ASSOCIATION OF SKELETAL MUSCLE FIBER TYPE DISTRIBUTION WITH LACTATE METABOLISM AND METABOLIC HEALTH

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## ABSTRACT

**Background:** The metabolic syndrome (MetS) is classified as a combination of risk factors of metabolic origins and increases the risk of cardiovascular disease, type 2 diabetes, atherosclerosis, stroke, and all-cause mortality. Treatment of MetS is expensive and early detection could help prevent the development. Blood lactate concentrations can be measured as lactate influences metabolic regulation inside and outside the cell. Higher levels of fasting blood lactate have been shown in people with obesity and type 2 diabetes. Skeletal muscle plays a major role in metabolic homeostasis and the production of lactate, and this is mediated by specialized muscle fibers. Skeletal muscle fiber type determines substrate use and possible metabolic health. Several studies have shown a link between a lower percentage of type I fibers and obesity and type 2 diabetes. Studies have also shown that a higher percentage of type I fibers can be associated with a healthier cardiometabolic phenotype. **Purpose:** Observe how muscle fiber type distribution relates to blood lactate levels and other factors of metabolic syndrome. **Methods:** Overweight subjects (n=32) were screened for any pre-existing conditions. Anthropometric and blood metabolite measures were

taken for each subject. During one of three visits a muscle biopsy was performed, the muscle was then placed in O.C.T and frozen. Muscle tissue was then sectioned, and fiber type protocol was followed to determine fiber-type distribution. After fluorescent imaging, each image was manually quantified. **Results:** There was a significant correlation between a higher percentage of type II fibers and higher lactate levels ($r=0.43$, $p=0.02$) There was a significant correlation between higher HDL levels and a higher percentage of type I fibers ($r=0.42$, $p=0.02$). Higher levels of triglycerides also showed a significant correlation with a higher percentage of type II fibers ($r=0.36$, $p=0.02$). Higher HOMA-IR levels have a significant relationship with a higher percentage of type II fibers ($r=0.53$ $p=0.002$). Although the correlation of systolic blood pressure to type I ($r=-0.24$, $p=0.2$) or type II fibers ($r=0.23$, $p=0.2$) showed no significance, higher diastolic blood pressure was significantly correlated with a higher percentage of type II fibers ($r=0.39$ $p=0.03$). A higher Android/Gynoid ratio was significantly correlated with a higher percentage of type II fibers ($r=0.49$, $p=0.01$). Higher VAT mass (g) was also significantly correlated with a higher percentage of type II fibers ($r=0.56$, $p=0.001$). The percentage of body fat did not show any significance in relation to type I ($r=-0.13$, $p=0.49$) or type II fibers ($r=0.11$, $p=0.54$). $VO_2$max also showed no significance in relation to type I ($r=0.32$, $p=0.08$) or type II ($r=-0.32$, $p=0.08$) fibers respectively.

**Conclusion:** The results of this study show that there is a significant association between fiber type distribution and lactate levels and other factors of metabolic syndrome. Future studies should explore metabolic enzymatic activity for different skeletal muscle compositions (fiber type distribution) and how it relates to the factors of the metabolic syndrome.

# ASSOCIATION OF SKELETAL MUSCLE FIBER TYPE DISTRIBUTION WITH LACTATE METABOLISM AND METABOLIC HEALTH

A Thesis

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# Chapter 1 Introduction

## Introduction

The metabolic syndrome is multifactorial and classified as increased waist circumference, triglycerides levels, blood pressure, fasting glucose levels, and reduced HDL cholesterol levels (Khan et al., 2015). Individuals with metabolic syndrome have an increased risk for type II diabetes, stroke, and cardiovascular disease. Insulin resistance is also a key element, which explains most of the implications of the metabolic syndrome (Eckel et al., 2010). Insulin is a hormone that stimulates the transport and disposal of glucose. Impairments in insulin action and disruptions in fuel selection contribute to insulin resistance, which is a metabolic condition that manifests as peripheral resistance to insulin in several tissues such as adipose, liver, and skeletal muscle resulting in increased levels of circulating glucose. In skeletal muscle, insulin resistance is viewed as a critical part of whole-body insulin resistance as it accounts for the majority of insulin-mediated glucose disposal in humans (Hickey et al., 1995). Muscles from obese or diabetic subjects showed a decreased capacity to switch between glucose and lipid substrates based on the physiological stimulus, termed metabolic inflexibility (Kelly et al., 1999). When individuals suffer from metabolic diseases such as obesity and/or diabetes, metabolic inflexibility may indicate mitochondrial dysfunction in the skeletal muscle. Mitochondrial dysfunction is broadly defined as a reduced capability to switch between fuel choices, thus limiting cellular glucose metabolism (San-Millan, & Brooks., 2017;2018). In his research on cell-cell shuttling, Brooks demonstrated how glycolytic and oxidative functions are connected. Lactate is a product in the glycolytic pathway and a substrate in the oxidative pathway.

This shows how fuel sources can switch depending on physiological needs. Mitochondrial function can be assessed by measuring lactate levels in relation to carbohydrate and fat oxidation as this could potentially indirectly assess metabolic flexibility (San-Millan, & Brooks., 2017;2018). At rest and during muscle contraction, lactate is readily oxidized by skeletal muscle. Carbohydrate oxidation rate and lactate concentration are related as they are both implicated with glycolytic pathways. Lactate is an intermediate of glucose metabolism that has been implicated in the pathogenesis of insulin resistance (Chondronikola et al., 2018). Lactate metabolism has three functions: 1. Is a major energy source. 2. Is a major gluconeogenesis precursor and 3. Is a signaling molecule (Brooks., 2020). Further and most importantly, lactate is thought to act as a bridge between glycolytic and aerobic pathways and is thus thought to play a role in metabolic flexibility (Brooks., 2020).

Skeletal muscle is a heterogeneous tissue composed of different fiber types with the ability to rapidly increase or decrease the rate of energy consumption. Muscle fiber types identified in humans are Type I, Type IIa, and Type IIx and are divided based on metabolic functions. Fiber type could indicate a potential development of metabolic syndrome as those individuals have fewer type I muscle fibers (Stuart et al.,2013). Type I muscle fibers likely play an important role in maintaining glucose homeostasis as they are considered highly oxidative (Albers et al., 2015). Glycolytic fibers primarily convert lactate to glycogen and may contribute to the retention of lactate (Gladden., 2000). Brooks (1998) presented a model of shuttling lactate from glycolytic to oxidative fibers. Oxidative fibers switch from lactate production to consumption at lower concentrations and maintain higher rates of lactate disposal at elevated lactate concentrations in

mammalian skeletal muscles (Donovan, & Pagliassotti., 2000). Further research should be conducted to determine if the same thing happens in human skeletal muscle. Elevated lactate concentrations in individuals at risk or with metabolic diseases would show a greater amount of type II fibers in their skeletal muscle and could explain differences in metabolic flexibility.

# Chapter 2 Literature Review

## Introduction

One of the indicators of being at risk for developing type 2 diabetes, stroke or cardiovascular disease is the metabolic syndrome. It is important to understand what influences metabolic health and how it can affect the quality of life. One of the most important factors that contribute to the metabolic syndrome is obesity, the inability of the tissues to respond to the actions of insulin, metabolic inflexibility, and mitochondrial dysfunction. Additionally, skeletal muscle composition (e.g., fiber types) has been known to play a part in insulin resistance. The literature below will explore current research on the metabolic syndrome, insulin resistance, metabolic flexibility, skeletal muscle fiber type, and lactate metabolism, as recent studies have found the association between elevated lactate levels and increased risk for the metabolic syndrome.

## Metabolic Syndrome

To define metabolic syndrome The International Diabetes Federation (IDF) and American Heart Association/ National Heart, Lung, and Blood Institute joined by the World Heart Federation, International Atherosclerosis Society, and International Association for the Study of Obesity (Khan et al., 2005) came up with a uniform definition for the metabolic syndrome. The definition of the metabolic syndrome states that a person must have increased waist circumference, triglycerides, blood pressure, fasting glucose, and reduced HDL cholesterol. Most of those values are uniform between countries, but the cut points for waist circumference are different between European and non-European people and they should adhere to those values until more

data is available (Eckel et al., 2010). Metabolic syndrome is a cluster of diseases of metabolic origin thus, people who suffer from it are at a higher risk for developing type 2 diabetes, stroke, and cardiovascular disease. Since the prevalence of obesity has increased worldwide, obesity has been recognized as a leading cause of metabolic syndrome (Wang et al., 2020). Obesity has more implications than excess amount of adipose tissue, obese individuals also exhibit diminished capacity to modulate glucose uptake in response to insulin. This lost capacity to switch between fuel sources is a hallmark of developing insulin resistance in skeletal muscle which is often associated with risk for the metabolic syndrome Eckel et al., 2010). These alterations could be a primary impairment that leads to obesity rather than merely resulting from obesity (Kelly et al., 19999).

Insulin Resistance

Insulin is a hormone that stimulates the transport and disposal of glucose. An increased amount of circulating glucose indicates that there is something wrong if a person has not been diagnosed with type I diabetes. Depending on the amount of blood glucose it can be classified as insulin resistance or hyperinsulinemia. Insulin resistance is a metabolic condition where one does not respond well to insulin and has trouble taking up glucose from the blood. A known cause of insulin resistance is disruptions in the appropriate fuel adjustments according to physiological needs (Muoio., 2014). Hyperinsulinemia is connected to insulin resistance. Prolonged insulin resistance can lead to hyperinsulinemia.

