Antioxidant Treatment of Muscle Wasting and Fatigue in Tumor-Bearing Mice

DISSERTATION

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By

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Abstract

Purpose: Fatigue is the most common and distressing symptom reported by cancer patients, significantly reducing quality of life. While the mechanisms of cancer-related fatigue (CRF) are poorly understood, evidence indicates that pro-inflammatory cytokines and reactive oxygen species, produced by the tumor and/or host, alter muscle metabolism to cause muscle wasting and cardiac dysfunction. Symptoms of muscle wasting and cardiac dysfunction are weakness and decreased effort tolerance, similar to patients with CRF. Ubiquinol, an endogenously produced, lipid soluble antioxidant, protects against lipid peroxidation to cellular lipid membranes, proteins and DNA, and regenerates active forms of other antioxidants such as vitamins C and E. The purpose of this study was to determine if treatment with ubiquinol, might preserve muscle mass, improve cardiac function, and reduce fatigue in an animal model of CRF. The following hypotheses were tested in mice bearing the colon26 (C26) adenocarcinoma: 1. Ubiquinol will reduce the expression of biomarkers of muscle protein degradation and increase protein synthesis in the gastrocnemius and cardiac muscles of tumor-bearing mice; 2. Ubiquinol will increase: (a) muscle mass, (b) fore-limb grip strength, (c) *in vivo* cardiac function, and (d) voluntary running activity (VRA) in tumor-bearing mice and; 3. Ubiquinol will not affect tumor growth.

Method: Eight week old adult female CD2F1 mice were acclimated to running wheels and grip strength 1 week prior to being inoculated with the tumor cells. Half of the control and half of the tumor bearing mice received ubiquinol 500 mg/kg/day in their drinking water. VRA and grip strength were measured on days 0, 8, 14 and 17 of tumor growth. Cardiac function was measured by echocardiography on day 18 or 19 after which the mice were euthanized. The heart and gastrocnemius muscles were weighed and normalized to body weight. Serum levels of proinflammatory cytokines were measured by ELISA. Oxidative stress was measured in skeletal and cardiac muscle homogenates. Skeletal and cardiac muscle expression of genes reflecting muscle inflammation and biomarkers associated with muscle metabolism were determined using real time-PCR.

Results: Tumor growth induced loss of muscle mass and decreased VRA and grip strength. Tumor growth also increased serum and muscle levels of pro-inflammatory cytokines, oxidative stress in cardiac muscle, altered cardiac function and biomarkers associated with muscle metabolism in skeletal muscle. Echocardiography revealed a diastolic dysfunction in the tumor-bearing mice. Treatment with ubiquinol did not alter levels of proinflammatory cytokines in serum and skeletal and cardiac muscle, oxidative stress in cardiac muscle, biomarkers associated with muscle metabolism, cardiac function, VRA or grip strength in the tumor-bearing mice, but did increase skeletal muscle mass.

Conclusion: These data confirm prior studies indicating tumor growth alone affects cardiac function, which could contribute to CRF. Because ubiquinol improved muscle mass without affecting measures of fatigue and weakness, inflammation, cardiac function

or muscle metabolism, we conclude that antioxidant treatment alone is not likely to

reverse CRF. These data also suggest that skeletal muscle wasting may not be a major

factor in reduced physical activity and weakness associated with CRF.

Dedication

This is dedicated to everyone who supported and helped me along my journey. This includes my children: Monica, Maria and Naman; my parents: Joshua and Kimie Bacon; my family members: to include Gloria, Sophia, Kimie and Dale Jr.; and a great friend:

Shiron Devere Jones

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Chapter 1: Introduction

It is estimated that more than 1.6 million people will be newly diagnosed with cancer in 2013 (ACS, 2013). Of that number 65% will be alive in 5 years (Morgan, 2009), due to earlier detection and more targeted aggressive treatment options. Fatigue, experienced by 70% to 100% of all patients with cancer, is the most commonly reported symptom (Durham, Dillon & Sheffield-Moore, 2009; Franklin & Packel, 2006). Cancer related fatigue (CRF) is more distressing than pain, nausea, or vomiting and may be a presenting symptom at diagnosis. It may increase during treatment for cancer and may continue for years after treatment is completed (Bower, Ganz, Desmond, Bernaards, Rowland, et al., 2006; Curigliano et al., 2012; Ryan, Carroll, Ryan, Mustian, & Fiscella, 2007). The causes of CRF are multimodal and not clearly understood. Several proposed mechanisms include disturbances in the hypothalamic pituitary adrenal (HPA) axis, serotonin synthesis, circadian rhythm, the immune system and alterations in sleep/awake patterns (reviewed in Barsevik et al., 2010).

CRF is experienced as an overwhelming sense of exhaustion with a reduction in capacity for physical and mental work. The impact of CRF on the ability to perform activities of daily living (ADL) is both profound and pervasive, with patients reporting significant impairment in their ability to perform household responsibilities and socialize (Hofman, Ryan, Figuero-Moseley, Jean-Pierre, & Morrow, 2007). In addition, many

employed patients with CRF have to alter their employment status, which often leads to negative economic consequences (Amir, Wilson, Hennings & Young, 2011).

Skeletal Muscle Wasting

Tumor-induced skeletal muscle wasting may be a significant factor in CRF, contributing to complaints of weakness and decreased effort tolerance (Dodson et al., 2011). In muscle wasting, there is a decrease in muscle protein synthesis, an increase in muscle protein degradation, or both (Tisdale, 2009). Several metabolic pathways contribute to muscle protein degradation, two of which are the ubiquitin proteosome ATP-dependent pathway (UPP) and the autophagy-lysosome system (ALS). The UPP is responsible for the degradation of the majority of normal and abnormal intracellular proteins. The ALS is important for removal of damaged proteins and organelles in cells. Both systems maintain cellular homeostasis in skeletal muscle. Two components of the UPP are the E3 ubiquitin ligases, MuRF1 and MAFbx. These ligases attach ubiquitin molecules to damaged proteins; the proteins are then recognized by the proteosome, which breaks the ubiquinated proteins down into smaller peptides (Lecker, Goldberg & Mitch, 2006). BNIP3 is a pro-apoptotic protein that is involved in the autophagic process (Azad, et al., 2008; Bellot et al., 2009). Skeletal muscle expression of MAFbx, MuRF1 and BNIP3 is upregulated in tumor bearing mice with skeletal muscle wasting (Asp, Tian Wendel & Belury, 2010), and components of the UPP were elevated in patients with cancer who developed skeletal muscle wasting (Khal, Hine, Fearon, DeJong & Tisdale, 2005a).

During the autophagic process, a vesicle (autophagosome) is formed around material in the cytoplasm to be degraded and then attaches to a lysosome for degradation of its contents (Lamark & Johansen, 2010). Higher rates of vesicle formation have been found in the skeletal muscles of mice during fasting and denervation conditions normally associated with muscle atrophy (Mizushima, Yamamoto, Matsui, Yoshimori & Ohsumi, 2004; Wang et al., 2005).

Pro-inflammatory cytokines

Muscle wasting is associated with tumor growth and is mediated in part by an increase in pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNFa) and interleukin-6 (IL-6), produced by the tumor and/or host (Acharyya & Guttridge, 2007; Aggarwal, Shishodia, Sandur, Pandey & Sethi, 2006; Carson & Baltgalvis, 2010; Durham et al., 2009). Tumor growth increases plasma levels (Chen & Qui, 2011; Krzysteck-Korpacka, et al., 2007; Takahashi et al., 2009), and muscle expression of these pro-inflammatory cytokines and the type I receptor for TNFa (TNRF1) (Catalano, et al., 2003; Graves, Ramsey & McCarthy, 2006; Hitt, Graves & McCarthy, 2005). TNFa acts via the TNFR1 on the muscle cell wall to activate multiple intracellular signaling pathways known to be involved in muscle wasting, including the UPP and ALS (Acharyya & Guttridge, 2007; Argiles, Busquets & Lopez-Soriano, 2009; Saini, Faulkner, Al-Shanti & Stewart, 2009).

A significant correlation between fatigue and circulating levels of inflammatory markers, such as IL-6, is found across various cancers (Schubert, Hong, Natarajan, Mills & Dimsdale, 2007). Treatment with a non steroidal anti-inflammatory drug, preserved

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skeletal muscle mass and reduced expression of MAFbx and MuRF1 in skeletal muscle in tumor bearing mice (Hitt et al., 2005; McCarthy, Whitney, Hitt, & Al-Majid, 2004). In patients with advanced cancer with weight loss and fatigue, the use of an anti-inflammatory drug reduced serum levels of TNFa and improved physiological measures of fatigue (Mantovani et al., 2010). These studies indicate the role of inflammation in skeletal muscle wasting.

Oxidative Stress

Proinflammatory cytokines increase the production of reactive oxygen species (ROS). ROS are normally generated during cellular metabolism and mitochondrial energy production, acting as second messengers for other cellular signaling responses (Valko et al., 2006). Normally ROS that are produced during metabolism are balanced by the intracellular antioxidant defense system. When there is an imbalance between the two systems, oxidative stress develops and damage to cellular membrane lipids, structural and regulatory proteins and DNA results (Moylan & Reid, 2007). Oxidative stress affects the cellular membranes by producing reactive intermediates that can cause the lipid peroxidation of polyunsaturated fatty acids, leading to the formation of lipoperoxide radicals (Barrera, 2012). Products of lipid peroxidation 4-hydroxy-2-noneal (HNE) and Malondialdehyde (MDA) can spread and react with and cross-link with other phospholipids, proteins and DNA, inducing damage (Bentiger, Brismar & Dallner, 2007). Oxidative stress damages proteins by altering amino acid residue side chains and protein backbones, causing protein fragmentation and the formation of protein carbonyl derivatives. Formation of protein carbonyl derivatives can occur from reactions with

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HNE and MDA or from reactive carbonyl derivatives such as ketoamines, ketoaldehydes and deoxyosones (Berlett & Stadtman, 1997). Exposure of DNA bases or the deoxyribosomal backbone to oxidative stress causes damage to the bases or cause strand breakage (Valko, Izakovik, Mazur, Rhodes, & Tessler, 2004).

Tumor growth is associated with increased oxidant activity (Montovani, et al., 2012). Pro-inflammatory cytokines such as TNFa and IL-6 induce oxidative stress (Moylan & Reid, 2007). In muscle cells, increased oxidant activity is associated with increased expression of MuRF-1, MAFbx, and Bnip3, suggesting that oxidative stress can induce skeletal muscle wasting (Aucello, Dobrowolny, & Musaro, 2009; McClung, Judge, Powers, & Yan, 2010). An impaired antioxidant defense system along with an increase in muscle expression of ROS, total protein carbonylation, indicators of lipid peroxidation, HNE and MDA protein adducts, MAFbx and MuRF1 have been found in tumor bearing rats with skeletal muscle wasting (Barriero et al., 2005; Mastrocola, et al., 2008). An antioxidant and anti-inflammatory agent used with advanced cancer patients who had CRF and skeletal muscle wasting reduced serum levels of ROS, IL-6, TNFa and fatigue and facilitated weight gain (Mantovani et al., 2006). These studies indicate the significance of oxidative stress on muscle metabolism and suggest that oxidative stress may be a potential target for preventing skeletal muscle wasting and perhaps reducing CRF.

Cardiac Dysfunction

People with cancer are at increased risk for cardiovascular dysfunction as a side effect of chemotherapy and/or radiotherapy treatments (Berry & Jorden, 2005; Petersen

&Wurschmidt, 2011; Sheppard, Berger & Sebag, 2013). The major effect of cardiac dysfunction is reduced effort tolerance, a major complaint of patients with CRF. While the cardiotoxic effects of chemotherapy have been known for some time (Shakir & Rasul, 2009), newer evidence suggests that tumor growth also has a direct effect on the myocardium, resulting in cardiac atrophy and dysfunction (Cosper & Leinwand, 2011; Tian, Asp, Nishijima, & Belury, 2011; Tian et al., 2010; Wysong et al., 2011; Xu et al., 2011). Heart and skeletal muscle wasting in tumor bearing rats has been correlated with oxidative damage to proteins involved in glycolysis, ATP production and distribution, muscle contraction and mitochondrial metabolism (Marin-Corral et al., 2010), indicating that the increase in oxidative damage from tumor growth not only occurs in skeletal muscle but also in the heart. Treatment with sodium selenate or omega-3 fish oil with vitamin E reduced the amount of oxidized protein in the myocardium and improved cardiac function in diabetic rats (Aydemir-Koksoy, Bilginoglu, Sariahmetoglu, Schulz & Turan, 2010). In diabetic mice, cardiac overexpression of the antioxidant catalase preserved normal cardiac morphology, prevented contractile dysfunction and the formation of MDA adducts in the heart (Ye et al., 2004). Cardiac overexpression of the mitochondrial antioxidant, manganese superoxide dismutase (MnSOD), prevented damage to the mitochondria, increased cardiac expression of the antioxidant catalase, and prevented contractile dysfunction in diabetic mice (Shen, Zheng, Metreveli, & Epstein, 2006). The effect of antioxidant treatment on oxidative damage in hearts of tumor bearing animals has not been determined.

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Ubiquinol

CoQ10 (ubiquinone) is well known for its bioenergetic and antioxidant functions. CoQ10 plays an important role in respiratory metabolism as a mobile electron and proton carrier in the mitochondrial electron transport chain producing ATP (Lenaz, Fato, Formigga, & Genova, 2007). CoQ10 shuttles electrons and protons from complex I and II to complex III existing in three redox states, oxidized ubiquinone, ubisemiquinone and the reduced form ubiquinol depending on the level of protonation (Bentinger, Tekle, & Dallner, 2010; Lenaz, Fato, Formigga, & Genova, 2007). A lipid soluble component of all cellular membranes, CoQ10 is the only lipid soluble antioxidant synthesized endogenously (Littaru & Tiano, 2007; Turunen, Olsson & Dallner, 2004). CoQ10 is decreased in the plasma of breast, lung, melanoma and cervical cancers patients as compared to healthy controls, and is a marker of disease occurrence in breast cancer and severity in melanoma, conditions in which CRF is a significant problem (Cooney et al., 2011; Cobanoglu et al., 2011; Folkers, Osterborg, Nylander, Morita & Mellstedt, 1997; Palan, Mikhail, Shaban & Romney, 2003; Rusciani et al., 2006;).

In a mouse model of Alzheimer's disease, treatment with CoQ10 reduced oxidative stress as measured by a reduction in protein carbonyl content in the brain, and improved cognitive function as compared to untreated animals (Dumont et al., 2011). CoQ10 supplementation has resulted in reduced levels of biomarkers associated with oxidative stress, increased expression of endogenous antioxidants, and improved systolic function in patients with cardiovascular disease (Belardinelli et. al, 2006; Langsjoen & Langsjoen, 2008; Lee, Huang, Chen, & Lin, 2012). Supplementation with CoQ10 has

been beneficial in alleviating symptoms of fatigue and improving exercise endurance in healthy mice and humans as evidenced by an increase in activity levels (Fu, Ji & Dam, 2010; Mizuno et al., 2008), and prevented cardiotoxicity and nephrotoxicity caused by the administration of chemotherapeutic agents to patients with cancer (Conklin, 2004; El-Sheikh, Morsey, Mahmoud, Rifaai & Abdelrahman, 2012; Fouad, Al-Sultan, Refaie & Yacoubi, 2010).

