. This can inhibit the TCA cycle in muscles. HMGB1 can also induce autophagy in muscles, which can increase proteolysis leading to the generation of glutamine. Glutamine can be taken up by tumors to generate glucose.
b. Silibinin

Silibinin is a natural product which has been demonstrated to have anti-cancer properties. Our lab demonstrated that silibinin successfully reduced the growth of pancreatic cancer cells by reducing glycolytic flux\textsuperscript{198}. Moreover, it was also demonstrated that silibinin reduces tumor growth and proliferation in an orthotopic mouse model of pancreatic cancer and prevents loss of body weight and muscle mass.

c. Etomoxir

In recent studies, Fukawa and colleagues reported that factors released by cachectic cancer cell lines induce fatty acid oxidation in human myotubes, which lead to oxidative stress, p38 activation and impaired muscle growth\textsuperscript{136}. Moreover, pharmacological blockade of fatty acid oxidation by administration of etoxomir not only prevented human myotube degradation but also improved muscle mass in murine models of cancer cachexia\textsuperscript{136}.

d. Antioxidants

Since oxidative stress-induced modifications are an important part of muscle wasting, antioxidants are being tested for their efficacy in combating cancer cachexia. It was reported that D-alpha-tocopherol, an antioxidant was successful in combating muscle wasting in a murine model of cancer cachexia by decreasing the oxidative stress\textsuperscript{86}. 
Figure 6: Targeting tumor metabolism in order to combat cachexia. Tumor metabolism can be altered by ketone bodies which inhibits glycolysis, by etoxomir which blocks fatty acid oxidation or by silibinin which inhibits c-myc thereby inhibiting transcription of glycolysis genes. Antioxidants can directly affect the muscles by reducing reactive oxygen species related damage, which can then inhibit the transcription of ubiquitin ligases.
10. Potential for new therapeutic approaches

Although a lot is known about the mechanisms of cachexia development, most of the therapies which includes anti-inflammatory drugs, nutrition support, hormonal supplements have not given promising results in clinical trials. As mentioned in this chapter, because cachexia is a product of inflammatory and metabolic alterations, a combination of drugs targeting both these pathways looks most promising. Since chemotherapy can also aggravate muscle wasting, compounds which combat muscle wasting can be administered along with chemotherapeutic drugs to ameliorate the effect of chemotherapy on the muscles. This is not a trivial pursuit since it is important to not decrease the efficacy of the chemotherapeutic drug by its combination with chemoprotectant drugs.

11. Conclusion and Perspectives

It has been three decades since the discovery of “cachectin”, and yet there has been no promising therapeutic approach to combat cachexia. As previously mentioned, cachexia is more than just a wasting disease; it reduces the patient’s ability to receive or respond to therapy and decreases survival. It is a multifactorial syndrome that is associated with anorexia and hyper catabolism due to pro-inflammatory cytokines, hormones, neuronal peptides, tumor-derived factors and altered tumor metabolism. A lot of research is still required to target these factors together in a multimodal therapeutic approach. This is challenging since each tumor varies genetically and metabolically and a common approach might not be
effective for all patients. This suggests that there is a need for the development of personalized medicine to combat cancer and also mitigate cachexia.

12. Goals of the Dissertation

The aim of this dissertation is to elucidate novel mechanisms involved in the manifestation of pancreatic cancer-cachexia. To this end, we utilized spontaneous pancreatic cancer mouse models, pancreatic cancer patient muscle samples and in-vitro models of muscle atrophy. Chapters 3 and 4 illustrate the role of Sirt1-Nox4 axis in inducing cachexia and Chapter 5 investigates the role of chemotherapy in muscle wasting in pancreatic cancer.
Chapter 2: Methods and Materials

All the chapters of this dissertation are majorly focused on methods involving muscle wasting. Since most of the projects utilize similar methods, they are described in this section:

Cell Culture and Reagents

Pancreatic cancer cell lines T3M4 and S2-013 were generous gifts from Dr. Michael A. Hollingsworth. Cell lines were validated by STR profiling by the Genetics Core at Arizona University. Mouse pancreatic cancer cell lines (KPC 1245, 1199 and 242) were derived from the transgenic mice models described below. Colon cancer cell line C26 was obtained from ATCC. All cell lines were cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Sigma-Aldrich D5648) with 10% fetal bovine serum (FBS), 100 I.U./ml penicillin, 100 μg/ml streptomycin, and incubated at 37°C in a humidified incubator with 5% CO2. C2C12 myoblasts were obtained from ATCC and cultured in DMEM with 10% FBS until confluent. After reaching confluency, the myoblasts were differentiated in DMEM with 2% horse serum and 1 μg/ml insulin for 72 hours, as previously described. Resveratrol for in-vitro studies was purchased from Sigma-Aldrich (R5010) and stocks were diluted in DMSO. Ex-527 was purchased from Santa Cruz Biotechnology (CAS 49843-98-3). NBD peptide / NF-κB blocker (ALX-163-011-1) was purchased from Enzo Life Sciences. FoxO1 inhibitor, AS-1842856 (16761) and Nox4 inhibitor, GKT 137831 (1218942218942-37-0) was purchased from Cayman Chemical. BMX-001 was a kind gift from Dr. Rebecca Oberley-Deegan.
Leucovorin, Oxaliplatin, 5-fluoroplatin, Irinotecan and Gemcitabine were purchased from Nebraska Medicine Pharmacy.

**Lentivirus and Adenovirus Transfection**

Cell transfections for producing replication-incompetent lentivirus were performed by utilizing Turbofect (Thermo Scientific), following the manufacturer's protocol. Stable short hairpin RNA (shRNA) constructs for *Sirt1* were obtained from Sigma-Aldrich. After transfection, cells were selected using puromycin. Human *NOX4* adenovirus was a kind gift from Dr. Melissa Teoh Fitzgerald (UNMC). GFP adenovirus was a kind gift from Dr. Matthew Zimmerman (UNMC).

Mouse *Sirt1* adenovirus was purchased from ABM (222151A). C2C12 myotubes were differentiated for 48 hours following which, a MOI (multiplicity of infection) of 200 was used to transfect the myotubes.

**Cancer Cell-conditioned Media Preparation**

For conditioned medium (CM) preparation, S2-013 and T3M4 cell lines were seeded as previously described\(^\text{198}\). After attachment, cells were washed twice with 1X phosphate buffered saline (PBS) and cultured in serum free DMEM for 24 hours. The media was then collected and centrifuged at 3000 RPM for 10 minutes and the supernatant was collected in a fresh tube to be either used immediately or stored in -80°C for future use.
Cell Viability Assays

Cell viability was determined by MTT assays as described previously\textsuperscript{198}. All the MTT assays in this study were carried out at 72 hours post-treatment.

RNA Isolation and qRT-PCR

Total RNA was extracted from cells or tissue lysates by using TRIzol reagent (Invitrogen, Carlsbad, USA) as previously discussed\textsuperscript{201}. Complementary DNA (cDNA) was synthesized using Verso-cDNA synthesis kit (Thermo fisher scientific, MA, USA) or High-Capacity Reverse Transcription Kit (Applied Biosystems) according to manufacturer’s protocol. qRT-PCR was performed using SYBR Green master mix (Applied Biosystems, NY, USA). Beta actin was used an internal control. Total RNA for RNA-seq analysis was isolated using RNAeasy columns (Qiagen) as per manufacturer’s protocol. Relative gene expression analysis were performed by utilizing ∆Ct method as described previously\textsuperscript{189}.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was performed as described previously\textsuperscript{189}. Briefly, chromatin immunoprecipitation was performed by utilizing NFκB p65 along with IgG control. 3 µl purified chromatin for each reaction were used for qPCR analysis. The threshold cycle (Ct), values obtained for each genomic region were utilized for further analysis. For ChIP qPCR analysis, Ct values were normalized to an input control and represented as a fold increase over enrichment detected using IgG.
Immunohistochemistry

Immunohistochemistry was performed as described previously. We utilized Novolink Polymer (Leica, Wetzlar, Germany), as per the manufacturer’s instructions. Muscle sections prepared from human patient tissues were stained with SIRT1 antibody (Cell Signaling Technology, Danvers, MA) and NOX4 antibody (Abcam, Cambridge, UK). Each muscle fiber in a cross-sectional field of view was given an intensity score by evaluating staining intensity of positive staining (0 = none, 1 = weak, 2 = intermediate, 3 = strong). The histoscore was calculated by multiplying the percentage of fibers (0-100) with the particular score by its corresponding intensity score (0-3). All the scores in a given section were added, and the value was then divided by 100 to attain a score between 1 and 3.

Immunoblotting

Protein isolation and western blotting were performed as described previously [49]. Briefly, cells were washed twice with PBS and lysed in radio-immunoprecipitation assay (RIPA) lysis buffer by incubating at 4°C shaker for 10 min. Then, the lysates were centrifuged at 13000 rpm for 5 min and supernatant was collected. Protein concentration was measured by Bradford assay. Equal amounts of protein were used for western blotting. Primary antibodies against MuRF1, ATROGIN-1, p65 (NF-κB) (Santa Cruz Biotechnology, Dallas, Texas, USA), Sirt1, FoxO3a, FoxO1, Acetyl-p65 (K310) (Cell Signaling Technology, Danvers, MA, USA), Tubulin and Myosin heavy chain 2 (Developmental Studies Hybridoma Bank, Iowa City, IA), Nox4 (Abcam) were utilized for probing specific proteins.
**SIRT1 Activity Assay**

SIRT1 activity was measured in myotubes by using Sirt1 activity assay kit following manufacturer’s protocol (Sigma Aldrich CS1040). C2C12 myotubes were treated with either control media, S2-013- orT3M4- conditioned media for 24 hours and Sirt1 activity was measured.

**NF-κB Reporter Assay**

NF-κB luciferase adenovirus was purchased from Vector Biolabs (1740). C2C12 cells were seeded and differentiated in 6-wells plates as previously mentioned. C2C12 myotubes were then treated with cancer-cell CM for 6 hours with and without resveratrol. After 6 hours, cells were harvested and assayed for luciferase activity using Promega Luciferase Reporter Assay according to manufacturer’s instructions (#E1690).

**Animal Studies**

**Orthotopic Studies**: All the animal experiments performed in this study were approved by University of Nebraska Medical Center Institutional Animal Care and Use Committee (IACUC). Athymic female nude mice (NCr-nu/nu) were bred in-house and 6–8-week-old mice were used for orthotopic implantations. $0.5 \times 10^6$ S2-013 scrambled control, S2-013 shSIRT1-A, or S2-013 shSIRT1-B cells were injected into the pancreas of female athymic nude mice and 7 days post-implantation mice were segregated into groups of 10 animals each. Age and gender-matched mice, without any tumor cell injection, were utilized as healthy controls. Trans-resveratrol (Cayman Chemical, Ann Arbor, MI, Catalog #70675)
was solubilized in 1.5% methylcellulose with vigorous vortexing\textsuperscript{202}. Beginning on
day 7, mice received a daily oral treatment of 200mg/kg delivered using a 20G
gavage needle. Tumor volumes and body weights were recorded regularly. After
14 days of treatment, all the mice were euthanized and tumor weight, tumor
volume, gastrocnemius muscle weight, and body weight were measured. Tumor
tissue, liver, spleen, and muscles were flash frozen in liquid nitrogen and formalin
fixed for further analysis.

For GKT studies, 0.25 X 10\textsuperscript{6} S2-013 cells were implanted in the pancreas of
athymic nude mice. The mice were randomly divided into two groups. GKT137831
was solubilized in 1.2% of methylcellulose and 0.1% of polysorbate 80 and was
administered to 10 tumor-bearing mice at a concentration of 60 mg/kg via daily
oral gavage. The rest were administered the vehicle solution. To test for toxicity,
we also injected 4 healthy mice with GKT137831.

**KPC tissues:** The muscles of 10, 15 and 25 weeks of \textit{Kras}\textsubscript{LSL,G12D/+}; \textit{p53}\textsubscript{R172H/+};
\textit{Pdx1-Cre}\textsuperscript{tg/+} (KPC) mouse spontaneous progression model of pancreatic cancer
were collected by euthanizing these mice along with littermate control.

