THE EFFECTS OF AGE ON ENDOTHELIAL RESPONSE TO HIGH INTENSITY INTERVAL TRAINING

by

Gabriel Gonzalez-Contreras

July, 2023

Director of Thesis: Dr. Theodore G. Graber, PhD.

Major Department: Department of Kinesiology

Purpose: The world population is growing exponentially older. The age-related loss of muscle mass and strength, termed sarcopenia, leads to loss of function and independence, disability, and increased mortality. Declining endothelial function during aging may increase dysfunction in muscle. Exercise may partly rescue muscle vascular function. High intensity interval training (HIIT) is a form of exercise that involves short bursts of intense exercise alternated with low intensity recovery periods. The main purpose of this study was to investigate the impact of age on endothelial response to HIIT. We also sought to determine the impact of HIIT on capillarization in both adult and older adult mice. Homogenized gastrocnemius C57BL/6 mice samples were used. The study randomly assigned C57BL/6 mice to a 13-week program of HIIT, with an additional sedentary control group. Randomized mice were adult ages (aged 6 months to 10 months) or older adult ages (aged 22 months to 26 months). Due to complications incurred during the SARS-CoV-2 epidemic, 28-months old mice served as the sedentary control group (28m). The functional ability of each mouse was measured pre- and post-training using our comprehensive functional

assessment battery (CFAB) composite scoring system, consisting of five validated tests. Sarcopenia was measured through analyses of body composition (EchoMRI) and post-training muscle wet mass. Western immunoblots were performed using gastrocnemius homogenates to determine expression of vascular endothelial growth factor (VEGF), a potent angiogenic promoter, and immunohistochemistry was performed to determine the capillary to fiber ratio. We observed no statistically significant differences in VEGF expression with either age or exercise status compared to controls two days after the final training session, comparing means with a 1-Way ANOVA. However, due to large individual sample variation limiting power in the ANOVA, we used independent samples t-test, finding older mice had significantly less baseline VEGF expression, VEGF in older mice HIIT group tended to be elevated compared to controls, and HIIT restored VEGF levels to baseline levels of young adult mice. Immunohistochemistry results showed no statistical difference in capillarization between groups (HIIT, CON) in the older adult mice, albeit with a low n=3 per group. Results from the present study suggest that a HIIT training mode may not lead to chronically elevated VEGF levels within muscle in adults but may do so in older adults. This suggests that incorporating HIIT may enhance endothelial health in older adults. Future work is needed to determine if VEGF increased transiently after acute exercise bouts, and whether age plays a role in this response.

THE EFFECTS OF AGE ON ENDOTHELIAL RESPONSE TO HIGH INTENSITY INTERVAL TRAINING

A Thesis

Presented to the Faculty of the Department of Kinesiology

East Carolina University

In Partial Fulfillment of the Requirements for the Degree

The Master of Science in Kinesiology

Exercise Physiology

by

Gabriel Gonzalez-Contreras

July 2023

THE EFFECTS OF AGE ON ENDOTHELIAL RESPONSE TO HIGH INTENSITY INTERVAL TRAINING

by

Gabriel Gonzalez-Contreras

APPROVED BY:

DIRECTOR OF THESIS: Dr. Theodore G. Graber, PhD.

COMMITTEE MEMBER: Dr. Nicholas T. Broskey, PhD.

COMMITTEE MEMBER: Dr. David A. Tulis, PhD.

CHAIR OF THE DEPARTMENT OF KINESIOLOGY: Dr. Joonkoo Yun, PhD.

DEAN OF THE GRADUATE SCHOOL: Dr. Kathy Cox PhD.

TABLE OF CONTENTS

List of Tables vi

List of Figures vii

List of Abbreviations viii

Chapter I: Introduction 1

Purpose 2

Hypothesis 3

Chapter II: Literature Review 4

Cardiovascular Aging 4

Endurance Exercise 5

Endothelial Function 6

Vascular Endothelial Growth Factor 8

The Effect of Exercise Type on VEGF Concentrations 9

Exercise Induced Capillary Growth 10

High Intensity Interval Training 13

Physiological Adaptations to Exercise Old vs Young 15

Chapter III: Methods 17

Experimental Strategy 17

Subjects 18

Study Design 18

Exercise Training 18

Control 19

Western Immunoblotting 20

Electrophoresis 20

Blotting 22

Immunodetection 22

Stripping 23

Immunohistochemistry 24

IHC-Fr: Capillary CD31, aSMA and Laminin on Frozen Muscle.....24

Statistical Analysis 25

Chapter IV: Results 26

VEGF Western Blots 26

Immunohistochemistry 27

Chapter V: Discussion 28

Conclusion 31

References.....33

Tables and Figures 43

Appendix A: IACUC Certification.....49

Appendix B: Western Blots.....50

Appendix C: Immunohistochemistry.....54

Appendix D: IACUC Approval Memo..55

List of Tables

1. Table 1: Blot 1 Data Analysis
2. Table 2: Blot 2 Data Analysis
3. Table 3: Blot 3 Data Analysis
4. Table 4: Blot 4 Data Analysis

LIST OF FIGURES

1. Figure 1: VEGF relative expression: age vs. HIIT
2. Figure 2: VEGF expression vs CFAB in 26m HIIT responders
3. Figure 3: VEGF relative expression: age vs CFAB difference in 26m HIIT mice
4. Figure 4: VEGF expression vs. CFAB in 10m CON and 28m CON mice
5. Figure 5: Capillary to fiber ratio in gastrocnemius
6. Figure 6: Representative immunohistochemistry images 26m HIIT and 28m CON
7. Figure 7: Representative immunohistochemistry image

LIST OF ABBREVIATIONS

1. Cardiovascular disease (CVD)
2. Cardiorespiratory fitness (CRF)
3. Control (CON)
4. Comprehensive Functional Assessment Battery (CFAB)
5. Heart rate (HR)
6. High intensity interval training (HIIT)
7. Moderate-intensity interval training (MICT)
8. Nitric oxide (NO)
9. Nitric oxide synthase (eNOS)
10. Rating of perceived exertion (RPE)
11. Vascular endothelial growth factor (VEGF)
12. Voluntary wheel running (VWR)

Chapter I: Introduction

The world population is steadily growing older. The number of individuals over the age of 60 was predicted to increase 10-fold from 1950 to 2050 to over 2 billion worldwide (United Nations, 2015), and currently there are roughly 703 million adults aged 60 or over. The dramatic increase in the proportion of older adults worldwide has brought attention to age-related impairments. While multimorbidity can develop at any age, the number and complexity of comorbid conditions usually increase with advancing age. These include increases in the risk of chronic diseases such as type 2 diabetes, arthritis, cancer, and cardiovascular disease (CVD). Cardiovascular disease ranks first in terms of global mortality and morbidity (Tsao et al., 2023). More than 70% of adults develop CVD by the age of 70 years, among whom more than two thirds also develop non-CVD comorbidities (Dunlay & Chamberlain, 2016). Older adults are more prone to developing CVD because age plays a key role in impairing the optimal functionality of the cardiovascular system. Other comorbidities may result from age-related declines in physiological and physical function. Exercise capacity also declines and together these factors increase risk for development of sarcopenia (age-related loss of muscle mass and strength) and frailty (inability of body to thrive and maintain homeostasis) (Fleg, 2012). This may contribute to increased sedentary time for older adults. A sedentary lifestyle increases all-cause mortality and the risks for diabetes mellitus, CVD, hypertension, and cancers that may accelerate a loss of independence (Patterson et al., 2018). It is important to find factors which reduce the risk of dependence in basic activities of daily living.

Chronic diseases create a burden for older adults, especially because they frequently suffer from multiple comorbidities. As the world population grows older, it becomes increasingly important to find ways to improve or mitigate chronic disease. Higher cardiorespiratory fitness

(CRF) is associated with improved survival and decreased incidence of CVD and other comorbidities (Al-Mallah et al., 2018). Endurance exercise has been demonstrated to improve cardiorespiratory fitness. Endurance exercise may also enhance endothelial health (Ferrari et al., 2019). Proper endothelial cell function is essential to ensure maintenance of vascular homeostasis. Through exercise, endothelial cells can signal the growth of new capillaries. The most central angiogenic factor in skeletal muscle capillary growth is VEGF. During muscle contraction, VEGF increases in the muscle interstitium, acts on VEGF receptors on the capillary endothelium, and thereby stimulates angiogenic processes (Gavin et al., 2017). This enhances the cardiovascular system as capillaries have the important function of delivering oxygen, nutrients, and hormones in tissues. Recently, HIIT has been recognized as a safe and effective alternative to moderate-intensity continuous training (MICT) for older patients with CVD in cardiac rehabilitation settings to improve health outcomes (Dun et al., 2019). The effect of HIIT on underlying mechanisms and specific HIIT protocols for older patients has not been adequately reviewed. By investigating the effects of HIIT, this study will contribute to the knowledge needed to effectively design and implement proper interventions of exercise for improving or mitigating endothelial dysfunction in older individuals.

Purpose

This study's main purpose was to investigate the impact of age on the vascular endothelial baseline response, including expression of the potent angiogenic factor VEGF to HIIT. Second, this study sought to determine the impact that age and training modes had on the amount of

(VEGF-mediated) capillarization in the gastrocnemius muscle. For this purpose, homogenized C57BL/6 mice samples from the study by Pajski et al. (2021) were used.

Hypothesis

We hypothesized that a prescribed endurance exercise program, in the form of HIIT, would better improve endothelial (vascular) health compared to sedentary controls. The parameter to measure vascular health will be VEGF expression and capillarization. Furthermore, our secondary hypothesis was that older mice would receive less benefit from a similar volume of exercise than adult mice. This study will examine the role of exercise intensity on vascularity (endothelial health) in muscle. A better understanding of how exercise intensity affects endothelial health would be expected to result not only in a better comprehension of physiological adaptations, but also improve the body of knowledge for prescribing more effective exercise interventions.

Chapter II: Review of the Literature

Life expectancy has increased over the past century (Crimmons, 2015). While this represents a fundamental triumph in human achievement, there are health challenges that impact older adults. Chronic diseases represent a major problem which is not only associated with health specific concerns, but which also impact quality of life and independence. Exercise interventions can improve CVD, but the best mode of exercise for enhanced vascular improvements is not well known. High intensity interval training is an underutilized tool for improving or mitigating CVD that may serve as an alternative to or in conjunction with endurance protocols. This study investigated the effects of age and HIIT on endothelial health (VEGF levels and capillarization). This review of literature will examine the following topics: cardiovascular aging, endurance exercise, endothelial function, VEGF, capillarization, influence of exercise intensity and difference in physiological adaptations to exercise in adults versus older adults.

Cardiovascular Aging

Cardiovascular aging is a highly dynamic process that has prominent effects on the heart, arterial system, and endothelium (Xu et al., 2017). Aging lowers the threshold for CVD by promoting adverse changes in cardiac function and structure, related to a decline in the cardioprotective molecular mechanisms (Xu et al., 2017). Vascular aging describes a process of endothelial dysfunction and vascular remodeling. Vascular aging is also characterized by pathological alterations including increased arterial stiffness, plaque formation luminal enlargement, wall thickening, hypertrophy, and more (Obas & Vasan, 2018). These detrimental effects may lead to hypertension, stroke, atherosclerosis, and myocardial infarction (Obas & Vasan, 2018). However, cardiovascular structural and functional changes can still be modulated

even in older adults. Lifelong exercise is associated with fewer age-related changes in the heart (Maessen et al., 2016). In available studies, vigorous physical activity was related to increased stroke volume and inversely correlated to vascular stiffness (Heiss & Haendeler, 2018). However, significant gaps remain in our understanding of the molecular mechanisms underlying the diverse functional and structural changes involved in cardiac aging.

Endurance Exercise

Exercise is known to positively affect endothelial function (Nystoriak et al., 2018). In older adults, prevention of cardiovascular disease by physical exercise plays not only a beneficial effect on physical function, but also adds to the preservation of overall quality of life. Aerobic exercise training leads to many physiological and biochemical adaptations in the cardiovascular system, endothelium, and skeletal muscle tissue. Major systemic cardiovascular adaptations include increased cardiac output through improvements in cardiac structure and function, and enhanced blood volume (Nystoriak et al., 2018). Peripheral adaptations in response to training include increases in oxidative and glycolytic enzymes, mitochondrial content, and increased capillarization in skeletal muscle (Nystoriak et al., 2018). The systemic cardiovascular adaptations increase maximal skeletal muscle perfusion capacity during whole body work, whereas the peripheral adaptations improve oxygen diffusion capacity and oxygen utilization. It has been proposed that a high proportion of cases of late life dementia can be attributed to cardiovascular risk factors (e.g., hypertension, diabetes, and obesity), which are the main modifiable factors that can be targeted for intervention. Endurance exercise has been shown to mitigate cardiovascular disease (Maessen et al., 2016).

Endothelial Function

Endothelial function is a common cardiovascular parameter to measure healthy vascular aging (Verma & Anderson, 2003). Endothelial function is essential in ensuring proper maintenance of vascular homeostasis (Godo & Shimokawa, 2017). The endothelium is a thin membrane that lines the inside of the heart and blood vessels. A healthy endothelium helps regulate cardiovascular physiology by fine tuning vascular tone, tissue perfusion and oxygenation, resistance to thrombosis, inhibition of underlying smooth muscle cell proliferation, and adhesion of inflammatory cells to vessel wall (Godo & Shimokawa, 2017).

