The Protective Effects of Omega-3 Fatty Acids Against Skeletal Muscle Cell Lipotoxicity

Bill Tachtsis

BExSSc (Hons), BBiomedSc

A thesis submitted in fulfilment of the requirements for the degree of:

Master of Philosophy

M.Phil.

Exercise and Nutrition Research Program
Mary MacKillop Institute for Health Research

Faculty of Health Sciences
Australian Catholic University

Graduate Research Office
250 Victoria Parade
Fitzroy, Victoria, 3065

ACU MARY MACKILLOP INSTITUTE FOR HEALTH RESEARCH

Statement of Authorship and Sources

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program and any editorial work, paid or unpaid, carried out by a third party is acknowledged, and ethics procedures and guidelines have been followed. Unless otherwise stated, all work comprising this thesis has been undertaken by the candidate. This candidature commenced Australian Catholic University (ACU) in June 2016.

Bill Tachtsis Date: 20/03/2020

Supervisory Panel

It is acknowledged that the work comprising this thesis was supervised by the following ACU staff members:

Principal Supervisor:	Prof. John A. Hawley (ACU, Melbourne, VIC)
Co-Supervisor:	Dr. Nolan J. Hoffman (ACU, Melbourne, VIC)
Associate Supervisor:	Dr. Donny M. Camera (Swinburne University, VIC)

Acknowledgments

The work presented in this thesis would not have been possible without the help and support of my close friends, family and colleagues.

Firstly, to my Mum and Sister Kristina, thank you for putting up with my mood swings throughout my candidature. Your unconditional love and support kept me going during the toughest of times.

To all the members of the ENRP past and present, thank you for your support throughout my time at ACU.

Nolan and Jamie. Your supervision and mentorship could not have come at a better time. I have learnt a lot from both of you and am grateful for the guidance and direction you have provided me.

To the Hamstring boys, thank you for making me feel welcome from my first day at ACU. It has been an absolute pleasure to have been able to share copious amounts of coffee with you.

To my American desk mate Bobby, our daily chats about powerlifting, science and the otherwise miscellaneous will be missed. Thank you for challenging me with constant thought-provoking conversation.

A special thanks to the breakdancing mad scientist Will Smiles, it has been a pleasure to work with you. Your encyclopaedic knowledge of cell signalling, and phosphorylation sites still baffles me to this day. Your enthusiasm for science never ceases to amaze me and will continue to inspire me.

Lastly, Professor John Hawley, thank you for giving me the opportunity to work in your lab, it has been an invaluable experience. I will forever associate vanilla coke with you and yes, I am eating enough protein.

Publications

Publications arising directly from this thesis:

1. **Tachtsis B**, Camera D, Lacham-Kaplan O. Potential Roles of n-3 PUFAs during Skeletal Muscle Growth and Regeneration. **Nutrients. 2018;10(3):309. Published 2018 Mar 5**. **This paper has been cited 13 times since its publication date.**

2. **Tachtsis B**, Whitfield J, Hawley JA and Hoffman NJ. Omega-3 Polyunsaturated Fatty Acids Mitigate Palmitate-Induced Impairments in Skeletal Muscle Cell Viability and Differentiation. **Frontiers in Physiology. Accepted for publication 2020 Jun 3.**

Conference Presentations

1. **Tachtsis B**, Camera D, Lacham-Kaplan O, Hawley JA and Hoffman NJ. Differential Protective Effects of Omega-3 Fatty Acids Against Palmitate-Induced Impairments in Skeletal Muscle Cell Viability and Differentiation. **Keystone Symposium, Immunometabolism, Metaflammation and Metabolic Disorders (D6)** – Vancouver, Canada, April 2019.

Table of Contents

List of Figures

List of Tables

Abbreviations

AICAR	5-Aminoimidazole-4-carboxamide ribonucleotide
ALA	α-linolenic acid
AMPK	5’ AMP-activated protein kinase
BCA	bicinchoninic acid
Bip	binding immunoglobulin protein
BMI	body mass index
BSA	bovine serum albumin
CHOP	CCAAT-enhancer-binding protein homologous protein
CNS	central nervous system
CPT1	carnitine palmitoyl transferase 1
DHA	docosahexaenoic acid
DMEM	Dulbecco’s modified eagle medium
DPA	docosapentaenoic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
EPA	eicosapentaenoic acid
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
FOXO	Forkhead box protein O
HS	horse serum
h	hour
IL-6	interleukin-6
MAPK	mitogen-activated protein kinase
MPS	muscle protein synthesis
MRF	myogenic regulatory factor
mTOR	mammalian target of rapamycin
p70S6K1	70 kDa ribosomal protein s6 kinase 1
PAL	palmitate
PBS	phosphate buffered saline
PGC-1α	peroxisome proliferator-activated receptor gamma co-activator 1-alpha
PMSF	phenylmethylsulfonyl fluoride
PPAR	peroxisome proliferator activated-receptor
PUFA	poly unsaturated fatty acid
PVDF	polyvinylidene fluoride
TNF-α	tumour necrosis factor α
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
UCP3	uncoupling protein-3
$\dot{V}O_{2peak}$	peak oxygen consumption

Wk	weeks
Yr	year

Abstract

Skeletal muscle is a highly malleable tissue with the capacity to alter its phenotype in response to exercise and nutrient availability. Adult skeletal muscle is primarily composed of terminally differentiated post-mitotic cells and relies on specialised muscle stem cells (satellite cells) to facilitate repair and regeneration. Satellite cell regenerative capacity is impaired by diets enriched with high concentrations of saturated fat as well as other environmental lifestyle factors such as obesity and physical inactivity. Omega-3 polyunsaturated fatty acids (n-3 PUFAs), commonly found in fish oil supplements, possess anti-inflammatory properties and have been shown to improve aspects of skeletal muscle function and metabolism. These protective properties have established n-3 PUFAs as a potential dietary supplement strategy to help combat muscle wasting and promote muscle growth and repair. However, the cell signalling pathways underlying their efficacy in skeletal muscle cells are not completely understood.

This Master's thesis comprises a published review of the literature pertaining to the potential roles of n-3 PUFAs during muscle growth and regeneration, and an original research study investigating and comparing the purported beneficial protective effects of n-3 PUFAs in skeletal muscle cells against lipotoxicity induced by the saturated fatty acid palmitate (PAL). The experimental data collected for this thesis demonstrate that the n-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), commonly found in fish oil

supplements, protect skeletal muscle cells against the deleterious effects of PAL. Furthermore, the work in this thesis provide novel data regarding the beneficial effects and potential mechanisms of Docosapentaenoic acid (DPA), a lesser studied intermediary n-3 PUFA, on maintaining skeletal muscle cell viability in an environment of high fat availability.

The results obtained demonstrate that all n-3 PUFAs induce protective effects against the deleterious effects of PAL on skeletal muscle cell viability and subsequent differentiation capacity. However, DPA confers the greatest protection against the induction of PAL-induced ER stress. Mechanistically, DPA protects against PAL-induced cytotoxicity via maintaining muscle cell mitochondrial membrane integrity.

In the context of the previous literature overviewed in the published review article, these data contribute important novel findings demonstrating that all n-3 PUFAs including DPA protect skeletal muscle cells *in vitro* against stimuli known to be deleterious to skeletal muscle growth and repair *in vivo*. These data also reveal that DPA has the potential to elicit superior protective effects against lipotoxicity compared to EPA and DHA, setting the stage for further research into the mechanisms underlying DPA's beneficial effects and potential future therapeutic applications in fish oil supplements to help promote muscle growth, maintenance and repair.

Chapter 1 : Introduction and Overview

Skeletal muscle constitutes approximately 40-50% of total body mass in humans and plays major roles in locomotion, thermoregulation and whole-body metabolic homeostasis [1]. However, the continuous use of skeletal muscle during daily living and particularly strenuous exercise makes it susceptible to injury and overuse. Biochemical, structural and phenotypic adaptations in skeletal muscle allow it to respond and help combat these potential deleterious effects of certain physiological stimuli [2]. During the ageing process, skeletal muscle becomes less responsive to mechanical stressors such as exercise [3, 4] and the anabolic effects of protein intake [5], both of which facilitate muscle growth. Blunted responses to both of these stimuli contribute to the loss of muscle mass over time with ageing, termed sarcopenia [3, 6]. Furthermore, exposure to Western diets rich in saturated fats can also negatively impact skeletal muscle by impairing its growth and repair capacity [7, 8]. While the precise mechanisms underpinning sarcopenia remain incompletely understood, accumulating evidence indicates that nutritional supplements such as fish oil may exert beneficial anabolic effects and potentially prevent and/or preserve muscle mass and function [9]. Omega-3 polyunsaturated fatty acids (n-3 PUFAs) found in fish oil supplements are known for their anti-inflammatory properties; however, they also have the potential to promote anabolism by increasing the intracellular activities of signalling molecules involved in re-programming cellular pathways to help maintain skeletal muscle mass [10, 11].

n-3 PUFAs are polyunsaturated fatty acids with numerous known biological actions and potential therapeutic applications including, but not limited to, the ability to reduce inflammation, and putative roles in cognition and development of the central nervous system (CNS) [12-14]. There are three major dietary n-3 PUFAs including α-linolenic (ALA; 18:3n-3), eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) (**Figure 1.1**). ALA is most commonly found in plant foods (e.g. flax seed and nuts). Conversely, cold-water fish such as salmon and tuna are rich sources of EPA and DHA. ALA is considered essential and has to be obtained via dietary sources, as humans lack the required delta-12 desaturase and delta-15 desaturase enzymes necessary to synthesise n-3 PUFAs from stearic acid [15]. While *de novo* synthesis of ALA is not possible in humans, EPA and DHA can be synthesised from ALA [16]. As highlighted in **Figure 1.2**, the same elongase and desaturase enzymes used to convert ALA to EPA are also used to synthesise omega-6 fatty acids. Therefore, the conversion of ALA to EPA and DHA is limited due to enzyme availability [17, 18].

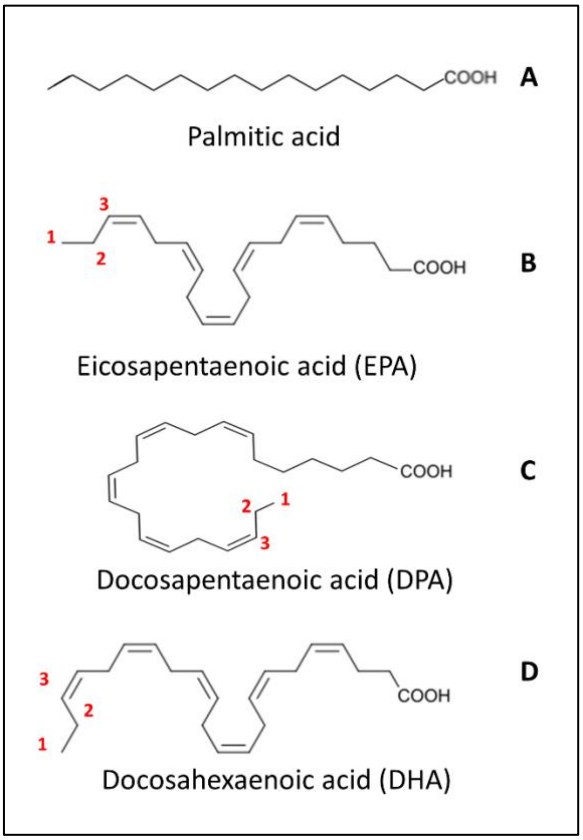

Figure 1.1. Chemical structures of the saturated fatty acid palmitic acid and omega-3 polyunsaturated fatty acids (n-3 PUFAs).
Palmitic acid (A) 16 carbons in its hydrocarbon chain that only contains single bonds as indicated by single lines above, whereas EPA (B), DPA (C) and DHA (D) contain multiple double bonds as indicated by double lines above. The double bonds of n-3 PUFAs begin on the 3rd carbon from the methyl (omega) end of each n-3 PUFA as numbered in red above.

Dietary supplementation with fish oil capsules and dietary consumption of seafood are the most efficient ways of increasing EPA and DHA levels in the blood and skeletal muscle [19, 20]. Docosapentaenoeic acid (DPA; 22:5n-3) is another form of marine n-3 PUFA that is structurally similar to EPA with the addition of two extra carbons within its hydrocarbon chain and consists of about 30% of daily n-3 PUFA intake in Australia [21]. Unlike EPA and DHA, DPA is not commonly found in current commercially available fish oil capsules, and its biological functions are only beginning to be elucidated [22].

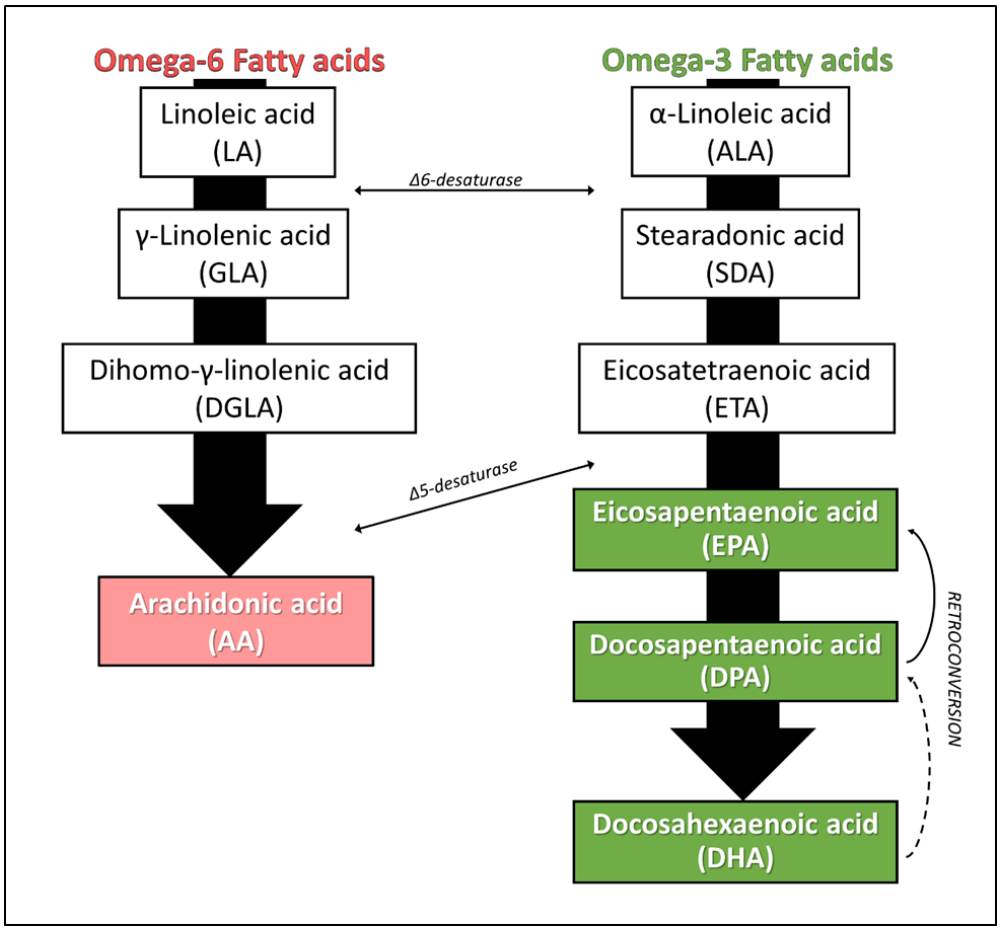

Figure 1.2. Biochemical pathways involved in the interconversion of n-6 and n-3 PUFAs. ALA, α-linolenic acid; AA, arachidonic acid; DGLA, dihomo--linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; GLA, -linolenic acid; LA, linoleic acid.

In contrast to n-3 PUFAs, the saturated fatty acid PAL has well-documented deleterious effects on skeletal muscle, including impaired rates of muscle protein synthesis [23] and reduced insulin sensitivity [24], leading to muscle atrophy [25]. PAL can also cause muscle cell death, further exacerbating muscle atrophy [26, 27] Some of these negative effects are due to excess saturated free fatty acid (FFA) uptake into cells resulting in the accumulation of intramyocellular diacylglycerol and ceramide lipid species that cause disruptions in intracellular signalling networks [28-30]. Ageing is often associated with elevations in circulating saturated FFA which can lead to increases in risk factors for all-

cause and cardiovascular mortality such as increased blood pressure, impaired endothelial cell function and increased circulating inflammatory markers [31]. Consequently, cellular PAL treatment is a commonly used experimental strategy *in vitro* to recapitulate the increased circulating free-fatty acid observed in older individuals and individuals with metabolic diseases such as insulin resistance and type 2 diabetes. Conversely, n-3 PUFAs have been shown to elicit beneficial effects on cellular signalling responses related to cell survival and muscle protein synthesis. Evidence also suggests that EPA and DHA can protect against the negative effects of PAL including ER stress, inflammation and cellular lipotoxicity [25, 32, 33].

The second chapter of this thesis is a peer-reviewed and published review article that critically examines the current literature pertaining to the roles of n-3 PUFAs in skeletal muscle, with a specific focus on muscle growth and repair. The fourth chapter describes an original research study that investigates the differential protective effects of EPA, DHA and the lesser studied DPA against PAL-induced cellular impairments in muscle cell viability and differentiation. As outlined in **Figure 1.3**, the following chapters comprise two independent yet complementary bodies of work, which critically examine the current literature and uncover novel protective roles of omega-3 fatty acids against skeletal muscle cell lipotoxicity.

Chapter 2 (Narrative review article): Potential roles of n-3 PUFAs during skeletal muscle growth and regeneration **(Figure 1.3 A)**.

Chapter 4 (Original research article): Differential protective effects of omega-3 fatty acids against PAL-induced impairments in skeletal muscle cell viability and differentiation (**Figure 1.3 B)**.

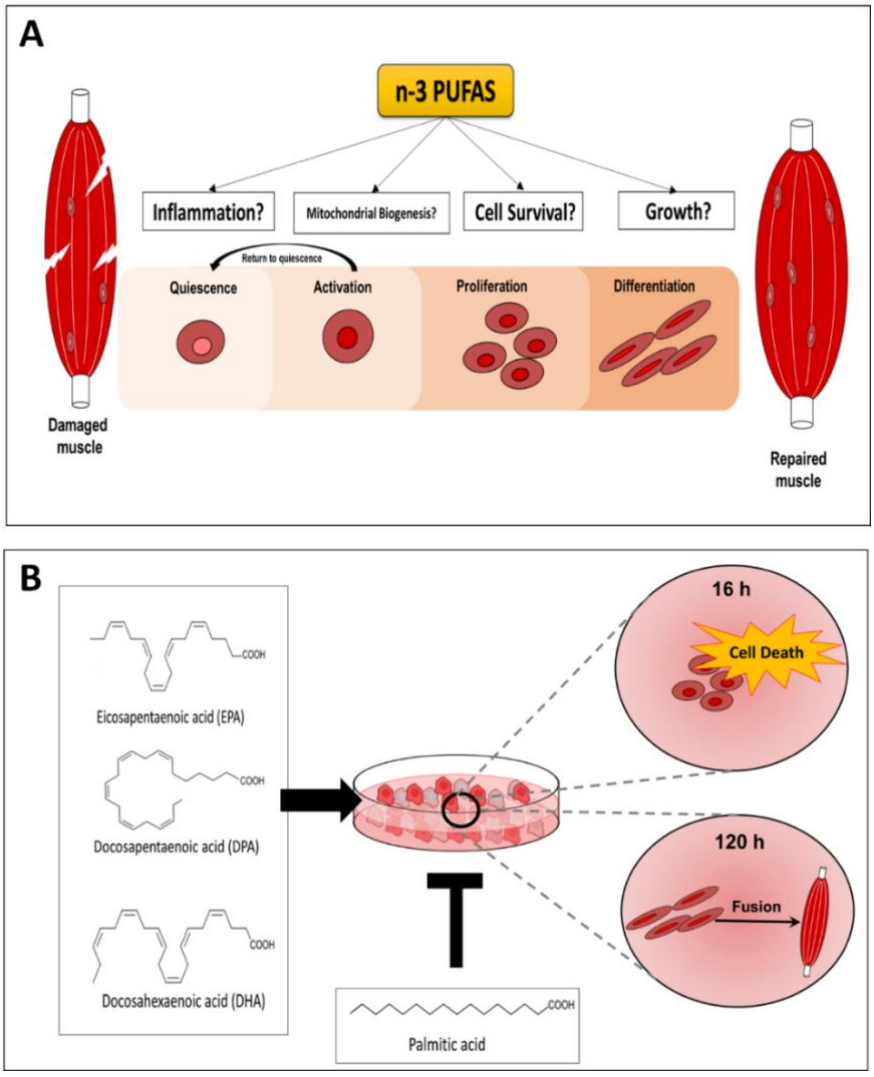

Figure 1.3. Schematic diagram of thesis outline.

The main topics discussed in the narrative literature review regarding potential mechanisms underlying the beneficial protective effects of n-3 PUFAs related to muscle growth and repair (A). Briefly, once incorporated into cellular membranes, n-3 PUFAs exert their beneficial effects by reducing inflammation, altering mitochondrial function, promoting cell survival and promoting cellular signalling associated with muscle growth. Schematic diagram of original research study in this thesis investigating the differential protective effects of EPA, DPA and DHA against PAL-induced impairments in skeletal muscle cell viability and differentiation (B). Briefly, skeletal muscle myoblasts were treated with the n-3 PUFAs EPA, DPA and DHA and/or the saturated fatty acid PAL for 16 h to compare their protective effects against PAL-induced lipotoxicity. Myoblasts were then differentiated to assess whether n-3 PUFA treated cells would efficiently differentiate following this PAL cellular insult.

Chapter 2 : Literature Review

This chapter has been adapted from the following published review:

1. **Tachtsis B**, Camera D, Lacham-Kaplan O. Potential Roles of n-3 PUFAs during Skeletal Muscle Growth and Regeneration. Nutrients. 2018 Mar 5;10(3):309.

The following publication has been **cited 13** times since being published.

Introduction

Skeletal muscle is a highly malleable tissue with the capacity to alter its phenotype in response to exercise and nutrient availability [2]. However, with old age skeletal muscle becomes less responsive to anabolic stimuli such as resistance exercise and protein feeding. It is thought that this reduced sensitivity to anabolic stimuli, termed 'anabolic resistance', is implicated in the aetiology of sarcopenia, the gradual loss of muscle mass with age [34-39]. Other factors known to contribute to sarcopenia include reductions in circulating sex hormones [40], physical inactivity [41], low grade inflammation [42, 43], impairments to neuromuscular junctions [44] and reduced muscle stem cell (i.e.: satellite cell) number and function [45]. Sarcopenia is phenotypically associated with decreased muscle fibre size and shifts in fibre type change from fast to slow, resulting in a decrease in maximal muscle force production [46, 47]. These changes are also accompanied by a diminished regenerative capacity of the muscle due to a loss in the number and activity of satellite cells in type II 'fast' fibres [46, 48, 49].

Satellite cells are muscle specific stem cells primarily responsible for the repair of muscle in response to injury and damage [50-52]. Upon their activation, satellite cells enter the cell cycle, proliferate, differentiate to myoblasts and myocytes in a process termed myogenesis, and fuse to damaged muscle fibres. Myogenesis is regulated by changes in the expression of myogenic transcriptional regulatory factors (MRF) that dictate whether satellite

cells are in a quiescent, activated, committed, or differentiated state [53, 54] **(Figure 2.1)**. Satellite cells play a role in skeletal muscle repair; however, their role in muscle hypertrophy is still equivocal (for reviews see [55, 56]).

