Cancer cachexia is a complex metabolic wasting syndrome that affects up to 80% of cancer patients and results in death in up to one-third of cancer patients. It is characterized by extreme, progressive skeletal muscle loss, cardiac dysfunction, as well as an abnormal metabolism. While research is growing in this field, there are still no clear diagnostic criteria or treatments for this condition and the mechanisms of cancer cachexia-associated muscle wasting are not well understood. Cancer cachexia remains an untreated condition with irreversible effects, poor prognosis of survival and a significantly reduced quality of life. Therefore, a great need exists to better understand this disease as well as possible treatment interventions. Research has shown that exercise interventions as part of cancer treatment have the potential to attenuate cachexia-associated muscle loss, inhibit tumor growth and improve quality of life. Most aerobic exercise interventions are easily accessible and affordable for cancer patients. Many questions remain regarding the most effective timing, extent, and intensity of exercise as a protective measure against cancer cachexia. Therefore, it is necessary to determine the effectiveness of different types and intensities of exercise to be able to develop a suitable exercise treatment to be used in conjunction with chemotherapy and nutritional interventions. Specifically, the effect of low intensity aerobic exercise as a treatment intervention needs to be assessed, as most cancer patients experience a lack of energy, physical fitness, self-esteem, and reduced quality of life, making it difficult for them to participate in high-intensity exercise. Therefore, the purpose of this study was to determine if low intensity treadmill exercise can act as a protective measure and treatment intervention against cancer cachexia in the male tumor bearing mouse.
To test this, 28 male mice were randomly selected and separated into four groups: sedentary non-tumor bearing (SED+NT; n=7), sedentary tumor bearing (SED+T; n=7), low intensity treadmill exercised non-tumor bearing (TM+NT; n=7), and low intensity treadmill exercised tumor bearing (TM+T; n=7). Mice were injected with tumor cells (T group; 5x10^5 LLC cells in flank) or remained non-tumor bearing (NT) for 4 weeks. During the 4 weeks, mice underwent a low intensity treadmill exercise training protocol (TM) or remained sedentary (SED). To examine the protective effects of exercise, grip strength, echocardiography, and tumor evaluations were taken at baseline and 4-week time points. Gastrocnemius, heart, and tumor tissues were collected 24 hours after the last exercise session of the protocol for further analyses. To better understand the molecular mechanisms and influence of autophagy behind cancer cachexia and exercise, Western Blotting and autophagic flux analyses were performed on these tissues.

SED+T mice exhibited the worst skeletal muscle function (grip strength: -23%) and cardiac function (fractional shortening: -8%) compared to all other groups, whereas TM+T mice showed a preservation of grip strength (-15%) and fractional shortening (-5%). Additionally, TM+T mice exhibited significantly smaller tumor masses and volumes (P < 0.05) compared to the SED+T group. Protein expression analysis via Western Blotting as well as autophagic flux analysis indicated potential influence of exercise on regulating autophagy, which is involved in regulating homeostasis between protein synthesis and degradation.

The data indicate potential effects of low intensity treadmill exercise on preserving muscle function and stunting tumor growth in cachectic mice. Low intensity exercise may be an effective and accessible treatment intervention for cancer patients. This information is crucial in understanding the significance of exercise in cancer patients and elucidating the importance of
timing and intensity of exercise as a protective measure against the detrimental effects of cancer cachexia.
EFFECTS OF LOW INTENSITY TREADMILL EXERCISE DURING CANCER CACHEXIA
IN THE MALE TUMOR-BEARING MOUSE

by

Louisa Tichy

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CHAPTER I: INTRODUCTION

Cancer cachexia is a complex metabolic wasting syndrome that affects up to 80% of cancer patients and results in death in more than 20% of cancer patients (Belloum et al., 2017). It is characterized by extreme, progressive skeletal muscle loss, with or without the loss of fat mass. Other symptoms include cardiac remodeling and dysfunction, as well as a negative protein and energy balance due to reduced food intake and abnormal metabolism (Fearon et al., 2011). Although low caloric intake can have a key role in cachexia-induced weight loss, the loss of skeletal muscle mass cannot be fully reversed by conventional nutritional support (Fearon et al., 2011). While research is growing in this field, the mechanisms of cancer cachexia-associated muscle wasting are not well understood and there are no clear diagnostic criteria or treatments for this condition. Cancer cachexia remains an untreated condition with irreversible effects, significantly reduced quality of life and if left untreated has a poor prognosis of survival.

Research has shown that exercise interventions as a part of cancer treatment can have a positive impact on physical fitness, attenuate muscle loss, inhibit tumor growth and improve quality of life (Fearon et al., 2011; Leal et al., 2020; Wasley et al., 2018). Most exercise treatments, especially aerobic exercise interventions, are easily accessible and affordable for cancer patients. Therefore, it is crucial to understand the metabolic alterations and molecular basis of cancer cachexia and to identify the importance of exercise as a protective and preventative measure.

However, even though research has indicated that exercise treatments have the ability to preserve muscle mass, inhibit tumor growth and positively impact quality of life, many questions remain regarding the most effective timing, extent and intensity of exercise as a protective measure against cancer cachexia. Each cancer development and progression is different and
dependent on the physical fitness, genetic constellation, nutrient intake, body composition and other characteristics of the cancer patients as well as the tumor type and stage. Therefore, it is necessary to determine the effectiveness of specific types and intensities of exercise to be able to configure a suitable exercise treatment in combination with chemotherapy and nutritional support for each individual cancer patient (Solheim et al., 2018).

**Problem Statement**

It is important to determine if aerobic endurance exercise training, in the form of structured, progressive exercise intervention concurrent with tumor bearing, can positively impact the progression of cancer cachexia. Exercise has the potential to act as an anti-inflammatory intervention against the negative characteristics of cancer cachexia, including muscle wasting, cardiac dysfunction, and decreased quality of life. Furthermore, it is important to determine the effectiveness of progressive, low intensity exercise interventions concurrent with tumor bearing as a protective measure against cancer cachexia, as some cancer patients may not be physically capable of sustaining a high-intensity exercise protocol.

**Purpose Statement**

This study was conducted to investigate the effects of low intensity treadmill exercise as a protective measure against cancer cachexia in mice compared to sedentary controls. Specifically, this study assessed the effects of the low intensity exercise intervention on muscle wasting, tumor growth, cardiac dysfunction and autophagic flux. Therefore, the purpose of this study was to determine if low intensity treadmill exercise can act as a protective measure and treatment intervention against cancer cachexia in male mice.
Variables

Independent Variables

1) The low intensity treadmill exercise protocol in the treadmill trained group or sedentary protocol in the sedentary group.
2) The injection of tumor cells in the tumor bearing group or saline sham injections in the non-tumor bearing group.

Dependent Variables

1) Muscle function measured as grip strength at baseline and sacrifice timepoints.
2) Body weight measured at baseline and sacrifice.
3) Absolute heart and gastrocnemius muscle weight and in relation to body weight.
4) Tumor mass (absolute and in relation to body weight).
5) Cardiac structure and function (Left Ventricular Diameter, Septal Wall thickness, Posterior Wall Thickness, and ejection fraction)
6) Autophagic flux measured via Fluorescent Microscopy.

Hypotheses

The primary hypothesis of this study is that exercise treatments in the form of endurance treadmill exercise training will have a positive impact on cancer cachexia by slowing its development. Specifically, we expect to find:

1) Low intensity exercise training will preserve skeletal muscle function in tumor bearing mice compared to sedentary counterparts.
2) Cardiac function will be preserved in treadmill exercised tumor bearing mice compared to sedentary tumor bearing mice.
3) Exercise training will inhibit tumor growth in tumor bearing mice.
Assumptions/Delimitations

The following assumptions are accepted for the purpose of this study:

1) The low intensity exercise protocol will represent an intensity of ~50% VO$_{2\text{max}}$ of each mouse in the specific intensity group.

2) The scale used for weight measurements is an accurate and reliable instrument for measuring body, muscle, or heart weight.

3) The BIOSEB Grip Strength Test is an accurate and reliable instrument for measuring grip strength of mice in grams.

4) Body weight, grip strength measurements as well as tumor evaluations will be measured under the same environmental circumstances each time.

5) The settings of the cell incubator for tumor cell growth are consistent throughout the process.

6) Tumor cell injections are of equal concentration for each animal.

7) Exercise performance is consistent throughout the 4-week treadmill protocol.

8) The echocardiography device is accurate and reliable in measuring heart structure and cardiac function of the mice.

Limitations

1) Animal research is not completely translatable to human research, especially since the tumor cells in this study will be injected subcutaneously instead of a spontaneously developing cancer as it is found in human cancer patients. Tumor development in human cancer patients would be more variable and less controllable.

2) The same tester will be used to obtain tumor evaluations, grip strength and body weight measurements, as there may be variations between testers.
3) Treadmill exercise training is considered involuntary exercise training and does not take psychological or other factors into consideration that could inhibit human cancer patients from partaking.

4) Food intake was not measured. While standard rodent chow was available *ad libitum*, exercise or tumor burden could have impacted daily food intake and therefore could have influenced cancer development.
CHAPTER II: LITERATURE REVIEW

Introduction

Cancer cachexia is a multifactorial wasting syndrome that is characterized by severe, ongoing muscle wasting. The mechanisms behind this condition are not well understood, there are no clear diagnostic criteria and no effective treatments. Therefore, the purpose of this literature review is to examine the known mechanisms of cancer cachexia, autophagy in muscle wasting and the effect of exercise on autophagy and cancer cachexia. First, the molecular mechanisms of autophagy at a basal level as well as during disease and cancer will be described. Then, cancer cachexia and the recent findings on its underlying mechanisms will be discussed, as well as the role of autophagy in cancer cachexia. Finally, the role of exercise as a potential protective intervention against cancer cachexia will be examined.

Autophagy

Regular Mechanisms of Autophagy

The term “Autophagy” is derived from the Ancient Greek terms “auto”, meaning “self”, and “phagein”, meaning “to eat” (Halling & Pilegaard, 2017). Autophagy is a normal physiological process that plays an essential role in protein homeostasis and cell survival through maintenance of cell bioenergetics and the clearance of damaged organelles and protein aggregates (Doherty & Baehrecke, 2018; Rabinowitz & White, 2010). By replacing outdated and damaged cellular components, autophagy can prevent the build-up of toxic protein aggregates while also recycling intracellular macromolecules, which can be reused for building new proteins or energy generation via cellular metabolism (Hansen et al., 2018; Rabinowitz & White, 2010). Therefore, the main purpose of autophagy is an intracellular quality control with constitutive autophagy occurring at a basal level, as a “housekeeping function”, and autophagy induced by
selective targets, for example invading bacteria or damaged proteins, so the cell can sustain its’ metabolism and survive during stress or starvation (X. Li et al., 2020; Mizushima, 2018).

Autophagy can be divided into three major types: Macroautophagy, Microautophagy and Chaperone-mediated Autophagy. These are three different processes with the same final result of delivering the cytoplasm to a degradative organelle and degrading the substrate proteins (Klionsky, 2005). Macroautophagy is the best studied type and is usually referred to as “Autophagy”, as it will be in this literature review (Parzych & Klionsky, 2014). Autophagy occurs in different steps that can be divided into the induction, assembly, and formation of autophagosomes, fusion, and degradation and recirculation stage. Under normal physiological conditions and short periods of starvation, the primary cell degradation system is the ubiquitin-proteasome system (Mizushima, 2007). The two main triggers for the induction phase of autophagy are prolonged nutrient starvation or oxidative stress, for example a depletion in leucine can trigger autophagy in the muscle and heart (Mizushima, 2007). Once the autophagic processes are induced, a double membrane bound vesicle, also known as the pre-autophagosome or phagophore, forms in the cytosol. The phagophore elongates and sequesters the cytoplasm or targeted cargoes in the cytoplasm, leading to the maturation of the autophagosome. The autophagosome then fuses with a lysosome, releasing the inner vesicle, also called the autophagic body, into the lumen where degradation can occur. The degradation process of the sequestered cargo can now occur in the fused autophagolysosome by resident hydrolases. Autophagosomal contents are broken down into amino acids or peptides and the resulting macromolecules can be recycled and reused by the cell (Hansen et al., 2018; X. Li et al., 2020; Mizushima, 2018).
Autophagy can be either non-selective, for example bulk degradation to support the metabolism during starvation, where autophagosome membranes cannot recognize what they enclose and sequestration happens in a random manner (Mizushima, 2007), or selective, for example the degradation of excess peroxisomes or mitophagy as the degradation of mitochondria (Klionsky, 2005; Rabinowitz & White, 2010).

