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Effects of an Acute High-Volume Isokinetic Intervention on Circulating Levels of TNF-α and STNFR: Influence of Age

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EFFECTS OF AN ACUTE HIGH-VOLUME ISOKINETIC INTERVENTION ON
CIRCULATING LEVELS OF TNF-α AND STNFR: INFLUENCE OF AGE

by

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ABSTRACT

The immune system has been implicated in recovery and muscle regeneration following exercise. In response to muscle damage, the immune system responds with an increase in circulating pro-inflammatory cytokines with the goal of recruiting leukocytes to the damaged area. Tumor Necrosis Factor-alpha (TNF-α), in particular, has been shown to be implicated in both muscle regeneration and muscle wasting. However, it remains unclear whether TNF-α is responsible for the age-related losses in muscle size and function. Also, due to the high clearance rate of TNF-α from circulation, analyzing the circulating levels of soluble TNF-α receptors 1 and 2 (STNFR1 and STNFR2) may provide a better indication of inflammatory events. Therefore, the purpose of this study was to compare changes in circulating levels of TNF-α, STNFR1, and STNFR2 following an acute muscle damaging intervention in young age (YA) and middle-aged (MA) males. Recreationally active young (YA; N=9, 21.8 ± 2.2 y, 179.5 ± 4.9 cm, 91.2 ± 12.2 kg, $21.8 \pm 4.3\%$ BF) and middle-aged (MA; N=10, 47.0 ± 4.4 y, 176.8 ± 7.6 cm; 96.0 ± 21.5 kg, $25.4 \pm 5.3\%$ BF) males completed an acute muscle damaging protocol (MDP). Blood samples were obtained at baseline (BL), immediately (IP), 30 minutes (30P), 60 minutes (60P), 120 minutes (120P), 24 hours (24H), and 48 hours (48H) post-MDP. Lower body performance was analyzed via isokinetic dynamometer at BL, IP, 120P, 24H, and 48H. No significant group x time interactions or main group effects were observed for TNF-α, STNFR1, STNFR2 or any marker of muscle damage. When collapsed across groups, plasma lactate was significantly elevated at IP ($p < 0.001$) and 30P ($p = 0.003$); serum myoglobin was increased at 30P ($p = 0.002$), 60P ($p = 0.001$), and 120P ($p = 0.007$); creatine kinase was elevated at 24H ($p = 0.001$)

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and 48H ($p = 0.005$). Plasma concentrations of TNF-α were unchanged following MDP. With both groups combined, serum STNFR1 was decreased at 30P ($p = 0.001$) and increased at 48H ($p = 0.028$). Serum STNFR2 was decreased at 30P ($p = 0.008$), 60P ($p = 0.003$), and 120P ($p = 0.002$). The results of this study indicate that the TNF-α and STNFRs response to exercise is similar between young and middle-aged males. Measuring STNFRs may be a more appropriate method of assessing the acute inflammatory response to muscle damage. In addition, an acute bout of exercise may attenuate ectodomain shedding of TNFR1 and TNFR2.

To my parents Eliott and Fausta, for all of the sacrifices you made to ensure my success. Thank you for supporting me in all my endeavors and teaching me the value of hard work.

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LIST OF ACRONYMS/ABBREVIATIONS

120P	120-minutes post-muscle damaging protocol
1-RM	1-Repetition Maximum
24H	24-hours post-muscle damaging protocol
30P	30-minutes post-muscle damaging protocol
48H	48-hours post-muscle damaging protocol
60P	60-minutes post-muscle damaging protocol
ADAM-17	A disintegrin and metalloproteinase-17
AIDS	Acquired Immunodeficiency Syndrome
ANOVA	Analysis of Variance
AU	Arbitrary Units
AUC	Area Under the Curve
BIA	Bioelectric Impedance Analysis
BL	Baseline
CK	Creatine Kinase
CSA	Cross-sectional Area
dB	Decibel
EI	Echo Intensity
EIMD	Exercise-Induced Muscle Damage
ELISA	Enzyme-linked Immunosorbent Assay
ES	Effect Size
HIV	Human Immunodeficiency Virus
HPL	Human Performance Laboratory

IGF-1	Insulin-like Growth Factor 1
IL-1ra	Interleukin-1 receptor antagonist
IL-1ß	Interleukin-1 beta
IL-6	Interleukin-6
IP	Immediately post-muscle damaging protocol
K_2EDTA	Ethylenediaminetetraacetic Acid
kDa	Kilodalton
LSD	Least Significant Difference
MA	Middle-Aged
MDP	Muscle Damaging Protocol
mP	Mean Power
mRNA	Messenger Ribonucleic Acid
MT	Muscle Thickness
mTNF-α	Membrane-bound Tumor Necrosis Factor-alpha
mTQ	Mean Torque
MVIC	Maximal Voluntary Isometric Contraction
NF-κB	Nuclear Factor- κB
PP	Peak Power
PTQ	Peak Torque
RF	Rectus Femoris
SD	Standard Deviation
SE	Standard Error
STNFR	Soluble Tumor Necrosis Factor Receptor

T1	Day 1 of testing
T2	Day 2 of testing
T3	Day 3 of testing
T4	Day 4 of testing
TACE	Tumor Necrosis Factor-alpha Converting Enzyme
TNFR	Tumor Necrosis Factor Receptor
TNF-α	Tumor Necrosis Factor-alpha
TRADD	TNFR1-associated Death Domain
TRAF	TNFR-associated Factor
TRAPS	TNF-Receptor-Associated Periodic Febrile Syndrome
VO$_2$max	Maximal Oxygen Uptake
YA	Young Age
η^2_p	Partial Eta Squared

CHAPTER ONE: INTRODUCTION

The term "sarcopenia" was initially used to describe the age-related loss of muscle mass. More recent definitions include measures of muscle mass, strength, and physical performance; all of which decline with age (Dodds, Roberts, Cooper, & Sayer, 2015). The number of people affected by sarcopenia is currently estimated at 50 million and is projected to increase to more than 200 million by 2050 (Cruz-Jentoft et al., 2010). The prevalence is reported to be 5-13% in individuals between the ages of 60 and 70 years old and 11-50% in individuals over the age of 80 (Cruz-Jentoft et al., 2010; Morley, 2008). As sarcopenia progresses, it may lead to disability and frailty, which in turn may lead to a loss of independence (Roubenoff, 2001). The significance of sarcopenia is evident in findings that show an association between weaker grip strength and higher risk of all-cause mortality in middle- and older-aged individuals (Cooper, Kuh, & Hardy, 2010). Age-associated decrements in both power and strength have been shown to begin as early as age 40 and continue to decline with increasing age (Metter, Conwit, Tobin, & Fozard, 1997). Although impaired movement is often the cynosure of sarcopenia, decreased muscle mass may also disrupt glucose regulation, hormone production, and communication between cells, which may lead to comorbidities and/or increased risk of mortality (Buford et al., 2010).

The immune system has been implicated in recovery and muscle regeneration following exercise (Hawke & Garry, 2001; Paulsen, Mikkelsen, Raastad, & Peake, 2012). When exercised muscle fibers are damaged, an inflammatory response is initiated with the goal of removing cellular debris and recruiting cells that aid in repair (Clarkson & Hubal, 2002). This process includes the recruitment and migration of immune cells, and muscle cell precursors in response

to cytokines and chemokines (Cannon & Pierre, 1998). These cell messengers are released by nearly all nucleated cells and can mediate both pro- and anti-inflammatory responses (Cannon & Pierre, 1998; Hirose et al., 2004; Pedersen & Hoffman-Goetz, 2000). Tumor necrosis factor-alpha (TNF-α) in particular is known to play a key role in controlling this response.

Chronic exposure to excessive TNF-α has been shown to result in whole-body protein decrements, which may adversely affect muscle function and possibly contribute to the onset of sarcopenia (Brüünsgaard & Pedersen, 2003; Tracey & Cerami, 1992). Consistent with this, increased basal concentrations of TNF-α have been reported in older adults compared to their younger counterparts (Bruunsgaard et al., 1999; Woods, Wilund, Martin, & Kistler, 2012). Chronically elevated levels of TNF-α have been shown to disrupt myofibrillar differentiation, stimulate catabolism, and induce apoptosis through the death domain receptors in muscle cells (Krabbe, Pedersen, & Bruunsgaard, 2004; Roubenoff, 2003). Furthermore, evidence suggests that elevated levels of TNF-α are inversely related to skeletal muscle protein synthesis, and may cause increased basal energy expenditure, as well as loss of bone and muscle mass (Greiwe, Cheng, Rubin, Yarasheski, & Semenkovich, 2001; Krabbe et al., 2004).

In contrast, the acute production of low concentrations of endogenous TNF-α appears to be beneficial for the coordination of tissue remodeling and recovery from muscle damage (Tracey & Cerami, 1992). Administration of low levels of recombinant TNF-α *in vitro* has been shown to play a role in the activation and proliferation of satellite cells (Li, 2003). Consistent with this, eliminating the effects of TNF-α in mice, either through double-knockout of its receptors or through neutralizing antibodies has been shown to negatively affect strength recovery following muscle damage when compared to wild-type mice (Warren et al., 2002). The acute production and autocrine signaling of TNF-α in macrophages following muscle damage

can trigger the release of growth factors that promote tissue remodeling (Noble, Lake, Henson, & Riches, 1993). This may explain the observed findings of a number of investigative groups whereby TNF-α expression in skeletal muscle and/or plasma is increased following resistance exercise (Buford, Cooke, & Willoughby, 2009; Townsend et al., 2013; Wells et al., 2016). Notwithstanding, research comparing circulating levels of TNF-α between middle-aged adults and younger adults is lacking. Limited evidence suggests that chronic elevations in basal TNF-α may contribute to the development of sarcopenia. However, evidence also suggests that there may be an age-associated blunting of the TNF-α response to acute exercise in older adults (Hamada, Vannier, Sacheck, Witsell, & Roubenoff, 2005). This blunted TNF-α response may lead to an age-associated decrement in strength recovery following an acute bout of resistance exercise. Whether differences in basal TNF-α concentrations and/or the acute TNF-α response to exercise become evident with advancement of chronological age is currently unclear.

TNF-α elicits its effects through two transmembrane tumor necrosis factor receptor (TNFR) molecules, TNFR1 and TNFR2 (Fiers, 1991). Ectodomain shedding of both of these receptors leads to release of their soluble forms (STNFR1 and STNFR2) into circulation (Buckley et al., 2005; Giai et al., 2013; Wallach et al., 1991), which can be found in small amounts in the serum and urine of healthy individuals (Nicod, 1993; Wallach et al., 1991). Similar to its ligand, the release of STNFRs appears to have several functions. A number of reports suggest that the ectodomains of TNFRs are shed to inhibit TNF-α signaling, protecting the cells from the harmful effects of excessive TNF-α (Aderka, 1996; Wallach et al., 1991). In contrast, others suggest that STNFRs serve to bind and sequester TNF-α, protecting it from denaturation and prolonging its effects (Aderka, Engelmann, Maor, Brakebusch, & Wallach, 1992; van Deuren, 1994). Despite some research indicating that serum levels of STNFR1 and

STNFR2 are elevated in conjunction with elevated levels of TNF-α following strenuous exercise, research observing the effects of muscle damage on circulating levels of STNFR1 and STNFR2 are lacking (Ostrowski, Rohde, Asp, Schjerling, & Pedersen, 1999).

To our knowledge, no study to date has investigated the TNF-α and STNFRs responses to muscle damage in middle-aged adults compared to their younger counterparts. Therefore, the purpose of this study was to compare changes in circulating levels of TNF-α and STNFRs following an acute muscle damaging intervention in young and middle-aged males. The relationship between circulating levels of TNF-α, STNFR1, and STNFR2 and isokinetic strength following muscle damage was also examined. A secondary purpose was to examine the relationship between basal circulating levels of TNF-α, STNFR1 and STNFR2 and muscle size and function in middle-aged adults to determine if TNF-α levels may indicate early signs of the age-associated loss of muscle mass and strength.

CHAPTER TWO: LITERATURE REVIEW

Skeletal muscle is capable of adapting to and recovering from a wide variety of physiological challenges including severe tissue injury (Hawke & Garry, 2001). Both direct and indirect evidence indicates that arduous, unaccustomed exercise leads to damage within muscle tissue, which provides a stimulus for repair and growth (Clarkson, Nosaka, & Braun, 1992; Hawke & Garry, 2001). The severity of the damage depends on the intensity, duration and type of exercise (Malm, 2001). Eccentric contractions, where muscles contract while lengthening, is known to cause more damage and inflammation than concentric exercise of the same intensity and duration (Friden, Sjöström, & Ekblom, 1983; Malm, 2001). Muscle damage is characterized by increased serum concentrations of creatine kinase and myoglobin, alterations in ultrastructural integrity, inflammation, increased proteolytic enzymes, and ultimately a decrease in force production (Brancaccio, Lippi, & Maffulli, 2010; Sorichter et al., 1997; Stupka, Tarnopolsky, Yardley, & Phillips, 2001; Warren, Lowe, & Armstrong, 1999). The initial response to skeletal muscle damage involves activation of the innate immune system (Tidball & Villalta, 2010). The primary role of the innate immune response is to clear cellular debris in preparation for regeneration and repair of damaged muscle fibers, blood vessels, nerve fibers, and the extracellular matrix (Clarkson & Hubal, 2002). The acute inflammatory response to exercise-induced muscle damage (EIMD) is similar to the response to infection (Tidball, 1995). Neutrophils are the first responders and begin to appear within 2 hours of muscle damage. Neutrophil infiltration is followed by the invasion of phagocytic macrophages (Aderem & Underhill, 1999; Tidball & Villalta, 2010; Wynn & Barron, 2010). Neutrophils and macrophages contribute to the lysis of damaged muscle tissue by releasing reactive oxygen and nitrogen

species (Nguyen & Tidball, 2003a; Nguyen & Tidball, 2003b). However, the functions of macrophages after EIMD go beyond phagocytosis of cellular debris, as evidence suggests that they remain present during the regenerative phase of muscle damage (Saclier, Cuvellier, Magnan, Mounier, & Chazaud, 2013).

Muscle Repair

Adult human skeletal muscle is a structurally unique tissue containing highly specialized fibers with the capacity to adapt to physiological demands such as postnatal growth, training, and damage (Hawke & Garry, 2001; Zammit & Beauchamp, 2001). These specialized functions are possible through terminal differentiation, which involves withdrawal from the cell cycle and establishment of a specific configuration of expressed genes that determine cellular function and identity within muscle (Zammit & Beauchamp, 2001). This high degree of specialization comes at the cost of the lost ability to proliferate. Therefore, muscle growth and repair depends on the persistence of a small population of cells located between the sarcolemma and the basal lamina, termed satellite cells (Mauro, 1961). These cells are undifferentiated myogenic precursors with the ability to re-enter the cell cycle in order to generate new muscle fibers and daughter cells that become new satellite cells (Kadi et al., 2005). Because skeletal muscle is a post-mitotic tissue containing hundreds of terminally differentiated nuclei per fiber (myonuclei), satellite cells also provide the only source of new myonuclei (Martin & Lewis, 2012). Satellite cells are mitotically quiescent, but are activated in response to increased workload, EIMD, exercise-induced release of inflammatory signals, and/or release of growth factors after exercise (Kadi et al., 2005). Once activated, satellite cells proliferate and either fuse to existing muscle fibers to provide new myonuclei or return to quiescence (Martin & Lewis, 2012). *In vitro* research has shown that macrophages release growth factors that stimulate the proliferation and differentiation of satellite

cells and myoblasts (Cantini et al., 1994; Cantini & Carraro, 1995). The importance of macrophages is highlighted by the fact that muscle regeneration cannot occur in the absence of macrophages (Lescaudron et al., 1999).Satellite cell proliferation and fusion is dependent on the release of growth factors, such as insulin-like growth factor (IGF)-1, which is upregulated in response to EIMD (Hawke & Garry, 2001). In response to these growth factors released from the injured muscle fibers and activated macrophages, satellite cells proliferate and then withdraw from the cell cycle and self-renew or form new centrally nucleated myotubes approximately 5 days after injury (Hawke & Garry, 2001). The process of myofiber damage, leukocyte infiltration, and satellite cell activation is depicted in Figure 1.
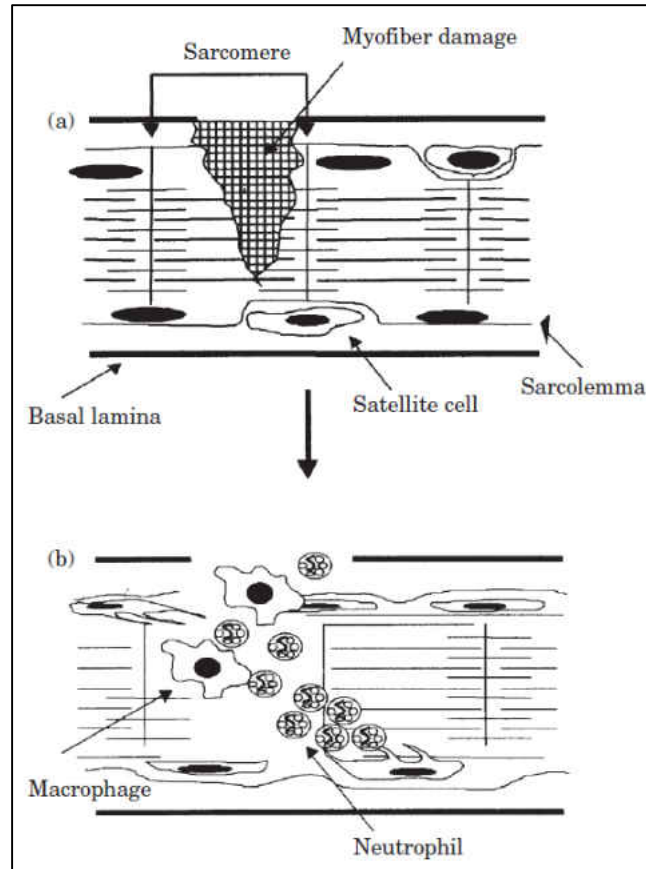
Figure 1: Leukocyte infiltration and satellite cell activation following muscle damage
(a)Exercise-induced muscle damage (EIMD) occurs. (b) Neutrophils and macrophages infiltrate the damaged area where they remove cellular debris and attract satellite cells to proliferate.
Adapted from: Vierck et al. (2000).

