

The impact of omega-3 polyunsaturated fatty acids on skeletal muscle regeneration in aged mice

Cassandra Chabert

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Department of Biochemistry, Microbiology, and Immunology
Faculty of Medicine
University of Ottawa

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Table of contents

Table of contents	2
Abstract	5
Résumé	6
Acknowledgments	7
List of tables	8
List of figures	8
List of abbreviations	10
Introduction	1
Adult skeletal muscle regeneration and repair	2
Skeletal muscle regeneration in aged individuals	5
Intrinsic changes in aged MuSCs	6
Age-related extrinsic factors affecting skeletal muscle regeneration	10
Potential therapies to improve aged skeletal muscle regeneration	13
Polyunsaturated fatty acids	15
Hypothesis	20
Materials and methods	21
C2C12 immortalized cell culture	21
Primary murine muscle stem cell isolation and culture	21
Real-time proliferation assay	22
Proliferation assay	22
Differentiation assay	23
MTS cell viability assay	23
Senescence assay	24
Animal care	24
Cardiotoxin injury	26
Body composition	26
Forelimb grip strength test	27
Contractile properties	27
Muscle freezing and blood collection	28
Immunofluorescence	28
Hematoxylin and Eosin Staining	30
Oil Red O staining	31

Picrosirius Red staining	31
Western blot.....	32
Cytokine and chemokine multiplex assay	34
Oxylipin analysis	34
Statistical analysis and figures.....	35
Results	36
1.0 Visual representation of <i>in vitro</i> experiments and workflow	36
1.1 Proof-of-concept experiments using C2C12 myoblasts	37
2.1 Aged myoblasts have a diminished proliferation capacity	38
2.2 DHA may improve proliferation capacity in aged myoblasts	39
3.1 DHA does not impact differentiation capacity on aged myoblasts	40
4.0 Visual representation of <i>in vivo</i> experiments and workflow	41
5.0 Effect of an enriched DHA diet on food consumption and mouse body composition	42
5.1 Assessment of muscle morphology	43
5.2 Effect of enriched DHA diet on early and late myogenic markers.....	46
5.3 Effect of enriched DHA diet effect on inflammation in the muscle	49
5.4 Enriched FA diet effect on metabolism	52
5.5 Effect of an enriched DHA diet on muscle contractile properties	54
6.1 DHA-enriched diet may affect muscle composition.....	57
Discussion	59
1.0 Effect of n-3 PUFAs on the Inflammatory Response.....	59
2.0 Effect of n-3 PUFAs on Skeletal Muscle Regeneration	62
3.0 Limitations and Future Directions	69
Conclusion.....	70
Appendix A: Supplementary Material	72
1.0 Cell culture media.....	72
1.1 Aged myoblast proliferation supplementary and supporting data.....	73
2.0 Specialized diets	74
2.1 Whole tissue sections	74
2.2 Immuno-regulation at the local and systemic level	76
3.0 Oxylipins cohort supporting data	76
Appendix B: Supplementary materials and methods	77
1.0 Image acquisition for immunofluorescence imaging	77
2.0 Image acquisition for brightfield imaging.....	77

3.0 Image acquisition for polarized light imaging.....	78
4.0 Representative images	78
References	79

Abstract

Skeletal muscle regeneration is a tightly regulated process mediated by the immune system and muscle stem cells (MuSCs). In aged skeletal muscle tissue, chronic low-grade inflammation drives maladaptive tissue remodeling. Currently, there are no approved therapies to attenuate age-related skeletal muscle disorders, leaving the elderly susceptible to loss of muscle mass/function, fragility, and a decline in quality of life. Omega-3 polyunsaturated fatty acids (n-3 PUFAs) are bioactive lipids with anti-inflammatory properties, primarily stemming from their metabolites. In this study, we have shown that n-3 PUFAs have a direct effect on myoblasts *in vitro*, and we have shown the effect of an enriched n-3 PUFA diet on skeletal muscle regeneration *in vivo* using a cardiotoxin injury model. Our results suggest that an enriched n-3 PUFA diet may increase anti-inflammatory markers in the blood, but the effect on inflammation in the tissue is limited, with the exception of inter-fiber lipid deposition.

Résumé

La régénération du muscle squelettique est un processus impliquant l'interaction entre le système immunitaire et les cellules souches musculaires (MuSC). En vieillissant, ce processus délicat se dérégule à cause de l'inflammation chronique. Actuellement, il n'existe aucun traitement approuvé pour atténuer les troubles musculo-squelettiques liés à l'âge, ce qui rend les personnes âgées vulnérables à une perte de masse et de fonction du muscle. Les acides gras polyinsaturés omega-3 (n-3 PUFAs) sont des lipides bioactifs qui produisent des effets anti-inflammatoires qui proviennent principalement de leurs métabolites (SPMs). Dans cette étude, nous avons démontré que les n-3 PUFAs ont un effet direct sur les myoblasts *in vitro* et nous avons démontré l'effet d'un régime enrichi en n-3 PUFAs sur la régénération du muscle squelettique *in vivo*. Nos résultats suggèrent qu'un régime riche en n-3 PUFAs augmente les marqueurs anti-inflammatoires sanguins, mais son effet sur l'inflammation tissulaire est limité, à l'exception d'une réduction du dépôt lipidique interfibre.

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Statement of contributions: Ryan Arsenault isolated and grew the primary myoblasts into their respective cell lines and contributed to the collection of the muscles during animal sacrifices. Anthony Ziadeh helped with the grip/strength test and also contributed to the collection of the muscles during animal sacrifices. I certify that all work is my own and that this thesis was written on my own, with feedback from Dr. Junio Dort.

List of tables

Table S1. Composition of cell culture media used for in vitro experiments	72
Table S2. Food composition of specialized diets, adapted from Inotiv (previously Teklad).	74

List of figures

Figure 1. Skeletal muscle architecture and organization	2
Figure 2. Adult skeletal muscle regeneration process and associated immuno-regulation	5
Figure 3. Skeletal muscle regeneration changes with aging.	12
Figure 4. Visual summary of in vitro experimental methods relating to aim 1 of this project.....	36
Figure 5. In vitro modelling of C2C12 myoblasts after 4 days of differentiation treated with either vehicle (abs. EtOH), alpha-linolenic acid (ALA, 50 nM), or docosahexaenoic acid (DHA, 50 nM).	38
Figure 6. Myoblast proliferation on untreated primary cells	39
Figure 7. Effect of DHA (50 nM) versus vehicle (abs. EtOH) on proliferation in aged myoblasts.....	40
Figure 8. Effect of DHA (50 nM) on differentiation of aged myoblasts	41
Figure 9. Schematic representations of two <i>in vivo</i> experiments completed.....	42
Figure 10. Effect of enriched DHA diet versus isoenergetic SBO diet on food consumption, body mass, and body composition in aged male and female mice	43
Figure 11. Histological and immunofluorescent validation of CTX injury at 21 DPI in male and female aged mice	44
Figure 12. Effect of DHA enriched diet versus isoenergetic SBO on CSA and minimum Feret's diameter in aged mice	45
Figure 13. Effect of DHA enriched diet versus isoenergetic SBO diet on CNF and eMyHC ⁺ fibers in aged mice.....	46
Figure 14. Effect of DHA enriched diet on early and late myogenic markers in aged mice at 21 DPI.....	47
Figure 15. Effect of DHA enriched diet versus isoenergetic SBO diet on proliferative capacity and distribution of myogenic markers in aged mice	49
Figure 16. Effect of DHA enriched diet versus isoenergetic SBO diet on tissue-resident macrophages in aged mice.....	50
Figure 17. Effect of DHA enriched diet versus isoenergetic SBO diet on neutrophils in aged mice.....	51

Figure 18. Effect of DHA enriched diet versus isoenergetic SBO diet on fibrosis in aged mice.....	52
Figure 19. Effect on myofiber type and distribution in DHA enriched diet versus isoenergetic SBO diet in aged mice.....	53
Figure 20. Effect of DHA enriched diet versus isoenergetic SBO diet on fatty lipid infiltration in aged mice.....	54
Figure 22. Oxylin concentration at 14 and 21 DPI in mice	58
Figure S1. Supporting data for aged myoblasts in proliferation media	73
Figure S2. Immunofluorescent staining and quantification of myofiber types (aged myoblasts)	73
Figure S3. Whole tissue cross-sections of fiber-type staining (21 DPI).	74
Figure S4. Picrosirius red tissue cross-sections	75
Figure S5. Validation of ORO kit.	75
Figure S6. Immuno-markers at the local and the systemic level in aged male mice 21 DPI.	76
Figure S7. Contractile and histological data for oxylin cohort.	76

List of abbreviations

ABBREVIATION	DEFINITION
13-HOTYEG	13-hydroxy-octadecatrienoic gamma-linolenic acid
14, 15 DIHETE	14,15-dihydroxy-5Z,8Z,11Z,17Z-eicosatetraenoic acid
15_KPGE2	15_keto prostaglandin E2
15_KPGE2A	15_keto prostaglandin E2 alpha
9,10_DIHOME	9,10-dihydroxy-12Z-octadecenoic acid, leukotoxin acid
9-HOTYE	9-hydroxy-octadecatrienoic acid
ABS. ETOH	Absolute ethanol
AGES	Advanced glycation endproducts
AKT	Protein kinase B
ALA	Alpha-linolenic acid
AMPK	Adenosine monophosphate (AMP) activated protein kinase
ARA	Arachidonic acid
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CCAC	Canadian Council on Animal Care
CD206	Macrophage mannose receptor
CNF	Centrally nucleated fibers
CO2	Carbon dioxide
COX	Cyclooxygenase
CSA	Cross-sectional area
CTX	Cardiotoxin
CYP450	Cytochrome P450
DAMPS	Damage-associated molecular patterns
DAPI	4',6-diamidino-2-phenylindole; Hoechst 33342
DH2O	Distilled water
DHA	Deoxyribonucleic acid
DI	Deionized water
DMD	Duchenne Muscular Dystrophy
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
D-PBS	Distilled-phosphate-buffered saline
DPI	Days post injury
DSHB	Developmental Studies Hybridoma Bank
ECM	Extracellular matrix
EMYHC	Embryonic myosin heavy chain
EPA	Eicosapentaenoic acid
ETOH	Ethanol
FAPS	Fibro-adipogenic progenitors
FBS	Fetal bovine serum

FGF	Fibroblast growth factor
FI	Fusion index
FO	Fish oil
GAS	Gastrocnemius muscle
GPR	G-coupled receptor protein
H&E	Hematoxylin and eosin
HCL	Hydrochloric acid
HRP	Horseradish peroxidase
HS	Horse serum
IF	Immunofluorescence
IFNB	Interferon beta
IFNY	Interferon gamma
IGG	Immunoglobulin G
IL	Interleukin
IM	Intramuscular
IP-10	Interferon γ -induced protein 10 kDA
IP	Intraperitoneal injection
KC	Casein protein
Lo	Optimal muscle length
LCMS⁺	Liquid chromatography mass spectrometry
LOX	Lipoxygenase
LY6G	Lymphocyte antigen 6 complex locus G6D
MAPK	Mitogen-activated protein kinases
MCP-5	Murine monocyte chemoattractant protein-5
MDC	Macrophage-derived chemokine
MOM	Mouse-on-Mouse
MRFS	Myogenic regulatory factors
MTORC	Mammalian target of rapamycin
MUSCS	Muscle stem cells
MYF5	Myogenic factor 5
MYHC	Myosin heavy chain
MYOD	Myoblast determination protein 1
N-3 PUFAS	Omega-3 polyunsaturated fatty acids
N-6 PUFAS	Omega-6 polyunsaturated fatty acids
NF-KB	Nuclear factor kappa B
NMJ	Neuromuscular junction
NMR	Nuclear magnetic resonance
NSAID	Non-steroidal anti-inflammatory drugs
OCT	Optimal cutting temperature compound
ORO	Oil red O
PAX7	Paired box 7
PBS	Phosphate buffered solution
PDGF-A	Platelet derived growth factor alpha

PFA	Paraformaldehyde
PGE2	Prostaglandin E2
PGF2-ALPHA	Prostaglandin F2 alpha
PPARY	Peroxisome proliferator-activated receptor gamma
PVDF	Polyvinylidene fluoride (membrane)
RI	Refractive index
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
ROS	Reactive oxidative species
RVD1	Resolvin D1
RVD2	Resolvin D2
RVE1	Resolvin E1
SA B-GAL	Senescence associated beta-galactosidase
SASP	Senescence-associated secretome pathway
SBO	Soybean oil
SDS	Sodium dodecyl sulfate
SEM	Standard error mean
SOL	Soleus (muscle)
SPMS	Specialized pro-resolving mediators
TA	Tibialis anterior
TARC	Thymus and activation-regulated chemokine
TBST	Tris-buffered saline with Tween 20
TGF-B	Transforming growth factor beta
TNF-A	Tumor necrosis factor alpha
UPLC	Ultra-performance liquid chromatography
UPLC-MS/MS	Ultra-performance liquid chromatography-tandem mass spectrometry
VEGF	Vascular endothelial growth factor
WNT	Wingless-related integration site
WT	Wild type

Introduction

The skeletal muscle accounts for ~40% of total body mass in healthy adults¹⁻³ and supports the human body through mundane activities from walking, breathing, and maintaining proper posture. The skeletal muscle is composed of multinucleated fibers, called myofibers, which are bundled into fascicles (**Figure 1**). Between the myofiber and basal lamina rests the muscle stem cells (MuSCs), also known as satellite cells, which typically remain dormant in adulthood^{4,5}. The muscle fascicles are surrounded by the tissue microenvironment, which is comprised of various cell types, including tissue-resident macrophages, adipocytes, and fibro-adipogenic progenitors (FAPs)⁶. The microenvironment also contains blood capillaries, which permit nutrients, hormones, and growth factors to be secreted within the tissue⁷⁻⁹, as well as the extracellular matrix (ECM), which itself contains proteins (e.g., collagen), sugars, ribonucleic acid (RNA), and offers structural support for the skeletal muscle^{10,11}. In adulthood, the skeletal muscle fibers are mature and remain plastic; skeletal muscle regeneration is triggered through the detection of damage-associated molecular patterns (DAMPs)^{12,13}, which can occur after an injury or after exercise^{5,14,15}. Skeletal muscle regeneration is compromised in elderly individuals⁵. In particular, chronic low-grade inflammation leads to dysregulated tissue repair, which contributes to the decline of muscle mass and the loss of muscle function^{1,9}. Omega-3 polyunsaturated fatty acids (n-3 PUFAs) are bioactive lipids that have previously been shown to limit excessive pro-inflammatory signaling through the production of specialized pro-resolving mediators (SPMs)¹⁶⁻¹⁸, and may be a promising therapeutic avenue to attenuate age-related skeletal muscle disorders¹⁹⁻²². Given the extensive role of the skeletal muscle and the increase in life expectancy¹², limiting chronic inflammation in the skeletal muscle during aging is essential to retain a good quality of life.

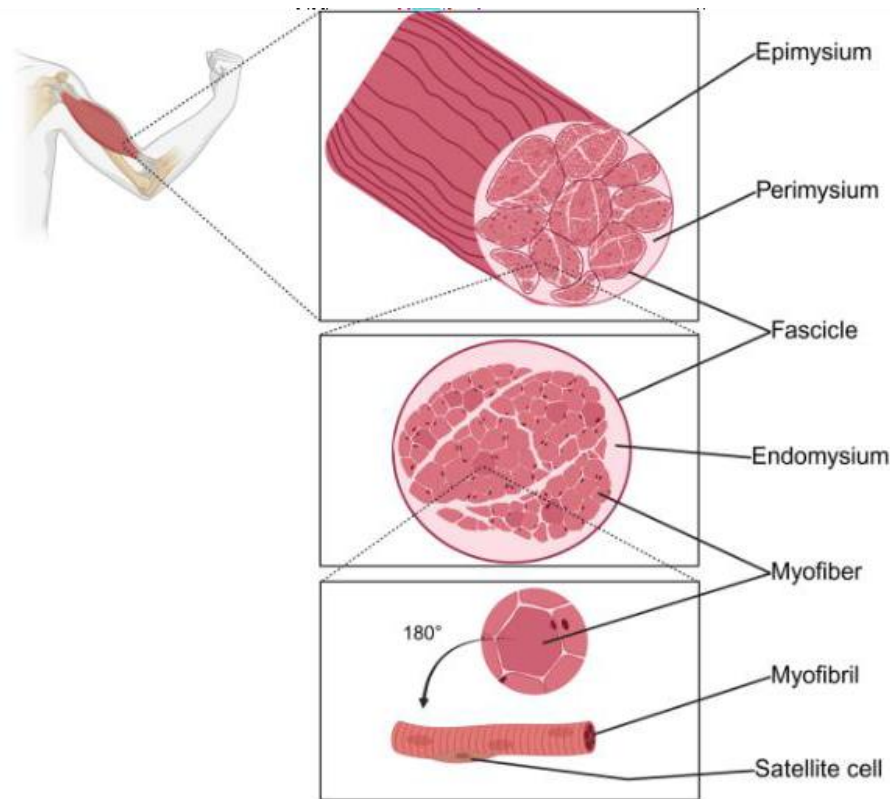


Figure 1. Skeletal muscle architecture and organization. The skeletal muscle epimysium is linked to a bone through a tendon (not shown). The epimysium protects the perimysium and the muscle fascicles, containing bundles of myofibers and the endomysium. Each myofiber within a fascicle contains myofibrils (muscle contractile unit) accompanied with satellite cells, located at the periphery of the fiber, among other cell types (e.g., FAPs, tissue-resident immune cells; not shown). Figure was made using BioRender.

Adult skeletal muscle regeneration and repair

In adulthood, skeletal muscle regeneration is initiated after there is an injury to the tissue. Damaged myofibers become necrotic, leading to the release of DAMPs (e.g., nitric oxide, heat-shock proteins, ECM fragments)^{5,13,15,23-25}. Upon DAMP detection, neutrophils (Ly6G⁺) are recruited and infiltrate the tissue, acting as the first responders in the inflammatory response^{25,26}. Neutrophils phagocytose cellular debris caused by the assault^{24,27} and release various factors (e.g., fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), and monocyte chemoattractant protein-1), allowing the continuation of the immune response cascade post-injury. Neutrophils are

typically cleared 4 days post-injury (DPI)^{24,25,27,28}, either by re-entering circulation or by undergoing apoptosis; failure clearing them may result in secondary damage to the tissue^{27,29,30}.

Shortly after neutrophils enter the site of injury, they stimulate tissue-resident macrophages and attract monocytes from the bloodstream (Ly6C^{high}), which are differentiated into M1 macrophages^{29,31} (CD68⁺ in humans, F4/80⁺ in mice). M1 macrophages (or classically activated macrophages) release pro-inflammatory molecules, including interleukin (IL)-6, tumor necrosis factor alpha (TNF- α), and prostaglandin E₂ (PGE 2), while simultaneously phagocytosing cellular debris^{26,32,33}. At the clinical level, the inflammatory response may exacerbate pain, swelling, and redness to the area of injury, which may be alleviated through consumption of non-steroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen. NSAIDs decrease pro-inflammatory signaling through inhibition of cyclooxygenase (COX)-2 enzymatic activity. Paradoxically, macrophage shift from their M1 pro-inflammatory state to their M2 anti-inflammatory state (pro-resolving state) and resolution of inflammation are partially mediated by the endogenous production of SPMs—molecules that are produced through COX enzymatic pathways^{28,34-36}.

Other notable cells involved in adult skeletal muscle regeneration include FAPs, denoted by the platelet-derived growth factor receptor alpha (PDGFR- α ⁺) marker, which are recruited by M2 macrophages, effectively overlapping muscle regeneration with the immune response^{37,38}. FAPs and M1 macrophages work together to activate the quiescent satellite cells (Pax7⁺MyoD⁻); satellite cells are typically dormant in adulthood and have low metabolic activity³⁹, which increases upon their activation and proliferation, and consequently causes deacetylation of histone H4 (e.g., increased accessibility to MyoD transcription)^{39,40}, favoring them into their active proliferative state (Pax7⁺MyoD⁺)^{31,41,42}. A portion of MuSCs will return to the quiescent state (Pax7^{high}MyoD⁻Myf5⁻) to repopulate the MuSC pool (self-renewal) through Notch⁴³⁻⁴⁵ and

Spry1^{23,46,47} signaling pathways while the other portion of MuSCs will exit the cell cycle and commit to the myogenic lineage (myoblasts; Pax7^{low}MyoD⁺Myf5⁺). The latter will eventually differentiate into myocytes (Myf5⁺Myogenin⁺),⁴² and fuse together to form myotubes, which is partially regulated through M2 anti-inflammatory signaling, SPM production, and the extracellular niche^{28,37,48,49}. The remaining M1 macrophages secrete TNF-type cytokines, resulting the in apoptosis of FAPs; this prevents them from differentiating into fibroblasts or adipocytes, ultimately limiting fibrosis formation and lipid accumulation in the tissue^{31,37,50,51}. M2 macrophages contribute to tissue re-vascularization, and initiate the tissue remodeling phase, where myotubes mature into newly formed myofibers (eMyHC⁺)³³. In the long term, regulatory T cells continue to promote tissue remodeling and tissue homeostasis,³³ and prepares the skeletal muscle to repeat this process in subsequent insults (**Figure 2**).

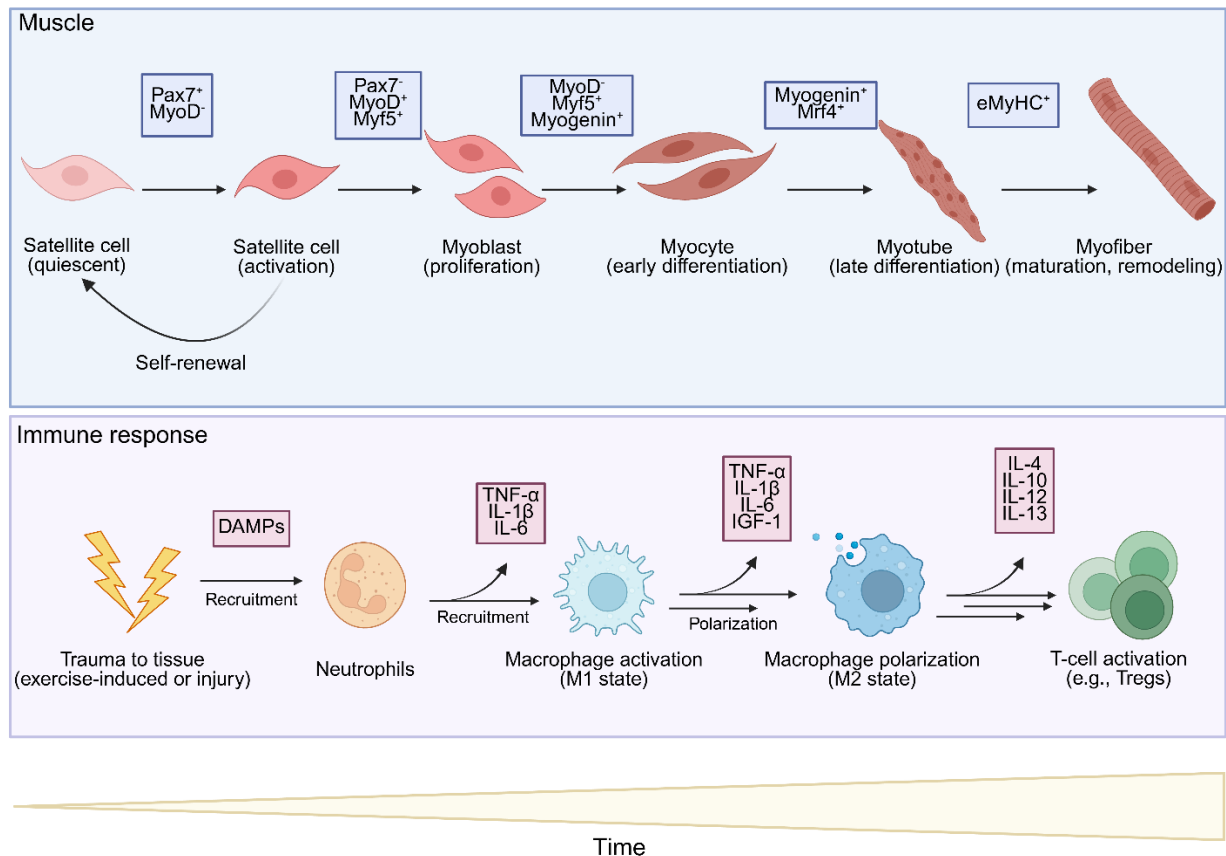


Figure 2. Adult skeletal muscle regeneration process and associated immuno-regulation. Briefly, satellite cells are activated upon trauma to tissue (e.g., injury), where DAMPs recruit neutrophils to the wounded area. Macrophages are then recruited and activated (“M1” pro-inflammatory state), and satellite cells enter the cell cycle (proliferate into myoblasts) or return to their quiescent state to replenish the stem cell pool. As debris from the injury is cleared, the macrophages polarize into their “M2” anti-inflammatory state, and myoblasts exit the cell cycle (differentiate into myocytes) and fuse together to form myotubes. Tissue remodeling then continues as other immune cells may be recruited to the tissue (e.g., T-cells) and myotubes mature into myofibers. Figure was made using BioRender.