The endothelium regulates vascular tone by synthesizing and releasing various endothelium-derived relaxing factors including nitric oxide (NO). Nitric oxide is a potent vasodilator that also plays roles in inhibiting vascular inflammation and preventing clots (Heffernan et al., 2010). As endothelial cells age, there is a reduction in endothelial nitric oxide synthetase (eNOS) activity, in turn reducing the abundance of endothelial-derived NO (Davies et al., 2009). The release of NO by the endothelial cells can be chronically upregulated by VEGF, dietary factors, and exercise (Davies et al., 2009). This can also be downregulated by oxidative stress, smoking, and pollution, and is also reduced with aging and vascular disease. Hemodynamic shear stress, the frictional force acting on endothelial cell surface from blood flow, is one of the most potent inducers of eNOS activity (Davies et al., 2009). As the blood vessels age, there is a decline in NO due to the endothelial cells becoming less responsive to shear stress (Seals et al., 2011). This is one of the many physiologic differences that may lead to enhanced adaptations from exercise in younger adults versus older adults.

The endothelium also plays a role in regulating inflammation. Low grade chronic inflammation can increase the risk of atherosclerosis and insulin resistance which are the leading

mechanisms in the development of frailty and CVD (Soysal et al., 2020). The risk of CVD is higher in older people with frailty and the risk of frailty is higher in patients with CVD, there may be relationship between inflammation and the development of CVD and frailty. Concurrent diseases like these may impair older population and lead to losing independence, increasing sedentary behavior (Hassan et al., 2021).

On the other hand, endothelial dysfunction is the hallmark of a wide range of cardiovascular diseases associated with pathological conditions that can cause vasoconstriction, thrombosis, and inflammatory state (Godo & Shimokawa, 2017). Endothelial dysfunction is caused, in part, by reduced production or the action of endothelium-derived relaxing factors and could be an initial step toward CVD. Endothelial dysfunction can also occur due to arterial thickening and stiffness that occurs with aging of the vasculature. Vascular dysfunction associated with aging leads to a variety of age-related pathologies, including loss of adequate tissue perfusion, insufficient vascular growth or regression, or excessive growth and remodeling (Rajendran et al., 2013).

Endothelial function is a common cardiovascular parameter to measure healthy vascular aging and was used for this study. A healthy endothelium helps regulate cardiovascular physiology by ensuring proper maintenance of vascular homeostasis. It does so by fine tuning vascular tone, tissue perfusion and oxygenation, resistance to thrombosis, and more. The endothelium plays a key role by synthesizing and releasing an array of endothelium-derived factors. These angiogenic factors like VEGF or NO can serve as indices of vascular health (Davies et al., 2009).

Vascular Endothelial Growth Factor

Vascular endothelial growth factor represents a potent angiogenic factor that is essential in the regulation of physiological angiogenesis like capillarization (Melincovici et al., 2018). Capillarization is the growth process of new capillaries from existing capillaries in response to a change in the mechanical and/or metabolic environment. Capillaries have a vital role in delivering oxygen, hormones, and nutrients throughout the body. Increasing capillary numbers are important for achieving full exercise capacity (Olfert et al., 2009). Higher cardiorespiratory fitness (CRF) is associated with improved survival and decreased incidence of CVD and other comorbidities (Al-Mallah et al., 2018). In this way, this expansion of the capillary network supports vascular health.

VEGF is necessary for regulating both cardiac and skeletal muscle capillarity, and a reduced number of VEGF-dependent muscle capillaries limits aerobic exercise capacity (Olfert et al., 2009). An early study researched the organ-specific functional role of VEGF. To explore this issue, Olfert et al. (2009) engineered skeletal muscle-targeted VEGF deficient mice. VEGF protein levels were reduced up to 90% in gastrocnemius muscle. This was accompanied by 48% and 39% decreases in the capillary-to-fiber ratio and capillary density, respectively, in the gastrocnemius and a 61% decrease in cardiac muscle capillary density. After the depletion, aerobic exercise capacity decreased. Maximal running speed and endurance running capacity were reduced by 34% and 81%, respectively, in mVEGF$^{-/-}$ mice compared to control mice. Since then, meta-analysis has supported the organ-specific role of VEGF in regulating cardiac and skeletal muscle capillarity (Hoier et al. 2014).

Muscle VEGF expression is upregulated by exercise. At least three physiological factors are thought to be involved in exercise-induced angiogenesis in skeletal muscle: (i) increased blood flow and thereby shear stress; (ii) mechanical stretch of the tissue; and (iii) enhanced metabolism

(Brown & Hudlicka, 2003). It is well established that aerobic training has an angiogenetic effect promoting skeletal muscle oxidative adaptation and increasing the expression of VEGF which is a major growth factor involved in the proliferation and stabilization of endothelial cells (Gavin, et al., 2017). Increased VEGF mRNA and protein expression are also stimulated by resistance exercise training, and the acute effect of resistance exercise training on angiogenic growth factors seems to be comparable to that of endurance exercise. The focus of this study was to investigate VEGF protein expression and capillarization within the muscle in response to HIIT.

The Effect of Exercise Type on VEGF Concentrations

Studies have analyzed the effects of exercise on VEGF concentrations in the older adult population (Adams et al., 2004; Park et al., 2010; Sandri et al., 2011, 2005). The results indicated exercise increases VEGF concentrations (Adams et al., 2004; Park et al., 2010; Sandri et al., 2011, 2005). Physical exercise promotes many alterations in the cardiovascular components that could be the mechanisms leading to increased VEGF expressions (Melincovici et al., 2018). These include increasing shear rate, tension, systolic volume, or local hyperemia, and an increase in nitric oxide. The type and duration of exercise will have different effects on VEGF concentrations.

Aerobic exercise promotes an increase in VEGF expression compared to resistance training. One study observed that resistance exercises of high and low intensity in a young population resulted in no increase in VEGF concentrations (Rojas et al., 2010). Comparable results were observed for an elderly group who performed a resistance training protocol. The study observed no increase in VEGF (Ogawa et al., 2010). Another study observed that a combined exercise training consisting of aerobic exercise, band exercise, and yoga exercise for 70 minutes three times per week observed an increase on VEGF concentrations (Park et al., 2010). Each

session reserved 40 minutes for aerobic exercise. Another study analyzed the effects of acute aerobic exercise on VEGF levels. This study found that 4-hours after a submaximal test, the VEGF levels were increased in sedentary men and women compared to baseline VEGF levels pre submaximal exercise test (Croley et al., 2005).

Acute aerobic exercise may promote an increase in VEGF concentrations. However, there is no consensus on whether VEGF levels are chronically elevated. An interesting observation was reported in a study on mice, where VEGF protein levels were elevated after seven days of voluntary treadmill running (Olenich et al., 2013). One study observed an increase in VEGF concentrations in humans following a short 4-week protocol consisting of endurance based aerobic activities (Sandri et al., 2005). However, another study in humans demonstrated that high intensity intermittent training induced capillary growth while finding no significant changes in VEGF levels after a 4-week training program (Jensen et al., 2004). On the other hand, Park et al. (2010) found an increase in levels of VEGF after 12 weeks (about 3 months) of physical exercise. Aerobic training protocols may increase VEGF concentrations, but the role of intensity on VEGF is still inconclusive. Further studies using an aerobic protocol should be done to elucidate the association between intensity of aerobic exercise and VEGF concentrations.

Exercise Induced Capillary Growth

Exercise training expands the capillary network (Hoier et al., 2014). The vasculature is normally quiescent. In skeletal muscles, an increase in capillary numbers is a hallmark of adaptation to endurance exercise training, contributing to increased aerobic capacity. This has been shown in both young and elderly humans based usually on the analysis of invasively obtained muscle biopsies. Higher cardiorespiratory fitness (CRF) is associated with improved survival and

decreased incidence of CVD and other comorbidities (Al-Mallah et al., 2018). This is an important adaptation in skeletal muscle that secures adequate diffusion capacity for oxygen and nutrients even at high-intensity exercise when increases in muscle blood flow are profound. Mechanical forces present during muscle activity, such as shear stress and passive stretch, lead to cellular signaling, enhanced expression of angiogenic factors, and initiation of capillary growth (Hoier et al., 2014). The most central angiogenic factor in skeletal muscle capillary growth is VEGF. During muscle contraction, VEGF increases in the muscle interstitium, acts on VEGF receptors on the capillary endothelium, and thereby stimulates angiogenic processes (Gavin et al., 2017).

The ability of the capillary network to expand is quite remarkable due to its plasticity. Well-trained endurance athletes may have three to four times more capillaries per muscle fiber than sedentary individuals (Hoier et al., 2014). Aerobic exercise training induces an increase in muscle VEGF levels. VEGF secretion from skeletal muscle is an essential step in capillary growth. Aerobic training may increase capillary to fiber ratio either with or without a parallel change in fiber area. The fiber area is usually unaltered. Resistance training differs as it may cause substantial change in fiber area that can be matched by an increase in capillary to fiber ratio. The adaptations to exercise training have been studied through different training/protocols. Future studies should focus on mechanisms underlying VEGF secretion in muscle. It would also be beneficial to further study the role the intensity of aerobic exercise may have in enhancing VEGF levels in skeletal muscle.

Adequate skeletal muscle capillarization can enhance muscle protein synthesis by ensuring the transport of amino acids and growth factors to muscle fibers (Moro et al., 2019). The progressive loss of muscle mass and function that occurs with aging is associated with morphological and structural alterations within skeletal muscle. For example, aged muscles have

smaller fibers, a phenomenon that is particularly evident in type II fibers, along with a decrease in capillary content (Miljkovic et al., 2015). The decline in skeletal muscle mass (sarcopenia) advances slowly with healthy aging but can be accelerated by concurrent diseases resulting in mobility limitation, disability, frailty, and loss of independence (Larsson et al., 2019). Appropriate nutritional interventions and habitual physical activity are often employed to counteract the onset of sarcopenia (Bosaeus & Rothenberg, 2016). Resistance exercise training is the most effective strategy to increase skeletal muscle mass and strength (Bechshøft et al., 2017). Adequate muscle perfusion supports the transport of nutrients, oxygen, and hormones into muscle fibers.

Exercise-induced angiogenesis normally occurs to increase oxygen diffusion and to enhance the removal of metabolites during muscle contraction (Hoier & Hellsten, 2014). Exercise programs with comparable intensity and volume have similar effects on muscle capillarization (Snijder et al., 2017). However, levels of shear stress are mediated by specific mechano-sensors, (Hoier & Hellsten, 2014), and thus, different contraction modalities may modify the tension applied from the sarcomere to the endothelial cell, resulting in different shear stress intensities. An early study suggests that muscle hypertrophy in trained individuals may occur without a parallel increase in muscle capillarization (Tesch et al., 1989). However, capillary density and capillary-to-fiber ratios are lower in untrained/sedentary individuals compared to trained individuals (Zoladz et al., 2005). Additionally, endurance athletes present higher capillarization when compared to power or strength athletes (Zoladz et al., 2005). To what extent the increase in capillarization occurs for the same functional purposes in response to moderate and high-intensity exercise remains unclear.

High Intensity Interval Training

Recently, HIIT has attracted growing attention. HIIT is characterized by repeated bouts of high-intensity exercise intermixed by low-intensity exercise or passive recovery. Meta-analyses have confirmed HIIT to be an appropriate training stimulus to improve cardiorespiratory fitness (Weston et al., 2014). Improving cardiorespiratory fitness can reduce the risk of all-cause mortality and CVD (Dun et al., 2019). It is widely accepted that lack of time is the most common barrier for people to consistently participate in regular exercise. By providing similar benefits to endurance-styled training in shorter durations, HIIT can serve as an alternative or supplemental training style for the public and special populations.

Increased cardiovascular fitness is associated with enhanced vascular function, and a decreased rate of mortality (Torma et al., 2019). Both endurance and HIIT types of training elicit large improvements in VO2max. High intensity interval training is one of the best ways to increase VO2max and endurance capacity for top athletes and the public. Meta-analyses have confirmed HIIT to be an appropriate training stimulus to improve cardiorespiratory fitness and reduce metabolic risk factors in patient populations (Weston et al., 2014). Improvements in fitness are obtainable with a shorter duration of exercise (i.e., 20 min), if the exercise session incorporates bouts of high-intensity exercise (Currie et al., 2015). In one study the difference in the training effect between HIIT and endurance was enhanced for older and less fit subjects, suggesting HIIT to have appeal for those involved in the fitness programming of older adults and patient populations, especially given that the safety concerns associated with HIIT are unfounded. When comparing the two modes of training, the gains in VO2max are greater following HIIT (Dun et al., 2019). Given the well-established link between aerobic fitness and mortality, further investigations

into HIIT are therefore recommended to enhance our understanding of the beneficial effects of HIIT.

The ratio and duration of high-intensity and low-intensity intervals are key parameters that differentiate MICT from HIIT. HIIT has a significant impact on the skeletal muscle. Studies have shown that HIIT is a powerful strategy to improve muscle total fiber amount and type proportions, capillary density, as well as the content and activity of glucose and fat oxidative metabolism markers (Dun et al., 2019). Interval training at the higher end aims for a peak power output that elicits ≥80% peak heart rate (HR). Three common forms of HIIT include intervals of short, medium, and long duration. Long-interval HIIT may include 4 sets of high-intensity intervals, each lasting 4 minutes interspersed with 3 sets of low-intensity intervals, each lasting 3 minutes. Medium-interval HIIT is also commonly used following 8x2 minute high-intensity intervals interspersed with 7x2 min low-intensity intervals. For older patients with heart failure, short intervals have been commonly used, including a 10x1-minute high-intensity intervals interspersed with 9x2-minute low-intensity intervals. Exercise training using high intensity (85-95% peak HR or peak VO2 and rating of perceived exertion (RPE) 15-17) with low intensity intervals (50-75% peak HR or peak VO2 and RPE 12-14) is proposed for older adults (Dun et al., 2019). However, there is no clear consensus on the optimal HIIT prescriptive variables that elicit the greatest benefits.