Cytokines

The process of muscle hypertrophy is highly dependent on the activity of cytokines. Cytokines are small messaging proteins that influence the survival, proliferation, differentiation and function of immune cells (Calle & Fernandez, 2010). They can be secreted by many nucleated cells including: neutrophils, activated macrophages, damaged myofibers and endothelial cells (Cannon & Pierre, 1998). Cytokines usually act in a paracrine or autocrine

manner at very low concentrations and elicit their effect through high affinity receptors on the surface of target cells (Nicod, 1993). Additionally, cytokines can stimulate further production of cytokines and/or hormones from the target cells (Cannon & Pierre, 1998). Though cytokines often exhibit both pro- and anti-inflammatory tendencies, certain cytokines are known to induce a primarily pro-inflammatory response. The pro-inflammatory cytokines include interleukin (IL)-1ß and TNF-α, which are known to promote inflammation following EIMD (Hirose et al., 2004). While TNF-α and IL-1ß are released following strenuous exercise, this release is counterbalanced with the release of IL-6, IL-1 receptor antagonist (IL-1ra) and soluble TNF-α receptors which inhibit the previously mentioned pro-inflammatory effects (Pedersen & Hoffman-Goetz, 2000). Despite TNF-α's close relationship to IL-1ß and other cytokines, this review will focus on TNF-α and its receptors.

<u>TNF-α</u>

TNF-α was discovered *in vitro* as an endotoxin-inducible substance produced by macrophages that caused necrosis of tumors in mice (Carswell et al., 1975). In other words, when the endotoxin was administered to the mice, macrophages produced TNF-α which worked as a toxin against the tumor. Further research on TNF-α showed that the administration of either recombinant TNF-α or lipopolysaccharide (LPS), a TNF-α-inducer, controlled tumor growth both *in vitro* and *in vivo* (Haranaka, Satomi, & Sakurai, 1984; Matthews, 1979; Prince, Anderson, & Tomasi, 1982; Sedger & McDermott, 2014). However, human clinical trials were not successful. The hopes of TNF-α being the cure for cancer were dismissed, as the results of human clinical trials showed that TNF-α treatment led to adverse effects and a lack of therapeutic benefit for tumor necrosis (Roberts, Zhou, Diaz, & Holdhoff, 2011; Sedger &

McDermott, 2014). Despite the lack of success of TNF-α as a treatment for cancer, TNF-α is now known to have pleiotropic functions (Tracey & Cerami, 1992).

TNF-α exists as a membrane-bound precursor (mTNF-α) with a molecular mass of 26 kDa ,expressed on the surface of activated monocytes/macrophages, natural killer cells, and T cells, but also on non-immune cells such as endothelial cells and fibroblasts (Moss et al., 1997; Sedger & McDermott, 2014). The 17 kDa soluble form of TNF-α is produced in response to the cleavage of mTNF-α from the cell membrane via the metalloprotease, TNF-α converting enzyme (TACE) (Black et al., 1997; Moss et al., 1997). Though mTNF-α is considered to be a precursor to TNF-α, there is evidence suggesting that mTNF-α can also mediate signaling through TNF-α receptors via cell-to-cell contact (Grell et al., 1995; Perez et al., 1990). In addition to serving as an activating ligand on surface receptors, some studies have revealed that mTNF-α also acts as a receptor that transmits reverse signals into the effector cells when activated with an antibody against TNF-α or with soluble TNF-α receptors (Eissner, Kolch, & Scheurich, 2004). TACE, also called a disintegrin and metalloproteinase (ADAM)-17, is a cell-surface protein with proteolytic properties expressed on various tissues including brain, heart, and skeletal muscle (Black et al., 1997; Gooz, 2010).Once TNF-α is released from the cell membrane, it can then bind to a receptor on the same cell; it can bind to receptors on neighboring cells; it can reach distant cells in the same tissue; or it may enter the bloodstream (Gooz, 2010). When TACE is not present, there is an 89-90% reduction in TNF-α release and a corresponding increase in mTNF-α (Black et al., 1997). TACE cleaves mTNF-α as well as TNF-α receptors in order to regulate cellular signaling and affect cell behavior (Figure 2). The cleavage of TNF-α receptors is discussed later on this review.
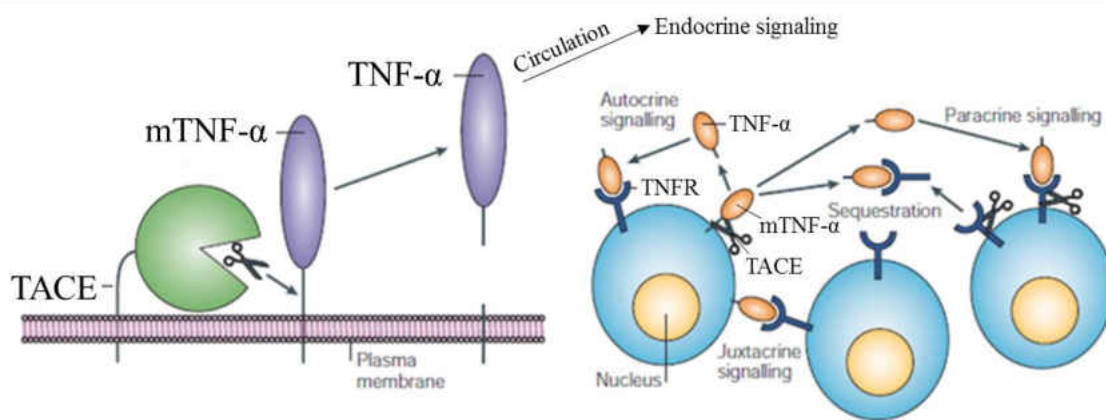
Figure 2*:* **Shedding of mTNF-α and/or TNF-α receptor**
Once the transmembrane precursor of Tumor Necrosis Factor-α (mTNF-α) is cleaved by TNF-α converting enzyme (TACE), it becomes a soluble TNF-α molecule than can then bind to its receptor on the same cell (autocrine signaling), neighboring cells (paracrine signaling), or enter the blood stream and bind to receptors on distant cells and initiate downstream signaling events (endocrine signaling). TACE can also cleave TNF-α receptors from the cell membrane, turning them into soluble TNF-α receptors which can then bind to TNF-α (sequestration; discussed later in this review).
Adapted from: Blobel (2005).

Functions of TNF-α

TNF-α is considered to be one of the most multifunctional, or pleiotropic, cytokines (Rothe, Gehr, Loetscher, & Lesslauer, 1992). Its functions range from mediating shock in sepsis to necrosis of tumors (Tracey & Cerami, 1992). Binding of TNF-α to a wide variety of cell types activates signaling pathways that regulate several different processes such as apoptosis, inflammation, immunity, metabolism, and disease (Chen & Goeddel, 2002; Liu, 2005). Depending on the cellular context and its concentration in the tissues, the net effect of TNF-α may be beneficial or injurious to the host. TNF-α has been shown to be one of the most abundant early mediators during inflammation (Parameswaran & Patial, 2010). It is considered to be a

"master regulator" of pro-inflammatory cytokine production due to its pivotal role in mediating the production of a pro-inflammatory cytokine cascade following trauma, infection, or exposure to bacterial-derived LPS (Parameswaran & Patial, 2010). TNF-α has also been shown to have effects on the vascular endothelium and circulating leukocytes. These effects include an elevation in the gene expression of adhesion molecules and chemokines by endothelial cells, and increased recruitment and adhesion of neutrophils and monocytes following muscle damage (Bevilacqua, Pober, Mendrick, Cotran, & Gimbrone, 1987; Peterson, Feeback, Baas, & Pizza, 2006; Weller, Isenmann, & Vestweber, 1992). Evidence suggests that chronic elevations of TNF-α may inhibit the removal of apoptotic neutrophils by monocyte-derived macrophages (Michlewska, Dransfield, Megson, & Rossi, 2009). Because apoptosis of neutrophils and subsequent removal by macrophages is a key step in the resolution of inflammation, this effect of chronically elevated TNF-α may contribute to the tissue damage associated with chronic inflammatory conditions. In addition to the recruitment and adherence of neutrophils, TNF-α has also been shown to prime neutrophils for enhanced phagocytosis and respiratory burst activity by triggering the release of reactive oxygen species and inducing degranulation (Atkinson, Marasco, Lopez, & Vadas, 1988; Nathan et al., 1989; Richter, Andersson, & Olsson, 1989; Shalaby et al., 1985). TNF-α has been shown to play an important role in the proliferation, apoptosis, and differentiation of macrophages (Parameswaran & Patial, 2010). Lombardo and colleagues (2007) demonstrated that long-term survival of macrophages requires autocrine signaling by TNF-α. Increased levels of circulating and/or muscle TNF-α have been linked to age-related muscle loss, insulin resistance, and increased mortality (Bruunsgaard, Andersen-Ranberg, Hjelmborg, Pedersen, & Jeune, 2003; Greiwe et al., 2001; Paolisso et al., 1998). TNF-α also modulates

growth and differentiation in many cell types (Li & Reid, 2001; Warren et al., 2002). Moreover, evidence suggests that TNF-α plays a large role in skeletal muscle metabolism.

TNF-α and muscle regeneration

TNF-α released following muscle damage has an important role in muscle repair (Li & Schwartz, 2001; Li, 2003; Warren et al., 2002). In addition to activated macrophages, muscle fibers have been shown to synthesize TNF-α, which perhaps functions in an autocrine fashion on the muscle cell itself (Saghizadeh, Ong, Garvey, Henry, & Kern, 1996). Mechanical damage to muscle causes a disruption in extracellular matrix, causing hyaluronate to leak out of the cell and activate the production of TNF-α and IL-1ß in macrophages. Evidence suggests that the binding of hyaluronate to the cell surface adhesion molecule CD44 on macrophages causes the production of TNF-α, which then causes the release of macrophage-derived IGF-1 via autocrine signaling of TNF-α (Noble et al., 1993). Noble et al (1993) noted that macrophage release of IGF-1 following hyaluronic acid stimulation of CD44 requires TNF-α production, since IGF-1 protein synthesis was inhibited when TNF-α signaling was prevented. Further, TNF-α may also recruit fibroblasts to proliferate and/or to synthesize extracellular matrix molecules (such as collagen), in order to repair and regenerate damaged connective tissue (Cannon & Pierre, 1998; Kovacs & DiPietro, 1994). Consequently, TNF-α appears to play a significant role in maintaining the integrity of skeletal muscle tissue.

The presence of TNF-α during muscle regeneration has been shown in several models. Collins & Grounds (2001) showed that TNF-α was highly expressed in injured and necrotic muscle fibers of mice. Uninjured skeletal muscle did not have an increased expression of TNF-α, indicating that significant muscle damage must occur in order for TNF-α to be present. Additionally, infiltrating macrophages, neutrophils, and activated mast cells were shown to be

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sources of TNF-α during injury. Normal muscle regeneration has been demonstrated in mice lacking TNF-α (TNF-α$^{-/-}$), indicating that other cytokines, perhaps IL-12, IL-1ß, and/or IL-6 may effectively and efficiently take over TNF-α's role in its complete absence. That is, the absence of TNF-α had no effect on the timing and extent of inflammation and muscle repair. This compensatory upregulation of other cytokines is not apparent when TNF-α is present, indicating that only mice completely lacking TNF-α may regenerate muscle through other cell mediators when TNF-α is not involved (Collins & Grounds, 2001). Additionally, TNF-α receptor signaling has been shown in the absence of TNF-α ligand binding, adding another possible explanation to the normal muscle regeneration seen in TNF-α$^{-/-}$ mice (Collins & Grounds, 2001; Engelmann et al., 1990). The investigators also observed thin, weak skin as well as poor capillary integrity in TNF-α$^{-/-}$mice, indicating TNF-α also has a role in skin healing, connective tissue regeneration, and angiogenesis (Collins & Grounds, 2001). Another study showed that traumatic freeze-induced skeletal muscle injury in adult mice was accompanied by an increase in TNF-α expression with a single peak at 24 h post-injury and a gradual reduction over 3-7 days post-injury (Warren et al., 2002). The increase in TNF-α coincided with the infiltration of activated monocytes and macrophages, highlighting TNF-α's role in regulating monocytes/macrophages during muscle damage. However, blocking the effects of TNF-α, either by double knockout of both TNF receptors (TNFR-DKO) or by neutralizing antibodies to TNF-α, did not significantly affect inflammatory cell infiltration; demonstrating the possible compensatory roles of other cytokines in leukocyte recruitment. Nevertheless, mice lacking the TNF-α receptors displayed 16-19% lower muscle strength than the wild-type mice despite similar body masses prior to muscle injury. In this study, no significant differences in strength recovery were noted between wild-type mice and TNF-deficient mice at day 5 post-injury. However, at 13 days post-injury,

strength for the wild-type mice had recovered considerably and was only 13% lower than pre-injury, while strength for the TNF-DKO mice and the TNF-α antibody-treated mice had not yet recovered and was still 27-31% lower than pre-injury (Warren et al., 2002). These results are in agreement with the findings of Spencer and colleagues (2000), who found that TNF- and dystrophin-deficient (TNF-/mdx) mice weighed significantly less than mice that were only dystrophin-deficient (TNF+/mdx) at 8 weeks of age. Furthermore, the quadriceps and diaphragm muscles of TNF-/mdx were significantly smaller than that of the TNF+/mdx mice at 8 weeks of age, indicating TNF-α's role in skeletal muscle development (Spencer et al., 2000; Warren et al., 2002). Zádor and colleagues (2001) found that mRNA and protein levels of TNF-α were dramatically increased following toxin-induced necrosis in the soleus and extensor digitorum longus of rats. This increase was seen at the onset of necrosis and invasion of macrophages, while a smaller increase in TNF-α was also seen during regeneration phase of the muscle. This finding further indicates the potential role of TNF-α in the regeneration process, though the investigators also suggested that the second wave of TNF-α may be due to newly formed muscle fibers that failed to fully develop and therefore became apoptotic (Zádor et al., 2001).

The TNF-α response to exercise has also been observed in humans. Buford and colleagues (2009) observed the effects of an acute bout of resistance exercise consisting of 3 sets of 10 repetitions on machine squat, leg press, and leg extension at 80% of one repetition maximum (1-RM) on several inflammatory-related genes in post-menopausal women. The investigators observed a significant up-regulation in mRNA content for TNF-α and IL-1ß in muscle tissue of the vastus lateralis 3-hours post-exercise. They also examined serum levels of TNF-α and IL-1ß at baseline as well as 3, 42, and 48 h post-exercise and found no significant changes between time-points. The investigators noted a limitation in the study was that they did

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not measure protein expression of the markers in the muscle, therefore the posttranscriptional events that took place following exercise were not accounted for in these findings. Additionally, the study did not include any measurements of muscle damage, therefore it is not known whether the protocol they used induced muscle damage or if the results indicate a response to muscle contraction rather than muscle injury (Buford et al., 2009). Hamada et al. (2005) found that an acute bout of eccentric exercise (downhill running) elevated gene expression of TNF-α and IL-1ß in the vastus lateralis of physically active young (age 23-35 years) and old (age 66-78 years) men during the repair phase of muscle damage (72 h post-exercise). These increases correlated strongly with the increased infiltration of leukocytes. TNF-α mRNA increased 2.8 and 1.8-fold in the young and old group, respectively. The investigators mentioned that the eccentric protocol did elicit muscle damage and systemic inflammation, determined by elevated levels of circulating creatine kinase and C-reactive protein, though the data was not shown (Hamada et al., 2005). The difference in the response to muscle damage between young and older adults will be discussed in more detail later in this review.

Other studies have reported increased TNF-α in circulation following resistance training or prolonged exercise. Townsend and colleagues (2013) found that circulating TNF-α was significantly elevated in young (age 22.3 ± 2.4 years) resistance-trained males immediately after an intense lower-body, muscle-damaging resistance training protocol. However, no significant increases were seen 30 minutes, 24 hours, or 48 hours post-exercise. Also, the significant increase in serum TNF-α was blunted with the consumption of a ß-Hydroxy-ß-methylbutyrate (HMB)-free acid supplement (Townsend et al., 2013). Another study comparing changes in markers of monocyte recruitment following an acute bout of high-intensity versus high-volume lower-body resistance exercise in young resistance-trained males reported that plasma

concentrations of TNF-α were significantly elevated immediately post-exercise and remained elevated 30 minutes, 1 hour, and 2 hours post-exercise for both trials combined. Interestingly, despite evidence of greater muscle damage caused by the high-intensity protocol compared to the high-volume protocol, no significant differences in plasma TNF-α levels were observed between the two trials (Wells et al., 2016). Ostrowski and colleagues (1999), found that plasma concentrations of TNF-α were elevated 2.3-fold in well-trained males (age 24-37 years; VO_2max 53.3-70.2ml $min^{-1} kg^{-1}$) 1 hour after running a marathon. Similarly, Starkie et al. (2001) also reported a significant increase in plasma levels of TNF-α immediately after and 2 hours after a competitive marathon. Interestingly, the investigators of that study found that circulating monocytes were not the source of TNF-α, providing evidence for other sources, perhaps damaged myocytes, of circulating TNF-α following exercise (Starkie et al., 2001).

In contrast, other studies have found no changes in circulating TNF-α following exercise. Smith and colleagues (2000), reported no significant elevations in circulating TNF-α at any time-point following an acute bout of high-intensity eccentric bench press and leg curl in young untrained males. A similar study found that an acute bout of 24 maximal eccentric bicep curls did not have an effect on plasma levels of TNF-α in untrained young males despite evidence of muscle damage (Nosaka & Clarkson, 1996). I. Brenner et al. (1999) compared the impact of strenuous all-out exercise ("all-out" cycling at 90% VO_2max for 5 minutes), circuit training (3 sets of 10 repetitions of biceps curl, knee extension, hamstring curl, bench press and leg press at 70% of 1-RM), and prolonged exercise (2 h of cycling at 60-65% VO_2max) on markers of inflammation in moderately fit young (age 24.9 ± 2.3 years) males. That study observed a significant increase in plasma TNF-α levels three hours after the prolonged exercise protocol but no significant changes in TNF-α levels following circuit training or the all-out exercise effort (I.

Brenner et al., 1999). Another study examining the cytokine response to eccentric exercise in recreationally active young (ages 20-27 years) and elderly (ages 37-75 years) individuals (gender not specified) found no effect of exercise on plasma TNF-α levels for either group (Toft et al., 2002).

Several explanations have been proposed for these conflicting findings in circulating levels of TNF-α following exercise. Due to rapid renal clearance and/or peripheral uptake, TNF-α has a short half-life of 14-18 minutes, therefore any increase in circulating TNF-α may not be accounted for in studies that did not collect blood within that time-frame following exercise (Bemelmans, Van Tits, & Buurman, 1996; Davies & Hagen, 1997; Suzuki et al., 2002). For instance, Sprenger and colleagues (1992) found only borderline plasma concentrations of no statistical significance in 22 trained, long-distance runners covering a distance of 22 kilometers in less than 2 hours. Yet, urine concentrations of TNF-α were significantly elevated immediately after exercise and remained elevated for an hour after exercise (Sprenger et al., 1992). Also, some assays fail to detect TNF-α when it is bound to soluble receptors, which have been shown to be released following exercise (Ostrowski et al., 1999; Tilz et al., 1993). Soluble receptors for TNF-α may inactivate circulating TNF-α and enhance its clearance from circulation, therefore accounting for the short half-life of TNF-α and failure to detect an increase following exercise (Bemelmans et al., 1996; Jablonska, Jablonski, & Holownia, 2000; Suzuki et al., 2002). It has also been suggested that resistance-trained individuals may have a more robust TNF-α response to exercise than untrained individuals, therefore accounting for the increases found in some studies but not others (Nosaka & Clarkson, 1996; Smith et al., 2000; Townsend et al., 2013; Wells et al., 2016). Taken together, evidence suggests that the production, action, and detection of circulating TNF-α can be blocked by multiple mechanisms.