Skeletal muscle regeneration in aged individuals

Overview: As early as the 1980s, it was observed that with age, there is a decline in skeletal muscle mass and function^{1,52}. There is increasing evidence that this phenomenon may be driven from the reduction in regenerative capacity of MuSCs^{46,53} in conjunction with persistent low-grade inflammation associated with aging, termed “inflammaging”,^{54,55} leading to maladaptive tissue repair/remodeling^{53,56}. While there are currently no viable therapies to attenuate age-related disorders in the skeletal muscle, previous research has shown increasing protein synthesis may

help to retain muscle mass, but the data remains inconclusive, suggesting there may be other underlying factors^{42,56-58}. Emerging evidence suggests that modulating the immune response, such as restoring the concentration of SPMs contained within the skeletal muscle, may be another promising therapeutic avenue for maintaining muscle mass and function in the elderly^{19,59,60}. To better understand how this may be the case, it is necessary to highlight the underlying mechanisms (intrinsic and extrinsic) leading to maladaptive tissue remodeling as we currently understand them.

Intrinsic changes in aged MuSCs

Changes in quiescent MuSC pool: In aged skeletal muscle, there is a decline in the number and function (e.g., decline in proliferation capacity) of MuSCs, leading to a loss in regenerative capacity^{31,46,61}. Maintenance of the quiescent stem cell pool is regulated by various signaling pathways, including Notch⁴³⁻⁴⁵, Spry1^{46,47}, and Protein Kinase B/mammalian target of rapamycin (Akt/mTOR)⁶². Disruption of these pathways (e.g., aging)^{15,63}, leads to a decline in the MuSC pool population, in part due to stem cell exhaustion and changes in chromatin accessibility^{41,62}. Additionally, there is a decline in MuSC heterogeneity (Pax7^{high} and Pax7^{low} MuSCs), which impacts the stem cell fate decision (commitment to myogenic lineage or replenish the stem cell pool)^{28,42}, further compromising muscle regeneration (e.g., delayed entry and exit of the cell cycle)^{57,60,64}. As the later stages of the cell cycle are dependent on successful completion of previous phases, including the stem cell fate decision⁶⁵, failure to maintain the quiescent stem cell pool and its heterogeneity leads to an undesirable feedback loop, resulting in a decline in skeletal muscle regeneration.

Changes in MuSC proliferation: Loss of proliferation capacity may lead to an increase in senescent cells and lead to changes in stem cell pool/stem cell pool dynamics^{66,67}. Cellular senescence refers to cells that are stuck in cell cycle arrest but do not undergo apoptosis^{66,68}. While cellular senescence and senolytics (therapies that attenuate cellular senescence) are severely understudied⁶⁹, it has previously been shown that senescent cells produce their own secretome—the senescence-associated secretory pathway (SASP)—which may contribute to aberrant tissue remodeling in aging⁶⁹. Emerging evidence suggests that the SASP may act in a paracrine manner^{66,70}, where healthy neighboring cells undergo senescence programming and may transdifferentiate into fibrotic cells⁵⁵, and contribute to maladaptive collagen remodeling^{32,66}. Although only a small proportion of cells within the tissue undergo this phenotypic switch,^{69,70} it is enough to promote age-related disorders *in vivo*^{71,72}.

Changes in cellular differentiation: After several rounds of proliferation, myoblasts will either return to their quiescent state, or they will commit to differentiation (myogenic lineage), defined by the co-expression of MyoD and Myf5^{5,9,13,73,74}. The *myod1* gene transcribes the MyoD protein, which has been shown to be the master regulator for myogenic commitment^{39,71,72}. Both gene and protein expression of *myod1* and MyoD, respectively, decline with age due to silencing of the gene promoter region and through histone modifications (e.g., DNA methylation)^{41,75-77}. A complete knock-out of MyoD protein expression demonstrated delayed myogenic progression, and consequently, poor myoblast fusion and a declined fusion rate^{28,29,57}. Additionally, aged MuSCs have an increased expression of cell-cycle inhibitors (e.g., p16lnk4a, p21) compared to young MuSCs⁷⁸, further highlighting the cell cycle dysfunction^{57,79,80}. Myogenic regulatory factor (MRF) expression (e.g., MyoD expression) is also regulated through the immune system²⁹. In aged tissue,

there is an increase in pro-inflammatory signaling, such as TNF- α expression, which may prevent myoblast fusion through activation of nuclear factor kappa B (NF- κ B) pathway, a known regulator of cyclin D1⁸¹⁻⁸³ (cell cycle regulator). Additionally, overexpression of transforming growth factor beta (TGF- β) superfamily, as seen in older adults⁸⁴⁻⁸⁷, has also been shown to block myoblast fusion^{84,88}.

Later differentiation markers include expression of myogenin (MyoG⁺) and the formation of newly formed myofibers (eMyHC⁺)⁷³, which develop post-myocyte fusion, through the assistance of integrins and catherins^{84,89}. In aged tissue, the overall myogenic commitment is dysregulated, leading to delays in myogenesis and consequently, maladaptive tissue repair. For example, late differentiation is partially regulated by myostatin, a myokine (cytokines released by muscle cells), that inhibits differentiation and may cause a decline in myotube size^{90,91}. Clinically, loss of myofiber size leads to loss of muscle mass and increased fragility, as seen in the elderly.

In the context of adult skeletal muscle regeneration, eMyHC has also been shown to affect myofiber size, myofiber type, the number of myogenic progenitors, and the rate of myogenic differentiation to facilitate the new formation of fibers⁶⁵. The non-intrinsic level of regulation of eMyHC is in part due to the secreted FGF, which was found to rescue myogenic differentiation in cultured eMyHC^{-/-} cells when supplemented to media⁶⁵. In aging, myofibers tend to be smaller in size and further apart (e.g., more intracellular space between fibers) which contributes to the loss of muscle density and muscle mass⁶⁴. With respect to fiber type, it has been previously shown that newly formed fibers typically exhibit the fast-twitch phenotype but may change into a different fiber type (e.g., slow-twitch fibers) based on the microenvironment⁹². Paradoxically, in aged

muscle, the fast-twitch fibers are the first fibers to waste away due to immobility^{58,93}, resulting in changes in contractile properties and in metabolic activity of the skeletal muscle.

Age-related changes in the metabolic roles of the skeletal muscle: The skeletal muscle is a known central regulator of metabolic pathways (e.g., the Cori cycle), allowing for metabolic flexibility (the use of various substrates to produce ATP)⁸. However, with aging, this metabolic flexibility decreases^{8,94,95} (e.g., increased insulin resistance, increased lipotoxicity, mitochondrial dysfunction)^{8,57,96,97} leading to an increase in reactive oxidative species (ROS)^{42,46,94} production, a decrease in mitochondrial biogenesis^{8,98}, and by consequence, a decrease in protein synthesis^{41,57,99}.

Metabolic flexibility in the skeletal muscle is partially due to the various, but distinct, fiber types found within the tissue. The fiber types are classified based on which myosin heavy chain (MyHC) isoform is expressed, and they are divided into two broad categories: slow-twitch and fast-twitch fibers. Slow fibers are typically higher in mitochondria content and number of blood capillaries, making them more resistant to muscular fatigue. Fast fibers (glycolytic and oxidative), on the other hand, contain less mitochondria and fewer blood capillaries, allowing for rapid ATP production and degradation⁵⁸. The latter fiber type muscles are typically shorter in length, allowing for explosive muscle movement. Fiber type partially depends on the microenvironment⁹² and remains plastic as a function of time; typically, newly formed fibers exhibit the fast-twitch fiber type and changes into slow-twitch fiber type over time. To maintain metabolic flexibility and to maintain MyHC isoforms, both of which play integral roles with respect to muscle regeneration, exercise seems to be the most effective form of therapy^{92,100,101}. However, approximately 19% of the Canadian population is classified as geriatric (adults aged 65+)¹², and may already experience limited mobility, which may hinder their ability to start or to continue exercising.

Age-related extrinsic factors affecting skeletal muscle regeneration

While age-related intrinsic changes in the skeletal muscle cells may impact tissue regeneration, emerging evidence suggests that changes within the tissue microenvironment—other cell types (e.g., immune cells) and structural components contained within the tissue—may also contribute to maladaptive skeletal muscle regeneration^{32,55,102,103}.

Changes in the skeletal muscle ECM: The ECM is composed of proteins (e.g., collagen), sugars, and RNA, and it supports the structural properties of the skeletal muscle¹⁰. The ECM alters with age. For example, *in vivo*, there is an increase in advanced glycation end products (AGEs) in the skeletal muscle due to aging, which allows an increase in AGE cross-links to be formed. The increase in cross-links leads to the formation of fibrotic fibers and increases myoblast trans-differentiation (e.g., formation of myofibroblasts), causing decreases in muscle elasticity due to increased ECM deposition (fibrosis)^{32,104}.

The ECM also facilitates contractile properties through the neuromuscular junction (NMJ), cellular migration, myotube formation, and intracellular signaling (e.g., adipogenic potential in cells)^{9,104}, which all impact skeletal muscle regeneration. For example, as a consequence of increased fibrotic fibers and AGEs, there is an increase in extracellular space between the skeletal muscle fibers¹⁰⁴, causing a loss of muscle contractile force, or fragility, due to aging^{104,105}. These effects may be exacerbated in the case of comorbidities (e.g., obesity, diabetes).

Changes in the inflammatory response: With age, whole-body homeostasis changes due to an increase and persistence of inflammation (“inflammaging”)^{106,107}. Within the skeletal muscle, an

elevation of inflammation impedes skeletal muscle regeneration due to changes in function observed in neutrophils, macrophages, and FAPs. Failure to clear neutrophils exacerbates damage to the tissue as neutrophils produce ROS, protein-degrading enzymes (e.g., matrix metalloproteinases), and increased production of pro-inflammatory cytokines^{27,29,108}. Additionally, failure to clear neutrophils in a timely manner causes delays in the immune response and creates an unfavorable environment for muscle regeneration^{41,109}. Macrophages also contribute to the elevation of inflammation in aged skeletal muscle. It has previously been demonstrated that endogenous production of SPMs in macrophages and in skeletal muscle composition declines in aged mice^{19,59}. Given that SPMs modulate M1-M2 polarization^{28,34-36}, which is one of the drivers needed to activate the quiescent satellite cells, a decline in SPM content delays M1-M2 polarization and leads to constitutive pro-inflammatory signaling from M1 macrophages^{35,49}. Additionally, M1 function is compromised (e.g., failure of FAP clearance), which leads to maturation of FAPs (adipocyte and fibroblast formation), and drives lipid and collagen deposition into the skeletal muscle^{110,111}. Consequently, the tissue microenvironment is unfavorable for skeletal muscle regeneration¹¹²⁻¹¹⁶. Other studies have demonstrated that an upregulation of interferon gamma (IFN γ), produced by natural killer and T cells, causes a downregulation of myogenin (late differentiation marker in the muscle) and simultaneously increases p16 expression, a well characterized marker of cellular senescence (e.g., a phenomenon that increases with age)¹¹⁷. Thus, the changes in the inflammatory response associated with tissue repair cause delays in activation of satellite cells and, consequently, delays in skeletal muscle regeneration^{46,118}.

Changes in skeletal muscle vasculature: Post-injury, endothelial cells and vascular cells transmit signaling molecules, (e.g., VEGF), to facilitate the formation of blood vessels within the tissue,

which permits nutrients to be delivered to newly formed fibers¹¹⁹⁻¹²³. However, with age, poor vasculature impedes nutrients from being delivered to the cells, exacerbating inflammation, hypoxia, and metabolic dysfunction^{7,120}.

Changes in the NMJ: The NMJ is the intersection between the brain and the muscle, involved in the contractile properties necessary for movement through a motor unit located at the periphery of the myofibers^{124,125}. The motor unit is related to fiber type (e.g., fast-twitch and slow-twitch); fast-twitch neurons are susceptible to cycles of denervation and reinnervation^{92,125}, leading to NMJ fragmentation and a decline in force output^{125,126}.

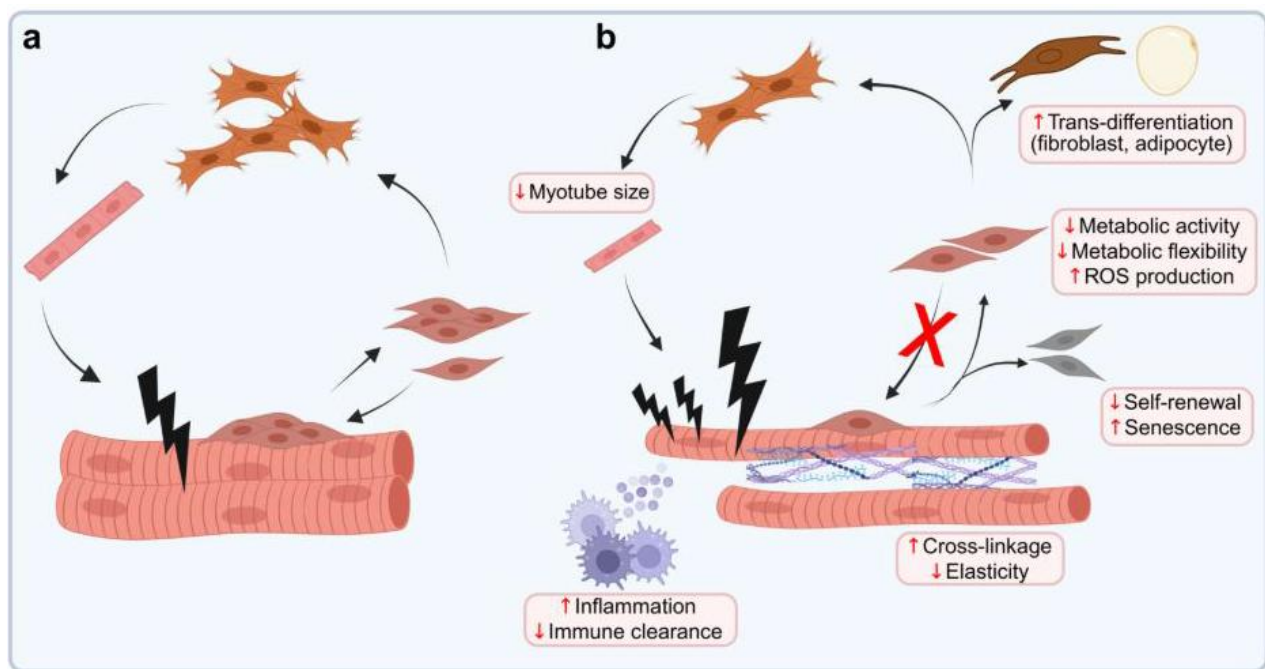


Figure 3. Skeletal muscle regeneration changes with aging. a) Adult skeletal muscle regeneration after trauma to the tissue (e.g., an injury). Briefly; satellite cells are activated, undergo a stem cell fate decision (return to quiescent state or commit to myogenesis). Committed cells undergo differentiation into myocytes and fuse together to form myotubes, which later mature into myofibers at the site of injury. **b)** Skeletal muscle regeneration in aging. Intrinsic and extrinsic changes in the skeletal muscle cause aberrant tissue remodeling, leading to a decline in skeletal muscle mass and function in the elderly. Intrinsic changes in the muscle include (but are not limited to): decrease in MuSC pool self-renewal, increase in senescence,

decrease in metabolic activity and metabolic flexibility, increase in ROS production, increase in trans-differentiation of myoblasts into fibroblasts or adipocytes, and decrease in myotube size. Extrinsic changes include (but not limited to): increase in inflammation/decrease in immune clearance, increase in cross-linkages (increased fibrosis), and decrease in tissue elasticity. Figure was made using BioRender.

Potential therapies to improve aged skeletal muscle regeneration

Currently, there are no viable therapies to attenuate age-related skeletal muscle disorders in the elderly. There is a growing body of research to better understand the underlying mechanisms, and how to delay or reverse them, associated with aberrant skeletal muscle regeneration and tissue repair in the elderly. Previous studies have been primarily focused on retention of muscle mass through resistance strength training paired with adequate protein intake in older adults^{8,39,41,118,127,128}. While resistance strength training promotes muscle regeneration through microscopic tears in muscle fibers, strength training on its own may not be sufficient or suitable after a severe muscle injury or for older adults with limited mobility^{129,130}. Additionally, it can be challenging to incorporate lifestyle changes (e.g., compliance with a strength training regime). Thus, there has been a recent shift in research towards modulation of the immune response (e.g., anti-inflammatory therapies) to improve skeletal muscle regeneration in the context of aging^{49,131,130,132}.

On a more cellular and molecular basis, there has been an effort to restore myogenic potential in aged MuSCs. These studies include cell-based therapies such as transplantation of MuSCs from a healthy donor to an unhealthy donor (may evoke ethical concerns) and the use of inducible pluripotent stem cells (expensive and requires further optimization)^{31,41}. Other approaches include modulation of key pathways (e.g., Notch signaling pathway) to address the biological changes associated with aging cells and cellular crosstalk, however many studies use C2C12 myoblasts (murine immortalized cell line), which may limit our understanding of potential therapies^{7,49,54,57,104,127}. Most older adults who experience chronic low-grade

inflammation are often prescribed with NSAIDs¹³² and this remains the most common over the counter therapy.

Prostaglandins are produced through the metabolism of arachidonic acid and converted by COX-2 into prostaglandin H₂, and then further converted into various prostaglandins subtypes (e.g., PGF₂ α and PGE₂). To induce their anti-inflammatory effects, NSAIDs inhibit COX-2 enzymatic activity, effectively reducing total prostaglandin production¹³²⁻¹³⁴. It has been demonstrated that complete inhibition of the COX-2 pathway may increase protein degradation (e.g., promote fiber atrophy) in the skeletal muscle of older adults, likely through reduction of PGF₂ α production^{14,135-137}. Long-term inhibition of COX-2 activity through NSAIDs has been shown to decrease the number of activated satellite cells and slows down muscle regeneration progression, likely due to an inadequate inflammatory response^{41,110,138,139}. For example, during the inflammatory stage of muscle repair, PGE₂ promotes myoblast proliferation while simultaneously reducing fibrosis^{110,140}; at later stages of tissue repair, PGE₂ has been shown to help promote re-vascularization¹⁴¹. Thus, decline of prostaglandin production through COX-2 inhibition may lead to aberrant tissue repair. In aged skeletal muscle tissue, there is a decline in PGE₂, which is exacerbated with the use of NSAIDs¹⁴²⁻¹⁴⁴. Finally, long term use of NSAIDs has been shown to impair muscle adaptation and hypertrophy in athletes^{145,146} and can lead to adverse effects systemically (e.g., liver and kidney failure), as seen in older adults¹⁴⁷. Therefore, NSAIDs are not suitable candidates to restore skeletal muscle regeneration in the elderly.

Over the past decade, there has been an increasing body of research investigating the anti-inflammatory properties of n-3 PUFAs^{98,130,148-150}. While most studies focus on the cardioprotective^{130,132,151} and neuroprotective¹⁵²⁻¹⁵³ effects of these lipids, several studies have also highlighted their roles within the skeletal muscle¹⁵⁴⁻¹⁵⁶. While there are still some uncertainties

with respect to what extent n-3 PUFAs modulate tissue repair, particularly in older adults (e.g., mechanistic approaches), these bioactive lipids may be a suitable candidate and a safer alternative compared to NSAIDs¹⁴⁷ to restore proper skeletal muscle regeneration in the elderly. Given the increase in life expectancy, restoring muscle regeneration through regulating age-related inflammation (inflammaging) may help to improve mobility and enhance the quality of life in the elderly.

Polyunsaturated fatty acids

n-3 PUFAs and n-6 PUFAs are bioactive lipids with a double carbon-carbon bond on the third or sixth methyl end of the chain, respectively. Typically, n-3 PUFAs promote anti-inflammatory pathways and resolution of inflammation^{98,130,148-150,156} whereas n-6 PUFAs are known to promote pro-inflammatory signaling, such as the release of prostaglandins^{7,130,157}. Given that age-related aberrant tissue repair arises in part by the dysregulated immune response, we have decided to focus on the effects of n-3 PUFAs in the context of the skeletal muscle. Often, n-3 PUFAs are acquired through diet; the most common dietary n-3 PUFAs are alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). ALA is an essential n-3 PUFA that is found in seeds, nuts, and algae. EPA and DHA can be synthesized from ALA and EPA, respectively (ALA → EPA → DHA)¹⁵⁸, but this conversion is quite inefficient in mammals, including humans^{159,160}. Therefore, DHA and EPA are often supplemented through diet (e.g., fatty fish) or as over-the-counter fish oil (FO) supplements^{161,162}.

From mouth to muscle: The skeletal muscle is a central compartment involved in regulation of metabolism, including lipid metabolism. Briefly, after digestion, n-3 PUFAs are absorbed in the small intestine, where they are packaged into lipoproteins and secreted into the bloodstream¹⁶³. Dietary n-3 PUFAs can either be taken up by certain tissues (e.g., the skeletal muscle) through fatty acid transporters or stored in adipose tissue as droplets, where they can be used to generate ATP when blood glucose is low (e.g., when fasting)^{8,163}. Within the skeletal muscle, n-3 PUFAs can be incorporated into the cell membrane^{141,150}, increase protein synthesis^{164,165}, and improve vasculature⁷, in addition to releasing anti-inflammatory molecules.

Anti-inflammatory properties of n-3 PUFAs: Emerging evidence demonstrates that the anti-inflammatory properties of n-3 PUFAs arise from a class of their metabolites called SPMs. The three main classifications of SPMs are resolvins, maresins, and protectins, which are further classified based on which n-3 PUFA they are derived from (e.g., the resolvin-D series are derived from DHA)¹⁶⁶⁻¹⁶⁸. SPMs are produced from the metabolism of n-3 PUFAs through the lipoxygenase (LOX), COX, and cytochrome P450 (CYP450) pathways^{19,169}. Endogenous SPM production has also previously been shown to decline with age and may contribute to maladaptive tissue remodeling, which can be alleviated through SPM supplementation¹⁹. Exogenous supplementation of resolvin-series SPMs improved tissue regeneration in injury and in Duchenne Muscular Dystrophy (DMD) murine models^{59,60}, through binding of G-coupled receptor proteins (GPRs)²⁰. Emerging evidence suggests that SPMs may be produced from both the immune response and from the muscle itself and may play a key role in facilitating cellular crosstalk for tissue repair^{14,133,170}. While these results are promising, these studies administer SPMs by intraperitoneal injection (IP) and SPMs have a short half-life, which limits their therapeutic

potential. Given that SPMs are derived from n-3 PUFAs, a dietary supplementation of n-3 PUFAs may upregulate SPM production within the skeletal muscle and may contribute to resolving inflammation by clearing neutrophils, improve macrophage polarization, and limit fibrosis^{28,98,130,148-150,156}.

n-3 PUFAs on myogenesis: The main intrinsic force behind age-related MuSC changes contributing to maladaptive tissue remodeling is the delay in cell cycle progression, and n-3 PUFA supplementation on myogenic regulation remains inconclusive¹⁷¹⁻¹⁷³. For example, in C2C12 myoblasts, n-3 PUFA supplementation led to a decline in myoblast proliferation through the inhibition of the mitogen-activated protein kinase (MAPK) phosphorylation cascade¹⁷⁴ and a decline in myoblast differentiation due to increased ROS production¹⁷⁵. Conversely, other studies have demonstrated a reversal in protein catabolism with n-3 PUFA supplementation in C2C12 myoblasts¹⁵⁰ as well as under the context of atrophic (dexamethasone-treated myoblasts)⁹⁸ and cachexic (proteolysis inducing factor-treated myoblasts)¹⁷⁶ cell models, as well as improved retention in sarcopenia aged mouse models through modulating mitochondrial dynamics¹⁶⁴. While these may not directly relate to myogenic progression, incorporation of n-3 PUFAs within the cell phospholipid membrane makes SPMs readily available upon cellular stress¹⁷⁵. For example, upon exposure of the pro-inflammatory cytokine TNF- α , EPA was shown to rescue myotube diameter through activation of peroxisome proliferator-activated receptor- γ (PPAR γ)^{177,178}. Furthermore, n-3 PUFAs can be incorporated in the membrane of other cell types (e.g., macrophages), facilitating cellular crosstalk during tissue regeneration¹⁶⁴.

The effects of n-3 PUFA supplementation on cells are dependent on the dose, time, and frequency of treatment^{150,153}. Additionally, most of these studies have been conducted in C2C12

myoblasts (murine immortalized cell line), which may limit translational work into aged murine models. With respect to myogenesis in aging, primary myoblasts isolated from aged murine models are more suitable models since they adequately represent the biological changes associated with aging^{171,179}. In the field, there is a lack of n-3 PUFA supplementation studies using primary myoblasts from aged models (e.g, aged mice) for *in vitro* work, which may limit our understanding of how these bioactive lipids may modulate myogenesis and muscle regeneration.

In vivo, previous work has demonstrated that n-3 PUFA supplementation may restore SPM concentrations within the muscle^{59,180}, which declines with age^{19,181}, indicating that an enriched fatty acid diet may modulate the local and systemic inflammatory response to promote tissue regeneration in the muscle. Other studies have shown that n-3 PUFA supplementation leads to more n-3 PUFAs (and consequently, their metabolites) to be incorporated into the cellular membrane, causing changes in membrane fluidity, membrane composition, and more readily available to be used^{182,183}. For example, in aged mice, n-3 PUFA supplementation improved mitochondrial oxidative capacity¹⁸⁴, reduced metabolic dysfunction^{185,186}, and decrease of fat accumulation in the muscle tissue^{187,188}. Consequently, n-3 PUFA supplementation has also been shown to directly modulate fiber type transition (fast to slow fiber types), which in turn may impact muscle quality and improve fatigue-resistance^{186,189}. However, other studies found opposite results (e.g., no change in fatigue resistance)¹⁸⁸⁻¹⁹¹. Additional studies have shown that n-3 PUFA supplementation promotes protective effects against protein degradation in the context of muscle atrophy (disuse of muscle)^{192,193}. While these studies were conducted in young mice (8-10 weeks) loss of muscle mass also affects aged populations. Finally, in addition to their metabolic and protein-protective effects, some studies have shown that n-3 PUFA supplementation may activate satellite cells and improve their myogenic capacity (e.g., activation of MRF transcription)^{183,188,190}.

