Whole-Body Metabolic Flexibility is Discordant with Skeletal Muscle Metabolism

By

Polina Krassovskaia

July 2024

Director of Dissertation: Dr. Nicholas Broskey

Major Department: Kinesiology

ABSTRACT

Metabolic disease continues to be a worldwide problem that affects all ages, sexes, and socioeconomic statuses. The development of metabolic disease is multifactorial with both genetic and environmental components. Because of this, interest has grown in the early identification of individuals susceptible towards the development of metabolic disease. Metabolic flexibility has gained standing as a metric of metabolic health and can be defined as the ability to appropriately adapt substrate utilization in response to substrate availability. Skeletal muscle (SkM) is a regulator of metabolic flexibility as it is the primary tissue responsible for post-prandial substrate handling, and metabolic inflexibility can be seen in the SkM of individuals with metabolic disease. As Western style diets are proportionally heavy in fats and implicated in the development of obesity, metabolic flexibility with lipids is of particular interest. High-fat diets (HFDs) are a common and

successful approach for distinguishing differences in metabolic flexibility with lipids across populations. Some evidence exists for metabolic inflexibility in healthy, lean individuals, which suggests metabolic flexibility may be an innate component of SkM. Studies of individuals with overweight allow insight into the intrinsic characteristics of SkM that promote the development of metabolic disease, yet this population remains largely understudied. The current study aimed to assess metabolic flexibility in a population of healthy individuals with overweight with a 3-day HFD.

In chapter 2, we show that the participants studied are sedentary, have overweight, and do not display evidence of dysglycemia or dyslipidemia. Participants did not differ in dietary habits before or during the diet. Metabolic flexibility was calculated as the change in SkM homogenate palmitate oxidation with the high-fat diet. Metabolically flexible individuals show almost a two-fold increase in palmitate oxidation with the diet, while inflexible individuals decrease oxidation by about one-third of pre-diet rates, and this occurs without any changes to pyruvate oxidation. Metabolic flexibility was not associated with skeletal muscle fiber type. The change in the respiratory quotient during a hyperinsulinemic-euglycemic clamp is a classical approach towards assessing metabolic flexibility; we found that this was not associated with metabolic flexibility when measured as the change in palmitate oxidation in homogenates with the diet. Additionally, flexible and inflexible participants did not differ in mitochondrial function. Taken together, this indicates that metabolic flexibility is an innate component of SkM and can be seen prior to any clinical evidence of metabolic disruption, and metabolic flexibility is not associated with either muscle fiber type or mitochondrial function.

In chapter 3, we utilized primary human skeletal muscle stem cells (HSkMCs) of the same cohort of participants studied in chapter 2. We show that a 3-day HFD does not result in changes in HSkMC metabolism measured by substrate oxidation, glycogen synthesis, and protein content of mitochondrial markers and PGC1a. Cells were additionally incubated with a lipid cocktail for 24 hours, which has been shown to help distinguish differences in metabolic flexibility in HSkMCs. Even with lipid treatment, no effect of the HFD was seen. Although participants were stratified based off the change in palmitate oxidation seen in SkM homogenates, a main finding was that HSkMCs of flexible individuals show enhanced glucose metabolism seen as higher glucose oxidation efficiency. Interestingly, measures of HSkMC lipid metabolism did not associate with similar measures of homogenate lipid metabolism. Together, these data provide novel findings demonstrating that metabolic inflexibility is present in SkM prior to detection at the whole-body level and occurs without any clinical markers of metabolic disturbance.

Whole-Body Metabolic Flexibility is Discordant with Skeletal Muscle Metabolism

A Dissertation

Presented to the Faculty of the Department of Kinesiology

East Carolina University

In Partial Fulfillment of the Requirements for the Degree

Doctor of Philosophy in Bioenergetics and Exercise Science

By

Polina Krassovskaia

July 2024

Director of Dissertation: Nicholas Broskey, Ph.D.

Dissertation Committee Members:

Joseph Houmard, Ph.D.

Donghai Zheng, Ph.D.

Kelsey Fisher-Wellman, Ph.D.

ACKNOWLEDGEMENTS

It would have been impossible to complete this dissertation without the many helping hands that have directly, or indirectly, contributed their time, energy, or support to this work. While this list is by no means all-encompassing, I would like to specifically thank some of these contributors.

First, I'd like to thank those in the Kinesiology department and the East Carolina Diabetes and Obesity Institute for their academic and scientific help, especially my committee members. Dr. Broskey – thank you for seeing something in me during my master's and giving me the chance to explore my favorite slice of science. I appreciate the support and numerous opportunities for professional and scientific growth that you've provided. Dr. Houmard – thank you for setting an incredible example for those of us who started with an interest in clinical exercise physiology but strive to go deeper. I appreciate your support through both my master's and doctoral studies as well as the opportunity to work on MoTrMyo and collect some fun data. Donghai – thank you for the never-ending support in the lab. You've provided priceless technical expertise and guidance in deciphering data, and you're always there with an open door for those of us in the lab. Kelsey – thank you for your guidance on the mitochondrial data, as well as the encouragement to think deeper about my project and push to make it better. While the mitochondrial component started off as the scariest part of my dissertation, I can confidently say that is no longer the case.

Second, I'd like to thank my family and friends for not only their support, but the memories and experience they have helped create while I worked on my doctorate. To my

parents – words will never be enough to capture the gratitude and love I have for you both. Thank you for always picking up the phone to offer words of guidance or support when I need it, even if I sometimes called multiple times a day. Thank you for learning a bit of science with me over these past few years so you could share in the excitement of my data. Thank you for everything you do and have done for me – I wouldn't be who or where I am without you. Boris – thank you for being the best big brother out there. Your growth not only personally but in your career has been incredible to see – thank you for setting such a great example for me and continuously reminding me of the important things in life. I'd be lying if I said your excitement to call me "Dr. Po" wasn't a motivating factor over these years. To the Wissemans – thank you for filling my time here with never-ending laughs and happiness. Scott – thank you for creating such an incredible DnD world for us to escape to, and the friendship you've shown me these past few years. Bree – if it wasn't for you, none of this would have been finished. You inspire me to be a better person, and I am so deeply thankful for you and our friendship. To all my Greenville friends – thank you for the support you've given me and for helping fill these four years with so much joy.

Lastly, I'd like to thank my dog, Smiley. Though you'll never read this, you have been my biggest supporter and the best boy. Thank you for always greeting me with a wagging tail, no matter how well my experiments go. Thank you for always being happy to go on a long walk, which helped remind me to separate myself from my work. Thank you for letting me practice dozens of presentations with you, and thank you for your contribution of laying your chin on the edge of my laptop while I work in the evenings. Most of all, thank you for being my best bud.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ΔRQ,	Change in the respiratory quotient
CPT1,	Carnitine palmitoyltransferase I
DEXA,	Dual-energy X-ray absorptiometry
DTNB,	Dithiobis 2-nitrobenzoic acid
FLEX,	Metabolically flexible participant
GLUT4,	Glucose transporter type 4
HFD,	High-fat diet
HSkMCs,	Primary human skeletal muscle stem cells
INFLEX,	Metabolically inflexible participant
MHC,	Myosin heavy chain
NIRS,	Near-infrared spectroscopy
NOGM,	Non-oxidized anionic glycolytic metabolite production
NRF,	Nuclear respiratory factor
PGC1α,	Peroxisome proliferator-activated receptor gamma coactivator alpha
PPARγ,	Peroxisome proliferator activated receptor gamma
REE,	Resting energy expenditure
ROS,	Reactive oxygen species
RPE,	Rate of perceived exertion
RQ,	Respiratory quotient
SIRT1,	Sirtuin 1
SkM,	Skeletal muscle
T2D,	Type 2 diabetes
VO_2max	Maximal aerobic exercise capacity

CHAPTER 1:

Introduction and Literature Review

Despite efforts in prevention, management, and treatment, metabolic diseases continue to be a worldwide problem affecting all ages, genders, and socioeconomic statuses[1–3]. Metabolic diseases include, but are not limited to, obesity, type 2 diabetes (T2D), hypertension, hyperlipidemia, and non-alcoholic fatty liver disease[4]. Not only do metabolic diseases present substantial costs to the individual affected, but they also exacerbate the strain on healthcare systems, public health, and global economies[5,6]. More than 1 in 5 individuals have obesity in the US as of 2023, and more than 1 in 10 individuals have diabetes[7,8] underlining the need for understanding and intervention. The development of metabolic disease is associated with a genetic predisposition[9,10] but manifestation is often spurred by environmental influences[11,12]. Because of this, there is a large interest in identifying susceptible individuals in hopes of preventing or delaying the development of metabolic disease.

Metabolic Flexibility

The term metabolic flexibility was coined by Kelley and Mandarino as "the capacity to switch from predominantly lipid oxidation and high rates of fatty acid uptake during fasting conditions to the suppression of lipid oxidation and increased glucose uptake, oxidation, and storage under insulin-stimulated conditions"[13]. With time, metabolic flexibility has more broadly come to be understood as "the ability of an organism to respond or adapt according to the changes in metabolic or energy demand as well as the

prevailing conditions or activity"[14]. There is growing interest and speculation in metabolic flexibility for its potentially causal role for metabolic pathologies, specifically in conditions of lipid oversupply. With lipid oversupply, metabolic inflexibility, seen as a mismatch of substrate oxidation to availability, is thought to result in ectopic lipid accumulation[15] and a shift towards an increasingly oxidative redox state which could result in insulin resistance[16] and weight gain. Metabolic inflexibility is well documented with obesity and T2D[17–20], but interestingly, evidence of metabolic inflexibility can be seen in lean, young, insulin-sensitive men with an acute HFD[21] suggesting metabolic flexibility may be an innate metabolic characteristic that predisposes certain individuals towards metabolic disease.

Skeletal muscle is a regulator of metabolism

Many factors affect an individual's ability to maintain homeostasis in the face of metabolic perturbations, and skeletal muscle (SkM) is a key regulator of this ability in both local and whole-body metabolism[22,23]. SkM makes up almost 40% of total body weight and is a main driver of both carbohydrate and lipid metabolism[24]. Further, a bidirectional correlation between metabolic disease and SkM health has been established, and alterations in SkM metabolism may precede any clinical detection of metabolic dysfunction[25]. Additionally, SkM holds an innate plasticity defined by its ability to adapt both its structural and functional properties in response to varying patterns of mechanical and metabolic loads[26–29]. This plasticity allows SkM to maintain homeostasis in the face of altered metabolic loads and energy states. Under conditions of lipid oversupply, SkM will undergo adaptations in its metabolism to mitigate the effects of the adverse load via

alterations in transcription, translation, enzyme activity, and cellular composition[30]. Thus, SkM is an important regulator of metabolic flexibility.

Individuals with overweight

As the development of metabolic disease is multifactorial and difficult to address, interest has grown in the identification of susceptible individuals. Extensive data collected in populations with obesity and T2D has clearly outlined hallmark signatures of metabolic disease, including metabolic flexibility. However, there is still little data surrounding metabolic flexibility in individuals with overweight. This population has been shown to be susceptible towards the development of metabolic disease[31–33], and investigation in individuals without overweight would allow for clarification on if metabolic flexibility is truly an innate characteristic of SkM and appears before the clinical diagnosis of metabolic disease. As few studies have been done exclusively in individuals with overweight, the literature outlined below is primarily based in individuals with obesity and T2D.

Assessments of metabolic flexibility

Hyperinsulinemic-euglycemic clamp

Traditionally, metabolic flexibility has been measured at the whole-body level during a hyperinsulinemic-euglycemic clamp using indirect calorimetry. The use of indirect calorimetry allows for the calculation of the respiratory quotient (RQ) which uses the ratio of VCO_2 to VO_2[34]. RQ is typically at 0.7 in the fasted state and represents predominantly fats being used for energy and can increase to 1.0 in the fed state representing reliance on predominantly carbohydrates[34]. Metabolic flexibility is measured as the change in the

respiratory quotient (ΔRQ) from the basal to insulin-stimulated condition which represents substrate switching from predominantly fat to carbohydrate oxidation with the appearance of insulin[13,35]. Two seminal papers by Kelley et al. established this technique when assessing fat oxidation between lean individuals and those with obesity and T2D[35,36]. The findings of these papers showed that individuals with metabolic disease have a higher resting RQ and do not increase RQ with insulin stimulation to the same extent as lean individuals establishing evidence of metabolic inflexibility with metabolic disease, and this has been largely corroborated by other studies[37–39].

Several considerations should be made for the implementation of a hyperinsulinemic-euglycemic clamp to measure metabolic flexibility. The major advantage is that plasma glucose and insulin levels are matched across participants which allows for comparison across disease states. However, several caveats exist. First, the hyperinsulinemic-euglycemic clamp is primarily a measure of flexibility with *carbohydrates* since the measurement is solely based off the ability to increase RQ with insulin stimulation. Indeed, several groups have shown that glucose uptake is the primary determinant of insulin-stimulated substrate handling[36,38–40] and differences between groups disappear when ΔRQ is normalized to glucose disposal rate[38,39]. Second, it is not a physiologically relevant assessment, as it does not reflect the dietary habits of individuals which include a mix of carbohydrates, fats, and proteins. Third, differences in ΔRQ establish a phenotypic difference between metabolically flexible and inflexible individuals, but studies rarely extend past this to show a mechanism driving differences in flexibility. Together, this suggests that the use of a hyperinsulinemic-euglycemic clamp may be a

biased assessment of metabolic flexibility in individuals and other, more physiologically relevant approaches should be considered.

High-fat diet

As individuals with obesity show an impairment in fatty acid oxidation at the whole-body, SkM tissue, and cellular level[17,18,18,35,41–43] investigation of metabolic flexibility with *lipids* is prudent. Administration of a high-fat diet (HFD) results in a physiologically relevant positive fat balance and serves as a metabolic challenge in which an increase in lipid oxidation should occur. An increase in fatty acid availability would signal SkM to shift towards increased fatty acid oxidation[44–46], and metabolically flexible individuals would appropriately upregulate lipid oxidation to match the increased availability. Inflexible individuals would fail to upregulate lipid oxidation and thus an increase in fatty acid oxidation intermediates and lipid storage would be seen. Metabolic flexibility measured as whole-body 24-hour ΔRQ during a 24-hour high-fat overfeeding study in healthy individuals was a determinant of weight gain at 6 months and 1 year after the diet independent from sex, age, ethnicity, or initial body weight[47]. Combined with evidence that 24-hour ΔRQ is strongly tied to familial factors[48,49] this suggests that metabolic flexibility is an innate component of SkM and when combined with a HFD could be used to identify individuals prone to the development of metabolic disease.

HFDs have largely been shown to induce changes in lipid metabolism in metabolically flexible individuals. Battaglia et al. show a 3-day HFD (70% of calories from fat) results in SkM of lean individuals increasing palmitate oxidation while individuals with obesity show no change[50]. Thomas et al. used a 7-day HFD with a lower proportion of

calories from fat (50%) but still showed that individuals with obesity show a depressed ability to increase fat oxidation in response to the increased supply of dietary fats[46]. While the results of these HFD studies is largely in agreement, some conflicting evidence exists. Bergouigan et al.[51] found no difference between lean individuals (n=10) and those with obesity (n=9) in carbohydrate or fat oxidation measured by room calorimetry after a HFD. The HFD implemented was eucaloric and comprised of 50% of energy from fat, and responses were measured over 24 hours, which may not have been a potent enough stimulus or long enough period to distinguish differences between groups of that sample size. In the US, the average dietary intake consists of 47% of calories from carbs, 16% from protein, and 36% from fat[52]. As this Western style diet is already comprised of a higher proportion of fats, HFD studies using a diet comprised of 50% fat and lasting <3 days with a small sample size may not be able to distinguish meaningful differences in lipid metabolism between groups. Astrup et al.[53] used a similar HFD composition, RQ measurement technique, and sample size but employed the HFD for 3 days and were able to conclude women with prior obesity display impaired metabolic flexibility. Blaak et al.[54] were able to distinguish meaningful differences in metabolic flexibility in healthy individuals and those with obesity with a single meal challenge, but the meal consisted of 95% calories from fat and was undertaken in 814 participants. Thus, we suggest three primary factors be considered for the implementation of a HFD to assess metabolic flexibility: first, the diet composition, second, the diet length, and third, the sample size. Of note, 3-day HFDs seem to be a successful timeframe for short-term diets as there is consistent evidence that they distinguish group differences across various compositions

(50-78% calories from fat) and sample sizes (n=7-17)[50,53,55–58]. An additional consideration that must be made when using RQ to assess metabolic flexibility during a HFD is that fasting RQ is largely influenced by dietary macronutrient composition and energy balance[59–61]. Thus, to reduce the variance pre-HFD dietary habits have on ΔRQ, standardization of pre-HFD meals should be implemented.

Lipid infusions

A concomitant infusion of lipid during a hyperinsulinemic-clamp has also been used as a model to study metabolic flexibility to lipids. Compared to HFDs, this model is less frequent, possibly due to the decreased physiological relevance, and most commonly used as a model to induce insulin resistance[62–64]. Nonetheless, Dube et al.[65], studied the effects of lipid infusion on substrate oxidation and mitochondrial respiration across individuals who were lean and trained, lean and sedentary, and sedentary with obesity. Interestingly, though differences in glucose oxidation were seen when calculated in proportion to total glucose infused, all groups showed a similar increase in fat oxidation with lipid infusion. Further, state 3, state 4, and maximal uncoupled respiration pre- and post-lipid infusion did not differ between groups either. While the mitochondrial data is in line with findings from Fisher-Wellman et al.[66], the fat oxidation data is conflicting with other literature of metabolic flexibility in individuals with obesity. This could be partly due to the model used. The infused lipid emulsion did not include heparin, which is shown to increase plasma free fatty acid levels to supraphysiological levels. While this change makes the lipid infusion more physiologically sound and results in circulating free fatty acids being closer to a level seen after a high-fat meal, this may reduce the potency of the

lipid infusion to distinguish differences in fat oxidation between groups. Comparison with other studies that have used similar methods to explain these discrepancies is currently limited, as the majority of studies using a lipid infusion to assess metabolic flexibility have been done in healthy and/or trained individuals[64,67,68].

Primary human SkM stem cells

Primary human SkM stem cells (HSkMCs) when cultured have been shown to retain morphological, metabolic, and biochemical properties of donors[69,70]. For example, compared to their lean counterparts, HSkMCs from individuals with obesity show depressed complete oxidation of glucose, oleate, and palmitate[43,71,72] and higher incomplete oxidation along with reduced rates of insulin-stimulated glycogen synthesis[72]. Retainment of donor characteristics despite HSkMCs being in a removed environment to close to one month underlines that HSkMCs offer a unique model to differentiate between the genetic and acquired deficiencies in the etiology of metabolic disease. HSkMCs have been used to study substrate flux, mitochondrial alterations, and cell signaling under conditions of lipid oversupply and metabolic disease[66,72–78]. HSkMCs from lean individuals and those with obesity show differences in metabolic flexibility when incubated with a lipid cocktail (oleate:palmitate in a 1:1 ratio at 100uM with 2mM carnitine) for 24 hours[79]. After lipid treatment, HSkMCs from lean individuals had almost double the mitochondrial state 3 respiration supported by lipid substrate (palmitoyl carnitine + malate) compared to HSkMCs of individuals with obesity. HSkMCs from individuals with obesity and T2D similarly show metabolic inflexibility when incubated with lipids[80]. ^{14}C-oleate oxidation

after lipid incubation (100uM oleic acid for 48 hours) was 50% lower compared to oxidation in HSkMCs from lean individuals with no difference in mitochondrial content between the groups. Together, this exemplifies the retainment of donor metabolic flexibility in HSkMCs and further underlines that metabolic flexibility is an innate characteristic of SkM. To the authors' knowledge, the effect of a HFD on metabolic flexibility in HSkMCs has yet to be investigated, and if done, would help fill the gap of a lack of mechanistic approaches to address metabolic flexibility differences across metabolic states.

SkM properties that could drive differences in metabolic flexibility

Several physiological differences could contribute to variations in metabolic flexibility. One difference could be SkM fiber type as it is a well-established influence on SkM substrate oxidation. Muscle fibers are commonly classified based off myosin heavy chain isoform expression. Broadly speaking, two primary fiber types exist: type I and type II muscle fibers. Though type II fibers can be further subdivided into type IIa, IIb, and IIx fibers, for the purposes of this review, a broad categorization will be used. Type I muscle fibers have a high capacity for oxidative metabolism due to a high mitochondrial density and high enzymatic activities of glycogen phosphorylase, succinate dehydrogenase, and citrate synthase[81,82]. Type II muscle fibers have a lower capacity for oxidative metabolism but have higher contraction velocity and a capacity to generate force[81,82]. A positive relationship between insulin-stimulated glucose uptake and type I fiber proportion is seen in human fibers[83], and with obesity, a decrease in type I fibers and an increase in type II fibers is seen[84–87]. Further, the proportion of type I fibers has been shown to vary between

15-85% of total fibers[88]. Thus, having a higher proportion of type I fibers may result in a more metabolically flexible phenotype.

Another potential explanation for differences in metabolic flexibility is mitochondrial capacity and/or content. Much debate exists about the role that mitochondria have in the development of metabolic disease. Mitochondria are central in dictating nutrient oxidation and cellular energy and are able to shift in structure, capacity, and efficiency in response to metabolic stress, and thus, intrinsic mitochondrial differences could explain variations in metabolic flexibility. Many studies suggest that mitochondrial function (i.e., respiratory capacity and efficiency) and content are decreased with adverse metabolic stress[89,90], yet there is evidence to suggest mitochondrial function and content are normal in individuals with insulin-resistance and obesity[65,66]. In healthy, lean adults, a lipid infusion does not impair oxidative phosphorylation or maximal respiration capacity[91]. Additionally, complex I and complex II linked respiration and maximal respiration are not altered with lipid oversupply[92]. Mitochondrial content[91,93] and mitochondrial enzyme activity[91,93–95] (i.e., citrate synthase activity) are similarly unaffected by lipid infusion. Interestingly, there is a significant increase in H_2O_2 emission and a decrease in total glutathione indicating an overall imbalance in the redox state[66]. The emission of reactive oxygen species (ROS) has been directly linked to the development of insulin resistance, and the resulting effect ROS has on the intracellular redox environment may explain differential outcomes. The intracellular redox status is tightly controlled and normally in the reduced state; however, metabolic imbalances due to an oversupply of lipids will favor H_2O_2 generation resulting in a greater

oxidizing condition[16]. Consistent low levels of H_2O_2 generation maintain and regulate this sensitive redox environment ensuring proper kinase activity and protein phosphorylation[96–98], or "phosphatase tone"[16]. The shift towards an increasingly oxidative redox environment will decrease "phosphatase tone" and result in disruptions of normally well-regulated patterns of glucose metabolism[16]. Additionally, mitochondrial dynamics, that is, the balance between fission and fusion, seems to be consistently altered with lipid infusion. With an oversupply of lipids, mitochondrial fission is increased, fusion is decreased, and membrane potential is decreased[89,91,93], which results in a shift towards an increasingly fragmented mitochondrial network. Taken together, this suggests that alterations in respiration do not precede metabolic disease, and a fragmentation of the mitochondrial network may serve to limit mitochondrial substrate flux and ensure a functional integrity of the remaining network in the face of chronic lipid oversupply. Evidence of mitochondrial dysfunction with metabolic inflexibility could thus be explained as a complication of lipotoxicity-induced ROS generation[89]. However, the extent and timeline of mitochondrial alterations under lipid-induced metabolic stress remains unclear.