In skeletal muscle, insulin resistance is viewed as a critical part of whole-body insulin resistance. One theory is that muscle insulin resistance arises from impaired

mitochondrial uptake and oxidation of fatty acids (Morino et al., 2006). This impairment could result in poor reliance on fatty acid oxidation by skeletal muscle during fasting conditions; in obesity, it has been known to significantly predict the severity of insulin-resistant glucose metabolism (Kelly et al., 1999). Insulin resistance is also characterized by limited mitochondrial protein expression (mitochondrial dysfunction) and poor capacity for cellular uptake and oxidation of glucose (Kelley et al., 2002). Additionally, lipid accumulation in the skeletal muscle of sedentary individuals can be associated with impaired insulin-stimulated glucose metabolism. Chronic lipid oversupply leads to impaired muscle glucose disposal (Sharma et al., 2019) resulting in increased glucose levels circulating in the blood. Patients with metabolic syndrome showed a dependence on carbohydrate-derived energy and have limited ability to switch between fuel energy choices.

Metabolic Flexibility

The simplest definition of metabolic flexibility is the ability to switch freely between key fuel energy substances in response to changing physiological conditions. If there is a disruption in the metabolic function, and one cannot freely switch between fuel choices it's termed metabolic inflexibility. One of the characteristics of skeletal muscle that exhibits metabolic flexibility is the capacity to modulate rates of energy production, blood flow, and substrate utilization (Kelly et al., 1999). Skeletal muscle can utilize either carbohydrates or fatty acids as fuel energy. When individuals suffer from metabolic diseases such as obesity and/or type II diabetes, metabolic inflexibility may indicate that there is a problem at the mitochondrial level proposing a problem of mitochondrial dysfunction. Mitochondrial dysfunction in the presence of elevated lactate levels

suggests that mitochondria have lost their capability to switch between fuel choices, thus limiting cellular glucose metabolism, leading to impaired fatty acid clearance and elevated plasma-free fatty acid concentration (San-Millan, & Brooks., 2017;2018). Assessment of mitochondrial function could help diagnose defects in individuals who are at risk of developing metabolic disease. Further, measuring lactate levels in addition to carbohydrate and fat oxidation could indirectly assess mitochondrial function and metabolic flexibility (San-Millan, & Brooks., 2017;2018). Carbohydrate oxidation rate and lactate concentration are related as they are both implicated with glycolytic pathways.

Lactate Metabolism

Lactate is an intermediate of glucose metabolism that has been implicated in the pathogenesis of insulin resistance (Chondronikola et al., 2018). At rest, in healthy individuals, the circulating levels of lactate remain low and lactate is constantly being formed and utilized (Brooks, 1985). Lactate metabolism is thought to have three major functions: 1. Is a major energy source. 2. Is a major gluconeogenesis precursor and 3. Is a signaling molecule (Brooks., 2020). Lactate production is increased by an increase in energy expenditure or a reduction in energy produced by aerobic oxidation. Lactate acts as a bridge between glycolytic and aerobic pathways and is thus thought to play a role in metabolic flexibility. Lactate enters the mitochondrial reticulum via oxidative phosphorylation to support energy homeostasis. Lactate production influences metabolic regulation inside and outside of cells. Lactate exchange within and outside the cell is called cell-to-cell shuttle which describes the role of lactate in the delivery of oxidative and gluconeogenic substrates as well as a signaling moiety (Brooks., 2020).

Important effects of lactate and lactate shuttling in healthy individuals could indicate when lactate signaling is impaired. For instance, lactate acts as a regulator of the cellular redox state by exchange and conversion to its more oxidized form, pyruvate, and has powerful effects on the cell redox balance (Brooks., 2009), thus influencing metabolic regulations at multiple levels in different cells showing that lactate plays a major role in the signaling mechanism. Additionally, mitochondria are necessary for the lactate shuttle to operate so impaired mitochondrial function could result in increased levels of circulating lactate levels. Elevated levels of fasting plasma lactate can occur due to two reasons: elevated lactate production and/or decrease in lactate use during gluconeogenesis in the liver. Aerobic glycolysis resulting in lactate production occurs in both resting and stressful conditions like exercise (Brooks., 2020). Studies in humans have shown that the major route of lactate disposal is oxidation in response to exercise (Mazzeo et al,1986). Crawford et al., in their ARICs study, showed that elevated lactate was found with higher levels of triglycerides, lower HDL, and higher fasting glucose among nondiabetic subjects. This coincides with the definition of metabolic syndrome previously discussed. Another study further supported this theory in that individuals with higher concentrations of lactate display a lower mitochondrial capacity and fatty acid oxidation in skeletal muscle (Broskey et al., 2020).

Fiber Type

Skeletal muscle is a heterogeneous tissue composed of different fiber types, the primary site for glucose disposal. Additionally, muscle is a site of lactate production and utilization occurring among (Brooks,. 1985) and within (Brooks,. 1985) muscle.

The availability of specific antibodies is an essential tool in recognizing different fiber types. MHC isoforms are type-specific markers and recent studies have confirmed the existence of four different fiber types (I, IIa, IIb, IIx) in mice and three different fiber types (I, IIa, IIb) in human skeletal muscle. Further immunohistochemical studies have shown an expression of MHC-2x which identifies Type IIx fiber, earlier classified as type IIb (Schiaffino., 2018). Historically, the literature used Type IIx interchangeably with Type 2b. This creates a discrepancy in the terminology. It is important to note that humans typically don't express IIb MHC in locomotor skeletal muscle (Smerdu et al., 1994) and its expression only refers to animal skeletal muscles. For this paper, the terminology of fiber type IIx will be used. Studies have shown that a single muscle fiber may express more than one myosin heavy chain (MHC). Those fibers are called hybrid fibers and have been shown to increase with exercise, aging (Moreillon et al., 2019), and pathological conditions. (Fronters, & Ochala., 2014). Examples of hybrid types include type IC, IIC, IIAC, and IIAB. (Types I, IC, IIC, IIAC, IIA, IIAB, and IIB arranged from slowest to fastest).

Whole muscle is classified as slow or fast twitch based on the speed of shortening, then skeletal muscle fibers are further classified by using three different methods: myosin ATPase histochemistry, myosin heavy chain identification, and biochemical identification of metabolic enzymes (Scott., 2001). Myosin ATPase histochemistry classifies the fibers into type 1 or type 2 which are known to correspond with slow and fast twitch muscle fibers. Myosin heavy chain isoform identifies MHCI, MHCIIa, and MHCIIx that correspond to isoforms identified by myosin ATPase (Staron., 1997). Biochemical classification is used to combine information on muscle fiber myosin

ATPase histochemistry and qualitative histochemistry for certain enzymes that reflect the energy metabolism of the fiber (Pette, et al., 1997). The analyzed enzyme reflects metabolic pathways are either aerobic/ oxidative or anaerobic/glycolytic (Pette et al., 1999) resulting in three fiber types: fast-twitch glycolytic, fast-twitch oxidative, and slow-twitch oxidative.

Oxidative muscle fibers are metabolically tuned for lactate oxidation and have a greater capacity for sarcolemmal lactate transport than glycolytic fibers do (Gladden., 2000) reflecting the role of lactate as an energy substrate. Glycolytic fibers primarily convert lactate to glycogen and may contribute to the higher levels of circulating lactate. (Gladden., 2000). Type I fibers have high volumes of mitochondria, increased lipid storage capacity and oxidation, and greater insulin sensitivity, and they consume ATP at a slow rate. Thus, they are referred to as slow-twitch oxidative muscle fibers. Type IIa are also considered oxidative but have a lower oxidative capacity and consume ATP at an intermediate rate. Thus, IIa are often referred to as fast-twitch oxidative. Together type I and type IIa fibers are sites for lactate oxidation (Brooks., 1985) Type IIx fibers are considered fast twitch as they consume ATP at the highest rate. They are also considered glycolytic. Type IIx fibers are a site for lactate formation (Brooks., 1985). Brooks presented a model of shuttling lactate from glycolytic to oxidative fibers. An elevated metabolic rate is associated with a faster glycolytic rate and concomitant lactate production (Gladden., 2000). However, the rate of lactate consumption depends on fiber type distribution. In healthy individuals, skeletal muscle is composed nearly of an equal number of slow-twitch and fast-twitch muscle fibers (Stuart et al., 2013). A shift in fiber distribution from slow twitch to fast twitch gives rise to altered activities of key

oxidative and glycolytic enzymes (Pette, & Hofer., 1980). That type of shift could impair metabolic activity.

Fiber type may be a risk factor in the development of obesity and/or type 2 diabetes as fewer type I fibers have been found in individuals with a metabolic disease. It is likely that type I muscle fibers play a more important role in maintaining glucose homeostasis. Studies suggest that there is a positive correlation between the proportion of type I fiber and whole-body insulin sensitivity (Albers et al., 2015). Decreased type I fibers have been found in patients with metabolic syndrome and obesity. A higher level of type IIa and IIx fibers could result in insulin resistance and/or explain the elevated lactate associated with these metabolic conditions. Therefore, the purpose of this study was to observe how lactate levels relate to muscle fiber type distribution and factors of metabolic syndrome. We hypothesized that individuals with fewer type I fibers will show higher levels of lactate and are more at risk for developing metabolic syndrome.

# Chapter 3 Methods

## Participants

This study recruited 32 individuals both men and women who were healthy and considered overweight (BMI 25.0-29.9 kg/m2), sedentary, and in the age range of 20-49 years old.

