UNDERSTANDING n-3 PUFA: A DOSING APPROACH TO STUDY INCORPORATION, WASHOUT, AND MOLECULAR MECHANISMS IN AGEING

by

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I dedicate this thesis to my late father. *Volim te*

Declaration

I declare that this thesis was composed by myself and that all the data were collected and analysed by myself, under the supervision of Dr Nidia Rodriguez-Sanchez, Professor Stuart Galloway, with the following exceptions:

i The protein synthesis data obtained in the cell experiment in Chapter 2 were conducted by Ms. Nerys Donker.

ii Lipid fatty acid profiling in the cell experiment in Chapter 2 was conducted by Mark Becker and Martin Balvers from the analytical department at Danone Nutricia Research.

iii Lipid fatty acid profiling in tissues described in Chapter 3 was conducted by the Institute of Aquaculture under the supervision of Mr. James Dick.

iv Dr. Iain Gallagher provided advice and the script to run the data analysis on the microarray data in Chapter 4.

Neither the thesis, nor the original work contained therein have been submitted to this or any other institution for a higher degree. This thesis does not infringe upon anyone's copyright and any material from the work of others included in this thesis are fully acknowledged in accordance with common reference practices.

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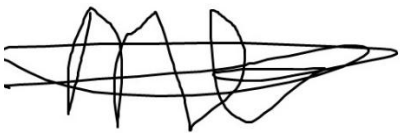

Milena Banic

Date: **12/09/2024**

Abstract

The global population is getting older. Ageing is associated with a loss in skeletal muscle mass (SMM) and strength. SMM, accounting for ~40% of total body weight, is crucial for health. Reductions in SMM result in increased frailty and decrease quality of life. Therefore, it is essential to elucidate new strategies that can counteract the loss of SMM with age. Accumulating evidence has demonstrated that omega-3 polyunsaturated fatty acids (n-3 PUFA) can positively regulate skeletal muscle health via incorporation into phospholipid membranes of skeletal muscle. However, the relationship between dietary intake of n-3 PUFA and its incorporation into phospholipid membranes, as well as its impact on SMM regulation, remains unclear. Additionally, the washout rates after stopping supplementation have not been determined. Hence, the overall aim of this thesis is to gain insight into the incorporation, washout, and underlying mechanisms of n-3 PUFA following different doses in young and older adults, with a focus on muscle and ageing.

Chapter 1 provides a comprehensive background to place the thesis in context, addresses gaps in the literature, and provides the reader with the aims and objectives of this thesis. **Chapter 2** assesses the incorporation and washout of EPA and DHA, administered in a 3:2 ratio, over time at low and high concentrations in cell lysates and phospholipid membranes of C2C12 myotubes, with measurements of muscle protein synthesis and associated signalling pathways. **Chapter 3** continues with a human trial investigating incorporation and washout of n-3 PUFA in erythrocytes, skeletal muscle phospholipid membranes, and adipose tissue, comparing two different dosing strategies in young and older adults. Finally, **Chapter 4** aims to identify global changes in skeletal muscle transcriptome in response to n-3 PUFA supplementation in young and older adults. **Chapter 5** discusses findings of this thesis in a broader context and addresses future directions.

Overall, the outcomes from this thesis contribute to the body of literature concerning n-3 PUFA supplementation, incorporation, and washout while also aiming to elucidate some of the underlying mechanisms.

Acknowledgements

I could not have done my PhD without the help of many throughout my journey. First, I would like to thank my academic supervisors, **Dr. Nidia Rodriguez-Sanchez** and **Prof. Stuart Galloway**. Your guidance, insights and kindness in your supervision have supported me throughout this PhD. You encouraged me to ask questions and think critically, which helped me become a better researcher. Next, I would like to thank **Dr. Oliver Witard**, your external supervision has far exceeded my expectations, and I learned a lot from you.

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'The road goes ever on and on, down from the door where it began. Now far ahead the road has gone, and I must follow if I can.'

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List of publications/ conference activity

M. Banic, M. van Dijk, F. J. Dijk, M. J. W. Furber, O. C. Witard, N. Donker, M. J. A. Becker, S. D. Galloway, N. Rodriguez-Sanchez (2024). Dose-dependency of a combined EPA:DHA mixture on incorporation, washout, and protein synthesis in C2C12 myotubes. Prostaglandins, Leukotrienes & Essential Fatty Acids.

O. Witard, **M. Banic**, N. Rodriguez-Sanchez, M. van Dijk, S.D.R. Galloway (2023). Long-chain n-3 PUFA ingestion for the stimulation of muscle protein synthesis in healthy older adults. Proceedings of the Nutrition Society. Published online 2023:1-11. doi:10.1017/S0029665123004834

July 2024 (**oral presentation**): European College of Sport Science in Glasgow, United Kingdom. Title: *Incorporation of n-3 PUFA into Skeletal Muscle Membranes Alters Global Gene Expression in Young and Older Adults.*

January 2024 (**online presentation**). Global Organization for EPA and DHA (GOED). Title: *The impact of n-3 PUFA on tissue content and function for healthy ageing*

June 2023 (**poster**): PhD course Skeletal Muscle Growth and Metabolism – Insights into Models and Mechanisms in Copenhagen, Denmark. Title: *Dose-dependent effect of omega-3 polyunsaturated fatty acid supplementation on lipid composition in human skeletal muscle: a pilot study.*

September 2022 (**poster**): Europhysiology in Copenhagen, Denmark. Title: *Temporal changes in the uptake and washout of EPA and DHA in C2C12 myotubes.*

List of Abbreviations

Thesis specific abbreviations

CD	Constant Dose
HC	High Concentration
LC	Low Concentration
LD	Loading Dose
LP	Loading Phase
MP	Maintenance Phase
SMM	Skeletal Muscle Mas

Other abbreviations

4E-BP1	4E-Binding Proteins
Akt	Protein Kinase B (PK)
ALA	Alpha-Linolenic Acid
ARA	Arachidonic Acid
c-AMP	Cyclic AMP
COX	Cyclooxygenase
CYP	Cytochrome P450
DHA	Docosahexaenoic Acid
DPA	Docosapentaenoic Acid
ECM	Extracellular Matrix
ELOVL2	Elongase 2
ELOVL5	Elongase 5
EMT	Epithelial-Mesenchymal Transition
ENSG	Ensembl Gene Ids
EPA	Eicosapentaenoic Acid
ETC	Electron Transport Chain
FA	Fatty Acid Profile
FADS1	Δ5-Desaturase
FADS2	Δ6-Desaturase
FAK	Focal Adhesion Kinase

FOXO1	Forkhead Box O 1,
GPR	G-Protein Coupled Receptors
GSEA	Gene Set Enrichment Analysis
HC	High Concentration
IFN-α	Interferon Alpha
IFN-γ	Interferon Gamma
LOX	Lipoxygenase
LTB_4	Leukotriene B4
LTB_5	Leukotriene B5
MPB	Muscle Protein Breakdown
MPS	Muscle Protein Synthesis
mTORC1	Mammalian Target Of Rapamycin Complex 1.
MuRF1	Muscle RING Finger
MVPA	Moderate To Vigorous Physical Activity
n-3 PUFA	Omega-3 Polyunsaturated Fatty Acids
NF-κB	Nuclear Factor Kappa Beta
p70S6K1	p70 Ribosomal Protein S6 Kinase 1
PC	Phosphatidylcholine
PDE4	Phosphodiesterase 4A
PE	Phosphatidylethanolamine
PGE_2	Prostaglandin E2
PGE_3	Prostaglandin E3
PPARγ	Peroxisome Proliferator-Activated Receptor Gamma
SCAN.UPC	Single Channel Array Normalization
SDA	Stearidonic Acid
SPM	Specialised Pro-Resolving Lipid Mediators
UPP	Ubiquitin-Proteasomal Pathway

Chapter 1: General Introduction

1.1: Overview of chapter

The world population is getting older. By the end of 2030, one in six people is expected to be over 60 years of age, and the number of people over 60 will have doubled by 2050 (World Health Organization, 2022). Ageing comes with several health complications and is associated with a loss of skeletal muscle mass (SMM) and strength (Mitchell *et al.*, 2012). SMM is crucial for health and comprises ~40% of total body weight (Frontera and Ochala, 2015). Reductions in SMM are detrimental to health and result in increased morbidity, frailty and decreased quality of life (Wolfe, 2006). The term 'sarcopenia' is used by the European Working Group on Sarcopenia in Older People (EWGSOP) to describe a syndrome characterised by progressive and generalised loss of SMM and strength with a risk of adverse outcomes such as disability, poor quality of life, and death (Cruz-Jentoft *et al.*, 2010). In 2019, the EWGSOP updated its consensus on sarcopenia, introducing additions to the diagnostic criteria: the updates emphasise low muscle strength as a key characteristic of sarcopenia, using the detection of low muscle quantity and quality to confirm a diagnosis of sarcopenia. Furthermore, Cruz-Jentoft *et al.,* (2019) proposed an algorithm for case-finding, diagnosis, and severity of determination, ensuring a systematic and consistent to identify individuals with sarcopenia or of risk at sarcopenia. (Cruz-Jentoft *et al.*, 2019). A recent meta-analysis concluded that age-related sarcopenia is prevalent among 10 – 27% of the global population over 60 years of age, with severe sarcopenia ranging between 2 – 9% (Petermann-Rocha *et al.*, 2022). Besides decreasing additional costs on healthcare, improving muscle quality can improve quality of life, and it is therefore essential to elucidate strategies that can counteract the loss of SMM with age. Sarcopenia is recognised as a significant contributor to frailty, a multidimensional geriatric syndrome that is characterised by cumulative decline in multiple body systems or functions (Fried *et al.*, 2001). While there is overlap between sarcopenia and frailty, such as low grip strength and slow gait speed, they remain distinct entities. Frailty is considered a geriatric syndrome, encompassing a broader systemic vulnerability, whereas sarcopenia is defined as a muscle-specific disease (Fried *et al.,* 2001).

Dietary strategies play a crucial role in counteracting sarcopenia. Omega (ω)-polyunsaturated fatty acids (n-3 PUFA) have been an emerging candidate of interest for positively regulating skeletal muscle health. N-3 PUFA have been extensively studied with regards to their health

effects on inflammation (Calder, 2020b), cardiovascular diseases (Harris, 2008) and neurocognition (Dighriri *et al.*, 2022). Recently, more studies have demonstrated the beneficial effects of n-3 PUFA on SMM via changes in skeletal muscle turnover (McGlory, Calder and Nunes, 2019). These effects occur through the incorporation of n-3 PUFA into cellular membranes. However, further research is necessary to investigate the effects of varying dietary dosing on the incorporation of fatty acids into phospholipid membranes of tissues, the subsequent washout rates, and the overall regulatory mechanisms by which n-3 PUFA can impact SMM. Understanding these dynamics is crucial for elucidating how dosing can impact muscle composition and function.

This chapter outlines the precedence for the thesis and provides a background to place the thesis in context. First, ageing and changes in muscle turnover and regeneration during ageing are covered. Then, a brief overview of n-3 PUFA will be given with relation to structure, metabolism and conversion, followed by incorporation and washout from tissues, with a subsection of EPA *vs.* DHA, two species of n-3 PUFA considered the most biologically active. Finally, the relationship between n-3 PUFA and muscle turnover will be examined, summarising current evidence and studies, followed by influences of n-3 PUFA on gene expression and the differential regulation of eicosapentaenoic acid (EPA) *vs.* docosahexaenoic acid (DHA). Building on this, the aims and objectives of the thesis will be outlined, followed by a brief overview of the subsequent chapters.

1.2: Ageing

1.2.1 Age-related declines in muscle mass

Due to improved environmental circumstances, the average human life expectancy worldwide has increased over the years (Roser, 2018). However, ageing introduces complexities as chronological age does not inherently guarantee the preservation of health (Hay *et al.*, 2017). Ageing is broadly defined as the time-dependent functional decline in adulthood that affects most living organisms. Currently, there are 12 hallmarks of ageing (López-Otín *et al.*, 2023), building up on previous hallmarks of ageing that have been extensively studied in ageing research (López-Otín *et al.*, 2013). The hallmarks of ageing fulfil the following criteria: (1) time-dependent manifestation of alterations accompanying the ageing process, (2) the possibility to accelerate ageing by experimentally accentuating the hallmark, and 3) the

opportunity to decelerate, halt, or reverse ageing by therapeutic interventions on the hallmark. The hallmarks of aging from López-Otín *et al.,* (2023) are defined as 1) genomic instability, 2) telomere attrition, 3) epigenetic alterations, 4) loss of proteostasis, 5) disabled macroautophagy, 6) deregulated nutrient sensing, 7) mitochondrial dysfunction, 8) cellular senescence, 9) stem cell exhaustion, 10) altered intercellular communication, 11) chronic inflammation, and 12) dysbiosis (López-Otín *et al.*, 2023). A review by Granic *et al.* (2023) focuses on the hallmarks of aging specifically in relation to skeletal muscle loss. The authors propose muscle-specific hallmarks associated with altered intercellular communication, including immunoaging, neural dysfunction, extracellular matrix (ECM) dysfunction, and reduced vascular perfusion. In the context of skeletal muscle, there is strong evidence linking epigenetic alterations, mitochondrial dysfunction, and neural dysfunction to muscle loss A review by Granic *et al.* (2023). Additionally, Granic *et al.,* 2023 propose there is moderate evidence supporting the roles of inflammation, deregulated nutrient sensing, chronic inflammation, ECM dysfunction, and reduced vascular perfusion as contributors to skeletal muscle aging (Granic et al., 2023). It is evident that the proposed hallmarks of ageing related to skeletal muscle are closely intertwined with each other (Granic *et al.*, 2023).

From a structural perspective, a noticeable reduction in SMM is observed from the 5th decade of life onwards (Janssen *et al.,* 2000), with declines between 0.2 – 0.5% per year (Mitchell *et al.,* 2012). Interestingly, although the loss of muscle mass is associated with the decline in strength, declines in muscle strength are more rapid than declines in muscle mass (Goodpaster *et al.,* 2006; Mitchell *et al.,* 2012). Human skeletal muscle tissue is composed of type I (oxidative) and type II (glycolytic) fibres. These fibres differ in their contractile, biochemical and metabolic phenotypes, which contributes to the different roles they play in making up the total human skeletal muscle tissue (Schiaffino and Reggiani, 2011). It is widely accepted that the decline in muscle mass is primarily due to loss of number and size of type II fibres, rather than type I fibres (Evans and Lexell, 1995). Furthermore, there is strong evidence for a loss of proteostasis (regulation and maintenance of cellular environment), deregulated nutrient sensing, and mitochondrial dysfunction in ageing skeletal muscle (Granic *et al.,* 2023).

1.2.2 Muscle protein turnover

Skeletal muscle is a highly adaptable tissue in response to different stimuli. Muscle hypertrophy occurs in response to anabolic stimuli, such as resistance exercise or nutritional

protein feeding (Drummond *et al.*, 2009). Conversely, a loss of muscle mass, known as muscle atrophy or muscle wasting, is associated with inactivity (McPhee *et al.*, 2016), disuse (Ferrando *et al.*, 1996), and ageing (Janssen *et al.*, 2000). On a mechanistic level, skeletal muscle and protein balance is regulated by muscle protein synthesis (MPS) and muscle protein breakdown (MPB). In ageing individuals, the same amount of protein feeding results in a suppressed MPS response, a term known as "anabolic resistance" (Cuthbertson *et al.*, 2005). This is widely considered a main contributor to declines in muscle mass (Breen and Phillips, 2011). MPS mainly revolves around the mammalian/mechanistic target of rapamycin (mTOR) complex protein pathway. Briefly, the pathway is described as follows: in response to outside stimuli, such as amino acid feeding or exercise, Akt is activated and subsequently phosphorylates mTORC1 either directly or via inhibiting the TSC1/2 complex (Drummond *et al.*, 2009; Papadopoli *et al.*, 2019). Phosphorylated mTORC1 promotes protein synthesis by phosphorylating eukaryotic initiation factor 4e-binding proteins (4E-BPs) and ribosomal protein S6 kinase beta-1 (p70S6K1) (Liu and Sabatini, 2020). Furthermore, there are some pathways that may regulate MPS independently of mTOR, such as MAPK/ERK1/2 Pathway (Hodson *et al.*, 2019). A schematic overview of the mTORC1 pathway to stimulate MPS is presented in **Figure 1.1**. Besides the presented overview, other factors can influence rates of protein synthesis. For example, chronic low-grade, age-related inflammation can contribute to muscle atrophy. Elevated levels of the pro-inflammatory cytokine IL-6 and TNF-α in older individuals were associated with less appendicular muscle mass and decreased strength (Visser *et al.*, 2002). Mechanistically, increased TNF-α levels observed in chronic low-grade inflammation can lead to protein degradation via upregulation of the NF-κB pathway and activation of the ubiquitin–proteasome system (Li, Malhotra and Kumar, 2008). Another important negative regulator of protein synthesis is myostatin, an extracellular myokine secreted by muscle cells and member of the transforming growth factor β (TGFβ) family (Elkina *et al.*, 2011). Myostatin inhibits the mTORC1 pathway by phosphorylating Smad2/3 receptors, which in its turn reduces Akt activity. During ageing, protein expression of myostatin was upregulated in older males compared to younger counterparts (McKay *et al.*, 2012). Furthermore, insulin appears to influence muscle protein synthesis (Fujita *et al.*, 2006). Early studies observed that insulin ingestion facilitates amino acid uptake and availability (Bennet *et al.*, 1990; Newman *et al.*, 1994; Biolo, Declan Fleming and Wolfe, 1995). The influence of insulin on muscle protein turnover is primarily mediated through the activation on key signalling pathways involved in protein synthesis, such as the PI3K-Akt-mTOR pathway (Ferreira and Duarte, 2023). Additionally, insulin resistance, commonly observed during

ageing, can impair muscle protein turnover, contributing to a progression in skeletal muscle atrophy (Shou, Chen and Xiao, 2020). Insulin resistance is strongly associated with sarcopenia, as described by Liu and Zhu (2023), who highlight that both conditions are significantly influenced by external factors such as age, gender, and physical activity levels.

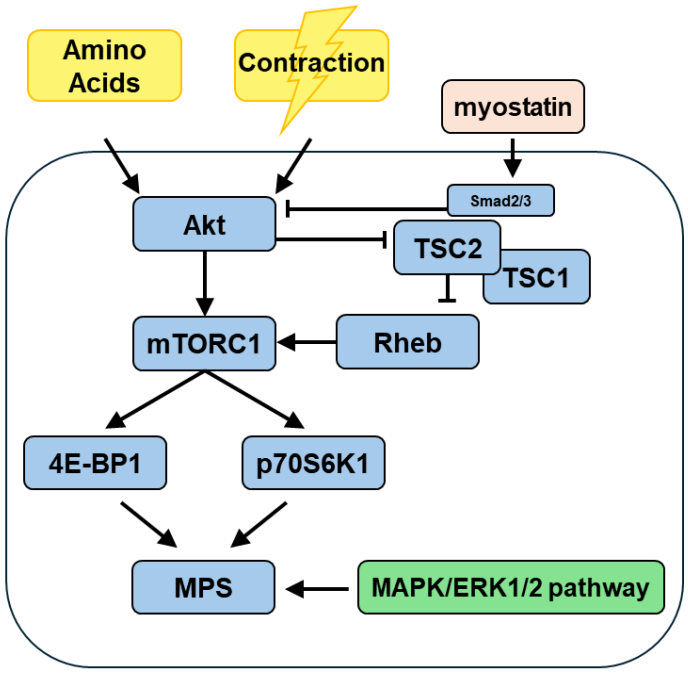

Figure 1.1: Schematic pathway of Muscle Protein Synthesis. Akt = Protein Kinase B (PKB), mTORC1 = Mechanistic Target of Rapamycin Complex 1, TSC = Tuberous Sclerosis Complex, Rheb = Ras Homolog Enriched in Brain, 4E-BP1 = Eukaryotic Translation Initiation Factor 4E-Binding Protein 1, p70S6K1 = 70 kDa Ribosomal Protein S6 Kinase 1, MAPK/ERK 1/2 = Mitogen-Activated Protein Kinase/Extracellular Signal-Regulated Kinase 1/2, MPS = Muscle Protein Synthesis.

Muscle Protein Breakdown occurs via three main systems; 1) the ubiquitin-proteasomal pathway (UPP), 2) autophagy, and 3) the calpain Ca2+ dependent cysteine proteases (Tipton, Hamilton and Gallagher, 2018). In the ubiquitin-proteasomal pathway, an ubiquitin molecule is attached to a target protein via several enzymatic steps. Once four molecules of ubiquitin are attached to a target protein, the protein is sent to the proteasome for degradation (Tipton, Hamilton and Gallagher, 2018). Two ubiquitin ligase genes that play a central role in models of skeletal muscle atrophy are Muscle RING Finger 1 (MuRF1), and Muscle Atrophy F-box (MAFbx) (Bodine *et al.*, 2001). Calpain Ca2+ dependent cysteine proteases are another complex that target substrates for degradation and are also associated with increased muscle wasting (Tidball and Spencer, 2002). Autophagy is a self-degradative process which serves many purposes in cellular homeostasis, such as clearance of misfolded proteins, clearing damaged organelles or eliminating intracellular pathogens (Glick, Barth and Macleod, 2010).

Although skeletal muscle atrophy seems largely attributed to UPP and calpain systems (Jackman and Kandarian, 2004), all three systems are essential and interconnected to promote MPB. Furthermore, many other factors play a role in declines in muscle mass and strength during ageing; physical inactivity (McPhee *et al.*, 2016), motor unit remodelling (Piasecki *et al.*, 2016), loss of fibres (Lexell, Taylor and Sjöström, 1988), fibre size (Nilwik *et al.*, 2013), or a decreased muscle regeneration in response to injury or as exercise (Sousa-Victor, García-Prat and Muñoz-Cánoves, 2022).

Both MPS and MPB regulate muscle protein turnover, playing essential roles in maintaining muscle mass. However, MPS appears to be more responsive to anabolic stimuli, such as resistance exercise (Phillips *et al.*, 1997) and amino acid ingestion (Biolo *et al.*, 1997). Ageing is primarily characterised by a diminished MPS response in response to anabolic stimuli, suggesting that this diminished MPS response is a key driver of the progression of age-related muscle atrophy (Paulussen *et al.*, 2021) (Shad, Thompson and Breen, 2016).

1.2.3 Muscle tissue regeneration

Muscle regeneration is a key component of muscle reorganisation, and its decline during ageing contributes to loss in muscle mass. Muscle regeneration can be divided into four phases; degeneration, inflammation, regeneration, and remodelling & repair (Carosio *et al.*, 2011; Forcina, Cosentino and Musarò, 2020). Injury of the muscle (degeneration) triggers a tightly regulated inflammatory process. Inflammation is critical in muscle regeneration, as multiple studies have shown that inhibiting inflammation impairs the muscle regeneration process (Summan *et al.*, 2006; Tidball and Wehling-Henricks, 2007). The infiltration of immune cells to the injured muscle triggers satellite cell activation through a precisely regulated mechanism (Tidball and Villalta, 2010). Typically, satellite cells reside quiescent between the basal lamina and plasmalemma. However, in response to muscle injury or exercise, satellite cells are activated due to the presence of immune cells (Anderson, 2022). Once activated, satellite cells enter the cell cycle, differentiate into myoblasts, and undergo proliferation (Sousa-Victor, García-Prat and Muñoz-Cánoves, 2022). Satellite cells can either return to quiescent state or differentiate to the formation or repair of myofibers. The final stage of remodelling & repair is characterised by the remodelling of connective tissue, including angiogenesis and recovery of the functional performance of the injured muscle. The extracellular matrix (ECM) plays a critical role in this process (Goetsch *et al.*, 2003; Carosio *et al.*, 2011). Interestingly, although some data in animals have demonstrated hypertrophy in the absence of satellite cells (McCarthy

et al., 2011), satellite cells have been implicated in regulating the ECM during hypertrophy and regenerative processes (Fry *et al.*, 2017), demonstrating that satellite cells play an important role in muscle maintenance and repair.

Multiple reviews have summarised the vast amount of research into the impact of ageing on skeletal muscle regeneration (Carosio *et al.*, 2011; Barberi *et al.*, 2013; Domingues-Faria *et al.*, 2016). Several factors contribute to an altered muscle regeneration during ageing. For example, the number of satellite cells is decreased, and satellite cells show altered proliferative potential (Lees, Rathbone and Booth, 2006; Shefer *et al.*, 2006). Other factors, such as telomere shortening in satellite cells (Decary *et al.*, 1997), delayed entrance of satellite cells into the proliferation phase (Barani *et al.*, 2003), reduced capillarization and increased distance between type II associated satellite cells and capillaries (Joanisse *et al.*, 2017), reduced innervations in senescent muscle (Larsson and Ansved, 1995), increased myonuclear apoptosis (Marzetti *et al.*, 2012), and increases in fibrotic tissue (O'Reilly, Tsou and Varga, 2024), are affected during the ageing process. Finally, alterations in the balance between the Notch and Wnt signaling pathways during ageing contribute to reduced skeletal muscle regeneration (Luo, Renault and Rando, 2005; Majchrzak *et al.*, 2024). In ageing, the reduction of the Notch signalling pathway decreases muscle regeneration (Conboy *et al.*, 2003), while the upregulation of the Wnt pathway is implicated in transferring cells to a fibrogenic state (Brack *et al.*, 2007), promoting the formation of fibrous tissue. Muscle regeneration and remodelling constitutes a complex, integrated system that is affected by ageing. To stimulate healthy ageing, it is essential to implement strategies that positively regulate muscle mass and muscle turnover.

1.2.4 Oxidation and Reactive Oxygen Species in ageing

Another factor contributing to the ageing process is through the accumulation of reactive oxygen species (ROS), which results in oxidative stress. ROS production in skeletal muscle is essential for various physiological processes, such as signal transduction in cell proliferation, the activation of antioxidant systems, and cellular apoptosis (Bou Saada *et al.*, 2017). However, an imbalance in ROS generation occurs with ageing (Maldonado *et al.*, 2023). A decline in ATP production in ageing is associated with an increase in mitochondrial ROS levels, leading to oxidative stress and causing significant damage to cellular components, including membranes, DNA, and proteins; hereby contributing to the ageing phenotype (Maldonado *et al.*, 2023). While this suggests that older individuals exhibit elevated ROS levels compared to their younger counterparts, a study in young and older trained and untrained individuals showed that markers related to mitochondrial ATP production rates differ between age groups in the

untrained individuals, whereas no differences were observed between trained young and trained older adults (Lanza *et al*., 2008). This suggests that physical activity (or a lack thereof) plays a more significant role in shaping the ageing phenotype. Within muscle cells specifically, the increased ROS observed in ageing can contribute to dysregulation of signalling pathways and oxidative damage to mitochondria, proteins, lipids, RNA, and DNA, playing a crucial role in the development of muscle atrophy and sarcopenia (Jackson *et al.*, 2022). This cascade of events underscores the importance of ROS in age-related muscle atrophy.

1.3: Overview n-3 PUFA

1.3.1 Structure and sources

Omega-3 fatty acids (n-3 PUFA) are a group of long-chain fatty acids with > 12 carbon atoms and ≥ 2 double bonds (Yaqoob, 2009). The first double bond starts on the position of the 3rd carbon atom counting the methyl carbon as number 1, hence the name omega-3 polyunsaturated fatty acid (Calder, 2012). The most studied omega-3 fatty acids with regards to health are eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), which are generally believed to be the most biologically active fatty acids (Calder, 2018), although more evidence is emerging for a beneficial role of DPA (Kaur *et al.*, 2011). EPA, DPA and DHA are marine derived n-3 PUFA, often referred to as *very long*-chain n-3 PUFA, because of their extended hydrocarbon chain (≥ 20 carbon atoms) (Calder, 2018). In contrast, plant-derived n-3 PUFA, such as ALA, have hydrocarbon chains consisting of 18-carbon atoms and could therefore be considered *short-chain* n-3 PUFA. EPA and DHA are primarily found in seafood, particularly in oily fish such as mackerel, sardines, salmon, trout, and herring (National Institutes of Health, 2023). The current recommendation of consuming EPA and DHA are 250 – 500mg EPA + DHA per day (European Food Safety Authority (EFSA), 2010), or 1 portion of oily fish per week (NHS, 2022). The Scientific Advisory Committee on Nutrition (SACN), an independent advisory committee in the UK, issued a report in 2004 recommending eating two portions of fish per week, including one portion of oily fish, to achieve an intake of approximately 450 mg/day of n-3 PUFA (Scientific Advisory Committee on Nutrition (SACN) and Committee on Toxicity (COT), 2004). Additionally, the report concluded that the average weekly consumption of oily fish in the UK population is 50 grams. However, among individuals who consume fish, the average intake increases to 194 g oily fish per week (Scientific Advisory Committee on Nutrition (SACN) and Committee on Toxicity (COT), 2004). This indicates a skewed distribution of fish consumption driven by a subset of

the populations consuming high amounts of fish. Furthermore, eating fish is not always sustainable or manageable, so the consumption of algae or microalgae could be an alternative source for consumption of EPA and DHA (Rizzo, Baroni and Lombardo, 2023). Plant-based sources of n-3 PUFA are green plant tissues, walnuts, rapeseed oil or soybean oil. These contain the fatty acid α-linolenic acid (ALA; 18:3 *n*-3), a pre-cursor for EPA and DHA (Baker *et al.*, 2016). Another option is echium oil which contains stearidonic acid (SDA; 18:4 *n*-3), which seem to be more readily converted to EPA and DHA than ALA (Kuhnt *et al.*, 2016; Greupner *et al.*, 2019). However, conversion of both ALA and SDA is limited in humans (Baker *et al.*, 2016). It is therefore most effective to consume EPA and DHA directly. An overview of n-3 PUFA sources and structure is visualised in **Figure 1.2**.

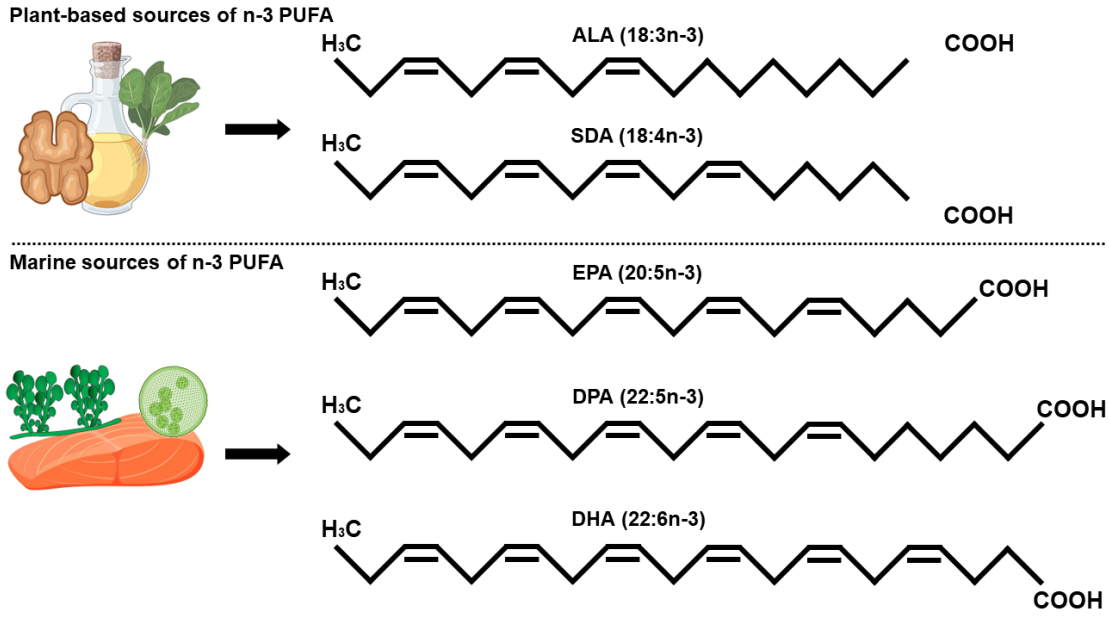

Figure 1.2: Structure and sources of polyunsaturated omega-3 fatty acids (n-3 PUFA). ALA = alpha-linolenic acid, SDA = stearidonic acid, EPA = eicosapentaenoic acid, DPA = docosapentaenoic acid, DHA = docosahexaenoic acid.

1.3.2 Absorption and metabolism

Before n-3 PUFA can affect cellular metabolism, they must be ingested, absorbed, digested and transported to target tissues. Absorption of fatty acids occurs mainly in the jejunum (Burdge and Calder, 2015). Due to their lipophilic and aquaphobic structure, n-3 PUFA cannot transport through the blood freely and thus are transported bound to albumin or via lipoproteins. There are several lipoproteins, which are classified depending on their size and lipid and protein composition into chylomicrons, Very Low Density Lipoproteins, (VLDL), Low Density

Lipoproteins (LDL) and High Density Lipoproteins (HDL) (Tulenko and Sumner, 2002). The distribution and ratio of these lipoproteins is an indicator for health. For example, high levels of LDL are associated with onset of atherosclerosis (Williams and Tabas, 1995), whereas higher levels of HDL are associated with a decreased risk of atherosclerosis (Gordon *et al.*, 2011). Although the ratio of lipoproteins may be partly based on genetic factors that result in a atherogenic lipoprotein phenotype (Austin, 2000), diet significantly influences lipoprotein composition and distribution (Feingold, 2000; Ooi *et al.*, 2015). The ingestion of n-3 PUFA is associated with a decreased risk of atherosclerosis (Yagi *et al.*, 2017). Once transported by lipoproteins to the target tissue of interest, n-3 PUFA then are taken up by the cell. This is a tightly regulated process, where a number of transporter proteins (CD36/FATP/FABPpm) and cytosolic proteins (FABPc) regulate uptake and subcellular localisation of fatty acids, allowing for them to be stored or metabolised effectively (Glatz, Luiken and Bonen, 2010). In the cell several intracellular fates are possible; n-3 PUFA can 1) be incorporated into the phospholipid bilayer of the cell membranes of the specific tissue of interest, 2) be oxidized for energy supplementation, or 3) stored (mainly in adipose tissue) to use for energy later (Burdge and Calder, 2015).

1.3.3 Metabolic pathway

As briefly described in section 1.3.1, n-3 PUFA undergo interconversion among each other. The conversion of ALA to EPA and DHA occurs primarily in the endoplasmic reticulum and is visualised in **Figure 1.3** (adapted from Calder 2012; Metherel *et al.,* 2017). In brief, ALA is converted to SDA through Δ6-desaturase (FADS2), followed by elongation to eicosatetraenoic acid (ETA; 20:4n-3) via Elongase 5 (ELOVL5). ETA is desaturated to EPA by Δ5-desaturase (FADS1), and further elongated to DPA (22:5n-3) by ELOVL5 and Elongase 2 (ELOVL2). DPA undergoes subsequent elongation to tetracosapentaenoic acid (TPAn-3, 24:5n-3) via ELOVL2, another desaturation to tetracosahexaenoic acid (THAn-3, 24:6n-3) via FADS2, and ultimately converts towards DHA through peroxisomal β-oxidation. Early studies have indicated that the conversion of ALA towards DHA is inefficient (Burdge, Jones and Wootton, 2002; Goyens *et al.*, 2005; Hussein *et al.*, 2005). This was supported by a review (Arterburn, Hall and Oken, 2006) and systematic review (Wood *et al.*, 2015), which both concluded that supplementation with ALA results in elevated levels of EPA, but not DHA. However, Metherel *et al.,* (2017) suggest that the rates of conversion to DHA are not as low as previously thought. Combining data from studies using oral dosing isotope tracers and stable isotope infusions,

they argue that: 1) much of the labelled ALA used in oral dose isotope studies is either β-oxidized or absorbed by other tissues and, therefore, not present in plasma and 2) the metabolic conversion pathway to DHA involves several precursors beyond ALA. Moreover, while multiple studies have shown that supplementation with DHA increases content of EPA which was attributed to retro-conversion (Schlenk, Sand and Gellerman, 1969; Brossard *et al.*, 1996; Park *et al.*, 2016), recent tracer studies have suggested that the accumulated EPA is due to the fact that supplementation with DHA slows the conversion from EPA to DHA (Metherel *et al.*, 2017). Finally, the enzymes involved in elongation and desaturation of n-3 PUFA play key roles in n-3 PUFA conversion. For example, both ALA → SDA and TPA → THA utilise the enzyme FADS2 for desaturation. One study in yeast observed that high levels of ALA decreased levels of DHA due to competition for this enzyme (Gregory *et al.*, 2011). In addition, they identified the rate-limiting step in n-3 PUFA conversion as the elongation of DPA → TPA, rather than the elongation of EPA → DPA, while both conversion steps use the ELOVL2 substrate. Other factors such as age (Plourde *et al.*, 2011), sex (Rotarescu *et al.*, 2022) or diet (Gonzalez-Soto and Mutch, 2021), may also impact the metabolism and conversion of n-3 PUFA in addition to substrate availability.

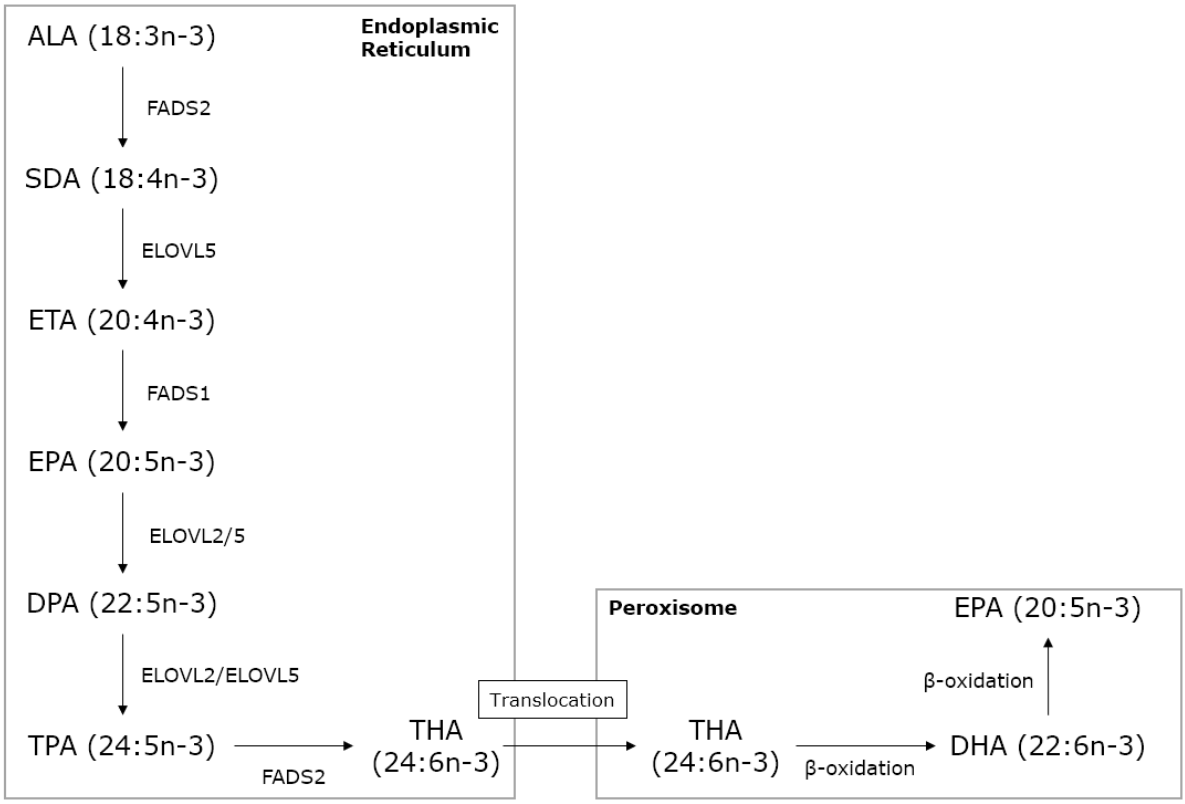

Figure 1.3: Metabolic conversion pathway of n-3 PUFA. ALA = alpha-linolenic acid, SDA = stearidonic acid, ETA = eicosatetraenoic acid, EPA = eicosapentaenoic acid, DPA = docosapentaenoic acid, TPA = tetracosapentaenoic acid, THA = tetracosahexaenoic acid, DHA = docosahexaenoic acid, FADS1 = Δ5-desaturase, FADS2 = Δ6-desaturase, ELOVL2 = elongase 2, ELOVL5 = elongase 5.

1.3.4 Eicosanoid and SPM production

The oxidation of polyunsaturated fatty acids results in the formation of oxylipins. Specifically, eicosanoids are oxylipins derived from 20-carbon PUFA, of which the n-6 PUFA arachidonic acid (ARA) and the n-3 PUFA EPA are the most extensively studied. Eicosanoids include prostaglandins and leukotrienes which play essential roles in inflammation, the immune response, and cellular signalling (Calder, 2017). While the inflammatory response is essential for wound healing and the immune response against infections, excessive or chronic inflammation is linked to adverse health outcomes (Calder, 2015; Serhan and Levy, 2018). The conversion of PUFA to eicosanoids occurs via 3 main enzymatic pathways: cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP) (Gabbs *et al.*, 2015). The consumption of ARA leads to production of pro-inflammatory eicosanoids, such as prostaglandin E2 (PGE_2) and leukotriene B4 (LTB_4) (Calder, 2017). Contrastingly, the consumption of EPA and DHA leads to secretion of specific oxylipins classed as specialised pro-resolving lipid mediators (SPM), which inhibit and decrease inflammation (Calder, 2020a).

The production of EPA and DHA derived SPM at the expense of ARA is well studied (Calder, 2020a). For example, 12 month supplementation with EPA and DHA decreased the n-3:n-6 ratio in several blood fractions (Walker *et al.*, 2015). Moreover, an *in vitro* study demonstrated that the enrichment of EPA and DHA led to decreased eicosanoids derived from ARA via the prostaglandin pathway (Wada *et al.*, 2007). EPA-derived eicosanoids are less potent than ARA-derived eicosanoids, and therefore EPA-derived eicosanoids are considered 'weaker'. For example, ARA derived eicosanoids LTB_4 and PGE_2 have been shown to bind with greater affinity to their specific receptors than EPA derived eicosanoids LTB_5 and PGE_3 (Goldman, Pickett and Goetzl, 1983; Lee *et al.*, 1984; Bagga *et al.*, 2003). Additionally, Wada *et al.,* (2007) demonstrated that prostaglandin receptors of eicosanoids showed less affinity for EPA derived mediators than ARA derived mediators (Wada *et al.*, 2007). In this study, PGE_3 had a 50 – 80% lower potency to bind to specific PGE receptors compared to PGE_2.

SPM secreted by n-3 PUFA can be categorised into resolvins, protectins and maresins. Resolvins produced from EPA are called E-series resolvins and those derived from DHA are D-series resolvins. Protectins, also known as neuroprotectins, are derived in neural tissue from DHA, and maresins are another class of SPM derived from DHA (Serhan, Chiang and Dalli, 2018; Calder, 2020a). The synthesis of SPM involves the COX and LOX conversion pathways, and they mediate their effects via binding to G-protein coupled receptors (GPR) on the phospholipid membrane. Specifically, GPR120 has been identified as a n-3 PUFA-specific receptor in mouse macrophages and adipocytes (Oh *et al.*, 2010). Since SPM play an important role in resolving inflammation, their presence in tissues can be an indicator for health status. The concentration of SPM in human tissues varies widely, with large differences observed between studies and across blood fractions (Calder, 2020a). It is reasonable to assume that the consumption of EPA and DHA increases the concentration of SPM. A study found that plasma concentrations of n-3 derived oxylipins, including SPM-precursors, increased linearly with an increased intake of EPA and DHA, highlighting a dose-dependent response (Ostermann *et al.*, 2019). Another study demonstrated the presence of resolvins in plasma and serum following n-3 PUFA supplementation (Mas *et al.*, 2012). Based on these findings, it is plausible that the production of SPM also occurs in muscle. It has been suggested that SPM can help mitigate skeletal muscle catabolism during critical care (Blaauw *et al.*, 2024). However, further studies are needed to explore the role of SPM in muscle turnover. A schematic overview of eicosanoids and SPM production can be found in **Figure 1.4**.

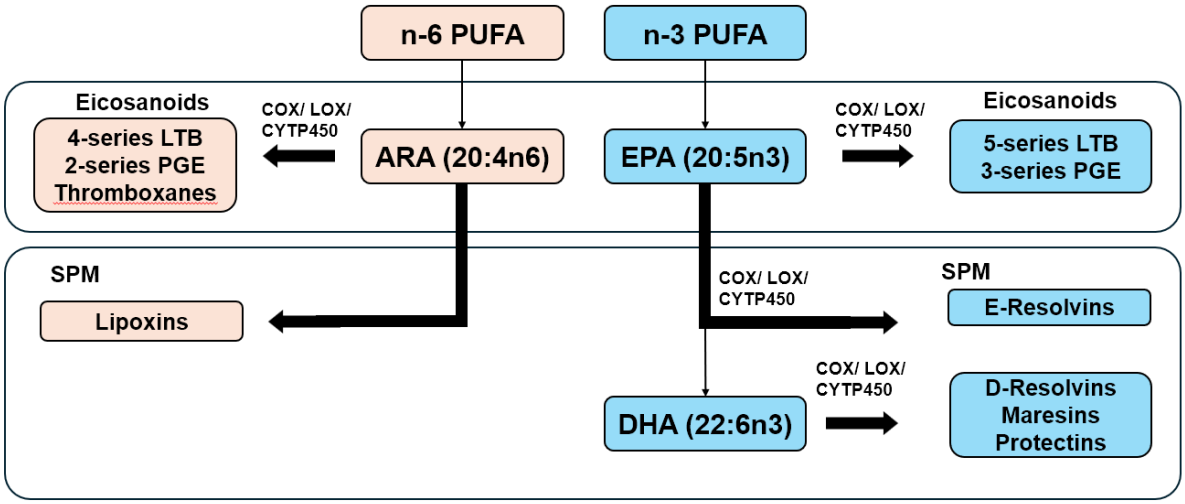

Figure 1.4: Schematic overview of eicosanoid and specialised pro-resolving mediator (SPM) production. ARA = arachidonic acid, EPA = eicosapentaenoic acid, DHA = docosahexaenoic acid, COX = cyclooxygenase, LOX = lipoxygenase, PYTP450 = cytochrome P450, LTB = Leukotrienes, PGE = Prostaglandins.

1.4: n-3 PUFA incorporation and washout

1.4.1 Incorporation of n-3 PUFA in blood, skeletal muscle, and adipose tissue.

Membranes composed of phospholipids with fatty acids without any double bonds have tightly packed chains, which reduces fluidity and increases rigidity (Holte *et al.*, 1995). Alterations in membrane structure can influence the fluidity and thereby the activity of membrane-associated proteins and cell signalling. Due to their unsaturated nature, n-3 PUFA increase the fluidity of the membrane. For example, adding EPA or DHA to palmitate treated cells increased fluidity of the cellular membrane in C2C12 cells, which was accompanied with increased glucose uptake and β-oxidation (Pinel *et al.*, 2016). Furthermore, incorporation of n-3 PUFA into phospholipid membranes is dose and time dependent. Using an animal model of tumour-bearing mice, it was demonstrated that the relative EPA and DHA content of a controlled diet was directly proportional to the subsequent uptake and incorporation of EPA and DHA into both plasma and skeletal muscle membranes following a 3-week experimental period (Dijk *et al.*, 2019). Similar findings were reported in human studies where the provision of a fish oil containing medical food formulation over 7 days resulted in an increased percent incorporation of EPA into the phospholipid membrane of white blood cells in healthy Caucasian adults (Faber *et al.*, 2011). However, the time-course of changes in n-3 PUFA incorporation appears to differ between blood, skeletal muscle tissue, and adipose tissue. With regards to skeletal muscle incorporation, McGlory *et al*., (2014) demonstrated that ≥2 weeks of n-3 PUFA intake was required to increase the EPA + DHA content of skeletal muscle in healthy young adults, whereas the incorporation of EPA and DHA into whole blood was increased after only 1 week of fish oil supplementation. In this study, a ~2-fold change in proportion of n-3 PUFA incorporated into the skeletal muscle cell was observed after 4 weeks of n-3 PUFA, although no plateau in incorporation was reached. Interestingly, a study in healthy females that administered 5 g/day of fish oil (3.50 g/d EPA, 0.90 g/d DHA) over an extended 8-week time course reported a plateau in muscle phospholipid n-3 PUFA incorporation after 6 weeks of fish oil supplementation (McGlory *et al.*, 2019). Moreover, other studies that provided lower doses of n-3 PUFA over an extended timeframe reported similar proportional increases in n-3 PUFA content at the end of an 8-week (Smith *et al.*, 2011a), and 12-week (Gerling *et al.*, 2019) supplementation period. In contrast, only 6 days of high dose n-3 PUFA ingestion resulted in increased muscle tissue n-3 PUFA content (Wardle *et al.*, 2020). An overview of significant changes in n-3 PUFA fatty acid composition in muscle in humans following different dose and time courses is provided in **Table 1.1**. In comparison to skeletal muscle, adipose tissue has an

ever slower turnover rate and has been suggested as a good marker for long-term habitual n-3 PUFA intake (Hodson, Skeaff and Fielding, 2008). An early study done by Katan *et al.,* (1997) showed that the half-life of incorporation of EPA and DHA into adipose tissue was over a year, and that this was dose dependent. Furthermore, in another study, in response to doses representing 1, 2, or 4 portions of oily fish per week, adipose tissue showed the lowest proportional increases in response to the different n-3 PUFA intakes (Browning *et al.*, 2012) compared to other tissue pools. From these studies it can be concluded that the duration and dose of n-3 PUFA supplementation modulate the magnitude of n-3 PUFA incorporation into blood, skeletal muscle, and adipose tissue, where the rate and content of incorporation varies between the tissues.

Table 1.1: Muscle incorporation of total n-3 PUFA of different studies

	Study population	Length and daily dose	Fraction	n-3 pre	n-3 post	Fold change
McGlory 2014	Young men	4wk 3.5g EPA + 0.9g DHA	WM	3.8 ± 0.22	6.79 ± 0.46	1.79
McGlory 2019	Young women	6wk 2.79g EPA + 2.03g DHA	WM	1848 ± 159*	5827 ± 424*	3.15
McGlory 2019	Young women	8wk 2.79g EPA + 2.03g DHA	WM	1848 ± 159*	5640 ± 625*	3.05
Smith 2011a	Young and middle-aged adults	8wk 1.86g EPA + 1.5g DHA	PL	4.38 ± 0.33	8.93 ± 0.37	2.04
Smith 2011b	Older adults	8wk 1.86g EPA + 1.5g DHA	PL	5.04 ± 0.45	9.03 ± 0.95	1.79
Gerling 2019	Young men	12wk 2g EPA + 1g DHA	WM	3.3 ± 0.2	8.3 ± 0.6	2.52
Wardle 2020	Young men	6d high fat diet with 10% n-3 PUFA	WM	5.73 ± 1.52	7.79 ± 3.22	1.36

EPA = eicosapentaenoic acid, DHA = docosahexaenoic acid, WM = whole muscle, PL = phospholipid membrane of skeletal muscle, n-3 = total omega-3 polyunsaturated fatty acids. *nM/g dry mass

1.4.2 EPA vs. DHA

Evidence from human studies suggests that EPA exhibits a faster rate of tissue incorporation compared to DHA. Accordingly, several studies (Browning *et al.*, 2012; Herbst *et al.*, 2014; Wardle *et al.*, 2020) have reported a more rapid uptake of EPA than DHA in blood. The differential incorporation profile of EPA and DHA in blood appears to follow a similar pattern in skeletal muscle (McGlory *et al.*, 2014), and adipose tissue (Katan *et al.*, 1997; Browning *et al.*, 2012) and could be attributed to a preferential incorporation of distinct n-3 PUFA species into different phospholipid fractions. For instance, EPA has been shown to be preferentially incorporated into phosphatidylcholine (PC) species of erythrocytes that are primarily located on the outer membrane, whereas DHA is preferentially incorporated into phosphatidylethanolamine (PE) species that are located on the inner membrane and requires

DHA to be transported through the cellular membrane for incorporation (Pal *et al.*, 2020). Although no confirmatory data exist, this has been proposed to underpin the consistent observation of slower incorporation rates of DHA *vs.* EPA into the phospholipid membrane (Pal *et al.*, 2020). Furthermore, phospholipid membranes consist of multiple domains. Lipid rafts are microdomains within the membrane that compartmentalise cellular processes and are platforms for facilitating cellular signalling and communication (Yaqoob and Shaikh, 2010). Studies investigating lipid raft modulation have found that EPA, DHA and DPA interact with lipid rafts differently (Williams *et al.*, 2012; Leng *et al.*, 2018). The differential incorporation patterns and interactions with membrane sections could explain the varying effects of different n-3 PUFA species on health outcomes.

1.4.4 Washout

The washout rate of n-3 PUFA from tissues is important for the maintenance of cellular physiological function within the phospholipid membrane and crucial for establishing crossover designs in future studies. Multiple human studies have investigated washout patterns of n-3 PUFA in blood fractions (Marangoni *et al.*, 1993; Prisco *et al.*, 1995; Katan *et al.*, 1997; Zuijdgeest-Van Leeuwen *et al.*, 1999; Di Stasi *et al.*, 2004; Cao *et al.*, 2006; Metherel *et al.*, 2009). One study observed that the washout of n-3 PUFA returned to baseline in platelets after three months of washout (Prisco *et al.*, 1995). In subsequent research, Cao *et al.,* (2006) observed that erythrocyte membrane n-3 PUFA remained higher than baseline 4 weeks after cessation of supplementation and only returned to baseline after 4 months of washout. When comparing washout rates between EPA and DHA, Studies have consistently revealed a more rapid washout of EPA compared to DHA in blood (Marangoni *et al.*, 1993; Katan *et al.*, 1997; Zuijdgeest-Van Leeuwen *et al.*, 1999; Cao *et al.*, 2006; Metherel *et al.*, 2009). In contrast to erythrocytes, washout kinetics in adipose tissue are remarkably slow. In gluteal adipose tissue, a 50% decline in EPA and 45% decline in DHA from peak levels were observed only after 6 months of cessation of n-3 PUFA (Katan *et al.*, 1997). Nevertheless, the washout kinetics of n-3 PUFA from skeletal muscle have yet to be characterised. Based on findings from McGlory *et al.,* (2014), which demonstrated slower incorporation in muscle compared to blood, it can be speculated that similar washout patterns may be observed for n-3 PUFA washout from muscle. However, research is necessary to confirm these speculations.

1.5: The impact of n-3 PUFA on skeletal muscle regulation

1.5.1 Overview of studies on n-3 PUFA and muscle protein turnover

Multiple studies investigating the impact of n-3 PUFA on muscle protein turnover suggest a beneficial role for n-3 PUFA in enhancing rates of MPS. The potential underlying mechanisms include impacting the anabolic mTORC1 signalling pathway and catabolic ubiquitin proteasome pathway. An early study examined the effects of n-3 PUFA on MPS in growing steers fed menhaden oil, which contained 13.5% EPA and 14.4% DHA (Gingras *et al.*, 2007). Here, they observed enhanced rates of MPS following n-3 PUFA supplementation, accompanied by an increase in the phosphorylation p70S6K1 and Akt, while mTOR and 4E-BP1 tended to increase. Building on this, a human study demonstrated that 8 weeks of 4g n-3 PUFA per day increased rates of MPS in response to a hyperaminoacidemic-hyperinsulinemic clamp in younger (Smith *et al.*, 2011b), and older (Smith *et al.*, 2011a) adults. These increases in MPS were accompanied by increases in the phosphorylation in mTORC1 and p70S6K1, with no effect on Akt. However, increases in rates of MPS were not observed in the basal state, suggesting that an anabolic stimulus is needed to observe the beneficial effects of n-3 PUFA on MPS. However, it is important to note that the studies conducted by Smith *et al.* (2011a, 2011b) utilised a hyperaminoacidemic-hyperinsulinemic clamp, reducing their physiological relevance and applicability. In our lab, McGlory *et al.,* (2014) observed that 4 weeks of 5g n- PUFA per day increased the MPS regulator focal adhesion kinase (FAK) content and mTORC1 content, with no impact on p70S6K1 and 4E-BP1 (McGlory *et al.*, 2014). In a follow-up study, resistance trained young men were supplemented with the same dose (5g/day) n-3 PUFA for 8 weeks, and rates of MPS were measured in a basal state and after a 30g whey protein bolus with and without RE (McGlory *et al.*, 2016). Contrasting to the results observed by Smith *et al.*, no additional benefit from n-3 PUFA supplementation on MPS was observed in any condition measured (basal, fed, fed + RE). Nevertheless, n-3 PUFA was found to suppress the activity of Akt at rest and the activity of p70S6K1 3h post-exercise and feeding, leading the authors to conclude that n-3 PUFA alters the kinase signalling response. Supporting this, Dalle *et al.*, (2021) observed after that 12 weeks of supplementation with 3g n-3 PUFA per day, older adults showed a tendency toward reduced Akt activation in response to feeding compared to controls, with no effect mTOR or p70S6K1. However, due to the lack of statistical significance and small sample size (n=23), definitive conclusions cannot be drawn. Moreover, Lalia *et al.*,

(2017) reported that 16 weeks of 3.9g n-3 PUFA per day intake increased basal and post-exercise MPS rates in older adults. Notably, the increases in basal rates of MPS observed after n-3 PUFA supplementation were not reported in the studies mentioned previously by Smith *et al.,* (2011a, 2011b) and McGlory *et al.,* (2016). Additionally, data values for MPS in this study were remarkably high (i.e. about 2-fold greater than values in older adults presented by Smith *et al.,* (2011b) and the study design did not include a control group. Additionally, Lalia *et al.,* (2017) reported several genes related to MPB and atrophy which were downregulated post-exercise, whereas genes related to MPS were upregulated. Similarly, a study in older females found that 6 weeks of 3.7g n-3 PUFA per day + single leg RE tended to increase rates of MPS after an acute exercise stimulus (Brook *et al.*, 2021). Here, they also observed an increase in 4E-BP1 expression after acute RE in the n-3 PUFA group compared to control. Contrastingly, Murphy *et al.,* (2021) did not observe an added increase in MPS after 24 weeks of supplementation with a leucine-protein drink + 4g n-3 PUFA in older adults (Murphy *et al.*, 2021).