Another potential source of discrepancy in metabolic flexibility across individuals could be the protein peroxisome proliferator-activated receptor-gamma (PPARγ) coactivator alpha (PGC1α). PGC1α is known to regulate mitochondrial mass through coactivation of the nuclear respiratory factor (NRF), increase GLUT4 expression via myocyte enhancer factor-2, regulate OXPHOS genes via NRF, and regulate fatty acid flux via PPARγ[99]. PGC1α expression has additionally been shown to decrease with a chronic oversupply of lipids[100,101]. Further, individuals with obesity have been shown to have a

reduced expression of PGC1 α, and this is associated with decreases in mitochondrial function[90,100–102]. L6 myotubes treated with increasing concentrations of lipids results in a disproportionate increase in incomplete fatty acid oxidation while rates of complete oxidation were unaffected[100]. When PGC1α was overexpressed, this mismatch was resolved exemplified by an increase in complete oxidation and a concomitant decrease in incomplete oxidation. Further, cDNA analyses showed that low PGC1α content combined with the lipid incubation resulted in an increase in an upregulation of β-oxidation associated genes without a parallel increase in downstream pathways (i.e., TCA cycle). Overexpression of PGC1α resulted in both β-oxidation and downstream pathway upregulation leading the investigators to propose that it is PGC1α expression that influences SkM to be able to appropriately adjust lipid oxidation. Together, this suggests that differences in PGC1α expression could be a potential explanation for variances in metabolic flexibility.

Conclusion

Accumulating evidence points to metabolic flexibility not only being an innate component of SkM but also important for preserving metabolic health. Metabolic inflexibility has been clearly outlined in individuals with obesity and T2D, yet it remains unclear if metabolic inflexibility can be seen prior to the diagnosis of metabolic disease. Several techniques can be used to assess metabolic flexibility but do so by testing differing components of flexibility. Individuals with metabolic disease present with impairments in lipid oxidation, and thus, HFDs present themselves as a powerful tool to assess metabolic

flexibility to lipids in SkM especially when combined with a mechanistic approach via HSkMCs. Several factors may influence variances in metabolic flexibility including muscle fiber type, mitochondrial capacity and/or content, and PGC1a expression and must be considered when making conclusions on metabolic flexibility. To address the gaps in the literature, **the objective of the current study is to assess metabolic flexibility in healthy individuals with overweight using a 3-day HFD via measures of substrate oxidation, mitochondrial content and function, muscle fiber typing, and PGC1α expression.** Our *central hypothesis* is that a 3-day HFD will result in the identification of metabolic flexible and inflexible individuals with overweight measured by an increase or no change in fatty acid oxidation, respectively, and this will occur independent from changes in mitochondrial function and content, differences in fiber type, and differences in PGC1a expression.

CHAPTER 2

Evidence for skeletal muscle metabolic inflexibility prior to markers of clinical metabolic disturbance in healthy individuals with overweight

ABSTRACT

Metabolic flexibility, defined as an increase in substrate oxidation in response to substrate availability, has gained traction as an accepted metric of metabolic health. Individuals with metabolic disease show metabolic inflexibility, but it remains unknown how early into the development of metabolic disease that metabolic inflexibility can be seen. This study aimed to assess metabolic flexibility in a population at risk of developing metabolic disease, individuals with overweight, in response to a 3-day HFD. Participants were stratified as metabolically flexible (FLEX) or inflexible (INFLEX) based off SkM fatty acid oxidation change with the HFD. Groups did not differ in age, BMI, insulin sensitivity, or VO_2max. FLEX participants increased fatty acid oxidation by an average of 93.5% ± 78.4 while INFLEX decreased by 30.6% ± 8.68. Groups did not differ in muscle fiber type nor mitochondrial function. These results suggest that metabolic flexibility is an innate component of skeletal muscle which can be observed before evidence of whole-body metabolic disturbances and occurs without alterations in mitochondrial function.

INTRODUCTION

Metabolic flexibility, or the ability to appropriately adjust substrate oxidation in response to availability, is becoming an increasingly accepted metric of metabolic health. Evidence of metabolic flexibility can be seen in lean individuals undergoing a HFD as both whole-body and SkM fat oxidation are increased. On the other hand, individuals with obesity and/or T2D show no change in fat oxidation under conditions of a HFD exemplifying metabolic inflexibility[35,50,53,103]. Metabolic inflexibility under conditions of lipid oversupply has been implicated in the excess accumulation of lipids and their intermediates which could result in the propagation of obesity[104]. Interestingly, some evidence of metabolic inflexibility exists in young, healthy individuals[21] which begs the question of whether metabolic flexibility is an innate component of SkM.

HFDs are a commonly used approach to assess differences of metabolic flexibility between populations, and there is a push in the field for their utilization over the classical approach of hyperinsulinemic-euglycemic clamps for their physiological relevance[15,105]. Short-term HFDs of three days have been shown to be effective at detecting differences in metabolic flexibility via increases in fatty acid oxidation in SkM[50,106]. Most data surrounding metabolic inflexibility has been collected in populations with obesity and T2D, and currently, little is known about individuals with overweight. Individuals with overweight are susceptible to the development of metabolic disease[31–33], and the investigation of metabolic flexibility in this population would allow insight into whether metabolic flexibility is truly an innate characteristic of SkM. Thus, the current study aimed to assess metabolic

flexibility in a population with overweight but no established metabolic disease and utilize the approach of a 3-day HFD.

METHODS

Participants

A sub-cohort of nineteen participants were chosen from the larger Metabolic Inflexibility is related to Elevated Muscle Anaerobic Glycolysis "MetFlex" study (ClinicalTrials.gov NCT4320264). A prescreening phone call established that participants met inclusion criteria of overweight BMI (25-29.9), age (18-55 y/o), weight stable for the past 3 months, and physically inactive. Exclusion criteria included smoking, established disease (i.e., cancer, uncontrolled hypertension, psychiatric disorder, type 1 or 2 diabetes), and taking medication known to influence carbohydrate or lipid metabolism. The experimental procedures and associated risks were explained through written and verbal format, and informed consent was obtained. The study was approved by the East Carolina University and Medical Center Institutional Review Board and was in accordance with the Declaration of Helsinki.

Study Design

At the screening visit, anthropometrics, a blood draw, and a medical history questionnaire were completed. At the first visit, a dual-energy X-ray absorptiometry (DEXA) scan, 3D body imaging, and maximal cycle ergometer exercise test were completed. At the second visit, resting energy expenditure (REE) was assessed as well as a submaximal cycle ergometer test. At the third visit, a hyperinsulinemic-euglycemic clamp, *vastus lateralis* muscle

biopsy, and indirect calorimetry was completed. The fourth and fifth study visits had similar measures of a venous blood draw, a *vastus lateralis* muscle biopsy, and near-infrared spectroscopy. Participants consumed a 3-day HFD after the fourth visit and the fifth visit was for post-HFD measures. For the diet, participants were instructed to increase fat intake up to 70% of their caloric intake. MyFitnessPal was used to track dietary intake for 3 days before and during the HFD.

Hyperinsulinemic-Euglycemic Clamp

A two-hour hyperinsulinemic-euglycemic clamp was utilized to assess insulin sensitivity with modifications from the method of DeFronzo et al[107]. Participants fasted overnight for 12 hours and arrived at 0630 at the research facility. Two catheters were placed: the first intravenously in an antecubital vein for insulin and glucose infusion and the second retrogradely in a dorsal hand vein for blood sampling. Participants kept this hand in a 60°C warming box for arterialized blood sampling. Four samples were obtained at 10-min increments to determine fasting glucose and insulin concentration. A primed continuous infusion of insulin (Humulin, Eli Lilly, Indianapolis, IN) at a submaximal dosage of 40 μU · m^{-2} · min^{-1} was then initiated; 4 min after the start of insulin infusion, a variable 10-20% dextrose (percentage dextrose used depended on availability) infusion was begun. Blood samples were obtained every 5 min, centrifuged, and autoanalyzed for arterialized whole blood glucose (HemoCue® Glucose 201 System; Brea, CA). Adjustments in glucose infusion rate were made as needed to maintain euglycemia. Samples for insulin were obtained every 10 min and centrifuged, and plasma was stored at −80°C for subsequent analysis. The M-Value was calculated as the quantity of average glucose metabolized per

unit of plasma insulin concentration per kg of lean body mass. RQ was measured using a canopy hood and metabolic cart (ParvoMedics TruOne 2000; Salt Lake City, UT) during the last 30 minutes of the basal and insulin phase of the clamp.

Maximal aerobic capacity and resting energy expenditure

REE was measured in the fasted state. Participants were fitted with a canopy hood connected to a metabolic cart (Parvo Medic's True 2400; East Sandy, UT) for the analysis of the O_2 and CO_2 flow to volume ratio. Participants were then instructed to lie quietly in a private room for 20 minutes during which inspired and expired respiratory gases were collected. A graded maximal exercise test was done on a stationary cycle ergometer (Excalibur Sports; Lode, Netherlands). Participants were fitted with a mouthpiece through which respiratory gases were used to calculate an exercise respiratory exchange ratio via a metabolic cart (Parvo Medic's True 2400). Participants were additionally fitted with a 12-lead EKG for the monitoring of heart rate and rhythm during the exercise test. A 5-minute warm up was completed, after which the maximal test began. The protocol consisted of 2-minute stages which started with a workload of 0 Watts; each stage increased in workload by 30 Watts. Participants were instructed to maintain approximately 50-70 revolutions per minute. Blood pressure and rate of perceived exertion (RPE) on the Borg's scale of 6-20 was recorded at the end of each stage, and participants continued until either reaching maximal effort (determined by two conditions met out of three: RER of 1.1, RPE >17, and peak heart rate within +/- 5% of age-predicted maximal heart rate) or revolutions per minute fell below 40. After the maximal test, participants completed an active recovery until near resting heart rate and blood pressure were met. Participants also completed a

submaximal exercise test done on a stationary cycle ergometer. Participants completed a 5-minute warm up, and then resistance was gradually increased in 6-minute intervals to reach 65% VO_2max while revolutions per minute were maintained at approximately 50-70. After the submaximal test, participants completed an active recovery until near resting heart rate and blood pressure were met.

Near-Infrared Spectroscopy (NIRS)

The NIRS system consisted of eight infrared diode lasers, with four emitting at 691 nm and four at 830 nm, along with a detector in each probe. The probes were secured to *the vastus lateralis* muscle of the dominant leg using a double-sided adhesive tape and Velcro straps. To determine the appropriate NIRS probe for each participant, subcutaneous adipose tissue thickness was measured using a skinfold caliper (~10 cm above the patella). Probe B was used for individuals with a skinfold thickness of 30 mm or less, whereas probe A was used for individuals with a skinfold thickness greater than 30 mm. During NIRS measurement, participants were positioned in a supine position on a medical examination table with both legs fully extended. After selecting the appropriate probe, the blood occlusion procedure was initiated. A blood pressure cuff was placed as close to the proximal area as anatomically possible, ensuring that the NIRS probe was not in contact with the cuff after inflation. A 15-gallon air compressor set to 30 psi was used to control the blood pressure cuff. Prior to the actual measurement, three 5-second practice occlusions were performed to familiarize the participants with the pressure around their leg and to ensure that there was no discomfort. The first arterial occlusion served as the baseline measurement, in which participants underwent a 60-second occlusion duration. After a

resting period of 2-3 minutes, the second occlusion was performed following an isometric contraction of the gastrocnemius muscle for 15-20 seconds. The cuff was then inflated to 300 mmHg for a 5-minute arterial occlusion to determine the total deoxygenated hemoglobin (deoxy Hb) level. The collected data was processed, and the rate of deoxy Hb was calculated by dividing the oxygen kinetics obtained during the one-minute occlusion by the total deoxy Hb oxygen kinetics obtained during the 5-minute occlusion. Percent-deoxy Hb was subsequently calculated by dividing the rate of deoxy Hb by the total deoxy Hb (umol/minute).

Substrate Oxidation

Substrate oxidation was measured as described previously[17,50,108,109]. Briefly, a portion (~50-60 mg) of the muscle tissue from the biopsy was collected in 200 µl of buffer (250 mM sucrose, 1 mM EDTA, and 10 mM Tris·HCl, pH 7.4). Scissors were used to mince the muscle tissue to remove excess fat and connective tissue, and then tissue was further diluted 20-fold with additional buffer. Tissue was homogenized on ice using a Teflon pestle for 30 s. A portion (40 µl) of this homogenate mixture was added to the top well of a customized, sealed 48-well plate which has a connecting channel to an adjacent trap well for the passage of CO_2 released by the complete oxidation of d-[1-^{14}C] palmitate (Perkin-Elmer; Waltham, MA) or d-[1-^{14}C] pyruvate (Perkin-Elmer). The bottom trap well contained 1 N sodium hydroxide to trap the $^{14}CO_2$ given off by the oxidation procedure. To initiate the reaction, 160 µl of a reaction buffer composed of the following was added to the top wells: 0.2 mM palmitate ([1-^{14}C]palmitate at 0.5 µCi/ml) or 1mM pyruvate ([1-^{14}C]pyruvate at 0.25 µCi/ml), 100 mM sucrose, 10 mM Tris·HCl, 5 mM potassium phosphate, 80 mM potassium

chloride, 1 mM magnesium chloride, 0.1 mM malate, 2 mM ATP, 1 mM dithiothreitol, 0.2 mM EDTA, 1 mM l-carnitine, 0.5 mM coenzyme A, and 0.5% fatty acid-free bovine serum albumin, pH 7.4. The samples were incubated in a 37°C water bath for 30 min, after which 100 µl of 70% perchloric acid was added to terminate the reaction. To ensure that CO_2 was completely transferred and trapped in sodium hydroxide, the plate was then placed on a shaker at RT for 1 h. Label incorporation into $^{14}CO_2$ was determined by liquid scintillation counting using 4 ml of Uniscient BD (National Diagnostics, Atlanta, GA). Incomplete oxidative products (acid-soluble metabolites; ASMs) remaining in the top well were measured as described previously (17).

Muscle Fiber Typing

A portion of the muscle biopsy was fixed in optimal cutting temperature compound and frozen at -80°C. Tissue was sectioned with a cryostat machine, mounted onto glass slides, and stored at -20°C. Slides were fixed in a 1:1 solution of methanol and acetone, rinsed with PBS, and blocked with 5% goat serum for 60-90 minutes at room temperature. Slides were incubated with primary antibodies myosin heavy chain (MHC) I (DSHB-#BA-FC, mouse IgG2b, Life Technologies 1:50), MHC IIa (DSHB-#SC-71C, mouse IgG1, Life Technologies 1:50), and Dystrophin (Ab275391, rabbit IgG, Abcam 1:100) overnight at 4°C. The next day, slides were rinsed with PBS and incubated with secondary antibodies Alexa Fluor 350 goat-anti-mouse IgG2b (#A-21140, Life Technologies, 1:250), Alexa Fluor 488 goat-anti-mouse IgG1 (#A-21121 Life Technologies, 1:250), and Alexa Fluor 555 goat-anti-rabbit IgG (#A27039 Invitrogen, 1:250) for 1 hour at room temperature. Slides were again

rinsed with PBS, Vectrashield was added on to the slide to preserve fluorescence. Slides were incubated at 4°C for 20 minutes, and then imaged with a Leica microscope.

Mitochondrial Respiration

A portion of muscle (~25mg) from the biopsy was separated for preparation of permeabilized fiber bundles. Muscle was dissected into bundles and trimmed of connective tissue and fat. Fiber bundles were separated along their longitudinal axis with a pair of needle-tipped forceps under magnification (MX6 Stereoscope; Leica Microsystems, Buffalo Grove, IL). Each bundle was treated with 30µg/ml saponin for 30 minutes at 4°C for permeabilization and subsequently washed in ice-cold buffer Z (105mM K-MES, 30mM KCl, 1mM EGTA, 10mM K_2HPO_4, 5mM $MgCl_2$-6 H_2O, 0.005mM glutamate, and 0.002mM malate with 5.0 mg/ml BSA; pH 7.4, 290 mOsm). High-resolution O_2 measurements were conducted at 30°C with the OROBOROS Oxygraph-2K (OROBOROS Instruments, Innsbruck, Austria) in buffer Z supplemented with 0.01mM Bleb to inhibit muscle contraction. Permeabilized fiber bundle respiration was assessed with three protocols. In protocol 1, CI/II/IV respiration was assessed; respiration was supported by 5 mM pyruvate, 0.5mM malate, and 5mM glutamate followed by additions of 4mM ADP, 10mM succinate, 0.002mM rotenone, 5mM malonate, 0.0025mM antimycin A, 2mM ascorbate, and 0.5mM TMPD. In protocol 2, lipid-supported TCA cycle respiration was assessed; respiration was supported by 0.02mM palmitoyl-CoA, 0.5mM malate, 0.018mM palmitoyl-L-carnitine chloride, and 5mM carnitine followed by additions of 4mM ADP, 10mM succinate, 5mM pyruvate, 5mM glutamate, 0.01mM cytochrome C, and titrations of 0.001mM FCCP until max respiration was reached. In protocol 3, CIII respiration was assessed; respiration was

supported by 4mM ADP followed by additions of 0.5mM duroquinol and 0.0025mM antimycin A. After assessments of respiration were concluded, muscle fibers were washed to remove salts, freeze-dried (Labconco; Kansas, MO), and weighed. Respiration rates were then normalized to muscle fiber weight.

Citrate Synthase Activity

Citrate synthase activity was determined as previously described[110]. Briefly, A colorimetric plate-based assay was used to measure the interaction between CoA-SH and 5', 5'-Dithiobis 2-nitrobenzoic acid (DTNB) to form TNB (OD: 412 nm). A 96-well plate was loaded with 200µL assay buffer (105 mM potassium-MES, 30 mM KCl, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 1 mM EGTA, 0.2mM DTNB, and 0.5mM acetyl-CoA; pH=7.2), 0.03 mg/mL alamethicin, and 10ug of homogenized muscle tissue. The plate was incubated for 5 minutes at 37°C, and then 1mM oxaloacetate was added to the sample wells. Absorbance at 412 nm was recorded every 30s for 20 min. Nonspecific activity calculated from one control well (no oxaloacetate) per sample was subtracted from sample rates. CS activity was determined using the Beer-Lambert Law and the molar absorption coefficient of TNB (13.6 mM/cm).

Statistical Analyses

Participants were stratified to either a metabolically flexible group “FLEX” or metabolically inflexible group “INFLEX” based on the change in palmitate oxidation with the HFD. If palmitate oxidation increased with the HFD, participants were stratified to the FLEX group. If palmitate oxidation decreased with the HFD, participants were stratified to the INFLEX group. Participants who had <15% change in palmitate oxidation were not included.

Unpaired or paired two-tailed t-test or two- or three-way ANOVA with Bonferroni correction were used to determine statistical significance. Factors were group (FLEX vs INFLEX) and condition (pre-HFD vs post-HFD). When significance was detected for either main effects or interactions between group and condition, a two-tailed t-test was performed for distinguishing meaningful differences. If data was not normally distributed, a Wilcoxon test was used. Associations between variables were tested using a Pearson correlation for normally distributed data or a Spearman correlation for non-normally distributed data. Analysis of covariance (ANCOVAs) were used to evaluate covariates. Statistical significance was set as $p \leq 0.05$. GraphPad Prism was used for ANOVAs and t-tests, JMP Statistics was used for regression analyses, and IMB SPSS Statistics was used for ANCOVA analyses. All data were expressed as mean ± SEM.

RESULTS

Participant characteristics and diet

Participant characteristics are reported in **Table 2.1**. FLEX and INFLEX participants did not differ in age, BMI, or ΔRQ. Participants did not present with hyperlipidemia, diabetes or pre-diabetes, or hypertension. On average, participants consumed 34.1% ± 9.0 of their calories from fats before the diet and increased to 50.2% ± 9.8 calories from fat on the HFD (**Figure 2.1A**). Participants significantly increased their percentage of calories from fats on the HFD while decreasing their percentage of calories from protein and carbohydrates (**Figure 2.1B**). FLEX and INFLEX did not differ in % calories from carbohydrates or fats before or on the HFD (**Figure 2.1C**). Though not different on the HFD, FLEX and INFLEX

differed in % calories from protein before the diet ($p<0.05$; 20.4% ± 2.66 vs 17.2% ± 2.45). FLEX participants trended towards decreasing the % calories from carbs with the HFD ($p=0.06$).

Divergent palmitate oxidation response to a HFD

Participants were stratified as "metabolically flexible" (FLEX) or "metabolically inflexible" (INFLEX) based off palmitate oxidation change with the HFD; those who increased palmitate oxidation were stratified to the FLEX group while participants who decreased palmitate oxidation were stratified to the INFLEX group. Participants who had <15% change in palmitate oxidation were not included (n=3). On average, FLEX increased palmitate oxidation by 93.5% ± 78.4 while INFLEX decreased by 30.6% ± 8.68 (**Figure 2.2A**). There was no difference between FLEX and INFLEX for pyruvate oxidation change with the HFD (**Figure 2.2B**). Metabolic flexibility is classically assessed using a hyperinsulinemic-euglycemic clamp and calculating the change in RQ from the basal to insulin state (ΔRQ). Interestingly, there was no association relationship between ΔRQ and the change in palmitate oxidation with HFD (**Figure 2.2C**). Additionally, there was no association between ΔRQ and palmitate oxidation, pyruvate oxidation, or ASM production before or after the HFD (HFD [**Table 2.2. (Supplemental Data)**]). There was also no association with ΔRQ and palmitate, pyruvate, or ASM change with the HFD [**Table 2.2. (Supplemental Data)**].

Substrate oxidation of ^{14}C-palmitate and -pyruvate were measured before and after the HFD in muscle homogenates. FLEX had lower pre-HFD palmitate oxidation than INFLEX ($p<0.05$) and increased with the HFD to the level of INFLEX pre-HFD (**Figure 2.3A**). INFLEX

decreased palmitate oxidation with the HFD to the level of FLEX pre-HFD (**Figure 2.3A**). Incomplete palmitate oxidation was measured using acid-soluble metabolites (ASMs). With the diet, only FLEX increased ASMs (**Figure 2.3B**). To examine efficiency of palmitate oxidation, a ratio of complete to incomplete oxidation was created and termed "Palmitate Partitioning". Groups did not differ in partitioning before or after the diet, and there was no effect of the diet on partitioning (**Figure 2.3C**). Additionally, pyruvate oxidation did not differ between groups or with the HFD (**Figure 2.3D**). Group differences remained significant when controlling for HFD composition (i.e., % calories from fat, increase in % fat from baseline) or whole-body measurements (i.e., age, BMI, M value, REE, ΔRQ) ($p<0.05$ for all listed) in multivariate modeling.

Muscle fiber type is not associated with measures of metabolic flexibility

As fiber type is known to influence substrate oxidation, especially with HFDs[111], fiber type composition was determined. Groups did not differ in percentage of type I or type II fibers (**Figure 2.4A-B**). Additionally, fiber type did not correlate with palmitate change with diet, BMI, insulin sensitivity, or ΔRQ (**Figure 2.4C-F**), nor with any measure of oxidation other than pre-HFD ASMs [**Table 2.3. (Supplemental Data)**].

Metabolic flexibility is not driven by differences in mitochondrial function

HFDs have been shown to induce changes in mitochondrial respiration and content[112,113], and their role in the development of metabolic disease has been largely contested[66,114–116]. Thus, both mitochondrial respiration and content were measured before and after the HFD. FLEX and INFLEX did not differ in Complex I, II, III, or IV supported respiration before or after the HFD (**Figure 2.5A, 2.5C**). Assessing the TCA cycle in the presence of fatty acids

showed no difference between FLEX and INFLEX before or after the HFD, and maximal electron transport system capacity was not different between the two groups ($p>0.05$) (**Figure 2.25B**). *In vivo* resting respiratory capacity was measured using NIRS, and there was similarly no difference between groups nor was there a diet effect (**Figure 2.5D**). Overall, there was no diet effect for FLEX or INFLEX for any state of mitochondrial respiration. Mitochondrial content, measured by citrate synthase activity, did not differ between groups or with HFD (**Figure 2.5E, F**).

DISCUSSION

Metabolic flexibility in substrate oxidation is a key component in preserving metabolic health as it aids in maintaining euglycemia and avoiding excess fat deposition[46,104], and increasing evidence supports metabolic inflexibility as a part of the "metabolic signature" that occurs in individuals with metabolic disease. It remains largely unknown, however, how early in the development of metabolic disease that metabolic inflexibility can be seen. The purpose of this study was to look at a population that is at risk of developing metabolic disease (individuals with overweight but otherwise healthy) and assess metabolic flexibility in response to a 3-day HFD. The main findings of the present study were that 1) metabolic inflexibility can be seen in overweight but healthy individuals before evidence of whole-body metabolic disturbances, and 2) metabolic inflexibility is not associated with measures of mitochondrial dysfunction.