## Materials

To determine plasma lactate concentrations a sterile lactate test strip was used to collect a sample by collecting a blood droplet from the participant's finger, which was then analyzed by a lactate meter (Lactate Plus, Waltham, MA) device. A Polar heart rate monitor (Kempele, Finland) was be used to record resting heart rate. Baseline blood pressure was manually assessed using the sphygmomanometer, (Welch Allyn, Skaneateles Falls, NY) and a stethoscope (Littmann, USA), and baseline pulse was determined by using a fingertip pulse oximeter (Fabrication Enterprises, Elmsford, NY). To measure REE and the respiratory exchange ratio ($VCO_2/VO_2$,), ParvoMedics True 2400 metabolic cart (East Sandy, UT) was used for indirect calorimetry. The metabolic software installed on the metabolic cart analyzed the $O_2$ and $CO_2$ flow/volume ratio. Dual-energy X-ray absorptiometry (DEXA; Horizon, Danbury, CT) was used to assess the body composition of the participant. An Excalibur Sports cycle ergometer (Lode, Netherlands) was used during exercise for $VO_2$ max test and submaximal exercise. 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, and a value of ≥12 μIU/ml indicated hyperinsulinemia. A muscle sample was collected via a *vastus lateralis* leg muscle biopsy. The frozen muscle sample was sectioned with

the use of a cryostat. The sections were then stained by following a fiber typing protocol. Slides were then imaged with the use of the fluorescent microscope (Leica DM4000B). The fluorescent images were used to count and differentiate fiber types manually.

**Protocol**

Screening

To recruit participants a flyer was posted in places like ECU Announce List, Pirate 411, Brody Graduate School, Pitt Community College, School of Allied Health, and the School of Nursing at ECU. These recruitment strategies resulted in positive responses indicating a willingness to participate in the study. The prospective participants were contacted via telephone to complete a prescreening to determine if they met the criteria for this study. The criteria for this study included having a Body Mass Index (BMI) 25.0-29.9 kg/m2 (classified as overweight) no changes in weight for the last 3 months, not currently following a specific diet, and not suffering from any health conditions such as cancer, uncontrolled hypertension, gastrointestinal (GI) disease, psychiatric disorders, or diabetes. Anxiety medications were included in the exclusion criteria. The subjects who fit the criteria for the study were invited to the Human Performance Lab (HPL) at East Carolina University where they filled out consent forms and different questionnaires such as an informed consent form, an IRB-approved personal family form, physical activity questionnaire, DEXA radiation consent form, and a menstrual cycle questionnaire when relevant. Height (m) and weight (kg) were measured to calculate BMI. Heart rate was measured via a finger pulse oximeter and blood pressure was taken manually using a stethoscope and a stationary

sphygmomanometer. A non-dominant hand was used to collect a blood sample for lactate analysis. A one-time use sterile lancet needle was used to puncture the fingertip, lactate strip was then used to collect the blood and placed into the Lactate meter (Lactate Plus, Waltham, MA) device for analysis. Levels of blood lactate were measured twice to ensure that the levels were within 0.3 mmol/L. If the difference between the two readings was greater than 0.3 mmol/L an additional measurement was taken. An additional blood sample was taken, which then was sent to LabCorp for analysis. Blood sample analysis included triglyceride, glucose, insulin, and HDL levels,

Visits

During the first visit, participants are asked to come in after fasting for at least 10-12 hours. This means the participants were to refrain from food, stimulants (including caffeine, nicotine, and certain medications), and alcohol before the visit. The first visit included body composition measurements via DEXA and 3-D scanning. The results of the DEXA scan provided an android/gynoid ratio and visceral adipose tissue (VAT) mass. The participants also performed a maximal exercise test on the cycle ergometer. The second visit included measuring the Resting Metabolic Rate (RMR), a submaximal test to measure the rate of oxygen consumption and carbon dioxide production. Blood samples were collected and processed via a Beckman-Coulter hematology analyzer to obtain concentrations of fasting insulin, glucose, and lactate. The oxygen utilization was recorded from the NIRS analysis which was transferred to Microsoft Excel for calculation. During the third visit, a muscle biopsy was performed.

Muscle biopsy

The participants were asked to dress in loose-fitting clothing for easier access to the biopsy site. During this visit, the weight of the participant was measured. The clinical site staff performed the Biospecimen Pre-Collection Assessment CRF to assess if a muscle biopsy can be performed. The participants were told to lay supine on the bed for at least 30min prior to the procedure. The biopsy was taken from the left of the participant approximately one hand width from the superior pole of the patella. The area for the biopsy was disinfected with an appropriate antiseptic. To numb the incision site lidocaine HCL was delivered by a certified operator into the skin/ subcutaneous tissue and muscle fascia. The clinical team waited 5 minutes to allow the anesthetic to numb the biopsy site. Using a no.11 scalpel, the operator made a small (appx. 5mm) incision through the top layers of skin, subcutaneous tissue, and muscle fascia. A sterile 5 or 6-mm Bergstrom biopsy needle was connected to a syringe for suction, and a muscle sample was collected from the belly of the muscle. The biopsy needle was removed and reported on the Muscle Connection CRF. A sterile gauze was placed over the incision site and firm pressure was applied for 5-10 minutes until hemostatic. After collection, the muscle sample was placed on a clean surface to avoid cross-contamination. Any leftover adipose/ connective tissue was removed. The muscle sample was placed on the gauze to remove excessive/ visible blood. The weight of the muscle sample was measured to determine if a second pass is necessary. If the first, try did not collect the required amount of 150g a second pass was required. The muscle sample was divided into aliquots. For this study, muscle samples were placed in a plastic mold filled with O.C.T (tissuetek, Fisher Scientific) and frozen using liquid nitrogen. Once the tissue was

frozen it was wrapped in tin foil or parafilm and was stored in a -80˚C freezer until sectioning.

Frozen Sectioning

Gloves were worn during the time tissue was handled in adherence to ECU bloodborne pathogen precautions. Attach the tissue block to the specimen holder, then place the holder on the microtome and tighten the knob to avoid movement. Adjust the orientation of the block with the loosening clamping lever and adjusting and retightening lever. Use the retraction button to move the specimen all the way back. The shorter the arm is, the more stable it is. Loosen the blade holder with a lever on the right side. Adjust the blade holder position so that it is close to the specimen, but not touching it. Tighten lever. Unlock the handle and turn it clockwise to advance the specimen. Start sectioning on Trim Thickness until you reach the desired area of your specimen. Switch back to “Sectioning Thickness” when you reach the tissue. Place the anti-roll plate against the blade and turn the handle clockwise in a slow smooth motion. The section should go between the anti-roll plate and the blade. Once a section is on the blade, gently pull back the anti-roll plate. Position the slide frosted slide toward the blade close enough to allow the section to melt onto it. Hold the slide steady to avoid wrinkles. If a section is messed up, just wipe it off and add another. The blade needs to be cleaned with a paintbrush after each section. Unfixed tissue slides should be placed in a small slide box inside the cryostat. All slides should be stored between -20 and -80$^{o}$C. (Joani Zary, BSOM Histology Core), Leftover O.C.T with the tissue “scrapes” was removed and the cryostat was fully cleaned to avoid cross-contamination. The cryostat was programmed to go into self-sterilizing mode.

Fiber Typing Protocol

Day 1

Slides were taken out of the -20˚C freezer and left at room temperature for 5 minutes. During that time mixture of 1:1 methanol and acetone was prepared. After 5 minutes slides were placed in a Coplin jar that contained a methanol and acetone mixture then placed in a -20˚C freezer for 5 minutes to fix. Slides were rinsed in 1x PBS and dried with a Kim wipe after. Slides were set in a slide box where 40ul/ section of blocking buffer (5% goat serum) was added and left at room temperature for 60-90 minutes. After blocking the blocking buffer was wiped off and all primary antibodies myosin heavy chain (MHC) I (DSHB-#BA-FC, mouse IgG2b, Life Technologies 1:50), IIa (DSHB-#SC-71C, mouse IgG1, Life Technologies 1:50), and Dystrophin (Ab275391 rabbit IgG Abcam1:100) were added to the slide. The slide box was carefully placed in a 4˚C fridge overnight with a sign that said “Do not move this box” as it is crucial to avoid any movement to the box.

Day 2

Slides were taken out of the fridge and placed in a Coplin jar where they were rinsed (3x5min) with 1x PBS and then dried with a Kim wipe. Slides were placed back in the slide box where all secondary antibodies Alexa Fluor 350 goat-anti-mouse IgG2b (#A-21140, Life Technologies, 1:250), Alexa Fluor 488 goat-anti-mouse IgG1 (#A-21121 Life Technologies, 1:250), Alexa Fluor 555 goat-anti-rabbit IgG (#A27039 Invitrogen, 1:250), will be added and left for 60min in the dark. After that, they will be placed back into a Coplin jar and rinsed (3x5min) with 1x PBS and dried with a Kim wipe. Then slides will be placed back into the slide box and 1 drop of Vectrashield was added to the

slide and a coverslip was placed on top. Slides were left in a 4˚C fridge and in the dark for 20 minutes before imaging.

Imaging

The slides were imaged via a Leica microscope with a camera attached. The cubes used were DAPI to distinguish type I fibers (blue), GFP to distinguish type 2 fibers (green), and CY3 to distinguish the outline of the fibers (red). The filters were merged in Image Pro Express software to show all the fiber types in one image. The brightness and contrast of the image or individual filters were adjusted as needed to get the best image possible.

Image Analysis

Each image was manually quantified. First, the number of all fibers present was counted and entered into Microsoft Excel, fibers that were stained for type I fiber (Blue color), were counted and entered into Microsoft Excel. Fibers that were stained for type II fiber (Green color) were counted and entered into Microsoft Excel. A percentage was taken for each fiber type to ensure accurate correlation.

Statistical Analysis

Each image was manually quantified, and the data was put into Microsoft Excel. To best predict fiber type, the statistical analysis of multiple regression and correlation was used. To check for normality Shapiro-Wilk test was performed. If the data seemed normal ($P<0.05$) Pearson correlation was then performed to assess associates between fiber types and factors of the metabolic syndrome and lactate. If the data was not normally disturbed($p>0.05$) the Spearman correlation was then performed. Statistical analysis was performed using JMP®, Version 17 PRO (SAS Institute Inc).

# Chapter 4 Results

## Results

Participants (n=32) consisted of both men (n=16) and women(n=16); the average age was 29±7 years. Subjects were classified as overweight with an average BMI of 27.2±1.9. Subject characteristics *(Table 1A)* and blood metabolite *(Table 1B)* profiles are available below. The distribution of fiber type *(Figure 1A and 1B)* showed no difference among genders; type I fibers (p=0.31) and type II fibers (p=0.27).