When taken orally, CoQ10 is converted to ubiquinol (its reduced antioxidant form) in the gastrointestinal (GI) tract. Ninety five percent of CoQ10 in the body exists as ubiquinol (Bhagavan & Chopra, 2007). Ubiquinol protects against lipid peroxidation to cellular lipid membranes, proteins and DNA, and regenerates active forms of other antioxidants such as vitamins C and E (Bentinger et al., 2007). CoQ10 in its pure form is poorly soluble in water and is slowly absorbed from the GI tract, affecting bioavailability (Lui, & Artman, 2009). Ubiquinol is more readily absorbed in the GI tract; in studies comparing the bioavailability of CoQ10 and ubiquinol, ubiquinol caused a significant increase in plasma concentrations (Bhagavan, & Chopra, 2007). Therefore, ubiquinol was used for this study because it is known to be more efficacious as an antioxidant than CoQ10 due to its better bioavailability.

Purpose of Study

The purpose of this study was to determine the effects of ubiquinol on muscle function in an animal model of cancer-related fatigue (***Figure 1***). In mice, fatigue is modeled as reduced voluntary running activity (VRA) and skeletal muscle weakness is modeled as reduced grip strength. Myocardial function was determined using

echocardiography. The specific aims of the study were to test the following hypotheses in mice bearing the colon26 (C26) carcinoma:

1. Ubiquinol will reduce the expression of biomarkers of muscle protein degradation (IL-6, TNFR1, MAFbx, MuRF1, and Bnip3) in the gastrocnemius and heart muscles of tumor-bearing mice.

2. Ubiquinol will increase (a) muscle mass, (b) expression of biomarker of protein synthesis (IGF-1, MyoD)

(c) fore-limb grip strength, (d) *in vivo* cardiac heart function, and (e) VRA in tumor-bearing mice.

3. Ubiquinol will not affect tumor growth.

It has been proposed that antioxidants, such as ubiquinol, might be useful for the treatment of skeletal muscle atrophy associated with aging and chronic inflammatory conditions (Bonetto et al., 2009b). We believe muscle wasting is a major factor in the pathobiology of CRF, a condition for which there currently are no effective treatments. Others have raised concerns that administration of anti-oxidants might favor tumor growth or alter the efficacy of anti-tumor therapies (Nicholson & Conklin, 2008). This study used an animal model of CRF to explore the associations between skeletal muscle wasting, myocardial function, and symptoms of CRF. The effects of ubiquinol on tumor growth were also explored. The findings will contribute to the understanding of the pathobiology of CRF, and more importantly, could lead to further studies to determine whether antioxidants are useful for the treatment of CRF in patients with persistent disease.
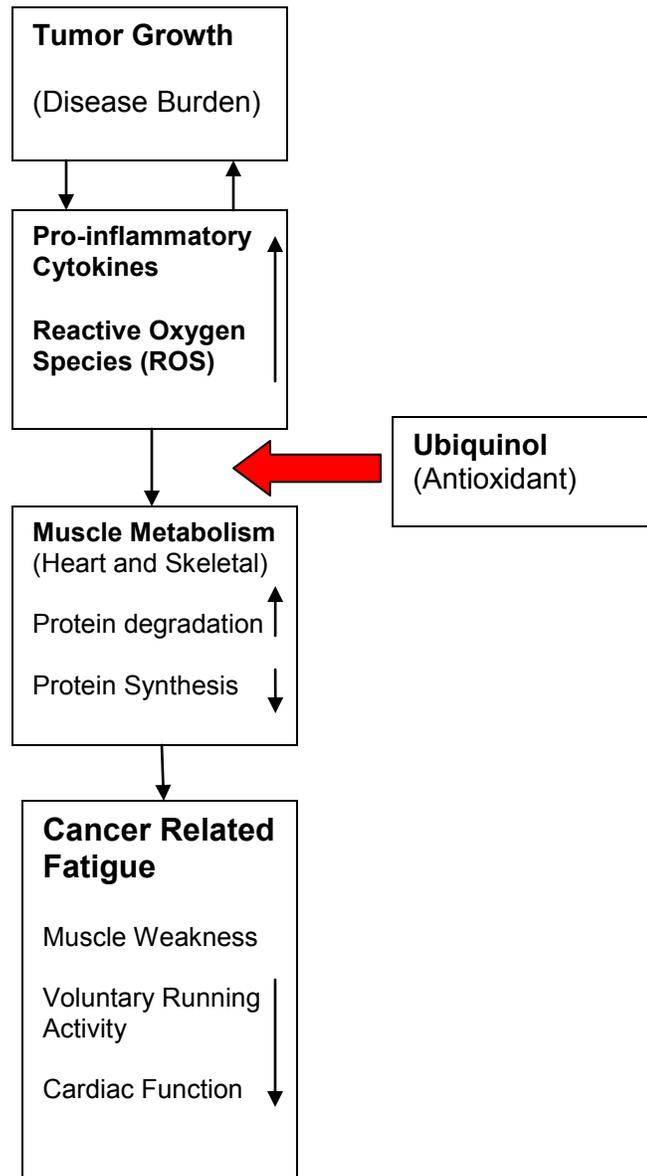
Conceptual Framework

Figure 1. Conceptual framework for relationship between tumor growth and fatigue.

This figure illustrates the relationship between tumor growth, proinflammatory cytokines, oxidative stress, muscle metabolism and symptoms of CRF.

Chapter 2: Review of Literature

The majority of patients with cancer experience cancer related fatigue (CRF), affecting their quality of life. The National Comprehensive Cancer Network (NCCN) defined CRF as "a distressing, persistent, subjective sense of physical, emotional and/or cognitive tiredness or exhaustion related to cancer or cancer treatment that is not proportional to recent activity and interferes with usual functioning" (NCCN, 2008, p. FT1). The causes of CRF are not clearly understood but prior research has implicated tumor induced skeletal muscle wasting and more recently myocardial dysfunction as possible mechanisms. A review of the literature of central and peripheral components of CRF, and the role of pro-inflammatory cytokines and oxidative stress in tumor-induced skeletal muscle wasting and myocardial dysfunction will follow. The potential importance of antioxidant therapy for treatment of CRF will also be discussed.

Cancer Related Fatigue

CRF has central and peripheral components. Central components of CRF include the effects of tumor growth or cancer treatments on the hypothalamic pituitary adrenal (HPA) axis, serotonin (5-HT) synthesis, circadian rhythm and the immune system, all of which affect mood and behavior. Peripheral components of fatigue include the effects of tumor growth and cancer treatment on muscle mass, which likely contributes to weakness and decreased effort tolerance. Alterations in cardiac function as a side effect of cancer

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treatment has been extensively studied, but new evidence suggests tumor growth alone can lead to cardiac dysfunction, a possible contributing factor to peripheral fatigue (Tian et al., 2011; Tian et al., 2010; Wysong et al., 2011; Xu et al., 2011).

Central Fatigue

HPA Axis

The HPA axis is the central regulatory system for the release of cortisol. Corticotropin releasing hormone (CRH) is released from the paraventricular nucleus of the hypothalamus and acts together with antidiuretic hormone (vasopressin) to release corticotrophin (adrenocorticotropic hormone [ACTH]) from the anterior pituitary (Ryan et al., 2007). The release of ACTH acts on the adrenal cortex to secrete cortisol. Cortisol influences metabolic, behavioral and immune processes that enable the body to meet the day-to-day demands and challenges. Circulating levels of cortisol act on the pituitary and hypothalamus to provide a negative feedback on further secretion of cortisol. Secretion of cortisol is greatest in the morning, and slowly declines over the course of the day, reaching its nadir with the onset of sleep.

When the individual perceives a threat to well-being, there is an additional release of CRH, ACTH and cortisol. Psychogenic and physical stress is relayed to the body via increased activity of the HPA axis. Increased secretion of cortisol results in metabolic responses that allow the individual to respond to the threat or to biological challenges (Silverman, Heim, Nater, Marques, & Sternberg, 2010). Increased circulating levels of cortisol or dysregulation of the HPA axis have been associated with increased immune activation and inflammatory responses associated with fatigue, anorexia, weight loss,

arthralgia, myalgia, sleep disturbance and mood disorders resembling CRF (Chrousos, 2009; Jager, Sleijfer, & Van der Rijt, 2008). Fatigued breast cancer survivors exposed to a psychological stressor exhibited a significantly blunted cortisol response, reflecting a dysregulation of the HPA axis in the fatigued survivors as compared to non-fatigued survivors (Bower, Ganz, & Aziz, 2005a). In a comparison of breast cancer survivors with fatigue to non-fatigued survivors, there were altered diurnal variations in cortisol expression with flatter cortisol slopes in the survivors with CRF (Bower, Ganz, Dickerson, Peterson, Aziz & Fahey, 2005b).

Serotonin

Serotonin is synthesized from the amino acid tryptophan. Tryptophan is introduced into the body by dietary proteins, crosses the blood brain barrier, and is converted to 5-hydroxytryptophan (5-HTP) by the enzyme tryptophan hydroxylase. 5-HTP is converted to serotonin by the enzyme aromatic amino acid decarboxylase. In the pineal gland, serotonin is converted to melatonin (Hensler, 2012). Serotonin (5-HT) has effects on sleep, appetite, mood, learning, memory and muscle contraction (Ryan et al., 2007). Symptoms of depressive disorders may be improved by treatment with selective serotonin reuptake inhibitor (SSRI) drugs. Decreased levels of serotonin are related to symptoms of depression which often co-occur with CRF (Roscoe et al., 2005). A review of the literature reflects that depression is consistently moderately correlated with CRF, with the average correlation between the two, weighted by sample size, being $r = .56$ (Brown & Kroenke, 2009).

Proinflammatory cytokines can affect mood through their effects on tryptophan metabolism and serotonin synthesis (as reviewed in Anisman, 2008). Proinflammatory cytokines such as IFN-alpha (IFNa), IFN-gamma (IFNy) and tumor necrosis factor alpha (TNFa) increase production of indoleamine 2,3 dioxygenase (IDO) through several inflammatory signaling pathways, such as nuclear factor-kB (NF-kB) and p38 mitogen activated protein kinase (p38MAPK) (Fujigaki et al., 2006). IDO competes with 5-HTP for metabolism of tryptophan. IDO converts tryptophan to kynurenine, which is then converted to either 3-hydroxykynurenine (3-HK) and quinolinic acid (QA) or kynurenic acid (KA) (Laviano, Meguid, Preziosa, & Fanelli, 2007). These metabolites of tryptophan have depressive effects on cognition and mood. In addition, proinflammatory cytokines interleukin-1 beta (IL-1β) and TNFa, via the p38MAPK pathway, upregulate serotonin membrane transporter activity, reducing concentrations of serotonin in the midbrain and striatal synaptosomes in healthy mice (Zhu, Blakely & Hewlett, 2006). In an animal model of chronic immune activation, IDO mRNA and depressed behavior were associated with elevated levels of mRNA for IFNy , IL-1β and TNFa in brain tissue (O'Connor et al., 2009a).

Tumor growth is associated with an increase in circulating levels of proinflammatory cytokines and could induce symptoms of depression by altering the metabolism of tryptophan and reducing serotonin activity in the brain **(*Figure 2*).** In patients with malignant melanoma treated with IFNa, a prolonged decrease in plasma tryptophan and increase in plasma kynurenine were seen in patients who developed severe depression compared to those who did not (Capuron et al., 2003). However,

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treatment of patients with CRF with an antidepressant, selective serotonin reuptake

inhibitor (SSRI), improved mood but did not improve symptoms of CRF, suggesting that

symptoms of depression are not a major contributor to the symptoms of CRF (Carroll,

Kohli, Mustian, Roscoe & Morrow, 2007).
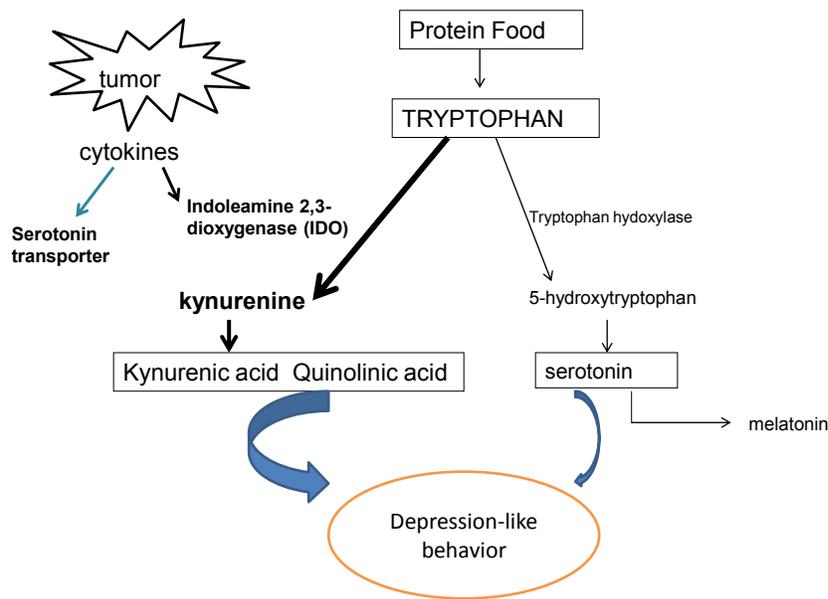
Figure 2. Effects of proinflammatory cytokines on serotonin synthesis.

This figure describes the proposed relationship between tumor growth, proinflammatory
cytokines, tryptophan degradation and decreased serotonin synthesis, all resulting in
depressive behavior.

Circadian Rhythms

Circadian rhythms are endogenous diurnal patterns based on a 24-hour cycle,

influenced by changes in environmental factors such as light and darkness and

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psychological factors such as stress (Barsevick et al., 2010). Patients with cancer often report sleep disturbances and alterations in rest/activity patterns, which are often associated with dysregulation of the HPA axis (Ryan et al., 2007). A higher level of fatigue and a reduction in time of uninterrupted sleep were seen in patients diagnosed with breast cancer before treatment (Ancoli-Israel et al., 2006; Berger, Farr, Kuhn, Fischer & Agrawal, 2007). Levin et al. (2005) found greater disturbances in circadian rhythms, with increased periods of wakefulness during sleeping hours and increased sleeping during the day, in fatigued patients with advanced lung cancer as compared to healthy controls.

Immune Function/Behavior

One explanation of CRF is the cytokine hypothesis of sickness behavior (Lee, et al., 2004). Infection by an invading microorganism triggers a set of immune, physiological, behavioral and metabolic responses essential for the survival of the host (Dantzer, 2004). When individuals develop an infection they experience weakness, fatigue, an inability to concentrate, depressed mood, reduced appetite and fever (Kelley et al., 2003). The physiological and behavioral responses displayed after exposure to specific proinflammatory cytokines is referred to as sickness behavior (Cleeland et al., 2003). Proinflammatory cytokines primarily involved in producing the characteristics of sickness behavior include IL-1B, IL-6 and TNFa (Vollmer et al., 2004, Myers, 2008). Tumor growth is thought to activate the immune response to tumor antigens, leading to the production of cytokines and development of sickness behaviors. A significant

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correlation between fatigue and circulating levels of inflammatory markers, such as interleukin-6 (IL-6), occurs with various types of cancers (Schubert et al., 2007).

Peripheral Fatigue

Skeletal Muscle Wasting

In animal models of sickness behavior, prolonged production of IL-6 and TNFa can lead to fat and muscle wasting (Wood et al., 2006). Prior research suggests that tumor-induced skeletal muscle wasting may be a significant factor in CRF, contributing to complaints of weakness and decreased effort tolerance (Dodson et al., 2011). Upregulation of two metabolic systems involved in muscle protein degradation, (UPP and ALS) results in increased protein degradation and myofiber loss. Both systems are responsible for degradation and removal of the majority of normal and abnormal intracellular proteins and organelles in the cells. Enzymatic ligases (E1, E2, and E3) attach chains of the polypeptide co-factor, Ub, onto proteins targeted for destruction by the 26S proteosome, resulting in smaller peptides (Lecker et al., 2006). Two E3 enzymatic ligases, Muscle RING-finger protein-1 (MuRF1) and MAFbx, are upregulated in the skeletal muscle of muscle wasting conditions associated with chronic diseases and disuse atrophy in humans and animals (as reviewed in Foletta, White, Larsen, Leger & Russell, 2011). An upregulation of UPP activity and increased expression of MAFbx and MuRF1 have been found in the skeletal muscle in several animal models of tumor growth and in patients with cancer with skeletal muscle wasting (Costelli et al., 2006; Khal, Wyke, Russell, Hine & Tisdale, 2005; Khal et al., 2005; McCarthy et al., 2004; Xu et al., 2011).

