The Effects of Aerobic Exercise and Lipolytic Stimulation on Skeletal Muscle and Adipose Expression of Perilipin 3 (Plin3) in Individuals With Type 2 Diabetes and Women With Polycystic Ovary Syndrome

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THE EFFECTS OF AEROBIC EXERCISE AND LIPOLYTIC STIMULATION ON SKELETAL MUSCLE AND ADIPOSE EXPRESSION OF PERILIPIN 3 (PLIN3) IN INDIVIDUALS WITH TYPE 2 DIABETES AND WOMEN WITH POLYCYSTIC OVARY SYNDROME

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Department of Biological Sciences

by

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August 2017
DEDICATION

This dissertation and all inspiration thereof for the pursuit of truth and knowledge is humbly dedicated to Baruch Spinoza.
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ABSTRACT

Ectopic lipid accumulation in skeletal muscle is linked with insulin resistance and type 2 diabetes (T2D). Neutral lipid is stored as lipid droplets, which are coated by lipid droplet coat proteins. One important class of these proteins are the perilipins. Little research has focused on perilipin 3 (PLIN3) and coatomer gtp-ases, theorized to be involved in PLIN3 directed lipid oxidation.

Under lipolytic stimulation with epinephrine or palmitate, forskolin, and ionomysin (PFI), primary human myotubes taken from health non-T2D donors showed increased protein content levels of PLIN3. Concordantly, following endurance exercise in non-T2D males—an experimental paradigm shown to increase lipolysis—levels of PLIN3 protein were increased and associated with changes in both in vivo and ex vivo lipid oxidation. In both primary myotubes and in muscle tissue from the exercise bout, several coatomer gtp-ases were significantly altered. From a second clinical investigation, PLIN3 muscle protein content was associated with in vivo and ex vivo lipid oxidation under resting conditions in non-T2D males. Similarly, knockdown of PLIN3 in primary human myotubes using siRNA showed frank reductions in lipid oxidation. Cross-sectionally, PFI treatment in myotubes from sedentary and T2D donors increased expression levels of PLIN3 and ARFRP1, while myotubes from active donors increased expression of PLIN5. When myotubes from all three cohorts were treated with brefeldin A, an inhibitor of the coatomer gtp-ase ARF1, lipid oxidation was decreased in sedentary and T2D donors, but not in active donors. This suggests a role for PLIN3 and coatomer-gtpases in lipid
oxidation and a potential alternate pathway for lipid oxidation in sedentary non-T2D and T2D individuals, not seen in active individuals.

In women with polycystic ovary syndrome (PCOS), a condition associated with insulin resistance, we discovered that PLIN3 expression in adipose is naturally knocked down when compared to weight-matched controls, along with reduced levels of lipid oxidation. Sixteen weeks of exercise training stimulated expression of PLIN3 in women with PCOS, concomitantly with increases in lipid oxidation. These results give credence to the myotube knockdown experiment that a naturally occurring absence of PLIN3 is associated with reduced lipid oxidation, which is rescued with aerobic exercise.
CHAPTER 1: INTRODUCTION

1.1 Type 2 Diabetes, Polycystic Ovary Syndrome, and Insulin Resistance

Type 2 Diabetes (T2D) is a metabolic disorder characterized by insulin resistance and hyperglycemia. In the United States, the prevalence has increased from 3.5 per 100 persons in 1990 to 8.3 per 100 persons in 2012, virtually doubling, with incidence rates increasing from 3.2 per 1000 persons per year to 7.1 per 1000 persons per year [1]. In particular, incidence and prevalence rates have significantly increased in adults aged 20 to 44 years over the past 20 years, thus affecting young aged individuals than in the past [1].

Though the clinical characteristics have been well defined as a fasting blood glucose (FBG) of $\geq 126$mg/dL or a hemoglobin A1c (HBA1c) of $\geq 6.5\%$, the specific etiology of the disease remains a mystery. Unlike type 1 diabetes, which has a sudden onset in youth, T2D typically has a longer, progressive, and insidious onset in which pancreatic beta cell function is preserved in the early stages of the disease, and is characterized with hyperinsulinemia in order to overcome tissue insulin resistance.

Though T2D is the principle disease associated with insulin resistance, other diseases have this as a significant hallmark. Polycystic ovary syndrome (PCOS) affects approximately 4-7% of women of reproductive age and serves as a main cause of infertility [2, 3]. Characterized by oligo- or amenorrhea, an important secondary feature of this illness is the presence of insulin resistance in approximately 20-43% of women with this disease [4, 5], and increased adiposity in approximately 70% of individuals [6].
Study into the direct pathogenesis of insulin resistance have, therefore, been of considerable importance into understanding both treatment and prevention of these diseases. As such, the three main body tissues that have been studied in the relation to these metabolic disorders have been the principal insulin stimulated tissues that mediate glucose metabolism: liver, adipose tissue, and skeletal muscle. One commonality found in the skeletal muscle and liver from biopsies of individuals with T2D is an over-abundance of lipid stores in these tissues (reviewed in [7]).

1.2 Ectopic Lipid Accumulation and Skeletal Muscle Insulin Resistance

One major theme that has thus far driven much investigation over the last two decades regarding the pathogenesis of T2D has been the “adipose tissue expandability hypothesis” where by larger quantities of lipid is deposited in skeletal muscle and liver as a result of an inability of adipose tissue expansion (reviewed in [8]). Within the framework of this theory, an unexplained etiology prevents the adipose tissue from expanding appropriately under conditions of positive energy balance. As such, since excess caloric intake of lipid cannot be properly deposited into adipose tissue, it ectopically accumulates within other insulin responsive tissues.

In addition to this ectopic lipid accumulation, the skeletal muscle from individuals with T2D has lower lipid oxidation levels [9]. This is coupled with the fact that insulin resistant individuals having lower levels of mitochondrial content for the processing of this excessively lipid overload [10, 11]. Thus, the accumulation of excess lipid is confounded by a reduced capacity to utilize it in cellular metabolism.
Focusing on skeletal muscle, it has been repeatedly demonstrated that intramyocellular lipid (IMCL) content as measured by $^1$H-Magnetic Resonance Spectroscopy (MRS) is inversely related to insulin sensitivity as measured by the gold-standard euglycemic-hyperinsulinemic clamp in both the soleus and tibialis anterior [12-14]. Nevertheless, this observation has been confounded with the phenomenon known as the athlete’s paradox, whereby highly insulin sensitive athletes possess significantly large levels of lipid within their skeletal muscle when compared to sedentary, but otherwise healthy counterparts [15]. Work from our group demonstrated that in primary skeletal muscle cultures from physically active individuals have higher levels of lipid over and above healthy, sedentary individuals [16].

1.3 Lipid Droplets as Stores for Intracellular Lipid

The major source of bioavailable nascent lipid stored intracellularly is in the form of lipid droplets. Lipid droplets represent the oldest, most basic form of intracellular organelle composed of a phospholipid monolayer surrounding a core composed primarily of triacylglycerides and cholesterol esters. Surrounded by a single phospholipid layer, lipid droplets serve as a reservoir for lipids needed for cellular functions whether they be metabolic, such as lipid oxidation, or constructive, such as the synthesis of membranes (reviewed in [17]). Because of this relation to both metabolism and membrane synthesis, lipid droplets have been implicated in pathological disease states ranging from obesity and T2D to the regulation of viral replication [18-20] and obscure inflammatory processes such as Reye syndrome [21].

In 2008, Guo et al published an important manuscript that highlighted the differences of lipid droplet associated protein species based on lipid droplet size [22]. As such, other variations in the lipid droplet coat protein proteome have been explored in relation to lipid
oxidation, lipid droplet biosynthesis, lipid droplet trafficking, and interaction with other organelles such as the mitochondria [23].

1.4 Perilipin Family of Proteins

The perilipin family of proteins represent a set of five proteins that to date have been shown to primarily function by localizing to lipid droplets. This group was originally referred to as the PAT proteins, taking on the first letter initials of the original names of the first three: perilipin (now PLIN1), adipocyte differentiation related protein (ADRP, now PLIN2), and tail interactive protein of 47 kDa (TIP47, now PLIN3) [24]. Perilipin 1 (PLIN1) was first discovered in 1991 [25] and was shown that through the process of alternate splicing consists of four protein isoforms (labeled A, B, C, and D), with A and B shown to be expressed in both the adipose tissue and steroidogenic cells, and with C and D expressed exclusively in steroidogenic cells such as the adrenal glands and gonads [26]. Perhaps most extensively investigated has been the mechanism behind the intracellular signaling between PLIN1 in the adipose as a recruiter of hormone sensitive lipase (HSL) and release of comparative gene identification-58 (CGI-58) to adipose triglyceride lipase (ATGL) following phosphorylation to facilitate the breakdown of triacylglycerides (reviewed in [27]). Importantly though, PLIN1 is not expressed in the skeletal muscle of mammalian species [28].

Further investigations in the skeletal muscle have focused on the associations between lipid oxidation and other perilipins, namely perilipin 2 (PLIN2) and perilipin 5 (PLIN5), which are both highly expressed in skeletal muscle [28]. Investigations have shown that PLIN2 is associated with insulin sensitivity of muscle tissue despite elevations in muscle lipid
accumulation [29]. PLIN2 expression has also increased in individuals with T2D following metformin therapy [30].

Investigations into PLIN5 in the skeletal muscle have perhaps been the most published and most extensive in diabetes literature. PLIN5 overexpression in mice greatly increases lipid oxidation [31], which may be in part explained by the observed direct protein-protein interaction with ATGL [32, 33]. It was shown also that PLIN5 localizes primarily to lipid droplets that contain almost exclusively triacylglycerols, while PLIN1, PLIN2, and PLIN3 localize to droplets that contain an even mixture of triacylglycerols and cholesterol esters with perilipin 4 (PLIN4) localizing to droplets composed almost entirely of cholesterol esters [34]. PLIN5 has been shown to localize not only to lipid droplets, but also to the mitochondria, which has led to hypotheses that PLIN5 might in some way signal mitochondrial translocation to lipid droplets for efficient lipid oxidation [35]. Finally, PLIN5 was shown to have phosphorylation sites on serine residues, but that theses phosphorylation sites are not altered with muscle contraction, stimulation with epinephrine, or increased lipolysis [36]; this is unlike the function of PLIN1.

Transcriptional control of perilipins 1, 2, 4, and 5 have been identified in both adipose tissue as well as skeletal muscle; and it has been shown that different peroxisome proliferator-activated receptors (PPARs) and co-factors of such, namely PPARγ coactivator 1α (PGC-1α), seem to be involved in their expression (Figure 1.1). Since these targets are involved in lipid metabolism as well as adipocyte differentiation, it would stand to reason that lipid droplet coat proteins would be under control of such transcription factors. The one perilipin where transcriptional response elements have not been confirmed is perilipin 3.
Perilipin 3 (PLIN3) is a 47kDa protein that has been described in detail through x-ray crystallography analysis by Hickenbottom et al. in 2004 [42]. Formally named both mannose-6-phosphate receptor binding protein 1 (M6PRBP1) and tail interacting protein of 47kDa (TIP47), has been extensively investigated as a cellular trafficking molecule involved in the transport of mannose-6-phosphate receptor between endosomes and the Golgi apparatus, which role at present remains outside of the scope of this investigation. Its role as a lipid droplet coat protein
started to fuel academic interest in relation to metabolism following the publication of Wolins et al. [28] when it was shown to be highly expressed in skeletal muscle along with PLIN2 and PLIN5.

Prior evidence has suggested that PLIN3 might serve some function with regards to lipid oxidation. Pratts et al. showed that HSL co-localizes to PLIN3 coated lipid droplets in rat skeletal muscle [43]. Smirnova et al. showed that ATGL co-localizes with PLIN3 [44]. Though it has been shown that there is not a direct protein-protein interaction between PLIN3 and ATGL [32], there has been demonstrated interaction between the co-activator of ATGL, CGI-58 [45]. Additionally, very recent discoveries have found that PLIN3 also interacts with the mitochondria just as PLIN5 has demonstrated [46]. However, just like PLIN5, it was recently shown that though PLIN3 has phosphorylation sites on serine residues, phosphorylation is not affected by muscle contraction of epinephrine stimulated lipolysis [36].

1.6 Coatomer GTPases interact with Perilipin 3

One important element that has seemed to be absent in the literature surrounding PLIN3 in relation to lipid metabolism in skeletal muscle is its interaction with coatomer GTPases that are involved in transport between the endoplasmic reticulum (ER) and the Golgi apparatus. Soni et al. showed that ATGL co-localizes to PLIN3 coated lipid droplets under lipolytic stimulation in HeLa cells, a phenomenon that was obliterated with treatment with brefeldin A [47]. Brefeldin A is an inhibitor of the coatomer GTPase ADP-ribosylation factor 1 (ARF1) [48]. Furthermore, when several other coatomers were knocked down, namely ARF1, Golgi-Brefeldin A-resistant factor 1 (GBF1), and the beta subunit of coatomer 1 (βCOP1), ATGL was not able to co-localize to PLIN3 coated lipid droplets [47].
1.7 Pilot Data concerning Perilipin 3 mRNA expression in Diabetes

In 2008, unpublished data from our group showed the marked increase in the expression of PLIN3 mRNA in the skeletal muscle of individuals with T2D over and above that of obese non-T2D individuals and lean non-T2D individuals. It was from here that initial hypotheses were formed that PLIN3 might in some way be involved in lipid oxidation in skeletal muscle.

![PLIN3 mRNA expression graph]

**Figure 1.2: Pilot data of skeletal muscle mRNA expression of perilipin 3.** Individuals with T2D had significantly higher levels of PLIN3 over both healthy weight (lean) insulin sensitive and insulin resistant individuals, but did not differ from obese individuals.

1.8 Validity of the use of primary skeletal muscle myotubes for these investigations

Several of the experiments planned for the following investigations relied on both the collection, availability, and use of experiments involving primary skeletal muscle cultures that
were differentiated into myotubes. By using both primary myotubes as well as skeletal muscle biopsy tissue, our experiments hoped to provide a more profound translatability to understanding the disease processes related to reduced lipid oxidation in skeletal muscle of T2D. Additionally, use of primary myotubes would allow for the addition of more interventionally manipulation that would otherwise be unethical if conducted on live human research participants, such as the direct treatment of pharmacologics on the skeletal muscle as well as instituting knockdown of genes through siRNA.

As such, performing investigations into the tissues collected from human biopsies serves as a robust enough paradigm to translate back to human physiologic adaptation, it was necessary to validate that use of primary human skeletal muscle cultures would indeed provide significant knowledge that could be translated back to the cohort population that they represent. Prior investigations from our lab already demonstrated that lipid oxidation in myotubes retain the same characteristics of the donor [49]. Further investigations were conducted by myself and other that showed the translatability of lipid content within myotubes from active, sedentary, and T2D donors to positively reflect certain physiologic characteristics such as activity levels, insulin sensitivity, and maximal mitochondrial capacity [16]. Additionally, primary myotube content of ceramides and diacylglycerol species was inversely related to insulin sensitivity and maximal mitochondrial capacity [16].

In a continuation of this theme, it was shown in Covington et al [50] that mitochondrial content within primary myotubes as measured by mitotraker positively correlated significantly back to both the ex vivo expression of mitochondrial electron transport chain complexes and to in vivo measures of maximal mitochondrial capacity using magnetic resonance spectroscopy.
Further, the presents of type-I muscle fibers in culture was positively correlated with the presence of type-I fibers in the donor muscle tissue. This investigation also showed that the reflection of these phenotypic characteristics was preserved through passage four and five of myotube culture processing and preservation. Due to the logistical fact that in order to make use of myotubes from so many different biopsy sessions from so many different donors, the earliest passage of primary myotubes that could be used for investigation occurs at the fourth passage. As best as can be shown from the investigations in our lab, it would seem that the use of primary muscle cultures is, in fact, a good and effective paradigm for providing translatable results back to the donor phenotypic characteristics for experiments that require more mechanistic understanding.

1.9 Aims of the Investigation

The investigations that follow shall endeavor to answer the following questions:

1) Does the expression of Perilipin 3 inhibit lipid oxidation? As such, would lipolytic stimulation, either through pharmacologic modification or through increased physical activity (i.e., endurance exercise), decrease the levels of perilipin 3?