**Transgenic Mice Model:** C57BL/6 Mice having Tamoxifen-inducible muscle
specific CRE was obtained from Jackson Laboratories (Tg(ACTA1-
cre/Esr1*)2Kesr/J ; Stock No. 025750 ). Mice having Nox4 floxed were a kind gift
from Dr. Junichi Sadoshima (Rutgers). These mice were crossbred and genotyped
to get the desired mice genotype (Nox4\textsuperscript{fl/fl}: ACTA1-cre). Nox4 knockout was
induced in the muscle by administration of Tamoxifen for 5 consecutive days at a
concentration of 75 mg/kg as directed by Jackson laboratories.
0.25 X 10^5 KPC 1245 cells were implanted in the pancreas of male age-matched Nox4^{fl/fl}: ACTA1-cre mice. Post-implantation, mice were segregated into two groups of 8 mice each and one group was injected with tamoxifen to induce Nox4 deletion in the muscles.

**Chemotherapy studies on Mice:** Male C57/BL6 mice were treated with FOLFIRINOX (100 mg/kg leucovorin, 5 mg/kg oxaliplatin, 50 mg/kg 5-fluorouracil, and 50 mg/kg irinotecan) once a week via tail vein injection. Mice were treated with gemcitabine at a concentration of 50 mg/kg twice a week via intraperitoneal injection (IP). BMX-001 was injected via IP at a concentration of 0.5 mg/kg.

**Mouse Body Fat Measurement**

Fat percent in each mouse was measured by Dual-energy X-ray absorptiometry (DEXA) scanning on the 18th day after implantation as previously mentioned. Mice were anesthetized using a mixture of isoflurane and oxygen and placed on imaging positioning tray. Mice were scanned using a Lunar PIXImus™ densitometer (GE Medical-Lunar, Madison, WI).

**Measurement of Grip Strength**

A grip strength meter (Columbus Instruments, OH, USA) was utilized to assess forelimb grip strength as previously mentioned. On the 18th day of treatment, we acclimatized mice to the procedure room for 15 minutes and measured grip strength as per manufacturer's instructions. We utilized the average value of three measurements for analysis.
**Patient Samples**

Skeletal muscle specimens were collected from pancreatic cancer patients under UNMC Rapid Autopsy Program (RAP).

**Detection of Reactive Oxygen Species in Muscles**

Electron paramagnetic resonance spectroscopy was utilized to detect oxygen radicals in the muscle tissues as previously described\(^{204}\). Briefly, gastrocnemius muscle tissues were harvested and incubated for 1 h at 37°C with the cell-permeable O2\(^{-}\)-sensitive spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH, 200 μmol/l, Noxygen Science Transfer and Diagnostics, Elzach, Germany) in a Krebs-HEPES buffer (pH 7.4) containing (in mmol/l) 99 NaCl, 4.69 KCl, 2.5 CaCl\(_2\), 1.2 MgSO\(_4\), 25 NaHCO\(_3\), 1.03 KH\(_2\)PO\(_4\), 5.6 d-glucose, 20 HEPES, and supplemented with the metal chelators DETC (5 μM) and deferoxamine (25 μM). The EPR-CMH spectra was normalized to the gastrocnemius muscle weight.

**ROS Assay**

Reactive oxygen species in cells were determined as described previously\(^{205}\). ROS levels were determined by using oxidation-sensitive fluorescent dye 2',7'-dichlorofluorescin diacetate (DCFDA). C2C12 cells were seeded at 3.0 × 10\(^4\) cells per well in a clear bottom black 96-well plate. The C2C12 myotubes were then differentiated and treated with the conditioned media for 8 hours. The media was replaced with fresh DMEM containing 20 μM DCFDA. H\(_2\)O\(_2\) along with DCFDA was used as a positive control and 2',7'-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein
was used as a negative control. Control, and treated cells were incubated at 37°C for 30 min. The cells were washed with PBS and 100 µL of PBS was added to the wells for measuring the emission of DCFDA using a Biotek Cytation 3 plate reader (BioTek Instruments Inc, Winooski, Vermont). DCFDA fluorescence was measured at 485 nm excitation wavelength and 529 nm emission wavelength.

**Glucose Uptake Assay**

Glucose uptake assay was performed as previously described. Briefly, S2-013 and T3M4 cells were seeded at a density of 5 x 10^4 cells/well in 12-well plates. After overnight attachment, the cells were treated with resveratrol (25 µM) or Ex-527 (0.5 µM) for 24 hours. The cells were then starved for 2 hours in DMEM without glucose, glutamine, pyruvate, and fetal bovine serum. [³H]2-deoxyglucose was then added to the wells, incubated for 20 minutes, washed once with 1X PBS and then lysed with 1% SDS (sodium dodecyl sulfate). The lysates were then counted for tritium incorporation by a scintillation counter, which was then normalized according to cell counts. For C2C12 myotubes, glucose uptake was performed after the differentiated myotubes were treated for 24 hours and normalization was done using protein content.

**RNA-seq Analysis**

RNA sequencing was performed on myotubes treated with S2-013 CM with and without resveratrol. All gene sets in GMT format with Entrez IDs for Mus musculus were downloaded from http://ge-lab.org/gskb/. The gene set for TFactS (http://www.tfacts.org/) was chosen from the list and was made into a separate file,
converted into gene symbols through ensembl biomart (https://www.ensembl.org/biomart), and predicted genes and pseudogene were removed (n=10) before being used by GSEA2 v2.2.3 with 1000 permutations in the classic scoring scheme. Heatmap of the normalized enrichment score was produced in R v3.3.2 with the gplots package after filtering for the top 25% percent most variable gene sets.

**Patient Data**

Our retrospective cohort study included all pancreatic cancer patients between 2009 and 2017 at University of Nebraska Medical Center, Omaha, Nebraska, USA who were recipients of chemotherapy and underwent an abdominal CT scan at the time of diagnosis and an interval scan at 8 weeks following chemotherapy. Prior to exclusion, we had 1280 patients with a diagnosis of pancreatic cancer at our institution. Exclusions included those who were treated at other institutions, those who did not have a follow-up CT scan and those who did not receive chemotherapy. After exclusion, we were left with a total of 162 patients.

The study was approved by the UNMC institutional review board. The UNMC electronic data source, including patients electronic medical record (EMR), and the cancer registry provided information on height, weight, disease stage, tumor characteristics, demographics and cancer treatment including surgery type (no surgery, pancreaticoduodenectomy or distal pancreatectomy) and the receipt of chemotherapy and/or radiation. Height and weight were measured at a clinical visit close to the CT scan performed at the time of diagnosis and at 8 weeks follow up.
Body composition/CT image analysis of muscle mass

Abdominal CT scans at cancer diagnosis (prior to therapy initiation) and at 2-month follow-up after treatment was retrieved from the picture archiving and communication system (PACS) in our institution. Slice-O-Matic software version 5.0 (Tomovision) was used to calculate muscle mass by selecting a single CT slice at the level of the third lumbar vertebrae (L3). Muscle mass was identified using the Hounsfield unit threshold of −29 to +150 and measured as cross-section surface area (cm²).
Chapter 3: Role of Sirtuin 1 in Pancreatic Cancer Cachexia

(Note – The data shown in this chapter has been submitted to a peer-reviewed journal as of March 2019)

Introduction

Pancreatic cancer is the third leading cause of cancer-related deaths in the United States. It is predicted that by the year 2030, it will be the second leading cause of cancer-related deaths. The dismal five-year survival rate of pancreatic cancer patients can be attributed to a variety of factors, including late diagnosis, early metastasis, resistance to chemotherapy, and cachexia. Cachexia is a metabolic syndrome that is observed in up to 80% of pancreatic cancer patients. One-third of pancreatic cancer patients die due to cachexia-associated complications, entailing respiratory or cardiac failure. In pancreatic cancer patients, cachexia is associated with poor response to chemotherapy and decreased overall survival.

Cachexia is defined as the loss in skeletal muscle mass that cannot be fully reversed by conventional nutritional therapy and leads to progressive functional impairment. Until now, the manifestation of cancer cachexia has been attributed to systemic inflammation caused by the host body and tumor-derived cytokines. Multiple clinical trials for combating cachexia with anti-inflammatory agents have demonstrated unsatisfactory results. Currently there are no FDA approved drugs to mitigate cancer-induced cachexia. Thus, there is an urgent need to find more efficient therapeutic targets.
Sirtuins are primarily NAD\(^+\)-dependent protein deacetylases and mono-[ADP-ribosyl] transferases\(^{213}\). They can sense energy fluctuations in the cells as their enzymatic activity is dependent on using NAD\(^+\) as a cofactor. There are seven mammalian sirtuins and they are also referred to as class III histone deacetylases\(^{214}\). The cellular localization of the sirtuins varies. Some sirtuins are present in the cytoplasm or the nucleus and the others in the mitochondria. Mammalian sirtuins are involved in various functions such as chromatin regulation, metabolic homeostasis, and cell survival under stress\(^{214}\).

It is now well-established that increased expression of muscle specific ubiquitin ligases (MuRF1 and MAFbx/Atrogin) are a hallmark of muscle atrophy\(^{215,216}\). These genes are activated by the FoxO family of transcription factors\(^{217,218}\). Activities of FoxO1 and FoxO3 are regulated by cycles of acetylation and deacetylation; the latter being mediated by a sirtuin family member SIRT1\(^{219}\).

The role of SIRT1 in cancer is very ambiguous. It could act either as a tumor suppressor or a tumor promoter, depending on its cellular context, and may have different signaling targets in different cancer types\(^{220,221}\). In pancreatic cancer, SIRT1-activating compounds (STACs) decrease tumor growth in vivo and in vitro through a lysosome dependent pathway\(^{222}\). Sirtuins have also been illustrated to play a role in muscle health\(^{223-225}\). To the best of our knowledge, the role of sirtuins in pancreatic cancer cachexia has not yet been evaluated.

In this chapter, we elucidated the potential utility of targeting sirtuins in combating pancreatic cancer cachexia. Moreover, we investigated whether inhibition or activation of sirtuins in the muscle, irrespective of its effect in the tumor,
would be sufficient to combat muscle wasting. Our findings demonstrate that SIRT1 stabilization in the skeletal muscles can mitigate pancreatic cancer-induced muscle wasting.
Results

