ABSTRACT

MITOCHONDRIAL OXIDATIVE CAPACITY IN HUMAN SKELETAL MUSCLE: Association with Plasma Lactate Concentration

By

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May, 2018

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Background: Metabolic disease is a growing concern for public health. Obesity and Type II Diabetes are an epidemic and a phenotype for insulin resistance in human skeletal muscle. A reduced mitochondrial function and oxidative capacity may be associated with impairments in fatty acid oxidation, which can lead to insulin resistance and metabolic dysfunction. While reductions in mitochondrial content may be a predictive variable, reductions in mitochondrial proteins and an increase in peroxisomal proteins may be contributing factors to increases in FAO and reductions in mitochondria oxidation. Furthermore, due to these reductions, a shift to anaerobic glycolysis has resulted in an increase in plasma lactate concentrations. **Purpose:** The purpose of this study was to determine if a reduced skeletal muscle mitochondrial oxidative capacity was due to a reduction in mitochondrial content. The main aim was to look at the association between mitochondrial protein content and plasma lactate levels. A subaim of this study was to investigate peroxisomal protein content in skeletal muscle to determine if this organelle is upregulated. **Methods:** This study used data from a previous Johnson & Johnson study. Premenopausal women (n=44), over the age of 21 were recruited. These subjects were separated by BMI, non-obese <30kg/m^2, obese ≥30 mg/m^2 and

diabetic. Following a muscle biopsy procedure, plasma lactate concentration was determined using a Coulter-Beckman clinical blood analyzer. Mitochondrial and peroxisomal protein content was determined and analyzed using Western Blot technique **Results:** Mitochondrial protein content, COXIV, was significantly higher in non-obese subjects compared to obese and diabetic. Peroxisomal protein content, PEX19, was also significantly higher in diabetic subjects compared to non-obese and obese. A significant ($P<0.05$) correlation was found between PEX19 and lactate ($p<0.0344$). There was a significant, positive correlation between FAO and lactate in non-obese subjects ($p<0.0471$). There was a significant difference in PMP70 protein content following a two-tailed t-test, and COXIV vs lactate following the Grubbs Test for outliers. There was no significance found between PMP70 vs lactate, COXIV vs FAO or PEX19 vs COXIV. **Conclusion:** Mitochondrial protein content was significantly reduced in obese and diabetic subjects with an upregulation of some peroxisomal proteins. Lactate was negatively associated with a reduced mitochondrial oxidative capacity. More research is needed to look at differences between protein content expression and the degree of adiposity.

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A Thesis

Presented to The Faculty of the Department of Kinesiology

East Carolina University

In Partial Fulfillment of the Requirements for the Degree

Masters of Science in Kinesiology

Exercise Physiology Concentration

By

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May, 2018

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ACKNOWLEDGEMENTS

Wow, this has been a long time coming. I don't even know where to begin. There are so many people that have helped me get to this point in my academic and professional career. Many late night calls and texts, and words of encouragement have helped me along the way. Without these people, I would not be where I am today.

First, I would like to thank my family, my mom, dad and brother, who have always supported me, encouraged me, and showed their unconditional love. They taught me to always give it your all and as long as you give your best effort, hard work will pay off. Thank you for always being patient with me and being proud of me.

I would also like to thank all of my professors, mentors, and committee members that have helped me and worked with me throughout my undergraduate and graduate career. They have taught me to give my best effort and to always strive for the best in order to achieve my goals. They have provided me with the best opportunities and the tools to succeed in whatever I want to do.

Lastly, I would like to thank my friends and classmates. They have always been there to give me advice when I needed it the most, and many times helped me get through school. Thank you for all the wonderful experiences I was able to have.

Thank you to everyone who has helped me achieve my goals personally and professionally, because of you, I am a better person.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

1. CS – **citrate syntha**se
2. COXIV- cytochrome oxidase subunit IV
3. PMP70 – peroxisomal membrane protein 70 kilo Dalton
4. FAO – fatty acid oxidation
5. ETC – electron transport chain
6. FFA – free fatty acid
7. PEXs – genes required for peroxisomal biogenesis and function
8. Peroxins - PEX gene encoded peroxisomal proteins conferring organelle creation and functions.
9. **PEX19**: a peroxisomal biogenesis factor/gene 19
10. **PMP70**: a 70kD peroxisomal membrane protein, marker of biogenesis

CHAPTER I

INTRODUCTION

Metabolic disease continues to be a growing concern in public health.[3] Consequently, both obesity and Type II Diabetes have become an epidemic in Western Societies, notably in the U.S. One of the overt phenotypes of obesity is skeletal muscle insulin resistance.[4,5,7,10] As such, individuals with insulin resistance demonstrate poor glycemic control (high blood glucose levels) which results in rapid blood sugar spikes following a meal. The earliest signs of the development of Type II Diabetes, is the reduced ability of insulin to stimulate glucose uptake into the skeletal muscle. Without the ability to oxidize, there is an accumulation of intra- and extra-cellular fatty acids which suggests there is a reduced fat-oxidative capacity of those who are insulin resistant.[37] In this regard, the implied defect in mitochondrial oxidative phosphorylation has resulted in an increased intracellular fat content in skeletal muscle, "forcing" skeletal muscle to metabolize glucose anaerobically and rely on the peroxisome to compensate for its dysfunction.[39] This may be due to many causative factors including our proposed hypotheses that reduced mitochondrial oxidative capacity and a greater preponderance of white, Type II muscle fibers (associated with a reduction in the proportion of slow, red, oxidative Type I fibers) are related to insulin resistance and consequential metabolic diseases such as those in obese and diabetic conditions. Among the many observed consequences of the heightened anaerobic oxidation of glucose is over production of lactic acid manifested by high levels of lactate in the blood, as we and others have demonstrated.[18,20,22,25] (preliminary data in the Appendix A)

Obesity was once not considered a disease or looked at simply as a problem of body image in the United States. Today obesity is considered as a standalone disease associated with

pathological, metabolic consequences.[24] As such, obesity continues to be a growing concern raising the morbidity and mortality of afflicted individuals. In addition, obesity is associated with a growing economic burden. For example, it has been estimated that the annual medical cost to combat obesity and consequential associated diseases in the United States is greater than $240 billion dollars.[1] (CDC). As such, more than one third of the adult population (36.5%) have been diagnosed as obese (Body Mass Index as weight in Kg/height^2; BMI) and 17% of our youth according to statistics from 2011-2014 (CDC). More so, obesity is associated with many other health concerns.[1] For example, obesity has been associated with various other diseases such as hypertension, insulin resistance, Type II Diabetes and other cardiovascular and metabolic diseases.[2] Thus, obesity as a disease has reached epidemic proportions in the U.S. and is pandemic worldwide. It is the 5th leading risk of global deaths and the leading cause of preventable deaths in the U.S. Due to these results and statistics, it is vital for scientists and health care providers to continue their research efforts to understand the factors (both biological, environmental and behavioral) that lead to the development and sustainability of obesity, in order to curtail the progression of obesity and associated metabolic diseases in Western societies.

Skeletal muscle metabolism occurs via two metabolic pathways, aerobically or anaerobically. The anaerobic pathway does not require oxygen, and muscle glycogen or blood glucose are the exclusive fuel source for energy production via glycolysis. This system is a 12 step process where derived pyruvate is converted to lactate. In comparison, aerobic metabolism utilizes glycolytic derived pyruvate and converts enzymatically to acetyl-coA in the mitochondria. Subsequently, acetyl-CoA is metabolized in the tricarboxylic cycle (TCA cycle; aka the Kreb's cycle). Thereafter, the 8 enzymatic reactions yield the reducing equivalents NADH and FADH2 to supply thermodynamic energy as electrons to be utilized by the inner

mitochondrial membrane system known as the electron transport chain. Thus, mitochondria can metabolize glucose derived pyruvate for energy transduction into adenosine triphosphate (ATP), the so called "universal energy substrate" for all cellular processes, including skeletal muscle contractions and maintenance of energy homeostasis. It should also be noted, that this energy transduction processes utilizes acetyl-CoA derived from lipid β-oxidation to achieve the same bioenergetic outcomes in an exclusive mitochondrial manner. To understand oxidative, aerobic processes, bioenergetisists can track substrate sources for oxidative metabolism using particular biomarkers. Examples of these markers of oxidative metabolism include content and activities of TCA cycle citrate synthase, changes in ETC mitochondrial respiratory capacity (a reduction in electron transport oxidative phosphorylation), mitochondrial organelle content, and consequential changes in both function and protein content of mitochondrial capacity.

The mitochondria are an important essential organelle in the human body with regard to producing energy, ATP, through respiration and regulating cellular metabolism. This is accomplished through the Krebs cycle derived reducing equivalents NADH and $FADH_2$ in the electron transport chain (ETC). However, with mitochondrial dysfunction many metabolic complications may arise. Mitochondrial dysfunction has been thought to be the link between insulin resistance in human skeletal muscle, being a key contributor to the obesity and metabolic syndrome crisis. When the mitochondria are less capable at oxidizing fatty acids, there is an increase in lipids resulting in a lipotoxic environment due to the lipid oversupply. When carnitine palmitoyl transferese I (CPT-1), the first step in fatty acid transport into the mitochondria, fails to move in to the mitochondria, the cell cannot oxidize fatty acids which results in an accumulation. A mitochondrial defect in oxidation of these fatty acids has been shown to contribute to this increase in intracellular fat.[39] This decline in the ability to oxidize fats results in an impairment in

insulin signaling transduction and leads to consequential insulin resistance.[38] Oxidative capacity of skeletal muscle may be of importance to boost lipid oxidation to the level of lipid supply and therefore, modulate substrate utilization. The reduction in oxidative capacity has been seen and consequently detrimental in those who are obese and who have Type II Diabetes, again contributing to the obesity and metabolic syndrome epidemic.[10] With a reduction in the mitochondria's ability to effectively oxidize lipids, there has been evidence to show an increase on the reliance of the peroxisomes to compensate for this reduction, while also seeing an increase in blood lactate production.

In addition to the mitochondria, it has been recently noted that peroxisomes are also essential for oxidizing fats. For example, they are capable of at least partially oxidizing long-chain fatty acids in an independent pathway from the mitochondria.[25,26] Peroxisomes are coupled to mitochondrial lipid metabolism, yet they also are distinct with their own β-oxidation system. In this regard, peroxisomes are the exclusive organelle for oxidizing very-long chain fatty acids. Consequently, peroxisomes are indispensable organelles for maintaining cellular lipid homeostasis, and their absence or dysfunction leads to metabolic pathologies which include adrenoleukodyrtropy and Zwellerger syndrome, both which are lethal in all mammalian species.[35,36] It is also known despite possessing a β-oxidation system, the process is incomplete resulting in partially oxidized fatty acid derived metabolites. These peroxisomal derived metabolites however, can be exported to the mitochondria for further, complete oxidation; the outcome of this metabolic "coupling" results in complete oxidation of these peroxisomal export products (as acyl-carnitine metabolites), thereby supporting lipid substrate oxidation during circumstances of cellular lipid overload and reductions in mitochondrial oxidative competency.[30] (Huang, Cortright 2017, published and one in review) Despite the ability of the peroxisomes to

increase mitochondrial lipid oxidation efficiency, these peroxisomal-mitochondrial interactions are also hypothesized to be essential for skeletal muscle lipid homeostasis given that peroxisomes do not possess an electron transport chain, which is exclusive to the mitochondria.. In summary, peroxisomal function in skeletal muscle is most important when the mitochondria are unable to support total oxidization of surplus fatty acids as observed with obesity and Type II Diabetes, and it has been noted that peroxisomal biogenesis is upregulated to compensate for mitochondrial incompetency.[30,34] (Huang, Cortright 2017, published and one in review)

As mentioned above, lactate production is the end-product of anaerobic metabolism by the cytoplasmic glycolytic pathway. Accordingly, we have noted that individuals with metabolic inflexibility (inability to switch to a predominant substrate source whether glucose or fatty acids) present with higher levels of plasma lactate levels in the basal state (see preliminary data in the appendix)[21,36]. Thus, blood/plasma lactate accumulation can be used as a relatively noninvasive marker for reductions in glucose oxidation and thus mitochondrial dysfunction (reductions in aerobic capacity); and by extension metabolic inflexibility. We therefore measured blood lactate levels among our experimental groups to determine if it is predictive of aerobic competency by the mitochondria. Skeletal muscle oxidative inflexibility has been found to have lowering fasting lipid oxidation and an inability to switch between lipid and glucose oxidation in insulin resistant individuals. Insulin resistant individuals have also shown to be associated with mitochondrial dysfunction and an increase in lactate reducing glucose oxidation. Insulin resistance and the inability to oxidize glucose has also led to an increase in lactate production.[18] Lactate production is likely to be higher in obese and diabetic individuals as opposed to non-obese as those who are obese or diabetic have a lower mitochondrial content and perhaps dysfunction of existing mitochondria. Lactate has been reported to be lower in non-obese individuals, higher in non-

diabetic obese individuals and even higher in Diabetics who were obese.[25] However, it is a secondary hypothesis of this research study that some lean or non-obese individuals (defined by BMI < 30) may also "suffer" from reduced mitochondrial oxidative potential thereby pre-disposing these individuals toward metabolic disease characterized by high resting blood/plasma lactate concentrations as described above.

Experimental Approach/Design

The primary focus of this thesis was the assessment of mitochondrial content in human skeletal muscle. However, given the above discussion, we also assessed peroxisomal content as well. This study recruited participants as 3 groups; 1) non-obese individuals who are both metabolically flexible and metabolically inflexible, 2) obese subjects who are metabolically flexible and inflexible, and 3) diabetic individuals. We looked at plasma and skeletal muscle protein extract samples collected from subjects recruited for a larger study funded by Johnson and Johnson. Protein content was determined using Western blotting technologies in vitro for both mitochondrial and peroxisomal content as a surrogate for capacities of oxidative metabolism. Measurements for mitochondrial aerobic capacity was done by assay for electron transport chain, cytochrome oxidase V. Peroxisomal content and function were selected organelle PEX genes (required for peroxisomal biogenesis and function) and associated peroxins (PEX gene encoded peroxisomal proteins conferring organelle creation and functions). In addition, plasma lactate levels were assessed to determine the extent of anaerobic metabolism in study participants of varying degrees of metabolic flexibility to establish associations of mitochondrial and peroxisomal function for oxidative metabolism and metabolic inflexibility

(the failure to shift substrate utilization between glucose and lipid in an aerobic vs anaerobic fashion).

