Summer 7-16-2018

Autophagy is stimulated by acute high intensity interval exercise in human skeletal muscle and electrical pulse stimulation in C2C12 myotubes in vitro

Kurt A. Escobar
University of New Mexico - Main Campus

Kurt A. Escobar
Candidate

Health, Exercise and Sports Sciences
Department

This dissertation is approved, and it is acceptable in quality and form for publication:

Approved by the Dissertation Committee:

Fabiano Amorim, Chairperson

Christine Mermier, Co-Chair person

Carlos Hiroshi Nitta

Trisha VanDusseldorp

Micah Zuhl

Autophagy is stimulated by acute high intensity interval exercise in human skeletal muscle and electrical pulse stimulation in C2C12 myotubes in vitro

BY

KURT A. ESCOBAR

B.S. Kinesiology, Exercise Science
California State University, Fresno, 2012

M.A. Kinesiology, Exercise Science
California State University, Fresno, 2014

DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

Doctor of Philosophy
Physical Education, Sports and Exercise Science

The University of New Mexico
Albuquerque, New Mexico

July 2018

Autophagy is stimulated by acute high intensity interval exercise in human skeletal muscle and electrical pulse stimulation in C2C12 myotubes in vitro

BY

KURT A. ESCOBAR

B.S. Kinesiology, Exercise Science
California State University, Fresno, 2012

M.A. Kinesiology, Exercise Science
California State University, Fresno, 2014

Ph.D., Physical Education, Sports and Exercise Sciences
University of New Mexico, 2018

ABSTRACT

Purpose: 1) To compare the effects of an acute bout of HIIT exercise (treadmill running) on autophagy to MICT exercise in human skeletal muscle 3 hours post exercise, and 2) to test an *in vitro* model of muscle contraction-induced autophagy using electrical pulse stimulation in C2C12 myotubes. **Methods:** Study 1: Ten recreationally active males and females completed a bout of high intensity interval training (HIIT) exercise and moderate intensity continuous exercise (MICT) exercise in a fasted state. Muscle biopsies from the *vastus lateralis* were taken pre and 3 hours post-exercise. Muscle tissue was analyzed for protein expression of markers of autophagy

(LC3I, LC3II) and autophagy signaling (p38MAPK). Study 2: C2C12 myoblasts were differentiated into myotubes and underwent 8 hours of low-frequency electrical pulse stimulation (EPS) and starvation (St) and EPS+St conditions with and without bafilomycin A1 (Baf) or non-treatment control conditions (Con). Cells were harvested immediately following EPS and analyzed for protein expression of markers of autophagy (LC3I, LC3II, and p62). **Results:** Study 1: No differences were detected for LC3I, LC3II, and p38MAPK at the 3h time point vs. pre-exercise in both HIIT and MICT conditions. The LC3II:LC3I ratio increased 3-h post exercise in the HIIT (162.4, SE: 45.9%), which was significantly higher than the 3h timepoint in the MICT (48.8, SE: 9.4%; $p<0.05$) Study 2: LC3II (324.4, SE: 29.8%) LC3II:I (258.2, SE: 323.3%), and p62 (437.8, SE: 9.7%) were significantly higher in EPS+Baf conditions compared to control (100%, SE: 9.3, 8.8, 22.6%, respectively) and EPS alone (127.7, SE: 24.3%; 95.5, SE: 13.2%, 251.2, SE: 33.2%) . p62 protein expression was also higher in EPS compared to Con and in St+Baf (320.9, SE: 65.9%) compared Con and St (91, SE: 20.3%). There were no differences between EPS and St alone vs. combined EPS+St conditions in LC3I, LC3II, LC3II:I or p62. **Conclusions:** HIIT stimulates autophagy in a distinct fashion compared to MICT. Additionally, EPS may serve as an *in vitro* model for muscle contraction-induced autophagy in C2C12 myotubes in fed and fasted conditions.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

SYMBOLS / ABBREVIATIONS

>: greater than

≤: less than or equal to

<: less than

±: plus or minus

~: approximately

°C: degrees Celsius

µg: microgram

µl: microliter

ml: milliliter

µmol: micromole

ANOVA: analysis of variance

Atg: autophagy-related

BF%: body fat percentage

BM: body mass

cm: centimeters

g/kg/d: grams per kilogram per day

kg: kilogram

LC3: microtubule-associated protein light chain 3

M: molar

mg: milligram

Mg: myoglobin

Mm: millimeter

mTOR: mammalian target of rapamycin

n= number of participants

p62: sequestosome 1

PBS: phosphate buffered saline

SD: standard deviation

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SE: standard error

Tris: tris (hydroxymethyl) aminomethane

Chapter 1: Introduction

It is well established that exercise serves as a robust means to promote health and longevity, as well as reduce the risk of chronic disease (Booth, Roberts, & Laye, 2012). However, the underlying cellular mechanisms governing this phenomenon remain largely unclear. Autophagy, an evolutionary-conserved cellular recycling system, has been implicated on mediating this adaptive response (Ghareghani et al., 2017; Mejias-Pena et al., 2016; Vainshtein, Grumati, Sandri, & Bonaldo, 2014). Autophagy is responsible for maintaining the proteome by the degradation of damaged, aged, and redundant cytosolic proteins and organelles (Todde, Veenhuis, & van der Klei, 2009). A number of degenerative and age-related diseases are associated with malfunctioning autophagy including type 2 diabetes, cardiovascular disease, neurodegenerative disease such as Alzheimer's and Parkinson's, and cancer (Cuervo, 2008; Todde et al., 2009). Compromised autophagy is also observed in aging (Salminen & Kaarniranta, 2009). Interestingly, autophagy is stimulated by acute exercise in a number of tissues (Dokladny et al., 2013; Ghareghani et al., 2017; He, Bassik, et al., 2012; He, Sumpter, & Levine, 2012; Li et al., 2016; Mejias-Pena et al., 2017a; Mejias-Pena et al., 2016), including skeletal muscle (Jamart, Benoit, et al., 2012; Jamart, Francaux, et al., 2012; Tam & Siu, 2014; Vainshtein et al., 2014) and evidences exist suggesting augmentation in autophagic function in response to chronic exercise (Lira et al., 2013; Mejias-Pena et al., 2017b; Mejias-Pena et al., 2016; Tam, Pei, Yu, et al., 2015; Tam, Pei, Yung, et al., 2015; Wohlgemuth, Seo, Marzetti, Lees, & Leeuwenburgh, 2010). Moreover, autophagy is required for several of the adaptive responses and adaptations to exercise and training (Grumati et al., 2011; He, Bassik, et al., 2012; Ju et al., 2016; Lira et al., 2013) including mitochondrial biogenesis and quality (Grumati et al., 2011; Ju et al., 2016; Lira et al., 2013). Health and population of mitochondria possess a number of positive outcomes in healthy and diseased conditions; improved aerobic metabolism and performance (Hawley, Hargreaves, Joyner, & Zierath, 2014), longevity (Hill & Van Remmen, 2014), as well as attenuated inflammation, oxidative stress, and insulin resistance (Martin & McGee, 2014). Thus, autophagy

may represent a key mechanism in potentiating the beneficial responses to exercise training that may be at least, in part, mediated by improved mitochondrial content and quality.

The exercise-autophagy response is regulated in a duration and intensity-dependent manner (Jamart, Benoit, et al., 2012; Jamart, Francaux, et al., 2012; Schwalm et al., 2015). Few data exist relating to acute exercise and skeletal muscle autophagy activity in humans; however, current evidences suggest exercise durations of greater than 60 minutes of moderate to moderately-high intensities (>55 – 70% VO_{2max}) are required for autophagy activation (Jamart, Benoit, et al., 2012; Jamart, Francaux, et al., 2012; Moller et al., 2015; Schwalm et al., 2015). It is unclear whether exercise of short duration and high intensity can elicit an autophagic response similar to that of longer duration, moderate intensity exercise.

Traditional exercise prescriptions are that of moderate intensity, continuous nature (L. Pescatello & Medicine, 2014). These exercise guidelines require significant training volumes and time commitment, resulting in low adherence (Qiu, Sun, Cai, Liu, & Yang, 2012; Statistics, 2012; Trost, Owen, Bauman, Sallis, & Brown, 2002). However, high intensity interval training (HIIT) is an increasingly practiced training method amongst recreationally active populations, in part due to its reduced time requirement. High intensity interval exercise is characterized by repeated bouts of brief (~30 sec – 3 min) high intensity (80 – 100% VO_{2max}, HR_{max}, peak power) exercise followed by periods of rest or low intensity exercise (~30 sec – 4 min) that result in ~10 minutes of overall work in training sessions of ~20 minutes (Gibala, Gillen, & Percival, 2014; Gillen & Gibala, 2014). -Several studies demonstrate that HIIT training can elicit equal or even superior physiological and health changes, including mitochondrial biogenesis, as traditional, moderate intensity, continuous training (MICT) when amounts of work are similar or even lower (Gibala et al., 2014; Gillen & Gibala, 2014; Little, Safdar, Bishop, Tarnopolsky, & Gibala, 2011). Therefore, this type of exercise may represent an efficacious and time efficient means to incur the benefits of exercise. This is significant insofar as that lack of time is often reported to be a barrier

to physical activity for individuals (Trost et al., 2002). The role of autophagy in mediating these robust health effects of HIIT is currently unknown. Given the shared myocellular signaling between MICT and HIIT that converge on key regulators of autophagy (i.e. AMPK, SIRT1, p53, p38MAPK) (Bartlett et al., 2012; Combes et al., 2015) as well as the common physiological outcomes to training (Gibala et al., 2006), HIIT may represent a means to stimulate muscle contraction-induced autophagy.

Studying muscle contraction-induced autophagy would shed light on the mechanisms underlying the health-positive effects of exercise. Currently, a basic approach does not exist. Electrical pulse stimulation (EPS) in cultured skeletal muscle cells (i.e. C2C12 cells) has recently emerged as an *in vitro* model of exercise (Evers-van Gogh et al., 2015; Nieuwoudt et al., 2017; Nikolic et al., 2012; Tarum, Folkesson, Atherton, & Kadi, 2017). This model has been employed to study molecular mechanisms of exercise-induced responses and adaptations in skeletal muscle by stimulating contraction of cultured myotubes through electrical pulse delivered by carbon electrodes immersed in cell culture medium (Nikolic et al., 2017). Canonical *in vivo* exercise signaling transduction has been shown to be produced by EPS including modulation of positive autophagy-related signals including AMPK, p38MAPK, and Ca++ and metabolic effects associated with autophagy such as enhanced insulin sensitivity, glucose uptake, GLUT4 translocation, and markers of mitochondrial biogenesis (Nikolic et al., 2017). Presently, however, the use of EPS to stimulate autophagy has not been documented. Acute starvation (4 hours) of C2C12 cells, however, has been shown to function as a model to stimulate autophagy (Desgeorges et al., 2014). The effects of starvation in combination of EPS are unknown.

Establishing an *in vitro* model of muscle contraction-induced autophagy would represent a means to understand the molecular underpinnings involved in the autophagy-mediated exercise adaptations and health outcomes. A functional model may be used to elucidate the underlying the metabolic responses and training outcomes, including in pathological conditions such as insulin

resistance and mitochondrial dysfunction. This has implications for understanding the health-positive effects of exercise as well as to identify molecular targets for pharmacological interventions.

Problem Statement

No data exist investigating autophagic activity following an acute bout of HIIT exercise. Additionally, an *in vitro* model of muscle contraction-induced autophagy using EPS does not exist.

Purpose of Study

The purpose of this investigation is twofold: firstly, to compare the autophagy responses of an acute bout of HIIT exercise (treadmill running) to MICT exercise in human skeletal muscle 3 hours post exercise; secondly, to test an in vitro model of muscle contraction-induced autophagy using electrical pulse stimulation in C2C12 myotubes.

Hypotheses

The following hypotheses will be tested in study 1: The effects of an acute bout of HIIT autophagy compared to MICT in human skeletal muscle

Hypothesis 1: HIIT and MICT will upregulate markers of autophagy activity (LC3) similarly in human skeletal muscle

The following hypotheses will be tested in study 2: Using electrical pulse stimulation as an *in vitro* model for muscle contraction-induced autophagy

Hypothesis 1: Short-term, low frequency EPS will upregulate markers of autophagy in C2C12 myotubes

Scope of Study

Study 1: The effects of an acute bout of HIIT on p53, autophagy, and mitochondrial biogenesis compared to MICT in human skeletal muscle

Using a crossover design, ten healthy, active young adult-aged males and females (18 – 30 years old) who have been engaging in regular physical activity (> 150 minutes of moderate to vigorous intensity aerobic activity per week for a minimum of 1 year) performed a bout of HIIT and MICT exercise separated by at least 72 hours in randomized order. Subjects were classified as "low risk" according to criteria put forth by the American College of Sports Medicine. Muscle biopsies were obtained from the thigh (*vastus lateralis* muscle) pre and 3 hours post-exercise. Subjects were allowed consume water ad libitum, but food intake was prohibited during the 3-hour post-exercise period as energy intake alters autophagic activity. Muscle tissue obtained from muscle biopsy was analyzed for markers of autophagy.

Study 2: Using electrical pulse stimulation as an *in vitro* model for muscle contraction-induced autophagy

Using a C2C12 model, myoblasts were differentiated into myotubes and underwent an 8 hour, low-frequency EPS in fed and starvation conditions with and without bafilomycin A1. Cells were collected immediately following the EPS protocol and analyzed for markers of autophagy.

Assumptions

The following assumptions will be made in this study:

1. Prior to the first visit, the participant will have refrained from exercise for 24 hours, alcohol for 24 hours, and caffeine for 8 hours.
2. Prior to the second and third visit, the participant will have refrained from exercise for 72 hours, alcohol for 24 hours, caffeine for 8 hours, and food for 8 hours.

3. Each participant performed VO_{2max} testing to their maximal capacity to yield a valid appropriate $\%VO_2max$ value used during HIIT and MICT exercise bouts.

Limitations

1. The study sample was comprised of healthy, active 18 – 30-year-old males and females; thus findings may not be generalized to individuals outside of these parameters.

2. The reproducibility and generalization of in vitro models, such as C2C12 model, may be limited in reflecting the phenomena in *in vivo*; thus, the use of EPS in C2C12 cells may be limited in its ability to represent what occurs in human skeletal muscle in response to exercise.

3. C2C12 myotubes are mice muscle cells, thus translating findings to human muscle may be limited.

Significance of the Study

Autophagy is an increasingly implicated mediator of the acute responses and the beneficial outcomes of exercise. Few data exist pertaining to the autophagic response to acute exercise in human skeletal muscle. Moreover, the evidences that do exist documenting exercise-induced autophagy in humans have used long duration, moderate intensity exercise protocols; the response to HIIT has not been investigated. Given the increasing prevalence in HIIT as a training method amongst active populations and the growing consensus of its efficacy as a mode of improving health, a characterization of the autophagic response to this mode of exercise is warranted. Findings of this study may elucidate the cellular underpinnings of the beneficial physiological adaptations of HIIT within skeletal muscle that facilitate human health and may better inform efficacious exercise programming.

Additionally, EPS has been used as an *in vitro* model to study the metabolic responses and molecular mechanisms of muscle contraction; however, an EPS protocol for an autophagic response does not exist. An *in vitro* muscle contraction-induced autophagy model provides a means to study autophagy signaling transduction as well as to study the mechanisms in underlying the metabolic responses and training outcomes, including in pathological conditions such as insulin resistance and mitochondrial dysfunction. This holds implications for understanding the health-positive effects of exercise as well as identify molecular targets for pharmacological interventions.

Definition of Terms

The terms in this study has been defined as follows:

Autophagy: An evolutionary-conserved cellular recycling system present in all eukaryotic cells responsible for the degradation of damaged, aged, and dysfunctional organelles and proteins.

Bafilomycin: A vacuolar-ATPase inhibitor used to neutralize the pH of the lysosome and inhibit the degradation of the autophagsome in autophagy.

C2C12: Immortalized mouse myoblast cell line capable of differentiation into myotubes. A common mechanistic model for the study of skeletal muscle.

Electrical Pulse Stimulation: An in vitro exercise model using electrical pulses stimulation via immersed electrodes in C2C12 cell media leading to the contraction of cultured myotubes. EPS has been shows to stimulate exercise-induced hormone secretion, substrate utilization and metabolic signaling, protein synthesis, and gene expression.

High-intensity interval training (HIIT): Repeated bouts of short duration (~30 sec – 3 min) maximal or near maximal intensity (80 – 100% VO_{2max}, HR_{max}, peak power) exercise followed by periods of rest or low intensity exercise (~30 sec – 4 min)

Oxygen uptake (VO_2): The rate of oxygen consumption and utilization

Maximal oxygen uptake (VO_{2max}): The maximal rate of oxygen consumption and utilization per minute of exercise

Mitochondrial biogenesis: The growth and division of pre-existing mitochondria involving coordination between nuclear DNA and mitochondrial DNA

Moderate intensity, continuous training (MICT): Uninterrupted, steady state, aerobic-based exercise at submaximal intensities (50 – 85% VO_{2max}, HR_{max}, peak power)

Western blot analysis: A technique used to detect specific proteins in a sample of tissue homogenate or extract.

References

Balaburski, G. M., Hontz, R. D., & Murphy, M. E. (2010). p53 and ARF: unexpected players in autophagy. *Trends Cell Biol, 20*(6), 363-369. doi:10.1016/j.tcb.2010.02.007

Bartlett, J. D., Close, G. L., Drust, B., & Morton, J. P. (2014). The emerging role of p53 in exercise metabolism. *Sports Med, 44*(3), 303-309. doi:10.1007/s40279-013-0127-9

Bartlett, J. D., Hwa Joo, C., Jeong, T. S., Louhelainen, J., Cochran, A. J., Gibala, M. J., . . . Morton, J. P. (2012). Matched work high-intensity interval and continuous running induce similar increases in PGC-1alpha mRNA, AMPK, p38, and p53 phosphorylation in human skeletal muscle. *J Appl Physiol (1985), 112*(7), 1135-1143. doi:10.1152/japplphysiol.01040.2011

Booth, F. W., Roberts, C. K., & Laye, M. J. (2012). Lack of exercise is a major cause of chronic diseases. *Compr Physiol, 2*(2), 1143-1211. doi:10.1002/cphy.c110025

Combes, A., Dekerle, J., Webborn, N., Watt, P., Bougault, V., & Daussin, F. N. (2015). Exercise-induced metabolic fluctuations influence AMPK, p38-MAPK and CaMKII phosphorylation in human skeletal muscle. *Physiol Rep, 3*(9). doi:10.14814/phy2.12462

Cuervo, A. M. (2008). Autophagy and aging: keeping that old broom working. *Trends Genet, 24*(12), 604-612. doi:10.1016/j.tig.2008.10.002

Dokladny, K., Zuhl, M. N., Mandell, M., Bhattacharya, D., Schneider, S., Deretic, V., & Moseley, P. L. (2013). Regulatory coordination between two major intracellular homeostatic systems: heat shock response and autophagy. *J Biol Chem, 288*(21), 14959-14972. doi:10.1074/jbc.M113.462408

Fiorini, C., Menegazzi, M., Padroni, C., Dando, I., Dalla Pozza, E., Gregorelli, A., . . . Donadelli, M. (2013). Autophagy induced by p53-reactivating molecules protects pancreatic cancer cells from apoptosis. *Apoptosis, 18*(3), 337-346. doi:10.1007/s10495-012-0790-6

Ghareghani, P., Shanaki, M., Ahmadi, S., Khoshdel, A. R., Rezvan, N., Meshkani, R., . . . Gorgani-Firuzjaee, S. (2017). Aerobic endurance training improves nonalcoholic fatty liver disease (NAFLD) features via miR-33 dependent autophagy induction in high fat diet fed mice. *Obes Res Clin Pract*. doi:10.1016/j.orcp.2017.01.004

Gibala, M. J., Gillen, J. B., & Percival, M. E. (2014). Physiological and health-related adaptations to low-volume interval training: influences of nutrition and sex. *Sports Med, 44 Suppl 2*, S127-137. doi:10.1007/s40279-014-0259-6

Gibala, M. J., Little, J. P., van Essen, M., Wilkin, G. P., Burgomaster, K. A., Safdar, A., . . . Tarnopolsky, M. A. (2006). Short-term sprint interval versus traditional endurance training: similar initial adaptations in human skeletal muscle and exercise performance. *J Physiol, 575*(Pt 3), 901-911. doi:10.1113/jphysiol.2006.112094

Gillen, J. B., & Gibala, M. J. (2014). Is high-intensity interval training a time-efficient exercise strategy to improve health and fitness? *Appl Physiol Nutr Metab, 39*(3), 409-412. doi:10.1139/apnm-2013-0187

Grumati, P., Coletto, L., Schiavinato, A., Castagnaro, S., Bertaggia, E., Sandri, M., & Bonaldo, P. (2011). Physical exercise stimulates autophagy in normal skeletal muscles but is detrimental for collagen VI-deficient muscles. *Autophagy, 7*(12), 1415-1423.

Hawley, J. A., Hargreaves, M., Joyner, M. J., & Zierath, J. R. (2014). Integrative biology of exercise. *Cell, 159*(4), 738-749. doi:10.1016/j.cell.2014.10.029

He, C., Bassik, M. C., Moresi, V., Sun, K., Wei, Y., Zou, Z., . . . Levine, B. (2012). Exercise-induced BCL2-regulated autophagy is required for muscle glucose homeostasis. *Nature, 481*(7382), 511-515. doi:10.1038/nature10758

He, C., Sumpter, R., Jr., & Levine, B. (2012). Exercise induces autophagy in peripheral tissues and in the brain. *Autophagy, 8*(10), 1548-1551. doi:10.4161/auto.21327

Hill, S., & Van Remmen, H. (2014). Mitochondrial stress signaling in longevity: a new role for mitochondrial function in aging. *Redox Biol, 2*, 936-944. doi:10.1016/j.redox.2014.07.005

Jamart, C., Benoit, N., Raymackers, J. M., Kim, H. J., Kim, C. K., & Francaux, M. (2012). Autophagy-related and autophagy-regulatory genes are induced in human muscle after ultraendurance exercise. *Eur J Appl Physiol, 112*(8), 3173-3177. doi:10.1007/s00421-011-2287-3

Jamart, C., Francaux, M., Millet, G. Y., Deldicque, L., Frere, D., & Feasson, L. (2012). Modulation of autophagy and ubiquitin-proteasome pathways during ultra-endurance running. *J Appl Physiol (1985), 112*(9), 1529-1537. doi:10.1152/japplphysiol.00952.2011

Ju, J. S., Jeon, S. I., Park, J. Y., Lee, J. Y., Lee, S. C., Cho, K. J., & Jeong, J. M. (2016). Autophagy plays a role in skeletal muscle mitochondrial biogenesis in an endurance exercise-trained condition. *J Physiol Sci, 66*(5), 417-430. doi:10.1007/s12576-016-0440-9

Li, H., Miao, W., Ma, J., Xv, Z., Bo, H., Li, J., . . . Ji, L. L. (2016). Acute Exercise-Induced Mitochondrial Stress Triggers an Inflammatory Response in the Myocardium via NLRP3 Inflammasome Activation with Mitophagy. *Oxid Med Cell Longev, 2016*, 1987149. doi:10.1155/2016/1987149

Lira, V. A., Okutsu, M., Zhang, M., Greene, N. P., Laker, R. C., Breen, D. S., . . . Yan, Z. (2013). Autophagy is required for exercise training-induced skeletal muscle adaptation and improvement of physical performance. *FASEB J, 27*(10), 4184-4193. doi:10.1096/fj.13-228486

Little, J. P., Safdar, A., Bishop, D., Tarnopolsky, M. A., & Gibala, M. J. (2011). An acute bout of high-intensity interval training increases the nuclear abundance of PGC-1alpha and activates mitochondrial biogenesis in human skeletal muscle. *Am J Physiol Regul Integr Comp Physiol, 300*(6), R1303-1310. doi:10.1152/ajpregu.00538.2010

Maiuri, M. C., Galluzzi, L., Morselli, E., Kepp, O., Malik, S. A., & Kroemer, G. (2010). Autophagy regulation by p53. *Curr Opin Cell Biol, 22*(2), 181-185. doi:10.1016/j.ceb.2009.12.001

Martin, S. D., & McGee, S. L. (2014). The role of mitochondria in the aetiology of insulin resistance and type 2 diabetes. *Biochim Biophys Acta, 1840*(4), 1303-1312. doi:10.1016/j.bbagen.2013.09.019

Mejias-Pena, Y., Estebanez, B., Rodriguez-Miguelez, P., Fernandez-Gonzalo, R., Almar, M., de Paz, J. A., . . . Cuevas, M. J. (2017a). Impact of resistance training on the autophagy-inflammation-apoptosis crosstalk in elderly subjects. *Aging (Albany NY)*. doi:10.18632/aging.101167

Mejias-Pena, Y., Estebanez, B., Rodriguez-Miguelez, P., Fernandez-Gonzalo, R., Almar, M., de Paz, J. A., . . . Cuevas, M. J. (2017b). Impact of resistance training on the autophagy-inflammation-apoptosis crosstalk in elderly subjects. *Aging (Albany NY), 9*(2), 408-418. doi:10.18632/aging.101167

Mejias-Pena, Y., Rodriguez-Miguelez, P., Fernandez-Gonzalo, R., Martinez-Florez, S., Almar, M., de Paz, J. A., . . . Gonzalez-Gallego, J. (2016). Effects of aerobic training on markers of autophagy in the elderly. *Age (Dordr), 38*(2), 33. doi:10.1007/s11357-016-9897-y

Moller, A. B., Vendelbo, M. H., Christensen, B., Clasen, B. F., Bak, A. M., Jorgensen, J. O., . . . Jessen, N. (2015). Physical exercise increases autophagic signaling through ULK1 in human skeletal muscle. *J Appl Physiol (1985), 118*(8), 971-979. doi:10.1152/japplphysiol.01116.2014

Pescatello, L., & Medicine, A. C. o. S. (2014). *ACSM's guidelines for exercise testing and prescription* (9th ed.). Philadelphia, pennsylvania: Wolters Kluwer/Lippincott Williams & Wilkins Health.