In addition to impacting the mTORC pathway, n-3 PUFA may alter muscle atrophy. One study involving twenty young women where one leg was immobilised in a cast for two weeks, found that 8 weeks of 5g n-3 PUFA per day increased MPS compared to the control group during the 2 weeks immobilisation period and after 2 weeks of remobilisation with RT, indicating a protective effect of n-3 PUFA during muscle atrophy (McGlory *et al.*, 2019). Additionally, the gene expression of ATF4, a transcription factor involved in protein turnover, was significantly greater in the n-3 PUFA group compared to control, while the expression of amino acid transporter LAT1 tended to be greater in the n-3 PUFA group compared to control (McGlory *et al.*, 2019). Furthermore, while the expression of ubiquitin ligase MuRF1 increased in both groups during immobilisation, this increase was less pronounced in the n-3 PUFA group (McGlory *et al.*, 2019). However, much more remains to be understood about the effects of n-3 PUFA on muscle atrophy, as a single study – particularly one with a relatively small sample size – is insufficient to draw definitive conclusions. Nevertheless, these studies demonstrate that there is a role for n-3 PUFA in muscle protein turnover. It is important to note that the studies described above utilised relatively high doses of fish oil, ranging from 3-5g of n-3 PUFA per day, exceeding the recommended daily intake (SACN). Furthermore, differences in study design, duration, dosage, and intervention may limit the applicability of these findings to daily life. An overview of studies investigating the impact of n-3 PUFA supplementation on skeletal muscle metabolism in humans is presented in **Table 1.2**.

Table 1.2: Summary of studies investigating the impact of n-3 PUFA on skeletal muscle metabolism and associated proteins

Study	Participants	Supplementation protocol	Impact on MPS	Impact on associated signalling proteins
Smith 2011	Older adults	8 wk of 1.86g EPA+1.50g DHA/ day or placebo	Increase in MPS under simulated fed conditions.	Increase in phosphorylation of mTOR, p70S6K1 under simulated fed conditions. No effect on Akt.
Smith 2011	Young and middle aged adults	8 wk of 1.86g EPA+1.50g DHA/ day or placebo	Increase in MPS under simulated fed conditions.	Increase in phosphorylation of mTOR, p70S6K1 under simulated fed conditions. No effect on Akt.
McGlory 2014	Young men	4 wk of 3.5g EPA + 0.9g DHA/ day	N/A	Increase in total FAK content (0-4w) and mTOR content (0-2w). No effect on p70S6K1 and 4E-BP1.
McGlory 2016	RT trained young men	8 wk of 3.5g EPA + 0.9g DHA/ day or placebo	No effect on MPS under basal conditions or after 30g whey protein with or without RE.	Compared to control, decrease in Akt at rest, decreased p70S6K1 after feeding + exercise.
Dalle 2020	Older adults	12wk FO (3g/day) or CO (placebo) + RT program	N/A	Tendency for less increase in Akt fasted - fed after n-3 PUFA supplementation. No effect on mTOR and p70S6K1.
Lalia 2017	Older adults	16 wk n-3 PUFA (3.9 g/d)	Increase in MPS under basal conditions and post RT.	After RE decrease of genes related to MPB and atrophy, increase of genes related to MPS.
Brook 2021	Older women	6 wk FO (3.7 g/d) or placebo (3.7g/d) + single leg RT	A trend for increases in MPS between 4-6wk after acute RE.	Increased 4E-BP1 content after acute RE in trained leg after fish oil compared to placebo.
Murphy 2021	Older adults	24 wk LEU-PRO, LEU-PRO+ 4g FO or placebo (CHO)	No effect on MPS.	N/A
McGlory 2019	Young women	8 wk FO (3.0 g EPA + 2.0 g/day DHA) or placebo	Increased basal MPS *vs.* control after 2wk immobilisation and 2wk of RT-induced remobilisation.	Increase in ATF and tendency for increase in LAT1 expression in n-3 group *vs.* control, impaired increase in MuRF1 in n-3 group *vs.* control.

1.5.2 The impact of n-3 PUFA on gene expression in skeletal muscle

In addition to signalling protein activity, n-3 PUFA can alter the skeletal muscle transcriptome. It has been well established that n-3 PUFA alter the NF-κB/PPARγ pathway, which are crucial transcription factors in regulating inflammation (Kliewer *et al.*, 1997; Calder, 2015). N-3 PUFA activate the expression PPARγ, which prevents the translocation of NF-κB to the nucleus. This inhibition of NF-κB translocation to the nucleus not only suppresses inflammation, but also prevents the activation of genes associated with MPB, such as MuRF1. In C2C12 myotubes, EPA inhibited the IκBα/NF-κB/MuRF1 pathway in a PPARγ dependent manner (Huang *et al.*, 2011). Moreover, animal studies have observed that n-3 PUFA supplementation resulted in decreased expression of muscle wasting genes atrogin-1 and MuRF1 in arthritic rats (Castillero *et al.*, 2009), affected genes related to muscle biology, muscle maintenance and muscle function in bovines (Hiller *et al.*, 2012), and decreased expression of MAFbx, MuRF1 and COX2 in immobilised rats, which was associated with preservation of muscle mass (You, Park and Lee, 2010). Despite the observed changes in the expression of muscle wasting genes in animal studies, further research is needed to understand how n-3 PUFA alter the human skeletal muscle transcriptome. It is important to consider species differences, as variations in physiology, metabolism, and response to n-3 PUFA supplementation may influence the outcomes and limit the direct applicability of findings to humans. Two human studies have performed whole genome analyses in skeletal muscle to investigate transcriptomic changes in response to n-3 PUFA supplementation. The first study used a subset of skeletal muscle biopsies from Smith *et al.*, (2015), where increases in grip strength and lean mass were observed in response to 6 months of n-3 PUFA supplementation in older adults. Analysis of the muscle transcriptome revealed small but coordinated transcriptomic changes in response to n-3 PUFA supplementation. Here, transcriptomic pathways related to mitochondrial function and extracellular matrix organisation increased, while expression in pathways related to calpain- and ubiquitin-mediated proteolysis and mTOR-inhibitor pathways decreased. However, these changes in gene expression were not significant at the level of individual genes. Furthermore, gene expression can be influenced by various factors, including diet and physical activity – variables that were not objectively measured in this study and may therefore act as confounding factors. Another study in older adults by Lalia *et al.*, (2017) demonstrated that four months of n-3 PUFA supplementation decreased genes related to oxidative phosphorylation and the TCA cycle in older adults compared to young adults. On an individual gene level, they identified genes that were uniquely

altered with exercise in response to n-3 PUFA supplementation. Here, several negative regulators of muscle growth and proliferation were downregulated, one atrophy inducing gene was downregulated, while genes promoting protein synthesis were upregulated. These changes in gene expression also resulted in increased protein synthesis, suggesting that alterations in protein synthesis are partly regulated on a transcriptomic level (Lalia *et al.*, 2017). An effect for n-3 PUFA in altering muscle transcriptome is indicated, but further research is needed to confirm these findings.

1.5.3 EPA vs DHA in skeletal muscle regulation

EPA and DHA exhibit different roles in the regulation of muscle protein turnover. It is generally thought that the anabolic action of n-3 PUFA is primarily ascribed to EPA, supported by *in vitro* studies conducted in C2C12 myotubes. First, Kamolrat *et al*., (2013) reported a 25% increase in MPS rates in the EPA condition *vs*. control cells, with no effect of DHA on MPS. Furthermore, MPB was 22% lower after incubation with EPA compared to control, with no changes in MPB in the DHA condition. This was accompanied with an increased phosphorylation of p70S6K1, with no significant effect on 4E-BP1 or Akt. Next, in our labs it was demonstrated that EPA treatment enhanced protein accretion via a suppression of MPB, with no effects of DHA on MPS or MPB (Jeromson *et al.*, 2018). Here, there were no differences observed in the phosphorylation status of p70S6K1 or 4E-BP1. Furthermore, EPA was shown to be effective in cachectic patients by downregulating proteolysis systems (Whitehouse and Tisdale, 2001). However, contrastingly to these results, Wang *et al.,* (2013) reported a greater attenuation of protein degradation with DHA compared to EPA in C2C12 myotubes, which was mediated via the upregulation of the PPARγ/NF-κB pathway. However, *in vitro* conditions do not account for the complexities and dynamic metabolic processed present in *in vivo* studies. Recently, a human study conducted in young males reported that either EPA or DHA alone, but not combined, attenuates exercise induced muscle damage (Heileson *et al.*, 2023). However, changes in muscle fatty acid composition or mechanistic underpinnings were not determined, as noted by the authors, meaning these changes are only observed at a level that is not fully conclusive. Nevertheless, from the studies above it appears that there are distinct roles for EPA and DHA in skeletal muscle regulation, and that not only dose, but also ratio may impact outcomes on health.

1.6 Limitations of existing research

There is considerable evidence that the incorporation of n-3 PUFA into cellular phospholipid membranes plays a beneficial role in skeletal muscle health by influencing health outcomes through several mechanisms, including the modulation of muscle protein turnover and affecting the activity and expression of signalling kinases and genes. However, as outlined in the previous paragraphs, research on these outcomes remains inconclusive. This disparity in outcomes is attributed to variations in study populations, as well as differences in dose and duration. A recent review critically evaluated the trial designs concerning n-3 PUFA supplementation and identified several methodological considerations that should be addressed when designing n-3 PUFA intervention studies (Anthony *et al.*, 2024). One key point was the need to thoroughly assess the dose and duration of the study design to ensure significant changes in phospholipid membranes leading to desirable outcomes on health markers. However, the relationship between dose, phospholipid membrane changes and functional outcomes is poorly understood. Most studies use high doses ≥3g n-3 PUFA daily to induce changes in membrane fatty acid composition (Metherel *et al.*, 2009; Smith *et al.*, 2011a, 2011b, 2015; McGlory *et al.*, 2014; Lalia *et al.*, 2017; McGlory *et al.*, 2019; Brook *et al.*, 2021; Murphy *et al.*, 2021). However, higher doses are not always feasible or sustainable due to potential side effects, such as foul breath or nausea (Borja-Hart and Marino, 2010; Schmidt *et al.*, 2020). Therefore, while studies using high doses serve as a proof of concept, more investigation is needed to explore whether lower doses can induce similar changes in membrane composition and subsequent functional outcomes. An initial higher loading dose followed by a lower maintenance could be a desirable strategy. Hence, it is important to investigate whether an initial high loading dose can be effectively followed by a lower maintenance dose while maintaining the initial changes in phospholipid membrane n-3 PUFA composition. Moreover, the skeletal muscle phenotype and genotype of older adults differs from those of young adults. Phospholipid membrane changes and subsequent outcomes in younger adults may not necessarily translate to the beneficial effects observed in older adults, which may account for some of the inconsistent findings regarding the effects of n-3 PUFA on health. Furthermore, washout is an important concept for both frequency of ingestion and designing cross-over designs. However, no studies have compared young and older individuals directly in terms of incorporation or examined the washout of n-3 PUFA from skeletal muscle tissue.

1.7 Aim and objectives of thesis

The overall aim of this thesis is to gain insight into the incorporation, washout, and underlying mechanisms of n-3 PUFA following different doses in young and older adults, with a focus on muscle and ageing. To investigate this, two lines of work are introduced: an *in vitro* study in C2C12 myotubes followed by a human trial in young and older adults. More specifically, the following aims will be addressed in the thesis in the following order:

Chapter 2:

1. To study the incorporation and washout responses of EPA and DHA over time in response to low and high concentrations of a combined EPA:DHA mixture in cell lysates and phospholipid membranes of C2C12 myotubes.
2. To measure how rates of MPS and associated mTORC1 signalling pathway proteins are impacted by incorporation and washout patterns of low and high concentrations of a combined EPA:DHA mixture.

Chapter 3:

3. To study the incorporation and washout responses of EPA + DHA in erythrocytes, skeletal muscle phospholipid membranes, and adipose tissue in response to two different dosing strategies in young and older adults.
4. To determine if a loading dose results in earlier higher incorporation of EPA + DHA in the aforementioned tissues, and whether this can be sustained by a maintenance dose.

Chapter 4:

5. To identify global changes in the skeletal muscle transcriptome in response to n-3 PUFA supplementation in young and older adults.
6. To investigate potential differences in the skeletal muscle transcriptome in response to n-3 PUFA supplementation between young and older adults.

Chapter 2: Dynamics of EPA and DHA Uptake, Washout, and Protein Synthesis in C2C12 Myotubes

2.1 Introduction

Age-related declines in skeletal muscle mass, starting from the fifth decade (Janssen *et al.*, 2000), have a detrimental effect on health. The underlying mechanisms are yet to be elucidated, but blunted stimulation of muscle protein synthesis (MPS) rates in response to anabolic stimuli is a contributing factor (Cuthbertson *et al.*, 2005). Emerging evidence highlights a therapeutic role for polyunsaturated omega-3 fatty acids (n-3 PUFA) in regulating skeletal muscle protein turnover. The first study demonstrating effects of n-3 PUFA on MPS showed an increased phosphorylation of p70S6K1$^{[Thr389]}$ and Akt$^{[Ser473]}$ in growing steers (Gingras *et al.*, 2007), two signalling kinases crucial in muscle protein turnover. In humans, n-3 PUFA have shown to; 1) enhance rates of MPS after a hyperinsulinemic/hyperaminoacidemic clamp in older and younger adults (Smith *et al.*, 2011a, 2011b), 2) increase rates of MPS accompanied by an upregulation of 4E-BP1$^{[Thr37/46]}$ in older women (Brook *et al.*, 2021), and 3) protect muscle mass during 2 weeks of leg immobilisation followed by 2 weeks of recovery in young women (McGlory *et al.*, 2019).

The n-3 PUFA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are widely recognised as the most biologically active forms but may exhibit different effects on muscle protein turnover. Incubating C2C12 myotubes with EPA resulted in a notable stimulation of protein synthesis, whereas DHA did not. However, both EPA and DHA increased the phosphorylation of p70S6K1$^{[Thr389]}$ (Kamolrat and Gray, 2013). Another study reported a more pronounced reduction in protein degradation with DHA compared to EPA in C2C12 myotubes, which was modulated via the upregulation of the PPARγ/NF-κB pathway (Wang *et al.*, 2013). These findings elucidate potential distinct roles for EPA and DHA on muscle protein turnover.

The biological actions of EPA and DHA on skeletal muscle metabolism are primarily mediated by their incorporation into the cellular membranes of skeletal muscle cells where they alter cell membrane phospholipid composition (Calder, 2012). The incorporation of EPA and DHA into skeletal muscle cells is time and concentration dependent,(McGlory *et al.*, 2014), with the rate of incorporation being influenced by the specific cellular fraction assessed. For example, increased levels of EPA and DHA were detected in mitochondrial membranes of skeletal

muscle after 12 weeks of fish oil supplementation (Herbst *et al.*, 2014; Gerling *et al.*, 2019), whereas only DHA was detected in the sarcolemmal membrane of skeletal muscle cells (Gerling *et al.*, 2019). Furthermore, higher doses of n-3 PUFA over a 4-week period induced similar incorporation increases compared to lower doses over extended periods in humans (Smith *et al.*, 2011a; Herbst *et al.*, 2014; McGlory *et al.*, 2014). In contrast, only 6 days of high dose n-3 PUFA ingestion led to an increased content in n-3 PUFA in the phospholipid membrane composition in muscle tissue (Wardle *et al.*, 2020). These data collectively suggest that higher doses lead to an earlier, stronger incorporation. In addition, *in vitro* studies have assessed membrane profile changes in response to EPA and DHA incubation. For example, 20 μM EPA or DHA modified membrane lipid composition during 2-4 day differentiation in L6 skeletal muscle cells (Briolay *et al.*, 2013). Likewise, incubating C2C12 myotubes for 72 h with 50 μM EPA or 50 μM DHA substantially altered membrane composition, while incubating with EPA also increased docosapentaenoic acid (DPA) content, accompanied by increased protein accretion (Jeromson *et al.*, 2018).

It is conceivable that manipulating the dose and duration of n-3 PUFA modulates the biological fate of EPA and DHA, particularly with regards to the incorporation of n-3 PUFA into the phospholipid membranes of skeletal muscle. However, the washout of n-3 PUFA within phospholipid membranes of skeletal muscle following the cessation of supplementation remains poorly understood. Besides dosage and duration, frequency of ingesting n-3 PUFA may impact dynamics of EPA and DHA incorporation and subsequent washout from skeletal muscle membranes. Hence, the washout of EPA and DHA from skeletal muscle fractions may depend on the specific supplementation regimen employed. Furthermore, the washout of EPA and DHA from skeletal muscle when transferring to a low EPA/DHA intake/dose offers valuable insights into the potential dosing frequency required for n-3 PUFA. The washout period of EPA and DHA after cessation of fish oil supplementation has been characterised in several blood fractions(Cao *et al.*, 2006; Metherel *et al.*, 2009). However, the washout of EPA and DHA from skeletal muscle has yet to be elucidated.

Despite accumulating evidence regarding the effects of n-3 PUFA ingestion in modulating skeletal muscle protein turnover, the dose and time-dependent rates of EPA and DHA incorporation and washout in relation to skeletal muscle remain poorly understood. Therefore, the aim of this study was to determine temporal changes in cell lysate and phospholipid membrane incorporation and washout of EPA and DHA in response to incubation with combined EPA + DHA at high or low concentrations in C2C12 myotubes over a 24 h period.

In addition, we assessed the impact of high and low concentrations of combined EPA and DHA incubation and washout on the phosphorylation status of associated mTORC1 pathway signaling proteins after a 16 h incorporation period and a 24 h washout period.

2.2 Materials and methods

2.2.1 Materials

Plastic ware for tissue culture was purchased from Corning Costar (6-well plates) and Greiner Bio-One (culture flasks). Fetal bovine serum (FBS; Cat.No: 10270-106) was purchased from ThermoFisher Scientific. Horse serum (HS-hi; Cat.No: VX16050122), phosphate buffered saline (PBS; Cat.No: VX14190169) and Trypsin-EDTA (trypsin; Cat.No: VX25300096) were purchased from Gibco. Penicillin-streptomycin (pen-strep; Cat.No: VX15140130) and Hanks' Balanced Salt Solution (HBSS; Cat.No: VX14175) were purchased from Invitrogen. Reagents for culture medium (DMEM powder; Cat.No: D5648, $NaHCO_3$), Insulin from bovine pancreas (Cat.No: I6634-100MG), L-Leucine BioUltra (Cat.No: 61819), EPA (cis-5,8,11,14,17, Cat.No: E2011-50MG) and DHA (cis-4,7,10,13,16,19, Cat.No: D2534-100MG) were purchased from Sigma-Aldrich. Puromycin (Cat.No: 540411-100) was purchased from Merck and anti-puromycin (Cat.No: EQ0001) was purchased from Kerafast. Anti-mouse Detection Module (Cat.No: DM-002), Anti-rabbit Detection module (Cat.No: DM-001), and 12-230 kDa Wes Separation Module (Cat.No: SM-W004) were purchased from Bio-Techne. Anti-4E-BP1 (Cat.No: CST9452), anti-phospho-4E-BP1 (Thr37/46) (Cat.No: CST2855), anti-p70S6K1 (Cat.No: CST34475), anti-phospho-p70S6K1 (Thr421/Ser424) (Cat.No: CST9204), Akt (pan) (40D4) Mouse mAb (Cat.No: CST2920240), and Phospho-Akt (Ser473) (D9E) XP(R) Rabbit mAb (Cat.No: CST4060P) were purchased from Cell Signaling Technology.

2.2.2 Cell culture and treatment

C2C12 myoblasts were cultured in T175 flasks containing growth medium and incubated at 37°C and 5% CO_2. The growth medium consisted of 10g Dulbecco's Modified Eagle Medium (DMEM) powder and 2.4g $NaHCO_3$ dissolved in 1L milliQ and supplemented with 10% FBS and 1% pen-strep. Myoblasts were maintained and passaged at ~60% confluence. For all experiments, 1.25×10^5 cells/mL C2C12 myoblasts were seeded in 6-well plates in 3 ml growth medium and incubated overnight. Differentiation was induced by changing growth medium

to a differentiation medium consisting of DMEM with 2% HS-Hi (Horse serum) and 1% pen-strep. The differentiation medium was refreshed every other day. Experimental treatments were initiated on day 7 of differentiation. Solutions of 100 mM EPA and DHA were prepared by dissolving EPA (≥99 %) and DHA (≥98 %) in 100 % ethanol, and further diluted in PBS + 2.5 % fatty acid free BSA to a final concentration of 10–20 mM. Exact fatty acid concentration was measured and aliquots were stored at -80 °C until further use. Cells were treated with EPA:DHA in a 3:2 ratio complexed with 2.5% albumin dissolved in PBS in the following concentrations: high concentration (HC): 50 µM EPA + 33.3 µM DHA or low concentration (LC): 12.5 µM EPA + 8.33 µM DHA. The selected EPA:DHA ratio and concentrations were chosen for several reasons. First, a 3:2 ratio of EPA:DHA reflects commercially available fish oil supplements. Secondly, an EPA-favourable ratio was chosen due to its proposed role as the primary driver behind MPS, where lastly, the concentration of 50 µM was selected based on previous findings where this concentration increased rates of MPS (Kamolrat and Gray, 2013) and protein accretion (Jeromson *et al.*, 2018). Differentiation medium was used as washout medium and medium control, and a differentiation medium containing 100% ethanol diluted to the same concentration as HC (1.28%) with PBS + 2.5% albumin was used as a vehicle control (VC). Treatments were freshly prepared on the morning of the experiment by adding stock EPA, DHA, or ethanol to the differentiation medium and heated at 37°C.

2.2.3 Experimental Design

C2C12 myotubes were treated with 3 mL of HC, LC, or VC. The uptake and washout rate of EPA, DHA and DPA were determined in two separate experiments. A third experiment was conducted to assess rates of MPS and the phosphorylation status of associated mTORC1 proteins. To assess the uptake of EPA and DHA into the cell (**Figure 2.1A**), myotubes were incubated for 1, 2, 4, 8, 16 or 24 h before whole cell lysates and phospholipid fractions were collected (as described below). To determine the washout rate (**Figure 2.1B**), C2C12 myotubes were treated with 3 mL of HC, LC, or VC or negative control and incubated 16 h (n=3) or 24 h (n=1). Treatments were then switched to a washout medium, and whole cell lysates and phospholipid fractions were collected at 0, 2, 4, 8, 16 or 24 h. To assess rates of MPS and the phosphorylation status of mTORC1 associated cell signaling proteins (**Figure 2.1C**), C2C12 myotubes were incubated for 16 h with HC, LC, or VC with the addition of an insulin/leucine (I/L) trigger. The treatments were then switched to a washout medium for a duration of 24 h. Measurement of MPS and anabolic cell signaling were determined after the 16 h incorporation

period and after a 24 h washout period. A schematic overview of the experimental design is displayed in **Figure 2.1**.

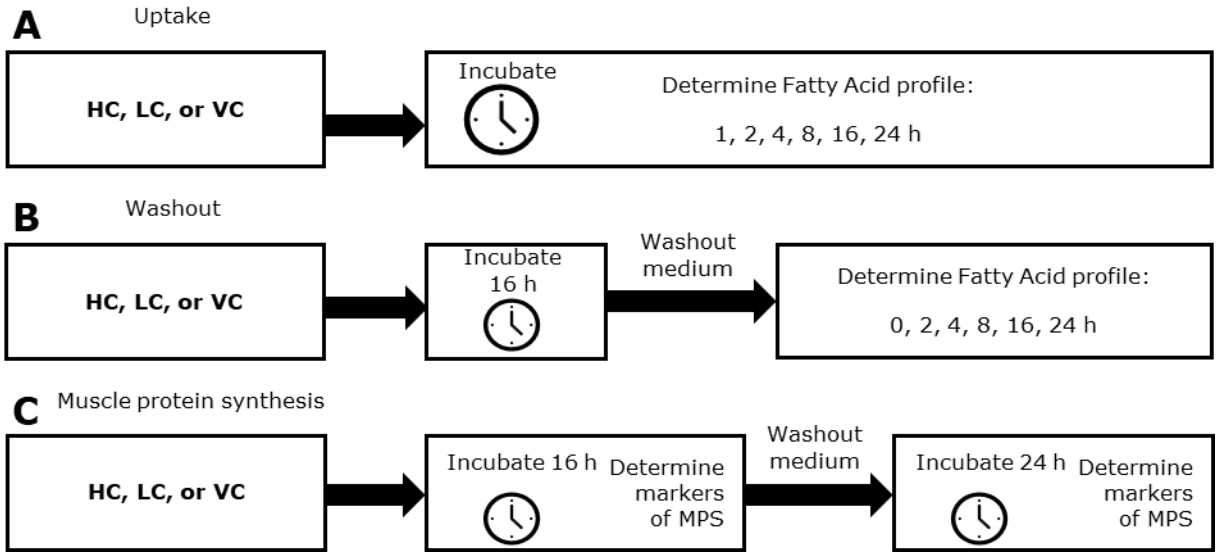

Figure 2.1: Schematic overview of incorporation (A), washout (B), and muscle protein synthesis experiments (C). HC = high concentration (50 µM EPA + 33.3 µM DHA), LC = low concentration (12.5 µM EPA + 8.33 µM DHA), VC = vehicle control (DM without EPA or DHA). EPA = Eicosapentaenoic acid, DHA = Docosahexaenoic acid. MPS = muscle protein synthesis. mTORC1 = mammalian target of rapamycin complex 1.

2.2.4 Cell collection and fatty acid profile analysis

Myotubes were washed twice with cold PBS and then lysed with 200 µl PBS + 1 % Triton X-100 (VWR). Cells were detached and resuspended following 5 min of incubation at 4°C. In total, 100µl for whole cell lysate or 150 µl for phospholipids from suspended cell lysates were collected in glass tubes and analysed by gas chromatography. In brief, a known amount of 1,2-dinonadecanoyl-sn-glycero-3-phosphocholine was added as an internal standard to 100 µl sample for total lipid 150 µl for phospholipids. The lipids were extracted according to a modified procedure of Bligh & Dyer (Bligh, E.G. and Dyer, 1959) (2 ml dichloromethane, 2 ml methanol and 2 ml 1% EDTA solution). After vortexing and centrifuging at 3000 RPM, the dichloromethane layer, containing the lipids, was collected in a new glass tube. For total lipids, they were then evaporated to dryness using a SpeedVac®. For phospholipid analysis, the phospholipid fraction was separated from the other lipid classes by Solid Phase Extraction (SPE). The phospholipids and dried total lipids were converted to fatty acid methyl esters (FAME) by adding 2% concentrated sulphuric acid in methanol and heated at 100 °C for 60 minutes (Christie, 1993). Once cooled, the fatty acid methyl esters were extracted with 2 ml hexane and 0.5 ml 2.5 mol/l sodium hydroxide solution. After vortexing, the upper layer,

hexane with FAME, was collected and dried using a SpeedVac®. Dried samples were subsequently dissolved in 80 µl iso-octane and analysed with an in-house validated analysis method using GC-FID (Shimadzu Corporation, Kyoto, Japan) with a CP-SIL88 for FAME column (60 m × 0.25 mm id. 0.20 µm film thickness; Agilent Technologies, Inc., Santa Clara, CA, USA). Fatty acids were identified based on retention time using reference standard GLC-569B (Nu-Chek Prep,Inc., Elysian, MN, USA). The relative concentration of the identified FAME in the samples was calculated via the peak area and total concentration of fatty acids in the sample was calculated via the internal standard.

2.2.5 Muscle Protein Synthesis measurements

2.2.5.1 Muscle protein synthesis treatment

After the treatment period, media was discarded, and cells were washed twice with 1 mL pre-warmed HBSS. Then, 3 mL of pre-warmed starvation medium (low glucose DMEM + 3.5g/L glucose) without insulin/leucine was added and C2C12 myotubes were incubated for 4 h at 37°C and 5% CO_2. Thereafter, DMEM + 1 mM leucine and 100 nM insulin or control was added and incubated at room temperature for 30 min. DMEM without I/L trigger was employed as a basal control to confirm the trigger worked. Next, 10 µl puromycin for a total concentration of 300 µM was added and cells were incubated for another 30 min. Cells were then washed with 1 mL 1x ice cold PBS, and 250 µl homogenisation^{+} buffer was added before cells were placed on ice. Cell lysates were collected and then centrifuged at 2000 RPM for 5 min at 4°C. The supernatant was collected and stored until further analysis on the WES™.

2.2.5.2 Protein Simple Western™ Analysis

Incorporation of puromycin into proteins was measured using Simple Western™ analysis on the Wes™ system (ProteinSimple, a Bio-Techne brand, San Jose, USA). Samples were diluted in 0.1x sample buffer to 0.5 g/L cell lysate and combined with Fluorescent Master Mix in a ratio of 4:1. Samples were vortexed, heated for 5 min at 100 °C and subsequently centrifuged at room temperature for 5 min at 10.000 RPM. Next, 4 µl of sample was loaded onto the WES plate and centrifuged for 5 min at 2500 RPM. The assay was run using the 12-230 kDa Separation Module (ProteinSimple). Anti-puromycin was diluted 5x and loaded on the WES plate and Anti-mouse Detection Module for Wes™ (ProteinSimple) was used as secondary antibody and detection. The run was performed using 30 min separation time, 375 V separation voltage, 30 min antibody diluent time, 30 min primary antibody time and 30 min secondary

antibody time. Total area under the curve of puromycin was calculated by the Compass for SW software (ProteinSimple), with ratios to the basal control group calculated as a measure of puromycin incorporation. MPS was then calculated by dividing puromycin by total protein and data was expressed as ratio to basal control.

2.2.5.3 Measurement of mTORC1 pathway proteins 4E-BP1, p70S6K1, Akt

The phosphorylation status of mTORC1 pathway proteins 4E-BP1, p70S6k, and Akt was determined with the Wes™ using the same settings as for MPS (see above). Anti-4E-BP1 (10× diluted) and anti-phospho-4E-BP1 (Thr37/46) (10× diluted) antibodies were used for 4E-BP1; anti-p70S6k (10× diluted) and anti-phospho-p70S6k (Thr421/Ser424) (10× diluted) antibodies were used for p70S6K1; Akt (pan) (40D4) Mouse mAb and Phospho-Akt (Ser473) (D9E) XP(R) Rabbit mAb were used for Akt. A concentration of 0.313 g/L protein for 4E-BP1[Thr37/46] and 0.625 g/L protein for Akt[Ser473] and p70S6K1[Thr421/Ser424] were loaded onto the Wes™. The total peak area was calculated by the Compass for SW software (ProteinSimple), with ratios to the basal control group calculated for each antibody. Signalling data were expressed as the ratio of phosphorylated proteins to unphosphorylated proteins. The 12–230 kDa Separation Module (ProteinSimple) was used for all signalling pathway proteins. The Anti-rabbit Detection Module or Anti-mouse Detection Module (ProteinSimple) was used for detection. The total peak area was calculated by the Compass for SW Software.

2.2.7 Statistical analysis

All statistical analyses were conducted in RStudio version 2023.12.1. At each timepoint, an independent samples Student's t-test was used to analyse differences in the presence of EPA and DHA in the whole cell lysate and phospholipid fraction between concentrations (HC and LC) and versus control (HC or LC *vs.* VC), and between the cell fractions (whole cell lysate *vs.* phospholipid fraction). Temporal changes in EPA, DHA and DPA uptake and washout into C2C12 myotubes were analysed using a linear mixed-effect model using 'lme4' package in R followed by Tukey's post-hoc analysis. To confirm that the MPS trigger worked but did not differ from VC, an independent samples t-test was used to test the I/L trigger compared to basal control and vehicle control. Changes in MPS ratio and phosphorylation status between the experimental conditions and control were tested by a linear mixed-effect model using 'lme4' package in R followed by Tukey's post-hoc analysis using the emm() package. All data are presented as mean ± SD and statistical significance was set at $P < 0.05$.

2.3 Results

2.3.1 Dose-response of EPA and DHA in C2C12 myotubes

2.3.1.1 Dose-response of EPA and DHA uptake into the whole cell lysate

A full breakdown of changed in fatty acid profile in whole cell lysates after incubation with HC and LC can be found in **Table 2.1.** EPA and DHA uptake into the whole cell lysate was observed at all timepoints *vs.* VC when treated with HC and LC ($P < 0.05$; **Figure 2.2A, 2.2B**), while VC showed a significant increase in EPA after 8 h compared to 1 h ($P < 0.05$). The proportion of EPA and DHA in the whole cell lysate was significantly higher in HC than LC at all time points ($P < 0.05$). After 24 h, EPA and DHA uptake into the whole cell lysate was 143.6 ± 1.8% and 105 ± 0.1% greater with HC than LC, respectively (**Figure 2.2A, 2.2B**).

EPA uptake into the whole cell lysate increased from 14.6 ± 1.5% of total FA profile at 1 h to 28.5 ± 3.3% at 24 h following incubation with HC (**Figure 2.2A**). Over time, EPA uptake increased between 1-2 h (5.5 ± 0.1%, 95% CI [3.3 - 7.8]), 2-4 h (5.0 ± 0.7%, 95% CI [3.2 – 7.8]), and 4-8 h (3.1 ± 0.7%, 95% CI [0.2, 4.9]), with no changes observed thereafter. Following incubation with LC, EPA uptake into the whole cell lysate increased from 7.6 ± 1.2% of total FA profile at 1 h to 11.7 ± 1.5% at 24 h (**Figure 2.2A**). EPA uptake increased between 1-2 h (2.1 ± 0.2%, 95% CI [1.2 – 2.9]), 2-4 h (1.9 ± 0.1%, 95% CI [1.0 – 2.7]), and 4-8 h (1.4 ± 0.4%, 95% CI [0.6 – 2.3]). No difference in EPA uptake was detected between 8-16 h (-0.1 ± -0.2%, 95% CI [-1.0 – 0.7]), however EPA uptake decreased between 16-24 h (-1.2 ± 0.2%, 95% CI [-2.1 – -0.4]).

DHA uptake into the whole cell lysate increased from 6.5 ± 0.9% of total FA profile at 1 h to 22.7 ± 1.2% after 24 h following incubation with HC (**Figure 2.2B)**. In comparison, DHA uptake into the whole cell lysate following incubation with LC increased from 2.8 ± 0.6% at 1 h to 11.0 ± 1.3% at 24 h. DHA uptake into the whole cell lysate continued to increase during the 24 h period for both HC and LC ($P < 0.05$).

Table 2.1: changes in fatty acid profile over time in C2C12 whole cell lysates following incubation with HC or LC

	HC (high concentration)						LC (low concentration)					
Fatty Acid	t1	t2	t4	t8	t16	t24	t1	t2	t4	t8	t16	t24
C14.0	1.13±0.69	0.93±0.52	0.75±0.41	0.70±0.33	0.68±0.33	0.66±0.27	1.30±0.81	1.15±0.61	1.09±0.61	1.00±0.56	0.98±0.50	1.02±0.55
C16.0	14.69±2.20	12.74±1.18	10.91±0.90	10.35±0.84	9.41±0.75	9.33±0.90	16.55±3.03	15.53±1.40	15.08±0.84	14.77±0.88	14.24±0.52	14.82±1.37
C18.0	12.38±0.86	10.71±0.71	8.83±0.76	8.49±0.56	7.79±0.39	7.94±0.87	14.01±1.28	13.03±0.88	12.45±0.84	12.70±0.55	13.08±0.34	13.62±0.44
C20.0	0.19±0.06	0.19±0.04	0.16±0.02	0.17±0.03	0.13±0.02	0.14±0.04	0.25±0.04	0.21±0.05	0.21±0.06	0.22±0.04	0.18±0.04	0.19±0.04
C22.0	0.15±0.05	0.14±0.03	0.12±0.03	0.12±0.03	0.10±0.02	0.11±0.03	0.17±0.04	0.12±0.09	0.15±0.04	0.14±0.03	0.14±0.01	0.14±0.02
C24.0	0.32±0.07	0.29±0.03	0.26±0.01	0.25±0.05	0.22±0.01	0.23±0.06	0.34±0.07	0.32±0.03	0.32±0.03	0.31±0.04	0.32±0.04	0.29±0.04
Saturated	**29.17±3.15 a**	**25.32±1.75 b**	**21.29±1.16^{c}**	**20.34±1.07^{c}**	**18.58±1.02^{c}**	**18.68±1.56^{c}**	**33.02±4.40 a**	**30.74±1.75 ab**	**29.67±0.62 b**	**29.57±0.85 b**	**29.36±0.67 b**	**30.50±1.65 ab**
C16.1n7	7.33±1.27	6.23±1.42	5.50±1.00	4.43±0.83	3.87±0.80	3.35±0.57	8.32±1.50	7.95±1.85	7.41±1.28	6.19±0.91	5.42±0.68	4.70±0.71
C18.1n9	15.61±0.85	13.71±0.81	11.81±1.02	10.23±1.16	9.09±1.04	8.55±1.04	17.85±1.25	17.20±0.92	16.17±1.27	15.13±1.19	13.95±0.88	13.48±0.98
C18.1n7	5.87±0.68	5.01±0.85	4.41±0.66	3.71±0.79	3.32±0.70	3.10±0.69	6.62±0.87	6.32±0.98	6.00±0.93	5.67±1.03	5.54±0.95	5.57±1.00
C20.1n9	0.18±0.04	0.17±0.04	0.14±0.04	0.12±0.03	0.11±0.03	0.14±0.09	0.22±0.05	0.21±0.04	0.18±0.03	0.20±0.04	0.18±0.03	0.15±0.01
C20.3n9	0.04±0.06	0.05±0.06	0.05±0.05	0.04±0.04	0.03±0.03	0.03±0.03	0.06±0.08	0.06±0.08	0.04±0.06	0.05±0.06	0.04±0.05	0.03±0.04
C24.1n9	0.44±0.06	0.44±0.04	0.41±0.03	0.37±0.08	0.33±0.03	0.32±0.05	0.52±0.06	0.51±0.08	0.50±0.03	0.51±0.07	0.48±0.06	0.53±0.07
Mono	**29.53±1.78 a**	**25.69±2.68 b**	**22.38±2.30^{c}**	**18.99±2.69 d**	**16.81±2.45^{e}**	**15.52±2.25^{f}**	**33.69±1.91 a**	**32.40±3.01 a**	**30.36±2.68 b**	**27.85±2.91^{c}**	**25.68±2.35 d**	**24.51±2.58 d**
C18.2n6	7.75±0.38	6.91±0.37	6.21±0.48	5.42±0.51	4.98±0.43	4.82±0.44	8.82±0.74	8.61±0.41	8.29±0.47	7.82±0.48	7.34±0.43	6.95±0.47
C18.3n6	0.25±0.07	0.21±0.04	0.18±0.04	0.16±0.03	0.18±0.02	0.16±0.03	0.30±0.08	0.25±0.05	0.26±0.06	0.23±0.06	0.25±0.06	0.21±0.06
C20.2n6	0.19±0.07	0.19±0.05	0.15±0.03	0.12±0.01	0.12±0.02	0.12±0.05	0.25±0.09	0.22±0.05	0.19±0.05	0.18±0.02	0.20±0.04	0.21±0.03
C20.3n6	1.26±0.15	1.11±0.09	0.94±0.09	0.83±0.06	0.75±0.03	0.69±0.05	1.43±0.22	1.38±0.14	1.30±0.12	1.18±0.12	1.09±0.08	1.00±0.10
C20.4n6	3.74±0.35	3.36±0.28	2.80±0.18	2.46±0.08	2.21±0.05	2.13±0.09	4.22±0.62	4.14±0.40	3.90±0.31	3.84±0.15	3.94±0.12	3.97±0.16
C22.4n6	0.23±0.05	0.22±0.02	0.18±0.02	0.15±0.02	0.16±0.03	0.14±0.03	0.28±0.09	0.27±0.04	0.23±0.04	0.23±0.03	0.24±0.03	0.26±0.03
C22.5n6	0.00±0.00	0.02±0.03	0.08±0.05	0.13±0.03	0.14±0.03	0.14±0.03	0.00±0.00	0.00±0.00	0.01±0.03	0.02±0.03	0.02±0.03	0.02±0.03
n-6 PUFA	**13.41±0.83^{a}**	**12.01±0.33^{b}**	**10.54±0.60^{c}**	**9.26±0.58^{d}**	**8.52±0.42^{de}**	**8.20±0.52^{e}**	**15.30±1.51 a**	**14.87±0.58 ab**	**14.18±0.48 bc**	**13.51±0.40^{c} d**	**13.07±0.44 d**	**12.62±0.69 d**
C18.3n3	1.20±0.03	1.28±0.06	1.30±0.05	1.31±0.06	1.26±0.06	1.24±0.08	1.32±0.04	1.49±0.10	1.59±0.12	1.59±0.14	1.29±0.20	1.07±0.18
C18.4n3	0.17±0.11	0.17±0.12	0.18±0.09	0.18±0.13	0.26±0.18	0.27±0.19	0.19±0.14	0.20±0.14	0.20±0.14	0.20±0.14	0.20±0.14	0.18±0.12
C20.3n3	0.11±0.01	0.12±0.03	0.10±0.02	0.09±0.01	0.08±0.01	0.08±0.01	0.13±0.02	0.15±0.03	0.13±0.02	0.15±0.02	0.14±0.01	0.15±0.02
C20.5n3	14.62±1.47	20.13±1.53	25.15±2.28	28.21±2.98	28.64±2.64	28.45±3.31	7.65±1.18	9.73±0.98	11.60±1.12	13.04±1.47	12.91±1.28	11.68±1.52
C22.3n3	0.04±0.05	0.03±0.03	0.04±0.03	0.03±0.03	0.02±0.02	0.01±0.02	0.02±0.04	0.04±0.05	0.03±0.05	0.01±0.02	0.02±0.03	0.01±0.03
C22.5n3	2.28±0.43	2.17±0.41	1.81±0.22	1.76±0.22	2.42±0.32	2.97±0.47	2.44±0.55	2.63±0.51	2.60±0.49	2.86±0.51	3.94±0.46	4.82±0.57
C22.6n3	6.49±0.93	10.35±0.94	14.78±1.21	17.84±1.22	21.42±1.32	22.66±1.25	2.82±0.59	4.27±0.42	6.32±0.77	8.23±0.88	10.12±0.84	11.03±1.27
n-3 PUFA	**24.90±2.58^{a}**	**34.25±2.33^{b}**	**43.34±3.39^{c}**	**49.41±4.25^{d}**	**54.10±3.82^{e}**	**55.69±4.31^{e}**	**14.57±2.25 a**	**18.51±1.66^{b}**	**22.46±2.29^{c}**	**26.07±2.85^{d}**	**28.61±2.46^{e}**	**28.94±3.23^{e}**

Total saturated fatty acids, total monounsaturated fatty acids, total n-6 PUFA, total n-3 PUFA. Mono = monounsaturated. Values are expressed as % of total fatty acids mean ± SD. Means that do not share a letter are significantly different from one another ($p < 0.05$). T = timepoint in hours.

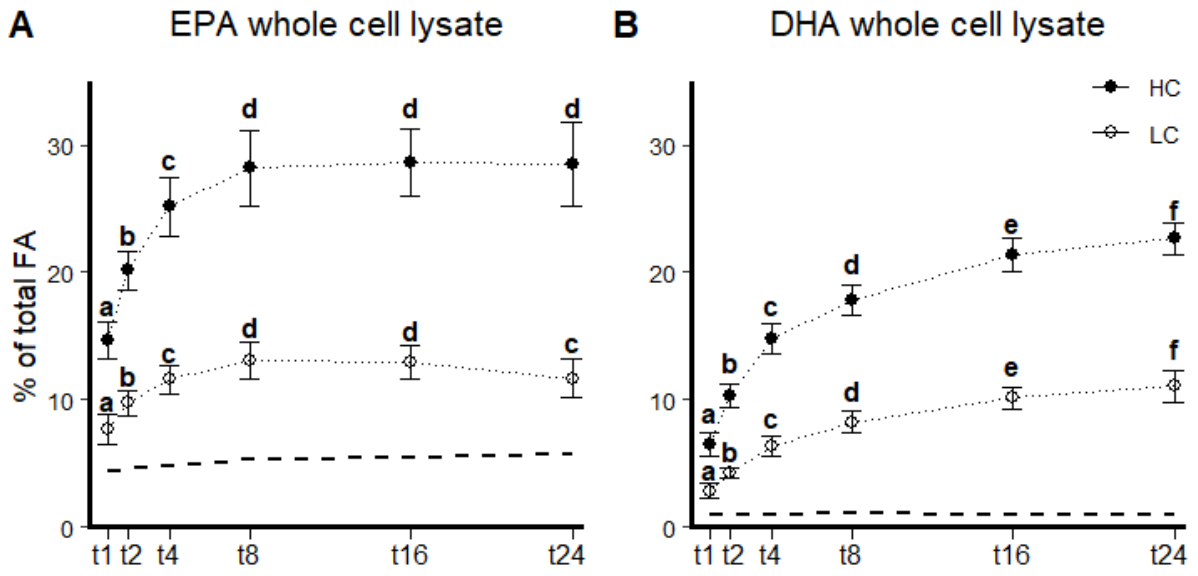

Figure 2.2: Temporal (24 h) changes in uptake of EPA (A) and DHA (B) into the whole cell lysates of C2C12 myotubes following incubation with HC (50µM EPA + 33.33µM DHA) and LC (12.5µM EPA + 8.33µM DHA). Dashed line represents vehicle control (VC) over time. Values are means ± SD (n=7). Mean values that do not share a common letter within HC or LC are statistically different from one another ($P < 0.05$). EPA and DHA uptake significantly differed from VC in both HC and LC at all timepoints ($P < 0.05$). Furthermore, EPA and DHA uptake differed significantly between HC and LC at all timepoints ($P < 0.05$).

2.3.1.2 Dose-response of EPA and DHA incorporation into the phospholipid fraction

A full breakdown of changed in fatty acid profile in whole cell lysates after incubation with HC and LC can be found in **Table 2.2.** The incorporation of EPA and DHA into the phospholipid fraction was detected at all time points *vs*. VC when treated with HC and LC ($P < 0.05$; **Figure 2.3A, 2.3B**). The proportion of EPA incorporated into the phospholipid fraction was higher in HC compared to LC ($P < 0.05$) at all time points with the exception of 16 h ($P > 0.05$), whereas no differences were detected in DHA incorporation between HC and LC at all timepoints ($P > 0.05$). After 24 h, the incorporation of EPA into the phospholipid fraction was 38.7 ± 0.1% greater in HC than LC, whereas the incorporation of DHA into the phospholipid fraction was 37.8 ± 0.2% greater in HC than LC although statistical significance was not reached. Compared to the whole cell lysate (**Figure 2.2**), the incorporation of EPA and DHA into the phospholipid fraction was lower at all timepoints following incubation with HC ($P < 0.05$). In contrast, a similar rate of EPA and DHA uptake into the whole cell lysate and incorporation into the phospholipid fraction was observed over a 24 h period following incubation with LC ($P > 0.05$).

Following incubation with HC, EPA incorporation into the phospholipid fraction increased starting from 10.3 ± 0.2% of total FA profile at 1 h to 18.1 ± 1.9% at 24 h (**Figure 2.3A**). Over time, EPA incorporation increased between 1-4 h (5.4 ± 1.3%, 95% CI [2.2 – 8.5]) and 4-8 h (3.2 ± 0.2%, 95% CI [0.0 – 6.3]), with no further incorporation thereafter. Following incubation with LC, EPA incorporation into the phospholipid fraction increased starting from 8.3 ± 0.8% of total FA profile at 1 h to 13.0 ± 2.0% at 24 h. Incubation with LC led to an increased EPA incorporation between 1-4 h (3.1 ± 0.6%, 95% CI [0.9 – 5.2]) and 4-16 h (2.4 ± 1.0%, 95% CI [0.3 – 4.5]), with no further changes thereafter.

DHA incorporation into the phospholipid fraction increased starting from 3.4 ± 0.6% of total FA profile at 1 h to 14.8± 2.8% at 24 h after incubation with HC (**Figure 2.3B**). DHA incorporation increased over time between 1-4 h (5.0 ± 0.9%, 95% CI [1.9 – 8.0]) and 4-16 h (4.7 ± 0.8%, 95% CI [1.6 – 7.7]), with no further incorporation thereafter. Following incubation with LC, DHA incorporation into the phospholipid fraction increased starting from 2.8 ± 0.6% of total FA profile at 1 h to 10.7± 2.6% at 24 h. DHA incorporation increased moderately over time between 1-8 h (4.3 ± 0.9%, 95% CI [1.5 – 7.2]) and 8-24 h (3.6 ± 1.1%, 95% CI [0.7 – 6.4]).

Table 2.2: changes in fatty acid profile over time in C2C12 phospholipid fractions following incubation with HC or LC

	HC (high concentration)						LC (low concentration)					
Fatty Acid	t1	t2	t4	t8	t16	t24	t1	t2	t4	t8	t16	t24
C14.0	1.47±0.29	1.27±0.30	1.16±0.09	0.97±0.14	0.98±0.06	0.86±0.03	1.43±0.38	1.30±0.32	1.31±0.18	1.33±0.09	1.13±0.08	1.24±0.15
C16.0	21.63±0.39	20.30±0.88	19.50±1.94	18.94±1.23	19.30±1.83	19.63±1.82	21.14±0.82	20.52±1.19	19.97±1.72	20.06±1.66	19.59±2.05	19.54±1.78
C18.0	9.06±0.74	8.89±0.94	8.58±1.01	9.60±1.59	10.25±1.16	11.08±2.13	8.66±0.88	8.81±0.70	8.64±0.67	8.99±0.55	9.48±0.38	9.86±1.38
C20.0	0.23±0.08	0.20±0.07	0.20±0.08	0.21±0.12	0.18±0.05	0.19±0.05	0.21±0.05	0.18±0.03	0.25±0.11	0.20±0.09	0.18±0.09	0.09±0.08
C22.0	0.19±0.06	0.16±0.05	0.16±0.06	0.17±0.04	0.16±0.06	0.19±0.02	0.18±0.01	0.16±0.01	0.15±0.04	0.16±0.04	0.17±0.06	0.15±0.04
C24.0	0.32±0.12	0.32±0.07	0.32±0.09	0.33±0.08	0.32±0.04	0.33±0.06	0.32±0.07	0.34±0.07	0.31±0.06	0.29±0.09	0.26±0.07	0.27±0.05
Saturated	**33.36±0.81**	**31.41±1.54**	**30.31±2.94**	**30.53±2.78**	**31.65±2.64**	**32.65±3.70**	**32.28±1.57**	**31.64±1.53**	**31.03±2.34**	**31.48±2.11**	**31.24±2.38**	**31.66±2.18**
C16.1n7	8.93±0.91	7.83±0.61	6.30±0.40	4.78±0.34	3.92±0.49	3.41±0.23	9.56±0.66	8.78±0.69	8.04±1.33	6.80±0.91	5.35±0.65	4.79±0.76
C18.1n9	16.13±0.23	15.23±0.30	13.79±0.34	11.73±0.61	10.02±0.77	9.24±0.77	17.03±0.30	16.38±0.31	15.70±0.15	14.27±0.50	12.70±1.04	12.15±1.14
C18.1n7	6.22±0.57	5.74±0.36	5.43±0.42	4.76±0.42	4.45±0.40	4.31±0.35	6.65±0.53	6.29±0.51	6.00±0.46	5.48±0.31	5.13±0.60	4.93±0.45
C20.1n9	0.18±0.06	0.14±0.03	0.15±0.02	0.15±0.05	0.17±0.07	0.14±0.05	0.20±0.05	0.16±0.04	0.20±0.09	0.15±0.03	0.12±0.02	0.13±0.03
C20.3n9	0.05±0.05	0.04±0.04	0.02±0.03	0.02±0.03	0.02±0.03	0.00±0.01	0.05±0.04	0.05±0.05	0.04±0.04	0.03±0.05	0.03±0.05	0.01±0.02
C24.1n9	0.66±0.22	0.66±0.21	0.59±0.08	0.72±0.23	0.55±0.06	0.70±0.12	0.74±0.21	0.65±0.16	0.62±0.11	0.60±0.20	0.53±0.14	0.58±0.17
Mono-unsaturated	**32.29±1.34^{a}**	**29.75±0.64^{b}**	**26.39±0.49^{c}**	**22.29±1.17^{d}**	**19.25±1.28^{e}**	**17.91±0.94^{e}**	**34.35±1.21^{a}**	**32.42±1.10^{a}^{b}**	**30.70±1.54^{b}**	**27.44±1.32^{c}**	**23.97±2.03^{d}**	**22.69±2.13^{d}**
C18.2n6	7.13±1.26	6.92±1.16	6.07±0.75	4.97±0.43	4.51±0.37	4.52±0.49	7.64±1.07	7.46±0.98	6.80±0.84	6.41±0.96	6.28±1.10	5.98±0.79
C18.3n6	0.16±0.05	0.16±0.04	0.13±0.02	0.09±0.02	0.06±0.06	0.07±0.01	0.19±0.07	0.16±0.05	0.16±0.04	0.16±0.03	0.14±0.01	0.12±0.02
C20.2n6	0.12±0.02	0.12±0.02	0.12±0.01	0.09±0.02	0.08±0.00	0.10±0.02	0.18±0.03	0.17±0.05	0.14±0.02	0.10±0.03	0.08±0.01	0.12±0.02
C20.3n6	0.88±0.10	0.86±0.08	0.74±0.11	0.64±0.07	0.54±0.05	0.45±0.03	0.91±0.14	0.88±0.08	0.81±0.09	0.71±0.05	0.66±0.06	0.59±0.05
C20.4n6	3.73±0.19	3.78±0.22	3.63±0.10	3.34±0.22	3.10±0.29	2.94±0.30	3.94±0.17	4.09±0.27	3.93±0.17	4.01±0.19	4.22±0.15	4.21±0.13
C22.4n6	0.24±0.07	0.26±0.03	0.24±0.08	0.25±0.08	0.20±0.06	0.19±0.05	0.30±0.06	0.26±0.07	0.26±0.06	0.24±0.06	0.26±0.04	0.26±0.02
Total n-6	**12.26±1.14^{a}**	**12.09±1.17^{a}**	**10.92±0.70^{ab}**	**9.37±0.38^{bc}**	**8.49±0.49^{c}**	**8.28±0.49^{c}**	**13.16±1.00^{a}**	**13.02±0.92^{a}**	**12.10±0.71^{b}**	**11.64±0.86^{bc}**	**11.63±1.24^{b}^{c}**	**11.28±0.91^{c}**
C18.3n3	1.02±0.09	1.04±0.10	1.07±0.02	0.97±0.05	0.83±0.05	0.80±0.08	1.13±0.07	1.21±0.05	1.16±0.06	1.10±0.07	0.83±0.09	0.73±0.07
C18.4n3	0.14±0.04	0.15±0.04	0.14±0.05	0.14±0.06	0.16±0.01	0.23±0.05	0.16±0.03	0.15±0.05	0.16±0.05	0.16±0.06	0.14±0.07	0.15±0.06
C20.3n3	0.13±0.01	0.12±0.02	0.11±0.00	0.12±0.04	0.12±0.04	0.11±0.02	0.15±0.03	0.12±0.02	0.13±0.00	0.12±0.02	0.10±0.02	0.12±0.02
C20.5n3	10.27±0.19	12.86±0.59	15.65±1.51	18.81±1.71	19.59±2.98	18.09±1.87	8.31±0.75	9.84±1.07	11.37±1.38	13.02±1.85	13.79±2.37	13.04±1.99
C22.5n3	2.25±0.43	2.26±0.42	2.12±0.31	1.97±0.39	2.04±0.43	2.24±0.61	2.45±0.49	2.56±0.42	2.53±0.50	2.51±0.35	3.32±0.30	4.30±0.41
C22.6n3	3.42±0.65	5.37±0.86	8.37±1.51	11.18±1.96	13.05±2.31	14.77±2.84	2.83±0.61	4.05±0.72	5.59±1.14	7.16±1.51	9.57±2.33	10.71±2.62
Total n-3	**17.24±1.10^{a}**	**21.80±1.57^{a}**	**27.46±2.80^{b}**	**33.19±2.00^{c}**	**35.79±4.11^{c}**	**36.23±3.10^{c}**	**15.02±1.70^{a}**	**17.92±2.04^{ab}**	**20.97±2.71^{bc}**	**24.08±3.58^{cd}**	**27.75±4.97^{d}**	**29.05±4.86^{d}**
C14.0	1.47±0.29	1.27±0.30	1.16±0.09	0.97±0.14	0.98±0.06	0.86±0.03	1.43±0.38	1.30±0.32	1.31±0.18	1.33±0.09	1.13±0.08	1.24±0.15
C16.0	21.63±0.39	20.30±0.88	19.50±1.94	18.94±1.23	19.30±1.83	19.63±1.82	21.14±0.82	20.52±1.19	19.97±1.72	20.06±1.66	19.59±2.05	19.54±1.78

Total saturated fatty acids, total monounsaturated fatty acids, total n-6 PUFA, total n-3 PUFA. Values are expressed as % of total fatty acids mean±SD. Means that do not share a letter are significantly different from one another ($p < 0.05$). T = timepoint in hours

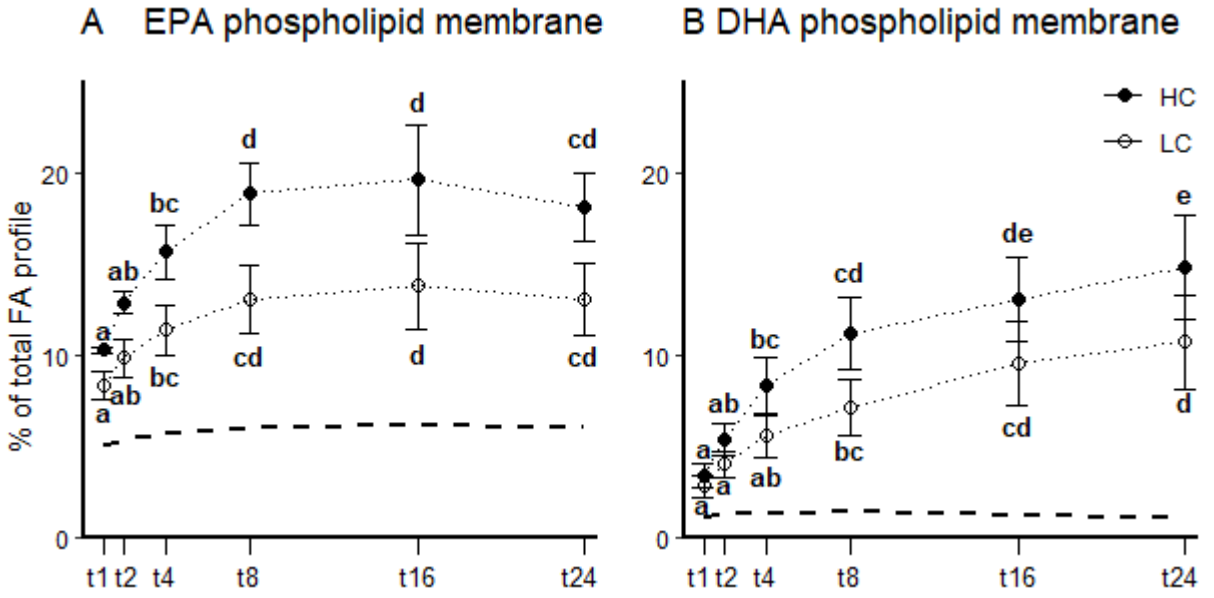

Figure 2.3: Temporal (24 h) changes in incorporation of EPA (A) and DHA (B) into the phospholipid fraction of C2C12 myotubes following incubation with HC (50µM EPA + 33.33µM DHA) and LC (12.5µM EPA + 8.33µM DHA). Dashed line represents vehicle control (VC) over time. Values are means ± SD (n=3). Mean values that do not share a common letter within HC or LC are statistically different from one another ($P < 0.05$). EPA and DHA uptake significantly differed from VC in both HC and LC at all timepoints ($P < 0.05$). Furthermore, EPA incorporation differed significantly between HC and LC at all timepoints ($P < 0.05$), except at 16 h for EPA ($P > 0.05$). whereas DHA incorporation did not differ between HC and LC ($P > 0.05$).