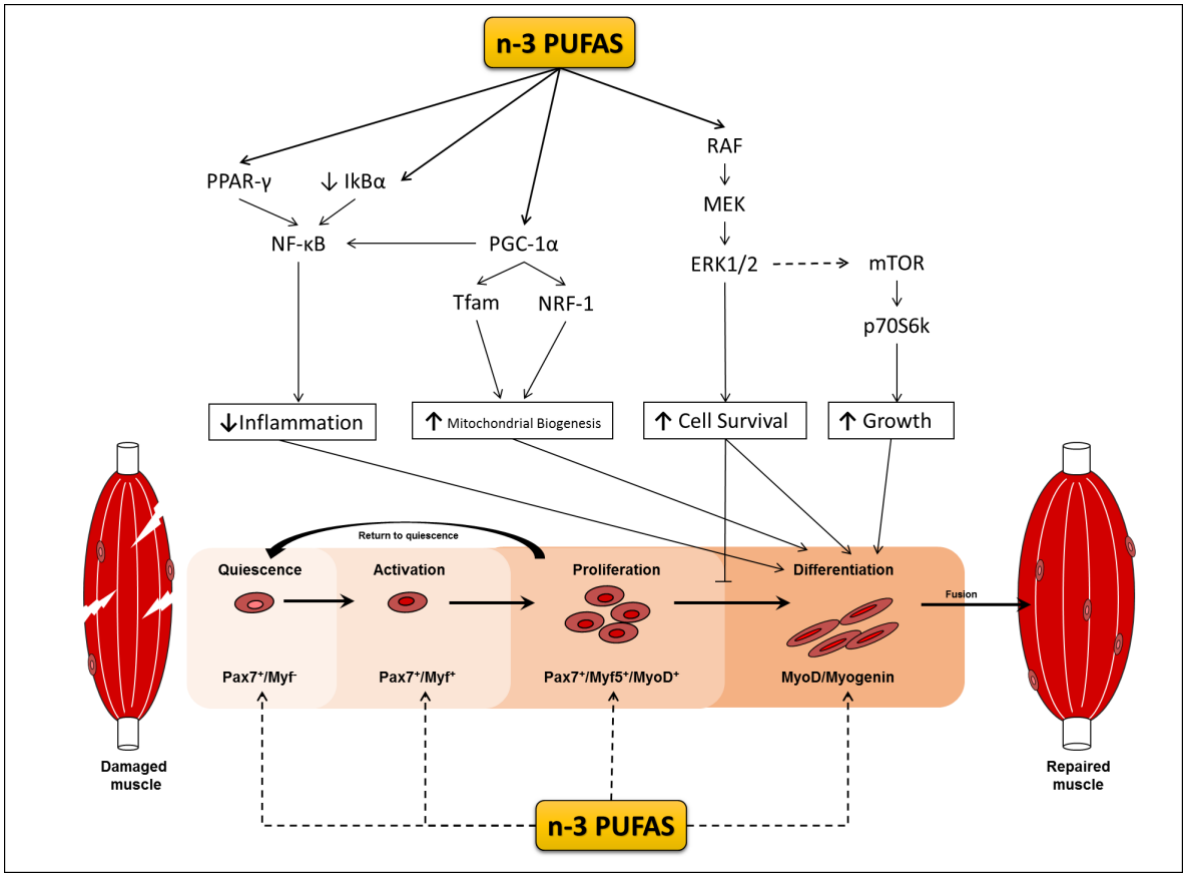

Figure 2.1. PAL and TNF-α elicit lipotoxic and cytotoxic deleterious effects on satellite cells during various stages of myogenesis.
N-3 PUFAs inhibit the negative effects of PAL and TNF-α by activating anti-inflammatory pathways within the cell thereby promoting differentiation. In isolation, it is currently unknown whether n-3 PUFAs are capable of modulating the expressions of key MRFs Pax7, MyoD and Myogenin that regulate myogenesis. Solid line: represents established role/pathway that n-3 PUFAs modulate during myogenic differentiation. Dotted line: limited or evidence no supporting the role of n-3 PUFAs during myogenesis. Solid line: substantial evidence for n-3 PUFAs effecting myogenesis via various pathways.

The results from several animal studies report skeletal muscle hypertrophy even in the absence of satellite cells [52, 57], whereas other studies provide evidence to support a role for satellite cells during hypertrophy [58, 59]. Although the extent to which satellite cells facilitate muscle hypertrophic response is still a topic of debate, most evidence indicates that

the presence and more likely the cells' activation and myogenic capacity are indispensable for supporting training-induced adaptations and may be implicated in the events leading to sarcopenia. However, throughout the lifespan of satellite cell depleted mice, sarcopenia is neither exacerbated nor accelerated [60, 61]. Notably, these mice are highly sedentary, and although this may be reflective of an elderly population it is still difficult to draw definitive conclusions in the context of exercise. While satellite cell depleted mice do not show signs of increased muscle loss, satellite cell depletion appears to cause a dysregulation in the surrounding muscle environment leading to increased fibrosis with ageing and a reduction in muscle quality and function. Indeed, satellite cells have been implicated in regulating extracellular matrix production during hypertrophy and regenerative processes and therefore do play an important role in the maintenance of muscle mass with age [58, 62, 63].

The precise molecular mechanisms responsible for sarcopenia are yet to be elucidated, however accumulating evidence indicate that nutritional supplements, in particular the n-3 PUFAs, have the potential to reduce muscle wasting and increase the functional capacity of muscle in older individuals by augmenting intracellular anabolic signalling [64-66]. EPA, DHA and ALA are the most commonly found n-3 PUFAs in the human diet [67]. ALA is most commonly abundant in plant foods, such as flax seed and nuts, while EPA and DHA are abundant in cold-water fish, such as salmon and tuna [68]. In addition, DPA is an emerging, but lesser known, n-3 PUFA that possesses a similar structure to EPA and is also biochemically active [69]. Only ALA is considered truly essential to

humans as both EPA and DHA can be synthesised in the endoplasmic reticulum in liver cells through conversion from ALA, though this conversion is limited due to enzyme availability [17, 18, 70].

The n-3 PUFAs EPA and DHA are known for their anti-inflammatory properties [71, 72]. They have also been reported to elicit positive effects on a wide range of other physiological processes and systems such as visual signalling [73, 74], insulin sensitivity and glucose tolerance [75]. Accumulating evidence indicates that n-3 PUFAs may also stimulate rates of muscle protein synthesis (MPS) by increasing both the intracellular activities of signalling molecules involved in the maintenance of skeletal muscle mass [11, 76] and possibly satellite cell activity [77]. Chronic low-grade inflammation associated with ageing and other comorbidities known to increase inflammation, such as obesity, often exacerbate the effects of sarcopenia [78]. As such, the anti-inflammatory effects of n-3 PUFAs on skeletal muscle may be most beneficial to older individuals with sarcopenia [64, 79, 80] and may complement other strategies, such as exercise and protein supplementation, to combat this diseases state. This review focuses on the dose and treatment periods required for n-3 PUFAs, EPA and DHA, to support differentiation of satellite cells into myogenic cells (myogenesis) and elicit protective ant-inflammatory effects in-vitro and in-vivo. The combined effects of n-3 PUFA supplementation, exercise, and protein ingestion, and their associated mechanisms of action on muscle metabolism in both young and older individuals, will also be discussed.

The Beneficial Effects of n-3 PUFAs in Skeletal Muscle

Reducing Inflammation

Muscle wasting is a hallmark of various disease states which are often associated with increased levels of inflammation [81, 82]. Acute transient increases in inflammation plays an important part in the early regenerative processes of muscle adaptation particularly in response to exercise [83, 84]. However, failure to effectively reduce inflammation at its onset results in a chronic state of inflammation and can be detrimental as it is associated with insulin resistance, obesity and muscle wasting [85-87]. Furthermore, elevated levels of inflammation during ageing may also exacerbate the effects of sarcopenia by impairing the regenerative capacity of muscle [88].

Upon their incorporation into membrane phospholipids, EPA and DHA act as substrates for the synthesis of lipid derived mediators of inflammation [89]. These mediators, termed eicosanoids, vary in their ability to mediate inflammation depending on the substrate used for their synthesis. For example, eicosanoids derived from arachidonic acid, an omega-6 fatty acid, are considered to be pro-inflammatory [90]. Conversely, eicosanoids derived from n-3 PUFAs are considered less potent, reducing the intensity and duration of the inflammatory responses by immune cells [91]. This divergent behaviour is due to the fact that eicosanoids derived from n-3 PUFAs are less biologically active than n-6 PUFAs and possess a weaker affinity for eicosanoid receptors [92-94]. EPA and DHA can also inhibit

the endogenous production of pro-inflammatory arachidonic acids by competing as substrates for the enzymes during eicosanoid synthesis [95, 96].

Inflammatory cytokines produced by immune cells post-exercise can have a profound effect on skeletal muscle protein turnover and myogenesis. In animal and cell models, cytokines such as tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6), bind to receptors on the muscle, activating the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) [97, 98]. Activation of NFκB subsequently increases the expression of the atrogenes muscle RING-finger protein-1 (MuRF1) and atrogin-1, promoting muscle wasting [97]. n-3 PUFAs, in addition to modulating eicosanoid synthesis, can also inhibit the activation of NFκB in skeletal muscle cells and other cell types *in vitro* [98-101]. Whether n-3 PUFAs can inhibit the activation of NFκB in human skeletal muscle in response to inflammation remains to be determined. Although considered pro-inflammatory, TNF-α and IL-6 also appear to play important role in the early stages of muscle regeneration following injury by enhancing myoblast proliferation and inhibiting differentiation [102-104]. This inhibition involves the down regulation of the MRFs, MyoD and Myogenin [102-104], with TNF-α receptor double knock out mice [103] and with IL-6 non-specific knock out mice [102] displaying impaired myogenic differentiation and hypertrophy compared to wild type mice during functional overloading. These findings highlight the importance of inflammatory processes during muscle regeneration following injury (i.e. exercise).

In contrast, chronic inflammation observed during ageing is associated with an increase in pro-inflammatory cytokines (compared to younger individuals) such as TNF-α and IL-6 due to possible dysregulation in immune cell function [88, 105]. Elevations in circulating pro-inflammatory cytokines reported in older individuals negatively impacts muscle regeneration by impairing satellite cell differentiation and fusion and by increasing NF-κB activation. These factors contribute to deterioration of muscle mass with age [88, 106]. Despite their purported anti-inflammatory effects which are thought to underlie the beneficial effects of n–3 PUFAs in muscle, Da Boit, Sibson [107] and others [65, 76], were unable to detect any differences in plasma TNF-α or IL-6 between a placebo and n-3 PUFA treated group post-intervention in both older and younger men and women. A possible explanation for this discrepancy is that the participants were healthy and had low levels of inflammation to begin with, making it difficult to detect any changes.

Mechanisms underlying the anti-inflammatory properties of n-3 PUFAs during myogenesis

The potential positive effects of n-3 PUFA's on myogenesis may relate to their capacity to alter the cell membrane lipid composition which in turn changes the profile of membrane bound proteins of lipid rafts [108, 109]. These changes impact membrane fluidity and assist in myoblast fusion during myotube formation [110], allowing n-3 PUFAs to simultaneously modulate several signalling pathways. One such pathway involves the Peroxisome proliferator-activated receptors (PPARs), a group of nuclear receptors well

characterised in preventing metabolic disorders, promoting adaptations to skeletal muscle following fasting and physical exercise, while also playing a novel role in regulating satellite cell activity [111-113]. PPARs are also critical regulators of genes involved in development, metabolism of lipids and carbohydrates, as well as inflammation [114]. Recent evidence suggests that lipids such as n-3 PUFAs can bind to PPARs inducing their activation and altering the expression of pro-inflammatory genes [115]. Results from several studies support the potential for n-3 PUFAs to regulate inflammatory events in muscle cells in association with the PPAR signalling pathway. TNF-α has been shown to elicit negative effects on differentiating myoblasts resulting in decreases in myotube size and number [116]. However, pre-treating or co-treating myoblasts with EPA prevents the cytotoxic effects of TNF-α [116]. These protective effects of EPA are associated with increases in PPARγ expression and decreases in NF-κB [117]. Moreover, treating C2C12 myotubes with high concentrations (400-600μM) of EPA or DHA has also been shown to increase the gene expression of PPARγ and reduce muscle breakdown. These findings collectively indicate that n-3 PUFAs are able to increase the expression of PPAR and reduce the expression of NF-κB in skeletal muscle resulting in reduced muscle wasting [98, 101].

PPARδ is another isoform of PPAR abundantly expressed in skeletal muscle that has a novel role in regulating satellite cell activity [118]. In response to injury PPARδ-KO mice when compared to aged-matched wild type mice, display reductions in proliferating satellite cell number and increases in differentiating cell number [118]. Whether n-3 PUFA

supplementation is able to augment satellite cell regeneration in a PPARδ regulated manner following injury or exercise remains to be determined but is possible considering that n-3 PUFAs are known PPARs agonists in skeletal muscle [98].

Like PPARs, the peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC-1α) is also capable of repressing the transcriptional activity of NF-kB and lowering inflammation in muscle cells [119]. In differentiated C2C12 myotubes, n-3 PUFAs increase the expression of PGC-1α in a dose and time dependent manner with 50 μM for 24h eliciting the greatest effect [120]. PGC-1α is also indirectly involved in regulating the expression of mitochondrial DNA (mtDNA) by increasing the transcription of mitochondrial transcription factor A (Tfam) and nuclear respiratory factor 1 (NRF1) [121]. In fully formed myotubes, the expression of both Tfam and NRF1 increase following both EPA and DHA treatment [120]. Moreover, overexpressing PGC-1α in C2C12 cells promotes differentiation and increase the expression of MyoD and Myogenin. However, it remains to be determined whether n-3 PUFAs can elicit similar responses in both PGC-1α and MRF expression during differentiation [122].

As fatty acid oxidation occurs within mitochondria, the increases in mitochondrial biogenesis seen following n-3 PUFA treatment could be a compensatory mechanism to dispose of excess fatty acids [123]. Indeed, EPA and DHA ameliorate the lipotoxic effects of other fatty acids such as PAL in fully formed myotubes and increase the expression of genes

associated with mitochondrial-β oxidation such as carnitine palmitoyl transferase 1α and β (CPT1α; CPT1β) [124]. Likewise, DHA treatment of C2C12 myoblasts attenuates the inhibitory effects of PAL on PGC-1α activity and preserves oxidative capacity by maintaining citrate synthase activity [125]. Thus, PGC-1α appears to play an important role overcoming the deleterious effects of PAL-induced lipotoxicity, though it is remains to be determined whether DHA or EPA can modulate the expression of PGC-1α during myoblast differentiation. In-vitro, both PPAR and PGC-1α are capable of attenuating inflammation via downregulating NF-kB following exposure to n-3 PUFAs during myogenic differentiation in C2C12 cells. More work is required to elucidate whether n-3 PUFAs can modulate the same pathways in human satellite cells.

In summary, the n-3 PUFAs EPA and DHA can reduce inflammation via their incorporation into membrane phospholipids where they inhibit the production of pro-inflammatory eicosanoids, reducing of activation of immune cells and the associated release of pro-inflammatory cytokines [100, 126, 127]. Therefore, n-3 PUFAs may assist in muscle regeneration in older individuals by reducing excessive inflammation and promoting a systemic environment conducive to growth [128]. The anti-inflammatory properties of n-3 PUFAs and their incorporation into membrane phospholipids may also underpin some of their other beneficial effects in muscle such as altering protein metabolism, increasing muscle strength and modulating myogenesis.

Regulation of Protein Metabolism

The combination of resistance exercise and protein ingestion is known to maximally stimulate rates of muscle protein synthesis (MPS) [129-131] and also lead to increases in satellite cell activity [132] and content in younger men [133]. Moreover, n-3 PUFA supplementation has been shown to exert a positive effect on rates of MPS in both young and old individuals. For example, when combined with amino acids, n-3 PUFAs enhance rates of MPS in both young and old people to a greater extent than amino acid ingestion alone [65, 76]. However, it should be noted that the findings were under non-physiological conditions of a hyperaminoacidemic-hyperinsulinemic clamp [65, 76]. These findings provide evidence that n-3 PUFAs may be an effective strategy to rescue anabolic sensitivity in healthy older individuals purported to be 'anabolic resistance' as observed by attenuated MPS responses compared to healthy young individuals in response to protein ingestion [35, 39] and exercise [3]. When acute resistance exercise, protein ingestion and n-3 PUFA supplementation (5 g/day for 8 weeks) are combined under physiological conditions, rates of MPS are not further enhanced compared to a placebo group in younger adults [11]. Interestingly, the activity of Akt and p70S6K1, signalling kinases involved in translation initiation in muscle, is attenuated following resistance exercise and n-3 PUFA supplementation. This could indicate that anabolic kinase efficiency is increased, suggesting less protein is required to elicit post translational modifications (e.g. phosphorylation) and that these posttranslational modifications occur at a faster rate with n-3 PUFA supplementation. This may be due to the increased incorporation of unsaturated fatty acids into membrane phospholipids thereby

changing their composition and altering the activities of proteins associated with stimulating MPS tethered to cell membrane [108, 109].

While it is possible that changes to protein signalling efficiency occur, a major limitation of the study conducted by McGlory et al. [11] is that they did not measure any changes in the total protein content of Akt and p70S6K1 nor did they measure any specific post-translational modifications of these proteins (i.e. altered phosphorylation at numerous sites). Measuring these parameters would confirm whether n-3 PUFAs are indeed capable of altering kinase efficiency and which post-translational modifications are altered following n-3 PUFA supplementation. Lastly, the supplementation protocol (dose and duration) used by McGlory et al. [11] should have been sufficient to induce increases in the muscle. Previously, the same group has shown that after four weeks of supplementation, concentrations of EPA and DHA continue to rise in muscle (~3.0% of total fatty acids in muscle at week 2 and ~5.0% of total fatty acids in muscle at week 4) whereas levels of EPA and DHA in blood plateau after two weeks with no further increase at 4 weeks (~8.0 % at week 2 and ~8.0% at week 4) [10]. Future research in older individuals is required to investigate whether the combined effects of protein ingestion, resistance exercise and n-3 PUFA supplementation is more beneficial than resistance exercise and/or protein ingestion in isolation. It also remains to be seen whether changes in inflammatory markers underlie changes to MPS following n-3 PUFA supplementation.

Increasing muscle strength

The CNS contains a high concentration of the unsaturated lipids, EPA and DHA, which define the structure and function of its cellular and subcellular components. These fatty acids are known to increase nerve conduction velocity in both young and older individuals through the modulation of sarcolemma ion channels which, in turn, improves contractile activity of the muscle [10, 134-137]. In older women, dietary n-3 PUFA supplementation of 2 g/day for 90 days in conjunction with strength training has been shown to exert additional increases to strength and functional capacity (i.e. increased peak torque and rate of torque development) than strength training alone [66]. Rodacki and co-workers [66] also found these increases to occur through changes in neuromuscular junction conductivity as evidenced by increases in electromyographic activation of the muscle and decreases in electromechanical delay. Even in the absence of regular resistance training, muscular strength and thigh volume can increase with n-3 PUFA supplementation in older men and women. Indeed, supplementation of 4 g/day of fish oil for six months results in increased whole thigh muscle area, 1-RM strength and isokinetic power [64]. However, in the study conducted by Smith et al. diet was not controlled which distort the true effect of n-3 PUFA supplementation on muscle area [64]. Furthermore, without the collection of muscle biopsies, it is difficult to determine the precise molecular mechanism underlying these responses as well as the potential for similar shifts in muscle fibre types which a known to occur in rat muscle as a result of n-3 PUFA supplementation [138].

There is also evidence to suggest that older women may respond better than men to combined n-3 PUFA supplementation and resistance exercise training. For example, when compared to both male and female placebo groups, Da Boit and co-workers observed greater improvement in muscle quality (strength / unit of muscle area) in the quadriceps of older women following combined resistance exercise and fish-oil supplementation [107]. A possible explanation for this sexual dimorphism is that older women do not respond as well to resistance exercise as men and also have absolute less muscle mass [107], potentially allowing a greater effect in women than men with n-3 PUFA supplementation due to their greater capacity for improvement. This theory however remains highly speculative as others have shown that older men and women respond equally as well to resistance exercise-based training and that no such sex differences exist in older individuals [139].

n-3 PUFAs have been shown to exert similar effects as the female sex hormone oestrogen in preventing bone loss in ovariectomised rats [140]. As such, another possible explanation regarding the sexual dimorphisms in fish oil efficacy is that n-3 PUFAs may function in a similar fashion to oestrogen promoting anabolism or at least preventing catabolism. However, it should be noted here that this study was conducted using an animal model and further human research is required as sexual dimorphisms in protein metabolism are not yet fully understood [141]. Whether n-3 PUFAs induce effects similar to male or female sex hormones in human skeletal muscle related to anabolism remain to be determined.

Potential to alter satellite cell activation

Satellite cells are primarily involved in muscle regeneration in response to muscle damage and injury sustained following resistance exercise and activities of unaccustomed intensity or duration [50]. Upon their activation, satellite cells enter the cell cycle, proliferate, and differentiate to myoblasts and myocytes in a process termed myogenesis, before fusing to existing muscle fibres. Circulating systemic factors such as hormones and inflammatory marker can positively or negatively influence satellite cell activation. For instance, circulating growth factors such as GDF11 and myostatin, as well as inflammatory markers, such as TNF-α and IL-6, increase with age impairing the regenerative capacity of satellite cells in human skeletal muscle [6, 42].

Though it is important to note that while GDF11 and myostatin have been associated with increased age in humans, no study to date has demonstrated a causal link between these circulating factors and their ability to affect the regenerative capacity of muscle. Further evidence from parabiosis experiments in mice indicate that ageing reduces satellite cell activity; however exposing cells to a more youthful systemic environment improves the regenerative capacity of satellite cells indicating that the age-related reduction in satellite cell activity can be modulated by systemic factors that change with age [142, 143]. Thus, n-3 PUFAs with their anti-inflammatory properties could promote a systemic environment that improves satellite cell responsiveness to injury during aging by attenuating systemic inflammation in a similar manner to nonsteroidal anti-inflammatory drugs (NSAIDs) [144-

146]. Currently, there is a paucity of human data to support the latter hypothesis, nevertheless studies investigating the influence of n-3 PUFAs myogenesis in-vitro have been conducted and these findings are summarised in **Table 2.1**.

The mechanisms underlying the modulatory effects of n-3 PUFAs on protein metabolism signalling and myogenesis

Activation of the mechanistic target of rapamycin (mTOR) and its downstream signalling targets the 70 kDa ribosomal protein S6 kinase-1 (p70S6K1) and eIF4E-binding protein 1 (4E-BP1) are involved in the regulation of translation initiation responses required for skeletal muscle hypertrophy and also have a role in myogenesis [147]. In isolation, n-3 PUFAs appear to have little effect on mTOR signalling pathway in fully formed C2C12 cells [109]. However, EPA exclusively augments leucine induced MPS in fully differentiated C2C12 myotubes and increases the phosphorylation of p70S6K1, with no changes in the phosphorylation of any of the other anabolic mTOR pathway members [148]. EPA and DHA induce similar increases in p70S6K1 phosphorylation without leucine stimulation during differentiation [108]. The activation of p70S6K1 following n-3 PUFA treatment in this case is not associated with an increase in Akt activity, an upstream effector of mTOR implicated in skeletal muscle hypertrophy [108, 149]. The fact that changes to p70S6K1 activity [11] and phosphorylation [108] can occur without changes to Akt signalling, suggests that n-3 PUFAs are capable of modulating differentiation and MPS independently of canonical mTOR signalling. This alternate activation may possibly come through signalling proteins

such as the mitogen-activated protein kinase (MAPKs), which phosphorylate members of the mTOR signalling pathway [150].

The MAPK cascade(s), particularly the extracellular signal-regulated kinases (ERK) 1 and 2 (ERK1/2), play a dual role in myogenesis [151, 152]. During proliferation, ERK1/2 expression is increased, promoting proliferation and inhibiting differentiation [153, 154]. In contrast to this inhibitory effect during the early stages of differentiation ERK1/2 becomes phosphorylated during the late stages of differentiation promoting the fusion and formation of myotubes [155, 156].

The actions of MAPK signalling in the regulation of myogenesis have been observed in cells treated with n-3 PUFAs. Exposing proliferating C2C12 cells to DHA and EPA for 24h decreases MAPK/ERK1/2 phosphorylation, preventing the progression of myoblasts from the G1 to S phase [157]. When the same cells are proliferated in the absence of n-3 PUFAs for 24 h, they continue through the cell cycle, which is important as it demonstrates that n-3 PUFAs can regulate a return to quiescence, keeping the cells in the G1 phase. This is noteworthy as there are particular muscle wasting conditions where satellite cells are constitutively active as inflammation is elevated, impairing their ability to self-renew [158, 159]. Constitutively active satellite cells also cause precocious differentiation (i.e.: premature and excessive differentiation), further reducing the satellite cell pool and regenerative capacity of muscle [158, 159]. Thus, n-3 PUFAs may have the potential to preserve muscle

mass by keeping cells in a quiescent state preserving the satellite pool or at least delay their inevitable activation following the induction of differentiation in-vitro. Whether n-3 PUFAs are able to prevent precocious differentiation under pathophysiological conditions in-vivo where inflammation is elevated remains to be determined.

