ASSOCIATION OF *IN VIVO*-SKELETAL MUSCLE OXIDATIVE CAPACITY AND CARDIOMETABOLIC HEALTH IN AN OVERWEIGHT POPULATION

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ABSTRACT

Background: Cardiometabolic health factors are becoming increasingly front and center in prevalence and cost of treatment in the U.S.. Incidence rates and complications from diseases related to these factors including obesity, heart disease, type 2 diabetes and certain types of cancers have not only cost over $1 trillion annually but are increasing mortality rates across the country. Skeletal muscle mitochondrial function has been connected to these factors, and research has shown that it can be used as an *in vivo* assessment of cardiometabolic health. Near Infrared Spectroscopy has recently seen an increase in interest for being used as a non-invasive method of assessing *in vivo*-mitochondrial function providing a simpler, cheaper and accurate way to determine cardiometabolic health both for practitioners and researchers. Studies have shown that NIRS assessments are similar in accuracy to other more expensive standards of assessment, such as ^{31}P-MRS. Thus, we aim to examine the relationship between factors of cardiometabolic health and assessment of mitochondrial function using NIRS. **Purpose:** To examine how indices of cardiometabolic health, and VO2max associate with NIRS assessment of

mitochondrial function. **Methods:** Overweight subjects (n=17) with a body mass index (BMI) of 25.0-29.9 kg/m2 were screened for varying factors of cardiometabolic health. Body composition was measured by a DEXA scan, 3-D body scan, and participants also underwent a maximal exercise test on a cycle ergometer and measures of resting metabolic rate. For a measure of mitochondrial function, subjects performed a NIRS assessment before and after three days of a high-fat diet. **Results:** Measures of cardiometabolic health were not statistically significant with mitochondrial function assessment with NIRS pre- or post- three day high-fat diet. **Conclusion:** The results from the current study show that mitochondrial function as assessed by NIRS has no statistically significant relationships with factors of cardiometabolic health or following an acute high-fat diet.

Association of Skeletal Muscle Oxidative Capacity and Cardiometabolic Health in an Overweight Population

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Chapter 1: Introduction

Introduction

Chronic diseases in the U.S. are on the rise, with obesity and type 2 diabetes (T2D) increasing each year. In 1997, almost 1 in 5 adults in the U.S. were classified as obese, and the World Health Organization declared obesity a global endemic (WHO, 2000). Since then, according to the CDC, obesity rates have increased to 42.4% in adults and 19.3% in children. Obesity has been linked to an increased incidence of T2D (Eckel et al., 2011), known cardiometabolic risk factors, and components of metabolic syndrome. More than 34 million Americans have diabetes with 90-95% of cases classified as T2D (CDC, 2020). As of 2018, 88 million adults are estimated to have prediabetes, which is when blood glucose levels are elevated above normal ranges. This condition has its own risks of developing T2D, increasing the risk of premature morbidity, greatly reduced quality of life due to kidney, eye, and nerve damage, and cardiovascular disease (Deshpande et al., 2008).

Whereas type 1 diabetes is when the pancreas has an impairment in the creation of insulin resulting in dysregulation of blood glucose levels, T2D is categorized as the loss of the ability to properly use insulin to regulate blood glucose levels. As the body requires more insulin to control blood glucose, it will become insulin resistant, and the body will not be able to move glucose out of the bloodstream and into the cells for use. This results in a condition called hyperglycemia, a medical condition characterized by abnormally high levels of glucose, which has been shown to impair glucose and fatty acid oxidation in cultured human myotubes (Lund et al., 2019). T2D has notably been shown to affect skeletal muscle, which acts as a reservoir for approximately 80% of insulin-mediated glucose disposal (Kelley & Mandarino, 2000; Fujimaki & Kuwabara, 2017). It

has been demonstrated that T2D leads to atrophy, a shift in fiber-type transition from oxidative to glycolytic, and impaired energy metabolism in skeletal muscle (Fujimaki & Kuwabara, 2017). These alterations give rise to skeletal muscle dysfunction, manifesting as muscular weakness and reduced capacity for exercise (Regensteiner et al., 1995). Furthermore, studies have revealed that individuals with T2D exhibit persistently elevated blood lactate levels, both at rest and during physical activity. (Jones et al. 2019; Huebschmann et al., 2015). Chronic elevated blood lactate levels in rats have been shown to decrease fatty acid transport and mitochondrial oxygen consumption, which could have implications for both metabolic health and flexibility. Because of this, a key marker for classifying early risk for metabolic disease, which drastically increases the risk of developing T2D, could be fasting plasma glucose levels. Genetic and environmental factors such as age, obesity, and physical inactivity are closely related to the onset of T2D, but their exact role is not yet fully understood. T2D is also closely linked to mitochondrial dysfunction; however, further research is needed to determine the exact relationship between these two conditions (Golvic et al., 2017).

Type 2 diabetes is associated with impaired metabolic flexibility, an impaired ability to switch from fatty acid to glucose oxidation in response to insulin in skeletal muscle. In the skeletal muscle energy production involves the tricarboxylic acid (TCA) cycle, also called the Krebs cycle, and the Cori Cycle. When the supply of oxygen to skeletal muscle is sufficient, the TCA cycle begins when glucose is converted to pyruvate and transported into the mitochondria as acetyl-CoA. This multistep process ends with the regeneration of oxaloacetate and the formation of two molecules of carbon dioxide. It is possible that problems may arise in the TCA cycle, which may be a marker for impairment in skeletal muscle metabolism. When the supply of energy from the TCA cycle to the skeletal muscle is insufficient, the Cori Cycle upregulates to

meet demand. The Cori Cycle involves conversion of lactate to pyruvate for gluconeogenesis in the liver. The created glucose is released into the blood for uptake by peripheral tissues in need of fuel. When energy demand has returned to normal, the pyruvate created by glycolysis will enter the TCA cycle, if energy supply remains low the Cori cycle will repeat.

Near Infrared Spectroscopy (NIRS) has recently been utilized more to examine the effects of metabolic diseases, such as obesity and T2D, on mitochondrial capacity in skeletal muscle (Ryan et al., 2013; Ryan et al., 2014). NIRS is a powerful noninvasive tool for studying the ratio of oxygen delivery to oxygen utilization using the concentration of oxygenation in heme compounds in skeletal muscle, specifically hemoglobin and myoglobin. Determining oxygenation typically involves expensive (e.g., MRI) or invasive techniques (e.g., catheters); however, NIRS offers a portable budget option for clinical, medical, and research purposes. NIRS has been shown to be a valid and repeatable method for measuring mitochondrial capacity (Sumner et al., 2020) and has been used to compare fasting lactate to the rate of deoxygenation of hemo/myoglobin (Broskey et al., 2020).

