

EXERCISE AND NUTRITIONAL STRATEGIES FOR HUMAN MUSCLE METABOLIC REGULATION WITH AGEING AND INACTIVITY

By

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DISCLAIMER

A large proportion of the work presented within the current thesis was conducted during the worldwide outbreak of the Sars-Cov-2 virus (2020-2021) and resulting >12-month pandemic. Unfortunately, due to government policy, national lockdowns, and limited access to research facilities, equipment, and participant recruitment, only the following chapters were possible to complete.

“You cannot get educated by this self-propagating system in which people study to pass exams, and teach others to pass exams, but nobody knows anything. You learn something by doing it yourself, by asking questions, by thinking, and by experimenting”

Dr. Richard Feynman, PhD. (1918-1988)

Professor of Theoretical Physics, California Institute of Technology, USA.

Nobel Prize, Physics, 1965.

The work presented in this thesis was performed within The University of Birmingham's School of Sport, Exercise and Rehabilitation Sciences and The University of Nottingham's School of Graduate Entry Medicine, Clinical, Metabolic and Molecular Physiology Group, which form part of The Medical Research Council & Versus Arthritis Centre for Musculoskeletal Ageing Research (CMAR).

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ABSTRACT

Age-related skeletal muscle wasting, or “sarcopenia”, negatively impacts physical function and health. The mechanisms relating to sarcopenia are complex and unclear but are thought to be underpinned by a blunted response of skeletal muscle to anabolic stimuli and the accumulation of dysfunctional mitochondria. Moreover, these issues may be further worsened with brief periods of disuse and inactivity. Therefore, resistance exercise (RE) and nutritional strategies are of paramount importance to offset the further decline. Accordingly, **Chapter 2** explored the molecular consequences of a brief five-day period of bed rest in older adults. Moreover, I explored how RE prehabilitation prior to bed rest *may* potentially offset declines in mitochondrial protein expression. However, I showed that neither 5-days of bed rest or prior RE prehabilitation affects markers of mitochondrial content, function, or fission-fusion dynamics. **Chapter 3** determined the effect of lifelong exercise on skeletal muscle mitochondrial content and quality control in a cohort of 'master athletes' and sedentary older adults in a rested and 1 hr post-RE. I observed significantly elevated abundance in a range of mitochondrial proteins in MA compared to untrained older adults, suggesting greater mitochondrial health and function. However, acute RE was unable to alter mitochondrial protein expression or phosphorylation status involved in biosynthesis and fission-fusion dynamics. **Chapter 4** investigated the acute neuromuscular response to a pragmatic RE mode, elastic band exercise (EB-RE), and how this compared to traditional machine RE (MN-RE) in younger and older adults. I observed no significant differences in EMG activity between EB-RE and MN-RE in either group. Thereby providing preliminary evidence for EB-RE as a potential RE stimulus in the absence of MN-RE equipment. Following this, **Chapter 5** assessed the integrated muscle protein synthetic (iMyoPS) response with an oral D₂O tracer methodology in younger and older adults. I observed a robust increase in iMyoPS following EB-RE in both groups. However, iMyoPS was blunted in older adults, independent of similarities in acute mTORC1 signalling. **Chapter 6** provides a study protocol for a currently ongoing randomised, double-blind, placebo-control study investigating the *potential* protective effects of a high-dose, leucine-enriched essential amino acids (Leu-EAA) at preventing muscle atrophy, muscle proteostatic imbalance, and mitochondrial dysfunction following period of reduced activity, in a cohort of healthy older adults. In conclusion, this thesis enhanced our understanding of the mechanisms underpinning ageing and sarcopenia by investigating the effects of disuse and inactivity and their effects on whole-body and muscle-specific metabolism/signalling, overall demonstrating the protective role of acute and lifelong exercise, as well as nutritional support on musculoskeletal health.

LIST OF PUBLICATIONS & AWARDS

(Obtained During PhD Studentship Only)

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- **Marshall RN**, McKendry J, Smeuninx B, Seabright AP, Morgan PT, Greig C and Breen L (2023) Acute resistance exercise training does not augment mitochondrial remodelling in master athletes or untrained older adults. *Front. Physiol.* 13:1097988
- **Marshall, R.N.**, Morgan, P.T. and Breen, L., 2022. Myofibrillar protein synthesis and acute intracellular signalling with elastic band resistance exercise in younger and older men. *Medicine in Science in Sport and Exercise*.
- **Marshall, R.N.**, Smeuninx, B., Seabright, A.P., Morgan, P.T., Atherton, P.J., Philp, A. and Breen, L., 2022. No effect of five days of bed rest or short-term resistance exercise prehabilitation on markers of skeletal muscle mitochondrial content and dynamics in older adults. *Physiological reports*, 10(13), p.e15345.
- Edwards, S.J., Shad, B.J., **Marshall, R.N.**, Morgan, P.T., Wallis, G.A. and Breen, L., 2021. Short-term step reduction reduces citrate synthase activity without altering skeletal muscle markers of oxidative metabolism or insulin-mediated signalling in young males. *Journal of Applied Physiology*, 131(6), pp.1653-1662.
- Allen, S.L., **Marshall, R.N.**, Edwards, S.J., Lord, J.M., Lavery, G.G. and Breen, L., 2021. The effect of young and old ex vivo human serum on cellular protein synthesis and growth in an in vitro model of aging. *American Journal of Physiology-Cell Physiology*, 321(1), pp.C26-C37.
- Morgan, P.T., Harris, D.O., **Marshall, R.N.**, Quinlan, J.I., Edwards, S.J., Allen, S.L. and Breen, L., 2021. Protein source and quality for skeletal muscle anabolism in young and older adults: a systematic review and meta-analysis. *The Journal of nutrition*, 151(7), pp.1901-1920.
- Edwards, S.J., Smeuninx, B., McKendry, J., Nishimura, Y., Luo, D., **Marshall, R.N.**, Perkins, M., Ramsay, J., Joannis, S., Philp, A. and Breen, L., 2020. High-dose leucine supplementation does not prevent muscle atrophy or strength loss over 7 days of immobilization in healthy young males. *The American journal of clinical nutrition*, 112(5), pp.1368-1381.
- **Marshall, R.N.**, Smeuninx, B., Morgan, P.T. and Breen, L., 2020. Nutritional Strategies to Offset Disuse-Induced Skeletal Muscle Atrophy and Anabolic Resistance in Older Adults: From Whole-Foods to Isolated Ingredients. *Nutrients*, 12(5), p.1533.
- **Marshall, R.N.**, Morgan, P.T., Martinez-Valdez, E. and Breen, L., 2020. Quadriceps muscle electromyography activity during physical activities and resistance exercise modes in younger and older adults. *Experimental Gerontology*, p.110965.

IN PREPARATION

- Morgan, P.T., Smeuninx, **Marshall, R.N.**, Quinlan, J., Shepherd, S., McPhee, J, Nielsen, J., Breen, L. Greater myofibrillar protein synthesis following weight bearing activity in obese old compared with non-obese old and young individuals. ([Under Review, Journal of Physiology](#)).
- **Marshall, R.N.**, Korzepa, M., Quinlan, J., Breen, L. The effects of disuse and unloading on human skeletal muscle mitochondrial respiration: A systematic review and meta-analysis.
- Korzepa, M., **Marshall, R.N.**, Smeuninx B., Quinlan, J, Breen, L. mTOR-TSC2-SESTRIN2 translocation and co-localization in human skeletal muscle following resistance exercise in younger and older adults. ([In Preparation, American Journal of Physiology-Cell Physiology](#)).

ABSTRACTS AND CONFERENCE PROCEEDINGS

- **Marshall, R.N.** Elastic band-based resistance exercise increases integrated rates of myofibrillar protein synthesis in younger and older males. *European College of Sports Science, 2022, Sevilla, Spain. ORAL COMMUNICATION.*
- Morgan, P.T., Smeuninx, **Marshall, R.N.**, Quinlan, J., Shepherd, S., McPhee, J, Nielsen, J., Breen, L. Greater muscle volume and load-induced myofibrillar protein synthesis, but poorer muscle quality in obese vs lean older adults. *European College of Sports Science, 2022, Sevilla, Spain. ORAL COMMUNICATION.*

- **Marshall, R.N.** Elastic band resistance exercise & protein turnover. *International Biochemistry of Exercise Conference, Toronto, Canada. POSTER COMMUNICATION.*
- **Marshall, R.N.** No effect of either of five days of bed rest or short-term resistance exercise prehabilitation on skeletal muscle mitochondrial dynamics in older adults. *British Society for Research on Ageing, 2022, Birmingham, UK. ORAL COMMUNICATION.*
- **Marshall, R.N.** Elastic band-based resistance exercise increases integrated rates of myofibrillar protein synthesis in younger and older males. *University of Birmingham Postgraduate Research Conference. ORAL COMMUNICATION.*

INVITED PRESENTATIONS

- **Can Elastic Band Exercise Induce Muscle Growth in Younger and Older Adults?**
University of Toronto, Department of Kinesiology, Protein Metabolism Research Group Seminar, Associate Professor Daniel Moore, PhD.
- **The Science of Protein, Muscle Growth and Muscle Loss.**
Black Steam 2021, Thinktank Birmingham Science Museum, Birmingham, UK.
- **Can We “Out Nutrition” Physical Inactivity? The Importance of Dietary Protein in Older Adults to Mitigate Muscle Loss.**
Halesowen Pensioners Convention, Halesowen Library, Birmingham, UK.
- **Is Increasing Protein Intake the Answer? The Role of Protein in Muscle Ageing.**
University of Birmingham Sports and Exercise Medicine Society, 2019, Birmingham, UK.
- **Ageing, Inactivity and Muscle Metabolic Deconditioning: The Importance of Resistance Exercise and Protein Nutrition.**
Sports and Exercise Medicine Society 2019, University of Birmingham, UK.
- **Inactivity and Accelerated Muscle Wasting: What Are the Consequences and How Can We Prevent Them?**
The Birmingham 1,000 Elders Group, 2021. University of Birmingham, UK.

SCIENTIFIC CONFERENCE ATTENDANCES

- European College of Sports Science (ECSS) 2022, Sevilla, Spain.
- International Biochemistry of Exercise Conference (IBEC), 2022, Toronto, Canada.
- British Society for Research on Ageing (BSRA) 2021 – Metabolism of Ageing.
- European College of Sports Science (ECSS) 2019, Prague, Czech Republic.

SCIENTIFIC WORKSHOPS / TRAINING

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- Agilent Seahorse Extracellular Flux Analysis Training Course, Mitochondrial Profiling Centre, University of Birmingham – Dr. Jonathan Barlow, PhD.

AWARDS

- **Research Fellowship**, ESPEN European Society for Clinical Nutrition and Metabolism. June 2022. “Determining the effects of two-weeks of step reduction on skeletal muscle proteome dynamics, proteostasis and energetics”.

DECLARATION

I declare that the research within this thesis is the author's original work (**Ryan N. Marshall**), other than the work mentioned below. This thesis has not been previously submitted to this or any other university for a degree and does not incorporate any material already submitted for a degree.

CHAPTER 2

In this chapter, analysed muscle samples were a sub-set of skeletal muscle biopsies samples collected by Dr. Benoit Smeuninx, PhD as part of a BBSRC funded postdoctoral fellowship (BB/N018214/1) and human clinical trial (NCT04422665). The study was conducted at the Wellcome Trust Clinical Research Facility, Queen Elizabeth Hospital, Birmingham.

CHAPTER 3

In this chapter, analysed muscle samples were a sub-set of skeletal muscle biopsies samples collected by Dr. James McKendry as part of his PhD studentship at The University of Birmingham.

CHAPTER 5

Dr. Leigh Breen, PhD and Dr. James McKendry, PhD (McMaster University, Canada) designed the study protocol utilised, including stable isotope tracer (D_2O) dosing protocol and the number and timing of skeletal muscle biopsies. Analysis of skeletal muscle myofibrillar protein synthesis for *Chapter 4* were performed in collaboration with Drs Daniel Wilkinson, PhD., Matthew Brook, PhD., and Professor Kenneth Smith, PhD., at the laboratory of Professor Philip Atherton, PhD., at the Centre for Molecular, Ageing and Physiology Group, University of Nottingham; MRC-Versus Arthritis Centre for Musculoskeletal Ageing.

CHAPTER 6

Data collection for the chapter is currently ongoing. Due to a ~18-month delay due to COVID-19, and the inability to undertake human metabolic physiology-based research, the project, and data collection were delayed and unable to be part of the current thesis. The project is partially funded by an ESPEN fellowship to Ryan Marshall (£40,000) and a PhD studentship from the MRC-Versus Arthritis Centre for Musculoskeletal Ageing Research.

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Signed

R.N. Marshall

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Not applicable – No tables in this chapter.

Chapter 3: Acute Resistance Exercise Training Does Not Augment Mitochondrial Remodelling in Master Athletes or Untrained Older Adults

- Table 1.** Participants anthropometric, body composition and exercise training characteristics.

Chapter 4: Quadriceps Muscle Electromyography Activity During Physical Activities and Resistance Exercise Modes in Younger and Older Adults

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Figure 7. Representative immunoblot images of upstream mTOR signalling. LAT-1, SNAT-2, Sestrin-2, IRS-1, P-IRS-1^{Ser636/639}, Akt, P-Akt^{Serine473}, TSC2, P-TSC2^{Ser939}, P-TSC2^{Ser1387}, P-TSC2^{Thr1462}, and Ponceau S.

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Chapter 6:

High-Dose, Leucine-Enriched Essential Amino Acids to Mitigate Step-Reduction-Induced Skeletal Muscle Atrophy and Proteostatic Dysfunction in Healthy Older Adults: Study Protocol for A Randomised, Placebo Control Trial.

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Figure 1. Age-Related alterations in mTOR expression. Protein expression and phosphorylation levels of p-mTOR (A) and total mTOR (B) before (Blue) and 1-hour post (Red) elastic band knee extension exercise in younger and older adults. Data are presented as fold-change relative to pre-RE young and mean $\pm$ SEM for n=8 subjects per group with individual data points. * Indicates significant time effects. # Indicates significant group effect (P<0.05).

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ABBREVIATIONS

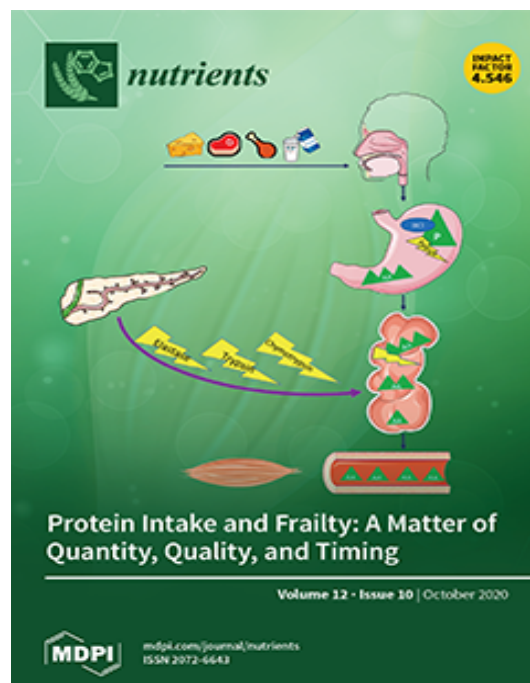
(In order of appearance)

- **RE** – Resistance Exercise
- **ELISA** – Enzyme Linked Immunosorbent Assay
- **NMJ** – Neuromuscular Junction
- **EWGSOP** – European Working Group Sarcopenia and Old People
- **VDBP** – Vitamin D Binding Protein
- **WHO** – World Health Organisation
- **DXA** – Dual X-ray Absorptiometry
- **ADP** - Adenosine Diphosphate
- **RDA** – Recommended Daily Allowance
- **CT** – Computerised Tomography
- **OXPHOS** – Oxidative Phosphorylation
- **DNA** - Deoxyribonucleic Acid
- **MRI** – Magnetic Resonance Imaging
- **mtDNA** – Mitochondrial DNA
- **RNA** - Ribonucleic acid
- **SPPB** – Short Performance Physical Battery
- **ATP** - Adenosine Triphosphate
- **MVC** – Maximum Voluntary Contraction
- **TUG** – Timed Up and Go
- **DRP1** - Dynamin-1-like protein
- **iMyoPS** – Integrated Myofibrillar Protein Synthesis
- **ASM** – Appendicular Skeletal Muscle
- **FIS1** - Mitochondrial fission 1 protein
- **TCA** - Tricarboxylic Acid
- **ROS** – Reactive Oxygen Species
- **MFN2** - Mitofusin-2
- **RAPTOR** - Regulatory-associated protein of mTOR
- **IL-6** – Interleukin-6
- **OPA1** - Optic atrophy 1
- **DEPTOR** - DEP Domain Containing MTOR Interacting Protein
- **TNF α** – Tumour Necrosis Factor Alpha
- **MCQ** – Mitochondrial Quality Control
- **PRAS40** - proline-rich Akt substrate of 40 kDa
- **CRP** – C-Reactive Protein
- **FGF21** - Fibroblast growth factor 21
- **TSC2** - Tuberous Sclerosis Complex 2
- **DHT** - Dihydrotestosterone
- **GDF15** - Growth Differentiation Factor 15
- **RHEB** - Ras homolog enriched in brain
- **RET** – Resistance Exercise Training
- **mTORC1** - mammalian target of rapamycin complex 1
- **RPS6** - Ribosomal protein S6
- **BMD** – Bone mineral Density
- **CSA** -Cross Sectional Area
- **EAA** – Essential Amino Acids
- **VDR** – Vitamin D Receptor
- **MU** – Motor Unit
- **4EBP1** - Eukaryotic translation initiation factor 4E-binding protein 1

- **FSR** – Fractional Synthesis Rate
- **FBR** – Fractional Breakdown Rate
- **IAAO** – Indicator Amino Acid Oxidation
- **D₂O** – Deuterium Oxide
- **APE** – Atom Percent Excess
- **3-MH** – 3-Methylhistidine
- **HIIT** – High Intensity Interval Training
- **T2D** – Type II Diabetes
- **MPB** – Muscle Protein Breakdown
- **BCAA** – Branch Chain Amino Acids
- **EB-RET** – Elastic Band Resistance Exercise
- **BR** – Bed Rest
- **ULI** – Unilateral Limb Immobilisation
- **SR** – Step Reduction
- **NMES** – Neuromuscular Electrical Stimulation
- **WP** – Whey Protein
- **CP** – Casein Protein
- **HMB** - Beta-Hydroxy-Beta-Methyl Butyrate
- **mRNA** – Messenger RNA
- **EPA** - Eicosatetraenoic
- **DHA** - Docosahexaenoic
- **n-3 FA** – Omega 3 Fatty Acids
- **MIS** – Multi Ingredient Supplement
- **EMG** – Electromyography
- **AMPK** - AMP-activated protein kinase
- **ACC** - Acetyl-CoA carboxylase
- **TFAM** - Mitochondrial transcription factor A
- **PGC1a** - Peroxisome proliferator-activated gamma coactivator 1-alpha
- **ULK1** - Unc51like kinase 1
- **CS** – Citrate Synthase
- **MFF** – Mitochondrial fission factor
- **MA** – Master Athlete
- **NAD⁺** - Nicotinamide adenine dinucleotide
- **ADL** – Activities of Daily Living
- **MN-RET** – Machine Resistance Exercise Training
- **BW** – Body Weight
- **FFM** – Fat Free Mass
- **SMM** – Skeletal Muscle Mass
- **BIA** – Bioelectrical Impedance Analysis
- **VL** – Vastus Lateralis
- **LAT1** – L-type amino transporter 1
- **SNAT2** - Sodium-dependent neutral amino acid transporter-2
- **MURF1** - Muscle-specific RING finger protein 1
- **Leu-EAA** – Leucine Enriched Essential Amino Acids
- **BMI** – Body Mass Index

CHAPTER 1

General Introduction



Published Peer-Reviewed Manuscripts from This Chapter: *Section 1.6. Nutritional Interventions in Inactivity and Disuse Atrophy.*

Marshall R.N., Smeuninx, B., Morgan, P., Breen, L. (2020) Nutritional Strategies for Disuse-Induced Skeletal Muscle Atrophy and Anabolic Resistance in Older Adults, from Whole-Foods to Isolated Ingredients: A Brief Review'. *Nutrients*.

1.1. Musculoskeletal Ageing & Sarcopenia

Skeletal muscle mass accounts for >40% of an individual's body mass and is essential for locomotion and cardiometabolic health ². As the largest tissue in the human body, skeletal muscle is the primary storage of amino acids (AA's), which are essential for the maintenance of muscle mass ³. Notably, skeletal muscle is in a constant state of turnover of 1-2% per day ⁴, with amino acids continuously being synthesised and broken down ³, with this process being facilitated by dietary protein intake ^{5,6} and resistance exercise (RE) (**see section 1.2**). The observed decrease in skeletal muscle mass places older adults at greater risk of developing a myriad of comorbidities (i.e., insulin resistance, [sarcopenic] obesity, hyperlipidaemia) and increased risk factors for falls and fractures ⁷. As such, several falls, or fractures in which bed-rest ⁸, cast-immobilisation ⁹, and/or a brief period of reduced activity ¹⁰ >60 years of age may punctuate the 'normal' decline in muscle mass and thereby result in an accelerated progression to functional disability (termed the 'catabolic crisis' model), in which tasks of daily living are no longer possible ¹¹.

1.1.1. Ageing Demographic

Worldwide, human lifespan is increasing at an alarming rate, with older adults in the UK, specifically, reaching an average of 81.2 years in 2021, representing a >5-year increase in average lifespan since 1990 ¹². Furthermore, the number of older adults aged >65 years as a percentage of the UK population is ~19% (~12.7 million), with this expected to increase to >22% within a decade ¹³. Notably, the fastest growing sub-population of older adults in the UK is the >85 years in which projected double from 1.6 million to 3.2 million from 2016-2040, with >5 million older adults aged >85 years by 2066 ¹³. There is also cause for

concern due to the increase in centenarians (i.e., >100 years of age), in which there has been a rapid increase of ~18% from 2019 to 2022, with >15,000 older adults living >100 years of age ¹⁴. The increase in human longevity can largely be attested to a radical improvement in healthcare, pharmacological therapies, and general living standards.

While the increase in human lifespan may initially seem positive, there is, however, a significant discord between an individual's lifespan (i.e., maximum years lived) versus their healthspan ¹⁵ (i.e., the number of years spent in good health) (**see section 1.1.4 lifespan versus healthspan in disease-free ageing**). The increase in the lifespan of humans is concurrent with a small and insidious reduction in skeletal muscle mass, strength, and function at a rate of 1-5% per annum (muscle mass; 1-2%, muscle strength; 1.5-5%, function, 3-4%) >50 years of age, termed 'sarcopenia' (**Figure 1**).

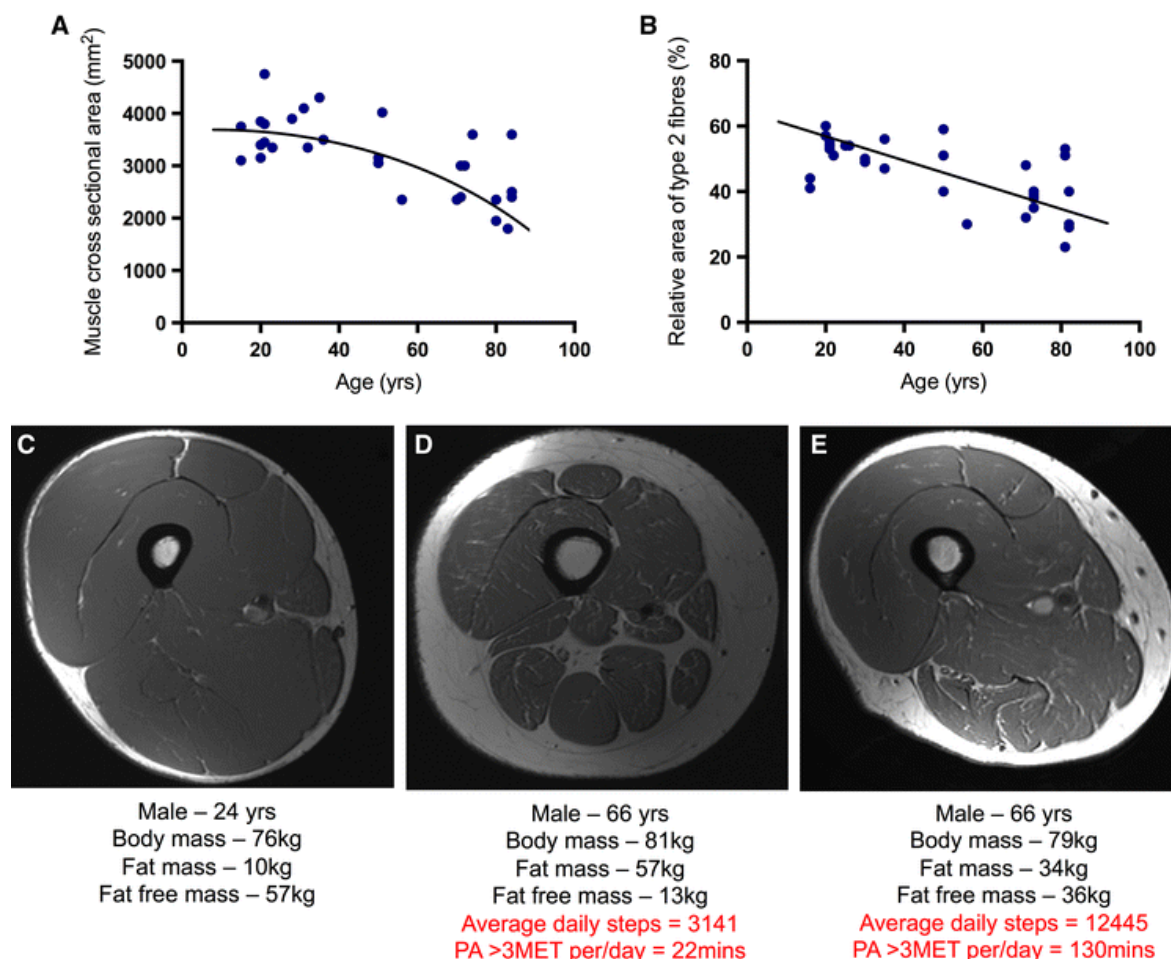


Figure 1. Skeletal muscle atrophy across the lifespan¹. Loss of skeletal muscle size and quality occurs during healthy ageing. Skeletal muscle cross sectional area (MRI^{CSA}) declines across lifespan (a) with a preferential decline observed in type 2 fibres (b). Representative MRI images depict skeletal muscle morphology in young (c), old-inactive (d) and old-active (e) males. a and b are adapted from (Lexell 1995)^{16,17}.

¹ This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. **McLeod, M., Breen, L., Hamilton, D.L. and Philp, A., 2016. Live strong and prosper: the importance of skeletal muscle strength for healthy ageing. *Biogerontology*, 17(3), pp.497-510.**

1.1.2. Sarcopenia: Clinical Definition, Prevalence and Cost

The term sarcopenia is derived from the Greek words 'sarco' and 'penia', meaning flesh and poverty, respectively. The term was first introduced by Rosenberg in 1989 to describe the process of age-related loss of skeletal muscle mass ¹⁸. Since its conception, the definition of sarcopenia has undergone several interpretations, with the current and most widely accepted clinical and practical definition developed by the European Working Group on Sarcopenia in Old People (EWGSOP) in 2010 ¹⁹, with a recent and notable updated definition in 2018 ²⁰. The EWGSOP define sarcopenia as 'low levels of measures for three parameters: (1) muscle strength, (2) muscle quantity/quality and (3) physical performance as an indicator of severity. Clinicians and healthcare practitioners can determine a case of sarcopenia with diagnostic tools for muscle strength (i.e., hand grip dynamometry, knee extensor torque or sit-to-stand test), muscle mass (i.e., dual-energy x-ray absorptiometry [DXA], computerized tomography [CT], magnetic resonance imaging [MRI] or bioelectrical impedance analysis [BIA]), and physical function (gait speed, short physical performance battery [SPPB], or timed up and go [TUG])²¹. Furthermore, the EWGSOP present cut-off points in which sarcopenia can be confirmed based on muscle strength (grip strength <27kg [male] or <17kg [female], or >15 seconds sit-to-stand), muscle mass (appendicular skeletal mass (ASM) <20kg [male] or <15kg [female], and physical function (<0.8m/second gait speed, or >20 second timed-up-and-go test). The guidelines set forth by the EWGSOP may help determine rates of prevalence of sarcopenia in community-dwelling and hospitalized older adults ²¹.

The prevalence of sarcopenia is difficult to determine due to variable classification and cut-off points. Early UK-based estimations range from 6.8% and 7.8% in men and women, respectively ²². However, recent estimations based on the EWGSOP suggest that 7.4% and 11% of men and women, respectively, are currently 'sarcopenic'. Furthermore, based on statistical modelling, the maximum probable prevalence of sarcopenia is 24.2% and 24.8% in men and women, respectively ²³. Global estimations following a meta-analysis of >150 studies suggest the prevalence of sarcopenia and severe sarcopenia ranged from 10-27% (i.e., 797,329,078 - 2,152,788,512) and 2-9% (i.e., 159,465,815 - 717,596,170) based on EWGSOP criteria and current global population of ~8 billion ²⁴. Considering the myriad of health consequences of sarcopenia, this places a significant strain on healthcare services and spending. Early US estimations in 2004 estimated the socio-economic cost of sarcopenia at ~\$18.5 billion per year ²⁵. However, recent estimates from both the UK and the US suggest the cost of sarcopenia to be ~£2.5 billion ²⁶ and ~\$40.4 billion ²⁷ respectively. Therefore, developing exercise and/or nutritional strategies to counteract this muscle loss is essential to extend the number of years lived in good health, as well as reduce the burden on health care systems and the global economy.

1.1.3. Overview of the Primary Aetiology of Sarcopenia

The mechanisms which contribute to the development of sarcopenia and age-related decline in skeletal muscle mass and function are undoubtedly multifactorial ²⁰. Several mechanisms have been hypothesised to accelerate sarcopenia such as endocrine alterations (i.e., declines in circulating

testosterone ²⁸, estrogen ²⁹, & vitamin-D ³⁰), mitochondrial dysfunction (i.e., decreased respiration, mitochondrial content and excessive ROS production) ³¹, chronic low-grade inflammation (i.e., elevated IL-6, TNF α & CRP) ³², neuromuscular (i.e., loss of motor units & fibre denervation) ³³, lifestyle factors (i.e., consumption of suboptimal protein [$<0.8\text{g/kg/day}^{-1}$] ³⁴, low (and over) nutritional intake, increased sedentary time [leading to sarcopenic-obesity ³⁵]), and altered proteostasis ³⁶ (i.e., blunted protein synthesis ^{5,37} and increased protein aggregation ³⁸). Ultimately, several of the aforementioned mechanisms occur simultaneously and often aggravate one another, overall accelerating age-related muscle deterioration and disability, morbidity and eventually death.

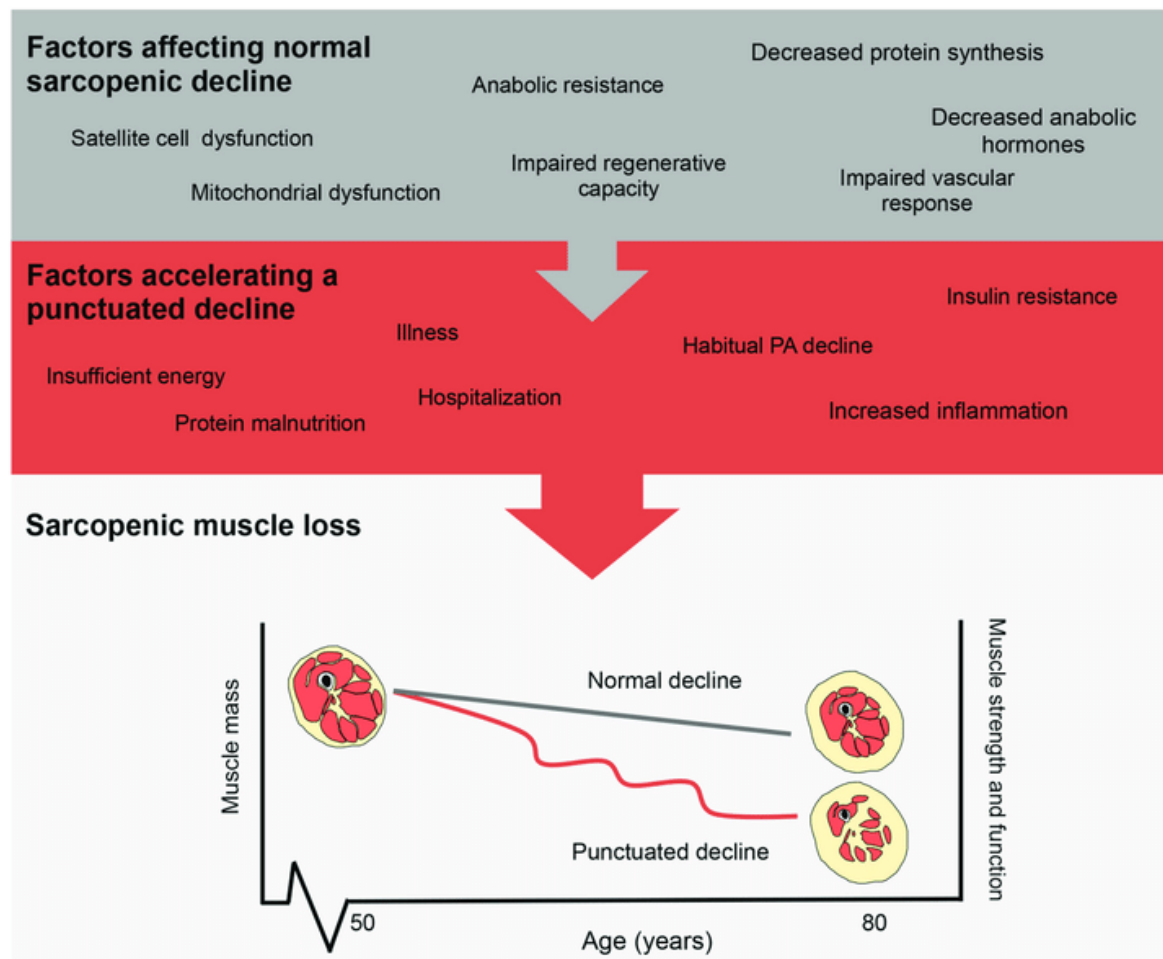


Figure 2. Factors influencing the progression of sarcopenia². Representations of normal sarcopenic muscle loss and accelerated muscle loss as shown by a punctuated decline³⁹.

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1.1.3.1. Endocrine

Testosterone

Levels of circulating bioavailable testosterone decrease at a rate of ~1% per year >40 years of age, termed 'andropause' ²⁸. The hypogonadal state in which older men exhibit results in an increased risk of cancer development (five-fold) and mortality (three-fold) ⁴⁰. Furthermore, testosterone plays a pivotal role in muscular development, in particular regulating muscle protein synthesis ⁴¹ via androgenic receptor signalling and the conversion of testosterone into steroidogenic enzymes 5 α -reductase and dihydrotestosterone (DHT) ^{42,43}. Older males may be prescribed exogenous testosterone therapy if circulating levels <231 ng/dL⁻¹ in order to restore 'youthful' levels of circulating testosterone to maintain levels of fat-free mass, strength, and muscle function in ^{40,42,43}. Indeed, several investigations have observed anabolic effects of daily transdermal ^{44,45} and/or biweekly intramuscular administration ^{46,47} of exogenous testosterone on muscle mass, strength, and function, with and without RET ^{48–51}. Indeed, the role of circulating physiological levels of endogenous testosterone as an anabolic component has recently been confirmed by Gharahdaghi et al., in a series of comprehensive metabolic investigations in younger ⁵² and older adults ⁵¹. Although therapeutic, low-dose, short-term testosterone therapy is known to be safe (100 mg/week⁻¹), the long-term cumulative effects of administration may have adverse and unwanted effects on prostate and cardiovascular health (i.e., erythrocytosis) ⁵³.

Estrogen

Similar to the aforementioned decline in testosterone in males, females undergo a comparable but abhorrent reduction in estradiol, termed 'menopause' between 40-50 years of age. Contrary to the small annual reductions in testosterone in males, the reduction in estrogen occurs much more rapidly, with a large reduction in circulating estrogen within the first year of menopause ⁵⁴. This process is well characterised by a reduction in bone mineral density (BMD) and an increase in whole-body adiposity ⁵⁵. Furthermore, decreased BMD places post-menopausal women at greater risk of falls-induced fractures ⁵⁶. Compared to testosterone, the role of estrogen on muscle protein turnover, muscle mass, and muscular strength in humans remains largely unanswered ⁵⁷. However, evidence suggests post-menopausal women display elevated basal rates of muscle protein synthesis compared to age-matched older males, and young, pre-menopausal women ^{29,58}. Interestingly, therapeutic administration of transdermal testosterone (1250 $\mu\text{g}/\text{day}^{-1}$) and progesterone (100mg/day⁻¹), but not transdermal estradiol (100 $\mu\text{g}/\text{day}^{-1}$) influences muscle protein synthesis ⁵⁸. Others have demonstrated a reduction, or perhaps a re-sensitizing effect on exercise-induced muscle protein synthesis rates with hormonal replacement therapy (i.e., 2 mg oral 17 β -estradiol) ⁵⁹, overall displaying the complexity in endogenous/exogenous hormonal milieu and the sexually dimorphic regulation of muscle mass during ageing and sarcopenia.

Vitamin-D (1,25-dihydroxyvitamin D₃)

Vitamin D is a fat-soluble vitamin associated with bone, immunity and skeletal muscle health and can be obtained via dietary consumption or exposure to ultraviolet B (UVB) rays. Activation of vitamin D₂ or D₃ into its active metabolite, 25-hydroxyvitamin D₃ (25, (OH)D₃), requires conversion via 25-hydroxy-lase in the liver. Following the conversion to 25, (OH)D₃, the metabolite goes through further hydroxylated into the biologically active hormone, 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) in the kidney where it can enter circulation and bind to vitamin D receptors (VDR) in other tissues (i.e., bone) and more recently, a focus on skeletal muscle has emerged ⁶⁰. Notably, however, 25, (OH)D₃ deficiency (i.e., <25 nmol/L) affects between 15-24% of UK adults, with a higher proportion of Asian (53.7%) and Black (34.9%) ethnicities being severely affected ⁶¹. Observational studies have consistently reported a causal relationship between low levels of circulating 25, (OH)D₃ and reduced muscular strength, lower extremity function in older adults, and sarcopenia. Recent advances have begun to observe the role of 25, (OH)D₃ and 1,25(OH)₂D₃ and VDR expression in skeletal muscle function, protein turnover, and mitochondrial function in pre-clinical models ^{60,62–64}. However, direct supplementation with vitamin D₃ has been observed to have little to no effect on skeletal muscle remodelling in older adults with and without vitamin D deficiency ^{65–67}, even when combined with RET ^{68,69}. Notably, there are large variances in dosing strategy in double-blind, placebo-control studies (400 IU/day⁻¹ - 4,000 IU/day⁻¹), and the measurement method (i.e., ELISA, UHP-LC-MS) ⁷⁰ and form of vitamin D (D₃, 25, (OH)D₃, 1,25(OH)₂D₃ or vitamin D₃ binding protein; VDBP) may influence the interpretation and translation into clinical practice/guidelines ^{71,72}. Though

vitamin D₃ supplementation does not significantly affect skeletal muscle mass and/or function in healthy older adults, it may, however, play a role in mitigating the musculoskeletal decline in metabolic or pathological disease states (i.e., liver disease & COPD) ^{73,74}.

1.1.3.2. Genetics

Environmental, and lifestyle influences such as reduced habitual activity ³⁹, excess calorie consumption ⁷⁵, decreased dietary protein intake ⁷⁶, and smoking ⁷⁷ are well-established variables in the onset and progression of sarcopenia. However, a genetic component and potential predisposition to sarcopenia may be evident, making an individual more or less susceptible to declines in muscle mass, strength, and function with age. Indeed, recent genome-wide association study (GWAS) meta-analyses with >6,000 Korean individuals observed three specific single nucleotide polymorphisms (SNP's), RPS10, NUDT3 and GPD1L, as genetic biomarkers of sarcopenia ⁷⁸. Interestingly, in larger European cohorts of >85,000 individuals, the top three identified SNP's related to muscle mass regulation during ageing were VCAN, ADAMTSL3 and IRS1 ⁷⁹. However, the calculated percentage variance of SNP's relating to lean body mass during ageing is <1%, suggesting it may be alterations in environmental and lifestyle factors modifying gene expression and transcription via the epigenome (i.e., DNA methylation, histone modification, and chromatin remodelling) ⁷⁸. Therefore, the role of the genome in the development of a pathological sarcopenic phenotype play little significance compared to the transcriptome, proteome, or metabolome ⁸⁰.

1.1.3.3. Mitochondrial Dysfunction

The decline in mitochondrial function has been extensively investigated during ageing and has been implicated in slower walking speed ⁸¹, fatigability ⁸² and development of sarcopenia ⁸³. Longitudinal data suggests the declines in cardiorespiratory fitness ($\text{VO}_2^{\text{peak}}$), phosphocreatine recovery rates and muscular strength correlate with declines in *ex vivo* mitochondrial respiratory function, in particular state-3 ADP-supported respiration ⁸⁴. Interestingly, there have been several inconsistencies with authors showing an age-related decline in mitochondrial function ^{31,85–87} while others do not ⁸⁸, which may be explained by technical differences in assessing mitochondrial function and/or normalisation methods ⁸⁹. Gold-standard measurements of mitochondrial respiratory function via high-resolution respirometry have observed no differences in state II respiration (residual, non-mitochondrial respiration), state III (ADP-supported) or maximal OXPHOS between younger and older adults ⁹⁰. However, upon titrations of ADP from 175 μM – 10,000 μM , there was an apparent decline in ADP sensitivity (i.e., the apparent K_m of ADP ~25% higher in older adults), which was concurrent with a reduction in complex II & III protein content of the electron transport chain ⁸⁷. Other than respiratory dysfunction ⁹¹, pre-clinical and human data suggest mitochondria also exhibit decreased mitochondrial volume/density (i.e., citrate synthase ⁹², mtDNA ³¹), increased reactive oxygen species (ROS) (i.e., H_2O_2 emissions) ⁸⁷, reduced biogenesis ⁹³, dysregulated fission-fusion dynamics ^{93,94}, and reduced mitophagy ⁹⁵ during ageing (**Figure 3**). In particular, relative to healthy younger adults, older adults report an 8%/decade⁻¹ decline in mitochondrial ATP production ³¹, and ~37% decline in fractional mitochondrial protein synthesis ⁹⁶. Therefore, as the critical

regulator of cellular energy homeostasis, any alterations in organelle function, morphology or turnover will likely have devastating effects on muscle and whole-body metabolism.

The dynamic role of mitochondrial dynamics (i.e., fission, fusion and subsequent mitophagy) is well established in pre-clinical mouse models observing altered fission-related protein expression (i.e., DRP1 and FIS1) and fusion (MFN2 and OPA1). In particular knockout of DRP1 results in severe muscle atrophy, decreased respiration, independent of increased mitochondrial content/volume ^{97,98}. Although rodent data supports a key role of mitochondrial quality control (MCQ), it's role in human skeletal muscle remains ill defined. However, several authors have observed declines in protein expression of DRP1 and OPA1 in sedentary older adults compared to younger adults speculating their role in human MCQ ^{99,100}. Unlike rodent models, determining the specific role of MCQ *in vivo*, in humans is unclear. Following mitochondrial fission, mitochondria undergo degradation by the autophagosome termed 'mitophagy' which are reliant on a process involving the PINK1-Parkin activation ^{101,102}. This pathway results in the recruitment of autophagy receptors p62 and Optineurin to the outer mitochondrial membrane where the recruitment of LC3B proteins to form the autophagosome ¹⁰³. Mitophagy can also be induced via mitophagy-specific receptors FUNDC1, BNIP-3 and NIX ¹⁰⁴. Similarly, to fission-fusion events, mitophagy is poorly characterised in humans and is limited to static markers of protein expression. Nevertheless, ageing has been shown to decrease PINK1 protein content by ~43% compared to younger adults ¹⁰⁵, which may explain poor mitochondrial quality control, and overall imbalance in mitochondrial proteostasis ¹⁰³.

The maintenance of mitochondrial health is fundamentally linked with physical activity levels ¹⁰⁶. Exercise, therefore, is a key regulator of mitochondrial function, biogenesis, and quality control. Notably, in older adults who have maintained high levels of physical activity and exercise throughout their life (termed 'lifelong exercisers' or 'master athletes') show significant mitigation of age-related decline in muscle mitochondrial function traditionally observed and, in some cases observe similar muscle morphology and metabolic function to younger adults ^{107–113} **(see 1.3.2. the role of mitochondria in ageing and sarcopenia)**.

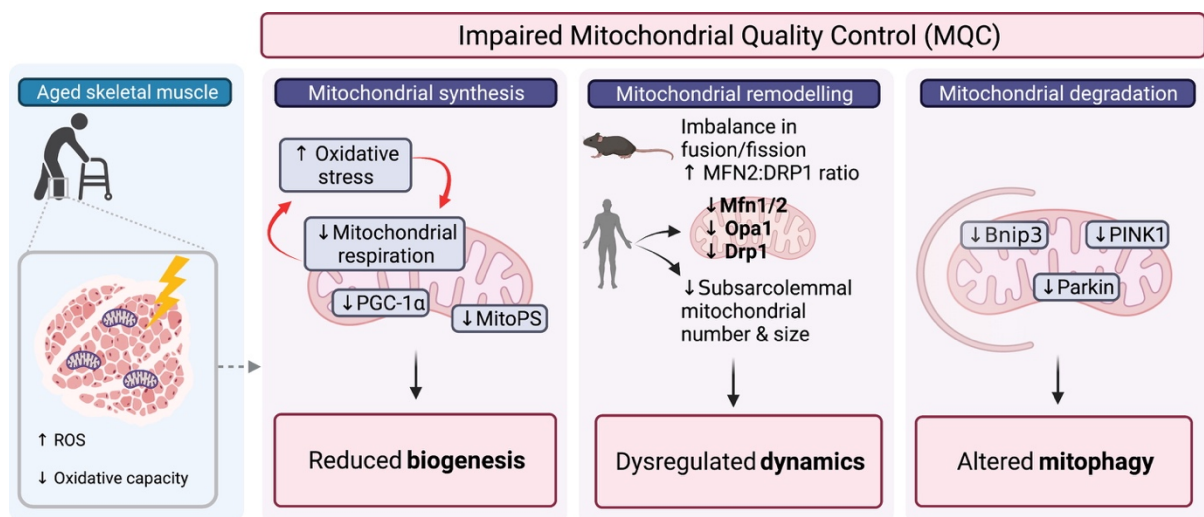


Figure 3. Conceptual model of mitochondrial dysregulation during ageing³. Representations of healthy mitochondrial quality control (mitophagy and fission-fusion dynamics) and sarcopenic muscle displaying impaired mitophagy, and fission resulting in impaired mitochondrial function and muscle health¹⁰⁴.

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1.1.3.4. Inflammation

Ageing results in a chronic state of low-grade inflammation termed 'inflammaging' which is primarily characterised by elevated levels of CRP ^{114,115}, IL-6 ¹¹⁶, and TNF α ¹¹⁷. However, other inflammatory markers have been implicated (i.e., FGF21 ^{118,119}, GDF15 ¹²⁰ and Irisin ¹²¹). Increased circulating cytokines result in an impairment in immunity, however, the exact mechanism and source of inflammatory cytokines remain unclear. Authors have speculated immunosenescence (i.e., increased cytotoxic CD8⁺ and CD28⁺ T-cells) ¹²², secretion of visceral adipokines (i.e., leptin ¹²³ and adiponectin ¹²⁴), gut dysbiosis (i.e., increased intestinal permeability and leaking of microbial bacteria into circulation) ¹²⁵, and accumulation of senescent cells ¹²⁶. From a muscle-centric point of view, an increase in circulating inflammatory markers, and in particular an increase in intramuscular inflammation (i.e., pro-inflammatory lipid species [ceramide and diacylglycerol ¹²⁷]) results in a blunted muscle protein synthetic response to exercise and protein feeding ^{127–131}. Therefore, several authors have sought to reduce chronic low-grade inflammation via nutritional (i.e., glycine) or pharmacological drugs (i.e., acetaminophen) in an attempt to offset or restore normal physiological function. Interestingly, pharmacological inhibition of cyclooxygenase (COX) results in a reduction in resistance exercise-induced muscle growth ¹³² and postprandial muscle protein synthesis ¹³³, with no protective effect on inactivity-induced muscle atrophy in older adults ¹³⁴. On the other hand, in pre-clinical *in vitro* and *in vivo* models of inflammation (induced via lipopolysaccharide [LPS]) the non-essential amino acid Glycine restored mTORC1 signalling and leucine-induced MPS, suggesting a potential role as to prevent inflammatory-related muscle

wasting^{135–137}. The role of inflammation in sarcopenia and ageing is in itself multifactorial due to the complex interplay between immunity, cytokines, and senescence. Future work should seek to clarify the precise role of each inflammatory variable, and the efficacy of exercise and/or nutritional strategies to attenuate inflammaging.

1.1.3.5. Neuromuscular

Concurrent with the absolute decrease in muscle mass, this is primarily due to a reduction in total fibre number within a muscle. Seminal studies observing muscle cross sections of human autopsies showed a ~18% reduction in *vastus lateralis* size in older adults compared to young¹³⁸. In particular, the authors undertook detailed anatomical counts of individual myofibers resulting in 478,000 vs. 364,000 fibres in young and older adults, respectively^{138,139}. More recent investigations often employ the use of muscle biopsy samples to determine fibre number, cross sectional area, and fibre-type.¹⁴⁰ Moreover, the decline in fibre CSA is predominantly due to a reduction in type II vs. type I fibres^{141,142}, which may be muscle dependant (i.e., *vastus lateralis* vs. *biceps brachii*)¹⁴³.

Muscle contraction is regulated by the motor unit (MU), comprising of the MU and the fibre it innervates. Ageing and sarcopenia-induced fibre atrophy has been associated with reduced MU and MU size. Seminal autopsy investigations observed ~30% fewer spinal cord bodies serving the lower limb musculature¹⁴⁴. Recent mechanistic investigations utilising surface and intramuscular electromyography (EMG) have observed 40-50% reduction in MU number within the *tibialis anterior* and *vastus lateralis* of older adults

compared to young ^{145–147}. The loss of MU results in the denervation of a muscle fibre, which in younger age, is reinverted by a nearby axon to maintain muscle mass in a process termed ‘MU remodelling’. During ageing, MU remodelling is dysregulated resulting in insufficient reinnervation of fibres resulting in fibre atrophy. Thus, old muscle compromises of fewer MU, but larger in size (i.e., MU/fibre ratio), until disease or disuse accelerates MU remodelling, resulting in fewer and smaller MU with reduced fibre CSA, morphology, and fibre type distribution (i.e., grouping) ¹⁴⁸. Interestingly, athletic older adults and lifelong exercise does not appear to mitigate neuromuscular decline ¹⁴⁶. Molecular mechanisms underpinning motor unit dysfunction is primarily driven by alteration in neuromuscular junction (NMJ); the synapse between the MU and muscle fibre ¹⁴⁹. Although a significant portion of our understanding of NMJ regulation of muscle fibre atrophy is from pre-clinical mouse models ^{150–152}, recent human investigations have begun to explore the NMJ via targeted gene expression and immunofluorescent analysis of key NMJ components across ageing/exercise ^{153–156}. These novel analytical techniques will undoubtedly shed light on the molecular regulation of the neuromuscular system to identify exercise and/or therapeutics to mitigate NMJ and ensuing motor unit dysfunction in older adults.

1.1.3.6. Lifestyle

The influence of lifestyle on sarcopenia is often overlooked in the search of defining key molecular regulatory mechanisms or pathways. However, lifestyle factors such as dietary protein intake, physical activity, smoking ⁷⁷ and alcohol

consumption are arguably *more* important as they will largely influence the physiological and molecular regulation of muscle mass, strength, and function.

1.1.3.6.1. Dietary Protein Intake & Malnutrition

Protein nutrition is essential for the maintenance of skeletal muscle mass and function. The World Health Organisation (WHO) recommended daily allowance (RDA) for protein is $0.8 \text{ g/kg}^{-1}/\text{day}^{-1}$, which is believed to adequately support metabolic homeostasis ¹⁵⁷. However, this reference value is based upon nitrogen balance (NB) investigations. The NB method is based upon urinary nitrogen being subtracted from consumed nitrogen (i.e., dietary protein) and a net positive or negative value dictates muscle mass maintenance. The development of sophisticated stable isotope tracer methodologies (i.e., Intravenous $^{13}\text{C}_6$ infusions, oral D_2O , D_3 -Creatinine, and D_3 -Methylhistidine ¹⁵⁸) have allowed us to determine acute and chronic rates of protein/amino acid metabolic flux and muscle mass following the provision of dietary protein (**see section 1.2.1 on ‘quantification of *in vivo* muscle protein synthesis and breakdown**). Intricate metabolic investigations have consistently observed an increased requirement of older adults beyond that of the RDA and exhibit a reduced sensitivity to dietary protein compared to younger adults, termed ‘anabolic resistance’ ^{159,160} (**see section 1.2.4 on ‘age-related anabolic resistance’**). Recent meta-analyses ^{161,162}, and expert position statements ^{76,163–166} recommend a dietary protein intake of $1.2\text{--}1.6 \text{ g/kg}^{-1}/\text{day}^{-1}$ to be ‘optimal’ for healthy older adults and maintain muscle mass and health in older age. However, many older adults often fall short of this recommendation ^{1,167}. Furthermore, cross-sectional data from UK (**Figure 4.**)

^{1,168}, Netherlands ^{169,170}, and North America ¹⁷¹ have observed a skewed dietary protein eating pattern in favour of a large protein serving consumed in the early evening. Meal-to-meal dietary protein recommendations have since been encouraged to positively influence 24-hour protein synthesis rates ¹⁷², and maintenance of lean body mass ¹⁷³.

Besides the daily dose and pattern of dietary protein intake, there is an increasing body of evidence to suggest the source of protein (i.e., animal vs. plant), and thereby the quality of protein (i.e., low vs. high quality) impacting rates of protein synthesis rates and muscle mass maintenance in older adults ¹⁷⁴. The extent to which dietary proteins stimulate muscle protein synthesis is largely dictated by the AA profile, in particular the leucine content, as well as its digestion and absorption kinetics resulting in rapid plasma AA bioavailability ¹⁷⁵. For example, it is generally accepted that most plant-based proteins (i.e., soy ¹⁷⁶, pea ^{177–179}, wheat ^{180–182}, potato ^{183,184}, mycoprotein ^{185–187}) exhibit a lower digestibility, deficient in one-or more AA's, and thus, not adequately support muscle anabolism compared to a dose-matched animal-based protein ^{175,188}. Indeed, a recent systematic review from our laboratory observed a favourable shift to high-quality proteins to support muscle anabolism and increases in lean body mass ¹⁶¹. Therefore, greater doses ^{180,185,187}, AA enrichment ^{189–191}, and/or blending ^{179,192,193} of plant-based sources are needed to facilitate a robust muscle anabolic response similar to animal-based proteins. Although the role of protein quality in muscle anabolism favours high-quality, there remains no significant differences between high- and low-quality proteins on changes in lean body mass when consuming $>1.2 \text{ g/kg}^{-1}/\text{day}^{-1}$ when combined with RE ¹⁶¹. The role of protein

quality in older adults consuming sub-optimal (i.e., 0.8-1.0 g/kg⁻¹/day⁻¹) protein warrants investigation. The role of dietary protein intake in older adults should offer pragmatic strategies to facilitate 'optimal' protein needs depending on dietary requirements (i.e., vegetarian/vegan) or physical activity levels, with a focus on higher-quality, evenly distributed protein meals.

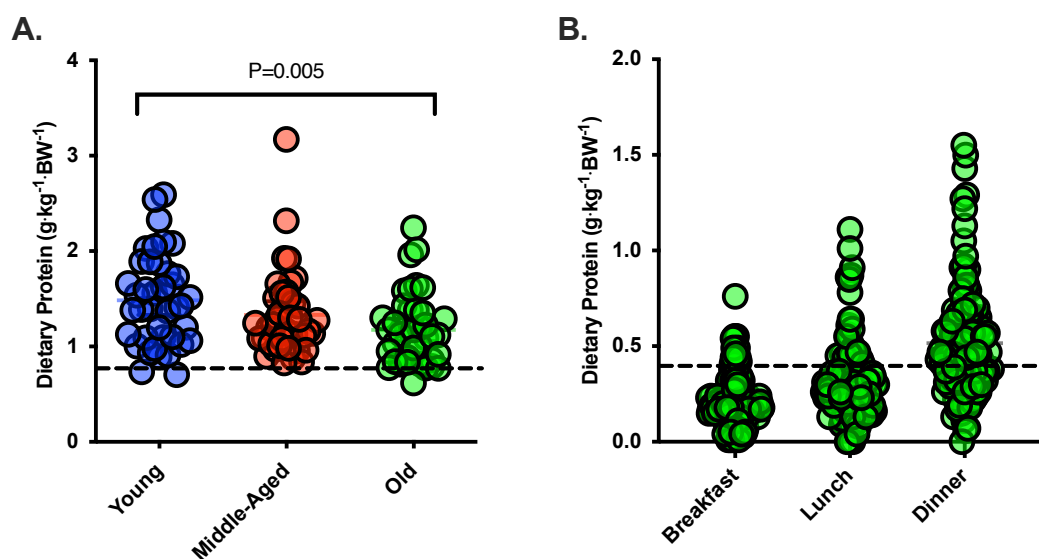


Figure 4. Cross sectional data of dietary protein intake of younger, middle-aged, and older adults in Birmingham, UK ⁴. (A) Average relative daily protein intake in young, middle-aged, and older individuals in Birmingham ¹. Scatter plots display mean with 95% confidence intervals. Dashed line indicates current recommended daily allowance for dietary protein allowance of 0.8 g·kg⁻¹·day⁻¹. (B) Meal-specific relative protein intakes for each meal consumed at breakfast, lunch, and dinner in older adults. Dashed lines represent threshold protein intakes 0.4 g·kg⁻¹, suggested for maximal stimulation of MPS in older adults ¹⁵⁹.

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1.1.3.6.2. Physical Inactivity

Maintaining sufficient levels of physical activity are essential for healthy musculoskeletal ageing. UK physical activity guidelines suggest an adult complete >150 minutes of moderate intensity exercise per week ¹⁹⁴. Unfortunately, older adults across the UK ¹⁹⁵, Europe ^{196,197}, and North America ^{198,199} are not meeting current physical guidelines. In the UK specifically, only 15% and 10% of older men and women, achieved ≥ 150 minutes per week ¹⁹⁵. Interestingly, independent of structured aerobic and RET, there is a substantial body of evidence in support of maintaining daily step count >10,000 steps/day¹ to mitigate disease risk (i.e., dementia ²⁰⁰, diabetes ^{201,202}, cancer ²⁰³, cardiovascular disease ²⁰⁴) and all-cause mortality ^{205,206}. Alarming, if inactivity was reduced by as little as 10% or 25%, more than >500,000 and >1.3 million deaths would be averted each year ²⁰⁷.

Mechanistically, the impact of even short-term periods of step reduction results in severe metabolic dysfunction and muscle atrophy in both younger and older adults ^{10,208–212} (**see section 1.4.3 on ‘reduced physical activity and step reduction’**). Seminal evidence by Olsen et al., observed large declines in insulin sensitivity and increased visceral fat accumulation following 2-weeks of reduced step count (<1,500/day⁻¹) in healthy, active younger males ²¹¹. This has since been investigated across younger ^{209,212}, older ^{10,213} and diseased individuals ²¹⁴, with stable isotope tracer methodologies, overall showing a rapid protein metabolic and musculoskeletal decline. Notably, when translated to a “catabolic crisis” model, these small, but intermittent periods of disuse, inactivity or step reduction across old age may result in a punctuated decline, leading to early physical disability.

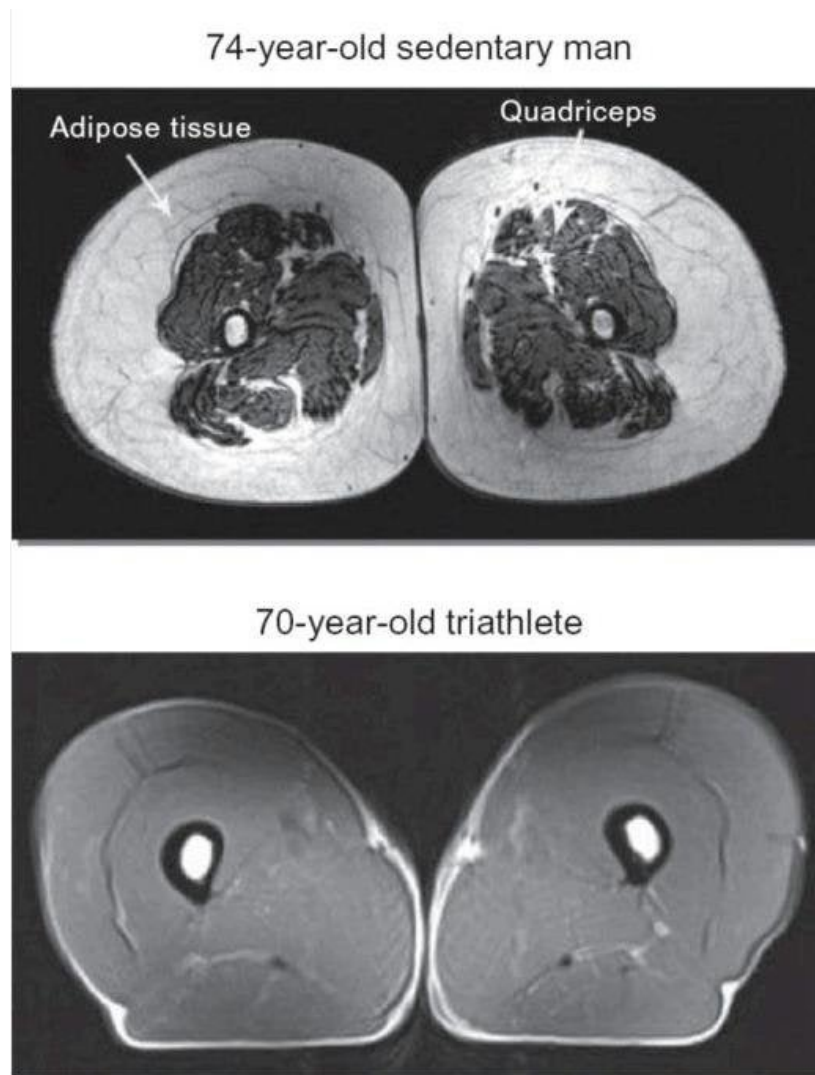


Figure 5. The influence of a sedentary lifestyle compared to lifelong exercise on muscle mass and quality ⁵. MRI CSA representations of an unhealthy 74-year-old male versus a highly active 70-year-old male. Note significant differences in muscle mass, fat mass, intramuscular fat ²¹⁵.