The effect of high intensity training on capillary growth, endothelial proliferation and VEGF levels has also been examined in human skeletal muscle (Jensen et al., 2004). For 4 weeks, two knee extensor training regimes were used. One group used 150% of leg VO2max while the second group used 90%. Muscle biopsies were obtained throughout the training periods. The

results demonstrated that high intensity intermittent training induces capillary growth, but results found no significant changes in VEGF levels.

People of all age groups can benefit from HIIT, and HIIT was recently considered safe for special populations including older adults and those with CVD. When prescribing HIIT for these groups, specific considerations are necessary as they may experience impaired balance, multimorbidity, frailty, and other side effects with age. Modifying HIIT to best accommodate each of these factors, making it safe and enjoyable, may facilitate the HIIT adherence in this population. One strategy is to include less strenuous exercise intensities for clinical populations compared to athletes. It may also be appropriate to use a combination of objective and subjective methods when prescribing HIIT. By accommodating for risk factors, more people may participate in HIIT that may result in similar or even superior physiological exercise training adaptations compared to MICT.

Difference in Physiological Adaptations to Exercise old vs Young

Aging is associated with a reduction of angiogenic factors with consequent reduced angiogenesis in muscle tissue and endothelial cells (Herrera et. al., 2010). The adverse effects of aging such as stiffening of large elastic arteries and endothelial dysfunction make aging the greatest risk factor for CVD. Vascular aging is driven by oxidative stress which can have detrimental effects on the endothelium like reducing NO bioavailability and stimulating changes in the extracellular matrix. Regular aerobic exercise is the most evidence-based strategy for reducing CVD risk associated with aging in both men and women. Much of this CV-protective effect of aerobic exercise is due to its vascular health-enhancing influence. Large elastic artery stiffening with advancing age is attenuated in healthy adults engaged in aerobic exercise training, and aerobic

exercise interventions improve arterial stiffness in previously sedentary middle-aged and older men and postmenopausal women. Regular aerobic exercise also enhances endothelial function with aging in men by reducing oxidative stress and preserving NO bioavailability.

There is lower skeletal muscle capillarization and VEGF expression in aged versus young men (Ryan et al., 2006). One study obtained biopsies of the vastus lateralis before and at 4 hours after a submaximal exercise bout to measure capillarization and VEGF. The older group had a mean age of 65 while the younger group had a mean age of 21 years of age. Both groups were previously sedentary. The results were that muscle VEGF mRNA and protein levels were lower 4 hours after post-exercise in the older adult group. The capillary-to-fiber perimeter exchange and muscle capillary contacts were lower in the aged group 4 hours after exercise (Ryan et al., 2006). These results confirm that VEGF expression and skeletal muscle capillarization are lower in older adult men compared to younger men.

Another study analyzed VEGF protein and mRNA as well as the VEGF receptor response to acute exercise in the vastus lateralis muscle of aged vs young women (Croley et al., 2005). The vastus lateralis muscle biopsies were obtained before and after a 4-hour submaximal exercise bout for the measurement of morphometry and VEGF and VEGF receptor expression. This study found that capillarization surrounding type II muscle fibers, resting VEGF protein, and the exercise-induced increase in VEGF mRNA are lower in older women when compared to younger women. Muscle VEGF was 35% lower in older women at rest, and the exercise induced increase in VEGF mRNA was 50% lower on older women compared with younger women. These results indicate there may be an aging-related impairment of the angiogenic response to exercise training. In our current study, though we did not measure VEGF receptor expression, we hypothesized the older adult mice would see lower VEGF protein levels than the younger mice.

Chapter III: Methods

Experimental strategy:

This study's main purpose was to investigate the impact of age on endothelial (vascular) response, in the form of baseline VEGF levels, to HIIT. Second, this study sought to determine the impact that age and training modes had on the amount of capillarization in the gastrocnemius muscle. For this purpose, homogenized C57BL/6 mice gastrocnemius muscle samples from the study by Pajski et al. (2021) were used to determine VEGF expression levels at two days following the last exercise bout of a 3-month training period. The Pajski et al. (2021) study's main purpose was to evaluate the effect of endurance exercise training protocols on the physical function of adult and older adult mice. Comprehensive Functional Assessment Battery (CFAB) is a composite mathematical construct that provides a sensitive assessment of the ability of an individual mouse to perform complex motor tasks and exercise capacity. This is in relation to the average of a control group which can provide a reliable, repeatable, noninvasive, and powerful numerical quantification to understand physical function. CFAB is comprised of the well-validated functional tests of: rotarod (overall motor function), grip meter (strength), inverted cling (strength/endurance), treadmill maximum speed (endurance), and activity/volitional exercise rate (using voluntary wheel running) (Graber et al., 2020). In the parent study, Pajski et al. (2021), mice were tested for physical function before and after the training period using the CFAB scoring system. After training and functional test completion the mice were humanely sacrificed and were homogenized. In this current study, western blotting was performed on the homogenate samples to detect changes in VEGF expression and immunohistochemistry was performed to determine capillarization of muscle cross-sections.

Subjects:

This study used male C57BL/6 mice, which are common in aging studies. The age equivalencies to humans are the following: 6-months old (6m) mice are equivalent to a young adult human (early to mid-20's) and 10-months old (10m) at the end of exercise training (equivalent to start of middle age in humans correlating to about mid- to late 30's); 22-months old at start (correlates to a human in early to mid-60's) and 26-months old (equivalent to early 70's human) at the end of training (Dutta & Sengupta, 2016). Due to complications incurred during the SARS-CoV-2 epidemic, we used a slightly older 28-months old control group (28m, equivalent to 78–80 year-old human). The mice were randomly divided into groups: sedentary control (CON, 10m, n=8, 6 survived to study end, and 28m, n=5), and high intensity interval training (HIIT,10m, n=8; 26m, n=10, 9 survived to study end). Sedentary mice were housed in groups in cages with environmental enrichment but with no exercise prescribed. Exercise mice followed the exercise programs described below. The mice were obtained from the NIH NIA Aging Rodent Colony and Charles River Laboratory and were treated humanely under approved IACUC protocols.

Study Design:

The mice underwent pre-training functional testing and body composition measurement. Mice were then randomized into groups (HIIT, CON) following the pre-training bout of functional testing (see below for details).

Exercise Training: Exercise Details: Details are published elsewhere (Pajski et al., 2021).

HIIT: Based off the maximum running speed at failure from pre-training treadmill functional testing, the HIIT mice were placed in similarly paced groups. This group, after acclimation, was run on an exercise protocol designed to mimic HIIT. The exercise intervention lasted 13 weeks. In brief: The mice warmed-up prior to HIIT by walking at 3 meters per minute for 2 minutes. This was followed by repeating intervals based upon a percentage of their maximum speed (determined during functional testing). The intervals were separated by 1-minute periods of active rest. Upon interval completion (mice started out with three intervals and progressed to five as the study went on), the mice had a 2-minute cool-down walk at 3 meters per minute velocity. Each interval began with a 30 second acceleration step to running speed (set to 75%, 80%, or 85% max speed to increase intensity as tolerated), which was maintained for 60 seconds, before ending with a 30 second deceleration back to walking speed (2 minute total intervals, separated by 1 minute of active recovery). At the study midpoint HIIT mice repeated the functional treadmill test and group speeds and assignments changed according to how the maximum running speed of each mouse had improved HIIT (treadmill running).

CON: Control mice did not exercise and were placed in environmentally enriched cages.

Western Immunoblotting

Western blots were used to analyze VEGF protein concentrations in the homogenized mixtures. The first step was to determine the volume that will be loaded into gel wells. A BCA protein assay (ThermoFisher Scientific, Product# A53226) was done using manufacturer's instructions to quantify the total concentration of protein in each homogenate sample. These concentrations were used for calculating appropriate amounts of homogenate in mixtures for Western blots. For this experiment, 12 µl were loaded into 15 µl wells. To get the best results it is best to use a protein load that gives a linear range. After optimization, this value was determined to be 23 µg. The total volume consisted of Laemmli buffer, β-mercaptoethanol (BME), protein homogenate and ddH2O. A quarter of the mixture loaded into the wells will be composed of a 9:1 mixture of 4x Laemmli buffer and BME. The remaining 75% will be a mixture of protein homogenate and ddH2O. Double-distilled water is used to dilute the protein homogenate to achieve the desired protein load concentration of 23 µg. These mixed samples will go into labeled 0.5 mL tubes.

Electrophoresis

The homogenate samples of interest and Spectra Multicolor Broad Range Protein Standard Kaleidoscope Product# 26634 (ThermoFisher Scientific) were thawed and placed on ice. After the homogenate samples have thawed, they were vortexed, spun down and stored on ice. Protein homogenate was then mixed with ddH2O, followed by appropriate 3:1 Laemmli buffer ratio to achieve the proper protein content per well (23 µg, which was determined using a linear range protocol-see Appendix B). This is a crucial step for accurate detection of target samples and the interpretation of results. The linear range is the span of signal intensities that displays a linear

relationship between the amount of target on the membrane and the signal intensity recorded by the detector. To find the proper protein content level, wells were loaded with a wide range of protein content per well. The signal was then measured to find linear range. The mixed samples were then heated for 5 minutes on the block heater at 95° C, briefly vortexed and spun down on a mini centrifuge.

We used premade SDS gels (15 well 4-15% Graduated Acrylamide Gels Biorad, Product# 4568086). Before the gel lanes were loaded, the comb and tape were gently removed. Schematics were created and noted in a notebook to keep a record of which sample was in each well.

To perform electrophoresis the electrophoresis cell must be set up in a particular way. Gels should be placed with the shorter plate facing inward in the cassette. This cassette is then placed in the Mini-PROTEAN Tetra cell (Biorad, Product# 552BR 226776). The cell was then filled with a running buffer (150 mL 10x Tris/glycine/SDS + ddH20 to 1.5 liter, Biorad Product# 1610772) and a stir bar was put into the cell. The cell was set in a shallow container filled with ice and placed on top of the stir plate. Once setup was complete, 5 µl of the protein ladder standard (Spectra Multicolor Broad Range Protein Standard Kaleidoscope Product# 26634) was loaded in the first well of each gel. Then 12 µl of the homogenate sample mixture were loaded into the 15 µl wells in the prior to electrophoresis. This was followed by loading 12 µl of the homogenate mixtures. Prior optimization determined that power requirements for the electrophoresis system would need to be at a constant 200 Volts for 40 minutes.

Blotting

To prepare for blotting, the 0.20 PVDF membrane (Immobilon-PSQ, Product# ISEQ00010) was first activated in 100% methanol for 1-2 minutes. All transfer sandwich materials were then equilibrated in the Towbin transfer buffer (100 mL 10x Tris/glycine/SDS + 100 mL 10x Tris/glycine then ddH2O to a final volume of 2 liters) for at least 15 minutes including the gel and PVDF. Sandwich materials include 2 foam pads per cassette and four pieces of filter paper (Biorad, Product# 1703932) per cassette. The transfer cassettes were also put in the transfer buffer before creating the transfer sandwich. Once all materials have equilibrated it is time to create the gel and membrane sandwich then carefully close it to avoid smearing. The sandwich was then slid into the transfer cassette (Biorad Mini Trans-Blot Cell Product# 153 BR112913) with the cathode side facing the back of the chamber. The transfer cell was placed in a shallow container filled with a transfer buffer to the blotting line. The cell was kept in a container surrounded by ice during electrophoresis. An ice pack was also placed in the open side of the chamber. A stir bar was added to the cell and stirred at a fast rate. The power settings, determined during optimization, were set to 200 constant Volts for 15 minutes.

Immunodetection

After the transfer step was completed, the membrane was then washed in 1xTBS (composed of 20mM Tris and 150 mM NaCl). The washing was done using a 5-4-3-2-1 process. The washing was done using an orbital shaker initially washing for 5 minutes then washed in clean 1 xTBS for 4 minutes and so forth. After the wash step the membrane was incubated in 25 mL of %3 BSA (blocking buffer) and sat on a rocker for an hour and a half. After incubation, the membrane was washed once again using the same 5-4-3-2-1 process but with TBST (TBS + 0.05%

tween20). Ten mL of blocking buffer (3% BSA in TBST) was used per membrane. The primary antibody (VEGF anti-rabbit PolyAb Proteintech Product# 19003-1-AP) was then diluted to a 1:2000 in the blocking buffer. The membrane was incubated overnight at 30-40 rpm on a rocker overnight at 4 degrees Celsius.

After the overnight incubation, the membrane was washed in TBST. The Goat Anti-Rabbit HRP (horseradish peroxidase)-conjugated secondary antibody (Biorad Product# 1706515) was diluted to the 1:20,000 dilution factor and used to incubate the membrane for 1 hour at room temperature (approximate 21 °C) with gentle rocking. After an hour of incubating in the secondary antibody the membrane was washed with TBST following the 5-4-3-2-1 wash protocol. The membrane was then washed once with TBS. Pierce Pico PLUS Product# 34578 was the developer used. To prepare the developer a 1:1 mixture was prepared with substrates A and B. These are sensitive to light, so foil was used to cover the conical tube from light. Ten mL were used for each membrane. After the wash step, the membrane was transferred to an incubation container with the developer (Pierce Pico PLUS Product# 34578). The incubation container was covered with a foil cap to protect it from ambient light and placed on a rocker for 4 minutes. After 4 minutes the membrane was placed in a clear sheet protector and taken to the Biorad ChemiDoc MP imager to measure chemiluminescence of the horseradish peroxidase. The membrane was then stripped and stored.

Stripping

The membrane was first incubated in mild stripping buffer (200 mM glycine + 0.1% SDS + 1% Tween-20) at room temperature for 10 minutes. The buffer was discarded, and the process was repeated with fresh buffer. The membrane was then washed twice with TBS for 10 minutes

each wash. Then the membrane was washed twice with TBST for 5 minutes each wash. The membrane was then dried and stored.