This study aimed to examine the association between the rate of deoxygenation in skeletal muscle using NIRS pre and post a 3-day high-fat diet in an overweight population and the relationship of NIRS values with HOMA-IR, BMI, VO_2max and other indices of cardiometabolic health. I hypothesize that assessment of hemoglobin/myoglobin deoxygenation through NIRS will be inversely associated with fasting lactate both pre- and post-HFD, as well as associate with VO_2max and time until exhaustion.

Research question

How are rates of hemoglobin/myoglobin deoxygenation associated with cardiometabolic factors pre- and post-HFD?

Hypothesis

Rates of hemoglobin/myoglobin deoxygenation will be inversely associated with fasting lactate, VO2max and time to exhaustion, both pre and post HFD however lactate and deoxygenation rates will be higher post HFD.

Purpose

To examine the association between blood lactate levels and the rate of deoxygenation of skeletal muscle using NIRS pre and post a 3-day high-fat diet (HFD) in an overweight population. A secondary purpose was to examine how HOMA-IR, BMI, other indices of cardiometabolic health, and VO2max associate with NIRS.

Chapter 2: Literature Review

Obesity

With levels of obesity on the rise in the U.S. and passing a prevalence rate of over 41% (NHANES, 2021), it is important to understand how this disease impacts factors related to metabolic health. Obesity is associated with an increased risk of several cardiometabolic risk factors, such as dyslipidemia, hypertension, and fasting glucose. Obesity is also a significant risk factor in the development of T2D, with both elevated body mass index (BMI) and waist circumference (WC) strongly associated with T2D. It has been found that as BMI exceeds 23, the occurrence of T2D increases linearly with BMI (Harris et al., 1998). T2D is the most common metabolic disease worldwide, and although its primary cause is unknown, it has been shown that insulin resistance plays an early role in its development (Lowell & Shulman, 2005).

Elevated fasting plasma lactate concentrations are evident in individuals who suffer from complications of metabolic diseases such as obesity (Broskey et al., 2021; Lovejoy et al., 1992). In a study examining the effects of high-fat diet-induced obesity in mice, it was found that decreased incremental aerobic exercise performance was associated with a faster accumulation of blood lactate during incremental exercise in the intervention group. Muscles of obese animals had greater glycolysis and decreased dependency on oxidative phosphorylation vs. glycolysis compared with that in control animals (Chen et al., 2017). This may be associated with a decrease in content of muscle protein that catalyzes the conversion of lactate to pyruvate in obese animals. It has also been shown in human populations that capacity for substrate oxidation is depressed in the skeletal muscle of individuals with severe obesity (Houmard et al., 2011).

Skeletal Muscle Metabolism

Skeletal muscle is the most abundant insulin-sensitive tissue, comprising 40%–50% of body mass, responsible for approximately 20%–30% of resting oxygen consumption, and handles ~80% of all insulin-mediated glucose disposal (Stump et al., 2006). Its contribution to the pathophysiology of metabolic syndrome and T2D has been studied for its importance in metabolic diseases such as obesity, T2D, and metabolic syndrome.

The ability of an organism to adapt its substrate for energy production in cellular respiration, based on the availability of the substrates is termed "metabolic flexibility" (Stump et al., 2006). The primary substrates are glucose and fatty acids, which are converted to acetyl-coenzyme A (acetyl-CoA) for use in the TCA cycle. Metabolic inflexibility, or the inability to switch from using carbohydrates to using fat and vice versa, is an early indicator of metabolic syndrome. In healthy skeletal muscle, when lean individuals are challenged by a hyper insulinemic euglycemic clamp, they are capable of drastically increasing the storage and oxidation of glucose compared to fasting conditions (Kelley et al., 1999). Conversely, fatty acid oxidation is noticeably reduced by insulin in these individuals. In obese insulin-resistant individuals, it was found that the ability to shift to glucose oxidation and storage in response to hyperinsulinemia lowered, while fatty acid uptake and oxidation remain relatively unchanged (Stump et al., 2006). In another study, when provided with a 3-day high-fat diet, lean individuals had increased fatty acid oxidation, whereas there was no change in fatty acid oxidation in obese individuals (Boyle et al., 2012). However, interventions that improve insulin sensitivity, such as weight loss have been shown to partially restore metabolic flexibility.

Mitochondria play a fundamental role in skeletal muscle oxidation. Mitochondria are known to be impaired in individuals with metabolic diseases such as obesity and T2D (Hesselink et al., 2016; Ritov et al., 2005). Defects in skeletal muscle mitochondria are thought to precede

the development of metabolic disease. Obesity has been shown to impair skeletal muscle mitochondrial function, which is reflected by impaired fatty acid metabolism, lower mitochondrial enzyme activity, and increased H2O2 emission (Pileggi et al., 2022; Kelley et al., 2002). This is related to dysfunction in the regulation of glucose and lipid metabolism, which are involved in insulin resistance in the skeletal muscle of obese individuals and those with type 2 diabetes. However, further research is needed to examine the association between BMI and insulin resistance with blood lactate and Hb levels before and after high-fat feeding in overweight individuals. Metabolic inflexibility has been implicated in obesity and insulin resistance and is linked with mitochondrial substrate utilization, with the mitochondrial capacity for substrate oxidation being depressed in subjects with metabolic disease (Houmard et al., 2011; Broskey et al., 2020). At rest, glucose can enter the cell and be converted to pyruvate and transported into the mitochondria as acetyl-CoA to be used in the TCA cycle. Glucose that is not fully oxidized then goes through glycolysis producing lactate through anaerobic glycolysis. Lactate is then transported to the liver to make glucose. Due to the higher levels of lactate in the skeletal muscle of obese individuals, it is hypothesized that an impairment in the TCA cycle could be a cause of metabolic inflexibility (Broskey et al., 2020). Thus, blood lactate could be used to detect this impairment in overweight individuals.

