2018

# VITAMIN D WORKS THROUGH THE LIPID DROPLET PROTEIN PLIN2 TO AUGMENT MITOCHONDRIAL FUNCTION IN SKELETAL MUSCLE

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Digital Object Identifier: https://doi.org/10.13023/etd.2018.390

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# VITAMIN D WORKS THROUGH THE LIPID DROPLET PROTEIN PLIN2 TO AUGMENT MITOCHONDRIAL FUNCTION IN SKELETAL MUSCLE

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DISSERTATION

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A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine
at the University of Kentucky

By
David Matthew Schnell

Lexington, KY

Director: Dr. D. Travis Thomas, Associate Professor of Clinical Nutrition

Lexington, Kentucky
2018

**ABSTRACT OF DISSERTATION**

VITAMIN D WORKS THROUGH THE LIPID DROPLET PROTEIN PLIN2 TO AUGMENT MITOCHONDRIAL FUNCTION IN SKELETAL MUSCLE

Vitamin D has been connected with increased intramyocellular lipid (IMCL) mitochondrial function in skeletal muscle. It is also shown to prevent lipotoxicity in several tissues, but this has not yet been examined in skeletal muscle. Perilipin 2 (PLIN2), a lipid droplet protein upregulated with vitamin D treatment, is integral to managing IMCL capacity and lipid oxidation in skeletal muscle. Increased lipid storage and oxidation is associated with increased tolerance to a hyperlipidic environment and resistance to lipotoxicity. Therefore, I hypothesized that vitamin D increases β-oxidation and lipid turnover though a PLIN2 mediated mechanism, thereby preventing lipotoxicity.

This hypothesis was divided into two specific aims: 1) Characterize the effect of vitamin D and PLIN2 on lipid turnover and β-oxidation in mature myotubes, and 2) Determine the role of vitamin D and PLIN2 in regulating key markers of lipotoxicity. To address these aims, cells were treated with or without vitamin D, palmitate, and PLIN2 siRNA in an eight group, 2x2x2 design. Key experiments included quantitative real time polymerase chain reaction for markers of lipid accumulation, lipolysis, and lipotoxicity; Seahorse oxygen consumption assay; $^{14}$C-palmitate oxidation assay; and analyses of lipid accumulation and profile.
Failure of the palmitate treatment to produce a reliable model for lipotoxicity resulted in negative data for Aim 2 of this dissertation and a focus on vitamin D and PLIN2 knockdown treatments as a four group, 2x2 model. Aim 1 showed that vitamin D reliably increases markers of lipolysis and lipid accumulation. Most of these markers were in turn decreased after PLIN2 knockdown, and DGAT2 exhibited an interaction effect between the two treatments. Contrary to our hypothesis and some published research, PLIN2 knockdown did not prevent lipid accumulation. Vitamin D increased oxygen consumption, especially consumption driven by mitochondrial complex II. PLIN2 knockdown decreased oxygen consumption and demonstrated an interaction effect specific to mitochondrial complex II.
Data in this dissertation show that vitamin D increases mitochondrial function, and these effects are at least in part accomplished through a PLIN2 mediated mechanism. However, this work lacks the data required to make specific claims regarding β-oxidation and lipid turnover. This research is some of the first to show that PLIN2 knockdown carries negative impacts for skeletal muscle mitochondria and makes valuable contributions to general knowledge of how vitamin D and lipid storage impact muscle health and function. This ultimately provides additional

evidence to advocate for vitamin D supplementation as a means of improving musculoskeletal health and function. Future research should investigate how vitamin D and PLIN2 impact markers of lipotoxicity in skeletal muscle.

Keywords: Vitamin D, PLIN2, Skeletal Muscle, Mitochondrial Metabolism, Lipid Droplets

David Matthew Schnell

10/11/2018

Date

VITAMIN D WORKS THROUGH THE LIPID DROPLET PROTEIN PLIN2 TO AUGMENT MITOCHONDRIAL FUNCTION IN SKELETAL MUSCLE

By

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Director of Graduate Studies

10/11/2018

- To my Family -

- for walking with me between the mountain tops -

**ACKNOWLEDGEMENTS**

The phrase “standing on the shoulders of giants” is commonly used to recognize how research can only be accomplished in light of the knowledge afforded by the many researchers who have laid the groundwork leading to a given project. I have certainly stood on the shoulders of giants, but I also recognize that I was only able to complete my work having been bolstered by a small army of family, friends, coworkers, and mentors. While this is certainly not an exhaustive list, I would like to take some time to acknowledge a few people who have played a particularly substantial role in bringing me to where I am today.

I would like to start by recognizing my advisor, Dr. Travis Thomas. Thank you for your guidance throughout my time in your lab and the many opportunities to grow intellectually, professionally, and personally that you have provided. I cannot thank you enough for your investment of time and intellect into my scholarly development and the knowledge and skills gained under your mentorship. Similarly, I would like to thank my fellow students in the Thomas laboratory, with special thanks to Maja Redzic, Hideat Abraha, and Danielle Jones. It has been a joy to work with and learn from all of you.

To each of the members of my committee, Drs. Charlotte Peterson, Geza Bruckner, Lance Bollinger, and Kevin Pearson, thank you for all of the time and energy you have given to committee meetings and keeping this project moving forward; the rigor with which you have evaluated my project was central to my development as a scientist. I would like to give a special thanks to Dr. Peterson, who graciously gave me a research bench and resources to in her laboratory. I also need to thank Dr. Grace Walton for coaching me through techniques and solving problems with me at the bench. Also, to the late Dr. Deneys van der Westhuyzen, than you for your service on my committee. You are dearly missed.

This project would not have been possible without the assistance of Drs. Patrick Sullivan and Hemendra Vekaria. Their experience and expertise was crucial to completing Seahorse experiments and analysis.

My graduate career would have been very different without the training I received from Dr. Daret St. Clair in the first three years of my graduate career. Thank you for your mentorship and support of my intellectual and career aspirations. I would also like to thank all the members of the St. Clair

lab, with special thanks to Dr. Sanjit Dhar for his guidance and patience as I learned the basics of molecular techniques. I must also acknowledge the members of my committee during the first half of my career: Drs. Edward Kasarskis, Howard Glauert, and Christian Paumi. Thank you for teaching me to refine ideas and ask good scientific questions.

Thank you to my friends and classmates past and present. It was a pleasure working with you towards success in classes, research, and the growth and development of NSPS. This department has changed substantially over the last six years, and I am proud to be a part of such a dedicated, talented, and ambitious group of students who are always looking for ways to improve our academic community. Keep doing great work.

Finally, I would like to thank my family. Thank you Chuck and Kari for bringing me into your family and the hundreds of hours watching Theo. Thank you to my parents for instilling in me the dedication required to make it through graduate school. A special thanks to Dad for the fervor with which you took a red pen to my papers with while I was in high school; the ability to write and a thick skin have both served me well! Most of all, thank you, Kim, for being my everything. Thank you for your patience during the many late nights and weekends that I was working. Thank you for supporting our family while I was in school. Thank you for being my rock when I was at my lowest and keeping my eyes on the finish line. I couldn't have done it without you.

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## 1.1 Vitamin D

### 1.1.1 History

Vitamin D is a secosteroid long recognized as a key regulator of blood calcium and phosphorus and a key player in supporting healthy bones [1]. This connection was first established in the early 20$^{th}$ century against the backdrop of a rickets epidemic in post-industrial London. In 1919, Sir Edward Mellanby of Emmanuel College, Cambridge, found that dogs fed a micronutrient deficient diet could be cured of rickets when their diet was supplemented with cod liver oil [2]. This rapid recovery of health and vigor was originally attributed to vitamin A, a recently discovered amine vital for health only available through the diet. However, this was proven false by American biochemist Elmer McCollum, who was a part of the group that originally proposed that rickets was a dietary deficiency. McCollum differentiated the unknown anti-rachitic from vitamin A by heating and aerating cod liver oil to degrade vitamin A and found that, although it could not treat night blindness, it did protect against rickets [3]. Simultaneously, researchers in England and Austria recognized that rickets was also prevented with exposure to summer sunlight or artificially generated ultraviolet (UV) rays [1]. These discoveries lead to a generation of children raised on cod liver oil and treated with UV lamps and a precipitous drop in the incidence of rickets. However, with the rise in rates of childhood obesity and lifestyle changes (e.g. less time playing outside), the incidence of rickets has increased dramatically since 2000 [4]. This is accompanied by increased rates of vitamin D deficiency in both children and adults at rates that some consider to be pandemic [5-10]. This is particularly concerning as vitamin D deficiencies have been connected with increased incidence of cancer, cardiovascular disease, and deficiencies in gross motor function among the elderly [11-14].

### 1.1.2 Vitamin D Synthesis and Metabolites

There are several molecules which fall into the vitamin D family and can be converted into functional vitamin D. Animals produce vitamin $D_3$ (cholecalciferol, $D_3$) from 7-dehydrocholesterol, while fungi produce vitamin $D_2$ from ergosterol. These molecules are very similar and undergo the same reactions during their activation, however, ergosterol and its downstream products contain a double bond on carbon 22 and an additional methyl group. In mammalian systems, 7-

dehydrocholesterol is photolyzed to $D_3$ in the skin by UV light between 270-300 nm. $D_3$ is carried by the blood to the liver where it is hydroxylated by vitamin D 25-hydroxylase (CYP2B1) into calcifediol, or 25-hydroxyvitamin $D_3$ ($25(OH)D_3$). A smaller amount of vitamin D metabolites bind to albumin, which offers similar protection. The final hydroxylation to activate vitamin $D_3$ occurs primarily in the kidney as vitamin D 1α-hydroxylase (CYP27B1) hydroxylates calcifediol to produce calcitriol, or 1α,25-dihydroxyvitamin $D_3$ ($1,25(OH)_2D_3$), the active form of vitamin $D_3$. Calcifediol (measured in ng/mL or nM) is 1,000 times more abundant in the serum than calcitriol (measured in ng/L or pM) [15], and as such, calcifediol is used as the clinically relevant marker of vitamin $D_3$ abundance. Both calcitriol and calcifediol are targeted for excretion by hydroxylation at carbon 24 into 24,25-dihydroxyvitmain $D_3$ by 25-hydroxyvitamin $D_3$ 24-hydroxylase (CYP24A1) [16]. This pathway is summarized in Figure 1.1.

Vitamin D metabolites are chaperoned throughout the circulation by vitamin D binding protein (DBP), a globulin protein in the albumin family [17, 18]. Approximately 88% of calcifediol and 85% of calcitriol are bound to DBP, with the vast majority of the remaining fraction bound to albumin; only 0.04% of calcifediol and 0.4% of calcitriol are free in serum [19]. The association of vitamin D metabolites to DBP serves several functions ranging from aiding solubility of hydrophobic vitamin metabolites to preventing renal excretion or vitamin D and mediating megalin-mediated endocytosis, the major pathway of preserving calcifediol and converting it into calcitriol [20].

Throughout this paper "vitamin D" references vitamin D and its metabolites in a nutritional or physiological sense (e.g. vitamin D deficiency, vitamin D synthesis). While vitamin D exists in multiple forms, the biological actions of vitamin D are produced exclusively through the actions of dihydroxylated vitamin D, whether $1,25(OH)_2D_2$ or $1,25(OH)_2D_3$. Because both $D_2$ and $D_3$ are present in humans and capable of eliciting biological responses, specific vitamin D metabolites will be named without reference to $D_2$ or $D_3$ when either molecule is applicable (e.g. 1,25(OH)D represents both $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$). Specific names of metabolites (e.g. calcitriol) will be used in reference to treatment with that metabolite.

### 1.1.3 Functions of Vitamin D

#### 1.1.3.1 Vitamin D Signaling

Vitamin D works primarily through its interaction with vitamin D receptor (VDR) to regulate gene expression. This mechanism is reviewed in detail by Haussler et al. [21] and Pike & Christakos [22].

VDR is a member of the nuclear receptor superfamily and is expressed in nearly every tissue in mammalian bodies. This transcription factor has three distinct domains: the c-terminal ligand binding domain, two zinc finger DNA-binding domains, and a hinge region which spans the two functional regions [23]. Upon activation by 1,25(OH)D, the α-helix rich ligand binding domain acts as a gating mechanism to retain the ligand and serve as a docking site for retinoid X receptor (RXR), the most common cofactor of VDR [24]. The VDR:RXR heterodimer then translocates to the nucleus where it binds to vitamin D response elements (VDREs) and associates with both gene and cell specific cofactors to activate gene transcription [21, 22]. In addition to VDREs, the VDR/RXR heterodimer has myriad additional cis-acting targets and DNA binding sequences, known as the cistrome (from *cis*-acting target and gen*ome*), which number from 1,000 to 8,000 per genome and vary as a function of cell type and epigenetic modification.

Vitamin D is also recognized to have non-genomic effects independent of nuclear activation and transcriptional regulation in multiple cell types [25]. Researchers have proposed that there is a secondary membrane VDR (mVDR), opposed to nuclear VDR (nVDR), which exerts non-genomic effects in cells. This mVDR is associated with the activation of multiple intracellular signaling cascades. Through mVDR, 1,25(OH)D can activate signaling molecules including phospholipase C and phospholipase $A_2$, phosphatidylinositol-3 kinase (PI3K) and p21ras and their second messengers. This in turn activates protein kinases including PKA, Src kinase, mitogen-activated protein (MAP) kinases, and PKC [25]. One study in C2C12 myoblasts found that VDR knockdown with short hairpin RNA (shRNA) abolished vitamin D-induced phosphorylation of p38 MAPK and Src, indicating rapid responses to vitamin D in survival/proliferation pathways [26]. mVDR is also connected to increased phosphoinositide turnover and production of second messengers inositol 1,3,4-triphosphate ($IP_3$) and diacylglycerol (DAG). Calcitriol is also shown to increase intracellular calcium through both activation of voltage-dependent calcium channels and release of intracellular calcium stores from sarcoplasmic reticulum and t-tubules [27, 28].

While the separate functions of mVDR and nVDR have substantial implications to the mechanisms of effects mediated by vitamin D, few researchers differentiate between these two pathways or consider the possibilities of each in experimental models. As such, most research investigating VDR does not differentiate between mVDR and nVDR and simply report the actions of "VDR." Descriptions of others' research in this paper do not imply mVDR or nVDR; however, original content reported in chapters 3, 4, and 5 specify nuclear and membrane VDR where appropriate.

#### 1.1.3.2 Calcium Regulation

Vitamin D is traditionally recognized as a modulator of serum calcium and regulates circulating calcium in three ways. First, it facilitates absorption of calcium in the intestines. About 70-80% of dietary calcium is absorbed in the intestines, primarily through the ileum and colon. Vitamin D modulates both transcellular calcium transport through intestinal epithelial cells and paracellular transport between tight junctions. Vitamin D-mediated transcellular calcium absorption is modulated by three key proteins: calcium transporter transient receptor potential vanilloid type 6 (TRPV6), intracellular transporter calbindin-$D_{9k}$ (CaBP), and transmembrane calcium pumps NCX1 and PMCA1b [29]. All of these proteins are induced by vitamin D genomic signaling and decreased, although not absent, in VDR-null animal models [30]. During transmembrane calcium transport, calcium is transported across the apical brush-border membrane into intestinal enterocytes through TRVP6. After transport through TRPV6, free intestinal intracellular calcium is captured by CaBP and ultimately pumped across the plasma membrane into circulation by calcium-ATPase pumps, primarily PMCA1b. It is worth noting that knockout mice of TRVP6 and CaBP have no dramatic phenotype, suggesting alternative pathways of calcium absorption. However, TRVP6 null mice do have decreased calcium absorption under conditions of low dietary calcium compared to wild type animals.

Vitamin D also regulates paracellular transport of calcium [29]. Paracellular transport occurs along the length of the intestines through tight junctions in a regulated fashion, likely coordinated with transcellular transport [31]. Vitamin D receptor knockout has been shown to decrease the expression of tight junction proteins claudin-2 and claudin-12, which facilitate calcium conductance [31]. Gene array studies have also shown that vitamin D suppresses cell adhesion protein cadherin-17 and tight junction channel protein aquaporin-18, expanding its function in paracellular calcium transport [32].

The second mechanism through which vitamin D regulates circulating calcium is through the mobilization of skeletal calcium by PTH. PTH is considered to be the most significant peptide hormone for regulating calcium homeostasis [33], and its function is closely tied to vitamin D. One of the most immediate effects of PTH secretion is increased expression and activity of CYP27B1 in the kidneys. This increases the 1α hydroxylation of 25(OH)D to 1,25(OH)D and thereby the bioavailability of vitamin D and intestinal calcium absorption[34]. However, when this is insufficient, PTH is released from the parathyroid glands and increases serum calcium in multiple

ways, one of which is the activation of osteoclasts and mobilization of skeletal calcium. PTH acts on osteoblasts and osteocytes to increase the surface expression of the receptor activator of nuclear factor-κB ligand (RANKL). Hematopoetic stem cells of the monocyte/macrophage line express RANK, the receptor for RANKL, and, upon binding to RANKL, differentiate into osteoclasts [35]. Osteoclasts in turn decrease the pH of the microenvironment immediately surrounding the bone, releasing calcium into the circulation. Because of this, chronically high levels of PTH can trigger dramatic demineralization of bone and are associated with osteopenia and osteoporosis.

Thirdly, vitamin D works through PTH to increase renal retention of calcium. As vitamin D decreases and PTH increases, tubular reabsorption of calcium increases and filtered load decreases. While the impact of vitamin D on renal regulation of calcium are well known, the molecular mechanisms through with vitamin D works to increase renal retention of calcium are not well understood [36].

#### 1.1.3.3 Lipid Management

Vitamin D has long been recognized as an important regulator of both lipid storage and lipid metabolism in a variety of tissues including liver [37-40], adipose [41-43], bone [44], kidney [45, 46], and skeletal muscle [47-49]. In fact, vitamin D is recognized as a necessary component for the normal accumulation and oxidation of lipids [50]. This is perhaps most evident in the liver. One study found that, of patients referred to a Diabetes and Metabolic Diseases clinic, individuals with non-alcoholic fatty liver disease (NAFLD) had decreased serum 25(OH)D [51]. This association was independent of age, sex, triglycerides, HDL, and blood glucose. A later meta-analysis concluded that low serum 25(OH)D likely contributes to the development of NAFLD in humans [40]. However, results in mice have suggested adverse effects of vitamin D and VDR on lipid management in the liver. One publication reported decreased accumulation of liver triglycerides and improved glucose tolerance in vitamin D deficient mice compared with vitamin D sufficient controls when fed a high fat diet [52], and another found that deletion of VDR prevented hepatosteatosis in $ApoE^{-/-}$ mice [38]. While responses may vary in different models, it is evident that vitamin D and VDR play a clear role in lipid management in the liver.

Adipose tissue and whole body adipose biology are also affected by vitamin D. Kang et al. [41] found that vitamin D treatment prevented weight gain in pregnant rats and reduced the amount of lipid in adipocytes. Vitamin D also negatively regulated lipolytic genes in both adipose and liver tissue including FAS, SCD1, ACC1, PPARγ, and INSIG2. The impact of vitamin D on PPARγ is

particularly important in regulating the differentiation of adipocytes. Treatment of preadipocytes with vitamin D prevents differentiation through PPARγ-mediated pathways [43, 47, 53]. Conversely, low levels of vitamin D upregulate PPARγ and have been connected to the transdifferentiation of C2C12 myoblasts into adipocytes [54]. This is a likely mechanism by which vitamin D deficiency contributes to the accumulation of EMCL. Vitamin D is also proposed to impact multiple organ systems through its effects on adipose tissue by regulating the expression of adipokines [55]. Mice without VDR or CYP27B1 genes have reduced serum leptin [56]. This result is mirrored in human studies; individuals with vitamin D deficiency and a normal BMI exhibit a negative correlation between vitamin D and leptin [57]. However, the mechanisms to support this correlation have not been established and there is limited support for the involvement of vitamin D-regulated adipokines [55].

Vitamin D has also been strongly associated with IMCL and muscle lipid oxidation. The relationship between vitamin D and muscle fat was first discovered by Gilsanz et al. in a cross-sectional study of young women that showed a strong inverse correlation between 25(OH)D and EMCL [58]. Another cross-sectional study of overweight women with polycystic ovarian syndrome (PCOS) demonstrated an inverse relationship between 25(OH)D and EMCL in the thigh [59]. Interestingly, this association was independent of visceral fat. This same study found that only women with 25(OH)D > 20 ng/mL had a significant decrease in thigh EMCL after a 12-week treadmill exercise program, suggesting that sufficient 25(OH)D may be required for efficient mobilization of EMCL and during exercise. Furthermore, high EMCL volume attenuates gains in muscle quality [60, 61] and is associated with increased morbidity in older men [62].

While these reports suggest that vitamin D decreases EMCL accumulation, there is evidence that vitamin D increases the accumulation of IMCL. A clinical study by Redzic et al. [49] showed a direct linear relationship between serum 25(OH)D and IMCL accumulation in older adults. In this study, vitamin D status was compared to IMCL, EMCL, physical activity, and markers of physical function in adults 65-80 years of age. Statistical analysis showed that the observed relationship between 25(OH)D and IMCL was independent of lifestyle factors or physical activity. The positive relationship between vitamin D and IMCL was reproduced in a C2C12 cell culture model by Jefferson et al. [48] using a C2C12 myotube model treated with 100 nM calcitriol for 96 hours and found an increase in neutral lipid accumulation as indicated by oil red O staining. In addition to decreased EMCL accumulation and increased IMCL accumulation, vitamin D has been shown to

prevent obesity in a high fat diet mouse model by increasing β-oxidation [63]. Together, these data suggest a model in which vitamin D supports lipolysis of EMCL and accumulation of IMCL in combination with increased mitochondrial activity and β-oxidation. This increase of lipid into and out of skeletal muscle improves muscular bioenergetics and may prevent lipotoxicity [64]. Both of these endpoints have beneficial implication for clinical populations at risk for sarcopenia, cachexia, or metabolic syndrome.

### 1.1.4 Clinical Implications

#### 1.1.4.1 Prevalence of Deficiency

Vitamin D deficiency, or hypovitaminosis D, is extremely prevalent and thought to affect over 1 billion individuals worldwide [7]. However, the clinical cutoff for vitamin D deficiency is a subject of debate, and exact rates of deficiency reported by different researchers or agencies vary widely. The Institute of Medicine defines vitamin D insufficiency as serum 25(OH)D less than 20 ng/mL (50 nmol/L) and deficiency as 25(OH)D levels below 12 ng/mL, while the Endocrine Society deems values less than 30 ng/mL (75 nmol/L) insufficient. Still, some researchers suggest that sufficiency is not reached until 40-60 ng/mL (150 nmol/L) [6]. Each of these cutoffs is set based on different biological outcomes. Defining deficiency as < 20 ng/mL is centered on improving bone health by minimizing serum PTH to prevent leaching of calcium from bone. Higher cutoffs recognize that intestinal calcium transport is not optimized until 30-32 ng/mL. Setting the lower limit of sufficiency at 40 considers recent evidence that higher vitamin D levels are associated with decreased rates of some cancers [6]. According to the most recent NHANES survey (2001-2006), 8% of Americans had 25(OH)D < 12 ng/mL, and 32% had 25(OH)D below 20 ng/mL. These rates increase with age and BMI.

Regardless of exact prevalence of vitamin D deficiency, there is little debate that rates of vitamin D deficiency are increasing around the world [7, 11-13, 65, 66]. There are likely multiple factors contributing to rising rates of deficiency. Foremost is a global trend towards decreased cutaneous exposure to UVB radiation and in turn decreased vitamin D synthesis [67]. This is associated with racial and geographic factors [68], as well as changing lifestyles among both adults and children [69, 70]. The association between skin cancer and UVB exposure has led to increased use of sunscreen, which also prevents cutaneous vitamin D synthesis, and may also be a contributing factor to increased vitamin D deficiency [71]. Increased rates of obesity contribute to vitamin D

deficiency; as BMI rises, more and more vitamin D is sequestered in adipose tissue, increasing the amount needed to maintain healthy levels in the blood. A recent study identified an independent association between serum 25(OH)D and obesity after controlling for racial, sociological, dietary, and environmental factors [72]. Because of the relatively low contribution of dietary vitamin D to serum 25(OH)D, dietary intervention is considered the least beneficial strategy for population-wide improvements in vitamin D levels [73].

#### 1.1.4.2 Symptoms of Deficiency Effecting Skeletal Muscle

Vitamin D deficiency is highly associated with musculoskeletal problems. One of the most commonly studied products of vitamin D deficiency is the risk of falls in persons over the age of 65. Multiple studies have found that vitamin D supplementation is associated with a decreased incidence of falls [74, 75]. Conversely, reduced vitamin D status is connected with decreased muscle mass and physical performance in the elderly [76]. While a 2012 meta-analysis published in the Cochrane Database of Systematic Reviews concluded that vitamin D did not reduce the rate of falls or risk of falling [77], the authors did note that there may be a benefit of vitamin D in people who are vitamin D deficient. Related to falls, vitamin D has been connected with sarcopenia in several studies [78-83]. While broad conclusions are difficult to draw, both molecular and clinical research have connected low levels of vitamin D to decreased muscle volume and function in the elderly [78, 79] and vitamin D supplementation with increased myofiber diameter [84-86]. However, vitamin D related deficiency and musculoskeletal deficit are not limited to aged populations. A study in sub-Himalayan India found a greatly increased rate of vitamin D deficiency in patients presenting with proximal muscle weakness as young as 22 years of age [65]. A meta-analysis investigating the overall effect of vitamin D on muscular strength did not find evidence that vitamin D supplementation has an effect on strength in adults with a baseline 25(OH)D > 25 ng/mL, but did suggest that multiple studies demonstrate a benefit in proximal muscle strength in adults with vitamin D deficiency [87]. Data seem to suggest that the majority of benefits of vitamin D treatment are observed when increasing 25(OH)D from deficient to sufficient levels, but it is thought that there are limited benefits to additional vitamin D supplementation once sufficiency is reached.

### 1.1.5 Vitamin D in Skeletal Muscle

Early in the new millennium, vitamin D was recognized to have a substantial impact on skeletal muscle. Visser et al. showed that low vitamin D and high parathyroid hormone (PTH) were predictive of sarcopenia [78], and Bischoff-Ferrari et al. associated high 25(OH)D with improved leg strength in inactive older adults [88]. In spite of multiple studies showing positive effects of vitamin D on skeletal muscle, many researchers remained skeptical of its direct effect on skeletal muscle. Many attributed musculoskeletal benefits associated with vitamin D to improved calcium and phosphate homeostasis, and a years' long debate surrounding the presence of VDR in skeletal muscle ensued. Research out of the University of Kentucky helped to solidify a direct role for vitamin D and VDR in skeletal muscle in 2012. Publication by Srikuea et al. [89] demonstrated that both VDR and CYP27B1 are expressed in C2C12 myoblasts and myotubes as well as adult muscle cells in rodents. Furthermore, both proteins localized to the nucleus of myoblasts upon stimulation with both 1,25(OH)D and 25(OH)D. These results were verified by cloning and DNA sequencing of transcribed VDR and CYP27B1 genes. However, it is of note that neither VDR nor CYP27B1 translocated to the nucleus upon treatment with calcitriol in differentiated myotubes. Further work has shown that VDR is expressed in human muscle biopsies [90, 91].

Functional effects of vitamin D in skeletal muscle have been well researched and generally show appreciable gains in measures of functional capacity among older adults [49, 80, 92-94]. These findings have been supported by multiple meta-analyses, each showing that vitamin D contributes to small but measurable gains in markers of muscle function [92, 93, 95, 96]. In addition to functional outcomes, vitamin D has been shown to increase type II muscle fiber cross sectional area in older women [97]. Similarly, meta-analysis has confirmed that vitamin D supplementation increases upper and lower limb power generation in healthy, untrained adults [95]. One randomized controlled trial has shown that vitamin D replenishment improves muscle recovery and hypertrophy after damaging eccentric exercise [98]. In contrast, quantifiable benefits of vitamin D supplementation in athletes remain elusive in randomized controlled trials [99, 100]. Correlative studies have shown an association between 25(OH)D status and several markers of athletic performance including $VO_2$max, sprinting and vertical jump [101]. One review notes that although there are multiple theoretical benefits of vitamin D for muscle growth and regeneration, recovery, and oxygen consumption, and vitamin D deficiency is associated with decreased performance and muscle function, the majority of studies on vitamin D in athletes have been

correlative; therefore, clear conclusions regarding the impact of vitamin D on the muscles of athletes cannot be drawn, and much more research is necessary to determine causation [102].