2) In relation to perilipin 3, how do other theorized lipid oxidation markers, notably the coatamer gtp-ases mentioned in Soni et al. [47], change in expression under lipolytic stimulation either through pharmacologic modification or through endurance exercise?

3) How does the knockdown of Perilipin 3 alter lipid oxidation in skeletal muscle tissue?

4) How is Perilipin 3 differentially expressed in the skeletal muscle among physically active individuals, healthy-sedentary individuals, and individuals with type 2 diabetes (T2D)? Likewise, how are coatamer gtp-ases expressed in these three cohorts? Further, what
happens to the expression of perilipin 3 and coatamer gtp-ases with pharmacological lipolytic stimulation? Similarly, what happens to perilipin 5 expression among these cohorts under lipolytic stimulation? What happens to lipid oxidation among these cohorts when treated with brefeldin A, the drug that blocks the action of ARF1?

5) Does a natural knockdown of Perilipin 3 exist in nature, and if so, what effect does it have on lipid oxidation? Likewise, how does exercise training influence the expression of perilipin 3?

In order to undertake the investigations in questions 1-4, we performed 1) experiments in primary human skeletal muscle myotubes from physically active healthy donors, sedentary non-T2D donors, and donors with T2D, 2) a clinical trial involving 20 healthy non-T2D males, who had skeletal muscle tissue biopsies taken before and immediately after a 650kcal endurance exercise bout in health non-T2D participants—an experimental paradigm shown to increase lipolysis both in vivo on the whole body levels and in vitro in skeletal muscle tissue, and 3) a clinical trial involving 29 health non-T2D male participants, who had biopsies of skeletal muscle tissue taken under resting conditions and who also underwent a 24 hour stent in a metabolic chamber. In order to investigate question 5, we discovered that a naturally occurring knockdown of PLIN3 exists in the adipose tissue of women with polycystic ovarian syndrome (PCOS). We performed a clinical trial in 8 women with PCOS and 8 weight matched non-PCOS control women as well as a 16-week exercise trial in the 8 women with PCOS, who had both adipose tissue biopsies and primary adipose tissue cultures established before and after aerobic exercise training.
It is hypothesized that PLIN3 expression in skeletal muscle is indeed associated with lipid oxidation and that differences will exist within primary skeletal muscle myotube cultures taken from sedentary/healthy donors, physically active donors, and donors with type 2 diabetes following lipolytic stimulation. Additionally, the investigation will seek to find if adipose tissue PLIN3 expression is associated with lipid oxidation in non-T2D insulin resistant females with PCOS, and what effects aerobic exercise would have on adipose tissue expression of PLIN3.
CHAPTER 2: SKELETAL MUSCLE PERILIPIN 3 AND COATOMER PROTEINS ARE INCREASED FOLLOWING EXERCISE AND ARE ASSOCIATED WITH FAT OXIDATION\(^1\)

2.1 Introduction

Ever since the discovery that intramyocellular lipid (IMCL) correlated inversely with insulin sensitivity in skeletal muscle, understanding lipid metabolism in skeletal muscle has been at the cusp of insulin resistance research. Lipid droplet dynamics is an integral part to understanding overall lipid metabolism. [12-14]. Paralleled to that, it has also been shown that endurance trained athletes as well as individuals with type 2 diabetes both have elevated IMCL, while the insulin sensitivities in these two groups remain polar opposites, [15] thus, indicating that merely having elevated IMCL does not necessarily result in insulin resistance. Therefore, understanding the regulation of lipid droplet dynamics within skeletal muscle would likely be an integral part to understanding overall lipid metabolism. Studies into the perilipin family of proteins have demonstrated that perilipin 2 (PLIN2, also known as ADRP) and perilipin 5 (PLIN5, also known as OXPAT) appear to be fundamental to lipid droplet oxidation and interaction of lipid droplets to mitochondria [29, 31, 35, 51-55]. However, few investigators have explored the regulation of perilipin 3 (PLIN3, also known as TIP47), a prominent, highly expressed lipid droplet-associated protein in skeletal muscle and its impact on lipid oxidation [45, 55, 56].

PLIN3 co-localizes to lipid droplets upon epinephrine stimulation and contraction in rat skeletal muscle [43]. Likewise, adipose triglyceride lipase (ATGL), a major lipase present in skeletal muscle, has been shown to co-localize to PLIN3 coated lipid droplets [44, 45]. Coatomer

\(^1\) Reprinted with permission from PLoS ONE March 2014, 9 (3): e91675. doi:10.1371/journal.pone.0091675. See Appendix A.
GTPases may have a possible regulatory role in the delivery of ATGL to PLIN3-coated lipid droplets via transport through the endoplasmic reticulum to Golgi apparatus [47]. Treatment with brefeldin A, a compound known to inhibit the ER-to-Golgi transport, prevents ATGL from localizing to PLIN3-coated lipid droplets in HeLa cells [47]. Furthermore, knockdown of ADP-ribosylation factor 1 (ARF1), ARF related protein 1 (ARFRP1), and Golgi-specific brefeldin A-resistance guanine nucleotide exchange factor 1 (GBF1) in HeLa cells and brown adipose tissue also prevents the co-localization of ATGL to PLIN3 coated lipid droplets [47, 57]. To date, only a few studies have investigated these pathways in skeletal muscle or in relation to exercise stimulation and therefore lipid availability for oxidative metabolism [45, 56]. Since endurance exercise is heavily dependent on lipid oxidation [58] and that endurance exercise trained athletes have high levels of IMCL [15], we hypothesized that perilipin 3 would be associated with exercise-induced lipolysis.

We therefore investigated the effect of exercise on PLIN3 protein and lipid droplet associated coatomers using both in vitro and in vivo approaches. In vitro experiments were performed using epinephrine [59] to stimulate lipolysis as well as a pharmacological cocktail of palmitate, forskolin, and ionomycin (PFI) to induce lipolysis in a primary human skeletal muscle cell culture model [60]. PLIN3 was also measured from human skeletal muscle biopsies taken before and after a long-duration endurance exercise bout. Furthermore, we investigated the expression of ATGL and the GTPases known to regulate ER-to-Golgi trafficking. These data suggest an important role of PLIN3 and ER-to-Golgi coatomers in relation to skeletal muscle lipid metabolism, and offers insight into potentially new lipolytic pathways for lipid metabolism in skeletal muscle.
2.2 Methods and Materials

2.2.1 Ethics Statement

The participants provided their written informed consent and all aspects of the study protocol were reviewed and approved by the Institutional Review Board at Pennington Biomedical Research Center.

2.2.2 Establishment of Primary Human Skeletal Muscle Cultures

Primary muscle cultures were established from muscle biopsies obtained from the vastus lateralis in five lean, healthy Caucasian male donors (Age 23.0 ± 1.9 yrs and BMI 24.2 ± 0.6 kg/m²). Establishment of human primary muscle culture has been modified from protocols as previously described [61]. Myoblast skeletal muscle progenitor cells were immuno-sorted using the 5.1H11 antibody provided by the Hybridoma Bank (University of Iowa) and the MACS cell sorting column system (Miltenyi Biotec, Auburn, CA). Myoblasts cultures from the five donors were grown simultaneously to approximately 90% confluence and then pooled together for experiments using protocols previously described [62]. Cells were further grown to approximately 80% confluence, and then treated with α-Minimum Essential Medium (Life Technologies, Grand Island, NY) supplemented with 2% fetal bovine serum (Life Technologies, Grand Island, NY), 1% bovine fetuin (Sigma, St. Louis, MO), and 1% Penstrep at 5 mg/mL (Life Technologies, Grand Island, NY) to trigger differentiation. Cells were maintained in differentiation media for 7 days and were considered myotubes upon visual assessment of fused, longitudinal, multinucleated cells.
2.2.3 *In vitro* Epinephrine and PFI Treatment with Primary Human Myotubes

Myotubes were treated using techniques adapted from Watt et al. [59] with 100 µM Epinephrine (Sigma, St. Louis, MO) for a time course of 15 minutes, 30 minutes, and 1 hour with collection of total protein at each time point. For PFI experiments, myotubes were treated with 30 µM palmitate, 4 µM forskolin, and 0.5 µM ionomycin (PFI) – all purchased from Sigma (St. Louis, MO). We previously showed that PFI-treatment in myotubes increased palmitate oxidation, increased mitochondrial oxidative phosphorylation complexes expression, and improved glucose uptake [60]. Briefly, myotubes were maintained in differentiation media for 4 days and then treated for 1 hour each day with PFI for 3 additional days. Differentiation media was similarly changed each day, without PFI, for control cells. Following 3 days of PFI treatment, total protein and mRNA were collected immediately following PFI (0 minutes) and for a time course of 15 minutes, 30 minutes, and 1 hour after PFI treatment. Total protein was collected using RIPA buffer (Sigma, St. Louis, MO) supplemented with 2% Protease Inhibitor Cocktail (Sigma, St. Louis, MO), 2% Phosphatase Inhibitor Cocktail 2 (Sigma, St. Louis, MO), and 2% Phosphatase Inhibitor Cocktail 3 (Sigma, St. Louis, MO). Total mRNA was collected using QIAzol (Qiagen, Germantown, MD).

2.2.4 Endurance Exercise Bout in Human Participants

Twenty healthy, normoglycemic male participants (16 Caucasian, 3 African American, 1 non-specified race) who were not engaged in sports at competitive level, were recruited to participate in this trial. Characteristics of these participants are provided in Table 1. Body composition was assessed by dual energy x-ray absorptiometry (DXA, QDR 4500A; Hologics, Waltham, MA) and VO2max was measured on a stationary bicycle ergometer (Lode Excalibur,
Groningen, The Netherlands) using an incremental workload protocol with simultaneous gas exchange measurements using a metabolic cart (TrueOne 2400; ParvoMedics, Sandy, UT). VO$_2$max and DXA measurements were assessed at a period of greater than 2 days before the exercise intervention in order to prevent any confounding acute effects of exercise on baseline measurements. Before the exercise bout, participants were admitted in the evening to the institutional in-patient unit. The following morning, after an overnight fast, resting metabolic rate was measured using a DeltaTrac metabolic cart and a percutaneous skeletal muscle biopsy of the vastus lateralis muscle was performed. Additional results of this exercise trial have already been published [63]. Gas exchange while exercising was assessed from expired air collected by mouthpiece using a Parvomedics TrueOne 2400 metabolic cart. Total energy expenditure and substrate oxidation were calculated as previously described [64]. Participants then exercised on a stationary bike at 50% their VO$_2$max until they had expended 650 kcals. Indirect calorimetry measures were performed after 8%, 20%, 40%, 60%, 80% and right before exercise completion in order to gage when 650 kcals of energy had been expended. Blood was drawn at regular intervals coupled to indirect calorimetry measures with epinephrine and norepinephrine determined by chemiluminescent immunoassay (Immulite 2000™, Siemens Healthcare Diagnostics, Deerfield, IL); serum glucose, insulin, and lipids by an enzymatic assay on a Beckman Coulter DXC 600 (Beckman Coulter, Brea, CA). Manufacturer’s protocols were followed for all the serum measurements. Here we have only reported serum measures from before and after exercise (Table 2). Immediately following the exercise bout, a second percutaneous skeletal muscle biopsy was obtained from the vastus lateralis proximal to the first biopsy.
2.2.5 Skeletal Muscle Biopsy Procedure

After local anesthesia with lidocaine/bupivacaine, skeletal muscle samples were collected using the Bergstrom technique with suction from the vastus lateralis (Propper Manufacturing Co., Long Island City, NY). Two separate incisions were made to collect tissues at baseline and post-exercise. The second biopsy collections were obtained within a time frame of no more than 3 minutes following the completion of exercise. All skeletal muscle samples were visually assessed and cleared for intramuscular adipose tissue, and then immediately snap frozen in liquid nitrogen for mRNA and Protein measures. Samples were blotted dry and then mounted in a mixture of Optimal Cutting Temperature (OCT, Thermo Scientific, Waltham, MA) and Tragacanth powder (Acros, Geel, Belgium) for immunohistochemical measures of glycogen, intramyocellular lipid, and fiber typing. Another sample was collected for measurements of ex vivo palmitate oxidation.

2.2.6 Immunohistochemical Measures

Measures of fiber typing and intramyocellular lipid (IMCL) were performed as previously described using immunofluorescence techniques [63]. Fiber typing was done by immunohistochemistry performed on 12 µm sections, obtained by using a Microm HM 550 (Thermo Scientific, Waltham, MA). A mouse monoclonal antibody specific for slow myosin heavy chain type 1 (MHC1) was used to detect type 1 fibers (MAB1628; Millipore, Burlington, MA), and a rat monoclonal antibody against laminin (AB2500; Abcam Inc, Cambridge, MA) was used to detect myofiber cell membranes. Sections were then counterstained with Bodipy 494 dye (Molecular Probes, Eugene, OR) to stain for IMCL. Images were taken using a confocal microscope (Leica TCS SPS AOBS resonant scanning multiphoton confocal microscope, Leica
Microsystems, Wetzlar, Germany) and type I fibers were counted. IMCL was determined using the Sigma Scan Pro 5 software (SPSS, Chicago, IL) by delineating Bodipy staining within myofibers. Glycogen content was measured using Periodic Acid, Shiff staining and analyzed using the Sigma Scan Pro 5 software [65]. Representative images from before and after the exercise intervention for IMCL, fiber typing, and glycogen are provided in supplemental figure 2. For all histology measures, three cross-sectional slices were obtained within the tissue. No less than 50 fibers were assessed from each cross-sectional slice for IMCL content, fiber type, and glycogen.

2.2.7 Ex vivo Palmitate Oxidation Measures in Skeletal Muscle

The palmitate oxidation assay was performed in skeletal muscle as previously described [63]. Briefly, approximately 75 mg of skeletal muscle tissue was homogenized and loaded into a trapping plate apparatus to assess gas exchange for fatty acid oxidation. 0.176 µM of total palmitate (0.088 µM of [1-14C]-palmitate in 0.088 µM of non-radiolabeled palmitate) was added to the muscle homogenate. Radiolabeled palmitate was obtained from American Radiolabeled Chemicals (St. Louis, MO). Radiolabeled 14CO2 and incomplete acid soluble intermediates from palmitate oxidation were assessed using scintillation counting. Data was adjusted to total protein content obtained from muscle homogenate as determined through the bicinchoninic acid assay (Pierce BCA, Thermo Scientific, Waltham, MA).

2.2.8 Gene Expression in Skeletal Muscle

Total mRNA from both in vivo and in vitro experiments was extracted using the miRNEasy Mini Kit (Qiagen, Germantown, MD), and cDNA was made using the High Capacity cDNA Kit (Applied Biosystems, Foster City, CA). Detection of gene expression was performed
using TaqMan Gene Expression Assays-on-Demand (Applied Biosystems, Foster City, CA); a list of catalogue numbers for each gene product is as follows: Ribosomal Protein, Large Protein O (RPLPO; Hs99999902_m1); Coatomer 1, beta subunit (βCOP1; Hs00200674_m1); Coatomer 2, sec23a subunit (Sec23a; Hs00197232_m1); ADP-Ribosylation Factor 1 (ARF1; Hs00796826_s1); Golgi Brefelden A resistant GTPase Exchange Factor 1 (GBF1; Hs00188327_m1); ARF related peptide 1 (ARFRP1; Hs00182389_m1); PPARgamma, co-activator 1 alpha (PGC1α; Hs01016719_m1); Peroxisome proliferative receptor alpha (PPARα; Hs00947538_m1); Pyruvate Dehydrogenase Kinase 4 (PDK4; Hs01037712_m1); and Beta-hydroxyacyl-CoA Dehydrogenase (β-HAD; Hs00193428_m1). Real-Time qPCR was carried out using the 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA), and expression levels were determined against a standard curve. Skeletal muscle and myotube gene expression was adjusted to the expression of RPLPO.