**Regulation and Signaling of Autophagy**

Many genes and proteins are involved in the signaling and regulation of autophagy. About 42 ATGs (autophagy-related genes) have been identified, that are involved in the regulation of the autophagic processes. Sixteen of these are classified as the “core” ATGs and are used by both non-selective and selective autophagy, whereas others are only specific to certain selective autophagic processes, for example mitophagy (Mizushima, 2018). One of the key players during autophagy is mTORC1, which is a central regulatory kinase regulating protein synthesis and cellular growth. It is a negative regulator of autophagy and inhibits the process at the transcriptional level (Escobar, 2018). During normal physiological, nutrient-rich conditions, active mTORC1 kinase phosphorylates ATG13 and inhibits an interaction of ATG14 with ULK1. ULK1, which is an essential positive regulator of autophagosome formation in mammals, binds to mTORC1. MTORC1 then inhibits the induction of autophagy by phosphorylating ULK1 (Rabinowitz & White, 2010). The endocrine system seems to regulate autophagy *in vivo*, with insulin being one of the primary endocrine regulators, as it is the master hormone of the fed state and can therefore block autophagy in nutrient-rich conditions. As mentioned above, starvation or external stressors are triggers for the induction phase of autophagy. When there is a starvation situation, numerous signaling pathways inactivate mTORC1 kinase activity to reduce energy demand by suppressing cell growth. Autophagy is induced for stress adaptation and survival,
mTORC1 dissociates from the ULK1 complex, which is then free and can trigger autophagosome nucleation and elongation (Rabinowitz & White, 2010). Besides mTORC1 as a key upstream regulator, AMPK activation also regulates the key upstream initiator of autophagy, ULK1 (Hansen et al., 2018). A series of phosphorylation processes then leads to the formation of a phagophore. In mammals, LC3 then conjugates to the membrane and controls the elongation of the phagophore, which then closes and forms an autophagosome. As mentioned earlier, the autophagosome fuses with a lysosome, which releases lysosomal hydrolases into the vesicle that results in the degradation of the sequestered content (Park et al., 2019). The primary signal for degradation is the ubiquitination, where ubiquitin is recognized and bound by autophagy receptors, such as p62. These receptors interact with LC3, which leads to the delivery of the cargo to the autophagosomes (Rabinowitz & White, 2010). LC3 is the most frequently used protein for detection of autophagic structures in mammalian tissues, because the entire pathway can be followed with the LC3 molecular marker (Mizushima, 2018). Another central regulator in the autophagic process is Beclin1, which regulates the size and number of autophagosomes needed in the degradation process. Beclin1 is therefore important for the phagophore and autophagosome formation, which will be of special interest when looking at the functions of autophagy in disease and cancer, as well as during exercise (Yun & Lee, 2018).

**Autophagy in Disease and Cancer**

As mentioned earlier, under normal physiological conditions, autophagy plays an important role in protein and organelle quality control, regulating organismal development, sustaining energy homeostasis and cooperating with the adaptive immune system (X. Li et al., 2020). Basal level autophagy prevents the toxic buildup of damaged proteins and organelles and ensures adequate nutrient availability, important for survival in stress situations (Rabinowitz &
Yet, in disease states autophagy seems to be dysfunctional, leading to the accumulation of abnormal and damaged organelles and proteins, which results in the formation of intracellular aggregates and an imbalance between protein synthesis and degradation. These aggregates of damaged material prevent autophagy from fighting against infectious pathogens and can disrupt cellular homeostasis (Escobar, 2018; X. Li et al., 2020; Liang et al., 2020). It has been suggested that failure of autophagosome fusion with lysosomes or the aggregation of misfolded proteins may be involved in myodegenerative disease, and the accumulation of autophagosomes has been reported in some cardiac diseases, such as coronary artery disease, hypertension and congestive heart failure in humans, rodents and isolated stressed cardiomyocytes (Levine & Kroemer, 2008). Research using mice models has shown that long-lived cells like neurons can be affected by dysfunctional autophagy, indicating a connection between defects in autophagy and neurodegenerative diseases, aging, and lifespan (Mizushima, 2018).

Autophagy’s role in cancer is complex and its function depends on tumor type, tumor stage, and driving oncogene and suppressor gene constellations of the tumor (Kimmelman & White, 2017). The role of autophagy in cancer remains controversial and it seems autophagy has a dual role in cancer, either tumor-suppressive or tumor-promoting, depending on the stages of the cancer development as well as the nutrient availability, immune system, and microenvironmental stress. The tumor-suppressive role during the early stages of tumorigenesis acts as a quality control mechanism to prevent tumor initiation and suppress the progression of the cancer. Intact autophagy can suppress the cancer development and prevent chronic tissue damage and inflammation by inhibiting the accumulation of oncogenic p62 protein aggregates, limiting genome damage and mutation and inducing tumor cell death. (Kimmelman & White,
Beclin1, important in the formation of phagophores, has been shown to play a role in the suppression of tumors. Studies using cancer cell lines and mouse models have shown that a loss of Beclin1 resulted in decreased autophagy levels and an increase in tumor cell proliferation (Yun & Lee, 2018). Another study found that Beclin1 heterozygous mice developed various neoplasms, indicating that the deletion of autophagy genes, like Beclin1, can result in the initiation of tumorigenesis in certain mouse models (Kimmelman & White, 2017). Excessive reactive oxygen species (ROS) production due to damage to the mitochondria can promote carcinogenesis, which can also be prevented by intact basal autophagy (Yun & Lee, 2018).

Although autophagy can have a tumor-suppressive function in some cancers, it may also be tumor-promoting and support tumor growth and tumor cell survival. Defects in the autophagic process are associated with increased tumorigenesis due to the inhibition of the degradation of damaged intracellular components, but tumor cells can also exploit autophagy, by increasing basal autophagy levels, to limit tumor necrosis, inflammation and increase nutrient availability needed for tumor progression and survival (Mathew et al., 2007). Especially in late stages of advanced cancers, autophagy can promote tumor survival and growth. Tumor cells are exposed to stressful microenvironmental conditions, such as hypoxia and nutrient deprivation due to under established blood supply to the tumor tissue. In these conditions, autophagy can help overcome the stresses and fulfill the high metabolic and energetic demands of tumor cells by recycling intracellular components via the autophagic processes and supply metabolic substrates as an energy fuel to the tumor cells. Besides providing nutrients to the tumor cells, autophagy can also help maintain mitochondrial function, prevent cancer cell damage, limit inflammation, and maintain genome stability. The adaptations of autophagy enhance stress tolerance in the
tumor cells, result in increased tumor proliferation and development and promote aggressiveness of cancers by facilitating metastasis of the tumor cells (Kimmelman & White, 2017; X. Li et al., 2020; Yun & Lee, 2018). One of the biomarkers detected during tumorigenesis is p62. The accumulation of p62 promotes cancer development and has been used as a marker for autophagy inhibition or defects in autophagic degradation as it is upregulated when autophagic processes are defective. Under normal conditions when autophagy is intact, LC3 directly interacts with p62, which would then be specifically degraded by autophagy. Patients with gastrointestinal cancer, prostate cancer or lung adenocarcinoma showed an abnormal accumulation of p62, suggesting that p62 is correlated with cancer development and progression and limiting the accumulation of p62 via autophagic processes may suppress tumorigenesis (X. Li et al., 2020).

As mentioned above, the role of autophagy in cancer is complex and the role of autophagy as a target for chemotherapeutic interventions needs further investigation. Studies have shown that some cancer cells, for example some breast cancer cells, keep growing even when autophagy was inhibited, whereas other cancer cells are dependent on autophagy for further development and cell survival. In some cancer cells, autophagy increased the resistance to chemotherapeutics and has been suggested to support survival of dormant tumor cells (Levy et al., 2017; X. Li et al., 2020). While other studies have shown that inhibiting autophagic processes in autophagy-dependent tumor cells could enhance and increase the efficacy of anticancer therapy (X. Li et al., 2020).

Overall, autophagy is an intracellular evolutionarily conserved process that, at basal levels, is necessary for intracellular quality control and protein homeostasis. The role of autophagy during cancer is very complex and the mechanisms behind it are not well understood.
More research in this area is required in order to leverage cancer treatments aimed at autophagy in a cancer specific and time dependent manner.

Cancer Cachexia

Definition, Symptoms and Statistics

Cancer cachexia is a multifactorial syndrome, which is characterized by involuntary and pathological ongoing loss of skeletal muscle - with or without the loss of fat mass - that cannot be fully reversed by nutritional support, and associated with anorexia, inflammation, reduced physical, emotional and social well-being and impaired quality of life (Baracos et al., 2018; Blauwhoff-Buskermolen et al., 2017; Dolly, 2020; Loumaye & Thissen, 2017). The prognosis for survival is poor and there are no clear diagnostic criteria for this condition. Early diagnosis is limited due to the lack of safe accessible and non-invasive tools to detect muscle wasting and the individual variability in development and progression of cancer, depending on tumor stage and nature, genetic predisposition, food intake and physical activity level (Loumaye & Thissen, 2017). Cancer cachexia is prevalent in about 35% of cancer patients but can be up to 80-90% for gastric and pancreatic cancers. It is highly associated with cancer of the liver, stomach, pancreas, lung, bowel and esophagus, especially in advanced stages of cancers (Baracos et al., 2018). Cancer cachexia results in the death of up to 20-25% of patients (Dalise et al., 2020; Dolly, 2020). The development of cancer cachexia can be divided into three stages: pre-cachexia, cachexia, and refractory cachexia. Research on animal models has indicated that minimizing or reversing muscle loss leads to prolonged survival, which suggests that the goal should be an early cancer cachexia detection and maintaining muscle mass in cancer patients (Loumaye & Thissen, 2017). Currently, there is no effective treatment or standard of care for cancer cachexia, rather cancer cachexia can impair the effectiveness of chemotherapy due to reduced quality of
life and abnormal metabolism. It has been suggested that a multimodal interventional approach with a combination of nutritional support, pharmaceuticals and a suitable program of physical activity could be effective in preserving muscle mass, increasing quality of life and prolonging survival (Argilés et al., 2019; Baracos et al., 2018; Dolly, 2020; Solheim et al., 2018).

**Molecular Mechanisms of Cancer Cachexia**

The molecular mechanisms of cancer cachexia are not well understood but anorexia, abnormal metabolism, inflammation induced by tumors, as well as an imbalance between protein synthesis and degradation seem to be key players in the progression of cancer cachexia. Most patients with cancer cachexia experience an increased resting energy expenditure accompanied by an energy intake that is typically lower than the energy expenditure. Tumors, organs and other tissues are actively competing for energy fuels and biosynthetic substrates, leading to an abnormal metabolic state (Baracos et al., 2018; Loumaye & Thissen, 2017). Increased systemic inflammation is one of the characteristics of cancer cachexia, suggesting cytokines and myokines are implicated in skeletal muscle loss (Daou, 2020). The tumor itself and the immune system in response to the tumor release proinflammatory cytokines which cause a systemic inflammatory state. The primary mediator of muscle cachexia is IL-6, which suppresses protein synthesis, promotes increased energy expenditure and decreases fat mass (Loumaye & Thissen, 2017; Siff et al., 2021). TNFa is another pro-inflammatory biomarker of the muscle wasting process found in cancer cachexia. Both cytokines are upregulated in different cancer cachexia animal models, which lead to cachexia and the early death of these animals. In humans, TNFa levels are not associated with survival in cancer patients but IL-6 levels have been shown to be a predictive factor for survival in advanced cancer patients (Loumaye & Thissen, 2017). These two cytokines as well as myostatin are involved in the primary pathways resulting in protein degradation and
muscle atrophy during cancer cachexia. IL-6, TNFα and myostatin bind to their respective receptor on the cell surface, which then activates NF-κB, STAT3 and SMAD2/3 pathways. The upregulation of these pathways leads to an increased expression of atrogin1 and MuRF1, which then induce protein degradation and muscle wasting via the ubiquitin-proteasome system (UPS) and autophagy (Daou, 2020; Siff et al., 2021; Yang et al., 2020).