Purpose of the Studies

The purpose of this study is to determine if a reduced skeletal muscle mitochondrial oxidative capacity is due to a reduced mitochondrial content. The main aim of the study is to associate mitochondrial protein content with plasma lactate levels; a surrogate marker of aerobic capacity. In addition, peroxisomes are intracellular organelles that are also capable of oxidizing lipid substrate. Therefore, a subaim of the present investigation is to determine the content of peroxisomes in skeletal muscle to determine if this organelle is upregulated to offset the reductions in mitochondrial oxidative capacity as expected in the obese, diabetic, and non-obese metabolically inflexible individuals. To address our aims, we utilized skeletal muscle obtained from biopsies of the vastus lateralis from both non-obese and obese individuals to determine both mitochondrial and peroxisomal oxidative protein levels.

Research Hypothesis

Globally, we predict that reductions in oxidative capacity are due to a lower mitochondrial content and overall reduced oxidative capacity of the mitochondria, and help explain why some individuals are more susceptible to metabolic diseases such as Obesity and Type II Diabetes. This is thought to predispose them to metabolic diseases, due in part to a reduced mitochondrial capacity to oxidize substrates (fat and carbohydrates). More specific to the present proposal, we hypothesize that these reductions will be associated with higher blood lactate levels. We further hypothesize that the protein content of peroxisomes will be elevated to partially offset reductions in mitochondrial oxidative capacity.

Significance of the Studies

The proposed studies are highly significant in that they will shed light on the relationship(s) between mitochondrial and peroxisomal content (indirect evidence for oxidative capacity) and the increase in lactate production. By understanding the mitigating biological factors contributory toward a mitochondrial dysfunction and decrease in mitochondrial content, health care providers will likely be able to devise new and more effective strategies for combating the rise in obesity, Type II Diabetes and associated metabolic diseases, which contribute to the current plague of declines in human health and the rise in the economic burden inflicted by these disease. Findings may also help to explain why some non-obese individuals are metabolically inflexible, which predispose them to the eventual development of metabolic diseases.

Delimitations

1. Participants were limited to those recruited from Greenville and surrounding area
2. Participants recruited are restricted only to Caucasian women
3. Obese and non-obese individuals are "all comers" regardless of demonstrating metabolic flexibility or not.
4. Assessments of skeletal muscle mitochondrial and peroxisomal oxidative capacity is by Western Blotting technologies for key proteins of mitochondria oxidation and the electron transport chain function, as well as key proteins involved with peroxisomal function.
5. The age range for participants is 21-55 years old.

Limitations

1. This study only looks at Caucasians without including those of Hispanic descent
2. Studies of organelle oxidative capacity are limited to protein content of mitochondria and peroxisomal content/function. No direct assessment of mitochondrial or peroxisomal oxidative function was performed. (e.g., Isolated mitochondria oximetry and permeabilized fiber high resolution oximetry)
3. The assessment of mitochondrial and peroxisomal oxidative capacities are limited to the proteins described above.
4. Subjects were not assessed for metabolic inflexibility.

Abbreviations:

1. CS – **citrate syntha**se
2. COXIV- cytochrome oxidase subunit IV
3. PMP70 – peroxisomal membrane protein 70 kilo Dalton
4. FAO – fatty acid oxidation
5. ETC – electron transport chain
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9. **PEX19**: a peroxisomal biogenesis factor/gene 19
10. **PMP70**: a 70kD peroxisomal membrane protein, marker of biogenesis

Operational definitions

1. RER – Respiratory exchange ratio
    a. Approximation of how much fat vs glucose is being utilized
2. Metabolic flexibility
    a. The ability to switch between two substrates, fat and carbohydrates, in response to a change in availability
3. Insulin resistance
    a. The blunted response of organs to insulin
4. Mitochondrial uncoupling
    a. Process by which the proton motive force built up by the electron transport chain is uncoupled from oxidative phosphorylation
5. Anaerobic – without using oxygen, causing an increase in lactate
6. Obesity - a metabolic disease defined as body mass index (BMI), greater than or equal to a BMI of 30 kg/m^2
7. Citrate synthase – an enzyme in the first step of the citric acid cycle
8. Electron Transport Chain protein COXIV
9. Peroxisomal membrane transporter protein PMP70
10. Beta-oxidation
    a. Catabolic process where fatty acid molecules are broken down
11. The electron transport chain
    a. Electron are donated and accepted through reduction and oxidation reactions and are transferred across the mitochondrial membrane
12. Oxidative phosphorylation

a. Metabolic pathway in the mitochondria where energy is released and ATP is produced

13. Peroxisomal beta-oxidation

a. Fatty acids are broken down in the peroxisome

CHAPTER II

LITERATURE REVIEW

Introduction

The purpose of this study was to determine if an association exits between mitochondrial oxidative capacity and plasma lactate levels. The following literature review will discuss: **1)** Obesity and its prevalence in the United States, **2)** metabolic flexibility and metabolic inflexibility and its effects on skeletal muscle in Obesity and Type II Diabetes, **3.)** mitochondrial function, **4.)** insulin resistance and its prevalence in individuals who are Obese or have Type II Diabetes, **5)** fatty acid oxidation, **6)** the accumulation of lactate, and **7)** the effect(s) of peroxisomes on mitochondrial function and lipid oxidation

Obesity

Obesity was once not considered a disease or looked at as a problem in the United States. Today it continues to be a growing concern and burden on many individuals, as well as the economy. It was estimated in 2008, the annual medical cost to combat obesity in the United States was $240 billion dollars.[49] More than one third of our adult population (36.5%) have been diagnosed as obese and 17% of our youth according to statistics from 2011-2014.[49] This prevalence continues to remain higher than the Healthy People 2020 goal of 30.5%.[49] These national medical costs have become a main reason for growing public health concerns.[70,71] Obesity is defined as an excess amount of body fat with a BMI (body mass index) over 30 kg/m^2. Obesity is not its own disease, but is also associated with many other health concerns and metabolic diseases.[49] Obesity has been found to have a very high correlation with various other diseases such as hypertension, insulin resistance, Type II Diabetes and other cardiovascular and

metabolic diseases.[3,9,62] Not only is obesity itself an epidemic in the U.S. and a pandemic worldwide, it is also the 5th leading risk of global deaths and the leading cause of preventable deaths in the U.S.[9] Due to these results and statistics, it is vital for more research to be conducted to solve numerous health, behavioral, cultural, biological, and additional variables that contribute to these statistics.

Metabolic Flexibility

Although the described study will not directly assess metabolic flexibility, because of the strong association with the metabolic phenomenon with the present thesis, a brief description is offered as follows. Metabolic flexibility is the ability of skeletal muscle to efficiently switch between two substrates, lipid and glucose in response to substrate availability.[38,65] There are differences in the state of the body, fasting, non-fasting and the individual themselves, lean or obese. In a presumably normal, healthy, insulin-sensitive individual, during the fasting stage the body will rely on lipid oxidation for metabolism. However, when we are in the non-fasting stage, our body primarily utilizes and oxidizes glucose metabolism. This process of utilizing lipid at first and then glucose is what occurs in a healthy individual. However, this does not regularly happen for those with some metabolic diseases such as obesity or Type II Diabetes. The impaired ability to switch from lipid to glucose oxidation and the reduced capacity to increase lipid oxidation during exercise is called metabolic inflexibility.[37] A reduced ability to switch between substrates when they predominate in the bioenergetic pathways of aerobic metabolism has been seen in some individuals who have an impaired glucose tolerance, signifying that this inflexibility plays a part in the development of Type II Diabetes.[37,65] Metabolic inflexibility is characterized as unhealthy skeletal muscle and is due to an impaired cellular glucose uptake.[21]

The inability to increase glucose oxidation and storage in insulin resistant individuals has shown an increase in RQ levels showcasing that there is more glucose entering cells and being available, but not being fully oxidized.[21] A low RQ is reflective of a high lipid oxidation and a high RQ is reflective of high glucose oxidation. The impaired ability for the body to increase glucose oxidation is indicative of metabolic inflexibility.[65] For example, in the healthy individual, aerobic exercise results in a decrease in the RER over prolonged periods of time. This change is not realized with metabolically inflexible individuals. Exercise stimulates key stress signals that control mitochondrial biogenesis in the skeletal muscle. Following endurance exercise, results showed an increase in mitochondrial content and a reduction in ROS production (reactive oxygen species) due to enhanced respiration.[41,42] Furthermore, a study by Holloszy found after an endurance training program, the mitochondria of the exercising group was able to utilize two times the amount of 0_2 as that of the sedentary subjects. In addition, the same study demonstrated an increase in total protein content of the mitochondria by 60% following prolonged endurance exercise, while also increasing the mitochondria's capacity for oxidative phosphorolation.[42] One study used a method of 7 consecutive days of exercise and found their subjects to have reductions in fasting and 2 hour insulin concentrations, improving insulin action. Exercise training increased complete FAO by 2 fold in lean and obese as well as incomplete oxidation being normalized following exercise.[73] This data demonstrates exercise as an effective tool at enhancing metabolic flexibility in those who are inflexible.

Mitochondrial Function

Mitochondrial function plays an essential role in the body staying healthy and being able to regulate insulin sensitivity, rather insulin resistant. When the mitochondria become

dysfunctional, metabolic inflexibility and insulin resistance are more likely to occur. Having an impaired mitochondrial function may lead to insulin resistance because of the impaired capacity of fat oxidation during the fasting state. The ensuing rise in intramyofibular lipid content is associated with reduced inulin signaling and glucose uptake thereafter. (Lowell and Shulman) Those with Type II Diabetes and obesity may have a reduced oxidative capacity due to a reduction in mitochondrial content or because their mitochondria cannot already effectively oxidize.[7] Improving β-oxidation and restricting fatty acid uptake into the mitochondria has been shown to improve insulin-sensitivity and protect against lipid accumulation.[19,42,65] β-oxidation is important because it occurs exclusively in the mitochondria and is responsible for metabolizing fatty acids. Restricting fatty acid uptake has allowed the mitochondria to not have to "over compensate" and therefore, will not result in damage or dysfunction.[19,22] As alluded to above, studies have shown an impairment in the ability of the mitochondria to successfully oxidize fatty acids, resulting in insulin resistance. A current hypothesis suggests these reductions in fatty acid oxidation potential are the result of an underutilized β-oxidation system due in part to reduced enzymatic activity of the enzyme carnitine palmitoyl transferase 1 (CPT-1), which is an enzyme complex responsible for fatty acid incorporation into the mitochondrial matrix leading to the first step of β-oxidation.[27,42] When CPT-1 activity is impaired, only a portion of the fatty acids were partially degraded and entering the mitochondria before being completely metabolized. It had been found that there was a reduction in CPT-1 in the mitochondria in those who were sedentary and obese.[21] When β-oxidation is overused there was a reduced CPT-1 activity which would result in reduced β-oxidation, and therefore a compromise in mitochondrial status and failure.[27,42] Defects in β-oxidation have also been shown to exist in the obese and diabetic states with a concomitant decrease in insulin sensitivity, which may account for a portion of metabolic

flexibility because of a decrease in glucose uptake.[70] The lipid oversupply seen in insulin resistance is in conjecture with an elevated incomplete β-oxidation, and a reduced FAO and increased lipotropic environment.[4,22] It was also reported that CPT-1 inhibition enhanced mitochondrial biogenesis demonstrating a 50% increase in mitochondrial content in CPT-1.[4] However, it is now suspected that this is not the only compromised condition as glucose can still be derived from stored glucose as glycogen, and yet oxidation of glucose is still reduced.[20] In this thesis, we scientifically conjecture that elevations in blood lactate will occur as a result in reduced oxidation of pyruvate. The function of the mitochondria is vital as it helps with coupling between phosphorylation and substrate oxidation through both the generation and utilization of the proton gradient over the mitochondrial membrane.[46]

Skeletal muscle has an abundant number of mitochondria and is dependent on oxidative phosphorylation for energy, in addition to being the site for fuel oxidation.[46] In a study by Kelley et.al., both the structure and the functional capability of the mitochondria were compromised.[36] Skeletal muscle mitochondria were found to be smaller in Type II Diabetic and obese participants compared to lean participants. Activity of NADH:O_2 was lowered by about 40% in skeletal muscle showing evidence of severely damaged mitochondria and a significant degree of insulin resistance. It was found in this study that there was an impaired bioenergenic capacity of skeletal muscle mitochondria in subjects with either disease, obesity or Type II Diabetes. Understanding why mitochondria are smaller in some individuals is important to recognize. One explanation may have been that during this specific study the subjects studied were sedentary. In addition, mitochondria biogenesis has been shown to increase with exercise training given sufficient intensity and while studying those who are sedentary, there would be an opposite effect.[36] According to Kelley et.al, the morphology of the mitochondria is changed in individuals

with obesity and Type II Diabetes. This finding was novel because the morphology of the mitochondria has been known to result in disorders of biochemical function of the mitochondria.[36] The area of their mitochondria was reduced by about 35%, and was also correlated with a reduced glucose disposal rate.[36] As for the lean individuals, their mitochondria was found to have a more clearly defined internal membrane structure. In contrast, the obese individuals had larger vacuoles in their mitochondria, which have multiple layers of a double membrane, as well as 30% less mitochondria than leans resulting in an impaired β-oxidation.[27] This has been suggestive of possible degenerated mitochondria. In this context, the excess of long-chain fatty acid CoA occurs due to an excess in caloric intake and can cause damage to the mitochondria.[36] Damage to the mitochondria has also been found in the ETC. A study by Ritov et al., demonstrated both obese and diabetic subjects had a deficiency in the activity of mitochondrial ETC activity and a disbalance between activity of ETC and activity of citrate synthase or β-Had.[56] Obese were significantly higher in CS activity, (3.7 ± 0.5 vs lean 2.7±0.28 and diabetic 3.28 ±0.49), and both obese and diabetics had significantly lower ETC activity than leans (obese, 0.18±0.2, diabetic 0.16±0.03, leans 0.44±0.12). Furthermore, Gaster et al., demonstrated an inhibition in the electron transport chain through an increase in glucose uptake, lactate production, reduced glycogen synthesis, and reduced and glucose oxidation.[22]