2.2.9 Protein Content in Skeletal Muscle

Protein content was measured from total protein extracts by western immunoblotting using the Criterion apparatus and 12.5% SDS-polyacrylamide gels (all purchased from Bio-Rad, Hercules, CA) and adjusted to either GAPDH (AB9484; AbCam, Cambridge, MA) or total protein assessed by Ponceau S stain (Sigma, St. Louis, MO). The antibody for PLIN3 was purchased from Novus Biologicals (Cat no. NB110-40764, Littleton, CO). The antibodies for GBF1 (AB86071), ATGL (AB109251), and ARFRP1 (AB108199) were purchased from AbCam (Cambridge, MA). The antibody for ARF1 was purchased from Epitomics (Cat no. 1635-1; Burlingame, CA).
2.2.10 Statistical Analysis

Data was analyzed using PRISM GraphPad Software, version 6.0 (GraphPad Software, La Jolla, CA). All data were found to be normally distributed using the Shapiro-Wilk Normality test. A paired, Student two-way t-test was used to assess baseline and intervention measures, and Pearson’s correlations were used in the exercise intervention. A one-way ANOVA was used to determine differences in gene expressions at different time points for the in vitro PFI experiments. A p value < 0.05 was considered statistically significant.

2.3 Results

2.3.1 PLIN3 changes in response to in vitro epinephrine and lipolytic cocktail (PFI treatment) in human myotubes.

The PLIN3 protein content increased steadily with epinephrine stimulation throughout the time course (Figure 2.1A). Likewise, the ER-to-Golgi coatomer GTPases ARF1 and ARFRP1 also increased (Figure 2.1A) 30 minutes and 1 hour respectively following the epinephrine stimulation. The coatomer GTPase, GBF1 was maximally expressed after 30 minutes of epinephrine prior to the noticeable maximal increases in PLIN3 and ARFRP1 (Figure 2.1A). ATGL levels were virtually not changed during the duration of epinephrine treatment (Figure 2.1A).
Figure 2.1. Epinephrine and lipolytic cocktail (PFI) in human primary myotubes increases perilipin 3 and coatomer proteins. A) Primary human skeletal muscle myotubes (n = 5) were stimulated with 100uM of epinephrine. PLIN3 expression as assessed by densitometry increased continuously with epinephrine. Additionally, GTPases ARF1 and GBF1 increased 30 minutes following epinephrine stimulation and ARFRP1 increased 1 hour following epinephrine stimulation. B) PLIN3, ATGL ARFRP1, GBF1 and ARF1 protein content increased after in vitro lipolytic stimulus (PFI) in primary human skeletal muscle myotube (n = 5).

In response to the lipolytic cocktail (PFI), PLIN3 protein content increased immediately following PFI treatment when compared to control conditions, achieving maximal values after 30 minutes (Figure 2.1B). Similarly, the coatomer GTPase GBF1 had maximal expression that coincided with PLIN3 maximal expression at the 30 minute time point (Figure 2.1B). PFI treatment increased the expression of the coatomer GTPase ARFRP1 (Figure 2.1B). Finally, ATGL expression was increased, albeit slightly, over controls immediately upon the completion of PFI treatment that steadily declined up to 1 hour after PFI treatment (Figure 2.1B).

Regarding mRNA expression levels, sec23a, a subunit of the coatomer 2 protein complex (COPII), and arf1 increased 2.5 and 3-fold respectively immediately after PFI treatment (Figure
2.2). The mRNA expression of gbf1 did not reach significant increase until 30 minutes and 1 hour following PFI treatment (Figure 2.2).

![Graph showing mRNA expression of Sec23a, ARF1, and GBF1](image)

**Figure 2.2. Coatomer GTPase gene expression changes with lipolytic cocktail (PFI) in human primary myotubes.** mRNA level of Sec23a, ARF1 and GBF1 in cultured human myotubes before and after PFI treatment (0, 30 min and 1h) (n = 5). *p < 0.05

2.3.2 PLIN3 changes in response to an endurance exercise bout in human muscle

The endurance exercise bout was successful at reducing whole-body respiratory exchange ratio (RER), increasing serum free fatty acid (FFA) concentration and *ex vivo* muscle palmitate oxidation, thus confirming that lipid oxidation was being favored over carbohydrate oxidation (Table 2.2). In parallel to the increased lipid oxidation, PLIN3 and ATGL protein content increased with exercise (Figure 2.3A, 2.3B and 2.3C). Similarly, the coatomer GTPase ARF1 increased at both protein and the mRNA level (Figure 2.3A and 2.3D); arfrp1, gbf1, sec23a, and βcop1 increased only at the mRNA level (Figure 2.3D). Both *ppara*, a transcription
factor known to regulate the expression of the perilipin protein family [66-68], and its co-activator \textit{pgc1α}.

\textbf{Table 2.1. Anthropometric Characteristics of Participants in the Single Endurance Exercise Bout.} BMI, body mass index; FM, fat mass; FFM, fat free mass; HOMA-IR, homeostatic model assessment for insulin resistance; FFA, free fatty acids.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Yr)</td>
<td>24.0 ± 4.5</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>76.7 ± 6.5</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>180.3 ± 5.4</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>23.6 ± 1.8</td>
</tr>
<tr>
<td>% Body Fat</td>
<td>16.6 ± 3.2</td>
</tr>
<tr>
<td>FM (kg)</td>
<td>12.8 ± 3.1</td>
</tr>
<tr>
<td>FFM (kg)</td>
<td>63.9 ± 4.7</td>
</tr>
<tr>
<td>$\text{VO}_{2}\text{Max}$ (mL/min/FFM)</td>
<td>47.2 ± 5.7</td>
</tr>
<tr>
<td>Fasting Glucose (mg/dL)</td>
<td>88.0 ± 4.6</td>
</tr>
<tr>
<td>Fasting Insulin (mU/dL)</td>
<td>3.6 ± 1.6</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.78 ± 0.37</td>
</tr>
<tr>
<td>Fasting FFA</td>
<td>0.45 ± 0.17</td>
</tr>
<tr>
<td>% of Type 1 fibers</td>
<td>35.9 ± 11.7</td>
</tr>
<tr>
<td>% of Type 2 fibers</td>
<td>64.2 ± 11.7</td>
</tr>
</tbody>
</table>

increased their mRNA expression with exercise. mRNA expression of $\beta$had and pdk4, two enzymes that favors fat oxidation, were also increased with exercise, further confirming lipid oxidation being favored in the skeletal muscle with exercise (Figure 2.3E).

Changes in PLIN3 protein with exercise were positively associated with both the change in \textit{ex vivo} muscle palmitate oxidation ($r = 0.49$, $p = 0.04$; Figure 2.4A) and with cumulative whole-body fat oxidation after adjusting for fat-free mass (FFM; $r = 0.52$, $p = 0.03$; Figure 2.4B).

We noted a slight decrease in IMCL content; however, despite increases in \textit{in vivo} and \textit{ex vivo} fat oxidations, the decrease in IMCL content did not reach significance ($p=0.21$, Table 2.2). We found that those individuals who increased PLIN3 with exercise tended to have an inverse
relationship to the change in IMCL ($r = -0.35, p = 0.10$). However, after adjusting for fiber-type cross-sectional area, a measure referred to as IMCL density, participants who increased PLIN3 protein content with exercise had a significant inverse correlation with IMCL density in type II fibers ($r = -0.58, p = 0.02$); whereas there was no relation with IMCL density in type I fibers (data not shown). As expected, glycogen content in the skeletal muscle significantly decreased after exercise ($p=0.001$, Table 2.2). Changes in PLIN3 protein content were positively associated with changes in glycogen ($r = -0.55, p = 0.01$; Figure 2.4C), suggesting that those participants who increased their PLIN3 favored lipid oxidation over carbohydrate oxidation during exercise.

Changes in PLIN3 protein, however, did not have any relation with changes in serum FFA, epinephrine or norepinephrine concentrations.

### Table 2.2. Clinical and Muscle Fiber Characteristics Before and After the Single Endurance Exercise Bout

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Post-Exercise</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RER</strong></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>p value</td>
</tr>
<tr>
<td></td>
<td>0.95 ± 0.04</td>
<td>0.89 ± 0.03</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Palmitate Oxidation, ex vivo (nmol/hr/mg protein)</strong></td>
<td>615.9 ± 375.9</td>
<td>887.3 ± 404.3</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>Total IMCL Content (AU)</strong></td>
<td>27.7 ± 27.5</td>
<td>21.3 ± 19.4</td>
<td>0.21</td>
</tr>
<tr>
<td><strong>IMCL Content in type 1 fibers (AU)</strong></td>
<td>29.3 ± 28.6</td>
<td>23.4 ± 22.6</td>
<td>0.21</td>
</tr>
<tr>
<td><strong>IMCL Content in type 2 fibers (AU)</strong></td>
<td>25.9 ± 25.9</td>
<td>20.1 ± 17.9</td>
<td>0.25</td>
</tr>
<tr>
<td><strong>IMCL Fiber type 1 density (AU)</strong></td>
<td>11.2 ± 13.6</td>
<td>7.9 ± 7.5</td>
<td>0.24</td>
</tr>
<tr>
<td><strong>IMCL Fiber type 2 density (AU)</strong></td>
<td>16.3 ± 16.9</td>
<td>13.3 ± 13.3</td>
<td>0.40</td>
</tr>
<tr>
<td><strong>Glycogen Content (AU)</strong></td>
<td>8.40 ± 0.79</td>
<td>7.32 ± 0.68</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>FFA (mmol/L)</strong></td>
<td>0.45 ± 0.17</td>
<td>0.73 ± 0.30</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Epinephrine (mg/dL)</strong></td>
<td>46.9 ± 17.8</td>
<td>193.4 ± 77.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Norepinephrine (mg/dL)</strong></td>
<td>302.2 ± 149.0</td>
<td>986.2 ± 347.6</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Figure 2.3. Changes in protein and gene expression relating to lipolysis with a single bout of endurance exercise in human skeletal muscle. A) Representative blots of PLIN3, ATGL, ARF1, ARFRP1 and loading control GAPDH. B) Quantitative bar graph of skeletal muscle PLIN3 protein after an acute exercise bout (n = 19). C) Quantitative bar graph of skeletal muscle ATGL protein after an acute exercise bout (n = 19). D) mRNA levels of lipid droplet coatamer genes (n=19), and (E) mRNA levels of oxidative genes, in skeletal muscle of healthy subjects in response to an acute exercise bout (n=14-19). *p < 0.05.
Figure 2.4. Associations between change in perilipin 3 (PLIN3) protein in skeletal muscle tissue and fat oxidation. A) Correlation between the change in PLIN3 protein expression and the change in ex vivo palmitate oxidation measured from skeletal muscle tissue homogenates (n = 18). B) Correlation between the percent change in PLIN3 protein expression and whole body cumulative fat oxidation measured by indirect calorimetry adjusted for fat free mass (n = 18). C) Correlation between the change in total glycogen content and the change in PLIN3 protein during exercise (n = 18).

2.4 Discussion

Perilipin 3 is a lipid droplet coat protein previously shown to co-localize to lipid droplets and with ATGL upon lipolytic stimulation [44, 45]. Our data shows for the first time in human primary muscle cells that PLIN3 expression increases in response to both epinephrine stimulation and to a pharmacological cocktail known to induce lipolysis. Importantly, we also show for the first time that PLIN3 expression increases in vivo, in skeletal muscle tissue of healthy, lean males following a long endurance exercise bout. The increase in PLIN3 protein correlated positively with ex vivo muscle homogenate palmitate oxidation as well as whole-body cumulative fat oxidation with exercise.

Our data supports the hypothesis that PLIN3 is involved in lipid oxidation within skeletal muscle. Prats et al. showed that in individual rat muscle fibers, PLIN3 co-localizes to lipid droplets after epinephrine administration [43]. Smirnova et al. showed that during lipolysis, ATGL co-localizes to lipid droplets coated with PLIN3 and replaces PLIN3 along the surface of
lipid droplets [44]. Our PFI treatment in human myotubes demonstrates that PLIN3 is increased immediately after the cessation of the lipolytic stimulus (Figure 2.1B). Additionally, PLIN3 expression increases from immediately following PFI treatment up to 30 minutes, while maximal expression of ATGL is immediately following PFI treatment with a steady decline in its expression following the lipolytic stimulation (Figure 2.1B). This supports the concept purported in Smirnova et al. stating that ATGL replaces PLIN3 during lipolysis. Furthermore, we show that PLIN3 in myotubes increases in response to in vitro epinephrine stimulation as well as in vivo after an endurance exercise bout (Figure 2.1A, 2.3A, and 2.3B). The increases in PLIN3 protein content in tissue were positively associated with changes in ex vivo palmitate oxidation in skeletal muscle (Figure 2.4A), cumulative whole-body fat oxidation (Figure 2.4B), and with changes in glycogen content in the muscle (Figure 2.4C). Those individuals who increased their PLIN3 protein content with exercise, had a trend towards an inverse relation to changes in IMCL content with exercise (data not shown). Together, these data suggest that PLIN3 protein is involved in lipolysis induced by either in vitro pharmacological stimuli or by endurance exercise in the skeletal muscle.

Our data also shows increases in several coatamer GTPases after lipolytic stimulation both in vitro and in vivo. Previous experiments have shown that knockdown of βCOP1, ARF1, GBF1, or Sec23a prevents the colocalization of ATGL to PLIN3 coated lipid droplets [47]. Guo et al. hypothesized that the coatamer GTPases are involved in the partitioning of lipid droplets for lipolysis and lipase interactions [22]. However, no direct protein-protein interaction between ATGL and PLIN3 has been observed [32]. Therefore, we speculate that an increase in PLIN3 along with coatomers GTPases suggests that PLIN3 facilitates lipolysis by serving as a targeting
signal for directing lipase delivery to lipid droplets. Our data reported here, though is novel, is observational and a true mechanism of these relationships cannot be fully elucidated from this study. Future proteomic investigations will be necessary to conclusively extrapolate a definitive mechanism for their interaction.

One of the limitations of our study is that although we recruited 20 male participants, we were only able to perform molecular investigations in 14 to 19 participants for some of our measures, due to limited amounts of skeletal muscle tissue. Additionally, since we collected biopsy samples at baseline and immediately after exercise bout, we are only able to provide the acute effect of exercise on PLIN3 content. An inverse correlation between changes in PLIN3 and IMCL density in type II fibers was observed but cannot be explained based on our current data. Future studies on fiber type specific IMCL and PLIN3 expression may provide insight to this correlation. Furthermore, based on these observational studies we cannot provide insight as to why the maximal expression of mRNA and protein level of GTPases investigated occur at different time points following the lipolytic stimulation (Figure 1A and 1B). We can merely state that increases in GBF1, ARFRP1, ARF1, Sec23a, and βCOP1 occur either at the mRNA level, the protein level or both at some time points following lipolytic stimulation. However, it is important to note that this is the first study that demonstrates an increase in PLIN3 and coatomer GTPases in human skeletal muscle following lipolytic stimulation both using in vitro and in vivo experiments. Future studies would be necessary to determine the effects of lipolytic stimulus duration, signal transduction, co-localization, and protein-protein interactions between PLIN3 and coatomer GTPases in skeletal muscle lipid metabolism. Interestingly, Louche et al. recently reported an upregulation of PLIN3 protein content after 8 weeks of
endurance exercise training [69]. Future studies should also be conducted to determine the chronic effects of exercise and various types of exercise training on the PLIN3 in relation to lipid oxidation.

In conclusion, our data demonstrates for the first time that the increase in expression of perilipin 3 and coatamer associated targets involved in ER-to-Golgi cargo transport with exercise are involved with exercise-stimulated lipolysis in skeletal muscle. These data offer a previously unexplored potential pathway for the regulation of lipase access to nascent or fragmenting lipid droplets and the regulation of lipolysis within skeletal muscle. This may provide further insight into potential aberrations in skeletal muscle fat oxidation, which is shown to occur in obesity, type 2 diabetes and lipodystrophy.
CHAPTER 3: PERILIPIN 3 DIFFERENTIALLY REGULATES SKELETAL MUSCLE LIPID OXIDATION IN ACTIVE, SEDENTARY AND TYPE 2 DIABETIC MALES

3.1 Introduction

Impaired lipid oxidation along with elevated levels of intramyocellular lipid (IMCL) are prominent characteristics of skeletal muscle pathophysiology associated with T2D [9]. It has been demonstrated that reduced insulin sensitivity in skeletal muscle is strongly associated with increased IMCL content [13]. However, Goodpaster et al. showed in 2001 that athletes, who are highly insulin sensitive also have high levels of IMCL known as the athlete’s paradox [15]. Efforts to understand lipid storage and utilization in skeletal muscle are important to provide insight into the athlete’s paradox.