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In mice subjected to nutritional starvation, an increase in autophagosomes was seen in the gastrocnemius and heart muscles (Mizushima et al., 2004). In an animal model of denervation atrophy, mice deficient in Runx1, a protein involved in muscle preservation, had increased skeletal muscle expression of MAFbx, MuRF1 and autophagic vacuoles (Wang et al., 2005). Skeletal muscle expression of MAFbx, MuRF1 and BNIP3 (a pro-apoptotic BH3-only protein that is involved in the autophagic process) has been found to be upregulated in tumor-bearing mice with skeletal muscle wasting (Asp et al., 2010; Bonetto et al., 2009a; Cosper & Leinwand, 2011; Costelli et al., 2006; Hitt et al., 2005; Penna et al., 2010; Siddiqui et al., 2009; Xu et al., 2011; Zhou et al., 2011).

Pro-inflammatory Cytokines

Mediators of increased UPP activity and autophagy in tumor-induced muscle wasting include proinflammatory cytokines IL-6 and TNFa and oxidative stress. Tumor growth is associated with an increase in TNFa and IL-6 produced by the tumor and/or host (Aggarwal et al., 2006; Chen & Qui, 2011; Krzysteck-Korpacka et al., 2007; Takahashi et al., 2009); both cytokines are key mediators in skeletal muscle wasting associated with cancer (Acharyya & Guttridge, 2007; Carson & Baltgalvis, 2010; Durham, et al., 2009). TNFa acts via the TNFR1 (type I receptor for TNFa) on the muscle cell wall to activate multiple intracellular signaling pathways known to be involved in muscle wasting, including the UPP and ALS (Acharyya & Guttridge, 2007; Argiles et al., 2009; Saini et al., 2009). Increased skeletal muscle expression of TNFR1 occurs with tumor growth (Catalano et al., 2003; Graves et al., 2006; Hitt et al., 2005).

The use of anti-inflammatory drugs has reduced skeletal muscle wasting in tumor-bearing animals and humans, indicating a role of proinflammatory cytokines in skeletal muscle wasting. Treatment with indomethacin, an anti-inflammatory drug, preserved skeletal muscle mass, prevented an increase in MAFbx and MuRF1, and reduced an increase in TNRF1 in tumor-bearing mice (Hitt et al., 2005). Treatment of tumor-bearing rats with inhibitors of TNFa and IL-6 demonstrated a decrease in proteasomal activity and a reduction in loss of skeletal muscle mass (Costelli et al., 2002). In patients with advanced cancer with skeletal muscle wasting, treatment with the anti-inflammatory drug celecoxib reduced serum levels of TNFa and increased lean body mass (Mantovani et al., 2010).

Oxidative Stress

Tumor growth is associated with increased oxidant activity (Montovani et al., 2012). One mechanism of increased oxidant activity is by pro-inflammatory cytokines such as TNFa binding to TNFR1 and increasing the production of reactive oxygen species (ROS) (Reid & Moylan, 2011). ROS includes the free radicals superoxide anion (O_2-) and hydroxyl (OH) as well as highly reactive hydrogen peroxide (H_2O_2) (Valko et al., 2006). Normally ROS that are produced are balanced by the intracellular antioxidant defense system. When there is an imbalance between the two systems, oxidative stress develops and cellular damage to membrane lipids, structural and regulatory proteins and DNA occurs (Moylan & Reid, 2007). Impairment of the antioxidant defense system with tumor growth is seen as an increase in ROS production and a decrease in antioxidant activity or an increase in ROS production and normal levels of antioxidants (Barriero et

al., 2005; Mastrocola et al., 2007; Montovani et al., 2003). Intensified oxidant activity is associated with increased expression of MuRF-1, MAFbx, and Bnip3 in muscle cells (Aucello et al., 2009; McClung et al., 2010). Increased expression of total protein carbonylation, and indicators of lipid peroxidation (4-hydroxy-2-noneal (HNE) and malondialdehyde (MDA) protein adducts) without impairment in antioxidant function indicates an ineffective antioxidant system in preventing oxidative damage to skeletal muscles proteins in tumor bearing rats (Barriero et al., 2005). Mastrocola et al. (2007) found increased skeletal muscle expression of MAFbx and MuRF1, ROS, HNE, GSSG/GSH ratio and decreased SOD in tumor bearing rats with skeletal muscle wasting. In patients with advanced cancer, there was an increase in serum levels of ROS and a decrease in serum levels of the antioxidant glutathione peroxidase (GPx) (Montovani et al., 2003).

Cardiac Dysfunction

Cardiac output (CO) is the amount of blood ejected from the heart in one minute, and ejection fraction (EF) is the portion of the diastolic volume of blood pumped out during each heartbeat. Fractional shortening (FS), an indicator of systolic function, is calculated during echocardiography as the difference between left ventricular diastolic and systolic diameters. Any decrements in systolic contraction can lead to reduced CO and symptoms of poor tissue perfusion, such as fatigue, reduced effort tolerance, and weakness; these are similar to symptoms experienced by cancer patients with CRF. While it is well know that cancer treatment affects myocardial function (Berry & Jordan, 2005; Curigliano et al., 2012), the effects of tumor growth on myocardial structure and function

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are largely unknown. Recent research has shown that tumor growth causes cardiac muscle wasting and/or alterations in cardiac structure and function (Tian et al., 2011; Tian et al., 2010; Shadfar et al., 2011; Wysong et al., 2011; Xu, et al., 2011).

Cardiac Muscle Wasting and Dysfunction

The myocardium can be affected by pro-inflammatory cytokines and oxidative stress. Animal models of tumor-induced skeletal muscle wasting have demonstrated that expression of MuRF1 and MAFbx mRNA is also increased in cardiac muscle and is associated with cardiac muscle wasting, contractile dysfunction and decreased systolic function (Tian et al., 2010; Wysong et al., 2011). Any alteration in contractile function alters the ability of the heart to adequately pump blood, resulting in a systolic or diastolic dysfunction.

Troponin I is involved in the contractile function of the cardiomyocyte. Kedar et al., (2004) found that an increase in the degradation of endogenous troponin I altered the shortening and lengthening of cardiomyocytes in cardiomyocytes with an increased expression of MuRF1. Increased degradation of troponin I, cardiac muscle expression of MAFbx and MuRF1 and decreased FS, were seen in tumor-bearing mice with cardiac muscle wasting (Tian et al., 2011; Xu et al., 2011). Decreased FS in hearts of tumor-bearing mice was associated with decreased cardiomyocyte sarcomere re-lengthening and contraction indicating impairment in systolic and diastolic dysfunction (Xu, et al., 2011).

Autophagy has been implicated in the progression of heart disease and can lead to the deterioration of cardiac function. In an animal model of dilated cardiomyopathy, autophagy was associated with cardiac remodeling, cardiomyocyte death, and cardiac

dysfunction (Miyata et al., 2006). Biomarkers of autophagy, such as BNIP3 are also upregulated in the hearts of tumor-bearing animals with cardiac dysfunction (Xu et al., 2011). Cosper & Leinwand (2011) found increased levels of cathepsin L and L3C, along with decreased cardiomyocyte size, increased fibrosis and myocellular disarray in tumor-bearing mice with cardiac muscle wasting.

The UP system and the ALS work together to maintain cellular homeostasis. Aggregates of misfolded proteins that are too large to be degraded by the proteosome can be degraded by autophagy (Rothermel & Hill, 2008). In cardiomyocytes treated with a proteosome inhibitor, autophagy was upregulated (Zheng, Su, Tian & Wang, 2011). Endomyocardial biopsy of a patient with a progressive decline in cardiac function revealed cardiomyocytes containing autophagic vacuoles in various stages, tubulofilamentous ubiquitin-positive inclusions, and an increased amount of fibrosis, suggesting that the degeneration of myocardial cells by autophagy leads to cardiomyocyte death (Fidzianska, Bilinska, Walczak, Witkowski & Chojnowska, 2010).

Pro-inflammatory Cytokines

Pro-inflammatory cytokines are implicated in cardiac dysfunction. TNFa and IL-6 are implicated as risk factors for the development and progression of heart failure, alterations in cardiac contractility, and the development of cardiac dysfunction in critically ill patients (as reviewed in El-Mentar et al., 2008 and Prabhu, 2004). Serum levels of TNFa and IL-6 were elevated in patients with impaired left ventricular diastolic diameter (LVDD) and left ventricular systolic diameter (LVSD) and in patients newly diagnosed with heart failure (Chrysohoou et al., 2009; Dinh et al., 2009). Elevated levels

of pro-inflammatory cytokines, cardiac atrophy and cardiac dysfunction have been found in animal models of tumor-induced skeletal muscle wasting. Muhfield et al., (2011) found elevated serum levels of TNFa and IL-6, and cardiac muscle expression of TNRF1 and TNFR2 receptors with a significant reduction in the myofibrillar volume and increased sarcoplasmic volume in tumor-bearing mice. An elevated level of IL-6 and the receptor for IL-6 (IL-6R) was found in cardiac muscle along with cardiac atrophy, cardiac remodeling and a decrease in contractile function (Tian et al., 2010). Elevated serum levels of TNFa and IL-6 and cardiac muscle expression of MAFbx and MURF-1 with decreased cardiac mass and cardiac dysfunction have also been found (Tian et al., 2011; Wysong et al., 2011; Zhou et al., 2010).

Oxidative Stress

Oxidative stress occurs when there is an imbalance between the production of ROS and the inability of antioxidants to neutralize this increase. Oxidative stress is implicated in the development and progression of cardiovascular disease. One mechanism, adverse cardiac remodeling, includes interstitial fibrosis, contractile dysfunction, cardiomyocyte death and ventricular enlargement, leading to cardiac dysfunction and heart failure (Nabeebaccus, Zhang & Shaw, 2011; Tsutsui, Kinugawa & Matsushima, 2009; Takimoto & Kass, 2007). In patients with congestive heart failure (CHF), the more severe the disease, the higher the increase in biomarkers of oxidative stress (plasma reactive protein carbonyl derivatives and MDA) and decrease in antioxidant activity (glutathione peroxidase) compared to healthy controls and patients with less severe heart disease. The elevations in reactive protein carbonyl derivatives

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correlated with the degree of left ventricular (LV) remodeling seen with echocardiography in these patients (Radovanovic et al., 2012), indicating a role of oxidative stress in adverse cardiac remodeling. Barriero et al. (2005) found decreased cardiac muscle mass in tumor-bearing rats with increased expression of total protein carbonylation and HNE and MDA protein adducts in the gastrocnemius muscle. In tumor-bearing rats, heart and skeletal muscle wasting correlated with oxidative damage to proteins involved in glycolysis, ATP production and distribution, muscle contraction and mitochondrial metabolism (Marin-Corral et al., 2010). Results of these studies suggest a role of oxidative stress as a mediator of skeletal and cardiac muscle wasting.

Antioxidants

Antioxidants are important in maintaining a defense against free radicals and in regeneration of other antioxidants. The harmful effects of ROS can be counteracted by an endogenous antioxidant system (Valko et al., 2006). Endogenously produced antioxidants are classified as enzymatic and non-enzymatic. Enzymatic antioxidants include (a) superoxide dismutase which exists in 3 forms: cytosolic CuZnSOD (SOD1), mitochondrial MnSOD(SOD2) and extracellular (ECSOD or SOD3); (b) catalase; and (c) glutathione peroxidase. Non-enzymatic antioxidants include: vitamins C, thiol containing compounds (glutathione, thioredoxin and lipoic acid) and natural flavenoids (Moylan & Reid, 2007). Lipid soluble non-enzymatic antioxidants include carotenoids, vitamin E, estrogens and Coenzyme Q10 (CoQ10) (Bentinger et al., 2007). Antioxidants have proven efficacious in reducing the damaging effects of increased oxidative activity in skeletal and cardiac muscle. Mice pretreated with the antioxidant Trolox had lower

levels of TNFa induced ROS and improved myofibrillar force production in diaphragm muscle (Hardin et al., 2008). Aged mice had a reduction in oxidative stress in skeletal muscle after exercise as evidenced by a reduction in H202, xanthine oxidase, GSH/GSSG ratio and lipid peroxidation when treated with the antioxidant resveratrol (Ryan et al., 2010). Treatment with sodium selenate or omega-3 polyunsaturated fatty acid with vitamin E, reduced the amount of oxidized protein, loss of troponin I, and alpha actinin protein in the myocardium, and improved cardiac function in diabetic rats (Aydemir-Koksoy et al., 2010). Treatment with grape seed proanthocyanidins, which have antioxidant properties, reduced cardiac remodeling, increased the serum levels of SOD, decreased the serum levels of MDA and prevented cardiac dysfunction in an animal model of isoproterenol induced cardiac remodeling (Zuo, Wang, Gao & Zhang, 2010).

Ubiquinol

CoQ10 (ubiquinone) is well known for its bioenergetic and antioxidant functions (Littaru & Tiano, 2007). It is a lipid soluble component of all cellular membranes and is the only lipid soluble antioxidant synthesized endogenously (Littaru & Tiano, 2007; Turunen et al., 2004). The majority of CoQ10 exists in the body in its antioxidant form, ubiquinol (Bhagavan & Chopra, 2007). CoQ10 plays an important role in respiratory metabolism as a mobile electron and proton carrier in the mitochondrial electron transport chain producing ATP (Lenaz et al., 2007). As an antioxidant, ubiquinol regenerates active forms of other antioxidants such as vitamins C and E and protects cellular lipid membranes against lipid peroxidation, and oxidization of proteins and DNA (Bentinger et al., 2007). Exogenous CoQ10 reduced oxidative stress as measured by a reduction in

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protein carbonyl content in the brain, and improved cognitive function in a mouse model of Alzheimer's disease (Dumont et al., 2011). Oral CoQ10 reduced MDA and increased catalase (CAT) and superoxide dismutase (SOD) activity in the plasma in patients with coronary artery disease (Lee et al., 2012), and improved left ventricular contractility and endothelial dysfunction in patients with congestive heart failure (CHF) (Belardinelli et. al, 2006). Ubiquinol supplementation in patients with CHF increased ejection fraction, reduced left ventricular end diastolic diameter (LVEDD) and improved New York Heart Association's functional classification of heart failure from a IV to a II on average (Langsjoen & Langsjoen, 2008). Cardiac tissue subjected to ischemia and then treated with CoQ10 had an increase in contractile function (Rosenfeldt et al., 2005).

Oxidative stress, reduced plasma levels of CoQ10 and cardiac dysfunction are seen in tumor-bearing animals and in patients with CRF (Barriero et al. 2005; Cobanoglu et al., 2011; Folkers et al., 1997; Marin-Corral et al., 2010; Montovani et al., 2012; Palan et al., 2003; Rusciani et al., 2006; Xu et al., 2011). The collective studies suggest that ubiquinol is an effective antioxidant that may be beneficial to those experiencing CRF. However, further research is needed before ubiquinol can be recommended to reduce the symptoms of CRF. Thus the purpose of this study was to determine the effects of ubiquinol on muscle wasting, cardiac function and physical activity in a mouse model of CRF.