Aside from regulating proliferation and differentiation, MAPKs can also initiate cell death responses in the face of noxious stimuli such as PAL and TNF-α [160, 161]. EPA combined with PAL and/or TNF-α treatment during C2 differentiation partially rescues cell death and differentiation via the suppression of MAPK [161]. Saini and co-workers [161] showed that EPA promotes differentiation by increasing the expression of MyoD and Myogenin. Whether the other n-3 PUFAs such as DHA and DPA elicit the same inhibitory effects on MAPK induced cell death and increase MRFs during differentiation remains unclear. The γ isoform of p38 can also modulate myogenesis by repressing the transcriptional activity of MyoD promoting proliferation [162] although it is unknown whether EPA and DHA alter the activation of p38 MAPK in the context of myogenesis. Future research incorporating in-vitro models is required to fully understand the effect that n-3 PUFAs exert on MAPKs during myogenesis and how this affects other pathways involved in growth such as mTOR signalling during this process.

Table 2.1. Summary of studies investigating n-3 PUFAs in proliferating and differentiating myoblasts.

Source	Treatment	Cell Line	Outcome
Smith, et al. [163]	50 μM EPA 1-10 nM PIF	C2C12	EPA ↓ protein degradation in PIF treated myoblasts
Magee, et al. [116]	50 μM EPA 20 ng/ml TNF-α	C2C12	EPA ↓ the negative effects TNF-α via ↓ NF-kB expression. EPA ↑ PPARγ expression
Lee, et al. [164]	1-10 μM DHA 48 h 1-10 μM EPA 48 h	C2C12	DHA ↑cell proliferation at 10μM concentration only. EPA has no effect on proliferation between 0-10μM. EPA and DHA were no investigated in the context of differentiation in this study
Magee, et al. [117]	50 μM EPA 24 h 20 TNF-α ng/ml 24 h	C2C12	EPA ↓ the negative effects TNFα has on cell death, apoptosis and myotube formation by ↑ PPARγ expression via the NfKB signalling pathway.
Peng, et al. [157]	10-100 μM DHA 24h 10-100 μM EPA 24h	C2C12	EPA and DHA ↓ cell proliferation in dose dependent manner DHA and EPA ↓ protein levels of Cyclin D and Cyclin E and CDK2. EPA and DHA ↓ ERK1/2 protein expression
Briolay, et al. [108]	20 μM DHA 72 h 20 μM EPA 72 h	L6	Treating cells with EPA and DHA ↑ fusion index. This is accompanied by ↑ in p70S6K1, and membrane bund protein caveolin-3 that is associated with fusion.
Luo, et la. [165]	400-600 μM EPA 10 days	C2C12	EPA in high doses of 400-600 μM induces transdifferentiation of myoblasts to adipocytes by ↓ Wnt/β-catanin signalling through PPARγ
Saini, et al. [161]	50 μM EPA 36 h & 72 h 750 μM PAL 36 h & 72 h	C2	EPA ↓ palmitate induced cell death via affecting the MAPK pathway. EPA can protect cells from MAPK induced cell death and myogenic suppression but not against of Id3/caspase induced apoptosis EPA ↑ MyoD

Abbreviations: Eicosapentaenoic acid (EPA), Docosahexanenoic acid (DHA) Alpha linolenic acid (ALA), Proteolysis inducing factor (PIF), tumour necrosis factor-alpha (TNF- α), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), Peroxisome proliferator activated-receptor gamma (PPARγ), Cyclin dependent kinase 2 (CDK2), Palmitate (PAL). ↑/↓ indicates the trend.

Table 2.2. Summary of studies investigating the effects of PUFAs in full differentiated myotubes.

Publication	Treatment	Outcome
Bryner, et al. [125]	100 μM DHA 500 μM PAL	DHA attenuates ↓ tube size by PAL
Wang, et al. [98]	300-700 μM DHA 300-700 μM EPA	EPA and DHA ↓ total protein degradation in a dose response manner DHA and EPA ↓ protein degradation by regulating the IkBα/NFkB signalling DHA is more effective in preventing degradation than EPA
Kamolrat and Gray [148]	50 μM DHA 50 μM EPA	EPA ↑ MPS and ↓ MPB DHA has no effect on MPS or MPB at 50 μM
Lee, et al.[166]	1-50 μM DHA, 1-50 μM EPA	DHA and EPA both ↑ UCP3 AICAR synergistically ↑ UCP3 activity when co-treated with DHA or EPA
Woodworth-Hobbs, et al. [167]	100 μM DHA 500 μM PAL	DHA ↓ proteolysis by palmitate in a time dependent manner and counteracts the effects of PAL by restoring Akt activity, ↓ FOXO activity ↓ and atrogin expression.
Lee, et al. [120]	1-50 μM DHA 1-50 μM EPA	EPA and DHA ↑ PGC-1α, NRF-1 and Tfam gene expression and ↑ PGC-1α promoter activity
Chen, et al. [168]	50 μM DHA 50 μM EPA, 50 μM AA 750 μM PAL	The negative effects of PAL on AMPK phosphorylation GLUT4 mRNA expression and basal glucose uptake were ↓ by AA, DHA and EPA. The expression of genes associated with protein degradation was ↓ by EPA, DHA and AA
Pinel, et al. [124]	50 μM DHA 50 μM EPA 50 μM ALA 500 μM PAL	EPA and DHA ↑ membrane fluidity which may improve glucose uptake. EPA and DHA ↑ key gene involved in β-oxidation.

Abbreviations: Eicosapentaenoic acid (EPA), Docosahexanenoic acid (DHA)), tumour necrosis factor-alpha (TNF- α), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), Peroxisome proliferator activated-receptor gamma (PPARγ), Palmitate (PAL), Muscle protein synthesis (MPS) Muscle protein breakdown (MPB), Uncoupling

protein-3 (UCP3), 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR), AMP-dependent protein kinase (AMPK), Forkhead box O (FOXO), Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α). ↑/↓ indicates the trend.

Table 2.3. Summary of human studies that have investigated the effects of n-3 PUFA supplementation combined with resistance exercise and/or protein supplementation on muscle anabolism and muscle function

Publication	Dose	Duration	Age	Sex	N	REX	Protein	Outcomes
Smith, et al. [65]	1.86 g/d EPA 1.5 g/d DHA	8 weeks	39.7 ± 1.7	M F	5 4	-	Insulin clamp Amino Acid clamp	Hyperaminoacidemia-hyperinsulinemia induced MPS ↑ after supplementation and ↑ p-mTORSer2448 p-p70S6k1Thr389.
Smith, et al. [76]	1.86 g/d EPA 1.5 g/d DHA	8 weeks	71 ± 1	M F	5 3	-	Insulin clamp Amino Acid clamp	Hyperaminoacidemia-hyperinsulinemia induced MPS ↑ after supplementation and ↑ p-mTORSer2448 p-p70S6k1Thr389.
Rodacki, et al. [66]	0.4 g/d EPA 0.3 g/d DHA	90 days 150 days	64 ± 1.4 64 ± 1.4	F F	45 45	3x/ week 3x/ week	-	Fish Oil supplementation and strength training results in ↑ improvements in peak torque and rate of torque development than strength training alone.
McGlory, et al. [10]	3.5 g/ EPA 0.9 g/d DHA	4 weeks	21.3 ± 3	M	10	-	-	EPA and DHA in the blood ↑ from week 0 to 2 and ↔ from 2 to 4 weeks. Levels of EPA and DHA in skeletal muscle are ↑ from 0 to 2 weeks and further ↑ 2 to 4 weeks.
Smith, et al. [64]	1.86 g/d EPA 1.5 g/day DHA	26 weeks	68 ± 5	M F	10 19	-	-	↑ thigh muscle volume, ↑ hand grip strength and ↑1RM
McGlory, et al. [11]	3.5 g/day EPA 0.9 g/day DHA 0.1 g/day DPA	8 weeks	24 ± 0	M	9	Acute bout	30 g Whey	↔ MPS following an acute bout of REX and protein ingestion ↓ p70S6K1 and Akt anabolic kinases following REX and protein ingestion.
Da Boit, et al. [107]	2.1 g/ day EPA 0.6 g/day DHA	18 weeks	70.6 ± 4.5 70.7 ± 3.3	M F	27 23	2x/ week	-	↑ Maximal isometric torque and ↑ muscle quality in women after exercise training in n–3 PUFA group than in the placebo group, with no such differences in men
Lalia, et al. [169]	0.675 g/day EPA 0.3 g/day DHA	16 weeks	27 ± 5 76 ± 5	M/F M/F	12 12	Acute bout	-	Myofibrillar MPS ↑ in both young and old after exercise and supplementation whereas mitoMPS and sarcoMPS ↑ in the older participants

Abbreviations: Eicosapentaenoic acid (EPA), Docosahexanenoic acid (DHA), Docosapentanoeic acid (DPA), Resistance exercise (REX), Muscle protein synthesis (MPS) Muscle protein breakdown (MPB), 1 Repetition Max (1RM), Male (M), Female (F). ↑/↓ indicates the trend.

Limitations and Differences Between *In Vitro* Cell Culture and *In Vivo* Human Studies

It is evident that EPA and DHA elicit differential effects during various stages of myogenesis (**Table 2.1**) but also in fully differentiated myotubes (**Table 2.2**). The differences identified could be due to structural characteristics of the two fatty acids as well as their ability to produce different metabolite species [170, 171]. It is important to highlight that the effects of n-3 PUFA in-vitro are dose and time dependent and an accurate balance between those parameters is required to significantly augment in-vitro proliferation, differentiation and post-differentiation events. Inconsistency in these parameters may also account for significant differences in results between published reports. For example, very high concentrations (200-600 μM) of EPA added to mice C2C12 myoblasts during proliferation for up to 10 days induce transdifferentiation to adipocytes [165]. In contrast, EPA has no effect on proliferation rate of C2C12 cells at concentration of 0.1-10 μM when treated for 48 h, while DHA proliferative stimulation starts at 10 μM [164]. Exposure to EPA and DHA at higher concentrations (20-100 μM) for 24 h attenuates the cell growth rate by preventing the transition of C2C12 cells from G1 to S phase of the cell cycle with DHA having a more pronounced effect [157].

Additionally, myotubes can be more resilient than myoblasts as concentrations of EPA and DHA ranging from 400-600 μM in myotubes do not cause protein degradation [98, 101] (Table 2.2). While 50 μM of both EPA and DHA promote myogenic responses during differentiation in C2C12 cells (Table 2.1), other cell lines such as L6 rat cells are sensitive to 20 μM of EPA and DHA. Furthermore, while studying in-vitro myogenesis provides mechanistic insight into the processes underlying muscle development and regeneration, it more closely resembles embryonic

muscle development and does not necessarily recapitulate what occurs during myogenesis in adult muscle following injury or exercise. Nevertheless *in vivo* human studies investigating the effects on n-3 PUFAs on satellite cell activation are warranted to compliment the in-vitro data.

One of the major discrepancies in human studies investigating the effects of n-3 PUFAs alone or combined with exercise and/or protein ingestion is the n-3 PUFA dosage and supplementation duration (Table 2.3). This discrepancy makes it difficult to elucidate the optimal supplementation duration and dosage required to effect on intracellular signalling and muscle anabolism responses. These dose discrepancies may arise due to regional differences in the availability of certain n-3 PUFA supplements whereas discrepancies in the duration of the supplementation may due to the research question being asked (i.e. acute MPS responses vs chronic changes in lean mass).

Future Directions

With respect to muscle fibre hypertrophy, studies concluding that satellite cells are necessary for muscle fibre hypertrophy utilise young or developing mice [172, 173] (<4 months of age). However, in models of fully grown/developed mice (>4 months of age), short-term hypertrophy occurs in the absence of satellite cells [52, 56, 174]. While satellite cells play an integral role in regulating the extracellular matrix during remodelling [58, 62, 63], it is yet to be determined whether excessive extracellular matrix (ECM) accumulation or a "myonuclear domain ceiling" limits prolonged hypertrophy in the absence of satellite cells. As such, it is likely that satellite cells are necessary for muscle fibre hypertrophy beyond a certain threshold. With regards to n-3 PUFAs, 6 months of supplementation in older individuals has been shown to increase gene expression of various pathways involved in growth and structural support (ECM organisation)

[175]. These small n-3 PUFA-induced transcriptional changes in pathways related to growth and ECM organization could modulate satellite cell function given the reciprocal relationship between fibroblasts and satellite cells [63]. Thus, n-3 PUFAs may possess the capacity to alter satellite cell activity directly by altering proliferation and differentiation or indirectly via changes to the ECM.

The *in vitro* effects of DPA on myogenesis remain largely understudied. This represents an area for future investigations as DPA is a biologically active n-3 PUFA with similar properties to DHA and EPA and can also be readily converted to EPA [69, 176, 177]. Thus, further research is required to elucidate whether DPA can also induce myogenesis in a dose dependent manner by regulating the same molecular targets as EPA and DHA. Exploring the effects of n-3 PUFAs on human satellite cell myogenesis *in vitro* as well as *ex vivo* would provide new insight into the effects timing and dose elicit during the muscle repair process. Understanding precisely how n-3 PUFAs are able to alter anabolic signalling following hypertrophic stimuli such as protein ingestion and resistance exercise could see novel n-3 PUFA supplementation strategies developed to maximises these responses increasing or at the very least maintaining skeletal muscle mass.

Summary and Conclusion

The mechanisms by which n-3 PUFAs exert their multiple beneficial effects centre largely on their increased incorporation into cellular membranes which induce changes to various phospholipid species used as substrates for various signalling cascades. n-3 PUFAs are also capable of reducing systemic inflammation by inhibiting the release of pro-inflammatory cytokines from immune cells [178-180] and improving the signalling efficiency of proteins involved in growth and hypertrophy [11, 101, 108]. However, several questions remain unanswered regarding

n-3 PUFA supplementation and human skeletal muscle metabolism. Firstly, the optimal dosage and duration required to elicit beneficial responses in skeletal muscle remains unclear. As it stands, supplementation dosages ranging from 2-5 g/day for a minimum of 4 wks results in improvements in anabolic signalling efficiency and muscle strength outcomes (Table 2.3). In older individuals sex differences in muscle quality have been observed following n-3 PUFA supplementation [107]. The mechanistic basis for these differences remains to be determined. Other sexual dimorphisms may also exist following n-3 PUFA supplementation such as changes to anabolic signalling activity and rates of MPS, though no study to date has been able to detect these changes.

Secondly, it is also possible that doses within ranges of 2-5 g/day may affect satellite cell activity particularly after exercise, although experimental evidence is required to confirm this hypothesis. The anti-lipotoxic and anti-cytotoxic properties of EPA and DHA during *in vitro* myogenesis have been established with 50 µM being an effective concentration to induce protective effects. Whether EPA, DHA and DPA similarly or differentially modulate the activity and expression of myogenic regulatory factors at 50 µM during myogenesis remains unclear.

Lastly, the capacity for n-3 PUFAs to activate satellite cells in human skeletal muscle also remains to be determined. Such information may possess important health and clinical implications by providing further support for n-3 PUFA supplementation to augment the growth, development and regenerative capabilities of skeletal muscle. Establishing how EPA, DHA and DPA modulate myogenesis and alters intracellular anabolic signalling would provide a greater understanding as to how n-3 PUFA supplementation can be used as an effective nutritional strategy in conjunction with exercise to increase and maintain muscle mass in “at-risk” populations.

Chapter 3 : Preface to Original Research Article

One of the major themes of the literature review chapter was the potential for n-3 PUFAs to support skeletal muscle growth and regeneration. In adults skeletal muscle growth and repair are facilitated by specialised cells called satellite cells. Following activation, these cells help repair muscle by proliferating, differentiating and fusing to existing muscle fibres [181]. Muscle cell growth and repair can be modelled *in vitro* by investigating surrogate markers of myoblast viability, proliferation and differentiation to myotubes after exposure to cellular insults, nutritional supplements and/or growth factors [182].

FFA serve important physiological roles as a critical source of energy during periods of starvation or prolonged exercise [183, 184]. As precursors to secondary messenger molecules, FFA can also affect cellular signalling thereby altering the expression of metabolic regulatory genes and enzymatic activities of metabolic biochemical reactions [185, 186]. PAL is one of the most abundant FFA in human plasma representing ~25% of the total circulating FFA pool [187]. Although elevations in FFA concentrations are of physiological importance to help meet energy requirements (e.g. during fasting and prolonged exercise), sustained elevations in FFA can contribute to and exacerbate obesity and associated metabolic diseases such as insulin resistance and type 2 diabetes [188]. Elevated FFA can also impair skeletal muscle function by disrupting intracellular signalling pathways required for protein synthesis [23, 189]. Consequently, cellular PAL exposure is commonly used *in vitro* to recapitulate the increased circulating FFA observed in individuals with obesity and metabolic diseases.

In contrast, unsaturated FFA such as the n-3 PUFAs EPA and DHA have been shown to induce beneficial effects on skeletal muscle by stimulating muscle protein synthesis [148] and preventing the lipotoxic effects of PAL that contribute to muscle atrophy [125]. In the current literature there is a paucity of studies examining the protective effects of n-3 PUFAs on skeletal muscle cells against PAL-induced impairments in cell viability and myogenesis. While two studies to date have investigated the protective effects of n-3 PUFAs against PAL exposure in myotubes [25, 125], no study to date has systematically compared the protective effects of the three n-3 PUFAs EPA, DPA and DHA in both undifferentiated myoblasts and differentiated myotubes.

An *in vitro* mouse C2C12 skeletal muscle cell model was employed (**Figure 3.1)**, to compare the protective effects and underlying mechanisms by which n-3 PUFAs protect against PAL-induced cell death and subsequently influence the differentiation process. An understanding of how marine derived n-3 PUFAs can potentially protect against PAL-induced cell death in myoblasts and thereby prevent defects in myotube differentiation may facilitate novel supplementation strategies to help maintain skeletal muscle mass and function in settings of elevated circulating FFA such as obesity, metabolic disease and ageing.

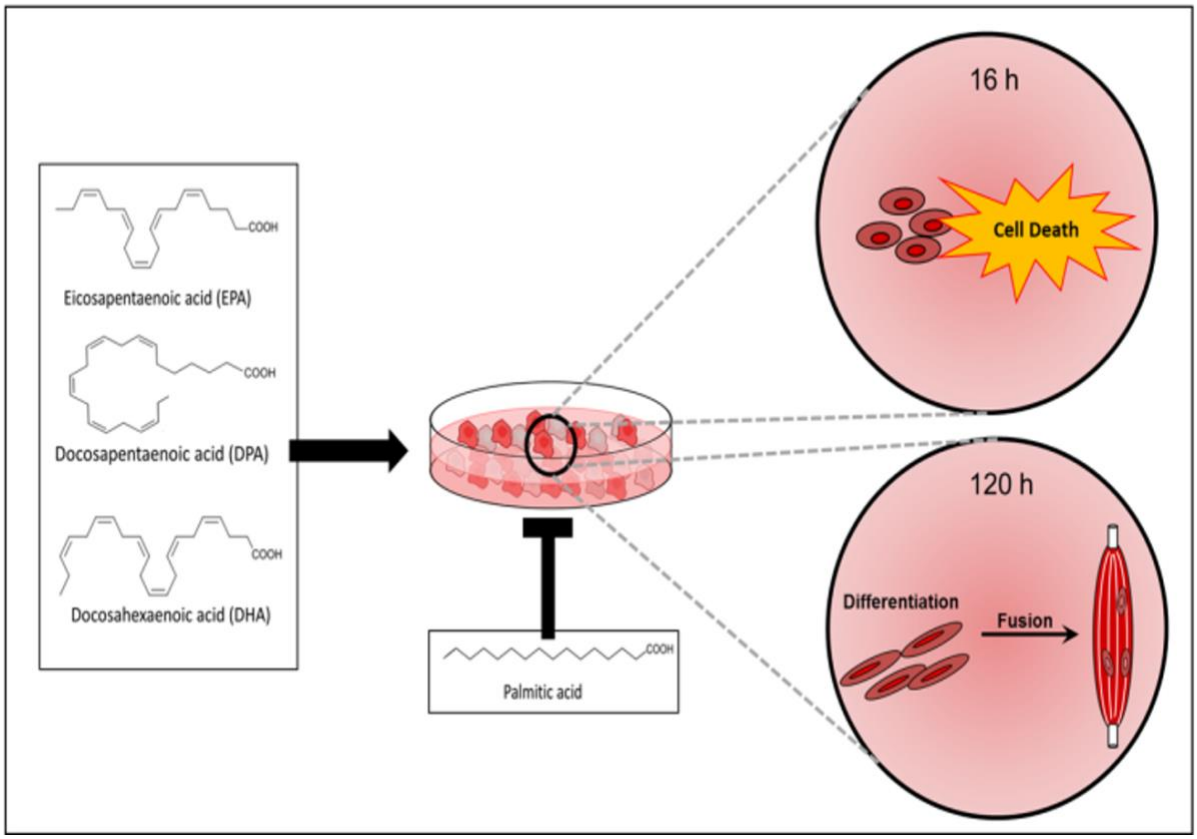

Figure 3.1. Schematic overview of study design in mouse C2C12 myoblasts (16 h, top panel) and myotubes (120 h, bottom panel) investigating the protective effects of EPA, DPA and DHA against the lipotoxic effects of palmitate.

Chapter 4 : Omega-3 Polyunsaturated Fatty Acids Mitigate Palmitate-Induced Impairments in Skeletal Muscle Cell Viability and Differentiation

This chapter has been adapted from the following peer-reviewed and published original research article:

1. **Tachtsis B**, Whitfield J, Hawley JA and Hoffman NJ (2020) Omega-3 Polyunsaturated Fatty Acids Mitigate Palmitate-Induced Impairments in Skeletal Muscle Cell Viability and Differentiation. Front. Physiol. 11:563. doi: 10.3389/fphys.2020.00563

Title:

Omega-3 Polyunsaturated Fatty Acids Mitigate Palmitate-Induced Impairments in Skeletal Muscle Cell Viability and Differentiation

Abstract

Accumulation of excess saturated free fatty acids such as palmitate (PAL) in skeletal muscle leads to reductions in mitochondrial integrity, cell viability and differentiation. Omega-3 polyunsaturated fatty acids (n-3 PUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) counteract PAL-induced lipid accumulation. EPA and DHA, as well as the n-3 PUFA docosapentaenoic acid (DPA), may therefore mitigate PAL-induced lipotoxicity to promote skeletal muscle cell survival and differentiation. C2C12 myoblasts were treated with 50 μM EPA, DPA or DHA in the absence or presence of 500 μM PAL for 16 h either prior to myoblast analysis or induction of differentiation. Myoblast viability and markers of apoptosis, ER stress and myotube differentiation capacity were investigated using fluorescence microscopy and immunoblotting. High-resolution respirometry was used to assess mitochondrial function and membrane integrity. PAL induced cell death via apoptosis and increased protein content of ER stress markers Binding immunoglobulin protein (BiP) and CCAAT-enhancer-binding protein homologous protein (CHOP). EPA, DPA and DHA co-treatment maintained cell viability, prevented PAL-induced apoptosis and attenuated PAL-induced increases in BiP, whereas only DPA prevented increases in CHOP. PAL subsequently reduced protein content of the differentiation marker myogenin and inhibited myotube formation, and all n-3 PUFAs promoted myotube formation in the presence of PAL. Furthermore, DPA prevented PAL-induced release of cytochrome c and maintained mitochondrial integrity. These findings demonstrate the n-3 PUFAs EPA, DPA and DHA elicit similar protective effects against PAL-induced impairments in muscle

cell viability and differentiation. Mechanistically, the protective effects of DPA against PAL lipotoxicity can be attributed in part to its ability to maintain mitochondrial respiratory capacity via mitigating PAL-induced loss of mitochondrial membrane integrity.

Introduction

Excess cellular accumulation of lipids such as PAL negatively impacts skeletal muscle cell viability. PAL is a dietary saturated fatty acid abundant in Western diets that is used to experimentally assess the lipotoxic effects of elevated saturated fatty acids in tissues such as skeletal muscle [190]. PAL exposure *in vitro* negatively impacts skeletal muscle cell metabolism by impairing insulin sensitivity [191], suppressing protein synthesis [23] and upregulating proteolytic machinery [167]. Furthermore, PAL has been shown to induce endoplasmic reticulum (ER) stress, which consequently activates the unfolded protein response (UPR), a series of coordinated signalling networks that collectively stimulate adaptive responses to re-establish cellular homeostasis [192]. In the event the UPR is unable to restore protein homeostasis in response to a cellular insult such as PAL, programmed cell death can be induced via apoptosis [193].