As mentioned in an earlier section, autophagy has a tumor-suppressive or tumor-promoting role in cancer and its mechanisms are poorly characterized. Cancer cachexia induced muscle wasting is associated with increased levels of autophagic processes leading to an imbalance between protein synthesis and protein degradation (Penna et al., 2019). On the other hand, another study on breast cancer patients showed that those with an increased number of LC3 puncta experienced an overall improvement and metastatic-free survival, suggesting that an increase rather than a decrease in autophagy might be beneficial during cancer therapy (Mulcahy Levy & Thorburn, 2020). When looking at muscle cells, most studies have shown that an upregulation of autophagy and UPS is directly activated in the muscle cells by the pro-inflammatory cytokines released from the immune system to fight the tumors, the pro-inflammatory cytokines released by the tumors themselves or a combination of both. Excessive activation of these two pathways leads to an increased breakdown of muscle tissue (Baracos et al., 2019; Loumaye & Thissen, 2017; Siff et al., 2021). Skeletal muscle wasting and atrophy during cancer cachexia can therefore be initiated by both UPS and autophagy. Increased proteolysis via UPS is characterized by an increased expression of atrogin1 and MuRF1, whereas cancer patients also experience an increased expression of autophagic genes, such as Beclin1, p62 or LC3B-II. TNFα and the activation of NF-κB pathways seem to stimulate autophagy in cachectic mice models (Miao et al., 2017; Siff et al., 2021). Tumor cells not only contribute to
muscle wasting by releasing pro-inflammatory cytokines leading to systemic inflammation and an increase in autophagy and UPS, but also by directly capturing nutrients and energy fuels, depriving other tissues of these nutrients, which leads to a shift in metabolism (Aquila et al., 2020; Baracos et al., 2019).

**Cancer Cachexia and Skeletal Muscle Wasting**

Skeletal muscle wasting is the main symptom associated with cancer cachexia. The major metabolic dysfunction leading to muscle atrophy during cancer cachexia is the imbalance between protein synthesis and protein degradation, with an increased degradation and a decreased protein synthesis. One of the characteristics in human gastrointestinal cancer patients is a significantly decreased cross-sectional area of muscle with cachexia and muscle loss, whereas other cancer patients for example with non-small-cell lung cancer did not experience any changes when comparing cachectic to non-cachectic patients (Dolly, 2020). Since cancer cachexia and the mechanisms behind muscle wasting due to cancer cachexia are not well understood, studies have focused on analyzing the skeletal muscle alterations in cancer cachexia mainly using animal models, with the two most studied preclinical models being Lewis Lung Carcinoma (LLC) and colon-26 adenocarcinoma (C26) in mouse models (Dolly, 2020). The major findings in preclinical studies are that proteolysis is increased, via autophagy and UPS, and protein synthesis is decreased. One of the main pathways, as mentioned earlier, that can lead to muscle wasting is the upregulation of TNFa and the activation of NF-kB by pro-inflammatory cytokines in the skeletal muscle (Yang et al., 2020). During muscle wasting, FoxO3 is another gene that is upregulated and promotes proteasomal degradation of proteins via the autophagic processes (Aquila et al., 2020). Additionally, mitochondrial dysfunction is another characteristic observed in cachectic rodent models, which is associated with muscle atrophy. Mitochondrial
quality and protein content are decreased leading to decreased ATP synthesis and the inability of
the mitochondria to provide the needed energy. Mitochondrial dysfunction ultimately leads to an
increase in mitophagy, selective autophagic processes that degrade mitochondria (Dolly, 2020;
Siff et al., 2021).

Due to the molecular mechanisms of cancer cachexia, there is an increase in systemic
inflammation which causes abnormal changes in metabolism. As mentioned earlier, tumor cells
deprive other tissues of necessary nutrients and energy fuels, which leads to an estimated
increase in muscle metabolism of about 40-50% in cachectic cancer patients (Aquila et al.,
2020). Overall, the alterations in muscle metabolism and the resulting skeletal muscle wasting
are characterized by decreased muscle strength, decreased movement, decreased energy,
increased hospitalization rates and a decreased quality of life (Patel & Patel, 2017). Therefore,
the management of muscle wasting and the maintenance or preservation of skeletal muscle mass
during cancer cachexia is crucial for survival. One study by Dalise et al. (2020) compared
oxidative metabolism, skeletal muscle strength and endurance of oncologic patients and controls
and found that cancer patients had significantly higher lactate levels while also performing
shorter exercise trials than controls. Maximum voluntary contraction during isometric exercises
was also significantly reduced and cancer patients fatigued more easily and earlier during
endurance trials than the control group. These data suggest that it is important to first assess
muscle function and develop a suitable rehabilitative intervention to manage muscle wasting
during cachexia. It also showed that muscle oxidative metabolism in cancer patients was
impaired and it is crucial to maintain physical fitness to reduce fatiguability and improve quality
of life (Dalise et al., 2020).
Cancer Cachexia and Cardiac Muscle Wasting

Besides skeletal muscle atrophy and wasting, most cancer patients and cachectic animals experience cardiac muscle wasting or cardiac atrophy as well, which is characterized by remodeling and dysfunction of the heart that can result in heart failure. Similar to skeletal muscle wasting, cardiac cachexia decreases quality of life and results in a reduced survival in about 40% of cachectic individuals (Aquila et al., 2020; Kazemi-Bajestani et al., 2014; Murphy, 2016; Rausch et al., 2021). Some of the symptoms of the cardiac abnormalities that patients experience are shortness of breath, fatigue, and impaired exercise tolerance. The molecular mechanisms behind cardiac muscle wasting are similar to skeletal muscle wasting, with an upregulation of autophagy markers such as LC3, Beclin1 and p62 noticeable in skeletal and cardiac muscles of cachectic rats (Rausch et al., 2021). In addition to cardiac muscle wasting occurring due to cancer cachexia itself, some chemotherapies may also result in cardiac dysfunction causing cardiotoxic effects. Cardiotoxicity limits the effectiveness of other anti-cancer treatments, leading to even more reduced quality of life and poor prognosis. Therefore, treatment of cardiac cachexia and cardiac dysfunction could lead to an improved effectiveness of chemotherapy (Kazemi-Bajestani et al., 2014; Murphy, 2016). As mentioned above, the underlying molecular mechanisms of cardiac muscle wasting are similar to skeletal muscle wasting, with the primary mechanism being the activation of autophagy pathways and the ubiquitin-proteasome system. The activation of both these pathways leads to an increased degradation of intracellular proteins leading to an imbalance of protein synthesis and protein degradation and therefore resulting in atrophy or wasting of the muscle. This abnormal metabolism leads to the typical phenotype of cardiac remodeling during cancer cachexia with the attributes of decreased heart mass due to atrophy, decreased left ventricular posterior wall thickness, and an impaired ejection fraction and
fractional shortening in mice (Belloum et al., 2017). The combination of cardiac muscle wasting, oxidative stress and inflammation during cancer cachexia will ultimately lead to heart failure (Belloum et al., 2017; Kazemi-Bajestani et al., 2014; Murphy, 2016). Similar to skeletal muscle wasting, elevated IL-6 levels are associated with left ventricular dysfunction and TNFa, which activates MuRF1 pathways, and can promote cardiac muscle atrophy (Belloum et al., 2017). One retrospective study by Barkhudaryan et al. (2017) found that BMI, heart weight and right ventricular wall thickness were significantly lower in cachectic gastrointestinal cancer patients compared to control groups (Barkhudaryan et al., 2017). Other studies have found that gastrointestinal, pancreatic and non-small-cell lung cancer patients, who had died of cardiac cachexia, experienced left ventricular remodeling, reduced heart masses, left ventricular wall thinning and increased fibrosis (Murphy, 2016). There are similar findings in preclinical mouse and rat models, which found that C26 tumor-bearing mice and Yoshida-130 tumor-bearing rats experienced left ventricular atrophy and cardiac remodeling. Cardiac remodeling was characterized by the dilation of the chambers and thinning of interventricular, septal and posterior walls, systolic and diastolic dysfunction as well as fibrosis with an ultimate result of heart failure (Kazemi-Bajestani et al., 2014; Murphy, 2016).

Overall, there are no current treatments or FDA approved drugs to counteract the progression of skeletal muscle and cardiac wasting during cancer cachexia. Since studies have shown that the maintenance of muscle mass and function and the management of muscle wasting are important for quality of life and survival, it is crucial to further investigate viable treatment options. One possible cost-effective and easily accessible treatment being aerobic exercise.
Effectiveness of Exercise Interventions during Cancer Cachexia

Exercise and Autophagy

A basal level of autophagy is necessary to preserve muscle mass and the integrity of the myofibers. Dysregulation or dysfunction of autophagy has been associated with myopathy and muscle atrophy, as is the case during cancer cachexia (Escobar, 2018; Halling & Pilegaard, 2017; Liang et al., 2020). ATG5 or ATG7-null mice, missing two of the autophagy-related genes, experienced an accumulation of abnormal mitochondria and ubiquitinated protein aggregates which resulted in muscle weakness and dysfunction (Rocchi & He, 2017). This shows that autophagy has an essential role in muscle function. Exercise may be a stress stimulus that can trigger autophagy and may induce non-selective autophagy and selective mitophagy in the skeletal and cardiac muscles of animal models as well as human cancer patients (Escobar, 2018; Rocchi & He, 2017). It has been shown that autophagic activity is required to acquire health benefits from exercise by degrading damaged proteins and organelles due to heat, pH changes, oxidative stress or mechanical stress during exercise. The extent to which autophagy is active depends on the magnitude of stress and protein damage within the cell, which is highly dependent on intensity and duration of the exercise intervention (Escobar, 2018). The upregulation of autophagy in skeletal and cardiac muscle after exercise can persist for several days. After just a single bout of treadmill exercise, several studies have found an increased content of autophagosomes and LC3-II in mouse skeletal muscles, as well as a decreased p62 content. In human models, the autophagic response is more complex with an increased content of LC3-II noticeable in ultra-endurance runners but a decreased LC3-II content in lower durations of running (Halling & Pilegaard, 2017). Another study found that in mice, a single bout of high intensity exercise is more efficient for inducing autophagy than moderate intensity exercise.
training of longer duration, whereas in humans only high intensity but not low intensity exercise interventions activated autophagic pathways (Rocchi & He, 2017). Therefore, it is important to find the appropriate duration and intensity level of exercise that can consistently induce autophagy in skeletal and cardiac muscle. Exercise-induced autophagy has been shown to be an effective treatment in slowing down myopathies like sarcopenia or muscle wasting by improving the quality of mitochondria, maintaining protein homeostasis and maintaining muscle function (Liang et al., 2020). The main pathways to initiate autophagy as an endurance exercise response is the exercise-induced activation of AMPK. With increasing exercise duration, AMPK has been reported to increase as well. AMPK activation leads to the phosphorylation of ULK1 in the muscle and the inhibition of Akt/mTORC1 signaling while also initiating formation of autophagosomes via AMPK/ULK1 activation. This process enhances the expression of MuRF1 and LC3-II which induces the upregulation of autophagic processes (Escobar, 2018; Liang et al., 2020; Park et al., 2019; Rocchi & He, 2017). Preclinical as well as clinical studies support this, as they have found increased LC3, Beclin1 protein content and autophagic flux in skeletal and cardiac muscle after endurance exercise (Brandt et al., 2018; Wu et al., 2019). FoxO3 signaling pathway has also been reported to play an important role in regulating the core ATGs and therefore the induction of autophagy, with an increased FoxO3 mRNA and protein content being observed in human and mouse skeletal muscle after an acute endurance exercise intervention (Halling & Pilegaard, 2017). The intensity and duration at which exercise influences autophagic processes is still poorly understood. Some studies indicate that aerobic exercise of 60min or more at 55-70% VO2max can stimulate autophagic activity, others suggest ultra-endurance exercises induce autophagy, while others say that moderate intensity bouts also promote autophagic activity but only when the duration is above 20min (Brandt et al., 2018; Escobar,
2018; Rocchi & He, 2017). One study even suggested that autophagy is more likely to be induced when intensities are sufficiently elevated, and exercise is performed in a fasted state. They found a reduced LC3-II protein amount and LC3-II/LC3-I ratio, which indicates a decreased autophagosome content, after moderate intensity endurance training of 55% and 70% VO2max for 120min in mice in both fasted and fed states. They also observed an increased phosphorylation of ULK1, with a greater effect when mice performed high intensity exercise in a fasted state (Martin-Rincon et al., 2017). Exercise could not only play a role in maintaining or improving muscle quality and function by inducing autophagy which leads to the upregulated degradation of damaged proteins, mitochondria, and other organelles, but also have a positive impact on age-related pathologies that autophagy has been reported to be involved in, for example cardiomyopathy, type 2 diabetes, or cancer. The most effective duration and intensity of exercise has yet to be determined based on the various changes in tumor progression and the influences of chemotherapy interventions used to treat the cancer (Escobar, 2018; Liang et al., 2020; Martin-Rincon et al., 2017; Wu et al., 2019).
Exercise and Cancer Cachexia

Endurance exercise has been known to be a potential anti-inflammatory stimulus by inducing the release of anti-inflammatory cytokines and inhibiting the release of pro-inflammatory cytokines. This leads to the establishment of an anti-inflammatory environment and the ability to reduce chronic inflammation, as it is found during cancer cachexia (Belloum et al., 2017; Niels et al., 2020). During cancer cachexia, maintenance of muscle mass and management of muscle wasting processes have been shown to be of importance to prolong chances of survival (Hardee et al., 2019). Exercise as an anti-inflammatory therapy might have the ability to attenuate or even reverse the processes of muscle atrophy and wasting by attenuating abnormalities in muscle metabolism, leading to an enhanced lifespan (Belloum et al., 2017; Solheim et al., 2018). Not only is cancer cachexia associated with muscle wasting and
strength loss, but endurance capacity is also significantly reduced in cachectic patients due to mitochondrial dysfunction in skeletal and cardiac muscle. Another benefit of aerobic exercise training is therefore the ability to prevent or restore the tolerance to physical activity, improve resistance to muscle fatigue, increase effectiveness of anti-cancer therapies and better quality of life (Aquila et al., 2020; Argilés et al., 2019; Belloum et al., 2017; Hardee et al., 2019).