In a study by Boyle et.al, it was found in obese individuals, their skeletal muscle was unable to upregulate mitochondrial respiration following a 24-h lipid incubation.[7] This finding displayed obese individuals not being able to increase lipid oxidation and to effectively use the substrate. It was found that obese subjects were metabolically inflexible due to a decrease in mitochondrial lipid oxidation in response to lipid exposure, which has shown an impaired ability to enhance lipid oxidation when there is an excess of lipid substrates. In addition, Ritov et al.,

demonstrated with an accumulation of high fats, there was a subsequent increase in citrate synthase activity and β-Had and a reduced ETC activity.[56] One explanation of this idea is due to a failure to increase mitochondrial content.[4,7,28] Houmard et al., not only found a greater partitioning of lipids towards storage, but a significant reduction in mitochondrial content and COXIV protein content in obese subjects.[28]

van de Weijer et. al., performed a study on muscle oxidative capacity. There were significant differences between insulin sensitive and insulin resistant subjects in the number, structure and function of their mitochondria.[70] In this study, they assessed PCr in exercise recovery and found that the capacity to oxidize fatty acids was decreased since the mitochondrial density and function was reduced. As a result, these subjects were unable to generate ATP effectively.[70] The reduced ability to synthesize ATP may be the result of increased mitochondrial uncoupling.[21]

Insulin Resistance:

Insulin resistance is the inability of the body to effectively or properly respond to insulin. Insulin itself is essential in maintaining and controlling blood glucose. This inability results in glucose not being able to easily enter the body's tissues that are dependent on insulin for glucose uptake, and therefore, resulting in excess glucose accumulating in the blood. Someone may become insulin resistant with an excess glucose found in the blood, which may lead to Type II Diabetes. Insulin resistance can result from an accumulation of lipid products in skeletal muscle that may obstruct insulin signaling and glucose uptake; therefore, insulin resistance may develop from the consequence of lipid accumulation in skeletal muscle especially in those who are obese and have Type II Diabetes.[36,38] This is caused from an impaired ability to upregulate lipid

oxidation when there is a an excess of lipids, which may lead to an increase in fat accumulation in the muscle.[21] Metabolic fitness in skeletal muscle is the capacity to switch between energy fuels which is not seen in individuals who are insulin resistant.[39] This was due to the reduced rates of lipid oxidation at the fasting conditions. It has been observed, that obese individuals had a noticeable decrease in fasting rates of lipid oxidation, whereas lean individuals were observed to utilize lipids as a fuel source and seen to have a sharp changeover from mainly using lipid oxidation to mainly using glucose as a fuel source.[39] The problem that obese subjects have is utilizing less lipid oxidation during fasting stages and more during insulin-stimulated stages. This higher rate of oxidation in the non-fasting stages causes disturbances in the ability to adapt to fasting rates (not being able to increase) and the rates of insulin (not being able to decrease).[39] However, an excess increase in lipid availability could interfere with muscle glucose metabolism in both diabetics and obese subjects. In a study by Kelley and Simoneau, a study done on rats, showed obese insulin-resistance increase in glucose oxidation in skeletal muscle whereas skeletal muscle in lean subjects were using lipid as the main source of fuel.[39] This increase in glucose metabolism was further looked at in a study by Winder et al. This increase in metabolism directed into an increase in malonyl CoA.[38] Malonyl CoA inhibited CPT-1 and also blocked the entry of FFA's into the mitochondria. Due to the buildup of excess triglycerides in the skeletal muscle, there might also be an increase in long-chain acyl CoA concentrations as well, which in turn may alter the insulin signaling inhibiting the stimulation of glycogen synthase and a signal for fatty acid splitting.[38,39] These defects in insulin action and abundance of lipids in the skeletal muscle have shown that long-chain fatty acyl-CoA's may in fact produce insulin resistance.[39] Muscles that have been found to have lower levels of CPT-1 in obesity have also had higher levels of triglycerides in skeletal muscle suggesting obesity has been partly caused by a reduction

in fat oxidation.[38] The increase in triglycerides has been suggestive of lipid accumulation may be the reason for insulin resistance. The buildup of triglycerides in obese skeletal muscle comes from a reduced ability for fat oxidation and diminished enzymes along with the inability to regulate fat oxidation when inflexible.[36,38] This accumulation of triglycerides, which is a metabolically inert substrate, does not directly induce insulin resistance in skeletal muscle. Rather the rise in their content is reflective of lipids species associated with increased triglyceride turnover related to reductions in mitochondrial oxidation of dietary lipid overload and reduced mitochondrial uptake and oxidation.

However, there are other factors to be looked at. The oxidative capacity in skeletal muscle is one such indicator. Individuals who are highly trained and not sedentary will have a higher type I muscle fiber count. Type I fibers are more aerobic, have a higher lipid content, higher rates of uptake in fatty acids and an overall greater insulin sensitivity.[28,30,38,39] This is not seen in type II fibers, which are in abundance in obese individuals. In a recent study, it was found that individuals who were Type II Diabetics had a 2 fold higher proportion of type IIx muscle fibers. In conjuncture, Hickey et al., demonstrated a significant decrease in type I fibers in those who were extremely obese, which indicated a reduction in fatty acid oxidation may be due to a loss in skeletal muscle oxidative capacity.[30] This was a novel finding because it presented a defect in the functional capacity of muscle mitochondria in Type II Diabetics due to skeletal muscle fibertype.[28,30,46] Insulin resistance has also been observed with a reduction in oxidative enzymes. In a study by Simoneau et al., muscle had a reduction in CPT activity in obese individuals. This reduction was found to be related to a decrease in activity in other enzymes such as hydroxyacyl dehydrogenase, and citrate synthase, which are important markers

for the β-oxidation and the TCA cycle respectively.[39] As stated above, reduced activity of CPT can also result in diminished fat oxidation and an accumulation of lipids in skeletal muscle.

Fatty Acid Oxidation

Through assessment of fatty acid oxidation, studies were able to predict the severity in which one of their subjects was insulin-resistant to glucose metabolism through their poor dependence on fatty acid oxidation. This had previously been seen during elevated lipid oxidation during insulin-stimulated conditions and through fasting conditions.[38,39] Some studies have found that myotubes are more metabolically flexible from tissues of lean vs. those obtained from insulin sensitive individuals in response to palmitate incubations. This data has suggested that there are defects in fat oxidation within the skeletal muscle of insulin resistant subjects that is translatable into cell culture.[21] It should be noted that an increase in fat oxidation is not always ideal as instead the cell would be less responsive to higher glucose oxidation. Fat oxidation is increased when there is a higher plasma lipid concentration. This increase in free fatty acids weakens the glucose disposal rate, which ultimately decreases glucose oxidation and develops lipid oxidation.[21] This suggests that the ability to oxidize fatty acids is "desirable", but can be counterproductive under conditions of lipid over supply as occurs on a high fat diet.

Regardless, skeletal muscle is vital for lipid oxidation. Lipid oxidation is the primary mechanism for the body to use at rest as about 90% of the energy comes from this oxidation. In contrast, reduced fatty acid oxidation is one way in which this has been observed as an increase of triglyceride content.[40,46] This increase is associated with a surplus of lipids and has therefore been linked to obesity. As described, with lipid oxidation, long-chain fatty acids must go through the mitochondria to be oxidized. In a study by Kim et al., it was found in obese individuals that

long chain fatty acid oxidation was decreased when it came to mitochondrial function.[40] The problem that arose was with the fatty acids not being able to successfully cross the mitochondrial membrane. As a reminder, the ability to cross the mitochondrial membrane stems from the assistance of the enzyme, CPT-1. CPT-1, carnitine palmitoyltransferase, is an enzyme that helps to regulate the transport of long-chain fatty acids across the mitochondrial membranes. However, this process has been shown to be difficult for obese individuals as CPT-1 activity has been inefficient. The decreased activity of CPT-1 contributes to obesity because with its decreased activity, long-chain fatty acids are not being oxidized and therefore, being stored within the muscle.[30,40] One explanation that may further explain why a reduction in CPT-1 occurs in obese individuals is due in part to the activity of another co-enzyme, malonyl-CoA. Malonyl-CoA inhibits the activity of CPT-1 and therefore directly reduces lipid oxidation.[40]

Further reductions in fatty acid oxidation were demonstrated in severe obesity. Kim et al., found a reduction in fatty acid oxidation in skeletal muscle from obese subjects compared to lean, which suggests a reduction in mitochondrial fatty acid oxidative capacity. In addition, fatty acid oxidation was 100% reduced in obese subjects, and an observed reduction of 276% in extreme obesity.[28,30] Hulver et al., further demonstrated a 60% decline in oxidative capacity in extreme obese compared to lean and obese.[30]

There are several other enzymes that have been reduced in obese individuals that correlate to mitochondrial activity and lipid oxidation. Mitochondrial content can be measured in correlation to citrate synthase. Citrate synthase is an enzyme that regulates the Krebs cycle, which takes place in the mitochondria. Citrate synthase along with Beta-HAD, an enzyme in beta-oxidation, were both significantly reduced by 35%.[40] In addition, there is evidence demonstrated that a dysfunction and inactivity of the mitochondria, as well as CPT-1 contribute

to the accumulation and build-up of fatty acids, and the subsequent decrease in lipid oxidation. CPT-1 activity is especially important in explaining the decrease in palmitate oxidation, which was decreased by 62% in obese individuals, as this mechanism is dependent on the activity of CPT-1.[40] Mitochondrial function was inhibited due to a decrease in function of these enzymes, CPT-1, citrate synthase, and beta-HAD. Reductions in fatty acid oxidation may be a result from numerous enzymes, and rate limiting steps, but a decrease in FAO would lead to an accumulation of fat, and therefore a preferential partitioning of lipids towards storage instead ofoxidation.[20,28]

Lactate:

Skeletal muscle oxidation has been found to have lowering fasting lipid oxidation and an inability to switch between lipid and glucose oxidation in insulin resistant individuals. Insulin resistant individuals have also shown an increase in mitochondrial dysfunction and an increase in lactate reducing glucose oxidation as stated above.[14] With a reduced mitochondria capacity, there is a reduced ability of maintaining lower levels of plasma lactate.[26] Insulin resistance and the inability to oxidize glucose has also led to an increase in lactate production.[1,18] Lactate production is likely to be higher in obese individuals as opposed to lean individuals as those who are obese have an elevated fat accumulation, larger fat cells and are utilizing anaerobic glycolysis.[14] Crawford et al., demonstrated a twofold increase in lactate in obese and diabetics (43.0 and 41.3 mg/dl), which is substantially higher than the recommended range of 4.5-19.8mg/dl.[39] Another study by Friedman et al., found similar results. Lactate production was found to be higher in Type II Diabetic subjects,[20] whereas other studies found plasma lactate to be 50% greater in Type II diabetic subjects vs leans (18.2±0.9 vs 12.6±0.7). The overall

incorporation of lactate into glucose was more than a twofold increase in diabetic subjects.[11,22] In comparison, lactate is lower in lean individuals, higher in non-diabetic obese individuals and even higher in Diabetics who were obese.[13] The accumulation of adipose tissue is detrimental as they have a large capacity to convert glucose to lactate as its major substrate, and therefore shifting towards anaerobic glycolysis. In humans lactate production can reach up to 50-70% of glucose that is metabolized which is a much larger amount for obese individuals.[33,44] This increase in adipose tissue is associated with increased hypoxia and poor vascularization.[13] These changes along with an increase in anaerobic glycolysis and glucose disposal have resulted in an elevation in fasting lactate levels.[11,33]

Blood lactate is the gap between energy expenditure and oxidative capacity.[13,33] Lactate is produced from pyruvate in anaerobic glycolysis and increases when energy demand surpasses the mitochondrial oxidative capacity within the skeletal muscle. The inability to switch between lipid and glucose substrates may in part be due to lactate and pyruvate, byproducts of glycolysis. In a recent study, it was found that lactate had a similar effect on insulin resistance as a high amount of glucose does, as well as a reduced substrate oxidation. With a reduced ability to utilize glucose, there is an ensuing increase in insulin resistance and shift towards anaerobic glycolysis, leading to an accumulation of lactate. Furthermore, lactate levels continued to increase following hyperglycemia.[10,13] The glycolytic process is a long pathway that involves many steps. Glucose at first enters the skeletal muscle and is phosphorylated to glucose 6-phosphate by the enzyme hexokinase. Then glucose can either be stored or enter glycolysis.[10] Glycolysis can either be converted to pyruvate where it will then enter the mitochondria for oxidation, or it can be converted to lactate by the enzyme lactate dehydrogenase through anaerobic metabolism. If the skeletal muscle continues to stay in anaerobic metabolism there will be an elevated lactate

response evidenced by a rise in the blood compartment, which is a new thought. The reduction of pyruvate to lactate has been seen as a metabolic dead end.[35] When pyruvate is reduced to lactate by LDH and oxidizes NADH, the production of lactate limits substrate entry when glycogen supply is limited due to lack of training and insulin resistance.[25,35]

Lactate, a byproduct of glycolysis, goes through the Cori Cycle once it is made. The Cori Cycle is a pathway that requires oxidation of fatty acids for lactic acid to `be converted to glucose. An increased lactate production may then result in excess glucose. Lactate production can also be produced by a decrease in pyruvate dehydrogenase activity or an increase in lactate dehydrogenase activity.[1] Lactate dehydrogenase is an enzyme that is responsible for breaking down lactate into pyruvic acid which will eventually be used for glycolysis. In the study by Consitt et al., LDH was found to increase phosphorylation by about 60%.[10] This is important because LDH is an enzyme responsible for the preferential conversion of pyruvate to lactate, causing a surplus of lactate. In relation the lactate shuttle is important at increasing LDH phosphorylation, as with high rates of glycolysis, lactate can be shuttled to adjacent sites within the skeletal muscle, to be used as a substrate for oxidation.