2.3.1.3 Formation of DPA

We assessed temporal changes in DPA expressed as %DPA of total fatty acid profile (**Figure 2.4**), given that previous research indicated the formation of DPA due to EPA supplementation (Jeromson *et al.*, 2018). DPA formation in the whole cell lysate was observed after 24 h (4.8 ± 0.6% of total FA profile) following incubation with LC compared to VC, whereas no significant DPA formation was detected at any timepoint following incubation with HC compared to VC (**Figure 2.4A**). Over time, DPA levels following incubation with HC decreased in the whole cell lysate between 1– 4 h (-0.5 ± 0.2%, 95% CI [-0.2 – -0.7]), after which levels increased between 4 – 16 h (0.6 ± 0.1%, 95% CI [0.3 – 0.8]), and 16 – 24 h (0.5 ± 0.2%, 95% CI [0.3 – -0.8]). Following LC, DPA levels in the whole cell lysate increased between 1-8 h (0.4 ± 0.1%, 95% CI [0.1 – 0.7]), with a further increase between 8-16 h (1.1 ± 0.1%, 95% CI [0.8 – 1.4]), and 16-24 h (0.9 ± 0.1%, 95% CI [0.6 – 1.2]).

Comparable to the whole cell lysate, a significant incorporation of DPA into the phospholipid fraction was detected at 24 h (4.3 ± 0.4% of total FA profile) following incubation with LC, whereas no detectable incorporation of DPA was observed following incubation with HC at any timepoint (**Figure 2.4B)**. The formation of DPA detected after 24 h did not differ between the whole cell lysate (**Figure 2.4A**) and the phospholipid fraction (**Figure 2.4B**) in LC at 24 h ($P > 0.05$). Over time, no changes in DPA incorporation into the phospholipid fraction were detected at any timepoints following incubation with HC ($P > 0.05$), whereas following incubation with LC, an increased DPA incorporation into the phospholipid fraction was detected between 1 – 16 h (0.9 ± 0.2%, 95% CI [0.5 – 1.3]), and 16 – 24 h (1.0 ± 0.1%, 95% CI [0.6 – 1.4]).

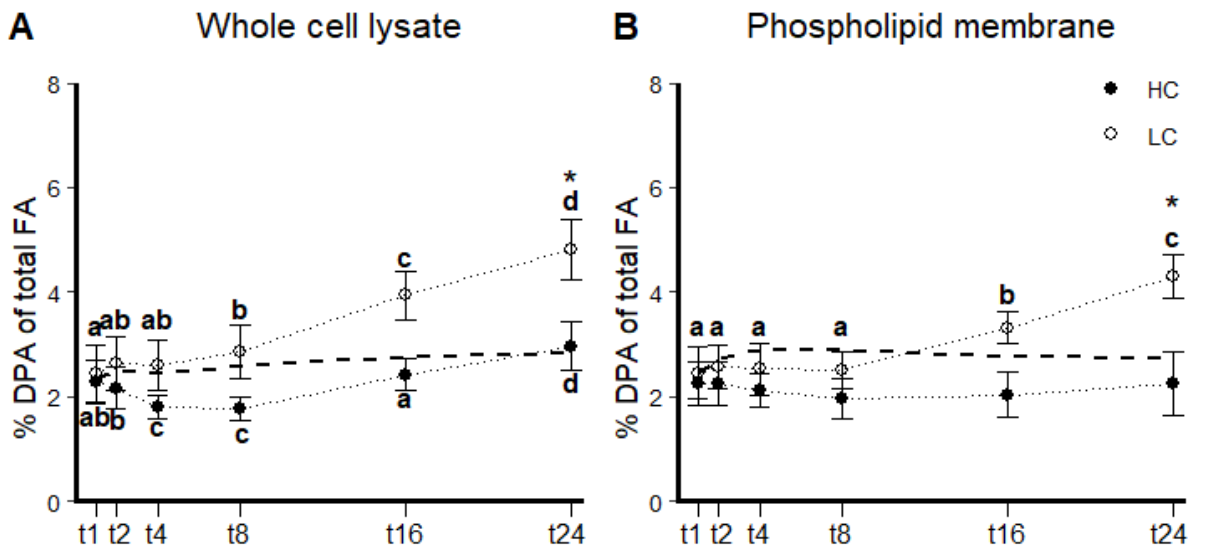

Figure 2.4: Temporal (24 h) changes in formation of DPA in the whole cell lysate (A, n=7) and phospholipid fraction (B, n=3) of C2C12 myotubes following incubation with HC (50µM EPA + 33.33µM DHA) or LC (12.5µM EPA + 8.33µM DHA). Values are means ± SD. Differences from vehicle control (VC; represented by a dashed line) are denoted by asterisks (*; $P < 0.05$). Mean values that do not share a common letter within HC or LC are statistically different from one another ($P < 0.05$). DPA formation at 24 h following incubation with LC did not differ between the whole cell lysate (A) and phospholipid fraction (B; $P > 0.05$).

2.3.2 Washout of EPA and DHA from C2C12 myotubes

2.3.2.1 Washout of EPA and DHA from the whole cell lysate

A full breakdown of changed in fatty acid profile in whole cell lysates after incubation with HC and LC can be found in **Table 2.3.** EPA and DHA levels remained above vehicle control

levels over the 24 h washout period when treated with HC and LC ($P < 0.05$; **Figure 2.5A, 2.5B;**). An increase in EPA in the vehicle control was detected from 8 h onwards compared to 1 h of incubation ($P < 0.05$). Furthermore, the uptake of EPA and DHA remained higher in HC compared to LC during the 24 h washout period ($P < 0.05$), except at 16 h for EPA ($P = 0.06$). After 24 h, EPA and DHA levels in the whole cell lysate were 120.3 ± 4.3% and 104.0 ± 0.9% greater with HC than LC, respectively.

Following overnight incubation with HC, EPA levels in the whole cell lysate **(Figure 2.5A)** decreased after 8 h (-3.6 ± 0.8%, 95% CI [-5.7 – -1.5]) when switching to the washout medium and continued to decrease until 16 h (-4.6 ± 0.4%, 95% CI [-5.5 – -0.9]). The overall decline in EPA over 24 h was 8.5 ± 1.8%. Following overnight incubation with LC, EPA levels decreased after 4 h (-0.8 ± 0.4%, 95% CI [-1.4 – -0.1]) when switching to the washout medium, with a further decrease observed between 4-16 h (-1.3 ± 0.3%, 95% CI [-1.7 – 0.2]**)**. The total decrease in EPA levels was 2.1 ± 0.5% over the 24 h period. No decline in DHA was observed from the whole cell lysate during the 24 h washout period when treated with HC or LC ($P > 0.10$; **Figure 2.5B**).

Table 2.3: changes in fatty acid profile over time in C2C12 whole cell lysates during 24h washout following incubation with HC or LC

	HC (high concentration)						LC (low concentration)					
Fatty Acid	t0	t2	t4	t8	t16	t24	t0	t2	t4	t8	t16	t24
C14.0	0.70±0.13	0.66±0.11	0.61±0.13	0.65±0.13	0.57±0.15	0.58±0.23	0.96±0.25	1.00±0.35	0.92±0.26	0.96±0.30	1.20±0.41	1.19±0.44
C16.0	10.13±0.29	10.41±0.78	10.51±0.69	11.59±0.57	13.02±0.96	12.84±1.07	15.62±0.85	16.03±0.56	16.16±0.36	16.49±0.85	17.83±0.62	19.49±1.40
C18.0	8.12±1.01	8.30±0.99	8.56±1.22	9.63±1.22	12.05±1.55	12.12±1.68	13.39±1.07	13.92±1.01	14.08±0.79	14.64±1.11	15.56±0.52	15.13±0.82
C20.0	0.13±0.04	0.09±0.07	0.10±0.03	0.14±0.06	0.14±0.03	0.11±0.02	0.14±0.03	0.15±0.03	0.16±0.04	0.15±0.04	0.14±0.03	0.11±0.02
C22.0	0.10±0.01	0.09±0.06	0.10±0.07	0.09±0.06	0.12±0.01	0.12±0.09	0.13±0.01	0.16±0.01	0.12±0.08	0.12±0.09	0.10±0.09	0.09±0.06
C24.0	0.22±0.02	0.22±0.02	0.19±0.03	0.21±0.07	0.23±0.05	0.25±0.07	0.26±0.06	0.30±0.10	0.29±0.02	0.28±0.09	0.23±0.03	0.28±0.08
Saturated	**19.59±1.45^{a}**	**19.94±1.77^{ab}**	**20.28±1.85^{ab}**	**22.53±1.82^{b}**	**26.40±2.44^{c}**	**26.25±2.82^{c}**	**30.76±1.58^{a}**	**31.89±1.49^{ab}**	**32.05±1.02^{ab}**	**32.97±1.68^{b}**	**35.42±1.51^{c}**	**36.60±1.10^{c}**
C16.1n7	3.64±1.19	3.43±1.08	3.20±0.89	2.40±1.85	1.49±1.41	2.30±0.74	4.64±1.50	4.37±1.26	3.97±0.93	3.17±2.22	3.62±0.80	3.77±0.77
C18.1n9	8.91±0.63	8.96±1.05	8.98±0.84	9.41±0.51	8.86±0.50	8.84±0.71	13.54±1.54	13.25±1.26	13.40±1.33	13.12±0.97	12.26±0.90	12.49±0.95
C18.1n7	3.27±0.58	3.28±0.76	3.31±0.75	3.52±0.71	3.65±1.02	3.83±0.86	5.82±1.12	5.67±1.01	5.69±1.07	5.69±1.07	5.28±0.90	5.58±0.89
C20.1n9	0.10±0.03	0.10±0.01	0.07±0.05	0.12±0.01	0.09±0.08	0.13±0.10	0.16±0.03	0.14±0.03	0.15±0.03	0.15±0.03	0.13±0.01	0.14±0.02
C20.3n9	0.02±0.04	0.02±0.04	0.02±0.04	0.02±0.04	0.02±0.03	0.01±0.02	0.02±0.04	0.02±0.04	0.02±0.04	0.02±0.04	0.03±0.05	0.01±0.03
C24.1n9	0.34±0.04	0.30±0.10	0.32±0.05	0.36±0.09	0.36±0.08	0.38±0.08	0.48±0.08	0.54±0.14	0.51±0.08	0.55±0.15	0.41±0.02	0.46±0.11
Mono	**16.32±2.12**	**16.13±2.63**	**15.97±2.24**	**15.86±3.15**	**14.55±2.85**	**15.55±2.02**	**24.73±4.16^{a}**	**24.11±3.53^{ab}**	**23.80±3.35^{ab}**	**22.76±4.37^{b}**	**21.76±2.52^{a} ^{b}**	**22.55±2.59^{b}**
C18.2n6	4.90±0.56	5.06±0.68	5.03±0.53	5.25±0.59	5.24±0.78	4.95±0.64	6.88±0.94	6.81±0.79	7.03±0.73	7.06±0.79	7.06±0.94	6.79±0.84
C18.3n6	0.13±0.02	0.12±0.03	0.13±0.03	0.12±0.03	0.13±0.02	0.10±0.09	0.15±0.05	0.18±0.06	0.15±0.06	0.14±0.05	0.13±0.05	0.12±0.03
C20.2n6	0.09±0.01	0.08±0.06	0.11±0.02	0.12±0.02	0.12±0.04	0.12±0.04	0.19±0.02	0.20±0.05	0.17±0.03	0.17±0.03	0.23±0.04	0.19±0.04
C20.3n6	0.66±0.07	0.67±0.06	0.68±0.07	0.71±0.09	0.71±0.08	0.63±0.06	0.92±0.08	0.92±0.04	0.88±0.06	0.85±0.06	0.91±0.05	0.92±0.06
C20.4n6	2.09±0.27	2.16±0.20	2.19±0.23	2.26±0.29	2.43±0.16	2.30±0.17	3.90±0.60	3.94±0.37	4.08±0.38	4.12±0.55	4.26±0.27	4.11±0.32
C22.4n6	0.12±0.07	0.17±0.07	0.12±0.09	0.14±0.06	0.14±0.06	0.10±0.08	0.23±0.10	0.24±0.05	0.19±0.10	0.24±0.09	0.21±0.08	0.21±0.06
n-6 PUFA	**8.08±0.61**	**8.35±0.62**	**8.35±0.55**	**8.66±0.74**	**8.87±0.69**	**8.29±0.63**	**12.26±0.79**	**12.29±0.52**	**12.49±0.57**	**12.60±0.96**	**12.80±0.85**	**12.34±0.73**
C18.3n3	1.29±0.14	1.45±0.13	1.57±0.14	1.72±0.29	1.74±0.57	1.54±0.52	1.10±0.19	1.32±0.19	1.48±0.23	1.41±0.28	1.09±0.14	0.86±0.15
C18.4n3	0.30±0.04	0.30±0.04	0.31±0.04	0.33±0.05	0.34±0.01	0.40±0.04	0.21±0.03	0.24±0.03	0.22±0.04	0.22±0.03	0.16±0.02	0.15±0.01
C20.3n3	0.06±0.04	0.08±0.06	0.11±0.01	0.08±0.06	0.12±0.02	0.12±0.02	0.16±0.03	0.15±0.03	0.17±0.03	0.18±0.03	0.17±0.04	0.18±0.04
C20.5n3	28.86±3.70	28.38±4.86	27.96±4.82	25.26±4.53	20.63±4.97	20.41±5.04	11.41±1.21	11.07±1.08	10.64±0.85	10.21±0.76	9.36±0.52	9.27±0.71
C22.5n3	2.75±1.60	2.80±1.63	2.94±1.68	3.22±1.64	3.75±1.32	4.06±1.44	4.41±1.14	4.35±0.95	4.52±0.86	4.70±0.76	5.23±0.68	5.29±0.75
C22.6n3	20.19±1.65	20.00±1.47	19.85±1.36	19.45±1.44	20.23±1.01	20.79±2.11	10.71±1.79	10.40±1.27	10.44±1.15	10.62±1.59	10.43±1.50	10.19±1.24
n-3 PUFA	**53.45±0.82^{a}**	**53.02±3.36^{ab}**	**52.74±2.88^{ab}**	**50.10±2.25^{bc}**	**46.82±3.96^{c}**	**47.31±3.35^{c}**	**28.01±2.85^{a}**	**27.55±2.00^{a}**	**27.48±1.86^{a}**	**27.34±2.48^{ab}**	**26.44±2.41^{b}**	**25.92±2.23^{b}**

Total saturated fatty acids, total monounsaturated fatty acids, total n-6 PUFA, total n-3 PUFA. Values are expressed as % of total fatty acids mean±SD. Means that do not share a letter are significantly different from one another ($p < 0.05$). T = timepoint in hours. HC = high concentration. LC = low concentration. Mono = mono-unsaturated

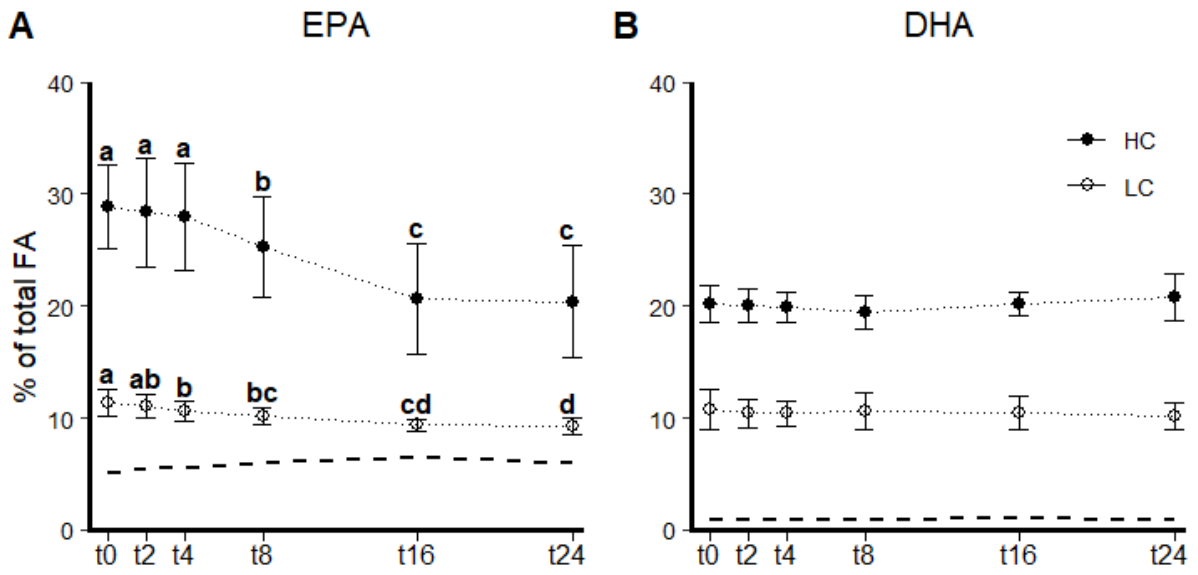

Figure 2.5: Temporal (24 h) changes in washout of EPA (A) and DHA (B) in C2C12 myotubes following overnight incubation with HC (50µM EPA + 33.33µM DHA) or LC (12.5µM EPA + 8.33µM DHA. Values are means ± SD (n=4, except HC 16 h, where n=3 due to a missing datapoint). Dashed line represents vehicle control over time. Mean values that do not share a common letter within HC or LC are statistically different from one another ($P < 0.05$). EPA and DHA levels differed significantly from VC in both HC and LC at all timepoints ($P < 0.05$), except t16 where $P = 0.06$. Furthermore, EPA and DHA levels differed significantly between HC and LC at all timepoints ($P < 0.05$).

2.3.2.2 Washout of EPA and DHA from the phospholipid fraction

A full breakdown of changes in fatty acid profile in phospholipid fractions after incubation with HC and LC can be found in **Table 2.4** EPA and DHA levels remained above VC in the phospholipid fraction over the 24 h washout period when treated with HC and LC ($P < 0.05$; **Figure 2.6**). An increase in EPA in the vehicle control was detected from 8 h onwards compared to 1 h of incubation ($P < 0.05$). Furthermore, the incorporation of EPA and DHA into the phospholipid fraction remained higher in HC compared to LC during the 24 h washout period ($P < 0.05$). After 24 h, EPA and DHA levels in the phospholipid fraction were 19.0 ± 0.4% and 136.9 ± 0.5% greater with HC than LC, respectively. Compared to the whole cell lysate, EPA and DHA levels in the phospholipid fraction were significantly lower following treatment with HC at 0, 2, 4 and 8 h after initiating the washout ($P < 0.05$). However, at 16 and 24 h after initiating washout, EPA and DHA levels were similar between the whole lysate and phospholipid fraction ($P > 0.05$). Following incubation with LC, EPA levels were similar between the whole cell lysate and phospholipid fraction at 0, 2, and 4 h ($P > 0.05$), however,

EPA levels were higher in the phospholipid fraction than the whole cell lysate after 8, 16, and 24 h ($P < 0.05$). Levels of DHA in the whole cell lysate were higher than in the phospholipid fraction at 2, 4, and 24 h ($P < 0.05$), while levels were similar at 0, 8, and 16 h ($P > 0.05$).

Following overnight incubation with HC, EPA levels in the phospholipid fraction decreased after 2 h (-1.0 ± 0.1%, 95% CI [-1.8 – -0.2]) when switching to the washout medium and continued to decrease at 16 h (-1.5 ± 0.4%, 95% CI [-2.5 – -0.7]; **Figure 2.6A**). However, no decline in EPA level was observed 8 h after switching to differentiation medium (-0.4 ± 0.7%, 95% CI [-1.2 – 0.4], $P = 0.61$). The overall decline in EPA over 24 h was 2.8 ± 0.6%. Likewise, EPA levels after overnight incubation with LC decreased after 2 h (-1.1 ± 0.1%, 95% CI [-1.1 –-0.1]) when switching to the washout medium, however, this decline in EPA was not observed 8 h after switching to washout medium (0.2 ± 0.4%, 95% CI [-0.3 – 0.7]). An increase in EPA incorporation was observed between 2 - 8 h (0.8 ± 0.3%, 95% CI [0.3 – 1.3]) whereas a decline in EPA levels was observed between 8 – 16h (-1.0 ± 0.1%, 95% CI [-1.5 – -0.5]), with no further changes observed thereafter. EPA levels decreased by 0.6 ± 0.3% over the 24 h washout period. Conversely, for DHA (**Figure 2.6B)**, an increase in incorporation in the phospholipid fraction of 2.9 ± 0.5% (95% CI [1.8 – 3.9]) was observed after 8 h when switching to washout medium following overnight incubation with HC. A further increase in DHA incorporation was detected between 8 – 16 h (3.9 ± 0.3%, 95% CI [2.4 – 4.8]). After incubation with LC, no differences in DHA incorporation in the phospholipid fraction were detected from the start of the washout period over 24 h. However, a modest increase in DHA incorporation was observed between 2 and 8 h (1.1 ± 0.5%, 95% CI [0.5 – 1.8]), with a subsequent decline between 8 – 24 h (-0.9 ± 0.6%, 95% CI [-1.6 – -0.3]).

Table 2.4: changes in fatty acid profile over time in C2C12 phospholipid fractions during 24h washout following incubation with HC or LC

	HC (high concentration)						LC (low concentration)					
Fatty Acid	t0	t2	t4	t8	t16	t24	t0	t2	t4	t8	t16	t24
C14.0	0.79±0.09	0.80±0.06	0.83±0.07	0.81±0.09	0.71±0.05	0.84±0.13	1.05±0.09	1.14±0.07	1.06±0.04	1.26±0.07	1.50±0.04	1.55±0.09
C16.0	24.26±1.45	25.77±0.66	26.21±1.13	20.79±0.37	21.59±0.65	22.01±0.89	24.91±1.22	26.71±0.97	26.61±1.10	22.60±0.49	24.60±0.31	24.92±0.72
C18.0	11.81±0.70	12.37±0.45	12.35±0.64	10.67±0.34	12.03±0.54	11.64±0.78	11.35±0.51	12.17±0.59	12.01±0.35	10.44±0.36	10.80±0.50	10.78±0.72
C20.0	0.22±0.04	0.19±0.03	0.19±0.04	0.20±0.04	0.18±0.04	0.18±0.02	0.21±0.04	0.19±0.03	0.16±0.04	0.20±0.06	0.17±0.02	0.18±0.04
C22.0	0.13±0.05	0.11±0.03	0.11±0.03	0.12±0.02	0.13±0.02	0.14±0.04	0.11±0.05	0.12±0.04	0.12±0.02	0.14±0.02	0.12±0.02	0.10±0.03
C24.0	0.30±0.04	0.29±0.09	0.33±0.11	0.35±0.04	0.34±0.01	0.34±0.10	0.31±0.05	0.29±0.09	0.32±0.08	0.38±0.05	0.32±0.05	0.32±0.08
Saturated	**37.87±2.08^{a}**	**39.92±1.21^{ab}**	**40.43±1.80^{b}**	**33.37±0.51^{c}**	**35.43±1.17^{cd}**	**35.63±1.34^{d}**	**38.33±1.82^{a}**	**41.04±1.49^{b}**	**40.69±1.08^{b}**	**35.52±0.59^{c}**	**37.98±0.78^{a}**	**38.31±1.19^{a}**
C16.1n7	3.00±0.34	2.68±0.31	2.52±0.44	2.54±0.41	1.69±0.36	1.92±0.51	4.42±0.51	4.01±0.56	3.79±0.59	3.92±0.78	3.85±0.56	3.99±0.63
C18.1n9	8.12±0.73	7.74±0.62	7.61±0.73	8.29±0.72	6.77±0.92	7.41±1.17	10.60±0.66	9.94±0.70	10.02±0.63	10.75±0.78	10.46±0.80	10.57±0.85
C18.1n7	3.83±0.69	3.58±0.61	3.43±0.71	3.75±0.74	3.09±0.46	3.59±0.77	4.32±0.69	4.25±0.75	4.08±0.81	4.29±0.96	4.23±0.75	4.21±0.84
C20.1n9	0.14±0.04	0.12±0.01	0.12±0.02	0.13±0.02	0.13±0.01	0.11±0.01	0.12±0.02	0.12±0.01	0.13±0.02	0.11±0.01	0.10±0.01	0.11±0.01
C20.3n9	0.02±0.02	0.01±0.01	0.02±0.02	0.01±0.02	0.00±0.00	0.01±0.01	0.03±0.02	0.04±0.03	0.03±0.02	0.02±0.03	0.02±0.02	0.03±0.04
C24.1n9	0.52±0.08	0.51±0.18	0.57±0.20	0.64±0.06	0.63±0.08	0.65±0.18	0.49±0.14	0.56±0.17	0.62±0.14	0.69±0.10	0.63±0.07	0.62±0.16
Mono	**15.73±1.57^{a}**	**14.73±1.32^{bc}**	**14.37±1.70^{b}**	**15.46±1.77^{c}**	**12.40±1.56^{d}**	**13.78±2.22^{bd}**	**20.08±1.65^{a}**	**19.00±1.83^{ab}**	**18.77±1.92 ^{b}**	**19.88±2.32^{ab}**	**19.40±1.92^{ab}**	**19.63±2.03^{ab}**
C18.2n6	3.73±0.35	3.83±0.30	3.79±0.26	4.22±0.23	3.63±0.38	3.71±0.32	5.00±0.43	5.10±0.33	5.13±0.26	5.73±0.23	5.78±0.31	5.68±0.32
C18.3n6	0.07±0.02	0.08±0.03	0.09±0.02	0.09±0.03	0.09±0.03	0.09±0.02	0.11±0.03	0.12±0.03	0.11±0.03	0.12±0.03	0.10±0.03	0.11±0.03
C20.2n6	0.09±0.03	0.10±0.03	0.11±0.03	0.09±0.03	0.08±0.02	0.09±0.02	0.11±0.01	0.10±0.03	0.08±0.01	0.12±0.04	0.11±0.02	0.13±0.03
C20.3n6	0.41±0.02	0.42±0.02	0.40±0.03	0.42±0.07	0.35±0.04	0.35±0.05	0.56±0.03	0.54±0.02	0.52±0.03	0.56±0.05	0.54±0.05	0.59±0.05
C20.4n6	3.14±0.11	3.06±0.08	3.07±0.11	3.36±0.21	3.33±0.12	3.29±0.16	3.89±0.22	3.83±0.19	3.79±0.24	4.21±0.36	3.89±0.25	3.89±0.35
C22.4n6	0.23±0.03	0.23±0.06	0.22±0.04	0.26±0.03	0.23±0.03	0.22±0.02	0.30±0.04	0.30±0.06	0.30±0.07	0.32±0.05	0.30±0.05	0.30±0.05
n-6 PUFA	**7.68±0.44^{a}**	**7.71±0.29^{a}**	**7.67±0.26^{a}**	**8.43±0.28^{b}**	**7.72±0.27^{a}**	**7.75±0.15^{a}**	**9.97±0.55^{a}**	**10.00±0.39^{a}**	**9.94±0.32^{a}**	**11.05±0.38 ^{b}**	**10.72±0.38^{b}**	**10.70±0.37^{b}**
C18.3n3	0.97±0.16	1.21±0.13	1.25±0.20	1.32±0.19	0.78±0.20	0.79±0.19	0.84±0.19	1.30±0.25	1.34±0.29	1.32±0.29	0.97±0.20	0.81±0.28
C20.3n3	0.13±0.01	0.14±0.04	0.14±0.02	0.17±0.01	0.14±0.03	0.15±0.04	0.15±0.04	0.18±0.03	0.18±0.03	0.19±0.04	0.21±0.01	0.20±0.04
C20.5n3	16.02±0.26	15.05±0.40	14.42±0.62	15.62±0.98	13.52±0.82	13.24±0.90	11.77±0.28	11.13±0.38	10.90±0.49	11.93±0.70	10.96±0.56	11.13±0.53
C22.3n3	0.03±0.04	0.04±0.05	0.04±0.05	0.03±0.04	0.05±0.05	0.04±0.05	0.04±0.05	0.03±0.04	0.05±0.06	0.04±0.05	0.04±0.06	0.05±0.07
C22.5n3	2.86±0.15	2.86±0.20	2.97±0.14	3.66±0.20	4.20±0.40	4.27±0.30	4.37±0.29	4.19±0.28	4.37±0.35	5.09±0.57	5.61±0.38	5.55±0.50
C22.6n3	13.58±0.87	13.38±0.89	13.74±0.62	16.44±1.33	20.36±1.04	19.16±0.94	8.45±0.64	7.86±0.55	8.12±0.69	9.00±1.02	8.50±0.64	8.09±0.47
n-3 PUFA	**33.59±0.82^{a}**	**32.69±0.93^{a}**	**32.56±0.49^{a}**	**37.24±1.75^{b}**	**39.04±0.94^{b}**	**37.65±1.13^{b}**	**25.63±0.86^{ab}**	**24.69±0.76^{a}**	**24.97±1.09^{a}**	**27.58±1.70^{c}**	**26.28±1.2^{b}**	**25.83±1.04^{ab}**

Total saturated fatty acids, total monounsaturated fatty acids, total n-6 PUFA, total n-3 PUFA. Values are expressed as % of total fatty acids mean±SD. Means that do not share a letter are significantly different from one another ($p < 0.05$). T = timepoint in hours. HC = high concentration. LC = low concentration. Mono = mono-unsaturated

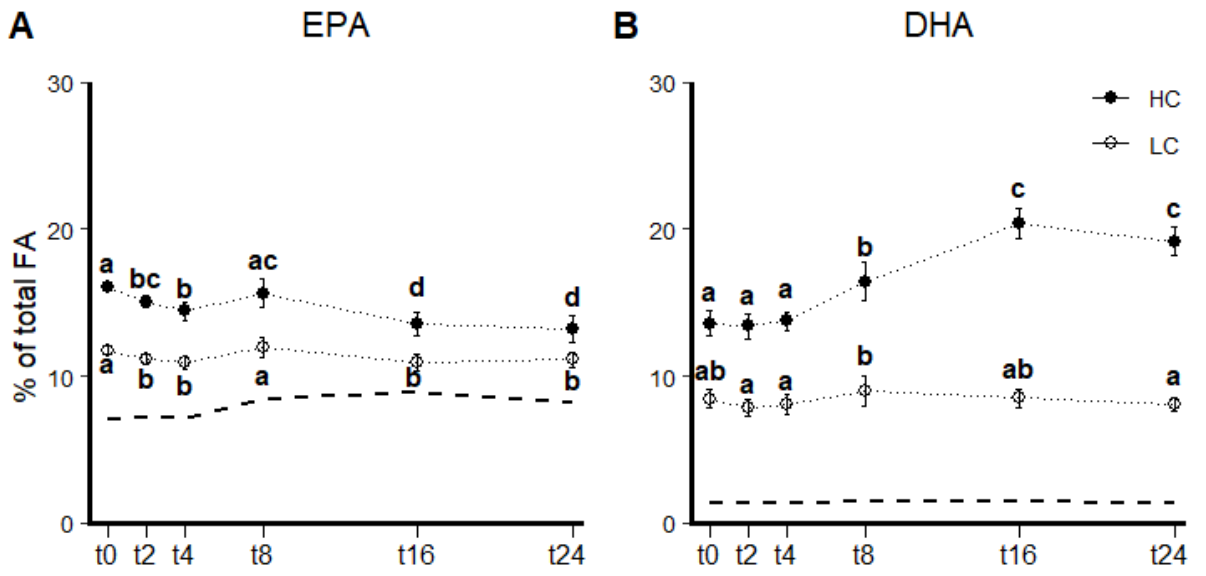

Figure 2.6: Temporal (24 h) changes in washout of EPA (A) and DHA (B) in the phospholipid fraction of C2C12 myotubes following overnight incubation with HC (50µM EPA + 33.33µM DHA) or LC (12.5µM EPA + 8.33µM DHA. Values are means ± SD (n=3). Dashed line represents vehicle control (VC) over time. Mean values that do not share a common letter within HC or LC are statistically different from one another ($P < 0.05$). EPA and DHA levels differed significantly from VC in HC and LC at all timepoints ($P < 0.05$). Furthermore, EPA and DHA levels differed significantly between HC and LC at all timepoints ($P < 0.05$), except at 16 h for EPA ($P = 0.06$).

2.3.2.3 Formation of DPA during washout in response to EPA:DHA supplementation

DPA levels in the whole cell lysate did not differ from the phospholipid fraction following incubation with HC or LC ($P > 0.05$). In the whole cell lysate (**Figure 2.7A**), an increase in DPA levels was detected in VC from 16 h onwards compared to start of washout ($P < 0.05$).

Following incubation with HC, no significant DPA formation was detected in the whole cell lysate at any timepoint compared to VC ($P > 0.05$; **Figure 2.7A**). However, DPA formation increased 1.3 ± 0.2% (95% CI [0.6 – 2.0]) after 24 h compared to the start of washout. After incubation with LC, significant levels of DPA in the whole cell lysate were detected at all timepoints compared to VC during the 24-hour washout period ($P < 0.05$; **Figure 2.7A**). In LC over time, DPA formation increased 0.9 ± 0.4%, 95% CI [0.4 – 1.3] after 24 h compared to the start of washout. There were no differences detected in DPA levels between HC and LC at any timepoint ($P > 0.05$).

In the phospholipid fraction (**Figure 2.7B**), an increase in DPA in the vehicle control was detected from 16 h onwards compared to start of washout ($P < 0.05$). Compared to VC, DPA levels were lower following treatment with HC after 0, 2 and 4 h after washout ($P < 0.05$), with no differences after 8 h ($P > 0.05$; **Figure 2.7B**). Over time, DPA levels increased between 0 –

8 h (0.8 ± 0.1%, 95% CI [0.4 – 1.2]), and 8 – 16 h (0.5 ± 0.2%, 95% CI [0.1 – 0.9]). Following incubation with LC, no additional formation of DPA was observed in the phospholipid fraction at any timepoint compared to VC ($P > 0.05$; **Figure 2.7B**). Over time, DPA formation increased after 8 h when switching to washout medium (0.7 ± 0.3%, 95% CI [0.3 – 1.1]), with a further increase detected between 8 – 16 h (0.5 ± 0.2%, 95% CI [0.1 – 0.9]). DPA incorporation differed between HC and LC at all timepoints ($P < 0.05$).

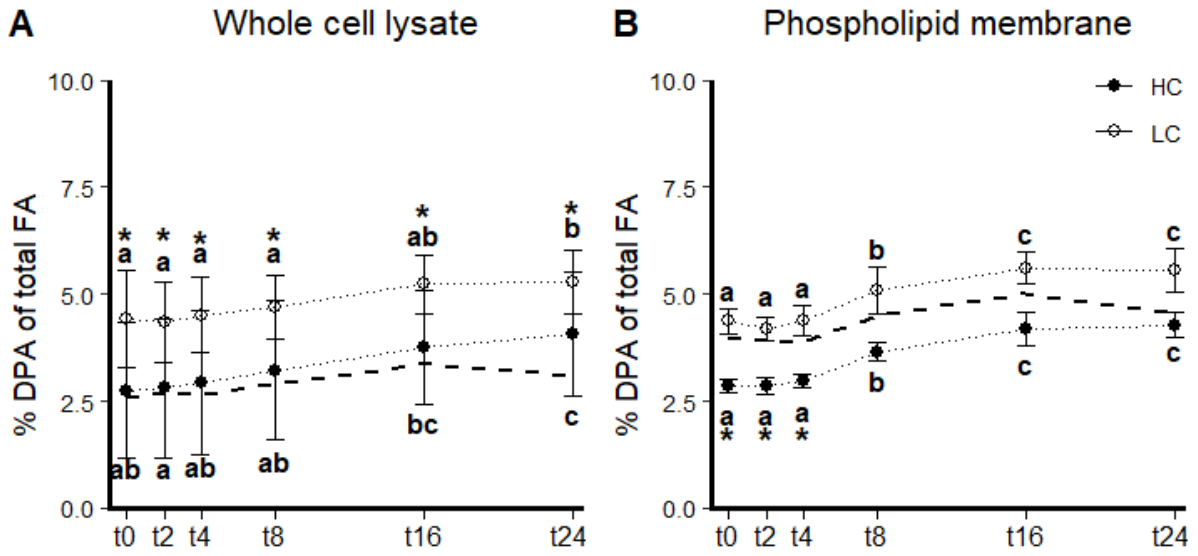

Figure 2.7: Temporal (24 h) changes in formation of DPA during the washout period in whole cell lysates of C2C12 myotubes (A, n=4) and phospholipid fraction (B, n=4, except t16 HC, where n=3) following overnight incubation with HC (50µM EPA + 33.33µM DHA) or LC (12.5µM EPA + 8.33µM DHA). Dashed line represents vehicle control (VC) over time. Values are means ± SD. Mean values that do not share a common letter within HC or LC are statistically different from one another ($P < 0.05$). Differences from VC are denoted by asterisks (*; $P < 0.05$). In the whole cell lysate (A), DPA formation in HC and LC did not differ significantly at any timepoint ($P > 0.05$). In the phospholipid fraction (B), DPA formation in HC and LC differed significantly at all timepoints ($P < 0.05$).

2.3.3 Muscle protein synthesis

2.3.3.1 Muscle protein synthesis in response to an insulin/leucine stimulus

The I/L trigger condition significantly upregulated protein synthesis rates compared to the basal control (CON) after the 16 h incubation and 24 h washout period ($P < 0.05$), confirming that the I/L trigger worked but did not significantly differ from VC ($P > 0.05$; **Figure 2.8**).

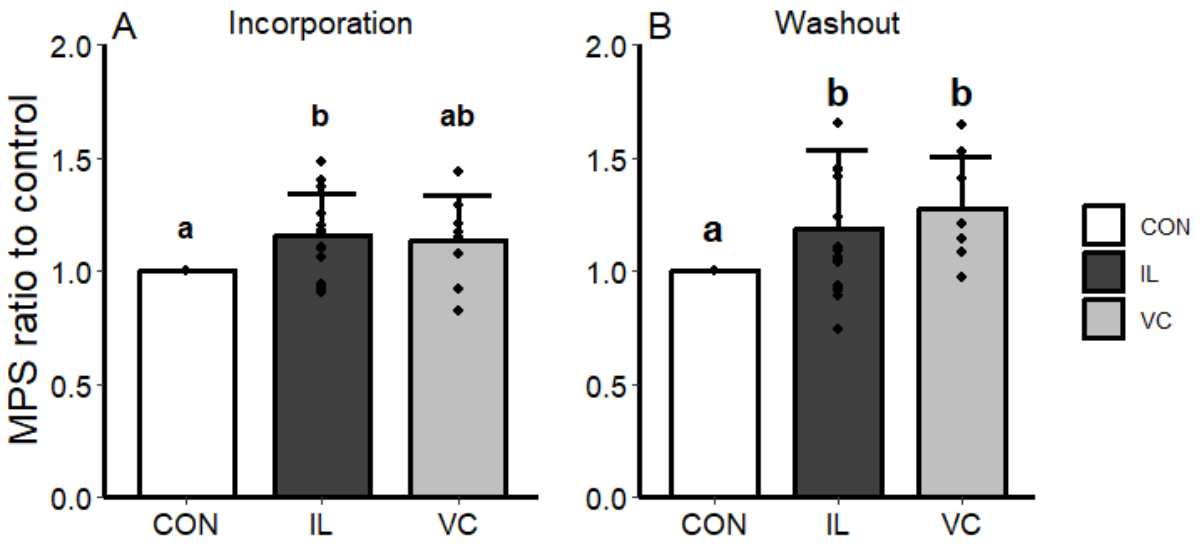

Figure 2.8: Protein synthesis in C2C12 myotubes following 16 h incubation (A) or 24 h washout (B) after incubation with CON (basal control), IL (1 mM leucine + 100 nM insulin trigger) or VC (vehicle control; IL + 100 % ethanol diluted to 1.28 % with PBS + 2.5 % albumin).). Protein synthesis is calculated as the area under the curve (AUC) for puromycin incorporation, with the AUC for CON set to 1. Data for IL and VC are expressed relative to CON, representing protein synthesis relative to the basal control. Values are means+ SD (n=4 in duplicate, except IL where n=4 in quadruplicate). Mean values that do not share a common letter are statistically different from one another ($P < 0.05$). MPS = muscle protein synthesis.

In the experimental conditions (**Figure 2.9A**), there were no additional rates of MPS observed after 16 h of incubation with HC or LC compared to VC ($P > 0.05$). However, following 24 h washout, the LC condition exhibited a 19.7 ± 1.2% higher MPS rate compared to VC (**Figure 2.9B**).

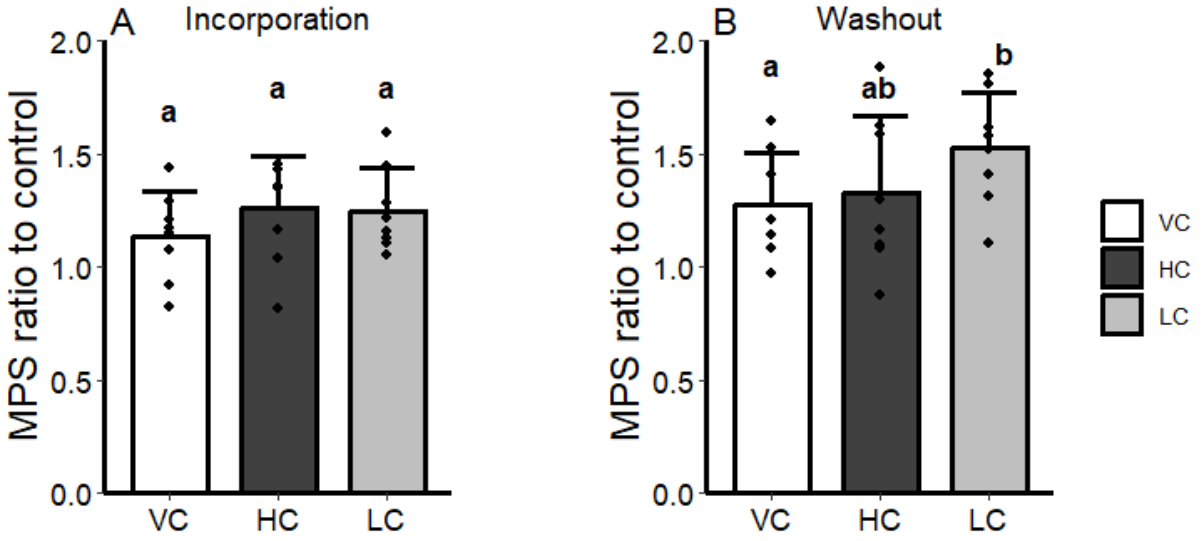

Figure 2.9: Protein synthesis in C2C12 myotubes following 16 h incubation (A) or 24 h washout (B) with VC (vehicle control; vehicle control; 100 % ethanol diluted to 1.28 % with PBS + 2.5 % albumin), HC (high concentration: 50µM EPA + 33.33µM DHA) or LC (12.5µM EPA + 8.33µM DHA). All conditions were treated with 1 mM leucine + 100 nM insulin trigger 60min prior to cell collection. Protein synthesis is calculated as the area under the curve (AUC) for puromycin incorporation, with the AUC for CON (Figure 2.8) set to 1. Data for IL and VC are expressed relative to CON, representing protein synthesis relative to the basal control. Values are means+ SD (n=4 in duplicate). Mean values that do not share a common letter are statistically different from one another ($P < 0.05$). EPA = Eicosapentaenoic acid, DHA = Docosahexaenoic acid, VC = vehicle control, HC = high concentration, LC = low concentration.

2.3.3.2 Signalling pathways in response to an insulin/leucine stimulus

The I/L trigger significantly upregulated phosphorylation of p70S6K1$^{[Thr421/Ser424]}$ and Akt$^{[Ser473]}$ after 16 h of incubation and 24 h washout compared to basal control ($P < 0.05$), but did not differ from VC ($P > 0.05$; **Figure 2.10B, C, E, F**). The phosphorylation status of 4E-BP1$^{[Thr37/46]}$ was upregulated by the I/L trigger after 16 h of incubation ($P < 0.05$; **Figure 2.10A**), however, no distinction in phosphorylation of 4E-BP1$^{[Thr37/46]}$ was observed between the I/L trigger and basal control after 24 h washout ($P > 0.05$; **Figure 2.10D**). The I/L trigger condition did not differ from VC for 4E-BP1$^{[Thr37/46]}$, after 16 h of incubation or 24 h washout (**Figure 2.10A, D**; $P > 0.05$).

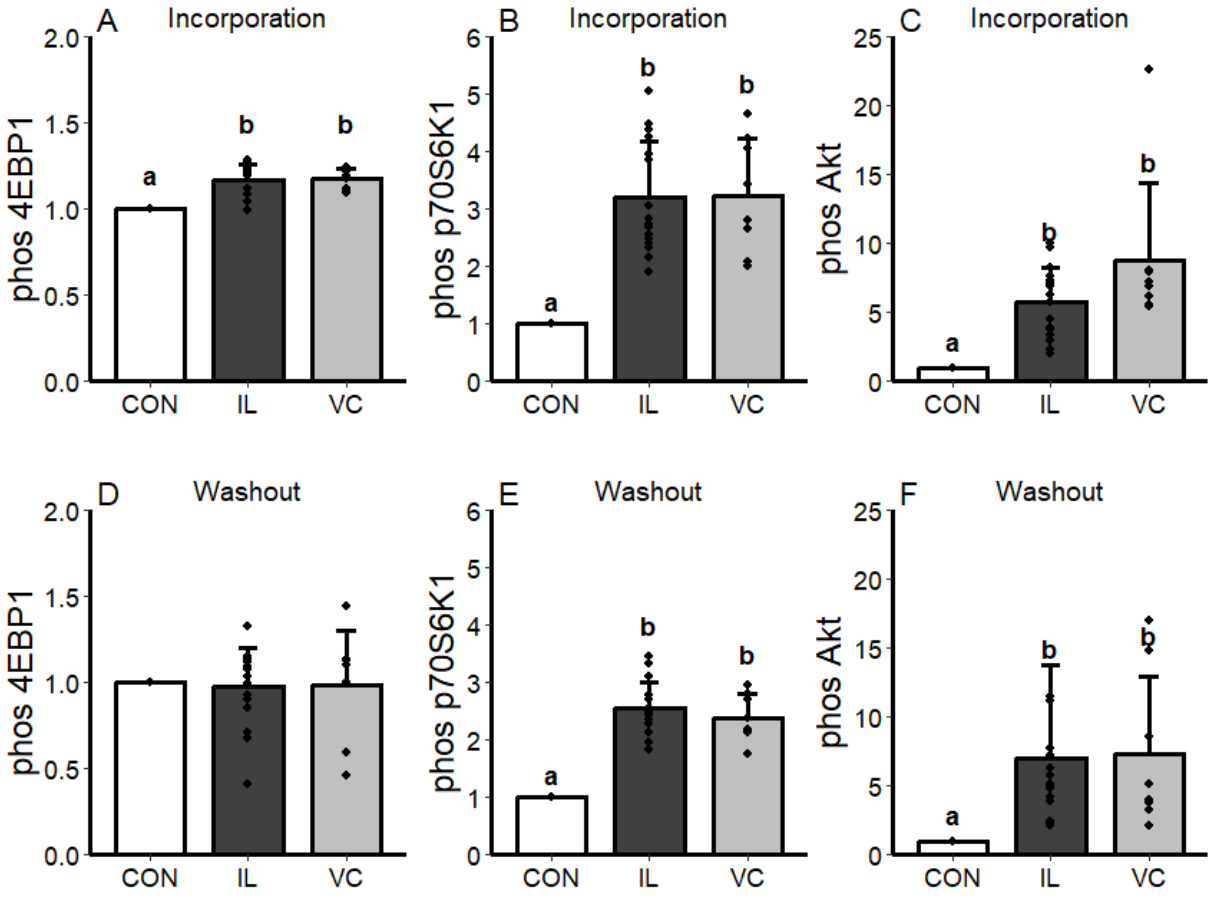

Figure 2.10: Phosphorylation status of signalling pathway proteins in in C2C12 myotubes following 16 h incubation (A, B, C) or 24 h washout (D, E, F) CON (basal control), IL (1 mM leucine + 100 nM insulin trigger) or VC (vehicle control; IL + 100 % ethanol diluted to 1.28 % with PBS + 2.5 % albumin). Data represent phosphorylated/total 4E-BP1$^{[Thr37/46]}$, p70S6K1$^{[Thr421/Ser424]}$, and Akt$^{[Ser473]}$. Values are mean + SD (n = 4 in duplicate for CON and VC, n = 4 in quadruplicate for IL). Mean values that do not share a common letter are statistically different from one another ($P < 0.05$). 4E-BP1 = eukaryotic translation initiation factor 4E-binding protein 1. Akt = Protein Kinase B. p70S6K1 = ribosomal protein S6 kinase beta-1.

In the experimental conditions there were no differences in the phosphorylation status of 4E-BP1[Thr37/46], p70S6K1[Thr421/Ser424] or Akt[Ser473] after 16 h of incubation with HC or LC ($P > 0.05$; **Figure 2.11**).

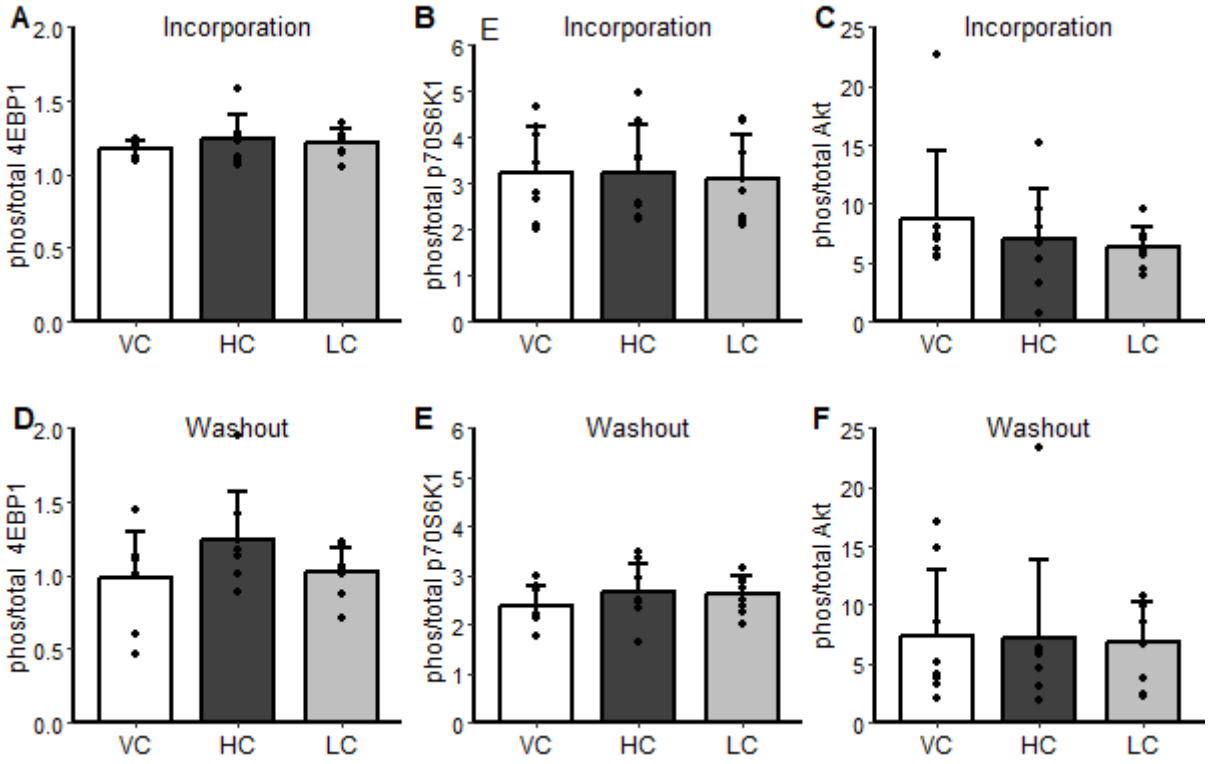

Figure 2.11: Phosphorylation status of signalling pathway proteins in C2C12 myotubes following 16 h incubation (A, B, C) or 24 h washout (D, E, F) after incubation with VC (vehicle control), HC (50µM EPA + 33.33µM DHA) or LC (12.5µM EPA + 8.33µM DHA). All conditions were treated with 1 mM leucine + 100 nM insulin trigger 60min prior to cell collection. Data represent phosphorylated/total 4E-BP1[Thr37/46], p70S6K1[Thr421/Ser424], and Akt[Ser473]. Values are mean + SD (n = 4 in duplicate). EPA = Eicosapentaenoic acid, DHA = Docosahexaenoic acid, HC = high concentration, LC = low concentration. 4E-BP1 = eukaryotic translation initiation factor 4E-binding protein 1. Akt = Protein Kinase B. p70S6K1 = ribosomal protein S6 kinase beta-1.

2.4 Discussion

This *in vitro* study examined the uptake and washout profiles of EPA and DHA in the whole cell lysate and phospholipid fraction of C2C12 myotubes during incubation with a low and high concentration EPA:DHA mixture, in combination with measurements of stimulated rates of MPS and associated mTORC1 signalling pathway proteins. The study revealed four main findings. First, EPA and DHA exhibit disparities in uptake, as evidenced by the stabilization of EPA uptake within 8 hours, while no comparable stabilization is observed for DHA uptake, seen in both the whole cell lysate and phospholipid fraction fraction. Second, over a 24-hour washout period a partial washout of EPA was observed in the whole cell lysate, with no washout of DHA. Likewise, within the phospholipid fraction, EPA exhibited a partial washout, whereas an increase in DHA level was observed during the washout phase. Third, DPA formation was observed in the whole cell lysate and phospholipid fraction after 24 h of incubation in LC, but not with HC. In the washout experiments, DPA was only significantly elevated in the whole cell lysate when incubating with LC, whereas pre-incubation with HC inhibited DPA formation in the phospholipid membrane during the washout phase. Finally, 16 h incubation with LC followed by a 24 h washout resulted in increased rates of insulin/leucine stimulated protein synthesis, independent of changes in the phosphorylation status of signalling kinases 4E-BP1$^{[Thr37/46]}$, p70S6K1$^{[Thr421/Ser424]}$, and Akt$^{[Ser473]}$. Taken together, these *in vitro* data indicate a finite effect of EPA:DHA incorporation into the phospholipid fraction in upregulating the stimulation of MPS in C2C12 myotubes.

2.4.2 Differences in uptake of EPA and DHA in C2C12 myotubes

Our findings indicate that EPA was fully incorporated into whole cell lysate and the phospholipid fraction 8 h after incubation with HC and LC, while no plateau in DHA was observed over the 24 h incubation period. This differential trend in incorporation pattern between EPA and DHA is consistent with *in vivo* studies, where EPA is incorporated more rapidly than DHA into erythrocytes, blood mononuclear cells, serum cholesteryl esters, plasma fractions, and adipose tissue (Katan *et al.*, 1997; Metherel *et al.*, 2009; Browning *et al.*, 2012). The differences in incorporation profile between EPA and DHA likely relates to their respective lipid structures and distributions within the phospholipid membrane. In this regard, DHA is primarily incorporated into phospholipids as a more stable lipid fraction, with a lower abundance in triacylglycerol and sterol esters, whereas EPA appears to be equally distributed between neutral lipids and phospholipids (Arterburn, Hall and Oken, 2006). In the phospholipid

fractions, EPA is preferentially incorporated into phosphatidylcholine (PC) species of erythrocytes that are primarily located on the outer segment of the membrane (Cartwright *et al.*, 1985). In contrast, DHA is preferentially incorporated into phosphatidylethanolamine (PE) species located on the inner segment of the membrane, requiring DHA to be transported through the cellular membrane for incorporation which may explain the slower incorporation of DHA into the cellular membranes of skeletal muscle cells (Pal *et al.*, 2020). Although the distribution of EPA and DHA within phospholipid membranes of total skeletal muscle tissue have not been extensively studied, a similar trend in skeletal muscle cells can be anticipated based on the observed pattern in the membranes of erythrocytes and aligns with observations in our C2C12 cell model.