One of the main anti-inflammatory cytokines released by the muscle, also called a myokine, as a result of exercise training is IL-6. While IL-6 is considered a pro-inflammatory cytokine when released by the tumor and immune system as a response to tumor cells, it is considered an anti-inflammatory myokine when release is induced by exercise interventions (Daou, 2020). When the myokine IL-6 is circulating it inhibits the TNFα pathway and stimulates AMPK signaling. Some of the effects associated with increased IL-6 myokine release are improved glucose uptake, insulin sensitivity, attenuation of the muscle wasting effects of cancer cachexia by stabilizing protein homeostasis and reduction of tumor growth (Daou, 2020; Ferreira et al., 2017; Leal et al., 2020).

Several studies indicated that exercise resulted in significant improvements in muscle mass and strength both before and during anti-cancer treatment (Niels et al., 2020; Solheim et al., 2018). However, the most effective mode, duration, and intensity of exercise as a therapy option during cancer cachexia has yet to be determined. Most studies focused on the effects of high or moderate intensity exercise, only a few studies considered looking at low-intensity exercise interventions. Two studies showed that short-term high intensity interval training on a treadmill resulted in the reduction of tumor volume, increased IL-6, improved running capacity and increased survival in LLC and C26 tumor-bearing mice (Alves et al., 2018; Leal et al., 2020). Some preclinical studies using moderate intensity exercise protocols showed that this type of
exercise can improve the regulation of autophagy, decrease oxidative stress, reduce tumor
growth, manage muscle atrophy and even stimulate hypertrophy, and decrease MuRF1 and
atrogin-1 gene expression in tumor-bearing mice and rats (Ballarò et al., 2019; Leal et al., 2020;
Parry & Hayward, 2018). Another study showed that moderate aerobic exercise lead to
significantly reduced necrosis, inflammation and fibrosis of cardiac muscle and reversed the
impairment of left ventricular ejection fraction in C26 tumor-bearing mice (Fernandes et al.,
2020). Only one study compared different treadmill exercise intensities and found that high
intensity exercise every two days was more efficient in limiting muscle atrophy and improving
quality of life in tumor-bearing mice compared to moderate intensity exercise every two days
(Jee et al., 2016). However, there was still a positive exercise effect on muscle function and
quality of life for both intensities. The researchers developed behavioral tests to determine
changes in quality of life based on step counts, food intake and body weight changes. (Jee et al.,
2016). Only one study looked at the effects of low-intensity exercise on cancer cachexia-induced
muscle atrophy in AH130 Yoshida tumor-bearing male Wistar rats (Tanaka et al., 2019). They
found that low-intensity treadmill exercise inhibited muscle mass loss through regulation of the
UPS, autophagy and AMPK signaling and suggested that low-intensity exercise could potentially
be an effective therapeutic intervention against cancer cachexia (Tanaka et al., 2019).

Overall, while these studies show that exercise has the potential to become a therapeutic
intervention, there is not enough evidence to determine which intensity and duration of
endurance exercise is the most efficient to counteract cancer cachexia (Niels et al., 2020).
Especially when cancer patients are already undergoing chemotherapy, they might be too busy or
overburdened to engage in additional treatment, may suffer from high psychological stress, and
may experience increased fatigue, which makes it difficult to determine the intensity and primary
target of exercise (Bayly et al., 2017). One study focused on the psychological adjustments and motivational attitudes of gastrointestinal and lung cancer patients with established cancer cachexia regarding regular exercise. The results of the questionnaire showed that the overall physical activity level of these cancer patients was low with only a few of them participating in regular structured exercise (Wasley et al., 2018). Most of the cachectic cancer patients in this study preferred very low intensity activities, which they could do on their own in a home setting, because they feared that symptoms could worsen, or higher intensity exercise could cause harm. This observation suggests that low intensity exercise interventions would address motivational and behavioral concerns better than higher intensities and there is a need to develop exercise interventions that match the perceived abilities and skill of the cancer patients (Wasley et al., 2018).

**Summary**

In summary, cancer cachexia is a complex condition with underlying mechanisms that are not well understood. Muscle wasting is one of the main symptoms that cachectic cancer patients experience. Many molecular pathways are activated or inhibited throughout the process of cachexia, which ultimately leads to abnormal metabolism, imbalance of protein homeostasis, increased systemic inflammation, loss of skeletal muscle mass and strength as well as a decreased quality of life. Autophagic pathways play an important role in the balance of protein synthesis and degradation. During cancer cachexia, there is a dysfunction in autophagy which is one of the reasons for increased inflammation and the muscle wasting symptoms. Exercise interventions have been shown to have an anti-inflammatory effect and the ability to counteract cancer cachexia by preserving muscle mass, decreasing systemic inflammation, regulating autophagic processes and increasing effectiveness of chemotherapy. Overall, exercise may
become a valuable therapeutic intervention that could improve quality of life and better the prognosis of survival in cachectic cancer patients. Even though research is growing in this field, questions still remain regarding the most effective duration, intensity and mode of exercise for it to have a beneficial effect. Most cachectic cancer patients experience low self-efficacy, increased fatiguability, low exercise tolerance and high psychological stress, which poses challenges for developing a suitable exercise protocol.
<table>
<thead>
<tr>
<th>Author</th>
<th>Model</th>
<th>Intensity</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alves et al. (2018)</td>
<td>Mice</td>
<td>HIIT: 5 intervals of 3min running at 18m/min followed by 4min running at 25m/min for 16 days</td>
<td>LLC+HIIT: -52% tumor mass, 6-fold increased CD274 mRNA expression, improved running capacity, skeletal muscle contractility, and survival rate</td>
</tr>
</tbody>
</table>
| Ballaro, et al. (2019) | Mice    | Motorized wheel running for 3 days out of 4 for 12 days at 11m/min for 45min/da | SED: muscle wasting and redox imbalance, ↑ROS, ↑ markers of autophagy Beclin1 and p62, ↓ mitochondrial mass  
EX: ↓ muscle wasting, prevention of muscle strength loss, ↓ levels of ROS, ↓ autophagy marker levels, ↑ mitochondrial mass |
| Brandt, et al. (2018) | Human   | 8-week exercise training: continuously moderate cycling (MOD) or continuously moderate cycling interspersed by 30-sec sprints (SPRINT) | Immediately after exercise: ↑AMPK and ULKSer317 phosphorylation in skeletal muscle  
Two hours after: ↑LC3-I and -II, and BNIP3 protein content  
MOD: ↑ Beclin1 |
| Fernandes, et al. (2020) | Mice    | Treadmill running for 45 days, 60min/day, 5 days/week, 60% of            | Tumor bearing mice: cardiac remodeling and dysfunction, cardiac atrophy, ↓ LV ejection fraction, cardiac necrosis, inflammation, and fibrosis  
Exercise: partially reversed LV ejection fraction, ↓ necrosis, inflammation, and cardiac collagen deposition, ↓ TGF-b1 mRNA levels, |
<table>
<thead>
<tr>
<th>Study</th>
<th>Species</th>
<th>Intervention</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>J.F. Halling &amp; H. Pilegaard. (2017)</td>
<td>Mice</td>
<td>Strenuous bout of Treadmill exercise (9h, 13.5m/min, 6° incline)</td>
<td>Autophagic vacuole formation 2-7 days after exercise, ↑ LC3-II and autophagosome content</td>
</tr>
<tr>
<td>Leal, et al. (2020)</td>
<td>Rats</td>
<td>Voluntary running</td>
<td>Positive regulation of pro- and anti-inflammatory cytokines, ↓ of tumor volume by 60%</td>
</tr>
<tr>
<td>Martin-Rincon, et al. (2017)</td>
<td>Human</td>
<td>Moderate intensity run (50-70% VO2max) for 60-120min</td>
<td>↓ Autophagosome content after endurance exercise</td>
</tr>
<tr>
<td>Parry, T.L., &amp; Hayward, R. (2018)</td>
<td>Rats</td>
<td>Voluntary wheel running exercise for 6 weeks (4 weeks before tumor, 2 weeks during)</td>
<td>SED+T: significantly lower left ventricular pressure, significant ↑ in cardiac autophagy, ↑ tumor LC3-II protein expression WR+T: significantly smaller tumors</td>
</tr>
<tr>
<td>Ranjbar, et al. (2019)</td>
<td>Mice</td>
<td>Combined exercise training</td>
<td>Muscle mass and strength improved Modulation of autophagy markers</td>
</tr>
</tbody>
</table>
for 6 weeks (4 before tumor, 2 during): resistance training included climbing 1 m ladder inclined at 85°, aerobic exercise on same day using motorized wheel - LC3-I/II ratio: ↑ in SED, ↓ in EX - P62: steadily ↑ in both SED and EX

<table>
<thead>
<tr>
<th>Study</th>
<th>Species</th>
<th>Protocol</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rocchi, A. &amp; He, C. (2017)</td>
<td>Mice</td>
<td>80min Treadmill running (17m/min)</td>
<td>Stimulated autophagosome formation (GFP-LC3 puncta) in skeletal muscle</td>
</tr>
<tr>
<td>Tanaka, et al. (2020)</td>
<td>Rats</td>
<td>Treadmill running at 20m/min at 20° incline for 30min (10 sessions over 2 weeks)</td>
<td>Inhibited muscle loss, rescued protein synthesis, prevented capillary regression, suppressed hypoxia, inhibited mitochondrial dysfunction in fast and slow twitch muscles</td>
</tr>
<tr>
<td>Tanaka, M. et al. (2019)</td>
<td>Rats</td>
<td>Low intensity treadmill running, 8 sessions in 10 days, 15m/min for 30min/session</td>
<td>EX: inhibited muscle loss through suppression of UPP, ↑ phosphorylation of AMPK, inhibited deactivation of mTOR in soleus muscle, prevention of cancer cachexia-induced muscle atrophy</td>
</tr>
</tbody>
</table>
CHAPTER III: METHODOLOGY

Research Design

Based on *a priori* statistical power analyses, 28 male mice were randomly selected from a convenience sample in the animal laboratory facility. The cohort was then separated into 4 test groups, independent of individual running performance: sedentary non-tumor bearing (SED+NT; n=7), sedentary tumor bearing (SED+T; n=7), low intensity treadmill exercised non-tumor bearing (TM+NT; n=7), and low intensity treadmill exercised tumor bearing (TM+T; n=7). Within each group, there was an equal number of LC3 Transgenic (n=3) and Wildtype (n=4) mice. Mice were implanted with tumor cells (T group; 5x10^5 LLC cells in flank) or remained non-tumor (NT) for 4 weeks. During those four weeks, mice underwent either a low intensity treadmill exercise training protocol (TM) or remained sedentary (SED). Other than these protocol differences, the environment, including food and water availability, and the dark/light cycle, was similar with the mice housed in individual cages within the same room.