In summary, muscle maintenance is a balance between pathways associated with muscle protein synthesis and muscle protein degradation (*Figure 3*). Muscle protein degradation has been associated with many of the symptoms of CRF. Muscle protein

synthesis involves IGF-1 binding to its receptor to activate the PI3K/AKt/mTOR pathway. MyoD is a protein involved in muscle satellite cell differentiation and is activated by AKt (Saini et al., 2009). TNFα binding to its receptor TNFR1, IL-6 and oxidative stress, all activate the NF-kB pathway of muscle protein degradation (Aggarwal et al., 2009; Moylan & Reid, 2007). NF-kB translocates to the nucleus and produces more of these proinflammatory cytokines. FOXO transcription factors are located in the nucleus and upregulates Mafbx, MuRF1 and BNIP3 (Mammucari, et al., 2007; Sandri, 2010). Activation of the PI3K/AKt/mTOR pathway blocks FOXO transcription and prevents muscle protein degradation. In the current study, we tested whether ubiquinol can reduce oxidative stress, prevent activation of the NF-kB pathway of muscle protein degradation and promote muscle protein synthesis in a mouse model of CRF.
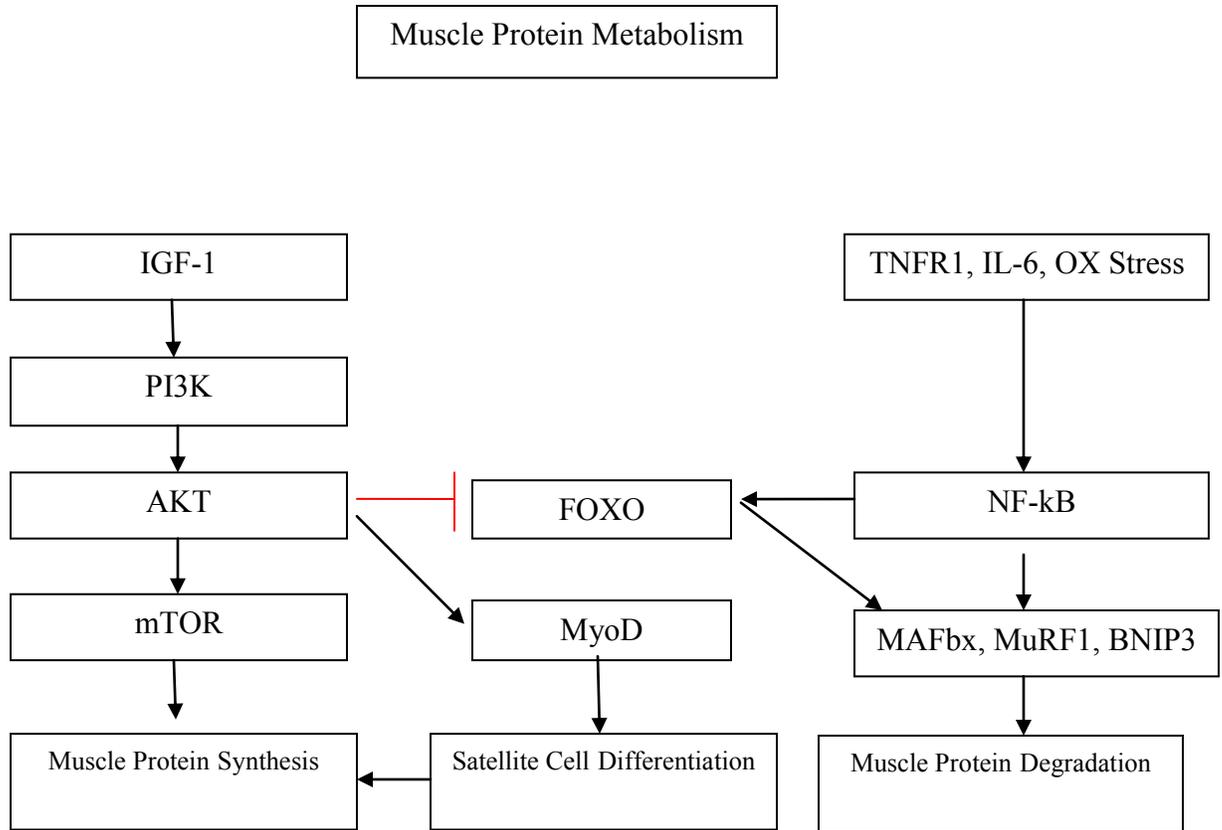
Figure 3. Pathway of muscle protein synthesis.

This figure illustrates the pathways involved in muscle protein synthesis and degradation. Insulin Growth Factor 1 (IGF-1); Phosphatidylinositide 3-kinases (PI3K); AKT (also known as protein kinase B); mammalian target of Rapamycin (mTOR); Forkhead box (FOXO); Nuclear Factor-Kappa B (NF-kB).

Chapter 3: Methods

Animal models are an excellent means by which to identify and examine the salient features of clinical phenomena that may be amenable to intervention and to provide proof-of-principle before testing an intervention with human subjects. An animal model of cancer related fatigue (CRF) was used in this study to ensure that (a) subjects have no co-morbid conditions or therapies that could affect muscle metabolism or function, (b) adequate tissue samples are available for analysis, (c) tumor growth can be closely monitored, and (d) treatment protocols are uniformly implemented. The purpose of this study was to determine the effects of the antioxidant ubiquinol on tumor induced skeletal muscle wasting and cardiac dysfunction in an animal model of CRF. A discussion of the methods used to examine the specific aims of the study and to test the hypothesized effects of ubiquinol on muscle mass, biomarkers of muscle metabolism, grip strength, VRA, *in vivo* heart function and tumor growth follow.

Animal model

The study was conducted with a total of 64, 20-22 gram (8-12 week old), adult CD2F1 female mice (Charles River) and maintained on a 12 hr-light and 12 hr-dark cycle. Eight to twelve week old mice were selected so there would be no morbidities associated with aging. Female mice were selected because in the presence of tumor growth or systemic infection, female mice maintain their body weight (Cosper &

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Leinwald, 2011; Kadioglu et al., 2011) and run for longer periods of time in darkness, their normally active period, compared to males (Kuljis et al., 2013). All mice were housed individually and maintained on standard rodent chow (Harlan) ad libitum.

Tumor growth was induced using the colon 26 adenocarcinoma (C26) murine tumor cell line. The C26 is a widely used animal model of tumor induced muscle wasting and has been used extensively in Dr McCarthy's lab. It has been shown to induce muscle wasting, increase pro-inflammatory cytokines (IL-6 and TNFR1), increase biomarkers of muscle wasting (MAFbx, MuRF1 and BNIP3) and induce cardiac dysfunction (Hitt et al., 2006; McCarthy et al., 2004; Tian et al., 2010; Tian et al., 2011; Xu et al., 2011).

The mice were inoculated subcutaneously between the scapulae with 5×10^5 tumor cells suspended in 0.2 ml saline. Growth of the tumor produces a subcutaneous nodule that is palpable within 7-10 days and can reach 10% of body weight in 21-23 days. Food intake was unaffected by tumor growth in these female mice, for up to 19 days. To rule out reduced food intake as a factor in weight loss and muscle wasting, the mice were euthanized on day 18 or 19 of tumor growth. Growth of the tumor between the scapulae did not impair mobility of the mice. This is important in order to rule out the effects of disuse atrophy of the hind limbs. Tumor growth was measured once a week using vernier calipers at the longest diameter (length) and perpendicular (width) and tumor size reported as Volume = ½ (length x width)^2 and reported as cm^4.

Fatigue was modeled as reduced voluntary running activity. Healthy mice normally run 2 hours a night (Brene et al., 2007; DeBono, Adlam, Paterson & Channon, 2006). A decline in VRA is a sensitive indicator of illness induced fatigue in mice and

rats (Skinner, Mitchell & Harden, 2009). In tumor bearing mice, tumor growth was associated with a progressive decline in VRA (Wood et al., 2006).

Weakness was modeled as reduced grip strength. Loss of muscle mass reduces muscle force generation in patients with cancer (Weber et al., 2009) and in tumor bearing mice (Gorselink et al., 2006) likely contributing to symptoms of weakness. Grip strength was reduced in patients with cancer (Brown, McMillan & Milroy, 2004; Norman et al., 2010) and in tumor bearing mice with muscle wasting (Murphy, Chee, Trieu, Naim & Lynch, 2012).

Drug Treatment

One half (n = 32) of the mice were injected subcutaneously between the scapulae with 5 x 10^5 of C26 adenocarcinoma tumor cells. Half of the control (n = 16) and half (n = 16) of the tumor-bearing mice received ubiquinol (Kaneka Nutrients L.P., Pasedena) in their drinking water (10% ubiquinol). The concentration was based on delivering a dose of 500mg/kg/day in a volume of 4mls, the average volume of fluid consumed by 20 gm mice. This dose decreased signs of senescence in aging mice (Schmelzer et al., 2010), and was shown in pilot work to reduce muscle wasting in our animal model of CRF. The other half of the tumor-bearing and control mice received a control solution in their drinking water.

Voluntary Running Activity (VRA)

VRA was measured on Days 0 (baseline), 8, 14 and 17 of tumor growth. Wheel turns were counted by a magnetic switch mounted outside the cage was tripped by a magnet imbedded in the front of the wheel (Columbus Instruments, Columbus, Ohio).

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Each turn of the wheel closed the switch and a turn was counted. The mechanical switches were connected to an input module, which in turn was connected to a peripheral processor. The computer was programmed to continuously record the number of wheel turns in a 24 hour interval.

Grip Strength

Grip strength was used to assess muscle *in vivo*. Forelimb grip strength was measured using an automated grip strength meter (Columbus Instruments, Columbus, Ohio), consisting of a strain gauge attached to a mesh grid. Each mouse was lowered vertically by its tail until the forelimbs rested on the grid. The mouse was then pulled firmly by its tail until dislodged from the grid. Three measurements, one minute apart, were averaged to determine the grams of force required to dislodge the mouse from the grid. Because smaller mice would be expected to generate less grip strength, this measure was normalized to body weight. Grip strength was measured on Days 0 (baseline), 8, 14 and 17 of tumor growth.

Echocardiography

A VisualSonics Vivo 2100 Ultra High Resolution In Vivo Imaging System (VisualSolics, Toronto, ON, Canada) was used to assess cardiac function in tumor-bearing and control mice. Mice were anesthetized with 1.5% isoflurane and placed on a warming pad. A 3-lead ECG was applied to monitor heart rate according to the manufacturer's instructions (VisualSonics). Scanning was performed at a frequency of 20MHz and three total measures were averaged from different points within the cardiac cycle according to standards set forth by the American Society for Echocardiography. M-

mode images were obtained at the level of the papillary muscles to assess end left ventricular systolic diameter (LVSD), end LV diastolic diameter (LVDD) and diastolic posterior wall thickness (PWT). M-mode calculations were used to derive fractional shortening (FS = LVDD-LVSD/LVDD). Pulsed wave Doppler in the four chamber apical view was used to assess diastolic mitral valve inflow velocities with the sample being taken at the point of maximal flow, as assessed by color Doppler. The ratio between early (diastolic ventricular filling) and late (atrial systole) ventricular filling (E/A ratio) was calculated from the mitral valve inflow velocities in order to assess diastolic function. Isovolumetric relaxation (IVRT), the period in the cardiac cycle from closure of the aortic valve and opening of the mitral value, was obtained from the same position. Total time for each individual echocardiographic analysis was approximately 20 minutes.

Euthanasia

The mice were euthanized on days 18 or 19 of tumor growth following echocardiography. The mice were deeply anesthetized by injection with 2mg of 1% ketamine/xylazine. Blood was drawn from the submandibular vein. The heart and right and left gastrocnemius muscles were dissected and the tumor was then removed.

Measures

Weight of the left and right gastrocnemius was averaged to determine gastrocnemius muscle weight and gastrocnemius and heart weight were normalized to body weight and expressed as muscle mass. Weight of the heart and average gastrocnemius was also normalized to carcass weight (bodyweight minus tumor weight).

Serum Cytokines

Blood was removed during euthanasia, placed into a microcentrifuge tube containing 100 ul of 0.1M EDTA, and centrifuged. The serum was collected and stored at -20ºC until analysis of cytokine levels. Serum concentrations of TNFa and IL-6 were measured using a commercially available mouse TNF-R1 and IL-6 Ultra-Sensitive ELISA kits (Meso Scale Discovery) (MSD) according to the manufacturer's instructions. All samples were tested in duplicate and results are expressed picograms protein per milliliter of serum (pg/ml).

Oxidative Stress

Oxidative stress was measured per protocol as described in Rahman et al., (2006). The most abundant antioxidant in aerobic cells is Glutathione (GSH) (Owen & Butterfield, 2010). In the cell, GSH exists in its reduced sulfhydryl and oxidized GSH disulfide (GSSG) forms and intracellular concentrations of GSH are an indicator of redox status. When cells are exposed to oxidative stress, an increase in GSSG occurs and the ratio of GSH/GSSG is decreased. Thirty mg of cardiac and gastrocnemius tissue was homogenized to measure GSH and GSSG concentrations. Results are reported as the GSH/GSSG ratio.

Extraction of Total RNA

A 30 mg sample of frozen gastrocnemius and heart tissue was disrupted and homogenized using the Tissue Lyser LT (Qiagen) at 40 HZ for 2-2.5 minutes (40 seconds x 3 for heart tissue and 40 seconds x 4 for gastrocnemius tissue) and Qiagen's Fibrous Tissue Mini Kit, per manufacturer's instructions, for extraction of total RNA. All tissue

was treated with RNase-Free DNase per manufacturer's instruction during the extraction process. Total RNA was quantified using the NanoDrop (Thermo Scientific) and the 260/280 ratio was obtained to check the purity of the RNA. All specimens had 260/280 ratios of ~ 2.0 nm. On an agarose gel, quality of skeletal and heart muscle total RNA from individual mice was verified by the discrete presence and brightness of 28S and 18S bands, with the 28S band being brighter.

Real Time Polymerase Chain Reaction (RT-PCR)

In principle, total RNA in tissue includes copies of mRNA from gene transcription activity at the time of tissue lysis. The cDNA can be probed for expression of genes of interest by methods of polymerase chain reaction (PCR) using primers specific for those genes. In the present study, the genes of interest were BNIP3, MAFbx, MuRF1, IL-6, TNFR1 (biomarkers of muscle protein degradation), MyoD and IGF-1 (biomarkers of muscle protein synthesis). Expression of each gene was normalized against the expression of a gene stably expressed in a cell such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH). In this study, one microgram of RNA was reverse-transcribed to cDNA using the iscript cDNA synthesis kit (BioRad). Skeletal and heart muscle expression of Bnip3, MAFbx, MuRF1, IL-6, TNFR1, MyoD and IGF-1 mRNA was measured using methods of quantitative real time PCR on a CFX96 thermocycler (BioRad). Appropriate primer pairs and SYBR super mix were used for MAFbx, MuRF1, IL-6, TNFR1, MyoD, IGF-1 and GAPDH (BioRad). BNIP3 was measured using Taqman Gene Expression Master Mix (AB Applied Science).

Table 1 Primers used for RT-PCR reactions

Gene of Interest	Forward	Reverse
MAFbx	5'GTG-CTT-ACA-ACT-GAA-CAT-CAT-GCA-3'	5'TGG-CCC-AGG-CTG-ACC A-3'
MuRF1	5'TGT-CTG-GAG-GTC-GTT-TCC G-3'	5'-TGC-CGG-TCC-ATG-ATC-ACT T-3'
IL-6	5'GCC-AGA-GTC-CTT-CAG-AGA-GAT-ACA-GA-3'	5'AGC-CAC-TCC-TTC-TGT-GAC-TCC-AGC T-3'
TNFR1	5'ACA-CGG-TGT-GTG-GCT-GTA-AG-3'	5'AGT-CCA-CGC-ACT-GGA-AGT-GT-3'
MyoD	5'GCT-TCT-ATC-GCC-GCC-ACT CC-3'	5'CGC-ACA-TGC-TCA-TCC-TCA CG-3'
IGF-1	5'GCT-CCG-GAA-GCA-ACA-CTC A-3'	5'GCT-ATG-GCT-CCA-GCA-TTC G-3'
GAPDH	5'ATG-GTG-AAG-GTC-GGT-GTG-AAC GG-3'	5'AGG-GGT-CGT-TGA-TGG-CAA-CAA-TCT-3'

Note. Forward and reverse primers are used to bind to the target DNA segment providing starting points for DNA synthesis.