Immunohistochemistry

Immunohistochemistry was performed to determine capillarization of muscle cross-sections. Skeletal muscles used for immunohistochemistry were trimmed, weighed, and then frozen in a liquified pool of isopentane. Most of the isopentane was previously frozen solid in liquid nitrogen. Prior to freezing, we set the muscle in optimal cutting temperature compound on a foil covered cork at relaxed length and straighten from origin myotendinous junction to insertion myotendinous junction. The muscle was then submerged in the isopentane for approximately 10 seconds. After freezing, we gently removed the muscle (embedded in the optimal cutting temperature compound) from the cork with forceps, placed it in a pre-chilled (in liquid nitrogen) externally treaded cryotube, and then submerged in liquid nitrogen. The muscle was then stored at -80°C until ready to be used.

IHC-Fr: Capillary CD31, αSMA and Laminin on Frozen Mouse Muscle

The first step was to cut the section into the appropriate size. These muscles were cut into 7µm thin sections and were air dried. The fixed sections were then placed in acetone at -20°C for 10 minutes. This was followed by a 3-minute wash in PBS and repeated 3 times. The sections were then blocked for one hour in MOM Ms IgG blocking (vector, Cat# MKB-2213) (1drop/ml of PBS). Using PBS 2 2-minute washes were completed. This was followed by another one-hour block in 2.5% NHS blocking solution at RT (Vector# S-2012). The primary antibody, Rt CD31 (1:100) (BD Biosciences Cat# 550274) was used for an overnight incubation; Ms IgG2a αSMA

(1:100) (Santa Cruz Cat# SC-130616); Rb laminin (1:100) (Sigma Cat #L9393) in 2.5% NHS at 4ºC. After the overnight incubation is complete PBS was used for a wash step lasting 3 minutes. This was repeated 3 times. This was then followed by a one-hour incubation step in 2ºAb Gt anti Rt IgG Cy3 (1:250) (Invitrogen, Cat# A-10522); Gt anti Ms IgG2a AF488 (1:250) (Invitrogen, Cat# A-21131); Gt anti Rb IgG AF647 (1:250) (Invitrogen, Cat# A-21244) in PBS at RT. The 3 washes in PBS were repeated 3 times. This was followed by an incubation step of 10min in DAPI (Invitrogen, Cat# D3571); dilute (100µm) stock (1:10,000 in PBS) at RT. Two washes 3 minutes each in PBS followed. The slides were then mounted with vector shield mounting media. The excess mounting media was drained. The slides were stored at 4°C.

Statistical Analysis

We presented the data as mean +/- SE (standard error). A one-way ANOVA was used to detect changes in means, with LSD (least significant differences) post hoc testing to avoid type 2 error (false negatives) with our small sample size. Because of our small sample size and low observed power, we also compared the two ages of CON, and CON vs. HIIT with independent samples Student's t-tests. Linear regression was used to determine relationships between the data. The statistical significance was set at $p<0.05$, and trends were reported at $0.05<p<0.10$. For statistical analysis SPSS v28 (IBM) was used.

Chapter IV: Results

VEGF Western Blots

Western immunoblotting was performed to detect baseline VEGF levels within the gastrocnemius in the sedentary control mice and two days after the final exercise intervention in HIIT. There appeared to be no statistical significance between groups HIIT, CON) with a one-way ANOVA ($F=1.262$, $p=0.312$). However, with a large amount of individual variability and low sample size leading to a low observed power post hoc testing was done with independent samples t-tests. The t-tests indicated no change detected between 10m CON (mean 1.687± 0.643 and 10m HIIT (mean 2.205± 1.242, though there was a trend ($p=0.075$) for increased VEGF in the 26m HIIT (mean 1.868± 1.066) versus the 28m CON (mean 1.068± 0.206). Age played a role in baseline VEGF content ($p=0.048$), with older adult control mice (mean = 1.068 ± 0.103) having 58% less VEGF protein expression than the 10m CON (mean 1.687 ± 0.243). Interestingly, exercise restored VEGF levels in 26m HIIT to comparable levels of the 10m groups. See Figure 1 for more details. Appendix B includes the blot data and images, including the total protein stain.

Linear regression was used to probe for relationships between functional outcome measures (CFAB and treadmill time) and VEGF expression. Notably, there was a decrease in CFAB score with increased VEGF levels. See Figure 2 for more details. The literature indicates there is a lack of consensus on VEGF levels following exercise training. One study found decreased levels of VEGF protein immediately after a one-hour submaximal cycle exercise bout and levels were returned to baseline at two hours after exercise (Gavin et al., 2004). Other interventions have also found increases in VEGF expression levels (Rullman et al., 2007; Gustafsson et al., 2002). For example, 4-hours after submaximal test, VEGF levels were found to be increased in sedentary men and women compared to baseline VEGF levels pre submaximal

exercise test (Croley et al., 2005). Others found no change in VEGF expression (Hoier et al., 2012; Gustafsson et al., 2007). The discrepant results of these studies reflect the complexity of VEGF expression. Future studies should focus on elucidating mechanisms and regulation of VEGF secretion.

Immunohistochemistry

In this study we hypothesized that the exercise group would see increased capillarization compared to the sedentary control group. The immunohistochemistry protocol was completed on the 26m HIIT and 28m CON groups. The representative images can be found in Figure 7 in Appendix C. Similar images were used to measure the number of capillaries per cell. The red represents CD31 which is a capillary and endothelial marker, the blue represents nuclei (DAPI counterstain), and the sarcolemma outline is represented by purple (laminin). The number of capillaries per cell was measured as the outcome. Similar images will be completed for the 10-month HIIT and 10-month control groups in the future for comparison.

Chapter V: Discussion

Vascular endothelial growth factor is a potent angiogenic factor for the growth of new capillaries (Gavin et al., 2017). The regulation of VEGF expression is complex. The literature on the effect of acute exercise and training on VEGF protein expression is conflicting. In this study, using a one-way ANOVA there appeared to be no statistically significant difference in the amount of VEGF protein expression levels between groups (10m and 26m HIIT, 10m and 28m CON). We observed no significant difference in VEGF concentrations with either age or exercise status two days after the final training session, comparing means with a 1-Way ANOVA. However, due to large individual variation, we did post-hoc analysis using independent samples t-test and found that older mice had significantly less baseline VEGF, VEGF in older HIIT mice tended to be elevated compared to controls, and HIIT restored VEGF levels in older mice to baseline levels of young adult mice. Immunohistochemistry results are pending, but we expect increased capillarization from HIIT training. Results from the present study suggest that a HIIT training mode may not lead to chronically elevated VEGF levels within muscle in adults but may do so in older adults. This could be due to the older sedentary mice needing less stimulus to provoke an upregulation in VEGF. These mice began the exercise intervention later in their lives than the younger adult mice. It is possible the younger adult mice may require more stimulus to see the same level of VEGF upregulation. This could be in the form of higher intensity treadmill running or increased number in intervals to see the same effect. Future work may investigate the role of increased volume or intensity may have on VEGF expression levels in the younger adult mice. Future work is also needed to determine if VEGF increased transiently after acute exercise bouts, and whether age plays a role in this response.

One reason no significant differences in VEGF concentration were observed between groups (HIIT, CON) could be that the upregulation of VEGF event was missed, where the changes only occurred acutely after a training session and not at baseline. A few studies have focused on the time course of VEGF expression in response to acute exercise in human skeletal muscle and found that VEGF is upregulated from between one and six hours after exercise with peak occurring between one- and two-hours post exercise (Hoier et al., 2013). However, inconsistent results have been observed when investigating protein levels of VEGF in human muscle in response to exercise. Hoier et al. (2007) analyzed VEGF protein levels at zero, one, and two hours after submaximal cycling and reported no change. These results are like another study that reported unchanged basal muscle VEGF protein levels after five weeks of training (Gustafsson et al., 2007). However, another study observed that the levels were increased at two hours after moderate- to high-intensity cycling (Rullman et al., 2007). Gustafsson et al. examined VEGF protein and mRNA expression after short-term exercise training and found both were elevated at 10 days (Gustafsson et al., 2002). In another study on mice, VEGF protein levels were elevated after seven days of voluntary treadmill running with levels being back to pretraining after 28 days of running (Olenich et al., 2013). Contrary to the findings discussed above, Gavin et al. (2007) found that immediately after a one-hour submaximal cycle exercise bout, levels of VEGF protein decreased, and levels were returned to baseline at two hours after exercise. This lack of consensus demonstrates the complexity of VEGF regulation. Although there is lack of consensus in the literature, it is possible that upregulation of VEGF expression is transient and occurs after exercise and could be the reason protein levels did not appear to increase with training in our mouse muscle. Further studies should focus on mechanisms underlying VEGF secretion in muscle.

In this study, we hypothesized that the exercise group would experience increased capillarization compared to the sedentary control group. Currently, the immunohistochemistry protocol has been completed on the 26-month HIIT and 28m CON groups. Three samples were analyzed per group (26m HIIT, 28m CON). The number of capillaries per cell was counted. A representative image was provided in Appendix C. No statistical differences were found in the number of capillaries per cell between groups as observed in Figure 5 (26m CON, 26m HIIT). The immunohistochemistry will be completed in the 10 months groups in a future study. We expect to see an increased capillarization among the exercise groups. We also expect that the older adult mice will see less capillarization compared to the younger mice groups, likely because aging is associated with reduced angiogenesis in muscle tissue and endothelial cells with consequent reduction of angiogenic factors (Herrera et. al., 2010). Studies have also observed lower skeletal muscle capillarization in aged versus young men after exercise (Ryan et al., 2006). Finding that an increased capillarization occurred without a rise in baseline VEGF expression could indicate there could have been an acute spike in VEGF shortly after exercise that returned to baseline. This finding would be similar to a study that looked at the effect of high intensity training on capillary growth, endothelial proliferation, and VEGF levels in human skeletal muscle (Jensen et al., 2004). The results demonstrated that high intensity intermittent training induced capillary growth without significant changes in baseline VEGF levels. However, further work is needed to determine if VEGF increased transiently after acute exercise bouts, and whether age plays a role in this response.

Conclusion

Aging is associated with a dominant risk factor for endothelial dysfunction. The fast growth of the older adult population in the world makes it increasingly important to find ways of mitigating age-related diseases. Improving or maintaining cardiovascular fitness can reduce the risk of all-cause CVD (Dun et al., 2019). Exercise plays a key role in reducing falls, maintaining good physical function, and maintaining a superior quality of life. In most cases exercise is safe and has been shown to decrease the risk for secondary cardiac events. Exercise training should therefore be emphasized as a part of prevention programs developed for cardiac patients to minimize the risk of cardiovascular events.

Cardiovascular fitness is a more powerful predictor of mortality when compared with other well established risk factors such as diabetes mellitus, hypertension, obesity, and smoking (Dun et al., 2019). High-intensity interval exercise training has been shown to elicit comparable and/or superior improvements in numerous cardiovascular disease risk factors when compared to the MICT most commonly in use in cardiac rehabilitation (Keteyian et al., 2014). Moreover, some authors claim that HIIT is a superior method compared to aerobic training, to improve diverse physiological functions. By providing comparable chronic physiological adaptations with less expenditure of time, HIIT style training protocols may enhance exercise benefits in the general population, older adults, and special populations such as individuals in a cardiac rehabilitation setting.

In this study, we hypothesized that a prescribed endurance exercise program, in the form of HIIT, would better improve endothelial (vascular) health compared to a sedentary control. The parameters for endothelial health were VEGF expression and capillarization. In this study there was no significant difference between the groups in VEGF expression. This could have occurred

due to missing the spiking event of VEGF expression similar to what has been observed in other studies. Future studies should focus on elucidating mechanisms and regulation of VEGF secretion. There was no significant difference in the amount of capillarization when comparing the older adult mice (26m, 28m) HIIT and CON groups. This may be due to our very small sample size. The capillaries per cell were analyzed by one person each. To ensure the accuracy of the data a second trial should be conducted to ensure accuracy. Increasing the sample size and repeating the capillary count per cell, may lead to a better representation of the data that might lead to finding a difference among groups. A better understanding of how exercise intensity affects endothelial health would be expected to result in a better comprehension of vascular health. This would be significant as endothelial cells play a key role in vascular homeostasis. Given the well-established link between aerobic fitness and mortality, further investigations on HIIT training can enhance our understanding of the beneficial effects of HIIT. Future studies should focus on elucidating mechanisms and regulation of VEGF secretion. Specifically, more models should investigate the acute VEGF expression levels shortly after exercise bouts and determine if there is a correlation between acute VEGF expression bouts and the amount of capillarization.