Many pre-clinical studies lack measurements of the contractile properties (e.g., grip strength, muscle force) thereby limiting our knowledge on the physical and functional properties of n-3 PUFA supplementation *in vivo*¹²⁴. Moreover, many age-related studies use mice between 18-20 months old (~50 years old in humans)²⁹, but very rarely will the mice be >20 months (>50-year-old in humans), where progressive loss of muscle mass can reach up to 2% per year^{35,52}. Therefore, examination of histological, immune, functional, and myogenic properties in appropriate pre-clinical models (e.g., >20-month mice) may serve as a better method to study age-related skeletal muscle regeneration and to ameliorate translational work from bench to bedside.

Clinically, a recent meta-analysis concluded that there is limited and inconclusive evidence demonstrating n-3 PUFAs may delay muscle wasting through improved muscle regeneration (e.g., rebuilding fibers) in older adults¹⁹⁴. These mixed findings may be due to inconsistencies with respect to choice of placebo, dosage, diets, and length of treatment from study to study¹⁵⁶. However, there is evidence that n-3 PUFA supplementation in conjunction with a resistance strength program may increase physical strength in older adults^{129,195,196}.

Therefore, to better understand the effects of n-3 PUFA supplementation on myogenesis and in skeletal muscle regeneration in the context of aging, murine-derived cells (primary cells) and aged mice (>20 months; with injury)¹⁹⁷ should be used as appropriate models to uncover mechanisms of regulation pertaining to both regulation of inflammation and modulation of myogenesis in the elderly. In turn, we may be able to improve the quality of regenerated tissue without completely dampening the crucial inflammatory response needed to facilitate tissue repair. Consequently, this may increase muscle mass and function in the elderly and lead to a higher quality of life, which is essential given that life expectancy is trending upwards, even in Canada¹².

Hypothesis

Given the upregulation of SPM production through n-3 PUFA supplementation, which are molecules involved in the resolution of inflammation, we hypothesize that a diet enriched in n-3 PUFAs may improve skeletal muscle regeneration by regulating the aberrant immune response associated with aging. To answer this, two main aims were developed;

1. Assess whether n-3 PUFA supplementation could modulate myogenic capacity in murine-derived aged myoblasts *in vitro*.
2. Assess the effects of a DHA-enriched diet on skeletal muscle regeneration *in vivo* after introducing a sterile IM CTX injury.

Materials and methods

C2C12 immortalized cell culture

C2C12 myoblasts were seeded in 6-well plates coated with rat-tail collagen type I (cat #354236, Corning®, USA) diluted in 0.02 M acetic acid (final concentration = 4.1 mg/mL). Cells were grown in Dulbecco's modified eagle's medium (DMEM) with 4.5g/L glucose and L-glutamine (cat #319-015-CL, Wisent INC., USA) supplemented with 10% (v/v) fetal bovine serum (FBS; cat #CA76327-086, VWR-Corning, Canada) and 1% (v/v) penicillin/streptomycin (100X, cat #450-201-EL, Wisent INC., USA). At 70-80% confluency, the cells were switched into differentiation media; DMEM with 4.5g/L glucose and L-glutamine glutamine (cat #319-015-CL, Wisent INC., USA) supplemented with 2% (v/v) horse serum (HS, cat #065-110, Wisent INC., USA) and 1% (v/v) penicillin/streptomycin (100X, cat #450-201-EL, Wisent INC., USA). Cells were treated with either 50 nM ALA (cat #90210-250, Cedarlane, Canada), 50 nM DHA (cat #90310-500, Cedarlane, Canada), or vehicle (absolute ethanol, abs. EtOH) in the differentiation media for 4 days (37°C, 5% CO₂), with fresh media (+treatment) added every 48 hours. Cells were then fixed in 4% paraformaldehyde (PFA, cat# AAJ61899AP, Fisher Scientific, USA) and stained (see **Immunofluorescence** for more detail).

Primary murine muscle stem cell isolation and culture

MuSCs were isolated from young (4 months) and geriatric (26-28 months) C57BL/6 mice (JAX, USA) using the Satellite Cell Isolation Kit for mice (cat #130-104-268, Miltenyi Biotec, Germany) according to the manufacturer's instructions. Briefly, the mice were administered sodium pentobarbital (120 mg/kg) by IP injection followed by cervical dislocation before retrieving the hindlimb muscles. The muscles were then minced into small pieces placed in gentleMACS C

Tubes (cat #130-093-237, Miltenyi Biotec, Germany) and digested in collagenase B (cat #11088831001, Millipore Sigma, Germany). The samples were gently inverted before being placed on the gentleMACS Octo Dissociator with Heaters (cat #130-096-427, Miltenyi Biotec, Germany) equipped with the 37_mr_SMDK_1 program. The homogenate was then quickly centrifuged and resuspended before filtering through a 70 μ m filter. The filtrate was then spun down at 3000 rpm for 5 mins and the supernatant was discarded. The pellet was resuspended and washed with MACs buffer (Miltenyi Biotech, Germany) and spun down for an additional 5 mins at 3000 rpm. The cells were then resuspended and then cultured in Hams F10 media (cat #318-050-CL, Wisent INC, USA) supplemented with 20% (v/v) FBS (cat #CA76327-086, VWR-Corning, Canada), 1% (v/v) penicillin/streptomycin (100X, cat #450-201-EL, Wisent INC., USA), and 10% (w/v) of human FGF (cat #511-126-QU, Wisent INC., USA) on collagen-coated plates, as previously described.

Real-time proliferation assay

Primary myoblasts were seeded at 10% confluency in a 24-well plate in proliferation media. The cells were then placed in the Incucyte® S3 Live-Cell Analysis System (Sartorius, Germany) incubator (37°C, 5% CO₂). Images were taken using the 20x objective, scanning every hour for 4 days (10 mins/scan). Media (no treatment) was changed approximately every 48 hours.

Proliferation assay

Young and aged myoblasts were cultured in a 12-well plate in proliferation media supplemented with 50 nM DHA (cat #90310-500, Cedarlane, Canada) or vehicle overnight (2, 4, and 6 days—supplementary material). Cells were then briefly washed with pre-warmed sterile Dulbecco's

phosphate buffered saline (1X, D-PBS, cat #311-425-CL, Wisent, USA) before fixing with 4% PFA (cat# AAJ61899AP, Fisher Scientific, USA) for 5 minutes. Cells were then stained according to the protocol described by Dumont and Rudnicki (2017)¹⁹⁸, further described under the **Immunofluorescence** section.

Differentiation assay

Aged myoblasts were cultured into 6-well dishes in proliferation media, without treatment. At 70-80% confluency, cells were placed in primary myoblast differentiation media; 50% (v/v) Hams F10 media (cat #318-050-CL, Wisent INC., USA), 50% (v/v) DMEM low glucose (cat #319-010-CL, Wisent INC., USA), 2.5% (v/v) HS (cat #065-110, Wisent INC., USA) and 1% (v/v) penicillin/streptomycin (100X, cat #450-201-EL, Wisent INC., USA). 50 nM DHA (cat #90310-500, Cedarlane, Canada) or vehicle was freshly prepared and added directly into the differentiation media, which was switched approximately every 48hrs for 4 or 6 days. Cells were then briefly washed with pre-warmed sterile D-PBS (1X, cat #311-425-CL, Wisent, USA) before fixing with 4% PFA (cat# AAJ61899AP, Fisher Scientific, USA) for 5 mins. Cells were then stained according to the protocol described by Dumont and Rudnicki (2017)¹⁹⁸, further described under the **Immunofluorescence** section below.

MTS cell viability assay

To measure cell viability, the CellTiter 96® AQueous One Solution Cell Proliferation Assay (cat #G3582, Promega Corporation, USA) was used according to the manufacturer's instructions. Briefly, aged myoblasts were seeded at 1000 cells/well in a 96-well plate and let to attach overnight. Fresh media with treatment (0 – 1000 nM) DHA or vehicle was added to the respective wells, with

the first row serving as a blank (proliferation media, no cells). After 48 hours, pre-warmed CellTiter 96® AQueous One Solution Reagent provided in the kit was added to the cells for 3hrs (separately optimized) before measuring the absorbance at 490 nm using a BioTek Synergy H1 multi-mode plate reader (Agilent Technologies, USA). % viability was calculated as shown below;

$$\% Viability = \frac{Treated (Measured\ abs - Blank\ abs)}{Control (Measured\ abs - Blank\ abs)} \times 100$$

Senescence assay

To measure the number of senescent cells, the Senescence β-Galactosidase Staining Kit (SA-β-gal, cat #9860, Cell Signaling Technology, USA) was used according to the manufacturer's instructions. Briefly, aged myoblasts were cultured in 12-well plate in proliferation media with either 50 nM DHA treatment (cat #90310-500, Cedarlane, Canada) or vehicle overnight in an incubator (37°C, 5% CO₂). Cells were then briefly washed with pre-warmed sterile D-PBS (1X, cat #311-425-CL, Wisent, USA) before fixing with the fixative solution provided in the kit for 10 minutes at room temperature. The staining solution was then added to the cells and placed in a dry incubator (no CO₂) at 37°C, until the cells turned blue (~24 hrs). The cells were then rinsed with PBS and stained with Hoechst 33342, Trihydrochloride, Trihydrate (DAPI, 1:10 000, cat #H3570, Invitrogen, USA).

Animal care

All experimental protocols were approved by to the University of Ottawa's Ethics Committee and adhere to the Canadian Council on Animal Care guidelines (CCAC). Aged male and female C57BL/6 mice (Charles River Laboratories, Canada) were individually housed in a 12:12 light:dark cycle and controlled temperature at the University of Ottawa Animal Facility. At 20-

months, the mice were randomly assigned to two experimental groups, receiving either a soybean oil diet (2020 + 19% SBO, lot #3718149 for male cohort, lot #4037071 for female cohort) or a FO diet (2020 + 9.5% FO + 9.5% SBO, lot #3718157 for male cohort, lot #4037062 for female cohort) (Inotivco, Madison, Wisconsin, USA), which contains 1% DHA. Diets were stored in a cold room to prevent pellet and nutrient degradation. See **Table S1 (Appendix A: Supplementary Material)** for the nutritional breakdown of each diet. The mice were acclimated from the chow diet to their respective experimental diet (SBO or 1% DHA) for up to two weeks before completely switching to their respective diets. Food was weighed every 2-3 days, and fresh pellets were added in bowls that were placed directly into the cage. Body weight was measured 2-3 times a week, including basal measurements before diet change. For the female cohort only, body composition (see below) and forelimb grip strength test (see below) were taken once a week throughout the course of the experiment.

In another cohort, aged male C57BL/6 mice (Charles River Laboratories, Canada) were subjected to the same conditions as described above, except they either received an SBO diet (2020 + 19% SBO, lot #3718149), a 1% DHA diet (2020 + 9.5% FO + 9.5% SBO, lot #3718157) or a 2% DHA diet (2020 + 19% FO, lot #230279) (Inotivco, Madison, Wisconsin, USA). Diets were stored in a cold room to prevent pellet and nutrient degradation (see **Table S1** for nutritional breakdown). The mice were acclimated from the chow diet to their respective experimental diet (SBO, 1% DHA, 2% DHA) for up to two weeks before completely switching to their respective diets. For oxylipin analysis (see **Oxylipin analysis** for details), an additional group of young C57BL/6 male mice on chow diet was included.

Cardiotoxin injury

Once acclimated to their respective diets, CTX (10 μ M diluted in 0.9% NaCl, cat# L8102, Latoxan, France) was administered directly into the tibias anterior (TA) by intramuscular (IM) injection. During this procedure, the mice were administered buprenorphine (0.1 mg/kg) by subcutaneous injection and administered isoflurane (1-2.5% flow rate) by inhalation. Mouse behaviour post-injury was assessed for four hours post-surgery and the subsequent three days. The mice were kept on their respective diets for 21 days (21 DPI). Data was collected and included up for grip test and body composition for this mouse, but all other data for SBO is n=7 (4 male, 3 female). At their endpoint (21 ± 1 DPI), mice were subjected to contractile properties measurement (see below) under anesthesia before harvesting the hindlimb muscles (TA; soleus, SOL; and gastrocnemius, GAS) and blood collection (see below for details) for further analysis. In the second *in vivo* study, mice were subjected to the same conditions as previously described. In this case, there were two timepoints, 14 DPI and 21 DPI (n=2 and n=1, respectively). Blood and muscle collection as well as subsequent experiments were completed for this cohort.

Body composition

Whole body composition was measured by nuclear magnetic resonance (NMR) using the Minispec LF50 Body Composition Mice Analyzer (Bruker, USA). The instrument was calibrated using a Daily Check sample provided by the manufacturer before placing the mice in a restraint tube (1 animal/tube) with an adjustable plunger, without anesthesia, and placed in the sample slot for detection of fat and lean mass at 7.5 MHz. Data was collected once a week throughout the course of the experiment, up to 18 DPI inclusively, in female mice only.

Forelimb grip strength test

Grip strength (forelimbs; uninjured) was measured using the Chatillon DFE II Grip Meter (Columbus Instruments, USA). Mice were held by the tail before being placed on a horizontal grid attached to a force transducer. Once the mouse gripped the grid, the mouse was pulled until the grip was released. Three consecutive trials were measured, and the average of the three replicates was used at each timepoint for each animal. Data was collected once per week throughout the course of the experiment, up to 19 DPI inclusively, in female mice only.

Contractile properties

The contractile properties of each leg were measured as previously described^{60,199}. Briefly, the mice were administered sodium pentobarbital (120 mg/kg) by IP injection. Once the mouse was unconscious but still alive, the hindlimb was carefully shaved and cut open to reveal the muscle, where the fascia was carefully removed with clean tweezers. The bottom tendon of the TA was tied with a small piece of thread on one end before placing the mouse on a pre-warmed platform. The other end of the thread was attached to the lever arm of the 300C Dual-Mode Lever System apparatus (Aurora Scientific, Aurora, Canada), equipped with the pre-warmed platform. Electrodes were directly placed in the sciatic nerve, near the knee. Optimal muscle length (L_0) was adjusted until the maximum isometric twitch tension was achieved. The force-frequency curve was determined by stimulating the muscles with a range of frequencies (10-150 Hz), with 1 min rest between each stimulation. Muscle fatigue was achieved by stimulating the muscles at 100 Hz every second for 18 (min) to 50 (max) stimulations. Muscle length and weight were also measured to calculate the specific force (N/cm^2) using the following equation;

$$\text{Specific force} = \frac{(L_o * L_f) * P_o * \text{Muscle density}}{\text{Muscle weight} * 10}$$

Where L_o is the optimal TA length (in mm, measured using calipers), L_f is fascicle length (standardized to 0.6), and P_o is the force exerted and measured by the apparatus (in mN). Muscle density is normalized to $1.062 \frac{g}{cm^3}$ ¹⁹⁹ and muscle weight was measured after isolating the TA (before freezing, in mg).

Muscle freezing and blood collection

The TA, SOL, and GAS muscles were carefully dissected and weighed on an analytical scale. The TA and the SOL of both legs were freshly frozen in optimal cutting temperature compound (OCT, cat #23730571, Fisher Scientific, USA) and carefully placed in cold 2-methyl-butane until the OCT became opaque before placing on dry ice. The GAS was placed in liquid nitrogen for quick-snap freezing. Blood (100 μ L) was collected using a sterile needle via cardiac puncture. The blood was left to separate on ice for at least 30 mins before being centrifuged at 10 000 xg for 10 min at 4°C (Micro Star 21R, VWR). The serum (supernatant) was carefully collected and placed in a fresh 1.5 mL centrifuge tube. The remaining blood fraction, blood serum, and muscles were then stored at -80°C.

Immunofluorescence

Cross-sections of the TA were cut into 10 μ m sections using the HM525 cryostat cutter (ThermoFisher Scientific). The tissues were then stained for immunodetection as described by Dumont and Rudnicki (2017)¹⁹⁸. Briefly, samples (cells and tissue) were fixed with 4% PFA (cat #AAJ61899AP, Fisher Scientific, USA) for 5 min, and permeabilized with 0.2% Triton X-100 (cat #BP151 100, Fisher Scientific, USA) for 10 min. Samples were blocked with mouse-on-mouse

(M.O.M®) blocking reagent (tissue only) (cat# VECTMKB22131, MJS Biolynx INC, Canada), 5% (w/v) bovine albumin serum (BSA, cat #800-095-EG, Wisent INC, USA), 5% (v/v) goat serum (cat #053-110, Wisent INC, USA), 4% (v/v) donkey serum (cat #035-110, Wisent INC, USA) in PBS for 60 min at room temperature and incubated overnight at 4°C with the following primary antibodies: rabbit anti-Ki67 (1:200, cat #MA5-14520, Invitrogen), mouse anti-MYH1E (1:15, cat #MF20-s, Developmental Studies Hybridoma Bank (DSHB)), rabbit anti-laminin (1:100, cat #AB11575, Abcam), mouse anti-Pax7 (1:10, cat #PAX7-s, (DSHB), rat anti-F4/80 (1:100, cat #MCA497R Bio-Rad), rabbit anti-CD206 (1:500, cat #AB64693, Abcam), rat anti-Ly-6G (1:100, cat #16-9668-82, Invitrogen), rabbit anti-myogenin (1:1000, cat #AB124800, Abcam), mouse anti-myosin heavy embryonic chain (1:10, cat #F1.652, DSHB), mouse anti-myosin heavy chain type IIA (1:20, cat #SC-71-s, DSHB), mouse anti-myosin heavy chain (slow, alpha, beta) (1:100, cat #BA-F8-c, DSHB), mouse anti-myosin heavy chain type IIB (1:10, cat #BF-F3-s DSHB), mouse anti-myoblast determination protein 1 (MyoD) (1:200, cat #sc-377460, Santa Cruz Biotechnology).

Samples were washed with PBS and incubated with appropriate cross-adsorbed secondary antibodies; goat anti-rabbit IgG H+L (1:1000, Alexa Fluor 488, cat #A11008, Invitrogen), goat anti-mouse IgG1 (1:1000, Alexa Fluor 594, cat #A21125, Invitrogen), donkey anti-rat IgG H+L (1:1000, Alexa Fluor 594, cat #A21209, Invitrogen), goat anti-rat IgG H+L (1:1000, Alexa Fluor 594, cat #A1107, Invitrogen), goat anti-mouse IgG1 (1:1000, Alexa Fluor 546, cat #A21123, Invitrogen), goat anti-mouse IgG2b (1:1000, Alexa Fluor 647, cat #A21242, Invitrogen), goat anti-mouse IgM (1:1000, Alexa Fluor 488, cat #A21042, Invitrogen), goat anti-mouse IgG H+L (1:1000, Alexa Fluor 488, cat #A11001, Invitrogen), and donkey anti-rabbit IgG H+L (1:1000, Alexa Fluor 488, cat #A21206, Invitrogen) for 1 h at room temperature. Samples were washed with PBS and counterstained with DAPI (1:10 000, cat #H3570, Invitrogen, USA). The samples

were then washed at least twice with PBS and immediately imaged (cells) or mounted with PermaFluor aqueous mounting media (tissue) (RI = 1.4, cat #TA-030-FM, ThermoFisher Scientific) under an uncoated #1.5 coverslip and left to dry at room temperature overnight before storing at -20°C (tissue) before imaging.

Cells were imaged using a commercial inverted EVOS M5000 or EVOS M7000 widefield microscope (ThermoFisher Scientific) and tissue sections were imaged using a commercial Upright Stand Leica DM6 B widefield microscope (Leica Microsystems, Germany). To enhance reproducibility of image acquisition, specific details are provided in the **Appendix B: Supplementary materials and methods** section of this report.

Hematoxylin and Eosin Staining

To assess muscle histology, the hematoxylin and eosin staining kit (H&E, cat #ab245880, Abcam) was used on TA tissue sections according to the manufacturer's instructions. Briefly, the sections were fixed using 4% PFA (cat #AAJ61899AP, Fisher Scientific, USA) for 5 min and then rinsed with distilled water (dH₂O). Sections were then covered with Mayer's Hematoxylin (Lillie's Modification) for 5 mins at room temperature and then rinsed twice with dH₂O. Bluing reagent was then added to the sections for 10-15 seconds and then immediately rinsed twice with dH₂O. The slides were dipped in absolute ethanol before covering the sections with Eosin Y Solution (Modified Alcoholic) for 2-3 mins at room temperature. The slides were rinsed with absolute ethanol followed by 3 changes to absolute ethanol (dehydration step). The slides were then mounted with the xylene-based EZ mount (RI = 1.495, cat #9999120, Fisher Scientific, USA) under an uncoated #1.5 coverslip and left to dry at room temperature before being stored at -20°C before imaging. Fields of view were acquired using the EVOS M5000 Imaging System

(ThermoFisher Scientific). To enhance reproducibility of image acquisition, specific details are provided in **Appendix B: Supplementary materials and methods**.

Oil Red O staining

To assess lipid infiltration, the Oil Red O Stain Kit (ORO, cat #ab150678, Abcam) was used on TA tissue sections according to the manufacturer's instructions. Briefly, the sections were fixed using 4% PFA (cat #AAJ61899AP, Fisher Scientific, USA) for 5 min and then rinsed with dH₂O. Sections were incubated in propylene glycol for 5 mins and then incubated in pre-warmed (60°C) ORO Solution for 10 min. Sections were then differentiated in 85% propylene glycol for 1 min, rinsed twice with dH₂O. The sections were then incubated in hematoxylin for 30 seconds before being thoroughly rinsed with tap water, followed by two rinses in dH₂O. The slides were then mounted with PermaFluor aqueous mounting media (RI = 1.4, cat #TA-030-FM, ThermoFisher Scientific) under an uncoated #1.5 coverslip and left to dry at room temperature overnight before storing them at -20°C. To validate this protocol, a liver section (10 µm) was stained using the protocol as described above (**Figure S5**). Fields of view were acquired using the EVOS M5000 Imaging System (ThermoFisher Scientific). To enhance reproducibility of image acquisition, specific details are provided in **Appendix B: Supplementary materials and methods**.

Picrosirius Red staining

To assess tissue fibrosis, the Picrosirius Red Stain Kit (cat #24901, Polysciences, USA) was used on TA tissue sections according to the manufacturer's instructions. Briefly, the sections were processed quickly using 100%-80%-40% EtOH and rinsed using deionized (DI) water before fixing with 4% PFA (cat# AAJ61899AP, Fisher Scientific, USA) for 30 min at room temperature. The sections were then rinsed again with DI water and incubated in Picrosirius Red for 90 minutes.

Then, the sections were rinsed twice for 1 min using 0.1 M hydrochloric acid (HCl) and rinsed again in DI water. The slides were dehydrated in 70% EtOH for 30 seconds and then mounted with the xylene-based EZ mount (RI = 1.495, cat #9999120, Fisher Scientific, USA) under an uncoated #1.5 coverslip and left to dry at room temperature. Slides were stored at -20°C until they were ready to be imaged. Images were acquired using a commercial Zeiss AxioObserver 7-APOLLO inverted microscope (Zeiss, Germany) with Koehler illumination settings (52.5° analyzer, 28° polarizer). To enhance reproducibility of image acquisition, specific details are provided in the **Appendix B: Supplementary materials and methods** section of this report.

Western blot

To collect protein, cells were gently washed using sterile PBS twice before adding ice-cold RIPA Lysis and Extraction Buffer (cat #89901, ThermoFisher Scientific). Protein was then collected and samples were immediately placed on ice before being placed in a tabletop centrifuge for 5 mins at 10 000 xg at 4°C. The supernatant was placed in a fresh 15 mL tube and placed on ice for protein quantification and the pellet was discarded. To extract protein from tissue, GAS muscles were homogenized using the Homogenization of Tissue for Protein Extraction kit (Miltenyi Biotec, Germany) according to the manufacturer's instructions. Briefly, the samples were thawed on ice before being minced into small pieces. Samples were then placed in gentleMACS™ M tubes (cat #130-093-236, Miltenyi Biotec, Germany) after adding ice-cold RIPA Lysis and Extraction Buffer (cat #89901, ThermoFisher Scientific) at a ratio of 1 mL of buffer for each 10 mg of tissue in the same tube. The samples were then quickly inverted and placed on the gentleMACS™ Octor Dissociator with Heaters and the Protein_01 protocol was initiated (cat #130-134-029, Miltenyi Biotec, Germany). After running the program, the samples were centrifuged for 5 mins at 4000 xg

at 4°C. The supernatant was placed in a fresh 15 mL tube and placed on dry ice for protein quantification and the pellet was discarded. Protein quantification was done using the Pierce™ BCA Protein Assay kit (cat #23227, ThermoFisher Scientific) was used according to the manufacturer's instructions.