A study by Consitt et al., found the skeletal muscle in aged individuals preferred to shuttle glucose to lactate. This was due to an impaired activity level of PDH.[10] By improving the activity of PDH the shuttle to create more lactate from glucose may decrease and shift back to more aerobic metabolism as opposed to anaerobic metabolism. Lactate functions in skeletal muscle to shuttle glycolytic fibers to oxidative fibers. This shift is carried through by MCT4 and MCT1. MCT1 is however decreased in skeletal muscle in those who are insulin resistant.[13]

In a study by Aas et al., lactate was found to possibly inhibit the actions of CPT-1 along with glucose oxidation. CPT-1 plays an important role in fatty acid metabolism within the

mitochondria as described above.[1] Lactate has also been shown to inhibit glucose uptake by halting glycolysis.[10]

Insulin sensitivity is altered due to these plasma lactate levels. A study by Watanabe et al., injected insulin to observe the change in blood lactate concentration. The highest rate of lactate production was after 20 minutes from receiving the injection. There was not much of any change before this point. This data suggests that insulin may have a significant impact on enhancing the conversion of glucose to lactate.[73]

Lactate is important to study as it is strongly related to metabolic diseases. Juraschek et al., observed a strong graded relationship between lactate production and the risk of Type II Diabetes and insulin resistance.[13,33] A higher lactate concentration was strongly correlated with a 2.4 times higher risk of Diabetes.[33] Crawford et al., found a correlation between lactate quartile and Type II Diabetes. Within the first quartile there was a 12% increase and then a 30% increase in the fourth quartile.[13] The decreased insulin signaling that causes insulin resistance, also causes a cascade of events. There is a decrease in blood flow, decrease in mitochondrial biogenesis and decreased oxidative capacity. This can all be improved by increasing insulin signaling activity.[33] Another factor increasing lactate production is GLUT4. GLUT4 is a glucose transporter in both fat and muscle cells. GLUT4 transports glucose into the cells where it will go through glycolysis. An increase in GLUT4 in the cells has been correlated with an increase in lactate production. Therefore, due to the suppression of GLUT 4, an insulin regulated glucose transporter, insulin resistance may result in lactate accumulation.[33] Furthermore, the greater the relative difference of lactate vs NAD+ suggests lactate can more readily diffuse from the cell when there is an increase in glycolytic flux allowing lactate to be directed toward the mitochondria for lactate oxidation.[35]

Peroxisomes:

Mitochondria function plays an important role in free fatty acid oxidation. When the mitochondria is impaired with respect to fatty acid oxidation, or dysfunctioning, insulin resistance may occur and the mitochondria will need other enzymes and organelles to help regulate fatty acid oxidation. (Huang, PGC1a published paper) Peroxisomes are an organelle that has been seen to help the mitochondria in this way. The peroxisome has been seen to be recruited to enhance peroxisomal fatty acid oxidation when there is an increase and accumulation in fatty acids.[6] When there is an overload of fatty acid oxidation and the mitochondria cannot compensate, the peroxisome is recruited to help upregulate fatty acid oxidation and slowdown the rise of intracellular lipid accumulation. A study by Wicks et al., demonstrated an increase in peroxisomal activity in order to make up for excess lipid accumulation.[71] With an accumulation of fatty acids in the mitochondria, the mitochondria are often at a dysfunction and exhibit an elevated incomplete β-oxidation, resulting in a need for the peroxisomes to be upregulate to utilize its partial β-oxidation.

Not only do peroxisomes help the mitochondria with FAO of LCFA, the peroxisomes are important in beta-oxidation as peroxisomes are the only organelle that are able to process very-long-chain fatty acids. The peroxisomes are preferred in the oxidation of VLCFA, and cannot be replaced by the mitochondria.[43] The mitochondria are unable to handle VLCFA, therefore the peroxisome assist in preventing lipotoxicity from the accumulation of fatty acids.[43,59,61,72] These are chains of at least 22 carbons.[27] The ability of the peroxisomes to metabolize these very-long-chain fatty acids is important as it has been found that skeletal muscle in obese individuals have carbon chains of C24. This data implies peroxisomes are involved in VLCFA metabolism in obese individuals.[13] Peroxisomal beta-oxidation cannot be solely reliant on as this oxidation is

often incomplete and cannot fully oxidize all of the fatty acids. Therefore, joining peroxisomes and mitochondrial function is essential in maintaining lipid homeostasis.[41,48,68] Peroxisomal function is important and necessary in helping to compensate the mitochondria in regards to lipid oversupply which is associated with Type II Diabetes and Obesity. For example, when acylcarnitines bypass CPT-1in the mitochondria, acylcarnitines are produced as products of incomplete mitochondrial fatty acid oxidation, and have been detected in obesity, and Type II Diabetes. This has resulted in an underproduction of acetyl-CoA and a dysfunction of the Krebs Cycle.

With an increase in adipose tissue peroxisomes have been seen to increase in number and these findings by (Novikoff and Novikoff, 1982) suggest peroxisomes are involved in lipid metabolism.[43] In order to regulate peroxisomal biogenesis, there are numerous proteins on the membrane of the peroxisome called peroxins which are a transport route between the mitochondria and peroxisome. These proteins are PMP's and Pex19 .[43,44] The expression of peroxisomal FAO can be seen through concentrations of PMP70, PEX19 and lignoceric acid. Lignoceric acid needs peroxisomes in order to be catabolized and Wicks et al showed lignoceric acid oxidation to be increased in obese subjects.[4] These new organelles coexist and cooperate with the beta oxidation system of the mitochondria, although they have different functions and benefit one another suggesting there is a link between the two organelles.

Summary

The purpose of this study is to determine if mitochondrial oxidative capacity in human skeletal muscle is associated with a compensation by peroxisomes and elevated plasma lactate concentration, determined by a reduction in mitochondrial protein content, in relation to a

subsequent reduced proportion of oxidative Type I muscle fibers. It is hypothesized that a reduction in mitochondrial capacity may explain why some individuals (whether they are lean or obese), are more susceptible to metabolic diseases such as Obesity and Type II Diabetes. It is further hypothesized that mitochondria content will be evident in skeletal muscle from subjects that are metabolically inflexible, as determined by higher blood lactate levels. We further hypothesize that the protein content of peroxisomes will be elevated to partially offset reductions in mitochondrial oxidative capacity.

CHAPTER III

METHODS

Human Subjects

Female, Caucasian subjects were recruited from the East Carolina University and the local Greenville and surrounding areas in North Carolina. Our subjects were 50 females. Inclusion criteria for our study were non-obese subjects with a BMI less than 30 kg/m^2 and obese subjects with a BMI over 30 kg/m^2. All the subjects were premenopausal, over the age of 21, and Caucasian. There was criteria for participants who were diagnosed with Type II Diabetes which is an impaired fasting hyperglycemia or diabetes. This is defined as a fasting glucose $\geq$7.0 mmol/L or $\geq$ 110 mg/dL. All participants were non-smokers and were not previously enrolled in an organized weight reduction program or diet regimen. Subjects were also sedentary and were not participating in organized physical activity regimens greater than 30 minutes per week. These samples were obtained from a J&J study where participants received an informed consent and all experimental procedures were approved by the Institutional Review Board of East Carolina University (UMCIRB 13-002234). Participants had their basal (fasting) blood lactate taken. Skeletal muscle biopsies were obtained from the lateral aspect of the vastus lateralis by the percutaneous needle biopsy technique. There were a total of 3 groups involved for this study. Subjects were separated into three distinct groups, 1) healthy non-obese with BMI <30 kg/m^2, 2) non-diabetic obese with BMI >30kg/m^2, and 3) diabetic, fasting glucose $\geq$7.0 mmol/L.

Muscle Biopsy

Muscle samples of approximately 80 – 120 mg were obtained from the lateral portion of the vastus lateralis of each participant in order to look for mitochondrial and peroxisomal protein content. One biopsy was completed for each subject. The muscle biopsy procedure used for this study involved first the removal of body hair from the area being used. The skin surface of the leg was sanitized with povidone and a sterile drape was then placed over the leg. Ethyl chloride was applied prior to injecting about 3.0 cc of 1% lidocaine without epinephrine. Next, a one centimeter incision was made through the skin and underlying fascia above the vastus lateralis. Afterward, a 5 mm Bergstrom needle was inserted through the fascia into the belly of the muscle. Once the needle was removed from the site, the muscle sample had pressure applied to the site of the incision. Pressure was continued for 10-15 minutes followed by an ice compact for an additional 5-10 minutes. Next, the incision was closed with steri-strips and a pressure bandage was fashioned around the thigh. All participants were directed to leave the pressure bandage in the same place for 12-24 hours and to contact the Human Performance Lab if any problems would come about.

Western Blotting Assessment

Western blotting assays were performed using PAGE-SDS procedures after whole cell tissue extraction for total protein for loading purposes for both the mitochondria and peroxisomes. Western blotting for mitochondrial and peroxisomal protein content was used to look at specific proteins. Proteins for both the mitochondria and peroxisomes was separated based on molecular weight and the antibodies that were used were specific for mitochondrial and peroxisomal specified proteins as verified previously in our laboratory. The specific antibodies

were used to specifically detect the presence of organelle proteins that are captured on the blotting membrane (Cell Signaling Technology, Danvers, MA, ab4844). This procedure is known as blocking for unspecified proteins that also bind to the blotting membrane. Then, the rest was washed away with multiple rinses to be cleared in order to add a second antibody that was specific to the primary antibody. The mitochondrial protein content that was assessed using western blotting was ETC function – cytochrome oxidase IV (COX IV). The peroxisomal protein content that was assessed using western blotting were PEX gene PEX 19, and peroxisomal membrane protein PMP70.

Details on tissue lysate preparation are as follows: Tissue was dissected as quickly as possible to prevent degradation by proteases. The tissue was then placed in microcentrifuge tubes and immersed in liquid nitrogen to snap freeze. Samples were stored at -80˚C for later use. For each ~5mg piece of tissue, ~300 μL ice cold lysis buffer was added, and homogenized with an electric homogenizer. The blade was rinsed twice with 2×300 μL lysis buffer. Then a constant agitation was maintained for 2 hours at 4˚C while on a shaker in the fridge. Next, the samples were centrifuged for 20 minutes at 12,000 rpm at 4˚C in a microcentrifuge. The tubes were then placed on ice, aspirated the supernatant and placed in a fresh tube kept on ice.

Details on the Western Blot quantification methods are as follows: *Western blotting assessment.* Western blotting assessments was using standard PAGE-SDS procedures. Briefly, 20 μg of protein from tissue cell lysates were suspended in sample buffer containing lammeli buffer (BIO-RAD 161-0710) and β-mercaptoethanol (9:1 ratio), are boiled at 95^0C for 5 minutes and then protein is equally loaded (verified by BCA protein determination) into wells of precast polyacrylamide 10% TRIS-HCl gels (Bio-Rad, Richmond, CA). Electrophoresis occurs at 100V for 30 minutes and then at 150V for 1 hr. Afterwards, proteins were transferred at 95V for 1.5 hr

onto a PVDF membrane (Millipore, Billerica, MA) pre-wetted with methanol and rinsed briefly with transfer buffer. Following membrane drying for 1.5hr, membranes were incubated in blocking buffer (Li-Cor 927-40000) for 1 hr and then incubated at 4^0C overnight in the blocking buffer with primary antibodies: anti-COX IV (ab4844), antianti-PEX19 (ab139684), anti-PMP70 (ab85550), antibodies were purchased from Abcam (Cambridge, MA). After five washes using Tris-buffered saline (TBS) with Tween (TBST) [(TBS 0.1% with Tween 20 (Bio-Rad, Hercules, CA)], membranes were incubated at room temperature for 1 hr in blocking buffer with appropriate secondary antibodies accordingly: goat anti-mouse 680LT (#926-68020), goat anti-mouse 800CW (#926-32210), donkey anti-rabbit 680LT (#926-68023), goat anti-rabbit 800CW (#926-32211), purchased from Li-Cor (Lincoln, NE). Following further washes in TBST, detection, quantification, and imaging was performed using an Odyssey infrared imaging system (Li-Cor, Lincoln, NE). All data were normalized to total ribosomal 18s protein for each sample blotted for.

Fatty Acid Oxidation

Mitochondrial fatty acid oxidation was measured in primary human skeletal muscle homogenates according to the methods of J.Y. Kim et al. (2000). Briefly, muscle fatty acid oxidation rate was determined in the fresh muscle homogenate. The oxidation rate was measured by collecting and counting the $^{14}CO_2$ produced during incubation. Forty micro-liters of a 20-fold diluted muscle homogenate were pre-incubated with a 95% O2 -5% CO2 mixture at 30°C for 15 min. A160-ml reaction mixture (pH 7.4) was then added to the preincubated muscle homogenate. Final concentrations of the incubation mixture were in millimoles per liter. The substrates used were 0.2 mM [1-14C] palmitate (0.5mCi), 0.2mM [1-14C] octanoate (0.5mCi), or 0.2 mM [1-14C] palmitoyl carnitine (0.5mCi) with 0.5% BSA. After 60 min of incubation at 30°C, 100ml of

4N sulfuric acid were injected to stop the reaction. CO2 produced during the 60-min incubation was trapped with 200 ml of 2M sodium hydroxide. Briefly, the incubation wells and $^{14}CO2$ trap consisted of a modified 48-well microtiter plate (Costar, Cambridge, MA). Each system consisted of two adjacent wells with a fabricated groove between each to allow the acid-driven $^{14}CO2$ from the homogenate to be trapped by the sodium hydroxide. Adjoining well pairs were sealed from each other by a rubber gasket. One hundred fifty microliters of the sodium hydroxide trap were counted for evolved $^{14}CO2$ by liquid scintillation and oxidation rate expressed as nano-moles of CO2 per gram of wet weight per hour.