In contrast to saturated fatty acids such as PAL, omega-3 polyunsaturated fatty acids (n-3 PUFAs) possess anti-inflammatory properties and can enhance skeletal muscle protein synthetic responses and metabolism by altering cellular membrane lipid composition [109, 194-197]. Furthermore, the n-3 PUFA docosahexaenoic acid (DHA) has been shown to ameliorate lipotoxic effects of PAL in skeletal muscle cell models by restoring insulin sensitivity [125] and preventing activation of the UPR in differentiated skeletal muscle myotubes [167]. In contrast to DHA, eicosapentaenoic acid (EPA) is the only n-3 PUFA shown to protect against the deleterious effects

of inflammation [198] and PAL exposure [161] by partially restoring the regenerative capacity of skeletal muscle. EPA and DHA are commonly found in fish oil supplements and have been demonstrated to improve markers of myogenic differentiation (i.e. myosin 4 expression and myotube fusion index) [108].

Docosapentaenoic acid (DPA) is a less-studied n-3 PUFA, but possesses similar bioactive properties to EPA and DHA [22, 199]. Cell based studies have shown that DPA is an intermediate n-3 PUFA and can be readily converted to EPA [200-202], while conversion to DHA is limited [22]. However, it remains to be determined whether DPA can also protect skeletal muscle against cellular insults such as PAL in a similar manner to these other n-3 PUFAs. Moreover, previous studies examining the effects of n-3 PUFAs in skeletal muscle cell models *in vitro* have utilised fully differentiated myotubes [9, 148], while little emphasis has been placed on comparing their effects in proliferating myoblasts and during the induction of myotube differentiation. Investigating the cellular events preceding myotube formation is important as myoblasts are the primary drivers of skeletal muscle embryonic development and skeletal muscle repair in adults [203, 204].

Accordingly, the primary aim of this study was to compare the efficacy of the n-3 PUFAs EPA, DPA and DHA in mitigating PAL-induced lipotoxicity in skeletal muscle cells. The secondary aims were to determine if n-3 PUFAs attenuate PAL-induced lipotoxic cellular mechanisms including ER stress induction and loss of mitochondrial integrity, as well as subsequent defects in myotube differentiation. It was hypothesised that EPA, DPA and DHA

would elicit similar protective effects against PAL-induced lipotoxicity and attenuate PAL-induced impairments in myotube formation to a similar extent.

Materials and Methods

C2C12 cell culture

Mouse C2C12 myoblasts (Lonza, Basel, Switzerland) were cultured in DMEM containing 4.5 g/L glucose and L-glutamine (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Life Technologies, Carlsbad, CA, USA) and 0.5% penicillin/streptomycin (Life Technologies, Carlsbad, CA, USA) in T75 cm^2 flasks at 37°C in a humidified 5% CO_2 incubator. At 70% confluency, myoblasts (passage numbers 7-14) were washed with 1× PBS and trypsinised using 0.25% trypsin solution (Life Technologies, Carlsbad, CA, USA). Dissociated cells were washed with 1× PBS solution (Life Technologies, Carlsbad, CA, USA), centrifuged (Heraeus Sepatech Megafuge 1.0R centrifuge, BS4402/A rotor #3360) at 3000 g for 3 min and re-seeded at a density of 3.0×10^5 mL onto 6 or 96 well plates for immunoblotting and fluorescence microscopy analysis. Each biological replicate experiment was performed with cells cultured and harvested independently, and where applicable technical replicates were also included within each biological replicate (as described in each analysis below).

Preparation of fatty acid treatments

DHA (cis-4, 7, 13, 16, 19-docosahexaenoic acid), EPA (cis-5, 8, 11, 14, 17-eicosapentaenoic acid) and PAL were obtained from Sigma-Aldrich (St. Louis, MO, USA). DPA (cis-7, 10, 13, 16, 19-Docosapentaenoic acid) was purchased from Nu-Chek Prep Inc (Elysian, MN, USA). Stock solutions of n-3 PUFAs and PAL were prepared by dissolving each fatty acid in 100% (v/v)

ethanol. Working solutions of n-3 PUFAs and PAL were made by diluting each stock solution in pre-warmed DMEM containing 2% (w/v) fatty acid free BSA (Sigma-Aldrich, St. Louis, MO, USA) to achieve a final concentration of 50 μM for each n-3 PUFA [25, 205] and 500 μM for PAL [23]. PAL stock solutions were sonicated at 50°C for 10 min to ensure PAL was completely solubilised. For cell treatments, working solutions were prepared fresh on the day of each experiment. All fatty acids were incubated at 37°C for approximately 30 min before being applied to cells. A final concentration of ethanol (< 0.01%) was used as a vehicle control and was identical between each treatment. Control treatment groups also contained 2% (w/v) fatty acid free BSA. The number of cells initially plated for each experiment and treatment condition was equal. At an equal level of 70% confluency between each treatment condition, cells were incubated with either vehicle, EPA, DHA, DPA and/or co-treated with PAL for 16 h. For assessment of differentiation capacity following this single overnight 16 h n-3 PUFA and/or PAL treatment(s), the following morning media was switched to DMEM containing 2% horse serum (Life Technologies, Carlsbad, CA, USA) to induce myotube differentiation. During the 5-day period of myotube differentiation, media was replaced every 2 days.

n-3 PUFA incorporation

After 16 h treatment with 50 μM EPA, DPA or DHA, media was aspirated and myoblasts were washed with ice-cold 1×PBS, pH 7.4 twice. 100 μL of trypsin EDTA was added to each well and incubated at 37 °C for 5 min. Cells from 3 wells were pooled together and 1.5 ml PBS was added to trypsinised cells. The cells were then transferred to 10 ml glass screw-capped (Teflon-lined) tubes, and lipids were extracted using a 2:1 chloroform:methanol mixture, as previously described in Kaur et al., [69] and Portolesi et al., [206]. In brief, after the addition of chloroform:methanol,

the samples were vortexed for 10 min and centrifuged (Heraeus Sepatech Megafuge 1.0R centrifuge, BS4402/A rotor #3360) at 1500 g for 10 min to separate the aqueous and organic phase at room temperature. The organic phase containing the lipid was removed and transferred to a new glass tube and evaporated under a stream of nitrogen. Fatty acids were derivatised with 2% H_2SO_4 in 100% methanol for 3 h at 80 °C to form the fatty acid methyl esters (FAME). The purified FAMEs were separated by capillary gas liquid chromatography (GLC) using a 50 m 0.32 mm (I.D.) fused silica bonded phase column (BPX70, SGE, Melbourne, Australia). Fatty acids were identified by comparison with standard mixtures of FAME as detailed by Kaur et al., [69] and results were calculated using response factors derived from FAME standards of known composition (Nu-Chek Prep, Inc., Elysian, MN, USA) and normalised to cell number.

Cell viability analysis

Following n-3 PUFA and/or PAL treatment(s), myoblasts were trypsinised (described above) and equal volumes of cell suspension and 0.4% trypan blue (Life Technologies, Carlsbad, CA, USA) were combined. To assess the number of remaining viable cells in each condition, 10 μL of this combined solution was loaded into an automated cell counting system (Countess II FL; Life Technologies, Carlsbad, CA, USA). DAPI staining (1:20,000, Life Technologies, Carlsbad, CA, USA) was also used to assess cell viability following n-3 PUFA and/or PAL treatment(s), as morphological changes to nuclei following PAL treatment are typically observed during the late stages of cell death [207]. Nuclei were defined as DAPI positive areas ranging from 10-1000 μm^2. By defining these size criteria, small nuclear fragments along with large nuclei clusters were manually excluded. The nuclear morphology parameters analysed include area (μm^2), perimeter (μm), and brightness (relative fluorescence units/nuclei). A total of 10 fields were randomly

selected from each treatment condition, and images were collected at 20× magnification. For all treatment conditions, parameters such as brightness and contrast were kept consistent during the acquisition and analysis of all images. Analysis was performed by converting images to 16-bit greyscale images and applying the threshold function within ImageJ software.

TUNEL assay apoptosis analysis

To assess apoptosis following n-3 PUFA and/or PAL treatment(s), TUNEL assay was performed using an In Situ Direct DNA Fragmentation (TUNEL) Assay Kit (Abcam, Cambridge, UK, ab66108) according to the manufacturer's instructions. Briefly, cell smears after paraformaldehyde fixation, blocking and permeabilisation were incubated with TUNEL reaction mixture for 1 h at 37 °C protected from light and counterstained with RNase/PI solution for 30 minutes. Cells were imaged using the EVOS FL Auto 2 Cell Imaging System at 20× magnification. A total of ten fields were randomly selected for imaging, and for each treatment condition TUNEL positive cells relative to total cells in these fields were counted (Life Technologies, Carlsbad, CA, USA). The same microscope settings (i.e. brightness and contrast) were used for imaging all treatment conditions. For all treatment conditions, parameters such as brightness and contrast were kept consistent during the acquisition and analysis of all images. Analysis was performed by converting images to 16-bit greyscale images and applying the threshold function within ImageJ software.

Immunofluorescence analysis of myotube differentiation

To assess myotube formation at 5 days following 16 h overnight n-3 PUFA and/or PAL treatment(s) and induction of differentiation the following morning, cells were washed twice with

ice-cold 1× PBS, fixed with 100% ethanol and permeabilised with 0.1% Triton X-100. Cells were blocked overnight with 2% BSA solution and incubated with desmin primary antibody (D33, MA5-13259, Life Technologies, Carlsbad, CA, USA) overnight at 4°C. Cells were then incubated for 2 h at room temperature in Alexa Fluor 488 secondary antibody (1:200, Life Technologies, Life Technologies, Carlsbad, CA, USA) protected from light. Nuclei were counterstained with DAPI. Images were captured using EVOS FL Auto 2 Cell Imaging System (Life Technologies, Carlsbad, CA, USA) at 10× magnification. A total of five fields were randomly selected from each treatment condition. The same microscope settings (i.e. brightness and contrast) were used for imaging all treatment conditions. Immunofluorescence analysis was performed using ImageJ software. For all treatment conditions, parameters such as brightness and contrast were kept consistent during the acquisition and analysis of all images. Analysis was performed using the manual cell counter function within ImageJ.

Immunoblotting

Following n-3 PUFA and/or PAL treatment(s) myoblasts and myotubes were rinsed twice with ice-cold 1× PBS and scraped in the presence of lysis buffer containing 50 mM Tris HCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM DTT, 10 $\mu g \cdot mL^{-1}$ trypsin inhibitor, 2 $\mu g \cdot mL^{-1}$ aprotinin, 1 mM benzamidine, and 1 mM PMSF (Sigma-Aldrich, St. Louis, MO, USA). Lysates were passed through a 22-gauge needle ten times for homogenisation and centrifuged (Eppendorf 5424 R centrifuge, F45-24-11 rotor) at 15,871 g for 15 min at 4°C to remove cellular debris. The resulting supernatant was analysed for total protein concentration using a BCA protein assay (Pierce, Rockford, IL, USA). Lysates were re-suspended in Laemmli sample buffer at 0.33 $\mu g \cdot \mu L^{-1}$. Proteins were separated by

SDS-PAGE using 4–20% Stain-Free Precast gels (Bio-Rad, Hercules, CA, USA) and transferred to polyvinylidene fluoride membranes, blocked with 5% non-fat milk, washed with 10 mM Tris·HCl, 100 mM NaCl, and 0.02% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) and incubated overnight at 4°C with primary antibody as specified by the respective supplier (1:1000, unless stated otherwise) including BiP (Cell Signaling Technology, Danvers, MA, USA, #3177, clone number C50B12), CHOP (Cell Signaling Technology, Danvers, MA, USA #5554, clone number D46F1), phospho-eIF2α Ser51 (Cell Signaling Technology, Danvers, MA, USA #9721), total eIF2α (Cell Signaling Technology, Danvers, MA, USA #9722), myosin heavy chain (Thermo Fisher, Waltham, MA, USA, PA5-31466), and myogenin [F5D] (Abcam, Cambridge, UK, ab1835). Membranes were then incubated with secondary antibody (anti-rabbit, HRP-linked, Cell Signaling Technology, Danvers, MA, USA 1:2000). All gels were equally loaded with 10 µg protein in each well. An identical pooled sample of cell lysate was run on every gel for each target analysed to normalise band intensity between gels, and all proteins were normalised to the total protein loaded in each lane using stain-free imaging technology (Bio-Rad, Hercules, CA, USA) as performed previously [8, 208].

Mitochondrial respiration

C2C12 myoblasts were incubated with 2% BSA (control), DPA or co-treated with PAL and DPA for 16 h. High-resolution respirometry experiments were performed in MiR05 respiration medium (0.5 mM EGTA, 3 mM MgCl2, 60 mM K-lactobionic acid, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 100 mM D-Sucrose and 1 g/L BSA, pH 7.1) at 37°C using an Oxygraph-2k FluoRespirometer (Series G) and DatLab Version 7.3 (Oroboros Instruments, Innsbruck, Austria). For all experiments, each chamber was loaded with 500 µL respiration medium

containing $1\times10_6$ viable cells (determined using a hemocytometer), which were permeabilised by the addition of 4.1 µM digitonin. Respiration was initiated with 10 mM pyruvate and 5 mM malate, followed by 5 mM ADP. Glutamate (10 mM) and succinate (10 mM) were added to determine maximal rates of respiration. Mitochondrial membrane integrity was tested through the addition of cytochrome c (10 µM), with <15% increase in respiration indicating preservation of outer mitochondrial membrane integrity [209, 210]. Finally, maximal electron transfer was stimulated through the titration of CCCP (sequential 0.5 µM additions). All experiments were performed in duplicate from cells of the same passage (technical replicates), with respiration rates averaged from both chambers of one Oxygraph-2K instrument. All treatments (with and without palmitate) and conditions (control and DPA) were performed on the same day. Cells were grown and harvested independently to generate a total sample size of n=4 biological replicates. Each biological replicate experiment consisted of two technical replicate measurements using separate chambers. For all experiments, rates of oxygen consumption (JO_2) were normalised to chamber volume (2 mL).

Statistical Analysis

Statistical analysis was conducted using Sigma Plot (Version 12.5). Data were analysed by a one-way or two-way analysis of variance (ANOVA). Where significant main effects were detected, Bonferroni *post hoc* analysis (condition × treatment) were used to determine differences within each group. All data are presented as mean ± SEM with p-values < 0.05 considered statistically significant. All results are representative of a minimum of three biological replicate experiments from independently cultured and harvested cells.

Results

EPA, DPA and DHA protect against PAL-induced cell death

Myoblasts were treated for 16 h with PAL (0-1000 μM dose response) to identify the concentration(s) at which PAL-induced lipotoxicity occurs. The lowest concentration tested at which significant reductions in cell viability occurred was 500 μM (~48% reduction compared to CON $p < 0.05$), and this concentration was selected for use in all subsequent experiments (**Figure 4.1 A**). There was no difference in viability observed between individual n-3 PUFAs alone relative to untreated control (CON) cells, while treatment of CON cells with PAL (CON-PAL) induced a ~60% reduction in viability ($p < 0.05$) (**Figure 4.1 B**). When myoblasts were co-treated with EPA or DHA in the presence of PAL, cell viability was significantly increased relative to CON-PAL (~55% and ~50% respectively, $p < 0.05$), although cell viability remained ~10% and ~20% lower than CON cells, respectively ($p < 0.05$). In contrast, co-treatment with DPA prevented PAL-induced decreases in cell viability, as DPA-PAL cell viability was not significantly different from CON or DPA cells alone.

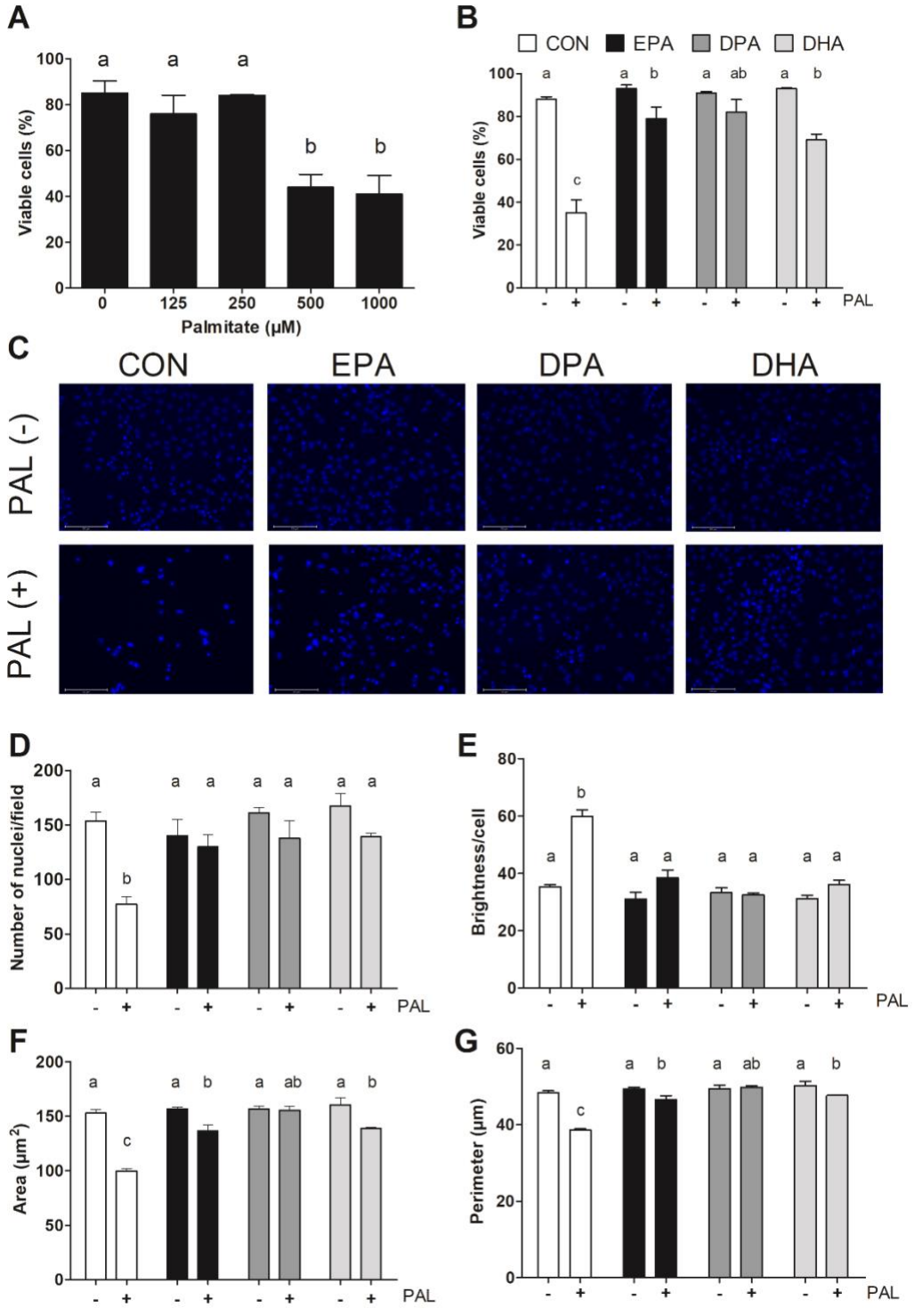

Figure 4.1. EPA, DPA and DHA protect against PAL-induced reductions in cell viability. Trypan blue exclusion test dose response experiment in myoblasts following 16 h of palmitate (PAL) treatment to assess cell viability (A). Trypan blue exclusion test of cell viability following treatment with 500 μM PAL and/or 50 μM EPA, DPA or DHA (B). Representative images of

nuclear DAPI staining following treatments (C). Average number of nuclei/field (D), brightness/nuclei (E), nuclear area (F) and nuclear perimeter (μm) (G). Treatments that do not share letters are considered statistically significant (p <0.05; myoblasts were cultured and harvested independently to generate a total sample size of 3-4 biological replicates). Scale bar represents 125 μm. Values are presented as mean ± SEM.

Nuclear staining with DAPI confirmed treatment with PAL induced cell death, as evidenced by reductions in the number of nuclei per field (~50%, $p < 0.05$; **Figure 4.1 D**), nuclear area (~35%, $p < 0.05$; **Figure 4.1 F**) and perimeter (~20%, $p < 0.05$; **Figure 4.1 G**) versus CON. An increase in nuclear brightness (~70% relative to CON, $p < 0.05$) was detected following PAL exposure, as demonstrated to be indicative of cell death [207] (**Figure 4.1 E**). However, there were no detectable changes between any of the PUFA treatments alone or PAL-PUFA co-treatments relative to CON in terms of nuclei number or brightness (**Figure 4.1 D-E**). Both EPA and DHA attenuated PAL-induced decreases in nuclei area ($p < 0.05$; **Figure 4.1 F**) and perimeter ($p < 0.05$; **Figure 4.1 G**) relative to CON-PAL, although these indices were still decreased relative to CON and PUFA alone. In contrast, DPA co-treatment completely protected against PAL-induced reductions in cell area and perimeter, as there were no differences detected in these measures between DPA-PAL compared to CON or DPA alone (**Figure 4.1 F-G**).

TUNEL assay staining of DNA fragmentation characteristic of the late phases of apoptosis confirmed that PAL treatment increased apoptosis (~60-fold increase in the percentage of TUNEL-positive cells relative to CON, $p < 0.05$). PAL-induced apoptotic cell death was reduced in the presence of EPA, DPA and DHA by ~60-fold, ~51-fold and ~54-fold relative to CON-PAL, respectively), with no detectable differences observed between PUFA treatments alone or PAL-PUFA co-treatments relative to CON (**Figure 4.2 A-B**).

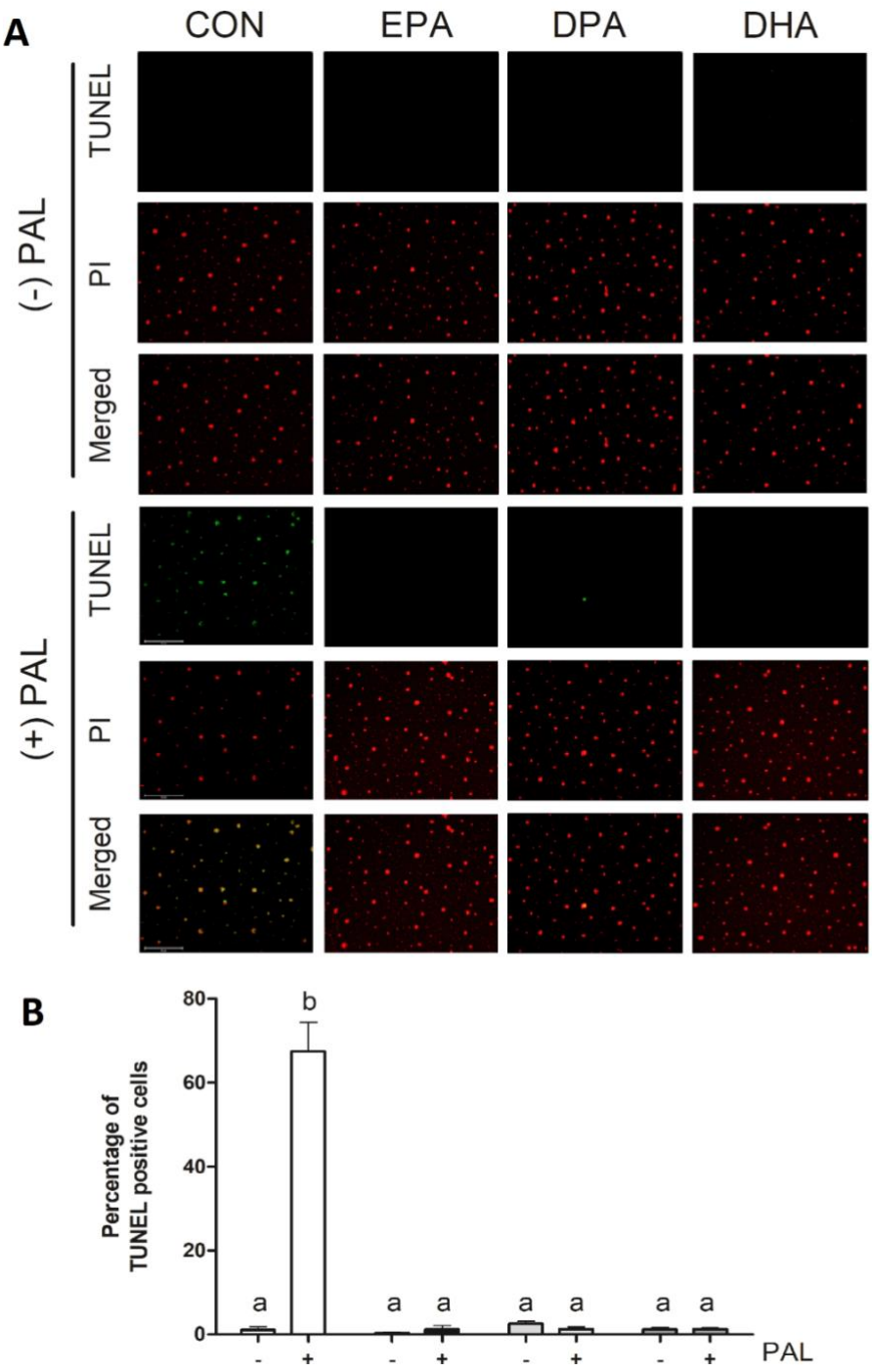

Figure 4.2. EPA, DPA and DHA attenuate PAL-induced increases in DNA fragmentation characteristic of apoptosis.