For the western blot, 40 ug of protein was diluted in Laemmli sample buffer (125 mM Tris-HCl at pH 6.8, 4% SDS, 20% glycerol, 0.05% bromophenol blue, and 200 mM dithiothreitol, which was then quickly vortexed before heating at 90°C for 10 mins to linearize the proteins. Proteins were separated on a 9% sodium dodecyl sulfate-polyacrylamide (SDS) gel, which was run at volts for ~1 hr. The gel was then gently removed transferred onto an Immuno-Blot polyvinylidene difluoride membrane (PVDF, cat #1620177, Bio-Rad Laboratories) using a semi-transfer standard protocol at 4°C. Once the transfer was complete, the membrane was briefly washed in TBST and then blocked for 2 hrs in 5% (w/v) BSA at room temperature on a plate shaker. The membrane was then briefly washed in TBST and incubated with either: mouse anti-MYH1E (1:200, cat #MF20-s, DSHB) or rabbit anti- β -actin (1:200, cat # PA1-183, Invitrogen) primary antibodies in 5% (w/v) BSA overnight on a shaker in a cold room (-20°C). Then, the membrane was rinsed a minimum of 3x 10 mins before incubating with either goat anti-mouse H+L (1:2000, cat # AB205719, Abcam) or goat anti-rabbit H+L (1:2000, cat #AB205718, Abcam) HRP-conjugated secondary antibodies in 5% (w/v) BSA for 1 hr at room temperature on a plate shaker. The membrane was quickly rinsed and placed in TBST before visualizing the bands using Clarity™ Western ECL Substrate (cat #1705060, Bio-Rad Laboratories) in combination with ChemiDoc MP (Bio-Rad Laboratories) under chemiluminescent light.

Cytokine and chemokine multiplex assay

To assess cytokine and chemokine markers in murine tissue and blood serum, the Mouse Cytokine 44-Plex Discovery Assay® (Eve Technologies Corporation, Canada) was used. Samples were prepared according to sample type, available on the Eve Technologies website. Samples were then analyzed according to the company's methodology. Briefly, 45 markers (cytokines, chemokines, growth factors) were multiplexed using the Luminex™ 200 system (Luminex, Austin, TX, USA) in conjunction with Eve Technologies' Mouse Cytokine 44-Plex Discovery Assay® which consists of two separate kits; one 32-plex and one 12-plex (MilliporeSigma, Burlington, Massachusetts, USA), which were run according to the manufacturer's protocol. The cytokines and chemokines analyzed include: Eotaxin, IL-1 α , IL-1 β , IL-6, IP-10, KC, VEGF, IFN β -1, IL-16, MCP-5, MDC, Fractalkine, and TARC (**Figure S6**).

Oxylipin analysis

In the second *in vivo* study, GAS muscles were sent to Creative Proteomics for oxylipin analysis (Creative Proteomics, USA). All samples were labelled blindly to avoid bias and processed according to the company's protocol. Briefly, GAS tissue samples were thawed before adding 100 mg of sample with 300 μ L methanol and quickly vortexed. 1 mL ethyl acetate was then added to the solution and vortexed for an additional 10 min at 25°C. Samples were then centrifuged at 12000 rpm and 4°C for 10 min. The supernatant was transferred into new tube, dry under the nitrogen. The dried extract was resuspended with 120 μ L methanol and then centrifuged at 12000 rpm and 4°C for 10 mins before transferring the supernatant into UPLC-MS/MS vials. The samples were analyzed with an AB SCIEX 5500 mass spectrometry connected with a Waters Acquity Ultra

Performance Liquid Chromatogram (UPLC) with the following conditions: Waters Acquity UPLC BEH C18 column (100 mm × 2.1 mm, 1.7 µm).

Statistical analysis and figures

Statistical analysis: All data is represented as mean ± standard error (SEM). All *in vitro* was done in at least three biological replicates (n=3), except for the C2C12 data, where the data is represented as technical replicates (n=1). *In vivo* work is represented as n=7-8 or n=3-4 (pooled data) for the first and second study, respectively. In the first study, one female mouse was found unexpectedly found dead in her cage at 11 DPI; only food consumption and body mass data was processed for analysis. All data was treated for outliers and normality using ROUT (Q=1%) and the Shapiro-Wilk test (alpha = 0.05), respectively. Statistical analysis was then applied, either using an unpaired Student's t-test with Welch's correction or an ordinary two-way ANOVA with uncorrected Fisher's LSD. All data was analyzed using GraphPad Prism Version 10.5.0 for Windows (GraphPad Software, USA).

Results

1.0 Visual representation of *in vitro* experiments and workflow

Three sets of *in vitro* experiments were conducted to complete the first objective of this project. First, a proof-of-concept experiment using immortalized C2C12 cells was used to establish whether ALA or DHA has a direct effect on cellular differentiation after 4 days of treatment (Figure 4a). Second, primary murine myoblasts from adult and aged mice were isolated and grown in the absence of treatment in real-time to establish the diminished myogenic capacity of myoblasts associated with aging (Figure 4b). Third, aged primary murine myoblasts were treated with DHA or control vehicle during proliferation and differentiation to determine whether DHA supplementation could restore the myogenic capacity of aged myoblasts (Figure 4c).

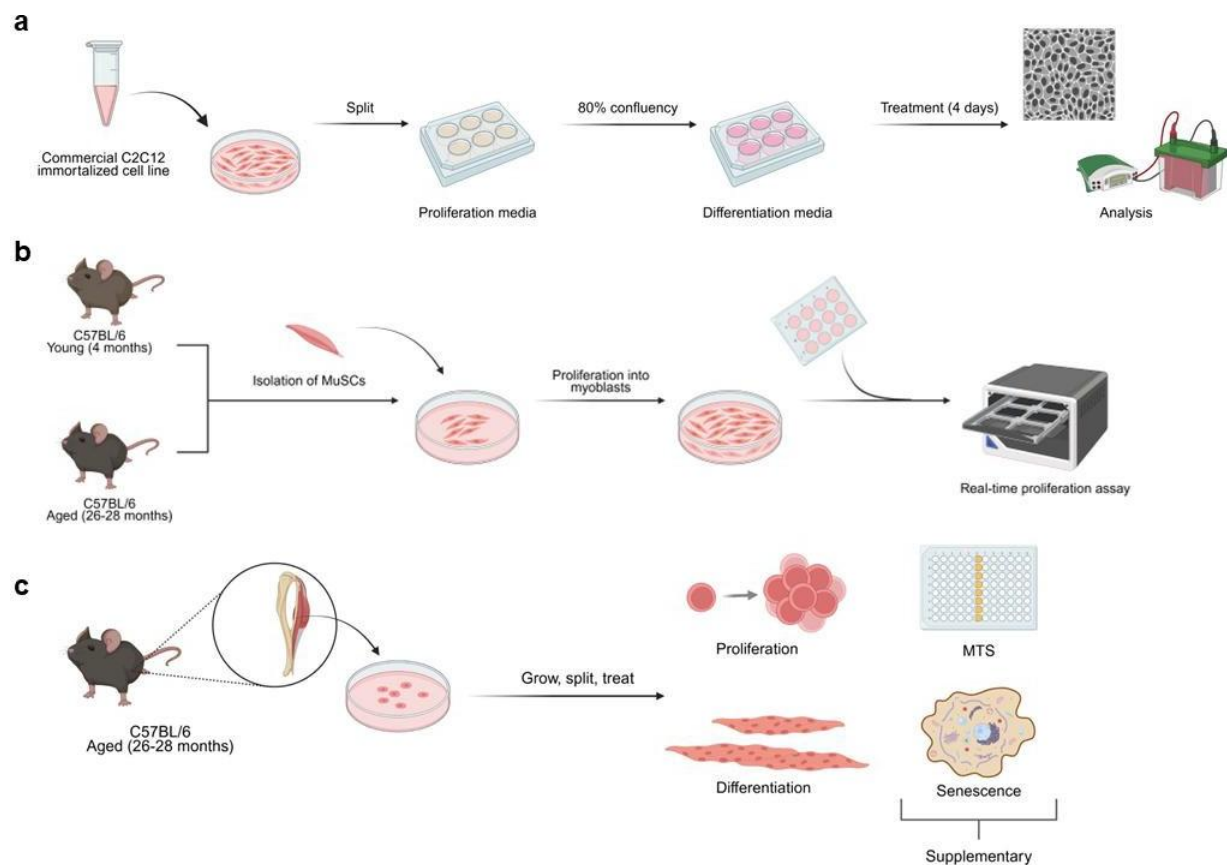


Figure 4. Visual summary of *in vitro* experimental methods relating to aim 1 of this project. a) Schematic representation of proof-of-concept experiment using C2C12 immortalized cells. **b)** Schematic

representation of untreated primary myoblast lines for real-time proliferation assay. **c)** Schematic representation of experiments on treated (50 nM DHA) versus untreated (vehicle) myoblasts during myogenesis.

1.1 Proof-of-concept experiments using C2C12 myoblasts

To establish a proof-of-concept, C2C12 myoblasts were treated with either vehicle (abs. EtOH), ALA or DHA at different concentrations (25-100 nM) for 4 days in differentiation media. From this initial screening (data not shown) in C2C12 myoblasts, we observed that 50 nM was the optimal concentration to use. At greater concentrations (>50 nM), we observed a decline in the phenotypic qualities of the myotubes (e.g., morphology, diameter). Using our primary aged myoblasts, we also conducted a viability screening, which exhibited similar findings (**Figure S1c**). Our results demonstrate that there is no effect on the differentiation and fusion indexes (Figure 5a-c) with either n-3 PUFA treatment compared to control. Furthermore, we do not observe any difference in MyHC protein level in C2C12 myoblasts treated with DHA for 4 days (Figure 5e, f). However, we observe that there is a significant increase in myofiber diameter (Figure 5a, d) in the DHA-treated myoblasts compared to both the vehicle and ALA-treated myoblasts. Smaller myotube size is typically associated with muscular atrophy, due to the decline in protein synthesis and loss of muscle^{58,64}. Based on these data, we decided to further investigate the effect of DHA at 50 nM for subsequent experiments.

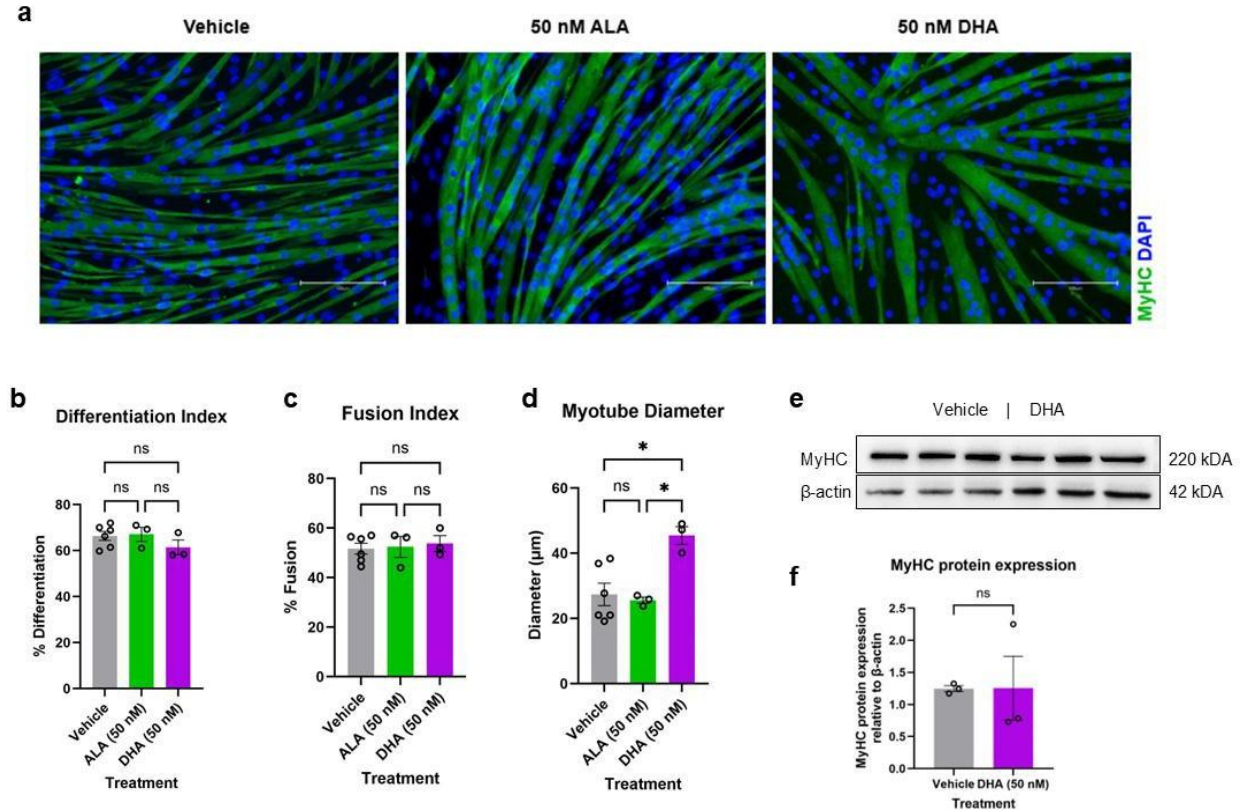


Figure 5. In vitro modelling of C2C12 myoblasts after 4 days of differentiation treated with either vehicle (abs. EtOH), alpha-linolenic acid (ALA, 50 nM), or docosahexaenoic acid (DHA, 50 nM). a) Representative images of MyHC + DAPI immunofluorescent (IF) staining (scale bar = 150 μm). Quantification of **b)** differentiation index, **c)** fusion index (minimum 2 nuclei), and **d)** myotube diameter (μm, minimum 2 nuclei). **e)** Representative western blots and **f)** quantification of MyHC (relative to β-actin as loading control). Data are represented as mean ± SEM (n=3 technical replicates), * p<0.05.

2.1 Aged myoblasts have a diminished proliferation capacity

While C2C12 myoblasts are widely used in muscle research for *in vitro* modelling, they are immortalized cells and therefore may not fully represent biological changes associated with aging. To rectify this, we isolated from adult (4 months, labeled as ‘young’) and geriatric (26-28 months) C57BL/6 mice to generate primary myoblast cell lines. Untreated young and aged myoblasts were plated at a low confluency (~10%) to monitor their growth in real-time using the Incucyte® S3 Live-Cell Analysis System (Sartorius) over the course of 4 days (Figure 6a). We observe a typical growth curve in the young myoblast line whereas the aged myoblasts remain at a low confluency

after 4 days (Figure 6a, b). Thus, we have demonstrated a decline in the rate of proliferation in aged myoblasts. Subsequently, we wanted to determine whether myoblast proliferation could be rescued with DHA (50 nM) supplementation.

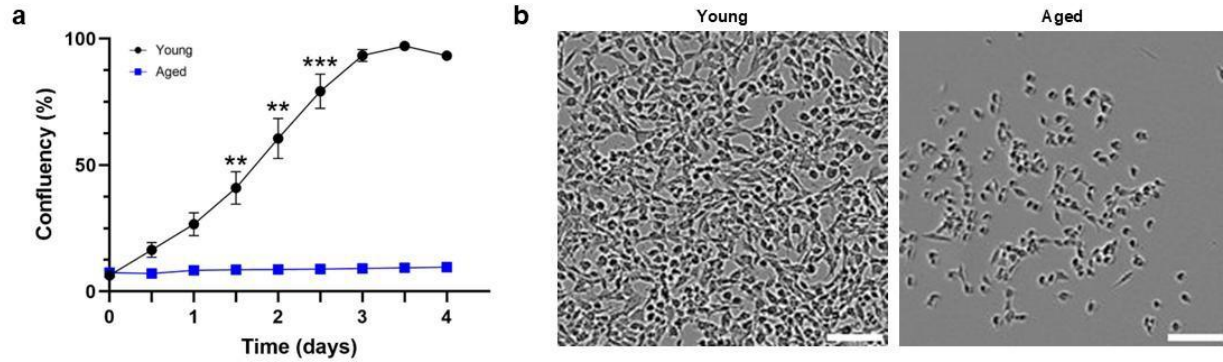


Figure 6. Myoblast proliferation on untreated primary cells. a) Real-time proliferation assay curve of untreated young (4 months) and aged (26-28 months) myoblasts. **b)** Representative images in phase contrast obtained using Incucyte® software (scale bar = 40 μm). Data are represented as mean ± SEM (n=3 technical replicates), ** p<0.005, *** p<0.001.

2.2 DHA may improve proliferation capacity in aged myoblasts

Based on our initial screening using C2C12 myoblasts, in conjunction with our real-time proliferation assay using murine primary myoblasts, we wanted to determine whether DHA at 50 nM could restore proliferative capacity and myogenic capacity. To do so, aged myoblasts at similar passage numbers were treated with either vehicle or DHA (50 nM) overnight (16 hrs) and then either fixed for IF staining or processed for protein collection. Our results by IF, show an increase in Ki67⁺ cells (proliferation marker, in green) in cells treated with DHA compared to vehicle (Figure 7a, b). Similarly, we see a tendency towards increased MyoD protein expression, denoting cells that have committed to the myogenic lineage (Figure 7c, d).

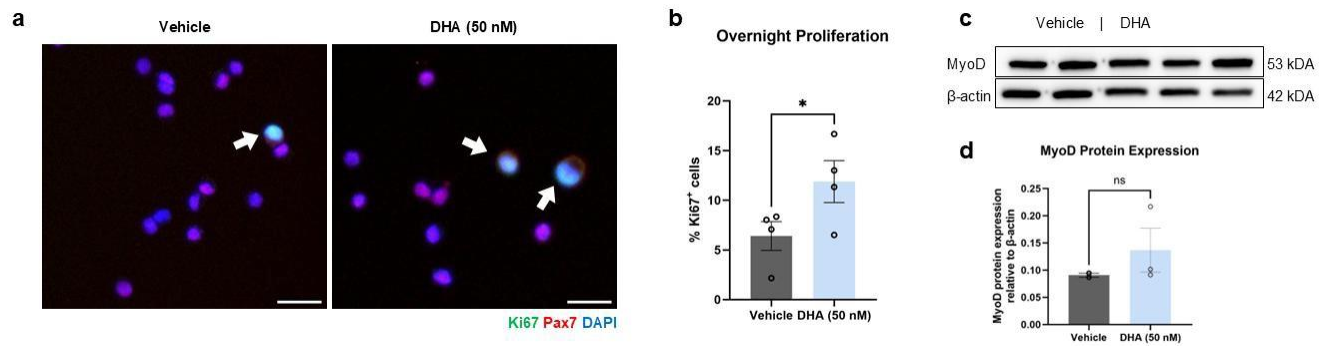


Figure 7. Effect of DHA (50 nM) versus vehicle (abs. EtOH) on proliferation in aged myoblasts. a) Representative images of Ki67 + Pax7 + DAPI staining on myoblasts (scale bar = 20 μ m). Arrows indicate Ki67⁺ cells. **b)** Quantification of Ki67⁺ cells. **c)** Representative western blots and **d)** quantification of MyoD (relative to β -actin as loading control). Data are represented as mean $\pm$ SEM (n=4 for IF; n=2-3 for WB). * $p < 0.05$

3.1 DHA does not impact differentiation capacity on aged myoblasts

Based on our findings using C2C12 myoblasts and our results on aged myoblast proliferation, we wanted to investigate whether DHA (50 nM) could improve differentiation of aged myoblasts. To do so, at 70-80% confluency, cells were switched from proliferation media into differentiation media, supplemented with either 50 nM DHA or control vehicle (abs. EtOH) for 4 days and 6 days (media changed every 48hrs) and then stained for MyHC (Figure 8a, g) or collected for protein (Figure 8e, k). After 4 days and 6 days of differentiation, we do not observe any differences in differentiation index (DI), fusion index (FI), or myotube diameter (Figure 8a-d, g-j) between the DHA-treated and the control group. Our results by western blot show a tendency towards an increase in MyHC protein level (relative to β -actin) after 4 days of differentiation but not at 6 days (Figure 8e, f, k, l). These results suggest that DHA (50 nM) may play a direct role in rescuing proliferation capacity in aged myoblasts but not differentiation capacity of aged myoblasts.

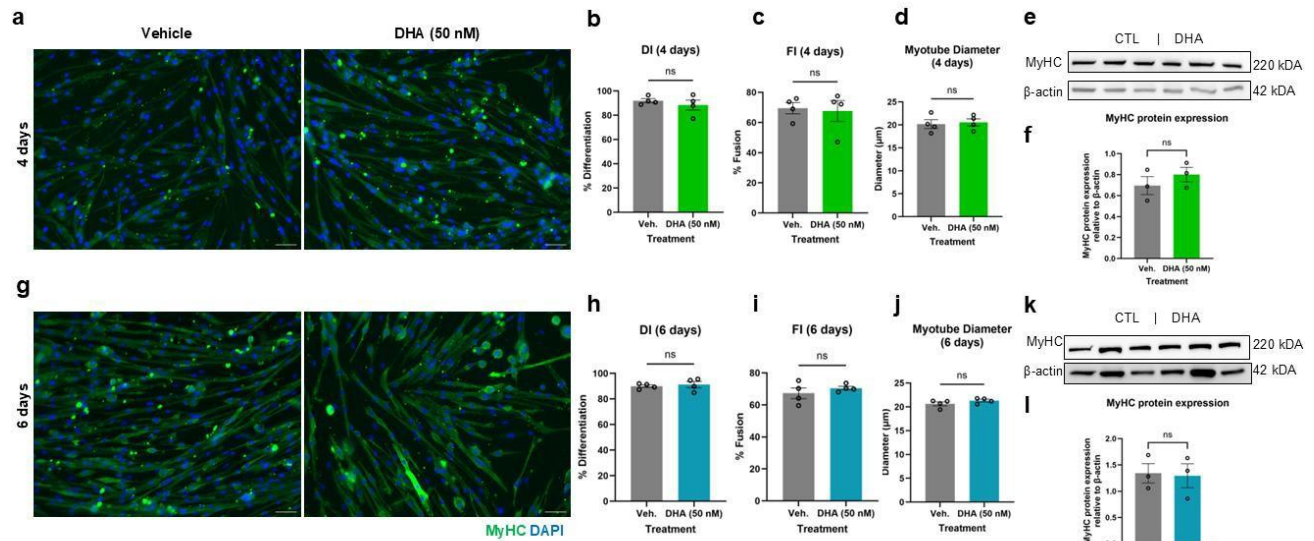


Figure 8. Effect of DHA (50 nM) on differentiation of aged myoblasts. **a+g)** Representative images of MyHC + DAPI staining (scale bar = 50 μm) after 4 (a) and 6 (g) days of differentiation. Quantification of **b+h)** differentiation index (DI), **c+i)** fusion index (FI), and **d+j)** myotube diameter after 4 and 6 days of differentiation, respectively. **e+k)** Representative western blots for MyHC and **f+i)** quantification of MyHC protein expression relative to β-actin after 4 and 6 days of differentiation, respectively. Data are represented as mean ± SEM (n=4 for IF data, n=3 for WB data).

4.0 Visual representation of *in vivo* experiments and workflow

Two separate *in vivo* experiments were conducted to complete the second objective of this project. First, an experiment with male and female aged C57BL/6 mice (22-24 months old) were acclimated to either an SBO or 1% (w/w) DHA, similar dose to previously published research, isoenergetic diet²⁰⁰⁻²⁰² (see **Table S2** for diet composition) before being subjected to IM injury in the TA using CTX. At 21 DPI, muscle contractility properties, histology/immuno-histochemistry, and inflammatory markers were assessed (Figure 9a). In the second experiment, male mice were subjected to one of three diets (SBO, 1% DHA or 2% DHA) before being subjected to IM injury in the TA using CTX. At 14 and 21 DPI, muscle contractility properties, and fiber size were assessed in TA cross-sections (supplementary data). Oxylipin concentrations in the gastrocnemius muscle (uninjured muscle samples) were measured by LCMS (Figure 9b).

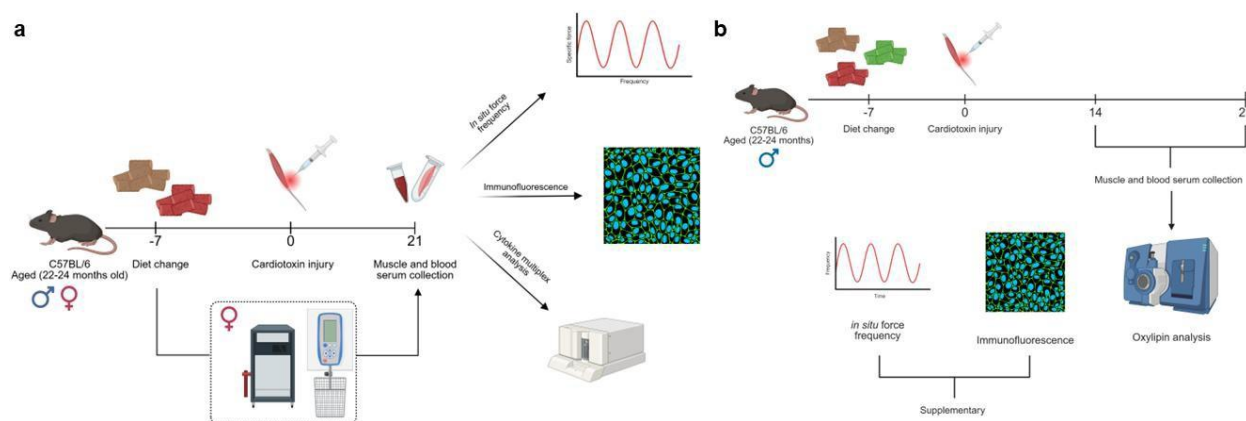


Figure 9. Schematic representations of two *in vivo* experiments completed; results are described below. **a)** Schematic representation of study 1: Aged male and female C57BL/6 mice were acclimated to their respective diets (SBO or 1% DHA) before being subjected to IM injury in the TA using CTX. At 21 DPI, *in situ* force measurements were taken followed by muscle and blood collection for further analysis. Female mice were subjected to body composition and grip strength tests once per week (done on separate days). **b)** Schematic representation of study 2: Aged male C57BL/6 mice were acclimated to their respective diets (SBO, 1-2% DHA) before being subjected to IM injury in the TA using CTX. At 14 and 21 DPI (n=1-3), *in situ* force measurements were taken followed by muscle and blood collection for further analysis (IF and oxylipin analysis). Not included in schematic representation: for the oxylipin analysis in study 2, an additional group, “young” (12-week C57BL/6 male mouse), was added for analysis.