Qiu, S. H., Sun, Z. L., Cai, X., Liu, L., & Yang, B. (2012). Improving patients' adherence to physical activity in diabetes mellitus: a review. *Diabetes Metab J, 36*(1), 1-5. doi:10.4093/dmj.2012.36.1.1

Salminen, A., & Kaarniranta, K. (2009). Regulation of the aging process by autophagy. *Trends Mol Med, 15*(5), 217-224. doi:10.1016/j.molmed.2009.03.004

Schwalm, C., Jamart, C., Benoit, N., Naslain, D., Premont, C., Prevet, J., . . . Francaux, M. (2015). Activation of autophagy in human skeletal muscle is dependent on exercise intensity and AMPK activation. *FASEB J, 29*(8), 3515-3526. doi:10.1096/fj.14-267187

Statistics, N. C. f. H. (2012). *Health, United States, 2011: With Special Feature on Socioeconomic Health*. Retrieved from Hyattsville, MD:

Tachtsis, B., Smiles, W. J., Lane, S. C., Hawley, J. A., & Camera, D. M. (2016). Acute Endurance Exercise Induces Nuclear p53 Abundance in Human Skeletal Muscle. *Front Physiol, 7*, 144. doi:10.3389/fphys.2016.00144

Tam, B. T., Pei, X. M., Yu, A. P., Sin, T. K., Leung, K. K., Au, K. K., . . . Siu, P. M. (2015). Autophagic adaptation is associated with exercise-induced fibre-type shifting in skeletal muscle. *Acta Physiol (Oxf), 214*(2), 221-236. doi:10.1111/apha.12503

Tam, B. T., Pei, X. M., Yung, B. Y., Yip, S. P., Chan, L. W., Wong, C. S., & Siu, P. M. (2015). Autophagic Adaptations to Long-term Habitual Exercise in Cardiac Muscle. *Int J Sports Med, 36*(7), 526-534. doi:10.1055/s-0034-1398494

Tam, B. T., & Siu, P. M. (2014). Autophagic cellular responses to physical exercise in skeletal muscle. *Sports Med, 44*(5), 625-640. doi:10.1007/s40279-013-0140-z

Todde, V., Veenhuis, M., & van der Klei, I. J. (2009). Autophagy: principles and significance in health and disease. *Biochim Biophys Acta, 1792*(1), 3-13. doi:10.1016/j.bbadis.2008.10.016

Trost, S. G., Owen, N., Bauman, A. E., Sallis, J. F., & Brown, W. (2002). Correlates of adults' participation in physical activity: review and update. *Med Sci Sports Exerc, 34*(12), 1996-2001. doi:10.1249/01.MSS.0000038974.76900.92

Vainshtein, A., Grumati, P., Sandri, M., & Bonaldo, P. (2014). Skeletal muscle, autophagy, and physical activity: the menage a trois of metabolic regulation in health and disease. *J Mol Med (Berl), 92*(2), 127-137. doi:10.1007/s00109-013-1096-z

Vainshtein, A., & Hood, D. A. (2016). The regulation of autophagy during exercise in skeletal muscle. *J Appl Physiol (1985), 120*(6), 664-673. doi:10.1152/japplphysiol.00550.2015

Wohlgemuth, S. E., Seo, A. Y., Marzetti, E., Lees, H. A., & Leeuwenburgh, C. (2010). Skeletal muscle autophagy and apoptosis during aging: effects of calorie restriction and life-long exercise. *Exp Gerontol, 45*(2), 138-148. doi:10.1016/j.exger.2009.11.002

Chapter 2: Review of Related Literature

Autophagy and Human Health

Autophagy is proteostatic system that has been highly conserved throughout evolution and is present in all known eukaryotic cells, from yeast to humans, and is involved in maintaining the intracellular environment (Cuervo, 2008; Levine & Klionsky, 2004; Most, Tosti, Redman, & Fontana, 2016). The umbrella term "autophagy" is often subdivided into three primary pathways, each dependent on lysosomal degradation, which are: chaperone mediated autophagy, microautophagy, and macroautophagy (Todde et al., 2009). Macroautophagy is currently best-understood and will serve as the focus in the current discussion, being referred to as autophagy hereafter. This process functions through nonselective bulk degradation of cellular material including organelles, cytosolic proteins, and protein aggregates; all of which are sequestered by double-membrane vesicles called autophagosomes and then transported to the lysosome for degradation where the constituent products may then be used to provision substrates (Rubinsztein, Marino, & Kroemer, 2011; Todde et al., 2009).

Cellular homeostasis involves management of the cytosolic proteome and organelle population via tightly-regulated biosynthesis and degradation processes (Todde et al., 2009). Autophagy serves as a primary mechanism, along with the ubiquitin proteome system (UPS), in regulating these processes (Dokladny, Myers, & Moseley, 2015) and is essential for cellular and organismal survival (Todde et al., 2009). Accrual and aggregation of defunct cytosolic components result in disruption of the proteome, progressive degeneration, and increases the risk of cell death (Cuervo et al., 2005; Terman, Gustafsson, & Brunk, 2007). Knockout mice, in which autophagy is systemically blocked, die within hours of birth (Komatsu et al., 2005). Accordingly, it has been proposed that escalating malfunction in this key regulatory process required for the maintenance, repair, and turnover of defective protein structures and organelles is likely to represent a primary cause of the cumulative cellular disorganization associated with chronic

disease and aging (Cuervo et al., 2005; Todde et al., 2009; Vellai, Takacs-Vellai, Sass, & Klionsky, 2009).

Malfunctioning autophagy is implicated in a number of degenerative conditions including type 2 diabetes (Gonzalez et al., 2011; Quan, Jung, & Lee, 2013), neurodegeneration (Komatsu et al., 2006; F. Yang et al., 2014), cardiomyopathy (Nair & Ren, 2012; Tanaka et al., 2000), cancer (Cao & Klionsky, 2007; Cecconi & Levine, 2008), chronic inflammation (Jo, Shin, & Choi, 2012; Levine, Mizushima, & Virgin, 2011) as well as attenuated muscle quality and function (Fan et al., 2016; Vainshtein et al., 2014). Further, impaired autophagy is associated with the aging process where autophagic function is attenuated in aged populations (Salminen & Kaarniranta, 2009). Moreover, in experimental conditions, compromised autophagic capability reduces lifespan and precipitates premature aging in numerous model species (Alvers et al., 2009; Hars et al., 2007; Juhasz, Erdi, Sass, & Neufeld, 2007; Kang, You, & Avery, 2007; Toth et al., 2008) while enhanced autophagy has been shown to promote longevity (Eisenberg et al., 2009; Pyo et al., 2013; Simonsen et al., 2008).

A decline in overall proteolytic activity instigates a progressive accumulation of damaged proteins in a broad range of organisms (Liang & Jung, 2010; Rajawat & Bossis, 2008; Ward, 2002). A loss of autophagic activity in cells is likely to increasingly constrain the ability of the cell to sustain a healthy proteome and organelle population, contributing to a progressive loss of cellular function, and eventually precipitating cell death (Cuervo & Macian, 2014; Rubinsztein et al., 2011). Though the mechanisms underlying the escalating impairment of autophagic function in degenerative conditions remain poorly understood, decreased autophagy-related protein (Atgs) expression at the mRNA and protein level has been implicated as a contributing factor in age-related pathologies (Carames, Taniguchi, Otsuki, Blanco, & Lotz, 2010; Lipinski et al., 2010; Rubinsztein et al., 2011). It has also been reported that ancillary proteins necessary for the induction of autophagy, such as Sirtuin 1 (SIRT1), display a similarly reduced expression in aged cells, concomitant with diminished autophagy (de Kreutzenberg et al., 2010; Rubinsztein et al.,

2011). At present, it remains unclear whether these decrements in Atgs and/or upstream signaling targets are the primary source of diseased or age-related autophagic malfunction (Rubinsztein et al., 2011), as it has also been suggested that the decline in basal autophagy may be at least partially mediated by excess mammalian target of rapamycin complex 1 (mTORC1) activity in some degenerative conditions, such as obesity, type 2 diabetes, cancer, and aging (Lee et al., 2010; Pani, 2011; S. Xu, Cai, & Wei, 2014). However, positively-dysregulated autophagy can facilitate and/or be present in certain diseased states and reducing autophagic activity can produce protective effects (Cherra, Dagda, & Chu, 2010; Kim et al., 2013; E. Y. Liu & Ryan, 2012).

The apparent role of autophagic function in diseased and aged conditions is interesting as dysregulation of 5' adenosine monophosphate-activated kinase (AMPK) and mammalian target of rapamycin complex 1 (mTORC1) activity is implicated in many of these degenerative diseases and aging and have become therapeutic targets in these conditions (Carling, 2017; Day, Ford, & Steinberg, 2017; Foretz, Guigas, Bertrand, Pollak, & Viollet, 2014; Johnson, Rabinovitch, & Kaeberlein, 2013; Richter & Ruderman, 2009; Zoncu, Efeyan, & Sabatini, 2011); this may, at least in part, be due to their regulatory control over autophagic activity (Hardie, 2011; Meijer, Lorin, Blommaart, & Codogno, 2015; Todde et al., 2009).

A potential key mechanism of autophagy may influence cellular function and human health is through the maintenance of mitochondrial function and content (Grumati et al., 2011; Ju et al., 2016; Lira et al., 2013; Shaik, Schiavi, & Ventura, 2016). Concurrent with defective autophagy, mitochondrial dysfunction is implicated in a number of degenerative diseases and occurs during aging (D. A. Hood, 2009; Jazwinski, 2015; Martin & McGee, 2014; Shaik et al., 2016). It has been proposed that a causative relationship between altered autophagy and mitochondrial dysfunction exists (Kobayashi & Liang, 2015). Indeed, transgenic mice models altering the expression of essential autophagy-related genes (Atgs) resulted in compromised mitochondrial function and reduced cytochrome protein expression (Lira et al., 2013; Masiero et al., 2009; Raben et al., 2008). Further, acute autophagy inhibition at the level of autophagosome

degradation abolished exercise training induced increases in mitochondrial proteins in mice (Ju et al., 2016). A potential link between autophagy and mitochondrial dysfunction may be the mitochondrial production of reactive oxygen species (ROS) whereby defective autophagy is associated with increased production and accumulation of ROS, a characteristic of several degenerative conditions, including insulin resistance (Gonzalez et al., 2011; Kobayashi & Liang, 2015; Wu et al., 2009). Additionally, mitochondrial dysfunction results in the accumulation of cytotoxic lipid intermediates which exerts deleterious effects on the insulin signaling pathway and promotes insulin resistance (Martin & McGee, 2014). These data suggest an inadequacy in autophagy to maintain proper turnover of mitochondria (mitophagy) and sustain a healthy mitochondrial population is likely involved in the development of degenerative conditions (Higgins & Coughlan, 2014; Shaik et al., 2016; Wu et al., 2009). Thus, the ability of autophagy to maintain mitochondrial function is likely key in its promotion of health.

Exercise Autophagy Signaling

The autophagic response to exercise appears to occur in a biphasic manner in that acute cellular perturbations induce a precipitous increase in autophagic flux occurring immediately in response to insult followed by posttranslational protein modification (Vainshtein & Hood, 2016). Moreover, autophagy appears to work in concert with another major proteolytic pathway, the UPS, whereby the immediate cellular degradation activity is mediated by the UPS, while autophagy activity demonstrates a slightly delayed response (Tam & Siu, 2014; Vainshtein & Hood, 2016); however, both systems have been shown to be activated simultaneously in some conditions (Jamart, Benoit, et al., 2012; Jamart, Francaux, et al., 2012). Various proteins, designated as Atgs, are required for autophagosome formation and sequestration of cytosolic components while separate proteins involved in other heterotypic and homotypic lysosomal membrane fusion facilitate the final step of delivery of cargo to into the lysosome (Todde et al., 2009). Key initial regulators of autophagy are AMPK and mTORC1, where AMPK serves as a

positive regulator (Hardie, 2014) and mTORC1 exerts inhibitory effects (Meijer et al., 2015). It is well established that acute exercise are potent modulators of AMPK and mTORC1 (Coffey & Hawley, 2007; Hawley et al., 2014) and thus play a major role in autophagy signaling during exercise (Vainshtein & Hood, 2016). Both kinases affect the immediate autophagy activation and the activation of autophagic transcriptional programs (Vainshtein & Hood, 2016)

Autophagy is sensitive to cellular energy status, where during states in which energy supply is in abundance or equilibrium with demand, mTORC1 negatively regulates the autophagic machinery by inhibiting autophagosome formation (Meijer et al., 2015). Conversely, when energy status is challenged, AMPK initiates autophagy (Hardie, 2011). Exercise instigates major energetic stress; accordingly, exercise has been shown to augment acute autophagic activity in skeletal muscle (Jamart, Benoit, et al., 2012; Jamart, Francaux, et al., 2012; Tam, Pei, Yu, et al., 2015; Vainshtein & Hood, 2016) as well as several other tissues (Dokladny et al., 2013; Ghareghani et al., 2017; He, Bassik, et al., 2012; He, Sumpter, et al., 2012; Li et al., 2016; Mejias-Pena et al., 2017a; Mejias-Pena et al., 2016). Alterations in ATP and AMP concentrations stimulates AMPK which functions to inhibit energy-consuming pathways (i.e. mTORC1 signaling pathway) and activates catabolic pathways to supply substrates for energy production (Aschenbach, Sakamoto, & Goodyear, 2004; J. Xu, Ji, & Yan, 2012). Autophagy functions to provision emergency alternative energy sources within skeletal muscle during exercise (Tam & Siu, 2014; Vainshtein et al., 2014). However, a number of other cellular challenges elicited by exercise promote increased autophagic activity in exercised muscle as well, including widespread protein and/or mitochondrial damage, elevated mitochondrial respiration, high concentrations of reactive oxygen species (ROS), the presence of certain cytokines, and various elements of the immune response (Tam, Pei, Yu, et al., 2015; Vainshtein & Hood, 2016).

In addition to providing substrates for ATP resynthesis during energetic challenge, autophagy mediates the clearance of proteins and organelles damaged by heat, pH changes, or mechanical stress which likely acts to prevent accumulation of these cytosolic components and

maintain myocyte function (Schwalm et al., 2015; Vainshtein et al., 2014). Moreover, alterations in calcium, NAD^+, and ROS levels also are strong instigators of autophagic activity (Vainshtein & Hood, 2016). As such, the magnitude of the autophagic response to exercise depends in part on the extent of cellular stress and protein damage (Schwalm et al., 2015; Vainshtein & Hood, 2016). Unlike other tissues such as the liver and pancreas, upregulation of autophagy in skeletal muscle persists for days, rather than hours, following a period of energy insufficiency, indicating an elevated importance of autophagic function in skeletal muscle proteostasis (Mizushima, Yamamoto, Matsui, Yoshimori, & Ohsumi, 2004; Sandri, 2010).

Exercise activates AMPK and SIRT1 which are sensitive to alterations in AMP and NAD^+, respectively (Hawley et al., 2014). AMPK and SIRT1 both act to upregulate expression of Atgs by activating several transcription factors increasing PGC-1α activity, as well as initiating autophagosome formation via AMPK activation and mTORC1 inhibition (Vainshtein & Hood, 2016). While the mechanisms underlying SIRT1 regulation of mTORC1 largely remain unclear, it is hypothesized that SIRT1 may act through interaction with tuberous sclerosis complex 2 (TSC2), a known mTORC1 inhibitor (Ghosh, McBurney, & Robbins, 2010; Ma et al., 2015). The relationship between AMPK and mTORC1 however, is well-characterized. AMPK acts to suppress mTORC1 activity in at least two ways: firstly, by activating TSC2, which prevents mTORC1 from binding to a key activator, ras homologue in brain (Rheb), on the lysosomal membrane (Inoki, Zhu, & Guan, 2003; Jung, Ro, Cao, Otto, & Kim, 2010); and secondly, through direct inhibitory phosphorylation of a primary regulatory protein complex of mTORC1, known as RAPTOR (Gwinn et al., 2008; Jung et al., 2010). The downregulation of mTORC1 results in the release of its inhibitory phosphosrylation of Atg1 (ULK in mammals) on Ser757 promoting phagophore induction (an immature precursor to the autophagosome) (Vainshtein & Hood, 2016). AMPK initiates the autophagic machinery by promoting phagophore induction via ULK1 Ser555 phosphorylation (Hardie, 2011; He, Bassik, et al., 2012; Mooren & Kruger, 2015).

Autophagosome nucleation involves the disassociation of the beclin-1 complex from Bcl-2 which is instigated by exercise through Bcl-2 phosphorylation (Vainshtein & Hood, 2016). Mutant mice which cannot disassociate belcin-1 from Bcl-2 demonstrated normal basal autophagy, but compromised exercise-induced autophagy and hindered exercise performance and exercise-mediated metabolic adaptations (He, Bassik, et al., 2012). Further, other genetically manipulated autophagy-deficient models have resulted in similar abrogated exercise performance and metabolic improvements (He, Bassik, et al., 2012; Lira et al., 2013).

Nucleation and expansion of the autophagosome requires LC3-I lipidation to LC3-II which is mediated by various conjugation reactions by Atg proteins (Martin-Rincon, Morales-Alamo, & Calbet, 2017). LC3 exists in its inactive form in the cytosol and is cleaved by the protease Atg4 to form LC3-I (Vainshtein & Hood, 2016). During autophagasome maturation, LC3-I is conjugated into LC3-II and is attached to the phagophore membrane and subsequently degraded upon interaction with cargo receptor complexes (i.e. p62) on the lysosomal membrane (Vainshtein & Hood, 2016). LC3 lipidation has been shown to increase during exercise while autophagosome content is reduced in human skeletal muscle following moderate intensity continuous exercise (MICT) (Martin-Rincon et al., 2017). Dynamics of LC3-I and LC3-II conversion and autophagosome status is difficult to assess as it is cell-type and stimulus-specific; however, it may be speculated the trend observed in skeletal muscle in response to exercise may indicate an elevation of autophagic flux.

Exercise also results in activation of autophagy-related transcription programs. Forkhead box protein 1 and 3 (FOXO1 and FOXO3, respectively) transcription factors are phosphorylated by AMPK resulting in their translocation from the cytoplasm to nucleus where they can activate the transcription of Atgs and lysosome-related proteins (Martin-Rincon et al., 2017; Vainshtein & Hood, 2016). Transcription factor EB (TFEB), the primary regulator of cellular recycling that coordinates the expression of lysosomal and autophagic genes via the CLEAR (coordinated lysosomal expression and regulation) network (Sardiello et al., 2009; Settembre et al., 2012) has

been shown to be activated during muscle contraction as well (Medina et al., 2015). At rest, mTORC1 phosphorylates TFEB on the lysosomal surface, confining it in the cytosol. During exercise, TFEB translocates to the nucleus as a result of the disassociation of mTORC1 from the lysosome and its dephosphorylation by Ca^{++}-dependent calcineurin where it then activates the CLEAR network and the transcription of autophagy-related genes and proteins (Medina et al., 2015). PGC-1α has also been shown to be required for the induction of genes LC3 and p62 following exercise (Vainshtein & Hood, 2016).

Recently, a tumor suppressor protein referred to as the *Guardian of the Genome*, p53 has been identified as a regulator of autophagy (Maiuri et al., 2010; Maiuri et al., 2009) and is likely involved in exercise-induced autophagy (Tachtsis, Smiles, Lane, Hawley, & Camera, 2016). p53 regulation of autophagy appears to be determined by post-translational modification (Loughery, Cox, Smith, & Meek, 2014) and/or subcellular location (Bartlett, Close, Drust, & Morton, 2014; Maiuri et al., 2010). Nuclear p53 induces autophagy, whereas cytosolic p53 inhibits autophagy (Maiuri et al., 2010). Acute exercise has been shown to increase nuclear p53 abundance (Tachtsis et al., 2016) as well as increase phosphorylation of Ser15, which is indicative of increased stability and activity (Bartlett et al., 2012). In the single human study investigating the effect of exercise on p53 and autophagy activity documented that 1 hour of cycling at 70% VO_{2max} in untrained males increased nuclear localization of p53, but showed no changes in markers of autophagy (Tachtsis et al., 2016).

While currently ill-characterized, muscle contraction-induced p53 activity appears to involve a bidirectional cross-talk with AMPK, where AMPK results an initial activation of p53 via phosphorylation of Ser15 (Balaburski, Hontz, & Murphy, 2010; Bartlett et al., 2014; Saleem, Adhihetty, & Hood, 2009) while downstream target gene Sestrin2 may act to further upregulate AMPK as well as inhibit mTORC1 (Budanov & Karin, 2008; Dagon, Mantzoros, & Kim, 2015; Li, Liu, Yuan, Niu, & Fu, 2017; X. Liu, Niu, Yuan, Huang, & Fu, 2015; Wolfson et al., 2016) which may result in autophagy activation. Sestrin2 has emerged as a key downstream target of

p53 in modulating the autophagy activity (Balaburski et al., 2010; Li et al., 2017; X. Liu et al., 2015; Maiuri et al., 2009) and has been shown to be coimmunoprecipitated with AMPKα2 in response to acute exercise in mice (X. Liu et al., 2015). Transfected C2C12 myotubes inducing overexpression of Sestrin2 demonstrated an increased phosphorylation of ULK Ser555 (Li et al., 2017), suggesting AMPK mediates Sestrin2 stimulated autophagy. Further, it has been documented that Sestrin2 also is capable of directly associating with ULK1 and p62 to promote autophagic flux in HEK293 cells (Ro et al., 2014). While it is unclear if the responses occurring *in vitro* reflect the signaling events that occur in exercised human skeletal muscle, it is increasingly understood that Sestrin2 likely serves the primary mode of p53-autophagy signaling, although p53 is also involved with the transcription of PGC-1α (Bartlett et al., 2014).

Autophagic Response to Exercise in Human Skeletal Muscle

The exercise-elicited autophagic response appears to be regulated in a duration and intensity-dependent manner (Jamart, Benoit, et al., 2012; Schwalm et al., 2015; Tachtsis et al., 2016) (Table 1). In humans, it has recently been reported that 2 hours of moderately-high-intensity cycling (70% VO_{2peak}) results in greater activation of autophagy than low-intensity cycling (55% VO_2 peak) in the vastus lateralis (Schwalm et al., 2015). While both the high- and low-intensity exercise protocols resulted in reductions of LC3B-II and the LC3B-II/LC3B-I ratio (Klionsky et al., 2012), only the high intensity bout showed decreased protein levels of sequestosome 1, also known as p62. p62 is a bridging protein involved in delivering substrates to the autophagosome, and decreased p62 concentration serves as an indicator of elevated autophagic flux. Additionally, the transcription of autophagy-related genes was found to be upregulated following only the high-intensity bout, as measured by LC3B p62, GABARAPL1, and Cathespin L mRNA, while ULK1 Ser317 phosphorylation was also significantly upregulated for the high-intensity group alone. Importantly, AMPK activity was determined to be

significantly increased following the high-intensity bout implying an upregulation of autophagy and a downregulation of mTORC1 (Schwalm et al., 2015).

Jamart and colleagues collected muscle biopsies from the vastus lateralis of 11 male ultra-endurance athletes 2 hours prior to and immediately following a mean of 24 hours of treadmill running (Jamart, Francaux, et al., 2012). Energy expenditure was calculated using run distance, running time, and running economy while energy intake was monitored throughout the bout. Energy intake during exercise covered 30% of energy needs resulting in caloric deficit over the 24 hours. Phosphorylation of Akt (an upstream activator of mTORC1), mTORC1, and 4E-BP1 was found to be decreased, while conversely, FOXO3a and AMPK activity was significantly increased. With regard to direct autophagic markers, LC3-II protein, an Atg8 homologue, was reported to have increased post-exercise by a remarkable ~550% of baseline. This was accompanied by a significant concomitant increase in Atg12-Atg5 conjugation, another indication of autophagosome formation (Jamart, Francaux, et al., 2012). Similarly, the same group reported in a separate study (without monitoring energetics) that free-running ultra-marathon performance (lasting ~ 28 hours) lead to increased transcription of a numerous Atgs (Atg4b, Atg12, GABARAPL1, and LC3) in the vastus lateralis, with the magnitude of the increase ranging from 59 to 286% (Jamart, Benoit, et al., 2012).

In contrast, Moller and colleagues (Moller et al., 2015) showed that 60 minutes of cycling exercise at 50% VO_{2max} resulted in a decreased LC3-II/LC3-I ratio but reported no reduction of p62 in the vastus lateralis 90 minutes following exercise. No changes in Atgs were noted despite an upregulation of AMPK and ULK1 Ser555.Similarly, muscle biopsies taken before and after 20 minutes of low intensity cycling (~50% VO2max) have been reported to reveal no significant impact on a variety of autophagic makers, including protein expression of p62, LC3-I, and LC3-II, with no associated change in phosphorylated AMPK (Masschelein et al., 2014). And whereas Schwalm et al. (Schwalm et al., 2015) showed 2 hours of cycling at 70% VO_{2max} was sufficient in eliciting increases in autophagic flux and a number of Atgs in well-trained athletes. Tachtsis et al.

(Tachtsis et al., 2016) reported no changes in ULK1, LC3B-I, LC3B-II, or p62 protein expression following 1 hour of cycling at 70% VO_{2max} in untrained males despite an increased nuclear localization of p53. These findings help highlight the importance of exercise duration and intensity in stimulating autophagic induction and point to a threshold for activation, likely involving AMPK-mediated determination of energy insufficiency. Importantly, the extreme elevations in autophagic activity observed with ultra-endurance performance are likely indicative of excessive muscle damage and energetic protein catabolism, thus offering intriguing implications regarding the J-shaped relationship observed between mortality and exercise participation (Arem et al., 2015; Kelly et al., 2014; Schnohr, O'Keefe, Marott, Lange, & Jensen, 2015). Data are needed characterizing the autophagic response to high and maximal intensity, short duration exercise, such as HIIT.