<u>TNF-α receptors</u>

The two tumor necrosis factor receptor (TNFR) molecules are TNFR1, a 55kDa receptor also called TNFR-P55; and TNFR2, a 75kDa receptor also called TNFR-P75 (Fiers, 1991). The expression of TNF-α receptors can range anywhere from 200-10,000 receptors per cell with 40% being TNFR1 and 60% being TNFR2 (murine model) (Fiers, 1991). Both of these can bind membrane-bound TNF-α as well as soluble TNF-α, however, TNFR2 can be fully activated only by mTNF-α and not by soluble TNF-α (Grell et al., 1995; Sedger & McDermott, 2014; Tartaglia et al., 1991). Mice lacking either TNFR1 or TNFR2 experience increased levels of TNF-α relative to controls in response to LPS. This observation indicates that both receptors play a role in regulating normal TNF-α levels (Peschon et al., 1998). Interestingly, there is evidence to suggest that the receptors for TNF-α provide the signal for the cellular response rather than the cytokine itself (Wallach et al., 1991). The wide range of TNF-α functions can be explained by the presence of these receptors on nearly all cell types. TNFR1 can be found on most human cells and is considered the receptor through which most of the pro-inflammatory effects are elicited; whereas TNFR2 is found on vascular endothelial cells and immune cells and has both pro- and anti-inflammatory roles (Aggarwal, 2003; MacEwan, 2002). Furthermore, some evidence suggests that TNFR2 may act as a "catcher" by binding TNF-α and delivering it to TNFR1 during low concentrations of TNF-α, given that TNFR2 has a higher affinity to its ligand (Bemelmans et al., 1996; Tartaglia et al., 1991).

Though the extracellular domains of each receptor are similar in structure and can both bind TNF-α, their intracellular domains are different in that TNFR1 contains a death domain that enables it to recruit the adaptor molecule TNFR1-associated death domain protein (TRADD) (Aggarwal, 2003; D. Brenner, Blaser, & Mak, 2015; Sedger & McDermott, 2014). TNFR2, on

the other hand, lacks the death domain and instead recruits TNFR-associated factor 1 (TRAF1) and TRAF2. A key difference in the binding of each receptor is that the TNFR2-TRAF1/TRAF2 signaling cascade promotes cell survival, while TNFR1-TRADD can result in either cell survival or cell death depending on the downstream signaling events and cellular context (D. Brenner et al., 2015). Mice deficient in TRAF2 are born with a systematically smaller muscle mass, indicating a role of TNFR2-TRAF2 in skeletal muscle development (MacLachlan & Giordano, 1998). Due to the complexity of the signaling events that occur after the binding of each molecule, predicting the outcome of TNF-α-mediated signaling is challenging.

Skeletal muscles have been shown to express both TNFR1 and TNFR2, and produce TNF-α (Li & Reid, 2001; Saghizadeh et al., 1996; Tartaglia & Goeddel, 1992). The binding of TNF-α to either TNFR1 or TNFR2 can activate nuclear factor-κB (NF-κB), however, the signaling cascades that lead from each receptor to the activation NF-κB are different (D. Brenner et al., 2015). TNF-α activation of NF-κB has been observed in differentiated myofibers; leading to reductions in total protein content and loss of adult myosin heavy chain content (Li, Schwartz, Waddell, Holloway, & Reid, 1998).

<div align="center">Soluble TNF-α Receptors</div>

Similar to the TNF-α cytokine, both TNFR1 and TNFR2 are cleaved from the cell surface by TACE, turning them into their soluble forms (STNFR1 and STNFR2) in response to stimulation of the cell (Giai et al., 2013; Gooz, 2010; Wallach et al., 1991). This process diminishes the amount of receptor available to respond to the ligand and can terminate TNF-α signaling. STNFR1 and STNFR2 retain the ability to bind to TNF-α and may function to sequester the TNF-α ligand and act as endogenous inhibitors of TNF-α (Blobel, 2005; Giai et al., 2013; Van Zee et al., 1992). In neutrophils, surface expression of both receptors decreases

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considerably when exposed to TNF-α due to both ectodomain shedding and internalization (Porteu & Nathan, 1990; Porteu & Hieblot, 1994; Schleiffenbaum & Fehr, 1990). Porteu & Hieblot (1994) found that surface expression of both TNFR1 and TNFR2 on human neutrophils is reduced when exposed to TNF-α *in vitro*, however, the mechanisms for this TNF-α-induced decrease in surface expression of each receptor differs in that it led to internalization of TNFR1 and ectodomain shedding of TNFR2. Both STNFR1 and STNFR2 are found in small amounts (1-3 ng/ml) in the serum of healthy individuals, with dramatic increases found in pathological states including: autoimmune diseases, infectious disease, and other inflammatory events (Nicod, 1993; Wallach et al., 1991). This indicates a way for cells to protect themselves from the harmful effects of excess TNF-α levels found in the serum of diseased patients (Wallach et al., 1991). One example of this is a condition known as TRAPS (TNF-receptor-associated periodic febrile syndrome). Individuals that suffer from TRAPS have genetic mutations in the cleavage site of TNFR1 that prevent or reduce ectodomain shedding and downregulation of the receptor (Blobel, 2005; McDermott et al., 1999). Consequently, TNFR1 accumulates on the cell surface and therefore the cell becomes more susceptible to TNF-α, which causes chronic fevers and inflammation (Blobel, 2005; McDermott et al., 1999).

Despite STNFR's ability to prevent overstimulation of the target cell by TNF-α, the "TNF-α-STNFR" complex remains in circulation, prolonging TNF-α's ability to bind once it's released from the complex (Aderka et al., 1992; van Deuren, 1994). Indeed, Aderka and colleagues (1992) suggested that both STNFR1 and STNFR2 may augment the effects of TNF-α by binding and preventing the denaturation of the molecule, thereby serving as a "slow release reservoir" of available TNF-α. This is of interest because, given that circulating concentrations of TNF-α decline rather quickly due to its short half-life, observing STNFR levels following muscle

damage may be of more value for monitoring the inflammatory response. The prolonged

elevation in serum STNFR following an inflammatory event may be due to either ongoing

production or slower clearance (van Deuren, 1994).

<div align="center">TNF-α receptors in exercise</div>

Research studies on the effects of exercise on TNFR expression are limited. The study by

Townsend and colleagues (2013) mentioned earlier reported a significant increase in TNFR1

expression on monocytes 30-minutes post-exercise. This increase in TNFR1 expression may

explain why there was an increase in plasma TNF-α immediately following exercise, but not at

30 minutes post-exercise (Townsend et al., 2013). Wells and colleagues (2016), on the other

hand, reported no change in TNFR1 expression on monocytes following resistance exercise

despite reporting significant increases in serum TNF-α. Human monocytes can be divided into

three subsets: classical, intermediate, and non-classical (Ziegler-Heitbrock et al., 2010). Research

suggests that intermediate monocytes express the highest surface expression of TNFR1

compared to the other monocyte subpopulations while non-classical monocytes express the

highest surface expression of TNFR2 (Hijdra, Vorselaars, Grutters, Claessen, & Rijkers, 2012).

Wells et al. (2016) observed changes in TNFR1 expression on classical monocytes only, while

Townsend et al. (2013) observed changes in TNFR1 expression on all monocyte subsets.

Therefore, it is possible that TNFR1 expression is not changed in classical monocytes as a

response to exercise, but it is in intermediate and/or in non-classical monocytes (Wells et al.,

2016).

Research studies on the effects of exercise on circulating levels of soluble TNF-α

receptors are lacking. A study mentioned earlier in this review by Ostrowski and colleagues

(1999) found that plasma concentrations of STNFR1 and STNFR2 increased 2.7- and 1.6-fold 1

hour after well-trained males ran a marathon, respectively. These increases in STNFRs paralleled the increase in circulating TNF-α, demonstrating a regulating mechanism for TNF-α (Ostrowski et al., 1999). Tilz et al. (1993) measured the plasma concentration of STNFR (specific type not known) in 18 young males with hiking experience during a hiking tour in the Austrian Alps. Compared to baseline, a significant increase in STNFR was observed 3 hours into the tour (at the top of the mountain), at the end of the tour (5 hours after the start), and 24 hours after the start of the tour. This study did not measure circulating levels of TNF-α, therefore no speculation can be made about the role of STNFRs in regulating inflammation (Tilz et al., 1993). Despite these findings, no study up to this point has observed the effects of resistance training and muscle damage on circulating levels of STNFRs.

<u>TNF-α and Aging</u>

The loss of muscle mass and function due to aging, termed sarcopenia, is linked to an increase in all-cause mortality (Cooper et al., 2010). Since skeletal muscle is the main determinant of resting metabolic rate and the primary site for glucose and fat disposal, the age-related loss of muscle mass may lead to increased adiposity, dyslipidemia, and insulin resistance (Buford et al., 2010; Greiwe et al., 2001). The age-associated decrements in muscle mass and function are a consequence of several distinct mechanisms. One such mechanism involves the immune system and its role in skeletal muscle metabolism. Aging adults are more susceptible to severe infections, take longer to recover from infections and are frequently less responsive to vaccination. This is partly due to immunosenescence, the functional deterioration of the immune system as a result of aging (Panda et al., 2009). The mechanisms underlying the age-associated detriments in immunity include decreased expression and functioning of receptors and defects in signaling pathways (Kohut & Senchina, 2004). Though T cell lymphocytes have been studied to

the greatest extent and appear to be most affected by aging, immunosenescence can affect both the adaptive and innate immune systems. Because aging leads to a functional deterioration of the innate immune system, and the innate immune system plays a significant role in recovery from exercise, immunosenescence may play a role in the age-associated loss of skeletal muscle mass and function.

<center>TNF-α and muscle loss</center>

While sarcopenia is characterized as the age-associated loss of muscle mass, cachexia is the cytokine-associated wasting of roughly equal amounts of fat and muscle mass and is associated with several pathologies such as cancer, HIV/AIDS, and rheumatoid arthritis among others (Thomas, 2007). However, elevated levels of pro-inflammatory cytokines have also been found in otherwise healthy older individuals. One study compared the plasma levels of TNF-α and TNFR2 between adults of four different age ranges and found that 100-year-olds have significantly higher levels of TNF-α and STNFR2 compared to 80-year-olds, 55-65-year-olds, and 18-30-year-olds. TNF-α and STNFR2 were also significantly higher in 80-year-olds compared to 18-30-year-olds (Bruunsgaard et al., 1999). Similar findings were observed by Rall and colleagues (1996). They found that stimulated production of TNF-α at baseline was 50% greater among healthy elderly individuals (65-80 years) compared to younger individuals (22-30 years). No differences in unstimulated production of TNF-α were observed between groups at baseline; however, the same pattern of higher production in older subjects compared with young subjects was observed (Rall et al., 1996). In muscle, TNF-α protein and mRNA content is significantly higher in frail elderly subjects compared to young subjects (Greiwe et al., 2001). Increased levels of TNF-α are associated with lower muscle mass and lower muscle strength in otherwise healthy older adults. Visser and colleagues (2002) observed a 1.2-1.3 kg decrease in

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grip strength for each standard deviation increase in plasma TNF-α. It appears that the mechanisms involved in cachexia include both reduced protein synthesis and increase protein degradation, though protein degradation is the major cause (Thomas, 2007).

Overnight infusion of murine TNF-α *in vivo* has been shown to reduce circulating levels of IGF-1, as well as decrease the local content of IGF-I in skeletal muscle in fasted rats (Fan, Char, Bagby, Gelato, & Lang, 1995). The infusion paradigm used in that study was chosen to simulate the relatively low levels of TNF-α detected in the circulation of humans during chronic inflammation or other catabolic illnesses. The TNF-α-induced decrease in IGF-1 may be responsible for the increase in protein degradation and reduced protein synthesis seen in conditions involving the overproduction of pro-inflammatory cytokines (Fan et al., 1995). Acute infusion of TNF-α in rats has also been shown to decrease muscle protein synthesis and increase protein breakdown (García-Martínez, López-Soriano, & Argilés, 1993). Frost and colleagues (1997) demonstrated that TNF-α directly inhibits protein synthesis in human myoblasts and myotubes; both at the basal level and when stimulated by IGF-1. Greiwe and colleagues (2001) reported an inverse relationship between muscle protein synthesis and muscle TNF-α protein content in frail elderly adults. The investigators also observed that resistance training coordinately decreased skeletal muscle TNF-α content and increased protein synthesis rate. These findings supports the hypothesis that elevated levels of TNF-α may negatively impact muscle hypertrophy, and that the positive effects of exercise on the elderly population may involve alterations in the concentration of a potentially detrimental cytokine in muscle (Greiwe et al., 2001).

Very few research studies have compared the acute response of TNF-α to exercise in older adults compared to younger adults. In a study by Hamada and colleagues (2005), there was

a trend toward a blunted accumulation of TNF-α in the muscle of the elderly men compared to the young men following an acute bout of eccentric exercise during the repair phase of muscle damage. Likewise, an age-associated decrease in leukocyte recruitment was reported in this study. Interestingly, the older group saw a significantly greater increase in IL-1ß gene expression compared to the younger group, suggesting a compensatory role of IL-1ß produced in muscle for the age-associated reduction of TNF-α (Hamada et al., 2005).

Conclusions

Despite our current knowledge of TNF-α's role in skeletal muscle regeneration following muscle damage, whether or not aging affects this response is unclear. Furthermore, although higher plasma concentrations of TNF-α are associated with lower muscle mass and lower muscle strength in healthy older men and may contribute to sarcopenia, whether this relationship is apparent earlier in life (middle-age) is unknown. Also, the relationship between STNFR1 and STNFR2 and muscle mass and strength has not been determined. Therefore, the purpose of the current study was to compare changes in plasma levels of TNF-α and STNFRs in response to muscle damage in young and middle-aged males. The relationship between circulating levels of TNF-α, STNFR1, and STNFR2 and isokinetic strength following muscle damage was also examined. A secondary purpose was to examine the relationship between baseline levels of TNF-α, STNFR1 and STNFR2 and muscle size and strength in middle-aged males to determine if TNF-α levels may indicate early signs of muscle wasting related to aging. We hypothesized that middle-aged males would show higher baseline levels of TNF-α, STNFR1, and STNFR2 compared to young males. We expected to see a negative relationship between TNF-α/STNFRs and muscle size/strength. We also anticipated a significant increase in circulating levels of TNF-α, STNFR1, and STNFR2 in both groups, though this response would be blunted in the middle-

aged group compared to the young group. Lastly, the middle-aged group would take longer to

recover their strength following muscle damage compared to the young group.

CHAPTER THREE: METHODOLOGY

Participants

Using the procedures described by Beck (2013), a minimum sample size of nine participants per group produced a statistical power $(1-\beta)$ of 0.80 at an alpha level of 0.05. Power calculations were made using G*Power statistical analyses software (Version 3.1.9.2, Düsseldorf, Germany) and were based upon an effect size (dz) of 1.51 generated from changes in plasma TNF-α from baseline to 30-minutes post resistance exercise reported by Wells et al. (2016) for the volume group. Twenty-five recreationally active males were recruited to participate in this experimental study. Study participants were recruited into two groups based on age. The young-age group (YA; N=12) consisted of males between the ages of 18 – 30 while the middle-aged group (MA; N=13) consisted of males between the ages of 40 – 60 years. Inclusion criteria required participants to be recreationally active during the past 6 months as defined by the American College of Sports Medicine (150 minutes of exercise/week) including resistance exercise (Garber et al., 2011). All participants were free of any physical limitations that may have affected performance. Additionally, all participants were free of any medications, performance enhancing drugs, and any dietary supplements that have antioxidant or recovery properties (creatine, beta-alanine etc.) as determined by a health and activity questionnaire. Following an explanation of all procedures, risks and benefits, each participant provided his informed consent prior to participation in this study. The research protocol and the informed consent document were approved by the New England Institutional Review Board prior to participant enrollment (NEIRB# 120160966, Approved 20 January 2017).

Study Design

Both groups reported to the human performance laboratory (HPL) on four separate occasions (Figure 3). On the first visit (T1), participants were assessed for anthropometrics including height, weight, and body fat percentage. Following anthropometric assessment, participants performed a standardized warm-up. After the warm-up, the participants completed a familiarization protocol on the isokinetic device with the purpose of familiarizing the participants with the motions involved in the lower body performance assessment. On the second visit (T2), participants arrived at the HPL following a 10-hour fast and completed baseline (BL) testing, which included an ultrasound assessment, a blood draw, and a lower body performance assessment. After a brief rest period, participants then completed a muscle damaging protocol (MDP). Immediately after the MDP (IP), participants again completed the lower body performance assessment to determine decrements in performance as a result of the MDP. Follow-up lower body performance assessments were also conducted 120-minutes (120P), 24-hours (24H), and 48-hours (48H) post-MDP. Follow-up blood samples were collected from each participant at IP, 30-minutes (30P), 60-minutes (60P), 120P, 24H, and 48H post-MDP.
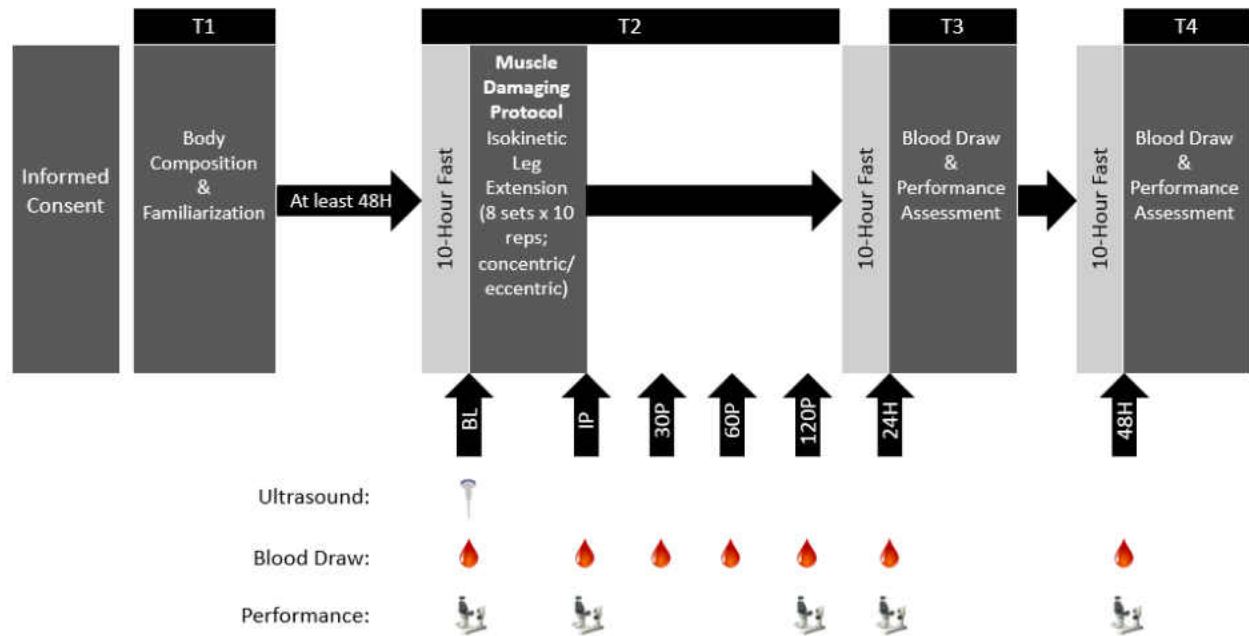
Figure 3: Study Design

Participants were assessed for anthropometrics and completed a familiarization protocol at least 48 hours prior to the muscle damaging protocol (T1). On visit 2 (T2), participants completed a muscle damaging protocol, provided blood samples at baseline (BL), immediately post- (IP), 30 minutes post- (30P), 60 minutes post- (60P), 120 minutes post- (120P), 24 hours (24H) post-, and 48 hours (48H) post-muscle damaging protocol. An ultrasound assessment was completed at BL. Lower body performance testing was completed at BL, IP, 120P, 24H, and 48H.