5.0 Effect of an enriched DHA diet on food consumption and mouse body composition

Aged mice were subjected to a specialized enriched FA diet, containing 1% (w/w) DHA, or an isoenergetic control diet (SBO) over the course of the experiment (Figure 10a). Given that these are specialized diets that differ from the regular chow diet, food consumption (g consumed/day/mouse) and mouse weight was recorded (Figure 10b-g). Our results show there is no difference in food consumption between male and female cohorts, and the overall average food consumption is similar between both diet groups (Figure 10b-d). There is a slight decrease in body mass in the mice fed the 1% DHA diet, which is observed in the female cohort but not in the male cohort (Figure 10e-g). Additionally, the female cohort was subjected to body composition measurements, which show a decrease in lean and fat mass in the 1% DHA diet, but this change is not significant even though overall body mass did decrease significantly (Figure 10h).

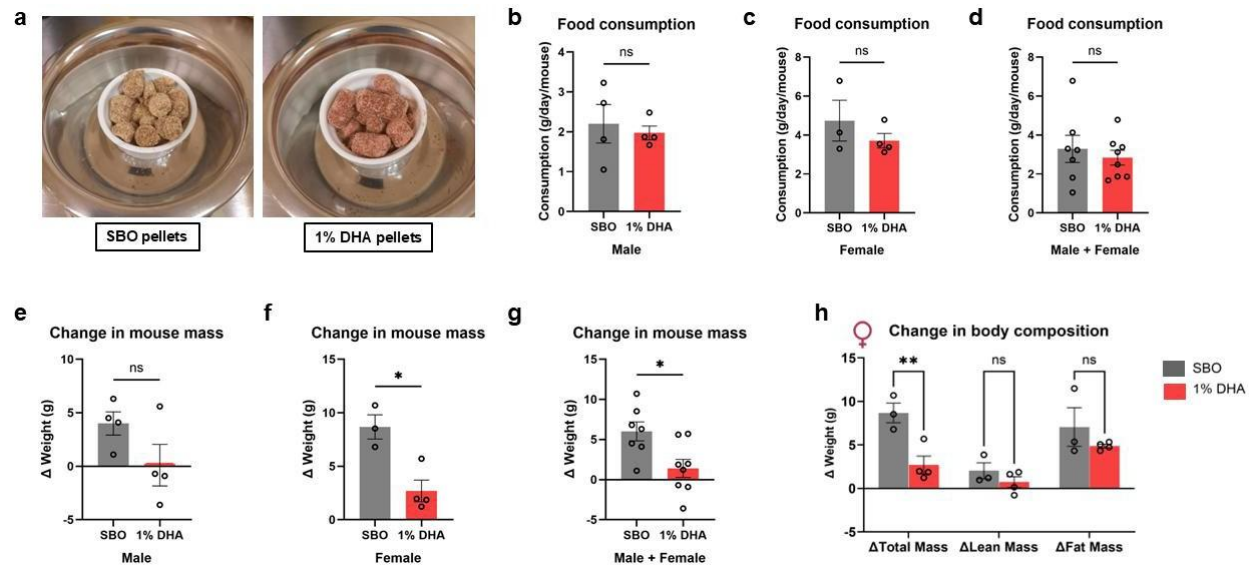


Figure 10. Effect of enriched DHA diet versus isoenergetic SBO diet on food consumption, body mass, and body composition in aged male and female mice. **a)** Representative images of the SBO and 1% DHA pellets. Food consumption (g/day/mouse) of **b)** male, **c)** female, and **d)** mixed cohorts for each diet group. Change in mouse mass of **e)** male, **f)** female, and **g)** mixed cohorts for each diet group. **h)** change in body composition (female only) for total mass, lean mass, and fat mass. Changes in mass were calculated as 21 DPI – basal mass (day 0). Data are represented as mean $\pm$ SEM ($n=7-8$; $n=4$ male, $n=3-4$ female), * $p<0.05$, ** $p<0.01$

5.1 Assessment of muscle morphology

Qualitative assessment

To induce muscle injury, an IM injection of CTX was used. To validate that the injury was successful, a H&E histology stain (Figure 11a) and IF stain of laminin and DAPI (Figure 11b) was used to show centrally nucleated fibers (CNF)^{47,203-206}. An average of 20% CNF has been previously reported as a typical baseline for aged muscle and in muscle pathologies, whereas younger muscle samples are closer to 0% CNF^{130,207}. At 21 days, in an injury model, muscle repair (e.g., immune infiltration and clearance, satellite cell activation and proliferation) has been completed, and the process of tissue remodeling has begun^{13,23}. Therefore, we do not expect regenerative myofibers to have become fully mature, where the nucleus is located at the periphery of the fiber²⁰⁴.

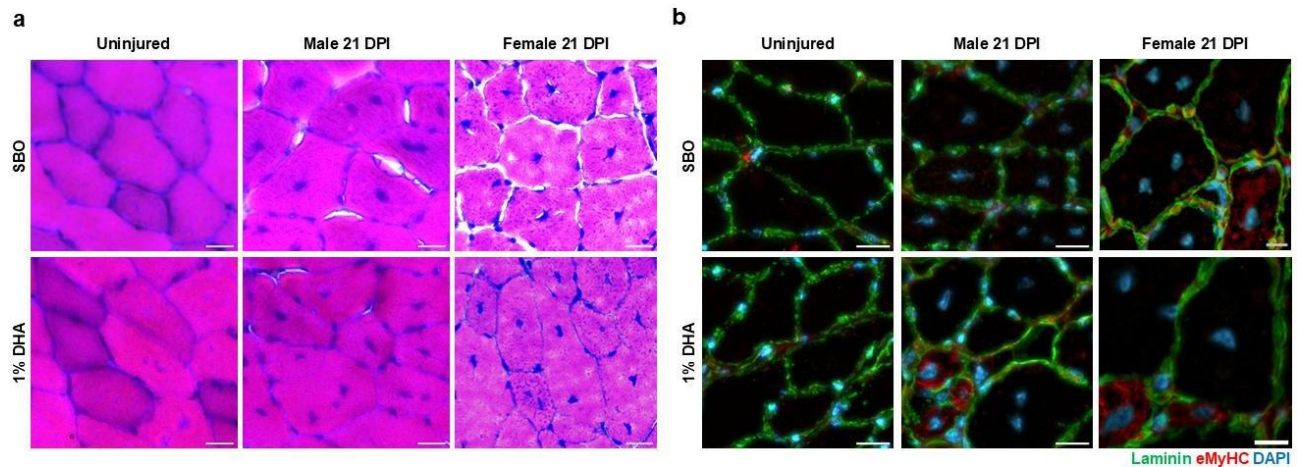


Figure 11. Histological and immunofluorescent validation of CTX injury at 21 DPI in male and female aged mice. a) Representative images of H&E histology stain (scale bar = 20 μ m). b) Representative images of Laminin (green), eMyHC (red), DAPI (blue) staining (scale bar = 200 μ m).

Quantitative assessment

To quantitatively assess whether the diet affected the size of the muscle fibers, CSA and minimum Feret's diameter were measured in uninjured and injured tissue. While CSA reports the total area of each fiber, the minimum Feret's diameter refers to the minimum width measured across each fiber²⁰⁸. These parameters may be used to identify muscle morphology, which may be affected in old animals (e.g. degradation of fibers, skeletal muscle atrophy)^{11,209,210}, and may be used to assess the extent of muscle repair or predict contractile force outcomes^{208,211,212}. In our findings, we do not observe any differences between the distribution of fiber size between diet groups pre- or post-injury (Figure 12a-c). There is also no significant difference in the average CSA in either diet group (Figure 12d-f). Similarly, our findings demonstrate no differences in the distribution of minimum Feret's diameter (Figure 12g-i) and the averages for the minimum Feret's diameter (Figure 12j-l) by diet group pre- and post- injury.

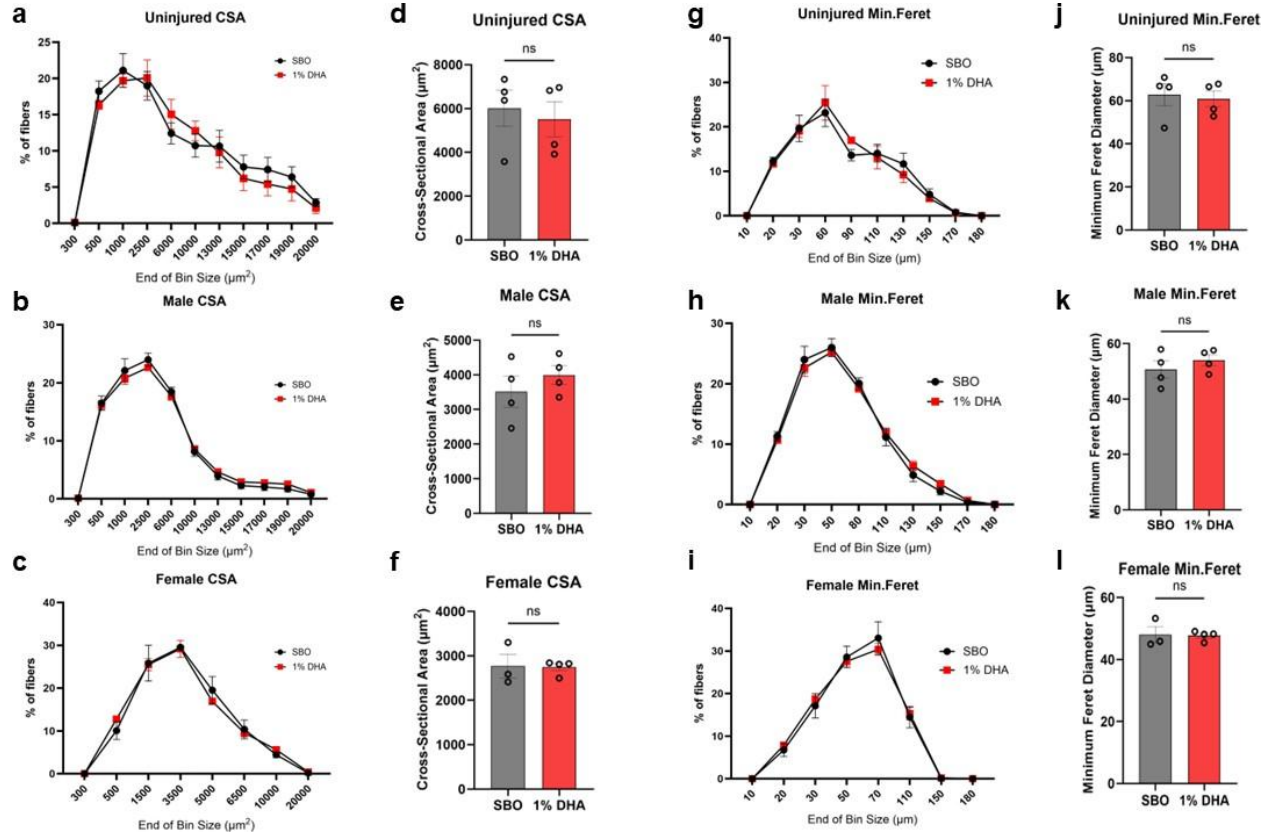


Figure 12. Effect of DHA enriched diet versus isoenergetic SBO on CSA and minimum Feret's diameter in aged mice. CSA distribution curve in **a)** uninjured, **b)** male, and **c)** female sections. Average CSA in **d)** uninjured, **e)** male, and **f)** female sections. Distribution curve of minimum Feret's diameter in **g)** uninjured, **h)** male, and **i)** female sections. Average Min. Feret diameter in **j)** uninjured, **k)** male, and **l)** female sections. Data are represented as mean $\pm$ SEM (n=4, uninjured, male injured; n=3-4 female injured).

Using an IF staining of laminin, eMyHC, and DAPI, the percent of CNF fibers and the percent of newly formed fibers (eMyHC⁺)²¹³ were quantified. Our results indicate no sex-specific differences in percentage of CNF between diet groups, even in uninjured tissue (Figure 13a-c). In both SBO and DHA diets, there is significantly more CNF post-injury (Figure 13d). Regarding newly formed fibers, there are no differences in percentage of eMyHC⁺ at basal level with either diet group, and there are no sex-specific differences observed either (Figure 13e-g). Overall, we observe a tendency towards more eMyHC⁺ fibers post-injury (Figure 13h), as expected at 21 DPI, though the overall percentage remains quite low.

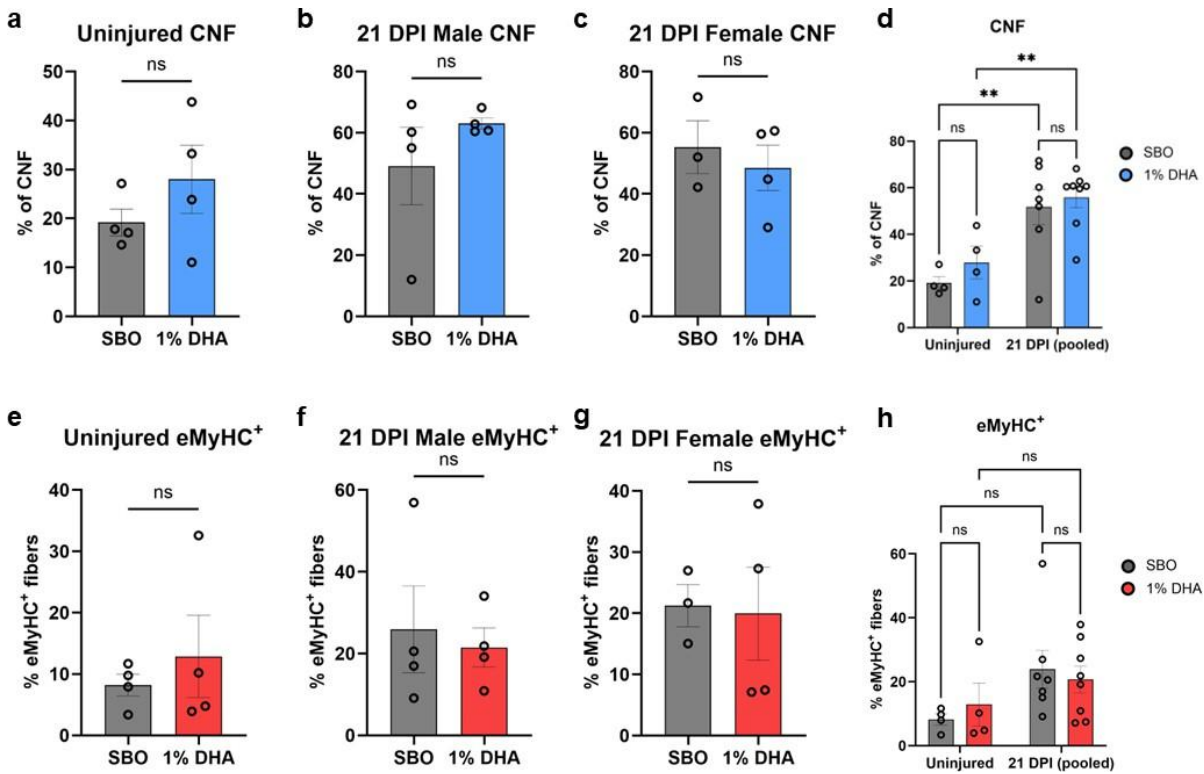


Figure 13. Effect of DHA enriched diet versus isoenergetic SBO diet on CNF and eMyHC⁺ fibers in aged mice. Percentage of CNF in **a)** uninjured, **b)** male, and **c)** female sections. **d)** Percentage of CNF in uninjured versus injured sections. Quantification of eMyHC⁺ fibers in **e)** uninjured, **f)** male, and **g)** female sections. **h)** Quantification of eMyHC⁺ fibers in uninjured versus injured sections. Data are represented as mean ± SEM (n=4, uninjured, male injured; n=3-4 female injured), **p<0.01

5.2 Effect of enriched DHA diet on early and late myogenic markers

We were interested in investigating whether there were any lasting differences in early (e.g., Pax7, MyoD) and late (e.g., myogenin) myogenic markers at 21 DPI, which could impact subsequent rounds of muscle regeneration, as seen in aged populations. To accomplish this, tissue cross-sections were co-stained with Pax7 (red; myogenic cells), myogenin (green; differentiating cells), and DAPI (blue; nuclear marker). We performed a co-staining of Pax7 and myogenin, allowing us to distinguish between differentiating and non-differentiation cells at 21 DPI (Figure 14a). At the basal level, before injury, we do not observe any differences between the diet groups in either

Pax7⁺ or myogenin⁺ cells (Figure 14b, f). Likewise, we do not observe any sex-specific differences in the number of Pax⁺ cells post-injury (Figure 14c, d). However, when pooling the data, we observe a significant decline in Pax7⁺ cells in the DHA diet group post-injury compared to SBO (Figure 14e). In the differentiating cells (myogenin⁺), we observe a significant decline in myogenin⁺ cells in DHA-fed female mice only, but no difference in the male mice (Figure 14g, h). However, when pooling the samples (eliminating sex-differences), we no longer observe any differences in myogenin⁺ cells after injury, though there is a tendency towards fewer differentiating cells in the DHA-fed group (Figure 14i).

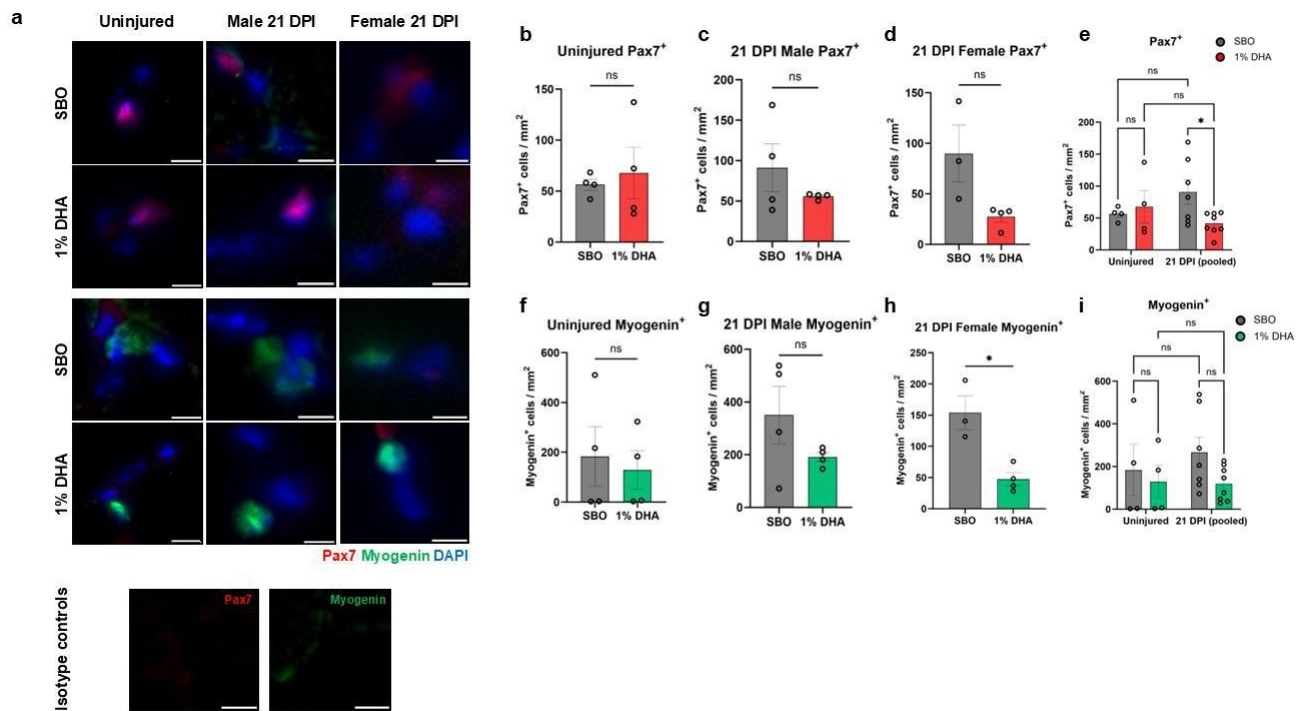


Figure 14. Effect of DHA enriched diet on early and late myogenic markers in aged mice at 21 DPI. **a)** Representative images of Pax7 (red), myogenin (green), and DAPI (blue) immunofluorescent staining (scale bar = 10 μ m). Quantification of Pax7⁺ cells in **b)** uninjured, **c)** male, **d)** female, and **e)** general effect (uninjured versus injured) tissue sections. Quantification of myogenin⁺ cells in **f)** uninjured, **g)** male, **h)** female, and **i)** general effect (uninjured versus injured) tissue sections. Data are represented as mean $\pm$ SEM (n=4, uninjured, male injured; n=3-4 female injured), *p<0.01.

Our previous results demonstrate that the number of Pax7⁺ cells remains unchanged after injury in both diet groups, however, these cells could either be part of the quiescent lineage for self-renewal or they could be part of the myogenic lineage to proliferation (MyoD⁺) and myogenic commitment. We therefore wanted to assess if DHA could influence myogenic potential in aged muscle tissue, even at 21 DPI when muscle regeneration should be complete. This was done by co-staining cross-sections with MyoD (purple) and DAPI (blue) to highlight cells committed to the myogenic lineage (Figure 15a). Our findings demonstrate there are no sex-specific differences in the proportion of MyoD⁺ cells at 21 DPI (Figure 15b-d). We observe a general reduction in MyoD⁺ cells post-injury, and this tendency is significant in the DHA diet group but not the SBO diet group (Figure 15e). With all tissue sections being stained with either Pax7/myogenin or MyoD, we determined the relative distribution of myogenic markers pre- and post-injury. In general, there is a larger distribution of myogenin⁺ cells post-injury, irrespective of diet, compared to basal status (Figure 15f-h).

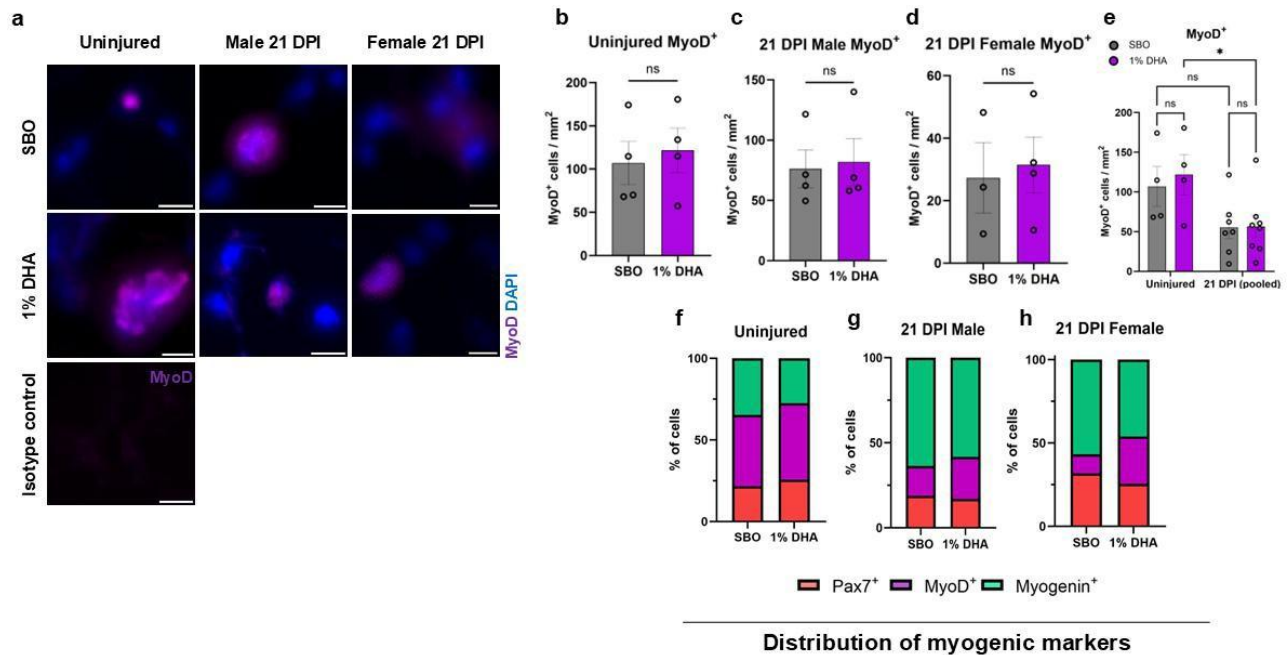


Figure 15. Effect of DHA enriched diet versus isoenergetic SBO diet on proliferative capacity and distribution of myogenic markers in aged mice. **a)** Representative images of MyoD (purple) and DAPI (blue) immunofluorescent staining (scale bar = 10 μ m). Quantification of MyoD⁺ cells in **b)** uninjured, **c)** male, **d)** female, and **e)** general effect (uninjured versus injured) tissue sections. Myogenic distribution in **f)** uninjured, **g)** male, and **h)** female tissue sections. Data are represented as mean $\pm$ SEM (n=4, uninjured, male injured; n=3-4 female injured), *p<0.01.

