Two-weeks of single-leg immobilization alters IMCL storage, mitochondrial content, and insulin signalling in healthy, young women

by

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Authour's Declaration

This thesis consists of material all of which I authoured or co-authoured: see Statement of Contributions included in this thesis. This is a true copy of the thesis, including any required final revision, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Statement of Contributions

M. Black and M. Devries-Aboud designed the research aims and experiments for the current study. C. McGlory and S. M. Phillips designed the immobilization trial. C. McGlory, R. Bahniwal, and M. Kamal performed the immobilization trial. M. Black performed all data and statistical analysis for the work presented in this thesis. M Black drafted this thesis.

Abstract

During limb immobilization and bed rest, there is a significant loss of muscle mass and strength, and an onset of insulin resistance (IR). Subcellular localization of intramyocellular lipids (IMCL) is suggested to regulate the metabolic role of IMCL within skeletal muscle. IMCL stored in the subsarcolemmal (SS) region of the muscle fibre is associated with IR. Total IMCL content is unchanged by immobilization, but endurance training has been found to redistribute IMCL from the SS to the intermyofibrillar (IMF) region. Disuse also decreases mitochondrial content and function and can contribute to IR. The aim of the research presented in this thesis was to determine the effects of disuse on IMCL storage location and apposition with mitochondria and whether these changes are related to disuse-induced defects in insulin signalling. We also determined whether omega-3 fatty acid (FA) supplementation alters the effect of disuse on these outcomes. Young, healthy (n=20) women received omega-3 FA supplement (2.97g of EPA and 2.03g of DHA) or a placebo (isoenergetic and volume equivalent of sunflower oil) for 4-weeks prior to and continuing through 2-weeks of leg immobilization and 2-weeks of recovery. Skeletal muscle biopsies were taken prior to supplementation, pre- and post-immobilization, and after recovery. Electron microscopy was used to determine IMCL size, number, area density, and subcellular localization, as well as mitochondrial area density and subcellular localization. Western blotting was used to examine proteins related to insulin signalling, IMCL metabolism, mitochondrial dynamics, and apoptosis-related signalling. There was no effect of omega-3 FA on any measured parameter. IMCL area density decreased in the SS region during immobilization and recovery due to reduced IMCL size. Central IMF IMCL area density increased with immobilization and returned to baseline during recovery. There was a corresponding decrease in mitochondrial content in the SS region during immobilization and in

both the SS region and central IMF during recovery. There was no effect of immobilization or recovery on IMCL or mitochondrial characteristics in the peripheral IMF region. PLIN5 and phosphorylated and total AKT content increased during immobilization and remained elevated in recovery. Accumulation of IMCL in the central IMF region is associated with decreased insulin sensitivity (IS). Previous work has found no relationship between SS IMCL content and IR in women, which is supported by the findings of the current study where SS IMCL area density decreased with immobilization. Surprisingly, there was no loss of insulin signalling protein content during immobilization. The differential effects induced by immobilization on insulin signalling in women may be mediated by estrogen and requires further examination.

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Dedication

This thesis is dedicated to my parents, Tamzen and Geoff Black, and my sisters, Rhea and Celeste. Thank you for being so supportive of me in whatever I chose to do. I am very grateful to have such a wonderful, supportive family and I couldn't have finished this degree without you.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

% BF	Percent body fat
ACC	Acetyl CoA carboxylase
AKT	Protein kinase B
AMPK	5’ adenosine monophosphate-activated protein kinase
AS160	AKT substrate 160
ATGL	Adipose triglyceride lipase
BAX	BCL2-associated X protein
BAK	BCL2 antagonist/killer 1
BCL2	B-cell lymphoma
COX	Cytochrome c oxidase
CS	Citrate synthase
DAG	Diacylglycerol
DGAT1	Diacylglycerol acyltransferase 1
DHA	Docosahexaenoic acid
DRP1	Dynamin related protein 1
EPA	Eicosapentanoic acid
FA	Fatty acids
FIS1	Fission 1
FOXO3	Forkhead box O3
GLUT4	Glucose transporter 4
GPAT	Glycerol-3-phosphate acyltransferase
H^+ MRS	Proton magnetic resonance spectroscopy
HSL	Hormone sensitive lipase
IMCL	Intramyocellular lipids
IMF	Intermyofibrillar region
IMTG	Intramuscular triacylglyceride
IR	Insulin resistance
IRS1	Insulin receptor substrate 1
IS	Insulin sensitivity
MAFBX	Muscle atrophy F-box protein
MFN	Mitofusin
mTOR	Mammalian target of rapamycin
MURF	Muscle RING-finger protein-1
PPAR	Peroxisome proliferator-activated receptor
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PKC	Protein kinase C θ
PLIN	Perilipin
PI3K	Phosphatidylinositol-3-kiase
ROS	Reactive oxygen species
SS	Subsarcolemmal region
T2D	Type 2 diabetics
TEM	Transmission electron microscopy

1.0 Literature Review

1.1 Brief introduction

Intramyocellular lipids (IMCL) are elevated in obesity and type 2 diabetes and are related to insulin resistance (IR); however, IMCL are also elevated in athletes who are insulin sensitive[1]. The physiological difference between the IMCL stored in trained versus insulin resistant muscle is unknown, but recent investigations suggest that it is not IMCL *per se*, but IMCL storage location that are related to IR[2]. Therefore, how and where IMCL are stored within skeletal muscle may be a better indicator of the physiological/pathological role of IMCL. Endurance training improves insulin sensitivity (IS) and fat oxidative capacity and induces a redistribution of IMCL from the subsarcolemmal (SS) to the intermyofibrillar (IMF) region of the muscle fibre[2,3]. Higher IMCL stored within the SS region of the muscle fibre is related to IR[2,4], thus a shift in IMCL storage from the SS to the IMF region may represent a shift towards available fuel to support exercising muscle metabolism. Muscle disuse is the antithesis of endurance training and induces IR in young, healthy individuals[5,6]. Short-term muscle disuse does not influence total IMCL content[7], yet currently there has been no investigation into how disuse influences subcellular localization of IMCL and whether this may contribute to IR. Disuse also decreases mitochondrial content and function, which can also contribute to IR[7–10]. Omega-3 fatty acids (FA) have been shown to attenuate the loss of muscle mass during immobilization[11–13], mainly due to its effects on muscle protein synthesis[12,13], but also perhaps due to its effect on maintaining insulin signalling[11]. Therefore, the purpose of this thesis was to examine how disuse and omega-3 FA supplementation influences IMCL and mitochondrial characteristics and its relationship to insulin signalling.

1.2 Intramyocellular lipids

1.2.1 Intramyocellular lipid synthesis and breakdown

Fat is stored in skeletal muscle in lipid droplets as IMCL. Typically, the lipids are stored in the droplet in a neutral form as intramuscular triacylglycerides (IMTG) that can be hydrolyzed to release free FA for oxidation as a fuel source or to act as a secondary messengers within the cell[14,15]. IMTG are composed of three fatty acyl CoA attached to a glycerol backbone. The glycerol backbone originates from glyercol-3-phosphate from glycolysis and is esterified with 2 fatty acyl CoA to produce phosphatidic acid; both esterifications are catalyzed by glycerol-3-phosphate acyltransferase (GPAT)[16]. The phosphate group gets removed to make a diacylglycerol (DAG) onto which a third fatty acyl CoA is esterified by diacylglycerol acyltransferase (DGAT), as seen in Figure 1[16]. These lipids are then stored in lipid droplets, which consists of a monolayer of phospholipids that encapsulate the lipids within the droplet[15]. The primary isoforms of GPAT and DGAT in skeletal muscle are located on the endoplasmic reticulum where lipid droplets are believed to be first formed[14]. However, little is understood as to how they expand once established[14].

For these FAs to be used as an energy source, the FAs of IMTG must be removed from the glycerol backbone in order to enter the mitochondria for β oxidation, as outlined in Figure 1[17]. Adipose triglyceride lipase (ATGL) preferentially removes the first FA from an IMTG molecule to form a DAG molecule. Hormone sensitive lipase (HSL) preferentially removes the second FA to form a monoacylglycerol molecule. Finally, monoacylglycerol lipase preferentially removes the final FA to release a free FA and a glycerol molecule[17]. From here, the fatty acyl CoAs are able to be transported into the mitochondria for oxidation[17].

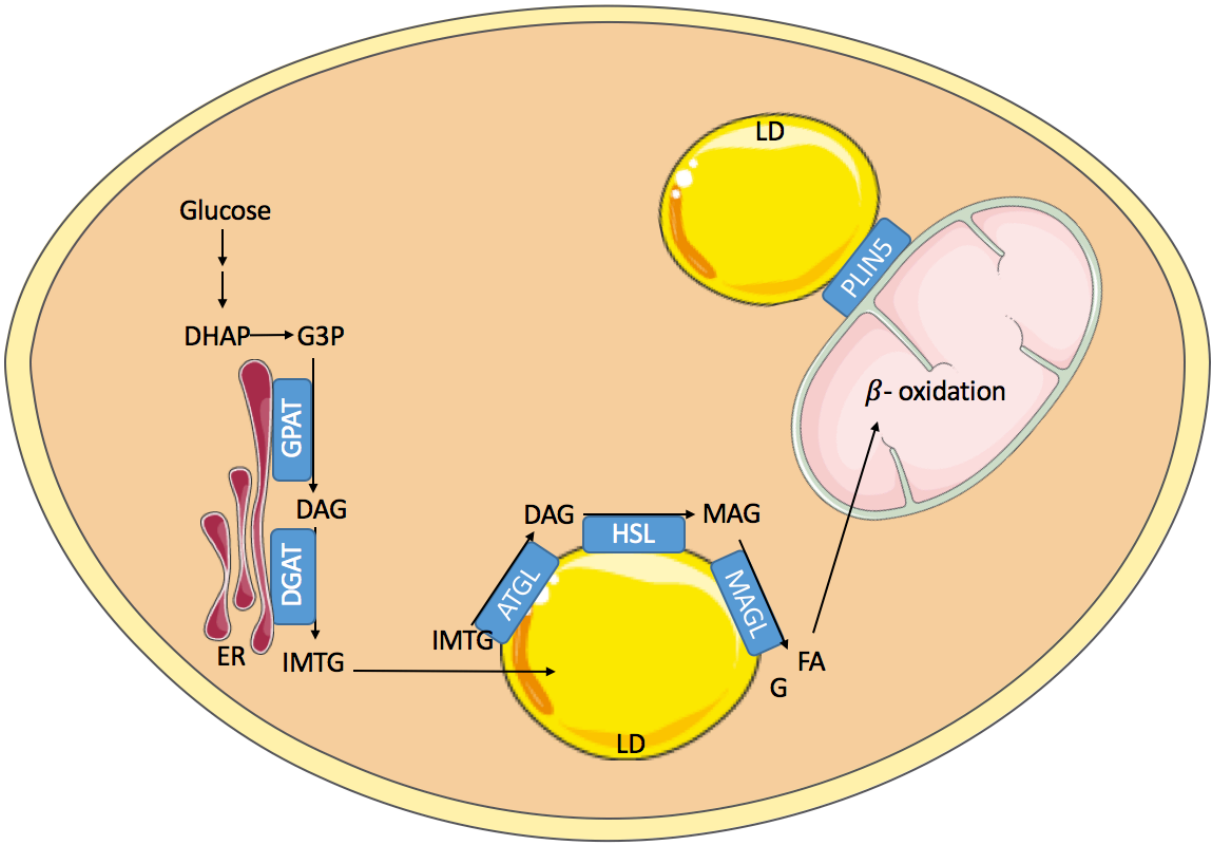


Figure 1: IMTG synthesis and degradation and the co-localization of PLIN5 on lipid droplets and mitochondria adapted from Watt *et al*[17]. DHAP: dihydroxyacetone phosphate. G3P: glycerol-3-phosphate. GPAT: glycerol-3-phosphate acyltransferase. DAG: Diacylglycerol. SR: sarcoplasmic reticulum. DGAT: diacylglycerol acyltransferase. IMTG: intramuscular triacylglycerol. ATGL: adipose triacylglycerol lipase. HSL: hormone sensitive lipase. LD: lipid droplet. MAGL: Monoacylglycerol lipase. G: glycerol. FA: fatty acyl CoA. PLIN5: perilipin 5. Mito: mitochondria.

1.2.2 Perilipin proteins

Originally, lipid droplets were believed to be stagnant storage facilities, but it is now known that they are highly metabolically active[18]. Amongst the monolayer of phospholipids are a family of proteins called perilipins that help dictate the metabolism of the lipid droplets[19,20]. There are three isoforms within skeletal muscle, all of which have been colocalized to lipid droplets: perilipin 2 (PLIN2), perilipin 3 (PLIN3), and perilipin 5 (PLIN5)[20,21]. PLIN2 and PLIN3 are ubiquitously expressed while PLIN5 is found in oxidative tissue[21]. All three proteins

have been shown to co-immunoprecipitate with ATGL and HSL and through these interactions mediate the metabolism of the lipid droplets[22].

PLIN2 overexpression increases IMCL content and favours a lipogenic gene expression[23]. It plays an important role in facilitating the storage of lipids in lipid droplets, specifically IMTGs[20]. Concurrently, PLIN2 decreases the association of ATGL with lipid droplets thus resulting in an accumulation of IMTGs[24]. Null PLIN2 mice have fewer, but larger lipid droplets and higher lipolysis[20,25]. This suggests that PLIN2 influences the stability of the lipid droplet while also acting as a regulator of their metabolism[20].

PLIN3 is the least studied of the PLIN proteins and therefore the least understood[26]. PLIN3 is commonly associated with smaller lipid droplets, but has been implicated in promoting lipolysis[27,28]. This is supported by its interaction with ATGL and HSL and its presence on the mitochondria[22,29]. In overfed mice, PLIN3 content dropped along with a decrease in smaller lipid droplets and an increase in larger lipid droplets[30].

In accordance with its anatomical location, PLIN5 is believed to play an important role in mediating lipolysis and oxidative control of lipid droplets[31]. PLIN5 localizes on both mitochondria and lipid droplets and its protein content has been highly correlated to oxidative phosphorylation protein content[32,33]. Using electron microscopy, PLIN5 has been found at the interface between lipid droplets and mitochondria, as seen in Figure 1[32]. Conversely, PLIN5 overexpression in the skeletal muscle of rats increases IMCL accumulation and lipid droplet size while promoting lipid droplet-mitochondria interaction[32]. This could be because under basal conditions, PLIN5 inhibits ATGL, but upon protein kinase A stimulation, such as that from exercise, PLIN5 stimulates lipolysis[33,34]. PLIN5 overexpression also increases the rate of fat oxidation in rats without changing mitochondria density, presumably due to an increase in the

efficiency of lipid transport rather than increasing oxidative capacity[23,32]. It has been suggested that PLIN5 couples IMTG degradation to oxidation by colocalizing IMCL to the mitochondria[32,33]. Overall, PLIN2 favours a lipogenic environment, PLIN3 favours a lipolytic environment, and PLIN5 can favour either scenario.

1.2.3 Factors influencing intramyocellular lipid and mitochondrial content and storage characteristics

1.2.3.1 Subcellular localization of intramyocellular lipids

There are two areas within the myofibre in which IMCL are located: the SS region and the IMF region. The SS region is located beneath the sarcolemma; it contains typical components of the cell, such as the nucleus, sarcoplasmic reticulum, and mitochondria, and is the primary location of signal transduction within the cell[35]. The IMF region of a myofibre contains the contractile proteins of the sarcomere from which contractile force is generated. Lipid droplets in the SS region are more commonly shown to have detrimental effects on IS[2,4,35] while lipid droplets in the IMF region are more often positively associated with IS[36]. However, the association between SS IMCL storage and IR has only been observed in men[2,4], not women[3].

1.2.3.2 Fibre type differences in intramyocellular lipid storage

The subcellular localization and total myocyte content of IMCL can be influenced by the fibre type of individual myofibres as their oxidative capacity greatly influences their preferential fuel source and thus the storage amount and localization of IMCL[36]. There are three types of muscle fibres in humans: type I, which has the highest oxidative capacity; type IIA, which more heavily relies on glycolytic fuel; and type IIX, which has the highest glycolytic power[37]. Type I

fibres are more highly recruited during aerobic activity while type II fibres are more highly recruited during anaerobic activity in agreement with their predominant fuel source. Type I fibres have a higher number of IMCL in either subcellular compartment than type II fibres[36]. The lipid droplet size in the SS region of type II fibres are 20% larger than those seen in type I fibres, potentially corresponding to a reduced oxidative capacity[36]. The content of IMCL is 1.5-fold higher in the IMF region of type I fibres than type II fibres in healthy, untrained men with no significant difference in the SS IMCL content, potentially corresponding to the higher reliance on fat metabolism in type I fibres[36]. Storage of larger IMCL in the SS region of type II muscle fibres has been associated with IR[4].

1.2.3.3 Effect of sex on intramyocellular lipid storage and mitochondrial content

IMCL storage characteristics and accumulation are influenced by sex[38–40]. Women have a higher area density of IMCL within their skeletal muscle than men, which is due to a higher number of IMCL, not because their IMCL are larger[40,41]. Women are also better protected against lipid infusion-induced insulin resistance than men[42], suggesting that women are better able to appropriately deal with increased lipid exposure. A potential mechanism by which women are better able to deal with increased lipid exposure is due to the fact that women have a greater capacity to oxidize lipids as evidenced by greater fat utilization during exercise[41,43], as well as, greater expression/protein content/activity of enzymes related to muscle fatty acid uptake, IMCL synthesis and breakdown, fatty acid entry into the mitochondria and beta-oxidation[41,44]. Furthermore, an acute bout of endurance exercise increases IMCL-mitochondria apposition in women, but not men[40], which is suggestive of a greater linkage between IMCL breakdown and fatty acid oxidation; however, women are not seen to rely more heavily on IMCL

during endurance exercise[40]. Given the difference in IMCL storage characteristics between the sexes and the difference in fat metabolism between the sexes, it is important to include women in trials as their response to various stimuli may differ from that of men.

1.2.3.4 Effect of age on intramyocellular lipid storage and mitochondrial content

Age also influences IMCL content with older individuals having a higher IMCL content than young[38,39]. The increase in IMCL content that occurs with age occurs at the same time as the development of IR and type II diabetes (T2D)[38]. Both older men and women have larger lipid droplets in the SS region than their younger counterparts[38], but women have a higher number of IMCL in their SS region than men in both young and old population[38]. Older women also have more IMCL touching mitochondria than older men, though this difference between the sexes was not seen in the younger population[38,40]. Younger individuals have a higher number and area density of mitochondria in both the SS region and the entire muscle fibre compared to older individuals, but there is no effect of age on mitochondrial size[38]. The higher number and increased apposition of IMCL to mitochondria in older women suggests that their IMCL are organized more favourably metabolically than older men. Older women are at a lower risk than older men for T2D[45] and IMCL accumulation has been linked to IR[1,46], as such perhaps the favourable storage characteristics of IMCL by older women contributes to their protection from T2D compared to older men.

1.2.3.5 Effect of obesity and type 2 diabetes on intramyocellular storage characteristics and mitochondrial content

IMCL accumulation is negatively associated with IR[1,2]. Obese individuals and those with T2D, both of which are conditions characterized by IR, have a higher IMCL content than lean

individuals[1,4,47]. T2D have more IMCL stored within skeletal muscle compared to lean individuals, regardless of the fibre type, which is due to the increased size of their IMCL[4]. Furthermore, T2D preferentially store their IMCL in the SS region of their muscle fibres as compared to lean, insulin sensitive individuals[4]. Obese individuals and T2D also have smaller mitochondria and reduced mitochondrial content[48], indicative of a lower oxidative capacity. Therefore, there are distinct IMCL and mitochondrial characteristics commonly associated with the IR state.

1.2.3.6 Effect of endurance training and sex on IMCL storage characteristics

Endurance training changes IMCL storage characteristics in a sex-dependent manner. Endurance training reduces IMCL area density in SS region by reducing IMCL size and number in men[2] and by reducing IMCL number in women[3]. The decrease in SS IMCL is positively correlated to improved IS in men[2], but no such relationship is found in women[3]. As SS IMCL decreases, there is a concomitant increase in IMCL area density in the IMF region in both men and women. For men, the increase in IMCL is due to an increase in IMCL number[2], while in women there is an increase in IMCL size[3]. Therefore, endurance training redistributes IMCL in both men and women from the SS to the IMF region, yet the morphological changes in IMCL induced by endurance training differ between the sexes. As such, these findings suggest there are sex specific differences in IMCL regulation.

Endurance training also increases mitochondria populations, which allows for greater fat oxidation[2,3,35,41]. The increased capacity for fat oxidation with endurance training corresponds with the increase in mitochondria size and area density, with both increases being greater in the IMF than the SS region[2,3,35,41]. Endurance training also increases IMCL-mitochondrial

apposition, which corresponds with the increased β-oxidation capacity induced by endurance training[2,3,41]. The increases in mitochondrial content, function and apposition with mitochondria induced by endurance training are not different between the sexes[41], however, IMCL-mitochondrial apposition is increased in women, but not men following an acute bout of exercise[40]. IMCL-mitochondrial apposition is thought to be mediated by PLIN5[32,33]. Endurance training has been found to increase PLIN5 content in both men and women, however, PLIN5 is higher in women than men[31]. These findings are further indicative of a greater capacity for women to oxidize lipid.

1.2.4 Intramyocellular lipid characteristics in relation to insulin resistance

1.2.4.1 Athlete's paradox

The responsiveness of skeletal muscle to insulin is a measure of skeletal muscle health. Ectopic accumulation of IMCL in skeletal muscle has been positively correlated to IR[18,49]. IMCL content is elevated in individuals who are obese or are T2D and the increased IMCL content is related to the extent of IR[15]. However, endurance athletes also have high concentrations of IMCL while exhibiting high sensitivity to insulin[1]. The term 'athlete's paradox' is used to describe the similarly high concentrations of IMCL found in athletes and obese/T2D, despite disparate levels of IS[1]. IMCL levels in endurance athletes are similar to those seen in T2D, which is 2-fold higher than the levels in lean, sedentary individuals[1]. Removing endurance athletes from the evaluation, there is an inverse relationship between IMCL content and IS for lean, obese, and T2D individuals[1]. Thus, there must be differences in how IMCL is stored within the myocyte in an athlete as compared to that stored in an obese/T2D individual. In fact, while endurance athletes and T2D have a similar volume of IMCL in the IMF region, T2D have a 3-fold greater IMCL

area density in the SS region as compared with endurance athletes[35]. Furthermore, trained athletes predominantly store their IMCL in small lipid droplets in the IMF region of type I fibres, while T2Ds predominantly store their IMCL in large lipid droplets in the SS region of type II fibres[4], as seen in Figure 2. With training, IMCL populations in T2D begin to develop lipid distribution patterns seen in athletes[4]. Furthermore, endurance training in lean and obese men can induce a shift in IMCL storage away from the SS and into the IMF region, which corresponds to an improvement in IS[2]. Therefore, it appears as though the relationship between IMCL and IS is influenced by the size, number, and location of IMCL within skeletal muscle and these characteristics can be modified by exercise to promote IS[1,18].

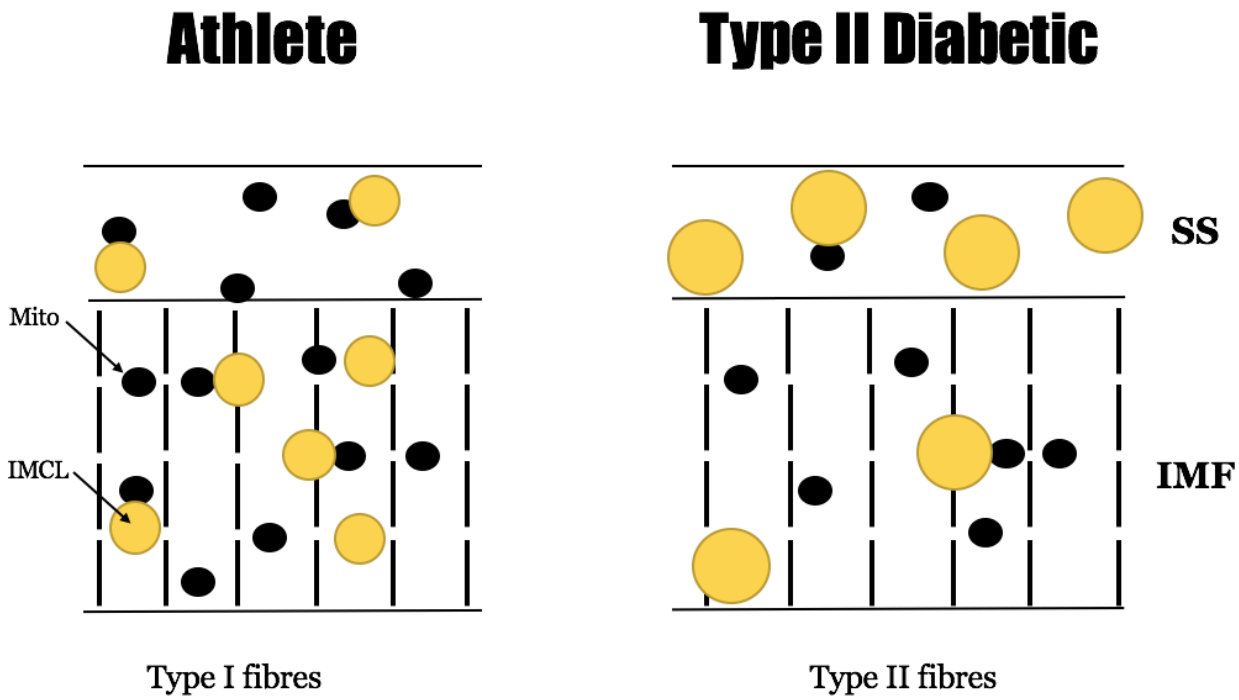


Figure 2: IMCL characteristics and distribution in skeletal muscle of an endurance athlete and an individual with type II diabetes. Black circles represent mitochondria. Yellow circles represent IMCL. Vertical black lines represent Z lines. The horizontal black lines separate the parts of the cell. Mito: mitochondria. IMCL: intramyocellular lipid. SS: subsarcolemmal region. IMF: intermyofibrillar region.

