Effects of Exercise Training on Subcutaneous Adipose Tissue in Obese Humans

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Abstract

Subcutaneous adipose tissue (SAT) is a dynamic organ that has a tremendous ability to remodel in the face of increased energy intake. In obesity, alterations to SAT may lead to metabolic dysfunction and increased risk for type 2 diabetes (T2D). Decrements in SAT mitochondrial function and the adipogenic capacity of SAT have been identified in previous investigations in obese humans. However, exercise training has been shown to improve metabolic function partly due to changes in SAT function.

In this dissertation report we investigated the effects of an aerobic interval training (AIT) exercise intervention in obese humans and its ability to modulate SAT function. The first main objective of the study was to delineate the impact of AIT on the mitochondrial content and function in SAT. The second main objective was to characterize the effects of AIT on the adipogenic capacity of preadipocytes isolated from SAT.

AIT was effective at increasing complex I-linked mitochondrial oxygen consumption rates in lower but not upper body SAT independent of changes to mitochondrial content. Increased complex I-linked flux may indicate an increased ability to oxidize fuels that may provide cytoprotection to adipocytes. Additionally, AIT reduced

the adipogenic capacity in preadipocytes isolated from SAT. This finding may help explain the ability of exercise to prevent weight gain and weight regain after weight loss.

Effects of Exercise Training on Subcutaneous Adipose Tissue in Obese Humans

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List of Symbols or Abbreviations

α	alpha
β	beta
γ	gamma
δ	delta
AB	Abdominal
ADSC	Adipose derived stem cell
AIT	Aerobic interval training
AMPK	Adenosine monophosphate activated protein kinase
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BMI	Body mass index
BSA	Bovine serum albumin
CEBP	CCAAT/enhancer-binding protein
CPT-1β	carnitine palmitoyltransferase 1B
CVD	Cardiovascular disease
CS	Citrate synthase
dl	Deciliter
DNA	Deoxyribonucleic acid
DXA	Dual-energy x-ray absorptiometry

FASN	Fatty acid synthase
FATP-1	Fatty acid transport protein 1
FFA	Free fatty acid
FNDC5	Fibronectin type III domain-containing protein 5
g	gram
GL	Gluteal
GLUT4	Glucose transporter type 4
HDL-C	High-density lipoprotein cholesterol
HIIT	High intensity interval training
HR	Heart rate
HR_{max}	Heart rate maximum
IBMX	3-Isobutyl-1-methylxanthine
kcal	Kilocalorie
kg	Kilogram
LDL-C	Low-density lipoprotein cholesterol
m	Meter
M	Molar
mg	Milligram
MHR	Maximum heart rate
min	Minute
ml	Milliliter
mM	Millimolar

mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial DNA
NADH	Nicotinamide adenine dinucleotide (reduced)
nM	Nanomolar
O_2	Molecular oxygen
OCR	Oxygen consumption rate
ORO	Oil red O
OXPHOS	Mitochondrial respiratory complex
PBS	Phosphate buffered saline
PGC1-α	Peroxisome proliferator-activated receptor-gamma coactivator 1-alpha
pM	Picomolar
PPARγ	Peroxisome proliferator-activated receptor gamma
ROS	Reactive oxygen species
SAT	Subcutaneous adipose tissue
SVF	Stromal vascular fraction
T2D	Type 2 diabetes
TC	Total-cholesterol
TG	Triglycerides
TZD's	Thiazolidinediones
VO_{2max}	Maximal aerobic capacity
VO_{2peak}	Peak aerobic capacity
UCP1	Uncoupling protein 1

µg	Microgram
µIU	Micro International Unit
µl	Microliter
µM	Micromolar

Chapter 1

Introduction

1.1 General Background

Ninety-eight percent of American adults do not achieve sufficient levels of physical activity (Troiano et al., 2008), which represents an opportunity for the treatment of the escalating obesity epidemic currently affecting over one third of the population in the United States (Flegal et al., 2016). Although the US has one of the highest rates of obesity, numerous countries around the world are now experiencing an escalation in obesity prevalence, a pandemic that has reached developing countries (Finucane et al., 2011). Obesity is a disease that results from an imbalance of energy intake and energy expenditure leading to the accumulation of excess body fat. Obesity represents a major risk factor for not only cardiovascular disease (CVD), but also insulin resistance and T2D.

Aerobic capacity is the greatest determinant of mortality and is highly modifiable with physical activity; obese and T2D patients with relatively high levels of fitness have a marked reduction in mortality risk compared to less fit patients (Myers et al., 2002). Although exercise training adaptations have been thoroughly examined in skeletal muscle and the cardiovascular system, very few investigations have been undertaken to delineate the effects of exercise training on SAT in humans leaving significant gaps in knowledge.

Of all the relevant metabolic organs SAT is the most plastic in terms of its ability to remodel and expand upon nutritional and environmental cues. From an energetic

viewpoint SAT functions to store excess calories in the form of triglycerides (TG's) after meals and to release free fatty acids (FFA's) that can be used for ATP production in other tissues during periods of fasting or physical activity. Proper SAT function is vital for a healthy metabolic profile and has been shown to be impacted in the obese state (Sun et al., 2013; Rosen et al., 2014).

Mitochondrial biogenesis, content, and function have all been shown to be downregulated in obesity in SAT (Chattopadhyay et al. 2011; Heinonen et al., 2015 & 2017; Semple et al., 2004; Yin et al., 2014). Therapeutic options may exist to treat this decrement as the drug class of thiazolidinediones (TZD's) has been shown to be effective at increasing mitochondrial biogenesis in SAT while simultaneously improving insulin sensitivity (Wilson-Fritch et al., 2004; Choo et al., 2006). However, TZD's unfortunately carry with them detrimental side effects including weight gain (Nichols et al., 2007), heart attack (Lipscombe et al., 2007), and bladder cancer (Turner et al., 2014), thus providing motivation to find alternative therapies to improve mitochondrial content and/or function in SAT. Exercise has been shown to stimulate mitochondrial biogenesis (Sutherland et al., 2009; Stallknecht et al., 1991; Xu et al., 2011; Peppler et al., 2016) and improvements to mitochondrial function (Stanford et al., 2015) in rodent models but there has been a limited number of human investigations to this point (Camera et al., 2010; Larsen et al., 2015; Pino et al., 2016).

SAT owes its tremendous plasticity and ability to expand in mass to the processes of hypertrophy of existing fat cells, as well as, through the formation of new fat cells termed adipogenesis. Recent investigations in the field of adipose biology have suggested that adipogenesis may be limited in the obese state (Danforth, 2000), and the

metabolic perturbations associated with this diminished capacity may lead to insulin resistance and T2D (Weyer et al., 2000). However, on the other hand an increase in adipogenesis may predispose individuals to weight gain over time as directly evidenced by patients on TZD therapy. Little is known concerning exercise and its effects on adipogenesis in humans, however, rodent models consistently show exercise training reduces adipogenesis in vivo, an effect that is sustained *in vitro* with cell culture of primary preadipocyte from the same animals (Zeve et al., 2016; Sakurai et al., 2010).

1.2 Purpose of the Study

The first purpose of this dissertation is to evaluate the effects of exercise training on mitochondrial function and content in SAT of obese participants. The second purpose is to evaluate the effects of exercise training on the adipogenic capacity of preadipocytes isolated from SAT of obese participants.

1.3 Dissertation Overview

The following three chapters in this dissertation document are aimed at investigating and evaluating the study purpose and are followed by a comprehensive overall discussion. The effects of exercise training on mitochondrial function and adipogenesis have not been thoroughly studied in humans, therefore, the first chapter is a literature review of the scientific basis for this study and the previous investigations in both rodent and human models. The third chapter of this document outlines the investigation of the effects of

exercise training on mitochondrial function and content of SAT of obese humans. The fourth chapter of this document outlines the investigation of the effects of exercise training on the adipogenic potential of preadipocytes isolated from SAT of obese humans. The fifth and last chapter of this dissertation document provides a summary of the four preceding chapters and potential future directions for these lines of research.

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Chapter 2

Literature Review

2.1 Exercise Training

Aerobic capacity is the greatest predictor of mortality in humans (Myers et al., 2002) and physical activity is one of the most effective ways at improving aerobic capacity. Lifestyle modifications with a focus on physical activity are considerably more successful at preventing T2D than pharmacological therapies; with both treatments having similar compliance rates (Diabetes Prevention Program., 2002). Exercise is a form of acute physical activity and when repeated over time in successive bouts is referred to as exercise training. Exercise is perhaps the lifestyle intervention with the greatest ability to protect against disease states as it alters the expression of hundreds of genes involved in homeostasis and tissue maintenance (Timmons et al., 2010). There is strong epidemiological evidence that exercise provides benefits that extend beyond traditional cardiovascular risk factors (Fiuza-Luces et al., 2013) that are often targets for medical treatment for diseases such as obesity and T2D.

2.2 High Intensity Interval Training

Although it has been performed in athletics since the early 20th century when it was first pioneered by in Finland by coach Lauri Pikhala, high intensity interval training (HIIT) has become prevalent in exercise science research investigations over the last several years

(Gibala et al., 2017). HIIT differs from continuous exercise training in that it is often broken up into periods of high intensity followed by low intensity periods. These blocks or intervals typically last between 30 seconds to several minutes. In shorter duration intervals intensity is normally determined by total work (i.e. watts), whereas in longer duration intervals intensity is usually determined by a percentage of maximum HR (%MHR).

HIIT has been shown to be as effective, and in some instances more effective, than continuous exercise training in its ability to improve aerobic capacity (Tjønna et al., 2008). In addition, HIIT induces similar skeletal muscle adaptations to continuous aerobic exercise (Gibala et al., 2006) and has also been shown to impact the content of lipogenic and fatty acid (FA) transport proteins, as well as, insulin sensitivity of SAT in patients with the metabolic syndrome (Tjønna et al., 2008). We have previously shown that HIIT is effective at improving aerobic capacity and skeletal muscle microvascular endothelial function (La Favor et al., 2016). Interestingly, anecdotal comments from participants from our exercise studies and others (Tjønna et al., 2008) have identified that HIIT may be a more enjoyable form of exercise compared to continuous moderate training.

2.3 Subcutaneous Adipose Tissue (SAT)

Once thought to be a static location for lipid storage, SAT is now appreciated as a dynamic organ with significant mitochondrial content (Kaaman et al., 2007) that plays a vital role in regulating whole body metabolism (Stanford et al., 2015). From an energetic viewpoint SAT functions to store excess calories in the form of triglycerides (TG's) after meals and to release free fatty acids (FFA) that can be used for ATP production in other tissues

during periods of fasting or physical activity. SAT is also considered a major secretory organ that releases cytokines or adipokines that affect insulin sensitivity and metabolism in other organs, as well as, pro- and anti-inflammatory proteins and peptides (Ronti et al., 2006).

SAT is an extremely plastic organ with the capacity to account for ~10% of body composition in lean humans to over 50% in the obese. The plasticity of SAT is possible through both the expansion in the size of existing cells (hypertrophy) or by an increase in the number of adipocytes (adipogenesis or hyperplasia). The functional integration of these processes is thought to function as follows: during periods of increased energy intake adipocytes first hypertrophy in size up to a certain threshold (~0.7–0.8 ug/cell), upon reaching this threshold adipogenic signals are transmitted leading to the proliferation and/or differentiation of preadipocytes (Krotkiewski et al., 1983).