Mouse models of vitamin D deficiency and VDR knockout add a greater degree of understanding to how vitamin D affects skeletal muscle. A recent study showed that mice fed a vitamin D deficient diet and protected from UV exposure had impaired performance including uphill sprint speed, stride length, and grip endurance [103]. VDR-null mice develop normally until weaning but later develop classical signs of rickets and spontaneous alopecia [104]. Additionally these mice show neuromuscular deficit evidenced most clearly by an abnormal, vertical swimming position and increased frequency of sinking episodes [105, 106]. A closer look at muscle morphology and physiology finds that VDR knockout mice show myofiber diameter decreased by approximately 20%, independent of hypocalcemia and hypophosphatemia [107]. Additionally, $VDR^{-/-}$ mice expressed chronically high levels of Myf5, myogenin, and E2A, although myocyte differentiation occurred normally. The authors hypothesize that the aberrant expression of these myogenic genes disrupts the strictly regulated progression of muscle differentiation and maturation, ultimately contributing to aberrant myosin heavy chain expression and atrophy [107]. Although skeletal muscle specific $VDR^{-/-}$ models are lacking, cardiomyocyte specific $VDR^{-/-}$ mice exhibit increased susceptibility to cardiac hypertrophy, myocyte enlargement, myocyte steatosis, and lipotoxicity [108, 109].

Positive correlations between vitamin D and markers of skeletal muscle health are generally accepted; however, direct mechanisms by which vitamin D enacts functional changes in muscle are still unknown. Multiple studies have attempted to delineate the mechanisms of vitamin D on skeletal muscle using the C2C12 mouse myoblast cell line with mixed results. Several studies have shown that vitamin D stimulates the Akt/mTOR signaling pathway [48, 84], suggesting increased anabolic signaling and increased fiber size, although fiber size was not directly measured. One study found that although supplementation decreased C2C12 differentiation, calcitriol increased average myotube diameter by 1.8x [86]. However, other researchers have failed to observe either anabolic signaling or myotube hypertrophy with 1,25(OH)D treatment [85]. Complicating the investigation of a vitamin D effect in skeletal muscle, VDR does not translocate to the nucleus upon stimulation with 1,25(OH)D, eliminating classical VDR/RXR nuclear signaling from potential mechanisms. This has led some researchers to suggest that vitamin D works in skeletal muscle through non-genomic signaling pathways [26, 110]. Vitamin D treatment causes rapid $Ca^{2+}$ influx

both *in vitro* and *in vivo* that are not prevented by inhibitors of RNA or protein synthesis [110]. Increases in intracellular $Ca^{2+}$ are also associated with vitamin D-induced activation of signaling cascades including calmodulin/calmodulin-dependent protein kinase II, PKA, and PKC. These pathways are associated with increased bioenergetic function [111], increased contractile force generation [112], and increased mitochondrial biogenesis [113]. In parallel, a study in the 1980s connected vitamin D deficiency with decreased oxygen consumption and lower calcium content in cardiomyocyte mitochondria [114].

More recent work has suggested connections between vitamin D and skeletal muscle bioenergetics. A clinical study by Sinha et al. [115] supplemented severely vitamin D deficient individuals (25(OH)D < 6 ng/mL) with 20,000 IU of vitamin D on alternate days for 10-12 weeks in a longitudinal design. This group used phosphorus-31 magnetic resonance spectroscopy ($^{31}$P-MRS) to measure phosphocreatine recovery half-time ($\tau_{1/2}$PCr), a surrogate for mitochondrial function, in the gastrocnemius during three minutes of a plantar flexion exercise. Results from this study showed that vitamin D supplementation increased 25(OH)D from 3.5 ng/mL to 45 ng/mL and decreased $\tau_{1/2}$PCr by 19%. However, some of these claims have encountered resistance [116], notably the failure to show functional improvement in proximal muscle, technical limitation of equipment used and practical limitations of examining short term impacts of exercise using NMR. Nevertheless, the results of Sinha et al. have been corroborated by additional research. A group operating out of Delhi, India reproduced the Sinha study with very similar results [117]. Additional work has continued to investigate the role of vitamin D in skeletal muscle bioenergetics through oxygen consumption.

#### 1.1.5.1 Oxygen Consumption

One of the leading hypotheses explaining how vitamin D improves muscle function is improved oxygen consumption and improved bioenergenic capacity of muscle fibers. Supporting this idea are multiple reports suggesting that vitamin D is a major contributor to mitochondrial function and oxygen consumption in a variety of tissues [63, 115, 118-120]. However, causal relationships between vitamin D and improved muscle function are yet to be established [121], and there is still a large body of research needed to determine how vitamin D improves skeletal muscle health and function [50].

Most of the research examining the effect of vitamin D on skeletal muscle oxygen consumption has focused at the whole body level during exercise. These studies consistently show that vitamin

D is directly correlated with $VO_2$max [93, 122] and inversely correlated with respiratory quotient (RQ) [123]. One interventional study in patients with type 2 diabetes mellitus (T2DM) found that 60,000 IU of vitamin D once weekly in conjunction with moderate exercise improved mitochondrial content in skeletal muscle and $VO_2$max and prevented simvastatin-associated decline in these markers [124].

Another group out of the Mayo Clinic has published work looking specifically at the role of vitamin D on skeletal muscle *in vitro*. Ryan et al. [118] demonstrated that calcitriol treatment increases the oxygen consumption rate (OCR) and mitochondrial metabolism of cultured human skeletal muscle cells in a dose-dependent manner from 10 pM to 1 nM. The authors primarily attributed this increase in OCR to changes in mitochondrial fission and fusion. Total mitochondrial DNA and volume were unaffected, but calcitriol increased the expression of pro-fusion protein OPA1, and decreased expression of pro-fission proteins Fis1 and Drp1. A secondary mechanism the authors propose is driven by an increase in pyruvate dehydrogenase activity; calcitriol treatment decreased protein expression of both pyruvate dehydrogenase kinase 4 (PDK4) and inactive, phosphorylated pyruvate dehydrogenase. PDK4 inactivates pyruvate dehydrogenase through phosphorylation, preventing the oxidation of pyruvate into acetyl-CoA and carbon dioxide. Therefore, by decreasing pyruvate dehydrogenase phosphorylation and PDK4 expression, calcitriol increased the production of acetyl-CoA for further oxidation in mitochondria and limited lactate production. Reinforcing the finding that vitamin D increases OCR, another study by the same group found that calcitriol prevents mitochondrial dysfunction associated with cancer conditioned media and corrects the associated decrease in OCR in primary human skeletal muscle cell culture [119].

#### 1.1.5.2 IMCL & EMCL

In recent years, vitamin D has been associated with increased IMCL content in both clinical and basic research [48, 49]. Increased muscle lipid, especially in association with increased body fat, is often associated with increased inflammation, oxidative and endoplasmic reticulum stress, and insulin resistance. This syndrome is seen in many tissues and collective referred to as lipotoxicity [125-128]. However, increased vitamin D is correlated with improved health outcomes and decreased symptoms of lipotoxicity in multiple tissues including liver [39], kidney [46], and bone [44] and has never been shown to produce lipotoxic effects. Furthermore, vitamin D has been shown in both basic science and clinical settings to improve insulin sensitivity, skeletal muscle

function, and skeletal muscle mitochondrial capacity [88, 115, 118, 129]. Many of the impacts of vitamin D on skeletal muscle mimic patterns seen in the athlete's paradox, a condition of high IMCL with simultaneously high levels of insulin sensitivity and mitochondrial function observed in endurance athletes [130-132].

Calcitriol modulates PLIN2 expression in mouse muscle [47, 48] and kidney [46]. Li et al. [47] created a mouse model of vitamin D deficiency using low dietary vitamin D (25 IU/kg/day) and UVB-free lighting compared with vitamin D sufficient (1,000 IU/kg/day) counterparts. The vitamin D deficient mice exhibited increased IMCL and EMCL accumulation and distorted longitudinal myotubes arrangement. The increase in IMCL was accompanied by increased expression of PLIN2 and PPAR-γ protein. MALDI-TOF/TOF mass spectroscopy revealed that vitamin D deficiency increased the abundance of select species of phospholipids and altered phospholipid profile. The authors noted that this was representative of changes to phospholipid membranes. A change in the ratio of saturated to unsaturated phospholipids in plasma membranes readily affects ER function and can trigger pro-apoptotic pathways [133]. In summary, Li et al. [47] showed that vitamin D deficiency resulted in pathological accumulation of both IMCL and EMCL in mouse skeletal muscle and is most likely driven by increased expression of PPAR-γ.

Alternatively, previous work from this group published by Jefferson et al. [48] showed that calcitriol supplementation in a C2C12 cell culture model increased PLIN2 mRNA and IMCL. This group used a 96-hour treatment with and 100 nM vitamin D and 250 µM of both palmitate and oleate to induce lipid accumulation. Supplementation yielded greater IMCL accumulation, particularly in myoblasts opposed to myotubes. This was accompanied by a two-fold increase in PLIN2 mRNA expression as well as significant increases in the expressions of CGI-58 and ATGL mRNA. Liquid chromatography mass spectroscopy revealed that calcitriol supplementation dramatically increased the expression of several species of DAG, most notably di-18:0, again showing that vitamin D affects lipid profile. Notably, this publication notes that calcitriol treated myotubes showed greater Akt phosphorylation in response to 15 minutes of insulin stimulation, suggesting that increased IMCL in response to calcitriol is associated with increased insulin sensitivity. This pattern is very similar to the findings of Bosma et al. who showed that PLIN2 overexpression increased both IMCL and insulin sensitivity [134]. While Li showed that vitamin D decreases PLIN2 and IMCL while Jefferson showed that vitamin D increases PLIN2 and IMCL, these results are not contradictory; one study examines vitamin D deficiency while the other models

vitamin D supplementation. These two studies may imply a complex mechanism of vitamin D-regulated lipid storage in skeletal muscle that involves a U-shaped curve of IMCL in relation to vitamin D with apparently similar but unique phenotypes at extremes: low vitamin D may trigger lipotoxic lipid accumulation, while high vitamin D triggers lipoexpedient lipid accumulation.

### 1.2 Lipid Storage in Skeletal Muscle

Skeletal muscle is one of the most metabolically active tissues in mammalian physiology. In endurance trained humans under high energetic demand, skeletal muscle can account for more than 90% of total oxygen consumption. However, at rest, skeletal muscle is relatively inactive. This incredible range of energy requirements mandates that skeletal muscle react quickly to bioenergetic demands. A rapid response is accomplished through a combination of increased macronutrient delivery, primarily glucose and fatty acids (FAs), via increased blood flow and mobilization of stored energy in the forms of muscle glycogen and triacylglycerides (TAG). Skeletal muscle has a remarkable capacity for macronutrient storage and can accumulate approximately 500 g of glycogen [135] and 750 g of lipid in an active, 70 kg male [136]. This equates to approximately 2,000 kCal of carbohydrate and 6,750 kCal of lipid. Although total intramyocellular lipid (IMCL) content pales in comparison to lipid stored in adipose tissue, skeletal muscle remains the second largest repository of TAG in humans. Because of skeletal muscle's capacity to store and oxidize lipid, IMCL content is directly associated with a variety of biological and lifestyle factors including fiber type [137], diet [138], exercise [130-132, 139, 140], and total body adiposity [139, 141, 142]. IMCL encompasses all lipid stored within muscle cells including TAG, DAG, cholesterol, phospholipids, and sphingolipids. Some researchers specifically discuss intramyocellular triglyceride (IMTG) because of its more direct impact on muscle bioenergetics. However, thinking specifically in terms of IMTG creates a tendency to overlook the contributions of other lipids in cellular systems. DAGs undergo lipolysis and, in addition to sphingolipids, are potent signaling molecules.

Although IMCL is the direct source of lipids oxidized in skeletal muscle, extramyocellular lipid (EMCL) is a much larger store of TAG and less susceptible to large swings in content. These two lipid storage sites are fundamentally different; they store and release lipid through different mechanisms under different stimuli from different tissues. IMCL exists within myocytes in the form of cytosolic lipid droplets (LDs), while EMCL is adipose tissue dispersed among muscle fibers.

While it is normal for some EMCL to accumulate in skeletal muscle, high levels of accumulation are considered pathological. Large depots of EMCL are sometimes called ectopic lipid. However, the term “ectopic lipid” is not specific to muscle and can describe adipose deposits in any tissue. Muscles of both predominantly type I and type II muscle fibers increase lipid content in response to a hypercaloric, high fat diet, but do so in different ways. Type I muscle fibers have a greater capacity for oxidative metabolism and, likewise, a greater capacity to store TAG for immediate oxidation than do type II fibers [137, 143]. A magnetic resonance spectroscopy study completed by Nagarajan et al. [144] suggested human muscles comprising primarily type I fibers (e.g. soleus) are more apt to accumulate IMCL as a result of obesity, while predominantly type II muscles (e.g. gastrocnemius) more readily accumulate EMCL. They also proposed that muscles containing primarily of type I fibers may be resistant to accumulating EMCL. Conversely, Covington et al. [145] showed that 8 weeks of overfeeding had no impact on total IMCL content in the soleus, tibialis anterior, or vastus lateralis, but rather increased the size of LDs. Increased LD size is associated with decreased glucose clearance rate and weight gain [145]. They went on to show that larger LDs were associated with increased reactive oxygen species production, decreased perilipin protein expression, and increased ceramide content.

The effect of training on IMCL is still the subject of some debate, with different interventions in different populations producing consistently different results. Well-trained endurance athletes have increased IMCL content compared with sedentary counterparts [131], and sedentary individuals have been shown to increase type I fiber IMCL with endurance training [146]. However, an endurance training regimen can decrease steady state IMCL in individuals with T2DM [147]. The ultimate effect of endurance training on IMCL likely depends on the metabolic and physiological status of the individual at the initiation of training [148]. Overall, the effect of training on IMCL appears to be dependent on the metabolic health and total fat mass of the individual. Training decreases IMCL content in those with high BMI or T2DM to mitigate effects of lipid overabundance, but produces a slow, adaptive accumulation of IMCL in lean individuals to foster greater energy availability.

### 1.2.1 Lipid Droplets

#### 1.2.1.1 LD Introduction

The vast majority of intramyocellular lipids are stored in LDs, which are sometimes called adiposomes [149]. Although once thought to be simple lipid-filled vacuoles, LDs are now recognized to be highly complex and metabolically active organelles that regulate lipid storage, trafficking, and oxidation. LDs are unique structures surrounded by a monolayer of phospholipids, primarily phosphatidylcholine and phosphatidylethanolamine, with a smaller amount of phosphatidylinositol [150]. This monolayer makes for interesting protein-membrane interactions; there is no set thickness of hydrophobicity as there is in a lipid bilayer. Therefore, many proteins embedded in the LD have an amphipathic α-helix domain or hydrophobic hairpins. These specialized domains are purported to assist the localization of proteins made in the cytosol to the LD [151].

The low density and hydrophobicity of LDs makes their isolation relatively easy, but simplistic techniques have led to facile analysis resulting in hundreds of proposed LD-binding proteins. Further research has suggested that many of these proteins are the product of contaminants from co-precipitated organelles (particularly the endoplasmic reticulum (ER) lumen) and plasma membrane fragments [152, 153]. These discoveries demonstrated the need for greater discretion when investigating LD proteomics. However, dozens of proteins have been positively identified as localized to the LD membrane and function to regulate membrane dynamics, metabolism, autophagy, and vesicular trafficking [152]. These proteins that coat the LD vary along with the morphology of LDs both between and within cells and contribute to specialized function of each droplet [154].

The LD membrane encapsulates a neutral lipid core comprised predominantly of TAG, DAG, and cholesterol esters (CE) [155, 156]. Although the details of their generation are still a subject of debate, LDs are known to be synthesized from the endoplasmic reticulum [151, 157]. Some propose that, similar to mitochondria, existing LDs can fuse to create new LDs. LDs are filled by a combination of TAG from the ER lumen and DAG converted to TAG by diglyceride O-acyltranferase (DGAT) on the LD surface in the presence of exogenous lipid [158].

#### 1.2.1.2 LDs are highly conserved

Lipid droplets are one of the oldest and most highly conserved organelles, maintained across all domains of life from archaea to bacteria to eukaryotes [159-161]. Spanning the monolayer is a network of highly specialized proteins that maintain droplets' structure, dock metabolic enzymes, and relay signals to and from LDs to other organelles. While individual proteins are not necessarily maintained between mammals, yeast, and prokaryotes, recombinant LD proteins commonly localize to LDs in other models [162-164]. For example, LD coating proteins from the yeast genome localize to lipid droplets in mammals and vice-versa. Moreover, recent studies reporting the presence of LD in bacterial nucleoli and hepatic nuclei, as well as histones H2A, H2B, and H2Av localized to lipid droplets, suggest a potential role for LDs in nucleic acid handling in both prokaryotes and eukaryotes [165].

#### 1.2.1.3 LD Organization and Morphology

LDs in skeletal muscle are highly organized at a subcellular level. The majority of skeletal muscle LDs are located in-between myofibrils, commonly found in pairs packed between two mitochondria along the I-band of sarcomeres [137]. A smaller subset of LD is found in the subsarcolemmal region. Similar to mitochondria, subsarcolemmal and intermyofibrillar LDs are thought to be both functionally and morphologically distinct with subsarcolemmal LDs more predictive of insulin resistance and lipotoxicity [166].

The size of LDs is extremely important in regulating skeletal muscle health and may play a larger role than total IMCL content [145]. In general, LDs in skeletal muscle are relatively small, ranging from 0.3 to 1.5 μm in diameter [167]. This is contrasted to LDs in adipocytes, which commonly reach 100 μm or more in diameter. Specialization of skeletal muscle LDs is further demonstrated as the size and abundance of LDs differs between fiber types. Type I fibers contain LDs that are both larger and more numerous than those found in type II fibers.

#### 1.2.1.4 LD Are Metabolically Active

Originally thought to serve only as a storage site for triglyceride, LDs are now known to be highly dynamic and metabolically complex organelles crucial to lipid trafficking and utilization in a variety of tissues [149]. This is especially true in highly oxidative tissues including the liver [168], heart [169], and skeletal muscle [167]. Lipid droplets are regulatory organelles that manage both lipid accumulation and lipolysis. In fact, very little lipid is oxidized without first passing through the lipid droplet. While muscle cells can directly import and oxidize fatty acids from circulation, the

majority of IMCL are first incorporated into TAG and stored in LDs before being hydrolyzed by lipases for oxidation in mitochondria [170, 171]. While strenuous exercise (45 minutes of cycling at 75% max power) acutely depletes the IMCL pool by more than 60% [172, 173], moderate exercise has been shown to have a different effect on IMCL pool. Using radioactive pulse-chase analysis, Guo et al. [171] found that exogenous fatty acids are incorporated into IMCL at a rate similar to IMCL oxidation during 90 minutes of cycling at ~45% $VO_2$max, thereby maintaining a relatively constant IMCL pool. These two studies demonstrate the importance of both acylation (the addition of an acyl chain to glycerol to) and lipolysis for the use of exogenous fatty acids.

#### 1.2.1.5 Lipid Accumulation

Lipid accumulation in LD can be categorized into two general strategies: accumulation of TAG in nascent LDs that have not separated from the endoplasmic reticulum (ER), and addition of TAG to cytosolic LDs [174, 175]. These two strategies are accomplished by two proteins of the DGAT family. DGAT1 and DGAT2 are transmembrane proteins that catalyze the esterification of acyl-coA to a DAG to form TAG. Although they are functionally similar, DGAT1 and DGAT2 are evolutionally distinct and share little homology [176]. Differences in structure are reflected in localization; DGAT1 localizes to the ER while DGAT2 also localizes to mitochondrial membranes and LDs [158, 177]. As a result, DGATs are thought to have independent roles in managing lipid accumulation and TAG acylation in LDs. DGAT1 mediates TAG acylation in the ER and nascent lipid droplets, while DGAT2 embeds into the phospholipid monolayer and produces TAG in pre-existing LDs [151, 177-179]. Both DGAT enzymes are important regulators of lipid metabolism, but DGAT2 plays a larger role in TAG homeostasis and likely contributes more directly to mitochondrial metabolism [180]. The importance of DGAT2 over DGAT1 is further evidenced in mouse knockout models. DGAT1$^{-/-}$ mice have reduced body fat and lactation defects [181]; however, DGAT2$^{-/-}$ mice suffer postnatally fatal derangements of bioenergetic and skin barrier function [182]. The mechanism by which TAG are incorporated into lipid droplets, especially nascent droplets on the ER, is still a topic of debate among biochemists and biophysicists.

When considering TAG accumulation in LD and lipid dynamics in skeletal muscle, it is important to recognize that *de novo* lipogenesis, the conversion of *acetyl-CoA* from non-lipid sources into *acyl-CoA*, only occurs in the liver and white adipose tissue. Lipid anabolism in skeletal muscle is limited to acyltransferase reactions and modification such as isomerizations, phosphorylations, and desaturations. There is no acyl-chain elongation in skeletal muscle.

#### 1.2.1.6 Lipolysis

Lipolysis is the hydrolysis of an acyl chain from a glycerolipid. Although most lipolysis in mammalian systems occurs in adipose tissue, lipolysis is a major component of lipid metabolism in skeletal muscle. The first and rate limiting step of lipolysis in most tissues, including skeletal muscle, is the hydrolysis of TAG to fatty acid and DAG by adipose triglyceride lipase (ATGL). Meex et al. [183] found that the overexpression of ATGL is sufficient to increase lipolysis and mitochondrial capacity *in vitro*, but these results were not observed *in vivo*. ATGL is found both free in the cytosol and bound to LDs, and its activity is regulated by a combination of inhibitory and activating binding driven by PKA/AMPK pathway phosphorylation. Lipid droplet proteins perilipin 1, 2 and 5 (PLIN1, PLIN2, and PLIN5), which are discussed in detail in the following section, and comparative gene identification-58 (CGI-58) all regulate ATGL activity [184]. PLIN1 is a major regulator of lipolysis in adipocytes, controlling as much as 95% of protein kinase A (PKA) mediated lipolysis [154] but plays little to no role in regulating lipolysis in skeletal muscle. Activation of PKA results in the phosphorylation of PLIN1 and association of hormone sensitive lipase (HSL) and ATGL, increasing lipolysis. However, the function of lipolysis in adipose tissue and skeletal muscle is considerably different; lipolysis in adipose tissue supplies fatty acids for other tissues, while lipolysis in skeletal muscle provides fatty acids for energy production in mitochondria, often in response to an immediate increase in energy requirements. In skeletal muscle, PLIN2 is reported to bind to ATGL in the cytosol and prevent association with the LD and CGI-58 [185]. Contractile stimulation or phosphorylation of PLIN2 and CGI-58 by PKA releases ATGL from PLIN2 and increases its affinity for CGI-58, increasing lipolysis [184, 186, 187]. PLIN3 and PLIN5 may also regulate lipolysis [188], although mechanisms behind these interactions are not well understood [154, 184].

Free fatty acids produced by lipolysis are activated through the action of acyl CoA synthetase and converted to acyl-CoA before import to the mitochondria. Transport into the mitochondria is mediated by carnitine palmitoyltransferase 1 (CPT1), the rate limiting enzyme of β-oxidation. CPT1 replaces the CoA group with a carnitine residue which makes the new acylcarnitine molecule available for translocation into the mitochondrial matrix. Upon entry into the matrix, acylcarnitine is reactivated into acyl CoA by CPT2.

### 1.2.2 Perilipins

Perilipins (PLINs) are a family of LD membrane proteins that function in fatty acid uptake, protein binding, lipolysis, and lipid oxidation. Perilipin proteins are essential for LD formation, and LDs lacking perilipins have not been identified [189]. PLINs are highly conserved and expressed in organisms from slime molds to insects to mammals [154]. There are five members of the PLIN family expressed in mammals known as PLIN1-5. For many years these highly related proteins had unrelated names: perilipin (PLIN1), adipophilin (PLIN2), TIP47 (PLIN3), S3-12 (PLIN4), and OXPAT (PLIN5). In 2010, leading perilipin researchers joined together to publish a new, unifying naming convention that highlights the common nature of PLINs {Kimmel, 2010 #1036}. These proteins share a high level of homology, and all but PLIN4 contain a conserved 100 amino acid domain in the amino terminus known as the "PAT domain" originally named to reflect the first three PLIN proteins identified: perilipin, adipophilin, and TIP47 [190]. However, this domain is not responsible for anchorage to the LD. Indeed, no single shared region has been identified to localize the PLIN family to the phospholipid monolayer [154]. Of the 5 members of the perilipin family, PLIN2, PLIN3, and PLIN5 are found in skeletal muscle, while PLIN1 is only expressed at high levels in adipose tissue. Although some have reported high levels of PLIN4 expression in skeletal muscle [191], its expression in skeletal muscle is not widely recognized [192].

#### 1.2.2.1 PLIN1

PLIN1 is the most abundant LD protein in adipose tissue where it is exclusively expressed and used as a marker for adipocyte differentiation [154]. It has at least 4 splice variants, known as PLIN1a-PLIN1d [156]. Of these variants, PLIN1a and PLIN1b have a preference for TAG accumulation, while PLIN1c has affinity for cholesterol esters. Currently, there is no known protein transcribed from the PLIN1d splice variant [156]. PLIN1 is associated with singular large lipid droplets. Like other perilipins, PLIN1 is primarily regulated by peroxisome proliferator-activated receptor gamma (PPARγ). Although primarily thought of as associated with lipid storage, PLIN1 is activated by polyphosphorylation by PKA and highly active in lipolysis. Upon activation, PLIN1 releases CGI-58 and binds with ATGL, increasing lipolysis by 50-fold [193, 194]. While PLIN1 is not expressed in skeletal muscle, PLIN1 knockdown has negative impacts on skeletal muscle health and metabolism. PLIN1$^{-/-}$ mice have dramatically reduced adipose stores, increased EMCL, and develop insulin resistance [189].

#### 1.2.2.2 PLIN2

PLIN2, also called adipose differentiation-related protein (ADRP) or adipophilin (ADPH), is the most highly expressed PLIN in skeletal muscle [192]. PLIN2 is thought to serve primarily as a scaffolding protein and, unlike PLIN1 or PLIN5, does not recruit lipases or stimulate lipolysis after activation by PKA [189]. While PLIN2 does bind with CGI-58 [195], it is not considered a powerful regulator of CGI-58 activity [154]. It is instead active in regulating LD stability, lipolysis, and esterification [192]. PLIN2 binds to several metabolically active proteins, including DGAT2 [179]. Most notably, PLIN2 attenuates lipolysis by reducing access of ATGL to LDs [185, 196], and loss of PLIN2 has been associated with increased rates of lipolysis and β-oxidation in skeletal muscle culture [134, 197].

Several studies have shown that PLIN2 may have a net negative effect on tissue health and disease, and that knockout may be beneficial. PLIN2 knockout mice are resistant to hepatosteatosis and insulin resistance in obesogenic models [198, 199], and some have reported that PLIN2 knockout is protective against diet induced obesity and fatty liver disease [200, 201]. A recent publication showed that PLIN2 knockdown in type 1 diabetic Akita mice alleviates hyperglycemia, unfolded protein response and ER stress and increased autophagic flux [202]. Conte et al. [201] wrote an excellent review outlining roles of PLIN2 in disease, and note its contribution to cancer, heart disease, obesity, insulin resistance, atherosclerosis, and lipotoxicity.

Although knockout appears to be protective against lipid accumulation and lipotoxicity in several tissues, PLIN2 expression may be beneficial in skeletal muscle. PLIN2 mRNA expression was found to be lower in the skeletal muscle of obese pigs compared to lean counterparts [203]. Others have suggested that overexpression of PLIN2 increases oxidative capacity and improve insulin sensitivity in skeletal muscle [134, 204]. Furthermore, PLIN2 expression has been connected with the increased insulin sensitivity and oxidative capacity in endurance athletes in a phenomenon known as “the athlete’s paradox”, and lipids from PLIN2-associated LDs are preferentially oxidized during endurance exercise [204]. These apparently contradictory findings suggest a complex relationship among PLIN2, lipid management, and health, with different implications from different stimuli in different tissues, further demonstrating the dynamic nature of lipid droplets. This recognition has lead researchers to question whether PLIN2 expression triggers lipid accumulation or if lipid abundance increases PLIN2 expression [192].

#### 1.2.2.3 PLIN5

PLIN5, also called as OXPAT, is recognized as the LD coating protein (LDCP) most closely associated with high fat-oxidative capacity and is hypothesized to be a key regulator in fatty acid oxidation. It is primarily expressed in highly oxidative tissues, including the heart, type I muscle, and brown fat [167]. A study of skeletal muscle specific overexpression of PLIN5 found decreased inflammation in the liver and increased browning of adipose tissue [205], suggesting systemic benefits of improved muscle lipid storage and utilization. The major factor that distinguishes PLIN5 from other PLIN proteins is its association with mitochondria [206], where it closely associates with mitochondrial complex I, II, IV and V. PLIN5 modulates the interaction of ATGL with the LD and its co-factor CGI-58 [207]. The Hesselink group has shown that overexpression of PLIN5 increases the expression of key lipolytic genes, especially ATGL [208], and suggests that PLIN5 increases the efficiency with which mitochondria oxidize lipid [206]. This has led the Hesselink group to hypothesize that PLIN5 increase is more beneficial to mitochondrial function than is PLIN2, as PLIN2 itself is not associated with increased lipid oxidation and may only be a storage mechanism, which will ultimately be overwhelmed if high lipid environment persists.