Procedures

Dietary Recall

Participants were asked to provide a 3-day dietary recall beginning on the day prior to T2 through T3 while maintaining the same caloric intake, macronutrient proportions and nutrient timing for the duration of the investigation. FoodWorks nutrient analysis software (McGraw-Hill, New York, N.Y., USA) was used to analyze the self-reported dietary recalls for total kilocalorie intake and macronutrient proportions (carbohydrate, protein, and fat). Additionally,

participants were asked to abstain from any nonsteroidal anti-inflammatory drugs, cryotherapy, or any other agents that may affect inflammation.

Anthropometric Measurements

At T1, body mass (±0.1 kg) and height (±0.1 cm) were measured using a Health-o-meter Professional scale (Patient Weighing Scale, Model 500 KL, Pelstar, Alsip, IL, USA). Body composition was assessed using a direct segmental multi-frequency bioelectrical impedance analyzer (BIA) via InBody (Model 770, InBody Co., Ltd, Cerritos, CA, USA) according to the manufacture's guidelines. BIA estimates body composition using the conductivity differences of the various tissues due to their tissue characteristics (water and electrolyte content). This analyzer processes 30 impedance measurements by using six different frequencies (1, 5, 50, 250, 500, 1000 kHz) at each of five segments of the body (right arm, left arm, trunk, right leg, left leg) using tetrapolar 8-point tactile electrodes (Kurinami et al., 2016). Participants were asked to remove all jewelry and footwear, including socks; and to void the bladder before performing the test. This assessment required the participants to wipe their hands and feet with an InBody tissue to enhance electrical conductivity and reduce surface bacteria before being assessed. The participants were then instructed to stand on the InBody platform electrodes while holding two hand electrodes out to the side. They held this position for one minute as the device conducted the electrical currents through the body to determine body composition. Values for total body fat percentage were recorded.

Familiarization

Following anthropometric testing, participants performed a familiarization protocol to become acquainted with the Biodex S4 isokinetic dynamometer (Biodex Medical System, Inc.,

New York, NY, USA). Prior to the familiarization protocol, participants performed a standardized warm-up consisting of five minutes on a cycle ergometer against a light resistance. Following the warm-up, participants were seated in the isokinetic dynamometer, positioned with a hip angle of 110° and strapped into the chair at the waist, shoulders, and across the thigh (Beyer et al., 2016). The chair and dynamometer settings were adjusted for each participant to properly align the axis of rotation with the lateral condyle of the femur. All participants were tested on their right leg which was secured to the dynamometer arm just above the medial and lateral malleoli. Isokinetic dynamometer settings for each individual were recorded and remained consistent throughout the study. The familiarization protocol included two maximal voluntary isometric contractions (MVIC) at a knee angle of 110° of extension (180° representing full extension); three sets of three repetitions of isokinetic concentric contractions at angular velocities of 60°/sec, 240°/sec, and 180°/sec, respectively; and 1 set of 10 repetitions of isokinetic concentric/eccentric contractions at angular velocities of 60°/sec for both the concentric and eccentric phase.

Ultrasound Assessment

Participants were assessed at BL for muscle size and quality using noninvasive ultrasonography of the rectus femoris (RF) muscle. Participants were asked to lie supine on an examination table for a minimum of fifteen minutes to allow for consistent muscle measurements (Berg, Tedner, & Tesch, 1993). The participants remained in a supine position, with their legs extended and a towel placed underneath the popliteal fossa of the right leg, allowing for a slight bend at the knee (Mangine et al., 2015). Ultrasound measurements were made using a 12-MHz linear probe (General Electric LOGIQe, Wauwatosa, WI, USA) coated with a water-soluble ultrasound gel (Aquasonic Clear, Parker Laboratories Inc., Fairfield, NJ, USA). Images were

captured at 50% of the straight line distance between the anterior inferior iliac crest and proximal border of the patella with gain set at 50 dB, dynamic range set at 72, and depth fixed at 6 cm (Boone, Stout, Beyer, Fukuda, & Hoffman, 2015; Mangine et al., 2015). Cross-sectional area (CSA) of the RF was measured using transverse panoramic images, while muscle thickness (MT) was measured using longitudinal still images. To ensure consistency, the same technician positioned each participant, collected all images, and analyzed each image.

ImageJ software (National Institute of Health, USA, version 1.48) was utilized to analyze the ultrasound images using a known distance of 1 cm on the ultrasound image to calibrate the software (Wells et al., 2014). To measure CSA, the polygon tool of the ImageJ software was used to trace the outline of the muscle from the transverse panoramic image while avoiding the surrounding bone and fascia. The ImageJ software calculated the area ($\pm$ 0.1 cm^2) contained within the traced muscle image. Echo intensity (EI) was measured from the transverse panoramic images using the standard histogram function, which measures the greyscale of each pixel in arbitrary units (AU). EI may provide a measure of muscle 'quality' by delineating the ratio of contractile to non-contractile tissues within the ultrasound image (Fukumoto et al., 2012). MT was determined as the straight-line distance ($\pm$0.1 cm) between the superior border of the deep aponeurosis and the inferior border of the superficial aponeurosis. Three images of each measurement were taken, and the mean of the three measurements was recorded. Intra-class correlation coefficients for the ultrasound technician were determined from a repeated-measures analysis of 10 individuals separated by 24 hours [CSA= 0.96, SE = 0.78 cm^2; MT = 0.94, SE = 0.21 cm; EI= 0.94, SE= 3.88 AU].

Blood Measurements

Blood samples were collected at seven time points throughout the study (BL, IP, 30P, 60P, 120P, 24H, and 48H). The blood samples on T2 (BL, IP, 30P, 60P and 120P) were obtained using a Teflon cannula placed in a superficial forearm vein and plastic syringe. The cannula was maintained patent using a non-heparinized isotonic saline solution (Becton Dickinson, Franklin Lakes, NJ, USA). The blood samples from 24H and 48H were collected using a 21 gauge, 1 ¼ inch Vacutainer® blood collection needle. Participants were instructed to lie supine for 15 minutes prior to each blood draw.

A total of 20 mL of blood was collected at each time-point in two, 10 mL Vacutainer® tubes: one containing K_2EDTA, which is an anticoagulant to prevent the blood from clotting, and a serum tube where the blood was allowed to clot. An aliquot of whole blood from the K_2EDTA tube was immediately utilized to assess hematocrit, hemoglobin, and red blood cell count. The remaining blood in the EDTA tube was subsequently centrifuged at 4, 000g for 15 minutes. Blood in the serum tube was allowed to clot at room temperature before being centrifuged. The resulting plasma and serum samples were placed into separate 1.8-mL microcentrifuge tubes and frozen at -80° C for later analysis.

Lower-Body Performance

The lower body performance assessment was performed on five occasions throughout the study (BL, IP, 120P, 24H, and 48H) and was composed of 1 set of 3 repetitions of isokinetic contractions. The isokinetic contractions were concentric knee extensions at an angular velocity of 180°/sec. Values of peak torque (PTQ), mean torque (mTQ), peak power (PP), and mean power (mP) were recorded for each contraction at each time point. The lever arm of the

dynamometer was programmed to extend the participant's leg from 95° of knee flexion to 155° of knee flexion (where 180° is full extension).

Muscle Damaging Protocol

After completing the initial lower-body performance assessment, participants were seated in the isokinetic dynamometer chair. Following a five-minute rest, participants performed the MDP. The MDP was composed of 8 sets of 10 repetitions, consisting of concentric knee extension (60°/sec) and eccentric knee flexion (60°/sec). The range of motion for each repetition of the MDP was from 95° of knee flexion to 155° of knee flexion. Participants were instructed to give maximal effort during this protocol. Participants were provided with one minute of rest between each set of the MDP. The lower body performance assessment at IP was performed five minutes after the conclusion of the MDP.

Biochemical Analysis

Blood lactate concentrations were analyzed from plasma using an automated analyzer (Analox GM7 enzymatic metabolite analyzer, Analox instruments USA, Lunenburg, MA, USA). Hematocrit and hemoglobin were analyzed in whole blood using a complete blood cell counter (Coulter ® AC ●T diff 2™ hematology analyzer). Plasma volume shifts were calculated using the formula established by Dill & Costill (1974). To eliminate inter-assay variance, all samples were analyzed in duplicate by a single technician. Coefficient of variation for each assay was 2.04% for blood lactate; 1.63% for hematocrit; and 1.10% for hemoglobin.

Serum concentrations of myoglobin (Calbiotech, Spring Valley, CA, USA), STNFR1, and STNFR2 (RayBiotech, Inc, GA, USA) were obtained via commercially available enzyme-linked immunosorbent assay (ELISA); while creatine kinase (CK) was analyzed using a

commercially available kinetic assay (Sekisui Diagnostics, Charlottetown, PE, Canada), per manufacturer's instructions. To limit inter-assay variability, all samples for a particular assay were thawed once, and analyzed in duplicate by the same technician using a BioTek Eon spectrophotometer (BioTek, Winooski, VT, USA). Coefficients of variation for each assay were 5.03% for myoglobin; 4.50% for CK; 2.91% for STNFR1; and 2.34% for STNFR2.

Plasma concentrations of TNF-α were analyzed via high-sensitivity cytokine multiplex assay (Luminex., Cat no. FCSTM09-02; R&D systems, Inc. Minneapolis, MN, USA) on a MAGPIX instrument (Luminex, Austin, TX), according to the manufacturer's instructions. All samples were run in duplicate with a mean intra-assay coefficient of variance of 3.32%.

Statistical Analysis

Prior to statistical procedures, all data was assessed for sphericity. If the assumption of sphericity was violated, a Greenhouse-Geisser correction was applied. Biochemical and performance changes were analyzed using a two factor (time x group) repeated measures analysis of variance (ANOVA). In the event of a significant F ratio, least significant difference (LSD) post-hoc analysis were used for pairwise comparisons. If a significant interaction was observed, follow-up one way repeated measures ANOVA were used to determine time effects for each treatment and independent t-tests were used for pairwise comparisons between groups at each time point. Comparisons between treatments were further analyzed using Cohen's d. Magnitudes of the standardized effects were interpreted using thresholds of <0.2, 0.2-0.6, 0.6-1.2, 1.2-2.0, 2.0-4.0. These values correspond to trivial, small, moderate, large and very large effect size (ES), respectively. Time effects were further analyzed using partial eta squared (η^2_p). Interpretations of η^2_p were evaluated in accordance with Cohen (1988) at the following levels: small effect (0.01-0.058), medium effect (0.059-0.137) and large effect (> 0.138). The net area

under the curve (AUC) was also calculated for biochemical measures from BL-120P using a standard trapezoidal technique, and was assessed using independent t-tests. Additionally, Pearson product moment correlations were calculated to examine selected bivariate relationships between biochemical markers, performance variables, age, and muscle morphological characteristics. Significance was accepted at an alpha level of $p \leq 0.05$. Data were analyzed using IBM SPSS Statistics for Windows (version 21.0; IBM Corp., Armonk, N.Y., USA). All data are reported as mean $\pm$ SD, unless otherwise stated.

CHAPTER FOUR: RESULTS

Participant Characteristics

Following an explanation of all procedures, risks and benefits, a total of 25 participants were enrolled in this study (YA = 12; MA = 13). Five participants were removed from the investigation prior to analysis (YA= 2; MA= 3). Of the five participants that were removed, two participants discontinued testing following the familiarization protocol due to knee discomfort (YA= 1, MA= 1), two reported recent or current consumption of recovery-enhancing supplements after being enrolled but prior to testing (YA= 1, MA=1), and one reported a metabolic disease after being enrolled but prior to testing (MA group). One participant completed testing but was excluded from the analysis due to indications of muscle damage unrelated to the intervention at baseline (YA group). Therefore, 9 participants were included in the analysis for YA (21.8 ± 2.2 y, 179.5 ± 4.9 cm, 91.2 ± 12.2 kg, $21.8 \pm 4.3\%$ BF) and 10 for MA (47.0 ± 4.4 y, 176.8 ± 7.6 cm; 96.0 ± 21.5 kg, $25.4 \pm 5.3\%$ BF) for a total of 19 participants. A graphical schematic of this is shown in Figure 4. Characteristics of the participants included in the analysis are listed in Table 1. Aside from age, no significant differences were observed between groups for participant characteristics.
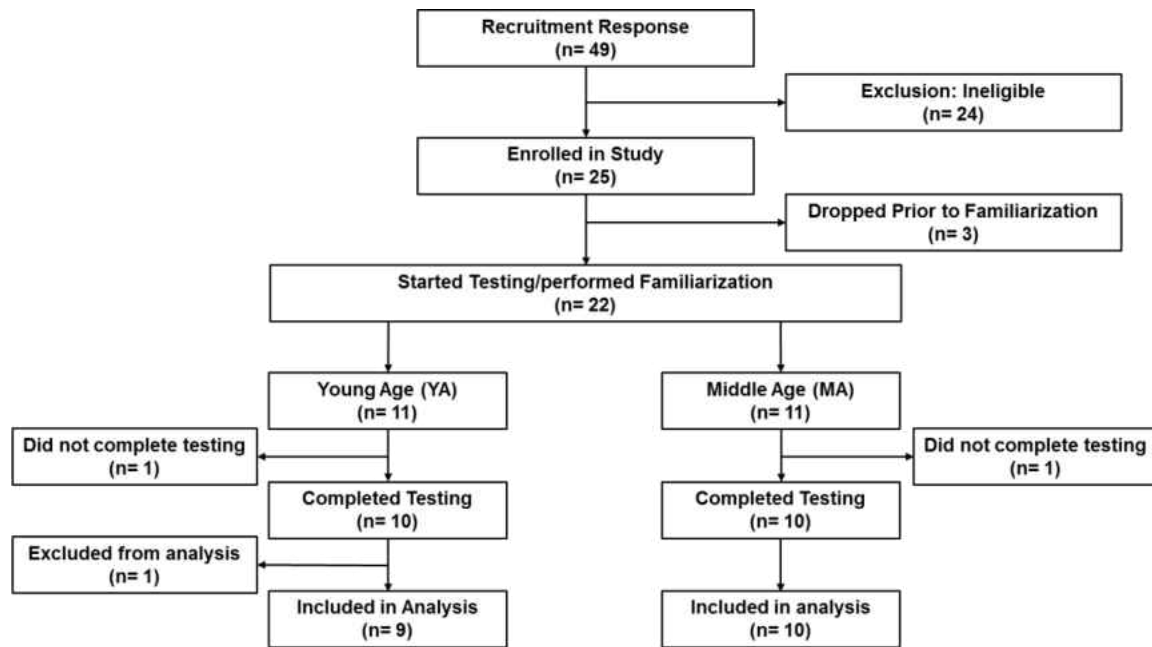
Figure 4: Participant recruitment
    Participant recruitment and enrollment.

Table 1: Participant Characteristics

Characteristics	Young Age (YA)	Middle-Aged (MA)	*p*
n	9	10	N/A
Age *(Yrs)*	21.8 (2.2)	47.0 (4.4)	< 0.001*
Height *(cm)*	179.5 (4.9)	176.8 (7.6)	0.39
Weight *(kg)*	91.2 (12.2)	96.0 (21.5)	0.559
Body Fat *(%)*	21.8 (4.3)	25.4 (5.3)	0.126
MT *(cm)*	2.62 (0.39)	2.37 (0.40)	0.182
CSA *(cm²)*	13.83 (2.32)	14.26 (1.61)	0.642
EI *(AU)*	84.68 (11.60)	95.23 (11.30)	0.061

Participant characteristics are shown as mean (SD). Muscle thickness (MT), cross-sectional area (CSA), and echo intensity (EI) of the rectus femoris were measured at baseline.
*Significant group difference ($p < 0.05$).

<u>Dietary Intake</u>

Dietary intake is presented in Table 2. No time x group interaction, main effect for group, or main effect for time was noted for caloric intake or macronutrient intake over the course of the three days (p's > 0.05). No significant differences were observed in mean or total caloric intake or any macronutrient intake (p's > 0.05).

Table 2: Dietary Intake

	Day 1	Day 2	Day 3	Mean	Total
Caloric Intake (kcal)					
YA (n=9)	2361.4 (820.0)	1836.0 (757.0)	2031.1 (757.0)	2076.2 (542.2)	6228.5 (1635.5)
MA (n=10)	1912.4 (862.0)	2019.5 (628.7)	1994.0 (516.7)	1975.3 (520.4)	5925.9 (1561.2)
Protein Intake (g)					
YA (n=9)	141.9 (70.8)	90.6 (50.1)	98.0 (62.8)	110.2 (43.8)	330.5 (131.5)
MA (n=10)	90.8 (39.5)	100.0 (29.9)	93.5 (23.7)	94.8 (21.6)	284.3 (64.7)
Carbohydrate Intake (g)					
YA (n=9)	220.4 (93.4)	206.3 (85.7)	234.3 (101.2)	220.3 (70.2)	661.0 (210.6)
MA (n=10)	224.5 (120.6)	207.6 (71.2)	242.4 (100.1)	224.8 (69.1)	674.5 (207.2)
Fat Intake (g)					
YA (n=9)	88.5 (56.1)	73.0 (37.5)	66.6 (25.9)	76.0 (27.4)	228.1 (82.2)
MA (n=10)	70.9 (48.4)	85.9 (44.2)	79.0 (32.8)	78.6 (32.9)	235.8 (98.6)

Groups: *YA* = Young Age; *MA* = Middle-Aged. Participants provided a 3-day dietary recall beginning on the day prior to T2 (Day 1) through T3 (Day 3). Daily, mean, and total caloric intake, protein intake, carbohydrate intake, and fat intake were analyzed and are shown as mean (SD).