2.4 Adipogenesis/Differentiation in SAT

Adipogenesis or differentiation of preadipocytes is a highly-regulated process that is sensitive to developmental, hormonal, nutritional, and physiological stimuli. Adipocyte progenitor cells or adipose derived stem cells (ADSC's) are pluripotent mesenchymal stem cells that reside along blood vessels surrounding SAT (Rodeheffer et al., 2008). Adipogenesis occurs in two stages, with the first phase termed determination in which mesenchymal stem cells become committed and lose their pluripotency. The second phase of adipogenesis is termed terminal differentiation; the regulation of this phase has been studied more extensively. During terminal differentiation preadipocytes acquire the

phenotype of mature adipocytes and gain the ability to respond to the metabolic hormone insulin. This process is initiated by the transient activation of CCAAT/enhancer-binding protein (CEBP) family members CEBP-β and CEBP-δ that stimulate the expression of CEBP-α and peroxisome proliferator-activated receptor-γ (PPARγ). PPARγ and CEBP-α are the principal transcriptional controllers in the process of adipogenesis and function to keep one another's expression at elevated levels (Christodoulides et al., 2008). PPARγ is responsible for regulating the expression of many genes involved in cellular actions such as FA uptake, lipogenesis, and mitochondrial biogenesis.

2.5 Mitochondrial Function in SAT

SAT requires proper mitochondrial function to carry out cellular processes that make it integral to whole body metabolism. Lipogenic potential in adipocytes is strongly correlated to mitochondrial DNA (mtDNA) content (Kaaman et al., 2007) and mitochondria provide several key intermediates for triglyceride synthesis (Kusminski et al., 2012). Additionally, adiponectin synthesis has been shown to be dependent on proper mitochondrial function (Koh et al., 2007). Interestingly, adipocyte mitochondria are able to alter their function in response to their current bioenergetic state. For instance, during lipolysis SAT mitochondria uncouple in response to liberated intracellular FFA's (Yehuda-Shnaidman et al., 2010) a process that may offer protection from lipotoxicity and increased oxidative stress, and may reduce circulating FFA's through an elevated oxidation rate. In another example of mitochondrial function affecting cellular processes, it was previously shown that reactive oxygen species (ROS) produced from the mitochondria of adipocytes are

required for the induction of adipogenesis (Tormos et al., 2012). Additionally, upon induction of the adipogenic program mitochondrial biogenesis increases 20-30-fold above basal levels in preadipocytes *in vitro* (Wilson-Fritch et al., 2003).

With the renewed interest in brown adipose tissue (BAT) and the introduction of the white fat cell 'beiging' phenomena, in which white fat cells may transdifferentiate into beige or 'brite' (brown in white) fat cells (Harms et al., 2013), numerous researchers have begun to analyze mitochondrial content and/or function in SAT in various experimental conditions (Yehuda-Shnaidman et al., 2010; Camera et al., 2010; Kraunsøe et al., 2010)

2.6 Mitochondrial Content and Function in SAT of Obese Humans

Over the last few years a significant amount of investigations have revealed that SAT mitochondrial function and/or content is impacted by obesity in humans. In one of the first reports on the topic, Semple and colleagues (Semple et al., 2004) displayed that the gene expression of Peroxisome proliferator-activated receptor-gamma coactivator 1-alpha (PGC1-α), a master transcriptional co-activator involved in mitochondrial biogenesis, was three-fold lower in morbidly obese (BMI = 40-70 kg/m^2) subjects compared to lean controls (BMI = 22-26 kg/m^2).

Since this novel finding several studies have been conducted that evaluate measures beyond gene expression that include indices of mitochondrial content and function. In an elegantly designed study investigating the effects of acquired obesity in SAT, healthy, weight-discordant monozygotic twin pairs were examined for a detailed analysis of their mitochondrial characteristics (Heinonen et al., 2015). The investigation

revealed that compared to their lean counterparts the obese twins displayed a reduction in mitochondrial biogenesis, mtDNA, oxidative metabolic pathways, and mitochondrial respiratory complex (OXPHOS) protein content, all of which were associated with impairments in their metabolic profile. Elements of this study were repeated using the same model in isolated adipocytes obtained from SAT through collagenase treatment (Heinonen et al., 2017). The findings were similar to the original investigation in that the heavier twins displayed a reduction in mitochondrial biogenesis and OXPHOS protein content.

In addition to studies investigating mitochondrial biogenesis and content, several reports have been conducted measuring the effects of obesity on mitochondrial function in SAT. Chattopadhyay and colleagues (Chattopadhyay et al., 2011) measured mitochondrial respiratory complex enzyme activity in isolated mitochondria and found that obese subjects had reduced activity of complexes I-V compared to lean controls despite no differences in citrate synthase (CS) enzyme activity. Furthermore, the investigation also revealed a reduction in mitochondrial membrane potential in the obese subjects. In a similar study researchers (Yin et al., 2014) compared maximal oxygen consumption rates (OCR) using high resolution respirometry, as well as, CS activity in isolated mitochondria from SAT of obese and lean humans. The authors discovered that OCR and CS activity were reduced in the obese compared to the lean subjects despite no differences in mitochondrial content as measured by mtDNA. The previous two studies differ in their opposing findings related to CS activity. The interpretation of this contradicting data is difficult as one could argue that similar CS activity between groups in isolated mitochondria would indicate proper recovery and isolation of mitochondria. On

the other hand, CS activity is considered a functional assay and differences could very well exist between groups. Lastly, a recent investigation by Fisher and colleagues (Fischer et al., 2015) revealed that maximal OCR in isolated mitochondria obtained from SAT as measured by Clark-type electrodes is inversely correlated to BMI. Furthermore, mtDNA and CS content were also found to be inversely correlated to BMI in this investigation. The results of these studies provide valuable information to the field of adipose biology. Although there is some disagreement in the results of the functional measurements performed, namely CS activity, these studies clearly point out that mitochondrial function is impacted in the obese state.

2.7 Effects of Exercise Training on Mitochondrial Function and Content in SAT

Rodent models have consistently shown that exercise training induces mitochondrial biogenesis in SAT (Sutherland et al., 2009; Xu et al., 2011; Peppler et al. 2016) and some studies have also shown improvements to mitochondrial function in various adipose tissue depots (Stanford et al., 2015; Stallknecht et al.,1991). To the best of our knowledge no human studies were conducted on the effects of exercise training on mitochondrial function prior to the seismic shift in adipose biology that occurred with the identification of adipose tissue ‘browning’ or ‘beiging’. The initial interest in studying this phenomena in humans was further bolstered by the discovery of the highly controversial myokine Irisin, a cleaved portion of Fibronectin type III domain-containing protein 5 (FNDC5) that gets its name from the Greek messenger goddess ‘Iris’ and functions to promote beiging of SAT (Boström et al., 2012).

In response to the potential for thermogenesis and/or the promotion of an oxidative phenotype in SAT, several exercise training investigations have been undertaken in humans to decipher changes to mitochondria. However, thus far very few investigations have examined changes to mitochondrial proteins or mitochondrial function and have largely focused on the abdominal SAT depot. Additionally, most studies conducted have only investigated lean and/or overweight subjects leaving significant gaps on the effects of training on obese humans, although these selection criteria may be designed to limit the confounding effects of obesity on study results. However, the argument could be made that obese subjects would benefit the most from increased thermogenesis and/or mitochondrial biogenesis in SAT. Lastly, to the best of our knowledge no studies have investigated the effects of exercise training on mitochondrial content and/or function in studies lasting longer than 6 weeks.

In one of the first studies conducted Camera and colleagues (Camera et al., 2010) measured the effects of 10 days of consecutive, alternating (continuous moderate and HIIT) exercise training in lean, untrained men on measures of mitochondrial gene expression and content, as well as, CS activity. Despite increases in skeletal muscle GLUT4 protein content, indicating an effective exercise training program, the investigators found no changes to mitochondrial gene expression or content combined with no changes to CS activity in SAT. In a study investigating the effects of low volume HIIT in overweight men researchers found no improvement to maximal OCR as measured by high resolution respirometry in SAT despite improvements in skeletal muscle OXPHOS capacity (Larsen et al., 2015). Lastly, in a study comparing exercise-trained subjects to overweight and lean sedentary controls, it was found that trained subjects displayed higher mitochondrial

content and mitochondrial gene expression compared to the sedentary cohort (Pino et al., 2016). Interestingly, the sedentary participants later performed a 3-week exercise training study that did not result in increases in mitochondrial content or mitochondrial gene expression.

2.8 Effects of Obesity on Adipogenesis

Investigating adipogenesis in humans is challenging as methods have not been developed to measure this process *in vivo*. Recently, mouse models have been developed to elegantly measure adipogenesis by employing the use of transgenic methodologies (Jeffery et al., 2016). Although these models may provide substantial mechanistic data, differences may exist between human and animal models in the study of adipogenesis.

Most human studies utilize *in vitro* models of adipogenesis in which cell lines, primary preadipocytes, or primary adipose-derived stem cells (ADSC's) are studied in cell culture. In this design, induction of adipogenesis is usually carried out by the addition of a differentiation 'cocktail' that traditionally includes TZD's, 3-Isobutyl-1-methylxanthine (IBMX), and dexamethasone amongst other compounds. Although this method employs non-physiological compounds measurable differences occur across treatments and in different subject populations or disease states. This model is often used to gather greater understanding of the mechanisms involved in the complex process of adipogenesis.

In recent years, possibly due to the discovery of metabolically healthy obese individuals, there has been great emphasis placed on the idea of the healthy expansion

of SAT. In the obese state mature adipocytes hypertrophy in response to increased energy intake, which has been shown to correlate with insulin resistance and T2DM (Weyer et al., 2010). Hypertrophy of mature adipocytes is associated with inflammation and fibrosis (Pellegrinelli et al., 2016) which has been suggested to reduce the adipogenic capability of preadipocytes in obesity (Isakson et al., 2009). Furthermore, obese individuals have been shown to have fewer numbers of committed preadipocytes in SAT (McLaughlin et al., 2007; Tchoukalova et al., 2007) and the capacity of preadipocytes to differentiate in vitro has been shown to be inversely correlated with obesity (Isakson et al., 2009). Interestingly, in a recent study (Rossmeislová et al., 2013) weight loss with a concomitant improvement in insulin sensitivity was shown to improve the adipogenic capacity of preadipocytes isolated from SAT in obese individuals.

2.9 Effects of Exercise Training on Adipocyte Size and Adipogenesis

The effects of exercise training on fat cell size have not been thoroughly studied. However, in the few studies that have been conducted there is a general consensus that exercise training reduces mature adipocyte cell size, although these results are often confounded by changes in body weight or composition (Despres et al., 1984 and 1985). Additionally, trained pre-menopausal women have been shown to have smaller mature adipocytes in both abdominal and gluteal depots compared to sedentary, age matched-controls (Mauriege et al., 1997); however, although not significant, there was a ~7kg difference in body fat between the two groups of women that may have affected the results.

To the best of our knowledge no studies have been performed on the effects of exercise training on adipogenesis in humans. In a study utilizing a 16-week aerobic interval training program in obese humans with the metabolic syndrome, endurance training significantly reduced the protein content of FATP-1, a fatty acid transporter, and fatty acid synthase (FASN), an important lipogenic protein, in SAT. Together the reduction of these proteins suggests a potential for reduced lipogenesis in SAT in response to training. Although this study only measured lipogenic proteins it provides valuable information on a topic related to hypertrophy and adipogenesis in SAT.

Several investigations have been undertaken on the effects of exercise training on adipogenesis in rodent models that have provided some valuable information to the field. Sakurai and colleagues (Sakurai et al., 2010) isolated the stromal vascular fraction (SVF), which contains preadipocytes, from an adipose depot in rats and found that PPARγ and PPARγ-regulated genes were downregulated. Interestingly, the authors cultured the SVF *in vitro* and revealed a reduction in lipogenesis upon adipogenic induction. Similar to the previous study, investigators utilized a transgenic *in vivo* model in mice to measure adipogenesis in response to exercise and found a reduction in the exercise trained cohort (Zeve et al., 2016). The authors revealed that these results were replicated *in vitro* when preadipocytes from trained mice displayed reductions in lipogenesis.