Storage and packaging of lipid droplets in skeletal muscle has recently become an important area of research. Much research has been extensively focused on perilipin 5 (PLIN5), expressed in skeletal muscle, [28] and has been shown to be associated with lipid metabolism [28, 28, 31-33, 35, 53, 56, 70]. Unfortunately, another lipid droplet protein highly expressed in skeletal muscle [28], perilipin 3 (PLIN3), has been neglected. Studies conducted in mouse skeletal muscle have shown PLIN3 to colocalize to lipid droplets during catecholamine stimulation and also after muscle contraction [43]. Other studies have shown that ATGL colocalizes to PLIN3-coated lipid droplets during lipolytic stimulation [44, 45]. Though ATGL does not directly interact with PLIN3 [32], other investigations have shown that coatamer GTPases, such as ADR-ribosylation factor 1 (ARF1), ARF-related peptide 1 (ARFRP1), and Golgi-

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2 Reprinted with permission from the Journal of Clinical Endocrinology and Metabolism. October 2015, 100 (10): 3683-3692. doi: 10.1210/JC.2014-4125. See Appendix B.
Brefeldin A resistant factor 1 (GBF1), are involved in pathways that deliver ATGL to PLIN3-coated lipid droplets, thus facilitating lipid oxidation [47, 71]. We have recently shown a possible role of PLIN3 on skeletal muscle lipid oxidation using both in vitro and ex vivo models of exercise [72]. However, apart from this observational study, the role of PLIN3 for skeletal muscle lipid oxidation has not been heavily investigated. To the best of our knowledge, no studies have examined skeletal muscle lipid oxidation following knockdown of PLIN3 in skeletal muscle cells, nor has the differential expression of PLIN3 and PLIN5 been investigated in myotubes from actives, T2Ds, or metabolically healthy sedentary lean subjects following lipolytic stimulation.

We aimed, therefore, to perform an investigation in three phases to examine the effects of PLIN3 on skeletal muscle lipid oxidation. First, we performed a clinical investigation in 29 sedentary, healthy, non-obese, normoglycemic males to examine the relations between the skeletal muscle protein expression of PLIN3 to both whole body fat oxidation as measured using a 24-hour metabolic chamber and ex vivo skeletal muscle specific lipid oxidation. Second, we cultured primary myotubes from five of these participants to determine the role of PLIN3 on lipid oxidation by knocking down PLIN3. Finally, we compared PLIN3 and PLIN5 protein content following in vitro pharmacological lipolytic stimulation (Palmitate, Forskolin, and Ionomycin, PFI cocktail), as well as lipid oxidation following brefeldin A (BFA) treatment, a pharmacologic compound show to inhibit ARF1 and coatomers that associate with PLIN3 [47] in primary skeletal muscle myotubes cultures from active, sedentary lean, and T2D donors. We hypothesized that PLIN3 would be associated with both whole body and muscle specific lipid oxidation, and that knockdown of PLIN3 in primary human myotubes would result in lower lipid
oxidation. We also hypothesized there would in fact be a differential expression of PLIN3 and PLIN5 in primary skeletal muscle cultures from these three cohorts, and that would be differential lipid oxidation response to BFA treatment from these cohorts.

3.2 Methods and Materials

3.2.1 Clinical Studies and Skeletal Muscle Biopsy Procedure

Twenty-nine sedentary, normoglycemic male participants were recruited into the EAT trial (Clinicaltrials.gov #: NCT01672632) and underwent body composition measures (dual energy x-ray absorptiometry [DXA], QDR 4500A; Hologics, Waltham, MA; and MRI, 3.0T magnet, Excite HD System; General Electric, Fairfield, CT), fasting blood serum measures, a euglycemic hyperinsulinemic clamp to measure insulin sensitivity, and a 24 hour respiratory chamber stay to measure 24 energy metabolism. All anthropometric and metabolic characteristics are provided in Table 1. Vastus lateralis muscle biopsies were obtained to measure protein content (n = 26), ex vivo palmitate oxidation (n = 15), and to establish primary myotube cultures in five lean, healthy male donors (lean). As a secondary study, skeletal muscle biopsies were obtained from the vastus lateralis from four lean, physically active male donors (actives), and four obese, type 2 diabetic male donors (T2D) to establish primary skeletal muscle cultures for cross-sectional comparison to sedentary, lean donors. Anthropometric and metabolic characteristics of the active, T2D, and the subset of five lean participants, in which primary muscle cultures were examined, are provided in Table 2. Active subjects were recruited based on their level of habitual physical activity level, if they were between 20-40 yrs of age, BMI 20-30 kg/m², were non-diabetic, were taking no medications, and were otherwise healthy. Physical activity level was calculated from a 7-day physical activity questionnaire recall and a
triaxial accelerometer worn for at least 4 days. Physical activity index (total daily energy expenditure/resting metabolic rate) was calculated using both methods and daily activity level was scrutinized from accelerometer data ensuring active subjects have an activity index of greater than 1.6. Another inclusion criterion for active participants was a VO$_{2_{\text{max}}}$ above 40 ml/kg/min. Active participants were enrolled in the ACTIV trial (Clinicaltrials.gov #: NCT00401791). T2D participants were enrolled in the TAKE TIME trial (Clinicaltrials.gov #: NCT00401791), if they had known type 2 diabetes mellitus, were weight stable, were otherwise healthy, and were permitted to be taking metformin, insulin and/or sulfonylureas, but not thiazolidinediones. All participants gave written informed consent, and all trials were reviewed and approved by the Institutional Review Board of Pennington Biomedical Research Center. Clinical anthropometric and metabolic characteristics of study participants were performed as follows: 24-hour whole body metabolism was measured using in a metabolic chamber as previously described [73]; basal and maximal in vivo assessment of skeletal muscle mitochondrial ATP production were performed under magnetic resonance spectroscopy (3T Signa Excite MRI; General Electric, Milwaukee, WI) as previously described [74, 75]; euglycemic-hyperinsulinemic clamps were performed for the ACTIV and TAKE TIME trials as previously described using a 120-minute protocol and an 80mU/min/m$^2$ insulin infusion [75] and for the EAT trial as previously described using a 120-minute protocol and a 50mU/min/m$^2$ insulin infusion [76]; fasting serum measures were assessed in a certified clinical chemistry laboratory. We reported cross-sectional measures of clinical data for GDR, 24hr energy expenditure, and mitochondrial ATP production (a surrogate marker for VO$_{2_{\text{max}}}$ [75]) to show that there were indeed differences between insulin sensitivity and physical fitness from the active participants.
compared to T2Ds. Skeletal muscle samples were collected after an overnight fast using the Bergstrom technique with suction (Proper Manufacturing Co., Long Island City, NY) from the vastus lateralis following administration of local anesthetic of lidocaine/bupivicaine. Clinical measures were performed in the same order for all participants with MRI/MRS measures performed first followed by a stent in the metabolic chamber for 24-hours. The participants then had skeletal muscle biopsies performed the morning that they emerged from the metabolic chamber, and then underwent the clamp procedure.

3.2.2 Assessment of in vivo Mitochondrial Capacity

Mitochondrial capacity was assessed both as the maximal rate of ATP production (ATPmax) and basal ATP demand/rate of ATPase (ATPase). These were both measured by $^{31}$P Magnetic Resonance Spectroscopy (MRS). Detailed descriptions of these methodologies have been previously described [75, 77]. ATPmax: Maximal mitochondrial capacity was assessed in the vastus lateralis by measuring the time constant (tau) required for Phosphocreatine (PCr) recovery after 45 seconds of isometric contractions of the quadriceps and the PCr level in resting oxygenated muscle (PCr$_{\text{rest}}$): ATPmax = PCr$_{\text{rest}}$/tau. Participants were asked to lie supine on the patient table of the 3.0 Tesla Magnetic Resonance Imager/Spectrometer (GE Excite) for approximately 45mins. After a scout scan to locate the vastus lateralis, $^{31}$P spectra were acquired every 60 seconds for 2 minutes to achieve baseline PCr, ATP, and Phosphate ion (Pi) concentrations; during acquiring $^{31}$P spectra, a blood pressure cuff was inflated around the upper thigh to 30mm Hg above systolic blood pressure. The cuff was inflated for approximately 16 minutes. The participants were then asked to perform isometric contractions of the quadriceps muscles for 45sec at a frequency of 1 contraction/sec in order to deplete PCr stores.
Replenishment of PCr stores were assessed following the 45sec isometric exercise. ATPase:

ATPase rate was measured by the rate of PCr breakdown in anoxic muscle, which represents the basal ATP demand of the cell. Anoxia in the vastus lateralis was induced by inflating a blood pressure cuff around the upper thigh to 50-60 mm Hg above systolic blood pressure, thus hauling blood flow and O₂ delivery, and therefore requiring the muscle to deplete stores of Mb-O₂ and Hb-O₂ to meet aerobic respiration needs. Cuff pressure was controlled with a Hokanson Rapid Inflator/Deflator system. Anoxia/ischemia was induced in the participant’s leg for 15 minutes with levels of PCr, ATP, and Pi assessed by ³¹P MRS in the vastus lateralis for 60sec every 2 minutes. In addition, glycolytic ATP production was assessed by measuring changes in PCr and cellular pH by ¹H MRS.

3.2.3 Measurement of Abdominal Subcutaneous and Visceral Adipose Tissue Volumes

Abdominal subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) volumes were assessed with a 3.0 T scanner (Excite HD System; General Electric, Milwaukee, WI). Between 240–340 images were obtained from the highest point of the liver to the pubic symphysis and analyzed by a single trained technician using Analyze software (AnalyzeDirect, Overland Park, KS). The mean coefficient of variation for three readings of the same scan was 9.9% for VAT and 1.8% for SAT. Estimates of MRI volumes were converted to mass using an assumed density of 0.92 kg/L.

3.2.4 Establishment of Primary Human Skeletal Muscle Cultures and in vitro Treatment with PFI, Brefeldin A, and Knockdown of PLIN3

Establishment of human primary muscle culture has been modified from protocols as previously described [61, 72], and distinct pooled cell lines for Lean, Active and T2D groups
were established for experiments using protocols previously described [62]. Myotubes were treated with 30μM palmitate, 4μM forskolin, and 0.5μM ionomycin (PFI cocktail) using techniques adapted as previously described [60, 72]. Brefeldin A (BFA) treatments were carried out in myotubes using techniques adapted from Soni et al [47]. Briefly, myotubes were maintained in differentiation media for 7 days and then treated for 30 minutes with Brefeldin A (BFA) at a concentration 1μg/mL (purchased from Sigma, St. Louis, MO). Knockdown of PLIN3 was achieved through siRNA silencing using Silencer Select Pre-designed siRNAs (Cat no. s19952) according to the manufacturer’s instructions (Life Technologies, Grand Island, NY). Myotubes were differentiated for 7 days and treated with lipofectamine in serum free media followed by siRNA incubation with either siPLIN3 or siScramble (siSCR, Life Technologies, Grand Island, NY), or exposed merely to serum free media for controls. Cultures were maintained under serum starvation for 24 hours per the manufacturer’s specifications.

3.2.5 Skeletal Muscle Tissue Measures of ex vivo and Primary Myotube Measures of in vitro Lipid Oxidation

Lipid oxidation was performed in skeletal muscle tissues as previously described [63]. Complete lipid oxidation in primary myotubes was measured as liberation of $^{14}$CO$_2$ using 100μM [1-$^{14}$C] oleate as previously described with slight modifications [78, 79]. Cells were treated with 500μL of radioactive media, which consisted of low glucose DMEM supplemented with 12.5mM HEPES, 1mM L-carnitine and 100μM [1-$^{14}$C] oleate (1μCi/mL), and maintained in an incubator (37°C, 5% CO$_2$) for 2h. At the end of the reaction period, the plates were placed on ice to stop the reaction, and 400μL of the reaction medium was transferred to plastic tubes, which were sealed with rubber caps, with the addition of 100μL of 70% perchloric acid to liberate $^{14}$CO$_2$, 

37
which was trapped in 200 µL of 1 N NaOH. NaOH trapped $^{14}$CO$_2$ was detected via scintillation counting. Data were normalized to protein content of each individual well.

3.2.6 Gene and Protein Expression Measures

Gene expression was performed as previously described [72] with Real-Time qPCR on a 7900HT Fast Real-Time PCR system (Life Technologies, Foster City, CA) using TaqMan Gene Expression Assays-on-Demand (Life Technologies, Foster City, CA). Catalogue numbers for each Assay-on-Demand product is provided as follows: ARF1 (Hs00796826_s1), Sec23a (Hs00197232_m1), CGI-58 (Hs01104373_m1), PLIN2 (Hs00765634_m1), and RPLPO (Hs99999902_m1). Expression levels were determined against a standard curve and adjusted to the expression of RPLPO. Total protein for all experiments (both in skeletal muscle tissue and in human primary myotubes) was collected using RIPA buffer (Sigma, St. Louis, MO) supplemented with 2% Protease Inhibitor Cocktail (Sigma, St. Louis, MO), 2% Phosphatase Inhibitor Cocktail 2 (Sigma, St. Louis, MO), and 2% Phosphatase Inhibitor Cocktail 3 (Sigma, St. Louis, MO). Protein content was assessed from total protein extracts using western immunoblotting adjusted to GAPDH (Cat no. AB9484; AbCam, Cambridge, MA). Imaging of western blots was facilitated on the Odyssey infrared imaging system (LiCor, Lincoln, Nebraska). Antibodies against PLIN3 (Cat no. NB110-40764) and PLIN5 (Cat no. NB110-60511) were obtained from Novus Biologicals (Littleton, CO); the antibodies for ATGL (Cat no. AB109251) and ARFRP1 (Cat no. AB08199) were obtained from AbCam (Cambridge, MA).

3.2.7 Statistical Analysis

Data was analyzed using the PRISM GraphPad Software, version 6.0 (GraphPad Software, La Jolla, CA). Pearson r correlations were used to assess associations between PLIN3
protein content and metabolic measures of lipid oxidation. One-way ANOVA with Tukey post-hoc tests were performed to compare differences between clinical and myotube measures across active, sedentary lean, and T2D individuals (Table 2 and Figure 4D). Two-way, paired student t-tests were used to assess differences between control and treatment measures in myotubes (Figure 4A, 4B, and 4C). Myotube experiments were performed in triplicate. All graphical data is presented as the mean ± SEM, and a p value < 0.05 was considered statistically significant.

### 3.3 Results

3.3.1 PLIN3 protein is associated with whole body and ex vivo lipid oxidation and PLIN3 knockdown results in marked decrease in complete lipid oxidation

The twenty-nine male participants (age: 26.8 ± 5.4 yrs, weight: 81.9 ± 10.3 kg, BMI: 25.5 ± 2.3 kg/m²) were considered on average insulin sensitive (glucose disposal rate (GDR) of 11.51 ± 2.54 mg/min/EMBS and fasting glucose level of 91.0 ± 6.7 mg/dL) and non-obese with a percent body fat of 19.4 ± 4.9. Fasting serum lipids showed a total triglyceride level of 87 ± 42 mg/dL, free fatty acids of 0.26 ± 0.08 nmol/L, total cholesterol of 171 ± 25 mg/dL, HDL-C of 55 ± 12 mg/dL, LDL-C of 99 ± 23 mg/dL, and the ratio of total cholesterol-to-HDL of 3.27 ± 0.96 as well as HDL-to-LDL ratio of 0.59 ± 0.20. These results have been provided in Table 3.1. Skeletal muscle protein content of PLIN3 was inversely related to 24 hour respiratory quotient (RQ) measured in a metabolic chamber (Figure 3.1A) and was positively associated with ex vivo palmitate oxidation in skeletal muscle tissue (Figure 3.1B). There were no other significant
Table 3.1: Anthropometric and Clinical Characteristics of Clinical Study. BMI, Body Mass Index; FM, Fat Mass; FFM, Fat Free Mass; SAT, Subcutaneous Adipose Tissue; VAT, Visceral Adipose Tissue; GDR, Glucose Disposal Rate; EMBS, Estimated Mean Body Size (FFM + 17.7); FFA, Free Fatty Acids

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<td>Weight (kg)</td>
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<td>% Fat</td>
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<td>VAT (kg)</td>
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<td>% Type-2a Fibers (vastus lateralis)</td>
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<td>% Type-2x Fibers (vastus lateralis)</td>
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<td>24 hour Respiratory Quotient</td>
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<td>GDR (mg/min/EMBS)</td>
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<tr>
<td>Insulin (μU/mL)</td>
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<tr>
<td>FFA (nmol/L)</td>
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<tr>
<td>Triglycerides (mg/dL)</td>
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<td>LDL-C (mg/dL)</td>
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<tr>
<td>Cholesterol/HDL</td>
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</tr>
<tr>
<td>HDL/LDL</td>
<td>0.59 ± 0.20</td>
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correlations between PLIN3 protein content and other clinical variables: GDR ($r = 0.14$, $p = 0.51$), Type-I fiber ($r = -0.32$, $p = 0.11$), body weight ($r = -0.12$, $p = 0.57$), fat free mass ($r = -0.09$, $p = 0.64$), fat mass ($r = -0.06$, $p = 0.77$), percent body fat ($r = -0.02$, $p = 0.91$), visceral adipose tissue ($r = 0.12$, $p = 0.59$), and subcutaneous adipose tissue ($r = 0.13$, $p = 0.53$). Knockdown of PLIN3 in myotubes from lean, sedentary donors revealed a ~85% reduction in complete oleate oxidation when compared to control and siSCR (Figure 3.1D). Figure 3.1C provides a representative immunoblot showing knockdown of PLIN3 in myotubes from lean donors.