Lactate Concentrations

Basal (fasting) plasma blood lactate was determined for all subjects prior to the skeletal muscle biopsy procedure. Blood lactate was determined using a Coulter-Beckman clinical blood analyzer. Subjects lactate concentrations were determined to be those with low to normal (< 0.6 mM) and high (> 1.0 mM). For assessing lactate, blood was drawn from a vein and plasma prepared.

Statistical Analysis

To examine various relationships between specific variables, a Pearson Product Moment Correlation and linear regression analysis were used to report the strength of the association between lactate concentrations (marker for anaerobic) and protein signals for organelle content and oxidative function [(Plasma lactate, DV, Y axis); (FAO, IV, X axis)]. Healthy non-obese, non-diabetic obese and diabetic subjects were compared and assessed using a One-way ANOVA. Correlations between lactate and FAO were determined using a One-way ANOVA among groups. The three groups for this group mean testing were non-obese who are metabolically

flexible, obese who are metabolically inflexible and diabetic. The statistical significance for this study was set *a priori* at a probability of $p < 0.05$ to reject the null and accept the alternative, experimental hypotheses.

CHAPTER IV

RESULTS

Overview

The major findings from this hypothesis driven research, conducted in human skeletal muscle homogenates obtained from Caucasian females are as follows: 1) PEX19 protein content was significantly higher (P = 0.0044) in diabetic subjects compared to non-obese and obese subjects. 2) Compared to obese and diabetic subjects, non-obese subjects demonstrated a significantly higher (P = 0.0069) COX IV protein content. 3) In relation to lactate (mmol) PEX19 protein content was significantly correlated to lactate. 4) Unexpectedly, PMP70 remained similar across all groups, 5) both PMP70 and COX IV in relation to lactate concentration remained relatively unchanged and were not significant, and finally 6) lactate was significantly lower in non-obese subjects when correlating lactate and fatty acid oxidation.

Participants

Participant's demographic data: age (yrs), height (in), weight (lbs), BMI (kg/m^2), waist (cm), hip (cm) and waist:hip from forty four Caucasian women are presented in Table 1 and are displayed in non-obese, obese and diabetic groups. Participants were between 21 and 55 years of age and were both non-obese (n=18), obese (n=26) and diabetic (n=9). On average the non-obese participants were 31.28 (±2.50) years of age compared to obese 44.81 (±2.19) and diabetic 49.67 (±2.96). The criteria for being non-obese vs obese was a BMI less than 30 kg/m^2 which constituted as being non-obese. On average non-obese participants had a BMI of 24.20 kg/m^2 (±0.80) whereas, obese were 41.06 kg/m^2 (±1.93) and diabetic 38.91 kg/m^2 (±2.82). Further detail can be found in Table 1.

All participants completed the personal history questionnaire and the responses garnered from the questionnaires confirmed that these participants were premenopausal, sedentary and were not taking medications known to affect skeletal muscle metabolism such as glucose oxidation. Each participant filled out and completed a menstrual cycle questionnaire which indicated the use and type of oral contraceptives during the study. In addition, blood measurements were obtained which included, cholesterol (mg/dl), triglycerides (mg/dl), HDL (mg/dl), VLDL (mg/dl), LDL (mg/dl), HDL:LDL, Total:LDL, assessment of fasting blood glucose (mg/dl), fasting insulin (µIU/mL), insulin resistance by homeostatic model assessment of insulin resistance (HOMA-IR), and lactate (mmol/L) were performed on eighteen non-obese, twenty-six obese and nine diabetic subjects, as shown in Table 2. According to the American Heart association (AHA) guidelines, cholesterol levels are healthy when total cholesterol is <200 mg/dl, HDL >40mg/dl, and LDL <130 mg/dl. Fasting blood glucose (mg/dl), insulin sensitivity levels (HOMA-IR) and insulin levels (µIU/mL), were determined based on criteria from the American Diabetes Association (ADA) guidelines. According to the ADA guidelines, a normal

fasting blood glucose is between 70 and 100 mg/dl, whereas a fasting blood glucose of >126mg/dl is the standard diagnosis for diabetes. The standard guidelines for HOMA-IR is <3.60 and the criteria for insulin sensitivity, however a HOMA-IR >3.60 is the criteria for insulin resistance. HOMA was calculated from fasting insulin and blood glucose by using the following formula: fasting blood glucose (mg/dl) X fasting insulin (μIU/mL) / 405. Finally, normal resting lactate levels are listed as 0.5-1mmol/L.

Participant's blood data was also collected and described in full in Table 2. The non-obese group was consistently lower in all values compared to the obese and diabetic group. Glucose values for non-obese were 85.61 (±2.15), obese 106.43 (±5.40) and diabetic 120.46 (±5.18). Insulin levels were 4.62 (±0.51) for non-obese, 19.92(±6.61) for obese and 14.21 (2.78). Insulin resistance was determined using HOMA-IR and was found to be 1.00 (±0.12) in non-obese, 6.93 (±3.46) and 4.29 (±0.96) in diabetic. Finally, lactate concentrations were determined and found to be significantly lower in non-obese participants compared to obese and diabetic, 0.93 (±0.07), 1.31 (±0.09) and 1.41 (±0.15) respectively. These demographic data demonstrate non-obese subjects were healthy, metabolically flexible with a lower degree of adiposity and lower lactate concentrations. This suggests non-obese do not have a significant shift towards anaerobic glycolysis, whereas obese and diabetic subjects did.

Participant's Characteristics

Caucasian Women

	Non-Obese (n=18)	Obese (n=26)	Diabetic (n=9)
Age	31.28 ± 2.50	44.81 ± 2.19	49.67 ± 2.96
Height (in)	65.22 ± 0.64	65.10 ± 0.79	66.78 ± 0.89
Weight (lbs)	145.98 ± 4.44	245.92 ± 10.50	247.39 ± 18.46
BMI (kg/m^2)	24.20 ± 0.80	41.06 ± 1.93*	38.91 ± 2.82*
Waist (cm)	77.64 ± 2.60	119.20 ± 3.79	127.11 ± 7.78
Hip (cm)	98.72 ± 1.87	135.32 ± 2.99	140.56 ± 5.40
Waist : Hip	0.79 ± 0.02	0.88 ± 0.02	0.90 ± 0.03

Table 1. Data are expressed as mean ± SEM for non-obese (n=18), obese (n=26) and diabetic (n=9) Caucasian females; age, height, weight, BMI (Body Mass Index), waist, hip and waist:hip * Significant difference ($P < 0.05$).

Cholesterol, Fasting Glucose, Insulin, Insulin Resistance (HOMA) and Lactate

Caucasian Women

	Non-Obese (n=18)	Obese (n=26)	Diabetic (n=9)
Cholesterol (mg/dl)	188.56 ± 11.28	205.54 ± 9.33	208.11 ± 19.57
Triglycerides (mg/dl)	89.22 ± 8.30	155.96 ± 14.25	182.11 ± 20.86
HDL (mg/dl)	62.44 ± 4.68	51.29 ± 2.08	50.00 ± 2.75
VLDL (mg/dl)	17.89 ± 1.63	31.18 ± 2.83	36.31 ± 4.14
LDL (mg/dl)	108.28 ± 8.50	123.08 ± 7.86	121.80 ± 16.43
HDL : LDL	0.63 ± 0.07	0.44 ± 0.02	0.44 ± 0.04
Total : LDL	3.18 ± 0.20	4.10 ± 0.20	4.17 ± 0.28
Glucose (mg/dl)	85.61 ± 2.15	106.43 ± 5.40*	120.46 ± 5.18*
Insulin (µIU/mL)	4.62 ± 0.51	19.92 ± 6.61	14.21 ± 2.78
HOMA IR	1.00 ± 0.12	6.93 ± 3.46	4.29 ± 0.96
Lactate (mmol/L)	0.93 ± 0.07	1.31 ± 0.09*	1.41 ± 0.15*

Table 2. Data are expressed as mean ± SEM. Data are expressed as cholesterol, triglycerides, HDL, LDL, and glucose (mg/dl), insulin (µIU/mL), insulin sensitivity (HOMA) and lactate (mmol/L) values in non-obese (n=18), obese (n=26) and diabetic (n=9) Caucasian females prior to muscle biopsy. * Significant difference ($P < 0.05$).

PEX19 Protein Content

PEX19 was chosen to look at whether the peroxisomal content was upregulated to assist the mitochondria in β-oxidation and VLCFA seen in obese and diabetic skeletal muscle. In order to determine PEX19 protein content in human skeletal muscle, the protein of interest was plotted several times using western blotting analysis technique. This was done in mom-obese, obese and diabetic subjects as shown in Figure 1. A one-way ANOVA was used to compare between groups of PEX19 protein. A significant difference was found between the diabetic subjects who had a significantly ($P < 0.05$) higher protein content than the non-obese group (0.88 ± 0.10 vs 1.53 ± 0.26) and obese group (1.08 ± 0.12 vs 1.53 ± 0.26) respectively.

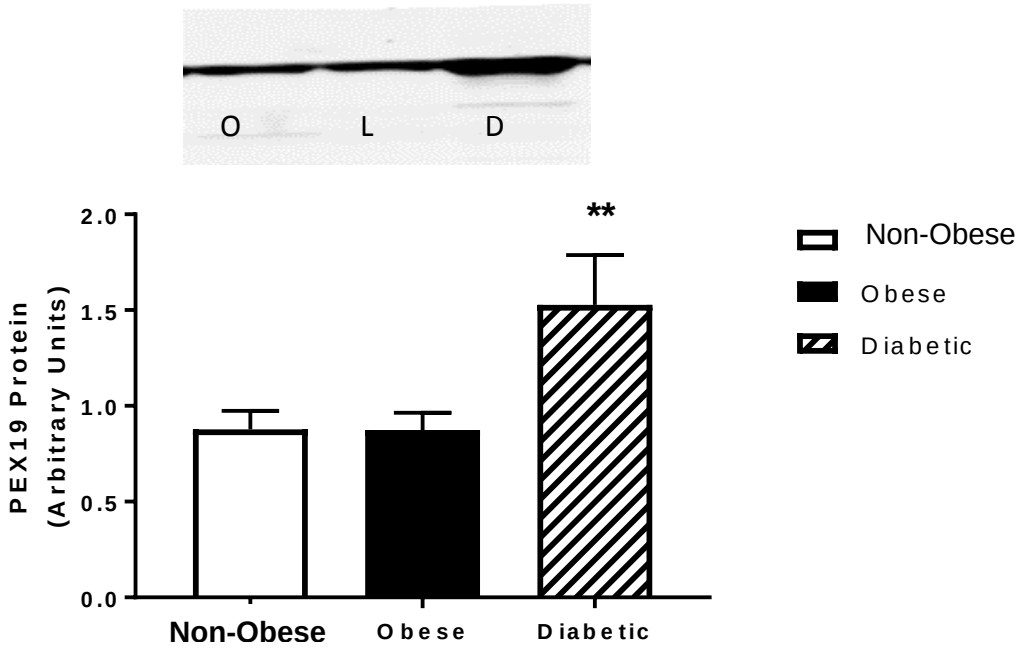
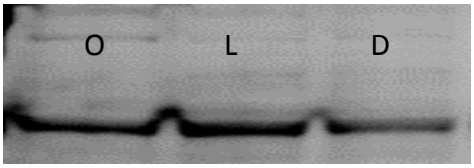


Figure 1. PEX19 Protein Content. Western blot analysis of PEX19 protein contents. Data are presented as non-obese (n=18), obese (n=26) and diabetic (n=9) in women. * Significant difference ($P < 0.05$) between non-obese, obese and diabetic women.

PMP70 Protein Content

PMP70 Protein content was determined by western blotting analysis. Protein bands were detected at the molecular weight for human skeletal muscle samples. Western blot analysis found a significant difference ($P < 0.05$) in diabetic women following a two-tailed t-test. Subsequently, we expected PMP70 to increase more for obese and diabetic subjects as they would have needed more assistance from the peroxisomes in relation to the mitochondria. A one-way ANOVA was used to compare between groups of PMP70 protein. Figure 2. demonstrates no significant difference ($P = 0.0741$) between the groups, non-obese (1.05 ± 0.09), obese (0.97 ± 0.11) and diabetic (0.66 ± 0.08).



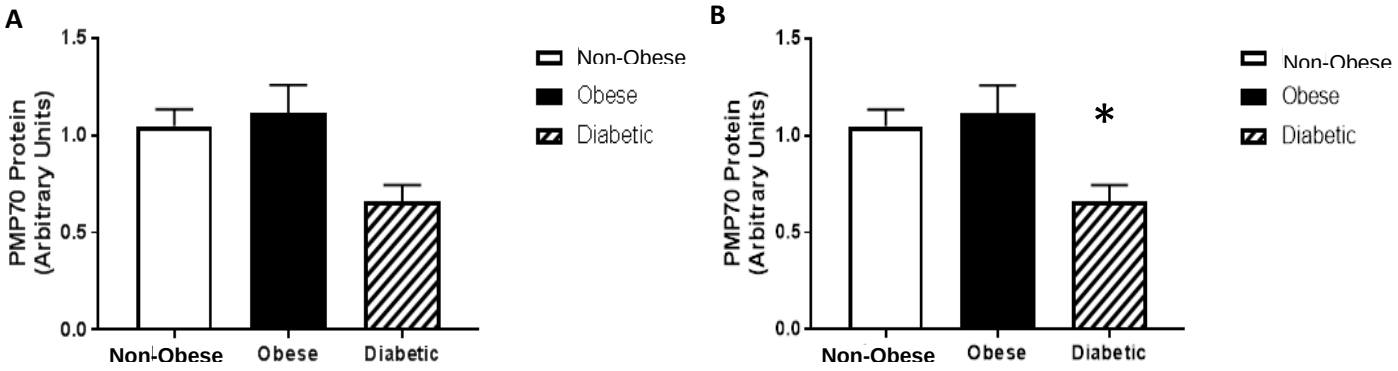


Figure 2. PMP70 Protein Content. PMP70 protein content as determined by western blot analysis in Caucasian women. Data are presented as non-obese (n=18), obese (n=26) and diabetic (n=9) in women. * Significant difference ($P < 0.05$) between non-obese, obese and diabetic. Figure 2a is the original analysis and 2b is following a two-tailed t-test, significance was found in diabetics.