1.2.4.2 Relationship between intramyocellular lipid size and intermyofibrillar storage location and insulin resistance

As discussed above, storage of IMCL in the IMF region appears to represent a site of readily available fuel for muscle metabolic needs. However, not all IMF IMCL are created equal as the number and size of lipid droplets within the IMF vary between individuals. A prospective study that evaluated the effect of IMCL accumulation during overfeeding on IR found that individuals who maintained more, smaller lipid droplets in the peripheral IMF region of the myocyte maintained greater IS than those who stored larger lipid droplets, suggesting that lipid droplet size and location, not overall IMCL content, play an important role in the development of IR[30]. Smaller lipid droplets have a higher surface area to volume ratio that is believed to be advantageous for higher FA oxidation[36]. Furthermore, large lipid droplets have impaired lipolysis[50]. The overfeeding protocol led to a significant reduction in the content of smaller sized lipid droplets around the periphery of the myocyte with a trend towards a reduction in the whole fibre ($p = 0.07$) and central IMF ($p = 0.09$) in young, healthy males[30]. Higher baseline physical activity protected against a loss of small peripherally located lipid droplets and smaller, peripherally located lipid droplets are also associated with higher whole body lipid oxidation[30]. Furthermore, highly trained endurance athletes have significantly higher IMCL content near the periphery of the myocyte compared to T2D, and weight matched sedentary individuals, though only in type I fibres[51]. Unfortunately, these trials did not use methods that were sensitive enough to differentiate between the peripheral IMF and the SS region of the myocyte (immunohistochemistry; oil red O staining)[30,51]. The peripheral IMF has the highest mitochondrial content and mitochondrial content increases with exercise, even in a T2D population[35]. Taken together with the positive association between IS and IMCL content in the IMF periphery, these finding suggest that peripheral IMF IMCL may be the least related to IR[4].

These findings agree with the IMCL distribution seen in athletes compared to obese individuals[4]. The higher mitochondrial content in the periphery of the myocyte is supportive of the hypothesis that peripheral IMCL are stored as readily available fuel to support energy needs and are not related to IR, whereas central IMCL are a site of storage during lipid oversupply and are related to IR[4].

1.2.4.3 Muscle fibre type storage of intramyocellular lipids is related to insulin resistance

IMCL area density, size, and number have all been negatively correlated with IS in the SS region in type II fibres, while in type I fibres only lipid droplet size is correlated negatively with IS[4], indicating that subcellular localization is necessary when evaluating IMCL characteristics between fibre types. Similarly, only IMCL area density and number, but not size, has been positively correlated to IS in the IMF region of type I fibres[4]. Together, the higher number and smaller size of the lipid droplets in type I compared to type II fibres may be indicative of its higher FA oxidative capacity[36] and may represent the optimal form of storing IMCL for oxidative purposes. The mechanistic link between IMCL stored in the SS and central IMF region and insulin resistance remains elusive, but may be related to the composition of the lipid and/or an influence of the lipids on muscle signalling pathways[1,52,53].

1.2.4.4 Influence of lipid intermediates on insulin sensitivity

As detailed above, endurance athletes and obese individuals have similar concentrations of IMCL in their skeletal muscle[1]. However, obese individuals and endurance athletes do not have the same lipid species in their muscles. Obese individuals have more DAG and ceramides than athletes, which have been shown to blunt insulin signalling[52–54].

DAG can attenuate insulin signalling by activating protein kinase C θ, which in turn phosphorylates insulin substrate receptor 1 (IRS1) at Ser 307, which prevents its interaction with the insulin receptor and blunts its downstream signalling pathway, as seen in Figure 3[52,53,55]. Despite this known association of DAG and IR, endurance athletes have double the DAG content as obese individuals and 50% more than lean individuals[56]. Similar to IMCL, subcellular localization is believed to influence DAG metabolism[57,58]. There is a significant inverse relationship between the percentage of total DAG localized to the membrane and IS while cytosolic DAG has no relationship to IS[57]. Di-saturated membrane DAG is also negatively associated with IR and is present in endurance athletes at lower concentrations than lean individuals, possibly because athletes have a higher protein concentration of stearoyl desaturase 1[56,57,59]. Membrane DAG is also related to protein kinase C θ activation while cytosolic DAG is inversely related to protein kinase C θ activation, suggesting the compartmentalization of DAG species affects its regulation[53,57]. Recent data has affirmed the need to evaluate DAG subcellular localization rather than total content to truly elucidate its relationship with IR[60].

Another prominent lipid intermediate with negative implications on IS is ceramide[52,53]. Ceramide content is higher in obese and IR individuals compared to sedentary or endurance athletes[46,56]. It can alter the signalling cascade for phosphatidylinositol-3-kinase (PI3K) by preventing the translocation and recruitment of protein kinase B (AKT) to the membrane, as seen in Figure 3[54]. Ceramide content is lower in endurance trained athletes[56] and decreases with exercise[46,61,62].

Athletes also have a higher IMTG fractional synthesis rate[58,59], which is suggested to protect against FA-induced lipotoxicity[63]. IMTG fractional synthesis has been positively related to IS and has an inverse relationship with specific, IR-related DAG and ceramide species[58,59].

Exercise can increase IMTG synthesis rates across several populations, including obese, T2D, and endurance athletes[58]. Exercise can also lead to a reduction in total DAG and ceramide content[46], as well as a shift in subcellular DAG populations that has been positively correlated with IS[58].

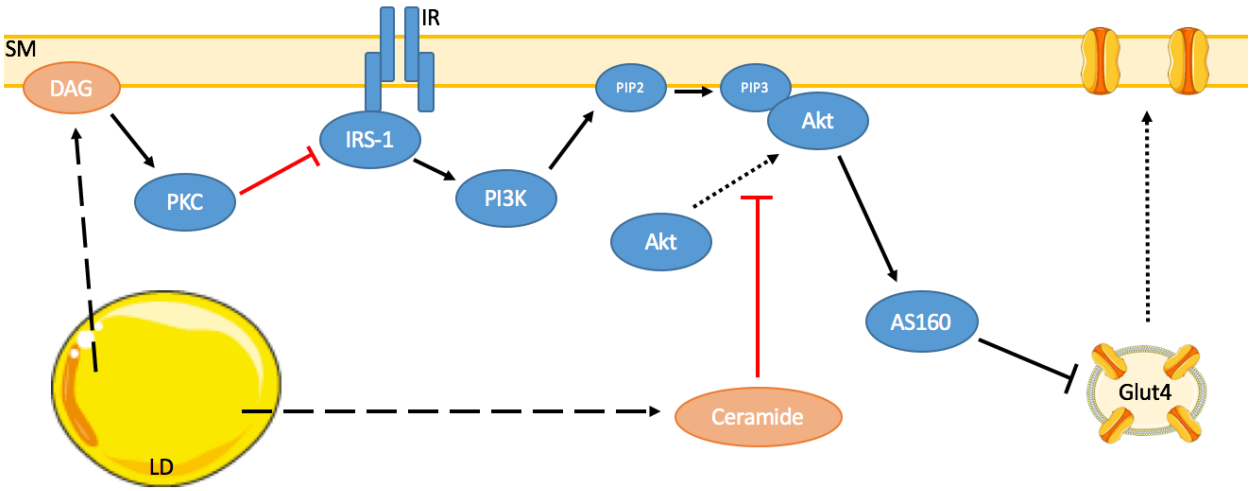


Figure 3: The insulin signalling pathway to induce GLUT4 translocation to the membrane and the locations at which lipid intermediates inhibit this adapted from Ritter *et al*[64]. Black arrows mean activation. Black lines mean inhibition. Red lines mean lipid intermediate inhibition. Dotted lines mean translocation. Dashed lines means source of the molecule. SM: Sarcolemmal membrane DAG: diacylglycerol. PKC: Protein kinase C θ. LD: Lipid droplet. IRS-1: insulin receptor substrate 1. IR: insulin receptor. PI3K: Phosphoinositol 3-kinase. PIP2: phosphatidylinositol 4,5-bisphosphate. PIP3: phosphatidylinositol 3,4,5-triphosphate. Akt: protein kinase B. AS160: Akt substrate 160. Glut4: glucose transporter 4.

1.2.4.5 Influence of perilipin proteins on insulin signalling

Perilipin proteins mediate the metabolism of lipid droplets, which can influence the accumulation or removal of lipid intermediates. PLINs also influence IS and can be influenced by exercise. As detailed earlier, PLIN2, PLIN3, and PLIN5 have all been shown to influence the metabolism of IMCL, yet different PLINs have opposing actions on IS. PLIN2 has been shown to both increase and decrease IS[20,25,65]. PLIN2 overexpression in rats leads to an increase in

IMTG content in skeletal muscle, but no change in DAG content, while improving IS[20]. Several other studies in mice have shown both positive[65] and negative[25] effects on IS when PLIN2 was knocked down in hepatocytes. Due to the ubiquitous nature of PLIN2, its deletion has been shown to lead to adaption in other PLINs, which influences its perceived impact on IS[66]. One study removed the compensatory PLIN3 upregulation that occurs when PLIN2 is knocked out and found IS was hindered[25], while another study allowed the compensatory PLIN3 upregulation and found that IS improved[65]. Meanwhile, in a recent human study, 8-weeks of overfeeding in healthy, young men decreased PLIN3 content and tended to decrease IS. This was accompanied by no change in PLIN2 content and a reduction in small lipid droplets[30], suggesting that PLIN3 positively impacts IS while the relationship between PLIN2 and IS remains unclear.

As PLIN5 can either inhibit or promote lipolysis, its influence on IS is also of interest. After a 60 hr fast in normoglycemic young men, PLIN5 redistributed from the cytosol to the lipid droplet surface[67]. This was accompanied by an increase in lipid droplet size and number, both of which occurred exclusively in lipid droplets coated with PLIN5. Furthermore, the participants who had the greatest increase in IMCL area density had the smallest reduction in IS[67]. Increased IMCL area density also correlated with blunted fasting-induced IR and mitochondria dysfunction, which may indicate a role for PLIN5 in mediating lipotoxicity[67]. Overall, while the role of the PLINs in regulating IS remains unresolved, it appears as though PLIN2 may improve IS by sequestering lipid intermediates, PLIN3 may enhance IS, and PLIN5, which favours lipolysis but can permit lipogenesis, may increase IS by sequestering IMCL at rest and promoting IMCL lipolysis during exercise.

1.3 Skeletal muscle disuse

There are various circumstances in an individual's life that may result in limb immobilization or bed rest, such as an injury, surgery, or prolonged illness. During these situations, there is a significant if not total elimination of movement, which induces unfavourable molecular and metabolic changes within skeletal muscle including the loss of muscle mass, lipid accumulation, mitochondrial dysfunction, and the onset of IR[6,8–10,68,69]. Skeletal muscle disuse is the antithesis to endurance training and therefore induces opposite effects on skeletal muscle as endurance training.

1.3.1 Insulin resistance during skeletal muscle disuse

In an insulin sensitive cell, insulin binds to the insulin receptor on the sarcolemma, initiating its tyrosine kinase activity to phosphorylate itself and its substrate, IRS1[53]. From here, IRS1 phosphorylates PI3K, from which a cascade of protein activations and deactivations results in numerous, varied effects within the muscle[53]. One such pathway leads to the phosphorylation and deactivation of AKT substrate 160 (AS160) by AKT, whose inactivation allows for the translocation of glucose transporter 4 (GLUT4) to the sarcolemmal membrane to bring glucose into the cell, as seen in Figure 3[53]. AS160 can also be phosphorylated by 5' adenosine monophosphate-activated protein kinase (AMPK) to promote GLUT4 translocation[70]. To further promote glucose uptake and utilization, AMPK phosphorylates, thereby inactivating, acetyl CoA carboxylase (ACC), which when active promotes FA synthesis[70]. As such, endurance training increases GLUT4 content and translocation to the membrane during and in the hours following exercise in skeletal muscle[71]. Endurance training also acutely increases the phosphorylation of

AS160, in accordance with the increased GLUT4 translocation[72,73]. However, interference in the signalling cascade can result in IR.

Skeletal muscle is responsible for 85% of glucose disposal within the body[74]. Limb immobilization, bed rest, and reduced step counts have been shown to reduce whole body IS, regardless of the mobility of the rest of the body[6,9,69,75]. IR has been positively correlated with reduced muscle mass and strength[76,77], yet changes in IS precede changes in body composition[5] such that the 3% loss of lean muscle mass seen after one week of bed rest cannot fully explain the 30% reduction in whole body IS[7]. In fact, a reduction in step count by ~80% can reduce whole body IS by 20% in 7 days[6,78,79]. This emphasizes the need for even basal activity to maintain IS. There are several structural and metabolic changes that occur during skeletal muscle disuse that invokes glucose intolerance to promote IR[7,8,80,81].

One of the primary actions of insulin on skeletal muscle is to increase glucose uptake[74]. As stated earlier, insulin induces a cascade of signalling that results in the translocation of GLUT4 to the sarcolemmal membrane[82]. A single day of inactivity can reduce glucose uptake by 39%[80] and one week of bed rest decreases whole body IS by 29%[7]. Bed rest leads to decreased GLUT4 content and therefore reduced glucose uptake and glycogen formation, as seen in Figure 4A[83–85]. Two weeks of immobilization has also been shown to reduce hexokinase content[85], AKT content, the upstream regulator of GLUT4 translocation[85], and reduce the activity of glycogen synthase[10,85,86]. Hexokinase converts glucose into glucose-6-phosphate, which is then converted to glycogen via glycogen synthase[87]. With a reduction in hexokinase and glycogen synthase activity, glucose content remains high in the cell, disrupting the concentration gradient between the blood and the muscle, reducing glucose uptake[87]. Taken together, these findings indicate that glucose metabolism is hindered at several steps with disuse leading to IR.

1.3.2 The effects of disuse on perilipin proteins

PLIN2 and PLIN5 are the most abundant PLIN proteins in skeletal muscle[23] and are therefore the most commonly studied. PLIN2 promotes the accumulation of IMCL and PLIN2 content increases with age[88,89]. In an older population, there is a significant increase in PLIN2 content with reduce mobility[10,89], which has a strong positive correlation with muscle atrophy proteins[89]. Even in a young population, PLIN2 content increases with reduced mobility[88], though this is not always seen[10]. These findings suggest that PLIN2 is involved in regulating the accumulation of ectopic lipids during disuse. Interestingly, PLIN2 content increases 3-fold with endurance training in obese, but not lean women[31], suggesting that adiposity may regulate changes in PLIN2. As PLIN2 specifically promotes the accumulation of IMTGs in lipid droplets and obese individuals tend to have higher concentrations of DAGs and ceramides, perhaps PLIN2 content increases in obese women as a mechanism by which to remove the lipid intermediates and may represent a potential mechanism by which endurance training increases IS. Therefore, PLIN2 content can increase as a result of two opposing stimuli that induce differential effects on IS. As higher PLIN2 content can result in both positive and negative effects on IS, the relationship between PLIN2 and IS is unclear and requires further investigation.

PLIN5 is commonly associated with lipolysis due to its adjacency to both IMCL and mitochondria[31–33]. Therefore, it would be expected that as disuse induces lipid accumulation[10,86,90], PLIN5 content would decrease. However, when not stimulated by exercise, PLIN5 can also support IMCL accumulation[32], thus during a period of disuse it is possible that PLIN5 could increase in order to allow for greater lipid synthesis. In young men, reduced mobility has no effect on PLIN5 content and in older men, PLIN5 content has been shown to

remain stable[10] or decrease with reduced mobility[10,89]. Since endurance training increases IMCL-mitochondrial apposition, it could be speculated that disuse would decrease the apposition of IMCL with mitochondria. Decreased IMCL-mitochondria apposition would support the reduced fat oxidation seen with disuse and would agree with the reduced PLIN5 content and may contribute to disuse-induced IR. PLIN5 overexpression has been shown to promote neutral IMCL accumulation in response to a high fat diet without compromising IS in rats[23], which is supportive of a role for PLIN5 to mediate lipid-induced IR. However, despite the implications of PLIN5 content on IS, no relationship has been found between PLIN5 content and IS, therefore any benefit of PLIN5 on IS appears to be indirect[91]. However, the current discrepancies in the literature suggest that further investigation is needed to understand the effect of disuse on PLIN5 content.

1.3.3 The effect of disuse on intramyocellular lipids

The effects of disuse on total IMCL content are conflicting, perhaps due to differences in the length of the disuse intervention. A recent study found that one week of bed rest induces significant whole body IR without the accumulation of IMCL[7]; however, an examination of subcellular IMCL localization was not performed. Alternatively, during longer periods of immobilization (> 2 weeks), there is an accumulation of IMCL within skeletal muscle along with the development of IR[10,86,90], but once again no examination of the subcellular localization was conducted. Furthermore, no study has examined the effect of disuse on IMCL size, another IMCL characteristic that has been shown to be related to IR. Since endurance training can alter IMCL storage characteristics and higher IMCL size and storage in the SS region of the myocyte are associated with IR, examining total IMCL content may be missing the critical effects of

disuse on IMCL in relation to IR. Using electron microscopy allows for the investigation of changes in IMCL subcellular localization and individual lipid characteristics induced by disuse that precede IMCL accumulation. Using electron microscopy we may be able to determine the critical effects of disuse on IMCL in relation to IR.

The accumulation of lipids seen in longer periods of disuse also suggests that there are increases in other lipid species[10,86,90]. Twenty-eight days of bed rest does not change total DAG content in skeletal muscle[92], but 7 days of bed rest has been shown to change specific DAG and ceramide species[7]. These findings suggest, in an opposing action to exercise, disuse can induce changes in specific populations of lipid intermediates that could blunt insulin signalling and result in IR. Therefore, the insulin signalling cascade could illuminate the points of intersection between disuse, IR, and IMCL metabolism.

1.3.4 Disuse decreases mitochondrial content and function

Disuse leads to a decrease in mitochondrial content and function[7–10]. Mitochondrial content can be measured directly with electron microscopy or indirectly through biochemical markers that represent mitochondrial content (i.e citrate synthase (CS)). In as little as 5 days disuse can reduce mitochondrial content as evidenced by the reduced expression of *Cs* mRNA[8,93]; this is corroborated by a corresponding decrease in CS content[7]. These results are further supported by electron microscopy analyses showing a decrease in mitochondrial number, size, and area density with disuse[94,95]. The reduction in mitochondrial content of oxidative proteins suggests that immobilized muscle has a lower capacity for oxidative metabolism. Reduced mitochondrial content and function are found in T2D and contribute to the development

of IR[82,96]. Therefore, similar alterations in mitochondrial content and function induced by disuse may contribute to the development of disuse-induced IR.

1.3.5 Decrease in mitochondrial dynamics with disuse

Mitochondria are dynamic structures that constantly undergo fission and fusion to maintain mitochondrial health[97]. Mitochondrial fission is mediated by two proteins, dynamin-related protein 1 (DRP1) and fission 1 (FIS1), whereas mitochondrial fusion is mediated by two different proteins, mitofusin 1/2 (MFN1/2) and optic atrophy 1 (OPA1). MFN1/2 fuse the outer membranes of two conjoining mitochondria and OPA1 fuses the inner membranes[98,99]. Fission is the division of one mitochondria into two smaller mitochondria. FIS1 is a receptor on the outer membrane of mitochondria that recruits DRP1[99]. DRP1 surrounds the mitochondria like a band and constricts and separates the mitochondria into two[98,99]. In healthy cells, mitochondrial fusion is used to counteract the effect of reactive oxygen species (ROS) on mitochondrial DNA[99]. By fusing mitochondria, the presence of more mitochondrial DNA can combat gene product deficiencies that may limit or hinder mitochondrial function[97,99]. Fission can be used to remove dysfunctional portions of mitochondria to contain and remove the damaged mitochondria effectively and efficiently[97]. Fission is also important during cytokinesis to ensure that both cells have mitochondria upon division[99].

During disuse, mitochondria increase their ROS production[75,100]. ROS induce changes in mitochondria morphology via increased fission, as evidenced by increases in the expression of DRP1 and FIS1, as seen in Figure 4B[101,102]. Higher mitochondrial fission is associated with mitochondrial autophagy and increased muscle atrophy[94,102,103]. Mitochondria fission can upregulate forkhead box O3 (FOXO3) protein expression through AMPK, independent of AKT,

which in turn induces the expression of genes for protein ligases[104]. The pro-apoptotic factor BCL2 associated X protein (BAX) inhibits MFN2 activity and MFN2 overexpression inhibits BAX expression[105]. Similarly, OPA1 can protect against apoptosis by preventing the release of cytochrome c induced by BAX[106]. While fission and fusion are necessary for normal mitochondrial function, an imbalance between the two with a preference for fission has been found to have negative implications on cell function[104,107]. However, the results are less definitive in a disuse environment. During hindlimb suspension, some animal studies have found increased fission proteins (DRP1 and FIS1) and decreased fusion protein expression (MFN2)[104,107], while others have shown a decrease in both fusion (MFN1/2, OPA) and fission (DRP1 and FIS1) proteins[94,108,109]. In older adults, 10-days of bed rest did not change either fusion or fission protein content[110]. Therefore, the relationship between fusion and fission on mitochondrial function in an immobilization environment is unclear and requires further examination.

1.3.6 The effect of disuse on apoptosis-related signalling

Apoptosis is the programmed death of a cell. Apoptosis is commonly shown to be upregulated with disuse[111] and one family known to help regulate apoptotic signalling is the β-cell lymphoma family of proteins[111,112]. In healthy cells, apoptosis is tightly controlled. One such protein that helps control apoptotic signalling is β cell lymphocyte 2 (BCL2)[113,114]. BCL2 is an anti-apoptotic factor that binds the pro-apoptotic factor BAX. BAX binds itself or another protein BCL2 antagonist/killer 1 (BAK) to form an oligomer. BAX/BAK oligomers will create pores in the outer membrane of mitochondria, which releases cytochrome c into the cytoplasm[115,116]. Cytochrome c can induce caspase activity, which will degrade proteins and

fragment DNA, thereby causing apoptosis[103,115,117]. When bound to BCL2, BAX cannot oligomerize, which will prevent apoptosis[115]. The ratio of BCL2 to BAX represents the integrity of the outer mitochondrial membrane[103,107] therefore their values are often reported as a ratio rather than individual content. Disuse decreases the ratio of BCL2 to BAX[107,108,118] as a result of BCL2 decreasing and BAX increasing[107,118]. As BAX is an upstream regulator of apoptosis, lower ratio of BCL2 to BAX may represent an increase in apoptosis-related signalling and can contribute to the loss of muscle mass seen with disuse[107].