Expression of sirtuins in cachectic muscles

We evaluated the sirtuins that are altered in the pancreatic cancer-induced cachectic muscle by determining the mRNA expression levels of all the seven sirtuins in the gastrocnemius muscles from 10, 15, and 25-weeks-old KPC spontaneous PDAC mice model and comparing them to the littermate controls. Sirt1 expression was significantly decreased in gastrocnemius muscles from KPC mice at 15 and 25 weeks of age, compared to that from the littermate controls (Figure 7). However, we observed no significant alterations in the other sirtuins, except for Sirt2, which was decreased in the KPC mice muscles only at 25-weeks of age (Figure 8). Therefore, we continued our investigation on the role of Sirt1.
Figure 7: Sirt1 expression in KPC muscles. Sirt1 mRNA expression in KPC mice gastrocnemius muscles (n=3) at 10, 15, and 25 weeks post birth. Data are mean ± S.E.M., compared with t-test.; p**<0.01; p***<0.001
Figure 8: Expression of all sirtuins in KPC muscles. Sirtuin mRNA expression in gastrocnemius muscles from KPC mice at 10, 15, and 25 weeks of age. Data are mean ± S.E.M., compared with t-test. p*<0.05.
We also wanted to evaluate if cachectic cancer patients had a similar decrease in SIRT1 expression in skeletal muscles. For this purpose, we utilized muscle sections from pancreatic cancer patients obtained from the Rapid Autopsy Program at the University of Nebraska Medical Center. We observed decreased muscle fiber cross-sectional area, along with a decrease in SIRT1 staining in pancreatic cancer patient skeletal muscle sections (Figure 9A). We scored 54 patient muscle samples and observed a significant positive correlation between muscle cross-section area and SIRT1 staining (Figure 9B, Table 2). We investigated whether this phenotype was evident in other models of cancer cachexia. We observed that muscles collected from C-26 colon tumor-bearing mice also demonstrated a decrease in Sirt1 expression, with no significant decrease in the other sirtuins (Figure 10).
Figure 9: Expression of SIRT1 in pancreatic cancer patients’ muscles. (A) IHC staining of Sirt1 in skeletal muscles of pancreatic cancer patients were scored according to the staining intensity. (B) Correlation of the average skeletal muscle fiber cross-section area with the SIRT1 histoscore in 54 muscle autopsy samples of pancreatic cancer patients. ‘R’ depicts Pearson’s correlation coefficient and p value denotes the significance of the correlation.
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Figure 10: Expression of Sirtuins in the C-26 model. Sirtuin mRNA expression in gastrocnemius muscles from C26 tumor-bearing mice at day 21 post implantation (n=5 in each group). Data are mean ± S.E.M., compared with t-test. *p<0.05.
In order to evaluate whether cancer cell-secreted factors could diminish Sirt1 expression in myotubes, we utilized the *in vitro* model of pancreatic cancer-induced muscle atrophy. We treated C2C12 myotubes with CM from human (S2-013 and T3M4) and mice (KPC 1245, 1199, and 242) pancreatic cancer cell lines. We observed that the mRNA levels of *Sirt1* decreased in C2C12 myotubes upon treatment with human and mice PDAC cell line-CM (Figure 11A). We also observed that expression of other sirtuins were not consistently altered (Figure 11B). Moreover, we also confirmed that the decreased expression correlated with decreased Sirt1 activity in the C2C12 myotubes upon treatment with cancer cell-CM (Figure 11C).
**Figure 11: Expression of sirtuins in CM-induced muscle atrophy.** (A) Sirt1 mRNA expression in myotubes treated with S2-013-, T3M4-, KPC 1245-, KPC 1199-, and KPC 242-conditioned media. (B) Sirt2-7 mRNA expression in myotubes treated with S2-013-, T3M4-, KPC 1245-, KPC 1199- and KPC 242-CM for 24 hours. Data are mean ± S.E.M., compared with one-way ANOVA with Bonferroni’s multiple comparisons. \( p^*<0.05; \ p^{**}<0.01; \ p^{***}<0.001 \). (C) Sirt1 activity in myotubes treated with S2-013- and T3M4-CM for 24 hours. Data are mean ± S.E.M., compared with one-way ANOVA with Bonferroni’s multiple comparisons. \( p^{**}<0.01; \ p^{***}<0.001 \).
Since we observed a decrease in Sirt1, we hypothesized that stabilization of this protein might lead to rescue of cancer induced muscle atrophy. Previous studies have established that resveratrol (3,5,4’-trihydroxystilbene), a natural phytoalexin and a polyphenol found in red wine increases the deacetylase activity of SIRT1\textsuperscript{226}. It has also been shown to increase the expression of SIRT1\textsuperscript{227-229}. Since we observed that Sirt1 levels were decreased in cachectic muscles, we next investigated the effect of resveratrol on the cachectic phenotype. Treatment with cancer cell-CM and resveratrol (50 µM) rescued C2C12 myotube degeneration in culture conditions, as measured by myotube thickness (Figure 12A, 12B). There was also rescue of the cancer cell-CM-induced decrease in myosin heavy chain expression. Resveratrol also diminished the cancer cell-CM-induced protein expression of Atrogin-1 and MuRF1 muscle-specific ubiquitin ligases, which mediate muscle breakdown (Figure 12C). The mRNA levels of MuRF1 (\textit{Trim63}) and Atrogin-1 (\textit{Fbxo32}) were also decreased upon addition of resveratrol to the CM (Figure 13). We also observed resveratrol-mediated rescue of Sirt1 mRNA levels in the myotubes upon treatment with conditioned-medium (Figure 13). It is important to mention that resveratrol did not alter the expression of other sirtuins (Data not shown).
**Figure 12 : Resveratrol rescues CM-induced atrophy in C2C12 myotubes.** (A) Brightfield microscopy images (at 200x) of myotubes treated with S2-013-CM and T3M4-CM with and without resveratrol (50 µM) treatment for 24 hours. Scale bars represent 50 µm. (B) Myotube width was measured by ImageJ. Five measurements were taken along the length of each myotube. (C) Immunoblots of MyHC, Atrogin-1, and MuRF-1 in myotubes treated with S2-013- and T3M4-CM along with resveratrol treatment for 24 hrs. Tubulin was used as a loading control. Data are mean ± S.E.M., One-way ANOVA with Bonferroni’s multiple comparisons. *p<0.05; ***p<0.001.
Figure 13: Resveratrol decreases CM-induced increase in atrophy genes. mRNA expression of Trim63 (MuRF-1), FBXO32 (Atrogin-1) and Sirt1 in myotubes treated with S2-013- and T3M4-CM along with resveratrol (50 µM) for 24 hrs. Data are mean ± S.E.M., One-way ANOVA with Bonferroni’s multiple comparisons. p*<0.05; p***<0.001
**SIRT1 activation in pancreatic cancer cell lines leads to a decrease in proliferation**

Next, we wanted to investigate the efficacy of systemic modulation of Sirt1 in diminishing muscle atrophy in mice. It was important to first evaluate the effect of resveratrol on pancreatic cancer cell lines, so that we could delineate its effect on muscles independent of its effect on tumor. Previous studies have demonstrated that resveratrol decreases proliferation of some pancreatic cancer cells\(^{230-232}\). We wanted to determine if treatment of pancreatic cancer cell lines with resveratrol would impact cell proliferation in our cell line models. Therefore, we treated S2-013 and T3M4 cells with multiple doses of resveratrol. We observed that treatment with resveratrol decreased cell proliferation in a dose-dependent manner (Figure 14A). Moreover, since pancreatic tumors have high rates of glycolysis\(^{233}\), we also evaluated whether resveratrol modified the glycolytic phenotype of pancreatic cancer cells. We observed that resveratrol decreased glucose uptake in pancreatic cancer cell lines (Figure 14B). We also observed that Ex-527, a SIRT1 inhibitor that has previously been shown to increase tumor growth rates *in vivo*\(^{234}\), increased glucose uptake in S2-013 and T3M4 PDAC cell lines (Figure 14C). Resveratrol treatment also increased glucose uptake in myotubes treated with cancer cell-CM (Figure 14D). These results demonstrated that resveratrol had an adverse effect on tumor growth, but it also rescued glucose uptake in myotubes which implies its role in reducing insulin resistance.
Figure 14: Effect of resveratrol on pancreatic cancer cells. (A) MTT assay demonstrating the dose-dependent effect of resveratrol on S2-013 and T3M4 cell survival 72 hours post treatment. (B) Relative glucose uptake in S2-013 and T3M4 cells upon resveratrol treatment (50 µM) for 24 hrs. (C) Glucose uptake in S2-013 and T3M4 cells upon Ex-527 (0.5 µM) treatment for 24 hrs. (D) Glucose uptake in C2C12 myotubes upon treatment with cancer cell-conditioned media (CM) and resveratrol for 24 hrs. Data are mean ± S.E.M., One-way ANOVA with Dunnett’s (A), Bonferroni’s (D) multiple comparisons; Student’s t-test (B-C); p*<0.05; p**<0.01; p***<0.001.
To develop a model to test the effect of resveratrol on the muscle, independent of its effect on the tumor, we generated *SIRT1* knockdowns in S2-013 cells by lentiviral-delivery of short hairpin RNA (shRNA) against *SIRT1*. Two constructs targeting independent regions of *SIRT1*, sh*SIRT1*-A and sh*SIRT1*-B were used for knocking down *SIRT1* expression (Figure 15A). We then investigated whether *SIRT1* knockdown altered the sensitivity of cancer cells to resveratrol (25 µM) by performing MTT assays. We observed that *SIRT1* knockdown cells were less sensitive to resveratrol (Figure 15B). Moreover, cancer cell-CM from the scrambled control and the *SIRT1* knockdown cell lines had similar effect on the protein content of the myotubes, implying that modulation of *SIRT1* in the tumor has no impact on the cachectic potential *in vitro* (Figure 15C).
Figure 15: Effect of Resveratrol on Sirt1 knockdown pancreatic cancer cell lines. (A) Immunoblot of SIRT1 in S2-013 cells. Tubulin was used as a loading control. (B) Relative survival of S2-013 shScr, shSIRT1-A and shSIRT1-B upon resveratrol treatment for 72 hours (25µM) by MTT assays. (C) Protein content in myotubes treated with S2-013 shScr-CM, S2-013 shSIRT1-A-CM, S2-013 shSIRT1-B-CM along with resveratrol (50 µM). Data are mean ± S.E.M., Two-way ANOVA with Bonferroni’s post-hoc analysis considering the interaction between treatment and cell line (B), One-way ANOVA with Bonferroni’s multiple comparisons (C), p*<0.05; p**<0.01; p***<0.001.
Resveratrol prevents pancreatic cancer-induced cachexia in vivo independent of its effect on the tumor.

To extrapolate our findings, we evaluated whether stabilizing the expression of Sirt1 in skeletal muscles by resveratrol would rescue muscle atrophy in animal models. We implanted scrambled control and SIRT1 knockdown S2-013 cells orthotopically into the pancreas of athymic nude mice and administered resveratrol (200 mg/kg) daily by oral gavage, starting at 6 days post-implantation (Figure 16A). We observed decreased tumor weight and volume upon necropsy in resveratrol-treated scrambled control tumor-bearing mice but not in the SIRT1 knockdown cells (Figure 16B, C). We also observed that resveratrol significantly diminished the tumor-induced body weight loss in mice at day 21 post-implantation (Figure 17A).
Figure 16: Resveratrol decreases growth of Sirt1 wildtype but not Sirt1 KD tumors (A) Schematic illustration of the treatment strategy. (B-C) Post necropsy measurements of S2-013 shScr (n=10), shSIRT1-A (n=10), and shSIRT1-B (n=10) tumor weights (B) and tumor volumes (C). Data are mean ± S.E.M., One-way ANOVA with Bonferroni’s multiple comparisons (B,C). $p^*<0.05; p^{**}<0.01; p^{***}<0.001$. 
Additionally, post-necropsy measurements demonstrated a significant rescue in gastrocnemius muscle weight upon resveratrol treatment (Figure 17B). We measured body fat percentage by performing DEXA scans at day 18 post-implantation and observed a significant increase in body fat percentage in tumor-bearing mice upon resveratrol treatment (Figure 17C). There was also a significant improvement in the forelimb grip strength of the mice cohorts treated with resveratrol (Figure 17D). Quantification of the gastrocnemius muscle fiber cross-sectional area in hematoxylin and eosin-stained tissue sections showed significant increase in the mice treated with resveratrol (Figure 17E). There was an increased expression of myosin heavy chain and decreased expression of MuRF1 and Atrogin-1 in the gastrocnemius muscles of the mice treated with resveratrol (Figure 17F). Importantly, SIRT1 immunoblotting and IHC staining of gastrocnemius muscle sections confirmed SIRT1 stabilization upon treatment with resveratrol (Figure 17F and 18).
Figure 17: Resveratrol rescues muscle wasting in tumor-bearing mice. (A) Change in body weight measurements of the tumor-bearing mice on day 21 post implantation. (B) Post necropsy gastrocnemius muscle weight from S2-013 shScr, S2-013 shSIRT1-A and S2-013 shSIRT1-B tumor-bearing mice. (C) Measurement of fat percentage of S2-013 shScr (n=8), S2-013 shSIRT1-A (n=8) and S2-013 shSIRT1-B (n=8) tumor-bearing mice by DEXA scanning on day 18th post implantation. (D) Measurement of grip strength of the tumor-bearing mice on day 18th post implantation. (E) Quantification of muscle fiber cross-sectional area in hematoxylin and eosin-stained muscle sections from the tumor-bearing mice. (F) Immunoblots of muscle tissue extracts from the tumor-bearing mice showing regulation of MyHC, Atrogin, MuRF1, FoxO1, and FoxO3. Tubulin was used as a loading control. Data are mean ± S.E.M., One-way ANOVA with Bonferroni’s multiple comparisons; p*<0.05; p***<0.001.
**Figure 18**: Resveratrol stabilizes SIRT1 in the gastrocnemius muscles of tumor-bearing mice. SIRT1 staining in gastrocnemius muscles of healthy controls and tumor-bearing mice treated with vehicle control or resveratrol. Scales represent 250 µm.
Resveratrol prevents muscle wasting by reducing ROS levels, and diminishing the expression and activity of FOXO transcription factors

In order to investigate the potential mechanism of action of resveratrol-induced protection against muscle wasting, we performed RNA-seq analysis on myotubes treated with S2-013-CM with or without resveratrol for 24 hours. Since Sirt1 is known to be involved in regulation of several transcription factors, we performed Gene Set Enrichment Analysis (GSEA) and determined the enrichment for transcription factors in the transcription factor gene set from TFactS (http://www.tfacts.org/) to evaluate the significantly altered pathways in all the cohorts (Figure 19A). We observed significant alterations in 9 out of 101 transcription factor pathways (Figure 19B). Interestingly, we observed enrichment of FoxO1 and FoxO3 transcription factor-regulated genes in the myotubes treated with S2-013-CM (Figure 19C). These set of genes also include Fbxo32 (Atrogin) and Trim63 (MuRF-1) which are muscle-specific ubiquitin ligases responsible for muscle atrophy. These enrichments were abolished by resveratrol treatment.