Table 1. A summary of studies investigating the autophagic response to acute endurance exercise in human skeletal muscle.

Author	Subjects	Exercise Protocol	Markers of Autophagic Activity	
Jamart et al. 2012	8 experienced ultra-endurance-trained males	200 km run (competitive race)	3 hr post-race: Atg4: ↑ 40% Atg12: ↑ 57% GABARAPL1: ↑ 286% LC3B: ↑ 103% Cathespin L: ↑ 123%	 BNIP3: ↑ 123% BNIP31: ↑123% Beclin1: ↔ ULK1: ↔
Jamart et al. 2012	11 experienced ultra-endurance-trained males	149.8 km run	10 min post-exercise: LC3B-II: ↑ 554% cAtg12: ↑ 36% Atg7: ↔ BNIP3: ↔	 Belcin1: ↔ AMPK: ↑ 247% FOXO3a: ↓ 49% mTOR: ↓ 32%
Masschelein et al. 2014	11 healthy monozygotic twins	20 min cycling ~50% VO_{2max}	Im post-exercise LC3-II (protein expression): ↔ LC3-I (protein expression): ↔ LC3-II:I (protein ratio): ↔ cATG12 (protein expression): ↔	 p62 (protein expression): ↔ BNIP3 (mRNA expression): ↔ FOXO1/3a (phosphorylation): ↔ AMPK (phosphorylation): ↔
Moller et al. 2015	8 recreationally-active males	60 min cycling ~50% VO_{2max}	90 min post-exericse AMPK (phosphorylation): ↑ mTOR (phosphorylation): ↔ ULK1 (phosphorylation): ↑ ULK1 (protein expression): ↔ LC3B-II (protein expression): ↓ GABARAP (protein expression): ↓	 Atg5 (protein expression): ↓ LC3B-I (protein expression): ↔ LC3B-II:I (protein ratio): ↓ p62 (protein expression): ↔ Beclin1 (protein expression): ↔
Tachtsis et al. 2016	16 healthy, untrained males	60 min cycling ~70% VO_{2max}	3 hr post-exercise p53 (nuclear protein localization) ↑ Atg5 (protein expression) ↓ ULK1 (protein expression) ↔ LC3B-I (protein expression) ↔	LC3B-II (protein expression) ↔ LC3B-II:I (protein ratio) ↔ p62 (protein expression) ↔
Schwalm et al. 2015	23 trained males	2 hr cycling: 55%VO_{2peak} (fasted and fed) or 70% VO_{2peak} (fasted and fed)	Im post, 1 hr post-exercise: $ULK1^{Ser757}$ (phosphorylation): 55% VO_{2peak} fasted: ↔ Im post; ↔ 1 hr 70%VO_{2peak} fasted: ↔ Im post; ↔ 1 hr 55% VO_{2peak} fed: ↓ Im post; ↓1 hr 70%VO_{2peak} fed: ↓Im post; ↓1 hr AMPK(phosphorylation): 55% VO_{2peak} fasted: ↔ Im post; ↔ 1 hr 70%VO_{2peak} fasted: ↑ Im post; ↔ 1 hr 55% VO_{2peak} fed: ↑ Im post; ↔ 1 hr 70%VO_{2peak} fed: ↑ Im post; ↔ 1 hr $ULK1^{Ser317}$(phosphorylation): 55% VO_{2peak} fasted: ↑ Im post; ↔ 1 hr 70%VO_{2peak} fasted: ↑ Im post; ↑ 1 hr 55% VO_{2peak} fed: ↑ Im post; ↑ 1 hr 70%VO_{2peak} fed: ↑ Im post; ↑ 1 hr LC3B-II (protein expression): 55% VO_{2peak} fasted: ↓ Im post; ↓ 1 hr 70%VO_{2peak} fasted: ↓ Im post; ↓ 1 hr 55% VO_{2peak} fed: ↓ Im post; ↔ 1 hr 70%VO_{2peak} fed: ↓ Im post; ↓ 1 hr LC3B-I (protein expression): 55% VO_{2peak} fasted: ↔ Im post; ↔ 1 hr 70%VO_{2peak} fasted: ↔ Im post; ↔ 1 hr 55% VO_{2peak} fed: ↔ Im post; ↔ 1 hr 70%VO_{2peak} fed: ↔ Im post; ↔ 1 hr	 LC3B-II:I (protein ratio): 55% VO_{2peak} fasted: ↓ Im post; ↓ 1 hr 70%VO_{2peak} fasted: ↓ Im post; ↓ 1 hr 55% VO_{2peak} fed: ↓ Im post; ↔ 1 hr 70%VO_{2peak} fed: ↓ Im post; ↓ 1 hr p62 (mRNA expression): 55% VO_{2peak} fasted: ↔ Im post; ↔ 1 hr 70%VO_{2peak} fasted: ↔ Im post; ↓ 1 hr 55% VO_{2peak} fed: ↔ Im post; ↔ 1 hr 70%VO_{2peak} fed: ↔ Im post; ↓ 1 hr p62 (protein expression): 55% VO_{2peak} fasted: ↔ Im post; ↑ 1 hr 70%VO_{2peak} fasted: ↑ Im post; ↑ 1 hr 55% VO_{2peak} fed: ↔Im post; ↑ 1 hr 70%VO_{2peak} fed: ↑ Im post; ↑ 1 hr GABARAPL1 (mRNA exrepression) 55% VO_{2peak} fasted: ↔ Im post; ↔1 hr 70%VO_{2peak} fasted: ↑ Im post; ↑ 1 hr 55% VO_{2peak} fed: ↔Im post; ↑ 1 hr 70%VO_{2peak} fed: ↑ Im post; ↑ 1 hr Cathespin L (mRNA expression) 55% VO_{2peak} fasted: ↔ Im post; ↔ 1 hr 70%VO_{2peak} fasted: ↑ Im post; ↑ 1 hr 55% VO_{2peak} fed: ↔ Im post; ↔ 1 hr 70%VO_{2peak} fed: ↑ Im post; ↑ 1 hr

km: kilometers; hr: hour(s); min: minute(s); Im: immediate; VO_{2peak}: peak oxygen consumption; VO_{2max}: maximum oxygen consumption

Autophagy is Involved in the Adaptive Responses to Exercise

In addition to serving as a means to meet the energetic demands of exercise, autophagy is understood to facilitate exercise in numerous ways in skeletal muscle (Dokladny et al., 2013; Grumati et al., 2011; He, Bassik, et al., 2012; Jamart, Francaux, et al., 2012; Masschelein et al., 2014; Schwalm et al., 2015). Using a mutant rodent model that inhibits exercise-induced autophagy, He and coworkers reported the autophagy-deficient mice demonstrated impaired glucose uptake, GLUT4 translocation, and AMPK activation during acute exercise (He, Bassik, et al., 2012). Moreover, data exist suggesting autophagy possesses a role in conferring the benefits of exercise, including shifting toward the oxidative phenotype (Lira et al., 2013), enhanced endurance (He, Bassik, et al., 2012; Lira et al., 2013), mitochondrial biogenesis (Grumati et al., 2011; Ju et al., 2016; Lira et al., 2013), and angiogenesis (Lira et al., 2013).

While exercise-induced skeletal muscle autophagy is presently the most studied, there are data showing enhanced autophagic activity in other tissues (Figure 1), thus demonstrating acute exercise is capable of instigating a global autophagic response (He, Bassik, et al., 2012; He, Sumpter, et al., 2012). In their study, He and colleagues reported acute endurance exercise increased autophagy activity in heart, liver, pancreatic β cells, and adipose tissue of wild-type mice but not in exercise-stimulated autophagy-deficient mutant mice (He, Bassik, et al., 2012). Moreover, the group showed acute exercise increased autophagic flux in the anterior cerebral cortex (He, Sumpter, et al., 2012). Li and coworkers showed a number of mitochondrial-autophagy (mitophagy) related proteins and flux were upregulated in myocardium of mice during exercise and up to 24 hours post-exercise; this paralleled an increase in inflammatory markers NLRP3 and IL1β (Li et al., 2016). Additionally, expression of several Atgs were rescued in mouse hepatocytes following a high-fat diet in response to 10 weeks of endurance exercise, and was associated with reduced lipid content and lipogenic gene expression (Ghareghani et al., 2017). Further, one hour of exercise in a warm environment (30°C) increased autophagy in peripheral blood mononuclear cells (PBMCs) (Dokladny et al., 2013). And notably, Miejas-Pena

and coworkers have shown 8 weeks of aerobic training (Mejias-Pena et al., 2016) and 8 weeks of resistance training (Mejias-Pena et al., 2017a) augment expression in several Atgs and basal autophagic activity in PBMCs in elderly subjects.

These noted systemic autophagic effects suggest exercise could possess a role in modulating some of the degenerative and age-related pathologies that autophagy has been reported to be implicated in, which include type 2 diabetes (Gonzalez et al., 2011; Quan et al., 2013), neurodegeneration (Komatsu et al., 2006; F. Yang et al., 2014), cardiomyopathy (Nair & Ren, 2012; Tanaka et al., 2000), cancer (Cao & Klionsky, 2007; Cecconi & Levine, 2008) and chronic inflammation, (Jo et al., 2012; Levine et al., 2011) while improving muscle quality and function (Fan et al., 2016; Vainshtein et al., 2014). Moreover, these autophagy-related conditions largely lie within the parameters of health benefits of exercise has been documented to augment (Atherton, Phillips, & Wilkinson, 2015; Moore et al., 2016; Sanchez, Bernardi, Py, & Candau, 2014; Vainshtein et al., 2014; Woods, Wilund, Martin, & Kistler, 2012).

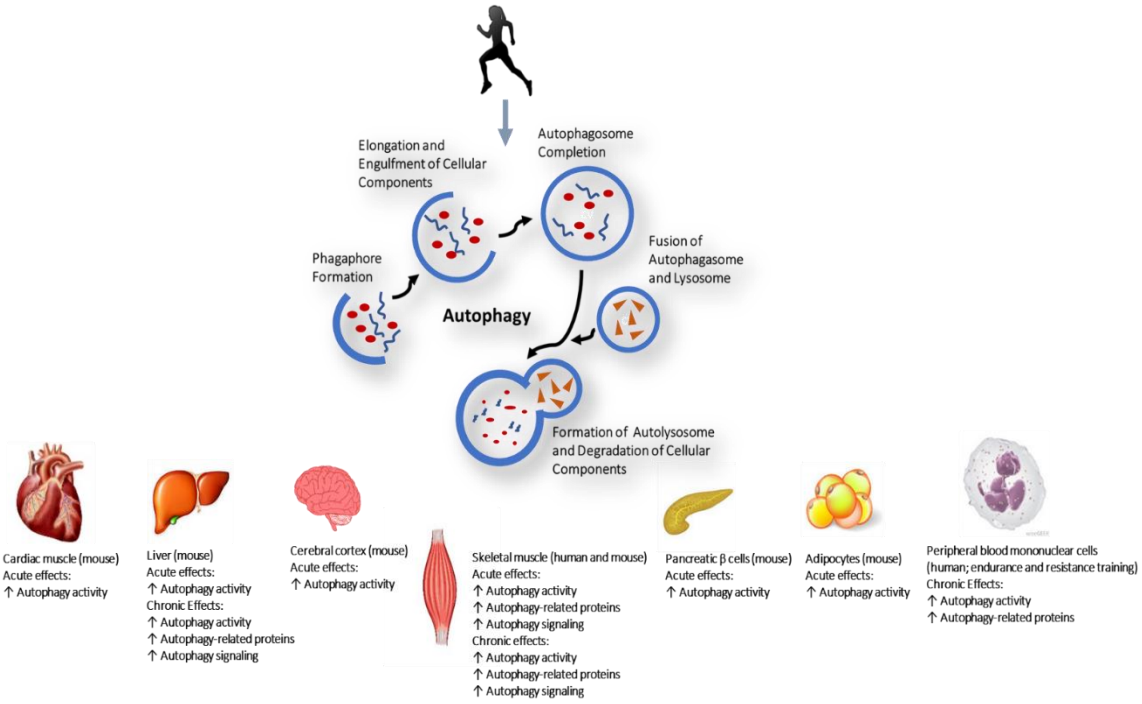

Figure 1. Effects of acute and chronic exercise on autophagy in multiple tissues.

High Intensity Interval Training

High-intensity interval training (HIIT) is an increasingly popular training method characterized by repeated bouts of brief (~30 sec – 3 min) high intensity (80 – 100% VO_{2max}, HR_{max}, peak power) exercise followed by periods of rest or low intensity exercise (~30 sec – 4 min) that result in ~10 minutes of overall work in training sessions of ~20 minutes (Gibala et al., 2014; Gillen & Gibala, 2014). Traditional exercise prescriptions for improvements of fitness and health are that of moderate intensity, continuous nature (i.e. 50 – 85% VO_{2max}, HR_{max}, peak power) (L. Pescatello & Medicine, 2014). However, increasing data demonstrate that HIIT training can elicit equal or even superior physiological and health changes as traditional, moderate intensity, continuous training (MICT) when amounts of work are similar or even lower (Gibala, Little, Macdonald, & Hawley, 2012). High intensity interval training has been shown to reduce fat mass (Heydari, Freund, & Boutcher, 2012), increase skeletal muscle GLUT4 content (M. S. Hood, Little, Tarnopolsky, Myslik, & Gibala, 2011; Little, Gillen, et al., 2011), improve insulin sensitivity and glucose regulation (Babraj et al., 2009; Metcalfe, Babraj, Fawkner, & Vollaard, 2012; Richards et al., 2010; Whyte, Gill, & Cathcart, 2010), increase VO_{2max}, stroke volume, cardiac output (Helgerud et al., 2007), improve endothelial function (Moholdt et al., 2009; Tjonna et al., 2008; Wisloff et al., 2007) and blood pressure (Rognmo, Hetland, Helgerud, Hoff, & Slordahl, 2004; Schjerve et al., 2008) and enhance oxidative capacity and mitochondrial biogenesis (Burgomaster, Hughes, Heigenhauser, Bradwell, & Gibala, 2005; Gibala et al., 2014; Gillen & Gibala, 2014; Little, Safdar, et al., 2011). Moreover, HIIT appears to be well-tolerated, even by individuals with low initial fitness levels (Astorino & Thum, 2016; Tjonna et al., 2008) and may facilitate adherence (Astorino & Thum, 2016) which is of interest given the poor physical activity adherence rates amongst the US population (Qiu et al., 2012; Statistics, 2012; Trost et al., 2002). Given these robust effects and that lack of time is a common obstacle to physical activity (Trost et al., 2002), HIIT may represent an efficacious and time efficient means to incur the benefits of exercise for a wide spectrum of populations; clinical, healthy, and athletic.

The molecular mechanisms underlying the physiological adaptations to HIIT overlap with those of MICT, namely through the upregulation of AMPK and p38MAPK in response to acute elevations of AMP and ROS (Gibala et al., 2012). AMPK is stimulated in a duration and intensity dependent manner insofar as either sustained energetic challenges to intracellular ATP concentrations (i.e. MICT) or brief and large fluctuations in ATP:AMP ratios can modulate its activity (Aschenbach et al., 2004; Combes et al., 2015; Gibala et al., 2012), thus HIIT is capable of eliciting profound activation of AMPK (Gibala et al., 2012). Additional signaling pathways include elevated SIRT1 activity via alterations in NAD^+ concentrations (Gurd, Perry, Heigenhauser, Spriet, & Bonen, 2010), and CaMKII, which may serve as an upstream regulator of p38MAPK, via changes in Ca^{++} (Combes et al., 2015).

References

Alvers, A. L., Fishwick, L. K., Wood, M. S., Hu, D., Chung, H. S., Dunn, W. A., Jr., & Aris, J. P. (2009). Autophagy and amino acid homeostasis are required for chronological longevity in Saccharomyces cerevisiae. *Aging Cell, 8*(4), 353-369. doi:10.1111/j.1474-9726.2009.00469.x

Aquilano, K., Baldelli, S., Pagliei, B., Cannata, S. M., Rotilio, G., & Ciriolo, M. R. (2013). p53 orchestrates the PGC-1alpha-mediated antioxidant response upon mild redox and metabolic imbalance. *Antioxid Redox Signal, 18*(4), 386-399. doi:10.1089/ars.2012.4615

Arem, H., Moore, S. C., Patel, A., Hartge, P., Berrington de Gonzalez, A., Visvanathan, K., . . . Matthews, C. E. (2015). Leisure time physical activity and mortality: a detailed pooled analysis of the dose-response relationship. *JAMA Intern Med, 175*(6), 959-967. doi:10.1001/jamainternmed.2015.0533

Aschenbach, W. G., Sakamoto, K., & Goodyear, L. J. (2004). 5' adenosine monophosphate-activated protein kinase, metabolism and exercise. *Sports Med, 34*(2), 91-103.

Astorino, T. A., & Thum, J. S. (2016). Response: Commentary: Why sprint interval training is inappropriate for a largely sedentary population. *Front Psychol, 7*, 746. doi:10.3389/fpsyg.2016.00746

Atherton, P. J., Phillips, B. E., & Wilkinson, D. J. (2015). Exercise and Regulation of Protein Metabolism. *Prog Mol Biol Transl Sci, 135*, 75-98. doi:10.1016/bs.pmbts.2015.06.015

Babraj, J. A., Vollaard, N. B., Keast, C., Guppy, F. M., Cottrell, G., & Timmons, J. A. (2009). Extremely short duration high intensity interval training substantially improves insulin action in young healthy males. *BMC Endocr Disord, 9*, 3. doi:10.1186/1472-6823-9-3

Balaburski, G. M., Hontz, R. D., & Murphy, M. E. (2010). p53 and ARF: unexpected players in autophagy. *Trends Cell Biol, 20*(6), 363-369. doi:10.1016/j.tcb.2010.02.007

Bartlett, J. D., Close, G. L., Drust, B., & Morton, J. P. (2014). The emerging role of p53 in exercise metabolism. *Sports Med, 44*(3), 303-309. doi:10.1007/s40279-013-0127-9

Bartlett, J. D., Hwa Joo, C., Jeong, T. S., Louhelainen, J., Cochran, A. J., Gibala, M. J., . . . Morton, J. P. (2012). Matched work high-intensity interval and continuous running induce similar increases in PGC-1alpha mRNA, AMPK, p38, and p53 phosphorylation in human skeletal muscle. *J Appl Physiol (1985), 112*(7), 1135-1143. doi:10.1152/japplphysiol.01040.2011

Bartlett, J. D., Louhelainen, J., Iqbal, Z., Cochran, A. J., Gibala, M. J., Gregson, W., . . . Morton, J. P. (2013). Reduced carbohydrate availability enhances exercise-induced p53 signaling in human skeletal muscle: implications for mitochondrial biogenesis. *Am J Physiol Regul Integr Comp Physiol, 304*(6), R450-458. doi:10.1152/ajpregu.00498.2012

Broatch, J. R., Petersen, A., & Bishop, D. J. (2017). Cold-water immersion following sprint interval training does not alter endurance signaling pathways or training adaptations in

human skeletal muscle. *Am J Physiol Regul Integr Comp Physiol, 313*(4), R372-R384. doi:10.1152/ajpregu.00434.2016

Budanov, A. V., & Karin, M. (2008). p53 target genes sestrin1 and sestrin2 connect genotoxic stress and mTOR signaling. *Cell, 134*(3), 451-460. doi:10.1016/j.cell.2008.06.028

Burgomaster, K. A., Hughes, S. C., Heigenhauser, G. J., Bradwell, S. N., & Gibala, M. J. (2005). Six sessions of sprint interval training increases muscle oxidative potential and cycle endurance capacity in humans. *J Appl Physiol (1985), 98*(6), 1985-1990. doi:10.1152/japplphysiol.01095.2004

Canto, C., & Auwerx, J. (2009). PGC-1alpha, SIRT1 and AMPK, an energy sensing network that controls energy expenditure. *Curr Opin Lipidol, 20*(2), 98-105. doi:10.1097/MOL.0b013e328328d0a4

Cao, Y., & Klionsky, D. J. (2007). Physiological functions of Atg6/Beclin 1: a unique autophagy-related protein. *Cell Res, 17*(10), 839-849. doi:10.1038/cr.2007.78

Carames, B., Taniguchi, N., Otsuki, S., Blanco, F. J., & Lotz, M. (2010). Autophagy is a protective mechanism in normal cartilage, and its aging-related loss is linked with cell death and osteoarthritis. *Arthritis Rheum, 62*(3), 791-801. doi:10.1002/art.27305

Carling, D. (2017). AMPK signalling in health and disease. *Curr Opin Cell Biol, 45*, 31-37. doi:10.1016/j.ceb.2017.01.005

Cecconi, F., & Levine, B. (2008). The role of autophagy in mammalian development: cell makeover rather than cell death. *Dev Cell, 15*(3), 344-357. doi:10.1016/j.devcel.2008.08.012

Chen, J., Wang, A., & Chen, Q. (2017). SirT3 and p53 Deacetylation in Aging and Cancer. *J Cell Physiol, 232*(9), 2308-2311. doi:10.1002/jcp.25669

Cherra, S. J., 3rd, Dagda, R. K., & Chu, C. T. (2010). Review: autophagy and neurodegeneration: survival at a cost? *Neuropathol Appl Neurobiol, 36*(2), 125-132. doi:10.1111/j.1365-2990.2010.01062.x

Coffey, V. G., & Hawley, J. A. (2007). The molecular bases of training adaptation. *Sports Med, 37*(9), 737-763.

Combes, A., Dekerle, J., Webborn, N., Watt, P., Bougault, V., & Daussin, F. N. (2015). Exercise-induced metabolic fluctuations influence AMPK, p38-MAPK and CaMKII phosphorylation in human skeletal muscle. *Physiol Rep, 3*(9). doi:10.14814/phy2.12462

Cuervo, A. M. (2008). Autophagy and aging: keeping that old broom working. *Trends Genet, 24*(12), 604-612. doi:10.1016/j.tig.2008.10.002

Cuervo, A. M., Bergamini, E., Brunk, U. T., Droge, W., Ffrench, M., & Terman, A. (2005). Autophagy and aging: the importance of maintaining "clean" cells. *Autophagy, 1*(3), 131-140.

Cuervo, A. M., & Macian, F. (2014). Autophagy and the immune function in aging. *Curr Opin Immunol, 29*, 97-104. doi:10.1016/j.coi.2014.05.006

Dagon, Y., Mantzoros, C., & Kim, Y. B. (2015). Exercising insulin sensitivity: AMPK turns on autophagy! *Metabolism, 64*(6), 655-657. doi:10.1016/j.metabol.2015.03.002

Day, E. A., Ford, R. J., & Steinberg, G. R. (2017). AMPK as a Therapeutic Target for Treating Metabolic Diseases. *Trends Endocrinol Metab, 28*(8), 545-560. doi:10.1016/j.tem.2017.05.004

de Kreutzenberg, S. V., Ceolotto, G., Papparella, I., Bortoluzzi, A., Semplicini, A., Dalla Man, C., . . . Avogaro, A. (2010). Downregulation of the longevity-associated protein sirtuin 1 in insulin resistance and metabolic syndrome: potential biochemical mechanisms. *Diabetes, 59*(4), 1006-1015. doi:10.2337/db09-1187

Dokladny, K., Myers, O. B., & Moseley, P. L. (2015). Heat shock response and autophagy--cooperation and control. *Autophagy, 11*(2), 200-213. doi:10.1080/15548627.2015.1009776

Dokladny, K., Zuhl, M. N., Mandell, M., Bhattacharya, D., Schneider, S., Deretic, V., & Moseley, P. L. (2013). Regulatory coordination between two major intracellular homeostatic systems: heat shock response and autophagy. *J Biol Chem, 288*(21), 14959-14972. doi:10.1074/jbc.M113.462408

Du, Y., Yang, D., Li, L., Luo, G., Li, T., Fan, X., . . . Le, W. (2009). An insight into the mechanistic role of p53-mediated autophagy induction in response to proteasomal inhibition-induced neurotoxicity. *Autophagy, 5*(5), 663-675.

Eisenberg, T., Knauer, H., Schauer, A., Buttner, S., Ruckenstuhl, C., Carmona-Gutierrez, D., . . . Madeo, F. (2009). Induction of autophagy by spermidine promotes longevity. *Nat Cell Biol, 11*(11), 1305-1314. doi:10.1038/ncb1975

Fan, J., Kou, X., Jia, S., Yang, X., Yang, Y., & Chen, N. (2016). Autophagy as a Potential Target for Sarcopenia. *J Cell Physiol, 231*(7), 1450-1459. doi:10.1002/jcp.25260

Foretz, M., Guigas, B., Bertrand, L., Pollak, M., & Viollet, B. (2014). Metformin: from mechanisms of action to therapies. *Cell Metab, 20*(6), 953-966. doi:10.1016/j.cmet.2014.09.018

Ghareghani, P., Shanaki, M., Ahmadi, S., Khoshdel, A. R., Rezvan, N., Meshkani, R., . . . Gorgani-Firuzjaee, S. (2017). Aerobic endurance training improves nonalcoholic fatty liver disease (NAFLD) features via miR-33 dependent autophagy induction in high fat diet fed mice. *Obes Res Clin Pract*. doi:10.1016/j.orcp.2017.01.004

Ghosh, H. S., McBurney, M., & Robbins, P. D. (2010). SIRT1 negatively regulates the mammalian target of rapamycin. *PLoS One, 5*(2), e9199. doi:10.1371/journal.pone.0009199

Gibala, M. J., Gillen, J. B., & Percival, M. E. (2014). Physiological and health-related adaptations to low-volume interval training: influences of nutrition and sex. *Sports Med, 44 Suppl 2*, S127-137. doi:10.1007/s40279-014-0259-6

Gibala, M. J., Little, J. P., Macdonald, M. J., & Hawley, J. A. (2012). Physiological adaptations to low-volume, high-intensity interval training in health and disease. *J Physiol, 590*(5), 1077-1084. doi:10.1113/jphysiol.2011.224725

Gillen, J. B., & Gibala, M. J. (2014). Is high-intensity interval training a time-efficient exercise strategy to improve health and fitness? *Appl Physiol Nutr Metab, 39*(3), 409-412. doi:10.1139/apnm-2013-0187

Gonzalez, C. D., Lee, M. S., Marchetti, P., Pietropaolo, M., Towns, R., Vaccaro, M. I., . . . Wiley, J. W. (2011). The emerging role of autophagy in the pathophysiology of diabetes mellitus. *Autophagy, 7*(1), 2-11.