All reactions were performed in duplicate using 25 ng of cDNA in a final reaction volume of 20 microliters under the following reaction conditions:

Table 2 Thermocycle Process in RT-PCR Reaction

Steps	Temperature	Time
1. Initiation	95ºC	3 minutes
2. Denaturization	95ºC	10 seconds
3. Annealing	60ºC	15 seconds
4. Extension/Elongation	72ºC	6 seconds

Note. From step 4, process is returned to step 2 and repeated 39 more times for amplification of gene of interest.
All results were normalized to the expression of GAPDH mRNA.

Procedures

For one week prior to the start of the experiment (days -7 to day 0), the mice were acclimated to handling and to running wheels. Mice were randomly assigned to one of four groups (tumor/no drug; tumor + drug; no tumor/no drug; no tumor + drug). On day 0, baseline measures of grip strength and VRA were obtained, after which the running wheels were removed from all cages. On day 1, half of the mice were inoculated with 5 x 10^5 tumor cells suspended in 0.2 ml saline. Half the tumor mice and half the controls were started on ubiquinol in their drinking water. On days 8, 14, and 17 of tumor growth, grip strength and VRA were measured in all of the mice. On days 18 and 19, echocardiography was performed on 42 mice under inhaled isoflurane anesthesia, after which the mice were injected with 2mg of 1% ketamine/xylazine. Blood was drawn from the submandibular vein, placed into a microcentrifuge tube containing 100 ul of 0.1M EDTA, and centrifuged. The serum was collected and stored at -20ºC until analyses of cytokine levels. The heart and right and left gastrocnemius muscles were dissected and individually weighed, frozen in liquid nitrogen, and stored at -80ºC for later analyses of biomarkers of muscle metabolism and oxidative stress. Weight of the left and right gastrocnemius was averaged to determine gastrocnemius muscle weight and gastrocnemius and heart weight were normalized to body weight and expressed as muscle mass. Lastly, the tumor was removed and weighed. The experiment was conducted three times using 18-24 animals at a time, (4-6 per group) and the data were pooled.

Statistical analyses

The independent variables were tumor (presence or not) and drug treatments (Ubiquinol or control solution). Dependent measures were VRA, grip strength, muscle mass, LVSD, LVDD, FS, PWT, IVRT, and expression of MyoD, IGF-1, TNFR1, IL-6, MAFbx, MuRF1, Bnip3 mRNA and oxidative stress in gastrocnemius and heart muscle. Descriptive statistics were used to examine the distribution of the group means. Data were analyzed using two-way analysis of variance (ANOVA) to determine main and interactive effects of ubiquinol and tumor growth on each dependent measure. Repeated measures ANOVA was performed for within subject changes in VRA and grip strength on days 0 (baseline), day 8, 14 and 17 due to tumor growth or ubiquinol. The Bonferroni post-hoc test was used to determine which groups were significantly different from each other on days 0, 8, 14 and 17. Independent samples t-test was performed to compare tumor weight. Statistical significance was set *a priori* at α=.05.

Chapter 4: Results

This chapter describes the result of the study to determine the effects of antioxidant treatment in a mouse model of cancer related fatigue. The independent variables were tumor growth and ubiquinol supplementation. The dependent variables were muscle mass (gastrocnemius and heart), grip strength, voluntary running activity (VRA), cardiac function, serum cytokines (IL-6 and TNFR1), biomarkers of muscle fiber degradation (MAFbx, MuRF1, BNIP3), inflammation (IL-6, TNFR1), biomarkers of protein synthesis (IGF-1, MyoD), oxidative stress (GSH.GSSG ratio) in gastrocnemius and heart muscle, and tumor growth.

Inflammation

Serum

There was a significant main effect of tumor growth on the serum expression of IL-6 ($F_{(1, 64)} = 62.82$, $p = < .01$). However there was no significant main effect of ubiquinol supplementation on serum expression of IL-6 ($F_{(1, 64)} = .00$, $p = .97$) (***Figure 4***) or significant interaction between tumor growth and ubiquinol supplementation ($F_{(1, 64)} = .00$, $p = .99$) on serum expression of IL-6.

Similarly, there was a significant main effect of tumor growth on the serum expression of TNFR1 ($F_{(1, 64)} = 33.83$, $p = < .01$). However there was no significant main effect of ubiquinol supplementation ($F_{(1, 64)} = .87$, $p = .35$) or significant interaction

between tumor growth and ubiquinol supplementation ($F_{(1, 64)} = .18$, $p = .73$) on serum expression of TNFR1.

Gastrocnemius

There was a significant main effect of tumor growth on gastrocnemius muscle expression of IL-6 mRNA ($F_{(1, 64)} = 4.20$, $p = .04$). However there was no significant main effect of ubiquinol supplementation ($F_{(1, 64)} = .02$, $p = .89$) (***Figure 5***) and no significant interaction between tumor growth and ubiquinol supplementation ($F_{(1, 64)} = 2.48$, $p = .12$) on gastrocnemius muscle expression of IL-6 mRNA.

Similarly, there was a significant main effect of tumor growth on gastrocnemius muscle expression of TNFR1 mRNA ($F_{(1, 64)} = 22.38$, $p < .01$). However there was no significant main effect of ubiquinol supplementation ($F_{(1, 64)} = .63$, $p = 43$) and no significant interaction between tumor growth and ubiquinol supplementation ($F_{(1, 64)} = .09$, $p = .77$) on gastrocnemius muscle expression of TNFR1 mRNA.

Heart

There was a significant main effect of tumor growth on heart muscle expression of IL-6 mRNA ($F_{(1, 64)} = 43.55$, $p < .01$). However there was no significant main effect of ubiquinol supplementation ($F_{(1, 64)} = .23$, $p = .63$) (***Figure 6***) or significant interaction between tumor growth and ubiquinol supplementation ($F_{(1, 64)} = 1.03$, $p = .31$) on heart muscle expression of IL-6 mRNA.

Similarly, there was a significant main effect of tumor growth on heart muscle expression of TNFR1 mRNA ($F_{(1, 64)} = 27.12$, $p < .01$). However there was no significant main effect of ubiquinol supplementation ($F_{(1, 64)} = 2.50$, $p = .12$) or significant

interaction between tumor growth and ubiquinol supplementation ($F_{(1, 64)} = 3.08, p = .08$)
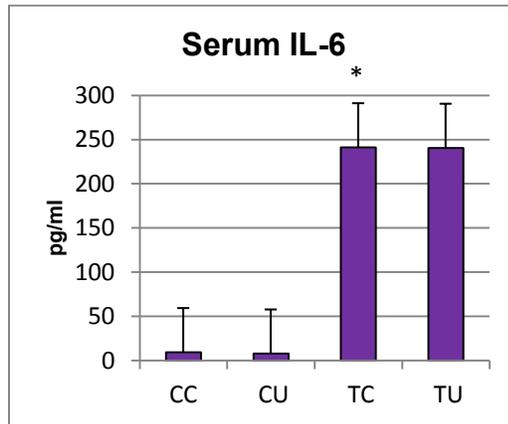
on heart muscle expression of TNFR1 mRNA.

Figure 4. Effects of tumor growth and ubiquinol supplementation on serum expression of IL-6.

This chart illustrates the results of tumor growth, *p = < .01 and ubiquinol supplementation, p = .97 on serum expression of IL-6.
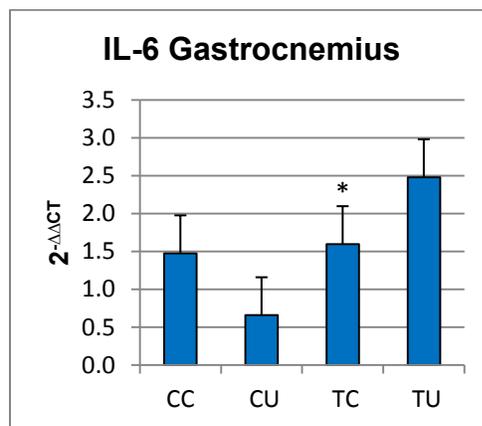

Figure 5. Effects of tumor growth and ubiquinol supplementation on gastrocnemius muscle expression of IL-6.

This chart illustrates the results of tumor growth, * p < .05 and ubiquinol supplementation, p = .89 on gastrocnemius muscle expression of IL-6.
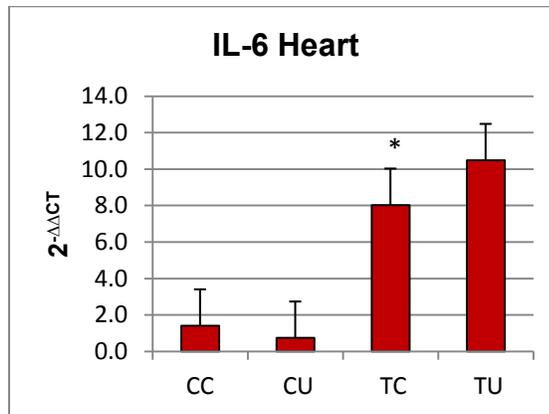

Figure 6. Effects of tumor growth and ubiquinol supplementation on heart muscle expression of IL-6.

This chart illustrates the results of tumor growth, *p < .01 and ubiquinol supplementation, p = .63 on heart muscle expression of IL-6. CC = Control No Drug: N=16; CU = Control/Ubiquinol: N=16; TC = Tumor No Drug: N=16; TD = Tumor/Ubiquinol: N=20.

Muscle Mass

Body Weight

There was a significant main effect of tumor growth on body weight at the time of sacrifice ($F_{(1, 64)} = 5.45, p = .02$) (**Figure 7**). There was no significant main effect of ubiquinol supplementation ($F_{(1, 64)} = .01, p = .93$) or significant interaction between tumor growth and ubiquinol supplementation ($F_{(1, 64)} = .01, p = .91$) on body weight.

Similarly there was a significant main effect of tumor growth on carcass body weight (body weight minus tumor weight) ($F_{(1, 64)} = 17.46, p < .01$) However there was no significant main effect of ubiquinol supplementation ($F_{(1, 64)} = .00, p = .98$) or

significant interaction between tumor growth and ubiquinol supplementation ($F_{(1, 64)}$ = .00, p = .99) on carcass weight.

Gastrocnemius Mass

When muscle mass was normalized to body weight, there was a significant main effect of tumor growth ($F_{(1, 64)}$ = 40.87, p < .01) and ubiquinol supplementation ($F_{(1, 64)}$ = 5.92, p = .02) on gastrocnemius mass (***Figure 8***). Although there was a significant increase in gastrocnemius muscle mass in the ubiquinol supplemented tumor and control mice, there was no significant interaction between tumor growth and ubiquinol supplementation ($F_{(1, 64)}$ = .38, p = .52) on gastrocnemius mass.

Similarly there was a significant main effect of tumor growth on muscle mass ($F_{(1, 64)}$ = 24.13, p < .01) and ubiquinol supplementation ($F_{(1, 64)}$ = 6.43, p = .01) when gastrocnemius weight was normalized to carcass weight (average gastrocnemius weight/carcass weight). There was no significant interaction between tumor growth and ubiquinol supplementation on gastrocnemius carcass mass ($F_{(1, 64)}$ = .23, p = .63).
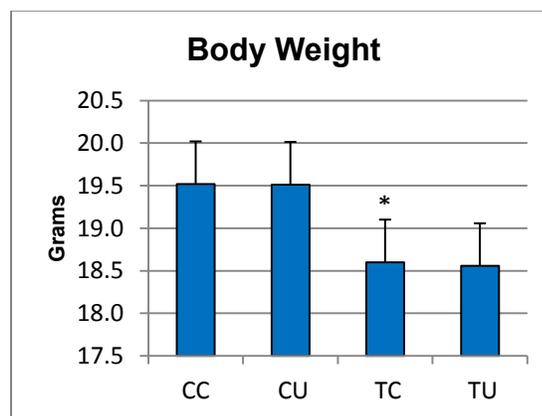

Figure 7. Effect of tumor growth and ubiquinol supplementation on body weight.

This chart illustrates results of tumor growth,*p = .02 and ubiquinol supplementation, p = .93 on body weight.
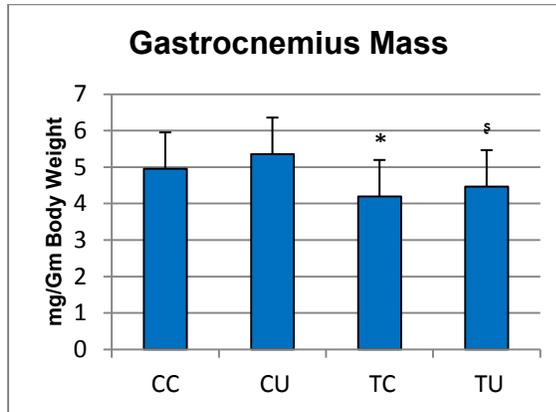
Gastrocnemius Mass

Figure 8. Effects of tumor growth and ubiquinol supplementation on gastrocnemius mass.

This chart illustrates the results of tumor growth, *p < .01 and ubiquinol supplementation, † p = .02 on gastrocnemius mass. CC=Control No Drug: N=16; CU=Control/Ubiquinol: N=16; TC=Tumor No Drug: N=16; TU=Tumor/Ubiquinol: N=20.

Heart Mass

There was no significant main effect of tumor growth ($F_{(1, 64)}$ = 3.03, p = .09) or ubiquinol supplementation ($F_{(1, 64)}$ = .07, p = .80) and no significant interaction between tumor growth and ubiquinol supplementation ($F_{(1, 64)}$ = .08, p = .78) on heart mass.

Heart Carcass Mass

There was a significant main effect of tumor growth on heart muscle mass when heart weight was normalized to carcass weight (heart weight/carcass weight) ($F_{(1, 64)}$ = 13.75, p = <.01). However there was no significant main effect of ubiquinol

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supplementation ($F_{(1, 64)}$ = .14, p = .71) or interaction between tumor growth and ubiquinol supplementation on heart carcass mass ($F_{(1, 64)}$ = .16, p = .69).

Grip Strength

Mauchley's test of grip strength data indicated that the assumption of sphericity was violated, $X^2(5)$ = 13.50, p = .02, therefore degrees of freedom was corrected using Huynh-Feldt estimates of sphericity (ε = .95). There was a significant main effect of tumor growth on grip strength within subjects over time ($F_{(2.84, 170.24)}$ = 4.33, p < .01), however there was no significant effect of ubiquinol supplementation on grip strength over time ($F_{(2.84, 170.24)}$ = .54, p = .64).