Metabolic flexibility is classically assessed using a hyperinsulinemic-euglycemic clamp and measuring the change in RQ between the basal and insulin-stimulated

conditions. Though this study did not stratify groups based off ΔRQ and instead used palmitate oxidation change with HFD, ΔRQ was measured in participants pre-HFD allowing us to evaluate associations between ΔRQ and oxidation measures. In the current study, there was no association between ΔRQ and palmitate, pyruvate, or pyruvate:palmitate oxidation at baseline, after the HFD, or change with the HFD [**Table 2.2 (Supplemental Data)**]. Additionally, when participants were stratified on ΔRQ rather than palmitate change with the HFD, no effect of the diet was seen on either group with any measure of oxidation (data not shown), showing whole-body metabolic flexibility is discordant from what is seen at the level of SkM substrate oxidation. The caveat of using a hyperinsulinemic-euglycemic clamp to assess metabolic flexibility lies behind the increasing body of evidence showing that glucose uptake is the primary determinant of insulin-stimulated substrate handling[36,38–40], and so, it may result in a biased assessment. Additionally, the use of clamp creates an artificial situation which may not reflect real-world physiological responses, as evident with our data [**Table 2.2 (Supplemental Data)**]. A strength of the current study, instead, is the use of a high-fat overfeeding diet, which aligns with the current push in the field for assessment[117] of metabolic flexibility under physiologically relevant conditions. In addition, a 3-day HFD has been shown to increase SkM palmitate oxidation in individuals with a lean body mass but not with obesity[50,54]. The present data showing a robust difference in palmitate oxidation response to a HFD suggests that metabolic inflexibility may be an inherent component of SkM in some individuals and predisposes them to the development of metabolic disease later in life.

The relationship between mitochondrial function and metabolic disease has been deliberated in the literature with supporting data overwhelmingly based in populations with obesity and type 2 diabetes[66,114,116]. In the overweight population, there have been few studies to assess mitochondrial function. Newcomer et al. utilized ^{31}P-MRS to evaluate *in vivo* mitochondrial function in overweight, nondiabetic women before and after weight loss. They found that weight loss did not affect mitochondrial ATP production rates (OxPHOS ATP production rate and V_{PCr}) but did improve mitochondrial rates dependent on muscle perfusion (TC_{ADP} and Q_{max}) indicating that blood flow may be the restricting factor which explains evidence of mitochondrial dysfunction with increased body weight rather than intrinsic OXPHOS maladaptations[118]. Two studies used the unique population of pre-pubertal children with overweight which allows for isolation of the effects of increased body fat from hormonal influences on mitochondrial function and found that weight status was not associated with depressed indices of mitochondrial function[119,120]. In the present study, no differences in mitochondrial content or respiration were seen. Respiratory capacity was assessed using two approaches (an uncoupler to assess maximal *in vitro* capacity and NIRS to assess resting *in vivo* capacity[121]), and neither showed that groups differ in respiratory capacity. It is important to note that a limitation of the mitochondrial respiration experiments is the small sample size obtained. Due to variations in the amount of SkM biopsy tissue collected, FLEX was limited to n=5 and INFLEX to n=5-6 for all protocols, making it conjecture to form conclusions based off the present study alone. However, when combined with the aforementioned studies, as well as other evidence supporting mitochondrial function does not influence metabolic flexibility[39], this

contributes to the case that mitochondrial function may not be altered with increased body fat.

Muscle fiber type is a well-established influence on SkM substrate oxidation and often implicated in the development of obesity[84,85,122]. Type I muscle fibers have the highest capacity for oxidative metabolism due to high oxidative enzyme activity and mitochondrial density, and individuals with obesity present with a lower proportion of type I fibers than individuals with a lean body weight[84–87,123,124]. A lower proportion of type I fibers is also associated with a lower propensity for weight loss[85,125]. With this, muscle fiber type seemingly presents itself as a plausible explanation for metabolic inflexibility as a lower proportion of type I muscle fibers would result in a decreased capacity to increase fat oxidation. Interestingly, in the current study, groups did not differ in their proportion of type I or type II fibers and yet, a robust difference in palmitate oxidation with the HFD was seen. Additionally, we found no associations between muscle fiber type and metabolic flexibility (**Figure 2.4C**) or BMI, insulin sensitivity, and ΔRQ (**Figure 2.4D-F**). Together, this suggests that muscle fiber type is not a driver of metabolic flexibility in individuals with overweight. Muscle fibers that express multiple MHC isoforms have been characterized as hybrid fibers, and on the spectrum of oxidative ability, type IIa and hybrid fibers lie between type I (high oxidative capacity) and type IIx (low oxidative capacity) fibers. The loss of type I and gain of type IIx fibers is seen with obesity, and type IIa and hybrid fibers could be contributors in this transition to a less oxidative muscle phenotype with obesity. More mechanistic studies into the role of hybrid fibers with obesity and longitudinal studies

assessing fiber type switching with the development of obesity are needed to fully understand the SkM phenotype with obesity.

We acknowledge that there are several limitations with the current study that should be considered for future directions. While the population studied was relatively healthy, we did not screen participants for a family history of metabolic disease. Several studies have shown that a family history of T2D results in impaired metabolic flexibility even in young, healthy, active individuals[126–128] though these measurements were limited to only whole-body measures. To the authors' knowledge, the present study is the first to show the existence of metabolic inflexibility existing at the level of SkM in individuals with overweight without the presence of any clinical measure of metabolic disturbance, and future studies are needed to assess the impact of a family history of metabolic disease on SkM metabolic inflexibility. Further, the relationship between insulin resistance and metabolic flexibility has largely been speculated in the literature. Evidence exists for insulin resistance being a direct contributor to[54,106,128,129] and independent from[38,39] metabolic flexibility. In the current study, our population did not show signs of insulin resistance, nor did groups differ in insulin sensitivity, and a large difference in palmitate oxidation was observed. Additionally, no measures of substrate oxidation were associated with any measures of insulin sensitivity (data not shown). It is important to note that the primary aim of this study was not to assess the relationship between insulin sensitivity and metabolic flexibility and so, our data does not rule out the possibility for an interdependent relationship between the two. With the present data, however, a need for further investigation is clearly needed for a true understanding of metabolic flexibility.

In conclusion, the current study found that 3-day HFD in a homogenous population of young, relatively healthy individuals with overweight results in divergent SkM substrate handling. An inability for SkM to adapt to an increased availability of dietary fats indicates the presence of metabolic inflexibility before any clinical markers of metabolic disturbance. In addition, differences in flexibility were not related to muscle fiber type, insulin sensitivity, or altered mitochondrial function indicating metabolic flexibility may be an innate trait of SkM that may predispose certain individuals towards metabolic disease.

CHAPTER 2 FIGURES

	FLEXIBLE N=9	INFLEXIBLE N=10	P-VALUE
GENDER	5 F / 4 M	2 F / 8 M	
ETHNICITY	9 C	6 C / 2 A / 2 O	
DELTA RQ	0.063 ± 0.04	0.061 ± 0.03	0.8830
BASAL RQ	0.743 ± 0.03	0.731 ± 0.04	0.4792
INSULIN RQ	0.807 ± 0.04	0.792 ± 0.05	0.5371
AGE (years)	26.3 ± 5.2	30.6 ± 9.1	0.2339
BMI (kg/m^2)	27.4 ± 1.8	28.0 ± 2.0	0.5008
% BODY FAT	29.4 ± 8.4	29.9 ± 7.6	0.9021
% LEAN MASS	67.3 ± 8.52	68.0 ± 7.0	0.8641
VAT Volume (cm^2)	454 ± 123	464 ± 122	0.8584
REE	1844 ± 299	1803 ± 260	0.7491
TOTAL CHOLESTEROL (mg/dL)	196 ± 44.8	175 ± 23.6	0.2246
TRIGLYCERIDES (mg/dL)	151 ± 145	89.1 ± 28.0	0.5621
LDL (mg/dL)	120 ± 27.3	113 ± 20.5	0.5320
HDL (mg/dL)	46.3 ± 9.45	46.2 ± 6.81	0.9720
GLUCOSE (mg/dL)	89.7 ± 4.95	93.7 ± 6.77	0.1604
INSULIN (mg/dL)	9.39 ± 4.29	9.56 ± 4.24	0.9314
HbA1c (%)	5.19 ± 0.302	5.23 ± 0.216	0.7350
HOMA-IR	2.03 ± 1.03	2.25 ± 1.11	0.6664
M VALUE	6.95 ± 2.14	8.71 ± 3.45	0.2342
LACTATE (mmol/L)	1.09 ± 0.431	0.950 ± 0.315	0.7634
VO_2MAX (mL/kg/min)	31.0 ± 8.84	35.3 ± 7.84	0.1128
SBP (mm)	115 ± 13.4	120 ± 13.0	0.4637
DBP (mm)	73.4 ± 6.88	75.4 ± 9.85	0.2862

Table 2.1. Participant characteristics of FLEX and INFLEX individuals. Data presented as mean ± SEM.

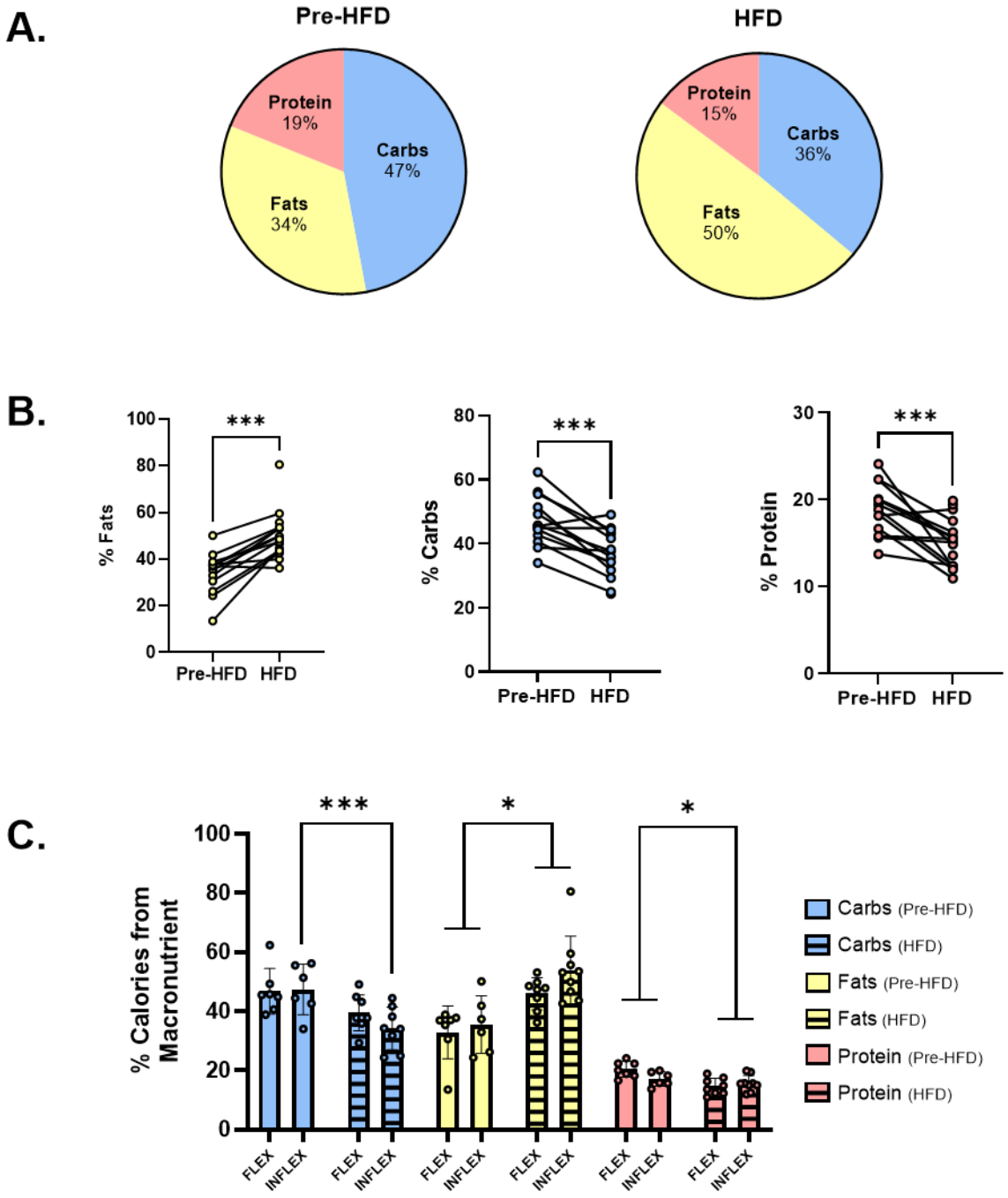

Figure 2.1. Pre-HFD and HFD composition breakdown. (A) Pre-HFD and HFD macronutrient breakdown by percentage calories from fats, carbohydrates, and protein as logged by participants in MyFitnessPal. Pre-HFD averages taken over three days. (B) Pre-HFD to HFD change in percent calories from fats, carbohydrates, and protein. (C) Pre-HFD and HFD macronutrient breakdown for FLEX (n=9) and INFLEX (n=10). Data presented as mean ± SEM. *p≤0.05; ***p≤0.001.

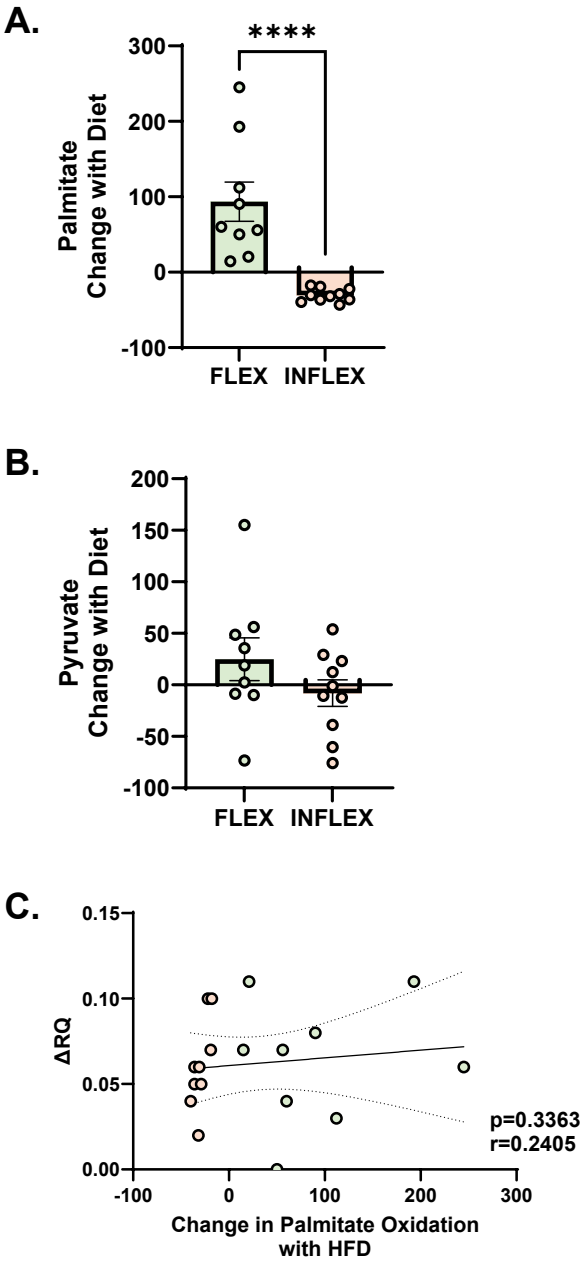

Figure 2.2. Healthy individuals with overweight have robustly different SkM fatty acid oxidation responses to a HFD. (A) Percent change of SkM homogenate ^{14}C-palmitate oxidation from FLEX and INFLEX. (B) Percent change of SkM homogenate ^{14}C-pyruvate oxidation from FLEX and INFLEX. (C) Spearman correlation between ΔRQ and the change in palmitate oxidation with HFD. Data presented as mean ± SEM. ****p≤0.0001.

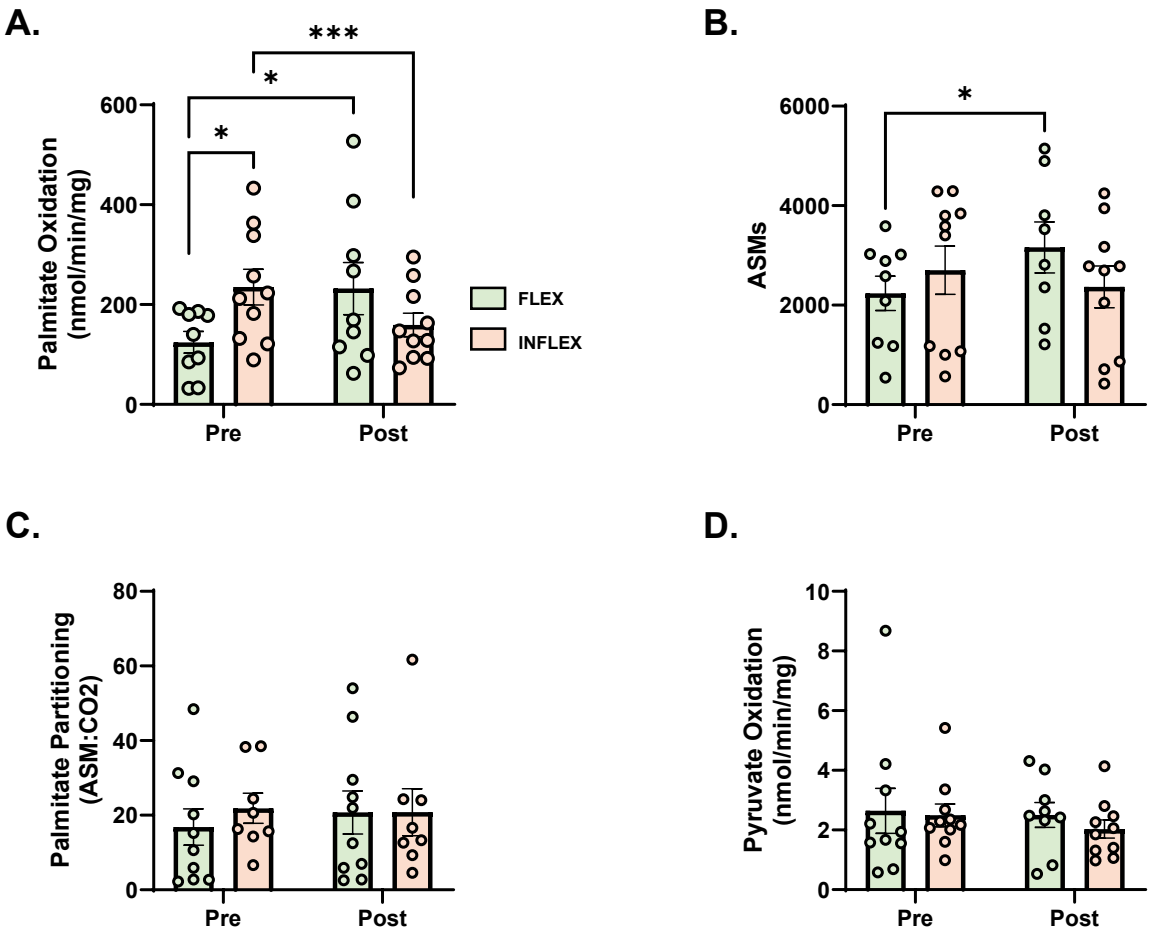

Figure 2.3. FLEX and INFLEX differ in SkM fatty acid oxidation before and in response to a HFD. (A) SkM homogenate ^{14}C-palmitate oxidation from FLEX and INFLEX pre- and post-HFD. (B) Incomplete ^{14}C-palmitate oxidation measured by acid-soluble metabolites (ASMs) pre- and post-HFD. (C) Partitioning of ^{14}C-palmitate to incomplete:complete oxidation pre- and post-HFD. (D) ^{14}C-pyruvate oxidation pre- and post-HFD. Data presented as mean ± SEM. *p≤0.05; ***p≤0.001.

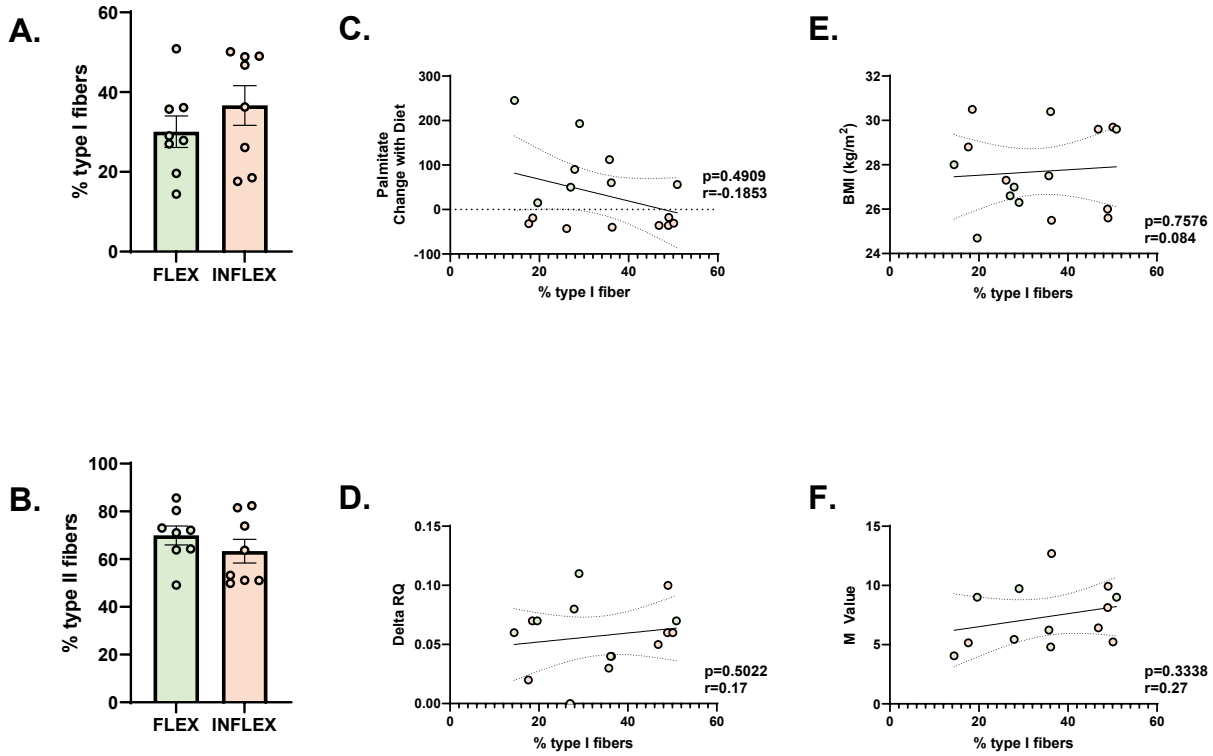

Figure 2.4. SkM fiber type is not associated with metabolic flexibility in healthy individuals with overweight. (A) Percentage of type I muscle fibers from biopsied *vastus lateralis* muscle. (B) Percentage of type II muscle fibers. (C) Spearman correlation between % type I muscle fibers and change in palmitate oxidation with HFD. (D) Pearson correlation between % type I muscle fibers and ΔRQ. (E) Pearson correlation between % type I muscle fibers and BMI. (F) Pearson correlation between % type I muscle fibers and M Value. Data presented as mean ± SEM.