Total Work Done

Total work done during MDP is shown in Figure 5. No significant difference was observed between groups on total work done during MDP ($p = 0.994$).
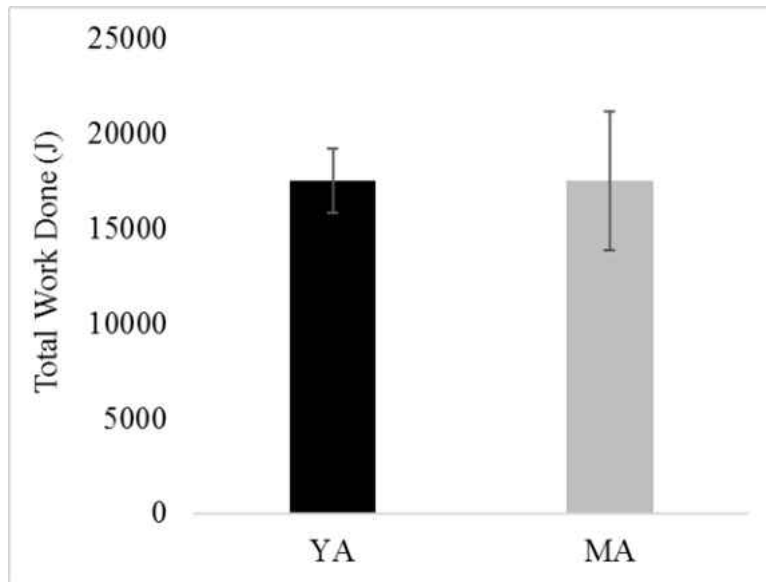
Figure 5: Total Work Done during MDP
Total work done during the muscle damaging protocol (MDP) was analyzed and is reported in joules (J) as means ± SD.

Peak Torque and Mean Torque

Changes in PTQ and mTQ in response to MDP are depicted in Figure 6. No significant group x time interaction ($p = 0.068$) or main group effect ($p = 0.071$) was observed for PTQ. When collapsed across groups, a significant time effect was observed (F = 14.3; $p < 0.001$; $\eta^2_p = 0.457$). With both groups combined, significant decreases in PTQ were observed at IP ($p < 0.001$), 120P ($p = 0.016$), 24H ($p = 0.012$) and 48H ($p = 0.006$) relative to BL.

A significant group x time interaction was observed for mTQ (F = 3.2; $p = 0.019$; $\eta^2_p = 0.156$). Significant decreases in mTQ were observed at IP ($p = 0.001$), 120P ($p = 0.031$), and 48H ($p = 0.046$) relative to BL for the YA group. Significant decreases were observed at IP ($p < 0.001$) and 24H ($p = 0.035$) relative to BL for the MA group. A significantly higher mTQ was observed in the YA group at BL compared to the MA group ($p = 0.050$). No other between group differences were noted.
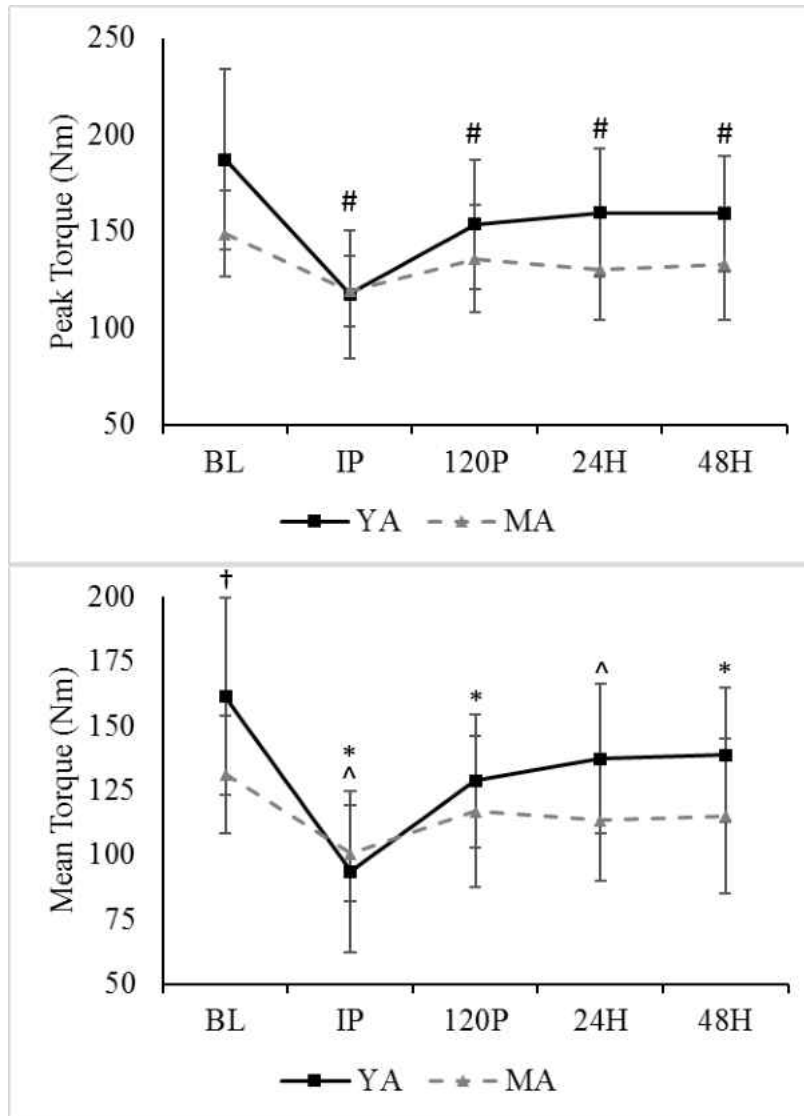
Figure 6: Changes in Peak Torque and Mean Torque

Young age (YA) and Middle-Aged (MA) groups were analyzed for changes in peak torque (PTQ) and mean torque (mTQ). Values are shown as newton meters (NM) at baseline (BL), immediately post- (IP), 120 minutes post-(120P), 24 hours (24H) post-, and 48 hours (48H) post-muscle damaging protocol. Data shown as means ± SD.

\# Significantly different from BL when collapsed across groups ($p < 0.05$)

† Significant difference between YA and MA

* Significantly different from BL for YA ($p < 0.05$)

^ Significantly different from BL for MA ($p < 0.05$)

Peak Power and Mean Power

Changes in PP and mP in response to MDP are depicted in Figure 7. No significant group x time interaction ($p = 0.156$) or main effect for group ($p = 0.116$) was observed for PP. When collapsed across groups, a significant time effect was observed for PP (F = 10.4; $p < 0.001$; $\eta^2_p = 0.379$). With both groups combined, a significant decrease in PP was observed at IP ($p < 0.001$) relative to BL.

No significant group x time interaction ($p = 0.093$) or main effect for group ($p = 0.208$) was observed for mP. When collapsed across groups, a significant time effect was observed for mP (F = 13.1; $p < 0.001$; $\eta^2_p = 0.434$). With both groups combined, significant decreases in mP were observed at IP ($p < 0.001$) and 120P ($p = 0.025$) relative to BL.
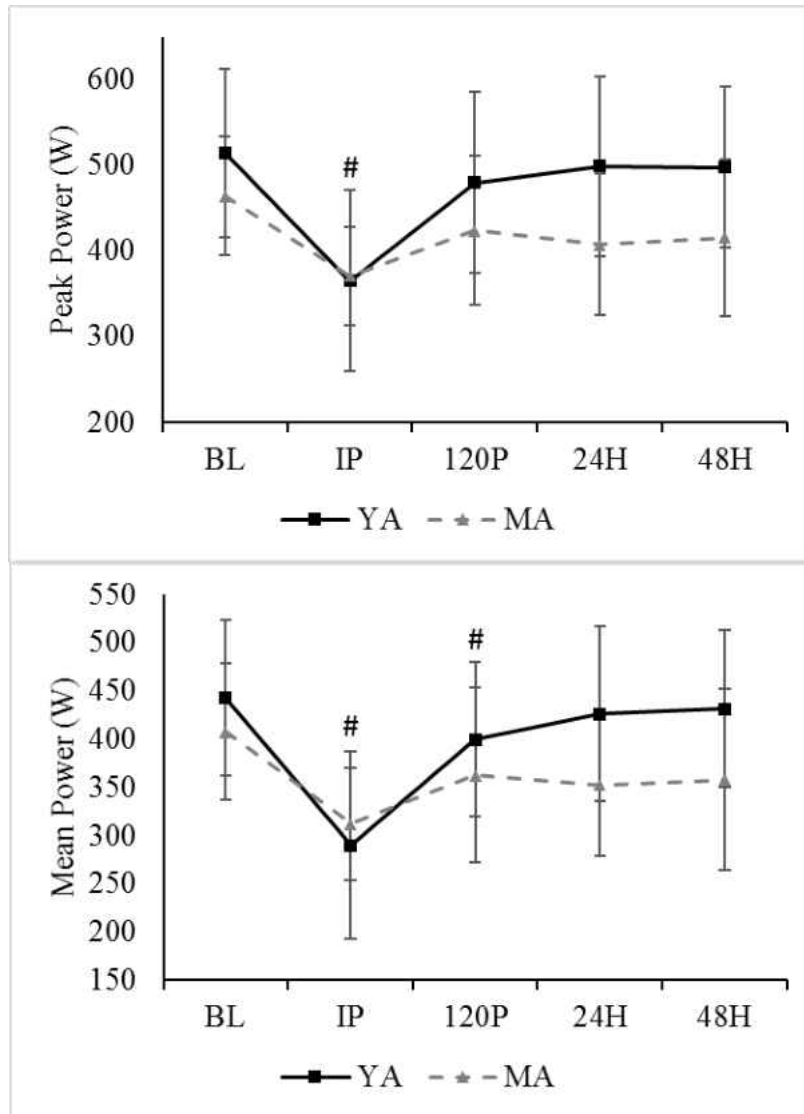
Figure 7: Changes in Peak Power and Mean Power
Young age (YA) and Middle-Aged (MA) groups were analyzed for changes in peak power (PP) and mean power (mP). Values are shown as watts (W) at baseline (BL), immediately post- (IP), 120 minutes post-(120P), 24 hours (24H) post-, and 48 hours (48H) post-muscle damaging protocol. Data shown as means ± SD.
Significantly different from BL when collapsed across groups ($p < 0.05$)

<center>Biochemical Analysis</center>

<center>Lactate</center>

Changes in plasma lactate concentrations are shown in Table 3. No significant time x group interaction ($p = 0.215$) or main effect for group was noted ($p = 0.096$); however, when collapsed across groups, a significant time effect was observed (F = 51.1; $p < 0.001$; $\eta^2_p = 0.773$). With both groups combined, significant elevations in plasma lactate concentrations were observed at IP ($p < 0.001$) and 30P ($p = 0.003$) relative to BL. AUC analysis revealed no significant difference between groups ($p = 0.362$)

Table 3: Changes in lactate concentrations

	Lactate (mmol/L)			
Group	BL	IP	30P	60P
YA	1.76 ± 0.53	$4.91 \pm 1.80\ddagger$	$2.59 \pm 0.82\ddagger$	1.79 ± 0.32
MA	1.54 ± 0.44	$3.77 \pm 1.10\ddagger$	$2.13 \pm 0.43\ddagger$	1.63 ± 0.33

Plasma lactate concentrations before (BL), immediately post- (IP), 30 minutes post- (30P), and 60 minutes post- (60P) muscle damaging protocol in the young age (YA) and middle-aged (MA) groups. ‡ = Significant increase relative to BL when groups are combined ($p < 0.05$). Data shown as means ± SD.

Myoglobin

Changes in circulating myoglobin concentrations are presented in Figure 8. No significant time x group interaction ($p = 0.640$) or main effect for group was noted ($p = 0.641$); however, a significant time effect was observed for circulating myoglobin concentrations (F = 8.7; $p = 0.005$; $\eta^2_p = 0.339$). With both groups combined, plasma myoglobin was significantly higher at 30P ($p = 0.002$), 60P ($p = 0.001$), and 120P ($p = 0.007$) relative to BL. AUC analysis revealed no significant differences between groups ($p = 0.710$)
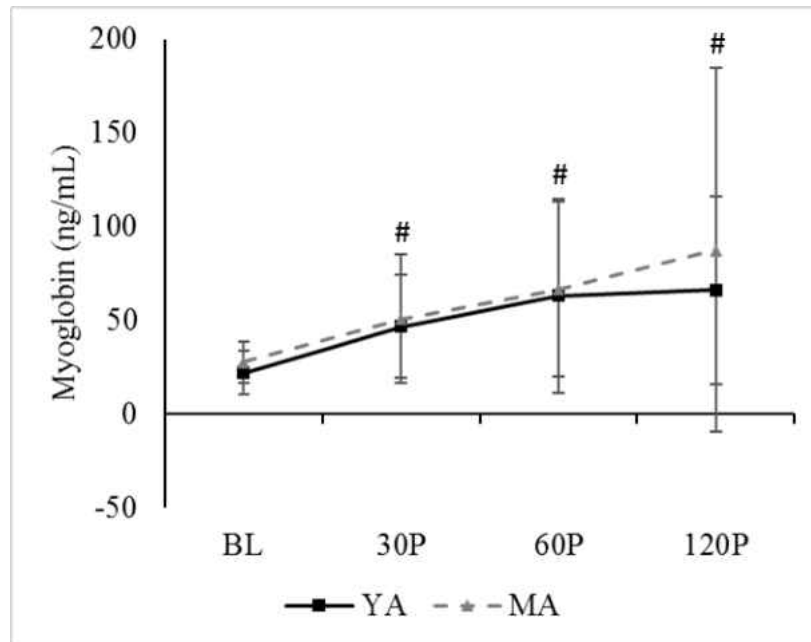
Figure 8: Changes in Myoglobin Concentrations
Plasma myoglobin concentrations before (BL), 30 minutes post- (30P), 60 minutes post- (60P), and 120 minutes post- (120P) muscle damaging protocol in the young age (YA) and middle-aged (MA) groups. Data shown as means ± SD.
Significantly different from BL when collapsed across groups (p < 0.05)

Creatine Kinase

Changes in circulating CK concentrations are presented in Figure 9. No significant time x group interaction ($p = 0.621$) or main effect for group was noted ($p = 0.654$). A significant time effect was observed for circulating CK concentrations (F = 8.2; $p = 0.001$; $\eta^2_p = 0.328$). With both groups combined, circulating CK was significantly higher at 24H ($p = 0.001$) and 48H ($p = 0.005$) relative to BL.
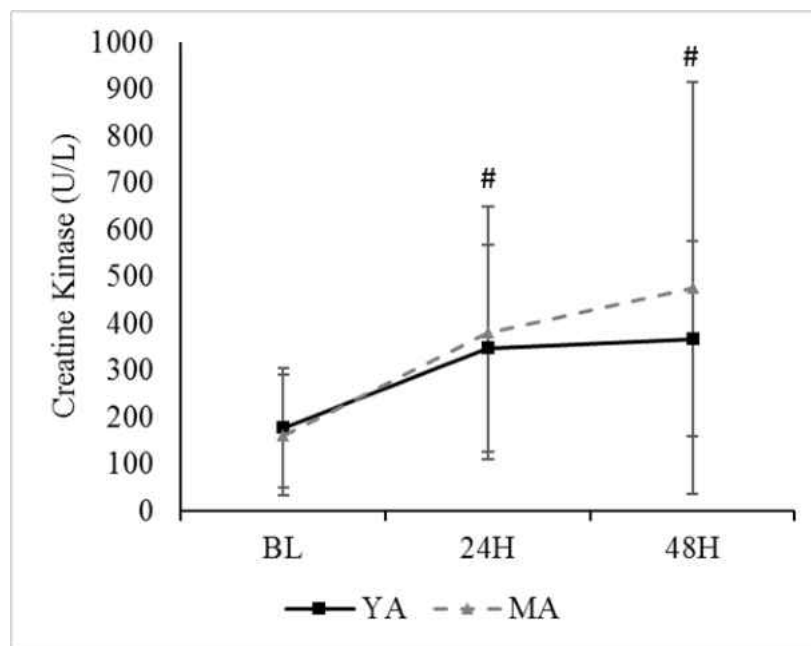
Figure 9: Changes in Creatine Kinase Concentrations
   Serum creatine kinase concentrations before (BL), 24 hours (24H) post-, and 48 hours (48H) post- muscle damaging protocol in the young age (YA) and middle-aged (MA) groups. Data shown as means ± SD.
   # Significantly different from BL when collapsed across groups (p < 0.05)

TNF-α

Changes in plasma TNF-α in response to MDP are depicted in Figure 10. No time x group interaction, main effect for group, or main effect for time was noted (p's > 0.05). TNF-α AUC is presented in Figure 11. AUC analysis revealed no significant differences between groups ($p = 0.289$).

Figure 10: Changes in plasma TNF-α
Plasma TNF-α concentrations before (BL), immediately post- (IP), 30 minutes post- (30P), 60 minutes post- (60P), 120 minutes post- (120P), 24 hours (24H) post-, and 48 hours (48H) post-muscle damaging protocol in the young age (YA) and middle-aged (MA) groups. Data shown as means ± SD.
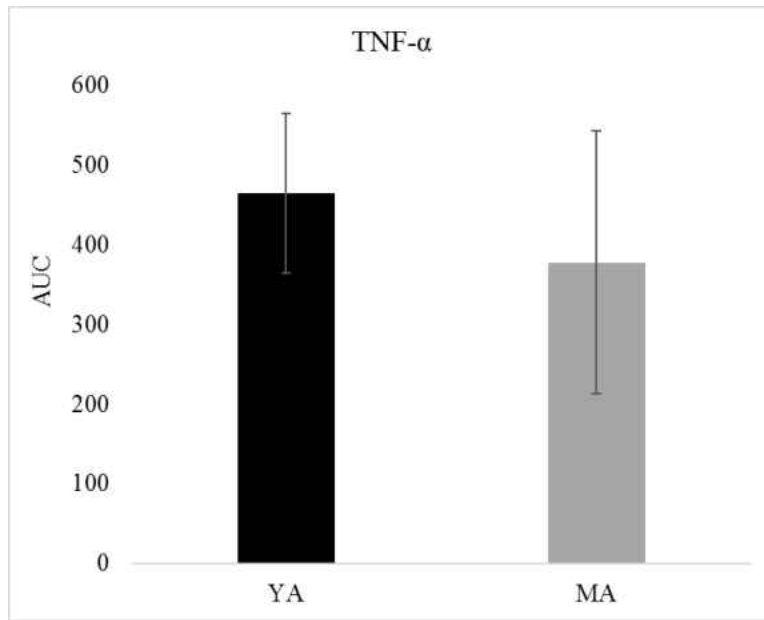
Figure 11: Tumor Necrosis Factor-alpha (TNF-α) area under the curve (AUC)
Groups: *YA* = Young Age; *MA* = Middle-Aged. Data reported as means ± SD.