At baseline/injection and the sacrifice timepoint after 4 weeks, grip strength and echocardiography measurements were taken, as well as tumor evaluations three times a week during the third and fourth week of the protocol, to examine the potential protective effects of exercise on tumor growth, muscle wasting, and cardiac function during cancer cachexia. At the end of the 4-week study, muscle tissue of the gastrocnemius mixed-fiber muscle, the heart as well as tumor tissue from the tumor bearing animals were collected, weighed, and either flash frozen in liquid nitrogen or embedded in OCT (optimal cutting temperature) gel and frozen on dry ice for further analyses.
Mice

This study was performed on inbred LC3 Transgenic (JAX stock #027139; (L. Li et al., 2014; Lin et al., 2014), and Wildtype mice between the ages of 8 and 12 weeks at the start of the activity and tumor protocols. All mice were bred in an accredited animal facility from the same distributor, and internally maintained from breeder rotation at the animal facility at the University of North Carolina at Greensboro. During the length of the protocol the mice were housed in a temperature-controlled room, maintained at 23-24°C, with a light-dark cycle of 12-12h (lights out at 18:00) and provided with tap water and standard rodent chow ad libitum. Experiments were approved by the Institutional Animal Care and Use (IACUC) Committee at UNCG. RFP (red fluorescent protein) – GFP (green fluorescent protein) – LC3 Transgenic mice were used due to their dual tagged LC3 protein characteristics. These mice produce autophagic vacuoles expressing RFP and GFP puncta, making it possible to analyze autophagic flux in cardiac and skeletal muscle tissue by using fluorescent microscopy (L. Li et al., 2014). This study focused on male mice to control for the potential influence of certain sex hormones and fluctuation throughout a four-week protocol.

Figure 2. Autophagic Flux Mechanism in vivo in GFP-RFP-LC3 Mouse Line.

Source: Joe Hill, UT Southwestern.
Tumor Model

Lewis Lung Carcinoma (LLC) cell line, obtained from American Type Culture Collection (ATCC; CRL-1642, Manassas, VA, USA), was used to grow a subcutaneous tumor in the flank of the tumor-bearing mice. This cell line was chosen in combination with the length of the protocol due to the ability of the subcutaneously injected LLC cells to induce a cachectic state in the mouse within a timeframe of three to four weeks, while not metastasizing to other parts of the body (Das et al., 2011). Although the cell growth of this cancer cell line is not spontaneous (as it would be seen in clinical studies), using the LLC model allowed for a more accurate control of the development of cancer cachexia and measurement of tumor growths than it would have been found in a spontaneously cancer growing mouse model. Therefore, the effects exercise had on the rate of cachexia could be examined more accurately.

Cells were grown in Dulbecco’s modified eagle medium (DMEM; ATCC, 30-2002, Manassas, VA, USA) supplemented with 10% fetal bovine serum, in an incubator that was set to 5% CO₂ and 37° Celsius. To ensure tumor implants were not disturbed by the rhythmic motions of exercise, 48-72 hours prior to starting the treadmill protocol, mice in the T groups were inoculated subcutaneously in the left flank with a concentration of $5 \times 10^5$ LLC cells in 100µL of sterile phosphate buffered saline (PBS). At this time, NT controls received an equivalent volume of PBS as a sham saline injection. Tumor measurements, body weight, and body condition were assessed three times a week during the third and fourth week of the protocol. Tumor length, width, and thickness were measured by using a Vernier caliper. The tumor measurements were used to estimate tumor mass, relative tumor mass, and tumor volume by using the following formulas:
1) \( \text{tumor mass}(g) = 0.79768 + (0.000456 \times \text{length} \times \text{width} \times \text{thickness of tumor in mm}) \)

2) \( \text{relative tumor mass} = \frac{\text{estimated tumor mass}}{\text{total body mass} - \text{estimated tumor mass}} \)

3) \( \text{tumor volume (mm}^3\) = \(\frac{a \times b^2}{2}\) ;(Parry & Hayward, 2018).

**Equipment/Instruments**

For this study, a motorized Collins Treadmill (Braintree, US) was used for the exercise protocol. The treadmill was equipped with a 12-lane divider cage on top of the belt to ensure enough individual running space for each animal. The treadmill could be adjusted for incline as well as speed (measured in m/min). To measure body weight, heart weight, and gastrocnemius muscle weight, a high precision laboratory scale (+/- 0.02g; U.S. Solid, Cleveland, US) was used. For grip strength measurements, a Grip Strength Test from the company BIOSEB was used. Tumor evaluations were performed by using a Vernier Caliper. Heart function was measured with a Vivid 7 Dimension (Horten, Norway) echocardiography device. Autophagic flux was measured by first cutting small samples of the heart and gastrocnemius tissues with a Cryostat machine and then taking images with a Confocal Fluorescent Microscope (Olympus Fluoview FV500/IX81) for further analyses.

**Treadmill Protocol**

Mice were divided into sedentary and low-intensity treadmill exercise groups. The low intensity exercise protocol lasted for 4 weeks and was progressive in nature to accommodate the learning curve associated with treadmill exercise for the mice to be able to tolerate and sustain the intensity throughout the four-week protocol. A progressive protocol was chosen over a static
protocol to represent an exercise intervention comparable to potential clinical interventions of
exercise protocols in human research models.

The exercise group started with an acclimation period consisting of 20min treadmill
running, starting with a low walking speed (5-8 m/min), and increasing to the specific speed of
the protocol (10m/min), on three days in the week before the start of the actual treadmill
protocol.

The low intensity treadmill protocol represented an intensity of approximately
50%VO2max (Guo et al., 2020; Landry et al., 2021; Schefer & Talan, 1996). While this protocol
is considered as low intensity for this study, when translated to human models the intensity
would equate to moderate exercise. During the first week, the protocol started out with an incline
of zero degrees and a speed of 10m/min for 45min on 5 days a week. During the second week,
the incline and speed stayed the same, but the duration was increased to 60min per session for 5
days a week. During week three and four, the mice were running at an incline of 2 degrees with a
speed of 10m/min for 60min per session on 5 days a week. Treadmill exercise was performed
during the light cycle, between 06:00 and 12:00.

**Grip Strength**

To measure grip strength as a measure of skeletal muscle function, mice grasped a metal
grid with their front and back legs. The metal grid was attached to a force meter (Harvard
Apparatus). Mice were pulled gently in the opposite direction of the force meter about 3 to 5
times per test, using the average of all trials (Bonetto et al., 2015). Mice were not given trial runs
for this test prior to data collection due to the noninvasive nature of this test and the proneness of
mice to becoming habituated and losing interest in the given task.
**Echocardiography**

Echocardiography was used to examine the effects of exercise and cancer on cardiac structure and function. At baseline and the timepoint of sacrifice after the four-week protocol, mice were loosely restrained in a supine position and hair was removed from the chest by means of a depilatory agent. Warm transduction jelly was applied to the chest. At the level of the papillary muscle, two-dimensional M-mode echocardiography (GE Vivid 7 Dimension) was performed in the parasternal long-axis view. Measurements represented the average of three cardiac cycles from each mouse (Parry et al., 2016). Cardiac structure and function were measured by Ultraql software (Durham, NC).

**Sacrifice and Tissue Harvest**

At the end of the four-week protocol, mice were sacrificed at least 24 hours after the last treadmill training session to evaluate basal biological marker levels. Sedentary mice were sacrificed at the same time as treadmill trained mice. Sacrifice was performed by anaesthetizing the mice using isoflurane, which was followed by cervical dislocation. Gastrocnemius, heart, and tumor tissues were harvested into 1.5mL Internally Threaded Cryogenic Storage Vials and flash frozen in liquid nitrogen or embedded in OCT (optimal cutting temperature) gel and frozen on dry ice. All samples were then stored at a temperature of -80°C until further analyses.

**Western Blotting**

Gastrocnemius mixed-fiber muscle tissue and cardiac muscle tissue from the apex of the heart were analyzed for Akt, P-NF-kB, IL-1b, Beclin1, and p62 protein content. Approximately 35mg of tissue was homogenized via 8M urea lysis buffer with a TissueLyser LT homogenizer (Hilden, Germany). Then, the homogenates were centrifuged at 4°C for 10min at 5000rpm and the supernatant was discarded.
Protein content was measured by Western Blotting using pre-casted 12% BisTris gels, then transferred to PVDF (Polyvinylidene Fluoride) membranes. Prior to the Western Blot sample preparation, Bradford Protein Assays were performed to analyze the protein content in each sample and to be able to account for an equal amount of protein content in each prepared sample for the Western Blot analysis. From the results of the Bradford Assays, 10-20µg of protein of the samples were loaded into each well of the gels. Additionally, into the first lane of each gel MagicMark XP Western Protein Standard (ThermoFisher; MA, USA) was loaded for molecular weight comparison. In a mini-gel NuPAGE gel apparatus (ThermoFisher; MA, USA) using NuPAGE MES running buffer (ThermoFisher; MA, USA), gel electrophoresis was performed for 30-40 minutes at constant 200 Volt. Using the same mini-gel NuPAGE gel apparatus and a sandwich apparatus, proteins were then transferred onto PVDF membranes for 60 minutes at constant 30 Volts. PVDF membranes were blocked in 5% skim milk and protein contents were determined using primary antibodies against Akt, P-NF-kB, IL-1b, Beclin1, and p62 (Cell Signaling; Danvers, USA). Primary antibodies anti-Akt, anti-Beclin1, and anti-p62 were prepared in 7.5mL of 5% milk solution, each at a 1:1000 dilution. Anti-P-NF-kb and anti-IL-1b primary antibodies were prepared in a 5% milk solution as well, each at a 1:200 dilution. The membranes were incubated in species-specific secondary antibodies. Band intensity was quantified using Quantity One (Bio Rad, US). Protein content was expressed in arbitrary units and normalized to control GAPDH protein content of each sample.
Figure 3. Autophagy in Cancer Signaling Pathways.

Note: Signaling molecules of interest for western blotting analysis in this thesis study are represented with a red rectangular border. Created with BioRender.com.

**Autophagy**

Autophagic flux in heart and gastrocnemius muscle tissue were analyzed via Confocal Fluorescent Microscopy. Approximately 8-12µm thick slices in OCT embedded and frozen tissues were cut by using a Cryotome machine and placed on slides. The sample slices on the slides were treated with Prolong Antifade to preserve the tissue. Then, images of the samples were obtained by using a Confocal Fluorescent Microscope. Images were taken of 10 random fields of the fixed tissue sample at 80x oil immersion. Green and Red Puncta Colocalization Macro for Image J (Shiwarski, 2015) was used to objectively quantify yellow and red puncta as a measure of autophagosome and autolysosome content in the sample. LC3 as one of the
autophagy proteins, is recruited by autophagosomes and can be analyzed in this mouse model by means of fluorescent microscopy. Generally, cells with enhanced autophagy express a larger number of LC3 puncta. As the LC3 protein starts to group up to form the early phase autophagosome, the red (RFP) and green (GFP) puncta together appear as a yellow color under the confocal fluorescent microscope. To form a late phase autolysosome, the autophagosome then fuses with a lysosome. The less stable GFP (green) signal is then quenched by the acidic hydrolases from the lysosome. This process leaves behind only the RFP or red signal (Xie et al., 2011).

**Statistical Analyses**

All data are presented as mean ± SEM. The statistical software GraphPad Prism (La Jolla, CA) was used for analyses. To determine interaction effects between activity and tumor treatments (SED+NT, SED+T, TM+NT, TM+T) for each variable, a two-way ANOVA was performed. Two-way repeated measures ANOVAs were performed to analyze the changes in grip strength, body weight, and fractional shortening of the heart between the baseline and sacrifice measurements in all four groups. Change score measurements were performed to calculate the percent change in grip strength, body mass, and fractional shortening from baseline to sacrifice. Independent t-tests were performed to analyze the differences in tumor characteristics between the two tumor bearing groups (SED+T, TM+T). All analyses were two-tailed, with an alpha level of 0.05 to define statistical significance. If a significant difference (P < 0.05) was identified, then a Tukey’s post hoc test was performed to identify where these significant differences occurred.
CHAPTER IV: RESULTS

Effects of Exercise and Tumor Burden on Body, Heart, and Gastrocnemius Mass

For four weeks, mice were either exercise trained on a treadmill (TM) or remained sedentary (SED). At the beginning of the 4-week protocol, mice were either inoculated with Lewis Lung Carcinoma (LLC) cells subcutaneously in the right flank, to grow localized tumor masses which allowed for convenient monitoring of the tumor growth and morphology (T) or remained non-tumor bearing (NT) and were injected with a sham saline injection. Body mass and echocardiography measurements were taken at the baseline time point and the end of the four-week protocol. Tumor evaluations were performed three times per week during the last two weeks of the protocol. All 28 animals that were selected for the study could sustain the tumor and exercise protocol as assigned to their specific groups. Therefore, the physiology data, echocardiography data, and tissue collections of all animals were included in all further procedures and the statistical analyses.