### 1.2.3 Lipotoxicity & Lipoexpediency

IMCL and myocellular LDs are closely associated with glucose homeostasis and metabolic function [209], but imbalances between lipid accumulation, lipolysis, and lipid oxidation can have detrimental effects on cells, tissues, and organisms as a whole. Lipotoxicity is a pathological accumulation of lipid in non-adipose tissue characterized by oxidative stress, mitochondrial dysfunction, ER stress, protein misfolding, inflammation, and ultimately contributes to cell dysfunction and apoptosis [210]. At a more macroscopic level, lipotoxicity in skeletal muscle is reported to contribute to insulin resistance and sarcopenia [211, 212]. In fact, IMCL content is inversely correlated with insulin sensitivity independent of visceral fat in obese adolescents [141]. However, not all lipids impact lipotoxicity equally. Beyond total IMCL content, lipid species within IMCL are an important contributor to skeletal muscle health. Increased ratio of saturated to unsaturated fatty acid content in IMCL is associated with insulin resistance and increased whole body adiposity [213]. TAGs are relatively stable, have no cellular signaling function, and their accumulation can paradoxically prevent lipotoxicity [214]. However, DAG and ceramides are highly active signaling molecules implicated in lipotoxic systems [215, 216]. In fact, the presence of these pro-inflammatory lipid species may be more important to the development of lipotoxicity

than total IMCL content. Covington et al. show increased ceramide, despite constant IMCL, after 8 weeks of overfeeding [145]. Moreover, even among DAG and ceramide, lipotoxic functions may be limited to specific species or subcellular localization. Clinical research has shown decreased DAG saturation in endurance athletes [217] and increased saturation of DAG is associated with insulin resistance and metabolic syndrome [218]. 1,2-DAGs can activate protein kinase C (PKC) to induce insulin resistance [219, 220], while 1,3-DAGs do not [219]. Perreault et al. [221] described strong, repeated correlations between ceramide 18:0 and insulin resistance, but noted that the subcellular localization – sarcolemmal, mitochondrial, endoplasmic, or nuclear – impacts the degree to which it impacts insulin resistance. These authors conclude that analyzing whole tissue lipid content may mask meaningful differences as a result of compartmentalization.

The importance of lipid specie and localization is echoed in healthy lipid accumulation. LDs are important not only for storing energy, but also for buffering free fatty acids in cells, because even low concentrations of FFA are toxic [214]. This sequestering of fatty acids prevents the accumulation of acylcarnitines, the accumulation of which supports production of DAG and ceramide and the development of lipotoxicity [222]. Another method of preventing lipotoxicity in a high IMCL environment is by a high rate of β-oxidation [64]. Exercise counteracts lipotoxicity by improving lipid turnover and lipid profile [223]. Vitamin D has also been shown to prevent or ameliorate symptoms of lipotoxicity in kidney, liver [39], bone [44], and kidney [46], however, strong connections between vitamin D and lipotoxicity in skeletal muscle has yet to be shown.

### 1.3 Conclusion

Skeletal muscle lipid accumulation can be either a sign of metabolic stress and functional impairment or a beneficial adaptation enabling greater use of lipid oxidation for ATP generation. The difference between these two conditions is largely dependent on the method by which the lipids are stored and the rate of lipid turnover. Lipids that are packaged in LDs and readily oxidized pose little threat to a cell while non-esterified lipids that accumulate in the ER can lead to ER stress, oxidative stress, and accumulation of bioactive lipid products including ceramides and DAG. PLIN2 appears to be a key component in lipid management in skeletal muscle and enables both efficient lipid storage and oxidation. There is substantial evidence that vitamin D acts beneficially in the lipid balance and metabolic profile of skeletal muscle, but the mechanisms behind this effect are not understood. Therefore, this dissertation sought to investigate the role

of vitamin D in lipid clearance in skeletal muscle as a possible mediating factor in the treatment and prevention of lipotoxicity. Our hypothesis was that vitamin D increases IMCL accumulation, β-oxidation, and lipid turnover through a PLIN2-mediated mechanism, thereby preventing lipotoxicity in skeletal muscle.

### 1.4 C2C12 *in vitro* model

The present project will address this hypothesis through the use of a C2C12 *in vitro* model. C2C12 cells are an immortalized line of myoblasts originally isolated from the leg of C3h mice 70 hours after crushing injury to induce myogenesis [224]. C2C12 cells have since become one of the most commonly used models for skeletal muscle *in vitro* and are regularly used in projects investigating vitamin D biology. These cells are passaged as undifferentiated myoblasts in a medium containing 10-20% fetal bovine serum to > 80% confluence and then differentiated into multinucleated myotubes by replacement of high-serum medium with low serum (2-5% horse or fetal bovine serum).

There are many advantages that come with using C2C12 cells. First, they differentiate relatively quickly when compared to human primary cells, usually completing differentiation in 4-6 days opposed to >7 days among human primary myoblast lines. C2C12 myoblasts also have the advantages that come with immortalized cell lines. Notably, they are more consistent than primary cell lines. They are also highly stable in culture and can be passaged for a month or more before failure opposed to days to weeks usually observed in primary cells. However, these advantages are not without tradeoffs. Differentiated cultures of cells invariably include a significant component of mononuclear cells, and C2C12 cells have a lower efficiency of differentiation than do primary cells. The proportion of differentiated to undifferentiated cells is affected by multiple factors including substrate composition, cell passage number, and medium contents. Another shortcoming of C2C12 cells is the limitation that comes with a lack of biological replicates in immortalized cell lines. As C2C12 cells are genetically homogenous, replicates are considered technical opposed to biological, and in a strict sense of data analysis, n = 1 no matter how many times an experiment is repeated. This reduces the strength of conclusions and the confidence of applicability to human physiology. As a result, findings in this project should be confirmed using a more physiologically relevant model; first in human primary cell culture to demonstrate relevance of mechanisms and patterns to human physiology, then *in vivo*.

## 1.5 Thesis Significance

The significance of this dissertation is twofold. First, although vitamin D is closely associated with skeletal muscle health, the mechanisms through which it works are largely unknown. Several studies point to improved mitochondrial bioenergetics as the driving factor. Better understanding how vitamin D works in skeletal muscle may increase the appreciation for its application in clinical settings. The second way in which this project contributes to the scientific community is through building upon our understanding of how PLIN2 impacts skeletal muscle health. Several studies have suggested that PLIN2 overexpression may be beneficial to skeletal muscle, while others show overexpression is harmful and knockdown prevents metabolic dysfunction. Additional research is needed in this field of study to better understand how PLIN2 impacts lipid regulation and metabolic health in skeletal muscle.

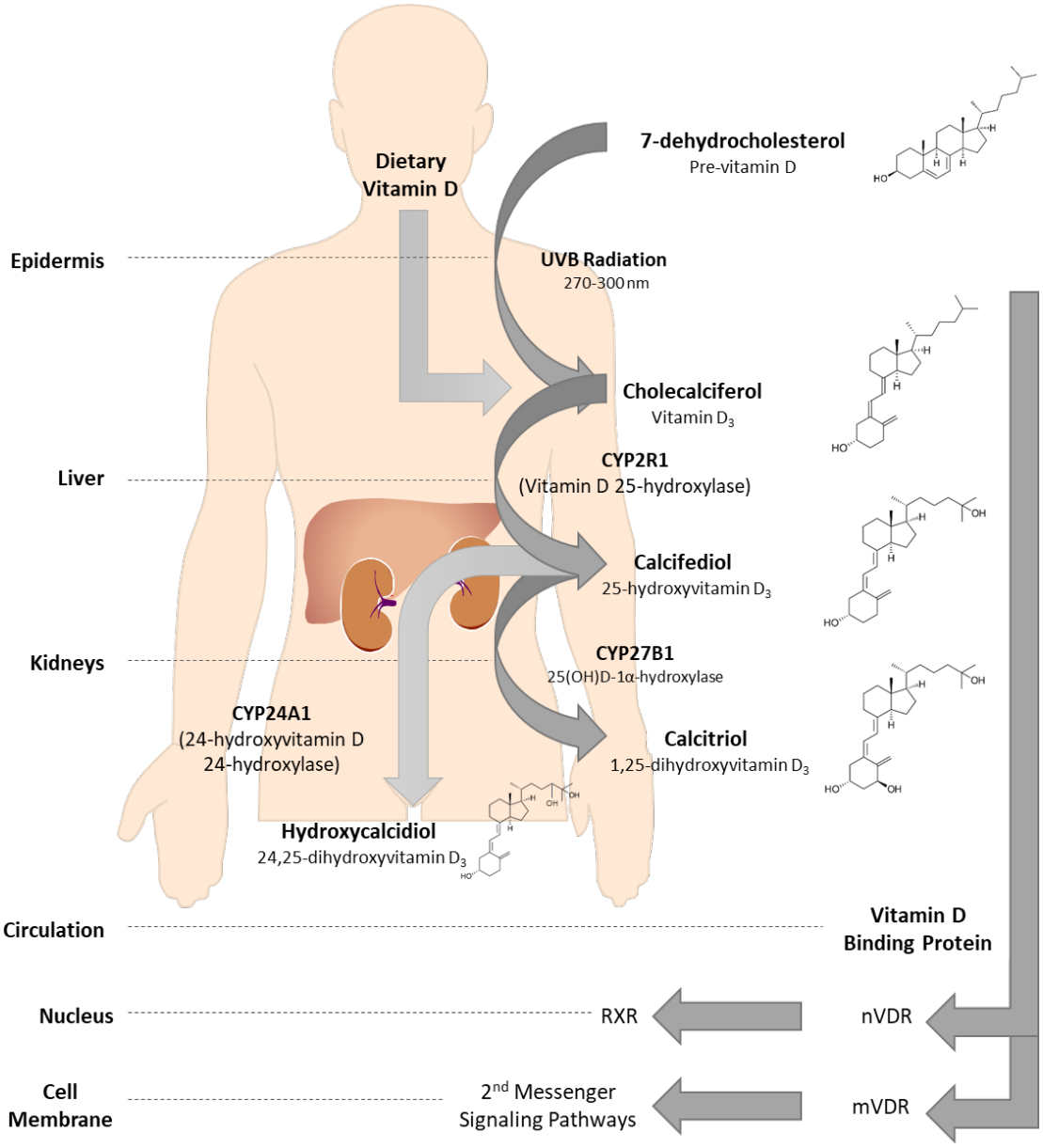

Dietary
Vitamin D
7-dehydrocholesterol
Pre-vitamin D
Epidermis
UVB Radiation
270-300 nm
Cholecalciferol
Vitamin D3
Liver
CYP2R1
(Vitamin D 25-hydroxylase)
Calcifediol
25-hydroxyvitamin D3
Kidneys
CYP27B1
25(OH)D-1α-hydroxylase
CYP24A1
(24-hydroxyvitamin D
24-hydroxylase)
Calcitriol
1,25-dihydroxyvitamin D3
Hydroxycalcidiol
24,25-dihydroxyvitamin D3
Circulation
Vitamin D
Binding Protein
Nucleus
RXR
nVDR
Cell
Membrane
2nd Messenger
Signaling Pathways
mVDR

**Figure 1.1. Vitamin D synthesis in mammalian systems.** 7-dehydrocholesterol is in converted to cholecalciferol (vitamin $D_3$) in the epidermis through a photorearrangement reaction after exposure to UVB radiation (270-300 nm). Cholecalciferol is also obtained through dietary consumption. Cholecalciferol and subsequent forms of vitamin D are bound by vitamin D binding protein (VDBP) to improve their chemical and biological stability. Cholecalciferol is hydroxylated into calcifediol (25-hydroxyvitamin $D_3$) by CYP2R1 (vitamin D 25-hydroxylase) in the liver. Calcifediol is hydroxylated at C1 into calcitriol (1,25-dihydroxyvitamin $D_3$) by CYP27B1 (25(OH)D-1α-hydroxylase) primarily in the kidney, although this reaction occurs in in various other tissues. Calcitriol can then bind to either nuclear vitamin D receptor (nVDR), which activates canonical gene regulation through interaction with retinoid X receptor (RXR), or membrane vitamin D receptor (mVDR), which activates a range of second messenger systems including protein kinase A, protein kinase C, and Akt. Calcifediol, in addition to calcitriol, can by hydroxylated at C24 into dihydroxycalcidiol (24,25-dihydroxyvitamin D) by CYP24A1 (25-hydroxyvitamin D 24-hydroxylase) for excretion in the urine.

# CHAPTER 2: METHODS

## 2.1 Cell Culture

C2C12 myoblasts were obtained from American Type Culture Consortium (ATCC; Manassas, Virginia, USA) and grown to a maximum of 60% confluence. At appropriate confluence, cells were trypsinized and seeded overnight in growth medium (GM) consisting of DMEM containing 1000 mg/L glucose with L-glutamine and sodium bicarbonate (MilliporeSigma, Burlington, MA, USA; #D6046) supplemented with 10% FBS (Gemini Bio-Products, West Sacramento, CA, USA; #100-106) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA, USA;Scientific, Waltham, MA, USA; #15140-122) in a humidified incubator kept at 37°C and 10% $CO_2$ (Day 0). Following overnight seeding, GM was replaced with differentiation medium (DM) consisting of DMEM (same as above) supplemented with 2% horse serum (Day 1). DM was changed every other day.

## 2.2 Treatment with PLIN2 siRNA

On Day 5 in DM, differentiated myotubes were treated with 10 nM Thermo Fisher Stealth siRNA against PLIN2 (Thermo Fisher Scientific Waltham, MA, USA; #132001) or medium GC content scramble Stealth siRNA (Thermo Fisher Scientific Waltham, MA, USA; #12935300) as previously published [134]. All siRNA was prepared in DM with 0.2% Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific Waltham, MA, USA; #13778) and 20% Opti-MEM (Thermo Fisher Scientific Waltham, MA, USA; #31985). Cells were treated with siRNA for a total of 48 hours. On Day 7, cells were treated with calcitriol and palmitate as described above. Cells with PLIN2 knockdown are represented in text as siCTL, siVitD, siPA, and siPA+VitD. Cell growth and treatment are summarized in Appendix 1.

## 2.3 Vitamin D and Palmitate Treatment

On Day 7, cells were treated with vector control (0.1% ethanol/0.2% BSA) (CTL), 100 µM palmitate (Cayman Chemical, #1006627) (PA), 100 nM calcitriol (Sigma, D1530) (VD), or both (PA+VD) for 24 hours. Treatments receiving PLIN2 knockdown are represented in text as siCTL, siPA, siVitD, and siPA+VitD.

### 2.4 Quantitative Real-Time Polymerase Chain Reaction

Cells were seeded 50,000/well in a 24 well culture plate and treated as described above. After treatment, media was removed and cells were washed in phosphate buffered saline (PBS) then scraped off the plate in 150 µL QIAzol Reagent (Qiagen, Hilden, Germany; #79306). Three wells receiving the same treatment were combined and lysed in a bead homogenizer. RNA was isolated using an ethanol precipitation on a RNA elution column (Enzymax, Lexington, KY, USA; #EZCR101). RNA was then reverse transcribed with a qScript cDNA synthesis kit (Quanta Biosciences, Beverly, MA; 101414-106) according to the manufacturer's recommendations. Relative gene expression was measured using PowerUp SYBR (Thermo Fisher Scientific, Waltham, MA; #A25778) in a QuantStudio 3 real time PCR machine (Thermo Fisher Scientific, Waltham, MA). The geometric mean of three housekeeping genes (RER1, VCP, and EMC7) was used as an endogenous control. Expression was quantified using the $2^{\Delta\Delta\text{-Ct}}$ method. Values were normalized to the CTL for each respective treatment and reported as fold change. Primers were purchased through Integrated DNA Technologies and primer sequences used in this study are listed in Appendix 2.

### 2.5 VDR/Myosin Heavy Chain (MyHC) Immunostaining

Cells were plated 25,000 per chamber in 4 well plastic chamber slides then differentiated and treated as described above. Following treatment, cells were fixed in 4% paraformaldehyde (PFA) and blocked with 10% normal goat serum. Cells were then incubated with Vitamin D3 Receptor (D2K6W) Rabbit mAb primary antibody (Cell Signaling, Danvers, MA USA; 12550) 1:200 in TBST with 5% normal goat serum overnight at 4°C with gentle rocking. Myotubes were then washed with PBS and incubated with anti-rabbit alkaline phosphatase polymer for 60 minutes, washed with PBS, then incubated with ImmPACT Vector Red substrate working solution for 10-30 minutes or until the desired intensity of color was reached. Cells were then washed with PBS and incubated with a primary antibody against pan myosin heavy chain (MyHC) (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, Iowa; A4.1025) in 5% normal goat serum for one hour at room temperature with gentle rocking. Myotubes were then incubated for 1 hour with an anti-mouse $IgG_{2a}$ Alexafluor488 secondary antibody at room temperature (Life Technologies, Carlsbad, CA, USA; A21121) and washed with PBS. Myotubes were finally incubated for 10 minutes in DAPI (Invitrogen, Cat# D3571) (1:10,000 dilution) at room temperature. Myotubes were imaged using a Zeiss AxioObserver D1 inverted fluorescent microscope (Jena, Germany).

Micrographs were taken using an AxioCam MR (Zeiss) camera then analyzed and annotated using AxioVision SE64 Rel. 4.9.1 software (Zeiss, Jena, Germany).

### 2.6 Oil Red O Staining

Cells were grown, plated, and treated as described above. Following treatment, cells were fixed in 4% PFA for 3 minutes then washed 2 times with PBS. Cells were then stained with 500 µL of Oil Red O (MilliporeSigma, Burlington, MA, USA; #O-0625) prepared in triethylphosphate according to the manufacturer's specifications for 30 minutes at 37°C with occasional rocking. Cells were then washed 3 x 5 minutes with PBS water at 37°C with occasional rocking. Following washing, 500 µL of PBS water was added to each well and cells were imaged using a Zeiss AxioObserver D1 inverted fluorescent microscope (Jena, Germany). Micrographs were taken using an AxioCam MR (Zeiss) camera then analyzed and annotated using AxioVision SE64 Rel. 4.9.1 software (Zeiss, Jena, Germany).

### 2.7 Succinate Dehydrogenase (SDH) Activity Staining

Cells were grown, plated, and treated as described above. Following treatment, myotubes were washed with PBS, fixed with 4% PFA and then incubated in 1.2 mM nitro blue tetrazolium chloride (NBT) (MilliporeSigma, Burlington, MA, USA; #N6876) with 275 mM succinic acid (MilliporeSigma, Burlington, MA, USA; #224731) in PBS at 37°C for 120 minutes. Cells were then washed 3 times with PBS and imaged using a Zeiss AxioObserver D1 inverted fluorescent microscope (Zeiss). Micrographs were taken using an AxioCam MR (Zeiss) camera then analyzed and annotated using AxioVision SE64 Rel. 4.9.1 software (Zeiss).

### 2.8 Seahorse OCR Assay

Myoblasts were plated 10,000 per well in a Seahorse XFe24 assay plate (Agilent, Santa Clara, CA, USA) overnight in GM, then differentiated, and treated as described above. On the day of the assay, fresh Seahorse XF Assay Medium (Agilent, Santa Clara, CA, USA; #102365) was supplemented with 5 mM glucose and 1 mM pyruvate and pH adjusted to 7.4. Myotubes were washed twice with XF Assay Medium then incubated for one hour in 100 µL of XF Assay Medium in a humidified chamber at 37°C with atmospheric $CO_2$. Vehicle and calcitriol treatments were maintained throughout incubation and the assay. Following incubation, XF Assay Medium was added to a final volume of 525 µL. OCR and Extracellular Acidification Rate (ECAR) were measured

at 37°C using Seahorse XFe24 Analyzer (Agilent, Santa Clara, CA, USA). During the assay, each treatment was injected sequentially to achieve the following final concentrations: 2.5 µM oligomycin (Biomol, Hamburg, Germany; #CM-111), 4 µM carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (Biomol, Hamburg, Germany; #CM120), 10 mM succinate (MilliporeSigma, Burlington, MA, USA; #S-7501) with 0.8 µM rotenone (Biomol, Hamburg, Germany; #CM-117), and 1 µM Antimycin A (MilliporeSigma, Burlington, MA, USA; #A8674). OCR was normalized to the average basal rate among all treatments for each given experiment. Specific rates based on equations suggested by Agilent Biosciences (listed in Table 2) were used to calculate the following OCRs: basal, oligomycin, FCCP, rotenone/succinate, Antimycin A, ATP-linked, maximum, reserve, complex I, complex II, leak, non-mitochondrial, and mitochondrial area under the curve (AUC).

### 2.9 Statistics

The Student's t-test was used to determine the effects of VitD alone vs vehicle control. A 2 x 2 factorial ANOVA was used to determine the effects of calcitriol (VitD effect) and palmitate (PA effect) or calcitriol and PLIN2 knock down (siPLIN2 effect) and respective interaction effects. When appropriate, a 3 x 3 factorial ANOVA was used to determine the effects of calcitriol, palmitate, PLIN2 knockdown and interactions between treatments. When there was a significant interaction between two treatments, the Fisher's LSD post-hoc test was applied. All tests were two-tailed with statistical significance defined as $p < 0.05$. All data were normally distributed. All quantitative results are shown as mean ± SEM of no fewer than 3 independent experiments. Statistical calculations were performed using JMP 12 (SAS Institute, Cary, NC, USA).

# CHAPTER 3: VITAMIN D PRODUCES A PERILIPIN 2-DEPENDENT INCREASE IN MITOCHONDRIAL FUNCTION IN C2C12 MYOTUBES

## 3.1 Abstract

Vitamin D has been connected with increased intramyocellular lipid (IMCL) and has also been shown to increase mitochondrial function and insulin sensitivity. Evidence suggests that perilipin 2 (PLIN2), a perilipin protein upregulated with calcitriol treatment, may be integral to managing increased IMCL capacity and lipid oxidation in skeletal muscle. Therefore, we hypothesized that PLIN2 is required for vitamin D induced IMCL accumulation and increased mitochondrial oxidative function. To address this hypothesis, we treated C2C12 myotubes with 100 nM calcitriol (the active form of vitamin D) and/or PLIN2 siRNA in a four group design and analyzed markers of IMCL accumulation and metabolism using qRT-PCR, cytochemistry, and oxygen consumption assay. Expression of PLIN2, but not PLIN3 or PLIN5 mRNA was increased with calcitriol, and PLIN2 induction was prevented with siRNA knockdown without compensation by other perilipins. PLIN2 knockdown did not appear to prevent lipid accumulation. Calcitriol treatment increased mRNA expression of triglyceride synthesizing genes DGAT1 and DGAT2 and also lipolytic genes ATGL and CGI-58. PLIN2 knockdown decreased the expression of CGI-58 and CPT1, and was required for calcitriol-induced upregulation of DGAT2. Calcitriol increased oxygen consumption rate while PLIN2 knockdown decreased oxygen consumption rate. PLIN2 was required for a calcitriol-induced increase in oxygen consumption driven by mitochondrial complex II. We conclude that calcitriol increases mitochondrial function in myotubes and that this increase is at least in part mediated by PLIN2.

## 3.2 Introduction

The ability to store lipid as potential energy is one of the oldest and most highly conserved adaptations of life on Earth [1-3]. In mammals, adipocytes are evolved to store large quantities of lipid, but appreciable lipid stores are also found in the skeletal muscle where they are made available for β-oxidation in mitochondria. Intramyocellular lipid (IMCL) is stored in lipid droplets (LD), highly specialized and tightly regulated organelles that play important roles in lipid accumulation, storage, and lipolysis. Lipid droplets are comprised of a phospholipid monolayer studded with several dozen different proteins, including perilipins (PLINs), that surrounds a neutral lipid core of triacylglycerides (TAG), diacylglycerides (DAG), and cholesteryl esters (CE) [4-

6]. PLINs are essential for LD formation and function [7]. Of the 5 members of the perilipin family, PLIN2, PLIN3, and PLIN5 (also referred to as ADRP, TIP47, and OXPAT, respectively) are found in skeletal muscle. Although some have reported high levels of PLIN4 expression in skeletal muscle [8], its expression is not widely recognized [9]. While each PLIN seems to play an important and independent role in IMCL regulation, PLIN2 is the most highly expressed PLIN in skeletal muscle and is thought to serve primarily as a scaffolding protein that modulates access of adipose triglyceride lipase (ATGL) to its enzymatic substrate, TAG [10, 11]. While some have reported that PLIN2 knockout is protective against pathological lipid accumulation in some tissues [12, 13], others have suggested that overexpression of PLIN2 increases oxidative capacity and improves metabolic function in skeletal muscle [14, 15]. Recent work from our group indicates that treatment with calcitriol (1,25-dihydroxyvitamin $D_3$, the active form of vitamin D) increases PLIN2 expression in muscle cells *in vitro* [16].

Dietary vitamin D supplementation and calcitriol treatment have both been shown to support healthy skeletal muscle function [17-19]. Vitamin D is associated with increased IMCL content in both clinical and basic research [16, 20]. Increased IMCL, especially in association with increased body fat mass, is often associated with increased inflammation, metabolic dysfunction, and insulin resistance. Pathological lipid processing is seen in many tissues and is broadly referred to as lipotoxicity [21-24]. Although vitamin D increases IMCL, it has also been associated with decreased inflammation and improved insulin sensitivity, mitochondrial activity, and functional capacity in skeletal muscle [18, 19, 25-27].

This paradoxical increase in IMCL and mitochondrial function following vitamin D supplementation may be a product of an increase in the efficiency with which muscle accumulates, stores, and oxidizes lipid, a process often referred to as “lipid flux”. Increased rates of lipid flux and well-regulated storage of lipids as TAG in lipid droplets may help to prevent lipotoxicity [28-30] as modeled in the athlete’s paradox, a condition observed in endurance athletes characterized by both increased IMCL, high mitochondrial efficiency, and insulin sensitivity [31-33]. Increased expression of PLIN2 has been connected to increased TAG storage and oxidation [14, 15], and may be a mechanism through which vitamin D increases lipid flux in skeletal muscle. This study sought to determine the role of PLIN2 in vitamin D mediated increases in IMCL accumulation and mitochondrial function. We hypothesized that PLIN2 is required for vitamin D-induced IMCL accumulation and increased mitochondrial oxidative function. To

investigate this, we used a C2C12 murine muscle cell line treated with calcitriol. The role of PLIN2 in changes in lipid content and metabolism was determined by knocking down PLIN2 expression using siRNA.

## 3.3 Methods

### 3.3.1 Cell culture

C2C12 myoblasts were obtained from American Type Culture Consortium (ATCC; Manassas, Virginia, USA) and grown to a maximum of 60% confluence. At appropriate confluence, cells were trypsinized and seeded overnight in growth medium (GM) consisting of DMEM containing 1000 mg/L glucose with L-glutamine and sodium bicarbonate (MilliporeSigma, Burlington, MA, USA; #D6046) supplemented with 10% FBS (Gemini Bio-Products, West Sacramento, CA, USA; #100-106) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA, USA;Scientific, Waltham, MA, USA; #15140-122) in a humidified incubator kept at 37°C and 10% $CO_2$ (Day 0). Following overnight seeding, GM was replaced with differentiation medium (DM) consisting of DMEM (same as above) supplemented with 2% horse serum (Day 1). DM was changed every other day.

### 3.3.2 Treatment with PLIN2 siRNA and Calcitriol

On Day 5 in DM, differentiated myotubes were treated with 10 nM Thermo Fisher Stealth siRNA against PLIN2 (Thermo Fisher Scientific Waltham, MA, USA; #132001) or medium GC content scramble Stealth siRNA (Thermo Fisher Scientific Waltham, MA, USA; #12935300) as previously published [134]. All siRNA was prepared in DM with 0.2% Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific Waltham, MA, USA; #13778) and 20% Opti-MEM (Thermo Fisher Scientific Waltham, MA, USA; #31985). Cells were treated with siRNA for a total of 48 hours. On Day 7, cells were treated with vehicle control (0.1% ethanol) (CTL) or 100 nM calcitriol (MilliporeSigma, Burlington, MA, USA; #D1530) (VitD) for 24 hours. Cells with PLIN2 knockdown are represented in text as siCTL and siVitD. Cell growth and treatment is summarized in Appendix 1.