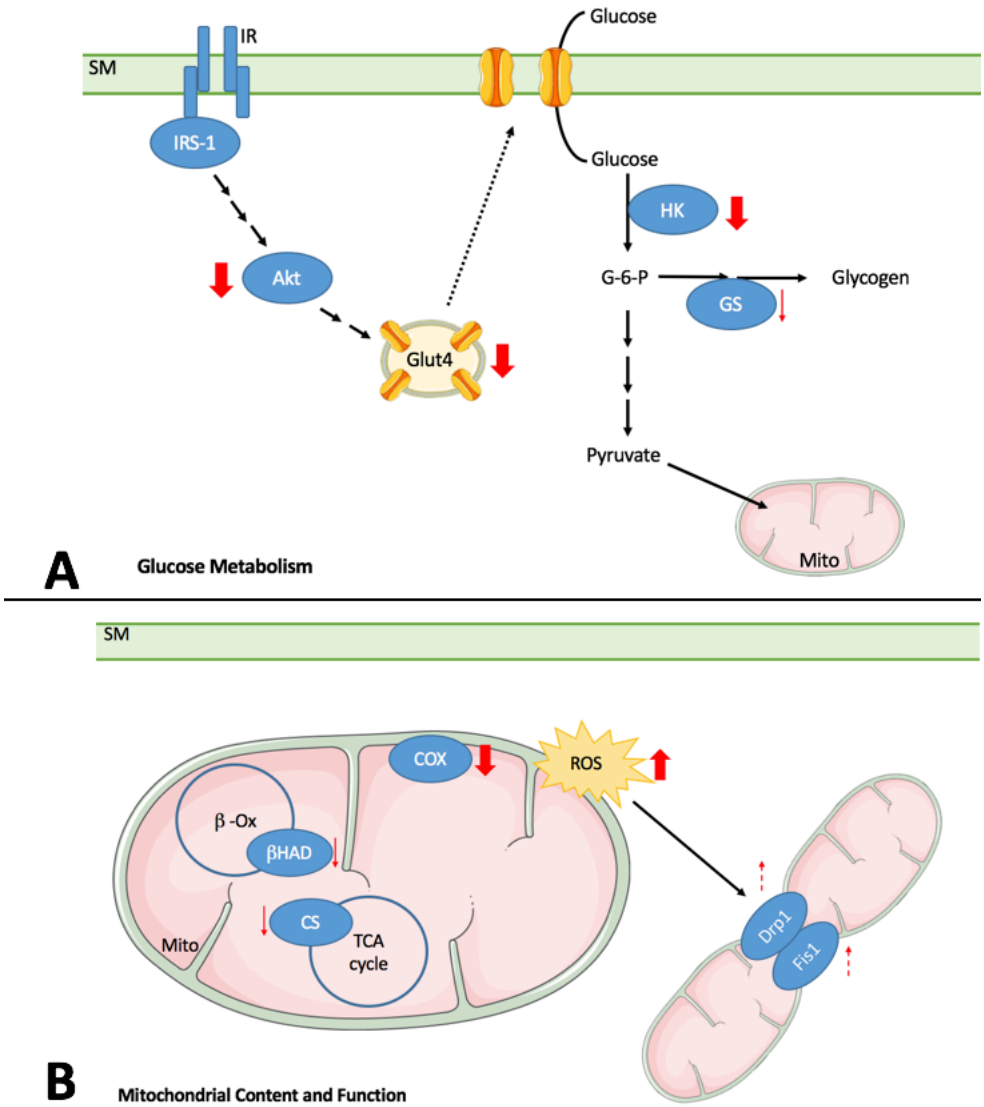


Figure 4: The effect of disuse on glucose metabolism (A) and mitochondrial content and function (B). Black lines mean transportation. Black arrows mean activation. Dotted black lines mean translocation. Red block arrows mean protein content. Red arrows mean enzymatic activity. Dashed red arrows mean gene expression. IR: Insulin receptor. IRS1: Insulin receptor substrate 1. Akt: Protein kinase B. Glut4: glucose transporter 4. HK: Hexokinase. G-6-P: glucose-6-phosphate. GS: Glycogen synthase. Mito: Mitochondria. β-Ox: β-oxidation. βHAD: β-hydroxyacyl CoA dehydrogenase. CS: Citrate synthase. TCA cycle: The citric acid cycle. COX: Cytochrome c oxidase proteins. ROS: Reactive oxygen species. DRP1: Dynamin-related protein 1. Fis1: Fission 1.

1.4 Omega-3 fatty acids

Omega-3 FA are polyunsaturated FA that are characterized by their first double bond at the third carbon from the methyl end of their hydrocarbon tail[119]. Humans are unable to synthesize omega-3 FA so they must be obtained from the diet[119]. One prominent source of omega-3 FA in the human diet is fish, who absorb them from the plankton and algae that they eat[119]. Omega-3 FA from fish is composed of two FA: eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). EPA and DHA have been shown to have positive impacts on the heart and brain, but recent studies suggest that it also positively impacts skeletal muscle by increasing muscle protein synthesis[12,13], muscle mass[120], and strength[121].

1.4.1 Omega-3 fatty acid supplementation impact on insulin sensitivity

The influence of omega-3 FA on IS is still debated. EPA increases basal glucose uptake by increasing *Glut1* expression in human myotubes[122]. A combined supplementation of EPA and DHA has been shown to increase *Glut4* and *Irs1* transcription in response to a high fat diet[123]; however, this is not always found[122,124]. Observational studies in humans support increased IS with omega-3 FA supplementation, yet several studies have failed to increase IS in T2D and obese individuals[125]. In a meta-analysis by Lalia *et al*. (2016), 10 out of 13 studies in non diabetic populations failed to improve IS across a range of omega-3 FA supplementation concentrations and durations ranging from 300 mg to 3.9 grams for 1 to 6 months, respectively[125]. Meanwhile in rats, omega-3 FA from fish oil normalized insulin action blunted by a high fat diet, which was highly correlated with the percentage of omega-3 FA in phospholipids[126]. In rats, EPA supplementation was shown to change the composition of phospholipids in the plasma membrane to prevent dietary-induced IR and increase glucose

infusion rate 3-fold[127]. The inconsistency between animal and human studies may be due to a preventative versus curative approach, respectively, and differential organismal responses to omega-3 FA supplementation[55,125]. The range of supplement duration, doses, and composition (EPA:DHA)nbetween human studies also makes it hard to draw comparisons[125].

1.4.2 Omega-3 fatty acid supplementation impact on intramyocellular lipid metabolism

Two studies involving protocols lasting 3 or 12-weeks found that omega-3 FA supplementation increased basal FA oxidation in young adults and older women[128,129]. Whole body resting metabolic rate was also increased, but only after 12-weeks of supplementation[128,129]. These findings suggest that perhaps IMCL content would decrease with omega-3 FA supplementation. This is supported by animal work which has shown that incubation of murine adipocyte cells with DHA can increase *Atgl*[130]. Furthermore, EPA and DHA also tend to increase *Hsl* in murine adipocytes, independently[130]. However, findings from a study using acute EPA exposure of human skeletal muscle cells suggest that EPA diverts FA to IMCL[122], but the effects of omega-3 FA supplementation on IMCL content remains unstudied.

Studies involving omega-3 FA supplementation have shown that EPA and DHA only minimally (~1%) get incorporated into IMCL in skeletal muscle; instead, EPA and DHA get incorporated into phospholipids or act as signalling molecules[131]. In human hepatoma cells and murine liver cells, phosphatidylcholine (PC) has been found to be the most abundant phospholipid class in lipid droplet monolayers followed by phosphatidylethanolamine (PE)[132,133]. Three months of omega-3 FA supplementation in men and women increased omega-3 incorporation into skeletal muscle phopholipids 2.5-fold[131]. Twelve weeks of EPA and DHA supplementation in men changed the profile of mitochondrial phospholipids by increasing the

incorporation of EPA and DHA into PC and PE species, while decreasing the presence of linoleic acid and arachidonic acid, two omega-6 FA[134]. The incorporation into phospholipids was accompanied by an increase in ADP sensitivity and submaximal oxidative activity[134]. Therefore, it may be possible that EPA and DHA are incorporated into the phospholipid classes also found in the lipid droplet monolayer and therefore alter lipid droplet protein functioning, as they do in the mitochondria[134]. With higher lipase content and activity, there is a potential for omega-3FA supplementation to prevent the accumulation of IMCL during disuse.

Omega-3 FA supplementation may also influence IMCL metabolism through cell signalling. Omega-3 FA are potent activators of transcription factors such as the peroxisome proliferator-activated receptor (PPAR) family[119]. PPAR α and PPARγ[135] control the expression of proteins involved in lipid metabolism[136]. During a high fat diet, supplementation with both DHA and EPA (~4:1) reduces lipogenic gene expression while increasing markers of FA oxidation and mitochondrial content in mice[137]. Supplementation also induces a significant increase in *Dgat1*, which could lead to increased protein content of DGAT1, the enzyme that catalyzes the addition of a free fatty acid to a DAG molecule to produce an IMTG[137]. Increases in IMTG synthesis have also been seen in human myotubes upon incubation with EPA[122]. While the relationship between DAG and disuse is unclear[92,138], IMTG synthesis has been positively linked to IS[59], so in a disuse model omega-3 FA induced upregulation of IMTG synthesis may prevent the accumulation of lipid intermediates and thereby positively influence IS[55]. Also, omega-3 FA supplementation reduces ceramide content in mice and improves IS[123]. Collectively, omega-3 FA supplementation could influence the phospholipid membrane of IMCL or cell signalling to mitigate the effects of disuse on skeletal muscle.

1.5 Techniques to assess intramyocellular lipid content

IMCL can be quantified using many different methods including biochemical techniques, histochemical methods, electron microscopy, and spectroscopy. Biochemical techniques indirectly measure IMCL content by measuring the glycerol content of a sample. Commonly, biochemical techniques are performed on whole muscle homogenate, which is a mix of both intramyocellular and extramyocellular lipid pools, therefore they often overestimate IMCL content[139]. As well, biochemical methods determine overall IMCL content, but give no indication as to where the IMCL are stored or their morphology, which can influence its relationship with IS[4].

Histochemical techniques use stains that target the neutral lipids found in the intracellular membranes of IMCL, which can then be visualized using fluorescence or bright field microscopy[140]. Two common stains for IMCL are Bodipy and oil red O. While both of these stains bind to the neutral lipids within IMCL, they also bind neutral lipids found in intracellular membranes[140]. Because the stains do not exclusively bind lipids in IMCL, it can be difficult to differentiate whether the fluorescent signal is from IMCL or other lipid populations within the cell[140]. One advantage of using histochemical IMCL analysis is that you can simultaneously stain for fibre type as well[139]. By staining for fibre type using myosin heavy chain antibodies, IMCL content, size, and localization can be determined based on fibre type. Fibre type influences IMCL storage and therefore provides valuable information for understanding the pathological and physiological storage of IMCL. However, histochemical techniques using traditional microscopy do not have the sensitivity to differentiate between the SS and the IMF regions, and these methods can only allow for determination of relative shifts of IMCL between the centre and periphery of the cell.

Electron microscopy is one of the most favourable techniques with which to analyze IMCL characteristics. Muscle samples are fixed in glutaraldehyde, embedded in resin from which very thin sections of sample are cut and stained with uranyl acetate and lead citrate to allow for visualization of muscle organelles and ultrastructure at very high magnifications using transmission electron microscopy (TEM). TEM is able to image samples with enough magnification and resolution to examine subcellular organelle organization and characteristics, including IMCL size and number, while differentiating between the SS region and the IMF regions. Unfortunately, TEM is not well suited to determining muscle fibre type unless immuno-electron microscopy methods are employed. However, immuno-electron microscopy requires that the sample be preserved in formaldehyde rather than glutaraldehyde, which does not preserve the muscle ultrastructure as well and reduces the ability to visualize muscle organelles, particularly mitochondria[141,142]. However, determining muscle fibre type can be achieved by using correlative electron microscopy. Correlative electron microscopy integrates the fluorescence of light microscopy with the magnification and resolution of TEM[143]. The fibre type is determined using fluorescent labels to illuminate fibre type specific proteins and the image can be overlaid with a TEM image. One disadvantage to correlative microscopy is that many of the labels used for fluorescence are green fluorescent proteins, whose fluorescence can be muted by heavy metals, yet heavy metals are necessary for electron microscopy preparation[144]. Therefore, TEM is ideally suited to provide data related to IMCL morphology and location, whereas correlative electron microscopy is ideally suited to determine muscle fibre type. Thus, the researcher needs to prioritize what outcomes are of most importance when planning their analyses as it is difficult to determine muscle fibre type and IMCL characteristics and storage location using one method.

Finally, proton magnetic resonance spectroscopy (H^+ MRS) is a non-invasive way to measure IMCL content. H^+ MRS is able to distinguish between IMCL and extramyocellular lipids because their morphologies produce differing resonances[145]. H^+ MRS also has the sensitivity to measure the degree of unsaturation of the lipids. Unfortunately, H^+ MRS is unable to distinguish the subcellular location of the IMCL, which is shown to have implications on IMCL metabolism and relate to IR[4]. Overall, electron microscopy is the technique best suited to identify changes in IMCL morphology and location in response to a stimulus.

2.0 Study Rationale

The size, number, and storage location of IMCL have been shown to influence IMCL metabolism[4]. T2D, a population with IR, predominantly store their IMCL in large lipid droplets in the SS region of type II fibres[4]. Athletes, an insulin sensitive population, predominantly store their IMCL in small lipid droplets in the peripheral IMF region of type I fibres[4]. IMCL content, size, and number is negatively associated with IS in type II fibres while IMCL content and number are positively associated with IS in type I fibres[4], suggesting that athletes store their IMCL in a metabolically favourable location while T2D do not. Endurance training redistributes IMCL from the SS region to the IMF region[2,3]. Muscle disuse is the antithesis of endurance training and periods of disuse have been shown to induce IR in as little as one week without the accumulation of IMCL[7]. However, longer periods of disuse have been shown to induce the accumulation of IMCL along with impaired glucose handling[10]. Therefore, there may be changes in the storage location of IMCL that precede IMCL accumulation that contribute to the IR seen during disuse. Since disuse is the opposite of endurance training, disuse may induce a redistribution of IMCL from the IMF to the SS region, which would have negative implications on IS without changing total IMCL content. To date, there has been no examination on the effect of immobilization of IMCL subcellular localization, which was one of the objectives of this study. As well, disuse has been shown to induce whole-body IR[6,9,69,75]. The accumulation of IMCL also results in accumulation of lipid intermediates that have been shown to negatively impact insulin signalling[53,55,135]. Thus, evaluation of the insulin signalling pathway may identify a role for IMCL accumulation in disrupting insulin signalling that thereby contributes to whole-body IR.

Disuse decreases mitochondrial content and function[7,8,94,95]. Mitochondrial fission and fusion has been implicated in contributing to mitochondrial dysfunction in immobilization[104,107]. Mitochondrial fission has also been shown to increase autophagy and therefore increase muscle atrophy[104]. Similarly, mitochondrial dysfunction has been shown to increase apoptosis[75,100]. Therefore, another objective of this study was to evaluate mitochondrial storage characteristics and mitochondria-related signalling following a period of disuse and remobilization.

Disuse leads to IMCL accumulation, which is believed to contribute to reduced IS. Omega-3 FA supplementation have been shown to increase the gene expression of proteins involved in fat transport, β-oxidation, and oxidative phosphorylation[129,134,137]. Omega-3 FA supplementation can also increase the gene expression of IRS1 and GLUT4[64]. Therefore, omega-3 FA supplementation may lower IMCL accumulation by increasing IMCL metabolism and potentially limit the impairments in insulin signalling seen with immobilization.

2.1 Objectives

The experiment detailed in this thesis examined the physiological effects of disuse on IMCL and mitochondria storage characteristics and location in relation to insulin signalling. As well, it examined whether omega-3 FA supplementation influenced IMCL characteristics and whether this relates to insulin signalling.

2.2 Hypotheses

Our *a priori* hypotheses were that:

1. Disuse would increase SS IMCL content due to an increase IMCL size, not number.
2. Disuse would decrease peripheral IMF IMCL content due to a decrease in the number of IMCL, not size.
3. IMCL content in the central IMF region would increase due to an increase in IMCL size, not number.
4. IMCL-mitochondria apposition would decrease in both the SS and the IMF regions as a result of disuse.
5. Protein content or phosphorylation status of enzymes of the insulin signalling pathway (IRS1, AKT, AS160, GLUT4, AMPK, ACC) would be blunted as a result of disuse.
6. Disuse would increase PLIN2 and decrease PLIN5 content.
7. Disuse would increase BAX and FIS1 expression and decrease OPA1 and BCL2 expression.
8. Omega-3 FA supplementation would mitigate the effect of disuse on IMCL storage location, IMCL-mitochondria apposition, mitochondrial content, insulin signalling, and PLIN proteins.

3.0 Methods

3.1 Participants

Twenty healthy, young women were recruited for this study. The trial was approved by the Hamilton Integrated Research Ethics Board (HIREB-1457) and met all the standards for the use of human participants by the Canadian Tri-Council Policy on the ethical use of human subjects in research[146] and conformed to the standard set by the latest version of the Declaration of Helsinki[147]. Participants provided written informed consent to participate. All participants were between the ages of 18 to 30 y, non-smokers, and had no history of lower limb injuries within one year of the study that necessitated a period of disuse. Participants were excluded if they were taking any analgesic or anti-inflammatory drugs chronically, had a history of neuromuscular problems or muscle and/or bone wasting diseases, had any acute or chronic illness, cardiac, pulmonary, liver, or kidney abnormalities, uncontrolled hypertension, insulin or non-insulin dependent diabetes or metabolic disorders, used medications known to affect metabolism, or were allergic to fish. All participants completed a general health screening questionnaire. Oral contraception was not an exclusion, though those using oral contraceptives were evenly distributed between the two groups.

3.2 Protocol

Following the initial meeting for consent, participants visited the lab 4 times over an 8-week period to complete this study, as outlined in Figure 5. On the first visit, participants underwent a DXA scan to determine body composition and were randomized by a computer generated sequence (*www.randomization.com*) into either the active intervention group (fish oil) or the control group (sunflower oil). Each group received 20 mL doses of either omega-3 FA

(2.97 g EPA and 2.03 g DHA) or sunflower oil (an isoenergetic and volume equivalent), which they continued to take for the duration of the study. Supplementation was provided as 5 capsules per day (Infinit Nutrition, Windsor, ON, Canada). The sunflower oil placebo had a high oleic acid content (75%). Both supplements had coconut flavouring to mask the participants to their supplementation group. After 4-weeks of supplementation, one of their legs was randomly assigned to immobilization for 2-weeks using a second randomization scheme (*www.randomization.com*). Their leg was held in a brace at a 60° angle of flexion using a Donjoy X-ACT knee brace (DJO LLC, Vista, CA, USA). Following two weeks of immobilization, participants underwent two weeks of recovery where they returned to baseline activities. Skeletal muscle biopsies of the immobilized leg were taken from the *vastus lateralis* muscle before supplementation (pre), before immobilization (D0), after immobilization (D14), and after recovery (D28). Samples from the biopsy were immediately stored in 2% gluteraldehyde for TEM analysis or flash frozen in liquid nitrogen and stored at -80 °C for western blotting analysis.

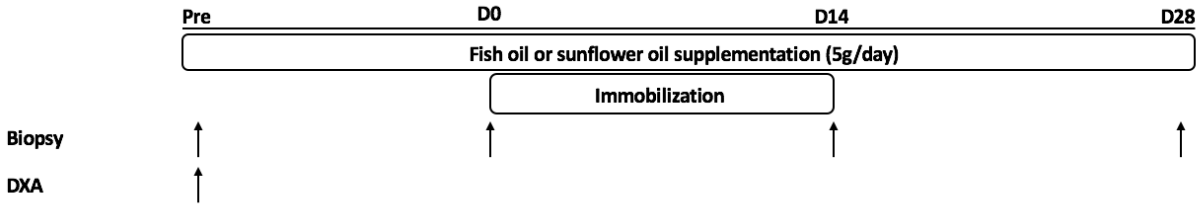


Figure 5: Schematic diagram of experimental design.

3.3 Electron microscopy analysis

Muscle samples were fixed in 2% glutaraldehyde for no more than 1 month before continuing the TEM sample preparation process. All sample preparation was done by trained technicians at the McMaster University Medical Centre Electron Microscopy facility. Samples

were post fixed in 1% osmium tetroxide, serially dehydrated with ethanol, and embedded in Spurr's resin. Thin sections (90 nm) were cut using a diamond knife ultramicrotome (Leica UCT microtime, Wetzler, Germany) and placed on Cu grids. Samples were stained with uranyl acetate followed by lead citrate and coated with a thin carbon layer. Sections were viewed on a transmission electron microscope (Phillips CM10 TEM, Eindhoven, Netherlands) at 5800X. Sixteen images were taken at each time point for each participant: 8 from the IMF region, 6 from the SS region with a nucleus, and 2 from the SS region without a nucleus (Fig 6). A total of 720 images were analyzed for this study.

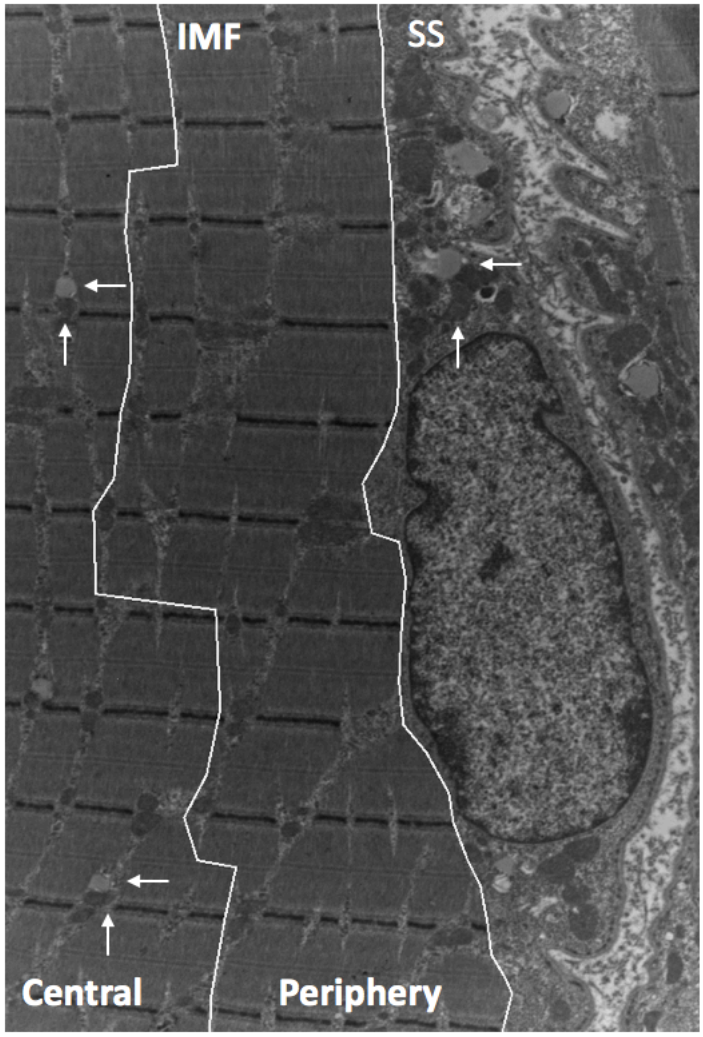


Figure 6: Representative image of a TEM image. The white line separates the SS region (right) from the IMF region (left). The IMF is further separated by a white line into the peripheral IMF and central IMF regions. IMCL (light grey) and mitochondria (dark grey) are identified with a white arrows.

TEM images were analyzed for lipid and mitochondria storage characteristics using ImageJ (Version 1.51a, National Institute of Health, USA). Lipid droplets and mitochondria were circled and converted to actual size using a scale bar from which the mean individual IMCL size (μm^2), number (#), and the area density (%) of IMCL were reported. Individual mitochondria number and size characteristics were not reported since mitochondria are a tubular network[148], and thus a 2 dimensional image is not an appropriate method to assess mitochondrial size/number; however, this information was used to determine mitochondrial area density (%).

IMCL and mitochondrial content was reported as area density because the images are 2-dimensional, which more accurately corresponds to the percentage of area covered by either IMCL or mitochondria rather than true volume. The proportion of IMCL in contact with mitochondria was reported as a percentage. All measures were analyzed relative to area such that every measure was assessed within the SS region, within the total IMF region, and within the IMF region when subdivided into the peripheral IMF region and the central IMF region. The peripheral IMF region was defined as within 3 sarcomeres from the SS region.

3.4 Western blotting analysis

Muscle samples were homogenized in ice cold 25 mM Tris buffer (25 mM Tris, 0.5% (v/v) Triton X-100, and protease/phosphatase inhibitor tablets (Roche Diagnostics, Laval, QC, Canada)) using a Tissuelyzer (Qiagen, Hilden, Germany). The samples were centrifuged at 1500 *g* at 4 °C for 10 min. The supernatant was removed, flash frozen, and stored at -80°C for future analysis. The protein content of each sample was determined using the bicinchoninic acid assay technique. From here, samples were prepared in Laemmli buffer (0.5 M Tris-HCl, glycerol, 10% SDS, 1% bromophenol blue, β-mercaptoethanol, and ddH_2O) and stored at -80 °C ready for western blotting analysis.

Proteins involved in insulin signalling, mitochondrial function, and IMCL metabolism were assessed using SDS-PAGE and western blotting. Equal amounts of protein (10 μg) of each sample were run on 4 - 15% Criterion TGX Stain-Free protein gels (BioRad, Hercules, CA, USA) at 200 V for 45 min. A protein ladder (Precision Plus Protein Standard, BioRad, Hercules, CA, USA) and standard curve (pooled from all samples) were run on every gel. Proteins were transferred to a PVDF membrane. Total protein was determined post transfer using a Chemidoc

MP imaging system (BioRad, Hercules, CA, USA). The membranes were blocked in blocking solution (TBST buffer: 20 mM Tris base, 137 mM NaCl, and 0.1% (v/v) Tween 20, pH 7.5, with either 5% (w/v) non-fat dry milk or bovine serum albumin) for 2 hr. Membranes were immunoprobed at 4°C for 12 hr with the corresponding primary antibody, detailed in Table 1. Next, the proteins were immunoprobed with horse-radish peroxidase-conjugated secondary antibodies, detailed in Table 1. Finally, the proteins were incubated with Clarity or Clarity Max Western ECL Blotting substrates (BioRad, Hercules, CA, USA) before being imaged on the Chemidoc imaging system (BioRad, Hercules, CA, USA). Bands were quantified using ImageLab (Version 6.0.1, BioRad, Hercules, CA, USA) and protein content was normalized using total protein and the standard curve obtained from the gel.