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Chapter 3

Exercise improves mitochondrial function in subcutaneous adipose tissue in a depot-specific manner

3.1 Abstract

Subcutaneous adipose tissue (SAT) is central to the regulation of systemic metabolism. In SAT mitochondrial biogenesis, content, and function have all previously been shown to be impacted in obesity. In rodent models, exercise training has previously been shown to induce beneficial adaptations to SAT including alterations to mitochondrial content and function. We evaluated the effects of 12 weeks of aerobic interval training (AIT) in obese participants on mitochondrial content and function, as assessed by high resolution respirometry, in both upper (abdominal) and lower (gluteal) body SAT. AIT was effective at increasing aerobic capacity (VO_{2peak}). AIT increased complex I-linked oxygen consumption rates (OCR) in gluteal SAT despite no increases in mitochondrial respiratory complex content or citrate synthase content while there were no changes to abdominal SAT in the same measurements. These results indicate that AIT is an effective stimulus to improve complex I-linked mitochondrial OCR in gluteal SAT of obese participants. Our findings are similar to previous investigations in abdominal SAT in relation to exercise training and mitochondrial function.

3.2 Introduction

Exercise is a therapy that can combat metabolic disease despite little to no weight loss, as exercise has favorable effects on body fat distribution (Ismail et al., 2012), insulin sensitivity (Houmard et al., 2004), and other components of metabolic disease. The improvements in metabolic fitness in response to exercise training are often attributed to skeletal muscle, the predominant site of glucose disposal in the body, however, exercise confers benefits to other metabolic organs in the body including adipose tissue. Once thought to be a static location for energy storage, subcutaneous adipose tissue (SAT) is now appreciated as a dynamic organ with significant mitochondrial content that plays a vital role in regulating whole body metabolism (Kahn et al., 2017; Stanford et al., 2015). Mitochondria are essential to adipocyte function as the metabolic processes of adipogenesis (Wilson-Fritch et al., 2003), lipogenesis (Kaaman et al., 2007), and adipokine release (Koh et al., 2007) are dependent on proper mitochondrial function. In the obese state these functions may become dysregulated resulting in elevated circulating free fatty acids, ectopic fat storage, and a concomitant reduction in insulin sensitivity.

Previous investigations have revealed that SAT mitochondrial function and content may be impacted in obesity. Mitochondrial respiratory chain complex activity (Chattopadhyay et al., 2011) and content (Heinonen et al., 2015 & 2017; Fischer et al., 2015), as well as, indices of mitochondrial biogenesis (Semple et al., 2004) have been shown to be reduced in SAT in obesity. Interestingly, despite no apparent differences in mitochondrial content between lean and obese humans, Yin et al. (Yin et al., 2014) found

that oxygen consumption rates (OCR) and citrate synthase (CS) activity were reduced in adipocytes from obese donors, independent of cell size. This discovery raises the possibility that mitochondrial function may be independent of mitochondrial content in SAT, a relationship important to investigate to support the development of therapies to counteract the observed decrement in obesity.

Rodent models have consistently demonstrated that exercise training induces mitochondrial biogenesis and functional improvements to mitochondria in SAT (Sutherland et al., 2009; Stallknecht et al., 1991; Xu et al., 2011; Peppler et al., 2016); as little as 11d of exercise training in mice is adequate to stimulate increases in PGC-1α expression in SAT (Stanford et al., 2015). However, very few human studies have been conducted investigating the effects of exercise on mitochondrial function and/or content (Camera et al., 2010; Larsen et al., 2015; Pino et al., 2016). Additionally, no studies thus far have performed mitochondrial analyses in response to exercise training in both upper and lower body SAT depots. Investigating different SAT depots is intriguing as upper body SAT has greater lipolytic activity *in vivo* (Martin et al., 1991) and adipogenic capacity *in vitro* (Tchoukalova et al., 2010) compared to lower body SAT. Furthermore, lower body SAT has been shown to be metabolically protective compared to upper body SAT (Snidjer et al., 2005). The purpose of this investigation was to evaluate mitochondrial function and indices of mitochondrial content in upper (abdominal) and lower (gluteal) body SAT in obese humans in response to a 12-week aerobic interval training (AIT) exercise intervention.

3.3 Research Design and Methods

Participants

Normoglycemic, obese (BMI > 30 kg/m^2) men and women (n=15; 4 men/12 women; ages 25-47) were recruited to participate in this study. Before inclusion into the study participants were not involved in any physical activity program (> 4 METS for > 30 minutes per day, > 1 day per week) for the previous 6 months. All participants were non-smokers with no known history of cardiovascular disease. Participants with a fasting blood glucose concentration greater than 125 mg/dl were excluded. Participants were not taking any medications for hypertension, hypercholesterolemia, type 2 diabetes (T2D) or insulin resistance. All procedures were approved by the University and Medical Center Institutional Review Board of East Carolina University.

Anthropometrics and Assessment of Aerobic Capacity

Height was measured with a stadiometer to the nearest 0.1 cm; body mass was measured with a digital electronic scale to the nearest 0.05 kg. Body fat percentage was determined using dual-energy x-ray absorptiometry (DXA; GE Lunar Prodigy Advance, Madison, WI, USA) as previously described (Pierce et al., 2015). At baseline and after exercise training subjects performed a standardized maximal exercise test to assess peak aerobic capacity (VO_{2peak}). VO_{2peak} was assessed via open circuit spirometry (TrueMax 2400; Parvomedics; Salt Lake City, UT, USA) using the Storie protocol, a treadmill ramp protocol where the speed or incline is increased every 2min until volitional fatigue is reached. Heart rate (HR) was recorded every minute throughout the test and immediately

upon fatigue to determine the maximum heart rate (HR_{max}) that was used to determine HR training zones for the exercise training program.

Blood Analyses

Before and after the training intervention a fasted blood draw was performed where blood was collected from the antecubital vein. Blood samples were allowed to clot and were then centrifuged at 3300 rpm for 10min. Serum glucose, insulin, triglycerides (TG's), total-cholesterol (TC), and high-density lipoprotein cholesterol (HDL-C) were assessed by a commercial laboratory (Laboratory Corporation of America). Low-density lipoprotein cholesterol (LDL-C) was calculated by the Friedewald formula (Friedewald et al., 1972) and the homeostatic model of insulin resistance (HOMA-IR) was calculated as previously described (Matthews et al., 1985).

Adipose Tissue Biopsies

Abdominal and gluteal SAT biopsies were performed as previously described (Gavin et al., 2013) after an overnight fast (≥10 hours) at baseline and after 12 weeks of exercise training. SAT samples were immediately rinsed with Krebs Ringer Bicarbonate buffer. Blood clots and blood vessels were then removed. The biopsy sample was then portioned for distribution to the subsequent analysis: one portion (~100mg) was placed in BIOPS (Kraunsøe et al., 2010) relaxing buffer for respirometry analysis while another portion (~150mg, when adequate SAT biopsy material was obtained) was immediately frozen in liquid nitrogen and stored at −80 °C.

Aerobic Interval Training

Participants performed an aerobic interval training (AIT) exercise intervention three days per week for 12 weeks. Exercise consisted of walking/running on a motor-driven treadmill. During each exercise session participants performed a 10-min warm-up at ~70% HR_{max}, followed by four 4-min intervals at 88-92% HR_{max} interspersed by 3-min active recovery periods at ~70% HR_{max}; intervals were followed by a 4-min cool down at ~60% HR_{max}, amounting to a total exercise time of 42 min. HR was monitored throughout each training session, and intensity (speed or incline) was increased when HR failed to reach at least 88% HR_{max} during the high-intensity interval. The fasting blood draw and SAT biopsies were repeated 2 days following the final exercise session to assess the impact of the exercise intervention and to avoid the acute effects of the last exercise bout on experiments. VO_{2peak} and anthropometric tests were repeated within one to two days of the blood draw and SAT biopsies.

Mitochondrial Respiration

Mitochondrial oxidative phosphorylation capacity was measured in abdominal and gluteal SAT. Approximately 50 mg (wet weight) of both abdominal and gluteal SAT were studied in duplicate in a respirometric chamber (Oroboros Instruments, Innsbruck, Austria) containing 2 ml of buffer Z (Perry et al., 2011) at 37°C. All respiratory measurements in SAT were conducted following hyperoxygenation to avoid any potential oxygen limitation to respiration. Digitonin (2μM) was added to the chamber to permeabilize the cells before addition of substrates and measurement of oxygen consumption rates (OCR). This concentration was chosen as higher concentrations have previously been shown to

damage adipose tissue (Kraunsøe et al., 2010). Substrates were added in the following order; succinate (10mM) to achieve state 2 complex II-linked respiration (CII) followed by ADP (5mM) allowing for state 3 complex II-linked respiration (CII(ADP)). Next glutamate (10mM) and malate (2mM) were added together to achieve state 3 complex I & II-linked respiration (CI + CII(ADP)). Lastly, the complex II inhibitor malonate (5mM) was added to identify state 3 complex I-linked respiration (CI(ADP)). In our pilot experiments cytochrome *c* was added to the chamber in state 3 to test whether our sample preparations damaged the mitochondrial outer membrane; a major increase in respiration with addition of cytochrome *c* indicates a defect in the outer mitochondrial membrane integrity. The change in respiration after addition of cytochrome *c* was -3.2% $\pm$ 2.9 (n=6). OCR rates were normalized to weight wet of SAT.

Western Blotting

SAT samples were homogenized using a TissueLyser (Qiagen) in a 4:1 (volume-to-weight) ratio of cold homogenization buffer (0.5% NP-40, 0.1% Na deoxycholate, 150mM NaCl, 50mM Tris-Cl pH 7.5, and protease inhibitor cocktail (Sigma P8340). Homogenized samples were rotated for 60min and centrifuged for 20min at 10,000×g twice at 4°C. The supernatant was then removed and protein concentration determined by the Pierce BCA protein assay (Thermo Scientific, Rockford, IL). Samples were prepared in Laemmli sample buffer (Bio-Rad, Hercules, CA) with 5% β-mercaptoethanol. 60µg of SAT protein and 20µg positive control (exercise trained rodent skeletal muscle) was loaded and separated on a 10% Bis/acrylamide gel and electrotransferred to a nitrocellulose membrane. Membranes were blocked for 1 hour at room temperature with TBS-T 0.1%

with 5% BSA and then probed overnight at 4 °C for Total OXPHOS (1:1000; Abcam) and CS (1:1000; Abcam). Primary antibodies were diluted in TBS-T 0.1% + 5% BSA + 0.1% NaN3. Blots were incubated for 1 hour at room temperature with appropriate HRP-conjugated secondary antibodies and visualized with enhanced chemiluminescence (ECL) and Chemidoc system (Bio-Rad, Hercules, CA). All samples were normalized to total protein (Ponceau) and presented in arbitrary units (AU/Total Protein).

Statistical Analysis

Data were analyzed using GraphPad Prism 5.0 software with Wilcoxon matched-pair signed rank or student's paired t-test as indicated. The level of significance was set at $P < 0.05$.

3.4 Results

Anthropometrics, Assessment of Aerobic Capacity

The characteristics of participants at baseline and after 12 weeks of AIT are listed in Table 1. The AIT intervention was effective at improving relative and absolute aerobic capacity (VO_{2peak}) by 11.5% ($p<0.001$) and 12.8% ($p<0.001$), respectively. Body weight (p=0.304) and body composition (p=0.375) remained stable in the participants in response to AIT.

Blood Analyses

The metabolic profile obtained from blood analyses of participants at baseline and after 12 weeks of exercise training are listed in Table 1. Exercise training did not greatly affect

the metabolic profile in participants as glucose (p=0.278), insulin (p=0.245), HOMA-IR (p=0.357), TC (p=0.206), HDL-C (p=0.124), LDL-C (p=0.423), and TG's (p=0.088) all remained similar before and after training.