### 3.3.3 Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

Cells were seeded 50,000/well in a 24 well culture plate and treated as described above. After treatment, media was removed and cells were washed in phosphate buffered saline (PBS) then scraped off the plate in 150 µL QIAzol Reagent (Qiagen, Hilden, Germany; #79306). Three wells receiving the same treatment were combined and lysed in a bead homogenizer. RNA was isolated using an ethanol precipitation on a RNA elution column (Enzymax, Lexington, KY, USA; #EZCR101). RNA was then reverse transcribed with a qScript cDNA synthesis kit (Quanta Biosciences, Beverly, MA; 101414-106) according to the manufacturer's recommendations. Relative gene expression was measured using PowerUp SYBR (Thermo Fisher Scientific, Waltham, MA; #A25778) in a QuantStudio 3 real time PCR machine (Thermo Fisher Scientific, Waltham, MA). The geometric mean of three housekeeping genes (RER1, VCP, and EMC7) was used as an endogenous control. Expression was quantified using the $2^{\Delta\Delta\text{-Ct}}$ method. Values were normalized to the CTL for each respective treatment and reported as fold change. Primers were purchased through Integrated DNA Technologies, and primer sequences used in this study are listed in Appendix 2.

### 3.3.4 Oil Red O Staining

To assess neutral lipid accumulation, myotubes were fixed in 4% paraformaldehyde (PFA) and washed with PBS, then stained with oil red O (ORO) (MilliporeSigma, Burlington, MA USA; #O-0625) prepared in triethylphosphate according to the manufacturer's specifications for 30 minutes at 37°C with occasional rocking. Cells were then washed and imaged. PLIN2 knockdown myotubes were treated with 100 µM palmitate for 3 hours prior to ORO staining. For all cytochemistry experiments, cells were imaged using a Zeiss AxioObserver D1 inverted fluorescent microscope (Jena, Germany). Micrographs were taken using a Zeiss AxioCam MR camera and analyzed and annotated using AxioVision SE64 Rel. 4.9.1 software (Zeiss, Jena, Germany).

### 3.3.5 Succinate Dehydrogenase Activity Staining

Myotubes were washed with PBS, fixed with 4% PFA and then incubated in 1.2 mM nitro blue tetrazolium chloride (NBT) (MilliporeSigma, Burlington, MA, USA; #N6876) with 275 mM succinic acid (MilliporeSigma, Burlington, MA, USA; #224731) in PBS at 37°C for 120 minutes. Cells were then washed 3 times with PBS and imaged as described above.

### 3.3.6 Seahorse Oxygen Consumption Rate (OCR) Assay

Myoblasts were plated 10,000 per well in a Seahorse XFe24 assay plate (Agilent, Santa Clara, CA, USA) overnight in GM, then differentiated, and treated as described above. On the day of the assay, fresh Seahorse XF Assay Medium (Agilent, Santa Clara, CA, USA; #102365) was supplemented with 5 mM glucose and 1 mM pyruvate and pH adjusted to 7.4. Myotubes were washed twice with XF Assay Medium then incubated for one hour in 100 µL of XF Assay Medium in a humidified chamber at 37°C with atmospheric $CO_2$. Vehicle and calcitriol treatments were maintained throughout incubation and the assay. Following incubation, XF Assay Medium was added to a final volume of 525 µL. OCR and Extracellular Acidification Rate (ECAR) were measured at 37°C using Seahorse XFe24 Analyzer (Agilent, Santa Clara, CA, USA). During the assay, each treatment was injected sequentially to achieve the following final concentrations: 2.5 µM oligomycin (Biomol, Hamburg, Germany; #CM-111), 4 µM carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (Biomol, Hamburg, Germany; #CM120), 10 mM succinate (MilliporeSigma, Burlington, MA, USA; #S-7501) with 0.8 µM rotenone (Biomol, Hamburg, Germany; #CM-117), and 1 µM Antimycin A (MilliporeSigma, Burlington, MA, USA; #A8674). OCR was normalized to the average basal rate among all treatments for each given experiment. Specific rates based on equations suggested by Agilent Biosciences (listed in Appendix 3) were used to calculate the following OCRs: basal, oligomycin, FCCP, rotenone/succinate, Antimycin A, ATP-linked, maximum, reserve, complex I, complex II, leak, non-mitochondrial, and mitochondrial area under the curve (AUC).

### 3.3.7 Statistical Analysis

The Student's t-test was used to determine the effects of calcitriol alone. A 2 x 2 factorial ANOVA was used to determine the effects of calcitriol (VitD effect), PLIN2 knockdown (siPLIN2 effect), and the interaction between treatments. When there was a significant interaction between VitD and siPLIN2, the Fisher's LSD post-hoc test was applied. All tests were two-tailed, with statistical significance defined as $p < 0.05$. All data were normally distributed. All quantitative results are shown as mean ± SEM of no fewer than 3 independent experiments. Statistical calculations were performed using JMP 12 (SAS Institute, Cary, NC, USA).

## 3.4 Results

### 3.4.1 Vitamin D drives changes in PLIN2 expression and mitochondrial activity in C2C12 myotubes consistent with increased lipid flux

We first examined the effect of VitD alone on the expression of genes associated with IMCL accumulation and mitochondrial metabolism. Using qRT-PCR, we measured gene expression of vitamin D receptor (VDR) and PLIN2 in C2C12 myotubes treated with VitD or CTL for 24 hours and found that the expression of both genes was increased after VitD treatment ($p < 0.05$) (Figure 3.1A). Because PLIN5 is commonly associated with increased lipid storage and increased mitochondrial function [206, 208], we also measured its expression with VitD treatment. RT-PCR analysis showed that PLIN5 did not change with VitD treatment (Figure 3.1A). To connect increased PLIN2 expression with increased IMCL accumulation, we labeled neutral lipid content in myotubes using ORO staining. Consistent with other publications [48, 49], we found that IMCL content in C2C12 myotubes appeared to be increased following VitD treatment (Figure 3.1B).

Having confirmed increased PLIN2 expression and IMCL accumulation, we next moved to measure changes in mitochondrial metabolism. We completed an initial assessment of mitochondrial function by SDH activity staining and found that VitD treatment appeared to increase the intensity of SDH activity staining in myotubes (Figure 3.1C). For a detailed, quantitative analysis of the VitD's impact on muscle cell mitochondrial metabolism, we analyzed OCR by Seahorse extracellular flux analyzer. Results show that VitD increased OCR throughout the experiment with statistically significant increases in OCR after the administration of FCCP (Figure 3.1D-E). To further characterize changes in OCR, OCR specific to ATP-production and mitochondrial complexes were measured. We found that increases were attributed to OCR driven by complex I and maximal OCR. These changes were accompanied by a trend to increase complex II OCR, however, this failed to reach statistical significance ($p = 0.15$) (Figure 3.1F).

### 3.4.2 PLIN2 knockdown does not impact mRNA expression of other perilipin genes

siRNA knockdown was used to investigate the role that PLIN2 plays in regulating VitD-induced changes in IMCL and mitochondrial function. RT-PCR quantification of mRNA expression verified that siRNA successfully decreased PLIN2 mRNA expression ($p < 0.01$); cells treated with siVitD decreased by 69% and siCTL decreased by 29% compared to their respective scramble controls

(Figure 3.2A). While VitD treatment caused increased VDR expression ($p < 0.001$), there was no effect of siRNA and no interaction between the treatments (Figure 3.2B). Perilipins other than PLIN2, namely PLIN3 and PLIN5, have been connected with increased mitochondrial activity in skeletal muscle [208, 226, 227]. We measured expression of these genes to ensure that these perilipins were not upregulated to compensate for decreased PLIN2 expression and found that neither was significantly increased under any treatment condition (Figure 3.2C-D).

### 3.4.3 PLIN2 knockdown does not prevent lipid accumulation

Overexpressing PLIN2 has been shown to increase lipid accumulation in skeletal muscle [134, 228], while knockdown prevents lipid accumulation in multiple tissues [200, 229, 230]. To see how PLIN2 knockdown impacts IMCL accumulation in our model, we treated differentiated myotubes with palmitate and assessed neutral lipid accumulation with ORO staining. Micrographs showed that PLIN2 knockdown did not appear to prevent lipid accumulation in C2C12 myotubes (Figure 3.3).

### 3.4.4 PLIN2 knockdown and VitD exert opposing effects on genes regulating lipid flux

IMCL storage and lipolysis are tightly regulated at the gene level and are associated with both the expression of PLIN2 [184, 192] and VDR [48]. We therefore measured the expression of key regulators of triglyceride storage and lipolysis to determine how they are impacted by simultaneous PLIN2 knockdown and VitD treatment. We found that VitD increased the expression of ATGL ($p < 0.001$) with no effect of siPLIN2 (Figure 3.4A). Comparative gene identification-58 (CGI-58) mRNA expression was upregulated by VitD but decreased after PLIN2 knockdown ($p = 0.045$) (Figure 3.4B). PLIN2 knockdown downregulated carnitine palmitoyltransferase 1 (CPT1) gene expression ($p = 0.003$) with VitD associated with a trend to slightly decrease expression ($p = 0.104$) (Figure 3.4C). These results show a pattern of VitD increasing the expression and PLIN2 knockdown decreasing the expression of key genes that regulate lipolysis and β-oxidation.

We next examined the expression of genes integral to triglyceride storage in lipid droplets. We found that VitD had a very strong positive effect on diglyceride O-acyltranferase 1 (DGAT1) expression ($p < 0.001$). While comparisons did not reach statistical significance, PLIN2 knockdown was associated with a mean increase in DGAT1 expression by approximately 500% compared to CTL and 50% compared to VitD (Figure 3.4D). Diglyceride O-acyltranferase 2 (DGAT2) was also

strongly upregulated with VitD treatment ($p < 0.001$). We observed an interaction effect between VitD and siPLIN2 ($p = 0.006$) (Figure 4E). Post-hoc analysis revealed that DGAT2 expression in VitD treated myotubes was decreased to CTL levels after PLIN2 knockdown.

### 3.4.5 PLIN2 knockdown prevents VitD induced increases in OCR

To obtain detailed, quantitative analysis of changes in mitochondrial function after treatment with VitD and PLIN2 knockdown, we measured OCR using a Seahorse XFe24 extracellular flux analyzer (Figure 3.5A). Simplified values obtained from the full OCR trace (Figure 3.5A) are shown as bar graphs for ease of interpretation (Figures 3.5B-F). At baseline, both VitD and siPLIN2 had significant effects on OCR. There was a significant interaction effect ($p = 0.029$) (Figure 3.5B). Post-hoc analysis indicated that OCR was decreased by siVitD, with no other differences between groups. Neither VitD nor siPLIN2 produced a significant effect in OCR following the administration of oligomycin (Figure 3.5C). VitD did not affect OCR following FCCP injection, but siPLIN2 caused a significant decrease in OCR (Figure 3.5D). VitD, but not PLIN2, produced a significant effect following the administration of rotenone and succinate with a significant interaction effect ($p = 0.022$) (Figure 3.5E). Post-hoc analysis indicated that VitD increased OCR in comparison to CTL, but the addition of siPLIN2 decreased OCR in the siVitD cells to levels equivalent to siCTL and CTL cells. There were no significant changes in response to either treatment after administration of Antimycin A (Figure 3.5F).

More specific analyses focusing on mitochondrial specific OCR were performed (Figures 3.5G-N). Following quantification of the area under the curve (AUC) specific to mitochondrial respiration, we found that VitD increased mitochondrial OCR while siPLIN2 decreased OCR (Figure 3.5G). VitD decreased basal mitochondrial respiration ($p = 0.049$), and siPLIN2 produced a trend towards decrease ($p = 0.066$) (Figure 3.5H). No significant responses to treatment were observed in ATP-Linked OCR, although siPLIN2 produced a trend towards decreased OCR ($p = 0.110$) (Figure 3.5I). VitD produced a trend towards increased Maximal OCR that failed to reach significance ($p = 0.084$) (Figure 3.5J). siPLIN2 significantly decreased Maximal OCR. Similarly, VitD yielded a trend towards increased Reserve OCR ($p = 0.052$), while siPLIN2 caused a significant decrease (Figure 5K). Complex I was unaffected by VitD but was decreased by siPLIN2 (Figure 3.5L). Both VitD and siPLIN2 had a significant effect on Complex II OCR. Co-treatment of VitD and siPLIN2 produced an interaction effect at complex II wherein VitD treatment alone increases OCR with either siCTL or

siRNA, but siPLIN2 treatment decreases the magnitude of the VitD effect (Figure 3.5M). VitD decreased mitochondrial leak, an indicator of mitochondrial uncoupling, while siPLIN2 caused a trend towards decreased mitochondrial leak ($p = 0.115$) (Figure 3.5N). There were no changes in extracellular acidification rate (ECAR), a measurement of glycolysis (Figures. 3.5O-P). In summary, VitD produced trends towards increased maximal and reserve mitochondrial OCR, a significant increase of OCR driven by complex II, and decreased basal OCR and mitochondrial leak. Conversely, siPLIN2 decreased maximal and reserve OCR, OCR driven by both complex I and complex II, and a trend towards decreased basal OCR. siPLIN2 reversed the VitD-mediated increase of OCR driven by complex II.

## 3.5 Discussion

This study was the first to examine the impact of calcitriol or PLIN2 knockout on mitochondrial function in skeletal muscle myotubes and advances our understanding of how these two factors modulate muscle bioenergetics. Data supported the hypothesis that calcitriol improves oxidative metabolism in differentiated skeletal muscle myotubes and that these benefits were partially mediated by the lipid packaging protein PLIN2. We showed that PLIN2 knockdown in myotubes decreased mitochondrial function, but did not appear to prevent IMCL accumulation. Our working hypothesis is illustrated in Figure 3.6. This study validates PLIN2 as an important component of mitochondrial metabolism in skeletal muscle, and bioenergic findings reported here should be considered in future studies investigating PLIN2 knockdown or knockout. We speculate that changes in mitochondrial metabolism were driven by increased fatty acid oxidation. Future investigations should examine the relationship between lipolysis, acyl chain import into mitochondria, and β-oxidation in reference to PLIN2 in skeletal muscle.

We first showed that calcitriol increases PLIN2 mRNA expression and neutral lipid accumulation in accord with previously published clinical and *in vitro* studies from our group [16, 20]. Curiously, this appears to be in opposition to a recent publication by Li et al. [42], who showed that a high-vitamin D dietary intervention in mice reduced PLIN2 expression and prevented IMCL accumulation. However, the observed difference reported by Li et al. may be a product of pathological lipid accumulation incited by vitamin D deficiency instead of decreased IMCL with vitamin D supplementation. While vitamin D deficiency and supplementation may have similar effects on PLIN2 expression, evidence suggests the physiological conditions that underlie these

scenarios are substantially different. Without markers of mitochondrial activity or β-oxidation, it is difficult to gauge the impact of IMCL on mitochondrial health.

Data obtained in this study through Seahorse oxygen consumption and SDH activity staining support the hypothesis that calcitriol treatment drives improved mitochondrial bioenergetics and that increased PLIN2 expression and IMCL accumulation after calcitriol treatment is not detrimental to mitochondrial function. Others have shown that vitamin D treatments improve mitochondrial function in both clinical and *in vitro* models. Sinha et al. [25] showed that calcitriol decreases the half time of creatine phosphorylation, a marker of mitochondrial function, in a clinical model. Ryan et al. have shown that calcitriol treatment in human primary myoblasts increases OCR in both healthy and cancerous models [26, 43]. However, differentiation of skeletal muscle triggers substantial changes in mitochondrial substrate management and bioenergetic remodeling [44], and mechanisms in myoblasts cannot be assumed to drive metabolic changes in myotubes after the same treatment.

Consistent with previous work form our group, we showed that calcitriol treatment increased the gene expression of ATLG, CGI-58, DGAT1, and DGAT2 [16]. These genes represent the rate limiting steps of both lipolysis and TAG acylation, and their upregulation suggests that calcitriol increases lipid flux [45]. This potential increase in lipid flux may have beneficial implications for muscle lipid storage that decrease the risk of lipotoxicity [28]. While vitamin D is generally accepted to contribute to the health and function of a variety of tissues, the role of PLIN2 in lipid homeostasis is hotly debated. Many studies suggest that PLIN2 enables steatosis and lipotoxicity [12, 39, 46], but others show that it may have a more beneficial effect, especially in skeletal muscle [14]. The divergence in the impact of PLIN2 may be associated with the role of lipids in the target tissues, specifically, how efficiently lipid is stored and used. We show that PLIN2 knockdown had no impact on the expression of several key lipid management genes when comparing CTL to siCTL myotubes, however, PLIN2 knockdown prevented calcitriol induced expression of CGI-58, CPT1, and DGAT2. CGI-58 is a potent regulator of lipolysis that acts both in conjunction with and independent from ATGL [47-49], suggesting that PLIN2 knockdown impairs lipolytic capacity. On the other hand, others have reported an increase in lipid oxidation in response to PLIN2 knockdown in skeletal muscle [14], which implies an increase in lipolysis. It is of note that PLIN2 also binds to ATGL and prevents the association of CGI-58, thereby decreasing ATGL activity [41]. However, excess ATGL activity is known to increase DAG and ceramide abundance and incite

metabolic dysfunction [29]. Therefore, it may be important to increase the expression of DGAT in harmony with ATGL to prevent the accumulation of bioactive signaling lipids.

Our results revealed that both DGAT1 and DGAT2 were upregulated with calcitriol treatment; however, PLIN2 knockdown prevented calcitriol-induced upregulation of DGAT2. Although both DGAT enzymes are important regulators of lipid metabolism, DGAT2 plays a larger role in TAG homeostasis [50] and likely contributes more directly to mitochondrial metabolism. DGAT1 is thought to mediate TAG acylation in the ER and nascent lipid droplets, whereas DGAT2 is found in the ER, cytosolic lipid droplets, and associated with mitochondria [51-53]. This, combined with decreased expression of CPT1, suggests that PLIN2 knockdown may make acyl chains less available for oxidation in mitochondria and exacerbate lipotoxic effects of aberrant ATGL activity.

Changes in the expression of lipolytic and lipid storage genes in response to PLIN2 knockdown and calcitriol imply changes in IMCL accumulation in myotubes. However, while not quantitative, ORO staining presented in this study did not support our hypothesis that PLIN2 is required for IMCL accumulation. Although previous studies have shown that PLIN2 knockout prevents lipid accumulation in multiple tissues [12, 39, 40], we note that many of studies are *in vivo* studies using complete knockout models. Also, mRNA expression of siCTL vs CTL is only reduced by approximately 30% in this study, a much less substantial decrease in expression than the approximately 75% knockdown observed in previous PLIN2 knockout in C2C12 cells [14]. This level of knockdown may not be sufficient to prevent new LD formation or dramatically reduce IMCL accumulation; however, it does allow us to interpret changes as those produced by the increase in PLIN2 expression opposed to the simple presence of PLIN2.

Our investigation into oxygen consumption uncovered dramatic effects of calcitriol supplementation that were highly dependent on PLIN2 upregulation. This is most easily observed in the increase on OCR measured by AUC after calcitriol treatment that returned to baseline levels with PLIN2 knockdown. Strong trends towards increase at maximal ($p = 0.084$) and reserve OCR ($p = 0.052$) provide evidence that calcitriol increases the mitochondrial oxidative capacity of C2C12 myotubes. These increases were both reduced to magnitudes below CTL after PLIN2 knockdown. Altogether, we observed siPLIN2 effects on basal mitochondrial OCR, maximal OCR, reserve OCR, and complexes I and II. Furthermore, all changes in mitochondrial respiration that failed to reach significance after PLIN2 knockdown trended to decease OCR. This provides evidence that increases in OCR observed after treatment with calcitriol are dependent on PLIN2

upregulation. Building on this, because vitamin D drives increases in ATGL and CGI-58 mRNA, and no change in ECAR in response to treatments, we hypothesize that increases in OCR are driven by increased rates of fatty acid oxidation.

The dramatic decrease in mitochondrial leak as a result of calcitriol treatment was combined with no change in the ATP-linked OCR, suggesting that the VitD effect observed at basal mitochondrial OCR may have been driven by decreases in proton leak. As a result, VitD increased the efficiency with which mitochondria use oxygen to produce ATP. Decreased rates of electron leak are also associated with decreased rates of oxidative stress in mitochondria [54], and calcitriol has been shown to decrease oxidative stress and damage in skeletal muscle [55, 56]. Future research should investigate molecular connections between calcitriol and mitochondrial oxidative stress.

We acknowledge the limitation that it is difficult to make strong claims regarding the direct impact of lipid flux in the observed changes in mitochondrial metabolism without direct measurement of fatty acid oxidation. There was also no notable increase in IMCL with ORO staining after VitD treatment as is claimed by previous research [16]. This could be a product of minor differences in lipid availability or more efficient lipid clearance with calcitriol treatment, resulting in less IMCL accumulation with a similar level of palmitate import. There is growing evidence that lipid species are perhaps more important to maintaining cellular function than total lipid abundance, and vitamin D has been shown to change the lipid profile in muscle [16, 42]. Finally, this study did not assess any markers of cellular stress, limiting our ability to make claims regarding the impact of shifts in lipid management after calcitriol treatment.

We conclude that calcitriol treatment in myotubes increases both IMCL storage and mitochondrial function, and that the upregulation of PLIN2 is required to realize metabolic improvements, but not IMCL accumulation. Although PLIN2 is not required for increased IMCL accumulation, mitochondrial function is markedly impaired after PLIN2 knockdown. Our data suggest that PLIN2 knockdown is detrimental to metabolic function in skeletal muscle. These findings contribute to the understanding of how vitamin D regulates mitochondrial function and the roles of PLIN2 in skeletal muscle mitochondrial metabolism.

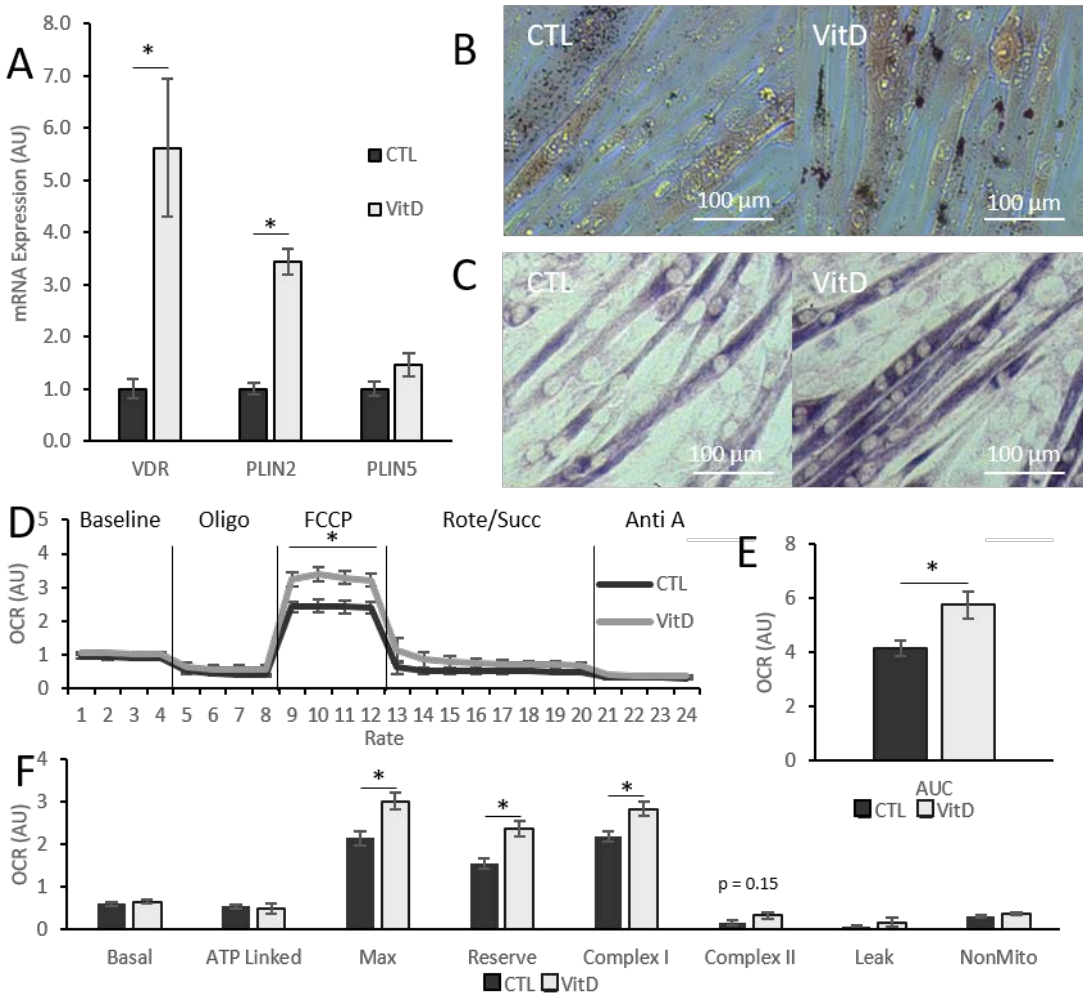



**Figure 3.1. Calcitriol increases lipid storage and mitochondrial activity in C2C12 myotubes**. (A) Gene expression of vitamin D receptor (VDR), perilipin 2 (PLIN2) and perilipin 5 (PLIN5) normalized to CTL in differentiated C2C12 myotubes. (B) Representative brightfield micrographs depicting oil red O staining. (C) Representative brightfield micrographs depicting succinate dehydrogenase activity staining. (D) Oxygen consumption rate (OCR) of myotubes throughout experiment with addition of oligomycin, FCCP, rotenone/succinate, and Antimycin A. (E) Total mitochondrial OCR. (F) OCR calculated using equations based on those provided by Agilent Biosciences. All data are represented as mean ± SEM, n = 5. All micrographs obtained at 32x magnification. Scale bars = 100 µm. CTL = 0.1% ethanol, 24 h ours; VitD = 100 nM calcitriol, 24 hours. n = 5; bars represent mean ± SEM; * $p < 0.05$, independent t-test. Oligo, Oligomycin; FCCP, Carbonyl cyanide-4-trifluoromethoxy)phenylhydrazone; Rote/Succ, Rotenenone & Succinate; Anti A, Antimycin A.

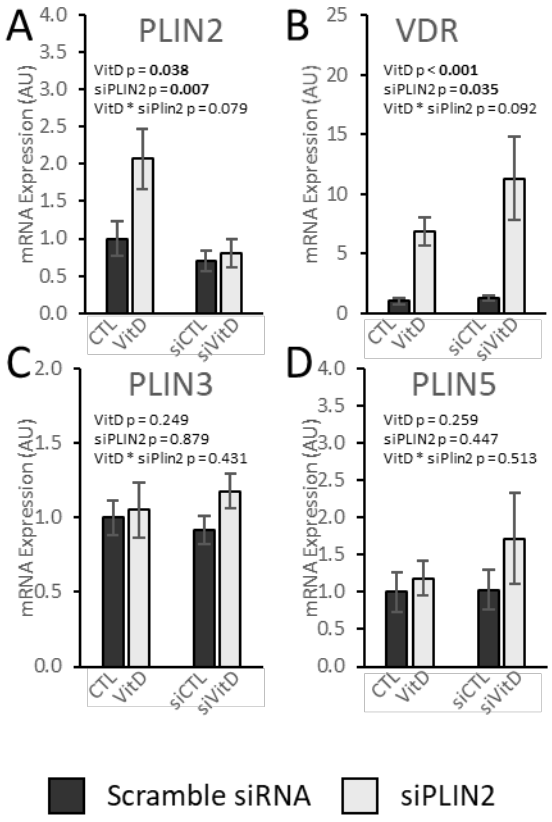



**Figure 3.2. PLIN2 knockdown decreases PLIN2 expression without compensation by other perilipin genes.** Gene expression in differentiated C2C12 myotubes relative to vehicle control with scramble siRNA. Cells were treated with either scramble siRNA and vehicle control (CTL), scramble siRNA and 100 nM calcitriol (VitD), PLIN2 siRNA and vehicle control (siCTL), or PLIN2 siRNA and 100 nM calcitriol (siVitD). All data are represented as mean ± SEM, n = 6. Values not sharing letters are significantly different (Fisher's LSD). p values represent 2-way ANOVA.

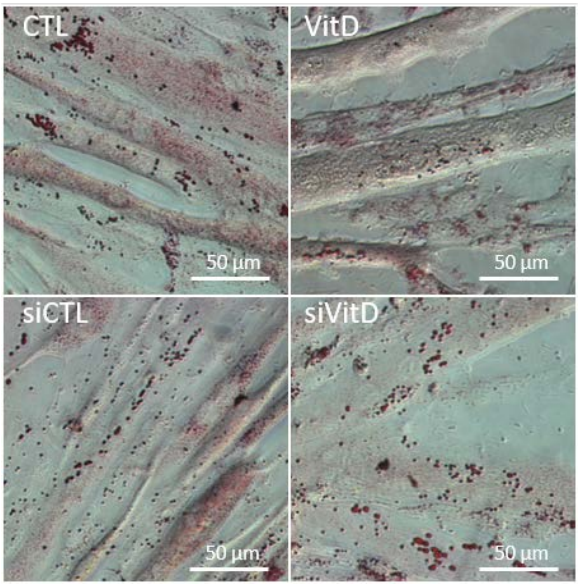



**Figure 3.3. PLIN2 knockdown does not appear to prevent neutral lipid accumulation in C2C12 myotubes**. Oil Red O micrographs show ample lipid accumulation in C2C12 myotubes after 3h of treatment with 100 µM palmitate despite PLIN2 knockdown. Images acquired at 40x magnification, scale bar = 50 µm.