Table 1: Antibodies for western blot analysis with the specifics for blocking, primary antibody, and secondary antibody incubations.

Pathway of interest	Protein	Ab Provider	Blocking Agent	1° Ab solution	2° Ab solution
IMCL Metabolism	PLIN2	Progen 610102	0.5% skim milk in 1X TBST	1: 1,000	1: 10,000
	PLIN5	Progen GP31S	5% skim milk in 1X TBST	1: 1,000	1: 10,000
	Total AMPK α	CS 2532S	5% skim milk in 1X TBST	1: 1,000	1: 10,000
	P-AMPK α (Thr 172)	CS 2531S			
	P-ACC (Ser79)	CS 3661			
Insulin Signalling	IRS1	CS 2382	5% skim milk in 1X TBST	1: 1,000	1: 10,000
	Total AKT	CS 4685			
	P-AKT (Ser473)	CS 9271S			
	Total AS160	CS 2447S			
	P-AS160 (Thr 642)	CS 4288S			
	GLUT4	Fisher PA1-1065	5% skim milk in 1X TBST	1: 2,000 in 5% BSA in 0.025% PBST	1: 10,000
Mitochondrial Function	BAX (N-20)	SC-493	2.5% skim milk in 1X TBST	1: 1,000	1: 5,000
	BCL2 (C-2)	SC-7382	5% skim milk in 1X TBST	1: 1,000	1: 10,000
	FIS1 (C-9)	SC-376469			
	OPA1 (D-10)	SC-393296	5% BSA in 1X TBST	1:100	1:500

All 1° and 2° antibodies solutions were the same as the blocking solutions unless otherwise stated. Ab: Antibody. CS: Cell Signalling. SC: Santa Cruz.

3.5 Statistical analyses

The data are presented as means ± standard error unless otherwise stated. All statistical analyses were performed as a two-way mixed model ANOVA with supplementation group as the between group factor and time as the within group factor using SPSS, version 25 (IBM Corp., Armonk, New York, USA). The statistical significance was defined as $p < 0.05$, while a trend

was defined as $0.05 < p < 0.1$. All final images were created using GraphPad Prism (GraphPad Software Inc, CA, USA).

4.0 Results

4.1 Participant characteristics

There were no differences in baseline characteristics between groups (Table 2).

Table 2: Participant characteristics

20 healthy, female participants			
	Placebo	Fish Oil	p value
Age (y)	22.6 ± 1	22.4 ± 1	0.900
Height (cm)	164.6 ± 1.7	165.8 ± 2.4	0.724
Weight (kg)	63.0 ± 2.3	63.7 ± 2.4	0.851
BMI (kg/cm^2)	23.3 ± 0.8	23.2 ± 0.7	0.930
% BF	28.7 ± 2.1	33.0 ± 1.8	0.157

Data are means ± SEM. BMI - body mass index, % BF - percent body fat.

4.2 Electron microscopy analysis

4.2.1 Pre-supplementation to pre-immobilization comparisons

Thirteen individuals had electron microscopy samples, 6 taking placebo and 7 taking fish oil. To evaluate the independent effect of the fish oil, all characteristics were first measured between the pre-supplementation and pre-immobilization time points. There was no effect of supplementation (main effect or interaction) on IMCL characteristics in the SS region after 4-weeks of supplementation (Supp. Fig 1). Similarly, there was also no significant effect of supplementation on the IMCL characteristics in the IMF region (Supp. Fig 2). There was also no change in the proximity of IMCL to mitochondria in the SS or the IMF regions (Supp. Fig 3). Finally, there was no change in the mitochondrial area density in the SS or IMF regions (Supp. Fig 4).

4.2.2 Pre-immobilization, post immobilization, and post recovery comparisons

As there was no effect of the supplementation on any measured characteristics before immobilization the pre-supplementation data was removed to focus on the effect of disuse. One individual only had pre-supplementation and pre-immobilization data so their data was excluded from these comparisons, making the total number of participants 12. There was no effect of omega-3 FA supplementation during immobilization or recovery (main effect or interaction) on IMCL or mitochondrial characteristics. Therefore, all data were graphed to focus on the effect of immobilization and recovery on IMCL and mitochondrial characteristics.

IMCL number had a strong tendency to decrease ($p = 0.053$, Fig 7A), though only during recovery, and IMCL size was unaffected by either immobilization or recovery (Fig 7B). However, there was a significant decrease in IMCL area density in the SS region as a result of immobilization ($p = 0.009$, Fig 7C), which remained reduced upon recovery. The lower IMCL area density following recovery was due to a decrease in IMCL number. As IMCL metabolism is closely linked to mitochondrial content and function, the proximity of IMCL to mitochondria was assessed during immobilization and recovery. There was no change in IMCL-mitochondrial proximity in the SS region (Fig 7D).

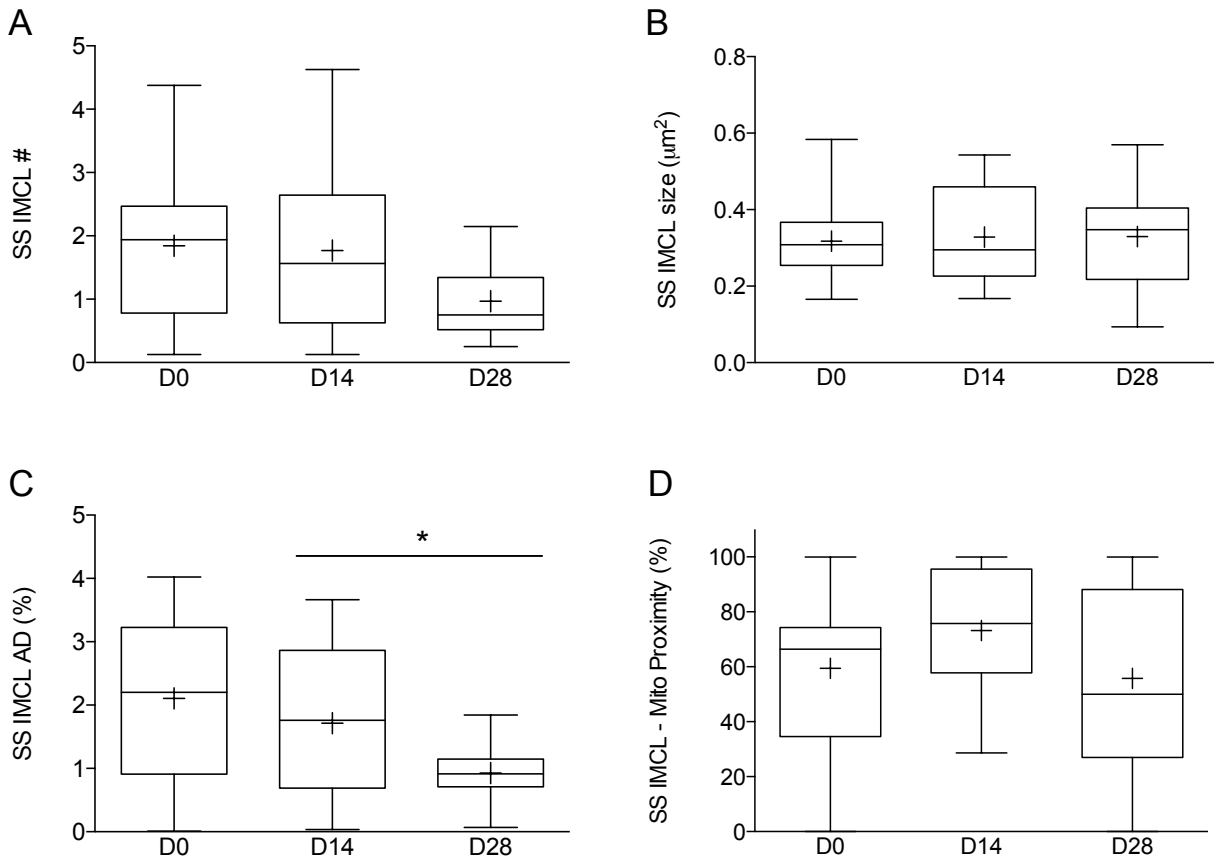


Figure 7: IMCL size (A), number (B), and area density (C) in the subsarcolemmal (SS) region of skeletal muscle before (D0) and after (D14) of immobilization and after recovery (D28). The box plot shows the median (line) and mean (+), with the box representing the IQR and the whiskers representing the maximum and minimum values. Data are reported for 12 participants. Analysis by 2-way ANOVA. * denotes a significant difference from D0, p = 0.009. AD: area density; IMCL: intramyocellular lipid.

In the IMF region there was no effect of immobilization or recovery on IMCL characteristics in the peripheral IMF region (Fig 8A,C,E, respectively). Alternatively, IMCL size increased significantly in the central IMF (p = 0.007; Fig 8B) region as a result of immobilization. IMCL size returned to baseline upon the reintroduction of mobility. Interestingly, the number of IMCL in the central IMF region did not change as a result of immobilization, but there was a significant decrease in the number of IMCL during recovery (p = 0.032, Fig 8D). Therefore, the IMCL area density tended to increase in the central IMF (p =

0.077; Fig 8F) region with immobilization, and tended to decrease back to near pre-immobilization levels upon recovery.

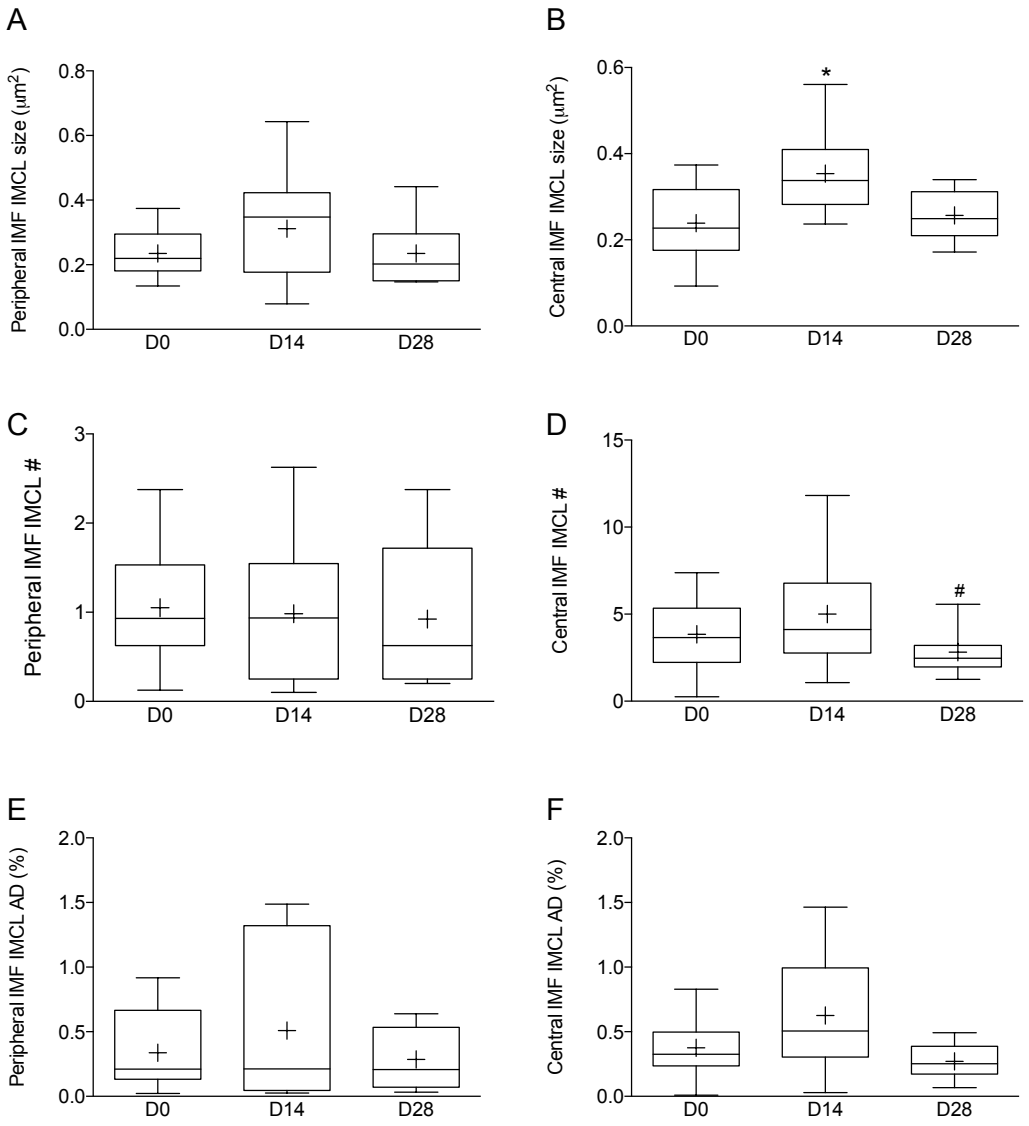


Figure 8: IMCL size (A,B), number (C,D), and area density (E,F) in the peripheral and central intermyofibrillar (IMF) region, respectively, of skeletal muscle before (D0) and after (D14) immobilization and after recovery (D28). The box plot shows the median (line) and mean (+), with the box representing the IQR and the whiskers representing the maximum and minimum values. Data are reported for 12 participants. Analysis by 2-way ANOVA. * denotes a significant increase from D0, $p = 0.007$. # denotes a significant decrease from D0, $p = 0.032$. IMCL: intramyocellular lipid.

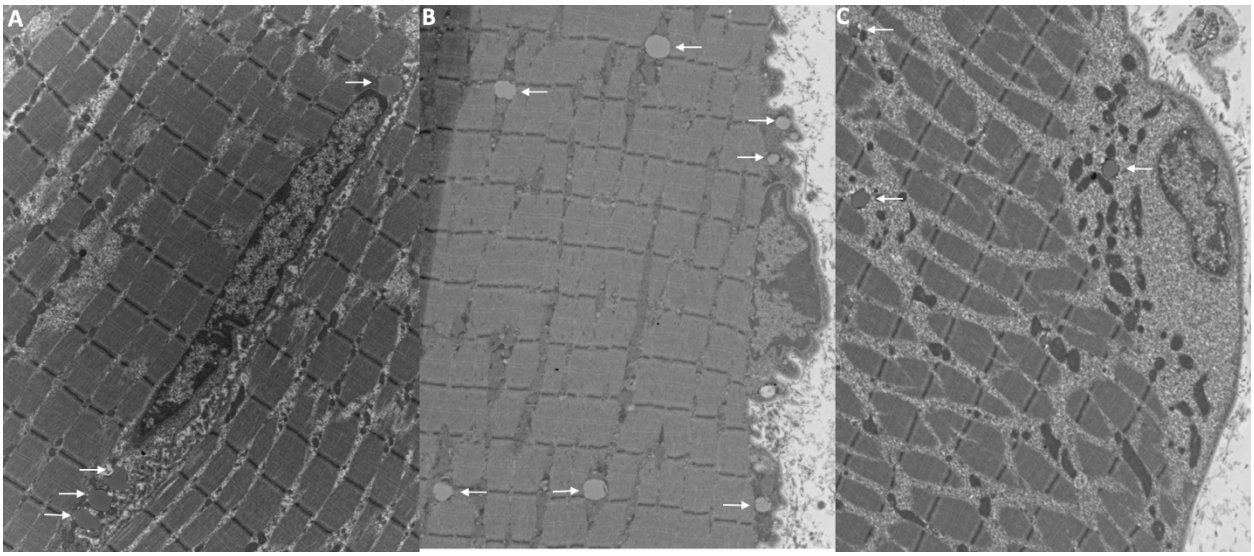


Figure 9: Representative images of IMCL before (D0; A) and after (D14; B) immobilization and after recovery (D28; C) taken at 5800x. The images show the decrease in IMCL in the SS region and the increase in IMCL in the central IMF during immobilization and the decrease in the number of SS IMCL and the decrease in IMCL in the central IMF during recovery. IMCL are denoted by white arrows.

Similar to the IMCL characteristics, there was no change in mitochondrial-IMCL proximity in the peripheral IMF region during immobilization or recovery (Fig 10A). In the central IMF region, despite there being an increase in IMCL area density with immobilization, there was no change in IMCL-mitochondrial apposition; however, there was a significant decrease in the proximity of IMCL to mitochondria in the central IMF region during recovery ($p = 0.002$; Fig 10B).

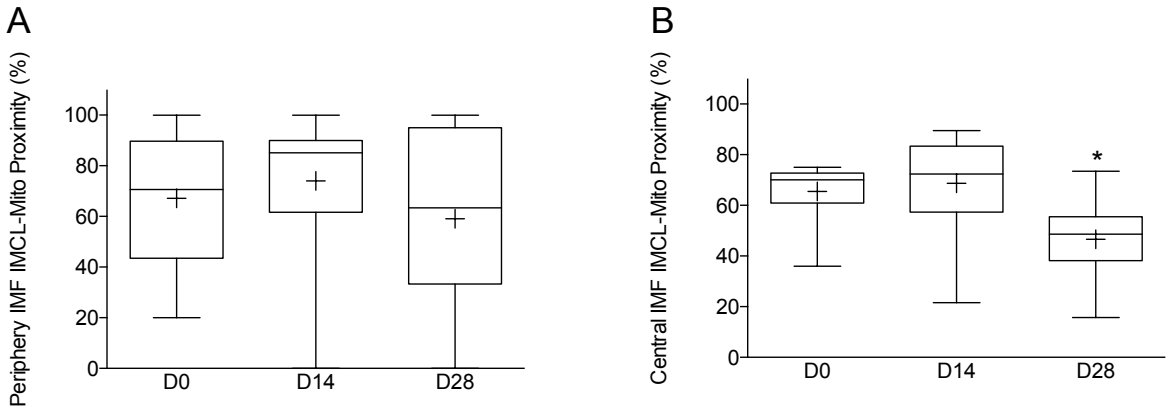


Figure 10: Proximity of IMCL and mitochondrial in the peripheral (A) and central (B) intermyofibrillar (IMF) region, represented as a percentage of IMCL that are touching at least 1 mitochondrion in skeletal muscle before (D0) and after (D14) immobilization and after recovery (D28). The box plot shows the median (line) and mean (+), with the box representing the IQR and the whiskers representing the maximum and minimum values. Data are reported for 12 participants. Analysis by 2-way ANOVA. * denotes a significant decrease from D0, $p = 0.002$. IMCL: intramyocellular lipid. Mito: mitochondria.

As with IMCL, immobilization and recovery altered mitochondrial area density. There was a significant decrease in mitochondrial area density in the SS region from pre-immobilization to recovery ($p = 0.002$; Fig 11A), yet pairwise analyses revealed only a trend towards a decrease during immobilization ($p = 0.087$). Similar to the IMCL, there was no effect of immobilization or recovery on mitochondrial area density in the peripheral IMF region (Fig 11B). While there was no effect of immobilization on mitochondrial area density in the central IMF region, mitochondrial area density decreased in the central IMF ($p = 0.001$; Fig 11C) region upon recovery. Therefore, the change in mitochondrial area density induced by immobilization only became significant after immobilization was completed.

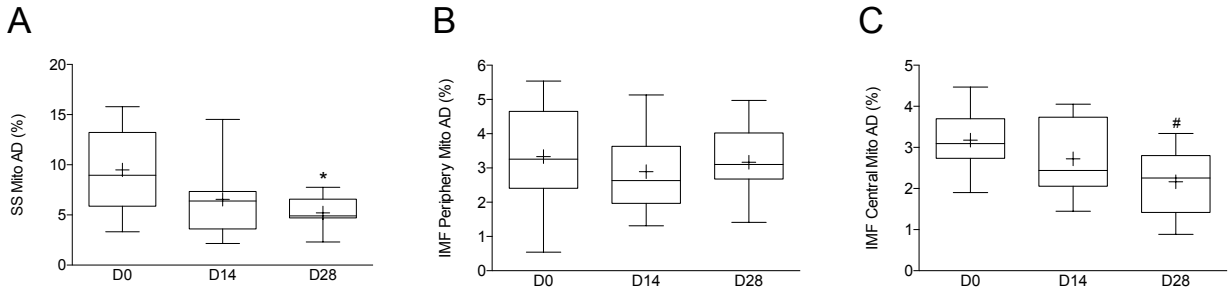


Figure 11: Mitochondrial area density in the subsarcolemmal (SS) region (A) and the peripheral (B), and central (C) intermyofibrillar (IMF) region of skeletal muscle before (D0) and after (D14) immobilization and after recovery (D28). The box plot shows the median (line) and mean (+), with the box representing the IQR and the whiskers representing the maximum and minimum values. Data are reported for 12 participants. Analysis by 2-way ANOVA. * denotes a significant difference from D0, p = 0.002. # denotes a significant decrease from D0 and D14, p = 0.001 Mito: mitochondria. AD: area density.

Finally, IMCL size, number, and area density of the entire fibre were determined regardless of its subcellular localization. IMCL number did not change during immobilization, but decreased during recovery (p = 0.039, Fig 12A). There was a trend towards an increase in IMCL size during immobilization and its return to baseline during recovery (p = 0.073, Fig 12B). Together, this equated to a trend towards increased IMCL area density during immobilization in the whole fibre and a return to baseline during recovery (p = 0.084, Fig 12C).

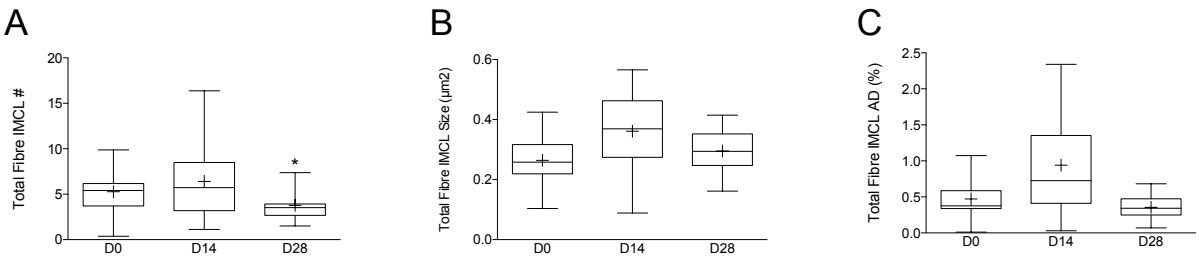


Figure 12: IMCL number (A), size (B), and area density (C) in the entire muscle fiber before (D0) and after (D14) immobilization and after recovery (D28). The box plot shows the median (line) and mean (+), with the box representing the IQR and the whiskers representing the maximum and minimum values. Data are reported for 12 participants. Analysis by 2-way ANOVA. * denotes a significant decrease from D0, p = 0.039. IMCL: intramyocellular lipid.

4.3 IMCL, mitochondria, and insulin signalling protein expression

Next, we evaluated changes in the content of proteins related to IMCL metabolism, mitochondrial function, and insulin signalling. There was no effect of omega-3 FA supplementation on any of the proteins related to IMCL metabolism, mitochondrial function, or insulin signalling, thus graphs are shown to highlight the effect of immobilization and remobilization.

We measured PLIN2 and PLIN5 as markers of IMCL synthesis and breakdown, respectively. PLIN2 did not change during immobilization, but tended to increase upon recovery ($p = 0.074$, Fig 13A). PLIN5 content increased during immobilization and remained elevated during recovery ($p = 0.011$, Figure 13B).