We validated the RNA-Seq results by evaluating the mRNA and protein levels of FoxO1 and FoxO3 in myotubes treated with cancer cell-CM with or without resveratrol. We observed that resveratrol decreased the cancer cell CM-induced expression of the FoxO genes (Figure 19D). Similar results were observed in the protein levels of FoxO1 and FoxO3 (Figure 19E), confirming that resveratrol prevents muscle wasting by modulating FoxO proteins.
Figure 19: Resveratrol reverts cancer cell-CM-induced upregulation of FoxO proteins via NF-κB. (A) Schematic illustration of the flow chart of RNA-Seq analysis. (B) Heatmap showing the Z-score changes in the 3 comparisons. 9 out of 101 pathways in the transcription factor database from TFactS were significantly altered. (C) GSEA plots of FoxO1 and FoxO3. The normalized enrichment scores (NES) for FoxO1 are 2.07 (CM-treated vs. control) and -1.9 (CM+Res-treated vs. CM-treated) pathway. The NES for FoxO3 are 2.5 (CM-treated vs. control) and 1.6 (CM+Res-treated vs. CM-treated). (D) mRNA levels of Foxo1 and Foxo3 in myotubes upon treatment with tumor cell-CM and resveratrol (50 µM). Data are mean ± S.E.M., One-way ANOVA with Bonferroni’s multiple comparisons (D). p*<0.05, p***<0.001.
Next, we evaluated the potential direct relationship between Sirt1 expression and muscle pathophysiology. For this purpose, we overexpressed Sirt1 by adenoviral infection of myotubes after 48 hours of differentiation, and then treated them with cancer cell-CM for 24 hours. Sirt1 overexpression attenuated the thinning of myotubes induced by cancer cell-CM (Figure 20A). Consequently, Sirt1 overexpression also rescued the loss of protein content in myotubes upon CM treatment (Figure 20B). Sirt1 overexpression also rescued the cancer CM-induced decrease in myosin heavy chain expression levels. (Figure 20C). We also observed a decrease in the mRNA levels of the FoxO1 and FoxO3 upon Sirt1 overexpression in CM-treated myotubes (Figure 21). Therefore, we concluded that Sirt1 overexpression was sufficient to abolish the CM-induced expression of FoxO proteins.
Figure 20: SIRT1 overexpression rescues atrophy in C2C12 myotubes. (A) Brightfield microscopy images (at 200x), protein content analysis (B), protein lysate immunoblot analysis (C) of GFP control or SIRT1 expressing myotubes, infected adenovirally (Ad), upon treatment with tumor cell-CM for 24 hours. Scale bars represent 75µm. Data are mean ± S.E.M., One-way ANOVA with Bonferroni's multiple comparisons (B). p*<0.05; p**<0.01.
Figure 21: SIRT1 regulates expression of FOXO proteins in C2C12 myotubes. Foxo1 and Foxo3 mRNA analysis (A) of GFP control or SIRT1 expressing myotubes, infected adenovirally (Ad), upon treatment with tumor cell-CM for 24 hours. Data are mean ± S.E.M., One-way ANOVA with Bonferroni's multiple comparisons (A). $p^*<0.05; p^{**}<0.01; p^{***}<0.001$. 
Additionally, the NF-κB pathway, which has been shown to be a major player in the development of cachexia\(^{235}\), was also significantly upregulated in myotubes treated with S2-013-CM, and this increase too was abrogated by resveratrol treatment (Figure 22A). These results are in line with previous studies demonstrating the regulation of NF-κB activity by Sirt1-mediated deacetylation\(^{236-238}\). Moreover, a promoter-reporter assay with an NF-κB-responsive promoter-luciferase reporter construct showed that resveratrol abolished the cancer cell-CM induced NF-κB transcriptional activity in myotubes (Figure 22B). Previous studies have demonstrated increased transcription activity of NF-κB upon acetylation of p65 at lysine 310\(^{239}\). Since K310 residue of p65 has been known to be deacetylated by SIRT1\(^{240}\), we evaluated K310 acetylation levels upon treatment of myotubes with cancer cell-CM with and without resveratrol. We observed increased acetylation of p65 upon cancer-cell CM treatment, and the effect was reduced by resveratrol treatment (Figure 22C). In the next chapter, we will illustrate the mechanisms via which NF-κB and FoxO pathways induce muscle wasting in pancreatic cancer.
Figure 22: Resveratrol rescues tumor-induced increase in NF-κB pathway. (A) GSEA plots for NF-κB. NES for NF-κB are 2.91 (CM-treated vs. control) and 1.6 (CM+Res-treated vs. CM-treated). (B) Luciferase assay to measure NF-κB-responsive promoter-luciferase reporter activity upon treatment of myotubes with cancer cell-CM with and without resveratrol (50 µM) for 6 hours. (C) Immunoblot analysis of acetylated (taken at high and low exposures) and total p65 subunit of NF-κB in myotubes treated with cancer cell-CM with and without resveratrol (50 µM) for 24 hours. Data are mean ± S.E.M., One-way ANOVA with Bonferroni's multiple comparisons (B). p*<0.05; p**<0.01; p***<0.001.
Discussion
In our study, we concluded that SIRT1 stabilization in the muscles successfully prevented muscle atrophy induced by pancreatic tumors in orthotopic mouse models. Additionally, the loss of SIRT1 in skeletal muscles was consistent across spontaneous progression models and human cancer patients. While, we did not observe a consistent alteration in the expression levels of other sirtuins in the cachectic muscles, we cannot rule out the roles played by other sirtuins in the tumor cells. Multiple sirtuins have been shown to regulate aggressiveness in tumor cells\textsuperscript{251,252} that may indirectly impart cachectic functions. Nonetheless, our studies establish that stabilizing/increasing SIRT1 expression may provide therapeutic opportunities for cancer cachexia.

Previous studies have demonstrated that resveratrol decreases tumor growth in multiple myeloma, cervix carcinoma, and chronic myeloid leukemia\textsuperscript{253-255}. While we observed similar anti-cancer response on pancreatic cancer cell lines in culture and in orthotopic tumor models, we noted a direct effect of resveratrol-mediated Sirt1 stabilization on preventing tumor-induced wasting in cultured myotubes and in mice muscles. Additionally, we demonstrated that resveratrol can abolish tumor-induced wasting in orthotopic models irrespective of its effects on the tumor and has the potential to be utilized as a therapy for cachexia.

In our studies with the \textit{in-vitro} models of cancer cell-CM-induced myodegeneration, RNA-Seq analysis demonstrated NF-\kappa B to be the most altered pathway. This was in line with classical studies in cachexia that demonstrated NF-\kappa B to be the master regulator of ubiquitin proteasome in cancer-induced muscle
atrophy\textsuperscript{235,256}. Even though previous studies have demonstrated an association of NF-κB activation and the proteins in the ubiquitin proteasome pathway\textsuperscript{256,257}, a direct mechanism was still not known. We also observed tumor cell-induced expression of FoxO proteins, which are known to be regulated by oxidative and metabolic stress\textsuperscript{258}. Based on the previous evidence highlighting the anti-oxidant functions of resveratrol\textsuperscript{238} and the influence of oxidative stress on muscle wasting\textsuperscript{259,260}, we next asked if the decrease in muscle atrophy was due to altered redox signaling via NF-κB activation. We observed that direct inhibition of NF-κB was able to abolish the tumor cell-CM-induced expression of MuRF1 and Atrogin-1 ubiquitin ligases that impart muscle wasting. Furthermore, we demonstrate that resveratrol can inhibit tumor cell-CM-induced activation of NF-κB. Hence, we establish that SIRT1 is an upstream regulator whose loss results in activation of NF-κB, which contributes to muscle wasting.
Chapter 4: Role of Nox4 in Cancer-Induced Cachexia

(Note – The data shown in this chapter has been submitted to a peer-reviewed journal as of March 2019)

Introduction

Reactive Oxygen Species (ROS) are highly unstable, reactive and short-lived molecules which regulate signaling pathways in all cells, including skeletal muscle cells\textsuperscript{241}. The major types of reactive oxygen species found in cells are superoxide, hydrogen peroxide and hydroxyl radical. At high concentrations, free radicals can damage macromolecules and organelles, which thereby leads to tissue degeneration or dysfunction\textsuperscript{84}. Studies have established the role of high oxidative stress or oxidative damage in the development of cancer-associated muscle wasting and the rescue of such wasting when treated with antioxidants\textsuperscript{86}. Similarly, reports have also demonstrated induction of the ubiquitin proteasome pathway by mild oxidative stress\textsuperscript{242}. In this chapter, we investigated the role of oxidative stress in the manifestation of pancreatic cancer cachexia. We evaluated the major enzymes involved in ROS homeostasis and observed the key role of NADPH oxidase 4 (Nox4) in the manifestation of cancer-cachexia. Nox enzymes catalyze the formation of free radicals by transferring an electron from NADPH to oxygen. The mammalian Nox enzymes are Nox1 to Nox5, and Duox1 and Duox2\textsuperscript{243}(Figure 23). Among them, Nox4 is the only one which is constitutively active, with others being activated by calcium or protein-protein interactions. This feature of Nox4 also implies the relevance of evaluating the expression of Nox4 as compared to its
activity, presumption being that any protein that is expressed is constitutively active\textsuperscript{243}. This chapter will illustrate the role of Nox4 in cachectic skeletal muscles and its potential of being a therapeutic target. This chapter will also link the findings of chapter 3 with the induction of oxidative stress in cachectic muscles.
**Figure 23 : Types of NADPH oxidases.** “Nox family NADPH oxidases: Molecular mechanisms of activation”.

“Nox family NADPH oxidases: Molecular mechanisms of activation”.