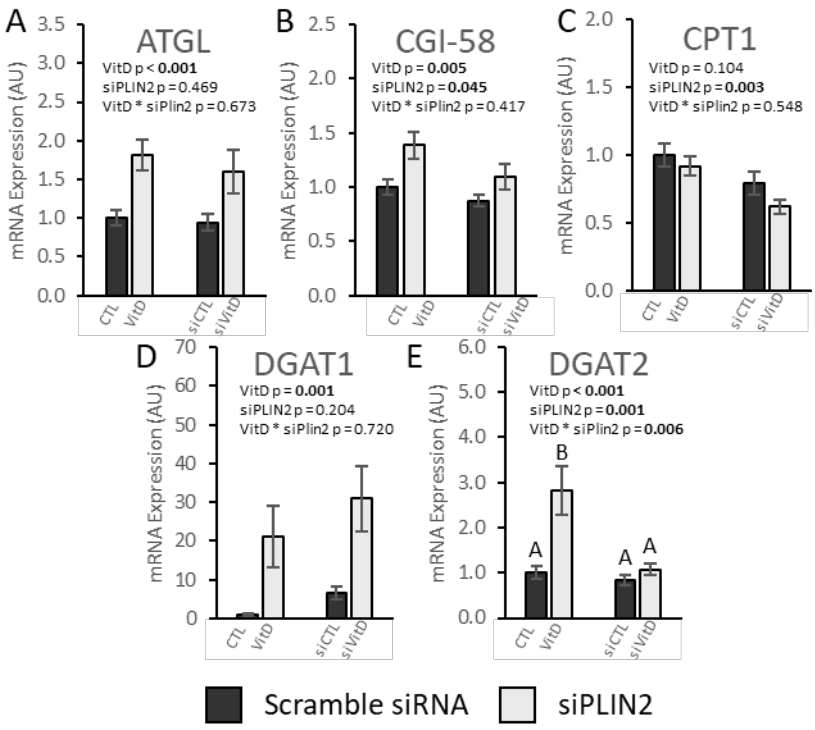



**Figure 3.4. PLIN2 knockdown and vitamin D exert opposing effects on genes regulating lipolysis and lipid storage.** Gene expression in differentiated C2C12 myotubes relative to vehicle control with scramble siRNA. Cells were treated with either scramble siRNA and vehicle control (CTL), scramble siRNA and 100 nM calcitriol (VitD), PLIN2 siRNA and vehicle control (siCTL), or PLIN2 siRNA and 100 nM calcitriol (siVitD). All data are represented as mean ± SEM, n = 6. Values not sharing letters are significantly different (Fisher's LSD). p values represent 2-way ANOVA.

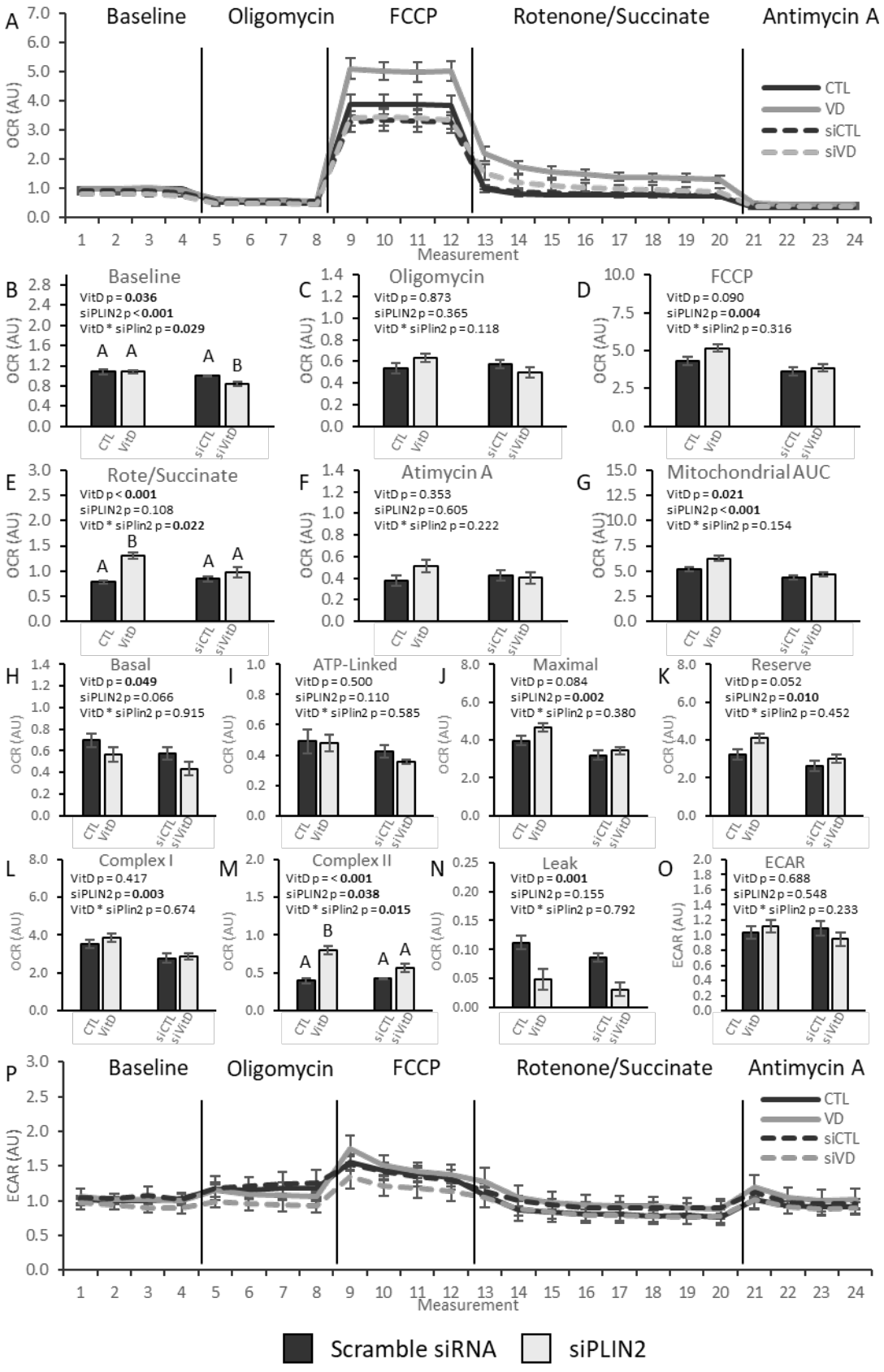

A
Baseline
Oligomycin
FCCP
Rotenone/Succinate
Antimycin A
OCR (AU)
Measurement
CTL
VD
siCTL
siVD
B
Baseline
VitD p = 0.036
siPLIN2 p < 0.001
VitD * siPlin2 p = 0.029
C
Oligomycin
VitD p = 0.873
siPLIN2 p = 0.365
VitD * siPlin2 p = 0.118
D
FCCP
VitD p = 0.090
siPLIN2 p = 0.004
VitD * siPlin2 p = 0.316
E
Rote/Succinate
VitD p < 0.001
siPLIN2 p = 0.108
VitD * siPlin2 p = 0.022
F
Atimycin A
VitD p = 0.353
siPLIN2 p = 0.605
VitD * siPlin2 p = 0.222
G
Mitochondrial AUC
VitD p = 0.021
siPLIN2 p < 0.001
VitD * siPlin2 p = 0.154
H
Basal
VitD p = 0.049
siPLIN2 p = 0.066
VitD * siPlin2 p = 0.915
I
ATP-Linked
VitD p = 0.500
siPLIN2 p = 0.110
VitD * siPlin2 p = 0.585
J
Maximal
VitD p = 0.084
siPLIN2 p = 0.002
VitD * siPlin2 p = 0.380
K
Reserve
VitD p = 0.052
siPLIN2 p = 0.010
VitD * siPlin2 p = 0.452
L
Complex I
VitD p = 0.417
siPLIN2 p = 0.003
VitD * siPlin2 p = 0.674
M
Complex II
VitD p = < 0.001
siPLIN2 p = 0.038
VitD * siPlin2 p = 0.015
N
Leak
VitD p = 0.001
siPLIN2 p = 0.155
VitD * siPlin2 p = 0.792
O
ECAR
VitD p = 0.688
siPLIN2 p = 0.548
VitD * siPlin2 p = 0.233
P
ECAR (AU)
Scramble siRNA
siPLIN2

**Figure 3.5. Vitamin D increases mitochondrial function and efficiency dependent on PLIN2 upregulation**. C2C12 myotubes were treated with 100 nM calcitriol and PLIN2 siRNA or their respective controls in a 2 x 2 design and OCR was measured by Seahorse XFe24 flux analyzer. (A) OCR was increased throughout measurements after treatment with calcitriol (grey lines) while siPLIN2 blunts OCR (hashed lines). Differences in OCR after each injection were quantified and compared for raw OCR (B-F), OCR specific to mitochondrial activity (H-N), and ECAR (O-P). All data are represented as mean ± SEM, n = 3-4. p values calculated by 2-way ANOVA. Bars not sharing letters are significantly different (Fisher's LSD). OCR, Oxygen Consumption Rate; ECAR, Extracellular Acidification Rate; FCCP, Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone.

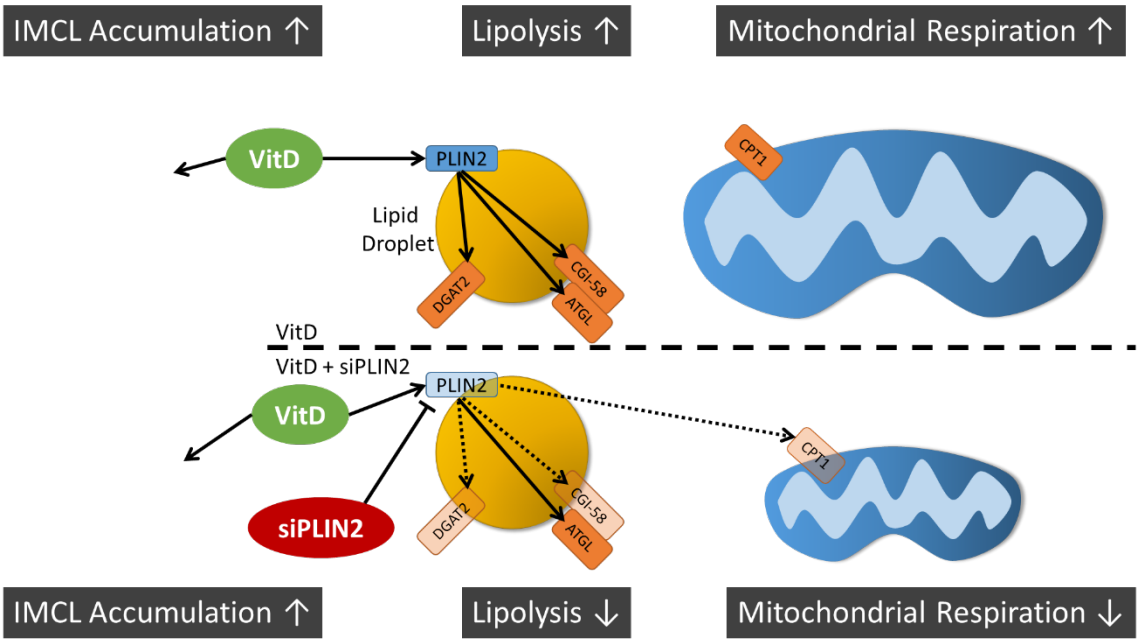



**Figure 3.6. Graphical abstract and working hypothesis**. VitD treatment increases IMCL accumulation, mRNA associated with both lipid storage and lipolysis, and mitochondrial respiration in skeletal muscle, but increases in lipolytic gene expression and mitochondrial respiration are dependent on PLIN2 expression. Calcitriol treatment (VitD) increases gene expression of lipid droplet proteins PLIN2, ATGL, and CGI-58 (top). VitD also increased mRNA expression for DGAT proteins that localize to both the endoplasmic reticulum for new lipid droplet synthesis (DGAT1) and to mature lipid droplets (DGAT2). These genes together are associated with increased capacity for IMCL accumulation and lipolysis and correspond with increased mitochondrial respiration. PLIN2 knockdown with siRNA before VitD treatment (bottom) decreases the expression of not only PLIN2 but also lipolytic cofactor CGI-58 and lipid droplet refilling gene DGAT2 (indicated by faded shapes and hashed lines). This is associated with decreases in CPT1 mRNA expression and mitochondrial respiration. VitD = Calcitriol; PLIN2 = Perilipin 2; DGAT2 = diglyceride O-acyltransferase 2; DGAT1 = diglyceride O-acyltransferase 1; ATGL = adipose triglyceride lipase; CGI-58 = comparative gene identifier 58; CPT-1 = carnitine palmitoyltransferase 1

# CHAPTER 4: C2C12 MYOTUBES ARE RESISISTANT TO PALMITATE-INDUCED LIPOTOXICITY WITH OR WITHOUT PLIN2 KNOCKDOWN

## 4.1 Abstract

Lipotoxicity is a pathological accumulation of lipid in non-adipose tissue connected with many obesity-associated co-morbidities. Signs of lipotoxicity include oxidative stress, endoplasmic reticulum (ER) stress, mitochondrial dysfunction, and apoptosis. One of the causes of lipotoxicity is an imbalance of lipid accumulation and oxidation favoring lipid accumulation. Lipotoxicity is associated with the toxic accumulation of bioactive lipids including diacylglycerols (DAG) and ceramides. Perilipin 2 (PLIN2) is a LD protein heavily implicated in the regulation of intramyocellular lipid (IMCL), and may be crucial in managing lipid storage and oxidation in lipotoxic settings. Additionally, there is evidence that vitamin D supports healthy lipid storage and increases lipid oxidation and may therefore prevent or ameliorate lipotoxicity. In this study, we combined a siRNA knockdown of PLIN2 in C2C12 myotubes with both calcitriol (VitD), the active form of vitamin D, and palmitate (PA) treatments to determine the combined effects of VitD and PLIN2 in preventing or ameliorating lipotoxicity. We hypothesized that treatment with PA would induce a lipotoxic phenotype in myotubes that would be ameliorated by VitD through increased storage and oxidation of TAG via the induction of PLIN2. Results showed that 24 hours of palmitate treatment (100 µM) increased IMCL accumulation; the addition of VitD augmented this result. Seventy-two hours of palmitate elicited myotube detachment from the growth surface, but this was prevented by co-treatment with VitD. Palmitate treatment for 24 hours did not decrease mitochondrial function or induce markers of inflammation or ER stress. We conclude that neither PLIN2 knockdown nor 100 µM palmitate for 24 hours produced a lipotoxic phenotype in C2C12 myotubes as determined by decreased mitochondrial function, increased inflammation, or ER stress. This suggests that PLIN2 knockdown does not induce lipotoxicity with moderate PA treatment. While, this model is unable to conclusively determine the effect of calcitriol on lipotoxicity, limited data from this project suggests that calcitriol prevents or ameliorates signs of lipotoxicity in C2C12 myotubes.

## 4.2 Introduction

Rates of obesity and T2DM are increasing around the world and is estimated to have a global economic burden of over $2.0 trillion [236]. Many of the morbidities associated with obesity have physiological origins in lipotoxicity. Lipotoxicity is the pathological accumulation of lipid in non-adipose tissue characterized by oxidative stress, mitochondrial dysfunction, ER stress, protein misfolding, inflammation, and ultimately contributes to cell dysfunction and apoptosis [210]. It has been hypothesized that lipotoxicity is not rooted simply in an overabundance of intracellular lipid, but an imbalance between lipid accumulation, storage, and oxidation. Research has shown that lipotoxicity is prevented by both sequestration of lipid as triacylglyceride (TAG) [225, 237] and increased fatty acid oxidation (FAO) [238, 239]. These findings suggest that lipid turnover is a key component to combating lipotoxicity [64, 223].

Lipid turnover is particularly important in skeletal muscle, the largest storage site of TAG outside of adipose tissue. Intramyocellular lipid (IMCL) is stored in lipid droplets, metabolically active organelles at the center of lipid accumulation, storage, and oxidation. Lipid droplets are enveloped by a phospholipid monolayer studded with proteins that maintain the structural integrity of the droplet and regulate both fatty acid acylation and lipolysis [188, 240]. Key to the LD proteome is the perilipin family of proteins. Perilipin 2 (PLIN2) is the most abundant perilipin protein in skeletal muscle, and may play a major role in regulating TAG storage and lipid flux [134, 184, 204]. However, there is a substantial debate as to whether PLIN2 prevents or enables lipotoxicity in skeletal muscle [47, 197, 228].

In the past decade, vitamin D has been recognized as a contributing factor to maintaining muscle health. Vitamin D deficiency is associated with increased incidence of falls and functional decline in the elderly [75, 77, 94]. One method that vitamin D may impact skeletal muscle function is through improved mitochondrial function. Researchers have shown that vitamin D increases mitochondrial activity in both clinical [115] and *in vitro* [118] studies. Calcitriol, the active form of vitamin D, is also associated with the prevention of lipotoxicity in the liver, kidney, and bone [39, 44, 46, 241]. Recent data from our group has also shown that vitamin D treatment increases the expression of PLIN2 *in vitro* [48], and other work has shown that PLIN2 upregulation increases IMCL accumulation and fatty acid oxidation without contributing to signs of lipotoxicity [134]. However, a direct connection from vitamin D to PLIN2 to lipotoxicity has not been established.

This study aimed to determine the role of vitamin D and PLIN2 in regulating key markers of lipotoxicity. We hypothesized that treatment with palmitate would induce a lipotoxic phenotype in myotubes that would be ameliorated by calcitriol through increased storage and oxidation of TAG via the induction of PLIN2. To test this hypothesis, we used a 2x2x2 *in vitro* model with or without PLIN2 siRNA knockdown in differentiated C2C12 myotubes treated with or without vitamin D and with or without palmitate.

## 4.3 Methods

### 4.3.1 Cell culture

C2C12 myoblasts were obtained from American Type Culture Consortium (ATCC; Manassas, Virginia, USA) and grown to a maximum of 60% confluence. At appropriate confluence, cells were trypsinized and seeded overnight in growth medium (GM) consisting of DMEM containing 1000 mg/L glucose with L-glutamine and sodium bicarbonate (MilliporeSigma, Burlington, MA, USA; #D6046) supplemented with 10% FBS (Gemini Bio-Products, West Sacramento, CA, USA; #100-106) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA, USA;Scientific, Waltham, MA, USA; #15140-122) in a humidified incubator kept at 37°C and 10% $CO_2$ (Day 0). Following overnight seeding, GM was replaced with differentiation medium (DM) consisting of DMEM (same as above) supplemented with 2% horse serum (Day 1). DM was changed every other day.

### 4.3.2 Treatment with calcitriol and palmitate

After 6 days of differentiation, DM was removed and myotubes were treated with either ethanol vehicle (0.1%) with BSA (CTL), 100 nM calcitriol (MilliporeSigma, Burlington, MA, USA; #D1530) with BSA (VitD), ethanol vehicle and 100 µM palmitate (PA) or 100 nM calcitriol with 100 µM palmitate (VitD+PA) for 24 hours.

### 4.3.3 Treatment with PLIN2 siRNA

On Day 5 in DM, differentiated myotubes were treated with 10 nM Thermo Fisher Stealth siRNA against PLIN2 (Thermo Fisher Scientific Waltham, MA, USA; #132001) or medium GC content scramble Stealth siRNA (Thermo Fisher Scientific Waltham, MA, USA; #12935300) as previously published [134]. All siRNA was prepared in DM with 0.2% Lipofectamine RNAiMAX transfection

reagent (Thermo Fisher Scientific Waltham, MA, USA; #13778) and 20% Opti-MEM (Thermo Fisher Scientific Waltham, MA, USA; #31985). Cells were treated with siRNA for a total of 48 hours. On Day 7, cells were treated with calcitriol and palmitate as described above. Cells with PLIN2 knockdown are represented in text as siCTL, siVitD, siPA, and siPA+VitD. Cell growth and treatment are summarized in Appendix 1.

### 4.3.4 Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

Cells were seeded 50,000/well in a 24 well culture plate and treated as described above. After treatment, media was removed and cells were washed in phosphate buffered saline (PBS) then scraped off the plate in 150 µL QIAzol Reagent (Qiagen, Hilden, Germany; #79306). Three wells receiving the same treatment were combined and lysed in a bead homogenizer. RNA was isolated using an ethanol precipitation on a RNA elution column (Enzymax, Lexington, KY, USA; #EZCR101). RNA was then reverse transcribed with a qScript cDNA synthesis kit (Quanta Biosciences, Beverly, MA; 101414-106) according to the manufacturer's recommendations. Relative gene expression was measured using PowerUp SYBR (Thermo Fisher Scientific, Waltham, MA; #A25778) in a QuantStudio 3 real time PCR machine (Thermo Fisher Scientific, Waltham, MA). The geometric mean of three housekeeping genes (RER1, VCP, and EMC7) was used as an endogenous control. Expression was quantified using the $2^{\Delta\Delta\text{-Ct}}$ method. Values were normalized to the CTL for each respective treatment and reported as fold change. Primers were purchased through Integrated DNA Technologies and primer sequences used in this study are listed in Appendix 2.

### 4.3.5 Oil Red O Staining

To assess neutral lipid accumulation, myotubes were treated as described above with or without VitD and 100 µM PA for either 24, 48, or 72 hours. Myotubes were fixed in 4% paraformaldehyde (PFA) and washed with PBS, then stained with oil red O (ORO) (MilliporeSigma, Burlington, MA USA; #O-0625) prepared in triethylphosphate according to the manufacturer's specifications for 30 minutes at 37°C with occasional rocking. Cells were then washed and imaged. For all cytochemistry experiments, cells were imaged using a Zeiss AxioObserver D1 inverted fluorescent microscope (Jena, Germany). Micrographs were taken using a Zeiss AxioCam MR camera and analyzed and annotated using AxioVision SE64 Rel. 4.9.1 software (Zeiss, Jena, Germany).

### 4.3.6 Succinate Dehydrogenase (SDH) Activity Staining

Myotubes were washed with PBS, fixed with 4% PFA and then incubated in 1.2 mM nitro blue tetrazolium chloride (NBT) (MilliporeSigma, Burlington, MA, USA; #N6876) with 275 mM succinic acid (MilliporeSigma, Burlington, MA, USA; #224731) in PBS at 37°C for 120 minutes. Cells were then washed 3 times with PBS and imaged as described above.

### 4.3.7 Seahorse Oxygen Consumption Rate (OCR) Assay

Myoblasts were plated 10,000 per well in a Seahorse XFe24 assay plate (Agilent, Santa Clara, CA, USA) overnight in GM, then differentiated, and treated as described above. On the day of the assay, fresh Seahorse XF Assay Medium (Agilent, Santa Clara, CA, USA; #102365) was supplemented with 5 mM glucose and 1 mM pyruvate and pH adjusted to 7.4. Myotubes were washed twice with XF Assay Medium then incubated for one hour in 100 µL of XF Assay Medium in a humidified chamber at 37°C with atmospheric $CO_2$. Vehicle and VitD treatments, but not PA, were maintained throughout incubation and the assay. Following incubation, XF Assay Medium was added to a final volume of 525 µL. OCR and Extracellular Acidification Rate (ECAR) were measured at 37°C using Seahorse XFe24 Analyzer (Agilent, Santa Clara, CA, USA). During the assay, each treatment was injected sequentially to achieve the following final concentrations: 2.5 µM oligomycin (Biomol, Hamburg, Germany; #CM-111), 4 µM carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (Biomol, Hamburg, Germany; #CM120), 10 mM succinate (MilliporeSigma, Burlington, MA, USA; #S-7501) with 0.8 µM rotenone (Biomol, Hamburg, Germany; #CM-117), and 1 µM Antimycin A (MilliporeSigma, Burlington, MA, USA; #A8674). OCR was normalized to the average basal rate among all treatments for each given experiment. Specific rates based on equations suggested by Agilent Biosciences (listed in Table A.2) were used to calculate the following OCRs: basal, oligomycin, FCCP, rotenone/succinate, Antimycin A, ATP-linked, maximum, reserve, complex I, complex II, leak, non-mitochondrial, and mitochondrial area under the curve (AUC).

### 4.3.8 Statistical Analysis

A 2 x 2 factorial ANOVA was used to determine the effects of calcitriol (VitD effect), palmitate (PA effect), and the interaction between treatments. The Student's t-test was used to determine the effects of calcitriol alone. When appropriate, a 3 x 3 factorial ANOVA was used to determine the effects of calcitriol (VitD effect), palmitate (PA effect), PLIN2 knockdown (siPLIN2 effect) and the

interaction between treatments. When there was a significant interaction between two treatments, the Fisher's LSD post-hoc test was applied. All tests were two-tailed, with statistical significance defined as $p < 0.05$. All quantitative results are shown as mean ± SEM of no fewer than 3 independent experiments. Statistical calculations were performed using JMP 12 (SAS Institute, Cary, NC, USA).

## 4.4 Results

### 4.4.1 Treatment with PA had no impact on the expression of select lipid storage genes in myotubes

To begin analysis of the impact of PA and VitD on lipid metabolism in myotubes, we measured the mRNA expression of several key lipid storage, accumulation, and lipolysis genes using quantitative real-time polymerase chain reaction (RT-PCR). Results showed that VitD significantly increased vitamin D receptor (VDR) expression by 7.8-fold when compared CTL ($p < 0.001$) (Figure 4.1A). PA treatment had no impact on VDR expression. We next examined the effect of VitD and PA on the expression of LD proteins PLIN2 and PLIN5. Treatment with VitD increased PLIN2 expression approximately 3.5-fold compared to CTL ($p < 0.001$) (Figure 4.1B), but PA treatment had no impact on PLIN2 expression. PLIN5 mRNA expression was unaffected by either VitD or PA treatment ($p > 0.05$) (Figure 4.1C). To complete an initial analysis of genes regulating TAG lipolysis and accumulation in myotubes, we examined the expression of ATGL and DGAT1 mRNA. Treatment with neither VitD nor PA impacted ATGL expression (Figure 4.1D). VitD increased DGAT1 expression by 2.8-fold compared to CTL (Figure 4.1E). There was no effect between VitD and PA in any of the genes examined.

### 4.4.2 VitD augments PA-induced IMCL accumulation and prevents PA-induced cell death

Myotubes were stained with oil red O (ORO) to determine the impact of VitD and PA on IMCL accumulation after treating with VitD and 100 µM PA for 24, 48, or 72 hours. Myotubes treated with PA showed time-dependent signs of cell death and detachment (Figure 4.2A). This was ameliorated with addition of VitD. There was no evidence of cellular stress with VitD alone.

To examine how PA and VitD impact IMCL storage, we treated cells with 100 nM VitD and 100 µM PA for 24 hours. Cells did not appear to show signs of myotube detachment in any treatment

(Figure 4.2B). Analysis of micrographs revealed that VitD appeared to produce a greater degree of IMCL accumulation than CTL and augmented IMCL accumulation with PA treatment (Figure 4.2C). All subsequent experiments were completed with 100 µM PA for 24 hours to prevent cell death.

### 4.4.3 PA treatment did not change mitochondrial metabolism

To determine the impact of 100 µM PA for 24 hours on mitochondrial metabolism in myotubes with and without VitD treatment, we used succinate dehydrogenase (SDH) staining assay. In accord with previous research discussed in Chapter 3, VitD appeared to increase the intensity of SDH staining in myotubes (Figure 4.3A). There was no clear effect of PA with or without VitD.

To quantify mitochondrial function in myotubes, we used a Seahorse oxygen consumption assay (Figure 4.3B). Analysis of the OCR trace found no impact of PA after injection of oligomycin, FCCP, rotenone/succinate, or Antimycin A (Figure 4.3C-G). Treatment with VitD increased OCR after injection of FCCP and produced a significant increase in OCR area under the curve (AUC) (Figure 4.3H).