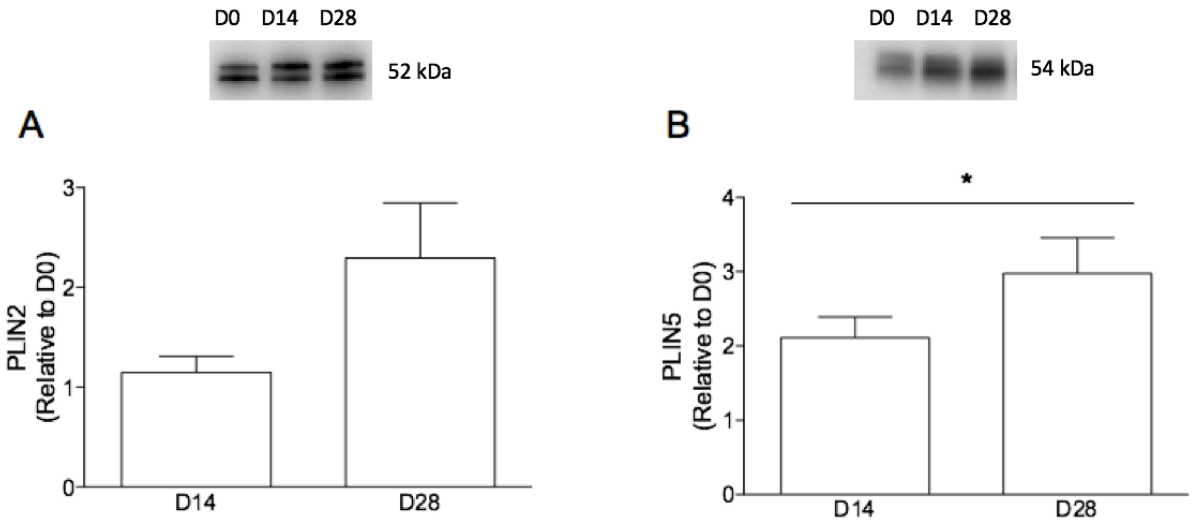


Figure 13: PLIN2 (A) and PLIN5 (B) protein expression of skeletal muscle after (D14) immobilization and after recovery (D28) relative to baseline (D0). Data are reported as the mean ± SEM for 20 participants. Analysis by 2-way ANOVA. * denotes difference from D0, $p = 0.011$.

We measured AMPK and ACC as links between fat metabolism and insulin signalling[149]. Total AMPK content tended to increase ($p = 0.071$; Fig 14A) upon immobilization and stay

elevated during recovery, yet there was no change in phosphorylated AMPK (Fig 14B), which is its enzymatically active state. Interestingly, its downstream effector, ACC, had significantly increased phosphorylation during immobilization and returned to baseline during recovery ($p = 0.021$, Fig 14C).

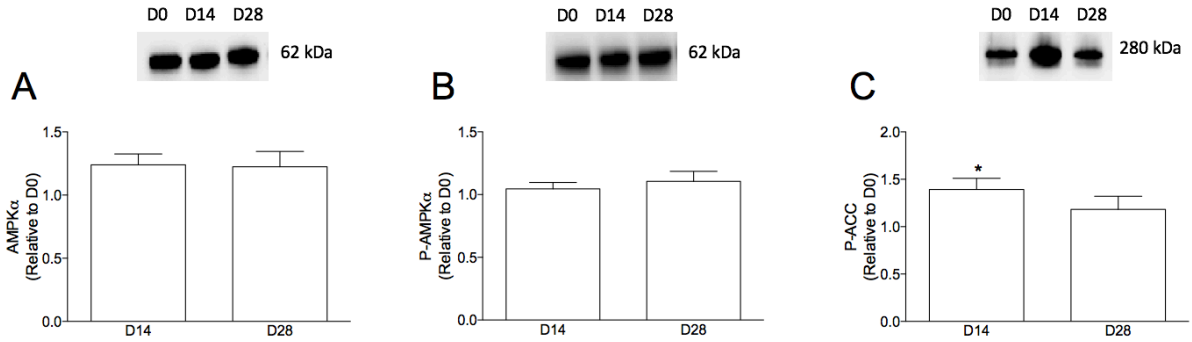


Figure 14: Total (A) and phosphorylated (B) AMPK α and phosphorylated ACC (C) protein expression of skeletal muscle after (D14) immobilization and after recovery (D28) relative to baseline (D0). Data are reported as the mean ± SEM for 20 participants. Analysis by 2-way ANOVA. * denotes a significant increase from D0, $p = 0.021$.

We evaluated mitochondrial function by determining mitochondrial fission and fusion patterns. There was a trend towards a decrease in FIS1 content ($p = 0.07$, Fig 15A) during immobilization as well as a strong trend for OPA1 content to increase ($p = 0.051$, Fig 15B) in recovery. However, when expressed as a ratio of OPA1/FIS1, there was a significant increase ($p = 0.035$, Fig 15C) during recovery, suggesting a preference for mitochondrial fusion. Apoptotic-related signalling was characterized by opposing apoptotic regulators. BCL2, an anti-apoptotic factor, significantly decreased upon immobilization and remained blunted during recovery ($p = 0.044$, Fig 15C). Conversely, BAX, a pro-apoptotic factor, significantly increased during both immobilization and recovery ($p = 0.001$, Fig 15D). Despite the significant changes in BCL2 and BAX content, when expressed as a ratio of BCL2/BAX, there was no effect of immobilization or recovery ($p = 0.50$, Fig 15F).

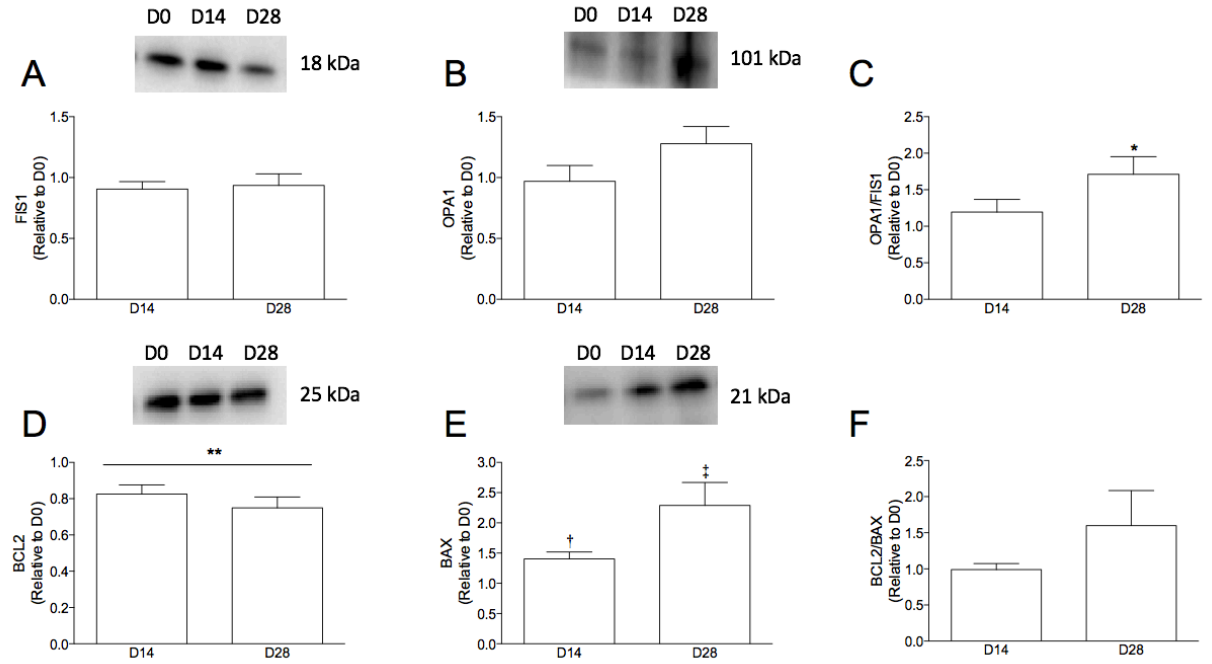


Figure 15: FIS1 (A) and OPA1 (B) protein expression, OPA1/FIS1 ratio BCL2 (D) and BAX (E) protein expression, and BCL2/BAX ratio (F) of skeletal muscle after (D14) immobilization and after recovery (D28) relative to baseline (D0). Data are reported as the mean ± SEM for 20 participants. Analysis by 2-way ANOVA. * denotes a significant increase from D0 and D14, p = 0.035. ** denotes a difference from D0, p = 0.044. † denotes a difference from D0, p = 0.001. ‡ denotes a difference from D0 and D14, p = 0.001.

Disuse negatively impacts insulin sensitivity[9,69,75] and IMCL is thought to play a role in the development of insulin resistance[18,49], therefore several proteins along the insulin signal pathway were analyzed. There was no change in IRS1 during immobilization, but there was a significant increase during recovery (p = 0.039, Fig 16A). GLUT4 content did not change as a result of immobilization or recovery (Fig 16B). Total AKT content significantly increased with immobilization and tended to continue increasing during recovery (p = 0.002, Fig 16C), while phosphorylated AKT increased with immobilization and remained elevated during recovery (p = 0.001, Fig 16D). The increase in AKT did not influence the total or phosphorylated content of its downstream substrate, AS160, during immobilization. However, there was a trend towards increased phosphorylated AS160 upon recovery (p = 0.075, Fig 16F).

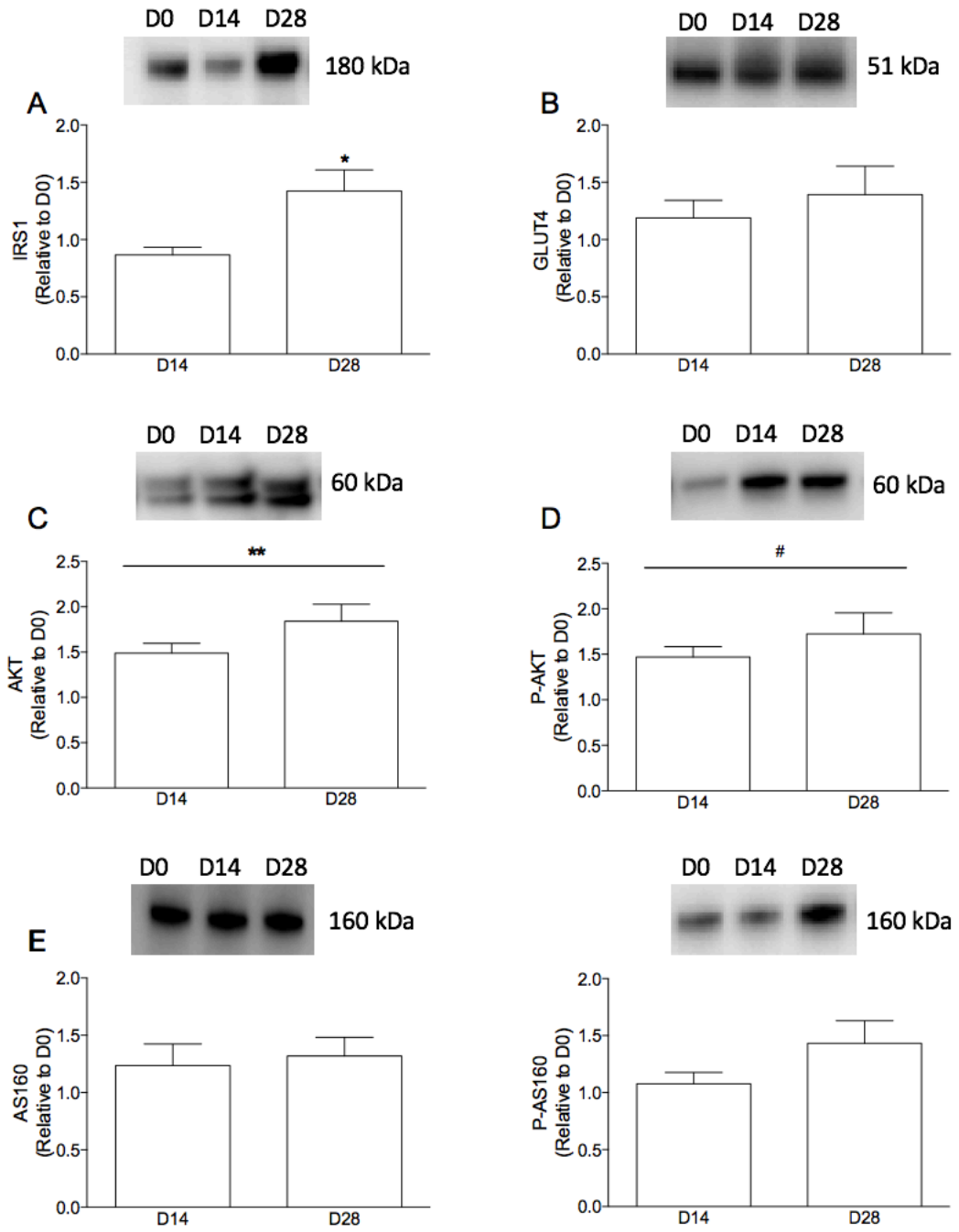


Figure 16: IRS1 (A), GLUT4 (B), total (C) and phosphorylated (D) AKT, and total (E) and phosphorylated (F) AS160 protein expression of skeletal muscle after (D14) immobilization and after recovery (D28) relative to baseline (D0). Data are reported as the mean ± SEM for 20 participants. Analysis by 2-way ANOVA. * denotes a significant increase from D0, $p = 0.039$. ** denotes a significant increase from D0, $p = 0.002$. # denotes a significant increase from D0, $p = 0.001$.

5.0 Discussion

This study aimed to assess changes in IMCL and mitochondrial characteristics during single leg immobilization and recovery and whether fish oil mitigated these effects. Based on the current literature, we believed that disuse would result in IMCL accumulation in the SS region and mitochondrial dysfunction, accompanied by decreased insulin signalling. These effects were expected to return to baseline during recovery. The key findings of this study are that: 1) immobilization increased IMCL content in the central IMF region and decreased content in the SS region, 2) mitochondrial content did not change during disuse, but decreased during recovery, and 3) immobilization increased total and phosphorylated AKT expression, which remained elevated in recovery. The disuse stimulus employed in this study was sufficient to influence muscle metabolism as evidenced by a decrease in muscle protein synthesis, muscle mass, and muscle function[150]. The novelty of this study is the examination of subcellular IMCL characteristics during disuse, not total content, as well as the continued assessment of all parameters following 2-weeks of recovery.

Disuse influences IMCL and mitochondria storage characteristics

IMCL area density significantly decreased in the SS region during immobilization and continued to decrease during recovery. While not significant, this change was mainly driven by a decrease in IMCL number ($p = 0.053$), not a decrease in IMCL size. These findings are contrary to our hypothesis that IMCL content would increase in the SS region during immobilization, antithetically to the effect of endurance training on SS IMCL content[2,3]. In fact, IMCL size and content in the SS region have been found to be negatively associated with IS[2,4], thus our finding that SS IMCL content decreased with disuse is hard to reconcile as it suggests that disuse

induced a favourable effect on SS IMCL storage. However, since SS IMCL size has been related to IR[4] and the decrease in SS IMCL content occurred without a change in IMCL size, it is likely that this was not a favourable change in IMCL storage, particularly considering that SS mitochondrial area density decreased during immobilization and remained lower during recovery. In T2D there is a 3-fold reduction in SS mitochondrial content and a 7-fold reduction in electron transport chain activity[151], which have been suggested to negatively impact IS[151,152]. Insulin resistant individuals also have reduced rates of fat oxidation as evidenced by reduced carnitine palmitoyltransferase 1 content, reduced oxidative enzyme activity, and a higher respiratory quotient[153,154]. Yet, despite the reduced oxidative capacity found in this population by a companion study[155], there was a decrease in SS IMCL content, which is hard to reconcile. However, decreased SS IMCL content is supported by the increase in ATGL seen during immobilization in the companion paper[155]. Several studies have found a negative association between SS IMCL content and IS in men[2,36], but in women this relationship has not been observed[3]. The decrease in mitochondrial content suggests a negative impact of disuse in the SS region, but the decrease in IMCL content in the SS region, while unexpected, may indicate that SS IMCL storage is not related to IR in women.

Unlike the SS region, disuse and recovery did not affect peripheral IMF IMCL characteristics. Higher habitual physical activity has been shown to protect against the loss of peripheral lipid droplets[30]. As this was a young, recreationally active population, perhaps they were active enough to be protected against disuse-induced peripheral IMCL changes. However, IMCL area density in the central IMF region tended to increase with immobilization due to a significant increase in IMCL size. Increased IMCL size is related to IR, particularly in the central IMF[4,30]. Previous literature has found that IMCL content increases during immobilization[10,86,90],

though the methods employed by these studies did not have the sensitivity to differentiate subcellular localization. Not all studies have found that IMCL content increases with disuse[7], however this may be due to a shorter period of immobilization, 7 days compared to ≥ 14 days. Recently, it has been shown that the subcellular localization of IMCL is important to the functionality of IMCL metabolism as trained individuals and those with T2D preferentially store their IMCL in different locations[4]. If IMCL content had not been separated based on subcellular localization, the whole fibre analysis would have missed the decrease in IMCL area density in the SS region and the increase in IMCL area density in the central IMF region. These findings emphasize the need to use methods, such as electron microscopy, that can determine IMCL characteristics and storage location when evaluating the effects of an intervention on IMCL content.

IMCL content is higher in the peripheral IMF region in athletes and is thought to represent a readily available fuel source to support ATP production due to the corresponding high mitochondrial content[4]. In untrained individuals IMCL content is greater in the central IMF region, which is in line with our results showing that with disuse IMCL accumulated exclusively in the central IMF and not the peripheral IMF. Furthermore, the increase in IMCL area density was due to increased IMCL size rather than number, and increased IMCL size in the IMF region has been negatively associated with IS, though this is seen only in type II fibres[4]. As such, fibre type has been implicated as an important factor when assessing IMCL quality[4]. Unfortunately, we were unable to determine fibre type in the current study to determine whether disuse had differing effects depending on fibre type. With recovery, the IMCL content returned to baseline levels in the central IMF region, due to a reduction in both IMCL size and number.

IMCL synthesis and breakdown are in part mediated by the PLINs. During disuse, IMCL area density in the SS region tended to decrease. At that time, there was a significant increase in PLIN5 content and no change in PLIN2 content. PLIN5 is thought to mediate IMCL-mitochondrial apposition and provide a physical link between IMCL and fat oxidation[26,33,156], thus the finding that PLIN5 and ATGL[155] increased during immobilization may explain the decreased SS IMCL content seen in the current study. The decrease in SS IMCL and increased ATGL may indicate the IMCL were being reduced as the lipids were metabolized for energy, however a companion paper found a 20% reduction in ADP-stimulated respiration and significant reduction in COX proteins [155], suggesting that immobilization decreased the oxidative capacity of the muscle. However, this same companion paper found there was no change in lipid oxidation in the whole muscle, as evidenced by no change in β-hydroxyacyl dehydrogenase content or lipid-supported mitochondrial respiration[155], suggesting that total lipid oxidation was unaffected by disuse. However, while total lipid oxidation may not have changed, the site of lipid utilization may have shifted as a decrease in IMCL in the SS region and an increase in IMCL in the IMF region would equate to a net zero change.

Alternatively, in the absence of stimulus from exercise, PLIN5 can promote lipogenesis[23]. Therefore, without exercise, PLIN5 may be promoting lipogenesis rather than lipolysis, which would correspond with the increase in central IMF IMCL size seen in the current study. PLIN2 favours a lipogenic phenotype[23], yet when IMCL area density increases in the central IMF region during immobilization, there is no concurrent increase in PLIN2 content. Therefore, perhaps PLIN5 is mediating IMCL accumulation in the IMF regions. However, this contradicts its proposed activity in the SS region. Furthermore, during recovery there was an increase in PLIN2 content at a point at which IMCL area density in the central IMF was decreasing, results which

are again hard to reconcile given the proposed role of PLIN2 to promote lipogenesis. During recovery, there was also a decrease in IMCL-mitochondrial apposition in the central IMF region despite elevated PLIN5 content. Acute and chronic exercise have been shown to increase IMCL-mitochondrial apposition[40,41], an effect thought to be mediated by PLIN5[33], yet apposition decreased in the IMF region during remobilization despite high PLIN5 content. Unfortunately, there was no evaluation on ATGL content during recovery to further evaluate IMCL metabolism during recovery[155]. Together, these findings raise several considerations that warrant further examination regarding the role of PLINs on IMCL content and apposition with mitochondria during and following disuse. However, it is important to note that while IMCL analyses were dependent on subcellular location, PLIN content was determined for the whole muscle homogenate. Similar to how the whole muscle analysis of IMCL content would have masked the decrease of IMCL in the SS region and the exclusive increase of IMCL in the central IMF, the analysis of PLIN content on whole muscle homogenate may be masking important subcellular influences of disuse on PLIN content/function. As such, separating the muscle into SS and IMF fractions may identify differential changes in PLINs induced by disuse that may align better with the changes seen in IMCL content and should be examined in future trials.

Several studies have shown that mitochondria area density decreases with disuse[7,93,95,157] and it significantly increases during recovery compared to immobilization, but does not return to pre-immobilization levels[95]. Disuse did not decrease mitochondrial area density in the IMF regions and it only tended to decrease in the SS region; however, similar to others[109,157], our study showed that SS mitochondria are more susceptible to degradation with immobilization. Reduced mitochondrial content and function is also found in T2D and has been linked to the development of IR[82,96]. Nielsen *et al*. found a spatial relevance to mitochondrial degradation

where the further into the myocyte the less susceptible the mitochondria are to degradation with SS > periphery > central[157]. Meanwhile, our data suggests SS > central > periphery. Nielsen *et al*. estimated volume of mitochondria using point counting whereas we calculated area density by individually outlining the mitochondria. Furthermore, their electron microscopy images were also taken at 40,000X compared to ours at 5,800X. Our lower magnification would produce a larger image, which may better represent changes to large areas and could account for the discrepancy in IMF regional mitochondrial susceptibility.

During recovery, mitochondrial content decreased significantly in the SS and central IMF regions. The effects of remobilization on mitochondrial content are controversial, with one study finding a partial recovery during remobilization[95], but another finding no improvement[158].The ubiquitin-protease system is not activated in recovery from immobilization as determined by the protein content of muscle RING-finger protein 1 (MURF) and muscle atrophy F-box protein (MAFBX)[150], suggesting that this system is not contributing to the reduction in mitochondrial content that occurs following immobilization. However, it has been found in rats that following 8 days of hindlimb unloading autophagy peaks at 6 days of recovery and is still significantly elevated at 10 days of recovery[159]. Based on these findings it has been suggested that remobilization increased autophagy to remove damaged organelles[159]. Damaged mitochondria produce more ROS, but a companion study found no changes in ROS production during immobilization, suggesting that mitochondrial damage was not occurring during immobilization. However, there was a significant increase in antioxidant protein content following immobilization[155], which could mask an increase in ROS production induced by immobilization. Unfortunately, there was no evaluation of ROS production or antioxidant status during the

recovery period to support the hypothesis that autophagy/mitophagy may increase during recovery.

Disuse influences apoptotic signalling and mitochondrial dynamics

BCL2 and BAX are anti- and pro-apoptotic factors, respectively, and their protein contents represents the balance of apoptotic signalling[160]. As expected and in agreement with other studies[118,161], their expressions were differentially influenced by disuse with BCL2 decreasing and BAX increasing during immobilization despite no change in the ratio of BCL2/BAX. During recovery, BCL2 remained in a depressed steady-state while BAX expression continued to increase, suggesting apoptosis-related signalling may still be elevated despite remobilization. The protein content of a downstream protease of BAX, caspase 9, is elevated in the tibialis anterior muscle of rats after 10 days of recovery from immobilization[159], while caspase 9 protein content returns to pre-immobilization levels within 6 days of recovery in the gastrocnemius muscle[159,162]. Caspase-9 activity represents the activation of apoptosis that is often activated by immobilization[159,162]. In rats, gastrocnemius is a mixed type muscle while tibialis anterior has very few type I muscle fibres. These findings suggest that there may be a fibre type specific difference in proteolytic pathway activation during immobilization. Interestingly, Slimani *et al*. suggested that the increased apoptotic susceptibility of the tibialis anterior could be due to the fibre type shift induced by immobilization whereby there was an increase in type I fibres, which are most susceptible to proteolysis with unloading[163]. Type I fibres are the most prominent and largest fibres in female human vastus lateralis[164], which may explain the increased BAX expression seen here during recovery. However, as BAX is early in

the apoptotic signalling pathway, it would be necessary to measure the proteins such as caspase-3 to definitively identify changes in apoptotic activity[111].

Mitochondria are dynamic structures that constantly undergo fission and fusion to maintain health. During hindlimb suspension, animal studies have shown downregulation of fission and fusion proteins[108,109] while others have shown increased fission protein content and decreased fusion protein content[104,107]. In humans, 10-days of bed rest did not change the content of fission or fusion proteins in older adults[110]. Mitochondrial remodeling has been shown to induce AMPK-FOXO3 signalling to induce atrophy, and FIS1 and DRP1 overexpression can exacerbate muscle atrophy. Despite no change in OPA1 during immobilization in the current study, there was a trend ($p = 0.07$) for FIS1 to decrease. A companion paper also found no significant changes in total content of mitochondrial fission and fusion during immobilization, as determined by MFN2 and DRP1 content, respectively, but there was a significant decrease in the ratio of MFN2/DRP1, suggesting a greater activation of fission[155]. We found no effect of immobilization on the ratio of OPA1/FIS1. Despite the tendency for FIS1 to decrease during immobilization, it was not powerful enough to change the balance between mitochondrial fusion to fission as determined by the ratio of OPA1/FIS1. Therefore the findings from our study and those of a companion paper both support no change in fission and fusion proteins during immobilization[110]; however, the ratio between fission and fusion may reveal more about mitochondrial dynamics. More research is needed to identify the cause of the discordance between the relative expression fission and fusion proteins with immobilization. While there was no evaluation of MFN2 or DRP1 during recovery, we found a strong trend ($p = 0.051$) for OPA1 content to increase during recovery as mitochondrial content decreased along with a significant preference towards mitochondrial fusion as evidenced by an increase in OPA1/FIS1. To my

knowledge, no other studies have evaluated OPA1 content during recovery from immobilization, but IS has a positive relationship with a mitochondrial fusion phenotype, which would agree with the increase in OPA1 expression during recovery seen here[165].