There was a significant main effect of tumor growth on grip strength between subjects ($F_{(1, 60)}$ = 3.97, p = .05) However there was no significant main effect of ubiquinol supplementation ($F_{(1, 60)}$ = .14, p = .71) (***Figure 9***) or significant interaction between tumor growth and ubiquinol supplementation on grip strength between subjects ($F_{(1, 60)}$ = .58, p = .45). Bonferroni adjusted pairwise comparisons of the tumor/control and tumor/ubiquinol mice indicate a 3% daily decrease in mean grip strength on day 0 vs day 17.
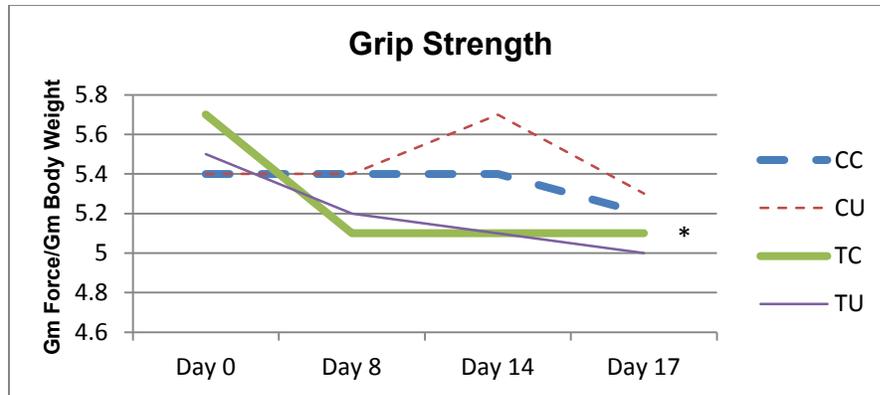
Figure 9. Effects of tumor growth and ubiquinol supplementation on grip strength.

This chart illustrates the results of tumor growth, *p = .05 and ubiquinol supplementation, p = .71 on grip strength. CC=Control No Drug: N=16; CU=Control/Ubiquinol: N=16; TC=Tumor No Drug: N=16; TU=Tumor/Ubiquinol: N=16.

Voluntary Running Activity (VRA)

Mauchley's test indicate that the assumption of sphericity was violated, $X^2(5) = 34.73$, p = .00, therefore degrees of freedom was corrected using Greenhouse-Geisser estimates of sphericity (ε = .63). There was a significant main effect of tumor growth on VRA within subjects over time ($F_{(1.88, 78.86)} = 3.39$, p = .04), however there was no significant effect of ubiquinol supplementation on VRA over time ($F_{(1.88, 78.86)} = .13$, p = .86).

There was a significant main effect of tumor growth on VRA between subjects ($F_{(1, 42)} = 7.40$, $p = .01$). There was no significant main effect of ubiquinol supplementation on VRA ($F_{(1, 42)} = .00$, $p = .97$) (*Figure 10*) or significant interaction between tumor growth and ubiquinol supplementation on VRA between subjects

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$F_{(1, 42)} = .16$, $p = .69$). Bonferroni adjusted pairwise comparisons of the tumor/control and tumor/ubiquinol mice on day 0 vs day 17 was non-significant.

Figure 10. Effects of tumor growth and ubiquinol supplementation on voluntary running activity (VRA).

This chart illustrates the results of tumor growth, *p = .01 and ubiquinol supplementation, p = .97 on VRA. Control No Drug: N=11; Control/Ubiquinol: N=12; Tumor No Drug: N=12; Tumor/Ubiquinol: N=11

Cardiac Function

Systolic Function

There was no significant main effect of tumor growth ($F_{(1, 38)} = .34$, $p = .56$), ubiquinol supplementation ($F_{(1, 38)} = 1.18$, $p = .29$) or significant interaction between tumor growth and ubiquinol supplementation ($F_{(1, 38)} = .42$, $p = .52$) on left ventricular diastolic diameter (LVDD).

Similarly, there was no significant main effect of tumor growth ($F_{(1, 38)} = .02$, $p = .89$), ubiquinol supplementation ($F_{(1, 38)} = .84$, $p = .37$) or significant interaction between

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tumor growth and ubiquinol supplementation ($F_{(1, 38)} = .12$, $p = .73$) on left ventricular systolic diameter (LVSD).

There was no significant main effect of tumor growth ($F_{(1, 38)} = .05$, $p = .83$), ubiquinol supplementation ($F_{(1, 38)} = .23$, $p = .63$) and no significant interaction between tumor growth and ubiquinol supplementation ($F_{(1, 38)} = 1.46$, $p = .24$) on posterior wall thickness (PWT).

Similarly, there was no significant main effect of tumor growth ($F_{(1, 38)} = .76$, $p = .39$), ubiquinol supplementation ($F_{(1, 38)} = .74$, $p = .40$) or significant interaction between tumor growth and ubiquinol supplementation ($F_{(1, 38)} = 1.02$, $p = .32$) on fractional shortening (FS).

Diastolic Function

There was a significant main effect of tumor growth on isovolumic relaxation time (IVRT) ($F_{(1, 38)} = 9.32$, $p = <.01$) (***Figure 11***). However, there was no significant main effect of ubiquinol supplementation ($F_{(1, 38)} = .18$, $p = .68$) or significant interaction between tumor growth and ubiquinol supplementation ($F_{(1, 38)} = .05$, $p = .83$) on IVRT.
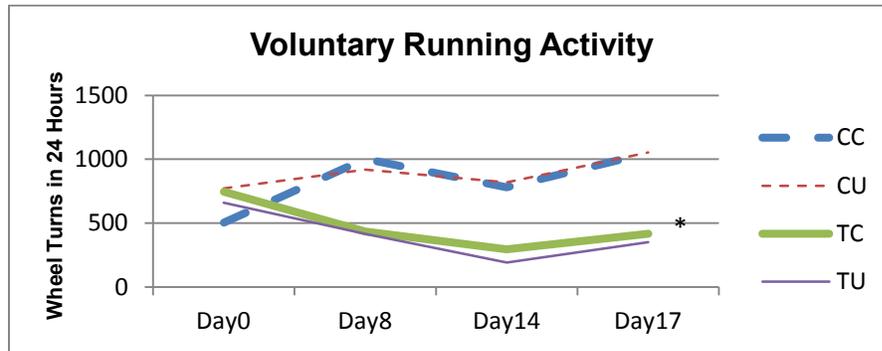
Figure 11. Effects of tumor growth and ubiquinol supplementation on isovolumic relaxation time (IVRT).

This chart illustrates the results of tumor growth, *p < .01 and ubiquinol supplementation, p = .68 on IVRT. CC = Control No Drug: N=10; CU = Control/Ubiquinol: N=10; TC = Tumor No Drug: N=11; TU = Tumor/Ubiquinol: N=11.

Muscle Metabolism

Protein Degradation

Gastrocnemius

There was a significant main effect of tumor growth on gastrocnemius muscle expression of MAFbx mRNA ($F_{(1, 64)}$ = 22.08, $p < .01$). However, there was no significant main effect of ubiquinol supplementation ($F_{(1, 64)}$ = .47, $p = .50$) (***Figure 12***) or significant interaction between tumor growth and ubiquinol supplementation ($F_{(1, 64)}$ = .74, $p = .39$) on gastrocnemius muscle expression of MAFbx mRNA.

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Figure 12. Effects of tumor growth and ubiquinol supplementation on gastrocnemius muscle expression of MAFbx.

This chart illustrates the results of tumor growth, *p < .01 and ubiquinol supplementation, p = .50 on gastrocnemius muscle expression of MAFbx. Control No Drug: N=16; Control/Ubiquinol: N=16; Tumor No Drug: N=16; Tumor/Ubiquinol: N=20

Similarly, there was a significant main effect of tumor growth on gastrocnemius muscle expression of MuRF1 mRNA ($F_{(1, 64)}$ = 14.94, p < .01). However, there was no significant main effect of ubiquinol supplementation ($F_{(1, 64)}$ = .00, p = 99) or significant interaction between tumor growth and ubiquinol supplementation ($F_{(1, 64)}$ = .17, p = .69) on gastrocnemius muscle expression of MuRF1 mRNA
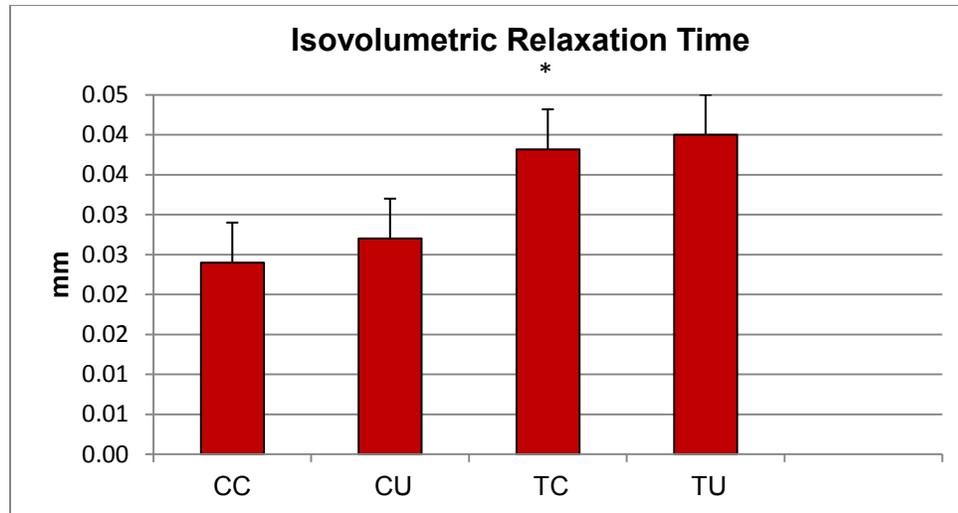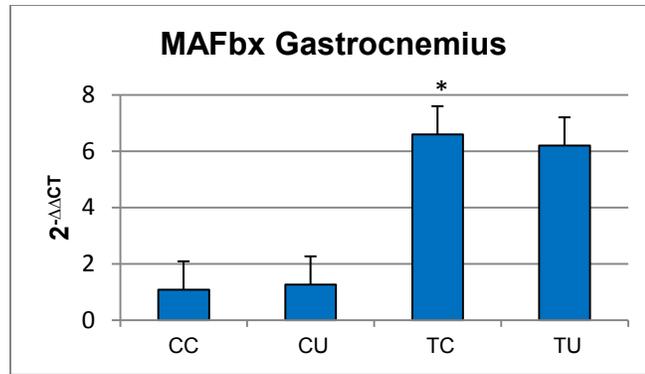
Similarly, there was a significant main effect of tumor growth on gastrocnemius muscle expression of BNIP3 mRNA ($F_{(1, 64)}$ = 13.21, p = < .01). However there was no significant main effect of ubiquinol supplementation ($F_{(1, 64)}$ = .01, p = .93) or significant interaction between tumor growth and ubiquinol supplementation ($F_{(1, 64)}$ = .06, p = .80) on gastrocnemius muscle expression of BNIP3 mRNA

Heart

There was no significant main effect of tumor growth ($F_{(1, 64)} = .32$, $p = .57$), or ubiquinol supplementation ($F_{(1, 64)} = .06$, $p = .80$) and no significant interaction between tumor growth and ubiquinol supplementation ($F_{(1, 64)} = .56$, $p = .46$) on heart muscle expression of MAFbx mRNA

Similarly, there was no significant main effect of tumor growth ($F_{(1, 64)} = .00$, $p = .98$), or ubiquinol supplementation ($F_{(1, 64)} = .02$, $p = .89$) and no significant interaction between tumor growth and ubiquinol supplementation ($F_{(1, 64)} = .11$, $p = .74$) on heart muscle expression of MuRF1 mRNA.

Similarly, there was no significant main effect of tumor growth ($F_{(1, 64)} = .79$, $p = .38$), or ubiquinol supplementation ($F_{(1, 64)} = .19$, $p = .66$) and no significant interaction between tumor growth and ubiquinol supplementation ($F_{(1, 64)} = .32$, $p = .57$) on heart muscle expression of BNIP3 mRNA.

Protein Synthesis

Gastrocnemius

There was a significant main effect of tumor growth on gastrocnemius muscle expression of IGF-1 mRNA ($F_{(1, 64)} = 6.96$, $p = .01$). However, there was no significant main effect of ubiquinol supplementation ($F_{(1, 64)} = .50$, $p = .48$) (***Figure 13***) or significant interaction between tumor growth and ubiquinol supplementation ($F_{(1, 64)} = 1.65$, $p = .20$) on gastrocnemius muscle expression of IGF-1 mRNA.

There was no significant main effect of tumor growth ($F_{(1, 64)} = 2.40$, $p = .13$) or ubiquinol supplementation ($F_{(1, 64)} = .36$, $p = .55$) and no significant interaction between

tumor growth and ubiquinol supplementation ($F_{(1, 64)} = .20, p = .65$) on gastrocnemius muscle expression of MyoD mRNA.

Heart

There was a significant main effect of tumor growth on heart muscle expression of IGF-1 mRNA ($F_{(1, 64)} = 34.10, p < .01$). However there was no significant main effect of ubiquinol supplementation ($F_{(1, 64)} = .07, p = .79$) (***Figure 14***) or significant interaction between tumor growth and ubiquinol supplementation ($F_{(1, 64)} = .14, p = .71$) on heart muscle expression of IGF-1 mRNA.

There was no significant main effect of tumor growth ($F_{(1, 64)} = 2.00, p = .16$) or ubiquinol supplementation ($F_{(1, 64)} = 1.72, p = .20$) and no significant interaction between tumor growth and ubiquinol supplementation ($F_{(1, 64)} = 2.03, p = .16$) on heart muscle expression of MyoD mRNA.
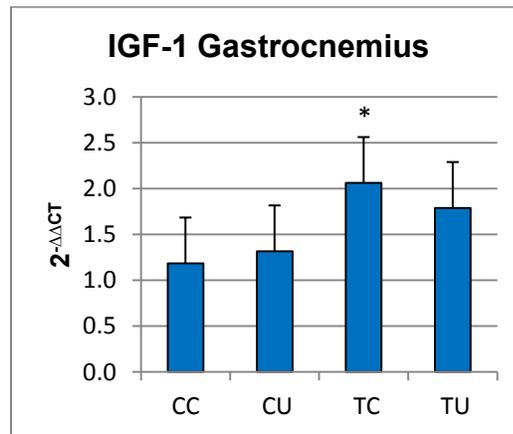

Figure 13. Effects of tumor growth and ubiquinol supplementation on gastrocnemius muscle expression of IGF-1.

This chart illustrates the results of tumor growth, * p = .01 and ubiquinol supplementation, p = .48 on gastrocnemius muscle expression of IGF-1.

IGF-1 Heart

Figure 14. Effects of tumor growth and ubiquinol supplementation on heart muscle expression of IGF-1.

This chart illustrates the results of tumor growth, *p < .01 and ubiquinol supplementation, p = .79 on heart muscle expression of IGF-1. CC = Control No Drug: N=16; CU = Control/Ubiquinol: N=16; TC = Tumor No Drug: N=16; TU = Tumor/Ubiquinol: N=20.

Oxidative Stress (GSH/GSSG)

Gastrocnemius

There was no significant main effect of tumor growth ($F_{(1, 64)}$ = 3.54, p = .06) or ubiquinol supplementation ($F_{(1, 64)}$ = .89, p = .35) and no significant interaction between tumor growth and ubiquinol supplementation ($F_{(1, 64)}$ = 3.54, p = .07) on GSH/GSSG in the gastrocnemius muscle.