Representative images of myoblast TUNEL staining 16 h following treatment with 500 μM PAL and/or 50 μM EPA, DPA or DHA (A). Percentage of TUNEL positive cells relative to total number of cells in the field (B). Treatments that do not share letters are considered statistically significant ($p < 0.05$; myoblasts were cultured and harvested independently to generate a total sample size of 3 biological replicates). Scale bar represents 125 μm. Values are presented as mean ± SEM.

EPA, DPA and DHA attenuate PAL-induced increases in the ER stress marker BiP, but only DPA prevents PAL-induced increases in CHOP.

As ER is involved in PAL-induced reductions in cell viability and potential mitigation by n-3 PUFAs [23, 211], markers of ER stress signalling were analysed in myoblasts to determine whether EPA, DHA and DPA also potentially attenuated ER stress induction responses to PAL. Relative to CON, increases in BiP (~8.4 fold, $p < 0.05$; **Figure 4.3 A-B**) and CHOP (~3.6 fold, $p < 0.05$; **Figure 4.3 A and 4.3 C**) protein content were observed with PAL treatment. Co-treatment with EPA, DPA or DHA attenuated PAL-induced increases in BiP by ~70%, ~80% and ~50%, respectively, compared to PAL ($p < 0.05$). However, only DPA prevented the PAL-induced increase in BiP protein content (**Figure 4.3 B**).

PAL-induced increases in CHOP protein content were still observed with EPA and DHA co-treatments (~64% and ~3.2 fold, respectively ($p < 0.05$), compared to the respective n-3 PUFA treatment alone). In contrast, CHOP protein content in DPA treated myoblasts remained at similar levels to CON following PAL co-treatment (**Figure 4.3 C**). There was a tendency for DPA to prevent the PAL-induced increase in CHOP expression (~66% reduction compared to PAL, $p = 0.06$; **Figure 4.3 C**). No changes in the ER stress marker eIF2α Ser51 phosphorylation or total protein content were detected between any of the PAL and/or n-3 PUFA treatments (**Figure 4.4 A-D**).

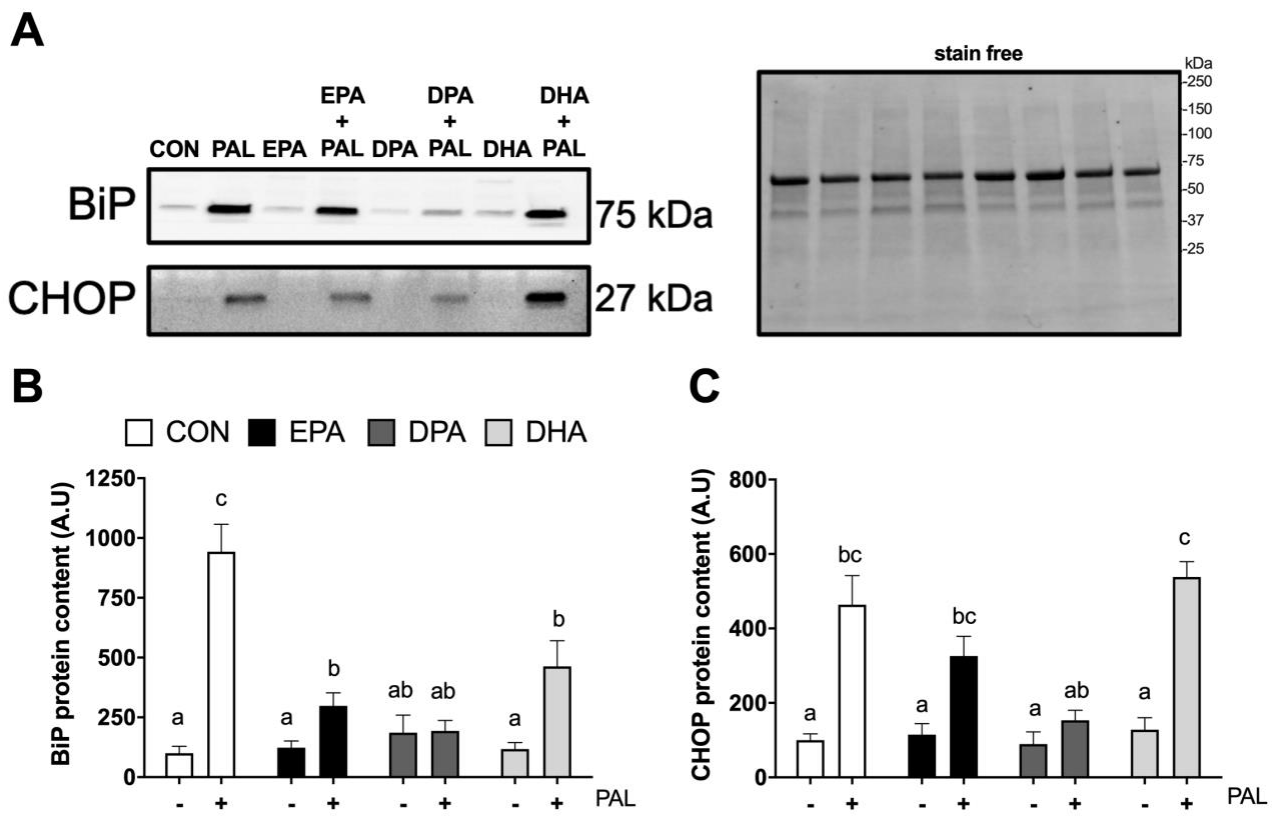

Figure 4.3. EPA, DPA and DHA attenuate PAL-induced increases in the ER stress markers BiP and CHOP.

Representative images of ER stress proteins BiP and CHOP following 16 h treatment with 500 μM PAL and/or 50 μM EPA, DPA or DHA (A) and representative image of a stain free blot used for normalisation with molecular weight markers indicated. Quantified protein content of BiP (B) and CHOP (C). In the absence of PAL, little to no CHOP protein was detectable. All densitometry values are expressed relative to protein content determined by stain free imaging and presented in arbitrary units relative to CON. Treatments that do not share letters are considered statistically significant ($p < 0.05$; myoblasts were cultured and harvested independently to generate a total sample size of five biological replicates). Values are presented as mean ± SEM.

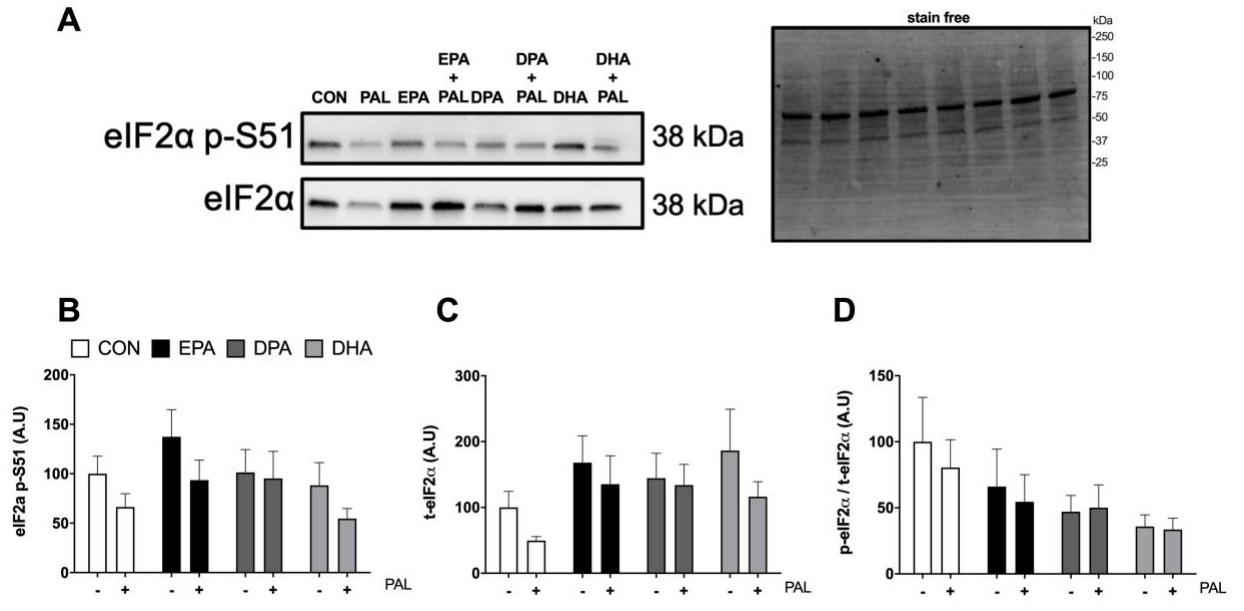

Figure 4.4. Total protein content and phosphorylation status of eIF2α is unchanged following PAL and/or n-3 PUFA treatment.
Representative images of total and phosphorylated eIF2α following 16h treatment with 500 μM PAL and/or 50 μM EPA, DPA or DHA, with a representative image of a stain free blot used for normalisation (A). Quantified phosphorylation of eIF2α (Ser51) (B) and total protein content of eIF2α (C). Levels of phosphorylation expressed relative to total protein content of eIF2α (D). All densitometry values are expressed relative to protein content determined by stain free imaging and presented in arbitrary units relative to CON. Myoblasts were cultured and harvested independently to generate a total sample size of six biological replicates. Values are presented as mean ± SEM.

EPA, DPA and DHA ameliorate the deleterious effects of PAL on myoblast differentiation.

To investigate the effects of n-3 PUFAs on the subsequent induction of muscle cell differentiation, myoblasts were differentiated for 5 days following exposure to the single treatment of PAL and/or each n-3 PUFA (EPA, DPA or DHA) and myogenic markers were assessed. Protein content of the myogenic marker myogenin was reduced following PAL treatment (~90% reduction compared to CON, $p < 0.05$; **Figure 4.5 A-B**). In contrast, EPA, DPA and DHA each protected against the deleterious effects of PAL on differentiation, as indicated by increased myogenin protein content (~50%, ~110%, and ~46%, respectively, $p < 0.05$ compared to CON-PAL; **Figure**

4.5 B). Consistent with these findings and the observed reductions in myoblast viability, PAL prevented the subsequent formation of myotubes (**Figure 4.5 C-E**). However, myoblasts treated with EPA, DPA and DHA effectively differentiated to myotubes following PAL co-treatment, with no changes observed in the average number of myotubes per field or myotube fusion index between each n-3 PUFA treatment either in the absence or presence of PAL (**Figure 4.5 C-E**).

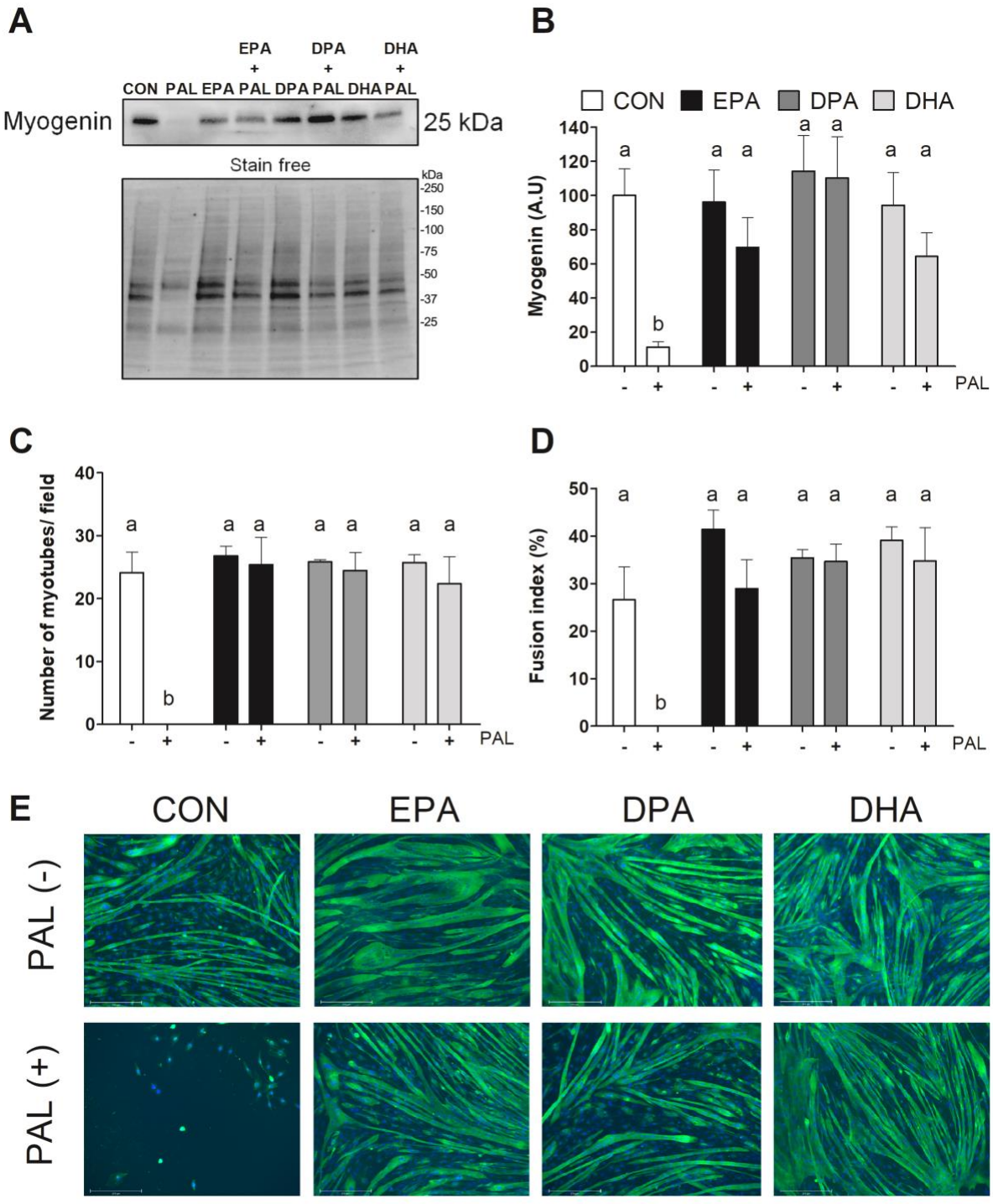

Figure 4.5. EPA, DPA and DHA protect against the inhibitory effects of PAL on myotube differentiation.

Protein expression of the myogenic protein myogenin 120 h following treatment with 500 μM PAL and/or 50 μM EPA, DPA or DHA, with a representative image of a stain free blot used for normalisation with molecular weight markers indicated (A). Quantified protein expression of the myogenic protein myogenin (B). All densitometry values are expressed relative to protein content

determined by stain free imaging and presented in arbitrary units relative to CON. Treatments that do not share letters are considered statistically significant (p <0.05; n=6 biological replicates). Average number of myotubes/field (C) and fusion index (number of nuclei in tubes relative to the the total number of nuclei expressed as a percentage) (D). Representative images of each treatment group at 120 h (n=3 biological replicates) (E). Scale bar represents 275 µm . Values are presented as mean ± SEM.

DPA protects against PAL-induced impairments in mitochondrial membrane integrity without altering mitochondrial content or maximal mitochondrial respiration

Given the known role of mitochondrial cytochrome c in the induction of cell death via apoptosis, mitochondrial function and content were evaluated following treatment with PAL and/or DPA, which provided the most robust attenuation of PAL-induced cell death and ER stress markers. Non-ADP-stimulated (pyruvate + malate; PM), ADP-stimulated (PMD), maximal complex I supported respiration (PMDG), maximal coupled (PMDGS) and uncoupled (CCCP) respiration were unchanged with PAL and/or n-3 PUFA treatment (**Figure 4.6 A**). However, CON myoblasts treated with PAL exhibited a significant increase in mitochondrial respiration when exogenous cytochrome c was added (~60%, $p < 0.05$), which was not observed when cells were co-treated with DPA (**Figure 4.6 B**). This response occurred independently of changes in mitochondrial content, as there were no detectable differences in protein content of any of the mitochondrial OXPHOS complexes in myoblasts treated with PAL and/or DPA (**Figure 4.7 A-F**).

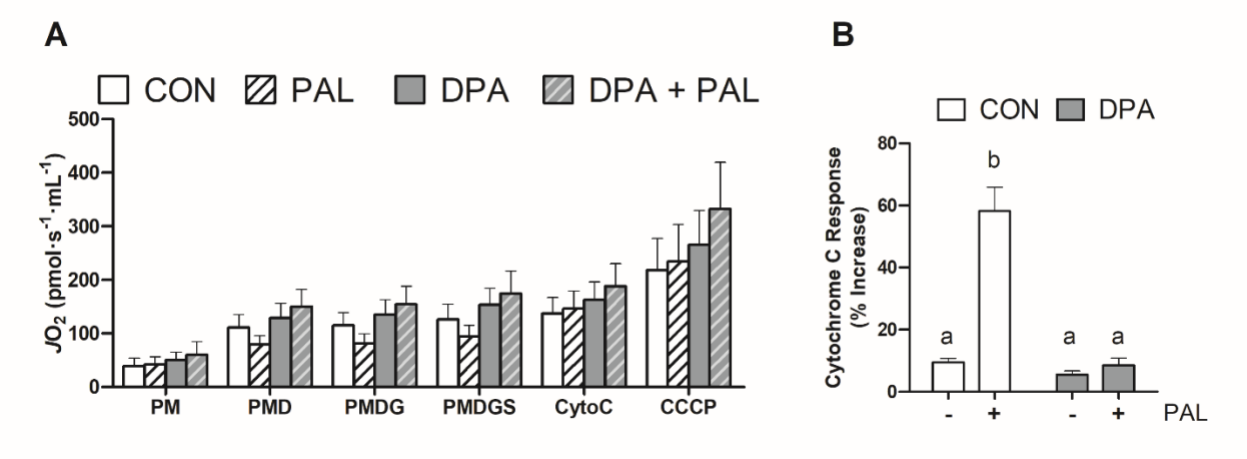

Figure 4.6. Treatment with DPA and/or PAL does not alter mitochondrial respiration, but DPA maintains mitochondrial membrane integrity in response to PAL.
In A, JO_2 represents mitochondrial O_2 flux. Cytochrome c retention test response expressed as (PMDGS-CytoC/CytoC) × 100 (B). Treatments that do not share letters are considered statistically significant (p <0.05). Myoblasts were cultured and harvested independently to generate a total sample size of four biological replicates. Values are presented as mean ± SEM.

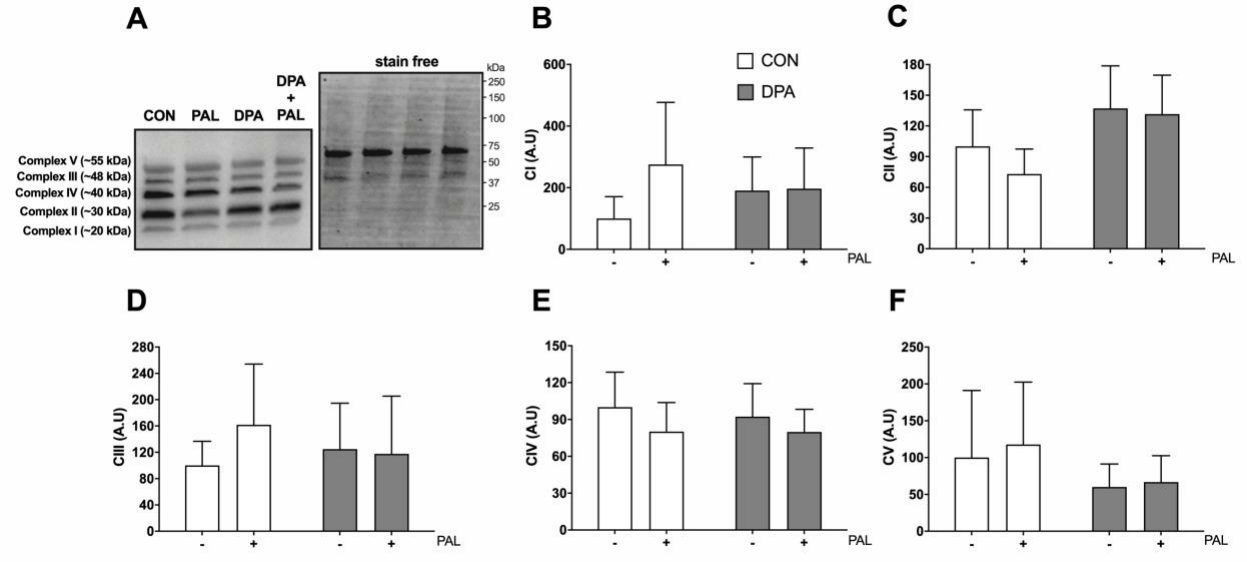

Figure 4.7. Treatment with DPA and/or PAL does not alter myoblast mitochondrial content. Protein content of mitochondrial OXPHOS complexes following 16 h treatment with 500 μM PAL and/or 50 μM EPA, DPA or DHA and representative images (A-F). All densitometry values are expressed relative to protein content determined by stain free imaging (A; representative image shown with molecular weight markers indicated) and presented in arbitrary units relative to CON. Myoblasts were cultured and harvested independently to generate a total sample size of six biological replicates. Values are presented as mean ± SEM.

EPA, DPA and DHA intracellular concentrations are increased in myoblasts after 16h treatment.

The intracellular concentration of EPA increased by ~17-fold (~1700% relative to CON, $p < 0.05$) following EPA treatment (**Figure 4.8 A**). DPA treatment increased intracellular DPA concentration of by ~4.2-fold (~427% relative to CON, $p < 0.05$). DPA treatment also increased intracellular EPA concentration by 1.8-fold (~180%) relative to CON that was not significantly different from DPA concentration ($p = 0.062$), indicating retro-conversion of DPA to EPA (**Figure 4.8 B**). DHA treatment increased DHA intracellular concentration by ~2.5-fold (256% (relative to CON, $p < 0.05$; **Figure 4.8 C**).

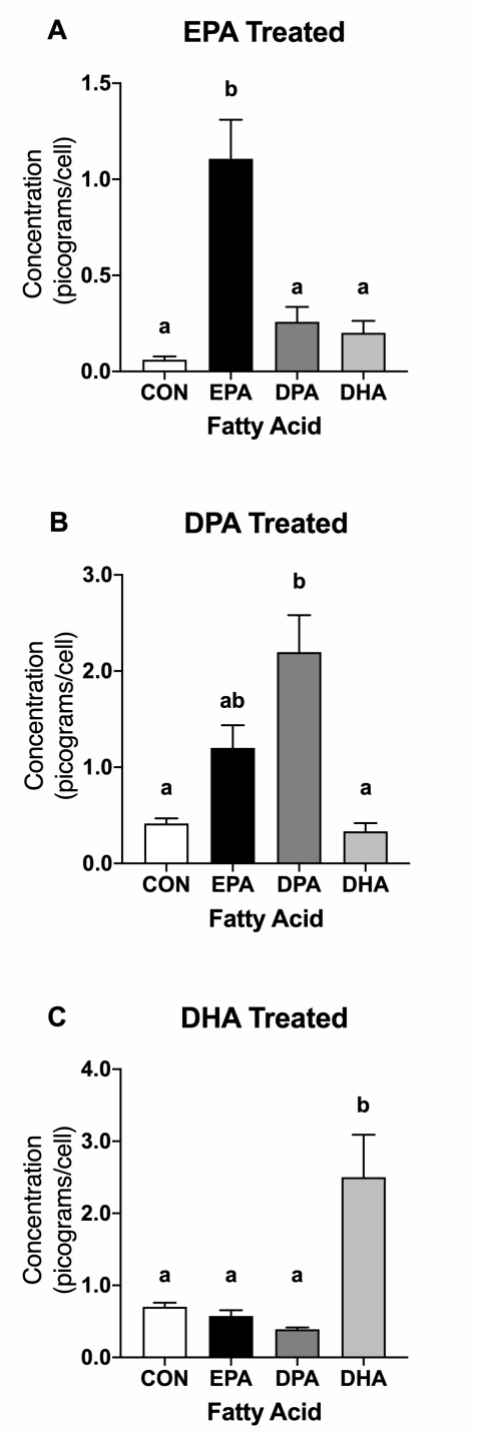

Figure 4.8. **Treatment of C2C12 myoblasts with 50 µM EPA, DPA or DHA increases the intracellular concentrations of each respective n-3 PUFA.**

Cellular concentrations of EPA, DPA and DHA following 16h treatment with either ethanol vehicle control (CON), 50 µM EPA (A), 50 µM DPA (B) or 50 µM DHA (C). Fatty acid analysis was carried out by fatty acid methyl esters (FAME) analysis and data are presented as absolute

concentrations relative to cell number. Treatments that do not share letters are considered statistically significant ($p < 0.05$). Myoblasts were cultured to generate a total sample size of four technical replicates. Values are presented as mean ± SEM.

