# The Importance of Dietary Protein Quality for Skeletal Muscle Anabolism in Older Adults

By

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**Thesis Disclaimers**

I (Marie Korzepa) declare that the work within this thesis is all of the Author's original work. No part of this thesis has been submitted to the University of Birmingham or any other academic institution and does not include material which has already been submitted for a degree.

The start date and recruitment in **Chapter 4** and **Chapter 5** were affected by the COVID-19 pandemic, where COVID-19 disruption statement to this thesis has been submitted and approved alongside this thesis submission.

In **Chapter 2**, samples used are from muscle biopsies collected as part of PhD work from Ryan N Marshall as part of MRC Versus Arthritis funded project. In **Chapter 4**, analysis of samples was completed by Ari Gritsas and Tyler Churchward-Venne at McGill University, Canada as part of a collaboration. In **Chapter 5** analysis of skeletal muscle biopsy samples and saliva deuterium enrichment was undertaken by myself with technical support from Joan Senden and Joy Goessens in Luc van Loon's lab in Maastricht University, The Netherlands as part of a collaborative visit. Funds to support all research was made possible as part of the £64,000 studentship awarded to Professor Leigh Breen and Marie Korzepa by Volac International LTD and University of Birmingham.

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**Thesis Abstract**

The age-related loss of skeletal muscle mass and strength, termed sarcopenia, is underscored by impairments in muscle protein turnover which presents as type II muscle fibre atrophy. Specifically, a blunted muscle anabolic response to dietary protein ingestion is suggested as a key driver of sarcopenia progression but the intracellular mechanisms remain unclear. Sarcopenia progression may be attenuated through dietary protein intake at or above 1.2 g·kg body mass[BM]$^{-1}$day$^{-1}$ and partaking in resistance exercise training (RET), but these strategies may not be feasible for many older adults. In this thesis, **Chapter 2** uses immunofluorescence microscopy (IF) to compare the localisation and abundance of mechanistic target of rapamycin (mTOR)-related intracellular signalling targets on a fibre type-specific basis in rested skeletal muscle of young (YM) and old (OM) men. The abundance of each target was similar between fibre types, although in OM mTOR abundance was lower than YM, whereas tuberous sclerosis complex 2 and Ras homolog enriched in brain (Rheb) were greater, whilst Rheb localisation at the muscle fibre periphery also differed between YM and OM. This chapter highlights that IF has the capacity to identify age-related alterations in intracellular signalling protein abundance and localisation, which may underly impairments in muscle anabolism implicated in the progression of sarcopenia. **Chapter 3** provides a narrative review highlighting barriers to increasing dietary protein intake beyond general population-wide guidelines and participating in RET. Considering these barriers, emphasising the consumption of higher quality protein sources (i.e., those with a complete essential amino acid (EAA) profile that is readily bioavailable) may support greater postprandial muscle anabolism, particularly in scenarios where protein intake is well below recommendations, such as with ill-health and disuse. Considering the proposed importance of dietary protein source,

**Chapter 4** explored the postprandial amino acid (AA) and appetite regulatory response to a very low protein-containing whole food mixed breakfast supplemented with a small bolus of higher quality whey protein concentrate (MB+WPC) or lower quality pea protein isolate (MB+PPI) smoothie in middle-to-older aged adults. Plasma total and EAA excursions and their overall availability over 180 minutes postprandially were not different between groups. However, plasma leucine availability was greater following MB+WPC. Perceived hunger and satiety responses, and plasma concentrations of appetite regulatory hormones were transiently altered following MB+WPC and MB+PPI but did not differ between groups. Greater postprandial plasma leucinemia with MB+WPC over MB+PPI could indicate a superior capacity for muscle anabolic stimulation in middle-to-older aged adults, without adverse effects on appetite. To investigate the suggestions of the preceding chapter, **Chapter 5** determined free-living daily rates muscle protein synthesis (iMyoPS) in middle-to-older aged adults consuming a 10-day diet containing ~1 g·kg $BM^{-1}$·$day^{-1}$ of protein. Meals and a snack were supplemented with a small bolus of higher-quality whey protein concentrate (HQ-D) or lower-quality pea protein isolate (LQ-D). Frequent unilateral RET sessions were performed over the 10-day dietary intervention. Daily rates of iMyoPS were similar between HQ-D and LQ-D in the untrained leg, but greater in the trained over the untrained leg in both groups. Changes in muscle adaptive remodelling, body composition and whole-body nitrogen balance were absent and/or indistinguishable between groups. In a typical lower protein-containing diet of healthy middle-to-older aged adults, the quality and source of ingested protein does not modulate rested or RET-induced iMyoPS rates over a 10-day period. Nonetheless, frequent RET augments daily rates of iMyoPS over a 10-day lower protein-containing diet and represents a potent strategy to mitigate age-related muscle deterioration.

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## Publications list

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Smeuninx, B., Elhassan, Y.S., Sapey, E., Rushton, A.B., Morgan, P.T., **Korzepa, M.**, Belfield, A.E., Philp, A., Brook, M.S., Gharahdaghi, N., Wilkinson, D., Smith, K., Atherton, P.J., Breen, L. A single bout of prior resistance exercise attenuates muscle atrophy and declines in myofibrillar protein synthesis during bed-rest in older men. J Physiol. (2023) doi: 10.1113/JP285130.

Lewgood, J., Oliveira, B., **Korzepa, M.**, Forbes, S.C., Little, J.P., Breen, L., Bailie, R., Candow, D.G. Efficacy of Dietary and Supplementation Interventions for Individuals with Type 2 Diabetes. Nutrients. (2021) doi: 10.3390/nu13072378.

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**Presentation list**

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European College of Sport and Exercise Science, *Seville, Spain,* 2022

International Biochemistry of Exercise Conference, *Toronto, Canada,* 2022

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**List of Abbreviations**

| | |
|---|---|
| 1RM | 1 repetition maximum |
| $^{2}H$ | Deuterium |
| 4E-BP1 | eucaryotic translation initiation factor 4E binding protein 1 |
| AAs | Amino acids |
| Akt | Protein kinase B |
| AMPK | 5' adenosine monophosphate-activated protein kinase |
| ANOVA | analysis of variance |
| APE | Atom percent excess |
| BMI | Body mass index |
| CONSORT | Consolidated standards of reporting trials |
| CR-10 | Category Ratio 10 scale |
| CSA | Cross-section area |
| $D_2O$ | Deuterated water |
| DIAAS | Digestible indispensable amino acid score |
| EAAs | Essential amino acids |
| EDTA | Ethylenediaminetetraacetic acid |
| eEF2 | Eucaryotic elongation factor 2 |
| eGFR | Estimated glomerular filtration rate |
| EI | Energy intake |
| FAK | Focal adhesion kinase |
| FAO | Food and Agriculture Organization |
| FFM | Fat free mass |
| GDPase | Guanosine diphosphate |
| GLP-1 | Glucagon-like peptide 1 (GLP-1) |
| GTPase | Guanosine triphosphate |
| HDL-c | High density lipoprotein cholesterol |
| HQ-D | Higher quality protein diet |

| | |
|---|---|
| iAUC | Incremental area under the curve |
| IF | Immunofluorescence microscopy |
| IMTG | Intramyocellular triglycerides |
| iMyoPS | Integrated myofibrillar protein synthesis rates |
| IR-P-MS | Isotope ratio mass spectrometer with pyrolysis oven |
| IRS-1 | Insulin receptor substrate 1 |
| IV | Intravenous |
| LDL-c | Low density lipoprotein cholesterol |
| LQ-D | Lower quality protein diet |
| MB | Mixed breakfast |
| MHC1 | Myosin heavy chain type I |
| MPB | Muscle protein synthesis |
| MPE | Mole percent excess |
| MPS | Muscle protein breakdown |
| MRI | Magnetic resonance imaging |
| mRNA | Messenger ribonucleic acid |
| MT | Muscle thickness |
| mTORC1 | Mechanistic target of rapamycin complex 1 |
| MVC | Maximum voluntary contraction |
| MVPA | Moderate and vigorous physical activity |
| NEAA | Non-essential amino acids |
| OCT | Optimum cutting temperature |
| OM | Older men |
| p70S6K | Ribosomal protein S6 kinase |
| PA | Pennation angle |
| PI3K | Phosphoinositide 3-kinase. |
| PPI | Pea protein isolate |
| PYY | Peptide tyrosine tyrosine (YY) |
| RDA | Recommended dietary allowance |

| | |
|---|---|
| REE | Resting energy expenditure |
| RER | Respiratory exchange ratio |
| RET | Resistance exercise training |
| Rheb | Ras homolog enriched in brain |
| RPS6 | Ribosomal protein S6 |
| SD | Standard deviation |
| SEM | Standard error of the mean |
| TAA | Total amino acids |
| TBW | Total body water |
| TEE | Total (daily) energy expenditure |
| tRNA | Transfer ribonucleic acid |
| TSC1 | Tuberous sclerosis complex 1 |
| TSC2 | Tuberous sclerosis complex 2 |
| UPLC-MS | Ultra-performance liquid chromatography mass spectrometry |
| VA | Voluntary activation |
| VAS | Visual analogue scale |
| WBNB | Whole body nitrogen balance |
| WGA | Wheat germ agglutinin |
| WHO | World Health Organisation |
| WPC | Whey protein concentrate |
| YM | Younger men |

# Chapter 1 General Introduction

## 1.1 Population ageing

Advancements in modern medicine and our scientific understanding of diseases and their treatment has meant that the human lifespan has increased significantly over the past 50 years. As of 2020, the average age, when combining males and females stood at 81 years old [1]. Indeed, the average life expectancy is projected to slow by 2070 increasing to 87 years (~5.7% increase) in comparison to the rises seen over the previous 50 years (~10.6% increase), showing we are near optimising the length of human life [1]. However, the increased population density means more people are projected to live over 60 years of age in the coming years. Importantly, these statistics only consider chronological ageing (lifespan). Alternatively, biological ageing is an important driver of overall healthy life expectancy (termed 'heath-span') and requires attention to ensure our ageing populations spend a greater portion of life free from disease and ill-health [2, 3]. A typical gap between lifespan and health-span is 10 years, meaning the last 10 years of life typically incur some health complications [2]. However, poor socioeconomic status, ethnicity and lifestyle choices can increase this gap [4]. From a governmental perspective, extending the health-span would improve the contribution to the workforce and economy up until retirement [5] and lower fiscal costs associated with poor health, estimated at £236 billion in the UK [6]. Consequently, extending the health-span is an important and increasingly popular locus of healthy ageing research [7]. Indeed, implications of poor health extend from direct care, with quality of life also affected. Notably, lower quality of life is associated with heightened rate of institutionalisation [8] with a decline in mental well-being. Consequently, research to support and extend healthy human ageing is of the utmost importance and relevance [9, 10].

## 1.2 Hallmarks of biological ageing

In order to understand how to extend lifespan in tandem with optimising health-span, it is important to identify inherent characteristics of ageing, which have been coined as 'hallmarks' [11]**.** Inflammation accentuates many hallmarks where exposure and risk of disease increases with age [12]. Factors which accelerate inflammation include exposure to environmental hazards (i.e., toxins, pollution and smoke) [13], periods of illness/non-communicable disease [14, 15], poorer diet [12], lowered activity [16] and increased adiposity [17]. Inflammation, which is an ageing hallmark in its own right, can also be exacerbated by and interact with age-related hormonal changes (i.e. menopause or hypogonadism) [18, 19] and changes in body composition (i.e., increased adiposity) [20]. Subsequently, such changes can compromise metabolic function and insulin sensitivity with advancing age [21–23]. Interestingly, all these facets are intertwined and are influenced by genetic and environmental factors [24]. For instance, beyond retirement age, physical activity levels generally decline and are associated with increased adiposity [25, 26]. Paired with alterations in the hormonal milieu, an elevated proinflammatory state impairs insulin sensitivity and normal metabolic function of peripheral tissues and organs [27] fuelling a vicious cycle in age-related hallmarks [11, 21, 28]. Commonly measured hallmarks of ageing which are affected by these age-related occurrences include dysbiosis, dysregulated nutrient-sensing, altered intercellular communication and chronic inflammation. These hallmarks are adversely affected by poor diet and physical inactivity [29], but may also be ameliorated through modifications to diet [30] and exercise training [11, 31]. Focusing on these lifestyle factors represents a cost-effective, non-pharmacological opportunity to target the hallmarks of ageing and extend health-span.

## 1.3 The importance of skeletal muscle

Skeletal muscle allows humans to perform locomotory tasks and serves as a reservoir for glycogen and lipid storage, which are essential for sustaining life [32] and energetic processes [33]. Consequently, skeletal muscle mass is conserved as an important metabolic regulator and is considered the primary contributor for overall metabolic rate [34]. Following adolescence and up to 60 years of age energy expenditure remains relatively stable, declining thereafter in a manner that coincides with declines in skeletal muscle mass [35]. Poor skeletal muscle health is implicated in insulin resistance and metabolic inflexibility, which in turn can have detrimental effects on the metabolic regulation of other tissues, further accelerating the decline in skeletal muscle health [36, 37]. In addition, low muscle mass and strength can limit the ability to complete activities of daily living, increase the risk of falls and reduce quality of life, as well as contribute to increased mortality rate [38–40].

## 1.4 Age-related sarcopenia

The decline in skeletal muscle mass and strength with advancing age has been termed ‘sarcopenia’ [41] and typically occurs >40 years of age [42]. The prevalence of sarcopenia diagnosis is estimated at ~5-10% of those aged 65 years and up to 50-60% of those aged 80 years and over. Alarmingly, these figures are projected to increase as a function of lifespan extension in global populations [43–45]. Sarcopenia increases the risk of falls and comorbidities, which ultimately reduces health-span and quality of life [46, 47]. This decline in skeletal muscle health has a colossal impact on an individual’s welfare and places a major strain on fiscal costs and healthcare expenditure if left unattended [48–50]. Furthermore, skeletal muscle health declines can be punctuated by falls, periods of inactivity and the presence of obesity from

middle age (50-60 years old) onwards [51–54]. Indeed, chronological ageing coincides with many factors which may exacerbate muscle health due to exposure and lifestyle (i.e., reduced activity following retirement). Considering the importance of skeletal muscle health as a strong prognosticator [55, 56], research efforts to understand mechanisms of muscle mass regulation with advancing age and develop strategies to support skeletal muscle health from middle age onwards are paramount. This introductory chapter will provide an overview of factors influencing skeletal muscle mass with ageing and support the rationale for subsequent experimental chapters within this thesis.

## 1.5 Morphological and functional changes in skeletal muscle with age

### *1.5.1 Skeletal muscle mass and fibre morphology*

On average, 0.5-1.0% of muscle mass is lost after the 5$^{th}$ decade of life [45, 57]. Henceforth, declines in skeletal muscle mass are used as key criteria in diagnosing sarcopenia, where appendicular muscle mass below 20 kg for males and below 15 kg for females confirms sarcopenia provided low muscle strength is present [42]. Skeletal muscle comprises bundles of muscle fibres which are responsible for the transduction of force once innervated by the central nervous system. These fibres are typically categorised as being oxidative slow twitch (type I fibres) or glycolytic fast twitch (type II fibres). Pertaining to the name, type II fibres use rapidly depleted glycogen stores to exert large forces quickly, but for a short period of time. Conversely, type I fibres are more mitochondria-dense to facilitate lower intensity activity patterns for a longer duration through increased reliance on oxidative processes. Interestingly, with advancing age, it has been repeatedly shown that type II muscle fibres display the greatest rate of atrophy [58, 59]. Furthermore, evidence suggests that muscles of the

lower limbs undergo greater atrophy than the upper limbs, likely because of the greater abundance of muscle fibres and the proportion of type II fibres [57, 60]. Age-related muscle fibre atrophy has been shown to be punctuated by inactivity [52] and periods of immobilisation such as bed rest [61, 62]. Alongside a reduction in type II fibre area, and a comparative increase in type I fibre area, the number of type II fibres has been suggested to decrease with age [63], albeit from data collected from diseased cadavers. More recently, studies have found most fibre type alterations with healthy ageing show a reduction in type II fibre atrophy rather than reduced fibre number [64]. The susceptibility of type II fibres to age-related atrophy and the comparative increase in type I fibre abundance remains to be fully understood. One possible explanation is the denervation of type II muscle fibres, where motor units innervating these fibres are reduced in size or firing capacity [65, 66]. Motor units which were innervating type II fibres instead behave like type I motor units, which have a lower firing rate or can even branch and innervate type I fibres [67], resulting in large groups of type I fibres and atrophied type II fibres [58, 68]. Additionally, the susceptibility of type II fibre atrophy may be related to altered proteostasis within fibres, since protein aggregation is common with ageing [69–71]. Dysregulated proteostasis could present as defect in proteins which regulate protein synthesis within muscle fibres, although this remains to be elucidated on a fibre type-specific basis with age. In summary, type II muscle fibre atrophy is synonymous with ageing and manifests as a reduction in overall muscle mass with implications for strength and function, and myriad health consequences. Henceforth, fibre type-specific investigations may shed light on regulatory mechanisms and potential defects that may underpin sarcopenia progression

### *1.5.2 Skeletal muscle quality and architecture*

Another, criterion for sarcopenia diagnosis is muscle quality, which is often described as the strength per area of a given contractile material. Indeed, muscle quality is an insightful assessment of muscle functionality, but shouldn't be used to categorise sarcopenia in isolation, as many factors can affect muscle strength and mass. Magnetic resonance imaging (MRI) scans have been instrumental in repeatedly highlighting that with increasing age, there is an increase in intramuscular and intermuscular fat infiltration. The general increase in fat and a reduction in skeletal muscle mass means there is less contractile material and force-generating potential for a given area [72]. Whilst MRI scans can provide holistic detail on specific muscle groups, fibre-type specific characteristics are challenging to investigate. The use of ultrasound has the capacity to identify within-muscle bundle characteristics which can change with age and ultimately affect functionality. Outcomes from ultrasound scans include muscle thickness, fascicle length, and the angle of fibres (pennation angle). In normal ageing, muscle thickness typically decreases as expected due to increased inter and intramuscular fat infiltration and loss of contractile material [73, 74]. Additionally, fascicle length and pennation angle can decrease [73, 75], resulting in less efficient force transduction in the direction of the muscle fibre bundles [76]. Skeletal muscle is highly placid, where ageing and exercise training influence structural and architectural changes. Such changes to skeletal muscle can be easily measured and provide insight into overall muscle functionality and health [73]

### *1.5.3 Muscle Strength and function*

A strong diagnostic tool for sarcopenia and frailty is muscle strength. Strength loss occurs at a rate 3-5% annually after 50 years of age, far exceeding rates of muscle loss from middle age [45, 77, 78]. Indeed, muscle fibre atrophy and muscle quality are associated with lower muscle strength and power [45, 79, 80], which can manifest as decreased voluntary muscle activation and force production [81, 82]. Considering the greater relative loss in muscle strength when compared to muscle mass with ageing [45, 57], deteriorations in strength are likely not exclusively explained by deteriorations in muscle proteostasis. Declines in neural function, which is a proposed mechanism for type II fibre denervation, may partly explain these greater strength reductions, as well as overall reduced neural drive, neural efficiency and motor unit size alterations [67, 78]. The consequences of reduced muscle strength and function are severe, and include markedly greater fall risk and a reduction in the capacity to undertake activities of daily living [83]. Subsequently, compromised muscle strength is implicit in sarcopenia diagnosis, with reduced strength and function (gait speed) being captured as key criteria important for health-span [49]. Henceforth, it is imperative that research studies look beyond alterations in muscle mass regulation, to skeletal muscle function and strength, since declines in strength cannot always be delineated from altered muscle mass.

## 1.6 Regulation of skeletal muscle proteostasis

### *1.6.1 Muscle protein turnover*

All cells within the body are made of and contain proteins. Skeletal muscle contains the highest proportion of protein of all body tissues, accounting for >50% of the total protein pool [84]. Indeed, the decline in muscle mass occurs when the rate of muscle protein breakdown (MPB) exceeds the rate of muscle protein synthesis (MPS) over a sustained period, creating a negative protein balance [85]. Conversely, skeletal muscle mass accretion is achieved when the rate of MPS frequently exceeds the rate of MPB [86]**.** MPB is required to sequester essential amino acids (EAAs) from other proteins and prevent uncontrolled expansion of the protein pool [87]. Studies have tried to capture MPB in humans [88, 89] but accurate and consistent measures are challenging since protein degradation is not linear, with variation in rate and fate of constituent elements which can be catabolic end-products and even recycled into new proteins [90, 91]. Outside of hypermetabolism where protein balance is moderately influenced by MPB [92], it is MPS which is considered the primary locus of regulation for overall net muscle protein balance. Henceforth, measuring MPS rates is more common in research and is instrumental for understanding the dysregulation in proteostasis with ageing.

### *1.6.2 Impact of protein ingestion and exercise on protein turnover*

An important prerequisite for tissue protein synthesis, including skeletal muscle, is the presence of amino acids (AAs). Peptide and polypeptide chains which form proteins once folded, are formed from a series of AAs with the largest protein (titin, within skeletal muscle) featuring ~34,000 AAs [93]. Given the large requirement, it is

imperative that all AAs are available for synthesis of endogenous proteins. Of particular importance EAAs, which need to be acquired exogenously as they cannot be synthesised within the body. The restriction of EAAs in animal models shows significantly blunted MPS rates, whereas provision of EAAs seems to be the primary determinant of postprandial MPS stimulation [94, 95]. Profound increases in MPS following EAA provision have also been shown in young humans with intravenous infusion [96] and greater MPS stimulation is apparent with increasing EAA provision [97]. In a day-to-day setting, EAAs are obtained via consumption of a variety of protein-containing foods or isolated supplements. A mixture of 10g of crystalline EAA was reported to maximally stimulate postprandial MPS in young adults, with no additional benefit with 20g of EAA [98]. Similarly, postprandial MPS has been reported to plateau after consumption of ~0.24 g·kg body mass $[BM]^{-1}$ protein in healthy young adults [99] representing an upper limit for protein-induced postprandial MPS stimulation in a typical eating pattern comprising 3-4 daily meals/snacks.

Another potent stimulus for MPS stimulation and net muscle protein accretion is resistance exercise. The impact of resistance exercise has long been seen to bolster muscle anabolism acutely [100, 101] and as much as 48-72h after exercise [101, 102]. Combined EAA provision and resistance exercise are synergistic for MPS stimulation and necessary to achieve a maximal muscle anabolic response [103, 104]. In healthy young adults, resistance exercise increases the upper limit for postprandial MPS stimulation. Some have observed that prior resistance exercise and consuming ~20g of protein maximises muscle anabolism, with 40g of protein offering no further augmentation in MPS [105]. In contrast, others report that 40g protein can further increase resistance exercise-stimulated MPS [106], which may be due to discordance in resistance exercise intensity. Irrespective, prior resistance exercise increases the

capacity for dietary protein-derived AAs to augment whole body protein synthesis, compared with protein ingested in the absence of resistance exercise [107]. Frequent distribution of adequate dietary protein (every 3-4 hours) appears to maximise the cumulative daily MPS response [108], although the additive effect of resistance exercise on postprandial MPS stimulation is sustained for at least 24 hours post-exercise [109]. Henceforth, frequent resistance exercise combined with protein feeding across daily feeding events equating to ~1.6 g·kg $BM^{-1}$·$day^{-1}$ is conducive to net muscle protein anabolism that underscores muscle hypertrophy, with associated benefits for strength accretion [110, 111].

### *1.6.3 Age-related dysregulation of muscle protein turnover*

Numerous studies show basal postabsorptive rates of MPS and MPB do not differ between younger and older adults [98, 112, 113]. Impairments in muscle protein turnover are instead concerned with blunted stimulation of MPS in response to anabolic stimuli of protein feeding and resistance exercise, which has been coined ‘anabolic resistance’ [114].

A seminal study by Cuthbertson et al. (2005) provided younger and older, apparently healthy adults with well digested crystalline EAAs at doses ranging from 1.5-20g [98]. Ingestion of EAAs increased MPS rates in both younger and older adults, although the magnitude of response was greater in younger adults at all doses. Interestingly, peak MPS was elicited with 10g of EAAs in young and 20g EAAs in old, with MPS rates plateauing with any increases in further doses. The substantial ~0.4%$h^{-1}$ higher maximal MPS response seen by Cuthbertson et al. (2005) in younger compared to older adults from very high doses of EAAs highlights a physiological impairment in the skeletal muscle of older adults. Indeed, the older adults recruited

within this paper exhibited significantly higher adiposity and inflammation than younger counterparts [98]. Whilst this is an expected difference with ageing, this metabolic alteration may partly explain the inferior MPS response with typical ageing and associated systemic changes. This impairment may be attributed to higher plasma and intramuscular concentrations of leucine in older adults, representing an impaired clearance and utilisation of leucine [98]. Nonetheless, impairments are partly ameliorated with the provision of greater protein doses. This blunted MPS response and requirement for greater protein intake to maximise postprandial MPS responses is well agreed [98, 114, 115]. As such, it has been delineated older adults require ~2-fold higher relative protein doses compared to their younger counterparts to maximise postprandial MPS, equating to ~0.4 g·kg $BM^{-1}$ [99].

As with EAA ingestion, older adults also display a blunted MPS response following resistance exercise, compared with younger adults. This was eloquently shown by [100] using a unilateral resistance exercise model at increasing percentages of 1 repetition maximum (1RM). The authors found older adults display a lowered MPS response compared to younger adults at each intensity [100], with 60% and 75% 1RM eliciting peak MPS for younger and older adults, respectively. Indeed, at all intensities, the MPS response over 4h was persistently blunted in older compared to younger adults. Studies have shown that promoting a higher training volume can augment the MPS response in older adults to elicit more comparable 'youthful' responses [116]. With regard to longer-term muscle adaptation to resistance exercise, the absolute increase in strength, muscle volume and type II fibre area are lower in older adults than young [117–119]. These data highlight that the blunted acute MPS impairments to resistance exercise underly a poorer adaptive remodelling of skeletal muscle in older compared with younger adults [120]. Notwithstanding, profound increases in

muscle mass and strength can be gained through regular high-volume resistance exercise in older adults [121, 122], which is likely imperative for preventing sarcopenia [123].

The blunted MPS response to resistance exercise and protein feeding in older adults compared with younger adults has been termed age-related muscle ‘anabolic resistance’ [114]. It is considered that the combination of high-volume resistance exercise and high protein intake is the most effective strategy to overcome age-related anabolic resistance where older adults can still achieve ‘youthful’ responses in MPS and adaptive remodelling [114, 124, 125]. In older adults, graded increases in supplemental whey protein paired with exercise show 30-40g protein can be ingested to maximise resistance exercise-stimulated MPS responses [126, 127]. The benefit of graded increased supplemental protein intake alongside resistance exercise is thought to be due to greater leucine availability, which is a key mobiliser for triggering MPS. As such, numerous studies have found emphasising leucine intake augments resistance exercise stimulated MPS in older adults [128–131]. However, the ability of resistance exercise to ‘sensitise’ skeletal muscle to higher protein doses and promote long-term adaptations in older adults remains unclear [86]. Nonetheless it is generally agreed that increasing protein intake with repeated resistance exercise training benefits skeletal muscle remodelling [114].

## 1.7 Methods for measuring *in vivo* human muscle protein turnover

### *1.7.1 Stable isotope tracer methodology*

All amino acids and thus all proteins contain 4 elements; carbon, hydrogen, oxygen and nitrogen, where each can be isotopically labelled. For protein turnover to be ascertained, at least one of these components needs to be labelled differently from its naturally occurring common form. Early utilisation of said isotopes to understand human physiology was seen following atomic bomb use which released carbon-14 [$^{14}C$] into the atmosphere and was found in tendons, where this incorporation has been used to evaluate slow tendon turnover rates [132, 133]. Notably, [$^{14}C$] is unstable and is a known carcinogen, which makes it and other unstable isotopes such as tritium [$^{3}H_2$] unsuitable for in vivo human application, even though they would be candidates to label protein components [134]. Instead, stable isotopes have been more widely used to explore metabolic flux, whole-body protein synthesis as well as muscle protein-specific turnover. The combination of stable isotopes and skeletal muscle biopsies allows for the determination of MPS, using sophisticated mass spectrometry instruments [134, 135]. Numerous studies have used a mixed muscle protein fraction to understand the bulk turnover of the protein pool within a muscle sample [101, 136, 137]. However, in doing this, differences in the turnover of specific protein fractions/pools are missed. It is understood that the muscle sarcoplasmic fraction, which comprises most of the organelles, turns over slower than the muscle myofibrillar fraction, which contains the contractile elements [138, 139]. As ageing may be partly characterised by an impairment in skeletal muscle function, examining alterations in the synthesis of muscle-derived myofibrillar proteins is particularly relevant.

### *1.7.2 Acute intravenous labelled isotope tracer infusion*

Routine in human physiology research, acute measurements of muscle protein turnover are often used to determine the response to a given stimulus or intervention. An intravenous (IV) infusion of isotope labelled AAs can be used to measure the rate of MPS as an index of tissue anabolism. Evaluating protein turnover through isotope tracer IV infusion requires an understanding of the substrate-specific isotope label, the rate of IV infusion, and the appearance of the labelled amino acids within a tissue of relevance (i.e., skeletal muscle) over a given timeframe [85, 140, 141]. By knowing the amount of tracer provided and taking muscle biopsy samples at set time points, the rate of MPS can be measured around an acute stimulus. For example, this approach has been used extensively to characterise temporal MPS rates at rest and in response to protein ingestion, and resistance exercise in younger and older adults. However, the time course of IV infusions is limited due to the supply of isotopically labelled amino acids and the reasonable time a cannula can be kept in place. Henceforth, IV infusions are excellent for understanding acute response to a physiological intervention, although must take place in controlled settings and cannot extend over multiple days.

Dual IV tracers have also been used to capture the flux of different proteins, where the incorporation of dietary protein-derived amino acids into skeletal muscle tissue can be quantified. Dual isotope methodologies typically involve consuming foodstuff with an endogenous AA, alongside a constant IV infusion or consumption of a separate labelled AA, which is used as a reference for digestibility and endogenous turnover rates [142, 143]. Numerous different foodstuffs have been isotopically labelled, for example, by infusing L-ring$^{13}C_6$ phenylalanine into dairy cows [144] in order to label milk and beef products which are subsequently fed to humans to

determine the postprandial MPS response [144, 145]. Furthermore, labelling different foodstuffs provides great insight into the rate of appearance and disappearance of endogenous and exogenous AAs to ascertain digestibility, Similarly, stable isotopes have been used to evaluate muscle protein turnover and/or digestibility of eggs [146], legumes [147] as well as insects [148]. Importantly, a finite number of stable AA isotopes, logistical challenges and cost, means that labelling multiple complex foods to gain insight into typical digestion rates and endogenous turnover from the diet is not feasible. Henceforth, alternative methods need to be used to ascertain habitual rates of protein turnover which may be modulated by food consumption.

### *1.7.3 Deuterium oxide isotope tracer methodologies*

Deuterium has been used over the past 100 years to understand metabolism and has been advanced in recent years for use in intricate investigations of free-living rates of MPS in humans [135]. Deuterium is a stable isotope of hydrogen, possessing the addition of one neutron within its nucleus. The addition of this neutron does not affect chemical charge, but does increase the molecular weight by 1, meaning deuterium can be presented as $^{2}H$. This increase in mass and rare typical abundance of deuterium in the body means that it can be detected in the body water pool if provided exogenously. In humans, deuterium stable isotope tracer has been provided orally as deuterated water ($D_2O$). The ability to provide an oral stable tracer capitalises on the time-restricted limitations and the high participant burden that are inherent to the IV tracer approach. Deuterium can endogenously label any hydrogen-containing molecule, including individual proteins or AAs within any tissue within the body. Theoretically, all AAs could be used to evaluate MPS, although this would require a large amount of sample and be associated with significant analysis costs. Instead, a

single amino acid; alanine, is commonly used to identify the incorporation of deuterium in the skeletal muscle protein pool. Alanine is selected because i) it is not oxidised [141], ii) its bioavailability is high but requires less tracer to overcome naturally occurring dilution, than the most abundant AA; glutamine [149], and iii) it has 4 hydrogens which are bound to carbon that can be substituted for deuterium, making a strong and sustained carbon-deuterium bond [150]. Regarding the latter, it has been found that an average of 3.7 deuterium are exchanged for hydrogens [151]. Henceforth, this value is used in the precursor-product calculation of MPS to indicate the average number of deuterium substituted for hydrogen.

As with the IV tracer approach, skeletal muscle biopsies are required alongside precursor tracer enrichment over the period of deuterium provision, to determine MPS using the $D_2O$ approach. Considering consumption of $D_2O$ is rapidly incorporated into the body water pool and tissue protein there are a variety of precursors that can be used. Deuterium tracer enrichment in the precursor pool should be at equilibrium in line with the amount of deuterium provided. During interventions, it is paramount that such measures are accurate but also do not create a burden to participants that would increase attrition. Indeed, common methods include blood draws to determine plasma deuterium enrichment, or saliva samples to infer deuterium enrichment in the body water pool. It has been shown there is a high agreement between both [152], with plasma-derived deuterium values typically higher than saliva due to the greater, but constant dilution of deuterium [151]. The use of $D_2O$ still requires a stable incorporation rate over the sampling period to allow for accuracy when comparing turnover between time points [153]. Providing a larger $D_2O$ bolus allows for more proteins to be labelled, however, an extended period of labelling may require lower deuterium doses as all proteins will eventually be turned over which may result in a plateau in calculated

synthesis rates [154]. Stable incorporation of deuterium via $D_2O$ into the total body water pool is commonly achieved by consuming one large bolus with subsequent smaller daily top-up boluses of deuterium, at a dose that is dependent on the length of the intervention and required level of enrichment for detection in precursor and muscle-fraction pools. The low burden and non-invasive nature of oral deuterium provision and saliva sampling mean that MPS from deuterium can be measured over multiple days [152, 155] and weeks [156, 157]. As a result, free-living daily rates of MPS can be determined, enabling measurement of dynamic changes in muscle protein turnover in response to activity level and food consumption over extended periods, capitalising on the shortcomings of acute IV infusions where techniques are compared in **Figure 1.1.** When using the $D_2O$ approach to determine free-living daily rates of MPS, it is fundamental for activity and eating patterns to be monitored/recorded to rule out any potential influence of these confounding variables that might influence the data and overall interpretation.