Upon associating fiber type with lactate and other factors of metabolic syndrome, the following results were present. Lactate (mmol/L) was negatively associated with type I fibers (r=-0.44, p=0.01) *(Figure 2A)* and positively associated with type II fibers (r=0.43, p=0.02) *(Figure 2B)*. Triglyceride levels (mg/dL) were negatively associated with type I fibers (r=-0.36, p=0.05) *(Figure 3A)* and positively correlated with type II fibers (r=0.36, p=0.04) *(Figure 3B).* HDL (mg/dL) was positively associated with type I fibers (r=0.42, p=0.02) *(Figure 4A)* and negatively associated with type II fibers (r=-0.41, p=0.02) *(Figure 4B)*, HOMA-IR was negatively associated with type I fibers (r=-0.54, p=0.002) *(Figure 5A)* and positively associated with type II fibers (r=0.53 p=0.002) *(Figure 5B)*. Although the measure of systolic blood pressure showed no significant correlation between either fiber type (type I fibers r=-0.24, p=0.19) *(Figure 7A)* (type II fibers r=0.23, p=0.20) *(Figure 7B)* measure of diastolic blood pressure showed a significant negative relationship between type I fibers (r=-0.39, p=0.03) *(Figure 6A)* and significant positive relationship between type II fibers (r=0.39, p=0.03) *(Figure 6B)*. As for body composition Android/ Gynoid Ratio was negatively associated with type I fibers

(r=-0.49, p=0.01) *(Figure 8A)* and positively associated with type II fibers (r=0.49, p=0.01) *(Figure 8B)*. VAT Mass (g) was negatively associated with type I fibers (r=-0.58, p=0.001) *(Figure 9A)* and positively associated with type II fibers (r=0.56, p=0.001) *(Figure 9B)*. Body fat percentage did not show any significance, (r=-0.13, p=0.49) *(Figure 10A)* and (r=0.11, p=0.54) *(Figure 10B)* respectively. $VO_2$ max (L/min) was positively associated with type I fibers (r=0.32 p=0.08) *(Figure 11A)* and negatively associated with type II fibers (r=-0.32, p=0.08) *(Figure 11B)* although those results were not significant.

| | Mean | Range |
|---|---|---|
| Sex | m=16, f=16 | |
| Age (yrs) | 29 ± 7 | 20 - 49 |
| BMI (KG/m2) | 27.2 ± 1.9 | 23.4 - 30.5 |
| Body Fat Percentage | 30.8 ± 7.7 | 19.6 - 46.0 |
| VAT Mass (g) | 404 ± 136.8 | 176 - 739 |
| Android/Gynoid Ratio | 0.94 ± 0.17 | 0.58 - 1.37 |
| VO2 Max (l/min) | 2.69 ± 0.82 | 1.26 - 4.56 |
| Systolic Blood Pressure (mmHg) | 119 ± 14 | 90 - 144 |
| Diastolic Blood Pressure (mmHg) | 75 ± 11 | 52 - 97 |
| Type I Fibers (%) | 36 ± 12.7 | 14-63 |
| Type II Fibers (%) | 63 ± 12.5 | 36-86 |
| Lactate (mmol/L) | 1 ± 0.56 | 0.20 - 3.21 |
| Triglyceride (mg/dL) | 104.28 ± 83.25 | 39 - 464 |
| HDL (mg/dL) | 52.97 ± 12.72 | 30 - 87 |
| HOMA-IR | 2.2 ± 1.3 | 0.6 - 6.1 |

*Table 1: Subject Characteristics and Blood Metabolites (n=32) (Mean ± SD)*

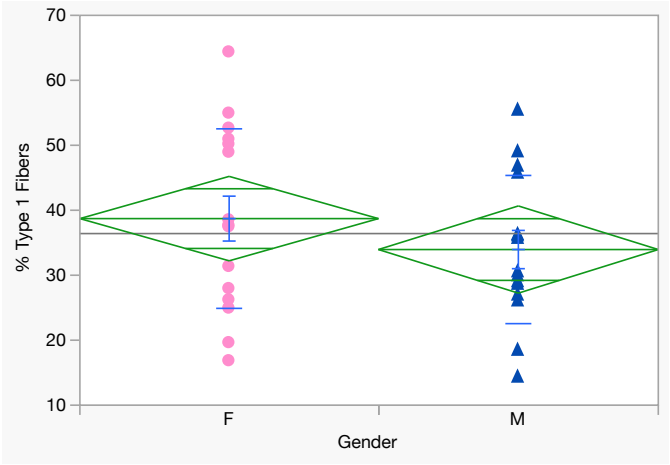


*Figure 1a Distribution of type I fibers among men (blue triangle), and women (pink circle). p=0.31, r=*

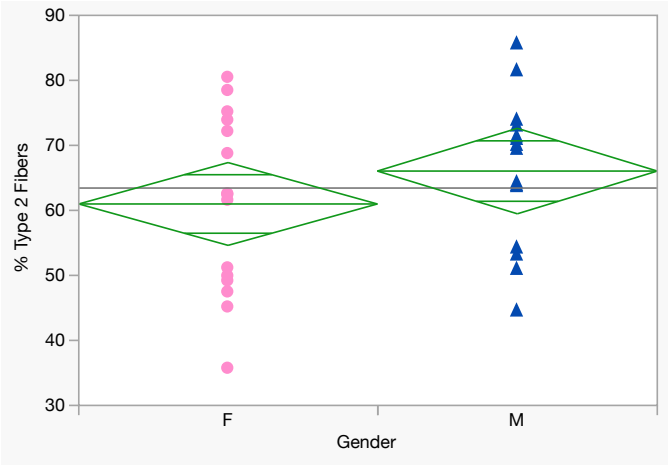


*Figure 1b Distribution of type II fiber among men (blue triangle) and women (pink circle) p=0.27, r=*

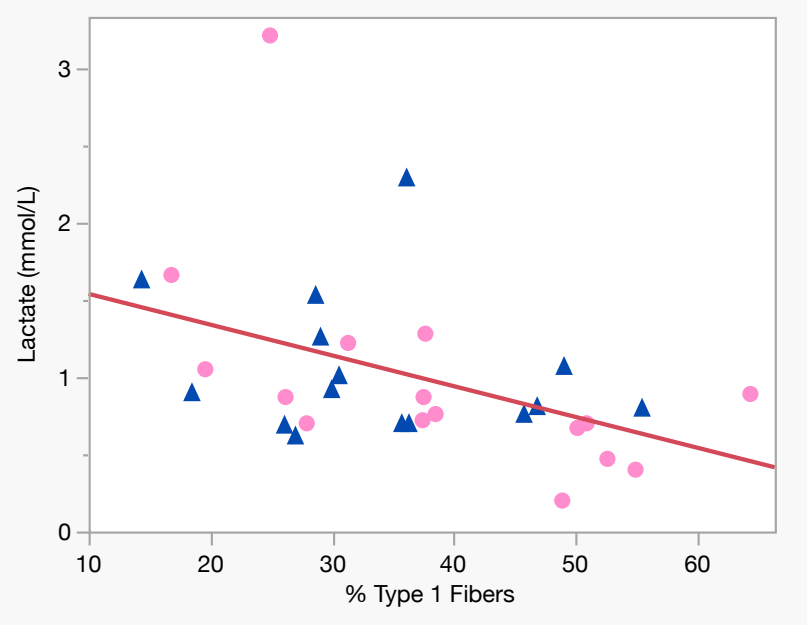


*Figure 2A: Strong negative correlation of lactate and percentage type I fibers. men (blue triangle), and women (pink circle). p=0.01, r=-0.44.*

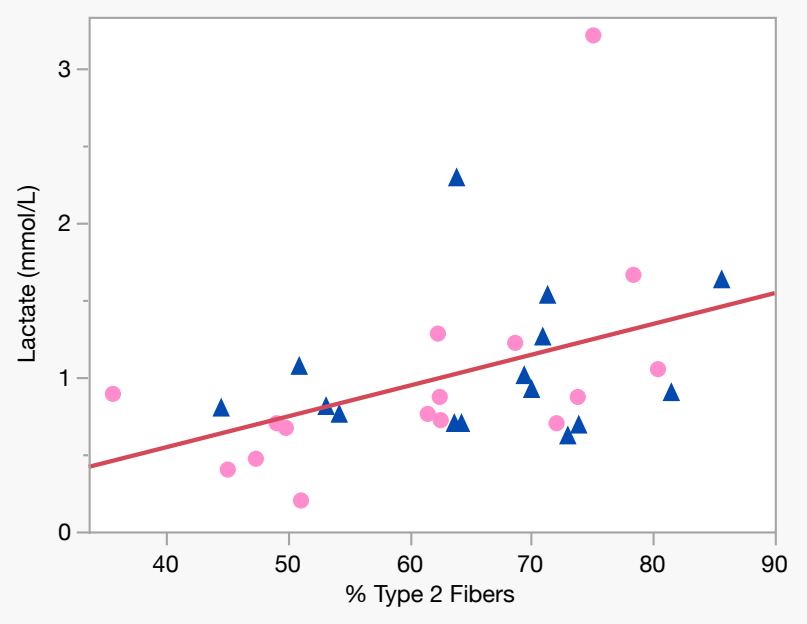


*Figure 2B: Strong positive correlation of lactate and percentage type II fibers. men (blue triangle), and women (pink circle. p=0.02, r=0.43.*