COX IV Protein Content

A one-way ANOVA was used to compare between groups and COX IV protein. As presented in Figure 3, there was a significant difference between the non-obese subjects as they possessed a significantly ($P < 0.05$) higher COX IV protein content than the obese and diabetic groups. Non-obese (1.15 ± 0.07), obese (0.90 ± 0.05) and diabetic (0.83 ± 0.08) respectively. These results were what we expected as COX IV is a mitochondrial protein. We expected COX IV to be higher in those who were non-obese as their mitochondrial content would be higher.

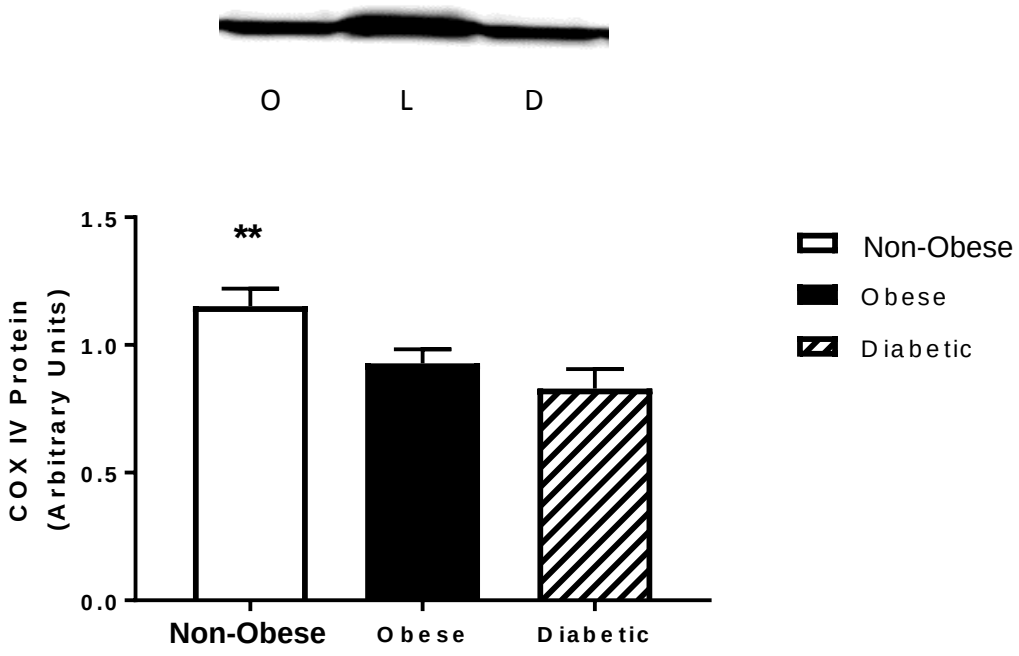


Figure 3. COX IV Protein Content. Comparison of COX IV protein content as determined by western blot analysis. Data are presented as non-obese (n=18), obese (n=26) and diabetic (n=9) in women. * Significant difference ($P < 0.05$) between non-obese, obese and diabetic Caucasian women.

Lactate Concentration in Relation to PEX19

There was a significant, positive correlation and interaction between PEX19 protein and lactate concentration (mmol/L). As we expected, those who had a higher PEX19 protein content, the subsequently higher lactate concentration they possessed. When comparing PEX19 protein content and lactate concentrations, lactate concentrations were significantly and positively correlated with PEX19. This can be seen in Figure 4. The significant association ($P < 0.05$) resulted in $P=0.0344$ and $r^2 = 0.1324$. Those with lower PEX19 content had lower lactate vs a higher PEX19 content had a higher lactate.

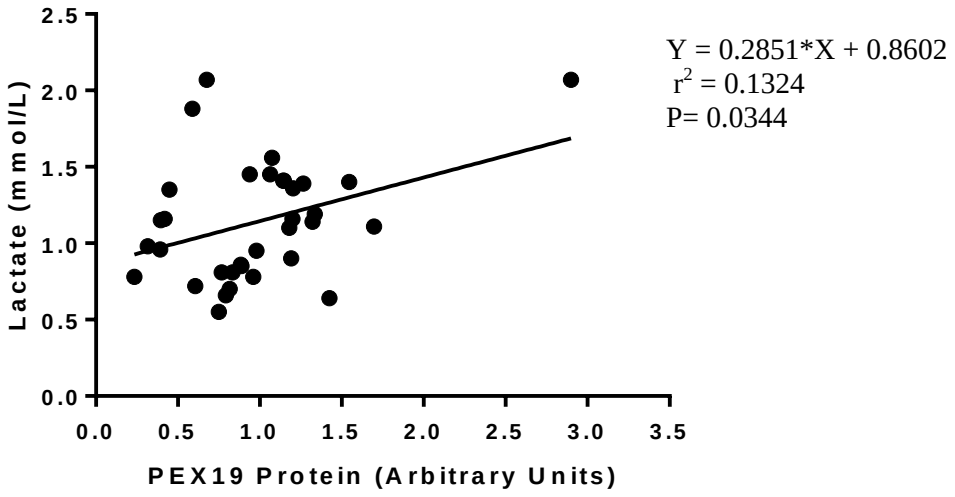


Figure 4. PEX19 Protein Content in Relation to Lactate Concentration. Relationship between PEX19 protein content and lactate (mmol/L) concentration in non-obese (n=18), obese (n=26) and diabetic (n=6).

COX IV in Relation to PEX19

There was no significant ($P < 0.05$) interaction between COX IV and PEX19 protein concentrations. This was not what we expected to see as we hoped to find that those individuals with a higher PEX19 content would present a lower COX IV content. We believed this to be the case due to PEX19 being higher in the diabetic subjects and COX IV being lower. However, this was not the result as demonstrated in Figure 5. The interaction was seen at $P = 0.8059$ and $r^2 = 0.001528$.

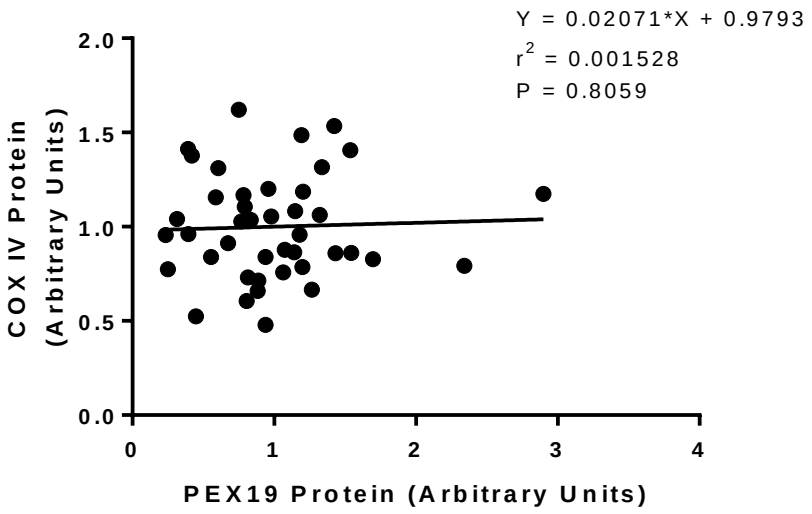


Figure 5. COX IV Protein Content in Relation to PEX19 Protein Content. Relationship between COX IV protein content and PEX19 protein content in non-obese (n=18), obese (n=26) and diabetic (n=9) Caucasian women.

Lactate Concentration in Relation to PMP70

There was no significant ($P < 0.05$) association between PMP70 protein and lactate concentration (mmol/L). This was not what we expected to see as we hoped to find that those individuals with a higher PMP70 protein content would also present a higher lactate concentration. We believed this to be the case due to PMP70 being a peroxisomal protein, and with a higher need for the peroxisome, the presumed greater shift from aerobic to anaerobic metabolism, therefore resulting in a greater lactate concentration. However, this was not the result as demonstrated in Figure 6. The interaction was seen at $P = 0.4880$ and $r^2 = 0.01515$ demonstrating those with a lower PMP70 content had lower lactate vs higher PMP70 content had a higher lactate, but were not significant.

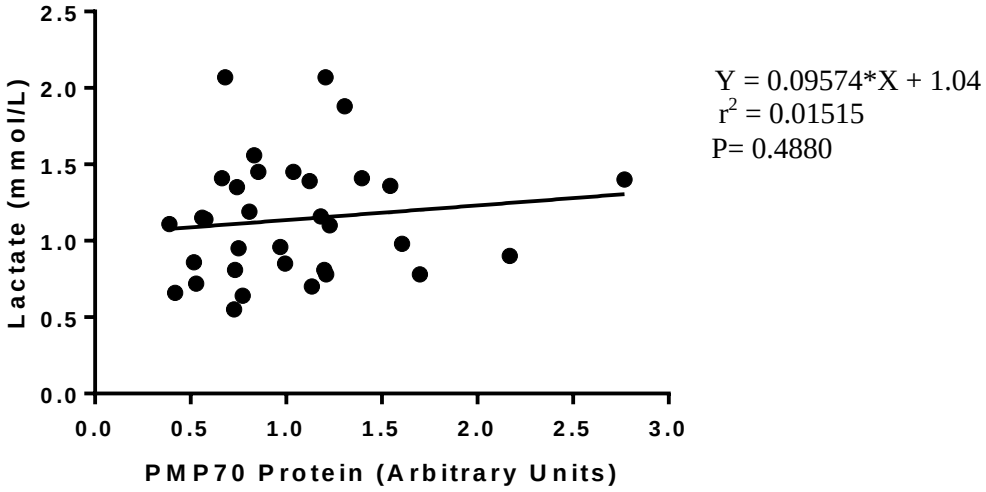


Figure 6. PMP70 Protein Content in Relation to Lactate Concentration. Relationship between PMP70 protein content and lactate (mmol/L) concentration in non-obese (n=18), obese (n=26) and diabetic (n=9) Caucasian women.

Lactate Concentration in Relation to COX IV

Following the Grubb's test for outliers, there was a significant ($P < 0.05$) association between COX IV protein and lactate concentration (mmol/L). With a continued reliance on the mitochondria and presumably normal functioning mitochondria, there would be a continued reliance on aerobic metabolism and therefore less lactate buildup. This was indeed the result as demonstrated in Figure 7. The interaction was seen at $P = 0.1148$ and $r^2 = 0.0759$, demonstrating those with a lower COX IV content had a higher lactate vs higher COX IV content and a lower lactate.

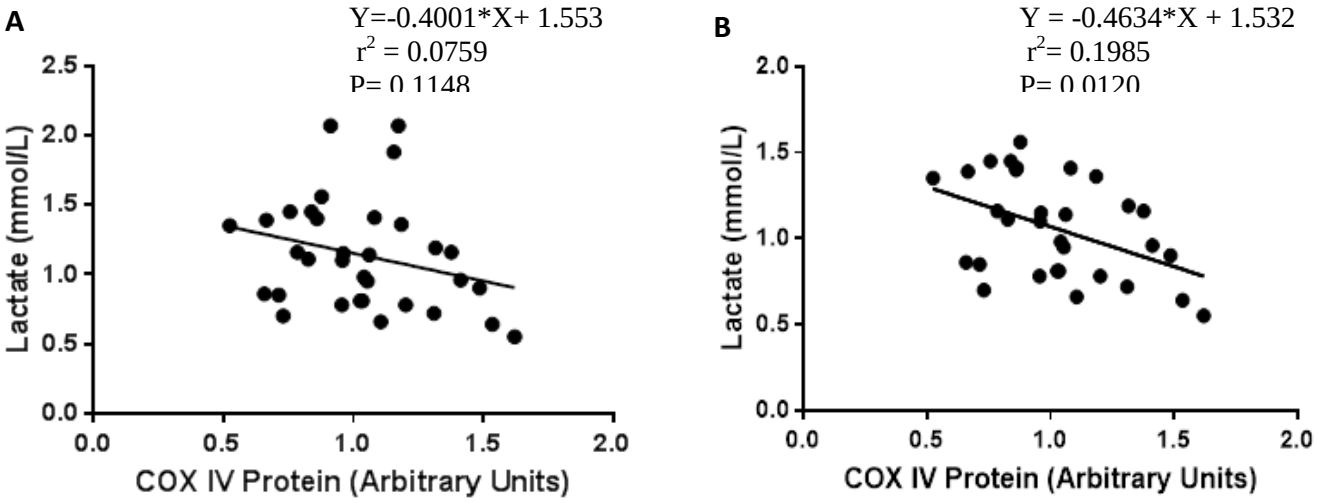


Figure 7. COX IV Protein Content in Relation to Lactate Concentration. Relationship between COX IV protein content and lactate (mmol/L) concentration in non-obese (n=18), obese (n=26) and diabetic (n=6) women. Figure 7a shows the original data analysis and 7b depicts the analysis following the Grubbs Test for outliers where two obese and one diabetic were found to be outliers.

Fatty Acid Oxidation in Relation to Lactate Concentration

There was a significant, positive correlation and interaction between fatty acid oxidation and lactate concentration (mmol/L) in non-obese subjects. As we expected, those who had a higher oxidation rate, the lower their lactate. This can be seen in Figure 8. The significant interaction ($P < 0.05$) resulted in P=0.0471 and $r^2 = 0.3387$. Those with lower oxidation rates had higher lactate vs a higher oxidation and a lower lactate.