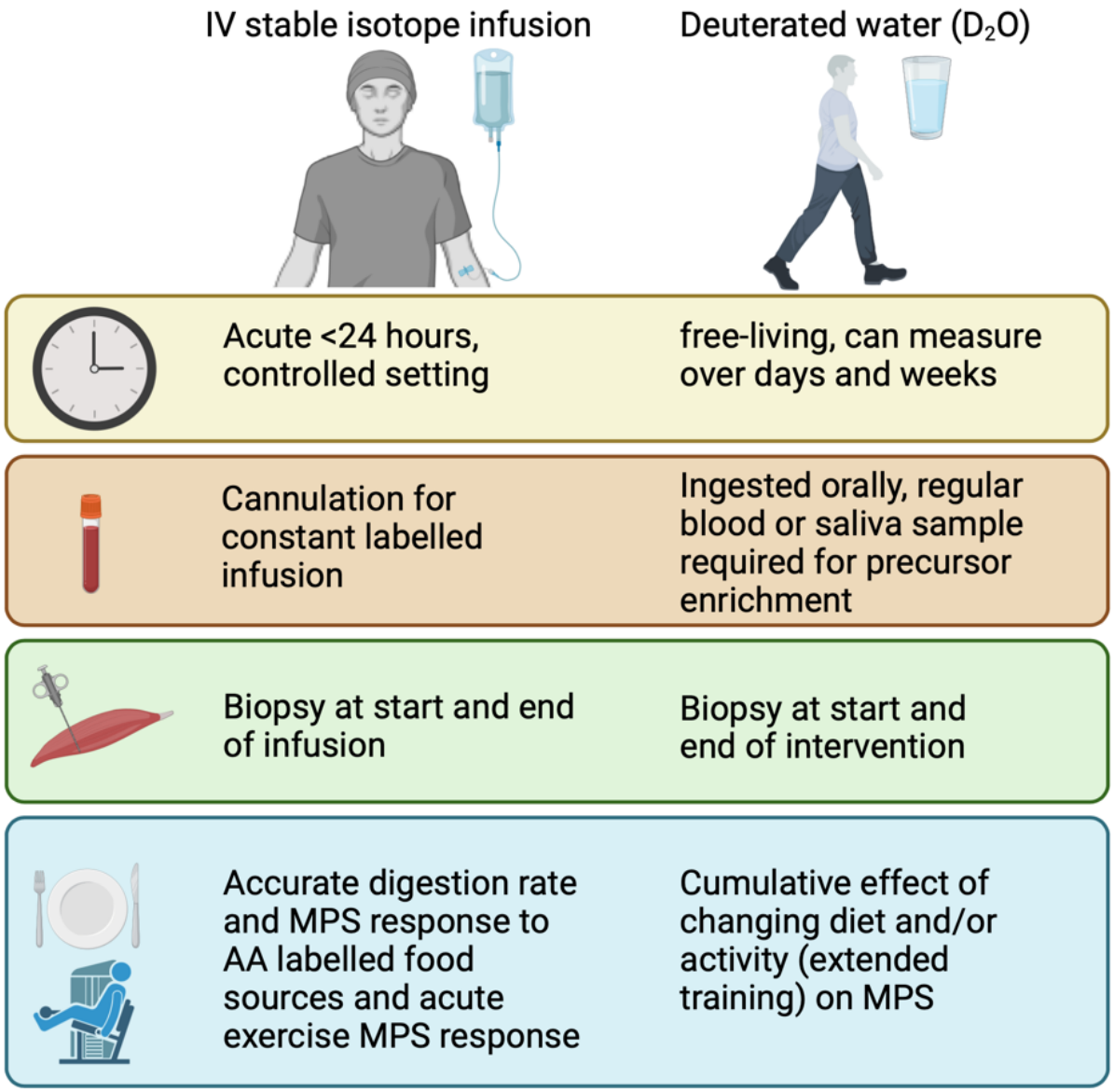


**Figure 1.1.** Comparison of intravenous infusion and deuterium oxide stable isotope tracer methodologies for determining muscle protein synthesis (MPS).

Yellow denotes the time frame for MPS measurements, orange denotes required administration and any precursors, green symbolises skeletal muscle biopsy requirements to capture differences in MPS between time points due to an intervention and blue highlights diet and exercise factors which can be best delineated from each technique

## 1.8 Molecular regulation of muscle protein synthesis

### *1.8.1 Exercise and amino acids*

To understand mechanisms of age-related skeletal muscle deterioration, it is important to first understand how muscle protein synthesis is regulated at the molecular level. The 289kDa mechanistic target of rapamycin complex 1 (mTORC1) is thought to possess a central role in muscle intracellular remodelling. mTORC1 activation evokes downstream signals fundamental for protein manufacturing, including i) translation initiation via eukaryotic translation initiation factors (i.e., 4E-BP1) responsible for assembling messenger RNA (mRNA) [158] and ii) ribosomal activation for translation elongation (i.e., via s6 kinase (p70S6K) phosphorylation activation and dephosphorylation activation of eucaryotic elongation factor (eEF2)) [159]. Fundamentally, ribosomes are the site where translated mRNA is read, and amino acids are assembled in a polypeptide chain before folding and assembly as functional proteins (including myofibrillar proteins) at the endoplasmic reticulum and Golgi [160]. Consequently, it is understood that mTORC1 signalling processes precipitate MPS, where experimental attention to intracellular signalling may be fundamental in uncovering strategies to improve MPS in older adults.

In younger healthy adults, stimuli including resistance exercise contraction, amino acids and growth factors such as insulin, can elicit phosphorylation and activation of mTORC1 and subsequent downstream effectors, such as p70S6K [161]. The convergence of signals on mTORC1 from these divergent ‘anabolic’ stimuli can synergistically accentuate mTORC1 phosphorylation and activation, more so than in isolation [162], likely through different upstream pathways [163]. With reference to resistance exercise, in cell-based muscle models its has been established that mechanotransducers at the sarcolemma, detect a contractile stimulus and innervate

factors such as paxillin and focal adhesion kinase (FAK) phosphorylation, which are thought to causally promote mTORC1 and related downstream target activation [164, 165]. In humans, this is supported since resistance exercise increases mTORC1 phosphorylation and MPS, whereas mTORC1 suppression using its inhibitor: rapamycin, diminishes the MPS response [166]. Further to this, rapamycin inhibits the phosphorylation of downstream mTORC1 effectors including p70S6K and eEF2, even with an exercise stimulus which ordinarily evokes phosphorylation of both downstream effectors [166].

The impact of protein ingestion on mTORC1 activation has been consistently demonstrated, with studies in isolated myotubes demonstrating that AAs enter the cytosolic space within myofibers via AA transporters (i.e., LAT1) [167]. The presence of AAs within the cytoplasm is imperative for transfer RNA (tRNA) to sequester and charge the AA for its corresponding anticodon [168]. This permits AAs to be arranged in a peptide chain in a sequence determined by the codons on the mRNA chain. Specific to AA-induced mTORC1 signalling, the branched chain amino acid leucine holds a position of prominence as a potent stimulus for mTOR phosphorylation [169, 170]. This process is thought to be mediated by leucine binding to an inhibitor of mTOR: Sestrin2 [171]. The presence of leucine therefore alleviates the Sestrin2 inhibition of mTORC1, facilitating the potential for greater mTOR phosphorylation [172]. Indeed, it has been shown removing or lowering leucine provision as part of an otherwise complete EAA mixture elicits suboptimal mTORC1-related signalling responses to feeding and exercise [173].

Other processes including insulin-mediated canonical signalling can upregulate proteins which are implicated in mTOR regulation. Animal models have elucidated insulin receptors are membrane-spanning sites, which when bound by insulin can

trigger a cascade of signals through activation of the insulin receptor substrate (IRS-1) and protein kinase B (Akt), through phosphoinositide 3-kinase (PI3K), upstream of mTOR [174, 175]. The upregulation of Akt inhibits a direct regulator of mTOR: tuberous sclerosis factor complex (TSC) 2 [176]. The inhibition of TSC2 dissociates this protein from its coregulator TSC1 and promotes the activation (changing from GDPase to GTPase form) of Ras homolog enriched in brain (Rheb), resulting in increased mTORC1 activation [177, 178]. Additionally, normal insulin signalling is required for regulating mTORC1 signalling. For instance, normal insulin signalling is required to upregulate to eukaryotic elongation factor-2 (eEF2) kinase [179] to inhibit eEF2. The inhibition of 95kDa eEF2 is a prerequisite for the addition of AAs to be encoded following translation initiation at the ribosome. Henceforth, the upregulation of eEF2 (as a result of impaired insulin signalling) could present issues for normal protein manufacturing and further exacerbate mTOR signalling. Importantly, negative feedback loops exist within the mTORC1 signalling pathway to switch off the process of protein manufacturing. This is important since the synthesis of new proteins is energy-demanding and an inability to turn off protein synthesis would dysregulate proteolysis. Such processes are turned off through phosphorylation of p70S6K which hyperphosphorylates mTOR at $Serine^{2448}$ to eventually deactivate mTORC1 [180]. Other proteins include 5'-adenosine monophosphate-activated kinase (AMPK), which is activated during energy depletion [181] and in cell models promotes TSC2 activation, thereby inhibiting mTORC1 phosphorylation and activation [182–184]. Indeed in human skeletal, activation of mTOR via AMPK-independent processes is thought to inhibit subsequent AMPK activation acutely (1-3h post exercise) [185]. The integrated regulation of intracellular mTORC1 signalling is depicted in **Figure 1.2**.

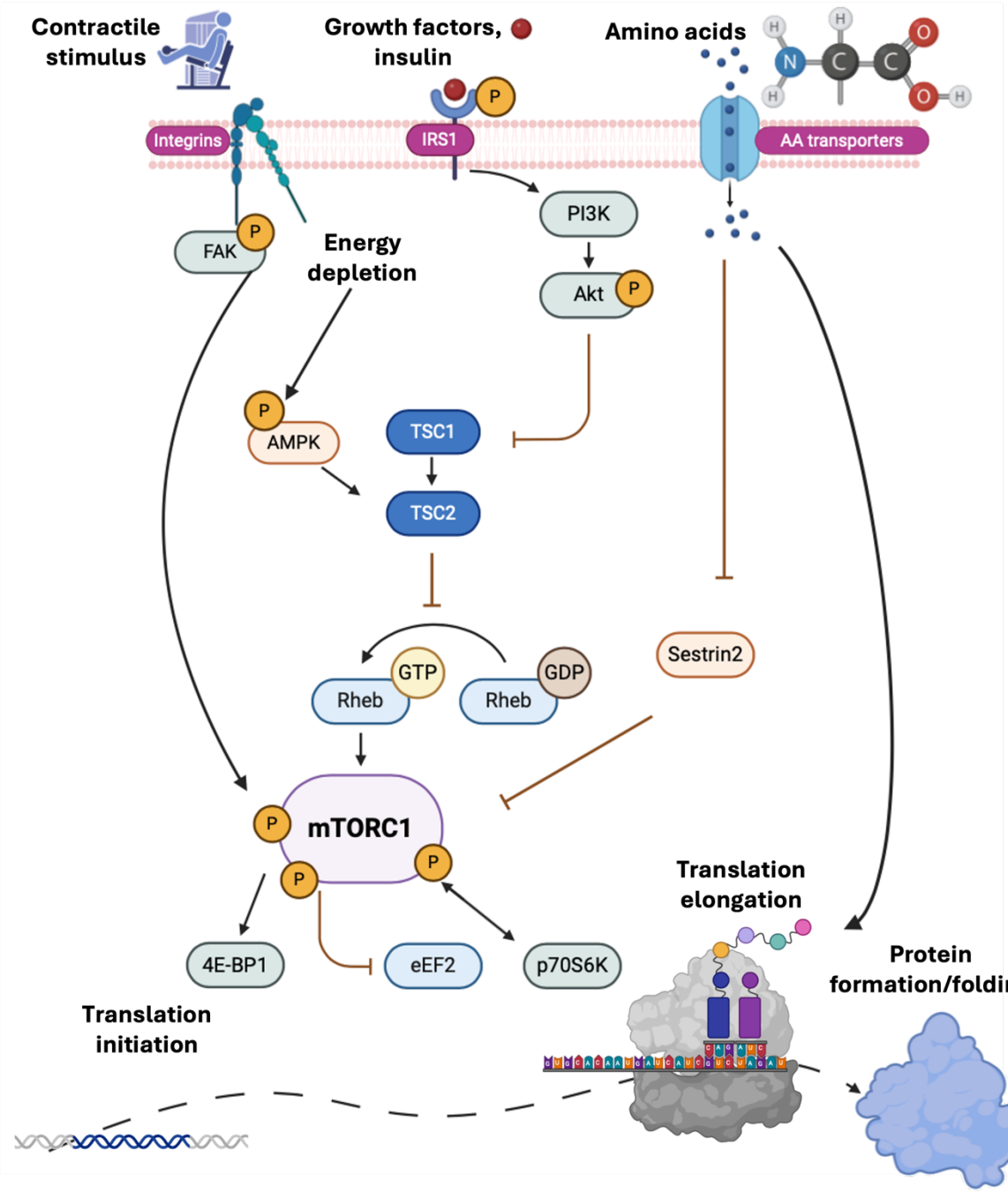


**Figure 1.2.** Synergistic effect of protein and exercise on stimulating mTORC1 signalling and protein synthesis

Simplified mTORC1 signalling pathway showing anabolic stimuli (contractile stimulus, growth factors and amino acids) signalling cascade through regulatory proteins which converge on mTORC1 and modulate protein manufacturing. A solid black arrow shows positive convergence, a brown boxed line denotes inhibition, and an orange P represents phosphorylation.

### *1.8.2 Dysregulated intracellular signalling in ageing*

As delineated from acute MPS studies, older adults exhibit a blunted MPS response to protein feeding and exercise compared to their younger counterparts. Considering MPS stimulation is the product of intracellular signalling events, the dysregulated MPS response may be due to age-related alterations or impairments in anabolic signalling within the mTORC1 pathway. In this regard, mTOR appears to be hyperphosphorylated, even at rest, in animal models of ageing [186, 187] as well as in older humans [113]. This hyperphosphorylation may be indicative of a dysregulation in the downstream effector pathway and is likely underpinned by upstream alterations in signals that communicate to mTORC1. Age-related differences in mTORC1 signalling were also reported by Kumar and colleagues, who showed that p70S6K phosphorylation and activation following resistance exercise peaks 2 hours later in older compared with younger adults [100]. Others have shown delayed and diminished mTORC1-mediated signalling in older adults following resistance exercise [188], protein ingestion [189] or their combination [190, 191] when compared to younger humans. These effects may be due to the hyperphosphorylation of mTORC1 at rest, whereby activation of this regulator of cell size requires a greater anabolic stimulus to catalyse mTORC1 response, although this remains to be elucidated.

The hyperphosphorylation of mTORC1 may also represent a mechanism for upregulation of protein manufacturing due to the accumulation of protein aggregates, which are non-functional within and around myofibers. Similarly, increased mTORC1 activation dispels an inability to activate autophagy, thus preventing the clearance of proteins which likely contributing to accumulation of protein aggregates [192, 193]. Concerning the resistance exercise-induced impairments in mTORC1-mediated signalling [100], older adults have more extracellular matrix proteins [194] which may

prevent the transduction of exercise/contractile signals to mTORC1, offering an explanation as to why a greater exercise volume stimulus may be necessary to increase MPS in older adults [116]. Other within fibre age-related alterations have also been observed which may contribute to defective mTOR signalling. Sestrin2 abundance within human skeletal muscle fibres has been found to be higher in older compared to younger adults [190], although transcriptomic data contradicts this observation. This lack of clarity highlights a requirement for more age-related research on Sestrin2 in skeletal muscle. Notwithstanding, an increase in Sestrin2 suggests more leucine may be required to overcome Sestrin2-mediated inhibition of mTORC1. If such age-related differences in Sestrin2 abundance do exist, this may explain the diminished response to protein ingestion displayed in older adults. This notion requires clarification as increased Sestrin2 could potentially represent a mechanism to compensate for dysregulated mTOR activity in aged skeletal muscle. Considering the type II muscle fibre atrophy which presents itself with ageing, and divergent metabolic properties between fibres, it is important to conceptualise any differences in regulatory protein abundance on a fibre-type-specific basis. Where it has been delineated that p70S6K and eEF2 signalling is divergent between type I and II fibres following resistance exercise and protein feeding in young adults [195, 196]. Evaluating regulators including Sestrin2 and mTOR on a fibre type-specific basis is yet to be comprehensibly conducted in older populations but may be an important line of enquiry to better understand the aetiology of type II fibre atrophy.

The aberrant age-related impairments in anabolic signalling defects in mTORC1-related signalling are thought to be at least partly accentuated by numerous systemic age-related alterations/hallmarks. For instance, older adults show markedly higher intramuscular inflammatory marker abundance compared to younger adults [23,

98]. Increased inflammation, shown in sepsis models, is thought to directly upregulate AMPK as well as inhibit the motility and activation of transcription and translation factors downstream of mTORC1, independent of TSC2 [197, 198]. Additionally, systemic inflammation can trigger a cascade of events which also inhibit IRS-1/Akt/TSC2-mediated regulation of mTOR, through ceramide accumulation [199]. The presence of this inflammation is precipitated by uncontrolled adipose tissue lipolysis which promotes the accumulation of intramyocellular triglycerides (IMTG) accumulation [200] . The expansion of IMTG pools causes an increase in lipid-derived metabolites and ceramides which inhibit Akt [201]. This ultimately lessens glucose clearance from the circulation and subsequent insulin sensitivity [202] as well as directly blunting protein synthesis signalling [203], through IRS-1 inhibition [28, 204]. Indeed, dysregulated mTOR signalling is thought to exacerbate itself with impaired insulin signalling [205–207], although the decline in insulin sensitivity with ageing can be attenuated through exercise [208–210]. Seemingly, older adults are likely more prone to dysregulated mTORC1, mediated by elevated inflammation and IMTG expansion due to the age-related decline in physical activity, insulin sensitivity and increased adiposity [22, 26]. In summary, alterations in mTORC1-related signalling partly underpin age-related anabolic resistance. Investigations to further elucidate the mechanisms driving this dysregulation of mTORC1 signalling are imperative to inform interventions with the potential to combat age-related anabolic resistance.

## 1.9 Methods for determining intracellular signalling regulation

### *1.9.1 Gene expression*

Gene expression captures the amount of mRNA present that encodes for a particular protein [211]. The upregulation of a gene sequence for a particular protein increases the likelihood of the corresponding protein being expressed. Consequently, gene expression data has been used as a proxy for intracellular protein expression following exercise and nutrition interventions in different age groups, though discordance between protein abundance and mRNA expression is widely observed [212]. For instance, whilst some report decreased TSC2 gene expression following exercise and amino acid ingestion in older compared to younger adults [213], others have observed an increased TSC2 protein abundance and divergent phosphorylation with age [214]. Discordance is likely due to the transient nature of signalling and gene upregulation, whereas protein expression is more stable. Additionally, phosphorylation is an insightful post-translation modification whereas alterations in gene expression cannot appropriately reflect this process. Additionally, gene expression data can infer the efficiency of existing protein functionality. For instance, a lower change in ribosomal gene expression was associated with the most profound increases in muscle mass following a resistance exercise training programme [215]. Seemingly, this suggests translation efficiency was already high and thus need to encode for new ribosomes is unnecessary. Nonetheless, the apparent discordance between gene expression and protein abundance, along with the inability to measure protein phosphorylation and activity, limits the inferences that can be made from gene expression data.

### *1.9.2 Immunoblot*

Much of the knowledge known about mTORC1-mediated regulation of cell size has been gleaned from immunoblotting (also known as Western blotting). Immunoblotting, holds the capacity to determine the phosphorylation status and the total abundance of a given protein target, including mTOR and its associated signalling intermediates that may be dysregulated in ageing and underpin muscle deterioration. Indeed, the bidirectional phosphorylation activation for key intracellular regulator proteins, such as serine$^{2448}$ phosphorylation on mTOR [180], can make it difficult to draw firm conclusions on the physiological significance of static (e.g., non-dynamic) signalling events. Henceforth, it is essential that multiple protein targets involved in metabolic signalling processes are blotted for, to gain holistic insight into alterations to intracellular signalling pathways.

With immunoblotting in human skeletal muscle experimental studies, it is common for mixed muscle fractions or sarcoplasmic fractions to be used, as the isolation of myofibrillar fractions from a limited tissue yield is often used to determine myofibrillar-specific protein synthesis rates. Recent evidence has emerged to suggest that mTOR-mediated signalling intermediates can be blotted even in lysosomal fractions, revealing discrete age-related differences in mTOR signalling in response to exercise and amino acid ingestion [216]. The presence of mTOR binding to the lysosome occurs only during mTOR activation [217]. However, the spatial proximity of intracellular signalling proteins that would shed light on the motility and biological action of the target protein, cannot be gleaned from immunoblotting. Additionally, immunoblotting is considered semi-quantitative and can change according to the subcellular fraction blotted for, as well as the time at which biopsies are obtained

around a given stimulus, given that intracellular signalling events are highly transient [218]. Immunoblotting can provide snapshot insights into intracellular signalling actions that are implicated in MPS stimulation, especially when measured in acute experimental settings with frequent biopsy sampling time points and a robust sample size to provide high temporal resolution and lower the inherent intra-individual variability.

Considering the deleterious effects of muscle mass loss in ageing appear mainly driven by type II fibre atrophy and/or loss, fibre-type specific analysis of intracellular signalling pathways is important and has recently been developed with immunoblotting [219]. This contemporary development has already identified fibre type-specific differences in protein and organelle abundance [220, 221], and mTOR-mediated signalling intermediates and phosphorylation [195, 196, 216]. However, in isolating and homogenising fibres for immunoblot analysis, the *in-situ* relevance and spatial occupancy of fibre type-specific signalling intermediates cannot be determined, and these parameters are likely important for understanding intracellular signalling activation and dysregulation in sarcopenia.

### *1.9.3 Immunofluorescence microscopy*

Immunofluorescence microscopy (IF) has the capacity to identify the localisation of intracellular signalling intermediates within and between skeletal muscle fibres. IF works through staining cryosections from intact skeletal muscle, where fibre type can be determined alongside intracellular signalling proteins of interest, relative to fibre area and in a fibre type-specific manner [222, 223]. In the context of skeletal muscle, visualisation of regulatory proteins with muscle fibres *in situ* provides a snapshot of signalling events at a given time, where intracellular localisation of

signalling proteins can be used to infer their activation status. For example, the activation status of mTOR can be inferred from its localisation at the fibre periphery, since mTOR phosphorylation drives lysosomal colocalisation and peripheral transport [224, 225]. In addition to localisation, the colocalisation to other signalling targets can also be measured with IF to understand acute signalling events in response to anabolic stimuli of interest. For example, the colocalisation (i.e., overlap of fluorophores tagged to each protein) of mTOR to Rheb has been reported following exercise and nutrient ingestion, highlighting that intracellular anabolic signalling is upregulated, where Rheb has been activated (through TSC2 dissociation) to activate mTOR signalling [226]. Indeed, colocalisation does not confirm interaction, where cross-section angle can affect interpretation. To be more certain of protein-protein interaction, proximity ligation assays can be performed where two proteins of interest are probed and if in close enough proximity, the associated secondary antibodies can be ligated together producing a colour fluorophore, again harnessing the power of IF.

The activation status of intracellular signalling intermediates can be measured by combining IF with immunoblotting. However, as both methods are semi-quantitative, findings can be discordant from each other. With IF, within fibre regions are quantified, which include sarcoplasmic, mitochondrial and myofibrillar proteins. Conversely, with immunoblotting, either a mixed muscle fraction which includes the extracellular matrix or just one subcellular fraction, typically the sarcoplasmic protein fraction, is used. This is noteworthy as protein assays show significant differences in protein content between myofibrillar and sarcoplasmic fractions (~2-fold higher in myofibrillar fraction), and that the protein content of each fraction is lower in older compared to younger adults [227]. It can be argued that insights from IF, which consider the whole intracellular space and account for differences in intracellular signalling proteins

relative to fibre area, would be the most appropriate approach to investigate potential age-related differences that may underpin impairments in muscle protein turnover. It is now emerging phosphorylation of mTOR targets can be visualised with IF, as seen with ribosomal protein S6 (RPS6) following exercise [228]. Although further efforts in this field are required, the available evidence suggests that the translocation of mTOR-mediated proteins within fibres coincides with increased phosphorylation *in situ*. IF represents a powerful tool to capture dysregulated intracellular signalling proteins in ageing in a fibre type-specific manner, further probing the mechanisms of impaired MPS and fibre-type atrophy that underlie sarcopenia. Additionally, measurement of the localisation and abundance of intracellular signalling proteins relative to fibre area allows for a more robust comparison between different populations, such as older and younger adults where there are established differences in muscle fibre size.

## 1.10 Protein nutrition for healthy muscles during ageing

### *1.10.1 Muscle anabolic resistance to protein nutrition*

The blunted MPS response to dietary protein ingestion in older adults suggests that there is a metabolic defect in the capacity to synthesise new muscle proteins with advancing age. These defects appear to be partially alleviated when very high doses of protein (and EAAs) are consumed. Indeed, position statements advocate for an increased protein recommended dietary allowance (RDA) for older adults compared to their younger counterparts, with 1.2 g·kg $BM^{-1}$·$day^{-1}$deemed *as sufficient to support daily muscle anabolism and net protein balance [229, 230].* Numerous studies have shown superior muscle health-related outcomes with dietary protein intake at the alternative recommended range in older adults, through acute experimental investigations of MPS [98, 231, 232] and longer-term observational and intervention studies [233–235]. Despite the importance of higher dietary protein intake for

supporting muscle mass retention in ageing, a large proportion of community-dwelling older adults do not typically consume protein at these levels. The reasons for low/inadequate dietary protein intake in older age are complex and multifaceted, but include lack of knowledge about protein requirements and good protein sources [236], sensory impairments [237], age-related appetite and digestion impairments [238, 239] as well as trends towards increased consumption of 'sustainable' plant-derived foods [240], where plant sources typically contain less protein and have poorer biodiversity of all the EAAs [241, 242]. An additional consideration towards achieving adequate dietary protein intake in older age is the increased likelihood of ill-health and periods of muscle disuse (e.g., during hospitalisation). Such debilitating events have been seen to rapidly punctuate muscle mass and strength loss [52, 243], thereby accelerating sarcopenia progression. It is evident that pre-operative and in-hospital food intake/provision is more restricted than in free-living conditions, with protein intakes typically falling well below the current RDA and alternative recommendations for older adults [229, 244–246]. Hence, in scenarios where the amount of protein consumed with meals and across the entire day is low, the source of protein consumed may become critical for preserving muscle health outcomes.

### 1.10.2 *Dietary protein quality for healthy muscle ageing*

Given the barriers to increasing dietary protein intake with advancing age, particularly in a clinical context, it is important to explore alternative approaches to support skeletal muscle health in older adults. The muscle anabolic properties of different protein sources are primarily determined by the abundance and availability of EAAs which are imperative for MPS stimulation. As previously highlighted, AAs are needed to bind to tRNA and form peptide chains and eventually functional proteins.

EAAs are especially important, as they need to be recycled from protein breakdown or sequestered from exogenous sources to ensure healthy turnover and even expansion of the protein pool in light of gradual EAA oxidation [247]. Importantly, some EAAs are in higher demand than others. For instance, all polypeptide chains require a start codon AUG which encodes for the EAA, methionine [248]. In many plant-derived proteins, methionine is often in lower abundance compared to other EAAs, whereas it is often markedly higher in animal-derived proteins [242]. Another EAA which is typically lower in plant proteins is leucine, which plays a critical role in signalling as well as being a substrate for muscle protein manufacturing. Indeed, high leucine provision within supplemental protein appears to be a key driver of MPS [128, 129, 249]. To overcome the deficiencies in EAAs in plant-derived proteins, higher doses or blends of lower-quality proteins are likely required, but this would come at the expense of greater total energy intake that might not be desirable for some populations. Alternatively, studies have shown that at high doses, isolated supplemental protein sources (i.e., animal-derived whey vs plant-derived pea or fungal-derived mycoprotein) can support equivalent MPS responses in younger [250, 251] and older adults [252, 253]. Importantly, isolated supplements are not typically consumed in the diet, particularly beyond middle age and so, increasing attention has turned to the muscle anabolic properties of protein-rich whole foods. The consumption of protein through complex whole food sources introduces more variance in digestion and absorption kinetics than isolated supplements, where variances in cooking method [254], fibre content [255–257] and reliance on mastication for digestion initiation [145] can affect postprandial plasma AA availability and appetite responses. Nonetheless, when sufficient protein is provided, whole food sources appear capable of supporting maximal postprandial MPS stimulation, with non-protein components of

the whole food matrix (i.e., bioactive compounds, fibre, calories) likely augmenting postprandial muscle anabolism [258–261]. However, in the typical lower protein-containing meals of older adults, the source and quality of protein that is ingested could have important implications for plasma AA availability, postprandial MPS stimulation and muscle mass regulation.

### *1.10.3 Dietary protein and appetite regulation*

The impact of dietary protein ingestion on appetite is important to consider when trying to achieve recommended daily protein intake targets to support muscle health in older adults. Specifically, foods which prolong fullness can limit energy/protein intake at subsequent feeding events, which may lower overall daily protein intake and impede muscle mass regulation. In healthy persons, feeding alters the secretion of appetite hormones. For instance, glucagon-like peptide 1 (GLP-1) is secreted from the gut following feeding and suppresses appetite by slowing gastric emptying whilst promoting insulin secretion [262, 263]. Similarly, peptide YY (PYY) secretion increases from the gut following feeding [264], mainly acting on the hypothalamus to suppress appetite [265]. Inversely, the postprandial secretion of the anorexigenic hormone ghrelin, implicated in increasing feelings of hunger, is lowered following feeding [266]. In isolated supplemental proteins, whey has been reported to evoke a significant rise in postprandial plasma GLP-1 responses compared with casein in young adults [267]. In a comparison between isolated sources of pea protein and whey protein, postprandial perceived appetite and plasma PYY excursions were similar and do not differ between young and older adults [268, 269]. Importantly, postprandial plasma GLP-1 and ghrelin were not evaluated in older populations and both studies involve consuming very large dose isolated proteins (>40g) that do not represent the typical eating habits of older adults. Humans typically consume mixed

meals multiple times a day, although research on acute appetite or anabolic responses from mixed meals has only been determined in the context of very high protein provision [256, 268, 270]. In the only study to compare divergent sources of protein from vegan and omnivorous diets mixed meals in older adults, the protein amount was very high (~0.45 g·kg $BM^{-1}$) and appetite responses were not ascertained [256]. It remains to be elucidated if mixed meals, or fortification of meals with pragmatic lower doses of protein from divergent sources offer any measurable benefit for postprandial muscle anabolism without compromising appetite responses in older adults. On one hand, with the greater compliment of all EAAs that can be provided with animal proteins, it stands to reason that their muscle anabolic potential would be greater than plant protein intake, particularly when consumed in amounts typically observed in the diet of older adults. Conversely, the slowed digestion of plant proteins may extend the delivery of EAAs [271]. Hence, even though EAAs are limiting, there may be more time for them to be periodically delivered and used within skeletal muscle. Testing the role of different protein sources/protein quality is likely logistically challenging, where altering protein sources (i.e., more plant than animal sources) from exclusively whole foods would affect antinutritive properties, not just EAAs [272]. Henceforth, providing supplemental proteins alongside a similar whole food diet may be valuable to investigate any potential impact of protein source on muscle anabolism due to divergent AA profiles. The provision of a small protein bolus, alongside typical low-protein meals may be a feasible way to increase protein intake and support MPS in older adults, although this needs to be considered alongside any possible influence on multiple indices appetite regulation.

### *1.10.4 Resistance exercise and protein source for muscle anabolism with age*

Undoubtedly, resistance exercise is beneficial as a stimulus for MPS and longer-term adaptive skeletal muscle remodelling, even in older adults who may display muscle anabolic resistance. As previously outlined, resistance exercise appears to sensitise skeletal muscle to the anabolic properties of amino acids from protein ingestion, resulting in a synergistic augmentation of MPS, particularly with higher protein doses (>20g) [106, 126, 127]. Consuming higher quality (i.e., well-digested, EAA complete) protein sources may mediate exercise-induced MPS responses in both older and younger adults [273]. The combination of resistance exercise with a high protein diet supplemented with high-quality whey protein resulted in superior MPS response compared with the same diet supplemented with low-quality collagen protein in postmenopausal women [155]. Indeed, collagen supplementation has a low and incomplete EAA content atypical of animal proteins and the total protein provision was at 1.6 g·kg $BM^{-1}$·$day^{-1}$ which is uncharacteristic of diets of older adults. In younger adults, it has been demonstrated that a mycoprotein- or omnivorous-based high protein-containing diet (~1.8 g·kg $BM^{-1}$·$day^{-1}$) supports equivalent changes in resistance exercise-induced MPS and longer-term adaptive remodelling [250]. Together, this could point toward an age-related nuance in the importance of protein quality for post-exercise anabolism or, alternatively, collagen is too deficient in all EAAs and that regardless of age, when total protein intake is high, quality becomes less important, permitting leucine and overall EAA availability is adequate [129, 274]. The importance of dietary protein source and quality (i.e., digestibility and EAA availability, including leucine) for resistance exercise-induced muscle anabolism may be more apparent with sub-optimal per meal and total daily protein intake, which are

more typical in diets of older adults. Future efforts in this space should seek to investigate how higher-quality animal-derived and lower-quality plant-derived proteins modulate resistance exercise-induced muscle adaptive remodelling when consumed in the typical lower protein-containing diet of older adults, over an extended period of free-living.

### 1.11 Aims and objectives

The overarching aims of this thesis are to improve the understanding of intracellular mechanisms that may underpin age-related alterations in skeletal muscle anabolic regulation and fibre type-specific atrophy, and to ascertain whether the source and quality of protein in a typical meal/diet influence the metabolic regulation of skeletal muscle anabolism and appetite with advancing age. To achieve this, the specific objectives of the thesis are as follows:

Firstly, given that methodological artefacts may have obscured the understanding of the aberrant mechanisms that underlie the age-related dysregulation of muscle protein turnover, **Chapter 2** presents a novel analysis of muscle biopsy tissue samples from younger and older adults utilising immunofluorescence microscopy to compare the fibre type-specific abundance, spatial occupancy and proximity of key intracellular regulators of muscle anabolism. Secondly, considering the absence of a clear consensus on the role of dietary protein source and quality for age-related muscle health, the objective of **Chapter 3** is to provide a comprehensive narrative review highlighting the barriers to increasing dietary protein intake with advancing age. This chapter outlines how higher-quality dietary protein intake in lower protein-containing diets might support postprandial muscle anabolism and longer-term muscle health outcomes in older adults, particularly during periods of ill-health and disuse (e.g., hospitalisation). Thirdly, given the barriers to consuming high dietary

protein with advancing age and limited understanding of circulating amino acid availability with mixed meals combined with supplemental protein from varied sources, **Chapter 4** investigated the acute postprandial plasma amino acid and appetite regulatory responses to a typical lower protein-containing whole food mixed breakfast supplemented with a small bolus of higher-quality whey protein or lower-quality pea protein. Finally, by utilising the combined meal-plus-supplement strategy described in the preceding chapter, **Chapter 5** investigated daily rates of myofibrillar protein synthesis under conditions of rest and resistance exercise in middle-to-older aged adults consuming a 10-day controlled lower protein-containing diet from mainly higher quality animal- or lower quality plant-derived sources. Finally, **Chapter 6** summarises the thesis findings and provides practical implications as well as further critical interpretation and insight into the data presented. Practical implications of the work as well as considerations for future work are outlined to enhance the understanding of mechanisms that underpin age-related muscle deterioration and the importance for dietary protein quality to support muscle health in older adults.