The magnitude of difference in EPA and DHA uptake between HC and LC was greater in the whole cell lysate than in the phospholipid fraction. This observation could be attributed, at least in part, to the regulatory mechanisms that govern the incorporation of EPA and DHA into the phospholipid membrane, resulting in an accumulation of excess EPA and DHA in other fractions of the cell. Accordingly, we detected a difference in EPA and DHA uptake between the whole cell lysate and phospholipid fraction when incubating C2C12 myotubes with HC. In humans, the provision of higher doses of n-3 PUFA increased EPA and DHA levels across several blood fractions, adipose, and muscle tissue (Katan *et al.*, 1997; Smith *et al.*, 2011a; Browning *et al.*, 2012; McGlory *et al.*, 2014). Notably, in blood, differences in relative abundance of EPA + DHA were dependent on which pool was measured (Browning *et al.*, 2012), thus confirming disparities between cellular fractions. In terms of skeletal muscle tissue, disparities also were apparent among cellular fractions. Gerling *et al.,* (2019) reported that EPA and DHA were enriched in the mitochondrial membrane, while only DHA was detected in the sarcolemmal fraction, suggesting divergent responses to EPA and DHA incorporation. In conclusion, recognizing the disparities between EPA and DHA, including their potential competition for incorporation and variations in incorporation across cellular fractions, is vital for comprehensive understanding of their divergent roles.

2.4.3 Differences in washout of EPA and DHA in C2C12 myotubes

Understanding the washout dynamics of individual omega-3 species is important for the maintenance of cellular physiological function associated with EPA and DHA within the phospholipid membrane. Multiple human studies have investigated washout patterns in erythrocytes and several plasma fractions following fish oil supplementation (Marangoni *et al.*,

1993; Katan *et al.*, 1997; Zuijdgeest-Van Leeuwen *et al.*, 1999; Metherel *et al.*, 2009). Indeed, these studies have consistently revealed a more rapid washout of EPA compared to DHA. However, in skeletal muscle the washout dynamics of EPA and DHA are yet to be characterised. Our findings in C2C12 myotubes are consistent with observations in blood, although we observed no washout of DHA from the whole cell lysate and noted an increase in DHA in the phospholipid fraction after washout when incubating with HC. One plausible explanation for the increased DHA content in the phospholipid fraction during washout relates to the notion that EPA undergoes metabolic conversion into DHA (Calder, 2017). However, Jeromson *et al.,* (2018) demonstrated that incubating C2C12 myotubes for 72 h with 50 μM EPA resulted in elevated DPA, but not DHA levels. While other human studies reported limited conversion rates of EPA to DHA during EPA supplementation when examining plasma and erythrocyte phospholipid levels (Ballantyne *et al.*, 2019; Guo *et al.*, 2020), a recent human tracer study attributed the increased DHA levels to exogenous EPA supplementation (Metherel *et al.*, 2019). Thus, it is plausible that EPA provided to our C2C12 myotubes is converted to DHA in the washout period, resulting in increased DHA in the phospholipid fraction. Future tracer studies in muscle tissue and C2C12 myotubes are warranted to confirm this notion.

While a decline in EPA was detected in the whole cell lysate during the washout phase, this decline was less pronounced in the phospholipid fraction, with an increase in EPA between 4 – 8 h in the washout phase. This increase in EPA can be attributed to the increased EPA levels in VC observed during the washout phase. It is noteworthy to mention that the increase in EPA in VC was not at the expense of any of its precursor compounds in the cell's lipid profile – namely alpha-linolenic acid (ALA), stearidonic acid (not detected), and eicosatetraenoic acid (**Appendix 2.9**). The growth medium supplemented to the cells contained 10% FBS, a serum known to contain EPA and DHA (Else, 2020), with concentrations varying between manufacturers (Koch *et al.*, 2021). While the specific fatty acid composition of the FBS used in this study was not analysed, it may have influenced the overall fatty acid profile. However, since the growth medium was added before differentiation, its impact was likely minimal. More notably, the differentiation medium for incubating C2C12 myotubes included ALA, a precursor that may have been converted to EPA, a process that has been observed in blood fractions in humans (Brenna *et al.*, 2009). Additionally, an increase in ALA was observed over time in VC (**Appendix 2.9**), supporting this notion.

Despite a more substantial absolute decrease in EPA levels during the washout period, a higher EPA level was consistently observed following the HC compared to LC, with the magnitude greater in the whole cell lysate. These findings suggest a propensity for the phospholipid

membrane to retain EPA, while the observed loss in the cell lysates may originate from other cellular fractions. Consequently, this implies that high concentrations are advantageous for retention of n-3 PUFA. In humans, a washout study that examined blood fractions observed a more rapid washout from the phospholipid fractions in plasma lipoproteins compared to the erythrocyte membrane (Cao *et al.*, 2006). Hence, it is plausible that the EPA loss from other cellular fractions, rather than the phospholipid membrane, may be associated with conversion to DHA, leading to increased DHA being observed during the washout period in the phospholipid membranes of C2C12 myotubes in our study.

2.4.4 DPA formation

Based on the decline of EPA in the whole cell lysate after 24 h of incubation with the low concentration, we hypothesized that the rise in DPA could be attributed to conversion from EPA. Similarly, Jeromson *et al.,* (2018) detected DPA formation in the phospholipid fraction following incubation with 50 μM EPA, but this was not observed when incubating with 50 μM DHA. Moreover, the presence of DPA was detected in plasma phospholipid membranes of humans that consumed DPA + EPA oil for 6 consecutive days (Guo *et al.*, 2020), and in skeletal muscle tissue of tumour-bearing mice following ingestion of fish oil, tuna oil, and isolated EPA (Dijk *et al.*, 2019). Collectively, these data indicate that the DPA detected in our study was primarily derived from exogenous EPA. Moreover, our data suggests that the DPA detected in the whole cell lysate was primarily located in the phospholipid fraction, given that no difference in DPA accumulation was observed between whole cell lysate and phospholipid fraction in the uptake and washout experiments (**Figure 2.4, 2.7**). Interestingly, formation of DPA was only observed after 24 h washout following incubation with LC, but not significantly with HC. One possible explanation is the competition between EPA and DHA, which proposes that accumulated EPA levels observed in plasma across studies are the result of a slowed metabolism of EPA due to accumulation of DHA, which in turn attenuates the rate of conversion from EPA to DPA (Pal *et al.*, 2020). The higher concentration of DHA in HC in the present study may have hindered the conversion rate of EPA to DPA. Aligned with this observation, a study conducted in rats using specific isotope analysis suggested that incorporation of DHA was responsible for a reduced conversion of EPA to DPA (Metherel *et al.*, 2017). In line with the uptake experiment, DPA formation was detected in the whole cell lysate at any timepoint during the washout period when treated with LC, but not HC. Intriguingly, no accumulation of DPA was observed in phospholipid fraction compared to VC

during the washout period, regardless of concentration administrated. In fact, administering HC seemed to prevent DPA accumulation in the first 4 h during washout compared to VC, consistent with the hypothesis that higher levels of DHA in HC may prevent metabolism of EPA to DPA.

2.4.5 Protein synthesis in response to incorporation and washout

The greater incorporation of EPA and DHA into the C2C12 lysates and phospholipid fractions with HC did not translate to a further increase in stimulation of MPS beyond VC. Instead, an increased stimulation of MPS was observed after 16 h of incorporation followed by a 24 h washout in the LC condition only. A potential explanation for this observation relates to the observation that the total concentration of EPA + DHA used in HC induced cellular stress, as evidenced by visual inspection of protein content. Consequently, this increased stress response may have negated any increased stimulation of MPS. In the present study, we administered a combination of EPA + DHA to simulate the n-3 PUFA content of common fish oil supplements administered in human studies (Witard *et al.*, 2023). Conversely, previous *in vitro* studies reported an increase in MPS and suppression of muscle protein breakdown (Kamolrat and Gray, 2013) or increased protein accretion (Jeromson *et al.*, 2018) after incubation with 50 μM EPA, whereas DHA had no effect. Hence, it is plausible that the higher dose of DHA in addition to EPA in HC in the present study attenuated the independent effect of EPA on muscle protein metabolism and that there may be interplay and/or competition between EPA and DHA in terms of regulating muscle protein metabolism. Moreover, an increased stimulation of MPS was observed after the 24 h washout phase, albeit not in response to the 16 h incorporation phase. Further analysis of our incorporation data revealed that the compositional profile of EPA + DHA in LC after the 24 h washout period was similar to the 16 h incorporation period, but with a marked rise in DPA levels (**Appendix 2.1 – 2.8**). Although EPA is commonly regarded as the most bioactive n-3 PUFA species in terms of stimulating MPS (Witard *et al.*, 2023), the data in this chapter suggest that the presence of DPA in the skeletal muscle membrane in addition to EPA could modulate the stimulation of MPS. However, this hypothesis is preliminary and requires further investigation. Taken together, the greater MPS response compared with VC following the 24 h washout phase with LC incubation may be attributed to factors such as the cumulative quantity of n-3 PUFA administered, the potential interaction between EPA and DHA that are likely less pronounced in LC, or the increased formation of DPA, which was markedly higher after LC compared to HC.

The mTORC1 pathway consists of cell signalling proteins known to regulate the stimulation of MPS (Zhou and Huang, 2010). Despite observing an increased stimulation of MPS after the 24 h washout period in LC, there were no changes in the phosphorylation status of 4E-BP1$^{[Thr37/46]}$, p70S6K1$^{[Thr421/Ser424]}$, or Akt$^{[Ser473]}$. This disassociation between molecular signals that regulate MPS has also been observed in previous *in vitro* studies (Kamolrat and Gray, 2013; Jeromson *et al.*, 2018), and in human studies (Greenhaff *et al.*, 2008; Holowaty *et al.*, 2023). In the present study, a possible explanation for the increased stimulation of MPS in the absence of an upregulation of mTORC1 signalling relates to the notion that incorporation of EPA and DHA after incubation with LC may have accelerated or optimized signalling. Accordingly, it was previously demonstrated that a lower and more efficient kinase activity was required to maximize MPS following an 8-week period of fish oil supplementation (McGlory *et al.*, 2016). These findings suggest that fish oil supplementation results in a shift in kinase signalling and MPS, suggesting that a reduced kinase activity is required to maximize MPS. This observation could explain, at least partially, the disconnect between the phosphorylation status of signalling proteins and MPS rates observed in our C2C12 myotubes, however this hypothesis warrants further investigation. It must be acknowledged that static measurements of kinase activity at a single time-point likely resulted in missing some peak readouts of the kinase activity. MPS and associated kinase phosphorylation were measured 30 minutes after exposure to an insulin/leucine trigger, following a 16 h incorporation + 24 h washout period with EPA and DHA. While the time-course of elevated MPS post anabolic trigger has been investigated in humans (MacDougall *et al.*, 1995), the reliance on a single time point within our *in vitro* model may have limited the potential to capture the optimal kinase signalling window in response to EPA and DHA. Furthermore, the focus of the present study was on MPS rather than MPB. Previous cell-based investigations in C2C12 myotubes have revealed a metabolic action of EPA (Kamolrat and Gray, 2013; Wang *et al.*, 2013; Jeromson *et al.*, 2018) and DHA (Wang *et al.*, 2013) in attenuating muscle protein breakdown rates. Moreover, microarray analysis technique in human skeletal muscle revealed that, among other pathways, pathways related to calpain, ubiquitin-mediated proteolysis, and inhibition of mTOR were decreased by n-3 PUFA supplementation (Yoshino *et al.*, 2016). Taken together in combination with findings from other animal (Castillero *et al.*, 2009; Marzuca-Nassr *et al.*, 2016) and cell (Huang *et al.*, 2011) studies, this observation emphasizes a potential role for n-3 PUFA in regulating muscle protein breakdown.

2.4.6 Practical implications & limitations

It is important to acknowledge that the observed uptake and washout dynamics and subsequent protein synthesis signalling are confined to a mouse muscle cell model and may not directly translate to human skeletal muscle. Concentrations of EPA and DHA used in *in vitro* studies range between 1 – 750 μM (Tachtsis, Camera and Lacham-Kaplan, 2018), depending on the experimental design and cell line. The shift in relative fatty acid profile change in EPA and DHA observed in C2C12 myotubes exceed those seen in a human population. For instance, we observed that the relative abundance in EPA of the total fatty acid profile increased by ~30% over the 24 h time period, whereas human skeletal muscle exhibits a rise in EPA levels ranging from 1.6 – 3 % depending on dose and supplementation period (Smith *et al.*, 2011b, 2011a; McGlory *et al.*, 2014, 2016). Moreover, while the current recommendation for eating oily fish rich in n-3 PUFA is at least once per week (NHS, 2022), it is essential to note that intermittent eating patterns deviate significantly from the practice of constant daily consumption (Browning *et al.*, 2014), emphasizing the importance of studying washout in human skeletal muscle.

2.4.7 Conclusion & future directions:

We characterized the uptake and washout dynamics of EPA and DHA using a C2C12 model in response to high and low concentration incubation with EPA + DHA. Our data suggest that that treatment with higher concentrations of EPA and DHA resulted in a greater incorporation of n-3 PUFA into the cell lysates and muscle phospholipid membrane and led to a greater preservation of n-3 PUFA following washout, with divergent patterns for EPA and DHA. However, only treatment with the low concentration of EPA and DHA stimulated increased rates of MPS without modulating the phosphorylation status of associated mTORC1 signalling proteins. The distinct dynamics of EPA and DHA in altering muscle cell lipid composition has the potential to impact physiological outcomes related to muscle protein turnover, with possible clinical implications for the prescription of omega-3 polyunsaturated fatty acid supplementation protocols to promote human health. Therefore, there is a need to establish a link between the incorporation of EPA and DHA into the skeletal muscle membranes and their impact on subsequent markers of muscle protein turnover in humans, ultimately contributing to the regulation or maintenance of skeletal muscle mass.

Chapter 3: Incorporation and washout of n-3 PUFA in healthy young and older adults following two different dosing strategies

3.1 Introduction

The beneficial impact of omega-3 polyunsaturated fatty acids (n-3 PUFA) on muscle protein synthesis is partly attributed to their incorporation into cell membranes across various tissues (McGlory, Calder and Nunes, 2019; Calder, 2020b). The incorporation of n-3 PUFA into phospholipid membranes modulates the structure and composition of cellular membranes, including lipid rafts, which are small dynamic microdomains organising cellular processes and influencing cellular signalling (Pike, 2006; Yaqoob and Shaikh, 2010). Furthermore, the incorporation of n-3 PUFA results leads to the secretion of specialised pro-resolving lipid mediators (Calder, 2020a), alters mitochondrial respiration kinetics (Herbst *et al.*, 2014; Miotto *et al.*, 2019), and decreases inflammation (Calder, 2017). Therefore, understanding n-3 PUFA incorporation into cellular membranes of their respective tissues in response to supplementation is pivotal for unravelling their effects on muscle protein turnover and health.

Several time-course studies conducted in blood (Metherel *et al.*, 2009; Browning *et al.*, 2012) and adipose tissue (Katan *et al.*, 1997; Browning *et al.*, 2012) have consistently shown time and dose-dependent increases in n-3 PUFA content. These studies reveal variations in the incorporation of n-3 PUFA into cellular membranes across different tissues. Additionally, in a study conducted in healthy young men (McGlory *et al.*, 2014), the presence of n-3 PUFA in blood was detected one week after a high dose of fish oil (5g/day). In contrast, detection of increases in n-3 PUFA content in muscle required a minimum of two weeks. These findings suggest that the time-course incorporation patterns observed in blood do not accurately reflect these patterns in skeletal muscle and adipose tissue.

Evidence supports dose-dependent increases in n-3 PUFA content in skeletal muscle. In **Chapter 2,** we observed a dose-dependent incorporation of EPA and DHA in cell lysates and phospholipid membranes of C2C12 myotubes. In human studies, a higher dose (5g/day) ingested over four weeks (McGlory *et al.*, 2014) revealed similar n-3 PUFA increases in muscle lipid content to those observed with a lower dose (4g n-3 PUFA per day) over eight weeks

(Smith *et al.*, 2011a). If a higher dose results in higher, earlier incorporation of n-3 PUFA into muscle tissue, there may be earlier benefits for muscle health and muscle protein turnover. Nonetheless, higher doses have been reported to result in gastrointestinal symptoms (bloating, fishy regurgitations) and are therefore not always inviting (Schmidt *et al.*, 2020). A higher loading dose followed by a lower maintenance dose may offer a viable solution. Thus, there is a need to examine whether high initial incorporation into tissues after an initial loading dose can be sustained by moving to a maintenance dose.

Washout of n-3 PUFA has been examined in various blood fractions (Zuijdgeest-Van Leeuwen *et al.*, 1999; Cao *et al.*, 2006; Metherel *et al.*, 2009). In **Chapter 2**, we identified the washout time courses of EPA and DHA in C2C12 myotubes in response to high and low concentrations. More specifically, we demonstrated that incubating C2C12 myotubes with a high concentration led to partial washout of EPA from the phospholipid membranes of C2C12 myotubes while DHA increased, with no formation of DPA. Conversely, incubating C2C12 myotubes with the low concentration also led partial washout of EPA, but DHA content did not change, and a significant increase in DPA formation was observed. This suggests that dosage is important when considering washout. However, these findings are confined to a cellular model and may not directly translate to washout from human skeletal muscle or other human tissues. The washout dynamics of n-3 PUFA from human skeletal muscle have yet to be characterised. Given the longer turnover time of skeletal muscle compared to blood (Goodman and Smith, 1961; Schmalbruch and Lewis, 2000), understanding the washout process from skeletal muscle is pivotal for designing future crossover studies and developing effective supplementation protocols. Furthermore, ageing is a human phenomenon that impacts tissue turnover time (Richardson, Allan and Le, 2014). Therefore, supplementation protocols based on younger participants may not necessarily be applicable to older populations. It is, therefore, essential to investigate washout in both young and older individuals.

Chapter 3 aims to investigate incorporation and washout responses in erythrocytes, skeletal muscle phospholipid membrane fractions, and adipose tissue in response to two different dosing strategies in young and older adults. We utilised a 12-week supplementation approach followed by an 8-week washout approach to determine if a loading dose leads to earlier, higher incorporation that can be sustained by a maintenance dose in young and older adults.

3.2 Methods

3.2.1 Recruitment of participants and ethical approval

Fourteen healthy volunteers aged 18-35 years and fourteen healthy volunteers over 60 were recruited to participate in this study. An overview of recruitment, and allocation to the intervention groups and analysis inclusion can be found in **Figure 3.1**. Volunteers were excluded from participation if they had a body mass index (BMI) >35 kg/m^2. Volunteers were excluded if they had a blood pressure >150/90 mmHg, an indication of high blood pressure. The cut-off of 150/90 rather than 140/90 was used, as blood pressure typically rises with age and levels >140/90 mmHg may occur naturally in otherwise healthy individuals over 60 (NHS, 2023). Volunteers were also excluded if they had musculoskeletal injuries, were smokers, were allergic to shellfish, nickel, or iodine, or were taking supplementation/ medication that could interfere with the outcome of study results. Women who were pregnant, intended to become pregnant during the duration of the study, or were lactating were excluded from the study. Following a health screening, omega-3 status was determined via a finger prick. An omega-3 index >6% of the total fatty acid profile excluded volunteers from the study. The average omega-3 index in the United Kingdom is 5.60% (Schuchardt *et al.*, 2024), suggesting that the average omega-3 index in Scotland falls within our inclusion criteria. Furthermore, an omega-3 index of 4-8% is validated as an 'intermediate risk', therefore using a 6% EPA + DHA in whole blood as a cut-off will identify individuals at the lower end of the spectrum (Harris and von Schacky, 2004). Ethical approval was granted by the NICR committee of Stirling (NICR 19/20 – 089) and NHS HRA authority (REF reference: 20/EM/0214).

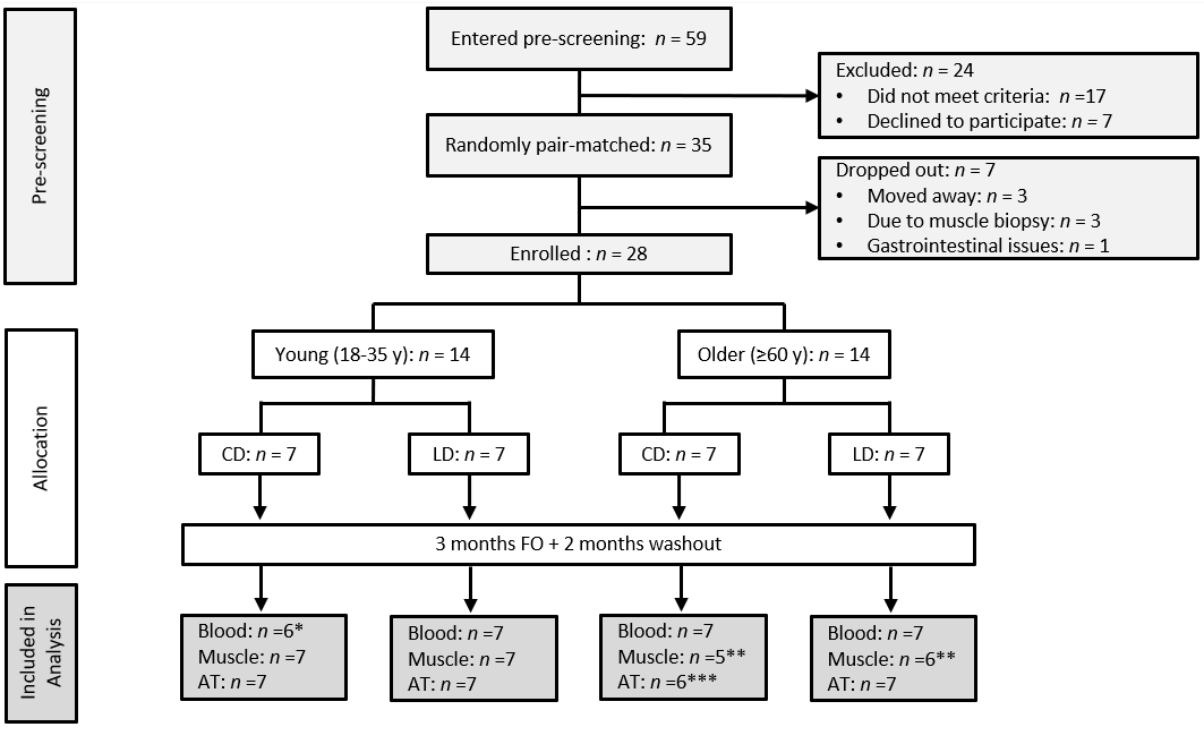

Figure 3.1: Recruitment, selection and pair-matched dosing allocation of participants. *One participant was excluded due to not being able to draw a venous blood sample. **Three participants were not included in muscle tissue biopsy, one participant entered the study without muscle biopsy, two participants did not want the biopsy. ***One participant did not want the biopsy. CD = constant dose, LD = loading dose, FO = fish oil supplementation, AT = adipose tissue.

3.2.2 Study design

Participants were randomly pair-matched on omega-3 status and total body mass by using RAND() in Microsoft® Excel® for Microsoft 365 MSOand allocated to one of the two dosing strategy groups: constant dose group (CD) or loading dose group (LD). Given the design of the study, blinding was not feasible. Consequently, both the participants and researchers were aware of the group allocation. Participants in the constant dose group received a dose of 720mg EPA + 540mg DHA/day (1.26g EPA + DHA/day) for 12 weeks. Participants in the loading dose groups received a loading dose of 1440mg EPA + 1080mg DHA/day (2.52g EPA + DHA/day) for the first four weeks and continued on a maintenance dose of 360 mg EPA + 270 DHA/day (630mg EPA + DHA/day) for eight weeks. Participants were instructed to ingest the fish oil capsules with a meal. At the end of the 12-week supplementation period, both groups had received the same amount of EPA + DHA. After the supplementation period, participants were subsequently followed for another eight weeks to track washout. Participants arrived after an overnight fast (10h) and abstained from alcohol, ibuprofen/paracetamol and strenuous exercise 48 hours before coming in. A venous blood

sample, and muscle and adipose tissue biopsies were taken at baseline, 4, 12 and 20 weeks. Additional blood samples were collected at 6, 8, 14, and 16- weeks. The study design is visualized in **2**.

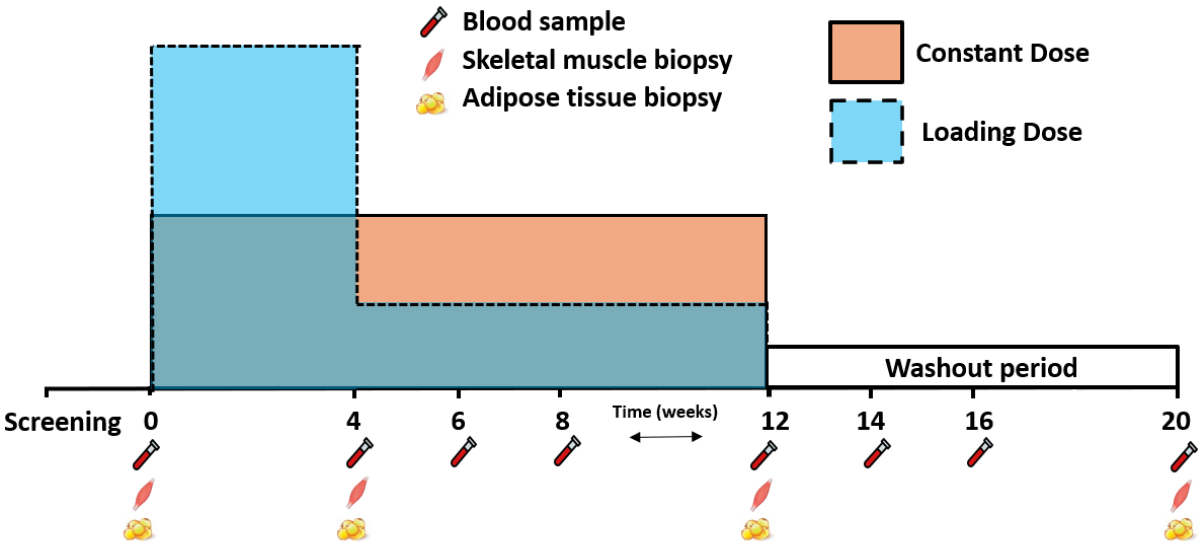

Figure 3.2: Schematic overview of the study.

3.2.2 Blood sample collection

Blood samples were collected from the antecubital forearm vein. All samples were drawn into 2 mL vacutainers containing ethylenediaminetetraacetic acid (EDTA), (Vacutainer Systems, Becton, Dickinson and Company. U.K). Blood samples were centrifuged for 20 minutes at 4°C ×1000g. The supernatant was discarded, and red blood cells were collected and stored in the -80 °C freezer for fatty acid profiling analysis.

3.2.3 Skeletal muscle tissue biopsy procedure

Muscle biopsies were obtained from the *vastus lateralis* using the Bergström biopsy technique. All muscle biopsies taken during the study were obtained from the same leg in each participant. After cleaning the area with iodine, an incision into the skin and fascia was made under local anaesthetic (2 % Lidocaine, B Braun Medical Ltd, UK). Then, a 5 mm Bergström needle was inserted to extract 60-200 mg of muscle. Muscle samples were rinsed with 0.9% saline, and visible connective/adipose tissue was removed. Next, muscle tissue was promptly aliquoted,

weighed, and snap frozen in liquid nitrogen prior to storage at a -80 °C freezer pending further analysis.

3.2.4 Adipose tissue biopsy procedure

Adipose tissue biopsies were obtained 5-10 cm lateral of the umbilicus. Like the muscle biopsy procedure, after cleaning the area with iodine, an incision into the skin was made under local anaesthetic (2 % Lidocaine, B Braun Medical Ltd, UK). Then, adipose tissue was collected according to the mini-liposuction technique (MacGregor *et al.*, 2021). Samples were promptly aliquoted, weighed and snap frozen in liquid nitrogen prior to storage at the -80 °C freezer, pending further analysis.

3.2.5 Bloodspot collection lipid extraction and fatty acid preparation

Blood was collected via a finger prick and placed onto two circular collection spots on Whatman 903 blood collection cards (GE Healthcare Ltd, Forest Farm Industrial Estate, Cardiff, CF 14 7YT, UK). Blood spots were dried for at least 3 hours before detached from the collection spots using forceps and subsequently placed into a screw-cap vial with 1 mL methylating solution (1.25M methanol/HCl). The vials were heated at 70°C for 1 h after which they were cooled to room temperature. Next, 2 mL distilled water and 2 mL KCl solution were added, and Fatty acid methyl esters (FAME) were extracted with 1 × 2 mL isohexane + BHT followed by another extraction with 2 mL isohexane.

3.2.6 Lipid extraction from erythrocytes and fatty acid preparation

Erythrocytes (25 µl) were added to a screw-top glass vial and 250 µl of 14% BF3-Methanol and 250 µl of isohexane were added, then sealed and vortexed for 30 seconds. Next, sealed vials were placed in a heat block for 10 minutes at 100°C and cooled to room temperature for another 10 min. Next, 250 µl of distilled water was added, vortexed for another 30 seconds, and centrifuged for 5 min at ×1000g. Finally, 60 µl of sample was removed from the top layer in the vial and transferred to a GC vial with a PTFE-lined septum, and Fatty Acid Methyl Esters (FAME) were analysed according to procedure below (3.2.7).

3.2.7 Lipid extraction from whole muscle and adipose tissue

Lipid extraction from muscle and adipose tissue was done using the Folch method (Folch, Lees and Stanley, 1957) and conducted at the Institute of Aquaculture, Stirling, whereas the fatty analyses conducted in **Chapter 2, section 2.2.4** were conducted at Danone Nutricia Research, the Netherlands. This has resulted in a slightly different fatty acid analysis methods. Briefly, muscle or adipose tissue was put in a vial and accurate tissue mass was weighed. Then, 1 mL of chloroform/methanol (C:M, 2:1 v/v) was added to each vial and samples were homogenised with a hand-held IKA-Werke Ultra-turrax T8 homogeniser (Fisher, Loughborough, UK). The probe was then rinsed with another 3 mL of C:M (2:1 v/v) and added to the reacti-vial. The reacti-vials were placed on ice for one h before 1 mL 0.88 % KCl was added to the solution, after which vials were shaken and incubated for 10 min to remove non-lipid impurities. The vials were centrifuged at ×400g for 5 min, after which the aqueous layer was removed. The lower solvent layer was removed using a Pasteur pipette and filtered through a 5.5 mm Whatman No. 1 filter, pre-washed with C:M, 2:1 into a 7 mL bottle. Samples were dried under N_2 for 40 min after which the lipid was re-dissolved in 1 mL of C:M, 2:1. Samples were transferred to a new pre-weighed 1.7 mL bottle. The 1.7 mL bottle was rinsed with 0.5 mL C:M, 2:1 and this was added to the 1.7 mL bottle to ensure all lipids were extracted. Lipids were dried under N_2 and desiccated overnight in a vacuum desiccator, after which the lipids were reweighed and dissolved in C:M, 2:1 + 0.01 % butylated hydroxyl toluene (BHT), at a concentration of 2 mg.mL-$_1$.

3.2.7.1 Phospholipid extraction and fatty acid preparation skeletal muscle

To determine phospholipid fraction, 0.5 mg total lipids were applied to a 20 × 20-cm silica gel 60 TLC plate (VWR, Lutterworth, Leicestershire, UK) and developed in isohexane–diethyl ether–acetic acid (80:20:1, by volume) before drying for ~3 min at room temperature. The plate was sprayed lightly with 2,7-dichlorofluorescein (0.1%) in 97% methanol, and the phospholipid bands were then scraped from the plate and placed in a 15-mL test tube. Fatty acid methyl esters (FAME) were prepared by acid-catalysed transesterification in 2 mL of 1% H_2SO_4 in methanol at 50°C overnight. Next, 2.5 mL of 2% $KHCO_3$ was added, and samples were extracted using 5 mL isohexane–diethyl ether (1:1) BHT. The samples then were re-extracted with 5 mL isohexane–diethyl ether (1:1) and the extracts were dried and dissolved in 0.3 mL of isohexane prior to FAME analysis.

3.2.8 Analysis of fatty acids methyl esters (FAME)

FAME were separated and quantified using gas-liquid chromatography (ThermoFisher Trace, Hemel Hempstead, England) with a 60 m x 0.32 mm x 0.25 μm film thickness capillary column (ZB Wax, Phenomenex, Macclesfield, UK). Hydrogen was used as carrier gas at a flow rate of 4.0 mL.min^{-1}. The temperature program was set from 50 to 150°C at 40°C·min^{-1} then to 195°C at 2°C·min^{-1} and finally to 215°C at 0.5°C·min^{-1}.

3.2.9 Statistical analysis

A priori power calculations were conducted in G*Power 3.1.9.7. Due to the absence of prior data on the statistical significance of differences in muscle lipid n-3 composition between different dosing strategies, power calculations were based on detecting significant changes in percentage n-3 of total fatty acids in muscle tissue from baseline. Data were based on a previous study by McGlory *et al.,* (2014), where the percentage n-3 of total fatty acids in muscle lipid composition significantly increased from 3.8 ± 0.70 to 5.14 ± 0.89% following two weeks of supplementation with 5.0g of n-3 PUFA per day. Based on these results, G*Power reported that n=7 participants per group were required to achieve 80% power ($P < 0.01$) to detect n-3 PUFA muscle incorporation changes. Data were analysed in RStudio version 2023.12.1. Participant characteristics at baseline are represented as mean ± SD and tested between age groups, and dosing groups within each age group with Welch's two-sample t-test. Differences in % EPA + DHA/total fatty acids were analysed using a linear mixed effect model using the lmer() package with main effects for time, dosing strategy, and age. Participant number was included as a random effect. If there was no main or interaction effect, groups were combined to increase statistical power. Changes over time were determined within CD and LD using a linear mixed model followed by Tukey's post-hoc analysis using the emm() package. To determine whether the dosing strategies resulted in differential changes in % EPA + DHA, the change in percentage EPA + DHA (Δ %EPA + DHA) was calculated for each participant. Welch's two-sample t-test was conducted to compare Δ %EPA + DHA between CD and LD during week 0 – 4 (loading phase), week 4 – 12 (maintenance phase), and week 12 – 20 (washout phase). Data are reported as mean + SD, with 95% confidence intervals calculated over each mean. Significance was set at $P < 0.05$.

3.3 Results:

3.3.1 Participant characteristics

Within the age groups, there were no differences between the constant and loading dose groups at baseline in age, body mass, BMI, systolic blood pressure, diastolic blood pressure, heart rate, and the sum of EPA + DHA expressed as a percentage of total fatty acids in erythrocytes ($P > 0.05$). Between the age groups, age ($P < 0.001$), systolic blood pressure ($P = 0.025$) and diastolic pressure ($P = 0.004$) differed significantly. An overview of baseline characteristics is presented in **Table 3.1**.

Table 3.1: Participants' characteristics at baseline. Data are presented as mean ± SD. No significant differences were observed between dosing strategies within the young and older age group ($P > 0.05$). '*' indicates significantly different from the young age groups (combined). Between the ages there was a significant difference in age, systolic blood pressure, and diastolic blood pressure ($P < 0.05$).

	Constant dose young (n=7)	Loading dose young (n=7)	Constant dose older (n=7)	Loading dose older (n=7)
Age (years)	22.7 ± 2.4	25.4 ± 5.8	68.1 ± 6.0*	66.0 ± 5.9*
Body mass (kg)	69.2 ± 8.3	66.7 ± 9.7	70.8 ± 13.0	65.9 ± 10.2
BMI (kg/m^2)	23.4 ± 2.3	24.1 ± 3.3	26.5 ± 3.2	24.0 ± 2.5
Systolic BP (mmHg)	121 ± 7	115 ± 13	134 ± 15*	123 ± 7*
Diastolic BP (mmHg)	75 ± 7	77 ± 9	88 ± 11*	83 ± 6*
Heart rate (BPM)	72 ± 8	75 ± 12	74 ± 7	68 ± 9
% EPA + DHA/ total fatty acids	4.89 ± 1.93	5.43 ± 0.95	5.88 ± 1.02	5.65 ± 1.16

BMI = body mass index, BP = blood pressure, EPA = eicosapentaenoic acid, DHA = docosahexaenoic acid. BPM = beats per minute

3.3.2 Erythrocyte incorporation and washout

There was a main effect of time on % EPA + DHA/total fatty acids incorporation ($P < 0.001$), as well as an interaction effect of time × dosing strategy ($P < 0.001$). Since there was no effect of age, young and older adults were grouped together to determine %EPA + DHA changes over time and Δ %EPA + DHA between the dosing strategies at the loading, maintenance, and

washout phase. A full breakdown of fatty acid profile in erythrocytes over time can be found in **Appendix 3.1** (CD) and **Appendix 3.2** (LD)**.**

Changes over time revealed that in CD (**Figure 3.2A**), % EPA + DHA/total fatty acids increased from week 0 to week 4 by 1.25 ± 0.68 % (95% CI [0.83 – 1.67]; $P < 0.001$), with a further increase to week 8 of 0.78 ± 0.51 % (95% CI [0.48 – 1.09]; $P = 0.004$), and further increased between week 8 – 12 with 0.69 ± 0.55 % (95% CI [0.35 – 1.02]; $P = 0.019$). In LD (**Figure 3.2A**), % EPA + DHA/total fatty acids increased from week 0 to week 4 by 2.49 ± 0.88 % (95% CI [1.98 – 3.00]; $P < 0.001$), with no further statistically significant difference during the supplementation period ($P > 0.05$). Differences in %EPA + DHA/total fatty acids between dosing strategies after the loading phase at week 4 were primarily due to changes in EPA (**Figure 3.2C**), whilst changes in DHA over time did not differ at any individual time point between dosing strategies (**Figure 3.2E;** $P > 0.05$).

Since there were no differences in incorporation between dosing strategies at week 12 and week 20 ($P > 0.05$; **Figure 3.2A**), CD and LD were grouped together to investigate washout (**Figure 3.2A**). A significant decline of -0.79 ± 0.53% (95% CI [-0.99 – -0.58]) was detected between week 12 – week 14 during the washout phase, with a further decline from week 14 – week 20 of -0.81 ± 0.54% (95% CI [-1.03 – -0.60]). Here, EPA + DHA content was significantly higher than baseline ($P < 0.001$).

LD resulted in a greater Δ %EPA + DHA compared to CD after the initial 4-week loading phase ($P < 0.001$; **Figure 3.2B**). Following the 4 – 12-week maintenance phase, CD resulted in a greater Δ %EPA + DHA compared to LD ($P < 0.001$; **Figure 3.2B**). There was no difference in Δ %EPA + DHA between the dosing strategies during the 12 – 20-week washout phase ($P > 0.05$; **Figure 3.2B**). Additionally, LD resulted in a greater Δ %EPA (**Figure 3.2D**) and Δ %DHA (**Figure 3.2F**) compared to CD after the initial 4-week loading phase ($P < 0.05$). Similarly, following the 4 – 12-week maintenance phase, CD resulted in a greater Δ %EPA (**Figure 3.2D**) and Δ %DHA (**Figure 3.2F**) compared to LD ($P < 0.05$). During the 12 – 20-week washout phase, there was no difference between dosing strategies in Δ %EPA (**Figure 3.2D;** $P > 0.05$) or Δ %DHA (**Figure 3.2F;** $P > 0.05$).

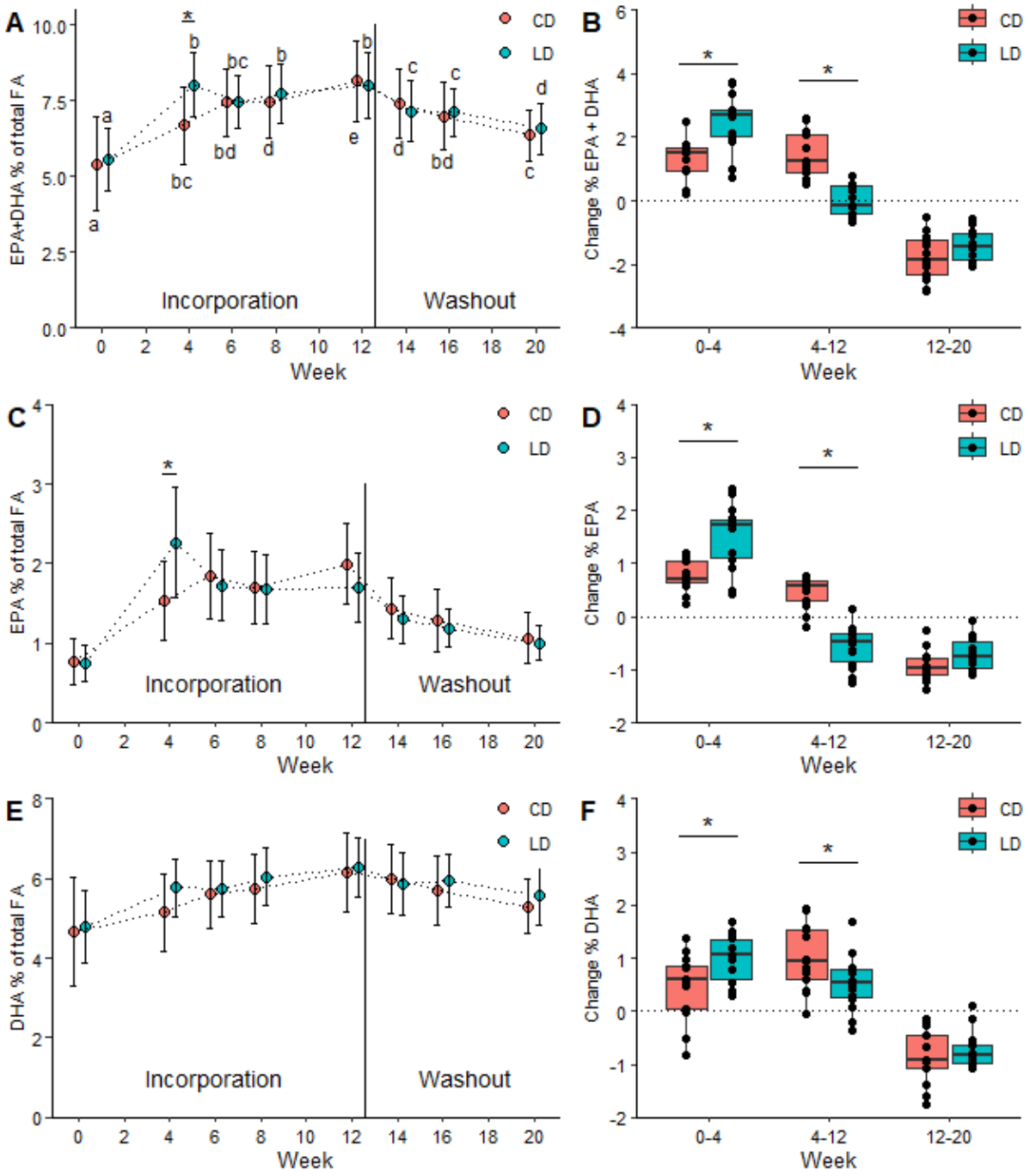

Figure 3.2: Changes in erythrocyte composition over time (**3.2A**) and between dosing strategies during loading, maintenance, and washout phase (**3.2B**), of the sum of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (**3.2A, 3.2B**), EPA (**3.2C, 3.2D**), and DHA (**3.2E, 3.2F**) expressed as percentage of total fatty acid profile (FA), , following a constant dose (CD) or loading dose (LD) strategy in young and older adults. Young and older adults were grouped together because there was no main effect of age on the change in % EPA + DHA. The CD group (n=13) received 720 mg EPA + 540 mg DHA /day for 12 weeks, the LD group (n=14) received a loading dose of 1440 mg EPA + 1080 mg DHA/ day for the first 4 weeks followed by 360 mg EPA + 270 mg DHA/ day for 8 weeks thereafter. Differences between strategies are indicated with an '*'. ($P < 0.05$). **Figure 3.2A, 3.2C, 3.2E**: Values are means ± SD (standard deviation). **Figure 3.2A:** Mean values that do not share a common letter within CD or LD are statistically different ($P < 0.05$). **Figure 3.2B, 3.2D, 3.2F**: Boxplot of change (median + interquartile

ranges and whiskers) in percentage EPA + DHA between week 0 – 4 (loading phase), week 4 – 12 (maintenance phase), and week 12 – 20 (washout phase). Mean values that do not share a symbol are statistically different ($P < 0.05$).

3.3.4 Skeletal muscle phospholipid membrane incorporation and washout

There was a main effect of time on % EPA + DHA/total fatty acids incorporation ($P < 0.001$), as well as an interaction effect of time × dosing strategy ($P = 0.006$). Since there was no effect of age, young and older adults were grouped together to determine % EPA + DHA changes over time and differences in Δ %EPA + DHA between the dosing strategies at the loading, maintenance, and washout phase. A full breakdown of fatty acid profile in skeletal muscle phospholipid membranes over time can be found in **Appendix 3.3.**

Changes over time revealed that in CD (**Figure 3.3A**), %EPA + DHA/total fatty acids increased from week 0 to week 4 by 0.61 ± 0.47 % (95% CI [0.31 – 0.90]; $P = 0.006$), with a further increase to week 12 of 0.73 ± 0.58% (95% CI [0.37 – 1.10]; $P = 0.002$). In LD (**Figure 3.3A**), %EPA + DHA/total fatty acids increased from week 0 to week 4 by 1.74 ± 1.35% (95% CI [0.93 – 2.56]; $P < 0.001$), with no further statistically significant difference during the supplementation period ($P > 0.05$). Given that there were no differences in incorporation between dosing strategies at week 12 and week 20 ($P > 0.05$; **Figure 3.3A**), CD and LD were grouped together to investigate washout. A significant decline of -1.05 ± 0.70% (95% CI [-1.34 – -0.76]) was detected during the washout phase, where EPA + DHA content did not differ from baseline ($P > 0.05$). Similarly to erythrocytes (**Figure 3.2**), differences in %EPA + DHA/total fatty acids in skeletal muscle phospholipid membranes between dosing strategies at week 4 were primarily due to changes in EPA (**Figure 3.3C**), whilst changes in DHA over time did not differ at any individual time point between dosing strategies (**Figure 3.3E;** $P > 0.05$).

LD resulted in a greater Δ %EPA + DHA compared to CD after the initial 4-week loading phase ($P < 0.05$; **Figure 3.3B**). Following the 4 – 12-week maintenance phase, CD resulted in a greater Δ %EPA + DHA compared to LD ($P < 0.05$; **Figure 3.3B**). There was no difference in Δ%EPA + DHA between the dosing strategies during the 12 – 20-week washout phase ($P > 0.05$; **Figure 3.3B**). Additionally, LD resulted in a greater Δ %EPA (**Figure 3.3D**) and Δ %DHA (**Figure 3.3F**) compared to CD after the initial 4-week loading phase ($P < 0.05$). Similarly, following the 4 – 12-week maintenance phase, CD resulted in a greater Δ %EPA (**Figure 3.3D**) and Δ %DHA (**Figure 3.3F**) compared to LD ($P < 0.05$). During the 12 – 20-week washout phase, there was no difference between dosing strategies in Δ %EPA (**Figure 3.3D;** $P > 0.05$) or Δ %DHA (**Figure 3.3F;** $P > 0.05$).

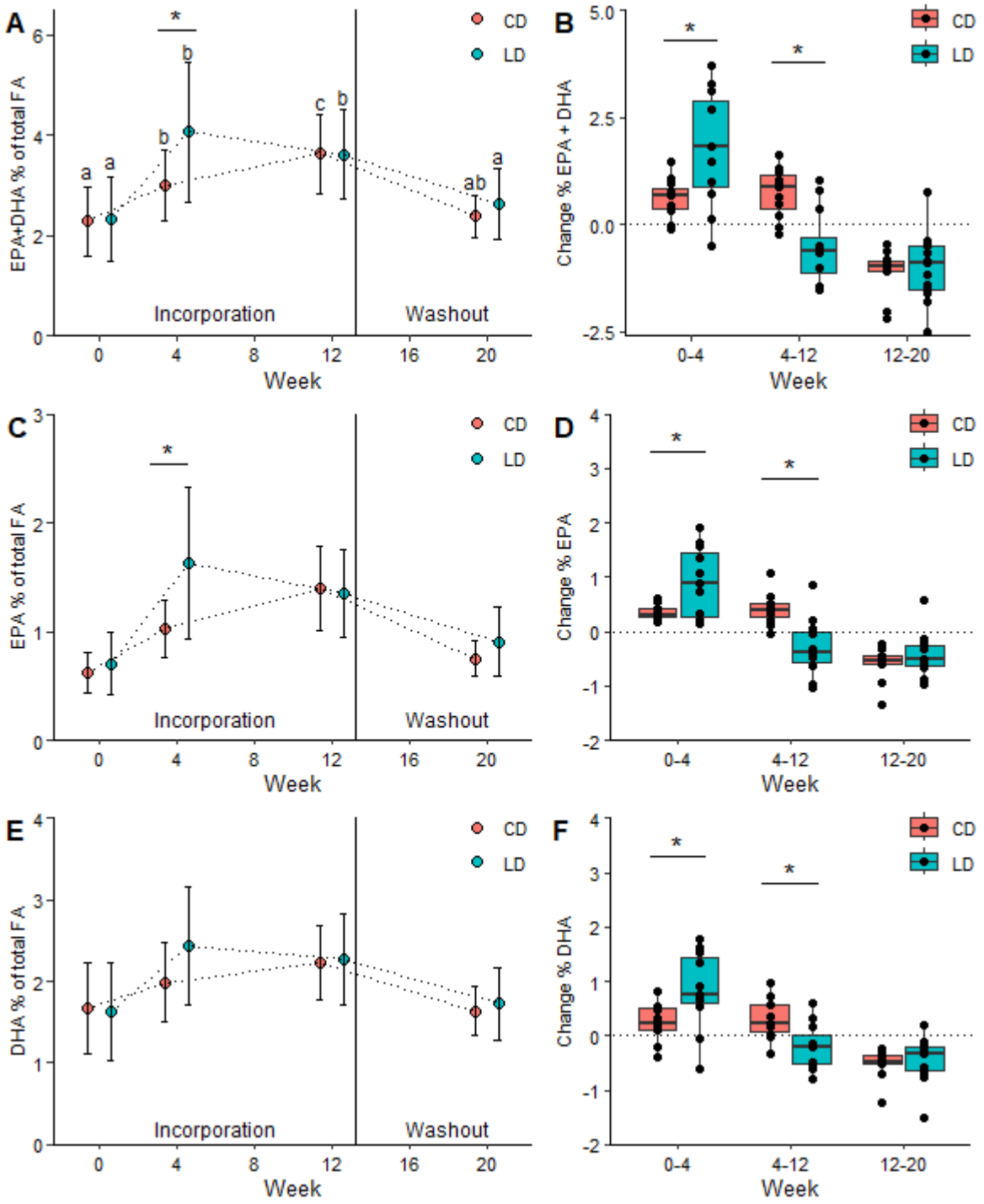

Figure 3.3: Changes in skeletal muscle phospholipid membrane composition over time (**3.5A**) and between dosing strategies during loading, maintenance, and washout phase (**3.3B**) of the sum of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) **(3.3A, 3.3B),** EPA **(3.3C, 3.3D),** and DHA **(3.3E, 3.3F)** expressed as percentage of total fatty acid profile (FA), following a constant dose (CD) or loading dose (LD) strategy in young and older adults. Young and older adults were grouped together since there was no main effect of age on the change in Δ%EPA + DHA. The CD group (n=12) received 720 mg EPA + 540 mg DHA /day for 12 weeks, the LD group (n=14) received a loading dose of 1440 mg EPA + 1080 mg DHA/ day for the first 4 weeks followed by 360 mg EPA + 270 mg DHA/ day for 8 weeks thereafter. Differences between strategies are indicated with an '*'. ($P < 0.05$). **Figure 3.3A, 3.3C, 3.3E**: Values are means ± SD (standard deviation). Mean values that do not share a common letter within CD or LD are statistically different ($P < 0.05$). **Figure 3.3B, 3.3D, 3.3F**: Boxplot of change

(median + interquartile ranges and whiskers) in percentage EPA + DHA between week 0 – 4 (loading phase), week 4 – 12 (maintenance phase), and week 12 – 20 (washout phase).

3.3.3 Adipose tissue incorporation and washout

There was a main effect of time ($P < 0.001$), and age ($P = 0.005$) on % EPA + DHA/total fatty acids incorporation, as well as an interaction effect of age × dosing strategy ($P = 0.026$). Due to the main effect of age and the interaction between age × dosing strategy, results for adipose tissue are separately presented for young and older adults. A full breakdown of fatty acid profile in adipose tissue in young and older adults over time can be found in **Appendix 3.4** (young) and **Appendix 3.5** (older).

Incorporation and washout in adipose tissue of young adults

Changes over time revealed that in CD (**Figure 3.4A**), %EPA + DHA/total fatty acids increased from week 0 to week 4 by 0.09 ± 0.08% (95% CI [0.01 – 0.16]; $P = 0.049$), with no further increase during the supplementation period ($P = 0.06$). In LD (**Figure 3.4A**), % EPA + DHA/total fatty acids increased from week 0 to week 12 by 0.13 ± 0.09% (95% CI [0.04 – 0.21]; $P = 0.013$). Given that there were no differences in incorporation between dosing strategies at week 12 and week 20 ($P > 0.05$; **Figure 3.4A**), CD and LD were combined to determine washout. No decline in %EPA + DHA/total fatty acids was detected during the washout phase, while EPA + DHA content was significantly higher compared to baseline ($P > 0.05$). However, if CD and LD are examined separately, EPA + DHA content in CD do not differ from baseline ($P > 0.05$), while EPA + DHA in LD are significantly elevated above baseline ($P < 0.05$). The observed changes in %EPA + DHA/total fatty acids in adipose tissue of young adults were primarily due to DHA (**Figure 3.4E**), while the %EPA in adipose tissue was very low and undetectable in some participants, resulting in minimal to no changes (**Figure 3.4C**).

No significant differences in Δ %EPA + DHA were observed between the dosing strategies during the 0 – 4 week loading phase, 4 – 12-week maintenance phase, or 12 – 20-week washout phase ($P > 0.05$; **Figure 3.4B**). Furthermore, no significant differences in Δ %EPA ($P > 0.05$; **Figure 3.4D**) or Δ %DHA ($P > 0.05$; **Figure 3.4F**) were observed between the dosing strategies during the 0 – 4 week loading phase, 4 – 12-week maintenance phase, or 12 – 20-week washout phase.

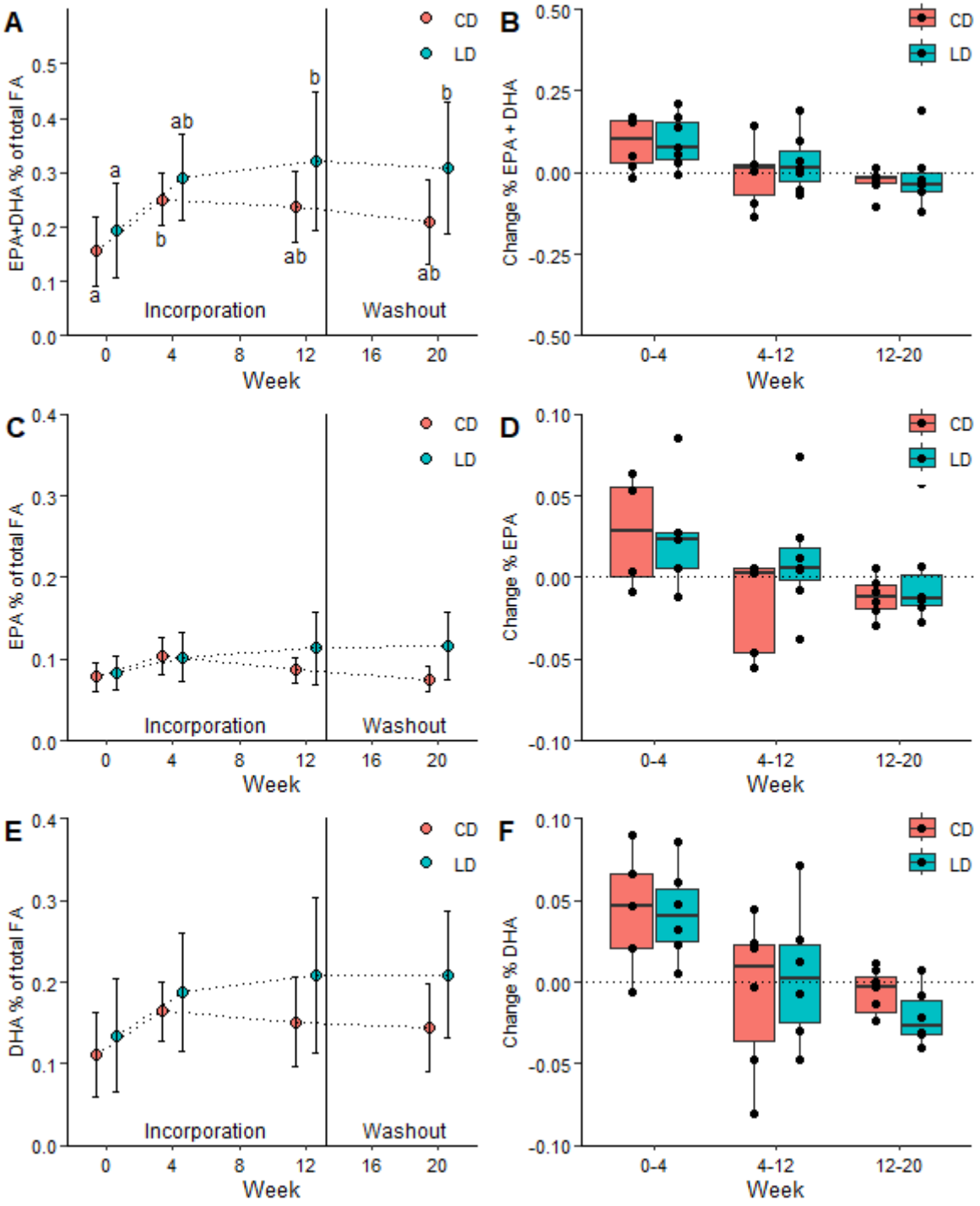

Figure 3.4: Changes in adipose tissue composition over time (**3.4A**) and between dosing strategies during loading, maintenance, and washout phase (**3.4B**) of the sum of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) **(3.4A, 3.4B)**, EPA **(3.4C, 3.4D)**, and DHA **(3.4E, 3.4F)** expressed as percentage of total fatty acid profile (FA), following a constant dose (CD) or loading dose (LD) strategy in young adults. The CD group (n=7) received 720 mg EPA + 540 mg DHA /day for 12 weeks, the LD group (n=7) received a loading dose of 1440 mg EPA + 1080 mg DHA/ day for the first 4 weeks followed by 360 mg EPA + 270 mg DHA/ day for 8 weeks thereafter. There were no differences between strategies at any timepoint. **Figure 3.4A, 3.4C, 3.4E**: Values are means ± SD (standard deviation). Mean values that do not share a common letter within CD or LD are statistically different ($P < 0.05$). **Figure 3.4B, 3.4D, 3.4F**: Boxplot of change (median + interquartile ranges and

whiskers) in percentage EPA + DHA between week 0 – 4 (loading phase), week 4 – 12 (maintenance phase), and week 12 – 20 (washout phase).