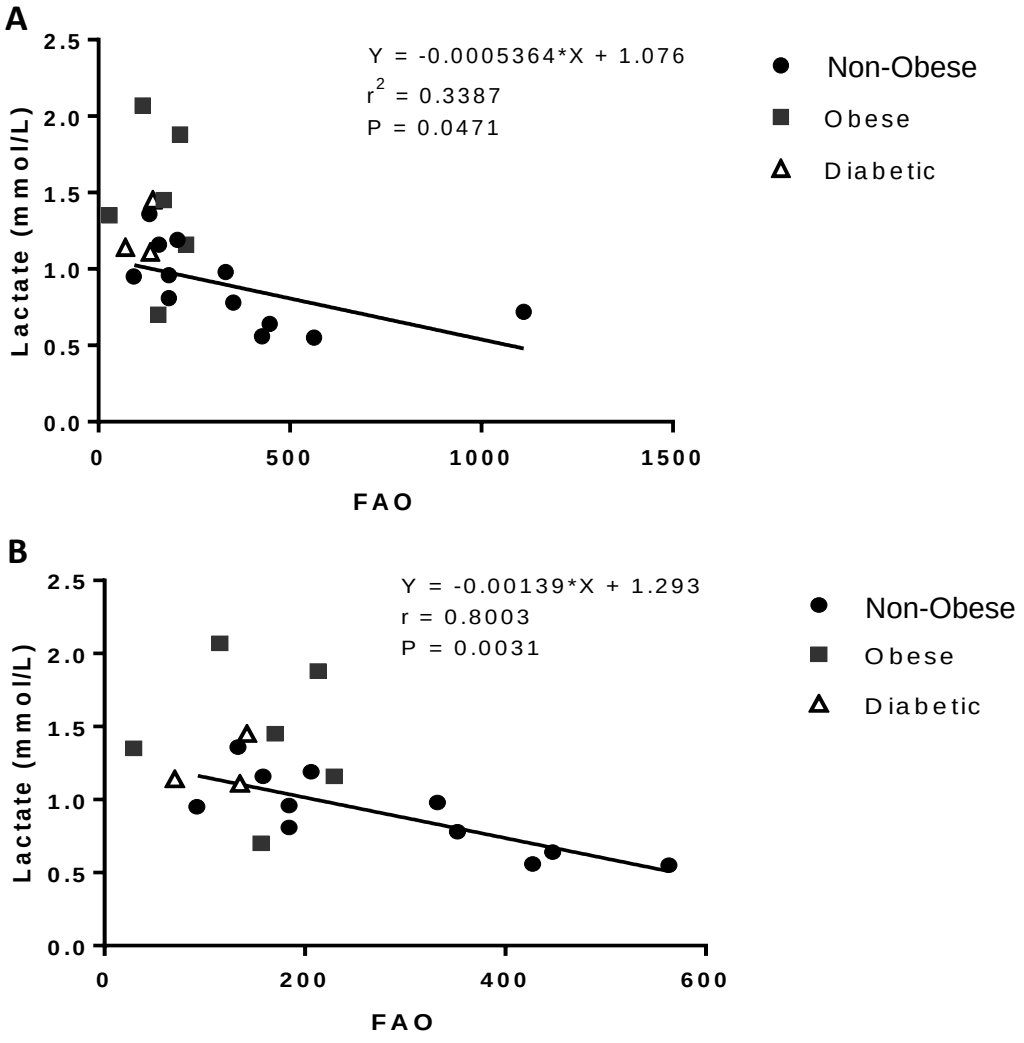


Figure 8. FAO in Relation to Lactate Concentration. Relationship between fatty acid oxidation and lactate (mmol/L) concentration in non-obese (n=12), obese (n=6) and diabetic (n=3) women. Figure 8a shows the original data analysis and 8b depicts the analysis following the Grubbs Test for outliers where one non-obese subject was found to be an outlier.

Fatty Acid Oxidation in Relation to COX IV

There was no significant ($P < 0.05$) interaction between fatty acid oxidation and COX IV protein content. This was not what we expected to see as we hoped to find that those individuals with a higher COXIV protein content would also present a higher fatty acid oxidation. However, this was not the result as demonstrated in Figure 9. The interaction was seen at $P = 0.1029$ and $r^2 = 0.107$ demonstrating those with a higher fatty acid oxidation also had a higher COXIV content, but was not significant.

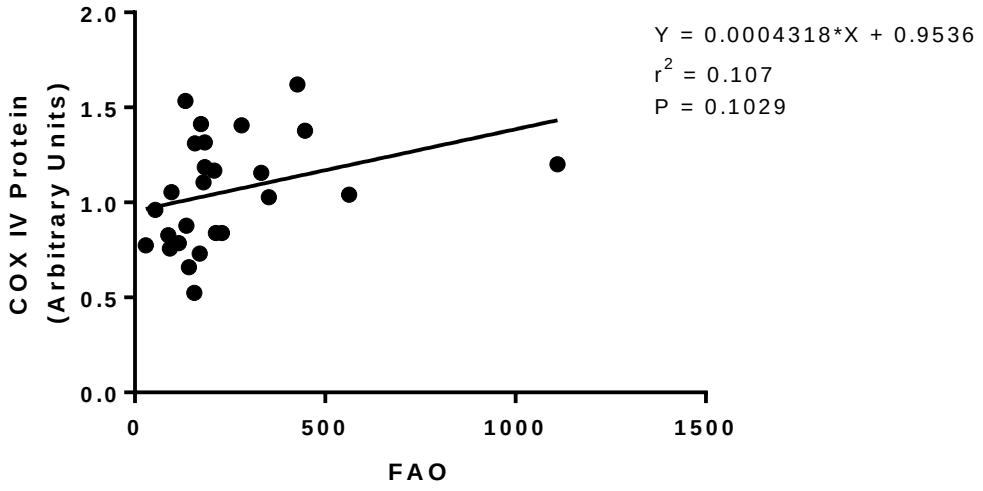


Figure 9. FAO in Relation to COX IV Protein Content. Relationship between fatty acid oxidation and COX IV in non-obese (n=12), obese (n=6) and diabetic (n=3) Caucasian women.

CHAPTER V

DISCUSSION

We predicted reductions in oxidative capacity are due to a lower mitochondrial content and overall dysfunction of the mitochondria. This in turn will hopefully indicate or provide some reasoning as to why some individuals are more susceptible to metabolic diseases such as Type II Diabetes and obesity. More specifically, we hypothesize these reductions in mitochondrial content will be evident in the skeletal muscle from subjects who are metabolically inflexible, which will be associated with higher blood lactate levels. Additionally, protein content of peroxisomes will be elevated to offset reductions in mitochondrial oxidative capacity. This study is a significant addition to previous research in that it will hopefully shed light on the relationship between mitochondrial and peroxisomal content and increased lactate production. With this information, more effective strategies for combatting obesity and Type II Diabetes may be implemented.

Obesity is defined for individuals as a BMI over 30 kg/m^2 and is continuing to be an issue globally. One idea to this being is a reduction in mitochondrial oxidative capacity and an increase in lactate. Previous related research have shown an increase in lactate concentration as there is a greater reliance on anaerobic glycolysis due to a depressed oxidation of glucose and fatty acids. The purpose of this study was to determine if a reduced skeletal muscle mitochondrial oxidative capacity is associated with a reduced mitochondrial content. Secondly, this study examined if mitochondrial protein content is associated with blood lactate levels as a surrogate for aerobic capacity. Finally, we determined if blood lactate is associated with measures *of in vitro* fatty acid oxidation.

Defects found in gene expression, muscle glycogen synthesis and an accumulation of intramyocellular triglycerides have all been identified as mediators for insulin resistance. To identify this we looked at various mitochondrial and peroxisomal protein content in human skeletal muscle in relation to blood lactate levels (marker for reductions in substrate oxidative capacity). Skeletal muscle dysfunction can be attributed to lower mitochondrial content and a reduced fatty acid oxidation suggests an abnormality in mitochondrial oxidative capacity.[4,17]

In a response to a reduction in mitochondrial content and oxidative capacity, the mitochondria will garner the help from its metabolically related organelle, the peroxisome. If lipids increase intracellularly, peroxisomal content will increase due to a need for oxidation of LCFA, which is employed to reduce the lipotoxic environment. Increasing peroxisomal content will activate its partial β-oxidation system and cause a reduction of lipotoxicity by chain shortening excess fatty acids and delivery of acylcarntine export products to the mitochondria to assist in complete oxidation of this substrate. Surprisingly, western blot analysis determined that the peroxisomal import protein, PMP70 did not increase any of the three groups studied. However, we did find that in diabetic subjects, there was a significant increase in PEX19 protein content.

PEX19 is critical for the peroxisomal membrane and import of peroxisomal membrane proteins (PMP's). PEX19 is also a soluble chaperone and import receptor for newly synthesized PMP's.[43] As a chaperone, PEX19 functions to bind to PMP's in the cytosol and is then recruited by the membrane receptor PEX3. A loss of PEX19 would be significant and result in the absence of detectable peroxisomal membranes. Similar to the mitochondria, the peroxisome has a transport system with the ER. A disruption between transporting among the ER and peroxisomes may be why PMP70 was not significantly higher in obese or diabetics.[60]

The endoplasmic reticulum and mitochondria share some regulatory factors such as assisting one another in a combined effort. One important aspect is that proteins reside in different parts of the organelles, but still interact and facilitate with each other to confer function. These organelles are approximately 10-25 nm from each other within the muscle cell which further explains how proteins on either the ER or the outer mitochondrial membrane are able to interact with one another. The continuously undergoing fusion and fission allow for the mitochondria to maintain their shape, size, and number. However, disruption of glucose homeostasis is the underlying principle of physiopathology of Type II Diabetes. The interaction between the ER and mitochondria is seen through contact points known as MAM's, mitochondria-associated membranes. If there is a miscommunication between the organelles, skeletal muscle insulin resistance may occur.[23,55] This interaction is important as some mitochondria are closely located to the Ca^{2+} release site of the ER. This is important as Ca^{2+} levels control the activity of the Krebs cycle. The increased interaction allowed for an increase in ER-mitochondrial coupling to enhance bioenergetics. This can only occur when the integrity of those MAM's are intact for insulin signaling. This idea is reciprocal as high glucose levels disrupted MAM integrity and function.

ER-mitochondrial miscommunication may contribute to metabolic disease. Therefore, MAMs could be a target to improve insulin action and improve glucose homeostasis. G. Hotamisligil, found MAM content was increased in obese and diabetic mice, which led to mitochondrial Ca^{2+} overload, mitochondrial dysfunction, and increased oxidative stress. Furthermore, the reduced expression of MAM improved mitochondrial oxidative capacity. In addition, an enhanced disruption of MAM's was associated with a loss of regulation, but further reductions were found in obese and diabetic mice due to a chronic disruption of MAM integrity.

This was further discussed with ER-mitochondria interactions in human myotubes from obese subjects who had altered insulin signaling compared to non-obese subjects. There are several contact sites along the ER and mitochondria, allowing different protein complexes to join. Based on our results it is plausible that the PEX19 gene is overcompensating for extremely high glucose levels compared to PMP70. Also, it would be worthwhile to look at ER-mitochondria interaction to address how ER stress is mediated and if this could be a reason why some proteins are more significantly increased than others.[23,45,58]

The peroxisomes are upregulated in response to a reduction or defect in mitochondrial oxidative capacity in order to compensate for this deficiency. While the decrease in mitochondrial oxidative capacity effects the peroxisomes it too effects the mitochondria's protein content. During a lipotoxic environment, the mitochondria does not function as efficiently and therefore results in a subsequent reduction in mitochondrial content. To test this we used COX IV protein and ran western blots for all subjects. What we found when we compared skeletal muscle from non-obese subjects to obese and diabetic, there was a significant reduction in obese and diabetic (Figure 5) subjects in COX IV content. Our results show that in response to a presumably greater lipotoxic environment/higher fat content in the tissues from obese and diabetic, COX IV overexpression was reduced vs. non-obese. This is in conjunction to previous studies by Houmard et al., where higher fat content was demonstrated in obese subjects.[29] This was also evident in a study by Kirwood et al., where it was demonstrated that central body fat was associated with a lower mitochondrial density, skeletal muscle mitochondria were smaller in size by ~35% in obese and Type II Diabetics, as well as a less defined inner membrane structure.[19,22] These results are consistent with extreme obese subjects (BMI >40kg/m^2)

demonstrating a significant reduction in FAO by ~60% compared to obese and lean subjects.[29] This is discussed in more detail below.

Our study showcased a higher COX IV protein content in skeletal muscle from the non-obese subjects suggesting a higher mitochondrial protein content and oxidative capacity. This data supports a previous study by Ritov et al., where they found a significant reduction in mitochondrial ETC activity in obese and diabetic subjects.[56] This study found the subsarcolemmal mitochondria electron transport chain activity was reduced in Type II Diabetic subjects (0.017 ± 0.003 vs 0.034±0.007 units/Mu) creatine kinase and a sevenfold decrease compared to lean subjects and a 3-4 fold reduction in obese subjects. In addition, in diabetics there was a significantly lower mitochondrial protein content compared to leans (0.8±0.1, 1.1 ±0.2 and 1.3±0.4 mg/g), as well as type II diabetics had a depletion of sarcolemma mitochondria thickness by 3 fold compared to lean. Furthermore, Boushel et al., found no significant differences in oxidative phosphorylation or ETC activity between type II diabetics and healthy control through citrate synthase activity or mitochondrial DNA content, which may be due to subjects not being significantly obese.[8] mtDNA content in skeletal muscle was reduced in obese and Type II Diabetics which shows a reduced mitochondrial content does not fully account for the overall reduction in ETC activity.[46]

Similarly, a study by Heilbronn et al., found an expression of PGC1 beta and COX1 to be significantly lower in an insulin resistant group. Furthermore, this study found citrate synthase activity and protein levels from mitochondrial complexes 1 and 3 of the respiratory chain to be lower in the insulin resistant group, CS (136 ± 8 vs 112 ± 6 nmol/mg protein*min) and COX1 (61 ±14 vs 38± 4 nmol/mg protein*min). A basic functional defect in mitochondrial activity in skeletal muscle is seen through a reduced amount of mitochondrial protein or citrate synthase

activity.[4] Our study also found a reduction in fatty acid oxidation in both obese and diabetic subjects compared to non-obese subjects. This has been supported by a previous study by Patti et al., where they found a decrease in the expression of genes that are involved in fatty acid oxidation in diabetic subjects.[51] These genes included mitochondrial 3,2-transenoyl-CoA isomere and 2,4-dienoyl CoA reductase 1 and were found on multiple components of the mitochondrial respiratory chain and a progressive decrease in expression genes, encoding key proteins in oxidative metabolism, were seen in insulin resistant and diabetic subjects. Furthermore, Turner et al., demonstrated insulin resistant subjects to have reductions in mRNA levels of mitochondrial genes and decreased mitochondrial DNA.[67]

Apart from protein content, we also measured plasma lactate concentrations in relation to PEX19, PMP70, and COX IV in non-obese, obese and diabetic subjects. Research has shown an increase in lactate as a result of an increase reliance on anaerobic glycolysis due to a depressed oxidation of glucose and fatty acids. Therefore, we looked to determine if lactate is a surrogate marker for aerobic capacity in relation to mitochondrial and peroxisomal protein content. As a result, we found a significant association in lactate in relation to PEX 19. However, we did not see any significance between lactate and PMP70 or COX IV. PMP70 is involved in the metabolic transport of long chain acyl-CoA across peroxisomal membranes and therefore, would expect to be higher in obese and diabetic subjects, as well as a significance between lactate and PMP70. The present study did not conclude this, but a study by Imanaka et al., established that PMP70 was present in the cytosol, suggesting the location of PMP70 to be significant.[32] PMP70's function is to transport proteins across the membrane, and therefore may not be significantly correlated to lactate accumulation. COXIV was also not significantly associated with lactate, and a study by Tran et al., found COXIV to be elevated in the mitochondria, but

lactate dehydrogenase to be elevated in the cytosol, suggesting location is a factor.[66] Future studies may be able to see a more significant association between a different cytochrome oxidase subunit with lactate.