Grumati, P., Coletto, L., Schiavinato, A., Castagnaro, S., Bertaggia, E., Sandri, M., & Bonaldo, P. (2011). Physical exercise stimulates autophagy in normal skeletal muscles but is detrimental for collagen VI-deficient muscles. *Autophagy, 7*(12), 1415-1423.

Gurd, B. J., Perry, C. G., Heigenhauser, G. J., Spriet, L. L., & Bonen, A. (2010). High-intensity interval training increases SIRT1 activity in human skeletal muscle. *Appl Physiol Nutr Metab, 35*(3), 350-357. doi:10.1139/H10-030

Gwinn, D. M., Shackelford, D. B., Egan, D. F., Mihaylova, M. M., Mery, A., Vasquez, D. S., . . . Shaw, R. J. (2008). AMPK phosphorylation of raptor mediates a metabolic checkpoint. *Mol Cell, 30*(2), 214-226. doi:10.1016/j.molcel.2008.03.003

Hardie, D. G. (2011). AMPK and autophagy get connected. *EMBO J, 30*(4), 634-635. doi:10.1038/emboj.2011.12

Hardie, D. G. (2014). AMPK--sensing energy while talking to other signaling pathways. *Cell Metab, 20*(6), 939-952. doi:10.1016/j.cmet.2014.09.013

Hars, E. S., Qi, H., Ryazanov, A. G., Jin, S., Cai, L., Hu, C., & Liu, L. F. (2007). Autophagy regulates ageing in C. elegans. *Autophagy, 3*(2), 93-95.

Hawley, J. A., Hargreaves, M., Joyner, M. J., & Zierath, J. R. (2014). Integrative biology of exercise. *Cell, 159*(4), 738-749. doi:10.1016/j.cell.2014.10.029

He, C., Bassik, M. C., Moresi, V., Sun, K., Wei, Y., Zou, Z., . . . Levine, B. (2012). Exercise-induced BCL2-regulated autophagy is required for muscle glucose homeostasis. *Nature, 481*(7382), 511-515. doi:10.1038/nature10758

He, C., Sumpter, R., Jr., & Levine, B. (2012). Exercise induces autophagy in peripheral tissues and in the brain. *Autophagy, 8*(10), 1548-1551. doi:10.4161/auto.21327

Helgerud, J., Hoydal, K., Wang, E., Karlsen, T., Berg, P., Bjerkaas, M., . . . Hoff, J. (2007). Aerobic high-intensity intervals improve VO2max more than moderate training. *Med Sci Sports Exerc, 39*(4), 665-671. doi:10.1249/mss.0b013e3180304570

Heydari, M., Freund, J., & Boutcher, S. H. (2012). The effect of high-intensity intermittent exercise on body composition of overweight young males. *J Obes, 2012*, 480467. doi:10.1155/2012/480467

Higgins, G. C., & Coughlan, M. T. (2014). Mitochondrial dysfunction and mitophagy: the beginning and end to diabetic nephropathy? *Br J Pharmacol, 171*(8), 1917-1942. doi:10.1111/bph.12503

Hood, D. A. (2009). Mechanisms of exercise-induced mitochondrial biogenesis in skeletal muscle. *Appl Physiol Nutr Metab, 34*(3), 465-472. doi:10.1139/H09-045

Hood, M. S., Little, J. P., Tarnopolsky, M. A., Myslik, F., & Gibala, M. J. (2011). Low-volume interval training improves muscle oxidative capacity in sedentary adults. *Med Sci Sports Exerc, 43*(10), 1849-1856. doi:10.1249/MSS.0b013e3182199834

Inoki, K., Zhu, T., & Guan, K. L. (2003). TSC2 mediates cellular energy response to control cell growth and survival. *Cell, 115*(5), 577-590.

Jamart, C., Benoit, N., Raymackers, J. M., Kim, H. J., Kim, C. K., & Francaux, M. (2012). Autophagy-related and autophagy-regulatory genes are induced in human muscle after ultraendurance exercise. *Eur J Appl Physiol, 112*(8), 3173-3177. doi:10.1007/s00421-011-2287-3

Jamart, C., Francaux, M., Millet, G. Y., Deldicque, L., Frere, D., & Feasson, L. (2012). Modulation of autophagy and ubiquitin-proteasome pathways during ultra-endurance running. *J Appl Physiol (1985), 112*(9), 1529-1537. doi:10.1152/japplphysiol.00952.2011

Jazwinski, S. M. (2015). Mitochondria to nucleus signaling and the role of ceramide in its integration into the suite of cell quality control processes during aging. *Ageing Res Rev, 23*(Pt A), 67-74. doi:10.1016/j.arr.2014.12.007

Jo, E. K., Shin, D. M., & Choi, A. M. (2012). Autophagy: cellular defense to excessive inflammation. *Microbes Infect, 14*(2), 119-125. doi:10.1016/j.micinf.2011.08.014

Johnson, S. C., Rabinovitch, P. S., & Kaeberlein, M. (2013). mTOR is a key modulator of ageing and age-related disease. *Nature, 493*(7432), 338-345. doi:10.1038/nature11861

Ju, J. S., Jeon, S. I., Park, J. Y., Lee, J. Y., Lee, S. C., Cho, K. J., & Jeong, J. M. (2016). Autophagy plays a role in skeletal muscle mitochondrial biogenesis in an endurance exercise-trained condition. *J Physiol Sci, 66*(5), 417-430. doi:10.1007/s12576-016-0440-9

Juhasz, G., Erdi, B., Sass, M., & Neufeld, T. P. (2007). Atg7-dependent autophagy promotes neuronal health, stress tolerance, and longevity but is dispensable for metamorphosis in Drosophila. *Genes Dev, 21*(23), 3061-3066. doi:10.1101/gad.1600707

Jung, C. H., Ro, S. H., Cao, J., Otto, N. M., & Kim, D. H. (2010). mTOR regulation of autophagy. *FEBS Lett, 584*(7), 1287-1295. doi:10.1016/j.febslet.2010.01.017

Kang, C., You, Y. J., & Avery, L. (2007). Dual roles of autophagy in the survival of Caenorhabditis elegans during starvation. *Genes Dev, 21*(17), 2161-2171. doi:10.1101/gad.1573107

Kelly, P., Kahlmeier, S., Gotschi, T., Orsini, N., Richards, J., Roberts, N., . . . Foster, C. (2014). Systematic review and meta-analysis of reduction in all-cause mortality from walking and cycling and shape of dose response relationship. *Int J Behav Nutr Phys Act, 11*, 132. doi:10.1186/s12966-014-0132-x

Kim, K. H., Jeong, Y. T., Oh, H., Kim, S. H., Cho, J. M., Kim, Y. N., . . . Lee, M. S. (2013). Autophagy deficiency leads to protection from obesity and insulin resistance by inducing Fgf21 as a mitokine. *Nat Med, 19*(1), 83-92. doi:10.1038/nm.3014

Klionsky, D. J., Abdalla, F. C., Abeliovich, H., Abraham, R. T., Acevedo-Arozena, A., Adeli, K., . . . Zuckerbraun, B. (2012). Guidelines for the use and interpretation of assays for monitoring autophagy. *Autophagy, 8*(4), 445-544.

Kobayashi, S., & Liang, Q. (2015). Autophagy and mitophagy in diabetic cardiomyopathy. *Biochim Biophys Acta, 1852*(2), 252-261. doi:10.1016/j.bbadis.2014.05.020

Komatsu, M., Waguri, S., Chiba, T., Murata, S., Iwata, J., Tanida, I., . . . Tanaka, K. (2006). Loss of autophagy in the central nervous system causes neurodegeneration in mice. *Nature, 441*(7095), 880-884. doi:10.1038/nature04723

Komatsu, M., Waguri, S., Ueno, T., Iwata, J., Murata, S., Tanida, I., . . . Chiba, T. (2005). Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. *J Cell Biol, 169*(3), 425-434. doi:10.1083/jcb.200412022

Lee, J. H., Budanov, A. V., Park, E. J., Birse, R., Kim, T. E., Perkins, G. A., . . . Karin, M. (2010). Sestrin as a feedback inhibitor of TOR that prevents age-related pathologies. *Science, 327*(5970), 1223-1228. doi:10.1126/science.1182228

Levine, B., & Klionsky, D. J. (2004). Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Dev Cell, 6*(4), 463-477.

Levine, B., Mizushima, N., & Virgin, H. W. (2011). Autophagy in immunity and inflammation. *Nature, 469*(7330), 323-335. doi:10.1038/nature09782

Li, H., Liu, S., Yuan, H., Niu, Y., & Fu, L. (2017). Sestrin 2 induces autophagy and attenuates insulin resistance by regulating AMPK signaling in C2C12 myotubes. *Exp Cell Res, 354*(1), 18-24. doi:10.1016/j.yexcr.2017.03.023

Li, H., Miao, W., Ma, J., Xv, Z., Bo, H., Li, J., . . . Ji, L. L. (2016). Acute Exercise-Induced Mitochondrial Stress Triggers an Inflammatory Response in the Myocardium via NLRP3 Inflammasome Activation with Mitophagy. *Oxid Med Cell Longev, 2016*, 1987149. doi:10.1155/2016/1987149

Liang, C., & Jung, J. U. (2010). Autophagy genes as tumor suppressors. *Curr Opin Cell Biol, 22*(2), 226-233. doi:10.1016/j.ceb.2009.11.003

Lipinski, M. M., Zheng, B., Lu, T., Yan, Z., Py, B. F., Ng, A., . . . Yuan, J. (2010). Genome-wide analysis reveals mechanisms modulating autophagy in normal brain aging and in Alzheimer's disease. *Proc Natl Acad Sci U S A, 107*(32), 14164-14169. doi:10.1073/pnas.1009485107

Lira, V. A., Okutsu, M., Zhang, M., Greene, N. P., Laker, R. C., Breen, D. S., . . . Yan, Z. (2013). Autophagy is required for exercise training-induced skeletal muscle adaptation and improvement of physical performance. *FASEB J, 27*(10), 4184-4193. doi:10.1096/fj.13-228486

Little, J. P., Gillen, J. B., Percival, M. E., Safdar, A., Tarnopolsky, M. A., Punthakee, Z., . . . Gibala, M. J. (2011). Low-volume high-intensity interval training reduces hyperglycemia and increases muscle mitochondrial capacity in patients with type 2 diabetes. *J Appl Physiol (1985), 111*(6), 1554-1560. doi:10.1152/japplphysiol.00921.2011

Little, J. P., Safdar, A., Bishop, D., Tarnopolsky, M. A., & Gibala, M. J. (2011). An acute bout of high-intensity interval training increases the nuclear abundance of PGC-1alpha and activates mitochondrial biogenesis in human skeletal muscle. *Am J Physiol Regul Integr Comp Physiol, 300*(6), R1303-1310. doi:10.1152/ajpregu.00538.2010

Liu, E. Y., & Ryan, K. M. (2012). Autophagy and cancer--issues we need to digest. *J Cell Sci, 125*(Pt 10), 2349-2358. doi:10.1242/jcs.093708

Liu, X., Niu, Y., Yuan, H., Huang, J., & Fu, L. (2015). AMPK binds to Sestrins and mediates the effect of exercise to increase insulin-sensitivity through autophagy. *Metabolism, 64*(6), 658-665. doi:10.1016/j.metabol.2015.01.015

Loughery, J., Cox, M., Smith, L. M., & Meek, D. W. (2014). Critical role for p53-serine 15 phosphorylation in stimulating transactivation at p53-responsive promoters. *Nucleic Acids Res, 42*(12), 7666-7680. doi:10.1093/nar/gku501

Ma, L., Dong, W., Wang, R., Li, Y., Xu, B., Zhang, J., . . . Wang, Y. (2015). Effect of caloric restriction on the SIRT1/mTOR signaling pathways in senile mice. *Brain Res Bull, 116*, 67-72. doi:10.1016/j.brainresbull.2015.06.004

Maiuri, M. C., Galluzzi, L., Morselli, E., Kepp, O., Malik, S. A., & Kroemer, G. (2010). Autophagy regulation by p53. *Curr Opin Cell Biol, 22*(2), 181-185. doi:10.1016/j.ceb.2009.12.001

Maiuri, M. C., Malik, S. A., Morselli, E., Kepp, O., Criollo, A., Mouchel, P. L., . . . Kroemer, G. (2009). Stimulation of autophagy by the p53 target gene Sestrin2. *Cell Cycle, 8*(10), 1571-1576. doi:10.4161/cc.8.10.8498

Martin-Rincon, M., Morales-Alamo, D., & Calbet, J. A. L. (2017). Exercise-mediated modulation of autophagy in skeletal muscle. *Scand J Med Sci Sports*. doi:10.1111/sms.12945

Martin, S. D., & McGee, S. L. (2014). The role of mitochondria in the aetiology of insulin resistance and type 2 diabetes. *Biochim Biophys Acta, 1840*(4), 1303-1312. doi:10.1016/j.bbagen.2013.09.019

Masiero, E., Agatea, L., Mammucari, C., Blaauw, B., Loro, E., Komatsu, M., . . . Sandri, M. (2009). Autophagy is required to maintain muscle mass. *Cell Metab, 10*(6), 507-515. doi:10.1016/j.cmet.2009.10.008

Masschelein, E., Van Thienen, R., D'Hulst, G., Hespel, P., Thomis, M., & Deldicque, L. (2014). Acute environmental hypoxia induces LC3 lipidation in a genotype-dependent manner. *FASEB J, 28*(2), 1022-1034. doi:10.1096/fj.13-239863

Medina, D. L., Di Paola, S., Peluso, I., Armani, A., De Stefani, D., Venditti, R., . . . Ballabio, A. (2015). Lysosomal calcium signalling regulates autophagy through calcineurin and TFEB. *Nat Cell Biol, 17*(3), 288-299. doi:10.1038/ncb3114

Meijer, A. J., Lorin, S., Blommaart, E. F., & Codogno, P. (2015). Regulation of autophagy by amino acids and MTOR-dependent signal transduction. *Amino Acids, 47*(10), 2037-2063. doi:10.1007/s00726-014-1765-4

Mejias-Pena, Y., Estebanez, B., Rodriguez-Miguelez, P., Fernandez-Gonzalo, R., Almar, M., de Paz, J. A., . . . Cuevas, M. J. (2017). Impact of resistance training on the autophagy-inflammation-apoptosis crosstalk in elderly subjects. *Aging (Albany NY)*. doi:10.18632/aging.101167

Mejias-Pena, Y., Rodriguez-Miguelez, P., Fernandez-Gonzalo, R., Martinez-Florez, S., Almar, M., de Paz, J. A., . . . Gonzalez-Gallego, J. (2016). Effects of aerobic training on markers of autophagy in the elderly. *Age (Dordr), 38*(2), 33. doi:10.1007/s11357-016-9897-y

Metcalfe, R. S., Babraj, J. A., Fawkner, S. G., & Vollaard, N. B. (2012). Towards the minimal amount of exercise for improving metabolic health: beneficial effects of reduced-exertion high-intensity interval training. *Eur J Appl Physiol, 112*(7), 2767-2775. doi:10.1007/s00421-011-2254-z

Mizushima, N., Yamamoto, A., Matsui, M., Yoshimori, T., & Ohsumi, Y. (2004). In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. *Mol Biol Cell, 15*(3), 1101-1111. doi:10.1091/mbc.E03-09-0704

Moholdt, T. T., Amundsen, B. H., Rustad, L. A., Wahba, A., Lovo, K. T., Gullikstad, L. R., . . . Slordahl, S. A. (2009). Aerobic interval training versus continuous moderate exercise after coronary artery bypass surgery: a randomized study of cardiovascular effects and quality of life. *Am Heart J, 158*(6), 1031-1037. doi:10.1016/j.ahj.2009.10.003

Moller, A. B., Vendelbo, M. H., Christensen, B., Clasen, B. F., Bak, A. M., Jorgensen, J. O., . . . Jessen, N. (2015). Physical exercise increases autophagic signaling through ULK1 in human skeletal muscle. *J Appl Physiol (1985), 118*(8), 971-979. doi:10.1152/japplphysiol.01116.2014

Moore, S. C., Lee, I. M., Weiderpass, E., Campbell, P. T., Sampson, J. N., Kitahara, C. M., . . . Patel, A. V. (2016). Association of Leisure-Time Physical Activity With Risk of 26 Types of Cancer in 1.44 Million Adults. *JAMA Intern Med, 176*(6), 816-825. doi:10.1001/jamainternmed.2016.1548

Mooren, F. C., & Kruger, K. (2015). Exercise, Autophagy, and Apoptosis. *Prog Mol Biol Transl Sci, 135*, 407-422. doi:10.1016/bs.pmbts.2015.07.023

Most, J., Tosti, V., Redman, L. M., & Fontana, L. (2016). Calorie restriction in humans: An update. *Ageing Res Rev*. doi:10.1016/j.arr.2016.08.005

Nair, S., & Ren, J. (2012). Autophagy and cardiovascular aging: lesson learned from rapamycin. *Cell Cycle, 11*(11), 2092-2099. doi:10.4161/cc.20317

Pani, G. (2011). From growing to secreting: new roles for mTOR in aging cells. *Cell Cycle, 10*(15), 2450-2453. doi:10.4161/cc.10.15.16886

Park, J. Y., Wang, P. Y., Matsumoto, T., Sung, H. J., Ma, W., Choi, J. W., . . . Hwang, P. M. (2009). p53 improves aerobic exercise capacity and augments skeletal muscle

mitochondrial DNA content. *Circ Res, 105*(7), 705-712, 711 p following 712. doi:10.1161/CIRCRESAHA.109.205310

Pescatello, L., & Medicine, A. C. o. S. (2014). *ACSM's guidelines for exercise testing and prescription* (9th ed.). Philadelphia, pennsylvania: Wolters Kluwer/Lippincott Williams & Wilkins Health.

Pyo, J. O., Yoo, S. M., Ahn, H. H., Nah, J., Hong, S. H., Kam, T. I., . . . Jung, Y. K. (2013). Overexpression of Atg5 in mice activates autophagy and extends lifespan. *Nat Commun, 4*, 2300. doi:10.1038/ncomms3300

Qiu, S. H., Sun, Z. L., Cai, X., Liu, L., & Yang, B. (2012). Improving patients' adherence to physical activity in diabetes mellitus: a review. *Diabetes Metab J, 36*(1), 1-5. doi:10.4093/dmj.2012.36.1.1

Quan, W., Jung, H. S., & Lee, M. S. (2013). Role of autophagy in the progression from obesity to diabetes and in the control of energy balance. *Arch Pharm Res, 36*(2), 223-229. doi:10.1007/s12272-013-0024-7

Raben, N., Hill, V., Shea, L., Takikita, S., Baum, R., Mizushima, N., . . . Plotz, P. (2008). Suppression of autophagy in skeletal muscle uncovers the accumulation of ubiquitinated proteins and their potential role in muscle damage in Pompe disease. *Hum Mol Genet, 17*(24), 3897-3908. doi:10.1093/hmg/ddn292

Rajawat, Y. S., & Bossis, I. (2008). Autophagy in aging and in neurodegenerative disorders. *Hormones (Athens), 7*(1), 46-61.

Richards, J. C., Johnson, T. K., Kuzma, J. N., Lonac, M. C., Schweder, M. M., Voyles, W. F., & Bell, C. (2010). Short-term sprint interval training increases insulin sensitivity in healthy adults but does not affect the thermogenic response to beta-adrenergic stimulation. *J Physiol, 588*(Pt 15), 2961-2972. doi:10.1113/jphysiol.2010.189886

Richter, E. A., & Ruderman, N. B. (2009). AMPK and the biochemistry of exercise: implications for human health and disease. *Biochem J, 418*(2), 261-275. doi:10.1042/BJ20082055

Ro, S. H., Semple, I. A., Park, H., Park, H., Park, H. W., Kim, M., . . . Lee, J. H. (2014). Sestrin2 promotes Unc-51-like kinase 1 mediated phosphorylation of p62/sequestosome-1. *FEBS J, 281*(17), 3816-3827. doi:10.1111/febs.12905

Rognmo, O., Hetland, E., Helgerud, J., Hoff, J., & Slordahl, S. A. (2004). High intensity aerobic interval exercise is superior to moderate intensity exercise for increasing aerobic capacity in patients with coronary artery disease. *Eur J Cardiovasc Prev Rehabil, 11*(3), 216-222.

Rubinsztein, D. C., Marino, G., & Kroemer, G. (2011). Autophagy and aging. *Cell, 146*(5), 682-695. doi:10.1016/j.cell.2011.07.030

Saleem, A., Adhihetty, P. J., & Hood, D. A. (2009). Role of p53 in mitochondrial biogenesis and apoptosis in skeletal muscle. *Physiol Genomics, 37*(1), 58-66. doi:10.1152/physiolgenomics.90346.2008

Saleem, A., & Hood, D. A. (2013). Acute exercise induces tumour suppressor protein p53 translocation to the mitochondria and promotes a p53-Tfam-mitochondrial DNA complex in skeletal muscle. *J Physiol, 591*(14), 3625-3636. doi:10.1113/jphysiol.2013.252791

Salminen, A., & Kaarniranta, K. (2009). Regulation of the aging process by autophagy. *Trends Mol Med, 15*(5), 217-224. doi:10.1016/j.molmed.2009.03.004

Sanchez, A. M., Bernardi, H., Py, G., & Candau, R. B. (2014). Autophagy is essential to support skeletal muscle plasticity in response to endurance exercise. *Am J Physiol Regul Integr Comp Physiol, 307*(8), R956-969. doi:10.1152/ajpregu.00187.2014

Sandri, M. (2010). Autophagy in skeletal muscle. *FEBS Lett, 584*(7), 1411-1416. doi:10.1016/j.febslet.2010.01.056

Sardiello, M., Palmieri, M., di Ronza, A., Medina, D. L., Valenza, M., Gennarino, V. A., . . . Ballabio, A. (2009). A gene network regulating lysosomal biogenesis and function. *Science, 325*(5939), 473-477. doi:10.1126/science.1174447

Schjerve, I. E., Tyldum, G. A., Tjonna, A. E., Stolen, T., Loennechen, J. P., Hansen, H. E., . . . Wisloff, U. (2008). Both aerobic endurance and strength training programmes improve cardiovascular health in obese adults. *Clin Sci (Lond), 115*(9), 283-293. doi:10.1042/CS20070332

Schnohr, P., O'Keefe, J. H., Marott, J. L., Lange, P., & Jensen, G. B. (2015). Dose of jogging and long-term mortality: the Copenhagen City Heart Study. *J Am Coll Cardiol, 65*(5), 411-419. doi:10.1016/j.jacc.2014.11.023

Schwalm, C., Jamart, C., Benoit, N., Naslain, D., Premont, C., Prevet, J., . . . Francaux, M. (2015). Activation of autophagy in human skeletal muscle is dependent on exercise intensity and AMPK activation. *FASEB J, 29*(8), 3515-3526. doi:10.1096/fj.14-267187

Settembre, C., Zoncu, R., Medina, D. L., Vetrini, F., Erdin, S., Erdin, S., . . . Ballabio, A. (2012). A lysosome-to-nucleus signalling mechanism senses and regulates the lysosome via mTOR and TFEB. *EMBO J, 31*(5), 1095-1108. doi:10.1038/emboj.2012.32

Shaik, A., Schiavi, A., & Ventura, N. (2016). Mitochondrial autophagy promotes healthy aging. *Cell Cycle, 15*(14), 1805-1806. doi:10.1080/15384101.2016.1181876

Simonsen, A., Cumming, R. C., Brech, A., Isakson, P., Schubert, D. R., & Finley, K. D. (2008). Promoting basal levels of autophagy in the nervous system enhances longevity and oxidant resistance in adult Drosophila. *Autophagy, 4*(2), 176-184.

Statistics, N. C. f. H. (2012). *Health, United States, 2011: With Special Feature on Socioeconomic Health*. Retrieved from Hyattsville, MD:

Tachtsis, B., Smiles, W. J., Lane, S. C., Hawley, J. A., & Camera, D. M. (2016). Acute Endurance Exercise Induces Nuclear p53 Abundance in Human Skeletal Muscle. *Front Physiol, 7*, 144. doi:10.3389/fphys.2016.00144

Tam, B. T., Pei, X. M., Yu, A. P., Sin, T. K., Leung, K. K., Au, K. K., . . . Siu, P. M. (2015). Autophagic adaptation is associated with exercise-induced fibre-type shifting in skeletal muscle. *Acta Physiol (Oxf), 214*(2), 221-236. doi:10.1111/apha.12503

Tam, B. T., & Siu, P. M. (2014). Autophagic cellular responses to physical exercise in skeletal muscle. *Sports Med, 44*(5), 625-640. doi:10.1007/s40279-013-0140-z

Tanaka, Y., Guhde, G., Suter, A., Eskelinen, E. L., Hartmann, D., Lullmann-Rauch, R., . . . Saftig, P. (2000). Accumulation of autophagic vacuoles and cardiomyopathy in LAMP-2-deficient mice. *Nature, 406*(6798), 902-906. doi:10.1038/35022595

Terman, A., Gustafsson, B., & Brunk, U. T. (2007). Autophagy, organelles and ageing. *J Pathol, 211*(2), 134-143. doi:10.1002/path.2094

Tjonna, A. E., Lee, S. J., Rognmo, O., Stolen, T. O., Bye, A., Haram, P. M., . . . Wisloff, U. (2008). Aerobic interval training versus continuous moderate exercise as a treatment for the metabolic syndrome: a pilot study. *Circulation, 118*(4), 346-354. doi:10.1161/CIRCULATIONAHA.108.772822

Todde, V., Veenhuis, M., & van der Klei, I. J. (2009). Autophagy: principles and significance in health and disease. *Biochim Biophys Acta, 1792*(1), 3-13. doi:10.1016/j.bbadis.2008.10.016

Toth, M. L., Sigmond, T., Borsos, E., Barna, J., Erdelyi, P., Takacs-Vellai, K., . . . Vellai, T. (2008). Longevity pathways converge on autophagy genes to regulate life span in Caenorhabditis elegans. *Autophagy, 4*(3), 330-338.