Incorporation and washout in adipose tissue of older adults

In older adults, changes over time revealed that in CD (**Figure 3.5A**), %EPA + DHA/total fatty acids increased from week 0 to week 12 by 0.07 ± 0.02 % (95% CI [0.05 – 0.09]; $P < 0.008$), where %EPA + DHA/total fatty acids remained above baseline levels during the washout phase at week 20 ($P > 0.05$). In LD, no changes in incorporation of %EPA + DHA/total fatty acids, %EPA/total fatty acids or %DHA of total fatty acids were observed ($P > 0.05$; **Figure 3.5A, 3.5C, 3.5E**). No significant differences in Δ%EPA + DHA were observed between the dosing strategies during the 0 – 4 week loading phase, 4 – 12 week maintenance phase, or 12 – 20 week washout phase ($P > 0.05$; **Figure 3.5B**). Furthermore, no significant differences in Δ %EPA ($P > 0.05$; **Figure 3.5D**) or Δ %DHA ($P > 0.05$; **Figure 3.5F**) were observed between the dosing strategies during the 0 – 4 week loading phase, 4 – 12-week maintenance phase, or 12 – 20-week washout phase.

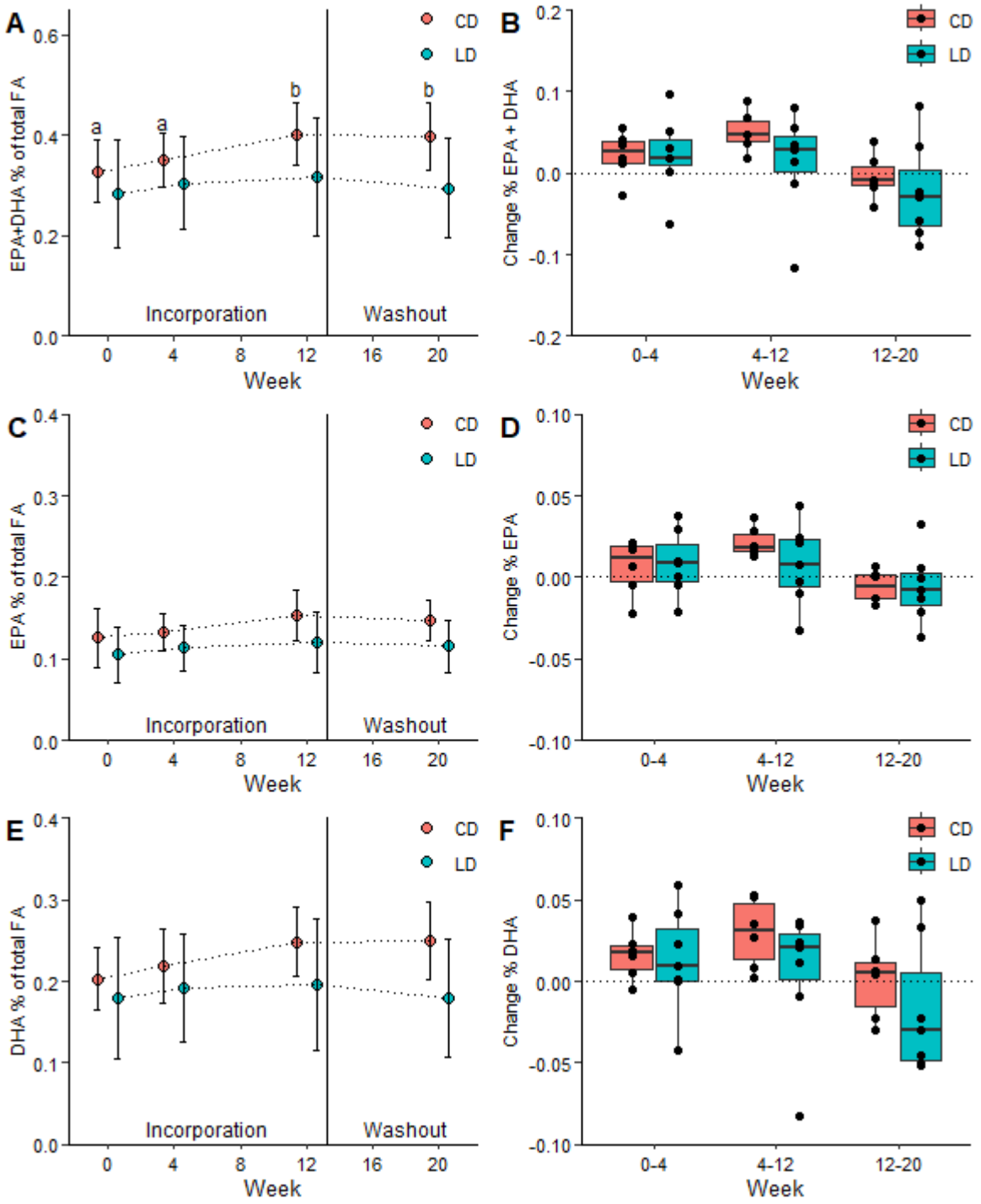

Figure 3.5: Changes in adipose tissue composition over time (**3.5A**) and between dosing strategies during loading, maintenance, and washout phase (**3.5B**) of the sum of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) **(3.5A, 3.5B),** EPA **(3.5C, 3.5D),** and DHA **(3.5E, 3.5F)** expressed as percentage of total fatty acid profile (FA), following a constant dose (CD) or loading dose (LD) strategy in older adults. The CD group (n=5 received 720 mg EPA + 540 mg DHA /day for 12 weeks, the LD group (n=6) received a loading dose of 1440 mg EPA + 1080 mg DHA/ day for the first 4 weeks followed by 360 mg EPA + 270 mg DHA/ day for 8 weeks thereafter. There were no differences between strategies at any timepoint. **Figure 3.5A, 3.5C, 3.5E**: Values are means ± SD (standard deviation). **Figure 3.5B, 3.5D, 3.5F**: Boxplot of change (median + interquartile ranges and whiskers) in percentage EPA + DHA between week 0 – 4 (loading phase), week 4 – 12 (maintenance phase), and week 12 – 20 (washout phase).

3.3.4 Correlation of EPA + DHA between erythrocytes and skeletal muscle phospholipid membranes

Correlation analysis revealed a significant association between erythrocyte %EPA + DHA/total FA and muscle phospholipid membrane %EPA + DHA/total fatty acids at baseline (**Figure 3.6A,** R = 0.52, P = 0.016), after week 4 (**Figure 3.6B,** R = 0.64, P = 0.011), week 12 (**Figure 3.6C,** R = 0.62, P = 0.0017), but not at the end of the washout period (**Figure 3.6D,** R = 0.21, P = 0.34).

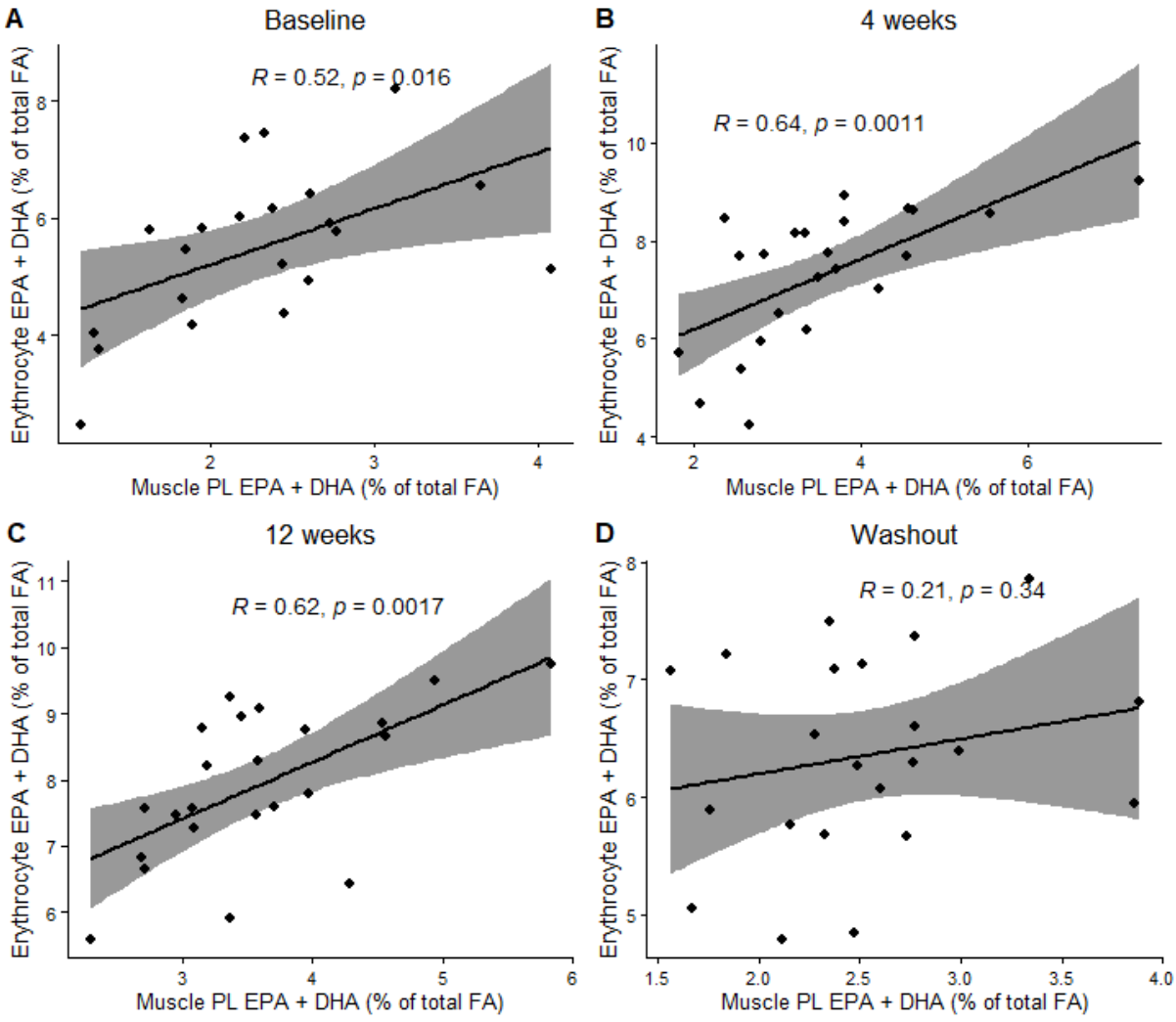

Figure 3.6: Correlation of the sum of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) between erythrocyte and skeletal muscle phospholipid membranes at baseline (**3.6A**), four weeks (**3.6B**), twelve weeks (**3.6C**) and after the washout period (**3.6D**). Significance was set at P < 0.05.

3.4 Discussion

This study examined the incorporation and washout of EPA + DHA as a percentage of total fatty acids over time in erythrocytes, skeletal muscle phospholipid membrane fractions, and adipose tissue in response to two different dosing strategies in young and older adults. Incorporation of EPA + DHA into erythrocytes and skeletal muscle phospholipid membranes followed a similar pattern. Here, a loading dose led to a significantly greater change in EPA + DHA during the 4-week loading phase. However, EPA + DHA content did not differ between the loading and constant dose after the total 12-week supplementation period, which resulted in similar washout patterns. In erythrocytes, EPA + DHA content was elevated above baseline following the 8-week washout phase. In skeletal muscle phospholipid membranes, EPA + DHA content returned to baseline after the 8-week washout phase. Finally, differential incorporation of EPA + DHA was observed between young and older adults in adipose tissue. However, in both young and older adults, the incorporation patterns of EPA + DHA did not differ in response to dosing strategies during the 4-week loading or subsequent 8-week maintenance phases. Additionally, no washout from adipose tissue was observed, which can attributed to limited incorporation and slow turnover.

3.4.1 Erythrocytes

Most studies investigating time-course changes in erythrocytes are conducted in either a young (Metherel *et al.*, 2009; McGlory *et al.*, 2014; McGlory *et al.*, 2016, 2019; Jannas-Vela *et al.*, 2017), or older cohort (Logan and Spriet, 2015; Da Boit *et al.*, 2017), making direct comparisons between age groups difficult due to variations in measured fractions, doses and study durations. The membrane composition of erythrocytes varies between young and older adults, with older adults having less fluid membranes (Goi *et al.*, 2005). However, we observed similar incorporation patterns between young and older adults, suggesting that the doses provided in this study did not alter erythrocyte composition differentially between young and older adults. Previous studies investigating dose-response relationships have employed higher doses (Katan *et al.*, 1997; Browning *et al.*, 2012; Witard *et al.*, 2023). However, doses used in these studies far exceed the recommended daily intake of 250 - 500mg EPA + DHA (European Food Safety Authority (EFSA), 2010), therefore it is essential to investigate incorporation of EPA and DHA into erythrocytes following lower doses. Even though the doses used in this study exceed the daily recommendations of 250 – 500mg EPA + DHA per day, we observed

that doses of 2.52g versus 1.26g EPA + DHA per day were sufficient to distinguish differential incorporation patterns in erythrocytes during the 4-week loading phase. Consequently, a 630mg/day maintenance dose was sufficient to conserve EPA + DHA content after the initial loading phase, suggesting that a loading dose is a beneficial strategy to accelerate greater incorporation. Future research is warranted to determine whether an initial high loading dose could be sustained by modification of diet alone.

Early studies examining washout of n-3 PUFA from blood have indicated that percentage of n-3 PUFA returned to baseline in platelets after 3 months of washout following a 4-month supplementation period with 4g n-3 PUFA per day (Prisco *et al.*, 1995), while another study showed levels of n-3 PUFA returned to baseline in plasma phospholipids, platelets and mononucleates after 1 month of washout following a 12 week supplementation period with 1-4g n-3 PUFA/day (Di Stasi *et al.*, 2004). In another early study conducted by Katan *et al.* (1997), levels of EPA in erythrocytes declined to 4% of peak values after 6 months following supplementation with 3, 6, or 9 g n-3 PUFA/day. In our study, the percentage of EPA + DHA of total fatty acids did not return to baseline in erythrocytes after the 8-week washout phase. Since erythrocytes are used as habitual intake markers of EPA + DHA (Harris, 2008), this is an important consideration when designing cross-over studies.

3.4.2 Skeletal muscle

There are numerous structural disparities in muscle tissue, myonuclei, and satellite cell nuclei between younger and older individuals (Cisterna and Malatesta, 2024) and several factors contribute to the ageing muscle phenotype, such as a decrease in growth, repair, and muscle innervation pathways (Kedlian *et al.*, 2024). Additionally, ageing impairs muscle regeneration by shifting muscle cells from myogenic to fibrogenic states (Carosio *et al.*, 2011). However, despite these changes in skeletal muscle tissue during ageing, we did not detect any differences in the incorporation of EPA and DHA into the skeletal muscle phospholipid membranes between the two age groups, suggesting that phospholipid membrane alterations in response to n-3 PUFA supplementation do not vary with ageing.

Although not a main objective of this study, an interesting observation was that the phospholipid membrane fractions of skeletal muscle had similar incorporation patterns during the loading and maintenance phase as erythrocytes (**Figure 3.2B, 3.5B**). If erythrocytes can serve as a marker for fatty acid composition in phospholipid membranes of skeletal muscle,

the need for invasive procedures such as skeletal muscle biopsies may be reduced. A previous study administering high doses of 5g n-3 PUFA per day found correlations between whole blood and total skeletal muscle composition at baseline and after two and four weeks of n-3 PUFA supplementation, but not after one week (McGlory *et al.*, 2014). In the present study, we measured incorporation in erythrocytes and phospholipid fractions of skeletal muscle and observed similar incorporation patterns after the 4-week and 12-week supplementation phases. However, contrastingly to erythrocytes, EPA + DHA content in the phospholipid membrane returned to baseline levels after an 8-week washout phase. This could be attributed to the lower increases in EPA + DHA content within skeletal muscle phospholipid membranes in response to supplementation, compared to erythrocytes. Our findings are consistent with the findings of McGlory *et al.,* (2014), where changes in whole blood were greater than in skeletal muscle. Taken together, these data indicate that erythrocytes have greater changes in fatty acid profile in response to n-3 PUFA supplementation leading to prolonged elevations in n-3 PUFA content. This should be considered when using erythrocyte fatty acid composition as a marker for changes in skeletal muscle phospholipid membrane composition, given that factors such as duration of supplementation and washout periods may influence outcomes of interest.

Finally, our data observed that a loading dose of 2.52g n-3 PUFA per day in this study was sufficient to provide significantly greater changes in phospholipid membrane composition compared to the constant dose of 1.26g n-3 PUFA per day, while changes in membrane composition after a loading dose can be sustained by a maintenance dose of 0.63g n-3 PUFA per day. Based on these findings, it is plausible that higher doses are advantageous in promoting earlier changes in skeletal muscle phospholipid membrane composition. As such, since changes in phospholipid membrane are dose dependent, it is important to investigate how these alterations in membrane composition may impact functional outcomes such as protein turnover signalling, or transcriptomic changes.

3.4.3 Adipose tissue

Despite historically being thought of as an inactive tissue, adipose tissue is now recognised as a metabolically active endocrine organ (Mohamed-Ali, Pinkney and Coppack, 1998), and the incorporation of EPA and DHA into adipose tissue may potentially exert anti-obesity, enhanced fatty acid oxidation, or anti-inflammatory effects (Todorčević and Hodson, 2015). In the current study, the adipose tissue content of EPA + DHA was markedly lower than in muscle

and erythrocytes. Our findings align with previous literature suggesting that EPA and DHA are not primarily stored in white adipose tissue, but rather in skeletal muscle, or undergo oxidation (Todorčević and Hodson 2015; Hodson, Skeaff, and Fielding 2008). Turnover of both lipids and cells within adipose tissue is slow (Strawford *et al.*, 2004). An early study conducted by Katan *et al.,* (1997) did not find a plateau in EPA or DHA incorporation after 1 year of supplementation with 3, 6, or 9g fish oil supplementation/day, and estimated a half-life of at least a year for EPA in subcutaneous adipose tissue, indicating a slow incorporation and washout of EPA and DHA in adipose tissue. Interestingly, in DHA-fed mice, a 16-day washout phase was sufficient for DHA levels to no longer differentiate from those in the control group (Lefils *et al.*, 2010), highlighting the importance of species-specific variations in turnover times. Our study detected a statistically significant elevation of EPA + DHA in adipose tissue during the supplementation period in all groups except within the older loading dose group. In this group, substantial individual variations were noted, likely contributing to an absence of detected incorporation over time. Due to the low incorporation, no washout was observed from the adipose tissue. The absence of observed washout, combined with sustained EPA + DHA content above baseline after cessation of supplementation, provides additional evidence on the slow incorporation and washout dynamics in adipose tissue. Furthermore, we did not detect any differences in the change of EPA + DHA between dosing strategies in the loading or maintenance phases. Aligned with this, a study conducted by Browning *et al.,* (2012) observed that the incorporation of n-3 PUFA into adipose tissue did not differ between doses equivalent to 1, 2, or 4 portions of oily fish per week.

Ageing impacts the function of adipose tissue by affecting the function of preadipocytes and mitochondrial bioenergetics (Schosserer *et al.*, 2018), as well as molecular mechanisms including transcriptomic, cellular and pro-inflammatory phenotypical metabolic changes (Trim *et al.*, 2022). While we did not see differential incorporation in response to the dosing strategies, we observed differential incorporation between the young and older age groups, as evidenced by a significant main effect for age and the interaction effect of dose by age. One possible explanation is that the baseline levels of EPA + DHA in the young age group were significantly lower than in the older age group (**Appendix 3.6;** $P < 0.001$). The slow turnover of adipose tissue may reflect an accumulation of EPA and DHA intake over several years. A review by Hodson *et al.,* (2008) has summarised studies investigating the association dietary intake and adipose tissue fatty acid composition, highlighting significant positive correlations between the relative intake of dietary PUFA and the relative content of adipose tissue n−6 and/or n−3, or

total PUFA (Hodson *et al.,* 2008). In contrast, reported associations between dietary intake and saturated or monounsaturated fatty acids content in adipose tissue was more variable (Hodson *et al.,* 2008). An explanation for the difference in association between fatty acids to dietary intake could be that n-3 PUFA cannot be synthesized de novo in humans, whereas other fatty acids, such as saturated fatty acids, can be produced from other physiological processes like de novo lipogenesis (Imamura *et al.*, 2020). Other dietary sources may therefore also influence fatty acid composition in adipose tissue. For instance, surplus intake of carbohydrates can lead to glucose storage through the formation of glycerol backbones (Richard *et al.*, 2000). Our findings indicate that higher doses and prolonged periods of supplementation are required to observe increases in EPA and DHA content in adipose tissue.

3.4.4 Limitations and future research

In considering the limitations for future research, it is important to note that this study did not investigate the phospholipid membrane composition of adipose tissue or erythrocytes, which could be a valuable area for exploration in future studies, given the differences in incorporation in whole muscle versus phospholipid membrane in skeletal muscle (**Appendix 3.7**), and the differential incorporation between cell lysates and phospholipid membranes in C2C12 myotubes observed in **Chapter 2**. Additionally, the observed individual variability in responses across some tissues suggests larger sample sizes are needed to achieve statistical power. Furthermore, the doses administered in this study were lower than doses used in other studies, and while we demonstrate a greater change in EPA + DHA incorporation in erythrocytes and skeletal muscle phospholipid membranes with the loading dose of 2.52g n-3 PUFA per day utilised in this study, future investigations may benefit from higher doses to detect stronger differences in incorporation in for example adipose tissue. However, our lower dosage approach is also a strength, offering valuable insights into more realistic dosing and eating patterns. The global average consumption worldwide is 163 mg EPA + DHA per day, with large variations across countries (Micha *et al.*, 2014), while the current recommendations are between 200 – 500 mg of EPA + DHA (European Food Safety Authority (EFSA), 2010).

3.4.5 Conclusion

This chapter demonstrates that the incorporation of EPA and DHA in response to n-3 PUFA supplementation varies by tissue and is influenced by age in adipose tissue, but not in

erythrocytes and skeletal muscle phospholipid membranes. Specifically, a loading dose leads to a greater change in % EPA + DHA within four weeks in erythrocytes and skeletal muscle phospholipid membrane fractions. This greater change can be sustained by a maintenance dose, resulting in similar levels of incorporation as the constant dose after 12 weeks, provided the total dose given over the supplementation period is equal. Contrastingly, in adipose tissue, supplementation doses used in this study could not detect differences in EPA + DHA incorporation in response to dosing strategy in young and older adults. Variations in incorporation between tissues can be attributed to the relatively quicker turnover time of erythrocytes compared to skeletal muscle and adipose tissue (Sender and Milo, 2021). Furthermore, an 8-week washout phase is not influenced by dosing strategy and results in a decline of EPA + DHA in erythrocytes (above baseline) and muscle phospholipid membranes (return to baseline) with the doses provided in this study, while adipose tissue warrants longer supplementation periods. This chapter has provided new knowledge to the body of literature concerning changes in lipid profile across tissues in response to different dosing regimens of n-3 PUFA supplementation.

Chapter 4: Impact of 12 weeks n-3 PUFA supplementation on skeletal muscle transcriptome in young and older adults

4.1 Introduction

During ageing, muscle mass and strength decline (Janssen *et al.*, 2000; Goodpaster *et al.*, 2006), which may result in negative consequences for health. The incorporation of omega-3 polyunsaturated fatty acids (n-3 PUFA) into the phospholipid membrane of skeletal muscle cells has been shown to impact muscle health by modulating muscle protein turnover (McGlory, *et al,* 2019), facilitate improvements in muscle strength in older adults (Phillips *et al.*, 2024), ameliorate increases in muscle soreness in competitive soccer players (Philpott *et al.*, 2018), improve lean mass in older women combined with resistance training (Brook *et al.*, 2021), and protect against muscle decline in younger women (McGlory *et al.*, 2019). Additionally, n-3 PUFA supplementation can impact bioenergetics such as an enhanced ADP sensitivity in young adults (Herbst *et al.*, 2014), a decreased oxygen demand in untrained (Kawabata *et al.*, 2014) and trained adults (Peoples *et al.*, 2008), and reduce mitochondrial ROS production in older adults (Lalia *et al.*, 2017).

The observed alterations in these outcomes by n-3 PUFA treatment may be, in part, influenced by modifications at the transcriptomic level. In animal studies, feeding bulls with a diet rich in n-3 PUFA affected the gene expression of genes related to muscle biology, muscle maintenance and muscle function (Hiller *et al.*, 2012). Additionally, feeding arthritic rats a dose of 1g/kg EPA daily attenuated muscle wasting and decreased expression of muscle wasting genes atrogin-1 and MuRF1 (Castillero *et al.*, 2009). A study in pigs fed with diets containing 5% fish oil showed an increase after an immunological challenge in the mRNA abundance of mTORC pathway kinase Akt, while genes related to muscle breakdown, including Forkhead Box O (FOXO) 1, FOXO4, and muscle RING finger, were decreased (Zhang *et al.*, 2019). These findings indicate that the incorporation of n-3 PUFA not only influences structural, neuromuscular, and inflammatory outcomes, but also regulates changes in skeletal muscle mass at the transcriptomic level.

Studies investigating human genome expression in response to n-3 PUFA supplementation are limited. Lalia *et al.,* (2017) demonstrated that four months of 3.9g n-3 PUFA/day altered expression of genes involved in muscle protein turnover in skeletal muscle of older adults. These alterations were accompanied by reduced mitochondrial ROS production and increased postabsorptive mitochondrial and sarcoplasmic protein synthesis. Another study conducted by Yoshino *et al.,* (2016) demonstrated that 6 months of n-3 PUFA supplementation with 1.86g EPA + 1.50g DHA/day in older adults resulted in small but coordinated changes in the muscle transcriptome. Here, expression in pathways related to mitochondrial function and extracellular matrix organisation increased, while expression in pathways related to calpain- and ubiquitin-mediated proteolysis and mTOR-inhibitor pathways decreased. However, these changes in gene expression were not significant at the level of individual genes, suggesting modest effects. Their study used a subset of skeletal muscle biopsies from Smith *et al.,* (2015). As an accompanying functional outcome, increases in thigh muscle volume, hand grip strength, and one repetition maximum were observed (Smith *et al.*, 2015). Alterations in global gene expression in response to long-term n-3 PUFA supplementation may partially explain the changes observed in muscle protein turnover (Lalia *et al.*, 2017), or changes in muscle mass and strength (Smith *et al.*, 2015; Yoshino *et al.*, 2016). However, whether more moderate doses can induce similar changes over a shorter period, and whether younger and older adults respond differentially to n-3 PUFA ingestion has not yet been elucidated.

Muscle between younger and older participants not only differs on a structural level but also on a transcriptomic level (Cisterna and Malatesta, 2024). Consequently, ageing muscle seems to respond uniquely to interventions compared to younger muscle. It is well-documented that older individuals exhibit a reduced sensitivity to anabolic stimuli compared to their younger counterparts (Dillon, 2013). At a genomic level, the muscle transcriptome of older adults has showed a greater response in terms of vascularization and strength to heavy load exercise compared to changes in muscle transcriptome of younger adults (Gautvik *et al.*, 2022). Moreover, a study involving a substantial cohort of healthy individuals spanning a broad age range (23-83 years old) revealed alterations in the muscle transcriptome with ageing, such as differential mRNA gene expression and splice variants that tended to cluster in proteins associated with oxidative phosphorylation, lipid metabolism and mTORC1 signalling (Tumasian *et al.*, 2021). The differential impact of n-3 PUFA supplementation on muscle transcriptome in young and older adults is yet to be fully understood and may contribute, in part, to age-related differences in n-3 PUFA impact on skeletal muscle.

The transcriptomic changes and their associated potential functional outcomes induced by n-3 PUFA may also be influenced by confounders such as variations in diet and physical activity. For instance, the skeletal muscle transcriptome between endurance-trained athletes differs significantly from sedentary individuals (Chapman *et al.*, 2020), and physical activity has been shown to modulate the skeletal muscle transcriptome after 8 weeks of endurance training (Beiter *et al.*, 2024). Additionally, dietary factors such as a higher protein intake in older adults have been found to alter gene expression in skeletal muscle (Riddle, Thalacker-Mercer and Campbell, 2019), and changes in body composition, such as weight loss due to caloric restrictions, have been linked to a shift in skeletal muscle gene expression toward oxidative metabolism (Larrouy *et al.*, 2008). Therefore, it can be challenging to determine whether alterations in the skeletal muscle transcriptome are directly attributable to n-3 PUFA supplementation or are instead influenced by confounders such as changes in diet, body composition, hand grip strength (as an indicator of overall strength), and physical activity. Consequently, these parameters are crucial to measure in intervention studies involving n-3 PUFA supplementation. Moreover, given that n-3 PUFA has been suggested to potentially impact grip strength (Smith *et al.*, 2015) and body composition (Noreen *et al.*, 2010), the measurement of these parameters is crucial for two key reasons: (1) to assess whether changes in body composition, diet, physical activity, and strength might confound the observed changes in the skeletal muscle transcriptome, and (2) to determine whether n-3 PUFA supplementation itself influences body composition and strength.

In **Chapter 3**, we observed similar incorporation in the skeletal muscle phospholipid membranes of young and older adults. However, similar changes in phospholipid fractions do not necessarily result in similar changes on transcriptomic expression, due to the differences in transcriptome between young and older adults. **In this chapter**, we aim to identify the global changes in skeletal muscle transcriptome in young and older adults following 12-weeks of moderate doses (1.26g/day EPA+DHA on average) of n-3 PUFA supplementation using the microarray technique, as well as the differential expression after treatment between the young and older adults. In **Chapter 3**, we established that after a 12-week supplementation of n-3 PUFA the loading and constant dose resulted in elevated but similar skeletal muscle EPA + DHA content, therefore both doses are grouped together in the present chapter to maximise statistical power. Changes in physical activity, grip strength, diet, and body composition are examined to determine whether any changes observed in gene expression are attributable to n-3 supplementation, rather than confounding factors.

4.2 Materials and Methods

4.2.1 Participants and study design

Recruitment of participants, study design, and muscle tissue biopsy procedure are as described in **Chapter 3, section 3.2**. Briefly, fourteen healthy volunteers between 18 – 35 years and fourteen healthy volunteers over 60 years of age were recruited and allocated to consume an average of 1.26g EPA + DHA/day for 12 weeks. Dosing strategies were grouped together as there was no difference in incorporation after 12 weeks (**Chapter 3, section 3.3.4**). Participants arrived after an overnight fast (10h) and abstained from alcohol, ibuprofen/paracetamol and strenuous exercise 48 hours before coming in. Skeletal muscle biopsies were obtained at baseline and after the 12-week supplementation period using the Bergström biopsy technique described in **Chapter 3, section 3.2.3**. For microarray analysis, an aliquot of 18 – 30mg muscle was placed into an RNA-free 1.5 mL Eppendorf tube containing 500 µl RNA*later*™ (Ca.No: AM7020, ThermoFisher Scientific (UK)) and stored in the fridge overnight. The next morning, samples were transferred to the -80 °C freezer pending RNA extraction. Global gene expression was measured at baseline and after 12-week supplementation period. Physical activity and a 3-day food diary were recorded at baseline, after 6 weeks and after 12 week of n-3 PUFA supplementation. Participants were instructed to repeat the last two days of their baseline 3-day food diary before the 12-week biopsy visit. Hand grip strength was measured during pre-screening and after eight weeks of n-3 PUFA supplementation. Body composition was measured at baseline and after twelve weeks of n-3 PUFA supplementation. The study design is visualised in **Figure 4.1**.

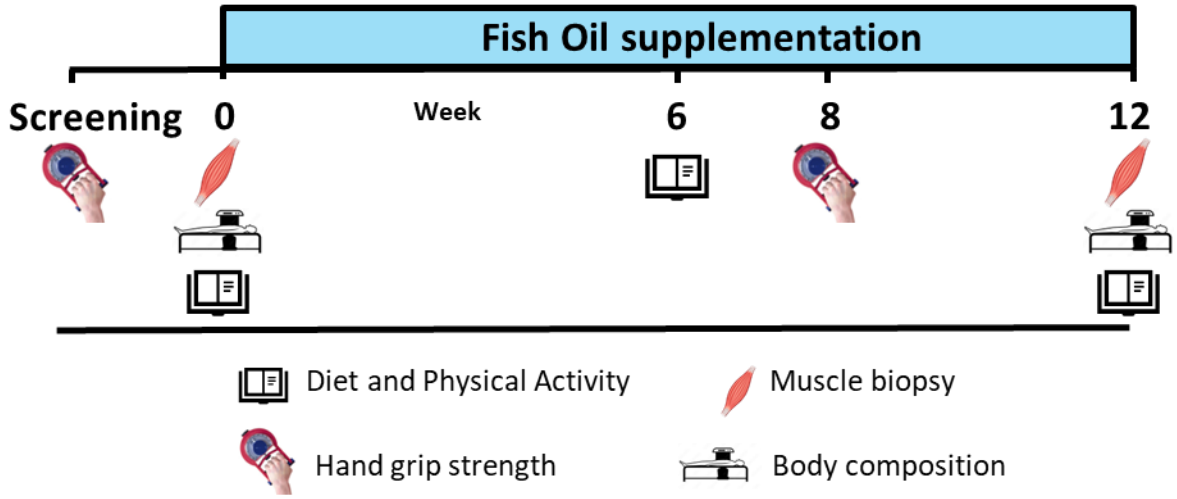

Figure 4.1: Schematic overview of the study.

4.2.2 Materials RNA extraction

RNeasy® Fibrous Tissue Mini kit (Cat.No: 74704) and PowerBead Tubes, Ceramic 1.4 mm (Cat.No: 13113-50) were purchased from Qiagen. RNA*later*™ Stabilization Solution (Cat.No: AM7020), GeneChip™ WT PLUS Reagent Kit (Cat.No: 902281), GeneChip™ WT Terminal Labeling and Controls Kit (Cat.No: 901524), GeneChip™ Hybridization, Wash, and Stain Kit (Cat.No: 900720), Clariom™ S Assay, human (Cat.No: 902927) were purchased from ThermoFisher Scientific (UK).

4.2.3 RNA extraction

Muscle samples were extracted according to the Fibrous Tissue method (Qiagen, 2020) with slight modifications for optimal yield (**Appendix 4.1**). In brief, 300 µl Buffer RLT was added to 1.4mm ceramic bead tubes, followed by the addition of 18 – 30mg of skeletal muscle tissue. The mixture was incubated for 10 minutes and then disrupted using the MagNA Lyser instrument (Roche, Switzerland), employing two cycles of 35 seconds each at 5000Hz, with brief intervals on ice between disruptions. RNA was then isolated from homogenised tissue using the adapted Fibrous Tissue mini kit. The final elution volume of RNA was 30 µl and RNA was placed on ice. Concentration (ng/mL) and purity (absorbance ratio 260:280 nm and 260:230 nm) were measured using DS-11 FX Spectrophotometer (DeNovix, Wilmington, DE, USA). Samples were stored in the -80 °C freezer pending microarray analysis.

4.2.4 Microarray procedure

Global gene expression was examined using microarrays and samples were prepared according to GeneChip™ WT PLUS Reagent Kit User Guide (Thermo and Scientific, 2020). In brief, 100ng of total RNA was amplified and prepared to complementary DNA (cDNA) using reagents from the GeneChip WT Plus Reagent kit.Subsequently, cDNA was purified, fragmented and labelled with biotin using the GeneChip™ WT Terminal Labeling and Controls Kit. Reactions were conducted on a Veriti™ 96-Well Fast Thermal Cycler (Thermo Fisher, 4375305, UK) as recommended by the kit manufacturer. A detailed overview of all the reactions (i.e., first-strand cDNA synthesis, second-strand cDNA synthesis, in vitro transcription cRNA synthesis, 2nd cycle primers-cRNA annealing, 2nd cycle ss-cDNA synthesis, RNA hydrolysis, fragmentation and labelling) can be found in **Table 4.1**. Hybridisation cocktails were prepared using the GeneChip™ Hybridization, Wash, and Stain Kit, and samples were overnight hybridised onto Clariom™ S human microarrays, containing over 20.000 well-annotated genes at 45°C for 16 hours in a GeneChip Hybridisation Oven 640, according to manufacturer's instructions (Thermo and Scientific, 2020). The next day, the arrays were washed and stained on the Affymetrix fluidics station 450, according to the manufacturer's instructions. Genechips were scanned, and images of the arrays were captured with CEL files were generated using the Affymetrix scanner 3000 7G.

Table 4.1. Thermal cycler reaction protocols according to (Thermo and Scientific, 2020).

Protocol	Lid temperature	Step 1	Step 2	Step 3	Step 4	Reaction volume
First-strand cDNA synthesis	42°C	25°C for 1 h	42°C for 1 h	4°C for 2 min		10µl
Second-strand cDNA synthesis	Disabled	16°C for 1 h	65°C for 10 min	4°C for 2 min		30µl
In vitro transcription cRNA synthesis	40°C	40°C for 16 h	Hold at 4°C			60µl
2nd cycle primers-cRNA annealing	70°C	70°C for 5 min	25°C for 5 min	4°C for 2 min		28µl
2nd cycle ss-cDNA synthesis	70°C	25°C for 10 min	42°C for 90 min	70°C for 10 min	Hold at 4°C	40µl
RNA hydrolysis	70°C	37°C for 45 min	95°C for 5 min	Hold at 4°C		44µl
Fragmentation	93°C	37°C for 60 min	93°C for 2 min	Hold at 4°C		48µl
Labelling	70°C	37°C for 60 min	70°C for 10 min	Hold at 4°C		60µl
Hybridisation control	65°C	65°C for 5 min				Variable
Hybridisation cocktail	99°C	95°C for 5 min	45°C for 5 min			Variable

Min = minutes; cDNA = complementary deoxyribonucleic acid; cRNA = complementary ribonucleic acid; ss-cDNA = single-stranded cDNA.

4.2.5 Physical Activity status

Physical activity status was monitored with a tri-axial ActiGraph GT3X+ accelerometer (Pensacola, Florida, USA). Participants were instructed to wear the accelerometer for five consecutive days, for at least 10 hours per day, at the time during which they were most active. The accelerometer was worn around the waist, on the right side of their body and initialised to collect data at 80 Hz. Data for total physical activity (light, moderate, vigorous) and moderate-vigorous activity were analysed using ActiLife software (v6.13.3, ActiGraph, Pensacola, 211Florida, USA). Physical Activity and moderate to vigorous physical activity were classified according to established cut-off points, as previously described by Freedson, Melanson and Sirard (1998). Activity data were included in the analysis if there were at least 3 successful days recorded of > 420 minutes (7 hours) per day (Prescott *et al.*, 2020).

4.2.6 Dietary intake analysis

Participants were instructed to complete a 3-day food diary (**Appendix 4.2**) at baseline, 6 weeks, and 12 weeks. Data were entered in Nutritics (Nutritics, 2024) and analysed for macronutrient content, n-3 PUFA intake, portions of oily fish, and energy intake. For analysis, only data from baseline and week six were considered, as participants were instructed to replicate their baseline food diary prior to the biopsy test visit at week 12.

4.2.7 Body composition

Lean mass and fat mass were quantified at baseline and after the 12-week supplementation period using dual energy X-ray absorptiometry (DEXA) (iDXA; GE Encore, version 13.40.038, GE Healthcare, Madison, WI, USA) according to standard procedure. Participants were instructed to drink 250 mL water 2 hours beforehand and arrived overnight fasted. Participants wore minimum clothing and removed any metallic objects and jewellery (if possible). Biologically assigned women at birth in the young age group underwent a pregnancy test before each scan.

4.2.8 Hand grip strength

Hand grip strength (kg) was measured with a handgrip dynamometer (T.K.K.5001, Grip-A, Takei, Tokyo, Japan). Instructions were provided based on the Southampton protocol (Roberts *et al.*, 2011). The participant was seated on a chair of adjustable height to ensure that the feet were positioned flat on the floor. The participants' arm was rested on a table with their elbow positioned approximately 90-degree angle (visually inspected) in a neutral position. The grip size of the dynamometer was adjusted so that the middle phalanx of the finger was horizontal when performing the test. Participants were instructed to squeeze it as hard as they could and were verbally encouraged to exert maximal grip strength. The procedure was repeated three times on both hands, with the participant alternating between hands. The combined maximum grip strength was used in the analysis to account for the differences in dominant hand strength between right- and left-handed individuals (Bohannon, 2003).

4.2.9 Statistical analysis

Analysis of global gene expression

Data were analysed in RStudio version 2023.12.1. The arrays were annotated using the latest BrainArray mapping and Ensembl Gene IDs (ENSG) were used for gene identification (Sandberg and Larsson, 2007). Data were normalised using The SCAN.UPC (Single Channel Array Normalization) method (Piccolo *et al.*, 2012), and probes were filtered and excluded where UPC (Universal expression Code) was <0.5 and Log2 expression was <0. Batch correction was carried out using the ComBat() package. Principal Component Analysis (PCA) was conducted before and after batch corrected and filtered data. Gene sets were differentially expressed using the limma() package (Smyth, no date). First, differences between the constant dose (CD) and loading dose (LD) groups were compared within both the young and older age groups. Contrasts were made to assess the difference in treatment effects between CD and LD within each age group. All analyses were adjusted for false discovery rate (FDR) using the Benjamini-Hochberg method (Benjamini & Hochberg, 1995). Next, results between doses were pooled with contrasts made for treatment effect in young, treatment effects in old, and the difference in treatment effects between young and old, and adjusted for false discovery rate (FDR) according to Benjamini and Hochberg's method (Benjamini and Hochberg, 1995). Gene set enrichment analysis (GSEA) was performed using Molecular Signatures Hallmark gene sets (Liberzon *et al.*, 2015), and differentially expressed pathways were adjusted for False Discovery Rate (FDR) according to Benjamini and Hochberg's method. Statistical significance was accepted when the FDR was less than 5%.

Analysis of physical activity, dietary intake, hand grip strength and body composition

Changes in physical activity, diet, hand grip strength, lean mass and fat mass were determined with the lmer() package using a linear mixed effects model. For physical activity and dietary intake, time and age were included as independent variables and interaction effects were examined. For grip strength and body composition, time, age, and sex were included as independent variables and interaction effects were examined. Participant number was included as a random effect. If a main effect was found, pairwise comparisons were conducted using Tukey's post-hoc analysis using the emm() package. If no main effect was found, paired t-tests were conducted to assess changes over time. Significance was set at $P < 0.05$.

4.3 Results

4.3.1 Muscle transcriptome GSEA changes between dosing groups in young and older adults.

Gene set enrichment analysis was performed to test whether there were differences in transcriptome responses to n-3 PUFA supplementation between dosing groups within the young and older age groups as described in **Chapter 3**.

In young adults, category enrichment analysis identified five gene sets that were differentially regulated between CD and LD (**Table 4.2**). Next, changes in gene sets from baseline to post-treatment were analysed separately for LD and CD. Here, category enrichment showed that, among the gene sets differentially regulated between CD and LD in **Table 4.2**, the direction of change in response to n-3 PUFA supplementation was significant in only one of the two dosing groups (**Table 4.3**).

Table 4.2: Gene sets that were significantly altered by 12-week n-3 PUFA supplementation in young adults. Significance was set at $P < 0.05$.

Gene set name	Genes expressed	Direction	P-val.
OXIDATIVE_PHOSPHORYLATION	171/200	Down	0.011
MYC_TARGETS_V1	149/200	Down	0.005
DNA_REPAIR	92/150	Down	0.005
UV_RESPONSE_DNA	96	Up	0.008
MYOGENESIS	104/120	Up	0.022

Table 4.3: Gene sets that were significantly altered in response to 12-week n-3 PUFA supplementation in CD (n=3) and LD (n=4) groups in young adults. Statistically significantly altered gene sets in response to n-3 PUFA supplementation are presented **in bold**. Significance was set at $P < 0.05$.

	Constant Dose		Loading Dose	
Pathway	**Direction**	**P-val.**	**Direction**	**P-val.**
OXIDATIVE_PHOSPHORYLATION	**Down**	**<0.001**	Down	0.68
MYC_TARGETS_V1	**Down**	**<0.001**	Down	0.92
DNA_REPAIR	**Down**	**<0.001**	Up	0.92
MITOTIC_SPINDLE	**Up**	**0.003**	Up	0.92
UV_RESPONSE_DN	**Up**	**0.010**	Down	0.92
MYOGENESIS	Up	0.688	**Down**	**<0.001**
INTERFERON_GAMMA_RESPONSE	Down	0.583	**Down**	**0.02**
INTERFERON_ALPHA_RESPONSE	Down	0.517	**Down**	**0.02**

In older adults, category enrichment analysis identified one gene set, DNA_REPAIR, that was significantly downregulated in CD compared to LD ($P = 0.044$). However, within CD and LD separately, there were no changes in the DNA_REPAIR over time in response no n-3 PUFA supplementation (**Table 4.4**).

Table 4.4: DNA_REPAIR alterations in response to 12-week n-3 PUFA supplementation in older adults. Summary of DNA_REPAIR pathway alterations following 12-week n-3 PUFA supplementation in CD (n=5) and LD (n=6) groups. No significant changes were observed in either group.

Pathway	**N_genes**	**Direction**	**P.val**
DNA_REPAIR_CD	92/150	Down	0.283
DNA_REPAIR_LD	92/150	Up	0.352

4.3.2 Muscle transcriptome GSEA changes in young and older adults

Category enrichment analysis identified one gene set related to bioenergetics that was downregulated in both young and older adults; oxidative phosphorylation (**Table 4.5, Figure 4.2A).** In the young age group, another pathway related to bioenergetics was downregulated; reactive oxygen species (ROS; **Table 4.5, Figure 4.2B**).

Five pathways related to muscle regeneration were altered after n-3 PUFA supplementation (**Table 4.5**). In the young age group, the mitotic spindle pathway was upregulated (**Figure**

4.2C), whereas two pathways related to muscle regeneration were downregulated: myc targets (**Figure 4.2D**) and DNA repair (**Figure 4.2E**). In contrast, in the older age group, two pathways related to muscle regeneration were upregulated; angiogenesis (**Figure 4.2F**) and epithelial-mesenchymal transition (EMT; **Figure 4.2G**).

Table 4.5: Gene sets that were significantly altered by 12-week n-3 PUFA supplementation in young and/or older adults. Significance was set at $P < 0.05$.

Gene set name	Genes expressed	Age group	Direction	P-val.
Bioenergetics				
OXIDATIVE_PHOSPHORYLATION	171/200	Young	Down	<0.001
		Older	Down	0.030
REACTIVE_OXYGEN_SPECIES	34/49	Young	Down	0.048
Muscle regeneration				
MITOTIC_SPINDLE	111/199	Young	Up	0.006
MYC_TARGETS_V1	149/200	Young	Down	<0.001
DNA_REPAIR	92/150	Young	Down	0.044
ANGIOGENESIS	18/36	Older	Up	0.004
EPITHELIAL_MESENCHYMAL_TRANS	104/120	Older	Up	0.003

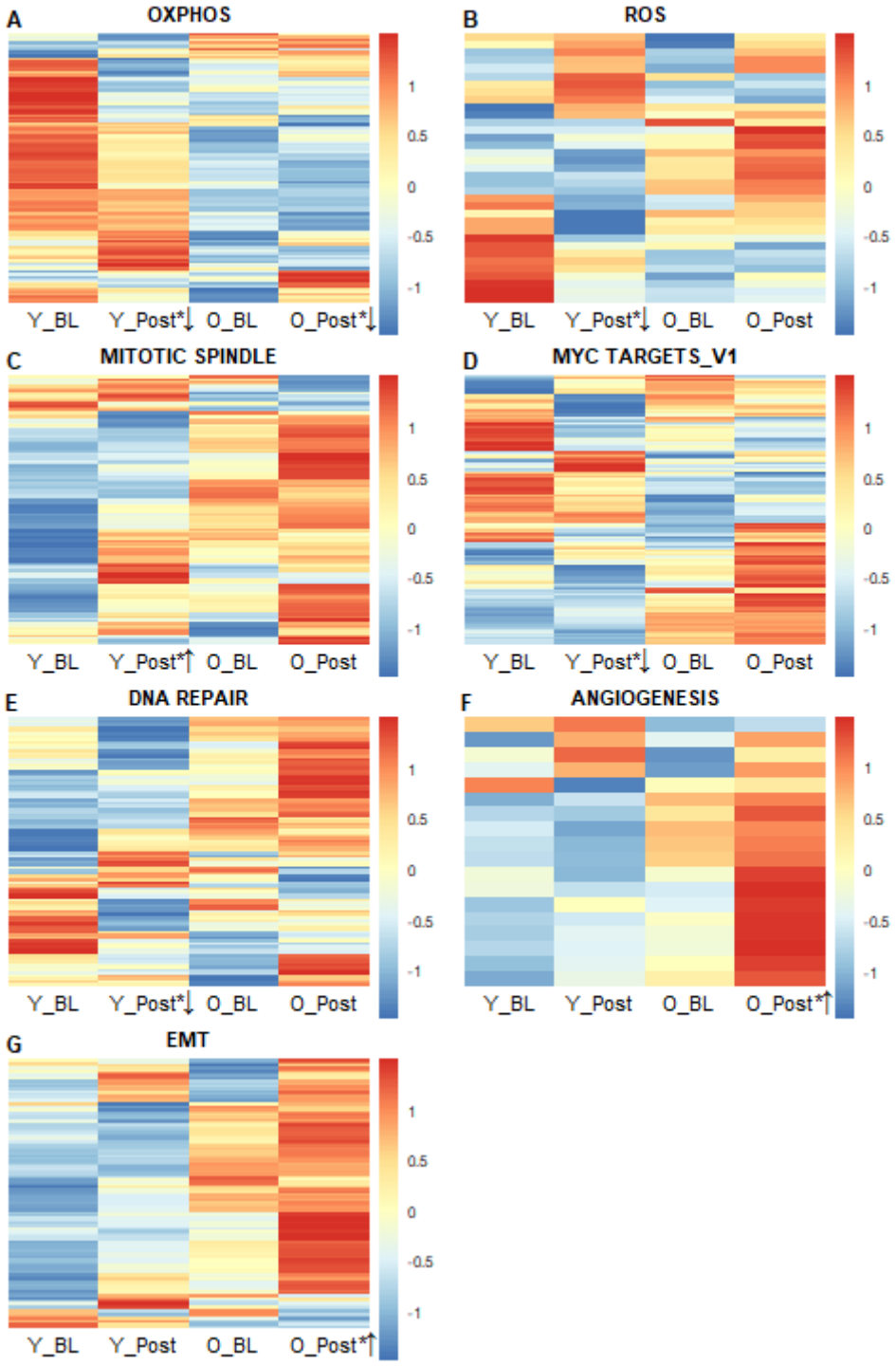

Figure 4.2: Heatmap of differentially expressed genes in significantly altered pathways resulting from gene set enrichment analysis (GSEA) at baseline (BL) and after 12-week of n-3 PUFA supplementation (Post) in young (Y) and Older (O) adults. **A**: Oxidative phosphorylation. **B**: Reactive Oxygen Species. **C**: Mitotic Spindle. **D**: Myc targets. **E**: DNA repair. **F**: Angiogenesis. **G**: Epithelial to mesenchymal transition. '*' indicates significance after correcting for false discovery rate (FDR). Upregulation from baseline is indicated with '↑' and downregulation with '↓'. Significance was set at $P < 0.05$. Red indicates relatively high expression, while blue indicates relatively low expression.

4.3.3 Differential regulation of muscle transcriptome between young and older adults in response to n-3 PUFA supplementation

No individual gene exhibited differential expression in response to n-3 P UFA supplementation between age groups after correcting for FDR ($P > 0.05$). Category enrichment analysis identified two gene sets that were differentially regulated between age groups in response to n-3 PUFA supplementation (**Table 4.6**). Both gene sets were associated with inflammation and regulate the IFN-γ and IFN-α pathways. These were upregulated in the older age group compared to the young age group (**Figure 4.3**).

Table 4.6: Gene sets that were significantly differently changed by n-3 PUFA supplementation between age groups. Significance was set at $P < 0.05$.

Gene set name	Genes expressed	Direction	P-val.
Inflammation			
INTERFERON_GAMMA_RESPONSE	75/200	Up Older Down Young	0.001
INTERFERON_ALPHA_RESPONSE	38/97	n.s Older Down Young	0.009

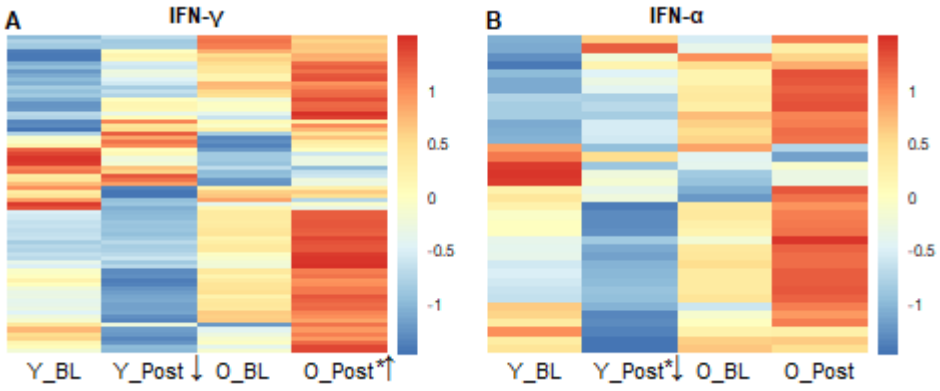

Figure 4.3: Heatmap of differentially expressed genes in significantly altered pathways resulting from gene set enrichment analysis (GSEA) at baseline (BL) and after 12-week of n-3 PUFA supplementation (Post) between young (Y) and Older (O) adults. **A**: IFN-γ. **B**: IFN-α. '*' indicates significance after correcting for false discovery rate (FDR). Upregulation from baseline is indicated with '↑' and downregulation with '↓'. Significance was set at $P < 0.05$. Red indicates relatively high expression, while blue indicates relatively low expression.

4.3.4 Individual genes

In the young age group, the gene encoding for Phosphodiesterase 4A (PDE4; ENSG00000065989) was differentially expressed post 12-week supplementation after correction for multiple testing (P = 0.049, fold change = 1.15; **Figure 4.4**). No individual gene was differentially expressed over the 12-week supplementation in the older age group after correction for multiple testing (P > 0.05).

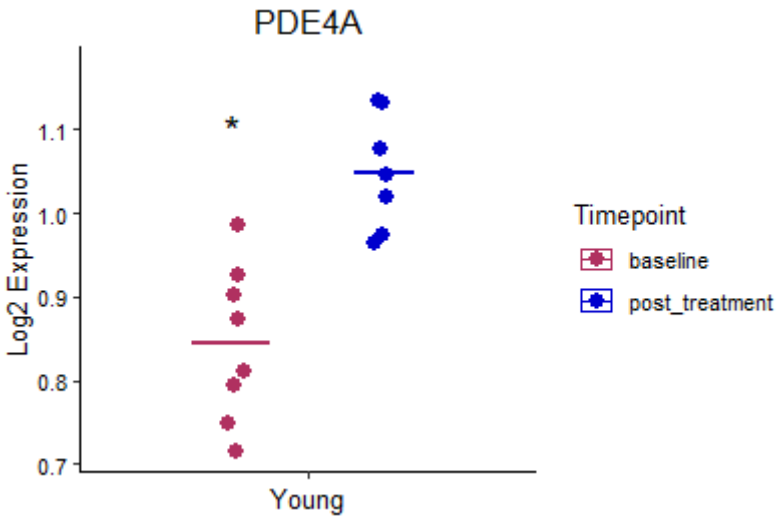

Figure 4.4: Log2 expression of differentially altered gene PDE4A in the young age group (n=8, except post-treatment where n=7). Bars represent mean values; dots represent individual participants. Significance between baseline and post-treatment is indicated with an '*'. Significance was set at P < 0.05. PDE4A = Phosphodiesterase 4A.

4.3.5 Physical Activity

Changes in total physical activity and moderate to vigorous physical activity (MVPA) were examined to assess its potential influence on the observed changes in gene expression. There were no main effects observed for total physical activity (P > 0.05; **Figure 4.5A**). There was a main effect of age on MVPA (P = 0.026). Post-hoc analysis revealed that the young age group had a significantly higher percentage of MVPA minutes compared to the older age group (**Figure 4.5B**). There was no statistically significant effect of time, confirming total physical activity and MVPA did not change during the 12-week supplementation period (P > 0.05; **Figure 4.5).**

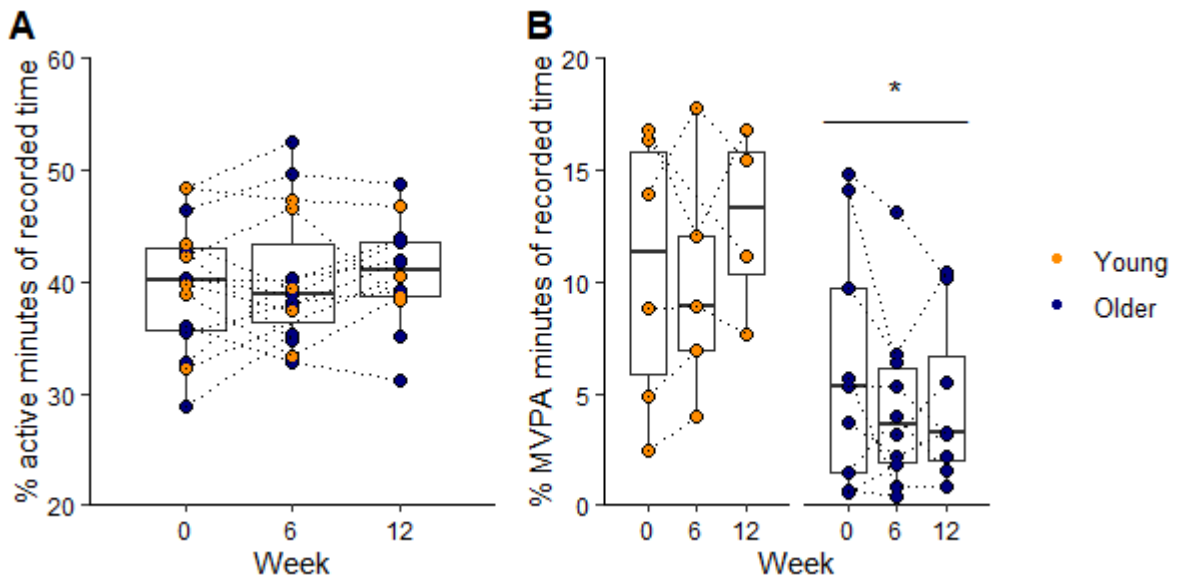

Figure 4.5: Total Physical activity (**A**) and moderate to vigorous (MVPA) physical activity (**B**) in young (n = 6, except week 6 where n = 5 and week 12 where n = 4) and older (n = 9, except week 6 where n = 10 and week 12 where n = 8) adults, expressed as the percentage of total recorded time at baseline, six, and twelve weeks of n-3 PUFA supplementation with 1.26g EPA + DHA per day on average. Boxplots represent median + interquartile ranges and whiskers, dots represent individual values. '*' indicates statistically significant difference from Young ($P < 0.05$).