Blood Lactate

With the incidence of chronic diseases, such as obesity and T2D on the rise it is important to find ways to examine the onset and effects of these conditions and how they associate with other health markers. Blood lactate was long assumed to only be a waste product of hypoxia due to conclusions that initially found elevated lactate levels in tissues and blood when O2 levels were lower than normal. This, combined with studies that found lactic acid levels increased with

stimulus-induced fatigue and decreased when fatigued muscles were placed in O2 rich environments, (Fletcher and Hopkins, 1907; Meyerhof, 1930), led to the assumption that because low O2 evokes high lactate, and O2 saturation lowered lactate, high lactate must also denote low O2. This argument was further supported by the fact that the study of lactate metabolism was tied to studies that examined exercise and fatigue (Wasserman and McIlroy, 1964; Wasserman et al., 1973).

It is now known that lactate is a major energy source and regulator of lipid and carbohydrate metabolism and is produced even under aerobic conditions. After further study, it was also established that an increase in lactate can represent a multitude of other causative factors besides low O2. Fasting lactate concentrations have been used to indicate the severity of acute illness, injury, and mortality (Andersen et al., 2013) to predict incidents of diabetes (Juraschek et al., 2013; Crawford et al., 2010), and research is ongoing to determine whether it can be used to predict early risk for metabolic diseases (Broskey et al., 2021). Lactate has been shown to have an association with many cardiometabolic risk factors that associate with metabolic syndrome and T2D (Crawford et al., 2008; Broskey et al., 2021). Because T2D impacts cardiometabolic heath, and fasting plasma lactate concentration is associated with various markers of cardiometabolic health, it is hypothesized that resting plasma lactate can potentially be used as a marker for mitochondrial function to help predict the onset of metabolic disease. However, the current body of research still needs to determine whether fasting plasma lactate levels are related to cardiometabolic factors in overweight populations.

Near-Infrared Spectroscopy (NIRS)

NIRS is a well-established noninvasive method for measuring tissue oxygenation, blood flow, and metabolism and has been increasingly used to study physiology in recent years.

(Barstow et al, 2019; Willingham et al, 2017). NIRS functions by using light absorbance of hemoglobin and myoglobin to provide a noninvasive examination of changes in tissue oxygen saturation. Recent studies using quantitative NIRS instruments and protocols, or morphometric modeling, have concluded that myoglobin likely contributes 60–90% to NIRS signals coming from skeletal muscle, as the wavelength of light absorbed by the chromophore is dependent on the oxygenation status of hemoglobin/myoglobin (Barstow et al., 2019). Skeletal muscle mitochondria in humans has traditionally been studied using tissue obtained from biopsies to sequence mitochondrial DNA using *in vitro* techniques to evaluate enzyme levels and quantify mitochondrial content in samples. These techniques provide information related to mutations, enzyme deficiencies and overall dysfunction, playing an important part in the diagnosis and characterization of various mitochondrial diseases (Pfeffer & Chinnery, 2013; Ahmed et al., 2018). However, these methods do not directly examine mitochondrial function or the ability of the mitochondria to perform oxidative phosphorylation. Mitochondrial function is typically evaluated *in vitro* by measuring the rate of oxygen consumption in muscle fibers or isolated mitochondria using fluorescent spectroscopy, respirometry, polarographic oxygen sensors, and phosphorescent oxygen-sensitive probes. While these methods have their uses in distinguishing respiratory steady-states, their ability to reflect physiological mitochondrial function is limited (Willingham & McCully, 2017). Not only is the structure of the interconnected skeletal muscle reticulum compromised in the preparation of the mitochondria, but isolated mitochondria are also exposed to environmental factors such as oxygen and temperatures, which can differ from the natural physiological environment of the mitochondria (Willingham & McCully, 2017). Assessments of mitochondrial function *in vivo* have also employed indirect measures of oxidative capacity. Indirect calorimetry using open-circuit spirometry has been used to quantify

aerobic metabolism in humans using whole-body oxygen consumption rate and onset kinetics during exercise to measure oxidative capacity (Bruce et al. 1973). However, oxygen consumption from this method is influenced by skeletal muscle, as well as cardiovascular and neurological systems, leading to debate about the interpretation of these measures. The use of NIRS to evaluate mitochondrial function *in vivo* allows for the preservation of the natural physiological environment, while being more cost-effective and accessible. Finally, magnetic resonance spectroscopy has been used to measure muscle-specific oxidative capacity by examining the recovery of phosphocreatine following acute exercise (Kumar et al., 2017). Due to regeneration of phosphor-creatine (PCr) being dependent on ATP production from aerobic metabolism, the rate of PCr recovery following exercise is an indicator of a muscle's mitochondrial oxidative capacity (Zanconato et al., 1993; Prompers et al., 2006). While MRS is a valid and accurate method of measuring mitochondrial function *in vivo*, it is confined by both the cost and accessibility of the method. Studies have shown that NIRS can be used to measure the recovery of metabolic metabolism following an acute bout of exercise as an assessment of mitochondrial oxidative capacity at a lower cost and with increased portability (Ryan et al., 2012, 2013, 2014). Lower costs and higher accessibility make NIRS a valid alternative for research, healthcare professionals, and clinical practices.

This study aims to fill the current gap in literature by using a novel NIRS protocol to examine mitochondrial function in relation to cardiometabolic health, VO_2max, and blood lactate which to our knowledge has not been done to date.

Chapter 3: Methods

Participants

A total of 17 subjects were recruited from East Carolina University and the Greenville area. Subjects' ages ranged from (20-48), with no diagnosis of metabolic or heart disease. None of the subjects were smokers, showed any physical impairment to exercise or pregnancy, or had any known cardiovascular or metabolic diseases.