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# Chapter 2 Age-related differences in basal human skeletal muscle fibre-type mTORC1 signalling abundance and localisation.

## 2.1 Abstract

Age-related atrophy of type II fibres may be underpinned by dysregulated mTOR-mediated signalling. Immunofluorescence microscopy (IF) was used to investigate basal age-related differences in fibre type-specific abundance and localisation of mTOR-related proteins. Rested *Vastus lateralis* biopsies were collected from eight young (YM; 24 ± 4 years) and seven older males (OM; 67 ± 5 years). Muscle cross-sections were stained for MHC-I, Rheb, TSC2 or WGA, mTOR and Sestrin2. IF images were analysed for colocalisation and fibre-type specific cross-sectional area (CSA), fluorescence intensity (abundance) and peripheral-to-central ratio of targets. CSA did not differ between groups although type II fibre proportion was lower in OM (-10%, $P$=0.020). Target abundance did not differ between type I and II fibres, within group. Pooling fibre types revealed greater TSC2 (+100%, $P$<0.001) and Rheb (+43%, $P$=0.013) and lower mTOR (-49%, $P$<0.001) abundance in OM than YM. Colocalisation did not differ between YM and OM for Sestrin2-WGA, mTOR-WGA, Sestrin2-mTOR or TSC2-Rheb. Rheb peripheral-to-central ratio was significantly higher in OM than YM, irrespective of fibre type (+13% $P$<0.001) but not different for mTOR, Sestrin2 or TSC2 within groups. Age-related alterations in basal mTOR, TSC2 and Rheb abundance, alongside Rheb localisation, may represent age-related impairments in muscle protein stasis.

## 2.2 Introduction

The age-related decline in skeletal muscle mass, strength and function (termed sarcopenia) is associated with an increased risk of frailty, falls, metabolic disease, and all-cause mortality [78]. This loss of muscle mass is typically greatest in the quadriceps, potentially due to the relatively large abundance of type II fibres and their susceptibility to atrophy [272]. Fibre-specific atrophy reduces the whole muscle cross-sectional area (CSA), leading to increased risk of falls/fractures, chronic illnesses, and a greater associated healthcare burden [59, 273]. Skeletal muscle mass is regulated through the dynamic balance between muscle protein synthesis (MPS) and breakdown (MPB). To date, the mechanisms that underpin age-related fibre-specific atrophy are poorly understood but appear to involve a diminished MPS response to anabolic stimuli underscored by impaired responsiveness of key molecular regulators [274].

It is well accepted that the activation of the mechanistic target of rapamycin (mTOR) and its associated downstream mediators [166] underscores cellular growth. In line with age-related reductions in MPS, mTOR signalling appears dysregulated at rest [186, 275], following protein ingestion [274] and contractile loading [9] in older adults. Age-related dysregulation may also occur upstream of mTOR, including tuberous sclerosis complex 2 (TSC2) and Ras homolog enriched in brain (Rheb). Ordinarily, the phosphorylation of TSC2 evokes Rheb activation which, in turn, promotes the phosphorylation and activation of mTOR complex 1. We and others have previously shown that TSC2 abundance is greater in older adults than young [9, 10], suggesting the possibility of divergent mTOR regulation in ageing. In addition to TSC2 and Rheb, Sestrin2, purported to have leucine sensing properties, can promote mTOR phosphorylation [11, 12]. Notably, Sestrin2 is reduced in atrophied fibres and thus may contribute to aberrant mTOR signalling in ageing [13].

Whilst an altered abundance of key regulatory proteins may partially explain the sarcopenia progression, the spatial occupancy and translocation of these proteins, suggested to be of central importance in muscle anabolism (i.e., growth/MPS), remains largely unexplored. Direct comparison between young and older individuals for the spatial occupancy of mTOR-mediated signalling intermediates within muscle fibre types would enhance our understanding of aberrant mechanisms of skeletal muscle mass regulation in older adults. Immunofluorescence microscopy (IF) is a sophisticated analytical tool quantifying muscle fibre-specific protein abundance and localisation. Hence, in comparison to conventional immunoblotting techniques alone, IF may provide greater insight into age-related alterations in anabolic signalling intermediates. Indeed, IF analysis of TSC2, Rheb and mTOR in younger adults has shown that translocation of these targets occurs toward and/or away from the periphery following protein ingestion, indicative of mTOR signalling 'activation' [14, 15]. In addition, IF approaches have revealed that downstream targets of mTOR are highly expressed in more atrophied fibres [16]. Therefore, an essential next step in uncovering molecular mechanisms of age-related muscle decline is to probe differences in basal fibre type-specific mTOR-mediated signalling between young and older adults. Our study used IF techniques to compare the abundance and localisation of mTOR and associated regulatory signalling proteins (TSC2, Rheb, and Sestrin2) in human skeletal muscle via a fibre-type-specific approach in young (YM) and older men (OM) in the rested state. We hypothesised that differences in the abundance and localisation of mTOR-mediated signalling intermediates would be apparent between YM and OM. We also posited that age-related differences in abundance and spatial proximity within fibre would be most apparent in type II muscle fibres, underlying the greater susceptibility to age-related atrophy.

## 2.3 Methodology

### *2.3.1 Participants*

Eight young (YM; 24 ± 4 years, BMI; 25 ± 2 kg·m$^2$) and seven older (OM; 67 ± 6 years, 26 ± 2 kg·m$^2$) healthy untrained males were recruited for the study following a general health screening questionnaire. Participants reported to the School of Sport, Exercise and Rehabilitation Science following an overnight fast and a 3-day diet and step count assessment (food diary and accelerometers, respectively). Habitual step count, BMI and grip strength were similar between groups (all $P$>0.05). A single resting muscle biopsy was taken from the m. *Vastus lateralis* using the adapted Bergström technique under local anaesthesia. Data contained in this present study were generated as part of a larger study [9]. All procedures were approved by the University of Birmingham and NHS East Midlands-Derby Research Ethics Committee (18/EM/0004) and were carried out in line with the Declaration of Helsinki.

### *2.3.2 Immunofluorescence staining*

Muscle tissue was embedded in Optimum Cutting Temperature (OCT) compound and frozen in liquid nitrogen-cooled isopentane (2-methyl butane, ThermoFisher, UK) in cryovials and stored at -80˚C until ready for sectioning. Embedded muscle fibres were orientated transversely, and sections were cut, in series, 7µm thick (Bright OFT 5040, UK) and collected on two separate uncoated glass slides (Superfrost™ Plus, Epredia, Germany) per participant. Samples were stored at -20˚C until all sections had been acquired, allowing analysis to be batch-completed. Before IF staining, slides were removed from the freezer and left to thaw for 30 minutes before being rocked in 3:1 acetone:ethanol mix for 5-minutes to fixate cryosections to the slides. Slides were washed for 3x5 minutes in phosphate-buffered saline (PBS) and blotted dry with a Kimtech® wipe. A glycerol-based PAP pen was used to draw around each section on the slide to minimise antibody runoff during application and

subsequent incubation. Secondary antibody specificity was achieved by omitting the primary and secondary antibodies, evaluating bleed-through across channels, and using serial dilution tests to ensure antibodies were not overexposed. Antibody cocktails were made so WGA, mTOR and Sestrin2 were co-stained on one slide and MHCI, TSC2 and Rheb on a separate slide (antibody characteristics and dilutions are displayed in **Table 2.1**. This process was carried out for each participant for a total of 15 slides. Dilutions were made with PBS and 5% normal goat serum to prevent unspecific antibody binding. Approximately 15μl of the primary antibody cocktail was added to each appropriate section and incubated for 2 hours (except WGA) in a damp box with a closed lid to prevent evaporation. A concoction of secondary antibodies specific to the primary target, including WGA, was added to each section (10μl) and incubated in the dark for 1 hour. Each target was conjugated to a different and contrasting fluorophore, enabling the identification of a different target in each channel on the microscope. Before applying a glass coverslip, all slides had 5μl of Mowiol to preserve the fluorophore. After that, slides were kept in a slide box, in darkness, ahead of image capturing.

**Table 2.1** Primary and Secondary Antibody Characteristics

| Target | Antibody | Source | Primary dilution | Secondary Antibody | Conjugation |
|---|---|---|---|---|---|
| TSC2 | Anti-TSC2 (SAB4503037) | Thermo Fisher Invitrogen | 1:100 | Goat anti-rabbit | 594 (red) |
| Rheb | Rheb monoclonal (MA527777) | Thermo Fisher Invitrogen | 1:100 | Goat anti-mouse | 488 (green) |
| MHC1 | MYH (sc-53089; A4. 840) | Santa Cruz Biotechnology | 1:25 | Goat anti-mouse IgM | 350 (blue) |
| mTOR | Anti-mTOR clone monoclonal (21D8.2) | Merck Millipore | 1:50 | Goat anti-mouse IgGgk | 594 (red) |
| Sestrin2 | SENS2 (10224310-1) | Abcam | 1:50 | Goat anti-rabbit IgG | 488 (green) |
| WGA | WGA (W11263) | Thermo Fisher Invitrogen | 1:10 | - | 350 (blue) |

TSC2: Tuberous Sclerosis Complex 2; Rheb: Ras Homologue Enriched in Brain; MHC1: Myosin Heavy Chain type I; mTOR: Mechanistic Target of Rapamycin; WGA: Wheat Germ Agglutinin.

### *2.3.3 Immunofluorescent microscopy image capture and analysis*

Images were captured on a 3-channel EVOS M5000 immunofluorescent microscope (Invitrogen, ThermoFisher Scientific, USA), with each target's gain and exposure identical. All images were analysed using Fiji ImageJ (V2.9) as 16-bit Tiffs with scale set before analysis. All fibres were manually drawn around to determine each target's fibre area and mean fluorescence for each colour channel. Analysis of fibre area was calculated using 10x images, and fibre-specific protein abundance, including peripheral abundance, was quantified with images captured at 20x magnification with fibres containing anomalous fluorophores excluded from the analysis. WGA intensity was quantified using thresholding to ascertain the mean grey area per image. Where targets were not co-stained with MHC1 (i.e., slides stained for WGA, mTOR and Sestrin2), sections were manually aligned with MHC1 positive stained sections, made possible due to the in-series nature slides being sectioned in.

An ImageJ plugin (JustAnotherColoclisationPlugin) was used for colocalisation analysis to correlate the degree to which two co-stained fluorophores overlapped as follows; Sestrin2 to WGA, mTOR to WGA, Sestrin2 to mTOR, and TSC2 to Rheb. Coloclaisiation data was reported as Pearson's coefficient for the whole image, rather than using a region of interest, to avoid any subjective interpretation. Identical thresholds were used within each channel, within each group. For peripheral analysis, a 5.5μm mask was made from the perimeter of each muscle fibre to define the periphery as previously described [14]. The difference between the peripheral (whole fibre without 5.5μm periphery) and central region (full fibre) for area and fluorescence was calculated by multiplying the area by the mean fluorescence for each fibre in both central and peripheral regions. The difference in total area and total fluorescence in peripheral regions were divided by each other, and this value was divided by the original peripheral fluorescence to ascertain the peripheral-to-central ratio, where a value above 1 indicated greater fluorescence at the periphery.

### *2.3.4 Statistical analysis*

For quantitative analysis, 847 fibres were analysed to ascertain fibre area at 10x magnification and 729 fibres for mean fluorescence at 20x magnification. A total of 36 fibres were excluded for violating circularity and considered longitudinal[17]. Statistical analysis was conducted on Prism V.10 (GraphPad Software, La Jolla, CA, USA). An independent samples t-test was used to evaluate differences in fibre area between YM and OM. Group effects for age and fibre type were examined using a 2-way repeated measures ANOVA with Bonferroni post-hoc analysis. All data are presented as mean ± SEM. Significance was set *a priori* as $P<0.05$, and effect sizes were examined using Cohen's d (d) with d=0.2, d=0.5 and d=0.8 considered as small, medium, and large effect size, respectively.

## 2.4 Results

### *2.4.1 Fibre type-specific area and distribution*

Fibre type distribution significantly differed between YM and OM, with OM having significantly fewer type II fibres (YM; 57.6%, OM; 47.6%, *P*=0.02, d=1.47). Mean fibre cross-sectional area (CSA) did not significantly differ with age for type I or type II fibres (type I, YM 4746 ± 260 $\mu m^2$ vs OM; 6133 ± 785 $\mu m^2$, *P*=0.095, d=0.93; type II YM; 6557 ± 558 $\mu m^2$ vs. OM; 5575 ± 723 $\mu m^2$, P=0.282, d=0.58).

### *2.4.2 Fibre type-specific mean fluorescence intensity*

When evaluating the effect of age (i.e., irrespective of fibre type), the total abundance of mTOR was significantly less in OM compared with YM (-48%, *P*=0.003, d=1.91, **Figure 2.1AB**). Sestrin2 was also markedly lower in OM than YM (-31%, *P*=0.021, d=0.91, **Figure 2.1C-D**), whereas TSC2 (*P*=0.013, d=4.16, **Figure 2.2A-B**) and Rheb (*P*<0.0001, d=1.68, **Figure 2.2C-D**) were 100% and 43% higher, respectively, in OM compared to YM. No differences in mean fluorescence between fibre types were apparent for mTOR, Sestrin2, TSC2 or Rheb in either YM or OM. Notably, OM displayed 31% higher WGA intensity than YM (YM; 38.4 ± 4.8 AU, OM; 50.19 ± 13.6 AU, *P*=0.038, d=1.19).

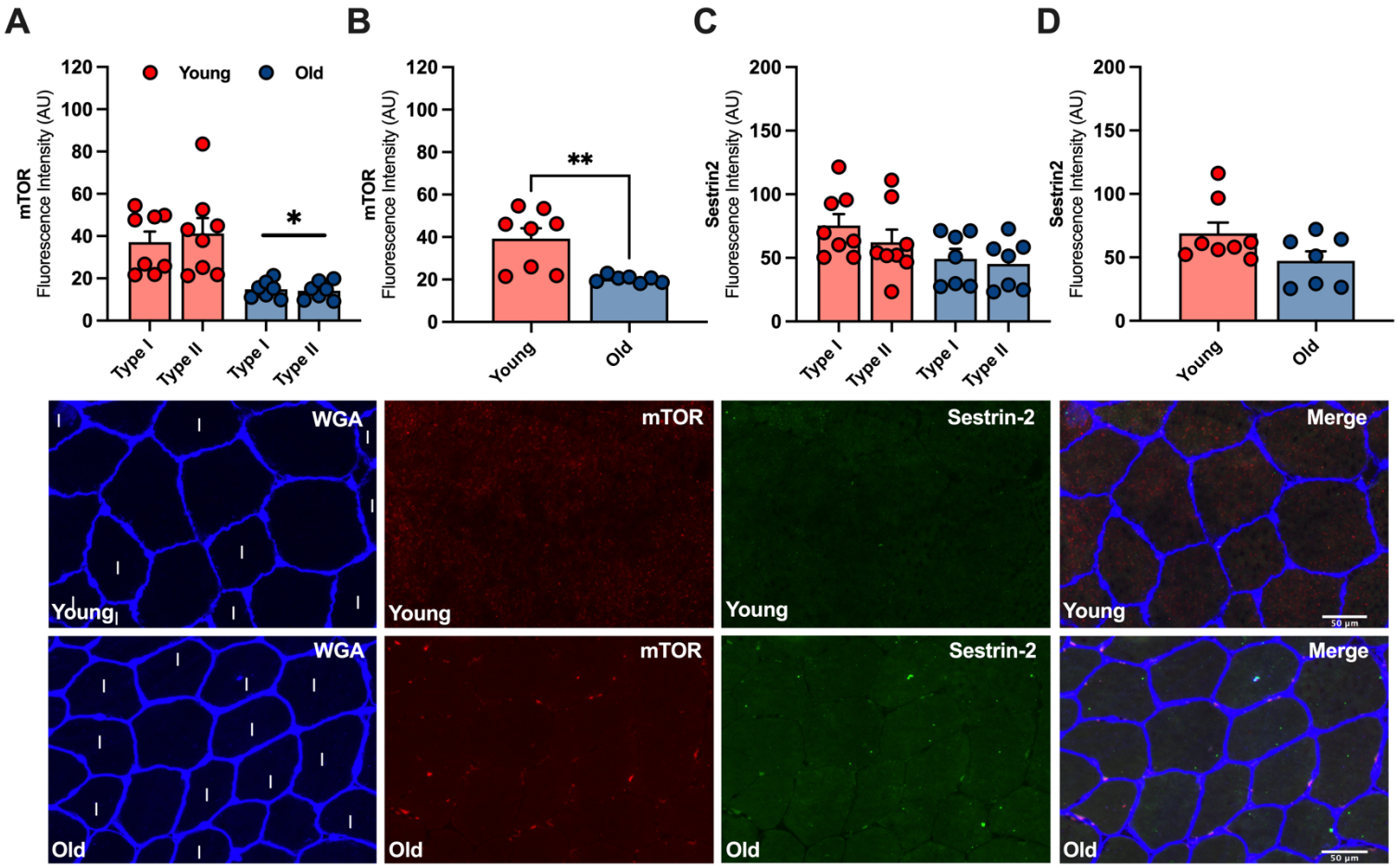


**Figure 2.1.** Skeletal muscle fibre-type specific mTOR and Sestrin2 immunofluorescent intensity.

Basal mean fluorescence relative to fibre area and fibre type for mTOR and Sestrin2, with representative images of WGA for the fibre border. Total mTOR fluorescence when separating type I and II fibres (**A**) and when pooling fibre types together (**B**) were significantly different between YM and OM *$P$<0.05,** $P$<0.01. Basal fluorescence of Sestrin2 when considering different fibre types (**C**) and pooling fibres together (**D**) were not significantly different between young and old. Representative images were captured at 20x for WGA, mTOR and Sestrin2 with **E-H** young and **I-J** old. White 'I' denotes type I fibres. Scale bar 50µm.

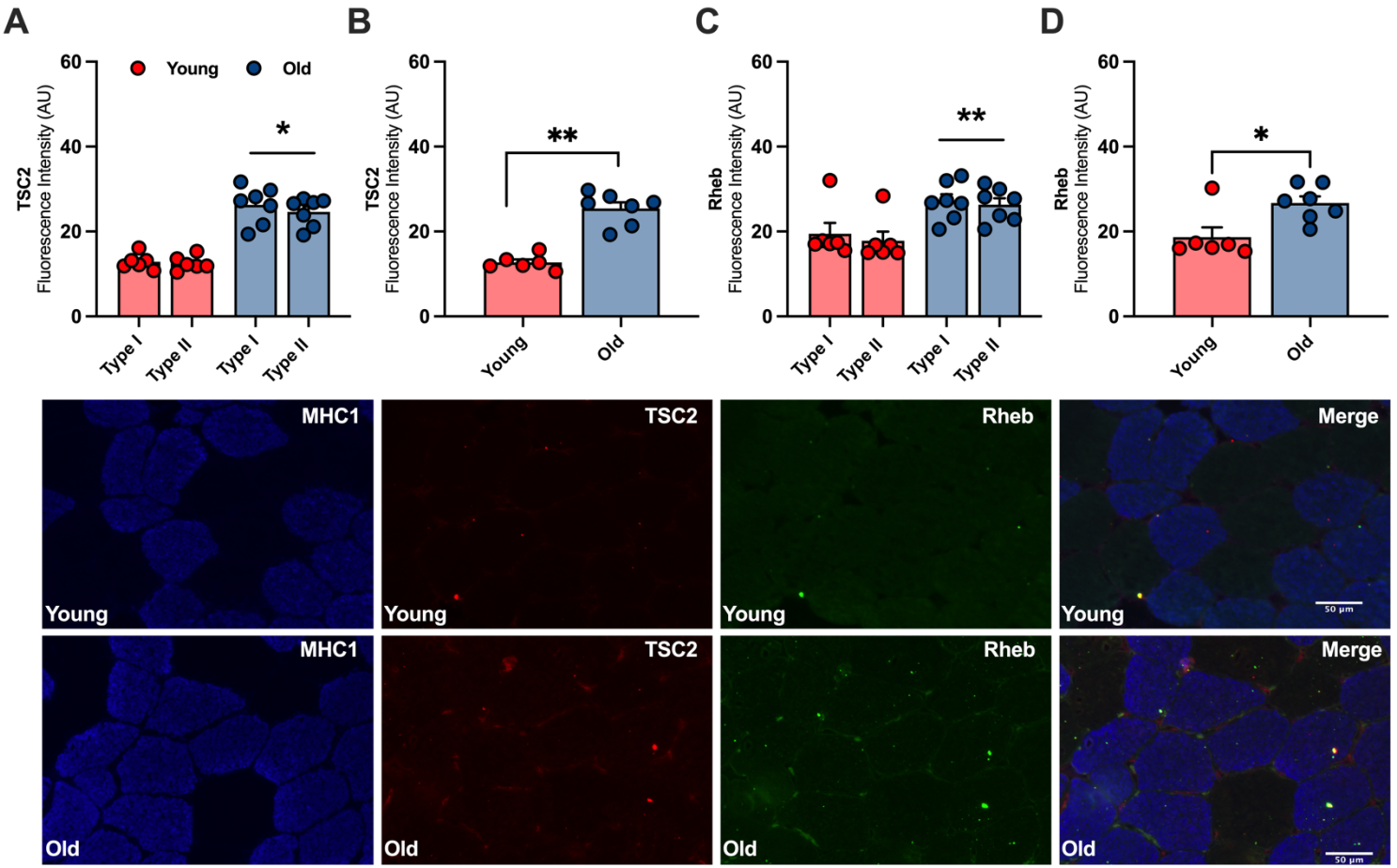


**Figure 2.2.** Skeletal muscle fibre-type specific TSC2 and Rheb immunofluorescent intensity.

Basal mean fluorescence relative to fibre area on a fibre type-specific basis for TSC2 and Rheb for YM and OM. Mean TSC2 fluorescence when comparing type I and II fibres (**A**) and when combining all fibres (**B**) revealed significant differences between YM and OM. Mean fluorescence for Rheb for both type I and II fibres (**C**) and when pooling fibres (**D**) were significantly different between YM and OM .(*$P$<0.05, ** $P$<0.01). Representative images were captured at 20x for MHC1, TSC2 and Rheb with **E-H** young and **I-J** old. White 'I' denotes type I fibres. Scale bar 50μm.

### *2.4.3 Protein-protein colocalisation and peripheral localisation*

To identify if the interaction between protein targets was different at rest between young and old, colocalisation analysis was used to measure the degree of overlap between costained targets (expressed as Pearson's coefficient). When evaluating protein-protein interactions, there was no significant difference between YM and OM in the colocalisation of WGA-Sestrin2 (**Figure 2.3A**), WGA-mTOR (**Figure 2.3B**), Sestrin2-mTOR (**Figure 2.3C**) or Rheb-TSC2 (**Figure 2.3D**).

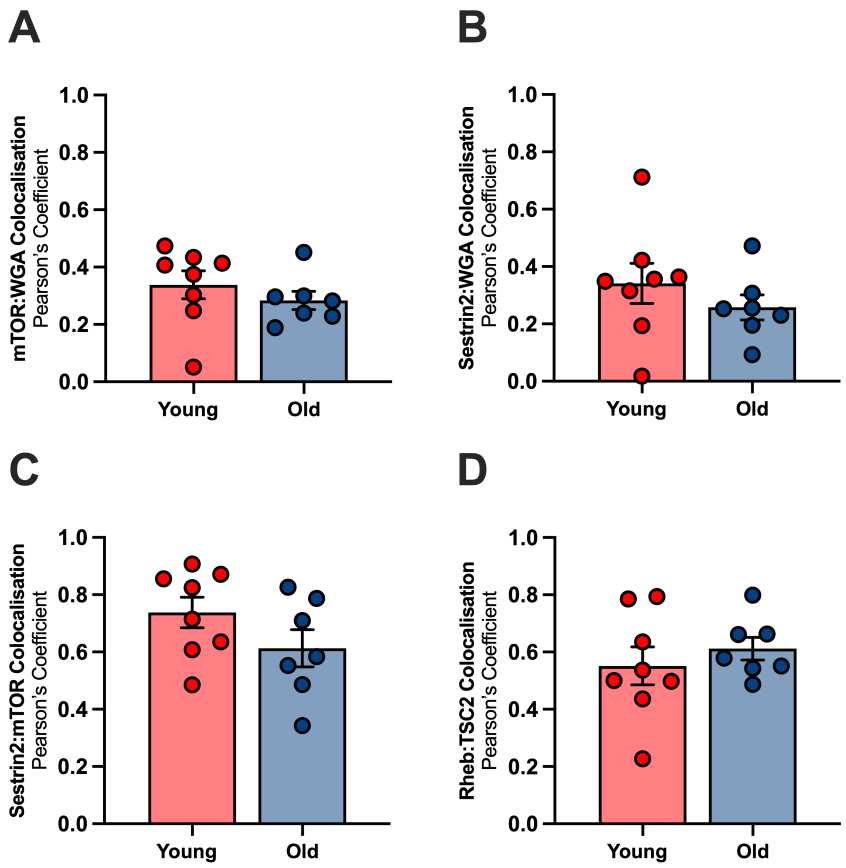


**Figure 2.3.** Protein-protein colocalisation for mTOR-related targets.

Pearson's correlation coefficient for co-stained targets comparison between YM and OM. There is no significant difference between YM and OM for colocalisation between mTOR and WGA (**A**), Sestrin2 and WGA (**B**), Sestrin2 and mTOR (**C**) or Rheb and TSC2 (**D**).

To identify if localisation of mTOR related targets differed between YM and OM and between fibre types, abundance in the central and peripheral regions were compared. Peripheral abundance did not differ between type I and II fibres for any target (**Figure 2.4A-C)** except Rheb (**Figure 2.4D**), which was significantly higher in OM compared with YM in the periphery of both type I (*P*=0.002, d=1.43) and type II fibres (*P*<0.001, d=1.85).

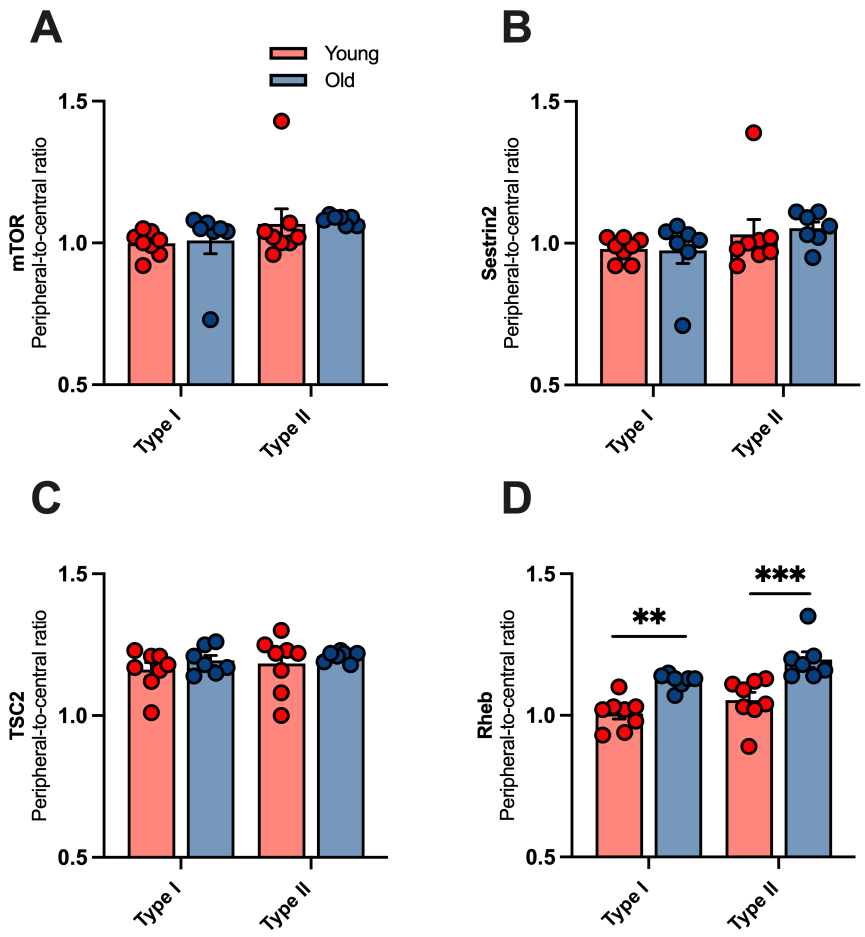


**Figure 2.4.** Peripheral to central ratio of mTOR related targets within skeletal muscle fibre types.

The fibre-specific peripheral-to-central ratio of stained targets. There is no significant difference between the central-to-peripheral ratio for YM and OM for mTOR (**A**), Sestrin2 (**B**) and TSC2 (**C**) in type I or II fibres. There is a significant difference in peripheral-to-central Rheb abundance (**D**) between YM and OM in both type I fibres (**$P$=0.002) and type II fibres (***$P$<0.001).

## 2.5 Discussion

Our study aimed to explore differences in abundance and spatial occupancy of mTOR and upstream mediators in YM and OM via a fibre-type specific IF approach. As such, we offer the first IF investigation of age-related differences in basal mTOR-mediated target abundance, localisation and colocalisation on a fibre type-specific basis.

### *2.5.1 Fibre type area and distribution differences with age*

OM had a significantly lower type II fibre proportion than YM (47.6% vs 57.6%), consistent with previous studies [2, 18]. The purported denervation of type II fibres, and their reinnervation by type I motor neurons [19] may explain this age-related reduction in type II fibre proportion, leading to larger type I fibres and smaller type II fibres, as reported elsewhere [2, 20]. Surprisingly, we did not detect any statistical difference in fibre CSA between YM and OM, despite a large effect size favouring higher CSA of type I (d=0.93) and smaller CSA of type II fibre (d=0.58), in OM compared with YM. It is worth noting that OM had a similar hand grip strength to YM (YM; 50.8kg ± 10.6 vs. OM; 49.1 ± 4.5, data not presented), suggesting that the severity of age-related muscle/type II fibre atrophy may have been relatively mild in OM [21]. Furthermore, using G*Power (V3.1) an estimated sample size of 24 (12 per group) would be required to detect a difference with meaningful effect size (1-β 0.95). We acknowledge that lower limb strength assessments would have permitted more specific inferences to quadriceps muscle fibre CSA from the m. *Vastus lateralis*. Nonetheless, the differences in muscle fibre-type composition between YM and OM largely reflect chronological ageing effects (i.e., little influence of biological ageing and lifestyle) and may offer a suitable platform to probe basal alterations in fibre-type mTOR signalling.

### *2.5.2 Age-related changes in the fibre-type abundance of key muscle anabolic regulatory proteins*

We quantified the abundance of key targets related to mTOR activation on a fibre-specific basis. In contrast to our hypothesis, we observed no difference in the abundance (measured by fluorescence intensity) of any measured target in either type I or type II fibres between YM or OM (**Figure 2.1**, **Figure 2.2**). Nonetheless, this observation is congruent with earlier work showing no difference in mTOR protein content between fibre types in healthy YM, determined by immunoblot [22]. Despite similar abundance between fibre types within age groups, we observed a striking difference in mean fluorescence intensity (a proxy marker for protein abundance) between YM and OM for all targets. Specifically, mTOR fluorescence was diminished in OM compared to YM, contradicting observations of increased abundance of mTOR and downstream targets in aged muscle as determined via immunoblot [8, 9]. It should be noted that the immunoblot analysis in these earlier studies would have likely been performed on mixed muscle protein fractions that integrated peripheral and/or membrane-bound mTOR in mixed type I and II fibres. Indeed, the existence of mTOR-related trafficking (lysosomes) bound to endothelial markers, between fibres, has been previously captured [23]. In contrast, the present IF analysis reflected the fibre-specific intracellular space. Our use of IF methodology (i.e., manually isolating fibre types) ensured that only fluorescence within the fibre was included to conclusively show age-related alterations, but no fibre-type difference, in the abundance of mTOR-mediated signalling.

Upstream mediators of mTOR signalling, Sestrin2, TSC2 and Rheb, also appeared to be altered by ageing. Sestrin2 is purported to have leucine-sensing properties and is a known negative regulator of mTOR [12, 24] that may be implicated in age-related muscle decline. Specifically, reduced Sestrin2 could theoretically be

linked to an impaired leucine-mediated dissociation from mTOR, and therefore impaired activation. To date, the influence of ageing on human skeletal muscle Sestrin2 expression has received little attention. Herein, we provide tentative evidence for an age-related decline in Sestrin2 abundance in human muscle fibres. This is concordant with transcriptomic data from others showing that Sestrin genes are downregulated in mouse models of muscle atrophy [13]. Reduced Sestrin2 in OM noted herein, may also be an adaptive response to persistently increased intracellular EAAs/leucine compared with YM, as has been reported previously [5, 12, 25].

Like Sestrin2, TSC2 negatively regulates mTOR by inhibiting Rheb-specific GTPase [26]. Whilst it has been shown in young adults that TSC2 interactions with Rheb are higher at rest (i.e., low mTOR activation) [15, 27] the impact of ageing upon TSC2/Rheb abundance across fibre types has not been explored. We show OM had 2-fold greater basal TSC2 and 1.4-fold higher Rheb fluorescence than YM. Elevated TSC2 and Rheb and lowered mTOR abundance may significantly reduce the capacity for mTOR activation and could indicate impaired MPS in OM. In partial support of this position, we previously reported numerically lower rates of free-living MPS in the current cohort of OM compared with YM (1.42 ± 0.48%·day$^{-1}$ vs. 1.61 ± 0.21%·day$^{-1}$, respectively, P=0.128, d=0.52). However. further work is required to establish links between age-related alterations in skeletal muscle mTOR signalling abundance and muscle protein turnover on a fibre-type basis.

### *2.5.3 Colocalisation between mTOR related targets does not differ with age in the resting state.*

For colocalisation analysis, no discernible differences were identified between OM and YM for any co-stained target (**Figure 2.3**). Indeed, this may be expected given that experiments were performed on skeletal muscle biopsies taken in the basal state (fasted, rested). However, there were trends for higher Rheb and TSC2 interaction in

OM. If such interactions are systemic (i.e., occurring in most muscles) this could evoke less conversion of Rheb GDPase to GTPase [26], at least in the basal state. Additional biopsy sampling following an acute anabolic stimulus would have permitted greater understanding of age-related colocalisation and translocaton differences within skeletal muscle. There was no effect of age for WGA-mTOR and WGA-Sestrin2 colocalisation. Although unexpected, the absence of an age-related difference in the colocalisation of these targets may be partially explained by the 31% greater intensity of WGA in OM compared with YM samples. WGA is known to bind to glycoprotein derivatives present in the extracellular matrix (ECM) [28] and, whilst less specific than other stains (e.g., picrosirius red, collagen II), can capture fibrotic-like collagen features in skeletal muscle cross sections [29]. The WGA stain presented herein showed a greater intensity in OM vs. YM, which could be explained by age-related ECM alterations (i.e., increased protein aggregation and glycation of ECM proteins [30, 31]). This greater WGA intensity between fibres could have resulted in peripheral identification errors that could not be accounted for. Consequentially, peripheral bound fluorophores may not be detected as being ‘within fibre’, which may have obscured potential differences between YM and OM. Finally, measures of colocalisation were limited to whole field of view images rather than fibre-specific cross-sectional images, leaving open the possibility that age-related in WGA colocalisation with mTOR and Sestrin2 could have occurred between differing fibre types, but were not measurable with the current methods employed. Further, accurate colocalisation analysis can only be undertaken on co-stained targets meaning potentially interesting interactions between Rheb and mTOR, which were not co-stained on the same cross section.