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Figure 23: Types of NADPH oxidases. This figure illustrates the different types of NADPH oxidases and their molecular mechanisms of activation.
Results

Resveratrol reduces ROS levels in cachectic muscles

Considering the established link between oxidative stress and muscle damage, continuing with the data presented in chapter 3, we wanted to evaluate whether reactive oxygen species (ROS) play a role downstream of Sirt1. On our advantage, previous studies have established an inverse relationship between SIRT1 and ROS levels in neuronal cells\(^ {244} \). Hence, we investigated whether diminished SIRT1 levels in cachectic muscles contributed to oxidative damage, resulting in muscle atrophy. We first tested our hypothesis in our in vitro model of cancer-induced muscle atrophy. Interestingly, we observed increased ROS levels in the myotubes treated with cancer cell-CM, which was abolished by resveratrol treatment (Figure 24).
Figure 24: Resveratrol decreases the tumor-induced ROS in C2C12 myotubes. ROS levels in C2C12 myotubes after treatment with cancer cell-CM with or without resveratrol for 8 hours. Data are mean ± S.E.M., One-way ANOVA with Bonferroni's multiple comparisons, $p^*<0.05; p^{**}<0.01; p^{***}<0.001$
We next hypothesized that since ROS is increased in cachectic myotubes, there would be an induction or reduction in the enzymes involved in ROS homeostasis. To that end, we evaluated the levels of the key enzymes responsible for reactive oxygen homeostasis in the muscles of the tumor-bearing mice treated with resveratrol (Figure 25A, 25B and 26). Interestingly, we observed that mRNA levels of NADPH oxidase 4 (Nox4) were increased in the muscles of the tumor-bearing mice but downregulated upon treatment of the tumor-bearing mice with resveratrol, implying its role in regulating oxidative stress in the cachectic muscles.
Figure 25: Expression of ROS enzymes in the muscles of tumor-bearing mice. (A) The mRNA levels of Nox4 in the gastrocnemius muscles from tumor cell-implanted mice (n=3 for each group) with and without resveratrol treatment. (B) The mRNA levels of redox regulators in gastrocnemius muscles of tumor-bearing mice with and without resveratrol treatment (n=3 for each group); Nox1-3 (NADPH oxidase1-3), Sod1-2 (Superoxide Dismutase1-3). Data are mean ± S.E.M., compared with one-way ANOVA with Bonferroni’s multiple comparisons. $p^*<0.05$; $p^{**}<0.001$. 

Nox4

A.

Nox1

Nox2

Nox3

Sod1

Sod2

Resveratrol

Resveratrol

Resveratrol

Resveratrol

Resveratrol
Figure 26: Expression of ROS enzymes in gastrocnemius muscles in tumor-bearing mice. The mRNA levels of redox regulators in gastrocnemius muscles of tumor-bearing mice with and without resveratrol treatment (n=3 for each group); Sod 3 (Superoxide Dismutase 3), Gpx1-4 (Glutathione peroxidase1-4), Catalase, Trdxn1-3 (Thioredoxin1-3). Data are mean ± S.E.M., compared with one-way ANOVA with Bonferroni’s multiple comparisons. p*<0.05.
Keeping in mind our results from Chapter 3, that Sirt1 regulates NF-κB and FoxO, we wanted to elucidate whether there was a redox link between the induction of NF-κB and Nox4. Intriguingly, previous reports have demonstrated the regulation of Nox4 by NF-κB\textsuperscript{245,246} in other models. To establish the direct regulation of Nox4 by NF-κB in cachectic skeletal muscles, we performed ChIP assays. The ChIP assays demonstrated increased occupancy of NF-κB (p65 subunit) at two consensus NF-κB response elements in the promoter region of Nox4 in C2C12 myotubes upon treatment with S2-013- and T3M4-CM. Moreover, the increased Nox4 promoter occupancy of NF-κB in response to CM treatment was abrogated upon treatment with resveratrol (Figure 27A). These results indicate that CM treatment facilitates NF-κB activation and binding to Nox4 promoter, likely inducing the Nox4 expression; these events can be abolished by resveratrol treatment. To prove this link via an independent method, we also utilized a cell permeable NF-κB essential modulator (NEMO)-binding domain (NBD) peptide that can block the activation of the IκB kinase (IKK) complex to inhibit NF-κB activity\textsuperscript{247}. We observed abrogation of the cancer cell-CM-induced expression of Nox4 and myotube atrophy by treating the myotubes with NBD (Figure 27B and 27C). These results establish that in cachectic muscles Nox4 expression is likely regulated by an increased activity of NF-κB.
Figure 27: NF-κB regulates the expression of Nox4 in cachectic muscles. (A). ChIP analyses demonstrating distal (-1047) and proximal (-369) Nox4 promoter region occupancy by NF-κB. (B-C) Nox4, Trim63 and Fbxo32 mRNA levels in C2C12 myotubes treated with cancer cell-CM and NBD. Data are mean ± S.E.M., One-way ANOVA with Bonferroni’s multiple comparisons. $p^*<0.05$; $p^{**}<0.01$; $p^{***}<0.001$. 
To validate our findings further, we wanted to evaluate the mRNA levels of Nox4 in the muscles from 10-, 15-, and 25-week-old KPC mice. We observed a significant increase in Nox4 levels in the muscles of 25-week-old KPC mice (Figure 28A). Moreover, overexpression of NOX4 in C2C12 myotubes via adenovirus infection directly led to myotube atrophy at 48 hours post-infection (Figure 28B), implying a causative link between Nox4 expression and myotube atrophy. NOX4 overexpression also decreased the levels of myosin heavy chain in the myotubes (Figure 28C). Additionally, we also observed that overexpression of NOX4 was sufficient to increase the expression of FoxO genes (Figure 28D). These results implied that FoxO is downstream of Nox4 induction.
**Figure 28: Nox4 induces atrophy in cachectic muscles.** (A) Nox4 mRNA levels in KPC mice muscles at 10-, 15- and 25-weeks of age (n=3). (B) Brightfield microscopy images (at 200x) demonstrating thinning of C2C12 myotubes upon Nox4 overexpression. Scale bars represent 250µm. (C) Immunoblot analysis of Nox4 overexpressing myotube extracts depicting decrease in the levels of myosin heavy chain. Tubulin was used as a loading control. (D) Foxo1 and Foxo3 mRNA levels in C2C12 myotubes upon Nox4 overexpression for 48 hours. Data are mean ± S.E.M., compare to the control using Student’s t-test (A, D). p<0.05; p***<0.001.
Validating our findings in another model of cancer cachexia we found that muscles from C26-bearing mice also demonstrated an increase in *Nox4* and *FoxO* genes, along with the induction of atrophy genes (Figure 29A,B). These results establish the critical role of *Nox4* in induction of the cachectic phenotype. We then wanted to come back to our original premise that decreased Sirt1 led to Nox4 induction. Consequently, overexpression of *Sirt1* was sufficient in downregulating the increased levels of *Nox4* in the myotubes treated with cancer cell-CM (Figure 29C). These results led us to hypothesize that Sirt1 downregulation leads to NF-κB activation, which can lead to increased expression of Nox4, an inducer of oxidative stress that can in turn induce the expression of the muscle-specific ubiquitin ligases to facilitate myopathy (Figure 30).
Figure 29: Expression of atrophy genes in the muscles of C26-bearing mice.

(A-B) mRNA levels of Nox4, Foxo1, Foxo3, Trim63, and Fbxo32 in the gastrocnemius muscles of healthy control and C26 bearing mice (n=5 in each group). (C) Nox4 mRNA level in C2C12 myotubes upon treatment with cancer cell-CM with or without adenoviral (Ad) expression of Sirt1. Data are mean ± S.E.M, compared with Student’s t-test (A-B), One-way ANOVA with Bonferroni’s multiple comparisons (C). p*<0.05; p**<0.01; p***<0.001.
Figure 30: Schematic illustration of the proposed pathway.
We wanted to illustrate whether a simplistic flow of events could explain the entire mechanism. To investigate this pathway further, we utilized, GKT137831 to inhibit Nox4 activity\textsuperscript{248}, BMX-001 to scavenge oxygen radicals\textsuperscript{249} and AS1842856 to inhibit FoxO1 activity\textsuperscript{250} (Figure 30). We observed that MuRF1 (\textit{Trim63}) and Atrogin-1 (\textit{Fbxo32}) were downregulated by targeting all the arms of the pathway (Figure 31). We would like to mention that our proposed mechanism in no way implies the absence of other branches and participants in the induction of cancer cachexia.
Figure 31: Expression of atrophy genes upon treatment with inhibitors. The mRNA levels of Trim63, Fbxo32 in C2C12 myotubes treated with control or cancer cell-CM with, GKT137831 (10µM), AS1842856 (0.1µM), NBD (5µM), and BMX-001 (1µM) for 24 hrs. Data are mean ± S.E.M., One-way ANOVA with Bonferroni’s multiple comparisons, treated samples are compared to the non-treated samples. \( p^*<0.05; \ p^{**}<0.01; \ p^{***}<0.001, \)
*Nox4 expression in skeletal muscles drives cancer-induced cachexia*

We next wanted to investigate whether Nox4 expression in muscles contributes to cancer cachexia. For this purpose, we generated mice with tamoxifen-inducible conditional knockout of Nox4 in the skeletal muscles (Nox4<sup>fl/fl</sup>; ACTA1-Cre/Esr1; Figure 32A). We confirmed the muscle-specific Nox4 knockout after tamoxifen injection in the gastrocnemius muscles via immunoblotting (Figure 32B).
Figure 32: Nox4 overexpression drives cachexia in orthotopic pancreatic tumor models. (A) Schematic illustration of pancreatic cancer-cachexia model utilizing Nox4^{fl/fl}; ACTA1-Cre/Esr mice. (B) Immunoblot analysis of muscle extracts from control and Nox4 knockout mice depicting deletion of Nox4 in the muscles. Actin was used as a loading control.
Next, we orthotopically implanted mouse pancreatic cancer cell line (KPC 1245) into the pancreas of Nox4fl/fl; ACTA1-Cre/Esr1 mice. We then randomly divided the mice into two groups- to be injected with Tamoxifen (Nox4-KO) or solvent control (Nox4-WT). Nox4 knockout in muscles significantly rescued tumor-induced body weight loss, compared to the Nox4-WT mice (Figure 33A). Additionally, we also observed that Nox4 knockout significantly rescued the tumor-induced loss in forelimb grip strength (Figure 33B) and gastrocnemius muscle weight (Figure 33C), as compared to the Nox4-WT mice.
**Figure 33**: *Nox4 Knockout in muscles rescues atrophy in tumor-bearing mice.* (A) Change in body weight of mice after 21 days of implantation. (B) Grip strength of tumor-bearing *Nox4 WT* and *Nox4* knockout mice on the 18th day post implantation. (C) Post necropsy gastrocnemius muscle weight of healthy control (n=10), tumor-bearing *Nox4 WT* (n=8) and tumor-bearing *Nox4* knockout (n=8) mice. Data are mean ± S.E.M., One-way ANOVA with Bonferroni’s multiple comparisons (A-C). *p*<0.05; **p**<0.01; ***p***<0.001.
It is important to mention here that there was no significant difference in tumor weights between the two groups (Figure 34A), implying that the changes in muscle, body weight and muscle function were predominantly due to changes in muscle physiology. Additionally, gastrocnemius muscle tissue sections stained with hematoxylin and eosin stain demonstrated a decrease in the muscle fiber cross-sectional area in the tumor-bearing mice, and these effects were rescued by knocking out Nox4 in the muscles (Figure 34B).
Figure 34: Nox4 knock out regulates muscle atrophy in tumor-bearing mice. (A) Post-necropsy tumor weights from tumor-implanted Nox4 WT and Nox4 KO mice. (B) Representative hematoxylin and eosin-stained muscle sections depicting changes in the muscle fiber cross-sectional area. Scale bars represent 25µm. Quantification of the gastrocnemius muscle cross-sectional area of healthy controls and tumor-implanted mice (n=3). Data are mean ± S.E.M., One-way ANOVA with Bonferroni’s multiple comparisons (B), Student’s t-test (A). $p^*<0.05$; $p^{**}<0.001$. 
Next, we also evaluated the molecular markers in the muscles of the tumor-bearing mice. We also observed that Nox4 knockout was able to rescue the decreased myosin heavy chain levels in the tumor-bearing mice. Consequently, the tumor-induced increase in MuRF1 and ATROGIN-1 levels in the mice muscles was also abolished by Nox4 knockout (Figure 35A). Interestingly, Nox4 knockout did not have an impact on SIRT1 stabilization, implying the importance of targeting Nox4 independent of SIRT1 (Figure 35A). This might also imply Nox4 acting downstream of Sirt1. Nox4 knockout also abrogated the tumor-induced mRNA levels of FoxO transcription factors (Figure 35B). Therefore, these studies suggest that targeting Nox4 in the skeletal muscles may combat cancer-induced cachexia.
**Figure 35**: *Nox4 regulates atrophy and FoxO genes in cachectic muscles.* (A) Immunoblot analysis of muscle extracts from healthy control, Nox4 WT and Nox4 KO tumor-bearing mice. (B) Foxo1 and Foxo3 mRNA levels in gastrocnemius muscles from control and tumor-implanted mice (n=5 for each group). Data are mean ± S.E.M., One-way ANOVA with Bonferroni’s multiple comparisons. $p^*<0.05$. 
**Pharmacological inhibition of Nox4 rescues cancer cachexia in mice models**