3.3.2 Differential expression and response of PLIN3 and intracellular transport proteins after PFI treatment in myotubes from active, lean, and T2D.

In terms of PLIN3 protein content, lean cells revealed an increase of PLIN3 following PFI treatment and up to 24 hours after PFI treatment, similar to our previous experiment [72], while T2D myotubes continuously increase levels of PLIN3 protein content up to 24 hour (Figure 3.2), both expressing higher levels than actives. Instead, PLIN5 protein content was upregulated in actives from immediately following PFI treatment up to 24 hours after (Figure 3.2). Lean and T2D myotubes displayed virtually no PLIN5 protein content with PFI treatment (Figure 3.2). ARFRP1 had the most prominent protein content following PFI treatment in leans above both T2Ds and actives (Figure 3.2). ATGL levels were increased in active myotubes following PFI treatment up to the 1-hour time point, with increases in lean myotubes only at 1-hour and 24-hours post-PFI treatment, and virtually no changes in T2D myotubes (Figure 3.2). At 24-hour post PFI treatment, PLIN3 and ARFRP1 had higher protein content in lean and T2D myotubes (Figure 3.2); PLIN5, however, was greatly expressed only in active myotubes 24 hours post PFI treatment (Figure 3.2).
Figure 3.1: Correlation between resting PLIN3 muscle protein content with \textit{in vivo} and \textit{ex vivo} whole body and muscle specific lipid oxidation and lipid oxidation in siRNA knockdown of PLIN3 myotubes. A) PLIN3 protein content from the vastus lateralis is inversely related to 24 hour respiratory quotient (RQ) indicating that increases in PLIN3 protein content is associated with increased whole body fat oxidation (Pearson $r = -0.44$, $p = 0.02$, $n = 26$). B) PLIN3 protein content from the vastus lateralis is positively associated with \textit{ex vivo} palmitate oxidation from muscle tissue from the vastus lateralis indicating that increases in PLIN3 protein content is associated with increased skeletal muscle specific fat oxidation (Pearson $r = 0.61$, $p = 0.02$, $n = 15$). C) Representative images of western blots probed for PLIN3 from primary human myotubes from lean, sedentary individuals under control conditions, following scramble siRNA treatment, and following siRNA knockdown of PLIN3. D) Complete lipid oxidation levels in myotubes established from lean participants following knockdown of PLIN3 as compared to control and treatment with a scramble siRNA (SCR). A distinct decrease in lipid oxidation is noted in cells from leans following PLIN3 knockdown. Data represents mean ± SEM of experiments performed in triplicate. $^{*}p<0.05$ vs. control and vs. SCR.
Figure 3.2: Protein expression following lipolytic stimulation with PFI in myotubes from active, sedentary, and type 2 diabetic donors. Primary myotubes taken from active (A); sedentary, lean (L); and type 2 diabetic (T or T2D) participants display differential protein content following PFI treatment (time course from immediately following 3 days of PFI treatment to 24 hours post PFI treatment).

The mRNA expression of ARF1, a coatamer protein that interacts with GBF1 to facilitate intracellular transport [80], was elevated above control conditions following PFI treatments in lean myotubes only, while both T2D and active cells decreased their ARF1 expression, notably actives reducing their expression more than T2Ds (Figure 3.3A). Sec23a, a subunit of the COPII complex responsible for retrograde transport from Golgi-to-ER [81], had increased expression in both lean and active myotubes following PFI treatment, while it remained only minimally elevated in T2D cells (Figure 3.3B). CGI-58, the coactivator of ATGL [82], was grossly elevated in active myotubes following PFI treatment over and above both T2Ds and lean myotubes (Figure 3.3C). Finally, PLIN2, a perilipin protein formerly known as ADRP, had drastic reduction in
mRNA expression in active myotubes followed by T2D after PFI treatment, while cells from lean increased PLIN2 expression (Figure 3.3D).

Figure 3.3: Differential mRNA expression following PFI treatment (time course) of myotubes collected from actives, leans and T2Ds. A) ARF1, a coatomer protein associated with ER-to-Golgi transport, is highly expressed in cells from lean following PFI with actives showing the highest decrease. B) Sec23a, part of the COPII complex, is differentially expressed in the three cohorts with actives having the highest increase in expression following PFI treatment. C) CGI-58, the co-activator of the lipase ATGL, is most highly expressed in actives following PFI treatment. D) PLIN2, a member of the perilipin family, displayed a distinct expression pattern among the three cohorts with leans increasing their expression following PFI with actives having a near ~80 % decrease in mRNA expression, and T2Ds having between a ~20 % to ~60 % decrease following the time course post-PFI treatment. All experiments were performed in triplicate and represented as mean ± SEM for a percent change.

3.3.3 Reduced in vitro lipid oxidation following brefeldin A treatment in lean and T2D myotubes but not in actives

Treatment of myotubes with brefeldin A (BFA), a drug that inhibits the activity of ARF1 [48], displayed reduced in vitro oleate oxidation for lean (Figure 3.4A) and T2D donors (Figure 3.4B). However, oleate oxidation was unaffected following BFA treatment in active myotubes
(Figure 3.4C). Percent change of *in vitro* oleate oxidation compared to control conditions revealed a significant difference between cells from lean and active (p = 0.02) and trended towards a difference between T2Ds and active (p = 0.08), with no differences in oxidation between lean and T2D myotubes (Figure 3.4D).

**Table 3.2: Anthropometric and Clinical Characteristics from Primary Myotubes.** FM, Fat Mass; FFM, Fat Free Mass; BMI, Body Mass Index; GDR, Glucose Disposal Rate; EMBS, Estimated Mean Body Size (FFM + 17.7). P values are provided from one-way ANOVA. Tukey post-hoc test was used to evaluate differences between groups: a, p < 0.05 compared to Lean; b, p < 0.05 compared to Type 2 Diabetics; c, p < 0.05 compared to Active

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<td>4</td>
<td>5</td>
<td>4</td>
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</tr>
<tr>
<td>Weight (kg)</td>
<td>79.98 ± 8.88^b</td>
<td>76.50 ± 8.19^b</td>
<td>110.53 ± 18.31^a,c</td>
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<tr>
<td>FM (kg)</td>
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<td>FFM (kg)</td>
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<td>62.14 ± 4.30</td>
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</tr>
<tr>
<td>% Fat</td>
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<td>BMI</td>
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<td>Serum Glucose (mg/dL)</td>
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<td>GDR (mg/min/EMBS)</td>
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<td>11.16 ± 3.01^b</td>
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<td>Maximal ATP production (mM/min)</td>
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<td>Basal ATP production (μM/min)</td>
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<td>24 hr Energy Expenditure (kcal)</td>
<td>3854 ± 339.79^a,b</td>
<td>2181 ± 132.23^e</td>
<td>2675 ± 201.23^c</td>
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**Figure 3.4: Lipid oxidation in myotubes with and without brefeldin A treatment.** Complete fatty acid oxidation (CO$_2$ release) in myotubes established from lean (A), T2Ds (B), and actives (C) under control conditions and following 30 min treatment with brefeldin A (BFA), a drug known to inhibit ARF1 and ER-to-Golgi transport. D) Percent changes in fatty acid oxidation as compared to control conditions between leans, T2Ds, and actives. All data represents mean ± SEM of experiments performed in triplicate. *p<0.05 vs. control

### 3.4 Discussion

The athlete’s paradox has represented a curiosity in skeletal muscle insulin resistance research whereby both athletes and T2Ds possess elevated IMCL, yet represent the bilateral extremes across the insulin sensitivity spectrum. Our study has shown for the first time a differential protein expression of perilipin 3 and perilipin 5 in primary skeletal myotubes from active, lean and T2D following *in vitro* lipolytic treatment. Furthermore, our study has shown the novel potential role PLIN3 might play in facilitating skeletal muscle lipid oxidation as evident by the reduced lipid oxidation levels in myotubes from lean donors following PLIN3 knockdown.
and its associations with both whole body in vivo lipid oxidation and skeletal muscle specific ex vivo lipid oxidation.

Prior investigations into the PAT family of proteins in human skeletal muscle have focused extensively on the function of PLIN5. Repeated investigations have shown a direct protein-protein interaction between PLIN5 and ATGL [32, 33], and that PLIN5 overexpression in mice has enhanced lipid oxidation [31]. Our investigation shows that PLIN5 is highly upregulated in myotubes derived from active donors following stimulation with palmitate, forskolin, and ionomycin (PFI) that has previously been shown to increase lipid oxidation and been referred to as an “exercise mimetic” (Figure 3.2) [60]. Our findings support the hypothesis that skeletal muscle from endurance trained actives utilizes PLIN5 for packaging of lipid droplets for faster lipid oxidation due perhaps in part to the direct interaction between PLIN5 and ATGL, which has been shown previously [32, 33]. As we show in Figure 3.2, ATGL is more responsive acutely to PFI treatment in active myotubes, and likewise, CGI-58, the co-activator of ATGL, greatly increases mRNA expression in active myotubes over and above sedentary, lean myotubes and T2D myotubes following PFI treatment (Figure 3.3C). Additionally, it has been shown that PLIN5 packages lipid droplets composed exclusively of triacylglycerides (TAGs) [34]. We have previously shown that primary myotubes from active donors have significantly elevated levels of TAGs over both lean and T2Ds [16], thus providing an additional explanation for elevated levels of PLIN5 in actives following lipolytic stimulus.

However, the abundance of data concerning PLIN5 and its association with lipid oxidation has perhaps overshadowed other targets that may be relevant to muscle lipid storage for oxidation. PLIN3 has been shown, in our previous study, to be positively associated with
both whole body *in vivo* and *ex vivo* skeletal muscle tissue fat oxidation following an endurance exercise bout in twenty healthy males [72]. We also showed an increase in PLIN3 protein following epinephrine stimulation of primary human myotubes [72]. Prior investigations have shown a colocalization of ATGL to PLIN3 coated lipid droplets in HeLa cells [44, 45], and a colocalization of PLIN3 to lipid droplets in isolated mouse skeletal muscle [43]. All of these have provided circumstantial evidence to the involvement of PLIN3 with the facilitation of lipid oxidation in skeletal muscle. Our present study independently repeated our findings of PLIN3 protein content being associated with both whole body *in vivo* and skeletal muscle specific *ex vivo* lipid oxidation; this time, though, using a 24 hour metabolic chamber (Figure 3.1A) and using skeletal muscle taken under resting, basal conditions (Figure 3.1B). Importantly, our study with human myotubes also demonstrated a robust reduction in lipid oxidation following knockdown of PLIN3 (Figure 3.1C and 3.1D). Previously, we have reported that in a natural occurring knockdown of PLIN3 protein in primary adipose cultures taken from women with polycystic ovary syndrome (PCOS), fat oxidation is reduced, but is increased following aerobic exercise, which is concomitantly associated with increases in PLIN3 protein content [83]. Here, we show that induction of knockdown of PLIN3 protein content likewise results in reductions in fat oxidation. This evidence demonstrates a significant possibility that PLIN3 serves as a target responsible for facilitating lipid oxidation in sedentary, lean and in insulin resistant individuals. We further investigated whether the effects of exercise mimetic stimulation on PLIN3 and PLIN5 protein content in actives, leans and T2D donors. Our findings show a remarkable difference in PLIN3 and PLIN5 response upon exercise mimetic stimulation among these three cohorts. Actives almost exclusively expressed PLIN5 following exercise stimulation (Figure 3.2C)
while leans and T2Ds favor the expression of PLIN3 (Figure 3.2B). In addition, we investigated the expression of ER-to-Golgi transport coatamer GTPases. Prior investigations in HeLa cells have suggested the dependence of coatamer proteins in facilitating ATGL delivery to PLIN3 coated lipid droplets [47, 71]. We previously reported an upregulation of several coatamer factors following a long bout of endurance exercise in human skeletal muscle tissue [72]. We also previously reported the same phenomenon to occur in adipose tissue from women with PCOS, a cohort that seemingly favors PLIN3 expression following aerobic exercise [83]. Here, we have demonstrated a differential response of coatamer targets among cells from active, lean and T2D after in vitro lipolytic stimulus at the protein level (ARFRP1, Figure 3.2) and mRNA level (ARF1 and Sec23a, Figure 3.3). Additionally, we demonstrate the differences in related targets involved in lipid packaging and lipolysis among these populations (CGI-58 and PLIN2, Figure 3.3). Though these differences are novel, they alone were not fully insightful. Thus, we further investigated lipid oxidation levels in primary myotubes cultured from these three cohorts after treatment with brefeldin A (BFA), an inhibitor of ARF1 activity [48]. BFA has previously been shown to block ATGL delivery to PLIN3 coated lipid droplets [47], but has similarly been shown not to inhibit intracellular transport of PLIN3 [84]. We demonstrated a reduction in lipid oxidation in myotubes from lean and in T2D donors following BFA treatment; therefore, suggesting that lipid oxidation is dependent in part on coatamer GTPases in these two groups (Figure 3.4A and 3.4B). On the other hand, myotubes from active donors did not show a reduction in lipid oxidation, suggesting a differing divergent pathway not requiring coatamer GTPases for facilitating lipid oxidation (Figure 3.4C). The novelty of this aspect of our study was the demonstration of differential upregulation of PLIN3 and PLIN5 following exercise mimetic
stimulation, the differential expression of coatomer GTPases, and the differential lipid oxidation levels following BFA treatment. These data demonstrate the possibility of previously unexplored lipid oxidation pathways dependent on PLIN3 and coatomer GTPases that is more readily utilized in skeletal muscle of sedentary, lean and possibly T2Ds compared to actives. Furthermore, these data highlight that PLIN3 would be pharmacological target in individuals with T2D due to the fact that they seem to favor lipolytic induced expression of PLIN3 and do not seem to express much PLIN5.

One of the major strengths of our study is the fact that we independently reproduced positive correlational associations between both whole body in vivo using results from a 24 hour metabolic chamber and skeletal muscle ex vivo lipid oxidation with PLIN3 protein content. This shows that in two separate, independent studies from non-actives that skeletal muscle expression of PLIN3 is associated with lipid oxidation [72]. To further strengthen those results, we successfully knocked down PLIN3 protein expression in primary human myotubes collected from individuals, who participated in the reported clinical investigation, and showed that lipid oxidation in vitro is reduced with reduced PLIN3 protein content. A second major strength of our study is the fact that our subsequent data presented here is exclusively performed in human primary myotubes, as opposed to cultured cell lines. We and others have shown that human myotubes reflect the phenotype of the donor [16, 49], thus allowing a powerful tool for investigating the skeletal muscle pathophysiology associated with T2D by utilizing primary myotubes obtained from donors with T2D. Though there is no substitute for a rigorous clinical investigation from direct skeletal muscle tissue in these cohorts, we hold that our experiments
in primary human myotubes offer conclusive insight into PLIN3 as facilitating muscle lipid oxidation.