### *2.5.4 Greater peripheral abundance of Rheb in older adults*

Whilst data pertaining to the abundance and colocalisation of mTOR signalling intermediates provide important mechanistic insight on the regulation of muscle protein turnover, isolating the spatial proximity of signalling proteins within myofibers could provide more revealing insights. It is well understood that protein targets, including mTOR-related intermediates, translocate towards/away from the cell periphery in response to anabolic stimuli to facilitate communication between cell signalling sites and to aid the transcription, translation and manufacturing of functional proteins [32]. We compared the mean fluorophore intensity for each target in central compared to peripheral myofiber regions. Significant differences with age ($P<0.001$) and fibre type ($P=0.014$) were identified for Rheb with OM possessing a peripheral-to-central ratio 12% higher for type I and 14% higher for type II fibres than YM (**Figure 2.4D)**. For other targets, no significant differences were observed between YM and OM, or between fibre types (**Figure 2.4A-C)**, suggesting the localisation of mTOR, TSC2 and Sestrin2, are conserved with age, at least in the basal state.

Central occupancy of mTOR related signalling proteins at the basal state have been suggested to allow for translocation to the periphery under anabolic stimulation[33, 34]. Hence, greater peripheral bound proteins within myofibers in the basal state could impede 'normal' protein trafficking. Interestingly in OM, when pooling type I and II fibres, peripheral-to-central ratio of Rheb is 14% higher than the peripheral-to-central of the regulator TSC2 (1.17 ± 0.08 vs. 1.02 ± 0.07) whereas peripheral Rheb is only 4% higher than TSC2 in YM (1.20 ± 0.04 vs. 1.16 ± 0.06). Sush disparages suggests more 'uninhibited' free Rheb (i.e., non-TSC2-bound) is located at peripheral region in OM. To confirm the existence of more unhibited Rheb at the periphery, within fibre colocalisation at a greater magnification could be employed in future research and would help decipher if mTOR activation could be

more profound at the periphery in OM in the basal state. Although similar across fibre types in OM and YM, the implications of regional mTOR signalling in myofibers could explain age-related alterations in muscle fibre morphology and should be considered for use in future investigations.

### *2.5.5 Considerations and Conclusions*

The present study provides the first evidence of skeletal muscle fibre-specific anabolic signalling abundance and localisation in YM and OM in the basal state. Notable differences in TSC2, Rheb and mTOR were found with age, alongside disagreements in Rheb localisation that may relate to deficits in MPS and age-related muscle decline. Future efforts in this space should seek to understand if age-related differences in the basal colocalisation and peripheral occupancy of these targets (relative to their abundance) are linked to impaired responses to anabolic stimuli. Further, this initial investigation only considers the total protein abundance for mTOR signalling intermediates, rather than the phosphorylation status that may ultimately dictate the capacity for MPS. Nonetheless, the present study establishes a methodological basis to address such questions. Finally, adding other relevant mTOR signalling intermediates, such as eEF2, S6K and RPS6 and their phosphorylation status using IF (as described elsewhere [14]) could reveal important age- and fibre-specific differences related to skeletal muscle decline.

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# Chapter 3 The Importance of Dietary Protein Quality for Skeletal Muscle Remodelling in Older Adults

## 3.1 Abstract

Skeletal muscle is imperative for human health, function and quality of life. Dietary protein plays an important role in skeletal muscle maintenance and remodelling through stimulating postprandial muscle protein synthesis (MPS) to promote net anabolism that is essential for muscle adaptive remodelling. The source and quality of ingested protein, determined primarily by constituent essential amino acids (EAAs), the rate of digestibility, and overall bioavailability, is implicated in postprandial MPS stimulation. Whilst per meal and total daily protein intake for muscle anabolism and adaptive remodelling has been relatively well defined in various populations, the importance of dietary protein quality on these parameters remains unclear. Numerous physiological states can influence the postprandial muscle anabolic response to protein ingestion. For example, age-related muscle deterioration (sarcopenia) is underpinned by a blunted MPS response to protein ingestion. Although this age-related muscle 'anabolic resistance' may be mitigated through higher per meal and total daily protein ingestion and/or introducing resistance exercise training (RET), such strategies may not be feasible for many older adults due to alterations in appetite regulation and the sensory system, as well as poor adherence to and understanding of RET guidance. The present review describes the theoretical of 'trade-off' between the amount and quality of dietary protein ingestion for MPS stimulation in older adults, where the source of ingested protein may be particularly important when per meal and total daily protein intake is limited and/or muscle anabolic resistance is present. We conclude that the quality of dietary protein intake in typical diet of community-dwelling older adults may be crucial for postprandial muscle anabolism, particularly during periods of critical illness and inactivity.

## 3.2 Introduction

Performing functional tasks with an objective is an evolutionarily conserved fundamental aspect of human nature [1]. Since hunter-gatherer days, activities of daily living have drastically changed, although completion of such tasks remains an important aspect of survival and livelihood. Functional capacity refers to the capability of completing tasks and activities of daily living which is largely underpinned by skeletal muscle mass and function, the importance of which becomes apparent when musculoskeletal injury negatively affects locomotion and productivity [2, 3]. Indeed, low skeletal muscle mass and poor function are prognostic markers for clinical outcomes across the lifespan and in patients suffering from a range of illnesses [4–8]. In addition, muscle mass and strength represent important predictors of independent living in older age [9] and strongly correlate with gait speed, physical functioning, and overall quality of life [10]. Thus, the natural, senescent decline in skeletal muscle mass and strength, termed sarcopenia, can lower healthy life expectancy in older age [11]. Whilst sarcopenia progression is inherent to chronological ageing, it can be exacerbated by lifestyle factors such as malnutrition [12], declining physical activity [13] and comorbidities [14].

Whilst the primary role of skeletal muscle is to support locomotion, it is also a highly metabolically active organ that interacts with other tissues through secretory proteins and is critical to metabolic homeostasis, including glycaemic control and lipid storage and oxidation [15, 16]. Indeed, low muscle mass is associated with an increased risk of developing type II diabetes and insulin resistance [17]. Further, insulin resistance is associated with dyslipidaemia and impaired glucose handling in musculoskeletal tissues [18, 19]. Skeletal muscle also has many other roles in health

and disease including, but not limited to, providing a protective effect during acute critical illness and chronic disease [20], physical inactivity [21], bone health [22] as well as being the primary determinant of whole-body metabolic rate [23, 24]. That low skeletal muscle mass and poor function also impedes physically active living, drives a vicious cycle of sedentarism, muscle deterioration and functional decline [25]. Hence, maintaining skeletal muscle health can help prevent the manifestation of myriad disease conditions which are accentuated with ageing [26].

The primary nutritional role of dietary protein is to provide amino acids (AA) for the synthesis of new, functional proteins, including skeletal muscle tissue. Current international recommendations for dietary protein intake (~0.8g·kg BM[body mass]$^{-1}$·day$^{-1}$) centre on the daily amount required to safely maintain whole-body nitrogen equilibrium, which are designed to meet basic nutritional requirements and avoid deficiencies in the majority of the population [27, 28]. In contrast, there has been continued debate around the requirement for higher dietary protein intakes to support skeletal muscle maintenance, health, and adaptive remodelling due to population-specific alterations to muscle protein turnover (e.g., older adults and critically ill patients; [29, 30]. In the context of ageing, evidence suggests the optimum daily protein intake for muscle mass maintenance in older adults exceeds the current Recommended Daily Allowance (RDA) [31, 32] and falls in the range of 1.0 – 1.2 g·kg BM$^{-1}$·day$^{-1}$ [33, 34], with an emphasis on higher-quality protein sources [35, 36]. However, whilst the current RDA works on the assumption that higher-quality protein sources are ingested [27], the role of dietary protein quality for muscle adaptive remodelling across the lifespan is not fully understood or integrated into current recommendations. Therefore, the aim of this review is to outline the importance of

dietary protein quality for muscle anabolism and adaptive remodelling in ageing. We outline the concept of a trade-off between dietary protein amount/dose and quality for muscle anabolism and discuss the utility of ingesting higher quality proteins to support muscle health in older adults.

### 3.3 Mechanisms of Skeletal Muscle Remodelling

Skeletal muscle mass is regulated by the net balance between rates of muscle protein synthesis (MPS) and muscle protein breakdown (MPB), the algebraic difference between which determines protein accretion (synthesis>breakdown) or loss (synthesis<breakdown). In the fasted resting state, rates of MPB are elevated above MPS resulting in a negative net protein balance [37]. In the fed-state, postprandial aminoacidemia stimulates MPS to promote a positive net protein balance that is the primary locus of regulation for muscle mass [38]. The consumption of protein-derived amino acids is the main modulator of MPS, with the dose, source and timing of protein ingestion suggested to influence longer-term skeletal muscle adaptive remodelling.

Leucine, one of the branched chain amino acids, holds a unique role as an intracellular signal and substrate for MPS [39–42]. The ‘leucine trigger’ hypothesis suggests that ingestion of sufficient leucine (generally 1-3g dependant on age, disease, and the level of physical activity) is necessary to surpass an intracellular leucine threshold to initiate a robust MPS response [43, 44]. However, in the face of insufficient quantities of the other EAAs, the effect of leucine ingestion on MPS is limited and may even impair MPS, as EAAs with common AA transporters compete with excess leucine for entry into the muscle [45, 46]. Hence, excessive consumption of leucine or disproportionate ingestion of other EAAs, may lower the availability of other EAAs

through the sarcolemma and limit incorporation into new muscle proteins [45]. By contrast, sustained and robust MPS responses are typically observed when sufficient quantities of all EAAs, including leucine, are present, [47–49].

### 3.4 Dietary Protein Requirements for Muscle Anabolism in Older Age

Seminal work by Rennie et al. (1982), suggested transient postprandial stimulation of MPS over a 2–5-hour period [50]. This response is primarily driven by EAAs and was suggested to occur in a dose dependant manner up to a point of saturation at ~10-15g of EAAs in young healthy adults, equivalent to ~20-30 g of high-quality protein [51]. These values have been reinforced by a retrospective analysis demonstrating a plateau in MPS after the ingestion of ~0.24 g·kg $BM^{-1}$ protein (CI: 0.18-0.30 g·kg $BM^{-1}$), equating to ~20 g (CI: 14.4-24.0 g) for an 80 kg young individual [52]. In older adults, postabsorptive rates of MPS under rested conditions do not seem to differ from healthy younger adults [53, 54]. However, there is consistent evidence of an age-related blunting in the postprandial MPS response to dietary protein ingestion (termed ‘anabolic resistance’) [53–56] which has been implicated in the development of sarcopenia [57]. This muscle anabolic resistance places a greater emphasis on increasing the amount of protein consumed, particularly the dose of EAA/leucine, for robust postprandial MPS stimulation in older adults [58, 59] which has been demonstrated in numerous studies [54–56, 60–63]. Instead, older adults may need to consume ~0.4 g·kg $BM^{-1}$ per meal (~32g of protein per meal weighing 80kg) to elicit comparable maximal MPS increases as younger adults [52] Indeed, there is a wealth of evidence to support the requirement for higher protein intakes for age-related muscle health. Most notably, dietary protein intake below the RDA results in muscle mass loss in older adults [64], whereas higher intakes (1.0-1.6 g·kg $BM^{-1}$ $day^{-1}$) support whole-body protein requirements in this population [65] and are

associated with greater lean tissue retention [66–68]. Overall, the support for higher protein intake from acute experimental studies utilising metabolic tracer techniques, alongside data from longer-term observational and interventional studies, supports the benefit of higher per meal and total daily protein intake for skeletal muscle protein anabolism.

### 3.5 Barriers to Dietary Protein Intake in Ageing

Considering the requirement for older adults to consume more protein than younger counterparts, it is interesting to note that per-meal protein intakes do not typically exceed 0.4 g·kg $BM^{-1}$ (1.2 g·kg $BM^{-1}$ day) in older adults [69–71]. There are several possible causes of reduced protein intake in older adults. Protein is highly satiating and lessens the desire to eat larger quantities of food (and by extension, protein-rich foods), particularly given age-related impairments in appetite [72], olfaction [73], mastication [74], dysphagia [75] and gustation [76, 77]. In addition, finances [78], insufficient education [79], health [80], culture and religion [81, 82], cooking ability [83] and loneliness [84] can all contribute to monotonous eating habits which restrict the amount and diversity of ingested protein consumed by older adults [76, 85]. The barriers to high protein intake with advancing age are summarised in **Figure 3.1**. Not only do ~30% of healthy community-dwelling older adults fail to consume the RDA for dietary protein [70, 86] but the likelihood of reaching sufficient levels of dietary protein intake to optimise muscle anabolism is perhaps worsened by the misinterpretation of the RDA as a target intake as opposed to a minimum requirement [31, 87].

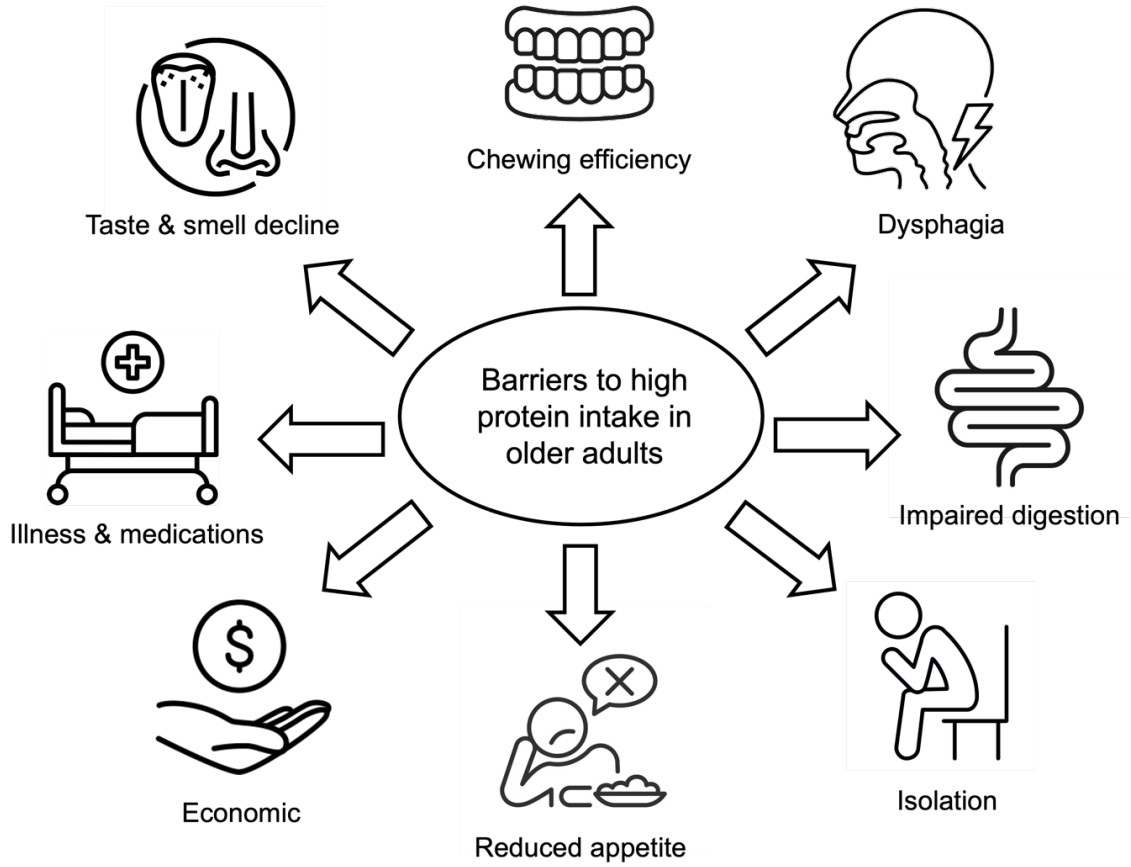


**Figure 3.1.** Summary of barriers which preclude protein intake in older adults.

Both physiological factors which are synonymous with ageing can preclude the capacity to consume high doses of protein which are thought to be required to maximise muscle anabolism in older adults. Such physiological factors include poorer gustation and olfaction (taste and smell of food), mastication (chewing), dysphagia (swallowing of food), impaired digestion and overall impaired appetite which can be accentuated by environmental and physiological factors. Environmental variables can also minimise protein intake, including isolation and monotonous eating habits, monetary value of high protein foods and thus being unaffordable, knowledge of protein intake and confounders of illness and hospitalisation which can limit food access and can also accentuate physiological responses which preclude food intake.

Irrespective of whether older adults can consume dietary protein at or above the current RDA, it is apparent that protein is typically consumed in an uneven or skewed fashion across the day, with emphasis on higher protein intake at the evening meal typically observed [70]. Such patterns of dietary protein intake likely do not facilitate robust postprandial MPS stimulation at each daily feeding opportunity, which is concerning for a population vulnerable to muscle deterioration. Whilst the notion of evenly distributed dietary protein intake across meals/servings to repeatedly stimulate a maximal MPS response has been demonstrated in an acute controlled experiments in younger adults [88, 89], the importance of this paradigm in the context of a typical free-living diet of older adults is not well understood. It was recently demonstrated that integrated rates of daily MPS do not differ between even or skewed protein distribution in older adults, albeit in a diet containing ~1.5 g·kg $BM^{-1}$ protein [90–92]. Longitudinal studies have reported that evenly distributed protein ingestion throughout the day is associated with greater lean muscle mass retention in older adults [93–95]. With reference to increasing dietary protein consumption at breakfast, which is typically low in protein for many older adults [93], Hilkens et al., demonstrated that replacing the carbohydrate-based component with dairy increased 5-hour postprandial aminoacidemia, perceived satiety and MPS in a dose-dependent manner [96]. Similarly, emphasising higher protein intake at breakfast has been associated with greater lean body mass accretion [97], especially during exercise training [98], suggested to be underpinned by a reduction in the time spent in negative net muscle protein balance across the day [99]. Furthermore, older adults who consume more protein at breakfast as opposed to dinner have greater indices of grip strength and skeletal muscle mass [100]. Thus, adequate dietary protein distribution across meals and snacks may be important for muscle anabolism and maintenance in older adults,

particularly at low-to-moderate protein intake levels. However, considering the numerous barriers to consuming sufficient per meal and total daily protein intakes for older adults, robust postprandial muscle anabolism and longer-term muscle maintenance may be difficult to achieve [101]. Thus, shifting the emphasis to the quality of dietary protein intake to support these parameters is an area of great interest.

### 3.6 Dietary Protein Quality and Muscle Anabolism

Currently, the Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO) recommend the Digestible Indispensable Amino Acid Score (DIAAS) as the preferred measure for dietary protein quality [36, 102]. DIASS assesses the quality of a protein based on the relative digestible content of the EAAs expressed relative to a theoretical reference protein that would provide EAA's at a level to meet whole-body human requirements [102, 103]. Typically, most animal-based proteins have a higher EAA content, superior digestibility and overall bioavailability for tissue anabolism compared with the majority of plant-based proteins [104–106]. However, DIAAS are not available for all proteins and do not consider ingestion of multiple protein sources or whole-foods and mixed meals [107]. Furthermore, the DIAAS of a given protein source is reflective of whole-body rather than tissue-specific (e.g., muscle) protein requirements [108, 109]. Hence, it is challenging to directly extrapolate the relevance of DIAAS for postprandial muscle anabolism. Instead, a subjective decision on the quality of protein for muscle anabolism is often made, typically by comparing divergent protein sources that broadly represent higher and lower quality (e.g., animal vs. plant) [110]. In a recent meta-analysis, we demonstrated that the ingestion of higher quality protein was associated with greater postprandial MPS stimulation at rest and following resistance exercise when compared with a dose-matched lower-quality protein [110]. However, it is

important to note that the studies included in this meta-analysis had measured MPS in a controlled acute postprandial period (e.g. 2-to-6-hours) following the ingestion of isolated protein supplements (free from other nutrients) consumed in drink form. Such conditions create an environment that would optimise protein digestion and absorption and lend towards a favourable muscle anabolic response to higher-quality sources. Based on such studies, a long-standing theory is that the rapidity and peak amplitude and EAA/leucine bioavailability following high-quality protein ingestion (e.g., whey protein) may be the crucial trigger for maximal acute postprandial muscle anabolism.

Recent work has challenged the EAA/leucine threshold concept, suggesting that the postprandial EAA/leucine availability following the ingestion of 'slower' or 'lower-quality' protein sources from whole-foods or isolated plant-based sources can elicit a favourably high MPS response, at least in younger adults [111–114]. Thus, it is possible that the threshold for EAA/leucine to trigger a maximal MPS response may be lower than first thought and/or that non-protein components of the whole-food matrix have the potential to support muscle anabolism [115]. For example, consuming milk, or a mixture of amino acids with an identical profile to milk devoid of the food matrix, elicits a comparable MPS response despite divergent patterns of postprandial EAA availability [116]. Similarly, despite inferior postprandial EAA from lower-quality plant-derived compared with higher-quality animal-derived proteins, comparable MPS responses have been reported when ingested in a dose considered sufficient to maximize MPS stimulation (e.g. >0.25 g·kg $BM^{-1}$) and/or as part of a blend that provides a full complement of EAA [111, 112, 117, 118]. In protein-rich whole foods, although the peak and rate of postprandial EAA appearance is attenuated compared with isolated or less complex whole foods, the capacity for MPS stimulation does not appear to be impeded provided that sufficient protein is ingested [119, 120]. It is

possible that the bioactive peptides, lipids and other non-protein components of complex protein-rich whole-foods (e.g., cheese, eggs, salmon) may facilitate MPS despite their relatively low postprandial EAA bioavailability [115, 121, 122]. Others have also shown that food processing and cooking methods may alter postprandial EAA availability but with little influence on the ensuing MPS response [123–125]. In summary, whilst numerous factors can dramatically alter postprandial EAA availability following protein ingestion, any differences in muscle anabolism appear negligible provided the EAA dose is not limiting for MPS. Indeed, of the ~50-60% of protein-derived amino acids that appear in circulation in the postprandial period, only ~20% are taken up into skeletal muscle [126]. **Figure 3.2** highlights the EAA content and digestibility (indicative of 'quality') of commonly consumed isolated protein supplements and whole foods. In the context of controlled experimental studies of acute MPS, this index of quality for isolated protein supplements ingested at a low-to-moderate dose (e.g., up to 0.25 g·kg $BM^{-1}$) may reasonably reflect their capacity for postprandial muscle anabolism. However, this index of quality may not necessarily indicate the capacity for postprandial muscle anabolism when isolated protein supplements are consumed in very large doses (e.g., >0.4 g·kg $BM^{-1}$), as part of a complementary blend, or as part of a nutrient-dense whole-food matrix.

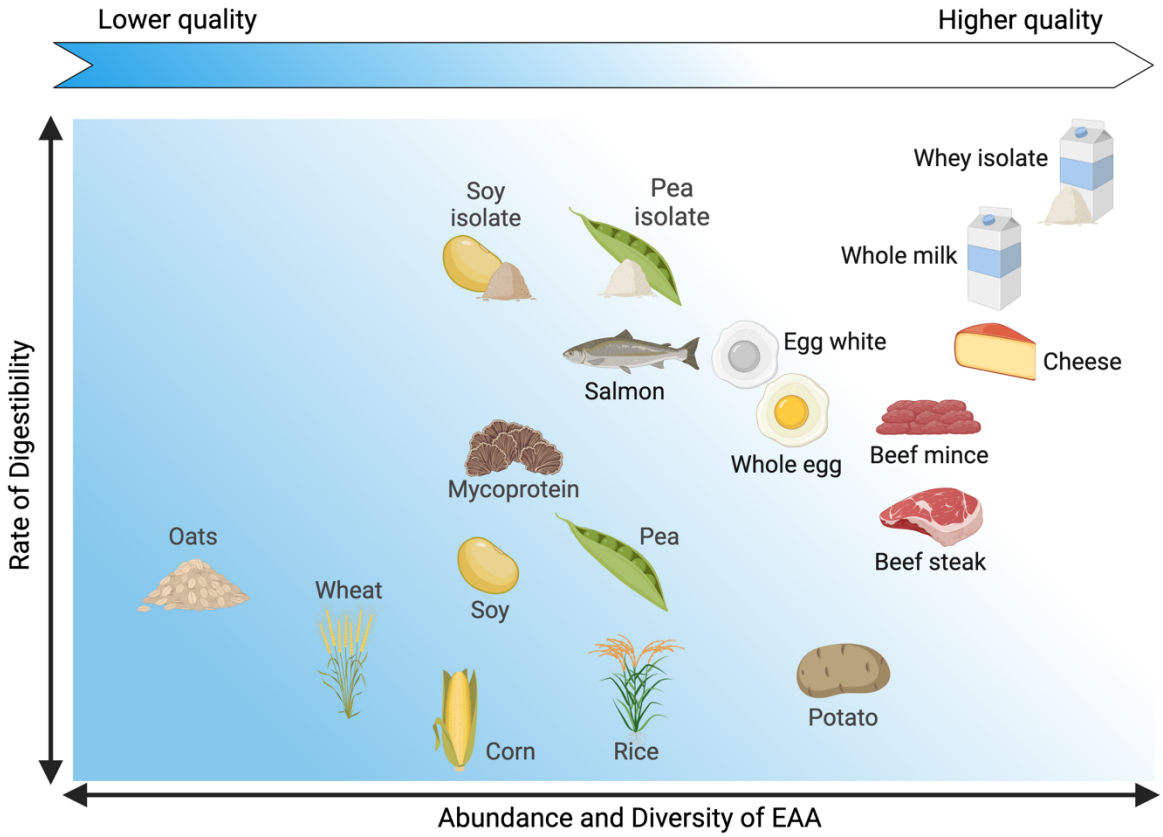


**Figure 3.2.** Illustration of animal and non-animal derived whole food and isolated proteins plotted for their quality according to digestibility and diversity of EAAs.

Highest quality proteins are depicted toward the top right whereas lowest quality proteins are located on the bottom left of the graph. Data obtained by cross-comparison of multiple studies; digestibility hierarchy was obtained from [119, 122, 123, 127–129] and EAA diversity and bioavailability obtained from [102, 106, 119, 122, 123, 129, 130].

### 3.7 Higher Protein Quality for Muscle Anabolism in Older Age

Considering the challenges associated with achieving adequate dietary protein intake to support muscle anabolism in older adults, several studies have compared the muscle anabolic response to protein sources with divergent EAA profiles and varying doses. Acute experimental studies have demonstrated that higher EAA/leucine availability may be necessary to overcome muscle anabolic resistance to the ingestion of low-to-moderate doses of isolated protein to stimulate a robust postprandial MPS response in older adults [55, 56, 59–63, 131, 132]. For example, Yang and colleagues showed that lower EAA containing soy protein needs to be consumed in higher doses (40g) to bolster MPS, whereas 20g whey protein is sufficient to promote significant change in MPS from basal in older adults [132]. Irrespective of the quality of protein consumed, very high dose protein ingestion from isolated supplemental sources appears to elicit comparable postprandial MPS response in older adults [60, 133]. As a specific example, Gorissen and colleagues [60] evaluated the MPS dose-response to ingestion of 35g of whey, casein or wheat protein hydrolysate in older adults with whey and casein increasing MPS beyond wheat protein, despite comparable EAA profile to whey. Only when dose was increased to 60g of wheat protein were increases in MPS observed, suggesting that a very high dose of lower quality protein is necessary to facilitate a rise in postprandial MPS in older adults, and overcome the inferior anabolic potential. Interestingly, the notion that the quality of ingested protein for maximal postprandial muscle anabolism is negligible at doses ≥0.4 g·kg $BM^{-1}$ may not necessarily hold true for whole-food mixed-meals. It was recently reported that ingestion of a high-protein (0.45 g·kg $BM^{-1}$) whole-food meal containing beef, resulted in a greater postprandial MPS response compared with the ingestion of an isonitrogenous whole-food plant-based meal in older adults [134]. Thus, the quality of ingested protein quality for postprandial muscle

anabolism in older adults may be particularly important in the context of whole-food mixed-meals, even when high amounts of protein are provided.

Consistent with the majority of acute experimental investigations, studies conducted under longer-term free-living conditions demonstrate that the quality of dietary protein ingestion does not seem to influence integrated daily MPS rates in older adults provided a high amount of daily protein is consumed (e.g., >1.4 g·kg $BM^{-1}$ $day^{-1}$) [133, 135, 136], unless a very low-quality (EAA-deficient) comparator such as collagen is used. For example, Oikawa and colleagues provided a cohort of older women with 60g of either whey protein or collagen protein on top of a RDA recommended (~0.8 g·kg $BM^{-1}$ $day^{-1}$) diet for 6 days to provide 1.6 g·kg $BM^{-1}$ $day^{-1}$ of protein [136]. At rest, only those supplementing with whey protein saw postprandial increases in MPS, with no significant change from baseline to postabsorptive MPS values with collagen consumption. The same group have more recently shown in older men, consuming 50g of whey or pea protein on top of a diet containing 0.8 g·kg $BM^{-1}$ $day^{-1}$ of protein for 7 days both provide greater postprandial MPS response than with consuming the RDA [133]. However, consumption of 50g collagen fails to elicits similar increases in MPS which are seen with consuming the RDA. To consolidate, the use of collagen proteins are not suitable for stimulating MPS even at very high doses, and their use will make comparator supplements appear more favourable, even when using plant-derived proteins although it remains to be elucidated if at low doses

Taken together, the available evidence suggests that consuming a greater proportion of higher quality protein within a lower protein-containing meal/snack may increase postprandial EAA availability to a level required for maximal postprandial muscle anabolic response in older adults, with important implications for longer-term muscle maintenance. Studies conducted over longer-term free-living conditions (e.g.,

days-to-weeks) incorporating commonly consumed whole food mixed-meals, which if featuring fortification, should avoid collagen protein, are required to understand the importance of dietary protein quality for muscle health outcomes in older adults.

### 3.8 Protein Quality for Muscle Anabolism in Illness and Disuse

Periods of disuse (i.e., bed rest, limb immobilisation), increased sedentary time, reduced physical activity and critical illness have implications for the regulation of muscle protein turnover, net muscle protein balance, strength and functional decline [137–141]. Additionally, during hospitalisation the additional burden of inflammation due to acute illness compounds the effects of disuse on muscle deterioration [142, 143]. Older adults undertaking ~1500 steps for 14-days had a resultant ~4% reduction of their leg lean mass and impaired postprandial MPS stimulation [138]. Hence, the removal of muscle mechanical loading diminishes MPS, particularly in the absence of any specific nutritional intervention to compensate [144–146]. Indeed, protein intake typically falls below 1 g·kg $BM^{-1}$·$day^{-1}$ during hospitalisation and even lower prior to surgery [141, 147, 148]. Inadequate protein nutrition is a prominent issue given 1.6 g·kg $BM^{-1}$·$day^{-1}$ is recommended to achieve energy balance during hospitalisation and minimise exacerbated skeletal muscle atrophy [149]. Authors have shown provision of a protein-dense pre-sleep snack can feasibly increase protein intake [150], although not above 1.6 g·kg $BM^{-1}$·$day^{-1}$, considering pre-sleep snack does not further increase protein feeding at other timepoints in the day. In part, this is unsurprising considering inactivity and illness which are causal of hospitalisation and admittance are associated with decreased appetite [151–153].

Considering the restricted food access during hospitalisation the perturbed nutritive state is unsurprising, although attention to the source of protein and

subsequently the EAAs provided may be of importance. Oikawa et al. (2018) [154] investigated the impact of twice daily supplementation of 30g of lower-quality collagen peptide protein or 30g of higher quality whey protein on integrated MPS rates in older adults during 14-days of step-reduction and energy restriction. Step reduction and energy restriction led to a reduction in integrated MPS and leg lean mass that was similar for both proteins. However, whey protein supplementation augmented MPS and leg lean mass over one week of return to normal activity. In another study, incorporating whey protein into a diet containing ~0.9 g·kg $BM^{-1}$ of protein attenuated lean body mass during 7-days of bed rest, compared with energy and macronutrient-matched mixed meals [155]. These data suggest that ingesting higher-quality protein may partially protect against disuse-induced muscle mass loss in older adults, when total daily protein intake is relatively low. It is pertinent to note that even in young healthy individuals, very-high EAA infusions following single-leg immobilisation does not completely abolish reductions in anabolic sensitivity [156], although perioperative AA infusion re-establishes muscle net balance during major surgery [157]. Hence, whilst high-dose and high-quality protein ingestion (or infusion) may offset a drastic reduction in MPS during disuse events, some loss of muscle mass and function is inevitable when normal contractile loading is perturbed [144–146]. Indeed, it appears that the quality of protein ingested is important for older adults, particularly in situations of perturbed activity and appetite suppression which is precipitated by illness and hospitalisation. The acceleration of skeletal muscle decline, in the absence of contractile stimulus, places greater emphasis on the importance of protein nutrition to provoke anabolic stimulus, which is even more crucial in the anabolically resistant older adult. Indeed, at very low protein intakes i.e., <0.6 g·kg $BM^{-1}$ which are typical in hospitalised settings, quality could be of particular importance to provide EAAs for

MPS stimulation, although the specific role of quality in such low doses, as seen during hospitalisation, remains largely unexplored.

### 3.9 Resistance exercise and protein quality

Resistance exercise training (RET) represents a potent stimulus for MPS and longer-term adaptive remodelling. Undoubtedly RET is the foremost intervention to mitigate sarcopenia progression in older adults, with the potential to promote muscle hypertrophy, increased strength and improved physical function, with myriad positive associated health benefits [158, 159]. Some have reported a diminished muscle anabolic and longer-term adaptive response to RET in older compared with younger adults [160, 161] which may be explained by inadequate RET volume [162] or insufficient per meal and total dietary protein around training. Unquestionably, RET holds the potential to promote significant benefit for muscle adaptation even at a very advanced stage of older age [163–165].

RET-induced MPS stimulation typically peaks in the initial hours of recovery and can remain elevated above resting rates for 48-72 hours afterwards [166]. The RET-induced increase in MPS can be further augmented through the provision of dietary protein [167–169]. Specifically, prior RE allows for greater use of dietary protein-derived EAA's for MPS [170]. The sustained sensitivity of skeletal muscle to EAA provision following RE may be due to factors such as, but not limited to, alterations to AA sensors/transporters [171] and increased blood flow enhancing nutritive delivery to skeletal muscle in both young and older adults [170, 172]. Hence, the consumption of protein beyond the immediate post-RET recovery period (e.g., >2-hours) is important for skeletal muscle adaptive remodelling [173, 174]. Although the duration of the post-RET 'anabolic window' is not well defined in older adults, the synergistic muscle anabolic actions of RET and protein ingestion are apparent across

age groups [51, 56, 62, 63]. Despite the suggestion that the RET-induced increase in MPS rates in older adults may be further augmented by large-dose protein ingestion (beyond that seen in younger adults [62, 132], what is clear that these combined stimuli can largely overcome age-related anabolic resistance and promote muscle mass accretion or maintenance [56]. Notwithstanding, as we have highlighted, consuming large doses of protein that may be necessary to maximise RET-induced MPS may be challenging for many older adults.