5.3 Effect of enriched DHA diet effect on inflammation in the muscle

Macrophages

As previously mentioned, n-3 PUFAs are bioactive lipids known for promoting anti-inflammatory signals. Given that the muscle regeneration process is partially mediated by the immune response post-injury, in conjunction with the anti-inflammatory properties of n-3 PUFAs, we wanted to establish whether these lipids could modulate the aberrant immune response associated with aged skeletal muscle repair. First, we wanted to investigate the effect of n-3 PUFAs in macrophages in the skeletal muscle tissue; to do so, an immunofluorescent staining of F4/80 (red), a macrophage marker, and CD206 (green), a “M2” or anti-inflammatory macrophage marker^{14,65,133} were co-stained with DAPI (blue) to verify the number of macrophages and their phenotype (Figure 16a).

Our findings show no statistically significant differences in the total number of macrophages ($F4/80^+$) present within the tissue at basal levels and there are no sex-specific differences in the total number of macrophages post-injury (Figure 16b-d). However, when comparing pre- and post-injury, there is an elevation of macrophages, regardless of diet (Figure 17e), which is expected^{5,13,23}. Of these macrophages, just under half possess the M2 (anti-inflammatory) marker, CD206, at basal level (Figure 16f). Moreover, we do not observe any sex-specific differences in the percent of M2 macrophages post-injury (Figure 16g, h). After pooling the samples together, we observe that the amount of M2 macrophages remains the same after injury in the SBO-fed mice, whereas the DHA-fed mice have a significant increase in M2 macrophages (Figure 16i).

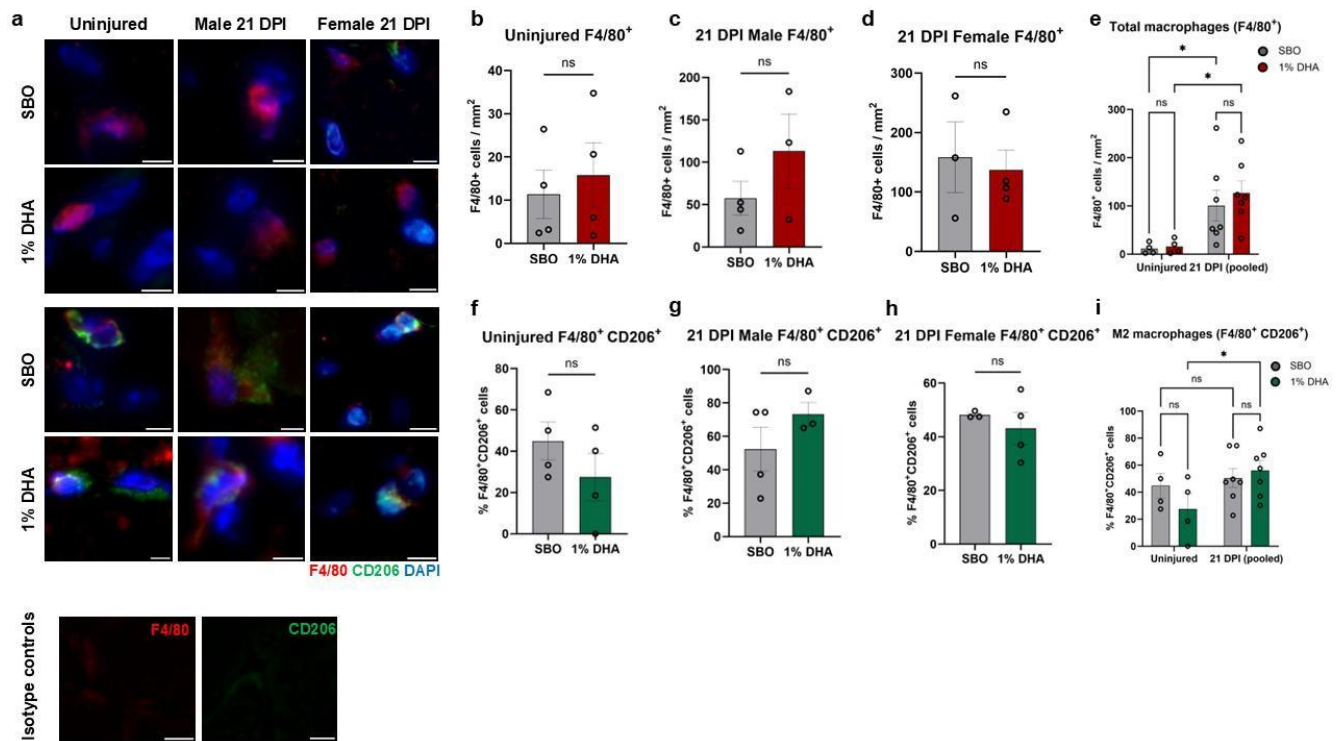


Figure 16. Effect of DHA enriched diet versus isoenergetic SBO diet on tissue-resident macrophages in aged mice. **a)** Representative images of F4/80 (red), CD206 (green), and DAPI (blue) immunofluorescent staining (scale bar = 10 μ m). Quantification of total macrophages ($F4/80^+$ cells) in **b)** uninjured, **c)** male, **d)** female, and **e)** general effect (uninjured versus injured) tissue sections. Quantification of M2 anti-inflammatory macrophages ($F4/80^+ CD206^+$ cells) in **f)** uninjured, **g)** male, **h)** female, and **i)** general effect (uninjured versus injured) tissue sections. Data are represented as mean $\pm$ SEM (n=4, uninjured; n=3-4 male or female injured), *p<0.01.

Neutrophils

Neutrophils act as the first responders after an injury; they promote the initial pro-inflammatory response and then facilitate tissue regeneration with the help of macrophages. A co-staining of Ly6G and DAPI was used to identify any neutrophils before and after injury (Figure 17a). Interestingly, there is a significant decrease in neutrophils at the basal level in the DHA group compared to the SBO group (Figure 17b). We do not observe any sex-differences post-injury, nor when the samples are pooled together (Figure 17c-e), indicating that the number of neutrophils remain near basal status at 21 DPI, regardless of diet.

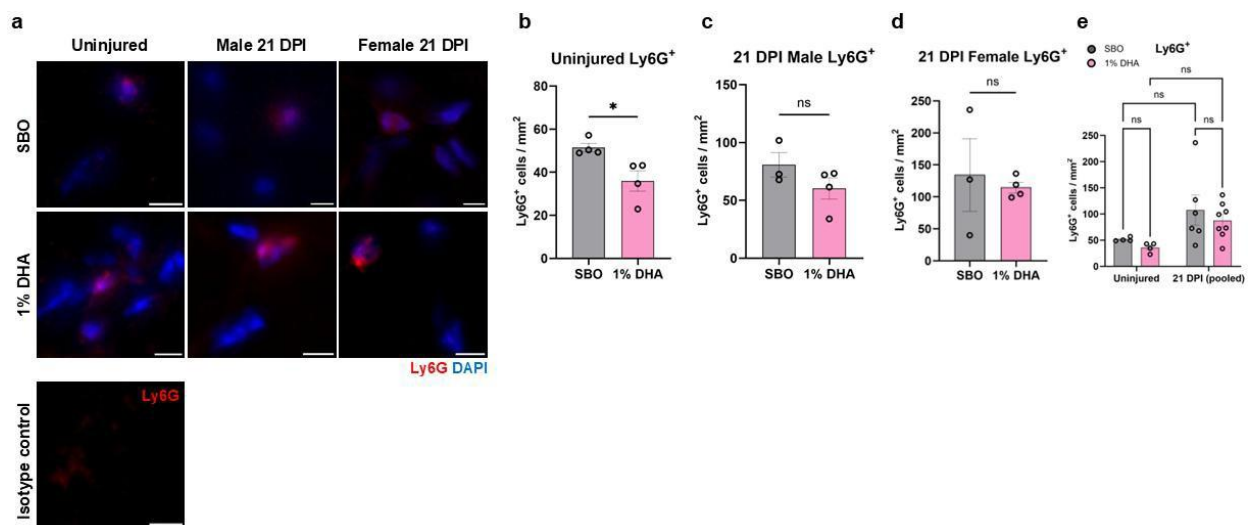


Figure 17. Effect of DHA enriched diet versus isoenergetic SBO diet on neutrophils in aged mice. a) Representative images of Ly6G (neutrophils; red) and DAPI (blue) immunofluorescent staining (scale bar = 10 μ m). Quantification of Ly6G⁺ cells in **b**) uninjured, **c**) male, **d**) female, and **e**) general effect (uninjured versus injured) tissue sections. Data are represented as mean $\pm$ SEM (n=4, uninjured; n=3-4 male or female injured), *p<0.01.

Fibrosis

To measure fibrosis, the amount and the type of collagen within the tissue was quantified after imaging under polarized light (Figure 19a). Given that collagen supports muscle fiber structural

integrity, there is a basal level of collagen present within the tissue (Figure 18a, b). At 21 DPI, we observe no significant differences in the amount of collagen present in the skeletal muscle from either diet or sex (Figure 18a, c, d). When comparing uninjured to injured, we observe no significant difference in the amount of collagen present after the CTX injury (Figure 18a, e).

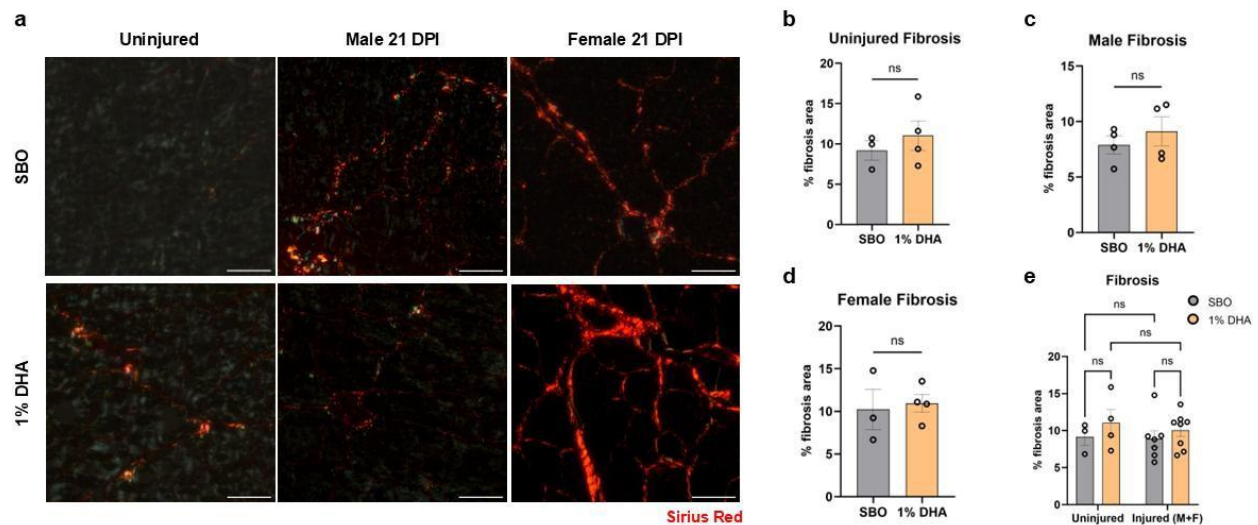


Figure 18. Effect of DHA enriched diet versus isoenergetic SBO diet on fibrosis in aged mice. a) Representative images of picrosirius red staining (scale bar = 50 μ m) under polarized light. Quantification of fibrotic area in **b)** uninjured, **c)** male, **d)** female, and **e)** general effect (uninjured versus injured) tissue sections. Data are represented as mean $\pm$ SEM (n=3-4, uninjured, female injured, n=4 male injured).

5.4 Enriched FA diet effect on metabolism

Fiber types

Myofibers are generally divided into slow or fast twitch fibers, partially based on their metabolic properties (e.g., substrate preference, number of mitochondria)²¹⁴. Fiber type is plastic and can change due the tissue microenvironment and use (or disuse) of the muscle^{213,215}. In older adults, as myofibers degrade (muscle wasting), fiber plasticity declines and leads to changes in mobility. We identified fiber types contained within the TA through IF staining to examine whether an enriched n-3 PUFA diet had an effect on restoring fiber diversity in aged mice (Figure 19a). Our findings indicate no statistical differences between fiber type pre- and post- injury in male and in female

mice for either diet-type (Figure 19b-d). Regarding the relative distribution of fiber types, we observe fewer MyHC IIA and MyHC IIB fibers post-injury compared to basal, irrespective of diet (Figure 19e). However, the approximate ratio of fast/slow (MyHC IIA/B/X to MyHC I) remains similar before and after injury. These findings complement the contractile properties (**Figure 21**), where the muscle function is similar to basal level, regardless of diet group.

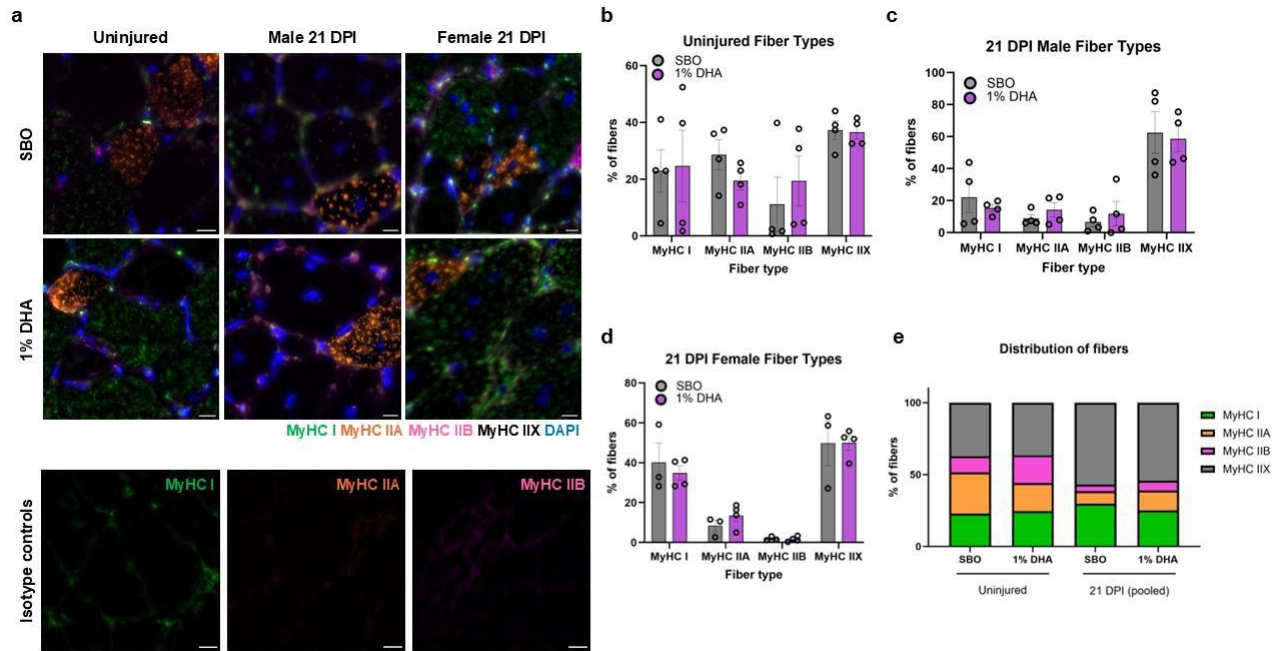


Figure 19. Effect on myofiber type and distribution in DHA enriched diet versus isoenergetic SBO diet in aged mice. **a)** Representative images MyHC I (green), MyHC IIA (orange), MyHC IIB (pink), MyHC IIX (black), DAPI (blue) immunofluorescent staining (scale bar = 10 μ m). Quantification of each fiber type in **b)** uninjured, **c)** male, and **d)** female tissue sections. **e)** Distribution of fibers between uninjured and injured sections. Data are represented as mean $\pm$ SEM (n=4, uninjured, male injured, n=3-4 female injured).

Lipid droplet infiltration

Given that lipid droplet infiltration in skeletal muscle is a known driver of age-related muscle wasting^{46,102,127}, we wanted to examine whether lipid droplets remain in the skeletal muscle. To do so, we used an ORO kit, which stains for neutral lipids (Figure 20a). Our findings demonstrate that

at basal levels, there is essentially no lipid accumulation within the tissue, irrespective of diet (Figure 20b, e). However, at 21 DPI, there is a significant increase in lipid droplets in the SBO diet group compared to the DHA group (Figure 20e), and this increase is specific to the male cohort compared to the female cohort (Figure 20c, d).

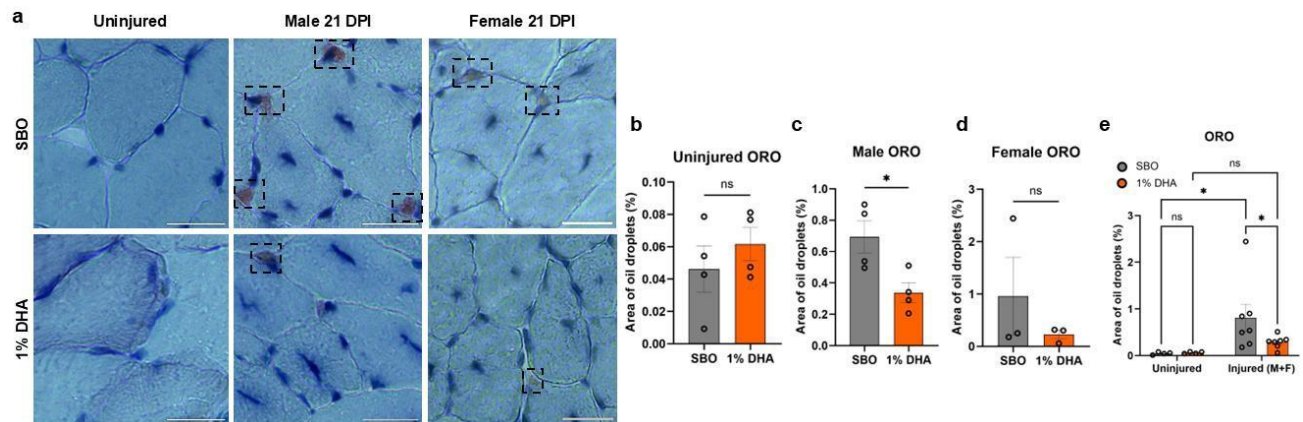


Figure 20. Effect of DHA enriched diet versus isoenergetic SBO diet on fatty lipid infiltration in aged mice. **a)** Representative images oil red o (ORO) staining in brightfield imaging (scale bar = 50 μ m). Quantification of oil droplet area in **b)** uninjured, **c)** male, **d)** female, and **e)** general effect (uninjured versus injured) in tissue sections. Data are represented as mean $\pm$ SEM (n=4, uninjured, male injured, n=3 female injured), *p < 0.01.

5.5 Effect of an enriched DHA diet on muscle contractile properties

Loss of muscle mass in aging is associated with a loss of muscle strength^{1,2,45}. Thus, we wanted to investigate whether an enriched DHA diet could restore muscle contractile properties (muscle force) after an IM injury at 21 DPI. To accomplish this, a known electric frequency can be applied through the sciatic nerve to stimulate the muscle *in situ* (Figure 21a). The force exerted by the muscle can then be measured (force frequency) and normalized to the TA length and weight (specific force frequency)¹⁶⁷. Regarding the muscle itself, we do not observe any sex-specific differences in TA weight post-injury (Figure 21b-d). Our results show no significant differences in force frequency or in specific force frequency between sexes and diet groups (Figure 21e-h, j-

m). We observe a slight decline in muscle contractile properties at 21 DPI compared to uninjured (Figure 21i, n). Finally, we measured muscle fatigue index (repeated muscle stimulation with short rest periods). Our results show similar trends in fatigue index between sexes and between injured versus uninjured (Figure 21o, p). The female cohort was subjected to a grip test once per week. Our results show an increase in grip strength in the 1% DHA diet group compared to the SBO diet at 21 DPI, but no differences in basal or pre-injury (Figure 21q).

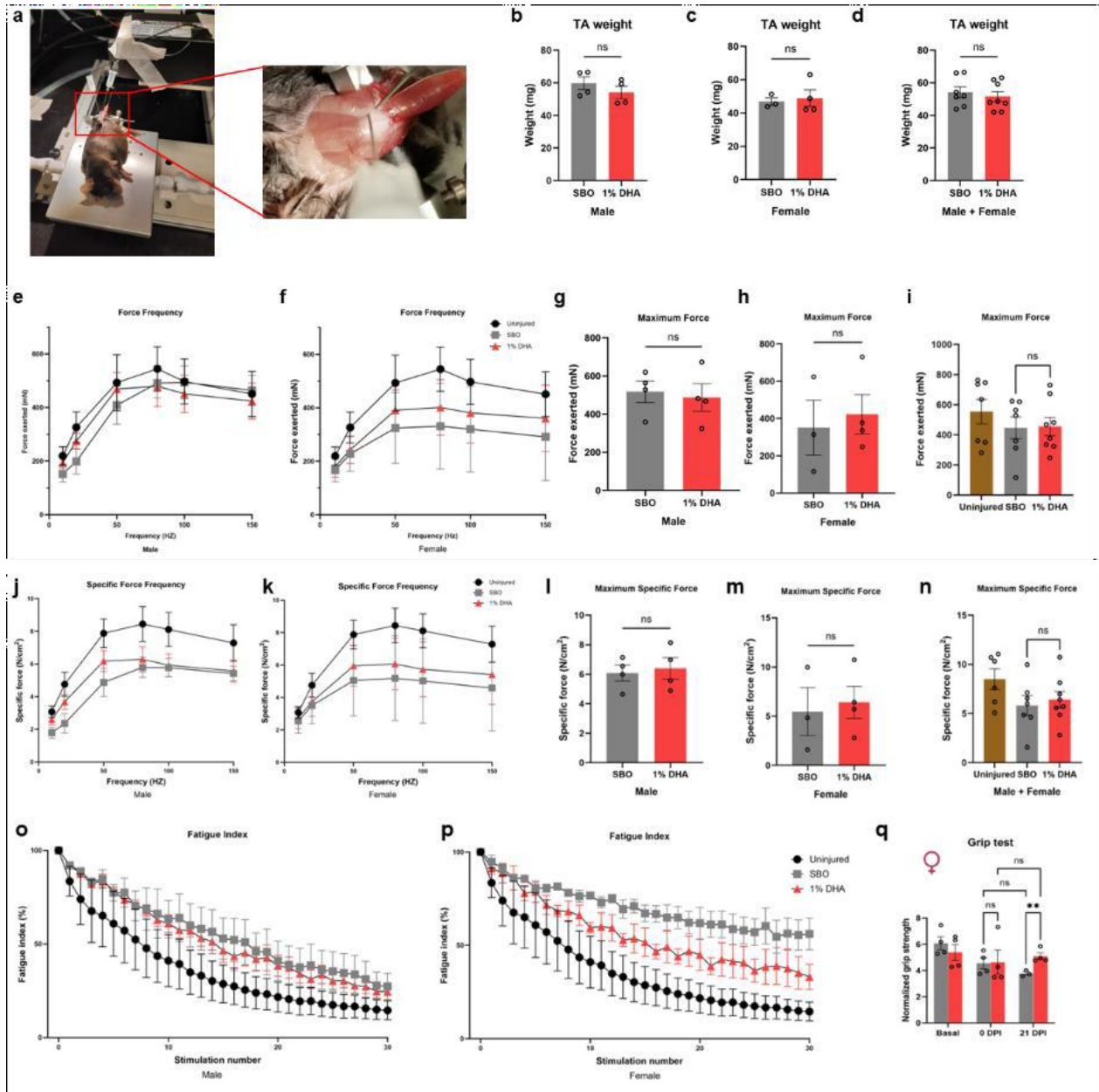


Figure 21. Effect of enriched DHA diet versus isoenergetic SBO diet on muscle function in aged male and female mice. **a**) Representative images of muscle stimulation using electric probes directly on the sciatic nerve, near the knee. TA weight of **b**) male, **c**) female, and **d**) pooled cohort for each diet group. Force frequency (10-150 Hz) graphs of **e**) male and **f**) female cohort. An uninjured group was added to assess basal force measurements. Maximum force of **g**) male, **h**) female, and **i**) uninjured and pooled cohorts for each diet group. Specific force frequency graphs in **j**) male and **k**) female cohorts. Specific force normalizes force exerted to the TA weight and length of each diet group. Maximum specific force of **l**) male, **m**) female, and **n**) uninjured and pooled cohorts for each diet group. Fatigue index (FI) for **o**) male and **p**) female cohort. **q**) Grip strength test (female cohort only) before diet change (basal), before injury (0 DPI), and post-injury (21 DPI). Data are represented as mean $\pm$ SEM (n=7-8; n=4 male, n=3-4 female), *p<0.05, **p<0.01

6.1 DHA-enriched diet may affect muscle composition

Previous data has demonstrated that with age, there is a decline in n-3 PUFA metabolites and SPMs in the muscle^{59,181}, which can be restored through dietary supplementation^{59,133}. In our second study *in vivo* study, we sought to confirm whether our specialized diets could restore the number and type of n-3 PUFA metabolites in the skeletal muscle. To do so, uninjured gastrocnemius muscle samples of young WT (chow diet) and aged mice fed either an SBO diet or 1-2% DHA diet (pooled samples) were collected and processed for analysis of oxylipins through LCMS. The results were normalized to the young WT muscle sample. While our results did not detect any DHA metabolites, we observed, in descending order, a large portion of ALA, ARA, and EPA-derived metabolites (Figure 22a). Of these data, one EPA-derived metabolite (14, 15 diHETE) was upregulated in the DHA-fed group compared to control (Figure 22a, b). In the ALA metabolites, we also observe a slight decrease in 15_kPGE2 accompanied with a slight increase in 15_kPGE2 α in the DHA-fed group compared to control (Figure 22a, b). In ARA metabolites, both diet groups are quite similar, except the DHA group has an increased trend in 9,10_DiHOME (p=0.0794) and 13-HOT γ Eg (p=0.0870) compared to the control group, and another ARA oxylipin (9-HOT γ E) remains quite elevated in both diet groups (Figure 22a, c).