Discussion

This is the first study to compare the protective effects of EPA, DHA, as well as DPA against the lipotoxic effects of PAL in skeletal muscle cells. We demonstrate that PAL induces cell death as determined by changes in myoblast viability, nuclei number and nuclei morphology, in support of previous findings [161, 207]. In addition, we determine that cell death occurs via apoptosis, as evidenced by increased TUNEL staining of PAL-treated myoblasts. Our results also indicate that EPA, DPA and DHA protect against these deleterious effects of PAL, thereby allowing differentiation to occur, despite differences observed in ER stress signalling responses between each n-3 PUFA. Furthermore, we demonstrate that DPA mitigates PAL-induced reductions in mitochondrial membrane integrity, which is a potential mechanism underlying the observed protective effects of n-3 PUFAs. While the results of the present investigation align with previous data in differentiated myotubes showing that EPA and DHA elicit protection against the detrimental cellular effects of PAL [124, 125, 167], we show for the first time that DPA may elicit additional protective effects against PAL lipotoxicity (i.e. cell death and associated ER stress markers) relative to EPA and DHA.

The mechanisms underlying cell death in response to PAL are complex, multifaceted and involve multiple cell signalling pathways as demonstrated by Saini and co-workers [161], who have shown that EPA protects against PAL-induced reductions in myoblast viability by reducing caspase activity. However, inhibiting caspase activity only partially prevents the cell death

response, indicating other pathways such as the MAPK and JNK signalling may be involved. Additional mechanisms that may play a role in PAL-induced cell death include the induction of ER-stress signalling [212], impaired autophagy processes involved in the removal of damaged mitochondria [213], as well as sustained impairments in mitochondrial function [27, 214]. Data in the present study align with the consensus that cellular PAL exposure results in lipotoxicity, and further demonstrates that n-3 PUFA treatments can ameliorate these deleterious effects thereby preventing cell death.

The present study also interrogated further mechanisms involving mitochondrial membrane integrity and cytochrome c release by which n-3 PUFAs may elicit protection against the lipotoxic effects of PAL leading to the observed reductions in myoblast viability, as investigated using complementary microscopy techniques to assess nuclear brightness and DNA fragmentation characteristic of apoptosis. Microscopy-based nuclei analysis confirmed that the PAL-induced reductions in nuclei per area occurred due to cell death and were not due to impairments in proliferation, as the number of cells initially plated was consistent between each experimental condition prior to analysis at ~70% confluency. TUNEL stain binds to 3'-hydroxyl termini of DNA double strand breaks, a hallmark of the late stages of apoptosis. Unlike the microscopy-based analyses of nuclear area and perimeter, TUNEL analysis did not detect any changes in the percentage of apoptotic cells between individual n-3 PUFA and PAL co-treated cells. A limitation of the present study is that TUNEL staining was only analysed at one time point. It is therefore difficult to ascertain precisely when apoptotic programmed cell death is occurring following PAL exposure. Nevertheless, the 16 h time point was selected as other investigations in

both muscle and non-muscle cell types have demonstrated that PAL induces significant cell death at this time point [23, 25, 191, 215, 216].

Results from studies undertaken in rodent and cell culture models both suggest that the provision of excess saturated fat can result in ER stress, leading to the activation of UPR signalling [25, 192, 217]. The UPR involves the release of BiP from ER transmembrane signal transducers PKR-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6). BiP is a key regulator of the UPR, since the activation of PERK, IRE1 and ATF6 is dependent on its dissociation from these proteins [218]. Ultimately, these three arms of the UPR converge downstream on the transcription factor C/EBP homologous protein (CHOP), triggering apoptosis [219]. Given that PAL is known to induce ER stress and stimulate UPR signalling, it is unsurprising that BiP protein expression was elevated in response to PAL exposure. In an attempt to relieve ER stress, PERK activation results in an increase in eIF2α phosphorylation, suppressing protein translation and promoting proper protein folding and export from the ER [220]. While no changes in the phosphorylation status or protein content of eIF2α were detectable, given the transient nature of phosphorylation it is possible that eIF2α phosphorylation may have occurred at an earlier timepoint in an attempt to re-establish homeostasis after PAL treatment [221]. Considering the overall lack of consistency observed between the effects of each n-3 PUFA with respect to cell viability and ER stress markers, the beneficial protective effects of n-3 PUFAs on cell viability in the present study's experimental design are unlikely to be due to protection against ER stress induction. The cell death and associated apoptosis responses observed do however align with that of the mitochondrial cytochrome c responses, indicating that PAL-induced cell death and the protective effects of n-3 PUFAs occur in a manner not solely dependent on the convergence of

the UPR on CHOP, suggesting that alternative apoptotic pathways could lead to the observed reductions in viability [222]. Nevertheless, PAL-induced cell death is attenuated when cells are co-treated with n-3 PUFAs. This response is most likely due to the inhibition of proteins responsible for the permeabilisation of the outer mitochondrial membrane preventing the release of cytochrome c from mitochondria [223].

The results of the present study also complement other reports regarding the effects of n-3 PUFAs in promoting differentiation of viable skeletal muscle myoblasts to myotubes [108, 161]. Despite differences in the timing of treatments and muscle cell line utilised, our results are in agreement with previous findings demonstrating that EPA and DHA promote differentiation of viable myoblasts to myotubes. Chronic treatment (up to 72 h) of differentiating L6 muscle cells with 20 μM EPA and DHA has also been shown to improve measures of differentiation [108]. In the current study further increases in myogenin were not observed. In contrast, Briolay and co-workers found that both EPA and DHA increased the expression of myosin and creatine kinase as well as myogenic index to a greater extent compared to untreated control cells. Likewise, Saini and co-workers [161] also provide evidence from mouse C2 cells that 50 μM EPA treatment for 72 h improves differentiation, as measured by creatine kinase and MyoD gene expression. In contrast, Zhang *et al*. [224] and Hsueh *et al*. [225] demonstrate that EPA and/or DHA can inhibit myoblast proliferation, reducing cell viability and myogenic potential resulting in attenuated expression of myogenin and myosin heavy chain mRNA expression as well as reduced myotube formation. There are a number of reasons why EPA and DHA in these two studies [224, 225] may show divergent effects to the results from the current investigation and previous work [161]. Earlier studies have differentiated C2C12 cells in the presence of 50 μM EPA or DHA either in

combination (100 µM PUFA total) [225], or separately for up to 72 h [224]. The 16 h single dose used in the current study allowed differentiation of viable myoblasts to occur, suggesting that chronic treatment with n-3 PUFAs in higher concentrations may have an inhibitory effect on differentiation. Nevertheless, the current study design is novel in that it directly compares the protective effects of the three n-3 PUFAs EPA, DPA and DHA against the lipotoxic effects of PAL in myoblasts, as well as the successive effects on remaining viable myotubes within the same set of experiments.

Considering that DPA-PAL resulted in the greatest magnitude of protection against loss of cell viability when compared to PAL treated cells, changes in mitochondrial function and content were investigated as mechanisms that could potentially explain why cell viability was maintained despite remaining increases in ER stress signalling following DPA treatment. Several studies have proposed that the biological effects of n-3 PUFAs occur via improvements in mitochondrial bioenergetics [210, 226-228]. The protective effect of DPA against PAL-induced cell death may be explained by the incorporation of DPA into cellular membranes, increasing the level of unsaturated lipid species (e.g. phospholipids) and consequently increasing the fluidity of mitochondrial membranes thereby altering mitochondrial function [229]. Increases in human mitochondrial membrane phospholipid species as a result of n-3 PUFA (EPA and DHA) supplementation have been shown to improve ADP kinetics rather than alter maximal mitochondrial respiration [197]. In support of these findings, no increases in maximal mitochondrial respiration following DPA treatment was observed. In contrast, the current study demonstrated that PAL compromises the integrity of outer mitochondrial membranes resulting in the release of cytochrome c. DPA attenuated this response, indicating that its incorporation into

lipid membranes maintains the integrity of mitochondrial membranes despite the presence of PAL and associated increases in ER stress-related signalling. This is consistent with n-3 PUFA incorporation into mitochondrial membranes in human skeletal muscle observed following 12-week supplementation with EPA and DHA [196]. Given our data show increased cellular incorporation of DPA following incubation with 50µM DPA, as well as elevated EPA levels that were not significantly different from DPA levels, further research focused on DPA's mitochondrial incorporation and biological actions is warranted to determine how this dose and level of incorporation translates to *in vivo* models.

Future studies are warranted to dissect the precise direct versus indirect cellular mechanisms underlying n-3 PUFA mediated protection against PAL-induced lipotoxicity. Evidence suggests that n-3 PUFAs prevent the accumulation of PAL-derived ceramide and diglyceride lipid species known to elicit cytotoxic effects by increasing fatty acid oxidation [124, 230]. One unexplored possibility of the current study is that the PAL treatment resulted in ceramide accumulation, causing excessive ROS production within the mitochondria. Consequently, this may have led to the activation of the mitochondrial permeability transition pore and release of cytochrome c responsible for the decrease in viability [231-233]. Additionally, n-3 PUFAs are known to be precursors to unique lipid mediators such as resolvins and protectins [234, 235]. Therefore, it is possible that the effects observed in the current study may have occurred indirectly via specific downstream cellular actions of their unique metabolites. Moreover, DPA may elicit distinct biological effects directly or via its conversion into lipid species that provide unique downstream actions and superior protection versus EPA and DHA against PAL-induced

lipotoxicity observed in the current study. This indirect biological action of DPA is consistent with findings from Suphioglu et al., [236] demonstrating that DHA protects against apoptosis in human neuronal cells indirectly via downstream actions on zinc transport that in turn reduce cellular zinc levels thereby promoting cell survival. It is possible that similar indirect cellular mechanism(s) could also be occurring in skeletal muscle in response to PAL-induced apoptosis. Considering the similar chemical structure of DPA relative to EPA and DHA, it is possible that the different biological effects of DPA can be explained by DPA being metabolised into different lipid species having unique properties and/or triggering distinct downstream cellular events to those produced by EPA and DHA. The current data supports this hypothesis, as an increase in DPA in response to EPA treatment was not observed, however retro-conversion of DPA to EPA following DPA treatment was observed, demonstrating potential for DPA's unique and superior biological effects.

In summary, the n-3 PUFAs EPA, DPA and DHA elicit similar protective effects against PAL-induced lipotoxicity, thereby preventing apoptosis and promoting cell viability and differentiation of viable myoblasts to myotubes. Furthermore, DPA maintains cell viability potentially via mitigating the loss of mitochondrial membrane integrity induced by PAL. Together, these data highlight the potential for n-3 PUFAs and specifically DPA to promote cell viability and combat deleterious effects of muscle cell lipid accumulation, as well as provides novel mechanistic underpinning for DPA's protective effects against PAL-induced lipotoxicity.

Chapter 5 : General Discussion and Conclusions

The primary aim of this thesis was to investigate the protective effects of the n-3 PUFAs EPA, DPA and DHA against the PAL-induced cellular lipotoxic effects in skeletal muscle cells including impairments in viability and differentiation capacity. Previous cell culture-based studies have demonstrated that the saturated fatty acid PAL potentiates ER stress leading to myotube atrophy and reduced regenerative capacity. Chapter 4 of this thesis highlights that DPA, similarly to EPA and DHA, offers protection against reductions in cell viability and induction of ER stress induced by cellular PAL exposure. Despite differences in their effects on ER stress signalling machinery this study is the first to compare the protective effects of these three PUFAs within the same experimental design.

Since mitochondria play a key role in the induction of apoptotic cell death and DPA elicited the greatest protection against PAL, mitochondrial respiration was assessed in the presence of PAL and DPA. Although DPA alone did not alter mitochondrial respiration, DPA maintained the integrity of mitochondrial membranes, preventing the release of cytochrome c that was observed in the presence of PAL. Thus, the findings reported in Chapter 4 and represented schematically in **Figure 5.1**, suggests a novel cellular mechanism by which n-3 PUFAs and specifically DPA, offer protection against PAL-induced skeletal muscle cell death.

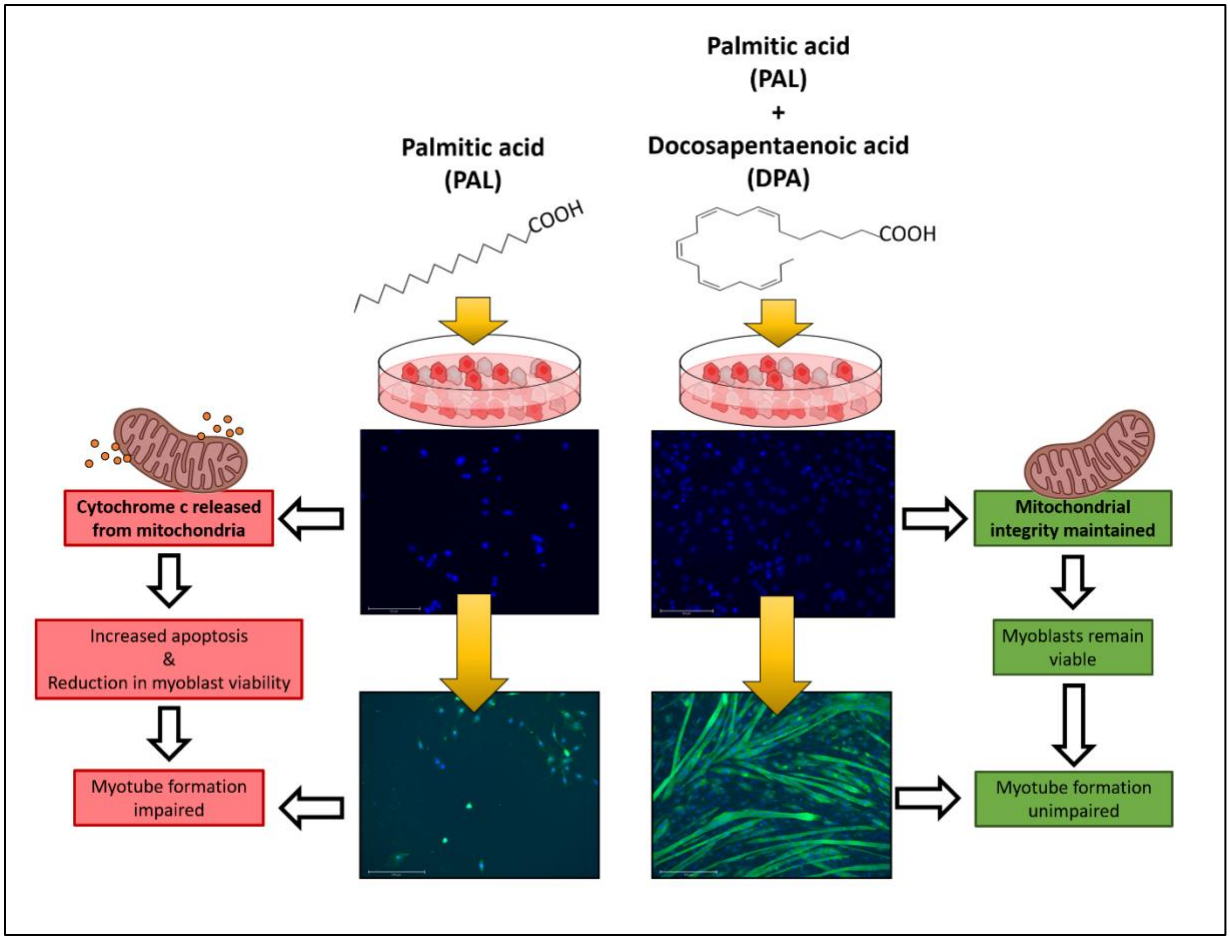

Figure 5.1. Schematic of the major novel findings from this thesis.
PAL induces apoptosis, reduces muscle cell viability and impairs myotube formation. As detailed in Chapter 3, all n-3 PUFAs (EPA, DPA and DHA) attenuated the lipotoxic effects of PAL and thereby facilitate myotube formation. The maintenance of mitochondrial integrity is one possible mechanism, as depicted above for DPA, explaining the protective effects of n-3 PUFAs against the lipotoxic effects of PAL.

There are several limitations that need to be acknowledged when interpreting the results reported in Chapter 4. Although the intracellular concentrations of n-3 PUFAs EPA, DPA and DHA following treatments were investigated, their incorporation into cellular membranes was not specifically assessed. Using liquid and or gas chromatography coupled with mass spectrometry, as well as membrane isolation and/or fractionation of various cellular components to assess differences in cellular incorporation before and after treatments, would allow confirmation of the relative uptake of these n-3 PUFAs in the absence or presence of PAL [22, 69, 205]. Without direct

measure of membrane n-3 PUFA incorporation before and after the fatty acid treatment(s) it is difficult to determine how and where these n-3 PUFAs are being distributed within the cell and whether differences exist in cellular uptake of saturated (PAL) versus unsaturated (n-3 PUFAs). However, the beneficial effects of DPA treatment observed on direct assessment of mitochondrial membrane integrity strongly suggest these fatty acid species enter and incorporate into mitochondrial membrane(s). The rise in intracellular n-3 PUFA concentration also suggests that n-3 PUFAs effectively enter the cell for incorporation and therefore have the capacity to potentially elicit the observed protective effects against PAL.

The 50 µM concentrations of n-3 PUFAs used in the study described in Chapter 4 are consistent with concentrations used in other *in vitro* studies [25, 148, 161, 198] supporting the results observed and reinforcing the novel and unique effects of n-3 PUFAs in skeletal muscle, specifically with regards to DPA. Presumably, the cellular uptake of both saturated and unsaturated fatty acids occurs through the fatty acid transporters CD36 and FAT1. It is possible that the uptake of unsaturated fatty acids occurs preferentially before that of saturated lipid species. If this is the case, then it may have contributed to the results observed as rate of uptake would dictate their cellular metabolism and ensuing cellular effects. Specifically, it is possible that unsaturated fatty acids are preferentially transported into cells, inhibiting the uptake of saturated fats and circumventing the negative effects of excess saturated fats accumulation within cells. While no confirmatory fatty acid uptake assays were conducted, this should be considered in future studies investigating co-treatments of saturated and unsaturated with regards to their uptake and downstream physiological effects in skeletal muscle. Another potential limitation is that mitochondrial respiration and/or mitochondrial membrane integrity was only assessed for DPA

and not assessed in the presence of EPA or DHA. Respiration and mitochondrial integrity analyses focused on DPA, as this n-3 PUFA induced the greatest protection against the induction of palmitate-induced ER stress. Considering that both EPA and DHA have been shown in other studies to elicit beneficial and/or protective effects in skeletal muscle, it is possible that EPA and DHA may also maintain mitochondrial integrity to a similar degree as DPA.

Excess PAL is known to elicit oxidative stress and calcium dysregulation, which if unresolved lead to cell death [233, 237]. Despite these cellular derangements involving different organelles (i.e. mitochondria and ER, respectively), they can both trigger apoptotic responses [222]. The regulation and storage of calcium occurs within the ER, whereas mitochondria are the primary site of intracellular ROS production. Considering the results presented in Chapter 4 and specifically the inability for n-3 PUFAs to totally ameliorate ER stress markers in the presence of PAL, it is unlikely that n-3 PUFAs are completely preventing cell death responses by inhibiting ER stress. Excess PAL can also result in the accumulation of ceramide lipid species, leading to oxidative stress and triggers ERK1/2 signalling pathways leading to apoptosis [238]. Further research is required to pinpoint which of the multiple pathways is the primary driver underpinning the observed cell death responses and moreover, which of these pathways is affected to the greatest extent by n-3 PUFAs in skeletal muscle [239, 240]. It is highly likely that the protective effects of n-3 PUFAs are due to their ability to affect multiple cell signalling process and organelles simultaneously. Regardless of the primary cell signalling mechanisms responsible for the protective effects of n-3 PUFAS, the results presented in Chapter 4 of this thesis reveals that they are all able to counteract the deleterious effects of PAL on cell viability and differentiation.

Chapter 2 primarily focused on the potential of n-3 PUFAs to modulate skeletal muscle growth and regeneration. However, a key understudied and clinically relevant concept not overviewed in Chapter 2 is the potential effects of n-3 PUFAs on muscle protein synthetic responses in the presence of PAL and/or high fat diet exposure characteristic of Western diets. Currently there is a paucity of studies assessing whether EPA, DPA and/or DHA are able to modulate muscle protein synthetic responses on their own or in the presence of PAL. Considering most humans do not consume individual PUFAs on a regular basis and that most fish oil supplements contain various ratios of EPA and DHA, it is difficult to determine which n-3 PUFA have the most profound modulatory effect on muscle protein synthetic responses. Studying which of these n-3 PUFAs induces the greatest changes in muscle protein synthetic responses alone and in the presence of lipid infusion and/or high fat diet would be clinically relevant, as ageing individuals who have blunted responses to anabolic stimuli are more susceptible to muscle wasting. Future studies should therefore consider investigating the differential effects of EPA and DHA and more importantly DPA on muscle protein synthesis using rodent and human models as n-3 PUFA supplementation remains understudied yet potentially clinically relevant in the context of muscle growth and regeneration. If DPA can increase rates of muscle protein synthesis in human models in addition to altering intracellular signalling to an equal or greater extent than EPA and DHA it warrants further investigation, especially since DPA is not commonly found in commercially available n-3 PUFA supplements to date.

In conclusion, the literature review and original research article presented in this thesis advance our understanding of the mechanisms underlying the protective effects of the n-3 PUFAs EPA, DPA and DHA against disruptions to cellular homeostasis that negatively impact skeletal

muscle and its regenerative capacity such as PAL exposure. The findings from the experiments conducted in this thesis also highlights the novel effects of the understudied n-3 PUFA, DPA, with respect to skeletal muscle viability, mitochondrial membrane integrity and differentiation. Collectively, the results presented provides a rationale for future cell-based and translational studies in rodents and humans to further investigate the protective effects of n-3 PUFAs against excess saturated fats such as PAL and discover the underlying mechanism(s) by which DPA elicits its superior beneficial cellular effects. Finally, DPA in conjunction with the n-3 PUFAs EPA and DHA, commonly found in current fish oil supplements, could be used as non-pharmacological means to help protect skeletal muscle mass and function against cellular insults and excessive circulating free fatty acids often associated with Western diets and the increased prevalence of obesity and associated metabolic diseases. Though several human trials investigating the latter are required to substantiate their therapeutic potential in humans. Nevertheless, the findings in this thesis set the stage for future studies into the therapeutic potential of n-3 PUFAs and specifically DPA in association with dietary interventions and resistance exercise, to help counteract the negative effects of muscle loss with metabolic disease and ageing.