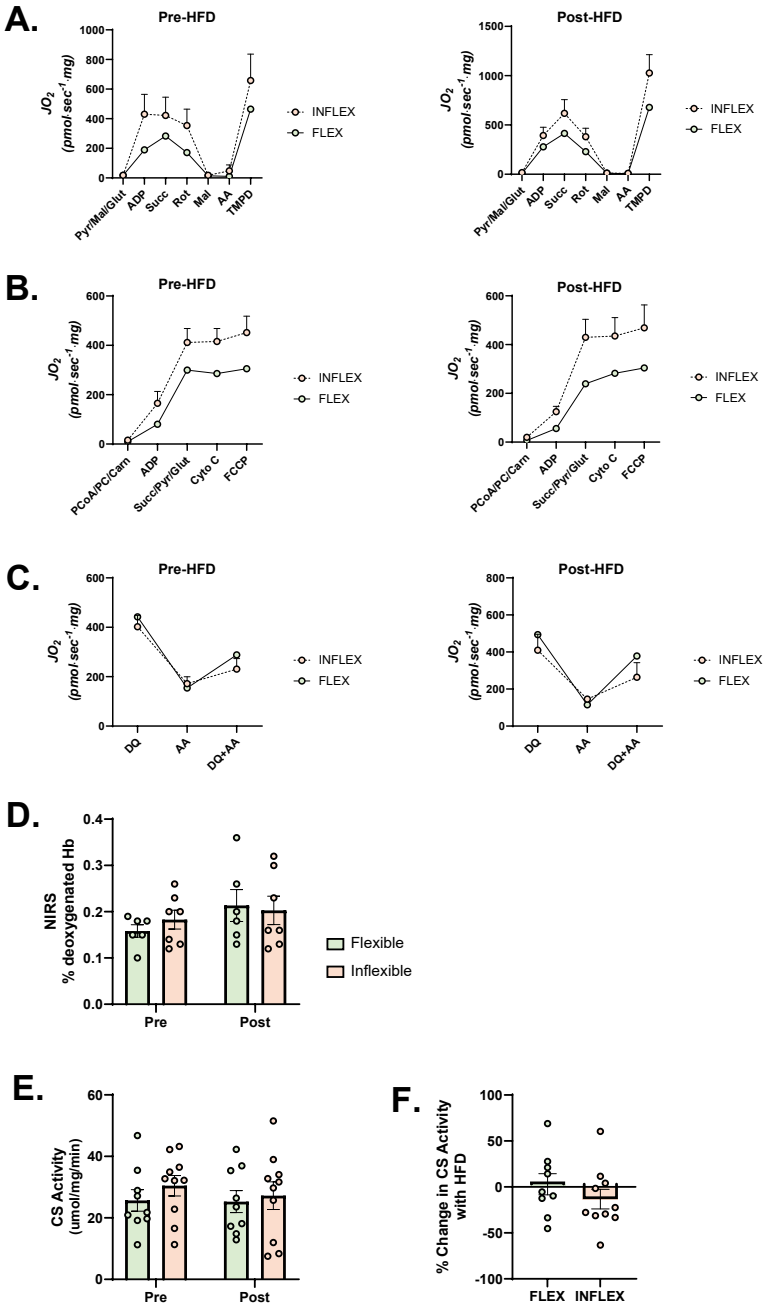

Figure 2.5. FLEX and INFLEX do not differ in mitochondrial function or content. (A) CI, II, IV respiration measured in permeabilized muscle fibers pre- and post-HFD. (B) TCA cycle respiration supported by lipid substrates measured pre- and post-HFD. (C) CIII respiration measured pre- and post-HFD. (D) Percent deoxygenated hemoglobin measured by near-infared spectroscopy (NIRS) pre- and post-HFD. (E) Citrate synthase activity measured in homogenate muscle tissue. (F) Percent change in citrate synthase activity with HFD. Data presented as mean ± SEM.

Participant Measure	Muscle Measure	Correlation coefficient	P value
ΔRQ	**Palmitate Oxidation**		
	Pre-HFD	-0.39	0.146
	Post-HFD	-0.23	0.421
	ASMs		
	Pre-HFD	0.39	0.166
	Post-HFD	0.51	0.074
	Pyruvate Oxidation		
	Pre-HFD	0.27	0.338
	Post-HFD	-0.052	0.859
	Change in ASMs with HFD	0.055	0.833
	Change in Pyruvate Oxidation with HFD	-0.34	0.235

Table 2.2. (Supplemental Data) Change in ΔRQ is not associated with SkM oxidation or oxidation change with HFD. Pearson's correlation used for normally distributed data, and Spearman's correlation used for non-normally distributed data.

Participant Measure	Muscle Measure	Correlation coefficient	P value
% type I fibers	**Palmitate Oxidation**		
	Pre-HFD	0.10	0.681
	Post-HFD	0.0072	0.977
	ASMs		
	Pre-HFD	0.69	*0.002**
	Post-HFD	0.38	0.128
	Pyruvate Oxidation		
	Pre-HFD	-0.14	0.570
	Post-HFD	-0.29	0.249

Table 2.3. (Supplemental Data) Muscle fiber type is not associated with measures of SkM substrate oxidation before or after HFD. Pearson's correlation used for normally distributed data, and Spearman's correlation used for non-normally distributed data.

CHAPTER 3

A 3-day HFD does not impact substrate metabolism of primary skeletal muscle cells from individuals with overweight

ABSTRACT

SkM of individuals with metabolic disease is shown to be metabolically inflexible, displayed as a lack of increased substrate oxidation in response to increased substrate availability. Individuals with overweight but no established metabolic disease also show metabolic inflexibility prior to any clinical signs of metabolic disturbance. Primary human skeletal muscle stem cells (HSkMCs) retain donor characteristics when cultured and allow for insight into the innate properties of SkM. This study aimed to assess the impacts of a 3-day HFD on HSkMCs of metabolic flexible (FLEX) and inflexible (INFLEX) individuals with overweight. Participants were stratified based on their SkM homogenate palmitate oxidation change with the diet; participants who increased oxidation were stratified to the FLEX group while those who decreased oxidation were stratified to the INFLEX group. Before the diet, body composition was assessed and a hyperinsulinemic-euglycemic clamp, blood draw, and maximal exercise test were performed. SkM biopsies were taken before and after the diet from which HSkMCs were isolated and cultured. HSkMCs were additionally treated with lipids for 24 hours to assess the impact of direct lipid exposure. FLEX and INFLEX did not differ in age, BMI (28 kg/m^2 ± 1.9 versus 28 ± 2.1, respectively), insulin sensitivity, or VO_2max. HSkMCs did not differ in palmitate, oleate, or glucose oxidation or partitioning with a 3-day HFD. Additionally, no diet effect on glycogen synthesis was seen. Groups did not show a difference in HSkMC metabolic flexibility, but

FLEX had higher glucose oxidation efficiency. HSkMC palmitate oxidation was not associated with any measure of SkM homogenate palmitate oxidation. These results suggest that a 3-day HFD does not result in changes to HSkMC substrate metabolism, and a metabolically flexible phenotype measured in SkM homogenates is not retained in HSkMCs.

INTRODUCTION

Metabolic flexibility measured as an increase in fatty acid oxidation in response to an increased availability of lipids can be seen in healthy individuals[46,50,53]. A loss of this flexibility is seen in individuals with metabolic disease as well as young individuals with a family history of metabolic disease[46,50,53,130]. In support of this, our data in chapter 2 shows that metabolic inflexibility can be seen in individuals with overweight prior to the appearance of any clinical markers of metabolic disturbance. Together, this suggests that metabolic inflexibility is part of the "metabolic signature" that occurs with metabolic disease and is an innate component of SkM.

HSkMCs when cultured have been shown to retain morphological, metabolic, and biochemical properties of donors[69,70,131,132]. For example, individuals with obesity show impairments in HSkMC oxidation of glucose, oleate, and palmitate as well as depressed rates of glycogen synthesis which is consistent with observations at the SkM tissue and whole-body level[43,71,72]. HSkMCs not only allow an understanding of innate characteristics of SkM separate from the influences of extracellular factors but also permit direct manipulation of the extracellular environment to highlight differences in metabolism. HSkMCs from individuals with obesity retain metabolic inflexibility; when treated with 100uM oleic acid for 48 hours, HSkMCs from individuals with obesity show fatty acid oxidation rates half that of HSkMCs from lean individuals[80]. HFDs are a commonly used approach to assess metabolic flexibility in individuals, and rodent models have shown SkM cells retain the effects of a HFD[133–135]. To the authors' knowledge, however, no data exists to the effect of a HFD on HSkMC outcomes. Thus, the purpose of this study was to assess

the impact of a 3-day HFD on HSkMCs and evaluate potential differences in HSkMC metabolic flexibility of individuals with overweight but no established metabolic disease.

METHODS

Participants

A subcohort of ten participants were chosen from the larger Metabolic Inflexibility is related to Elevated Muscle Anaerobic Glycolysis "MetFlex" study (ClinicalTrials.gov NCT4320264). A prescreening phone call established participants met inclusion criteria of overweight BMI (25-29.9), age (18-55 y/o), weight stable for the past 3 months, and physically inactive. Exclusion criteria included smoking, established disease (i.e., cancer, uncontrolled hypertension, psychiatric disorder, type 1 or 2 diabetes), and taking medication known to influence carbohydrate or lipid metabolism. The experimental procedure and associated risks were explained through written and verbal format, and informed consent was obtained. The study was approved by the East Carolina University and Medical Center Institutional Review Board and was in accordance with the Declaration of Helsinki.

Study Design

At the screening visit, anthropometrics, a blood draw, and a medical history questionnaire were completed. At the first visit, a DEXA scan, 3D body imaging, and maximal cycle ergometer exercise test were completed. At the second visit, REE was assessed as well as a submaximal cycle ergometer test. At the third visit, a hyperinsulinemic-euglycemic clamp, *vastus lateralis* muscle biopsy, and indirect calorimetry was completed. The fourth

and fifth study visits had similar measures of a venous blood draw, a *vastus lateralis* muscle biopsy, and NIRS. Participants consumed a 3-day HFD after the fourth visit and the fifth visit was for post-HFD measures. For the diet, participants were instructed to increase fat intake up to 70% of their caloric intake. MyFitnessPal was used to track dietary intake for 3 days before and during the HFD.

Hyperinsulinemic-Euglycemic Clamp

A two-hour hyperinsulinemic-euglycemic clamp was utilized to assess insulin sensitivity with modifications from the method of DeFronzo et al[107]. Participants fasted overnight for 12 hours and arrived at 0630 at the research facility. Two catheters were placed: the first intravenously in an antecubital for insulin and glucose infusion and the second retrogradely in a dorsal hand vein for blood sampling. Participants kept this hand in a 60°C warming box for arterialized blood sampling. Four samples were obtained at 10-min increments to determine fasting glucose and insulin concentration. A primed continuous infusion of insulin (Humulin, Eli Lilly, Indianapolis, IN) at a submaximal dosage of 40 μU · m^{-2} · min^{-1} was then initiated; 4 min after the start of insulin infusion, a variable 10-20% dextrose (percentage dextrose used depended on availability) infusion was begun. Blood samples were obtained every 5 min, centrifuged, and autoanalyzed for arterialized whole blood glucose (HemoCue® Glucose 201 System; Brea, CA). Adjustments in glucose infusion rate were made as needed to maintain euglycemia. Samples for insulin were obtained every 10 min and centrifuged, and plasma was stored at −80°C for subsequent analysis. The M-Value was calculated as the quantity of average glucose metabolized per unit of plasma insulin concentration per kg of lean body mass. RQ was measured using a

canopy hood and metabolic cart (ParvoMedics TruOne 2000; Salt Lake City, UT) during the last 30 minutes of the basal and insulin phase of the clamp.

REE was measured in the fasted state. Participants were fitted with a canopy hood connected to a metabolic cart (Parvo Medic's True 2400; East Sandy, UT) for the analysis of the O_2 and CO_2 flow to volume ratio. Participants were then instructed to lie quietly in a private room for 20 minutes during which inspired and expired respiratory gases were collected. A graded maximal exercise test was done on a stationary cycle ergometer (Excalibur Sports; Lode, Netherlands). Participants were fitted with a mouthpiece through which respiratory gases were used to calculate an exercise respiratory exchange ratio via a metabolic cart (Parvo Medic's True 2400). Participants were additionally fitted with a 10-lead EKG for the monitoring of heart rate and rhythm during the exercise test. A 5-minute warm up was completed, after which the maximal test began. The protocol consisted of 2-minute stages which started with a workload of 0 Watts; each stage increased in workload by 30 Watts. Participants were instructed to maintain approximately 50-70 revolutions per minute. Blood pressure and rate of perceived exertion (RPE) on the Borg's scale of 6-20 was recorded at the end of each stage, and participants continued until either reaching maximal effort (determined by two conditions met out of three: RER of 1.1, RPE >17, and peak heart rate within +/- 5% of age-predicted maximal heart rate) or revolutions per minute fell below 40. After the maximal test, participants completed an active recovery until near resting heart rate and blood pressure were met. Participants also completed a submaximal exercise test done on a stationary cycle ergometer. Participants completed a 5-minute warm up, and then resistance was gradually increased in 6-minute intervals to

reach 65% VO_2max while revolutions per minute were maintained at approximately 50-70. After the submaximal test, participants completed an active recovery until near resting heart rate and blood pressure were met.

SkM Cell Culture

A portion of the skeletal muscle biopsy (~50mg) was minced and then digested for 30 minutes in a 0.25% trypsin/0.068% collagenase type IV solution supplemented with 0.05% EDTA and 0.1% BSA at 37°C in a shaking water bath. Digested muscle was pre-plated in a 60mm dish for 1 hour at 37°C with 5% CO2 for removal of fibroblasts, and then transferred into a collagen-coated T-25 in 16% growth media (low-glucose DMEM, 16% FBS, 0.05% BSA, 0.1% 50mg/mL Gentamicin, 0.1% 1uM Dexamethasone, 0.1% human EGF, 0.02% 250 ug/mL Amphotericin B). After 70% confluency, myoblasts were expanded in non-collagen coated T-75 cell culture flasks in 10% growth media (low-glucose DMEM, 10% FBS, 0.05% BSA, 0.1% 50mg/mL Gentamicin, 0.1% 1uM Dexamethasone, 0.1% human EGF, 0.02% 250 ug/mL Amphotericin B. At 70% confluency, cells were split to 12-well plates coated with collagen type I (Greiner Bio-One; North Carolina, USA) and switched to differentiation media (low-glucose DMEM, 2% FBS, 0.2% BSA, 2% penicillin/streptomycin) at 90% confluency. Starting on day 5 of differentiation, myotubes were incubated in serum-starvation media (low-glucose DMEM with 1% BSA) with or without 250uM oleate:palmitate (1:1 ratio) with 2mM carnitine for 24 hours. All experiments were done on day 6 of differentiation and between passages 4-5.

Flow Cytometry

HSkMCs (n=4) were seeded on non-collagen coated plates and grown in 10% growth media until 50-70% confluency. HSkMCs were lifted with 0.25% Trypsin-EDTA (Thermo Fisher Scientific; Waltham, MA), centrifuged at 350x *g* for 5 min, and resuspended in staining buffer (0.2% BSA in PBS) at a concentration of 1 million cells per 100uL. BD Pharmingen Human BD Fc Block (BD Biosciences; Franklin Lakes, NJ) was added and solution was incubated for 10 min at RT. Anti-CD56 antibody (Miltenyi Biotec; Bergisch Gladback, Germany) concentration was optimized to 0.1uL Ab/100uL cell solution. The solution was then incubated for 20 minutes at RT in the dark then washed with cold stain buffer twice. DAPI stain was used to assess viability of stained cells and optimized to 10ng/ml. Assessment of viability and CD56 purity in HSkMCs was performed using a 5-laser Aurora spectral analyzer with SpectroFlow acquisition software (V2.2; Cytek; Fremont, CA)[136].

Substrate Oxidation

HSkMCs were washed with DPBS and incubated in d-[1-^{14}C] glucose (1.6μCi/ml, 5.0mM glucose; Perkin-Elmer) and insulin (100nM), d-[1-^{14}C] palmitate (1.0μCi/ml, 5.0mM glucose, 200μM palmitate, 1mM carnitine; Perkin-Elmer), d-[1-^{14}C] palmitate and insulin, or d-[1-^{14}C] oleate (1.0μCi/ml, 5.0mM glucose, 200μM oleate, 1mM carnitine; Perkin-Elmer) containing media for 2 hours at 37°C. The experimental media was then transferred into a customized 48-well trapping plate with grooves fabricated between two continuous wells. CO_2 in the media was trapped in 1N NaOH with the addition of 70% perchloric acid. Complete oxidation of ^{14}C-glucose, -oleate, and -palmitate was measured with liquid scintillation counting of the conditioned 1N NaOH. Acidified media from the ^{14}C-palmitate

and ^{14}C-oleate wells was collected post-CO_2 trapping and centrifuged at 12,000g at 4°C. As described previously[17,137], an aliquot of the supernatant was used for liquid scintillation counting to determine levels of ^{14}C-acid soluble metabolites (ASMs) (i.e., oleoyl-carnitine, acetyl-carnitine, acetyl-coenzyme A, and short chain fatty acyl coenzyme A). From ^{14}C-glucose and ^{14}C-glucose and insulin wells, unacidified media was collected to measure non-oxidized anionic glycolytic metabolite production (NOGM) (i.e., lactate) as previously published by our group[72,137]. Briefly, collected media was loaded onto ion-exchange cellulose paper, dried, and washed with DPBS four times. Liquid scintillation counting of cellulose paper was used to determine ^{14}C-glucose incorporation into NOGMs. Data was normalized to total protein content measured by a BCA assay (Pierce Biotechnology, Inc.; Rockford, IL) and internal controls.

Glycogen Synthesis

Insulin-stimulated glycogen synthesis was measured in HSkMCs as an index of insulin sensitivity as previously published from our laboratory[75,137,138]. HSkMCs were incubated in media containing d-[1-^{14}C] glucose (1.6μCi/ml, 5.0mM glucose; Perkin-Elmer) with or without insulin (100nM) for two hours, washed twice with DPBS, and then lysed with 0.5% SDS. An aliquot of the lysate was combined with carrier glycogen (1mg) and denatured at 100°C for one hour. Ice-cold ethanol was then added to the denatured lysates, and samples spun overnight at 4°C for glycogen precipitation. The next day, glycogen pellets were centrifuged at 11,100x *g* for 15 minutes at 4°C, washed with 70% ethanol, and centrifuged again. The glycogen pellets were then resuspended in dH2O, and the

incorporation of ^{14}C-glucose into glycogen was determined with liquid scintillation counting.

Immunoblotting

HSkMC lysates were used for immunoblot analysis as described previously[132,137]. Briefly, HSkMCs were treated with or without insulin (100nM) for 10 min then harvested in lysis buffer (M-PER; Thermo Fisher Scientific; Waltham, MA) supplemented with protease and phosphatase inhibitors (Sigma-Aldrich; St. Louis, MO). Lysates were sonicated for 5 sec, spun end-over-end for 2 hours at 4°C, and spun at 15,000 x g for 15 minutes. Supernatant was collected and total protein content was measured using a BCA assay (Pierce Biotechnology, Inc). Lysates were aliquoted at identical protein concentrations and combined with Laemmli Sample Buffer (Bio-Rad; Hercules, CA) and 5-5'-Dithiobis (2-nitrobenzoic acid) (Sigma-Aldrich). Lysates used to probe for OXPHOS complex V were supplemented with 1% Triton X-100 (Sigma-Aldrich). Samples were separated using SDS-PAGE with 7.5% or 8-16% Tris-HCl gels and transferred to nitrocellulose membranes for probing with appropriate antibodies. Primary antibodies were used: citrate synthase (CS) (ab96600; Abcam; Cambridge, MA; 1:1000 dilution), peroxisome proliferator-activated receptor γ coactivator α (PGC1α) (SAB2500781; Sigma-Aldrich; 1:500 dilution), and total OXPHOS (ab110411; Abcam; 1:1000 dilution). Following overnight incubation with primary antibodies, membranes were probed with IRDye secondary antibodies (LI-COR Biosciences; Lincoln, NE) and scanned (Odyssey; LI-COR Biosciences). Protein expression was normalized to β-actin protein expression and a gel-to-gel control sample.

Statistical Analyses

Participants were grouped by metabolic flexibility in SkM homogenate tissue assessed by a 3-day HFD. If palmitate oxidation increased with the HFD, participants were stratified to the FLEX group. If palmitate oxidation decreased with the HFD, participants were stratified to the INFLEX group. Participants who had <15% change in palmitate oxidation were not included.

Unpaired or paired two-tailed t-test or two- or three-way ANOVA with Bonferroni correction were used to determine statistical significance. Factors were group (FLEX vs INFLEX) and condition (pre-HFD vs post-HFD). When significance was detected for either main effects or interactions between group and condition, a two-tailed t-test was performed for distinguishing meaningful differences. If data was not normally distributed, a Wilcoxon test was used. Associations between variables were tested using a Pearson correlation for normally distributed data or a Spearman correlation for non-normally distributed data. Statistical significance was set as $p \leq 0.05$. GraphPad Prism was used for all statistical analyses. All data were expressed as mean ± SEM.

RESULTS

Participant Characteristics and Diet

Participant characteristics are reported in **Table 3.1**. FLEX and INFLEX participants did not differ in age, BMI, or any whole-body measure of metabolic status. On average, participants consumed 39.3% ± 6.4 of their calories from fats before the diet and increased to 55.1% ± 11.1 calories from fat on the HFD (**Figure 3.1A**). Participants significantly increased their percentage of calories from fats on the HFD while decreasing

their percentage of calories from protein and carbohydrates (**Figure 3.1B**). FLEX and INFLEX did not differ in % calories from carbohydrates, fats, or protein before or on the HFD (**Figure 3.1C**).

Lipid metabolism of HSkMCs does not differ with a 3-day HFD

HSkMCs were initially tested for purity of CD56, a reliable molecular marker of myoblasts in SkM[139]. Flow cytometry revealed HSkMCs had an average purity of 94% for CD56 [**Figure 3.6 (Supplemental Data)**].

As the effect of HFDs has yet to be tested in HSkMCs, the addition of a lipid treatment was included. Lipid treatment of HSkMCs has not only been shown to distinguish differences between groups and increase substrate oxidation[79,80], but in this case, allows us to understand the effects of "direct" lipid exposure and "indirect" (dietary lipids) exposure on HSkMCs. Overall, there was no diet effect seen on lipid metabolism of HSkMCs, but there was a significant 24hFA treatment effect (**Figure 3.2A-D**). When participants were stratified as either FLEX or INFLEX, 24hFA treatment had a significant effect on both palmitate and oleate oxidation and partitioning (**Figures 3.2E-H**). FLEX had higher palmitate oxidation than INFLEX before but not after the HFD, and this difference went away with 24hFA treatment (**Figure 3.2E**). Palmitate partitioning did not differ between groups in any condition (**Figure 3.2F**) though FLEX trended towards having higher partitioning towards complete oxidation in the untreated condition before the diet ($p=0.0952$) and after ($p=0.1508$). Groups did not differ in oxidation of oleate (**Figure 3.2G**), but FLEX had higher oleate partitioning towards complete oxidation before the diet in the untreated and 24hFA condition (**Figure 3.2H**).

Efficiency of glucose oxidation differs across metabolically flexible and inflexible individuals

Overall, there was neither a diet nor a 24hFA treatment effect seen on glucose metabolism of HSkMCs (**Figure 3.3A-C**), and an insulin effect was only seen in glycogen synthesis (**Figure 3.3C**). When participants were stratified as FLEX or INFLEX, groups did not differ in glucose oxidation at any timepoint or condition (**Figure 3.3D**), but FLEX trended towards having higher basal and insulin-stimulated glucose oxidation in both conditions before the diet (BASAL: untreated p=0.1173, 24hFA p=0.0952; INSULIN: untreated p=0.1487; 24hFA p=0.1294). A significant effect of insulin was seen for the untreated condition only. Before the diet, FLEX had higher partitioning towards complete glucose oxidation in both the untreated and 24hFA condition (**Figure 3.3E**), but after the diet, this was only present in the insulin-stimulated, 24hFA treated state. INFLEX increased partitioning with the diet but only in the basal, 24hFA treated state. Groups did not differ in glycogen synthesis rates (**Figure 3.3F**), but FLEX trended towards having higher insulin-stimulated rates in both the untreated (p=0.1664) and 24hFA (p=0.1215) condition before the diet. Post-HFD insulin-stimulated rates also trended to be higher in FLEX in the untreated condition (p=0.1626). Insulin had a significant effect across both conditions. Whole-body insulin sensitivity, or the M value, did not correlate with HSkMC glycogen synthesis rates (**Table 3.2**).