Mitochondrial Respiration

To evaluate whether SAT mitochondrial function is impacted in response to exercise training we measured oxidative phosphorylation capacity in abdominal (n=7) and gluteal (n=8) SAT at baseline and after 12 weeks of AIT. In response to AIT CII, CII(ADP), and CI + CII(ADP) did not change in either abdominal (Fig.1a, CII, p=0.189; CII(ADP), p=0.498; and CI + CII(ADP), p=0.946) or gluteal SAT (Fig.1b, CII, p=0.958; CII(ADP), p=0.945; and CI + CII(ADP), p=0.410), however CI(ADP) increased in gluteal SAT (Fig.1b, p=0.001) while remaining similar in abdominal SAT (Fig.1a, p=0.213).

Western Blotting

To identify whether improvements in mitochondrial function were related to changes to indices of mitochondrial content, we measured the protein content of mitochondrial respiratory complexes I-V, as well as, CS content before and after 12 weeks of AIT in abdominal (n=7) and gluteal (n=6) SAT. Mitochondrial respiratory complex content remained similar in response to AIT for all complexes detectable in both abdominal (Fig.2c, CI, p=0.280; CIII, p=0.828; CIV, p=0.130; CV, p=0.961) and gluteal (Fig.2f, CI, p=0.158; CIII, p=0.238; CIV, p=0.940; CV, p=0.182) SAT. Complex II was not detectable through ECL in both abdominal and gluteal SAT (Fig.2a and 2d, respectively). CS content

also remained similar in both abdominal (Fig.2c, p=0.128) and gluteal SAT (Fig.2f, p=0.059) before and after AIT.

3.5 Discussion

Mitochondrial function and/or content has been shown to be important for proper SAT function (Wilson-Fritch et al., 2003; DePauw et al., 2009; Koh et al., 2007; Kaaman et al., 2007) and is affected by obesity in humans. In this investigation we performed measurements of mitochondrial function and content in response to 12 weeks of AIT in abdominal and gluteal SAT of obese humans. Our results revealed that gluteal SAT mitochondrial complex I-linked OCR is improved despite no changes in respiratory complex and CS content whereas abdominal SAT mitochondrial complex I-linked OCR remained similar with no changes to content. These findings not only detail depot-specific changes to SAT in response to exercise training, but also expand upon previous investigations exhibiting a dissociation between mitochondrial function and content in SAT. In a previous study comparing mitochondrial function and content in SAT of obese and lean humans, researchers found that lean participants displayed greater maximal OCR and CS activity compared to obese subjects despite no differences in mitochondrial content (Yin et al., 2014). This relationship indicates that alterations in mitochondrial OCR may be related to changes in respiratory complex enzyme activity or remodeling of mitochondrial architecture, areas that certainly warrant further investigation in response to exercise training.

Our findings represent the first investigation performed in which mitochondrial function has been altered in lower body SAT in response to exercise training in humans. Previous studies conducted in humans measuring mitochondrial function and/or mitochondrial content in abdominal SAT have been unable to show changes in response to exercise training interventions of shorter duration (2-6 weeks). In a study of 10 days of consecutive alternating high intensity interval training (HIIT) or moderate intensity cycling exercise in lean humans mitochondrial content as assessed by electron microscopy, CS activity, and gene expression of mitochondrial markers did not change in abdominal SAT despite adaptations to skeletal muscle (Camera et al., 2010). In another study conducted by Larsen and colleagues (Larsen et al., 2015) overweight participants performed 6 weeks of low volume HIIT cycling exercise and mitochondrial function was measured in both SAT and skeletal muscle. Despite increases in maximal complex I & II-linked OCR in skeletal muscle, no changes were observed in SAT. Lastly, in a 3-week training study in sedentary lean and overweight participants Pino and colleagues (Pino et al., 2016) measured mitochondrial content (mtDNA copy number/cell) and gene expression of PGC1-α and CPT-1β and found no changes in content or expression in response to the intervention. Additionally, this investigation compared the sedentary group at baseline to an exercise trained group and found that the trained participants displayed greater mitochondrial content and greater gene expression of mitochondrial markers. The aforementioned studies are in agreement with our results in that mitochondrial function and content are unaltered in the abdominal SAT depot in response to exercise training. Collectively, these findings indicate that longer term training may be required to induce

mitochondrial content and/or improvements to mitochondrial function in the abdominal SAT depot.

The divergent response to exercise training in abdominal and gluteal SAT in this investigation is an attractive and novel finding as to the best of our knowledge no previous studies have been undertaken in the gluteal SAT depot in relation to exercise training and mitochondrial content and/or function in humans. Several physiological mechanisms may explain the stimulus for an increase in complex I-linked mitochondrial OCR in gluteal SAT. Although the ability of SAT to oxidize lipids is limited, a moderate improvement may offer cytoprotection to adipocytes during times of increased intracellular fatty acids (FA's) released from lipid droplets during lipolysis (Yehuda-Shnaidman et al., 2010). Reducing equivalents produced from β-oxidation and the TCA cycle flow through both complex I and II in the mitochondrial respiratory chain and an increase in complex I-linked OCR may indicate an increased ability to oxidize the reducing equivalent NADH.

Another potential mechanism for the increase in complex I-linked mitochondrial OCR is for an improved capacity for FA synthesis during times of caloric intake. In the energy-consuming process of lipogenesis acetyl-CoA must be supplied via the mitochondria from the metabolic breakdown of glucose. Additionally, glycerol-3-phosphate, a metabolite required for FA esterification and formation of TG's is also produced in the mitochondria. During lipolysis, FA's can become re-esterified and form TG's to be packaged back into the lipid droplet. Interestingly, FA re-esterification increases linearly and proportionally with exercise intensity (Townsend et al., 2017) and TG-FA recycling has been shown to be 4-fold higher in exercise-trained subjects compared to sedentary controls (Romijn et al., 1993).

Without increases in respiratory complexes and CS content as evidenced by western blotting the increased OCR derived from complex-I linked substrates is unlikely to be explained by SAT ‘beiging’ as beige adipocytes have higher mitochondrial density than white adipocytes (Harms et al., 2013). In addition, UCP1, a traditional marker of beige and brown adipocytes, was not detectable by western blotting both before and after training (data not shown).

In conclusion, our results revealed that mitochondrial function and content is not impacted in abdominal SAT in response to 12 weeks of AIT, which is in agreement with previous investigations. However, our data also revealed that in response to exercise training complex I-linked mitochondrial OCR is increased in gluteal SAT despite no increases in indices of mitochondrial content. Investigating the correlation between mitochondrial adaptations and alterations in SAT functions such as lipolysis, lipogenesis, and FA re-esterification will provide valuable insights for the effects of exercise training in SAT.

3.6 Tables and Figures

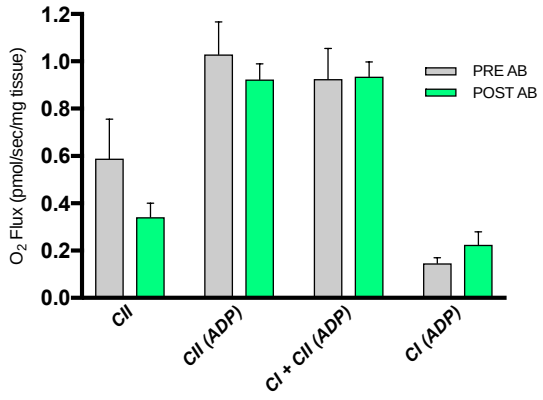
Table 1. Participant characteristics and metabolic parameters before and after training.

Measurement	Pre	Post	p-Value
N = 15 (3 M, 12 F)			
Age	*32.9 ± 1.6*		
Weight (**kg**)	*108.3 ± 4.9*	*107.4 ± 5.0*	*0.304*
BMI (kg/m^2)	*38.4 ± 1.4*	*38.0 ± 1.4*	*0.295*
Body Fat %	*44.1 ± 1.4*	*44.5 ± 1.4*	*0.375*
VO_2 (**l/min**)	*2.6 ± 0.2*	*2.9 ± 0.2*	***<0.001***
VO_2 (ml/kg/min)	*24.2 ± 1.4*	*27.3 ± 1.6*	***<0.001***
Glucose (mg/dl)	*90 ± 2.4*	*91.4 ± 2.5*	*0.278*
Insulin (uIU/ml)	*15.8 ± 2.4*	*14.1 ± 2.5*	*0.245*
HOMA-IR	*3.6 ± 0.6*	*3.3 ± 0.6*	*0.357*
Total C (mg/dl)	*182.5 ± 8.2*	*172.8 ± 7.2*	*0.206*
TG's (mg/dl)	*105.5 ± 12.7*	*91.5 ± 9.3*	*0.088*
HDL-C (mg/dl)	*50.2 ± 2.8*	*48.1 ± 3.1*	*0.124*
LDL-C (mg/dl)	*111.2 ± 7.5*	*106.4 ± 6.1*	*0.423*

Values are means ± SEM. n=15 (*serum blood analyses were not performed in n=2 participants). P values in bold reached the level of significance ($P < 0.05$). Student's paired t-test was performed for statistical analysis.

Figure 1. Mitochondrial respiration before and after 3 months of interval training in SAT.

A



B

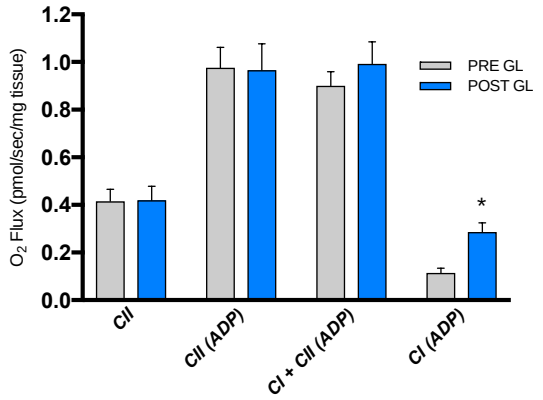
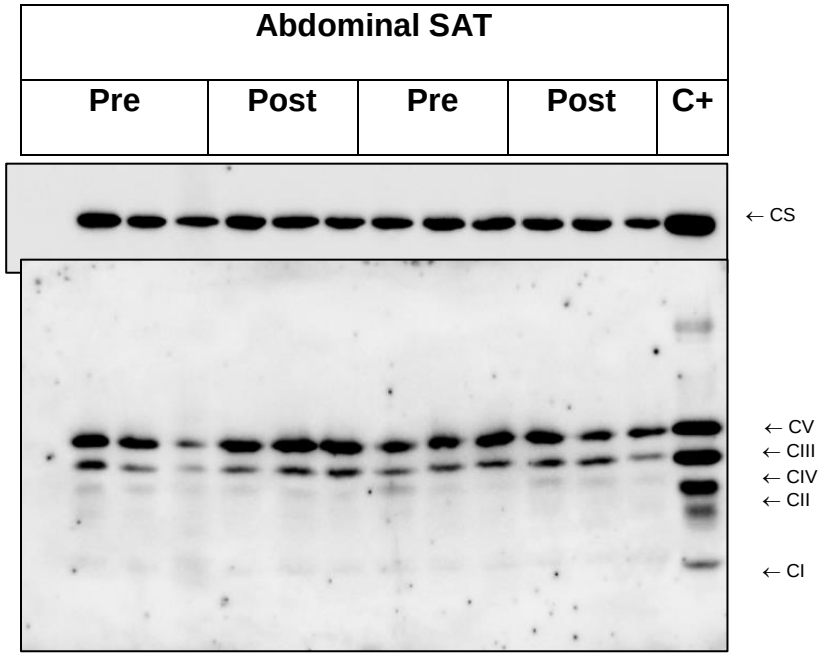


Figure 1. Mitochondrial respiration before and after 3 months of interval training in SAT. A. Oxygen flux before and after 12 weeks of exercise training in abdominal SAT B. Oxygen flux before and after 12 weeks of exercise training in gluteal SAT. CII = state 2 respiration with succinate (10mM, complex II substrate), CII(ADP) = state 3 respiration with succinate and ADP (5mM), CI + CII(ADP) = state 3 respiration with succinate and G/M = glutamate/malate (10mM/2mM complex I substrates), CI(ADP) = state 3 respiration with succinate, glutamate/malate and malonate (5mM, complex II inhibitor), PRE GLUT

= Gluteal SAT at baseline, POST GL = Gluteal SAT after 12 weeks of training. PRE AB = Abdominal SAT at baseline, POST AB = Abdominal SAT after 12 weeks of training, * P < 0.05 vs. PRE. Values are means ± SEM. Student's paired t-test was performed for statistical analysis.