STNFR1

Changes in serum STNFR1 in response to MDP are shown in Figure 12. No significant

group x time interaction ($p = 0.175$) or main effect for group ($p = 0.052$) was observed for serum

STNFR1. When collapsed across groups, a significant time effect was observed (F = 6.2; $p =$

0.001; $\eta^2_p = 0.294$). With both groups combined, STNFR1 significantly decreased from BL at

30P ($p = 0.001$), and was significantly increased relative to BL at 48H ($p = 0.028$). STNFR1

AUC is presented in Figure 13. AUC analysis revealed no significant differences between groups

($p = 0.170$).
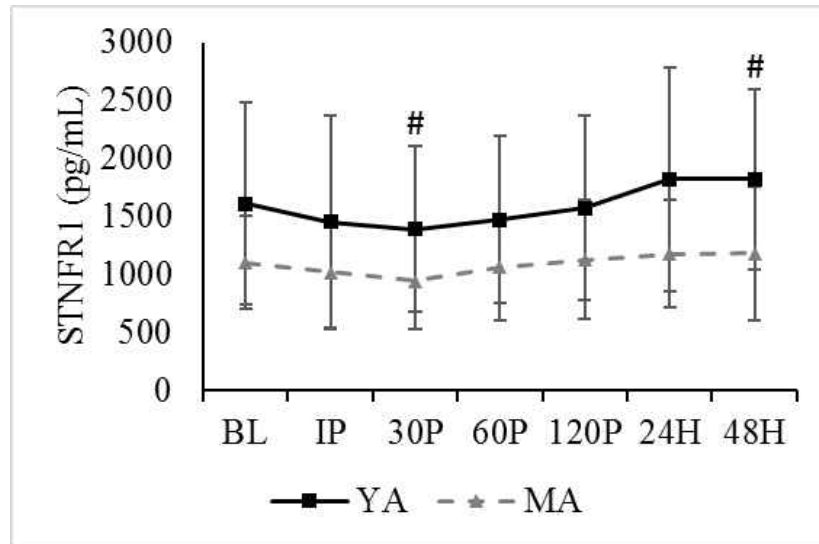
Figure 12: Changes in serum STNFR1
Serum STNFR1 concentrations before (BL), immediately post- (IP), 30 minutes post-
(30P), 60 minutes post- (60P), 120 minutes post- (120P), 24 hours (24H) post-, and 48
hours (48H) post-muscle damaging protocol in the young age (YA) and middle-aged
(MA) groups. Data shown as means ± SD.
Significantly different from BL when collapsed across groups ($p < 0.05$)
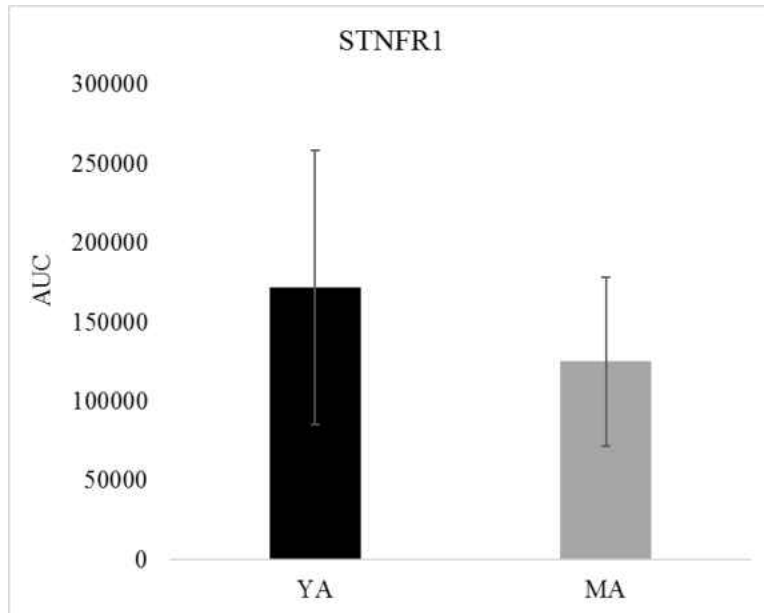
Figure 13: Soluble Tumor Necrosis Factor-alpha Receptor 1 (STNFR1) area under the curve (AUC)
Groups: *YA* = Young Age; *MA* = Middle-Aged. Data reported as means ± SD.

Changes in serum STNFR2 in response to MDP are shown in Figure 14. No significant group x time interaction ($p = 0.213$) or main effect for group ($p = 0.057$) was observed for serum STNFR2. When collapsed across groups, a significant time effect was observed (F = 6.2; $p = 0.013$; $\eta^2_p = 0.292$). With both groups combined, STNFR2 significantly decreased from BL at 30P ($p = 0.008$), 60P ($p = 0.003$), and 120P ($p = 0.002$). STNFR2 AUC is presented in Figure 15. AUC analysis revealed no significant differences between groups ($p = 0.139$).
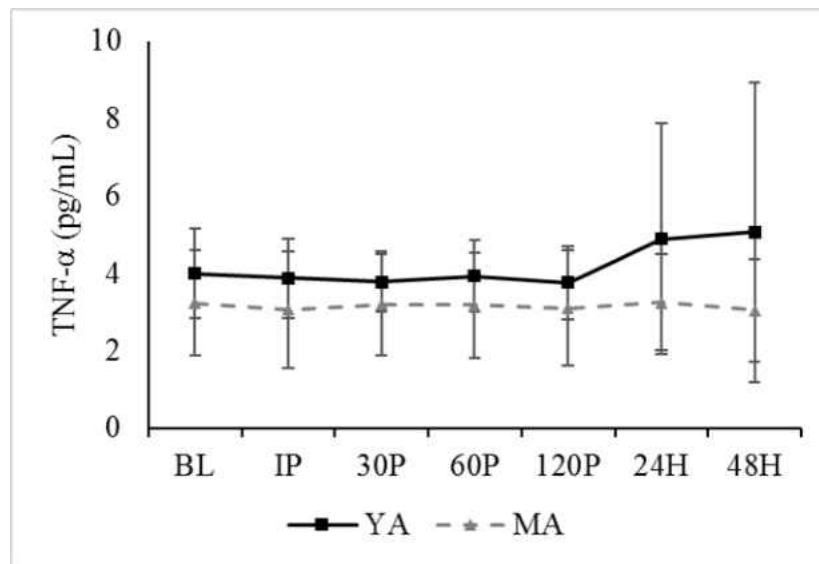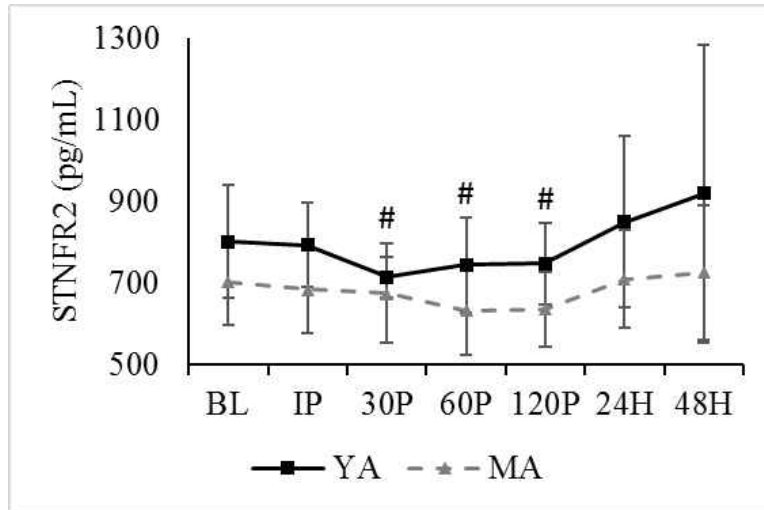

Figure 14: Changes in serum STNFR2
Serum STNFR2 concentrations before (BL), immediately post- (IP), 30 minutes post- (30P), 60 minutes post- (60P), 120 minutes post- (120P), 24 hours (24H) post-, and 48 hours (48H) post-muscle damaging protocol in the young age (YA) and middle-aged (MA) groups. Data shown as means ± SD.
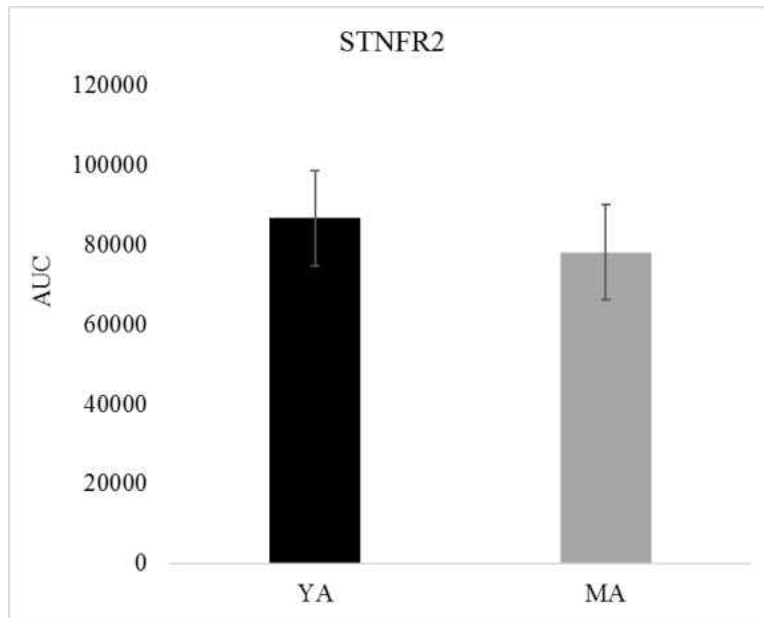
Figure 15: Soluble Tumor Necrosis Factor-alpha Receptor 2 (STNFR2) area under the curve (AUC)
Groups: *YA* = Young Age; *MA* = Middle-Aged. Data reported as means ± SD.

<u>Plasma Volume Shifts</u>

No significant group x time interaction ($p = 0.470$) or main effect for group ($p = 0.778$) was observed for plasma volume fluid shifts. When collapsed across groups, a significant time effect was observed (F = 47.7; $p < 0.001$; $\eta^2_p = 0.773$). With both groups combined, plasma volume decreased at IP (-7.6 ± 2.7%), decreased at 30P (-0.3 ± 3.8%), increased at 60P (1.2 ± 3.5%), and decreased at 120P (-1.1± 2.8%). Circulating markers were not corrected for changes in plasma volume.

A negative correlation was observed between age and STNFR1 at BL ($r = -0.462$, $p = 0.046$). Age was also negatively correlated with PTQ ($r = -0.527$, $p = 0.020$) and mTQ ($r = -0.508$, $p = 0.026$). Age was positively correlated with echo intensity of the rectus femoris at BL ($r = 0.468$, $p = 0.043$). Concentrations of TNF-α were positively correlated with concentrations of STNFR1 ($r = 0.481$, $p = 0.037$) and STNFR2 ($r = 0.692$, $p = 0.001$) at BL. A positive correlation was observed between concentrations of STNFR1 and STNFR2 at BL ($r = 0.480$, $p = 0.037$), IP ($r = 0.490$, $p = 0.046$), 60P ($r = 0.466$, $p = 0.044$), 120P ($r = 0.485$, $p = 0.035$), 24H ($r = 0.658$, $p = 0.002$), and 48H ($r = 0.599$, $p = 0.007$). No correlations were observed between any of the lower body performance variables and circulating levels of TNF-α, STNFR1, or STNFR2 (p's > 0.05).

CHAPTER FIVE: DISCUSSION

The results of this study indicate that plasma TNF-α and serum STNFRs responses to muscle damage are similar between young males and middle-aged males. Significant elevations in myoglobin were observed at 30P, 60P, and 120P relative to BL, while CK was significantly elevated at 24H and 48H. These changes were not significantly different between groups, indicating that muscle damage was similar irrespective of age. Muscle damage did not appear to have an effect on plasma levels of TNF-α. When collapsed across groups, serum STNFR1 concentrations were decreased 30 minutes post-MDP, and were increased 48 hours post-MDP relative to baseline. Serum STNFR2 concentrations were decreased relative to BL at 30, 60, and 120 minutes post-MDP. Our results indicate that ectodomain shedding of TNFR1 and TNFR2 appears to be attenuated following isokinetic eccentric exercise. This finding indicates that measuring circulating STNFRs following muscle damage may be a more appropriate method of assessing the acute inflammatory response compared to measuring plasma TNF-α. Levels of STNFR1, but not STNFR2, were elevated relative to BL at 48H. Since TNFR1 is the receptor through which most of TNF-α's pro-inflammatory signals occur, this finding may indicate a protective mechanism for cells to defend against excessive inflammatory signaling at 48-hours post-exercise.

Consistent with others (Nosaka & Clarkson, 1996; Smith et al., 2000), we observed no significant elevations in plasma levels of TNF-α following exercise. This lack of response may be due to the rapid peripheral uptake and/or renal clearance that is typical of TNF-α (Bemelmans et al., 1996; Davies & Hagen, 1997; Suzuki et al., 2002). An IP blood draw was included in the current study in order to account for TNF-α's short half-life. It is possible that an increase in rate of TNF-α synthesis may have been matched by the clearance and/or cell uptake rate and thus no

increases in TNF-α concentrations were detected. It is also conceivable that the stimulus provided in this study was not sufficiently robust to evoke an increase in plasma TNF-α. Previous research from our laboratory has shown significant increases in TNF-α using lower-body resistance training protocols comprised of multiple sets and multiple exercises at intensities of 70-90% 1-RM (Townsend et al., 2015; Wells et al., 2016). These protocols utilized larger amounts of muscle and elicited greater systemic increases in creatine kinase, myoglobin, and lactate relative to the those observed in the current study, which utilized an isokinetic protocol involving the knee extensors only. Consequently, it is likely that the volume and amount of muscle mass involved in exercise is a factor that directly dictates the ensuing inflammatory response (Nosaka & Clarkson, 1996; Townsend et al., 2013). Taken together, changes in circulating TNF-α may be difficult to detect, which explains why recent studies observing the effects of resistance training on inflammatory markers would choose to measure circulating STNFR1 and/or other pro-inflammatory signals, such as IL-6, in lieu of circulating TNF-α (Forti et al., 2017).

Serum concentrations of STNFR1 were decreased at 30P relative to baseline. This finding is in contrast to previous research noting significant elevations in STNFR1 following exercise (Ostrowski et al., 1999; Tilz et al., 1993). Ostrowoski et al. (1999) and Tilz et al. (1993) observed significant changes in circulating STNFRs following both a marathon and a hiking tour, respectively. Thus, it appears that the STNFR1 response to resistance exercise may differ from that of prolonged endurance exercise. The exact role of STNFR1 is not clear. While some suggest that ectodomain shedding of TNFRs may act as a method to prevent TNF-α from binding to the cells, others suggest that STNFRs help prolong the effects of TNF-α by protecting it from denaturation and maintaining it in circulation (Aderka et al., 1992; Wallach et al., 1991). The

findings from the present study suggest that an acute bout of exercise may attenuate shedding of TNFR1 from the cell surface. Pathological conditions characterized by an inhibition of TNFR1 ectodomain shedding have been shown to result in TNF-α-mediated chronic inflammation (McDermott et al., 1999). Accordingly, the acute decrease in STNFR1 following muscle damage observed in the present study may be a temporal change that allows for greater TNF-α signaling (Blobel, 2005; McDermott et al., 1999). Previous research from our laboratory has reported increased TNFR1 surface expression on monocytes following resistance training (Townsend et al., 2013). This increase in TNFR1 surface expression may result from a decreased ectodomain shedding of TNFR1. These results, in combination with the results found in the current study, indicate a possible role of ectodomain shedding of TNFR1 at regulating the inflammatory response to muscle damage. Furthermore, STNFR1 was significantly elevated relative to BL at 48H. This finding suggests that increased ectodomain shedding of TNFR1 may occur as a means of terminating TNF-α signaling in order to protect the host from excessive inflammation (Wallach et al., 1991).

Serum concentrations of STNFR2 were decreased at 30P, 60P, and 120P relative to baseline. However, the role of TNFR2 following EIMD is not clear. While TNFR1 is considered to be the primary pro-inflammatory receptor for TNF-α, TNFR2 is said to have both pro- and anti-inflammatory roles (Aggarwal, 2003; MacEwan, 2002). Similar to TNFR1, TNFR2 can be found on immune cells and it has been suggested that TNFR2 serves to facilitate the association between TNF-α and TNFR1 (Tartaglia et al., 1991). Therefore, attenuating ectodomain shedding of TNFR2 following exercise may serve to preserve TNFR2 on the cell surface and facilitate TNF-α signaling through TNFR1. Consistent with this, significant positive correlations between serum STNFR1 and serum STNFR2 were observed in all but one time-point. Unlike STNFR1,

STNFR2 was not significantly elevated relative to BL at any time-point. Since TNFR2 does not contain a death domain and therefore does not directly lead to cell death, it is plausible that increased ectodomain shedding of this receptor is not necessary (D. Brenner et al., 2015). Future research examining the effects of resistance training on the acute inflammatory response should aim to incorporate surface expressions of TNFR2 on immune cells in conjunction with serum STNFR2 to better understand the role of this receptor.

No significant differences were observed between groups for TNF-α, STNFR1, or STNFR2 at BL. A study by Brüünsgaard et al. (1999) showed that significant elevations in TNF-α and STNFR2 are only apparent in individuals who were 80-years-old or older, with no differences between 18-30-year-olds and 55-65 year olds. Our findings are consistent with this notion, since BL TNF-α concentrations were not different between groups. Interestingly however, a trend towards higher concentrations of both STNFR1 and STNFR2 were observed in the YA group compared to the MA group. Though not significant, this finding may suggest that the ability of cells to shed TNFR1 and TNFR2 may decline with age. This speculation is further reinforced by the negative relationship found between age and STNFR1 concentrations. Previous research indicates that STNFR1 may act as a natural inhibitor of TNF-α (Giai et al., 2013; Van Zee et al., 1992). Consistent with this, a recent study by Forti et al. (2017) found that 9 weeks of resistance training resulted in significant increases in serum concentrations of STNFR1 in healthy young adults. These authors concluded that increased levels of STNFR1 in response to resistance training may be beneficial in preventing age-related low-grade inflammation (Forti et al., 2017; Franceschi et al., 2007). Notwithstanding, future research should focus on clarifying the role of STNFRs as either beneficial or detrimental for health. Future research should also compare circulating levels of TNF-α and STNFRs between sedentary and recreationally trained

individuals to determine the role of training status and the effect of resistance training on these markers.