Trost, S. G., Owen, N., Bauman, A. E., Sallis, J. F., & Brown, W. (2002). Correlates of adults' participation in physical activity: review and update. *Med Sci Sports Exerc, 34*(12), 1996-2001. doi:10.1249/01.MSS.0000038974.76900.92

Vainshtein, A., Grumati, P., Sandri, M., & Bonaldo, P. (2014). Skeletal muscle, autophagy, and physical activity: the menage a trois of metabolic regulation in health and disease. *J Mol Med (Berl), 92*(2), 127-137. doi:10.1007/s00109-013-1096-z

Vainshtein, A., & Hood, D. A. (2016). The regulation of autophagy during exercise in skeletal muscle. *J Appl Physiol (1985), 120*(6), 664-673. doi:10.1152/japplphysiol.00550.2015

Vellai, T., Takacs-Vellai, K., Sass, M., & Klionsky, D. J. (2009). The regulation of aging: does autophagy underlie longevity? *Trends Cell Biol, 19*(10), 487-494. doi:10.1016/j.tcb.2009.07.007

Vousden, K. H., & Ryan, K. M. (2009). p53 and metabolism. *Nat Rev Cancer, 9*(10), 691-700. doi:10.1038/nrc2715

Ward, W. F. (2002). Protein degradation in the aging organism. *Prog Mol Subcell Biol, 29*, 35-42.

Whyte, L. J., Gill, J. M., & Cathcart, A. J. (2010). Effect of 2 weeks of sprint interval training on health-related outcomes in sedentary overweight/obese men. *Metabolism, 59*(10), 1421-1428. doi:10.1016/j.metabol.2010.01.002

Wisloff, U., Stoylen, A., Loennechen, J. P., Bruvold, M., Rognmo, O., Haram, P. M., . . . Skjaerpe, T. (2007). Superior cardiovascular effect of aerobic interval training versus moderate continuous training in heart failure patients: a randomized study. *Circulation, 115*(24), 3086-3094. doi:10.1161/CIRCULATIONAHA.106.675041

Wolfson, R. L., Chantranupong, L., Saxton, R. A., Shen, K., Scaria, S. M., Cantor, J. R., & Sabatini, D. M. (2016). Sestrin2 is a leucine sensor for the mTORC1 pathway. *Science, 351*(6268), 43-48. doi:10.1126/science.aab2674

Woods, J. A., Wilund, K. R., Martin, S. A., & Kistler, B. M. (2012). Exercise, inflammation and aging. *Aging Dis, 3*(1), 130-140.

Wu, J. J., Quijano, C., Chen, E., Liu, H., Cao, L., Fergusson, M. M., . . . Finkel, T. (2009). Mitochondrial dysfunction and oxidative stress mediate the physiological impairment induced by the disruption of autophagy. *Aging (Albany NY), 1*(4), 425-437. doi:10.18632/aging.100038

Xu, J., Ji, J., & Yan, X. H. (2012). Cross-talk between AMPK and mTOR in regulating energy balance. *Crit Rev Food Sci Nutr, 52*(5), 373-381. doi:10.1080/10408398.2010.500245

Xu, S., Cai, Y., & Wei, Y. (2014). mTOR Signaling from Cellular Senescence to Organismal Aging. *Aging Dis, 5*(4), 263-273. doi:10.14336/AD.2014.0500263

Yang, F., Chu, X., Yin, M., Liu, X., Yuan, H., Niu, Y., & Fu, L. (2014). mTOR and autophagy in normal brain aging and caloric restriction ameliorating age-related cognition deficits. *Behav Brain Res, 264*, 82-90. doi:10.1016/j.bbr.2014.02.005

Zoncu, R., Efeyan, A., & Sabatini, D. M. (2011). mTOR: from growth signal integration to cancer, diabetes and ageing. *Nat Rev Mol Cell Biol, 12*(1), 21-35. doi:10.1038/nrm3025

Chapter 3: Methodology

Study 1: The effects of an acute bout of HIIT autophagy compared to MICT in human skeletal muscle

METHODS

Ten recreationally active males and females completed a bout of high intensity interval training (HIIT) exercise and moderate intensity continuous exercise (MICT) exercise in a fasted state separated by at least 72 hours. Muscle biopsies from the *vastus lateralis* were taken pre and 3 hours post-exercise. Subjects remained fasted during the 3-hour post-exercise period. Muscle tissue was analyzed for protein expression of markers of autophagy (LC3I, LC3II) and autophagy signaling (p38MAPK). Mean differences in LC3I, LC3II, LC3II:I, and p38MAPK in skeletal muscle between HIIT and MICT exercise were assessed using a 2 (HIIT and MICT) x 2 (2 time points – pre and 3 hours post-exercise) repeated measures ANOVA.

Subjects

Ten healthy, active young adult-aged males (n = 5; age 25.2 ± 1.1 yr, height 180.2 ± 6.4 cm, and body mass 78.8 ± 10.6 kg; VO_{2max}: 48.0 ± 4.9 ml/kg/min :) and females (n = 5; age 21.6 ± 3.6 yr, height 162.5± 11.7 cm, and body mass 58.8 ± 11.7 kg; VO_{2max}: 39.4 ± 7.7 ml/kg/min) who have been engaging in regular physical activity (> 150 minutes of moderate to vigorous intensity aerobic activity per week for a minimum of 1 year) were recruited for the study. The study was approved by the institution's Institutional Review Board (IRB). Participants completed a health history questionnaire (see appendix) and a physical activity history questionnaire (see appendix) to determine training and health status. Subjects were classified as "low risk" according to criteria put forth by the American College of Sports Medicine (L. Pescatello & Medicine, 2014). Participants were excluded if they were smokers, currently diagnosed with any disease, currently taking any medications known to impact muscle metabolism (e.g., statins, COX inhibitors,

AMPK activators, etc.), had a bleeding or blood clotting disorder, had any physical injury, physical limitations, or were currently under the active care of a physician for any condition that may interfere with their safe participation in the study protocol, Participants were made aware of all procedures, including the risks and benefits, gave written consent and completed health history and physical activity questionnaires.

Table 2. Subject characteristics.

	Males (n = 5)	**Females (n = 5)**
Age (yrs)	25.2 ± 1.1	21.6 ± 3.6
Height (cm)	180.2 ± 6.4	162.5± 11.7
Body Mass (kg)	78.8 ± 10.6 kg	58.8 ± 11.7 kg
Body Fat (%)	9.2 ± 2.6	18.1 ± 5.0
VO_{2max} ml/kg/min	48.0 ± 4.9	39.4 ± 7.7

Testing Procedures

The study design required three visits to the Exercise Physiology Laboratory; one visit to determine VO_2max followed by two randomized experimental visits (HIIT and MICT). All visits were separated by at least 72 hours. Experimental exercise bouts (HIIT and MICT) were performed in the fasted state (≥8 hour overnight fast). Muscle biopsies were obtained from the thigh (*vastus lateralis* muscle) pre and 3 hours post-exercise following HIIT and MICT using fine needle microbiopsy to extract muscle tissue for analysis. Subjects remained fasted during the 3 hour post-exercise period.

Exercise Testing and Screening

Subjects arrived at the laboratory after abstaining from alcohol for 24 hours, exercise for 24 hours, and caffeine for 8 hours for visit 1. This visit included filling out health/exercise history and consent forms, a measurement of resting blood pressure, 3 site skinfold body composition measurement and a maximal graded exercise test on a treadmill. The maximal graded exercise test was used to assess VO2max, as well as Vmax to determine running speeds for HIIT (Vmax)

and MICT (55% Vmax) exercise. High intensity interval training and MICT exercise were performed at a 3% grade, therefore the graded maximal exercise test was performed at 3% through the entirety of the test. The test was individually designed using a valid equation established by Jurca et al. 2005 (Jurca et al., 2005) to estimate VO2max. Using the ACSM running metabolic equation (L. Pescatello & Medicine, 2014), estimated VO2max was used to determine estimated Vmax, which was then divided by 10 mins to determine an individualized speed increment per stage for the 10-minute maximal graded exercise test. Subjects warmed up with 3 minutes at 3 mph at 3% grade. Exercise started at minute 3 at 4 mph at 3% grade and speed was increased by the determined value every minute. During the maximal graded running exercise test, heart rate was measured. At maximal exercise, the test was terminated when subjects reached any criteria for absolute or relative indications for terminating exercise testing (L. S. Pescatello & American College of Sports Medicine., 2014). Maximal speed achieved during the graded exercise test was used as V_{max} for HIIT exercise and to determine MICT speed (55% V_{max}). Expired gases were collected via a metabolic gas analyzer (Parvomedics, TrueOne 2400, Sandy, UT) to assess VO_2max. Termination of the maximal graded exercise test occurred upon the subject reaching maximal exertion, which is the point of which the subject was no longer capable of running at the current speed and grade.

Experimental Design

In a randomized crossover design subjects performed either HIIT or MICT running exercise on a treadmill on visit 1 and 2. Trials were separated by at least 72 hours. Subjects arrived at the laboratory after abstaining from exercise for 72 hours, alcohol for 24 hours, caffeine and food for 8 hours (i.e. overnight fast). Heart rate was measured continuously during both exercise bouts using a heart rate monitor (Polar V800, Bethpage, NY). Muscle biopsies were obtained from the thigh (*vastus lateralis* muscle) pre and 3 hours post-exercise. Subjects were allowed consume water ad libitum, but food intake was prohibited during the 3-hour post-exercise period as energy

intake alters autophagic activity. From overnight to fast to 3 hours post-exercise, subjects were fasted ~12 – 15 hours. Muscle tissue obtained from muscle biopsy was analyzed for markers of autophagy.

Exercise Protocols

The HITT bout consisted of a 2 minute warmup at 5 mph followed by 6 bouts of 1 minute at 100% of maximum velocity (V_{max}) at VO_{2max} at 3% grade and 1 minute of 3 mph. Subjects then had a 5 minute recovery period of walking 3 mph prior to another set of 6 bouts of 1 minute at 100% of Vmax at 3% grade and 1 minute at 3 mph. This was followed by 2 minutes of cooldown at 3 mph. The MICT bout consisted of a 2-minute warm up at 5 mph, 60 minutes at 55% Vmax, and 2 minutes of cooldown at 3 mph. Including the warm up and cooldown, the high intensity interval running exercise was 31 minutes in duration; the moderate continuous exercise was 64 minutes in duration. Heart rate was measured throughout both HITT and MICT exercise bouts.

Skeletal Muscle Biopsy Procedures

The fine needle biopsy (i.e. 14-gauge needle microbiopsy) involved the extraction of a small piece (~10-20 mg) of muscle tissue from the m. vastus lateralis using a sterile hollow needle performed by IRB-approved biopsy technicians. The participant's self-reported dominant leg was used for all biopsies. The area over the m. vastus lateralis (outside of the lower thigh muscle) was carefully cleaned with 70% isopropyl alcohol followed by an antiseptic solution (betadine) and covered with a fenestrated drape. Through the access hole of the drape, approximately 3 cc of Lidocaine anesthetic was injected into and under the skin in circular (360˚) fashion. After 2 – 3 minutes and verification that the participant did not have superficial sensation at the region of interest, a small puncture to the skin was made using a 14-gauge pilot needle and inserted into the leg until the superior fascia of the superficial muscle was pierced. A 14-gauge hollow biopsy needle was loaded into the biopsy instrument and inserted into the leg via the pilot incision to

extract skeletal muscle tissue. The needle was removed from the leg and the tissue sample was removed from the biopsy needle using a sterile instrument and placed into a sterile and sealed tube. Approximately, 10 mg of muscle tissue was extracted. The biopsy needle was reloaded in the biopsy instrument and reinserted into the pilot incision for an additional extraction of muscle tissue for a total of approximately 20 mg of muscle tissue. Following the biopsy, the incision was cleaned with 70% isopropyl alcohol, treated with a sterile dressing, and a wrapped in a bandage.

All tissue samples were immediately frozen in liquid nitrogen and stored in a -80°C freezer for subsequent analysis. Samples were analyzed for protein expression of total p38MAPK and LC3.

Protein Quantification

Skeletal muscle was homogenized in 1 ml Lysis Buffer 3 (Cloud Clone ,Katy, TX) per 50 mg of tissue using BeadBug zirconium prefilled tubes (Sigma Aldrich, St. Louise, MO). Muscle tissue was spun in a BeadBug microtube homogenizer (Sigma Aldrich, St. Louise, MO) twice for 40 seconds at 4,000 rpm. Samples were then spun for 2 minutes at 13,000 rpm at 4°C in a microcentrifuge. Supernatant was then collected and stored for protein quantification. Supernatant was diluted 1:20 with Cloud Clone Lysis Buffer 3 for protein quantification which was performed using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). A 2X Laemmli buffer with 5% β-mercaptoethanol was added to the protein lysate and incubated at 100°C for 10 minutes before gel loading for protein separation. Fifteen ug of protein per sample was loaded into the gel for protein separation.

Western blot protein analysis

Proteins were separated by electrophoresis on a resolving and stacking sodium dodecyl sulfate polyacrylamide gel. Separated proteins were transferred to a Trans-blot Turbo transfer PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked for 30 min in

5% dry milk and Tris buffered saline Tween 20 buffer solution, washed in Tris-buffered saline (TBS), and incubated overnight in the LC3 (Sigma Aldrich, St. Louise, MO), p62 (Cell Signaling Technology, Danvers, MA) and p38 (Cell Signaling Technology, Danvers, MA) primary antibodies at 4°C. All membranes were washed in TBS with 0.05% Tween 20 (TBS-tween) and incubated with a horseradish peroxidase-conjugated secondary antibody (Invitrogen, Carlsbad, CA) and incubated for 1 hour at room temperature. Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Santa Cruz, CA) using the ChemiDoc Touch Imaging System (Bio-Rad, Hercules, CA) was used to develop and record the membrane. Image Lab software (Bio-Rad, Hercules, CA) was used to quantify protein expression by determining densitometric values. All proteins were normalized to total protein loaded.

Statistical Analysis

A sample of 10 subjects was selected based of previous related work (Bartlet et al. 2014). Mean differences in LC3I, LC3II, LC3II:I, and p38MAPK in skeletal muscle between HIIT and MICT exercise were assessed using a 2 (HIIT and MICT) x 2 (2 time points – pre and 3 hours post-exercise) repeated measures ANOVA using SPSS (Version 20.0, Chicago, IL, USA). All experimental conditions were compared to Con which was given a standard value of 100%. A student's T Test was used to determine mean differences in HR between HIIT and MICT. The threshold for statistical significance was set *a priori* at a p-value of ≤ 0.05 for all tests.

Study 2: Using electrical pulse stimulation as an in vitro model for muscle contraction-induced autophagy

METHODS

C2C12 myoblasts were differentiated into myotubes and underwent a long duration (8 hours), low frequency (1Hz, 2ms pulse width, 1.5 V/mm) EPS protocol (EPS) using C-Pace EP multichannel

culture pacer (IonixOptix, Westwood, MA) or a non-EPS control condition (Con). Differentiation medium was replenished 24 hours before the experiment for EPS and Con cells. Immediately prior to the experiment, bafilomycin A1 was added to differentiation medium in EPS cells (EPS+Baf) or differentiation medium was replaced with glucose-free and amino acid-free starvation medium in EPS (EPS+St) and Con (St) cells with (EPS+St+Baf) and without (St+Baf) bafilomycin A1 (InvivoGen, San Diego,CA) immediately prior to EPS. The EPS protocol employed has been shown to elicit a reliable and predictable response to muscle contraction (Nieuwoudt et al., 2017). Cells were collected immediately following the 8 hour protocol. Cells were analyzed for markers of autophagy.

Cell Culture

C2C12 myoblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS) and passed into 6-well C-Dish cell culture plates (IonixOptix, Westwood, MA) at 70 – 80 % confluence. Medium was changed on a 2 – 3-day basis. To differentiate myoblasts into myotubes, cells were grown to 100% confluence and maintained with supplemented DMEM for 3 days upon reaching 100% confluence. At day 3 of confluence, medium was replaced with DMEM supplemented with 2% horse serum (HS) and 1% P) to initiate myoblast differentiation into myotubes. Differentiation media was changed on a 2 – 3-day basis for 4 – 6 days, until visibly differentiated. Twenty-four hours prior to EPS, differentiation medium was replaced for non-St condition cells while for St condition cells, differentiation medium was replaced with glucose-free and amino acid-free starvation medium of PBS with 2% HS and 1% PS starvation medium with and without bafilomycin (1 uM). Cells then underwent EPS or non-stimulated control condition (incubation).

Electrical Pulse Stimulation

C2C12 myotubes grown in 6-well C-Dish cell culture plates (Ionix Optix, Westwood, MA) were stimulated via carbon electrodes by applying low frequency EPS (1Hz, 2ms pulse width, 1.5 V/mm [11.5 V] for 8 hours) using a multichannel culture pacer (C-Pace EP ,IonixOptix, Westwood, MA). Cells were placed in an incubator (37°C and 5 % CO_2) during the 8 hours of EPS. Non-EPS Con cells were incubated for 8 hours. Differentiation medium was replaced 24 hours prior to the experiment for both stimulated (EPS) and non-EPS (Con) cells. To study the effect of energy status on autophagy in C2C12 cells undergoing EPS, cells were starved during the experimental protocol by replacing differentiation medium with starvation medium in EPS (EPS+St) and non-EPS cells (St). Starvation of C2C12 cells has been shown to augment autophagic activity (Desgeorges et al., 2014). To control for the dynamic LC3 and p62 activity during increased autophagy, 1 uM bafilomycin (Invivogen, San Diego, CA) was added to differentiation and starvation medium at a known to effectively inhibit autophagasome degradation, 1:4000 (Drew et al. 2014) immediately before the experiment in EPS (EPS+Baf, EPS+St+Baf) and non-EPS (St+Baf). Bafilomycin terminates the degradation of the autophagasome by inhibiting the lysosomal proton pump, neutralizing the acidic pH of the lysosome (Klionsky et al., 2016). Thus, autophagic activity may be discerned more clearly as proteins that may be simultaneously transcribed and degraded during increased autophagic flux accumulate and measurement of overall protein content provides a more robust assessment of autophagy activity. Cells were collected immediately following EPS and stored in a -80°C freezer for subsequent analysis. Samples were analyzed for LC3 and p62.

Protein Quantification

C2C12 cells were homogenized with 150 µl of RIPA commercial lysis buffer. The protein-containing supernatant was collected and stored in 1.5 ml Eppendorf tubes for protein quantification. Protein quantification was performed using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA). A 2X Laemmli buffer with 5% β-mercaptoethanol was added

to the protein lysate and incubated at 100°C for 10 minutes before gel loading for protein separation.

Western blot protein analysis

Proteins were separated by electrophoresis on a resolving and stacking sodium dodecyl sulfate polyacrylamide gel. Separated proteins were transferred to a Trans-blot Turbo transfer PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked for 30 min in 5% dry milk and Tris buffered saline Tween 20 buffer solution, washed in Tris-buffered saline (TBS), and incubated overnight in the LC3 (Sigma Aldrich, St. Louise, MO), and p62 (Cell Signaling Technology, Danvers, MA) primary antibodies at 4°C. All membranes were washed in TBS with 0.05% Tween 20 (TBS-tween) and incubated with a horseradish peroxidase-conjugated secondary antibody (Invitrogen, Carlsbad, CA) and incubated for 1 hour at room temperature. Santa Cruz Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Santa Cruz, CA) using the ChemiDoc Touch Imaging System (Bio-Rad, Hercules, CA) was used to develop and record the membrane. Image Lab software (Bio-Rad, Hercules, CA) was used to quantify protein expression by determining densitometric values. All proteins were normalized to total protein loaded.

Statistical Analysis

A one-way analysis (ANOVA) was used to probe for differences in LC3I, LC3II, LC3II:I, and p62 in C2C12 myotubes between experimental EPS conditions (Con, EPS, EPS+Baf), experimental St conditions (Con, St, St+Baf) and combined experimental conditions (Con, EPS, EPS+Baf, St, St+Baf, EPS+St, EPS+St+Baf) using SPSS (Version 20.0, Chicago, IL, USA). When appropriate, a Tukey Honest Significant Difference (HSD) was performed. All experimental conditions were compared to Con which was given a standard value of 100%. The threshold for statistical significance was set *a priori* at a p-value of ≤ 0.05 for all tests.

References

Balaburski, G. M., Hontz, R. D., & Murphy, M. E. (2010). p53 and ARF: unexpected players in autophagy. *Trends Cell Biol, 20*(6), 363-369. doi:10.1016/j.tcb.2010.02.007

Booth, F. W., Roberts, C. K., & Laye, M. J. (2012). Lack of exercise is a major cause of chronic diseases. *Compr Physiol, 2*(2), 1143-1211. doi:10.1002/cphy.c110025

Desgeorges, M. M., Freyssenet, D., Chanon, S., Castells, J., Pugniere, P., Bechet, D., . . . Defour, A. (2014). Post-transcriptional regulation of autophagy in C2C12 myotubes following starvation and nutrient restoration. *Int J Biochem Cell Biol, 54*, 208-216. doi:10.1016/j.biocel.2014.07.008

Dokladny, K., Myers, O. B., & Moseley, P. L. (2015). Heat shock response and autophagy--cooperation and control. *Autophagy, 11*(2), 200-213. doi:10.1080/15548627.2015.1009776

Egan, D. F., Shackelford, D. B., Mihaylova, M. M., Gelino, S., Kohnz, R. A., Mair, W., . . . Shaw, R. J. (2011). Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy. *Science, 331*(6016), 456-461. doi:10.1126/science.1196371

Evers-van Gogh, I. J., Alex, S., Stienstra, R., Brenkman, A. B., Kersten, S., & Kalkhoven, E. (2015). Electric Pulse Stimulation of Myotubes as an In Vitro Exercise Model: Cell-Mediated and Non-Cell-Mediated Effects. *Sci Rep, 5*, 10944. doi:10.1038/srep10944

Gibala, M. J., Gillen, J. B., & Percival, M. E. (2014). Physiological and health-related adaptations to low-volume interval training: influences of nutrition and sex. *Sports Med, 44 Suppl 2*, S127-137. doi:10.1007/s40279-014-0259-6

Gibala, M. J., Little, J. P., Macdonald, M. J., & Hawley, J. A. (2012). Physiological adaptations to low-volume, high-intensity interval training in health and disease. *J Physiol, 590*(5), 1077-1084. doi:10.1113/jphysiol.2011.224725

Gillen, J. B., & Gibala, M. J. (2014). Is high-intensity interval training a time-efficient exercise strategy to improve health and fitness? *Appl Physiol Nutr Metab, 39*(3), 409-412. doi:10.1139/apnm-2013-0187

Grumati, P., Coletto, L., Schiavinato, A., Castagnaro, S., Bertaggia, E., Sandri, M., & Bonaldo, P. (2011). Physical exercise stimulates autophagy in normal skeletal muscles but is detrimental for collagen VI-deficient muscles. *Autophagy, 7*(12), 1415-1423.