For clinical relevance of this study, we wanted to target Nox4 pharmacologically. We next investigated the potential of GKT137831 (henceforth referred to as GKT), a NOX4/NOX1 inhibitor to rescue cachexia in pancreatic cancer mouse models. This compound was our best option since it has already passed the Phase I safety trial and is currently in Phase II for diabetes (NCT02010242) and primary biliary cholangitis (NCT03226067). To evaluate the efficacy of GKT on cancer cachexia, we implanted human pancreatic cell line S2-013 in 20 athymic nude mice and randomly segregated them into two groups of 10 mice each. We started daily treatment with GKT or vehicle control via oral gavage after 6 days post-implantation (Figure 36A). Three weeks after implantation, mice were sacrificed and analyzed. We observed body weight loss in S2-013 tumor-bearing mice that was rescued by treatment with GKT (Figure 36B). There was no difference in tumor weight upon treatment with GKT, implying that these effects were muscle-specific (Figure 36C).
Figure 36: Pharmacological inhibition of Nox4 rescues muscle wasting in a cancer cachexia model. (A) Schematic illustration of S2-013 tumor cell implantation and GKT therapy initiation in athymic nude mice. (B) Change in the mouse body weight at day 21 post implantation for healthy controls (n=9), healthy controls with GKT (n=4), S2-013-implanted (n=9), and S2-013-implanted with GKT treatment (n=10). (C) Post-necropsy tumor weight for S2-013-implanted mice with and without GKT treatments. Data are mean ± S.E.M. One-way ANOVA with Bonferroni’s multiple comparisons (B,C). $p^{*}<0.05; p^{***}<0.001$.  

**Legend:**

- **A.** Schematic illustration of tumor cell implantation and GKT therapy initiation.
- **B.** Change in body weight for different conditions.
- **C.** Tumor weight for S2-013-implanted mice with and without GKT treatment.
We observed that GKT could rescue tumor-mediated decrease in gastrocnemius muscle weight, body fat content, and grip strength (Figure 37A-C). We also observed an increase in the reactive oxygen free radicals in the gastrocnemius muscles of tumor-bearing mice by performing EPR spectroscopy; the increase was abrogated by treatment with GKT (Figure 37D).
**Figure 37 : GKT rescues muscle wasting in tumor-bearing mice.** (A) Gastrocnemius muscles for S2-013-implanted mice with and without GKT treatments. (B) Body fat percent at day 18 in tumor-bearing mice with and without GKT treatments. (C) Grip strength measurements on day 18 post implantation. (D). Gastrocnemius muscle ROS measurement by EPR in tumor-bearing mice with and without GKT treatment (n=5 for each group). Data are mean ± S.E.M., One-way ANOVA with Bonferroni’s multiple comparisons (A-D). p*<0.05; p**<0.01; p***<0.001.
GKT treatment also abrogated the tumor-induced decrease in gastrocnemius muscle fiber cross-section area, as observed by performing tissue staining with hematoxylin and eosin (Figure 38A). Analysis of molecular markers of atrophy by immunoblotting demonstrated GKT-mediated rescue in the levels of myosin heavy chain, ATROGIN-1 and MURF1 (Figure 38B). Moreover, SIRT1 levels were not stabilized up to control levels upon GKT treatment, implying again that SIRT1 is an upstream regulator of Nox4 (Figure 38B). GKT treatment also diminished the tumor-induced mRNA expression of FoxO transcription factors (Figure 38C). Therefore, we concluded that pharmacological inhibition of NOX4 in the PDAC tumor models can combat muscle wasting.
Figure 38: GKT rescues muscle atrophy via FoxO pathway. (A) Representative images of hematoxylin and eosin-stained gastrocnemius muscle cross-sections. Quantification of the cross-sectional area measured via ImageJ (n=3). Scale bars represent 100µm. (B) Immunoblot analysis of gastrocnemius muscle extracts from mice with indicated treatments. (C) FoxO1 and FoxO3 mRNA levels in gastrocnemius muscles from mice with indicated treatments (n=3 for each group). Data are mean ± S.E.M., One-way ANOVA with Bonferroni’s multiple comparisons (A,C). p*<0.05; p**<0.01; p***<0.001.
To validate whether this phenotype was clinically significant, we performed Nox4 staining on skeletal muscle tissue sections from human pancreatic cancer patients obtained from the Rapid Autopsy Program and observed a significant negative correlation between the muscle fiber cross-section area and staining intensity (Figure 39A and 39B). This demonstrated that cachectic patients have an increased expression of Nox4 in their muscles.

We concluded our study by establishing the pathway illustrated in Figure 40 and demonstrated the prevention of cachexia by inhibiting the key players of this pathway.
Figure 39: Nox4 expression in human PDAC muscle samples. (A) Representative images of NOX4-stained human muscle specimens. (B) Correlation of Nox4 staining histoscore and muscle fiber cross-sectional area in skeletal muscles from human pancreatic cancer patients (n=47).
Figure 40: Schematic illustration of the action of resveratrol and GKT as potential therapeutic interventions in combating muscle wasting in cancer.
Discussion

Since SIRT1 is a known regulator of oxidative stress\textsuperscript{244}, we screened for the redox regulators that were induced by cancer cell-CM. Our screening identified Nox4 to be the key oxidative stress regulator that was upregulated in the muscles of tumor-bearing mice, an effect that was abrogated by resveratrol treatment. Notably, Nox4 is also demonstrated to be upregulated in angiotensin II-induced muscle wasting, implying the possibility of a common pathway\textsuperscript{261} in multiple muscle disorders. Moreover, NOX4 expression also demonstrated to have a significant negative correlation with the skeletal muscle fiber cross-sectional area in human pancreatic cancer patients. Previous studies have demonstrated that Nox4 is regulated by NF-κB in different models\textsuperscript{245,246}. Herein, we demonstrate that Nox4 promoter occupancy by NF-κB and NF-κB activity are significantly induced by tumor cell-CM, and the effect is abrogated by resveratrol. Furthermore, muscle-specific Nox4 knockout and pharmacological blockade of Nox4 in tumor-bearing mice abolished tumor-induced muscle atrophy. We also observed a decrease in the expression of Nox4-induced FoxO expression. These studies demonstrate that SIRT1 regulates oxidative stress and atrophy in skeletal muscles by NF-κB-mediated expression of FoxO. Interestingly, GKT137831, a Nox1/Nox4 inhibitor, which is in clinical trials for various diseases, can potentially be utilized in a muscle wasting syndrome.

Overall, this study demonstrates that tumor-induced muscle wasting was mediated by the SIRT1 loss-triggered activation of NF-κB, which in turn induces the expression of Nox4 in skeletal muscles, leading to induction of the protein
degradation pathways. These findings provide new insights into the mechanisms that underlie weight and muscle loss in cancer cachexia. These studies also provide novel therapeutic opportunities for targeting tumor-induced skeletal muscle wasting.
Chapter 5: Role of Oxidative Stress in Chemotherapy-Induced Muscle Atrophy in Pancreatic Cancer

(Note- The data in this chapter is under preparation to be submitted to a peer-reviewed journal as of March 2019)

Introduction

Cancer-associated cachexia limits cancer therapy, quality of life and survival\textsuperscript{209,210}. In 2011, the international consensus defined cancer cachexia as a multifactorial syndrome with ongoing loss of skeletal muscle mass, with or without loss of fat mass. This weight loss cannot be fully reversed by conventional nutritional support and leads to progressive functional impairment\textsuperscript{3}. To update the definition of cachexia, Vagnildhaug \textit{et al.} have recently established a weight loss grading system (WLGS) which is related to survival and progression of the disease in cancer patients\textsuperscript{262}. This grading system considered the body mass index (BMI) and the degree of weight loss of the patient and is now included in the international practice guidelines of the European Society for Clinical Nutrition and Metabolism (ESPEN). However, even though BMI has been demonstrated to play a role in determining the prognosis of the patient, studies have also established skeletal muscle mass loss as a negative prognostic factor, regardless of overall body weight\textsuperscript{263}. Onesti \textit{et al.} (2016) have found sarcopenia to be a predictor of overall survival (OS) in male patients undergoing pancreatic resection\textsuperscript{264}. Similarly, a study conducted in Japan by Ninomiya \textit{et al.} (2017) showed sarcopenia to be a
valid prognostic factor as tumor size and resection margins in patients undergoing surgical resection for pancreatic ductal adenocarcinoma.

Additionally, studies have also demonstrated that chemotherapy alone can induce muscle wasting in experimental mice models. In 2016, Baretto et al. also reported that cancer and chemotherapy contribute to weight loss by activating the same pathways in colon tumor-bearing mice models. These pathways included mitochondrial dysfunction, alteration in the TCA cycle, fatty acid metabolism and modification of reactive oxygen species, to name a few. These studies have shed light on the importance of elucidating the mechanism of chemotherapy-induced muscle wasting.

The goal of our study was to determine the correlation between skeletal muscle depletion and survival outcomes in patients with pancreatic cancer. Since our patient cohort was either treated with gemcitabine or FOLFIRINOX, we wanted to evaluate the effect of these chemotherapeutic agents on body weight loss and muscle wasting in an experimental murine model. Moreover, we also wanted to elucidate the pathway which leads to muscle wasting and attempt to combat the wasting via pharmacological intervention.

Since chemotherapy has been demonstrated to increase ROS in cancer cells in its process to abolish it, we hypothesized that chemotherapy would be increasing ROS in other tissues, including skeletal muscles. In this chapter, we will illustrate our findings on the role of oxidative stress in chemotherapy-induced skeletal muscle wasting.
Results

**Muscle-loss correlates negatively with survival in pancreatic cancer patients after therapy**

We collected the clinical data for the patients at two separate time points: at diagnosis and at eight weeks after the start of therapy. At the same timepoints calculation of the muscle mass value was performed for each patient using the Slice-O-Matic software (Figure 41A). We subdivided the patients into three groups based on the degree of muscle loss at 8 weeks compared to the time of diagnosis. Majority of the patients (57%) had less than 10% muscle loss or had muscle gain, twenty-nine percent had 10-20% muscle loss, and the remaining fourteen percent had muscle loss that was greater than 20%. Kaplan-Meier curves demonstrate that patients with continuous muscle loss during the first eight weeks of therapy have inferior PFS (p-value = 0.027), Figure 1, and OS (the p-value = 0.038). Adjusting for a list of confounders, the p-value is 0.038, indicating a significant risk of death associated with higher percentage of muscle loss. (Figure 41B).
Figure 41: Muscle loss in Pancreatic Cancer Patients leads to poor prognosis. (A) Representative images of abdominal muscles around the L3 lumbar vertebrae before and after 8 weeks of chemotherapy. (B-C) Overall and Progression Free Survival curves.
Table 3: Patient characteristics for L3 muscle measurement.