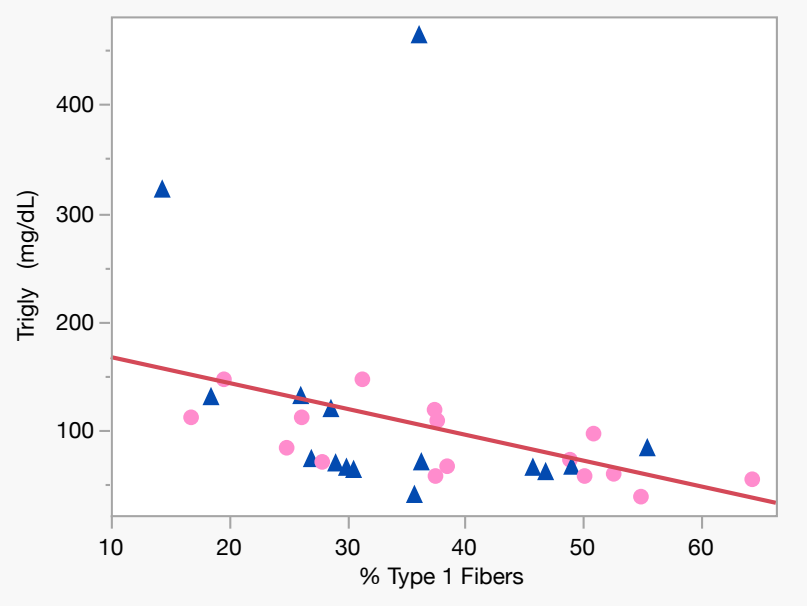


*Figure 3A: Strong negative correlation of triglyceride level (mg/dL) and percentage of type I fibers. men (blue triangle), and women (pink circle). p=0.05, r=-0.36.*

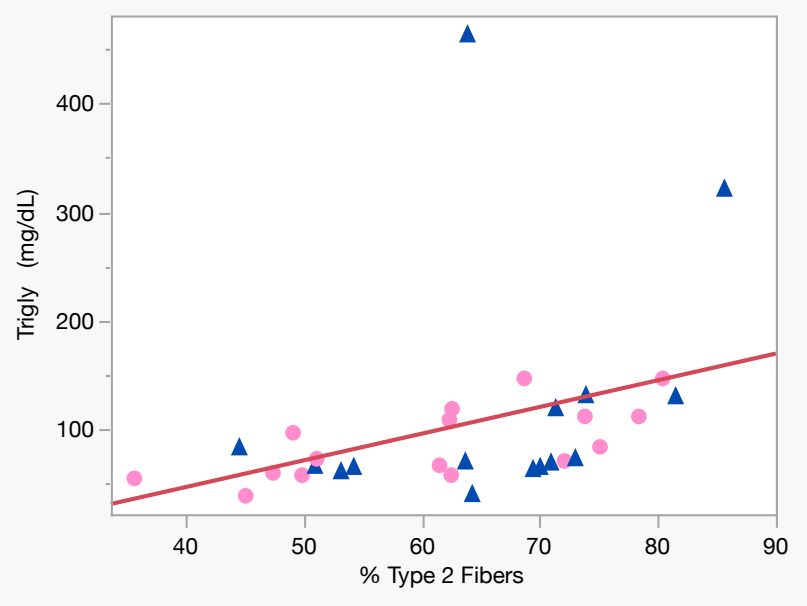


*Figure 3B: Strong positive correlation of triglyceride level (mg/dL) and percentage type II fibers. men (blue triangle), and women (pink circle). p=0.04, r=0.36.*

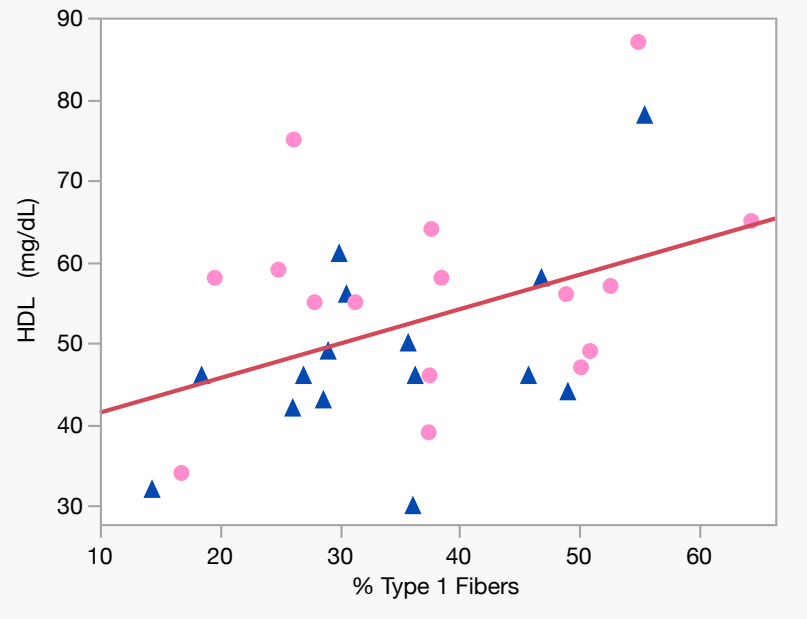


*Figure 4A: Strong positive correlation of HDL levels (mg/dL) and percentage of type I fibers. men (blue triangle), and women (pink circle). p=0.02, r=0.42*

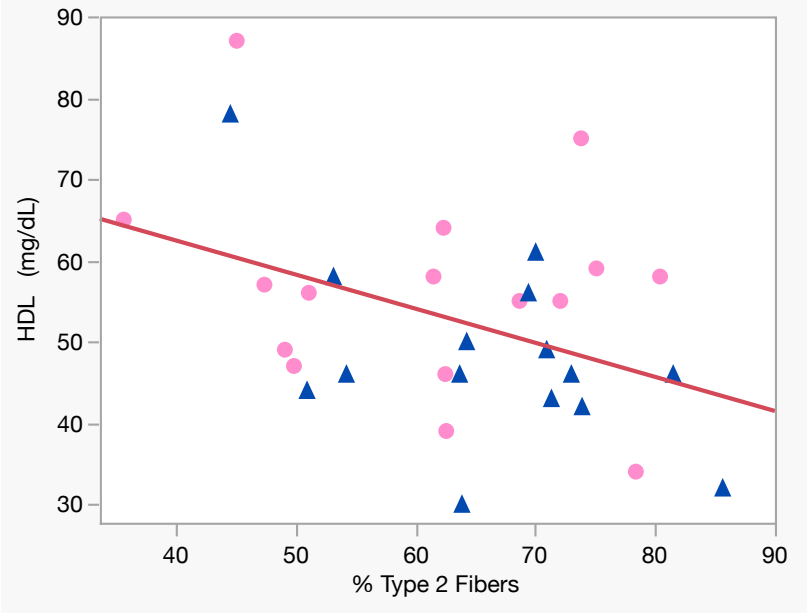


*Figure 4B: Strong negative correlation of HDL levels (mg/dL) and percentage of type II fibers. men (blue triangle), and women (pink circle). p=0.02, r=-0.41.*

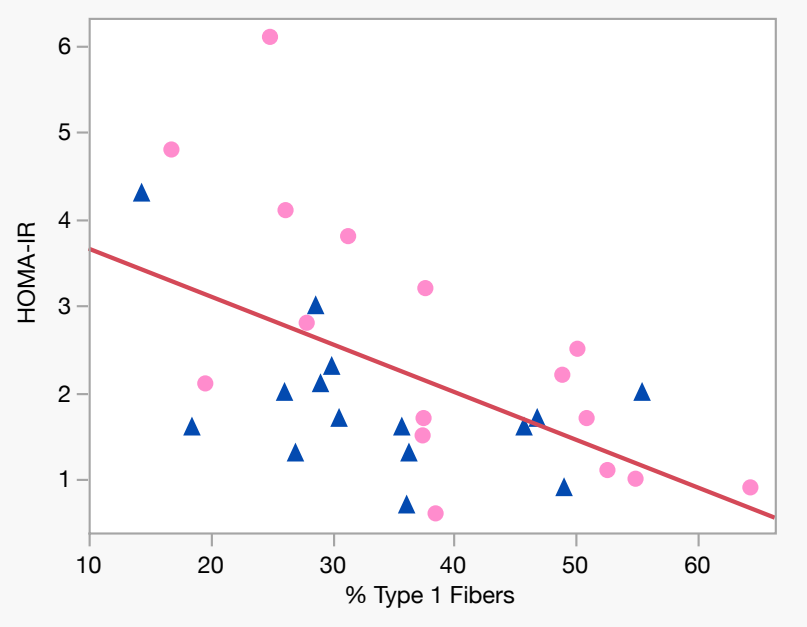


*Figure 5A: Strong negative correlation of HOMA-IR and percentage of type I fibers. men (blue triangle), and women (pink circle). p=0.002, r=-0.54.*

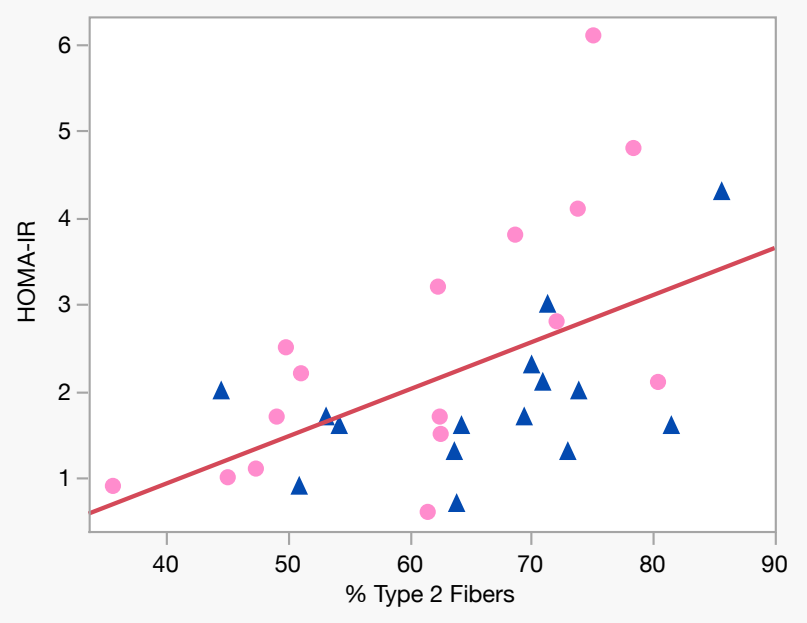


*Figure 5B: Strong positive correlation of HOMA-IR and percentage of type II fibers. men (blue triangle), and women (pink circle). p=0.002, r=0.53.*