References

Adams, V., Lenk, K., Linke, A., Lenz, D., Erbs, S., Sandri, M., Tarnok, A., Gielen, S., Emmrich, F., Schuler, G., & Hambrecht, R. (2004). Increase of Circulating Endothelial Progenitor Cells in Patients with Coronary Artery Disease After Exercise-Induced Ischemia. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *24*(4), 684–690. https://doi.org/10.1161/01.atv.0000124104.23702.a0

Al-Mallah, M. H., Sakr, S., & Al-Qunaibet, A. (2018). Cardiorespiratory Fitness and Cardiovascular Disease Prevention: an Update. *Current Atherosclerosis Reports*, *20*(1), 1. https://doi.org/10.1007/s11883-018-0711-4

Bechshøft, R. L., Malmgaard-Clausen, N. M., Gliese, B., Beyer, N., Mackey, A. L., Andersen, J. L., Kjær, M., & Holm, L. (2017). Improved skeletal muscle mass and strength after heavy strength training in very old individuals. *Experimental Gerontology*, *92*, 96–105. https://doi.org/10.1016/j.exger.2017.03.014

Bosaeus, I., & Rothenberg, E. (2015). Nutrition and physical activity for the prevention and treatment of age-related sarcopenia. *Proceedings of the Nutrition Society*, *75*(2), 174–180. https://doi.org/10.1017/s002966511500422x

Crimmins, E. M. (2015). Lifespan and Healthspan: Past, Present, and Promise. *The Gerontologist*, *55*(6), 901–911. https://doi.org/10.1093/geront/gnv130

Croley, A. N., Zwetsloot, K. A., Westerkamp, L. M., Ryan, N. A., Pendergast, A. M., Hickner, R. C., Pofahl, W. E., & Gavin, T. P. (2005). Lower capillarization, VEGF protein, and VEGF mRNA response to acute exercise in the vastus lateralis muscle of aged vs. young women. *Journal of Applied Physiology*, *99*(5), 1872–1879. https://doi.org/10.1152/japplphysiol.00498.2005

Currie, K. D., Bailey, K. J., Jung, M. E., McKelvie, R. S., & MacDonald, M. J. (2015). Effects of resistance training combined with moderate-intensity endurance or low-volume high-intensity interval exercise on cardiovascular risk factors in patients with coronary artery disease. *Journal of Science and Medicine in Sport*, *18*(6), 637–642. https://doi.org/10.1016/j.jsams.2014.09.013

Davies, P. F. (2009). Hemodynamic shear stress and the endothelium in cardiovascular pathophysiology. *Nature Clinical Practice. Cardiovascular Medicine*, *6*(1), 16–26. https://doi.org/10.1038/ncpcardio1397

Dun, Y., Smith, J. R., Liu, S., & Olson, T. P. (2019). High-Intensity Interval Training in Cardiac Rehabilitation. *Clinics in Geriatric Medicine*, *35*(4), 469–487. https://doi.org/10.1016/j.cger.2019.07.011

Dunlay, S. M., & Chamberlain, A. M. (2016). Multimorbidity in Older Patients with Cardiovascular Disease. *Current Cardiovascular Risk Reports*, *10*(1). https://doi.org/10.1007/s12170-016-0491-8

Dutta, S., & Sengupta, P. (2016). Men and mice: Relating their ages. *Life Sciences*, *152*, 244–248. https://doi.org/10.1016/j.lfs.2015.10.025

Ferrari, L., Vicenzi, M., Tarantini, L., Barretta, F., Sironi, S., Baccarelli, A. A., Guazzi, M., & Bollati, V. (2019). Effects of Physical Exercise on Endothelial Function and DNA Methylation. *International Journal of Environmental Research and Public Health*, *16*(14), 2530. https://doi.org/10.3390/ijerph16142530

Fleg J. L. (2012). Aerobic exercise in the elderly: a key to successful aging. Discovery medicine, 13(70), 223–228.

Gavin, T. P., Robinson, C. B., Yeager, R. C., England, J. A., Nifong, L. W., & Hickner, R. C. (2004). Angiogenic growth factor response to acute systemic exercise in human skeletal muscle. *Journal of Applied Physiology*, *96*(1), 19–24. https://doi.org/10.1152/japplphysiol.00748.2003

Godo, S., & Shimokawa, H. (2017). Endothelial Functions. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *37*(9). https://doi.org/10.1161/atvbaha.117.309813

Graber, T. G., Maroto, R., Fry, C. S., Brightwell, C. R., & Rasmussen, B. B. (2020). Measuring Exercise Capacity and Physical Function in Adult and Older Mice. [published online ahead of print, 2020 Aug 21]. *The Journals of Gerontology. Series A, Biological Sciences and Medical Sciences*, 2021 April 30; *76*(5), 819–824. https://doi.org/10.1093/gerona/glaa205

Gustafsson, T., Knutsson, A., Puntschart, A., Kaijser, L., Nordqvist, A. C., Sundberg, C. J., & Jansson, E. (2002). Increased expression of vascular endothelial growth factor in human skeletal muscle in response to short-term one-legged exercise training. *Pflugers Archiv : European journal of physiology*, *444*(6), 752–759. https://doi.org/10.1007/s00424-002-0845-6

Gustafsson, T., Rundqvist, H., Norrbom, J., Rullman, E., Jansson, E., & Sundberg, C. J. (2007). The influence of physical training on the angiopoietin and VEGF-A systems in human skeletal muscle. *Journal of Applied Physiology*, *103*(3), 1012–1020. https://doi.org/10.1152/japplphysiol.01103.2006

Hassan, E. B., Phu, S., Warburton, E., Humaith, N., & Wijeratne, T. (2021). Frailty in Stroke—A Narrated Review. *Life*, *11*(9), 891. https://doi.org/10.3390/life11090891

Heffernan, K. S., Fahs, C. A., Ranadive, S. M., & Eshan Patvardhan. (2010). *Review Article: L-Arginine as a Nutritional Prophylaxis Against Vascular Endothelial Dysfunction With Aging*. *15*(1), 17–23. https://doi.org/10.1177/1074248409354599

Heiss, C., Spyridopoulos, I., & Haendeler, J. (2018). Interventions to slow cardiovascular aging: Dietary restriction, drugs and novel molecules. *Experimental Gerontology*, *109*, 108–118. https://doi.org/10.1016/j.exger.2017.06.015

Herrera, M. D., Mingorance, C., Rodríguez-Rodríguez, R., & Alvarez de Sotomayor, M. (2010). Endothelial dysfunction and aging: An update. *Ageing Research Reviews*, *9*(2), 142–152. https://doi.org/10.1016/j.arr.2009.07.002

Hoier, B., & Hellsten, Y. (2014). Exercise-Induced Capillary Growth in Human Skeletal Muscle and the Dynamics of VEGF. *Microcirculation*, *21*(4), 301–314. https://doi.org/10.1111/micc.12117

Hoier, B., Nordsborg, N., Andersen, S., Jensen, L., Nybo, L., Bangsbo, J., & Hellsten, Y. (2012). Pro- and anti-angiogenic factors in human skeletal muscle in response to acute exercise and training. *The Journal of Physiology*, *590*(3), 595–606. https://doi.org/10.1113/jphysiol.2011.216135

Hoier, B., Prats, C., Qvortrup, K., Pilegaard, H., Bangsbo, J., & Hellsten, Y. (2013). Subcellular localization and mechanism of secretion of vascular endothelial growth factor in human skeletal muscle. *The FASEB Journal*, *27*(9), 3496–3504. https://doi.org/10.1096/fj.12-224618

Jensen, L., Bangsbo, J., & Hellsten, Y. (2004). Effect of high intensity training on capillarization and presence of angiogenic factors in human skeletal muscle. *The Journal of Physiology*, *557*(2), 571–582. https://doi.org/10.1113/jphysiol.2003.057711

Keteyian, S. J., Hibner, B. A., Bronsteen, K., Kerrigan, D., Aldred, H. A., Reasons, L. M., Saval, M. A., Brawner, C. A., Schairer, J. R., Thompson, T. M. S., Hill, J., McCulloch, D., & Ehrman, J. K. (2014). Greater Improvement in Cardiorespiratory Fitness Using Higher-Intensity Interval Training in the Standard Cardiac Rehabilitation Setting. *Journal of Cardiopulmonary Rehabilitation and Prevention*, *34*(2), 98–105. https://doi.org/10.1097/hcr.0000000000000049

Larsson, L., Degens, H., Li, M., Salviati, L., Lee, Y. il, Thompson, W., Kirkland, J. L., & Sandri, M. (2019). Sarcopenia: Aging-Related Loss of Muscle Mass and Function. *Physiological Reviews*, *99*(1), 427–511. https://doi.org/10.1152/physrev.00061.2017

Maessen, M. F. H., Verbeek, A. L. M., Bakker, E. A., Thompson, P. D., Hopman, M. T. E., & Eijsvogels, T. M. H. (2016). Lifelong Exercise Patterns and Cardiovascular Health. *Mayo Clinic Proceedings*, *91*(6), 745–754. https://doi.org/10.1016/j.mayocp.2016.02.028

Melincovici, C. S., Boşca, A. B., Şuşman, S., Mărginean, M., Mihu, C., Istrate, M., Moldovan, I. M., Roman, A. L., & Mihu, C. M. (2018). Vascular endothelial growth factor (VEGF) - key factor in normal and pathological angiogenesis. *Romanian journal of morphology and embryology = Revue roumaine de morphologie et embryologie*, *59*(2), 455–467.

Miljkovic, N., Lim, J.-Y., Miljkovic, I., & Frontera, W. R. (2015). Aging of Skeletal Muscle Fibers. *Annals of Rehabilitation Medicine*, *39*(2), 155. https://doi.org/10.5535/arm.2015.39.2.155

Milkiewicz, M., Hudlicka, O., Brown, M., & Silgram, H. (2005). *Nitric oxide, VEGF, and VEGFR-2: interactions in activity-induced angiogenesis in rat skeletal muscle*. *289*(1), H336–H343. https://doi.org/10.1152/ajpheart.01105.2004

Moro, T., Brightwell, C. R., Phalen, D. E., McKenna, C. F., Lane, S. J., Porter, C., Volpi, E., Rasmussen, B. B., & Fry, C. S. (2019). Low skeletal muscle capillarization limits muscle adaptation to resistance exercise training in older adults. *Experimental Gerontology*, *127*, 110723. https://doi.org/10.1016/j.exger.2019.110723

Nystoriak, M. A., & Bhatnagar, A. (2018). Cardiovascular Effects and Benefits of Exercise. *Frontiers in Cardiovascular Medicine*, *5*(135). https://doi.org/10.3389/fcvm.2018.00135

Obas, V., & Vasan, Ramachandran S. (2018). The aging heart. *Clinical Science*, *132*(13), 1367–1382. https://doi.org/10.1042/cs20171156

Olenich, S. A., Gutierrez-Reed, N., Audet, G. N., & Olfert, I. M. (2013). Temporal response of positive and negative regulators in response to acute and chronic exercise training in mice. *The Journal of Physiology*, *591*(Pt 20), 5157–5169. https://doi.org/10.1113/jphysiol.2013.254979

Olfert, I. M., Howlett, R. A., Tang, K., Dalton, N. D., Gu, Y., Peterson, K. L., Wagner, P. D., & Breen, E. C. (2009). Muscle-specific VEGF deficiency greatly reduces exercise endurance in mice. *The Journal of Physiology*, *587*(8), 1755–1767. https://doi.org/10.1113/jphysiol.2008.164384

Pajski Ml, Byrd C, Nandigama N, Seguin, E., Seguin, A., Fennell, A., & Graber, T. G. (2021). Endurance Exercise to Improve Physical Function in Adult and Older Mice: High Intensity Interval Training (HIIT) versus Voluntary Wheel Running (VWR). Preprint at https://doi.org/10.1101/2021.05.20.445050

Park, J., Nakamura, Y., Kwon, Y., Park, H., Kim, E., & Park, S. (2010). The Effect of Combined Exercise Training on Carotid Artery Structure and Function, and Vascular Endothelial Growth Factor (VEGF) in Obese Older Women. *Japanese Journal of Physical Fitness and Sports Medicine*, *59*(5), 495–504. https://doi.org/10.7600/jspfsm.59.495

Patterson, R., McNamara, E., Tainio, M., de Sá, T. H., Smith, A. D., Sharp, S. J., Edwards, P., Woodcock, J., Brage, S., & Wijndaele, K. (2018). Sedentary behavior and risk of all-cause, cardiovascular and cancer mortality, and incident type 2 diabetes: a systematic review and dose response meta-analysis. *European Journal of Epidemiology*, *33*(9), 811–829. https://doi.org/10.1007/s10654-018-0380-1

Rajendran, P., Rengarajan, T., Thangavel, J., Nishigaki, Y., Sakthisekaran, D., Sethi, G., & Nishigaki, I. (2013). The Vascular Endothelium and Human Diseases. *International Journal of Biological Sciences*, *9*(10), 1057–1069. https://doi.org/10.7150/ijbs.7502

Rojas Vega, S., Knicker, A., Hollmann, W., Bloch, W., & Strüder, H. K. (2010). Effect of Resistance Exercise on Serum Levels of Growth Factors in Humans. *Hormone and Metabolic Research*, *42*(13), 982–986. https://doi.org/10.1055/s-0030-1267950

Rullman, E., Rundqvist, H., Wågsäter, D., Fischer, H., Eriksson, P., Sundberg, C. J., Jansson, E., & Gustafsson, T. (2007). A single bout of exercise activates matrix metalloproteinase in human skeletal muscle. *Journal of Applied Physiology*, *102*(6), 2346–2351. https://doi.org/10.1152/japplphysiol.00822.2006

Ryan, N. A., Zwetsloot, K. A., Westerkamp, L. M., Hickner, R. C., Pofahl, W. E., & Gavin, T. P. (2006). Lower skeletal muscle capillarization and VEGF expression in aged vs. young men. *Journal of Applied Physiology (Bethesda, Md.: 1985)*, *100*(1), 178–185. https://doi.org/10.1152/japplphysiol.00827.2005

Sandri, M., Adams, V., Gielen, S., Linke, A., Lenk, K., Kränkel, N., Lenz, D., Erbs, S., Scheinert, D., Mohr, F.-W., Schuler, G., & Hambrecht, R. (2005). Effects of Exercise and Ischemia on Mobilization and Functional Activation of Blood-Derived Progenitor Cells

in Patients With Ischemic Syndromes. *Circulation*, *111*(25), 3391–3399. https://doi.org/10.1161/circulationaha.104.527135

Sandri, M., Beck, E. B., Adams, V., Gielen, S., Lenk, K., Höllriegel, R., Mangner, N., Linke, A., Erbs, S., Möbius-Winkler, S., Scheinert, D., Hambrecht, R., & Schuler, G. (2010). Maximal exercise, limb ischemia, and endothelial progenitor cells. *European Journal of Cardiovascular Prevention & Rehabilitation*, *18*(1), 55–64. https://doi.org/10.1097/hjr.0b013e32833ba654