Figure 2. Protein content of OXPHOS before and after 12 weeks of exercise training.

A



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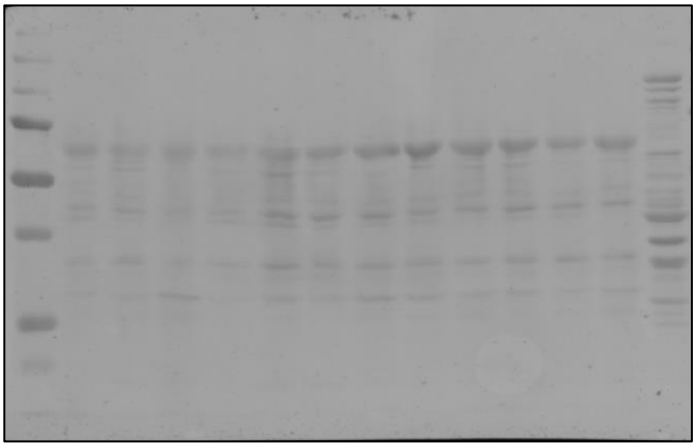
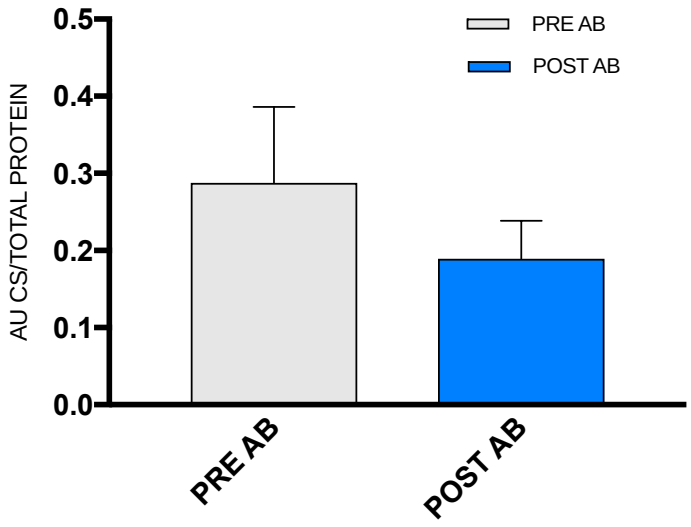
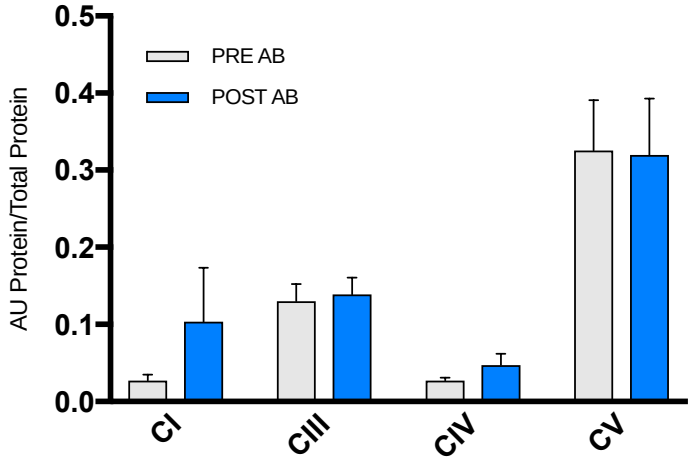


C


PRE AB
POST AB
AU CS/TOTAL PROTEIN
0.5
0.4
0.3
0.2
0.1
0.0
PRE AB
POST AB


PRE AB
POST AB
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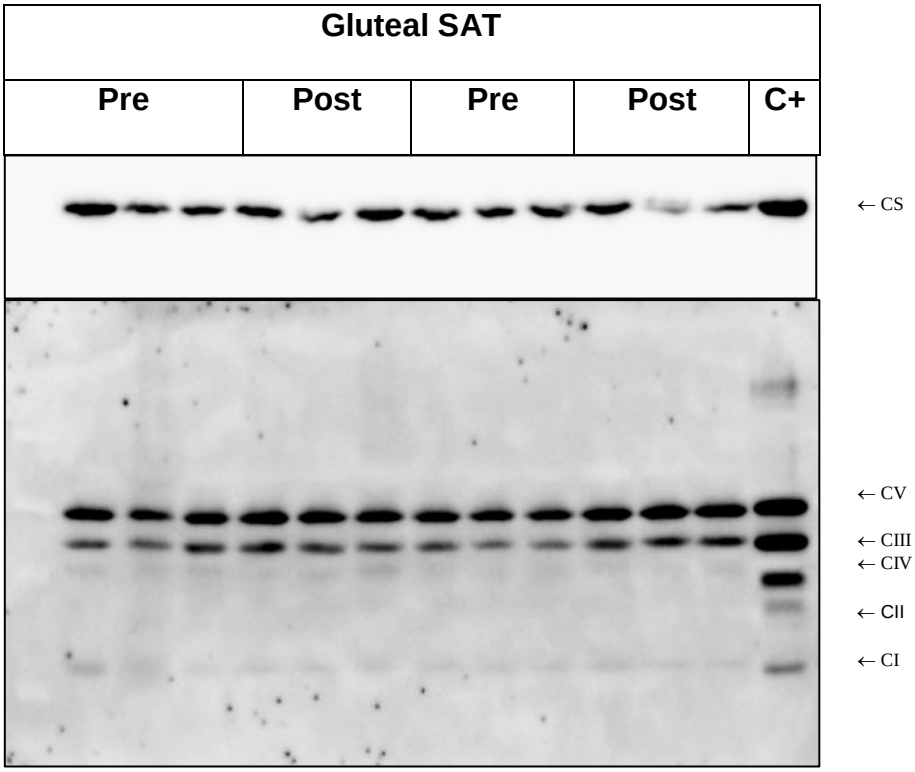
0.4
0.3
0.2
0.1
0.0
CI
CIII
CIV
CV

D



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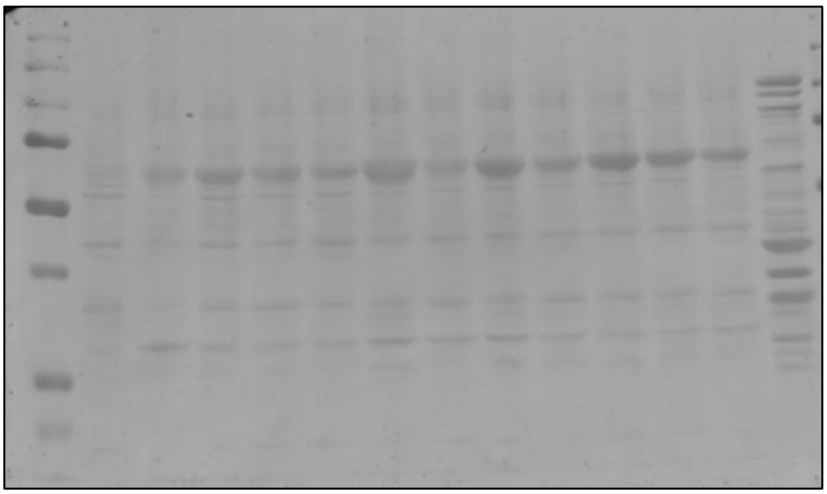

F

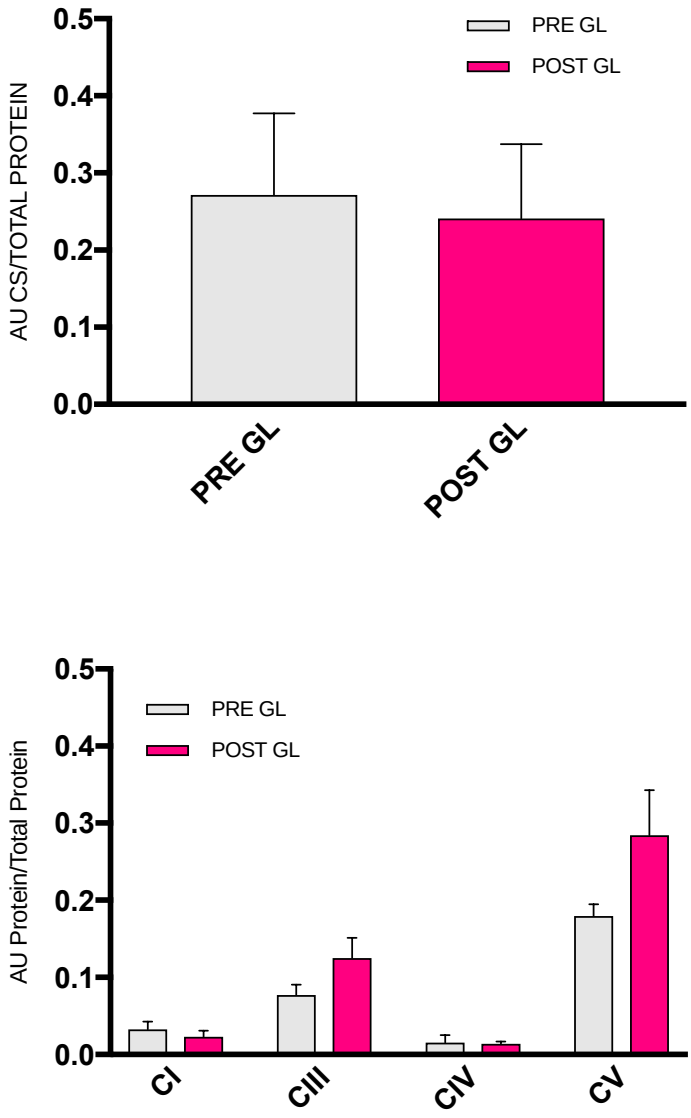

Figure 2. Protein content of OXPHOS before and after 12 weeks of exercise training. (A) Representative Western blot images for OXPHOS (respiratory complex content I, III, IV, V) and citrate synthase (CS) from abdominal SAT obtained from obese subjects before (PRE AB) and after (POST AB) 12 weeks of exercise training. (B) Representative Ponceau images for total protein, AB samples (C) Densitometry analysis OXPHOS and

CS normalized to total protein in abdominal SAT (n = 7). (D) Representative Western blot images for OXPHOS and CS from gluteal SAT obtained from obese subjects before (PRE GL) and after (POST GL) 12 weeks of exercise training. (E) Representative Ponceau images for total protein, GL samples (F) Densitometry analysis of OXPHOS and CS normalized to total protein in gluteal SAT (n = 6). Values are means ± SEM.

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Chapter 4

Exercise reduces the adipogenic capacity of preadipocytes from subcutaneous adipose tissue of obese humans

4.1 Abstract

Exercise training has been shown to impact metabolic organs including subcutaneous adipose tissue (SAT). To determine the effects of exercise training on the *in vitro* adipogenic capacity of preadipocytes isolated from SAT we enrolled 8 (4 male/4 female) normoglycemic, obese humans in a 12-week aerobic interval training (AIT) exercise program and obtained paired SAT biopsies at baseline and after the intervention. AIT was effective at improving aerobic capacity (VO_{2peak}) and the metabolic profile of participants including improvements in insulin sensitivity and lipid profile. The intervention diminished the adipogenic capacity of preadipocytes evidenced by a reduction in gene expression of PPARγ, PPARγ-related genes, and lipogenic genes in response to adipogenic induction *in vitro*. Interestingly, PPARγ and several genes under its regulation displayed decreased expression even before the induction of adipogenesis. Gene expression revealed the reduction in adipogenic potential was likely unrelated to cell senescence. The findings in this investigation illustrate exercise imprinting as seen in previous investigations evaluating the effects of exercise on progenitor cell function. The ability of preadipocytes to maintain their phenotype after extensive cell passaging indicates that these effects may be mediated by epigenetic mechanisms. A reduction in adipogenic capacity of

preadipocytes may be a mechanism of the prevention of weight gain consistently seen with exercise training.