The importance of higher-quality protein ingestion to support post-RET MPS rates and long-term adaptive remodelling may be important for older adults, due to the presence of muscle anabolic resistance and the reported higher post-RET protein dose threshold for MPS stimulation (described above [62, 175] When sufficient protein is consumed (≥1.4 g·kg $BM^{-1}$·$day^{-1}$) and an abundant supply of EAA are present for postprandial MPS stimulation at each meal (e.g., ≥0.4 g·kg $BM^{-1}$), the quality of ingested protein for RET-induced muscle anabolism in older individuals may only be marginal additive [135], as theorised in **Figure 3.3**. In our recent meta-analysis, we observed that with sub-optimal protein intake following RET in older adults, higher-quality sources elicit a greater MPS response than lower-quality sources [110]. This is gleaned from the notion that higher quality proteins (i.e., whey protein rather than collagen protein), combined with longer term, twice weekly, RET is the only way to see profound structural remodelling in older adults [176] which is consistent with others showing blunted RET response in older adults [160, 177], with benefits apparent with protein supplementation [178]. Subsequently, sequestering sufficient EAAs wither through high enough protein dose or in the absence of sufficient dose, higher protein quality, is likely needed to maximise benefits of RET in older populations.

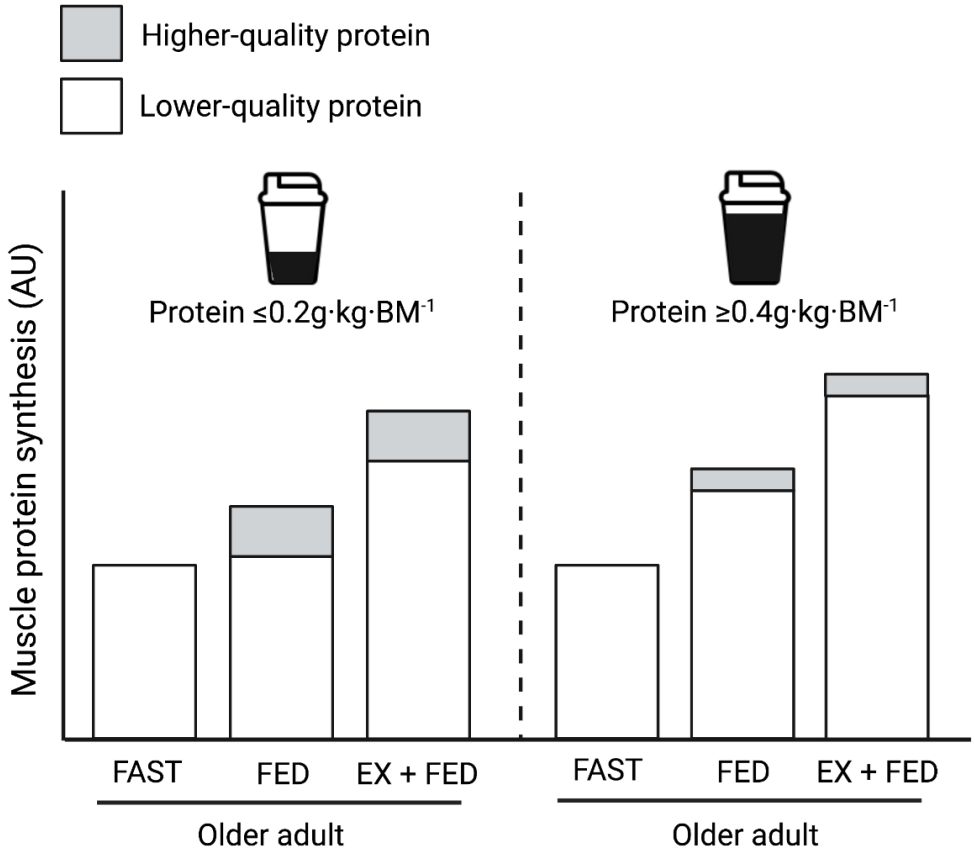


**Figure 3.3.** Theoretical illustration of the role of protein quality on rested and post-exercise muscle protein synthesis in older adults ingesting small or larger protein doses.

**Left:** With small dose (≤0.2 g·kg $BM^{-1}$**)** lower quality protein ingestion (FED; white bar), MPS is not increased above fasted rates (FAST), whereas the same small dose of higher quality protein ingestion increases MPS above fasted rates (FED; grey bar). Small-dose higher quality protein following resistance exercise results in a greater synergistic increase in MPS (EX+FED; grey bar) compared with the same small dose of lower quality protein (EX+FED; grey bar). **Right:** Large dose (≤0.4 g·kg $BM^{-1}$**)** low-quality protein ingestion (FED; white bar) increases MPS above fasted rates (FAST), with a minor to negligible potentiating effect of the same small dose of higher quality protein (FED; grey bar). Larger dose lower quality protein following resistance exercise results in a synergistic increase in MPS (EX+FED; white bar) with minor to negligible potentiation with the same large dose of higher quality protein (EX+FED; grey bar).

## 3.10 Conclusions

In healthy older adults, the ingestion of ≥0.4 g·kg $BM^{-1}$ of dietary protein with each meal/snack, or ≥1.2 g·kg $BM^{-1}$·$day^{-1}$ may be sufficient support postprandial MPS stimulation and longer-term muscle mass retention, with the source and quality of the protein playing a lesser role. However, age-related muscle anabolic resistance, impaired appetite/satiety, sensory issues and food availability are barriers to increased protein intake in older adults, in whom levels often fall below recommended guidelines to support muscle health. In such scenarios, emphasising highly digestible, EAA-rich protein sources may better support postprandial MPS stimulation and longer-term muscle maintenance. Specifically, the addition of higher quality protein to lower protein-containing meals, typical in diet of many older adults, may increase postprandial EAA availability and enhance muscle anabolism. The intake of higher quality protein for age-related muscle health may be particularly important during periods of ill-health and inactivity (e.g., during hospitalisation) where appetite is further diminished, muscle anabolic resistance is exacerbated through unloading and inflammation, and sarcopenia progression is acutely accelerated. Older adults who struggle to consume higher recommended amounts of dietary protein (≥1.4 g·kg $BM^{-1}$·$day^{-1}$) to maximize muscle adaptive remodelling with RET, may reap some benefit from emphasizing higher-quality protein ingestion. Combined with global population ageing and trends towards a greater contribution of plant-based protein sources in the diet, resolving the importance of dietary protein quality for muscle-centric outcomes in older adults is a priority.

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# Chapter 4 Postprandial plasma amino acid and appetite response to a low protein breakfast supplemented with whey or pea protein in middle-to-older aged adults.

## 4.1 Abstract

The addition of low-dose protein to lower protein-containing meals in middle-to-older aged adults has the potential to promote greater postprandial plasma aminoacidemia and mitigate declines in muscle health but may be dependent on the source and quality of protein consumed. The present study investigated postprandial plasma aminoacidemia and appetite regulatory responses to a typical lower protein-containing mixed breakfast supplemented with animal-derived whey protein concentrate (MB+WPC) or pea protein isolate (MB+PPI) in middle-to-older aged adults. Participants were randomised to MB+WPC (male: n=6, females n=7; aged 64 ± 4 yrs) or MB+PPI (male: n=5, females n=4; aged 57 ± 7 yrs) and consumed an identical breakfast containing ~0.07 g·kg body mass[BM]$^{-1}$ of protein supplemented with ~0.13 g·kg BM$^{-1}$ of protein from whey or pea sources. Venous blood samples were collected whilst fasted and over a 180 min postprandial period and analysed for plasma amino acid concentrations via mass spectrometry. Perceived appetite ratings were measured periodically via Visual Analogue Scales. Plasma total amino acids and essential amino acids increased over time (both $P$<0.05) with no between-group differences at any time point, or overall availability (incremental area under the curve (iAUC)). Plasma leucine increased over time (both $P$<0.05) with a greater peak concentration ($P$=0.032) and iAUC (P=0.012) in MB+WPC compared with MB+PPI. Plasma total ghrelin and total GLP-1 concentrations and perceived ratings of hunger, fullness and satiety were transiently altered following MB+WPC and MB+PPI ($P$<0.05 for all), with no differences between groups except for the desire for fatty food intake which was greater in MB+PPI than MB+WPC ($P$=0.011). In middle-to-older age adults, co-ingesting a small bolus of whey protein alongside a typical lower protein-containing breakfast elicits greater plasma leucinemia than co-ingestion of pea protein, with no

influence on indices of appetite regulation. Whether the addition of low-dose whey protein to typical meals of middle-to-older aged adults influences can enhance muscle anabolism without adversely affecting appetite remains to be determined.

## 4.2 Introduction

Age-related skeletal muscle mass and strength loss (termed ‘sarcopenia’) [1], is associated with numerous comorbidities, loss of independence and high socio-economic burden. Sarcopenia progression is underpinned by a diminished muscle protein synthesis (MPS) response to dietary protein ingestion [2, 3], particularly at low-to-moderate protein doses [4–6]. It is suggested that dysregulation of MPS [7, 8] and the onset of sarcopenia [9] may commence in middle age. In light of the increasing prevalence of sarcopenia in globally ageing societies [10] the development of dietary approaches from middle age onwards are paramount to preserve muscle health with advancing age.

Compared with younger adults, it is recommended that older adults need to consume more protein with each meal/snack to maximally stimulate MPS [4, 11, 12]. This notion is reinforced by evidence linking higher daily dietary protein intake with superior muscle health outcomes in older adults [13]. However, increasing dietary protein intake in older adults is often impeded by alterations in appetite [14, 15], taste and sensory impairments [16], digestive issues [17], as well as psycho-socio and economic factors [18, 19]. Frail and hospitalized older adults, as well as community-dwelling middle-to-older aged adults, do not typically meet recommendations for dietary protein intake to support muscle anabolism [20, 21], and consume dietary protein in an uneven pattern of distribution throughout the day [22, 23]. For example, typical protein intake at breakfast is below the threshold required for robust MPS stimulation [22, 24], which would theoretically prolong overnight fasted negative net muscle protein balance, with implications for muscle maintenance [25]. Conversely, higher protein containing breakfasts have been associated with greater overall daily protein intake [26] and superior muscle health outcomes in older adults [11, 27].

Given the difficulties middle-to-older aged adults may have consuming sufficient protein for MPS stimulation, the source and quality of ingested protein may have an important influence on postprandial muscle anabolism in this population [28]. Dietary protein quality is determined by the constituent amino acid profile, particularly the essential amino acids (EAA) and leucine, and the overall rate of digestibility [29]. Most plant-based protein sources are deficient in one or more of the EAAs and generally exhibit slower rates of digestion [30]. Indeed, postprandial MPS stimulation is generally greater with animal-derived compared with plant-derived proteins, when consumed in isolated supplemental form [31, 32] or as part of a whole-food mixed meal [33]. Importantly, many traditional breakfasts contain relatively more lower quality plant-based proteins (i.e., cereals, breads, fruit) than other meals [34, 35]. Considering the amount and source of ingested protein at breakfast in middle-to-older aged adults may provide insufficient EAA/leucine for postprandial MPS stimulation, supplementation approaches could represent a means to overcome this issue [36]. Given evidence that postprandial aminoacidemia may influence perceived hunger and satiety responses [37, 38], the appetite response to lower protein-containing meals with additional protein supplementation requires consideration. Specifically, increasing meal protein intake may promote greater satiety [39, 40] and reduce food/protein intake at subsequent meals, which may negate any muscle anabolic benefit at the subsequent meal.

The aim of the present study was to investigate postprandial plasma aminoacidemia and indices of appetite regulation following the ingestion of a lower protein-containing whole food mixed breakfast, supplemented with a small bolus of whey or pea protein in middle-to-older aged adults. We hypothesised that the addition of a small bolus of high-quality whey protein to a lower protein-containing breakfast

would elicit a more pronounced postprandial EAA and leucine response than an isonitrogenous amount of pea protein. Furthermore, we posited that indices of appetite regulation and associated hormonal mechanisms would be transiently yet similarly altered following breakfast consumption with whey and pea protein supplementation.

## 4.3 Methods

### *4.3.1 Participants*

Twenty-seven active and otherwise healthy males and females aged 50–70-years old volunteered to participate in a single-blind parallel designed study, in which they were randomly assigned to consume a low-protein containing mixed breakfast supplemented with a small bolus of whey protein concentrate (MB+WPC) or pea protein isolate (MB+PPI). Complete plasma samples were collected for 22 participants (MB+WPC; n=13, MB+PPI; n=9). Ethical approval was obtained by the NHS Surrey Borders Research Ethics Committee (REC21/LO/0401) with all procedures conducted in accordance with the Declaration of Helsinki. The present study was part of a larger experimental trial, which was prospectively registered at ClinicalTrials.gov (NCT05574205). Participant flow through the study protocol is outlined in a CONSORT diagram in **Figure 4.1.**

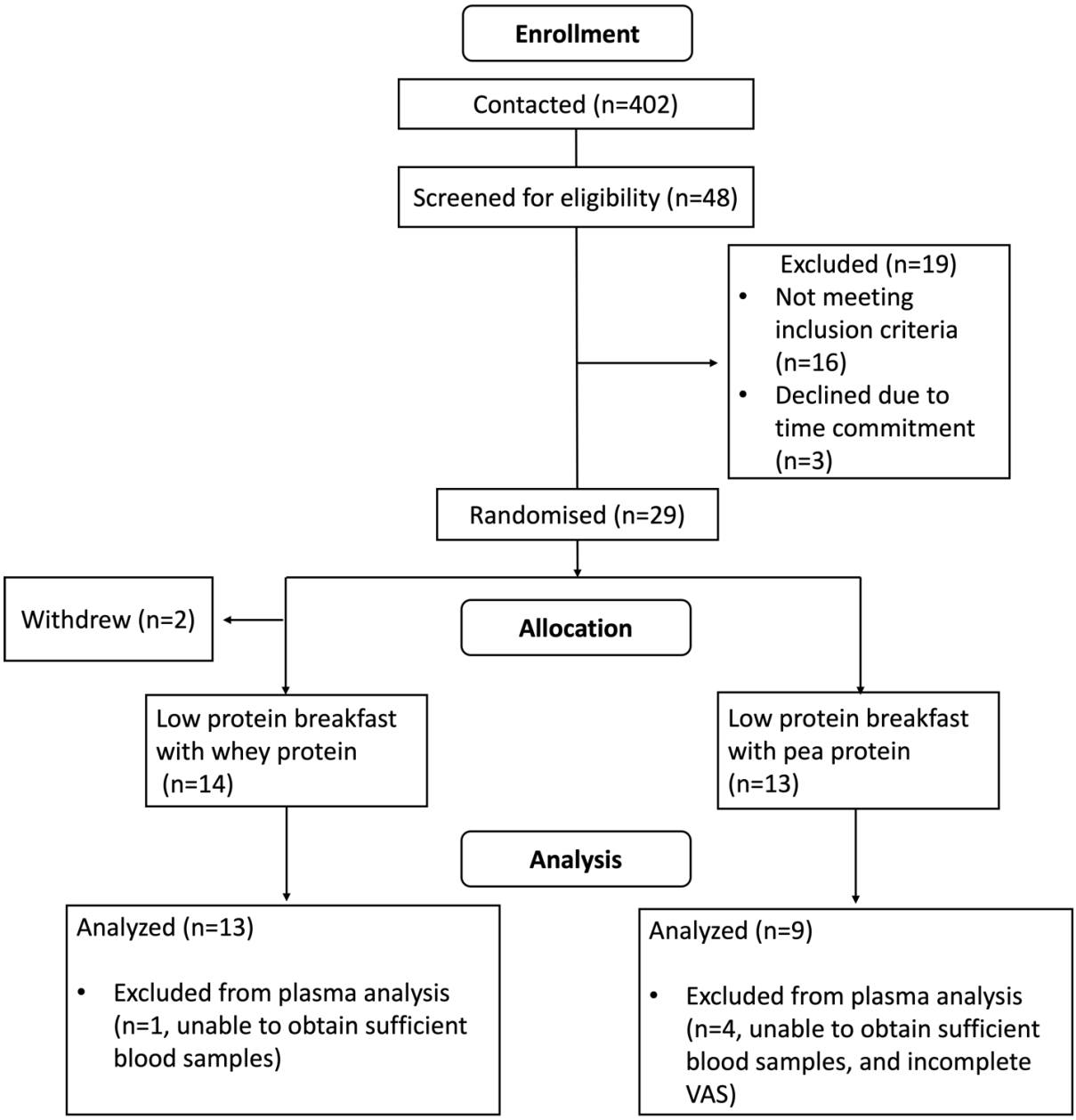


**Figure 4.1** CONSORT diagram

Consolidated standards of reporting trials (CONSORT) diagram indicating the number of participants enrolled, allocated and then analysed for each outcome measure.

### *4.3.2 Preliminary assessments*

Participants were screened for eligibility before providing written informed consent to participate. Participants were excluded if they had a BMI >30kg·m$^2$, any underlying health conditions or dietary intolerances which may cause allergic reactions, adverse appetite or metabolic responses to the breakfast meals or supplements. Anthropometrics were collected for height using a stadiometer (SECA, Hamburg, Germany) and body mass by digital scale (OHAUS, Champ II, Switzerland). Following preliminary testing, participants were asked to keep a diet diary for at least 3 days prior to the intervention (including 1 weekend day) and were provided with a wrist worn accelerometer (GENEActiv, ActivInsights, UK) to characterise habitual dietary intake and physical activity.

### *4.3.3 Experimental testing*

A schematic overview of the experimental period can be seen in **Figure 4.2**. Participants arrived at the School of Sport, Exercise and Rehabilitation Sciences at University of Birmingham having fasted for >10 hours and abstained from strenuous exercise for 24h prior. Anthropometric measurements of height and weight were reassessed to confirm initial measurements made during preliminary testing (**Table 4.3**). Whilst fasted, body composition was analysed using a dual-x-ray absorptiometry scanner (DXA; Discovery A, Hologic, Bedford, MA). Resting metabolic rate (RMR; CPX Jaeger, Vyntus) was also evaluated with the participant laying supine whilst wearing a Hans Rudolf face mask (Cranlea, Birmingham, UK) during 20 minutes for data collection, as described previously [41]. Thereafter, a cannula (BD Venflon$^{TM}$, USA) was inserted antegrade into an antecubital forearm vein to obtain a fasted blood sample (t=-20). The cannula device was flushed with 5 mL sterile NaCl 0.9% (BD PosiFlushTM, USA) after each blood sample to maintain patency. Participants were

then accompanied to a research kitchen where they consumed a whole-food mixed breakfast alongside a small bolus of supplemental whey or pea protein smoothie. Following breakfast consumption, further blood samples were obtained at 0, 20, 40, 60, 90, 120 and 180 min of the postprandial phase. In the postabsorptive state and over the 180 min postprandial phase, a nine-question visual analogue scale (VAS; Flint et al., 2000) was used to measure perceived appetite, hunger, and taste. Participants were instructed to draw a vertical line through a horizontal 0-100mm VAS for 9 questions at each measurement time-point. A composite appetite score was calculated using the first 4 questions on the VAS: 'How hungry do you feel?', 'How full do you feel?', 'How satisfied do you feel?', and 'How much do you think you can eat?' as previously described by [43] and shown in the equation below. For statistical analysis, all VAS-derived measures, including appetite composite score, were expressed as change from baseline (t=-20).

$$Composite\ appetite\ score = desire\ to\ eat\ (mm) + hunger\ (mm) + \big(100 - fullness(mm)\big) + percieved\ eating\ capacity\ (mm)/4$$

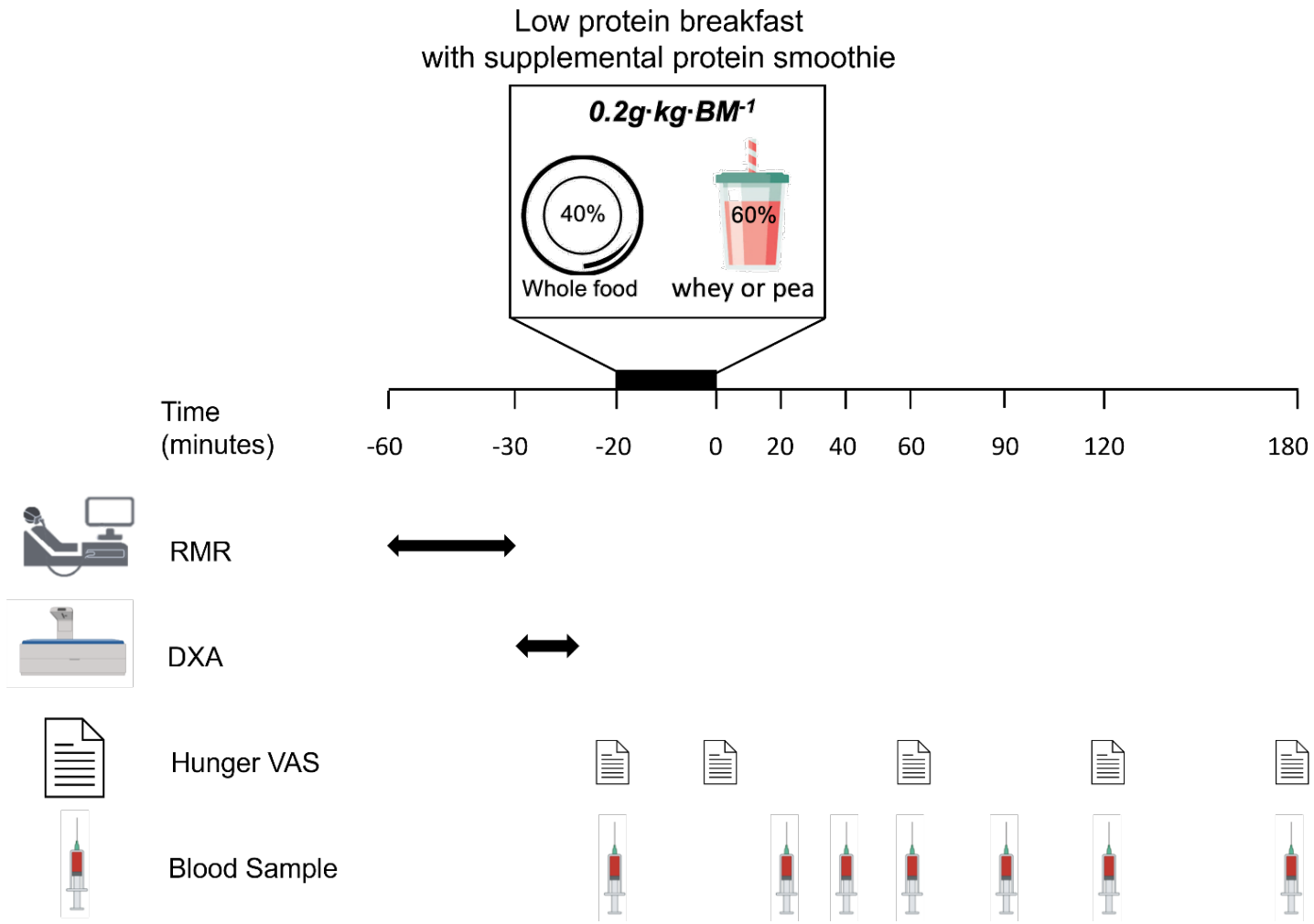


**Figure 4.2.** Study Schematic

RMR: Resting Metabolic Rate; DXA: Dual-energy x-ray absorptiometry; VAS: Visual Analogue Scale.

*Whole-food breakfast and protein supplements*

The lower protein-containing whole-food breakfast provided 0.07 g·kg body mass[BM]$^{-1}$ of protein for both groups (MB+WPC; 6.5 ± 0.6 g, MB+PPI; 6.8 ± 1.0 g, *P*=0.364). Foods consumed for the breakfast test meal were identical for each condition (muesli, yoghurt and blueberries), in which 66-80% of protein was provided from plant-derived sources, which is comparable to typical omnivorous breakfasts consumed by community-dwelling older adults [44]. Alongside the breakfast meal, participants consumed a small bolus (<150ml) protein smoothie beverage, containing 0.13 g·kg$^{-1}$ BM$^{-1}$ of protein from whey protein concentrate (Volactive® UltraWhey Sugar Free WPC, Volac, Hertfordshire, UK) or pea protein isolate (MyProtein™, The Hut Group, Manchester, UK). This supplemental protein dose was selected to identify the role of protein source with a very low protein mixed breakfast typical, whilst achieving an overall typical protein intake for older adults at 0.2 g·kg$^{-1}$ BM$^{-1}$. The 0.13 g·kg$^{-1}$ BM$^{-1}$ equated to 7.89 ± 1.55 g (range: 5.62 – 11.65 g) of protein for both treatments, or 8.00 ± 1.10 g (range: 6.39 – 9.73 g) and 7.74 ± 2.11 g (range: 5.62 – 11.65 g) of supplement material for WPC and PPI, respectively. Total breakfast *plus* supplemental smoothie protein intake was 15.88 ± 2.67 g (range: 12.50 – 23.20 g) of protein for both groups (MB+WPC; 15.4 ± 1.6 g, MB+PPI; 16.5 ± 3.7 g). The constituent elements of the protein smoothie were identical for each condition, apart from the supplemental powder used. The details of the amino acid profile of supplements used are provided in **Table 4.1** with the macronutrient and energy of the WPC and PPI supplemented breakfasts provided in **Table 4.2.** The details of the protein smoothie recipe are provided in **Supplemental Figures 4.1** and **4.2.**

**Table 4.1.** Amino acid content of protein supplements

| Content per 100g of powder (g) | Whey Protein Concentrate (Volactive Sugar Free) | Pea Protein Isolate (MyProtein) |
|---|---|---|
| Aspartic Acid | 9.15 | 8.99 |
| Serine | 4.50 | 4.18 |
| Glutamic acid | 14.50 | 12.5 |
| Glycine | 1.65 | 3.13 |
| Histidine | 1.45 | 1.78 |
| Arginine | 2.00 | 6.12 |
| Threonine | 5.70 | 2.69 |
| Alanine | 4.20 | 3.17 |
| Proline | 5.00 | 3.46 |
| Cystine | 2.00 | 0.67 |
| Tyrosine | 2.00 | 2.77 |
| Valine | 4.00 | 3.04 |
| Methionine | 2.00 | 0.80 |
| Lysine | 8.10 | 5.90 |
| Isoleucine | 4.50 | 2.57 |
| Leucine | 8.10 | 5.75 |
| Phenylalanine | 2.50 | 3.74 |
| Tryptophan | 1.40 | - |
| **∑ Determined amino acids** | **82.75** | **71.26** |
| **∑ EAA** | **37.75** | **26.27** |
| **∑ NEAA** | **45.00** | **44.99** |

EAA. essential amino acids; NEAA: non-essential amino acids.

**Table 4.2.** Energy and macronutrient content of breakfast meals

| | MB+WPC | MB+PPI | *P value* |
|---|---|---|---|
| Carbohydrate (g) | 35.8 ± 5.3 | 38.4 ± 5.7 | 0.295 |
| Carbohydrate (g·kg $BM^{-1}$) | 0.51 ± 0.05 | 0.53 ± 0.08 | 0.276 |
| Fat (g) | 7.65 ± 0.71 | 6.87 ± 1.27 | 0.080 |
| Fat (g·kg·$BM^{-1}$) | 0.11 ± 0.01 | 0.09 ± 0.01 | **0.013*** |
| Protein (g) | 15.80 ± 1.98 | 16.56 ± 3.67 | 0.092 |
| Protein (g·kg $BM^{-1}$) | 0.22 ± 0.01 | 0.23 ± 0.01 | 0.538 |
| Supplemental protein (g) | 8.00 ± 1.10 | 7.74 ± 2.11 | 0.705 |
| Supplemental protein (g·kg $BM^{-1}$) | 0.13 ± 0.0 | 0.13 ± 0.0 | 0.990 |
| Proportion animal protein (%) | 70.94 ± 2.09 | 10.07 ± 1.28 | **<0.001**** |
| Fibre (g) | 5.24 ± 0.66 | 5.85 ± 0.83 | 0.071 |
| Fibre (g·kg $BM^{-1}$) | 0.07 ± 0.01 | 0.08 ± 0.01 | 0.070 |
| Total Energy (Kcal) | 303 ± 34 | 311 ± 49 | 0.683 |

### *4.3.4 Plasma analysis*

Blood samples were collected in EDTA-coated tubes for plasma (BD Vacutainer®, USA) were placed on ice until the end of the collection and then centrifuged at 3500*g* for 15 minutes at 4°C with collected plasma stored in -80°C freezers until all participant data was collected for analysis. Plasma samples were aliquoted out separately for later plasma glucose determination, amino acid concentration and appetite hormone analysis to minimise repeated freeze-thaw cycles of samples.

Plasma glucose was determined on the automated Daytona RX+ using the glucose hexokinase kit (Randox Laboratories, UK) with all samples run in duplicate. Enzyme-linked immunosorbent assay (ELISA) kits were used to measure plasma derived insulin (Mercodia, Uppsala, Sweden), total GLP-1 and total ghrelin (Merck Millipore, Darmstadft, Germany), with all samples run in duplicate and within participant samples run on the same plate for timepoints -20, 20, 40, 60, 90, 120 and 180.

Amino acid concentrations within plasma were analysed using reversed phase ultra-performance liquid chromatography mass spectrometry (UPLC-MS) on an Agilent 6460 triple quadrupole mass spectrometer coupled with an Agilent 1290 UPLC system (Agilent CA, USA) using methods as previously described [38]. Plasma AA data are presented in $\mu mol \cdot L^{-1}$.

### *4.3.5 Statistical Analysis*

Participant characteristics and amino acid breakdown comparison tables were created on Excel (Microsoft, USA). Figures were produced using GraphPad Prism with statistical significance set *a priori* as $P \leq 0.05$. For all time-dependant and time-

independent variables of plasma and VAS-derived outcomes over the 180 min postprandial period, a 2-way repeated measures analysis of variance (ANOVA) was completed. In the instance of missing timepoints, a mixed-effect model was performed. The fasted baseline value was used as the reference point for statistical comparisons when running time-course ANOVAs. Incremental area under the curve (iAUC) and where appropriate, total area under the curve (AUC) for postprandial metabolite, appetite hormone and VAS responses were calculated with the trapezoid method using the Time Series Response Analyser [45]. In instances where there were statistically significant interaction effects, Bonferroni post-hoc test was run to locate statistical differences between specific parameters. Data are presented as mean ± standard error of the mean (SEM) unless otherwise stated and effect sizes were calculated where appropriate using Cohens d (d) where d=0.2, d=0.5 and d=0.8 was considered as small, medium and large effect size, respectively.

## 4.4 Results

### *4.4.1 Participant characteristics*

Participant characteristics are presented in **Table 4.3**. The mean age of participants was significantly lower for MB+PPI compared with WPC (*P*=0.008). There was no difference in sedentary or moderate and vigorous activity (MVPA) time between groups. RMR, BMR, estimated TEE and body composition measures did not differ between groups.

**Table 4.3.** Participant characteristics

| | MB+WPC (n=13) | MB+PPI (n=9) | Pooled (n=22) | *P value* |
|---|---|---|---|---|
| Male/Female | 6/7 | 5/4 | 11/11 | |
| Age (y) | 64.2 ± 4.4 | 57.1 ± 7.0 | 61.3 ± 6.5 | **0.008*** |
| Height (m) | 1.69 ± 0.10 | 1.71 ± 0.13 | 1.70 ± 0.11 | 0.769 |
| Mass (Kg) | 71.6 ± 9.8 | 74.4 ± 20.3 | 72.7 ± 14.7 | 0.668 |
| BMI ($Kg/m^2$) | 24.9 ± 1.8 | 25.1 ± 3.7 | 25.0 ± 2.7 | 0.866 |
| Body fat (%) | 26.1 ± 7.1 | 28.2 ± 8.8 | 27.0 ± 7.7 | 0.192 |
| RER | 0.89 ± 0.1 | 0.81 ± 0.1 | 0.86 ± 0.1 | 0.998 |
| REE (kcal) | 1474 ± 337 | 1621 ± 618 | 1576 ± 479 | 0.456 |
| Estimated TEE (kcal) | 2167 ± 307 | 2187 ± 564 | 2175 ± 418 | 0.912 |
| Sedentary time (%) | 72.2 ± 11.2 | 65.6 ± 13.3 | 70.0 ± 12.3 | 0.250 |
| MVPA (%) | 17.7 ± 7.3 | 24.1 ± 8.1 | 20.5 ± .7.7 | 0.082 |

MB+WPC: mixed breakfast with whey protein concentrate; MB+PPI; mixed breakfast with pea protein isolate; BMI: Body mass Index; RER: respiratory exchange ratio; MVPA: moderate and vigorous physical activity. Data presented as mean ± SD. Independent samples t-test was used to test significant difference between PPI and WPC. * Denotes significant differences between groups (*P*<0.05).

### *4.4.2 Plasma aminoacidemia*

Plasma total amino acid (TAA) concentrations significantly increased over time from basal (t=-20 mins) (**Figure 4.3A**; *P*<0.001) with no significant main effect of group (*P*=0.247), or group x time interaction (*P*=0.915). There were no between-group differences in peak plasma TAA concentrations (*P*=0.517) or time-to-peak (*P*=0.377). The plasma TAA iAUC was not significantly different between groups (**Figure 4.3B**; *P*=0.865).

Plasma essential amino acid (EAA) concentrations significantly increased over time following baseline fast values (t=-20) (**Figure 4.3C;** *P*<0.001) with no significant main effect of group (*P*=0.531), or group and time interaction (*P*=0.368). There were no between-group differences in peak plasma EAA concentrations (*P*=0.366) or time-to-peak (*P*=0.757). The plasma EAA iAUC was not significantly different between groups (**Figure 4.3D**; *P*=0.190).

Plasma leucine concentrations significantly increased over time (**Figure 4.3E**; *P*<0.001), with no significant main effect of group (*P*=0.258) or group and time interaction (*P*=0.057, d=0.29). Considering the strong trend for group and time interaction it was noted that plasma leucine concentrations were significantly greater with MB+WPC compared with MB+PPI at the 40 min of the postprandial phase (WPC: 230 ± 40 μmol·$L^{-1}$; PPI: 190 ± 23 μmol·$L^{-1}$, *P*=0.006). There were no between-group differences in peak plasma leucine concentrations (*P*=0.081) or time-to-peak (*P*=0.784). The plasma leucine iAUC was greater in MB+WPC compared with MB+PPI (**Figure 4.3F**; *P*=0.032, d=0.96).