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1.1.3.7. Proteostasis

The maintenance of protein homeostasis, termed 'proteostasis', refers to the process of protein synthesis, protein breakdown, protein folding and protein aggregation ²¹⁶. It is often assumed, and potentially oversimplified, that protein turnover is just the delicate balance of protein synthesis and breakdown, overall creating a net positive or negative balance dictating muscle mass accretion or loss. However, two key aspects of proteostasis are often overlooked in this equation, cell proliferation and protein aggregation (**see section 1.2. on 'regulation of muscle mass and protein turnover'**). Measurements of cell proliferation (i.e., DNA synthesis) are an important variable in determining muscle proteostasis (i.e., protein:DNA ratio) ²¹⁷. Therefore, an increased protein synthesis relative to DNA synthesis is indicative of a greater proportion of protein synthesis related to proteome turnover, and less to cell proliferation ²¹⁸. Lastly, protein aggregates and 'inactive proteins' are a result of misfolding that are resistant to turnover ²¹⁹, which may in part be due to alterations in autophagy flux with ageing ²²⁰. Therefore, the accumulation of damaged, misfolded, or aggregated proteins within skeletal muscle alters the rate in which the proteome can dynamically turnover ²²¹.

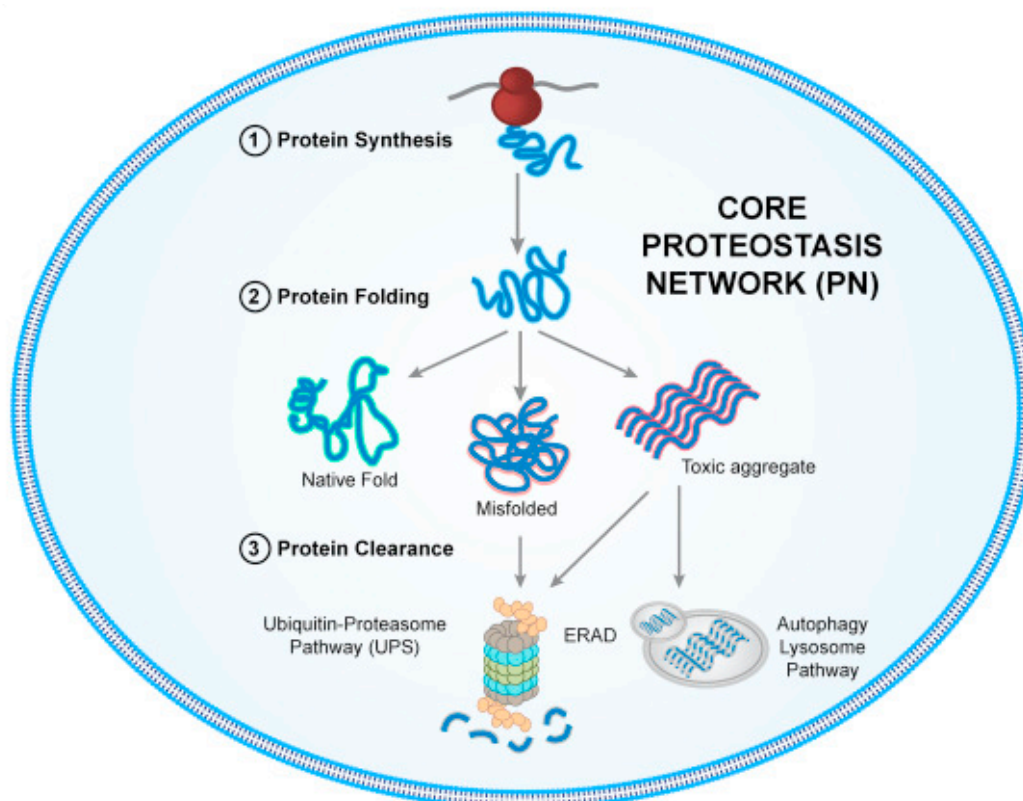


Figure 6. Overview of key proteostatic mechanisms ⁶. The three core processes contributing to proteostasis; protein synthesis, protein folding, and protein degradation of misfolded, excess, or aggregated protein. These processes tightly interact to maintain healthy proteostasis and cellular function.

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During ageing, it well accepted that the declines in muscle mass are due to the decreases in postprandial protein synthesis, and less so changes in postabsorptive protein synthesis, or elevations in protein breakdown (see **section 1.2.4 ‘age-related anabolic resistance’**). The differences in the daily oscillations of protein synthesis and breakdown following meal-time dietary protein consumption (*potentially* due to skewed protein intake ^{168,172,222}) may be important for muscle mass maintenance ²²³. For example, the repeated blunting of postprandial MyoPS following breakfast, lunch and dinner may result in a negative net balance and loss of muscle protein over a 24hr period ¹⁷².

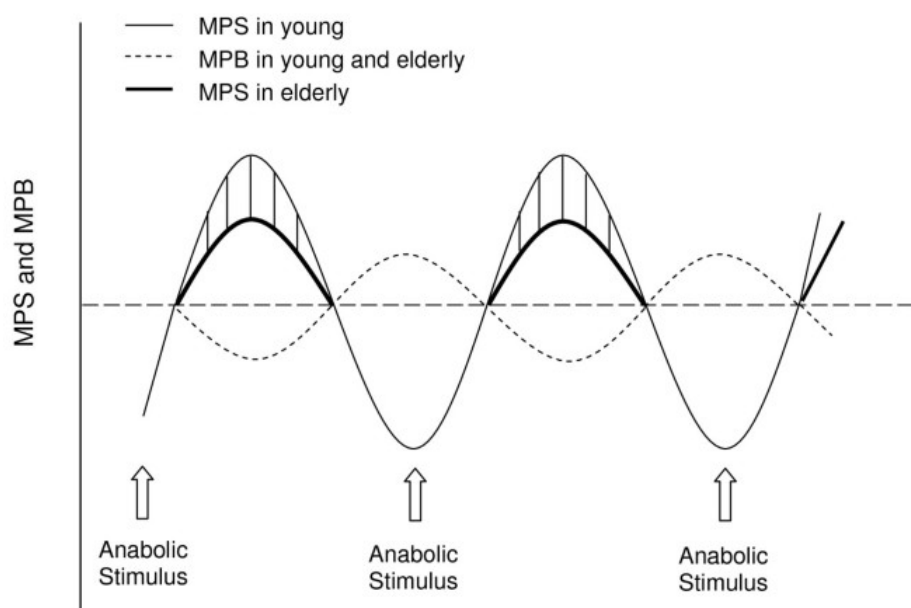


Figure 7. Schematic representation of muscle protein metabolism in response to anabolic stimuli in younger and older adults ⁷. Healthy older adults display an inability to mount a robust protein synthetic response to feeding above that seen in the basal-state.

⁷ Reproduced with permission from Springer Nature. Springer Nature is pleased to offer reuses of its content for a thesis or dissertation free of charge contingent on resubmission of permission request if work is published. **Breen, L. and Phillips, S.M., 2011. Skeletal muscle protein metabolism in the elderly: Interventions to counteract the ‘anabolic resistance’ of ageing. *Nutrition & metabolism*, 8(1), pp.1-11.**

When accounting for the aforementioned variables, pre-clinical mouse models coupled with sophisticated longer-term D₂O tracer methodologies over multiple weeks, ageing results in no blunting of muscle protein synthesis, although this varies by muscle (i.e., tibialis anterior, gastrocnemius, soleus) and subcellular fraction (i.e., myofibrillar, mitochondrial, collagen) ²²⁴. This has recently been observed in human data suggesting younger and older adults do not differ in rested rates of muscle protein synthesis (Young; $1.35 \pm 0.08\% \text{ day}^{-1}$ vs. Old; $1.34 \pm 0.09\% \text{ day}^{-1}$) or indeed following 6-weeks of RET (Young; $1.45 \pm 0.05\% \text{ day}^{-1}$ vs. Old; $1.39 \pm 0.1\% \text{ day}^{-1}$) ²²⁵. This is likely due to the choice of stable isotopic tracer (¹³C₆ Phenylalanine vs. D₂O), labelling period (5 hrs vs days/weeks), and development of more sensitive mass spectrometry capabilities ^{219,226} (**see section 1.2.1 on ‘quantification of *in vivo* muscle protein synthesis & breakdown’**). The same study observed no alterations in proteostasis (i.e., protein:DNA ratio) between older adults, and that the muscle growth response in younger adults is likely due to greater ribosomal capacity per unit of DNA (i.e., RNA:DNA ratio: Young, +32% vs. Old, +9%) ²²⁵. In brief, ageing undoubtedly alters muscle proteostasis (i.e., synthesis, breakdown, proliferation, and aggregation) ^{218,221}. However, human metabolic research typically focuses on solely synthesis rates, therefore the exact mechanisms of proteostatic dysfunction remain unclear during ageing and sarcopenia.

1.1.4. Lifespan Versus Healthspan in Disease-Free Ageing

1.1.4.1. The ‘Master Athlete’ Model of Ageing

Our current experimental model of ‘healthy ageing’ is limited by the study older adults in good general health and free from non-communicable disease. However, the typical study of ageing often does not account for lifelong physical activity levels ²²⁷. Notably, it’s the study of lifelong exercise in cohorts termed ‘master athletes’ that are arguably a true study of optimal musculoskeletal ageing ²²⁸. Master athletes (MA) are typically characterised as an older adult who has participated in structured athletic training (endurance or RE) and/or athletic competition their entire adult life ²²⁸. This proposes the question as to whether lifelong exercise can mitigate the physiological impairments in muscle mass, strength, and function.

MA have been observed to maintain high levels of skeletal muscle mass, strength, and metabolic function, relative to healthy older adults, and in some cases, younger adults ^{110,153,229–233}. Performance in athletic competition is undoubtably the greatest indicator of physiological function. Current world records for 100 m sprint and 10,000 km distances are 13.49 & 15.03, and 42:39 & 51:46 seconds in men and women, respectively, for >75 years old ²³⁴. This maintenance of exercise performance can generally be attributed to greater muscle mass and quality (**Figure 5.**). A recent systematic review and meta-analysis from our group including >50 studies and >900 lifelong exercisers have observed similar muscle characteristics (i.e., muscle CSA, fibre type & area), body composition (i.e., fat-free mass, fat mass) and muscle function (VO_{2max} and MVC) between MA and healthy young controls, and greater than age-matched ‘untrained’ older adults, overall displaying an alleviation of traditional

age-related decline ¹¹². Further work by our laboratory has also determined the turnover rates of MA ¹¹³. Overall, showing untrained older adults have similar iMyoPS rates in rested ($\sim 1.47\%/day^{-1}$ vs. $\sim 1.46\%/day^{-1}$), and post-exercise state ($\sim 1.60\%/day^{-1}$ vs. $\sim 1.61\%/day^{-1}$), to endurance-trained MA, suggesting muscle protein turnover rates are not the primary mechanism by which MA display greater muscle mass and quality ¹¹³. Recent in-depth, unbiased skeletal muscle proteomic analysis of 14 elite-level octogenarian athletes (8 world-record holders), identifying >6,000 proteins, observed 880 differently regulated proteins compared to untrained older adults ²³⁵. Notably, of the proteins identified, 369 were mitochondrial, with TCA cycle, OXPHOS and electron transport proteins being significantly greater in MA, and comparable to healthy younger individuals ²³⁵. Overall, MA, and older adults who have participated in lifelong exercise display greater physiological and metabolic health compared to age-matched untrained older adults. However, based upon the 'Set Point Theory' of ageing by Professors Steve Harridge and Norman Lazarus ²³⁴, MA are not entirely resistant to the ageing process and are merely delaying the inevitable physiological decline (i.e., increased healthspan) and will still display a period of disability and ill health prior to death ²³⁴. However, the socioeconomic burden of MA are significantly lower, relative to sedentary adults. This is primarily due to the compressed period of morbidity in later-life, and reduced reliance on social care and prescription medications ²⁷.

1.2. Regulation of Skeletal Muscle Mass & Protein Turnover

1.2.1. Quantification of *In Vivo* Muscle Protein Synthesis & Breakdown

Since the implementation of stable isotopic tracer techniques in humans, and the rapid improvements in mass spectrometry instruments, the rate and

resolution by which we can quantify muscle protein synthesis ²³⁶, breakdown ^{237,238}, and even to the extent in which in certain subcellular protein pools (i.e., myofibrillar ²³⁹, mitochondrial ²⁴⁰, cytosolic ²⁴¹ and collagen ²⁴²), RNA/DNA synthesis ^{243,244}, and even individual turnover rates of proteins can be determined ^{245,246}. However, prior to these advancements, the signal transduction and phosphorylation of mTORC1 intermediates could, and still is determined via immunoblotting in exercise/muscle physiology research ²⁴⁷. Here I provide a brief overview of western blot techniques to study muscle molecular signalling, and the role of oral stable isotopic tracers to quantify muscle protein anabolism *in vivo*.

1.2.1.1. Immunoblotting of mTORC1 Signalling Proteins

Western blots have traditionally been utilised in biomedical & physiological research to determine regulatory events underpinning the adaptive response to therapeutics. However, this method has been utilised in physiology research to investigate protein abundance, subcellular localisation, protein-protein interactions, and post-translational modifications (i.e., phosphorylation, ubiquitylation, methylation and SUMOlation) of proteins in response to exercise, ageing, nutrition, and disease ²⁴⁷. There has been a widespread incorporation of western blot techniques to study the muscle-specific molecular signalling in response to exercise science, with a particular focus on protein phosphorylation status of mTORC1-related proteins that are involved in regulating muscle protein synthesis, and subsequent muscle growth following acute or chronic resistance/endurance training. In brief, the process of western blots relies on six major steps (**Figure 7**) that rely on (1) biological sample, (2)

homogenisation and fractionation, (3) SDS-Page, (4) electro-transfer, (5) antibody probing, and (6) chemiluminescent imaging and visualisation. Overall, this process allows us to quantify the protein band intensity of a protein of interest to determine how it responds to certain physiological stress such as exercise. A detailed outline of western blot technique in human physiological and metabolic research has been described elsewhere ²⁴⁷. Below, we will discuss the role of mTORC1 signal transduction and its pertinent role in muscle mass maintenance, and remodelling process.

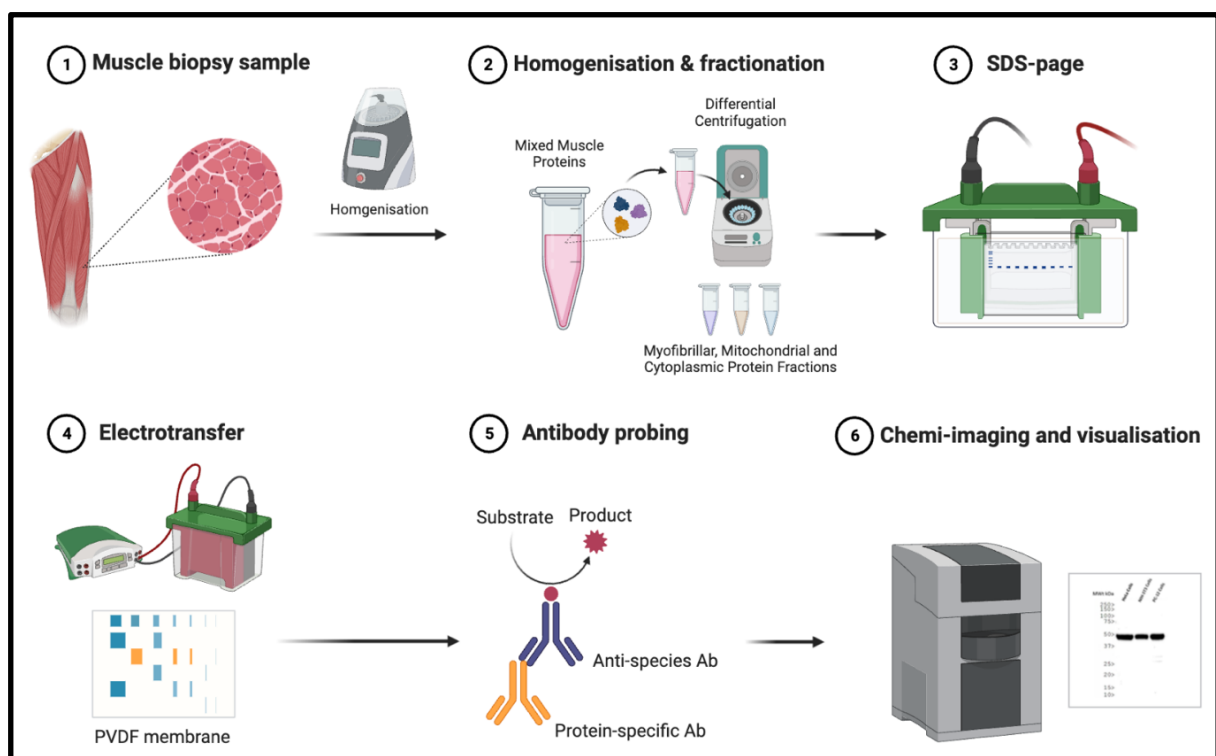


Figure 8. Western blot experimental procedure⁸. Skeletal muscle biopsy harvested from human vastus lateralis muscle (1), homogenisation of snap-frozen tissue in specific detergent-based buffer and isolation of subcellular protein pools within skeletal muscle via differential centrifugation (2). Prepared protein loaded into tris-glycine gels to separate protein molecular weights from ~250-10kD (3), transfer of protein from gel to PVDF membrane (4), and incubation in primary/secondary antibody to determine protein abundance (5). Finally, the protein of interest can be detected following incubation in chemiluminescent substrate to detect protein band.

⁸ Created using BioRender .

RE is a potent stimulator of muscle growth, and thereby results in large post-exercise increases in protein signalling (i.e., phosphorylation) to facilitate elevated protein synthesis ^{248,249}, which when practised multiple times per week, will lead to the accrual of muscle mass over time. Since its discovery ~30 years ago in yeast by Professor David Sabatini at Johns Hopkins University ²⁵⁰, the serine/threonine mTOR protein kinase has been observed to be a highly nutrient and mechanically sensitive pathway controlling cellular growth ²⁵¹, thereby crucial in the maintenance of skeletal muscle, and its adaptive growth or atrophy following exercise and disuse. Interestingly, recent evidence is emerging questioning its role in muscle mass maintenance in which protein synthesis and muscle maintenance can be achieved independent of mTOR itself ^{252–254}. Nevertheless, the characterisation of exercise and/or protein-induced alterations in the mTORC1 signalling pathways is well established and will be discussed in **section 1.2.4**.

Briefly, mTORC1 contains several signalling proteins that converge on mTOR kinase to alter its translocation and subsequent phosphorylation of downstream intermediates to facilitate translation initiation, and synthesising of proteins. Unlike its sister complex mTORC2, mTORC1 consists of regulatory-associated protein of mTOR (RAPTOR), DEP domain-containing mTOR-interacting protein (DEPTOR) and proline-rich Akt substrate 40 kDa (PRAS40) which regulate mTOR protein kinase activity (**Figure 8**). An important and often overlooked activator of mTOR activation is ras homolog enriched brain (RHEB), which in its GTP-bound state, binds to mTOR, and activates the complex. Contrary, tuberous sclerosis factor 2 (TSC2) initiates GTP hydrolysis, and therefore, inhibits mTORC1 activation when bound to RHEB. Following a

nutrient and/or mechanical stimulus, the phosphorylation of TSC1/2 subsequently dissociates from RHEB allowing direct activation of downstream proteins (i.e., P70S6k, RPS6, 4EBP-1) (**Figure 8**). P70S6 Kinase 1 (P70S6K) is the first and perhaps the most well characterised mTOR substrate, in which its phosphorylation at Threonine 389 activates its kinase activity, allowing the phosphorylation of downstream ribosomal protein S6 (RPS6), a component of the 40S ribosomal unit, and thereby activating protein translation. P70S6K and RPS6 have been extensively studied in human skeletal muscle as they are highly sensitive to both mechanical loading and nutrient provision (EAA and dietary protein) ^{255–258}. Both the acute protein abundance of phosphorylated P70S6^{Thr389} and RPS6^{Ser240/244}, as well as its kinase activity via [γ -³²P] ATP kinase assay ²⁵⁹, are key readouts correlating with acute rates of MyoPS and long-term accrual of muscle mass ^{260,261}. The second major substrate of mTOR is eukaryotic translation initiation factor 4E-binding protein 1 (4-EBP1) ²⁶². mTOR dependant phosphorylation of 4-EBP1 occurs at four sites, which all result in the eventual inhibition of 4-EBP-1 towards eIF4E. First, 4-EBP1 is phosphorylated at Threonine 37 and Threonine 46, which prepares the protein for further phosphorylation at Serine 67 and Serine 70 ²⁶². The phosphorylation of 4-EBP1, and subsequent disassociation from eIF4E allows for the unblocking of eIF4G. The preinitiation complex of eIF4G and eIF4A can then be recruited to the mRNA stand before initiation (**Figure 8.**) ²⁶³. In human skeletal muscle 4-EBP1 phosphorylation is poorly understood with some authors displaying increases ^{249,257,258,264}, with others displaying a reduction following an anabolic stimulus ²⁶⁵. This may be due to difference in the chosen phosphorylation site and sufficient anabolic stimulus.

The role of mTORC1 protein signalling and phosphorylation undoubtedly warrant further investigation (via unbiased phosphoproteomics) to determine their exact role in muscle mass regulation and discover novel growth and/or exercise-related signalling proteins ²⁶⁶ (i.e., TRIM28 ²⁶⁷, Obscurin ²⁶⁸, Isthmin-1 ²⁶⁹, and C18ORF25 ²⁷⁰). This is particularly applicable as there is emerging evidence regarding the discord between traditional mTOR signaling and muscle protein synthesis, with authors showing blunted mTORC1 phosphorylation ²⁷¹ and inhibited mTOR activation signature following chronic RET ²⁷². Further review of mTORC1-associated and ribosomal proteins (i.e., ERK1/2, P90RSK, MAPK, UBF, TIF1a, c-Myc) are beyond the scope of this thesis, however, have been reviewed in detail elsewhere ^{273,274}.

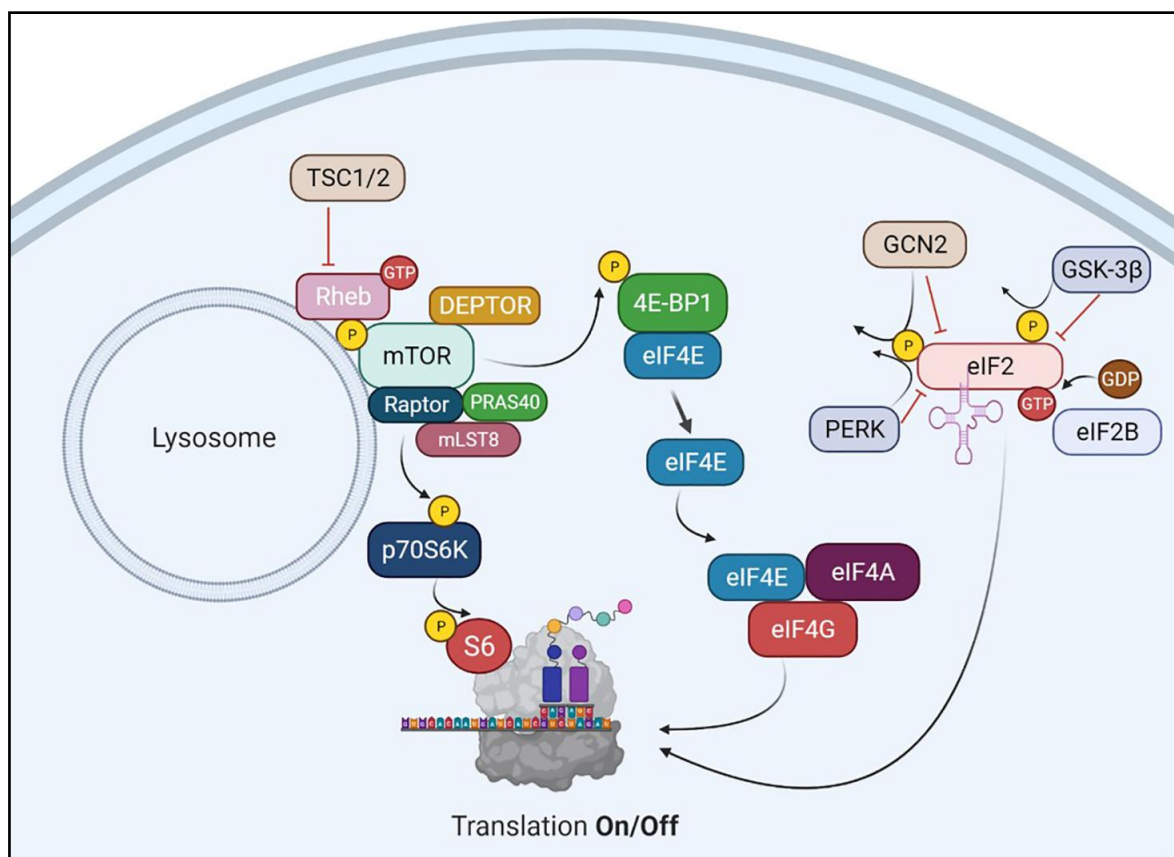


Figure 9. An overview of mTOR complex 1 signaling⁹. General mTORC1 overview of conical signaling proteins P70S6K, RPS6 and 4EBP1. mTOR activation is achieved through its phosphorylation at Serine 2448, the inactivation of TSC1/2, binding of RHEB, and translocation to the lysosome. Upon binding to the lysosome, mTOR directly phosphorylates P70S6K at Serine Threonine 389, and subsequently RPS6 at Serine 240/244, 4EBP1 at Threonine 37/46.

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1.2.1.2. Oral Deuterated Water (D₂O, ²H₂O, ‘Heavy Water’)

The use of deuterium oxide can be traced back as far as the 1930's when it was used in metabolic investigations focusing on bile acid and cholesterol metabolism in canines and rodents ^{275–277}. One of the first studies to measure protein turnover was Ussing et al. 1941, who observed to the incorporation of D₂O into liver, kidney, and muscle protein. He speculated that this process was due to the transfer of ²H with the H of amino acids via transamination. In 1961, this was followed up with the study of protein synthesis in 3C57BL/6J mice, which determined diet-induced obesity impairs nutrient-stimulated muscle protein synthesis ²⁷⁸. Since the 1960's, D₂O vanished from the scientific literature until its emergence in the late 2000's with several rodent models of resistance exercise and dietary manipulations to measure muscle, and in particular myofibrillar protein synthesis rates ^{279,280}. Notably, compared to intravenously administered amino acid stable isotope tracers that have typically been used to characterise the response of muscle protein turnover to various stimuli ²⁸¹, the use of orally administered D₂O in humans is minimally invasive and rapidly equilibrates in the body water pool within 2-3hrs. As alanine provides four potential hydrogens to be exchanged with deuterium, this allows for the α and β hydrogens of alanine to be labelled with 3.7 times more than the hydrogen molecules of D₂O ²⁸². The day-to-day validation of D₂O in humans by Wilkinson et al., was the first to compare non-exercised vs exercised over eight days with a unilateral study design ⁴. This study confirmed the use of D₂O to detect measurable changes in skeletal muscle myofibrillar, sarcoplasmic and collagen synthesis rates in younger adults ⁴. A follow-up investigation by the same group utilised D₂O to measure rates of myofibrillar protein synthesis over

6hrs (3hr postabsorptive and 3hr postprandial [20g EAA])²⁸³. When compared to a traditional primed continuous $^{13}\text{C}_6$ phenylalanine there was no significant differences between methodologies²⁸³. This highlighted the use of D_2O as a cost effective (1L 70% APE D_2O , £405 vs 1g $^{13}\text{C}_6$ Phenylalanine, £1,600), and minimally invasive methodology to study protein turnover *in vivo*.

Since the validation of D_2O to study rates of protein synthesis in humans, several research groups have published prolifically utilising this methodology to determine the effects of ageing, inactivity, exercise, nutrition, and pharmacological agents on protein turnover. These studies are typically limited to measuring rates of mixed muscle or subcellular protein fractions (myofibrillar, mitochondrial, sarcoplasmic and collagen). Recent advances in mass spectrometry instruments now allow for the quantification and measurement of individual protein synthesis rates within a whole muscle or subcellular proteome (**Figure 10**). In brief, this is the combination of D_2O and proteomic platforms. State-of-the-art, mass spectrometry instrumentation (i.e., nano-flow HPLC and Q Exactive MS) coupled to powerful bioinformatic platforms (i.e., maxquant, Perseus, and proteome discover) has the ability to identify thousands (>10,000) of proteins in skeletal muscle²⁸⁴. Although label-free proteomics is a powerful tool to determine proteome signatures, or identify novel proteins associated with exercise adaptation and/or disease progression, this unfortunately, merely provides 'static' measures of protein abundance at a given timepoint in which the sample was obtained. The addition of an isotopic label (i.e., D_2O) allows for the simultaneous measurement protein abundance, whilst also allowing for the dynamic measurement of protein synthesis of individual muscle proteins (i.e., myosin, actin, tropomyosin) (**Figure 10**). Dynamic proteomics has since been

implemented using human plasma ^{285,286}, with relatively few in human skeletal muscle ^{287–289}. Utilising the previously mentioned high sensitivity MS instrumentation, coupled with open access bioinformatic analytic software (i.e., D2Ome ²⁹⁰, DeuteRater ²⁹¹, ProTurn ²⁹², and TurnoveR ²⁹³) should allow future research to measure, calculate, and interpretate the dynamic flux of individual skeletal muscle proteins over a given time period (i.e., hours, days, weeks, or months). Overall, D₂O provides researchers with a cheap, minimally invasive, and incredibly powerful isotope tracing methodology to determine bulk and individual rates of protein turnover in skeletal muscle, and other obtainable tissues/biofluids (i.e., plasma, adipose, urine and faeces) in humans.

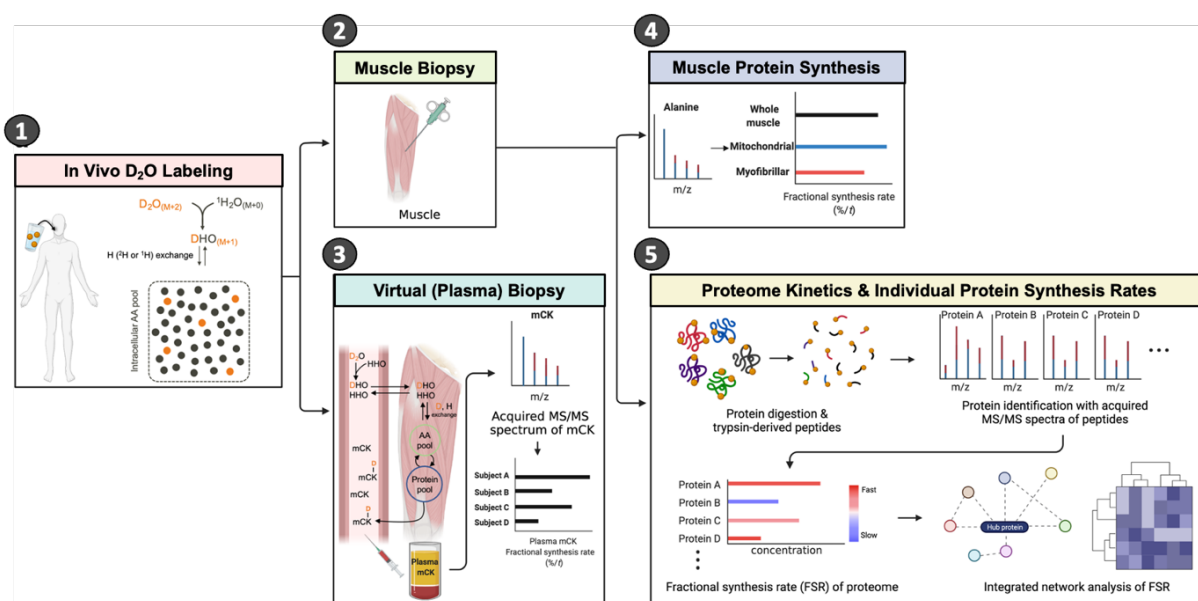


Figure 10. Overview of oral deuterium oxide methods to determine muscle protein synthesis & breakdown rates¹⁰. A single bolus oral ingestion of 70% D₂O (either 400ml or body mass specific [3ml/kg/BM] followed by daily top-ups [50ml/day⁻¹ or 0.3ml/kg/BM/day⁻¹] allows for enrichment of D₂O into body water (1). Following incorporation, muscle biopsies (2) and/or plasma (3) synthesis rates can be determined following the sampling over days/weeks/months to establish mixed muscle, or subcellular-specific synthesis rates (i.e., myofibrillar, mitochondrial, collagen) via incorporation of D₂O into ²H-Alanine. Furthermore, D₂O also allows for the analysis of individual protein synthesis, and proteome dynamics/networks for greater metabolic flux analysis.

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1.2.1.3. D₃-3Methylhistidine (D₃-3MH)

D₃-3Methylhistidine (D₃-3MH) approach has been gaining interest as a cheap and minimally invasive methodology to determine *in vivo* rates of protein breakdown.

3-MH was initially discovered in the 1950's in which it was found in human urine, but its origin was unknown ²⁹⁴. Later studies determined 3-MH was largely a by-product of contractile protein fragmentation (in particular actin), and is, therefore, released from the breakdown of contractile proteins ²⁹⁵. Hence, its use as a measurement marker of protein catabolism in human metabolic research ²⁹⁶. The ingestion of a stable isotopically labelled 3-MH with deuterium (D₃-3MH), therefore, allows for the quantification of a fractional breakdown rate (FBR%/hr⁻¹) due to the dilution of exogenously D₃-3MH with endogenous 3-MH (**Figure 11**). A recent validation of this approach utilising orally ingested D₃-3MH observed in younger and older adults, with and without a meat-free to calculate decay rates, and thereby FBR%/hr⁻¹ over several hours ²⁹⁷. The authors observed no differences in meat vs meat-free diet, albeit urinary FBR%/hr⁻¹ to be higher compared to plasma, potentially due to increased contribution of endogenous 3-MH in non-skeletal muscle tissues ²⁹⁷. The contribution of other organs to this method is well documented, with conflicting data. Since its validation, its use in human metabolic research has been slow, with relatively few studies. However, this method has been observed to measure in FBR%/hr⁻¹ following RET and testosterone therapy ⁵¹, biological sex ²⁹⁸, and limb-immobilisation ²⁹⁹. Future research with this methodology, across cohorts (i.e., young, old, male, female) exercise training (i.e., RE, aerobic, HIIT) and disease (i.e., obesity, T2D, cachexia) warrants further

investigation. The kinetic analysis of 3-MH across multiple compartments (i.e., muscle, plasma, urine) may allow us to determine muscle-specific 3-MH flux as previously reported in canines, cattle, and pigs ^{300–302}. Overall, this method allows for a cheap, minimally invasive technique to study rates of protein breakdown.

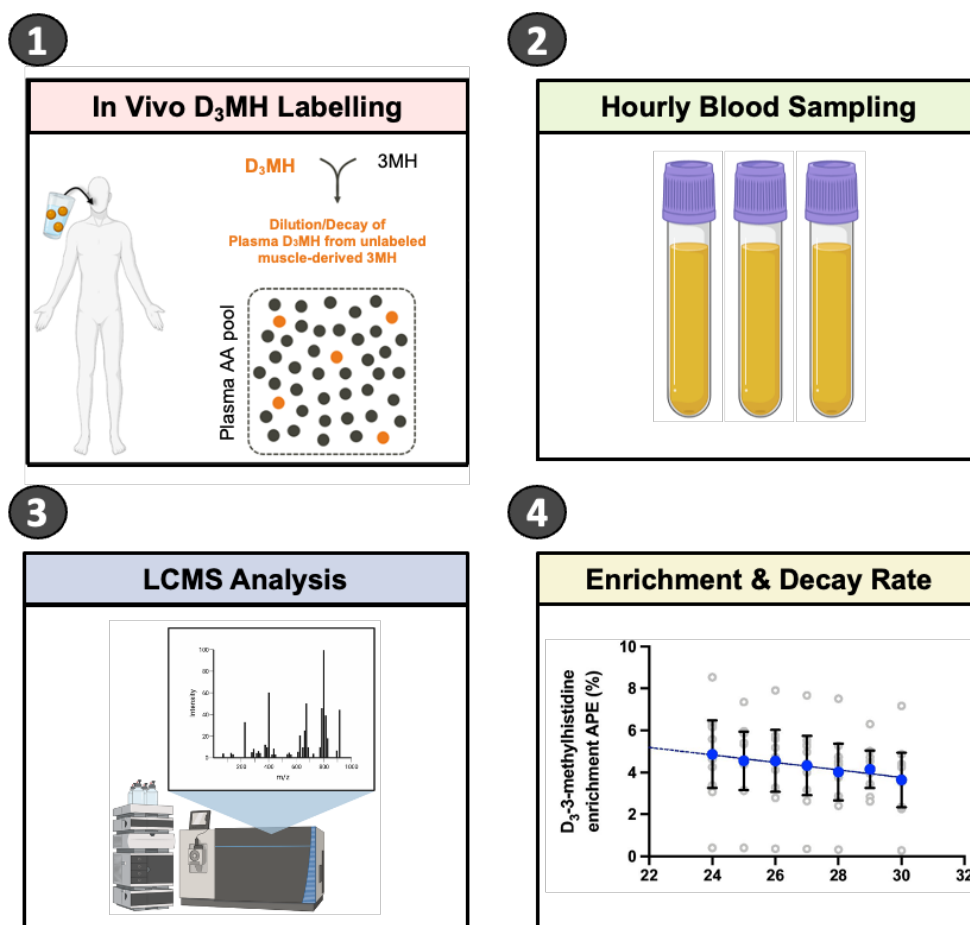


Figure 11. Oral D₃-Methylhistidine to study whole body protein breakdown¹¹. A single bolus oral ingestion of 10mg D₃MH is consumed 24hr prior to assessment of protein breakdown. Following incorporation into plasma, the tracee is diluted by endogenous unlabelled 3MH. Serial blood samples over several hours allows for the quantification of the isotopic decay constant rate per hour (k/hr^{-1}).

¹¹ Created using BioRender .

1.2.2. Protein and Amino Acid Nutrition-Induced Muscle Anabolism

It is well established RE is the single most potent stimulator of MPS in humans. However, in the absence of RE, consumption of dietary protein and amino acids facilitates a postprandial rise in rates of MPS. In a postabsorptive fasted state, rates of MPS have been reported between 0.03-0.07%/hr ⁵, with MPB at 0.08-0.11%/hr ⁶, representing a negative protein balance, and loss of muscle protein.

A potent regulator of MPS following food-intake, is the dietary protein content of a meal. Following the ingestion of a protein rich food component, MPS transiently rises over a 3-5hr period to a greater extent of MPB, and therefore, creates a positive protein balance to ensure muscle mass is maintained in healthy adults. The driver of postprandial increases in MPS are exclusively driven by the EAA content ³⁰³, with the BCAA leucine, playing a critical role in 'triggering' mTORC1 signalling and improving EAA transport/uptake into muscle ³⁰⁴. Notably. Seminal work from Atherton et al. observed a primary role of leucine at stimulating mTORC1 substrate phosphorylation compared to other EAA's ³⁰⁵.

The anabolic response to dietary protein is dose-dependent, with a maximal MPS response occurring upon the provision of ~20g of dietary protein presenting an EAA content of ~10g in healthy younger adults. In particular, seminal work by Cuthbertson et al., determined the muscle anabolic response to increasing dosages (2.5g, 5g, 10g, 20g) of orally ingested crystalline, free-form EAA's in younger and older adults displaying the dose-response in MyoPS, and was the first to display 'anabolic resistance' to EAA provision (**see section 1.2.4.1 for anabolic resistance**). Since crystalline EAA's do not reflect what is traditional consumed in an individual's diet, authors have since followed

up with dose-response investigations on 'real world' equivalents of protein-based drinks (whey, casein, egg, milk etc) ¹⁶⁰, and whole-food sources ^{306,307}. Instead of utilising arbitrary protein doses, a compilation of studies from six MPS feeding studies at McMaster University with ~65 younger adults sought to determine the 'optimal' protein dose based on an individual's body mass, lean body mass ¹⁵⁹. Following breakpoint analysis of linear and biphasic regression models, the authors determine a protein dose of $0.24 \pm 0.6\text{g/kg}$ ¹⁵⁹. The response of older adults will be discussed in **section 1.2.4** and the role of anabolic resistance to dietary protein and RE.

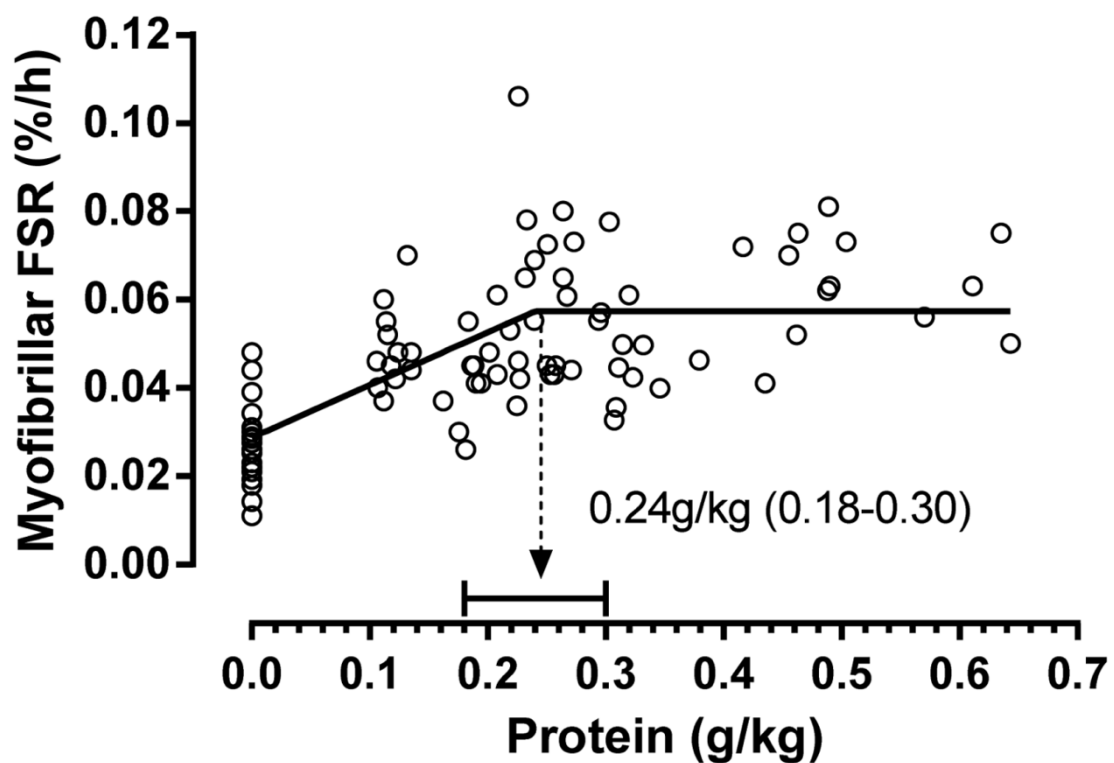


Figure 12. ‘Optimal’ feeding-induced protein dose in younger adults relative to whole body mass ¹². Biphasic linear regression of six studies on relative protein intake per kg body mass and the rested & postprandial rates of protein synthesis in response to 0-40g protein (whey, n=5., egg, n=1).

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1.2.3. Resistance Exercise-Induced Muscle Anabolism

RE is the most potent stimulator of MPS and has been shown to induce a 100-200% increase in MPS across mixed muscle ^{236,308}, myofibrillar ²⁴⁸, mitochondrial ³⁰⁹, and collagen ³¹⁰ protein pools. Following RE, MPS can be sustained for up to 24hr post-exercise. Several authors have sought to manipulate RE variables, (i.e., high-load ³¹¹, low-load ³¹², time-under-tension ³¹³, blood flow restriction ³¹⁴, eccentric-loading ³¹⁵) to maximise MPS.

A common misconception is the dose-response relationship with RE training load, and ensuing MPS. In the absence of protein and EAA's, RE-induced MPS peaks at 1-2hrs before returning to baseline at 4hrs. In younger adults, RE-induced MPS is maximised with loads between 60-90% 1RM ^{316,317}. However, a seminal investigation by Burd et al. observed no significant differences in RE-induced MPS between groups training with 90% 1RM or 30% 1RM, when trained to volitional failure ³¹¹. The notion of low-load training has also translated into similar muscle hypertrophy following a RET intervention, although, low-load does not improve muscle strength to the same extent as high-load training ³¹⁸. The large proportion of acute and chronic RET interventions have focussed on machine knee extension and/or leg press variations typically seen in gym facilities. The role of pragmatic bodyweight squat ³¹⁹, elastic band RE (EB-RE) (**see chapter 4 & 5**), or even walking ³²⁰, is not well characterised, as these modes of exercise can be competed without access to gym facilities, and instead, at home with zero to minimal equipment and improve rates of MPS.

The cellular anabolic signalling following RE is more complex than that of solely amino acid nutrition. However, similar to nutrient provision, exercise

induces a coordination of mTORC1 phosphorylation (i.e., P70S6K, RSP6) and protein translocation/co-localisation (i.e., mTOR:Lysosome ^{321,322} and TSC2:Rheb ^{323,324}). However, following RE, mTORC1 is further activated by mechanically-sensitive signalling proteins (i.e., phosphatidic acid ³²⁵, focal adhesion kinase ³²⁶). The combination of a mechanical stimuli and protein provision increases MPS greater than that of protein alone. In particular, the provision of 25g whey protein, MyoPS rates increase ~103% above postabsorptive, whereas exercise increases MyoPS ~204% over a 5hr period ³²⁷. The combined postprandial and RE-induced increases in MyoPS do not however, correlate with long-term changes in muscle mass and strength ³²⁸, with integrated daily rates of MyoPS providing a more robust indicator of long-term muscle mass accrual ³²⁹.

1.2.4. Age-Related Anabolic Resistance

The tightly regulated cellular signalling of mTORC1 and MPS in repose to nutrient provision (EAA's, dietary protein [drink, whole-food]) and RE observed in younger adults, is severely blunted in older adults, with evidence suggesting it may be apparent at middle-aged. This is concurrent with the age-related loss of muscle mass and strength, suggesting alteration in protein synthesis and breakdown kinetics are altered during ageing exacerbating muscle loss. This section will discuss the notion of 'anabolic resistance' and how older adults observe a blunted response to both the anabolic stimuli of amino acids and RE. Furthermore, I will discuss how older adults can overcome 'anabolic resistance'.

1.2.4.1. Anabolic Resistance to Protein Nutrition

In the mid-to-late 1990's several authors observed a reduction in basal, postabsorptive rates of MPS in older compared to younger adults ^{330,331}. However, those studies have since been disproven, and was likely due to methodological errors in the tracer methodology. It was between early 2000's when the seminal investigations of Volpi et al. (2000) ³³², Guillet et al. (2004) ^{333,334}, and Cuthbertson et al. (2005) ⁵, observed what we now term 'anabolic resistance' to EAA's. Both were conducted Volpi et al. (2000) ³³², Guillet et al. (2004) ^{333,334} were conducted under supraphysiological euglycemic hyperinsulinemic hyperaminoacidemic clamp methodologies via an intravenous infusion, which do not reflect normal *in vivo* conditions. Furthermore, Cuthbertson et al. (2005) provided participants with an oral drink of free-form crystalline EAA's, which do not reflect habitual intake, or consumption of dietary protein ⁵. Nevertheless, these studies observed a ~47% ³³², ~29% ³³⁴, and ~40% ⁵ lower maximal MPS response in older compared to younger adults. Therefore, it wasn't until 2015 when two retrospective cross-sectional studies from the Netherlands ³⁷ and North America ¹⁵⁹, displayed 'anabolic resistance' to orally consumed 20-40g of dietary protein (i.e., whey, egg, casein, and beef ^{10,335-338}). These data suggest a ~16% lower absolute rate of MPS in older adults compared to younger adults (**See Figure 12**) ³⁷, with older adults needing ~66% greater dose of protein (per kg/BM⁻¹) to maximise MyoPS ¹⁵⁹.

In an effort to maximise the MPS in older adults, several authors have sought to improve the MPS by feeding greater amounts of dietary

protein, which may not be a pragmatic approach due to large disease or volumes of whole-foods. Another perspective is to fortify or enrichment dietary protein sources with additional Leucine to 'trigger' a greater MPS response. Indeed, a seminal from Katsanos et al., (2006) observed older adults need a greater proportion of Leucine (41% vs 26% Leu) compared to maximise MPS to similar levels to that of younger adults ³³⁹. Notably, this led to the development of the leucine 'threshold' in older of >2.5g leucine per meal ²²³. Since then, several authors have observed significant effects of leucine-enrichment to bolster the MPS response in older adults to overcome the blunted anabolic response ^{190,340–344}. Notably, low-dose (i.e., 1.5g or 6g) of leucine-enriched EAA's appear to facilitate a robust MyoPS response similar to that of 20g of whey protein in older adults ³⁴¹. Leucine-enriched EAA's may, therefore, provide a potential nutritional strategy to maintain muscle mass during periods of disuse when provided in higher doses to maximise MyoPS.

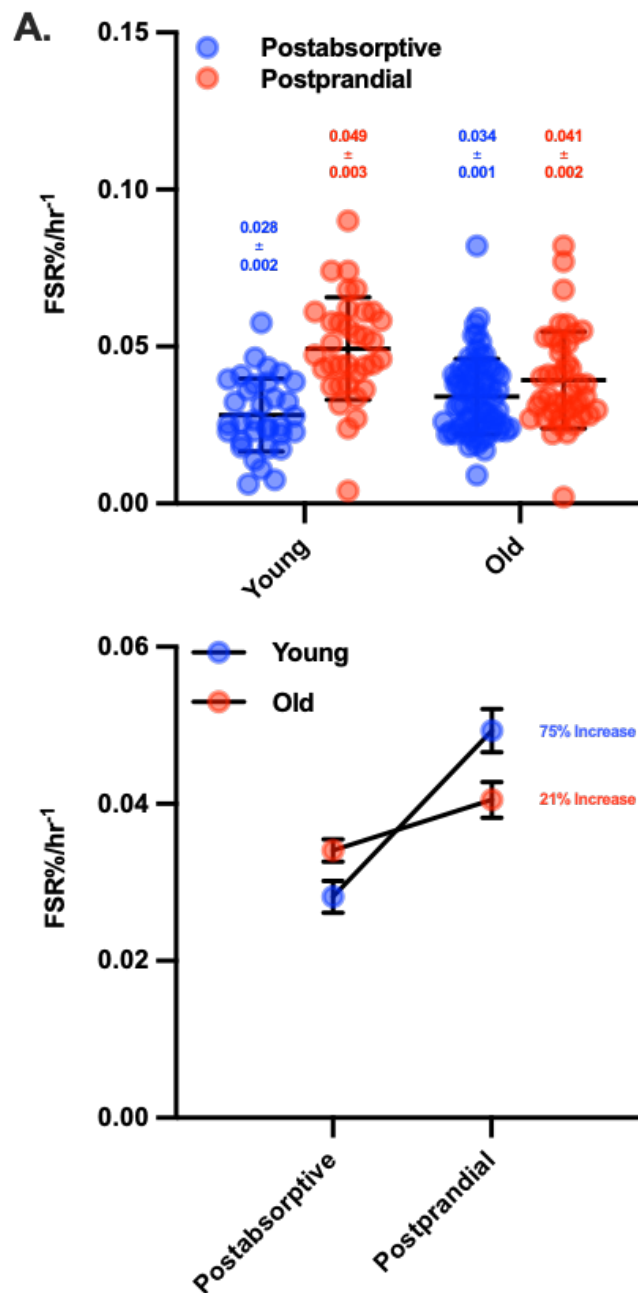


Figure 13. Postabsorptive and postprandial mixed muscle fractional synthesis rates in younger and older adults¹³. Cross sectional analysis of six studies in n=34 young, and n=72 older adults. (A) individual data points with mean $\pm$ SD of MyoPS. (B) Change in MyoPS from postabsorptive to postprandial, with % change in MyoPS from postabsorptive. Data reinterpreted and re-drawn on Prism GraphPad from raw data provided by Wall et al. (2015).

¹³ This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. Wall, B.T., Gorissen, S.H., Pennings, B., Koopman, R., Groen, B.B., Verdijk, L.B. and van Loon, L.J., 2015. Aging is accompanied by a blunted muscle protein synthetic response to protein ingestion. PloS one, 10(11), p.e0140903.

1.2.4.2. Anabolic Resistance to Resistance Exercise Training

Concurrent with 'anabolic resistance' to dietary protein provision, older adults also display a blunted response to RE. Several investigations have observed a blunted response to RE across various RE loads and intensities (30-90% 1RM) ^{316,317,345}. The first study to observe RE-induced anabolic resistance displayed a ~30% reduction in MyoPS across all ranges of RET intensities, which the authors speculated was due to impairments in phosphorylation of P70S6K and 4EBP-1 ³¹⁶. The authors provided a follow-up in which doubling the RE volume (i.e., from 3 to 6 sets of RE) improved MPS to similar rates observed in younger adults ³¹⁷. Prior to this, 'anabolic resistance' was observed in older adults following 6 sets of knee extension exercise, albeit during an A-V balance model ^{346,347}. The authors reported a ~41% increase in mixed muscle MPS in younger adults, whereas older adults only increased MPS by ~3.9% over a 3hr post-exercise period ³⁴⁷. Others have not observed anabolic resistance when directly comparing younger and older adults ^{249,348}, however, this is due to the concurrent consumption of crystalline EAA's (20g) or dietary protein ³⁴⁸ (~340g beef [~90g protein]) following RE, thereby maximising MPS and masking the ability to detect differences in MPS. Furthermore, following a RET intervention, the acute anabolic resistance in older adults blunts the longer-term hypertrophic adaptation in muscle mass accrual compared to younger adults ²²⁵. Interestingly, when utilising D₂O over the training period, no anabolic resistance was observed in MyoPS rates between younger and older adults in the untrained or trained-state following 6-weeks of RET ²²⁵.

1.2. Regulation of Skeletal Muscle Mitochondria

The maintenance and health of mitochondria are critical for skeletal muscle health and functional capacity. Under normal resting physiological conditions, mitochondria respire (i.e., consume oxygen) to support energy demand via oxidative phosphorylation and the conversion of metabolic fuel sources such as carbohydrates and lipids into cellular energy (i.e., ATP). Furthermore, mitochondria undergo a continuous quality control via the simultaneous synthesis and degradation of organelles to maintain cellular health and integrity. These quality control mechanisms include the synthesis of mitochondrial proteins ²⁴⁰, fission & fusion ¹⁰⁶, and the tagging of damaged organelles for subsequent mitochondrial-specific autophagy (termed, mitophagy) ¹⁰³. However, the most well characterised mechanism is the regulation on mitochondrial health and function is the biogenesis of new mitochondria and thereby improving mitochondrial content/volume/density within a cell or tissue ³⁴⁹. Here we provide a brief overview of these mechanisms, and how they are altered by ageing and the development of sarcopenia.

1.3.1. Respiratory Capacity

All substrates that enter oxidative metabolism undergo oxidation reactions within the TCA cycle in order to provide the reducing intermediates for successful energy production. In brief, following the combination of oxaloacetate and acetyl-CoA to form citrate (via citrate synthase), eight enzymatic reactions occur in the TCA cycle producing NADH, NAD⁺+H⁺ and FADH₂, leaving CO₂ and oxaloacetate as by products. As the primary driver of

the TCA cycle, acetyl-CoA are derived from pyruvate (via glycolysis) and fatty acids from beta-oxidation. The TCA cycle converges with the electron transport chain, where reducing substrates (NADH, NAD⁺H⁺ and FADH₂) donate electrons to complex I and II to create a proton gradient to facilitate energy production at the end of the complex at complex V (i.e., ATP synthase). Notably, only complex I, III, IV possess the ability to “pump” protons across the inner mitochondrial membrane. Overall, this creates a positively charged inner mitochondrial space, which drives an increase in proton motive force in the intermembrane space to diffuse H⁺ ions through ATP synthase, driving the conversion of ADP to ATP.

During ageing, the process of substrate conversion in the TCA cycle and subsequent mitochondrial respiratory function and capacity to produce ATP are impaired. Experiments involving the respiratory analysis of tissues (i.e., isolated liver mitochondria) goes back to 1955³⁵⁰. However, was not until 1987 in which a method for the analysis of permeabilised muscle fibres was established³⁵¹. Since then, authors have used sophisticated high-resolution respirometers (HRR) to determine the metabolic flux of muscle fibres^{352,353}. In the context of ageing, HRR have allowed researchers to establish age-related alterations in respiratory function⁸⁵, ADP sensitivity⁸⁷, ATP production, and H₂O₂ emissions. These experiments have consistently observed a lower rate of respiratory function compared to younger adults, with a reported ~8% decline per decade in mitochondrial complex I+II function³¹. Furthermore, the decline in mitochondrial function is associated with reduced exercise capacity, gait speed, muscle function and habitual step count/daily activity³⁵⁴. The maintenance of adequate mitochondrial respiratory function and/or capacity are, therefore,

essential for the maintenance of whole-body health, attenuation of disease, and risk of disability.

1.3.2. Mitochondrial Turnover and Quality Control

Here I briefly discuss the relevant mechanisms involved in the maintenance of a healthy mitochondrial pool, and how ageing alters these tightly orchestrated systems.

1.3.2.1. Mitochondrial Protein Synthesis

Similar to the measurement of contractile proteins mentioned previously to determine myofibrillar protein synthesis, mitochondria undergo similar synthesis and degradation to maintain a healthy protein pool. Early *in vitro* and pre-clinical rodent models observed a ~30% decline in mitochondrial protein synthesis (MitoPS). The first human investigation utilising stable isotope tracer methodologies reported a ~40% lower rate of MitoPS in older compared to younger adults ⁹⁶. However, others have reported no differences in MitoPS between younger and older adults ³⁵⁵, which may be due to differences in isotope tracer methodologies. Interestingly, no authors to date have directly compared the daily rates of MitoPS between younger and older adults in free living conditions, to our knowledge. Although, pre-clinical rodent data suggests MitoPS to be increased in older age compared you younger mice ³⁵⁶, therefore warrants further investigation in human skeletal muscle.

1.3.2.2. Fission and Fusion

The dynamic remodelling of mitochondrial morphology via fission and fusion is a tightly orchestrated signalling cascade involving inner and outer mitochondrial proteins ¹⁰⁶. Mitochondrial fission involves the fragmentation of damaged or dysfunctional sections of the organelle for subsequent degradation via mitophagy. The process of fission involves outer (i.e., DRP1 and MFF) and inner (i.e., FIS1) membrane proteins, which bind, and dock to the mitochondrial membrane to facilitate sequestration of the mitochondria ^{98,357,358}. On the contrary, mitochondrial fusion involves the binding of outer (i.e., MFN1 and MFN2) and inner mitochondrial proteins (i.e., OPA1) to coordinate the tethering, and merging of several smaller organelles, to form larger, more extended mitochondrial networks ^{359,360}. Pre-clinical rodent data utilising knockout or overexpression models have consistently observed the important role in DRP1 ^{97,98,100}, OPA1 ^{99,361}, and FIS1³⁵⁸ in mitochondrial morphology, and their role in muscle mass regulation. In humans, the dynamic measurement of mitochondrial fission and fusion are poorly characterised, with limitations to gene expression, protein content, and TEM images of mitochondrial networks. In particular, ageing induces a reduction in both gene and protein expression compared to younger adults ^{93,362}. However, physical activity and exercise plays a critical role in regulating mitochondrial quality control. Notably, Tezze et al. observed reductions in fission/fusion markers in sedentary older adults, with senior sportsmen (i.e., MA) displaying ~50% increased expression of OPA1, MFN2, and DRP1 ³⁶². Furthermore, following 12-weeks of endurance exercise training, older adults have reported increases of ~93%, ~36%, and ~201% in MFN1, MFN2, and FIS1, respectively ³⁶³. Therefore, maintaining sufficiently

high levels of habitual and/or structured exercise training may maintain or improve mitochondrial morphology during ageing.

1.3.2.3. Biogenesis

Since its discovery in 1998 by Professor Bruce Spiegelman ³⁶⁴, PGC1 α has become one of, if not the most widely studied signalling molecules in biological, and in particular exercise physiology research. PGC1 α has been publicised as the 'master regulator' of mitochondrial biogenesis (i.e., 'the synthesis of new mitochondria' or 'new components of the mitochondrial reticulum') ^{365,366}. The transgenic overexpression of this protein results in mouse models results in astonishing phenotypical changes to muscle fibre composition and exercise capacity ³⁶⁷. PGC1 α was then discovered to be highly exercise-sensitive, with a reported 40-fold and 10-fold increase in transcription and mRNA abundance, respectively, following 3-hrs of leg extension exercise ³⁶⁸. As a critical regulator of whole-body and skeletal muscle metabolic health, maintaining sufficient expression of PGC1 α , and inducing its transcription is essential for mitochondrial function. It's worth of note, mitochondrial biogenesis can be induced in the absence of PGC1 α , with other key molecular markers contributing to biogenesis (i.e., Nrf2, ERR γ , PPAR β , and LRP130 ³⁶⁹).

During ageing, skeletal muscle mitochondrial content declines ³⁷⁰, and authors have suggested this may be due to declines in molecular regulation of biogenesis. Interestingly, older adults report diminished mRNA expression, but elevated levels of PGC1 α ^{232,371}. The cross sectional investigation from Joannis et al. observed significantly greater expression of PGC1 α mRNA and protein content in a cohort of MA, compared to untrained younger and older

adults when sampled in a basal rested state ²³². These data suggest PGC1 α may not decline as a result of ageing, but as a consequence of reduced activity and contractile stimuli; however, further evidence is needed in humans to confirm the role of PGC1 α in age-related muscle loss.

1.3.2.4. Mitophagy

The process of mitophagy (i.e., mitochondrial-specific autophagy), play an important role in the regulation and health of skeletal muscle proteostasis ¹⁰³. The process involves several receptors and signalling molecules on the outer mitochondrial membrane for the targeted, and selective degradation via the autophagosome. The activation of the PINK1/Parkin and recruitment of autophagy-related receptors (i.e., optineurin and P62) is the most well characterised process of mitophagy, but recent evidence also suggests PINK1/Parkin independent mitophagy via specific signalling receptors FUNDC1, BNIP3 and cardiolipin. A comprehensive review of the molecular underpinnings of mitophagy in skeletal muscle are beyond the scope of this thesis and can be found elsewhere ¹⁰⁴.

The gold-standard measurement of mitophagy requires the use of novel florescent microscopy techniques to study the dynamics of mitochondrial proteins, in particular the tagging of mitochondrial proteins with mCherry-GFP ^{372,373}. However, this technique is limited to *in vitro* and pre-clinical rodent models due to the modification of genetic material; nevertheless, provide us with incredible detail in to the mitophagic response to physiological (i.e., exercise) ³⁷⁴ or pathophysiological states. Investigations in human skeletal muscle is, unfortunately, limited to the assessment of molecular markers

associated with mitophagy. During ageing, the process of mitophagy is thought to blunted, resulting in the accumulation of damaged and/or dysfunctional mitochondria. Indeed, older adults report a ~43% reduction in Parkin protein content compared to younger adults ³⁷⁵. This was supported with mRNA expression in frail older adults, presenting a 30-40% lower abundance compared to healthy older controls ³⁷⁶. Regular exercise, and maintenance of a habitually high-levels of physical activity during older age has been observed to maintain higher protein abundance of mitochondrial Parkin protein, to levels ~145% greater than sedentary older adults ⁹³. Collectively, improving mitophagy in older adults may present a therapeutic strategy which can be attained via exercise, or even supplementation of purported 'mitophagy activating' compounds ³⁷⁷.