Materials

In order to assess plasma lactate concentrations, a sterile lancet was used to perform a finger prick and obtain a blood sample. The collected blood droplet was then placed on a lactate test strip, which was used to obtain a lactate reading. The lactate reading was analyzed using a lactate meter device (Lactate Plus, Waltham, MA.) Resting heart rate measurements were recorded with a Polar heart rate monitor (Kempele, Finland) along with a baseline fingertip pulse oximeter (Fabrication Enterprises, Elmsford, NY). To evaluate blood pressure, a mobile sphygmomanometer (Welch Allyn Skaneateles Falls, NY) in conjunction with a stethoscope (Littmann, USA) were manually used. For the measurement of resting energy expenditure (REE) and the determination of the respiratory exchange ratio (VCO_2/VO_2), indirect calorimetry was performed (Parvo Medic's True 2400 metabolic cart, East Sandy, UT). The metabolic measurement software with the Parvo metabolic cart analyzed the O2 and CO2 flow/volume ratio. To analyze the body composition of the subjects, a Dual-energy X-ray absorptiometry (DEXA) scanner (Horizon, Danbury, CT) was used. An Excalibur Sports Cycle Ergometer (Lode, Netherlands) was used as the exercise modality during the VO2 max test. To indirectly measure the oxidative capacity of the muscles, the recovery rate of muscle VO_2 (mVO_2) saturation was determined using OxiplexTS Near Infrared Spectroscopy (NIRS) (ISS,

Champaign, IL). Finally, blood analysis was conducted using a hematology analyzer (Beckman Coulter, USA).

Protocol

Screening:

Following multiple recruitment strategies, such as the ECU Announce List, Pirate 411, Brody Graduate School, Pitt Community College, School of Allied Health, and the School of Nursing at ECU, potential participants responded to study advertisements expressing their interest. Subsequently, a prescreening process was conducted via telephone or email to determine if they met the inclusion criteria, which included being overweight (classified as having a Body Mass Index of 25.0-29.9 kg/m2), maintenance of a stable weight for the past 3 months, not following a specific meal plan, and not having any health conditions such as cancer, uncontrolled hypertension or GI disease, psychiatric disorders, or diabetes. Qualified participants were then scheduled to visit the Human Performance Lab/FITT building at East Carolina University. Prior to their arrival, participants were instructed to come in a fasted state, with no more than a light snack and water consumed within 4 hours prior to the appointment. They were also advised to refrain from exercising for three days prior, as well as abstain from stimulants (such as caffeine, nicotine, or stimulant medication) and alcohol for a minimum of 10-12 hours. Upon arrival, the study staff, approved by the Institutional Review Board (IRB), obtained informed consent from each participant verbally and in writing. The participants were required to complete various IRB-approved forms, including a personal family history form, a physical activity questionnaire, a DEXA radiation consent form, and a menstrual cycle questionnaire, before proceeding with measurements of resting energy expenditure (REE) and the VO_2 max test. At the beginning of the session, the participant's weight (in kilograms) and height (in meters) were measured to

calculate Body Mass Index (BMI). Resting heart rate was recorded using a finger pulse oximeter, and blood pressure was manually assessed using a mobile sphygmomanometer and stethoscope. To obtain lactate levels, a lactate meter was used for blood lactate assessment. The third or "ring" finger of the non-dominant hand was selected for lactate sampling. The finger was sterilized with a 70% isopropanol wipe, and a sterile, single-use lancet needle was used to puncture the fingertip and expose the venous blood. Blood was collected using a lactate strip and inserted into the Lactate Plus device for measurement. Blood lactate levels were analyzed twice to ensure accuracy within a range of 0.3 mmol/L. If the two readings differed by more than 0.3 mmol/L, an additional lactate measurement was taken, and the value within 0.3 mmol/L of the previous measurement was used. In the event of lactate meter error, the measurements were repeated. Excessive blood on the finger was wiped off using sterile gauze and a bandage was applied to cover the puncture site after the completion of lactate analysis. For the measurement of other blood metabolites, blood samples were drawn from the antecubital vein during the screening visit, and the blood cells were separated through centrifugation. Participants who still met the inclusion criteria were invited for two additional visits. During the first visit, body composition was assessed using DEXA and 3-D scanning techniques. In addition, they performed a maximal exercise test using a cycle ergometer. Upon their return for the second visit, the participants underwent Resting Metabolic Rate measurements in a quiet, controlled environment.

Visit 1

Subject:

Before the first visit, participants were provided with a food diary to record their dietary intake for seven days. They were instructed to arrive at the laboratory in a fasted state.

Visit:

During the first visit, several measurements were taken, including body composition assessment and a VO2 max test. Resting measurements were obtained, including resting heart rate recorded using a pulse oximeter and resting blood pressure measured manually with a stethoscope and a sphygmomanometer.

To evaluate body composition, Dual-Energy X-Ray Absorptiometry (DEXA) and 3-D body scans were performed. Before the scans, participants removed shoes, jewelry, and any items in their pockets and informed the staff of any metal in their body. The subjects were then instructed to lie on the scanning bed with their head on the right side and feet on the left. The research staff ensured proper alignment of the body and limbs, and secured the subject's toes together with a Velcro strap. Once in the correct position, the participants were asked to remain still for approximately two and a half minutes while the scan was conducted. Afterward, they could relax while the results were collected. The 3-D body scan required participants to undress in a private space, follow instructions to stand correctly in the 3-D scanner for approximately one minute, and then dress before proceeding to the maximal exercise test.

Next, participants performed a VO2 max test on a cycle ergometer, and indirect calorimetry was used to measure aerobic capacity. The cycle seat and mouthpiece used for data collection were adjusted to ensure participant comfort. A member of the research staff explained the testing procedure. The participants were connected to a 10-lead EKG to capture their heart rate and EKG data. Before the test began, resting heart rate and blood pressure were recorded. Once the test began, the baseline gas exchange was measured, and the workload was programmed into the computer. Participants were instructed to maintain a revolutions per minute (rpm) of approximately 50-70rpm and were given a 10-second warning before proceeding to the next stage. Near the end of each stage, the Rate of Perceived Exertion (RPE) on the Borg 6-20 scale,

heart rate, and blood pressure were measured and recorded. Participants progressed through each stage until they achieved maximum effort or cycled at 40rpm or below. Maximum effort was determined by meeting two of three criteria: a respiratory exchange ratio (RER) of 1.10, an RPE greater than 17 on the Borg Scale, and a peak heart rate within ± 5% of the age-predicted maximum heart rate. After completing the test, the heart rate and blood pressure were immediately recorded, and participants entered the active recovery phase to reduce their heart rate and blood pressure. The mouthpiece was then removed. Heart rate was recorded every minute, and blood pressure every two minutes during the recovery period. The cycle ergometer was set to zero watts during active recovery. Once heart rate and blood pressure approached the participant's resting state, the test was concluded.