The T2D participants that we were able to recruit for this study were taking medications that assist in regulating hyperglycemia (e.g. metformin, sulfonylurea, and insulin). Therefore, the effects of medication on any differences in the results from T2D participants cannot be completely ruled out. We acknowledge that inclusion of an obese, non diabetic cohort as well as T2D subjects without medication could have provided further insight to our results. However, based on the fact that our aims were to explore the effects of lipolytic stimulation on PLIN3 content and the inhibition of coatamer function on levels of lipolysis in myotubes from the three cohorts to see if there were in fact differences, our data presented here are of great importance.

To conclude, the skeletal muscle expression of perilipin 3 is associated with both \textit{in vivo} and \textit{ex vivo} lipid oxidation, and has been shown as a target for the facilitation of lipid oxidation in primary human myotubes taken from healthy, lean, sedentary male donors. PLIN3 is more highly up regulated in myotubes taken from lean and T2D donors following \textit{in vitro} exercise mimetic stimulation. The ER-to-Golgi coatamer target ARF1 similarly seems to be involved with facilitating lipid oxidation in primary myotubes from lean and T2D donors. In contrast, primary myotubes from active donors do not seem to express much PLIN3, but rather express PLIN5, following \textit{in vitro} lipolytic stimulation. Additionally, active donors do not seem to be dependent on ARF1 or related coatamer proteins to stimulate lipid oxidation whereas sedentary leans and patients with type 2 diabetes did. These data indicate a potential for two separate pathways (PLIN3 and PLIN5) in regulation of lipid oxidation in skeletal muscle that may depend upon
training status of the individual, and thus may in part offer insight into the existence of the athlete's paradox.
CHAPTER 4: POTENTIAL EFFECTS OF AEROBIC EXERCISE ON THE EXPRESSION OF PERILIPIN 3 IN THE ADIPOSE TISSUE OF WOMEN WITH POLYCYSTIC OVARY SYNDROME: A PILOT STUDY

4.1 Introduction

Polycystic Ovary Syndrome (PCOS) is a complex endocrine and reproductive disorder affecting approximately 4-7% of women of reproductive age [2, 3]. As a principle cause of infertility in reproductive aged women, PCOS is characterized by the presence of menstrual disturbances, hyperandrogenemia, and ovarian cysts [85]. Similarly, approximately 70% of women with PCOS have increased adiposity [6], and between 20-43% have insulin resistance and reduced glucose control [4, 5]. One possible culprit speculated to contribute to this irregular metabolic phenomena is defects within the adipose tissue [86].

Adipose tissue functions as a storage reservoir for excess lipid, a reserve that must be readily lipolyzed upon increased energy demand. Inefficient ability of the adipose tissue to mobilize and secrete free fatty acids during conditions of energy demand have been linked to conditions of impaired glucose tolerance and type 2 diabetes [87-89]. It has been previously documented that women with PCOS have dysfunctions in lipase activity and catecholamine-mediated lipolysis [90-92]. Furthermore it has been reported that testosterone can reduce: 1) catecholamine-stimulated adipose tissue lipolysis [93, 94]; 2) expression of beta-adrenergic receptors in adipose tissue [93, 94], and 3) adrenergic stimulated lipolysis in the brown adipose tissue [95]. Possible molecular targets, such as the expression of lipases, have been implicated

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as factors regulated by testosterone [94, 96, 97], thus offering a possible relationship between impaired lipolysis and the hyperadrogenemic states seen in women with PCOS.

Exercise training has been shown to improve several metabolic impairments typical of PCOS [98-100], and has been shown to enhance adipose tissue lipolysis [101, 102]. Previous studies from our group revealed an increase in basal and pharmacologically stimulated lipolysis with isoproterenol from the adipose tissue following exercise training [103]. The molecular markers that regulate lipolysis in the adipose tissue of women with PCOS however have not been thoroughly studied.

Prior investigations have shown that a single nucleotide polymorphism within the perilipin gene (PLIN1) that exists in women with PCOS is associated with impaired glucose tolerance and increased LDL [104], perhaps implicating a potential role for the perilipin family of proteins, lipases, and related factors effecting adipose tissue lipolysis. Recent investigations have also identified several coatamer GTPase proteins (ARF1, Sec23a, βCOP, GBF1, ARFRP1), which are usually involved in ER-to-Golgi transport, and that are involved in the delivery of adipose tissue triglyceride lipase (ATGL) to lipid droplets, particularly those lipid droplets coated by perilipin 3 (PLIN3) [47]. We have recently shown that PLIN3 and coatamer GTPases in skeletal muscle tissue and in primary human skeletal muscle cultures were associated with increased fat oxidation following exercise and lipolytic stimulation [105]. We, therefore, hypothesized that these novel mediators of lipolysis might also be differentially expressed in the adipose tissue of women with PCOS, where lipolysis is impaired, and upregulated following aerobic exercise training. We investigated the expression of lipases, perilipins, and coatamer GTPases from adipose tissue in a cross-sectional cohort of 8 women with PCOS compared to
women with normal menses matched for age, BMI, and percent body fat. Furthermore, in the women with PCOS, we investigated the effects of a 16-week aerobic exercise training program on lipolysis both in adipose tissue and in stromal-derived adipose cultures. Our results indicate previously unexplored potential roles for perilipin 3 (PLIN3) and coatomers, (ADP-ribosylation factor 1 (ARF1), ARF related peptide 1 (ARFRP1), coatomer complex 1 subunit beta (β-COP1), and coatomer complex 2 subunit 23a (Sec23a)) in the regulation of lipolysis in the adipose tissue.

4.2 Materials and Methods

4.2.1 Participants and study design

Eight obese women with PCOS and eight age, BMI, and percent fat matched healthy women without clinical signs of abnormal menses or hyperandrogenemia were recruited in this study (anthropometric characteristics are provided in Table 1). The results reported for this investigation were an ancillary project to a clinical study, which was designed and powered to determine the effect of an aerobic exercise program on body composition and whole body insulin resistance in obese women diagnosed with PCOS. The original study was powered using the mean and standard deviation of glucose disposal rate (GDR) to estimate sample sizes required to measure a minimum 20% change in GDR from baseline. With a prospective study design (paired) with a target power of 80% and a significance level set to α=0.05, we can conclude that only 6 subjects would be needed to detect a 20% change in GDR from baseline. The main outcomes of this study were previously reported in Moro et al. [103] and in Redman et al. [106] The diagnosis of PCOS was assessed by the Rotterdam criteria. [107, 108] Women with PCOS had to possess two of the following criteria: confirmation by medical history of
menstrual irregularity (oligo- or amenorrhea), presence of more than 10 ovarian follicles 2-9mm in diameter as assessed by MRI, or either clinical (hirsutism score) or serum measures of androgen excess (elevated free androgen index, FAI). Other causes of oligomenorrhea (hyperprolactinemia, congenital adrenal hyperplasia, Cushing’s syndrome, hyperthyroidism) were excluded by medical history. Women in the control group were excluded from participation in our study for exercise training, use of contraceptive medications, and menstrual cycle irregularity together with androgen excess. All women in our control group had FAI values below 3.6 as defined as a cut-off value for FAI in the assessment of PCOS according to Hahn et al. [109] Additionally, all women in our control group reported regular menstruation, thus confirming that they did not have PCOS based on the Rotterdam guidelines. Potential subjects were excluded from participating in either group if they smoked, were taking any medications, had current or past history of cardiovascular disease, hypertension (>140/90 mmHg), diabetes (type 1 or type 2), kidney, liver or heart disease, alcoholism or substance abuse, were pregnant or trying to become pregnant and were unable to comply with exercise training program.

Adipose tissue measures are reported in all participants included in this study. Fat cell size was determined using osmium fixation and counted using a multisizer 3 Coulter counter (Beckman Coulter, Brea, CA) as previously described [110]. In vitro measures of stromal-derived adipocytes are reported from five women with PCOS before and after exercise due to availability of adipose tissue material for the isolation and culturing of stromal-vascular cells. For immunocytochemistry analysis of lipid droplet comparisons and protein immunoblotting, controls were taken from 5 women with normal menstrual cycling unrelated to the original study that were matched for age (24.2 ± 2.3 yrs, p = 0.37 compared to women with PCOS) and
BMI \((26.1 \pm 2.5 \text{ kg/m}^2, p = 0.83\) compared to women with PCOS)—this was necessary because the collection of stromal-derived adipose cultures was not part of the initial study protocol for the control subjects. The study design and protocol was approved by the institutional review board of Pennington Biomedical Research Center, and all volunteers gave written informed consent. This study was registered at clinicaltrials.gov (NCT01150539).

All women were examined at baseline, and the women with PCOS were reexamined after 16 weeks of aerobic exercise training. At each time-point, study testing occurred over 3 days. In order to reduce confounding factors associated with dietary differences and metabolic testing, we provided participants a standard diet of 50% carbohydrate, 35% fat, and 15% protein 2 days prior to testing and throughout the duration of testing. Following an overnight fast, blood and subcutaneous abdominal adipose tissue samples were collected, body composition (percent body fat surmised from all regions of the body) was assessed by dual energy x-ray absorptiometry (DXA, QDR 4500A; Hologic, Bedford, MA), and insulin sensitivity was determined by a hyperinsulinemic-euglycemic clamp (120-minutes at 80mU/min/m\(^2\)) as previously described [106]. Aerobic capacity (\(\text{VO}_2\max\)) was measured during a graded treadmill test (TrueMax 2400; ParvoMedics, Salt Lake City, UT). Serum testosterone and sex hormone binding globulin were determined by an automated chemiluminescent immunoassay on the Immulite 2000 (Siemens Healthcare Diagnostics, Deerfield, IL). Serum glucose and serum insulin were measured by an enzymatic assay on a Beckman Coulter DXC 600 (Beckman Coulter, Brea, CA). Ovarian morphology, abdominal subcutaneous adipose tissue (SAT), and visceral adipose tissue (VAT) as well as intrahepatic lipid and intramyocellular lipid (IMCL) of the soleus was
examined before and after 16 weeks of aerobic exercise training using a 3T MRI/MRS (GE 3.0T Signa EXCITE MRI, GE Healthcare, Pittsburgh, PA; results provided in Table 1).

4.2.2 Magnetic Resonance Imaging and Spectroscopy

Abdominal fat: An 8 channel torso-array coil was placed over the chest/abdomen area and 3.4 mm slices (1.7 mm intersection gap) were acquired from the highest point of the liver to the inferior pole of the right kidney. A total of approximately 220 images were acquired on each participant. Total (TAT), visceral (VAT) and subcutaneous (SAT) abdominal adipose tissue mass were calculated using the Analyze™ software package (CNSoftware, Rochester, MN).

Muscle and hepatic lipid content: For the muscle intramyocellular lipid measures, the right leg was positioned inside a $^1$H knee coil with the knee in extension and the ankle in a neutral position. Separate water suppressed PRESS boxes (10 x 7.5 x 7.5 mm voxels) were collected from the tibialis anterior (n=1), soleus (n=3) and peanut oil phantom (n=1). For the liver lipid content, with the participant lying prone, a $^1$H body coil was placed over the torso and a single PRESS box (30 x 30 x 30 mm) was collected in an area of the liver free from heavy vascularization. Data were analyzed using the jMRUi software package. Ovarian morphology: An 8 channel torso array coil was used to acquire coronal (T2 weighted fast spin echo (FSE), short T1 inversion recovery (STIR), and T1 weighted localizer), sagittal (T1 weighted localizer only) and axial (T2 weighted FSE and STIR) sequences. Images were acquired from the highest point of the uterus or ovaries through the bottom of the ovaries with 4mm thick slices and 1 mm intersection gap for a total of approximately 40 images. Images were analyzed using Analyze™ 8.1 (CNSoftware, Rochester, MN) by a trained analyst. Ovaries, as well as ovarian follicles, were located and quantified on each coronal FSE image. Follicles were located on an image and followed down
through the remaining consecutive images to ensure that each individual follicle was identified as such. The total number of follicles in each ovary was calculated using this technique. The volume of each follicle was calculated based on the number of pixels measured in each image and the number of consecutive images in which the follicle was located.

4.2.3 Subcutaneous Adipose Tissue Biopsy

Subcutaneous adipose tissue was obtained from the left upper quadrant of the abdomen with a 5-mm Bergstrom needle using the Bergstrom technique. The skin was cleansed with povidone-iodine solution, a sterile drape was placed over the incision site, and local anesthesia (5 mL 1:1 mixture of 0.5% bupivicaine and 2% lidocaine) was administered. An approximate 1cm incision was made and adipose tissue was collected.

4.2.4 Exercise training program

Aerobic exercise was performed under supervision at the Pennington Health and Fitness Center, five times per week. Aerobic exercise was prescribed on an individual basis with the objective to achieve specified exercise energy expenditure (ExEE) in each session. During the first four weeks, the target ExEE was 4% of the participants’ estimated energy requirement for weight maintenance, and was incremented to 6% for weeks 5-8, to 8% for weeks 9-12 and to 10% for weeks 13-16. All exercises were performed on a treadmill at 55% VO$_{2max}$, a moderate intensity. The necessary speed and gradient to achieve the ExEE was estimated from a linear regression of O$_2$ uptake and workload during the VO$_{2max}$ test and the ExEE was confirmed once during each 4-week interval by indirect calorimetry. Heart rate was monitored during all sessions to verify ExEE. The exercise time necessary to complete the energy expenditure target was 23±1 minutes per session during weeks 1-4, 35±1 minutes per session during weeks 5-8,
47±2 minutes per session during weeks 9-12 and 58±2 minutes per session during weeks 13-16. In order to prevent variation in results due to dietary influences during the exercise intervention, we asked participants to maintain their dietary habits throughout the 16-weeks of intervention as they had prior to study enrollment. Additionally, we requested updates on food intake during site visits for exercise sessions.

4.2.5 Stromal-derived adipocyte cultures, immunofluorencenc staining, lipolysis, triglyceride determination, and oleate oxidation

Adipose tissue samples from subcutaneous abdominal depot were collected under aseptic conditions and isolated stromovascular (SV) cells were cultured as previously described [111] and differentiated as previously described [112] with modifications: DMEM-F12 (1:1) medium plus 10 mg/ml transferin, 33 μM biotin, 17 μM calcium pantothenate, 0.5 μM insulin, 0.1 μM dexamethasone, 0.2 nM triiodothyronin, as well as 0.5 μM Roziglitazone, and 540 μM IBMX during the last 48 hours of culture. All experiments were performed on cells following 9 days of differentiation. Cultures were stained for lipids (BODIPY, 10 μg/ml) and DNA (DAPI, 300 nM) (Invitrogen, Carlsbad, CA). Images were obtained using the Leica TCS SP5 AOBS resonant scanning confocal microscope (Leica AG, Wetzlar, Germany). The lipolysis assay was performed over 3 hr by adding 0.2 ml of HBSS + BSA 2%. At the end of incubation, medium was collected for measurement of glycerol, performed in duplicate using the free glycerol reagent (Sigma-Aldrich, St Louis, MO) and adjusted for the triglyceride content. Triglyceride levels were then measured using a glycerol phosphate oxidase triglyceride determination kit (Sigma-Aldrich, St Louis, MO) and normalized to the protein content. Cultures were preincubated for 3 hr with [1-14C] oleic acid (1 μCi/ml; PerkinElmer, Boston, MA) and non-labeled (cold) oleic acid (100 μM).
Oleic acid was coupled to fatty acid-free BSA in a molar ratio of 5:1. Following incubation, $^{14}$CO$_2$ was measured as previously described [113] The cells were then lysed in 0.2 ml of SDS 0.1% for determination of cell-associated label uptake and protein content for normalization. All assays were performed in duplicate.