He, C., Bassik, M. C., Moresi, V., Sun, K., Wei, Y., Zou, Z., . . . Levine, B. (2012). Exercise-induced BCL2-regulated autophagy is required for muscle glucose homeostasis. *Nature, 481*(7382), 511-515. doi:10.1038/nature10758

Jamart, C., Benoit, N., Raymackers, J. M., Kim, H. J., Kim, C. K., & Francaux, M. (2012). Autophagy-related and autophagy-regulatory genes are induced in human muscle after ultraendurance exercise. *Eur J Appl Physiol, 112*(8), 3173-3177. doi:10.1007/s00421-011-2287-3

Jamart, C., Francaux, M., Millet, G. Y., Deldicque, L., Frere, D., & Feasson, L. (2012). Modulation of autophagy and ubiquitin-proteasome pathways during ultra-endurance running. *J Appl Physiol (1985), 112*(9), 1529-1537. doi:10.1152/japplphysiol.00952.2011

Ju, J. S., Jeon, S. I., Park, J. Y., Lee, J. Y., Lee, S. C., Cho, K. J., & Jeong, J. M. (2016). Autophagy plays a role in skeletal muscle mitochondrial biogenesis in an endurance exercise-trained condition. *J Physiol Sci, 66*(5), 417-430. doi:10.1007/s12576-016-0440-9

Jurca, R., Jackson, A. S., LaMonte, M. J., Morrow, J. R., Jr., Blair, S. N., Wareham, N. J., . . . Laukkanen, R. (2005). Assessing cardiorespiratory fitness without performing exercise testing. *Am J Prev Med, 29*(3), 185-193. doi:10.1016/j.amepre.2005.06.004

Klionsky, D. J., Abdelmohsen, K., Abe, A., Abedin, M. J., Abeliovich, H., Acevedo Arozena, A., . . . Zughaier, S. M. (2016). Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy, 12*(1), 1-222. doi:10.1080/15548627.2015.1100356

Li, H., Liu, S., Yuan, H., Niu, Y., & Fu, L. (2017). Sestrin 2 induces autophagy and attenuates insulin resistance by regulating AMPK signaling in C2C12 myotubes. *Exp Cell Res, 354*(1), 18-24. doi:10.1016/j.yexcr.2017.03.023

Lira, V. A., Okutsu, M., Zhang, M., Greene, N. P., Laker, R. C., Breen, D. S., . . . Yan, Z. (2013). Autophagy is required for exercise training-induced skeletal muscle adaptation and improvement of physical performance. *FASEB J, 27*(10), 4184-4193. doi:10.1096/fj.13-228486

Liu, X., Niu, Y., Yuan, H., Huang, J., & Fu, L. (2015). AMPK binds to Sestrins and mediates the effect of exercise to increase insulin-sensitivity through autophagy. *Metabolism, 64*(6), 658-665. doi:10.1016/j.metabol.2015.01.015

Maiuri, M. C., Malik, S. A., Morselli, E., Kepp, O., Criollo, A., Mouchel, P. L., . . . Kroemer, G. (2009). Stimulation of autophagy by the p53 target gene Sestrin2. *Cell Cycle, 8*(10), 1571-1576. doi:10.4161/cc.8.10.8498

Masiero, E., Agatea, L., Mammucari, C., Blaauw, B., Loro, E., Komatsu, M., . . . Sandri, M. (2009). Autophagy is required to maintain muscle mass. *Cell Metab, 10*(6), 507-515. doi:10.1016/j.cmet.2009.10.008

Meijer, A. J., Lorin, S., Blommaart, E. F., & Codogno, P. (2015). Regulation of autophagy by amino acids and MTOR-dependent signal transduction. *Amino Acids, 47*(10), 2037-2063. doi:10.1007/s00726-014-1765-4

Mercken, E. M., Crosby, S. D., Lamming, D. W., JeBailey, L., Krzysik-Walker, S., Villareal, D. T., . . . Fontana, L. (2013). Calorie restriction in humans inhibits the PI3K/AKT pathway and induces a younger transcription profile. *Aging Cell, 12*(4), 645-651. doi:10.1111/acel.12088

Moller, A. B., Vendelbo, M. H., Christensen, B., Clasen, B. F., Bak, A. M., Jorgensen, J. O., . . . Jessen, N. (2015). Physical exercise increases autophagic signaling through ULK1 in human skeletal muscle. *J Appl Physiol (1985), 118*(8), 971-979. doi:10.1152/japplphysiol.01116.2014

Ng, F., & Tang, B. L. (2013). Sirtuins' modulation of autophagy. *J Cell Physiol, 228*(12), 2262-2270. doi:10.1002/jcp.24399

Nieuwoudt, S., Mulya, A., Fealy, C. E., Martelli, E., Dasarathy, S., Naga Prasad, S. V., & Kirwan, J. P. (2017). In vitro contraction protects against palmitate-induced insulin resistance in C2C12 myotubes. *Am J Physiol Cell Physiol, 313*(5), C575-C583. doi:10.1152/ajpcell.00123.2017

Nikolic, N., Bakke, S. S., Kase, E. T., Rudberg, I., Flo Halle, I., Rustan, A. C., . . . Aas, V. (2012). Electrical pulse stimulation of cultured human skeletal muscle cells as an in vitro model of exercise. *PLoS One, 7*(3), e33203. doi:10.1371/journal.pone.0033203

Nikolic, N., Gorgens, S. W., Thoresen, G. H., Aas, V., Eckel, J., & Eckardt, K. (2017). Electrical pulse stimulation of cultured skeletal muscle cells as a model for in vitro exercise - possibilities and limitations. *Acta Physiol (Oxf), 220*(3), 310-331. doi:10.1111/apha.12830

Pescatello, L., & Medicine, A. C. o. S. (2014). *ACSM's guidelines for exercise testing and prescription* (9th ed.). Philadelphia, pennsylvania: Wolters Kluwer/Lippincott Williams & Wilkins Health.

Pescatello, L. S., & American College of Sports Medicine. (2014). *ACSM's guidelines for exercise testing and prescription* (9th ed.). Philadelphia: Wolters Kluwer/Lippincott Williams & Wilkins Health.

Raben, N., Hill, V., Shea, L., Takikita, S., Baum, R., Mizushima, N., . . . Plotz, P. (2008). Suppression of autophagy in skeletal muscle uncovers the accumulation of ubiquitinated

proteins and their potential role in muscle damage in Pompe disease. *Hum Mol Genet, 17*(24), 3897-3908. doi:10.1093/hmg/ddn292

Sahani, M. H., Itakura, E., & Mizushima, N. (2014). Expression of the autophagy substrate SQSTM1/p62 is restored during prolonged starvation depending on transcriptional upregulation and autophagy-derived amino acids. *Autophagy, 10*(3), 431-441. doi:10.4161/auto.27344

Salminen, A., & Kaarniranta, K. (2009). Regulation of the aging process by autophagy. *Trends Mol Med, 15*(5), 217-224. doi:10.1016/j.molmed.2009.03.004

Schwalm, C., Jamart, C., Benoit, N., Naslain, D., Premont, C., Prevet, J., . . . Francaux, M. (2015). Activation of autophagy in human skeletal muscle is dependent on exercise intensity and AMPK activation. *FASEB J, 29*(8), 3515-3526. doi:10.1096/fj.14-267187

Tachtsis, B., Smiles, W. J., Lane, S. C., Hawley, J. A., & Camera, D. M. (2016). Acute Endurance Exercise Induces Nuclear p53 Abundance in Human Skeletal Muscle. *Front Physiol, 7*, 144. doi:10.3389/fphys.2016.00144

Tam, B. T., & Siu, P. M. (2014). Autophagic cellular responses to physical exercise in skeletal muscle. *Sports Med, 44*(5), 625-640. doi:10.1007/s40279-013-0140-z

Tarum, J., Folkesson, M., Atherton, P. J., & Kadi, F. (2017). Electrical pulse stimulation: an in vitro exercise model for the induction of human skeletal muscle cell hypertrophy. A proof-of-concept study. *Exp Physiol, 102*(11), 1405-1413. doi:10.1113/EP086581

Todde, V., Veenhuis, M., & van der Klei, I. J. (2009). Autophagy: principles and significance in health and disease. *Biochim Biophys Acta, 1792*(1), 3-13. doi:10.1016/j.bbadis.2008.10.016

Vainshtein, A., Grumati, P., Sandri, M., & Bonaldo, P. (2014). Skeletal muscle, autophagy, and physical activity: the menage a trois of metabolic regulation in health and disease. *J Mol Med (Berl), 92*(2), 127-137. doi:10.1007/s00109-013-1096-z

Vainshtein, A., & Hood, D. A. (2016). The regulation of autophagy during exercise in skeletal muscle. *J Appl Physiol (1985), 120*(6), 664-673. doi:10.1152/japplphysiol.00550.2015

Yang, L., Licastro, D., Cava, E., Veronese, N., Spelta, F., Rizza, W., . . . Fontana, L. (2016). Long-Term Calorie Restriction Enhances Cellular Quality-Control Processes in Human Skeletal Muscle. *Cell Rep, 14*(3), 422-428. doi:10.1016/j.celrep.2015.12.042

Chapter 4: Results

Study 1: The effects of an acute bout of HIIT on autophagy compared to MICT in human skeletal muscle

Results

Protein Expression of Markers of Autophagy

There were no significant differences time or condition effects in protein expression of LC3I (Figure 1) or LC3II (Figure 1) in skeletal muscle at 3 hours post-exercise compared to pre-exercise ($p > 0.05$) or between conditions (HIIT and MICT; $p > 0.05$). However, there was a time x condition interaction for LC3II:I. ($p = 0.045$). A Tukey Post Hoc test revealed a statistical difference between HIIT and MICT 3 hours post-exercise ($p = 0.038$). LC3II:I 3 hours post HIIT increased to 162.4% (SE: 45.9%) of pre-exercise and decreased to 48.8 ± 29.8% (SE: 9.4%) of pre-exercise 3 hours post MICT (Figure 1).There were no significant differences between protein expression of total p38MAPK at 3 hours post-exercise compared to pre-exercise ($p > 0.05$) or between conditions ($p > 0.05$; Figure 1).

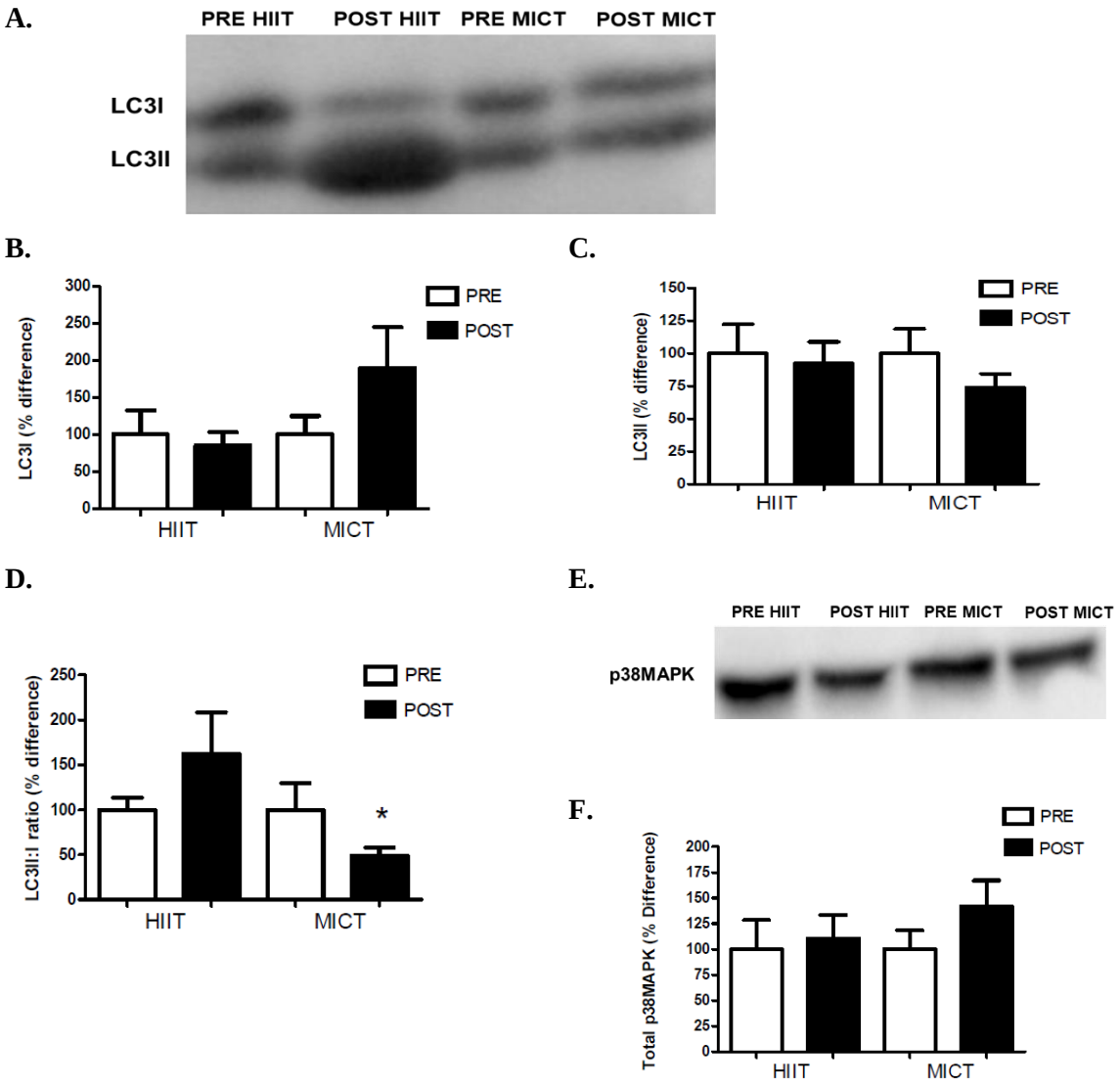

Figure 2. Relative quantification of autophagy-related proteins in skeletal muscle of physically active males (n = 5) and females (n =5) pre-exercise (PRE) and 3 hours post-exercise (POST) following an acute bout of high intensity interval training (HIIT) or moderate intensity continuous training (MICT). A. Representative blot for LC3I, LC3II, and LC3II:I in human skeletal muscle. Percent change from pre HIIT and pre MICT for B. LC3I, C. LC3II, D. LC3II:I, E. Representative blot for total p38MAPK. F. Percent change from pre HIIT and pre MICT for total p38MAPK. Quantification of relative protein content was completed using densitometric values obtained using Image Lab software and normalized to total protein loaded and set to 100 for control condition. Data represent means and standard error. * indicates statistically significant ($p \leq 0.05$) difference compared to POST HIIT.

Heart Rate

Mean HR during the HITT bout was 159 ± 14 bpm and 154 ± 8 bpm in the MICT bout (Figure 2).

A T Test revealed a statistical difference ($p < 0.00$) between mean HR in the two bouts.

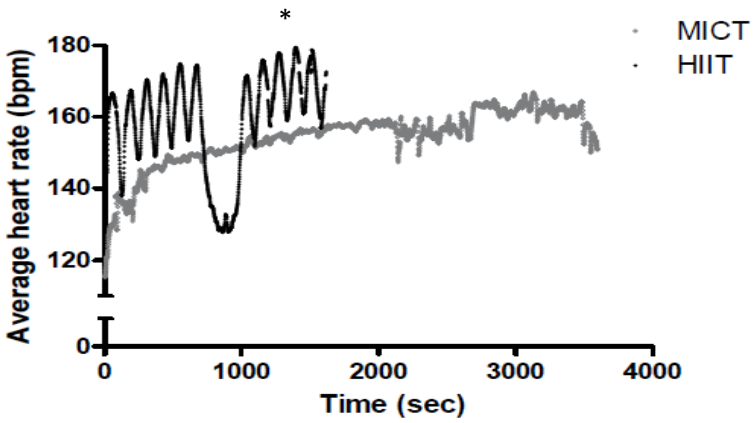

Figure 3. Mean heart rate (HR) in beats per minute (bpm) during high intensity interval (HIIT) exercise and moderate intensity continuous (MICT) exercise. Data represent means and standard error. * signifies a statistically significant difference ($p \leq 0.05$) between HIIT and MICT.

Study 2: Using electrical pulse stimulation as an *in vitro* model for muscle contraction-induced autophagy.

RESULTS

EPS Conditions

An analysis of variance between EPS conditions found no statistically significant differences for LC3I protein expression (Figure 3). LC3II was significantly greater in EPS+Baf (324.4%, SE: 29.3%) compared to Con (100%, SE: 9.3%; $p < 0.001$) and EPS (127.7%, SE: 24.3%; $p < 0.001$). LC3II:I was higher in EPS+Baf (258.2%, SE: 32.3%) compared to Con (100%, SE: 8.8%;$p <$

0.001) and EPS (95.5%, SE: 13.2%). p62 was greater in EPS+Baf, (437%, SE: 9.7%) compared to Con (100%, SE: 22.6; p = 0.001) and EPS (251.2%, SE: 33.2%; p = 0.020) and EPS was greater than Con.

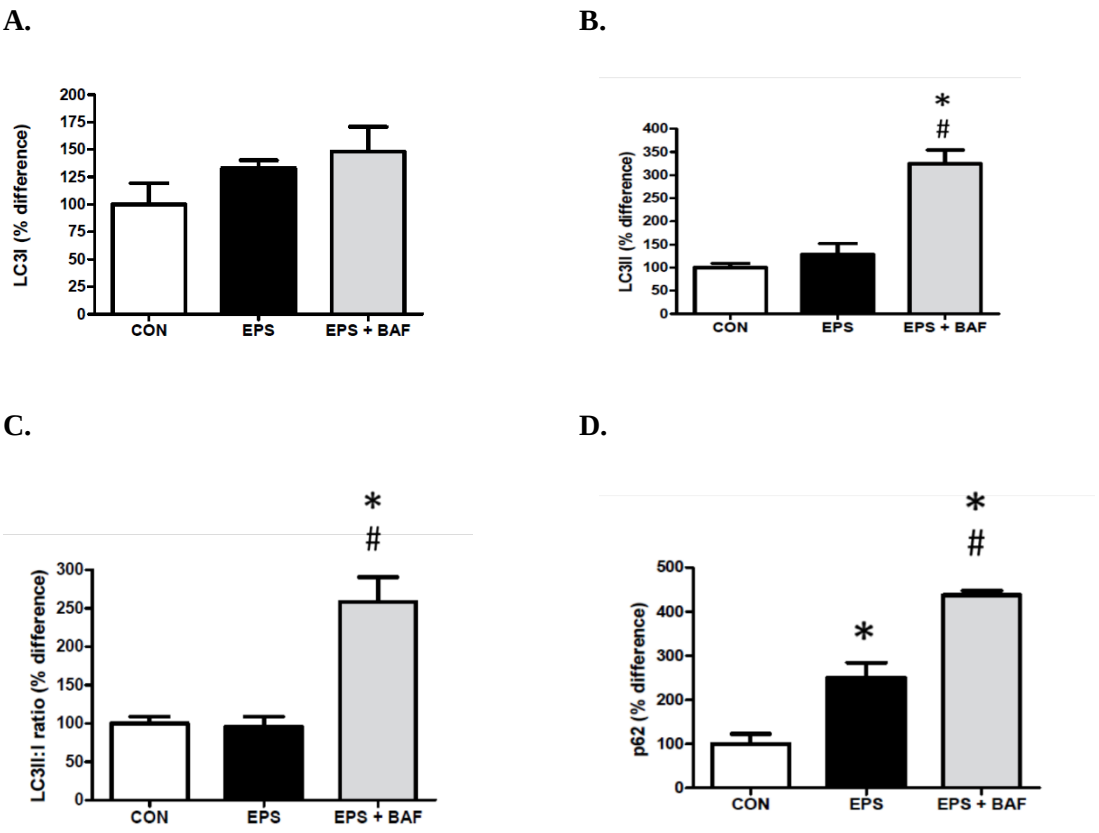

Figure 4. Relative quantification of autophagy-related proteins A. LC3I, B. LC3II, C. LC3II:I, D. p62 in C2C12 myotubes following 8 hours of electrical pulse stimulation conditions (EPS), EPS with bafilomycin treatment (EPS+Baf), and control condition (Con). Quantification of relative protein content was completed using densitometric values obtained using Image Lab software and normalized to total protein loaded and set to 100 for control condition. Data represent means and standard error. * indicates significantly ($p \leq 0.05$) different from Con; # from EPS. (n = 3 – 5).

Starvation Conditions

There were no statistically significant differences between starvation conditions (Con, St, St+Baf) LC3I, LC3II, or LC3II:I (Figure 4). p62 protein expression was significantly greater in St+Baf

(320.9%, SE: 65.9%) compared to Con (100%, SE: 22.6%; p = 0.027), and St (91.3%, SE: 20.3%; p = 0.032).

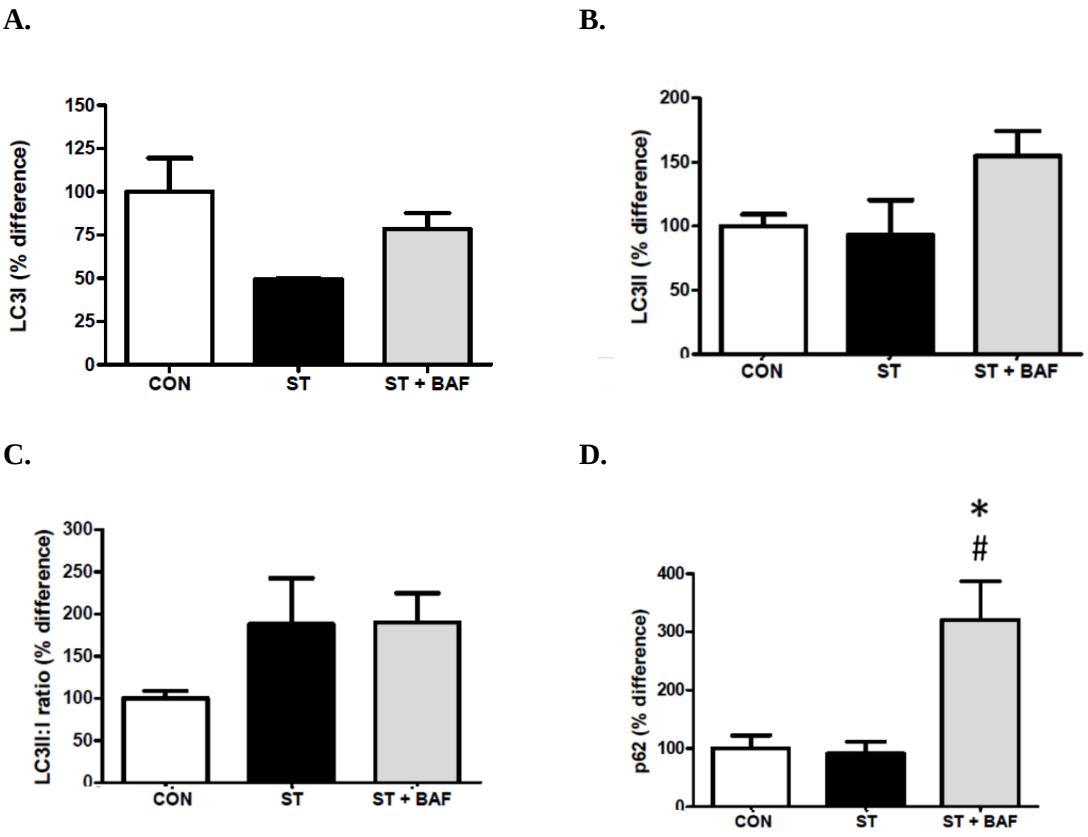

Figure 5. Relative quantification of autophagy-related proteins A. LC3I, B. LC3II, C. LC3II:I, D. p62 in C2C12 myotubes following 8 hours of starvation (St), starvation with bafilomycin treatment (EPS+Baf), and control condition (Con). Quantification of relative protein content was completed using densitometric values obtained using Image Lab software and normalized to total protein loaded and set to 100 for control condition. Data represent means and standard error. * indicates significantly ($p \leq 0.05$) different from Con; # from EPS. (n = 3 – 5).

Combined Experimental Conditions

LC3I protein expression in EPS+Baf (148.0%, SE: 22.7%) and EPS (132.9%, SE: 7.4%) were significantly greater than St (49.3%, SE:0.3%) and EPS+St (57.6%, SE: 9.8%) ($p < 0.05$) (Figure 5).

LC3II protein expression was significantly greater in EPS+Baf (324.4%, SE: 66.7%) compared to Con (100%, SE 9.3%), EPS (127%, SE: 24.3%), St (92.9%, SE: 27.3%), St+Baf (154.8%, SE:

19.5%) and EPS+St (92.0%, SE: 19.8%) ($p < 0.05$). LC3I was higher in EPS+St+Baf (263.4 SE: 56.4%) compared to Con, EPS, St, and EPS+St ($p < 0.05$).

LC3II:I ratio was significantly higher in EPS+Baf (258.2%, SE: 32.3%) and EPS+St+Baf (302.1%, SE: 27.7%) compared to Con (100%, SE: 8.8%) and EPS (95.2%, SE: 13.2%) ($p < 0.05$).

Protein expression of p62 was significantly greater in EPS+Baf (437.9%, SE: 9.7%) and EPS+St+Baf (394.1%, SE: 93.8%) compared to Con (100%, SE: 22.6%), St (91.3%, SE: 20.3%), and EPS+St (187.4%, SE: 6.9%) ($p < 0.05$). p62 was also higher in St+Baf (320.9%, SE: 65.9%) compared to Con and St ($p < 0.05$).