LODE Bike VO_2 Max Test Protocol (Leg Cycle Ergometer)

Minutes	**Stages**	**Workload (Watts)**
1	1	0
2		0
3	2	30
4		30
5	3	60
6		60
7	4	90
8		90
9	5	120
10		120
11	6	150
12		150
13	7	180
14		180
15	8	210
16		210
17	9	240
18		240
19	10	270
20		270

Visit 2

Upon arrival, the subject was guided to the Resting Energy Expenditure (REE) room, where they assumed a reclined position. They were required to arrive in a fasted state, abstaining from exercise for 72 hours, and refrain from consuming food, stimulants (such as caffeine, nicotine, and stimulant medication), and alcohol for a minimum of 10-12 hours prior to the test. The testing room was maintained at approximately 70 degrees Fahrenheit (21 degrees Celsius) with subdued lighting to promote a relaxed environment. The subject was instructed to remain awake and minimize movement throughout the test. Following a resting period of approximately 10-15 minutes to ensure a stable baseline, an open circuit canopy hood, equipped for measuring REE using indirect calorimetry, was placed on the subject. The research staff adjusted the levels of carbon dioxide (CO2) to stabilize them at around 1.0% throughout the test. At times, the subject may have been left alone for several minutes to ensure that they reached an optimal resting state. After a 20-minute data collection period, the test was concluded, and the canopy hood was removed from the subject. The results were then recorded for further analysis.

Near-Infrared Spectroscopy

The NIRS protocol was used to assess mitochondrial respiratory capacity at the whole-body level. The NIRS system consisted of eight infrared diode lasers, with four emitting at 691 nm and four at 830 nm, along with a detector in each probe. The probes were secured to the vastus lateralis muscle of the dominant leg using a double-sided adhesive tape and Velcro straps. To determine the appropriate NIRS probe for each participant, subcutaneous adipose tissue thickness was measured using a skinfold caliper (~10 cm above the patella). Probe B was used for individuals with a skinfold thickness of 30 mm or less, whereas probe A was used for individuals with a skinfold thickness greater than 30 mm. During NIRS measurement,

participants were positioned in a supine position on a medical examination table with both legs fully extended. After selecting the appropriate probe, the blood occlusion procedure was initiated. A blood pressure cuff was placed as close to the proximal area as anatomically possible, ensuring that the NIRS probe was not in contact with the cuff after inflation. A 15-gallon air compressor set to 30 psi was used to control the blood pressure cuff. Prior to the actual measurement, three 5-second practice occlusions were performed to familiarize the participants with the pressure around their leg and to ensure that there was no discomfort. The first arterial occlusion served as the baseline measurement, in which participants underwent a 60-second occlusion duration. After a resting period of 2-3 minutes, the second occlusion was performed following an isometric contraction of the gastrocnemius muscle for 15-20 seconds. The cuff was then inflated to 300 mmHg for a 5-minute arterial occlusion to determine the total deoxygenated hemoglobin (deoxy Hb) level. The collected data was processed, and the rate of deoxy Hb was calculated by dividing the oxygen kinetics obtained during the one-minute occlusion by the total deoxy Hb oxygen kinetics obtained during the 5-minute occlusion. Percent-deoxy Hb was subsequently calculated by dividing the rate of deoxy Hb by the total deoxy Hb (umol/minute).

High-Fat Diet

Participants followed an eucaloric diet consisting of 70% fat, 20% carbs, and 10% protein. Subjects tracked their diet for 3 days using the My Fitness Pal website.

Data Processing

BMI was determined by dividing the participant's weight by the square of their height (kg/m^2). Exercise energy expenditure, as well as resting and exercise respiratory exchange ratio (RER), were obtained from the metabolic cart during the study. Body composition data, including fat mass (FM) and fat-free mass (FFM), were acquired using the DEXA scanner.

Plasma and serum samples were collected and sent to Labcorp for analysis of glucose, lactate, insulin, and lipids. Homeostatic model assessment of insulin resistance (HOMA-IR) was calculated using the formula (glucose * insulin) / 405. A HOMA-IR value of <2 was considered normal, whereas a value of ≥12 μIU/ml indicated hyperinsulinemia.

Statistical Analysis

The normality of the data was assessed using the Shapiro-Wilks Test. The strength of the association between the variables of skeletal muscle oxidative capacity and cardiometabolic health were measured using Pearson product-moment correlations. Statistical significance was determined using a p-value of ≤ 0.05. All statistical analyses were performed using JMP®, Version 14, developed by SAS Institute, Inc.

Chapter 4: Results

Participants (n=17) were both men and women (n=13, n=4), respectively, ages averaged 28 ± 7 years, with most subjects classified as overweight BMI (27.5 ± 2.1 kg/m^2). The subjects were insulin sensitive using HOMA-IR (1.9 ± 0.91) with fasting glucose levels of 90.6 ± 5.7 mg/dl and insulin values of 8.3 ± 3.6 μIU/ml. Blood Cholesterol (182 ± 35.2 mg/dl), HDL (47.4 ± 8.1 mg/dl) and triglycerides (105.6 ± 97.2 mg/dl) were all in normal ranges. LDL was slightly elevated (114.6 ± 24.4 mg/dl), and cohort was prehypertensive with Systolic blood pressure values of 120 ± 12 mmHg and diastolic blood pressure values of 73 ± 8 mmHg. The average pre-high-fat diet percent-deoxygenated hemoglobin was 0.22 ± 0.15 and post-high-fat diet percent-deoxygenated hemoglobin was 0.29 ± 0.36.

Using a bivariate fit model between factors of cardiometabolic health and percent deoxygenated hemoglobin pre- and post-high-fat diet no statistical significance was found.