4.2.6 Real-time qRT-PCR and Western Blotting

Total RNA was extracted from approximately 100 mg of adipose tissue using miRNEasy Kits (Qiagen, Valencia, CA) according to the manufacturer’s specifications. RNA extracts were converted into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) and stored at -20°C until Real Time-PCR was performed. Gene expression was carried out using Real Time-PCR with TaqMan gene expression assays-on-demand on the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Gene expression assays were performed for the following genes: PLIN1 (Hs00160173_m1), PLIN2 (Hs00765634_m1), PLIN3 (Hs00998421_m1), PLIN4 (Hs00287411_m1), PLIN5 (Hs00965990_m1), GBF1 (Hs00188327_m1), ARF1 (Hs00796826_s1), ARFRP1 (Hs00182389_m1), βCOP (Hs00200674_m1), Sec23a (Hs00197232_m1), ATGL (Hs00386101_m1), CGI-58 (Hs01104373_m1), MGL (Hs00200752_m1), DGAT2 (Hs01045913_m1), mtGPAT (Hs00326039_m1), PPIA (Hs99999904_m1). Relative gene expression was assessed using a standard curve of known concentrations of mRNA and normalized to Cyclophilin A (PPIA) gene expression. Protein extracts from adipose culture were immunoblotted and probed with an antibody against PLIN3 (Novus Biologicals, Littleton, CO) and normalized against the loading control GAPDH (AbCam, Cambridge, MA).
4.2.7 Statistical analyses

All analyses were performed using GraphPad Prism Software, version 5.0 (GraphPad Software, La Jolla, CA). The Mann-Whitney test was used for cross-sectional comparisons between PCOS and Obese Control women when data was not normally distributed, and an Independent Samples t-test was used when the data was normally distributed. Paired t-tests were used to compare variables from baseline to the end of the exercise training intervention; except when the data was not normally distributed, Wilcoxon Signed Ranked Paired tests were used. P < 0.05 was considered statistically significant. All graphical data is presented as mean ± SEM.

4.3 Results

4.3.1 Comparison of controls vs. women with PCOS

Compared to the control participants, women with PCOS had elevated serum testosterone and free androgen index, as expected, but they did not differ in terms of sex hormone binding globulin (SHGB) (Table 4.1). Otherwise, control participants were effectively matched to women with PCOS for body composition, insulin sensitivity (fasting glucose, fasting insulin, glucose disposal rate, HOMA-IR), and energy metabolism (resting metabolic rate, fasting respiratory quotient (RQ), metabolic flexibility (i.e. changes between the fasting RQ and RQ during the steady state of the hyperinsulinemic euglycemic clamp) and VO$_{2\text{Max}}$ (Table 4.1). Importantly, women with PCOS did not differ from control participants with regard to mean subcutaneous fat cell size (Table 4.1).
Table 4.1: Anthropometric, metabolic, and serum markers for both the cross-sectional study and exercise training intervention. This data reflects the 5 females with PCOS for whom we were able to obtain primary stromal-derived adipose cultures both before and after exercise intervention. All follicles measured between 0.5-9 mm in diameter were only detectable in 3 of the 8 control participants, but all women with PCOS had greater than 10 follicles measuring between 2-9 mm in diameter. EMBS: Estimated Mean Body Size, FM: Fat Mass, FFM: Fat Free Mass, GDR: Glucose Disposal Rate, RQ: Respiratory Quotient, DHEA-S: Dehydroepiandrosterone Sulfate, SHBG: Sex Hormone Binding Globulin, FAI: Free Androgen Index, AT: Adipose Tissue, IHL: Intrahepatic lipid, IMCL: Intramyocellular

<table>
<thead>
<tr>
<th>Women with PCOS</th>
<th>Control</th>
<th>Pre-Exercise</th>
<th>Post-Exercise</th>
<th>Control vs PCOS</th>
<th>Pre- vs Post-exercise</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>29.7 ± 12.4</td>
<td>27.0 ± 2.9</td>
<td>--</td>
<td>0.65</td>
<td>--</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>78.9 ± 15.7</td>
<td>82.2 ± 18.2</td>
<td>81.4 ± 21.6</td>
<td>0.74</td>
<td>0.69</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.5 ± 5.4</td>
<td>30.8 ± 4.2</td>
<td>30.4 ± 5.8</td>
<td>0.17</td>
<td>0.66</td>
</tr>
<tr>
<td>Total body fat (%)</td>
<td>29.9 ± 9.8</td>
<td>36.8 ± 5.0</td>
<td>34.8 ± 6.1</td>
<td>0.19</td>
<td>0.21</td>
</tr>
<tr>
<td>FM (kg)</td>
<td>23.3 ± 9.3</td>
<td>30.8 ± 11.1</td>
<td>29.3 ± 13</td>
<td>0.23</td>
<td>0.39</td>
</tr>
<tr>
<td>FFM (kg)</td>
<td>55.6 ± 15.9</td>
<td>51.4 ± 7.9</td>
<td>52.1 ± 9.0</td>
<td>0.60</td>
<td>0.24</td>
</tr>
<tr>
<td>Visceral AT (kg)</td>
<td>1.4 ± 0.8</td>
<td>1.2 ± 0.5</td>
<td>1.1 ± 0.7</td>
<td>0.75</td>
<td>0.64</td>
</tr>
<tr>
<td>Subcutaneous AT (kg)</td>
<td>11.4 ± 1.8</td>
<td>10.9 ± 4.8</td>
<td>10.5 ± 5.7</td>
<td>0.86</td>
<td>0.47</td>
</tr>
<tr>
<td>IHL (AU)</td>
<td>0.013 ± 0.017</td>
<td>0.13 ± 0.17</td>
<td>0.03 ± 0.05</td>
<td>0.16</td>
<td>0.20</td>
</tr>
<tr>
<td>IMCL (soleus, AU)</td>
<td>0.006 ± 0.003</td>
<td>0.006 ± 0.004</td>
<td>0.007 ± 0.005</td>
<td>0.89</td>
<td>0.27</td>
</tr>
<tr>
<td>Glucose, fasting (mg/dL)</td>
<td>103.5 ± 28.6</td>
<td>82.2 ± 6.3</td>
<td>89.6 ± 5.5</td>
<td>0.14</td>
<td>0.08</td>
</tr>
<tr>
<td>Insulin, fasting (mg/dL)</td>
<td>11.0 ± 10.2</td>
<td>9.8 ± 3.9</td>
<td>12.4 ± 5.6</td>
<td>0.81</td>
<td>0.51</td>
</tr>
<tr>
<td>HOMA-IR (AU)</td>
<td>3.4 ± 4.2</td>
<td>2.0 ± 0.8</td>
<td>2.7 ± 1.2</td>
<td>0.50</td>
<td>0.42</td>
</tr>
<tr>
<td>GDR/EMBS (mg/kgFFM +17.7)</td>
<td>6.8 ± 4.3</td>
<td>6.3 ± 1.2</td>
<td>7.6 ± 1.6</td>
<td>0.79</td>
<td><strong>0.02</strong></td>
</tr>
<tr>
<td>Resting Metabolic Rate/FFM (kcal/day/kg)</td>
<td>30.3 ± 4.8</td>
<td>30.7 ± 5.0</td>
<td>30.0 ± 2.7</td>
<td>0.91</td>
<td>0.62</td>
</tr>
<tr>
<td>Resting RQ</td>
<td>0.80 ± 0.03</td>
<td>0.81 ± 0.02</td>
<td>0.80 ± 0.01</td>
<td>0.37</td>
<td>0.21</td>
</tr>
<tr>
<td>Clamp RQ</td>
<td>0.93 ± 0.07</td>
<td>0.90 ± 0.04</td>
<td>0.90 ± 0.05</td>
<td>0.50</td>
<td>0.83</td>
</tr>
<tr>
<td>ΔRQ (Clamp vs Resting)</td>
<td>0.13 ± 0.07</td>
<td>0.09 ± 0.02</td>
<td>0.10 ± 0.03</td>
<td>0.26</td>
<td>0.72</td>
</tr>
<tr>
<td>VO₂max (mL/min/kg)</td>
<td>27.7 ± 10.0</td>
<td>29.5 ± 3.0</td>
<td>33.6 ± 4.3</td>
<td>0.71</td>
<td><strong>0.04</strong></td>
</tr>
<tr>
<td>Fat Cell Size (nL)</td>
<td>0.69 ± 0.31</td>
<td>0.68 ± 0.13</td>
<td>0.76 ± 0.25</td>
<td>0.92</td>
<td>0.40</td>
</tr>
<tr>
<td>Testosterone (ng/dL)</td>
<td>35.4 ± 11.7</td>
<td>92.4 ± 41.2</td>
<td>64.8 ± 25.3</td>
<td><strong>0.002</strong></td>
<td>0.61</td>
</tr>
<tr>
<td>SHBG (nmol/L)</td>
<td>24.1 ± 4.6</td>
<td>30.9 ± 31.7</td>
<td>34.2 ± 19.3</td>
<td>0.56</td>
<td>0.75</td>
</tr>
<tr>
<td>FAI (AU)</td>
<td>5.1 ± 1.4</td>
<td>18.8 ± 14.2</td>
<td>13.9 ± 17.5</td>
<td><strong>0.02</strong></td>
<td>0.67</td>
</tr>
<tr>
<td>Number of ovarian cysts (2-9mm in diameter)</td>
<td>29.4 ± 46.1</td>
<td>97 ± 29.7</td>
<td>119.4 ± 28.6</td>
<td><strong>0.008</strong></td>
<td>0.10</td>
</tr>
</tbody>
</table>
Women with PCOS did however differ significantly from controls in terms of subcutaneous adipose tissue gene expression for candidates responsible for lipolysis, lipid droplet perilipin proteins, and coatomer proteins. As shown in Figure 4.1A, perilipin 1, 3, and 5 (PLIN1, PLIN3, and PLIN5, respectively) were ~80-90% lower in women with PCOS when compared to controls, while perilipin 2 (PLIN2) and perilipin 4 (PLIN4) were approximately 3-fold higher. Expression mRNA levels of coatomer GTPases ARF1, ARFRP1, and βCOP (Figure 4.1B) were ~80% lower in women with PCOS compared to controls, with GBF1 and Sec23a being expressed ~9- and 7-fold higher, respectively. Finally, lipase mRNA expression of ATGL and monoglycerol lipase (MGL) were reduced by 90% and 65% respectively, with no difference in the expression of CGI-58 (Figure 4.1C).

Figure 4.1: Gene expression from abdominal subcutaneous adipose tissue from women with PCOS versus age, BMI, and percent fat matched controls (cross-sectional study). A) Perilipin family of protein gene expression of all five perilipin proteins shows drastic differences in expression. Perilipin 1 (PLIN1), perilipin 3 (PLIN3), and perilipin 5 (PLIN5) are all expressed approximately 90% lower in females with PCOS compared to controls. Perilipin 2 (PLIN2) and perilipin 4 (PLIN4) are expressed about 3 times higher when compared to controls. B) Coatomer proteins involved in ER-to-Golgi transport were also differentially regulated on the gene expression level. ADP-ribosylation Factor 1 (ARF1), ARF related protein 1 (ARFRP1), and Beta-coatomer (βCOP, part of the COPI complex) were drastically reduced in women with PCOS. GDP-exchange Brefeldin A resistant Factor 1 (GBF1, which operated in conjunction with ARF1) and Sec23a (part of the COPII complex) were both expressed approximately 8-fold higher in the women with PCOS. C) Expression of Adipose Triglyceride Lipase (ATGL) and Monoglyceride Lipase (MGL) were both reduced in women with PCOS on the gene expression level, while there was no statistical difference in the expression of the ATGL co-activator CGI-58. *p < 0.05; **p < 0.01; ***p < 0.001
4.3.2 Effect of Sixteen-Weeks of Aerobic Exercise Training in Women with PCOS

Aerobic exercise resulted in a ~10% improvement in maximal aerobic capacity (VO\(_{2\text{Max}}\), \(p=0.04\)) and a ~20% improvement in glucose disposal rate (\(p=0.02\)) (Table 4.1). No other changes were noted in terms of body composition, abdominal adipose depot size (visceral or subcutaneous depots), ectopic lipid accumulation (in the liver or muscle), or in fat cell size (Table 4.1). However, alterations were seen in the gene expression in the adipose tissue following exercise. Of all the measured perilipins, only expression of PLIN3 increased significantly (\(p<0.05\); Figure 4.2A), whereas PLIN1, PLIN2, PLIN4 and PLIN5 did not change. On average, ARF1, ARFRP1, βCOP, and Sec23a expressions were increased by approximately 5-, 8-, 7-, and 4- fold, respectively (\(p<0.05\)), with no changes in GBF1 (Figure 4.2B). Additionally, the lipases ATGL, MGL and the ATGL co-activator CGI-58, increased significantly after sixteen weeks of exercise training (\(p<0.05\); Figure 4.2C).

**Figure 4.2:** Gene expression targets in adipose tissue known to be involved in lipid droplet regulation and lipolysis were altered at 16 weeks of exercise training in the women with PCOS (Exercise training intervention, women with PCOS only). A) Gene expression of the perilipin family of proteins from before to after exercise demonstrates that only PLIN3 is significantly elevated following exercise training. B) Coatomer gene expression reveals that ARF1, ARFRP1, βCOP, which were grossly decreased when compared to controls before exercise training, revealed an increase in their expression. Sec23a was also increased following exercise training. C) Gene expression of ATGL and MGL, which was blunted when compared to controls at baseline, increased with exercise training. Additionally, CGI-58 increased following exercise training. *\(p < 0.05\)
4.3.3 Primary Adipose Culture Lipid Droplet Morphology and Lipolysis

As shown in Figure 4.3, primary adipocyte cultures revealed larger lipid droplet morphology in women with PCOS compared to control, which decreased in size after exercise training, and thus more closely resembled the morphology of control females (Figure 4.3). *In vitro* measures of fat oxidation and lipolysis in primary cultured adipocytes showed that oleate oxidation and glycerol release were increased following exercise training (p<0.05, Figure 4.4A and 4.4B). Likewise, total triglyceride content was reduced after exercise (p<0.05, Figure 4.4C). Since PLIN3 was the only perilipin to significantly increase in adipose tissue on the mRNA level, we measured the protein level of PLIN3 in primary adipose culture. We found virtually nonexistent levels of PLIN3 in 4 out of 5 women with PCOS, while the expression of PLIN3 was present in controls (Figure 4.4D). Exercise induced protein expression of PLIN3 in those 4 women with PCOS, who had no protein expression of PLIN3 before exercise, and was increased.
compared to pre-exercise expression in the one woman with PCOS, who had PLIN3 protein expression present before exercise training (Figure 4.4D).

![Graph showing results](image.png)

**Figure 4.4: Ex vivo oleate oxidation studies in stromal derived adipose cultures from women with PCOS and PLIN3 protein content.** *Ex vivo* oleate oxidation studies revealed that stromal derived adipose cultures from women with PCOS had increased completed oleate oxidation as measured by $^{14}$C labeled CO$_2$ (A) after 16 weeks of exercise training. Furthermore, glycerol release into the culture media was increased (B) and total triglyceride content was reduced (C) in the adipose cultures of women with PCOS after exercise training. D) Both cross-sectional and exercise intervention expression of PLIN3, the only perilipin protein to increase in adipose tissue in the women with PCOS following exercise training on the gene expression level, were found to be virtually nonexistent in 4 out of the 5 females with PCOS donors when compared to controls in their primary adipocyte cultures. Likewise, expression levels increased in all 5 females with PCOS after exercise training in their primary adipose cultures. *p < 0.05

### 4.4 Discussion

Our data highlights a previously unrecognized, potential role of perilipin 3 in adipose tissue lipolysis in women with PCOS. We show for the first time that the expressions of PLIN3, along with PLIN1 and PLIN5, is greatly reduced in adipose tissue of women with PCOS when
compared to age- and body composition- and metabolically-matched females. PLIN3 mRNA expression is increased—the only perilipin protein to significantly increase—following 16 weeks of aerobic exercise training in women with PCOS. Additionally, stromal-derived primary adipose cultures from PCOS women revealed that virtually no PLIN3 protein is expressed before exercise training, but becomes expressed following exercise training coupled with increases in oleate oxidation. We previously reported that exercise training in this cohort increased adipose tissue lipolysis under adrenergic stimulation [103]. Our data suggest that PLIN3 may in part contribute to enhanced adipose tissue lipolytic stimulation.