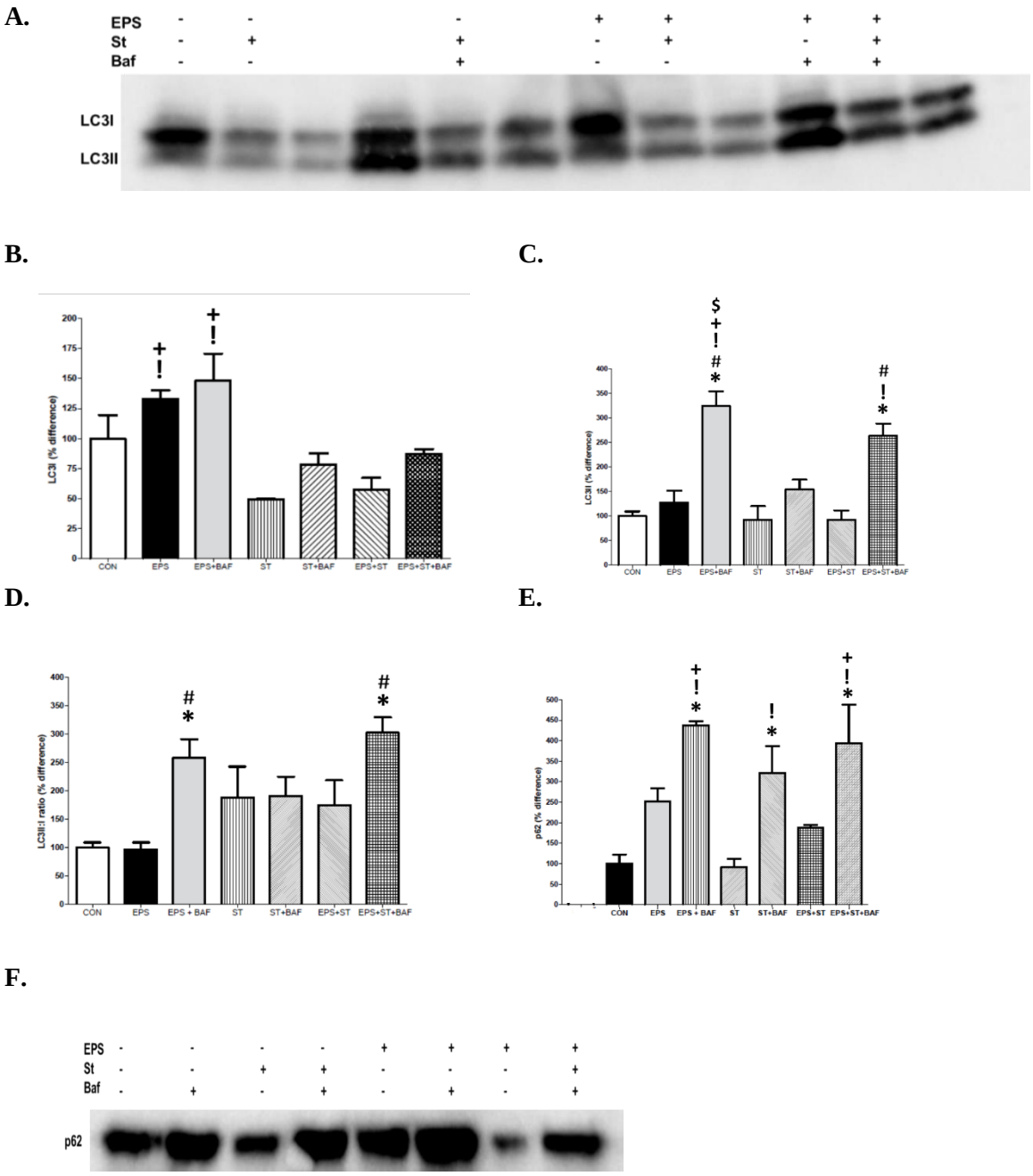

Figure 6. Relative quantification of autophagy-related proteins following 8 hours of electrical pulse stimulation (EPS), starvation (St), EPS and St combined, with and without bafilomycin treatment (Baf), and control condition (Con) in C2C12 myotubes. A. representative blot for LC3I, LC3II, LC3II:I. B – D. Percent change from Con in EPS, St, and combined EPS and St conditions with and without bafilomycin (Baf) for LC3I, LC3II, and LC3II:I. E. Representative blot for p62. F. Percent change from Con in EPS, St, and combined EPS and St conditions with and without bafilomycin (Baf) for p62. Quantification of relative protein content was completed using densitometric values obtained using Image Lab software and normalized to total protein loaded and set to 100 for control condition. Data represent means and standard error. Indicates significantly different from * indicates significantly ($p \leq 0.05$) different from Con; # from EPS; ! from St; $ from St+Baf; + from EPS+St.

Chapter 5: Discussion

Summary

The purpose of this investigation was twofold: firstly, to compare the effects of an acute bout of HIIT (treadmill running) on the autophagy responses to MICT in human skeletal muscle 3 hours post exercise.

Secondly, to test an *in vitro* model for muscle contraction-induced autophagy using electrical pulse stimulation in C2C12 myotubes.

Study 1: The effects of an acute bout of HIIT autophagy compared to MICT in human skeletal muscle

Autophagy was activated following an acute bout of HIIT and MICT exercise in human skeletal muscle of physically active males and females. No significant differences were observed in LC3I or LC3II between conditions or from pre-exercise to 3 hours post-exercise. LC3II:I ratio was statistically higher 3 hours post-exercise in HIIT (162.4%, SE: 45.9%) compared to MICT (48.8%, SE: 9.4%). No significant differences were observed in total p38MAPK between pre and 3 hours post-exercise or between conditions.

These results are the first to show an acute bout of HIIT activates autophagy as represented by a change in LC3II:I. Moreover, HIIT elicited a distinct response in LC3II:I ratio 3 hours post-exercise from MICT; increasing from pre-exercise, while decreasing in MICT. These findings also demonstrate 60 minutes of running at 55% VO_{2max} alter autophagy activity, which has not demonstrated before. This is consistent with previous literature showing a minimum of 60 minutes of exercise at a minimum of 55% VO_{2max} are required for autophagy activation in skeletal muscle (Jamart, Benoit, et al., 2012; Jamart, Francaux, et al., 2012; Moller et al., 2015; Schwalm et al., 2015).

These data show that both HIIT and MICT are capable of activating autophagy which is central to human health and possesses a role in promoting chronic adaptations to exercise. Thus, HIIT may serve as a means to elicit autophagy in similar fashion to MICT.

Study 2: Using electrical pulse stimulation as an *in vitro* model for muscle contraction-induced autophagy

Eight hours of low frequency EPS, starvation, and combined EPS and starvation conditions increased autophagy activity in C2C12 myotubes. In EPS only conditions, LC3II protein expression and LC3II:I ratio in EPS+Baf was significantly greater than Con and EPS. Protein expression of p62 in EPS+Baf and EPS was greater than Con and EPS+Baf was greater than EPS. In starvation conditions, no statistically significant differences were found in LC3I, LC3II, and LC3II:I between Con, St, and S+Baf. p62 in EPS+St was greater than Con and St. In combined conditions, LC3I protein expression was significantly greater in EPS than Con, St, and EPS+St. EPS+Baf produced greater LC3I content than St and EPS+St. LC3II in EPS+Baf was greater than Con, EPS, St, EPS+St, and St+Baf. EPS+St+Baf produced greater LC3II protein expression than Con, EPS, and St. LC3II:I ratio was greater in EPS+Baf compared to Con and EPS and EPS+St+Baf was also greater than Con and EPS. Protein expression of p62 in EPS+Baf was greater than Con, St, and EPS+St. St+Baf was greater than Con and St, and EPS+St+Baf was greater than Con, St, and EPS+St.

These results demonstrate EPS in fed and starvation conditions may be used as an *in vitro* muscle contraction-induced autophagy model in C2C12 myotubes. Electrical pulse stimulation in cultured skeletal muscle cells has produced *in vivo* exercise signaling transduction and metabolic responses including modulation of Ca++ transients, AMPK, p38MAPK, mTOR and increased fatty acid and glucose uptake and oxidation, GLUT4 translocation, and increased insulin sensitivity in C2C12 cells (Nieuwoudt et al., 2017; Nikolic et al., 2017; Tarum, Folkesson, Atherton, & Kadi, 2017). Autophagy is known to be regulated by these signaling pathways

(Vainshtein & Hood, 2016) and implicated in mediating these metabolic responses (Halling & Pilegaard, 2017). Acute starvation (i.e. 4 hours) has been shown to elicit autophgagic activity in C2C12 cells (Desgeorges et al., 2014)

Indeed, EPS and starvation induced changes in a number of autophagic makers including LC3I, LC3II, LC3II:I, and p62. This response was punctuated with the addition of bafilomycin which inhibits autophagasome degradation leading to a significant increase in autophagasome-associtated proteins LC3II and p62 and an increase in LC3II:I ratio compared to non-bafilomycin treated cells.

DISCUSSION OF RESULTS

The present studies demonstrate an autophagic response to muscle contraction in human skeletal muscle following acute exercise and in C2C12 myotubes following electrical pulse stimulation.

Autophagy was stimulated by acute bouts of HIIT and MICT exercise in human skeletal muscle and by EPS in fed and starvation conditions in C2C12 myotubes. Importantly, study 2 did not specifically aim at mimicking study 1 (i.e. design, approach). These studies differ in several meaningful ways. The experimental stresses, species tissue, and duration of protocols in each model are distinct and cannot be extrapolated from one model to the other. Thus, each study employed its own approach and design. Results in study 1 were not intended to be replicated study 2.

<u>Study 1: The effects of an acute bout of HIIT autophagy compared to MICT in human skeletal muscle</u>

This study demonstrated that an acute bout of HIIT exercise performed in a fasted state elicits an autophagic response in skeletal muscle, an observation not documented before. An acute bout of MICT exercise amid a fast also produced changes in autophagic activity. Interestingly, HIIT and MICT exercise produced distinct autophagic response as measured by LC3II:I ratio 3 hours post-

exercise. High intensity interval training exercise increased LC3II:I to 162.4 ± 145.3% of pre-exercise while MICT decreased LC3II:I to 48.8 ± 29.8% of pre-exercise. These results support our hypothesis that HIIT exercise stimulates autophagy as does MICT exercise; however, these exercise bouts elicited divergent responses.

A discrepant LC3II:I response between HIIT and MICT bouts is a novel finding; however, LC3 is known to be highly-dynamic in its forms (LC3I and LC3II) and content (Klionsky et al., 2016). During autophagic flux, LC3-I, the cytosolic form of LC3, is lipidated to the autophagasome membrane-bound form, LC3-II which is associated with the completed autophagasome. The autophagasome is then degraded at the lysosome along with LC3II. (Klionsky et al., 2016). Thus, content of LC3I and LC3II is predicated on baseline transcriptional activity, the conversion of LC3I to LC3II, and the clearance of LC3II. Importantly, with regard to lipidation, a clear precursor/product relationship between LC3I and LC3II does not always exist and has not been discerned in human skeletal muscle (Klionsky et al., 2016). LC3 may also be transcriptionally upregulated during cellular stress (Settembre et al., 2011). Thus, content and form of LC3 is volatile and increased autophagy may be marked by a number of different LC3 compositions. Moreover, LC3 dynamics during and time course of increased levels of autophagy are variable and dependent on species, tissue, and type of cellular stress including lipidation and autophagasome formation and degradation (Klionsky et al., 2016). Presently, scarce data exist describing the autophagy response to acute exercise in human skeletal muscle and results describing LC3 are conflicting. However, an inflammatory response as a results of tissue damage from muscle biopsy could also elicit effects on autophagy activity. No studies have used HIIT exercise or MICT exercise of 60 minutes at 55% VO_{2max} using running as the mode of exercise.

Investigations of the autophagic response to acute exercise in human skeletal muscle has produced discrepant results, particularly with respect to LC3. Existing literature suggests exercise durations greater than 60 – 120 minutes of continuous moderate to moderately-high intensities

(>55 – 70% VO2max) are required for autophagy activation in skeletal muscle (Jamart, Benoit, et al., 2012; Jamart, Francaux, et al., 2012; Moller et al., 2015; Schwalm et al., 2015) as demonstrated by changes in LC3II and/or LC3II:I. In contrast, 20 minutes cycling at ~50% VO_{2max} (Masschelein et al., 2014) and 60 minutes of cycling at ~70% VO_{2max} (Tachtsis, Smiles, Lane, Hawley, & Camera, 2016) did not produce any changes in LC3 immediately post-exercise and 3 hours post-exercise, respectively. Interestingly, however, while 60 minutes of cycling at ~70% VO_{2max} resulted in no changes in LC3II or LC3II:I 3 hours post-exercise, 60 minutes of cycling at ~50% VO_{2max} (Moller et al., 2015) resulted in a decreased LC3II and LC3II:I 90 minutes post-exercise. Moreover, and though the data are scarce, when changes do occur in LC3II, both a decrease (Moller et al., 2015; Schwalm et al., 2015) and an increase (Jamart, Francaux, et al., 2012) were observed. Decreases in LC3II were observed 90 minutes post-exercise after 60 minutes of cycling at ~50% VO_{2max} (Moller et al., 2015) and immediately and 1 hour post-exercise following 2 hours of cycling at 55%VO_{2peak} or 70% VO_{2peak} in a fasted and fed state; LC3II changes returned back to baseline 1 hour post-exercise when cycling at 55%VO_{2peak} was performed in the fed state (Schwalm et al., 2015). These changes were accompanied by a decreased LC3II:I ratio. An increase in LC3II occurred following a 200 km competitive running race at 3 hours post-exercise; LC3II:I was not reported (Jamart, Francaux, et al., 2012). These data imply the status of LC3II in skeletal muscle is highly dynamic and changes may be dependent on exercise duration and intensity as well as time of sampling. Given that basal turnover of LC3II (i.e. relationship of rate of autophagasome formation and degradation) is not known in skeletal muscle (Klionsky et al., 2016), turnover amid stresses of acute exercise are far from characterized nor are the differences between mode, duration, and type of exercise in addition to the time course of the turnover effect post-exercise. Our data add to the sparse but divergent exercise-LC3II response in skeletal muscle. With regard to LC3I, no studies have reported changes in LC3I, which is consistent with our results, despite changes in LC3II. This may imply that decreases in LC3II and LC3II:I ratio occur as a function of clearance of existing

autophagasomes or it may be that LC3I is replenished at an equal rate of lipidation. No data depicting LC3I mRNA abundance in response to exercise exist.

Our data depict a non-significant reduction in LC3II in both HIIT and MICT post-exercise periods ($p = 0.169$). However, MICT exercise incurred a greater decrement (73.7 ± 34.3%) to that of HIIT exercise (92.4 ± 52.4%). Although not significant, LC3I protein expression trended toward divergent responses ($p = 0.092$) where 3 hours post-HIIT exercise LC3I was lower than pre-exercise (84.9 ± 57.8%) while it was elevated post-MICT exercise (189.4 ± 174.7%). While these differences are not statistically significant, a significant difference does manifest in the resultant LC3II:I ratios between exercise conditions where HIIT exercise increased LC3II:I to 162.4 ± 145.3% of pre-exercise while MICT exercise decreased LC3II:I to 48.8 ± 29.8% of pre-exercise. It may be speculated that the observed trends of differences between LC3I and LC3II may stem from differences in LC3I to LC3II conversion via lipidation and/or autophagasome degradation between exercise bouts. An increased LC3II:I following HIIT exercise may have resulted from an increased lipidation rate as a result of greater autophagic flux as observed by a trend toward of reduced LC3I content. A decreased LC3II:I ratio following MICT exercise may be a result of replenishment of LC3I amid reduced lipidation to LC3II which may suggest lesser autophagic signaling and flux. Measurement of an autophagic substrate, such as p62, would aid to elucidate this, although changes of p62 do not always correlate with that of LC3II in that clearance of autophagic substrates (i.e. p62) may occur at slower rate than changes in LC3II (Klionsky et al., 2016). p62 was not successfully measured in this study, Measurement of regulators of autophagy would give insight into differences in autophagic signaling; however, no such markers were successfully measured.

A number of canonical exercise-induced cellular signaling proteins regulate autophagy, including AMPK, SIRT1, p38MAPK, and CaMKII (Vainshtein & Hood, 2016). Tumor suppressor protein p53 (Balaburski, Hontz, & Murphy, 2010; Du et al., 2009; Maiuri et al., 2010; Maiuri et al., 2009)

and Sestrin2 (Balaburski et al., 2010; Li, Liu, Yuan, Niu, & Fu, 2017; Liu, Niu, Yuan, Huang, & Fu, 2015; Maiuri et al., 2009) have been recently implicated in regulating autophagy and may possess a role in exercise-induced autophagy (Liu et al., 2015; Tachtsis et al., 2016). This study aimed, but failed to measure AMPK activity, CaMKII, p53, and Sestrin2 due to technical difficualties. Total p38MAPK was measured and while a trend was observed in the positive direction ($p = 0.177$), no differences between pre and post-exercise or between exercise conditions were observed. Most studies measuring p38MAPK activity in response to exerciseadditionally measure phosphorylated p38MAPK and derive a ratio of phosphorylation to total p38MAPK. Both HIIT and MICT exercise increase this ratio (Gibala et al., 2009; Hawley, Hargreaves, Joyner, & Zierath, 2014) This study also failed to produce these measures. p53, AMPK, and p38MAPK are also implicated in mitochondrial biogenesis, namely, by activating PGC1-α (Hawley et al., 2014). A detailed assessment of activity of these autophagic regulators may have provided some explanation in LC3II:I differences between exercise bouts as they may have elucidated discrepancies in activity and thus, potential discrepancies in autophagic flux.

Autophagy is key to human health and longevity (Cuervo, 2008; Todde, Veenhuis, & van der Klei, 2009). Exercise is known to serve as a robust means to promote health and longevity, as well as reduce the risk of chronic disease (Booth, Roberts, & Laye, 2012), and autophagy has been increasingly implicated in meditating this response (Halling & Pilegaard, 2017; Vainshtein, Grumati, Sandri, & Bonaldo, 2014). A characterization of the nature (i.e. type, duration, and intensity) of exercise that elicits an autophagic response, to potentially facilitate autophagy-mediated exercise adaptations and health effect, has yet to established. That is to say the dose of exercise required to stimulate autophagy remains unclear. Our data from the MICT exercise are in agreement with the current literature suggesting exercise durations of greater than 60 minutes of moderate to moderately-high intensities (>55 – 70% VO_{2max}) are required for autophagy activation (Jamart, Benoit, et al., 2012; Jamart, Francaux, et al., 2012; Moller et al., 2015;

Schwalm et al., 2015). Notably, our results at the low end of these duration and intensity parameters, pointing to a potential minimum exercise dose to augment autophagy activity. Additionally, our study demonstrates that HIIT exercise modulates autophagy activity. High intensity interval exercise is a popular and efficacious mode of exercise capable of eliciting equal or even superior physiological and health changes as traditional, moderate intensity, continuous training (MICT) when amounts of work are similar or even lower (Gibala, Little, Macdonald, & Hawley, 2012). Given the growing role of autophagy in mediating training outcomes, we speculate autophagy may be involved in HIIT-induced adaptations. Indeed, we show autophagy is stimulated by an acute bout of HIIT. The role of autophagy in chronic HIIT outcomes in humans remains unknown; however, data from a rodent model demonstrate 10 weeks of HIIT produce changes in basal autophagy-related proteins (F. H. Li et al., 2018).

In total, this study produced three novel findings; firstly, HIIT exercise performed in a fasted state elicits an autophagic response 3 hours post-exercise in skeletal muscle. Secondly, MICT exercise comprised of 60 minutes of running at 55% of VO_{2max} performed in a fasted state produces changes in autophagy 3 hours post-exercise. And lastly, these exercise bouts produced distinct autophagic responses as demonstrated by changes in LC3II:I. These data add to the currently scarce literature pertaining to exercise-induced autophagy in skeletal muscle and may be used to characterize the nature of exercise required to elicit an autophagic response which may possess implications on human health.

Study 2: Using electrical pulse stimulation as an *in vitro* model for muscle contraction-induced autophagy

Results from our study demonstrate electrical pulse stimulation (EPS) is capable of eliciting a muscle contraction-induced autophagic response in C2C12 myotubes in fed and starvation conditions. This response was made manifest with the addition of bafiloycin A1 which inhibits the degradation of the autophagasome at the lysosome precipitating an accumulation of

autophagasome-associated proteins and substrates. It is interesting to note that EPS in combination with starvation (EPS+St, EPS+St+Baf) did not produce greater changes than EPS or starvation alone conditions (EPS, EPS+Baf, St, St+Baf).

Electrical pulse stimulation has been shown to activate pro-autophagy signaling including increases in Ca++ transients, AMPK, p38MAPK, and PGC1α via distinct EPS protocols (i.e. short-term, long-term, low frequency, high frequency) (Nieuwoudt et al., 2017; Nikolic et al., 2017; Tarum et al., 2017). Both short-term (≤ 8 hours) and long-term (≥ 24 hours) low frequency (≤ 5 Hz) EPS protocols have been shown to activate these positive autophagic regulators (Nikolic et al., 2017). Here we show short-term (8 hours), low frequency EPS (1 Hz) increases autophagy activity. Protein expression of LC3II and LC3II:I ratio were elevated in response to EPS with bafilomycin A1 treatment (EPS+Baf). Treatment with bafilomycin inhibits the degradation of the autophagsome-associated LC3II. Thus, an increase in LC3II and LC3II:I ratio in bafilomycin treated conditions indicates an accumulation of the protein that would otherwise be degraded amid increased flux, as observed in EPS alone. Protein expression of autophagy substrate p62 was increased in both EPS and EPS+Baf. While p62 is used as a marker of autophagic flux as it is degraded with the autophagasome, its status does not always correlate with LC3II changes which is also degraded by the lysosome (Klionsky et al., 2016). Whereas LC3 changes may occur rapidly, degradation of autophagy substrates may occur over a longer time course (Klionsky et al., 2016). Moreover, p62 is upregulated during sustained periods of elevated clearance after an initial decrease (Sahani, Itakura, & Mizushima, 2014). It may be that transcription of p62 is increased during the 8 hour EPS protocol even through likely elevated degradation as seen in EPS. This may be supported by a lack of increased LC3II in EPS in an addition to the significant increase in p62 with the addition of bafilomycin (EPS+Baf) compared to EPS alone. This potentially suggests our EPS protocol instigated increased autophagic flux as well as upregulation

of the autophagy protein-related transcriptional program. Measurement of p62 mRNA and transcriptional program regulators (i.e. FOXOs, p53) could be used to elucidate this.

Acute starvation of C2C12 myotubes has been shown to in increase autophagy activity (Desgeorges et al., 2014). Four hours of starvation using the same glucose and amino acid-free starvation medium as used in our study resulted in an increase in LC3I and LC3II content and increased LC3II:I ratio (Desgeorges et al., 2014). Autophagy signaling was also elevated via reduced target of rapamycin (mTOR) activity and increased AMPK and ULK1 activity. Expression of autophagy-related proteins Ulk1, Atg13, Vps34, and Atg5–Atg12 conjugate were also increased. p62 was not measured in this study (Desgeorges et al., 2014). Our data from our starvation protocol diverge from these results as we observed no differences between LC3I, LC3II, or LC3II:I in response to the 8 hour protocol, although p62 was significantly increased in St+Baf. Discrepancies in LC3 responses in our study compared to the Desgeorges et al. study may stem from differences in duration of protocol. It has been observed that LC3 is transcriptionally upregulated in starvation conditions (Klionsky et al., 2016). Upregulation of LC3 transcription occurred in HeLa cells during 2 hours of starvation (Settembre et al., 2011). And while LC3 response is species, cell, and stress-dependent, may be that 8 hours of starvation in C2C12 cells increases transcriptional activity of LC3; however, during a rate of degradation that precludes an increase in LC3II and LC3II:I.

Our results for p62 expression are consistent with other work showing that p62 is upregulated during starvation conditions. Starvation-induced transcription and translation stimulated by autophagy-provisioned amino acids increased p62 mRNA and restore protein expression to baseline levels during 4 to 8 hours of starvation in MEF and HepG2 cells following an initial and rapid degradation (Sahani et al., 2014). Our study found p62 unchanged from control (91.3% ± 28.7) following 8 hours of starvation (St) despite a likely increased rate of clearance. The addition of bafilomycin (St+Baf) resulted in a significant increase in p62 (320.9 ± 93.3% of Con). This

suggests transcription of p62 is upregulated during our starvation protocol, facilitating maintenance of baseline levels in the absence of bafilomycin and an elevation with bafilomycin treatment. Quantifying mRNA abundance would discern this speculation.

It is interesting to note that the combination of EPS and starvation did not produce additive effects on autophagic activity. No differences were observed between EPS and St to EPS+St or EPS+Baf and St+Baf to EPS+St+Baf in LC3II, LC3II:I, or p62. This may have resulted from a reaching of maximal autophagic flux and/or transcriptional activity. Measuring activity of regulators of autophagy (AMPK, SIRT1, p38MAPK, p53, PGC1α, etc.) would provide insight into magnitude of autophagic signaling between EPS, St, and EPS+St conditions. Quantifying mRNA abundance of LC3 and p62 would also elucidate transcriptional activity between conditions.

These data present an *in vitro* model to study muscle contraction-induced autophagy in C2C12 myotubes. This model may be used in fed or starvation conditions. This provides a means to study autophagy signaling transduction in response to muscle contraction. Moreover, given that autophagy has been implicated in mediating a number of exercise responses and adaptations including the improvement of human health and longevity, this model may be used to study the mechanisms underlying the metabolic responses and training outcomes, including in pathological conditions such as insulin resistance and mitochondrial dysfunction. This holds implications for understanding the health-positive effects of exercise as well as identifying molecular targets for pharmacological interventions.

Limitations

1. The study sample was comprised of healthy, active 18 – 30-year-old males and females; thus, findings may not be generalized to individuals outside of these parameters.

2. Performing the post-exercise biopsy 3 hours-post exercise may have resulted in missing autophagy activity in the immediate post-exercise period.

3. Lacking of bafilomycin only data in C2C12 experiments limits the ability to discern the effects of bafilomycin in the absence of experimental conditions.

4. The reproducibility and generalization of in vitro models, such as C2C12 model, may be limited in reflecting the phenomena in *in vivo*; thus, the use of EPS in C2C12 cells may be limited in its ability to represent what occurs in human skeletal muscle in response to exercise.

5. C2C12 myotubes are mice muscle cells, thus translating findings to human muscle may be limited.

Conclusions

Our studies produced a number of novel findings relating to muscle contraction-induced autophagy using a human and cell model. Firstly, we demonstrate the HIIT exercise stimulates autophagy in human skeletal muscle of active male and females. We also showed that 60 minutes of MICT running exercise at 55% VO_2max elicits an autophagy response. Next, we demonstrated that HIIT and MICT exercise elicit distinct responses in LC3II:I ratio. We also test an *in vitro* model of muscle contraction-induced autophagy using EPS in C2C12 myotubes.

Our data carry several implications in the understanding of exercise physiology. Exercise is known to improve health and longevity and reduce the risk of chronic degenerative disease (Booth, Roberts, & Laye, 2012); however, the underlying mechanisms are currently not fully understood. A growing body of evidence suggest autophagy is key in mediating these health-positive responses (Halling & Pilegaard, 2017; Vainshtein, Grumati, Sandri, & Bonaldo, 2014). A characterization of efficacious “doses” of exercise have yet to be established. Our data add to the sparse literature documenting acute exercise stimulating autophagy in human skeletal muscle and

are the first to demonstrate an autophagic response to HIIT exercise. This is novel in that HIIT a popular mode of exercise that promotes robust health effects, equal and even superior to traditional MICT exercise (Halling & Pilegaard, 2017; Vainshtein, Grumati, Sandri, & Bonaldo, 2014). Our results allow for speculation that autophagy may be involved in the chronic outcomes to HIIT. We also test an *in vitro* model for muscle contraction-induced autophagy in C2C12 myotubes. This is significant in that EPS may be used to study the mechanisms associated with autophagy-mediated exercise responses and respective signaling transduction. This includes investigating the role of autophagy in ameliorating symptoms of pathological conditions including insulin resistance and mitochondrial dysfunction and furthering our understanding of how "exercise is medicine."