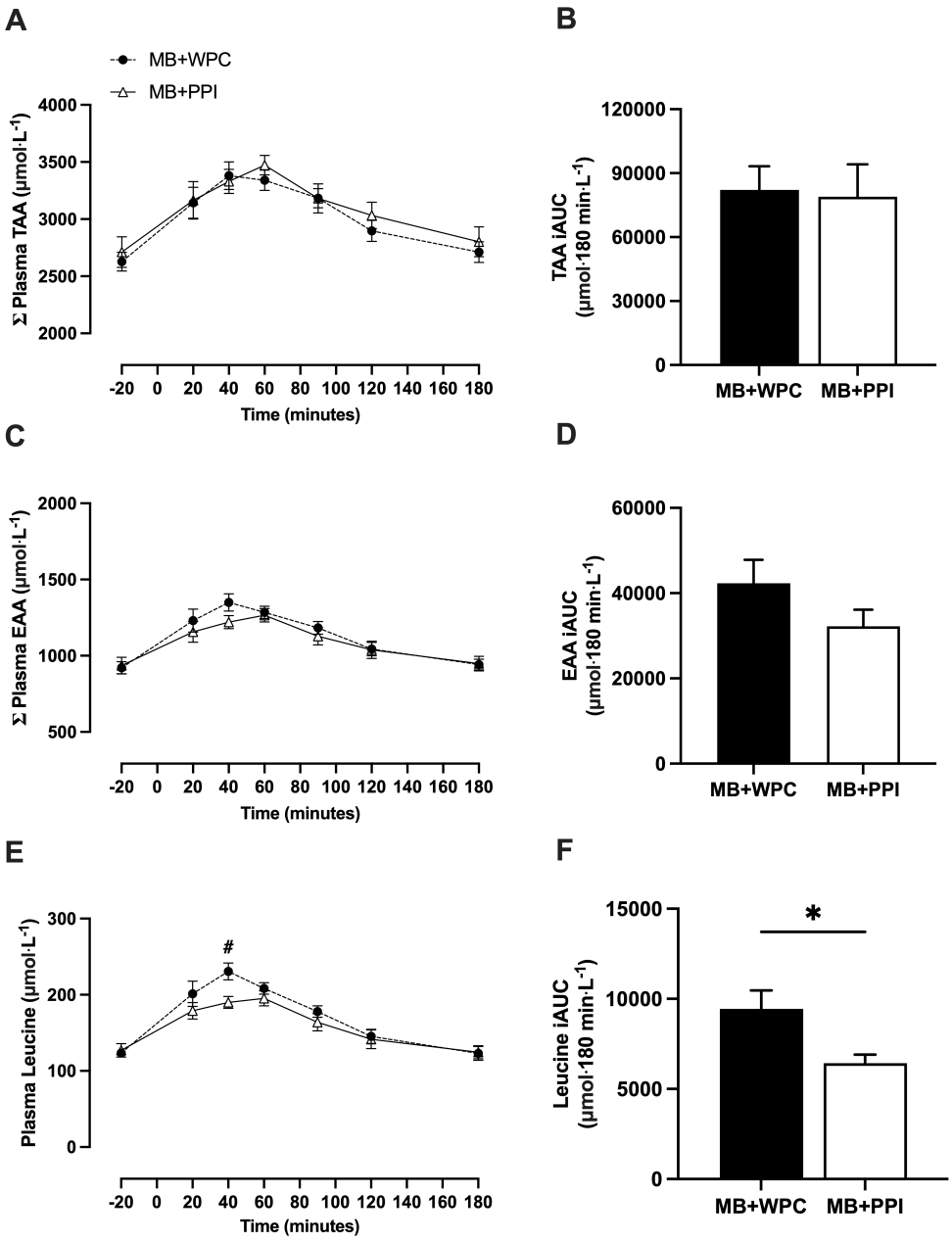


**Figure 4.3.** Plasma total amino acid (TAA), essential amino acid (EAA) and leucine concentrations

Postprandial plasma TAA (**A**) and incremental area under the curve (iAUC) (**B**) over 180 minutes. Postprandial EAA excursions (**C**) and corresponding iAUC over 180 minutes (**D**). Plasma leucine excursions (**E**) and iAUC response (**F**). Data are presented as mean and SEM with n=13 for mixed meal with whey protein concentrate (MB+WPC) and n=9 for mixed breakfast with pea protein isolate (MB+PPI). Two-way ANOVA showed a significant effect of time for **A**, **C** and **E** (P<0.001) but no group or clear interaction effect, apart from close interaction for **E** (*P*=0.057). Independent samples t-test was used to compare between groups iAUC for TAA (**B**), EAA, (**D**) and leucine (**F**). *Denotes significant difference (*P*<0.05) and # denotes significant difference between groups at indicated timepoint due to close interaction effect in **E**.

The remaining plasma AA concentrations and the corresponding iAUC are presented in **Supplemental Figures 4.3** and **4.4.** Briefly, postprandial plasma availability significantly increased over time from fasted values for all amino acids with the exception of cystine and glycine (both $P>0.05$). During the 180 min timecourse response, two-way ANOVA revealed time x group interaction effect for plasma arginine ($P=0.012$) and isoleucine ($P=0.044$), with main group differences for plasma phenylalanine ($P<0.001$) and tyrosine (P=0.008) (**Supplemental Figure 4.3)**. Bonferroni post hoc analysis from interaction effect unveiled plasma arginine concentration was significantly ($P>0.05$) greater in MB+PPI than MB+WPC at t=60 and t=120, whereas plasma isoleucine concentrations were significantly greater in MB+WPC than MB+PPI at t=40 (**Supplemental Figure 4.3**). Peak plasma phenylalanine ($P<0.001$) and asparagine ($P=0.048$) concentrations were greater in MB+PPI than MB+WPC, whereas peak plasma aspartic acid ($P=0.038$) concentration was greater in MB+WPC than MB+PPI. The iAUC for plasma arginine ($P=0.004$), glycine ($P=0.022$), methionine ($P=0.026$) and phenylalanine ($P=0.018$) was significantly different between groups (**Supplemental Figure 4.4**).

### *4.4.3 Plasma glucose and plasma insulin*

Significant increases over time were seen for plasma insulin (**Figure 4.4A**; $P<0.001$) and plasma glucose (**Figure 4.4C**; $P<0.001$) with no significant main effect of group for insulin ($P=0.844$) and glucose ($P=0.650$) or group and time interaction for plasma insulin ($P=0.791$) and glucose ($P=0.483$). There were no between-group differences in peak plasma insulin ($P=0.203$) or glucose concentrations ($P=0.109$) or time-to-peak plasma insulin ($P=0.980$) or plasma glucose ($P=0.946$). There was no significant difference between groups in iAUC for plasma insulin (**Figure 4.4B**, $P=0.797$) or plasma glucose (**Figure 4.4D,** $P=0.764$).

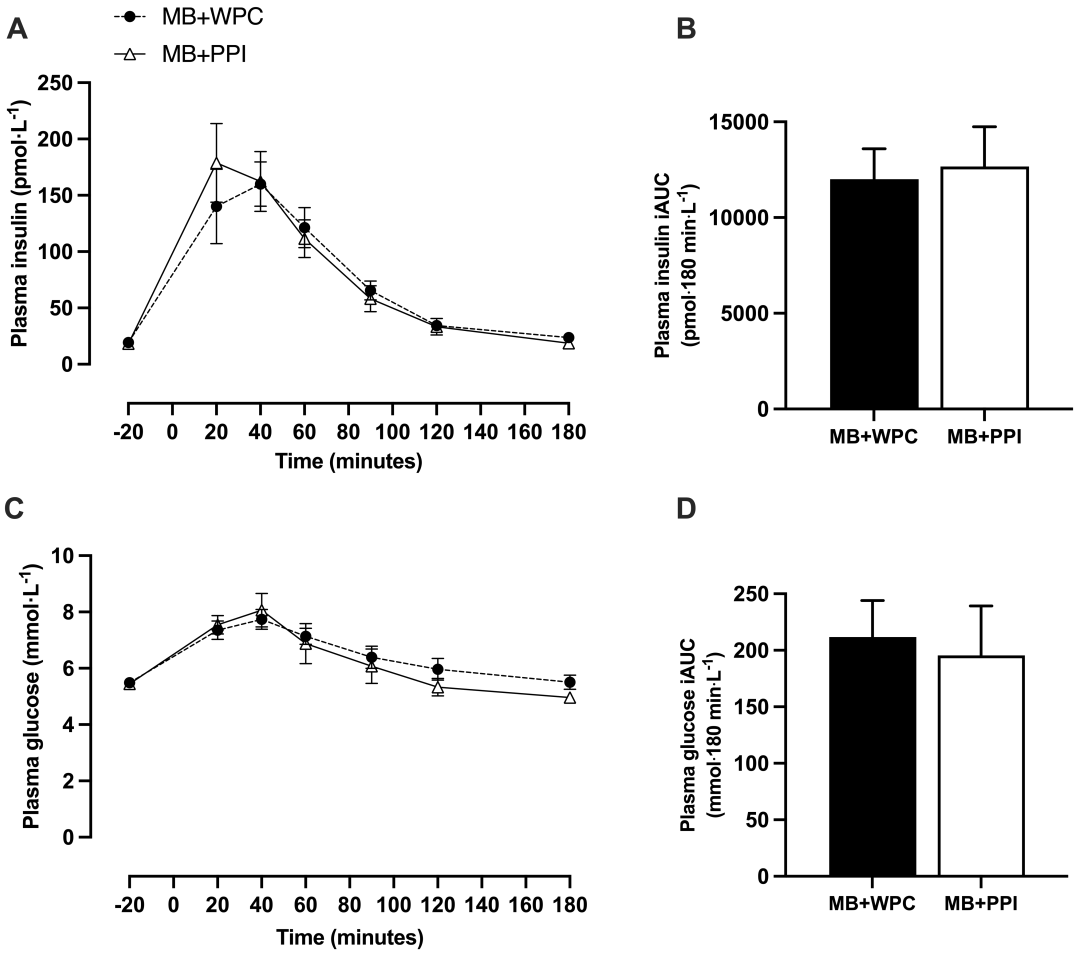


**Figure 4.4.** Insulin and Glucose excursions and area under the curve

Postprandial plasma insulin excursions (**A**) and incremental area under the curve (iAUC) (**B**) over 180 minutes and postprandial glucose excursions (**C**) and iAUC over 180 minutes (**D**). Data are presented at mean and SEM with n=13 for mixed breakfast with whey protein concentrate (WPC) and n=9 for mixed breakfast with pea protein isolate (PPI). Two-way ANOVA was run to test for significant difference between groups and timepoints, with baseline value (t=-20) within condition set as the reference point. Independent samples t-test was used to compare insulin iAUC (**B**) and Glucose iAUC (**D**). *Denotes significant difference from baseline for both groups ($P$ <0.05).

### *4.4.4 Plasma appetite hormones*

Plasma total GLP-1 concentrations were significantly increased with time compared to baseline (**Figure 4.5A**; $P<0.001$) with no significant main effect of group ($P=0.212$), or group and time interaction ($P=0.131$). There were no between-group differences in peak plasma total GLP-1 concentrations ($P=0.187$) or time-to-peak ($P=0.533$). The plasma total GLP-1 iAUC was not significantly different between groups (**Figure 4.5B**; $P=0.134$). Plasma total ghrelin concentrations significantly increased over time (**Figure 4.5C**; $P<0.001$) with no significant main effect of group ($P=0.316$), or group and time interaction ($P=0.592$). There were no between-group differences in nadir plasma total ghrelin concentrations ($P=0.496$) or time-to-nadir ($P=0.561$). The plasma total ghrelin iAUC was not significantly different between groups (**Figure 4.5D;** $P=0.345$).

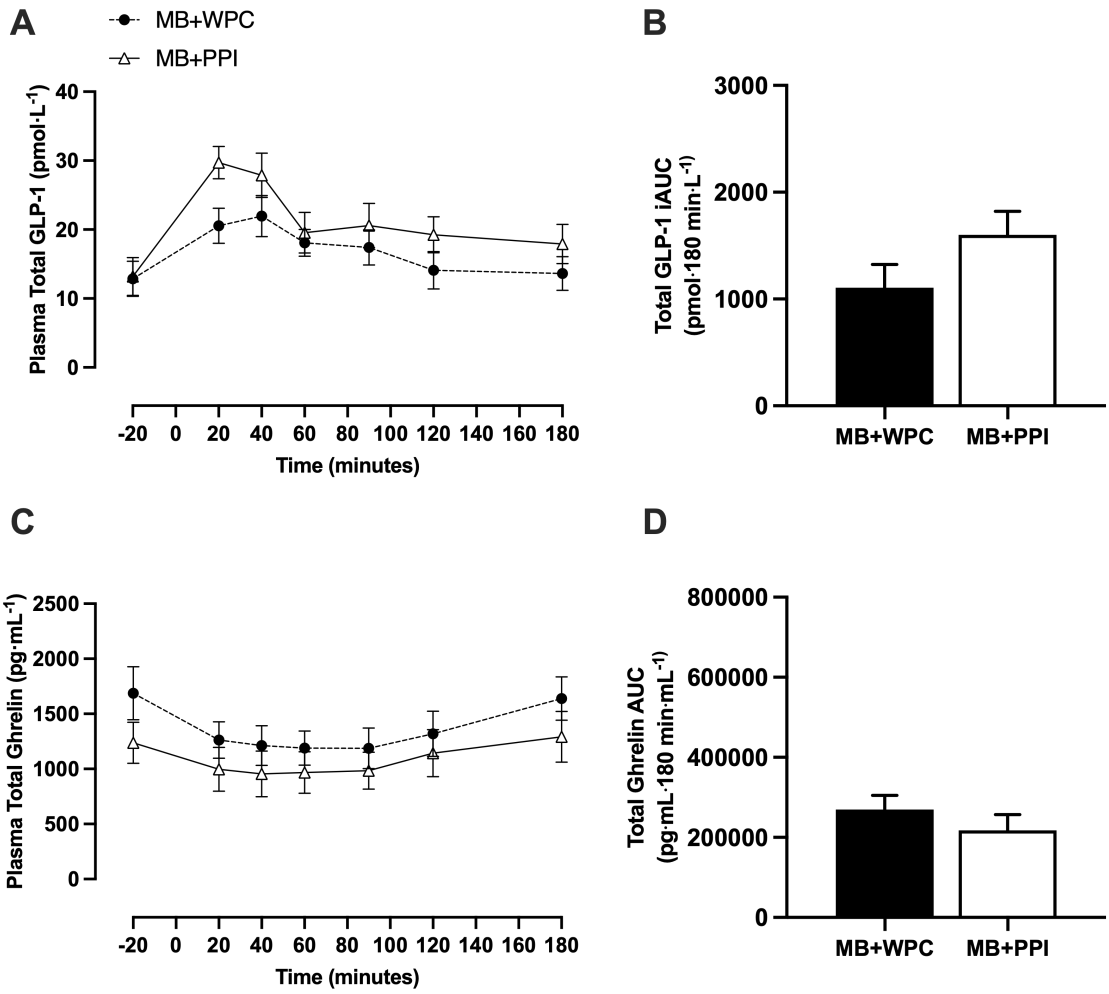


**Figure 4.5.** Postprandial appetite hormone response following pea or whey protein supplemented mixed breakfast

Postprandial total GLP-1 excursions (**A**) and area under the curve (iAUC) (**B**) over 180 minutes and postprandial total ghrelin excursions (**C**) and area under the curve (AUC) over 180 min (**D**). Data are presented at mean and SEM with n=13 for mixed breakfast with whey protein concentrate (MB+WPC) and n=9 for mixed breakfast with pea protein isolate (MB+PPI). 2-way ANOVA was run to test for significant differences between groups and timepoints and overall interaction with baseline value (t=-20) within condition set as the reference point for **A** and **C**. Independent samples t-test was used to compare total GLP-1 iAUC (**B**) and total Ghrelin AUC (**D**).

### *4.4.5 Perceived appetite responses*

Perceived appetite responses from VAS-derived outcomes showed there a main effect of time for thirst ($P$=0.003), hunger ($P$<0.001), satisfaction ($P$=0.001), fullness ($P$<0.001), eating capacity ($P$<0.001) (**Figure 4.6A-E)** and the composite appetite score ($P$<0.001) (**Figure 4.6J**), but no main effect of time for any taste preferences (**Figure 4.6F-I**, all $P$>0.05). Main group effects were found for fatty food desire ($P$=0.005), and group and time interactions were found for fullness ($P$=0.036), desire for sweet food ($P$=0.045) and desire for fatty food ($P$=0.011). Following post hoc analysis, only significant differences between group and time remained for fatty food desire which was significantly greater for MB+PPI at 60, 120 and 180 min compared with MB-WPC ($P$<0.05 for all).

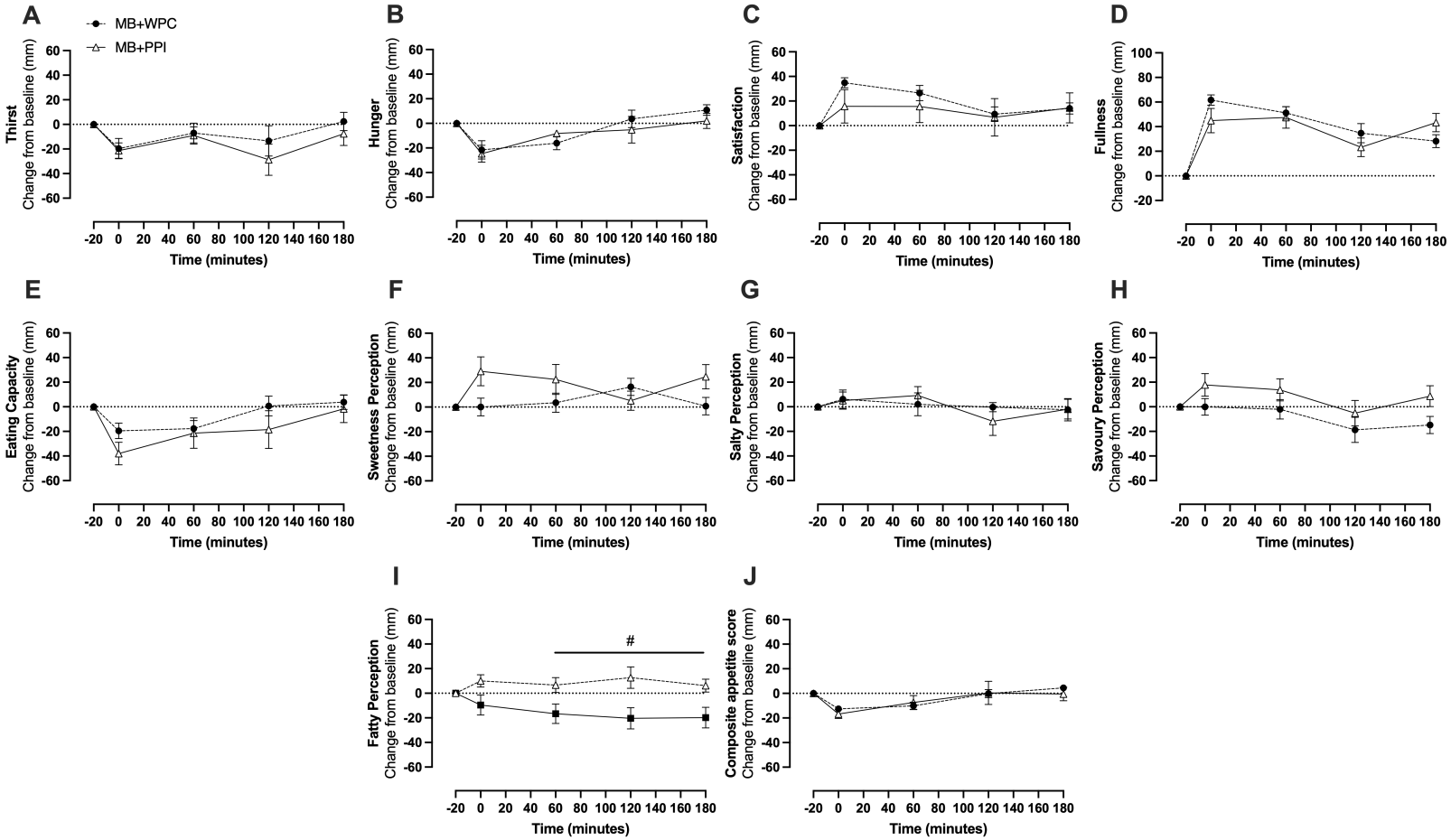


**Figure 4.6.** Visual analogue scales (VAS) for perceived appetite expressed as change from baseline over 180 minutes postprandially following mixed meal breakfast and whey or pea protein supplementation

Data are presented as mean ± SD for mixed breakfast with whey protein concentrate (MB+WPC) n = 14 and n = 11 for mixed breakfast with pea protein isolate (MB+PPI). Thirst (**A**), appetite related scores (**B-E**), taste perception scores (**F-I**) and composite appetite score (**J**). 2-way ANOVA was run to test for significant difference between groups and timepoints, with baseline value (t=-20) within condition set as the reference point. Significant main effect for time was seen for A, B, C, D, E, I and J (all $P<0.05$) and significant main effect difference between groups for I ($P<0.05$). Main interaction for D, F and I (all P<0.05). # Denotes significant difference ($P<0.05$). found between groups over indicated timepoints following significant interaction effect which remained following post hoc analysis.

## 4.5 Discussion

Achieving sufficient protein intake to maximise postprandial muscle anabolism in middle-to-older adults is challenging given the higher muscle anabolic protein dose requirement and barriers to consuming such intakes. Pragmatic nutritional strategies including the provision of higher quality protein sources to typical lower protein-containing meals have the potential to augment postprandial MPS stimulation and support longer-term muscle health with advancing age. The present study is the first to characterise the postprandial plasma AA and appetite regulatory response to the ingestion of a lower protein-containing whole food mixed breakfast supplemented with a small bolus of high-quality whey protein concentrate (MB+WPC) or isonitrogenous lower-quality pea protein isolate (MB+PPI) in middle-to-older aged adults. Our findings show that MB+WPC and MB+PPI resulted in a comparable postprandial increase in plasma TAA and EAA concentrations, whereas the overall availability and peak concentration of plasma leucine was greater for MB+WPC than MB+PPI, corroborating the findings of others on isolated pea vs whey protein-containing drinks [46]. Ingestion of MB+WPC and MB+PPI transiently altered concentrations of plasma glucose, plasma insulin, plasma total GLP-1, and total ghrelin as well as perceived hunger and appetite sensations, with no differences between conditions. Collectively, these findings suggest that the postprandial plasma AA response, in particular leucine, with MB+WPC could potentially support a greater muscle anabolic response than MB+PPI in middle-to-older aged adults, without adversely affecting appetite regulation.

Leucine is important in the regulation of intracellular anabolic signalling and as a substrate for MPS [47, 48]. Postprandial plasma leucine availability has been implicated

as a critical driver for MPS stimulation [49]. Furthermore, leucine fortification of lower-dose amino acid provision has been previously shown to augment postprandial MPS in older adults [50–52]. Based on the divergent leucine profile of the whey and pea protein supplements used herein, we hypothesised that postprandial plasma leucinemia would be greater following ingestion of MB+WPC than MB+PPI, indicative of a more favourable condition for postprandial muscle anabolism. In line with our hypothesis, postprandial plasma leucine availability and peak plasma leucine concentration was greater with MB+WPC than MB+PPI. Given our suggestion that the superior plasma leucine response to MB+WPC could be associated with a greater postprandial MPS response, it is possible that this strategy could have implications for net muscle protein balance and muscle maintenance in middle-to-older aged adults when repeated over multiple feeding events. It has recently been suggested that leucine content is subordinate for MPS when combined with whole foods, where additive properties of whole foods may prevail as more beneficial for MPS or lower the amount of leucine needed to maximise anabolism and surpass the 'leucine threshold' (>2.5g) to trigger MPS [53–55]. Indeed, long-term leucine supplementation (2.5 g/day for 3 months) in healthy older men with a daily protein intake similar to the present study did not augment skeletal muscle mass or strength over placebo [56]. Conversely, the addition of 5g leucine with low protein meals for 3 days augments muscle protein synthesis rates in older men [57], suggesting high dose leucine dose may be required to stimulate anabolism. Undoubtedly, the benefit of small increases in leucine (when below the ~2.5g 'leucine threshold') alongside whole foods remains to be elucidated. In the present study, MB+WPI were provided with 0.60-0.92g leucine from WPC supplement and MB+PPI were provided with 0.40-0.84g leucine from the PPI

supplement, although the leucine contribution from whole-food and the magnitude under the 'leucine threshold' is unknown. Henceforth, it stands to reason if the ~29% greater leucine provision with WPC over PPI in the present study, could offer a superior anabolic response over an extended period of supplementation.

Indeed, postprandial MPS is regulated by factors other than plasma leucine availability [55]. Specifically, the plasma availability of all EAAs (i.e., not just leucine) may be necessary for a robust and sustained postprandial MPS response [58, 59] and longer-term maintenance of muscle remodelling in older adults [56, 60]. In contrast to our hypothesis, there were no significant differences in postprandial EAA availability or peak plasma EAA concentration between MB+WPC and MB+PPI, despite divergent amino acid profiles of the whey and pea protein supplements. It is possible that the composition of the MB+WPC and MB+PPI may have masked any difference in postprandial EAA concentrations. Ingesting a low-dose of whey and pea protein in smoothie form alongside a whole food mixed breakfast would slow the rate of digestion and lower postprandial plasma aminoacidemia [33, 61–64], compared with ingesting supplements in isolated drink form alone [31, 46, 65, 66]. Indeed, McKendry and colleagues [46] recently demonstrated that the co-ingestion of a 25g whey protein isolate drink with a mixed breakfast meal elicited greater postprandial plasma EAA and leucine availability than ingestion of a 25g of pea protein isolate drink in older adults. Nonetheless, our decision to provide protein supplements in smoothie form alongside the whole food mixed breakfast was taken to represent pragmatic and acceptable means of delivering dietary protein and other important nutrients in a population at increased risk of nutrient deficiencies [67]. Notwithstanding, the comparable postprandial plasma EAA

concentrations between groups could indicate a similar capacity for MPS stimulation. It is also worth noting that other non-protein components of the whole food matrix in mixed breakfast meals provided could influence the postprandial muscle anabolic response [54, 68]. We also acknowledge that the small sample size may have impaired our ability to detect a significant difference in postprandial EAA concentrations between groups, the mean of which was ~27% greater in MB+WPC than MB+PPI. Using power calculations (G*Power V3.1), it was determined the present study with participants recruited was slightly underpowered (1-β=0.60) whereby 32 participants would be needed (16 per group) to adequately power (1-β=0.80) the study using total iAUC leucine availability. Future studies should seek to understand how the addition of low-dose protein from different sources to meals and snacks in the typical diet of middle-to-older aged adults influences muscle anabolism over an extended free-living period.

Consuming higher protein foods supresses appetite and hunger more so than lower protein foods [39, 69], thought to be underpinned by alterations in appetite regulating hormones [37, 38]. Whilst the satiating properties of dietary protein hold benefits for weight loss [40, 69] the potential reduction in energy/protein intake at subsequent meals [70] could have a detrimental effect on postprandial muscle anabolism and net muscle protein balance in middle-to-older aged adults, who may already experience age-related impairments in appetite regulation [15]. The satiating effects of protein ingestion may be dictated by the pattern of postprandial amino acid response [71, 72], particularly the rise in leucine concentrations [73]. In contrast, others have reported no difference in perceived appetite or regulatory hormonal concentrations in response to isolated protein sources that elicit divergent postprandial plasma TAA, EAA and leucine

concentrations in younger adults [74, 75]. In line with the present hypothesis, we observed similar transient suppression of perceived hunger and an increase in fullness and satiety over a 180 min postprandial period despite the divergent AA content of whey and pea protein supplements and postprandial leucine concentrations following MB+WPC and MB+PPI. Moreover, the postprandial rise in plasma glucose, plasma insulin, plasma GLP-1 and the decrease in plasma ghrelin did not differ between groups. The only between-group difference observed was a greater desire for fatty food intake in MB+WPC over MB+PPI. It appears that the amount of protein consumed (which was identical between groups in the present study) may be the more important determinant of postprandial appetite regulation [76]. Components of the whole food matrix of MB+WPC and MB+PPI may also have influenced appetite regulation, obviating any effect of subtle differences in postprandial aminoacidemia on these parameters. Unfortunately, we were unable to measure how the addition of low-dose whey and pea protein smoothies to the whole food mixed breakfast influenced indices of appetite regulation, which would have required a mixed breakfast-only condition devoid of supplemental protein. Similarly, providing a higher dose of supplemental protein than 0.13 g·kg $BM^{-1}$ to achieve a total meal intake of ~0.4 g·kg $BM^{1}$ to theoretically maximize MPS stimulation in older adults [4] may result in higher satiety and fullness. Addressing these unresolved questions will demonstrate the feasibility of supplementing low protein-containing meals and snacks with additional protein in middle-to-older aged adults.

In conclusion, the present study demonstrates that the addition of a small bolus of high-quality whey protein to a lower protein-containing whole food mixed breakfast (MB+WPC) elicited a greater postprandial plasma leucine response compared with the

addition of an isonitrogenous amount of lower quality pea protein (MB+PPI) in middle-to-older aged adults. However, postprandial plasma TAA and EAA response to MP+WPC and MB+PPI was equivalent. Indices of perceived appetite and regulatory hormone concentrations did not differ between MB+WPC and MB+PPI. Collectively, these findings suggest that MB+WPC is a pragmatic strategy to augment postprandial plasma leucinemia in middle-to-older aged adults, with potential benefits for muscle anabolic stimulation. This suggestion has important implications for longer-term muscle health in middle-to-older aged adults and requires further clarification, particularly in the context of ill-health and hospitalisation where access to, and consumption of, higher protein-containing foods is limited.

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## 4.7 Supplementary Material

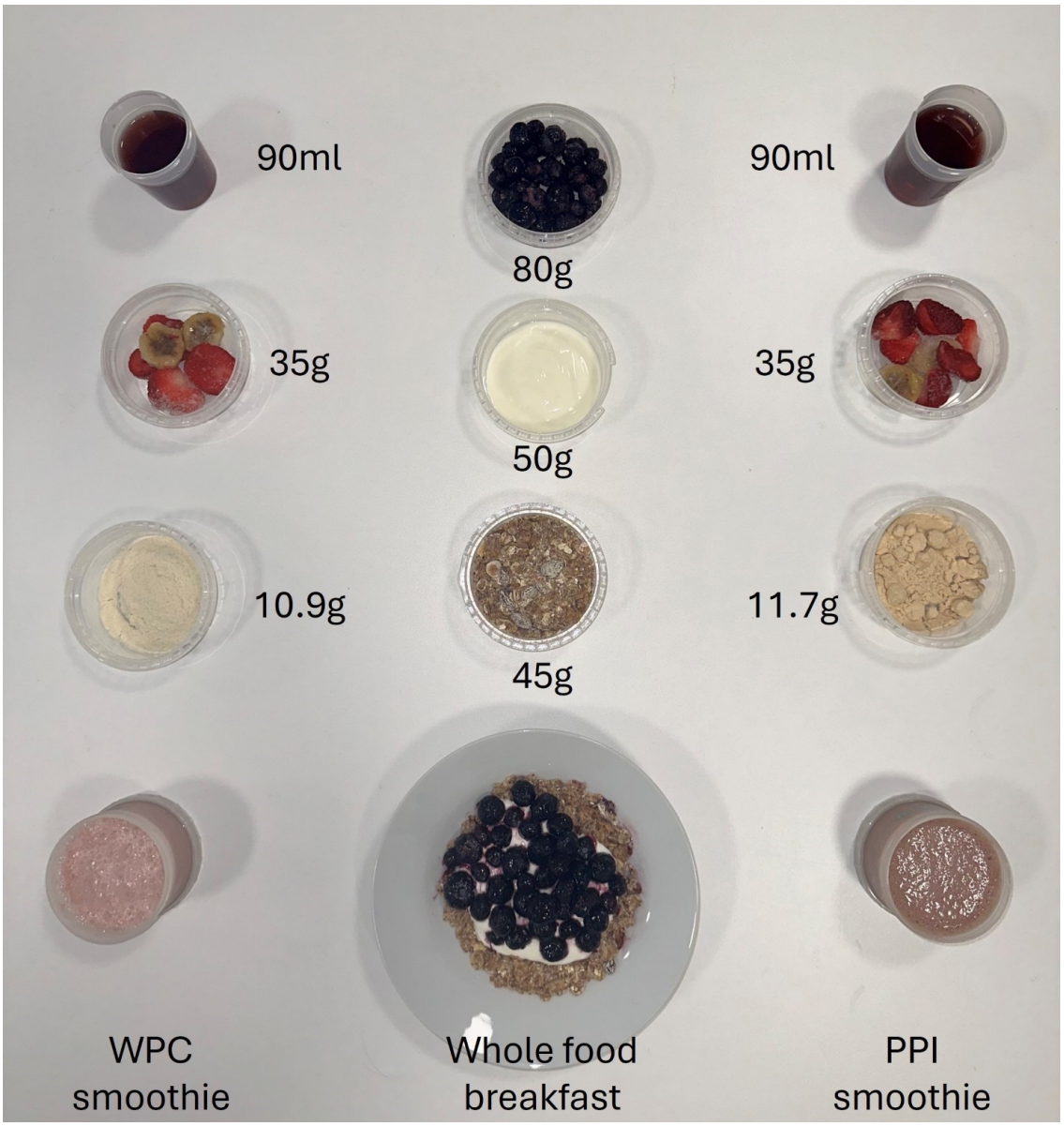
**Supplemental Table 4.1**. Relative amino acid contribution from supplemental protein

| Relative protein (0.13 g·kg BM$^{-1}$) | Whey Protein Concentrate | Pea Protein Isolate | *P* value |
|---|---|---|---|
| Aspartic Acid | 0.85 ± 0.12 | 0.87 ± 0.24 | 0.814 |
| Serine | 0.42 ± 0.06 | 0.40 ±0.11 | 0.692 |
| Glutamic acid | 1.33 ± 0.19 | 1.21 ± 0.33 | 0.217 |
| Glycine | 0.15 ± 0.02 | 0.30 ± 0.08 | **<0.001*** |
| Histidine | 0.13 ± 0.02 | 0.17 ± 0.05 | **0.017*** |
| Arginine | 0.18 ± 0.03 | 0.59 ± 0.14 | **<0.001*** |
| Threonine | 0.53 ±0.07 | 0.26 ± 0.07 | **<0.001*** |
| Alanine | 0.39 ± 0.05 | 0.31 ±0.08 | **0.009*** |
| Proline | 0.47 ± 0.06 | 0.33 ± 0.09 | **<0.001*** |
| Cystine | 0.19 ± 0.03 | 0.06 ±0.02 | **<0.001*** |
| Tyrosine | 0.19 ± 0.03 | 0.27 ±0.07 | **<0.001*** |
| Valine | 0.36 ±0.05 | 0.29 ± 0.07 | **0.011*** |
| Methionine | 0.19 ± 0.03 | 0.08 ±0.02 | **<0.001*** |
| Lysine | 0.75 ± 0.10 | 0.57 ±0.16 | **0.003** |
| Isoleucine | 0.42 ± 0.06 | 0.25 ±0.07 | **<0.001*** |
| Leucine | 0.75 ± 0.10 | 0.56 ±0.15 | **0.002*** |
| Phenylalanine | 0.23 ± 0.03 | 0.36 ±0.10 | **<0.001*** |
| Tryptophan | 0.13 ± 0.02 | N/A | - |

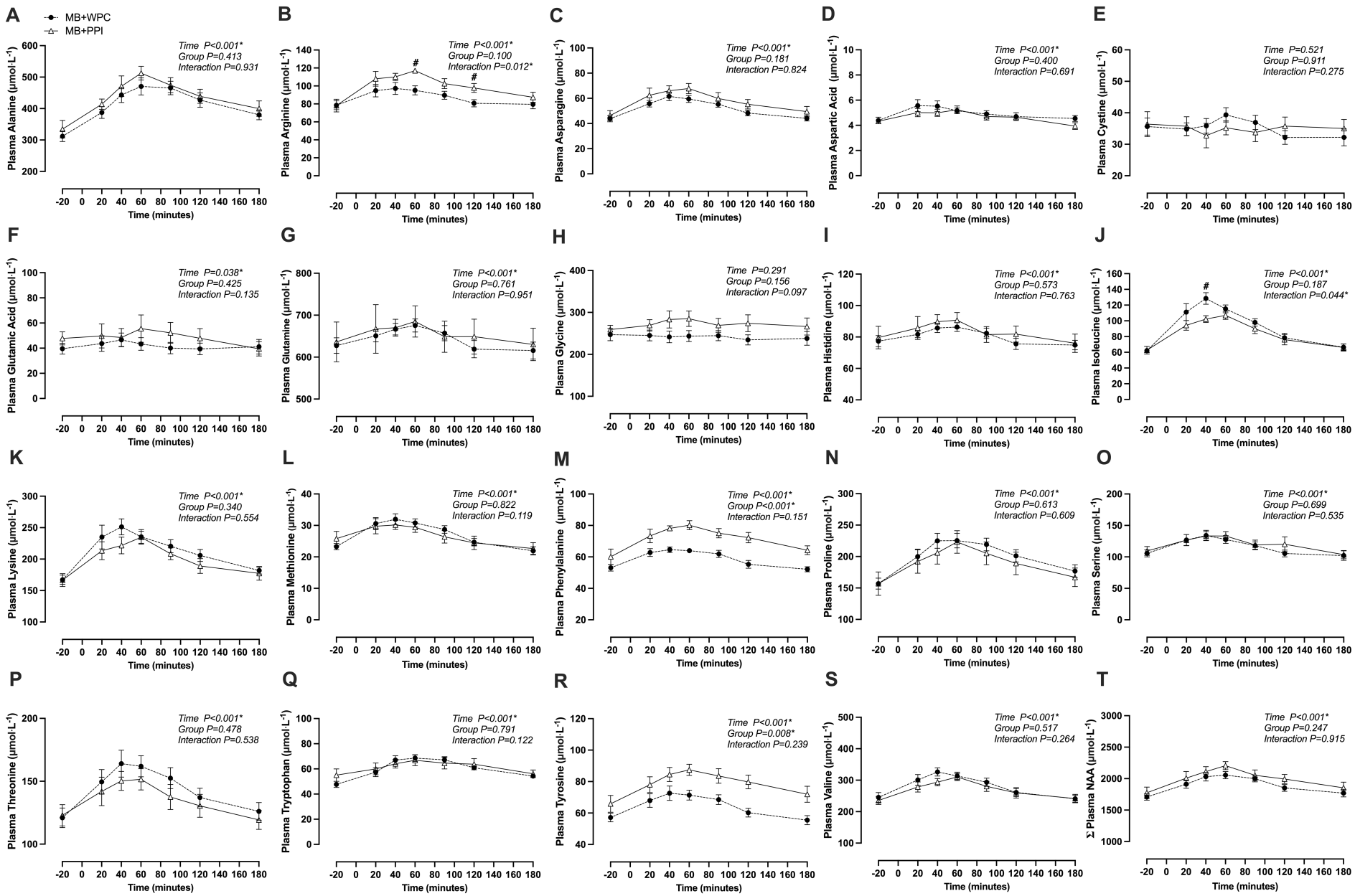
Amino acid dose (g) provided from supplemental whey protein concentrate (WPC) and pea protein isolate (PPI) as part of smoothie beverages providing 0.13 g·kg BM$^{-1}$ of protein. Data are presented as mean ± SD for low protein mixed breakfast with either WPC (n=13) or PPI (n=9). Independent samples t-test was used to statistically compare relative amino acid content between supplemental protein groups. * Denotes statistical significance between groups ($P$<0.05).