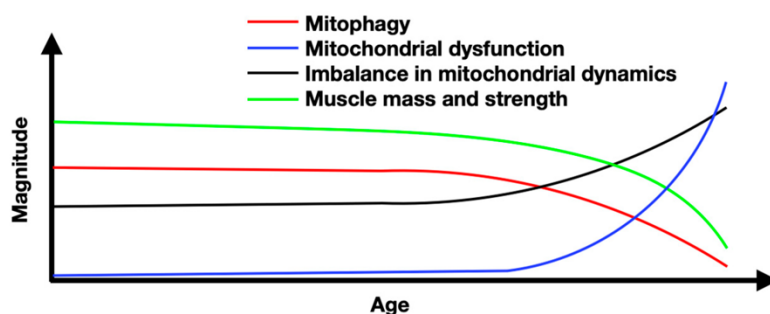
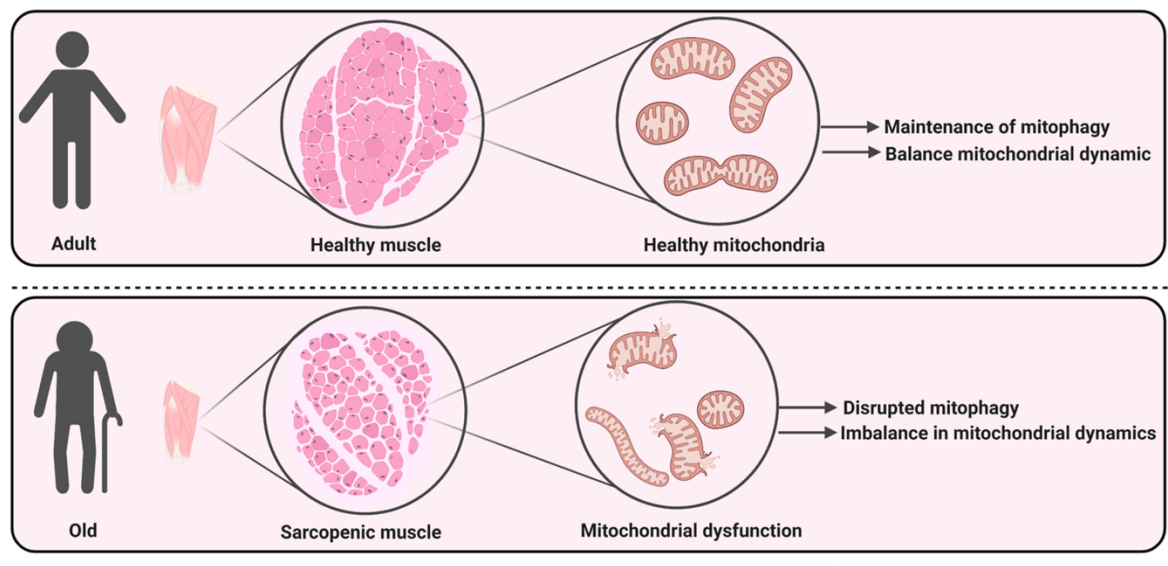


Figure 14. Conceptual model of impaired mitochondrial dynamics and mitophagy in ageing skeletal muscle¹⁴. In healthy younger muscle, mitochondrial quality control is well maintained via the biogenesis, and dynamic fission and fusion of damaged organelles for degradation. Ageing results in impairments in fusion and fusion, leading to the accumulation of damaged mitochondria due to blunted degradation signalling and mitophagy (Leduc-Gudet et al. 2021)

¹⁴ This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. **Leduc-Gaudet, J.P., Hussain, S.N., Barreiro, E. and Gouspillou, G., 2021. Mitochondrial dynamics and mitophagy in skeletal muscle health and aging. *International Journal of Molecular Sciences*, 22(15), p.8179.**

1.4. Experimental Models of Disuse in Human Clinical Research

In the scientific literature, musculoskeletal disuse in humans is generally studied utilising bed rest (BR) (i.e., severe whole-body unloading), unilateral limb-immobilisation (ULI) (i.e., limb-specific [leg or arm] unloading), or reduced physical activity (i.e., step count $<1,500/\text{day}^{-1}$). Although these models do not include the impact injury, disease, or illness, they provide a controlled setting in which to study 'uncomplicated/simple' disuse. It is worthy of note, there has been a recent advancement in which to determine 'complicated' disuse, by which muscle damage or inflammation is superimposed on the disuse model ³⁷⁸; however, is beyond the scope of the thesis. Here I provide a brief overview of all three experimental models, with a particular focus on their impact on older adults.

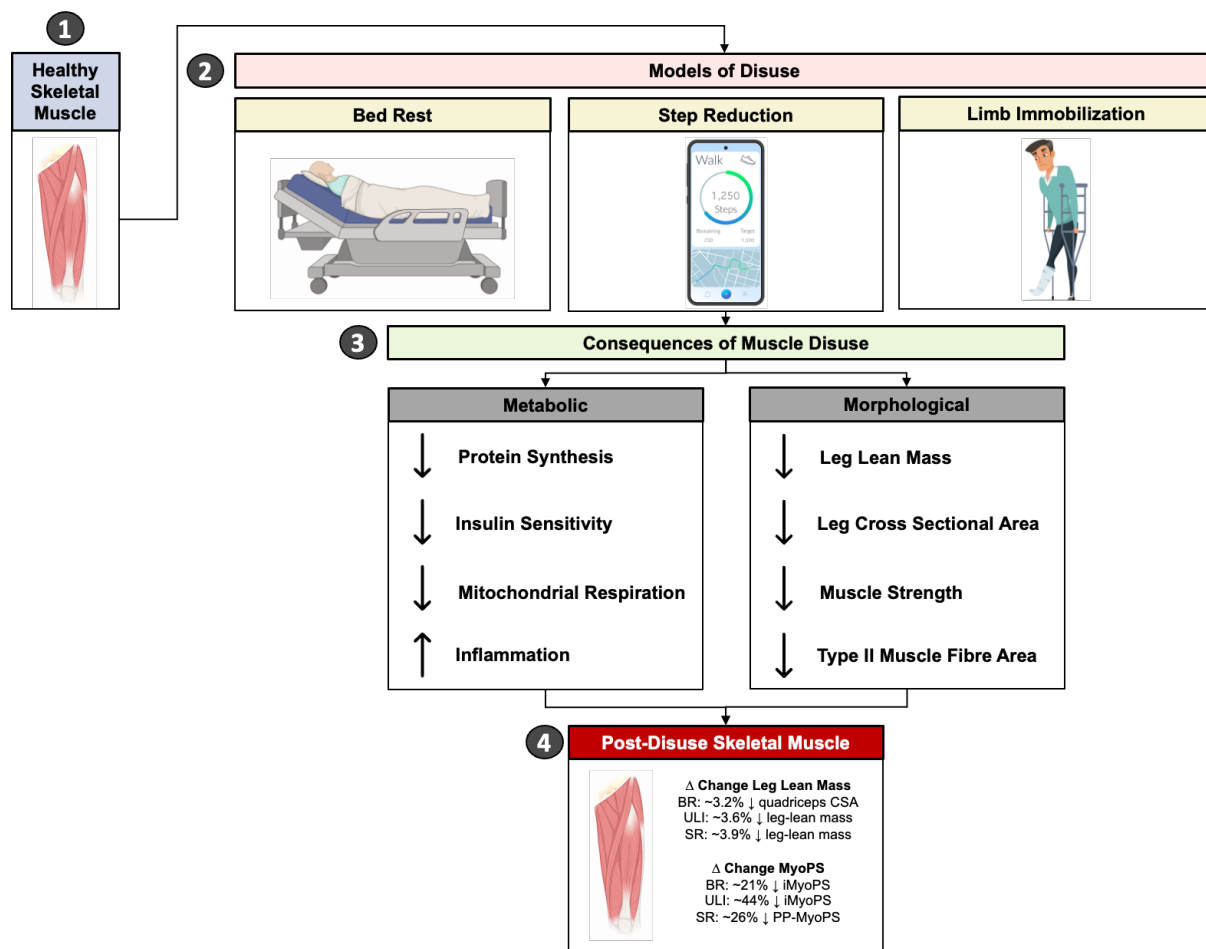


Figure 15. General overview of disuse models and their effects on morphology metabolic function¹⁵. In healthy young and old muscle (1), muscle is well regulated by the dynamic synthesis and breakdown of muscle proteins. During a period of disuse, ranging from 3-14 days of BR, ULI or SR (2), skeletal muscle undergoes drastic changes in its physiological profile. All models of disuse alter muscle mass, strength, and fibre type morphology (3). Furthermore, the morphological changes are concurrent with declines in protein synthesis, insulin sensitivity, and mitochondrial respiration (3). Following disuse, this results in catastrophic changes to the phenotype, which, in older adults, may not return to pre-disuse levels, thereby, exacerbating age-related trajectory to functional impairment and disability.

¹⁵ Created using BioRender . In sections (4) of Figure 12, the ∇ change in leg lean mass & MyoPS data relate to published observations from Dirks et al. (2020), Edwards et al. (2020), Breen et al. (2013), Smeuninx et al. (2021), and Kilroe et al. (2020).

1.4.1. Bed Rest

Experimental bed rest (BR) as a disuse model allows researchers to mimic the physical activity levels during hospitalisation, illness, or injury in the absence of catabolic disease pathologies (i.e., inflammation, infection [bacterial/viral]). Clinical investigations using BR in healthy younger and older adults have consistently observed substantial whole-body and leg-specific declines in muscle mass and strength across various BR durations (i.e., 5-28 days) ^{8,379–385}. Two recent systematic review and meta-analysis comprehensively evaluated the effects of BR in younger ³⁸⁶ (18-50 yrs) and older adults ³⁸⁷ (>65 yrs) across various durations. Across all studies, there was a significant effect on muscle atrophy in the early periods of disuse, which plateau into longer durations (>20 days). This is particularly relevant given the average duration of BR in the UK is as short as ~4.5 days ³⁸⁸, with older adults reporting a rapid loss of ~3% in leg muscle mass in 5-days ³⁸⁹.

BR-induced loss of skeletal muscle mass is also associated with impairments in metabolic health, i.e., glucose tolerance and insulin sensitivity ³⁸⁵, mitochondrial ROS ³⁹⁰, mitochondrial respiration ³⁸⁴, and protein turnover ³⁸⁹. As declines in muscle mass and metabolic health predispose older adults to greater risk of morbidity and mortality, understanding the molecular response to BR may allow us to determine therapeutic strategies to mitigate the decline. Several authors have attempted to maintain muscle mass and function with nutritional interventions (EAA ³⁹¹, Leucine ³⁹², HMB ³⁹³), albeit with limited success (**see table 2**). The use of nutritional strategies is framed in **section 1.6** and published elsewhere ³⁹⁴.

1.4.2. Unilateral Limb Immobilization

Unilateral limb immobilisation (ULI) is a disuse model to mimic real-world scenarios of sports injury, or local trauma associated with falls, fractures, and sprains. Although ULI and BR induce similar reductions in leg muscle mass, the ULI model does this in a limb-specific manner, with a particular focus on restricting the knee and ankle movement. ULI, therefore, results in localised atrophy of the quadriceps and gastrocnemius, with minimal atrophy in hamstring and tibialis anterior (recently termed ‘atrophy susceptible’ and ‘atrophy resistant’ muscles ³⁹⁵). The ULI model allows for the study of localised muscle atrophy without the systemic factors associated with whole-body disuse via BR (i.e., elevated systemic inflammation ³⁹⁶, microvascular dysfunction ³⁹⁷, and whole-body insulin resistance ³⁹⁰). Notably, compared to BR, the ULI model allows the participant are able to carry out their habitual activities of daily life and maintain their normal dietary intake. Typically, ULI models induce a disuse period of 7-14 days, which may not reflect the immobilisation period associated with a fall, fracture, or sprain to the ankle or knee (4-6 weeks), which may reduce its ecological validity as a disuse model. As severe local atrophy occurs in as little as 3-days ^{398,399}, several authors have sought to attenuate and prevent musculoskeletal decline with nutritional, (whey protein ⁴⁰⁰, leucine ⁴⁰¹, n-3 FA’s ⁴⁰²) and even pharmacological agents (i.e., nandrolone deaconate ⁴⁰³). Nutritional countermeasures across ULI will be discussed in **section 1.6** and are presented in **table 2**.

1.4.3. Reduced Physical Activity & Step Reduction

Compared to models of BR and ULI, the study of SR is relatively new, with very few invasive metabolic investigation ^{10,208–213,404–412}. Nonetheless, the model has observed significant alterations in muscle mass, strength, and metabolic function (i.e., insulin sensitivity and protein synthesis) in both younger and older cohorts, across varying ranges of duration and physical activity levels (**see table 1**). Interestingly, only two of the studies (to date) have explored interventions (exercise/nutritional) to mitigate SR-induced atrophy and metabolic decline (whey protein ²¹³ and citrulline malate ⁴¹³, **see table 2**). Compared to the whole-body and localised muscle atrophy from BR and ULI, SR results in a reduction in physical activity without the need for complete unloading ³⁹. During and SR investigation, volunteers are required to lower their daily step count to between 750-1,500. The lower range of ~750 steps/day⁻¹ mimics the average daily step count of a patient within an hospital facility ⁴¹⁴. As BR and ULI occur relatively infrequently throughout the lifespan, the SR model may better reflect the intermittent periods of reduced activity as a result of illness (i.e., influenza) or periods of poor weather which effect a greater proportion of the population ³⁹.

In 1953, a seminal study from Morris et al., observed the role of London bus drivers and conductors, in which conductors (high physical activity levels) reported a ~30% lower incidence of coronary heart disease, compared to highly sedentary and inactive bus drivers ^{415,416}. Since then, the laboratory of Frank Booth has developed rat models specifically bred for low and high voluntary wheel running ⁴¹⁷. These models allow the group to investigate chronic behavioural and physiological determinants of physical (in)activity. The human

equivalent would be the comparison of previously mentioned life-long exercisers/MA to non-trained sedentary older adults. The first comprehensive metabolic investigation of SR directly manipulated daily step count from ~10,500 to ~1,300 in a cohort of healthy younger adults ²⁰⁹. This brief period of physical inactivity displayed a ~5.4% decrease in VO_2^{peak} , ~2.6% decrease in leg-lean mass, and ~17% decrease in whole-body insulin sensitivity ²⁰⁹. A subsequent follow-up from Breen et al. (2013), this time in healthy older adults, reported similar declines in leg-lean mass, insulin sensitivity, and muscle protein synthesis ¹⁰. Since then, a number of authors have characterised the physiological and metabolic effect of short-term SR (**see table 1**). However, since the recent COVID-19 pandemic, and the chronic reduced ambulatory activity associated with self-isolation, future research should seek to clarify nutritional and/or exercise interventions to mitigate muscle mass and metabolic decline during a pandemic.

Table 1. Overview of reduced activity & step reduction models in younger, middle-aged, and older adults.

Reference	Mode of Disuse	Participant Population (Age)	Duration of Disuse	Changes in Body Composition	Changes in Strength & Function	Changes in Muscle Anabolism	Changes in Mitochondrial Function	Changes in Glycemic Control	Changes in Lipid-Adipose Metabolism
Olsen et al., (2008) ^(A)	Step Reduction ~1,394 steps per day	Healthy Young Adults (24 ± 2 yr)	21 days	-	-	-	-	↑ ~52% postprandial Insulin AUC OGTT (+180mins)	-
								↑ ~60% postprandial Insulin AUC OGTT (+180mins)	
								↑ ~78.5% postprandial Insulin AUC OGTT (+180mins)	
Olsen et al., (2008) ^(B)	Step Reduction ~1,157 steps per day	Healthy Young Adults (24 ± 2 yr)	14 days	↔ ~0.8% TFM	-	-	-	↑ 57.2% postprandial Insulin AUC OGTT (+180mins)	↑ 49.5% postprandial Insulin AUC OFTT (+480mins)
				↑ ~6.7% IAFM					↑ ~21% in postprandial triglycerides AUC OFTT (480mins)
				↓ ~2.1% TFFM					↑ ~27.7% postprandial C-Peptide AUC OFTT (480mins)

Krogh-Madsen et al., (2010)	Step Reduction ~1,334 steps per day	Healthy Young Adults (24 ± 2 yr)	14 days	↓ ~1.7% TBLM ↓ ~2.6% TLM ↓ ~2.7% LLM	↓ VO ₂ MAX, ~7%	-	-	↓ ~17% Peripheral Insulin Sensitivity	-
Stephens et al., (2011)	Step Reduction ~264 steps	Healthy Young Adults (26 ± 5 yr)	1 day (24 hrs)	-	-	-	-	↓ ~39% Whole body insulin action ↑ 19% fasting insulin	-
Knudsen et al., (2012)	Step Reduction ~1,521 steps per day + High fat overfeeding (+50% Kcal)	Healthy Young Adults (24 ± 3 yr)	14 days	↑ ~13.2% TBF ↑ ~14.2% TFM ↑ ~49.6% VF	↓ VO ₂ MAX, ~4%	-	-	↓ ~37% MI after 3 days ↓ ~43% MI after 7 days ↓ ~26% MI after 14 days	↑ ~22% in circulating triglycerides ↓ ~35% in circulating FFA
Mikus et al., (2012)	Step Reduction ~4,319 steps per day	Healthy Younger Adults (29 ± 1 yr)	3 days	-	-	-	-	↑ 46.7% fasting insulin ↑ 53% fasting C-Peptide ↔ fasting glucose ↓ ~30% MI ↑ ~49% HOMA-IR	-
Dixon et al., (2012)	Step Reduction <4,000 steps per day	Healthy and Overweight Middle-aged Adults (51 ± 1 yr) (49 ± 1 yr)	7 days	-	-	-	-	↑ Glucose and Insulin AUC OGTT	↑ Fasting Triglycerides ↔ LDL or HDL

Breen et al., (2013)	Step Reduction ~1,413 steps per day	Healthy Older Adults (72 ± 1 yr)	14 days	↑ TBF ~2.5% ↑ TFM ~1.9% ↓ LLM ~3.6% ↓ LSM ~3.6%	-	↓ ~26% in postprandial MyoPS ↔ in p70S6K phosphorylation ↓ Akt phosphorylation ↓ 4E-BP1 phosphorylation ↔ total mTOR, eEF2	-	↑ ~9.2% postprandial glucose AUC OGTT (+120min) ↑ ~11.5% postprandial insulin AUC OGTT (+120mins) ↑ 13.2% HOMA- IR ↓ ~22.5% MI	-
Walhin et al., (2013)	Step Reduction <4,000 steps per day + Caloric Surplus (+50% Kcal)	Healthy Young Adults (25 ± 7 yr)	7 days	↑ ~1.9 % FM ↑ ~4.4% LM ↑ ~3.5% AFM	-	-	-	↑ ~95% HOMA- IR ↑ ~88% HOMA- β	↔ GLUT4 ↓ AMPK, AKT1, AKT2
Krogh-Madsen et al., (2014)	Step Reduction ~1,796 steps per day + 2,00Kcal over feeding	Healthy Young Adults (22 ± 4 yr)	14 days	↑ ~12.2 % TFM ↑ ~8.7% ASF ↑ ~29.5% VFM	↓ ~6.3% VO ₂ MAX	-	-	↑ ~33.2% in fasting insulin ↑ ~10.2% in mean 24hr glucose	↓ ~30.8% in fasting FFA ↓ ~23.8% in FFA AUC OGTT (+180mins)
McGlory et al., (2018)	Step Reduction ~991 steps per day	Overweight, Prediabetic Older Adults (69 ± 4 yr)	14 days	↔ in BM, TBF, TLM, ↓ 0.6% LLM	↔ MVC-ISO	↓ ~12% MyoPS	↔ in mitochondrial protein complexes ↓ in mRNA abundance related to mitochondrial function & OXPHOS	↓ ~25.6% MI ↑ ~46.1% HOMA-IR	-
Bowden Davies et al., (2018)	Step Reduction ~2,000 step per day	Healthy Adults with and Without Family History of T2D	14 days	↓ ~0.3kg LBM ↓ ~0.2KG LLM	-	-	-	↑ ~85% fasting Insulin	↑ ~46% LDL Cholesterol

		(36 ± 14 yr)		↑ ~0.9% TBF				↑ ~9.3% HOMA-IR	↔ HDL Cholesterol
				↑ ~1.7% AF				↓ ~28% MI	
				↑ ~0.7% LF					
Moore et al., (2018)	Step Reduction ~1,500 steps per day	Healthy Older Adults (71 ± 5 yr)	14 days	↓ ~12% Type I Fibres ↓ ~29% Type II Fibres	-	↓ PAX7 Cells in Type I & II Fibres ↔ Capillary Density	-	-	-
Reidy et al., (2018)	Step Reduction ~2,994 steps per day	Healthy Older Adults (70 ± 2 yr)	14 days	↓ ~1.3% LLM ↓ ~2.4% Plantar extensor CSA ↔ Type I & II fibre CSA	↓ ~8% MVC-ISO	-	-	↓ ~15% Insulin sensitivity	↑ Circulating and intramuscular ceramides
Oikawa et al., (2019)	Step Reduction ~920 steps per day	Healthy Older Adults (69 ± 2 yr)	14 days	↓ ~1.4% LBM	↓ ~5.5% MVC-ISO	-	-	-	-
Saoi et al., (2019)	Step Reduction ~980 steps per day	Overweight, Prediabetic Older Adults (69 ± 24 yr)	14 days	-	-	↓ ~11.3% MyoPS ↑ 1.3-fold plasma glutamine (OMICS) ↑ 1.57-fold plasma creatine (OMICS) ↑ 1.2-fold plasma methionine (OMICS)	↑ 1.2-fold plasma carnitine (OMICS) ↑ 1.16-fold plasma deoxy carnitine (OMICS) ↓ 0.67-fold plasma oxoproline (OMICS)	↑ ~18.4% postprandial glucose OGTT (+120mins)	-

Shad et al., (2019)	Step Reduction ~1,192 steps per day	Healthy Young Adults (22 ± 2 yr)	7 days	-	-	↓ 26% MyoPS	-	↑ ~12% postprandial glucose AUC OGTT ↑ ~36.9% postprandial insulin AUC OGTT ↓ ~30.7% MI	-
Edwards et al., (2021)	Step Reduction ~1,192 steps per day	Healthy Young Adults (22 ± 2 yr)	7 days	-	-	-	↓ in Citrate Synthase Activity ↔ OXPHOS protein content ↔ Fission and Fusion Dynamics	-	-

1.5. Contractile Interventions in Inactivity & Disuse Atrophy

1.5.1. Resistance Exercise Training

As the most potent stimulator of muscle mass accrual, RE during disuse may be able to offset muscle atrophy, although not always possible due to specific contraindications. During clinical disuse events in which the severity is minor, the patient may be able to undertake RE. For example, during limb-immobilisation, if the ankle is injured, the individual may be able to undertake knee extension/flexion exercises to maintain upper thigh muscle mass. Similarly, bed-rest patients who have undergone elective surgery may be able to conduct RE within the inpatient facility. Furthermore, the notion of RET prior to elective surgery may be an alternative to 'get fit for surgery' in which individuals undertake a period of 7-30 days of training in an effort to improve physiological function and capacity to offset anticipated decline in function during hospitalisation.

Seminal RE interventions involving leg press exercise (3 sets of 10-12 repetitions) every other day during a 14-day of bed rest showed significant promise as a clinical intervention ⁴¹⁸. In the control (non-exercise group) rates of muscle protein synthesis decreased by ~46%, whereas the RE group increased by ~42% ⁴¹⁸. Although no measure of muscle mass was obtained, the authors observed a ~3% increase in muscle strength, compared to the ~9% decrease in the non-exercise group ⁴¹⁸. RE has also been utilised during models of limb-immobilisation ⁴¹⁹ and step reduction ⁴¹³, with similar positive effects. Although the least ecologically valid, Oates et al., 2010 observed a significant protective effect of one singular set of knee extension exercise at 80% 1-RM every other day during a 14-day leg-brace immobilisation ⁴¹⁹. The

MRI-assessed quadriceps CSA in the non-exercise control group reduced by ~7.6%, compared to no changes in the RE group ⁴¹⁹. Similarly, Devries et al., 2013 showed a mitigation of muscle atrophy, and development of 'anabolic resistance' following 14-days of step reduction with low-load RE (thrice weekly) in older males ⁴¹³. In brief, non-exercise control group observed a ~124g loss in leg-lean mass, compared to ~126g increase in leg-lean mass in the RE group ⁴¹³. This was concurrent with a ~73% (+111% within group) and ~109% (~155% within group) difference between groups in postabsorptive and postprandial MyoPS ⁴¹³. This selection of studies clearly show a protective effect of maintaining a contractile stimulus during disuse; however, this may not always be possible. Other contractile or nutritional interventions are also needed. Instead of undertaking peri or post-operative RET, the notion of prehabilitation has been proposed. Notably, our laboratory recently examined the *potential* protective effects of short-term (i.e., 7-days, 4 sessions) RE prehabilitation at mitigating muscle atrophy and anabolic resistance from 5-days of bed rest ³⁸⁹. Interestingly, I observed no protective effect of RE-prehabilitation compared to non-exercise controls ³⁸⁹. In particular, both groups observed large declines in quadriceps CSA (-2.3% vs -2.4%), integrated MyoPS (-26% vs -21%) and acute postprandial MyoPS ³⁸⁹. Future research on pragmatic and cost-effective RE warrant further investigation to mitigate disuse-related muscle atrophy in clinical settings.

1.5.2. Neuromuscular Electrical Stimulation (NMES)

In the absence, or inability to undertake RE, the role of NMES has been observed to induce significant increases in muscle anabolism across a range

of cohorts and clinical settings^{420–429}. NMES involves the use of skin electrodes to evoke strong, but submaximal muscle contractions when compared to voluntary contractions. The implementation of NMES for between 40-80 mins per day, on a specific muscle and/or limb, has been observed to increase rates of muscle protein synthesis and mTORC1 signal phosphorylation in healthy young, old, diseased, and even critically ill individuals^{427–431}. Therefore, applied during a period of disuse (i.e., bed rest or limb immobilisation [quadriceps/calves]), several authors observing a protection of muscle mass, with no protective effect on muscle strength^{422–426}. Seminal observations from Gibson et al., displayed 6-weeks of limb immobilisation following tibial fracture resulted in ~17% and 23% reduction in quadriceps CSA and protein synthesis respectively⁴²⁵. However, 60 minutes/day of low voltage NMES of the quadriceps muscles preserved muscle mass and rates of protein synthesis to that of the non-immobilised control leg⁴²⁵. Interestingly, to date, there has been no observations utilising D₂O as the stable isotope tracer to measured rates of protein synthesis over days, instead of hours^{420,421}. Nevertheless, NMES may provide clinicians, physiotherapists, and pro-active individuals a novel and potent anabolic stimulus to maintain muscle mass during a period of disuse⁴³².

1.6. Nutritional Interventions in Inactivity and Disuse Atrophy

1.6.1. Isolated Protein

Whey protein (WP) and casein protein (CP) nutritional supplements are produced by separating proteins from their naturally occurring whole-food derivative (i.e., milk). Studies investigating the metabolic profiles of dairy-derived proteins demonstrate the rapid digestion and absorption kinetics of

isolated WP compared with the more slowly digested casein ^{433–435} and complete whole-milk proteins ^{436,437}. Indeed, evidence exists to suggest WP possesses superior muscle anabolic properties compared with casein. Specifically, following the consumption of WP, the increase in MPS is greater than casein, potentially due to the superior AA profile and postprandial leucinemia of WP ⁴³⁴. However, not all authors have observed a benefit of WP over its whole-milk component for muscle anabolism, despite temporal differences in aminoacidemia ^{438,439}. This may be partly due to the whole-food matrix and added macro-micronutrient value of whole milk, which may facilitate the potent and rapid increase in MPS. Moreover, the measurement duration, protein dosage and synergistic effect of RE likely influences the subtle differences or similarities observed between whole milk protein and isolated WP and CP derivatives on acute measures of postprandial MPS. Nevertheless, high-quality leucine-rich WP supplementation has been shown to induce a greater postprandial MPS response in both younger and older adults compared with other dose-matched isolated protein sources (e.g., soy, casein, and wheat) ^{435,440} under conditions of rest and exercise ⁴³⁵. Evidence also exists to support the use of WP during inactivity and disuse. Indeed, recent investigations have found WP to be effective in offsetting BR-induced muscle deterioration in older adults ⁴⁴¹. Furthermore, Arentson-Lantz et al. (2019) demonstrated that replacing whole-food mixed meal proteins with WP (i.e., improving protein *quality*), attenuated the decline in whole-body lean tissue during seven days of BR. Importantly, dietary protein intake was relatively low in this study ($\sim 0.9 \text{ g}\cdot\text{kg}^{-1}\cdot\text{BW}\cdot\text{day}^{-1}$) and, therefore, more representative of the eating habits of older adults within inpatient facilities ⁴⁴². Others have attempted to navigate the

issue of dietary protein malnutrition during BR with the implementation of blended mixed protein complete meal replacements [WP, CP, pea protein and soy protein] ⁴⁴³. Specifically, Dirks et al. (2019) provided volunteers with a standardised diet of $\sim 1.2 \text{ g} \cdot \text{kg}^{-1} \cdot \text{BW} \cdot \text{day}^{-1}$ of dietary protein, introduced via nasogastric tube by four intermittent protein boluses or via a 24-h continuous feeding approach ⁴⁴³. Interestingly, the findings suggested that the delivery method of dietary protein did not influence the negative consequences of BR on skeletal muscle mass, strength, and metabolic function. In addition, it is of interest to note that the total amount of dietary protein ($\sim 1.2 \text{ g} \cdot \text{kg}^{-1} \cdot \text{BW} \cdot \text{day}^{-1}$) was insufficient at protecting losses in muscle mass ⁴⁴³.

During ULI, the use of isolated dietary proteins has been reported to have no effect on skeletal muscle mass and strength loss in healthy older males when compared with a placebo ⁴⁴⁴. Specifically, Dirks et al. (2014) used a five-day whole-leg cast immobilisation approach with and without WP supplementation ($2 \times \sim 21 \text{ g}$ daily [10.6 g EAAs, 2.8 g leucine per drink]), which increased daily protein intake from ~ 1.1 to $\sim 1.6 \text{ g} \cdot \text{kg}^{-1} \cdot \text{BW} \cdot \text{day}^{-1}$. Others have also shown no beneficial effect of protein supplementation during more extended periods of ULI (~ 14 days), in healthy middle-aged males ⁴⁴⁵, albeit with lower daily supplemental strategies ($1 \times 20 \text{ g}$). Mitchell et al. (2018) observed no benefit of a single daily bolus of $\sim 20 \text{ g}$ milk protein with breakfast on preventing loss of muscle mass and strength during a habitually skewed dietary protein intake of $1.0 \text{ g} \cdot \text{kg}^{-1} \cdot \text{BW} \cdot \text{day}^{-1}$ (breakfast 25%, lunch, 25% and dinner 50% of energy and protein requirements) to represent typical eating habits of older individuals ⁴⁴⁵. Although current data are inconclusive on the effects of WP during ULI, further investigation into other WP strategies (e.g.,

dosage (~40 g)¹⁶⁰, timing⁴⁴⁶, pre-sleep consumption⁴⁴⁷, pattern distribution¹⁷² and additional free-form leucine⁴⁴⁸) could offer protection against disuse atrophy.

To date, there has only currently been one randomised controlled trial (RCT) of the influence of dietary protein during an SR model⁴⁴⁹. Oikawa et al. (2018) found that a high quality, high-protein diet (~1.6 g·kg⁻¹·BW·day⁻¹) during SR (~750 steps day⁻¹) and a ~500 Kcal energy deficit for two weeks, failed to attenuate muscle atrophy in older adults. Specifically, the provision of two daily WP drinks (providing ~30 g protein each (15.4 g EAA, 4.3 g leucine) or a protein-matched hydrolysed collagen peptide drink (4.5 g EAA, 0.8 g leucine) during SR failed to attenuate the reduction (~14%) in free-living MPS⁴⁴⁹. An important observation from this study is that a seven-day transition from energy balance to energy restriction evoked a decline in MPS (~12%), even in the presence of double the current protein RDA (~1.6 g·kg⁻¹·BW·day⁻¹). Alarming, Oikawa et al. (2018) also found that, following a seven-day period of re-ambulation, MPS was not fully restored to levels pre-intervention levels, highlighting the importance for an appropriately prescribed rehabilitation and nutrition program following a period of disuse in older adults⁴⁴⁹. Nonetheless, whilst WP supplementation failed to prevent the loss of lean tissue during SR, WP did promote a greater recovery of lean tissue upon re-ambulation⁴⁴⁹.

Although isolated proteins have been shown acutely to stimulate postprandial MPS and augment the muscle anabolic response to RE, these isolated ingredients, in particular high-quality WP, may be relatively ineffective at attenuating muscle atrophy during disuse in older adults. This may be due to the relatively good health of older volunteers in these studies, or possibly the

relatively high level of total combined daily dietary protein intake (meals, plus supplementation) provided in these studies ($\sim 1.6 \text{ g} \cdot \text{kg}^{-1} \cdot \text{BW} \cdot \text{day}^{-1}$ ^{444,449}). Therefore, greater relative daily amounts ($> 2 \text{ g} \cdot \text{kg}^{-1} \cdot \text{BW} \cdot \text{day}^{-1}$), combined with novel isolated intact protein blends as well as protein sources containing high relative proportions of di- and tripeptides (i.e., protein hydrolysates), may be needed to facilitate greater protective effects on muscle during disuse. Indeed, whilst evidence is limited, protein hydrolysates have been observed to be superior to intact proteins, largely due to decreased splanchnic extraction, increased postprandial AA bioavailability and rapid appearance of EAAs, in particular leucine, into circulation ^{435,450–452}. Moreover, WP hydrolysates have also been observed to augment a greater acute postprandial MPS response compared to intact protein sources in younger and older adults^{435,450,452}. However, ultimately, more chronic investigations are required to assess the longer-term anabolic potential of protein hydrolysates compared with intact (whole) proteins, particularly during periods of disuse and inactivity. Nevertheless, protein supplementation may, however, be of use to older adults with compromised health status (i.e., pre-existing anabolic resistance) or those who consume low levels of dietary protein (i.e., $< 0.8 \text{ g} \cdot \text{kg}^{-1} \cdot \text{BW} \cdot \text{day}^{-1}$)⁴⁵³, which is particularly prevalent during illness/hospitalisation.

1.6.2. Crystalline Free Form Amino Acids

During periods of inactivity and disuse, the consumption of whole-food mixed meals or even isolated protein sources may not be feasible, primarily due to the aforementioned issues of appetite and satiety regulation that are common in older adults. Therefore, constituent free-form AA's as either a complete EAA

profile (i.e., all nine EAAs) or individual AA's (i.e., leucine) that possess anabolic and anti-catabolic properties may be an effective and energetically efficient in offsetting muscle atrophy. The following section discusses the efficacy of AA supplementation in a range of modes of disuse and inactivity.

1.6.2.1. Essential Amino Acids (EAA's)

Iso-caloric consumption (15 g) of EAAs (2.79 g leucine) in older individuals has been shown to result in a >30% increase in MPS compared to a protein-matched ~15 g WP (1.75 g leucine) ⁴⁵⁴, likely due to the higher proportion of EAAs and more rapid digestion and absorption of crystalline EAAs. A follow-up investigation found that, when matched for EAA content, ~15 g of WP resulted in a greater accrual of muscle proteins compared to 6.7 g of EAA, overall indicating that the content of EAA within WP is likely not the sole explanation for its anabolic superiority to pure EAAs ⁴⁵⁵. As discussed above, an important consideration regarding protein nutritional strategies in older adults is the dosage required for maximal MPS stimulation (EAA, ~15 g, WP, >30 g), and their respective effects on appetite and satiety ⁴⁵⁶. Recent evidence comparing an EAA-matched dose of WP (~15 g (7.5 g EAA, 1.9 g leucine) to crystalline EAA (7.5 g, 3.0 g leucine) prior to breakfast in older adults, found that EAA supplementation resulted in greater protein and energy intake compared to WP. In addition, consumption of EAAs did not affect appetite ratings or satiety hormones (i.e., PYY), compared with WP ⁴⁵⁷. Furthermore, it is pertinent to note that EAA, when ingested with meals, is known to significantly increase

the postprandial MPS response by ~30%, but without affecting circulating glucose or insulinemia ⁴⁵⁸.

In human metabolic research, much like isolated proteins, dosing strategies for EAAs range considerably: from ~1.5 g ³⁴¹, ~3 g ^{344,459}, ~10 g ³⁴³, ~15 g ⁴⁶⁰ and ~18 g ³⁰³ to >20 g ^{257,264}. Early studies showed that consumption of ~15 g EAAs thrice daily for ~28 days of BR in young males resulted in maintenance of whole-body and leg lean mass compared with the placebo control ⁴⁶¹, which was associated with a maintenance of MPS. Importantly, similar observations have also been found following ~10 days of BR in older adults ³⁹¹. Thus, supplementing with EAAs in-between meals during periods of BR, as previously established in both younger ^{461,462} and older adults ³⁹¹, may offer a viable option for repeated and frequent MPS stimulation, without impacting satiety (Figure 2) ⁴⁵⁸.

In models of ULI, recent evidence regarding AA's, particularly a novel AA blend with additional non-essential AA's (NEAA)—arginine, glutamine, and n-acetylcysteine—has been shown to mitigate muscle atrophy associated with seven days of ULI in healthy young men ⁴⁶³. Indeed, as the authors stated, it is possible that certain NEAAs may function by attenuating catabolism (i.e., glutamine), maintaining capillary perfusion (i.e., arginine) and reducing oxidative stress (i.e., n-acetylcysteine), which have all been implicated in disuse atrophy ^{464–468}. Specifically, the consumption of an EAA:NEAA supplement in between meals ($3 \times 23.7 \text{ g} = \sim 71.1 \text{ g}$ EAA:NEAA, totalling ~12 g leucine per day) attenuated muscle atrophy and increased intramuscular lipid

accumulation compared with a placebo ⁴⁶³. It is, however, important to note that this study implemented a seven-day loading period of the high-dose EAA:NEAA blend, prior to and during the seven-day period of ULI, which, whilst possible for older adults prior to anticipated disuse events (i.e., elective surgery), would not be possible in acutely hospitalised patients. Furthermore, participants were provided with >70 g AA's on top of a standardised $1.0 \text{ g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ of dietary protein. The large amounts of AA's provided by Holloway and colleagues, whilst providing proof of concept, lack real-world practicality ⁴⁶³. Interestingly, however, leucine-enriched (40%) low EAA dosages of ~3 g (1.2 g leucine, 1.8 g EAA) has been shown to augment similar increases in MPS to that of ~20 g WP (2 g leucine, 7.6 g EAA) in older women ³⁴⁴. Further, dosages as low as ~1.5 g EAA (0.6 g leucine, 0.9 g EAA) have been reported to stimulate an equivalent MPS stimulation to a large (~40 g) bolus of WP (4 g leucine, 15.2 g EAA) in healthy older women ³⁴¹. The addition of ~15 g of EAAs in between, and more importantly, to sub-optimal protein containing meals may therefore be a strategy to mitigate negative effects of disuse and inactivity (Figure 2). This is particularly applicable within in-patient facilities where low-protein consumption and satiety may be of particular concern for older adults.

1.6.2.2. Branch Chain Amino Acids (BCAA's)

Of the EAAs, the branched-chain AA leucine, has been shown in multiple human models to be a key mediator of postprandial MPS in healthy younger and older adults ^{341,448,469–471}, and also in response to acute

bouts of RE in the presence of a complete protein source ^{337,340,341,344}. Leucine has previously been found to have a protective effect on skeletal muscle mass and function during 14 and 7 days of BR in healthy middle-aged ³⁹² and older adults ⁴⁷², respectively, in the presence of a complete protein source. English et al. (2016) reported that supplementation of a high dose of leucine ($0.06 \text{ g} \cdot \text{kg}^{-1} \cdot \text{BW} \cdot \text{meal}^{-1}$ or $\sim 5 \text{ g}$) per meal, attenuated the decline in postabsorptive MPS, muscle mass and muscle strength following 14 days of BR compared with a placebo ³⁹². Similarly, following seven days of BR in older adults, the same previously mentioned leucine strategy attenuated the loss of leg lean mass; however, no effect was observed for muscle strength or function. Moreover, leucine did not mitigate the effects of BR on muscle fibre CSA or intracellular catabolic protein expression ⁴⁷². However, these findings are in agreement with other seven-day ULI protocols which also found no protective effect of leucine supplementation on quadriceps CSA or leg strength ⁴⁷³. It remains unclear whether the disuse model (BR, ULI or SR), dosage (13.2 g vs. 7.5 g per day) and/or timing of leucine intake (i.e., with or without meals) is attributable to these observed differences and warrants further research.

The role of leucine as a stand-alone nutritional strategy therefore displays equivocal effects at maintaining muscle mass and anabolism. This is particularly important as it is impossible for MPS to exceed MPB when the AA substrates are derived entirely from MPB, and therefore a net positive protein balance cannot occur in the absence of an exogenous supply of a complete EAA profile ^{474,475}. Nevertheless, $\sim 6 \text{ g}$

of leucine containing BCAAs have been previously shown to trigger intracellular anabolic signalling, and augment a small, but significant increase in short-term MPS in younger ⁴⁷⁶ and older adults ⁴⁷⁷. However, in the absence of a full EAA profile, leucine fails to provoke a robust and prolonged state of postprandial MPS as displayed by a traditional ~20 g dose of WP ⁴⁷⁵. Therefore, the role of leucine may not be as a stand-alone supplement, but in fortifying complete proteins with additional free-form leucine to augment a greater anabolic effect. Indeed, this has been observed in fortifying low, suboptimal dosages of protein that have been observed to elicit similar acute increases in MPS to that of >25 g of protein ³⁴¹. Therefore, it is plausible to suggest that a lower quantity, leucine-enriched protein dose may alleviate some of the satiating effects of a larger protein bolus, as discussed above, whilst still facilitating a net positive protein balance ⁴⁷⁸, thus supporting the maintenance and/or development of muscle mass in older adults ^{470,471}.

1.6.2.3. Non-Essential Amino Acids (Non-EAA's)

With a lack of evidence to support the use of non-essential AA's (NEAA) in supporting protein accretion, particularly models of disuse ^{10,208,209,212,449,479–481}, there is currently only one study to have utilised NEAA, albeit in the form of the single non-essential AA, citrulline ⁴⁷⁹. Devries and colleagues postulated that, as a precursor of nitric-oxide, the consumption of citrulline would enhance vasodilatory nutrient delivery to skeletal muscle and attenuate inactivity-induced impairments in MPS in older men ⁴⁷⁹. However, purported vasodilatory nutrients have

shown little success in augmenting vasodilation, and evidence suggests that even potent pharmacological manipulations of blood flow and tissue-specific microvascular circulation do not enhance postprandial MPS^{482–484}. Therefore, it is unsurprising that nutrient derived vasodilatory compounds; AA's (i.e., citrulline and arginine)^{465,479,485–487} and other nutraceuticals with purported vasodilatory properties (i.e., cacao flavanols and Montmorency tart cherry)^{488,489} have failed to elicit any notable muscle anabolic effects. Indeed, Devries et al. were unable to demonstrate any effect of daily supplementation of citrulline on femoral blood flow or MPS⁴⁷⁹. Nevertheless, one study has found a beneficial effect of pharmacologically manipulating vasodilation on MPS under conditions of a hyperinsulinemic clamp in older adults⁴⁹⁰. Notably, under normo-insulinemic conditions, such beneficial effects on MPS have not been replicated⁴⁹¹. The implementation of purported vasodilatory nutrient compounds, therefore, may not be beneficial for enhancement or maintenance of MPS and muscle mass during inactivity and/or disuse in older adults. By direct contrast, vasoconstriction (induced via cold water immersion) has been shown to attenuate the post-exercise increase in MPS by ~20%⁴⁹², which may have important implications during disuse and/or periods of hospital stay but warrants further investigation.

1.6.3. Beta-Hydroxy Methyl Butyric Acid (HMB)

Beta-Hydroxy-Beta-Methyl Butyrate (or HMB) is a downstream metabolite of leucine metabolism in skeletal muscle, with a physiological turnover rate of

~0.2–0.4 mg per day or 0.66% of total leucine turnover ⁴⁹³. HMB, as an oral nutritional supplement, is commercially available in both calcium-salt (Ca-HMB) and free-acid forms ⁴⁹⁴, both of which have been shown in acute in vitro ^{495–497} and in vivo human models to stimulate MPS and dampen MPB ^{494,498}. Furthermore, it is also pertinent to note that use of HMB may also be associated with anti-inflammatory effects, although such findings are equivocal ^{499,500}.

Early studies investigating the use of HMB in healthy individuals undertaking RE, demonstrated a highly potent anabolic effect ^{501–504}. Specifically, one study observed significant improvements in body composition and muscle strength, which were associated with a ~20% reduction in MPB (assessed via urinary 3-Methylhistidine excretion) and reductions of indirect markers of muscle damage (i.e., creatine kinase and lactate dehydrogenase) ⁵⁰⁵. However, more recent RCTs ^{506–509} and meta-analyses ^{510,511} have failed to detect any benefit (i.e., increases in muscle mass, strength, or function) of HMB in trained ⁵¹² or clinical populations ⁵¹¹. Nevertheless, HMB is still considered a nutritional compound that may possess the potential to attenuate the rate muscle loss during ageing and disuse ^{393,510,513–515}.

The use of HMB supplementation during 10-days BR (1.5 g Ca-HMB, twice daily) has been shown to attenuate muscle anabolic resistance and atrophy compared with a placebo in healthy older adults ³⁹³. Importantly, Ca-HMB in this study, attenuated BR-induced muscle atrophy under conditions of a sub-optimal dietary protein intake ³⁹³. Further, following 10-day BR, the placebo group reported a significant loss of whole-body (~5% vs. 1%), and lower limb (~7% vs. <1%) skeletal muscle mass compared with Ca-HMB, respectively. Similarly, following a standardised liquid meal, whilst postprandial

MPS remained unchanged in the Ca-HMB group following 10-day BR, a ~23% reduction in postprandial mixed-MPS was observed in the placebo group ³⁹³. Subsequent follow-up investigations by Standley et al. sought to determine the effects of Ca-HMB on skeletal muscle oxidative function from a sub-sample of older participants, in which mediators of mitochondrial and lipid metabolism were assessed ^{393,515}. In contrast to previous investigations, Ca-HMB displayed no effect on mitochondrial oxidative phosphorylation, dynamics (*fission and fusion*) or autophagy between Ca-HMB or placebo supplemented groups ⁵¹⁵. However, Ca-HMB supplementation did increase the intramyocellular lipid pool, particularly triacylglycerol lipids, which the authors speculated may have protected skeletal muscle from inflammatory and reactive oxygen species-stimulatory lipids. In vitro data suggests HMB alters myotube lipid biosynthesis and upregulation of fatty acid synthase, which may facilitate anabolic processes and offer protection of muscle cells from other bioactive lipid species ⁵¹⁶. To date, however, the exact mechanisms through which supplemental HMB may act to protect muscle mass during disuse events in older adults remain unclear.

Studies investigating the effect HMB on disuse atrophy, unlike many nutritional strategies, have transitioned into clinically relevant models of critically ill older adults, across a range of patient populations, including patients suffering with malnutrition ⁵¹⁷, within intensive care units ⁵¹⁸, cachexia patients ⁵¹⁹ and patients with HIV ⁵²⁰. A recent systematic review and meta-analysis conducted by Bear et al. (2019) showed that the data surrounding HMB supplementation are relatively limited ($n = 13$ studies), particularly when provided in isolation and not as part of a mixed nutrient “cocktail” ($n = 3$). For example, HMB is often consumed with other AA’s (i.e., arginine and glutamine)

or as part of a multi-ingredient oral nutritional supplement (e.g., with additional protein and vitamin D) ^{511,521,522}. Therefore, as a stand-alone nutritional supplement, more high-quality RCTs are needed to establish HMB's role in mitigating the loss of muscle mass and strength during disuse events, particularly in older clinical populations.

1.6.4. Creatine Monohydrate

Creatine monohydrate (CM) has been a popular sports supplement with athletic populations for over three decades, with purported significant improvements in muscle mass and strength when combined with RE in young and older adults ^{523–525}. The potential mechanisms through which creatine exerts its beneficial effects are founded on three underlying anabolic constituents. Firstly, CM has been reported to increase osmo-sensing proteins related to signal transduction ⁵²⁶ and targeted mRNA gene expression ⁵²⁷. Secondly, CM is purported to have an indirect role in muscle anabolism through the phosphorylation of primary downstream anabolic signalling proteins and inhibition of the myokine, myostatin ⁵²⁸. However, isotopically labelled tracer studies have failed to detect any increases in mixed-muscle, myofibrillar and/or sarcoplasmic MPS following RE and CM supplementation ^{529–531}. CM has been reported to have anti-catabolic properties via a reduction in leucine oxidation ⁵³⁰ and urinary 3-methylhistidine excretion ⁵³². Finally, CM has been suggested to augment muscle satellite cell activation ^{533,534}. Therefore, with a growing body of literature suggesting that CM can increase muscle mass and strength in older adults ^{524,525}, it has been postulated that CM may also provide some muscle anabolic benefit during periods of musculoskeletal disuse ^{535,536}.

During periods of disuse, Johnston et al. showed that oral CM supplementation (~20 g per day) provided up to three weeks of beneficial effects during a period of upper-arm ULI in healthy younger adults by supporting the maintenance of lean muscle mass, strength, and endurance to a greater extent than a placebo ⁵³⁵. The potential for CM to attenuate muscle deterioration has also been studied in the context of lower-limb ULI in younger adults ⁵³⁶. Specifically, following a seven-day loading phase and seven-day maintenance dose pattern of oral CM supplementation (loading Phase: 4 × 5 g day., maintenance: 1 × 5 g day), CM did not attenuate the loss of muscle mass or strength following ~7 days of ULI compared to a placebo control ⁵³⁶. Whilst the mechanisms of CM in models of disuse remain unclear, an exploration into the effects of in vivo skeletal muscle creatine sensitivity and transportation may offer insight into the uptake of creatine into muscle cells ^{537,538}. Nevertheless, the current available evidence regarding its lack of stimulatory effect on MPS, combined with the requirement of a loading phase, suggests that CM supplementation is likely an inadequate and impractical nutritional intervention, at least in isolation, during models of disuse. However, MPS methodologies and disuse interventions have yet to be established in older adults, therefore, we cannot rule out a potential therapeutic benefit of CM in such populations.

1.6.5. Omega 3 Fatty Acids (n-3 FA's)

Marine fish oils, particularly n-3 FA's rich in Eicosatetraenoic (EPA) and Docosahexaenoic (DHA), have been shown in both young and older adults to be key signalling mediators of skeletal muscle anabolism ^{355,539,540}, insulin sensitivity ^{541,542} and inflammation ⁵⁴³. Human studies utilising intravenous

hyperinsulinemic and hyperaminoacidemic clamps have shown a moderate dose (~4 g) of n-3 FA supplementation, augments MPS rates in healthy young⁵³⁹, middle-aged⁵³⁹ and older adults⁵⁴⁰. Further, McGlory et al. demonstrated that eight weeks of daily n-3 FA supplementation resulted in a ~23% increase in myofibrillar MPS, compared with a placebo, following a bolus feeding of WP in young men, although this was not statistically significant⁵⁴⁴. However, n-3 FA supplementation was associated with a suppression of resistance exercise and feeding-induced increases in anabolic signalling phosphorylation⁵⁴⁴. In older adults, data suggest ~16 weeks of moderate-to-high dosage of n-3 FA (~4 g·day⁻¹) increase the postabsorptive and post-RE (~15–18 h) rates of mitochondrial and myofibrillar protein fractions in healthy older adults³⁵⁵. Moreover, long-term supplementation of n-3 FA (~6 months) resulted in significant increase in thigh muscle volume and strength in the absence of RE⁵⁴⁵. Collectively, evidence from these studies forms the basis of n-3 FA supplementation as a potential strategy to counteract disuse atrophy in older adults.

During a 14-day period of ULI, n-3 FA have been shown to attenuate declines in muscle mass in healthy young women compared with an isoenergetic placebo (i.e., sunflower oil)⁴⁰². In addition, daily supplementation of dosages of ~5 g·day⁻¹ of n-3 FA resulted enhanced myofibrillar MPS and restored muscle mass over 14 days of re-ambulation following disuse⁴⁰². The mechanism through which n-3 FA may protect against disuse atrophy might be, in part, explained by an increase in the total phospholipid content of the muscle cell⁵⁴⁶. It is, however, pertinent to note the methodological considerations in the implementation of n-3 FA, in particular, the fundamental loading phase prior

to a period disuse. Whilst McGlory and colleagues observed temporal changes in skeletal muscle phospholipid content, a minimum of two weeks (and up to four weeks) of supplementation was required to sufficiently elevate and saturate muscle cell membranes with EPA:DHA ⁵⁴⁷. Importantly, it would seem that higher doses of EPA+DHA are required to achieve a more rapid saturation (~2-fold increase in four weeks) ^{547,548} of muscle cell phospholipids than previous used lower doses (~2-fold increase in eight weeks) ^{539,540}. However, the temporal changes in skeletal muscle EPA:DHA content have yet to be established in older adults ⁵⁴⁹. Moreover, n-3 FA have currently yet to begin translation into older adults and across other experimental models of disuse and inactivity ⁵⁴⁹.

Interestingly, when considering a food-first approach to nutrient delivery in older adults undergoing disuse events, recent evidence suggests that consuming n-3 FA rich foods offer similar benefits to EPA:DHA status as dietary n-3 FA supplementation ⁵⁵⁰. Carboni et al. recently reported thrice-weekly feeding of cooked mussels consumed for lunch over four weeks, resulted in an ~18% increase in n-3 index, as well as a ~40% increase in whole-blood levels of EPA:DHA ⁵⁵⁰. Importantly, the EPA:DHA content of cooked mussels in this study was considerably lower (~300 mg) compared with previous investigations (>3000 mg) ^{355,539,546,547}, suggesting that a substantially longer period of time and/or larger doses of n-3 FA rich meals would be required to achieve an optimal n-3 status. Taken together, whilst there is a lack of research across different models of disuse/inactivity, particularly in older patient populations ^{549,551}, the available data suggest a potential benefit of daily n-3 FA supplementation for maintenance of muscle mass in older adults during periods

of disuse and/or inactivity, with the caveat that any protective effect may depend on whether a loading phase is possible prior to disuse (i.e., elective surgery patients). However, it is pertinent to note that n-3 FA are also anti-inflammatory and thus, may provide additional benefit to inflammatory disease patients^{552,553}. Indeed, lowering inflammation would have a plethora of potential beneficial effects besides maintaining muscle mass^{552,553}. However, more studies are required to investigate this further, particularly in models of disuse.

1.6.6. Multi-Ingredient Supplements

In isolation, the use of single-ingredient nutritional compounds, previously mentioned, may not be sufficient to fully attenuate disuse atrophy in both younger and older adults. Nevertheless, a more comprehensive multi-ingredient supplement (MIS) with several purportedly beneficial compounds (i.e., WP, EAAs, creatine, vitamin D, leucine, glutamine, HMB and n-3 FA's) [207], may be a superior alternative to help support the maintenance of muscle mass and strength during ageing. However, whilst a recent systematic review and meta-analysis encompassing ~35 RCTs utilising MIS as part of a ~6-week training intervention observed MIS as not superior to WP alone in healthy trained populations; MIS was shown to be beneficial in older and untrained adults undertaking a period of multi-modal concurrent exercise [208–210]. Furthermore, the majority of studies investigating the use of MIS also implement RE as part of the intervention, and therefore, this may mask any beneficial effects that might be seen with the supplement alone. Nevertheless, evidence has emerged to demonstrate that a MIS comprising of WP (30 g), creatine (2.5 g), vitamin D (500 IU), calcium (400 mg) and n-3 FA's (1500 mg)

may have a beneficial effect on skeletal muscle mass and strength [208,211], inflammation [212] and cognition [213] in older adults, although no effect on exercise-induced myogenesis was observed [214]. MIS, however, did not benefit the adaptive remodelling response to a 12-week multi-modal, concurrent RET and high-intensity interval training intervention in older men [215]. Specifically, Bell and colleagues observed no benefit of twice-daily consumption of a WP-based MIS (WP 30 g, creatine 2.5 g, vitamin D 500 IU, calcium 1500 mg, n-3 polyunsaturated fatty acid 1500 mg) on integrated measures of myofibrillar MPS compared with a carbohydrate-based placebo. This is intriguing as the treatment group consumed ~30–40 g ($0.4\text{--}0.5\text{ g}\cdot\text{kg}^{-1}\cdot\text{BW}\cdot\text{day}^{-1}$) more protein over the experimental period than the carbohydrate control group [208,215]. Although there is currently no evidence supporting the MIS approach both acutely and in older adults during disuse, it is plausible that a blend of multiple beneficial compounds (i.e., EAA, creatine, leucine, HMB, vitamin D and n-3 FA's) may be advantageous in targeting multiple dysfunctional signalling mechanisms associated with disuse and/or inactivity, when combined.

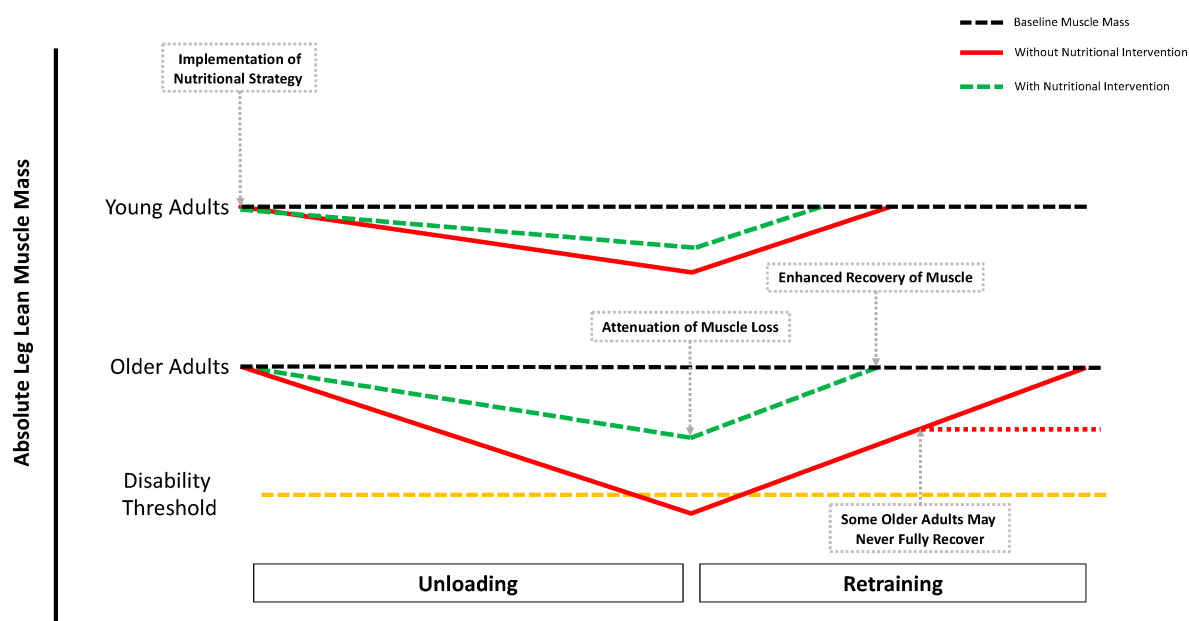


Figure 16. Skeletal muscle atrophy younger and older adults in response to a period of unloading and a subsequent period of exercise rehabilitation¹⁶. The **black dotted lines** indicate phenotypical differences in absolute levels of muscle mass. The **yellow dotted line** indicates a level in which low levels of leg lean mass results in disability and reduced functional ability (i.e., disability threshold). The **red solid line** indicates traditional trajectory in loss of muscle mass observed by disuse. The **green dotted line** indicates the potential of a nutritional strategy to offset muscle loss during disuse and improve rates of recovery following reambulation and rehabilitation. Importantly, the **red dotted line** indicates a proportion of older adults may not return to pre-disuse levels of muscle mass, particularly in the absence of aggressive retraining (Adapted from Perkin et al.)⁵⁵⁴.

¹⁶ This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. Marshall, R.N., Smeuninx, B., Morgan, P.T. and Breen, L., 2020. Nutritional strategies to offset disuse-induced skeletal muscle atrophy and anabolic resistance in older adults: from whole-foods to isolated ingredients. *Nutrients*, 12(5), p.1533.

Table 2. Overview of nutritional strategies during bed rest, limb-immobilization, and reduced activity in healthy younger and older adults to prevent muscle loss and metabolic dysfunction.