Funding acknowledgment: This work was conducted as part of the University of New Mexico Women in STEM Faculty Development Fund. Additional funding was provided by the New Mexico Graduate and Professional Student Association.

References

Balaburski, G. M., Hontz, R. D., & Murphy, M. E. (2010). p53 and ARF: unexpected players in autophagy. *Trends Cell Biol, 20*(6), 363-369. doi:10.1016/j.tcb.2010.02.007

Booth, F. W., Roberts, C. K., & Laye, M. J. (2012). Lack of exercise is a major cause of chronic diseases. *Compr Physiol, 2*(2), 1143-1211. doi:10.1002/cphy.c110025

Cuervo, A. M. (2008). Autophagy and aging: keeping that old broom working. *Trends Genet, 24*(12), 604-612. doi:10.1016/j.tig.2008.10.002

Desgeorges, M. M., Freyssenet, D., Chanon, S., Castells, J., Pugniere, P., Bechet, D., . . . Defour, A. (2014). Post-transcriptional regulation of autophagy in C2C12 myotubes following starvation and nutrient restoration. *Int J Biochem Cell Biol, 54*, 208-216. doi:10.1016/j.biocel.2014.07.008

Du, Y., Yang, D., Li, L., Luo, G., Li, T., Fan, X., . . . Le, W. (2009). An insight into the mechanistic role of p53-mediated autophagy induction in response to proteasomal inhibition-induced neurotoxicity. *Autophagy, 5*(5), 663-675.

Gibala, M. J., Little, J. P., Macdonald, M. J., & Hawley, J. A. (2012). Physiological adaptations to low-volume, high-intensity interval training in health and disease. *J Physiol, 590*(5), 1077-1084. doi:10.1113/jphysiol.2011.224725

Gibala, M. J., McGee, S. L., Garnham, A. P., Howlett, K. F., Snow, R. J., & Hargreaves, M. (2009). Brief intense interval exercise activates AMPK and p38 MAPK signaling and increases the expression of PGC-1alpha in human skeletal muscle. *J Appl Physiol (1985), 106*(3), 929-934. doi:10.1152/japplphysiol.90880.2008

Halling, J. F., & Pilegaard, H. (2017). Autophagy-Dependent Beneficial Effects of Exercise. *Cold Spring Harb Perspect Med, 7*(8). doi:10.1101/cshperspect.a029777

Hawley, J. A., Hargreaves, M., Joyner, M. J., & Zierath, J. R. (2014). Integrative biology of exercise. *Cell, 159*(4), 738-749. doi:10.1016/j.cell.2014.10.029

Jamart, C., Benoit, N., Raymackers, J. M., Kim, H. J., Kim, C. K., & Francaux, M. (2012). Autophagy-related and autophagy-regulatory genes are induced in human muscle after ultraendurance exercise. *Eur J Appl Physiol, 112*(8), 3173-3177. doi:10.1007/s00421-011-2287-3

Jamart, C., Francaux, M., Millet, G. Y., Deldicque, L., Frere, D., & Feasson, L. (2012). Modulation of autophagy and ubiquitin-proteasome pathways during ultra-endurance running. *J Appl Physiol (1985), 112*(9), 1529-1537. doi:10.1152/japplphysiol.00952.2011

Klionsky, D. J., Abdelmohsen, K., Abe, A., Abedin, M. J., Abeliovich, H., Acevedo Arozena, A., . . . Zughaier, S. M. (2016). Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy, 12*(1), 1-222. doi:10.1080/15548627.2015.1100356

Li, H., Liu, S., Yuan, H., Niu, Y., & Fu, L. (2017). Sestrin 2 induces autophagy and attenuates insulin resistance by regulating AMPK signaling in C2C12 myotubes. *Exp Cell Res, 354*(1), 18-24. doi:10.1016/j.yexcr.2017.03.023

Liu, X., Niu, Y., Yuan, H., Huang, J., & Fu, L. (2015). AMPK binds to Sestrins and mediates the effect of exercise to increase insulin-sensitivity through autophagy. *Metabolism, 64*(6), 658-665. doi:10.1016/j.metabol.2015.01.015

Maiuri, M. C., Galluzzi, L., Morselli, E., Kepp, O., Malik, S. A., & Kroemer, G. (2010). Autophagy regulation by p53. *Curr Opin Cell Biol, 22*(2), 181-185. doi:10.1016/j.ceb.2009.12.001

Maiuri, M. C., Malik, S. A., Morselli, E., Kepp, O., Criollo, A., Mouchel, P. L., . . . Kroemer, G. (2009). Stimulation of autophagy by the p53 target gene Sestrin2. *Cell Cycle, 8*(10), 1571-1576. doi:10.4161/cc.8.10.8498

Masschelein, E., Van Thienen, R., D'Hulst, G., Hespel, P., Thomis, M., & Deldicque, L. (2014). Acute environmental hypoxia induces LC3 lipidation in a genotype-dependent manner. *FASEB J, 28*(2), 1022-1034. doi:10.1096/fj.13-239863

Moller, A. B., Vendelbo, M. H., Christensen, B., Clasen, B. F., Bak, A. M., Jorgensen, J. O., . . . Jessen, N. (2015). Physical exercise increases autophagic signaling through ULK1 in human skeletal muscle. *J Appl Physiol (1985), 118*(8), 971-979. doi:10.1152/japplphysiol.01116.2014

Nieuwoudt, S., Mulya, A., Fealy, C. E., Martelli, E., Dasarathy, S., Naga Prasad, S. V., & Kirwan, J. P. (2017). In vitro contraction protects against palmitate-induced insulin resistance in C2C12 myotubes. *Am J Physiol Cell Physiol, 313*(5), C575-C583. doi:10.1152/ajpcell.00123.2017

Nikolic, N., Gorgens, S. W., Thoresen, G. H., Aas, V., Eckel, J., & Eckardt, K. (2017). Electrical pulse stimulation of cultured skeletal muscle cells as a model for in vitro exercise - possibilities and limitations. *Acta Physiol (Oxf), 220*(3), 310-331. doi:10.1111/apha.12830

Sahani, M. H., Itakura, E., & Mizushima, N. (2014). Expression of the autophagy substrate SQSTM1/p62 is restored during prolonged starvation depending on transcriptional upregulation and autophagy-derived amino acids. *Autophagy, 10*(3), 431-441. doi:10.4161/auto.27344

Schwalm, C., Jamart, C., Benoit, N., Naslain, D., Premont, C., Prevet, J., . . . Francaux, M. (2015). Activation of autophagy in human skeletal muscle is dependent on exercise intensity and AMPK activation. *FASEB J, 29*(8), 3515-3526. doi:10.1096/fj.14-267187

Settembre, C., Di Malta, C., Polito, V. A., Garcia Arencibia, M., Vetrini, F., Erdin, S., . . . Ballabio, A. (2011). TFEB links autophagy to lysosomal biogenesis. *Science, 332*(6036), 1429-1433. doi:10.1126/science.1204592

Tachtsis, B., Smiles, W. J., Lane, S. C., Hawley, J. A., & Camera, D. M. (2016). Acute Endurance Exercise Induces Nuclear p53 Abundance in Human Skeletal Muscle. *Front Physiol, 7*, 144. doi:10.3389/fphys.2016.00144

Tarum, J., Folkesson, M., Atherton, P. J., & Kadi, F. (2017). Electrical pulse stimulation: an in vitro exercise model for the induction of human skeletal muscle cell hypertrophy. A proof-of-concept study. *Exp Physiol, 102*(11), 1405-1413. doi:10.1113/EP086581

Todde, V., Veenhuis, M., & van der Klei, I. J. (2009). Autophagy: principles and significance in health and disease. *Biochim Biophys Acta, 1792*(1), 3-13. doi:10.1016/j.bbadis.2008.10.016

Vainshtein, A., Grumati, P., Sandri, M., & Bonaldo, P. (2014). Skeletal muscle, autophagy, and physical activity: the menage a trois of metabolic regulation in health and disease. *J Mol Med (Berl), 92*(2), 127-137. doi:10.1007/s00109-013-1096-z

Vainshtein, A., & Hood, D. A. (2016). The regulation of autophagy during exercise in skeletal muscle. *J Appl Physiol (1985), 120*(6), 664-673. doi:10.1152/japplphysiol.00550.2015

Appendices

A. Informed Consent

B. Health History Questionnaire

C. Flyer

D. Data Collection Sheets

1. Skinfold Data Sheet

2. VO_2max Data Sheet

Appendix A: Informed Consent

Consent Form:
Comparing the effects of high intensity interval exercise to moderate intensity continuous exercise on autophagy in human skeletal muscle
Version date: 3/09/2018
1163636-3

Purpose of the study: You are being asked to participate in a research study that is being done by Fabiano Amorim, Ph.D. and Christine Mermier, Ph.D., who are the Principal Investigators, and Kurt Escobar from the Department of Health, Exercise & Sport Sciences and their associates. The purpose of this study is to investigate the role of exercise intensity on autophagy and mitochondrial synthesis. Autophagy is a cellular recycling system involved in promoting health. Mitochondria are energy producing organelles also involved in maintaining health. We are studying whether there is a difference between high intensity interval exercise and moderate intensity continuous exercise in stimulating autophagy activity and new mitochondria mitochondrial post-exercise.

You are being asked to participate in this study because you are a healthy (no heart or lung disease or disorders such as diabetes, kidney disease and other diagnosed medical conditions, and no current bone or joint injury) individual between the ages of 18 – 30 years old currently engaging in regular physical activity (> 150 minutes of moderate to vigorous intensity aerobic activity per week for a minimum of 1 year). You must also not have a bleeding disorder or blood clotting problem or be allergic to Lidocaine. Up to 25 people will be recruited for this study at the University of New Mexico.

This form will explain what to expect when joining the research, as well as the possible risks and benefits of participation. If you have any questions, please ask one of the study researchers.

What you will do in the study:

- After reading the consent form and discussing the details with the research team, you will be asked to sign this consent form if you decide to participate in the study and before any study activities take place.
- You will then complete a Health History and Exercise History Questionnaire to assess your current health status and training history. You will be required to visit the Exercise Physiology Laboratory three times. We will request that you wear exercise attire for the exercise bouts. Upon arrival, we will measure resting blood pressure which will involve you sitting upright while a blood pressure cuff is wrapped around your upper arm. A stethoscope will be placed against your arm under the cuff which will be inflated and then deflated while systolic and diastolic blood pressure is measured. You will also have your body composition estimated, using the skinfold caliper technique. This will require a slight pinching of the skin at three sites of the body.
- During first visit you will perform a maximal graded exercise test on a treadmill. The speed and/or grade is increased periodically during the test until volitional exhaustion (until you fatigue) or if the researchers determine it is not safe for you to continue based on your blood pressure, heart rate, etc. This test assesses the

maximum ability of the body to use oxygen to support physical activity (VO_{2max}) which is measured by a gas analyzer by collecting expired air from your lungs. This will require you to wear a mouthpiece that feels like a swimming snorkel and a nose-clamp during the test. Your heart rate will be measured throughout the test and your blood pressure will be measured before exercise and immediately after exercise. Test will take approximately 8 – 12 minutes.

- You will have refrained from alcohol for 24 hours, exercise for 24 hours, and caffeine for 8 hours. Women who are of child bearing age/potential will be asked to take a urine pregnancy test.
- The second visit will occur at least 72 hours after the first visit.
- The second and third visits will take will take up to 5 hours and will be separated by at least 7 days. Subjects will arrive to the laboratory in the morning after abstaining from exercise for 72 hours, alcohol for 24 hours, caffeine for 8 hours, and food for 8 hours (i.e. overnight fast).
- You will have your urine specific gravity measured to assess hydration status. You will urinate at least 2 – 3 ml into a plastic urine collection receptacle. If urine specific gravity is greater than 1.030, you will be given ≥500 ml of water to drink and urine specific gravity will be measured again after 30 minutes. If your urine specific gravity remains over 1.030, you will be given another ≥500 ml of water and wait another 30 minutes for reassessment. This may be repeated until your urine specific gravity is below 1.030, which ensures you are adequately hydrated.
- You will perform a bout of either a high intensity interval exercise or a moderate intensity continuous exercise. The high intensity running exercise will consist of a 2 minute warmup at 5 mph followed by 6 bouts of 1 minute at 100% of the maximum velocity (V_{max}) you reached during your VO_{2max} test. You will run on the treadmill at 3% grade for 1 minute of 3 mph, 5 minutes of recovery at 3 mph, and 6 bouts of 1 minute at 100% of Vmax at 3% grade and 1 minute at 3 mph, followed by 2 minutes of cooldown at 3 mph. The moderate-intensity continuous running exercise will consist of a 2 minute warm up at 5 mph, 60 minutes at 55% V_{max} at 3% grade, and 2 minutes of cooldown at 3 mph. Including the warm up and cooldown, the high intensity interval running exercise will be 30 minutes in duration; the moderate continuous exercise will be 64 minutes in duration. You will wear a heart rate monitor around your torso during both exercise bouts.
- A blood draw (2 tablespoons) from a vein in your arm will be performed by a trained phlebotomist before the exercise bout, and 3 hours post-exercise. Your blood may be used to assess total antioxidant capacity and/or autophagy in white blood cells.
- A small skeletal muscle tissue sample will be collected via biopsy taken from your upper leg/thigh area before the exercise bout and 3 hour post-exercise. You will be placed on an examination table lying down on your back (supine) so that the muscles of the leg are relaxed. The skin will be cleaned and prepared with surgical antiseptic after which a surgical cover will be placed around the sampling site. A local anaesthetic (Lidocaine) will be injected into the tissue under the skin around the site to be sampled. You may experience a slight pinching sensation while the anaesthetic is injected. If you are allergic to the local anaesthetic Lidocaine, you will be disqualified from the study. Once the area has been completely numbed, a small incision will then be made in the skin overlying the muscle using a pilot needle (16-gauge) and a small (14-gauge) biopsy needle will be inserted into the incision. There may be some minimal bleeding. If you have a bleeding disorder or blood clotting problem, you will inform the lab personnel and

not participate in the biopsy procedure. Sterile disposable instruments will be used for the preparation of the site and tissue sampling. Approximately 10 mg (size of a sesame seed) of skeletal muscle tissue will be removed. You may feel a brief sensation of pressure in the leg and potentially some moderate pain. This pain will quickly dissipate and you will likely be able to perform exercise and normal daily activities unhindered. This technique is minimally invasive; however, it is common for participants to feel a sense of tightness and potentially may feel a sensation of a deep bruise or "Charlie Horse" at the biopsy site, although this should be alleviated within 2 days and you may begin exercising immediately. There may be a possibility of a small scar forming. There have been no other major complications reported as a result of taking small tissue samples from the skeletal muscle. Following the biopsy, the incision will be cleaned, treated with a sterile dressing, and a wrapped in a bandage to minimize the possibility of a blood related infection. You will also be given instructions for the proper care of the biopsy site.

- You will be asked to stay in the Exercise Physiology Laboratory for the duration of the 3 hours after exercise and before the second muscle biopsy. You may leave the laboratory only in the case if you have class that is inside Johnson Center. You will not be able to consume food or calorie-containing beverages or coffee during the 3 hour period between exercise and the second muscle biopsy. You will be given a snack (granola bar, sports bar, etc.) following the second biopsy if you would like one.

Participation in this study will take a total 11 hours over a period of three laboratory visits.

OPTIONAL: The UNM Exercise Physiology Laboratory conducts studies that may include tests and procedures that are involved in this study. Data from this study may be useable in other studies. Your data will be de-identified (your name will not be associated with any data) and contain only variables such as sex, age, VO_{2max}, body composition, etc. If you are willing to allow the results from this study to be used in other future studies, please initial below. By initialing, you are not required to perform any additional tasks, tests, or paperwork. The laboratory will only use your data.

I agree to allow my data to be used in other studies _____

Initials

Risks:

There are risks associated with maximal graded exercise test including the following: muscle soreness, fatigue, nausea, or dizziness during or after completion of exercise. The incidence of risk of fatal and nonfatal events during maximal exercise testing are very low, approximately <0.8 per 10,000 tests or 1 per 10,000 hours of testing. We will minimize these risks by checking your medical history questionnaire for any medical conditions or history that could increase your risk, and by using trained personnel to conduct your testing.

There are minor risks associated with blood draws including temporary pain and discomfort from the needle stick, a risk of bruising, and feeling faint or light-headed. This risk will be minimized by using trained and experienced phlebotomists who will draw your blood with sterile technique.

The risks associated with a muscle biopsy include momentary discomfort or moderate pain during the time the needle is inserted. To minimize the occurrence of discomfort and pain, an effective numbing agent (Lidocaine) will be used to numb the area to sampled. You will likely experience a brief and small pinching sensation while the numbing agent (Lidocaine) is injected. A minimal amount of muscle tissue (10 mg) will be extracted from your leg. You may feel a brief sensation of pressure in the leg and potentially some moderate pain during the tissue sampling. This pain will quickly dissipate and you will likely be able to perform exercise and normal daily activities unhindered. There is a risk that you may feel a sense of dizziness or feeling faint. Your leg may feel tight and you may feel a sensation of a deep bruise or "Charlie Horse" afterwards; however, this tightness in the muscle typically dissipates within 2 days and you may begin exercising immediately, and routinely begin exercising at normal capacity within 2 days. There is also a risk of the possible appearance of a scar, bruising or soreness, and infection. To limit the potential risks, only trained technicians using sterilized instruments will perform the biopsy procedure. Additionally, the sampling site will be sterilized prior to the procedure.

There are risks of stress, emotional distress, inconvenience and possible loss of privacy and confidentiality associated with participating in a research study.

Benefits: You will also be provided with the results of your body composition assessment and VO_{2max} test, services which normally cost over $100. Knowledge of body composition and VO_{2max} are of benefit in that they are indicative of health and aerobic fitness which can be helpful in directing an individual exercise program. Additionally, it is hoped that the information gained from this study will help us understand the relationship of exercise intensity and autophagy and mitochondrial biogenesis which may impact human health and aging.

Confidentiality of your information: Privacy will be maintained by conducting screening and testing in
private room in the Johnson Center Exercise Physiology lab with no access to anyone but the study team. You will be given a random number for confidentiality of your data. Only approved research team members will have access to your information through a password protected computer, with hard copies stored in a locked file cabinet. We will take measures to protect the security of all your personal information, but we cannot guarantee confidentiality of your data. The University of New Mexico Institutional Review Board (IRB) that oversees human subject research may be permitted to access your records. Your name will not be used in any published reports about this study.

What will happen if I am injured or become sick because I took part in this study?
If you are injured or become sick as a result of this study, UNM Health Science Center will provide you with emergency treatment, at your cost. No commitment is made by the University of New Mexico Health Sciences Center (UNMHSC) to provide free medical care or money for injuries to participants in this study. In the event that you have an injury or illness that is caused by your participation in this study, reimbursement for all related costs of care will be sought from your insurer, managed care plan, or other

benefits program. If you do not have insurance, you may be responsible for these costs. You will also be responsible for any associated co-payments or deductibles required by your insurance.
It is important for you to tell the investigator immediately if you have been injured or become sick because of taking part in this study. If you have any questions about these issues,or believe that you have been treated carelessly in the study, please contact the Office of the Institutional Review Board (OIRB) at the (505) 277-2644 for more information.

Payment: You will be compensated with a $50 gift card for successful completion of the study.

Right to withdraw from the study: Your participation in this study is completely voluntary. You have the right to choose not to participate or to withdraw your participation at any point in this study without penalty.

If you have any questions, concerns, or complaints about the research study, please contact the principal investigators: Fabiano Amorim, PhD and Christine Mermier, Ph.D., Department of Health, Exercise & Sport Sciences, 1 University of New Mexico, Albuquerque, NM, 87131. They may be reached Monday-Friday 8:00 a.m. – 5:00 p.m. at (505) 277-2658, or anytime via email at amorim@unm.edu and cmermier@unm.edu.

If you would like to speak with someone other than the research team or have questions regarding your rights as a research participant, please contact the IRB. The IRB is a group of people from UNM and the community who provide independent oversight of safety and ethical issues related to research involving people:

UNM Office of the IRB, (505) 277-2644, irbmaincampus@unm.edu. Website: http://irb.unm.edu/

CONSENT

You are making a decision whether to participate in this study. Your signature below indicates that you have read this form (or the form was read to you) and that all questions have been answered to your satisfaction. By signing this consent form, you are not waiving any of your legal rights as a research participant. A copy of this consent form will be provided to you.

I agree to participate in this study.

_____ _____

Name of Adult Participant Signature of Adult Participant
Date

Researcher Signature (to be completed at time of informed consent)

I have explained the research to the participant and answered all of his/her questions. I believe that he/she understands the information described in this consent form and freely consents to participate.

_____	_____
Name of Research Team Member	Signature of Research Team Member Date

Appendix B: Health & Physical Activity Questionnaire

HEALTH & PHYSICAL ACTIVITY QUESTIONNAIRE

Family history questions are included because certain conditions of your first degree relatives can incur risk to you during maximal exercise.

Subject #______________________________ Date___/___/___

Phone (H or cell)____________________

Date of Birth ___/___/___ Age____ Sex_____ Ethnicity_______

Emergency contact (name, phone #)____

♦♦

MEDICAL HISTORY

Physical injuries:____

Limitations____

Have you ever had any of the following cardiovascular problems? Please check all that apply.

Heart attack/Myocardial Infarction____ Heart surgery ____ Valve problems _____

Chest pain or pressure ____ Swollen ankles ____ Dizziness _____

Arrhythmias/Palpitations ____ Heart murmur ____ Shortness of breath _____ Congestive heart failure ____

Have you ever had any of the following? Please check all that apply.

High blood pressure _____
Asthma _____ Total cholesterol >200 mg/dl _____
Diabetes (specify type) _____ HDL cholesterol <35 mg/dl _____
Emphysema _____
Stroke _____ LDL cholesterol >135 mg/dl _____
Triglycerides>150 mg/dl _____

Do immediate blood relatives (biological parents & siblings **only**) have any of the conditions listed above? If yes, list the problem, and family member age at diagnosis.

____
____

Do you currently have any other medical condition not listed?
Details____
____

____
____

Indicate level of your overall health. Excellent ____ Good ____ Fair ____ Poor_____

Are you taking any medications, vitamins or dietary supplements now? Y N

If yes, what are they?____
_

Are you allergic to latex? Y N

Have you ever experienced any adverse effects during or after exercise (fainting, vomiting, shock, palpitations, hyperventilation)? Y N If yes, elaborate.____

____
____

♦♦♦

LIFESTYLE FACTORS

Do you now or have you ever used tobacco? Y N If yes: type ________________

How long?______ Quantity____/day Years since quitting______________

♦♦♦

EXERCISE HISTORY

Endurance training

Days per week (circle one): <3 3-5 6-7

Minutes per day (circle one): 30-60 60-240 240-360 >360

Hours per week (circle one): 1-2 3-5 6-8 >8

Training background (in years) (circle one): <1 1-3 4-5 6-15 >15

Race days/yr (circle one): 0-10 10-20 20-100 >100

Resistance training

Times per week (circle one): <3 3-5 6-7

Minutes per day (circle one): 30-60 60-240 240-360 >360

Hours per week (circle one): 1-2 3-5 6-8 >8

Training background (in years) (circle one): <1 1-3 4-5 6-15 >15

Do you participate in other sports? If so, how often? (describe)

_____

Appendix C: Flyer

Body Composition Test
by a trained professional

Health and Fitness Tests

Men and women ages 18-30 needed for research project at the UNM Exercise Physiology Lab

VO_2max Test

What is the study about? This research is studying the effect of high intensity interval training on a cell process that protects against cancer, diabetes, and Alzheimer's disease.

Who can volunteer? Physically fit men and women between the ages of 18-30.

What will I be asked to do if I participate? You will perform a session of high intensity interval training and a session of moderate intensity continuous exercise. You will also have blood drawn and a small amount of muscle (size of a sunflower seed) removed from your leg. Additionally, you will have your body composition (body fat percentage) assessed, and aerobic fitness tested (running until exhaustion) Total time required: 5 hrs. over a period of three days.

Who can I contact for more information?

Kurt Escobar, 559-281-2220, kaescobar@unm.edu

Fabiano Amorim, amorim@unm.edu

Appendix D: Data Collection Sheets

Appendix D.1: Skinfold Data Sheet

Exercise Physiology Laboratory Skinfold Data Sheet

Subject Number_____ Date_____

Technician_____

Skinfolds	1	2	3	Avg
Subscapular	____	____	____	____
Tricep	____	____	____	____
Bicep	____	____	____	____
Chest (women too)	____	____	____	____
Midaxillary	____	____	____	____
Suprailiac	____	____	____	____
Abdomen	____	____	____	____
Thigh	____	____	____	____
Calf	____	____	____	____

Body Density_____ Equation_____

Body Fat % _____ Equation_____

Appendix D.2: VO_2max Data Sheet

EXERCISE PHYSIOLOGY LABORATORY
VO2 MAX DATA SHEET

Subject Number_____ DATE:_____

AGE:_____ WT: _____(lbs)_____(kg) HT:_____(in.) _____(cm)

Technicians(s):_____

Mode: _____ Protocol: _____ Total Exer. Time: _____

Resting Data **Exercise Data**

HR/BP (sit): _____ ____ / ____ Max HR: _____

HR/BP (stand): _____ ____ / ____ Max METS/Vo2: _____

Stage	Time (min)	Speed/Grade Or Watts/RPM	HR	BP	RPE	Comments
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						
12						
13						
14						
15						
16						
17						
18						
	Recovery 0-1					
	1-2					
	2-3					