No significant time x group interactions were observed between YA and MA for PTQ. Since no significant difference was observed between the total work done during the damaging protocol, it appears that the ability to recover PTQ following muscle damage does not significantly decrease from young to middle age when individuals are recreationally trained. While no differences in PTQ were observed between the two groups at any time-point, the YA group showed a significantly higher mTQ at BL compared to the MA group. This finding indicates a decreased ability to generate torque throughout the entire range of motion of the knee extension with age. Both groups exhibited declines in mTQ at IP. mTQ in the YA group remained significantly decreased at 120P and 48H while mTQ in the MA group was only significantly decreased at 24H. The lower mTQ exhibited by MA at BL may account for the quicker recovery of mTQ when compared to YA. When covaried for BL difference, no time x group interaction is observed (data not shown).

No significant group x time interaction was observed for PP or mP. This finding indicates that middle-aged males may be able to maintain muscle power if they meet the ACSM guidelines for physical activity (Garber et al., 2011). Consistent with this, a recent meta-analysis found that resistance training is an efficient strategy for improving lower body power in adults 50 years or older (Straight, Lindheimer, Brady, Dishman, & Evans, 2016). When collapsed across groups, PP was significantly decreased at IP relative to BL with no significant differences at any other time point. A significant decrease in mP was also observed at IP and 120P relative to BL. The exercise stimulus provided in the present study appeared to result in a decline in both peak power and mean power; however, these values returned to BL values after 120 minutes and 24 hours,

respectively. Future research comparing recovery of performance following muscle damage between middle-aged and young age males may benefit from including sedentary counterparts in order to determine the effect of training status on power recovery.

Previous research indicates that individuals tend to lose muscle mass at a rate of 1-2% per year beginning as early as middle age (Buford et al., 2010; Lauretani et al., 2003) Therefore, we anticipated that middle-aged males would have smaller muscle mass and lower muscle quality when compared to young males However, no significant group differences were observed for CSA, MT, or EI of the rectus femoris at BL. Previous research evaluating the effects of resistance training on muscle CSA in middle-aged and older people demonstrated significant increases in the CSA of the leg extensors after a 6-month training protocol (Hakkinen et al., 1998). Since the middle-aged males recruited in the current study were recreationally trained, it is possible that the age-related decline in muscle size and quality is delayed through the use of recreational exercise including resistance training. Nonetheless, a positive correlation was observed between age and EI of the RF. Additionally, a negative relationship was observed between age and BL values of PTQ and mTQ. These findings are consistent with previous research by Fukumoto et al. (2012), who found that muscle quality of the quadriceps assessed from EI is positively correlated with age and negatively correlated with muscle strength in middle-aged and elderly persons. Taken together, our findings indicate that muscle quality may begin to decline as early as middle age and may serve as a possible reason behind the lower mTQ observed in MA compared to YA at BL.

<div align="center">Limitations</div>

Circulating STNFRs exist as either free STNFRs or as STNFR/TNF-α complexes (Aderka, 1996). One of the limitations of the present study was that the assays used to measure

STNFR1 and STNFR2 in serum were not specific to either bound or free form. Therefore, it is unclear whether the binding of TNF-α to STNFRs in circulation interfered with the quantification of total STNFRs via ELISA. It is conceivable that the serum concentrations of STNFR1 and STNFR2 observed in the present study did not account for the STNFRs that were bound to TNF-α. Moreover, the present study did not measure surface expression of TNFR1 or TNFR2 on immune cells. Therefore, it is not clear whether the decreases observed in serum concentrations of STNFR1 and STNFR2 were in fact a result of attenuated ectodomain shedding from immune cells. Future studies may benefit from analyzing changes in circulating STNFRs in conjunction with membrane TNFR receptor expressions. Lastly, the exercise intervention used in the present study may not have been sufficiently vigorous to elicit an appreciable increase in circulating TNF-α. A more intense resistance training protocol may be required to assess the differences in the inflammatory response between young and middle-aged adults.

Conclusions

The present study compared the effects of an acute isokinetic muscle damaging intervention on circulating levels of TNF-α and STNFRs between middle-aged and young age males. Markers of muscle damage were similar between the two groups. No group differences were observed in plasma TNF-α, STNFR1, or STNFR2 at BL. Therefore, our results indicate that changes in circulating levels of TNF-α and STNFRs are not present at middle age but rather may only be seen in older age (i.e. 65 years and older). However, a negative correlation was observed between age and baseline values of STNFR1. Consequently, our results suggest that natural inhibitors of TNF-α may decline as early as middle age. No group differences were observed in the acute response of TNF-α and STNFRs to muscle damage. This finding indicates that the pro-inflammatory response to muscle damage does not decline at middle age when

individuals are recreationally trained. Aside from mTQ, no significant differences were observed in lower body performance between YA and MA. Similarly, no significant differences in muscle size or quality were observed between the two groups. Therefore, the age-related decline in muscle size and function may not commence until later in life; or perhaps maintaining a recreationally active lifestyle may delay these effects of aging.

APPENDIX A: UCF IRB APPROVAL LETTER

Notice that UCF will Rely Upon Other IRB for Review and Approval

From :   **UCF Institutional Review Board**
                FWA00000351, IRB00001138

To   :   **Adam J. Wells**

Date :   **December 05, 2016**

IRB Number: **SBE-16-12594**

Study Title: **Effects of an Acute High-Volume Isokinetic Intervention on Inflammatory and Strength Changes: Influence of Age**

Dear Researcher:

The research protocol noted above was reviewed by the University of Central Florida IRB Designated Reviewer on **December 05, 2016**. The UCF IRB accepts the New England's Institutional Review Board review and approval of this study for the protection of human subjects in research. **The expiration date will be the date assigned by** the New England's **Institutional Review Board and the consent process will be the process approved by that IRB.**

This project may move forward as described in the protocol. It is understood that the New England's IRB is the IRB of Record for this study, but local issues involving the UCF population should be brought to the attention of the UCF IRB as well for local oversight, if needed.

All data, including signed consent forms if applicable, must be retained and secured per protocol for a minimum of five years (six if HIPAA applies) past the completion of this research. Any links to the identification of participants should be maintained and secured per protocol. Additional requirements may be imposed by your funding agency, your department, or other entities. Access to data is limited to authorized individuals listed as key study personnel.

Failure to provide a continuing review report for renewal of the study to the New England's IRB could lead to study suspension, a loss of funding and/or publication possibilities, or a report of noncompliance to sponsors or funding agencies. If this study is funded by any branch of the Department of Health and Human Services (DHHS), an Office for Human Research Protections (OHRP) IRB Authorization form must be signed by the signatory officials of both institutions and a copy of the form must be kept on file at the IRB office of both institutions.

On behalf of Sophia Dziegielewski, Ph.D., L.C.S.W., UCF IRB Chair, this letter is signed by:

Signature applied by Patria Davis on 12/05/2016 09:51:38 AM EST

IRB Coordinator

APPENDIX B: NEIRB APPROVAL LETTER

January 20, 2017

Adam Wells, PhD
University of Central Florida
12494 University Boulevard
Orlando, FL 32816, United States

NEIRB: 120160966
Study Title: Effects of an Acute High-Volume Isokinetic Intervention on Inflammatory and Strength Changes: Influence of Age

Notification of Approval

This is to inform you that New England Independent Review Board (NEIRB) has approved the following for the above referenced research.

Date of Review: 01/20/2017

Approval Includes:
Protocol (submitted on 01-19-2017)
Main Consent (01-20-2017), NEIRB Version 5.0

> *Please have all current and future subjects sign the revised Consent Form(s) specified in this approval.*

NEIRB has approved the following locations to be used in the research:
- University of Central Florida Human Performance Lab, 12494 University Boulevard, Orlando, Florida 32816

Continued approval is conditional upon your compliance with the following requirements:
- Compliance with all aspects of NEIRB's Investigator Responsibilities (available at www.neirb.com).
- The study cannot continue after the End Approval Date unless re-approved by NEIRB. A Study Continuing Review Report must be completed and returned to NEIRB prior to the study end date of the approval period.
- Compliance with all federal, state and local laws pertaining to this research.
- Any and all necessary FDA approvals must be received prior to your initiation of the trial.

Please contact NEIRB with any questions about this determination.
Thank you.

Copy:

    NEIRB study file
    Enclosures

APPENDIX C: INFORMED CONSENT

Effects of an Acute High-Volume Isokinetic Intervention on Inflammatory and Strength Changes: Influence of Age

Informed Consent

Principal Investigator(s):	Dr. Adam J. Wells, Ph.D.
Sub-Investigators:	Dr. Jeffrey R. Stout, Ph.D. Dr. Jay R. Hoffman, Ph.D. Joseph A. Gordon III, B.S. Eliott Arroyo, B.S. Alyssa Varanoske, M.S.
Phone number:	(407) 823-2367
Sponsor:	UCF Institute of Exercise Physiology and Wellness
Investigational Site(s):	University of Central Florida College of Education and Human Performance Institute of Exercise Physiology and Wellness

Introduction:

You are being invited to take part in a research study which will recruit about 20 people at UCF and its surrounding areas. You have been asked to take part in this research study because you are a recreationally active male between the ages of 18 and 30 years or between the ages of 40 and 60 years.

The investigators conducting the research are Dr. Jay R. Hoffman, Dr. Jeffrey R. Stout, (Professors of Sport and Exercise Science in the College of Education and Human Performance), Dr. Adam J. Wells (Assistant Professor of Sport and Exercise Science in the College of Education and Human Performance), Mr. Joseph A. Gordon III, Mr. Eliott Arroyo, and Ms. Alyssa N. Varanoske (Graduate Students of Sport and Exercise Science in the College of Education and Human Performance).

What you should know about a research study:

- Someone will explain this research study to you.
- A research study is something you volunteer for.
- Whether or not you take part is up to you.
- You should take part in this study only because you want to.

- You can choose not to take part in the research study.
- You can agree to take part now and later change your mind.
- Whatever you decide it will not be held against you.
- Feel free to ask all the questions you want before you decide.

Purpose of the research study:

The purpose of this study is to compare the effects of an acute, high-volume isokinetic exercise intervention on the rate of recovery in younger versus older recreationally-trained males. Recovery parameters include lower-body performance and markers of muscle damage and inflammation, which will be assessed in the subsequent 48 hours following a muscle-damaging protocol.

What you will be asked to do in the study:

Screening Visit:

You will be asked to read and sign this consent form before any study-related procedures are performed. During the screening visit, the following will be done:
- Physical activity readiness questionnaire (PAR-Q)
- Your age, race and gender will be collected
- Self-reported confidential medical and activity history questionnaire

During the screening visit, we will review the inclusion/exclusion criteria with you. We will also inform you of the requirements of the study and determine whether you have any intolerance to the exercise.

Study Protocol:

Investigator expectations are the same for all participants. All procedures are being done solely for research purposes. Following enrollment into the study, you will report to the Human Performance Lab for assessment on four separate occasions:

- Day 1: On the first visit, you will report to the Human Performance Laboratory (HPL) following a 10-hour fast. Upon arrival, your weight and height will be measured using a standard scale and measuring tape, respectively. Additionally, your body composition will be assessed using bioelectrical impedance analysis. Following a brief warmup, you will be familiarized with the lower body performance assessments (isokinetic device and isometric mid-thigh pull).
- Day 2: On the second visit, again following a 10-hour fast, you will outfitted with a near-infrared spectroscopy (NIRS) optode. Following this, you will undergo baseline (BL) measurements including: an ultrasound assessment, a blood sample collection, a lower-body performance assessments protocol, and a visual analog scale (VAS) to indicate your current level of pain and soreness. After a brief rest period following the

lower-body performance assessment, you will undergo the acute muscle-damaging protocol (MDP).

- o Immediately upon completion of the MDP (IP), you will complete a blood sample collection, a second lower-body assessment protocol followed by an ultrasound assessment.
- o Thirty minutes post MDP (30P), you will complete another ultrasound assessment, a blood sample collection, and a VAS.
- o At sixty minutes (60P) and 120 minutes (120P) post MDP, additional blood samples will be collected.
- o A third lower-body performance assessment protocol (BioDex S4 isokinetic dynamometer, and mid-thigh pull) and an additional ultrasound assessment will be completed at 120P.
- Day 3: On the third visit, you will report to the HPL again following a 10-hour fast and perform the 24-hour post (24H) ultrasound assessment, a blood sample collection, and lower-body performance assessment protocol. Additionally, you will indicate your level of pain and soreness using the VAS.
- Day 4: On the fourth and final visit, you will again report to the HPL following a 10-hour fast. You will then complete the 48-hour post (48H) assessment, which includes an ultrasound assessment, a blood sample collection, and the lower-body performance assessment protocol. Additionally, you will indicate your level of pain and soreness using the VAS.

The figure below outlines the daily procedures throughout the investigation:

72
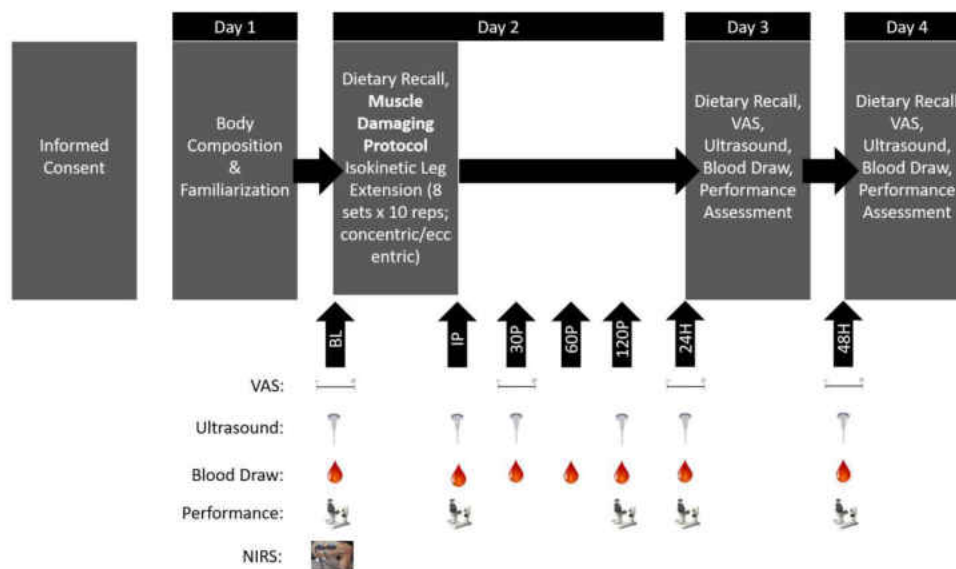
<u>Anthropometric Measurements</u>

Body mass and height will be measured using a standard professional scale. Body composition will be assessed using a bioelectrical impedance analysis device. You will be asked to remove your footwear and socks before performing the test. This assessment requires you to wash your hands and feet with a wet tissue prior to analysis. Next, you will stand on the platform while holding the two handles out to the side. You will hold this position for one minute as the device conducts an electrical current through your body to determine body composition. Values for total and segmental body fat percentage will be recorded. The test will be completed in approximately 5 minutes. There are no risks or discomforts associated with the use of bioelectrical impedance analysis.

<u>Lower-Body Performance Assessment Protocol</u>

The lower body performance assessment protocol will consist of three assessments. Two assessments will be performed on an isokinetic dynamometer, and one on a force platform (mid-thigh pull).

Mid-thigh pull

Following a general warm-up, you will complete a two mid-thigh pulls to evaluate isometric strength of the lower body, two isometric mid-thigh pulls will be assessed. You will be instructed to stand on a force platform, bent slightly at the knees and hip. A barbell will be adjusted so that it is fixed at mid-thigh position. You will be instructed to pull upwards on the barbell as hard and forcefully as you can for 6 seconds. Between isometric mid-thigh pull assessment sets, you will be provided with three minutes of rest. Potential risks and/or discomforts associated with the isometric mid-thigh pull assessment may include muscle pain and/or soreness, similar to those experienced when exercising.

Isokinetic Dynamometer

Following the mid-thigh pull, you will be seated in an isokinetic device, positioned with a hip angle of 110° and strapped into the chair at the waist, shoulders, and across the thigh to complete two maximal voluntary isometric contractions (MVIC) where you will be required to kick maximally against a fixed resistance for 5-seconds as your leg stays in one position. Between MVIC sets, you will be provided with three minutes of rest. Potential risks and/or discomforts associated with the lower-body isokinetic and isometric assessment may include muscle pain and/or soreness, similar to those experienced when exercising.

After completing the MVIC test, you will then complete a series of isokinetic contractions on the BioDex S4 isokinetic dynamometer. The seat and leg setup will be the same as it was for the MVIC test. you will complete 3 sets of isokinetic leg extension at 60, 180, and 240 degrees per second, respectively. The order of the sets will be randomized to account for any fatigue from each set. The starting point for a repetition will be when the knee is at 90 degrees, with 180 degrees representing full extension. you will be given two practice attempts at each speed before recording begins. You will be instructed to give maximal effort as you extend the knee joint to 180 degrees. Then they will relax the muscles to allow the leg to return to the starting position. This process of starting at 90 degrees, extending to 180 degrees and then returning

back to 90 degrees will be considered 1 repetition. You will be given 3 minutes of rest between each set. This test should last approximately 10 minutes.

Acute Lower-Body Muscle-Damaging Protocol

After completing the lower-body assessment protocol, you will remain seated in the isokinetic dynamometer chair. Following a five-minute rest, you will perform the MDP. The MDP is composed of eight sets of 10 repetitions. You will be instructed to give maximal effort during this protocol. You will be provided with one minute of rest between each set of the MDP. Potential risks and/or discomforts associated with the lower-body MDP are the same as associated with the lower-body isokinetic and isometric assessment.

Visual Analog Soreness Questionnaire

You will be asked to quantify your degree of lower-body muscle soreness and pain using a 15-cm visual analog scale (VAS). You will provide your levels of pain and soreness by making a mark on a horizontal line with words anchored at each end of the VAS.

Blood Measurements

You will report to the HPL at the same time on each day following a 10-hour fast. The blood samples collected at BL, IP, 30P, 60P, and 120P will be drawn from a forearm vein using a Teflon™ cannula by personnel trained in phlebotomy with extensive experience in both research and clinical settings. A cannula is a hollow tube, which can be inserted into the opening of a vein and serve as a channel for the transport of fluid. The cannula prevents the need for multiple needle pricks from being performed. The cannula will be kept open following each blood draw with an infusion of a saline solution. This solution contains salt that is similar to the osmolality of the blood and acts to minimize potential blood clotting within the cannula that may occur with prolonged use. The cannula placement will not interfere with your ability to perform the exercise routine. The blood draws at 24 and 48H will be made using a 21 gauge, 1 ¼ inch Vacutainer® blood collection needle. The total amount of blood that will be taken during the study as a whole will not exceed 140 ml (20 ml per blood draw). This is approximately 9.5 tablespoons. To put the total volume of blood being drawn in proper perspective, one pint (475 ml) of blood is typically drawn when donating blood. All blood draws will be conducted under sterile conditions. As an additional safeguard in preventing contamination, new disposable gloves will be used for all blood draws. The discomforts associated with the blood drawing procedures are minimal, but sometimes bruising and infection may occur, as well as soreness in the area.