Reference	Mode of Disuse	Participant Population	Duration of Disuse	Nutritional Intervention	Changes in Muscle Mass	Changes in Muscle Strength	Changes in MPS	Changes in Mitochondrial Function	Changes in Glycemic Control	Changes in Lipid Metabolism
Paddon-Jones et al., (2004)	Bed Rest	Healthy Young Adults	28 days	16.5 g, 3 x per day, EAA	↑ ~200g LLM in EAA group ↓ ~400g LLM in Placebo group	↓ ~8.8kg in 1RM in EAA group ↓ ~17.8kg in 1RM in Placebo group	↓ ~6% in MixedMPS in EAA group ↓ ~27% in MixedMPS in Placebo group	-	-	-
Paddon-Jones et al., (2005)	Bed Rest	Healthy Young Adults	28 days	16.5 g, 3 x per day, EAA	↑ ~210g in LLM in EAA group ↓ ~346g in LLM in Placebo group	↓ ~9.1kg in 1RM in EAA group ↓ ~17.8kg in 1RM in Placebo group	↑ ~2.7% in MixedMPS in EAA group ↓ ~31% in MixedMPS in Placebo group	-	-	-
Ferrando et al., (2010)	Bed Rest	Healthy Older Adults	10 days	15 g, 3 x per day, EAA	↓ ~6.3% in LLM in EAA group ↓ ~5.9% in LLM in Placebo group	↓ ~2% in SPT in the EAA group ↓ ~14% in SPT in the Placebo group	↑ ~1.4% in MixedMPS in the EAA group ↓ ~30% in MixedMPS in the Placebo group	-	↑ ~9% GLUT4 in Creatine group ↓ ~20% GLUT4 in Placebo group ↔ Glycogen or Creatine content	-

Deutz et al. (2013)	Bed Rest	Healthy Older Adults	10 days	3 g per day, HMB 3 g per day, Placebo	↓ ~0.7% in LLM in HMB group ↓ ~7.9% in LLM in Placebo group	↔ No change in HMB group (ISO) ↓ ~8.6% in Placebo group (ISO)	↓ ~1.6% in HMB group ↓ ~21% in Placebo group	-	-	-
English et al., (2015)	Bed Rest	Healthy Middle-Aged Adults	14 days	0.06g·kg ⁻¹ ·meal ⁻¹ Leucine 0.06g·kg ⁻¹ ·meal ⁻¹ Alanine	↓ 900g of LLM in Leucine group ↓ 1,200g of LLM in Placebo group	↓ ~10% in Leucine group (ISO) ↓ ~24% in Placebo group (ISO)	↓ ~10% in postabsorptive MPS in Leucine group ↓ ~30% in postabsorptive MPS in the Placebo group. ↔ between postprandial MPS between groups	-	-	-
Standley et al., (2017)	Bed Rest	Healthy Older Adults	10 days	3 g per day, HMB 3 g per day, Placebo	-	-	↓ In ribosomal gene transcription ↔ OXPHOS Proteins ↔ Fission and fusion Proteins ↔ Autophagy and proteolysis ↓ HMB blunted decreases in gene transcripts of OXPHOS	-	-	-
Arentson-Lantz et al., (2019)	Bed Rest	Healthy Older Adults	7 days	Whey Protein, dosage N/A	↓ 680g of LLM in Whey group ↓ 1,035g in Control group	↓ ~9.1% in Whey group ↓ ~16.3% in Control group	-	-	-	-

Standley et al., (2020)	Bed Rest	Healthy Older Adults	10 days	3 g per day, HMB 3 g per day, Placebo	-	-	-	↓ in mitochondrial content, no difference between groups ↓ in mitochondrial respiration, no differences between groups ↑ in H ₂ O ₂ production, no difference between groups	-	-
Arentson-Lantz et al., (2020)	Bed Rest	Healthy Older Adults	7 days	0.06g·kg ⁻¹ ·meal ⁻¹ Leucine 0.06g·kg ⁻¹ ·meal ⁻¹ Alanine	↓ ~423g of LLM in Leucine group ↓ ~1,035g of LLM in Placebo group	↓ ~7.1% in Leucine group (ISO) ↓ ~0.4% in Placebo group (ISO) ↓ ~24.8% in Leucine group (KIN) ↓ ~16.3% in Placebo group (KIN)	↓ in mTORC1 phosphorylation in both groups	-	-	-
Arentson-Lantz et al., (2021)	Bed Rest	Healthy Older Adults	7 days	0.06g·kg ⁻¹ ·meal ⁻¹ Leucine 0.06g·kg ⁻¹ ·meal ⁻¹ Alanine	-	-	-	↓ ~27% in state 2 (residual oxygen consumption) respiration in Placebo group ↑ ~6.5% increase in state 2 (residual oxygen consumption) respiration in Leucin group ↓ ~24% decrease in state 3 (ADP stimulated)	-	-

										respiration in Placebo group
										↑ 9% increase in state 3 (ADP stimulated) respiration in Leucine group.
										↓ ~7% in oxidative phosphorylation in Placebo group
										↑ ~25% in oxidative phosphorylation in Leucine group.
										↓ ~15% decrease in state 4 respiration in Placebo group.
										↑ 3% increase in state 4 respiration in Leucine group.
										↔ in Electron Transport Chain Complex, I-V protein abundance.
Hespel et al., (2001)	Leg Immobilization	Healthy Young Adults	14 days	20 g, per day, 7-day loading period. 5 g daily, Creatine Monohydrate	↓ ~11% in CSA in Creatine group	↓ ~25.6% in Creatine group (KIN)	-	-	-	-
				20 g, per day, 7-day loading period. 5 g daily, Dextrose	↓ ~9.4% in CSA in Placebo group	↓ ~23.7% in Placebo group				

Op't et al., (2001)	Leg Immobilization	Healthy Young Adults	14 days	20 g, per day, 7-day loading period. 5 g daily, Creatine Monohydrate 20 g, per day, 7-day loading period. 5 g daily, Dextrose							↑ ~9% GLUT4 in Creatine group ↓ ~20% GLUT4 in Placebo group ↔ Glycogen or Creatine content
Dirks et al., (2014)	Leg Immobilization	Healthy Young Adults	5 days	20 g x 2 per day, Whey protein No supplement, Control	↓ ~1.6% in CSA, Whey group ↓ ~0.7% in CSA, Control	↓ ~9.3% in 1RM, Whey group ↓ ~8.3% in 1RM, Control	↑ Myostatin, myogenin and MuRF-1 mRNA in both groups ↑ MAFBx in Protein group only	-	-	-	
Backx et al., (2018)	Leg Immobilization	Healthy Young Adults	7 days	2.5 g, 3 x per day, Leucine 2.5g, 3 x per day, CHO	↓ ~6.2% in LLM in Leucine group ↓ ~5.5% in LLM in Placebo group	↓ ~12.7% 1RM in Leucine group ↓ ~5.3% 1RM in Placebo group	-	-	-	-	
Backx et al., (2017)	Leg Immobilization	Healthy Young Adults	7 days	20 g, per day, 7-day loading period. 5 g daily, Creatine Monohydrate 20 g, per day, 7-day loading period. 5 g daily, Dextrose	↓ ~5.6% in CSA in Creatine group ↓ ~5.5% in CSA in Placebo group	↓ ~9.2% 1RM in Creatine group ↓ ~5.6% 1RM in Placebo group	-	-	-	-	

Mitchell et al., (2018)	Leg Immobilization	Healthy Young Adults	14 days	20 g, once per day, Milk Protein	↓ ~1.9% in LLM, Milk group	↓ ~25% in ISO, Milk group	MyoPS not reported ↑ in MuRF-1, Atrogin-1, no difference between groups	MitoPS not reported ↔ Citrate synthase between groups	-	-
				20 g, once per day, Maltodextrin	↓ ~1.2% in LLM, Placebo group	↓ ~25% in ISO, Placebo group				
Holloway et al., (2019)	Leg Immobilization	Healthy Young Adults	7 days	23.7g, 3 x per day, Novel AA blend	↓ ~0.7% in CSA, AA group	↓ ~7.5% in both AA and Placebo groups	-	-	-	-
				23.7g, 3 x per day, Maltodextrin	↓ ~3.1% in CSA in Placebo group					
McGlory et al., (2019)	Leg Immobilization	Healthy Young Adults	14 days	5 g EPA: DHA loading phase (28 days); 5 g EPA: DHA per day	↓ ~8% in CSA, Omega 3 group	↓ ~35% in Omega 3 group (ISO)	↓ ~9% in MyoPS, Omega 3 group ↓ ~14% in MyoPS, Placebo group	-	-	-
				5 g Sunflower oil, Placebo	↓ ~14% in CSA, Placebo group	↓ ~50% in Placebo group (ISO)				
Miotto al., (2019)	Leg Immobilization	Healthy Young Adults	14 days	5 g EPA: DHA loading phase (28 days); 5 g EPA: DHA per day	-	-	-	↓ 20% reduction in ADP stimulated respiration in control	-	-
				5 g Sunflower oil, Placebo				↔ No change in mitochondrial respiration with omega-3 supplementation ↓ in OXPHOS protein content in placebo but not omega-3 group ↓ in MFN-2/DRP-1 ratio in placebo but not omega-3 group		

									protein abundance.	↑ ~87% increase in PLIN5 protein abundance
									↓ ~56% decrease in GLUT4 protein abundance.	
Zeng et al., (2021)	Leg Immobilization	Healthy Young Adults	14 days	20g Milk Protein Concentrate per day. Isocaloric carbohydrate placebo.	-	-	↔ No effect or differences between groups on DEPTOR, REDD1, p-mTOR, Total mTOR, protein abundance. ↓ ~37% in Phos-P70S6K expression in control group. ↓ ~14% in Phos-P70S6K expression in Milk group. ↓ ~38% in Phos-RPS6 expression in control group. ↓ ~25% in Phos-RSP6 expression in Milk group.	-	-	-
Devries et al., (2015)	Step Reduction (<1,500 steps ^{day-1})	Healthy Older Adults	14 days	5 g, 1 x per day, Citrulline 5 g, 1 x per day, Glycine	↔ Difference between groups ↓ ~1.3% LLM ↓ ~1% SMM	↔ No change over the intervention	↔ Difference between groups ↓ ~42% in postabsorptive MyoPS ↓ ~52% in postprandial MyoPS	-	-	-

Oikawa et al., (2018)	Step Reduction (<1,500 steps ^{day-1})	Healthy Older Adults	14 days	30 g, 2 x per day, Whey protein	↓ ~2.7% LLM, Whey group	-	↓ ~13.9% in MyoPS (D ₂ O)	-	-	-
				30 g, 2 x per day, Collagen protein	↓ ~2.3% LLM, Collagen group		↓ ~15.6% in MyoPS (D ₂ O)			

1.7. Summary

Skeletal muscle is critical for the maintenance of whole-body metabolic health and function. As ageing is associated with the slow and insidious loss of skeletal muscle mass, this results in an increased risk of morbidity and mortality ²³. Furthermore, intermittent periods of muscle disuse either by bed rest ⁸, limb-immobilisation ⁹, or reduced activity ¹⁰, accelerate this decline into functional disability. RE is the single most potent strategy to maintain, or at least offset the rate of muscle atrophy in older adults. As such, the development of pragmatic RE such as elastic band RE, and RE prior to disuse situations may offer older adults an alternative to gym-based RE to maintain muscle mass and strength ^{389,555–559}. These RE modalities can (in theory) be undertaken at home, and still augments a muscle building response similar to that of free-weight or machine-based RE. Interestingly, the role of RE on muscle anabolic signalling and protein synthesis is well characterised ²⁷⁴, but it's role in mitochondrial remodelling, in particular RE-induced alterations in mitochondrial content and quality control remain poorly defined ⁵⁶⁰. Lastly, in the absence of, or the inability to undertake RE to maintain muscle mass, nutritional interventions may play a key role in muscle maintenance. Several nutritional ingredients have purported anti-catabolic and pro-anabolic effect (whey protein, EAA's, Leucine, HMB, n-3 FA's, Vitamin-D), however, relatively few offer significant 'protection' against disuse atrophy ³⁹⁴. The daily consumption of leucine-enriched EAA's may offer older adults a cheap and pragmatic strategy to maintain muscle mass during periods of reduced activity ³⁴¹. As such, a further hypothesis of this thesis is RE and nutritional approached may alleviate expected physiological and metabolic decline with disuse.

1.8. Aims and Objectives of the Thesis

The aims of this thesis are as follows:

- 1. Chapter 2. Determine the role of resistance exercise prehabilitation and 5-days of bed rest on mitochondrial content and quality control.**

To assess the molecular response in mitochondrial content, and quality control following a 7-day period of resistance exercise prehabilitation, prior to undergoing 5-days of clinical bed-rest in healthy older males. In particular, determine the role of mitochondrial fission and fusion dynamics as molecular markers of mitochondrial quality control.

- 2. Chapter 3. Investigate the effects of lifelong exercise on resistance exercise induced mitochondrial remodelling.**

Lifelong exercisers (termed ‘master athletes’) maintain greater whole-body and muscle metabolic function. This chapter aims to explore the effects of resistance exercise training to induce mitochondrial content and improve mitochondrial quality control mechanisms.

- 3. Chapter 4. Asses the acute neuromuscular response to elastic band resistance exercise training and activities of daily living.**

To compare the neuromuscular response (via surface EMG) of undertaking activities of daily living (walking, stair climbing, and sit-to-stand), elastic band resistance exercise training to traditional machine-based exercise training in healthy older male and females.

- 4. Chapter 5. Determine the muscle anabolic and protein synthetic response to elastic band resistance exercise training.**

Assess the molecular and metabolic response to a single session of elastic band resistance exercise training in younger and older males. Notably, to assess the cell

signalling response of mTORC1 protein expression and phosphorylation, and post-exercise-induced increases in integrated rates of myofibrillar protein synthesis over 48-hours via D₂O tracer methodology.

5. Chapter 6. Investigate the effectiveness of leucine-enriched essential amino in older adults undergoing short-term reduced physical activity.

Determine the role of high-dose, leucine-enriched essential amino acids at mitigating loss of skeletal muscle mass, and alterations in muscle protein synthesis in healthy older adults. In particular, determine the dynamic changes in the muscle proteome, and mitochondrial functioning following 14-days of step reduction.

CHAPTER 2

No Effect of Five Days of Bed Rest or Short-Term Resistance Exercise Prehabilitation on Markers of Skeletal Muscle Mitochondrial Content and Dynamics in Older Adults



Published Peer-Reviewed Manuscripts from This Chapter ⁵⁶¹:

Marshall, R.N., Smeuninx, B., Seabright, A.P., Morgan, P.T., Atherton, P.J., Philp, A. and Breen, L., 2022. No effect of five days of bed rest or short-term resistance exercise prehabilitation on markers of skeletal muscle mitochondrial content and dynamics in older adults. *Physiological reports*, 10(13), p.e15345.

2.1 ABSTRACT

Background: Bed rest (BR) results in significant impairments in skeletal muscle metabolism. Mitochondrial metabolism is reportedly highly sensitive to disuse, with dysregulated fission-fusion events and impaired oxidative function previously reported. The effects of clinically relevant short-term BR (≤ 5 days) on mitochondrial protein expression are presently unclear, as are the effects of exercise prehabilitation as a potential counteractive intervention. The present study aim was to examine the effects of a 5-day period of BR and short-term resistance exercise prehabilitation (ST-REP) on mitochondrial protein content. **Methods:** Ten older adults underwent 5-days of BR, completing four sessions of high-volume unilateral resistance exercise prehabilitation over 7-days beforehand. Muscle biopsies were obtained from the *vastus lateralis* in the non-exercised control and exercised legs, both pre- and post-prehabilitation and pre- and post-BR, to determine changes in citrate synthase enzyme activity and the expression of key proteins in the mitochondrial electron transport chain and molecular regulators of fission-fusion dynamics, biosynthesis, and mitophagy. **Results:** We observed no significant effect of either BR or ST-REP on citrate synthase protein content, enzyme activity, or ETC complex I-V protein content. Moreover, we observed no significant changes in markers of mitochondrial fission-fusion dynamics (Phos-DRP-1^{S616}, Phos-DRP-1^{S637}, Phos-DRP-1^{S616/S637} ratio, Phos-MFF^{S146}, Mitofillin, OPA-1, MFN-2, DRP-1, or MFN-2/DRP-1 ratio. Finally, we observed no differences in markers of biosynthesis (Phos-AMPK^{T172}, Phos-ACC^{S79}, PGC1a, TFAM) or mitophagy-related signalling (ULK-1, BNIP3/NIX, LC3B I/II) ($P > 0.05$ for all). **Conclusion:** In contrast to previous longer-term periods of musculoskeletal disuse (i.e., 7-14 days), a clinically relevant, 5-day period of BR resulted in no significant perturbation in muscle mitochondrial proteostasis in healthy older adults, with no effect of ST-REP in the week prior to BR. Accordingly, disuse-induced muscle atrophy may precede alterations in mitochondrial content.

2.2. INTRODUCTION

Mitochondrial content and function (i.e., mitochondrial O₂ consumption or respiration) decline during ageing⁸⁷ and may lead to impaired glucose and lipid utilisation, insulin resistance and increased adiposity⁵⁶². Mitochondrial metabolism is highly sensitive to physical inactivity and unloading⁵⁶³ and may accelerate age-related mitochondrial dysfunction⁸⁷. Pre-clinical *in vivo* models have suggested that mitochondrial dysfunction, and accrual of damaged proteins may precede disuse-induced skeletal muscle atrophy via the early and rapid declines in respiratory capacity and protein content^{564,565}. Human models of disuse have also demonstrated significant effects of unloading on mitochondrial respiratory capacity³⁹⁰, enzyme activity^{566,567}, protein synthesis^{400,401} and protein expression^{515,568,569} (i.e., electron transport chain [ETC], biosynthesis, fission-fusion dynamics, and mitophagy). Observable declines in mitochondrial function, but not protein expression, are apparent following as little as 3-days of disuse⁵⁷⁰, with others observing declines in protein expression following 7-14 days across varying disuse models (i.e., BR, immobilisation, and step reduction) and population cohorts^{385,515,570}. However, little is known about the impact of shorter, clinically relevant durations of BR (i.e., ≤5-days) on markers of mitochondrial function but is essential to understand given that the average length hospital stay in the UK is ~4.5 days. Understanding the temporal change in muscle mitochondrial content and function during disuse/bed rest in older adults, is essential to inform strategies to preserve cellular metabolic health of older patients upon discharge.

Mitochondrial morphology (i.e., the dynamic fission and fusion of organelles) has emerged as an essential regulator of skeletal muscle mass and longevity *in vivo*^{99,571,572}. As ageing is associated with an intrinsic increase in oxidative stress, accrual of mitochondrial DNA damage and dysfunctional mitochondria³⁷⁰, the targeted

removal of damaged mitochondria (termed 'mitophagy') is crucial in maintaining cellular health ⁵⁷³. Fission and fusion of mitochondria are tightly orchestrated by specific outer mitochondrial membrane (OMM) and inner mitochondrial membrane (IMM) proteins. DRP1 induced mitochondrial fission and mitophagy are essential in the removal, fragmentation, and subsequent degradation of dysfunctional components of organelles via the lysosome ⁵⁷⁴. Contrary to this, MFN2 & OPA1 mediated fusion may coordinate the merging of multiple healthy organelles to form larger, spherical, or elongated tubular-shaped mitochondria, depending on subcellular-specific localisation ^{359,575}. The dynamic remodelling favouring mitochondrial fission has been implicated in disuse-atrophy ^{94,563}. Indeed, human data indicates fission dynamics; in particular, increases DRP1 and MFN2 are altered following longer periods (i.e., 10-14 days) of disuse in both younger and older adults, causing a removal of dysfunctional organelles and subsequent reduced mitochondrial density and respiratory function ^{515,570}. Therefore, given the increases in mitochondrial fission, declines in mitochondrial content, and ensuing muscle atrophy during disuse, implementing strategies to mitigate the deterioration of mitochondrial metabolism are essential for maintaining mitochondrial health and function both during and following disuse events.

Endurance exercise (EE) or high-intensity interval training (HIIT) induces mitochondrial remodelling in skeletal muscle ⁵⁷⁶ and can attenuate the age-associated declines in mitochondrial function ^{231,232}. In addition, EE and HIIT induce rapid adaptations in mitochondrial density, respiratory capacity, and tissue substrate utilisation ⁵⁷⁶. Emerging data suggests acute and chronic resistance exercise (RE) training can augment skeletal muscle mitochondrial protein turnover ^{577,578}, function ^{578,579} and remodelling ⁵⁸⁰. Indeed, EE, HIIT, and RE have all been utilised as part of traditional post-disuse rehabilitation to restore muscle mass, strength, and function

^{384,515}. However, exercise strategies prior to BR (termed 'prehabilitation') have the potential to improve pre-and peri-operative physical condition and improve post-BR recovery of physical function ⁵⁸¹. Typically, prehabilitative strategies require a minimum of ~30 days to induce detectable changes in muscle morphology ⁵⁵⁸ and metabolic function ⁵⁸² prior to elective surgery. In contrast, shorter-term interventions may be necessary in scenarios where the waiting period is substantially shorter, and longer-term interventions are not possible. In this regard, we recently observed that short-term (7-day) RE prehabilitation did not offset BR-induced impairments in muscle anabolism or quadriceps atrophy in older adults ³⁸⁹. Despite this, short-term resistance exercise rehabilitation (ST-REP) may still effectively mitigate mitochondrial dysfunction that may occur in the early phase of BR, although this has yet to be explored.

The primary aim of this study was, therefore, to determine the effects of a clinically relevant, 5-day period of BR on skeletal muscle mitochondrial content and enzyme activity in older adults and to establish whether, and to what extent, ST-REP would influence these parameters. The second aim of this study was to determine if the anticipated loss in mitochondrial content is mediated by an increase in DRP1 phosphorylation and parallel reductions in OPA1 and MFN2. Utilising a unilateral, within-subject design, we hypothesised that ST-REP will facilitate an increase in mitochondrial density, electron transport chain (ETC) protein expression, and augment mitochondrial fusion in the exercised leg, thereby effectively counteracting any observable decline in mitochondrial content associated with BR when compared to non-exercised control leg.

2.3. METHODOLOGY

2.3.1. Participants

The present study was a retrospective analysis of skeletal muscle biopsy samples obtained as part of a larger clinical trial on the effects of short-term RE prehabilitation on muscle mass and myofibrillar protein synthesis ³⁸⁹. Briefly, ten healthy older men (age; 71.5 ± 4.0 years, height; 1.77 ± 0.07 m, weight; 79.6 ± 9.0 kg) were recruited to the study through local advertisements and research recruitment databases. All participants were non-sarcopenic and non-obese as determined through the completion of a general health questionnaire, a score of >9 on the short physical performance battery test, appendicular lean mass of >7.0 kg/m² and a BMI <30 kg/m². Body composition, blood chemistry, activity status, and dietary consumption for this participant cohort have previously been reported ³⁸⁹. Ethical approval was obtained through the West Midlands – Black Country Research Ethics Committee (16/WM/0483) and was registered at Clinicaltrials.gov (NCT04422665; RG_16-100). The study conformed to the standards outlined by the Declaration of Helsinki (7th Edition).

2.3.2. Study Design

As previously described ³⁸⁹, participants visited the Clinical Research Facility (CRF) at the Queen Elizabeth Hospital Birmingham for (i) a preliminary testing phase [day 1], (ii) prehabilitation phase [days 2-7], (iii) mid-phase testing [day 8], and (iv) BR phase [day 8-13]. Briefly, participants reported to the CRF at 0800h in an overnight fasted state, where a skeletal muscle biopsy was obtained from the vastus lateralis under 1% lidocaine using the modified Bergstrom biopsy needle technique. Participants then returned to the CRF to complete a bout of unilateral knee extension and flexion on

days 2, 4, 6, and 7. RE was undertaken on the dominant leg, as determined by a previously established one repetition maximum (1RM) test during the preliminary testing visit. Each RE session commenced with two warm-up sets at 50% of 1RM, followed by six sets of twelve repetitions at 75% of 1RM of machine-based knee extension and knee flexion. An intra-set recovery period of 120 seconds was utilised to ensure sufficient recovery in between sets. The exercise load was adjusted to maintain a rate of perceived exertion of 8-9 on the modified-Borg CR-10 scale ⁵⁸³. On the morning of day 8 [post-prehabilitation, pre-BR], participants reported to the CRF in an overnight fasted state, where a muscle biopsy was obtained from both the exercised (EX) and the non-exercised control leg (CTRL). Following this, participants then undertook five days of strict BR to mimic a typical inpatient hospital stay. Further details of the BR protocol, dietary intake and physical activity parameters have been described elsewhere ³⁸⁹. On the morning after the final day of BR (i.e., day 14), final dual biopsies of the EX and CTRL legs were obtained in the fasted state. Skeletal muscle biopsy samples from the CTRL and EX legs from pre-BR and post-BR periods underwent biochemical analysis (N=4, per participant) as previously reported ³⁸⁹.

2.3.3. Mitochondrial Enzyme Activity Assays

Citrate Synthase (CS) enzyme activity was determined following the protocols of Spinazzi et al. ⁵⁸⁴ and adapted to 96-well plate format as previously described ⁵⁸⁵. In brief, 20-30 mg of skeletal muscle was homogenised in 10 $\mu\text{L}/\mu\text{g}$ of ice-cool sucrose lysis buffer (50 mM Tris, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5 mM $\text{Na}_4\text{P}_2\text{O}_7$ - $^{10}\text{H}_2\text{O}$, 270 mM sucrose, 1 M Triton-X, 25 mM β -glycerophosphate, 1 μM Trichostatin A, 10 mM Nicotinamide, 1 mM 1,4-Dithiothreitol, 1% Phosphatase Inhibitor Cocktail 2; Sigma, 1% Sigma Phosphatase Inhibitor Cocktail 2; Sigma, 4.8% complete Mini

Protease Inhibitor Cocktail; Roche) using a TissueLyser II, and 5-mm stainless steel beads (Qiagen, Hannover, Germany). Samples were disrupted following 3 x 2-minute cycles at 20 Hz, followed by centrifugation at 13,000 RPM for 10 mins. The protein content of the resulting supernatant was determined via DC protein assay (Bio-Rad, Hercules, California, USA) and prepared at 2 µg/µL in ddH₂O. For determination of CS activity, 10 µL of sample (total of 20 µg of protein) was diluted in 186 µL of assay solution (50 mM Kpi buffer (pH 7.4) with 100 µM DTNB, 115 µM acetyl-CoA in ddH₂O) to a total well volume of 196 µL, as previously described⁵⁸⁵. Baseline absorbance was read every 15 s for three minutes in a microplate reader (FLUOstar Omega, BMG Labtech, Aylesbury, UK). Following the baseline reading, 4 µL of 5 mM oxaloacetate (100 µM final concentration) was added to start the reaction and absorbance was read again every 15 s for three minutes. CS activity was calculated using the extinction coefficient (CS ϵ = 13.6). Measurement CV% within-plate was 7.01 ± 0.9% for three technical repeats⁸⁹.

2.3.4. Western Blotting

Immunoblotting was performed on the sarcoplasmic fraction of skeletal muscle biopsy samples as a result of myofibrillar protein extraction, as previously reported³⁸⁹. Briefly, 20-30 mg of snap-frozen skeletal muscle was homogenised in ice-cold homogenisation buffer [50 mM Tris HCL (pH 7.4), 50 mM NaF, 10 mM β -glycerophosphate disodium salt, 1 mM Ethylenediaminetetraacetic acid [EDTA], 1 mM Ethyleneglycoltetraacetic acid [EGTA], 1 mM activated sodium orthovanadate [Na₃VO₄], and a complete protease inhibitor cocktail tablet (Roche, West Sussex, UK) at 10 µL/µg per tissue and shaken on a vibrax shaker at 4°C for 10 mins. Homogenates

were spun at 11,000g for 10 min at 4°C, and the supernatant was collected and frozen at -80°C for subsequent western blot analysis.

Protein content was determined by DC Protein Assay (Bio-Rad, Hertfordshire, UK), with protein content measured at $6.02 \pm 0.78 \mu\text{g}/\mu\text{L}$. Western blot aliquots were subsequently prepared at $3 \mu\text{g}/\mu\text{L}$ in 4 x Laemmli sample buffer and ddH₂O. Following preparation, samples were left at room temperature for ~24 hr to denature as previously described⁴⁰¹. Equal amounts of protein (30 μg) were loaded into 4-15% Tris-Glycine precast gels (BioRad, Hertfordshire, UK) and separated by SDS-PAGE for ~1 hr at 140V in pre-made tris-glycine running buffer (24mM Tris, 192mM Glycine, pH 8.3). Proteins were transferred to a polyvinylidene difluoride (PVDF; Whatman, Dassel, Germany) membrane at 100V for 1hr in a pre-made transfer buffer. Membranes were subsequently blocked in 5% low-fat milk (diluted in Tris-buffered saline and 0.1% Tween-20 (TBS-T)) for 1h at room temperature. The membranes were then incubated overnight at 4°C with appropriate antibodies; total OXPHOS human antibody cocktail (Manufacturer; Abcam, Catalogue Number; 110411, diluted 1:1000 in tris-buffered saline with 0.1% Tween 20 (TBST), Citrate Synthase (CS; CST, 14309, 1:1000), MFN2 (CST, 9482, 1:1,000), DRP1 (CST, 8570, 1:1,000), Phosphorylated DRP1^{Serine616} (CST, 4494, 1:1,000), Phosphorylated DRP1^{Serine637} (CST, 6319, 1:1,000), OPA-1 (BD Bioscience, 612607, 1:1,000), Mitochondrial Fission Factor; MFF (CST, 84580S, 1:1000), Phosphorylated Mitochondrial Fission Factor; MFF^{Serine146} (CST, 49281, 1:1000), Mitofilin (ProteinTech, 10179-AP, 1:1,000), Total AMP-activated protein kinase; AMPK (CST, 2532S, 1:1000), Phosphorylated AMPK^{Threonine172} (CST, 2535L, 1:1000), Total Acetyl-CoA carboxylase; ACC (Dundee MRC DSTT, N/A [custom made], 1:1000), Phosphorylated ACC^{Serine79} (CST, 3661L, 1:1000), Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-

1 α ; Abcam, 3242, 1:1000), mitochondrial transcription factor A [TFAM] (Sigma-Aldrich, SAB1401383, 1:1,000), Total UNC-51 like kinase; ULK-1 (CST, 4773S, 1:1000), BNIP3L/Nix (CST, 12396, 1:1,000), LC3B I/II (CST, 4108, 1:1,000). Following overnight incubation, membranes were washed 3 $\times$ 5 min in TBS-T, incubated in horseradish peroxidase (Mouse/Rabbit-HRP)-conjugated secondary antibody (CST, 7076 & 7074, 1:10,000 in 2.5% BSA in TBS-T) at room temperature for 1 h, before the last 3 $\times$ 5 min washes in TBS-T. Membranes were exposed to Chemiluminescent HRP Substrate (Millipore Corp., Billerica, MA, USA) for 2 mins and bands visualised by G:BOX Chemi XT4 imager with GeneSys capture software (Syngene UK, Cambridge, UK). Quantification was performed using ImageJ/Fiji (NIH, Bethesda, MD, USA). Relative arbitrary units were normalised to the total amount of protein loaded as visualised via Ponceau S staining, and an internal gel control to account for gel-to-gel differences^{586,587}. Following normalisation, where relevant, the phosphorylation of proteins, as a proxy of their activation, was expressed relative to the total amount of each protein (phos/total)⁵⁸⁷.

2.3.5. Statistical Analysis

Markers associated with (I) mitochondrial content & enzyme activity, (II) fission & fusion dynamics, (III) bioenergetics, and (IV) mitophagy were analyzed using a two-way repeated-measures ANOVA (group $\times$ time), with a group (CTRL vs EX) and time (pre-BR vs post-BR). All analysis was conducted using GraphPad Software Inc Prism version 8. Significance was set at $P \leq 0.05$. All data are presented as fold-change from their CTRL baseline leg along with mean $\pm$ SEM unless otherwise stated.

2.4. RESULTS

2.4.1. Citrate Synthase Enzyme Activity and ETC Complex Content

Citrate Synthase activity was not significantly altered by RE-prehabilitation or five-days of BR ($P=0.94$) (**Figure 1A**). Furthermore, there was no significant changes in citrate synthase protein expression (**Figure 1B**) (Group, $P=0.88$, Time, $P=0.42$, Interaction, $P=0.57$). No changes in mitochondrial ETC complex I-V protein expression were observed (**Figure 2**); (**A**) Complex I (*NDUFB8*) (Group, $P=0.96$, Time, $P=0.42$, Interaction [Group x Time], $P=0.27$), (**B**) Complex II (*SDHB*) (Group, $P=0.78$, Time, $P=0.37$, Interaction, $P=0.19$), (**C**) Complex III (*UCQRC2*) (Group, $P=0.99$, Time, $P=0.34$, Interaction, $P=0.13$), (**D**) Complex IV (*COX2*) (Group, $P=0.76$, Time, $P=0.09$, Interaction, $P=0.23$), I Complex V (*ATP5A*) (Group, $P=0.88$, Time, $P=0.23$, Interaction, $P=0.28$), or (**F**) total OXPHOS (Group, $P=0.97$, Time, $P=0.23$, Interaction, $P=0.27$).

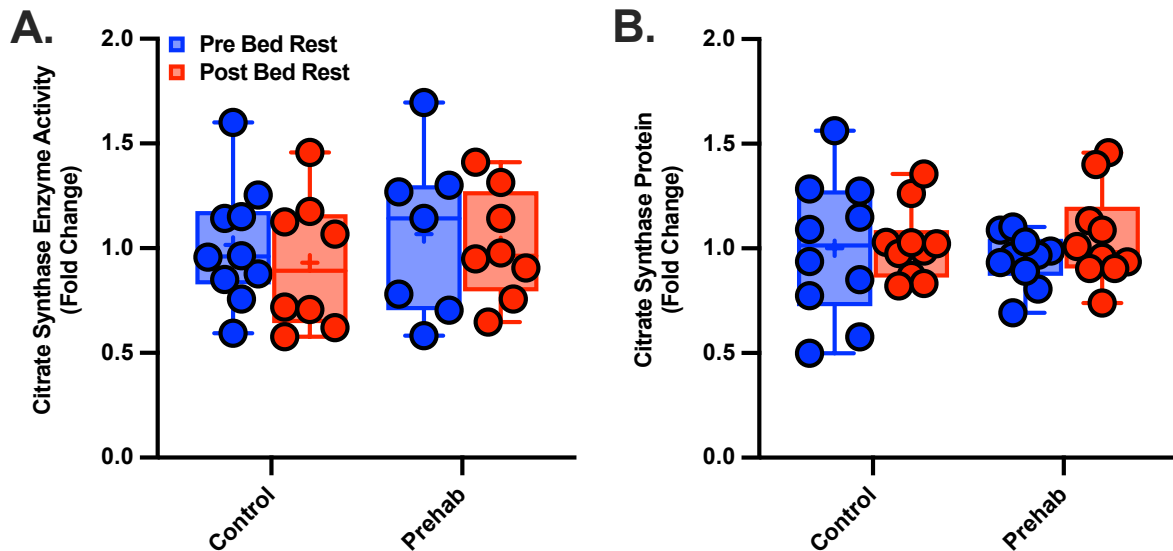


Figure 1. Bed rest does not alter citrate synthase enzyme activity or protein expression. (A) No changes in skeletal muscle enzymatic activity of citrate synthase (CS) or (B) protein abundance was observed pre-bed rest (**BLUE**) and post-bed rest (**RED**) with (Prehab) and without (Control) resistance exercise prehabilitation. Data presented as fold-change from pre-BR CTRL with individual data points (min to max) (n=10 per group). Statistical significance was set at $P < 0.05$.

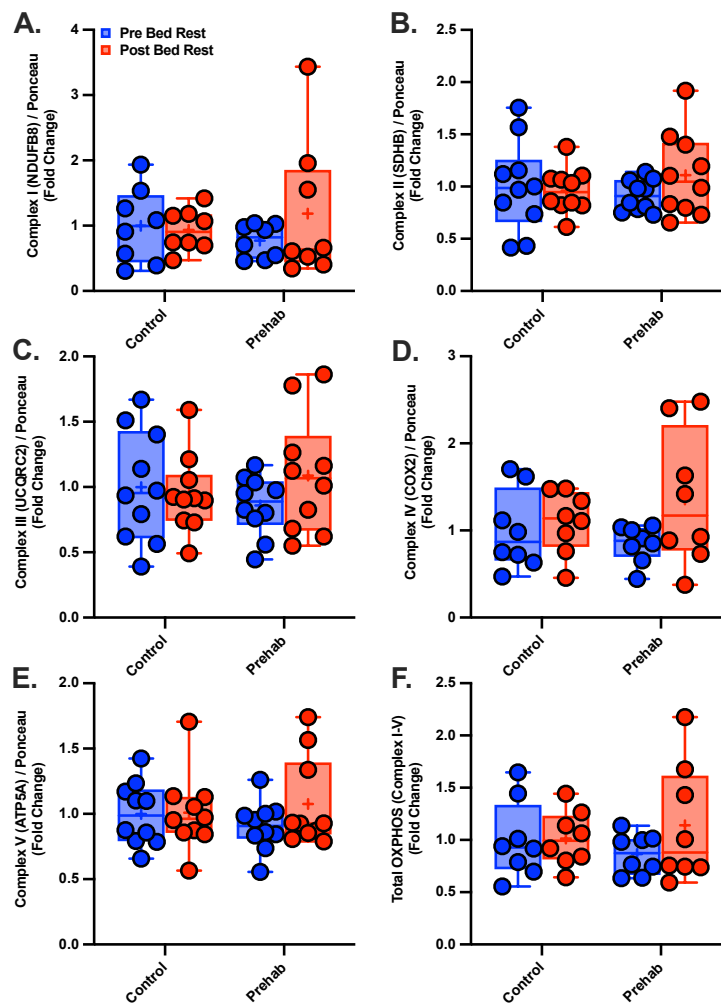


Figure 2. No change in mitochondrial electron transport chain protein content following short term bed rest. No changes in the expression of skeletal muscle oxidative phosphorylation electron transport Complex I (A), Complex II (B), Complex III (C), Complex IV (D), Complex V (E) or total OXPHOS (F) were observed pre-bed rest (BLUE) and post-bed rest (RED) with (Prehab) and without (Control) resistance exercise prehabilitation. Data presented as fold-change from pre-BR CTRL with individual data points (min to max) (n=10 per group). Statistical significance was set at $P < 0.05$.

2.4.2. Mitochondrial Fission and Fusion Dynamics

We observed no significant changes in markers associated with mitochondrial fission and fusion dynamics (**Figure 3**); **(A)** p-DRP1^{S616}:total DRP1 (Group, $P=0.81$, Time, $P=0.37$, Interaction, $P=0.80$), **(B)** p-DRP1^{S637}:total DRP1 (Group, $P=0.89$, Time, $P=0.19$, Interaction, $P=0.67$), **(C)** p-DRP1^{S616}:p-DRP1^{S637} (Group, $P=0.27$, Time, $P=0.52$, Interaction, $P=0.99$), **(D)** p-MFF^{S146}:total MFF (Group, $P=0.90$, Time, $P=0.64$, Interaction, $P=0.34$), **(E)** Mitofillin (Group, $P=0.78$, Time, $P=0.45$, Interaction, $P=0.43$), **(F)** OPA-1, (Group, $P=0.29$, Time, $P=0.84$, Interaction, $P=0.62$), **(G)** MFN2 (Group, $P=0.34$, Time, $P=0.26$, Interaction, $P=0.58$).

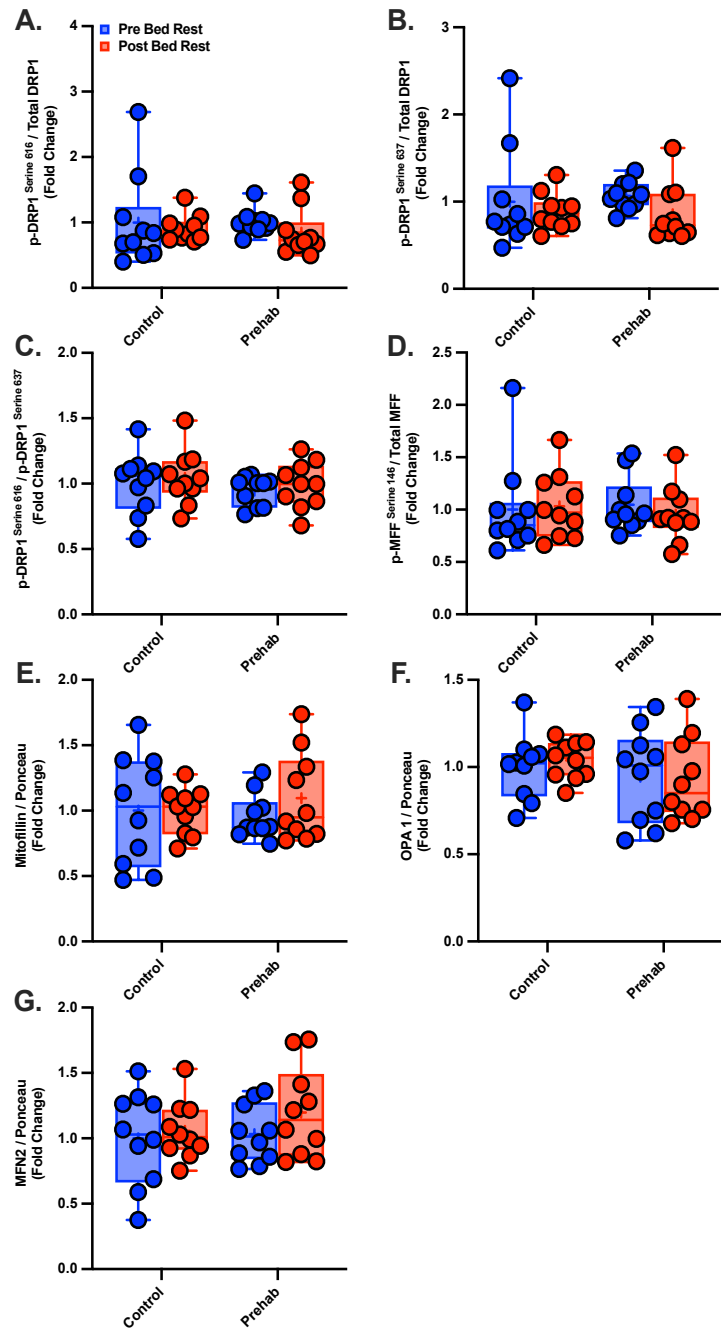


Figure 3. Markers of mitochondrial fission and fusion were unchanged following short term bed rest. No changes in expression of p-DRP1^{S616}: total DRP1 (A), p-DRP1^{S637}: total DRP-1 (B), p-DRP1^{S616}: p-DRP1^{S637} (C), p-MFF^{S146}: total MFF (D), Mitofillin I, OPA1 (F) or MFN2 (G) expression were observed pre-bed rest (BLUE) and post-bed rest (RED) with (Prehab) and without (Control) resistance exercise prehabilitation. Data presented as fold-change from pre-BR CTRL with individual data points (min to max) (n=10 per group). Statistical significance was set at P<0.05.

2.4.3. Mitochondrial Biogenesis and Mitophagy

The expression of proteins involved in mitochondrial biogenesis or mitophagy (**Figure 4**) remained unchanged following prehabilitation and BR; **(A)** p-AMPK^{T172}:total AMPK α 1 (Group, $P=0.25$, Time, $P=0.13$, Interaction, $P=0.28$), **(B)** p-ACC^{S79}:total ACC (Group, $P=0.94$, Time, $P=0.60$, Interaction, $P=0.52$), **(C)** PGC1 α (Group, $P=0.47$, Time, $P=0.92$, Interaction, $P=0.24$), **(D)** TFAM (Group, $P=0.052$, Time, $P=0.16$, Interaction, $P=0.28$), **(E)** ULK1 (Group, $P=0.98$, Time, $P=0.73$, Interaction, $P=0.62$), **(F)** BNIP3L/Nix (Group, $P=0.26$, Time, $P=0.58$, Interaction, $P=0.45$), and **(G)** LC3B I/II (Group, $P=0.844$, Time, $P=0.61$, Interaction, $P=0.76$).

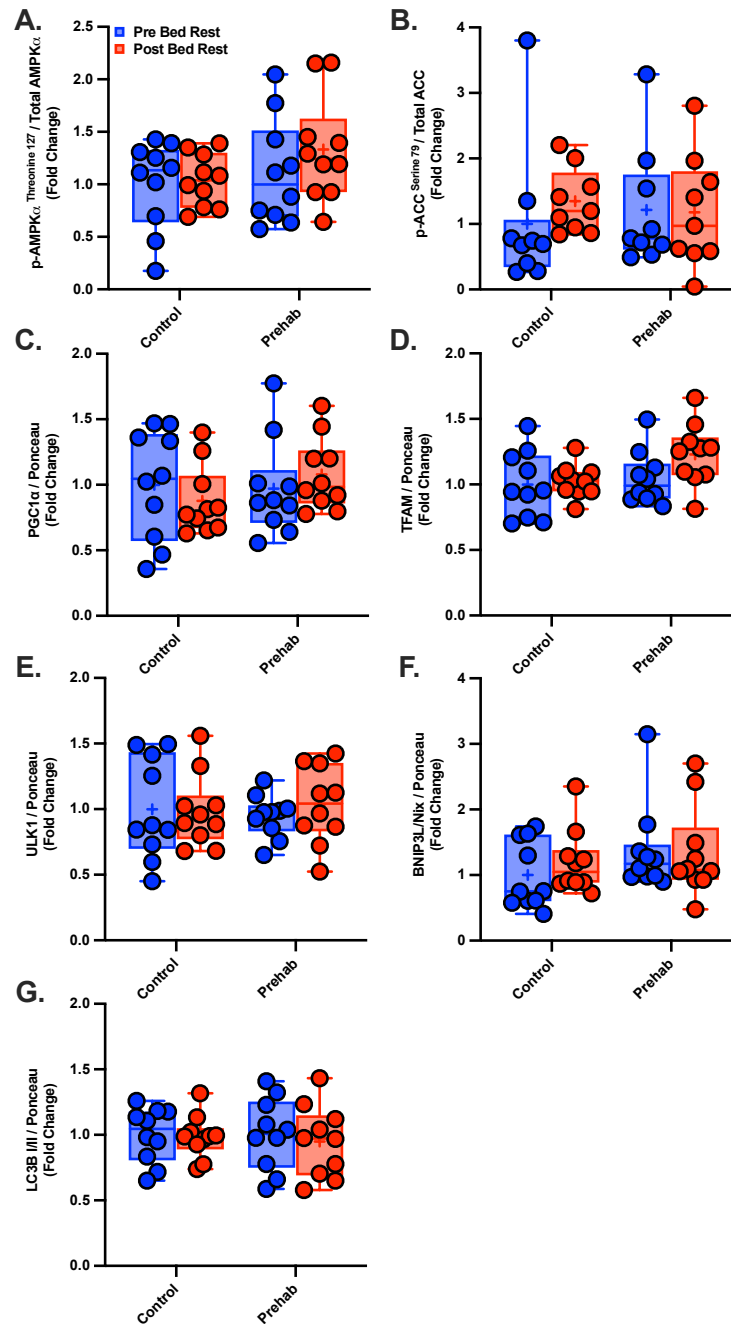


Figure 4. Short term bed rest did not alter the protein content of regulators of mitochondrial biogenesis or mitophagy. No changes in skeletal muscle protein expression of p-AMPK^{T172}: total AMPK α 1 (A), p-ACC^{S79}: total ACC (B), PGC1 α (C), or TFAM (D), ULK1 I, BNIP3L/Nix (F), or LC3BI/II (G) were observed pre-bed rest (BLUE) and post-bed rest (RED) with (Prehab) and without (Control) resistance exercise prehabilitation. Data presented as fold-change from pre-BR CTRL with individual data points (min to max) (n=10 per group). Statistical significance was set at P<0.05.

2.5. DISCUSSION

Periods of musculoskeletal disuse as a consequence of elective surgery have consistently reported a dysregulation in several indices of skeletal muscle mitochondrial metabolism (i.e., respiratory capacity, enzyme activity, protein content, biosynthesis, and quality control)^{381,384,390,401,481,566,568,570,588}. However, in contrast to previous longer duration models of disuse (i.e., 7-14 days), the present 5-day BR model resulted in no detectable alterations in mitochondrial enzyme activity and protein expression related to regulators of mitochondrial content, dynamics, biogenesis, and mitophagy in older adults. Furthermore, a 7-day period of high-volume ST-REP immediately prior to BR did not modulate mitochondrial protein abundance or phosphorylation status in older adults. Overall, the BR-induced decline in MRI-assessed quadriceps cross-sectional area (CTRL; -3.5% & EX; -3.0%) and integrated myofibrillar protein synthesis (CTRL: -21.3% & EX, -26.1%) we have previously reported in this cohort³⁸⁹ occurred independently of changes in static markers of mitochondrial protein content, dynamics, or biosynthesis.

2.5.1. Mitochondrial Content and Function

Previous studies suggest that proxy markers of mitochondrial density (i.e., CS activity) and ETC protein content are highly sensitive to protracted periods of disuse^{390,515,567,568,570}. However, we were unable to identify any changes in CS activity or protein content (**Figure 1**) and individual ETC complex proteins following a 5-day period of BR in older adults (**Figure 2**). Compared with previous studies, differences in participant age (young³⁸⁵, middle-aged⁵⁸⁹, older adults⁵¹⁵), duration of BR (5-day³⁹⁰, 7-day^{401,566}, 10-day^{393,568}, 14-day^{384,402,481}), disuse model (BR³⁹⁰, unilateral limb immobilization [ULI]⁴⁰¹, step-reduction [SR]⁴⁸¹) may explain the absence of any

apparent changes in mitochondrial-protein content and density in the present study. In older adults specifically, the regulation of mitochondrial function, CS activity, and ETC complex I-V abundance following BR may vary due to the conflicting disparity in the duration of BR models, including, 4³⁸¹, 7,⁵⁹⁰ 10^{515,568}, and 14-days³⁸⁴. It has been reported that as little as 4-days of strict BR in older men resulted in an ~18% decline in CS activity³⁸¹. In contrast, we were unable to detect any changes in CS activity following 5-days of BR in our cohort of older men (**Figure 1**). In support of our findings, and contrary to data in younger adults, 7-days of BR in older adults does not appear to affect mitochondrial function, ETC protein abundance⁵⁹⁰, and 10-days of BR does not alter mitochondrial capacity⁵¹⁵, overall suggesting older adults may be less susceptible to declines in mitochondrial metabolism. Interestingly, Standley et al. reported an increase in complex I expression and a *trend* for other ETC protein complexes to increase post-BR in older adults⁵¹⁵. An identical 10-day BR design in older adults from the same group showed a marked reduction in ETC protein content and respiratory capacity, which may be due to the intrinsic functional capacity and health status of older participants in this study⁵⁶⁸. Lastly, longer-term 14-day models of BR in older adults have observed no significant changes in complexes I and II, but large declines in complexes III, IV and V³⁸⁴. Collectively, the available data suggests that relatively short periods of bed rest (e.g., ≤ 7 days) do not alter markers of mitochondrial-protein content in otherwise healthy older adults. In contrast, alterations to mitochondrial-protein content with shorter-term periods of disuse may be more apparent in younger adults, or with more severe models of unloading. This is particularly pertinent as other have speculated the slower rate of change in skeletal muscle mass in older adults during disuse and exercise is largely due to slower

synthesis rates and subsequent delayed or inhibited adaptative response compared to younger adults ⁹.

2.5.2. Mitochondrial Quality Control

Fission and fusion of mitochondria are required to prevent the accumulation of damaged/dysfunctional mitochondria and maintain a healthy mitochondrial protein pool ⁵⁷⁵. Limited data in a ULI model suggests that a 14-day period of disuse in younger adults' results in significant increases in protein expression of DRP1 but no changes in MFN2 ⁵⁷⁰. On the contrary, 10-days of BR in older adults did not significantly alter any key regulatory fission-fusion proteins ⁵¹⁵. The data presented here extend on these previous investigations by demonstrating no alteration in DRP1, MFN2 or OPA1 protein expression following 10-days of BR in older adults ⁵¹⁵. However, our shorter-duration BR model and addition of MFF and the activation/inhibitory sites of DRP1 allowed us to further probe DRP1-mediated fission signalling (**Figure 3**). The phosphorylation of DRP1 at serine 616 has been found to activate mitochondrial fission via the accumulation and oligomerisation of DRP1 around mitochondrial constriction sites, facilitating scission and fragmentation ⁵⁹¹. Importantly, phosphorylation of MFF at serine 146 facilitates recruitment of DRP1 to the OMM ^{357,358,592}. On the contrary, the reversible phosphorylation at serine 637 orchestrates inhibition of DRP1 binding, leading to mitochondrial fusion and elongation ⁵⁹³; however, the precise regulation of these phosphorylation sites remains largely unclear in human skeletal muscle with ageing and disuse. Interestingly, we observed no changes in either p-DRP1^{S616} and p-DRP1^{S637}, p-MFF^{S146}, suggesting that 5-days of BR does not alter the phosphorylation status of DRP1 mediated fission signalling in older adults (**Figure 3**). However, it is important to note that DRP1 may bind to other

associated receptors on the OMM (i.e., MIEF2/MiD49 and MIEF1/MiD51) to initiate fission^{594,595} and warrants further exploration. Taken together, these data demonstrate the absence of any change in protein regulators of mitochondrial dynamics following short-term BR.

2.5.3. Mitochondrial Biogenesis and Mitophagy

The biosynthesis of nuclear and mitochondrial-encoded proteins is integral to the maintenance of the mitochondrial protein pool and overall cellular health⁵⁹⁶. Notably, the transcriptional co-activator PGC1 α , has previously been shown to be downregulated following a period of disuse alongside a reduction in mitochondrial content and/or function^{384,597}. Furthermore, transgenically overexpressing PGC1 α *in vivo* prevents disuse atrophy⁵⁹⁸. In line with the mitochondrial ETC content data, we observed no changes in markers associated with mitochondrial biogenesis (PGC1 α , TFAM) or central energy-sensing regulatory proteins (p-AMPK^{T172}, p-ACC^{S79}) (**Figure 4**). This is in disagreement with several studies displaying large and significant decreases in mRNA or protein expression of PGC1 α in young and older adults following BR or ULI^{384,401,568}. The discrepancy may, in part, relate to the duration of BR in the current study (i.e., 5-days) being too short to induce the dysregulation in mitochondrial biosynthesis in older adults.

The targeted and selective removal of damaged proteins and organelles is orchestrated by a tightly regulated process of autophagy/mitophagy⁵⁹⁹. Recently, ULK1 has been shown to be a critical regulator of mitophagy in skeletal muscle following its activation via p-AMPK^{T172}^{102,374,600}. However, at a transcriptional level (i.e., mRNA), ULK1 is not altered following a 7-day period of BR in younger adults⁶⁰¹. Similarly, we report no significant changes in ULK-1 protein expression (**Figure 4**).

This observation may be partially explained by an increase in mTOR^{S2448}, reported in our previous work from the same cohort³⁸⁹, which may act to attenuate ULK-mediated mitophagy signalling⁶⁰⁰. Indeed, ULK1 is inactive under nutrient sufficiency (i.e., feeding) when mTOR is active via mTOR-mediated phosphorylation of ULK1^{S757} to prevent its interaction with AMPK to initiate autophagy/mitophagy⁶⁰⁰. However, ULK1 becomes active under nutrient and energetic stress (i.e., fasting and exercise) to phosphorylate AMPK^{T172}, and subsequently phosphorylates ULK1 at serine 555 to mediate mitophagy via LC3B-I/II^{374,600,602}. Mitophagy may also be initiated by several receptor-mediated signalling proteins (i.e., FUNDC1, BNIP3 & BNIP3L/Nix), which regulate the binding of damaged proteins and organelles to LC3B-I/II and engulfment by the autophagosome for lysosomal degradation⁶⁰³. In the present study, and in agreement with a previous BR study in older adults⁵¹⁵, we did not observe any increases in mitophagy receptors, BNIP3L/Nix or LC3B-I/II signalling following BR (**Figure 4**). Overall, a 5-day period of BR is not long enough to alter AMPK-ULK1-mediated mitophagy signalling in older adults.

2.5.4. Resistance Exercise Prehabilitation

It is well established that RE training augments rapid increases in skeletal muscle mass and strength⁶⁰⁴. However, less is known regarding RE-induced adaptation of mitochondrial metabolism, particularly in older adults⁵⁶⁰. Nonetheless, acute, and chronic RE have been reported to increase mitochondrial protein synthesis^{309,578,605}, respiratory capacity^{578,579}, enzyme activity, and quality control mechanisms^{580,606}. In the present study, however, four high-load, high-volume RE training sessions performed over 7-days did not induce a change in CS enzyme activity or protein abundance (**Figure 1**), ETC complex I-V abundance (**Figure 2**), or mitochondrial

proteins related to fission-fusion (p-DRP1^{S616}, p-DRP1^{S637}, OPA1, MFN2 or Mitofillin) (**Figure 3**), biosynthesis (p-AMPK^{T172}, p-ACC^{S79}, PGC1a, TFAM) (**Figure 4**), or mitophagy (ULK1, LCBI/II, BNIP3L/Nix) (**Figure 4**). Overall, suggesting the duration, intensity, and/or volume of RE was not a sufficient stimulus to augment mitochondrial remodelling and morphology.

In addition to the effects of ST-REP as a means to augment muscle mitochondrial parameters, we were also interested to understand whether this strategy could offset disuse-induced loss of mitochondria. However, given the absence of any detectable change in CS enzyme activity, ETC protein abundance, fission-fusion dynamics, and biosynthesis following 5-days of BR, we were unable to resolve this. Furthermore, declines in function are largely dependent on the age of cohorts, in which younger adults display significantly greater alterations in mitochondrial content and function ^{390,401,570} compared to older adults ^{515,568,590} following a period of disuse >5-days. Crucially, RE-induced changes in the mitochondrial protein pool and phosphorylation status may be load and repetition-dependent and favour a higher-volume, low-load RE beyond the current ST-REP intervention ^{313,318,607,608}. Low-load RE (~30% 1-RM) alone and in combination with increased time-under-tension increases acute mitochondrial protein synthesis rates for up to ~24 hr post-RE ^{311,313}. Furthermore, as RE can increase in both myofibrillar and mitochondrial protein turnover following lower loads ^{311,313,317,609}, and subsequent long-term changes in lean mass are independent of exercise load ^{610,611}, lower-loads may, potentially, be more beneficial to simultaneously facilitate both accrual of muscle mass and strength, while simultaneously improving oxidative capacity and should form the basis of future investigations.

2.6. CONCLUSION

In summary, neither 5-days of BR or ST-REP induces changes in mitochondrial density and intracellular protein expression related to mitochondrial health and function in healthy older adults. These data further our insights into the time-course of changes in the protein content of regulators of mitochondrial dynamics and morphology during a clinically relevant period of BR in older adults. Overall, it appears that a short-duration BR is not sufficiently long enough to alter the mitochondrial content in healthy older adults, although the same may not be true for older adults with poorer physiological health. Furthermore, whilst ST-REP did not induce any change in regulators of mitochondrial dynamics, it remains possible that such interventions could mitigate the decline in mitochondrial function in longer duration (i.e., >5-days) or more severe (e.g., ULI) disuse events or in older adults with poorer physiological health, where mitochondrial metabolism may be compromised.

SUPPLEMENTARY FIGURES

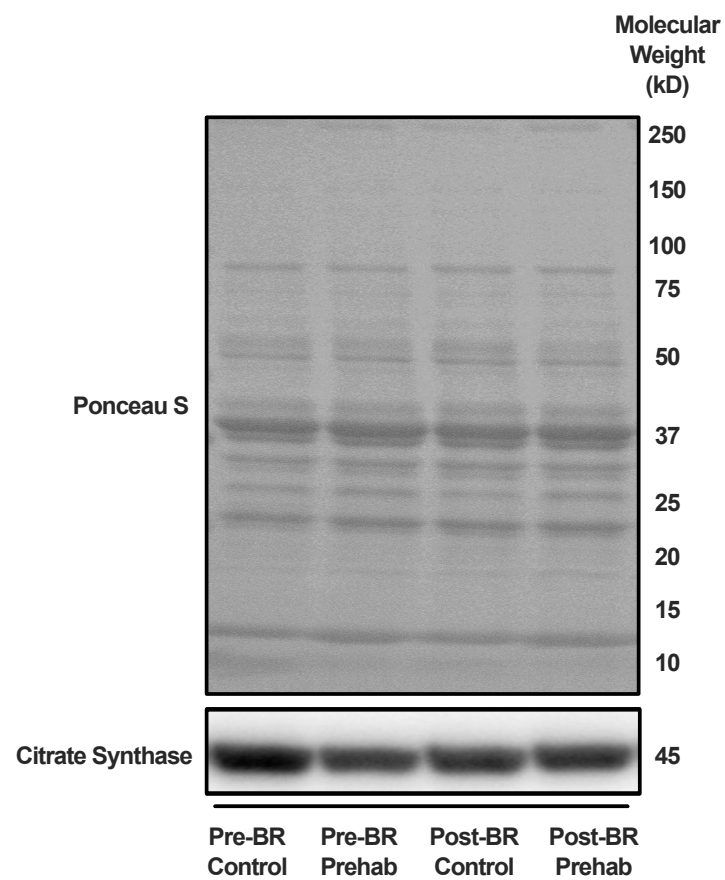


Figure 5. Representative immunoblot image. Citrate Synthase and Ponceau S.

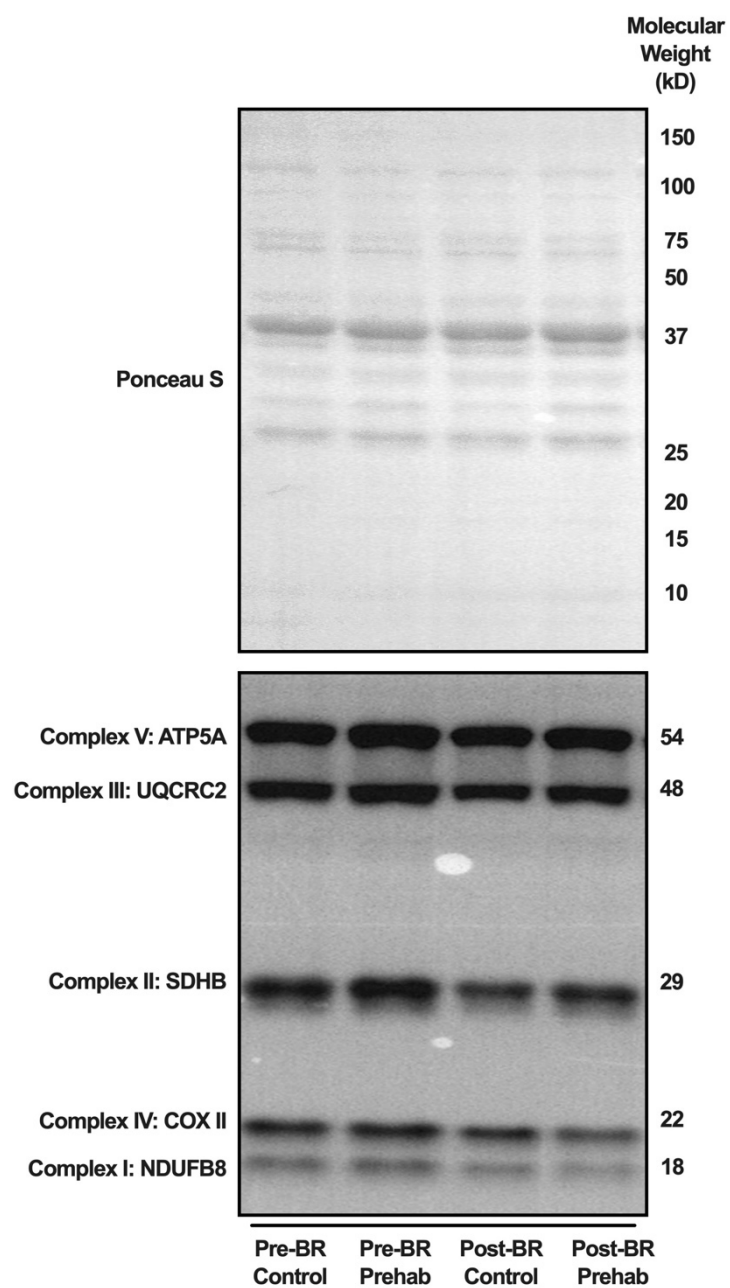


Figure 6. Representative immunoblot image. Human OXPHOS cocktail of electron transport chain proteins I-V and Ponceau S.

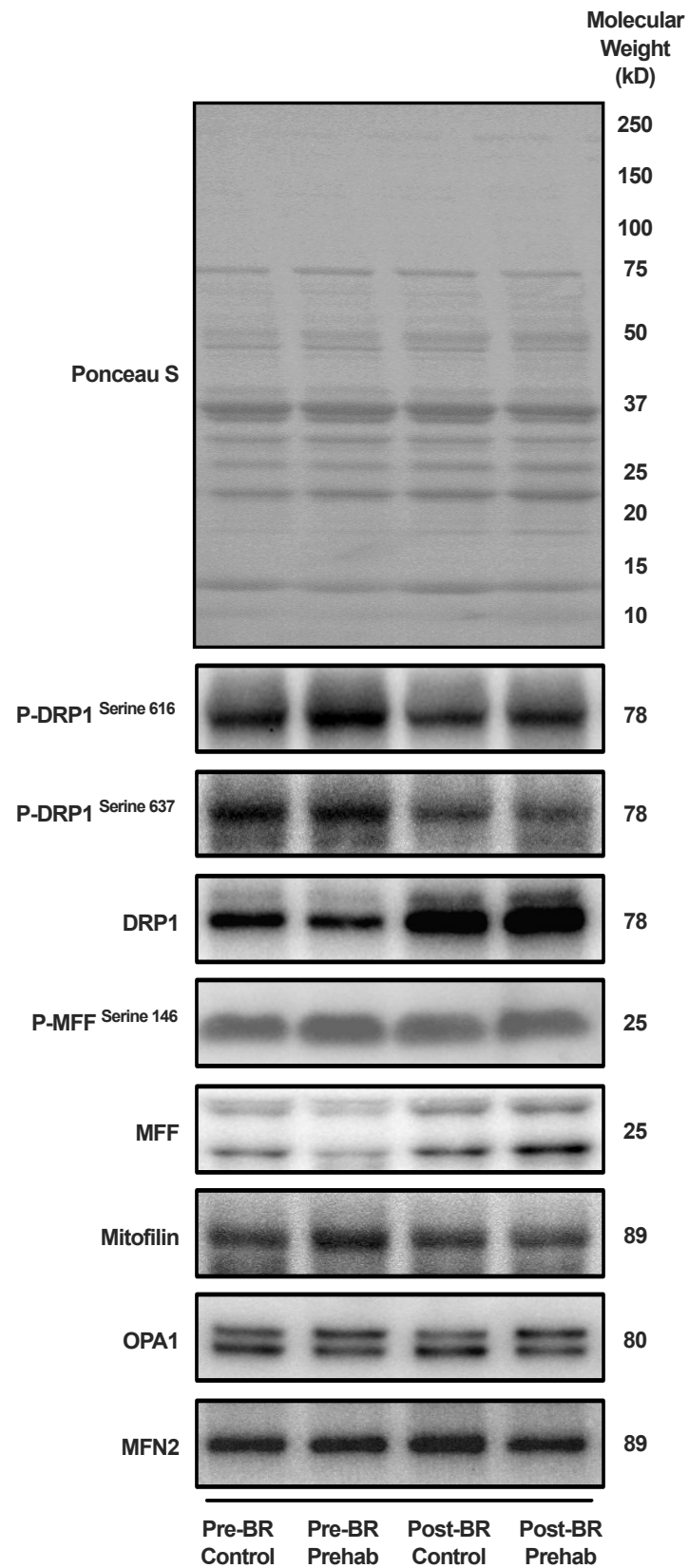


Figure 7. Representative immunoblot images. P-DRP1^{Ser616}, P-DRP1^{Ser637}, DRP1, P-MFF^{Ser146}, MFF, Mitofilin, OPA1, MFN-2 and Ponceau S.