Whole-body metabolic flexibility associates with glucose handling in HSkMCs

Whole-body ΔRQ is measured during a hyperinsulinemic-euglycemic clamp as the change in RQ from the basal state, when fat oxidation should be predominant, to the insulin-stimulated state, when carbohydrate oxidation should be predominant. In attempt to

measure an "*in vitro* ΔRQ", palmitate oxidation was tested with the addition of insulin. This was calculated as the percent inhibition of palmitate oxidation with the presence of insulin. Overall, there was no diet or 24hFA treatment effect (**Figure 3.4A**). When participants were stratified as FLEX or INFLEX, groups did not differ in rates (**Figure 3.4B**), but FLEX trended towards having higher inhibition of palmitate oxidation by insulin before the diet in the untreated condition (p=0.0793). Whole-body ΔRQ did not correlate with any measure of "*in vitro* ΔRQ". Interestingly, ΔRQ correlated with post-HFD glucose oxidation and glycogen synthesis rates across all states and conditions (**Table 3.2**).

Mitochondrial content does not change with a 3-day HFD

Mitochondrial content was assessed using citrate synthase and OXPHOS complex V content. Overall, there was no diet effect or 24hFA treatment effect for citrate synthase, complex V, or PGC1α expression (**Figure 3.5A-C**). FLEX and INFLEX did not differ before or after HFD in citrate synthase content in the untreated condition (**Figure 3.5D).** In the 24hFA condition, FLEX had lower citrate synthase before and after the HFD (**Figure 3.5D**). Complex V content was higher in FLEX before and after the HFD in the untreated condition (**Figure 3.5E**), but there were no differences between groups in the 24hFA condition. Groups did not differ in PGC1α in any condition or state (**Figure 3.5F**).

No association seen between HSkMC and SkM tissue palmitate oxidation

To assess the association between HSkMC and muscle tissue fatty acid oxidation, measures of cellular palmitate oxidation were correlated with measures of homogenate palmitate oxidation. Both HSkMCs and muscle homogenate samples were derived from the same muscle biopsy sample. There was no association between the two levels for

complete palmitate oxidation nor partitioning nor the percent change in palmitate oxidation with HFD (**Table 3.3**).

DISCUSSION

The metabolic inflexibility phenotype can be seen at the whole-body, SkM homogenate, and SkM fiber level[50,54,106]. Additionally, individuals with obesity and/or T2D have been shown to retain this phenotype in HSkMCs[140], but it remains unclear if metabolic inflexibility can be seen at the cellular level in individuals with overweight. HSkMCs grant control over the extracellular environment allowing for insight into the innate properties of SkM separate from the metabolic influences of hormones and nervous system control. The purpose of this study was to assess the impact of a 3-day HFD on HSkMC metabolism of healthy individuals with overweight. The main findings of the present study were that: 1) a 3-day HFD does not result in changes to HSkMC metabolism, and 2) the HSkMC phenotype of metabolically flexible and inflexible individuals is discordant with the muscle tissue phenotype.

Three-day HFDs result in changes to whole-body and *in vitro* muscle substrate metabolism and are a useful tool to distinguish differences in metabolic flexibility between individuals[50,55,56,106,141]. To the authors' knowledge, this is the first study to look at the effects of a 3-day HFD in HSkMCs. No differences were seen in palmitate, oleate, or glucose oxidation or partitioning with the HFD; glycogen synthesis rates and indices of mitochondrial content did not change with the HFD. Additionally, HSkMCs were incubated with fatty acids for 24 hours, which has been shown to increase substrate oxidation and

distinguish group differences[79]. With 24hFA treatment, no differences were seen between pre- and post-HFD measures. Together, these results suggest that a 3-day HFD is not enough of a stimulus to result in adaptations in HSkMC metabolism, and the temporal changes of HSkMC metabolism in response to a HFD needs more investigation. Some molecular markers have been shown to be affected by a HFD. For example, sirtuin 1 (SIRT1) regulates lipid oxidative gene transcription through PGC1α, and SIRT1 activity and content are shown to be increased with a 3-day HFD[58]. In the current study, we found no changes in PGC1α content with the diet which would suggest no change to SIRT1 and is consistent with findings by Bergouigan et al[51]. However, a limitation of the current study is only PGC1α content, and not acetylation, was measured, which has been shown to change with short-term HFDs[51,142]. In the current study, mitochondrial content was assessed using citrate synthase and complex V content as markers. Interestingly, in the untreated condition, FLEX and INFLEX differed in complex V content but not citrate synthase; in the 24hFA condition, groups differed in citrate synthase content but not complex V. As the differences between FLEX and INFLEX in complex V are unsubstantial and do not align with citrate synthase, this suggests these differences are not meaningfully significant and not a driving factor for differences between groups. The differences in citrate synthase content in the 24hFA condition may suggest that groups may respond differently to metabolic stress, but since this is not supported by differences in complex V, more investigation is needed for conclusive interpretation. Neither marker was changed with the HFD, which aligns with other studies that have shown neither citrate synthase activity and/or content [19,112,113,141,143–145] nor OXPHOS complex content[44,50,113] changes with a HFD. Together, the

observation of no differences in any functional measurements of substrate metabolism or protein content of PGC1α suggests a 3-day HFD is not a potent stimulus for adaptation in HSkMCs.

A large strength of the current study was measurement of substrate oxidation at the whole-body, SkM homogenate, and HSkMC level in the same individual. To the authors' knowledge, direct comparison of SkM tissue and HSkMC substrate oxidation in individuals with overweight has not been studied before. Interestingly, we found no association between SkM homogenate and HSkMC palmitate oxidation, partitioning, or the % change in palmitate oxidation with HFD. In SkM homogenates before the diet, FLEX showed lower palmitate oxidation, while in HSkMCs FLEX showed higher palmitate oxidation and higher oleate partitioning. Together, our results suggest that SkM tissue and HSkMCs substrate oxidation are not associated in this population, but this may be partly due to the different assay conditions between muscle homogenates and HSkMC. The base medium used for the ^{14}C-palmitate cocktail with HSkMCs is low-glucose DMEM and thus, measures palmitate oxidation in the presence of glucose in intact cells. Meanwhile, the base medium used for SkM tissue does not contain glucose resulting in no substrate competition and is performed in a homogenized sample. To fully understand the association between HSkMC and SkM tissue substrate oxidation, more investigation is needed, and the use of similar assay conditions should be utilized. Interestingly, strong positive associations between whole-body ΔRQ and HSkMC glucose metabolism were seen; post-HFD glucose oxidation and glycogen synthesis in both the untreated and 24hFA treated condition correlated with ΔRQ. While glucose uptake was not directly measured in HSkMCs, glucose oxidation and

glycogen synthesis are the two primary pathways of glucose disposal in cells. As there is increasing evidence for glucose uptake being the primary determinant of insulin-stimulated substrate handling during a hyperinsulinemic-euglycemic clamp[36,38–40], our data supports this notion that measurement of ΔRQ via a hyperinsulinemic-euglycemic clamp is more indicative of insulin-stimulated substrate handling rather than metabolic flexibility. With glucose, FLEX showed a trend for increased oxidation and consistently had significantly higher partitioning towards complete oxidation compared to INFLEX. FLEX also trended towards having higher glycogen synthesis rates and flexibility to switch from palmitate to glucose oxidation with insulin-stimulation. Together, this suggests that FLEX HSkMCs have enhanced glucose handling even after a HFD. This is surprising considering that participants were stratified to FLEX due to their increase in SkM homogenate palmitate oxidation with the HFD, and groups showed no difference in pyruvate oxidation in SkM homogenates (**Figure 2.3D**) and warrants further investigation to understand these discrepancies.

A limitation of the current study was the small sample size (n=5 per group). While similarly small sample sizes have been previously effective at distinguishing group differences in HSkMCs[74,75,77,79,140], this has been primarily in groups with large phenotypic differences (e.g., lean individuals vs individuals with severe obesity). In the current study, there were no whole-body differences between FLEX and INFLEX (**Table 3.1**). Interestingly, the primary distinction between FLEX and INFLEX in HSkMCs was in glucose partitioning though participants were grouped based off their SkM homogenate change in palmitate oxidation with HFD. Our findings of numerous trending p-values (palmitate partitioning,

glucose oxidation, insulin-stimulated glycogen synthesis, and change in palmitate with insulin addition) suggests there may be clear HSkMC differences between FLEX and INFLEX, but a larger sample size is needed to distinguish meaningful differences.

In conclusion, the current study found that a 3-day HFD in a population of young, relatively healthy individuals with overweight does not result in changes to HSkMC substrate handling. Further, when participants were stratified by metabolic flexibility measured in SkM homogenate tissue, the HSkMC phenotype was discordant with muscle tissue and instead associated with whole-body measures of metabolic flexibility. More investigation is needed in this population to result in meaningful conclusions on the effects of a 3-day HFD on HSkMC metabolism and to distinguish potential differences in cellular metabolic flexibility.

CHAPTER 3 FIGURES

	FLEXIBLE N=5	INFLEXIBLE N=5	P-VALUE
GENDER	1 F / 4 M	2 F / 3 M	
ETHNICITY	1 Other / 4 White	5 White	
DELTA RQ	0.046 ± 0.045	0.064 ± 0.035	0.5009
BASAL RQ	0.72 ± 0.018	0.73 ± 0.048	0.8021
INSULIN RQ	0.77 ± 0.045	0.79 ± 0.061	0.5003
AGE (years)	27 ± 4.6	32 ± 12	0.4147
BMI (kg/m^2)	28 ± 1.9	28 ± 2.1	0.9261
% BODY FAT	25.9 ± 9.2	29.6 ± 3.0	0.5299
% LEAN MASS	72.4 ± 9.01	68.6 ± 8.63	0.5085
VAT Volume (cm^2)	448 ± 154	516 ± 132	0.4750
REE	2130 ± 273	1831 ± 273	0.1211
TOTAL CHOLESTEROL (mg/dL)	192 ± 53	164 ± 28	0.3228
TRIGLYCERIDES (mg/dL)	160 ± 170	85.2 ± 21.3	0.6429
LDL (mg/dL)	117 ± 28	103 ± 24	0.4030
HDL (mg/dL)	43 ± 7.7	45 ± 8.6	0.7368
GLUCOSE (mg/dL)	87.8 ± 3.49	94.8 ± 6.3	0.2143
INSULIN (mg/dL)	6.0 ± 1.9	11 ± 5.8	0.1252
HbA1c (%)	5.3 ± 1.25	5.3 ± 0.13	0.6479
HOMA-IR	1.3 ± 0.42	2.6 ± 1.5	0.1065
M VALUE	7.0 ± 1.7	9.1 ± 3.9	0.3516
LACTATE (mmol/L)	1.1 ± 0.55	1.1 ± 0.36	>0.9999
VO_2max (mL/kg/min)	37.0 ± 7.51	37.4 ± 9.03	0.9500
SBP (mm)	118 ± 17..6	126 ± 13.3	0.3333
DBP (mm)	70 ± 5.2	76 ± 11	0.3480

Table 3.1. HSkMC donor characteristics. Data presented as mean ± SEM.

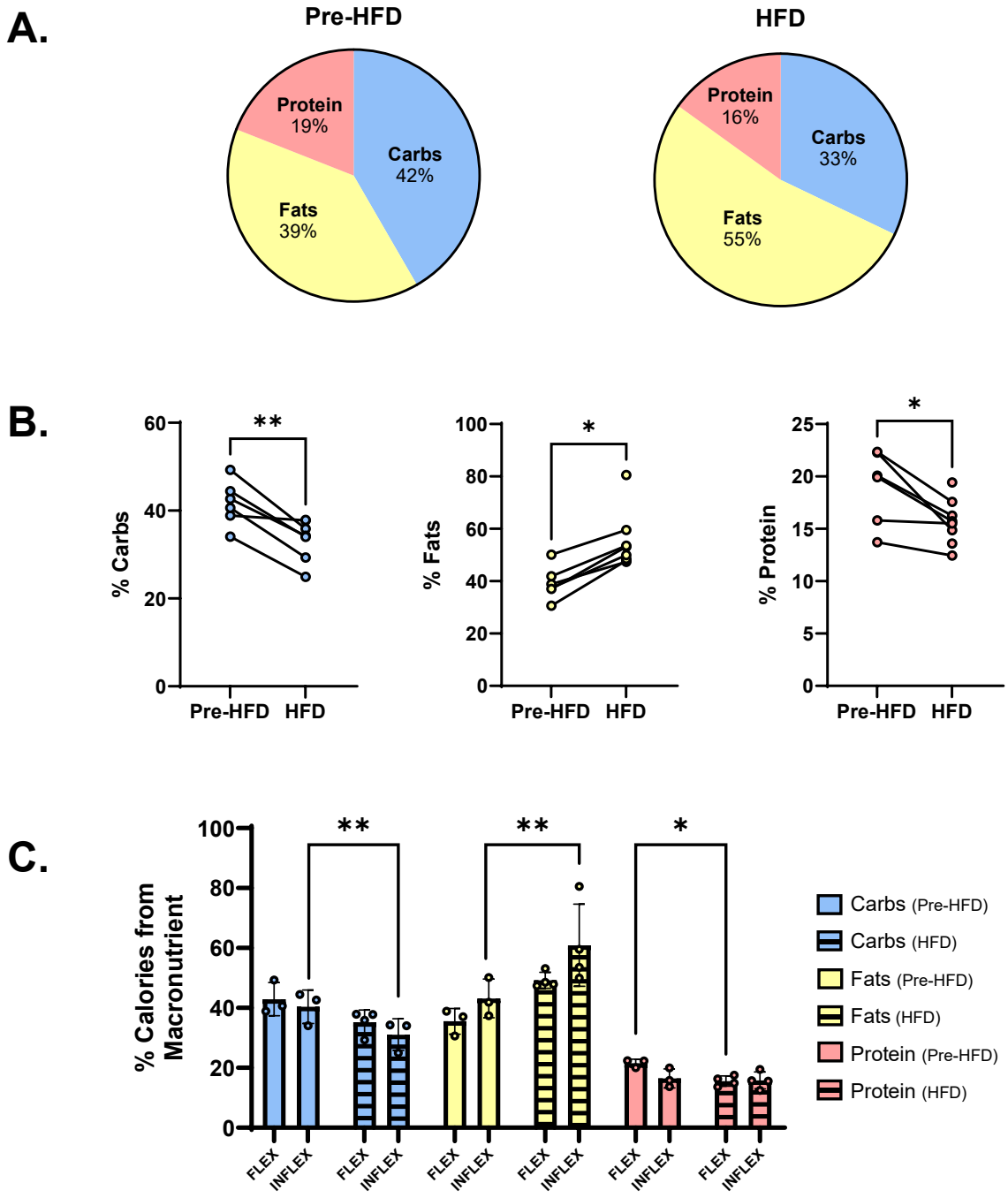

Figure 3.1. Pre-HFD and HFD composition breakdown of HSkMC donors. (A) Pre-HFD and HFD macronutrient breakdown by percentage calories from fats, carbohydrates, and protein as logged by participants in MyFitnessPal. Pre-HFD averages taken over three days. (B) Pre-HFD to HFD change in percent calories from fats, carbohydrates, and protein. (C) Pre-HFD and HFD macronutrient breakdown for FLEX (n=3-4) and INFLEX (n=3-4). Data presented as mean ± SEM. *p≤0.05; **p≤0.01.

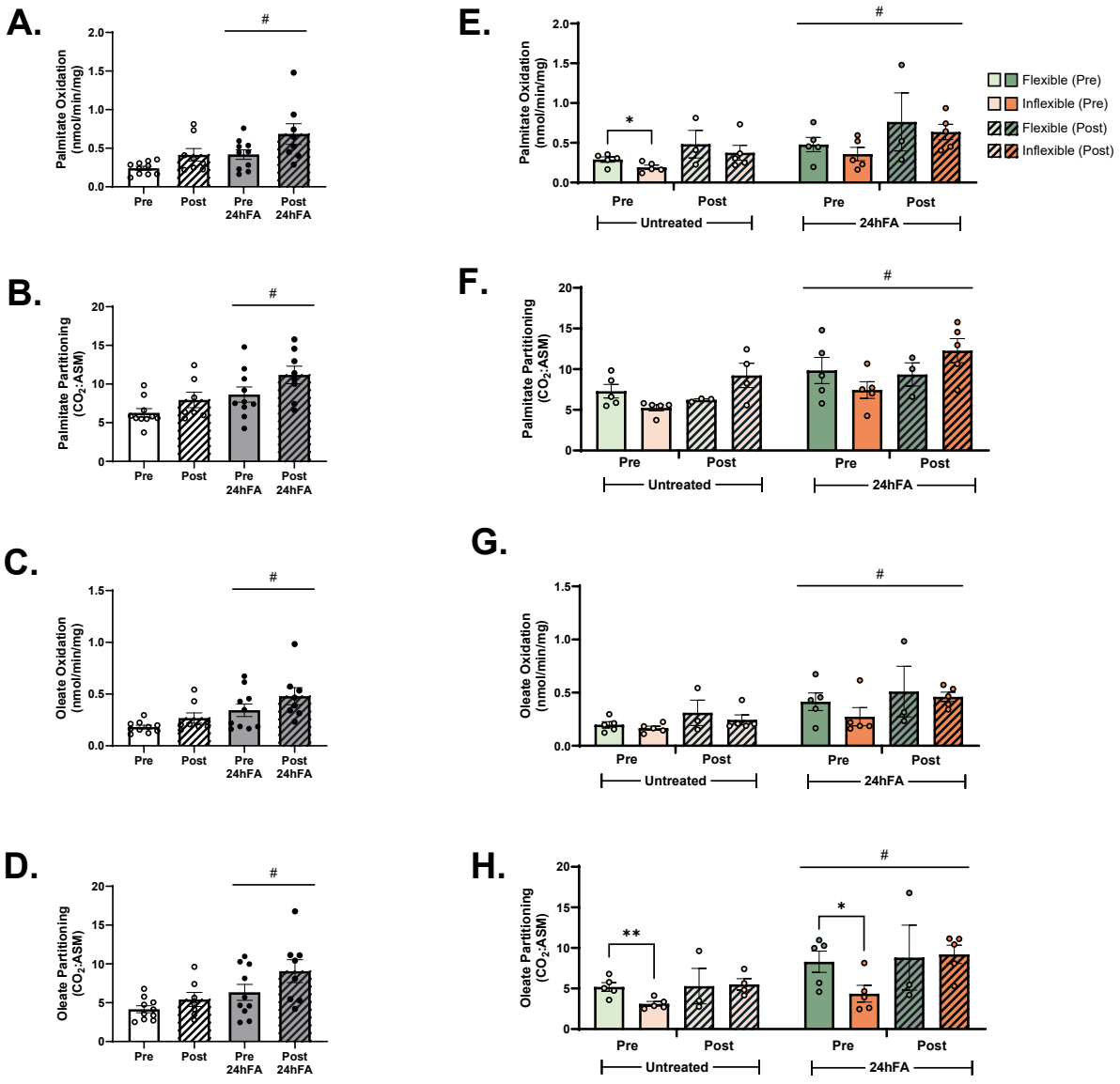

Figure 3.2. Lipid metabolism of HSkMCs is not affected by a 3-day HFD. (A) ^{14}C-palmitate oxidation from all participants pre- and post-HFD. (B) Partitioning of ^{14}C-palmitate to complete (CO_2): incomplete (ASM) oxidation pre- and post-HFD. (C) ^{14}C-oleate oxidation from all participants pre- and post-HFD. (D) Partitioning of ^{14}C-oletate to complete (CO_2): incomplete (ASM) oxidation pre- and post-HFD. (E) ^{14}C-palmitate oxidation of metabolically flexible (FLEX) and inflexible (INFLEX) individuals pre- and post-HFD. HSkMCs were assessed in the untreated state or after treatment with a 1:1 palmitate:oleate cocktail for 24 hours (24hFA). (F) Partitioning of ^{14}C-palmitate to complete (CO_2): incomplete (ASM) oxidation in FLEX and INFLEX participants. (G) ^{14}C-oleate oxidation from FLEX and INFLEX participants. (H) Partitioning of ^{14}C-oleate to complete (CO_2): incomplete (ASM) oxidation in FLEX and INFLEX participants. # denotes significant effect of lipid treatment. Data presented as mean ± SEM. *p≤0.05; **p≤0.01.

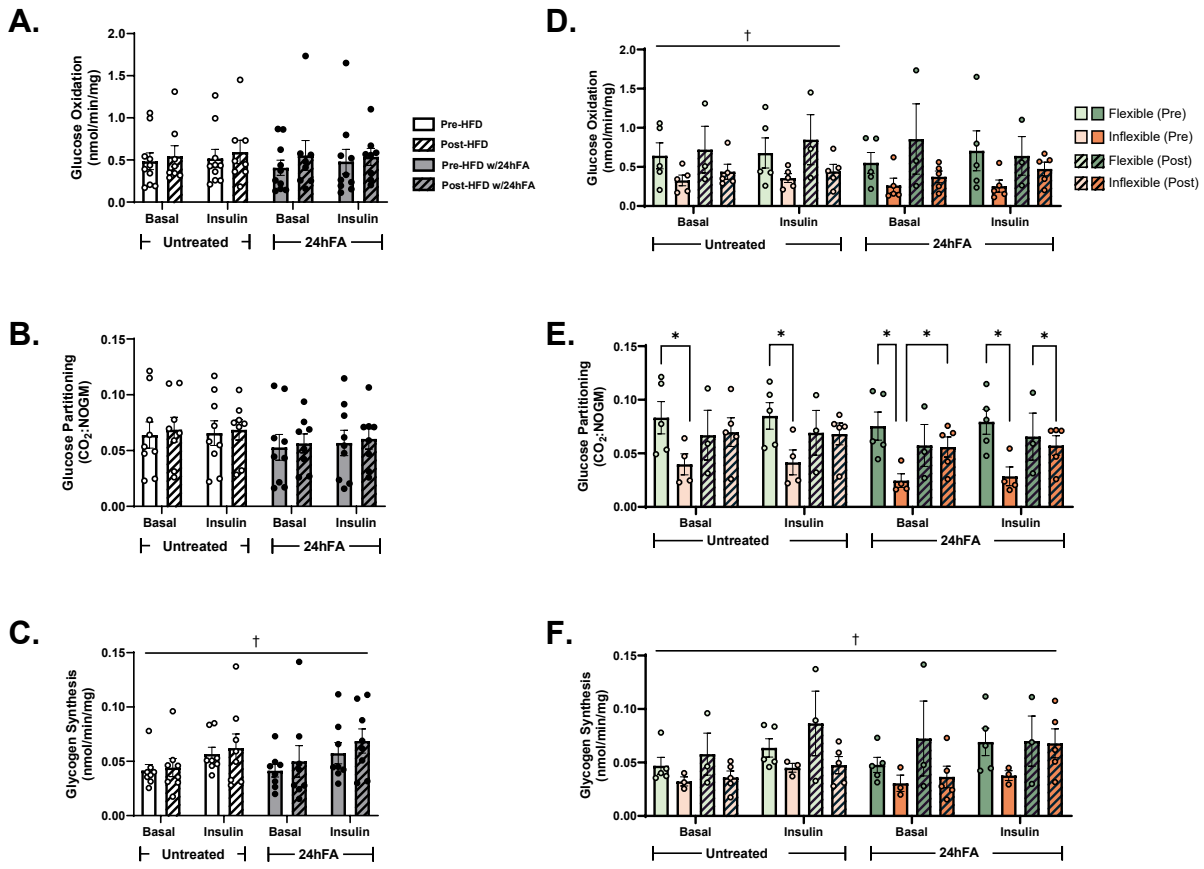

Figure 3.3. Glucose metabolism of HSkMCs is not affected by a 3-day HFD. (A) ^{14}C-glucose oxidation from all participants pre- and post-HFD. (B) Partitioning of ^{14}C-glucose to complete (CO_2): incomplete (non-oxidized glucose metabolites; NOGM) oxidation pre- and post-HFD. (C) ^{14}C-glucose incorporation into glycogen from all participants pre- and post-HFD. (D) ^{14}C-glucose oxidation from FLEX and INFLEX participants pre- and post-HFD. (E) Partitioning of ^{14}C-glucose to complete (CO_2): incomplete (non-oxidized glucose metabolites; NOGM) oxidation pre- and post-HFD in FLEX and INFLEX participants. (C) ^{14}C-glucose incorporation into glycogen from FLEX and INFLEX participants pre- and post-HFD. † denotes significant effect of insulin. Data presented as mean ± SEM. *p≤0.05.