Table 1: Subject Characteristics (n=17)

	Mean	Range
Sex (M/F)	13/4	
Age (years)	28 ± 7	28 ± 7
BMI (kg/m^2)	27.5 ± 2.1	30.5 - 24.7
Systolic Blood Pressure (mmHg)	120 ± 12	137 - 95
Diastolic Blood Pressure (mmHg)	73 ± 8	85 - 56
Percent Body Fat (%)	26.2 ± 6.1	37.8 – 19.6
Waist Circumference (cm)	38.01 ± 2.9	44.7 – 33.2
Hip Circumference (cm)	43.2 ± 2.2	47 – 39.6
Waist-to-Hip Ratio	0.88 ± 0.04	0.95 – 0.80
Android/Gynoid Ratio	0.98 ± 0.14	1.26 – 0.76
VO_2Max (mL/kg/min)	35.47 ± 8.24	49.8 – 18.3
Time to Exhaustion (min)	15.28 ± 3.43	22.9 – 8.3
Pre-HFF [Hb] B (%physiological calibration/min)	0.22 ± 0.15	0.72 – 0.1
Post-HFF [Hb] B (%physiological calibration/min)	0.29 ± 0.36	1.66 – 0.1

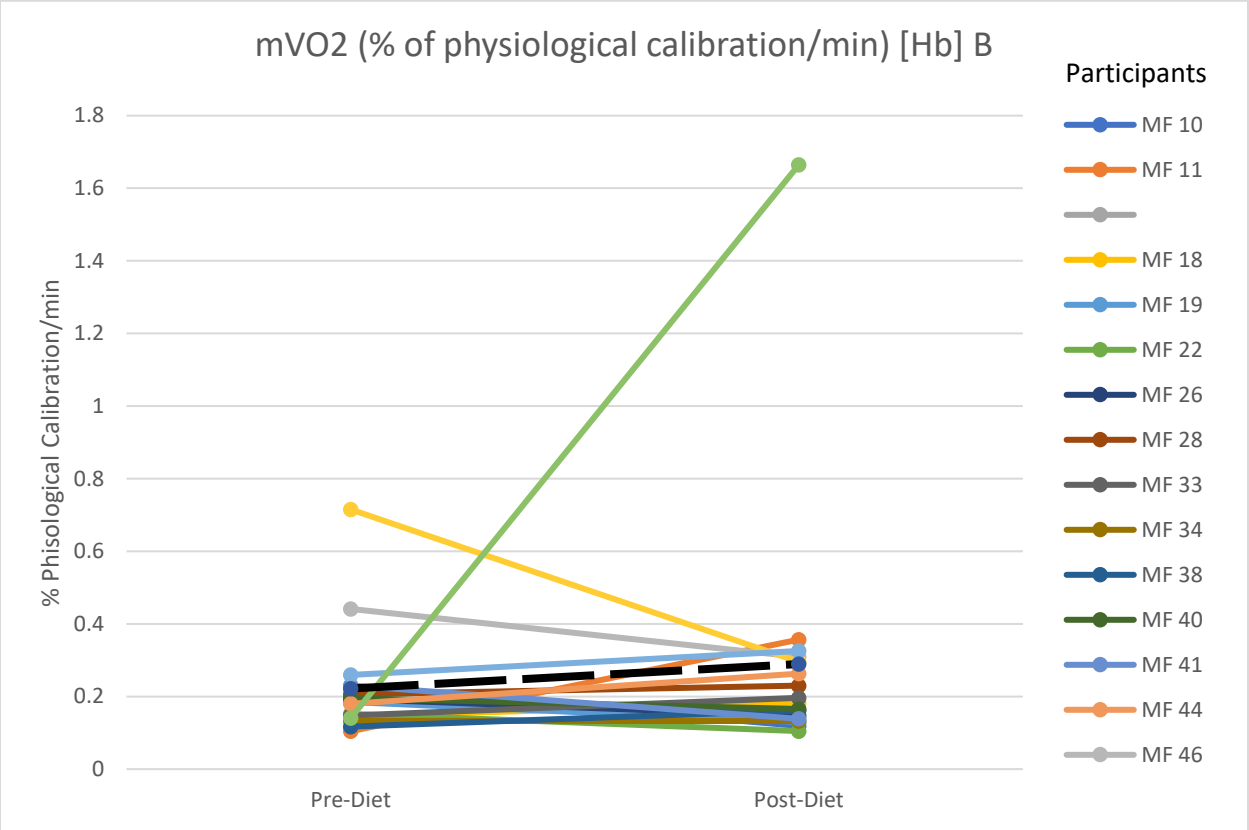
Mean ± SD

Table 2: Blood Metabolites

	Mean	Range
Lactate (mmol/L)	0.98 ± 0.45	2.29 – 0.47
Total Cholesterol (mg/dl)	182.1 ± 35.3	283 - 129
Triglycerides (mg/dl)	105.6 ± 97.2	464 - 41
HLD (mg/dl)	47.4 ± 8.1	61 - 30
LDL (mg/dl)	114.6 ± 24.4	162 - 66
LDL:HDL (mg/dl)	0.44 ± 0.14	0.76 – 0.19
Glucose (mg/dl)	90.6 ± 5.7	101 - 83
Insulin (µIU/ml)	8.3 ± 3.6	19.1 – 3.6
HOMA-IR	1.9 ± 0.91	4.8 – 0.7

Mean ± SD

Figure 1: Change in % Physiological Calibration Pre and Post High Fat Diet



Near infrared spectroscopy (NIRS) data before and after a 3-day high-fat diet (HFF). Subjects followed a eucaloric diet composed of 70% fat 20% carbohydrates and 10% protein. NIRS procedure was done before diet and participants followed up with another post diet. NIRS was performed on gastrocnemius muscle following blood occlusion in conjunction with resistance exercise. No statistical significance between pre and post NIRS measurements $p = 0.85$.

Table 3: Correlation and Significance of Cardiometabolic Health Factors with Mitochondrial Function

Factor	r	p
Age	-0.15521	0.2608
BMI	0.279	0.7576
LDL	-0.084	0.7082
HDL	-0.101	0.9925
HDL:LDL	0.004	0.8298
Cholesterol	-0.289	0.8738
HOMA-IR	0.058	0.5555
Systolic Blood Pressure	0.249	0.9478
Diastolic Blood Pressure	-0.531	0.2724
VO_2Max	-0.211	0.8153
Time to Exhaustion	-0.159	0.8296
Lactate	-0.079	0.7645

Statistically Significant at $p<0.05$

Chapter 5: Discussion

Cardiometabolic health factors are measures or indicators of an individual's overall cardiovascular and metabolic health. These factors provide insights into the risk of developing cardiometabolic conditions, such as metabolic syndrome, T2D, coronary heart disease, and cardiovascular disease (Papakonstantinou et al., 2014), and can help guide preventive measures and treatment strategies (Pereira et al., 2009). Previous research has shown that NIRS can be used as a noninvasive method to determine skeletal muscle oxidative capacity (Ryan et al., 2014), which has been associated with cardiometabolic factors, such as VO_2max and blood lactate levels (Broskey, 2021). These markers could potentially be used as early biomarkers for predicting metabolic diseases (Broskey et al., 2020). Our study aimed to answer whether a relationship exists between skeletal muscle oxidative capacity as measured by NIRS and factors of cardiometabolic health in men (n=13) and women (n=4) with overweight. The study demonstrated that there was no relationship between skeletal muscle oxidative capacity and cardiometabolic factors, including BMI, waist-to-hip ratio, blood pressure, total cholesterol, triglycerides, LDL and HDL levels, HOMA-IR, Android/Gynoid ratio, and VO_2max, when outliers were controlled for.