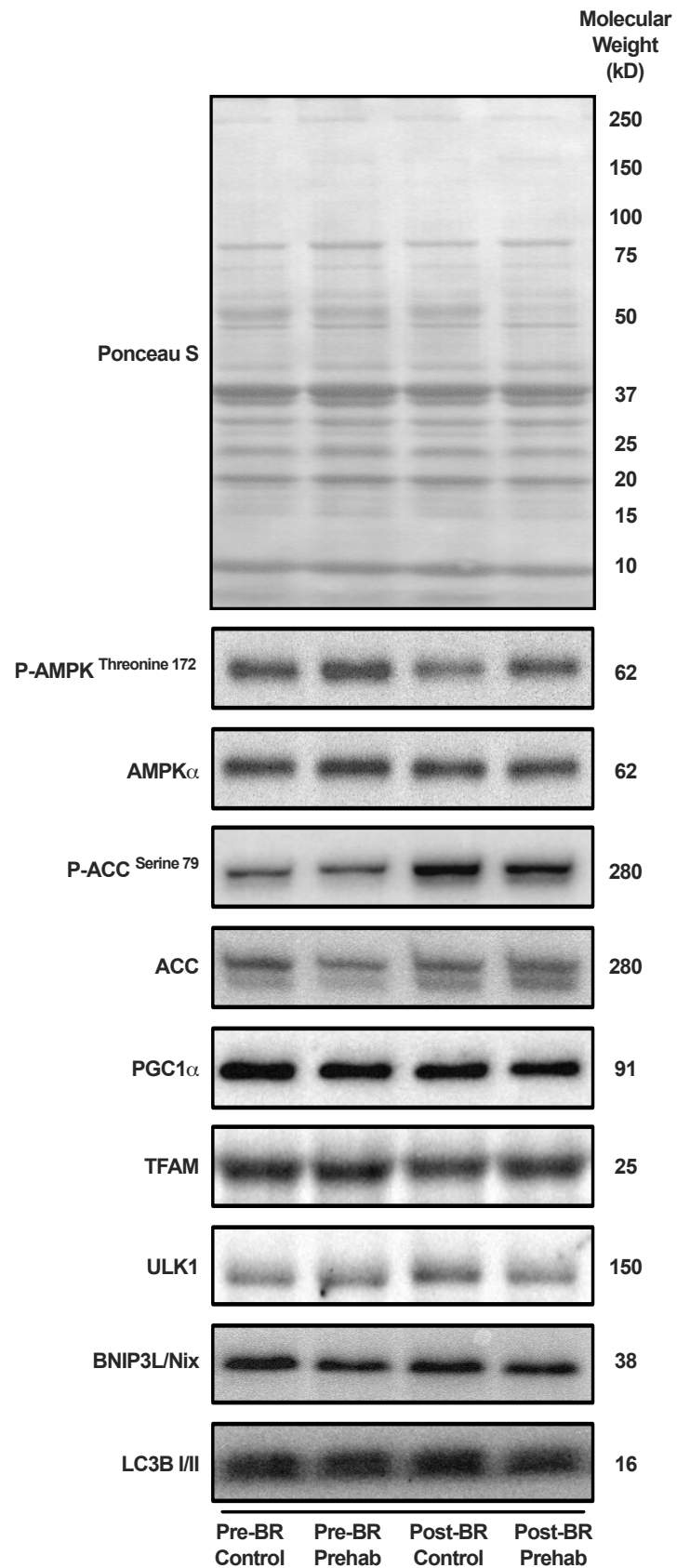


Figure 8. Representative immunoblot images. P-AMPK^{Thr172}, AMPK, P-ACC^{Ser79}, ACC, PGC1 α , TFAM, ULK1, BNIP3/Nix, LC3B I/II, and Ponceau S.

CHAPTER 3

Acute Resistance Exercise Training Does Not Augment Mitochondrial Remodelling in Master Athletes or Untrained Older Adults



Published Peer-Reviewed Manuscripts from This Chapter:

Marshall RN, McKendry J, Smeuninx B, Seabright AP, Morgan PT, Greig C and Breen L (2023) Acute resistance exercise training does not augment mitochondrial remodelling in master athletes or untrained older adults. *Front. Physiol.* 13:1097988.

3.1. ABSTRACT

Background: Ageing is associated with alterations to skeletal muscle oxidative metabolism that may be influenced by physical activity status, although the mechanisms underlying these changes have not been unravelled. Similarly, the effect of resistance exercise (RE) on skeletal muscle mitochondrial regulation is unclear.

Methods: Endurance-trained masters athletes ([MA], 74 ± 3 yrs) and untrained older adults ([OC], 69 ± 6 yrs) completed a single session of knee extension RE (6 x 12 repetitions, 75% 1-RM, 120-second intra-set recovery). Vastus lateralis muscle biopsies were collected pre-RE, 1hr post-RE, and 48hr post-RE. Skeletal muscle biopsies were analyzed for citrate synthase (CS) enzyme activity, mitochondrial content, and markers of mitochondrial quality control via immunoblotting. **Results:** Pre-RE CS activity and protein content were ~45% ($P < 0.001$) and ~74% greater in MA compared with OC ($P = 0.006$). There was a significant reduction (~18%) in CS activity 48hr post-RE ($P < 0.05$) in OC, but not MA. Pre-RE levels of individual and combined mitochondrial electron transport chain (ETC) complexes I-V were significantly greater in MA compared with OC, as were markers of mitochondrial fission and fusion dynamics (p-DRP-1^{Ser616}, p-MFF^{Ser146}, OPA-1 & FIS-1, $P < 0.05$ for all). Moreover, MA displayed greater expression of p-AMPK^{Thr172}, PGC1 α , TFAM and SIRT-3 ($P < 0.05$ for all). Notably, RE did not alter the expression of any marker of mitochondrial content, biogenesis, or quality control in both OC and MA. **Conclusion:** The present data suggest that long-term aerobic exercise training supports superior skeletal muscle mitochondrial density and protein content into later life, which may be regulated by greater mitochondrial quality control mechanisms and supported via superior fission-fusion dynamics. However, a single session of RE is unable to induce mitochondrial remodelling in the acute (1hr post-RE) and delayed (48hr post-RE) recovery period in OC and MA.

3.2. INTRODUCTION

The number of older adults (>65 years of age) is growing, and predictions indicate that the worldwide population of this sub-group of adults will reach >2 billion by 2050 ⁶¹². This demographic shift presents a substantial burden for healthcare providers and national healthcare expenditure ⁶¹³. Ageing is associated with a gradual decline in skeletal muscle mass and function, termed 'sarcopenia' ⁶¹⁴. The loss of skeletal muscle mass proceeds at a rate of 1-2% per year, whereas muscle strength is lost more rapidly (1-5% per year) ⁶¹⁵. In parallel with the declines in skeletal muscle mass and function, there is also an age-related deterioration in mitochondrial function at a rate of ~8% per decade ³¹. Furthermore, poor mitochondrial energetics has been associated with greater functional impairment in day-to-day tasks, resulting in more severe declines in mobility across the lifespan ⁸⁴. However, participating in regular physical activity and exercise throughout adult life may preserve or at least attenuate the rate of decline in muscle mass, function, and cardiorespiratory fitness ⁶¹⁶, with implications for extending healthspan and minimizing years spent in poor health ¹⁵.

Older adults who have maintained structured endurance exercise training over an extended period of their life (termed 'Master Athletes' [MA], lifelong exercisers and/or high-functioning older adults) display consistently greater indices of whole-body and tissue-specific health and exhibit similar metabolic and phenotypic profiles to healthy younger adults (YA) ^{229,231,616}. As higher mitochondrial content and oxidative capacity have been implicated in a lower disease prevalence ³⁷⁰, undertaking endurance exercise training across the lifespan may offset age-related mitochondrial dysfunction ⁶¹⁷. Indeed, compared with healthy age-matched adults, MA display superior mitochondrial function, content, biogenesis, and quality control ^{231,617-620}.

Therefore, the maintenance of physical activity, and structured exercise training across the lifespan may contribute to the attenuation of mitochondrial function and muscle health decline with ageing.

The single most potent strategy to maintain skeletal muscle mass, strength, and function in older age is resistance exercise (RE) ^{604,621}. However, to date, research investigating RE-induced molecular signalling and adaptative remodelling in YA and older adults (OA) have primarily centred around the muscle anabolic role of mTORC1 (mechanistic target of rapamycin complex 1), the acute elevations in muscle protein synthesis, and the long-term accrual of lean muscle mass ⁶²². Recent advances have begun to evaluate the mechanistic underpinnings of how RE augments mitochondrial remodelling utilizing acute and chronic exercise training models ^{580,623}. Mitochondria undergo dynamic remodelling via biosynthesis, fission, fusion, and degradation of dysfunctional organelles ⁶²⁴. Given that RE may be beneficial to facilitate the simultaneous accrual of myofibrillar and mitochondrial proteins, with concurrent improvements in mitochondrial function ^{560,579,608,623}, understanding how acute RE modulates the mitochondrial regulatory signalling pathways is imperative for tailored exercise prescription to improve the musculoskeletal and general health of older adults. However, our understating of how RE augments mitochondrial remodelling lifelong exercisers remain unanswered.

Therefore, the first aim of the present study was to comprehensively assess molecular signalling regulating skeletal muscle mitochondrial and substrate metabolism (i.e., content, fission-fusion, and biogenesis) in endurance-trained MA compared with untrained healthy age-matched older adults (OC). Secondly, we aimed to determine the effect of a single bout of RE on muscle mitochondrial-mediated

signalling in the early (1hr) and late-phase (48hr) of recovery in MA and OC. We hypothesized that endurance-trained MA would display a greater abundance and activity of mitochondrial protein expression. Secondly, we hypothesized RE would increase post-RE mitochondrial remodelling via protein phosphorylation in both cohorts of participants, with a blunted response in MA.

3.3. METHODOLOGY

3.3.1. Ethical Approval

The present study was a retrospective analysis of skeletal muscle biopsy samples obtained as part of a larger trial on the effects of RE on integrated myofibrillar protein synthesis between endurance-trained MA and untrained older individuals ¹¹³. Briefly, prior to participation in the study, written informed consent was obtained from all subjects. All procedures were approved by the National Health Service East Midlands Research Ethics Committee (18/EM/0004) and the University of Birmingham Governance and Research Ethics Committee (RG_17-187).

3.3.2. Participants

Seven untrained older males (OC; 74) $\pm$ 3 years, BMI; 24.8 $\pm$ 3.3 kg/m²) and seven endurance-trained master athletes (MA; 69 $\pm$ 6 years, BMI; 22.0 $\pm$ 1.6kg/m²) were recruited as previously described ¹¹³. Briefly, OC were deemed eligible for participation if they were habitually physically active (i.e., >7,000 steps/day⁻¹) and had not previously participated in or followed any structured exercise training. MA were eligible if they had maintained continuous endurance exercise training at least twice per week for >20 years preceding the study. The endurance training history of MA has been reported

previously (exercise experience; 48 ± 15 years, sessions per week; 4.3 ± 1.6 , training volume; 8.4 ± 6.6 hours) ¹¹³.

Table 1. Participants anthropometric, body composition and exercise training characteristics.

Anthropometrics			
	Old Control (N=7)	Master Athlete (N=7)	P Value
Age (years)	73.5 ± 3.3	68.9 ± 5.7	0.071
Height (cm)	173 ± 0.6	176 ± 0.8	0.362
Body Mass (Kg)	73.8 ± 8.5	70.0 ± 4.5	0.131
BMI ($\text{Kg}\cdot\text{m}^{-2}$)	24.8 ± 3.3	22.0 ± 1.6	0.059
Body Composition			
Whole Body FFM (Kg)	52.9 ± 3.6	52.0 ± 3.8	0.628
Whole Body FM (Kg)	20.9 ± 6.9	16.0 ± 1.5	0.092
Body Fat (%)	27.8 ± 6.7	23.6 ± 1.9	0.134
Training History			
Training Experience (Years)	-	47.7 ± 14.8	-
Training Frequency (Sessions $\cdot\text{week}^{-1}$)	-	4.3 ± 1.6	-
Training Duration (Hours $\cdot\text{week}^{-1}$)	-	8.4 ± 6.6	-
Training Distance (Km $\cdot\text{week}^{-1}$)	-	180 ± 29 55 ± 15	-

BMI, Body Mass Index; FFM, Fat Free Mass; FM, Fat Mass; FFM = fat-free mass; FM = fat mass. Data presented as mean $\pm$ SD. Adapted from McKendry et al., 2018.

3.3.3. Study Design

In a parallel study design, as previously described ¹¹³, OC and MA were recruited to determine the acute (1hr) and delayed (48hr) post-exercise molecular signalling regulating skeletal muscle mitochondrial quality control. Participants reported to The School of Sport, Exercise and Rehabilitation Sciences (University of Birmingham) in an overnight fasted state. A single skeletal muscle biopsy was collected upon arrival, followed by the completion of a single bout of knee extension RE (6 x 12 repetitions, 75% 1-RM, 120-second intra-set recovery). Muscle biopsy samples were obtained from the quadriceps *vastus lateralis* under local anaesthesia (1% lidocaine) using the Bergström needle technique. Muscle biopsy tissue was quickly rinsed in ice-cold saline and blotted to remove any visible fat and connective tissue before being frozen in liquid nitrogen. Following cessation of RE, participants rested for 1hr before another muscle biopsy was sampled from the same leg. Finally, 48hr post-exercise, participants returned to the laboratory following an overnight fast for further muscle biopsy sampling ~3cm proximal to the previous sampling site. RE was selected as the exercise mode as both OC and MA would be largely unaccustomed to the nature of the contractile stimulus

3.3.4. Immunoblotting

Immunoblotting was performed on the sarcoplasmic fraction of skeletal muscle biopsy samples that remained following myofibrillar protein extraction, as previously reported ¹¹³. Briefly, 30-35mg of snap-frozen vastus lateralis skeletal muscle was mechanically homogenized using a TissueLyser II and 5 mm stainless steel beads (Qiagen, Hannover, Germany). Samples were disrupted following 3 x 2-minute cycles at 20 Hz

in ice-cold homogenization buffer (25 mM Tris buffer [Tris-HCl, Trizma Base, 25 ml of Milli-Q H₂O, pH 7.2], 100 µl TritonX-100, one PhosSTOP Tablet (Roche, Switzerland), one complete protease inhibitor tab) (Roche, West Sussex, UK) at 10µl/µg per tissue and shaken on a vibrax shaker at 4°C for 10 mins. Homogenates were spun at 4,500g for 10 min at 4°C, and the sarcoplasmic supernatant was collected and frozen at -80°C for subsequent western blot analysis. Protein content was determined by DC Protein Assay (Bio-Rad, Hertfordshire, UK). Western blot aliquots were prepared at 3µg/1µL in 4 x Laemmli sample buffer and ddH₂O. Following preparation, samples were left at room temperature for 24hr to denature. Equal amounts of protein (30µg) were loaded into 4-15% Tris-Glycine precast gels (BioRad, Hertfordshire, UK) and separated by SDS-PAGE for 1hr at 140V in pre-made tris-glycine running buffer (24mM Tris, 192mM Glycine, pH 8.3). Proteins were transferred to a polyvinylidene difluoride (PVDF; Whatman, Dassel, Germany) membrane at 100V for 1hr in a pre-made transfer buffer. Membranes were blocked in 5% low-fat milk (diluted in Tris-buffered saline and 0.1% Tween-20 (TBS-T)) for 1h at room temperature. The membranes were incubated overnight (4°C) with appropriate antibodies (see 'Antibodies'). Following overnight incubation, membranes were washed 3×5 min in TBS-T, incubated in horseradish peroxidase (Mouse/Rabbit-HRP)-conjugated secondary antibody (Cell Signaling, 7076/7074, 1:10,000 in 2.5% BSA in TBS-T) at room temperature for 1 h, before the last 3 × 5 min washes in TBS-T. Membranes were exposed to Chemiluminescent HRP Substrate (Millipore Corp., Billerica, MA, USA) for 2-minutes and bands were visualized by a Chemi XT4 imager with GeneSys capture software (Syngene UK, Cambridge, UK). Quantification was performed using ImageJ/Fiji (NIH, Bethesda, MD, USA). Relative arbitrary units were normalized to the total amount of protein loaded as

visualized via Ponceau S staining ⁵⁸⁶. Specifically, bands were normalized to the ~45kDa actin band of the Ponceau S stain as a loading control ⁶²⁵. No difference was detected between the ~45kDa actin band of the Ponceau S stain across all samples. Following these corrections, where appropriate, the phosphorylation of proteins as a proxy of their activation is expressed relative to each protein's total abundance ⁵⁸⁷.

3.3.5. Antibodies

All primary antibodies were prepared in TBST. Antibodies were as follows; total OXPHOS human antibody cocktail (Manufacturer; Abcam, Catalogue Number; 110411, diluted 1:1000 in tris-buffered saline with 0.1% Tween 20 (TBST), Citrate Synthase (CS; Cell Signaling Technology [CST], 14309, 1:1000), Mitofusin-2 (MFN2; CST, 9482, 1:1000), Dynamin-Related Protein-1 (DRP-1; CST, 8570, 1:1000), Phosphorylated DRP-1^{Ser616} (p-DRP-1^{Ser616}, CST, 4494, 1:1,000), Optic Atrophy-1 (OPA-1; BD Bioscience, 612607, 1:1000), Mitochondrial Fission Factor (MFF; CST, 84580, 1:1000), Phosphorylated Mitochondrial Fission Factor (p-MFF^{Ser146}; CST, 49281, 1:1000), Mitochondrial Fission Protein-1 (FIS-1; Atlas Antibodies, HPA017430, 1:1000), Mitofilin (ProteinTech, 10179-AP, 1:1000), Total AMP-activated protein kinase, (AMPK; CST, 2532, 1:1000), Phosphorylated AMPK^{T172} (p-AMPK^{Thr172}, CST, 2535, 1:1000), Total Acetyl-CoA carboxylase, (ACC; Dundee MRC DSTT, N/A [custom made], 1:1000), Phosphorylated ACC^{Ser79} (p-ACC^{Ser79}, Dundee MRC DSTT, N/A [custom made], 1:1000), Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α ; Abcam, 3242, 1:1000), mitochondrial transcription factor A (TFAM; Sigma-Aldrich, SAB1401383, 1:1000), Sirtuin-3 (SIRT3; CST, D22A3, 1:1000), Poly-ADP Ribose Polymerase 1 (PARP1; CST, 9542, 1:1000), Nicotinamide

Riboside kinase 1/2 (NMRK1 and NMRK2; BioGenes, Berlin, Germany, N/A [custom made], 1:1000). Appropriate (Mouse/Rabbit) HRP conjugated secondary antibody (CST, 7076 & 7074, 1:10,000 in 2.5% BSA in TBS-T) following overnight incubations.

3.3.6. Enzyme Activity

Citrate Synthase (CS) enzyme activity was determined following the protocols of Spinazzi et al. ⁵⁸⁴ and adapted to 96-well plate format as previously described ⁵⁸⁵. In brief, 20-30 mg of skeletal muscle was homogenized in 10 μ L/ μ g of ice-cool sucrose lysis buffer (50 mM Tris, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5 mM Na₄P₂O₇·10H₂O, 270 mM sucrose, 1 M Triton-X, 25 mM β -glycerophosphate, 1 μ M Trichostatin A, 10 mM Nicotinamide, 1 mM 1,4-Dithiothreitol, 1% Phosphatase Inhibitor Cocktail 2; Sigma, 1% Sigma Phosphatase Inhibitor Cocktail 2; Sigma, 4.8% complete Mini Protease Inhibitor Cocktail; Roche) using a TissueLyser II, and 5 mm stainless steel beads (Qiagen, Hannover, Germany). Samples were disrupted following 3 x 2-minute cycles at 20 Hz, followed by centrifugation at 13,000 RPM for 10 minutes. The protein content of the resulting supernatant was determined via DC protein assay (Bio-Rad, Hercules, California, USA) and prepared at 2 μ g/ μ L in ddH₂O. For determination of CS activity, 10 μ L of sample (total of 20 μ g of protein) was diluted in 186 μ L of assay solution (50 mM Tris buffer (pH 7.4) with 100 μ M DTNB, 115 μ M acetyl-CoA in ddH₂O) to a total well volume of 196 μ L, as previously described ⁵⁸⁵. Baseline absorbance was read every 15 s for three minutes in a microplate reader (FLUOstar Omega, BMG Labtech, Aylesbury, UK). Following the baseline reading, 4 μ L of 5 mM oxaloacetate (100 μ M final concentration) was added to start the reaction and absorbance was read again every 15 s for three minutes. CS activity was calculated using the extinction coefficient

(CS ϵ = 13.6). Measurement CV% within-plate was $6.6 \pm 1.7\%$ for three technical repeats⁸⁹.

3.3.7. Statistical Analysis

Enzyme activity and immunoblotting data were compared using a Two-Way ANOVA with a between-group factor (Group [OC vs MA]) and within-group factor (Time [0hr vs 1hr vs 48hr post-RE]). Bonferroni post hoc analysis was applied to correct for multiple comparisons. Significance was set at $P < 0.05$. Data are presented as mean $\pm$ SEM unless otherwise stated, and displayed as fold-change relative to the pre-exercise OC. All analysis was conducted using GraphPad Software Inc Prism version 8.

3.4. RESULTS

3.4.1. Mitochondrial Content and Volume

CS enzyme activity was ~65% greater CS enzyme activity in MA (221.5 ± 56.8 nmol/min/mg) compared with OC (152.08 ± 32.6 nmol/min/mg) ($P < 0.001$). Post hoc analysis also revealed a significant decline (~18%) in CS enzyme activity 48hr post-exercise in OC compared with pre-RE (pre-RE: 152 nmol/min/mg vs 48hr post-RE: 124 nmol/min/mg) ($P = 0.025$) (**Figure 1A**). CS protein expression was ~74% greater in MA compared with OC ($P = 0.006$), with no effect of RE ($P = 0.362$) (**Figure 1B**). The protein abundance of electron transport chain (ETC) proteins (I-IV) was significantly greater in MA compared with OC (Complex I [NDUFB8], ~265%, $P = 0.021$, Complex II [SDHB], ~24%, $P = 0.040$, Complex III [UQCRC2], ~104%, $P = 0.010$, Complex IV [COXII], ~162%, $P = 0.005$, and Total ETC, ~80%, $P = 0.007$), with no differences in Complex V protein abundance (ATP5A, $P = 0.064$) between groups (**Figure 1C-H**). No effect of time effect was observed in the 1hr or 48hr post-RE period for ETC proteins between groups (Complex I, $P = 0.171$, Complex II, $P = 0.070$, Complex IV, $P = 0.082$, Complex V, $P = 0.413$, Total ETC, $P = 0.81$) (**Figure 1C-H**). However, there was a significant time effect of RE in Complex III expression ($P = 0.033$), with post hoc analysis revealing a small (+8%), but significant ($P = 0.046$) increase in the 48hr post-RE in MA only.

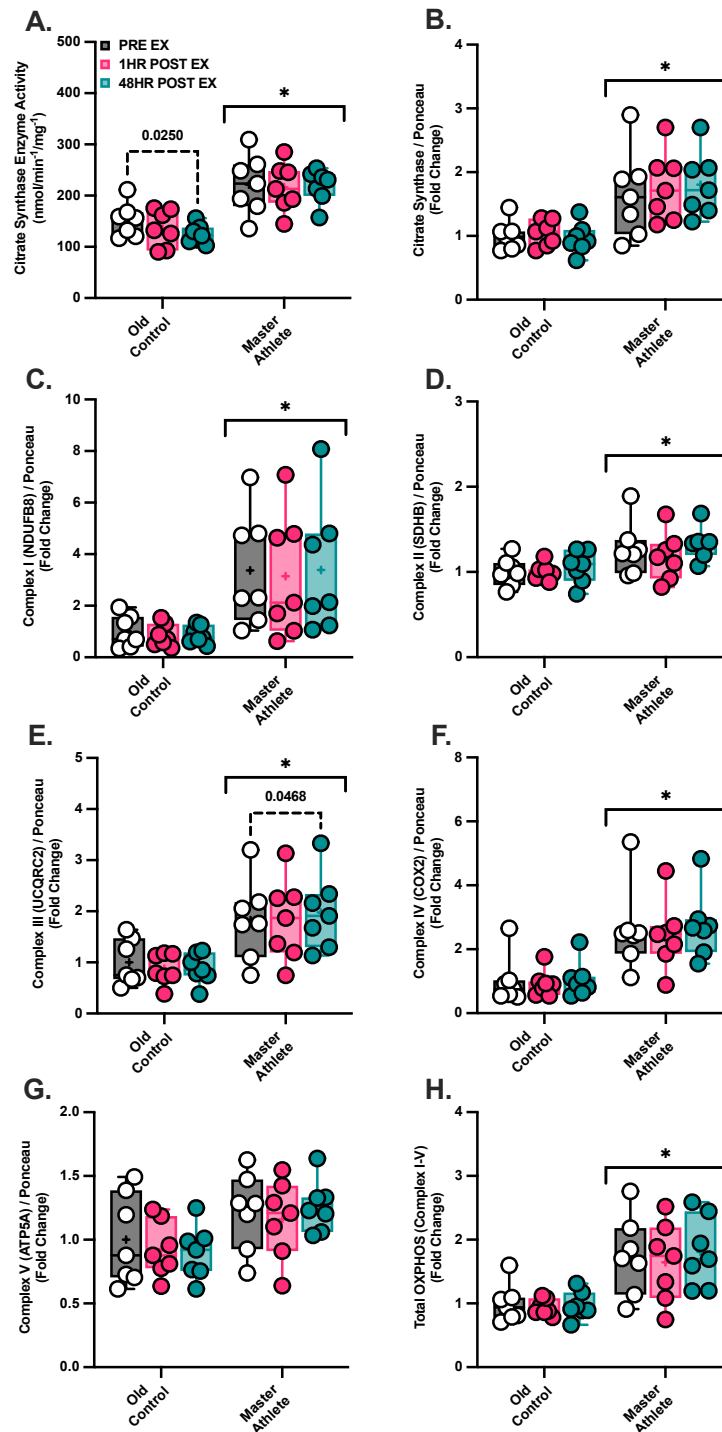


Figure 1. Master athletes display greater citrate synthase enzyme activity and mitochondrial content compared to old controls. Citrate synthase enzyme activity (A) citrate synthase protein expression (B) and protein expression of electron transport chain complexes I-V; NDUFB8 (C), SDHB (D), UQCRC I, COX II (F), ATP5A (G), Total OXPHOS (H) at rest (**BLACK**), 1 h post-resistance exercise (**PINK**), and 48 h post-resistance exercise (**GREEN**) in older untrained controls (n=7) and master athletes (n=7).

3.4.2. Mitochondrial Fission and Fusion Dynamics

MA displayed significantly greater abundance of; p-MFF^{Ser146} (~17%, P=0.042, **Figure 2D**), OPA-1 (~55%, P=0.019, **Figure 2G**), FIS-1 (~41%, P=0.031, **Figure 2H**) and Mitofilin (~270%, P=0.003, **Figure 2I**). However, we observed no significant differences between groups in the abundance of p-DRP-1^{Ser616} (P=0.437, **Figure 2A**), total DRP-1 (P=0.274, **Figure 2B**), phospho/total DRP-1 (P=0.744, **Figure 2C**), total MFF (P=0.435, **Figure 2E**), phospho/total MFF (P=0.950, **Figure 2F**), MFN-2 (P=0.350, **Figure 2J**). We observed an interaction effect on the expression of phospho/total DRP-1 (P=0.037, **Figure 2C**), with no effect of RE on any other markers of fission/fusion dynamics. Further posthoc analysis of phospho/total DRP-1 revealed a ~54% increase in the 1hr post-RE period in MA only (P=0.035, **Figure 2C**).

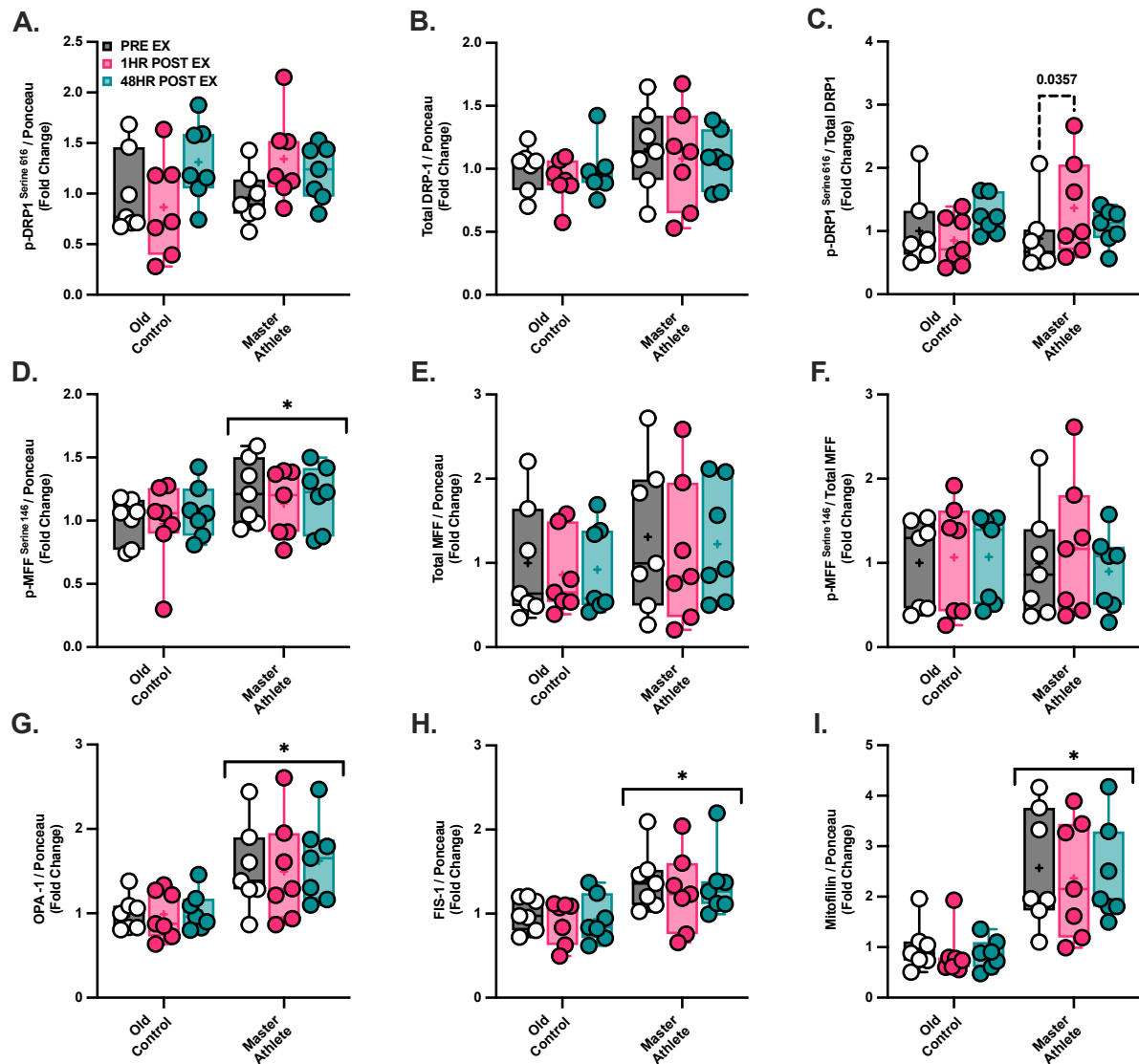


Figure 2. Master athletes display greater abundance of proteins involved in fission and fusion dynamics. P-DRP1^{Ser616} (A) total DRP1 (B) phosphorylated: total DRP1 (C), P-MFF^{Ser146} (D), total MFF I, Phosphorylated: total MFF (F), OPA-1 (G), FIS1 (H), Mitofilin (I), MFN-2 (J) at rest (**BLACK**), 1 h post-resistance exercise (**PINK**), and 48 h post-resistance exercise (**GREEN**) in older untrained controls (n=7) and master athletes (n=7).

3.4.3. Mitochondrial Energy Metabolism

We observed significant group differences between MA and OC, with MA displaying a greater abundance of; p-AMPK α ^{Thr172} (~37%, P=0.043, **Figure 3A**), phospho/total AMPK α (~28%, P=0.013, **Figure 3C**), PGC1 α (~42%, P=0.028, **Figure 3G**), TFAM (~43%, P=0.03, **Figure 3H**), and SIRT-3 (~43%, P=0.02, **Figure 3I**). We observed no differences in abundance of, total AMPK α (P=0.531, **Figure 3A**), p-ACC^{Ser79} (P=0.395, **Figure 3D**), total ACC (P=0.585, **Figure 3E**), phospho/total ACC (P=0.966, **Figure 3F**), PARP1 (P=0.936, **Figure 3J**), NMRK1 (P=0.456, **Figure 3K**) and NMRK2 (P=0.572, **Figure 3L**) between groups. Post-hoc analysis revealed a small but significant 1hr post-RE increase in phospho/total ACC in OC (~22%, P=0.034, **Figure 3F**), as well as a reduction in expression in TFAM in MA at 48hr post-RE (~5%, P=0.027, **Figure 3H**).

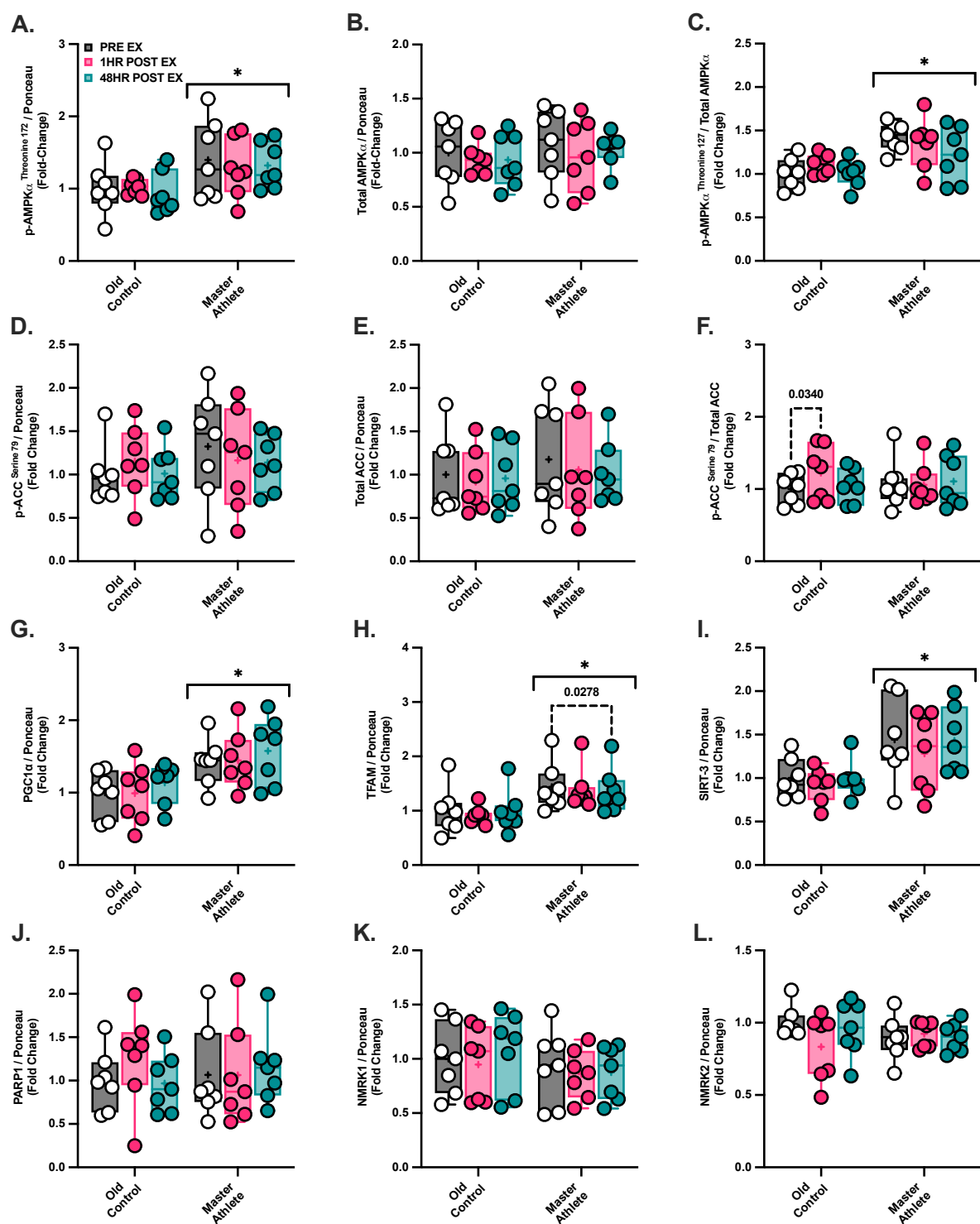


Figure 3. Master athletes display greater levels of proteins involved in mitochondrial biosynthesis. P-AMPK $_{\alpha}$ ^{Thr172} (a) total AMPK $_{\alpha}$ (b) phosphorylated: total AMPK $_{\alpha}$ (c), P-ACC^{Ser79} (d), total ACC I, phosphorylated: total ACC (f), PGC1 α (g), TFAM (h), SIRT-3 (i), PARP-1 (j), NMRK1 (k), and NMRK2 at rest (**BLACK**), 1 h post-resistance exercise (**PINK**), and 48 h post-resistance exercise (**GREEN**) in older untrained controls (n=7) and master athletes (n=7).

3.5. DISCUSSION

The contribution of skeletal muscle mitochondrial metabolism to the progression of sarcopenia is a highly debated topic., with several authors reporting substantial declines in mitochondrial function with ageing ^{231,232}. By contrast, endurance-trained MA cyclists display a greater abundance of mitochondrial-related proteins and show higher mRNA gene expression of MFN-2, ETC complexes, and PGC1 α compared with untrained OC and YA ^{231,232}. The current data support and extend on recent findings by Joannis et al. ²³² by comprehensively assessing mitochondrial signalling at a protein-specific level in endurance-trained MA and untrained age-matched OC. This is particularly important because there are reported divergences between mRNA levels (transcription) and the subsequent downstream changes in protein expression (translation) ^{626,627}. In addition, we determined the responsiveness of mitochondrial protein expression during recovery from RE, a known potent stimulus for myofibrillar remodelling and muscle hypertrophy with unclear effects on mitochondrial signalling. We observed significantly greater CS activity (~45%) and mitochondrial protein expression (OXPHOS, OPA-1, Mitofilin, PGC1 α & SIRT-3) in MA compared with OC. Interestingly, we detected a significant decrease in CS activity (~18%) in OC but not MA following RE. Furthermore, we demonstrated an upregulation of p-DRP-1^{Ser616} in MA at 1hr post-RE, with an elevated abundance of p-MFF^{Ser146}, OPA-1, FIS-1 and Mitofilin. However, we detected no other differences between OC and MA in the abundance of selected fission-fusion markers (MFN-2, DRP-1, MFF), energetic markers (AMPK, ACC, PGC1 α , PARP1, NMRK1/2) at rest or 1hr following RE. Overall, these data show that MA maintain greater levels of mitochondrial-related protein expression compared with OC, and that RE did not alter acute mitochondrial

remodelling in the acute (1hr post-RE) and delayed (48hr post-RE) recovery period in OC and endurance-trained MA.

3.5.1. Mitochondrial Content and Volume

Intrinsic mitochondrial volume (assessed herein by CS activity) is a key determinant of skeletal muscle mitochondrial health and a validated marker of mitochondrial content⁶²⁸. Metabolic profiling of >140 adults (aged 19-89) has demonstrated a 5-8% decline per year in CS protein content and activity³⁷⁰. Here we report ~45% and ~74% higher resting levels of CS activity and CS protein abundance in MA compared with OC (**Figure 1A-B**). These data are similar to Gries et al., who report that CS activity in endurance-trained MA was ~47% greater than OC and ~4% greater than healthy, active YA⁶²⁹. Increased CS activity has been reported following acute and chronic endurance/interval-based exercise⁶³⁰. However, the activity of CS is less well understood in models of RE, and to a lesser extent, in cohorts of older adults⁵⁶⁰. Modest increases in CS activity (in YA and OC) have been reported with chronic RE^{631–636}, with others reporting no change^{51,609,637,638} or even a decline in CS activity⁶³⁹. However, the lack of heterogeneity between RE studies (e.g., RE protocols, experimental approaches) may explain, at least in part, these equivocal observations. Specifically, due to an appreciable increase in myofibrillar and sarcoplasmic protein fractions following RE, mitochondrial density and/or content may appear unchanged, or reduced following a RE intervention⁶⁴⁰, as this is not observed in models of endurance exercise when negligible hypertrophy is detected⁵⁶⁰. To date, CS activity has not been established in the acute or delayed post-RE period in human skeletal muscle. Here, we observed a significant ~18% decrease in CS activity in OC 48hr post-

RE but no change in CS activity in MA (**Figure 1A**). Interestingly, Ogborn et al. observed a ~32% decline in CS mRNA following acute RE in untrained OC unaccustomed to RE ⁶⁴¹. This decline may reflect a preferential upregulation in muscle protein anabolism to support tissue remodelling/recovery after a novel contractile stimulus, which may not be the case in long-term endurance-trained MA. In support of this notion, we previously observed a ~9% increase in myofibrillar protein synthesis over the 48hr post-RE in both OC and MA ¹¹³. Therefore, further evidence is needed to determine the temporal changes in CS enzyme activity and other mitochondrial complexes with ageing and exercise training status.

The expression of proteins involved in oxidative phosphorylation within the ETC represent key markers of mitochondrial content and have previously been shown to be markedly decreased in OC compared with MA ^{91,231,232,580}. Endurance exercise positively influences ETC protein expression; however, the acute and chronic impacts of RE on ETC protein adaptation remains inconclusive. Chronic RE interventions have been reported to increase ETC protein expression in older adults ^{90,580,642}. However, studies of the acute regulatory changes to these ETC proteins with RE are lacking. It is possible that acute changes in ETC expression may not be sensitive to RE ^{580,641} and/or the timing of muscle biopsy sampling has not been appropriate to detect any potential changes in ETC protein expression ⁵⁶⁰. In agreement with this notion, we did not observe any acute changes in ETC protein expression ^{643,644}. Despite detecting no effect of RE, we observed a significantly greater abundance of all the individual ETC complexes (I-V) in MA compared with OC (**Figure 1C-H**). Moreover, combined ETC protein complexes were ~83% greater in MA compared with OC, suggesting greater overall mitochondrial health. However, further detailed assessments of permeabilized

skeletal muscle fibres via high-resolution respirometry are necessary to determine if the observed elevated CS activity and ETC protein content in MA translate into greater respiratory function and bioenergetic health.

3.5.2. Mitochondrial Fission and Fusion

Mitochondrial quality control relies on several tightly orchestrated signalling proteins involved in the fission and fusion of damaged organelles to maintain cellular health, function, and muscle mass^{98,99,360,361,571,574}. Fission events are coordinated by AMPK-mediated phosphorylation of MFF^{Ser146} to act as a DRP-1 adaptor and allow the binding of DRP-1 to the outer mitochondrial membrane^{357,592}. On the contrary, mitochondrial fusion is synchronized by MFN-2 and OPA-1 on the inner mitochondrial membrane^{361,362}. Human data on fission-fusion dynamics suggests MA maintain greater expression of OPA1-, MFN-1, MFN-2, and DRP-1 compared with OC and is similar to that of YA^{99,100}. In the present study, whilst we did not observe greater expression of MFN-2 and DRP-1 (**Figure 3B&J**), we did detect significantly greater levels of OPA-1 (~36%), FIS-1 (~36%) and Mitofilin (~133%) in MA (**Figure 3G, H & I**). Interestingly, OPA-1 has recently been implicated in inner mitochondrial membrane cristae formation and remodelling, and therefore, represents a crucial marker of fusion dynamics and overall mitochondrial functioning^{645,646}. Greater expression of OPA-1 is also associated with muscle mass protection *in vivo* when transgenically overexpressed following denervation-induced atrophy^{361,645}. Further, when probing post-translational modifications of fission-fusion dynamics, we observed ~15% greater expression of p-MFF^{Ser146} in MA compared with OC, with only the MA group demonstrating an ~57% increase in post-RE activation of p-DRP-1^{Ser616}. On the contrary, p-DRP-1^{Ser616}

decreased by ~32% in OC following RE. *In vivo* models have determined the critical role of DRP-1 following exercise and that muscle-specific knockdown/knockout results in blunted exercise adaptation, muscle atrophy, mitochondrial dysfunction, and premature animal death ^{97,98}. Taken together, these data suggest that MA maintain greater skeletal muscle mitochondrial quality control mechanisms to more efficiently fragment damaged and defective organelles for degradation to maintain mitochondrial proteostasis, function and muscle mass.

3.5.3. Mitochondrial Biosynthesis

RE can augment mitochondrial turnover and protein content ^{91,580}. and, therefore, mitochondrial biosynthesis markers are also expected to increase with RE ⁶⁴⁷. Mitochondrial biogenesis is controlled by the transcription of nuclear and mitochondrial encoded genes, with PGC1 α and TFAM being central regulators ⁶⁴⁸. However, the role of PGC1 α for training-induced mitochondrial biogenesis *in vivo* has recently been questioned ^{369,649}, with other markers being identified as critical regulators (e.g., Nrf2, ERR γ , PPAR β , LRP130) ³⁶⁹. Whilst endurance and high-intensity interval training (i.e., HIIT) exercise upregulates the PGC1 α -TFAM pathway ⁶⁵⁰, less is understood in models of RE. Herein, MA displayed a greater expression of p-AMPK^{Thr172}, PGC1 α , and TFAM compared with OC (**Figure 3C, G, H**). However, in our study, RE was not sufficient to augment any increase in biosynthetic signalling. This is not entirely surprising as others have observed inconsistencies in the response of regulators of mitochondrial biogenesis following RE, with no changes in mRNA or protein, despite reported increases in mitochondrial protein turnover ^{313,577,605}. Interestingly, our cohort of MA displayed a ~42% greater abundance of PGC1 α protein expression compared

with OC. Interestingly, in previous studies, the abundance of PGC1 α protein expression in MA has been reported to be as much as ~190% greater than OC and YA²³², despite similar weekly exercise training history, training volume and phenotype to the current MA cohort. Others have observed a similarly greater abundance of PGC1 α in 'high' compared with 'low' functioning older adults⁹⁹. Since PGC1 α is a key signalling node in initiating mitochondrial biogenesis, the increased protein content may reflect the greater mitochondrial mass and content observed in the MA. Thus, mitochondrial biogenesis may be exercise and/or activity-dependent and not affected by ageing *per se*.

PGC1 α also directly regulates sirtuin and NAD⁺-dependant signalling, such as SIRT-3, PARP1, and NMRK1/2. Here we observed that MA displayed greater expression (~38%) of SIRT-3, similar to previously reported data⁶⁵¹. However, we observed no differences in PARP1 or NMRK1/2 protein expression between groups. Interestingly, chronic RE has recently been reported to reverse age-related declines in NAD⁺ salvage signalling, particularly NAMPT expression^{636,651}. Therefore, further investigation into tissue-specific and circulating NAD⁺ metabolites as previously undertaken in healthy older adults⁶⁵² warrants investigation in MA to determine if these populations maintain the NAD⁺ metabolome and potential resistance to the age-associated decline in NAD⁺ metabolites.

Although the present study provides important insights into the regulation of mitochondrial activity and bioenergetics, we acknowledge there are limitations. Our interpretation is limited by the timing of skeletal muscle biopsy sampling. In contrast, the time course in mRNA, protein expression, and phosphorylation status of mitochondrial signalling cascades has been extensively examined in the context of

endurance and HIIT-based exercise^{643,644}. Therefore, the present data adds to the paucity of data on RE-induced mitochondrial signalling regulation and suggests MA display greater mitochondrial signalling health compared with age-matched controls. It is worthy of note, there was a 'trend' for significant differences in age between MA and OC (**Table 1**). This may influence our molecular outcomes due to greater mitochondrial decline during ageing. Nevertheless, more research employing stable isotopic tracers (mitochondrial protein synthesis), high-resolution respirometry (mitochondrial respiration, ATP production and H₂O₂ emission) and detailed biochemical analysis of molecular signalling events post-RE are needed to improve our understanding of mitochondrial remodelling following single or repeated bouts of RE.

3.6. CONCLUSION

In summary, we observed greater CS activity and expression of proteins involved in mitochondrial ETC and quality control signalling in long-term endurance-trained MA compared with age-matched untrained individuals. However, mitochondrial protein expression and/or phosphorylation status remained largely unchanged in the early (1hr) and late (48hr) phase of recovery following an acute bout of RE. Collectively, these data suggest that participating in regular aerobic exercise training across adulthood may support mitochondrial health by bolstering levels of mitochondrial enzyme activity and expression of proteins involved in ETC and fission-fusion dynamics. In contrast, RE has little effect on acute mitochondrial-related signalling or enzyme activity.

SUPPLEMENTARY FIGURES

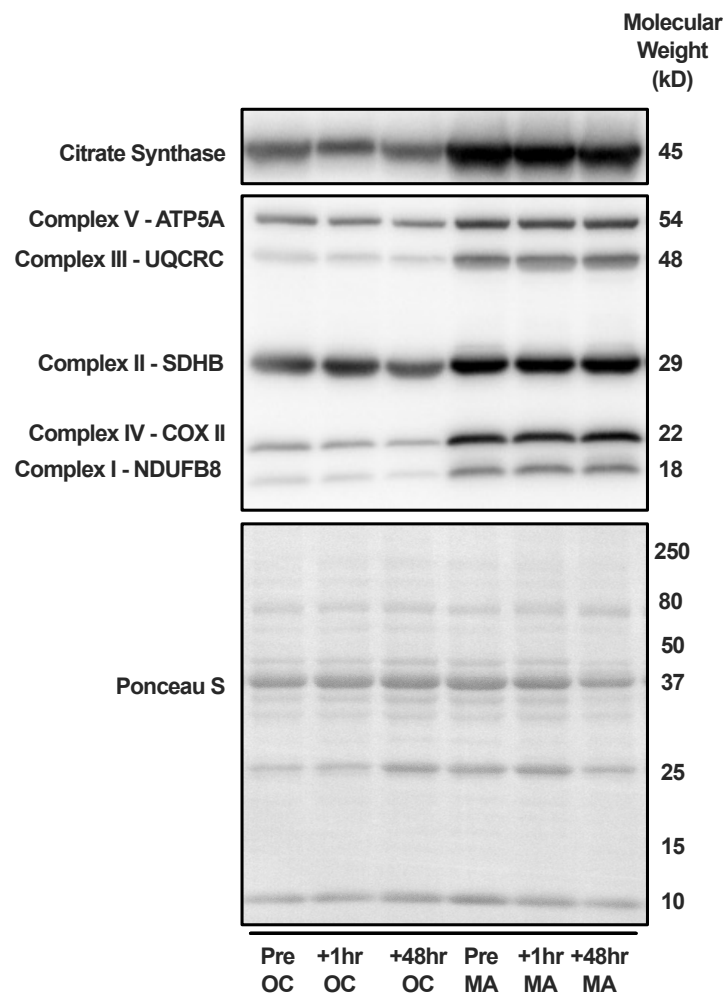


Figure 4. Representative immunoblot image. Citrate synthase, human OXPHOS cocktail of electron transport chain proteins I-V and Ponceau S.

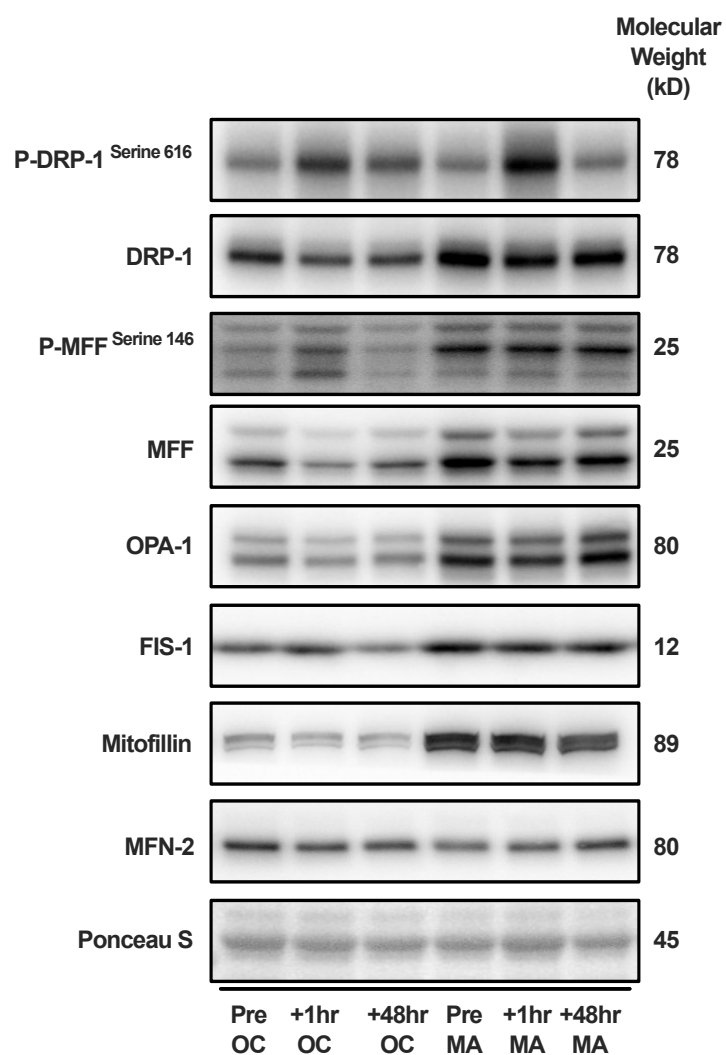


Figure 5. Representative immunoblot image. P-DRP1^{Ser616}, DRP1, P-MFF^{Ser146}, MFF, OPA-1, FIS-1, Mitofillin, MFN-2 and Ponceau S.

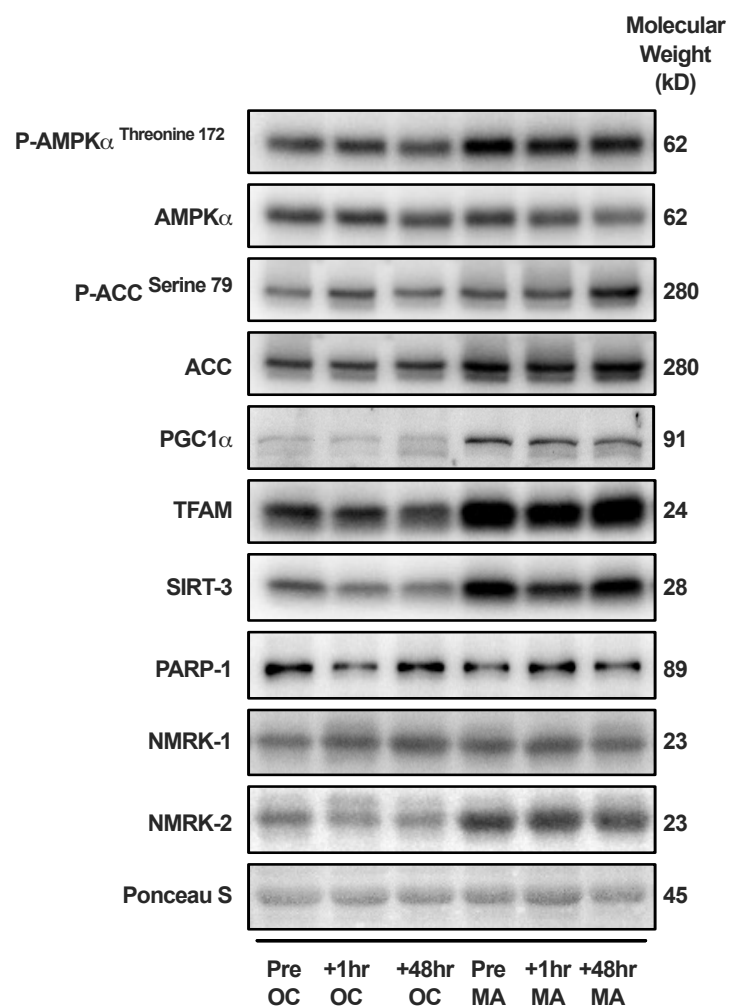
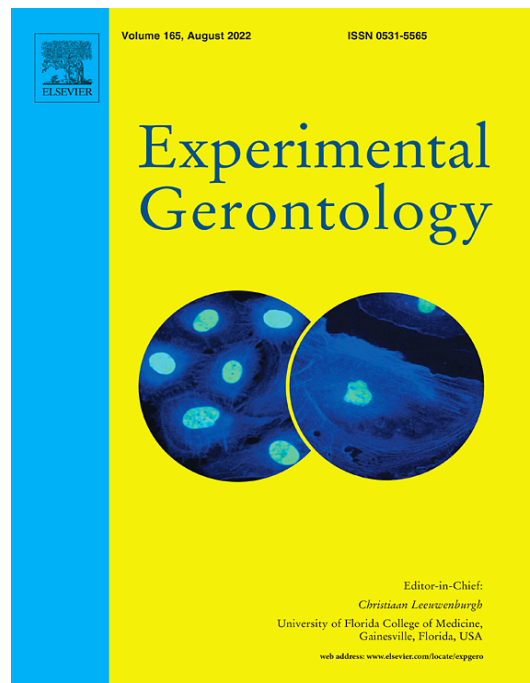


Figure 6. Representative immunoblot image. P-AMPK^{Thr172}, AMPK, P-ACC^{Ser79}, ACC, PGC1 α , TFAM, SIRT-3, PARP-1, NMRK1, NMRK2 and Ponceau S.

CHAPTER 4

Quadriceps Muscle Electromyography Activity During Physical Activities and Resistance Exercise Modes in Younger and Older Adults



Published Peer-Reviewed Manuscripts from This Chapter:

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4.1. ABSTRACT

Background: Understanding the root cause of the age-related impairment in muscle adaptive remodeling with resistance exercise (RE) and developing pragmatic and accessible resistance exercise for older adults, are essential research directives. **Methods:** We sought to determine whether indices of quadriceps muscle EMG activity in response to different modes of RE and activities of daily living (ADL), differed between 15 healthy younger (25 ± 3 years) and 15 older (70 ± 5 years) adults. On four separate days, participants completed a maximal voluntary contraction (MVC) of the knee extensors, followed by a 15 m walking task, stair climbing task (i.e., ADL) and lower-limb RE through body-weight squats (BW-RE) and seated knee extensions on a machine (MN-RE) or via elastic bands (EB-RE). Quadriceps electromyography (EMG) was measured throughout all tasks to provide indirect estimates of changes in muscle activity. **Results:** MVC was significantly greater in young vs. older adults (Young: 256 ± 72 vs. Old: 137 ± 48 N·m, $P < 0.001$). EMG activity during all exercise tasks was significantly higher in older vs. younger adults when expressed relative to maximal EMG achieved during MVC ($P < 0.01$, *for all*). In addition, relative quadriceps muscle EMG activity was significantly greater in EB-RE and MN-RE compared with BW-RE in young and older adults ($P < 0.001$). However, there was no significant difference in quadriceps EMG between EB-RE and MN-RE ($P > 0.05$). **Conclusions:** In conclusion, relative quadriceps muscle EMG activity was higher across a range of activities/exercise modes in older vs. younger adults. The similar quadriceps muscle EMG activity between EB-RE and MN-RE provides a platform for detailed investigation of the neuromuscular and muscle metabolic responses to such pragmatic forms of RE to strengthen the evidence-base for this mode of RE as a potential countermeasure to sarcopenia.

4.2. INTRODUCTION

Ageing is associated with a precipitous loss of skeletal muscle strength, quantity/quality and functional capacity (termed sarcopenia) ^{18,20}. Sarcopenia progresses at a rate of ~0.7–1.2%/year after the 5th decade of life ⁶⁵³, and is associated with a loss of independence ⁶⁵⁴ and increased morbidity and mortality risk ⁶⁵⁵. The prevalence of sarcopenia within the UK, in community-dwelling older adults (>65 years), has been reported to be >12.5% ²², rising to >20% in those aged >85 years ⁶⁵⁶. Alarming, >78% of older adults admitted to primary care and inpatient facilities are diagnosed with this condition ⁶⁵⁷. Ultimately, sarcopenia presents a considerable burden on healthcare resources ²⁷ that will continue to rise as prevalence increases ⁶⁵⁸.

Evidence from randomised control trials ^{659–661}, systematic reviews and meta-analyses ^{621,662} demonstrates that regular, progressive resistance exercise (RE) can effectively attenuate/counteract sarcopenia and alleviate many of its adverse health consequences. However, the muscle adaptive remodelling response to RE may be compromised in older age ⁶⁶³. Specifically, there is strong evidence that the muscle protein synthetic (MPS) response to RE is blunted in older, compared with younger, adults ⁶⁶⁴. This is important as RE-induced MPS stimulation is a key determinant of the overall net protein balance/accretion for muscle hypertrophy ⁶⁰⁴. The underlying cause of this muscle anabolic resistance in older age is unclear but requires further investigation in order to maximize the health benefits of RE in older adults.

Contraction-induced MPS stimulation is dependent on a number of highly coordinated neurophysiological and muscle metabolic processes ⁶⁶⁵. Motor unit recruitment and maximal fibre activation are thought to be crucial for RE-induced MPS stimulation ^{313,666}. Indeed, the compromised properties of the ageing neuromuscular

system are suggested as key factors in the loss of muscle fibre size, fibre number and motor performance⁶⁶⁷ and, thus, could be implicated in the attenuated MPS response to RE. To date, very few studies have investigated how RE-induced neurophysiological responses are altered by ageing, partly due to the technical challenges of measuring these parameters in vivo under exercise conditions⁶⁶⁸. Surface electromyography (EMG) is an accessible, non-invasive tool that can be used to measure the transmembrane current of muscle fibres (or 'muscle activity'), that, when applied in such a way to overcome the well-described shortcomings of this method⁶⁶⁹, can be useful for gaining insight into neuromuscular responses to ageing, disease, and exercise⁶⁶⁹. As such, EMG application may enhance our understanding of potential age-related changes in RE-induced muscle activation.