4.3.6 Dietary intake analysis

No main effects of time or age were observed on energy intake (**Figure 4.6A**), n-3 PUFA intake (**Figure 4.6B**), or macronutrient composition (**Figure 4.6C**), confirming dietary intake did not change during the 12-week supplementation period ($P > 0.05$). Due to an outlier for n-3 PUFA intake at 6 weeks (**Figure 4.6B**), portions of oily fish were determined of all participants. There were no reports of oily fish intake at baseline or at six weeks (data not shown).

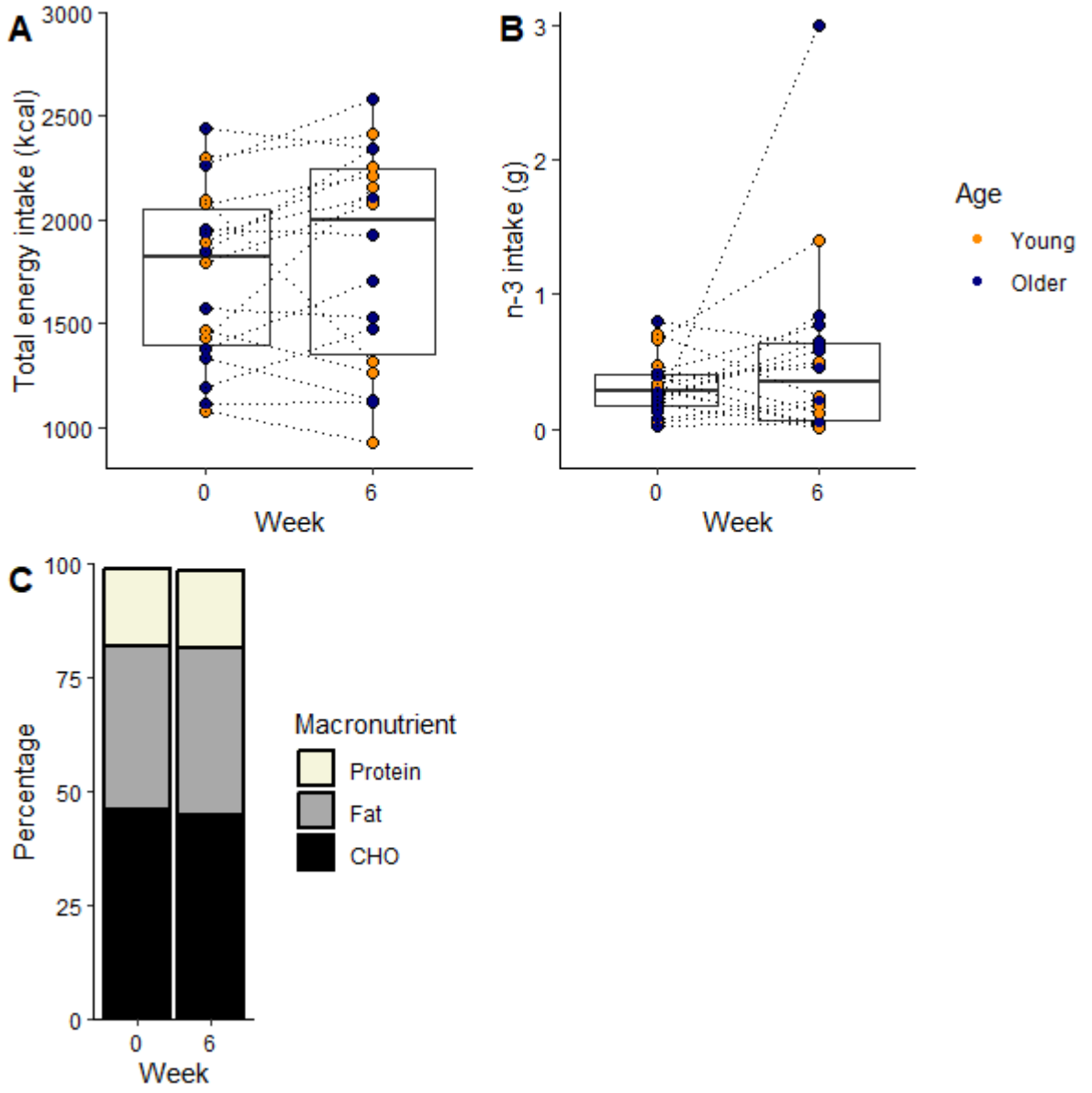

Figure 4.6: Total energy intake in kcal (**A**), n-3 intake in grams (**B**), and macronutrient composition expressed as the percentage of total energy intake (**C**) in young (n = 8) and older (n = 10) adults at baseline and 6 weeks of n-3 PUFA supplementation with 1.26g EPA + DHA per day on average. **A, B**: Boxplots represent median + interquartile ranges and whiskers, dots represent individual values. No significant changes were found between age groups or time points ($P > 0.05$).

4.3.7 Hand grip strength

There was a main effect of sex ($P < 0.001$) on combined hand grip strength, and a trend for an interaction effect of age × sex ($P = 0.05$). Post-hoc analysis revealed that women had a significantly lower combined hand grip strength compared to men ($P < 0.001$). There was no statistically significant effect of time, confirming that grip strength did not change during the 12-week n-3 PUFA supplementation period ($P > 0.05$; **Figure 4.7).**

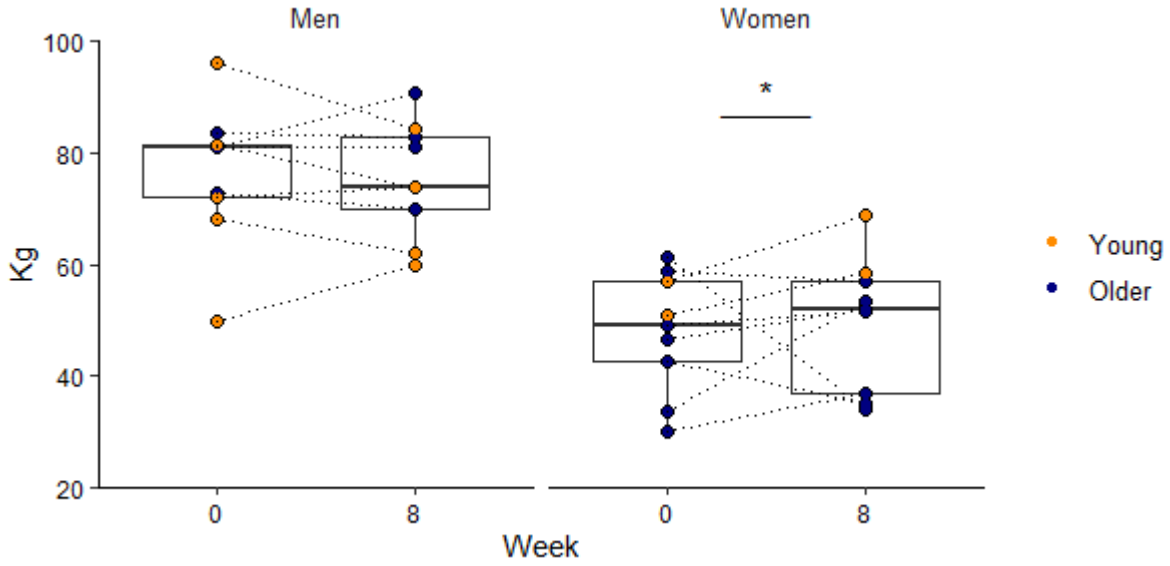

Figure 4.7: Combined hand grip strength (kg) of men (n=5 young, n=4 older) and women (n=2 young, n=7 older) at baseline and after 8 weeks of n-3 PUFA supplementation with 1.26g EPA + DHA per day on average. Boxplots represent median + interquartile ranges, dots represent individual values. '*' indicates statistically significant difference from men ($P < 0.05$).

4.3.8 Total lean mass and total fat mass

There was a main effect of sex on total lean mass (P = 0.001), while there were no main effects for total fat mass. The main effect of sex on total fat mass did not reach statistical significance, due to an outlier in the male group (P = 0.08). Post-hoc analysis revealed that women had a significantly lower total lean mass compared to men (P < 0.001). There was no statistically significant effect of time on total lean or total fat mass, confirming that lean mass and fat mass were not altered during the 12-week n-3 PUFA supplementation period (P > 0.05, **Figure 4.8**).

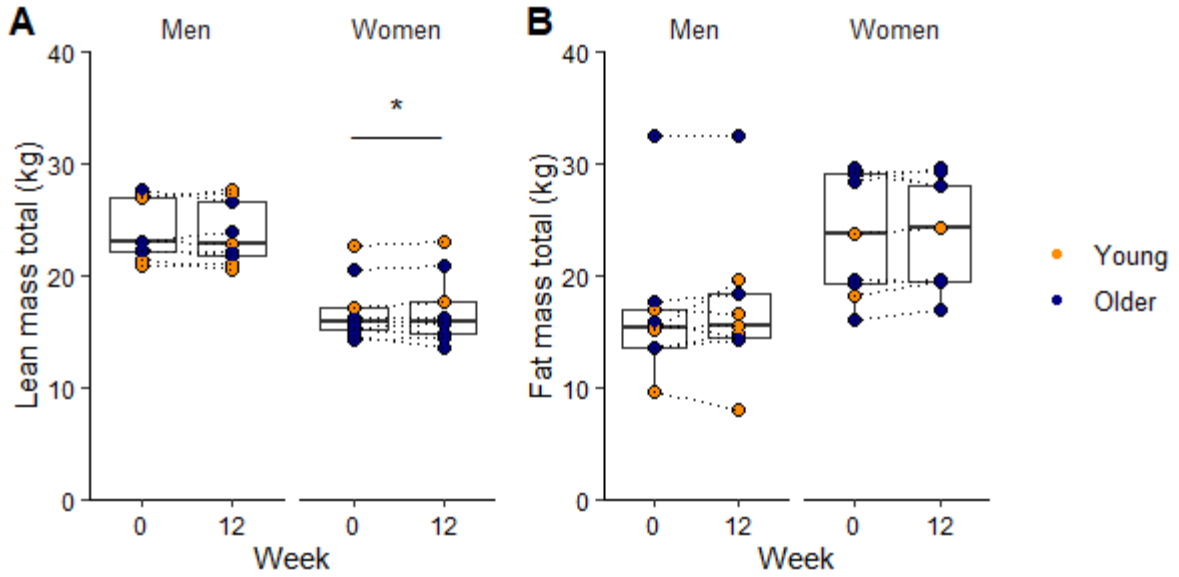

Figure 4.8: Total lean mass (A) and total fat mass (B) of men of men (n=5 young, n=4 older) and women (n=2 young, n=7 older) at baseline and after 12 weeks of n-3 PUFA supplementation with 1.26g EPA + DHA per day on average. Boxplots represent median + interquartile ranges, dots represent individual values. '*' indicates statistically significant difference from men (P < 0.05).

4.4 Discussion

This chapter sought to explore the changes in muscle transcriptome in response to n-3 PUFA supplementation in young and older adults, as well as the differential expression after treatment between the two age groups. Twelve weeks of supplementation with 1.26g n-3 PUFA per day on average resulted in differential changes in skeletal muscle transcriptome expression in young and older adults in pathways related to bioenergetics, muscle regeneration, and inflammation. In young adults, one individual gene (PDE4A) was differentially expressed in the young age group in response to n-3 PUFA supplementation. Physical activity, diet, hand grip strength, diet, and body composition did not change over the 12-week supplementation period, indicating that the observed changes in muscle transcriptome were most likely due to n-3 PUFA supplementation and confounding factors.

4.4.1 Differential regulation between dosing groups

In **Chapter 4,** the underlying hypothesis for the transcriptome analysis was that changes in the phospholipid membrane composition would result in changes in transcriptomic expression. Although the phospholipid membrane composition was similar between the CD and LD groups after 12 weeks, the results from dosing group analysis suggests that the dosing strategy may influence differential expression. However, when examining the changes in gene sets in response to n-3 PUFA supplementation separately for LD and CD over time (**Table 4.3, Table 4.4**), it was demonstrated that these gene sets were only significant in one of the two dosing groups, leading to apparent differential regulation. Furthermore, it is well-established that gene expression varies between individuals (GTEx Consortium, 2015), which may influence how genes are regulated by intervention trials. Given the small sample sizes in the current study, where n = 3, 4, 5 and 6, individual differences may be exaggerated, leading to a lack of significant differential regulation when analysed separately. Based on these preliminary observations, particularly in the skeletal muscle of younger adults, larger studies with bigger sample sized are needed to examine the impact of dosing approaches on the transcriptomic expression of pathways involved in muscle regeneration and repair.

4.4.2 Bioenergetics changes

The impact of n-3 PUFA on bioenergetics has been extensively studied, where studies show an impact of n-3 PUFA on bioenergetic variables, such as an increased resting VO_2 and resting metabolic rate (Gerling *et al.*, 2014), improved ADP sensitivity (Herbst *et al.*, 2014) and

reduced mitochondrial oxidant emission rates (Lalia *et al.*, 2017). However, not all studies report changes in mitochondrial function in response to n-3 PUFA supplementation (Kunz *et al.*, 2022). In the present study, we observed a downregulation in the oxidative phosphorylation pathway across both age groups in the muscle transcriptome. Aligned with this, Lalia *et al.,* (2017) found that individual genes related to oxidative phosphorylation were more downregulated in older individuals compared to young individuals after four months of 3.9g/day n-3 PUFA supplementation compared to baseline, using mRNA sequencing. Conversely, Yoshino *et al.,* (2016), observed an increase in the oxidative phosphorylation pathway in skeletal muscle after 6 months of supplementation with 3.4g n-3 PUFA per day. These discrepancies may be attributable to differences in study design. The study by Yoshino *et al.,* study included a control group that matched the intervention group, where the intervention group comprised the strongest 10 responders in changes in thigh muscle volume. Furthermore, while participants were being asked to maintain their physical activity, this was not measured and could have changed during the supplementation period. Differences in study duration — 6 months versus 3 months — and dosage — 3.4g/day versus 1.26g/day — as reported in Yoshino *et al.,* compared to our design, might also contribute to the divergent outcomes. From a mechanistic standpoint, the higher dose over a longer period in Yoshino *et al.,* may result in more pronounced mitochondrial membrane alterations. This prolonged exposure could allow gene expression to eventually align with these changes, potentially resulting in upregulation. However, this hypothesis is speculative and underscores the necessity for further research. Moreover, Yoshino *et al*., employed the KEGG pathway database, whereas we used GSEA. The overlap in genes between the GSEA (200 genes) and KEGG (132 genes) is merely 83 genes (**Appendix 4.4**), which may also contribute to a disparity in the observed differences.

Oxidative phosphorylation includes the transfer of electrons across the electron transport chain (ETC), which is located on the inner mitochondrial membrane. Here, the transfer of electrons creates a proton gradient which is utilised by ATP synthase to generate ATP (Papa *et al.*, 2012). The incorporation of n-3 PUFA into the mitochondrial membrane may impact the efficiency of the electron transport across the ETC and resulting proton gradient, possibly enhancing the energy production. For example, Peoples and McLennan (2010) demonstrated that rats fed n-3 PUFA utilised less oxygen for a given twitch during and recovering from multiple bouts of prolonged contraction. Following that, Herbst *et al.,* (2014) showed that n-3 PUFA supplementation increased mitochondrial ADP sensitivity, sub-maximal ADP-stimulated

respiration, and maximal mitochondrial ROS emission, without altering the capacity of oxidative phosphorylation or the proteins in the ETC complexes in human skeletal muscle. In the present study, although speculative, a compositional change in the membrane might have resulted in a heightened sensitivity to ADP uptake and subsequently reduced the expression of genes related to oxidative phosphorylation pathway. Gerling *et al.,* (2019) observed that the percentage of n-3 PUFA in both whole muscle phospholipid membrane and mitochondria phospholipid membrane increased by 5% following a 12-week supplementation period with high doses (2 g EPA + 1 g DHA per day), indicating that similar proportions of n-3 PUFA were directed towards the whole muscle membrane and the mitochondrial membrane. In our study, we noted an increase in EPA + DHA of the whole muscle phospholipid membrane ranged between 1.15 – 1.57 % (**Chapter 3, section 3.3.4**). Therefore, it is likely that the composition of mitochondrial membranes in our study are altered in response to n-3 PUFA supplementation.

Aligned with a reduction in the oxidative phosphorylation pathway, a reduction in the reactive oxygen species (ROS) pathway was observed in the young age group, while no such effects were observed in the older age group. Mitochondrial ROS productions typically increase with age (Maldonado *et al.,* 2023), while ageing is also associated with decreased mitochondrial content and function (Johnson, Robinson and Nair, 2013). However, there is also evidence that rather than ageing, a decrease in physical activity is more likely the reason to these changes in ROS production. For example, one study in young and older trained and untrained individuals showed that markers related to mitochondrial ATP production rates did only differ between ages in the untrained individuals, whereas no differences were observed between trained young and trained older adults (Lanza *et al.*, 2008). Interestingly, Lalia *et al.*, (2017) reported no differences in mitochondrial ROS production between young and older adults at baseline. They followed up the older adults after 16 weeks of 3.9/day n-3 PUFA supplementation and observed a reduction in ROS emissions in skeletal muscle. However, when the same group repeated the research in healthy older adults over a six month supplementation period using the same dose, no differences in ROS generation were observed following n-3 PUFA supplementation (Kunz *et al.*, 2022). In our study, the downregulation we observed in the young age group was at a transcriptomic level, which may not necessarily translate to changes in ROS production. Collectively, our findings suggest a role for n-3 PUFA in modulating pathways linked to muscle bioenergetics; however, the specific mechanisms remain to be fully elucidated. Our data indicate that this modulation is partially regulated at the transcriptomic level.

4.4.3 Muscle regeneration

Several pathways related to muscle regeneration were differentially altered in young and older adults. A recent comprehensive study determined differences in integrated cellular and molecular pathways in the context of ageing (Lai *et al.*, 2024), which can suggest a differential role for n-3 PUFA in whole transcriptome modulation with respect to age. In the young age group, there was an observed increase in the mitotic spindle pathway, a crucial pathway in cell proliferation (Petry, 2016), which can potentially enhance muscle regeneration. Two other pathways related to cell proliferation were downregulated: Myc targets and DNA repair. The role of Myc and related genes in cell proliferation and muscle tissue are dual; c-Myc is a transcription factor known to both inhibit but also promote myoblast proliferation and thereby muscle hypertrophy (Luo *et al.*, 2019). While often studied in the context as a master regulator of oncogenesis (Meškytė, Keskas and Ciribilli, 2020), overexpression of c-Myc in mice increased skeletal muscle ribosome biogenesis and protein synthesis without activation of mTORC1 (Mori *et al.*, 2021), emphasizing its dual role. DNA repair mechanisms in skeletal muscle are essential for restoration of broken or damaged DNA (Jackson and Bartek, 2009). Given the observed reduction in gene expression of ROS pathway within the young age group, it is plausible that this decrease may have contributed to a decrease in DNA damage. Consequently, this potential decrease in DNA damage might have led to a downregulation in the gene expression of DNA repair mechanisms, since ROS are a form of damage that could activate DNA repair mechanisms (Srinivas *et al.*, 2019).

In the older age group, two pathways related to muscle regeneration were upregulated: angiogenesis and epithelial to mesenchymal transition. Angiogenesis refers to the formation of new blood vessels. Although angiogenesis is associated with tumour formation and progression (Carmeliet, 2003), angiogenesis is also notably augmented in response to exercise stimuli (Holloway *et al.*, 2018), and considered a favourable attribute within skeletal muscle physiology (Olfert *et al.*, 2016). Epithelial to mesenchymal transition (EMT) describes dynamic changes between the epithelial to mesenchymal phenotypes in cells, leading to functional changes in cell migration and invasion which are often associated with cancer biology (Yang *et al.*, 2020). However, it must be noted that ‘type 3 EMT’ only is associated with cancer, whereas ‘type 1 EMT’ is associated with embryogenesis and organ development, and ‘type 2 EMT’ plays a crucial role in wound healing and tissue repair (Kalluri and Weinberg, 2009). Given that skeletal muscle regeneration follows the same process as wound healing and tissue repair –degeneration, inflammation, remodelling & repair – taken together with the

alterations observed in angiogenesis, it is more likely that the upregulation in the EMT pathway observed in our study is related to type 2 EMT: wound healing, regeneration, and fibrosis (Marconi *et al.*, 2021). Angiogenesis and EMT are regulated by the extracellular matrix (Kim *et al.*, 2006; Mongiat *et al.*, 2016). The extracellular matrix (ECM) is a complex network of proteins and (signalling) molecules surrounding cells and is involved in many processes related to structural support, cellular communication, tissue repair, and regulation of cellular functions (Lu *et al.*, 2011). Therefore, it can be assumed that the altered pathways observed in the young age group, mitotic spindle, myc targets, DNA repair, also interact with and, at least to a certain extent, are regulated by the ECM. Alterations in skeletal muscle transcriptome pathways related to ECM by n-3 PUFA have been observed by Yoshino *et al*., (2016). However, Yoshino *et al.,* (2016) observed reduction in protein breakdown pathways related to calpain/proteasome, whereas we used the Hallmark gene set database, which does not contain pathways directly related to protein breakdown and therefore we cannot make direct comparisons. However, Hallmark gene sets summarise the most relevant information from the original founder sets, providing a more refined and concise input for GSEA (Liberzon *et al.*, 2015). Furthermore, the Hallmark gene sets are a combination of an automated computational procedure followed by manual expert curation, enhancing their reliability and utility in genomic analyses. In conclusion, the alterations in muscle transcriptome observed in our study might suggest additional pathways to protein synthesis or breakdown that are impacted by moderate doses of n-3 PUFA. Overall, our findings suggest that n-3 PUFA plays a role in skeletal muscle regeneration, with distinct effects observed between young and older adults.

4.4.4 Differential regulation of IFN-γ and IFN-α between young and older adults in response to n-3 PUFA supplementation

Two pathways, IFN-γ and IFN-α, related to inflammation (Liberzon *et al.*, 2015), were differentially altered between age groups in response to n-3 PUFA treatment; these were upregulated in the older age group and downregulated (IFN-γ) or unchanged (IFN-α) in the younger age group. Interferons (IFN) are a class of cytokines that have a central role in inflammation and are known for their modulation of immune cell function (Pestka, Krause and Walter, 2004). In the context of inflammation, n-3 PUFA are recognised for their established anti-inflammatory properties (Calder, 2020b), and therefore would be expected to decrease the gene expression of IFN. However, in skeletal muscle, IFN-γ is also involved in tissue repair. For example, Cheng *et al.*, (2008) demonstrated that endogenous IFN-γ promotes muscle

regeneration, where they proposed a dual role for IFN-γ in skeletal muscle, since other findings showed a decreased muscle proliferation at higher, exogenous concentrations of IFN-γ (Shelton *et al.*, 1999). Given the observed upregulation we detected in molecular pathways with regards to muscle regeneration such as angiogenesis and EMT in the older age group, it is plausible that the alterations in these pathways are accompanied by an increase in endogenously expressed IFN-γ. In line with this, a study in mice demonstrated that gene expression of IFN-γ was downregulated in ageing muscle compared to younger muscle (Zhang *et al.*, 2020). This suggests that the lower genomic expression of IFN-γ in ageing tissue may result in an increased expression when muscle repair is accelerated, for example by n-3 PUFA treatment. However, the role of IFN-γ and its regulation in human skeletal mass warrants further investigation (Peake, Gatta and Cameron-Smith, 2010). Moreover, IFN-α, a different type of IFN (Pestka, Krause and Walter, 2004), also exhibited differential expression across age groups following n-3 PUFA supplementation, and has been closely involved in IFN-γ production (Brinkmann *et al.*, 1993). The interplay between n-3 PUFA and IFN pathways related to inflammation in skeletal muscle suggests broader implications, such as possible skeletal muscle regeneration.

4.4.5 Changes in PDE4A expression in the young age group

The gene encoding for PDE4A was the only individual gene upregulated after n-3 PUFA supplementation in the young age group, albeit with a small fold-change. PDE4 is a subclass of phosphodiesterase and is involved in deactivating cyclic AMP (c-AMP), an important second messenger involved in many cellular processes (Serezani *et al.*, 2008), including hypertrophy and cell proliferation (Berdeaux and Stewart, 2012). High expression of PDE4A has been observed in cells of patients with inflammatory diseases (Schafer *et al.*, 2016), while PDE4A inhibitors restored c-AMP levels which subsequently induced mitochondrial biogenesis (Hamidie *et al.*, 2021). We did not observe any changes in c-AMP related genes, and only a few genes related to c-AMP were expressed following GSEA (Data not shown), therefore it is unlikely that the upregulation of PDE4A in this study is a response to elevated levels of c-AMP. It is possible that the upregulation of PDE4A provides greater control over cAMP dependent pathways, but further research beyond the scope of this thesis is warranted to explore this.

4.4.6 Physical activity, dietary intake, hand grip strength and body composition

There were no changes in physical activity, diet, hand grip strength or body composition during the supplementation period, strengthening the argument that the changes observed in transcriptomic regulation are attributed to n-3 PUFA supplementation. When analysing the n-3 PUFA intake from the 3-day food diary, one person was a clear outlier (**Figure 4.6C**). Upon inspecting their food diary, it was observed that three teaspoons of chia seeds were added to their breakfast at six weeks, resulting in an increased n-3 PUFA intake. Chia seeds are high in the n-3 PUFA alpha-linolenic acid, a precursor for EPA and DHA (Baker *et al.*, 2016). Nutritics® analytics software does not separate between the various species of n-3 PUFA. No foods containing high amounts of EPA or DHA (oily fish) were consumed in this person's diet. Furthermore, the individual's incorporation response was a 2.39% increase in EPA + DHA in erythrocytes after six weeks, indicating that the increased n-3 PUFA intake at week six did not lead to a significant additional formation of EPA and DHA.

No changes in physical activity over time were observed, while there only was an effect of age on MVPA (**Figure 4.5B**). This difference aligns with literature suggesting that a decrease in physical activity occurs in ageing (McPhee *et al.*, 2016). Although we did not test skeletal muscle transcriptome expression at baseline, as this was not an aim of this thesis, the heatmap in **Figure 4.2A** suggests that older adults exhibited a lower gene expression of the oxidative phosphorylation pathway. Another indicator of changes in physical activity are changes in hand grip strength, as hand grip strength is a good indicator of overall muscle strength (Bohannon, 2003). Another indicator of changing habitual dietary patterns are changes in body composition, which could indicate changes not captured by the 3-day food diary. However, not only are strength and body composition indicators of changes physical activity and diet, but there might also be an effect of n-3 PUFA on changes in strength and body composition. Studies have observed that n-3 PUFA can enhance muscle strength (Smith *et al.*, 2015; Da Boit *et al.*, 2017; Alkhedhairi *et al.*, 2022) and reduce fat mass (Couet *et al.*, 1997; Noreen *et al.*, 2010). However, recent meta-analyses yielded more equivocal findings. One analysis reported that n-3 PUFA positively impacts lower body strength but not upper body strength or lean mass (Cornish *et al.*, 2022). Another study observed no effects of n-3 PUFA on hand grip strength, and although they suggested there might be a positive influence of n-3 PUFA on lean mass, it was noted that these results should be interpreted with caution (Timraz *et al.*, 2023). It is important to note that both meta-analyses from Cornish *et al.,* and Timraz *et al.,* reported high heterogeneity among the studies conducted which contributed to lack of significant outcomes.

Aligned with these meta-analyses, we did not observe changes in hand grip strength or lean mass and fat mass during the n-3 PUFA supplementation period. When analysing hand grip strength data, we observed a trend for sex × age, but no significant difference between young and older age groups. Given that hand grip strength typically declines with age (Frederiksen *et al.*, 2006; Stenholm *et al.*, 2012), it is notable that this decline was not observed in the present study. This may be attributed to the overall health of our older cohort: despite lower MVPA levels, total physical activity levels were similar across age groups, and our cohort was generally healthy. Overall, the absence of changes over time in measures of physical activity, diet, hand grip strength, and body composition supports the validity of our findings regarding the alterations in the skeletal muscle transcriptome in response to 12 weeks of n-3 PUFA supplementation.

4.4.7 Strengths and limitations

Several limitations warrant consideration. Firstly, the observed alterations in gene expression were confined to a limited subset of molecular pathways. This could be due to the moderate intervention of 1.26g EPA + DHA per day, with no training component, or the usage of the Hallmark gene sets rather than KEGG pathway analysis. Additionally, the study lacked sufficient statistical power to discern potential sex differences in response to n-3 PUFA treatment, thus potentially overlooking differential gene expression patterns between sexes. Previous research has demonstrated sex-specific increases in muscle function and quality in older populations receiving fish oil treatment (Da Boit *et al.*, 2017), while biological sex also influences the skeletal muscle transcriptome in ageing (Huang *et al.*, 2023). Moreover, the translation of observed changes in these molecular pathways into corresponding changes in protein expression or clinically meaningful outcomes remains to be elucidated. However, a strength of this study was the analysis of physical activity, grip strength, diet, and body composition to examine whether the observed changes in skeletal muscle transcriptome were attributed to n-3 PUFA ingestion or influenced by the aforementioned factors.

4.4.8 Conclusion

In summary, this chapter reveals that 12 weeks with moderate doses of n-3 PUFA supplementation differentially alters global transcriptomic expression in the skeletal muscle of young and older adults. Although the observed alterations in transcriptomic expression are

limited, they likely reflect interconnected mechanisms involving bioenergetics, inflammation, and muscle regeneration. However, larger sample sizes are required to evaluate changes in muscle tissue. These findings provide preliminary evidence of the potential for n-3 PUFA to modulate skeletal muscle in a partially transcriptomic manner, supporting existing mechanisms of action but also providing insights into potential new pathways involved.

Chapter 5: General discussion & synthesis of findings

5.1 Summary aims and objectives

The incorporation of n-3 PUFA into their respective tissues can alter phospholipid membrane composition and subsequently cell signalling. N-3 PUFA have been an emerging candidate in skeletal muscle mass (Brook *et al.*, 2021), muscle strength (Phillips *et al.*, 2024), and protein turnover (McGlory, Calder and Nunes, 2019). There is considerable evidence that the incorporation of n-3 PUFA into the cellular phospholipid membranes of skeletal muscle plays a beneficial role in regulating skeletal muscle health. Here, n-3 PUFA may influence health outcomes through several mechanisms, including the modulation of muscle protein turnover and affecting the activity and expression of signalling kinases and genes (Witard *et al.*, 2023). However, research on these outcomes remains inconclusive. This disparity in outcomes is attributed to variations in study populations, as well as differences in dose and duration (Anthony *et al.*, 2024). One key point addressed by Anthony *et al.*, (2024) was the importance to thoroughly assess the dose and duration of the study design. This evaluation is crucial to ensure significant changes in phospholipid membranes, leading to desirable outcomes on health markers. However, the relationship between dose, phospholipid membrane changes and functional outcomes are poorly characterised. While studies using high doses serve as a proof of concept, more investigation is needed to explore whether lower doses can induce similar changes in phospholipid membrane composition and subsequent alterations in transcriptomic regulation and cell signalling. An initial higher loading dose followed by a lower maintenance dose could be a desirable strategy. This strategy may allow for an initial high increase in n-3 PUFA content in the phospholipid membrane, which could then be maintained through diet alone. The current recommendation by the NHS in one portion of oily fish per week (NHS, 2022). One portion of salmon of 140g results in approximately 3.5g EPA + DHA (Scientific Advisory Committee on Nutrition (SACN) and Committee on Toxicity (COT), 2004), which equates to 500mg EPA + DHA per day. Moreover, ageing has an impact on numerous aspects of skeletal muscle physiology, such as a decreased number and ratio type II to type I fibres (Lexell, Taylor and Sjöström, 1988), a blunted muscle protein synthesis response to anabolic stimuli (Dillon, 2013), or changes in the skeletal muscle transcriptome (Cisterna and Malatesta, 2024). Phospholipid membrane changes and subsequent markers of health in young adults may not necessarily translate to the beneficial effects observed in older adults. Furthermore, washout of n-3 PUFA from tissue membranes is important to examine, not only to gain insights on the

frequency of ingestion, but also to determine whether cross-over designs are achievable. To our knowledge, no studies have directly compared the incorporation and washout of n-3 PUFA in a combined EPA + DHA mixture in a C2C12 cell model, or in multiple tissues (erythrocytes, skeletal muscle phospholipid membranes and subcutaneous adipose tissue) in a human model with young and older individuals. Additionally, the impact of n-3 PUFA supplementation on the differential transcriptomic regulation of skeletal muscle in young and older individuals has yet to be elucidated.

The overall aim of this thesis was to gain insights into the **incorporation**, **washout**, and **underlying mechanisms** of n-3 PUFA following different dosing strategies in a cell model and a human study in young and older adults, with a focus on muscle and ageing. After a comprehensive discussion of the literature and setting a general introduction for the thesis, the overall aim was successfully achieved by completing the following objectives:

1. To study the incorporation and washout responses of EPA and DHA over time in response to low and high concentrations of a combined EPA:DHA mixture in cell lysates and phospholipid membranes of C2C12 myotubes (**Chapter 2**).
2. To measure how rates of muscle protein synthesis (MPS) and associated mTORC1 signalling proteins are impacted by incorporation and washout patterns of EPA and DHA in C2C12 myotubes (**Chapter 2**).
3. To study the incorporation and washout responses of EPA + DHA in erythrocytes, skeletal muscle phospholipid membranes, and adipose tissue in humans in response to two different dosing strategies in young and older adults (**Chapter 3**).
4. To determine if a loading dose results in a significantly greater incorporation of EPA + DHA during the loading phase in the aforementioned tissues of young and older adults, and whether this can be sustained by a maintenance dose (**Chapter 3**).
5. To identify global changes in the skeletal muscle transcriptome in response to n-3 PUFA supplementation in young and older adults and to investigate potential differences between young and older adults (**Chapter 4**).

Findings have been thoroughly discussed within each respective experimental chapter, summarised in **Figure 5.1** and are briefly synthesised as follows:

1. In C2C12 myotubes, incorporation of EPA into the phospholipid membrane was higher in HC than LC at all timepoints except 16h, with a plateau observed from 8h. In contrast, DHA incorporation did not differ between concentrations, whereas a

significant incorporation of DPA was observed at 24h in LC. EPA declined after 2h of washout in HC and LC and continued to decline until 16h in HC, whereas DHA levels remained constant over time for LC and increased for HC while HC inhibited DPA formation during the initial 4h of washout. EPA and DHA exhibit distinct concentration-dependent incorporation and washout profiles.

2. MPS was increased following a 24h washout period with LC, but this was independent of changes in 4E-BP1$^{[Thr37/46]}$, p70S6K1$^{[Thr421/Ser424]}$, and Akt$^{[Ser473]}$ phosphorylation.
3. Incorporation of EPA + DHA into erythrocytes and skeletal muscle phospholipid membranes followed a similar pattern between young and older adults where EPA + DHA content increased significantly over time during the 12-week supplementation period, with different patterns observed between the loading and constant dose. In erythrocytes, EPA + DHA content was elevated above baseline following the 8-week washout phase, whereas in skeletal muscle phospholipid membranes, EPA + DHA content returned to baseline after the 8-week washout phase. In adipose tissue, differential incorporation of EPA + DHA was observed between young and older adults. Additionally, no washout from adipose tissue was observed, which is attributed to limited incorporation.
4. A loading dose led to a significantly greater change in EPA + DHA during the 4-week loading phase in erythrocytes and skeletal muscle phospholipid membranes. Here, after the subsequent 8-week maintenance phase, EPA + DHA content did not differ between the loading and constant dose. This resulted in similar incorporation of EPA + DHA after the 12-week supplementation period, subsequently resulting in similar washout patterns between the two dosing strategies. However, in adipose tissue of young and older adults, the incorporation patterns of EPA + DHA did not differ in response to dosing strategies during the 4-week loading or subsequent 8-week maintenance phases. Additionally, no washout from adipose tissue was observed, which is partly attributed to limited incorporation.
5. One individual gene (PDE4A) was differentially expressed in the young age group in response to n-3 PUFA supplementation. In young adults, n-3 PUFA ingestion downregulated two pathways related to bioenergetics (OXPHOS and ROS) and two pathways related to tissue regeneration (MYC targets, DNA repair), while one pathway linked to tissue regeneration (Mitotic spindle) was upregulated. In older adults, two pathways related to tissue regeneration were upregulated (Angiogenesis and EMT), while one gene set related to bioenergetics (OXPHOS) was downregulated.

6. Differential regulation of IFN-α, and IFN-γ gene sets was observed between age groups, which were upregulated in the older age group and downregulated (IFN-γ) or unchanged (IFN-α) in the younger age group.

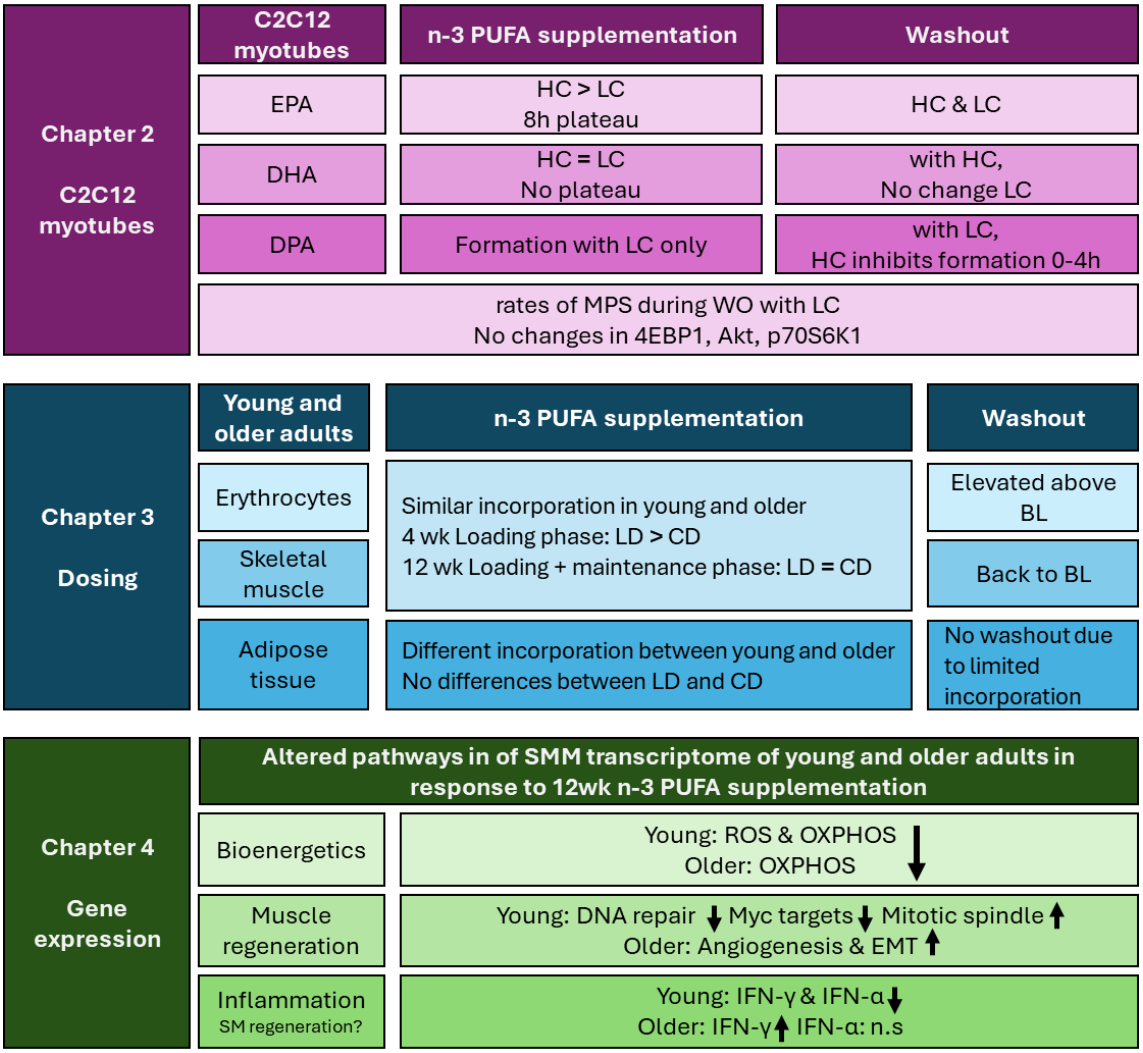

Figure 5.1: Diagram of key findings of thesis summarised per chapter. **Chapter 2**: LC = low concentration, HC = high concentration, EPA = eicosapentaenoic acid, DPA = docosapentaenoic acid, DHA = docosahexaenoic acid, MPS = muscle protein synthesis, 4E-BP1 = eukaryotic translation initiation factor 4E-binding protein 1. Akt = Protein Kinase B. p70S6K1 = ribosomal protein S6 kinase beta-1. **Chapter 3:** LD = loading dose, CD = constant dose, BL = baseline. **Chapter 4:** ROS = reactive oxygen Species, OXPHOS = oxidative phosphorylation, EMT = epithelial to mesenchymal transition, SM = skeletal muscle, IFN = interferon.

5.2 Time-course and washout changes of n-3 PUFA related to dosing

C2C12 myotubes are a common model used to study the impact of n-3 PUFA on markers of proliferation, differentiation and muscle metabolism (Tachtsis, Camera and Lacham-Kaplan, 2018). C2C12 myotubes are a suitable approach to study muscle metabolism as they seem appropriate for studies of exercise/stress responses (Abdelmoez *et al.*, 2020). Indeed, some findings related to the impact n-3 PUFA on skeletal muscle metabolism, in particular the hypothesis that EPA is the main driver behind MPS, are based on C2C12 data (Kamolrat and Gray, 2013; Jeromson *et al.*, 2018). The initial goal of the cell study model in this thesis was to investigate the effect of different dosing strategies on the incorporation and washout of EPA, DHA and DPA over time and its subsequent effect on MPS and signalling mTORC1 associated proteins in response to incubation with an EPA:DHA mixture. However, given the link between n-3 PUFA incorporation into cellular membranes and cell signalling, it was first crucial to investigate the time-course changes of EPA, DHA, and DPA as a percentage of total fatty acids during incubation with n-3 PUFA, particularly as the uptake dynamics of these fatty acids over time had not been previously characterised in C2C12 myotubes. While previous studies have employed incubation times of 72 hr (Jeromson *et al.*, 2018) and 24 hr (Kamolrat and Gray, 2013), here we show that 8 hours of incubation with EPA + DHA led to a plateau in EPA incorporation following high and low concentrations, whereas DHA did not reach a plateau within the 24 hr period (**Figure 5.1**). Moreover, given the interconversion between the fatty acids as explained in **Chapter 1** and demonstrated by multiple studies (Achard, Bénistant and Lagarde, 1995; Gregory *et al.*, 2011; Calder, 2012; Metherel *et al.*, 2019), it is likely that the combination of EPA and DHA in a mixture provided to C2C12 myotubes influences the incorporation and washout dynamics of EPA and DHA. In **Chapter 2,** we found that the incorporation dynamics differed not only per fatty acid but were also influenced by the total concentration provided. In addition to incorporation, diverse washout patterns for EPA and DHA were observed. An interesting observation was the formation of DPA, which was only detected after incubation with the low concentration (**Figure 5.1**). The increase of DPA formation with the low, but not high concentration is likely a result of conversion kinetics. A recent study utilising compound specific isotope analysis found that DHA supplementation in humans resulted in increased plasma EPA and DPA levels, but not δ13C-EPA or δ13C-DPA levels (Metherel *et al.*, 2019), where the accumulation of plasma EPA and DPA was attributed to a slowed down metabolism of EPA to DPA/DHA rather than retro-conversion. The results from the cell study conducted in **Chapter 2** provide additional support for this hypothesis,

where the higher concentration provided in our study may have led to 'competition' between EPA and DHA: the total amount of DHA may have slowed down the metabolism of EPA to DPA and DHA. However, this remains speculative, and since it was not the focus of this thesis, we did not implement methods to test this hypothesis. DHA has been proposed to be a crucial component in human evolution with respect to neuronal and brain development (Crawford and Broadhurst, 2012), and is essential during pregnancy for foetal brain development (Lauritzen *et al.*, 2016). In humans, DHA content exceeds that of EPA in erythrocytes and skeletal muscle (**Appendix 3.1 – 3.3**), and DHA is also higher than EPA in the majority of the marine sources that we eat (National Institutes of Health, 2023). Based on these arguments, one could argue that DHA is the more critical fatty acid for promoting health outcomes. However, compared to DHA, EPA is more effective in improving cardiovascular outcomes (Sweeney, Gaine and Michos, 2023), increasing MPS (Kamolrat and Gray, 2013; Jeromson *et al.*, 2018), and increasing glucose uptake (Jeromson *et al.*, 2018). The dynamics of EPA and DHA described in **Chapter 2** appear to be influenced by their structure (**Figure 1.2**), preferential incorporation into specific phospholipid membranes regions (Pal *et al.*, 2020), and conversion kinetics (Metherel *et al.*, 2024). Moreover, **Chapter 2** additionally demonstrated that incubating C2C12 myotubes with the high concentration led to an increased DHA content in the phospholipid membranes during the washout period. It is an attractive hypothesis that some of the EPA loss observed in the washout study was converted into DHA. However, since we did not use compound specific isotope analysis, or measurement of EPA and DHA in the medium, this speculation cannot be confirmed. Further investigation into the metabolism and interconversion of n-3 PUFA are warranted, where the absolute dose and ratios of EPA:DHA are important considerations.

The cell study was subsequently followed up by a randomised controlled human clinical trial. Here, a ratio of 3:2 EPA:DHA was provided to the participants, consistent with the ratio of the EPA:DHA mixture used in the cell study. An EPA rich supplement was chosen based on evidence in cell models that EPA is the primary driver of MPS (Kamolrat and Gray, 2013; Jeromson *et al.*, 2018). The main aim of **Chapter 3** was to study the incorporation of EPA + DHA in blood, skeletal muscle phospholipid membranes, and adipose tissue in response to two different dosing strategies. A major objective of this study was to investigate whether higher doses lead to an earlier, higher incorporation during the loading phase and whether this incorporation can be sustained by switching to a maintenance dose. We observed tissue- and dosing strategy- specific differences, where doses of 2.52g *vs.* 1.26g n-3 PUFA per day were

sufficient to induce significant differences in EPA + DHA incorporation in erythrocytes and skeletal muscle phospholipid membranes, where age did not affect the changes in fatty acid profile. Conversely, in adipose tissue, incorporation – and thus washout – differed between age groups, where no differences between dosing strategies was observed in both young and older adults. These findings have been thoroughly discussed in **Chapter 3** and are visualised in **Figure 5.1**. Time-course changes of n-3 PUFA content in muscle were first assessed by McGlory *et al.,* (2014), where a ~1.79-fold change in n-3 PUFA content was observed in whole muscle after 4 weeks of high dose feeding with 5g n-3 PUFA (3.5g EPA + 0.9g DHA) per day. In a follow-up study, McGlory *et al.,* (2016) observed a ~2-fold change in skeletal muscle phospholipid fractions, indicating that the plateau of n-3 PUFA incorporation in muscle is somewhere between 4 – 8 weeks when providing high doses of 5g n-3 PUFA per day. Smith *et al.,* reported a ~2 fold change (Smith *et al.*, 2011b) and ~1.79-fold change (Smith *et al.*, 2011a) in skeletal muscle phospholipid membranes over 8 weeks using lower doses of 1.86g EPA + 1.5g DHA per day. The comparable fold changes in skeletal muscle phospholipid membranes reported by Smith *et al.* and McGlory *et al.* following different doses suggest a limit to the amount of n-3 PUFA that can be incorporated into skeletal muscle phospholipid membranes. Moreover, another study investigating whole muscle fatty acid profile changes observed a ~2.52-fold change in n-3 PUFA content after 12 weeks of supplementation with 3g n-3 PUFA (2g EPA + 1g DHA) per day (Gerling *et al.*, 2019). In our study, when grouping both doses together, the total n-3 content changed from 2.3 ± 0.8% to 3.6 ± 0.8% over 12 weeks of n-3 PUFA supplementation, with a ~1.57-fold change. Both the fold change and total n-3 PUFA content in our study were lower compared to those observed in previous studies. When investigating changes during the 4-week loading phase in our study, the loading dose (2.52g n-3 PUFA/day) led to an ~1.74-fold change, whereas the constant dose (1.26g n-3 PUFA/day) resulted in an ~1.31-fold change. While our loading dose led to a similar fold-change as observed by McGlory *et al.,* (2014) over four weeks using lower doses, this discrepancy can be attributed to the difference in muscle fraction measured; whole muscle in the study by McGlory *et al.,* (2014) *vs.* phospholipid membrane in our study. Our data, combined with fold changes reported in other studies, indicate that muscle fraction, along with dose and duration are critical factors in modulating changes in the fatty acid composition in skeletal muscle. An overview of changes in n-3 PUFA fatty acid composition in muscle in humans is provided in **Table 5.1**.

Table 5.1: Muscle incorporation of total n-3 PUFA of different studies

	Study population	Length and daily dose	Fraction	n-3 pre	n-3 post	Fold change
McGlory 2014	Young men	4wk 3.5g EPA + 0.9g DHA	WM	3.8 ± 0.22	6.79 ± 0.46	1.79
McGlory 2016	Young men	8wk 3.5g EPA + 0.9g DHA	PL	5.53 ± 0.30	11.16 ± 0.45	2.02
Smith 2011a	Young and middle-aged adults	8wk 1.86g EPA + 1.5g DHA	PL	4.38 ± 0.33	8.93 ± 0.37	2.04
Smith 2011b	Older adults	8wk 1.86g EPA + 1.5g DHA	PL	5.04 ± 0.45	9.03 ± 0.95	1.79
Gerling 2019	Young men	12wk 2g EPA + 1g DHA	WM	3.3 ± 0.2	8.3 ± 0.6	2.52
Banic 2024*	Young and older adults	4wk 0.72g EPA + 0.54g DHA (CD)	PL	3.8 ± 0.7	4.6 ± 0.8	1.20
Banic 2024*	Young and older adults	4wk 1.44g EPA + 1.08g DHA (LD)	PL	3.9 ± 1.0	5.8 ± 1.5	1.49
Banic 2024*	Young and older adults	12wk 0.72g EPA + 0.54g DHA on average	PL	3.8 ± 0.8	5.2 ± 0.9	1.37

EPA = eicosapentaenoic acid, DHA = docosahexaenoic acid, WM = whole muscle, PL = phospholipid membrane of skeletal muscle, n-3 = total omega-3 polyunsaturated fatty acids.

The link between phospholipid membrane composition and beneficial effects of EPA and DHA remains to be elucidated. For example, how much EPA and DHA incorporation is required to elucidate beneficial effects on muscle protein turnover or other beneficial effects ascribed to them? What is the maximal incorporation of EPA and DHA in the membrane? And subsequently, are there potential negative consequences if the phospholipid membrane is altered too much? Harris (2008) defined the omega-3 index (EPA + DHA) risk zones in blood for cardiovascular diseases as follows (in percentages of total erythrocyte fatty acids): high risk, <4%; intermediate risk, 4-8%; and low risk, >8%. In the studies by Smith *et al.*, changes in skeletal muscle phospholipid composition led to an n-3 PUFA content of 8.93 ± 0.67% in younger (Smith *et al.*, 2011b) and 9.03 ± 0.95% in older (Smith *et al.*, 2011a) adults, which was associated with increased rates of MPS following a hyperaminoacidemic-hyperinsulinemic clamp. In our human study in **Chapter 3**, the percentage n-3 PUFA of total fatty acids after 12 weeks of supplementation was 5.2 ± 0.95%, which resulted in coordinated changes in the skeletal muscle transcriptome described in **Chapter 4**. The findings from **Chapter 4** suggest that smaller changes in phospholipid membrane n-3 PUFA composition are sufficient to influence changes in the skeletal muscle transcriptome. Relating this back to the literature, the comparable fold changes observed by McGlory *et al*. and Smith *et al.* over the same period using different doses indicates that not all n-3 PUFA provided by McGlory *et al.,* (2016) were incorporated into the phospholipid membrane. This suggests that the remaining n-3 PUFA not incorporated can either be stored elsewhere, such as in adipose tissue, or may

undergo β-oxidation. As described in **Chapter 1**, SPM are anti-inflammatory and work on the COX and LOX pathways where they compete with the pro-inflammatory eicosanoids produced by ARA (Blaauw *et al.*, 2024). The impact of dosing on the proportion of n-3 PUFA that are incorporated into membranes or undergo β-oxidation warrants further investigation.

Regarding potential negative consequences of high EPA + DHA levels in the human body, an early study demonstrated that 4g and 9g of n-3 PUFA supplementation daily for six weeks was associated with an increased time of bleeding (Schmidt *et al,.* 1990). However, analysis of multiple clinical studies in over 600 subjects did not find evidence to support concerns about adverse bleeding with doses ranging from 1.5g n-3 PUFA per day (orally administered as supplement) up to 10g n-3 PUFA daily (via tube feeding) (Jeansen *et al.*, 2018). Given the anti-inflammatory effects of n-3 PUFA via the production of eicosanoids or binding to transcription factors in the PPARγ/NF-κB pathways, involved in the inflammation pathway (Calder, 2017), it could furthermore be argued that high doses of n-3 PUFA supplementation suppress the required inflammation response essential in acute infections (Fenton *et al.*, 2013). For example, fish oil feeding administered as 17g per 100g diet in mice delayed the clearance of the influenza virus in mice (Byleveld *et al.*, 1999), and increased viral load in the lungs post-infection and the mortality rate in another study in mice administering 4g/100g diet (Schwerbrock *et al.*, 2009). However, it must be noted that doses used in the above animal studies translate to 4% and 17% of total diet weight as fish oils, which is significantly higher than the recommended daily dose of 250mg per day (European Food Safety Authority (EFSA), 2010), or doses typically used in human studies. This may explain that there is no evidence of delayed viral clearance after n-3 PUFA supplementation in humans. In fact, n-3 PUFA were regarded beneficial for improving clinical outcomes in critically ill patients during the COVID-19 pandemic (Lampova *et al.*, 2022). The human body appears to be well-adapted to physiological doses of n-3 PUFA, though further research is needed to understand the long-term effects.

The washout of n-3 PUFA from skeletal muscle membranes after cessation of supplementation had not been investigated before but has implications for designing randomised controlled cross-over studies. The data from **Chapter 3** demonstrate that content of EPA + DHA as a percentage of total fatty acids returned to baseline in skeletal muscle phospholipid membranes after the 8-week washout period. However, in erythrocytes levels of EPA and DHA were still elevated after the 8-week washout period (**Figure 5.1**). These discrepancies between tissues are discussed in **Chapter 3** but could result in different baseline fatty acid composition in cross-over designs, and therefore longer washout periods may be required. There is cross-talk

between tissues, but whether outcomes on muscle health could be impacted by erythrocyte or adipose tissue n-3 PUFA content is unclear. Furthermore, the higher total EPA + DHA content in erythrocytes compared to muscle after 12 weeks of supplementation (**Appendix 3.1 – 3.3**), along with the low fold change observed in SMM phospholipid membranes in our study relative to others due to lower dose provided (**Table 1**), highlights that the total daily dose administered significantly influences the duration of the washout period required for fatty acid profiles to return to baseline. Moreover, adipose tissue appears to need even longer washout periods, suggesting that accounting for fatty acid profile changes in adipose tissue may not be feasible when evaluating muscle outcomes in cross-over designs. The hypothesis that the alteration of membrane fluidity is the primary mechanism for subsequent cell signalling is compelling. However, the relationship between total unsaturation and outcomes of muscle health, as well as the dose-dependent effect on these outcomes, needs to be elucidated further.

5.3 Role of n-3 PUFA in molecular signalling pathways on positive ageing and muscle outcomes.

Ultimately, the objective is to preserve muscle strength and muscle mass during ageing, as these are important indicators of quality of life. In this thesis, we employed an *in vitro* cell line using C2C12 myotubes to study rates of MPS after incubation with 3:2 EPA:DHA mixture in response to high and low concentrations (**Chapter 2**), followed by a randomised controlled human trial where the impact of 12-week of n-3 PUFA supplementation on changes in skeletal muscle transcriptome in young and older adults was assessed (**Chapter 4**). In **Chapter 2**, increased rates of MPS were observed after incubation with the low, but not high concentration of n-3 PUFA (**Figure 5.1**). Three potential explanations were given: the competition between the EPA and DHA, the stress on cells after incubation with HC as observed by visual inspection, or the contribution of DPA, only detected after incubation with LC. The changes in fatty acid profile of the phospholipid membrane in C2C12 myotubes were much higher than observed in human studies (**Table 1, Chapter 2.3, 3.3**). Contrastingly, as explained above, changes in the skeletal phospholipid membranes in our human study were relatively moderate (1.37-fold change). Whether greater alterations in phospholipid membrane in humans would lead to more pronounced changes level in the skeletal muscle transcriptome or subsequent functional outcomes in a dose-dependent manner is not known. However, in humans it appears that relatively small changes in phospholipid membrane composition in skeletal muscle is sufficient to induce changes in skeletal muscle transcriptome (Yoshino *et al.*, 2016) or MPS (**Table 5.1**).