Disuse does not change or increases insulin signalling proteins in young women

Immobilization induces insulin resistance[9,69,75], thus we hypothesized that there would be a decrease in insulin signalling pathway proteins. Despite the acceptance that disuse induces insulin resistance[6,9,69,75], there has been almost no examination of the effect of disuse on the insulin receptor or its substrate IRS1. In the current study, we found no effect of disuse on IRS1; however, IRS1 increased during recovery. Previous studies have shown that unloading can induce increased phosphorylation of the serine residues on IRS1 in as little as two weeks in mice[166,167]. Serine phosphorylation increases over time concurrently with IRS1 degradation[166]. When IRS1 is serine phosphorylated instead of tyrosine phosphorylated, it is unable to interact with the insulin receptor[168]. IRS1-serine phosphorylation is also seen in insulin resistance[169]. Therefore, even though we found no change in IRS1 content during disuse, there may be a change in its activity levels and this should be explored in future studies.

One of the outcomes of insulin signalling is to increase GLUT4 translocation to the membrane. GLUT4 translocation is inhibited by AS160 and this inhibition can be removed by phosphorylating and thereby inhibiting AS160, which can be done by either AMPK or AKT. Yet despite both AKT and AMPK increasing or tending to increase, respectively, during immobilization, there was no increase in AS160 phosphorylation during immobilization; however, it did tend to increase upon recovery. When AS160 phosphorylation increased in recovery, so did IRS1 content, which may represent an increase in insulin signalling during

recovery. These findings suggest that there may be changes in signalling with disuse that mediate or interrupt AS160 phosphorylation. This is corroborated by Biensø *et al.* who also found no increase in AS160 content with 12-days of bed rest[85]; however, they did not explore its expression during recovery. GLUT4, whose translocation is controlled by AS160, also did not change with immobilization. GLUT4 has been shown to increase[167], decrease[85], and not change[170] with hindlimb unloading, 12-days of bed rest, and 9-days of bed rest, respectively. During recovery when AS160 phosphorylation increased, GLUT4 content did not increase, but it was measured in whole muscle homogenate, and so no comment can be made on whether disuse or recovery influenced its translocation to the sarcolemma.

One of the proteins responsible for GLUT4 subcellular location is AMPK. AMPK activity is regulated and increased by phosphorylation and allosterically via AMP[149]. An energy imbalance in ATP/AMP is a potent activator of AMPK and has been seen in disuse[118]. We found that total AMPK tended to increase and remain elevated during recovery, but there was no concomitant increase in AMPK phosphorylation status at any time point. Due to the dramatic decrease in mitochondrial content during immobilization (SS) and recovery (SS & IMF), an imbalance of ATP/AMP is likely occurring. Yet, while some studies support increased AMPK expression during disuse[107,171], there are also studies that show no change in total or phosphorylated AMPK[170,172,173]. Unfortunately, there have been a variety of immobilization conditions tested in both human and animal trials, and as such the source of the discrepancy is unclear. ACC is a downstream effector of AMPK so its increased phosphorylation, indicating lower activity, during immobilization is in line with our AMPK findings. However, ACC phosphorylation status returned to pre-immobilization levels during recovery despite total AMPK expression tending to remain elevated. When active (dephosphorylated), ACC promotes

fatty acid synthesis, which is favourable for PLIN2 expression[23]. PLIN2 content did not change with immobilization, in agreement with previous findings[86], but tended to increase with recovery. The dephosphorylation of ACC during recovery despite no IMCL accumulation also suggests a high IMCL turnover, in agreement with the increase of PLIN2 and PLIN5 content seen during recovery.

Another prominent regulator of AS160 phosphorylation and therefore GLUT4 translocation is AKT. In the current study total and phosphorylated AKT expression increased with immobilization and remained elevated during recovery. The effect of disuse on AKT content appears to be time-dependent with Liu *et al*. finding that AKT phosphorylation decreased during the first 3 – 7 days of hindlimb unloading in males rats, yet it was elevated above pre-immobilization levels at day 14, though not significantly[108]. Therefore, it is necessary to factor in the length of disuse when evaluating AKT status across studies. However, the findings from the current study conflict with those from many studies in humans[85,170,174] and animals[167,173,175] that have found that total and/or phosphorylated AKT decreases in response to a variety of immobilization protocols. AKT is a potent regulator of the mammalian target of rapamycin (mTOR), which initiates protein synthesis[176–178]. Both AKT and mTOR respond to feeding and in the current study the muscle biopsies were taken in the fasted state[179]. As such, it would be important to also evaluate the effect of immobilization on AKT and mTOR in the fed state in order identify changes to protein content once the proteins have been properly activated[179]. Regardless, an increase in AKT is unexpected; however, an increase in AKT has been seen in animal studies during immobilization[108,118,180]. While a previous study found that both mTOR and AKT increased during disuse, the increase in mTOR was found to be independent of AKT, indicating that the activation of AKT will not reverse muscle atrophy during disuse[11].

Furthermore, a companion study found no change in mTOR gene expression despite a decrease in muscle protein synthesis and a loss in muscle mass[150]. The finding that mTOR did not change despite an increase in AKT content is supportive of a disassociation between AKT and mTOR during disuse.

Interestingly, two of the three studies that showed an increase in AKT during disuse were done in females rodents[118,180], while only one of six studies that exhibited a decrease in AKT was done in female rodents[167]. Estrogen has been shown to increase GLUT4 content and IS in male mice and is believed to protect premenopausal women against T2D[45]. Ovariectomy reduces whole body insulin-stimulated glucose uptake and decreases GLUT4 content in skeletal muscle[181,182]. Disuse is known to induce IR[9,69,75], yet in the current study we did not find an effect of disuse on insulin signalling in skeletal muscle, the largest storage depot for glucose within the body[74]. To my knowledge, the only study that compared the effect of disuse on IS in men and women was a step reduction study where the evaluation of IS was collapsed because the population was older men and post-menopausal women[183]. As such, it is possible that young women are protected against the deleterious effects of disuse on insulin sensitivity due to estrogen. Importantly, the aforementioned human studies that found no increase in total or phosphorylated AKT during recovery were done exclusively in men and none of them examined IRS1 content or AS160 phosphorylation status[174,175,184]. Thus, estrogen may prevent the deleterious effects of disuse by preventing declines in insulin signaling during disuse and perhaps even speeding recovery and warrants further examination.

Omega-3 FA supplementation did not influence IMCL or mitochondrial storage characteristics, apoptosis-related signalling, or insulin signalling

Omega-3 supplementation has been found to increase muscle protein synthesis[12,13] and can attenuate the loss of muscle mass during disuse[150]. Given that muscle mass is the largest storage depot for glucose, we hypothesized that omega-3 FA supplementation would attenuate declines in insulin signalling, which may be mediated by its effect on IMCL storage characteristics. Contrary to our hypothesis, there was no effect of omega-3 FA supplementation on IMCL storage characteristics or insulin signalling. The effectiveness of omega-3 FA supplements has been shown to be reliant on several factors including the dose administered, the length of the intervention, and habitual omega-3 FA consumption. Furthermore, whether the supplement actually contains the appropriate level of EPA and DHA and participant compliance will also influence the effectiveness of omega-3 FA supplementation. In the current trial supplementation adherence was confirmed by determining levels of EPA and DHA in the phospholipids of red blood cells, which were found to increase ~3-fold in the first 2-weeks of supplementation, prior to immobilization, and remained constant throughout the duration of the study[150]. EPA has been found to rapidly incorporate itself into the membranes of red blood cells and have been suggested to mask non-adherence[185]. These findings also support that the omega-3 FA supplement contained the appropriate levels of EPA and DHA. Despite no effects of omega-3 FA on any of the outcomes measured in the current trial, two companion papers found significant effects of omega-3 FA supplementation suggesting that the dose and length of supplementation was sufficient to induce metabolic effects within skeletal muscle. As well, the dose and length of intervention employed by the current trial was longer than other studies that have shown that omega-3 FA supplementation during disuse can attenuate declines in muscle

protein synthesis[150,186], suggesting that the lack of effect of omega-3 FA supplementation on IMCL metabolism and insulin signalling is a valid finding. The habitual diet of the individuals can influence the effectiveness of a supplementation trial. High habitual omega-3 FA intake or the supplementation with omega-3 FA could prevent an effect of supplementation as participants may have already been consuming adequate amounts of omega-3 FA. In the current trial participants were excluded from the trial if they had supplemented with omega-3 FA within the past 6 months. Furthermore, the fact that our two companion studies did find an effect of supplementation on outcomes related to muscle protein synthesis and mitochondrial function suggest that our participants benefited from omega-3 FA supplementation. Together, these findings suggest that the lack of effect of omega-3 FA supplementation seen in the current trial was due to the fact that omega-3 FA do not influence IMCL, not due to an inadequate supplementation regime.

There are conflicting reports of the effects of omega-3 FA supplementation in humans[125], however many long term supplementation trials have found that omega-3 FA increases mRNA expression across a host of metabolic avenues, including the insulin signalling pathway and IMCL synthesis [122,123,137]. These findings suggest that omega-3 FA influence gene expression, which is supported by findings in the liver and brain where omega-3 FA increased the gene expression and regulated the activity of gene transcription factors[187,188]. However, increased gene expression does not always correspond with increased protein content[137], which may explain why we did not see an effect of omega-3 FA supplementation in the current trial.

Omega-3 FA also incorporate themselves into membranes where they influence the activity of proteins[134]. Many of the proteins evaluated in the current study, particularly all of the insulin signalling pathway proteins, are cytosolic and their protein content, not activity, was

measured. In a companion study[150], omega-3 FA supplementation increased muscle protein synthesis and blunted the immobilization-induced increase in ubiquitin ligase gene expression. Similarly, in another companion paper, omega-3 FA supplementation preserved the protein content of all the COX proteins and preserved mitochondrial sensitivity to ADP[155]. Given that these analyses were conducted in the same group of participants, these findings may suggest a preference for EPA and DHA to regulate oxidative metabolism and muscle protein synthesis over insulin signalling and IMCL metabolism. This is supported by multiple studies involving omega-3 FA supplementation for 6 months that failed to see a change in whole body insulin signalling in healthy populations[125].

6.0 Future Directions and Conclusions

In this study there were several findings that varied from previous literature as well as findings contrary to our hypotheses that warrant further investigation. SS IMCL area density decreased during immobilization and it, along with IMCL size, continued to decrease during recovery. SS IMCL accumulation is associated with IR[2,4]. Endurance training induces a shift in IMCL storage from the SS region to the IMF region[2,3], a shift that has been found to be associated with improved IS in men[2], but not women[3]. Since disuse is the antithesis to endurance training and induces insulin resistance[6,9,75], it was believed that IMCL would accumulate in the SS region as this is negatively associated with IR[4]. Instead, SS IMCL decreased during disuse, a change that is thought to have a positive impact on IS[4]. Women are inherently more insulin sensitive than men[189,190] and in the current study AKT content increased in our female population, whereas in previous studies conducted in men AKT decreased with disuse[85,170,174]. Perhaps estrogen mitigated the negative effects of disuse on IMCL metabolism and insulin signalling, with particular emphasis on the AKT pathway. Interestingly, high intensity interval training has been shown to improve IS in men, but not women[191,192]. Furthermore, it has been suggested that the insulin-sensitizing effect of exercise in women may be blunted[191]. Therefore, perhaps the effect of disuse on insulin signalling and insulin sensitivity is also blunted in women and requires further examination.

The current study undertook a careful examination of the effects of disuse on IMCL and mitochondrial storage characteristics in multiple subcellular locations within the myocyte. IMCL and mitochondrial content decreased, as did their apposition, during recovery in the IMF region. There was no change in PLIN2 content during disuse, but IMF IMCL content increased as did PLIN5 content. As PLIN5 promotes lipolysis and PLIN2 promotes lipid accumulation, these

findings seem contradictory. Unfortunately, while we were able to characterize the effects of disuse on IMCL and mitochondria within different myocyte regions, we were only able to determine whole muscle PLIN content. As the subcellular regions of IMCL storage had opposing reactions to disuse, assessment of PLIN proteins in subfractionated muscle homogenate (myofibrillar vs. sarcolemmal) may have provided insight into how IMCL content was decreased in the SS region and increased in the IMF region.

Often, studies that evaluate the effects of disuse do not continue to study the effect of disuse when mobility is resumed[7,8,86,155]. Our analyses show that many of the effects of disuse become apparent during recovery from immobilization. Indeed, mitochondrial content did not decrease during immobilization, but did decrease during recovery in the SS and central IMF regions. Furthermore, IRS1 did not change during immobilization but increased during recovery. Finally, AKT, P-AKT, and PLIN5 increased and BCL2 decreased during immobilization and remained higher/lower during recovery. As such, these findings highlight the prolonged effect of disuse on muscle metabolism and the importance of continuing to study the effects of disuse past the return of mobility.

One defining aspect of this study was its population. We chose to examine the effects of disuse in women because previous research in older adults found that women may experience greater rates of muscle mass decline during disuse, and thus potentially a greater induction of insulin resistance, as compared with men. However, the findings of the current study suggest that women are protected against disuse-induced impairments in insulin signalling as there was no decrease in any insulin signalling proteins with disuse and AKT actually increased. Given that most studies that have been conducted looking at the effects of disuse on muscle metabolism have included men, and not women, as research participants it is difficult to reconcile whether

our results are contrary to what has been previously found or if they are due to the inclusion of women as participants. Regardless, our findings highlight the importance of including women as research participants. A follow up study that compares the effects of disuse on IMCL storage characteristics, fat metabolism and insulin signalling in both sexes is needed to determine whether sex influences the effect of disuse on these outcomes.

In conclusion, two weeks of unilateral limb immobilization in young, healthy females induced central IMF IMCL accumulation and maintained the protein content of the insulin signalling pathway. Two weeks of recovery was sufficient to normalize IMCL accumulation and ACC phosphorylation status, but FIS1 content remained lower and BAX content remained elevated, suggesting that two weeks of recovery is insufficient to completely reverse the effects of immobilization. Given that women have a lower risk of developing type 2 diabetes than men[181], despite greater adiposity, an effect thought to be mediated by estrogen, it is hypothesized that estrogen may be mediating the effect of disuse on insulin signalling proteins in young women.

References

1. Goodpaster BH, He J, Watkins S, Kelley DE. Skeletal Muscle Lipid Content and Insulin Resistance: Evidence for a Paradox in Endurance-Trained Athletes. *J Clin Endocrinol Metab*. 2001;86(12):5755-5816. doi:10.1210/jc.86.12.5755.
2. Samjoo IA, Safdar A, Hamadeh MJ, et al. Markers of Skeletal Muscle Mitochondrial Function and Lipid Accumulation Are Moderately Associated with the Homeostasis Model Assessment Index of Insulin Resistance in Obese Men. *PLoS One*. 2013;8(6):e66322. doi:10.1371/journal.pone.0066322.
3. Devries MC, Samjoo IA, Hamadeh MJ, et al. Endurance training modulates intramyocellular lipid compartmentalization and morphology in skeletal muscle of lean and obese women. *J Clin Endocrinol Metab*. 2013;98(12):4852-4862. doi:10.1210/jc.2013-2044.
4. Daemen S, Gemmink A, Brouwers B, et al. Distinct lipid droplet characteristics and distribution unmask the apparent contradiction of the athlete's paradox. *Mol Metab*. 2018;17:71-81. doi:10.1016/j.molmet.2018.08.004.
5. Knudsen SH, Hansen LS, Pedersen M, et al. Changes in insulin sensitivity precede changes in body composition during 14 days of step reduction combined with overfeeding in healthy young men. *J Appl Physiol*. 2012;113(1):7-15. doi:10.1152/japplphysiol.00189.2011.
6. Krogh-Madsen R, Thyfault JP, Broholm C, et al. A 2-wk reduction of ambulatory activity attenuates peripheral insulin sensitivity. *J Appl Physiol*. 2010;108(5):1034-1040. doi:00977.2009 [pii]\r10.1152/japplphysiol.00977.2009.
7. Dirks ML, Wall BT, Van De Valk B, et al. One week of bed rest leads to substantial muscle atrophy and induces whole-body insulin resistance in the absence of skeletal muscle lipid accumulation. *Diabetes*. 2016;65(10):2862-2875. doi:10.2337/db15-1661.
8. Wall BT, Dirks ML, Snijders T, Senden JMG, Dolmans J, Van Loon LJC. Substantial skeletal muscle loss occurs during only 5 days of disuse. *Acta Physiol*. 2014;210(3):600-611. doi:10.1111/apha.12190.
9. Gram M, Vigelsø A, Yokota T, et al. Two weeks of one-leg immobilization decreases skeletal muscle respiratory capacity equally in young and elderly men. *Exp Gerontol*. 2014;58:269-278. doi:10.1016/j.exger.2014.08.013.
10. Vigelsø A, Gram M, Wiuff C, et al. Effects of immobilization and aerobic training on proteins related to intramuscular substrate storage and metabolism in young and older men. *Eur J Appl Physiol*. 2016;116(3):481-494. doi:10.1007/s00421-015-3302-x.
11. You J-S, Park M-N, Song W, Lee Y-S. Dietary fish oil alleviates soleus atrophy during immobilization in association with Akt signaling to p70s6k and E3 ubiquitin ligases in rats. *Appl Physiol Nutr Metab*. 2010;35(3):310-318. doi:10.1139/H10-022.
12. Smith GI, Atherton P, Reed DN, et al. Dietary omega-3 fatty acid supplementation increases the rate of muscle protein synthesis in older adults: a randomized controlled trial. *Am J Clin Nutr*. 2011;93(2):402-412. doi:10.3945/ajcn.110.005611.INTRODUCTION.
13. Smith GI, Atherton P, Reeds DN, et al. Omega-3 polyunsaturated fatty acids augment the muscle protein anabolic response to hyperaminoacidemia -hyperinsulinemia in healthy young and middle aged men and women. *Clin Scinence*. 2011;121(6):267-278.

14. Khor VK, Shen W-J, Kraemer FB. Lipid droplet metabolism. *Curr Opin Clin Nutr Metab Care*. 2013;16(6):632-637. doi:10.1097/MCO.0b013e3283651106.Lipid.
15. Gemmink A, Hesselink M. Intramyocellular lipid storage and insulin resistance. *Diaspedia*. 2015;7.
16. MacLaren D, Morton J. Chapter 6 Lipids. In: *Biochemistry for Sport and Exercise Metabolism*. ; 2012:115-118.
17. Watt MJ, Hoy AJ. Lipid metabolism in skeletal muscle: generation of adaptive and maladaptive intracellular signals for cellular function. *AJP Endocrinol Metab*. 2012;302(11):E1315-E1328. doi:10.1152/ajpendo.00561.2011.
18. Gemmink A, Goodpaster BH, Schrauwen P, Hesselink MKC. Intramyocellular lipid droplets and insulin sensitivity, the human perspective. *Biochim Biophys Acta - Mol Cell Biol Lipids*. 2017;1862(10):1242-1249. doi:10.1016/j.bbalip.2017.07.010.
19. Kimmel AR, Sztalryd C. Perilipin 5, a lipid droplet protein adapted to mitochondrial energy utilization. *Curr Opin Lipidol*. 2014;25(2):110-117. doi:10.1038/nmeth.2839.A.
20. Bosma M, Hesselink MKC, Sparks LM, et al. Perilipin 2 improves insulin sensitivity in skeletal muscle despite elevated intramuscular lipid levels. *Diabetes*. 2012;61(11):2679-2690. doi:10.2337/db11-1402.
21. Sztalryd C, Kimmel AR. Perilipins: lipid droplet coat proteins adapted for tissue-specific energy storage and utilization, and lipid cytoprotection. *Biochimie*. 2014;96:96-101. doi:10.1016/j.clinbiochem.2015.06.023.Gut-Liver.
22. MacPherson REK, Vandenboom R, Roy BD, Peters SJ. Skeletal muscle PLIN3 and PLIN5 are serine phosphorylated at rest and following lipolysis during adrenergic or contractile stimulation. *Physiol Rep*. 2013;1(4):n/a-n/a. doi:10.1002/phy2.84.
23. Bosma M, Sparks LM, Hooiveld GJ, et al. Overexpression of PLIN5 in skeletal muscle promotes oxidative gene expression and intramyocellular lipid content without compromising insulin sensitivity. *Biochim Biophys Acta - Mol Cell Biol Lipids*. 2013;1831(4):844-852. doi:10.1016/j.bbalip.2013.01.007.
24. Listenberger LL, Ostermeyer-Fay AG, Goldberg EB, Brown WJ, Brown DA. Adipocyte differentiation-related protein reduces the lipid droplet association of adipose triglyceride lipase and slows triacylglycerol turnover. *J Lipid Res*. 2007;48(12):2751-2761. doi:10.1194/jlr.M700359-JLR200.
25. Bell M, Wang H, Chen H, et al. Consequences of Lipid Droplet Coat Protein Downregulation in Liver Cells. *Diabetes*. 2008;57(8):2037-2045. doi:10.2337/db07-1383.
26. MacPherson REK, Ramos S V., Vandenboom R, Roy BD, Peters SJ. Skeletal muscle PLIN proteins, ATGL and CGI-58, interactions at rest and following stimulated contraction. *AJP Regul Integr Comp Physiol*. 2013;304(8):R644-R650. doi:10.1152/ajpregu.00418.2012.
27. Covington JD, Galgani JE, Moro C, et al. Skeletal muscle perilipin 3 and coatomer proteins are increased following exercise and are associated with fat oxidation. *PLoS One*. 2014;9(3):1-8. doi:10.1371/journal.pone.0091675.
28. Louche K, Badin PM, Montastier E, et al. Endurance exercise training up-regulates lipolytic proteins and reduces triglyceride content in skeletal muscle of obese subjects. *J Clin Endocrinol Metab*. 2013;98(12):4863-4871. doi:10.1210/jc.2013-2058.
29. Ramos S V., MacPherson REK, Turnbull PC, et al. Higher PLIN5 but not PLIN3 content in isolated skeletal muscle mitochondria following acute in vivo contraction in rat hindlimb. *Physiol Rep*. 2014;2(10):1-12. doi:10.14814/phy2.12154.

30. Covington JD, Johannsen DL, Coen PM, et al. Intramyocellular Lipid Droplet Size Rather Than Total Lipid Content is Related to Insulin Sensitivity After 8 Weeks of Overfeeding. *Obesity*. 2017;25(12):2079-2087. doi:10.1002/oby.21980.
31. Peters SJ, Samjoo IA, Devries MC, Stevic I, Robertshaw HA, Tarnopolsky MA. Perilipin family (PLIN) proteins in human skeletal muscle: the effect of sex, obesity, and endurance training. *Appl Physiol Nutr Metab*. 2012;37(4):724-735. doi:10.1139/h2012-059.
32. Bosma M, Minnaard R, Sparks LM, et al. The lipid droplet coat protein perilipin 5 also localizes to muscle mitochondria. *Histochem Cell Biol*. 2012;137(2):205-216. doi:10.1007/s00418-011-0888-x.
33. Wang H, Sreenivasan U, Hu H, et al. Perilipin 5, a lipid droplet-associated protein, provides physical and metabolic linkage to mitochondria. *J Lipid Res*. 2011;52(12):2159-2168. doi:10.1194/jlr.M017939.
34. Wang H, Bell M, Sreenevasan U, et al. Unique regulation of adipose triglyceride lipase (ATGL) by perilipin 5, a lipid droplet-associated protein. *J Biol Chem*. 2011;286(18):15707-15715. doi:10.1074/jbc.M110.207779.
35. Nielsen J, Mogensen M, Vind BF, et al. Increased subsarcolemmal lipids in type 2 diabetes: effect of training on localization of lipids, mitochondria, and glycogen in sedentary human skeletal muscle. *AJP Endocrinol Metab*. 2010;298(3):E706-E713. doi:10.1152/ajpendo.00692.2009.
36. Nielsen J, Christensen AE, Nellemann B, Christensen B. Lipid droplet size and location in human skeletal muscle fibers are associated with insulin sensitivity. *Am J Physiol - Endocrinol Metab*. 2017;313:E721-E730. doi:10.1152/ajpendo.00062.2017.
37. Schiaffino S. Fibre types in skeletal muscle: A personal account. *Acta Physiol*. 2010;199(4):451-463. doi:10.1111/j.1748-1716.2010.02130.x.
38. Crane JD, Devries MC, Safdar A, Hamadeh MJ, Tarnopolsky MA. The Effect of Aging on Human Skeletal Muscle Mitochondrial and Intramyocellular Lipid Ultrastructure. *J Gerontol A Biol Sci Med Sci*. 2010;65(2):119-128. doi:10.1093/gerona/glp179.
39. Chee C, Shannon CE, Burns A, et al. The relative contribution of intramyocellular lipid to whole body fat oxidation is reduced with age, but subsarcolemmal lipid accumulation and insulin resistance are only associated with overweight individuals. *Diabetes*. 2016;65(4):840-850. doi:10.2337/db15-1383.
40. Devries MC, Lowther SA, Glover AW, Hamadeh MJ, Tarnopolsky MA. IMCL area density, but not IMCL utilization, is higher in women during moderate-intensity endurance exercise, compared with men. *Am J Physiol Regul Integr Comp Physiol*. 2007;293(6):R2336-42. doi:10.1152/ajpregu.00510.2007.
41. Tarnopolsky MA, Rennie CD, Robertshaw HA, Fedak-Tarnopolsky SN, Devries MC, Hamadeh MJ. Influence of endurance exercise training and sex on intramyocellular lipid and mitochondrial ultrastructure, substrate use, and mitochondrial enzyme activity. *AJP Regul Integr Comp Physiol*. 2007;292(3):R1271-R1278. doi:10.1152/ajpregu.00472.2006.
42. Høeg LD, Sjøberg KA, Jeppesen J, et al. Lipid-induced insulin resistance affects women less than men and is not accompanied by inflammation or impaired proximal insulin signaling. *Diabetes*. 2011;60(1):64-73. doi:10.2337/db10-0698.
43. Tarnopolsky LJ, MacDougall JD, Atkinson SA, Tarnopolsky MA, Sutton JR. Gender differences in substrate for endurance exercise. *J Appl Physiol*. 1990;68(1):302-308. doi:10.1152/jappl.1990.68.1.302.