The exercise benefits associated with improving the symptomatology of PCOS has been thoroughly investigated. Studies have shown improvements for women with PCOS in insulin resistance[98, 99, 114, 115], serum lipids[114, 116], and cardiovascular disease risk[98, 99, 114, 117]. Additionally, menstrual cycle improvements have been shown following exercise intervention in women with PCOS[98, 114, 118]. Though some studies show reductions in body weight with exercise[98, 99, 114], several studies have shown that benefits occur without weight loss[106, 115, 119] and suggest that exercise for PCOS would be recommended even if weight loss were not achieved. Our study showed improvements in insulin resistance as measured by euglycemic-hyperinsulinemic clamps (Table 4.1) and improvements in menstrual function[106]. However, despite all these benefits of exercise, few studies have investigated molecular targets in the adipose tissue from women with PCOS. This seems important given the reported defects in adipose tissue function previously shown in women with PCOS (reviewed in [86]). We previously reported that 16 weeks of aerobic exercise can improve basal and catecholamine stimulated lipolysis of adipose tissue[103]. A prior investigation showed a single
nucleotide polymorphism found in women with PCOS for the perilipin gene[104], indicating a potential for defects in the perilipin family of proteins, which are involved in lipolysis. Here, we have investigated targets that regulate adipose tissue lipolysis, with an emphasis on PLIN3 given the upregulation of gene expression seen in the adipose tissue with exercise.

Studies centered on the perilipin family of proteins have focused heavily on the involvement of PLIN1 in the regulation of lipolysis in adipose tissue (reviewed in [120]) via a coupling of PLIN1 phosphorylation and ATGL activation [121, 122]. However, the possible role of PLIN3 regulation in adipose lipolysis has been for the most part overlooked. Studies have shown PLIN3 to be highly expressed in adipocytes and described to have a preference for PLIN3 to coat smaller lipid droplets [123]. Additionally, studies in HeLa cells have revealed a colocalization of ATGL to PLIN3 coated lipid droplets during lipolytic stimulus [44, 47]. Furthermore, a handful of studies investigating the role of PLIN3 in skeletal muscle lipolysis demonstrated that PLIN3 colocalizes to lipid droplets in rats during epinephrine and muscle contractile stimulation [43, 45]. Work from our group has recently shown that PLIN3 expression is upregulated following aerobic exercise in skeletal muscle, upregulated in response to lipolytic stimulation in human primary skeletal myotube cultures, and is positively associated with both ex vivo skeletal muscle and in vivo whole body fat oxidation [105]. Our data here, using both in vivo and in vitro systems, shows that the association between PLIN3 and lipolysis is evident in the adipose tissue of women with PCOS, indicating PLIN3 as a potential novel mediator of lipolysis.

Our data also highlights previously uninvestigated coatomer GTPases that might potentially be involved in mediating lipolysis. We show a lower expression of ARF1, ARFRP1,
and βCOP1 in women with PCOS compared to controls; the expression of these targets increases greatly following 16 weeks of aerobic exercise training in the women with PCOS. Soni et al. showed that ATGL was delivered to PLIN3 coated lipid droplets: a phenomenon that was inhibited when either treated with brefeldin A, a compound known to inhibit ARF1, or when several of the coatamer GTPases, such as ARF1, Sec23a, βCOP, and GBF1 were knocked down [47]. Studies from our group have shown that ARF1, Sec23a, and ARFRP1 increase their expression with aerobic exercise in skeletal muscle and with lipolytic stimulation in primary skeletal muscle cultures [105]. Finally, Guo et al. found differential expression of coatamer GTPases with regards to differences in lipid droplet morphology [22]. Likewise, we show that independent of differences in fat cell size, women with PCOS possess larger lipid droplet morphology when compared to their age and body composition matched controls. Lipid droplet morphology more closely resembled control females following 16 weeks of aerobic exercise in the women with PCOS. Alterations in these coatamer GTPases may perhaps be involved in regulating lipolysis not only as a mediator of lipase delivery to lipid droplets, but also as a regulator of lipid droplet morphology. Further functional investigations are warranted to determine these mechanisms.

We also observed reduced mRNA expression of lipases (both ATGL and MGL) in the adipose tissue of women with PCOS, compared to controls. The expression of lipases increased with exercise, which would be expected given the increase in both in vivo and in vitro lipolysis. One aspect of lipase expression that remains elusive is that prior investigations have shown that testosterone mediates the expression of lipases [96, 97]. Our data shows that lipase expression is increased following exercise training with no decreases in circulating
concentrations of total testosterone or free androgen index, perhaps indicating that aerobic exercise can increase adipose tissue lipolysis despite altering testosterone expression. Furthermore, we speculate that the increases in lipase expression we observed are not solely responsible for the increased lipolysis following exercise training. In fact, previous reports show that lipases do not act alone on lipid droplets to facilitate lipolysis, but require a chaperone such as the perilipin proteins (reviewed in [120]) or certain coatomer GTPases [47].

We recognize that our results and conclusions are based on observational data and associations between increases in lipolysis, and expression of PLIN3 and coatomer GTPases following exercise, and does not establish causative results. However, given the preponderance of our findings and data presented in prior investigations from our group and others, we believe that these associations are novel and relevant to highlight potentially new, unrecognized targets that may in part mediate adipose tissue lipolysis. Additionally, it is noted that we only conducted the exercise intervention in the women with PCOS, since our original design was a prospective exercise intervention study for the women with PCOS. Although we did not conduct an exercise intervention in our control group since the cross-section component was added later, the goal of the study involving the control group was to perform the same set of state-of-the-art assessments (clamp, MRI, fat cell size, DXA) and to obtain a control group matched on the basis of metabolic phenotype. The cross-sectional comparison demonstrates novel and robust differences between the women with PCOS and controls and is able to further demonstrate that exercise in women with PCOS can rescue defects in lipolysis to levels observed in control subjects. Due to our rigorous study design, supervised and carefully monitored exercise interventions, and extensive phenotyping analyses of the women with PCOS
before and after their exercise intervention, we are confident that we were adequately able to identify the upregulation of PLIN3 gene expression as well as the coatomer GTPases, thus demonstrating their potential role in the improvements in adipose tissue lipolysis following exercise training. Further investigations would be necessary to understand specific mechanistic roles of PLIN3 in adipose tissue lipolysis.

In conclusion, based on prior evidence from single nucleotide polymorphisms in the perilipin gene [104], we investigated the expression of the perilipin family of proteins in the adipose tissue from women with PCOS, and have shown that several perilipin proteins are differentially expressed in women with PCOS versus age- and body composition-matched control females. We have also shown that coatomer GTPases (ARF1, Sec23a, βCOP, GBF1, ARFRP1) are differentially expressed in women with PCOS. Sixteen weeks of aerobic exercise training significantly increased PLIN3 expression as well as coatomer GTPases (ARF1, Sec23a, βCOP, GBF1, ARFRP1). Stromal-derived primary adipose cultures showed increases in \textit{in vitro} lipolysis, oleate oxidation, and reductions in triglyceride content following exercise training. Additionally, adipose cultures revealed virtually no PLIN3 protein expression before exercise, which was then increased/became expressed following exercise training. Finally, primary adipose cultures demonstrated a large lipid droplet morphology, which was altered by exercise training, despite the fact that there were no differences in fat cell size from adipose tissue cross-sectionally. These data highlights previously unrecognized and novel potential targets that might be responsible for improving exercise mediated lipolysis in the adipose tissue of women with PCOS.
CHAPTER 5: GENERAL DISCUSSION

The overarching themes of these investigations highlights a previously unrecognized potential of perilipin 3 (PLIN3) as a facilitator of lipid oxidation in sedentary, but otherwise healthy individuals, individuals with type 2 diabetes (T2D), and women with polycystic ovary syndrome (PCOS). Additionally, coatomer gtp-ase proteins that have been investigated in stable cell lines before, have now for the first time been measured in relation to lipolytic stimulation in human skeletal muscle and adipose tissue. As discussed earlier, current research focus in this field rests heavily on other perilipin proteins, namely perilipin 1 (PLIN1) in the adipose tissue as well as perilipin 2 (PLIN2) and perilipin 5 (PLIN5) in the skeletal muscle. As such until now, the potential of PLIN3 to serve as a target for inducing lipid oxidation in individuals with T2D has been overlooked. However, the research provided here should serve as a catalyst for future investigations into the therapeutic opportunity that PLIN3 and the coatomer gtp-ases might have to offer for improving lipid metabolism in the skeletal muscle of individuals with T2D.

The data presented in chapter 2, which was originally published in *PLoS ONE* in 2014, shows that in primary skeletal muscle myotubes donated from sedentary, healthy males, epinephrine treatment as well as treatment with the pharmacologic cocktail of palmitate, forskolin, and ionomycin (PFI), both known to increase lipolysis, increased the expression of PLIN3 protein content. Coatomer gtp-ases, ARF1, ARFR1, and GBF1, increased in protein content, and ARF1, Sec23a, and GBF1, increased in mRNA expression following PFI treatment. These data were combined with a clinical trial involving 20 healthy male participants, who underwent a single endurance exercise bout expending 650kcal of energy with biopsies taken before and immediately after exercise. The clinical results showed an increase in PLIN3 skeletal
muscle protein content following exercise as well as significant increases in the mRNA levels of ARF1, GBF1, and Sec23a. Changes in the expression of PLIN3 were significantly, positively correlated with changes in both whole body in vivo lipid oxidation and skeletal muscle specific ex vivo palmitate oxidation. The combined results of these study show that PLIN3 levels are increased with lipolytic stimulation, whether they be pharmacologic or physiologic, in the skeletal muscle of humans both on the in vitro and in vivo levels.

In chapter 3, utilizing a second clinical trial involving 29 sedentary males, which was originally published in the Journal of Clinical Endocrinology and Metabolism in 2015, it was found that under resting conditions, skeletal muscle protein levels of PLIN3 were correlated with increased lipid oxidation on both the whole-body level in vivo from 24-hr respiratory quotient and with ex vivo skeletal muscle tissue specific palmitate oxidation. This evidence independently reproduced the data that was shown in chapter 2. Utilizing primary skeletal muscle myotube cultures, PLIN3 was artificially knocked down using siRNA, and levels of lipid oxidation were drastically reduced. The combination of these data from both chapters 2 and 3 display evidence that human skeletal muscle PLIN3 is involved in lipid oxidation that is reproducible from two separate, independent clinical trials, on both the whole-body and tissue-specific levels, and that artificial knockdown of PLIN3 by siRNA in primary muscle culture reduces lipid oxidation in vitro.

The remainder of chapter 3 highlighted the possibility that the functions of PLIN3 mediated lipid oxidation is a phenomenon seen only in primary muscle culture taken from sedentary healthy donors and donors with T2D. PFI treatments were used on myotube cultures from donors who were physically active, donors who were sedentary but otherwise healthy,
and donors with T2D. In a time-course following 3 days of PFI treatments, PLIN3 protein levels as well as protein levels of the coatamer gtp-ase ARFRP1 increased in the myotubes from donors who were healthy and sedentary and donors with T2D. However, myotubes from active donors did not express much of either PLIN3 or ARFRP1, but rather expressed increased protein levels of PLIN5. Further, utilizing the pharmacologic brefeldin A, a drug that inhibits the action of ARF1, in vitro lipid oxidation levels were significantly decreased in the myotubes from both the sedentary individuals and the individuals with T2D. However, brefeldin A did not have a significant impact on the lipid oxidations levels of myotubes from physically active donors. These results reveal the potential of two separate metabolic pathways for lipid oxidation of lipid droplets that is mediated either by PLIN3 and coatamer gtp-ases or by PLIN5. With the evidence presented, it would seem at this point that individuals with T2D are more dependent on the PLIN3 mediated pathway, which would suggest that further investigations into PLIN3 might uncover a potential therapeutic agent for increasing skeletal muscle lipid oxidation in T2D.

Two questions remained following the investigations in chapters 2 and 3. First, though it was shown that when PLIN3 was knocked down artificially in primary skeletal muscle culture using siRNA, lipid oxidation levels decreased, does such a knockdown exist in nature and what effects does it have on lipid oxidation? Furthermore, all the investigations heretofore have looked into the skeletal muscle expression of PLIN3 and the coatamer gtp-ases; but what about in human adipose tissue? In order to answer these questions, it was shown in chapter 4, which was originally published in the European Journal of Endocrinology in 2015, that women with polycystic ovary syndrome, an illness characterized by infertility, hyperandrogenism, and insulin
resistance, naturally had nonexistent protein levels of PLIN3 in their subcutaneous adipose.
Likewise, mRNA levels of other key coatmer gtp-ases were significantly different when compared to age- and weight-matched control women without PCOS, as well as wide variation in lipid droplet morphology in primary adipose cultures. The women with PCOS underwent a 16-week aerobic exercise trial, with repeated subcutaneous adipose biopsies and whole body lipid oxidation measured. Following exercise training, PLIN3 protein expression was evident in the adipose tissue, and whole body lipid oxidation increased. Furthermore, mRNA expression levels of coatmer gtp-ases ARF1, ARFRP1, βCOP1, and Sec23a also significantly increased, and lipid droplet morphology in primary adipose cultures resembled that of the control women from the beginning of the trial. These data show that PLIN3 mediated lipid oxidation potentially occurs in adipose tissue following aerobic exercise and that a naturally occurring knockdown of PLIN3 is associated with reduced levels of lipid oxidation.

Continued research would be needed to understand the alterations and mechanisms between the PLIN3 mediated lipid oxidation pathway that is seen in sedentary and T2D individuals and the PLIN5 mediated lipid oxidation pathway seen in active individuals.

Importantly, the results presented here are shown only on the in vitro level in primary muscle cultures. Regardless of how robust a model of primary tissue culture might be, it is important to see if these same results are translatable to the in vivo skeletal muscle tissue in individuals with T2D. Additionally, continued research into the adipose tissue of women with PCOS concerning PLIN3 mediated lipid oxidation might also help to improve metabolic outcomes of this insulin resistant disease.
Further research could also be extended to identifying transcription factors that would regulate the increase in expression of PLIN3, and the testing of pharmacologic agonists of such transcription factors. As described in the introductory chapters, the transcription factors that control PLIN3 have not been described. However, if pharmacologics could be targeted to increase its expression, more clinical investigations could be utilized to provide therapeutic benefit of patients with T2D.
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APPENDIX C: PERMISSION TO REPRINT CHAPTER 4

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Authors:
Jeffrey D. Covington, Sudip Bajpeyi, Cedric Moro, Yourka D. Tchoukalova, Philip J. Ebenezer, David H. Burk, Eric Ravussin, Leanne M. Redman

Issue Details:
2015, 172, 47-58
Doi: 10.1530/EJE-14-0492

Kind Regards,
Jeff

Jeffrey D. Covington
4th Year Medical Student
Louisiana State University
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APPENDIX D: GRANTS AND FUNDING SOURCE ACKNOWLEDGEMENTS

The works presented herein have been funded, and utilized the Genomics Core Facility and Cell Biology and Bioimaging Core at Pennington Biomedical Research Center, in part by the Nutrition Obesity Research Center (NORC) grant NIH 1P30 DK072476, as well as COBRE (NIH P20 GM103528). The clinical trial presented in chapter 2 was supported in part by a young investigator award from The Obesity Society. The clinical trial and studies that provided primary myotubes presented in chapter 3 were supported in part by the EAT Study Grant NIH R01-DK060412, the ACTIV Study Grant NIH 1R01AG030226-01A2, and unrestricted research grants from Novartis, Novartis Clinical Innovation Fund and Takeda Pharmaceuticals North America. The clinical trial presented in chapter 4 was supported in part by a grant from the Health and Performance Enhancement Division of Pennington Biomedical Research Center.
VITA

Jeffrey Daniel Covington was born on July 20, 1985 in Baton Rouge, LA. As native and long-time resident of Baton Rouge, he attended elementary school at Riveroaks Elementary, middle school at Starkey Academy, and graduated high school in 2003 from Baton Rouge Magnet High School. Jeffrey pursued undergraduate studies at LSU from 2003 to 2007 earning a Bachelor of Arts degree in Philosophy and a Bachelor of Science degree in Psychology with a minor in Biological Sciences. Jeffrey began working at Pennington Biomedical Research Center in the Spring of 2004 as a student intern, and was hired as a full-time Research Associate in 2008 to the lab of Dr. Eric Ravussin. He entered graduate school at LSU in 2010 in the department of Biological Sciences. In 2013, Jeffrey was accepted into Medical School at LSU Health Sciences Center in New Orleans, LA. Jeffrey anticipates graduating in May 2017 from the Graduate School at LSU with a PhD in Biological Sciences and will graduate from Medical School in May 2017 with a MD from LSU School of Medicine in New Orleans. Following graduation, Jeffrey will start residency training in Anatomic and Clinical Pathology at the University of Vermont in Burlington, Vermont, and will be appointed as a clinical instructor to the College of Medicine at the University of Vermont starting in June of 2017.