4.2 Introduction

Subcutaneous adipose tissue (SAT) orchestrates the bidirectional flux of fatty acids (FA's) ensuring that energy demand of peripheral tissues including skeletal muscle is met. During the postprandial period SAT extracts FA's from the systemic circulation for esterification and storage as triacylglycerol (TG) (Frayn et al., 1998). Inversely, during the post-absorptive state and during physical activity SAT releases the stored FA's for use by peripheral tissues (Frayn et al., 2010).

During periods of chronic positive energy imbalance SAT expands primarily through an increase in the size of existing mature adipocytes (hypertrophy) and to some extent via an increase in preadipocyte and adipocyte numbers (adipogenesis). Adipocyte hypertrophy is positively correlated with insulin resistance and longitudinally with the development of type 2 diabetes (T2D) (Weyer et al., 2000). The limited expandability hypothesis is the primary mechanism to explain this relationship (Danforth et al., 2000). This hypothesis states that due to a reduced adipogenic potential, existing adipocytes hypertrophy reducing their ability to buffer FA's leading to systemic spillover. This hypothesis is supported by investigations that display a reduction in the number of committed preadipocytes in SAT of obese humans, as well as, an accumulation of small adipocytes that exhibit impaired lipogenesis in obese and insulin resistant subjects (McLaughlin et al., 2007; Tchoukalova et al., 2007). The limited expandability concept is

also supported by studies showing lower expression of adipogenic genes in SAT of obese and insulin-resistant subjects (Dubois et al., 2006; Rogers et al., 2012).

However, alternative hypotheses exist in the discussion of SAT dysfunction and the development of insulin resistance. Firstly, the maladaptive response to inflammatory stimuli hypothesis (Gregor et al., 2011; Reilly et al., 2017) proposes that metabolic stresses activate immune cells as an adaptive mechanism that orchestrate SAT expansion. However, hypertrophied adipocytes and unresolved inflammation cause, or exacerbate, insulin resistance. The second is the endocrine hypothesis which suggests that a reduction of the anti-inflammatory and insulin-sensitizing adipokine, adiponectin, is central to the development of both inflammation and insulin resistance (Kern et al., 2003). Lastly, the preadipocyte senescence hypothesis that stipulates that preadipocytes can acquire a senescent phenotype which may impact their ability to differentiate (Tchkonia et al., 2010).

It is generally accepted that low levels of daily physical activity are associated with poor metabolic health and that lifestyle interventions that include an increase in physical activity improve insulin-sensitivity and delay or prevent the development of T2D (Yamaoka et al., 2005). A meta-analysis of the effect of moderate aerobic exercise prescribed for T2D prevention on body weight showed that it is usually insufficient to reduce weight (Boule et al., 2001). This is because below a certain threshold many mechanisms exist to counterbalance mild negative energy imbalances. A randomized clinical trial showed that at least 7 hours of continuous moderate to vigorous physical activity per week is required to lose and maintain a significant amount of weight loss and prevent weight regain (Donnelly et al., 2009). Nevertheless, meta-analyses established

that 150 minutes of continuous moderate to vigorous exercise per week is the threshold at which a significant reduction of HbA1c is established (Umpierre et al., 2011). Therefore, the benefits of physical activity appear to be independent of significant changes to body weight. A mode of exercise training that has recently received significant interest is high-intensity interval training (HIIT). Aerobic interval training (AIT) is a type of HIIT that was adapted for patients at risk for cardiovascular diseases. AIT protocols are variable but generally consist of interval blocks of 3-4 minutes of a high intensity period (85-90% of max heart rate (MHR)), followed by 3-4 minutes of active recovery (~60-70% of MHR). The impact of AIT on metabolic heath has not been thoroughly studied compared to continuous moderate exercise but AIT has been shown to be more effective than continuous training at improving insulin signaling in adipose tissue in subjects with the metabolic syndrome (Tjønna et al., 2008).

A recent investigation identified that weight loss through caloric restriction is associated with an increased adipogenic potential *in vitro* (Rossmeislová et al., 2013). As both exercise and weight loss have been shown to improve insulin sensitivity, a characteristic associated with adipogenic capacity, we hypothesized that AIT would improve the ability of preadipocytes to differentiate into mature adipocytes. Thus, we studied the adipogenic capacity of preadipocytes isolated from SAT samples of obese participants in response to 12 weeks of AIT.

4.3 Research Design and Methods

Participants

Normoglycemic, obese (BMI > 30 kg/m^2) men and women (n=8; 4 males/4 females; ages 25-39) were recruited to participate in this study. Before inclusion into the study participants were not involved in any physical activity program (> 4 METS for > 30 minutes per day, > 1 day per week) for the previous 6 months. All participants were non-smokers with no known history of cardiovascular disease. Participants with a fasting blood glucose concentration greater than 125 mg/dl were excluded. Participants were not taking any medications for hypertension, hypercholesterolemia, type 2 diabetes (T2D) or insulin resistance. All procedures were approved by the University and Medical Center Institutional Review Board of East Carolina University.

Participant Characteristics and Assessment of Aerobic Capacity

Height was measured with a stadiometer to the nearest 0.1 cm; body mass was measured with a digital electronic scale to the nearest 0.05 kg. Body fat percentage was determined using dual-energy x-ray absorptiometry (DXA) (GE Lunar Prodigy Advance, Madison, WI, USA) as previously described (Pierce et al., 2015). At baseline and after exercise training subjects performed a standardized maximal exercise test to assess peak aerobic capacity (VO_{2peak}). VO_{2peak} was assessed via open circuit spirometry (TrueMax 2400; Parvomedics; Salt Lake City, UT, USA) using the Storie protocol, a treadmill ramp protocol where the speed or incline is increased every 2 minutes until volitional fatigue is reached. Heart rate (HR) was recorded every minute throughout the test and immediately

upon fatigue to determine the maximum heart rate (HR_{max}) for the exercise training intervention.

Blood Analyses

Before and after the training intervention a fasted blood draw was obtained from the antecubital vein. Blood samples were allowed to clot and were then centrifuged at 3300 rpm for 10min. Serum glucose, insulin, triglycerides (TG's), total-cholesterol (TC), and high-density lipoprotein cholesterol (HDL-C) were assessed using commercial laboratory (Laboratory Corporation of America). Low-density lipoprotein cholesterol (LDL-C) was calculated using Friedewald formula (Friedewald et al., 1972) and the homeostatic model assessment of the insulin resistance index (HOMA-IR) was calculated as previously described (Matthews et al., 1985).

Adipose Tissue Biopsies

Abdominal SAT biopsies were performed as previously described (Gavin et al., 2013) after an overnight fast (≥10 hours) at baseline and after 3 months of exercise training. Post-training biopsies were performed 2 days after the last exercise training session to avoid the acute effects of exercise on subsequent analyses.

Isolation, Culture, and Differentiation of Preadipocytes

Upon collection abdominal SAT biopsy samples were immediately placed in DMEM-F12. The samples were digested in DMEM-F12 in the presence of type 1 collagenase (1mg/ml) for 60-75min at 37˚C. Preadipocytes were separated from mature fat cells through

filtration and centrifugation at 600g for 10min and further separated from erythrocytes through selective lysis and additional filtration and centrifugation. Preadipocytes were then plated and subcultured to the 4th passage in preadipocyte media (DMEM-F12 supplemented with 10% fetal bovine serum (FBS) (Thermo Scientific HyClone, Logan, UT)), 10 mM glucose, 1 mM pyruvate, 33 μM Biotin, 17 μM D-Pantothenate, 1 μM epidermal growth factor, 1 μM basic fibroblast growth factor, 100 nM insulin, and 10 nM hydrocortisone was used for propagation (growth factors were from Gemini Bio-Products, West Sacramento, CA; chemicals from Sigma-Aldrich, St-Louis, MO). Subculturing to the 4th passage has been shown to remove contamination from immune cells and macrophages (Isakson et al., 2009; Mitchell et al., 2006).

Differentiation of preadipocytes at 2d post-confluence was induced by differentiation media (DMEM-F12 medium supplemented with 3% FBS, 15mM HEPES, 25mM $NaHCO_3$, 100 units/ml penicillin/ 100 μg/ml streptomycin, 33μM d-biotin, 17μM pantothenate, 100nM dexamethasone, 100nM insulin, 1μM rosiglitazone, 0.5mM IBMX, 2nM T_3, 10μg/ml transferrin) for 7d and maintained in maintenance media for 9d (DMEM-F12 medium supplemented with 15mM HEPES, 25mM $NaHCO_3$, 100 units/ml penicillin/ 100 μg/ml streptomycin, 33μM d-biotin, 17μM pantothenate, 100nM dexamethasone, and 100nM insulin) for 9d until Day 16 of differentiation when cells were harvested for RNA and fixed for Oil red O (ORO) staining.

Oil Red O Staining and Quantification

Cells were fixed with 10% formalin and stained with ORO working solution for 30min. ORO was eluted with 100% isopropanol. Optical density (OD) was measured at 500 nm

in a plate reader. OD of the samples was normalized to a standard curve of ORO working solution.

Gene Expression Analysis

Total RNA was isolated using a Direct – zol RNA MiniPrep Kit (Genesee Scientific, San Diego, CA, USA). mRNA levels were measured using reverse transcription quantitative PCR (Applied Biosystems, Carlsbad, CA, USA). GAPDH was used as an endogenous control for gene expression analysis. Results are expressed as $\Delta\Delta C_t$ (threshold cycle) values.

Aerobic Interval Training

Participants performed an aerobic interval exercise training intervention 3 days per week for 12 wks. Exercise consisted of walking and/or running on a motor-driven treadmill. During each exercise session participants performed a 10min warm-up at ~70% HR_{max}, followed by four 4min intervals at 88-92% HR_{max} interspersed by 3-min active recovery periods at ~70% HR_{max}; intervals were followed by a 4min cool down at ~60% HR_{max}, amounting to a total exercise time of 42 min. HR was monitored throughout each training session, and intensity (speed or incline) was increased when HR failed to reach at least 88% HR_{max} during the high-intensity phase of the intervals. The fasting blood draw and SAT biopsies were repeated 2 days following the final exercise session to assess the impact of the exercise intervention and to avoid the acute effects of the last exercise bout on experiments. VO_{2peak} and anthropometric tests were repeated within 1-2 days of the blood draw and adipose biopsies.

Statistical Analysis

Data were analyzed using GraphPad Prism 5.0 software with Wilcoxon matched-pair signed rank or student's paired t-test as indicated. The level of significance was set at $P < 0.05$.

4.4 Results

Participant Characteristics Assessment of Aerobic Capacity

The characteristics of participants at baseline and after 12 weeks of exercise training are listed in Table 1. The exercise training intervention was effective at significantly improving relative and absolute aerobic capacity (VO_{2peak}) by 19.1% (p=0.003) and 17.2% (p=0.002), respectively, while body weight (p=0.860) and body composition (p=0.520) remained stable.

Blood Analyses

The metabolic profile of participants at baseline and after 12 weeks of exercise training are listed in Table 1. Exercise training was successful at improving metabolic health in participants as insulin resistance as assessed by HOMA-IR (p=0.032) was reduced by 30.1% while fasting insulin was decreased by 29.8% (p=0.014). Fasting blood glucose remained similar (p=0.465). The exercise intervention improved the lipid profile of participants as TC (p=0.010) and LDL-C (p=0.012) were reduced by 12.0 and 14.4%, respectively; TG's (p=0.459) remained similar after the exercise training intervention.