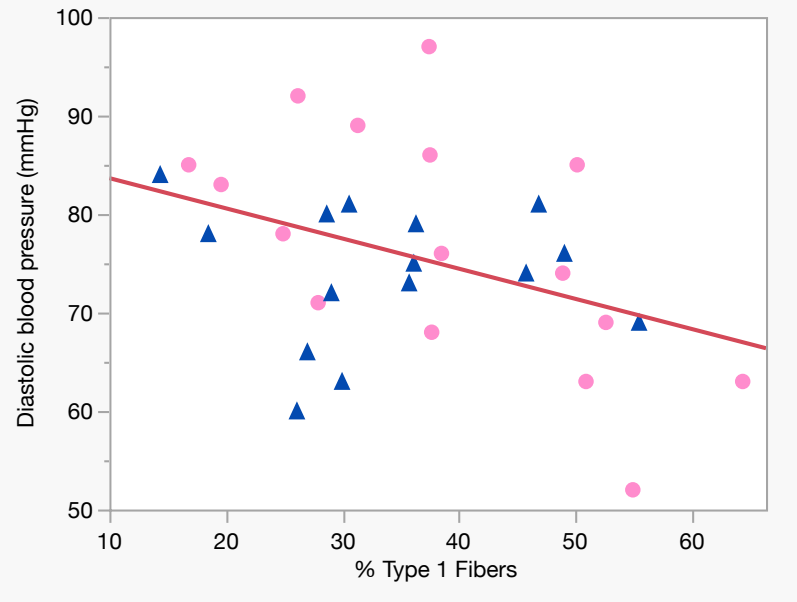


*Figure 6A: Strong negative correlation of diastolic blood pressure and percentage of type I fibers. men (blue triangle), and women (pink circle). p=0.03, r=-0.39.*

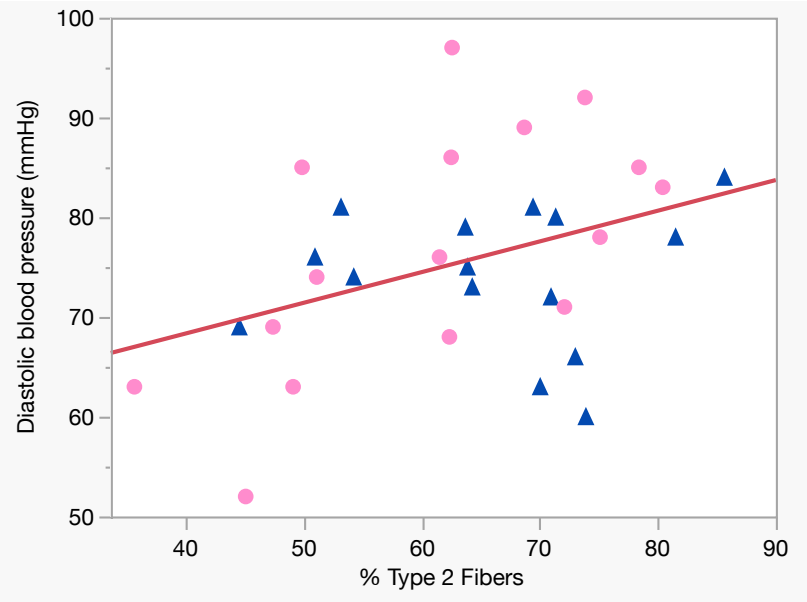


*Figure 6B: Strong positive correlation of diastolic blood pressure and percentage of type II fibers. men (blue triangle), and women (pink circle). p=0.03, r=0.39.*

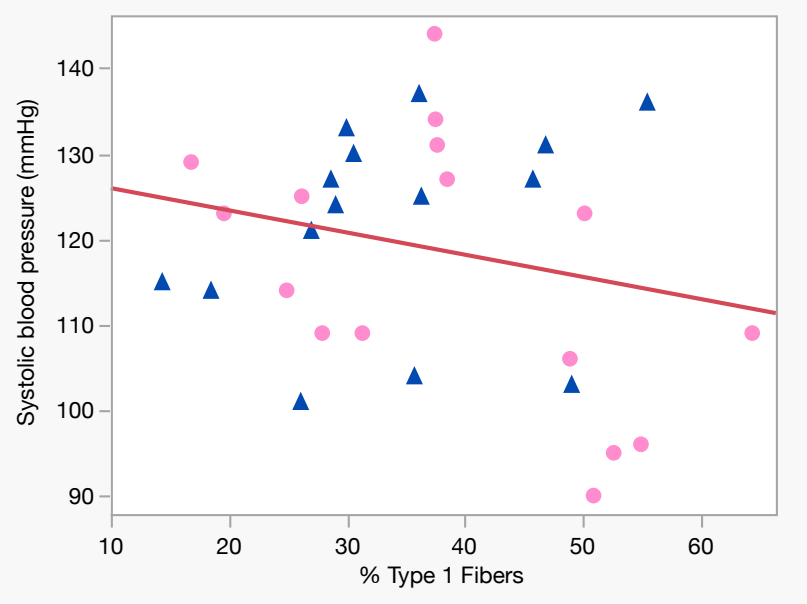


*Figure 7A: Nonsignificant correlation between systolic blood pressure and percentage of type I fibers. men (blue triangle), and women (pink circle). p=0.19, r=-0.24.*

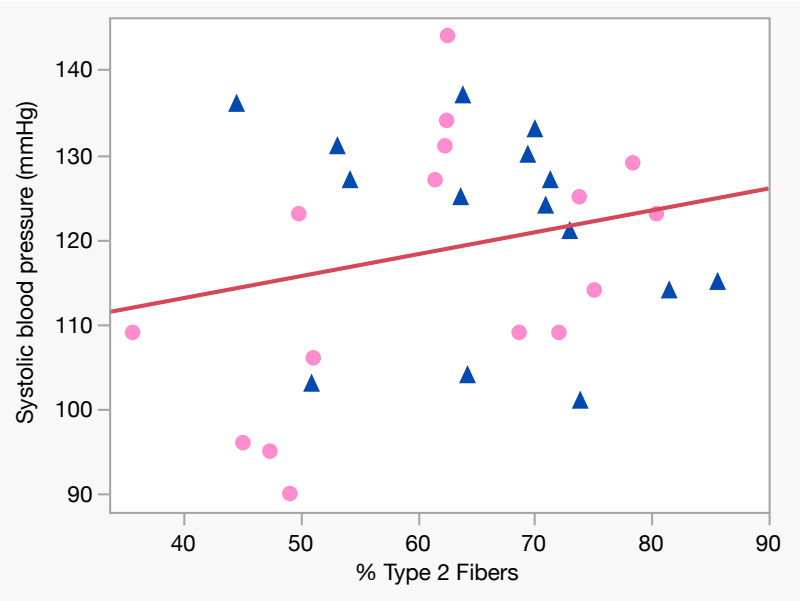


*Figure 7B: Nonsignificant correlation between systolic blood pressure and percentage of type II fibers. men (blue triangle), and women (pink circle). p=0.20, r=0.23.*

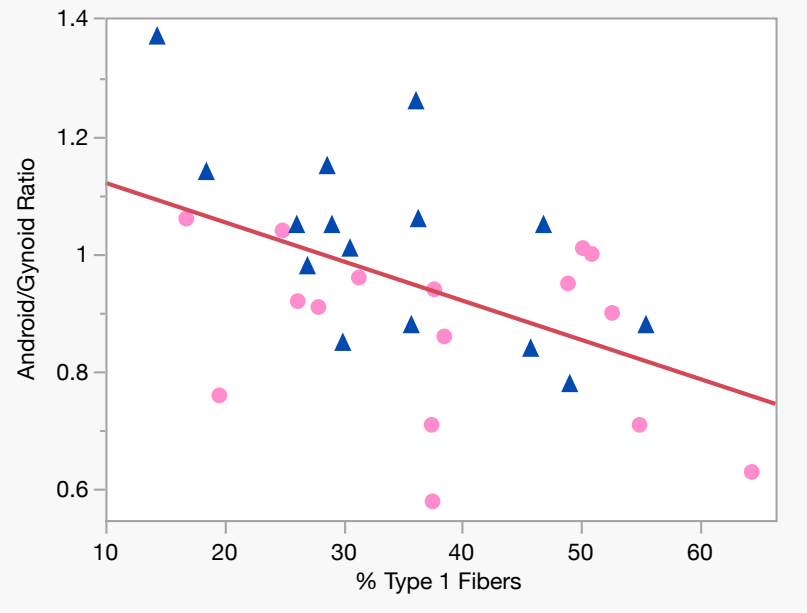


*Figure 8A: Strong negative correlation of Android/Gynoid ratio and percentage of type I fibers. men (blue triangle), and women (pink circle). p=0.01, r=-0.49.*