**Supplemental Figure 4.1.**

- Add 90mL of whole cranberry juice to blender ahead of other ingredients to minimise residual substance on walls of blender container. To the cranberry juice add 15g of pre chopped frozen banana and 20g of frozen strawberry to provide 35g of whole fruit. Add 0.13 g·kg $BM^{-1}$ of protein powder to the blender container.
- Given that the protein content of the powder differs (80% PPI, 86% for WPC), the absolute amount of powder to be weighed will vary. For a 72kg individual to achieve a serve of 0.13 g·kg $BM^{-1}$ the amount of PPI would be 11.7g and WPC would be 10.9g.
- Once the powder is added, secure the lid of the blender container and place in the machine and blitz at full speed for 10-15 seconds until smooth.
- Decant into a container for consumption or into a screw top secure container and refrigerate. If refrigerated, ensure to shake well before consumption and consume within 2 days once blended.

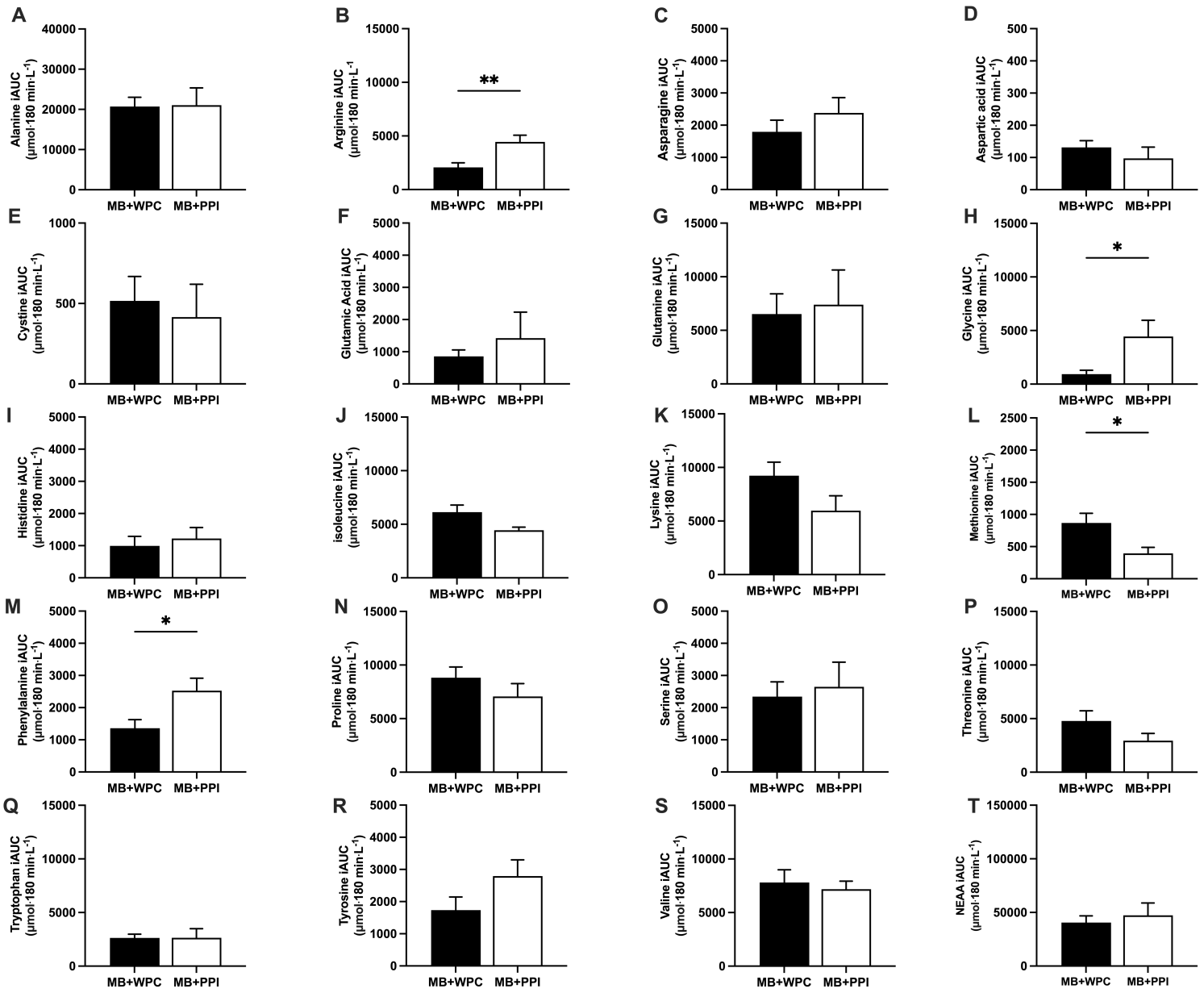



**Supplemental Figure 4.2.**
Image of blended whey protein concentrate (WPC; left) and pea protein isolate (PPI; Right) smoothies with their constituent elements. Both conditions consumed the same whole-breakfast test meal (centre) with variations in volume of food according to relative protein intake. Values here are for a 72kg individual where 0.13 g·kg $BM^{-1}$ is contributed from one of the supplements and the remaining 0.07 g·kg $BM^{-1}$ is from the whole food breakfast.


A
MB+WPC
MB+PPI
Plasma Alanine (μmol·L-1)
Time P<0.001*
Group P=0.413
Interaction P=0.931
B
Plasma Arginine (μmol·L-1)
Time P<0.001*
Group P=0.100
Interaction P=0.012*
C
Plasma Asparagine (μmol·L-1)
Time P<0.001*
Group P=0.181
Interaction P=0.824
D
Plasma Aspartic Acid (μmol·L-1)
Time P<0.001*
Group P=0.400
Interaction P=0.691
E
Plasma Cystine (μmol·L-1)
Time P=0.521
Group P=0.911
Interaction P=0.275
F
Plasma Glutamic Acid (μmol·L-1)
Time P=0.038*
Group P=0.425
Interaction P=0.135
G
Plasma Glutamine (μmol·L-1)
Time P<0.001*
Group P=0.761
Interaction P=0.951
H
Plasma Glycine (μmol·L-1)
Time P=0.291
Group P=0.156
Interaction P=0.097
I
Plasma Histidine (μmol·L-1)
Time P<0.001*
Group P=0.573
Interaction P=0.763
J
Plasma Isoleucine (μmol·L-1)
Time P<0.001*
Group P=0.187
Interaction P=0.044*
K
Plasma Lysine (μmol·L-1)
Time P<0.001*
Group P=0.340
Interaction P=0.554
L
Plasma Methionine (μmol·L-1)
Time P<0.001*
Group P=0.822
Interaction P=0.119
M
Plasma Phenylalanine (μmol·L-1)
Time P<0.001*
Group P<0.001*
Interaction P=0.151
N
Plasma Proline (μmol·L-1)
Time P<0.001*
Group P=0.613
Interaction P=0.609
O
Plasma Serine (μmol·L-1)
Time P<0.001*
Group P=0.699
Interaction P=0.535
P
Plasma Threonine (μmol·L-1)
Time P<0.001*
Group P=0.478
Interaction P=0.538
Q
Plasma Tryptophan (μmol·L-1)
Time P<0.001*
Group P=0.791
Interaction P=0.122
R
Plasma Tyrosine (μmol·L-1)
Time P<0.001*
Group P=0.008*
Interaction P=0.239
S
Plasma Valine (μmol·L-1)
Time P<0.001*
Group P=0.517
Interaction P=0.264
T
Σ Plasma NAA (μmol·L-1)
Time P<0.001*
Group P=0.247
Interaction P=0.915
Time (minutes)

**Supplemental Figure 4.3.** Time course for plasma amino acid appearance

Postprandial plasma concentrations for individual amino acids (**A-S**) and the sum of all non-essential amino acids (NEAA) (**T**) over 180 minutes postprandially following ingestion of MB+WPC or MB+PPI . Data are presented at mean and SEM with n=13 for mixed meal with whey protein concentrate (WPC) and n=9 for mixed breakfast with pea protein isolate (PPI). 2-way ANOVA tested for significant main effect difference for time, group and interaction within each figure with baseline value (t=-20) within condition set as the reference value for time differences. *Denotes significant main effect difference from baseline ($P$<0.05) and # for differences between groups ($P$<0.05) designated above each timepoint.


A
Alanine iAUC (µmol·180 min·L-1)
40000
30000
20000
10000
0
MB+WPC
MB+PPI
B
Arginine iAUC (µmol·180 min·L-1)
15000
10000
5000
0
**
MB+WPC
MB+PPI
C
Asparagine iAUC (µmol·180 min·L-1)
5000
4000
3000
2000
1000
0
MB+WPC
MB+PPI
D
Aspartic acid iAUC (µmol·180 min·L-1)
500
400
300
200
100
0
MB+WPC
MB+PPI
E
Cystine iAUC (µmol·180 min·L-1)
1000
500
0
MB+WPC
MB+PPI
F
Glutamic Acid iAUC (µmol·180 min·L-1)
5000
4000
3000
2000
1000
0
MB+WPC
MB+PPI
G
Glutamine iAUC (µmol·180 min·L-1)
15000
10000
5000
0
MB+WPC
MB+PPI
H
Glycine iAUC (µmol·180 min·L-1)
15000
10000
5000
0
*
MB+WPC
MB+PPI
I
Histidine iAUC (µmol·180 min·L-1)
5000
4000
3000
2000
1000
0
MB+WPC
MB+PPI
J
isoleucine iAUC (µmol·180 min·L-1)
15000
10000
5000
0
MB+WPC
MB+PPI
K
Lysine iAUC (µmol·180 min·L-1)
15000
10000
5000
0
MB+WPC
MB+PPI
L
Methionine iAUC (µmol·180 min·L-1)
2500
2000
1500
1000
500
0
*
MB+WPC
MB+PPI
M
Phenylalanine iAUC (µmol·180 min·L-1)
5000
4000
3000
2000
1000
0
*
MB+WPC
MB+PPI
N
Proline iAUC (µmol·180 min·L-1)
15000
10000
5000
0
MB+WPC
MB+PPI
O
Serine iAUC (µmol·180 min·L-1)
5000
4000
3000
2000
1000
0
MB+WPC
MB+PPI
P
Threonine iAUC (µmol·180 min·L-1)
15000
10000
5000
0
MB+WPC
MB+PPI
Q
Tryptophan iAUC (µmol·180 min·L-1)
15000
10000
5000
0
MB+WPC
MB+PPI
R
Tyrosine iAUC (µmol·180 min·L-1)
5000
4000
3000
2000
1000
0
MB+WPC
MB+PPI
S
Valine iAUC (µmol·180 min·L-1)
15000
10000
5000
0
MB+WPC
MB+PPI
T
NEAA iAUC (µmol·180 min·L-1)
150000
100000
50000
0
MB+WPC
MB+PPI

**Supplemental Figure 4.4.** Incremental area under the curve for plasma amino acids over 180min postprandial period.

Incremental area under the curve (iAUC) over 180 minutes for individual amino acids (**A-S**) and sum of non-essential amino acids (NEAA) (**T**) captured in the postprandial period following ingestion of mixed breakfast with whey protein concentrate (MB+WPC) or pea protein isolate (MB+PPI). Data are presented at mean and SEM with n=13 for MB+WPC and n=9 for MB+PPI. Independent samples t-test was used to compare between-group differences in iAUC. *Denotes significant difference between groups (*$P$<0.05, **P<0.01).

# Chapter 5 Resistance training increases myofibrillar protein synthesis rates in middle-to-older aged adults consuming a typical protein-containing diet, with no influence of protein quality

## 5.1 Abstract

**Introduction:** The protein quality of the diet may impact muscle maintenance in middle-aged to older adults. The impact of a diet providing lower quality plant- versus higher quality animal-derived protein on muscle protein synthesis rates is presently unclear. **Methods:** In a 10-day intervention, twenty-seven males and females (50-70 years) were randomised to consume a controlled diet containing 1 g·kg $BM^{-1}$·$day^{-1}$ of protein from either higher-quality animal-derived (HQ-D) or lower-quality plant-derived (LQ-D) sources, primarily through supplementation of whey protein concentrate or pea protein isolate with meals. Five unilateral knee extensor resistance exercise training (RET) sessions were completed over the 10-day intervention. Habitual diet and physical activity were measured and metabolic rate, body composition, lipid and renal profiles, whole-body nitrogen balance (WBNB), and muscle strength and architecture were also determined. Deuterated water ingestion and skeletal muscle biopsies were used to measure daily integrated myofibrillar protein synthesis (iMyoPS) rates in the trained and untrained leg. **Results:** Daily iMyoPS rates were significantly greater ($P$<0.001) in the trained compared to the untrained leg for HQ-D (1.44 ± 0.25 vs 1.28 ± 0.2 %·$day^{-1}$) and LQ-D (1.50 ± 0.17 vs 1.34 ± 0.21 %·$day^{-1}$) with no differences between HQ-D and LQ-D for either leg. There were no effect of training or dietary intervention on markers of intracellular anabolic signalling, muscle architecture, muscle strength, metabolic rate or lipid/renal profiles or WBNB. Serum Non-HDL-c was significantly different with time between groups ($P$=0.011) with no other changes in lipid profiles and kidney function due to the intervention and condition. **Conclusions:** The provision of lower quality plant-derived protein as opposed to higher quality animal-derived protein in a diet providing 1.0 g·kg $BM^{-1}$·$day^{-1}$ protein does not compromise daily myofibrillar protein synthesis rates in middle-aged to older

adults and has little impact on metabolic and renal health parameters. Resistance exercise training enhances rates of daily iMyoPS in middle-aged to older adults consuming 1 g·kg $BM^{-1}$·$day^{-1}$ of protein, irrespective of the quality of the proteins ingested.

## 5.2 Introduction

The loss of skeletal muscle mass and function with ageing, termed sarcopenia, reduces quality of life and increases the risk of falls, comorbidities, and mortality [1–3]. Sarcopenia is thought to commence from the 4$^{th}$ decade of life [4], although most research studies have primarily focused on those aged ≥60 years. Resistance exercise training (RET) and adequate dietary protein nutrition are well characterised muscle anabolic stimuli to combat sarcopenia progression [5, 6]. However, compared with younger adults, the ensuing muscle protein synthesis (MPS) in response to these anabolic stimuli is blunted in older adults [7–11]; and may underpin progressive muscle deterioration with advancing age. Evidence suggests the dysregulation of MPS may begin to manifest in middle age [12, 13]. Therefore, feasible dietary protein and RET strategies with the potential to elicit robust muscle anabolic responses in middle-to-older aged adults are of paramount importance.

To overcome postprandial age-related muscle anabolic resistance, it is suggested that older adults require a higher per-meal protein intake compared with younger adults [7, 14, 15]. In agreement with population-specific dietary protein recommendations [16, 17], higher total daily and per-meal protein intakes are associated with greater muscle mass retention in older adults [18, 19]. Further to this, the potency of RET as a stimulus for muscle mass maintenance with advancing age is underscored by enhanced utilisation of dietary protein-derived amino acids for MPS [20–22]. Importantly, RET participation is low amongst older adults owning to lack of knowledge and preference for expert supervision [23, 24]. Similarly, consuming a higher protein diet in older age is challenging for numerous reasons. These include i) a lack of awareness of protein requirements [25], ii) the satiating properties of protein-

dense foods [26] and iii) impairments in age-related appetite [27, 28] and olfactory and gustatory functions [29, 30].

Considering the challenges associated with combined high protein diets and RET participation, focus has shifted to the importance of protein source and quality to support muscle anabolism and maintenance in older age. The quality of protein-containing whole foods and supplements is primarily based on their capacity to support whole-body amino acid requirements [31, 32]. The importance of protein source/quality for muscle anabolism centres on the bioavailability of essential amino acids (EAAs); determined by the constituent EAA profile and digestibility of the protein source [33]. Animal-derived proteins have been considered higher quality than most plant-derived proteins in their capacity for MPS stimulation, due to greater EAA abundance and digestibility [34, 35]. The superiority of animal- over plant-derived protein intake for MPS stimulation in older adults has been demonstrated with isolated supplements [36–38] and a whole-food mixed meal [39], albeit under tightly controlled acute experimental conditions. Over longer-term 'free-living' conditions, Monteyne et al. (2020) demonstrated equivalent daily rates of integrated myofibrillar protein synthesis (iMyoPS) in healthy older adults consuming a high-protein intake diet (~1.8 g·kg body mass $[BM]^{-1}\cdot day^{-1}$) from omnivorous- or vegan-derived sources [40], whereas Oikawa et al. (2020) demonstrated superior iMyoPS rates with supplementation of whey protein over collagen protein in a high-protein diet (~1.6 g·kg $BM^{-1}\cdot day^{-1}$) for 6 days [38]. Currently, it is unclear whether the source and quality of dietary protein impact daily iMyoPS stimulation in middle-to-older aged adults consuming a diet providing a more generic amount of protein in the normal range of 1.0-1.1 g·kg $BM^{-1}\cdot day^{-1}$ [41, 42].

Therefore, the primary aim of the present study was to determine the impact of protein quality when consuming a diet providing 1.0 g·kg $BM^{-1}$·$day^{-1}$ of protein on daily myofibrillar protein synthesis rates in middle-aged to older adults, alone and in combination with RET. Specifically, participants consumed a 10-day controlled diet containing 1.0 g·kg $BM^{-1}$·$day^{-1}$ protein from mainly higher quality animal-derived (HQ-D) or lower quality (LQ-D) plant-derived sources, and performed unilateral leg RET every other day. HQ-D and LQ-D interventions were achieved largely through supplementing selected low-to-moderate protein-containing breakfast, lunch and snacks with whey protein concentrate and pea protein isolate, respectively. Exploratory secondary outcomes included measures of body composition, muscle morphology, strength, and markers of metabolic and renal health. We hypothesised that HQ-D would support greater rates of daily iMyoPS rates in middle-to-older aged individuals compared with LQ-D with no adverse or differential effects in secondary outcomes. Additionally, due to the sensitising effects of exercise for protein utilisation, iMyoPS was expected greatest following RET but any superiority of HQ-D would be less apparent.

## 5.3 Methods

### 5.3.1 *Participants and Ethical Approval*

Twenty-seven middle-to-older aged volunteers volunteered to participate in the study. Initial recruitment was conducted through local advertisements and participants were screened for eligibility via email or telephone. Exclusion criteria included i) aged <50 or >70 y, ii) recent engagement in structured exercise training, iii) metabolic conditions, respiratory disease, or chronic illness, iv) habitual smoking, v) allergies or

intolerances to study materials and supplements, vi) use of medications known to affect muscle protein metabolism and vii) following an exclusively animal- or plant-based diet. Ethical approval was acquired from Surrey Boarders Research Ethics Committee (IRAS: 291370, REC references: 21/LO/0401). This trial was registered with ClinicalTrials.gov (NCT05574205). All procedures were carried out in line with the Deceleration of Helsinki (7$^{th}$ Ed). Participants were informed of the study purpose, experimental procedures and potential risks associated with participating before they provided written informed consent.

### 5.3.2 *Study design*

Upon study induction, participants were randomised, according to a predefined sex-stratified participant code, to a 10-day controlled dietary intervention providing ~1 g·kg BM$^{-1}$·day$^{-1}$ of protein predominantly from higher-quality animal-derived (HQ-D; n=14) or lower-quality plant-derived (LQ-D; n=13) sources, which was achieved largely through supplementing meals/snacks with whey or pea protein concentrates or isolates, respectively. Throughout the intervention, supervised unilateral leg extension RET was undertaken every other day. See the CONSORT diagram in **Figure 5.1** for participant numbers for enrolment, allocation and analysis and **Figure 5.2** for study schematic for the dietary and exercise intervention.

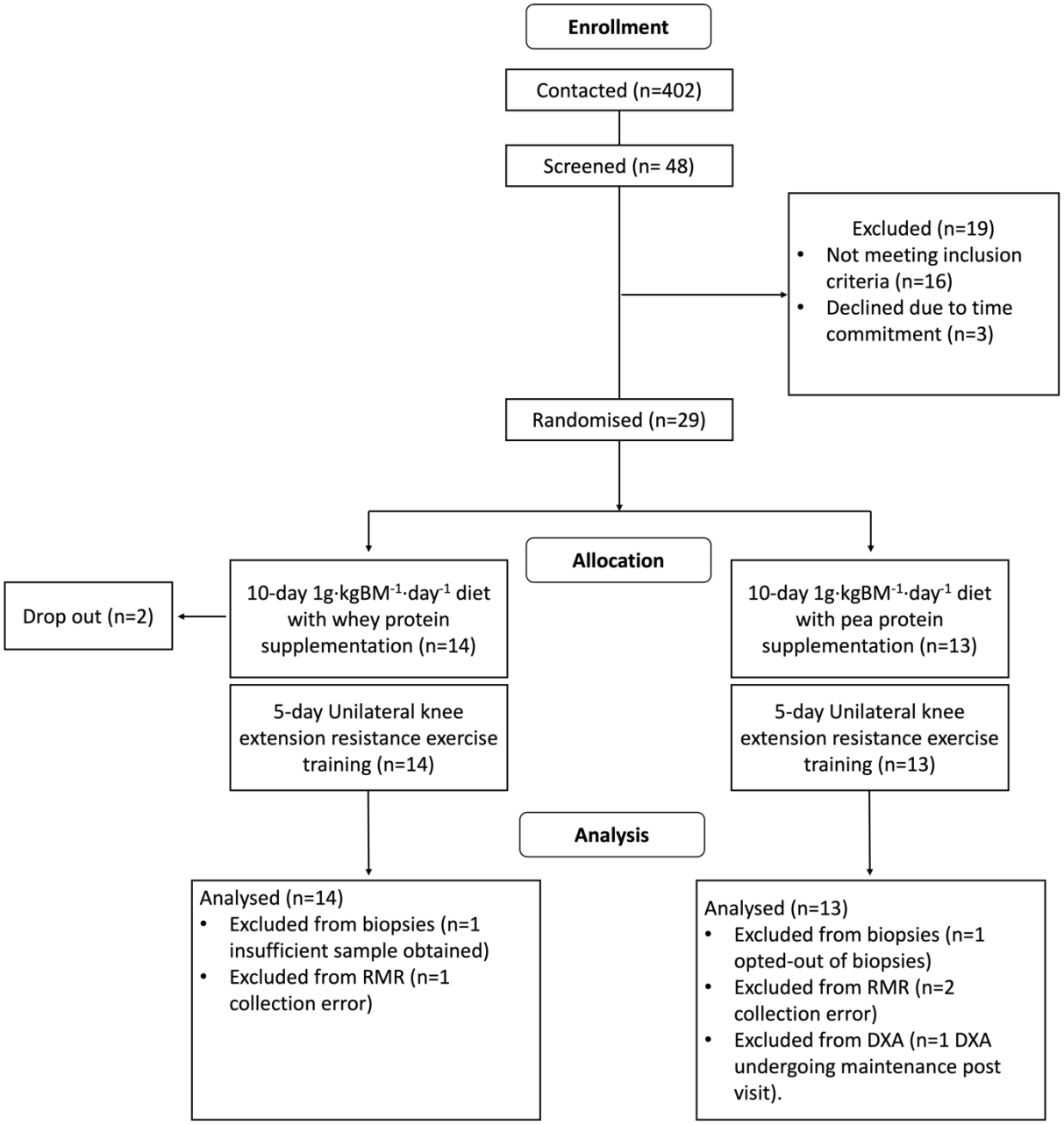



**Figure 5.1.** CONSORT flow diagram

Consolidated standards of reporting trials (CONSORT) diagram showing the number of participants enrolled and randomised to each condition and breakdown of participants analysed for each outcome.

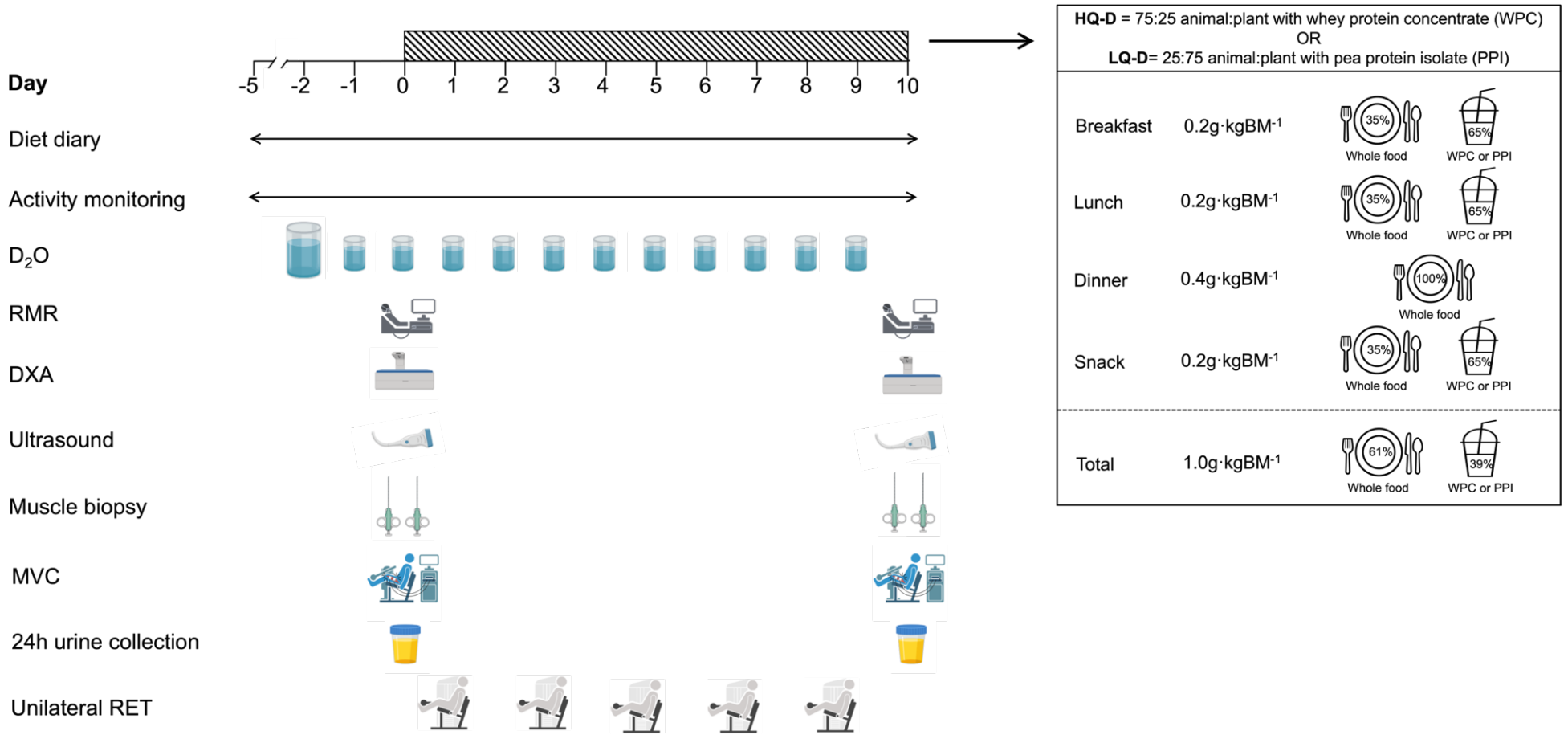



**Figure 5.2.** Schematic overview of the study timeline.
$D_2O$: deuterated water; RMR: resting metabolic rate; DXA: dual-energy x-ray absorptiometry; MVC: maximal voluntary contraction; RET: resistance exercise training. Overview of the study design with 5-day habitual measurement phase and 10-day intervention phase of controlled diet and RET.

### 5.3.3 *Habitual characteristics (Day -5)*

Following study induction, participants were provided with a wrist worn accelerometer (GENEActiv, ActivInsights, Cambridgeshire, UK) set to record activity at 60Hz over 15 days to determine physical activity prior-to and throughout the 10-day intervention. Participants were also instructed to fill out a detailed weighed food diary for a minimum of 3 days (including the day before the intervention and one weekend day) to evaluate habitual dietary intake. Anthropometrics were measured for body mass (OHAUS, Champ II, Switzerland) and height (SECA, Hamburg, Germany), whilst body fat percentage was determined using bioelectrical impedance analysis (BIA; BC-420MA, TANITA, Japan). Preliminary anthropometrics were used to calculate dietary protein intake at 1.0 $g \cdot kg^{-1} day^{-1}$ for the subsequent 10-day intervention phase and estimated daily energy requirements according to the Harris Benedict equation [43] and multiplied by a physical activity correction factor rated for each participant.

### *5.3.4 Preliminary assessments (Day -2)*

Knee Extensor strength: After 3-days of dietary intake and physical activity monitoring, participants reported to the laboratory for an assessment of unilateral maximal knee extensor strength on a knee extension machine (Matrix Fitness, Wisconsin, USA). For all participants, the leg to be trained during the 10-day diet intervention was randomised and counterbalanced between HQ-D and LQ-D groups according to leg dominance. Participants were instructed on the correct technique to encourage a full range of motion to promote full concentric to eccentric contraction of the knee extensors. Participants initially carried out a warm-up set of 10 repetitions at a low load (14-29 kg). The estimation of 1 repetition maximum (1RM) was determined by progressively increasing the load by 7-9 kg and asking the participant to complete

3 full extensions with 2 min between each weight increase. Estimated 1RM was determined when the load where full extension was not achieved, or 3 repetitions could not be completed [44]. The machine set-up for each participant was documented for replication in later training visits.

Deuterium oxide tracer protocol: Deuterium oxide ($D_2O$) tracer (70% $^2H_2O$, Cambridge Isotope Laboratories, Massachusetts, USA) was consumed daily over a 12-day period to achieve $^2H$ enrichment of the body water pool at ~0.6 atom percent excess (APE; [45]) for the calculation of free-living daily iMyoPS rates (described below). The use of $D_2O$ has been validated as a measure of free living daily iMyoPS and for detecting between leg differences with short-term unilateral RET [45, 46]. Following 1RM strength assessment, participants provided a baseline (i.e., unenriched) saliva sample, and then consumed a 400 mL $D_2O$ loading dose provided in 8x50 mL boluses, with the first three doses given at least 60 min apart to avoid mitigating potential side effects (i.e., nausea, dizziness, blurred vision), which can occur with sudden and drastic incorporation of $D_2O$ into the body water pool. Thereafter, participants were instructed to consume the remaining 5x50mL $D_2O$ doses at home, spaced 60 min apart. Participants were asked to document the time $D_2O$ consumption was completed and any side effects. For the next 11 days (ending on Day 10 of the intervention), participants provided a daily saliva sample (~10 mL) upon waking before consuming a 50 mL top-up dose of $D_2O$ (until day 9). A slight upward drift in $^2H$ enrichment was expected given the absolute $D_2O$ dose that was provided, as previously observed [45]. $D_2O$ consumption was well tolerated with no side effects reported beyond minor transient dizziness.