To quantify OCR specific to individual mitochondrial complexes, OCR was measured using equations described in Appendix 3. Analysis showed that neither basal (Figure 4.3I) nor ATP linked (Figure 4.3J) were significantly affected by either 24 hours of either VitD or PA. Both maximal mitochondrial OCR (Figure 4.3K) and reserve OCR (Figure 4.3L) were increased with VitD treatment, but were unaffected by PA. There was no interaction effect at either measurement. There were no responses to individual or combined treatment with VitD or PA when OCR was measured at Complex I (Figure 4.3M), Complex II (Figure 4.3N), mitochondrial leak (Figure 4.3O), or non-mitochondrial respiration (Figure 4.3P).

### 4.4.4 PA, but not VitD, increased baseline lactate production

While measuring OCR using the Seahorse extracellular flux analyzer, we simultaneously measured extracellular acidification rate (ECAR), a marker of glycolysis (Figure 4.4A). Analysis showed that 100 µM of PA for 24 hours, but not VitD, increased ECAR at baseline measurements (Figure 4.4B). However, there were no changes after the addition of oligomycin (Figure 4.4C), FCCP (Figure 4.4D), rotenone/succinate (Figure 4.4E), Antimycin A (Figure 4.4F), or in the AUC (Figure 4.4G).

### 4.4.5 PLIN2 knockdown does not interact with PA to regulate VDR or perilipin gene expression

To examine the effect of PLIN2 knockout on VDR-mediated lipid management in myotubes, we measured the expression of VDR and several perilipin proteins after treatment with 100 µM PA for 24 hours. In depth analysis of the effect of VitD in reference to siPLIN2 has been reported in Chapter 3. Analysis of the role of PA in lipid management showed that PA did not impact the expression of VDR nor any perilipin mRNAs with or without PLIN2 knockdown (Figure 4.3A-D).

We then examined the expression of several genes key to regulating lipid metabolism in myotubes after PLIN2 knockdown. We found that while PA does not have a main effect on ATGL expression, it does have an interaction effect with VitD in which VitD abrogates PA-induced ATGL expression (Figure 4.3E). PA did not affect the expression of CGI-58 (Figure 4.3F), DGAT1 (Figure 4.3G), or DGAT2 (Figure 4.3H). PA significantly increased the expression of CTP1. P Values for all main and interaction effects of lipid management genes are reported in Table 1.

> *Note: VitD and CTL data presented here and in Figure 4.3 are the same as that presented in Chapter 3 and Figure 4. However, this analysis includes comparisons with PA not reported in Chapter 3.*

### 4.4.6 PA did not impact markers of inflammation or endoplasmic reticulum stress

To determine the effect of PLIN2 on the role of PLIN2 in VitD-mediated changes in lipid management to prevent lipotoxicity, we measured the expression of genes associated with inflammation and endoplasmic reticulum (ER) stress associated with lipotoxicity after treatment with 100 µM PA for 24 hours. We found that VitD increased the expression of IL-1β (Figure 4.6A), and siPLIN2 decreased the expression of IL-1β. PA had no effect on the expression of IL-1β. VitD decreased the expression of IL-6 with no effect of siPLIN2 of PA (Figure 4.6B). VitD had a strong trend towards increased the expression of TNF-α, although this failed to reach statistical significance (Figure 4.6C). There was no effect in response to siPLIN2 or PA. We then measured the expression of several markers of ER stress. No treatments had a significant effect on the expression of GRP78 (Figure 4.6D), GRP94 (Figure 4.6E), ATF4 (Figure 4.6F), or the ratio of spliced to unspliced XBP1 (Figure 4.6G). The main and interaction effects of genes associated with lipotoxicity are reported in Table 2.

## 4.5 Discussion

This project aimed to determine the role of VitD and PLIN2 in the regulation of lipid metabolism and prevention of lipotoxicity in a hyperlipidic environment. We hypothesized that treatment with PA would induce a lipotoxic phenotype in myotubes that would be ameliorated by VitD through increased storage and oxidation of TAG via the induction of PLIN2. Results were not sufficient to support this hypothesis as the PA treatment used for the majority of this project, 100 µM for 24 hours, did not consistently induce lipotoxicity. However, 48 and 72 hour treatments with PA did induce cell death consistent with lipotoxicity; this lipotoxicity was prevented by co-treatment with 100 nM calcitriol. These data indicate that treatment with PA alone is not sufficient to induce a lipotoxic phenotype, modulate lipid metabolism, or drive gene expression in C2C12 myotubes at the concentration used.

Initial RT-PCR analysis showed that treatment with PA did not affect the expression of VDR or key lipid regulatory genes. Treatment with VitD increased the expression of VDR and PLIN2 consistent with previous research [48] and upregulated DGAT1 as discussed in depth in Chapter 3. However, we did not observe an increase in ATGL as previously published [48] and seen in scramble siRNA control samples treated with VitD (Figure 4.3E). These data further support the hypothesis that VitD supports lipid accumulation in myotubes. However, it is curious that this response is not impacted by PA. Research by de Wilde et al. [242] showed that PA produces a dose dependent increase in PLIN2 protein abundance that reached statistical significance at 200 µM for 15 hours. There was a dramatic increase in the expression of PLIN2 when PA was increased from 100 µM to 200 µM. Similarly, Chen et al. observed an increase in PLIN2 expression with a 500 µM treatment of PA for 12 hours. The lack of an increase of PLIN2 in response to PA in this study may be associated with the relatively low concentration of PA used.

While there is some debate regarding the ability of PA to induce lipid accumulation in skeletal muscle, most research indicates it is a weak inducer of LD formation in comparison to oleate as indicated by PLIN2 protein accumulation [238, 242, 243]. Our data support this conclusion. PA effectively induced a visibly evident increase in IMCL accumulation as indicated by ORO staining. This increase in IMCL was clear at 100 µM PA for 24 hours and was augmented by the addition of VitD. The increase in IMCL without a corresponding increase in PLIN2 expression suggests decreased PLIN2 abundance in each LD or a decrease in the PLIN2 to IMCL ratio. The decrease of this ratio may be associated with an inability to produce LDs to adequately store IMCL is

associated with DAG and non-esterified fatty acid accumulation and ER stress [238]. The presence of this stress is evidenced in the present model with PA treatment of 48 or 72 hours, which produced a clear pathological phenotype in myotubes consistent with lipotoxicity that was ameliorated by co-treatment with VitD. Prevention of lipotoxicity with VitD is well documented in a range of tissues including kidney and liver [39, 244], although it has not been well investigated in skeletal muscle. After the discovery of a clear adverse effect of 100 µM PA after 72 hours, we chose a 24-hour treatment to maintain cell integrity and prevent off target effects produced an excessively stressful environment. Unfortunately, the lipotoxic phenotype was not maintained throughout the duration of the study. This ORO experiment was completed before all minor adjustments to cell culture conditions were optimized for myotube growth and differentiation. As a result of media conditions catered to robust, healthy cells, myotubes tolerated the 100 µM palmitate treatment well and did not show signs of lipotoxicity at later points in the study.

This lack of lipotoxicity was further evidenced by analysis of mitochondrial metabolism. SDH staining appeared to show an increase SDH activity with VitD treatment, but there was no evident response to PA. Verifying these results, we saw no response to PA throughout the Seahorse OCR assay. This is particularly interesting as PA is a well-documented uncoupler of mitochondrial respiration in C2C12 myotubes [245, 246]. We would therefore expect to see either increased OCR after the addition of oligomycin indicative of mitochondrial leak and uncoupled respiration. However, the effect of PA differs from myoblasts to myotubes and may be highly sensitive to differences in lipid profile of both media and the cell itself as well as oxidative environment [215]. One mechanism through which PA decreases mitochondrial function is through increased ceramide abundance [247]. Therefore, one may not observe mitochondrial deficiency if myotubes are not treated with a dose of palmitate sufficient to induce ceramide production. As noted previously, 100 µM is a relatively low treatment with palmitate and may have been insufficient to induce the stress hypothesized. Indeed, Patkova et al. did not note significant mitochondrial effect in myotubes below 200 µM palmitate [245]. Together, these data suggest that our model of palmitate treatment did not induce a lipotoxic phenotype, and that the dose of palmitate employed was likely too low.

Concurrent with OCR measurements, the Seahorse XF analyzer measured ECAR, and these data can be used to supplement OCR to ascertain a more complete picture of bioenergetic phenotype. However, it is important to recognize that the mitochondrial stress test used to generate these

data is not designed to specifically diagnose differences in glycolytic metabolism, and results should be interpreted with some degree of caution and only to supplement primary outcome data. Data revealed an increase in OCR in response to VitD consistent with what was reported in Chapter 3, but limited effect PA throughout the assay. However, we did record a significant increase in baseline OCR after PA treatment. The most evident analysis of this increase in light of our hypothesis is a compensatory response of glycolytic ATP production to account for reduced oxidative function. However, OCR analysis does not indicate any significant decrease in ATP-linked OCR after PA treatment. One could argue that the low dose PA produced a level of mitochondrial stress not clearly evident when examining OCR, but, as glycolysis is much less efficient at producing ATP, the effect on glycolytic metabolism is magnified.

Knockdown of PLIN2 had limited effect on gene expression in relation to PA, but some responses were observed in the expressions of ATGL and CPT1. The PLIN2-dependent increase in ATGL expression with PA treatment may be associated with the inhibitory function of PLIN2 in ATGL function. Because PLIN2 binds ATGL and prevents its association with CGI-58, decreasing the amount of PLIN2 in a myotube likely decreases the amount of ATGL that is inhibited at any given time. Therefore, less ATGL is required for the same amount of lipolysis when PLIN2 expression is suppressed. The increase in CPT1 expression is supported by previous research indicating increased CPT1 activity after treatment with 50-100 μM PA [248]. This is likely associated with increased lipid clearance in response to increased abundance of fatty acids.

Post-hoc analysis revealed a PLIN2-dependent increase in ATGL mRNA expression after treatment with PA. However, there was no independent PA effect or siPLIN2 effect. This can be compared to a PLIN2-independent increase in ATGL after VitD treatment. PLIN2 binds to ATGL and prevents its association with CGI-58 to upregulate lipolysis [184]. We speculate that if PLIN2 is knocked down, there may be an abundance of free ATGL to associate with CGI-58 and respond to the increased palmitate abundance in the cell with increased lipolysis [184]. However, this suggests that PLIN2 is not required for an increased lipolytic response to a high amount of exogenous lipid after VitD treatment.

Interestingly, the response of mRNA expression to PA after treatment with lipofectamine and scramble RNA (Figure 4.3) differed from that observed without lipofectamine (Figure 4.1). This effect was independent of siPLIN2, and previous researchers have not reported a lipofectamine effect in lipid metabolism studies. However, lipofectamine may have unexamined effects on

metabolic pathways in cell culture; it is conceivable that cationic lipofectamine liposomes form complexes with anionic non-esterified fatty acids and mediate their import into the cell during the siRNA treatment window, effectively increasing the abundance of lipid in the cell media. However, to our knowledge, this hypothesis is untested.

While PA treatment failed to elicit a substantial effect on markers of lipotoxicity, VitD increased TNF-α gene expression ($p = 0.054$). Although the magnitude of change and SEM similar to that of treatment with VitD, the PA effect on TNF-α expression did not approach significance ($p = 0.384$). PA is a well-documented inducer of inflammatory cytokines, and 200 μM palmitate can induce IL-6, TNFα and CCL2 expression in muscle cells through activation of the NF-κB pathway [249]. Further research has shown that TNF-α knockdown prevents palmitate-induced signs of lipotoxicity [250]. However, others have shown that 200 μM of PA in C2C12 myotubes is not sufficient to induce TNF-α protein or mRNA expression and that a secondary stressor, such as glucose, is required to induce inflammation [128, 251, 252]. We attribute the lack of an inflammatory, lipotoxic phenotype to a combination of insufficient PA concentration in a cellular environment optimized for health. Future work would benefit from a higher dose of PA or glucose to adequately stress cells and produce inflammation and endoplasmic reticulum stress.

The mechanisms controlling VitD-regulated modulation of cytokines are not fully understood. In a clinical study examining the relationship between the cytokines IL-6 and TNF-α with 25(OH)D and VDR, Pojednic et al. [253] found that VDR protein was negatively associated with intramuscular IL-6 protein but, contrary to results shown here, positively associated with IL-6 gene expression. This increase in IL-6 gene expression may be a product of endocrine or paracrine signaling not present in cell culture models. However, the authors concluded that VitD ultimately contributes to decreased IL-6 protein expression. In accord with the present study, they found no correlation between any marker of VitD and TNF-α.

The only recorded effect of PLIN2 knockdown on inflammatory cytokine mRNA expression was observed as a PA or VitD-independent reduction of IL-1β. Limited research has investigated the interaction between perilipin proteins and cytokines, but one study found that PLIN2 overexpression in C2C12 cells led to an increased expression of several markers of inflammation including NLRP3, caspase-1, and IL-1β [254]. This is curious as IL-1β is a classic marker of the inflammasome associated with lipotoxicity [255, 256].

Contrary to our hypothesis, there was no effect of any treatment on markers of ER stress, as both PA overabundance and PLIN2 deficiency are associated with ER stress and unfolded protein response [202, 238]. Furthermore, sequestration of fatty acids in TAG, a process mediated by PLIN2, is known to prevent ER stress [237].

The results of this study offer limited insight into how vitamin D and PLIN2 relate to the regulation of lipid oxidation in a high lipid environment. We conclude that 100 μM palmitate does not induce lipotoxicity but is instead well-tolerated by C2C12 myotubes in an otherwise healthy environment. Future work should revisit this hypothesis after defining a consistent model of lipotoxicity using a higher dose of palmitate in addition to a secondary stressor. However, OCR experiments were consistent with results reported in Chapter 3 and further support the hypothesis that calcitriol increases mitochondrial function in skeletal muscle mytoubes.

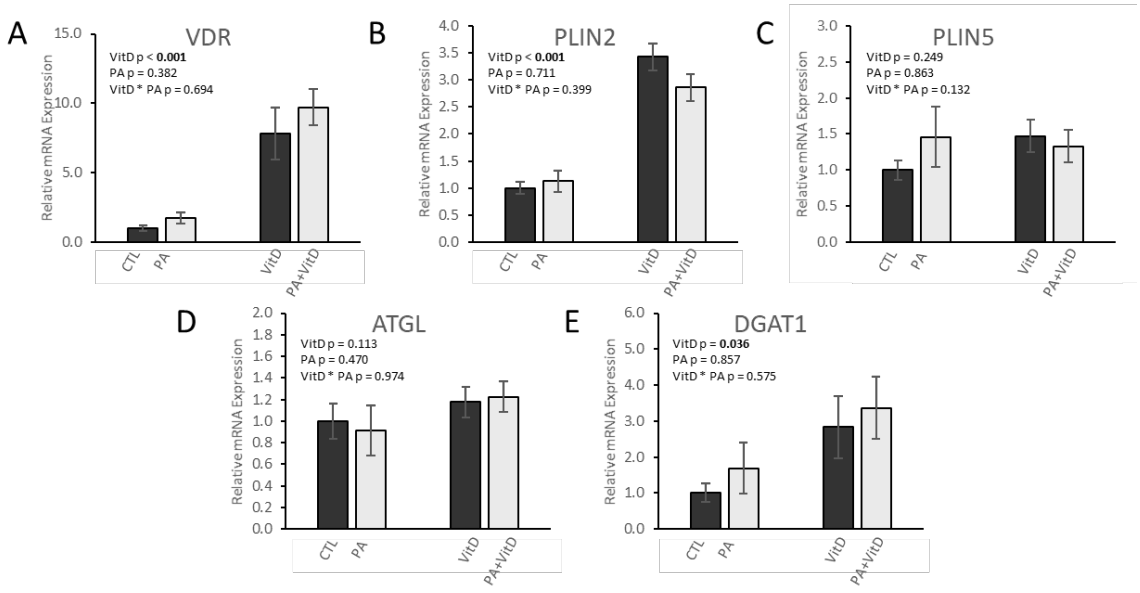



**Figure 4.1. Palmitate does not impact mRNA VDR-induced expression of lipid management genes.** Gene expression of vitamin D receptor (VDR) (A) and key lipid storage and lipolysis genes perilipin 2 (PLIN2) (B), perilipin 5 (PLIN5) (C), adipose triglyceride lipase (ATGL) (D), and diglyceride acyl-O-transferase (E). Cells were treated with vehicle controls (CTL), 100 µM palmitate (PA), 100 nM calcitriol (VitD), or 100 µM palmitate and 100 nM calcitriol PA+VitD for 24 hours. Bars represent mean ± SEM, n = 5; p calculated by 2-way ANOVA.

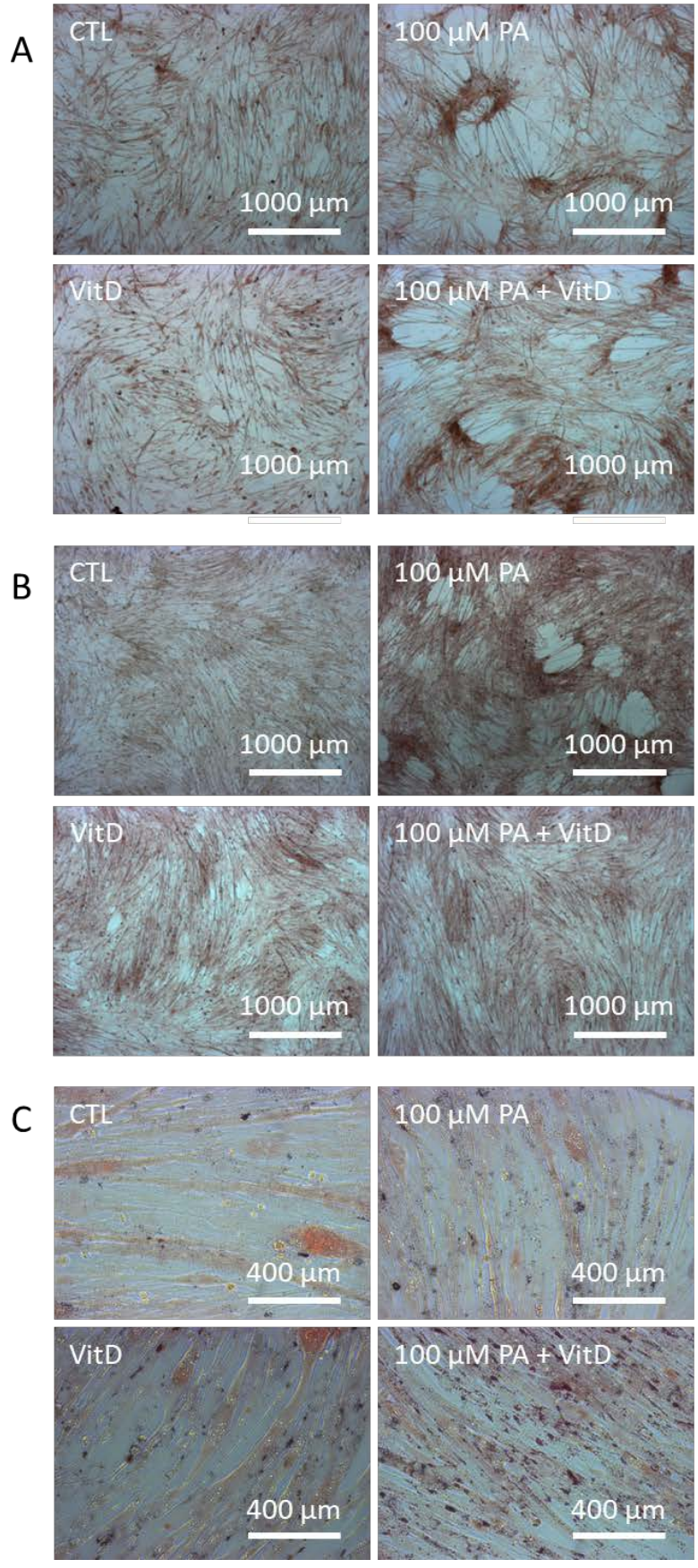

A
CTL
100 µM PA
1000 µm
1000 µm
VitD
100 µM PA + VitD
1000 µm
1000 µm
B
CTL
100 µM PA
1000 µm
1000 µm
VitD
100 µM PA + VitD
1000 µm
1000 µm
C
CTL
100 µM PA
400 µm
400 µm
VitD
100 µM PA + VitD
400 µm
400 µm

**Figure 4.2. VitD augments PA mediated IMCL accumulation and prevents myotube macrostructure derangement.** Myotubes were treated with 0, 100 μM palmitate and 0 or 100 nM calcitriol for 72 hours (A) 48 hours (B) or 24 hours (C) and stained with oil red O

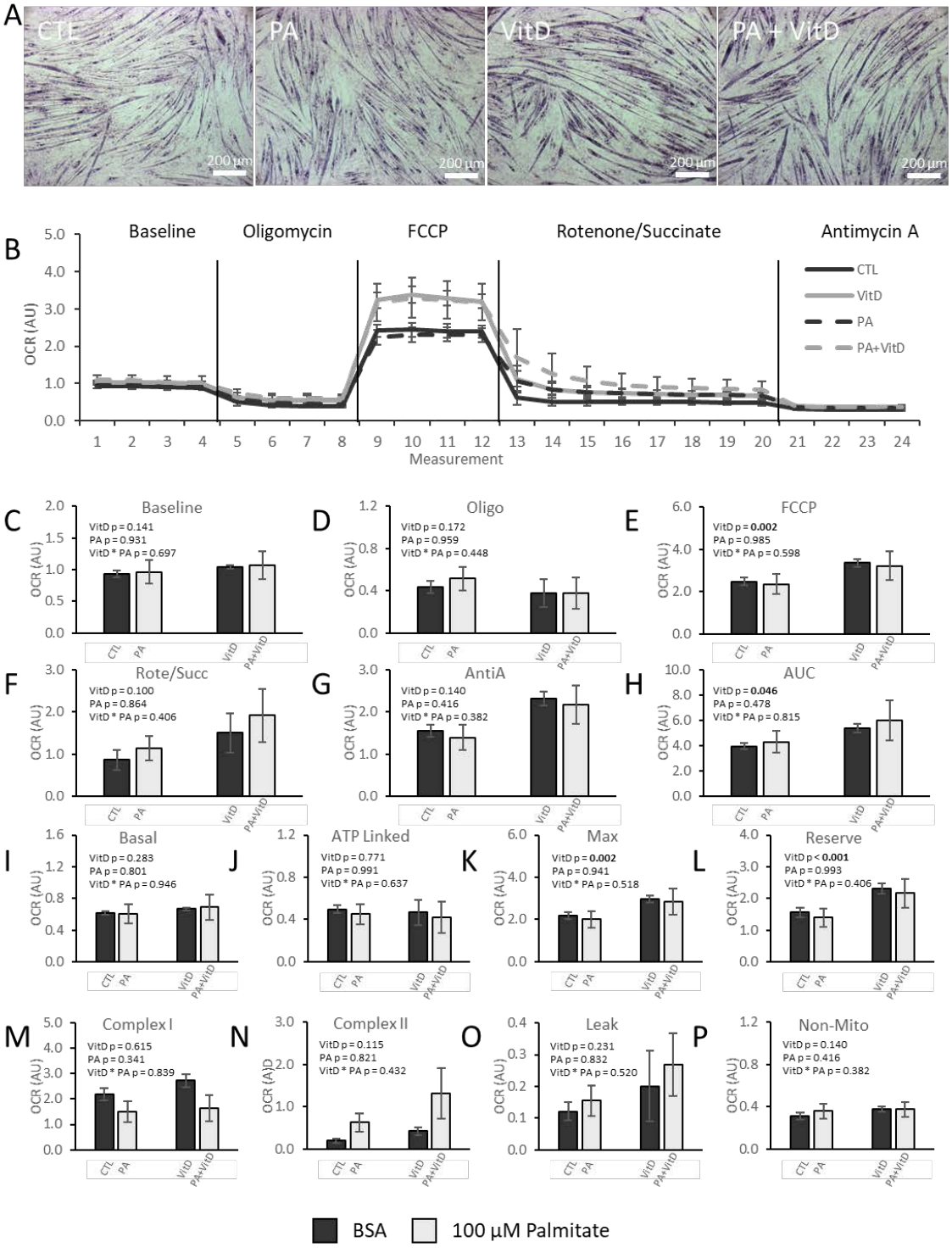

A
CTL
PA
VitD
PA + VitD
200 µm
B
Baseline
Oligomycin
FCCP
Rotenone/Succinate
Antimycin A
CTL
VitD
PA
PA+VitD
OCR (AU)
Measurement
C
Baseline
VitD p = 0.141
PA p = 0.931
VitD * PA p = 0.697
D
Oligo
VitD p = 0.172
PA p = 0.959
VitD * PA p = 0.448
E
FCCP
VitD p = 0.002
PA p = 0.985
VitD * PA p = 0.598
F
Rote/Succ
VitD p = 0.100
PA p = 0.864
VitD * PA p = 0.406
G
AntiA
VitD p = 0.140
PA p = 0.416
VitD * PA p = 0.382
H
AUC
VitD p = 0.046
PA p = 0.478
VitD * PA p = 0.815
I
Basal
VitD p = 0.283
PA p = 0.801
VitD * PA p = 0.946
J
ATP Linked
VitD p = 0.771
PA p = 0.991
VitD * PA p = 0.637
K
Max
VitD p = 0.002
PA p = 0.941
VitD * PA p = 0.518
L
Reserve
VitD p < 0.001
PA p = 0.993
VitD * PA p = 0.406
M
Complex I
VitD p = 0.615
PA p = 0.341
VitD * PA p = 0.839
N
Complex II
VitD p = 0.115
PA p = 0.821
VitD * PA p = 0.432
O
Leak
VitD p = 0.231
PA p = 0.832
VitD * PA p = 0.520
P
Non-Mito
VitD p = 0.140
PA p = 0.416
VitD * PA p = 0.382
BSA
100 µM Palmitate

**Figure 4.3. Calcitriol, but not palmitate, increased mitochondrial metabolism.** Myotubes stained for succinate dehydrogenase activity (A). Seahorse mitochondrial stress test (B) and quantized oxygen consumption rate (OCR) at baseline (C) and after the injection of oligomycin (D), carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (E), rotenone with succinate (F), and Antimycin A (G). Mitochondrial specific OCR derived from equations described in Appendix 3 (H-P). Bars represent mean ± SEM, n = 4-5; statistical significance defined as $p < 0.05$, 2-way ANOVA.

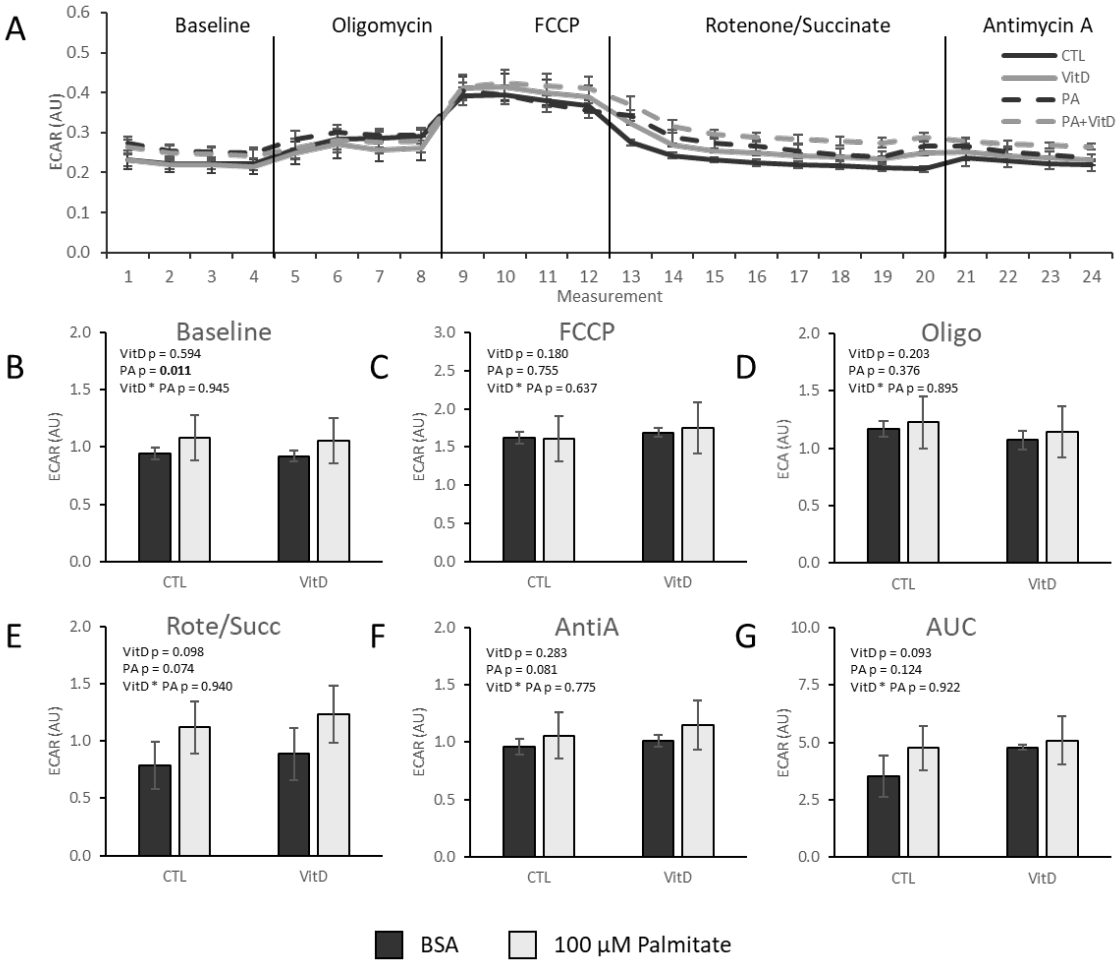



**Figure 4.4. Palmitate, but not calcitriol, increases baseline extracellular acidification rate.** Extracellular acidification rate (ECAR) measured during Seahorse mitochondrial stress test (A) and simplified values at baseline (B) and after the injection of oligomycin (C), carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (D), rotenone with succinate (E), Antimycin A (F), and the area under the curve (G). Bars represent mean ± SEM, n = 4-5; statistical significance defined as $p < 0.05$, 2-way ANOVA.