Oil Red O Staining and Quantification

To assess the lipogenic capability of differentiating preadipocytes we performed ORO staining on Day 16 of differentiation. ORO staining was reduced by 55.2% (Figure 1) after training although this effect did not reach statistical significance ($p = 0.088$).

Gene Expression Analysis

At Day 16 of differentiation expression of adipogenic transcription factors PPARγ and CEBPα was decreased in response to exercise training, 4.6 and 6.7-fold, respectively (Figure 2a). Following the pattern of reduced PPARγ expression after exercise training, the expression of PPARγ-regulated genes ADIPOQ (40-fold), LPL (48-fold), glycerol-3-phosphate dehydrogenase 1 (GPD-1; 32-fold), and DGAT-1 (3-fold) decreased in response to exercise training. Additional genes involved in lipid uptake (CD36; 5.6-fold) and *de novo* lipogenesis (FASN; 13-fold) decreased in response to the exercise training intervention as well.

To further characterize the effects of exercise on preadipocytes we measured gene expression at Day 0, before adipogenic induction, to decipher if preadipocytes displayed exercise imprinting that may have affected their ability to differentiate (Figure 2b). At Day 0, gene expression of PPARγ was reduced by 4.3-fold after the training intervention compared to baseline. In addition, the expression of GPD-1 was significantly reduced 6.7-fold. Wnt10b, a secreted glycoprotein involved in the inhibition of adipogenesis, increased 43% in expression in response to exercise training but did not reach statistical significance. FASN decreased by 50% and CD36 decreased 4-fold but did not reach statistical significance.

To explain the differences seen in adipogenic and lipogenic gene expression at Day 0 we measured the gene expression of pro-inflammatory factors. At Day 0, both IL-6 and IL-1β gene expression increased (~7 fold) in response to exercise training; IL-8 increased by 4.7-fold but this effect did not reach statistical significance (Figure 2c). Interestingly while there was an increase in pro-inflammatory factors CCL2 did not change and CD68, a macrophage marker, decreased by 3-fold. This finding may rule out that the preadipocytes isolated after exercise training had increased contamination from macrophages in culture at Day 0. Therefore, we hypothesized that the increased gene expression of pro-inflammatory factors may indicate increased cell-senescence, as preadipocytes isolated from human samples often contain senescent cells (Tchkonia et al., 2010). We thus measured gene expression of p53 and p21 as markers of cell senescence. The gene expression of both p53 and p21 remained similar before and after training indicating that increased cell senescence may not explain the decrease in adipogenic potential.

4.5 Discussion

Exercise training has consistently been shown to improve metabolic health, which may be mediated in part by adaptations to SAT function (Stanford et al., 2015). However, it is not known whether AIT impacts preadipocytes located in SAT of subjects with obesity and insulin resistance. In this investigation we utilized an *in vitro* model of adipogenesis and showed that 12 weeks of AIT reduced their adipogenic and lipogenic capacities.

Repeated bouts of exercise over time may alter the microenvironment of preadipocytes leading to changes in adipogenic potential. This effect is likely mediated through epigenetic mechanisms as differences in adipogenic potential at baseline and in response to exercise training were apparent after 4 cellular passages in our model. In a recent investigation in SAT by Rönn et al. (Rönn et al., 2013) the authors found that 7,663 unique genes showed differences in levels of DNA methylation after exercise training and mRNA expression was changed in 1/3 of gene regions with altered DNA methylation.

The exercise imprinting displayed in this investigation may be due to several mechanisms. Exercise stimulates exposure to systemic factors (i.e. hormones and metabolites) both during and after exercise. Additionally, signals exchanged between mature fat cells and preadipocytes may represent a potential mechanism as exercise has been shown to reduce adipocyte apoptosis (Sertie et al., 2013), an effect that may decrease adipocyte turnover and impact the adipogenic capacity of preadipocytes.

Alterations in bioenergetics may also lead to a reduction in adipogenesis; a reduction in adipogenic potential may represent a mechanism of the prevention of weight gain or weight re-gain after weight loss. In rodent models investigating the mechanisms of weight re-gain after weight loss, overfeeding results in a marked increase in small adipocytes, possibly indicating an increase in adipogenesis (Jackman et al., 2008). Exercise has been shown to be a highly effective therapy for preventing weight gain and weight re-gain after weight loss. In rodent models investigating the effects of exercise on weight regain (Steig et al., 2011, Giles et al., 2016) exercised animals display an increased propensity for oxidation of dietary fat and a reduction in lipid storage in SAT. These effects are most likely mediated by a bioenergetic shift with elevated expression of genes involved in

mitochondrial biogenesis, beta-oxidation, as well as, lipid uptake and utilization in skeletal muscle (Steig et al., 2011) with a concomitant reduction in genes involved in lipid uptake, TG synthesis, and de novo lipogenesis in SAT (Giles et al., 2016).

Our data reveal that after training preadipocytes had a marked increase in inflammatory markers IL-6 and IL-1β. We originally hypothesized that this could be indicative of the inflammation associated with the cell senescent phenotype, however, cell senescent markers did not increase in expression in response to training. Another possible explanation could be ascribed to macrophage contamination in the preadipocyte cell culture. This hypothesis does not seem to be a plausible explanation for the increase in inflammation as CD-68, a macrophage marker, decreased in expression in response to the training intervention. Preadipocytes are inherently pro-inflammatory and release cytokines both *in vivo* and *in vitro*. The increased expression of inflammatory markers may be related to the reduction in PPARγ expression after training as PPAR's are traditionally associated with the repression of inflammatory pathways (Daynes et al., 2002). PPARγ-specific ligands have previously been shown to prevent the production of inflammatory cytokines IL-6 and IL-1β in several cell types (Delerive et al., 2001; Willson et al., 2000). A reduction in PPARγ expression and/or activation in response to training may have led to maintenance of the pro-inflammatory phenotype traditionally associated with preadipocytes.

Adipogenic experiments performed in cell culture may not fully represent *in vivo* conditions as differentiation is often induced with non-physiological compounds present in the media including rosiglitazone, a potent PPARγ agonist. However, we measured gene expression at Day 0 of differentiation (before addition of differentiation media) and

found that PPARγ, GPD-1, and genes involved in lipid uptake and lipogenesis were decreased. This may indicate that the differentiation capacity of preadipocytes was impacted before the induction of differentiation through mechanisms associated with exercise training. In a study conducted by Sakurai et al. (Sakurai et al., 2010) the investigators isolated and measured the gene expression of the stromal vascular fraction of adipose tissue in rats. They revealed that PPARγ and PPARγ-regulated genes were downregulated in response to exercise training. Interestingly preadipocyte factor-1 (pref-1), a preadipocyte marker that is also an inhibitor of adipogenesis, was upregulated in response to training. Furthermore, the investigators performed measurements of *in vitro* adipogenesis and displayed a reduction in lipogenesis as measured by ORO. This investigation indicated that gene expression in the stromal vascular fraction was impacted in response to exercise training and may have led to a reduced ability to differentiate *in vitro*. In a similar study Zeve et al. (Zeve et al., 2016) utilized a transgenic model in mice to display a reduction of adipogenesis in vivo, an effect that was mirrored *in vitro*. The cessation of exercise training has also been shown to impact adipogenic capacity as upon detraining, previously exercise-trained rats experience increases in lipogenic capacity and adipogenic gene expression (Sertie et al., 2013). Collectively, these data illustrate that exercise training reduces adipogenic capacity *in vivo* and the effects are retained *in vitro*, thereby adding validity to our findings.

In summary, our investigation reveals that exercise training reduces the adipogenic capacity of preadipocytes isolated from SAT of obese humans. This finding may have important implications for the bioenergetics involved in the prevention of weight gain. The participants in the investigation greatly improved their insulin sensitivity and overall

metabolic profile possibly indicating that a reduction in adipogenesis may be associated with reductions in the risk for metabolic disease. A limitation of our study was that we did not perform *in vivo* or *ex vivo* measures of adipogenesis and lipogenesis; it will be important for future studies to relate the effects of exercise imprinting on preadipocytes to functional measures related to whole-body metabolism in humans. This study represents an exciting introduction to the effects of exercise training on preadipocyte function in humans, an understudied area that will likely provide valuable information for the prevention of metabolic disease.

4.6 Tables and Figures

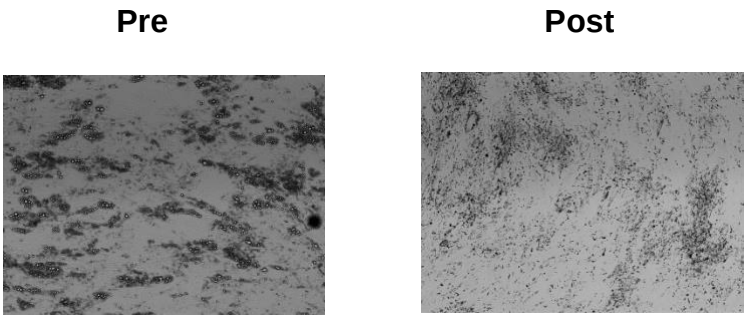
Table 1. Participant characteristics and metabolic parameters before and after training.

Measurement	Pre	Post	p-Value
N = 8 (4 M, 4 F)			
Age	*30.3 ± 1.7*		
Weight (kg)	*106.1 ± 6.2*	*106.3 ± 6.3*	*0.860*
BMI (kg/m^2)	*35.8 ± 1.2*	*35.9 ± 1.2*	*0.817*
Body Fat (%)	*41.4 ± 1.7*	*41.9 ± 1.5*	*0.520*
VO_2 (l/min)	*2.9 ± 0.4*	*3.4 ± 0.3*	***0.003***
VO_2 (ml/kg/min)	*26.7 ± 2.0*	*31.8 ± 1.6*	***0.002***
Glucose (mg/dl)	*89.5 ± 3.2*	*87.9 ± 2.6*	*0.465*
Insulin (uIU/ml)	*16.1 ± 2.8*	*11.3 ± 1.7*	***0.014***
HOMA-IR	*3.6 ± 0.7*	*2.5 ± 0.4*	***0.032***
Total-C (mg/dl)	*179.8 ± 8.9*	*158.3 ± 6.7*	***0.010***
TG'S (mg/dl)	*134.6 ± 22.0*	*123.5 ± 23.9*	*0.459*
HDL-C (mg/dl)	*43.6 ± 3.9*	*40.1 ± 2.9*	***0.011***
LDL-C (mg/dl)	*109.1 ± 7.8*	*93.4 ± 7.8*	***0.012***

Values are means ± SEM (n=8). P values in bold reached the level of significance ($P < 0.05$). Student's paired t-test was performed for statistical analysis. Total C = total cholesterol, HDL-C = high density lipoprotein cholesterol, LDL-C = low density lipoprotein, TG's = triglycerides

Figure 1. Lipogenic capacity before and after training

A



B

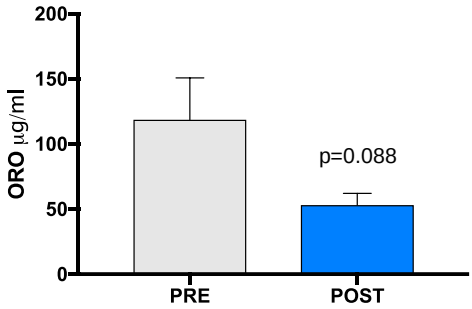
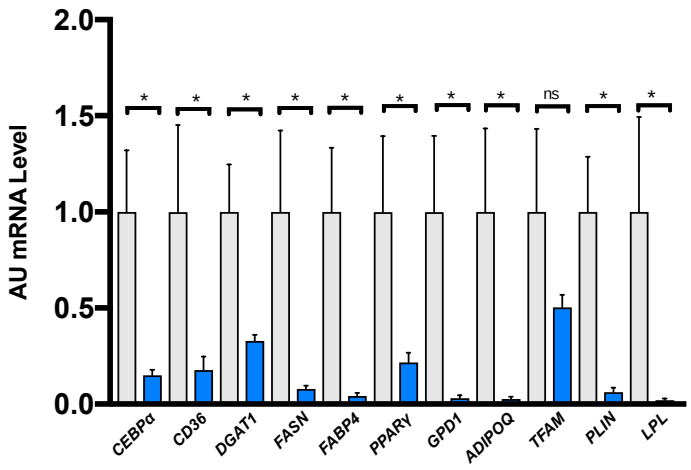