Chapter 6 : List of References

1. Zierath, J.R. and J.A. Hawley, *Skeletal muscle fiber type: influence on contractile and metabolic properties.* PLoS Biol, 2004. **2**(10): p. e348.
2. Hawley, J.A., et al., *Integrative biology of exercise.* Cell, 2014. **159**(4): p. 738-49.
3. Kumar, V., et al., *Age-related differences in the dose-response relationship of muscle protein synthesis to resistance exercise in young and old men.* J Physiol, 2009. **587**(1): p. 211-7.
4. Greig, C.A., et al., *Blunting of adaptive responses to resistance exercise training in women over 75y.* Exp Gerontol, 2011. **46**(11): p. 884-90.
5. Moore, D.R., et al., *Protein ingestion to stimulate myofibrillar protein synthesis requires greater relative protein intakes in healthy older versus younger men.* J Gerontol A Biol Sci Med Sci, 2015. **70**(1): p. 57-62.
6. Egerman, M.A., et al., *GDF11 Increases with Age and Inhibits Skeletal Muscle Regeneration.* Cell Metab, 2015. **22**(1): p. 164-74.
7. D'Souza, D.M., et al., *Diet-induced obesity impairs muscle satellite cell activation and muscle repair through alterations in hepatocyte growth factor signaling.* Physiol Rep, 2015. **3**(8).
8. Smiles, W.J., et al., *A single bout of strenuous exercise overcomes lipid-induced anabolic resistance to protein ingestion in overweight, middle-aged men.* Faseb j, 2019. **33**(6): p. 7009-7017.
9. Tachtsis, B., D. Camera, and O. Lacham-Kaplan, *Potential Roles of n-3 PUFAs during Skeletal Muscle Growth and Regeneration.* Nutrients, 2018. **10**(3).
10. McGlory, C., et al., *Temporal changes in human skeletal muscle and blood lipid composition with fish oil supplementation.* Prostaglandins Leukot Essent Fatty Acids, 2014. **90**(6): p. 199-206.
11. McGlory, C., et al., *Fish oil supplementation suppresses resistance exercise and feeding-induced increases in anabolic signaling without affecting myofibrillar protein synthesis in young men.* Physiol Rep, 2016. **4**(6).

12. Davidson, M.H., *Omega-3 fatty acids: new insights into the pharmacology and biology of docosahexaenoic acid, docosapentaenoic acid, and eicosapentaenoic acid.* Curr Opin Lipidol, 2013. **24**(6): p. 467-74.

13. Cutuli, D., *Functional and structural benefits induced by omega-3 polyunsaturated fatty acids during aging.* Curr Neuropharmacol, 2016.

14. Guesnet, P. and J.M. Alessandri, *Docosahexaenoic acid (DHA) and the developing central nervous system (CNS) - Implications for dietary recommendations.* Biochimie, 2011. **93**(1): p. 7-12.

15. Sprecher, H., *Metabolism of highly unsaturated n-3 and n-6 fatty acids.* Biochim Biophys Acta, 2000. **1486**(2-3): p. 219-31.

16. Plourde, M. and S.C. Cunnane, *Extremely limited synthesis of long chain polyunsaturates in adults: implications for their dietary essentiality and use as supplements.* Appl Physiol Nutr Metab, 2007. **32**(4): p. 619-34.

17. Burdge, G.C., A.E. Jones, and S.A. Wootton, *Eicosapentaenoic and docosapentaenoic acids are the principal products of alpha-linolenic acid metabolism in young men**. Br J Nutr, 2002. **88**(4): p. 355-63.

18. Burdge, G.C. and S.A. Wootton, *Conversion of alpha-linolenic acid to eicosapentaenoic, docosapentaenoic and docosahexaenoic acids in young women.* Br J Nutr, 2002. **88**(4): p. 411-20.

19. Tur, J.A., et al., *Dietary sources of omega 3 fatty acids: public health risks and benefits.* Br J Nutr, 2012. **107 Suppl 2**: p. S23-52.

20. Carboni, S., et al., *Mussel Consumption as a "Food First" Approach to Improve Omega-3 Status.* Nutrients, 2019. **11**(6).

21. Howe, P., et al., *Dietary intake of long-chain omega-3 polyunsaturated fatty acids: contribution of meat sources.* Nutrition, 2006. **22**(1): p. 47-53.

22. Kaur, G., et al., *Short-term docosapentaenoic acid (22:5 n-3) supplementation increases tissue docosapentaenoic acid, DHA and EPA concentrations in rats.* Br J Nutr, 2010. **103**(1): p. 32-7.

23. Perry, B.D., et al., *Palmitate-induced ER stress and inhibition of protein synthesis in cultured myotubes does not require Toll-like receptor 4.* PLoS One, 2018. **13**(1): p. e0191313.

24. Nieuwoudt, S., et al., *In vitro contraction protects against palmitate-induced insulin resistance in C2C12 myotubes.* Am J Physiol Cell Physiol, 2017. **313**(5): p. C575-C583.

25. Woodworth-Hobbs, M.E., et al., *Docosahexaenoic acid counteracts palmitate-induced endoplasmic reticulum stress in C2C12 myotubes: Impact on muscle atrophy.* Physiol Rep, 2017. **5**(23).

26. Patkova, J., M. Andel, and J. Trnka, *Palmitate-induced cell death and mitochondrial respiratory dysfunction in myoblasts are not prevented by mitochondria-targeted antioxidants.* Cell Physiol Biochem, 2014. **33**(5): p. 1439-51.

27. Rachek, L.I., et al., *Palmitate induced mitochondrial deoxyribonucleic acid damage and apoptosis in l6 rat skeletal muscle cells.* Endocrinology, 2007. **148**(1): p. 293-9.

28. Skovbro, M., et al., *High-fat feeding inhibits exercise-induced increase in mitochondrial respiratory flux in skeletal muscle.* J Appl Physiol (1985), 2011. **110**(6): p. 1607-14.

29. Boon, M.R., et al., *Short-term high-fat diet increases macrophage markers in skeletal muscle accompanied by impaired insulin signalling in healthy male subjects.* Clin Sci (Lond), 2015. **128**(2): p. 143-51.

30. Ellis, B.A., et al., *Long-chain acyl-CoA esters as indicators of lipid metabolism and insulin sensitivity in rat and human muscle.* Am J Physiol Endocrinol Metab, 2000. **279**(3): p. E554-60.

31. Miedema, M.D., et al., *Plasma-free fatty acids, fatty acid-binding protein 4, and mortality in older adults (from the Cardiovascular Health Study).* Am J Cardiol, 2014. **114**(6): p. 843-8.

32. Sakamoto, A., et al., *Eicosapentaenoic acid ameliorates palmitate-induced lipotoxicity via the AMP kinase/dynamin-related protein-1 signaling pathway in differentiated H9c2 myocytes.* Exp Cell Res, 2017. **351**(1): p. 109-120.

33. Yamada, H., et al., *Eicosapentaenoic acid shows anti-inflammatory effect via GPR120 in 3T3-L1 adipocytes and attenuates adipose tissue inflammation in diet-induced obese mice.* Nutr Metab (Lond), 2017. **14**: p. 33.

34. Cuthbertson, D., et al., *Anabolic signaling deficits underlie amino acid resistance of wasting, aging muscle.* FASEB J, 2005. **19**(3): p. 422-4.

35. Wall, B.T., et al., *Aging Is Accompanied by a Blunted Muscle Protein Synthetic Response to Protein Ingestion.* PLoS One, 2015. **10**(11): p. e0140903.

36. Brook, M.S., et al., *Synchronous deficits in cumulative muscle protein synthesis and ribosomal biogenesis underlie age-related anabolic resistance to exercise in humans.* J Physiol, 2016. **594**(24): p. 7399-7417.

37. Fry, C.S., et al., *Aging impairs contraction-induced human skeletal muscle mTORC1 signaling and protein synthesis.* Skelet Muscle, 2011. **1**(1): p. 11.

38. Rosenberg, I.H., *Sarcopenia: origins and clinical relevance.* J Nutr, 1997. **127**(5 Suppl): p. 990S-991S.

39. Volpi, E., et al., *The response of muscle protein anabolism to combined hyperaminoacidemia and glucose-induced hyperinsulinemia is impaired in the elderly.* J Clin Endocrinol Metab, 2000. **85**(12): p. 4481-90.

40. Janssen, H.C., M.M. Samson, and H.J. Verhaar, *Vitamin D deficiency, muscle function, and falls in elderly people.* Am J Clin Nutr, 2002. **75**(4): p. 611-5.

41. Szulc, P., et al., *Hormonal and lifestyle determinants of appendicular skeletal muscle mass in men: the MINOS study.* Am J Clin Nutr, 2004. **80**(2): p. 496-503.

42. Visser, M., et al., *Relationship of interleukin-6 and tumor necrosis factor-alpha with muscle mass and muscle strength in elderly men and women: the Health ABC Study.* J Gerontol A Biol Sci Med Sci, 2002. **57**(5): p. M326-32.

43. McKay, B.R., et al., *Elevated SOCS3 and altered IL-6 signaling is associated with age-related human muscle stem cell dysfunction.* Am J Physiol Cell Physiol, 2013. **304**(8): p. C717-28.

44. Vandervoort, A.A., *Aging of the human neuromuscular system.* Muscle Nerve, 2002. **25**(1): p. 17-25.

45. Verdijk, L.B., et al., *Satellite cells in human skeletal muscle; from birth to old age.* Age (Dordr), 2014. **36**(2): p. 545-7.

46. Verdijk, L.B., et al., *Satellite cell content is specifically reduced in type II skeletal muscle fibers in the elderly.* Am J Physiol Endocrinol Metab, 2007. **292**(1): p. E151-7.

47. Larsson, L., B. Sjodin, and J. Karlsson, *Histochemical and biochemical changes in human skeletal muscle with age in sedentary males, age 22--65 years.* Acta Physiol Scand, 1978. **103**(1): p. 31-9.

48. Frontera, W.R., A.R. Zayas, and N. Rodriguez, *Aging of human muscle: understanding sarcopenia at the single muscle cell level.* Phys Med Rehabil Clin N Am, 2012. **23**(1): p. 201-7, xiii.

49. Miljkovic, N., et al., *Aging of skeletal muscle fibers.* Ann Rehabil Med, 2015. **39**(2): p. 155-62.

50. Relaix, F. and C. Marcelle, *Muscle stem cells.* Curr Opin Cell Biol, 2009. **21**(6): p. 748-53.

51. Sambasivan, R., et al., *Pax7-expressing satellite cells are indispensable for adult skeletal muscle regeneration.* Development, 2011. **138**(17): p. 3647-56.

52. McCarthy, J.J., et al., *Effective fiber hypertrophy in satellite cell-depleted skeletal muscle.* Development, 2011. **138**(17): p. 3657-66.

53. Olguin, H.C. and B.B. Olwin, *Pax-7 up-regulation inhibits myogenesis and cell cycle progression in satellite cells: a potential mechanism for self-renewal.* Dev Biol, 2004. **275**(2): p. 375-88.

54. Venuti, J.M., et al., *Myogenin is required for late but not early aspects of myogenesis during mouse development.* J Cell Biol, 1995. **128**(4): p. 563-76.

55. Fukada, S.I., *The roles of muscle stem cells in muscle injury, atrophy, and hypertrophy.* J Biochem, 2018.

56. Murach, K.A., et al., *Starring or Supporting Role? Satellite Cells and Skeletal Muscle Fiber Size Regulation.* Physiology (Bethesda), 2018. **33**(1): p. 26-38.

57. Murach, K.A., et al., *Differential requirement for satellite cells during overload-induced muscle hypertrophy in growing versus mature mice.* Skelet Muscle, 2017. **7**.

58. Fry, C.S., et al., *Regulation of the muscle fiber microenvironment by activated satellite cells during hypertrophy.* FASEB J, 2014. **28**(4): p. 1654-65.

59. Egner, I.M., J.C. Bruusgaard, and K. Gundersen, *Satellite cell depletion prevents fiber hypertrophy in skeletal muscle.* Development, 2016. **143**(16): p. 2898-906.

60. Keefe, A.C., et al., *Muscle stem cells contribute to myofibers in sedentary adult mice.* Nat Commun, 2015. **6**: p. 7087.

61. Fry, C.S., et al., *Inducible depletion of satellite cells in adult, sedentary mice impairs muscle regenerative capacity but does not contribute to sarcopenia.* Nat Med, 2015. **21**(1): p. 76-80.

62. Fry, C.S., et al., *Myogenic Progenitor Cells Control Extracellular Matrix Production by Fibroblasts during Skeletal Muscle Hypertrophy.* Cell Stem Cell, 2017. **20**(1): p. 56-69.

63. Murphy, M.M., et al., *Satellite cells, connective tissue fibroblasts and their interactions are crucial for muscle regeneration.* Development, 2011. **138**(17): p. 3625-37.

64. Smith, G.I., et al., *Fish oil-derived n-3 PUFA therapy increases muscle mass and function in healthy older adults.* Am J Clin Nutr, 2015. **102**(1): p. 115-22.

65. Smith, G.I., et al., *Omega-3 polyunsaturated fatty acids augment the muscle protein anabolic response to hyperinsulinaemia-hyperaminoacidaemia in healthy young and middle-aged men and women.* Clin Sci (Lond), 2011. **121**(6): p. 267-78.

66. Rodacki, C.L., et al., *Fish-oil supplementation enhances the effects of strength training in elderly women.* Am J Clin Nutr, 2012. **95**(2): p. 428-36.

67. Simopoulos, A.P., *Human requirement for N-3 polyunsaturated fatty acids.* Poult Sci, 2000. **79**(7): p. 961-70.

68. Baker, E.J., et al., *Metabolism and functional effects of plant-derived omega-3 fatty acids in humans.* Prog Lipid Res, 2016. **64**: p. 30-56.

69. Kaur, G., et al., *Docosapentaenoic acid (22:5n-3) down-regulates the expression of genes involved in fat synthesis in liver cells.* Prostaglandins Leukot Essent Fatty Acids, 2011. **85**(3-4): p. 155-61.

70. Arterburn, L.M., E.B. Hall, and H. Oken, *Distribution, interconversion, and dose response of n-3 fatty acids in humans.* Am J Clin Nutr, 2006. **83**(6 Suppl): p. 1467S-1476S.

71. Morin, C., et al., *Eicosapentaenoic acid monoglyceride resolves inflammation in an ex vivo model of human peripheral blood mononuclear cell.* Eur J Pharmacol, 2017. **807**: p. 205-211.

72. Vedin, I., et al., *Effects of docosahexaenoic acid-rich n-3 fatty acid supplementation on cytokine release from blood mononuclear leukocytes: the OmegAD study.* Am J Clin Nutr, 2008. **87**(6): p. 1616-22.

73. Shindou, H., et al., *Docosahexaenoic acid preserves visual function by maintaining correct disc morphology in retinal photoreceptor cells.* J Biol Chem, 2017. **292**(29): p. 12054-12064.

74. Litman, B.J., et al., *The role of docosahexaenoic acid containing phospholipids in modulating G protein-coupled signaling pathways: visual transduction.* J Mol Neurosci, 2001. **16**(2-3): p. 237-42; discussion 279-84.

75. Albert, B.B., et al., *Higher omega-3 index is associated with increased insulin sensitivity and more favourable metabolic profile in middle-aged overweight men.* Sci Rep, 2014. **4**: p. 6697.

76. Smith, G.I., 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): p. 402-12.

77. Bhullar, A.S., C.T. Putman, and V.C. Mazurak, *Potential Role of Omega-3 Fatty Acids on the Myogenic Program of Satellite Cells.* Nutr Metab Insights, 2016. **9**: p. 1-10.

78. Kalinkovich, A. and G. Livshits, *Sarcopenic obesity or obese sarcopenia: A cross talk between age-associated adipose tissue and skeletal muscle inflammation as a main mechanism of the pathogenesis.* Ageing Res Rev, 2017. **35**: p. 200-221.

79. Millward, D.J., *Nutrition and sarcopenia: evidence for an interaction.* Proc Nutr Soc, 2012. **71**(4): p. 566-75.

80. Phillips, S.M., *Nutritional Supplements in Support of Resistance Exercise to Counter Age-Related Sarcopenia.* Adv Nutr, 2015. **6**(4): p. 452-60.

81. Soysal, P., et al., *Inflammation and frailty in the elderly: A systematic review and meta-analysis.* Ageing Res Rev, 2016. **31**: p. 1-8.

82. Bano, G., et al., *Inflammation and sarcopenia: A systematic review and meta-analysis.* Maturitas, 2017. **96**: p. 10-15.

83. McKay, B.R., et al., *Association of interleukin-6 signalling with the muscle stem cell response following muscle-lengthening contractions in humans.* PLoS One, 2009. **4**(6): p. e6027.

84. Zhang, C., et al., *Interleukin-6/signal transducer and activator of transcription 3 (STAT3) pathway is essential for macrophage infiltration and myoblast proliferation during muscle regeneration.* J Biol Chem, 2013. **288**(3): p. 1489-99.

85. Sindhu, S., et al., *Obesity Is a Positive Modulator of IL-6R and IL-6 Expression in the Subcutaneous Adipose Tissue: Significance for Metabolic Inflammation.* PLoS One, 2015. **10**(7): p. e0133494.

86. Feinstein, R., et al., *Tumor necrosis factor-alpha suppresses insulin-induced tyrosine phosphorylation of insulin receptor and its substrates.* J Biol Chem, 1993. **268**(35): p. 26055-8.

87. Hotamisligil, G.S., N.S. Shargill, and B.M. Spiegelman, *Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance.* Science, 1993. **259**(5091): p. 87-91.

88. Merritt, E.K., et al., *Heightened muscle inflammation susceptibility may impair regenerative capacity in aging humans.* J Appl Physiol (1985), 2013. **115**(6): p. 937-48.

89. Calder, P.C., *Long-chain fatty acids and inflammation.* Proc Nutr Soc, 2012. **71**(2): p. 284-9.

90. Calder, P.C., *n-3 fatty acids, inflammation and immunity: new mechanisms to explain old actions.* Proc Nutr Soc, 2013. **72**(3): p. 326-36.

91. Wada, M., et al., *Enzymes and receptors of prostaglandin pathways with arachidonic acid-derived versus eicosapentaenoic acid-derived substrates and products.* J Biol Chem, 2007. **282**(31): p. 22254-66.

92. Kelley, D.S., et al., *Effects of dietary arachidonic acid on human immune response.* Lipids, 1997. **32**(4): p. 449-56.

93. Kelley, D.S., et al., *Arachidonic acid supplementation enhances synthesis of eicosanoids without suppressing immune functions in young healthy men.* Lipids, 1998. **33**(2): p. 125-30.

94. Phang, M., M.L. Garg, and A.J. Sinclair, *Inhibition of platelet aggregation by omega-3 polyunsaturated fatty acids is gender specific-Redefining platelet response to fish oils.* Prostaglandins Leukot Essent Fatty Acids, 2009. **81**(1): p. 35-40.

95. Bartram, H.P., et al., *Effects of fish oil on rectal cell proliferation, mucosal fatty acids, and prostaglandin E2 release in healthy subjects.* Gastroenterology, 1993. **105**(5): p. 1317-22.

96. Calder, P.C., *Polyunsaturated fatty acids and inflammatory processes: New twists in an old tale.* Biochimie, 2009. **91**(6): p. 791-5.

97. Cai, D., et al., *IKKbeta/NF-kappaB activation causes severe muscle wasting in mice.* Cell, 2004. **119**(2): p. 285-98.

98. Wang, Y., et al., *DHA inhibits protein degradation more efficiently than EPA by regulating the PPARgamma/NFkappaB pathway in C2C12 myotubes.* Biomed Res Int, 2013. **2013**: p. 318981.

99. Camandola, S., et al., *Nuclear factor kB is activated by arachidonic acid but not by eicosapentaenoic acid.* Biochem Biophys Res Commun, 1996. **229**(2): p. 643-7.

100. Williams-Bey, Y., et al., *Omega-3 Free Fatty Acids Suppress Macrophage Inflammasome Activation by Inhibiting NF-κB Activation and Enhancing Autophagy.* PLoS One, 2014. **9**(6).

101. Huang, F., et al., *EPA inhibits the inhibitor of kappaBalpha (IkappaBalpha)/NF-kappaB/muscle RING finger 1 pathway in C2C12 myotubes in a PPARgamma-dependent manner.* Br J Nutr, 2011. **105**(3): p. 348-56.

102. Serrano, A.L., et al., *Interleukin-6 is an essential regulator of satellite cell-mediated skeletal muscle hypertrophy.* Cell Metab, 2008. **7**(1): p. 33-44.

103. Chen, S.E., et al., *Role of TNF-{alpha} signaling in regeneration of cardiotoxin-injured muscle.* Am J Physiol Cell Physiol, 2005. **289**(5): p. C1179-87.

104. Langen, R.C., et al., *Tumor necrosis factor-alpha inhibits myogenic differentiation through MyoD protein destabilization.* FASEB J, 2004. **18**(2): p. 227-37.

105. Domingues-Faria, C., et al., *Skeletal muscle regeneration and impact of aging and nutrition.* Ageing Res Rev, 2016. **26**: p. 22-36.

106. Wahlin-Larsson, B., G. Carnac, and F. Kadi, *The influence of systemic inflammation on skeletal muscle in physically active elderly women.* Age (Dordr), 2014. **36**(5): p. 9718.

107. Da Boit, M., et al., *Sex differences in the effect of fish-oil supplementation on the adaptive response to resistance exercise training in older people: a randomized controlled trial.* Am J Clin Nutr, 2017. **105**(1): p. 151-8.

108. Briolay, A., et al., *Myogenic differentiation and lipid-raft composition of L6 skeletal muscle cells are modulated by PUFAs.* Biochim Biophys Acta, 2013. **1828**(2): p. 602-13.

109. Jeromson, S., et al., *Lipid remodelling and an altered membrane proteome may drive the effects of EPA and DHA treatment on skeletal muscle glucose uptake and protein accretion.* Am J Physiol Endocrinol Metab, 2017: p. ajpendo 00438 2015.

110. Mukai, A., et al., *Dynamic clustering and dispersion of lipid rafts contribute to fusion competence of myogenic cells.* Exp Cell Res, 2009. **315**(17): p. 3052-63.

111. Grimaldi, P.A., *Regulatory role of peroxisome proliferator-activated receptor delta (PPAR delta) in muscle metabolism. A new target for metabolic syndrome treatment?* Biochimie, 2005. **87**(1): p. 5-8.

112. Chandrashekar, P., et al., *Inactivation of PPARbeta/delta adversely affects satellite cells and reduces postnatal myogenesis.* Am J Physiol Endocrinol Metab, 2015. **309**(2): p. E122-31.

113. Deng, B., et al., *MyoD promotes porcine PPARgamma gene expression through an E-box and a MyoD-binding site in the PPARgamma promoter region.* Cell Tissue Res, 2016. **365**(2): p. 381-91.

114. Varga, T., Z. Czimmerer, and L. Nagy, *PPARs are a unique set of fatty acid regulated transcription factors controlling both lipid metabolism and inflammation().* Biochim Biophys Acta, 2011. **1812**(8): p. 1007-22.

115. Fritsche, K.L., *The Science of Fatty Acids and Inflammation.* Adv Nutr, 2015. **6**(3): p. 293S-301S.

116. Magee, P., S. Pearson, and J. Allen, *The omega-3 fatty acid, eicosapentaenoic acid (EPA), prevents the damaging effects of tumour necrosis factor (TNF)-alpha during murine skeletal muscle cell differentiation.* Lipids in Health and Disease, 2008. **7**(1): p. 24.

117. Magee, P., et al., *PPARgamma as a molecular target of EPA anti-inflammatory activity during TNF-alpha-impaired skeletal muscle cell differentiation.* J Nutr Biochem, 2012. **23**(11): p. 1440-8.

118. Angione, A.R., et al., *PPARδ regulates satellite cell proliferation and skeletal muscle regeneration.* Skeletal Muscle, 2011. **1**(1): p. 33.

119. Eisele, P.S., et al., *The peroxisome proliferator-activated receptor gamma coactivator 1alpha/beta (PGC-1) coactivators repress the transcriptional activity of NF-kappaB in skeletal muscle cells.* J Biol Chem, 2013. **288**(4): p. 2246-60.

120. Lee, M.S., et al., *Effects of Eicosapentaenoic Acid and Docosahexaenoic Acid on Mitochondrial DNA Replication and PGC-1α Gene Expression in C(2)C(12) Muscle Cells.* Prev Nutr Food Sci, 2016. **21**(4): p. 317-22.

121. Virbasius, J.V. and R.C. Scarpulla, *Activation of the human mitochondrial transcription.* Proc Natl Acad Sci U S A, 1994. **91**(4): p. 1309-13.

122. Lin, Y., et al., *PGC-1α is associated with C2C12 Myoblast differentiation.* Central European Journal of Biology, 2014. **9**(11): p. 1030-1036.

123. Hancock, C.R., et al., *High-fat diets cause insulin resistance despite an increase in muscle mitochondria.* Proc Natl Acad Sci U S A, 2008. **105**(22): p. 7815-20.

124. Pinel, A., et al., *N-3PUFA differentially modulate palmitate-induced lipotoxicity through alterations of its metabolism in C2C12 muscle cells.* Biochim Biophys Acta, 2016. **1861**(1): p. 12-20.

125. Bryner, R.W., et al., *Docosahexaenoic Acid protects muscle cells from palmitate-induced atrophy*. ISRN Obes, 2012. **2012**: p. 647348.