Elevated plasma lactate is present in insulin – resistant conditions, including obesity and type II diabetes. This has been shown in association with anaerobic metabolism and an exaggerated anaerobic response.[10] A study by Crawford et al., found similar results to our study. They found lactate to be elevated among obese, insulin resistant subjects, as well as a reduced oxidative capacity associated with higher levels of plasma lactate in type II diabetics.[14] Lactate was higher in association to a higher BMI, which was also evident in our study. Lactate was also elevated in relation to fatty acid oxidation for both obese and diabetic subjects compared to non-obese. Overall, lactate was lower in non-obese subjects, higher in obese and even more elevated in diabetics. Another study by Consoli et al., supports our data as they found plasma lactate appearance to be 50% greater in type II diabetic subjects (18.2 ± 0.9 umoles/kg/min) vs 12.6 ± 0.7 umoles/kg/min) in leans.[11] There should be no storage of glucose in the post absorptive state and when glucose oxidation is reduced as in in diabetic subjects, one may be able to speculate an increase in muscle glucose uptake would result in an increase in glycolytic products. As oxidation decreases and is less efficient there is a subsequent shift from aerobic to anaerobic metabolism and therefore the rise in lactate concentrations, due to glucose entering the cell and immediately shifting toward anaerobic glycolysis.[73]

A reduced mitochondrial oxidative capacity and reduction in mitochondrial content results in an accumulation of fatty acids. Due to this there is a higher reliance on the peroxisome as well as a shift towards anaerobic glycolysis. As a result, the present study looked at the effect of FAO and lactate in regard to non-obese, obese and diabetic subjects. What we found was a

significant, positive correlation between FAO and lactate concentration in the subjects. We observed as the FAO increased, lactate was much lower. This was the case only for non-obese subjects, but due to obese and diabetic subjects having an increased accumulation of fatty acids, their FAO rate remained low and therefore maintained a higher lactate concentration. However, there were non-obese that had low FAO rates and high lactates. Similar results were found by Utzschneider et al., where lean, insulin resistant subjects demonstrated more diabetes, higher fasting glucose and insulin, and a greater risk for the metabolic syndrome than obese, insulin sensitive subjects.[69] Those who were lean, but insulin resistant were metabolically less healthy.[63] Furthermore, a study by Primeau at al., demonstrated obese who were insulin sensitive.[53] The metabolically healthy obese subjects revealed a 54% less fat accumulation, lower muscle fat, lowered plasma glucose, higher insulin clearance, and high insulin sensitivity. These data suggest some leans and non-obese are predisposed toward metabolic disease and lactate may be able to track who are lean and non-obese, but metabolically unhealthy, and obese who are metabolically healthy.

A plausible explanation for some results not being significantly different may be due to the degree of adiposity. This study only looked at non-obese vs obese by BMI. However, a study by Hulver et al., compared palmitate oxidation rates between non-obese, obese and extremely obese (BMI >40 kg/m^2).[31] Their study found palmitate oxidation was 58 and 83% lower in skeletal muscle from extremely obese (44.9±5.2 nmol/g/h) compared to normal (71±5 nmol/g/h) and non-morbidly obese (82.2±8.7 nmol/g/h). In addition, Kim et al., also found a 276% lower fatty acid oxidation rate in extremely obese subjects compared to a 100% reduction in obese.[40] Our results along with these studies suggest the degree of adiposity may play a significant role in why PMP70 was not significantly higher in obese and diabetic subjects. PMP70 may have been

significantly higher in those who were extremely obese rather than obese or diabetic. (Huang, paper in review as unpublished data)

Although we found some significance with our study, there were still some limitations associated with the present study. One limitation to this study was we only looked at females who were 21 years of age or older. Another limitation to this study was we only looked at Caucasian females. It would be beneficial to look at differences across ethnicities as obesity has a higher prevalence among African Americans. Cortright et al., demonstrated lower rates of skeletal muscle oxidation of fatty acids in AAW compared to CW. AAW had a significantly reduced capacity to oxidize palmitic acid by 22% compared to CW, (64.2 ± 5.2 nmol/g tissue/hr vs 82.1± 5.0).[12] Hickner et al., showed that lean AAW were similar to obese women in that they were metabolically inflexible, but did not occur in CW. These results show AAW did not have the ability to shift towards fat. Future studies may find more significant results than those found among Caucasian as well as in other proteins due to other ethnicities being more susceptible to obesity and diabetes.

Another limitation to this study was we were only able to look at these specific proteins, PEX19, PMP70, and COX IV. As shown previously, some of these proteins were more significant than others, and may be interesting to continue to look at in future studies along with other proteins; mitochondrial as well a peroxisomal. Future findings could indicate a higher activity of a protein at a specific section. Kim et al., demonstrated reductions in various areas, such as CPT-1 activity, citrate synthase, and β-oxidation.[40] Palmitate oxidation, which is partially dependent on CPT-1, was depressed by 50% with obesity, palmitoyl carnitine oxidation was also depressed by 45%, and CPT-1 and citrate synthase activity, an index of mitochondrial

content was significantly reduced by 35% with obesity. This data suggests mitochondrial content may contribute to the reliance on fat oxidation.

Within this study, there were many missing values that we were not able to use when doing our analysis and later our graphs. This was seen while comparing FAO and lactate between the groups, as some of the subjects only had lactate data, but no matching FAO data and vice versa. Due to this limitation, we were only able to see a handful of obese data points and only three diabetic data points. Finally, the present study only divided BMI's into non-obese ($<30kg/m^2$) and obese ($>30kg/m^2$). As described above, previous research had found significant changes in those who were extremely obese rather than simply obese. Future studies may want to distinguish the addition of having another group of extreme obesity to look at the protein content of mitochondrial and peroxisomal proteins.

In conclusion, we found a significantly higher protein levels of COX IV mitochondrial content in non-obese subjects ($P<0.05$) compared to obese and diabetic as well as a significantly higher protein levels of the peroxisomal content, PEX 19, in diabetic subjects. Furthermore, we found a significant association between lactate and PEX19. The present data suggests the individuals who are non-obese, but have a high lactate may be "sick" and on their way to being Type II Diabetic. The discrepancies in the data suggest that those who have a higher mitochondrial content may have a higher number of mitochondria rather than a higher functioning mitochondria.

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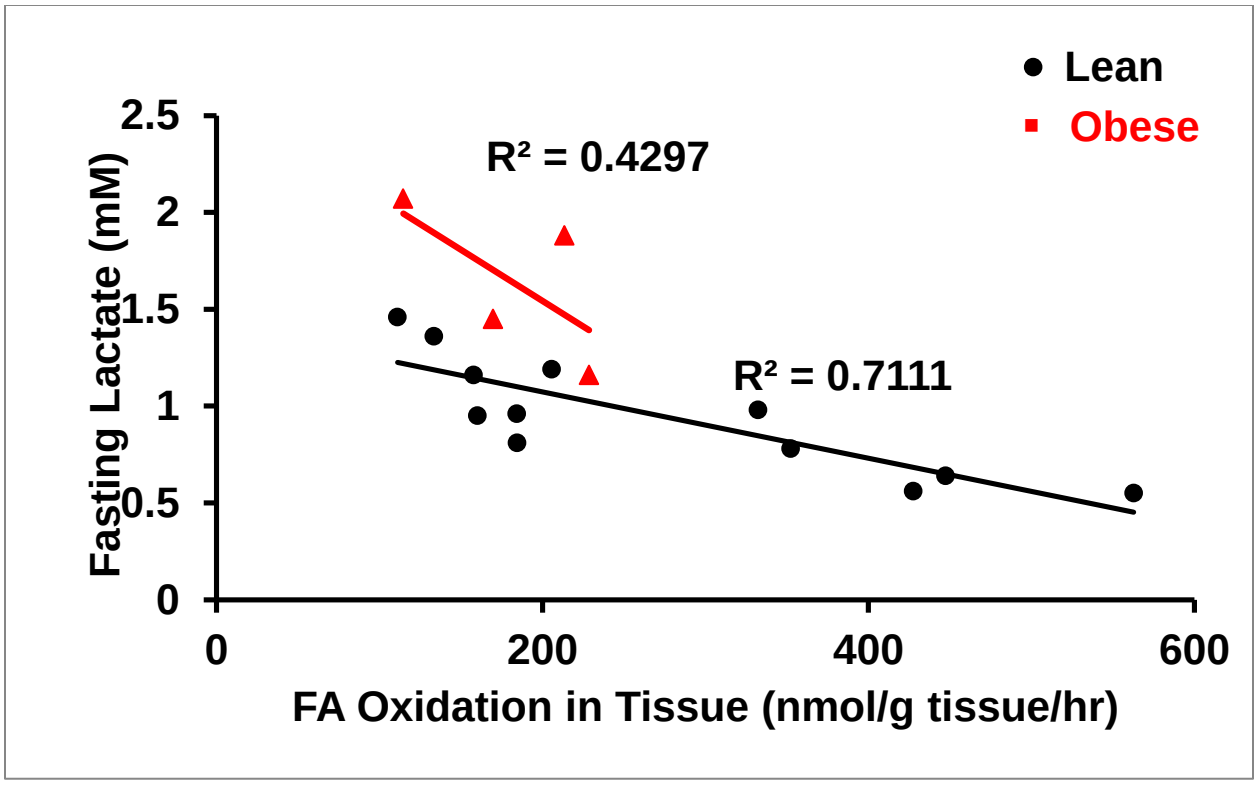
APPENDIX A: PRELIMINARY DATA



Preliminary data courtesy of G. Lynis Dohm, ECU shows the correlation of Fatty Acid Oxidation and Fasting Lactate in Lean and Obese subjects.

APPENDIX B: 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** ·
www.ecu.edu/ORIC/irb

Notification of Continuing Review Approval

From: Biomedical IRB
To: Joseph Houmard
CC:

Josh Yang
Date: 3/16/2018
Re: CR00006737
UMCIRB 13-002234
Characterization of Myotubes to be used for a Bioassay.

I am pleased to inform you that at the convened meeting on 3/14/2018 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 3/14/2018 to 3/13/2019.

The Biomedical IRB deemed this study Greater than Minimal Risk.

Changes to this approved research may not be initiated without UMCIRB review except when necessary to eliminate an apparent immediate hazard to the participant. All unanticipated problems involving risks to participants and others must be promptly reported to the UMCIRB. The investigator must submit a continuing review/closure application to the UMCIRB prior to the date of study expiration. The investigator must adhere to all reporting requirements for this study.

Approved consent documents with the IRB approval date stamped on the document should be used to consent participants (consent documents with the IRB approval date stamp are found under the Documents tab in the study workspace).

The approval includes the following items:

Document	Description
Cell studies medical history form(0.01)	Data Collection Sheet
Consent document (approval for use of stored samples)(0.01)	Dataset Use Approval/Permission
ECU J&J main consent 5-5-15 clean copy with corrections(0.01)	Consent Forms
ECU JJ Genetic consent V.2 May 2015.doc(0.02)	Consent Forms
ECU JJ Main consent V.3 5-5-15 mods.doc(0.02)	Consent Forms
ECU/ J&J Protocol June 2014(0.01)	Study Protocol or Grant Application
ECU/J&J PTC Oct. 5, 2016(0.01)	Study Protocol or Grant Application
ECU-J&J Obese non-surgery flyer(0.01)	Recruitment Documents/Scripts
ECU-J&J PTC V.3 may 5, 2015(0.01)	Study Protocol or Grant Application
Flyer to focus on diabetic enrollment for females 20-65, BMI over 40.(0.01)	Recruitment Documents/Scripts
Houmard Cell Culture Consent (covers future research)(0.01)	Consent Forms
J&J Myotubes Flyer Non-surgery group Oct 2015(0.01)	Recruitment Documents/Scripts
J&J Myotubes Main consent V.4 4/19/16(0.01)	Consent Forms
J&J Myotubes PTC V.4 4/19/16(0.01)	Study Protocol or Grant Application
J&J Proposal April 2014(0.01)	Study Protocol or Grant Application
J&J Student group consent (updated locations)(0.01)	Consent Forms

Document	Description
Menstrual Cycle Recall:(0.01)	Surveys and Questionnaires
Recruitment flyer (lean group)(0.01)	Recruitment Documents/Scripts
Recruitment flyer: Add Virginia Koonce as coordinator(0.01)	Recruitment Documents/Scripts

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

J. Yang

M. L. Pories

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

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IRB00000705 East Carolina U IRB #1 (Biomedical) IORG0000418
IRB00003781 East Carolina U IRB #2 (Behavioral/SS) IORG0000418