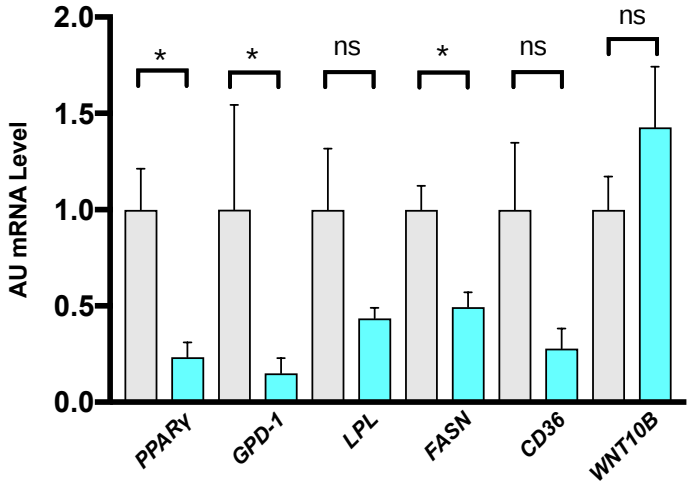
Figure 1. Lipogenic capacity before and after training. A: Representative images of differentiated preadipocytes at Day 16 stained with ORO from one participant before and after training. **B:** Quantification of ORO via absorbance; quantification was performed and normalized to a standard curve of ORO stock and expressed as ug/ml of stock ORO (n=8). Student's paired t-test was performed for statistical analysis.

Figure 2. Gene expression before and after training.

A



B



C

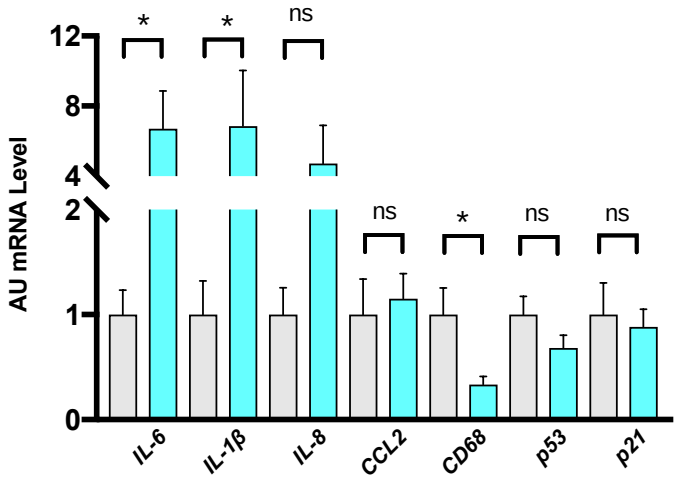


Figure 2. Gene expression before and after training. A-C: mRNA expression (arbitrary units [AU mRNA]) in adipocytes normalized to GAPDH expression (n=8). * $P < 0.05$; ns = not significant ($P > 0.05$).

4.7 References

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Chapter 5

Overall Discussion

5.1 Summary

The purpose of the overall dissertation was to evaluate the effects of exercise training in obese participants on mitochondrial function and content in SAT, as well as, the adipogenic capacity of preadipocytes isolated from SAT. The global results from this project indicate that exercise training provides a sufficient stimulus to impact SAT function in obese humans. This is an exciting conclusion as SAT plays a vital role in metabolism and represents an exciting therapeutic target due to the renewed interest in thermogenesis in SAT. Additionally, due to the low side effects with exercise training this may represent an ideal therapy to improve SAT function compared to TZD therapy, which has been shown to have life threatening side effects. Our findings provide valuable information in the understudied areas of mitochondrial function and adipogenesis in SAT and how they are impacted by exercise in obese humans.

The findings in Chapters 3 and 4 were obtained from *ex vivo* and *in vitro* experiments that were performed in response to the same exercise training intervention in obese humans from paired SAT biopsies. The two chapters investigated the effects of exercise on processes that are both impacted in the obese state. The findings in both Chapters were most likely affected by the alterations to the microenvironment around SAT provided by repeated bouts of exercise over time. In our investigation we were able to show alterations to existing mature adipocytes as well as preadipocytes. It is

reasonable to hypothesize that the changes in mature adipocytes may have impacted preadipocyte function possibly through a mechanism of crosstalk between the two cell types. One possible mechanism is that exercise has been shown to reduce apoptosis in mature adipocytes (Sertie et al., 2013). This may have impacted preadipocyte adipogenic capacity due to the reduction in cell turnover. Additionally, changes to the stromal vascular fraction (SVF) may have impacted mature adipocyte function as well due to the pro-inflammatory nature of this fraction. Although these findings are important and novel, further experiments should be conducted to elucidate the mechanisms leading to the changes to SAT function experienced with exercise training.

5.2 Future Research Directions

In our findings from Chapter 3 we displayed an improved mitochondrial function in gluteal SAT of obese humans in response to exercise training. The improvement in mitochondrial function in this depot was independent of increases in mitochondrial content as evidenced by similar content of respiratory complexes and CS content before and after exercise training. One potential mechanism for the improvement in mitochondrial function may be related to increases in specific mitochondrial enzyme activities, namely complex I activity. The enzyme activity of respiratory complexes, including complex I, have been shown to be reduced in obese compared to lean subjects in isolated mitochondria obtained from SAT (Chattopadhyay et al., 2011). Obtaining enough SAT sample (~5-7g) from biopsy methods to perform mitochondrial isolation is challenging, however, mitochondrial activity assays have been performed in SAT homogenates in previous studies (Bogacka et al.,

2005; Camera et al., 2010). Although these are maximal activity assays, they may provide further information for the improvement in mitochondrial function in response to exercise training.

A limitation of our findings in Chapter 3 is that the experiments we conducted in relation to mitochondrial function and content were performed in whole tissue in SAT, which is composed of various cell types including immune cells. To achieve a clearer perspective on the effects of exercise training in SAT these experiments should be repeated in isolated adipocytes from SAT that can be obtained through collagenase treatment. Isolated mature adipocytes have been shown to have higher mtDNA copy number per cell than whole tissue in SAT (Kaaman et al., 2007). Experiments conducted in these cells may provide clearer results with less variability between paired biopsy samples. It is very likely that isolated adipocytes would have higher mitochondrial content in relation to total protein compared to whole tissue in SAT.

An intriguing topic in the field of adipose biology that we did not investigate in this dissertation are the effects of macrophages and other immune cells on SAT. Macrophages and immune cells account for a large percentage of the total cell composition in SAT. Recently it was shown that macrophages impact mitochondrial function in SAT through secreted factors in human cells *in vitro* (Keuper et al., 2017). With our frozen SAT samples we would be able to perform gene analysis of macrophage markers to investigate whether the expression is affected by exercise training. If gene expression is shown to be altered by exercise training we would then be able to perform correlations between changes in macrophage gene expression and changes in mitochondrial function in response to exercise training. Although these findings would be

correlational in nature, they would provide valuable information in relation to the mechanism of improved mitochondrial function in response to exercise training evidenced in our findings.

In our findings from Chapter 4 we displayed that the adipogenic potential of preadipocytes isolated from SAT of obese humans is reduced in response to 12 weeks of exercise training. Because this effect was evaluated *in vitro* after four rounds of cell passaging in culture, we hypothesized that the mechanism behind the exercise imprinting in this study may be epigenetic in nature. Epigenetics is a topic that has begun to be investigated in exercise science with many of the investigations being conducted in skeletal muscle. However, recently Rönn and colleagues (Rönn et al., 2013) performed an investigation that evaluated DNA methylation patterns in SAT in response to a 6-month exercise intervention and found that a vast number of genes displayed differential methylation in response to exercise training and that gene expression was altered in 1/3 of genes with changes to DNA methylation. It would be interesting to perform DNA methylation analysis of adipogenic and lipogenic genes on the stromal vascular fraction of SAT that contains preadipocytes. This type of investigation may reveal mechanistic changes to preadipocytes would explain the reduction in adipogenic capacity evidenced in our findings.

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adipocyte cellularity of the periepididymal white adipose tissue in rats. J Appl Physiol (1985). 2013; 115(3):394–402. doi: 10.1152/japplphysiol. 01272.2012 PMID: 23703117.

EAST CAROLINA UNIVERSITY
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TO: Justin LaFavor, MS, ECU, Department of Exercise and Sport Science, Mailstop 158

FROM: UMCIRB KwB

E MAILED 6-1-11 PI & RespFac

DATE: May 26, 2011

RE: Full Committee Approval of a Study

TITLE: "Role of NADPH Oxidase Activity on Muscle Microvascular Endothelial Function in Human Obesity"

UMCIRB #11-0262

The above referenced research study was initially reviewed by the convened University and Medical Center Institutional Review Board (UMCIRB) on **04/27/2011.** The research study underwent a review and approval of requested modifications on **05/17/2011** by expedited review. The UMCIRB deemed this **American College of Sports Medicine** (pending award) sponsored study **more than minimal risk** requiring a continuing review in **12 months**. Changes to this approved research may not be initiated without UMCIRB review except when necessary to eliminate an apparent immediate hazard to the participant. All unanticipated problems involving risks to participants and others must be promptly reported to the UMCIRB. The investigator must submit a continuing review/closure application to the UMCIRB prior to the date of study expiration. The investigator must adhere to all reporting requirements for this study.

The above referenced research study has been given approval for the period of **04/27/2011** to **04/26/2012**. The approval includes the following items:

- Internal Processing Form – revised (UMCIRB receipt date 05/16/11)
- Protocol (dated 4/18/11)
- Informed consent – revised (dated 05/16/11)
- Medical history questionnaire
- 3 day food record with directions
- Recruitment flyer

The following UMCIRB members were recused for reasons of potential for Conflict of Interest on this research study: None

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

The UMCIRB applies 45 CFR 46, Subparts A-D, to all research reviewed by the UMCIRB regardless of the funding source. 21 CFR 50 and 21 CFR 56 are applied to all research studies under the Food and Drug Administration regulation. The UMCIRB follows applicable International Conference on Harmonisation Good Clinical Practice guidelines.

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Notification of Amendment Approval

From: Biomedical IRB
To: Robert Hickner
CC: Gabriel Dubis
Date: 3/9/2017
Re:
Ame26_UMCIRB 11-0262
UMCIRB 11-0262
[IMPORTED] Role of NADPH Oxidase Activity on Muscle Microvascular Endothelial Function in Human Obesity

Your Amendment has been reviewed and approved at the convened meeting on 3/8/2017 12:15 PM of Biomedical IRB.

Approval of the amended study and any consent form(s) is for the period of 3/8/2017 12:15 PM to 3/7/2018. The Biomedical IRB determined that this revision does not change the overall risk/benefit ratio of the study.

Please note that any further changes to this approved research may not be initiated without UMCIRB review except when necessary to eliminate an apparent immediate hazard to the participant. All unanticipated problems involving risks to participants and others must be promptly reported to the UMCIRB. A continuing or final review must be submitted to the UMCIRB prior to the date of study expiration. The investigator must adhere to all reporting requirements for this study.

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

The approval includes the following items:

Document	Description
Informed Consent Clean(0.13)	Consent Forms

The following UMCIRB members were recused for reasons of potential for Conflict of Interest on this research study: None

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