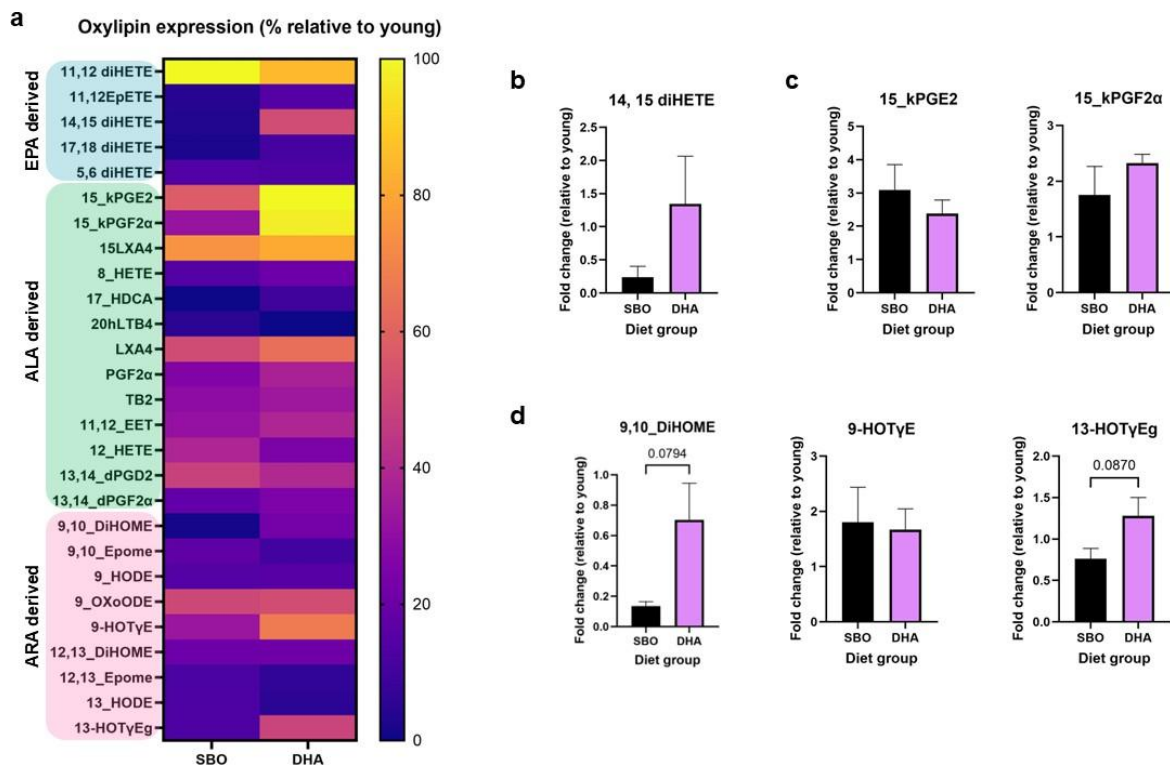


Figure 22. Oxylin concentration at 14 and 21 DPI in mice. a) Heatmap of EPA (blue), ALA (green), and ARA (red) metabolites present in gastrocnemius muscle samples of SBO and 1-2% DHA diet-fed aged mice. Heatmap is normalized to young WT (chow diet). One EPA-derived **b)**, two ALA-derived **c)**, and **d)** three ARA-derived metabolites called out from the heatmap. Data are represented as mean fold change. n=1 (young WT), n=3 SBO, n=4 1-2% DHA pooled samples.

Discussion

In this study, we have demonstrated the impact of n-3 PUFA supplementation on myogenesis and skeletal muscle regeneration in aged mice. We sought to assess whether n-3 PUFAs could directly modulate myogenic capacity in murine-derived aged myoblasts *in vitro* and delay age-derived changes, such as failure of resolving inflammation, in skeletal muscle regeneration using a CTX injury model *in vivo*. Our main findings are 1) at 21 DPI, there is an elevation of immune cells (neutrophils, macrophages), which suggests that there is a dysfunction in immune response (or a delay in immune response) post-injury in both diets and sexes and 2) myogenesis was activated, albeit delayed and muscle regeneration is incomplete, in both diet groups and sexes.

1.0 Effect of n-3 PUFAs on the Inflammatory Response

It is well established that the immune system and skeletal muscle regeneration are deeply intertwined together, and its meticulous regulation becomes dysfunctional with age^{27,55,216}. Previous work has shown that n-3 PUFA supplementation may modulate the aberrant immune response through modulating cytokine and chemokine production, which in turn affects immune recruitment²¹⁷, and recently, there is evidence that n-3 PUFA supplementation may impact muscle composition and myokine production within the skeletal muscle itself^{133,187,218}.

Given that neutrophils act as the first responders, typically infiltrating within the first couple of hours and resolving within the first few days in murine injury models^{133,203}, we did not expect to detect any significant differences in the total number of neutrophils between basal state and 21 DPI. However, our results revealed a slight increase in neutrophils in the uninjured tissue in the SBO diet group compared to DHA diet; overall, the number of neutrophils remain at similar levels—albeit trending upwards—at 21 DPI compared to basal, irrespective of diet or sex. These

findings could be due to a decline in chemoattraction of neutrophils due to a reduced production of pro-inflammatory cytokines (e.g., IL-1) and due to a reduced production of lipid mediator leukotriene B₄,²¹⁹⁻²²¹ as previously published. Similar results (reduction of neutrophils) were reported with RvD2 supplementation, DHA-derived SPM, in the blood of human monocytes and in the skeletal muscle in a hind limb ischemia mouse model^{48,222}. Our 1% DHA diet contains a mixture of DHA and SBO (**Table S2**), suggesting that there is an elevation of n-6 PUFA metabolites present from the SBO diet, which may compete metabolically with n-3 PUFAs^{30,223} post-injury, leading to an elevation of inflammation and a delayed resolution response in aged mice¹³³. Interestingly, we also report a significant decline of neutrophils in the uninjured TA in the DHA diet group compared to the SBO diet group. Presence of neutrophils in uninjured tissue is consistent with the concept of inflammaging, even at 21 DPI, and our findings suggest that DHA may improve the inflammatory cascade associated with tissue repair^{157,224,225}.

Following neutrophil infiltration, macrophages are recruited from the blood to the site of injury and polarized into their M1 pro-inflammatory state, allowing for phagocytosis of cellular debris and regulating activation of FAPs and satellite cells³⁸. Subsequently, M1 macrophages undergo a class-switch into their M2 anti-inflammatory status, allowing for differentiation and maturation of myocytes and myotubes (e.g., inhibit proliferation of satellite cells) to promote skeletal muscle regeneration^{27,34,226}. At 21 DPI, we do not expect to observe any macrophages (F4/80⁺ cells), indicating that immune-clearance post-injury was successful and tissue remodeling begins, as seen in previous studies using a CTX sterile injury^{109,227}. Our results demonstrate that at 21 DPI, only around half of the total number of macrophages are marked as M2, irrespective of diet or sex. These findings further suggest there is a delay in resolution of inflammation, which may exacerbate low-grade chronic inflammation, particularly for subsequent insults^{228,229}.

However, this conflicts with our cytokine and chemokine panel, which demonstrates a reduction of pro-inflammatory cytokines and chemokines in the muscle and in the blood (**Figure S6**). Notably, we observe a significant decline in IL-16, which has been proposed to be a marker for sarcopenia (age-related muscle wasting) and promotes M1 macrophage polarization²³⁰⁻²³².

Formation of fibrotic tissue and a clearance of intramuscular lipid droplets are processes that are partially modulated by macrophages during wound-healing and immune-regulation in the skeletal muscle after injury^{25,38,109,233}. we observe that post-injury, fibrosis remains at basal state (~10% of total area), irrespective of diet and sex. These findings are surprising as we expect a higher percentage of fibrotic area in skeletal muscle samples from aged mice due to inflammaging and increased muscle stiffness from collagen cross-linkages²³⁴⁻²³⁶. Our lab has previously shown that SPM supplementation (e.g., RvE1) decreases TGF- β levels released by macrophages *in vitro*^{10,104}. Prolonged expression of TGF- β promotes fibrosis, as seen in aged animals^{84,234,237,238}. Increased SPM production through n-3 PUFA supplementation may aid in mitigating pro-fibrotic pathways post-injury in the skeletal muscle. Another group demonstrated RvE1 and RvD1 treatment dampened fibrosis in the kidney through suppression of fibroblast proliferation in a unilateral ureteric obstruction mouse model^{239,240}.

In aged muscle, fat accumulates between the muscle fibers (myosteatorsis), causing a decline in muscle fiber density and loss of muscle force^{241,242}. We showed a significant reduction of lipid droplets at 21 DPI in the DHA diet group, particularly in male mice, compared to the SBO diet group. This is consistent with previous findings, where n-3 PUFA supplementation was shown to decrease fat accumulation in young male mice in the skeletal muscle¹⁸⁷ and in the liver²⁴³. Furthermore, an enriched n-3 PUFA diet, using menhaden oil (EPA and DHA enriched)¹⁸⁷ and camelina oil (ALA enriched)⁵⁶, have shown to facilitate clearance of lipid accumulation and

promote lipid metabolism, which had become dysregulated in the n-6 PUFA control diet in young mice¹⁸⁷ and in aged female mice⁵⁶. Given that MuSCs can trans-differentiate into FAPs through delayed M1-M2 macrophage polarization^{109,230,244} and promote intramuscular fat in the skeletal muscle after an injury^{109,245}, our findings suggest that temporal phenotypic switching of macrophages *in vivo* remains intact in the DHA diet group post-injury despite the failure to return to homeostatic level 21 DPI. Thus, the microenvironment (e.g., increase in n-3 PUFAs or SPM concentration) post-injury may promote or prioritize certain macrophage effector functions resulting in improved metabolic flexibility (e.g., fatty acid oxidation)^{201,243,246,247} and protection against protein degradation (atrophy)^{193,202} within the skeletal muscle itself^{248,249}.

Overall, it appears that a dietary intervention of n-3 PUFA supplementation in aged mice may not produce immediate, striking effects in terms of anti-inflammatory properties and immune regulation after a single IM CTX injury. In fact, we mainly observe the opposite effect (increased inflammatory status), which may be due to several reasons, including intrinsic fiber damage²⁵⁰, increased ROS³³, and dysregulated metabolic pathways^{173,251}, though additional work would be required to explore these avenues.

2.0 Effect of n-3 PUFAs on Skeletal Muscle Regeneration

It is well demonstrated that adult mice recover quickly after a CTX sterile intramuscular injury, typically completing muscle regeneration around 21 days^{15,252,253}. Herein, we have demonstrated that in aged mice, there is a failure in immune clearance (returning to homeostasis) at 21 DPI, suggesting that skeletal muscle regeneration may also be delayed in the SBO and the DHA diet groups given the interconnection between the immune system and skeletal muscle regeneration. Failure of resolving inflammation in aged skeletal muscle tissue may impact the number and

function of MuSCs^{45,46,254-256}, ultimately leading to delays in cell cycle regulation (e.g., entry and exit of the cell cycle)^{9,57,257}. Conjunctly, aged tissue is associated with a dysfunctional immune response (e.g., unresolved inflammation), as we have previously shown, ultimately driving delays in skeletal muscle regeneration and promote aberrant tissue remodeling. With respect to the myogenic markers, we report a decline in Pax7⁺ expressing cells at 21 DPI in the DHA diet group compared to the SBO diet group, however there is no significant difference in Pax7-expressing cells between uninjured and injured state for both diets and sexes. DHA has previously been shown to suppress Pax7 expression to promote activation of satellite cells (MyoD⁺)¹⁷⁵. However, we also observe a significant decline in MyoD⁺ expressing cells at post-injury in the DHA diet group and a similar downward trend (not statistically significant) in the SBO diet group compared to basal status. Downregulation of both Pax7 and MyoD may be a protective mechanism to prevent muscle degradation during the early stages of myogenesis in the presence of high levels of inflammation^{164,258-260}.

Finally, we report no changes in myogenin⁺ cells post-injury for both diet groups. When comparing the relative distribution of Pax7, MyoD, and myogenin expression, we observe a shift towards more myogenin⁺ cells post-injury in both diets and sexes. The shift in differentiating cells post-injury is expected due to the increase proportion of M2 pro-regenerative macrophages remaining in the tissue, which have been identified to promoter myoblast differentiation^{109,261}. Additionally, while there is a trend towards more newly formed myofibers (eMyHC⁺) at 21 DPI compared to uninjured, irrespective of diet and sex, it is not statistically significant, which further highlights the delayed timing of skeletal muscle regeneration *in vivo* at 21 DPI, which is consistent with the dysregulated immune response, as previously described^{252,253}.

Conversely, in our *in vitro* experiments, we have first demonstrated a decline in proliferative capacity in primary aged myoblasts compared to primary young myoblasts, which was rescued through DHA supplementation (50 nM) through increased Ki67 (proliferation marker) expression compared to control. Although we discovered an increase in myoblast proliferation, we did not detect any changes regarding the senescent pathway (**Figure S1**). Additionally, our *in vitro* experiments show that DHA at 50 nM showed no effect in our differentiation experiments in aged myoblasts, but we observe hypertrophic myotubes in our experiments using C2C12 myoblasts. Previous studies have shown that, in C2C12 myoblasts, n-3 PUFA supplementation led to hypertrophic fibers, through upregulation of AMPK signaling and through the phosphorylation of kinase p70S6K1^{98,246,262} and an increase in differentiation capacity of C2C12 myoblasts through limiting damage from ROS²¹⁸. Conversely, other studies have shown that n-3 PUFA supplementation led to a reduction of myotube fusion due to delayed entry and exit of the cell cycle entry or due to changes to various signaling pathways (e.g., protein synthesis/degradation pathways, Wnt signaling pathways)^{99,174,263,264}. However, these studies use a significantly higher dose of DHA (e.g. 50 μ M) compared to our work (50 nM; higher doses negatively affect cell viability in our pre-liminary work, **Figure S1**). Our conflicting *in vitro* data from C2C12s versus primary aged myoblasts may be due to the intrinsic biological changes associated with aging, further highlighting the need to use appropriate models for age-related studies and to improve translational work. One study found that treatment of EPA rescued skeletal muscle differentiation in the presence of the pro-inflammatory marker TNF- α in C2C12 myoblasts¹⁷⁷. Thus, investigation of myoblasts in conjunction with immune cells (e.g., co-culture of myoblasts with macrophages) may be worth investigating to further understand the effects of n-3 PUFA supplementation on myogenic capacity of aged myoblasts, as previously published²⁵⁸.

Interestingly, we observed an abundance of MyoD-expressing cells in the uninjured tissue compared to injured), suggesting that more cells may be available to commit to the myogenic lineage in absence of injury or to replenish the stem cell pool into a steady-state²⁶⁵. Previous research has indicated that an increase in MyoD expression in uninjured tissue is due to changes in structural integrity from aging¹⁰⁴, but this conflicts with our findings on fibrosis, which remains unchanged before and after injury. Without a younger age group, we cannot efficiently assess whether there were any significant structural changes within the skeletal muscle, resulting in the abundance of MyoD expression within the aged skeletal muscle. MyoD is the key myogenic marker in the muscle stem cell fate decision (myogenic commitment or return to quiescent state) and it has been previously shown that delays in cell cycle progression leads to a decline in myocyte fusion, myotube formation, and smaller fiber diameter, which further contributes to the loss of muscle mass and muscle atrophy^{58,64}. In this present study, we have shown at 21 DPI, the average CSA and diameter decline in both sexes declines by ~50% and ~10%, respectively compared to basal for both diet groups. While this is expected as part of the tissue remodeling process, where fibers are initially small and become larger as they mature, fibers with a minimum Feret's diameter of less than or equal to ~40 μm are considered to be atrophic^{266,267}, however we have shown that the fiber size exceeds the minimum threshold in this study. Previous studies have shown that EPA and DHA inhibit muscle atrophy through regulation of metabolic activity, increased protein synthesis, and improved controlled calcium release during muscular contraction^{193,201,202}, which may offer some protection against atrophy in this present study. Since we have shown that muscle regeneration is incomplete, we expect average CSA and minimum Feret's diameter to further increase upon successful completion, especially since fibrosis and fat deposition—factors that

have previously been shown to have an inverse relationship with successful skeletal muscle regeneration and fiber size^{11,268,269}—remained relatively low at 21 DPI in the DHA diet group.

We did not expect to find similar results between both diet groups since soybean oil produces more n-6 PUFA metabolites, typically associated with prolonged pro-inflammatory signaling, whereas DHA produces more anti-inflammatory metabolites^{8,67,197}. *In vitro*, most studies focus on myotube formation (e.g., late myogenic markers) as opposed to MuSC proliferation^{172,270}, though one study demonstrated an increase in myogenic capacity in both aged and in obese cell models with n-3 PUFA supplementation²⁷¹. In C2C12 myoblasts, n-3 PUFA supplementation has been shown to promote myoblast proliferation and myogenic commitment through modulation of the MAPK signaling pathway^{174,272}. While this is consistent with our *in vitro* work using primary myoblasts, this did not translate into our *in vivo* work—we saw a delay in muscle regeneration, likely due to the dysregulated immune response post-injury. Failure to resolve inflammation after a single bout of injury may result in aberrant tissue repair in subsequent bouts of injury, which in turn may promote inflammaging, fibrosis, and decline of muscle mass.

Initially, we expected to find an increase in muscle contractile properties since we hypothesize that a DHA-enriched diet would resolve inflammation and restore the intricate balance of myocyte-monocyte crosstalk, ultimately leading to improved muscle repair and consequently, increase in muscle force. Correspondingly, previous research demonstrates that n-3 PUFA supplementation may improve muscle contractile properties without changes in muscle mass^{182,190,273}. We demonstrated that there is no difference in maximum force or maximum specific force for either diet or sex group at 21 DPI and we also observe no changes in TA weight from either diet group. Notably, food consumption of the specialized diets remained within the average chow consumption (4 ± 1 g/day) for both sexes²⁷⁴. However, we observe an increase in total body

mass in the SBO-fed mice, particularly in the female mice, which we revealed is not due to an increase in fat mass²⁷⁵, as per our body composition measurements. Thus, these findings suggest that female mice may be more sensitive to diets containing more fat content compared to male mice. Interestingly, we observe an increase in fatigue resistance in female mice compared to male mice post-injury, further suggesting that aged female mice may be more susceptible to metabolic changes (increased bioenergetics) compared to males. Furthermore, we observe an increase in grip strength in female mice at 21 DPI in the DHA diet group compared to the SBO diet group, albeit grip strength at 0 DPI and 21 DPI did not change significantly. While these were measured using the forelimbs (uninjured), this increase in grip strength suggests that in the absence of an injury, a n-3 PUFA enriched diet may help to improve muscle function in aging, consistent with clinical studies^{129,154,276,277}.

Previous research suggests that fiber arrangement and organization could be an indicator of muscle strength in aged animal models²¹⁷. We observe a small clustering of type I fibers on the outer portion and a clustering of type IIA fibers in the center of tissue cross-sections of the DHA diet group (**Figure S3**). The opposite pattern in SBO diet cross-sections; however, the relative proportion of fiber types (I, IIA, IIB, IIX) remains unchanged, regardless of diet or sex. We also observe a shift towards more type IIX (intermediate fatigue resistance) fibers in both diet groups at 21 DPI, although this is not statistically significant and is more likely a consequence of the injury. Based on our results from the contractile properties, we expected to find an increase in slow-twitch fibers (type I fibers), however, that is not the case. Thus, the increased fatigue resistance may be due to the shift in increased type IIX fibers, rather than the slow twitch fibers. Previous studies have shown that aging is associated with a decline in type II fibers and an increase in type I fibers^{278,279}. Thus, while the relative proportion of type I fibers remains similar, the

increase in type IIX fibers at 21 DPI indicate that both the SBO and DHA enriched diets may be able to retain fiber type diversity and fiber plasticity in aging, which in turn, may play a role in maintaining muscle contractile properties and improving fatigue resistance in aged mice.

Post-injury, the nuclei are at the center of the myofiber whereas they remain in the periphery in an uninjured state^{204,205}. In aged muscle, a CNF of ~20% has been shown to be a typical baseline in uninjured muscle (no comorbidities), which is typically more elevated than young muscle^{126,207}. We also reported that the percentage of CNF in uninjured muscle remained at ~20%. At 21 DPI, we reported a statistically significant elevation in the percentage of CNF in both diet groups and sexes, further indicating that the muscle has not reached homeostasis (e.g., incomplete muscle regeneration). In a CTX injury model, homeostasis is typically reached at 28 DPI²⁰⁴, therefore an elevation of CNF is expected. This may also explain the decline in average CSA and minimum Feret's diameter post-injury, which may increase from 21 DPI to 28 DPI. One study proposed that an elevation of CNF three weeks post-injury may further promote damage, leading to a decline in contractile properties²⁸⁰, based on the elevation of immune cells present in the tissue at 21 DPI, we would expect to observe a similar phenomenon. In the future, multiple timepoints may aid in further understanding the effect of SBO and DHA enriched diets on muscle regeneration in aged mice, potentially even extending beyond 28 DPI with a CTX injury model. Other injury models, such freeze-injury, the percentage of CNF remains approximately 50-60% at 30 DPI in young and in aged mice²⁸¹, highlighting the differences in skeletal muscle regeneration timelines using various *in vivo* injury models. Further investigation (e.g., comparison of 21 DPI with an earlier timepoint, young mouse control, perform a second bout of injuries at 21 DPI) may help to indicate whether the elevation of CNF in aged muscles at 21 DPI (and beyond) may be due

to the CTX model itself, due to age factor (e.g., intrinsic repeated rounds of regeneration/degeneration²⁸⁰), or a combination of these factors.

3.0 Limitations and Future Directions

We chose to treat the mice through dietary intervention to improve translational work from pre-clinical animal work to clinical work, as n-3 PUFA supplementation is typically administered through the oral route. Other oral routes of administration (e.g., gavage) may cause negative side effects if poorly administered, thus a dietary intervention was more suitable given that aged animals are more susceptible to other health-related (non-study related) complications (e.g., skin lesions, dermatitis, tumor growth)²⁸². Although the mice were slowly acclimated to the specialized diets, the food consumption was monitored over the course of the experiment, and the average food consumption remained at ~4g/day/mouse, some individual data points are quite low, at 1-2 g/day/mouse. This could be due to an aversion to the specialized diets²⁸³ or due to changes in mouse activity; declined activity would be associated with decreased appetite. However, we did not monitor mouse activity or include an exercise control in this study, which may limit the effects of the DHA-enriched diet post-injury on contractile properties, as previously described by Russ et al. (2020)²⁷³. Additionally, to further our understanding of biological variation and to enhance the statistical power of this study (e.g., more definitive effect of control versus treated), more biological replicates may be used in future studies.

Furthermore, we did not investigate any metabolic-related markers or metabolic pathways that may have been affected *in vivo*. A deeper investigation on metabolic enzymatic activity could offer some insight into changes of basal glycolic to oxidative phosphorylation ratio and metabolic flexibility, which both have previously been shown to decline with aging²⁸⁴⁻²⁸⁶.

Similarly, *in vitro*, we did not conduct any pathway analysis, but based on our results, we hypothesize that metabolically driven pathways, such as protein synthesis, cellular division, and limitation of ROS may be affected, as previous research has demonstrated these effects^{127,150,164}. Consequently, DHA may be incorporated into the membrane, thereby affecting membrane fluidity in conjunction with these processes, however we did not collect media to verify which factors were released by the myoblasts in culture.

Finally, loss of muscle mass and muscular atrophy is exacerbated with cycles of regeneration and degeneration, as seen in other myopathies (e.g., DMD). In a similar fashion, age-related loss of muscle mass and function is a progressive problem, partially caused by the decline of immuno-regulation and producing an atrophic-like microenvironment²⁹. Thus, it is possible that introducing an n-3 PUFA supplementation in an aged mouse may be too little exposure to derive any meaningful results; however, the cost of a long-term study (e.g., feeding for 12+ months) is quite high and the animal welfare may suffer. Another study proposes that an intact neurovascular system permits the inflammatory response to proceed as normal post-injury, thereby leading to proper muscle regeneration²⁸¹, which may act as a confounding factor in CTX injury studies. Thus, to improve our study model for future age-related studies, it may be more appropriate to perform multiple rounds of injury, or a different injury model (e.g., devascularization) to emphasize the effects of an aged tissue microenvironment, which could offer an improved insight into the roles of n-3 PUFAs on skeletal muscle regeneration in aging.

Conclusion

Skeletal muscle regeneration is a carefully regulated process primarily involving myogenic regulatory factors and the immune system. In aged skeletal muscle, satellite cell dysfunction and

chronic low-grade inflammation impedes skeletal muscle regeneration, leading to the loss of muscle mass and strength, and increased fragility in the elderly. Currently, there are no approved therapies to attenuate age-related muscle disorders, despite the global increase in life expectancy. In this study, we have demonstrated that *in vitro* DHA may improve proliferative capacity of aged myoblasts but not differentiation capacity—both of which have been previously reported to decline with age^{31,46}. *In vivo*, we have shown that at 21 DPI, the myogenic program was activated, but not completed, and we denote a failure in resolving inflammation in the SBO and DHA diets. While these findings conflict with the well-characterized anti-inflammatory properties of n-3 PUFAs^{156,158}, we observed an improved regulation of fat deposition in the DHA enriched diet, which may indicate different roles of n-3 PUFAs in the skeletal muscle. We expect that over the course of multiple/repeated injuries, the characteristics associated with aberrant tissue repair (e.g., lipid infiltration, increased inflammation and fibrosis, smaller fiber density) may be more evident as a function of time, but that n-3 PUFA supplementation alone may not be sufficient to dampen these effects^{188,287}.