Seals, Douglas R., Jablonski, Kristen L., & Donato, Anthony J. (2011). Aging and vascular endothelial function in humans. *Clinical Science*, *120*(9), 357–375. https://doi.org/10.1042/cs20100476

Soysal, P., Arik, F., Smith, L., Jackson, S. E., & Isik, A. T. (2020). Inflammation, Frailty and Cardiovascular Disease. *Advances in Experimental Medicine and Biology*, 55–64. https://doi.org/10.1007/978-3-030-33330-0_7

Tesch, P. A., Thorsson, A., & Essén-Gustavsson, B. (1989). Enzyme activities of FT and ST muscle fibers in heavy-resistance trained athletes. *Journal of applied physiology (Bethesda, Md. : 1985)*, *67*(1), 83–87. https://doi.org/10.1152/jappl.1989.67.1.83

Torma, F., Gombos, Z., Jokai, M., Takeda, M., Mimura, T., & Radak, Z. (2019). High intensity interval training and molecular adaptive response of skeletal muscle. *Sports medicine and health science, 1(1)*, 24–32. https://doi.org/10.1016/j.smhs.2019.08.003

Tsao, C. W., Aday, A. W., Almarzooq, Z. I., Anderson, C. A. M., Arora, P., Avery, C. L., Baker-Smith, C. M., Beaton, A. Z., Boehme, A. K., Buxton, A. E., Commodore-Mensah, Y., Elkind, M. S. V., Evenson, K. R., Eze-Nliam, C., Fugar, S., Generoso, G., Heard, D. G., Hiremath, S., Ho, J. E., Kalani, R., … American Heart Association Council on Epidemiology and Prevention Statistics Committee and Stroke Statistics Subcommittee (2023). Heart Disease and Stroke Statistics-2023 Update: A Report From the American Heart Association. *Circulation*, *147*(8), e93–e621. https://doi.org/10.1161/CIR.0000000000001123

United Nations, Department of Economic and Social Affairs, Population Division (2015). World Population Ageing 2015 (ST/ESA/SER.A/390)

Verma, S., Buchanan, M. R., & Anderson, T. J. (2003). Endothelial Function Testing as a Biomarker of Vascular Disease. *Circulation*, *108*(17), 2054–2059. https://doi.org/10.1161/01.cir.0000089191.72957.ed

Weston, K. S., Wisløff, U., & Coombes, J. S. (2014). High-intensity interval training in patients with lifestyle-induced cardiometabolic disease: a systematic review and meta-analysis. *British journal of sports medicine*, *48*(16), 1227–1234. https://doi.org/10.1136/bjsports-2013-092576

Wisløff, U., Ellingsen, Ø., & Kemi, O. J. (2009). High-intensity interval training to maximize cardiac benefits of exercise training?. *Exercise and sport sciences reviews*, *37*(3), 139–146. https://doi.org/10.1097/JES.0b013e3181aa65fc

Xu, X., Wang, B., Ren, C., Hu, J., Greenberg, D. A., Chen, T., Xie, L., & Jin, K. (2017). Age-related Impairment of Vascular Structure and Functions. *Aging and Disease*, *8*(5), 590. https://doi.org/10.14336/ad.2017.0430

Zoladz, J. A., Semik, D., Zawadowska, B., Majerczak, J., Karasinski, J., Kolodziejski, L., Duda, K., & Kilarski, W. M. (2005). Capillary density and capillary-to-fibre ratio in vastus lateralis muscle of untrained and trained men. *Folia histochemica et cytobiologica*, *43*(1), 11–17.

Tables and Figures

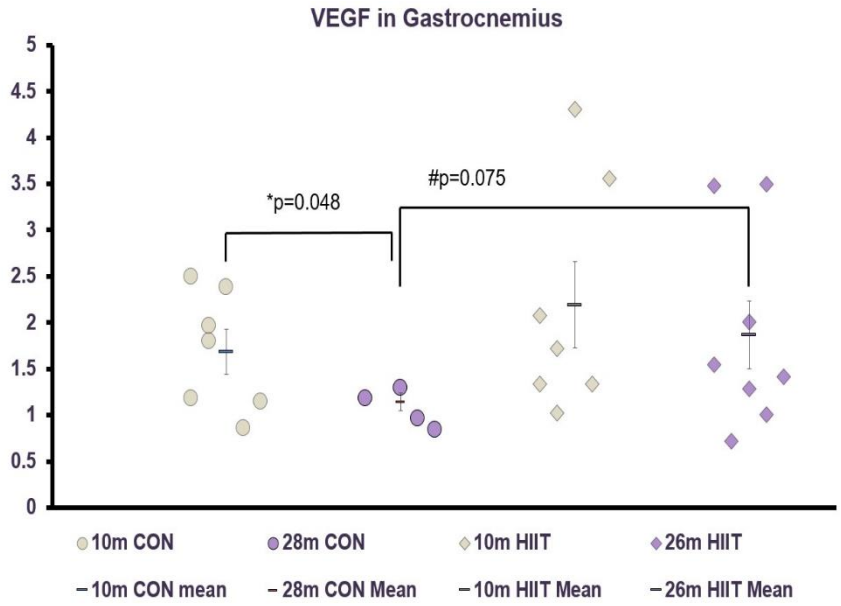


Figure 1: VEGF relative expression: age vs. HIIT. Key: circles = sedentary controls (CON), diamonds = high intensity interval training (HIIT). Grey = adults (10m, months) and purple = older adults (26m or 28m as listed). * = statistically significant by independent sample T-Test with $p<0.05$; # = trend, $0.05<p<0.10$.

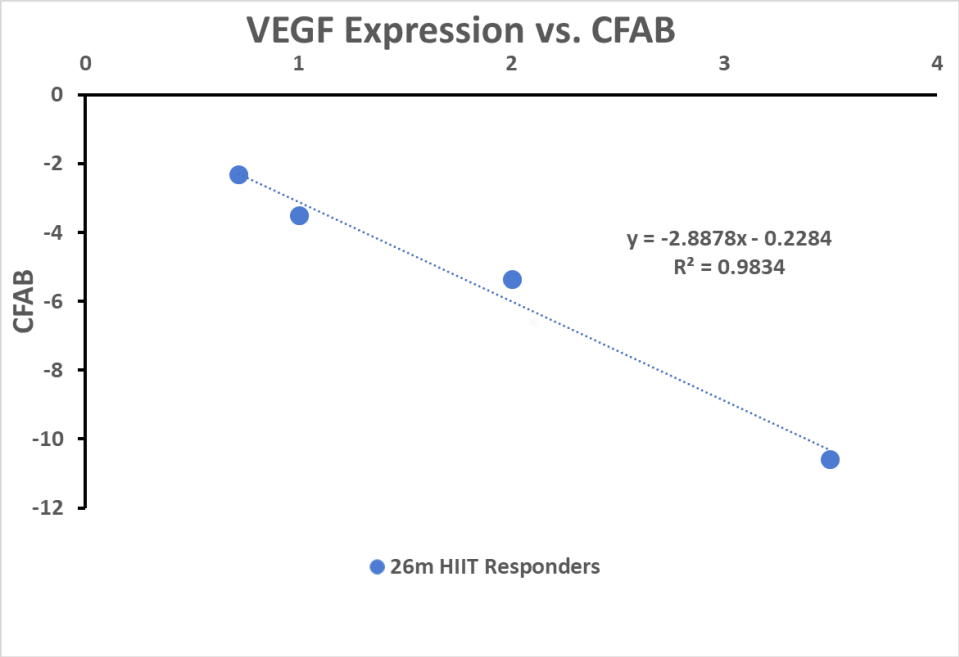


Figure 2: VEGF expression vs CFAB in 26m HIIT responders. Key: Circles = 26m high intensity interval training (HIIT).

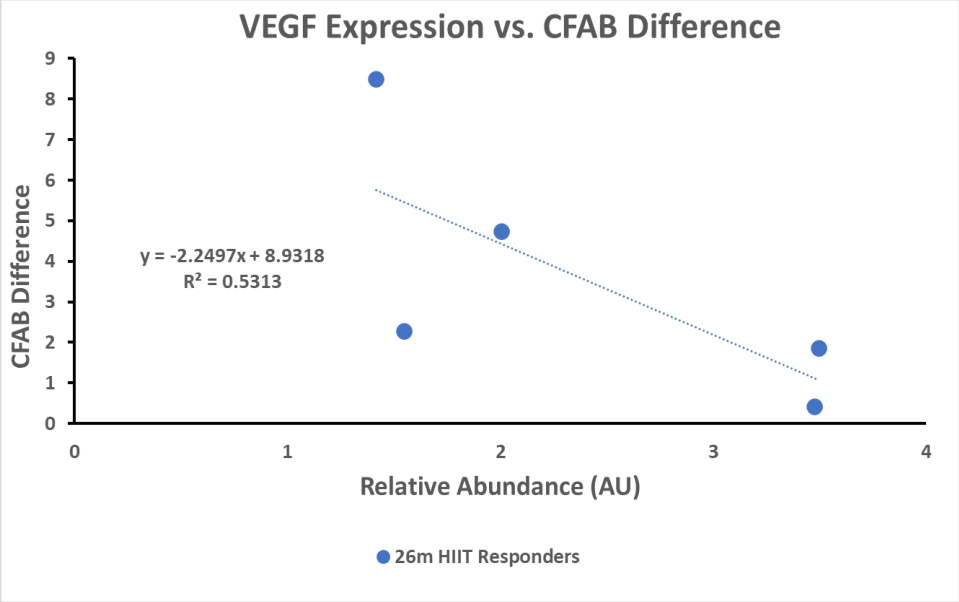


Figure 3: VEGF relative expression: age vs. CFAB difference in 26m HIIT mice. Key: Circles = high intensity interval training (HIIT). (AU) = arbitrary units.

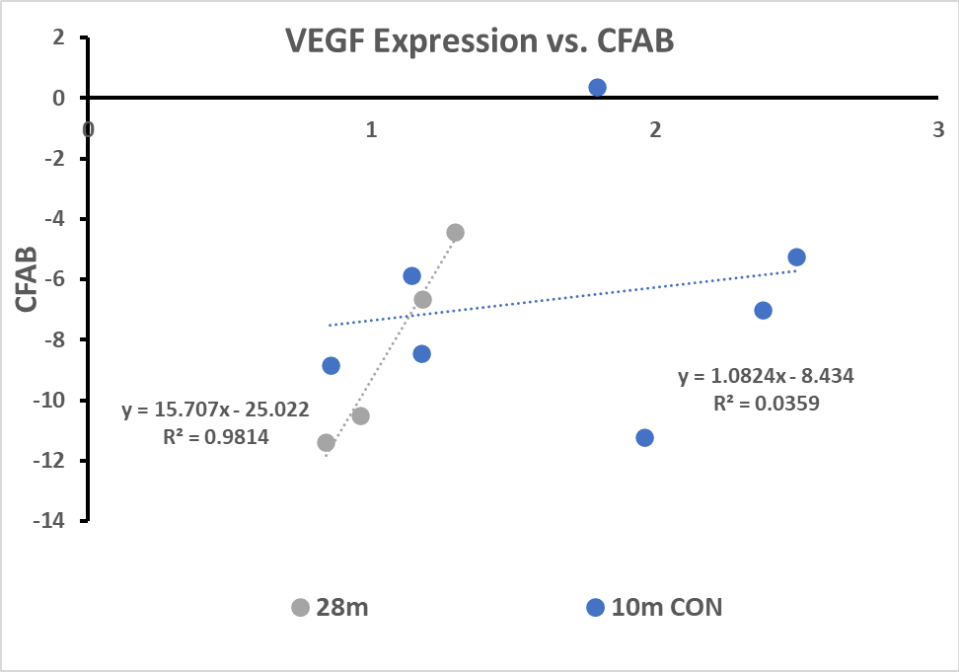


Figure 4: VEGF expression vs. CFAB in 10m CON and 28m CON mice. VEGF relative Expression: age vs. CFAB in 10m CON and 28m CON. Key: Blue circles = sedentary controls (CON), grey circles = high intensity interval training (HIIT).

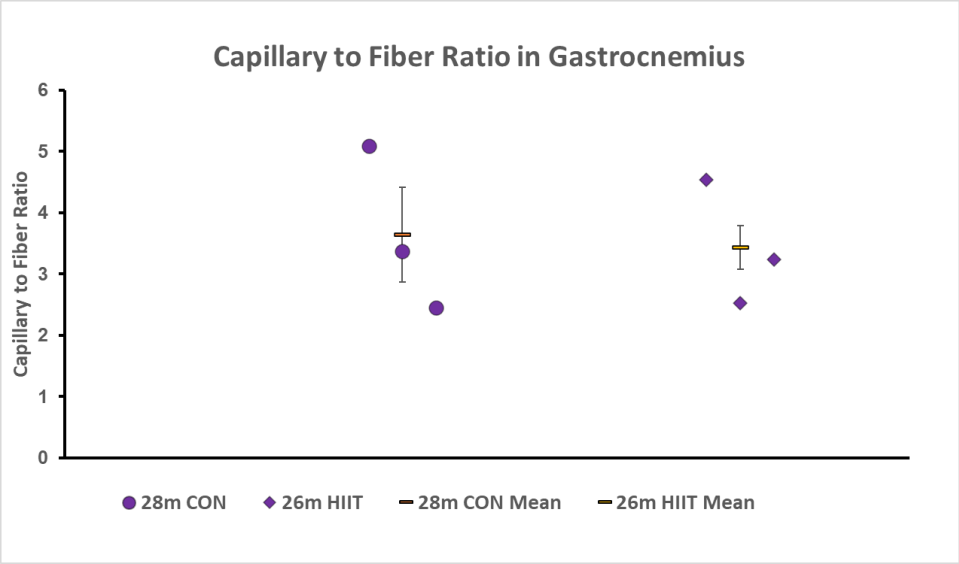


Figure 5: Capillary to fiber ratio in gastrocnemius. Key: circles = 28 month old sedentary controls (CON), diamonds = 26 month old mice who completed high intensity interval training (HIIT).