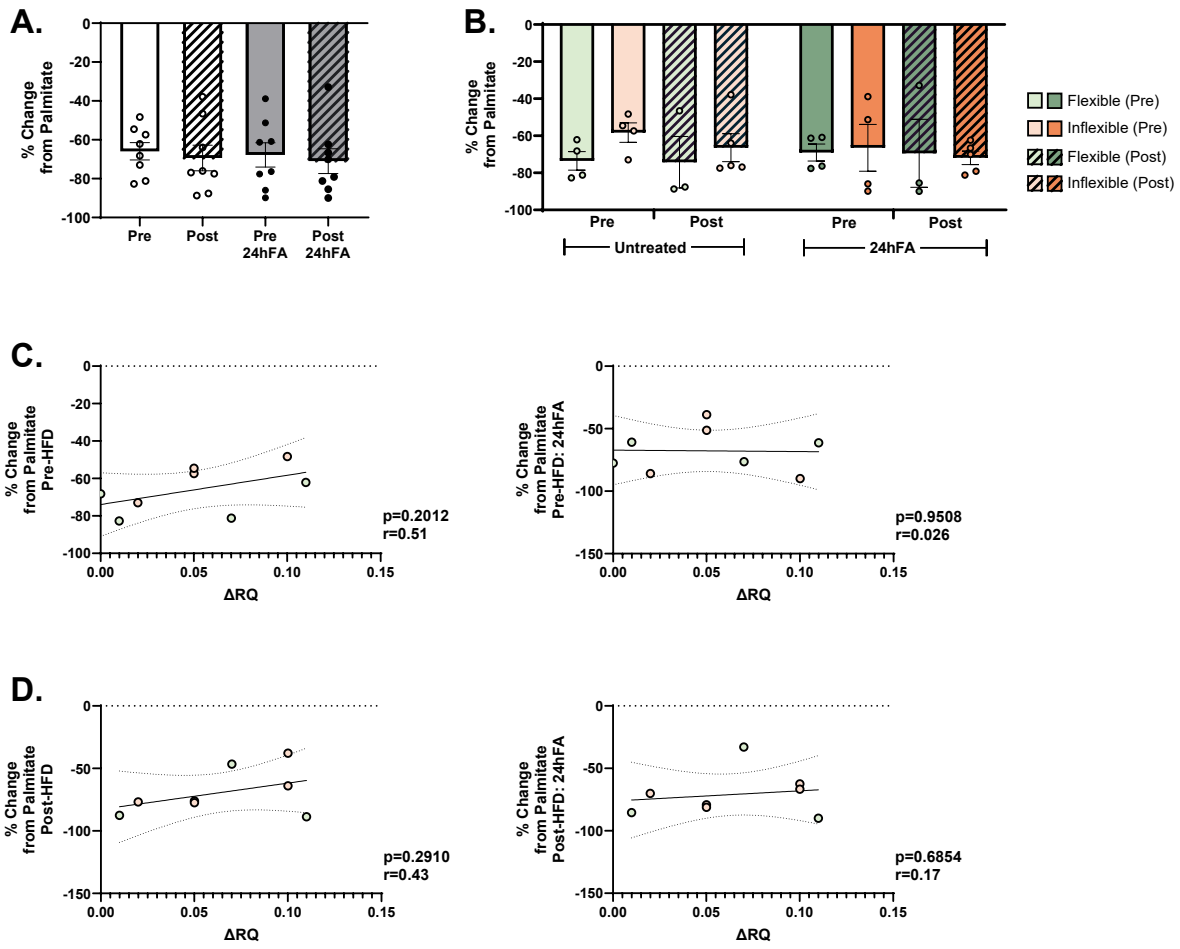

Figure 3.4. Whole-body metabolic flexibility is not associated with *in vitro* metabolic flexibility. (A) HSkMC percent decrease of ^{14}C-palmitate oxidation with the addition of insulin before and after HFD in all participants. (B) Percent decrease of ^{14}C-palmitate oxidation with the addition of insulin before and after HFD separated by FLEX and INFLEX. (C) Pearson correlation between ΔRQ measured by a hyperinsulinemic-clamp and HSkMC percent decrease of palmitate oxidation in the presence of insulin before the HFD. (D) Pearson correlation between ΔRQ measured by a hyperinsulinemic-clamp and HSkMC percent decrease of palmitate oxidation in the presence of insulin after the HFD. Data presented as mean ± SEM.

Participant Measure	Post-HFD Measure	Correlation Coefficient	P value
ΔRQ	**Glucose Oxidation** (nmol/min/mg)		
	Basal	0.7591	0.0289
	Basal, 24hFA treated	0.7229	0.0427
	Insulin	0.6930	0.0567
	Insulin, 24hFA treated	0.7122	0.0474
	Glycogen Synthesis (nmol/min/mg)		
	Basal	0.7952	0.0183
	Basal, 24hFA treated	0.7350	0.0378
	Insulin	0.7641	0.0273
	Insulin, 24hFA treated	0.9067	0.0019
M Value (mg/min/kg lean mass)	**Glycogen Synthesis** (nmol/min/mg)		
	Insulin	0.07143	0.9063
	Insulin, 24hFA treated	0.1007	0.8300

Table 3.2. Whole-body metabolic flexibility is associated with post-HFD glucose metabolism in HSkMCs. Pearson's correlation used for normally distributed data, and Spearman's correlation used for non-normally distributed data.

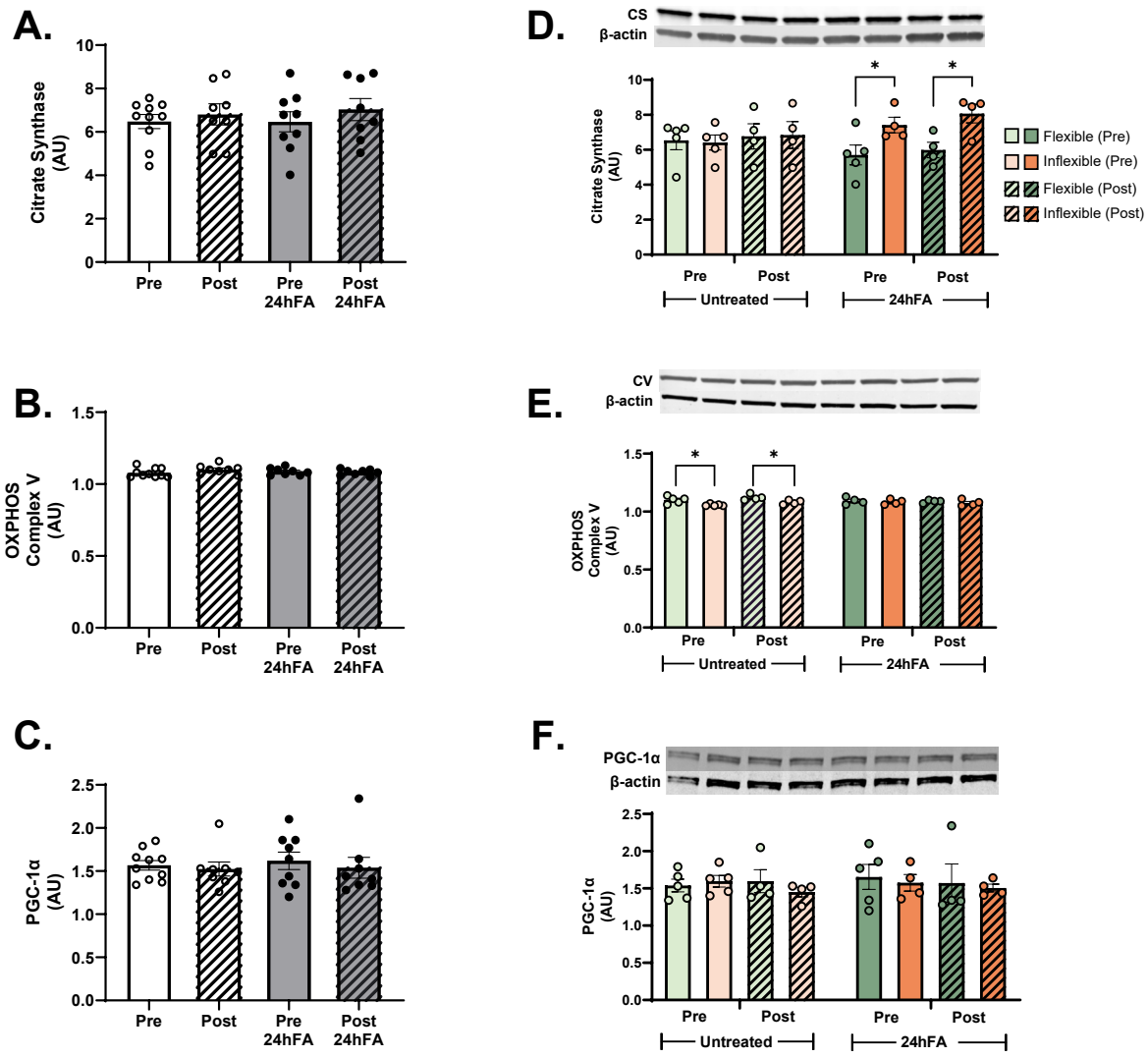

Figure 3.5. Markers of mitochondrial content and PGC1a do not differ with a 3-day HFD in HSkMCs. (A) Citrate synthase content measured in all participants before and after HFD. (B) OXPHOS complex V content measured in all participants before and after HFD. (C) PGC1a content measured in all participants before and after HFD. (D) Citrate synthase content before and after HFD separated by FLEX and INFLEX grouping. (E) OXPHOS complex V content before and after HFD separated by FLEX and INFLEX grouping. (F) PGC1a content before and after HFD separated by FLEX and INFLEX grouping. Data presented as mean ± SEM. *p≤0.05.

Cellular Measure	Homogenate Measure	Correlation coefficient	P value
Palmitate Oxidation	**Palmitate Oxidation**		
	Pre-HFD	-0.031	0.9316
	Post-HFD	0.55	0.1710
Palmitate Partitioning	**Palmitate Partitioning**		
(CO_2:ASM)	*Pre-HFD*	-0.35	0.3586
	Post-HFD	-0.071	0.9063
% Change in Palmitate Oxidation with HFD	***% Change in Palmitate***	-0.43	0.2928

Table 3.3. HSkMC measures of palmitate oxidation do not associate with SkM homogenate measures of palmitate oxidation from the same individuals. Pearson's correlation used for normally distributed data, and Spearman's correlation used for non-normally distributed data.

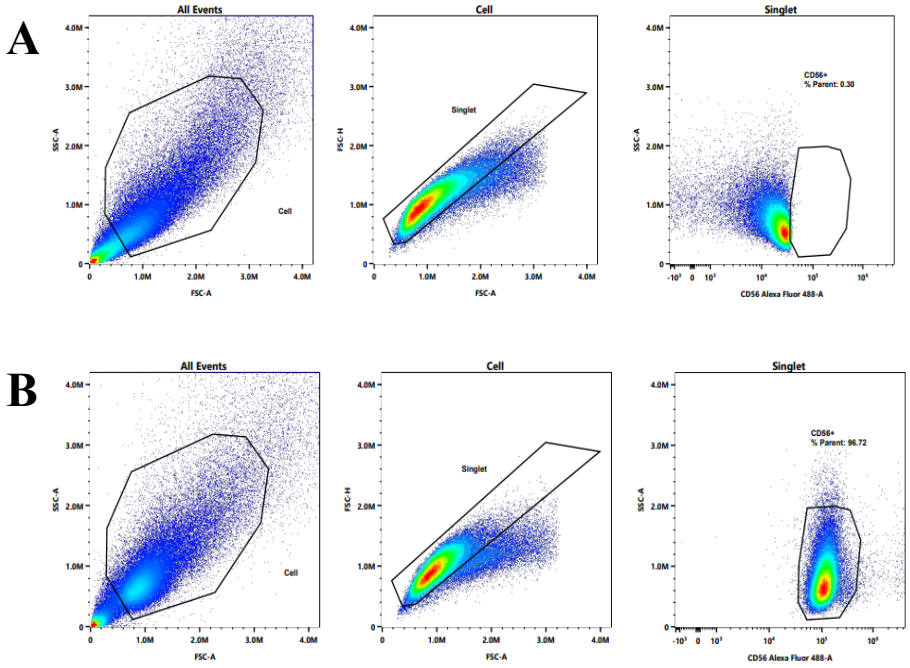

Figure 3.6. (Supplemental Data) HSkMCs show high purity for CD56. (A) Representative flow cytometry images of unstained HSkMCs. (B) Representative flow cytometry images of HSkMCs expressing CD56.

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

A growing body of evidence suggests that metabolic flexibility is an innate component of SkM and part of the metabolic signature seen with metabolic disease. However, it remains not well defined how early into the development of metabolic disease that metabolic inflexibility can be seen. The primary goal of this study was to test metabolic inflexibility in healthy individuals with overweight using a 3-day HFD, and if differences were seen, to understand their relationship with muscle fiber type, mitochondrial content and function, and SkM metabolism.

In summary, in chapter 2 we show that metabolic inflexibility can be seen in individuals with overweight despite having healthy blood lipids and no insulin resistance. Metabolic flexibility was measured by the change in palmitate oxidation with a 3-day HFD with flexible individuals increasing oxidation and inflexible individuals decreasing oxidation. Groups did not differ in diet composition before or during the HFD. Additionally, changes in palmitate oxidation occurred without any changes to efficiency of palmitate oxidation or absolute pyruvate oxidation, and this did not correlate to ΔRQ measured by a hyperinsulinemic clamp or muscle fiber type. Flexible and inflexible individuals did not differ in mitochondrial function, measured by content, capacity, and varying states of respiration, which supports the growing evidence that mitochondrial function is not associated with impairments in metabolic flexibility[39]. In chapter 3, we show that a 3-day HFD does not result in changes to HSkMC metabolism measured by substrate oxidation, glycogen synthesis, and protein content of mitochondrial markers and PGC1α, and no

effect of the diet is seen even after a 24-hour incubation of HSkMCs with lipids. Interestingly, though flexible individuals were defined by their SkM homogenate change in palmitate oxidation, HSkMCs of flexible individuals show higher efficiency with glucose oxidation and trend towards having glycogen synthesis rates. HSkMCs of flexible individuals additionally show trends for higher fatty acid oxidation and partitioning, and this occurs without differences in mitochondrial content or PGC1α.

Is an ability to increase fatty acid oxidation a positive adaptation?

In chapter 2, we show a clear distinction in SkM of individuals with overweight to adjust fatty acid oxidation in response to lipid oversupply. An interesting point of conversation is if increasing fatty acid oxidation is a positive adaptation, or if in fact, decreasing fatty acid oxidation would be more metabolically beneficial. The suggestion for decreased fatty acid oxidation being more metabolically favorable lies in studies showing inhibition of fatty acid oxidation results in a reciprocal increase in glucose utilization[146,147],improved[148] or unchanged[147] insulin sensitivity, and increased mitochondrial biogenesis[147], and this occurs despite increases in diacylglycerol[149]. While most of this data has been collected using animal models, some human data exists to support this. Timmers et al. show that 5-day etomoxir, a pharmacological inhibitor of carnitine palmitoyltransferase I (CPT1), treatment in combination with a 3-day HFD results in increased glucose oxidation, decreased fat oxidation, improved HOMA-IR, and increased sarcolemma glucose transporter type 4 (GLUT4) content[149]. However, it is important to note that human data is currently limited, and more investigation and long-

term studies are needed to understand the implications of CPT1 inhibition in humans. Fatty acid oxidation can additionally be inhibited through CPT2 and allow for the accumulation of long-chain acyl-carnitines rather than acyl-CoAs, which may be more metabolically favorable. Pereyra et al. show that a SkM-specific CPT2 knockout model in combination with a HFD results in mice that with lower weight and adiposity and improved insulin sensitivity and signaling[150]. Some studies have shown that while initial inhibition of fatty acid oxidation results in improved glucose handling, long-term inhibition results in whole-body insulin resistance and hepatic steatosis, which is shown to be a predictor for the development of T2D independent of glucose disposal rate[151,152]. Interestingly, Pereyra et al., did not see an increase in liver triacylglycerols despite using a 16-week HFD. Work by Koves et al. suggests that an ability to increase flux through β-oxidation may reach a point at which flux through the TCA cycle and electron transport system is exceeded[153,154]. At this point, incomplete fatty acid oxidation occurs and results in the accumulation of its intermediates and subsequent mitochondrial dysfunction and insulin resistance. Together, this suggests that an ability to increase fat oxidation may not be a positive adaptation. On the other hand, human data has shown an inverse relationship between fasting fatty acid oxidation and insulin-stimulated glucose disposal rate in lean individuals and those with obesity and T2D suggesting dysregulation of fasting fatty acid oxidation is associated with decrements in insulin action[105]. Dobbins et al. show evidence contradicting previously mentioned CPT1 knockout studies and argue that prolonged inhibition of fatty acid oxidation results in intramyocellular lipid accumulation and insulin resistance[155]. Intramyocellular lipid content has been shown to be a strong predictor of

insulin resistance in sedentary individuals[156–158]. A large body of work done in humans supports that an ability to increase fatty acid oxidation is protective for insulin action. HFDs ranging from 2 days to 6 weeks consistently show that an ability to increase fatty acid oxidation results in the preservation or improvement of insulin action rather than impairment[44,112,159–168]. Taken together, while the evidence for inhibition of fatty acid oxidation being metabolically favorable is intriguing, a lack of human data currently limits considerations for metabolic inflexibility to be a positive adaptation in humans.

Future directions

In chapter 2 we show evidence of metabolic inflexibility in SkM of individuals with overweight prior to the appearance of any clinical marker of metabolic disease. To the authors' knowledge, this is the first study to use the change in palmitate oxidation with a HFD to define metabolic flexibility. Though we show a robust difference between FLEX and INFLEX, it remains unclear how this measure extrapolates as an indicator of long-term metabolic health and should be validated in future studies. Metabolic flexibility measured using whole-body 24h ΔRQ during a HFD has been shown to be an independent predictor of weight gain when participants are followed up to 5 years later[47,169]. With this, we suggest longitudinal studies to be a future direction, which would allow for several novel findings. 1) How is the change in SkM homogenate palmitate oxidation with a HFD related to long-term metabolic health? Assessment of participants' change in body composition and markers of whole-body metabolic health (i.e., HbA1c, blood lipids, fasting lactate) over time and their association with SkM homogenate oxidation would validate if this measure

of metabolic flexibility is predictive of a metabolic disruption. 2) Is an ability to increase or decrease fatty acid oxidation more metabolically favorable? As individuals with overweight have an increased likelihood of developing metabolic disease[31–33], it is highly likely that several of these participants will do so with time. A longitudinal study would allow assessment if there is a higher rate of incidence in the FLEX or INFLEX group and provide novel human data for this argument.

The large novelty of our data lies in the population studied – young, healthy individuals with overweight. Studies including individuals with overweight primarily consist of grouping this population together with individuals with obesity making it unclear if some of the metabolic signatures seen with obesity occur at a lower adiposity. In chapter 2, we confirm that metabolic inflexibility can be seen prior to the development of obesity, and thus arises the question of if metabolic inflexibility can be improved with intervention. Two primary interventions have been studied surrounding metabolic flexibility, weight loss and exercise, with mixed results. Data surrounding weight loss via dietary intervention as a method of improving metabolic flexibility is not compelling. Astrup et al. studied women with obesity who underwent weight loss using a dietary intervention to reach a healthy BMI and continued to be weight stable for 6 months prior to data collection[53]. Compared to lean counterparts who never had obesity, the women who previously had obesity did not increase fat oxidation appropriately in response to a medium- or HFD indicating that metabolic inflexibility is not ameliorated by weight loss. Results from Larson et al. confirms that in a mix of men and women, weight-reduced individuals show an impaired ability to increase fat oxidation[170]. Together, these studies show that weight loss via dietary

intervention is not a viable strategy of increasing metabolic flexibility. Participants in the study by Astrup et al. had a confirmed history of metabolic disease and when taken into consideration with other data supporting that a family history of metabolic disease is a potent factor affecting metabolic flexibility, this strongly points towards metabolic inflexibility having a genetic or epigenetic component and being unrelated to body fat composition. Exercise interventions, on the other hand, have been shown to be a powerful strategy of increasing metabolic flexibility. As outlined in the review by Fritzen et al.[45], adaptations from exercise training largely act on the same pathways that HFDs impact. For example, exercise training results in an increase in fatty acid uptake and oxidation capacity, which is similar to the adaptations of metabolically flexible individuals undergoing a HFD. Additionally, in mice undergoing a HFD with or without the addition of exercise, proteomics reveals a significant overlap between proteins regulated by the HFD and exercise[171]. Thus, exercise training could be a viable strategy for improving metabolic flexibility. Indeed, many studies have shown that exercise training results in improvements in fatty acid metabolism in metabolically flexible individuals. Battaglia et al. show that in individuals with metabolic inflexibility, 7-days of endurance exercise (70% VO_2max for 1h/day) training results in palmitate oxidation increasing to the level of lean individuals pre-exercise intervention[50]. Even individuals with extreme obesity (BMI>40) show improvements in fatty acid metabolism in response to exercise training; 10-days of endurance exercise training (75% VO_2max for 1h/day) resulted in palmitate oxidation almost doubling that of pre-exercise rates despite no change in body weight[172], which confirms that exercise training is a successful means of improving metabolic flexibility.

Overall, there has been extensive data to outline that metabolic inflexibility is an innate component of SkM, exists across a range of metabolic statuses, and can be improved with intervention; however, few studies have used a mechanistic approach to address the reasons behind these differences in substrate oxidation. We propose that future studies utilize transcriptomics and proteomics in a study of metabolically flexible and inflexible individuals undergoing a HFD, as this would allow for understanding into the coordinated molecular efforts to adjust fat oxidation in conditions of oversupply, and if used in a population with overweight, these approaches could help identify targets for intervention to slow the development of metabolic disease. A potential cause of metabolic inflexibility is an inability to appropriately adapt flux through the TCA cycle[153,154]. With a HFD, this results in a supply that exceeds demand and the unbalanced bioenergetics results in insulin resistance and metabolic disease[16,173]. Zou et al. show that in HSkMCs from individuals with extreme obesity, flux through the TCA cycle is impaired resulting in depressed TCA cycle intermediates[174]. To investigate whether this could be a potential explanation for substrate handling differences between metabolically flexible and inflexible individuals, ^{13}C metabolic flux assays should be implemented in future studies to identify the specific points at which substrate oxidation is differentially regulated. Additionally, if combined with an exercise intervention, this would help to answer the question if increasing demand to match substrate supply ameliorates differences in metabolic inflexibility, and if this occurs via similar mechanisms in flexible and inflexible individuals.

In conclusion, the current study provides several novel findings to contribute to the field of metabolic flexibility in humans. First, healthy individuals with overweight exhibit metabolic inflexibility which occurs prior to the appearance of any clinical markers of metabolic disturbance. Second, despite no differences in whole-body ΔRQ, metabolic inflexibility is seen at the SkM level. Together, this exemplifies that metabolic inflexibility is an innate component of SkM. Future studies should focus on several unknowns about metabolic flexibility. First, whether an ability to increase or decrease fatty acid oxidation is a positive adaptation; second, the mechanisms that drive differences in metabolic flexibility; third, whether increasing demand to match substrate supply occurs through similar mechanisms in individuals with differing metabolic flexibility.