Appendix A: Supplementary Material

1.0 Cell culture media

Table S1. Composition of cell culture media used for *in vitro* experiments.

Media + Cell type	Reagent	Concentration	Catalog # and company
C2C12 myoblasts; Proliferation Media	Dulbecco's modified eagle's medium (DMEM) with 4.5g/L glucose and L-Glutamine	500 mL bottle	319-015-CL, Wisent INC (USA)
	Fetal bovine serum (FBS)	10% (v/v)	CA76327-086, Corning (CAN)
	Penicillin/Streptomycin	1% (v/v)	450-201-EL, Wisent INC (USA)
C2C12 myoblasts; Differentiation Media	DMEM with 4.5g/L glucose and L-Glutamine	500 mL bottle	319-015-CL, Wisent INC (USA)
	Horse serum (HS)	2% (v/v)	065-110, Wisent INC (USA)
	Penicillin/Streptomycin	1% (v/v)	450-201-EL, Wisent INC (USA)
Aged myoblasts; Proliferation Media	Hams F10	500 mL bottle	318-050-CL, Wisent INC (USA)
	FBS	20% (v/v)	CA76327-086, Corning, (CAN)
	Pen/strep	1% (v/v)	450-201-EL, Wisent INC (USA)
	FGF 50 ug	10% (w/v)	511-126-QU, Wisent INC (USA)
Aged myoblasts; Differentiation media	Hams F10	50% (v/v)	318-050-CL, Wisent INC (USA)
	DMEM low glucose	50% (v/v)	319-010-CL, Wisent INC (USA)
	Horse serum	2.5% (v/v)	065-110, Wisent INC (USA)
	Pen/strep	1% (v/v)	450-201-EL Wisent INC (USA)

1.1 Aged myoblast proliferation supplementary and supporting data

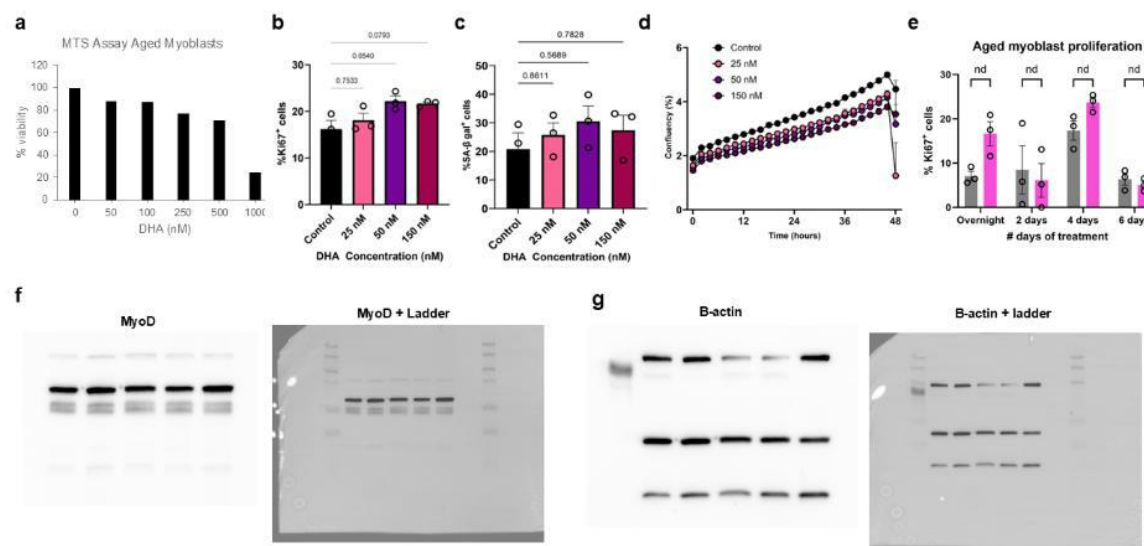


Figure S1. Supporting data for aged myoblasts in proliferation media. a) MTS viability assay. b) Ki67⁺ quantification of aged myoblasts treated with 0-150 nM DHA c) SA β-gal quantification of aged myoblasts treated with 0-150 nM DHA d) 48-hr real-time proliferation with aged myoblasts treated with 0-150 nM DHA e) time-course of aged myoblasts treated with 50 nM or vehicle overnight, 2 days, 4 days, and 6 days (media changed every 48hrs). f+g) raw data for western blots for MyoD and β-actin, respectively. b-e data are represented as mean of 3 technical replicates. For c, data was not reproducible using the senescence detection kit.

1.2 Myofiber types *in vitro*

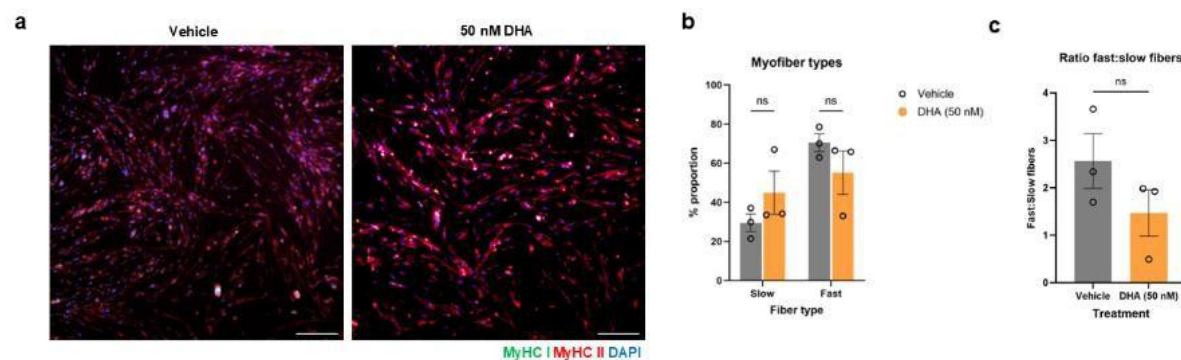


Figure S2. Immunofluorescent staining and quantification of myofiber types (aged myoblasts). a) Representative images of slow (green) and fast (red) fiber types. Fields of interest were taken on the Zeiss Observer 7 microscope at 10x magnification. b) Quantification of each fiber type c) Ratio of fast:slow fibers. Data are represented as the mean of 3 technical replicates; cells were treated for 4 days in differentiation media with either DHA (50 nM) or control vehicle.

2.0 Specialized diets

Table S2. Food composition of specialized diets, adapted from Inotiv (previously Teklad).

Diet name	Formula	Macromolecule	% by weight	% kcal from
Soybean oil (SBO)	- 2020X basal mix without fat - Soybean oil, 19% (w/w)	Protein	15.9	15.3
		Carbohydrate	40.9	39.3
		Fat	21.0	45.4
1% Fish oil (1% DHA)	- 2020X basal mix without fat - Soybean oil, 9.5% (w/w) - Fish oil, 9.5% (w/w) - Red food colour	Protein	15.9	15.3
		Carbohydrate	40.9	39.3
		Fat	21.0	45.4
2% Fish oil (2% DHA)	- 2020X basal mix without fat - Fish oil, 19% (w/w) - Blue food colour	Protein	15.9	15.3
		Carbohydrate	40.9	39.3
		Fat	21.0	45.4

2.1 Whole tissue sections

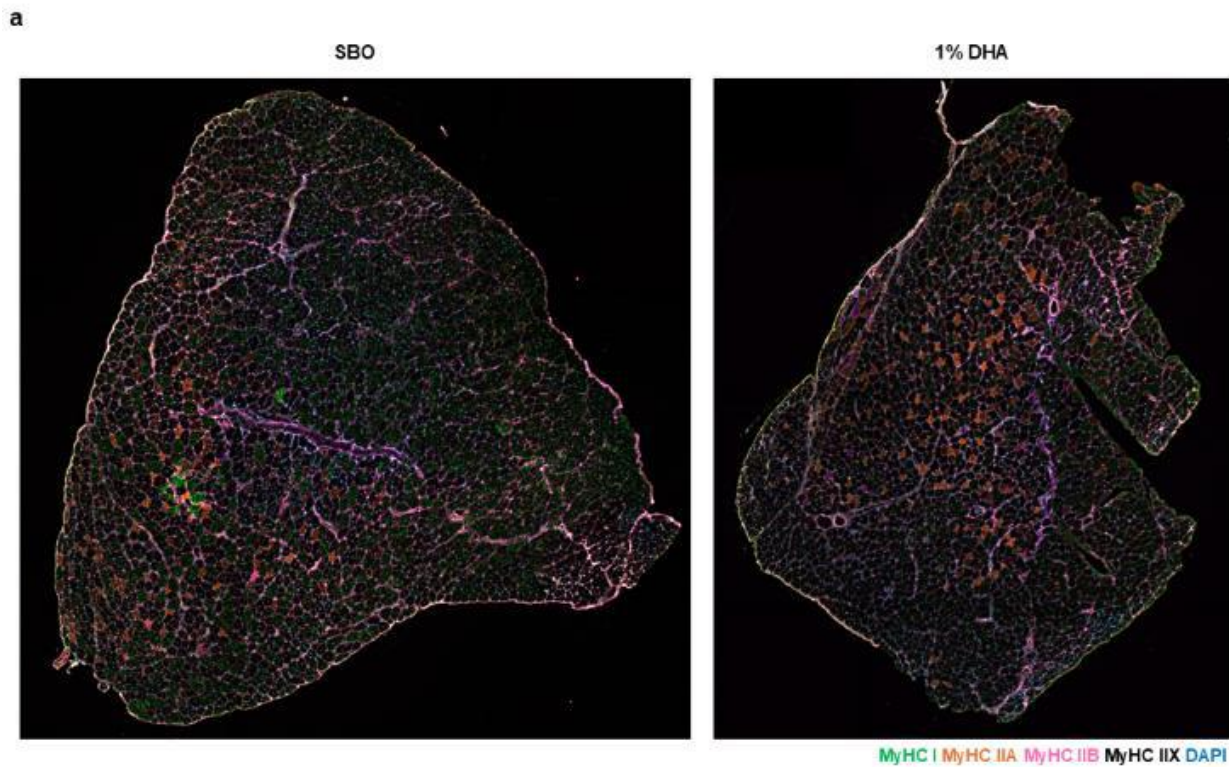


Figure S3. Whole tissue cross-sections of fiber-type staining (21 DPI). a) Representative images of whole tissue sections at 21 DPI for SBO and 1% DHA.

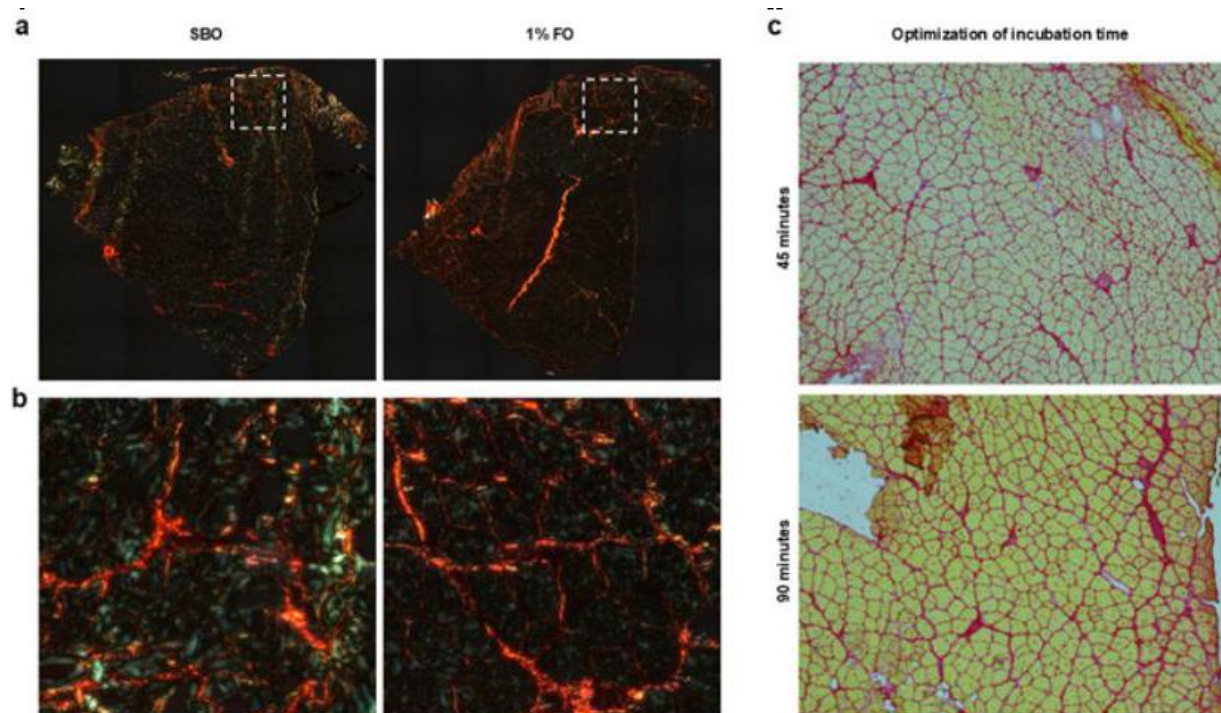


Figure S4. Picrosirius red tissue cross-sections. a) Representative images of whole tissue sections b) and fields of interest of fibrosis staining. Images were taken on the Zeiss Observer 7 microscope at 10x magnification under polarized light (Koheller illumination). c) Optimization of incubation time for the staining. Fields of interest were taken at 10x on the EVOS M5000 in Brightfield.

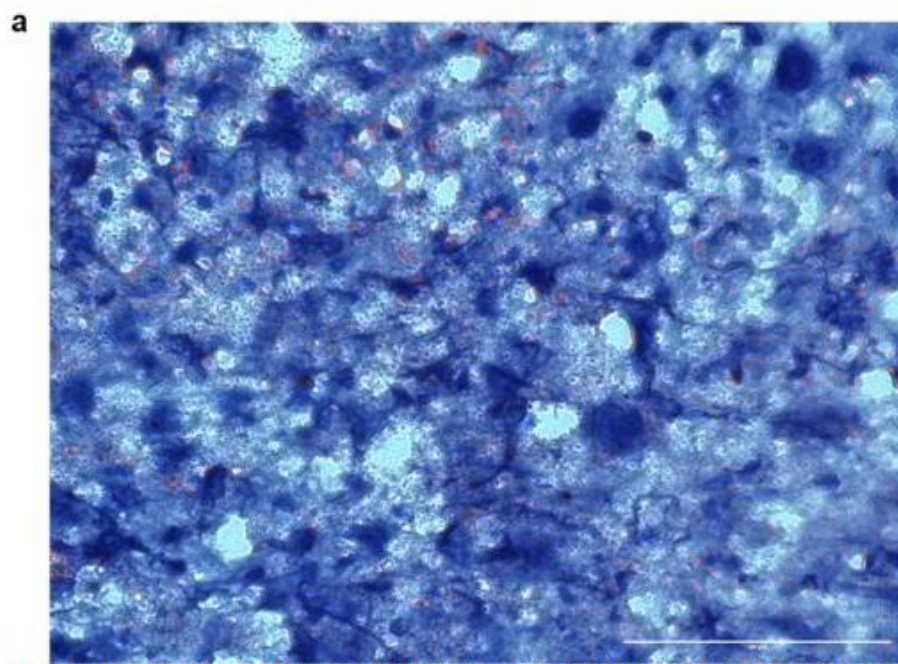


Figure S5. Validation of ORO kit. a) Representative image of murine liver section. Oil droplets (in pink) can be seen in this tissue. Image was taken at 10x on the EVOS M5000 in brightfield.

2.2 Immuno-regulation at the local and systemic level

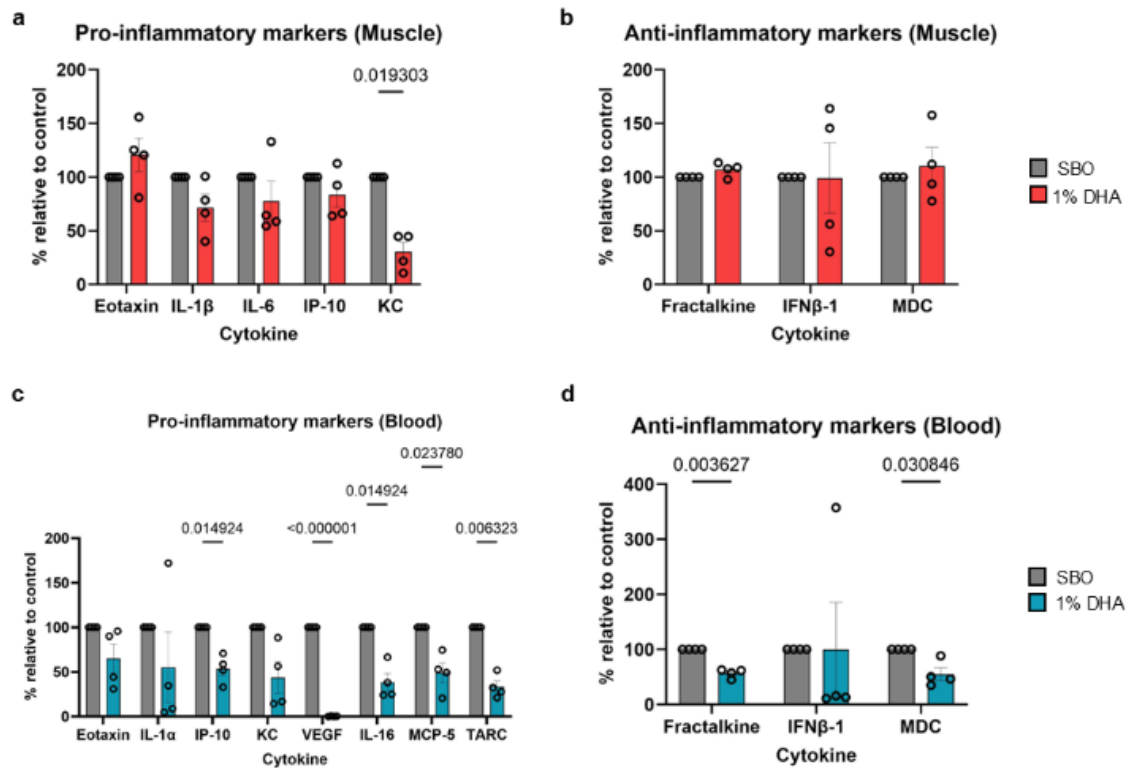


Figure S6. Immuno-markers at the local and the systemic level in aged male mice 21 DPI. a+b) Pro-inflammatory (a) and anti-inflammatory (b) markers in the skeletal muscle. c+d) Pro-inflammatory (c) and anti-inflammatory (d) markers in the blood serum of aged male mice. Data are represented as mean $\pm$ S.E.M, n=4.

3.0 Oxylinpins cohort supporting data

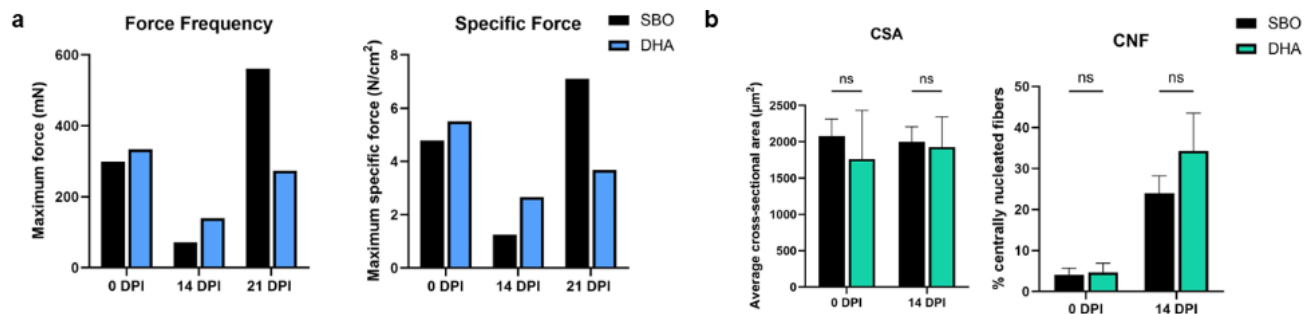


Figure S7. Contractile and histological data for oxylinpins cohort. a) Force and specific force frequency at 0, 14, and 21 DPI. b) Mean cross-sectional area (CSA) and percentage of centrally nucleated fibers (CNF) at 0 and 21 DPI for each diet group. n=1-3; DHA is 1-2% fish oil diet pooled together.

Appendix B: Supplementary materials and methods

1.0 Image acquisition for immunofluorescence imaging

IF imaging of cells: Images were acquired using a commercial inverted EVOS M5000 or EVOS M7000 widefield microscope (ThermoFisher Scientific) using a fully motorized stage and focus control (mechanical for M5000, motorized for M7000). The LED light source equipped with the CMOS sensor monochrome camera was used with the following filter sets: DAPI, GFP, TX red, and Cy5 at 20x/0.40 NA (dry objective). 5-10 random fields of view (FoVs) were acquired using the EVOS M5000 model with manual focus, and 5% of the well (from the center) were acquired using the EVOS M7000 model with tiling and scanning function. The scans were then stitched together automatically by the software. Raw files (both models) were exported as tiffs. For proliferation, channels were split in FIJI ImageJ into their respective pseudo-colour channels before applying a threshold (green and blue channel) to convert into a binary image. With the binary image, the watershed function was applied and then the particles were analyzed using count masks. All of these functions are pre-installed in the program. For differentiation, the same protocol was used to count the total number of nuclei. For differentiation index, cells that were not colocalized were counted and then subtracted from the total number of cells. For the fusion index, cells contained within a myotube (green) with at least 2 nuclei were counted manually. Myotube diameter was taken manually using the “straight” tool applied to the thickest part of the myotube containing a minimum of 2 nuclei. The average thickness for each FoV was then recorded.

IF imaging for tissue cross-sections: Images were acquired using a commercial Upright Stand Leica DM6 B widefield microscope (Leica Microsystems, Germany). Sections were imaged using a fully motorized stage and focus control. The EL6000 light source was used in combination with the following fluorescent filter sets: DAPI, GFP, Cy3, TX red, Cy5 using the Leica K5 sCMOS monochrome camera at either 10x/0.32 NA (dry objective), 20x/0.75 NA (multi-immersion objective) with Type G immersion oil (cat #11513910, Leica Microsystems, Germany), or 40x/0.95 NA (dry objective). Images of whole tissue sections were acquired using the tiling function, Z-stack function (120 μ m steps), set with 16-bit digitizer and no binning. Images were immediately processed using the instant computational clearing (ICC) function and tile stitching. All image acquisition and processing were done using the LasX Software (Leica Microsystems, Germany). The raw files and processed files were exported as lifs and tiffs, respectively. For tissue analysis, approximately 10% of area of each section (fields of view with centrally located nuclei) was analyzed and counted manually using the FIJI ImageJ software, with the exception of % CNF, minimum Feret’s diameter, and CSA. For these data, whole tissue section analysis was completed using the MorphoLibJ segmentation²⁸⁸ with the macrofibers plug-in, with the tolerance threshold being set between 2-10.

2.0 Image acquisition for brightfield imaging

Tissue sections only: Images were acquired using a commercial EVOS M5000 Imaging System (Thermofisher Scientific), using a fully motorized stage (mechanical) and focus control. The LED light source was used with the RGB translucent cube in combination with the CMOS monochrome camera at 20x/0.40 NA (dry objective) for FoV and 40x/0.60 NA (dry objective for ROIs with manual focus. Images were immediately exported by the M5000 software into tiffs. To analyze the lipid infiltration, the area covered by lipid droplets was divided by the total area of the FoV and multiplied by 100 (percent of area covered by lipid). This was done manually using the FIJI ImageJ software.

3.0 Image acquisition for polarized light imaging

Tissue sections only: Images were acquired using a commercial Zeiss AxioObserver 7-APOLLO inverted microscope (Zeiss, Germany) equipped with a fully motorized stage and focus control at 1.11 μm resolution. Whole tissue sections were imaged at 10x/0.30 NA (dry objective) using the transmitted light LED set with Koehler illumination settings (52.5° analyzer, 28° polarizer) in combination with the Zeiss AxioCam 705 colour camera. Whole tissue sections were acquired using the tiling function and then immediately stitched directly in the Zen 3.1 Pro (Zeiss, Germany) software. Raw files were then exported as czi and tiffs for analysis. To analyze the area of red collagen, the images were converted into 8-bit images using the FIJI ImageJ software before setting a threshold. The area (limited to threshold) was measured, and the total area of the tissue was measured to obtain the percentage of red collagen for each section.

4.0 Representative images

To whole fields of view (linear application) of representative images were adjusted to enhance the representative data. Colour balance on whole images was adjusted to minimize background staining and the channels tool was used to toggle between channels (for pseudo-colour colocalization). Crops of areas of interest were taken using the region of interest (ROI) manager tool and the crop tool. If needed, image contrast and brightness were also adjusted (linear) to improve clarity. A scale bar was added to the representative images and the length of each scale bar is indicated in the associated figure caption.

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