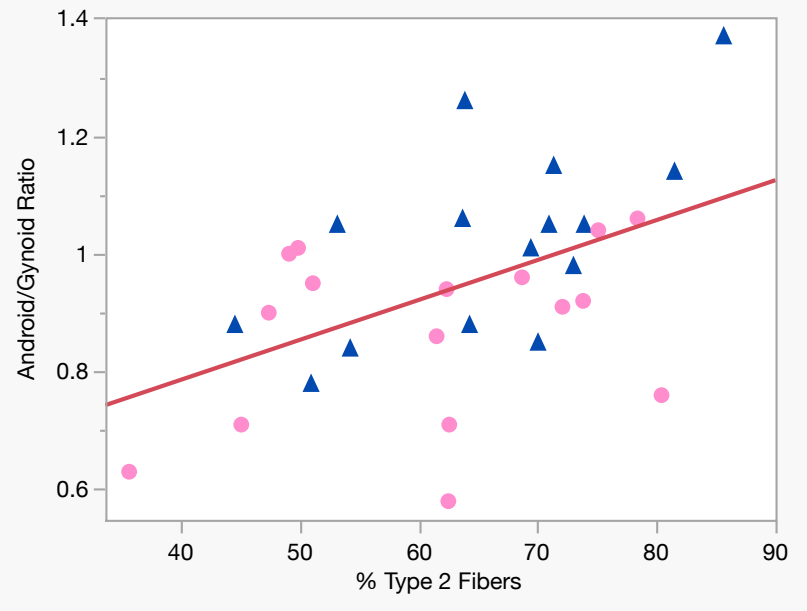


*Figure 8B: Strong positive correlation of Android/Gynoid ratio and percentage of type II fibers. men (blue triangle), and women (pink circle). p=0.01, r=0.49.*

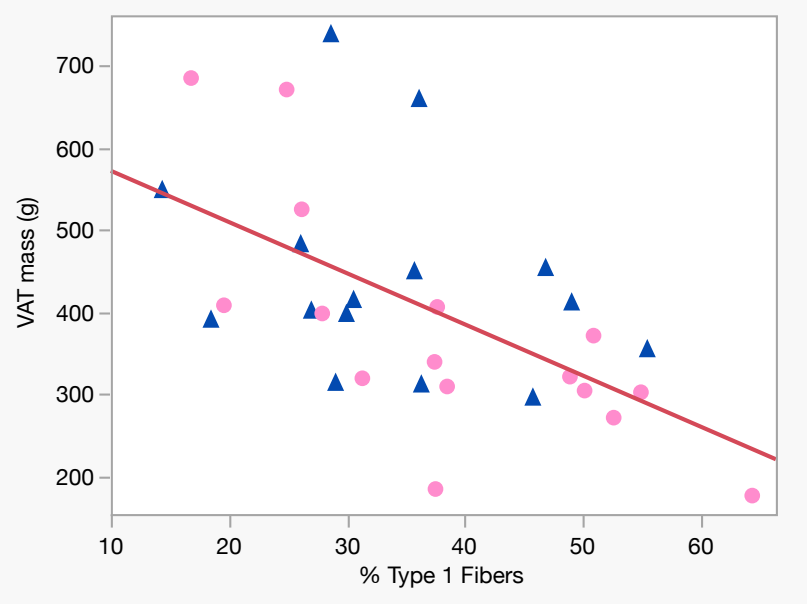


*Figure 9A: Strong negative correlation of VAT mass (g) and percentage of type I fibers. men (blue triangle), and women (pink circle). p=0.001, r=-0.58.*

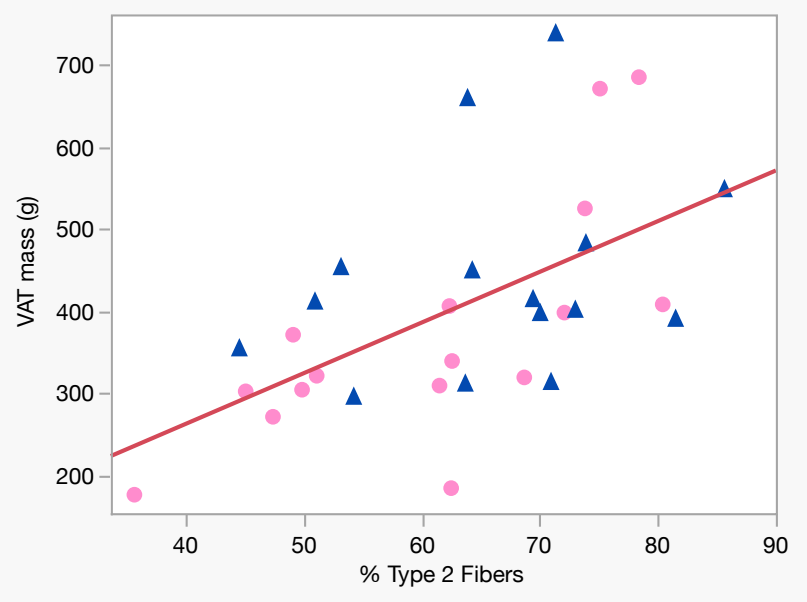


*Figure 9B: Strong positive correlation of VAT mass (g) and percentage of type II fibers. men (blue triangle), and women (pink circle). p=0.001, r=0.56.*

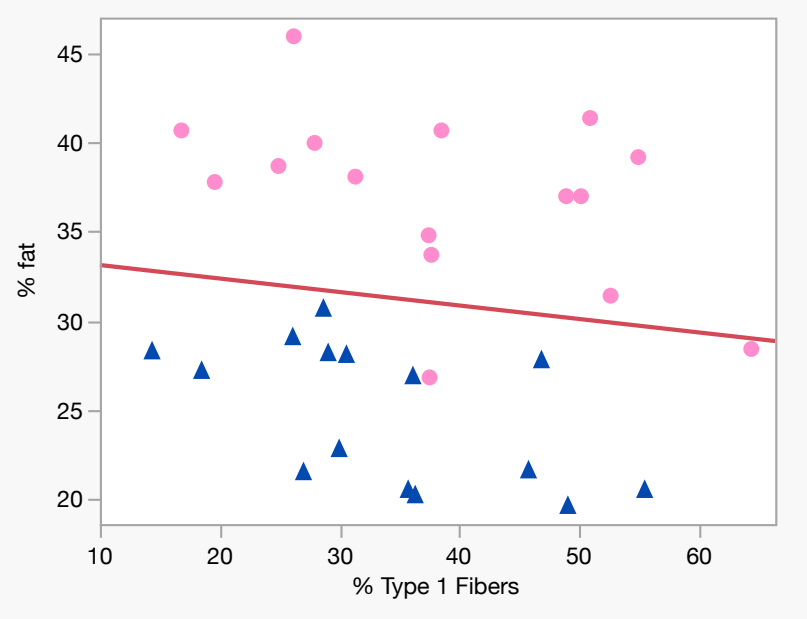


*Figure 10A: No correlation between the percentage of body fat and the percentage of type I fibers. men (blue triangle), and women (pink circle). p=0.49, r=-0.13.*

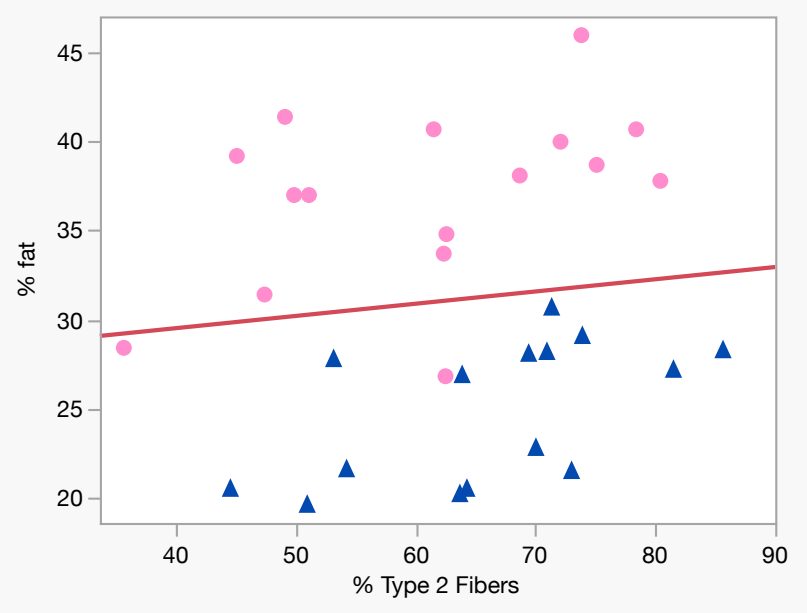


*Figure 10B: No correlation between the percentage of body fat and the percentage of type II fibers. men (blue triangle), and women (pink circle). p=0.54, r=0.11.*

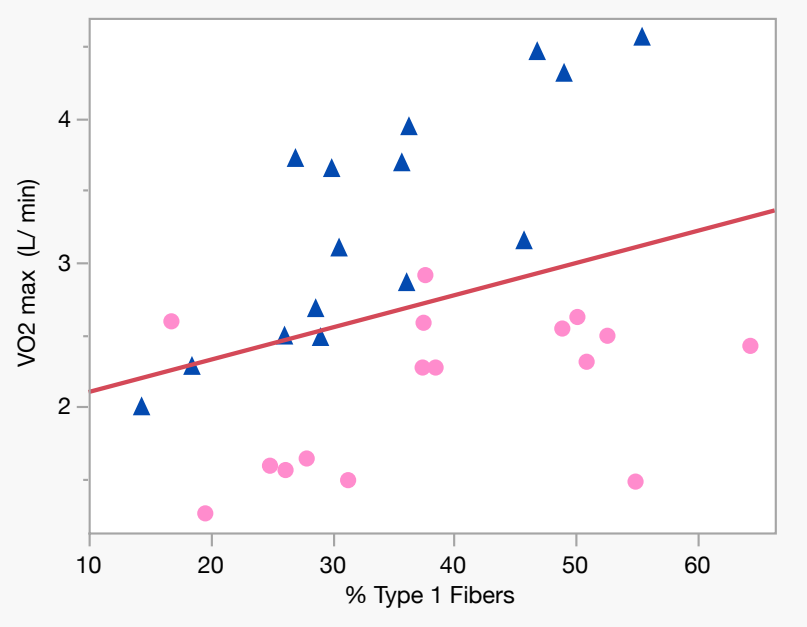


*Figure 11A: Positive non-significant correlation of VO2 max (L/min) and percentage of type I fibers. men (blue triangle), and women (pink circle). p=0.08, r=0.32.*