REFERENCES

1. Arroyo-Johnson C, Mincey KD. Obesity Epidemiology Worldwide. *Gastroenterol Clin North Am*. 2016;45(4):571-579. doi:10.1016/j.gtc.2016.07.012

2. Eckel RH, Grundy SM, Zimmet PZ. The metabolic syndrome. *Lancet Lond Engl*. 2005;365(9468):1415-1428. doi:10.1016/S0140-6736(05)66378-7

3. Zheng Y, Ley SH, Hu FB. Global aetiology and epidemiology of type 2 diabetes mellitus and its complications. *Nat Rev Endocrinol*. 2018;14(2):88-98. doi:10.1038/nrendo.2017.151

4. Chew NWS, Ng CH, Tan DJH, et al. The global burden of metabolic disease: Data from 2000 to 2019. *Cell Metab*. 2023;35(3):414-428.e3. doi:10.1016/j.cmet.2023.02.003

5. Seuring T, Archangelidi O, Suhrcke M. The Economic Costs of Type 2 Diabetes: A Global Systematic Review. *PharmacoEconomics*. 2015;33(8):811-831. doi:10.1007/s40273-015-0268-9

6. Tremmel M, Gerdtham UG, Nilsson PM, Saha S. Economic Burden of Obesity: A Systematic Literature Review. *Int J Environ Res Public Health*. 2017;14(4):435. doi:10.3390/ijerph14040435

7. CDC. New Adult Obesity Maps. Centers for Disease Control and Prevention. Published September 21, 2023. Accessed March 23, 2024. https://www.cdc.gov/obesity/data/prevalence-maps.html

8. National Diabetes Statistics Report | Diabetes | CDC. Published November 29, 2023. Accessed March 23, 2024. https://www.cdc.gov/diabetes/data/statistics-report/index.html

9. Claussnitzer M, Susztak K. Gaining insight into metabolic diseases from human genetic discoveries. *Trends Genet TIG*. 2021;37(12):1081-1094. doi:10.1016/j.tig.2021.07.005

10. van Dijk SJ, Tellam RL, Morrison JL, Muhlhausler BS, Molloy PL. Recent developments on the role of epigenetics in obesity and metabolic disease. *Clin Epigenetics*. 2015;7:66. doi:10.1186/s13148-015-0101-5

11. Blüher M. Obesity: global epidemiology and pathogenesis. *Nat Rev Endocrinol*. 2019;15(5):288-298. doi:10.1038/s41574-019-0176-8

12. Leitner DR, Frühbeck G, Yumuk V, et al. Obesity and Type 2 Diabetes: Two Diseases with a Need for Combined Treatment Strategies - EASO Can Lead the Way. *Obes Facts*. 2017;10(5):483-492. doi:10.1159/000480525

13. Kelley DE, Mandarino LJ. Fuel selection in human skeletal muscle in insulin resistance: a reexamination. *Diabetes*. 2000;49(5):677-683. doi:10.2337/diabetes.49.5.677

14. Goodpaster BH, Sparks LM. Metabolic Flexibility in Health and Disease. *Cell Metab*. 2017;25(5):1027-1036. doi:10.1016/j.cmet.2017.04.015

15. Galgani JE, Fernández-Verdejo R. Pathophysiological role of metabolic flexibility on metabolic health. *Obes Rev Off J Int Assoc Study Obes*. 2021;22(2):e13131. doi:10.1111/obr.13131

16. Fisher-Wellman KH, Neufer PD. Linking mitochondrial bioenergetics to insulin resistance via redox biology. *Trends Endocrinol Metab TEM*. 2012;23(3):142-153. doi:10.1016/j.tem.2011.12.008

17. Kim JY, Hickner RC, Cortright RL, Dohm GL, Houmard JA. Lipid oxidation is reduced in obese human skeletal muscle. *Am J Physiol Endocrinol Metab*. 2000;279(5):E1039-1044. doi:10.1152/ajpendo.2000.279.5.E1039

18. Hulver MW, Berggren JR, Cortright RN, et al. Skeletal muscle lipid metabolism with obesity. *Am J Physiol Endocrinol Metab*. 2003;284(4):E741-747. doi:10.1152/ajpendo.00514.2002

19. Anderson AS, Haynie KR, McMillan RP, et al. Early skeletal muscle adaptations to short-term high-fat diet in humans before changes in insulin sensitivity. *Obes Silver Spring Md*. 2015;23(4):720-724. doi:10.1002/oby.21031

20. Anderson EJ, Lustig ME, Boyle KE, et al. Mitochondrial H2O2 emission and cellular redox state link excess fat intake to insulin resistance in both rodents and humans. *J Clin Invest*. 2009;119(3):573-581. doi:10.1172/JCI37048

21. Smith SR, de Jonge L, Zachwieja JJ, et al. Fat and carbohydrate balances during adaptation to a high-fat. *Am J Clin Nutr*. 2000;71(2):450-457. doi:10.1093/ajcn/71.2.450

22. Argilés JM, Campos N, Lopez-Pedrosa JM, Rueda R, Rodriguez-Mañas L. Skeletal Muscle Regulates Metabolism via Interorgan Crosstalk: Roles in Health and Disease. *J Am Med Dir Assoc*. 2016;17(9):789-796. doi:10.1016/j.jamda.2016.04.019

23. Merz KE, Thurmond DC. Role of Skeletal Muscle in Insulin Resistance and Glucose Uptake. *Compr Physiol*. 2020;10(3):785-809. doi:10.1002/cphy.c190029

24. Sylow L, Tokarz VL, Richter EA, Klip A. The many actions of insulin in skeletal muscle, the paramount tissue determining glycemia. *Cell Metab*. 2021;33(4):758-780. doi:10.1016/j.cmet.2021.03.020

25. Richter-Stretton GL, Fenning AS, Vella RK. Skeletal muscle - A bystander or influencer of metabolic syndrome? *Diabetes Metab Syndr*. 2020;14(5):867-875. doi:10.1016/j.dsx.2020.06.006

26. Frontera WR, Ochala J. Skeletal muscle: a brief review of structure and function. *Calcif Tissue Int*. 2015;96(3):183-195. doi:10.1007/s00223-014-9915-y

27. Tomlinson DJ, Erskine RM, Morse CI, Winwood K, Onambélé-Pearson G. The impact of obesity on skeletal muscle strength and structure through adolescence to old age. *Biogerontology*. 2016;17(3):467-483. doi:10.1007/s10522-015-9626-4

28. Flück M, Hoppeler H. Molecular basis of skeletal muscle plasticity--from gene to form and function. *Rev Physiol Biochem Pharmacol*. 2003;146:159-216. doi:10.1007/s10254-002-0004-7

29. Nielsen J, Gejl KD, Hey-Mogensen M, et al. Plasticity in mitochondrial cristae density allows metabolic capacity modulation in human skeletal muscle. *J Physiol*. 2017;595(9):2839-2847. doi:10.1113/JP273040

30. Gram M, Dahl R, Dela F. Physical inactivity and muscle oxidative capacity in humans. *Eur J Sport Sci*. 2014;14(4):376-383. doi:10.1080/17461391.2013.823466

31. Delpino FM, Dos Santos Rodrigues AP, Petarli GB, et al. Overweight, obesity and risk of multimorbidity: A systematic review and meta-analysis of longitudinal studies. *Obes Rev Off J Int Assoc Study Obes*. 2023;24(6):e13562. doi:10.1111/obr.13562

32. Bogers RP, Bemelmans WJE, Hoogenveen RT, et al. Association of overweight with increased risk of coronary heart disease partly independent of blood pressure and cholesterol levels: a meta-analysis of 21 cohort studies including more than 300 000 persons. *Arch Intern Med*. 2007;167(16):1720-1728. doi:10.1001/archinte.167.16.1720

33. Meigs JB, Wilson PWF, Fox CS, et al. Body Mass Index, Metabolic Syndrome, and Risk of Type 2 Diabetes or Cardiovascular Disease. *J Clin Endocrinol Metab*. 2006;91(8):2906-2912. doi:10.1210/jc.2006-0594

34. Livesey G, Elia M. Estimation of energy expenditure, net carbohydrate utilization, and net fat oxidation and synthesis by indirect calorimetry: evaluation of errors with special reference to the detailed composition of fuels. *Am J Clin Nutr*. 1988;47(4):608-628. doi:10.1093/ajcn/47.4.608

35. Kelley DE, Goodpaster B, Wing RR, Simoneau JA. Skeletal muscle fatty acid metabolism in association with insulin resistance, obesity, and weight loss. *Am J Physiol*. 1999;277(6):E1130-1141. doi:10.1152/ajpendo.1999.277.6.E1130

36. Kelley DE, Mandarino LJ. Hyperglycemia normalizes insulin-stimulated skeletal muscle glucose oxidation and storage in noninsulin-dependent diabetes mellitus. *J Clin Invest*. 1990;86(6):1999-2007. doi:10.1172/JCI114935

37. Wohl P, Wohl P, Girman P, Pelikánová T. Inflexibility of energy substrate oxidation in type 1 diabetic patients. *Metabolism*. 2004;53(5):655-659. doi:10.1016/j.metabol.2003.12.013

38. Galgani JE, Heilbronn LK, Azuma K, et al. Metabolic flexibility in response to glucose is not impaired in people with type 2 diabetes after controlling for glucose disposal rate. *Diabetes*. 2008;57(4):841-845. doi:10.2337/db08-0043

39. van de Weijer T, Sparks LM, Phielix E, et al. Relationships between mitochondrial function and metabolic flexibility in type 2 diabetes mellitus. *PloS One*. 2013;8(2):e51648. doi:10.1371/journal.pone.0051648

40. Yki-Järvinen H, Sahlin K, Ren JM, Koivisto VA. Localization of rate-limiting defect for glucose disposal in skeletal muscle of insulin-resistant type I diabetic patients. *Diabetes*. 1990;39(2):157-167. doi:10.2337/diab.39.2.157

41. Thyfault JP, Kraus RM, Hickner RC, Howell AW, Wolfe RR, Dohm GL. Impaired plasma fatty acid oxidation in extremely obese women. *Am J Physiol Endocrinol Metab*. 2004;287(6):E1076-1081. doi:10.1152/ajpendo.00177.2004

42. Mengeste AM, Rustan AC, Lund J. Skeletal muscle energy metabolism in obesity. *Obes Silver Spring Md*. 2021;29(10):1582-1595. doi:10.1002/oby.23227

43. Hulver MW, Berggren JR, Carper MJ, et al. Elevated stearoyl-CoA desaturase-1 expression in skeletal muscle contributes to abnormal fatty acid partitioning in obese humans. *Cell Metab*. 2005;2(4):251-261. doi:10.1016/j.cmet.2005.09.002

44. Lundsgaard AM, Holm JB, Sjøberg KA, et al. Mechanisms Preserving Insulin Action during High Dietary Fat Intake. *Cell Metab*. 2019;29(1):50-63.e4. doi:10.1016/j.cmet.2018.08.022

45. Fritzen AM, Lundsgaard AM, Kiens B. Tuning fatty acid oxidation in skeletal muscle with dietary fat and exercise. *Nat Rev Endocrinol*. 2020;16(12):683-696. doi:10.1038/s41574-020-0405-1

46. Thomas CD, Peters JC, Reed GW, Abumrad NN, Sun M, Hill JO. Nutrient balance and energy expenditure during ad libitum feeding of high-fat and high-carbohydrate diets in humans. *Am J Clin Nutr*. 1992;55(5):934-942. doi:10.1093/ajcn/55.5.934

47. Begaye B, Vinales KL, Hollstein T, et al. Impaired Metabolic Flexibility to High-Fat Overfeeding Predicts Future Weight Gain in Healthy Adults. *Diabetes*. 2020;69(2):181-192. doi:10.2337/db19-0719

48. Zurlo F, Lillioja S, Esposito-Del Puente A, et al. Low ratio of fat to carbohydrate oxidation as predictor of weight gain: study of 24-h RQ. *Am J Physiol*. 1990;259(5 Pt 1):E650-657. doi:10.1152/ajpendo.1990.259.5.E650

49. Toubro S, Sørensen TI, Hindsberger C, Christensen NJ, Astrup A. Twenty-four-hour respiratory quotient: the role of diet and familial resemblance. *J Clin Endocrinol Metab*. 1998;83(8):2758-2764. doi:10.1210/jcem.83.8.5044

50. Battaglia GM, Zheng D, Hickner RC, Houmard JA. Effect of exercise training on metabolic flexibility in response to a high-fat diet in obese individuals. *Am J Physiol Endocrinol Metab*. 2012;303(12):E1440-1445. doi:10.1152/ajpendo.00355.2012

51. Bergouignan A, Gozansky WS, Barry DW, et al. Increasing dietary fat elicits similar changes in fat oxidation and markers of muscle oxidative capacity in lean and obese humans. *PloS One*. 2012;7(1):e30164. doi:10.1371/journal.pone.0030164

52. Centers for Disease Control and Prevention. Dietary intake for adults aged 20 and older. Published December 27, 2023. Accessed May 8, 2024. https://www.cdc.gov/nchs/fastats/diet.htm

53. Astrup A, Buemann B, Christensen NJ, Toubro S. Failure to increase lipid oxidation in response to increasing dietary fat content in formerly obese women. *Am J Physiol*. 1994;266(4 Pt 1):E592-599. doi:10.1152/ajpendo.1994.266.4.E592

54. Blaak EE, Hul G, Verdich C, et al. Fat oxidation before and after a high fat load in the obese insulin-resistant state. *J Clin Endocrinol Metab*. 2006;91(4):1462-1469. doi:10.1210/jc.2005-1598

55. Lithell H, Jacobs I, Vessby B, Hellsing K, Karlsson J. Decrease of lipoprotein lipase activity in skeletal muscle in man during a short-term carbohydrate-rich dietary regime. With special reference to HDL-cholesterol, apolipoprotein and insulin concentrations. *Metabolism*. 1982;31(10):994-998. doi:10.1016/0026-0495(82)90141-x

56. Jordy AB, Serup AK, Karstoft K, Pilegaard H, Kiens B, Jeppesen J. Insulin sensitivity is independent of lipid binding protein trafficking at the plasma membrane in human skeletal muscle: effect of a 3-day, high-fat diet. *Am J Physiol Regul Integr Comp Physiol*. 2014;307(9):R1136-1145. doi:10.1152/ajpregu.00124.2014

57. Lundsgaard AM, Sjøberg KA, Høeg LD, et al. Opposite Regulation of Insulin Sensitivity by Dietary Lipid Versus Carbohydrate Excess. *Diabetes*. 2017;66(10):2583-2595. doi:10.2337/db17-0046

58. Fritzen AM, Lundsgaard AM, Jeppesen JF, et al. Fatty acid type-specific regulation of SIRT1 does not affect insulin sensitivity in human skeletal muscle. *FASEB J Off Publ Fed Am Soc Exp Biol*. 2019;33(4):5510-5519. doi:10.1096/fj.201801950R

59. Hurni M, Burnand B, Pittet P, Jequier E. Metabolic effects of a mixed and a high-carbohydrate low-fat diet in man, measured over 24 h in a respiration chamber. *Br J Nutr*. 1982;47(1):33-43. doi:10.1079/bjn19820006

60. McNeill G, Bruce A, Ralph A, James W. Inter-individual differences in fasting nutrient oxidation and the influence of diet composition. *Int J Obes*. 1988;12(5). Accessed May 9, 2024. https://pubmed.ncbi.nlm.nih.gov/3235264/

61. Schutz Y. The adjustment of energy expenditure and oxidation to energy intake: the role of carbohydrate and fat balance. *Int J Obes Relat Metab Disord J Int Assoc Study Obes*. 1993;17 Suppl 3:S23-27; discussion S41-42.

62. Chow LS, Seaquist ER, Eberly LE, et al. Acute free fatty acid elevation eliminates endurance training effect on insulin sensitivity. *J Clin Endocrinol Metab*. 2012;97(8):2890-2897. doi:10.1210/jc.2012-1515

63. Itani SI, Ruderman NB, Schmieder F, Boden G. Lipid-induced insulin resistance in human muscle is associated with changes in diacylglycerol, protein kinase C, and IkappaB-alpha. *Diabetes*. 2002;51(7):2005-2011. doi:10.2337/diabetes.51.7.2005

64. Phielix E, Meex R, Ouwens DM, et al. High oxidative capacity due to chronic exercise training attenuates lipid-induced insulin resistance. *Diabetes*. 2012;61(10):2472-2478. doi:10.2337/db11-1832

65. Dubé JJ, Coen PM, DiStefano G, et al. Effects of acute lipid overload on skeletal muscle insulin resistance, metabolic flexibility, and mitochondrial performance. *Am J Physiol - Endocrinol Metab*. 2014;307(12):E1117-E1124. doi:10.1152/ajpendo.00257.2014

66. Fisher-Wellman KH, Weber TM, Cathey BL, et al. Mitochondrial respiratory capacity and content are normal in young insulin-resistant obese humans. *Diabetes*. 2014;63(1):132-141. doi:10.2337/db13-0940

67. Bruls YMH, Op den Kamp YJM, Phielix E, et al. L-carnitine infusion does not alleviate lipid-induced insulin resistance and metabolic inflexibility. *PloS One*. 2020;15(9):e0239506. doi:10.1371/journal.pone.0239506

68. Meex RCR, Phielix E, Moonen-Kornips E, Schrauwen P, Hesselink MKC. Stimulation of human whole-body energy expenditure by salsalate is fueled by higher lipid oxidation under fasting conditions and by higher oxidative glucose disposal under insulin-stimulated conditions. *J Clin Endocrinol Metab*. 2011;96(5):1415-1423. doi:10.1210/jc.2010-1816

69. Gaster M. Metabolic flexibility is conserved in diabetic myotubes. *J Lipid Res*. 2007;48(1):207-217. doi:10.1194/jlr.M600319-JLR200

70. Gaster M, Kristensen SR, Beck-Nielsen H, Schrøder HD. A cellular model system of differentiated human myotubes. *APMIS Acta Pathol Microbiol Immunol Scand*. 2001;109(11):735-744. doi:10.1034/j.1600-0463.2001.d01-140.x

71. Gaster M. Reduced lipid oxidation in myotubes established from obese and type 2 diabetic subjects. *Biochem Biophys Res Commun*. 2009;382(4):766-770. doi:10.1016/j.bbrc.2009.03.102

72. Zou K, Turner K, Zheng D, et al. Impaired glucose partitioning in primary myotubes from severely obese women with type 2 diabetes. *Am J Physiol Cell Physiol*. 2020;319(6):C1011-C1019. doi:10.1152/ajpcell.00157.2020

73. Zou K, Hinkley JM, Park S, et al. Altered tricarboxylic acid cycle flux in primary myotubes from severely obese humans. *Int J Obes 2005*. 2019;43(4):895-905. doi:10.1038/s41366-018-0137-7

74. Kugler BA, Gundersen AE, Li J, et al. Roux-en-Y gastric bypass surgery restores insulin-mediated glucose partitioning and mitochondrial dynamics in primary myotubes from severely obese humans. *Int J Obes 2005*. 2020;44(3):684-696. doi:10.1038/s41366-019-0469-y

75. Hinkley JM, Zou K, Park S, Turner K, Zheng D, Houmard JA. Roux-en-Y gastric bypass surgery enhances contraction-mediated glucose metabolism in primary human myotubes. *Am J Physiol Endocrinol Metab*. 2017;313(2):E195-E202. doi:10.1152/ajpendo.00413.2016

76. Park S, Jevtovic F, Krassovskaia PM, et al. Effect of resveratrol on insulin action in primary myotubes from lean individuals and individuals with severe obesity. *Am J Physiol Endocrinol Metab*. 2024;326(3):E398-E406. doi:10.1152/ajpendo.00299.2023

77. Huang TY, Zheng D, Houmard JA, Brault JJ, Hickner RC, Cortright RN. Overexpression of PGC-1α increases peroxisomal activity and mitochondrial fatty acid oxidation in human primary myotubes. *Am J Physiol Endocrinol Metab*. 2017;312(4):E253-E263. doi:10.1152/ajpendo.00331.2016

78. Sparks LM, Moro C, Ukropcova B, et al. Remodeling lipid metabolism and improving insulin responsiveness in human primary myotubes. *PloS One*. 2011;6(7):e21068. doi:10.1371/journal.pone.0021068

79. Boyle KE, Zheng D, Anderson EJ, Neufer PD, Houmard JA. Mitochondrial lipid oxidation is impaired in cultured myotubes from obese humans. *Int J Obes 2005*. 2012;36(8):1025-1031. doi:10.1038/ijo.2011.201

80. Corpeleijn E, Hessvik NP, Bakke SS, et al. Oxidation of intramyocellular lipids is dependent on mitochondrial function and the availability of extracellular fatty acids. *Am J Physiol Endocrinol Metab*. 2010;299(1):E14-22. doi:10.1152/ajpendo.00187.2010

81. Qaisar R, Bhaskaran S, Van Remmen H. Muscle fiber type diversification during exercise and regeneration. *Free Radic Biol Med*. 2016;98:56-67. doi:10.1016/j.freeradbiomed.2016.03.025

82. Schiaffino S, Reggiani C. Fiber types in mammalian skeletal muscles. *Physiol Rev*. 2011;91(4):1447-1531. doi:10.1152/physrev.00031.2010

83. Zierath JR, He L, Gumà A, Odegoard Wahlström E, Klip A, Wallberg-Henriksson H. Insulin action on glucose transport and plasma membrane GLUT4 content in skeletal muscle from patients with NIDDM. *Diabetologia*. 1996;39(10):1180-1189. doi:10.1007/BF02658504

84. Serrano N, Hyatt JPK, Houmard JA, Murgia M, Katsanos CS. Muscle fiber phenotype: a culprit of abnormal metabolism and function in skeletal muscle of humans with obesity. *Am J Physiol Endocrinol Metab*. 2023;325(6):E723-E733. doi:10.1152/ajpendo.00190.2023

85. Tanner CJ, Barakat HA, Dohm GL, et al. Muscle fiber type is associated with obesity and weight loss. *Am J Physiol Endocrinol Metab*. 2002;282(6):E1191-1196. doi:10.1152/ajpendo.00416.2001

86. Hickey MS, Carey JO, Azevedo JL, et al. Skeletal muscle fiber composition is related to adiposity and in vitro glucose transport rate in humans. *Am J Physiol*. 1995;268(3 Pt 1):E453-457. doi:10.1152/ajpendo.1995.268.3.E453

87. Helge JW, Fraser AM, Kriketos AD, et al. Interrelationships between muscle fibre type, substrate oxidation and body fat. *Int J Obes Relat Metab Disord J Int Assoc Study Obes*. 1999;23(9):986-991. doi:10.1038/sj.ijo.0801030

88. Simoneau JA, Bouchard C. Human variation in skeletal muscle fiber-type proportion and enzyme activities. *Am J Physiol*. 1989;257(4 Pt 1):E567-572. doi:10.1152/ajpendo.1989.257.4.E567

89. Bonnard C, Durand A, Peyrol S, et al. Mitochondrial dysfunction results from oxidative stress in the skeletal muscle of diet-induced insulin-resistant mice. *J Clin Invest*. 2008;118(2):789-800. doi:10.1172/JCI32601

90. Lowell BB, Shulman GI. Mitochondrial dysfunction and type 2 diabetes. *Science*. 2005;307(5708):384-387. doi:10.1126/science.1104343

91. Axelrod CL, Fealy CE, Erickson ML, et al. Lipids activate skeletal muscle mitochondrial fission and quality control networks to induce insulin resistance in humans. *Metabolism*. 2021;121:154803. doi:10.1016/j.metabol.2021.154803

92. Brands M, Hoeks J, Sauerwein HP, et al. Short-term increase of plasma free fatty acids does not interfere with intrinsic mitochondrial function in healthy young men. *Metabolism*. 2011;60(10):1398-1405. doi:10.1016/j.metabol.2011.02.006

93. Chavez AO, Kamath S, Jani R, et al. Effect of Short-Term Free Fatty Acids Elevation on Mitochondrial Function in Skeletal Muscle of Healthy Individuals. *J Clin Endocrinol Metab*. 2010;95(1):422-429. doi:10.1210/jc.2009-1387

94. Wardle SL, Macnaughton LS, McGlory C, et al. Human skeletal muscle metabolic responses to 6 days of high-fat overfeeding are associated with dietary n-3PUFA content and muscle oxidative capacity. *Physiol Rep*. 2020;8(16):e14529. doi:10.14814/phy2.14529

95. Barazzoni R, Zanetti M, Gortan Cappellari G, et al. Fatty acids acutely enhance insulin-induced oxidative stress and cause insulin resistance by increasing mitochondrial reactive oxygen species (ROS) generation and nuclear factor-κB inhibitor (IκB)-nuclear factor-κB (NFκB) activation in rat muscle, in the absence of mitochondrial dysfunction. *Diabetologia*. 2012;55(3):773-782. doi:10.1007/s00125-011-2396-x

96. Alway SE, Mohamed JS, Myers MJ. Mitochondria Initiate and Regulate Sarcopenia. *Exerc Sport Sci Rev*. 2017;45(2):58-69. doi:10.1249/JES.0000000000000101

97. Forman HJ, Maiorino M, Ursini F. Signaling functions of reactive oxygen species. *Biochemistry*. 2010;49(5):835-842. doi:10.1021/bi9020378

98. Jones DP. Radical-free biology of oxidative stress. *Am J Physiol Cell Physiol*. 2008;295(4):C849-868. doi:10.1152/ajpcell.00283.2008

99. Liang H, Ward WF. PGC-1alpha: a key regulator of energy metabolism. *Adv Physiol Educ*. 2006;30(4):145-151. doi:10.1152/advan.00052.2006

100. Koves TR, Li P, An J, et al. Peroxisome proliferator-activated receptor-gamma co-activator 1alpha-mediated metabolic remodeling of skeletal myocytes mimics exercise training and reverses lipid-induced mitochondrial inefficiency. *J Biol Chem*. 2005;280(39):33588-33598. doi:10.1074/jbc.M507621200

101. Sparks LM, Xie H, Koza RA, et al. A high-fat diet coordinately downregulates genes required for mitochondrial oxidative phosphorylation in skeletal muscle. *Diabetes*. 2005;54(7):1926-1933. doi:10.2337/diabetes.54.7.1926

102. Mootha VK, Lindgren CM, Eriksson KF, et al. PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet*. 2003;34(3):267-273. doi:10.1038/ng1180

103. Kelley DE, Simoneau JA. Impaired free fatty acid utilization by skeletal muscle in non-insulin-dependent diabetes mellitus. *J Clin Invest*. 1994;94(6):2349-2356.