126. Endres, S., et al., *The effect of dietary supplementation with n-3 polyunsaturated fatty acids on the synthesis of interleukin-1 and tumor necrosis factor by mononuclear cells.* N Engl J Med, 1989. **320**(5): p. 265-71.

127. Oh, D.Y., et al., *GPR120 Is an Omega-3 Fatty Acid Receptor Mediating Potent Anti-inflammatory and Insulin-Sensitizing Effects.* Cell, 2010. **142**(5): p. 687-698.

128. Rieu, I., et al., *Reduction of low grade inflammation restores blunting of postprandial muscle anabolism and limits sarcopenia in old rats.* J Physiol, 2009. **587**(Pt 22): p. 5483-92.

129. Moore, D.R., et al., *Ingested protein dose response of muscle and albumin protein synthesis after resistance exercise in young men.* Am J Clin Nutr, 2009. **89**(1): p. 161-8.

130. MacDougall, J.D., et al., *The time course for elevated muscle protein synthesis following heavy resistance exercise.* Can J Appl Physiol, 1995. **20**(4): p. 480-6.

131. Witard, O.C., et al., *Myofibrillar muscle protein synthesis rates subsequent to a meal in response to increasing doses of whey protein at rest and after resistance exercise.* Am J Clin Nutr, 2014. **99**(1): p. 86-95.

132. Hulmi, J.J., et al., *The effects of whey protein on myostatin and cell cycle-related gene expression responses to a single heavy resistance exercise bout in trained older men.* Eur J Appl Physiol, 2008. **102**(2): p. 205-13.

133. Farup, J., et al., *Whey protein supplementation accelerates satellite cell proliferation during recovery from eccentric exercise.* Amino Acids, 2014. **46**(11): p. 2503-16.

134. Lauretani, F., et al., *Omega-6 and omega-3 fatty acids predict accelerated decline of peripheral nerve function in older persons.* Eur J Neurol, 2007. **14**(7): p. 801-8.

135. Kang, J.X. and A. Leaf, *Effects of long-chain polyunsaturated fatty acids on the contraction of neonatal rat cardiac myocytes.* Proc Natl Acad Sci U S A, 1994. **91**(21): p. 9886-90.

136. O'Neill, S.C., et al., *Direct and indirect modulation of rat cardiac sarcoplasmic reticulum function by n-3 polyunsaturated fatty acids.* J Physiol, 2002. **538**(Pt 1): p. 179-84.

137. Lewis, E.J., et al., *21 days of mammalian omega-3 fatty acid supplementation improves aspects of neuromuscular function and performance in male athletes compared to olive oil placebo.* J Int Soc Sports Nutr, 2015. **12**: p. 28.

138. Mizunoya, W., et al., *Dietary fat influences the expression of contractile and metabolic genes in rat skeletal muscle.* PLoS One, 2013. **8**(11): p. e80152.

139. Leenders, M., et al., *Elderly men and women benefit equally from prolonged resistance-type exercise training.* J Gerontol A Biol Sci Med Sci, 2013. **68**(7): p. 769-79.

140. Jin, Y., M. Lee, and Y. Park, *Synergistic attenuation of ovariectomy-induced bone loss by combined use of fish oil and 17beta-oestradiol.* Br J Nutr, 2017. **117**(4): p. 479-489.

141. Smith, G.I. and B. Mittendorfer, *Sexual dimorphism in skeletal muscle protein turnover.* J Appl Physiol (1985), 2016. **120**(6): p. 674-82.

142. Conboy, I.M., et al., *Rejuvenation of aged progenitor cells by exposure to a young systemic environment.* Nature, 2005. **433**(7027): p. 760-4.

143. Calder, P.C., et al., *Health relevance of the modification of low grade inflammation in ageing (inflammageing) and the role of nutrition.* Ageing Res Rev, 2017. **40**: p. 95-119.

144. Apolinario, L.M., et al., *Long-Term Therapy With Omega-3 Ameliorates Myonecrosis and Benefits Skeletal Muscle Regeneration in Mdx Mice.* Anat Rec (Hoboken), 2015. **298**(9): p. 1589-96.

145. Mackey, A.L., et al., *Activation of satellite cells and the regeneration of human skeletal muscle are expedited by ingestion of nonsteroidal anti-inflammatory medication.* FASEB J, 2016. **30**(6): p. 2266-81.

146. Landi, F., et al., *Nonsteroidal anti-inflammatory drug (NSAID) use and sarcopenia in older people: results from the ilSIRENTE study*. J Am Med Dir Assoc, 2013. **14**(8): p. 626 e9-13.

147. Zhang, P., et al., *mTOR is necessary for proper satellite cell activity and skeletal muscle regeneration.* Biochem Biophys Res Commun, 2015. **463**(1-2): p. 102-8.

148. Kamolrat, T. and S.R. Gray, *The effect of eicosapentaenoic and docosahexaenoic acid on protein synthesis and breakdown in murine C2C12 myotubes.* Biochem Biophys Res Commun, 2013. **432**(4): p. 593-8.

149. Bodine, S.C., 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**(11): p. 1014-9.

150. Martin, T.D., et al., *mTORC1 and JNK coordinate phosphorylation of the p70S6K1 autoinhibitory domain in skeletal muscle following functional overloading.* Am J Physiol Endocrinol Metab, 2014. **306**(12): p. E1397-405.

151. Bennett, A.M. and N.K. Tonks, *Regulation of distinct stages of skeletal muscle differentiation by mitogen-activated protein kinases.* Science, 1997. **278**(5341): p. 1288-91.

152. Kramer, H.F. and L.J. Goodyear, *Exercise, MAPK, and NF-kappaB signaling in skeletal muscle.* J Appl Physiol (1985), 2007. **103**(1): p. 388-95.

153. Volonte, D., Y. Liu, and F. Galbiati, *The modulation of caveolin-1 expression controls satellite cell activation during muscle repair.* FASEB J, 2005. **19**(2): p. 237-9.

154. Kook, S.H., et al., *Cyclic mechanical stress suppresses myogenic differentiation of adult bovine satellite cells through activation of extracellular signal-regulated kinase*. Mol Cell Biochem, 2008. **309**(1-2): p. 133-41.

155. Cho, Y.Y., et al., *RSK2 mediates muscle cell differentiation through regulation of NFAT3.* J Biol Chem, 2007. **282**(11): p. 8380-92.

156. Li, J. and S.E. Johnson, *ERK2 is required for efficient terminal differentiation of skeletal myoblasts.* Biochem Biophys Res Commun, 2006. **345**(4): p. 1425-33.

157. Peng, Y., et al., *Different effects of omega-3 fatty acids on the cell cycle in C2C12 myoblast proliferation.* Mol Cell Biochem, 2012. **367**(1-2): p. 165-73.

158. Day, K., et al., *The depletion of skeletal muscle satellite cells with age is concomitant with reduced capacity of single progenitors to produce reserve progeny.* Dev Biol, 2010. **340**(2): p. 330-43.

159. He, W.A., et al., *NF-kappaB-mediated Pax7 dysregulation in the muscle microenvironment promotes cancer cachexia.* J Clin Invest, 2013. **123**(11): p. 4821-35.

160. Murphy, L.O. and J. Blenis, *MAPK signal specificity: the right place at the right time.* Trends Biochem Sci, 2006. **31**(5): p. 268-75.

161. Saini, A., et al., *Omega-3 fatty acid EPA improves regenerative capacity of mouse skeletal muscle cells exposed to saturated fat and inflammation.* Biogerontology, 2017. **18**(1): p. 109-129.

162. Gillespie, M.A., et al., *p38-γ–dependent gene silencing restricts entry into the myogenic differentiation program.* J Cell Biol, 2009. **187**(7): p. 991-1005.

163. Smith, H.J., M.J. Lorite, and M.J. Tisdale, *Effect of a cancer cachectic factor on protein synthesis/degradation in murine C2C12 myoblasts: modulation by eicosapentaenoic acid.* Cancer Res, 1999. **59**(21): p. 5507-13.

164. Lee, J.H., et al., *Modulation of proliferation and differentiation of C2C12 skeletal muscle cells by fatty acids.* Life Sci, 2009. **84**(13-14): p. 415-20.

165. Luo, H., et al., *Activation of PPARgamma2 by PPARgamma1 through a functional PPRE in transdifferentiation of myoblasts to adipocytes induced by EPA.* Cell Cycle, 2015. **14**(12): p. 1830-41.

166. Lee, M.S., I.H. Kim, and Y. Kim, *Effects of eicosapentaenoic acid and docosahexaenoic acid on uncoupling protein 3 gene expression in C(2)C(12) muscle cells.* Nutrients, 2013. **5**(5): p. 1660-71.

167. Woodworth-Hobbs, M.E., et al., *Docosahexaenoic acid prevents palmitate-induced activation of proteolytic systems in C2C12 myotubes.* J Nutr Biochem, 2014. **25**(8): p. 868-74.

168. Chen, S.C., et al., *Long-chain polyunsaturated fatty acids amend palmitate-induced inflammation and insulin resistance in mouse C2C12 myotubes.* Food Funct, 2016. **7**(1): p. 270-8.

169. Lalia, A.Z., et al., *Influence of omega-3 fatty acids on skeletal muscle protein metabolism and mitochondrial bioenergetics in older adults.* Aging (Albany NY), 2017. **9**(4): p. 1096-115.

170. Serhan, C.N., et al., *Maresins: novel macrophage mediators with potent antiinflammatory and proresolving actions.* J Exp Med, 2009. **206**(1): p. 15-23.

171. Lee, S., et al., *Current clinical applications of omega-6 and omega-3 fatty acids.* Nutr Clin Pract, 2006. **21**(4): p. 323-41.

172. Schiaffino, S., S.P. Bormioli, and M. Aloisi, *The fate of newly formed satellite cells during compensatory muscle hypertrophy.* Virchows Arch B Cell Pathol, 1976. **21**(2): p. 113-8.

173. Moss, F.P. and C.P. Leblond, *NATURE OF DIVIDING NUCLEI IN SKELETAL MUSCLE OF GROWING RATS.* J Cell Biol, 1970. **44**(2): p. 459-61.

174. Murach, K.A., et al., *Differential requirement for satellite cells during overload-induced muscle hypertrophy in growing versus mature mice.* Skelet Muscle, 2017. **7**(1): p. 14.

175. Yoshino, J., et al., *Effect of dietary n-3 PUFA supplementation on the muscle transcriptome in older adults.* Physiol Rep, 2016. **4**(11).

176. Pawar, A. and D.B. Jump, *Unsaturated fatty acid regulation of peroxisome proliferator-activated receptor alpha activity in rat primary hepatocytes.* J Biol Chem, 2003. **278**(38): p. 35931-9.

177. Kishida, E., M. Tajiri, and Y. Masuzawa, *Docosahexaenoic acid enrichment can reduce L929 cell necrosis induced by tumor necrosis factor.* Biochim Biophys Acta, 2006. **1761**(4): p. 454-62.

178. Lo, C.J., et al., *Fish oil modulates macrophage P44/P42 mitogen-activated protein kinase activity induced by lipopolysaccharide.* JPEN J Parenter Enteral Nutr, 2000. **24**(3): p. 159-63.

179. Weldon, S.M., et al., *Docosahexaenoic acid induces an anti-inflammatory profile in lipopolysaccharide-stimulated human THP-1 macrophages more effectively than eicosapentaenoic acid.* J Nutr Biochem, 2007. **18**(4): p. 250-8.

180. Mullen, A., C.E. Loscher, and H.M. Roche, *Anti-inflammatory effects of EPA and DHA are dependent upon time and dose-response elements associated with LPS stimulation in THP-1-derived macrophages.* J Nutr Biochem, 2010. **21**(5): p. 444-50.

181. Dhawan, J. and T.A. Rando, *Stem cells in postnatal myogenesis: molecular mechanisms of satellite cell quiescence, activation and replenishment.* Trends Cell Biol, 2005. **15**(12): p. 666-73.

182. Khodabukus, A., et al., *In Vitro Tissue-Engineered Skeletal Muscle Models for Studying Muscle Physiology and Disease.* Adv Healthc Mater, 2018. **7**(15): p. e1701498.

183. Magkos, F., et al., *Free fatty acid kinetics in the late phase of postexercise recovery: importance of resting fatty acid metabolism and exercise-induced energy deficit.* Metabolism, 2009. **58**(9): p. 1248-55.

184. Kiens, B., et al., *Skeletal muscle substrate utilization during submaximal exercise in man: effect of endurance training.* J Physiol, 1993. **469**: p. 459-78.

185. Itoh, Y., et al., *Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40.* Nature, 2003. **422**(6928): p. 173-6.

186. Gemeinhardt, A., et al., *The influence of linoleic and linolenic acid on the activity and intracellular localisation of phospholipase D in COS-1 cells.* Biol Chem, 2009. **390**(3): p. 253-8.

187. Richieri, G.V. and A.M. Kleinfeld, *Unbound free fatty acid levels in human serum.* J Lipid Res, 1995. **36**(2): p. 229-40.

188. Boden, G. and G.I. Shulman, *Free fatty acids in obesity and type 2 diabetes: defining their role in the development of insulin resistance and beta-cell dysfunction.* Eur J Clin Invest, 2002. **32 Suppl 3**: p. 14-23.

189. Stephens, F.B., et al., *Lipid-induced insulin resistance is associated with an impaired skeletal muscle protein synthetic response to amino acid ingestion in healthy young men.* Diabetes, 2015. **64**(5): p. 1615-20.

190. Alsabeeh, N., et al., *Cell culture models of fatty acid overload: Problems and solutions.* Biochim Biophys Acta Mol Cell Biol Lipids, 2018. **1863**(2): p. 143-151.

191. Hage Hassan, R., et al., *Endoplasmic reticulum stress does not mediate palmitate-induced insulin resistance in mouse and human muscle cells.* Diabetologia, 2012. **55**(1): p. 204-14.

192. Deldicque, L., et al., *The unfolded protein response is activated in skeletal muscle by high-fat feeding: potential role in the downregulation of protein synthesis.* Am J Physiol Endocrinol Metab, 2010. **299**(5): p. E695-705.

193. Bohnert, K.R., J.D. McMillan, and A. Kumar, *Emerging roles of ER stress and unfolded protein response pathways in skeletal muscle health and disease.* J Cell Physiol, 2018. **233**(1): p. 67-78.

194. Di Girolamo, F.G., et al., *Omega-3 fatty acids and protein metabolism: enhancement of anabolic interventions for sarcopenia.* Curr Opin Clin Nutr Metab Care, 2014. **17**(2): p. 145-50.

195. McGlory, C., et al., *Fish oil supplementation suppresses resistance exercise and feeding-induced increases in anabolic signaling without affecting myofibrillar protein synthesis in young men.* Physiological Reports, 2016. **4**(6): p. e12715.

196. Gerling, C.J., et al., *Incorporation of Omega-3 Fatty Acids Into Human Skeletal Muscle Sarcolemmal and Mitochondrial Membranes Following 12 Weeks of Fish Oil Supplementation.* Front Physiol, 2019. **10**: p. 348.

197. Herbst, E.A., et al., *Omega-3 supplementation alters mitochondrial membrane composition and respiration kinetics in human skeletal muscle.* J Physiol, 2014. **592**(6): p. 1341-52.

198. Magee, P., S. Pearson, and J. Allen, *The omega-3 fatty acid, eicosapentaenoic acid (EPA), prevents the damaging effects of tumour necrosis factor (TNF)-alpha during murine skeletal muscle cell differentiation.* Lipids Health Dis, 2008. **7**: p. 24.

199. Kaur, G., X.F. Guo, and A.J. Sinclair, *Short update on docosapentaenoic acid: a bioactive long-chain n-3 fatty acid.* Curr Opin Clin Nutr Metab Care, 2016. **19**(2): p. 88-91.

200. Achard, F., C. Benistant, and M. Lagarde, *Interconversions and distinct metabolic fate of eicosapentaenoic, docosapentaenoic and docosahexaenoic acids in bovine aortic endothelial cells.* Biochim Biophys Acta, 1995. **1255**(3): p. 260-6.

201. Kaur, G., et al., *Docosapentaenoic acid (22:5n-3): a review of its biological effects.* Prog Lipid Res, 2011. **50**(1): p. 28-34.

202. Norris, P.C. and E.A. Dennis, *Omega-3 fatty acids cause dramatic changes in TLR4 and purinergic eicosanoid signaling.* Proc Natl Acad Sci U S A, 2012. **109**(22): p. 8517-22.

203. Wosczyna, M.N. and T.A. Rando, *A Muscle Stem Cell Support Group: Coordinated Cellular Responses in Muscle Regeneration.* Dev Cell, 2018. **46**(2): p. 135-143.

204. Feige, P., et al., *Orienting Muscle Stem Cells for Regeneration in Homeostasis, Aging, and Disease.* Cell Stem Cell, 2018. **23**(5): p. 653-664.

205. Jeromson, S., et al., *Lipid remodeling and an altered membrane-associated proteome may drive the differential effects of EPA and DHA treatment on skeletal muscle glucose uptake and protein accretion.* Am J Physiol Endocrinol Metab, 2018. **314**(6): p. E605-e619.

206. Portolesi, R., B.C. Powell, and R.A. Gibson, *Competition between 24:5n-3 and ALA for Delta 6 desaturase may limit the accumulation of DHA in HepG2 cell membranes.* J Lipid Res, 2007. **48**(7): p. 1592-8.

207. Mandelkow, R., et al., *Detection and Quantification of Nuclear Morphology Changes in Apoptotic Cells by Fluorescence Microscopy and Subsequent Analysis of Visualized Fluorescent Signals.* Anticancer Res, 2017. **37**(5): p. 2239-2244.

208. Tachtsis, B., et al., *Acute Endurance Exercise Induces Nuclear p53 Abundance in Human Skeletal Muscle.* Front Physiol, 2016. **7**: p. 144.

209. Leckey, J.J., et al., *High dietary fat intake increases fat oxidation and reduces skeletal muscle mitochondrial respiration in trained humans.* Faseb j, 2018. **32**(6): p. 2979-2991.

210. Johnson, M.L., et al., *Eicosapentaenoic acid but not docosahexaenoic acid restores skeletal muscle mitochondrial oxidative capacity in old mice.* Aging Cell, 2015. **14**(5): p. 734-43.

211. Chen, K., et al., *Artemisinin and dihydroartemisinin promote beta-cell apoptosis induced by palmitate via enhancing ER stress.* Apoptosis, 2020.

212. Zhang, E., et al., *The functional role of Bax/Bak in palmitate-induced lipoapoptosis.* Food Chem Toxicol, 2019. **123**: p. 268-274.

213. Sadeghi, A., et al., *Interplay between oxidative stress and autophagy function and its role in inflammatory cytokine expression induced by palmitate in skeletal muscle cells.* Cytokine, 2020. **125**: p. 154835.

214. Yuzefovych, L., G. Wilson, and L. Rachek, *Different effects of oleate vs. palmitate on mitochondrial function, apoptosis, and insulin signaling in L6 skeletal muscle cells: role of oxidative stress.* Am J Physiol Endocrinol Metab, 2010. **299**(6): p. E1096-105.

215. Listenberger, L.L., D.S. Ory, and J.E. Schaffer, *Palmitate-induced apoptosis can occur through a ceramide-independent pathway*. J Biol Chem, 2001. **276**(18): p. 14890-5.

216. Salvado, L., et al., *Oleate prevents saturated-fatty-acid-induced ER stress, inflammation and insulin resistance in skeletal muscle cells through an AMPK-dependent mechanism.* Diabetologia, 2013. **56**(6): p. 1372-82.

217. Yuzefovych, L.V., et al., *Mitochondrial DNA damage via augmented oxidative stress regulates endoplasmic reticulum stress and autophagy: crosstalk, links and signaling.* PLoS One, 2013. **8**(12): p. e83349.

218. Lee, A.S., *The ER chaperone and signaling regulator GRP78/BiP as a monitor of endoplasmic reticulum stress.* Methods, 2005. **35**(4): p. 373-81.

219. Li, Y., et al., *New insights into the roles of CHOP-induced apoptosis in ER stress.* Acta Biochim Biophys Sin (Shanghai), 2014. **46**(8): p. 629-40.

220. Harding, H.P., et al., *Perk is essential for translational regulation and cell survival during the unfolded protein response.* Mol Cell, 2000. **5**(5): p. 897-904.

221. Saelens, X., M. Kalai, and P. Vandenabeele, *Translation inhibition in apoptosis: caspase-dependent PKR activation and eIF2-alpha phosphorylation.* J Biol Chem, 2001. **276**(45): p. 41620-8.

222. Green, D.R. and F. Llambi, *Cell Death Signaling.* Cold Spring Harb Perspect Biol, 2015. **7**(12).

223. Ott, M., et al., *The mitochondrial TOM complex is required for tBid/Bax-induced cytochrome c release.* J Biol Chem, 2007. **282**(38): p. 27633-9.

224. Zhang, J., et al., *EPA and DHA Inhibit Myogenesis and Downregulate the Expression of Muscle-related Genes in C2C12 Myoblasts.* Genes, 2019. **10**(1): p. 64.

225. Hsueh, T.-Y., J.I. Baum, and Y. Huang, *Effect of Eicosapentaenoic Acid and Docosahexaenoic Acid on Myogenesis and Mitochondrial Biosynthesis during Murine Skeletal Muscle Cell Differentiation.* Frontiers in Nutrition, 2018. **5**(15).

226. Lanza, I.R., 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): p. E1391-403.

227. Aas, V., et al., *Eicosapentaenoic acid (20:5 n-3) increases fatty acid and glucose uptake in cultured human skeletal muscle cells.* J Lipid Res, 2006. **47**(2): p. 366-74.

228. Vaughan, R.A., et al., *Conjugated linoleic acid or omega 3 fatty acids increase mitochondrial biosynthesis and metabolism in skeletal muscle cells.* Lipids Health Dis, 2012. **11**: p. 142.

229. Sullivan, E.M., et al., *Mechanisms by Which Dietary Fatty Acids Regulate Mitochondrial Structure-Function in Health and Disease.* Adv Nutr, 2018. **9**(3): p. 247-262.

230. Capel, F., et al., *DHA at nutritional doses restores insulin sensitivity in skeletal muscle by preventing lipotoxicity and inflammation.* J Nutr Biochem, 2015. **26**(9): p. 949-59.

231. Yuzefovych, L.V., et al., *Protection from palmitate-induced mitochondrial DNA damage prevents from mitochondrial oxidative stress, mitochondrial dysfunction, apoptosis, and impaired insulin signaling in rat L6 skeletal muscle cells.* Endocrinology, 2012. **153**(1): p. 92-100.

232. Kim, S., C. Kim, and S. Park, *Mdivi-1 Protects Adult Rat Hippocampal Neural Stem Cells against Palmitate-Induced Oxidative Stress and Apoptosis.* Int J Mol Sci, 2017. **18**(9).

233. Law, B.A., et al., *Lipotoxic very-long-chain ceramides cause mitochondrial dysfunction, oxidative stress, and cell death in cardiomyocytes.* Faseb j, 2018. **32**(3): p. 1403-1416.

234. Markworth, J.F., et al., *Divergent shifts in lipid mediator profile following supplementation with n-3 docosapentaenoic acid and eicosapentaenoic acid.* Faseb j, 2016. **30**(11): p. 3714-3725.

235. Serhan, C.N., N. Chiang, and J. Dalli, *New pro-resolving n-3 mediators bridge resolution of infectious inflammation to tissue regeneration.* Mol Aspects Med, 2018. **64**: p. 1-17.

236. Suphioglu, C., et al., *Zinc and DHA have opposing effects on the expression levels of histones H3 and H4 in human neuronal cells.* Br J Nutr, 2010. **103**(3): p. 344-51.

237. Xu, S., et al., *Palmitate induces ER calcium depletion and apoptosis in mouse podocytes subsequent to mitochondrial oxidative stress.* Cell Death Dis, 2015. **6**: p. e1976.

238. Ueda, N., *Ceramide-induced apoptosis in renal tubular cells: a role of mitochondria and sphingosine-1-phoshate.* Int J Mol Sci, 2015. **16**(3): p. 5076-124.

239. Graciano, M.F., et al., *Omega-3 fatty acids control productions of superoxide and nitrogen oxide and insulin content in INS-1E cells.* J Physiol Biochem, 2016. **72**(4): p. 699-710.

240. Lucena, C.F., et al., *Omega-3 supplementation improves pancreatic islet redox status: in vivo and in vitro studies.* Pancreas, 2015. **44**(2): p. 287-95.