From the muscle tissue collected in the human intervention study outlined in **Chapter 3**, we examined changes in skeletal muscle transcriptome rather than assessing changes in protein expression, kinase activity or rates of MPS and MPB. These findings are discussed in **Chapter 4** and summarised in **Figure 5.1**. Our approach highlighted long-term alterations over acute protein signalling effects. A rationale for focusing on investigating changes in transcriptomic expression was that the n-3 PUFA supplementation intervention did not involve an acute feeding or exercise training stimulus. Since no alterations in rates of MPS or associated kinase activity were observed in a fasted state in the early human intervention trials (Smith *et al.*, 2011b, 2011a), it was unlikely that we would capture any changes in altered rates of basal MPS or kinase signalling activity. This approach led to formulation of hypothesis-generating results rather than hypothesis testing.

Twelve weeks with 1.26g n-3 PUFA per day on average resulted in transcriptomic changes in muscle related to bioenergetics, muscle tissue regeneration, and inflammation differentially in young and older adults. The pathways related to skeletal muscle regeneration, such as angiogenesis and EMT, were altered in the older age group in response to n-3 PUFA supplementation, which may elucidate the role of n-3 PUFA in tissue regeneration, a process that diminishes with ageing (Carosio *et al.*, 2011). In this context, the extracellular matrix (ECM) appears to play a role in most of, if not all the pathways altered in **Chapter 4**, highlighting the importance of the ECM in regulation of cellular metabolism. Given that compositional changes in skeletal muscle phospholipid membranes lead to alterations in cellular signalling, it is highly plausible that the metabolites regulated by the ECM are also affected. One study showed that 24 weeks of supplementation with n-3 PUFA altered changes in proteins associated with ECM in patients with psoriatic arthritis but not healthy volunteers (Holm Nielsen *et al.*, 2021), suggesting n-3 PUFA supplementation may improve tissue turnover in patients with inflammatory diseases. The pathways altered by n-3 PUFA in this thesis may provide further insights into the role of the ECM in cellular signalling and its potential as a target for n-3 PUFA's mechanism of action. The microarray technique used in **Chapter 4** enables investigation into the whole skeletal muscle transcriptome, allowing for the identification of potential pathways beyond those already known (Yoshino *et al.*, 2016; McGlory, Calder and Nunes, 2019). Discrepancies in our outcomes and those in other studies studying the impact of n-3 PUFA on skeletal muscle transcriptome (Yoshino *et al.*, 2016; Lalia *et al.*, 2017) could be attributed to multiple factors, which are thoroughly discussed in **Chapter 4.**

Furthermore, we used microarray analysis as opposed to RNA sequencing to capture the transcriptomic profile. Microarray analysis used a set of pre-defined probes whereas RNA sequencing is a newer technique that is often employed to discover novel transcripts. However, RNA sequence analyses are often based on the known transcriptome and may miss lower expressed genes (Stokes *et al.*, 2023). Beyond alterations in molecular pathways described by the Hallmark Gene set (Liberzon *et al.*, 2015), we observed minimal changes in the expression of individual genes after correcting for FDR, except PDE4A, which was upregulated in the young age group in response to 12-week n-3 PUFA supplementation (**Chapter 4**). A similar absence of individual gene alterations was observed in the study by Yoshino *et al.,* (2016), suggesting that the impact of n-3 PUFA on changes in muscle turnover might be moderate. Nevertheless, since multiple studies have shown effects of n-3 PUFA on measures of mass, strength, and protein turnover, there is a clear connection between the molecular, cellular and physiological level. Large, multi-faceted studies are needed to further elucidate this connection.

Age-related differences in skeletal muscle are related to transcriptomic, proteomic, and structural differences. Specifically, the differential regulation of the interferon pathways between the age groups in our study was remarkable, as interferons are typically associated with inflammation (Pestka, Krause and Walter, 2004). In **Chapter 4** the hypothesis was proposed that levels of IFN-γ are decreased in ageing muscle and that IFN-γ plays a role in accelerating skeletal muscle regeneration. This could potentially explain why the IFN-γ pathway was upregulated in our older age group in response to n-3 PUFA supplementation. To the best of our knowledge, no other studies have observed this differential regulation of the IFN-γ pathway in the skeletal muscle transcriptome in ageing. Additionally, it remains unclear whether the changes seen in the transcriptomic pathway in **Chapter 4** translate into differential protein expression of IFN-γ. These uncertainties highlight the need for further investigation into the role of IFN-γ in skeletal muscle regeneration.

Although one might consider other factors responsible for the upregulation of genes associated to the IFN-γ pathway, there were no changes in physical activity or diet, as assessed by activity monitoring, grip strength measurements, 3-day food diary, or body composition analysis (**Chapter 4, section 3.4 – 3.7**). Literature suggests that n-3 PUFA interventions might be particularly beneficial for older individuals (Witard *et al.*, 2023). However, there are numerous studies showing beneficial effects of n-3 PUFA in young adults (Smith *et al.,* 2011a; McGlory *et al.,* 2014; McGlory *et al., 2*019), summarised in **Table 1.2** in **Chapter 1.** Our study reveals differential effects of n-3 PUFA supplementation on the skeletal muscle transcriptome between

young and older adults, indicating different roles for n-3 PUFA in affecting health outcomes in young and older adults. Finally, there may be a different role for EPA and DHA in regulation of skeletal muscle turnover. The effects of MPS are mainly attributed to EPA and based on previous C2C12 studies (Kamolrat and Gray, 2013; Jeromson *et al.*, 2018). However, Wang *et al.,* (2013) reported a greater attenuation of protein degradation with DHA compared to EPA, which was mediated via the upregulation of the PPARγ/ NF-κB pathway. In a human study, the provision of EPA or DHA individually elicited a beneficial effect on markers of muscle damage, but not when provided in combination (Heileson *et al.*, 2023). Although the Heileson *et al.,* (2023) did not investigate the underlying mechanisms, they recommended that future studies focus on measuring more direct metabolites. The impact of competition between EPA and DHA regarding their effects on muscle protein turnover need to be investigated. Additionally, further research is needed to understand how variations in n-3 PUFA dose and ratio of EPA:DHA influence their incorporation into tissues, eicosanoid production, transcriptomic regulation, and markers of kinase signalling activity.

5.4 Limitations and practical applications of the thesis

The data generated from this thesis contributes to the literature concerning n-3 PUFA incorporation in response to dosing, washout, and underlying molecular mechanisms. However, it is important to consider the limitations. Firstly, while the findings in **Chapter 2** are essential and contribute to the literature, they do not directly translate to humans. While the fatty acid profile of EPA and DHA were elevated up to 30% with the high concentration, a similar amount does not seem to reach the phospholipid membranes in humans (**Table 5.1**). Next, while **Chapter 2** discusses that the dynamics of EPA, DHA and DPA incorporation and washout are influenced by their conversion kinetics, we did not employ methods to directly assess this. Compound specific isotope analysis (CSIA) offers a valuable tool for gaining more insight into the conversion kinetics of n-3 PUFA (Lacombe and Bazinet, 2021). A recent study demonstrated that DHA feeding resulted in decreased DHA synthesis in the liver via inhibition of EPA elongation (Metherel *et al.*, 2024). The liver plays an important role in the elongation and conversion of n-3 PUFA. Therefore, a cell model may not accurately reflect washout of n-3 PUFA in human models. Exploring the conversion kinetics in humans during washout following n-3 PUFA supplementation using CSIA would provide valuable insights. Furthermore, we measured rates of MPS but had no measures on MPB. MPB was affected by incorporation of EPA in previous C2C12 studies (Kamolrat and Gray, 2013; Jeromson *et al.*,

2018). Other studies have shown the impact of n-3 PUFA on measures related to breakdown, such as impaired increase in MuRF1 during an immobilisation period (McGlory *et al.*, 2019), or via the changes in the expression of ubiquitin proteasome system (Yoshino *et al.*, 2016). This indicates that there is a role for n-3 PUFA both in regulating MPS and MPB. Furthermore, in **Chapter 3**, the fatty acid profile of phospholipid membranes was only examined in skeletal muscle, not in erythrocytes or adipose tissue. We observed differences in time-course responses of EPA + DHA in whole cell lysates versus phospholipid membrane in C2C12 myotubes in **Chapter 2**. Moreover, fatty acid profiling in whole muscle tissue in humans followed a different pattern of incorporation compared to the phospholipid membrane in **Chapter 3** (**Appendix 3.7**). This suggests that it is important to consider the fraction measured when assessing changes in lipid profile. Erythrocytes are a good measure of long-term dietary n-3 PUFA intake (Harris, 2008). Due to differences in n-3 content across blood fractions (Metherel *et al.*, 2009), the phospholipid membrane profile of erythrocytes may exhibit a distinct incorporation pattern for EPA and DHA compared to the whole erythrocyte. However, there is a strong correlation between n-3 PUFA levels in whole blood and in phospholipid membrane of erythrocyte levels of EPA and DHA (Bell *et al.*, 2011), arguing that whole blood levels could be used as a marker for erythrocyte level, if differences in content are accounted for. In adipose tissue, there was no successful extraction of phospholipid membranes as described in **Chapter 3, section 2.7.1**. The reason for this remains unknown but may be related to technical issues. For lipid profiling, a sample of 40mg adipose tissue was analysed, which may have resulted in an undetectable amount of phospholipid membrane fraction due to the nature of the tissue. Analyses in the future should use larger quantities of adipose tissue and determine the minimum amount of adipose tissue required to successfully extract phospholipid membranes. While an early study in rats showed that the membrane phospholipids of the adipocyte membrane plasma was altered by a high n-3 PUFA diet (Field *et al.*, 1990), it would be valuable to examine how the fatty acid composition of adipocyte phospholipid membranes is altered by n-3 PUFA supplementation in humans. Finally, the human trial employed in this thesis had four arms of study; there were two different dosing strategies (CD and LD) and two different age groups (young and older adults). One the on hand, this can be considered a strength as we not only compared the differences in incorporation between dosing strategies but were also able to investigate how ageing may affect the process of incorporation and subsequent skeletal muscle transcriptomic regulation. However, on the other hand, due to time and resources, this has also resulted in smaller sample sizes for each group. Moreover, muscle biopsies were not successfully taken for all participants at each timepoint, further diminishing the group sizes

(Figure 3.1). Next, there were large variations in incorporation responses into the tissues. To address this, correlations between the ΔEPA + DHA in skeletal muscle phospholipid membranes after 12 weeks of n-3 PUFA supplementation and total body mass, total lean mass, appendicular skeletal muscle mass, total fat mass, body mass index (BMI) and body fat percentage (BFP) were assessed. Upon analysis, there was a correlation between change in ΔEPA + DHA and total fat mass, BMI, and bodyfat percentage (**Appendix 5.1**), where a higher fat mass, BMI and BFP were negatively associated with increases in EPA + DHA incorporation over the 12-week supplementation period. Although studies have not investigated weight adjusted doses, this could be an important consideration when analysing incorporation responses in muscle. In the study outlined in **Chapter 3**, there were no differences in average body mass between the dosing groups or age groups at baseline (**Table 3.1**).

Moreover, the human trial data comprised males (n=11), females (n=16) and 1 male transgender person (left out from the sex-specific analyses in **Chapter 4**). It is widely accepted that the physiology and thereby underpinning molecular mechanisms differ between men and women. However, the studies in this thesis were not powered sufficiently to distinguish between sexes with regards to fatty acid profile changes in n-3 PUFA or the skeletal muscle transcriptome. As a result, we may have missed sex-specific incorporation or regulation. For example, higher DHA baseline levels in erythrocytes were observed in women compared to men (Metherel *et al.*, 2009). Moreover, a more pronounced increase in DHA incorporation has been observed in women following n-3 PUFA supplementation (Metherel *et al.*, 2009; Patterson *et al.*, 2015), which may be linked to a higher conversion of ALA to EPA and DHA in the liver of women. A recent study demonstrated that women had higher levels of TPA and THA precursors –precursors for DHA synthesis (**Figure 1.3**)– following EPA supplementation than men (Rotarescu *et al.*, 2022). Conversely, Da Boit *et al.*, (2017) reported no sex differences in the incorporation of EPA and DHA into blood and skeletal muscle following 12 weeks of n-3 PUFA supplementation, despite observing improvements in maximal isometric torque and muscle quality in older women only (Da Boit *et al.*, 2017). However, given that previous studies did not adjust the dose of ingested n-3 PUFA for individual body mass, the greater incorporation of n-3 PUFA in women may merely be a function of the smaller stature and subsequent higher relative dose of n-3 PUFA in women compared to men. **Appendix 5.1** shows that the incorporation response n-3 PUFA in the study outlined in **Chapter 3** was negatively associated with total fat mass, BMI and body fat percentage. Although not an aim in this study, there was no difference in n-3 PUFA response between men and women during the 12-week

supplementation period (**Appendix 5.2**). The author is aware of sex-specific studies currently being conducted by McGlory at Queens University in Ontario, which may provide additional insights on sex-specific incorporation of n-3 PUFA in skeletal muscle.

An important practical consideration relates to the sustainability of sources rich in EPA and DHA. As briefly explained in **Chapter 1**, the primary source of EPA and DHA is marine life, particularly oily fish (National Institutes of Health, 2023). Given that the world population currently exceeds 8 billion people and will continue to rise (Ritchie *et al.*, 2023), the current recommendations of 500mg EPA + DHA per day would result in a global consumption of 4000 tonnes of EPA + DHA per day. However, the supply of EPA and DHA from natural sources is not sufficient to address this demand (Naylor *et al.*, 2009). Aquaculture plays a crucial role in the production of EPA and DHA. However, farmed fish themselves require dietary sources rich in EPA and DHA. This makes aquaculture not only the largest provider of EPA and DHA, but also the largest consumer (Tocher *et al.*, 2019). Tocher *et al.,* (2019) have highlighted that as aquaculture production increased, the n-3 PUFA content in fish feed was partially replaced by n-6 PUFA, leading to a decreased n-3 PUFA content and thus quality of farmed fish. It is evident that aquaculture alone does not offer a sustainable solution to the global food challenge. Krill oil has been proposed as an alternative source of n-3 PUFA due to its more efficient incorporation compared to fish oil, attributed to its phospholipid content (Tou, Jaczynski and Chen, 2008; Ramprasath *et al.*, 2013). Consequently, humans would require lower amounts of krill oil to induce similar metabolic effects as fish oil (Ulven *et al.*, 2011). However, harvesting krill oil on a large scale seems unsustainable and may pose environmental and ecological concerns, particularly regarding its impact the marine ecosystem (Hill *et al.*, 2006). Therefore, there is a need to investigate alternative, more sustainable sources in aquaculture. Tocher *et al.,* (2019) summarised alternative sources of EPA and DHA, highlighting microalgae, genetically modified (GM) camelina, GM canola, and GM yeast as potential options. Microalgae seem to have the highest content of EPA and DHA and offer the advantage of not being genetically modified (Napier and Betancor, 2023). However, developing methods for mass production of microalgae remains challenging (Ahmad, W. Hassan and Banat, 2022). Mass production of microalgae for fish feed or human consumption faces technical and economic challenges, particularly because some microalgae strains have thick cell walls that makes them difficult to digest and impairs nutrient absorption and increases production costs, limiting its practicality (Ahmad, W. Hassan and Banat, 2022). Another promising alternative solution are genetically modified (GM) crops, which have been extensively researched and summarised (Napier and

Betancor, 2023). Furthermore, regulatory affairs surrounding GM crops or consumer acceptance of GM crops may hinder its production as a wide-scale source. The alternative sources discussed in this paragraph have primarily been investigated for their potential use in fish feed in aquaculture, increasing the quality of n-3 PUFA marine sources. Further research is warranted to understand how alternative sources of n-3 PUFA are absorbed and utilised in human tissues. A combination of multiple approaches is required to develop sustainable solutions for bridging the gap between supply and demand of EPA and DHA (Tocher *et al.*, 2019).

5.5 Future directions of research

Taken together, the findings in this thesis contribute novel insights into the incorporation of n-3 PUFA in erythrocytes, skeletal muscle phospholipid membranes and adipose tissue in young and older adults, and how this subsequently impacts transcriptomic changes in skeletal muscle. The human body comprises many tissues and different cell types with cellular membranes, that all may be altered by n-3 PUFA ingestion. The incorporation of EPA and DHA into all these tissues may potentially influence skeletal muscle turnover, given the extensive cross-talk between tissues. The integrative cellular networks within the human system are incredibly complex, and extensive research is needed to fully understand the role of n-3 PUFA on markers of skeletal muscle health. Future research should focus on large-scale clinical trials, employing techniques to study bioavailability across various tissues, while simultaneously conducting molecular pathway analyses within these tissues, for example via omics techniques. Additionally, more research is required focussing on pathways involved in muscle protein breakdown, providing a more comprehensive understanding of metabolic effects in muscle. Using multi-integrative approaches to study these aspects will provide a comprehensive understanding of the metabolic fate of n-3 PUFA in humans. While the data presented in this thesis contribute to the existing literature, they also raise additional questions for future investigation:

1. Could higher loading doses, such as 5g n-3 PUFA per day, result in an even stronger incorporation of EPA + DHA, and can this be effectively maintained with a subsequent maintenance dose of 500mg per day?

2. How would rates of washout of n-3 PUFA from tissues be impacted by higher doses?

3. What is the optimal EPA:DHA ratio in altering the phospholipid membrane composition, considering the competition between the two fatty acids?

4. How do different doses and ratios of EPA and DHA impact subsequent cellular signalling?

5. How are changes in the skeletal muscle transcriptome associated with functional outcomes in muscle mass, strength and turnover?

6. How can sustainable sources of EPA and DHA be produced to meet the demands on a population level of 250 – 500mg as the recommended target?

7. Are there differences in incorporation of EPA and DHA from different sources, e.g. fish oil, krill oil, microalgae, GM camelina and GM canola?

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Appendix 3.1: Table of changes in fatty acid profile in erythrocytes in the constant dose group

Fatty Acid	Week 0	Week 4	Week 6	Week 8	Week 12	Week 14	Week 16	Week 20
C14.0	0.27 ± 0.08	0.29 ± 0.09	0.30 ± 0.07	0.28 ± 0.08	0.25 ± 0.06	0.28 ± 0.11	0.28 ± 0.09	0.28 ± 0.10
C16.0	21.78 ± 1.37	21.61 ± 1.48	22.06 ± 1.10	21.96 ± 1.40	22.02 ± 1.49	21.88 ± 1.25	22.00 ± 1.31	21.89 ± 1.59
C18.0	16.88 ± 1.06	16.75 ± 1.15	16.68 ± 1.10	16.87 ± 1.01	16.54 ± 0.97	16.68 ± 0.92	16.64 ± 1.04	16.74 ± 1.03
C20.0	0.16 ± 0.02	0.18 ± 0.04	0.15 ± 0.03	0.15 ± 0.03	0.16 ± 0.04	0.15 ± 0.03	0.15 ± 0.04	0.16 ± 0.06
C22.0	0.37 ± 0.11	0.37 ± 0.12	0.31 ± 0.11	0.32 ± 0.12	0.32 ± 0.12	0.33 ± 0.11	0.36 ± 0.13	0.33 ± 0.13
C24.0	0.87 ± 0.33	0.96 ± 0.32	0.73 ± 0.30	0.75 ± 0.31	0.74 ± 0.31	0.79 ± 0.29	0.84 ± 0.37	0.68 ± 0.29
Saturated	**40.31 ± 0.99**	**40.16 ± 1.09**	**40.24 ± 0.90**	**40.34 ± 0.99**	**40.04 ± 0.73**	**40.11 ± 0.83**	**40.26 ± 1.14**	**40.08 ± 1.29**
C16.1n7	0.35 ± 0.16	0.35 ± 0.18	0.37 ± 0.17	0.36 ± 0.21	0.36 ± 0.21	0.36 ± 0.15	0.35 ± 0.20	0.34 ± 0.22
C18.1n9	15.43 ± 0.89	15.43 ± 1.02	15.34 ± 0.96	15.32 ± 1.10	15.39 ± 0.84	15.41 ± 0.99	15.46 ± 1.07	15.94 ± 0.86
C20.1n9	0.27 ± 0.06	0.28 ± 0.06	0.27 ± 0.05	0.26 ± 0.06	0.26 ± 0.06	0.26 ± 0.06	0.25 ± 0.05	0.28 ± 0.08
C24.1n9	0.95 ± 0.45	1.03 ± 0.41	0.85 ± 0.41	0.84 ± 0.42	0.83 ± 0.31	0.85 ± 0.26	0.93 ± 0.42	0.76 ± 0.31
Monounsaturated	**17.00 ± 0.85**	**17.09 ± 1.08**	**16.81 ± 1.00**	**16.78 ± 1.18**	**16.84 ± 0.91**	**16.88 ± 0.96**	**16.99 ± 1.02**	**17.32 ± 0.97**
C18.2n6	11.70 ± 1.78	11.32 ± 1.72	11.56 ± 2.10	11.38 ± 1.90	11.49 ± 1.58	11.71 ± 1.48	11.74 ± 1.66	11.75 ± 1.84
C18.3n6	0.10 ± 0.03	0.11 ± 0.03	0.10 ± 0.02	0.11 ± 0.03	0.11 ± 0.03	0.12 ± 0.03	0.13 ± 0.04	0.12 ± 0.03
C20.3n6	1.78 ± 0.27	1.66 ± 0.23	1.62 ± 0.20	1.60 ± 0.21	1.63 ± 0.19	1.71 ± 0.24	1.76 ± 0.25	1.84 ± 0.25
C20.2n6	0.26 ± 0.08	0.25 ± 0.07	0.25 ± 0.07	0.24 ± 0.07	0.25 ± 0.06	0.25 ± 0.06	0.24 ± 0.06	0.25 ± 0.07
C20.4n6	16.04 ± 1.42	15.51 ± 1.46	14.84 ± 1.18	14.93 ± 1.44	14.50 ± 1.33	14.69 ± 1.41	14.77 ± 1.46	15.20 ± 1.33
C22.4n6	3.04 ± 0.81	2.79 ± 0.68	2.58 ± 0.63	2.60 ± 0.59	2.33 ± 0.50	2.34 ± 0.52	2.38 ± 0.52	2.50 ± 0.66
C22.5n6	0.61 ± 0.14	0.57 ± 0.14	0.50 ± 0.10	0.49 ± 0.12	0.45 ± 0.12	0.45 ± 0.11	0.46 ± 0.14	0.46 ± 0.11
n-6 PUFA	**33.54 ± 2.33^{a}**	**32.22 ± 2.40^{b}**	**31.45 ± 1.83^{bc}**	**31.35 ± 2.18^{cd}**	**30.77 ± 2.05d**	**31.30 ± 1.67^{cd}**	**31.47 ± 1.97^{bd}**	**32.11 ± 2.02^{bc}**
C18.3n3	0.16 ± 0.05	0.16 ± 0.04	0.17 ± 0.05	0.17 ± 0.05	0.17 ± 0.06	0.19 ± 0.07	0.19 ± 0.07	0.17 ± 0.05
C20.5n3	0.77 ± 0.29	1.53 ± 0.50	1.84 ± 0.54	1.70 ± 0.45	2.00 ± 0.51	1.43 ± 0.38	1.29 ± 0.39	1.07 ± 0.31
C22.5n3	2.81 ± 0.43	2.91 ± 0.50	3.08 ± 0.54	3.11 ± 0.41	3.25 ± 0.48	3.26 ± 0.50	3.24 ± 0.40	3.09 ± 0.49
C22.6n3	4.66 ± 1.35	5.14 ± 0.99	5.61 ± 0.84	5.75 ± 0.87	6.15 ± 0.98	5.98 ± 0.88	5.69 ± 0.86	5.30 ± 0.67
n-3 PUFA	**8.40 ± 1.47^{a}**	**9.75 ± 1.36^{bc}**	**10.70 ± 1.21^{d}**	**10.73 ± 1.32^{d}**	**11.56 ± 1.66^{e}**	**10.86 ± 1.38^{de}**	**10.42 ± 1.32^{bd}**	**9.63 ± 1.02^{c}**
ARA/EPA	**24.86 ± 12.30^{a}**	**11.54 ± 5.46^{bcd}**	**8.63 ± 2.41^{bc}**	**9.54 ± 3.46^{bc}**	**8.05 ± 3.75^{b}**	**11.15 ± 4.13^{bc}**	**12.75 ± 5.28^{cd}**	**15.54 ± 5.37^{d}**

Total saturated fatty acids, total monounsaturated fatty acids, total n-6 PUFA, total n-3 PUFA. Values are expressed as % of total fatty acids mean ± SD. Means that do not share a letter are significantly different from one another ($p < 0.05$).

Appendix 3.2: Table of changes in fatty acid profile in erythrocytes in the loading dose group

Fatty Acid	Week 0	Week 4	Week 6	Week 8	Week 12	Week 14	Week 16	Week 20
C14.0	0.28 ± 0.06	0.29 ± 0.10	0.30 ± 0.08	0.27 ± 0.07	0.26 ± 0.08	0.30 ± 0.10	0.27 ± 0.07	0.27 ± 0.07
C16.0	21.47 ± 0.63	21.46 ± 0.76	21.43 ± 0.50	21.41 ± 0.69	21.37 ± 0.57	21.61 ± 0.88	21.60 ± 0.61	21.61 ± 0.78
C18.0	16.87 ± 0.63	17.28 ± 0.58	16.97 ± 0.76	16.95 ± 0.55	16.93 ± 0.53	16.49 ± 0.94	16.90 ± 0.71	16.97 ± 0.77
C20.0	0.15 ± 0.03	0.16 ± 0.03	0.16 ± 0.03	0.15 ± 0.03	0.16 ± 0.02	0.15 ± 0.04	0.14 ± 0.02	0.14 ± 0.02
C22.0	0.37 ± 0.09	0.33 ± 0.11	0.35 ± 0.12	0.34 ± 0.13	0.34 ± 0.07	0.32 ± 0.07	0.30 ± 0.06	0.29 ± 0.06
C24.0	0.74 ± 0.25	0.73 ± 0.29	0.79 ± 0.29	0.76 ± 0.32	0.77 ± 0.16	0.69 ± 0.11	0.66 ± 0.18	0.60 ± 0.21
Saturated	**39.88 ± 0.67**	**40.26 ± 0.70**	**39.99 ± 0.66**	**39.88 ± 0.56**	**39.83 ± 0.7**	**39.57 ± 0.93**	**39.87 ± 0.99**	**39.88 ± 0.96**
C16.1n7	0.24 ± 0.08	0.24 ± 0.13	0.25 ± 0.07	0.23 ± 0.08	0.25 ± 0.10	0.30 ± 0.15	0.26 ± 0.11	0.28 ± 0.13
C18.1n9	15.73 ± 1.37	15.25 ± 1.26	15.59 ± 1.26	15.45 ± 1.18	15.58 ± 1.24	16.00 ± 1.13	15.91 ± 1.27	15.72 ± 1.23
C20.1n9	0.25 ± 0.05	0.26 ± 0.03	0.26 ± 0.04	0.25 ± 0.04	0.26 ± 0.04	0.25 ± 0.04	0.26 ± 0.04	0.26 ± 0.04
C24.1n9	0.86 ± 0.29	0.81 ± 0.37	0.84 ± 0.36	0.81 ± 0.36	0.80 ± 0.19	0.75 ± 0.16	0.70 ± 0.23	0.66 ± 0.21
Monounsaturated	**17.08 ± 1.41^{ab}**	**16.55 ± 1.35^{a}**	**16.94 ± 1.26^{ab}**	**16.74 ± 1.19^{ac}**	**16.89 ± 1.26^{ab}**	**17.31 ± 1.22^{b}**	**17.13 ± 1.24^{bc}**	**16.92 ± 1.27^{ab}**
C18.2n6	12.19 ± 1.49	11.00 ± 1.37	12.04 ± 1.41	11.68 ± 1.30	11.63 ± 1.37	12.88 ± 1.99	12.22 ± 1.28	12.39 ± 1.34
C18.3n6	0.09 ± 0.02	0.08 ± 0.02	0.09 ± 0.02	0.09 ± 0.01	0.09 ± 0.03	0.12 ± 0.03	0.11 ± 0.02	0.11 ± 0.03
C20.3n6	1.80 ± 0.42	1.54 ± 0.37	1.66 ± 0.32	1.63 ± 0.38	1.60 ± 0.38	1.73 ± 0.42	1.73 ± 0.41	1.77 ± 0.45
C20.2n6	0.24 ± 0.03	0.23 ± 0.02	0.23 ± 0.02	0.23 ± 0.02	0.23 ± 0.02	0.23 ± 0.02	0.23 ± 0.02	0.24 ± 0.02
C20.4n6	15.77 ± 1.54	14.84 ± 1.73	14.30 ± 1.61	14.69 ± 1.77	14.50 ± 1.62	14.14 ± 1.61	14.44 ± 1.47	14.90 ± 1.48
C22.4n6	3.02 ± 0.63	2.78 ± 0.57	2.58 ± 0.57	2.54 ± 0.48	2.42 ± 0.43	2.25 ± 0.46	2.42 ± 0.46	2.57 ± 0.49
C22.5n6	0.59 ± 0.15	0.51 ± 0.14	0.48 ± 0.12	0.49 ± 0.12	0.45 ± 0.08	0.42 ± 0.08	0.43 ± 0.08	0.46 ± 0.10
n-6 PUFA	**33.71 ± 1.64^{a}**	**30.99 ± 1.75^{bc}**	**31.39 ± 1.54^{bc}**	**31.35 ± 1.68^{bc}**	**30.92 ± 1.60^{b}**	**31.78 ± 1.64^{cd}**	**31.58 ± 1.32^{bc}**	**32.44 ± 1.40^{d}**
C18.3n3	0.15 ± 0.04	0.13 ± 0.04	0.16 ± 0.08	0.14 ± 0.03	0.15 ± 0.04	0.16 ± 0.05	0.16 ± 0.04	0.16 ± 0.04
C20.5n3	0.75 ± 0.23	2.27 ± 0.70	1.72 ± 0.44	1.68 ± 0.43	1.71 ± 0.43	1.30 ± 0.30	1.19 ± 0.23	1.00 ± 0.22
C22.5n3	2.78 ± 0.42	3.14 ± 0.43	3.16 ± 0.37	3.24 ± 0.39	3.30 ± 0.43	3.07 ± 0.53	3.19 ± 0.43	3.07 ± 0.48
C22.6n3	4.79 ± 0.90	5.76 ± 0.72	5.73 ± 0.70	6.04 ± 0.73	6.29 ± 0.74	5.86 ± 0.78	5.93 ± 0.66	5.57 ± 0.74
n-3 PUFA	**8.46 ± 1.08^{a}**	**11.31 ± 1.15^{b}**	**10.77 ± 0.92^{bc}**	**11.11 ± 1.14^{bd}**	**11.44 ± 1.28^{b}**	**10.40 ± 1.34^{ce}**	**10.48 ± 0.97^{cde}**	**9.80 ± 1.01^{e}**
ARA/EPA	**23.49 ± 8.61^{a}**	**7.47 ± 3.39^{b}**	**9.17 ± 3.77^{bc}**	**9.50 ± 3.44^{bc}**	**9.19 ± 3.18^{bc}**	**11.63 ± 3.62^{c}**	**12.62 ± 3.08^{cd}**	**15.69 ± 3.91^{d}**

Total saturated fatty acids, total monounsaturated fatty acids, total n-6 PUFA, total n-3 PUFA. Values are expressed as % of total fatty acids mean ± SD. Means that do not share a letter are significantly different from one another ($p < 0.05$).

Appendix 3.3: Table of changes in fatty acid profile in skeletal muscle phospholipid membranes in constant dose and loading dose group

	Constant Dose				Loading Dose			
Fatty Acid	Week 0	Week 4	Week 12	Week 20	Week 0	Week 4	Week 12	Week 20
C14.0	0.38±0.08	0.37±0.13	0.35±0.07	0.35±0.09	0.41±0.06	0.37±0.09	0.36±0.08	0.41±0.16
C15.0	0.17±0.06	0.16±0.05	0.17±0.07	0.18±0.09	0.19±0.06	0.17±0.05	0.18±0.06	0.20±0.08
C16.0	16.62±1.26	16.64±1.47	16.95±1.19	16.65±1.24	16.82±1.90	16.27±1.71	16.36±1.83	16.79±2.02
C18.0	13.27±0.83	13.51±0.82	13.44±0.80	13.58±0.73	13.57±0.59	13.43±0.97	13.61±0.56	13.73±0.55
C20.0	0.09±0.01	0.11±0.03	0.10±0.02	0.13±0.02	0.09±0.02	0.10±0.04	0.17±0.23	0.14±0.15
C22.0	0.20±0.07	0.23±0.10	0.17±0.03	0.21±0.07	0.27±0.33	0.18±0.06	0.17±0.04	0.17±0.04
C24.0	0.19±0.09	0.23±0.12	0.18±0.04	0.21±0.08	0.19±0.06	0.18±0.06	0.16±0.05	0.21±0.16
Saturated	**30.89±1.85**	**31.24±2.26**	**31.32±1.71**	**31.27±1.75**	**31.54±2.36**	**30.70±2.49**	**30.98±2.04**	**31.61±2.32**
16.1n.9	0.16±0.06	0.14±0.04	0.17±0.07	0.18±0.06	0.14±0.03	0.13±0.04	0.22±0.22	0.15±0.07
16.1n.7	0.55±0.15	0.84±1.09	0.53±0.19	0.61±0.28	0.54±0.18	0.55±0.22	0.60±0.26	0.76±0.48
18.1n.9	7.15±1.24	7.08±1.20	7.30±1.37	7.29±0.70	6.82±0.91	6.36±1.50	6.56±1.60	7.46±2.72
18.1n.7	1.96±0.31	1.89±0.26	2.07±0.38	1.94±0.25	1.95±0.26	1.79±0.29	1.96±0.25	1.96±0.26
20.1n.9	0.12±0.02	0.11±0.03	0.13±0.05	0.15±0.06	0.13±0.05	0.10±0.02	0.11±0.03	0.15±0.08
24.1n.9	0.19±0.08	0.23±0.11	0.22±0.07	0.23±0.08	0.22±0.08	0.23±0.13	0.22±0.07	0.25±0.15
Monounsaturated	**10.13±1.63**	**10.27±1.52**	**10.39±1.87**	**10.36±0.55**	**9.77±1.27**	**9.15±1.84**	**9.65±2.06**	**10.71±3.39**
18.2n.6	30.07±2.78	28.92±3.22	29.38±2.91	30.31±2.40	30.58±1.97	29.08±2.53	29.23±3.48	30.30±4.03
18.3n.6	0.09±0.02	0.09±0.02	0.10±0.02	0.10±0.03	0.08±0.02	0.10±0.05	0.11±0.06	0.11±0.05
20.3n.6	1.17±0.18	1.21±0.26	1.18±0.25	1.30±0.25	1.23±0.26	1.30±0.28	1.32±0.45	1.29±0.35
20.4n.6	11.87±1.61	11.59±1.40	10.97±1.05	11.17±1.49	11.47±1.27	11.13±0.93	11.36±1.79	10.61±1.34
22.4n.6	0.59±0.14	0.57±0.18	0.48±0.14	0.60±0.18	0.58±0.15	0.53±0.18	0.56±0.29	0.54±0.22
22.5n.6	0.35±0.10	0.43±0.29	0.32±0.09	0.38±0.15	0.33±0.10	0.38±0.18	0.32±0.07	0.35±0.15
n-6 PUFA	**44.12±1.99^{a}**	**42.94±2.64^{a}**	**42.40±2.35^{b}**	**44.04±1.76^{ab}**	**44.26±1.37**	**44.02±3.87**	**43.61±1.90**	**43.46±4.49**
18.3n.3	0.29±0.07	0.27±0.08	0.30±0.10	0.31±0.09	0.32±0.07	0.26±0.06	0.28±0.11	0.34±0.07
20.5n.3	0.62±0.19	1.02±0.26	1.40±0.39	0.75±0.16	0.71±0.28	1.63±0.70	1.35±0.41	0.91±0.32
22.5n.3	1.20±0.24	1.25±0.23	1.28±0.26	1.20±0.25	1.21±0.25	1.42±0.21	1.36±0.15	1.20±0.26
22.6n.3	1.67±0.55	1.99±0.49	2.23±0.45	1.64±0.31	1.63±0.59	2.44±0.72	2.28±0.55	1.72±0.45
n-3 PUFA	**3.79±0.74^{a}**	**4.55±0.83^{b}**	**5.23±0.97^{c}**	**3.90±0.59a^{b}**	**3.86±0.96^{a}**	**5.77±1.53^{b}**	**5.27±0.97^{b}**	**4.18±0.86^{a}**
16.0DMA	6.46±1.00	6.39±0.78	6.16±0.91	5.99±0.80	6.15±0.68	5.95±0.81	6.08±0.64	5.73±0.38
18.0DMA	2.42±0.35	2.46±0.30	2.36±0.35	2.36±0.33	2.41±0.41	2.46±0.46	2.45±0.38	2.40±0.41
18.1DMA	2.19±0.38	2.15±0.34	2.14±0.43	2.09±0.28	2.01±0.27	1.95±0.36	1.96±0.24	1.92±0.26
DMA	**11.08±1.38^{a}**	**11.00±0.97^{ab}**	**10.66±1.33^{ab}**	**10.43±1.02^{b}**	**10.57±1.12**	**10.36±1.43**	**10.49±1.01**	**10.04±0.79**
ARA/EPA	**21.66±10.92^{a}**	**12.27±4.72^{bc}**	**8.48±3.15^{b}**	**15.79±5.78^{c}**	**18.62±6.87^{a}**	**8.23±4.05^{b}**	**9.35±3.76^{bc}**	**12.76±3.75^{c}**

Total saturated fatty acids, total monounsaturated fatty acids, total n-6 PUFA, total n-3 PUFA, and total dimethyl aldehyde (DMA). Values are expressed as % of total fatty acids mean ± SD. Means that do not share a letter are significantly different from one another ($p < 0.05$).

Appendix 3.4: Table of changes in fatty acid profile in adipose tissue in the constant dose and loading dose group in young adults

	Constant Dose				Loading Dose			
visit	Week 0	Week 4	Week 12	Week 20	Week 0	Week 4	Week 12	Week 20
C14.0	2.50±0.53	2.50±0.66	2.49±0.64	2.47±0.51	2.63±0.35	2.62±0.45	2.74±0.50	2.68±0.43
C15.0	0.25±0.08	0.25±0.11	0.25±0.10	0.28±0.08	0.29±0.04	0.30±0.05	0.31±0.05	0.30±0.04
C16.0	20.14±2.17	19.94±2.27	19.81±2.62	19.57±1.96	21.32±1.28	21.25±1.80	21.36±1.79	21.18±1.87
C18.0	4.69±0.75	4.39±0.87	4.32±0.87	4.33±0.93	4.70±0.96	4.78±1.16	4.41±1.10	4.73±1.12
C20.0	0.25±0.05	0.24±0.06	0.23±0.07	0.25±0.07	0.21±0.02	0.22±0.04	0.19±0.03	0.21±0.03
C22.0	0.08±0.02	0.09±0.02	0.12±0.03	0.09±0.04	NaN±NA	NaN±NA	NaN±NA	NaN±NA
Saturated	**27.86±3.01**	**27.37±3.04**	**27.14±3.21**	**26.90±2.24**	**29.15±2.07**	**29.17±2.99**	**29.01±2.66**	**29.10±3.00**
C16.1n.9	0.55±0.06	0.56±0.08	0.56±0.06	0.57±0.05	0.60±0.06	0.61±0.09	0.62±0.06	0.60±0.09
C16.1n.7	3.32±0.69	3.41±0.69	3.41±0.73	3.51±0.75	3.51±1.06	3.50±1.30	3.82±0.99	3.60±1.20
C18.1n.9	47.77±1.64	47.94±1.69	48.42±1.91	48.73±1.72	47.09±2.24	47.22±2.29	46.96±2.32	47.29±2.27
C18.1n.7	2.14±0.34	2.28±0.44	2.15±0.19	2.17±0.29	2.43±0.27	2.29±0.31	2.36±0.39	2.37±0.25
C20.1n.9	0.78±0.05	0.82±0.05	0.81±0.10	0.84±0.09	0.79±0.06	0.80±0.07	0.77±0.09	0.78±0.08
Monounsaturated	**54.58±1.80**	**55.00±1.64**	**55.35±1.57**	**55.81±1.60**	**54.42±2.65**	**54.41±2.97**	**54.53±2.72**	**54.64±2.91**
C18.2n.6	15.39±3.41	15.48±3.68	15.33±3.41	15.02±3.08	14.09±1.80	14.04±1.58	14.05±1.57	13.82±1.46
C18.3n.6	0.15±0.13	0.08±0.02	0.10±0.04	0.10±0.04	0.12±0.09	0.13±0.05	0.09±0.02	0.11±0.02
C20.2n.6	0.23±0.04	0.23±0.04	0.25±0.03	0.24±0.03	0.23±0.05	0.23±0.05	0.22±0.05	0.22±0.05
C20.3n.6	0.15±0.03	0.16±0.03	0.16±0.02	0.17±0.03	0.18±0.05	0.18±0.06	0.18±0.05	0.18±0.05
C20.4n.6	0.28±0.06	0.28±0.05	0.29±0.06	0.29±0.06	0.29±0.07	0.30±0.07	0.30±0.08	0.29±0.10
C22.4n.6	0.10±0.03	0.10±0.02	0.10±0.02	0.10±0.02	0.11±0.03	0.11±0.02	0.11±0.02	0.11±0.03
n-6 PUFA	**16.23±3.42**	**16.32±3.72**	**16.18±3.47**	**15.90±3.07**	**15.00±1.76**	**14.93±1.54**	**14.93±1.52**	**14.70±1.43**
C18.3n.3	1.06±0.29	0.97±0.25	0.98±0.23	1.06±0.32	1.08±0.22	1.03±0.23	1.02±0.17	1.06±0.19
C20.5n.3	0.08±0.02	0.10±0.02	0.09±0.02	0.08±0.02	0.08±0.02	0.10±0.03	0.11±0.05	0.12±0.04
C22.5n.3	0.11±0.02	0.12±0.02	0.13±0.02	0.14±0.02	0.14±0.06	0.16±0.05	0.18±0.05	0.17±0.05
C22.6n.3	0.11±0.05	0.16±0.04	0.15±0.05	0.14±0.05	0.13±0.07	0.19±0.07	0.21±0.10	0.21±0.08
n-3 PUFA	**1.33±0.28**	**1.31±0.20**	**1.33±0.19**	**1.40±0.27**	**1.42±0.24**	**1.49±0.19**	**1.53±0.27**	**1.56±0.23**
ARA/EPA	**4.12±1.05**	**2.96±0.67**	**3.57±1.22**	**4.26±1.58**	**3.83±1.13**	**3.05±1.07**	**2.94±1.22**	**2.91±0.99**

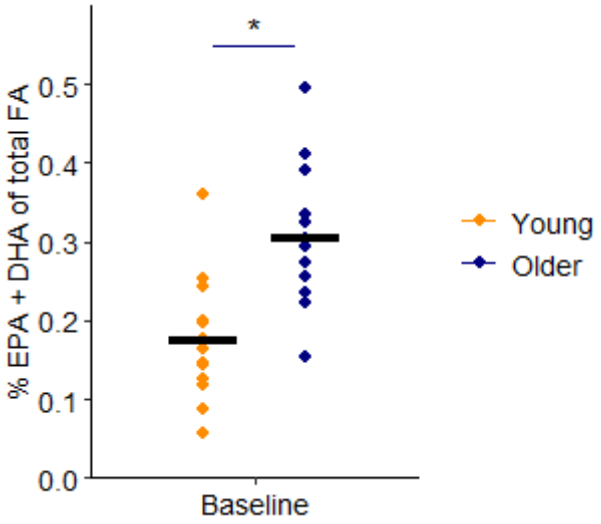
Total saturated fatty acids, total monounsaturated fatty acids, total n-6 PUFA, total n-3 PUFA, and total dimethyl aldehyde (DMA). Values are expressed as % of total fatty acids mean ± SD. Means that do not share a letter are significantly different from one another ($p < 0.05$).

Appendix 3.5: Table of changes in fatty acid profile in adipose tissue in the constant dose and loading dose group in older adults

	Constant Dose				Loading Dose			
visit	Week 0	Week 4	Week 12	Week 20	Week 0	Week 4	Week 12	Week 20
C14.0	2.77±0.53	2.71±0.55	2.72±0.46	2.75±0.47	3.20±0.61	3.20±0.54	3.21±0.58	3.17±0.56
C15.0	0.31±0.05	0.30±0.05	0.31±0.07	0.31±0.06	0.38±0.05	0.37±0.05	0.38±0.06	0.39±0.04
C16.0	21.14±1.81	20.79±1.79	21.03±1.79	20.91±1.87	19.62±1.41	19.63±1.18	19.67±1.44	19.46±1.42
C18.0	3.98±1.27	3.70±1.21	3.78±1.16	3.64±1.12	4.36±1.12	4.31±1.01	4.43±1.09	4.33±1.18
C20.0	0.21±0.07	0.18±0.05	0.18±0.06	0.18±0.05	0.24±0.09	0.24±0.08	0.25±0.08	0.25±0.09
C22.0	0.10±NA	NaN±NA	0.09±NA	0.08±NA	0.10±0.01	0.09±NA	0.10±0.01	0.10±0.00
Saturated	**28.42±3.46**	**27.67±3.45**	**28.03±3.27**	**27.80±3.27**	**27.84±2.71**	**27.77±2.13**	**27.97±2.44**	**27.63±2.70**
C16.1n.9	0.69±0.16	0.71±0.17	0.69±0.15	0.71±0.15	0.63±0.10	0.63±0.12	0.64±0.10	0.66±0.11
C16.1n.7	5.74±1.12	6.04±1.10	5.86±1.26	6.01±1.09	5.05±1.54	5.13±1.58	4.91±1.51	5.14±1.77
C18.1n.9	47.21±2.14	47.47±2.38	47.17±2.49	47.15±2.47	49.10±1.16	49.11±0.98	49.03±1.34	48.91±1.03
C18.1n.7	2.58±0.27	2.64±0.22	2.61±0.22	2.66±0.24	2.42±0.38	2.37±0.41	2.40±0.38	2.53±0.46
C20.1n.9	0.79±0.10	0.76±0.07	0.77±0.09	0.75±0.07	0.83±0.07	0.83±0.09	0.84±0.09	0.81±0.08
Monounsaturated	**57.01±2.90**	**57.62±3.00**	**57.10±2.90**	**57.29±2.81**	**58.04±2.74**	**58.07±2.41**	**57.82±2.62**	**58.05±2.61**
C18.2n.6	11.84±0.84	11.95±0.76	12.01±0.84	12.03±0.69	11.67±0.91	11.68±0.93	11.71±1.03	11.82±1.09
C18.3n.6	0.09±0.01	0.09±0.01	0.09±0.01	0.10±0.02	0.08±0.01	0.08±0.01	0.08±0.01	0.08±0.01
C20.2n.6	0.20±0.02	0.20±0.03	0.21±0.02	0.18±0.05	0.18±0.03	0.18±0.02	0.19±0.02	0.19±0.02
C20.3n.6	0.27±0.08	0.27±0.08	0.26±0.07	0.27±0.07	0.22±0.15	0.21±0.13	0.21±0.12	0.22±0.11
C20.4n.6	0.49±0.14	0.51±0.16	0.50±0.12	0.51±0.12	0.35±0.18	0.34±0.19	0.35±0.18	0.37±0.19
C22.4n.6	0.16±0.05	0.16±0.07	0.16±0.05	0.15±0.06	0.12±0.09	0.12±0.08	0.12±0.09	0.11±0.07
n-6 PUFA	**13.05±0.93**	**13.16±0.88**	**13.23±0.94**	**13.24±0.80**	**12.61±0.66**	**12.62±0.70**	**12.65±0.83**	**12.79±0.90**
C18.3n.3	0.90±0.19	0.89±0.20	0.93±0.22	0.95±0.21	1.00±0.23	0.99±0.21	0.99±0.21	1.00±0.21
C20.5n.3	0.13±0.04	0.13±0.02	0.15±0.03	0.15±0.02	0.11±0.03	0.11±0.03	0.12±0.04	0.12±0.03
C22.5n.3	0.26±0.05	0.26±0.06	0.27±0.04	0.27±0.05	0.21±0.11	0.22±0.09	0.22±0.09	0.21±0.09
C22.6n.3	0.20±0.04	0.22±0.04	0.25±0.04	0.25±0.05	0.18±0.07	0.19±0.07	0.20±0.08	0.18±0.07
n-3 PUFA	**1.52±0.28[a]**	**1.54±0.27[a]**	**1.64±0.32[b]**	**1.67±0.30[b]**	**1.51±0.30**	**1.54±0.30**	**1.55±0.36**	**1.53±0.35**
ARA/EPA	**4.00±1.18[a]**	**3.87±1.22[a]**	**3.32±0.78[b]**	**3.53±0.86[ab]**	**3.36±1.09**	**3.12±1.50**	**3.03±1.17**	**3.34±1.39**

Total saturated fatty acids, total monounsaturated fatty acids, total n-6 PUFA, total n-3 PUFA, and total dimethyl aldehyde (DMA). Values are expressed as % of total fatty acids mean ± SD. Means that do not share a letter are significantly different from one another ($p < 0.05$).

Appendix 3.6 Baseline levels of EPA + DHA in adipose tissue of young and older adults



Appendix 3.6: Baseline % EPA + DHA of total FA in adipose tissue of young (0.18 ± 0.07% (95% CI [0.13 – 0.22) and older (0.30 ± 0.09% (95% CI [0.25 – 0.36) and adults. Baseline levels in the young age group were significantly lower compared to the older age group ($P < 0.001$, 95% CI = [-0.20 – -0.06]).

Appendix 3.7: Whole muscle incorporation and washout in young and older adults

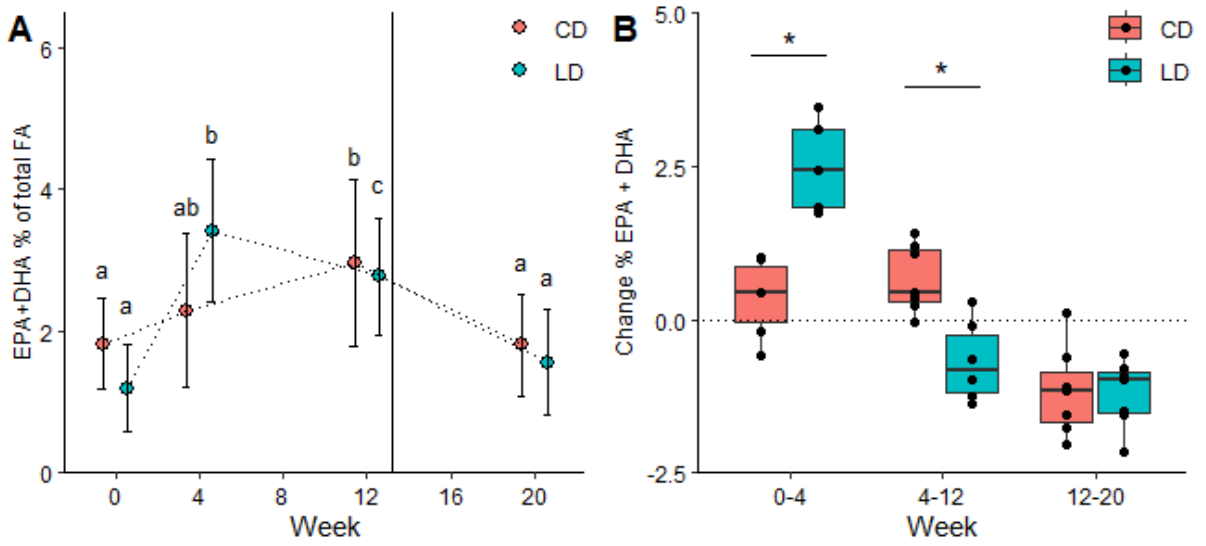
This thesis initially included an analysis of changes in the fatty acid profile of whole muscle tissue. However, to maintain the focus of the thesis, these results are moved to the Appendix. Here, it is shown that changes in whole muscle tissue differ from those in the fatty acid profile of the skeletal muscle's phospholipid membrane.

There was a main effect of time on % EPA + DHA/total fatty acids incorporation ($P < 0.001$), as well as an interaction effect between time × age ($P < 0.001$), time × dosing strategy ($P < 0.001$) and time × age × dosing strategy ($P = 0.025$). Due to the interaction effect involving age, results for whole muscle are separately presented for young and older adults.

Incorporation and washout in whole muscle of young adults

Changes over time revealed that in CD (**Appendix 3.7.1A**), %EPA + DHA/total fatty acids increased from week 0 to week 12 by 0.89 ± 0.75 % (95% CI [0.19 – 1.59]; $P = 0.008$). In LD (**Appendix 3.7.1A**), %EPA + DHA/total fatty acids increased from week 0 to week 4 by 2.50 ± 0.76 % (95% CI [1.80 – 3.21]; $P < 0.001$), whereas a decline was detected from week 4 – week 12 of -0.68 ± 0.68 % (95% CI [-1.29 – -0.07]; $P = 0.028$). Since there were no differences in incorporation between dosing strategies at week 12 and week 20 ($P > 0.05$; **Appendix 3.7.1A**), CD and LD were grouped together to determine washout. A significant decline of -1.18 ± 0.62% (95% CI [-1.55 – -0.82]) was detected during the washout phase, where levels did not differ from baseline ($P > 0.05$).

LD resulted in a greater Δ%EPA + DHA compared to CD after the initial 4-week loading phase ($P = 0.001$; **Appendix 3.7.1B**). Following the 4 – 12 week maintenance phase, CD resulted in a greater Δ %EPA + DHA compared to LD ($P = 0.001$; **Appendix 3.7.1A**). There was no difference in Δ%EPA + DHA between the dosing strategies during the 12 – 20-week washout phase ($P > 0.05$; **Appendix 3.7.1A**).

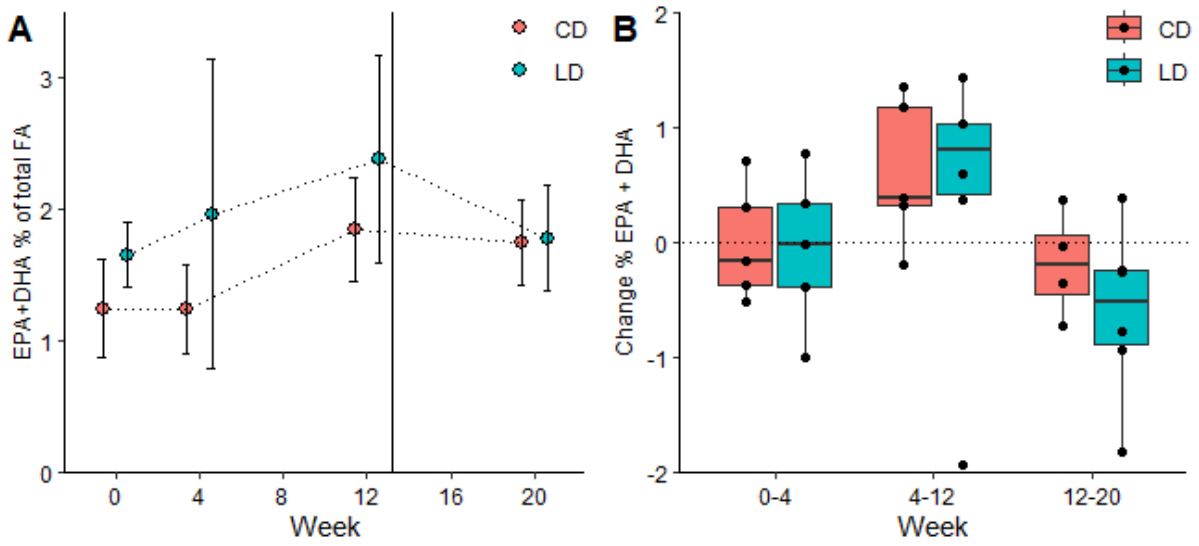


Appendix 3.7.1) Changes in skeletal muscle composition over time (**3.7.1A**) and between dosing strategies during loading, maintenance, and washout phase (**3.7.1B**) of the sum of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) following a constant dose (CD) or loading dose (LD) strategy in young adults. The CD group (n=7) received 720 mg EPA + 540 mg DHA /day for 12 weeks, the LD group (n=7) received a loading dose of 1440 mg EPA + 1080 mg DHA/ day for the first 4 weeks followed by 360 mg EPA + 270 mg DHA/ day for 8 weeks thereafter. Differences between strategies are indicated with an '*'. ($P < 0.05$). **Appendix 3.7.1A**: Values are means ± SD (standard deviation). Mean values that do not share a common letter within CD or LD are statistically different ($P < 0.05$). **Appendix 3.7.1B**: Boxplot of change (median + interquartile ranges and whiskers) in percentage EPA + DHA between week 0 – 4 (loading phase), week 4 – 12 (maintenance phase), and week 12 – 20 (washout phase).

Incorporation and washout in whole muscle of older adults

In older adults receiving the constant dose (CD), a statistically significant main effect of time was observed for %EPA + DHA/total fatty acids. However, no significant differences were detected between time points (**Appendix 3.7.2A**). In LD (**Appendix 3.7.2A**), no statistically significant main effect of time on %EPA + DHA/total fatty acids was observed, precluding further post-hoc analyses.

No significant differences in Δ%EPA + DHA were observed between the dosing strategies during the 0 – 4 week loading phase, 4 – 12 week maintenance phase, or 12 – 20 week washout phase ($P > 0.05$; **Appendix 3.7.2B**).



Appendix 3.7.2: Changes in skeletal muscle composition over time (**3.7.2A**) and between dosing strategies during loading, maintenance, and washout phase (**3.7.2B**) of the sum of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) following a constant dose (CD) or loading dose (LD) strategy in older adults. The CD group (n=5) received 720 mg EPA + 540 mg DHA/day for 12 weeks, the LD group (n=6) received a loading dose of 1440 mg EPA + 1080 mg DHA/ day for the first 4 weeks, followed by 360 mg EPA + 270 mg DHA/ day for 8 weeks thereafter. There were no differences between strategies at any time point. **Appendix 3.7.2A**: Values are means ± SD (standard deviation). **Appendix 3.7.2B**: Boxplot of change (median + interquartile ranges and whiskers) in percentage EPA + DHA between week 0 – 4 (loading phase), week 4 – 12 (maintenance phase), and week 12 – 20 (washout phase).