Despite the well-established effects of RE, adherence, and compliance to such gym-based RE in an unsupervised environment is underwhelming⁶⁷⁰. This may, in part, be due to cost and aversion to commercial gym facilities in older adults⁶⁷¹. As such, there is a need to develop pragmatic and accessible RE interventions that can effectively support the maintenance or, indeed, enhancement of muscle mass, strength, and function in older age⁶⁷¹. Elastic resistance bands (EB) and body weight (BW) lifting are proficient methods of RE, capable of increasing muscle mass, strength, and functional capacity in community-dwelling^{672,673}, institutionalized^{674,675}, sarcopenic-obese older adults⁶⁷⁶, and older adults with cognitive impairment⁶⁷⁷. Compared with traditional machine-based (MN) RE, EB have also been shown to generate an 'ascending' (or 'linear variable') resistance training load, providing an increasing tensile load due to the stretch properties of the EB⁶⁷⁸. In addition, EB-RE demonstrates similar EMG activity when compared with machine and free-weight alternatives during multi-joint movements in younger adults^{679–682}, and clinical

rehabilitation^{683,684}. Given the dearth of work in this area, there is a need for improving understanding of the neuromuscular and muscle metabolic response to EB-RE and BW-RE in older adults, particularly in comparison to traditional machine-based RE and the response in younger adults. Addressing these important questions will enhance the evidence-base for RE recommendations and prescription in older adults.

The primary purpose of the present study was, therefore, to assess quadriceps muscle EMG activity using textile embedded EMG shorts during BW-RE, EB-RE and MN-RE and activities of daily living (ADL) in younger and older adults to understand the effects of ageing and contractile mode on muscle activity. We hypothesized that BW-RE would result in lower relative EMG activity compared to EB-RE and MN-RE in younger adults, however, that there would be no difference between RE tasks in older adults. Moreover, EB-RE would elicit similar quadriceps muscle EMG activity to that observed with traditional MN-RE, irrespective of age. Subsequently, the secondary purpose of the study was to determine the effects of ADL (activities of daily living: walking and stair climbing), and their respective effects on quadriceps muscle EMG activity in younger and older adults. We hypothesized that quadriceps muscle EMG activity relative to that achieved during a maximal voluntary contraction (MVC) would be greater in older vs. younger adults across all RE modalities and ADL.

4.3. METHODOLOGY

4.3.1. Participants

Fifteen healthy younger (25 ± 3 years, 8 men, 7 women, BMI: 24.3 ± 3.0 kg/m²) and fifteen older (70 ± 5 years, 8 men, 7 women, BMI: 23.6 ± 3.4 kg/m²) volunteers participated in this study. All study participants were recreationally active, and defined as healthy, as determined via a general health screening and medical questionnaire. Briefly, inclusion criteria included being habitually or recreationally active, non-smoker, non-diabetic and normotensive ($<140/90$). Exclusion criteria included a highly sedentary lifestyle ($<5,000$ steps per day), hypertensive ($>140/90$), the consumption of non-steroidal anti-inflammatory drugs, and the consumption of caffeine ~12 hours prior to an experimental visit. This study was conducted in accordance with the declaration of Helsinki, and ethical approval was obtained from the University of Birmingham (ERN_19-0220). After being informed of the experimental procedures and associated risks, all participants provided written informed consent prior to engaging in the study.

Table 1. Participants anthropometric, body composition and strength characteristics in young and old adults.

Anthropometrics				
	Young (N=15)	Old (N=15)	P Value	Effect Size
Age (years)	25.1 ± 3.0	70.8 ± 5.4	‡ <0.001	-
Height (cm)	171.8 ± 7.0	167.9 ± 8.5	0.18	-
Body Mass (Kg)	72.3 ± 12.2	67.0 ± 12.3	0.25	-
BMI (Kg·m ⁻²)	24.4 ± 3.0	23.6 ± 3.5	0.54	-
Knee Extensor Maximal and Submaximal Strength				
MVC (N·m)	256 ± 72	137 ± 48	‡ <0.001	2.01
KET/BM (N·m·kg ⁻¹)	3.5 ± 0.7	2.1 ± 0.6	‡ <0.001	2.16
EB 12-RM (kg)	17 ± 3	12 ± 3	* 0.002	1.25
MN 12-RM (kg)	17 ± 7	10 ± 4	‡ <0.001	1.54
Body Composition				
Whole Body FFM (Kg)	55.6 ± 10.9	43.4 ± 9.4	0.06	0.71
Whole Body FM (Kg)	16.7 ± 5.1	20.9 ± 14.2	0.47	0.27
Body Fat (%)	23.2 ± 6.6	27.3 ± 9.6	0.17	0.51
Skeletal Muscle Mass (Kg)	27.4 ± 6.3	21.7 ± 5.2	† 0.01	0.91

BMI, Body Mass Index; MVC, Maximal Voluntary Contraction; KET/BM, Knee Extension Torque Relative to Body Mass; EB, Elastic Band, MN, Machine; FFM, Fat Free Mass; FM, Fat Mass; FFM = fat-free mass; FM = fat mass. Data presented as mean ± SD. * Significantly different from young (P<0.05); † Significantly different from young (P<0.01); ‡ Significantly different from young (P<0.001).

4.3.2. Experimental Design

Participants visited the laboratory on four separate occasions over a 2-week period with all tests separated by at least 48 hours and conducted at a similar time of day ($\pm$ 90 min) to limit potential changes in quadriceps strength and to account for diurnal variations in neuromuscular excitability⁶⁸⁵. Using a within-subject design and following familiarization and strength testing (see 'Familiarization and Strength Testing'), participants visited the lab on three separate occasions to perform BW-RE, EB-RE, and MN-RE (see 'Experimental Trials'). Prior to each experimental visit, participants consumed their habitual diet and were instructed not to participate in any rigorous physical activity or consume alcohol ~48hrs and ~24hrs, respectively, prior to the visit. Briefly, on each of the trial days, participants wore textile surface EMG shorts (see 'Surface Electromyography') to provide an indirect measure of muscle activity during each exercise task. Following a standardised warm-up, participants performed six sets of twelve repetitions at ~70% of their 1 repetition maximum (1-RM), with a 120 s intra-set recovery period. Immediately after each set, perceived exertion (RPE) was determined using a modified Borg scale (Borg, 1998). This method of assessing perceived exertion during resistance exercises is comparable to other RPE scales currently validated for monitoring intensity during resistance exercises performed with elastic bands in older populations^{686,687}.

4.3.3. Familiarization and Strength Testing

The first laboratory visit was used to assess body composition, as determined via bioelectrical impedance analysis (mBCA 525, SECA, Hamburg, Germany), incorporating assessments of fat mass (FM), body fat percentage (BF%), fat-free mass (FFM) and skeletal muscle mass (SMM). BIA has previously been observed to be a

reliable and valid measure of body composition ⁶⁸⁸. In addition, MVC of the knee extensors was assessed using an isokinetic dynamometer (KinCom 125AP, KinCom, USA) for evaluation of quadriceps isometric muscle strength and maximal EMG activity of both limbs. Briefly, participants were seated with the hip and knee joints at relative angles of 90° and 110°, respectively, with the tested limb, chest, and hips, stabilised ⁶⁸⁹ and adjusted so that the axis of rotation of the lever arm was in line with the lateral epicondyle of the femur. All participants completed 3 × 3 s MVC of the knee extensors with each contraction separated by a 60 s passive recovery period. Participants were given standardized verbal encouragement for the duration of each 3 s contraction. All participants then completed 3 × 15 m walk at their regular walking pace, followed by the completion of the descent and ascent of a flight of stairs (12 steps, 30 cm each in height) to determine the effects on quadriceps muscle EMG.

Participants were then familiarised to the exercise tasks for the experimental trials, as described below, which included the assessment of a 12-repetition maximum (12-RM) for EB-RE and MN-RE. Subsequently, participants performed three experimental trials: (1) BW-RE, (2) EB-RE and (3) MN-RE. MN-RE 12-RM was determined using a knee extension strength training machine (Elite Series Leg Extension/Curl, Fitness Warehouse Ltd., Preston, Lancashire, UK), starting with a self-selected load estimated to be ~50% of the participants' 1-RM. Following the completion of a standardized warm-up and 12 successful repetitions, the load was increased by ~5-10 kg increments until the participant reached the desired load representing their 12-RM (~70% 1-RM). The 'desired' load was estimated based upon participants' rating of perceived exertion (RPE), between 7 and 8 ⁶⁹⁰. For EB-RE, the 12-RM load was assessed using an elastic resistance band (PeakSupps, Bridgend, UK) with loads

ranging from 'light' to 'very heavy' (6 kg, 12 kg, 20 kg, and 28 kg at 100% elongation of manufacturer guidelines). Using the machine-based variation as a guide, the participants commenced knee extension exercise testing with a band representing ~50% of their 1-RM. Following two initial warm-up sets, the load was increased via the increased elongation of the band in 20 cm increments and/or an increase in band resistance (i.e., band colour) until the participant reached a load representing their 12-RM, as described above. Participants were instructed to perform each repetition over a total of 3 s (eccentric phase: ~1.5 s and concentric phase: ~1.5 s), timed via a visual prompt to 'push' and 'relax', accompanied by the same verbal instructions from the experimenter. The starting position of EB-RE was subject to an initial pre-stretch of the band, providing a load at the start of each contraction and was, therefore, comparable to the starting position of MN-RE.

4.3.4. Experimental Trials

Visits 2-4 were completed in a randomised order. For BW-RE (chair squats), participants were required to stand shoulder width apart in front of a standardised chair (height: 43cm) and squat down to lightly touch the chair before ascending to a standing position with each repetition being completed within ~3 seconds and with a 120 s intra-set recovery period. The second and third experimental RE conditions required participants to undertake a single bout of EB and MN knee extension resistance exercise, respectively. For EB-RE and MN-RE, the trials started with a standardised warm-up routine consisting of two sets of twelve repetitions at 50% and 75% of their 12-RM, respectively. Participants then undertook a bout of knee extension exercise comprising of the same training volume, intra-set recovery and exercise cadence as BW-RE at their 12-RM. Figures 4 and 5 provide a visual example of a participant

undertaking the ADL and RE tasks, respectively, and are available as an online supplement.

4.3.5. Surface Electromyography

EMG was used to quantify the electric potential of quadriceps and referred to as 'quadriceps muscle EMG activity' throughout (108). Specifically, surface EMG was recorded from the *m. vastus lateralis* and *m. vastus medialis* of the right and left leg quadriceps, using textile shorts made of polyamide (71%) and elastane (29%) with bipolar electrodes integrated into the fabric (Mbody, Myontec Ltd, Kuopio, Finland). The electrodes connected wirelessly to an electronics module attached to the front of the shorts (MCell, Myontec Ltd, Kuopio, Finland), as previous described ⁶⁹¹. The relative position of the two quadriceps electrodes were such that they were located over the distal portion of the quadriceps muscle, superficial to the *m. vastus lateralis* and *m. vastus medialis*, as previously described ⁶⁹¹. The sizes of the electrodes are 2.5cm x 9-14cm depending on the size of the shorts. The reference electrodes are placed longitudinally over the tractus iliotibialis on either side of the shorts. The MCell contains the signal amplifiers, microprocessor, and data memory. A range of sizes were used to ensure close proximity of the electrodes to the skin and to maximise the sensitivity of the EMG signal. Where appropriate, compression sleeves were used to ensure sufficient electrode contact with the skin superficial to the muscles of interest. Furthermore, the skin area underneath each EMG electrode was sprayed with water to optimise conductivity between the skin and electrodes. This method of EMG assessment has previously been established to be a valid and feasible method of assessing rectified values of muscle EMG activity during day-to-day functional

exercise tasks ^{692–694}. Figure 6 provides an annotated image of the textile EMG shorts used in the current study and is available as an online supplement.

4.3.6. Data analysis

The EMG signals were pre-amplified (1000 Hz) and then band-pass filtered (2nd order) with cut-off frequencies of ~40 – 300 Hz. EMG data were recorded continuously and digitised synchronously with 12-bit resolution via an A/D converter. The 1000 Hz signal was first rectified and then averaged over non-overlapping 100 ms intervals, similar to the root mean square method. The averaged data was stored in the memory of the MCell at 25 Hz, from which the data was extracted and analysed using customised software (Muscle Monitor, Myontec Ltd, Kuopio, Finland) (Supplementary Figure 7.). All EMG data was visually assessed and corrected for artefacts, as previously described ⁶⁹¹. Signal noise was determined via a test repetition during the warm-up sets prior to the main bout of exercise. To obtain an average rectified value of EMG amplitude, the average EMG signal amplitude was taken from the onset of the exercise task (or 'set') until task end, for both young and older groups. Following completing of each individual exercise session (i.e., walking, stair climb, BW-RE, EB-RE, and MN-RE) the mean EMG amplitude was determined (i.e., the mean EMG amplitude of all 6 sets of 12 repetitions during RE) and presented relative to the maximal EMG amplitude achieved from a 1-s peak period during MVC.

4.3.7. Statistical analyses

A power analysis with an error = 0.05, power = 0.95, and effect size = 6.57, was performed using G x Power 3.1 analysis software (Heinrich Hein University, Duesseldorf, Germany) based on the differences in walking EMG activity between

younger and older adults ⁶⁹⁵. However, an additional power analysis with an error = 0.05, power = 0.95, and effect size = 1.32, was performed based on the differences in RMS EMG activity during knee extension contractions between younger and older adults ⁶⁹⁶. This produced a minimum sample size of 14 participants. A total of 15 per group were recruited to account for differences between measurement methods, age, and activities of daily living and RE modes to provide a more detailed characterisation of our outcome measures. Independent samples *t*-tests were used to assess differences in components of body composition (i.e., body fat %, fat mass, fat-free mass, and skeletal muscle mass) and EMG amplitude (i.e., muscle activity) between groups (i.e., young, and old). In addition, differences in maximal (i.e., knee extensor MVC torque) and sub-maximal (i.e., EB 12-RM, MN 12-RM) strength were also assessed via independent samples *t*-tests. Statistical analysis of young vs old comparisons for ADL (i.e., walking stair climbing and chair squats) and both EB-RE and MN-RE were assessed via independent samples *t*-tests. Lastly, a two-way ANOVA analysis of group (i.e., within-factor, young vs old) and exercise (i.e., between factor, EB-RE vs MN-RE). Normal distribution was assessed using the Shapiro-Wilk test. For calculation of effect size, partial eta squared (η^2) was used for omnibus tests and Cohen's *d* was used for *t*-tests and post-hoc comparisons. All statistical tests were performed on relative and raw absolute data. However, unless otherwise stated, relative data is presented for all variables. Where sphericity was violated, a Greenhouse-Geisser correction factor was applied. For all tests, results were considered statistically significant when $P < 0.05$. Data are presented as mean $\pm$ standard deviation, unless otherwise stated. All statistical analyses were conducted using IBM SPSS Statistics version 25 (IBM SPSS inc. Chicago, Illinois, USA).

4.4. RESULTS

4.4.1. Body composition

Components of body composition for both groups can be seen in table 1. No differences were observed between groups for FM ($P=0.46$, $d=0.27$) or BF% ($P=0.17$, $d=0.51$). However, whilst there was no significant difference between groups for FFM, there was a strong effect size, implying a higher FFM in the young group ($P=0.06$, $d=0.71$). In addition, SMM was greater in the young compared with the old group ($P=0.01$, $d=0.91$). The coefficient of variation of BIA in our lab measurements of body composition for each group were FM (Young, $0.9 \pm 0.2\%$ vs Old, $0.4 \pm 0.4\%$), BF% (Young, $0.8 \pm 1.2\%$ vs Old, $0.5 \pm 0.4\%$), FFM (Young, $0.2 \pm 0.4\%$ vs Old, $0.1 \pm 0.1\%$), SMM (Young, $0.3 \pm 0.4\%$ vs Old, $0.2 \pm 0.2\%$), respectively, with no differences observed between groups ($P>0.5$ for all).

Table 2. Electromyography amplitude and perceived exertion during activities of daily living and resistance exercise training modalities.

	Young (n=15)	Young RPE	Old (n=15)	Old RPE
MVC μ V	685 $\pm$ 52	-	*273 $\pm$ 103	-
15 m Walk μ V (%EMG)	15.8 $\pm$ 8.2 (2.3 $\pm$ 1.0)	-	28.0 $\pm$ 11.5 *(11 $\pm$ 5)	-
Stair Climb μ V (%EMG)	36.3 $\pm$ 7.4 (5.4 $\pm$ 1.1)	-	44.2 $\pm$ 8.6 *(20.8 $\pm$ 7.4)	-
Chair Squat μ V (%EMG)	56.4 $\pm$ 16.2 (8.6 $\pm$ 2.9)	3.5 $\pm$ 1.3	67.3 $\pm$ 12.4 *(27.0 $\pm$ 9.3)	4.4 $\pm$ 1.7
Elastic Band μ V (%EMG)	132.9 $\pm$ 52.5 (20.3 $\pm$ 8.7)	6.8 $\pm$ 0.8	95.4 $\pm$ 21.9 *(37.0 $\pm$ 10.7)	7.2 $\pm$ 0.7
Machine μ V (%EMG)	149.5 $\pm$ 54.8 (22.9 $\pm$ 10.3)	7.1 $\pm$ 1.0	95.3 $\pm$ 20.6 *(37.8 $\pm$ 10.8)	6.7 $\pm$ 1.2

EMG, Electromyography; MVC, Maximal Voluntary Contraction. * Significantly different from young ($P < 0.001$). Data presented as mean $\pm$ SD. EMG (%) is expressed relative to maximal EMG achieved during MVC.

4.4.2. Knee extensor strength

Data for maximal and 5 sub-maximal knee extensor strength are seen in table 1. Knee extensor MVC torque was ~46% lower in the old compared with the young group ($P<0.001$, $d=2.01$, Fig. 1A). In addition, knee extension torque (KET), relative to body mass (N·m/kg), was ~66% greater in the young group ($P<0.001$, $d=2.16$, Fig. 1B). 12-RM strength was higher in the young group for EB ($P=0.002$, $d=1.25$) and MN (Young, $P<0.001$, $d=1.54$), with a 40% and 27% difference in 12-RM strength, respectively, between groups.

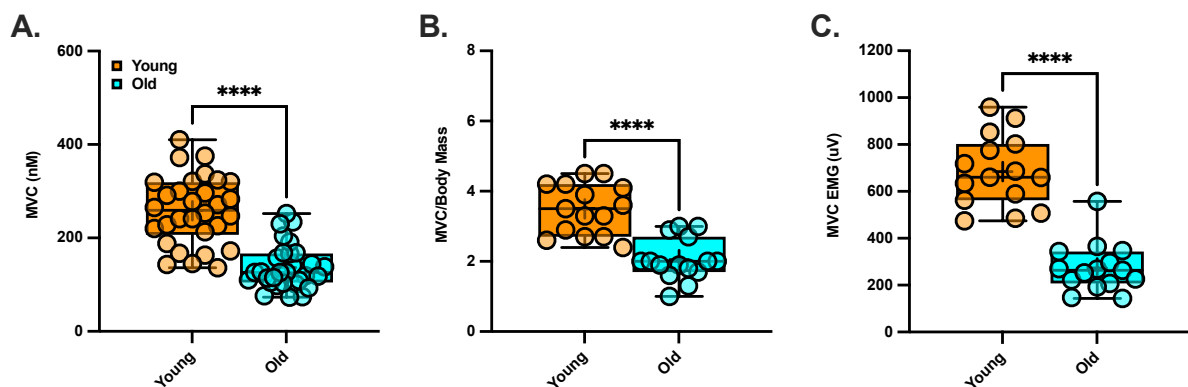


Figure 1. Maximal knee extensor torque and peak EMG activity. Individual data points for (A) Maximal quadriceps knee extension torque (N·m), (B) maximal quadriceps knee extension torque relative to bodyweight (N·m·kg⁻¹); and (C) peak electromyography (EMG) activity (μV) achieved during a 90° isometric knee extensor contraction for young (ORANGE) and old (BLUE) adults. * Indicate significant (P < 0.05) differences between groups. MVC, Maximal voluntary contraction; KET, Knee-extension torque; BM, Body mass.

4.4.3. Muscle EMG activity

Maximal quadriceps muscle EMG activity, as measured during MVC, was higher in the young compared with the old group ($P<0.001$, $d=3.23$, Table 2, Fig. 1C), with older adults exhibiting a ~60% difference in maximal quadriceps muscle EMG activity compared with their younger counterparts. We also observed significant main effects of group (i.e., old vs. young) and resistance exercise modality (i.e., BW-RE, EB-RE, MN-RE) on muscle activity (Table 2, Fig. 3, *both* $P<0.001$, *both* $\eta^2>0.55$). Similarly, we also observed significant main effects of group and ADL task on muscle activity (Table 2, Fig. 2, *both* $P<0.01$, *both* $\eta^2>0.62$). Specifically, during a 15 m walk, we observed a >3-fold difference in relative quadriceps muscle EMG activity in the older compared with younger adults (Young: $2.3 \pm 1.0\%$ vs. Old: $11.0 \pm 5.0\%$, $P<0.001$, $d=2.91$, Table 2, Fig. 2A). In addition, older adults displayed a >2-fold difference in quadriceps muscle EMG activity compared with their younger counterparts during the descent (Young: $4.5 \pm 1.0\%$ vs. Old: $14.8 \pm 5.0\%$, $P<0.001$, $d=3.12$, Table 2, Fig. 2B), ascent (Young: $6.3 \pm 1.8\%$ vs. Old: $20.8 \pm 7.4\%$, $P<0.001$, $d=3.43$, Table 2, Fig. 2C), and mean (ascent and decent) of the stair climb (Young: $5.4 \pm 1.1\%$ vs. Old: $17.8 \pm 6.1\%$, $P<0.001$, $d=3.44$, Table 2, Fig. 2D). Post-hoc tests revealed significant differences in quadriceps muscle EMG activity between all ADL tasks (i.e., 15 m walk, ascent, and descent), irrespective of the group (all $P<0.01$, Table 2, Fig. 2).

During BW-RE, older adults exhibited ~3-fold greater quadriceps muscle EMG activity than that of younger adults (Young: $8.6 \pm 2.9\%$ vs. Old: $27.0 \pm 9.3\%$, $P<0.001$, $d=3.02$, Table 2, Fig. 3A). In addition, quadriceps muscle EMG activity during EB-RE (Young: $20.3 \pm 8.7\%$ vs. Old: $37.0 \pm 10.7\%$) and MN-RE (Young: $22.9 \pm 10.3\%$, vs. Old: $37.8 \pm 10.8\%$) were greater in older compared with younger adults (Table 2, Fig. 3B&C, *both* $P<0.001$, $d>1.40$). In older adults, BW-RE was associated with higher

muscle EMG activity when compared with younger adults (Older: $73.4 \pm 19.3\%$ vs. Young: $42.3 \pm 14.4\%$, when expressed relative to EB-RE and MN-RE). A significant difference between younger and older adults was observed between EB-RE and MN-RE ($P < 0.001$, Fig. 4). However, no differences were observed between EB-RE and MN-RE ($P = 0.52$, Fig. 4).

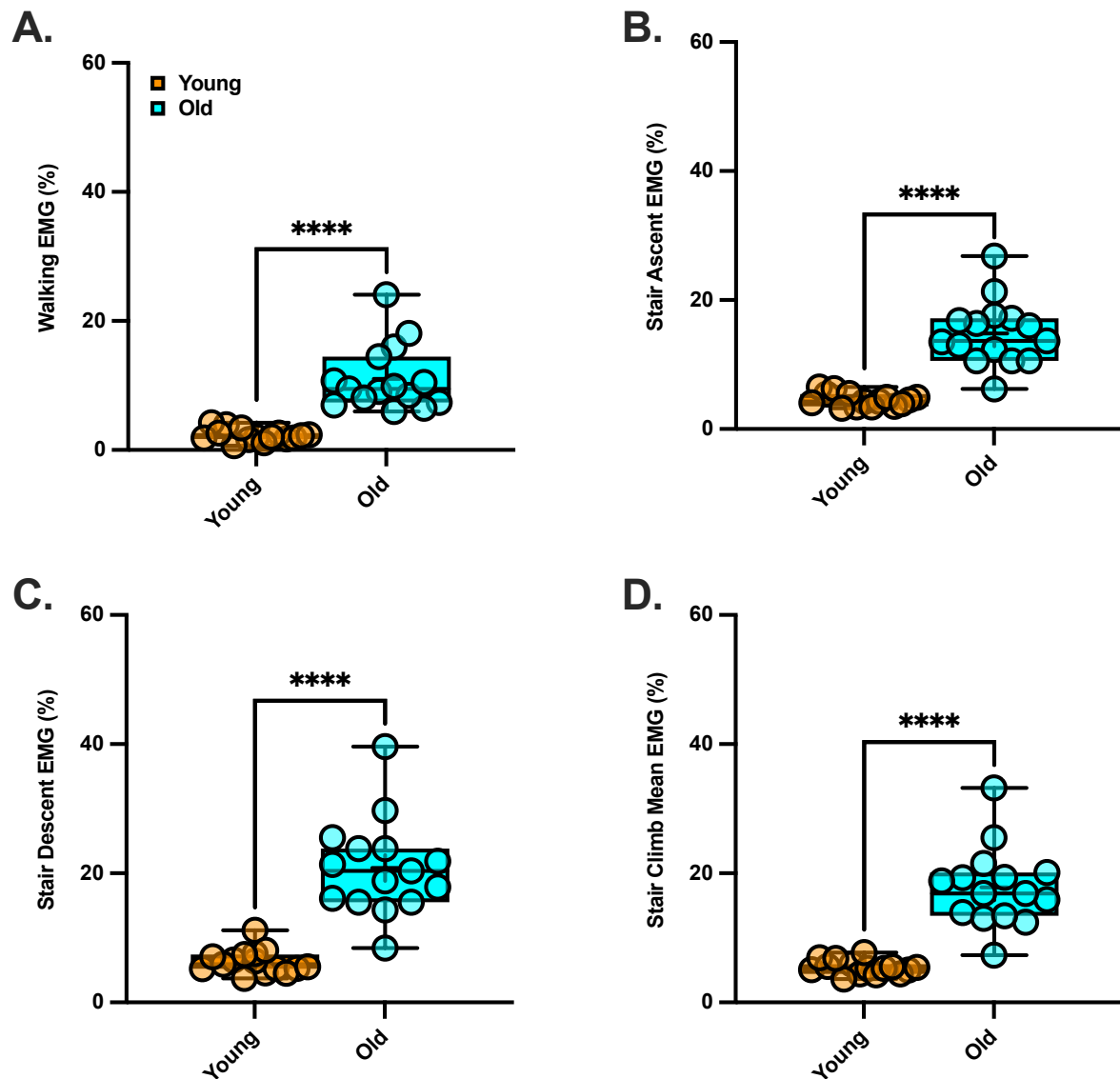


Figure 2. Quadriceps muscle EMG activity during activities of daily living. Peak muscle EMG activity of the quadriceps during activities of daily living (walking, stair descent, stair ascent and stair mean) in young (ORANGE) and old (BLUE) adults, with individual datapoints. * Indicate significant ($P < 0.05$) differences in muscle EMG activity (EMG, %) between groups. EMG, Electromyography.

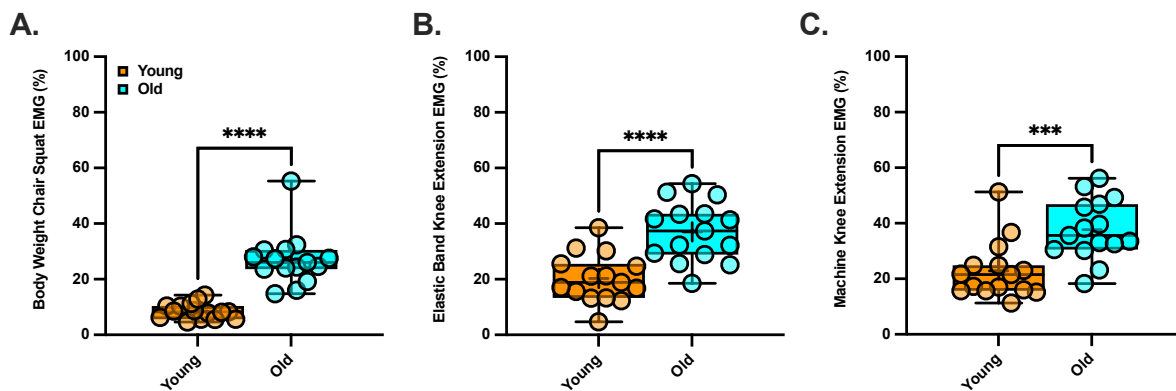


Figure 3. Quadriceps muscle EMG activity during chair squats, elastic band, and machine resistance exercise. EMG activity of the quadriceps during, **(A)** bodyweight (BW-RE), **(B)** elastic band (EB-RE) and **(C)** machine (MN-RE) resistance exercise modalities in young (ORANGE) and old (BLUE) adults, with individual datapoints. * Indicates significant ($P < 0.001$) differences in muscle activity between groups.

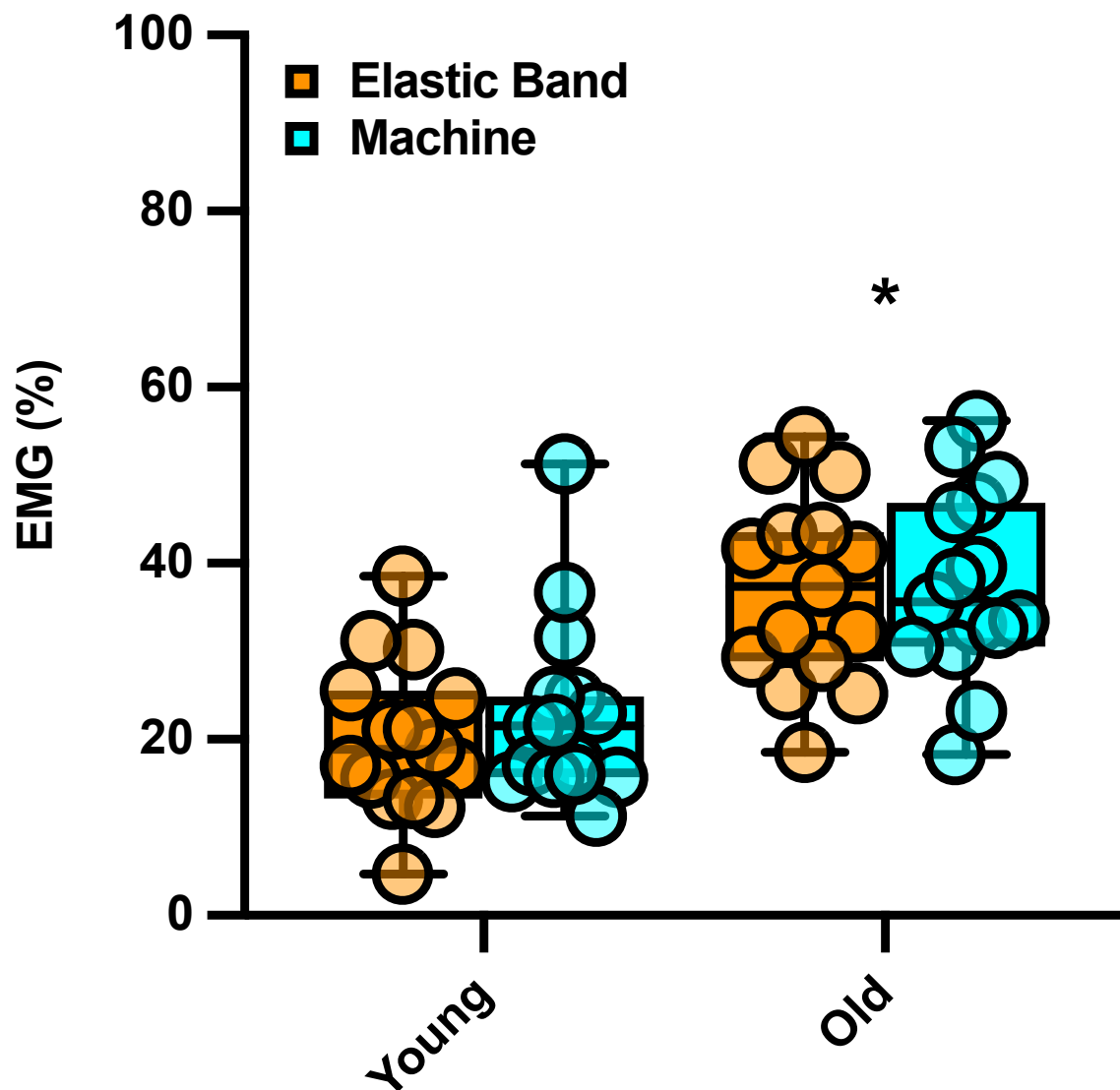


Figure 4. No differences in quadriceps muscle EMG activity between elastic and machine resistance exercise. EMG activity of the quadriceps during elastic band (EB-RE, **ORANGE**) and machine (MN-RE, **BLUE**) resistance exercise modalities in younger and old adults, with individual datapoints.

4.4.4. Perceived exertion

A significant main effect of RE modality was observed on RPE ($P < 0.01$, $\eta^2 = 0.76$, Table 2). However, no differences were found between young and older adults, irrespective of the RE modality ($P = 0.86$, $\eta^2 = 0.13$, Table 2). Specifically, no differences in RPE between EB-RE and MN-RE were observed in the young (EB: 6.8 ± 0.8 , vs. MN: 7.1 ± 1.0 , $P = 0.40$, $d = 0.31$, Table 2) or older group (EB: 7.1 ± 1.0 , vs. MN: 6.7 ± 1.2 , $P = 0.23$, $d = 0.47$, Table 2). However, BW-RE resulted in significantly lower RPE in both groups when compared with EB-RE and MN-RE (Young: 3.5 ± 1.3 vs. Old: 4.5 ± 1.8 , $P = 0.12$, $d = 0.59$, Table 2).

4.5. DISCUSSION

The aim of this study was to determine quadriceps muscle EMG activity in response to various RE modalities and ADL in healthy younger and older adults. In agreement with our primary hypothesis, we demonstrated EB-RE to elicit comparable quadriceps muscle EMG activity to that of MN-RE in both younger and older adults when exercise volume and intensity was matched. However, whilst, in older adults, BW-RE (i.e., chair squats) elicited ~27% of maximal quadriceps muscle EMG activity, this was significantly lower (~73%) than the quadriceps muscle EMG activity evoked by EB-RE and MN-RE. Finally, and in agreement with our secondary hypothesis, we observed significantly greater quadriceps muscle EMG activity during all RE modalities and ADL (walking and stair climbing) in older compared with younger adults when expressed relative to maximal muscle EMG. Taken together, these data suggest that relative muscular effort (of the quadriceps) required to complete RE and ADL is greater in older compared with younger adults, and that EB-RE, but not BW-RE, can modulate quadriceps muscle EMG activity to a similar extent to that of traditional MN-RE, in

healthy younger and older adults. Finally, and as expected, quadriceps muscle strength was ~46% lower in older compared with younger adults and this was associated with a ~60% lower peak quadriceps muscle EMG, used as an indirect measure of maximal muscle EMG activity.

Our observation that EB-RE, but not BW-RE, elicited similar increases in quadriceps muscle EMG activity compared with traditional MN-RE in healthy younger and older adults is in line with previous findings ^{683,697}. However, whilst our data suggest EB-RE and MN-RE elicit similar quadriceps muscle EMG activity, recent evidence suggests EB may, instead, provoke a greater increase in muscle activity compared with leg extension (~11%) and leg press (~27%) machine-based exercise, respectively, during recovery from total knee arthroplasty ⁶⁸³. Nevertheless, recent systematic reviews have shown no differences in voluntary muscle activity ⁶⁹⁸, muscle strength and function ⁶⁹⁹, between EB-RE and MN-RE in healthy populations. Indeed, longitudinal evidence regarding the use of EB-RE has shown significant improvements in body composition, strength and physical function in older, overweight adults following short-term training interventions (8-12 weeks) of a similar magnitude to that reported with MN-RE ⁷⁰⁰. This is consistent with MN-RE and EB-RE comparisons, overall observing similar increases in muscle strength and function in both healthy and sedentary females ^{556,701}. Nonetheless, collectively, the available data suggest EB-RE as a viable stimulus to support longer-term muscle remodelling in older adults, with implications for maintenance of strength, function, and quality of life.

Unsurprisingly, BW-RE elicited a lower degree of quadriceps muscle EMG activity compared with EB-RE and MN-RE in younger and older adults. Nonetheless, BW-RE may still provide a potent RE stimulus for older adults, particularly as this method of RE induced a 10-16% greater EMG response (relative to maximal EMG)

compared with all ADL (BW-RE: ~27% vs. Walking: ~11% vs. Stair Climbing: ~17%). Indeed, quadriceps muscle EMG activity during BW-RE, in older adults, represented ~73% of that observed during EB-RE and MN-RE, compared with ~42% in younger adults. Moreover, it is likely that prescribing BW-RE at an equivalent relative load to EB-ME and MN-RE (i.e., 12-RM), would lead to greater quadriceps EMG activity which, potentially, may translate to higher EMG activity across all muscles of the lower limb. Indeed, recent recommendations suggest bodyweight exercise to be performed to volitional fatigue ⁷⁰². In addition, it is likely that BW-RE may be more beneficial for adults with lower relative lean and concomitant higher levels of inert mass, contributing to a higher resistive load. BW-RE may, therefore, represent a feasible loading intervention to be completed as part of a home or community-based RE programme to increase muscle strength and function in older adults ⁶⁷⁴.

Irrespective of potential limitations, the potency of BW-RE as a contractile stimulus for muscle activation have been demonstrated in older adults ⁶⁸³. Specifically, Jakobsen and colleagues recently reported bodyweight sit-to-stand exercise resulted in a ~14% and 8% greater increase in voluntary muscle activity of the quadriceps compared with both leg press and knee extension machine exercises, respectively ⁶⁸³. In contrast, the present study did not achieve such high levels of quadriceps muscle EMG activity during BW-RE. This discrepancy is likely explained by differences in the study population. Specifically, the higher muscle EMG may be explained by atrogenic inhibition of the quadriceps muscles following knee surgery rehabilitation ⁶⁸³. Indeed, our data are in agreement with Fujita and colleagues who revealed that, with increasing age, muscle activity substantially increases during a 'sit-to-stand' squat exercise (Young, ~14%; Middle-aged, ~25%; Older adults ~52%; Frail older adults ~72%) ⁷⁰³. Furthermore, recent longitudinal evidence from the same group

demonstrated marked improvements in muscle strength (>22%) following a sit-to-stand exercise regime in frail institutionalised older adults ⁶⁷⁴. This, in combination with our data, suggests that BW-RE may be a potent and pragmatic RE stimulus particularly in older adults.

In younger adults, we reported quadriceps muscle EMG activity during a 15 m walk at ~2% relative to that achieved during MVC, which was significantly lower than quadriceps muscle EMG in older adults (~11%). These observations are comparable to observations in middle-aged adults (~10%) using similar EMG technology during ADL ⁶⁹¹. The differences in muscle EMG activity of older adults may be explained by lower relative lean mass, reduced muscular metabolic efficiency (i.e. increased consumption of ~7-20% more metabolic energy to walk a given distance compared to younger adults) ⁶⁹⁵, increased leg muscle coactivation (i.e. increased activation of agonist [quadriceps] and antagonist muscles [bicep femoris, gastrocnemius and tibialis anterior] during walking) ^{695,704,705} and impaired neuromuscular function (i.e. motor unit potential, compound muscle action potential) ⁷⁰⁶. This may be partially explained via the increase in muscle activation prior to foot contact when walking, which is accompanied by coactivation of antagonist muscles ⁷⁰⁷. This occurrence is favourable in older adults to maintain joint stability during gait and stair climbing as a method to avoid potential falls ⁷⁰⁸. Nevertheless, considering the relatively low muscle activation response to walking, relative to RE modes, it is likely that this mode of activity does not provide a sufficient stimulus in attenuating muscle loss with ageing. Furthermore, increased ambulation during disuse events (i.e., 2000 steps per/day on a hospital ward) was reported to have no effect on muscle mass in older adults ³⁸³. Moreover, maintaining or increasing habitual physical activity levels may be key to maintaining whole muscle neuromuscular characteristics as even brief periods of

disuse and lower limb immobilisation have been shown to result in catastrophic declines in neuromuscular function ⁷⁰⁹.

Quadriceps muscle EMG activity during the ascent of a stair climb task in older adults was ~20%, which is in agreement with previous research ⁶⁹¹. Interestingly, quadriceps muscle EMG activity was >3-fold greater in older compared with younger adults (~6%). As mentioned previously above, the increase in muscle EMG activity may be due to reduced muscle metabolic and neuromuscular efficiency. Indeed, stair climbing is strongly associated with functional performance in community-dwelling ⁷¹⁰ and functionally impaired institutionalized older adults. In addition, previous research has observed significant improvements in cardiorespiratory fitness and muscle power ⁷¹¹ following a stair climbing programme, suggesting that this type of exercise task may provide an important stimulus for maintenance of muscle mass, strength, and endurance in older adults ⁷¹². Indeed, a twelve-week bodyweight stepping training intervention in older women was associated significant improvements in thigh cross-sectional area volume (~2.8%) and isometric MVC strength (~9-15%) ⁷¹³, similar to that observed following a high intensity (2 x 10-15 repetitions, ~80% 1RM, 3d per week) RE programme ⁷¹⁴. The present study adds to the current body of evidence on muscle activity during stair climbing by characterising both the descending and ascending loading component. Specifically, we observed lower quadriceps muscle EMG activity during the descending loading pattern (Young, ~4%, Old, ~14%) compared with the stair ascent (Young, ~6%, Old, ~20%). The lower EMG activity during the descent may be partially explained by the heightened antagonistic muscle pre-activation and co-activation of biceps femoris and tibialis anterior associated with age-related leg stiffness ⁷⁰⁴. Nevertheless, descending stair walking has recently been observed to be superior to ascending stair walking following a 12-week training

intervention on markers of metabolic health ⁷¹⁵, quadriceps isometric strength and functional fitness ⁷¹⁶ in older adults with metabolic syndrome. Whilst the evidence regarding stair climbing as a potential exercise stimulus certainly lacks substantial acute and longitudinal physiological evidence, the current body of literature suggests that stair climbing should be incorporated habitually into ADL to help support the maintenance of muscle mass in older age. Moving forward, it would be prudent to understand the long-term muscle-specific and whole-body health benefits of stair climbing exercise in older individuals when compared directly to other accessible exercise modes (i.e., chair-based sit-to-stand and elastic bands).

Whilst textile embedded EMG shorts has previously been used in a variety of experimental settings ^{691,694,717}, we acknowledge that the sensitivity of the surface EMG technique does not allow us to explore complex neuromuscular patterns, compared with methods such as the application of indwelling intramuscular ^{667,718} and high-density EMG ⁷¹⁹, nor does it allow us to determine the EMG activity of the whole quadriceps muscle. The normalization of muscle EMG activity was conducted during knee extensor MVC at a 90° knee angle, comparable to the starting position of MN-RE and EB-RE. Whilst it has long been disputed whether to normalize EMG to isometric ⁷²⁰, dynamic-isokinetic ⁷²¹ and/or during locomotion ⁷²², it is pertinent to note that the most appropriate method of EMG normalization is lacking consensus ⁷²⁰. The present investigation also utilised an acute within-subject, within-muscle approach to compare and contrast muscle EMG activity across a range of activities and exercise modes to account for inter-individual variability and heterogeneity ⁶⁶⁹. However, despite similar investigations finding no differences between EB and MN-RE ^{679,683,723}, we are not able to infer whether EB-RE would be superior at supporting longitudinal

gains in skeletal muscle mass and strength compared with machine-based alternatives⁶⁶⁹.

4.6. CONCLUSION

In conclusion, the present study suggests that performing EB-RE, but not BW-RE, elicits similar increases in quadriceps muscle EMG activity to that of traditional machine-based resistance exercise in both younger and older adults. Conversely, precise exercise prescription, either by training to volitional failure, or adding external load may be necessary to augment quadriceps specific muscle activity in BW-RE in older adults. Moreover, EB and BW modes of RE do not require specialised equipment and/or facilities, and therefore, may be prescribed alone and/or in combination as a pragmatic and feasible home-based RE alternative to traditional machines for older adults to maintain and improve skeletal muscle mass, strength, and function. Finally, it is apparent that the declines in muscle mass and strength associated with ageing significantly increase the muscular effort of ADL and RE in older adults.

SUPPLEMENTARY DATA



Figure 5. Example of participant undertaking functional exercise tasks. Images from left to right: 15 m Walk (A, B, C), Stair Assent (D, E, F), Stair Descent (G, H, I)

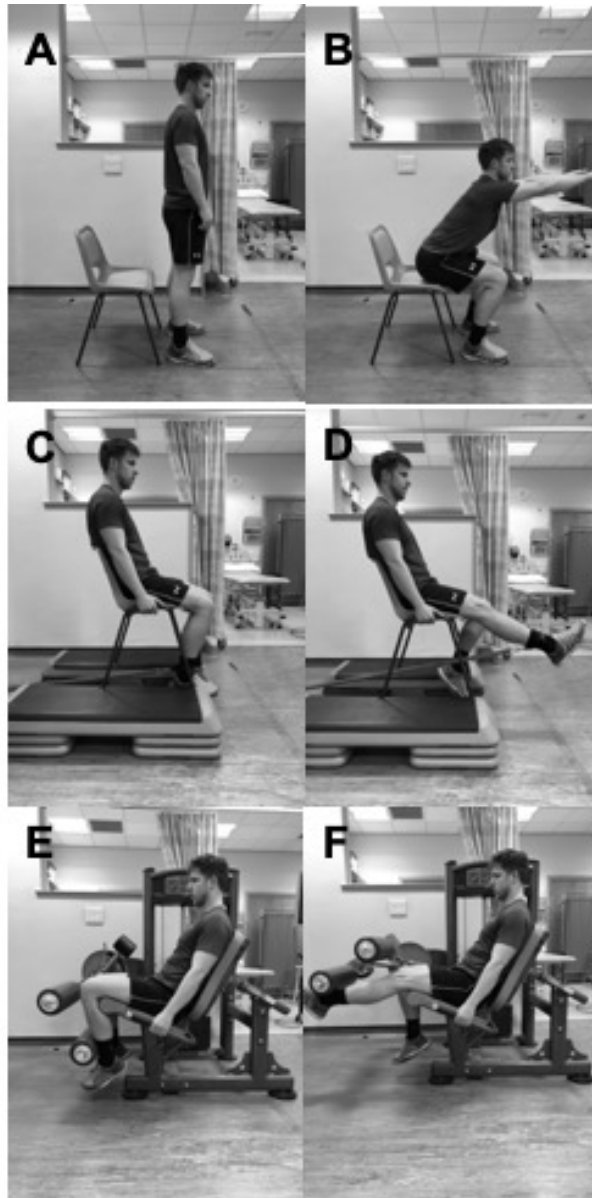


Figure 6. Example of resistance exercise modes. Start (A, C and E) and end position (B, D and F) for bodyweight chair squat (A, B), elastic band knee extension exercise (C, D), machine knee extension exercise (E, F), respectively.

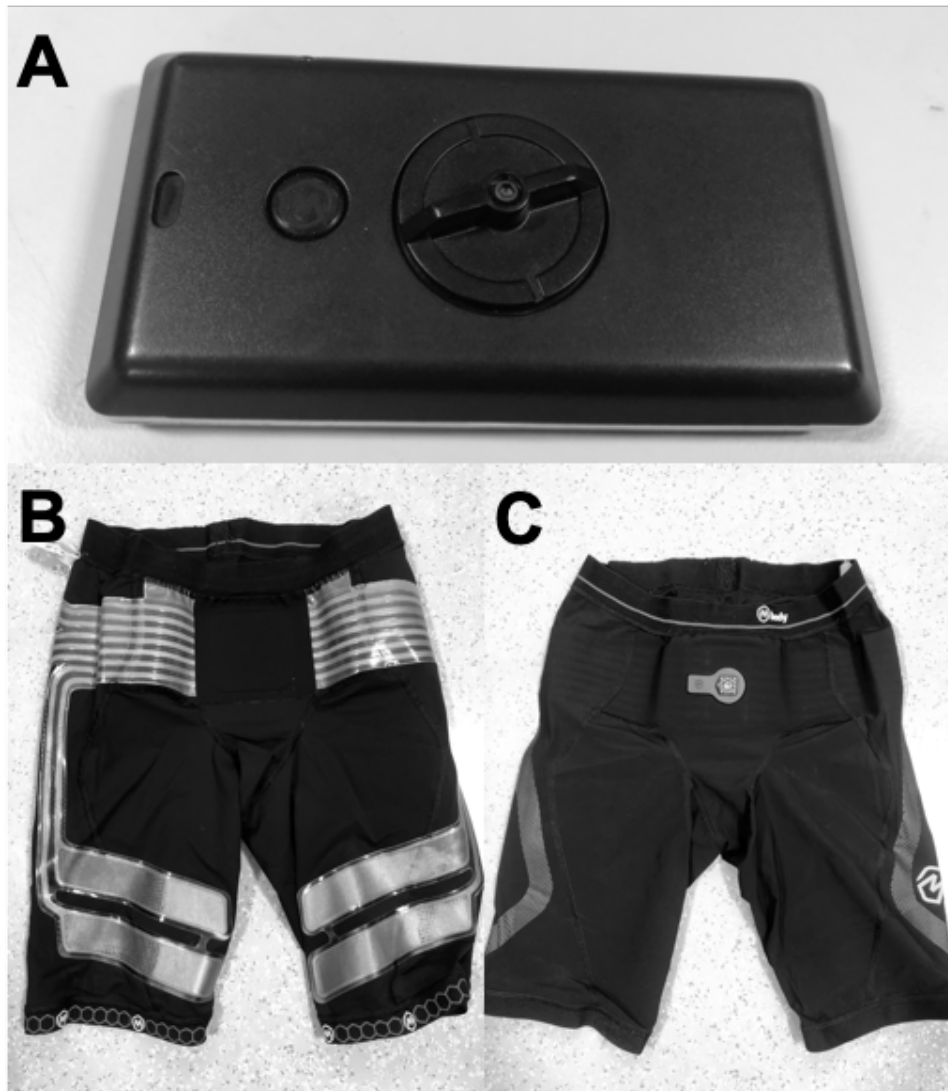


Figure 7. Image of textile EMG shorts. Textile-embedded EMG shorts from front (A) and inside Out (B) perspective.

CHAPTER 5

Myofibrillar Protein Synthesis and Acute Intracellular Signalling with Elastic Band Resistance Exercise in Young and Older Men

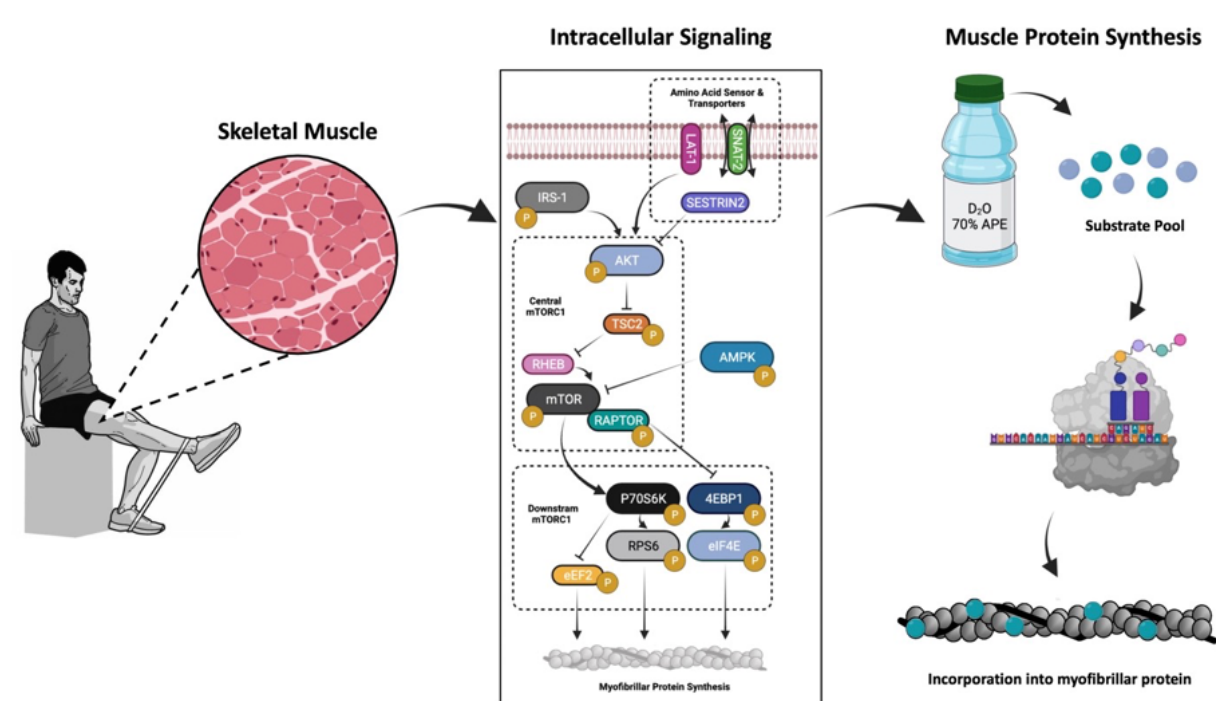


Published Peer-Reviewed Manuscripts from This Chapter:

Marshall, R.N., Morgan, P.T., Smeuninx, B., Quinlan, J.I., Brook, M.S., Atherton, P.J., Smith, K., Wilkinson, D.J. and Breen, L., Myofibrillar Protein Synthesis and Acute Intracellular Signaling with Elastic Band Resistance Exercise in Young and Older Men. *Medicine & Science in Sports & Exercise*, pp.10-1249.

5.1 ABSTRACT

Background: Resistance exercise training (RE) attenuates age-related declines in muscle mass and strength ('sarcopenia'). Compared with typical machine-based RE, the efficacy of cost-effective, accessible elastic band RE (EB-RE) for muscle adaptive remodelling lacks supporting mechanistic evidence. **Methods:** In a parallel study design of 8 young (YM; 24 ± 4 yrs) and 8 older (OM; 68 ± 6 yrs) males consumed an oral stable isotope tracer (D_2O) combined with serial vastus lateralis muscle biopsies to measure integrated myofibrillar protein synthesis (iMyoPS) and regulatory signalling over ~48hrs prior to (habitual) and following an acute bout of EB-RE (6 x 12 repetitions at ~70% of one-repetition maximum). iMyoPS was determined via gas chromatography-pyrolysis-isotope ratio mass spectroscopy and regulatory signalling expression by immunoblot. **Results:** EB-RE induced a significant ~32% increase in iMyoPS (Rested; $1.52 \pm 0.62\% \cdot \text{day}^{-1}$ vs Exercised; $1.99 \pm 0.73\% \cdot \text{day}^{-1}$, $P=0.007$), with no significant group (Rested; YM $1.62 \pm 0.08\% \cdot \text{day}^{-1}$ vs OM, $1.43 \pm 0.17\% \cdot \text{day}^{-1}$ Exercised; YM, $2.23 \pm 0.25\% \cdot \text{day}^{-1}$ vs OM $1.75 \pm 0.19\% \cdot \text{day}^{-1}$ $P=0.12$) or interaction effect ($P=0.35$). EB-RE increased the phosphorylation of key anabolic signalling proteins similarly in YM and OM at 1h post-exercise, including p-IRS-1^{Ser636/639}, p-Akt^{Ser473}, p-4EBP-1^{Thr37/46}, p-P70S6K^{Thr389} and p-RPS6^{Ser240/244}, whereas p-TSC2^{Thr1462} and p-mTOR^{Ser2448} increased only in YM (all $P<0.05$). There were no differences in expression of amino acid transporters/sensors or proteolytic markers following EB-RE. **Conclusion:** iMyoPS following EB-RE was elevated in both YM and OM. Furthermore, the increase in acute anabolic signalling following EB-RE was largely similar between YM and OM. In conclusion, EB-RE may support muscle mass and remodeling in younger and older adults. However, further work may be necessary to optimise its prescriptive programming.



5.2. INTRODUCTION

Ageing is associated with a loss of skeletal muscle mass, strength, and functional capacity (termed 'sarcopenia')²¹. Sarcopenia is associated with a loss of independence and increased morbidity and mortality risk⁶⁵⁵. Given the pivotal role of skeletal muscle mass in whole-body metabolic health, sarcopenia progression impairs basal and/or postprandial macronutrient handling⁵⁶⁶ and exacerbates age-related co-morbidities and chronic disease. Whilst age-associated muscle deterioration is evident in all humans, the prevalence of diagnosable sarcopenia (i.e., accelerated muscle loss) and musculoskeletal weakness in community-dwelling older adults (>65 years) in the UK has been reported to be >12.5%, rising to >20% in those aged >85 years⁶⁵⁶. Combined with a shift in population ageing demographics, sarcopenia presents an uncompromising economic burden on global healthcare services and resources²⁷.

It is the dynamic balance between muscle protein synthesis (MPS) and muscle protein breakdown (MPB) rates that results in either a net gain (synthesis>breakdown) or net loss (breakdown>synthesis) of muscle mass. Importantly, impairments in the exercise-induced stimulation of MPS have been reported with ageing (termed 'anabolic resistance') and have been suggested to underpin age-related muscle loss²⁴⁸. Resistance exercise (RE) is a potent non-pharmacological stimulus for muscle protein synthesis (MPS) and, therefore, is paramount to mitigate losses of skeletal muscle mass, metabolic dysfunction and physical disability associated with ageing. However, despite the well-established effects of RE on muscle mass, strength, and function, only ~10-15% of those aged 60 and over and even fewer of those aged 75 and over adhere to RE recommendations⁷²⁴. Importantly, machine-based RE in a typical gym environment presents numerous barriers to many older adults⁶⁷⁰. Hence,

pragmatic, and accessible RE interventions that can effectively maintain or increase muscle mass, strength and function in older age are required.

Elastic band RE (EB-RE) has been reported to increase muscle strength and functional capacity in healthy, institutionalized, and clinical populations ⁷²⁵. However, the potential of EB-RE to support robust muscle adaptive remodelling has been questioned, as the changing tensile properties of the band across the range-of-motion results in a variable load stimulus ⁷²⁶. This is noteworthy as older adults display a blunted acute MPS response to RE-induced mechanical contraction compared with younger adults ²⁴⁸ and require a greater RE stimulus to facilitate an MPS response that is comparable to the young ³¹⁷. We recently demonstrated that quadriceps muscle EMG activity increased with high-volume EB-RE in younger and older adults and was equivalent to machine-based RE ⁵⁵⁵. However, muscle EMG activity may not readily translate to true motor unit recruitment/activation, that is thought to be crucial for MPS and muscle hypertrophy ⁷²⁷. Currently, our understanding of the intramuscular metabolic and molecular response to EB-RE is limited to circulating plasma biomarkers of myogenic growth factors ⁷²⁵ and inflammatory proteins ⁷²⁸, whilst effects on MPS and associated regulatory signalling have yet to be investigated.

Therefore, the purpose of the present study was to assess the acute molecular and metabolic response to high-volume EB-RE in healthy untrained younger (YM) and older males (OM). Through combined oral deuterium oxide (D₂O) isotope tracer consumption and serial skeletal muscle biopsies, we determined the rates of integrated myofibrillar protein synthesis (iMyoPS) over 48h prior-to and following a bout of moderate-load, high-volume EB-RE. We also determined acute intracellular protein expression and phosphorylation of anabolic and proteolytic markers in YM and OM following EB-RE to probe possible underpinning mechanisms of iMyoPS

responsiveness. We hypothesized that high-volume EB-RE would elicit significant and equivalent increases in iMyoPS rates and intracellular regulatory signalling responses in YM and OM.

5.3. METHODOLOGY

5.3.1. Participants

Eight young (YM; 24 ± 4 years, BMI; 24 ± 4 kg·m²) and eight old (OM; 67 ± 5 years, BMI; 25 ± 2 kg·m²) untrained male volunteers provided written, informed consent to participate in the present study (**Table 1**). Participants were deemed healthy by the completion of a general health questionnaire. Briefly, participants were required to have normal blood chemistry, be normotensive (<140/90 mmHg), and not be prescribed medications. In addition, all participants had no prior RE experience (i.e., not having undertaken structured RE for at least 6-months previously). Ethical approval was obtained through the East Midlands – Derby Research Ethics Committee (18/EM/0004), conformed to the requirements of Research Governance at the University of Birmingham and was conducted in accordance with the Declaration of Helsinki.

5.3.2. Experimental Design

In a parallel study design, YM and OM were recruited to investigate iMyoPS in the habitual state and in response to a single bout of moderate-load, high-volume EB-RE on iMyoPS, alongside markers of intracellular regulatory protein expression. Following initial study screening and consenting, participants reported to the School of Sport, Exercise and Rehabilitation Sciences (SportExR) laboratory on four separate occasions over a seven-day period (**Figure 1**). For each visit, participants reported to

the laboratory at ~08:00 in an overnight fasted state, having been asked to refrain from caffeine consumption on the day of the trial. In addition, participants were asked to refrain from any strenuous physical activity and alcohol consumption for the duration of study involvement.

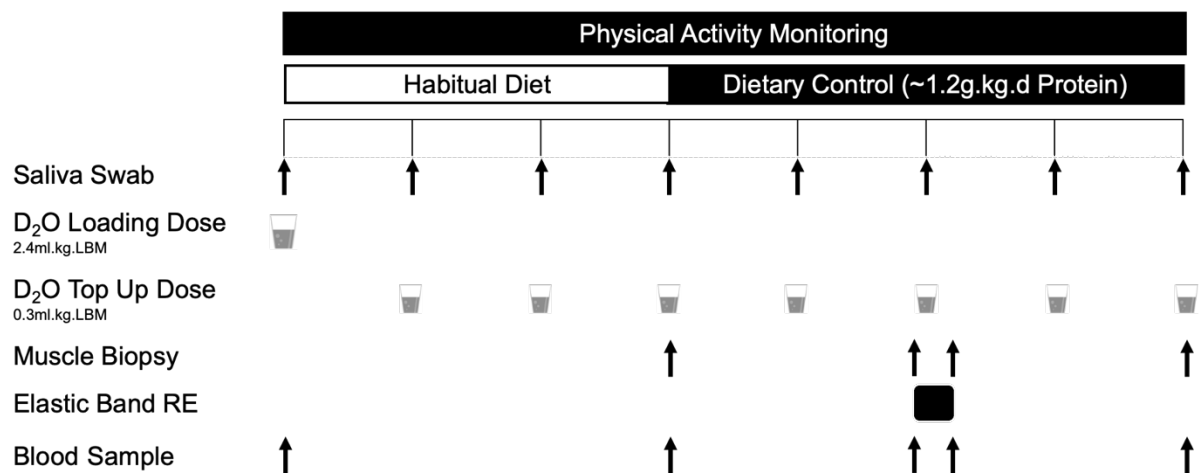


Figure 1. Study Schematic and Overview of Experiential Procedures.