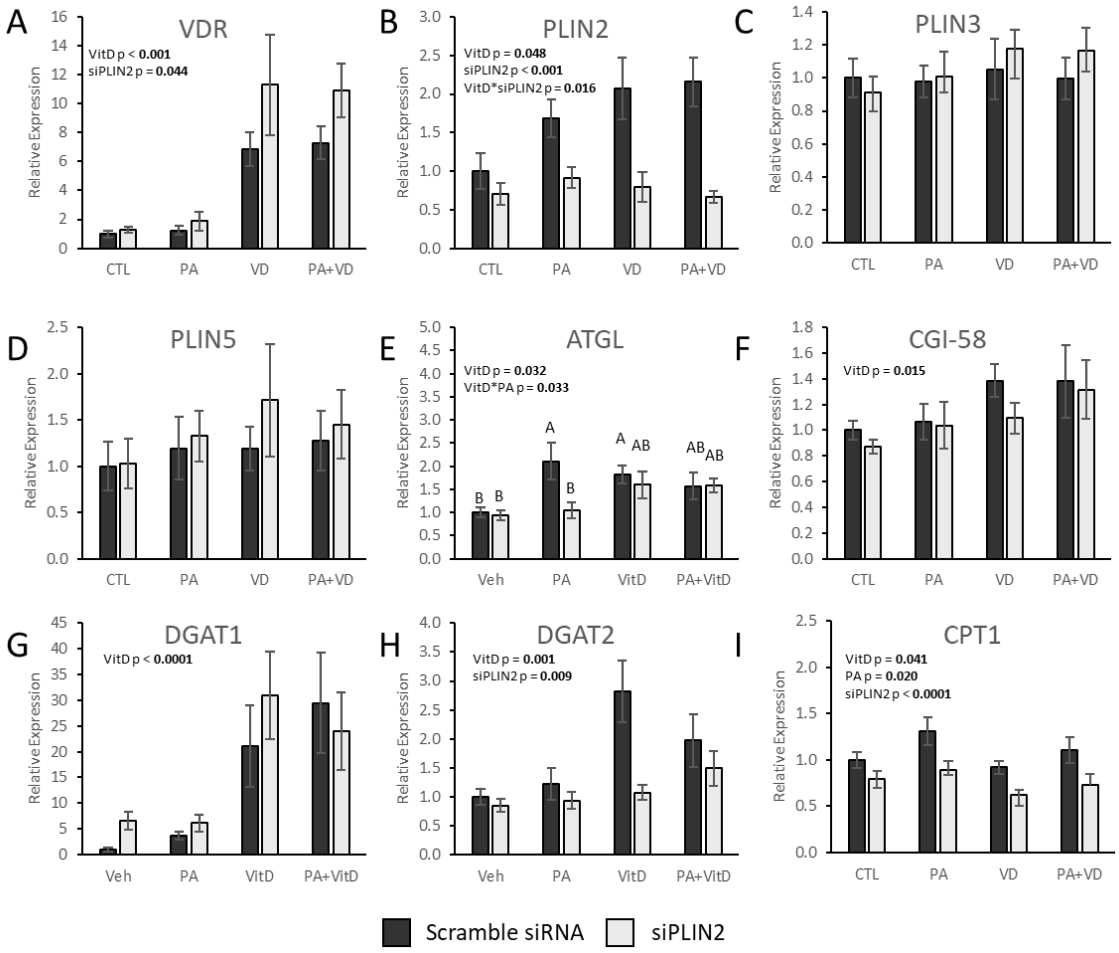



**Figure 4.5**. Palmitate had limited effect on calcitriol-induced changes to genes involved in lipid storage and metabolism with or without PLIN2 knockdown. Gene expression in myotubes was analyzed for genes associated with lipid storage and lipolysis using qRT-PCR after treatment with or without 100 nM calcitriol (VitD) and 100 µM palmitate (PA) for 24 hours with or without PLIN2 knockdown. VDR = vitamin D receptor; PLIN2 = perilipin 2; PLIN3 = perilipin 3; PLIN5 = perilipin 5; ATGL = adipose triglyceride lipase; CGI-58 = comparative gene identification-58; DGAT1 = diglyceride acyl O-transferase 1; DGAT2 = diglyceride acyl O-transferase 2; CPT1 = carnitine palmitoyltransferase 1. Black bars = scramble siRNA, Grey bars = PLIN2 knockdown. Bars represent mean ± SEM, n = 6; statistical significance defined as $p < 0.05$, 3-way ANOVA. Bars not sharing a letter are significantly different (Fishers LSD). *NB*: This figure contains VitD and Veh data also published in Chapter 3 of this dissertation.

**Table 1** Complete statistical analysis of 3 x 3 ANOVA for the expression of genes associated with lipid flux

| Term | VDR | PLIN2 | PLIN3 | PLIN5 | ATGL | DGAT1 | DGAT2 | CGI-58 | CPT1 |
|---|---|---|---|---|---|---|---|---|---|
| VitD | **<0.0001** | **0.047** | 0.195 | 0.284 | **0.032** | **<0.001** | **0.001** | **0.015** | 0.041 |
| PA | 0.838 | 0.220 | 0.988 | 0.751 | 0.156 | 0.827 | 0.826 | 0.348 | **0.020** |
| siPLIN2 | **0.044** | **<0.0001** | 0.523 | 0.394 | 0.053 | 0.470 | **0.009** | 0.284 | **<0.0001** |
| VitD*PA | 0.858 | 0.171 | 0.710 | 0.510 | **0.033** | 0.969 | 0.629 | 0.973 | 0.704 |
| VitD*siPLIN2 | 0.108 | 0.016 | 0.338 | 0.590 | 0.178 | 0.838 | 0.098 | 0.682 | 0.872 |
| PA*siPLIN2 | 0.922 | 0.312 | 0.674 | 0.800 | 0.251 | 0.288 | 0.351 | 0.496 | 0.345 |
| VitD*PA*siPLIN2 | 0.784 | 0.712 | 0.861 | 0.649 | 0.071 | 0.484 | 0.214 | 0.785 | 0.647 |

VitD = 100 nm calcitriol; PA = 100 µM palmitate; siPLIN2 = perilipin 2 siRNA knockdown. n = 6.

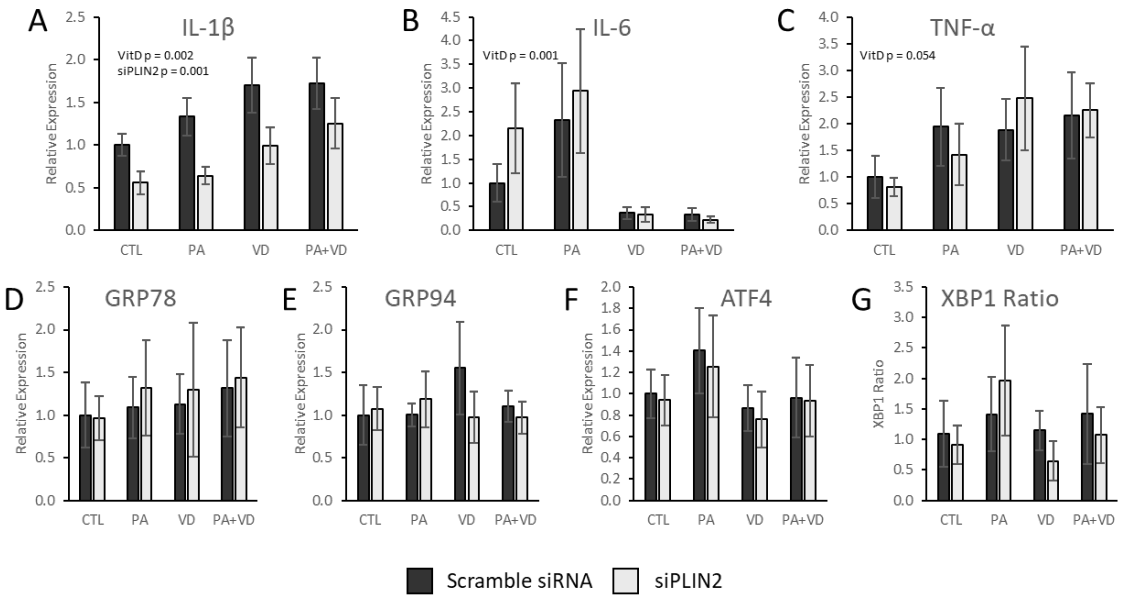



**Figure 4.6. Neither palmitate treatment nor PLIN2 knockdown induced the transcription of genes associated with lipotoxicity.** Gene expression in myotubes was analyzed for genes associated with inflammation (A-C) and endoplasmic reticulum (ER) stress (D-G) indicative of lipotoxicity using qRT-PCR after treatment with or without 100 nM calcitriol (VitD) and 100 µM palmitate (PA) for 24 hours with or without PLIN2 knockdown. IL-1β = interleukin 1β; IL-6 = interleukin 6; TNF-α = tumor necrosis factor α; GRP78 = glucose-regulated protein, 78 kDa; GRP96 = glucose-regulated protein, 96 kDa; ATF4 = activating transcription factor 4; XBP1 ratio = spliced X-box protein 1/unspliced X-box protein 1. Black bars = scramble siRNA, Grey bars = PLIN2 knockdown. Bars represent mean ± SEM, n = 6; statistical significance defined as $p < 0.05$, 3-way ANOVA.

**Table 2** Complete statistical analysis of 3 x 3 ANOVA for expression of genes associated with lipotoxicity

| Term | IL-1β | IL-6 | TNF-α | ATF4 | GRP78 | GRP96 | XBP1 Ratio |
|---|---|---|---|---|---|---|---|
| VitD | **0.002** | **0.001** | 0.054 | 0.381 | 0.574 | 0.706 | 0.519 |
| PA | 0.290 | 0.341 | 0.384 | 0.377 | 0.590 | 0.699 | 0.229 |
| siPLIN2 | **0.001** | 0.437 | 0.997 | 0.329 | 0.735 | 0.606 | 0.779 |
| VitD*PA | 0.840 | 0.283 | 0.411 | 0.326 | 0.938 | 0.524 | 0.693 |
| VitD*siPLIN2 | 0.954 | 0.360 | 0.444 | 0.344 | 0.948 | 0.273 | 0.477 |
| PA*siPLIN2 | 0.990 | 0.769 | 0.650 | 0.341 | 0.876 | 0.530 | 0.605 |
| VitD*PA*siPLIN2 | 0.461 | 0.825 | 0.932 | 0.335 | 0.827 | 0.699 | 0.731 |

VitD = 100 nm calcitriol; PA = 100 µM palmitate; siPLIN2 = perilipin 2 siRNA knockdown. n = 6.

# CHAPTER 5: DISCUSSION

## 5.1 Summary of Research Problem & Hypothesis

Vitamin D and PLIN2 each play an important role in the accumulation and oxidation of intramyocellular lipid (IMCL). Although vitamin D is widely considered to be beneficial to lipid metabolism and management in a variety of tissues, the benefit of PLIN2 is a subject of debate [134, 200, 202, 204, 229, 230]. Furthermore, PLIN2 and vitamin D are both implicated in the development or prevention of obesity and lipotoxicity. I hypothesized that **vitamin D increases IMCL accumulation, β-oxidation, and lipid turnover through a PLIN2-mediated mechanism, thereby preventing lipotoxicity in skeletal muscle**. This hypothesis was addressed in the following in two aims: (1) characterize the effect of vitamin D and PLIN2 on lipid turnover and β-oxidation in mature myotubes and (2) determine the role of vitamin D and PLIN2 in regulating key markers of lipotoxicity.

## 5.2 Aim 1: PLIN2 is required for increased mitochondrial oxygen consumption

Aim 1 was designed to characterize the effect of vitamin D and PLIN2 on lipid turnover and β-oxidation in mature myotubes. This aim showed that calcitriol, the active form of vitamin D, increased mitochondrial metabolism, most notably through complex II of the electron transport chain, succinate dehydrogenase (SDH) and that PLIN2 knockdown decreased mitochondrial function. Of particular note, our statistical model revealed that PLIN2 knockdown decreased calcitriol-induced oxygen consumption by SDH, suggesting that PLIN2 upregulation is necessary for calcitriol-induced increase of SDH activity. While these data support my hypothesis, the lack of data directly measuring fatty acid oxidation precludes definitive claims regarding the role β-oxidation or lipid turnover in this mechanism.

The original design of this study included a $^{14}$C-palmitate oxidation assay intended to directly measure β-oxidation (Figure 5.1). However, after 6 attempts, data showed no change in fatty acid oxidation in response to treatment with either siRNA or calcitriol. Because previous research has shown changes in response to both calcitriol and PLIN2 modulation as well as mRNA data showing metabolic changes at the gene expression level, I am highly suspicious of the veracity of this data and did not include it in previous chapters. The ratios of $^{14}CO_2$ to acid soluble metabolites (ASM) observed in this experiment were higher (1:5) than published by others in a study PLIN2

overexpression (1:40) [134]. This may be a product of the increased incubation time (5 hours vs 2 hours) that was employed to increase signal of $^{14}C$ in the $CO_2$ fraction. An alternative technique to measure fatty acid oxidation using the Seahorse XFe24 in combination with a palmitate-enriched media with 2-deoxyglucose is currently underway in a second attempt to measure fatty acid oxidation after calcitriol treatment and PLIN2 knockdown.

Another noteworthy experiment not shown in Chapter 3 was a fluorescent labeling of vitamin D receptor (VDR) and myosin heavy chain MyHC in C2C12 myotubes (Figure 5.2). Images were not included for publication because of the generally low quality of fluorescence and the complicating implications of these images; micrographs showed a discrepancy in subcellular location of VDR protein between mature myotubes and undifferentiated myoblasts where VDR localized to the nucleus in myoblasts (i.e. cells not expressing MyHC) while remaining cytosolic in myotubes. These data aligned with those published by Esser and colleagues [89] and raise the important question of whether vitamin D signaling in skeletal muscle is caused by canonical nuclear VDR-driven gene transcription (nVDR), or second messenger systems initiated by membrane VDR (mVDR). If results in this project were driven by nVDR-mediated gene transcription in myoblasts, it dramatically weakens physiological implications of this work as they are relatively rare in skeletal muscle tissue and contribute negligibly to lipid storage and metabolic activity. However, because of the strong induction of VDR and high abundance of myotubes observed throughout this project, I believe that downstream effects of mVDR are a more likely mechanism for the effects produced by calcitriol in this project. If this mechanism indeed drives cellular response in the present model, treatment effects may be far downstream from the initial VDR activation [26, 27, 110].

In spite of the aforementioned limitations, metabolic and gene expression data imply that calcitriol does induce metabolic changes that favor both lipid accumulation and lipolysis, while PLIN2 knockdown results in changes that promote lipid accumulation but limit mitochondrial activity. This supports the hypothesis that vitamin D influences lipid flux while showing that PLIN2 is required for robust lipid clearance. This beneficial role of PLIN2 in lipid clearance should be taken into consideration by researchers investigating PLIN2 knockout models as a means of investigating obesity, T2DM, or fatty liver disease, as effects produced by impaired lipid flux in skeletal muscle may contribute to off-target consequences and systemic maladaptations not predicted in organ-specific models [125].

## 5.3 Aim 2: Palmitate alone may not be enough to produce lipotoxicity in C2C12 myotube culture

Aim 2 sought to determine the role of vitamin D and PLIN2 in the prevention of palmitate-induced lipotoxicity and address the hypothesis that a palmitate-induced lipotoxic phenotype would be ameliorated by VitD through increased storage and oxidation of triacylglyceride (TAG) via the induction of PLIN2. While preliminary oil red O experiments showed a strong protective effect of calcitriol in response to cellular dysfunction caused by 100 µM palmitate, later experiments showed no effect of palmitate on cellular attachment, mitochondrial function, endoplasmic reticulum (ER) stress, or inflammation. Thus, the model employed failed to produce lipotoxicity and data were unable to support the hypothesis. However, important conclusions can be drawn from these negative outcomes.

Signs of lipotoxicity are not limited to mitochondrial function and inflammation. I attempted a liquid chromatography mass spectroscopic analysis to measure changes in lipid species abundance, particularly DAG and ceramides, after treatment with palmitate, calcitriol, or PLIN2 knockdown. However, I was unable to adequately normalize the raw data and was therefore unable to interpret meaningfully the results. This experiment was intended not only to quantify lipid abundance more reliably than the oil red O experiments discussed in Chapter 3 but also to determine if lipid species associated with lipotoxicity were impacted by any treatments. Analyzing the lipid profile is an exceedingly important aspect of evaluating lipotoxicity as data continue to show that it is not the quantity of lipid that produces a lipotoxicity but instead the quality of lipids; that is, the species in a lipid profile have a greater influence on the development of lipotoxicity than the total abundance of lipid [131, 139, 247, 257, 258]. The failure of this experiment substantially limits interpretation of results, and positive results could have suggested subtle changes in lipid metabolism in response to palmitate treatment that did not produce measurable changes in other experiments focused on characterizing gene expression or oxygen consumption.

The most important implications of Aim 2 data stem from the lack of stress after either treatment with palmitate or PLIN2 knockdown. Palmitate treatment is considered a standard model for lipotoxicity in *in vitro* studies [215, 259, 260]. However, these results suggest that physiological doses of palmitate are not sufficient to produce metabolic, inflammatory, or endoplasmic reticulum stress in C2C12 myotubes, indicating that palmitate-based models of lipotoxicity may be either insufficient in C2C12 culture or rely on supraphysiological concentrations. Researchers

should evaluate both species-specific abundance of fatty acids and the concentration of glucose in research using palmitate as a model of lipotoxicity [215, 252]**.** Several publications have suggested that lipotoxicity is dependent on excessive glucose and rebranded the pathology glucolipotoxicity [128, 252]. The most prominent hypothesis for this interplay between glucose and fatty acids is tied to the glucose-fatty acid cycle, which was first proposed by Randle in 1963 [261] and revisited recently by Hue and Taegtmeyer [262]. Randle's hypothesis states that a high concentration of both glucose and fatty acid produces an oversupply of substrate to the Krebs cycle. Subsequently, surplus acetyl-CoA accumulates in the cytosol and is converted to malonyl-CoA through acetyl-CoA carboxylase, and Malonyl-CoA in turn inhibits the transport of acyl-CoA into mitochondria by inhibiting carnitine palmityltransferase-1. The accumulation of acyl-CoA precipitates the accumulation of bioactive, lipotoxic lipids including DAG and ceramides.

### 5.4 Overall Conclusion

Collectively, the findings of my work suggest that calcitriol treatment increases oxidative metabolism in myotubes and that this increase is at least in part attributable to the increased expression of PLIN2. While β-oxidation was not directly measured, data presented in this study give credence to the speculation that calcitriol produces a PLIN2-dependent increase in lipid oxidation. The present study shows that calcitriol induced the expression of several genes associated with the mobilization of lipids for β-oxidation including ATGL and CGI-58. Similarly, calcitriol treatment resulted in an increase in oxygen consumption that was dependent on PLIN2 expression. Several studies have shown that vitamin D treatment increases whole body fatty acid oxidation in both humans and rats [37, 63], while Seahorse analysis of glycolysis by Calton et al. [263] revealed that supplementation with vitamin D decreases glycolysis in blood mononuclear cells. To my knowledge, there are no studies suggesting that treatment with vitamin D increases the rate of glycolysis. In light of this previous research, these findings suggest that the PLIN2-dependent increase in oxygen consumption after calcitriol treatment is associated with an increase in β-oxidation.

Expanding upon this line of reasoning, data presented in this dissertation support the hypothesis that vitamin D and PLIN2 play a role in the prevention of lipotoxicity in skeletal muscle. However, I make these speculations cautiously, as I did not induce lipotoxicity and therefore could not measure the effects of my treatments on lipotoxicity. Because increased lipid oxidation

ameliorates lipotoxicity [239], the increase in oxygen consumption, which was most likely driven by β-oxidation, can be loosely tied to a decreased risk of lipotoxicity. In parallel, one can reasonably suspect that PLIN2 knockdown increases risk of lipotoxicity through decreased oxygen consumption. In opposition to this, several researchers have found that PLIN2 knockdown or knockout increases β-oxidation [134, 197]. Ultimately, this study is not sufficient to draw any concrete conclusions regarding the roles of PLIN2 knockdown and vitamin D in lipotoxicity.

In spite of this shortcoming, there are sufficient data to conclude that PLIN2 knockdown decreases mitochondrial function in C2C12 myotubes. While there is a large body of literature that suggests that PLIN2 knockout is protective against lipotoxicity and obesity, a claim that PLIN2 enables lipotoxicity may be overly simplistic. I propose that PLIN2 plays a beneficial role in lipid metabolism in skeletal muscle and that without PLIN2, bioenergetic benefits of vitamin D supplementation may not be realized. My work suggests that PLIN2 upregulation in response to healthy stimuli, such as exercise or high levels of vitamin D, enables greater lipid storage and β-oxidation. The beneficial upregulation of PLIN2 is accompanied by increased insulin sensitivity and increased mitochondrial function without increased markers of oxidative stress or mitochondrial dysfunction [48, 208]. This pattern is observed in both skeletal muscle [264, 265] and the liver [266] in response to several stimuli including endurance, sprint interval, and resistance exercise as well as vitamin D supplementation in projects shown here and published by Jefferson et al. [48].

### 5.5 Significance & Impact

The significance of these findings can be divided into three main contributions to the fields of vitamin D and muscle lipid biology. First, these results provide a more detailed description of cellular processes underlying vitamin D-induced increases in mitochondrial activity functional capacity in skeletal muscle. For several years, researchers have had evidence that vitamin D increased mitochondrial function; there is now strong evidence implicating SDH in the observed increase. This discovery was verified by modifying the Seahorse protocol to add rotenone and succinate before adding Antimycin A. This allowed for observation of oxygen consumption driven by SDH. Future researchers should consider including this minor change to the protocol of future oxygen consumption rate (OCR) analyses and include it as a new standard of the mitochondrial stress test protocol.

Secondly, this dissertation provides evidence that PLIN2 knockdown negatively impacts skeletal muscle oxidative metabolism under healthy conditions. There is a multitude of studies investigating the role of PLIN2 in lipid management in both healthy and pathological environments, the results of which are highly contradictory. Some studies suggest that increased PLIN2 expression is associated with increased oxygen consumption and protection against symptoms of lipotoxicity [48, 204, 208, 242, 264, 265, 267], while others show that PLIN2 increases with dysfunction and pathology [228]. A large cohort of knockout studies in mice suggest that the absence of PLIN2 prevents signs of ectopic fat accumulation [197, 200, 229, 230]. This is a long and ongoing conversation within the scientific community, and the role of PLIN2 in lipid storage and metabolism is likely nuanced, multifaceted, and context dependent. While my results do not solve the puzzle of PLIN2 in lipid metabolism, the information presented here is an important contribution to the debate surrounding the nature of PLIN2 abundance in relation to metabolic health of skeletal muscle.

Finally, this project demonstrates that the entire cellular environment must be considered when implementing a palmitate-based model of lipotoxicity and that minor changes to cell culture models can have a dramatic impact on cellular health and function. To aid future researchers in reproducing results, publications investigating the presence or absence of toxic effects in response to treatment with palmitate, and lipids in general, should be reported very carefully. Methods sections of these publications should include a high degree of specificity when describing the composition of cell media, fatty acid preparation and conjugation, cell confluence, time allowed for differentiation, and cell passage number. Researchers designing lipotoxicity studies should, in turn, pay careful attention to characterizing cell models and pay special attention to reproducing results of others before applying previously published models to new experiments.

### 5.6 Strengths & Limitations

The greatest strength of this study is the comprehensive approach to key outcomes. Similar metabolic outcomes were examined in three ways; SDH activity, Seahorse XF assay, and fatty acid oxidation assay all yield consistent results. It is because of these parallel methods that I can confidently reject the results of the $^{14}$C-palmitate fatty acid oxidation assay. Without multiple experiments in the same model suggesting a phenotype contrary to that demonstrated in one experiment, it is exceedingly difficult to dismiss the results.

Another strength is the depth of analysis of mitochondrial metabolism afforded by the methods used in the Seahorse assay. General Agilent protocols do not include the rotenone/succinate injection and, therefore, do show oxygen consumption specific to SDH. Because I completed a more exhaustive analysis at the suggestion of the laboratory of Dr. Patrick Sullivan, I was able to identify the mitochondrial complex driving PLIN2-dependant differences in oxygen consumption after calcitriol treatment.

Perhaps the greatest weakness of this study was the lack of lipotoxicity in my palmitate treatment model. Early in data acquisition, model design, and oil red O experimentation using 100 and 200 µM palmitate with or without 100 nM calcitriol for 48 or 72 hours produced a dose and time dependent effect evidenced by cell detachment and morphological signs of stress in myotubes treated with palmitate. This response was ameliorated with co-treatment with 100 nM calcitriol. This experiment served as a proof-of-concept that calcitriol prevented palmitate-induced lipotoxicity in differentiated C2C12 cells. From these results, I established a 100 µM palmitate treatment for 24 hours to shorten treatment durations and prevent off target effects from excessive stress. After this experiment was completed, cell culture medium was changed from 4500 mg/L glucose DMEM to 1000 mg/L glucose DMEM to support more consistent, higher quality myotube formation. Subsequent ORO experiments and palmitate treatments did not produce the previously observed stress response, but it was not until a significant percentage of the project was completed that I recognized this lack of stress. I hypothesize that this was a product of a decreased concentration of glucose in the cell medium in alignment with the Randle hypothesis of glucolipotoxicity.

Another limitation of this project was the reliance on mRNA to infer metabolic changes with calcitriol treatment. Metabolic endpoints were reliably drawn from the measurement of oxygen consumption. However, the mechanisms behind these changes were inferred from the interpretation of patterns of mRNA expression, which are not as reliable as those drawn from protein content or enzyme activity. Furthermore, several of the proteins implicated in this study are post-translationally regulated or vary activity based on sub-cellular localization or protein-protein interactions. The results obtained from mRNA analysis provide clues to mechanisms underlying changes in metabolism, but cannot be assumed to represent changes at the level of enzyme activity.

Another limitation of this study is the heterogeneous nature of C2C12 cell culture. Because differentiation is not perfectly efficient, undifferentiated myoblasts in each experiment contribute to two potential problems. First, as myoblasts and myotubes respond differently to calcitriol [89], changes observed with calcitriol treatment may be a product of response by myoblasts and not myotubes. Secondly, variability of the rate and efficiency of differentiation between experiments likely resulted in a difference in the relative abundance of myoblasts and myotubes in each given replicate. This could contribute to increased variability, underpowered experiments, and type II error. Future experiments in differentiated myocyte culture could employ several strategies to minimize these problems. First, using a cell model that differentiates more efficiently, such as human primary cells, could prevent myoblast contamination in experiments. Further measures could include pharmacological interventions during experiments to increase efficiency of differentiation and encourage trimming on undifferentiated cells. Such approaches could include promoting differentiation with Insulin-Transferring-Selenium solution or targeting actively proliferating myoblasts with cytosine arabinoside (Ara-C).

### 5.7 Future Directions

While this project provides strong evidence that calcitriol increases mitochondrial function in skeletal muscle dependent on PLIN2, several questions remain unanswered. These unanswered questions can be addressed through future experimentation divided into short term and long term goals. Short term questions include projects that could be addressed in one or two experiments over the course of several months. Long term questions serve as the foundation of future research projects and would likely require years to investigate fully.

Were this project to continue for another six months, I would focus on three experiments: 1) accurate analysis of β-oxidation in response to treatment with calcitriol, 2) mass spectroscopic analysis of DAG and ceramide in myotubes treated with 100 µM palmitate to determine if there are low level lipotoxic responses in lipid profile in response to the treatment used in project and 3) reproducing key metabolic measurements in human primary myotube culture to demonstrate applicability to human physiology.