44. Tarnopolsky MA. Sex Differences in Exercise Metabolism and the Role of 17-Beta Estradiol. *Med Sci Sports Exerc*. 2008;40(4):648-654. doi:10.1249/MSS.0b013e31816212ff.
45. Mauvais-jarvis F. Estrogen and androgen receptors : regulators of fuel homeostasis and emerging targets for diabetes and obesity. *Trends Endocrinol Metab*. 2011;22(1):24-33. doi:10.1016/j.tem.2010.10.002.
46. Dubé JJ, Amati F, Stefanovic-Racic M, Toledo FGS, Sauers SE, Goodpaster BH. Exercise-induced alterations in intramyocellular lipids and insulin resistance: the athlete's paradox revisited. *AJP Endocrinol Metab*. 2008;294(5):E882-E888. doi:10.1152/ajpendo.00769.2007.
47. Van Loon LJC, Goodpaster BH. Increased intramuscular lipid storage in the insulin-resistant and endurance-trained state. *Pflugers Arch Eur J Physiol*. 2006;451(5):606-616. doi:10.1007/s00424-005-1509-0.
48. Kelley DE, He J, Menshikova E, Ritov VB. Dysfunction of Mitochondria in Human Skeletal Muscle in Type 2 Diabetes. *Diabetes*. 2002;51(10):2944-2950.
49. Li Y, Xu S, Zhang X, Yi Z, Cichello S. Skeletal intramyocellular lipid metabolism and insulin resistance. *Biophys reports*. 2015;1(2):90-98. doi:10.1007/s41048-015-0013-0.
50. Fei W, Shui G, Zhang Y, et al. A role for phosphatidic acid in the formation of "supersized" Lipid droplets. *PLoS Genet*. 2011;7(7). doi:10.1371/journal.pgen.1002201.
51. Van Loon LJC, Koopman R, Manders R, et al. Intramyocellular lipid content in type 2 diabetes patients compared with overweight sedentary men and highly trained endurance athletes. *Am J Physiol - Endocrinol Metab*. 2004;287:558-565. doi:10.1152/ajpendo.00464.2003.
52. Coen PM, Goodpaster BH. Role of intramyocelluar lipids in human health. *Trends Endocrinol Metab*. 2012;23(8):391-398. doi:10.1016/j.tem.2012.05.009.
53. Samuel VT, Petersen KF, Shulman GI. Lipid-induced insulin resistance: unravelling the mechanism. *Lancet*. 2010;375:2267-2277. doi:10.1016/S0140-6736(10)60408-4.
54. Stratford S, Wald DBDE, Summers SA. Ceramide dissociates 3'-phosphoinositide production from pleckstrin homology domain translocation. *Biochem J*. 2001;368(Pt 2):359-368.
55. Jeromson S, Gallagher I, Galloway S, Hamilton D. Omega-3 Fatty Acids and Skeletal Muscle Health. *Mar Drugs*. 2015;13(12):6977-7004. doi:10.3390/md13116977.
56. Amati F, Dubé JJ, Alvarez-Carnero E, et al. Skeletal muscle triglycerides, diacylglycerols, and ceramides in insulin resistance: Another paradox in endurance-trained athletes? *Diabetes*. 2011;60(10):2588-2597. doi:10.2337/db10-1221.
57. Bergman BC, Hunerdosse DM, Kerege A, Playdon MC, Perreault L. Localization and composition of skeletal muscle diacylglcerol predicts insulin resistance in humans. *Diabetologia*. 2012;55(4):1140-1150. doi:10.1007/s00125-011-2419-7.Localisation.
58. Bergman BC, Perreault L, Strauss A, et al. Intramuscular triglyceride synthesis - importance in partitioning muscle lipids in humans. *Am J Physiol - Endocrinol Metab*. 2017;314:E152-D164. doi:10.1152/ajpendo.00142.2017.
59. Bergman BC, Perreault L, Hunerdosse DM, Koehler MC, Samek AM, Eckel RH. Increased intramuscular lipid synthesis and low saturation relate to insulin sensitivity in endurance-trained athletes. *J Appl Physiol*. 2010;108(5):1134-1141. doi:10.1152/japplphysiol.00684.2009.

60. Perreault L, Newsom SA, Strauss A, et al. Intracellular localization of diacylglycerols and sphingolipids influences insulin sensitivity and mitochondrial function in human skeletal muscle. *JCI Insight*. 2018;3(3):1-21. doi:10.1172/JCI.INSIGHT.96805.
61. Dubé JJ, Amati F, Toledo FGS, et al. Effects of weight loss and exercise on insulin resistance, and intramyocellular triacylglycerol, diacylglycerol and ceramide. *Diabetologia*. 2011;54(5):1147-1156. doi:10.1007/s00125-011-2065-0.
62. Bruce CR, Thrush AB, Mertz VA, et al. Endurance training in obese humans improves glucose tolerance and mitochondrial fatty acid oxidation and alters muscle lipid content. *Am J Physiol Endocrinol Metab*. 2006;291:99-107. doi:10.1152/ajpendo.00587.2005.
63. Listenberger LL, Han X, Lewis SE, et al. Triglyceride accumulation protects against fatty acid-induced lipotoxicity. *Proc Natl Acad Sci*. 2003;100(6):3077-3082.
64. Ritter O, Jelenik T, Roden M. Lipid-mediated muscle insulin resistance: different fat, different pathways? *J Mol Med*. 2015;93(8):831-843. doi:10.1007/s00109-015-1310-2.
65. Chang BH-J, Li L, Saha P, Chan L. Absence of adipose differentiation related protein upregulates hepatic VLDL secretion, relieves hepatosteatosis, and improves whole body insulin resistance in leptin-deficient mice. *J Lipid Res*. 2010;51(8):2132-2142. doi:10.1194/jlr.M004515.
66. Bosma M, Kersten S, Hesselink MKC, Schrauwen P. Re-evaluating lipotoxic triggers in skeletal muscle: Relating intramyocellular lipid metabolism to insulin sensitivity. *Prog Lipid Res*. 2012;51(1):36-49. doi:10.1016/j.plipres.2011.11.003.
67. Gemmink A, Bosma M, Kuijpers HJH, et al. Decoration of intramyocellular lipid droplets with PLIN5 modulates fasting-induced insulin resistance and lipotoxicity in humans. *Diabetologia*. 2016;59(5):1040-1048. doi:10.1007/s00125-016-3865-z.
68. de Boer MD, Selby A, Atherton P, et al. The temporal responses of protein synthesis, gene expression and cell signalling in human quadriceps muscle and patellar tendon to disuse. *J Physiol*. 2007;585(Pt 1):241-251. doi:10.1113/jphysiol.2007.142828.
69. Breen L, Stokes KA, Churchward-Venne TA, et al. Two weeks of reduced activity decreases leg lean mass and induces “anabolic resistance” of myofibrillar protein synthesis in healthy elderly. *J Clin Endocrinol Metab*. 2013;98(6):2604-2612. doi:10.1210/jc.2013-1502.
70. Janzen N, Whitfield J, Hoffman N. Interactive Roles for AMPK and Glycogen from Cellular Energy Sensing to Exercise Metabolism. *Int J Mol Sci*. 2018;19(11):3344. doi:10.3390/ijms19113344.
71. Cartee GD. Roles of TBC1D1 and TBC1D4 in insulin- and exercise-stimulated glucose tranpsort of skeletal muscle. *Diabetologia*. 2014;58(1):19-30. doi:10.1088/1367-2630/15/1/015008.Fluid.
72. Pehmøller C, Brandt N, Birk JB, et al. Exercise alleviates lipid-induced insulin resistance in human skeletal muscle-signaling interaction at the level of TBC1 domain family member 4. *Diabetes*. 2012;61(11):2743-2752. doi:10.2337/db11-1572.
73. Treebak JT, Frøsig C, Pehmøller C, et al. Potential role of TBC1D4 in enhanced post-exercise insulin action in human skeletal muscle. *Diabetologia*. 2009;52(5):891-900. doi:10.1007/s00125-009-1294-y.
74. Defronzo RA, Jacot E, Jequier E, Maeder E, Wahren J, Felber JP. The effect of insulin on the disposal of intravenous glucose. 1981;30(December):1000-1007.

75. Gram M, Vigelsø A, Yokota T, Helge JW, Dela F, Hey-Mogensen M. Skeletal muscle mitochondrial H2O2 emission increases with immobilization and decreases after aerobic training in young and older men. *J Physiol*. 2015;593(17):4011-4027. doi:10.1113/JP270211.
76. Kalyani RR, Tra Y, Yeh H, Egan JM, Ferrucci L, Brancati FL. Quadriceps strength, quadriceps power, and gait speed in older U.S. adults with diabetes: results from the National Health and Nutrition Examination survey (NHANES), 1999-2002. *J Am Geriatr Soc*. 2013;61(5):769-775. doi:10.1111/jgs.12204.Quadriceps.
77. Leenders M, Verdijk LB, van der Hoeven L, et al. Patients with type 2 diabetes show a greater decline in muscle mass, muscle strength, and functional capacity with aging. *J Am Med Dir Assoc*. 2013;14(8):585-592. doi:10.1016/j.jamda.2013.02.006.
78. Olsen R, Krogh-Madsen R. Metabolic Responses to Reduced Daily Steps. *Am Med Assoc*. 2008;299(11):1261-1263.
79. Walhin J, Richardson JD, Betts JA, Thompson D. Exercise counteracts the effets of short-term overfeeding and reduced physical activity independent of energy imbalance in healthy young men. *J Physiol*. 2013;591(24):1-38. doi:10.1113/jphysiol.2013.262709.This.
80. Stephens BR, Granados K, Zderic TW, Hamilton MT, Braun B. Effects of 1 day of inactivity on insulin action in healthy men and women: Interaction with energy intake. *Metabolism*. 2011;60(7):941-949. doi:10.1016/j.metabol.2010.08.014.
81. Blanc S, Normand S, Pachiaudi C, Fortrat JO, Laville M, Gharib C. Fuel homeostasis during physical inactivity induced by bed rest. *J Clin Endocrinol Metab*. 2000;85(6):2223-2233. doi:10.1210/jc.85.6.2223.
82. Di Meo S, Iossa S, Venditti P. Skeletal muscle insulin resistance: Role of mitochondria and other ROS sources. *J Endocrinol*. 2017;233(1):R15-R42. doi:10.1530/JOE-16-0598.
83. Mikines KJ, Richter E a, Dela F, Galbo H. Seven days of bed rest decrease insulin action on glucose uptake in leg and whole body. *J Appl Physiol*. 1991;70(3):1245-1254. http://www.ncbi.nlm.nih.gov/pubmed/2032990.
84. Tabata I, Suzuki Y, Fukunaga T, Yokozeki T, Akima H, Funato K. Resistance training affects GLUT-4 content in skeletal muscle of humans after 19 days of head-down bed rest. *J Appl Physiol*. 1999;86(3):909-914. doi:10.1152/jappl.1999.86.3.909.
85. Biensø RS, Ringholm S, Kiilerich K, et al. GLUT4 and glycogen synthase are key players in bed rest-induced insulin resistance. *Diabetes*. 2012;61(5):1090-1099. doi:10.2337/db11-0884.
86. Vigelsø A, Gram M, Dybboe R, et al. The effect of age and unilateral leg immobilization for 2 weeks on substrate utilization during moderate-intensity exercise in human skeletal muscle. *J Physiol*. 2016;594(8):2339-2358. doi:10.1113/JP271712.
87. Sanderson A, Radda GK, Leighton B. Abnormal Regulation of Hexokinase in Insulin-Resistant Skeletal Muscle. *Biochem Mol Med*. 1996;59:80-86.
88. Conte M, Vasuri F, Trisolino G, et al. Increased Plin2 Expression in Human Skeletal Muscle Is Associated with Sarcopenia and Muscle Weakness. *PLoS One*. 2013;8(8):1-10. doi:10.1371/journal.pone.0073709.
89. Conte M, Vasuri F, Bertaggia E, Armani A. Differential expression of perilipin 2 and 5 in human skeletal muscle during aging and their association with atrophy- related genes. *Biogeronotology*. 2015;16:329-340. doi:10.1007/s10522-014-9549-5.

90. Stettler R, Binnert C, Acheson K, Decombaz J, Boesch C, Tappy L. Interaction between physical activity and dietary lipids on insulin sensitivity in healthy man. *Diabetes*. 2004;53-Suppl2:A570.
91. Gemmink A, Daemen S, Brouwers B, et al. Dissociation of intramyocellular lipid storage and insulin resistance in trained athletes and type 2 diabetes patients; involvement of perilipin 5? *J Physiol*. 2018;596(5):857-868. doi:10.1113/JP275182.
92. Cree MG, Paddon-jones D, Newcomer BR, Ronsen O, Wolfe RR, Ferrando A. 28 day bed rest with hypercortisolemia induces peripheral insulin resistance and increases intramuscular triglycerides. *Metabolism*. 2010;59(5):703-710. doi:10.1016/j.metabol.2009.09.014.28.
93. Wall BT, Dirks ML, Snijders T, et al. Short-term muscle disuse atrophy is not associated with increased intramuscular lipid deposition or a decline in the maximal activity of key mitochondrial enzymes in young and older males. *Exp Gerontol*. 2015;61:76-83. doi:10.1016/j.exger.2014.11.019.
94. Iqbal S, Ostojic O, Singh K, Joseph A-M, Hood DA. Expression of mitochondrial fission and fusion regulatory proteins in skeletal muscle during chronic use and disuse. *Muscle Nerve*. 2013;48:963-970. doi:10.1002/mus.23838.
95. Liu J, Peng Y, Feng Z, et al. Reloading functionally ameliorates disuse-induced muscle atrophy by reversing mitochondrial dysfunction, and similar benefits are gained by administering a combination of mitochondrial nutrients. *Free Radic Biol Med*. 2014;69:116-128. doi:10.1016/j.freeradbiomed.2014.01.003.
96. Schrauwen P, Schrauwen-Hinderling V, Hoeks J, Hesselink MKC. Mitochondrial dysfunction and lipotoxicity. *Biochim Biophys Acta - Mol Cell Biol Lipids*. 2010;1801(3):266-271. doi:10.1016/j.bbalip.2009.09.011.
97. Iqbal S, Hood DA. The role of mitochondrial fusion and fission in skeletal muscle function and dysfunction. *Front Biosci*. 2015;20:157-172.
98. Youle RJ, Karbowski M. Mitochondrial fission in apoptosis. *Nat Rev Mol Cell Biol*. 2005;6:657-663.
99. Westermann B. Molecular Machinery of Mitochondrial Fusion and. *J Biol Chem*. 2008;283(20):13501-13505. doi:10.1074/jbc.R800011200.
100. Talbert EE, Smuder AJ, Min K, Kwon OS, Szeto HH, Powers SK. Immobilization-induced activation of key proteolytic systems in skeletal muscles is prevented by a mitochondria-targeted antioxidant. *J Appl Physiol*. 2013;115(4):529-538. doi:10.1152/japplphysiol.00471.2013.
101. Wu S, Zhou F, Zhang Z, Xing D. Mitochondrial oxidative stress causes mitochondrial fragmentation via differential modulation of mitochondrial fission-fusion proteins. *FEBS J*. 2011;278(6):941-954. doi:10.1111/j.1742-4658.2011.08010.x.
102. Powers SK, Wiggs MP, Duarte JA, Zergeroglu AM, Demirel HA. Mitochondrial signaling contributes to disuse muscle atrophy. *AJP Endocrinol Metab*. 2012;303(1):E31-E39. doi:10.1152/ajpendo.00609.2011.
103. Calvani R, Joseph A, Bossola M. Mitochondrial pathways in sarcopenia of aging and disuse muscle atrophy. *Biol Chem*. 2013;394(3):393-414. doi:10.1515/hsz-2012-0247.Mitochondrial.
104. Romanello V, Guadagnin E, Gomes L, et al. Mitochondrial fission and remodelling contributes to muscle atrophy. *EMBO J*. 2010;29(10):1774-1785. doi:10.1038/emboj.2010.60.

105. Karbowski M, Norris KL, Cleland MM, Jeong S, Youle RJ. Role of Bax and Bak in mitochondrial morphogenesis. 2006;443(October). doi:10.1038/nature05111.
106. Frezza C, Cipolat S, Brito OM De, et al. OPA1 Controls Apoptotic Cristae Remodeling Independently from Mitochondrial Fusion. 2006:177-189. doi:10.1016/j.cell.2006.06.025.
107. Zhang S, Zhang Y, Li B, Chen N. Physical inactivity induces the atrophy of skeletal muscle of rats through activating AMPK / FoxO3 signal pathway. *Eur Rev Med Pharmocol Sci*. 2018;22(1):199-209.
108. Liu J, Peng Y, Cui Z, et al. Depressed Mitochondrial Biogenesis and Dynamic Remodeling in Mouse Tibialis Anterior and Gastrocnemius Induced by 4-week Hindlimb Unloading. *IUBMB Life*. 2012;64(11):901-910. doi:10.1002/iub.1087.
109. Wagatsuma A, Kotake N, Kawachi T, Shiozuka M, Yamada S, Matsuda R. Mitochondrial adaptations in skeletal muscle to hindlimb unloading. *Mol Cell Biochem*. 2011;350:1-11. doi:10.1007/s11010-010-0677-1.
110. Standley RA, Distefano G, Pereira SL, et al. Effects of β-hydroxy-β-methylbutyrate (HMB) on skeletal muscle mitochondrial content and dynamics, and lipids after 10 days of bed rest in older adults. *J Appl Physiol*. 2017;(3):jap.00192.2017. doi:10.1152/japplphysiol.00192.2017.
111. Dupont-versteegden EE. Apoptosis in skeletal muscle and its relevance to atrophy. *World J Gastroenterol*. 2006;12(46):7463-7466.
112. Willis S, Day CL, Hinds MG, et al. The Bcl-2-regulated apoptotic pathway. *J Cell Sci*. 2003;116(20):4053-4056. doi:10.1242/jcs.00754.
113. Sanchez AMJ, Bernardi H, Py G, Candau RB. Autophagy is essential to support skeletal muscle plasticity in response to endurance exercise. *Am J Physiol - Regul Integr Comp Physiol*. 2014;307:R956-R969. doi:10.1152/ajpregu.00187.2014.
114. Kaur J, Debnath J. Autophagy at the crossroads of catabolism and anabolism. *Nat Rev Mol Cell Biol*. 2015;16(8):461-472. doi:10.1038/nrm4024.
115. Keane KN, Cruzat VF, Carlessi R, De Bittencourt PIH, Newsholme P. Molecular Events Linking Oxidative Stress and Inflammation to Insulin Resistance and β-Cell Dysfunction. *Oxid Med Cell Longev*. 2015;2015. doi:10.1155/2015/181643.
116. Jeng PS, Inoue-yamauchi A, Hsieh JJ, Cheng EH. BH3-dependent and independent activation of BAX and BAK in mitochondrial apoptosis. *Curr Opin Physiol*. 2018;3:71-81.
117. Pierre N, Appriou Z, Gratas-Delamarche A, Derbré F. From physical inactivity to immobilization: Dissecting the role of oxidative stress in skeletal muscle insulin resistance and atrophy. *Free Radic Biol Med*. 2016;98:197-207. doi:10.1016/j.freeradbiomed.2015.12.028.
118. Hu N, Chang H, Du B, et al. Tetramethylpyrazine ameliorated disuse-induced gastrocnemius muscle atrophy in hindlimb unloading rats through suppression of Ca 2 + / ROS-mediated apoptosis. *Appl Physiol Nutr Metab*. 2017;42(2):117-127.
119. Poudyal H, Panchal SK, Diwan V, Brown L. Omega-3 fatty acids and metabolic syndrome: Effects and emerging mechanisms of action. *Prog Lipid Res*. 2011;50(4):372-387. doi:10.1016/j.plipres.2011.06.003.
120. Smith G, Julliand S. Fish oil–derived n–3 PUFA therapy increases muscle mass and function in healthy older adults. *Am J Clin Nutr*. 2015;102:115-122. doi:10.3945/ajcn.114.105833.INTRODUCTION.

121. Da Boit M, Sibson R, Sivasubramaniam S, et al. Sex differences in the effect of fish-oil supplementation on the adaptive response to resistance exercise training in older people : a randomized. 2016;105:151-158. doi:10.3945/ajcn.116.140780.151.
122. Aas V, Rokling-Andersen MH, Kase ET, Thoresen GH, Rustan AC. Eicosapentaenoic acid (20:5 n-3) increases fatty acid and glucose uptake in cultured human skeletal muscle cells. *J Lipid Res*. 2006;47(2):366-374. doi:10.1194/jlr.M500300-JLR200.
123. Lanza IR, Blachnio-Zabielska A, Johnson ML, et al. Influence of fish oil on skeletal muscle mitochondrial energetics and lipid metabolites during high-fat diet. *Am J Physiol - Endocrinol Metab*. 2013;304(12):1391-1403.
124. Le Foll C, Corporeau C, Le Guen V, Gouygou J, Berge J, Delarue J. Long chain n-3 polyunsaturated fatty acids dissociate phosphorylation of Akt from phosphatidylinositol 3'-kinase activity in rats. *Am J Physiol - Endocrinol Metab*. 2007;292(4):1223-1230.
125. Lalia AZ, Lanza IR. Insulin-sensitizing effects of omega-3 fatty acids: Lost in translation? *Nutrients*. 2016;8(6). doi:10.3390/nu8060329.
126. Storlien LH, Jenkins a B, Chisholm DJ, Pascoe WS, Khouri S, Kraegen EW. Influence of dietary fat composition on development of insulin resistance in rats. Relationship to muscle triglyceride and omega-3 fatty acids in muscle phospholipid. *Diabetes*. 1991;40(2):280-289. doi:10.2337/diab.40.2.280.
127. Mori Y, Murakawa Y, Yokoyama J, et al. Effect of highly purified eicosapentaenoic acid ethyl ester on insulin resistance and hypertension in Dahl salt-sensitive rats. *Metabolism*. 1999;48(9):1089-1095.
128. Couet C, Delarue J, Ritz P, Antoine J, Lamisse F. Effect of dietary fish oil on body fat mass and basal fat oxidation in healthy adults. *Int J Obes*. 1997;21:637-643. doi:10.1038/sj.ijo.0800451.
129. Logan SL, Spriet LL. Omega-3 fatty acid supplementation for 12 weeks increases resting and exercise metabolic rate in healthy community- dwelling older females. *PLoS One*. 2015;10(12):1-18. doi:10.1371/journal.pone.0144828.
130. Barber E, Sinclair AJ, Cameron-smith D. Comparative actions of omega-3 fatty acids on in-vitro lipid droplet formation. *Prostaglandins Leukot Essent Fat Acids*. 2013;89(5):359-366. doi:10.1016/j.plefa.2013.07.006.
131. Andersson A, Nalsen C, Tengblad S, Vessby B. Fatty acid composition of skeletal muscle reflects dietary fat composition in humans. *Am J Clin Nutr*. 2002;76(6):1222-1229.
132. Tauchi-Sato K, Ozeki S, Houjou T, Taguchi R, Fujimoto T. The Surface of Lipid Droplets Is a Phospholipid Monolayer with a Unique Fatty Acid Composition. *J Biol Chem*. 2002;277(46):44507-44512. doi:10.1074/jbc.M207712200.
133. Bartz R, Li W, Venables B, et al. Lipidomics reveals that adiposomes store ether lipids and mediate phospholipid traffic. *J Lipid Res*. 2007;48:837-847. doi:10.1194/jlr.M600413-JLR200.
134. Herbst EAF, Paglialunga S, Gerling C, et al. Omega-3 supplementation alters mitochondrial membrane composition and respiration kinetics in human skeletal muscle. *J Physiol*. 2014;592(6):1341-1352. doi:10.1113/jphysiol.2013.267336.
135. Coen PM, Dubé JJ, Amati F, et al. Insulin resistance is associated with higher intramyocellular triglycerides in type I but not type II myocytes concomitant with higher ceramide content. *Diabetes*. 2010;59(1):80-88. doi:10.2337/db09-0988.
136. Ahmadian M, Suh JM, Hah N, et al. PPARγ signaling and metabolism: the good, the bad, and the future. *Nat Med*. 2013;19(5). doi:10.1038/nm.3159.PPAR.