While two-way repeated measures ANOVA indicated no significant interaction between the exercise and tumor protocols, post hoc testing and change score measurements, as summarized in Figure 4, showed that when comparing baseline body weight measures to body mass at the sacrifice timepoint, sedentary tumor bearing animals experienced the greatest decrease in body mass. While not significant, treadmill exercise training seemed to abolish body mass loss in the treadmill trained tumor bearing group compared to the sedentary counterpart. This data indicates the potential of low intensity treadmill exercise to preserve body weight during cancer cachexia.
Figure 4. Comparison of Average Body Mass in Grams Between All Four Groups at Baseline and Sacrifice, and Percent Change in Body Mass.

Note: Graph a) represents the average body weight measurements in grams (g) taken at the initial baseline time point and the sacrifice time point after the four-week protocol for each group. b) Change scores were calculated by subtracting the body mass in grams at baseline from the body mass in grams at the sacrifice timepoint and then dividing the result by the body mass in grams at baseline. Results are represented as percent change (%).

Figure 5 shows the heart mass and gastrocnemius mass relative to body mass at the end of the four-week intervention. Two-way ANOVA indicated no interaction between the two protocols. Interestingly, when looking at the post hoc analysis of heart weight comparisons, the sedentary tumor bearing group experienced a significantly greater heart weight when compared to the SED+NT and TM+T groups. The reason for this is not well understood but could be due to fibrosis of the heart as a possible symptom of cancer cachexia.

Looking at the post hoc results for skeletal muscle mass, while insignificant, treadmill trained non-tumor bearing mice showed the greatest gastrocnemius muscle weights compared to
all other groups, indicating a potential training effect due to the low intensity treadmill intervention.

**Figure 5. Normalized Heart and Gastrocnemius Muscle Weights at Sacrifice.**

Note: Significant difference with a p-value smaller than 0.05 is indicated as (*); significant difference with a p-value smaller than 0.01 is indicated as (**).

**Effects of Exercise and Tumor Burden on Skeletal Muscle Function**

A two-way ANOVA and change score analyses were performed to analyze the differences between initial grip strength measurements and muscle function at the end of the four-week protocol. The results of the grip strength measurements are summarized in Figure 6. The graph of the analyzed data shows that the TM+NT mice were the only group that demonstrated an increase in grip strength compared to baseline, while all other groups experienced a negative percent change in grip strength as a measure of muscle function from baseline to the sacrifice time point. While insignificant, these findings indicate that the low
intensity treadmill exercise might have had a positive impact on improving muscle function. Specifically, a significant difference ($P < 0.05$) is noticeable when comparing grip strength percent changes between SED+T mice and TM+NT mice, as well as an insignificant decrease in grip strength when comparing SED+T to the sedentary non-tumor bearing group. While a decrease in grip strength was experienced by the TM+T group overtime as well, the results are not significantly different from the non-tumor bearing groups, and overall, the decrease in grip strength was less than the decrease experienced by the sedentary tumor bearing group. The analyzed data indicates that tumor bearing alone can negatively affect grip strength as a measure of muscle function, but the low intensity treadmill intervention seemed to attenuate this loss in muscle function.

**Figure 6. Comparison of Percent Changes in Grip Strength from Baseline to Sacrifice.**

**Percent Change in Grip Strength from Baseline to Sacrifice**

Note: The averages of three grip strength trials were used to calculate these changes.

Percent change in grip strength was calculated by subtracting the average grip strength at
baseline from the average grip strength at sacrifice and then dividing the result by the average grip strength measure at baseline.

Two-way repeated measures ANOVA indicated no interaction between the two treatments. Post hoc testing (Figure 7) showed a significant decrease in grip strength from baseline to sacrifice in the sedentary tumor bearing group (P < 0.05) but not in the treadmill exercised tumor bearing group or the two non-tumor bearing controls. This data also suggests that low intensity treadmill exercise may have a positive impact on preserving muscle function in the tumor bearing mouse.

Figure 7. Comparison of Baseline and Sacrifice Grip Strength Measures Between the Four Study Groups.

Grip Strength Relative to Body Weight at Baseline and Sacrifice

![Graph showing grip strength relative to body weight at baseline and sacrifice for different groups.]

Note: Grip strength measurements in grams were normalized to body weight of each mouse in grams to allow for more accurate inter- and intragroup comparisons.
Western Blotting on Gastrocnemius Tissue

Effects on Expression of Pro-Inflammatory Cytokines in Skeletal Muscle

Western Blotting analyses were performed on gastrocnemius mixed-fiber skeletal muscle to analyze differences in protein expression between the different tumor and exercise protocols and determine if tumor burden or exercise influenced these expressions. The protein expression of both pro-inflammatory cytokines, NF-kB and IL-1β, was analyzed in this study. No interaction between the tumor and exercise interventions was determined by two-way ANOVA. Figure 8 depicts the results of the post hoc analysis of NF-kB and IL-1β Western Blotting analyses and compares the protein expressions of each group. While none of the statistical analyses showed significant findings, tumor bearing seemed to upregulate P-NF-kB protein expression. No significant differences in any group comparisons were found when looking at the IL-1b protein expression. These data indicates that inflammation might not be a strong indicator for mixed fiber muscle tissue with this model.

Figure 8. Comparisons of Pro-Inflammatory Protein Expressions in Skeletal Muscle Tissue.
Effects on Metabolic Protein Expression in Skeletal Muscle

Figure 6 shows the comparisons of AKT protein content in the gastrocnemius muscle tissue based on densitometry analysis of the Western Blot membranes. Statistical analyses showed no significant findings, but post hoc analysis (Figure 9) showed that tumor bearing groups experienced an upregulation in AKT protein expression. While insignificant, AKT expression in TM+T mice seemed to be lower than expression in SED+T mice, indicating potential regulatory effects by the exercise intervention.

Figure 9. AKT Protein Expression Comparison in Skeletal Muscle Tissue.

Effects on Autophagic Protein Expression in Skeletal Muscle

Figure 10 shows the comparison of Beclin1 protein expression in skeletal muscle tissue between all four study groups. While two-way ANOVA did not show an interaction effect between the interventions, post hoc testing did show significant findings. Interestingly, tumor bearing did not seem to influence protein expression as there was no significant difference in protein content when comparing both sedentary groups. Significant differences (P < 0.05), based on post hoc testing, were found when comparing the Beclin1 protein content of both treadmill-
trained groups to both sedentary groups, indicating that low intensity treadmill exercise might influence the regulation of autophagic processes and suppress this signaling molecule.

**Figure 10. Beclin1 Protein Expression in Skeletal Muscle.**

Two-way ANOVA showed no interaction effect between exercise and tumor bearing. While insignificant, the post hoc analysis of p62 protein expression (Figure 11) indicated an increase in p62 protein expression in the gastrocnemius muscle when comparing sedentary tumor bearing animals to SED+NT animals. This increase in p62 expression was abolished by treadmill exercise, indicated by the significant differences when comparing the SED+T group to the treadmill trained non-tumor bearing and tumor bearing groups. Both tumor bearing groups indicated insignificantly lower p62 protein expression when compared to SED+NT mice. This data also indicates that the low intensity treadmill intervention had an influence on autophagy in the skeletal muscle and potentially resulted in a better regulation of autophagic processes and tumor suppression.
Figure 11. Skeletal Muscle p62 Protein Expression.

Autophagic Flux Analysis

Findings of Fluorescent Microscopy Analysis of Skeletal Muscle Tissue

Figure 12 summarizes the findings of early phase autophagosome (yellow puncta) and late phase autolysosome (red puncta) analyses. There was no interaction effect between the two treatment interventions regarding the early phase autophagosomes (yellow puncta) in the gastrocnemius tissue. When looking at the post hoc analysis of yellow puncta, or early phase autophagosomes, exercise seemed to increase early phase autophagy depicted by the significantly (P < 0.05) greater numbers of yellow puncta in the treadmill exercised groups compared to the sedentary counterparts.

Two-way ANOVA of the late phase autolysosome analysis (red puncta) in the gastrocnemius tissue did show an interaction effect between the exercise and tumor intervention, with a main exercise effect. Post hoc testing of the late phase autolysosomes indicated that tumor bearing increased late phase autophagy, which was not abolished by the exercise intervention,
shown by the significant (P < 0.05) differences between the tumor bearing and non-tumor bearing groups. This supported the findings of increased p62 and NF-kB protein expression in sedentary tumor bearing animals and indicated a cancer-mediated increase in autophagy in mixed-fiber skeletal muscle tissue.

**Figure 12. Autophagic Flux Analyses in Skeletal Muscle Tissue.**

Findings of Fluorescent Microscopy Analysis of Cardiac Muscle Tissue

Autophagic flux analyses were also performed on cardiac muscle tissue to identify the influences of tumor burden and exercise on early and late phase autophagy in the heart. Two-way ANOVAs were performed to indicate interaction effects between the two treatment interventions. Interaction effects of both interventions were found for both early phase autophagosome and late phase autolysosome analysis. Two-way ANOVAs were followed by post hoc testing as summarized in Figure 13.

Based on post hoc testing, yellow puncta analysis in the heart showed similar yet different findings when compared to the findings in the skeletal muscle. Numbers in yellow puncta per field were significantly (P < 0.05) greater in the tumor bearing groups when compared to the non-tumor bearing groups. SED+T animals showed a significantly (P < 0.05) greater
number of yellow puncta when compared to the treadmill trained tumor bearing animals, suggesting that while early phase autophagy was increased with tumor burden, exercise seemed to attenuate this early phase autophagy.

The post hoc analysis of red puncta, or late phase autolysosomes, showed significantly (P < 0.05) more red puncta per field in the sedentary non-tumor bearing group compared to all other groups. Additionally, TM+T mice showed significantly (P < 0.05) less late phase autolysosomes when compared to SED+T mice, which supports the findings of the yellow puncta analysis. Interestingly, sedentary non-tumor bearing groups experienced the highest number of red puncta when compared to all other group (P < 0.05).

**Figure 13. Autophagic Flux Analyses in Cardiac Muscle Tissue.**

A two-way repeated measures ANOVA was performed to compare the baseline and sacrifice measurements of fractional shortening as a measure of cardiac function within and between each group. Figure 14 summarizes the post hoc results of average fractional shortening compared between all four study groups. Figure 14a represents the change in fractional
shortening at baseline and after the four-week protocol, whereas Figure 14b shows the change score from baseline to sacrifice. When looking at the percent change in fractional shortening (Figure 11b), the treadmill non-tumor bearing group was the only group experiencing an increase in fractional shortening, indicating potential positive impacts of low intensity treadmill exercise on cardiac function. Sedentary tumor bearing mice showed the greatest decrease in fractional shortening from baseline to sacrifice, with significantly (P < 0.05) less fractional shortening at sacrifice when compared to the TM+NT group. While insignificant, exercise seemed to attenuate the loss in cardiac function, indicated by the less negative change in fractional shortening when comparing TM+T mice to SED+T mice, with the treadmill trained tumor bearing group experiencing insignificantly better fractional shortening results compared to the sedentary non-tumor bearing group. This data indicates that low intensity treadmill exercise could have potential protective effects on cardiac structure and function, and potentially preserve cardiac function during tumor burden.
Figure 14. Change in Fractional Shortening as a Measure of Cardiac Function.

Note: Fractional shortening was calculated by using the average left ventricular diameters at systole and diastole of three cardiac cycles and using the following formula: 

\[ FS = \frac{LVDd - LVDs}{LVDd} \]

Western Blotting on Heart Tissue

Effects on Expression of Pro-Inflammatory Cytokines in Cardiac Tissue

Western blotting analyses were performed to analyze the same protein expressions in cardiac tissue as in skeletal muscle tissue. Pro-inflammatory cytokines P-NF-kB and IL-1b analyses did not show any interaction effects or significant differences between tumor and non-tumor bearing groups, as well as sedentary versus exercised groups. Nonetheless, the trends noticeable based on the post hoc testing summarized in figure 15a) indicate an increase in P-NF-kB in the sedentary tumor bearing mice compared to all other groups. Treadmill training seemed to attenuate protein expression of P-NF-kB. While treadmill trained tumor bearing mice experienced greater protein expression than the treadmill trained non-tumor bearing group, both tumor bearing groups exhibited lower protein expression than both sedentary counterparts.
Similar findings can be seen in figure 15b), representing post hoc testing of IL-1β expression, indicating an insignificant increase in IL-1β in the SED+T group when compared to the SED+NT group. While treadmill trained groups experienced greater protein expression compared to SED+NT animals, exercised mice still exhibited slightly less IL-1b expression than the SED+T group.