5.3.3. Experimental Protocol

Visit One: An overview of the study timeline is provided in **Figure 1**. Upon arrival at the laboratory, participants underwent anthropometric and body composition assessments followed by a measurement of blood pressure. Thereafter a single baseline saliva sample was obtained, with further saliva samples collected daily throughout the duration of the study. Participants then completed a maximal isometric contraction (MVC) of the right and left quadriceps, bilateral 12 repetition maximum (12-RM) strength testing of the knee extensors and familiarization to the exercise protocol, as described below. Participants were then given a bolus of D₂O and daily top-up doses to measure iMyoPS, as per our previous work ¹¹³ and described below. Before departure, participants were provided with a wrist-worn pedometer to record their average total daily step count over the duration of the study.

Visit Two: Seventy-two hours following Visit 1 and following the provision of daily single saliva samples and consumption of a dose of D₂O, a muscle biopsy was collected from the quadriceps vastus lateralis (VL) of a single leg. Participants were provided with a weight maintenance food parcel, matched for total calories and macronutrient content for the subsequent 4 days to standardize dietary conditions, and reduce the influence of dietary variances between individuals (See 'Dietary Control').

Visits Three and Four: Forty-eight hours following Visit 2, participants provided a VL muscle biopsy (as described below). Participants then completed a single bout of leg RE, which involved performing 6 sets of 12 repetitions of leg extension exercise at ~70% (12 repetition maximum [12-RM]), as previously described ^{113,317}, with each set separated by 2 min of passive recovery. Immediately following the completion of the exercise bout, participants consumed a single 30 g bolus of whey protein concentrate and then rested for 1 h before another VL muscle biopsy was obtained to examine the

acute post-exercise anabolic signal molecule phosphorylation response. Forty-eight hours following visit 3, participants returned to the lab to provide a final saliva and VL muscle biopsy sample.

5.3.4. Experimental Procedures

5.3.4.1. Body Composition

Body composition was determined via bioelectrical impedance analysis, incorporating assessments of fat mass (FM), body fat percentage (BF%), fat-free mass (FFM) and skeletal muscle mass (SMM) (mBCA 525, SECA, Hamburg, Germany). Individuals were instructed to lay in a supine position for ~10 min to allow for any fluid shifts, and two electrodes were attached to each hand and foot.

5.3.4.2. Resistance Exercise Familiarization

The 12-RM load was assessed as previously described ⁵⁵⁵. Briefly, the 12-RM load was assessed using an elastic resistance band (PeakSupps, Bridgend, UK) with loads ranging from 'light' to 'very heavy' (6 kg, 12 kg, 20 kg, and 28 kg at 100% elongation of manufacturer guidelines), starting with a self-selected load estimated to be ~50% of the participants' 1-RM. Following the completion of the warm-up, the load was increased via the increased elongation of the band in 20 cm increments and/or an increase in band resistance (i.e., band colour) until the participant reached a load representing their estimated 12-RM (~70% 1-RM), as described previously. Participants were instructed to perform each repetition over a total of 3 s (eccentric phase: ~1.5 s and concentric phase: ~1.5 s), timed via a visual prompt to 'push' and 'relax', accompanied by the same verbal instructions from the experimenter.

5.3.4.3. Strength Testing

Maximal voluntary contraction (MVC) of the knee extensors were assessed as previously described using an isokinetic dynamometer (Cybex, division of Lumex, Inc., Ronkonkoma, New York, USA) for evaluation of quadriceps isometric muscle strength of both limbs. Briefly, participants were seated with the hip and knee joints at relative angles of 90° and 110°, respectively, with the tested limb, chest, and hips stabilized and adjusted so that the axis of rotation of the lever arm was in line with the lateral epicondyle of the femur. All participants completed 3 × 3 s MVC of the knee extensors, with each contraction separated by a 60 s passive recovery period. Participants were given standardized verbal encouragement for the duration of each 3 s contraction.

5.3.4.4. Dietary Control

Participants were provided with a standardized, weight-maintenance diet (~50% carbohydrate, ~30% fat and ~20% protein) to consume during the study (**Table 1**). Energy requirements were calculated using the Harris-Benedict equation multiplied by an activity factor of 1.35⁷²⁹. Participants could choose from a selection of food options, including several pre-packaged fresh, frozen, and microwavable options for lunch and dinner, along with pre-weighed breakfast ingredients (e.g., porridge oats/cereal, milk) and pre-packaged mid-meal snacks (snack bars, nuts, fruit). Water was consumed ad libitum, and pre-weighed fruit juice beverages were provided. Food was provided to participants between Visits 2 and 4 for consumption over the course of iMyoPS measurements. The content of the controlled diet was representative of values from our previous work on the habitual dietary protein amount and pattern of intake of community-dwelling OM¹. Participants also consumed a commercially available flavored protein beverage containing 30g of whey protein concentrate (Myprotein,

Northwich, United Kingdom) immediately following EB-RE at Visit 3 to facilitate exercise recovery.

5.3.4.5. Isotope Tracer Protocol

Stable isotopically labelled D₂O (70 atom%; Cambridge Isotope Laboratories, Inc, Tewksbury, Massachusetts, USA) was provided to participants throughout the duration of the study as previously described ¹¹³. Briefly, to rapidly increase the body water enrichment of deuterium (²H) to ~0.2% atom percent excess (APE), participants consumed a bolus dose of D₂O on day 1 (2.4 mL·kg lean body mass [LBM]⁻¹). Due to the current lack of consensus on D₂O dosing strategies, we sought to mimic steady-state enrichment often used with standard AA infusion studies and in line with recent recommendations and guidelines on the use of D₂O methodology in human research ²¹⁹. Therefore, to maintain a pseudo 'steady-state' of ~0.2% APE enrichment of the body water pool throughout the study, participants consumed a daily 'top-up' dose of D₂O of 0.3 mL·kg LBM⁻¹. Total body water enrichment was used as a surrogate for deuterated alanine labelling. All doses were consumed in the morning, immediately following saliva sample provision. These saliva samples were collected each morning immediately on waking in sterile plastic tubes (~5-10 ml). Samples were temporarily stored in the fridge (~5°C) and returned to the investigator at the next experimental visit. Saliva samples were subsequently centrifuged to separate salivary debris and pipetted into a 1.5 ml Eppendorf, sealed, and stored at -80°C until later analysis. Across the study, percutaneous skeletal muscle biopsy samples were obtained (Visits 2, 3 and 4) from the vastus lateralis under local anesthesia (5-10ml, 1% lidocaine) using the Bergström needle technique ⁷³⁰. Muscle biopsy tissue was quickly rinsed in ice-cold saline and blotted to remove any visible blood, fat, and connective tissue

before being immediately snap-frozen in liquid nitrogen (^2N) and stored at -80°C until further analysis.

5.3.5. Data Analysis

5.3.5.1. Body Water Deuterium Enrichment

Body water enrichment of deuterium was measured as previously described ⁴. Briefly, pure fractions of body water were extracted by heating 100 μl of saliva in an inverted 2 ml autosampler vial for 4 h at 100°C . Vials were then placed upright on ice to condense extracted body water and transferred to a clean autosampler vial ready for injection. Body water (0.1 μl) was injected into a high-temperature conversion elemental analyzer (Thermo Finnigan, Thermo Scientific, Hemel Hempstead, UK) connected to an isotope ratio mass spectrometer (Delta V Advantage, Thermo Scientific). For high-temperature conversion elemental analyzer (TC/EA) analysis of body water enrichment, following direct liquid injection into the TC/EA, where samples were immediately converted to H_2 gas, sample gases were directed to the isotope-ratio mass spectrometer, where the $^2\text{H}/^1\text{H}$ ratio was determined. The deuterium isotopic enrichment provided as $\delta^2\text{H}$ was converted to atom% using the following equation:

$$\text{Atom\%} = \frac{100 \times AR \times (\delta^2\text{H} \times 0.001 + 1)}{1 + AR (\delta^2\text{H} \times 0.001 + 1)}$$

where AR represents the absolute ratio constant for deuterium based on the Vienna standard mean ocean water (VSMOW), an isotopic standard, and equates to 0.00015595. This was then converted to APE by correcting for baseline sample, i.e.,

background enrichment. If any sample fell outside the instrument's dynamic range, these were re-injected or re-prepared.

5.3.5.2. Myofibrillar Bound Alanine Enrichment

Muscle protein-bound [^2H]-alanine enrichments were measured in muscle biopsy samples collected on days 3, 5 and day 7 of the experimental protocol in the rested (days 3-5) and post resistance exercise (days 5-7). Assessment of myofibrillar bound ^2H -Alanine was measured as previously described ⁴. Briefly, for isolation of myofibrillar protein, ~30mg of muscle was homogenized in ice-cold homogenization buffer (50mM Tris-HCL, 1mM EDTA, 1mM EGTA, 10mM B-glycerophosphate, 50mM NaF, 0.5mM Sodium Orthovanadate and a single EDTA-free protease inhibitor per 10mL of buffer). Samples were then placed on a vibrax shaker for 10 min at 1000rpm at room temperature before the sarcoplasmic supernatant was collected following centrifugation at 13,000 g for 15 min at 4°C. The myofibrillar pellet was solubilized in 0.3 M sodium hydroxide (NaOH) and separated from the insoluble collagen by centrifugation. The myofibrillar protein was precipitated using 1 M perchloric acid (PCA) and separated by centrifugation. Protein-bound amino acids were then released using acid hydrolysis by incubating at 110°C in 0.1 M HCl in Dowex H⁺ resin slurry overnight before being eluted from the resin with 2 M ammonium hydroxide (NH₄OH) and evaporated to dryness; amino acids were then derivatized as their n-methoxycarbonyl methyl esters. Dried samples were suspended in a solution composed of 60 µl distilled water and 32 µl methanol, and following vortex, 10 µl of pyridine and 8 µl of methyl chloroformate were added to the solution. Samples were vortexed for 30 s and left at room temperature for 5 minutes. The newly formed n-methoxycarbonyl methyl esters of amino acids were then extracted into 100 µl of

chloroform. A molecular sieve was added to each sample for ~20 s before being transferred to a clean glass gas chromatography insert, removing any remaining water by size exclusion absorption. Incorporation of deuterium into protein-bound alanine was determined by gas chromatography–pyrolysis–isotope ratio mass spectrometry (Delta V Advantage), as aforementioned, alongside a standard curve of known L-alanine-2,3,3,3-d4 enrichment to validate measurement accuracy of the instrument.

5.3.5.3. Calculation of Fractional Synthetic Rate

iMyoPS was calculated from the incorporation of deuterium-labelled alanine into protein, using the enrichment of body water (corrected for the mean number of deuterium moieties incorporated per alanine, 3.7, and the dilution from the total number of hydrogens in the derivative, i.e., 11) as the surrogate precursor labelling between subsequent biopsies ⁴. FSR was calculated as follows:

$$FSR = -\ln \left(\frac{1 - \left[\frac{(APE_{Ala})}{(APE_P)} \right]}{t} \right)$$

where APE_{Ala} is deuterium enrichment of protein-bound alanine, APE_P is mean precursor enrichment over the time period, and t is the time between biopsies.

5.3.6. Immunoblotting

Immunoblotting was performed on the sarcoplasmic fraction after extraction of the myofibrillar protein pellet, as previously described ²²⁵. The protein concentration of samples was determined by DC (detergent compatible) protein assay (Bio-Rad, Hercules, California, USA) using FLUOstar Omega (BMG Labtech, Aylesbury, UK) at

an absorbance of 750nm; samples were standardized to 3 ug/ul⁻¹ by dilution with 4× Laemmli loading buffer and heated at 95°C for 5 min. Precisely, 30 µg of proteins were loaded onto Criterion TGX Tris-Glycine–20-4% SDS-PAGE gels (Bio-Rad, Hercules, California, USA) for electrophoresis at 150 V for ~1 h. Samples were then transferred to polyvinylidene difluoride (PVDF; Whatman, Dassel, Germany) membranes for 60 min at 100 V. Membranes were subsequently blocked in 5% low-fat milk (diluted in Tris-buffered saline and 0.1% Tween-20 [TBS-T]) for 1 h at room temperature and then incubated in a rocking device overnight at 4°C in the presence of the following primary antibodies (1:1000 dilution in 2.5% BSA in TBS-T; see supplementary methods for primary antibodies). For each, membranes were washed 3 × 5 min in TBS-T, incubated in HRP-linked anti-rabbit (CST7074, 1:10000 in TBST) or anti-mouse (CST7076, 1:10000 TBST) IgG at room temperature for 1 h, before 3 × 5 min washes in TBS-T. Finally, membranes were exposed to chemiluminescent HRP Substrate (Millipore Corp., Billerica, MA, USA) for 2-3 minutes and visualized using a BOX Chemi XT4 Imager with GeneSys capture software (Syngene, Cambridge, UK). Bands were quantified using ImageJ software (National Institute of Health, Bethesda, MD). Relative arbitrary units were normalized to the total amount of protein loaded as visualized via Ponceau S staining ⁵⁸⁶. Specifically, bands were normalized to the ~45kDa actin band of Ponceau S stain as a loading control ⁶²⁵. No difference was detected between the ~45kDa actin band of Ponceau S stain across all samples. Following these corrections, where appropriate, the phosphorylation of proteins as a proxy of their activation is expressed relative to the total abundance of protein ⁵⁸⁷.

5.3.7. Statistical Analysis

Power analysis with an error of 0.05, power of 0.95, and effect size of 2.02, was performed using G x Power 3.1 analysis software (Heinrich Hein University, Duesseldorf, Germany) based on the RE-induced increases (~10%) in ~48hr iMyoPS in OM, as previously described ¹¹³. This produced a minimum sample size of eight participants per group. Differences in participant physical characteristics between groups were measured via independent t-tests. All other data sets (body water enrichment, iMyoPS%day⁻¹ and anabolic/catabolic signal transduction) were analysed by two-way, repeated-measures ANOVA with a Bonferroni correction using Prism v. 5 (GraphPad Software, La Jolla, CA, USA). Where the ANOVA revealed a significant interaction effect, post-hoc t-tests were completed using Bonferroni. Normal distribution was assessed using the Shapiro-Wilk test. Where sphericity was violated, a Greenhouse-Geisser correction factor was applied. The effect size (ES) of the changes in iMyoPS FSR were examined using Cohen's D. For all tests, results were considered statistically significant when $P < 0.05$. Data are presented as mean $\pm$ SEM unless otherwise stated, with individual data points presented on each figure.

5.4. RESULTS

5.4.1. Anthropometric, Activity, Exercise and Dietary Characteristics

Data are presented in Table 1. Skeletal muscle mass index ($P=0.029$), MVC ($P=0.004$) and MVC relative to skeletal muscle mass ($P=0.01$) were lower in OM compared with YM. There was no significant difference between YM and OM in fat mass, body fat percentage, fat-free mass, appendicular skeletal muscle index, absolute skeletal muscle mass, handgrip strength, 12-RM strength, or average habitual step count. With EB-RE, there was no difference in average Borg-CR10 rating or per set or the total training volume between groups. Average total energy intake was significantly lower in OM compared with YM ($P=0.048$), as was the absolute and relative carbohydrate intake ($P=0.001$ and 0.01 , respectively). There were no differences in absolute or relative dietary protein or fat between groups.

Table 1. Participants characteristics, exercise characteristics and dietary intake during experimental testing.

	Young (N=8)	Old (N=8)
Anthropometric and Strength Characteristics		
Age (Years)	24.3 ± 3.7	67.7 ± 5.6*
Body Mass (kg)	77.2 ± 10.7	76.8 ± 7.3
Height (cm)	176.9 ± 10.3	173.3 ± 7.1
BMI (kg.m ⁻²)	24.5 ± 1.9	25.5 ± 2.6
Fat Mass (kg)	19.8 ± 6.8	21.3 ± 6.8
Fat (%)	25.2 ± 5.9	27.3 ± 7.0
Fat Free Mass (kg)	57.3 ± 5.9	55.5 ± 4.1
Skeletal Muscle Mass (kg)	28.8 ± 3.3	26.0 ± 1.5
Skeletal Muscle Mass Index (%)	37.4 ± 2.4	34.0 ± 3.1*
ASMI/Height ² (Kg.m ⁻²)	12.0 ± 1.6	11.3 ± 1.0
Knee Extensor MVC (Nm)	288 ± 38	205 ± 33*
Hand Grip Strength (Kg)	51 ± 10	49 ± 4
Average Daily Step-Count	8524 ± 3363	7594 ± 2884
Exercise Characteristics		
Unilateral EB Knee Extension 12-RM (Kg)	23.4 ± 2.2	21.1 ± 3.0
Predicted EB Knee Extension 1-RM (Kg)	33.7 ± 3.2	30.9 ± 4.9
Training Volume (Kg)	1683 ± 160	1522 ± 220
Volume Per Set (Kg)	280.6 ± 27	254 ± 37
Average Borg CR-10 Rating	8.6 ± 0.8	8.2 ± 0.7
Experimental Dietary Intake		
Total Energy (Kcal.day ⁻¹)	2228 ± 249	1985 ± 193*
CHO (kcal.day ⁻¹)	1130 ± 86	943 ± 91*
CHO (g.kg.BM ⁻¹ .day ⁻¹)	3.71 ± 0.50	3.08 ± 0.33*
CHO (% Energy Intake)	51 ± 4	47 ± 5
Fat (kcal.day ⁻¹)	682 ± 110	661 ± 83
Fat (g.kg.BM ⁻¹ .day ⁻¹)	0.97 ± 0.16	0.96 ± 0.12
Fat (% Energy Intake)	31 ± 5	33 ± 4
Protein (kcal.day ⁻¹)	394 ± 48	381 ± 39
Protein (g.kg.BM ⁻¹ .day ⁻¹)	1.24 ± 0.03	1.24 ± 0.04
Protein (% Energy Intake)	18 ± 2	19 ± 2

Values are means ± SD for N=8 participants per group. MVC, Maximal Voluntary Contraction; 12-RM, 12 Repetition Maximum; 1-RM, 1 Repetition Maximum; CHO, Carbohydrate. Daily step count was recorded over the entire study period. * Indicates significantly different from young adults (P<0.05).

5.4.2. Integrated Myofibrillar Protein Synthesis Rates

Saliva ^2H enrichment increased significantly above basal values on Day 1, at 24h after D_2O consumption ($P < 0.05$ for YM and OM; **Table 2 and Figure 2**). Average Body water ^2H enrichment during both habitual (Days 3-5) and post-exercise (Days 5-7) phases of iMyoPS measurement were higher in OM compared with YM ($P < 0.01$ for both phases; **Table 2**). Steady-state enrichments were not achieved, with a significant drop in ^2H enrichments at day 2 and 5 in YM ($P < 0.05$, **Figure 2A**). Fitting of a Michaelis-Menten curve displayed a significantly lower k_m and $V_{\max}$ between YM and OM ($P < 0.05$) **Figure 2B**). EB-RE induced a significant $\sim 32\%$ increase in iMyoPS (Rested; $1.52 \pm 0.62\% \cdot \text{day}^{-1}$ vs Exercised; $1.99 \pm 0.73\% \cdot \text{day}^{-1}$, $P = 0.007$), with no significant group (Rested; YM $1.62 \pm 0.08\% \cdot \text{day}^{-1}$ vs OM, $1.43 \pm 0.17\% \cdot \text{day}^{-1}$ Exercised; YM, $2.23 \pm 0.25\% \cdot \text{day}^{-1}$ vs OM $1.75 \pm 0.19\% \cdot \text{day}^{-1}$ $P = 0.12$) or interaction effect (Age $\times$ Exercise $P = 0.35$). Exploratory post hoc analysis revealed rates of iMyoPS were significantly elevated above habitual rates following EB-RE in YM ($2.23 \pm 0.69\%/\text{day}^{-1}$; $P = 0.024$, ES 1.2) but not OM ($1.75 \pm 0.54\%/\text{day}^{-1}$; $P = 0.302$, ES 0.67) during the 48 h post-exercise period.

Table 2. Mean body water enrichment (APE)

	YL	OM #
Day		
1 *	0.180 ± 0.027	0.210 ± 0.053
3 – 5	0.182 ± 0.040	0.228 ± 0.032
5 – 7	0.197 ± 0.036	0.245 ± 0.034

Values are means ± SD. YM, Young Males, OM, Older Males. 1 represents mean body water enrichment 24-h after the first dose of deuterated water (D₂O). 3 – 5 and 5 – 7 represent the habitual and post-exercise phases, respectively. *Significantly above basal values at 24h after consumption of the first D₂O dose (P < 0.05). #OM significantly different from YL (P < 0.01).

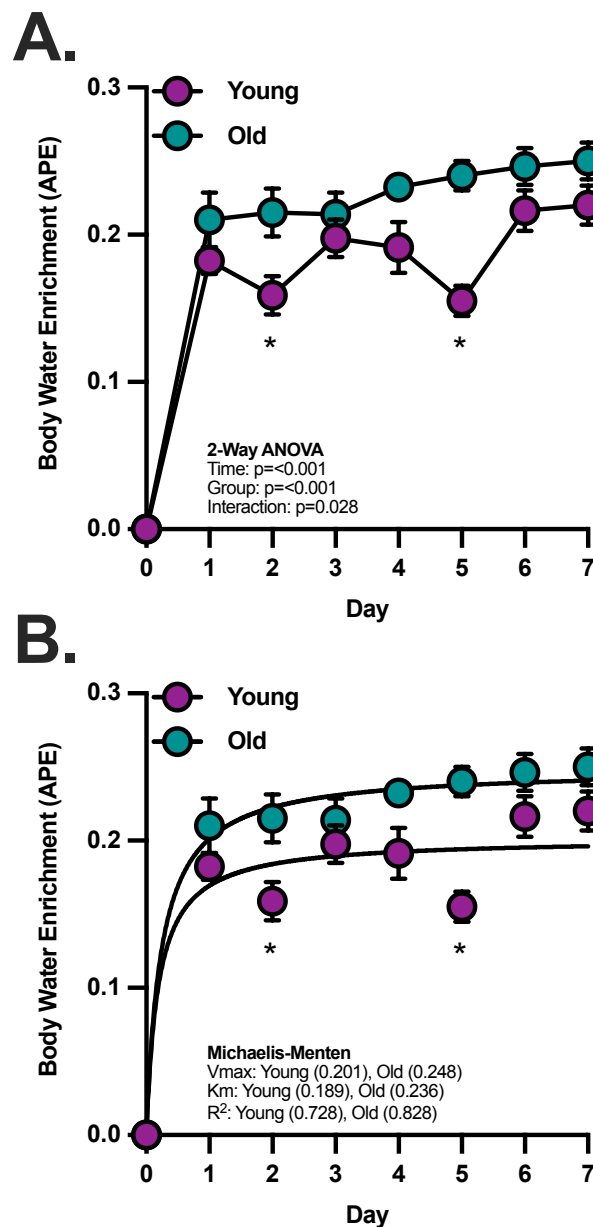


Figure 2. Saliva Body Water Enrichment. Deuterium enrichment of the body water, measured in APE from daily morning saliva samples following deuterium oxide ingestion expressed as absolute daily enrichments (A) and fitted Michaelis-Menten curve (km) (B) in younger adults (Purple) and older adults (Green) in 48hr rested (days 3-5) and 48hr following EB-RE (days 5-7). Values are presented as mean $\pm$ SEM for $n=8$ subjects per group. * Indicates significant group effect ($P < 0.05$).

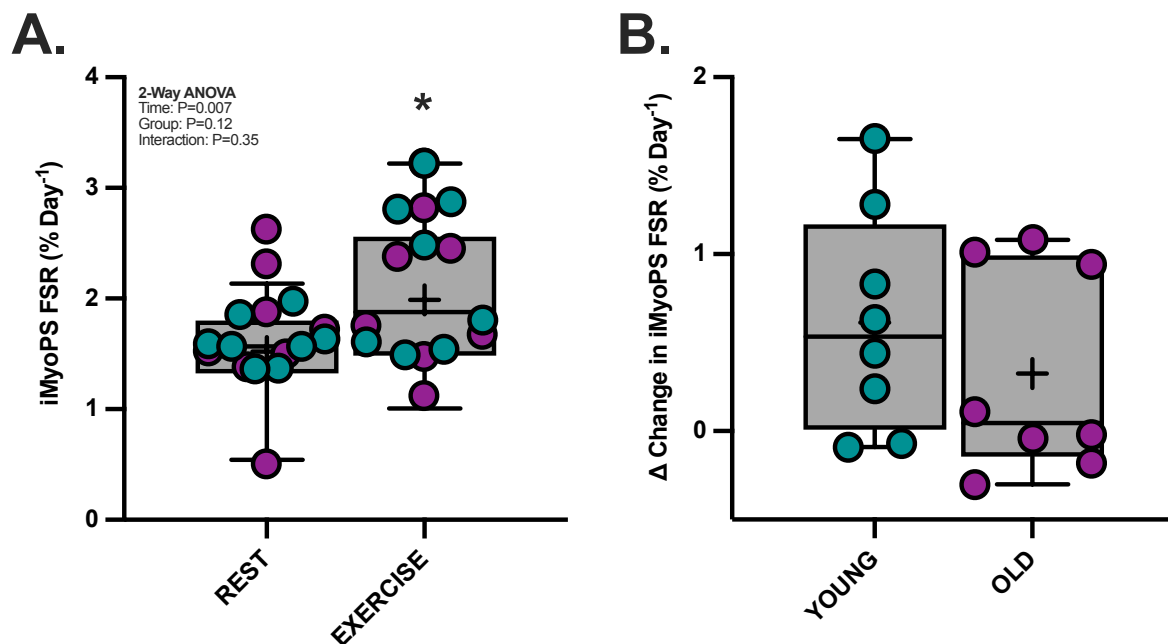


Figure 3. Myofibrillar protein synthesis. Integrated myofibrillar protein synthesis rates in a rested and exercised state following a single bout of elastic band resistance exercise in untrained younger (**Green Circles**) and older adults (**Purple Circles**) (A). Delta change in integrated myofibrillar protein fractional synthesis rates from habitual to 48h post-exercise (B). Values are presented as mean $\pm$ 9-95 percentile for $n=8$ subjects per group with individual data points. * Indicates significant time effect ($P<0.05$).

5.4.3. Intracellular Protein Expression and Phosphorylation

5.4.3.1. Amino Acid Transporters & Upstream mTORC1

No differences were detected in the expression of LAT1, SNAT-2 and Sestrin-2 between YM and OM or with EB-RE (**Figure 4A-C**). The expression of p-IRS1^{Ser636/639} increased following EB-RE in YM (~106%, $P<0.001$) and OM (~79%, $P<0.001$) with no difference between groups (**Figure 4D**). The expression of p-Akt^{Ser473} increased following EB-RE in YM (~181%, $P<0.001$) and OM (~124%, $P=0.001$) and was lower across rest and post-exercise in OM compared with YM (~33%, $P=0.008$; **Figure 4E**). The expression of p-TSC2^{Ser939} was lower in OM than YM (~52%; $P=0.003$), with no effect of EB-RE in either group ($P=0.73$, **Figure 4F**). The expression of p-TSC2^{Thr1462} increased following EB-RE in YM (~65%, $P=0.027$), but not OM (**Figure 4H**). No differences were detected in the expression of p-TSC2^{Ser1387} between groups or with EB-RE (**Figure 4G**).

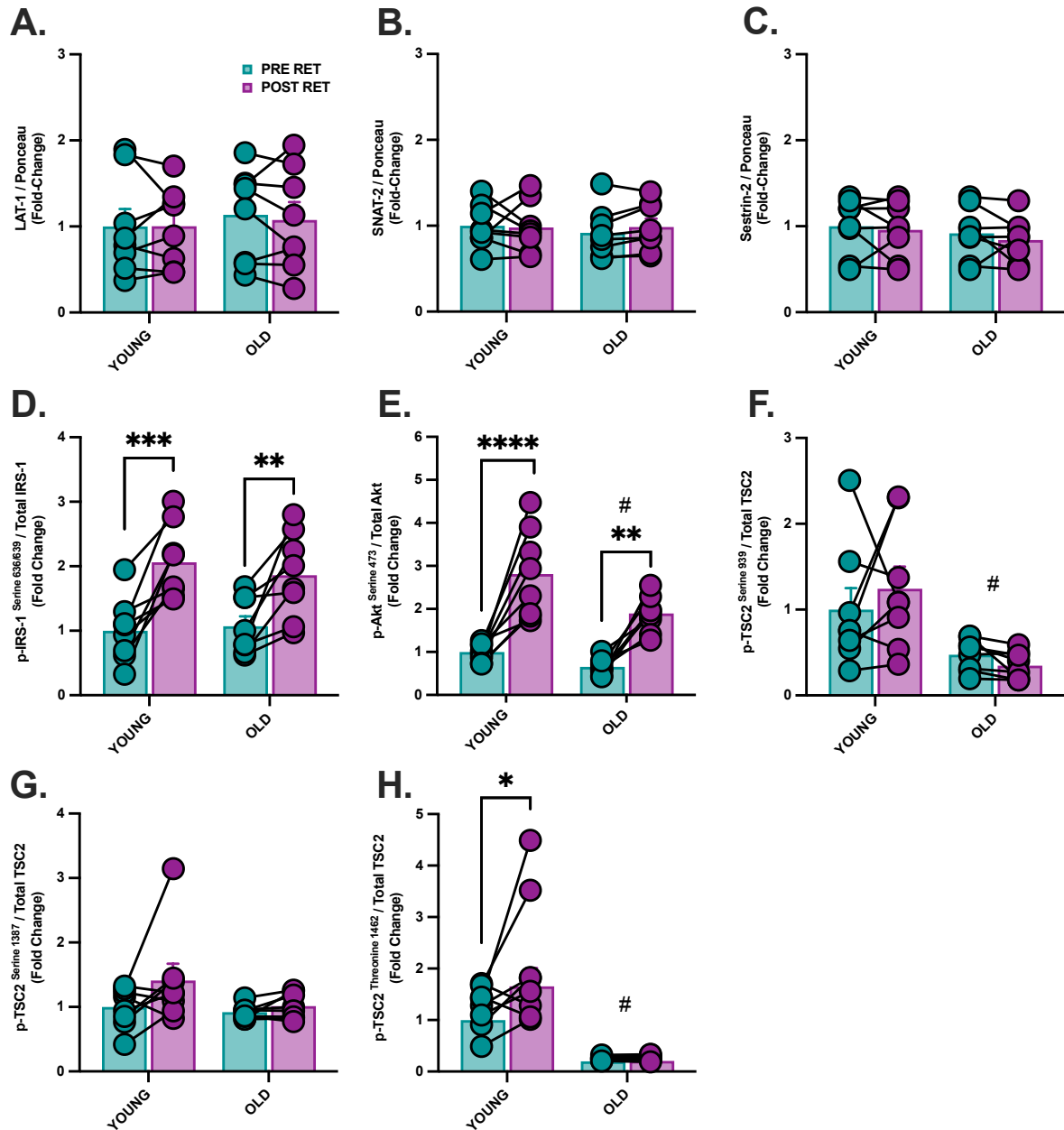


Figure 4. Amino acid transporters and upstream mTOR signalling. Protein expression of LAT-1 (A), SNAT-2 (B), Sestrin-2 (C), p-IRS1^{Ser636/639}/total-IRS1 (D), p-Akt^{Ser473}/total-Akt (E), p-TSC2^{Ser939}/total-TSC2 (F), p-TSC2^{Ser1387}/total-TSC2 (G), p-TSC2^{Thr1462}/total-TSC2 (H), before (Green) and 1-hour post (Purple) elastic band knee extension exercise in younger and older adults. Data are presented as fold-change relative to pre-RE for young and mean $\pm$ SEM for $n=8$ subjects per group with individual data points. * Indicates significant time effects. # Indicates significant group effect ($P<0.05$).

5.4.3.2. Central & Downstream mTORC1

The expression of Rheb expression decreased following EB-RE in OM only (~9%, $P=0.024$; **Figure 5A**) but did not differ between groups. The expression of p-Raptor^{Ser792} did not differ between groups or with EB-RE (**Figure 5B**). The expression of p-mTOR^{Ser2448} increased following EB-RE in YM (~51%, $P=0.027$), but not OM (**Figure 5C**). The expression of p-AMPK α ^{Thr172} did not differ between groups or with EB-RE (**Figure 5D**). The expression of p-4EBP-1^{Thr37/46} increased following EB-RE in YM (~48%, $P<0.001$) and OM (~59%, $P<0.001$) with no difference between groups (**Figure 5E**). The expression of p-P70S6K^{Thr389} increased following EB-RE in YM (~550%, $P<0.001$) and OM (~424%, $P<0.001$) with no difference between groups (**Figure 5F**). The expression of p-RPS6^{Ser240/244} increased following EB-RE in YM (~1030%, $P<0.001$) and OM (~1005%, $P<0.001$) with no difference between groups (**Figure 5G**). The expression of p-eIF4E^{Ser209} did not differ between groups or with EB-RE (**Figure 5H**). The expression of p-eEF2^{Thr56} did not differ between groups or with EB-RE (**Figure 5I**).

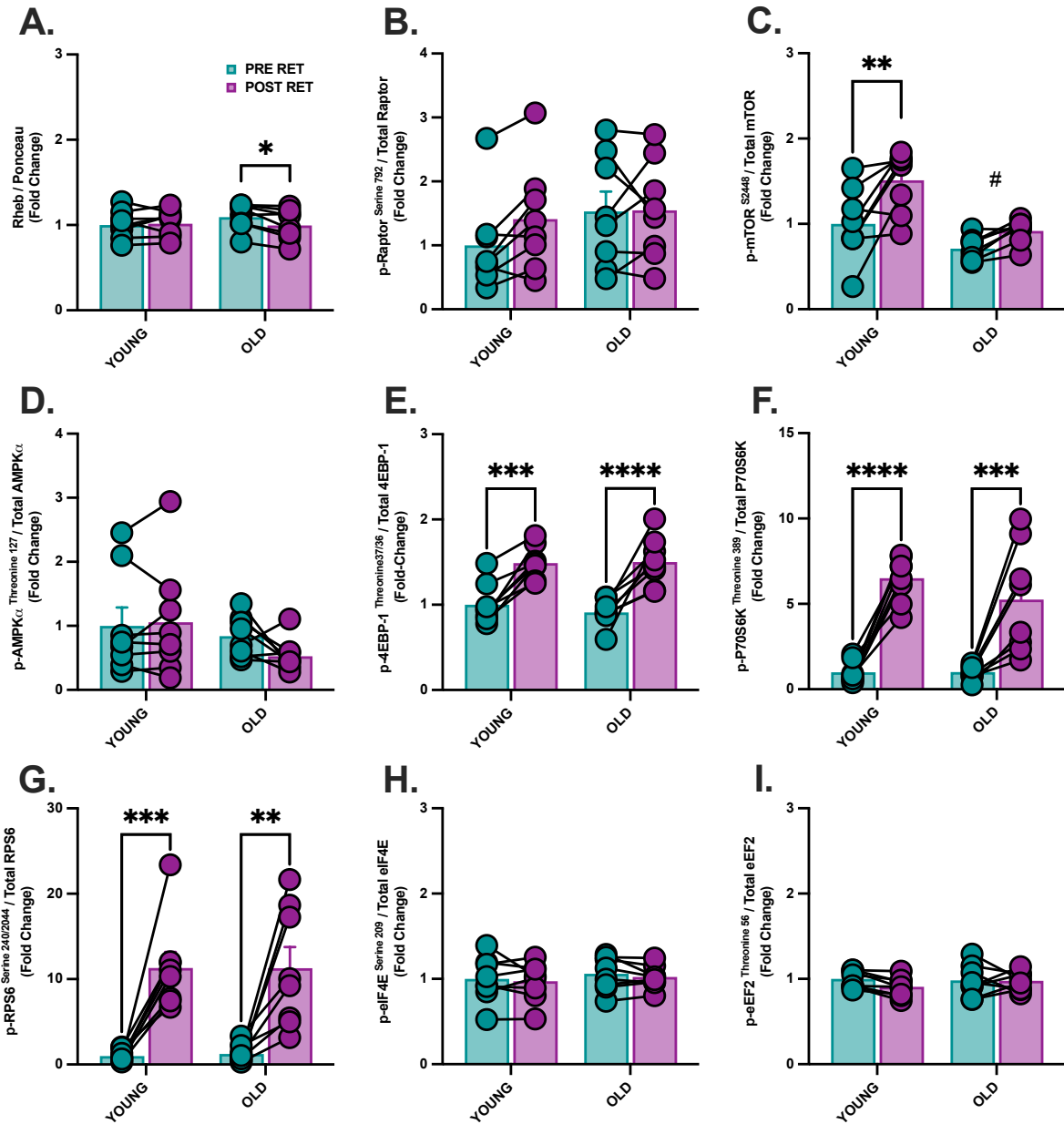


Figure 5. Central and Downstream mTOR Signalling. Protein expression and phosphorylation levels of Rheb (A), p-Raptor^{Ser792}/total-Raptor (B) p-mTOR^{Ser2448}/total-mTOR (C), p-AMPK^{Thr172}/total-AMPK α (D), p-4EBP-1^{Thr37/46}/total-4EBP-1 (E), p-P70S6K^{Thr389}/total-P70S6K (F), p-RPS6^{Ser240/244}/total-RPS6 (G), p-eIF4E^{Ser209}/total-eIF4E (H) p-eEF2^{Thr56}/total-eEF2 (I) before (Green) and 1-hour post (Purple) elastic band knee extension exercise in younger and older adults. Data are presented as fold-change relative to pre-RE young and mean $\pm$ SEM for n=8 subjects per group with individual data points. * Indicates significant time effects. # Indicates significant group effect (P<0.05).

5.4.3.3. Proteolytic Signalling

The expression of proteins involved in protein ubiquitylation and proteasomal degradation, MuRF1, MuRF2, MuRF3, Atrogin-1, Caspase-3, Proteasome 19S ATPase subunit, 19S RPT5/S6A subunit, or 20S B5 subunit were not significantly altered following EB-RE in YM or OM (**Figure 6A-H**). In OM, the expression of Proteasomal 19S ATPase subunit (~30%, $P=0.002$) and 19S RPT5/S6A subunit (~41%, $P=0.001$) were greater, and MuRF2 expression lower (~43%, $P=0.004$) than YM.

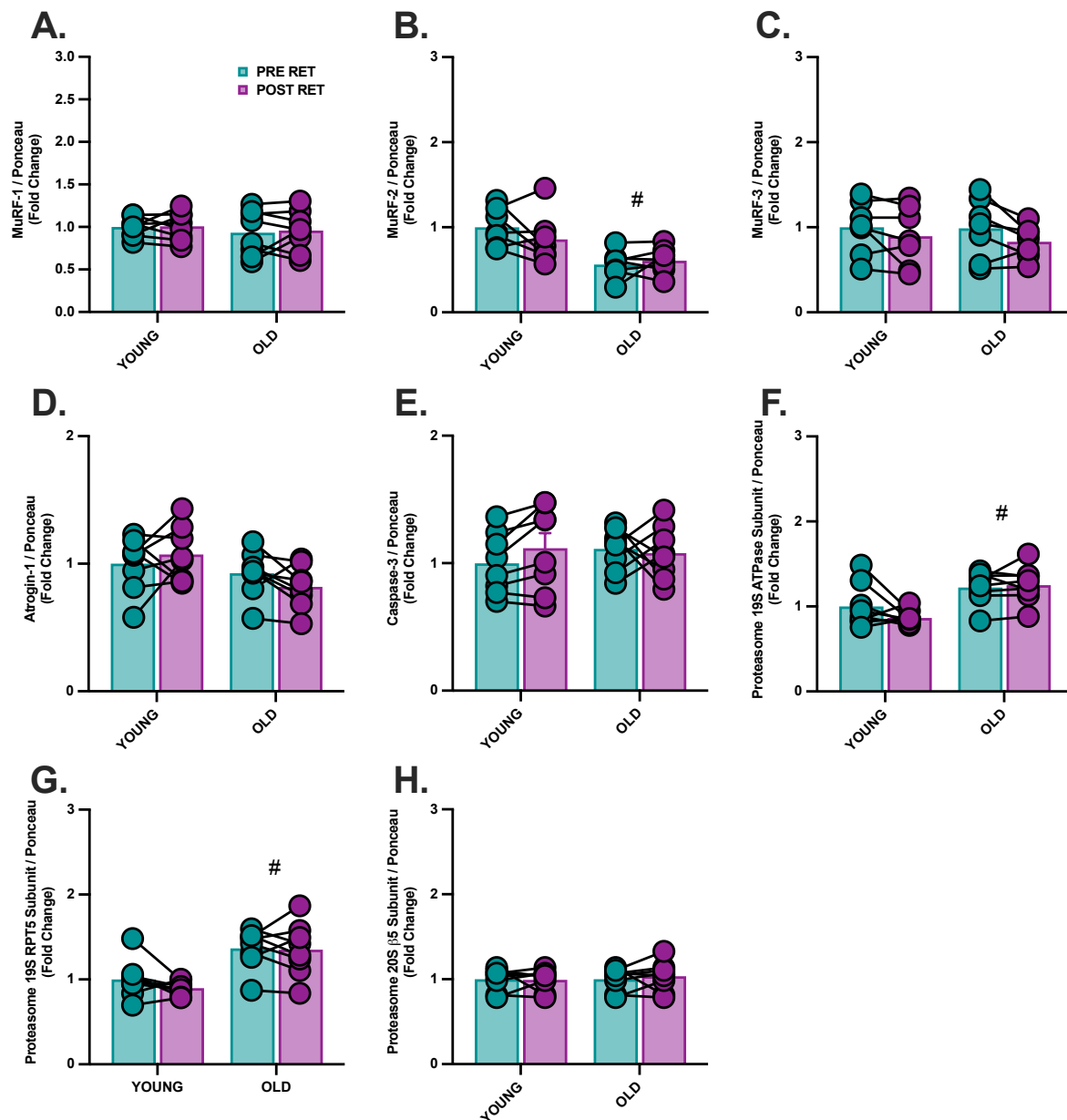


Figure 6. Proteolytic and Catabolic Markers. Protein expression levels of MuRF1 (A), MuRF2 (B), MuRF3 (C), Atrogin-1 (D), Caspase-3 (E), Proteasome 19S ATPase Subunit (F), Proteasome 19S RPT5/S6A Subunit (G), Proteasome 20S B5 Subunit (H), before (Green) and 1-hour post (Purple) elastic band knee extension exercise in younger and older adults. Data are presented as fold-change relative to pre-RE for young and mean $\pm$ SEM for n=8 subjects per group with individual data points. # Indicates significant group effect (P<0.05).

5.5. DISCUSSION

Elastic band resistance exercise (EB-RE) represents a cost-effective, accessible means to support or augment muscle mass and function with advancing age and/or scenarios of clinical rehabilitation. In some instances, longer-term EB-RE has been shown to induce morphological and metabolic changes ^{557,676}. However, mechanistic evidence to support the muscle anabolic potential of EB-RE in young and older adults is lacking. Herein, we provide the first investigation of integrated myofibrillar protein synthesis (iMyoPS) and regulatory intramuscular signaling following a single bout of EB-RE in younger (YM) and older males (OM). Rates of iMyoPS following EB-RE were significantly increased above rested values in both YM and OM. There was also evidence of anabolic signaling augmentation at 1h following EB-RE in YM and OM, with only minor differences between groups. Taken together, our data suggest that EB-RE may provide a stimulus for muscle adaptive remodeling.

In contrast to the constant load-resistance of machine-based RE, the variable load-resistance and mechanical stimulus with EB-RE ⁷³² may be sub-optimal to stimulate muscle anabolism and support longer-term muscle adaptive remodeling. This may be particularly relevant for older adults, who display an impaired acute and iMyoPS response to machine-based RE contractions ²²⁵. We previously demonstrated that the present EB-RE protocol augmented quadriceps EMG activity in young and older adults ⁵⁵⁵, to a level that was comparable with machine leg extension RE of similar volume-load, despite the target load of ~70% 1RM only being reached at the top of the concentric phase with EB-RE ⁵⁵⁵. Extending on these findings, we demonstrate that high-volume EB-RE performed to a high degree of effort elicits a muscle anabolic response in both YM and OM. Given EB-RE elicited a significant iMyoPS response in YM and OM was not surprising given that this protocol was

standardized equivalent to, or more than, previous machine-based RE regimens that elicited robust acute MyoPS and 48 h iMyoPS responses in OM ²²⁵. The observed iMyoPS response to EB-RE in YM and OM aligns to rates reported over 48h following high-volume machine-based RE in YM (~30-40% above rest) ⁷³³, demonstrating the muscle anabolic potential of this exercise modality. EB-RE variables may require manipulation to induce greater mechanical/metabolic stress to maximize iMyoPS in younger and older adults, for example by increasing total volume with additional sets/repetitions, although this conjecture requires further investigation. Similarly, future studies should compare the muscle remodelling response to longer-term, and repeated bouts of EB-RE and machine-based RE in young and older adults, which should be considered alongside age- and mode-specific strength and functional adaptations.

The muscle anabolic response to RE contractile loading and protein ingestion is thought to be underpinned by alterations in proteins implicated in the cellular growth signalling cascade of mTOR, and the ubiquitin-proteasomal degradation system, with suggestions that signal transduction is impaired in OM compared with YM ²⁴⁸. In the present study, we observed a robust acute regulatory signalling response at 1 h after EB-RE (combined with whey protein ingestion) that was largely similar between in YM and OM, despite an absent iMyoPS response in OM. The sensitivity of skeletal muscle and the ability to transport circulating amino acids (AA) into the cytosol has been reported to be impaired in OM, resulting in a blunted MyoPS capacity ⁷³⁴. Here we report no differences in the expression of key AA transporters/sensors, LAT-1, SNAT-2, or Sestrin-2 with age or 1 h after EB-RE and whey protein ingestion, similar to recent work from others in YM following combined RE and EAA ingestion ⁷³⁵. Although Sestrin-2 has been reported to be a key leucine sensor *in vitro*, there is conflicting

evidence around the response of Sestrin-2 with ageing, acute RE, and protein feeding⁷³⁶. Our data add to the conflicting evidence of amino acid transporters/sensors and their putative role in exercise and feeding-induced muscle anabolism. Indeed, whilst we observed minimal differences in protein expression, age-related impairment in activation and/or cellular location of these AA transporters/sensors with RE and feeding requires further investigation.

In upstream mTOR signaling, we observed a robust increase in p-IRS-1^{Ser636/639} and p-Akt^{Ser473} 1 h after EB-RE in YM and OM. Interestingly, there was evidence of divergent p-TSC2 and p-mTOR signalling 1 h after EB-RE between YM and OM. TSC2 phosphorylation is thought to be essential for mTOR co-localization to the lysosome and subsequent activation of downstream mTOR signaling intermediates for skeletal muscle remodeling⁷³⁷. However, the role of TSC2 in human skeletal muscle is mainly confined to pre-clinical models⁷³⁸ or RE and feeding studies in YM only³²⁴, and precise changes in mTOR expression with ageing and/or RE are still largely debated⁷³⁹. It is also worth noting that the absent p-mTOR response to EB-RE in OM was only evident when p-mTOR was expressed against a background of greater total mTOR protein compared with YM (data not reported) as has been reported by others^{740–742}. In any case, the minor variations in central mediators of mTORC1 expression did not alter downstream S6K, rpS6, and 4EBP-1 phosphorylation, and were all similarly increased above resting levels in YM and OM at 1 h after EB-RE. Taken together, these data demonstrate a largely similar anabolic signaling response to high volume EB-RE in YM and OM, despite some difference in the 48-hour iMyoPS, congruent with findings from machine-based RE of similar volume-load to the present study^{248,317}. Whilst we acknowledge some discrepant findings between markers of anabolic signaling and iMyoPS, it is worthy of note that the acute (1 h) signaling response to

RE (with or without feeding) does not always correspond with longer-term changes in MPS and functional outcomes ²⁷¹ and, thus, may partly explain the discord between acute anabolic signaling and iMyoPS in the present study.

Changes in proteolysis and associated signalling events may play an important role in the overall net muscle protein balance to RE ⁷⁴³. There is also a suggestion that proteins involved in the ubiquitin-proteasome system (i.e., MuRF1 and Atrogin-1) are elevated with ageing ⁷⁴⁴. Therefore, we were interested in how EB-RE and ageing altered proteolytic signalling expression. The present data suggest that the proteolytic signalling was not altered over 1 h after EB-RE and was largely similar between YM and OM. Whilst our findings are congruent with earlier work showing no differences in MuRF1 protein expression between YM and OM at rest or following machine-based RE ⁷⁴⁵, others have reported alterations in proteolytic signalling following machine-based RE, albeit with limited differences between younger and older adults ⁷⁴⁶. Any divergence in proteolytic signalling between the present study and that of Fry et al ⁷⁴⁶ could be due to differences training mode/volume or the timeframe and technique of signalling analysis (mRNA expression vs protein expression). It is also possible that whey protein ingestion immediately following EB-RE in the present study may have altered proteolytic signalling ⁷⁴⁵ and masked any potential influence of EB-RE and/or ageing. Nonetheless, our analysis is confined to protein rather than mRNA expression, making direct comparisons to earlier work challenging. Given the reported discord between acute proteolytic signalling events and dynamic rates of MPB ⁷⁴³, we cannot rule out that MPB was altered by EB-RE and/or ageing. However, others have reported that MPB rates were not altered at 24 h post-RE and did not differ between young and older adults ⁷⁴⁶. Interestingly, we did observe age-related alterations in MuRF2 and proteasomal subunits; 19S ATPase and 19S RPT5. Greater temporal resolution

through serial biopsy sampling is required to better understand the proteolytic response to exercise stimuli and potential influence of ageing and should allied to more advanced techniques to study dynamic proteolytic signaling processes.

Whilst the present study sheds important light on the mechanisms of muscle adaptive remodeling with EB-RE, we acknowledge several limitations. Firstly, the measurement of iMyoPS using orally ingested D₂O does not account for temporal changes in iMyoPS in the earlier recovery phase after EB-RE (e.g., <48h). Indeed, age-related differences in acute MyoPS have been reported over several hours after machine-based RE using isotopically labelled AA tracer infusion ^{317,731}. Nonetheless, compared with acutely measured MyoPS, the measurement of iMyoPS via D₂O under free-living conditions may better reflect longer-term muscle adaptive remodeling ⁷⁴⁷. We were unable to detect a significant *group x time* interaction in iMyoPS, therefore, suggesting underpowered sample size to determine age-related differences in iMyoPS between YM and OM. Additionally, we were unable to achieve steady-state ²H enrichments in YM saliva, which may reflect the higher observed iMyoPS in YM. We were unable to determine changes in regulatory signaling expression beyond 1h of recovery from EB-RE and protein feeding, which may have differed between YM and OM, as reported elsewhere ²⁴⁸. Finally, it is possible that the standardized dietary protein intake (~1.24g/kg/day) may have been insufficient to support maximal iMyoPS stimulation over the 48h after EB-RE. For example, RE-induced fat-free mass accretion has been reported to increase up to ~1.6g/kg/day of dietary protein ⁶⁰⁴, and the protein dose requirement for maximal post-RE MyoPS stimulation is greater in OM vs. YM ¹⁵⁹. Furthermore, dietary control may minimise the ecological validity of the present study, as we may have provided more or indeed less protein than the participant habitually consumes, thereby influencing daily MyoPS rates. The

implementation of dietary monitoring, to allow research volunteers to consume a habitual eating pattern may have improved the translation of the present investigation. Interestingly, no difference in rested or post-RE iMyoPS have previously reported between older individuals consuming 0.8 or 1.2 g/kg/day of dietary protein ⁴⁷¹. Hence, it is possible that dietary protein intake beyond that provided in the present study could have augmented iMyoPS stimulation following EB-RE, particularly in OM. However, it is important to note that dietary protein intake in the present study was standardized at levels typical of community-dwelling OM ¹.

5.6. CONCLUSION

In conclusion, a single bout of high-volume EB-RE significantly increased the signalling phosphorylation of mTORC1 intermediates in YM and OM, with only minor differences between groups. Post-exercise rates of iMyoPS were elevated above habitual values in both YM and OM. These findings suggest that a single bout of high-volume EB-RE may promote robust muscle adaptive remodelling in YM and OM to facilitate muscle mass accrual. However, further work is required to refine EB-RE strategies for optimal muscle mass outcomes for young and older adults across the health spectrum, in particular, the use of EB-RE during periods of musculoskeletal disuse.

SUPPLEMENTARY DATA

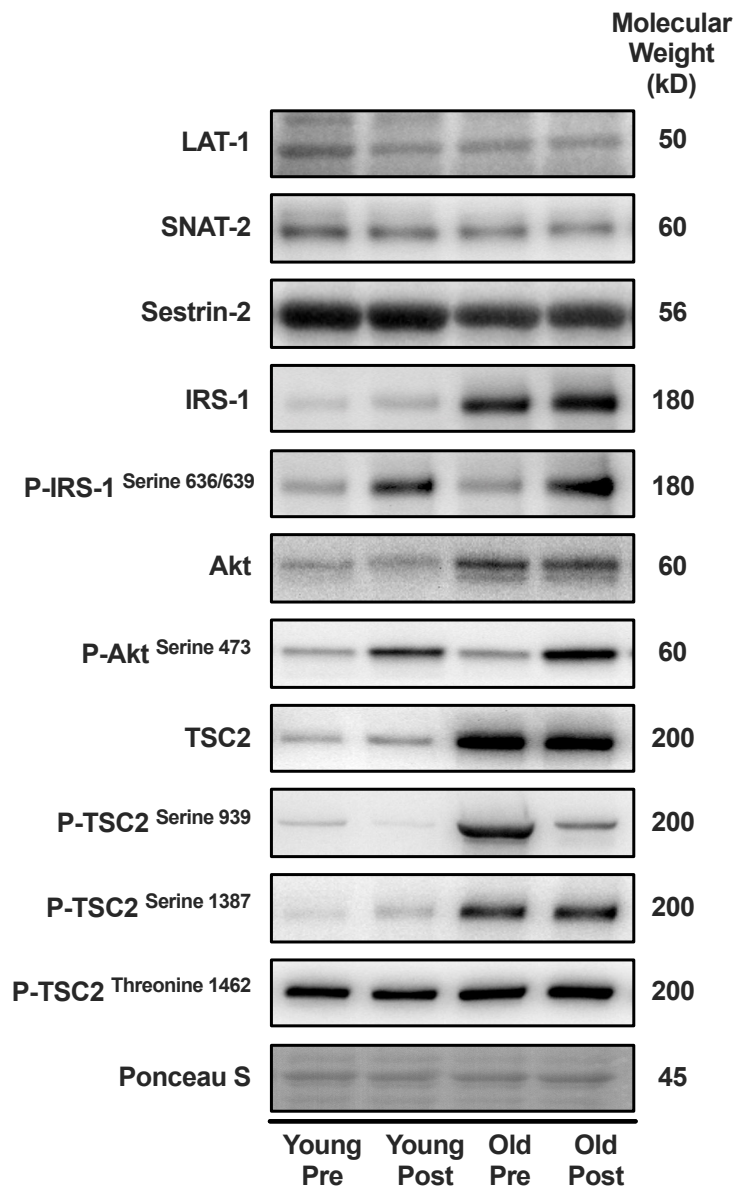


Figure 7: Representative Immunoblot Image. LAT-1, SNAT-2, Sestrin-2, IRS-1, P-IRS1^{Ser636/639}, Akt, P-Akt^{Ser473}, TSC2, P-TSC2^{Ser939}, P-TSC2^{Ser1387}, P-TSC2^{Thr1462}, Ponceau S.

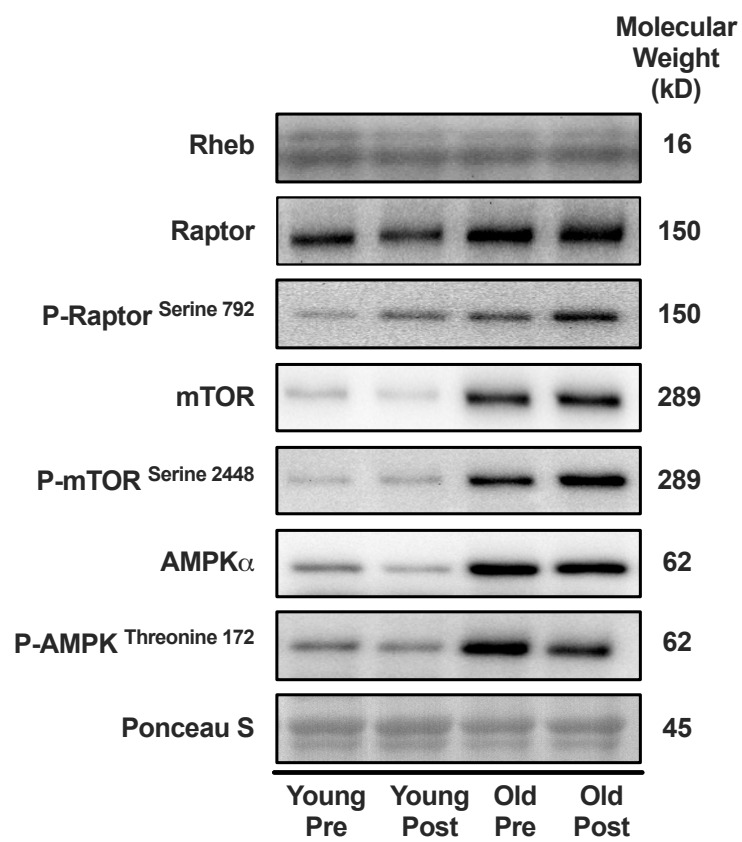


Figure 8: Representative Immunoblot Image. Rheb, Raptor, P-Raptor^{Ser792}, mTOR, P-mTOR^{Ser2448}, AMPK, P-AMPK^{Thr172}, Ponceau S.

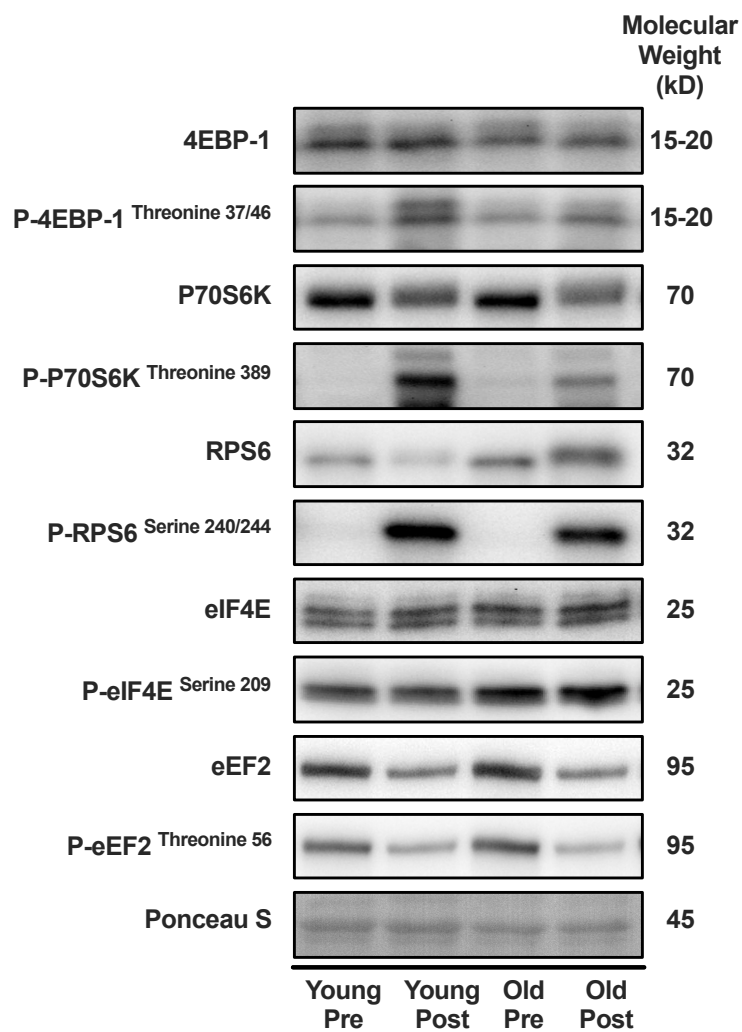


Figure 9: Representative Immunoblot Image. 4EBP-1, P-4EBP-1^{Thr37/46}, P70S6K, P-P70S6K^{Thr389}, RPS6, P-RPS6^{Ser240/244}, eIF4E, P-eIF4E^{Ser209}, eEF2, eEF2^{Thr56}, Ponceau S.

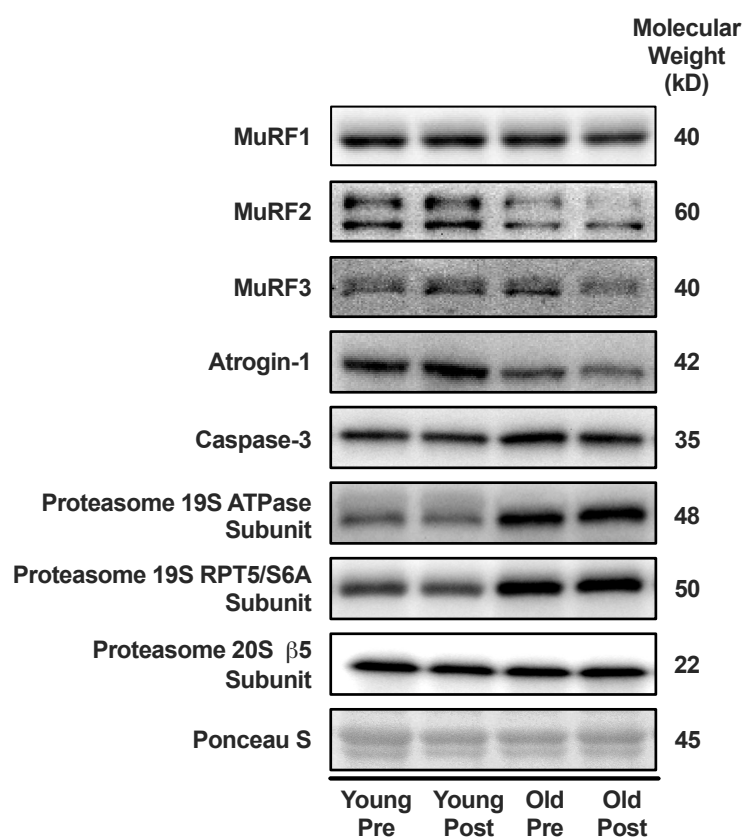


Figure 10: Representative Immunoblot Image. MuRF1, MuRF2, MuRF3, Atrogin-1, Caspase-3, Proteasome 19S ATPase Subunit, Proteasome 19S RPT5/S6A Subunit, Proteasome 20S B5 Subunit, Ponceau S.

SUPPLEMENTARY METHODS

Primary antibodies for immunoblotting

LAT-1 (Abcam, 85226, 1:1000), SNAT-2 (Santa Cruz, 166366, 1:1000), Sestrin-2 (CST, 8487, 1:1000), p-IRS-1^{Ser636/639} (CST, 2388, 1:1000), IRS-1 (CST, 2382, 1:1000), p-Akt^{Ser473} (CST, 4051, 1:1000), Akt (CST, 4691, 1:1000), p-TSC2^{Ser939} (CST, 3615, 1:1000), p-TSC2^{Ser1387} (CST, 5584, 1:1000), p-TSC2^{Thr1462} (CST, 3617, 1:1000), TSC2 (CST, 4308, 1:1000), p-Raptor^{Ser92} (CST, 2083, 1:1000), Raptor (CST, 2280, 1:1000), p-mTOR^{Ser2448} (CST, 2971, 1:1000), mTOR (CST, 2983, 1:1000), p-AMPK α ^{Thr172} (CST, 2353, 1:1000), AMPK α (CST, 2532, 1:1000), p-4EBP1^{Thr37/46} (CST, 2855, 1:1000), 4EBP1 (CST, 9644, 1:1000), p-P70S6K^{Thr389} (CST, 9205, 1:1000), P70S6K (CST, 9202, 1:1000), p-RPS6^{Ser240/244} (CST, 2215, 1:1000), RPS6 (CST, 2217, 1:1000), p-eIF4E^{Ser209} (CST, 9741, 1:1000), eIF4E (CST, 2067, 1:1000), p-eEF2^{Thr56} (CST, 2331, 1:1000), eEF2 (CST, 2332, 1:1000), MuRF-1 (SC, 398608, 1:1000), MuRF-2 (SC, 517149, 1:1000), MuRF-3 (SC, 166137, 1:1000), Atrogin-1 (SC, 166806, 1:1000), Caspase-3 (CST, 9665, 1:500), Proteasome 19S ATPase Subunit (Enzo, PW9265, 1:1000), Proteasome 19S RPT5/S6A Subunit (Enzo, PW8310, 1:1000), Proteasome 20S β 5 Subunit (Enzo, PW8895, 1:1000).