137. Philp LK, Heilbronn LK, Janovska A, Wittert GA. Dietary enrichment with fish oil prevents high fat-induced metabolic dysfunction in skeletal muscle in mice. *PLoS One*. 2015;10(2):1-19. doi:10.1371/journal.pone.0117494.
138. Bergouignan A, Rudwill F, Simon C, Blanc S. Physical inactivity as the culprit of metabolic inflexibility: evidence from bed-rest studies. *J Appl Physiol*. 2011;111(4):1201-1210. doi:10.1152/japplphysiol.00698.2011.
139. De Bock K, Dresselaers T, Kiens B, Richter EA, Hecke P Van, Hespel P. Evaluation of intramyocellular lipid breakdown during exercise by biochemical assay , NMR spectroscopy , and Oil Red O staining. *Am J Physiol - Endocrinol Metab*. 2007;293:428-434. doi:10.1152/ajpendo.00112.2007.
140. Prats C, Gomez-cabello A, Nordby P, et al. An Optimized Histochemical Method to Assess Skeletal Muscle Glycogen and Lipid Stores Reveals Two Metabolically Distinct Populations of Type I Muscle Fibers. *PLoS One*. 2013;8(10). doi:10.1371/journal.pone.0077774.
141. Namimatsu S, Ghazizadeh M, Sugisaki Y. Reversing the Effects of Formalin Fixation with Citraconic Anhydride and Heat : A Universal Antigen Retrieval Method The Journal of Histochemistry & Cytochemistry. 2005;53(1):3-11. doi:10.1369/jhc.4C6466.2005.
142. Park C, Kim HL, Chang B, et al. Overview of Immunoelectron Microscopy. *Appl Microsc*. 2018;48(4):87-95.
143. De Boer P, Hoogenboom JP, Giepmans BNG. Correlated light and electron microscopy : ultrastructure lights up! *Nat Methods*. 2015;12(6):503-513. doi:10.1038/nmeth.3400.
144. Perkovic M, Kunz M, Endesfelder U, et al. Correlative Light- and Electron Microscopy with chemical tags. *J Struct Biol*. 2014;186(2):205-213. doi:10.1016/j.jsb.2014.03.018.
145. Velan SS, Durst C, Lemieux SK, et al. Investigation of Muscle Lipid Metabolism by Localized One- and Two-Dimensional MRS Techniques using a Clinical 3T MRI / MRS Scanner. *J Magn Reson Imaging*. 2007;24:192-199. doi:10.1002/jmri.20786.
146. Research CI of H, Natural Sciences and Engineering Council of Canada, Social Sciences and Humanities Research Council of Canada. Tri-Council Policy Sstatement: Ethical Conduct for Research Involving Humans. 2014.
147. WMA General Assembly. WMA DECLARATION OF HELSINKI – ETHICAL PRINCIPLES FOR Scienti c Requirements and Research Protocols. *World Med Assoc*. 2013;64.
148. Dahl R, Larsen S, Dohlmann TL, et al. Three-dimensional reconstruction of the human skeletal muscle mitochondrial network as a tool to assess mitochondrial content and structural organization. *Acta Physiol*. 2015;213(1):145-155. doi:10.1111/apha.12289.
149. Steinberg GR, Kemp BE. AMPK in Health and Disease. *Physiol Rev*. 2009;89:1025-1078. doi:10.1152/physrev.00011.2008.
150. Mcglory C, Gorissen SHM, Kamal M, et al. Omega-3 fatty acid supplementation attenuates skeletal muscle disuse atrophy during two weeks of unilateral leg immobilization in healthy young women. *FASEB J*. 2019;33(3):4586-4597. doi:10.1096/fj.201801857RRR.
151. Ritov VB, Menshikova E V, He J, Ferrell RE, Goodpaster BH, Kelley DE. Deficiency of Subsarcolemmal Mitochondria in Obesity and Type 2 Diabetes. *Diabetes*. 2005;54(1):8 LP-14. http://diabetes.diabetesjournals.org/content/54/1/8.abstract.

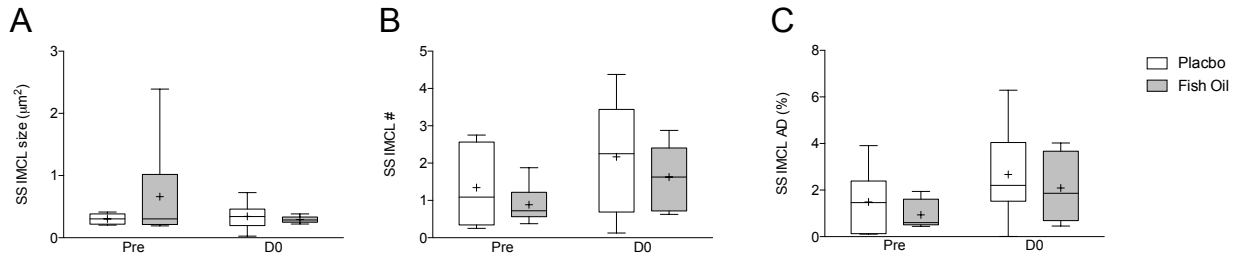
152. Morino K, Petersen KF, Dufour S, et al. Reduced mitochondrial density and increased IRS-1 serine phosphorylation in muscle of insulin-resistant offspring of type 2 diabetic parents. *J Clin Invest*. 2005;115(12):3587-3593. doi:10.1172/JCI25151.
153. Kelley DE, Goodpaster B, Wing RR, Simoneau J, David E. Skeletal muscle fatty acid metabolism in association with insulin resistance , obesity , and weight loss. *Am J Physiol*. 1999;277(6 Pt 1):E1130-41.
154. Simoneau JA, Veerkamp JH, Turcotte LP, Kelley DE. Markers of capacity to utilize fatty acids in human skeletal muscle: relation to insulin resistance and obesity and effects of weight loss. *FASEB J*. 1999;13(14):2051-2060.
155. Miotto PM, Mcglory C, Bahniwal R, Kamal M, Phillips SM, Holloway GP. Supplementation with dietary w-3 mitigates immobilization-induced reductions in skeletal muscle mitochondrial respiration in young women. *FASEB J*. 2019:1-9. doi:10.1096/fj.201900095R.
156. MacPherson REK, Peters SJ. Piecing together the puzzle of perilipin proteins and skeletal muscle lipolysis. *Appl Physiol Nutr Metab*. 2015;40(7):641-651. doi:10.1139/apnm-2014-0485.
157. Nielsen J, Suetta C, Hvid LG, Schrøder HD, Aagaard P, Ørtenblad N. Subcellular localization-dependent decrements in skeletal muscle glycogen and mitochondria content following short-term disuse in young and old men. *Am J Physiol - Endocrinol Metab*. 2010;299:1053-1060. doi:10.1152/ajpendo.00324.2010.
158. Mitchell CJ, D'Souza RF, Mitchell SM, et al. Impact of dairy protein during limb immobilization and recovery on muscle size and protein synthesis; a randomized controlled trial. *J Appl Physiol*. 2018;124:717-728. doi:10.1152/japplphysiol.00803.2017.
159. Slimani L, Micol D, Amat J, et al. The worsening of tibialis anterior muscle atrophy during recovery post-immobilization correlates with enhanced connective tissue area , proteolysis , and apoptosis. *Am J Physiol - Endocrinol Metab*. 2012;303:1335-1347. doi:10.1152/ajpendo.00379.2012.
160. Giotakis AI, Kontos CK, Manolopoulos LD, Sismanis A, Konstadoulakis MM, Scorilas A. High BAX / BCL2 mRNA ratio predicts favorable prognosis in laryngeal squamous cell carcinoma , particularly in patients with negative lymph nodes at the time of diagnosis. *Clin Biochem*. 2016;49(12):890-896. doi:10.1016/j.clinbiochem.2016.04.010.
161. Adhihetty PJ, Leary MFNO, Chabi B, Wicks KL, Hood DA. Effect of denervation on mitochondrially mediated apoptosis in skeletal muscle. *J Appl Physiol*. 2019;3:1143-1151. doi:10.1152/japplphysiol.00768.2006.
162. Vazeille E, Codran A, Claustre A, et al. The ubiquitin-proteasome and the mitochondria-associated apoptotic pathways are sequentially downregulated during recovery after immobilization-induced muscle atrophy. *Am J Physiol - Endocrinol Metab*. 2008;295:1181-1190. doi:10.1152/ajpendo.90532.2008.
163. Baldwin KM, Haddad F. Skeletal muscle plasticity: cellular and molecular responses to altered physical activity paradigms. *Am J Phys Med Rehabil*. 2002;81(11):40-51. doi:10.1097/01.PHM.0000029723.36419.0D.
164. Staron R, Hagerman F, Hikida R, et al. Fiber Type Composition of the Vastus Lateralis Muscle of Young Men and Women. *J Histochem Cytochem*. 2000;48(5):623-629.
165. Lionetti L, Mollica MP, Donizzetti I, et al. High-Lard and High-Fish-Oil Diets Differ in Their Effects on Function and Dynamic Behaviour of Rat Hepatic Mitochondria. *PLoS Genet*. 2014;9(3):e92753. doi:10.1371/journal.pone.0092753.

166. Hilder TL, Baer LA, Fuller PM, et al. Insulin-independent pathways mediating glucose uptake in hindlimb-suspended skeletal muscle. *J Appl Physiol*. 2005;99:2181-2188. doi:10.1152/japplphysiol.00743.2005.
167. Hilder TL, Tou JCL, Grindeland RE, Wade CE, Graves LM. Phosphorylation of insulin receptor substrate-1 serine 307 correlates with JNK activity in atrophic skeletal muscle. *FEBS Lett*. 2003;553:63-67. doi:10.1016/S0014-5793(03)00972-4.
168. Kiens B. Skeletal Muscle Lipid Metabolism in Exercise and Insulin Resistance. *Physiol Rev*. 2006:205-243. doi:10.1152/physrev.00023.2004.
169. Hirosumi J, Tuncman G, Chang L, et al. A central role for JNK in obesity and insulin resistance. *Nature*. 2002;420:333-336.
170. Mortensen B, Friedrichsen M, Andersen NR, et al. Physical inactivity affects skeletal muscle insulin signaling in a birth-weight dependent manner. *J Diabetes Complications*. 2014;28(1):71-78. doi:10.1016/j.jdiacomp.2013.09.002.
171. Cannavino J, Brocca L, Sandri M, Grassi B, Bottinelli R, Pellegrino MA. The role of alterations in mitochondrial dynamics and PGC-1α over-expression in fast muscle atrophy following hindlimb unloading. *J Physiol*. 2015;593(8):1981-1995. doi:10.1113/jphysiol.2014.286740.
172. Eijnde BO, Derave W, Wojtaszewski JFP, et al. AMP kinase expression and activity in human skeletal muscle : effects of immobilization , retraining , and creatine supplementation. 2019:1228-1233. doi:10.1152/japplphysiol.00665.2004.
173. Mirzoev T, Tyganov S, Vilchinskaya N, Lomonosova Y, Shenkman B. Key Markers of mTORC1-Dependent and mTORC1-Independent Signaling Pathways Regulating Protein Synthesis in Rat Soleus Muscle during Early Stages of Hindlimb Unloading. *Cell Physiol Biochem*. 2016;39(3):1011-1020. doi:10.1159/000447808.
174. Nedergaard A, Jespersen JG, Pingel J, et al. Effects of 2 weeks lower limb immobilization and two separate rehabilitation regimens on gastrocnemius muscle protein turnover signaling and normalization genes. *BMC Res Notes*. 2012;5:166.
175. Lang SM, Kazi AA, Hong-brown L, Lang CH. Delayed Recovery of Skeletal Muscle Mass following Hindlimb Immobilization in mTOR Heterozygous Mice. *PLoS One*. 2012;7(6). doi:10.1371/journal.pone.0038910.
176. Whiteman EL, Cho H, Birnbaum MJ. Role of Akt / protein kinase B in metabolism. *Trends Endocrinol Metab*. 2002;13(10):444-451.
177. Bilodeau PA, Coyne ES, Wing SS. The ubiquitin proteasome system in atrophying skeletal muscle : roles and regulation. *Am J Physiol - Cell Physiol*. 2016;311:392-403. doi:10.1152/ajpcell.00125.2016.
178. Bodine SC, Stitt TN, Gonzalez M, et al. Akt mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. *Nat Cell Biol*. 2001;3(November).
179. Saxton RA, Sabatini DM. mTOR Signaling in Growth, Metabolism, and Disease. *Cell*. 2018;168(6):960-976. doi:10.1016/j.cell.2017.02.004.mTOR.
180. You J, Anderson GB, Dooley MS, Hornberger TA. The role of mTOR signaling in the regulation of protein synthesis and muscle mass during immobilization in mice. *Dis Model Mech*. 2015;8:1059-1069. doi:10.1242/dmm.019414.
181. Saengsirisuwan V, Pongseeda S, Prasannarong M, Vichaiwong K, Toskulkao C. Modulation of insulin resistance in ovariectomized rats by endurance exercise training and estrogen replacement. *Metabolism*. 2009;58(1):38-47. doi:10.1016/j.metabol.2008.08.004.

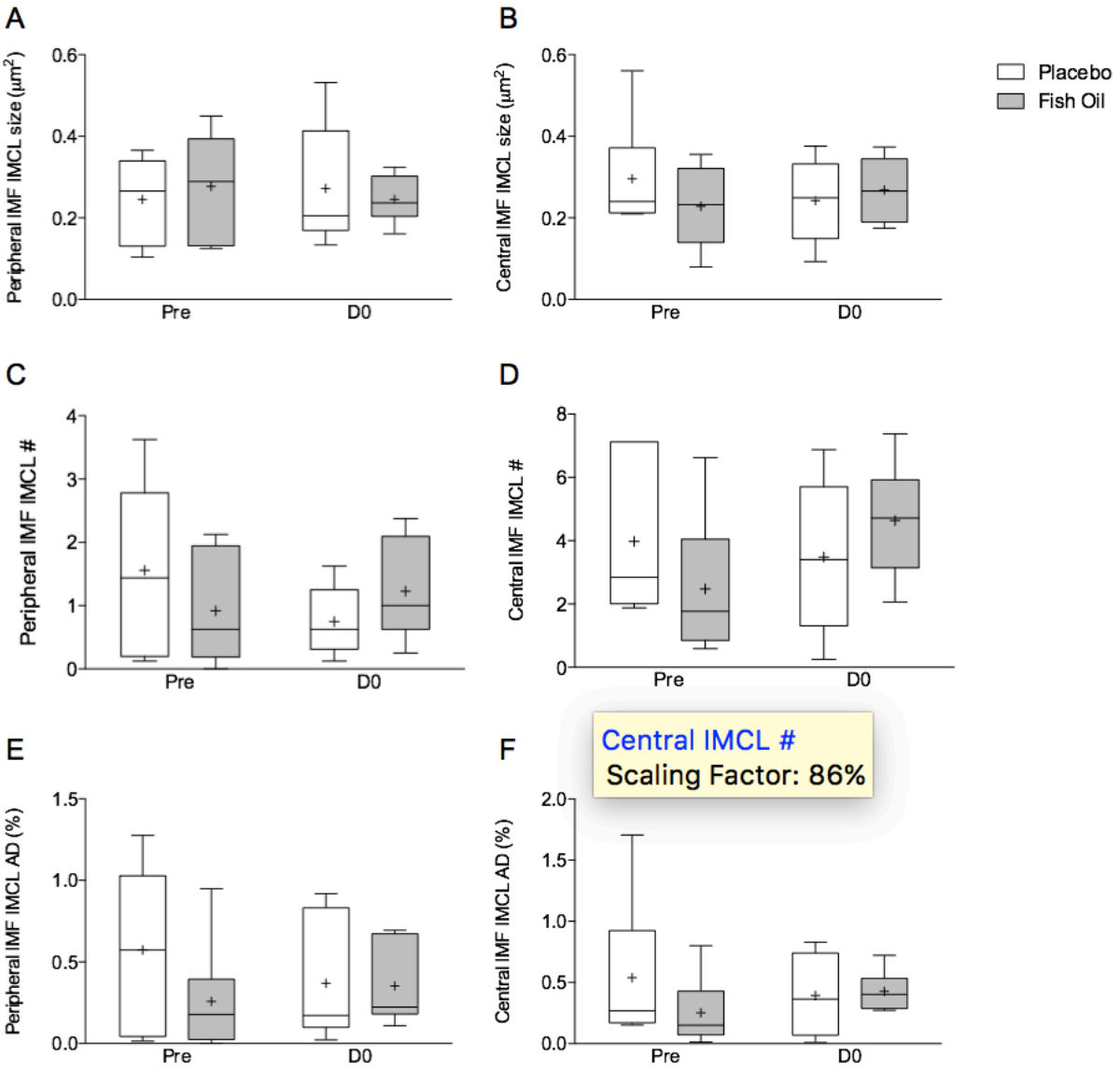
182. Kumagai S, Holmang A, Bjorntorp P. The effects of oestrogen and progesterone on insulin sensitivity in female rats. *Acta Physiol Scand*. 1993;149:91-97.
183. Mcglory C, Allmen MT Von, Stokes T, et al. Failed recovery of glycemic control and myofibrillar protein synthesis with two weeks of physical inactivity in overweight, pre-diabetetic older adults. *Journals Gerontol Ser A*. 2017;00(00):1-8. doi:10.1093/gerona/glx203/4583629.
184. Zhang X, Trevino MB, Wang M, et al. Impaired Mitochondrial Energetics Characterize Poor Early Recovery of Muscle Mass Following Hind Limb Unloading in Old Mice. *Gerontol Soc Am*. 2018;73(10):1313-1322. doi:10.1093/gerona/gly051.
185. Brenna JT, Plourde M, Stark KD, Jones PJ, Lin Y. Best practices for the design, laboratory analysis, and reporting of trials involving fatty acids. *Am J Clin Nutr*. 2018;108:211-227. doi:10.1093/ajcn/nqy089.
186. Bostock EL, Morse CI, Winwood K, McEwan IM, Onambélé-Pearson GL. Omega-3 fatty acids and vitamin D in immobilisation: Part B- Modulation of muscle functional, vascular and activation profiles. *J Nutr Heal Aging*. 2017;21(1):59-66. doi:10.1007/s12603-016-0711-4.
187. Ghadge A, Harsulkar A, Karandikar M, Pandit V, Kuvalekar A. Comparative anti-inflammatory and lipid- normalizing effects of metformin and omega-3 fatty acids through modulation of transcription factors in diabetic rats. *Genes Nutr*. 2016;11(10):1-12. doi:10.1186/s12263-016-0518-4.
188. Kitajka K, Sinclair AJ, Weisinger RS, Weisinger HS, Mathai M. Effects of dietary omega-3 polyunsaturated fatty acids on brain gene expression. *Proc Natl Acad Sci*. 2004;101(30):10931-10936.
189. Magkos F, Wang X, Mittendorfer B. Metabolic actions of insulin in men and women. *Nutrition*. 2010;26(7-8):686-693. doi:10.1016/j.nut.2009.10.013.
190. Kautzky-Willer A, Harreiter J, Pacini G. Sex and gender differences in risk, pathophysiology and complications of type 2 diabetes mellitus. *Endocr Rev*. 2016;37(3):278-316. doi:10.1210/er.2015-1137.
191. Gillen JB, Percival ME, Ludzki A, Tarnopolsky MA, Gibala MJ. Interval Training in the Fed or Fasted State Improves Body Composition and Muscle Oxidative Capacity in Overweight Women. *Clin Trials Behav Pharmacother Devices, Surg*. 2013;21(11):2249-2255. doi:10.1002/oby.20379.
192. Arad AD, Dimenna FJ, Thomas N, et al. High-intensity interval training without weight loss improves exercise but not basal or insulin-induced metabolism in overweight / obese African American women. *J Appl Physiol*. 2015;119:352-362. doi:10.1152/japplphysiol.00306.2015.

Appendix

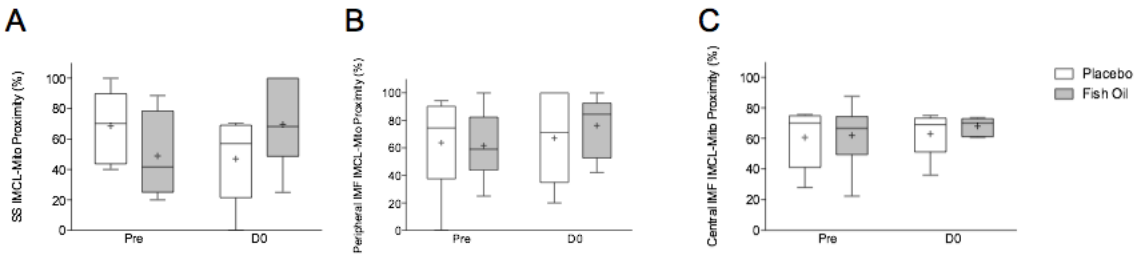
Supplementary data examining the effect of 4-weeks of omega-3 supplementation on IMCL and mitochondrial characteristics.



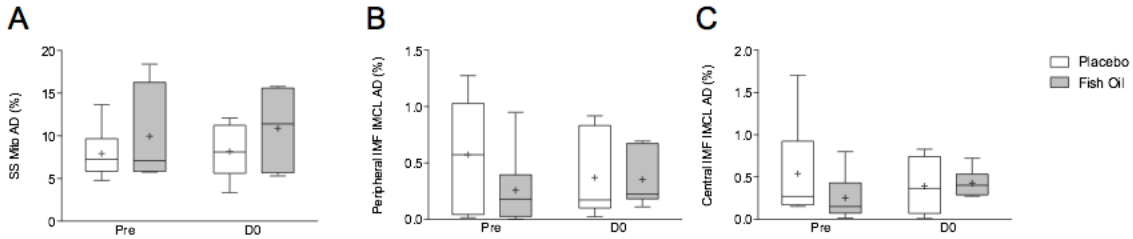
Supplementary figure 1 IMCL size (A), number (B), and area density (C) in the subsarcolemmal (SS) region of skeletal muscle before supplementation (Pre) and before immobilization (D0) after 4-weeks of supplementation. The box plot shows the median (line) and mean (+), with the box representing the IQR and the whiskers representing the maximum and minimum values. Data are 13 participants. Analysis by 2-way ANOVA. Clear: Placebo. Grey: Fish Oil. Pre: Before supplementation. D0: Before immobilization, after 4-weeks of supplementation.AD: area density; IMCL: intramyocellular lipid.



Supplementary figure 2 IMCL size (A,B), number (D,E), and area density (G,H) in the peripheral, central, and total intermyofibrillar (IMF) regions, respectively, of skeletal muscle after 4-weeks of supplementation. The box plot shows the median (line) and mean (+), with the box representing the IQR and the whiskers representing the maximum and minimum values. Data are 13 participants. Analysis by 2-way ANOVA. Clear: Placebo. Grey: Fish Oil. Pre: Before supplementation. D0: Before immobilization, after 4-weeks of supplementation. AD: area density; IMCL: intramyocellular lipid.



Supplementary figure 3 Proximity of IMCL and mitochondrial in the subsarcolemmal (SS) region (A) and the peripheral (B), and central (C) intermyofibrillar (IMF) region, represented as a percentage of IMCL that are touching at least 1 mitochondrion in skeletal muscle after 4-weeks of supplementation. The box plot shows the median (line) and mean (+), with the box representing the IQR and the whiskers representing the maximum and minimum values. Data are 13 participants. Analysis by 2-way ANOVA. Clear: Placebo. Grey: Fish Oil. Pre: Before supplementation. D0: Before immobilization, after 4-weeks of supplementation. IMCL: intramyocellular lipid; Mito: mitochondria.



Supplementary figure 4 Mitochondrial area density in the subsarcolemmal (SS) region (A) and the peripheral (B), and central (C) intermyofibrillar (IMF) region of skeletal muscle after 4-weeks of supplementation. The box plot shows the median (line) and mean (+), with the box representing the IQR and the whiskers representing the maximum and minimum values. Data are 13 participants. Analysis by 2-way ANOVA. Clear: Placebo. Grey: Fish Oil. Pre: Before supplementation. D0: Before immobilization, after 4-weeks of supplementation. IMCL: intramyocellular lipid; Mito: mitochondria.