Appendix 4.1: RNA extraction adjusted protocol Fibrous Tissue Kit

- Unless otherwise indicated, perform the procedure, as well as all centrifugation steps, at room temperature (15–25°C).
- Add 10 µl β-mercaptoethanol (β-ME), per 1 ml Buffer RLT. Buffer RLT containing DTT or β-ME can be stored at room temperature for up to 1 month.

1. Heat heating block to 55°C.
2. Add 300µl Buffer RLT bead tubes and then add ≤30 mg tissue.
3. Incubate for **10 minutes** and disrupt in the Magnalyser (2x35s at 5000Hz). Put on ice few seconds in between disruptions.
4. Add 590 µl RNase-free water, then 10 µl proteinase K,
5. Mix (invert 5-6 times) and incubate at 55°C for **20 min**.
6. Centrifuge at 18,000 x *g* for **3 min**.
7. Transfer 850 µl supernatant to new tube. Add 425 µl 96–100% ethanol, and mix. *Do not centrifuge.*
8. Transfer 350 µl of sample to RNeasy column in a 2 ml collection tube and pre-incubate for **5 min**.
9. Close lid, centrifuge for **30s** at ≥13,000*g* and discard flow-through. Repeat step until complete lysate is used (without pre-incubation).

DNAse step:

10. Add 350 µl Buffer RW1 to RNeasy column and pre-incubate for **5min**. Close lid, centrifuge for **15s** at 18,000*g* and discard flow-through.
    a. During this: Mix 10µl DNase stock solution with 70 µl Buffer RDD
11. Add DNase mix to RNeasy membrane and incubate for **15 min** at 20–30°C.
12. Add 350 µl Buffer RW1 to RNeasy column and pre-incubate for **5min**. Close lid, centrifuge for **15s** at 18,000*g* and discard flow-through.

Wash:

13. Add 500 µl Buffer RPE to RNeasy column and preincubate for **5min**. Close lid, centrifuge for **15s** at 18,000*g* and discard flow-through.
14. Add another 500 µl Buffer RPE to RNeasy column. Close lid, centrifuge for **2min** at 13,000*g*.
15. Place RNeasy column in new 2 ml tube, close lid and centrifuge at 18,000g for **1 min**.
16. Place RNeasy column in a a new 1.5mL tube and air-dry on heatblock for **3-5min**.
17. Add 30µl pre-heated RNAse-free water and preincubate for **5min.** Centrifuge for **1min** at 18,000*g*.
18. Put collected RNA on ice and measure using Nanodrop.
19. Turn off heatblock, clean up.

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Appendix 4.2: 3-day Food and activity Diary

Name: .....

- Record everything you eat and drink the 3 days before you come in for your visit.
- Record all activity you do the 3 days before you come in for your visit.
- Write down what, how much, and brands (if applicable).
- Try to be as specific as possible! Pictures are welcome.

Food and activity diary explained

Please provide detailed information when recording food and drinks intake. Include supplements e.g. vitamin C.

Try to provide precise weights using scales (grams) or measure fluid volumes (ml). If this is not possible, describe the amount of food/fluid according to plate, portion and cup size etc.
Try to specify the brand (e.g. Uncle Ben's, Tesco etc.) and how it was prepared (if you used a preparation method). For prepared food you can write raw weight (pre-cooked food weight) or prepared (prepared weight). If you cook meals with different components, please list every single item of what you've eaten.

It is extremely important to be completely honest when you are keeping this diary. The more detailed information you provide, the more detailed analysis can be undertaken. Please use the **example** below to help you fill out this diary:

Meal	Time	Brand (if known)	Type of food	Quantity	Preparation
Breakfast	0800	Kellogg's	Cornflakes	45 g	
Breakfast	0800	Tesco	Semi skimmed milk	120 ml	
Breakfast	0800		Eggs	2 large	Fried
Snack	1110	Aldi	Sugar free orange juice	Pint	
Lunch	1300	Tesco	Cheddar cheese, pre-grated	20 g	Melted
Lunch	1300	Warburton	Whole meal bread	2 slices	Toasted
Snack	1530		Monster munch – pickled onion	1 packet	
Dinner	1900		Pasta (uncooked)	70g	Boiled
Dinner	1900	Dolmio	Dolmio original pasta sauce	130g	Boiled
Dinner	1900	BenGJerry	Cookie Dough Ice Cream	160g	

Time	Activity	Intensity	Duration
08.30	Cycle to work – 3 miles	Low – flat roads and slow pace	30 min
18.00	Gym session – strength 3 sets of 5 reps for squat, deadlift, bench press and bent over row	High	45 min

Day 1: Day of the week:_____Date:_____

Food and fluid diary

Meal	Time	Brand (if known)	Type of food/fluid	Quantity	Preparation

Activity

Time	Activity	Intensity	Duration

Day 2: Day of the week:_____Date:_____

Food and fluid diary

Meal	Time	Brand (if known)	Type of food/fluid	Quantity	Preparation

Activity

Time	Activity	Intensity	Duration

Day 3: Day of the week:_____Date:_____

Food and fluid diary

Meal	Time	Brand (if known)	Type of food/fluid	Quantity	Preparation

Activity

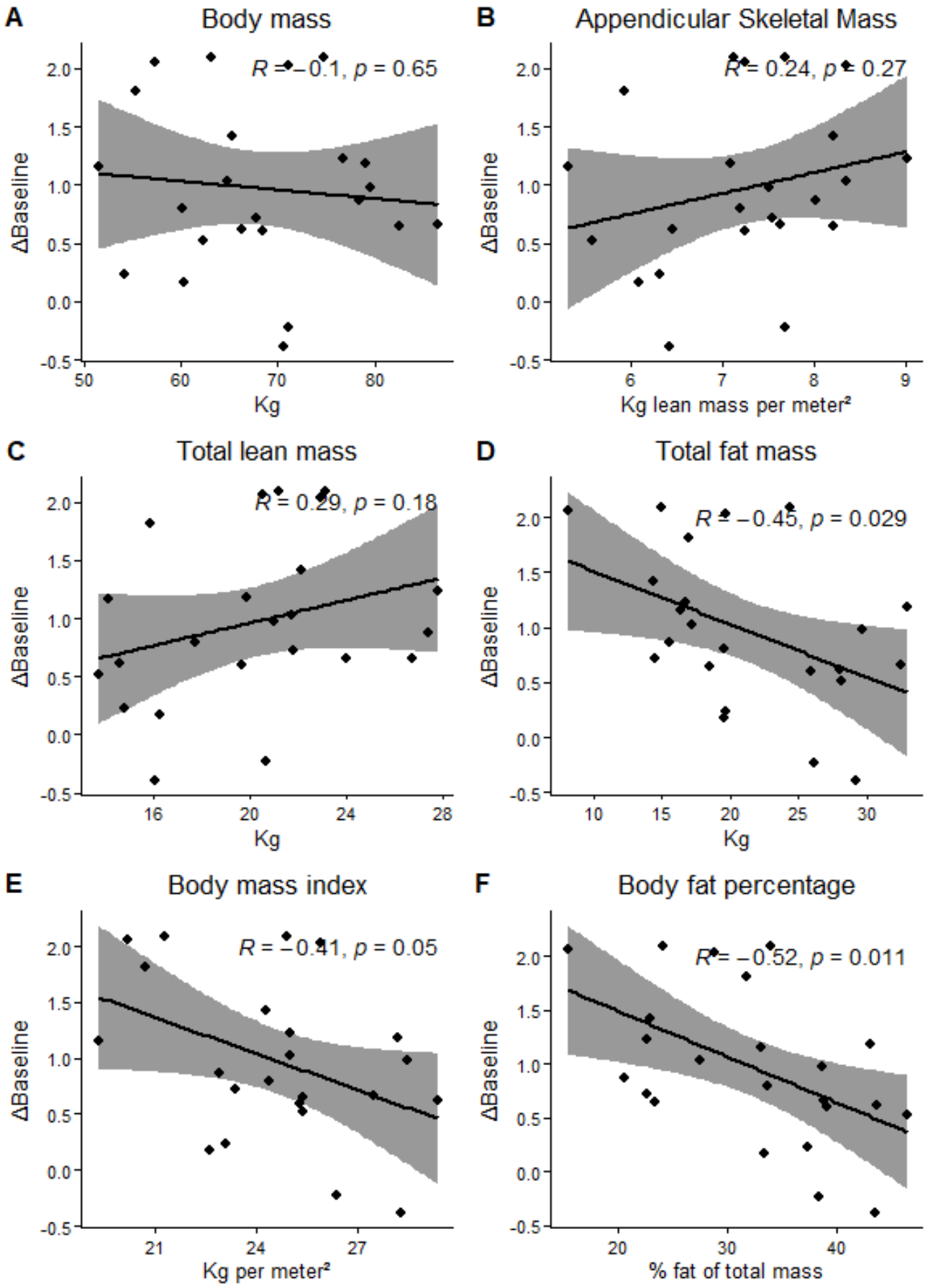
Time	Activity	Intensity	Duration

Appendix 4.3 Overlap genes in oxidative phosphorylation pathways between KEGG and GSEA

Gene Set Name [# Genes (K)]	Description	# Genes in Overlap
HALLMARK_OXIDATIVE_PHOSPHORYLATION [200]	Genes encoding proteins involved in oxidative phosphorylation.	200
KEGG_OXIDATIVE_PHOSPHORYLATION [132]	Oxidative phosphorylation	83

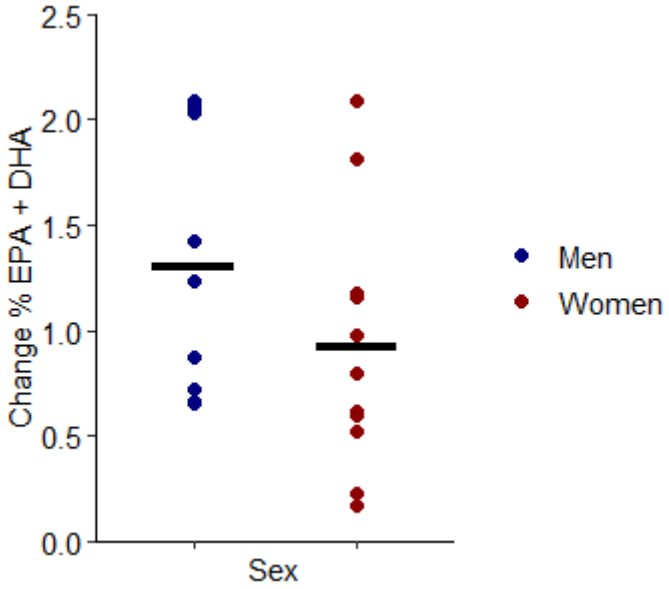
Gene Set Name [# Genes (K)]	Description	# Genes in Overlap (k)	k/K	p-value	FDRq-value
HALLMARK_OXIDATIVE_PHOSPHORYLATION [200]	Genes encoding proteins involved in oxidative phosphorylation.	200		$0\ e^{0}$	$0\ e^{0}$
KEGG_OXIDATIVE_PHOSPHORYLATION [132]	Oxidative phosphorylation	83		$3.97\ e^{-166}$	$4.69\ e^{-164}$

Appendix 5.1 Correlation between ΔEPA + DHA and measured of body composition



Appendix 5.1: Correlation between the ΔEPA + DHA as percentage of total fatty acid in skeletal muscle phospholipid membranes and total body mass (**A**), Appendicular Skeletal Mass (**B**), total lean mass (**C**), total fat mass (**D**), body mass index (**E**) and body fat percentage (**F**) after 12 weeks of n-3 PUFA supplementation with 1.26g EPA + DHA per day on average.

Appendix 5.2 Difference in ΔEPA + DHA between 0 and 12 weeks in men and women.



Appendix 5.2: Change in percentage EPA + DHA from baseline in men (1.30 ± 0.62 % (95% CI [0.82 – 1.78])) and women (0.73 ± 0.72% (95% CI [0.30 – 1.17])) after 12 weeks of supplementation with 720 mg EPA + 540 mg DHA /day on average for 12 weeks. There were no differences in incorporation response (P = 0.063, 95% CI [-0.04 - 1.17]). n = 9 men, n = 13 women.

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Original research article

Dose-dependency of a combined EPA:DHA mixture on incorporation, washout, and protein synthesis in C2C12 myotubes

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SUMMARY

We demonstrate divergent incorporation and washout patterns for EPA and DHA following high and low-dose EPA+DHA incubation in C2C12 myotubes, with higher concentrations favoring *n*-3 PUFA incorporation. Lower *n*-3 PUFA concentrations increased MPS without further upregulating the mTORC1 signaling pathway. Our study provides novel insights into the temporal incorporation and washout dynamics of EPA and DHA and, specifically, their combined effect on MPS, thereby advancing knowledge regarding dietary *n*-3 PUFA prescription to promote skeletal muscle health in humans.

1. Introduction

A key mechanism that underpins the pathophysiology of muscle atrophy in older adults is anabolic resistance which describes the age associated impaired stimulation of muscle protein synthesis (MPS) in response to anabolic stimuli, namely protein feeding and muscle loading [1]. Emerging evidence exists to suggest a therapeutic role of long chain omega-3 polyunsaturated fatty acids (*n*-3 PUFA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), in modulating skeletal muscle protein turnover [2–5]. For instance, seminal *in vivo* studies have reported chronic (8 weeks) fish oil-derived *n*-3 PUFA supplementation to potentiate rates of MPS and molecular readouts of anabolic cell signaling in response to a hyperinsulinemic / hyperaminoacidemic clamp in young and older adults [4,5]. The biological actions of *n*-3 PUFA in skeletal muscle are primarily mediated by the incorporation of EPA and DHA into the cellular membranes of skeletal muscle cells, thus altering cell membrane phospholipid composition [6,7]. Additionally, the ingestion of *n*-3 PUFA leads to the secretion of specialized pro-resolving mediators (SPM), which compete with pro-inflammatory eicosanoids derived from *n*-6 PUFA arachidonic acid [8]. These SPM help mitigate the upregulation of catabolic signaling pathways by resolving inflammation, specifically in critical care settings [9]. However, the optimal dosing strategy for *n*-3 PUFA ingestion to maximize EPA and DHA membrane phospholipid composition and upregulate the stimulation of MPS and associated signaling proteins remains unclear.

The dynamics of EPA and DHA incorporation into blood, adipose tissue and skeletal muscle is dose and time-course dependent, as evidenced by multiple human studies [10–12]. Accordingly, high dose (4.4 g EPA + DHA) *n*-3 PUFA ingestion over a 4-week period induced a similar increase in muscle EPA and DHA incorporation compared to low dose (3 – 3.36 g EPA + DHA) *n*-3 PUFA ingestion over extended time periods (8–12 weeks) in humans [5,11,13]. In contrast, only 6 days of high dose (6 % of total energy) *n*-3 PUFA ingestion led to an increased composition of *n*-3 PUFA in the phospholipid membrane composition of muscle tissue [14]. Collectively, these data suggest that higher doses of ingested *n*-3 PUFA lead to a more rapid and pronounced incorporation of EPA and DHA into skeletal muscle tissue. In addition, several *in vitro* studies have assessed changes in *n*-3 PUFA membrane composition in response to EPA or DHA incubation. For example, incubation with 20 μM EPA or DHA modified membrane lipid composition during 2–4 days of differentiation in L6 skeletal muscle cells [15]. Similarly, incubating C2C12 myotubes for 72 h with 50 μM EPA or 50 μM DHA markedly altered membrane *n*-3 PUFA composition, while incubation with EPA also increased docosapentaenoic acid (DPA) content and resulted in an increased protein accretion [16]. However, the time-course of changes in *n*-3 PUFA composition in an *in vitro* model in response to different

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doses of combined EPA/DHA remains unclear.

The dose and duration of *n*-3 PUFA ingestion may impact the washout of EPA and DHA from the skeletal muscle phospholipid membrane. The washout time course of EPA and DHA after cessation of n-3 PUFA rich fish oil supplementation has been characterized in several blood fractions, including erythrocytes [10,17,18], whole blood [18], and plasma [17–20] in human studies. However, the washout dynamics of EPA and DHA following cessation of *n*-3 PUFA supplementation has yet to be elucidated in any skeletal muscle model. Given that distinct differences exist in the incorporation rates of EPA and DHA into erythrocytes and skeletal muscle tissue [11], it is plausible that the washout profile of EPA and DHA measured in blood does not translate to skeletal muscle tissue. Hence, it is crucial to investigate washout profiles of EPA and DHA from skeletal muscle.

The incorporation of EPA and DHA into the phospholipid membrane of skeletal muscle cells results in the modulation of muscle protein turnover, as evidenced by findings from *in vivo* human studies [3–5] and *in vitro* cell models [16,21,22]. While some studies report increased rates of MPS following *n*-3 PUFA supplementation is accompanied by the increased phosphorylation of associated signaling proteins [4,5], this finding is not universal [23]. Preliminary evidence in human muscle suggests that changes in skeletal muscle phospholipid membrane composition resulting from the incorporation of EPA and DHA may enhance signaling efficiency [23].

Moreover, there is evidence that EPA and DHA exhibit differential effects on muscle protein turnover. For instance, incubation of C2C12 myotubes with EPA resulted in a 25 % greater stimulation of MPS, with no effect of DHA incubation on MPS rates [21]. Nevertheless, both EPA and DHA incubation increased the phosphorylation status of $p70S6K1^{[Thr389]}$. However, Wang et al. [24] observed a more pronounced reduction in protein breakdown with DHA compared to EPA incubation in C2C12 myotubes, as mediated by an upregulation of signaling proteins involved in the PPARγ/NFκB pathway. Hence, these studies indicate distinct roles for EPA and DHA in regulating muscle protein turnover when administered independently in cellular models. However, EPA and DHA are commonly co-administered in commercially available products, which emphasizes the need to understand the dynamics of EPA and DHA uptake into the phospholipid membrane and subsequent effects on muscle protein metabolism when EPA and DHA are administered in combination at different doses. To this end, no *in vitro* experiment has incubated cells with high and low doses of combined EPA and DHA.

Despite accumulating evidence regarding the effects of *n*-3 PUFA ingestion in modulating skeletal muscle protein turnover [2–5], the dose and time-course dependent rates of EPA and DHA incorporation and washout in relation to skeletal muscle remain poorly understood. Therefore, the aim of this study was to determine temporal changes in phospholipid membrane incorporation and washout of EPA and DHA, in concert with measured MPS rates, in response to incubation with combined EPA + DHA at high or low concentration in C2C12 myotubes. In addition, we assessed the impact of high and low concentrations of combined EPA and DHA incubation and washout on the phosphorylation status of associated mTORC1 pathway signaling proteins after a 16 h incorporation period and a 24 h washout period.

2. Material and methods

2.1. Chemicals and reagents

Fetal bovine serum (FBS; Cat.No: 10,270–106) was purchased from ThermoFisher Scientific. Horse serum (HS-hi; Cat.No: VX16050122), phosphate buffered saline (PBS; Cat.No: VX14190169) and Trypsin-EDTA (trypsin; Cat.No: VX25300096) were purchased from Gibco. Dulbecco's MEM (DMEM) Low Glucose, w/o Amino Acids, Pyruvic Acid (Powder, Cat.No: D9800-13) was purchased from US Biological Life Sciences. Penicillin-streptomycin (pen-strep; Cat.No: VX15140130) and Hanks' Balanced Salt Solution (HBSS; Cat.No: VX14175) were purchased from Invitrogen. EPA (≥99 %, cis-5,8,11,14,17-Eicosapentaenoic acid, Cat.No: E2011–50MG) and DHA (≥98 %, cis-4,7,10,13,16,19-Docohexaenoic acid, Cat.No: D2534–100MG) were purchased from Sigma-Aldrich. Reagents for culture medium (DMEM powder; Cat.No: D5648, $NaHCO_3$), Insulin from bovine pancreas (Cat.No: I6634–100MG), L-Leucine BioUltra (Cat.No: 61,819), were purchased from Sigma-Aldrich. Puromycin (Cat.No: 540,411–100) was purchased from Merck and anti-puromycin (Cat.No: EQ0001) was purchased from Kerafast. Anti-mouse Detection Module (Cat.No: DM-002), Anti-rabbit Detection module (Cat.No: DM-001), and 12–230 kDa Wes Separation Module (Cat.No: SM-W004) were purchased from Bio-Techne. Anti-4EBP1 (Cat.No: CST9452), anti-phospho-4EBP1 (Thr37/46) (Cat.No: CST2855), anti-p70S6K1 (Cat.No: CST34475), anti-phospho-p70S6K1 (Thr421/Ser424) (Cat.No: CST9204), Akt (pan) (40D4) Mouse mAb (Cat.No: CST2920240), and Phospho-Akt (Ser473) (D9E) XP(R) Rabbit mAb (Cat.No: CST4060P) were purchased from Cell Signaling Technology.

2.2. Cell lines, cell culture and treatment

For all experiments, 1.25×10^5 cells/mL C2C12 myoblasts (Maastricht University) were seeded in 6-well plates in 3 mL DMEM containing 10 % FBS and 1 % pen-strep and incubated overnight at 37 °C and 5 % CO_2. Differentiation was induced by changing medium to DMEM with 2 % HS-Hi and 1 % pen-strep and refreshed every other day. Experimental treatments were initiated on day 7 of differentiation. Solutions of 100 mM EPA and DHA were prepared by dissolving EPA (≥99 %) and DHA (≥98 %) in 100 % ethanol, and further diluted in PBS + 2.5 % fatty acid free BSA to a final concentration of 10–20 mM. Exact fatty acid concentration was measured and aliquots were stored at -80 °C until further use. Cells were treated with EPA:DHA in a 3:2 ratio complexed with 2.5 % albumin dissolved in PBS in the following concentrations: high concentration (HC): 50 μM EPA + 33.3 μM DHA or low concentration (LC): 12.5 μM EPA + 8.33 μM DHA. This ratio was chosen based on commercially available products, and previous studies using concentrations of 50 μM EPA or DHA to study rates of muscle protein synthesis [16,21]. Differentiation medium was used as washout medium, and differentiation medium containing 100 % ethanol diluted to the same concentration as HC (1.28 %) with PBS + 2.5 % albumin was used as a vehicle control (VC). Treatments were freshly prepared on the morning of the experiment by adding stock EPA, DHA, or ethanol-PBS-albumin to the differentiation medium and heated at 37 °C.

2.3. Experimental design

C2C12 myotubes were treated with 3 mL of HC, LC, or VC. The incorporation and washout rate of EPA, DHA and DPA were determined in two separate experiments. A third experiment was conducted to assess rates of MPS and the phosphorylation status of associated mTORC1 proteins. To assess the incorporation of EPA, DHA and DPA (Fig. 1**A**), myotubes were incubated for 1, 2, 4, 8, 16 or 24 h before phospholipid membrane fractions were collected (as described below). To determine the washout rate (Fig. 1**B**), C2C12 myotubes were treated with 3 mL of HC, LC, or VC and incubated 16 h ($n = 3$) or 24 h ($n = 1$). Treatments were then switched to a washout medium, and cells were collected at 0, 2, 4, 8, 16 or 24 h. To assess rates of MPS and the phosphorylation status of mTORC1 associated cell signaling proteins (Fig. 1**C**), C2C12 myotubes were incubated for 16 h with HC, LC, or VC. Treatments were then switched to a washout medium for 24 h. Measurement of MPS and anabolic cell signaling were determined after the 16 h incorporation period and after a 24 h washout period. A schematic overview of the experimental design is displayed in Fig. 1).

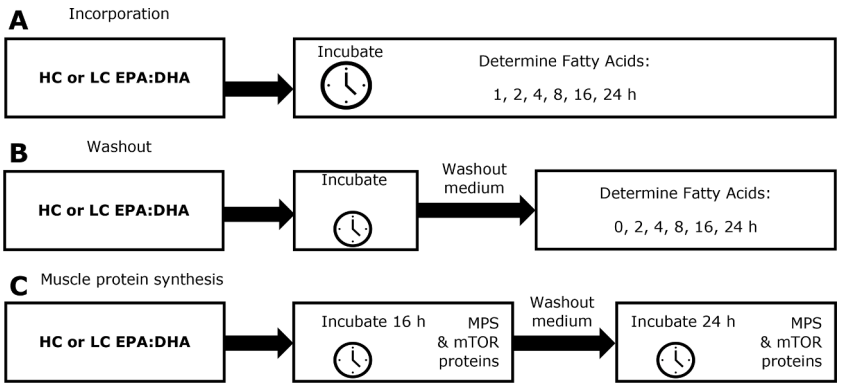

Fig. 1. Schematic overview of incorporation (A), washout (B), and muscle protein synthesis experiments (C). HC = high concentration (50 μM EPA + 33.3 μM DHA), LC = low concentration (12.5 μM EPA + 8.33 μM DHA). EPA = Eicosapentaenoic acid, DHA = Docosahexaenoic acid. MPS = muscle protein synthesis. mTORC1 = mammalian target of rapamycin complex 1.

2.4. Cell collection and fatty acid analysis

Myotubes were washed twice with cold PBS and then lysed with 200 μL PBS + 1 % Triton X-100 (VWR). Cells were detached and resuspended following 5 min of incubation at 4 °C. In total, 150 μL from suspended cell lysates were collected in glass tubes and analyzed by gas chromatography with flame ionization detection (GC-FID). In brief, a known amount of 1,2-dinonadecanoyl-sn-glycero-3-phosphocholine was added as an internal standard to 150 μl for phospholipid analysis. The lipids were extracted according to a modified procedure of Bligh and Dyer (2 mL dichloromethane, 2 mL methanol and 2 mL 1 % EDTA solution) [25]. After vortexing and centrifuging at 3000 RPM, the dichloromethane layer containing the lipids was collected in a new glass tube. The phospholipid fraction was separated from the other lipid classes by SPE (Solid Phase Extraction). To convert the fatty acids into fatty acid methyl esters (FAME), 80 μl of concentrated sulphuric acid was added to the extracted phospholipids in 4 mL methanol to yield a 2 % concentrated sulphuric acid in methanol solution and heated at 100 °C for 60 minutes [26]. Once cooled, the fatty acid methyl esters were extracted with 2 mL hexane and 0.5 mL 2.5 mol/L sodium hydroxide solution. After vortexing, the upper layer, hexane with FAME, was collected and dried using a SpeedVac®. Dried samples were subsequently dissolved in 80 μl isooctane and analyzed with an in-house validated analysis method using GC-FID (Shimadzu Corporation, Kyoto, Japan) with a CP-SIL88 for FAME column (60 m × 0.25 mm id. 0.20 μm film thickness; Agilent Technologies, Inc., Santa Clara, CA, USA). Fatty acids were identified based on retention time using reference standard GLC-569B (Nu-Chek Prep,Inc., Elysian, MN, USA). The relative concentration of the identified FAME in the samples was calculated via the peak area and total concentration of fatty acids in the sample was calculated via the internal standard.

2.5. Muscle protein synthesis treatment

Cells were washed with 1 mL pre-warmed HBSS and incubated in starvation medium (low glucose DMEM + 3.5g/L glucose) without insulin/leucine for 4 h at 37°C and 5 % CO_2. Thereafter, DMEM + 1 mM leucine and 100 nM insulin or control was added and incubated at room temperature for 30 min. DMEM without I/L trigger was employed as a basal control to confirm the trigger worked. Next, 10 μL puromycin for a total concentration of 300 μM was added and incubated for another 30 min. Cells were washed with PBS and homogenized with 250 μL cell lysis buffer. Cell lysates were collected and centrifuged at 2000 RPM for 5 min at 4 °C. The supernatant was collected for analysis on the Wes™ system.

2.6. Protein Simple Western™ analysis

Incorporation of puromycin into proteins was measured using Simple Western™ analysis on the Wes™ system (ProteinSimple, a Bio-Techne brand, San Jose, USA). Samples were diluted in 0.1 × sample buffer to 0.5 g/L cell lysate and combined with Fluorescent Master Mix in a ratio of 4:1. Samples were vortexed, heated for 5 min at 100 °C and subsequently centrifuged at room temperature for 5 min at 10,000 RPM. Next, 4 μL of sample was loaded onto the WES plate and centrifuged for 5 min at 2500 RPM. The assay was run using the 12–230 kDa Separation Module (ProteinSimple). Anti-puromycin was diluted 5x and loaded on the WES plate and Anti-mouse Detection Module for Wes™ (ProteinSimple) was used as secondary antibody and detection. The run was performed using 30 min separation time, 375 V separation voltage, 30 min antibody diluent time, 30 min primary antibody time and 30 min secondary antibody time. Total area under the curve of puromycin was calculated by the Compass for SW software (ProteinSimple), with ratios to the basal control group calculated as a measure of puromycin incorporation.

2.7. Measurement of mTORC1 pathway proteins 4EBP1, p70S6k, Akt

The phosphorylation status of mTORC1 pathway proteins 4EBP1, p70S6k, and Akt was determined with the Wes™ using the same settings as for MPS (see above). Anti-4EBP1 (10 × diluted) and anti-phospho-4EBP1 $^{(Thr37/46)}$ (10 × diluted) antibodies were used for 4EBP1; anti-p70S6k (10 × diluted) and anti-phospho-p70S6k $^{(Thr421/Ser424)}$ (10 × diluted) antibodies were used for p70S6K1; Akt (pan) (40D4) Mouse mAb and Phospho-Akt $^{(Ser473)}$ (D9E) XP(R) Rabbit mAb were used for Akt. A concentration of 0.313 g/L protein for 4EBP1$^{[Thr37/46]}$ and 0.625 g/L protein for Akt$^{[Ser473]}$ and p70S6K1$^{[Thr421/Ser424]}$ were loaded onto the Wes™. The total peak area was calculated by the Compass for SW software (ProteinSimple), with ratios to the basal control group calculated for each antibody. Signaling data were expressed as the ratio of phosphorylated proteins to unphosphorylated proteins. The 12–230 kDa Separation Module (ProteinSimple) was used for all signaling pathway proteins. The Anti-rabbit Detection Module or Anti-mouse Detection Module (ProteinSimple) was used for detection. The total peak area was calculated by the Compass for SW Software.

2.8. Statistical analysis

All statistical analyses were conducted in R (version 4.3.1). An independent samples Student's *t*-test was used to analyze differences in EPA, DHA and DPA uptake at each timepoint between concentrations. Temporal changes in EPA, DHA and DPA incorporation into C2C12 myotubes, MPS rates and the phosphorylation status of mTORC1

pathway proteins were analyzed using linear mixed-effect models followed by Tukey's post-hoc analysis. Statistical significance was set at $P < 0.05$. Data are expressed as the mean ± standard deviation (SD).

3. Results

3.1. Dose-response of EPA and DHA incorporation into the phospholipid membrane

The proportion of EPA incorporated into the phospholipid membrane was higher in HC compared to LC ($P < 0.05$) at all time points except for 16 h ($P = 0.06$). No changes in EPA incorporation were detected from 8 h onwards after incubation with HC and LC ($P > 0.05$; Fig. 2**A**). EPA incorporation increased from 10.3 ± 0.2 % of total FA profile at 1 h to 18.1 ± 1.9 % at 24 h after incubation with HC, while after incubation with LC, EPA incorporation increased from 8.3 ± 0.8 % of total FA profile at 1 h to 13.0 ± 2.0 % at 24 h. The proportion of DHA incorporated into the phospholipid membrane was similar between HC and LC at all timepoints ($P > 0.05$, Fig. 2**B**). In contrast to EPA, the incorporation of DHA increased moderately during the 24 h period after incubation with HC and LC and did not reach a plateau. DHA incorporation increased from 3.4 ± 0.6 % of total FA profile at 1 h to 14.8 ± 2.8 % at 24 h after incubation with HC, and from 2.8 ± 0.6 % of total FA profile at 1 h to 10.7 ± 2.6 % at 24 h after incubation with LC.

3.2. Dose-response of DPA incorporation into the phospholipid membrane

We assessed temporal changes in DPA expressed as %DPA of total fatty acid profile (Fig. 3), given that previous research indicated the formation of DPA due to EPA supplementation [16]. We observed no detectable incorporation of DPA after incubation with HC. However, we detected a significant incorporation of DPA into the phospholipid membrane after 24 h incubation with LC ($P = 0.01$), whereby DPA levels increased from 2.5 ± 0.5 % of total FA profile at 1 h to 4.3 ± 0.4 % at 24 h.

3.3. Washout of EPA and DHA from the phospholipid membrane

After the overnight incubation period, incorporation of EPA was 16.0 ± 0.3 % of total FA profile in HC and 11.8 ± 0.3 % of total FA profile in LC at the onset of the washout period (t0). Similarly, DHA incorporation was 13.6 ± 0.9 % of total FA profile in HC and 8.5 ± 0.6 % of total FA profile in LC at the onset of the washout period following overnight incubation. Incubation with HC and LC led to sustained EPA and DHA levels above the vehicle control throughout the 24 h washout period ($P < 0.05$; Fig. 4). Additionally, HC resulted in consistently higher EPA and DHA incorporation into the phospholipid membrane compared to LC during the 24 h washout period ($P < 0.05$). After 24 h of washout, EPA levels in the phospholipid membrane were 19.0 ± 0.4 % greater with HC than LC (Fig. 4**A**).Following overnight incubation with HC, EPA levels in the phospholipid membrane decreased after 2 h when switching to the washout medium ($P = 0.02$) and continued to decrease at 16 h ($P < 0.001$). Likewise, EPA levels after overnight incubation with LC decreased 2 h after switching to washout medium ($P = 0.01$), but with no further decline thereafter ($P > 0.05$). No decline in EPA level was observed between 0–8 h in either condition. The overall decline in EPA over 24 h was 2.8 ± 0.6 % after treatment with HC and 0.6 ± 0.3 % after treatment with LC. DHA incorporation into the phospholipid membrane was 136.9 ± 0.5 % greater with HC than LC after a 24 h washout (Fig. 4**B**). Conversely, an increase in DHA incorporation was observed after 8 h when switching to washout medium after overnight incubation

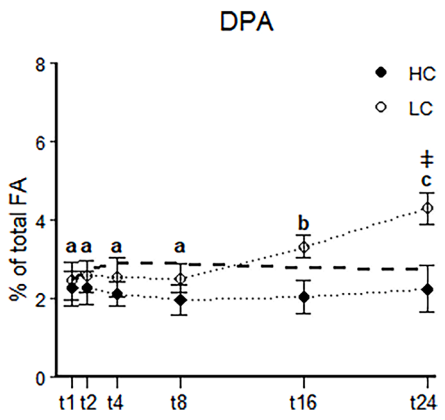

Fig. 3. Temporal (24 h) changes in incorporation of DPA into the phospholipid membrane of C2C12 myotubes following incubation with HC (50 μM EPA + 33.33 μM DHA) or LC (12.5 μM EPA + 8.33 μM DHA) treatments. Values are means ± SD ($n = 3$). Differences from vehicle control (VC; represented by a dashed line) are denoted by '‡' ($P < 0.05$). Mean values that do not share a common letter within HC or LC are statistically different from one another ($P < 0.05$). Levels of DPA did not differ between HC and LC at any timepoint ($P > 0.05$). DPA = docosapentaenoic acid, HC = high concentration, LC = low concentration, FA = fatty acids.

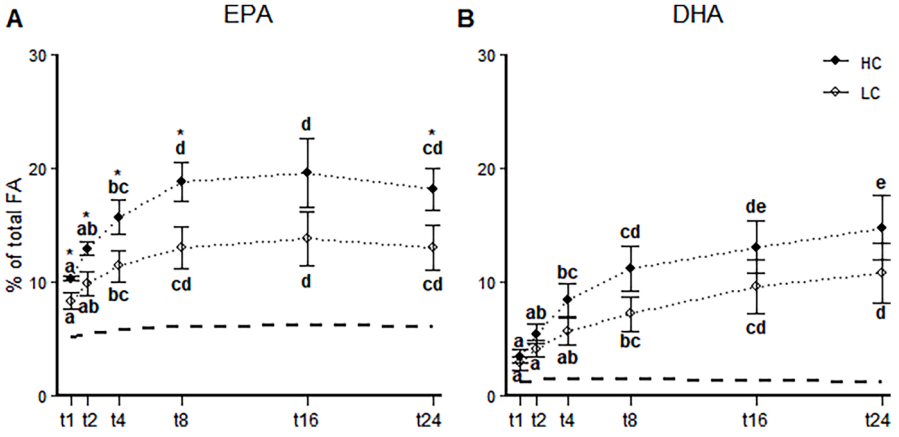

Fig. 2. Temporal (24 h) changes in incorporation of EPA (A) and DHA (B) into the phospholipid membrane of C2C12 myotubes following incubation with HC (50 μM EPA + 33.3 μM DHA) or LC (12.5 μM EPA + 8.33 μM DHA) treatments. Values are means ± SD ($n = 3$). Dashed line represents vehicle control (VC) over time. Mean values that do not share a common letter within HC or LC are statistically different from one another ($P < 0.05$). Significant differences between HC and LC at each timepoint are denoted by an asterisk (*; $P < 0.05$). EPA = Eicosapentaenoic acid, DHA = Docosahexaenoic acid, HC = high concentration, LC = low concentration.

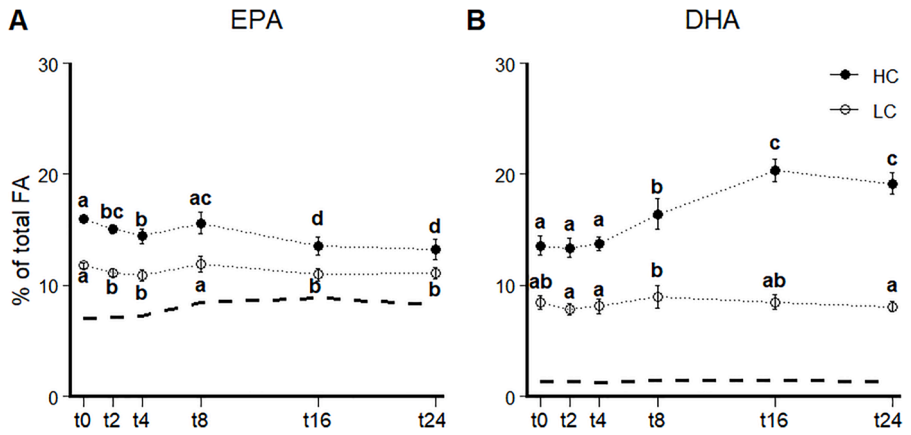

Fig. 4. Temporal (24 h) changes in washout of EPA (A) and DHA (B) from the phospholipid membrane of C2C12 myotubes following overnight incubation with HC (50 μM EPA + 33.33 μM DHA) or LC (12.5 μM EPA + 8.33 μM DHA). Values are means ± SD ($n = 4$, except t16 HC, where $n = 3$). Dashed line represents vehicle control (VC) over time. Mean values that do not share a common letter within HC or LC are statistically different from one another ($P < 0.05$). EPA and DHA levels differed significantly between HC and LC treatments and from VC at all timepoints ($P < 0.05$). EPA = Eicosapentaenoic acid, DHA = Docosahexaenoic acid, HC = high concentration, LC = low concentration, FA = fatty acids.

with HC ($P < 0.001$), and further increased between 8–16 h ($P < 0.001$). After incubation with LC, no differences in DHA incorporation in the phospholipid membrane were detected from the start of the washout period ($P > 0.05$).

3.4. Formation of DPA during washout in response to EPA:DHA supplementation

We observed an increase in DPA levels between 1–8 h of washout ($P < 0.001$; Fig. 5) in both concentrations, and an increase in DPA in the vehicle control from 16 h onwards compared to start of washout ($P < 0.001$). DPA incorporation differed at all timepoints between HC and LC ($P < 0.05$). Compared to VC, DPA levels were lower following treatment with HC after 0, 2 and 4 h after washout ($P < 0.05$), with no differences after 8 h ($P > 0.05$). No additional DPA formation compared to VC was observed at any timepoint after incubation with LC ($P > 0.05$).

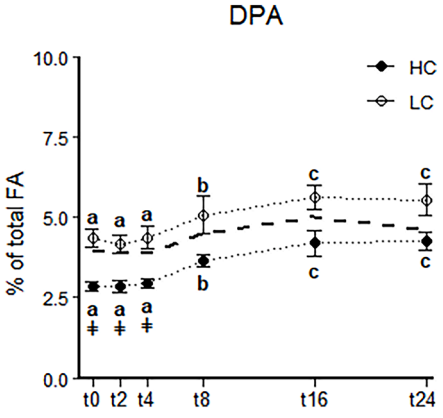

Fig. 5. Temporal (24 h) changes in incorporation of DPA during the washout period in the phospholipid membrane of C2C12 myotubes following overnight incubation with HC (50 μM EPA + 33.33 μM DHA) or LC (12.5 μM EPA + 8.33 μM DHA). Values are means ± SD ($n = 4$, except t16 HC, where $n = 3$). Differences from vehicle control (VC; represented by a dashed line) are denoted by '‡' ($P < 0.05$). Mean values that do not share a common letter within HC or LC are statistically different from one another ($P < 0.05$). Levels of DPA differed at all timepoints between HC and LC ($P < 0.05$). DPA = docosapentaenoic acid, HC = high concentration, LC = low concentration, FA = fatty acids.

3.5. Protein synthesis rates in response to an insulin/leucine stimulus

The insulin/leucine stimulated condition significantly upregulated protein synthesis rates and the phosphorylation status of mTORC1 pathway proteins compared to the basal control after the 16 h incorporation and 24 h washout period ($P < 0.05$), with the exception of 4EBP1$^{[Thr37/46]}$ phosphorylation after the 24 h washout period, whereby no difference was detected between conditions (**Supplemental Figure 1**). We report no further increase in MPS after 16 h of incubation with HC or LC compared to VC ($P > 0.05$; Fig. 6**A**). Moreover, there were no differences in the phosphorylation status of 4EBP1$^{[Thr37/46]}$, p70S6K1$^{[Thr421/Ser424]}$, or Akt$^{[Ser473]}$ after 16 h of incubation with HC or LC (Fig. 6**C, E and G**). The LC condition exhibited a 19.7 ± 1.2 % higher MPS rate compared to VC after the 24 h washout period ($P = 0.01$; Fig. 6**B**). However, there were no differences in the phosphorylation status of 4EBP1$^{[Thr37/46]}$, p70S6K1$^{[Thr421/Ser424]}$, or Akt$^{[Ser473]}$ after 24 h washout (Fig. 6**D, F** and **H**).

4. Discussion

This *in vitro* study examined the incorporation and washout profiles of EPA and DHA in the phospholipid membrane of C2C12 myotubes during incubation with a low or high concentration EPA:DHA mixture, in combination with measurements of stimulated rates of MPS and mTORC1 signaling. The study revealed four main findings. First, the higher concentration EPA:DHA mixture resulted in a greater incorporation of EPA into the phospholipid membrane over the 24 h incubation period in C2C12 myotubes, with no differences in DHA incorporation between HC and LC treatments. Second, EPA and DHA exhibited a distinct temporal pattern of uptake and washout, whereby a plateau in EPA uptake was observed after 8 h of incubation and exhibited a partial washout, whereas DHA uptake increased over the entire 24 h incubation period in both conditions, and during washout in HC. Third, DPA formation was observed after 24 h of incubation in LC, whereas pre-incubation with HC inhibited DPA formation during the washout phase. Finally, incubation with LC followed by a 24 h washout resulted in increased insulin/leucine stimulated MPS rates above VC, albeit independently of changes in the phosphorylation status of 4EBP1$^{[Thr37/46]}$, p70S6K1$^{[Thr421/Ser424]}$, and Akt$^{[Ser473]}$. Taken together, these *in vitro* data indicate a finite effect of EPA:DHA incorporation into the phospholipid membrane in upregulating the stimulation of MPS in C2C12 myotubes.

Our cell culture model revealed a dose-dependent, *n*-3 PUFA-specific, pattern of *n*-3 PUFA uptake into the phospholipid membrane

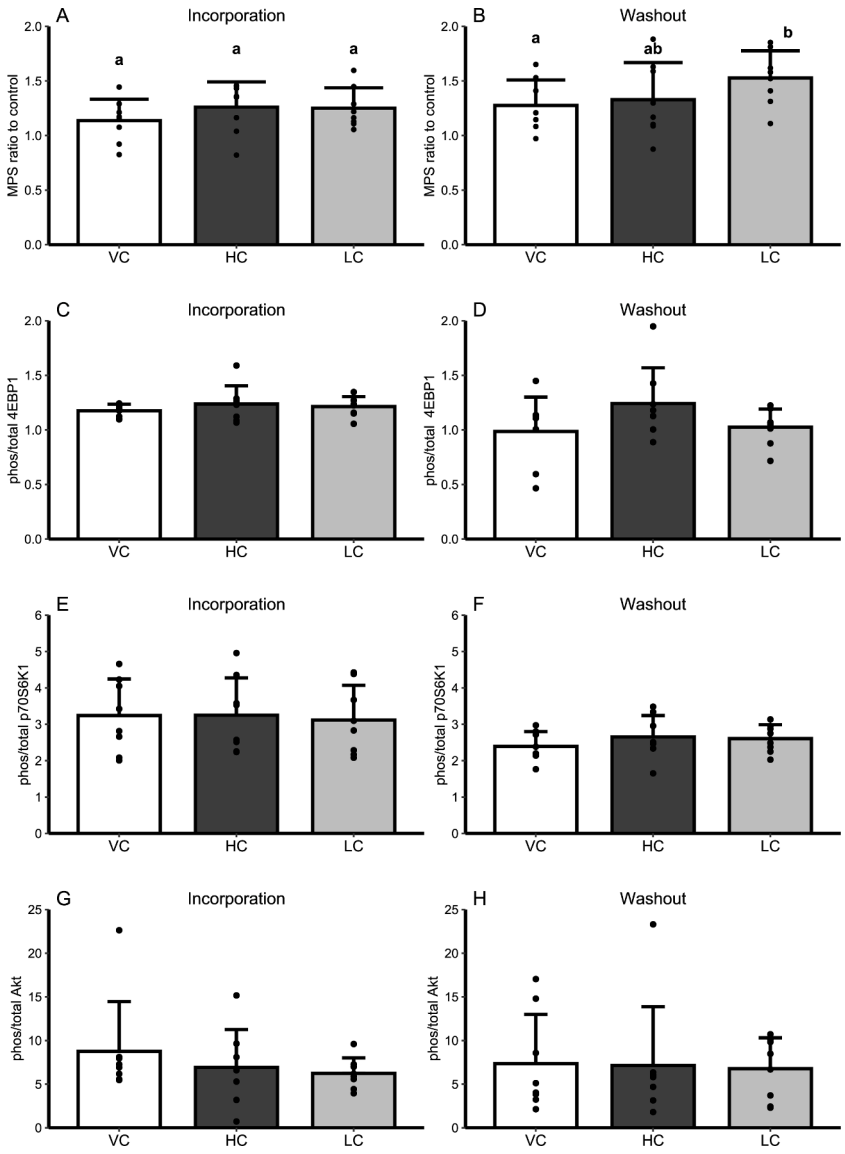

Fig. 6. Protein synthesis following 16 h incorporation (A) or 24 h washout (B) and phosphorylation status of signaling pathway proteins following 16 h incorporation (C, E, G) or 24 h washout (D, F, H) in C2C12 myotubes after incubation with VC (vehicle control), HC (50 μM EPA + 33.33 μM DHA) or LC (12.5 μM EPA + 8.33 μM DHA). Data represents protein synthesis relative to basal control (A, B) and phosphorylated/total 4EBP1$^{[Thr37/46]}$, p70S6K1$^{[Thr421/Ser424]}$, and Akt$^{[Ser473]}$ (C, D, E, F, G, H). Values are means + SD ($n = 4$ in duplicate). Mean values that do not share a common letter are statistically different from one another ($P < 0.05$). MPS = Muscle Protein Synthesis. EPA = Eicosapentaenoic acid, DHA = Docosahexaenoic acid, HC = high concentration, LC = low concentration. 4EBP1 = eukaryotic translation initiation factor 4E-binding protein 1. Akt = Protein Kinase B. p70S6K1 = ribosomal protein S6 kinase beta-1.

of C2C12 myotubes and indicate that provision of a high dose EPA:DHA mixture elicits a greater uptake of EPA into the muscle phospholipid membrane, with no apparent dose effect of *n*-3 PUFA provision on DHA uptake into the phospholipid membrane fraction. The differential incorporation and washout profile of *n*-3 PUFA with EPA + DHA incubated was likely mediated, at least in part, by the distinct lipid structures of EPA and DHA and variable distributions within the phospholipid membrane. Consistent with this notion, EPA is preferentially incorporated into phosphatidylcholine species of erythrocytes that are primarily located on the outer segment of the membrane [27]. In contrast, DHA is preferentially incorporated into phosphatidylethanolamine species located on the inner segment of the phospholipid membrane and requires the transport of DHA across the cellular membrane for incorporation [28,29]. These divergent physical properties of *n*-3 PUFA species may explain the slower incorporation of DHA into the cellular membranes of skeletal muscle cells [29]. Moreover, it is plausible that the distinct structural characteristics of EPA and DHA also play a role in modulating the washout profile of *n*-3 PUFA. Accordingly, multiple human studies have investigated the washout profile of *n*-3 PUFA in erythrocytes [10,17,18], whole blood [18], and plasma [17–20] and revealed a more rapid washout of EPA compared to DHA. In humans, the skeletal muscle washout profile of EPA and DHA in response to different doses of *n*-3 PUFA are yet to be characterized. However, in the present *in vitro* study we observed a similar washout pattern for EPA between dose

conditions. Despite a more pronounced decline in absolute EPA levels, the incorporation of EPA into the phospholipid membrane remained higher in HC compared to LC, indicating that a higher *n*-3 PUFA concentration is required to preserve the EPA content of the skeletal muscle phospholipid membrane.

In contrast to EPA, no decline in DHA was observed during the washout period in the present experiment. In fact, we observed an increase in the muscle phospholipid membrane content of DHA during the washout period in HC. One plausible explanation for this increase in DHA content during the washout period relates to the observation that EPA undergoes metabolic conversion to DHA [30]. While several studies in humans have reported a limited conversion rate of EPA to DHA in plasma and erythrocyte phospholipids following EPA supplementation [31,32], a recent tracer study demonstrated an increase in plasma DHA level that was primarily attributed to exogenous EPA supplementation [33]. Moreover, an *in vitro* study demonstrated that incubation of C2C12 myotubes with 50 μM EPA for 72 h failed to elevate DHA levels in the phospholipid membrane [16]. However, it is plausible that the EPA: DHA mixture administered in the present study facilitated an increased DHA content during the washout period by converting a proportion of EPA to DHA. Interestingly, the amount of DHA formed during the washout is not linear to the loss of EPA from the phospholipid membrane observed during the washout period. Hence, it is plausible that the EPA loss from other cellular fractions, rather than the phospholipid membrane, are elongated, desaturated and β-oxidized to DHA, leading to increased DHA being observed in the phospholipid membrane during the washout phase in our study. However, since we did not use compound specific isotope analysis in our experiment, we cannot confirm this hypothesis. Future tracer studies in muscle tissue and C2C12 myotubes are warranted to confirm this notion. Nevertheless, our data indicate that the metabolic interplay between EPA and DHA modulates their incorporation and washout profiles, and this process appears to be influenced by the administered dose of *n*-3 PUFA.

The formation of DPA following EPA administration has previously been demonstrated in C2C12 myotubes [16] and the skeletal muscle tissue of tumor-bearing mice [34]. Consistent with these observations, we observed an increase in DPA formation after 24 h of incubation with LC whereas DPA formation was negligible in HC. Although speculative, we suggest that competition between EPA and DHA in HC could explain this observation, suggesting that the accumulation of DHA impairs the conversion of EPA to DPA [29]. Accordingly, findings from tracer studies in rodents [35] and humans [33] suggest that DHA accumulation mediates a reduced conversion of EPA to DPA. Moreover, a recent comprehensive study from the same group confirmed that increasing DHA levels impaired elongation of EPA to DPA, as mediated by a negative feedback system via the elongation enzyme ELOVL2 [36]. Taken together, these findings suggest that the higher *n*-3 PUFA concentration provided in our study may have led to competition between EPA and DHA, slowing down the conversion of EPA to DPA and DHA. Moreover, in the present study DPA accumulation was blunted during the initial 4 h period in HC which further supports the hypothesis that higher DHA levels impair the conversion of EPA to DPA.

The greater incorporation of EPA and DHA into the muscle phospholipid membrane with HC did not translate to a further increase in stimulation of MPS beyond VC. Instead, we observed an increased stimulation of MPS after 16 h of incorporation followed by a 24 h washout in the LC condition only. A potential explanation for this observation relates to the observation that the total concentration of EPA + DHA in HC induced cellular stress, as evidenced by visual inspection of protein content. Consequently, this increased stress response may have negated any increased stimulation of MPS. In the present study, we administered a combination of EPA + DHA to simulate the *n*-3 PUFA content of common fish oil supplements administered in human studies [37]. Conversely, previous *in vitro* studies reported an increase in MPS and suppression of muscle protein breakdown [21] or increased protein accretion [16] after incubation with 50 μM EPA, whereas DHA had no effect. Hence, we postulate that the higher dose of DHA in addition to EPA in HC in our study attenuated the independent effect of EPA on muscle protein metabolism and that there may be interplay and/or competition between EPA and DHA in terms of regulating muscle protein metabolism. Moreover, we observed an increased stimulation of MPS after the 24 h washout phase, albeit not in response to the 16 h incorporation phase. Further analysis of our incorporation data revealed that the compositional profile of EPA + DHA in LC after the 24 h washout period was similar to the 16 h incorporation period, but with a marked rise in DPA levels. Although EPA is commonly regarded as the most bioactive *n*-3 PUFA species in terms of stimulating MPS [37], our data suggest that the presence of DPA in the skeletal muscle membrane in addition to EPA could modulate the stimulation of MPS. Taken together, the greater MPS response compared with VC following the 24 h washout phase with LC incubation may be attributed to factors such as the cumulative quantity of *n*-3 PUFA administered, the potential interaction between EPA and DHA that are likely less pronounced in LC, or the increased formation of DPA, which was markedly higher after LC compared to HC.

The mTORC1 pathway consists of cell signaling proteins known to regulate the stimulation of MPS [38]. Despite observing an increased stimulation of MPS after the 24 h washout period in LC, we failed to detect any changes in the phosphorylation status of 4EBP1$^{[Thr37/46]}$, p70S6K1$^{[Thr421/Ser424]}$, or Akt$^{[Ser473]}$. This disassociation between molecular signals that regulate MPS has also been observed in previous *in vitro* studies [16,21]. In the present study, a possible explanation for the increased stimulation of MPS in the absence of an upregulation of mTORC1 signaling relates to the notion that incorporation of EPA and DHA after incubation with LC may have accelerated or optimized signaling. Accordingly, we previously demonstrated that a lower and more efficient kinase activity was required to maximize MPS following an 8-week period of fish oil supplementation [23]. These findings indicate that fish oil supplementation results in a shift in kinase signaling and MPS, suggesting that a reduced kinase activity is required to maximize MPS. This observation could explain, at least partially, the disconnect between the phosphorylation status of signaling proteins and MPS rates observed in our C2C12 myotubes, however this hypothesis warrants further investigation.

We acknowledge that static measurements of kinase activity at a single time-point likely resulted in missing some peak readouts of the kinase activity. We measured MPS and associated kinase phosphorylation 30 minutes after exposure to an insulin/leucine trigger, following a 16 h incorporation + 24 h washout period with EPA and DHA. While the time-course of elevated MPS post anabolic trigger has been investigated in humans [39], our reliance on a single time point within our *vitro* model may have limited our potential to capture the optimal kinase signaling window in response to EPA and DHA.

It is important to acknowledge that the observed incorporation and washout profile of EPA and DHA, and subsequent stimulation of MPS and molecular signaling, are confined to a mouse muscle cell model, and may not directly translate to human skeletal muscle. The concentrations of EPA and DHA utilized *in vitro* studies range between 1–750 μM [40], and the shift in relative fatty acid profile observed in our C2C12 myotubes exceeded those observed in a human population [37]. Moreover, given the current recommendation to consume at least one portion of oily *n*-3 PUFA rich fish per week [41], it is noteworthy that intermittent eating patterns deviate significantly from the practice of constant daily consumption [42], emphasizing the importance of studying the washout profile of *n*-3 PUFA species in human skeletal muscle. Furthermore, the focus of the present study was on MPS rather than muscle protein breakdown. Previous cell-based investigations in C2C12 myotubes have revealed a metabolic action of EPA [16,21,24] and DHA [24] in attenuating muscle protein breakdown rates. Taken together in combination with findings from other animal [43,44] and cell [45] studies, this observation emphasizes a potential role for *n*-3 PUFA in regulating muscle protein breakdown. Future investigations are warranted to

investigate the time-dependent uptake and washout dynamics of EPA, DHA, DPA, and related lipid classes in human skeletal muscle. Additionally, it is important to examine the influence of different EPA + DHA concentrations on subsequent measurements of MPS and anabolic cell signaling pathways.

In conclusion, our data demonstrate that treatment with higher concentrations of EPA and DHA resulted in a greater incorporation of *n*-3 PUFA into the muscle phospholipid membrane and led to a greater preservation of *n*-3 PUFA following washout, with divergent patterns for EPA and DHA. However, only treatment with the low concentration of EPA and DHA stimulated increased rates of MPS without modulating the phosphorylation status of mTORC1 signaling proteins. The distinct dynamics of EPA and DHA in altering muscle cell lipid composition have the potential to impact physiological outcomes related to muscle protein turnover, with possible clinical implications for the prescription of omega-3 polyunsaturated fatty acid supplementation protocols to promote human health.

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Data availability

Data presented in this study are available on request from the corresponding author upon reasonable request.

CRediT authorship contribution statement

M. Banic: Conceptualization, Validation, Formal analysis, Investigation, Writing – original draft, Visualization, Project administration. **M. van Dijk:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration. **F.J. Dijk:** Methodology, Validation, Investigation, Writing – review & editing. **M.J.W. Furber:** Conceptualization, Resources, Writing – review & editing, Funding acquisition. **O.C. Witard:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition. **N. Donker:** Validation, Investigation, Writing – review & editing. **M.J.A. Becker:** Validation, Investigation, Writing – review & editing. **S.D. Galloway:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition. **N. Rodriguez-Sanchez:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors have no relevant conflicts of interest to disclose. F.J.D., M.v.D., M.J.W.F, and M.J.A.B., are employees of Danone Global Research & Innovation Center B.V., Utrecht, the Netherlands.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.plefa.2024.102651.

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