CHAPTER 6

High-Dose, Leucine-Enriched Essential Amino Acids to Mitigate Step-Reduction-Induced Skeletal Muscle Atrophy and Proteostatic Dysfunction in Healthy Older Adults: Study Protocol for A Randomised, Placebo Control Trial



THE EUROPEAN
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NUTRITION
AND
METABOLISM

Data collection for the current chapter is on-going:

Due to >18-months of delays with COVID-19, the present chapter is currently ongoing. As such, the following chapter provides the study outline & protocol. The study is jointly funded by the MRC-Versus Arthritis Centre for Musculoskeletal Ageing and recent ESPEN Clinical Nutrition and Metabolism Fellowship.

6.1. ABSTRACT

Background: Skeletal muscle mass and function progressively deteriorates as we age. The rate of muscle atrophy and metabolic deconditioning is exaggerated when intermittent periods of reduced activity and musculoskeletal unloading. The present research protocol was designed to investigate the potential protective effects of a nutritional supplement to mitigate the loss of skeletal muscle mass and metabolic function following 14 days of reduced physical activity in healthy older adults.

Methods: In a double-blind, randomised, placebo-control trial, two groups of older adults will undertake 14-days of reduced physical activity via the reduction in daily step counts to $<1,500/\text{day}^{-1}$ with wrist and hip-worn pedometers. One of the groups will receive a nutritional intervention ($3 \times 15\text{g}/\text{day}^{-1}$, leucine-enriched EAA's) or an isocaloric placebo ($3 \times 15\text{g}/\text{day}^{-1}$, maltodextrin) to consume daily for the entire period of reduced activity. Pre (day 0), during (day 7) and post step-reduction assessments of body composition (whole-body, leg-lean-mass, fat-mass) will be assessed via DXA. Daily rates of muscle protein synthesis (bulk fraction & individual proteins [FSR%/day⁻¹]) over the entire interventional period will be measured using oral D₂O stable isotope tracer methodology to determine protein synthesis over habitual activity and the early (days 0-7) and later phase (days 7-14) of reduced activity. Metabolic measures of mitochondrial respiration via high-resolution respirometry will be conducted from muscle biopsies, alongside indices of glycaemic control and insulin sensitivity from blood samples obtained during an oral glucose tolerance test. **Discussion:** The study aims to identify novel nutritional strategies that could be implemented to protect against the loss of skeletal muscle and metabolic function during a period of 2-weeks of step reduction in older adults. Furthermore, we aim to probe the molecular and mechanistic basis associated with inactivity to determine key metabolic pathways associated with atrophy and development of anabolic resistance. **Conclusion:** The present study will add to the minimal nutritional research in the model of step reduction and advance our understanding of the mechanistic basis of atrophy in older adults.

6.2. INTRODUCTION

Ageing is associated with a slow and precipitous loss of skeletal muscle mass and strength at a rate of between 1-2% per year after the age of 50. The loss of lean mass and strength are further exaggerated with intermittent periods of disuse and/or physical inactivity throughout an individual's lifespan ¹¹. Short-term periods of inactivity as a result of bed rest ⁸ and limb-immobilisation ⁹ as a result of elective surgery, or fracture result in catastrophic whole-body ³⁸⁶ and limb-specific ⁹ muscle atrophy. However, these disuse situations may occur relatively infrequently throughout life. The simplistic model of reduced activity via step reduction may offer researchers a real-world approach to study more frequent periods of disuse as result of illness via bacterial/viral infections and a combination of poor weather ⁵⁵⁴. Scenarios such as these result in a severe reduction in daily step counts (i.e., <1,500 steps/day⁻¹), compared to the total unloading associated with bed rest. Models of step reduction have observed large declines in leg-lean mass ¹⁰, protein synthesis ^{10,213}, insulin sensitivity ^{208,209,211} across younger and older adults.

The investigation into nutritional interventions to mitigate muscle atrophy and metabolic function in models of bed rest and limb-immobilisation are well established, with very little during models of step reduction, which are limited to whey protein ⁷⁴⁸, and the non-essential amino acid, Citrulline ⁴¹³. Evidence suggests the consumption of EAA's mitigate disuse-induced muscle loss during bed rest ⁴⁶¹, limb immobilisation ⁴⁶³ and following elective surgery ^{749,750}. The fortification or enrichment of the branch chained amino acid, Leucine, has been observed to enhance to muscle anabolic response to feeding and exercise ^{189,340–343,751}. Notably, a single low-dose (1.5-6g), leucine-enriched EAA (Leu-EAA) drink displays a robust MyoPS response similar to that of 40g whey protein in a cohort of healthy older women ^{341,344}. Therefore, in a

disuse situation, may offer a protective effect on skeletal muscle mass in older adults when provided in sufficient volume and frequency.

6.2.1. Study Objectives

The primary aim of the study is to determine the protective effects of a high-dose (15g), leucine-enriched EAA intervention to mitigate the loss of whole-body and leg-lean mass associated with 14-days of reduced physical activity ($<1,500$ steps/day⁻¹). Secondary outcomes aim to determine the influence of reduced activity on free-living rates of muscle protein synthesis (bulk & individual proteins), mitochondrial respiratory function, insulin sensitivity, protein breakdown (3-MH) at baseline (day 0), mid-point (day 7) and post-intervention (day 14) (**Figure 1**).

6.2.2. Study Design

The study has been ethically approved by the University of Birmingham Research Governance (RG_20-171) and National Health Service (NHS) Black Country Research Ethics Committee (21/WM/0219). The study will incorporate a double-blind, placebo-controlled trial, in which one group receive Leu-EAA, and the other an isocaloric placebo to consume thrice daily throughout the 14-day period of reduced activity (**Figure 1**). Hypotheses: (1) reduced activity will result in loss of whole-body, leg-lean mass and protein turnover, and (2) Leu-EAA will offset any declines in muscle mass and protein synthesis from the reduced activity period.

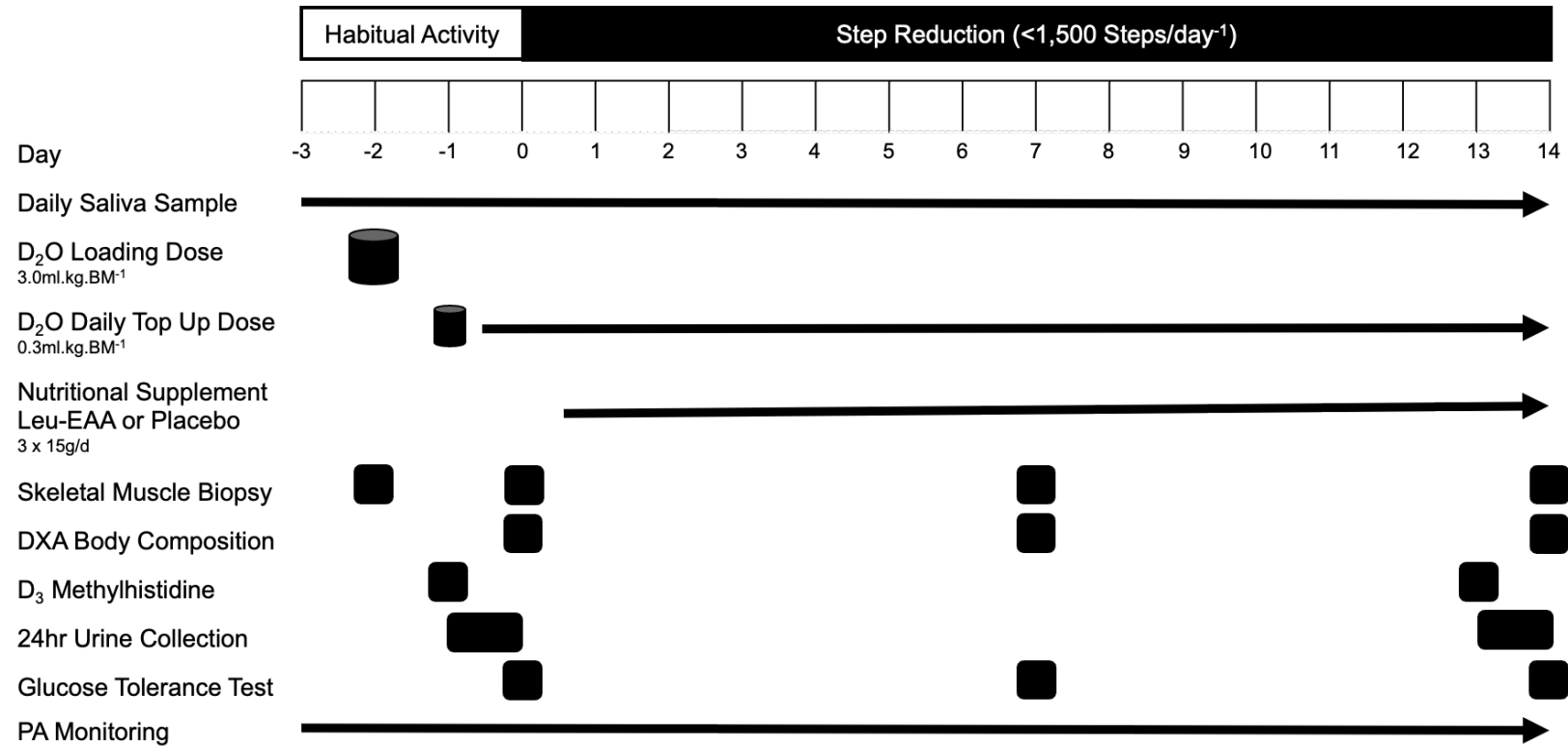


Figure 1. A schematic overview of the study indicating stable isotope tracer consumption, muscle biopsies, blood sampling and body composition measures.

6.3 METHODOLOGY

6.3.1. Participants

The study will be advertised on the University of Birmingham web pages, and on posters placed around the University of Birmingham campus and distributed to local community clubs for older people in the Birmingham area.

A targeted recruitment poster will be advertised on social media (i.e., Facebook) within a 20km distance.

6.3.1.1. Inclusion Criteria

- Aged 60-85 years.
- Male and female.
- BMI (body mass index) between 18-29 kg/m².
- Good general health defined as (i) no known cardiovascular or metabolic disease, ii) non-smokers, iii) accustomed to normal levels of physical activity (at least 30 mins of moderate physical activity a day/ >7,000 steps per day [assessed during screening]).

6.3.1.2. Exclusion Criteria

- Have a BMI (body mass index) above 29 kg/m².
- Health problems such as i) heart disease, ii) rheumatoid arthritis, iii) diabetes, iv) poor lung function, v) uncontrolled hypertension.
- Regular consumption of any analgesic or anti-inflammatory drug(s), either prescription or non-prescription.
- Lidocaine allergy

- Taking any medications known to affect muscle protein metabolism (e.g., beta-blockers, corticosteroids, non-steroidal anti-inflammatories).

6.3.1.3. Population and Sample Size

A total of 24 healthy (12 in EAA treatment group, and 12 in the placebo group) older male and female participants (aged 60-85 years) will be recruited to achieve the trial objectives. In accordance with G*power, a sample size of 12 will provide 80% statistical power to detect a pre-intervention difference in thigh lean mass means of 0.01 (e.g., a first condition mean of 0.01 and a second condition mean of 0.02) using a mixed-model ANOVA with a 0.05 significance level. The sample size is based on a previous study that was conducted where a significant decline in leg-lean mass was noted following 14-days of reduced activity in healthy older adults ¹⁰. Moreover, sample size was based upon previous nutritional interventions over a similar duration which attenuated losses of leg lean mass and anabolic resistance compared to an isocaloric placebo ⁴⁰². Therefore, we believe our sample size to be justified.

6.3.2. Study Procedures

6.3.2.1. Main Metabolic Trials

Main trials will take place on day pre (day 0), during (day 7), and at the end of the 14-days of step reduction (day 14). See **figure 1** for schematic overview. Participants will be asked to report to the laboratory after a 10 hour fast wearing lightweight clothing with no metal components. Bioelectrical impedance balance scales and stadiometer will be used to determine the participants body mass and height for BMI. Following this, a whole-body DXA will be performed

to determine body composition (i.e., lean-mass, fat-mass, and bone mineral density). A resting venous blood sample, and percutaneous skeletal muscle biopsy from the vastus lateralis via the Bergstrom needle biopsy technique will be sampled. Muscle biopsies are used for measurement of muscle protein synthesis and mitochondrial respiration (see **primary outcomes**). Participants will then ingest a loading dose of D₂O water (3mL/kg⁻¹/BM⁻¹), with daily top-ups (0.3mL/kg⁻¹/BM⁻¹) to be consumed every morning until the end of the study. An oral glucose tolerance test (OGTT) will be conducted following the oral consumption of 75g glucose (82.5g dextrose monohydrate), with blood samples taken at 0, 15, 30, 45, 60, 75, 90, 120 mins after consumption of the drink.

6.3.2.2. Step Reduction Intervention

For the 14 day period of inactivity, participants will be asked to restrict their daily step count to <1500 steps per day. A wrist and hip-worn pedometer will be worn by participants at all times throughout the day to allow monitoring and feedback of step count. Step count will be recorded each evening prior to bed by the participant in a provided log-book. The pedometer will also automatically record daily step count and will reset at midnight. Participants will be asked to record their dietary intake on the first, seventh and 13th days of the step-reduction intervention using a dietary record sheet. For the two visits to the laboratory during the step-reduction period, transport arrangements will be made to minimise walking for the participant for the sake of the visits (i.e., door-to-door transport, and transport within the facility/campus by wheelchair).

6.3.2.3. Nutritional Intervention

Supplements contain 15g of leucine-enriched essential amino acids (Impact EAA's, MyProtein, UK), or isocaloric maltodextrin (maltodextrin, MyProtein, UK). Individual servings are identically flavoured and packaged in powdered form. Participants are instructed to mix each package with ~300 mL of water before ingestion and are asked to consume the beverage within a 5-min period three times per day, in-between main meals to support repeated stimulation of protein synthesis throughout the day when between-meal periods where a net loss of muscle protein is often observed. Supplements were isocaloric and energy-matched; their contents appear in **table 1**.

Table 1. Contents of nutritional supplements consumed three times daily with 250-300 mL water during the 14-d step reduction period.

	Leu-EAA (g/15g serving)	PLA (g/15g serving)
Histidine, g	0.42 (1.67%)	-
Isoleucine, g	1.57 (10.67%)	-
Leucine, g	6.28 (40.00%)	-
Lysine, g	1.93 (16.67%)	-
Methionine, g	0.46 (3.33%)	-
Phenylalanine, g	0.75 (6.67%)	-
Threonine, g	1.53 (9.33%)	-
Valine, g	1.57% (11.00%)	-
Tryptophan, g	0.48 (0.67%)	-
Maltodextrin, g	-	15
Total EAA (g)	15	-
Flavouring (g)	1.5	1.5

6.3.3. Sample Processing and Storage

Skeletal muscle tissue samples will be processed and analysed immediately on the day of collection or stored at -80°C for later batch analysis. Blood samples collected into EDTA, and serum separator tubes (5 ml) will be centrifuged to obtain plasma and serum, respectively. Urine will be collected in a 3 litre sealed container over a 24hr period and stored in the participants fridge until returning to the laboratory. All samples will be stored at -80°C for later analysis.

6.3.4. Potential Harms

Blood and tissue sampling techniques used in the study also carry a risk of discomfort to the participant; however, all study staff are fully trained, experienced, and adhere to good clinical practice at all times to minimise this risk. All tissue sampling procedures will be performed in sterile conditions, and participants will be provided with extensive advice on aftercare of biopsy sites to minimise risk of infection. Any adverse events will be recorded, assessed, and monitored in line with the University of Birmingham sponsorship agreement and the Adverse Event Reporting Standard Operating Procedure. All potential risks have been reported to, assessed, and approved by the study sponsor, NHS Black Country Research Ethics Approval Committee for Health, and the University of Birmingham Research Governance.

Body composition has previously been identified as a key determinant of the metabolic capacity, and therefore a highly accurate assessment of this variable is necessary. DXA scanning is considered the most appropriate in this instance. DXA does involve exposure to ionizing radiation. Ionizing Radiation

(Medical Exposure) Regulations 2000 (IRMER) procedures are in place at The University of Birmingham. The dose constraints will be 0.015mSv per participant, and the risk will be explained on the participant information sheet. The procedures will exceed normal care as participants would not normally expect to receive any radiation during normal care at the research site. However, the amount of radiation exposure from DXA scanning is extremely low (i.e., less than a chest x-ray).

Oral consumption of the stable isotope tracer deuterated oxide (D_2O) share the same place in the periodic table and thus have the same chemistry but have a different mass. Stable isotopes are non-toxic and occur naturally, at varying amounts, in the body. The D_2O 'heavy water' tracer can cause feelings of dizziness or nausea when consumed in a large bolus in one go (>300mL). However, these issues will be minimised by asking participants to consume $3\text{mL/kg}^{-1}/\text{BM}^{-1}$, with smaller daily top-ups of $0.3\text{mL/kg}^{-1}/\text{BM}^{-1}$.

6.3.5. Primary Outcome Measure

6.3.5.1. Changes in Whole-body and Leg Lean Mass

Body composition will be assessed following an overnight fast (12 h). DXA measurements will be conducted using a Discovery W (S/N 82161) whole body scanner (Discovery DXA Systems, Hologic Inc., Bedford, MA, USA) and analyzed via manufacturers software (Hologic APEX software, version 13.5.2.1). The machine will be calibrated each testing day with a lumbar spine phantom provided by Hologic Inc. The analysis regions are standard regions where the head, torso, arms, and legs are automatically subdivided by the software, but will be subsequently checked manually to ensure consistency.

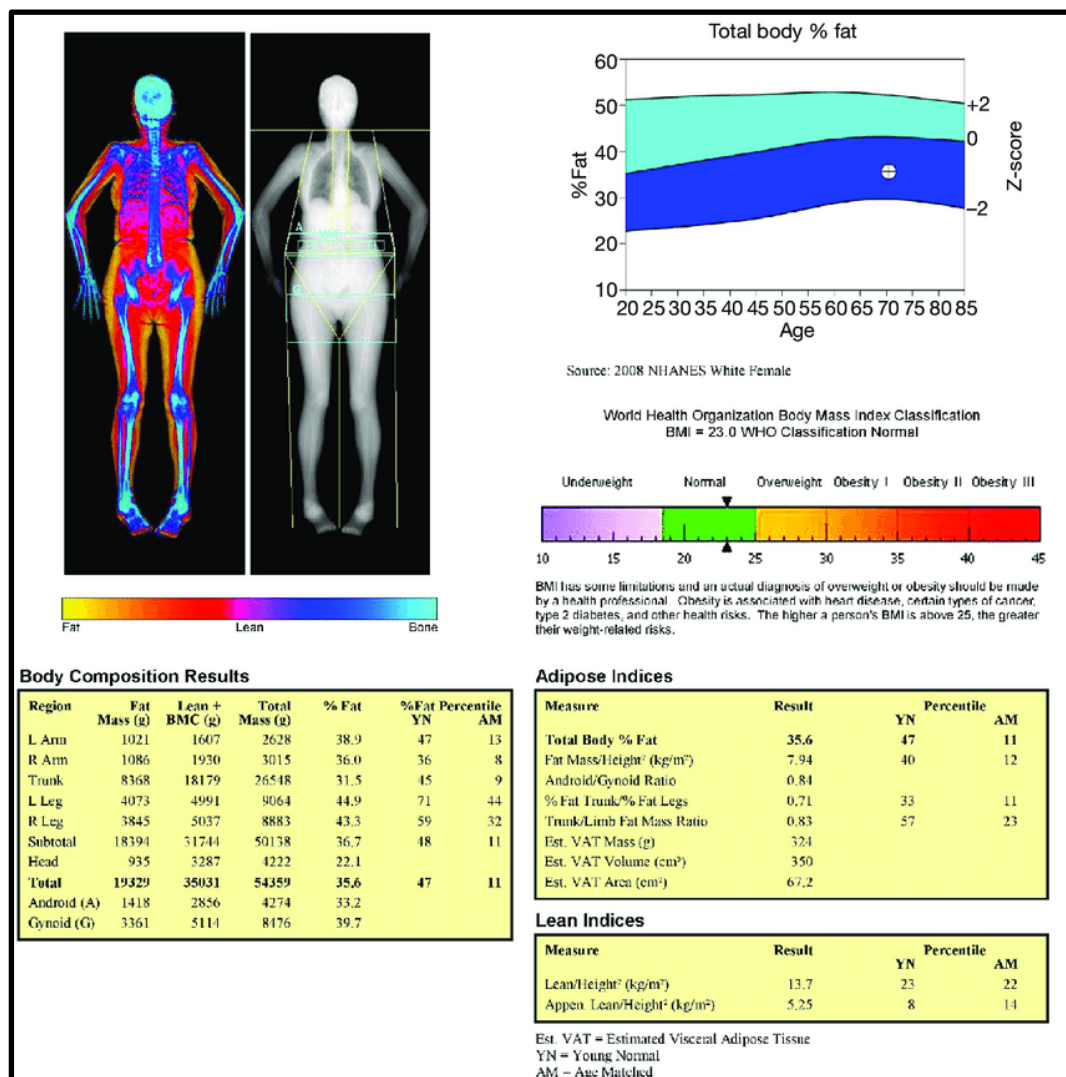


Figure 2. Example dual x-ray absorptiometry scan of a 70 year old white female. Body composition analysis of fat mass (android and gynoid), lean mass, and bone mineral density are expressed as whole-body, or segmental (i.e., leg, arms, trunk, and head).

6.3.6. Secondary Outcomes

6.3.6.1. Bulk and Individual Muscle Protein Synthesis Rates

Bulk mixed muscle protein synthesis and rates of individual proteins within myofibrillar, mitochondrial and collagen fractions will be examined via both GC-pyrolysis-MS and Q-TOF LC-MS with ESI to determine the static abundance and protein flux over the habitual and reduced activity period, as previously described ⁷⁵². Together, along with mass isotope distribution analysis (MIDA) ⁷⁵³, and bioinformatic network analysis, allows for the quantification of proteome dynamics (**Figure 3**). Cytosolic protein fraction will be used to determine intracellular protein aggregation, and immunoblotting of proteostatic signalling markers.

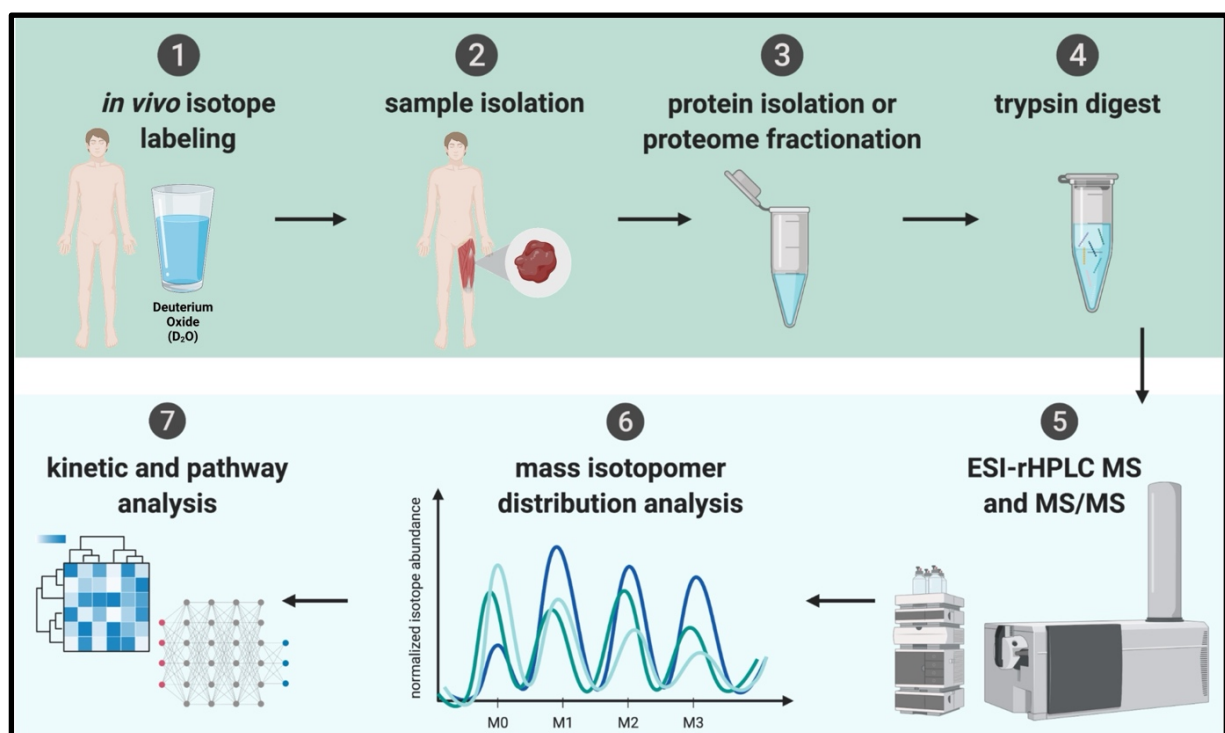


Figure 3. Simplified schematic of study workflow and steps to determine proteome dynamics in skeletal muscle. (1) Participants ingest D_2O as the isotope tracer. (2) Muscle biopsy sampling various timepoint to determine synthesis rates between timepoints (i.e., intervention). (3) Protein isolation with homogenisation buffer, or separation into subcellular pools via differential centrifugation protocols. (4) Overnight trypsin digest of isolated proteins, followed by ESI-HPLC-MS & MS/MS. Raw data of isotopomers (i.e., M_0 , M_1 , M_2 , M_3) can be analysed via mass isotopomer distribution analysis

6.3.6.2. Mitochondrial Respiratory Function

Ex vivo mitochondrial respiratory function will be assessed in permeabilised muscle fibres from muscle biopsy samples. Samples will undergo a standard SUIT-008 protocol with the use of pyruvate & malate, ADP, cytochrome-C, glutamate, succinate, CCCP, rotenone, and antimycin-A (see **table 2** for full protocol). This protocol will utilise an ADP titration step across varying concentrations of ADP from 100-16000 μ M to determine ADP sensitivity as previously described ⁸⁷, and has been utilised in BR and ULI models of disuse ^{390,397,568,570}. This protocol allows the measurement of leak respiration (-ADP), complex I coupled respiration (+ADP), complex I+II coupled respiration (+ADP Succinate), and uncoupled (maximal) respiration (+CCCP).

Table 2. High resolution respirometry protocol (SUIT-008 + ADP Titration) for Oroboros O2K Fluro-Respirometer analysis of mitochondrial respiration in human skeletal muscle biopsy samples.

Compound	Mark	Stock Concentration	Injection Volume	Final Concentration
MIR05	-	-	2mL	-
(-)-Blebistatin	<i>B</i>	10 mM	1 µl	5 µM
Muscle Fibres	<i>Pfi</i>	-	-	2.5-3.5 mg
100% Oxygen	<i>O₂</i>	-	-	400-450 µM
Pyruvate	<i>P</i>	2 M	10 µl	10 mM
Malate	<i>M</i>	400 mM	10 µl	2 mM
ADP	<i>D1</i>	50 mM	1 µl	25 µM
ADP	<i>D2</i>	50 mM	1 µl	50 µM
ADP	<i>D3</i>	50 mM	3 µl	125 µM
ADP	<i>D4</i>	50 mM	3 µl	200 µM
ADP	<i>D5</i>	50 mM	3 µl	275 µM
ADP	<i>D6</i>	500 mM	1 µl	500 µM
ADP	<i>D7</i>	500 mM	2 µl	1000 µM
ADP	<i>D8</i>	500 mM	4 µl	2000 µM
ADP	<i>D9</i>	500 mM	8 µl	4000 µM
ADP	<i>D10</i>	500 mM	16 µl	8000 µM
ADP	<i>D11</i>	500 mM	32µl	16000 µM
Cytochrome c	<i>C</i>	4 mM	5 µl	10 µM
Glutamate	<i>G</i>	2 M	10 µl	10 mM
Succinate	<i>S</i>	2 M	20 µl	10 mM
CCCP	<i>U</i>	1 mM	1 µl	0.5 µM
CCCP	<i>U</i>	1 mM	1 µl	0.5 µM
CCCP	<i>U</i>	1 mM	1 µl	0.5 µM
Rotenone	<i>R</i>	5 mM	1 µl	2.5 µM
Antimycin A	<i>Ama</i>	5 mM	1 µl	2.5 µM

6.3.6.3. Other Secondary Outcomes

Muscle protein breakdown will be determined via D₃-3-Methylhistidine methodology as described in **section 1.2.1.4**. In brief, the provision of 10mg of D₃-3-MH the day before the start of inactivity, and the day prior to the last day of step reduction to determine in 3-MH flux in a habitual and following 14-days of step reduction. Plasma and urine will be analysed for detectable D₃-3-MH to determine hourly and daily rates of protein breakdown.

Oral glucose tolerance tests at day 0, 7 and 14 will allow us to determine changes in whole-body glycaemic control and insulin sensitivity during and following inactivity. Analysis of blood glucose, insulin and c-peptide allow the quantification of various index's related to whole-body metabolic health ^{754–756}.

6.3.7. Statistical Analysis

Repeated measures analysis of variance (ANOVA, Treatment: Leu-EAA vs Placebo, Time: day 0, day 7, day 14, Interaction: Treatment x Time) with appropriate post-hoc tests adjusted for multiple comparisons will examine changes over time (day 0, day 7, day 14 of inactivity) in the primary (whole-body and leg-lean mass) and secondary (muscle protein synthesis, mitochondrial respiration, and glycaemic control) outcomes variables. Statistical significance will be accepted at $P < 0.05$.

6.4. DISCUSSION

The study aims to evaluate the effectiveness of a novel nutritional intervention at mitigating inactivity-induced muscle loss and metabolic deconditioning. Based on previous studies in older adults, it is expected that there will be a significant loss of leg-lean mass ^{10,213}, and declines in various metabolic parameters (i.e., protein synthesis, insulin sensitivity and mitochondrial function) ^{10,213,480,481}. The nutritional strategy deployed in the study (leucine-enriched EAA's) was chosen based upon the strong evidence of it preventing muscle atrophy in models of BR ⁴⁶¹, ULI ⁴⁶³, and following knee arthroplasty ^{749,757}. Furthermore, acute postprandial feeding investigations have observed leucine-enriched EAA's to provide a robust stimulation of muscle protein synthesis, similar to that of 40g high-quality whey protein ^{341,344}. It is hoped that in the absence of a contractile stimulus such as RE ⁴⁷⁹ or NMES ⁴²³, leucine-enriched EAA's may be an effective countermeasure against step-reduction-induced muscle loss. Furthermore, the use of D₂O, coupled with high-sensitivity mass spectrometry may allow us to develop a better mechanistic understanding of inactivity-related changes in skeletal muscle protein synthesis at a single protein level ^{289,752}. Therefore, given exercise may not always be feasible during these disuse and inactivity periods, nutritional strategies are strongly needed.

6.4.1. Study Limitations

When utilising D₂O and calculating protein synthesis rates via the plateau in fraction new method (i.e., k/day^{-1} fraction new plotted across time and curves fitted using one-phase associations), a complete control group would be beneficial ^{218,219}. Therefore, a group which maintained their normal physical activity levels for the entire study duration is the most effective control instead

of both groups having a habitual period prior to step reduction. This method would allow for the estimation of the protein pool which is actively turning over (i.e., dynamic protein pool), with 1.0 equal to 100% of the protein pool ²¹⁸. For example, in pre-clinical models, mouse gastrocnemius myofibrillar protein has a plateau rate of ~ 0.8 , whereas collagen protein is ~ 0.2 , representing differences in subcellular protein pool and turnover dynamics ⁷⁵⁸.

A common limitation in human metabolic investigations is the lack of dietary control during interventions. Dietary control from investigators often provide the research volunteers with more, or in some cases less food volume than they typically consume, with significantly altered macronutrient compositions to their habitual dietary intake. These alterations to the dietary intake during an intervention may mask, or over overstate the effect (or lack of effect) during of the intervention. In the present study, we sought to improve the ecological validity, and allow habitual dietary intake with monitoring (via 3-day food diaries) at the beginning, middle and end of the intervention.

Lastly, mitochondrial function assessed via high resolution respirometry is the gold-standard measurement to assess skeletal muscle mitochondria. However, this requires small fibre bundles from muscle biopsies to be chemically permeabilised and oxygen consumption ($\text{pmol/second/mg}^{-1}$) measured in response to supraphysiological concentrations of substrates, uncouplers, and inhibitors. Recently, researchers have been opting for non-invasive *in vivo* measures of mitochondrial function via ³¹P-MRS ^{759–761} and even NIRS ^{397,761}. In particular, ³¹P-MRS allows for the quantification of phosphocreatine, pH, ADP, and ATP in real-time, under physiological conditions, without the need for a muscle biopsy. These non-invasive

methodologies may offer researchers novel mechanistic insights into muscle metabolism under various experimental conditions or participant cohorts.

6.5. CONCLUSION

The present study will add to the limited data on step reduction and reduced activity models in older adults. Furthermore, it will add novel mechanistic insights into the temporal changes in lean-mass, proteome dynamics and mitochondrial capacity in this cohort. Lastly, the development of nutritional components to mitigate muscle atrophy during a period of inactivity is paramount to prevent the acceleration to the 'disability threshold' of low muscle mass, strength, and function.

CHAPTER 7

General Discussion

7.1 Introduction

Since the consequences of age-related muscle loss, and clinical definition of 'sarcopenia' ²⁰, there has been a plethora of scientific investigations to determine the metabolic and molecular mechanisms by which sarcopenia occurs, and the development of therapies (i.e., exercise, nutritional and pharmacological) to prevent this disease condition. Although the mechanisms of sarcopenia are multifactorial, they ultimately manifest in cellular changes in protein synthesis (and to some extent breakdown), resulting in a net loss of muscle protein mass over time. Central to this notion, the reduction in skeletal muscle energy metabolism, in particular mitochondrial content and function are thought to provoke changes in muscle protein synthesis via reduced ATP synthesis, to facilitate the high-energy demand required for synthesising new muscle proteins. Furthermore, these are concurrent with elevations in ROS, causing cellular protein damage, and accumulation of dysfunctional organelles.

Older adults who have participated in life-long exercise display exceptionally greater body composition, and whole-body metabolic health compared to age-matched untrained older adults ^{215,616}. The greater physiological and metabolic health places them at a higher 'set point' and allows for a prolonged period of time spent in good health during ageing ²³⁴. In a rested and post-RE state, we previously reported untrained older adult display identical rates of iMyoPS, and mTORC1-related signalling compared to endurance-trained MA ¹¹³. The greater muscle metabolic health has, therefore, been suggested to be differences in mitochondrial health, function, and quality control ²³². Few authors have observed greater mitochondrial health in MA compared to age-matched and even younger adults. Moreover, the mitochondrial signalling and remodelling response (i.e., quality control) to RE is poorly understood.

Musculoskeletal disuse such as BR, ULI and SR results exacerbate the age-related decline in muscle mass and compromise metabolic health, thereby increasing the rate at which an older individual would reach a 'disability threshold' for functional dependency ³⁹. Although the underpinning molecular mechanisms of disuse remain unclear, disuse results in severe declines in muscle mass, protein synthesis, and cellular health in as little as 3-days ^{398,399}. For a large proportion of older adults, the access to traditional gym-based facilities necessary to support muscle mass and metabolic health during free-living and disuse events may not be feasible due to internal (i.e., physical, psychological, social) or external (i.e., illness, weather, injury) environmental barriers ⁶⁷⁰. Therefore, the development and practice of pragmatic, easily accessible exercise strategies are needed. Elastic band exercise can be implemented at home, or in group exercise classes with minimal cost and space, which may offer a strategy to maintain muscle mass and strength in older adults ⁷⁶².

To date, the neuromuscular, metabolic, and molecular mechanisms associated with; RE prehabilitation, lifelong-exercise, elastic band resistance exercise, and nutritional supplementation are not fully explored. Investigating these factors are essential in these to develop successful strategies to maintain musculoskeletal health and function during ageing. Therefore, the primary focus of this thesis was:

- I. Determine the effects of a resistance exercise prehabilitation intervention at mitigating mitochondrial declines following a clinically relevant duration of bed rest in older adults.
- II. Investigate the role of life-long exercise and RE on the expression of molecular markers associated with mitochondrial content and quality control.

- III. Determine the neuromuscular effect of activities of daily living, and pragmatic, resistance exercise modes (i.e., bodyweight squat & elastic band knee extension) in younger and older adults.
- IV. Establish the role of elastic band resistance exercise on acute intracellular mTORC1 signalling, and subsequent exercise-induced changes in integrated MyoPS.
- V. Investigate the role of leucine-enriched essential amino acids a potential nutritional intervention to maintain muscle mass and metabolic function during a period of reduced activity in healthy older adults.

The present chapter will present a brief summary of the key findings from the studies described in previous chapters of the thesis. Based on **Chapter 2**, I will discuss the impact of bed rest, and the implementation of resistance exercise prehabilitation in clinical bed rest. Based on **Chapter 3**, I will discuss how life-long exercise maintains mitochondrial content, quality control, and mechanism by which RE may (or may not) alter mitochondrial remodelling processes. Based on data provided in **Chapter 4 & 5**, I will discuss the practicality of implementing elastic band RE to induce muscle anabolic remodelling and improvements in muscle protein turnover. Finally, from **Chapter 6**, I will discuss the therapeutic potential of high-dose leucine-enriched EAA's as a nutritional intervention to maintain muscle mass and function in older adults following a 14-day period of reduced activity.

7.2 Bed Rest and Skeletal Muscle Mitochondria

Bed rest results in declines in mitochondrial function, content, and alterations in fission and fusion dynamics in both younger and older adults ^{384,390,397,515,568}. Furthermore, prehabilitation prior to elective surgery has been reported to prevent, or at least offset disuse-induced alteration in whole-body or tissue-specific metabolism ^{558,559,581,763}. Here we show neither 5-days of bed rest, or a 7-day period of RE prehabilitation alter the enzyme activity of citrate synthase, ETC complex I-V, or markers associated with fission/fusion (i.e., DRP1, MFF, OPA1, FIS1), biogenesis (i.e., AMPK, ACC, PGC1 α , TFAM), or mitophagy (i.e., ULK1, LC3B or BNIP3) ⁵⁶¹. While initially observed as a 'negative outcome', this is, in fact, a positive observation showing no decline in mitochondrial protein abundance following 5-days of bed rest. This is important, as longer periods of bed rest (i.e., 7, 10 ⁵⁶⁸ or 14-days ³⁸⁴) results in significant decreases in mitochondrial respiratory function, content, and morphology-related protein expression. Notably, in younger adults, mitochondrial respiratory capacity, and ETC protein expression are decreased ~13%, independent of changes in mitochondrial density (i.e., citrate synthase activity) following 7-days of bed rest ³⁹⁰. In older adults, similar declines are observed following 10-days of bed rest ⁵⁶⁸, which may reflect the longer duration (≥ 7 -days) needed to observe declines in mitochondrial function, content, or protein expression. It is noteworthy, at least in younger adults, that muscle mitochondrial function is decreased in as little as 3-days following ULI, with no declines in ETC observable until 14-days ⁵⁷⁰. Similarly, 4-days of bed rest in younger adults have observed a decrease in mitochondrial density, but an increase in 'intrinsic' mitochondrial respiration ³⁸¹. Therefore, the severe consequences observed following disuse maybe age-dependant, with younger adults displaying larger dysregulation due to a higher starting physiological setpoint.

7.3. Resistance Exercise and Mitochondrial Remodelling

Resistance exercise induced muscle anabolism is well characterised with >1,300 publications indexed on PubMed mentioning “resistance exercise”, “skeletal muscle”, “mTOR” and “protein synthesis”. However, the role of RE on muscle mitochondrial signalling and adaptation are poorly understood. Here I report RE induced little, to no alterations in mitochondrial density (i.e., CS activity), or markers associated with mitochondrial quality control (i.e., DRP1, MFN2, OPA1, FIS1) in cohorts of MA or sedentary older adults in the acute (1hr) and delayed (48hr) post exercise recovery periods. In a comprehensive review by Parry et al. the authors discussed the role of RE in mitochondrial remodelling, which are primarily limited to prolonged training interventions⁵⁶⁰. These studies often report only minor improvements in mitochondrial protein content and/or function, which may be an artefact of an increase in cell size, protein accretion, and thereby, a ‘dilution’ of mitochondrial protein or enzyme activity when expressed relative to mg/μg of muscle protein⁵⁶⁰. The process of muscle protein synthesis, and the building of muscle proteins following resistance exercise may favour the process of mTORC1 signalling, and muscle protein synthesis, over increased mitochondrial remodelling, and changes to oxidative function.

The present study also sought to characterise the differences in markers associated with mitochondrial health and quality in older endurance-trained MA. In line with previously published data^{229,231,232}, I observed drastically greater mitochondrial density, content, and quality control mechanisms in MA compared with age-matched untrained controls (OC). These data suggest the importance of maintenance of high levels of structured physical activity and exercise across the lifespan for overall greater muscle health. Although the measurement of mitochondrial protein synthesis in the present study was not achievable (i.e., limited biopsies, low tracer enrichment, cellular

material used primarily for measurement of MyoPS), we would *speculate* due to the high-volume of weekly endurance training (4 session per week), and >40yrs of exercise training experience of our MA cohort ¹¹³, they would observe greater rates of mitochondrial protein synthesis would be observed compared to sedentary older adults. The influence of lifelong exercise on whole-body and musculoskeletal health are evident, however, the molecular mechanisms remain poorly understood. Future investigations should seek to utilise proteomic techniques to characterise, and profile tissue specific proteome. These types of analytical techniques have allowed researchers to discover ~176 skeletal muscle mitochondrial proteins (from 6176 quantified) which are differentially expressed in world-class, elite level octogenarian athletes compared to age-matched controls ⁶²⁰.

7.4. Is Elastic Band Training a Pragmatic Exercise Alternative?

Inability or lack of motivation to undertake traditional gym or machine-based exercise training is highly prevalent in older adults. In an attempt to provide evidence of accessible, pragmatic, and home-based exercise alternatives, this thesis investigated the acute neuromuscular ⁵⁵⁵ and muscle anabolic response ⁷⁶⁴ to elastic band knee extension in younger and older adults. In **Chapter 4** we observed elastic band knee extension exercise to induce a similar level of muscle activity compared to machine-based knee extension in both younger and adults ⁵⁵⁵. In the same study also characterised the neuromuscular demand of activities of daily living (i.e., walking, stair climbing and chair squats) ⁵⁵⁵. Although not seen as exercise, these brief intermittent periods of short 'exercise snacks' throughout the day play an important role in regulating postprandial glycaemia ⁷⁶⁵, and rates of muscle protein synthesis ⁷⁶⁶. Notably, the relevant effort (i.e., %EMG^{MVC}) is markedly higher in older adults across

all activities, and exercise modes due to lower absolute muscle mass and strength. Our findings corroborate previous data observing similar EMG activity between elastic band and machine-based exercise training ⁵⁵⁶. It is worth of note, the EMG activity in **Chapter 4** was established with standard EMG, whereas the development of novel high-density surface EMG, and indwelling needle electrode techniques allows for incredibly detailed assessment of neuromuscular function and motor unit morphology during exercise ⁷⁶⁷, ageing ⁷⁶⁸, and disuse ⁷⁶⁷.

A follow-up in investigation in **Chapter 5** was the first investigate the muscle anabolic response to elastic band resistance exercise in younger and older adults. Previously reported investigations had been limited to circulating anabolic hormonal milieu (i.e., IGF-1, myostatin, testosterone) ⁷²⁸, which have limited, if any purported effect on post-exercise muscle anabolism ^{769,770}. Here we provided evidence suggesting a single session of high-volume elastic-band RE induced large increases in mTORC1 signalling (p-AKT^{Ser473}, p-P70S6K^{Thr308}, p-RPS6^{Ser240/244}, p-4EBP1^{Thr37/46}) that were broadly comparable in young and old ⁷⁶⁴. An interesting observation was the increased expression of mTOR and the abundance of p-mTOR^{Ser2448} (**Chapter 6, Supplementary Figure 7**) in older adults. Here we observed a ~146% greater p-mTOR and ~272% total mTOR in older compared to younger adults (**Figure 1**). However, when expressed relative to the protein (i.e., Phospho/Total), older adults report a ~54% lower phospho/total mTOR ratio. Previous pre-clinical observations in OB/OB mice have showed elevated mTOR and activation of its phosphorylation site in a basal, non-stimulated conditions are linked to lower skeletal muscle mass, strength, and fibre area ⁷⁷¹. Furthermore, given mTOR is chronically activated in ageing skeletal muscle, the ability for further activation following dietary protein or resistance exercise training is limited, which likely plays a role in the blunted response

observed in the **Chapter 5**. The concept of normalising mTOR expression and activation to youthful levels has been attempted with mTOR inhibitors, which have resulted in increased lifespan, and reverse the sarcopenic phenotype of pre-clinical models ⁷⁷¹. In humans, it has been observed following 20-weeks of RE training that mRNA signature of mTORC1 is decreased, leading to greater exercise-induced activation, and greater gains in muscle mass ²⁷².

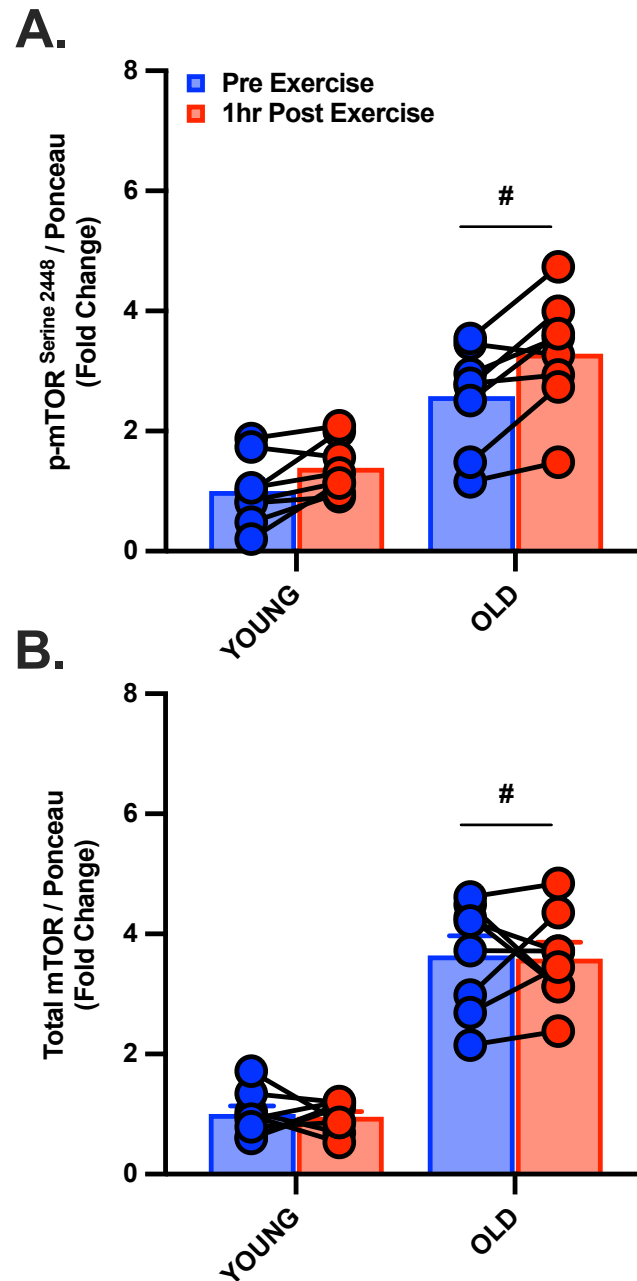


Figure 1. Age-related alterations in mTOR expression. Protein expression and phosphorylation levels of p-mTOR (A) and total mTOR (B) before (Blue) and 1-hour post (Red) elastic band knee extension exercise in younger and older adults. Data are presented as fold-change relative to pre-RE young and mean $\pm$ SEM for n=8 subjects per group with individual data points. * Indicates significant time effects. # Indicates significant group effect (P<0.05).

The iMyoPS response to EB-RE was blunted in older adults compared to younger adults over the 48hr recovery period ⁷⁶⁴. This may be explained by (1) the variable load of resistance over a full range of motion, (2) sub-optimal dietary intake to maximise MyoPS and muscle adaptive response in older, but not younger adults ($\sim 1.2\text{g/kg/BM}^{\text{day}^{-1}}$), and (3) reduced total energy intake ($\sim 11\%$ lower in older adults). Due to the variable resistance observed in EB-RE, it may be more beneficial to train closer to volitional failure/fatigue than a standardised number of sets and repetitions by increasing training volume (e.g., added load, sets, repetitions). Previous evidence suggests MyoPS and long-term gains in muscle mass can be attained with low-load machine-based RE ($\sim 30\%$ 1RM) and induce a similar adaptative response to that of high-load RE ($>80\%$ 1RM) ^{607,772}, suggesting training to failure may be an important training variable in older adults. Although the MyoPS response was blunted in older adults in **Chapter 5**, this does not necessarily negate any potential other positive effects this may have on other adaptive changes in muscle remodelling (i.e., muscle strength and function). Previous authors have observed positive increases in muscle mass, strength, and metabolic variables following training interventions with EB-RE ^{556,557,676,773,774}. Furthermore, longer durations of exercise interventions (i.e., 7-10 days), or repeated daily exercise bouts of EB-RE, may be needed to elicit an increase in MyoPS in older adults. This exercise mode, could therefore, be utilised in inpatient facilities in adults undergoing elective surgery, and as part of pre, peri and post-operative care to mitigate muscle atrophy and deconditioning associated with surgery and disuse (**Figure 2**).

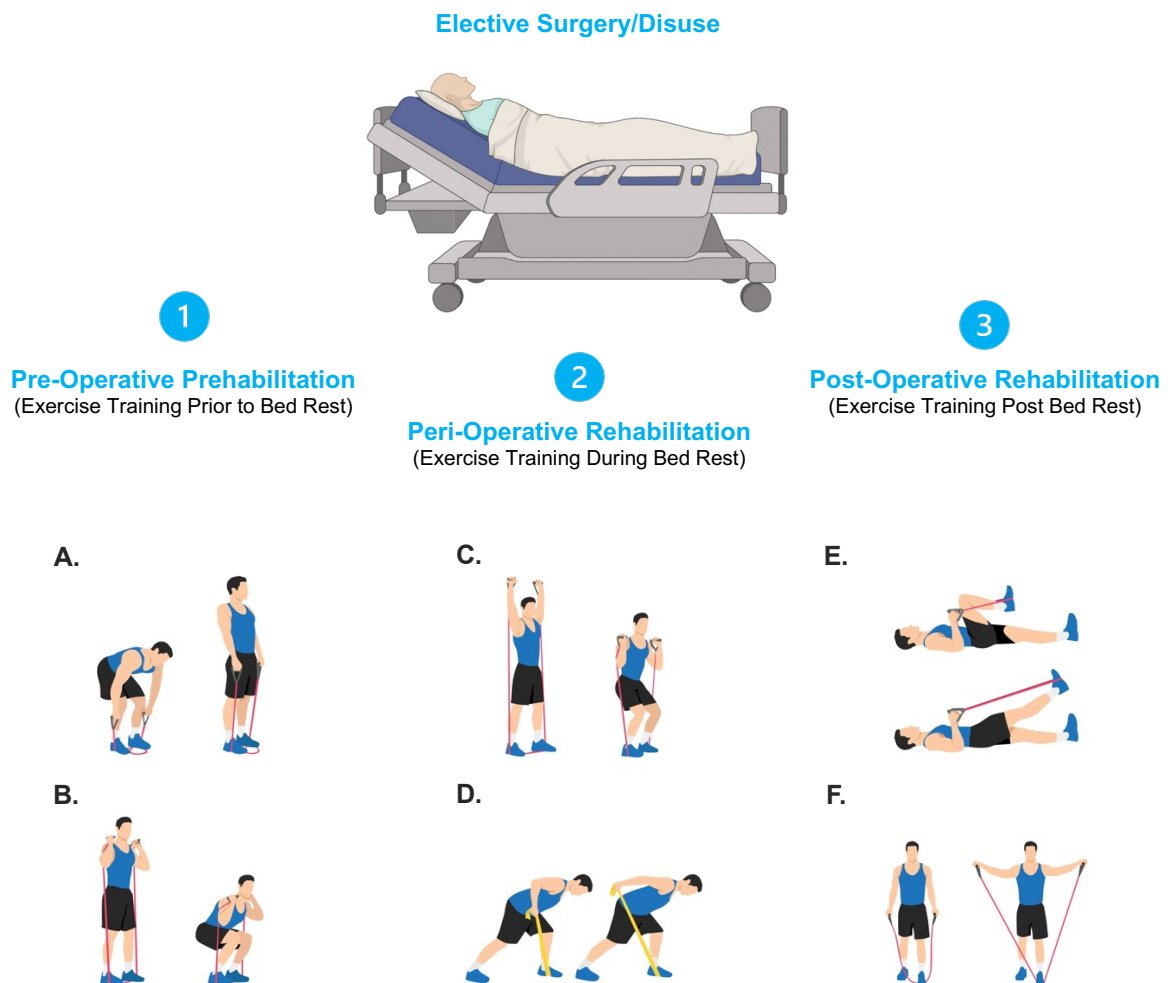


Figure 2. Elastic band resistance exercise as an intervention pre, during and post Bed Rest. The use of EB-RE could be implemented at home in the pre-operative phase in the run-in to surgery to improve musculoskeletal outcomes (1), during the inpatient stay at bed-side (2), or in the rehabilitation period post-BR to regain lost muscle mass and strength. EB-RE is able to target multiple muscles with one single elastic band making it capable of training large and small muscles of the upper and lower body in a single session.

7.5. Can We ‘Out Nutrition’ Physical Inactivity in Older Adults?

Nutritional strategies during musculoskeletal disuse in younger and older adults have had mixed success³⁹⁴. The use of EAA's^{391,463,750}, n-3 FA's⁴⁰², and HMB^{393,515} have been observed to have a positive effect on muscle mass during BR and ULI by maintaining amino acid sensitivity, MyoPS rates and offsetting muscle atrophy . However, the role of nutrition during a model of reduced activity and step reduction has been limited to whey protein²¹³ and L-Citrulline⁴⁷⁹, with no investigation, to date, on the influence of EAA's during disuse. Hence **Chapter 6** provides the study outline to determine the effectiveness of EAA's, with a 40% leucine content (e.g., 'leucine-enriched') at mitigating muscle atrophy and declines in muscle protein synthesis. Based on the previously reported pro-anabolic properties of Leu-EAA's in younger³³⁹ and older adults^{341,344}, the thrice daily oral consumption of high-dose (15g), Leu-EAA during inactivity has the potential to maintain muscle mass and musculoskeletal health (or perhaps offset the decline). Intriguingly, there has been several indications that excessive exposure to EAA's, in particular BCAA's, may induce insulin resistance, diabetes and increase mortality in pre-clinical models and longitudinal human investigation⁷⁷⁵. An over-exposure to dietary EAA's and BCAA's may also chronically elevate mTORC1 signalling molecules, leading to altered translational control and/or efficiency, thereby translating poorly functional proteins, and reducing the muscle protein synthetic response to dietary protein⁵ and/or resistance exercise²⁴⁸. Musculoskeletal disuse has also been associated with the development of insulin resistance via elevated circulatory and intracellular BCAA's, which, when combined with excessive consumption of Leu-EAA's, *may* potentially exacerbate metabolic dysfunction associated with reduced activity and disuse.

7.6. Limitations

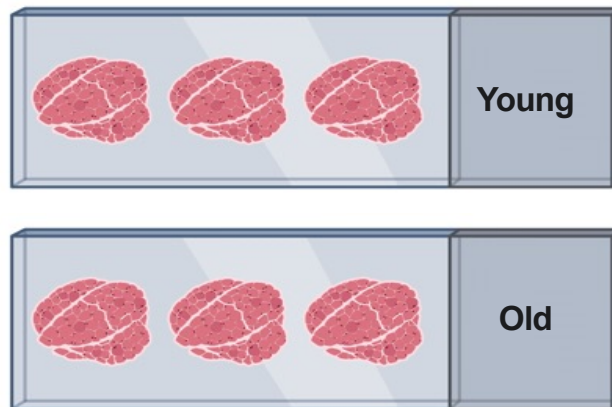
As with all research, the studies conducted in this thesis should be considered within context. Notably, the investigations in **Chapter 2, 3, and 5** are limited by sample size due to the invasive nature of undertaking muscle biopsies in older adults. Nevertheless, based on previous literature, the present set of studies were adequate to detect changes in molecular signalling and iMyoPS. The present thesis was unable to identify sex-specific metabolic alterations due to the recruitment of males. This is critically important as female research has, and still is, chronically overlooked in human metabolic research. Considering the evidence suggesting females are more susceptible to age-related, and disuse-induced muscle atrophy, these replication of studies in females, and comparison to male cohorts are needed. Furthermore, due to limitations in biopsy time-point sampling, we are unable to determine the effects of disuse or resistance exercise in greater resolution. Lastly, from an analytical perspective, the use of immunoblotting techniques are 'semi-quantitative', which may limit the sensitivity of detecting subtle changes in protein abundance, or its post-translational modifications. Therefore, it is worthy of note that changes in mRNA and transcriptome precede changes in protein abundance, or indeed, the phosphorylation of candidate proteins observed in Chapters 2, 3, and 5 ⁷⁷⁶. Detailed RNA sequencing and/or targeted PCR techniques may have allowed the identification and changes in regulatory gene expression of known and potentially unknown protein-protein interactions. Furthermore, the use of D₂O requires careful consideration regarding its dosing (i.e., 400ml vs relative to BM), the duration of measurement (i.e., days, weeks, months), instrumentation analysis (i.e., GC-MS, GC-pyrolysis-IRMS, LC-MS), and the calculations to determine rates of protein synthesis (i.e., body water enrichment of 3.7 or MIDA calculation) ²¹⁹. The use of highly sensitive analytical mass spectrometry

instruments may have allowed us to identify a 'proteomic signature' of ageing in the context of inactivity and exercise. Nano-UHPLC is capable of quantifying of 6-10,000 proteins within a biological sample and may offer even greater metabolic insight when coupled D₂O to accurately measure metabolic flux of individual proteins.

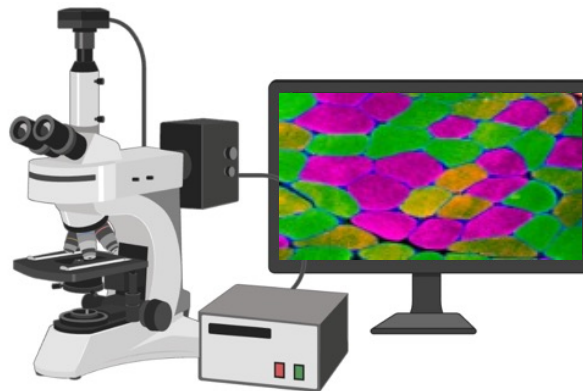
7.7. Future Directions

Future investigations should seek to determine the molecular and metabolic consequences of bed rest and exercise cohorts of older females. Older women lose muscle at a rate of 0.8-0.98%/year⁻¹, which is significantly greater than that of older males (0.64-0.7%/year⁻¹)⁷⁷⁷. The underpinning metabolic and molecular regulation of older females muscle metabolism therefore warrants future investigation. Further analysis on skeletal muscle samples collected from these PhD studies will hopefully aid in our understanding of the molecular regulation of muscle mass and add important pieces to the complex puzzle of musculoskeletal ageing. Notably, determining the influence of age, exercise and protein nutrition on protein content, localisation, and translocation within a cell via immunofluorescent microscopy techniques may aid our understanding of protein-protein interactions. Exploring the nutrient and contractile regulation mTOR, TSC2, RHEB and Sestrin-2 co-localisation in samples from previously collected samples⁷⁶⁴, may offer an insight into 'potential' alerted mTOR signalling in older adults. The rise in prevalence of OMIC's technology, in particular proteomics and metabolomics allows for the unbiased exploration of the complete proteome/metabolome to identify novel, and potentially unexplored protein/metabolites and determine their physiological importance. The application of these platforms in muscle and plasma samples from human exercise, and ageing research will undoubtedly improve our understanding of muscle and ageing biology.

A.



B.



C.

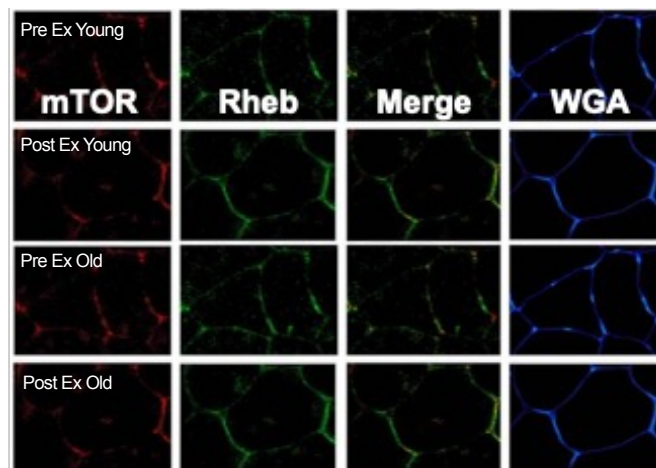


Figure 3. Determining mTOR-RHEB protein expression, co-localisation, and translocation pre and post elastic band resistance exercise. Muscle cross sections (A), confocal microscopy to determine fibre type morphology (B), and the staining of mTOR-RHEB via immunofluorescent antibodies (C).

7.8. Conclusions

This thesis explores some of the potential mediators (i.e., disuse, physical activity), mechanisms (i.e., mitochondrial quality control, mTORC1 signalling, and MyoPS) and countermeasures (i.e., RE prehabilitation, elastic band RE, and Leu-EAA) to age-related muscle loss. We observed no effect of RE prehabilitation, or a subsequent 5-day period of BR on markers of skeletal muscle mitochondrial content and quality control ⁵⁶¹. Furthermore, older adults who have maintained habitually high levels of structured exercise over much of their lives, display greater indices of mitochondrial content (i.e., mitochondrial content, density, and quality control). However, a single bout of RE is insufficient at facilitating acute (i.e., 1hr post) changes in molecular markers associated with mitochondrial fission and fusion dynamics, as well as mitochondrial biogenesis. In response to a single bout of elastic band RE, muscle EMG activity was similar to that observed during traditional machine-based exercise ⁵⁵⁵. The MyoPS response following elastic band resistance exercise was elevated in younger adults, but blunted in older adults, independent of similar mTORC1 signal phosphorylation ⁷⁶⁴. Finally, leucine-enriched EAA's may provide a robust nutritional stimulus to facilitate the maintenance of muscle mass and proteostatic function during a period of reduced activity in older adults.

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