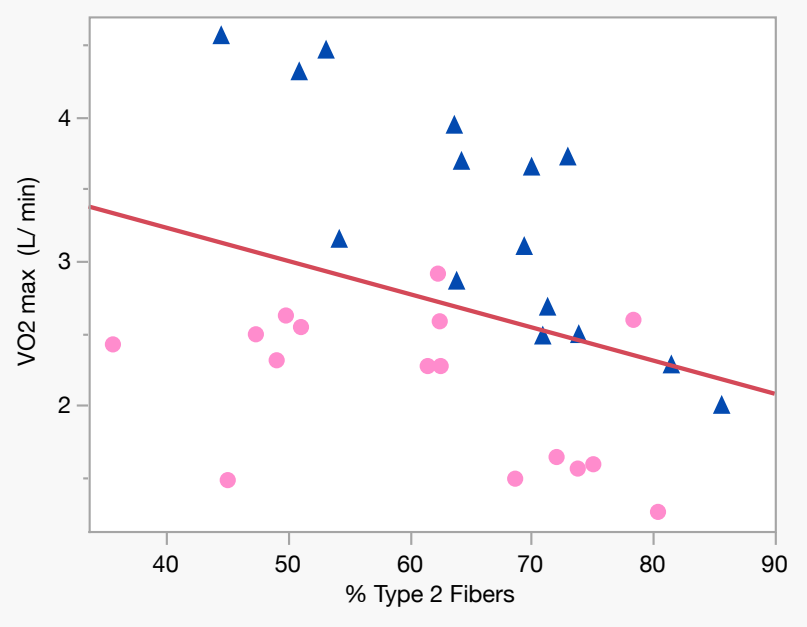


*Figure 11B: Negative non-significant correlation of VO2 max and percentage of type II fibers. men (blue triangle), and women (pink circle). p=0.08, r=-0.32.*

# Chapter 5 Discussion

## Discussion

The purpose of this study was to examine the association of fiber type distribution with lactate levels and other factors of the metabolic syndrome. It found that there is a statistically significant relationship between fiber type distribution with lactate levels, and several factors of the MetS. The results showed that there is a significant relationship between fiber type distribution and lactate. Results showed a positive correlation with the percentage of type II fibers and a negative correlation with the percentage of type I fibers. Additional findings demonstrated a significant negative relationship between higher levels of HDL and the percentage of type II fibers. Another significant finding displayed a positive relationship between the percentage of type II fibers with factors of MetS such as triglyceride levels, HOMA-IR levels, diastolic blood pressure, android/gynoid ratio, and VAT mass. Supplementary results that did not show significance included body fat percentage, $VO_2$max, and systolic blood pressure. Body fat percentage did not show any relationship regarding fiber type distribution. $VO_2$max exhibited a negative relationship with the percentage of type II fibers. Systolic blood pressure showed a positive relationship with the percentage of type II fibers.

### The relationship between fiber types and lactate

The relationship between plasma lactate concentrations and fiber type distribution is an interesting one. Studies have shown that both lactate and skeletal muscle play a key role in metabolism. Elevated plasma lactate levels are evident in cardiometabolic disease (Crowford et al., 2010) and a shift in fiber type distribution (type I to type II) can affect muscles' metabolic activity (Pette, & Hofer., 1980). That is why the hypothesis

that individuals with higher plasma lactate levels will have fewer type I fibers was formed. The results of this study support our hypothesis. This is the first study to examine the association of lactate with fiber type distribution in an overweight but relatively healthy population of men and women. Another study to examine subjects who are at a higher risk of developing metabolic disease indicated that metabolically impaired individuals (i.e overweight, sedentary) had significantly higher fasting plasma lactate levels than compared non-obese group (Broskey et al., 2019). Previous studies have also shown that higher levels of lactate are strongly correlated with markers of insulin resistance and play a role in the pathogenesis of type 2 diabetes (Juraschek et al., 2013).

The relationship between fiber types and HOMA-IR

Previous studies on fiber type distribution focused mostly on its association with other cardiometabolic factors, which showed that a higher number of type I fibers is associated with healthier cardiometabolic phenotype (Fisher et al., 2017) and type I fibers are associated with greater insulin sensitivity and lower blood pressure. Our results support this statement. Our study found that higher HOMA-IR values were correlated with a higher percentage of type II fibers indicating that type II fibers are associated with weaker insulin sensitivity. In their research, De Jalon and colleagues (2022) indicated that over time there was a sustained relationship between HOMA levels and MetS. Another study reported that even non-diabetic insulin resistant MetS subjects had significantly fewer type I fibers when compared with the control group (Stuart et al, 2013). So far, most studies done on insulin resistance focused on either a population that had MetS or a population that was obese. Since our study focused on

an at-risk population, it is interesting to see a similar correlation and it has been shown that HOMA-IR can be a useful tool in identifying patients with an increased metabolic and cardiovascular risk (De Jalon et al., 2022).

The relationship between fiber type and adiposity.

A lot of available literature focuses on obesity and its effects on factors of the MetS as well as fiber type. To define the interrelation between muscle fiber type, insulin action, and obesity, researchers revealed a weak relationship between insulin action and the percentage of type II fibers, a correlation between insulin action and oxidative capacity showing that the percentage of type I fibers was significantly related to the oxidative capacity of the muscle. They also found that the degree of obesity correlated negatively with the percentage of type I fibers. (Kierotkos et al., 2006). Our research could potentially extend these findings as it focused on a different population that had a similar fiber type distribution profile. Another study (Tanner et all, 2002) showed that there is a relationship between fiber type and obesity. Their study also found that BMI was positively related to a higher percentage of type II fibers, although our study did not find the same association between BMI and the percentage of type II fibers. However, our study supported the evidence that adiposity especially the android/gynoid ratio and VAT mass are positively related to a higher percentage of type II fibers. It is important to add that visceral fat accumulation plays a role in the development of MetS through insulin resistance and chronic low-grade inflammation (Son et al., 2022). The difference in these results could come from the different populations being studied, as we focused on a population classified as overweight, and Tanner (et al., 2002) focused on a population with obesity. A study on exercise training and insulin action in men with

morbid obesity demonstrated that exercise training alone reduced fasting and two-hour insulin concentration in obese men indicating an improvement in insulin action (Hickey et al., 1999). It is possible that these results could be relevant to our population. Since our subject pool was sedentary, an increase in physical activity could help reduce insulin concentration, and it has other various health benefits that could help our population in the prevention of developing MetS.

The relationship between fiber type blood pressure.

Another important aspect of MetS is elevated blood pressure. Hypertension affects approximately 50 million individuals in the United States (NHLBI., 2003) and it substantially increases the risk of developing cardiovascular disease (CVD). Our study found that systolic blood pressure was negatively correlated with type I fibers showing that individuals who had a higher number of type I fibers had lower systolic blood pressure. These results were not significant, however when looking at diastolic blood pressure that association was significant, meaning the higher number of type II fibers was correlated with higher diastolic blood pressure. This is an interesting finding as many studies like The Multiple Risk Factor Intervention Trial found that despite the DBP level, the SBP levels was the main indicator of cardiovascular risk (Leonetti et al., 2000). Another study showed that greater slow twitch muscle fiber percentage was associated with lower blood pressure (Hall et at., 2021), although this cohort was primarily athletes. However, another study (Perry et al., 2000) found that in subjects under 40 years, pretreatment of DBP was a greater predictor of cardiovascular risk than SBP. Further examination of this association should be explored.

The relationship between lactate and aerobic fitness

A sedentary lifestyle and poor cardiovascular fitness (CVF) have been associated with the core components of MetS (Lakka et al., 2003). The benefits of participation in physical activity are well established and include an improvement in blood metabolites profile and a decreased risk for CVD. Researchers found that $VO_2$ max had a strong, inverse, and graded association with the risk of having MetS independently of confounders. They indicated that sedentary, least fit men were almost seven times more likely to have MetS than more fit men. Findings suggest that a sedentary lifestyle and CVF are not only associated with MetS but should be considered as features (Lakka et al., 2003). Our study did not show significance when examining $VO_2$ max; however, the results revealed a correlation with fiber type distribution and showed that individuals who had higher aerobic capacity had a higher percentage of type I fibers, this finding is supported by the result of the study done by Stuart and colleagues (2013) who demonstrated that $VO_2$ max was positively correlated with the proportion of type I fibers.

## Chapter 6 Conclusion

Despite significant results, our study is not without limitations. The sample size may be too small to generalize the results. Immunofluorescent staining does not provide any information regarding the metabolic properties of the fiber, further studies with the use of different enzymatic staining should be done. It is important to know that a small cross-section used for this research only represents a small portion of the length of the muscle fiber. Further research should focus on how those results compare with other populations (ex. Obese, healthy). Additional studies should look at the effects of GLP-1 drugs on lactate levels, and the long-term effects on skeletal muscle metabolism.

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# Appendix: IRB Approval Letter

**EAST CAROLINA UNIVERSITY**
**University & Medical Center Institutional Review Board**
Willis 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: 2/14/2024
Re: CR00010384
UMCIRB 19-000914
Metabolic inflexibility is related to elevated muscle anaerobic glycolysis

I am pleased to inform you that at the convened meeting on 2/14/2024 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 2/14/2024 to 2/13/2025.

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.8 05.23.23-Clean.doc(0.17) | Consent Forms |
| MetFlex Main ICF v.9 05.23.23-Clean.doc(0.15) | 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.8.0 05.23.23-Clean.docx(0.14) | 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: M.L. Pories and N. Broskey.

The following UMCIRB members with a potential Conflict of Interest did not attend this IRB meeting: None.

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