BMI has been linked to skeletal muscle mitochondrial oxidative capacity using Phosphorous-Magnetic Resonance Spectroscopy (P-MRS), to assess phosphocreatine recovery after exercise (Wells et al., 2017). Wells et al., determined that obese and overweight participants had longer PCr recovery than non-obese controls following 5x30s of moderate-intensity exercise showing that higher BMI correlated to lower oxidative capacity. Despite it being suggested that NIRS is a valid method for assessing mitochondrial function, and that direct comparisons between NIRS and P-MRS measurements may be possible (Ryan et al., 2013). Our findings

failed to find a significant relationship between BMI and skeletal muscle mitochondrial oxidative capacity. Notably, both studies had small sample sizes (n=17 and n=16), which may limit the applicability of these results comparing measurement to a wider population. However, a study conducted in children showed that there was no difference in skeletal muscle oxidative capacity using P-MRS between overweight and normal-weight children, matching the results of our study (Fleischman et al., 2009).

Mitochondria

Mitochondrial function has been linked to many factors of cardiometabolic health including VO_2max, blood lactate, blood pressure, insulin sensitivity, and obesity. Previous studies have established that reductions in mitochondrial oxidative capacity can contribute to a reduction in VO_2max, as shown in a study that matched muscle convective O_2 delivery in young adult and late middle-aged hybrid rats (Hepple et al., 2003). The main finding of this study was that VO_2max was reduced independent of muscle convective O_2 delivery in late middle-aged animals. This demonstrates that alterations within the skeletal muscles contribute significantly to the decline of VO_2max (Hepple et al., 2003). This study differed from ours using rats as opposed to overweight humans, but coincides with another study that examined the association between skeletal muscle mitochondrial capacity and efficiency with walking performance in a group of older adults (Coen et al., 2013). Coen et al. found that lower mitochondrial capacity and efficiency were both associated with lower VO_2peak and slower walking speed within a group of older participants with a wide range of BMIs. This study examined a larger and different population than our study with older adults whose weights ranged from healthy to slightly overweight, possibly explaining their different conclusions. Finally, a study by Zwaard et al. found a relation between mitochondrial capacity in skeletal muscle and $\dot{V}O_2$max when

examining subjects ranging from patients with chronic heart failure to professional cyclists (Van Der Zwaard et al., 2016).

In a study examining whether the variance in fasting lactate concentrations was associated with factors linked to cardiometabolic health in a young, lean cohort, it was found that there is an inverse relationship between plasma lactate and muscle oxidative capacity, which is indicative of mitochondrial function. (Broskey et al., 2021). Another study which looked to determine whether fasting plasma lactate is related to metabolic status examined the effects of 6 months of exercise training on blood lactate levels in subjects with components of metabolic syndrome and discovered that post-exercise, participants had lower levels of fasting lactate (Jones et al., 2019). One explanation for this may be attributed to the mitochondrial adaptations induced by exercise, as explored by Drake et al. Their study reaffirmed that exercise has been known to promote mitochondrial biogenesis, as well as examined its impact on mitochondrial dynamics and clearance (Drake et al., 2016). These adaptations resulted in increased mitochondrial function and thus, lower blood lactate levels. Blood lactate has also been used to link mitochondrial function to hypertension. In a 2008 study by Crawford et al., researchers examined whether insufficient oxidative capacity played a central role in the development of insulin resistance and hypertension. They found that associations between lactate and diastolic blood pressure suggest that insufficient oxidative capacity may play a role in obesity-related hypertension (Crawford et al., 2008). The proposed mechanism that researchers gave for this is a mass-specific decrease in oxidative capacity that occurs with obesity, combined with a global increase in energy expenditure, may play complementary roles in the development of obesity-related hypertension (Crawford et al., 2008). The main difference between this study and ours is in the population that was examined. While our study examined overweight individuals on a

high-fat diet, this study examined a larger number (n=40) of obese individuals on a longer low-calorie diet versus a lean cohort.

Insulin resistance is another cardiometabolic risk factor that has been linked to mitochondrial function in previous research. In a study examining mitochondrial networks in skeletal muscle with compromised oxidative capacity and insulin sensitivity in four groups of participants, researchers found that mitochondrial dynamics and quality control in skeletal muscle are linked to oxidative capacity in humans (Houzelle et al., 2021). Using ^{31}P-MRS measured oxidative capacity, researchers have examined the relationship between insulin resistance and mitochondrial oxidative capacity in patients without diabetes (Fabbri et al., 2017). This study found that, independent of all confounders in their population, mitochondrial function was significantly and positively correlated with both prediabetes and HOMA. The results of this study suggest that mitochondrial impairment occurs before IR progression to type 2 diabetes; however, the mechanism by which mitochondrial impairment could lead to IR is unclear (Fabbri et al., 2017). This study differs from our study in several ways. In addition to using 31P-MRS to measure mitochondrial function, researchers did not use an euglycemic clamp to determine insulin sensitivity for financial reasons and used oral glucose tolerance tests 2-h glucose levels rather than fasting glucose levels. Another study examining the relationship between insulin resistance and mitochondrial function in patients with type 2 diabetes and BMI-matched control subjects found that PCr half-life was prolonged in diabetic patients, but intramyocellular lipid content was not different between the two groups. (Schrauwen-Hinderling et al., 2007). A prolonged PCr half-life suggests impaired mitochondrial function is an important player in the development of type 2 diabetes mellitus (Schrauwen-Hinderling et al., 2007). Rodent studies have also shown that even in high-fat diets resulting in increased saturation of skeletal muscle

mitochondrial phospholipids, there was no impact on mitochondrial oxidation and no statistical significance between mitochondrial fat oxidative capacity and skeletal muscle insulin resistance (Hoeks et al., 2011), further implying that it is mitochondrial function and not intramyocellular lipid content that results in insulin resistance.