**Figure 15. Pro-Inflammatory Protein Expression in Heart Tissues of All Study Groups.**

\[\text{Cardiac P-NF-kB Protein Expression} \]

\[\text{Cardiac IL-1β Protein Expression} \]

*Note:* Protein analyses were performed in Cardiac Tissue from the apex region of the Heart. Protein expression was normalized to the housekeeping protein GAPDH. a) P-NF-kB protein expression in the heart. b) IL-1b protein expression in the heart.

**Effects on Expression of Metabolic Proteins in Cardiac Tissue**

AKT protein expression was analyzed in cardiac tissue and protein content, measured via densitometry analysis of Western Blot membranes, was compared between each group. Statistical analyses did not show any significant findings, but post hoc results are summarized in Figure 16. Similar findings as in skeletal muscle tissue (see Figure 9) were found when comparing tumor bearing groups to the sedentary non-tumor bearing control group, with both
tumor bearing groups experiencing elevated AKT expression compared to SED+NT mice. While insignificant, these findings indicated that tumor bearing increases AKT protein expression. Interestingly, cardiac AKT expression was also insignificantly elevated in the treadmill trained non-tumor bearing group which needs to be further analyzed.

**Figure 16. Comparison of AKT Protein Expression in Cardiac Tissue.**

![Cardiac AKT Protein Expression](image)

**Effects on Expression of Autophagy Proteins in Cardiac Tissue**

For neither Beclin1 nor p62 protein expression, there was no interaction effect between the interventions based on two-way ANOVA testing. The autophagic proteins Beclin1 and p62 were analyzed in heart tissue and protein expression was compared between each group based on post hoc testing.

Post hoc analysis found opposite effects on Beclin1 protein expression when comparing the results for skeletal muscle tissue (see Figure 10) and cardiac muscle tissue in Figure 17. Similar to the findings in skeletal muscle tissue, tumor burden did not seem to have an influence on Beclin1 protein expression as there was no significant difference between SED+T and SED+NT animals and between TM+T and TM+T animals. While there was no treatment effect, Beclin1 protein expression was significantly (P < 0.05) elevated in the TM+NT group compared
to SED+NT animals and in the TM+T group when compared to both sedentary groups. This suggests that exercise seems to upregulate Beclin1 protein expression in the heart, and potentially has a modulating effect on autophagy, which could result in potential cardioprotective effects.

**Figure 17. Comparison of Beclin1 Protein Expression in Cardiac Tissue.**

![Cardiac Beclin1 Protein Expression](image)

While insignificant, similar trends as seen in the skeletal muscle tissue (see Figure 11) can be seen in the post hoc analyses results of p62 protein expression in cardiac tissue. Sedentary tumor bearing mice experienced the greatest protein expression in p62 levels when compared to all other groups. Exercise seemed to decrease p62 expression, indicated by lower p62 protein levels in the TM+NT group compared to the SED+NT group. While protein expression in the treadmill trained tumor bearing group was greater than in the TM+NT group, p62 expression was similar to the SED+NT group and less than in the SED+T group. This data also indicates that exercise seemed to have potential influences on modulating autophagy.
Figure 18. Comparison of p62 Protein Expression Between All Study Groups.

![Cardiac p62 Protein Expression Graph]

Effects of Exercise and Cancer on Tumor Characteristics

Figure 19 shows that treadmill trained tumor bearing mice showed significantly (P < 0.05) smaller estimated tumor mass, relative tumor mass, and estimated tumor volumes when compared to the sedentary tumor bearing group. While not significant, tumor wet mass relative to body mass analysis still showed a smaller tumor mass in the TM+T group compared to the SED+T, which coincides with the findings of the calculated measures.

These data indicate that the low intensity treadmill exercise intervention had inhibitory effects on the tumor characteristics by slowing the cancer development and inhibiting tumor growth.
Figure 19. Tumor Characteristics of Sedentary and Exercised Tumor Bearing Mice.

(a) Tumor Wet Mass/BM

(b) Estimated Tumor Mass

(c) Relative Tumor Mass

(d) Estimated Tumor Volume
CHAPTER V: DISCUSSION

Exercise interventions as part of cancer treatment have been investigated in animals and humans with varying degrees of success and there has not yet been clinical evidence of any consistent strategy to treat the detrimental effects of cancer cachexia in human cancer patients. While research is growing, the most effective and beneficial duration, mode, and intensity of exercise as a protective and preventative measure against the cancer-mediated physiological and psychological effects has not yet been identified. The aim of this study was to identify if low intensity treadmill training can act as a protective measure and treatment intervention against cancer-mediated muscle wasting in male mice.

The four-week tumor bearing, and exercise protocols were successfully finished by all 28 animals, none of the mice had to be removed from the study early, and all measurements and analyses were qualified to be used for further analyses.

Cancer cachexia is characterized by severe progressive skeletal muscle loss, with or without the loss of fat mass (Dolly, 2020). To identify if tumor burden and exercise had an effect on weight loss, body mass measurements were taken at baseline and the sacrifice time point at the end of the four-week protocol. While insignificant, sedentary tumor bearing mice experienced the greatest loss in body mass (-8%) compared to all other groups, indicating that tumor burden indeed resulted in body weight loss. This loss in weight was abolished by exercise as treadmill trained tumor bearing mice exhibited almost no difference in body mass overtime (+0.1%), indicating potential exercise influences on cancer cachexia by slowing its development and maintaining body mass. The prevention of body mass loss by exercise interventions during tumor bearing has been reported in previous cancer studies in mice and rats (Ballarò et al., 2019; Ranjbar et al., 2019)
Effects of Exercise and Tumor Burden on Skeletal Muscle

Tumor bearing showed a significant reduction in grip strength as a measure of skeletal muscle function, which was preserved by low intensity treadmill exercise. Tumor bearing itself resulted in the greatest loss in muscle function when compared to all other groups, with a significant difference in percent change when compared to the TM+NT group (SED+T: -23%; TM+NT: +6%). This finding coincides with previous research that has found increased muscle loss, muscle wasting and decreased muscle function in sedentary tumor bearing groups (Alves et al., 2018; Ranjbar et al., 2019; Tanaka et al., 2019).

While insignificant, the cancer-mediated loss in muscle function seemed to be preserved by the exercise intervention as TM+T mice exhibited a smaller loss in percent change of grip strength overtime (-15%) than their sedentary counterpart (SED+T: -23%). Compared to other studies, the findings of this thesis study were less significant but showed similar trends when looking at the effects of exercise on skeletal muscle function during tumor burden. One study has found significant increases in skeletal muscle contractility and survival rate after high intensity interval training concurrent with tumor bearing (Alves et al., 2018). When looking at endurance exercise interventions of higher intensities than this thesis study, researchers have found similar findings compared to this low intensity protocol, with decreased muscle wasting, prevention of muscle strength loss, and improved muscle function after the exercise intervention compared to the sedentary tumor bearing counterparts (Ballarò et al., 2019; Ranjbar et al., 2019; Tanaka et al., 2019, 2020).

Western Blot analysis of P-NF-kB and IL-1b proteins indicated that inflammatory pathways might not be primarily involved in preserving skeletal muscle function in this mouse model in combination with the specific tumor and exercise protocol.
Similar trends as indicated by the P-NF-kB Western Blot analysis were found when looking at the AKT protein expressions in gastrocnemius mixed-fiber muscle as a representation of skeletal muscle tissue. Tumor bearing seemed to insignificantly elevate AKT expression, which was insignificantly reduced by low intensity treadmill exercise. AKT has been shown to be involved in multiple cellular functions, including protein synthesis, proliferation, cell survival, metabolism, as well as tumorigenesis when dysfunctional (Sakamoto et al., 2003). Research has shown that inhibiting AKT pathways, for example by using analogs of rapamycin to inhibit mTOR, can show potential anti-tumor effects in human patients (Pathuri et al., 2014). Therefore, further research in this area would be interesting, for example by analyzing the phosphorylated AKT. When phosphorylated, P-AKT is in the activated state. Therefore, an interesting future evaluation for this study would be to identify the ratio of phosphorylated AKT to the total AKT values and determine if tumor burden or exercise had an influence on activated AKT protein expression.

Worse grip strength in sedentary tumor bearing mice, as indicated above, appears to be related to increased autophagy in the skeletal muscle. This is supported by an upregulation of p62 protein expression in the SED+T mice, which was abolished by low intensity treadmill exercise as TM+T and TM+NT mice indicated significantly lower protein expressions compared to the sedentary tumor bearing group. P62 is one of the oncogenic biomarkers that has been detected during tumorigenesis. Cancer development is promoted when there is an accumulation of p62 protein aggregates. This upregulation can also lead to dysfunctional autophagic processes, which also explains the supporting role of p62 during cancer development and tumor progression (X. Li et al., 2020). Therefore, in this study p62 protein expression was used as a marker for autophagy inhibition or dysfunctional autophagy when upregulated. While insignificant when
compared to sedentary non-tumor bearing controls, the SED+T still experienced an elevation in p62 expression that was significantly higher than p62 expression in the treadmill trained groups. This suggests that tumor bearing alone results in an elevation in p62 protein expression, which indicates upregulated and dysfunctional autophagic pathways. Abnormally upregulated autophagy could accelerate tumor development and increase cancer-mediated loss in skeletal muscle function, which coincides with the findings of greatest loss in grip strength as a measure of skeletal muscle function in the SED+T group.

Beclin1 is another protein involved in autophagic pathways that was analyzed in this study. Opposite to p62, Beclin1 is involved in the formation of phagophores and has been shown to be a key player in the suppression of tumors (Yun & Lee, 2018). Anyhow, other studies have found increased Beclin1 expression in cancer patients, suggesting a potential dual function as a tumor suppressor and promoter during cancer cachexia (Miao et al., 2017). Interestingly, in our study skeletal muscle analysis showed significantly lower Beclin1 protein expressions in both exercised groups when compared to both sedentary groups, which indicates an influence of the low intensity treadmill exercise on potentially modulating autophagic processes. Tumor bearing alone did not seem to upregulate this protein expression since SED+T mice did not experience a significant difference when compared to SED+NT mice. Further investigations need to be done on the influence of exercise on Beclin1 expression and the potential influence on modulating and regulating autophagy during cancer cachexia.

The findings of this thesis study regarding protein expression of different autophagy-related proteins via western blotting coincides with findings of previous research. Ballaro et al. (2019) have also found increased Beclin1 and p62 protein expression in skeletal muscle tissue of sedentary tumor bearing groups, whereas exercise significantly downregulated expression of
these autophagy markers in mice models. Other research studies have found steadily increased p62 protein levels in both sedentary and exercise trained tumor bearing mice, differing from what was found in this thesis study (Ranjbar et al., 2019). One study that looked at different exercise modalities in humans and the effect on autophagy found that continuous moderate cycling led to an increase in Beclin1 protein expression in skeletal muscle, which shows opposite effects of what was found in this thesis study, as Beclin1 protein expression was significantly downregulated in the treadmill trained groups compared to the sedentary counterparts (Brandt et al., 2018).

Finally, autophagic flux analyses coincide with the findings of elevated p62 autophagic protein expression and the suggestion that tumor bearing seems to abnormally upregulate autophagy. Red puncta analysis, representing late phase autolysosome numbers, indicated tumor-mediated elevated late phase autophagy, which did not seem to be abolished by exercise. These findings coincide with elevated p62 protein expression in the gastrocnemius determined via western blotting. Coinciding with the findings of this study, previous research has found that aerobic exercise can induce and upregulate autophagic processes which can persist multiple days. It has also been shown that even a single bout of treadmill exercise could increase autophagosome content, as well as decrease p62 content in skeletal muscle (Halling & Pilegaard, 2017). Other research studies showed increased autophagosome content after a strenuous bout of treadmill exercise in mice, as well as after an ultra-endurance run in humans, whereas other human model studies found decreased autophagosome content after moderate endurance exercise (Halling & Pilegaard, 2017; Martin-Rincon et al., 2017). These findings in previous studies coincide with the p62 protein expression and autophagic flux results of skeletal muscle tissue.
found in this research study, indicating potential regulating or modulating effects of low intensity treadmill exercise on autophagy concurrent with tumor bearing.