To investigate β-oxidation in myotubes, would employ two simultaneous techniques. I would work with a collaborator to troubleshoot the [1-$^{14}$C]-palmitate fatty acid oxidation assay. This assay has been used by multiple researchers and supplies detailed information regarding the rate

of β-oxidation and the completeness of said oxidation. For this reason, this is my preferred method for measuring β-oxidation. However, I would simultaneously work to gain data through a modified Seahorse oxygen consumption assay. This method would include treatment with 100 μM and no glucose for 2 hours prior to assay and the addition of 50 mM 2-deoxyglucose to prevent glucose metabolism. In this condition, fatty acids would be the only substrate available for large amounts of oxidation as amino acid oxidation generally contributes negligibly to oxygen consumption and ATP production. This approach is not as detailed as the $^{14}$C-palmitate approach, but it would provide valuable information regarding the relative rates of fatty acid oxidation with and without vitamin D treatment and PLIN2 knockdown.

A reliable LC-MS analysis of DAG and ceramide, two families of lipid species most commonly associated with lipotoxicity, would provide great insight into subtle changes that may be occurring in response to vitamin D treatment and PLIN2 knockdown. This would therefore be a substantial focus were this project to extend for six months. A similar approach would be used as was reported in Chapter 4, however, a small sample of cell suspension would be taken from each sample for BCA assay and normalization of spectra to protein quantification. This is important as phospholipid profile is reported to change in response to vitamin D treatment [47].

The final short term experiment would aim to reproduce metabolic outcomes in human primary myotubes. Experiments would repeat seahorse analyses with vitamin D and PLIN2 knockdown treatments to verify that responses in human primary cells are similar to those observed in immortalized mouse cells. I would complete Seahorse analysis using the exact procedures and treatment described in Chapter 3. Two-way factorial ANOVA would again be used to determine significant effects of each treatment and trends would be compared to those observed in C2C12 cells.

Two of the primary questions raised by this project to be addressed with a long term approach are 1) what are the mechanisms by which vitamin D increases OCR, and 2) does the observed PLIN2-dependent increase in mitochondrial activity ameliorate symptoms of lipotoxicity?

Future research should focus on more closely examining the effects of calcitriol on various stages of lipid metabolism such as TAG acylation, LD formation, and lipolysis from myocellular LDs. Experiments to investigate these areas of cellular metabolism should include mass spectroscopic analysis of lipids focusing on the abundance of non-esterified fatty acid, monoacylglyceride, DAG,

and TAG after the addition of a tracer isotope such as $^{13}$C-palmitate. Schweiger has summarized advanced methods for measuring lipolysis through protein quantification, phosphorylation analysis, protein co-localization, and enzyme activities [268]. Each of these methods should be employed to determine how calcitriol impacts lipolysis. Lipid droplet formation can be tracked by using fluorescent microscopy markers for ER and neutral lipid in combination with an antibody to PLIN2. Localization of PLIN2 to the ER suggests early-stage LD formation, and counting the number of LDs after 0, 1, 3, 6, and 24 hours of treatment provides means for a direct analysis of the effect of calcitriol on LD formation. Finally, β-oxidation should be measured through one of several methods. Use of dually labeled, tritiated $^{14}$C-palmitate, as employed by Broderick [269], may provide a better internal control than using $^{14}$C-palmitate alone. Alternatively, β-oxidation can be measured through Seahorse oxygen consumption assay by comparing the respiration of cells treated with etomoxir, a CPT-1 inhibitor, with those receiving a vehicle control.

As the lipotoxic model in this project was not efficacious, future research should improve this model and complete the investigation of whether PLIN2 upregulation prevents or ameliorates symptoms of lipotoxicity. In accord with the aforementioned attention to cellular environment, this research should first take a systematic approach to inducing lipotoxicity in skeletal muscle. Treatment should vary the concentrations of palmitate and glucose in media with either horse or fetal bovine serum to comprehensively analyze the response of myotubes to palmitate under these conditions. For continuity of research and the most effective application of these results to future interpretations, time of treatment should be 24 hours. Lipotoxicity should be quantified in reference to inflammation (cytokine mRNA and protein expression), ER stress (CHOP, XBP1 splicing, ATF4 expression), oxidative stress (dichlorofluorescein, dihydroethidium, and 4-hydroxynonenal staining), and liquid chromatography mass spectroscopic analysis of lipid profile (quantification and qualification of ceramide and DAG).

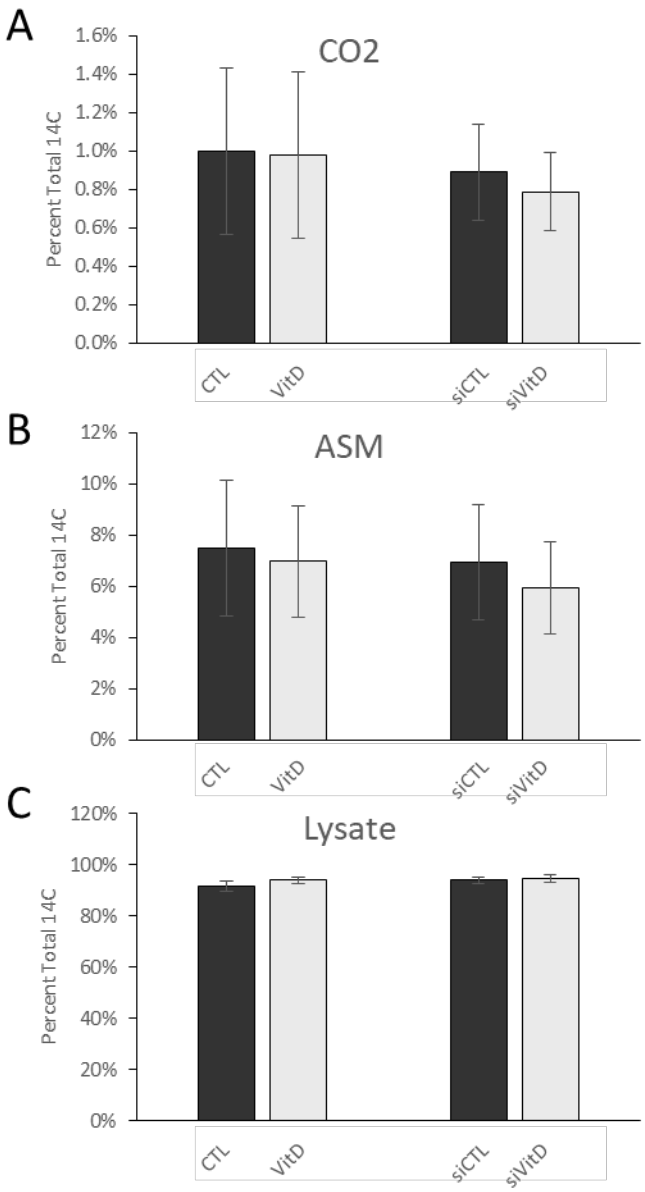



**Figure 5.1. $^{14}$C-palmitate pulse-chase analysis showed that treatment with neither VitD nor siPLIN2 significantly impacted fatty acid oxidation.** Myotubes treated with or without 100 nM VitD and with or without PLIN2 knockdown showed no change in 14C-labeled CO2 (A), acid soluble metabolites (B), or cellular lysate after 5 hours of incubation with 100 µM palmitate.

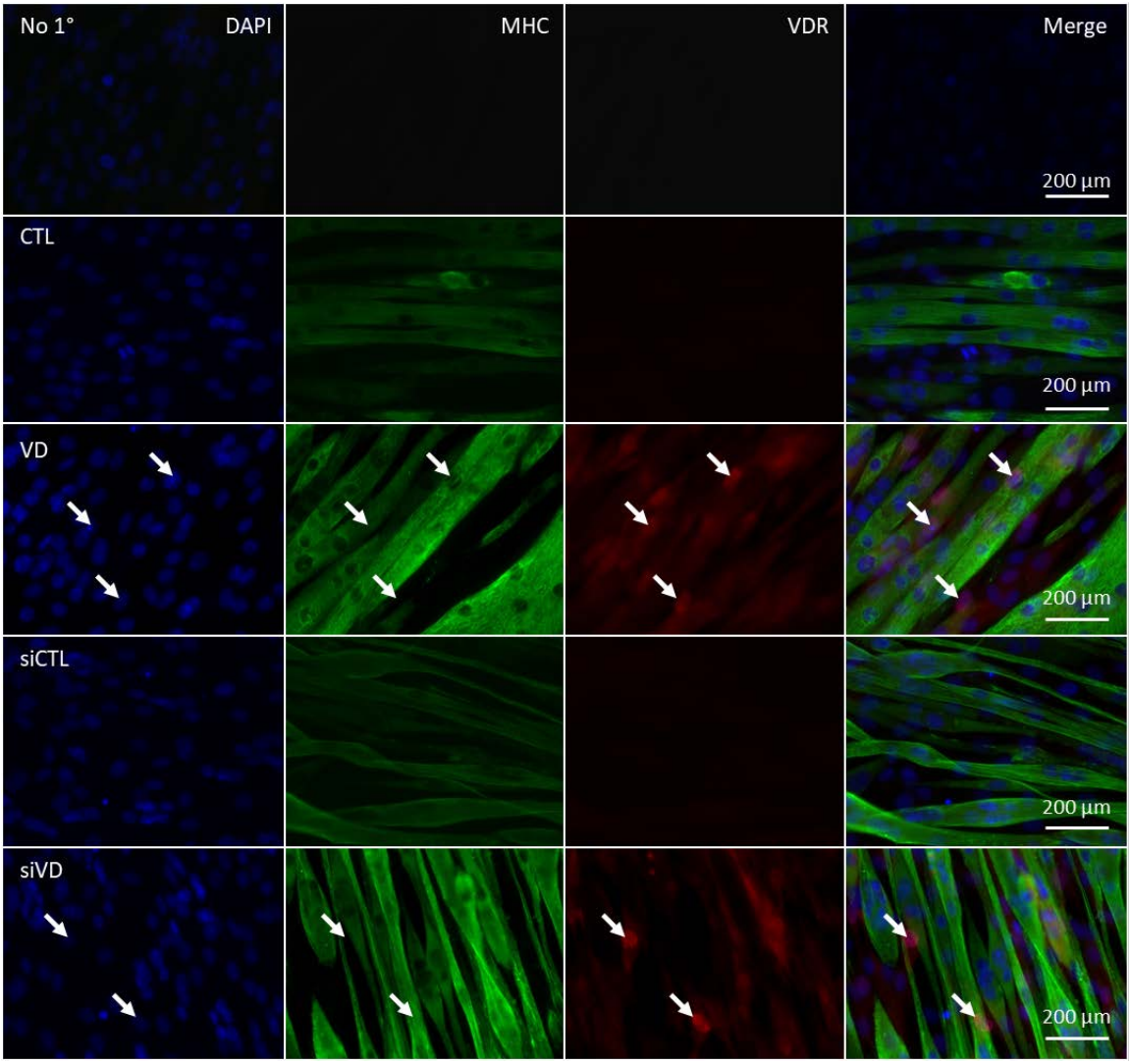
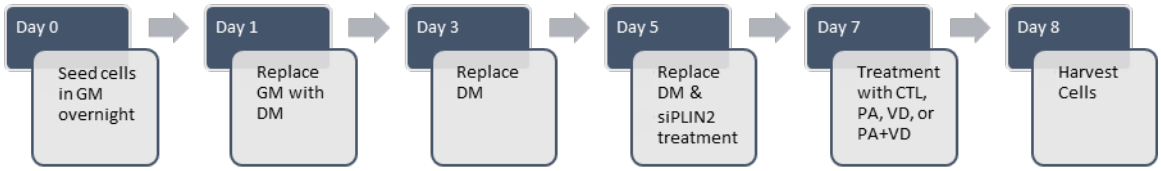



**Figure 5.2. Fluorescent labeling shows that calcitriol increases VDR expression in the nucleus of myoblasts and cytosol of myotubes.** Fluorescent labeling of myosin heavy chain (MyHC, green) and vitamin D receptor (VDR, red) in C2C12 culture. White arrows show localization of VDR expression in the nuclei of cells not expressing MyHC (e.g. myoblasts). Nuclei stained with DAPI. Scale bars = 200 µm.

## APPENDICES




***Appendix 1. Schematic of cell growth and treatment.*** C2C12 cells are seeded in growth media (GM) overnight in a 24 well plate and differentiated for a total of 6 days in DMEM with 2% horse serum (DM) before treatment with palmitate (PA), calcitriol (VitD), both (PA+VitD), or vehicle controls (CTL) for 24 hours. All cells are treated with either 10 nM PLIN2 siRNA or scrambled control siRNA with 1:500 Lipofectamine RNAiMAX starting day 5 for a total of 48 hours.

**Appendix 2.** List of primer pairs used for RT-PCR.

| Gene | Forward | Reverse |
|---|---|---|
| RER1 | CGG AGC TGC GAG TTA CAG AA | ATC TGT CCA AGC CGT GAG AA |
| VCP | TGA CCC TCA TGG ATG GCC TA | TGT CAA AGC GAC CAA ATC GC |
| EMC7 | CGA GTG CTG GTA GAC GGA GA | CCA CTA CAT AAG AGC CAG AGG G |
| VDR | CCA CCA CAA GAC CTA CGA CC | ATC ATG TCC AGT GAG GGG GT |
| ATGL | GAG GAA TGG CCT ACT GAA CC | AGG CTG CAA TTG ATC CTC CT |
| DGAT1 | AAG AGG ACG AGG TGC GAG AC | GAT GGC ACC TCA GAT CCC AGT A |
| DGAT2 | CCC TAC TCC AAG CCC ATC AC | GGC ATG GTA CAG GTC GAT GT |
| CGI-58 | CGG TGG AGG GTC AGG ATG G | TAT GCG CAC AGG CTC TTT CT |
| CPT-1 | TGG CTA CGG GGT CTC TTA CA | GGG CGT TCG TCT CTG AAC TT |
| PLIN2 | TCA GCT CTC CTG TTA GGC GT | TTC TCG GCC ATC TCA CAC AC |
| PLIN3 | ATG AAC ACT CCC TCG GCA AG | CCT GTG GGG TCT TCT GGT TC |
| PLIN5 | TAC TTT GTG CGT CTG GGG TC | CTG GGT ACG GTG TTT GCT CT |
| XBP-1 (Reverse) | - | GTC CAT GGG AAG ATG TTC TGG |
| Spliced XBP1 | CTG AGT CCG AAT CAG GTG CAG | - |
| Unspliced XBP1 | CAG CAC TCA GAC TAT GTG CA | - |
| ATF4 | CGC TGC GGT AGG ATC ACG | TGG ATT TCG TGA AGA GCG CCA T |
| GRP78 | GTG TGT GAG ACC AGA ACC GT | TAG GTG GTC CCC AAG TCG AT |
| GRP94 | AAG AAT GAA GGA AAA ACA GGA CAA AA | CAA ATG GAG AAG ATT CCG CC |
| IL-1β | CTA CAG GCT CCG AGA TGA ACA AC | TCC ATT GAG GTG GAG AGC TTT C |
| IL-6 | TTC CAT CCA GTT GCC TTC TTG | GGG AGT GGT ATC CTC TGT GAA GTC |
| TNF-α | ATC CGC GAC GTG GAA CTG | ACC GCC AGT TCT GGA A |

**Appendix 2. List of primer pairs used for RT-PCR.** RER1 = Retention in Endoplasmic Reticulum Sorting Receptor 1; VCP = Valosin Containing Protein; EMC7 = Endoplasmic Reticulum Membrane Protein Complex Subunit 7; VDR = Vitamin D Receptor; ATGL = Adipose Triglyceride Lipase; DGAT1 = Diacylglyceride O-Acyltransferase 1; CGI-58 = Comparative Gene Identification-58; CPT-1 = Carnitine Palmitoyltransferase 1; SCD-1 = Steroyl-CoA Desaturase 1; PLIN2 = Perilipin 2; PLIN3 = Perilipin 3; PLIN5 = Perilipin 5. ATF4 = Activating Transcription Factor 4; GRP78 = Glucose Responsive Protein, 78 kDa; GRP94 = Glucose Responsive Protein, 94 kDa; IL-1β = Interleukin-1β; IL-6 = Interleukin-6; TNF-α = Tumor Necrosis Factor-α

**Appendix 3.** Calculations used to derive oxygen consumption rates from Seahorse XFe24.

| Measurement | Equation |
|---|---|
| Baseline | Average(Rates 1-4) |
| Oligomycin | Minimum(Rates 5-8) |
| FCCP | Maximum(Rates 9-12) |
| Rotenone/Succinate | Minimum (Rates 13-20) |
| Antimycin A | Average(Rates 21-24) |
| Basal | [Baseline] – [Antimycin A] |
| ATP-Linked | [Baseline] – [Oligomycin] |
| Maximum | [FCCP] – [Antimycin A] |
| Reserve | [FCCP] – [Basal] |
| Complex I | [FCCP] – [Rotenone/Succinate] |
| Complex II | [Rotenone/Succinate] – [Antimycin A] |
| Leak | [Oligomycin] – [Antimycin A] |
| Non-Mitochondrial | [Antimycin A] |
| Mitochondrial AUC | [Average(Baseline, FCCP, Rotenone/Succinate)] – Antimycin A |

**Appendix 3. Calculations to derive OCR from Seahorse XFe24.** Equations used to determine oxygen consumption rates (OCR) after analysis with Seahorse Biosciences XFe24 Extracellular Flux Analyzer.

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# VITA

David M. Schnell

EDUCATION

Degrees

| | |
|---|---|
| 2018 | PhD, Nutritional Sciences<br>Graduate Center for Nutritional Sciences<br>College of Medicine<br>University of Kentucky |
| May 2012 | Bachelor of Science, Exercise Physiology<br>Department of Exercise Physiology<br>School of Medicine<br>West Virginia University |
| May 2012 | Minor, Biology<br>Department of Biology<br>Eberly College of Arts and Sciences<br>West Virginia University |
| May 2012 | Minor, Sport and Exercise Psychology<br>Department of Sport and Exercise Psychology<br>College of Physical Activity and Sport Science<br>West Virginia University |
| 2010 | Study Abroad<br>University of Sydney<br>Sydney, NSW, Australia |

## Research

*Research Positions*

| | |
|---|---|
| 2015-Present | Graduate Research Assistant, Laboratory of Dr. D. Travis Thomas, Department of Clinical Sciences, College of Health Sciences, University of Kentucky<br>Experienced in: Grant Writing, Primary Cell Culture, Nutritional Science (emphasis: Vitamin D), Cellular Biology Techniques, Clinical Research and Institutional Review Board. |

2012-2015 Graduate Research Assistant, Laboratory of Dr. Daret St. Clair, Department of Toxicology, College of Medicine, University of Kentucky.
Experienced in: Cell Culture, Stable Isotope Resolved Metabolomics (SIRM), Molecular Techniques, Free Radical Biology, Metabolism, Cancer Biology, Grant Writing.

2010 Undergraduate Research Assistant, Hollander Lab, Department of Exercise Physiology, School of Medicine, West Virginia University
Experienced in: Molecular Techniques, Organelle Isolation, Animal Injections, Glucose Testing, Animal Organ Harvesting

*Peer-Reviewed Articles*

1. **David M. Schnell**, R. Grace Walton, Hemendra J. Vekaria, Patrick G. Sullivan, Lance M. Bollinger, Charlotte A. Peterson, D. Travis Thomas. *Vitamin D produces a perilipin 2-dependent increase in mitochondrial function in C2C12 myotubes*. (Under Review)
2. D. Travis Thomas, **David M. Schnell**, Maja Redzic, Mingjun Zhao, Hideat Abraha, Danielle Jones, Howard Brim, Guoqiang Yu. *The Effects of Vitamin D and Aerobic Exercise on local in vivo measures of Muscle Lipid and Oxygen Consumption*. (In Submission)
3. Grace Jefferson, **David Schnell**, D Travis Thomas, Lance Bollinger. Calcitriol concomitantly enhances insulin sensitivity and alters myocellular lipid partitioning in high-fat treated skeletal muscle cells. Journal of Physiological Biochemistry. 2017 November 73(4):613-621. doi: 10.1007/s13105-017-0595-8
4. Xiaowei Wei, Yong Xu, Fang Fang, Luksana Chaiswing, **David Schnell**, Teresa Noel, Chi Wang, Jinfei Chen, Daret K. St. Clair, and William H. St. Clair. *RelB Expression Determines the Differential Effects of Ascorbic Acid in Normal and Cancer Cells*. Cancer Research. 2017 Mar 15;77(6):1345-1356. doi: 10.1158/0008-5472.CAN-16-0785
5. Xu Y, Miriyala S, Fang F, Bakthavatchalu V, Noel T, **Schell DM**, Wang C, St. Clair WH, St. Clair DK. *Manganese superoxide dismutase deficiency triggers mitochondrial uncoupling and the Warburg effect.* Oncogene. 2015 Nov 13. doi: 10.1038/onc.2014.355.
6. Zhao Y, Miriyala S, Miao L, Mitov M, **Schnell D**, Dhar S, Sultana R, Butterfield DA and St.Clair DK. *Proteomic identification of HNE-bound mitochondrial proteins in Cardiomyocytes after Doxorubicin treatment in mice*. Free Radic Biol Med. 2014 Mar 12. pii: S0891-5849(14)00107-5. doi: 10.1016/j.freeradbiomed.2014.03.001.
7. **Schnell DM**, St. Clair DK. *Redox Pioneer: Joe M. McCord*. Antioxidant and Redox Signaling. 2014 Jan 1;20(1):183-8. Epub 2013 Nov 12.
8. Baseler WA, Dabkowski ER, Jagannathan R, Thapa D, Nichols CE, Shepherd DL, Croston TL, Powell M, Razunguzwa TT, Lewis SE, **Schnell DM**, Hollander JM. *Reversal of Mitochondrial Proteomic Loss in Type 1 Diabetic Heart with Overexpression of Phospholipid Hydroperoxide Glutathione Peroxidase*. American Journal of Physiology – Regulatory, Integrative and Comparative Physiology. Vol. 302: R553–R565.

*Abstracts*

1. **David M. Schnell**, Lance Bollinger, Charlotte Peterson, D. Travis Thomas. *Vitamin D may promote a PLIN2-dependent increase in lipid flux of C2C12 myotubes*. University of Kentucky Center for Clinical and Translational Research Day 2018.
2. Vitamin D induced increase in lipid storage and oxidation may be regulated by PLIN2 in C2C12 myotubes
3. **Schnell DM**, Bollinger L, Thomas DT. *Vitamin D Modulated Lipid Storage and Mitochondrial Function in Skeletal Muscle.* American Association for the Advancement of Science Annual Meeting 2017.
4. Luksana Chaiswing, Fang Fang, Teresa J Noel, **David M Schnell**, Jon S Thorson, Yong Xu, William H St. Clair, Daret K St. Clair. *Repurposing azithromycin for treating aggressive prostate cancer.* Markey Cancer Center Research Day 2016.
5. **Schnell DM**, D Travis Thomas. Calcitriol treatment increases oxygen consumption and blunts lipid accumulation in human myotubes. Center for Clinical and Translational Research Conference 2016.
6. **Schnell DM**, Dhar SK, Holley AK, Fan T, St. Clair DK. MnSOD deficiency triggers pre-carcinogenesis metabolic switch and aerobic glycolysis in healthy cells. Markey Cancer Center Research Day 2015.
7. **Schnell DM**, Dhar SK, Holley AK, Fan T, St. Clair DK. *MnSOD deficiency triggers pre-oncogenesis metabolic switch*. Markey Cancer Center Research Day 2014.
8. Walter A. Baseler, Erinne R. Dabkowski, Rajaganapathi Jagannathan, Dharendra Thapa, Cody E. Nichols, Danielle L. Shepherd, Tara L. Croston, **David M. Schnell**, John M. Hollander. *Overexpression of phospholipid hydroperoxide glutathione peroxidase (MPHGPx) attenuates cardiac mitochondrial proteomic loss and reverses protein import detriments observed with type 1 diabetes mellitus*. Experimental Biology 2012.

*Book Chapters*

1. Thomas, D. Travis and David M Schnell. "Chapter 4: Dietary Fat and Exercise." Sports Nutrition: A Practice Manual for Professionals. 6th ed. September, 2017.

*Grants*

1. *National Institute of Health* Kirschstein-*NRSA* 1F31GM117894-01 "MnSOD deficiency triggers a pre-carcinogenesis metabolic switch and aerobic glycolysis in healthy cells" *F31 Individual Pre-doctoral Fellowship – Submitted 04/06/15 (Rejected, scored 27th percentile)*
2. National Institute of Health Kirschstein-NRSA Training Grant 5T32DK007778-15 "Training Program in Oxidative Stress and Nutrition." Nancy Webb, Program Director. Pre-Doctoral Fellow. Support from 10/2014-09/2015.
3. National Institute of Health Kirschstein-NRSA Training Grant 5T32DK007778-14. "Training Program in Oxidative Stress and Nutrition" Lisa Cassis, Program Director. Pre-Doctoral Fellow. Support from 10/2013-09/2014.

*Invited Presentations*

1. 2014 SFRBM Annual Conference, Seattle, WA: "MnSOD deficiency triggers pre-carcinogenesis metabolic switch"
2. 2014 Invited Student Speaker, Markey Cancer Center Research Day, Lexington, KY: "MnSOD deficiency triggers pre-carcinogenesis metabolic switch"

## Professional experience

*Positions*

6/2015-7/2018 Kentucky Science and Technology Corporation – Exomedicine Institute
Biomedical Sciences Consultant
Facilitate advancement of basic biomedical research on the International Space Station (ISS) in partnership with Kentucky Space and Space Tango, INC. Communicate biological needs of model systems and contribute to design of automated laboratories installed on the ISS for commercial and academic research.

## Teaching Experience

*Courses Taught*

Fall 2015 IS/ICT 200 – Information Literacy and Critical Thinking
Lecturer
General education course focusing on developing Information Literacy skills such as identifying an informational need, finding, evaluating, organizing, and presenting information.

*Courses Facilitated*

Fall 2014 Integrated Biomedical Sciences 610 – Critical Scientific Reading
Senior Student Facilitator
Assisted primary instructor to teach first year PhD students critical reading skills for scientific literature. Class focused on discussion and understanding scientific techniques employed in landmark studies of contemporary molecular biology.

*Students Mentored*

2013 **Mentor** - Mr. Alexander Johnson, Research Observer
Aug-Dec Provided shadowing experience for collegiate graduate before applying to a graduate program.

2013 **Mentor** - Ms. Katelyn Robertson, Undergraduate Research Project
May-Dec Mentored undergraduate student in research project investigating the role of MESNA in abrogating TNF-$\alpha$ induced apoptosis in murine brain tissue during Doxorubicin treatment. Student became proficient in basic scientific techniques and Western blot

*Teaching-Specific Courses Taken*

Fall 2014 GS610 – College Teaching
Spring 2015 GS650 – Preparing Future Faculty

Honors and Awards

*Scholarships and Awards*

2014 Society for Free Radical Biology Research and Medicine Young Investigator Award
2007-2012 West Virginia PROMISE Scholarship
2007-2012 West Virginia University Mountaineer Scholarship

*Honors*

2007-2012 West Virginia University Dean's List
2007-2009 West Virginia University Honor's College

## Academic Service

*Professional Societies*

2018-Present American Medical Writers Association
2016-Present American Association for the Advancement of Science
2014-2016 Society for Free Radical Biology and Medicine
2009-2011 National Strength and Conditioning Association

*Leadership Positions*

2016-2017 Secretary, University of Kentucky Pharmacology and Nutritional Sciences Graduate Student Association.
2015-2016 President, University of Kentucky Pharmacology and Nutritional Sciences Graduate Student Association.
2015-2016 Member, Society for Free Radical Biology and Medicine Trainee Council
2014-2015 Vice President, University of Kentucky Pharmacology and Nutritional Sciences Graduate Student Association.

## Scientific Advocacy

April 2016 Biotechnology Industry Organization Washington DC Fly-In
Joined scientific professionals from across the country to meet with congressmen and discuss the importance of federal support for biotechnology and biomedical research and development.

## WORKSHOPS and WEBINARS

April 2015 Science Philosophy and Grant Writing with Aruni Bhatnagar, PhD - NSPS
May 2016 Derby Summit Career Workshop – KY Life Sciences Council
May 2017 Derby Summit Career Workshop – KY Life Sciences Council
May 2016 Time Management Workshop – UK Human Resources
June 2016 Office Communication Workshop – UK Human Resources
June 2016 Common grant-writing errors – AAAS Web-based Class
July 2018 Scientific Communication Workshop – COMPASS Scientific Communications