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Effect of Chemotherapy on Muscle Mass and Function in Mice

To generate an experimental model to study the effect of chemotherapy on muscles, we treated normal mice with Gemcitabine or FOLFIRINOX for a period of 8 weeks (Figure 42A). The doses of gemcitabine and FOLFIRINOX were similar to those used in experimental cancer models\textsuperscript{201,267}. We observed a decrease in body weight, grip strength and rotarod performance upon treatment with chemotherapy (Figure 42B-D).
Figure 42: Chemotherapy induces loss in body weight and muscle function in mice. (A) Schematic illustration of treatment of C57/BL6 with gemcitabine (n=12) and FOLFIRINOX (n=12). (B) Measurement of change in body weight after 8 weeks of chemotherapy treatment; control (n=6), gemcitabine (n=12), FOLFIRINOX (n=12). (C) Change in Grip Strength of control, gemcitabine-, FOLFIRINOX- treated mice. (D) Change in Rotarod Performance of control, gemcitabine-, FOLFIRINOX-treated mice. Data are mean ± S.E.M. One-way ANOVA was performed and Tukey’s post-hoc analysis was performed to compare all the groups. p<0.001 is indicated as *** to compare gemcitabine and FOLFIRINOX with the control group.
Post-necropsy measurements of gastrocnemius muscles depicted a decrease in muscle weight (Figure 43A). As mentioned in chapters 3 and 4, muscle wasting is characterized by the increase in muscle specific ubiquitin ligases, MuRF1 and Atrogin-1, which are responsible for the protein turn over in muscles\(^{215,216}\). Therefore, we also measured the mRNA levels of these two genes in the muscles of the mice treated with chemotherapy. We observed an increase in the ubiquitin ligase expression implying an increase in protein ubiquitination in the muscles (Figure 43B-C).
Figure 43: Chemotherapy induces muscle atrophy in mice. (A) Post-necropsy measurement of gastrocnemius muscle of control (n=6), gemcitabine- (n=12), FOLFIRINOX (n=12)- treated mice. (B-C) mRNA expression of Trim63 (MuRF1) and Fbxo32 (Atrogin-1) in gastrocnemius muscles of control (n=3), gemcitabine- (n=3), FOLFIRINOX- (n=3) treated mice. Data are mean ± S.E.M. One-way ANOVA was performed and Tukey’s post-hoc analysis was performed to compare all the groups (A). Student’s t-test (B-C) was performed. p<0.05 is indicated as *, p<0.01 as **, p<0.001 as ***.
As previously mentioned, studies have demonstrated modification in ROS pathways upon treatment with chemotherapy such as FOLFIRI\textsuperscript{176}. We wanted to evaluate whether similar pathways were also activated upon treatment with gemcitabine and FOLFIRINOX. To this end, we measured the levels of reactive oxygen species in the gastrocnemius muscles via Electron Paramagnetic Resonance (EPR) by utilizing a superoxide-sensitive probe as demonstrated in previous studies\textsuperscript{268}. We observed a significant increase in ROS in the muscles of the mice treated with chemotherapy (Figure 44).
Figure 44: Chemotherapy induces ROS in gastrocnemius muscles in mice. EPR measurement of gastrocnemius muscle of control (n=5), gemcitabine- (n =5), FOLFIRINOX (n =5)-treated mice. Data are mean ± S.E.M. Student’s t-test was performed. p<0.05 is indicated as *, p<0.01 as **.
**Pharmacological blockade of oxidative stress rescues chemotherapy-induced muscle wasting**

To combat the increased levels of ROS in the muscles of the chemotherapy-treated mice, we utilized a superoxide dismutase mimetic, BMX-001 (hereafter termed as BMX). This drug acts as a mimic of SOD and is present in the nucleus, mitochondria, cytoplasm and the extracellular matrix. This compound has been approved by the FDA and is now currently in clinical trials for high grade glioma (NCT02655601), head and neck cancer (NCT03386500), multiple brain metastasis (NCT03608020) primarily as a radio-protectant. After two weeks of treatment with chemotherapy, mice were divided into two groups, and one group (n=5) was given BMX and the other vehicle control (n=5) (Figure 45A). We observed that the decrease in body weight, grip strength and rotarod performance was rescued in mice treated with BMX along with chemotherapy (Figure 45B, 46 A-D).
Pharmacological blockade of ROS rescues chemotherapy-induced body weight loss and muscle function. Schematic illustration of treatment of C57/BL6 with Gemcitabine (n=10) and FOLFIRINOX (n=10). After two rounds of chemotherapy, 5 mice in each group were treated with BMX-001. (B) Measurement of change in body weight after 8 weeks of chemotherapy treatment; control (n=10), gemcitabine- (n=5), gemcitabine and BMX- (n=5), FOLFIRINOX- (n=5), FOLFIRINOX and BMX- (n=5) treated mice. Data are mean ± S.E.M. One-way ANOVA was performed and Tukey’s post-hoc analysis was performed to compare all the groups. p<0.01 as **.
Figure 46: BMX rescues chemotherapy-induced muscle wasting. (A) Change in Rotarod Performance of control, gemcitabine-, gemcitabine and BMX-treated mice. (B) Change in Grip Strength of control, gemcitabine-, gemcitabine and BMX-treated mice. (C) Change in Rotarod Performance of control, FOLFIRINOX-, FOLFIRINOX and BMX-treated mice. (D) Change in Grip Strength of control, FOLFIRINOX-, FOLFIRINOX and BMX-treated mice. Data are mean ± S.E.M One-way ANOVA was performed and Tukey’s post-hoc analysis was performed to compare all the groups. p<0.05 is indicated as *, p<0.01 as **, p<0.001 as ***.
We observed a rescue in gastrocnemius muscle weight upon treatment with BMX and chemotherapy (Figure 47A). Hemotoxylin and Eosin (H&E) staining of gastrocnemius muscle sections demonstrated decrease in cross sectional area with chemotherapy which was rescued upon treatment with BMX (Figure 47B).
Figure 47: BMX rescues chemotherapy-induced muscle atrophy. (A) Post-necropsy measurement of gastrocnemius muscle of control (n=10), FOLFIRINOX- (n=5), FOLFIRINOX and BMX treated (n=5)- treated mice. (B) Representative images of H&E staining of gastrocnemius muscle sections of control (n=3), FOLFIRINOX- (n=3), FOLFIRINOX and BMX- (n=3), gemcitabine- (n=3), gemcitabine and BMX- (n=3) treated mice. Quantification of gastrocnemius muscle cross sections. Data are mean ± S.E. One-way ANOVA was performed and Tukey’s post-hoc analysis was performed to compare all the groups. p<0.05 is indicated as *, p<0.001 as ***.
The increase in MuRF1 and Atrogin-1 was abrogated with the use of BMX along with chemotherapy (Figure 48A). Moreover, the EPR studies also demonstrated a decrease in ROS in the muscles of the mice treated with BMX-001 along with chemotherapy (Figure 48B). Therefore, we concluded that chemotherapy-induced muscle wasting can be abrogated by scavenging reactive oxygen species in the muscles.
Figure 48: BMX decreases chemotherapy-induced ROS in gastrocnemius muscles. (A) mRNA expression of Trim63 (MuRF1) and FBXO3 (Atrogin-1) in gastrocnemius muscles of control (n=3), FOLFIRINOX-(n=3), FOLFIRINOX and BMX- (n=3), gemcitabine- (n=3), gemcitabine and BMX- (n=3) treated mice. (B) EPR measurement of gastrocnemius muscle of control (n=5), gemcitabine- (n=5), gemcitabine and BMX, FOLFIRINOX (n=3), FOLFIRINOX and BMX- (n=5) treated mice. Data are mean ± S.E.M. One-way ANOVA was performed and Tukey’s post-hoc analysis was performed to compare all the groups. p<0.05 is indicated as *, p<0.01 as **, p<0.001 as ***.
**Pharmacological Blockade of oxidative stress in tumor-bearing mice rescues chemotherapy-induced muscle wasting**

In order to have translational relevance, we wanted to investigate whether BMX would be successful in rescuing chemotherapy-induced wasting in tumor-bearing mice. We orthotopically implanted 40 C57 mice with KPC 1245 cells. We then randomly divided them into four groups of 10 mice. We injected one group with FOLFIRINOX, second with BMX and the third with FOLFIRINOX and BMX. We compared all these groups to the untreated tumor-bearing group. We started the BMX treatment at the same time as FOLFIRINOX, after 4 days of tumor-implantation. Unfortunately, this study did not show us the expected results and there was no rescue in body weight or muscle loss with BMX in the tumor-bearing mice treated with FOLFIRINOX (data not shown). We re-evaluated our treatment strategy and designed another one according to the ongoing clinical trials. We pretreated the mice twice with double dose (1mg/kg) of BMX, 4 days and 2 days before the start of the chemotherapy. FOLFIRINOX injections started from 4 days post implantation. 21 days-post implantation, we observed a significant rescue with BMX in body weight of the tumor-bearing mice which were treated with FOLFIRINOX (Figure 49). We also observed that FOLFIRINOX treatment of tumor-bearing mice caused more weight loss than just tumor-bearing mice, confirming our hypothesis that chemotherapy exacerbates cancer induced muscle wasting.
**Figure 49: BMX rescues chemotherapy-induced wasting in tumor-bearing mice.** Change in body weight of healthy control (n=8), KPC tumor-bearing (n=10), KPC tumor-bearing treated with FOLFIRINOX (n=10), KPC tumor-bearing treated with BMX (n=10), KPC tumor-bearing treated with FOLFIRINOX and BMX (n=10). Data are mean ± S.E.M One-way ANOVA was performed and Bonferroni’s post-hoc analysis was performed to compare all the groups. p<0.05 is indicated as *, p<0.01 as **, p<0.001 as ***.
On the 18\textsuperscript{th} day-post implantation, we recorded the grip strength and latency to fall of the mice. We observed that FOLFIRINOX treatment of the tumor-bearing mice did not decrease the grip strength of the mice significantly as that compared to untreated tumor-bearing mice. However, the decrease in grip strength was rescued by BMX (Figure 50A). On the other hand, latency to fall showed a trend to decrease in tumor-bearing mice treated with FOLFIRINOX as compared to the untreated group. This decreased was rescued by BMX treatment (Figure 50B).
Figure 50: BMX rescues muscle function in chemotherapy-induced cachexia in tumor-bearing mice. Decrease in grip strength (A), rotarod performance (B) of healthy control (n=8), KPC tumor-bearing (n=10), KPC tumor-bearing treated with FOLFIRINOX (n=10), KPC tumor-bearing treated with BMX (n=10), KPC tumor-bearing treated with FOLFIRINOX and BMX (n=10). Data are mean ± S.E.M One-way ANOVA was performed and Bonferroni’s post-hoc analysis was performed to compare all the groups. p<0.05 is indicated as *, p<0.01 as **.
Post-necropsy measurements demonstrated decrease in muscle weight of tumor-bearing mice treated with FOLFIRINOX compared to the untreated group. This decrease in muscle weight was rescued by BMX treatment (Figure 51A). It is worthwhile to mention that the tumor burden was significantly decreased in the group treated with FOLFIRINOX and BMX (Figure 51B). Therefore, we cannot conclude that the effect of BMX is strictly due to its effect on the muscles. However, due to the lack of better models to study the effect of a compound on skeletal muscle wasting independent of its effect on the tumor, we have to depend on the results of this study. We were successful in addressing this in chapter 3 but in this case, this is the best model available. We believe since BMX is not aggravating the tumor, but in turn is working synergistically to shrink it, this compound might be an effective therapeutic intervention to prevent chemotherapy-related wasting.
**Figure 51**: BMX rescues chemotherapy-induced wasting in tumor-bearing mice. Decrease in muscle weight (A) and tumor weight (B) of healthy control (n=5), KPC tumor-bearing (n=5), KPC tumor-bearing treated with FOLFIRINOX (n=5), KPC tumor-bearing treated with BMX (n=5), KPC tumor-bearing treated with FOLFIRINOX and BMX (n=5). Data are mean ± S.E.M. One-way ANOVA was performed and Bonferroni’s post-hoc analysis was performed to compare all the groups. p<0.05 is indicated as *, p<0.01 as **, p<0.001 as ***.
Upon evaluating the molecular markers, we observed an increase in the atrophy genes (MuRF1 and Atrogin) in the muscles of the tumor-bearing mice and those treated with FOLFIRINOX (Figure 5A and 5B). This increase in atrophy genes was rescued upon treating with BMX. We also evaluated the reactive oxygen species in the muscles of the mice via EPR and observed an increase in ROS in the muscles of the tumor-bearing mice with FOLFIRINOX and a reduction in ROS with BMX treatment (Figure 5C).
Figure 52: BMX rescues chemotherapy-induced ROS in the muscles of tumor-bearing mice. mRNA levels of Trim63 (A), Fbxo32 (B) and EPR measurements (C) in the muscles of healthy control (n=5), KPC tumor-bearing (n=5), KPC tumor-bearing treated with FOLFIRINOX (n=5), KPC tumor-bearing treated with BMX (n=5), KPC tumor-bearing treated with FOLFIRINOX and BMX (n=5). Data are mean ± S.E. One-way ANOVA was performed and Bonferroni’s post-hoc analysis was performed to compare all the groups. p<0.05 is indicated as *, p<0.01 as **, p<0.001 as ***.
Discussion

FOLFIRINOX and gemcitabine are two first line therapies for pancreatic cancer patients, which have significantly improved survival of patients\textsuperscript{271,272}. However, studies have established that chemotherapy can induce muscle and body weight loss in patients.