Overall, specifically the findings of body mass changes, grip strength changes as a measure of skeletal muscle function, p62 and Beclin1 protein expression, as well as autophagic flux analyses suggest that the low intensity treadmill exercise intervention had positive effects on cancer cachexia by slowing its development and preserving skeletal muscle function.

**Effects of Exercise and Tumor Burden on Cardiac Muscle**

Echocardiography measurements were used to analyze the effects of exercise and tumor burden on cardiac structure and function by taking real time two-dimensional M-mode echocardiography images. Images were analyzed for left ventricular septal thickness, left ventricular internal dimensions, and posterior wall thickness at diastole and systole using the Ultralinq software (Durham, NC) to determine fractional shortening as a measure of cardiac function. Decreased fractional shortening overtime has been associated with cardiac remodeling and dysfunction as it is an indicator of less efficient contractility of the heart. Additionally, an increase in fractional shortening indicates improvement in contractility (Gao et al., 2011).

In this study, echocardiography was performed at baseline and on the day of harvest, which was approximately 24 hours after the last treadmill exercise session for the TM groups to allow for recovery and no acute exercise effects that could influence heart rate or contractility of the cardiac muscle. During the time course of this study, sedentary non-tumor bearing controls seemed to experience an insignificant decline in fractional shortening (-3%), measured as percent change from baseline to sacrifice, which could be due to aging or acute response differences due to stress on both evaluation days. Interestingly, TM+NT mice were the only study subjects that experienced an increase in fractional shortening overtime (+4%). While insignificant, this could
indicate a potential positive exercise effect on cardiac muscle and improved cardiac structure and function as it has also been shown in previous research done by Fernandes et al. (2020). Tumor bearing itself seemed to negatively affect cardiac function indicated by the greatest decline in fractional shortening in the SED+T group (-11%) compared to all other groups. Low intensity treadmill exercise seemed to attenuate this decline in fractional shortening. TM+T mice exhibited a percent change difference from baseline fractional shortening to sacrifice by -1%. These findings of preserved cardiac function are similar to what has been found in previous cardiac cachexia research (Fernandes et al., 2020; Parry & Hayward, 2018), indicating that the low intensity treadmill protocol could alleviate cancer-mediated cardiac dysfunction and preserve cardiac structure and function during cancer cachexia.

Western Blotting was also performed on cardiac tissue from the apex of the heart to better understand the protein pathways involved during tumor burden and exercise and to identify certain regulators that led to the potential preservation of cardiac function. Similar to what was found in skeletal muscle tissue, inflammatory pathways, including P-NF-kB and IL-1b protein expression, may not be a key player in this model. This is different to the findings of other researchers, which have showed significant increases in TNFα and IL-6 protein expressions, as well as increased inflammation, necrosis, and fibrosis of the heart (Fernandes et al., 2020; Leal et al., 2020).

AKT analysis showed similar findings to the analysis of the two above mentioned inflammatory proteins. Sedentary tumor bearing mice exhibited the greatest (P > 0.05) expression of AKT when compared to all other groups, whereas AKT expression in the TM+T group seemed to be insignificantly downregulated compared to the SED+T group. While all findings were insignificant, trends are noticeable showing that tumor bearing potentially leads to
an increase in inflammation and dysfunctional cell growth, but this needs to be further evaluated with a greater sample size.

Autophagic protein expression analysis in the heart showed interesting findings. Opposite from what was found in the Beclin1 protein expression analysis in the gastrocnemius, low intensity treadmill exercise seemed to significantly upregulate Beclin1 expression in cardiac tissue. As mentioned earlier, Beclin1 seems to be involved in the formation of phagophores and autophagosomes and is therefore a key player in the autophagic process. Beclin1 also seems to be involved in the suppression of tumor development and upregulation of this protein could potentially lead to inhibition of tumor growth and regulation of autophagy (Yun & Lee, 2018). Increased Beclin1 expression in the exercised groups could suggest increased modulating and regulating effects of Beclin1 pathways on autophagy and therefore potential cardioprotective effects initiated by the low intensity treadmill intervention. This is supported by previous research suggesting regulatory effects of exercise on autophagic pathways by increasing cardiac Beclin1 and LC3 protein expression (Brandt et al., 2018; Halling & Pilegaard, 2017).

P62 analysis, as the other protein involved in the autophagy processes, did not show any significant differences between the groups. Still, similar trends as the other protein expressions were noticeable, with sedentary tumor bearing mice experiencing the greatest p62 protein expression compared to all other groups. Treadmill training seemed to downregulate p62 protein expression, as TM+NT experienced the lowest p62 protein expression compared to all other groups, and TM+T mice showed p62 protein levels that were lower than the expression in SED+T mice and closer to the protein expression of sedentary non-tumor bearing mice.

Findings of yellow puncta analysis, as a representation of early phase autophagy, coincide with the overall findings of preserved cardiac function and modulated autophagic
processes. Sedentary tumor bearing mice exhibited significantly more yellow puncta compared to all other groups. While there were significantly more yellow puncta found in the samples of the treadmill trained tumor bearing group compared to non-tumor bearing groups, the number of early phase autophagosomes was also significantly lower than in the SED+T group. This indicates that tumor bearing significantly upregulates autophagy to abnormal levels, but this upregulation was abolished by exercise. Red puncta analysis indicated similar findings, with the SED+T group exhibiting significantly greater numbers in late phase autolysosomes when compared to the TM+T group. Low intensity treadmill exercise seemed to have potential positive effects on regulating and modulating autophagy. These findings agree with previous preclinical and human research models looking at the effects of different exercise modalities, for example voluntary wheel-running or moderate to high intensity exercise protocols, on cancer-mediated cardiac cachexia. Research has shown that sedentary tumor bearing animals experienced cardiac remodeling and dysfunction, cardiac atrophy, decreased fractional shortening, as well as increased cardiac autophagy, which all seemed to be attenuated with the respective exercise intervention (Fernandes et al., 2020; Parry et al., 2016; Tanaka et al., 2020).

Overall, fractional shortening, autophagic flux, and protein expression analyses suggest potential cardioprotective effects of the low intensity treadmill intervention by regulating autophagy and preserving cardiac structure and function.

**Effects of Exercise and Tumor Burden on Cancer Development**

Research has shown that intratumoral signaling pathways and growth characteristics can be influenced and modulated by extrinsic factors such as exercise interventions (Yang et al., 2020). Potential exercise effects regulating tumorigenesis include physiological changes, such as increased blood flow and pH regulation, as well as endocrine effects, such as regulation of stress
hormones, myokines, and circulating exosomes, may regulate cancer progression and reduce the rate of tumor growth by regulating dysfunctional tumor metabolism (Hojman et al., 2018; Martinez-Outschoorn et al., 2017; Schneider et al., 2017).

One of the most interesting findings of this study was the effect of the low intensity treadmill intervention on tumor characteristics compared to sedentary tumors. While the inhibition of tumor growth in clinical settings could be because of pharmacological interventions, in this study the differences in tumor characteristics are solely due to the exercise intervention as no nutritional support, chemotherapy drugs, or other additional interventions were assigned to any of the two tumor bearing groups. As mentioned earlier in this study, exercise interventions have been shown to have potential anti-inflammatory effects and the ability to regulate autophagy (Belloum et al., 2017; Hardee et al., 2019; Niels et al., 2020). Exercise-mediated regulation of autophagy could therefore also regulate protein homeostasis by regulating the degradation and synthesis of proteins (Escobar, 2018; Wu et al., 2019).

Based on the length, width, and thickness tumor measurements and the calculations followed by these measurements, treadmill exercised mice experienced significantly smaller tumors when compared to the sedentary tumor bearing group. Exercised estimated tumor volumes were reduced by -52%, estimated tumor masses were reduced by -38%, and relative tumor masses were -39% smaller than sedentary tumors. These findings show similar or greater reductions in tumor masses and volumes compared to previous research. Alves et al. showed that mice experienced decreased tumor growth by -52% after 18 days of HIIT. Hojman et al. showed decreased tumor mass by -56% after free wheel running exercise and stated that reduction in tumor growth due to exercise interventions can be as high as 67%, whereas Penna et al. showed a decrease in tumor mass by only -20% after a moderate intensity aerobic exercise protocol.
While the underlying mechanism behind cancer-mediated or exercise-mediated autophagy are still not well understood, the findings of this thesis study, indicating significant reductions in tumor growth, coincide with the other findings of this study, suggesting exercise-mediated regulation of autophagy and therefore stunting of tumor growth and attenuation of cancer development in low intensity treadmill trained tumor bearing mice. Therefore, the beneficial effects of this low intensity treadmill protocol conform with most previously conducted research which have mostly looked at higher exercise intensities than this thesis study.

Overall, the findings of this study provided evidence that low intensity treadmill exercise of 4-weeks could act as a protective measure and treatment intervention against cancer cachexia in male mice. This suggestion was associated with the findings of preserved grip strength in the treadmill trained mice as a measure of skeletal muscle function, as well as preserved cardiac structure and function, regulated autophagy, and inhibited tumor growth in the exercised group compared to sedentary counterparts. While further studies need to be performed to evaluate the translatability to human cancer patients, the data of this study indicate that low intensity exercise interventions may potentially serve as an easily affordable and accessible cancer treatment, in addition to pharmaceutical interventions, that could slow the development of cancer, attenuate the detrimental effects of cancer cachexia, and increase quality of life.

Limitations

While some of the limitations have already been addressed, for example comparing phosphorylated AKT as the active form to total AKT to identify the ratio of active versus inactive AKT protein expression in each group, there are other limitations that need to be addressed for consideration.
First, this study was performed in a preclinical laboratory setting using a mouse model for the intervention. While variance of cancer development in this setting is rather small because each mouse was inoculated with the same tumor cell concentration, in the same location, and at the same time points, the translatability to human cancer models is limited. The development of human tumors is more spontaneous, the types of tumors may differ for each individual, and effects of cancer cachexia on each individual are not as predictable in the clinical setting. Tumor development in human cancer patients would be more variable and less controllable.

Second, while the treadmill trained mice were physically encouraged to continue their running sessions, this type of exercise is considered involuntary. Throughout the treadmill protocol, differences in consistency and willingness to perform the exercise session was observed, both when comparing mice’s performance to each other as well as individual performance differences between different days of exercise. These variances in exercise performance have not been used for validation. Therefore, individual differences in performance could have resulted in smaller exercise effects in some animals, which would contribute to greater variability within and between the two exercised groups. Future studies should include measurements of aerobic adaptations, for example Cytochrome C analysis, to 1) determine if the low intensity treadmill exercise protocol resulted in exercise adaptations, and 2) if the adaptations due to this protocol influence the results.

Lastly, food intake was not controlled for in this study. Research has shown that high intensity treadmill interventions in mice can lead to suppressed food intake and caloric deficits (Landry et al., 2021). While this treadmill protocol was representative for low intensity exercise, food intake measurements would still be an interesting analysis and could potentially reveal nutritional effects on cancer development and cancer-mediated muscle loss. Especially because
there have been recent successful interventions combining calorie restriction with other treatment options to prevent and treat cancer in human cancer patients (Champ & Klement, 2020).

**Future Directions**

While the field of cancer cachexia research is relatively new, it is rapidly growing and of high interest due to the detrimental and irreversible effects that affect an immense amount of cancer patients. This study underlines the importance of better understanding the benefits of exercise for cancer patients and to understand the underlying mechanisms. The findings of this study support the concept that exercise as an additional cancer intervention may not only attenuate the detrimental effects of cancer cachexia but also improve quality of life. Future studies should investigate the combined effects of low intensity treadmill interventions in mice in combination with pharmaceutical chemotherapy drugs as this maintains to be the primary treatment option for cancer patients. The combination of these two interventions in preclinical settings could aid in determining the potential advantages or disadvantages of exercise in combination with pharmaceutical interventions, as well as determining the most beneficial ratio or involvement of each intervention throughout the treatment period. Additionally, food intake and caloric restriction should be assessed during exercise, tumor burden, as well as during the combination of both low intensity treadmill exercise concurrent with tumor burden to identify potential benefits of nutritional aspects in combination with exercise. Throughout the process, future studies should further investigate the role of autophagy regulation by analyzing autophagic flux and autophagic protein analysis to determine if there are specific pathways that, when controlled, could lead to the regulation of autophagy and potentially slow or inhibit cancer development.
Lastly, once the beneficial effects of low intensity exercise in combination with other treatment interventions have been assessed, future studies should examine the most beneficial timing, duration, and intensity that can be translated to clinical settings, which will ultimately lead to low intensity exercise interventions implemented into treatment of human cancer patients suffering from the detrimental effects of cancer cachexia.
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