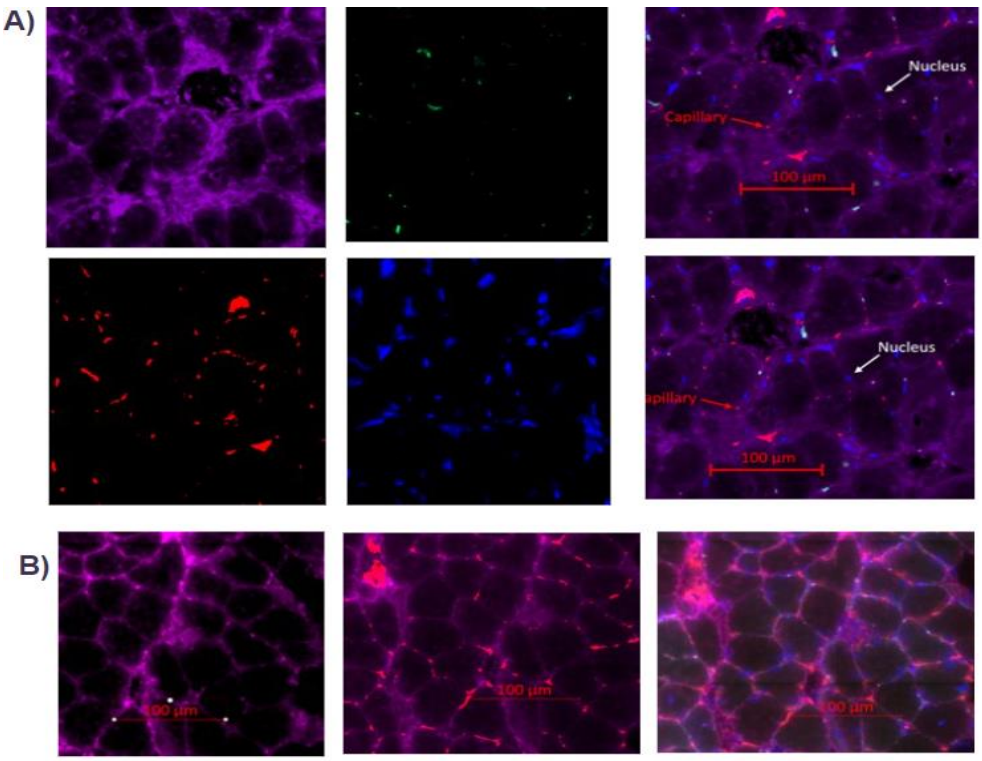
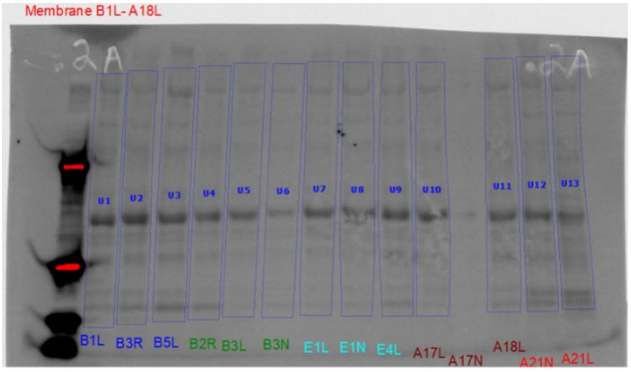


Figure 6: Representative immunohistochemistry images 26m HIIT and 28m CON. Representative images: A) 26m HIIT. Upper left = laminin (cell membrane). Upper middle = alpha-smooth muscle actin. Bottom left = CD-31 (endothelial marker). Bottom middle = DAPI (nuclei). Upper right combined laminin, CD31, and DAPI, Bottom right = all combined B) 28m CON. Left = laminin. Middle = laminin + CD-31. Right = All. KEY: red = CD31 (capillary/endothelial marker), blue = nuclei (DAPI counterstain), purple = laminin (sarcolemma outline), green = a-SMA (alpha-smooth muscle actin, arteriole marker). Orange = arteriole (overlap of a-SMA and CD-31). The number of capillaries per cell were measured as the outcome.

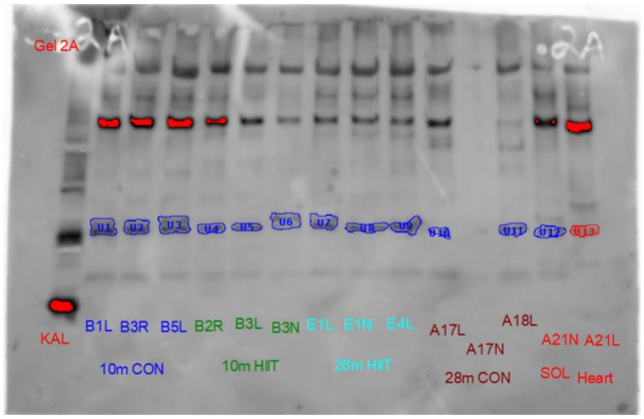
APPENDIX A: IACUC Certification

APPENDIX B: Western Blot

Blot 1 Total Protein



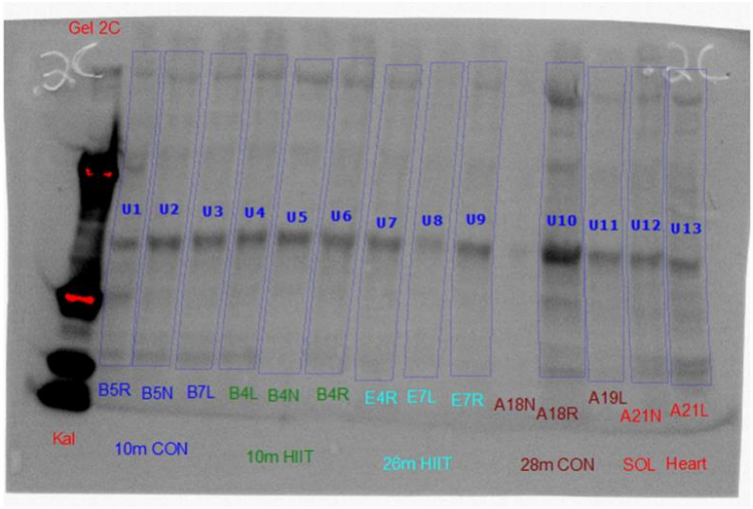
Blot 1 VEGF Expression



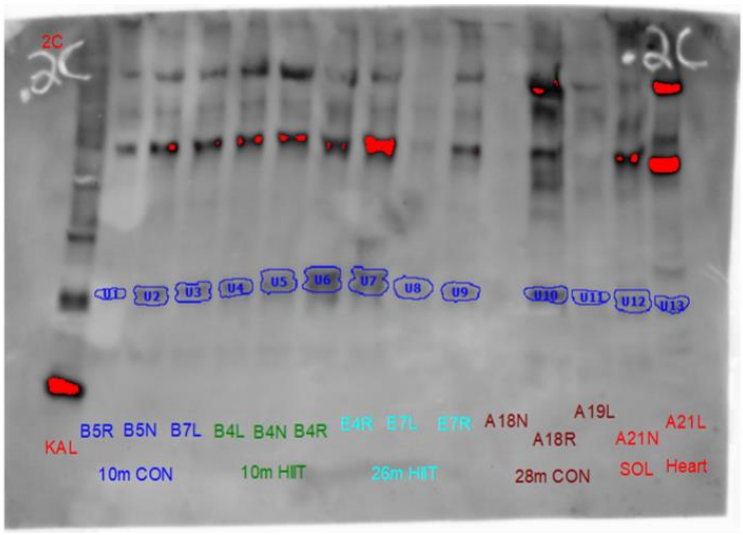
Blot 1 Data Analysis

Before			Trial											
Type	Label	1	2	3	SD	Mean	CV	STM	Name	Total Protein		Protein	adj_total protein	adj by blot control
40 kDa	U1	3,262,104.75	3,024,126.15	3,080,047.00	124,436.05	3,122,092.63	3.25427913	6m Heart	B1L	10,241,306.92	U1	0.304852951	0.304852951	2.498216037
40 kDa	U2	2,558,492.04	2,859,644.04	2,904,690.13	188,226.28	2,774,275.41	5.539684818	10m CON	B3R	12,653,670.38	U2	0.219246695	0.219246695	1.796687904
40 kDa	U3	4,049,915.37	3,815,798.84	3,678,161.36	187,951.97	3,847,958.53	3.988144452	10m CON	B5L	13,241,833.89	U3	0.290591058	0.290591058	2.381342353
40 kDa	U4	1,562,243.00	1,462,550.00	1,251,345.31	158,746.91	1,425,379.44	9.093460172	10m CON	B2R	8,718,040.22	U4	0.16349769	0.16349769	1.339834664
40 kDa	U5	1,295,036.50	1,366,603.17	1,442,650.45	73,818.31	1,368,096.71	4.405565574	10m HIIT	B3L	6,517,598.13	U5	0.209908111	0.209908111	1.720159859
40 kDa	U6	1,737,541.10	1,981,106.83	1,742,360.38	139,252.38	1,820,336.11	6.246049604	10m HIIT	B3N	3,466,988.90	U6	0.525048149	0.525048149	4.302676763
40 kDa	U7	2,166,414.78	2,333,336.86	2,052,496.02	141,251.57	2,184,082.55	5.280543437	10m HIIT	E1L	5,151,743.57	U7	0.423950168	0.423950168	3.474196683
40 kDa	U8	2,127,879.18	1,988,879.25	1,921,375.75	105,294.32	2,012,711.39	4.271474567	26m HIIT	E1N	1,580,704.86	U8	1.273299934	1.273299934	10.43446786
40 kDa	U9	2,367,613.72	2,345,492.27	2,223,259.21	77,748.00	2,312,121.73	2.745572335	26m HIIT	E4L	9,456,704.97	U9	0.244495492	0.244495492	2.003597333
40 kDa	U10	582,324.00	601,915.59	671,313.15	46,759.89	618,517.58	6.172708495	26m HIIT	A17L	6,594,688.96	U10	0.093790258	0.093790258	0.768594587
40 kDa	U11	1,763,552.29	1,705,183.88	1,592,374.17	87,019.97	1,687,036.78	4.211615982	28m CON	A18L	11,724,660.67	U11	0.143887898	0.143887898	1.179135889
40 kDa	U12	1,272,371.73	1,330,303.84	1,179,416.57	76,118.07	1,260,697.38	4.92982234	28m CON	A21N SOL	10,331,192.17	U12	0.122028258	0.122028258	1
40 kDa	U13	1,072,924.40	1,196,480.69	1,003,987.47	97,529.57	1,091,130.85	7.298167472	28m CON	A21L Heart	12,344,908.03	U13	0.088387119	0.088387119	0.724316813