104. Palmer BF, Clegg DJ. Metabolic Flexibility and Its Impact on Health Outcomes. *Mayo Clin Proc*. 2022;97(4):761-776. doi:10.1016/j.mayocp.2022.01.012

105. Galgani JE, Moro C, Ravussin E. Metabolic flexibility and insulin resistance. *Am J Physiol Endocrinol Metab*. 2008;295(5):E1009-1017. doi:10.1152/ajpendo.90558.2008

106. Ukropcova B, Sereda O, de Jonge L, et al. Family history of diabetes links impaired substrate switching and reduced mitochondrial content in skeletal muscle. *Diabetes*. 2007;56(3):720-727. doi:10.2337/db06-0521

107. DeFronzo RA, Tobin JD, Andres R. Glucose clamp technique: a method for quantifying insulin secretion and resistance. *Am J Physiol*. 1979;237(3):E214-223. doi:10.1152/ajpendo.1979.237.3.E214

108. Berggren JR, Boyle KE, Chapman WH, Houmard JA. Skeletal muscle lipid oxidation and obesity: influence of weight loss and exercise. *Am J Physiol Endocrinol Metab*. 2008;294(4):E726-732. doi:10.1152/ajpendo.00354.2007

109. Cortright RN, Sandhoff KM, Basilio JL, et al. Skeletal muscle fat oxidation is increased in African-American and white women after 10 days of endurance exercise training. *Obes Silver Spring Md*. 2006;14(7):1201-1210. doi:10.1038/oby.2006.137

110. McLaughlin KL, Hagen JT, Coalson HS, et al. Novel approach to quantify mitochondrial content and intrinsic bioenergetic efficiency across organs. *Sci Rep*. 2020;10(1):17599. doi:10.1038/s41598-020-74718-1

111. Schrauwen P, Hoppeler H, Billeter R, Bakker AH, Pendergast DR. Fiber type dependent upregulation of human skeletal muscle UCP2 and UCP3 mRNA expression by high-fat diet. *Int J Obes Relat Metab Disord J Int Assoc Study Obes*. 2001;25(4):449-456. doi:10.1038/sj.ijo.0801566

112. Skovbro M, Boushel R, Hansen CN, Helge JW, Dela F. High-fat feeding inhibits exercise-induced increase in mitochondrial respiratory flux in skeletal muscle. *J Appl Physiol Bethesda Md 1985*. 2011;110(6):1607-1614. doi:10.1152/japplphysiol.01341.2010

113. Leckey JJ, Hoffman NJ, Parr EB, et al. High dietary fat intake increases fat oxidation and reduces skeletal muscle mitochondrial respiration in trained humans. *FASEB J Off Publ Fed Am Soc Exp Biol*. 2018;32(6):2979-2991. doi:10.1096/fj.201700993R

114. Kelley DE, He J, Menshikova EV, Ritov VB. Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. *Diabetes*. 2002;51(10):2944-2950. doi:10.2337/diabetes.51.10.2944

115. Ritov VB, Menshikova EV, Azuma K, et al. Deficiency of electron transport chain in human skeletal muscle mitochondria in type 2 diabetes mellitus and obesity. *Am J Physiol Endocrinol Metab*. 2010;298(1):E49-58. doi:10.1152/ajpendo.00317.2009

116. Petersen KF, Dufour S, Befroy D, Garcia R, Shulman GI. Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes. *N Engl J Med*. 2004;350(7):664-671. doi:10.1056/NEJMoa031314

117. McDougal DH, Marlatt KL, Beyl RA, Redman LM, Ravussin E. A Novel Approach to Assess Metabolic Flexibility Overnight in a Whole-Body Room Calorimeter. *Obes Silver Spring Md*. 2020;28(11):2073-2077. doi:10.1002/oby.22982

118. Newcomer BR, Larson-Meyer DE, Hunter GR, Weinsier RL. Skeletal muscle metabolism in overweight and post-overweight women: an isometric exercise study using (31)P magnetic resonance spectroscopy. *Int J Obes Relat Metab Disord J Int Assoc Study Obes*. 2001;25(9):1309-1315. doi:10.1038/sj.ijo.0801673

119. Fleischman A, Kron M, Systrom DM, Hrovat M, Grinspoon SK. Mitochondrial function and insulin resistance in overweight and normal-weight children. *J Clin Endocrinol Metab*. 2009;94(12):4923-4930. doi:10.1210/jc.2009-1590

120. Chu L, Riddell MC, Schneiderman JE, McCrindle BW, Hamilton JK. The effect of puberty on fat oxidation rates during exercise in overweight and normal-weight girls. *J Appl Physiol Bethesda Md 1985*. 2014;116(1):76-82. doi:10.1152/japplphysiol.00888.2013

121. Ryan TE, Brophy P, Lin CT, Hickner RC, Neufer PD. Assessment of in vivo skeletal muscle mitochondrial respiratory capacity in humans by near-infrared spectroscopy: a comparison with in situ measurements. *J Physiol*. 2014;592(15):3231-3241. doi:10.1113/jphysiol.2014.274456

122. Pileggi CA, Hooks BG, McPherson R, Dent RRM, Harper ME. Targeting skeletal muscle mitochondrial health in obesity. *Clin Sci Lond Engl 1979*. 2022;136(14):1081-1110. doi:10.1042/CS20210506

123. Kriketos AD, Pan DA, Lillioja S, et al. Interrelationships between muscle morphology, insulin action, and adiposity. *Am J Physiol*. 1996;270(6 Pt 2):R1332-1339. doi:10.1152/ajpregu.1996.270.6.R1332

124. Methenitis S, Nomikos T, Kontou E, et al. Skeletal muscle fiber composition may modify the effect of nutrition on body composition in young females. *Nutr Metab Cardiovasc Dis NMCD*. 2023;33(4):817-825. doi:10.1016/j.numecd.2022.12.027

125. Gerrits MF, Ghosh S, Kavaslar N, et al. Distinct skeletal muscle fiber characteristics and gene expression in diet-sensitive versus diet-resistant obesity. *J Lipid Res*. 2010;51(8):2394-2404. doi:10.1194/jlr.P005298

126. Russell RD, Roberts-Thomson KM, Hu D, et al. Impaired postprandial skeletal muscle vascular responses to a mixed meal challenge in normoglycaemic people with a parent with type 2 diabetes. *Diabetologia*. 2022;65(1):216-225. doi:10.1007/s00125-021-05572-7

127. Russell RD, Kraemer RR, Nelson AG. Metabolic dysfunction in diabetic offspring: deviations in metabolic flexibility. *Med Sci Sports Exerc*. 2013;45(1):8-15. doi:10.1249/MSS.0b013e31826909d3

128. Allerton TD, Irving BA, Spielmann G, et al. Metabolic flexibility is impaired in response to acute exercise in the young offspring of mothers with type 2 diabetes. *Physiol Rep*. 2019;7(17):e14189. doi:10.14814/phy2.14189

129. Turner N, Kowalski GM, Leslie SJ, et al. Distinct patterns of tissue-specific lipid accumulation during the induction of insulin resistance in mice by high-fat feeding. *Diabetologia*. 2013;56(7):1638-1648. doi:10.1007/s00125-013-2913-1

130. Smith SR, de Jonge L, Zachwieja JJ, et al. Fat and carbohydrate balances during adaptation to a high-fat. *Am J Clin Nutr*. 2000;71(2):450-457. doi:10.1093/ajcn/71.2.450

131. Bourlier V, Saint-Laurent C, Louche K, et al. Enhanced glucose metabolism is preserved in cultured primary myotubes from obese donors in response to exercise training. *J Clin Endocrinol Metab*. 2013;98(9):3739-3747. doi:10.1210/jc.2013-1727

132. Bikman BT, Zheng D, Reed MA, Hickner RC, Houmard JA, Dohm GL. Lipid-induced insulin resistance is prevented in lean and obese myotubes by AICAR treatment. *Am J Physiol Regul Integr Comp Physiol*. 2010;298(6):R1692-1699. doi:10.1152/ajpregu.00190.2009

133. Choi JW, Ohn JH, Jung HS, et al. Carnitine induces autophagy and restores high-fat diet-induced mitochondrial dysfunction. *Metabolism*. 2018;78:43-51. doi:10.1016/j.metabol.2017.09.005

134. Heo JW, No MH, Cho J, et al. Moderate aerobic exercise training ameliorates impairment of mitochondrial function and dynamics in skeletal muscle of high-fat diet-induced obese mice. *FASEB J Off Publ Fed Am Soc Exp Biol*. 2021;35(2):e21340. doi:10.1096/fj.202002394R

135. Kyriazis ID, Vassi E, Alvanou M, et al. The impact of diet upon mitochondrial physiology (Review). *Int J Mol Med*. 2022;50(5):135. doi:10.3892/ijmm.2022.5191

136. Park LM, Lannigan J, Jaimes MC. OMIP-069: Forty-Color Full Spectrum Flow Cytometry Panel for Deep Immunophenotyping of Major Cell Subsets in Human Peripheral Blood. *Cytom Part J Int Soc Anal Cytol*. 2020;97(10):1044-1051. doi:10.1002/cyto.a.24213

137. Chaves A, Weyrauch LA, Zheng D, et al. Influence of Maternal Exercise on Glucose and Lipid Metabolism in Offspring Stem Cells: ENHANCED by Mom. *J Clin Endocrinol Metab*. 2022;107(8):e3353-e3365. doi:10.1210/clinem/dgac270

138. Park S, Turner KD, Zheng D, et al. Electrical pulse stimulation induces differential responses in insulin action in myotubes from severely obese individuals. *J Physiol*. 2019;597(2):449-466. doi:10.1113/JP276990

139. Capkovic KL, Stevenson S, Johnson MC, Thelen JJ, Cornelison D. Neural cell adhesion molecule (NCAM) marks adult myogenic cells committed to differentiation. *Exp Cell Res*. 2008;314(7):1553-1565. doi:10.1016/j.yexcr.2008.01.021

140. Løvsletten NG, Rustan AC, Laurens C, Thoresen GH, Moro C, Nikolić N. Primary defects in lipid handling and resistance to exercise in myotubes from obese donors with and without type 2 diabetes. *Appl Physiol Nutr Metab Physiol Appl Nutr Metab*. 2020;45(2):169-179. doi:10.1139/apnm-2019-0265

141. Peters SJ, Harris RA, Wu P, Pehleman TL, Heigenhauser GJ, Spriet LL. Human skeletal muscle PDH kinase activity and isoform expression during a 3-day high-fat/low-carbohydrate diet. *Am J Physiol Endocrinol Metab*. 2001;281(6):E1151-1158. doi:10.1152/ajpendo.2001.281.6.E1151

142. Draznin B, Wang C, Adochio R, Leitner JW, Cornier MA. Effect of dietary macronutrient composition on AMPK and SIRT1 expression and activity in human skeletal muscle. *Horm Metab Res Horm Stoffwechselforschung Horm Metab*. 2012;44(9):650-655. doi:10.1055/s-0032-1312656

143. Goedecke JH, Christie C, Wilson G, et al. Metabolic adaptations to a high-fat diet in endurance cyclists. *Metabolism*. 1999;48(12):1509-1517. doi:10.1016/s0026-0495(99)90238-x

144. Kiens B, Essen-Gustavsson B, Gad P, Lithell H. Lipoprotein lipase activity and intramuscular triglyceride stores after long-term high-fat and high-carbohydrate diets in physically trained men. *Clin Physiol Oxf Engl*. 1987;7(1):1-9. doi:10.1111/j.1475-097x.1987.tb00628.x

145. Helge JW, Bentley D, Schjerling P, et al. Four weeks one-leg training and high fat diet does not alter PPARalpha protein or mRNA expression in human skeletal muscle. *Eur J Appl Physiol*. 2007;101(1):105-114. doi:10.1007/s00421-007-0479-7

146. Hübinger A, Knode O, Susanto F, Reinauer H, Gries FA. Effects of the carnitine-acyltransferase inhibitor etomoxir on insulin sensitivity, energy expenditure and substrate oxidation in NIDDM. *Horm Metab Res Horm Stoffwechselforschung Horm Metab*. 1997;29(9):436-439. doi:10.1055/s-2007-979072

147. Wicks SE, Vandanmagsar B, Haynie KR, et al. Impaired mitochondrial fat oxidation induces adaptive remodeling of muscle metabolism. *Proc Natl Acad Sci*. 2015;112(25):E3300-E3309. doi:10.1073/pnas.1418560112

148. Keung W, Ussher JR, Jaswal JS, et al. Inhibition of carnitine palmitoyltransferase-1 activity alleviates insulin resistance in diet-induced obese mice. *Diabetes*. 2013;62(3):711-720. doi:10.2337/db12-0259

149. Timmers S, Nabben M, Bosma M, et al. Augmenting muscle diacylglycerol and triacylglycerol content by blocking fatty acid oxidation does not impede insulin sensitivity. *Proc Natl Acad Sci U S A*. 2012;109(29):11711-11716. doi:10.1073/pnas.1206868109

150. Pereyra AS, Rajan A, Ferreira CR, Ellis JM. Loss of Muscle Carnitine Palmitoyltransferase 2 Prevents Diet-Induced Obesity and Insulin Resistance despite Long-Chain Acylcarnitine Accumulation. *Cell Rep*. 2020;33(6):108374. doi:10.1016/j.celrep.2020.108374

151. Vozarova B, Stefan N, Lindsay RS, et al. High alanine aminotransferase is associated with decreased hepatic insulin sensitivity and predicts the development of type 2 diabetes. *Diabetes*. 2002;51(6):1889-1895. doi:10.2337/diabetes.51.6.1889

152. Lundsgaard AM, Fritzen AM, Nicolaisen TS, et al. Glucometabolic consequences of acute and prolonged inhibition of fatty acid oxidation. *J Lipid Res*. 2020;61(1):10-19. doi:10.1194/jlr.RA119000177

153. Koves TR, Ussher JR, Noland RC, et al. Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. *Cell Metab*. 2008;7(1):45-56. doi:10.1016/j.cmet.2007.10.013

154. Watt MJ, Hevener AL. Fluxing the mitochondria to insulin resistance. *Cell Metab*. 2008;7(1):5-6. doi:10.1016/j.cmet.2007.12.002

155. Dobbins RL, Szczepaniak LS, Bentley B, Esser V, Myhill J, McGarry JD. Prolonged inhibition of muscle carnitine palmitoyltransferase-1 promotes intramyocellular lipid accumulation and insulin resistance in rats. *Diabetes*. 2001;50(1):123-130. doi:10.2337/diabetes.50.1.123

156. Perseghin G, Scifo P, De Cobelli F, et al. Intramyocellular triglyceride content is a determinant of in vivo insulin resistance in humans: a 1H-13C nuclear magnetic resonance spectroscopy assessment in offspring of type 2 diabetic parents. *Diabetes*. 1999;48(8):1600-1606. doi:10.2337/diabetes.48.8.1600

157. Krssak M, Falk Petersen K, Dresner A, et al. Intramyocellular lipid concentrations are correlated with insulin sensitivity in humans: a 1H NMR spectroscopy study. *Diabetologia*. 1999;42(1):113-116. doi:10.1007/s001250051123

158. Jacob S, Machann J, Rett K, et al. Association of increased intramyocellular lipid content with insulin resistance in lean nondiabetic offspring of type 2 diabetic subjects. *Diabetes*. 1999;48(5):1113-1119. doi:10.2337/diabetes.48.5.1113

159. Chokkalingam K, Jewell K, Norton L, et al. High-fat/low-carbohydrate diet reduces insulin-stimulated carbohydrate oxidation but stimulates nonoxidative glucose disposal in humans: An important role for skeletal muscle pyruvate dehydrogenase kinase 4. *J Clin Endocrinol Metab*. 2007;92(1):284-292. doi:10.1210/jc.2006-1592

160. Allick G, Bisschop PH, Ackermans MT, et al. A low-carbohydrate/high-fat diet improves glucoregulation in type 2 diabetes mellitus by reducing postabsorptive glycogenolysis. *J Clin Endocrinol Metab*. 2004;89(12):6193-6197. doi:10.1210/jc.2004-1041

161. Bisschop PH, de Metz J, Ackermans MT, et al. Dietary fat content alters insulin-mediated glucose metabolism in healthy men. *Am J Clin Nutr*. 2001;73(3):554-559. doi:10.1093/ajcn/73.3.554

162. Cutler DL, Gray CG, Park SW, Hickman MG, Bell JM, Kolterman OG. Low-carbohydrate diet alters intracellular glucose metabolism but not overall glucose disposal in exercise-trained subjects. *Metabolism*. 1995;44(10):1264-1270. doi:10.1016/0026-0495(95)90027-6

163. Yost TJ, Jensen DR, Haugen BR, Eckel RH. Effect of dietary macronutrient composition on tissue-specific lipoprotein lipase activity and insulin action in normal-weight subjects. *Am J Clin Nutr*. 1998;68(2):296-302. doi:10.1093/ajcn/68.2.296

164. Branis NM, Etesami M, Walker RW, Berk ES, Albu JB. Effect of a 1-week, eucaloric, moderately high-fat diet on peripheral insulin sensitivity in healthy premenopausal women. *BMJ Open Diabetes Res Care*. 2015;3(1):e000100. doi:10.1136/bmjdrc-2015-000100

165. von Frankenberg AD, Marina A, Song X, Callahan HS, Kratz M, Utzschneider KM. A high-fat, high-saturated fat diet decreases insulin sensitivity without changing intra-abdominal fat in weight-stable overweight and obese adults. *Eur J Nutr*. 2017;56(1):431-443. doi:10.1007/s00394-015-1108-6

166. van Herpen NA, Schrauwen-Hinderling VB, Schaart G, Mensink RP, Schrauwen P. Three weeks on a high-fat diet increases intrahepatic lipid accumulation and decreases metabolic flexibility in healthy overweight men. *J Clin Endocrinol Metab*. 2011;96(4):E691-695. doi:10.1210/jc.2010-2243

167. Stettler R, Ith M, Acheson KJ, et al. Interaction between dietary lipids and physical inactivity on insulin sensitivity and on intramyocellular lipids in healthy men. *Diabetes Care*. 2005;28(6):1404-1409. doi:10.2337/diacare.28.6.1404

168. Borkman M, Campbell LV, Chisholm DJ, Storlien LH. Comparison of the effects on insulin sensitivity of high carbohydrate and high fat diets in normal subjects. *J Clin Endocrinol Metab*. 1991;72(2):432-437. doi:10.1210/jcem-72-2-432

169. Rynders CA, Pereira RI, Bergouignan A, Kealey EH, Bessesen DH. Associations among dietary fat oxidation responses to overfeeding and weight gain in obesity-prone and resistant adults. *Obes Silver Spring Md*. 2018;26(11):1758-1766. doi:10.1002/oby.22321

170. Larson DE, Ferraro RT, Robertson DS, Ravussin E. Energy metabolism in weight-stable postobese individuals. *Am J Clin Nutr*. 1995;62(4):735-739. doi:10.1093/ajcn/62.4.735

171. Kleinert M, Parker BL, Jensen TE, et al. Quantitative proteomic characterization of cellular pathways associated with altered insulin sensitivity in skeletal muscle following high-fat diet feeding and exercise training. *Sci Rep*. 2018;8(1):10723. doi:10.1038/s41598-018-28540-5

172. Berggren JR, Hulver MW, Dohm GL, Houmard JA. Weight Loss and Exercise: Implications for Muscle Lipid Metabolism and Insulin Action. *Med Sci Sports Exerc*. 2004;36(7):1191. doi:10.1249/01.MSS.0000074670.03001.98

173. Muoio DM, Neufer PD. Lipid-induced mitochondrial stress and insulin action in muscle. *Cell Metab*. 2012;15(5):595-605. doi:10.1016/j.cmet.2012.04.010

174. Zou K, Hinkley JM, Park S, et al. Altered tricarboxylic acid cycle flux in primary myotubes from severely obese humans. *Int J Obes 2005*. 2019;43(4):895-905. doi:10.1038/s41366-018-0137-7

Appendix A: University and Medical Center Institutional Review Board (UMCIRB) Approval

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

Notification of Continuing Review Approval

From: Biomedical IRB
To: Joseph Houmard
CC: Elizabeth Gates
Date: 2/14/2024
Re: CR00010384
UMCIRB 19-000914
Metabolic inflexibility is related to elevated muscle anaerobic glycolysis

I am pleased to inform you that at the convened meeting on 2/14/2024 12:15 PM of the Biomedical IRB, this research study underwent a continuing review and the committee voted to approve the study. Approval of the study and the consent form(s) is for the period of 2/14/2024 to 2/13/2025.

The Biomedical IRB deemed this study Greater than Minimal Risk .

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

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

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

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

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

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

The approval includes the following items:

Document	Description
APPHoumard-Dohm_NIH_R01_12 (1).pdf(0.01)	Study Protocol or Grant Application
Flyer and email(0.07)	Recruitment Documents/Scripts
Flyer Canvas distribution(0.01)	Recruitment Documents/Scripts
Group 1 and 2 Information Sheet V.1.docx(0.02)	Recruitment Documents/Scripts
Group 3 Information Sheet V.3 1.20.22(0.03)	Recruitment Documents/Scripts

Document	Description
Health history 08.13.20.docx(0.02)	Surveys and Questionnaires
Met Flex ppt Script.docx(0.01)	Recruitment Documents/Scripts
MetFlex Bariatric Surgery ICF v.8 05.23.23-Clean.doc(0.17)	Consent Forms
MetFlex Main ICF v.9 05.23.23-Clean.doc(0.15)	Consent Forms
MetFlex Participant website.docx(0.01)	Recruitment Documents/Scripts
MetFlex Protocol v.4.0 02.17.22.docx(0.06)	Study Protocol or Grant Application
MetFlex Protocol v.5.0 07.26.22.docx(0.01)	Study Protocol or Grant Application
MetFlex Screening ICF v.8.0 05.23.23-Clean.docx(0.14)	Consent Forms
PARQPlus2019ImageVersion2.pdf(0.01)	Surveys and Questionnaires
Participant questionnaire 01.13.20.docx(0.01)	Surveys and Questionnaires
Phone screen final 08.13.20.docx(0.02)	Recruitment Documents/Scripts

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

The following UMCIRB members were recused for reasons of potential for Conflict of Interest on this research study: M.L. Pories and N. Broskey.

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