Heart

There was a significant main effect of tumor growth ($F_{(1, 60)}$ = 34.54, p < .01) on GSH/GSSG in the heart muscle. However there was no significant main effect of ubiquinol supplementation ($F_{(1, 60)}$ = .62, p = .43) (***Figure 15***) or significant interaction

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between tumor growth and ubiquinol supplementation ($F_{(1, 60)} = .92$, $p = .34$) on
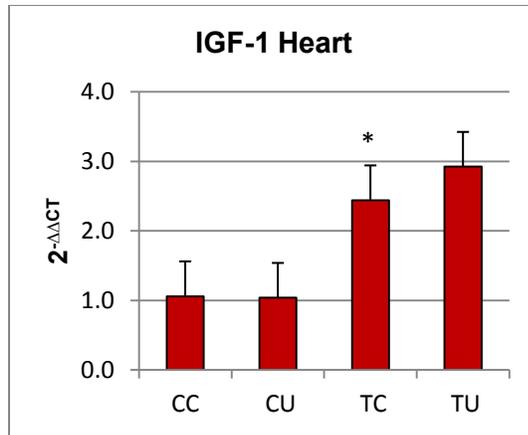
GSH/GSSG in the heart muscle.
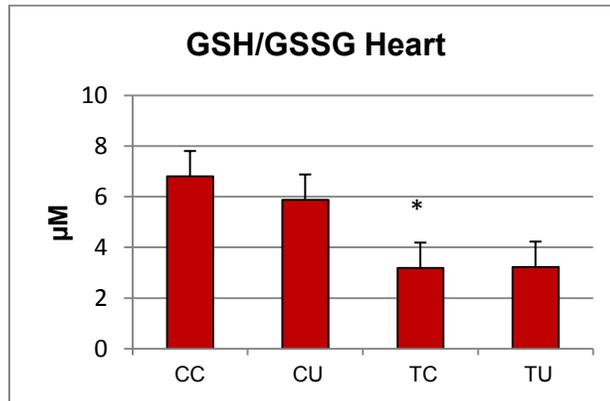
Figure 15. Effects of tumor growth and ubiquinol supplementation on heart muscle expression of GSH/GSSG.

This chart illustrates the results of tumor growth, *p < .01 and ubiquinol supplementation, p = .43 on heart muscle expression of GSH/GSSG. Control No Drug: N=16; Control/Ubiquinol: N=16; Tumor No Drug: N=16; Tumor/Ubiquinol: N=20

Tumor Mass

Tumor growth was palpable by day 7 and increased in size over the course of 18-19 days as measured by calipers (***Figure 16***).

An independent samples t-test was conducted to compare mean tumor weights in the tumor no drug group (M = .72, SD = .30) and the tumor/ubiquinol groups (M = .82, SD = .23). There was no significant difference between tumor weights in the tumor no drug group and the tumor group supplemented with ubiquinol, t(34) = -1.04, p = .30 (***Figure 17***).

Tumor Volume

Figure 16. Comparison of tumor growth between tumor mice.

This chart illustrates the difference between tumor growth between control and ubiquinol treated mice. Formula used: $V = 1/2\ (L*W)\ ^2$.

Tumor Weight

Figure 17. Comparison of tumor weight between tumor mice.

This chart illustrates the difference between tumor weight in grams between control and ubiquinol treated mice. TC = Tumor No Drug: N=16; TU = Tumor/Ubiquinol: N=20.

Chapter 5: Discussion

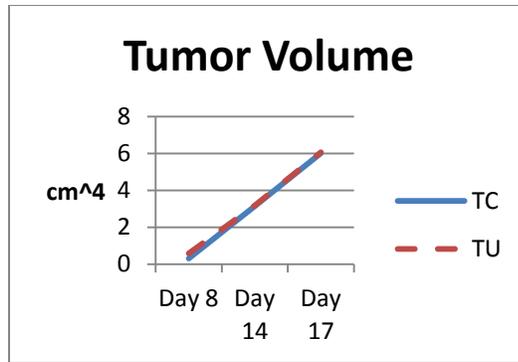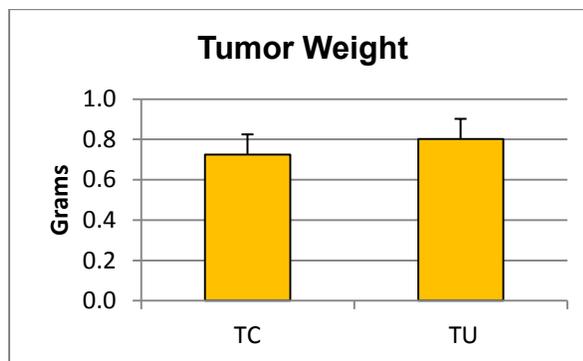Cancer related fatigue (CRF) is a significant problem in people with cancer. The causes of CRF are multimodal and not clearly understood; no effective treatments are available. Tumor-induced skeletal muscle wasting, one possible cause of CRF, is mediated in part by proinflammatory cytokines and oxidative stress, activating pathways associated with muscle wasting. Recent evidence indicates that these pathways are also activated in cardiac muscle suggesting that cardiac dysfunction might also contribute to CRF (Tian et al., 2011; Wysong et al., 2011; Xu et al., 2011). There is evidence that antioxidant supplementation can decrease muscle wasting (Bonetto et al., 2009) improve cardiac function (Aydemir-Koksoy et al., 2010; Huynh et al., 2012), and reduce circulating levels of proinflammatory cytokines such as IL-6 (Lee et al., 2010). The purpose of this study was to determine if treatment with ubiquinol, an antioxidant, might preserve skeletal muscle mass, improve cardiac function, and reduce behavioral measures of fatigue in an animal model of CRF.

The following hypotheses were tested in mice bearing the colon26 (C26) adenocarcinoma: 1) Ubiquinol will reduce the expression of biomarkers of muscle protein degradation (IL-6, TNFR1, MAFbx, MuRF1, and BNIP3) and protein synthesis (IGF-1 and MyoD) in the gastrocnemius and cardiac muscles of tumor-bearing mice. 2) Ubiquinol will increase: (a) muscle mass, (b) fore-limb grip strength, (c) *in vivo* cardiac

function, and (d) voluntary running activity (VRA) in tumor-bearing mice. 3) Ubiquinol will not affect tumor growth.

As expected with this model, we found that tumor growth increased serum and muscle levels of proinflammatory cytokines IL-6 and TNFR1, decreased body weight, skeletal muscle mass, voluntary running activity (VRA) and grip strength in the tumor-bearing mice. In skeletal muscle tumor growth increased biomarkers of muscle protein degradation MAFbx, MuRF1 and BNIP3, but did not increase oxidative stress. In contrast, tumor growth did not increase biomarkers of protein degradation in the heart muscle but did increase oxidative stress. Echocardiography revealed an increase in isovolumic relaxation time (IVRT), indicating a diastolic dysfunction in the tumor-bearing mice. Ubiquinol supplementation improved skeletal muscle mass in the tumor-bearing mice, but had no effect on serum or muscle expression of proinflammatory cytokines, biomarkers of protein degradation, oxidative stress or cardiac function. While ubiquinol did improve skeletal muscle mass in the tumor-bearing mice, it had no effect on VRA or grip strength. Study findings are discussed relative to the hypotheses and findings from previous research. Limitations of the study and suggestion for future research are also included.

Effect of Tumor Growth/Ubiquinol on Inflammation

Tumor growth is associated with increased tumor/ and or host production of IL-6 and TNFa (Aggarwal et al., 2006; Chen & Qui, 2011; Krzystek-Kopacka et al., 2007). In the present study, tumor growth increased serum levels of IL-6 and TNFR1, and expression of IL-6 and TNFR1 mRNA in skeletal and cardiac muscles of the tumor-

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bearing mice. These findings are consistent with other studies showing increased serum levels (Baltgalvis et al., 2008; Catalano et al., 2003; Montovani et al, 2010; Takahashi, et al., 2009; Tian et al., 2011; White et al., 2011; Wysong et al., 2011; Zhou et al., 2011), skeletal muscle expression (Figueras et al., 2005; Graves et al., 2006; Hitt et al., 2005) and cardiac muscle expression of these proinflammatory cytokines (Tian et al., 2010; Muhlfeld et al., 2011).

Treatment of the tumor-bearing mice with ubiquinol had no effect on expression of these pro-inflammatory cytokines in the serum, skeletal or cardiac muscle. Other models of chronic inflammatory conditions have shown that administration of ubiquinol can reduce cytokine production. CoQ10 (the oxidized form of ubiquinol) reduced the inflammatory score (a sum of inflammatory biomarkers such as IL-6 and TNFa mRNA) in the liver of obese mice (Sohet et al., 2009). After oral ingestion of CoQ10, it is stored in the liver until released into the blood (Miles, 2007). Sohet et al, (2009) reported a 40 fold increase in CoQ10 in the liver of the supplemented mice which could account for the decreased cytokine levels in the liver. Similarly Lee et al., (2012) reported that CoQ10 reduced serum levels of IL-6 in patients with coronary artery disease (CAD), but others found no effect of CoQ10 on serum levels of IL-6 and TNFa in patients with end stage heart failure (Berman et al., 2004). Lee et al., (2012) used a higher dosage of CoQ10 used than in Berman et al., (2004) which could account for conflicting results between these 2 studies.

Thus it is possible that the lack of effect of ubiquinol on cytokine expression in tumor bearing mice may represent an inadequate dose of ubiquinol in the context of

progressive tumor growth and production of IL-6. Therefore inadequate dosing of ubiquinol is a possible explanation for the lack of effect of ubiquinol on serum and tissue expression of cytokines in tumor bearing mice.

Effect of Tumor Growth/Ubiquinol on Oxidative Stress

Although others found that tumor growth increased oxidant activity in muscle (Barriero et al., 2005), we observed no change in oxidant activity, measured as the ratio of reduced sulfhydryl glutathione (GSH)/ and oxidized GSH disulfide (GSSG), in the gastrocnemius muscle of the tumor-bearing mice compared to muscles from the healthy control mice. It is possible there were no changes in oxidant status between our study and Barriero et al., (2005) due to tumor type, degree of muscle wasting and method of oxidative stress evaluation used. Barriero et al., (2005) induced tumor burden with the AH-130 Yoshida ascites hepatoma cells. The rats were euthanized on days 4 and 7, with a 12% and 26% reduction in gastrocnemius weight, which indicates a more virulent strain of tumor cells and muscle wasting than our C26 inoculated mice who lost 20% gastrocnemius weight by day 18. In addition, for oxidative stress evaluation, indicators of lipid peroxidation, 4-hydroxy-2-noneal (HNE) and malondialdehyde (MDA) assays were used, along with western blotting analysis of protein carbonylation in gastrocnemius muscle, whereas we used just one indicator of oxidative stress, the GSH/GSSG assay.

However, we did observe a significant decrease in the GSH/GSSG ratio in cardiac tissue, indicating oxidative stress in the cardiac muscle of the tumor-bearing mice. Others using a different biomarker of oxidative stress, found no significant effect of tumor growth on oxidant activity in the heart (Muhlfeld et al., 2011). Although there were

similar characteristics between the tumor-bearing mice in both studies (no loss of heart mass), the lack of oxidative stress induction in the cardiac muscle may be due to use of different assays to detect oxidative stress. Muhlfeld et al., (2011) used MDA as an indicator of oxidative stress while we used the GSH/GSSG assay. Based upon results of oxidative stress evaluation in these studies, it is possible the GSH/GSSG assay is a more sensitive measure of oxidative stress in the cardiac muscle than in skeletal muscle, while MDA may be a more sensitive measure in skeletal than cardiac muscle. Alternative measures of oxidative stress evaluation should be considered including, HNE and western blotting for evaluation of protein carbonylation in tissue in subsequent studies.

In this study, we found ubiquinol supplementation had no effect on the GSH/GSSG ratio in cardiac muscle. These findings suggest that the dose of ubiquinol administered in the present study was not sufficient to reduce oxidative stress in the heart of the tumor-bearing mice. Tumor growth is associated with increase in cytokine activity and oxidative stress. In the previously cited studies in which CoQ10 supplementation affected inflammation, serum levels of oxidative stress were also reduced (Sohet et al, 2009; Lee et al., 2012). Ubiquinol had no effect on tumor growth or cytokine expression in our study, such that continued tumor growth increased cytokine expression and production of oxidative stress in the heart. This lack of effect of ubiquinol on oxidative stress may be due to tumor growth overwelming the antioxidant capacity of exogenously administered ubiquinol.

<center>**Effect of Tumor Growth/Ubiquinol on Muscle Metabolism**</center>

Protein Degradation

Both proinflammatory cytokines and oxidative stress increase activity of the ubiquitin proteasome pathway (UPP) of muscle protein degradation and activity of the autophagy lysosome system (ALS) for removal of damaged proteins and organelles in cells (Aucello et al., 2009; McClung et al., 2010; Saini et al., 2009). In this study, tumor growth significantly increased skeletal muscle expression of MAFbx and MuRF1 (biomarkers of UPP) and BNIP3 (biomarker of autophagy) mRNA. Similar findings have been reported by others (Asp et al., 2010; Bonetto et al., 2009a; Cosper & Leinwand, 2011; Costelli et al., 2006; Hitt et al., 2005; Penna et al., 2010; Siddiqui et al., 2009; Xu et al., 2011; Zhou et al., 2011). We found that ubiquinol had no effect on expression of MAFbx, MuRF1 and BNIP3 mRNA in the skeletal muscle of the tumor bearing mice. The inability of ubiquinol to affect UPP activity in the skeletal muscle might be explained by the failure of ubiquinol to reduce tumor-induced serum levels and skeletal muscle expression of proinflammatory cytokines. In addition, there may have been inadequate skeletal tissue concentrations of ubiquinol due to inadequate dosing of ubiquinol to affect UPP and ALS activity.

Tumor growth had no effect on expression of MAFbx, MuRF1 and BNIP3 mRNA in heart muscle. While this is consistent with findings of prior studies of tumor-bearing mice (Cosper & Leinwand, 2011; Zhou et al., 2011), others found an increase in cardiac muscle expression of MAFbx and MuRF1 and BNIP3 mRNA (Tian et al., 2011; Shadfar et al., 2011; Wysong et al., 2011; Xu et al., 2011). The prior studies in tumor

<center>61</center>

bearing mice that had increased cardiac muscle expression of MAFbx and MuRF1 mRNA; there were also increased serum levels of IL-6 and TNFa (Tian et al., 2011; Wysong et al., 2011). Oxidative stress is implicated in activation of UPP and autophagy (Aucello et al., 2009). In the present study, although there was an increase in serum and cardiac muscle expression of IL-6 and TNFR1 and a decrease in the GSH/GSSG ratio in the cardiac muscle it is possible that levels of inflammation and oxidative stress may not have been significant enough to activate UPP and ALS in cardiac muscle. It is also possible that if muscle wasting was more severe in the tumor-bearing mice or tumor burden was greater, changes in UPP and ALS activity may have been seen as in Xu et al., (2011).

Muscle Protein Synthesis

Tumor growth is associated with an increase in pathways associated with muscle wasting (UPP and ALS) and a decrease in pathways associated with muscle synthesis (Dodson et al., 2011; Saini et al., 2009). IGF-1 activates the PI3K/AKt/mTOR pathway of protein synthesis. Prior research has found that tumor growth decreased expression of IGF-1 mRNA in skeletal muscle (Costelli et al., 2006; Penna et al., 2010a; White et al., 2011). Contrary to these finding, in this study, tumor growth increased skeletal and cardiac muscle expression of IGF-1 mRNA. There was also no effect of ubiquinol supplementation on cardiac and skeletal muscle expression of IGF-1 mRNA. There may have been inadequate tissue concentrations of ubiquinol due to inadequate dosing to affect IGF-1 mRNA in cardiac and skeletal muscle. Further research is need to elucidate signaling pathways responsible for the increase in IGF-1 mRNA expression and the

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simultaneous increase in biomarkers associated with muscle wasting in skeletal muscle of tumor-bearing mice seen in our tumor-bearing mice.