Muscle Ultrasound

To evaluate the muscle architecture and morphology of your upper thigh, non-invasive ultrasound measurements will be made using a linear probe ultrasound. The probe will be coated with a water-based conduction gel. You will be positioned on your non-dominant leg side, with your hips perpendicular to the examination table. You will be instructed to keep you legs, knees, and ankles stacked together. A mark will be made at on your thigh at a specified location to guide location of the image capture. Ultrasound images will be later analyzed for muscle morphological characteristics. There are no potential risks associated with ultrasonography.

Near Infrared Spectroscopy

To assess tissue oxygenation during the acute muscle damage protocol, a near infrared spectroscopy (NIRS) optode (PortaLite, Artinis Medical Systems, Gelderland, the Netherlands) will be placed over the vastus lateralis muscle of your right leg. The optode will be secured using a self-adhering bandage. There are no risks or discomforts associated with the use of near infrared spectroscopy.

Dietary Recall

You will be instructed to remember as accurately as possible everything they consumed during the 24-hours preceding BL assessments. You will also be required to provide a dietary recall upon arrival to the HPL on Day 3(24h) and Day 4 (48h). be required to ou will be interviewed at each visit, and asked to recall all food consumed on each of these days.

Location:
All testing will be performed in the Institute of Exercise Physiology and Wellness at the Human Performance and Strength and Conditioning Laboratories at the University of Central Florida.

Time required:
We expect that the time requirements for participation in this study will be 4 days, which will consist of a total of 4 visits to the HPL. The familiarization visit will last approximately 60 minutes, the intervention visit will last approximately 240 minutes, and the subsequent two consecutive days following the intervention visit will be approximately 60 minutes each. The total time that you will be in the laboratory will be approximately 7 hours over a period of 4 days.

Funding for this study:
There will be no funding provided for the completion of this study.

Risks:

- The intervention protocol consists of both concentric and eccentric muscle contractions at a high volume. It is expected that you will experience the normal soreness that accompanies a bout of resistance exercise.

- The risks associated with the blood draw include some momentary pain at the time the needle is inserted into the vein, but other discomfort should be minimal. It is also possible for a bruise to develop at the needle site or for individuals to report dizziness and faint after the blood is drawn. It is also rare, but possible to develop minor infections and pain after the blood draw. To minimize the risks, the skin area where the needle or catheter is to be inserted will be cleaned and prepared with a disinfectant wipe before the needle or catheter is inserted. In addition, the catheter will be inserted while the participant is lying supine and all blood draws will be performed with the subject in a supine position.

- There are no risks or discomforts associated with any of the ultrasound or anthropometric measures.

- All testing will be overseen by individuals certified in CPR and AED. An AED is located in the Wellness Research Center within the Education Building (approximately 200 feet from the Human Performance Laboratory).

- If at any time during the study, you feel discomfort or do not wish to continue, you are encouraged to inform the researcher. Discontinuation of participation may occur at any time. Any discomforts should be immediately reported to Joseph Gordon III (407-823-2367), Alyssa Varanoske (407-823-2367) or to the professional performing the test and later to the principal investigator (Dr. Adam Wells, 407-823-3906).

Benefits:

There are no expected benefits to you for taking part in this study.

Compensation or payment:

There is no compensation or other payment to you for taking part in this study.

Medical care and compensation for injury:

This is a minimal-risk study and it is unlikely that you will experience adverse effects. However, in the event that an adverse effect occurs, you will be instructed to immediately report any discomforts or adverse effects to the principal investigator. An adverse effect is defined as an intolerable response, perceived to be a direct consequence of participation in this study. If immediate assistance is needed it will be provided via the emergency medical system. For non-emergency injuries, you must seek treatment from your own physician. If you suffer a physical injury as a result of participation in this study, you may be reimbursed for medical expenses to treat the injury, to the extent not paid by your insurance. You should receive medical care in the same way as you would normally. No funds have been set aside for payments or other forms of compensation (such as for lost wages, lost time, or discomfort). You do not give up any of your legal rights by signing this consent form. Adverse events/side effects will be reported to the New England Independent Review Board (NEIRB) immediately upon notification.

Alternative:

The alternative is to not participate in the study.

Cost:

There is no cost to you to be in the study.

Confidentiality:

Records of your participation in this study will be held confidential so far as permitted by law. The study investigator or it's designee, and, under certain circumstances, the Institutional Review Board will be able to inspect and have access to confidential data that identifies you by name. Any

publication or presentation of the data will not identify you. By signing this consent form, you authorize the study investigator to release your medical records to the IRB.

The results of this study will be published as a group as part of a scientific publication. No individual results will be published or shared with any person or party. All information attained from the medical and activity questionnaire or performance tests will be held in strict confidence. Individual results will remain confidential. Individual test results will be shared with the participant only at their request. Test results will not be shared with participants. All medical and activity questionnaires, as well as data collection sheets will be kept in a locked cabinet during and following the study. Your names will be kept separately from the study results as a separate electronic file under password protection. This file will be stored on a computer in the Education and Human Performance building. All information will be destroyed 5 years from the end of the study. Your folders and blood storage tubes will be marked with an I.D. number to protect against a breach of confidentiality and the I.D. number will be removed upon disposal. Your names will be stored apart from the blood samples. The identifiers will be removed from the samples and destroyed when the samples are disposed of. All the medical information taken during the study will not be useful for you or cannot be used to supplement/replace medical care.

Study contact for questions about the study or to report a problem:
If you have questions, concerns, or complaints, or think the research has hurt you, contact Dr. Adam Wells, Human Performance Laboratory, Sport and Exercise Science (407) 823-3906 or by email at adam.wells@ucf.edu.

IRB contact about your rights in the study or to report a complaint:
For information about the rights of people who take part in research or if you have questions, complaints or concerns, please contact New England Independent Review Board at (800) 232-9570 or email at info@NEIRB.com.

Withdrawing from the study:
You have the right to discontinue participation without penalty, regardless of the status of the study. Your participation in the study may also be terminated at any time by the researchers in charge of the project for the following reasons.
- Inability to adhere to the study protocol. This includes:
    - Failure to adhere to requirements
    - Failure to complete all visits to the human performance lab
    - Failure to provide a fasted blood draws at the start of the control or exercise trial
- Cancellation of the study.

The investigator or the New England IRB can also stop your participation in this study at any time.

For Students and Employees of UCF:

Your participation in this study is voluntary. You are free to withdraw your consent and discontinue participation in this study at any time without prejudice or penalty. Your decision to participate or not participate in this study will in no way affect your continued employment or your relationship with individuals who may have an interest in this study. Employees of UCF cannot participate to the study

during work hours. In addition, if you are a student you have the right to withdraw at any time and without any negative affect on your grade or on your ability to continue your education at the University.

_____initials.

Results of the research:

Biologic specimens obtained from this research are not expected to be part of or lead to the development of a commercial product. As previously stated, individual results will remain confidential, and you will be informed of only your results upon request.

VOLUNTEER'S STATEMENT

I have been given a chance to ask questions about this research study. These questions have been answered to my satisfaction. I may contact Joseph Gordon or Eliott Arroyo (student investigators), or Adam Wells, PhD., if I have any more questions about taking part in the study.

I understand that my participation in this research project is voluntary. I know that I may quit the study at any time without harming my future medical care or losing any benefits to which I might be entitled. I also understand that the investigator in charge of this study may decide at any time that I should no longer participate in the study.

If I have any questions about my rights as a research subject in this study I may contact:

New England Independent Review Board, 197 First Avenue, Suite 250, Needham, MA, 02494
E-Mail: info@neirb.com
Telephone 1.800.232.9570

By signing this form, I have not waived any on my legal rights.

I have read and understand the above information. I agree to participate in this study. I understand that I will be given a copy of this signed and dated form for my own records.

_____
Name of participant

_____   _____
Signature of participant   Date

_____   _____
Signature of person obtaining consent   Date

_____
Printed name of person obtaining consent

APPENDIX D: MEDICAL HEALTH QUESTIONNAIRE AND PAR-Q

Human Performance Laboratory
University of Central Florida

Confidential Medical and Activity History Questionnaire

Participant #_____

When was your last physical examination? _____

1. **List any medications (prescription or over-the-counter), herbals or supplements you currently take or have taken the last month:**

   Medication   Reason for medication
   _____   _____
   _____   _____
   _____   _____
   _____   _____

2. **Are you allergic to any medications? If yes, please list medications and reaction.**

3. **Please list any allergies, including food allergies that you may have?**

4. **Are you a current or former smoker? If former, how long has it been since you quit?**

5. **Are you currently enrolled in another clinical research study?**

6. **Do you currently drink > 8oz/day of either green or black tea?**

7. **In the past two years have you been diagnosed with cancer? If so what type?**

8. **Are you currently on a diet regimen including but not limited to, Atkins, South Beach, Intermittent Fasting, etc?**

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9. **Have you donated blood or plasma recently? If so when?**

10. **Do you currently have any chronic illness that causes continuous medical care? If so what is the illness?**

11. **Have you ever been hospitalized? If yes, please explain.**

Year of hospitalization   Reason

_____   _____

_____   _____

_____   _____

12. **Illnesses and other Health Issues**

List any chronic (long-term) illnesses that have caused you to seek medical care.

Have you ever had (or do you have now) any of the following. Please circle questions that you do not know the answer to.

Sickle cell anemia	yes	no
Cystic fibrosis	yes	no
Water retention problems	yes	no
Heart pacemaker	yes	no
Epilepsy	yes	no
Convulsions	yes	no
Dizziness/fainting/unconsciousness	yes	no
Asthma	yes	no
Shortness of breath	yes	no
Chronic respiratory disorder	yes	no
Chronic headaches	yes	no
Chronic cough	yes	no
Chronic sinus problem	yes	no
High blood pressure	yes	no
Heart murmur	yes	no

2

Heart attack	yes	no
High cholesterol	yes	no
Diabetes mellitus or insipidus	yes	no
Rheumatic fever	yes	no
Emphysema	yes	no
Bronchitis	yes	no
Hepatitis	yes	no
Kidney disease	yes	no
Bladder problems	yes	no
Tuberculosis (positive skin test)	yes	no
Yellow jaundice	yes	no
Auto immune deficiency	yes	no
Anemia	yes	no
Endotoxemia	yes	no
Thyroid problems	yes	no
Hyperprolactinemia	yes	no
Anorexia nervosa	yes	no
Bulimia	yes	no
Stomach/intestinal problems	yes	no
Arthritis	yes	no
Back pain	yes	no
Gout	yes	no
Hepatic encephalopathy	yes	no
Mania	yes	no
Hypermania	yes	no
Monosodium glutamate hypersensitivity	yes	no
Seizure disorders	yes	no

Any others (specify):_____

_____

_____

Do you smoke cigarettes or use any other tobacco products?	yes	no
Do you have a history of drug or alcohol dependency?	yes	no
Do you ever have any pain in your chest?	yes	no
Are you ever bothered by racing of your heart?	yes	no
Do you ever notice abnormal or skipped heartbeats?	yes	no
Do you ever have any arm or jaw discomfort, nausea, Or vomiting associated with cardiac symptoms?	yes	no
Do you ever have difficulty breathing?	yes	no
Do you ever experience shortness of breath?	yes	no
Do you ever become dizzy during exercise?	yes	no
Are you pregnant?	yes	no

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Is there a chance that you may be pregnant?	yes	no
Have you ever had any tingling or numbness in your arms or legs?	yes	no
Has a member of your family or close relative died of heart problems or sudden death before the age of 50?	yes	no
Has a health care practitioner ever denied or restricted your participation in sports for any problem	yes	no

If yes, please explain: _____

What is your current level of physical activity (hours per week, intensity, mode of exercise)?

_____
_____
_____
_____

Are you presently taking any nutritional supplements or ergogenic aids? (if yes, please detail. _____

_____
_____
_____
_____

4

PAR-Q & YOU

(A Questionnaire for People Aged 15 to 69)

Regular physical activity is fun and healthy, and increasingly more people are starting to become more active every day. Being more active is very safe for most people. However, some people should check with their doctor before they start becoming much more physically active.

If you are planning to become much more physically active than you are now, start by answering the seven questions in the box below. If you are between the ages of 15 and 69, the PAR-Q will tell you if you should check with your doctor before you start. If you are over 69 years of age, and you are not used to being very active, check with your doctor.

Common sense is your best guide when you answer these questions. Please read the questions carefully and answer each one honestly: check YES or NO.

YES	NO		
☐	☐	1.	Has your doctor ever said that you have a heart condition <u>and</u> that you should only do physical activity recommended by a doctor?
☐	☐	2.	Do you feel pain in your chest when you do physical activity?
☐	☐	3.	In the past month, have you had chest pain when you were not doing physical activity?
☐	☐	4.	Do you lose your balance because of dizziness or do you ever lose consciousness?
☐	☐	5.	Do you have a bone or joint problem (for example, back, knee or hip) that could be made worse by a change in your physical activity?
☐	☐	6.	Is your doctor currently prescribing drugs (for example, water pills) for your blood pressure or heart condition?
☐	☐	7.	Do you know of <u>any other reason</u> why you should not do physical activity?

If you answered

YES to one or more questions

Talk with your doctor by phone or in person BEFORE you start becoming much more physically active or BEFORE you have a fitness appraisal. Tell your doctor about the PAR-Q and which questions you answered YES.

- You may be able to do any activity you want — as long as you start slowly and build up gradually. Or, you may need to restrict your activities to those which are safe for you. Talk with your doctor about the kinds of activities you wish to participate in and follow his/her advice.
- Find out which community programs are safe and helpful for you.

NO to all questions

If you answered NO honestly to <u>all</u> PAR-Q questions, you can be reasonably sure that you can:
- start becoming much more physically active — begin slowly and build up gradually. This is the safest and easiest way to go.
- take part in a fitness appraisal — this is an excellent way to determine your basic fitness so that you can plan the best way for you to live actively. It is also highly recommended that you have your blood pressure evaluated. If your reading is over 144/94, talk with your doctor before you start becoming much more physically active.

DELAY BECOMING MUCH MORE ACTIVE:
- If you are not feeling well because of a temporary illness such as a cold or a fever — wait until you feel better; or
- if you are or may be pregnant — talk to your doctor before you start becoming more active.

PLEASE NOTE: If your health changes so that you then answer YES to any of the above questions, tell your fitness or health professional. Ask whether you should change your physical activity plan.

<u>Informed Use of the PAR-Q</u>: The Canadian Society for Exercise Physiology, Health Canada, and their agents assume no liability for persons who undertake physical activity, and if in doubt after completing this questionnaire, consult your doctor prior to physical activity.

No changes permitted. You are encouraged to photocopy the PAR-Q but only if you use the entire form.

NOTE: If the PAR-Q is being given to a person before he or she participates in a physical activity program or a fitness appraisal, this section may be used for legal or administrative purposes.

"I have read, understood and completed this questionnaire. Any questions I had were answered to my full satisfaction."

NAME _____

SIGNATURE _____ DATE_____

SIGNATURE OF PARENT _____ WITNESS _____
or GUARDIAN (for participants under the age of majority)

Note: This physical activity clearance is valid for a maximum of 12 months from the date it is completed and becomes invalid if your condition changes so that you would answer YES to any of the seven questions.

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APPENDIX E: DIETARY RECALL FORM

Dietary Recall Instructions

1. Use the forms provided to record everything you eat or drink on the days instructed.

2. Indicate the name of the FOOD ITEM, the AMOUNT eaten, how it was PREPARED (fried, boiled, etc.), and the TIME the food was eaten. If the item was a brand name product, please include the name. Try to be accurate about the amounts eaten. Measuring with measuring cups and spoons is best, but if you must make estimates, use the following guidelines:
   - A fist is about 1 cup
   - Tip of your thumb is about 1 teaspoon
   - A thumb represents about 1 ounce of cheese
   - A golf ball represents about 2 tablespoons
   - The palm is about 3 ounces of meat (roughly the size of a deck of cards)

3. Try to maintain your normal diet, and be honest about what you eat. The information you provide is confidential.

4. Follow the specific instructions below when reporting foods:
   - MILK – indicate % fat, source (e.g., cow, almond, coconut), and flavoring (if any).
   - FRUITS & VEGETABLES – an average serving size of cooked or canned fruits and vegetables is ½ cup. Fresh, whole fruits and vegetables should be listed as small, medium, or large. Be sure to indicate sugar or syrup is added to fruit and list if any margarine, butter, cheese sauce, or cream sauce is added to vegetables. When recording salad, list items comprising the salad separately and be sure to include salad dressing used.
   - EGGS – indicate whole or whites only, method of preparation (e.g., scrambled, fried, poached), and number eaten.
   - MEAT/POULTRY/FISH – indicate approximate size or weight, in ounces, of the serving. Be sure to include any gravy, sauce, or breading added and preparation method.
   - CHEESE – indicate kind, number of ounces or slices, and whether it is made from whole milk, part skim, or is low calorie.
   - CEREAL – specify kind, brand, whether cooked or dry, and measure in terms of cups or ounces. *Consuming 8 oz. of cereal is not the same as consuming 1 cup of cereal. Be sure to include any milk consumed with cereal (see MILK).
   - BREADS – specify kind (e.g., whole wheat, enriched wheat, white) and number of slices.
   - BEVERAGES – include everything drink, excluding water. Be sure to record cream and sugar used in tea and coffee, whether juices are sweetened or unsweetened, and whether soft drinks are diet or regular.
   - FATS - record any butter, margarine, oil, or other fats used in cooking or on food.
   - PREPARED DISHES/CASSEROLES – list the main ingredients, approximate amount of each ingredient to the best of your ability, and brand (if applicable).

5. Express approximate measures in cups (c), tablespoons (T), teaspoons (t), grams (g), ounces (oz), pieces, etc.

6. If you are unsure of how to report any food items you consume, please take pictures of the packaging and Nutrition Facts panel, when possible.

Subject #:_____   **Date:_____**

Day 1

Meal	Time	FOOD/BEVERAGE DESCRIPTION	AMOUNT	Total kcal *(from label)*
Breakfast				
Lunch				
Dinner				
Snacks				

Day 2   **Date:_____**

Meal	Time	FOOD/BEVERAGE DESCRIPTION	AMOUNT	Total kcal *(from label)*
Breakfast				
Lunch				
Dinner				
Snacks				

Day 3   **Date:**_____

Meal	Time	FOOD/BEVERAGE DESCRIPTION	AMOUNT	Total kcal *(from label)*
Breakfast				
Lunch				
Dinner				
Snacks				

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