We measured the L3 muscle mass in pancreatic cancer patients at diagnosis and after treatment and correlated this mass with overall and progression free survival. We demonstrated that decreased muscle mass is highly prevalent among patients with pancreatic cancer and outperforms BMI as a prognostic indicator. Given the retrospective nature of our study, causal relationships cannot be determined definitively, and unmeasured confounding is possible. However, we tried to minimize this with our use of a well-characterized data set and correcting for all known potentially significant confounders. Next, selection bias may affect the results, as patients who did not have available CT imaging at diagnosis and at the time of next restaging were excluded from analysis. Lastly, while we use a data-driven approach to define cachexia at a single time point based on survival outcomes, this approach has been widely used in many studies with remarkably similar cut points for cachexia across various populations. To the best of our knowledge, this is the largest study evaluating the prognostic role of muscle wasting in pancreatic cancer patients with significant results.

Furthermore, our group wanted to evaluate the effect of chemotherapy alone on muscle and body weight. Experimental mouse models helped us to
demonstrate that FOLFIRINOX and gemcitabine induced body weight and muscle weight loss via increasing oxidative stress in the muscles. We validated this finding by inhibiting oxidative stress by utilizing a superoxide dismutase mimetic, BMX-001. This compound was preferred as it is already in clinical trials as a radioprotector in cancer treatment. It is important to mention that though using an antioxidant was sufficient to prevent chemotherapy-induced wasting in mice muscles, studies have shown antioxidants to increase tumor growth in experimental models. Therefore, this observation validated our use of BMX-001, which has been demonstrated to be a pro-oxidant in tumors while being antioxidants in other tissues. However, we observed that FOLFIRINOX did not increase the ROS levels in the muscles above the level of just the tumor-bearing mice. We, therefore hypothesized that there might be an increased oxidative damage which was not measured in the snapshot of the EPR measurement. This might be because EPR only measures free oxygen radicals present in the tissues and not damage that has already taken place. For this purpose, we are currently evaluating oxidative damage in the muscle tissues. We observed that BMX was able to rescue FOLFIRINOX induced wasting in pancreatic cancer models. Future studies are needed to demonstrate the effect of similar compounds in cancer models, but we believe our study is the first to provide understanding of the role of oxidative stress-mediated muscle wasting induced by chemotherapy.
Chapter 6: Discussion and Perspectives

Cachexia has been identified as a syndrome for centuries. Hippocrates had eloquently described this syndrome, “The flesh is consumed and becomes water…the shoulders, clavicles, chest and thighs melt away…the patient becomes thin and dry…this illness is fatal. [Regardless of cause] the treatment is similar; but few survive…” Even though there has been evidence of this syndrome in the medical literature for centuries, it has been shunned by the scientific community as a symptom of the disease and not a disease itself. This idea has changed immensely in the last couple of years wherein groups have demonstrated an increase in survival rates in preclinical models just by preventing cachexia and not decreasing the tumor burden. Our study adds to the increasing knowledge and understanding of this field and establishes unexplored avenues, to make a difference in combating this syndrome.

Our study revolved mainly around pancreatic cancer, since it is characterized by a dismal rate of survival and poor quality of life. Even after therapy, the five-year survival rate is one of the least amongst all cancers. The low survival rate is attributed to late diagnosis and early metastasis. Moreover, this dismal survival can also, in part, be attributed to cachexia since about 80% of pancreatic cancer patients suffer from this syndrome. The diagnosis of muscle wasting is a part of the manifestation of pancreatic cancer and studies have attributed inflammatory cytokines released by the tumor and the host body to be the main culprit behind muscle wasting. Based on this hypothesis, clinical trials have been performed targeting inflammatory cytokine but without any significant
success. This has led to a lot of curiosity and urgency in finding newer targets and therapeutic interventions and each study tells us a little more about this devastating syndrome.

Chapters 3 and 4 of this dissertation deal with investigating novel targets other than the inflammatory pathways, which are involved in the induction of cachexia. We focused on sirtuins because they are the switches between signaling pathways and metabolic networks and have been shown to play a role under energy modulations in the cell. Consequently, we did find a sirtuin, Sirt1, to be altered in all our cachectic models. Upon further analysis, we found that one of its downstream targets was NF-κB. This was again intriguing, since NF-κB is also regulated via cytokines. But clinical trials using anti-inflammatory agents did not show any success. This raises many questions - do other signaling pathways work through the same axis as cytokines but have different downstream mechanistic regulators? In that case, would it be necessary to target both arms of the pathway? We would also like to mention here that we did not observe a consistent difference in cytokine profiles in our models of cachexia as compared to the pancreatic epithelial cells or non-cachectic pancreatic cancer (data not shown). This observation also led us to speculate the validity of exploring inflammatory pathways as a therapeutic intervention for cancer cachexia.

We followed the axis downstream from NF-κB hypothesizing the role of ROS in induction of muscle atrophy. Subsequently, we found Nox4 to be modulated in cachectic muscles. Nox4 is also found to be upregulated in other disorders such as muscular dystrophy and hypertension. This led us to another
question – if the presence of a tumor is not necessary for the induction of this protein in the muscles, then can this phenotype be attributed to tumor-secreted factors or if is this a common defense mechanism of the body against any kind of disorder, inflammation or injury? Cachexia or muscle wasting is not only seen in cancer but also in diseases such as heart failure, chronic obstructive pulmonary disorder (COPD) and tuberculosis. All of these diseases lack an uncontrolled growing mass of cells. Therefore, the question is what is the systemic assault which is common amongst all these disorders that leads to the same phenotypic appearance and sometimes, also the same markers such as upregulation of Nox4? We hypothesize that the liver and the hypothalamus may be playing a role in this process. As previously mentioned in the Introduction of this dissertation, hepatic metabolism is closely linked to skeletal muscle metabolism and this might be common factor in all the diseases. There are liver-associated CD68+ macrophages which might be playing a role as well. Another important player in wasting is the hypothalamus, which has receptors for adipokines and is prone to inflammation via cytokines\textsuperscript{278}. It is possible that an inflamed hypothalamus elicits its effects in a similar way in all these diseases. However, due to time constraints we did not attempt to answer these questions.

The fifth chapter of this dissertation investigates the effect of chemotherapy on body and muscle weight loss. We found that chemotherapy on its own has a negative effect on body and muscle weight loss without the presence of a tumor. This result was neither counterintuitive or surprising, since the chemotherapies used to treat pancreatic cancer are extremely toxic agents. They are almost
equivalent to being called the necessary evil in the context of cancer treatment. These agents are known to increase ROS in the tumor cells in order to destroy them, therefore we hypothesized the same pathway of degeneration in the muscle cells. We did observe an increase in ROS in the muscles of the mice treated with chemotherapy. Consequently, our rationale pointed to the same pathway as described in chapter 4. Did we just unravel a common pathway via which both the tumor and chemotherapy are inducing muscle atrophy? Yes, we observed Nox4 to be upregulated in these muscles too (Data not shown). We pursued it and treated the mice with FOLFIRINOX and GKT, hoping to rescue the wasting similar to the tumor-bearing mice. Unfortunately, GKT was unable to rescue chemotherapy-induced cachexia. This might imply that though Nox4 is upregulated in the muscles, it might not be one of the main regulators of ROS induction. There might also be more confounders, such as compensation from other enzymes or drug interactions. Next, we attempted to utilize BMX to rescue chemotherapy induced wasting, the successful results of which, are discussed in chapter 5. BMX also proved to be able to combat chemotherapy-induced cachexia in tumor-bearing mice and might be a potential contender for clinical trials.

We would also like to mention that our studies focused mainly on skeletal muscle wasting in cachexia. Although we did measure fat content via DEXA scanning and post-necropsy adipose tissue weight, we did not investigate the mechanisms leading to adipose tissue wasting. To assess muscle function, we performed grip-strength and rotarod measurements. These are the gold-standard measurements for cachexia studies. Post-necropsy, we measured the
gastrocnemius muscles of the mice to assess muscle weight change. In future studies, we plan to measure more of the muscle groups of the leg – the quadriceps, external digitalis and the soleus. We utilized CT scans of patients to measure the muscle mass around the L3 vertebrae, which is currently the most accurate method of detecting loss of muscle mass. Similarly, we also plan to measure the lean body mass of mice via Magnetic Resonance Imaging (MRI) for a more accurate measurement of muscle mass.

Moreover, we focused on the mechanisms leading to protein degradation in the cachectic muscles. There are also studies which focus on the role of muscle stem cells in cancer-cachexia. It has been demonstrated that there is impaired regeneration of muscle cells in cachectic muscles. Therefore, in addition to protein degradation, the cachectic muscles possess the inability to regenerate the muscle fibers. It would be interesting to study the role and expression of Sirt1 and Nox4 in the muscle stem cells in the cachectic muscles. We could also determine the status of muscle stem cells in our models and treatments and, investigate whether oxidative stress plays a role in reprogramming the muscle stem cell fate.

The use of antioxidants has been controversial in the treatment of cancer since it is shown to exacerbate the disease\textsuperscript{273,279}. Therefore, it is not recommended that any antioxidant be used to scavenge the ROS generated in the muscles due to the tumor or chemotherapy. Interestingly, BMX-001 has been shown to act as an antioxidant in normal tissues and as a pro-oxidant in tumor tissues (Data generated by collaborators, not shown). We demonstrated that this compound was
successful in attenuating the high levels of ROS in cachectic muscles. However, we have not examined its effect on the oxidative state of the tumor.

Furthermore, our laboratory has observed distinct metabolic profiles in cachectic and non-cachectic pancreatic cancer patients. We have already demonstrated that SIRT1 and Nox4, two genes which can regulate various metabolic homeostasis pathways, are differentially expression in cachectic and non-cachectic PDAC patients. Studying the metabolomic profiles further can reveal the metabolic cues which might be regulating Sirt1 and Nox4. Since, we have access to patient samples of various stages, it would also be intriguing to map the metabolic and the genomic profile of these patients and analyze the pathways and genes, which give rise to this syndrome at various stages of cancer progression.

Our studies with two different models of cancer and chemotherapy-induced cachexia point towards a serious concern in cachexia research. The ongoing research attempting to combat cachexia in preclinical models do not consider the involvement of chemotherapy. Though these studies are important to elucidate the pathways and mechanisms involved in the induction of cachexia, their translational value cannot be truly assessed unless the effect of chemotherapy is also considered. This is because it would be highly unlikely for patients to be treated for cachexia and not for tumor burden.

There is still much to be done in the field of cancer cachexia. We have been successful in establishing novel targets, such as SIRT1 and Nox4, in cancer-induced cachexia. It is also important to mention that our studies in the chapters 3
and 4 established that the alterations in Sirt1 and Nox4 were the cause of the syndrome and not the result of the tissue damage. This is because overexpressing both these proteins rescued or degraded myotubes, respectively. We have also illustrated the role of oxidative stress in the induction of chemotherapy-induced cachexia which can be reverted with the use of BMX-001. The most promising factor of our studies is that all our therapeutic interventions have already passed the first phase of clinical trials and have been deemed safe for use. Our ongoing studies include investigating the effect of tumor and chemotherapy on cardiac muscle and function and, the role of post-translational modifications in cachectic muscles. We continue to work towards the understanding and treatment of this devastating syndrome.
References