Blot 2 Total Protein



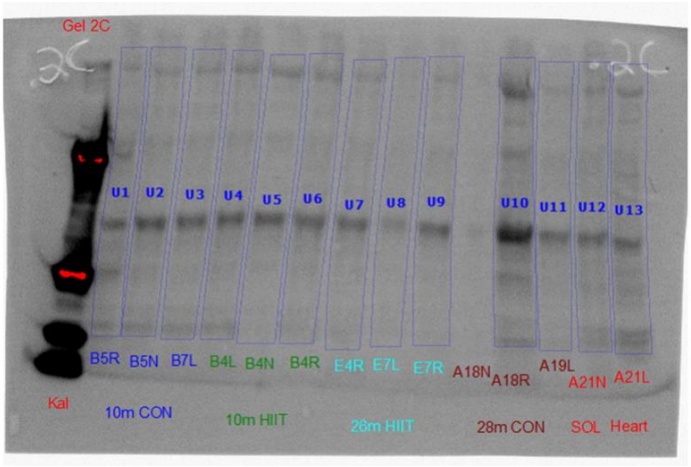
Blot 2 VEGF Expression



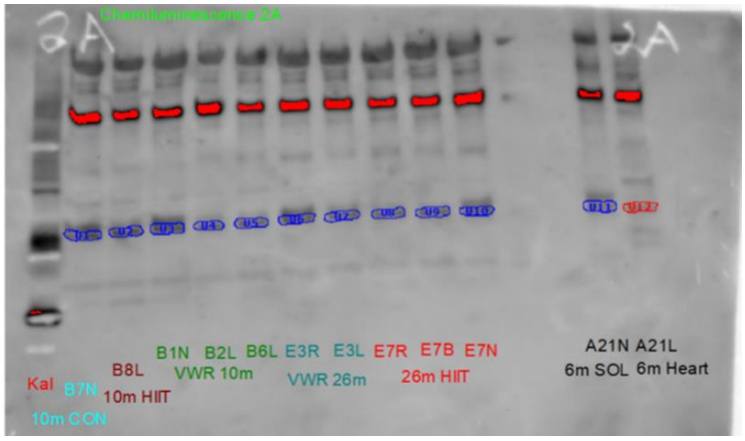
Blot 2 Data Analysis

Before			Trial										
Type	Label	1	2	3	SD	Mean	CV	STM	Name	Total Protein	Name	adj by total protein	adj by blot control
40 kDa	U1	386,774.21	313,055.20	384,770.85	41,995.32	361,533.42	9.484332628	10m CON	B5R	3,563,039.09	U1	0.10147	0.800920959
40 kDa	U2	1,128,491.48	1,280,918.75	1,773,702.82	337,235.79	1,394,371.02	19.7473893	10m CON	B5N	8,334,520.20	U2	0.16730	1.320564258
40 kDa	U3	1,315,920.56	1,313,506.37	1,616,482.43	174,230.57	1,415,303.12	10.05146292	10m CON	B7L	8,819,535.14	U3	0.16047	1.266676066
40 kDa	U4	1,405,013.91	1,569,606.44	1,572,936.76	96,003.37	1,515,852.37	5.171111844	10m HIIT	B4L	8,974,444.89	U4	0.16891	1.333248512
40 kDa	U5	1,564,572.59	2,214,275.99	2,072,215.91	341,564.37	1,950,354.83	14.2992514	10m HIIT	B4N	8,140,515.91	U5	0.23959	1.891139196
40 kDa	U6	3,909,641.10	3,868,251.32	4,682,644.85	458,709.19	4,153,512.43	9.017295446	10m HIIT	B4R	8,633,865.90	U6	0.48107	3.797274654
40 kDa	U7	3,396,295.28	3,412,222.91	3,575,276.03	99,057.32	3,461,264.74	2.336717106	26m HIIT	E4R	7,818,942.63	U7	0.44268	3.494206659
40 kDa	U8	820,420.11	712,394.67	755,930.36	54,350.37	762,915.05	5.816754294	26m HIIT	E7L	4,255,629.36	U8	0.17927	1.415057798
40 kDa	U9	1,452,620.66	1,219,189.22	1,519,335.38	157,601.19	1,397,048.42	9.210907148	26m HIIT	E7R	7,583,248.69	U9	0.18423	1.454179292
40 kDa	U10	3,777,667.59	4,122,783.02	3,030,311.46	558,441.28	3,643,587.36	12.51418866	28m CON	A18R	24,096,792.53	U10	0.15121	1.193525614
40 kDa	U11	763,455.15	816,077.65	699,952.65	58,147.38	759,828.48	6.248402253	28m CON	A19L	6,252,138.88	U11	0.12153	0.959287418
40 kDa	U12	1,139,373.00	1,137,661.33	1,230,057.89	52,857.99	1,169,030.74	3.691808001	6m SOL	A21N SOL	9,227,578.06	U12	0.12669	1
40 kDa	U13	1,067,956.54	1,250,771.00	1,023,744.55	120,358.38	1,114,157.36	8.820316455	6m Heart	A21L Heart	11,588,869.16	U13	0.09614	0.7588698

Blot 3 Total Protein



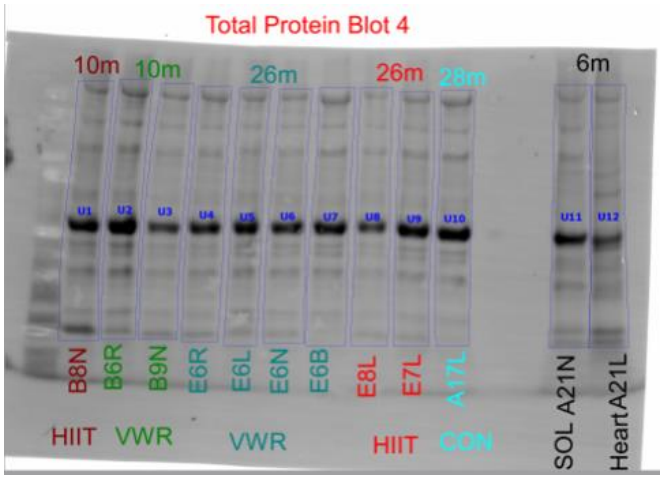
Blot 3 VEGF Expression



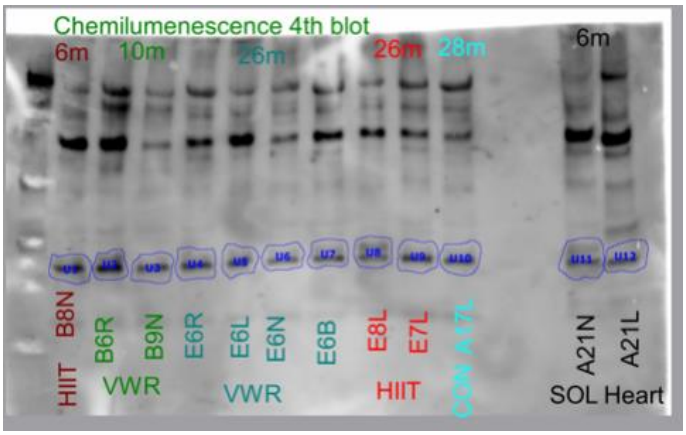
Blot 3 Data Analysis

Before			Trial											
Type	Label	1	2	3	SD	Mean	CV	STM	Name	Total Protein		adj total protein	adj by total protein	adj by blot control
40 kDa	U1	1,093,627.00	1,179,953.41	1,166,148.45	46,372.02	1,146,576.29	3.302231099	10m CON	B7N	5,836,691.89	U1	0.196442833	0.19644	1.877021006
40 kDa	U2	1,611,148.61	1,436,906.99	1,607,464.87	99,552.08	1,551,840.15	5.23790639	10m HIIT	B8L	8,945,744.44	U2	0.173472444	0.17347	1.657537805
40 kDa	U3	1,381,444.89	1,350,648.67	1,357,272.35	16,210.04	1,363,121.97	0.970965459	VWR 10m	B1N	9,412,623.14	U3	0.144818501	0.14482	1.383747953
40 kDa	U4	992,012.00	1,060,469.12	1,012,812.26	35,095.59	1,021,764.46	2.804504786	VWR 10m	B2L	7,775,332.78	U4	0.131411026	0.13141	1.255638873
40 kDa	U5	1,404,499.31	1,388,238.00	1,398,636.80	8,235.43	1,397,124.70	0.481288649	VWR 10m	B6L	9,078,270.50	U5	0.153897673	0.15390	1.470499893
40 kDa	U6	1,111,285.00	1,290,580.95	1,259,027.30	95,716.99	1,220,297.75	6.404387602	VWR 26m	E3R	8,752,844.10	U6	0.139417284	0.13942	1.332139059
40 kDa	U7	1,099,771.67	1,141,339.43	1,126,818.39	21,096.07	1,122,643.16	1.534313856	VWR 26m	E3L	8,310,159.75	U7	0.13509285	0.13509	1.290818879
40 kDa	U8	1,285,908.78	1,203,035.70	1,142,218.29	72,126.81	1,210,387.59	4.865490551	26m HIIT	E7R	8,957,278.89	U8	0.135128939	0.13513	1.291163708
40 kDa	U9	1,382,835.42	1,383,254.69	1,385,518.00	1,443.06	1,383,869.37	0.085142052	26m HIIT	E7B	9,201,777.46	U9	0.150391528	0.15039	1.436998506
40 kDa	U10	1,106,913.31	1,069,291.05	1,105,604.00	21,353.30	1,093,936.12	1.593776205	26m HIIT	E7N	8,964,426.45	U10	0.122030799	0.12203	1.166010331
40 kDa	U11	1,058,866.87	1,023,801.27	1,029,149.82	18,891.38	1,037,272.65	1.487048752	6m SOL	A21N Sol	9,911,191.60	U11	0.104656704	0.10466	1
40 kDa	U12	958,821.33	921,323.70	937,523.09	18,806.50	939,222.71	1.634910049	6m Heart	A21L Heart	11,928,387.88	U12	0.078738445	0.07874	0.752349743

Blot 4 Total Protein



Blot 4 VEGF Expression



Blot 4 Data Analysis

	Label	1	2	3	SD	Mean	CV	STM	Name	Total Protein	adj by tota	adj by blot control	
40 kDa	U1	23,447,299.84	22,538,047.79	24,665,142.47	1,067,271.58	23,550,163.37	3.700287	10m HIIT	B8N	506,741,770.52	0.04647	0.7687804	
40 kDa	U2		29,592,151.76	31,530,928.72	1,370,922.34	30,561,540.24	3.171923	VWR 10m	B6R	597,239,041.67	0.05117	0.8464906	
40 kDa	U3	18,596,389.88	18,886,563.32	18,264,305.30	311,364.16	18,582,419.50	1.368109	VWR 10m	B9N	288,210,802.03	0.06448	1.0665644	
40 kDa	U4	23,617,054.44	25,214,523.97	23,124,374.22	1,092,654.92	23,985,317.54	3.719563	VWR 26m	E6R	368,050,109.56	0.06517	1.0780368	
40 kDa	U5	19,725,742.46	19,456,429.27	20,207,802.19	380,673.18	19,796,657.97	1.570055	VWR 26m	E6L	368,011,958.07	0.05379	0.8898668	
40 kDa	U6	16,129,013.56	15,638,386.74	16,364,136.78	370,295.09	16,043,845.69	1.88449	VWR 26m	E6N	352,381,086.54	0.04553	0.7531664	
40 kDa	U7	20,602,911.97	20,983,051.53	22,550,314.28	1,032,246.29	21,378,759.26	3.94235	26m HIIT	E6B	430,155,695.35	0.04970	0.8221516	
40 kDa	U8	21,762,439.82	23,910,768.68	23,863,438.96	1,226,903.58	23,178,882.49	4.321876	26m HIIT	E8L	258,469,139.18	0.08968	1.4834703	
40 kDa	U9	22,704,577.99	21,411,988.92	22,476,517.23	689,929.80	22,197,694.71	2.537765	28m CON	E7L	419,549,749.96	0.05291	0.8752245	
40 kDa	U10	21,319,171.78	21,999,941.00	20,825,993.85	589,466.30	21,381,702.21	2.250977	28m CON	A17L	414,226,600.54	0.05162	0.853885	
40 kDa	U11	27,007,262.81		26,359,564.45	457,991.90	26,683,413.63	1.213672	6m SOL	A21N Sol	441,404,198.30	0.06045	1	
40 kDa	U12	15,508,901.62		16,446,041.00	662,657.61	15,977,471.31	2.93269	6m Heart	A21L Heart	518,035,108.47	0.03084	0.5102041	

APPENDIX C: Immunohistochemistry

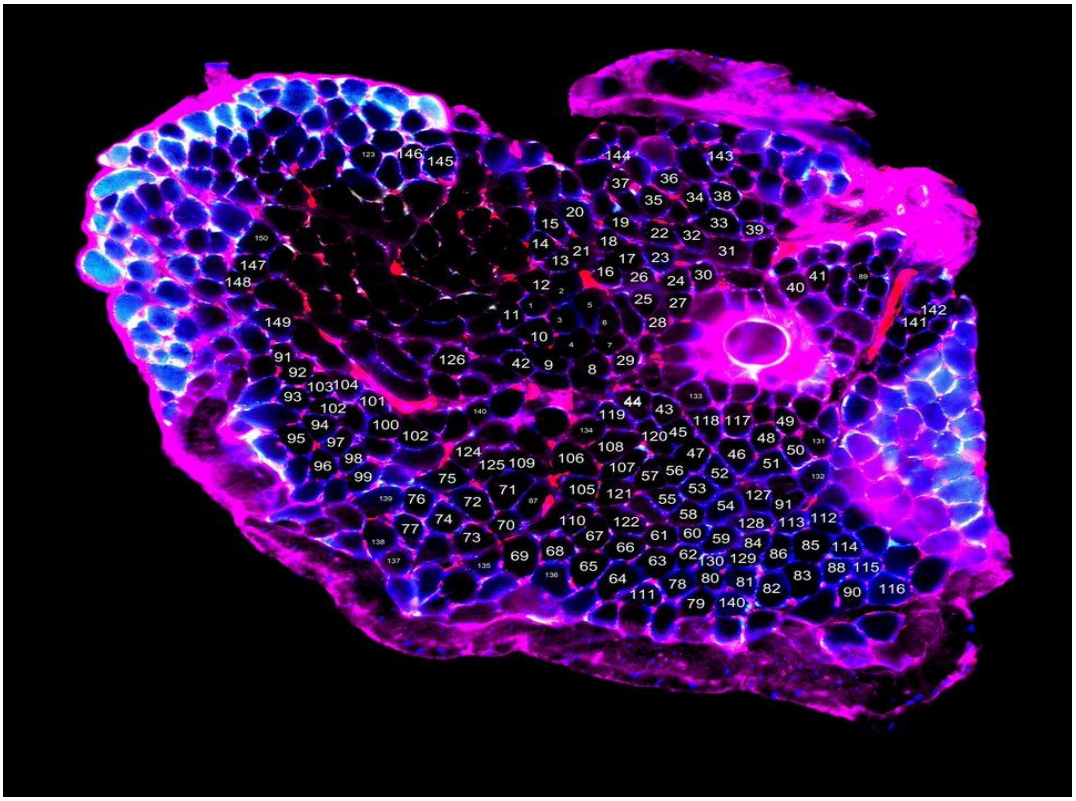


Figure 7: Representative immunohistochemistry image. Representative of a gastrocnemius muscle slice image used to measure the number of capillaries per cell. KEY: red = CD31 (capillary/endothelial marker), blue = nuclei (DAPI counterstain), purple = laminin (sarcolemma outline). The number of capillaries per cell was measured as outcomes.

APPENDIX D: IACUC Approval Memo

ECU
Animal Care and Use Committee
003 Ed Warren Life Sciences Building | East Carolina University | Greenville NC 27834 – 4354
252-744-2436 office | 252-744-2355 fax

July 27, 2022

Ted Graber, Ph.D.
Department of Physical Therapy, ECU

Dear Dr. Graber:

Your Animal Use Protocol entitled "Mechanisms of Age-Related Functional Decline and Loss of Exercise Capacity" (AUP#P106a) was reviewed by this institution's Animal Care and Use Committee on 07/26/2022. The following action was taken by the Committee:

"Approved as submitted"

****Please contact Aaron Hinkle prior to any hazard use****

A copy of the protocols is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP/Amendment and are familiar with its contents.**

Sincerely yours,

Jamie DeWitt, Ph.D.
Vice-Chair Animal Care and Use Committee

JD/GD

enclosure