IGF-1 signaling is also responsible for the transcription of MyoD a protein involved in muscle satellite cell differentiation. Tumor growth had no effect on expression of MyoD mRNA in cardiac or skeletal muscle. Similarly, no change in expression of this biomarker has been previously reported in skeletal muscle (Penna et al., 2010b), while others have found decreased expression of MyoD in skeletal muscle in tumor-bearing animals (Costelli et al., 2005; Mastrocola et al., 2008; Moore-Carrasco et al., 2007). A possible rationale for the conflicting results between these studies and ours is tumor cell line and animal type. In this study and in Penna et al., (2010), tumor-bearing mice were inoculated with the C26 cell line. Others used rats inoculated with the AH-130 Yoshida ascites hepatoma in their tumor-bearing animals (Costelli et al., 2005; Mastrocola et al., 2008; Moore-Carrasco et al., 2007). This information suggest that tumor cell line and animal species makes a difference in expression of MyoD in muscle.

Effect of Tumor Growth/Ubiquinol on Muscle Mass

In animal models of tumor-induced muscle wasting, tumor growth causes a reduction in body weight and loss of muscle mass. In our present study, tumor growth reduced body weight and gastrocnemius muscle mass. This finding is consistent with findings from other animal models of tumor-induced muscle wasting (Graves et al., 2006; Hitt et al., 2004; McCarthy et al., 2004). In this study, ubiquinol preserved gastrocnemius muscle mass but had no effect on body weight. Tumor-bearing mice loose muscle mass and adipose tissue. Adipose tissue is preferentially lost while muscle mass is less affected

in patients with GI cancer (Dahlman et al., 2010). It is possible ubiquinol had an effect on skeletal muscle mass but not adipose tissue, thereby preserving skeletal muscle mass but not body weight.

Muscle mass is a balance between muscle protein synthesis and degradation. Factors associated with muscle protein degradation include proinflammatory cytokines and activation of UPP and ALS activity. While factors associated with muscle protein synthesis involves IGF-1 signaling. In the present study, tumor-induced increases in cytokine expression and activation of UPP, ALS and IGF-1 was found in the gastrocnemius of the tumor bearing animals, however, ubiquinol had no effect on any of these biomarkers. Therefore it is unclear as to why muscle mass was preserved in the tumor-bearing mice. Further studies to determine the biological mechanism contributing to improved muscle mass in the ubiquinol treated mice are needed, including downstream effects of the IGF-1/PI3K/AKt/mTOR pathway of muscle protein synthesis. Although Penna et al., (2010a) found a decrease in muscle expression of IGF-1, there was no change in the activated or phosphorylated AKt (pAKt) activity compared to controls indicating that the IGF-1 signaling pathway was not down-regulated in tumor-bearing animals. Similarly, White et al., (2011) a decrease in IGF-1 signaling and an increase in pAKt in the gastrocnemius muscle of tumor-bearing mice, beginning when the mice lost 6-19% of body weight. Activity of pAKt should be examined as an explanation for improved muscle mass in the ubiquinol-treated tumor-bearing mice.

In the present study, tumor growth had no effect on heart mass. Findings from other studies differ, some reporting no effect (Muhlfeld et al., 2011; Xu et al., 2011),

while others reported a decrease in heart mass in tumor-bearing animals (Cosper & Leinwand, 2011; Shadfar et al., 2011; Tian et al., 2010; Wysong et al., 2011; Zhou et al., 2010). Serum and/or muscle expression of cytokines and/or biomarkers of UPP and ALS activity were increased in these tumor-bearing animals so it is unsure why there are conflicting results on maintenance or loss of heart mass. One possible cause may be the virulence of the tumor cells themselves. In Murphy et al., (2012), C26 cell lines from 2 different laboratories were used, with one causing severe muscle wasting while the other caused a mild muscle wasting. Most of the studies used the C26 cell line (Cosper & Leinwand, 2011; Shadfar et al., 2011; Tian et al., 2010; Wysong et al., 2011; Xu et al., 2011; Zhou et al., 2010), while one used the Lewis lung carcinoma (Muhlfeld et al., 2011). In Xu et al., (2011), C26 tumor-bearing mice have been shown to cause a milder muscle wasting than Tian et al., (2010), which was the C26 cell line used to induce the severe muscle wasting seen in Murphy et al., (2012). It is possible that tumor-bearing mice bearing the Lewis lung carcinoma also induce a milder muscle wasting. In Muhlfeld et al., (2011), there was no loss of heart mass but there was a decrease in myofibril volume and an increase in sarcoplasmic volume fraction seen with electron microscope. Therefore, tissue histological examination may be useful to determine if there are tumor-induced changes in the morphology of the cardiac muscle. In addition, IGF-1 was increased in the tumor-bearing mice hearts; this may have prevented heart muscle wasting.

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Effect of Tumor Growth/Ubiquinol on Grip Strength

Weakness is a major feature of CRF and loss of muscle mass correlates with decreased contractile muscle force in tumor-bearing mice (Gorselink, 2006). In our animal model of CRF, weakness is modeled as reduced grip strength. In the present study, grip strength progressively declined in the tumor-bearing mice over the course of tumor growth. Similarly, Toledo et al., (2011) found a decline in grip strength in tumor-bearing mice over time. Ubiquinol supplementation had no effect on grip strength in the tumor-bearing mice. There are conflicting reports on the effects of CoQ10 on grip strength in animal models of Huntington's disease; no effect was found by Menalled et al., (2010), Smith et al., (2006) reported improved grip strength in mice with Huntington's disease. In this study, the ubiquinol supplemented tumor bearing mice had an increase in skeletal muscle mass. Even though skeletal muscle mass was increased in tumor-bearing mice treated with ubiquinol, grip strength was not improved. These findings suggest that maintenance of skeletal muscle mass was not enough to prevent the tumor-induced decline in grip strength seen in our animal model of CRF.

Effect of Tumor Growth/Ubiquinol on Voluntary Running Activity

Normal healthy mice can run miles a day on a running wheel. Decreased voluntary running activity (VRA) has been used as a valid measure of malaise and fatigue in rodents **(**Skinner et al, 2009: Wood et al., 2006). VRA progressively declined with no significant change in heart mass and minimal changes in echocardiographic measures of cardiac function over the course of tumor growth in this study. Ubiquinol preserved muscle mass in the tumor-bearing mice, but there was no improvement on VRA.

Reduced VRA has been reported in other studies of tumor-induced muscle wasting and fatigue. Baltgalvis et al., (2010) measured VRA for 21 weeks in tumor-bearing mice with a reduction in activity correlating with the severity of muscle wasting. These data suggest that cardiac dysfunction and preserved muscle mass are not major factors in the decreased VRA in this animal model of CRF.

In this present study, improved muscle mass in the ubiquinol-treated mice with tumors did not improve grip strength or VRA. These data suggest that tumor-induced muscle wasting is not a major factor in the decline in physical strength or activity associated with CRF. Tumor growth is associated with an increase in circulating levels of proinflammatory cytokines (Aggarwal et al., 2006) and could induce symptoms of depression by altering the metabolism of tryptophan, increasing serotonin membrane transporter activity (Zhu et al., 2006) and reducing serotonin activity in the brain (Anisman, 2008). Decreased levels of serotonin are related to symptoms of depression which often co-occur with CRF (Roscoe et al., 2005). Anhedonia is the inability to enjoy activities that are normally considered pleasurable; in animal models this includes a reduction in consumption of sucrose and VRA (Brene et al., 2007; De La Garza, 2005). Although muscle mass was maintained in the ubiquinol-supplemented mice, ubiquinol had no effect on elevated serum and muscle expression of IL-6 and TNFa. The lack of reduction of proinflammatory cytokines could be a potential reason why pleasurable and motivational activities such as VRA and grip strength did not improve.

In an animal model of chronic immune activation, depressed behavior was associated with elevated levels of proinflammatory cytokines in brain tissue with a

significant increase in the duration of immobility during the forced swim test (FST) (O'Connor et al., 2009b). Conducting a FST in the ubiquinol-supplemented mice could be used to determine if decreased VRA was due to reduced effort tolerance or to depressive mood. It is possible that VRA and Grip strength are not sensitive enough measures for fatigue and weakness in our model of CRF. Investigation of different measurements of physical strength and activity is warranted.

Effect of Tumor Growth/Ubiquinol on Cardiac Function

The direct effects of tumor growth on cardiac function have only recently been reported in the literature with differing findings. There have been reports of increased left ventricular systolic diameter (LVSD) and a decreased fractional shortening (FS) and posterior wall thickness (PWT) (Shadfar et al., 2011; Tian et al., 2011; Tian et al., 2010; Wysong et al., 2011; Xu et al., 2011), others have reported no effects of tumor growth on cardiac function in tumor-bearing mice (Muhlfeld, et al., 2011; Cosper & Leinwand, 2011). In this study, echocardiography was performed on the mice on day 18 or 19 of tumor growth, when VRA and grip strength were significantly reduced. No effects of tumor growth on left ventricular diastolic diameter (LVDD), LVSD, PWT and FS were found. However, isovolumic relaxation time (IVRT), an indicator of diastolic dysfunction was significantly increased in the tumor-bearing mice. Increased IVRT indicates poor myocardial relaxation, affecting cardiac output (CO) and producing symptoms of fatigue and weakness similar to CRF. A possible mechanism for this increase in myocardial relaxation is due to an alteration of the contractile proteins. A prior study in our lab showed increased sarcomere re-lengthening in cardiomyocytes isolated from tumor-

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bearing mice indicating a diastolic dysfunction (Xu et al., 2011). This is congruent with the increase in IVRT seen in this study. Others have also found alterations in contractile proteins in the hearts of tumor-bearing mice. Tian et al., (2010) found a decrease in Troponin 1 mRNA, and Cosper & Leinwand, (2011) found a reduction in actin mRNA in the hearts of tumor-bearing mice. In addition, a reduction in adult myosin heavy chain (α-MyHC) mRNA, and increased fetal isoform of MyHC (β-MyHC) mRNA was seen in the hearts of tumor-bearing mice (Cosper & Leinwand, 2011; Tian et al., 2010, Tian et al., 2011).

Although CO was not evaluated (there is conflicting evidence in the literature concerning accuracy of this measurement in mice) (Tournoux et al., 2011), ubiquinol had no effect on any parameters of cardiac function by echocardiography. Tumor growth increased heart muscle expression of IL-6 and TNFa and oxidative stress. Inflammation and oxidative stress are implicated in cardiac dysfunction (El-Menyar, 2008; Nabeebaccus et al., 2011; Prabhu, 2004). Ubiquinol had no effect on these biomarkers so an improvement in diastolic dysfunction would not be expected. Inadequate tissue concentration of ubiquinol due to inadequate dosing may be a possible rationale for the lack of improvement in inflammation, oxidative stress and diastolic dysfunction in the tumor-bearing mice. In addition, reduced antioxidant capacity and inadequate anti-inflammatory ability of ubiquinol are also possible causes as might be the physical condition of the mice compared to other studies. In the presence of tumor growth, female mice maintain their body weight, skeletal muscle mass and heart mass better than males, due to estrogen signaling (Cosper & Leinwald, 2011). In this study, the female tumor-

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bearing mice lost an average of 5% more body weight (with tumor) than the control mice at time of euthanasia and from baseline.

A more significant loss of body weight in tumor-bearers with cardiac dysfunction and/or cardiac muscle wasting has been reported. Tian et al., (2010) reported a 23% weight loss between control mice and tumor-bearing mice at end of study. Shadfar et al., (2011) reported $8.8 \pm 1.7\%$ decrease in body weight from baseline in tumor-bearing mice. Zhou et al., (2011) reported a > 20% reduction in body weight from baseline within 2-3 weeks in tumor-bearing mice. However, in our tumor-bearing mice, if skeletal muscle wasting had been more severe, it is possible additional tumor-induced changes in echocardiographic measures of cardiac function and cardiac muscle wasting may have occurred.

Limitations and Suggestions for Future Research

Limitations to this study include using an animal model of CRF, because the results are not immediately applicable to care of patients with CRF. However, the findings will provide proof of principle for translational research to determine if ubiquinol will preserve muscle mass in patients with cancer.

The length of time for ubiquinol supplementation was a limitation possibly affecting the results. Prior studies demonstrating beneficial effects of CoQ10 or ubiquinol supplementation involved 4 to 26 weeks of supplementation. In an animal model of metabolic syndrome, CoQ10 levels were increased in the serum after 2 weeks of supplementation, but serum biomarkers of oxidative stress were not reduced for 10 weeks, suggesting CoQ10 rises in serum quickly but much slower in the tissues.

(Kunitomo et al, 2008). Diabetic mice supplemented with CoQ10 for 10 weeks demonstrated improvement in diastolic dysfunction, left ventricular end diastolic diameter (LVEDD), left ventricular (LV) mass and cardiac fibrosis (Huynh et al., 2012). In an animal model of Huntington's disease, mice showed a significant increase in grip strength after 7 weeks of treatment with CoQ10 (Smith et al., 2006). In rats supplemented with CoQ10, concentrations of total CoQ10 were approximately 23% in heart and 45% higher in skeletal tissue at 4 weeks (Kwong et al., 2002). Therefore supplementing the mice with ubiquinol 4-5 weeks prior to tumor inoculation would be an option for future studies to ensure adequate tissue concentrations of the drug. Verification of plasma and tissue levels of ubiquinol in the experimental animals is an option to ensure adequate tissue concentrations, in addition to trying a higher dose of the drug.

Another limitation is the health of the mice. The tumor-bearing mice did not lose as much weight as expected. Overall the tumor-bearing mice lost 5% body weight and 9% carcass body weight on average from baseline. During the study, the mice either became very sick or not sick at all with tumor sizes varying in the animals from days 14 to 18. Data from 2 mice that became sick before 14 days or died early were eliminated. However, the initial power analysis (52 mice needed to detect a significant effect at $\alpha =$ 0.5 and power of 80%) obtained from the pilot study was not affected due to additional mice being used for data collection (total 64-68 mice). The decision to eliminate sick mice from data collection was due to abnormal values being seen in the expression of proinflammatory cytokines and biomarkers of protein degradation mRNA with RT-PCR.

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A lack of tumor-induced changes in echocardiography parameters previously seen in our tumor-bearing mice is an additional limitation. Future research is needed looking at cardiomyocyte function and cardiac muscle histological examination, in the tumor-bearing and ubiquinol treated animals to determine if there are changes at the cellular level before it is seen by echocardiography and if ubiquinol supplementation has an effect.

Summary

Concerns have been raised that administration of antioxidants might favor tumor growth (Nicholson & Conklin, 2008). This study used an animal model to explore the associations between tumor growth and antioxidant usage. Results indicate no effect of ubiquinol supplementation on tumor growth. In addition, a diastolic dysfunction was seen in the tumor-bearing mice, adding to the growing knowledge that tumor growth alone affects cardiac muscle and cardiac function prior to cancer treatment. Precautions can be then be taken to closely monitor cardiac status for these patients. The preservation of skeletal muscle mass in the tumor-bearing mice was an additional strength of this study. This finding will provide proof of principle for additional research for using ubiquinol in patients with cancer.

Conclusion

In this mouse model of CRF, tumor growth induced loss of muscle mass and decreased VRA and grip strength. Tumor growth also increased cardiac, skeletal muscle and serum levels of pro-inflammatory cytokines, oxidative stress in the heart, and biomarkers associated with muscle wasting in skeletal muscle and synthesis in cardiac

and skeletal muscle. Echocardiography revealed a diastolic dysfunction in the tumor-bearing mice. These data confirm prior studies indicating tumor growth alone affects cardiac function, which could contribute to CRF. Treatment with the antioxidant agent ubiquinol did not improve levels of proinflammatory cytokines in serum and muscle, oxidative stress in cardiac muscle, cardiac function, biomarkers associated with muscle metabolism, VRA or grip strength in the tumor-bearing mice, even though it did improve skeletal muscle mass. Because it did improve muscle mass without changing measures of fatigue, inflammation, cardiac dysfunction or muscle metabolism, a conclusion is that antioxidant treatment alone is not likely to reverse CRF. These data also suggest that skeletal muscle wasting may not be a major factor in reduced physical activity and weakness associated with CRF.

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