There are limitations to this study, however. One limitation would be the sample size, as there were only 17 subjects, which limits the generalizability to a wider population. Adding to this, a confounding variable may be that we did not time the data collection with the female menstrual cycle. We also only analyzed those with an overweight BMI so for a future study it is recommended to look at both lean and obese cohorts to compare differences between all three groups. Furthermore, standardization in the application of NIRS testing and subject familiarization can help ensure accurate measurements for data analysis.

To conclude, this study showed that there was no statistically significant evidence that mitochondrial function as measured by NIRS, is associated with measures of cardiometabolic health in an overweight population.

Chapter 6: Conclusion

This study aimed to determine the relationship between skeletal muscle oxidative capacity and cardiometabolic health factors in overweight men and women. It can be concluded that there is no statistically significant relationship between skeletal muscle oxidative capacity, as measured using NIRS, and cardiometabolic health factors. Several limitations may have contributed to this conclusion. The sample size poses an issue regarding the generalizability of the results. In future studies, a larger sample size with a larger range of BMI values should be used to increase the applicability of these results to a wider population. Second, the reliability of the testing procedures used in this study is another aspect that should be considered. Ensuring consistent and accurate measurements using NIRS is crucial to obtain reliable results. Any potential inconsistencies in the testing procedures, such as subject familiarity with the NIRS protocol between visits 4 and 5, cuff placement, probe placement, or staff changeover, may have influenced the outcomes and contributed to the lack of significant findings. Finally, the duration of the high-fat diet followed by participants could have impacted the results. In the future, research could aim to follow a longer dieting period to help determine potential relationships between oxidative capacity and cardiometabolic health. Despite the non-significant findings, this study provides valuable insights for practitioners and clinicians. It is important that future research aims to address the limitations mentioned. By doing so, a better understanding of the relationship between skeletal muscle oxidative capacity and cardiometabolic health can be achieved.

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APPENDIX: IRB APPROVAL LETTER

EAST CAROLINA UNIVERSITY
University & Medical Center Institutional Review Board
4N-64 Brody Medical Sciences Building· Mail Stop 682
600 Moye Boulevard · Greenville, NC 27834
Office **252-744-2914** · Fax **252-744-2284** ·
rede.ecu.edu/umcirb/

Notification of Continuing Review Approval

From: Biomedical IRB
To: Joseph Houmard
CC:

Elizabeth Gates
Date: 2/27/2023
Re: CR00010015
UMCIRB 19-000914
Metabolic inflexibility is related to elevated muscle anaerobic glycolysis

I am pleased to inform you that at the convened meeting on 2/22/2023 at 12:15 PM of the Biomedical IRB, this research study underwent a continuing review and the committee voted to approve the study. Approval of the study and the consent form(s) is for the period of 2/22/2023 to 2/21/2024.

The Biomedical IRB deemed this study Greater than Minimal Risk .

As the Principal Investigator you are explicitly responsible for the conduct of all aspects of this study and must adhere to all reporting requirements for the study. Your responsibilities include but are not limited to:

1. Ensuring changes to the approved research (including the UMCIRB approved consent document) are only initiated with UMCIRB review and approval except when necessary to eliminate an apparent immediate hazard to the participant. All changes (e.g. a change in procedure, number of participants, personnel, study locations, new recruitment materials, study instruments, etc.) must be prospectively reviewed and approved by the UMCIRB before they are implemented;

2. Ensuring that only valid versions of the UMCIRB approved, date-stamped informed consent document(s) are used for obtaining informed consent (consent documents with the IRB approval date stamp are found under the Documents tab in the ePIRATE study workspace);

3. Promptly reporting to the UMCIRB all unanticipated problems involving risks to participants and others;

4. Applying for continuing review and receive approval of continuation of the study prior to the study's current expiration date. Application for continuing review should be submitted no less than 30 days prior to the expiration date. Lapses in approval (i.e. study expiration) should be avoided to protect the safety and welfare of enrolled participants and liability to the University; and

5. Submission of a final report when the study meets the UMCIRB criteria for closure. Study approval should not be allowed to expire simply because the study is completed, rather the UMCIRB should be formally notified of study completion via the final report process.

The approval includes the following items:

Document	Description
APPHoumard-Dohm_NIH_R01_12 (1).pdf(0.01)	Study Protocol or Grant Application
Flyer and email(0.07)	Recruitment Documents/Scripts
Flyer Canvas distribution(0.01)	Recruitment Documents/Scripts
Group 1 and 2 Information Sheet V.1.docx(0.02)	Recruitment Documents/Scripts
Group 3 Information Sheet V.3 1.20.22(0.03)	Recruitment Documents/Scripts
Health history 08.13.20.docx(0.02)	Surveys and Questionnaires
Met Flex ppt Script.docx(0.01)	Recruitment Documents/Scripts
MetFlex Bariatric Surgery ICF v.7 07.13.22-Clean.doc(0.16)	Consent Forms
MetFlex Main ICF v.8 07.08.22-Clean.doc(0.14)	Consent Forms
MetFlex Participant website.docx(0.01)	Recruitment Documents/Scripts
MetFlex Protocol v.4.0 02.17.22.docx(0.06)	Study Protocol or Grant Application
MetFlex Protocol v.5.0 07.26.22.docx(0.01)	Study Protocol or Grant Application
MetFlex Screening ICF v.7-0 1.25.22.docx(0.13)	Consent Forms
PARQPlus2019ImageVersion2.pdf(0.01)	Surveys and Questionnaires
Participant questionnaire 01.13.20.docx(0.01)	Surveys and Questionnaires
Phone screen final 08.13.20.docx(0.02)	Recruitment Documents/Scripts

For research studies where a waiver or alteration of HIPAA Authorization has been approved, the IRB states that each of the waiver criteria in 45 CFR 164.512(i)(1)(i)(A) and (2)(i) through (v) have been met. Additionally, the elements of PHI to be collected as described in items 1 and 2 of the Application for Waiver of Authorization have been determined to be the minimal necessary for the specified research.

The following UMCIRB members were recused for reasons of potential for Conflict of Interest on this research study:

None

The following UMCIRB members with a potential Conflict of Interest did not attend this IRB meeting: M. L. Pories