### *5.3.5 Pre- and post-intervention measures (Day 0 and Day 10)*

*Resting Metabolic Rate:* Two days following the 1RM strength assessment, participants reported to the laboratory after a >10-h overnight fast and abstaining from strenuous exercise for the prior 24 h to complete a series of measures and begin the 10-day diet intervention. All measures described below were repeated the day immediately following the 10-day diet intervention. Participants first had resting energy expenditure (REE) and respiratory exchange ratio (RER) measured by laying in a supine position whilst continuously wearing a Hans-Rudolf face mask (Cranlea Birmingham, UK) over a 20-minute period (following acclimation) to collect breath-by-breath measurements through a Vyntus Jaeger CPX metabolic cart (Vyarie Medical, Illinois, USA), as previously described [47].

*Muscle architecture:* Ultrasonography of both legs was undertaken to determine structural alterations of the m.*Vastus Lateralis*. Laying supine, femur length was measured by locating the lateral epicondyle of the knee and protrusion from the greater trochanter, and the midpoint marked. The ultrasound probe (LOGIQ; M-18, 5cm) was moved around this midpoint to find the borders of the m.*Vastus Lateralis.* Once the borders were located, the probe was placed parallel to the leg and manipulated, and depth adjusted to ensure clear aponeurosis and fascicles of the m.*Vastus Lateralis* were visible. A series of images were captured on LOGIQ S8 (General Electric, Milwaukee County, US) ultrasound at a scan depth of 4-5cm and frequency of 3-13MHz and saved for each leg when there was minimal pressure from the probe onto the leg. Images with clear aponeurosis and fascicles were exported for analysis of muscle thickness and pennation angle. All representative images were analysed on semi-automated analysis on Fiji ImageJ (V1.54, US National Institutes of Health, Bethesda, UK) using Simple Muscle Architecture (SMA) plugin (Ver 2.21) [48].

*Dual X-ray absorptiometry (DXA) scanning:* DXA scanning (Discovery A; Hologic, Bedford, MA), was used to determine whole-body and regional fat mass and fat-free mass. Participants were laid in the supine position with feet taped to point toward the mid-sagittal plane to maximise the length of the femur head on both legs. Prior to DXA-derived body composition analysis, body mass and total body water were measured through BIA, using a 4-compartment model to account for any body mass difference due to total body water pre- and post-intervention.

*Skeletal muscle biopsies:* Percutaneous skeletal muscle biopsies were taken from the *m.Vastus Lateralis* of both legs to allow for within, and between leg comparisons in daily iMyoPS rates with 10-day diet interventions under conditions of rest and exercise. Skeletal muscle biopsy procedures were performed under local anaesthetic (1% Lidocaine Hydrochloride) using the Bergström technique adapted for suction [49]. The yield of skeletal muscle tissue for iMyoPS fractional synthesis rate (~50-80 mg) was freed of visible connective tissue and fat, placed into a cryovial and immediately snap frozen in liquid nitrogen. Snap frozen tissue was stored at -80°C until later analysis (detailed below).

*Neural activation and dynamometry: The s*trength of both legs was assessed by isometric maximal voluntary contraction using Biodex dynamometer (Biodex Medical Systems, Shirley, New York, USA). Participants were seated at 85˚ with chair height adjusted until the lateral epicondyle of the knee was in line with the pivot point of the leg attachment. The positioning of the Biodex was recorded for each participant and replicated for post-intervention measures. To carry out the interpolated twitch, disposable electrodes were placed on and adjacent to the superficial femoral nerve and voltage set to evoke a small involuntary contraction upon short-term stimulation. Participants carried out a short warm up followed by three short maximal voluntary

contractions (MVCs) each >45 seconds apart to allow for phosphocreatine resynthesis and minimise acute fatigue. During and immediately after each MVC, an electrical impulse was delivered to ascertain the superimposed and potentiated twitch to find voluntary activation, as previously described [50]. All dynamometry assessments were performed following muscle biopsy procedures so as not to interfere with acute intramuscular signalling events.

*24 h urine collection:* Participants were provided with two urine containers (3L capacity) to collect all passes in their own home over Day 0-1 and Day 9-10 of the intervention. Urine collection at these timepoints was used to estimate whole-body nitrogen balance (WBNB) change over the course of the 10-day diet interventions. The time between the first and last pass of urine collection over the 24 h period was recorded by the participant and the total urine volume measured in the laboratory. Two 15 mL urine samples were aliquoted, treated with 2M sodium azide (75 μL per 2 mL of sample) and centrifuged at 4000*g* for 20 min. From this, aliquots of 2 mL were frozen at -80°C to minimise bacterial growth ready for later analysis of urea and creatinine to calculate WBNB (described below).

### *5.3.6 Dietary Intervention*

HQ-D and LQ-D were designed to provide ~1.0 g·kg $BM^{-1}$·$day^{-1}$ of protein in line with the average dietary protein intakes of middle-to-older aged adults [42, 51], which are beyond the RDA of 0.8 g·kg $BM^{-1}$·$day^{-1}$ but below the optimum thought to be required to maximise muscle health these individuals [17]. The distribution of dietary protein in the diets was 0.2 g·kg $BM^{-1}$ at breakfast, lunch, and mid-afternoon snack, and 0.4 g·kg $BM^{-1}$ at dinner. The total daily protein intake and distribution were designed to mimic the typical skewed pattern, whereby a larger proportion of total daily

protein intake is consumed in the evening, particularly among older adults [42]. At breakfast, lunch and mid-afternoon snack, ~0.13 g·kg $BM^{-1}$ (65%) of the ingested protein was provided in the form of a smoothie containing supplemental whey protein concentrate (WPC; Volactive® UltraWhey Sugar Free, Volac, Hertfordshire, UK) for HQ-D or pea protein isolate (PPI; MyProtein™, The Hut Group, Manchester, UK) for LQ-D. Both supplemental sources are relatively high quality considering the refined protein content and higher digestibility due to their powdered form. However, PPI icreases the proportion of plant provision which contained fewer EAAs compared with animal derived WPC when provided as a relative daily dose **Table 5.3**. Participants were shown typical food items which would be consumed as part of the 10-day dietary intervention, where they had the opportunity to outline their food preferences for the duration of the intervention. The aim of allowing some flexibility in elements of the dietary control meant that the adherence would be maximized whilst allowing for the inclusion of representative and diverse dietary preferences. The specific timing of meal/snack consumption was at the discretion of the participants to minimise attrition. Timings of food consumption were documented by participants in a written dietary log, where participants could also disclose is any additional foods were consumed or if any of the provided foods were left. To enforce free-living conditions, drinks/liquids were not restricted during the diet intervention (except for alcohol) to minimise adverse and unrepresentative effects on RET-induced iMyoPS. Caffeinated warm beverages were permitted if they were part of the habitual diet to maintain adherence. To control for protein, participants were provided with a measured amount of milk to add to these drinks to consume each day which was factored into daily protein provision.

The HQ-D and LQ-D interventions differed in the proportion of food from animal and plant sources, respectively, for the main evening meal (e.g., pasta with meatballs or vegan meatballs for HQ-D and LQ-D, respectively). Over the four daily meals the target ratio of animal- to plant-based protein was 75:25 for HQ-D and 25:75 for LQ-D. Detailed information of HQ-D and LQ-D are provided in **Table 5.2** and example meal plans for both groups provided in **Supplementary Materials 1.** Ingredients for all meals were individually weighed by a member of the research team, according to individual protein requirements and predicted TEE. The diets were designed to allow participants to prepare and heat meals in their own home, without being overly arduous, to maximise adherence to the intervention. For HQ-D and LQ-D, participants were given two optional meal plans for each meal/snack, which were rotated daily (i.e., both meal plans were consumed on 5 occasions during the 10-day intervention). Meals and smoothies were all labelled to ensure consumption on the correct days and were given at each RET visit to provide 2 days worth of food (containing 8 meals and 6 smoothies).

### *5.3.7 Resistance Exercise Training*

Participants underwent supervised unilateral knee extension RET every other day over the course of the 10-day dietary intervention. The leg to be trained during the 5 RET sessions was randomly selected and counterbalanced for leg dominance within groups. The knee extension machine set up at each visit was identical to that used during familiarisation on the same machine, on Day -2. For each RET session, participants reported to the laboratory after having consumed their breakfast and supplemental smoothie at home at least 2-h prior. The time of each RET session was kept similar for each participant, with the final RET session performed at least 18-h

before the final biopsy on the morning following completion of the intervention. For RET sessions, participants completed 10-12 repetitions at ~75% of their pre-determined 1RM for 8 sets on each visit. A member of the research team supervised RET sessions to encourage full range-of-motion in knee extension/flexion and controlled eccentric and concentric movement. Collectively, participants completed 415 ± 20 extensions over the 10-day intervention. Following the completion of each set, participants were asked to report a rating of perceived exertion (RPE) using the CR-10 Borg scale [52] with 1 indicating 'no exertion' and 10 indicating 'maximal exertion'. Additional information on pain and comfort with each RET set was monitored to minimise injury and ensure an appropriate muscle anabolic stimulus was delivered. Participants were given 2-min rest between the each set to allow for ATP resynthesis and minimise acute post-exercise anabolic blunting that may be apparent with very short rest periods [53].

### *5.3.8 Analysis*

*Habitual diet:* All habitual dietary intake was recorded in weighted food diaries by participants and analysed using Nutritics (V6.0, Dublin, Ireland). Food diary entries were spot-checked by an independent researcher for accuracy. Data for all macronutrients and micronutrients was exported into Microsoft Excel (V.16.8 Microsoft®, USA) and protein quality/contribution from animal or plant sources was manually delineated by a member of the research team by classifying each food according to if the constituent elements were primarily animal (higher quality) or plant (lower quality).

*Physical activity monitoring:* Data collected over the habitual and intervention period were extracted using ActivInsights software and exported into a Macro file (V.16.8 Microsoft®, USA) to determine physical activity levels during the habitual pre-intervention phase and the 10-day intervention phase.

*Resting Metabolic Rate:* All data for RER and REE was smoothed to 5-second increments and exported as XLS files. Any data deviating by the mean by more than two standard deviations was excluded from analysis.

*Dual x-ray absorptiometry scanning:* DXA outputs for whole-body and *lower-limb* segmental analysis were manually exported into *Microsoft* Excel (V.16.8 Microsoft®, USA).

*Saliva enrichment:* Deuterium ($^2H$) enrichment in the body water pool was determined by analysis of daily saliva samples on a Delta V Advantage Isotope Ratio Mass Spectrometer (IR-MS) (Thermo Fisher, Massachusetts, USA). Saliva samples from days -2, 0, 2, 4, 6, 8 and 10 of the intervention were prepared for $^2H$ enrichment analysis. Baseline samples (day -2), taken before $D_2O$ loading were prepared neat, without any dilution. The remaining samples, containing deuterium oxide, were diluted 70x in miliQ water to account for the 70% deuterium oxide tracer (Cambridge Isotope Laboratories, USA). Standards ranging from 147.5 ppm to 304.0 ppm of deuterium were used on each run in addition to blanks to find the standard curve. Samples were run in duplicate for each timepoint after filling each tube with 98% helium gas. Saliva $^2H$ enrichment data are presented as APE and expressed relative to background enrichment.

*Myofibrillar Protein Extraction:* Snap frozen skeletal muscle samples were weighed (Mettler Toledo, XS205, DualRange, USA) and kept in Eppendorfs on liquid nitrogen. An isolation buffer (2.29 g sucrose, 0.373 g Tris, 0.372 g EDTA in 80 mL miliQ water) was prepared. Phosphatase inhibitor cocktail (PhosSTOP, Roche, Germany) and protease inhibitor (cOmplete mini, Roche, Germany) were added for every 10 mL of isolation buffer to prevent unrepresentative phosphorylation changes. Isolation buffer was added in quantities equating to 10x the weight of each muscle sample. Samples were manually homogenised in the detergent-free isolation buffer to minimise destruction to the mitochondrial membrane, preventing contamination of other fractions. Samples were kept on ice and transferred to a centrifuge (Universal 320R, Hettich, Germany) cooled to 4°C at 700*g* for 10 min. The resulting supernatant comprised of sarcoplasmic proteins was aliquoted into separate Eppendorfs for later immunoblot analysis (described below). To separate out the insoluble (extracellular matrix components) and soluble proteins (myofibrillar) from the resulting supernatant, the pellet was washed to remove inhibitors and 1 mL 0.3M NaOH was added and heated to 50°C to cause myofibrillar proteins to unravel and solubilise. Samples were spun at 11,000*g* for 5 min and the resulting soluble myofibrillar supernatant was transferred to glass tubes. Another 1mL of NaOH was added to the pellet and centrifuged 1000*g* to suspend any residual soluble proteins (i.e., myofibrillar) which were added to the same glass tube. From each glass tube, 50 μL of sample was removed and stored for protein assay to determine protein content of each myofibrillar fraction prior to amino acid derivatisation. To denature the myofibrillar proteins, 1mL of 1M perchloric acid (PCA; VWR Avantor, USA) was added to each glass tube to form a pellet. To minimise later contamination with the mass spectrometer, the pellet was cleaned with 1mL of 70% ethanol and centrifuged (Rotanta 460R, Hettich, Germany)

at 2000 RPM for 5 minutes with the supernatant discarded, and wash step repeated to leave a clean pellet. To each tube, 2mL of 6M hydrochloric acid (HCl) was then added. Each glass tube was closed with a screw top lid under a nitrogen gas flow to minimize oxidation of samples and vortexed before being placed on a heating block for 16 h at 110°C. Samples were then dried by heating with open lids at 110°C (QBD4, Grant) under a constant nitrogen stream to leave the resulting amino acids.

*Myofibrillar bound alanine determination:* Derivatisation of amino acids and deuterium bound alanine were carried out at Maastricht University, Stable Isotope Research Centre (SIRC), Netherlands. Prior to insertion on the mass spectrometer, samples were purified by placing on columns filled with a negatively charged ion exchange resin. Subsequently, 2M ammonia was eluted through the column to invert the ionic state of the resin and allow purified amino acids to be collected in glass tubes. Following purification, samples were prepared for insertion on the gas chromatographer (Trace 1310, Thermo Fisher, USA) to separate out amino acids and fed into the isotope ratio mass spectrometer with pyrolysis oven (IR-P-MS, 253 plus, Thermo Fisher, USA). A known standard of the amino acid of interest, alanine, was inserted into the column and ionised creating a reference peak. The ratio of $H_2$ and deuterium ($^2H$) (pulled through at different detectors from the magnetic pull) at the known peak on the spectra was evaluated for each myofibrillar sample to determine the amount of deuterium-bound alanine in the trained and untrained leg, pre and post-intervention.

*Myofibrillar fractional synthesis rate:* The fractional synthesis rate (FSR) of myofibrillar protein was determined using the standard precursor-product method, as we have described previously [45]. Total body water (saliva) $^2H$ enrichment was used as a surrogate for plasma alanine labelling (precursor) with a correction factor of 3.7

applied as an approximation of the number of hydrogens on alanine which have been substituted as deuterium [46]. The within-leg change in myofibrillar bound $^{2}H$ enrichment of alanine between Day 0 and 10 of the intervention was used to calculate myofibrillar FSR using the following equation:

$$FSR\ (\%\ day) = \left(\frac{\mathrm{Em2} - \mathrm{Em1}}{E\mathrm{precursor} \times\ \Delta\mathrm{t}}\right) \mathrm{x}\ 100$$

Where $E_{m1}$ and $E_{m2}$ is the muscle protein-bound deuterated alanine enrichments on days 0 and 10, respectively; $E_{precursor}$ is mean body water enrichment with 3.7 multiplication factor over the incorporation period (day -2 to day 9); and *t* represents the time between muscle biopsy sampling, which was first determined in hours to account for subtle differences in the deuterium incorporation period. FSR in hours was multiplied by 24 to determine daily iMyoPS FSR (%·day$^{-1}$).

*Immunoblotting:* Western blot analysis was performed on the sarcoplasmic fraction obtained during myofibrillar protein extraction (described above). Samples were boiled and concentrated to 2μg/μL, in accordance with DC protein assay concentrations, in 4x Laemmli sample buffer equally loaded (15μL per lane) onto 4-15% gradient precast gels (Criterion™ TGX™, Bio-Rad, California, USA). Gels were run at 200 V for 1 h in electrophoresis tanks (Bio-Rad, California, USA). Gels were transferred onto polyvinylidene difluoride (PVDF; Amersham™ Cytiva, UK) membranes at 100 V for 1 h. Membranes were blocked in 5% milk, washed in tris-buffered saline with 0.1% Tween® (TBST) before being incubated overnight (4°C) with appropriate primary antibodies. The following primary antibodies were used; total mechanistic target of rapamycin (mTOR; CST2983, 1:1000 in 5% BSA TBST), phospho (p)-mTOR$^{Ser2448}$ (CST2971, 1:1000 in 5% BSA TBST), total protein kinase B

(Akt; CST9272, 1:1000 in 5% BSA TBST), p-Akt$^{Ser473}$ (CST4060, 1:1000 in 5% BSA TBST), total eukaryotic elongation factor 2 (eEF2; CST2332, 1:1000 in 5% BSA TBST), and p-eEF2$^{Thr56}$ (CST2331, 1:1000 in 5% BSA TBST), all purchased from Cell Signaling Technology (Hitchin, United Kingdom). The following horseradish peroxidase (HRP)-linked anti-rabbit (CST7074) IgG dilutions were used; 1:5000 in 3% BSA TBST dilution for total and p-mTOR$^{Ser2448}$, and 1:10000 in TBST dilution for total Akt, p-Akt$^{Ser473}$, total eEF2, and p-eEF2$^{Thr56}$. Imaging was undertaken using a G:Box Chemi-XR5 (Syngene, Cambridge, United Kingdom), and bands were quantified using Fiji ImageJ. Values were corrected to a gel control in the first instance before being corrected to the loading control (Ponceau S). The phosphorylation of each target protein, as a proxy of their activation, was expressed relative to its corresponding total protein signal, with all changes presented relative to the respective pre-intervention leg.

*Urinary analysis:* Urea and creatinine concentrations were diluted 30-fold and analysed on the semi-automated Daytona RX+ using the Creatinine and Urea kits (Randox Laboratories, UK). Samples were run in duplicate, and values provided as mmol/L for urea and nmol/L for creatine. To estimate nitrogen loss, derived concentrations of urinary creatinine and urea were expressed relative to their respective molecular weights, converted to g/L and multiplied by the measured volume of urine excreted. To this, a multiplication factor of 0.37 and 0.46 was applied to creatinine and urea values, respectively, to account for the proportion of the molar mass which is comprised of nitrogen. After combining the absolute urea and creatinine values, a further 10% was added to account for nitrogen losses by other means [54]. The amount of protein consumed in the diet was divided by 6.25 as a crude estimate of the nitrogen content of protein. Overall WBNB was determined as the difference

between the calculated nitrogen intake and excretion [55] between Day 0 and Day 10 of the intervention.

*Statistical analysis:* Data analysis was performed on Prism GraphPad (V11, LA Jolla, California, USA) and checked for normality using Shapiro-Wilk on SPSS (Ver 27**,** IBM, Chicago, USA). Between group baseline characteristics were compared using independent samples t-test. Characterisation data was compared between HQ-D and LQ-D and where appropriate within groups for pre-to-post intervention changes using a 2-way repeated measured ANOVA (time x group). For all time-based comparisons between groups (i.e., trained vs. untrained for iMyoPS, intracellular signalling, leg strength, architecture, and body composition) a 2-way ANOVA was used. Statistical significance was set *a priori* as $P \leq 0.05$. Any statistically significant main interaction effects were inspected using Bonferroni post-hoc analysis to identify where statistical significance lay. All values are presented as mean ± standard deviation (SD) unless otherwise stated.

## 5.4 Results

### *5.4.1 Anthropometrics and physical activity*

Anthropometrics and physical activity characteristics are presented in **Table 5.1**. The mean age of participants was significantly lower in LQ-D compared with HQ-D ($P$=0.035). Two-way ANOVA revealed there was no interaction (group x time) for DXA-derived whole-body fat mass, whole-body fat-free mass (FFM) or region-specific FFM (all $P$>0.05). Similarly, interaction effects (time x group) were absent for RER, REE and time spent in non-sedentary time and completing moderate and vigorous physical activity (MVPA). Irrespective of the intervention, there were main group differences for whole body FFM, trained and untrained leg FFM and REE (all $P$>0.05).

**Table 5.1.** Participant Characteristics from pre and post-intervention.

| | **HQ-D** | | **LQ-D** | | ***P* Value** | | |
|---|---|---|---|---|---|---|---|
| | Pre intervention | Post intervention | Pre intervention | Post intervention | *Interaction* | *Time* | *Group* |
| Age (y) | 63.9 ± 4.4 | - | 58.9 ± 7.1* | - | - | - | **0.035*** |
| Height (m) | 1.68 ± 0.1 | - | 1.70 ± 0.1 | - | - | - | 0.645 |
| Body Mass (kg) | 74.4 ± 17.1 | 73.4 ± 17.3 | 73.7 ± 17.8 | 72.8 ± 18.1 | 0.991 | 0.843 | 0.893 |
| BMI (kg/m$^2$) | 24.2 ± 2.2 | 23.9 ± 2.2 | 24.9 ± 3.6 | 24.1 ± 4.0 | 0.767 | 0.515 | 0.564 |
| Body fat (%) | 31.3 ± 8.3 | 31.8 ± 8.4 | 28.7 ± 8.4 | 29.5 ± 8.8 | 0.948 | 0.779 | 0.293 |
| Whole body FFM (kg) | 44.3 ± 9.5 | 43.4 ± 9.3 | 50.7 ± 13.6 | 50.5 ± 14.1 | 0.917 | 0.869 | **0.048*** |
| Trained leg FFM (kg) | 6.93 ± 1.66 | 6.96 ± 1.62 | 8.47 ± 2.68 | 8.46 ± 2.80 | 0.975 | 0.988 | **0.020*** |
| Untrained leg FFM (kg) | 6.99 ± 1.62 | 6.88 ± 1.60 | 8.31 ± 2.60 | 8.27 ± 2.68 | 0.955 | 0.903 | **0.032*** |
| RER | 0.87 ± 0.06 | 0.88 ± 0.06 | 0.87 ± 0.06 | 0.89 ± 0.07 | 0.779 | 0.401 | 0.779 |
| REE (Kcal) | 1446 ± 331 | 1382 ± 351 | 1627 ± 579 | 1596 ± 607 | 0.330 | 0.453 | **0.014*** |
| Estimated TEE (Kcal) | 2161 ± 296 | - | 2189 ± 465 | - | - | - | 0.850 |
| MVPA (%) | 17.8 ± 6.3 | 17.4 ± 8.0 | 22.0 ± 7.9 | 20.0 ± 7.2 | 0.684 | 0.593 | 0.107 |
| Non-sedentary time (%) | 27.3 ± 15.8 | 27.6 ± 10.6 | 31.7 ± 16.4 | 30.8 ± 10.4 | 0.873 | 0.936 | 0.312 |

BMI: body mass index; FFM: fat-free mass; RER: resting respiratory exchange ratio; REE: resting energy expenditure; TEE: total energy expenditure; MVPA: moderate and vigorous physical activity. Significance was set at $P \leq 0.05$. Values are presented as means ± SD for $n = 14$ participants for higher quality protein diet (HQ-D) and $n = 13$ participants for lower quality protein diet (LQ-D). t-test was used to test for between-group differences for age height and TEE. Two-way ANOVA was used to evaluate interaction, group and time effects. Asterix (*) denotes significant difference (*$P < 0.05$, ** $P < 0.01$).

### 5.4.2 *Dietary intake*

Habitual and 10-day intervention dietary intake data are presented in **Table 5.2**, the relative amino acid content of each supplement serving is presented in **Table 5.3** and the macronutrient composition of intervention diet meals is presented in **Table 5.4**. Dietary protein over the intervention was 1.04 ± 0.05 g·kg $BM^{-1}$·$day^{-1}$ for HQ-D and 1.05 ± 0.04 g·kg $BM^{-1}$·$day^{-1}$ for LQ-D, with no between habitual and intervention protein provision between groups (interaction: *P*=0.831). The provision of the animal-derived (WPC) and plant-derived (PPI) protein supplementation for HQ-D and LQ-D, respectively, in addition to discrete differences in whole-food protein sources, ensured the proportion of animal protein intake was greater for HQ-D over LQ-D (~75% vs. ~22%, respectively; *P*<0.001). Conversely, the proportion of plant-based protein intake was significantly greater for LQ-D over HQ-D (~78% vs. ~25%, respectively; *P*<0.001). By controlling for the amount and source of protein, the contribution of animal protein (%) significantly differed from habitual for both groups (time x group interaction: *P*<0.001), and during the intervention differed between groups (+33% HQ-D, -57%, LQ-D, both *P*<0.001).

In matching for estimated total daily energy requirements without compromising protein intake and protein source, there was no interaction (all *P*>0.05) or group effect (all *P*>0.05) for energy, carbohydrate, fat, protein, fibre or salt. Irrespective of group, there was a significant main effect for time (difference between habitual and intervention) for carbohydrate (P<0.01) and salt intake (P<0.01). The relative total amino acids provided daily from WPC and PPI (e.g., in HQ-D and LQ-D, respectively) presented in **Table 5.3** were comparable (P>0.05) but daily EAA provision was significantly (*P*<0.001) 37% higher with WPC than PPI, even when excluding tryptophan which was not measured in PPI.

**Table 5.2.** Comparison of habitual and controlled intervention dietary intake

| | HQ-D | | LQ-D | | *P* Value | | |
|---|---|---|---|---|---|---|---|
| | Habitual | Intervention | Habitual | Intervention | *Interaction* | *Time* | *Group* |
| Energy (kcal) | 1981 ± 468 | 2133 ± 316 | 1878 ± 675 | 2244 ± 429 | 0.405 | 0.050 | 0.978 |
| Carbohydrate (g) | 198 ± 48 | 254.6 ± 41.3 | 214 ± 81 | 286 ± 65 | 0.633 | **<0.001*** | 0.156 |
| Fat (g) | 86 ± 27 | 81.3 ± 15.9 | 66 ± 42 | 72 ± 21 | 0.453 | 0.954 | 0.076 |
| Protein (g) | 80 ± 31 | 74.7 ± 9.7 | 87 ± 56 | 78 ± 27 | 0.831 | 0.413 | 0.586 |
| Protein (g·kg $BM^{-1}$) | 1.14 ± 0.46 | 1.04 ± 0.05 | 1.18 ± 0.6 | 1.05 ± 0.04 | 0.906 | 0.290 | 0.869 |
| Animal protein (%) | 56.3 ± 13.5 | 74.7 ± 2.0 **#** | 58.1 ± 16.7 | 24.9 ± 1.6 **#§** | **<0.001*** | **0.018*** | **<0.001*** |
| Fibre (g) | 26 ± 6 | 18 ± 9 | 24 ± 14 | 26 ± 7 | 0.065 | 0.274 | 0.182 |
| Salt (g) | 2.0 ± 0.7 | 4.5 ± 1.1 | 1.5 ± 0.9 | 5.0 ± 1.3 | 0.075 | **<0.001*** | 0.956 |

Habitual dietary intake recorded on weighed food diaries and intervention diet is from provided diet for 10 days of the intervention. Values are means ± SD for $n$ = 14 participants for HQ-D and $n$ = 13 participants for LQ-D. Significance was set at $P \leq 0.05$. Two-way ANOVA was used to test significant difference between groups and timepoints. Asterix (*) denotes significant main effect from ANOVA (*P < 0.05, **P < 0.01). **#** Denotes significant difference between groups within timepoint when there is an interaction effect (**#** $P< 0.05$), § denotes significant difference between groups at that timepoint (§ $P<0.05$).

**Table 5.3.** Relative daily amino acid provision from supplemental source

| **Daily relative intake** (g) | **Whey Protein Concentrate** | **Pea Protein Isolate** | *P* value |
|---|---|---|---|
| Aspartic Acid | 2.52 ± 0.35 | 2.61 ± 0.60 | 0.653 |
| Serine | 1.24 ± 0.17 | 1.21 ± 0.28 | 0.753 |
| Glutamic acid | 4.0 ± 0.56 | 3.63 ± 0.83 | 0.182 |
| Glycine | 0.46 ± 0.06 | 0.91 ± 0.21 | **<0.001*** |
| Histidine | 0.40 ± 0.06 | 0.52 ± 0.12 | **0.003*** |
| Arginine | 0.55 ± 0.08 | 1.78 ± 0.41 | **<0.001*** |
| Threonine | 1.57 ± 0.22 | 0.78 ± 0.18 | **<0.001*** |
| Alanine | 1.16 ± 0.16 | 0.92 ± 0.21 | **0.003*** |
| Proline | 1.38 ± 0.19 | 1.01 ± 0.23 | **<0.001*** |
| Cystine | 0.55 ± 0.08 | 0.19 ± 0.04 | **<0.001*** |
| Tyrosine | 0.55 ± 0.08 | 0.80 ± 0.18 | **<0.001*** |
| Valine | 1.10 ± 0.15 | 0.88 ± 0.20 | **0.004*** |
| Methionine | 0.55 ± 0.08 | 0.23 ± 0.05 | **<0.001*** |
| Lysine | 2.24 ± 0.31 | 1.71 ± 0.39 | **<0.001*** |
| Isoleucine | 1.24 ± 0.17 | 0.75 ± 0.17 | **<0.001*** |
| Leucine | 2.24 ± 0.31 | 1.67 ± 0.38 | **<0.001*** |
| Phenylalanine | 0.69 ± 0.10 | 1.09 ± 0.25 | **<0.001*** |
| Tryptophan | 0.39 ± 0.05 | - | - |
| **∑ AAs** | 22.47 ± 3.13 | 20.71 ± 4.75 | 0.263 |
| **∑ EAAs** | 10.04 ± 1.40 | 7.63 ± 1.75 | **<0.001*** |
| **∑ NEAAs** | 12.43 ± 1.73 | 13.07 ± 3.0 | 0.496 |

AA: Amino acids; EAA: essential amino acids; NEAA: non-essential amino acids. Amino acid content provided by supplemental protein powders relative to body mass per day (3 daily 0.13 g·kg $BM^{-1}$). Significance was set at $P \leq 0.05$. Values are means ± SD for $n$ = 14 in HQ-D consuming Whey Protein Concentrate (WPC) and $n$ = 13 in LQ-D consuming Pea Protein Isolate (PPI). Tryptophan not measured in PPI and so is removed from the sum comparisons. Independent samples t-test was run to test for significant differences between groups where significance is denoted *$P < 0.05$, ** $P < 0.01$.

**Table 5.4.** 10-day dietary intervention provision between group comparison

| **HQ-D** | CHO (g) | Fat (g) | Pro (g) | Pro/kg (g) | Fibre (g) | Salt (g) | Energy (Kcal) | AML Pro (%) | PLA Pro (%) |
|---|---|---|---|---|---|---|---|---|---|
| Meal rotation 1 | 262 ± 40 | 80.3 ± 14.1 | 74.6 ± 9.3 | 1.04 ± 0.05 | 25.5 ± 2.8 | 4.10 ± 1.09 | 2136 ± 302 | 73.7 ± 3.3 | 26.3 ± 3.3 |
| Meal rotation 2 | 248 ± 43 | 82.3 ± 18.1 | 74.7 ± 10.3 | 1.05 ± 0.05 | 11.7 ± 7.7 | 4.85 ± 0.89 | 2131 ± 341 | 75.1 ± 1.3 | 24.9 ± 1.3 |
| Average | 255 ± 41 | 81.3 ± 15.9 | 74.7 ± 9.7 | 1.04 ± 0.05 | 18.6 ± 9.0 | 4.48 ± 1.05 | 2133 ± 316 | 74.8 ± 2.7 | 25.2 ± 2.3 |
| **LQ-D** | CHO (g) | Fat (g) | Pro (g) | Pro/kg (g) | Fibre (g) | Salt (g) | Energy (Kcal) | AML Pro (%) | PLA Pro (%) |
| Meal rotation 1 | 281 ± 98 | 69.2 ± 29.5 | 78.0 ± 26.9 | 1.05 ± 0.28 | 30.0 ± 10.3 | 4.67 ± 1.88 | 2275 ± 718 | 20.8 ± 4.0 | 79.2 ± 4.0 |
| Meal rotation 2 | 292 ± 67 | 74.5 ± 25.9 | 78.0 ± 27.1 | 1.05 ± 0.28 | 22.1 ± 7.2 | 5.24 ± 1.78 | 2213 ± 746 | 22.1 ± 2.3 | 77.9 ± 2.3 |
| Average | 286 ± 65 | 71.9 ± 20.6 | 78.0 ± 17.6 | 1.05 ± 0.04 | 26.0 ± 6.8 | 4.95 ± 1.32 | 2244 ± 429 | 21.5 ± 3.3 | 78.6 ± 3.3 |
| **Between groups** (*P* value) | 0.134 | 0.157 | 0.551 | 0.811 | **<0.001**** | 0.253 | 0.443 | **<0.001**** | **<0.001**** |

CHO: Carbohydrates; Pro: Protein; HQ pro: higher quality protein; LQ pro: Lower quality protein. AML Pro: animal protein; PLA Pro: plant protein. Meal rotation 1; was provided on day 0, 2, 4, 6 and 8 of the intervention, comprised of 4 meals (breakfast, lunch, dinner, and snack, including 3 daily protein supplemented smoothies). Meal rotation 2 also contains 4 meals with the same 3 daily protein supplemented smoothies and were provided on days 1, 3, 5, 7 and 9 of the intervention. Significance was set at $P \leq 0.05$. Data is presented as values from Values are means ± SD for dietary provision values for n = 14 participants in HQ-D and n = 13 for LQ-D. Independent samples t-test was run to compare differences between groups. Asterix (*) denotes that the average of meal rotations 1 and 2 is significantly different between groups (** P < 0.01).

### *5.4.1 Deuterium enrichment and myofibrillar protein synthesis*

Deuterium body water APE from saliva sampling over the 10-day period following $D_2O$ loading, significantly increased over time ($P$<0.001), with no difference between groups ($P$=0.910) or overall interaction ($P$=0.214) **Figure 5.3A**. Mean deuterated body water APE over the 10 day period did not differ between groups ($P$>0.05) **Figure 5.3B**. Myofibrillar-bound deuterated alanine was significantly increased in the trained leg (T) compared to the untrained leg (UT) ($P$<0.001), with no difference between group ($P$=0.733) or overall interaction ($P$=0.964) **Figure 5.3C.** Myofibrillar deuterated alanine and saliva precursor enrichments enabled the calculation of iMyoPS in trained and untrained legs over the 10-day diet intervention, where there was no group x time interaction for iMyoPS ($P$=0.956) and no main group effect ($P$=0.527) **Figure 5.4A**. Specifically, rates of iMyoPS were significantly different between T and UT, irrespective of the group (main effect of training: $P$<0.001). Subsequently, the absolute difference in iMyoPS between trained and untrained legs was similar between groups **Figure 5.4B**.

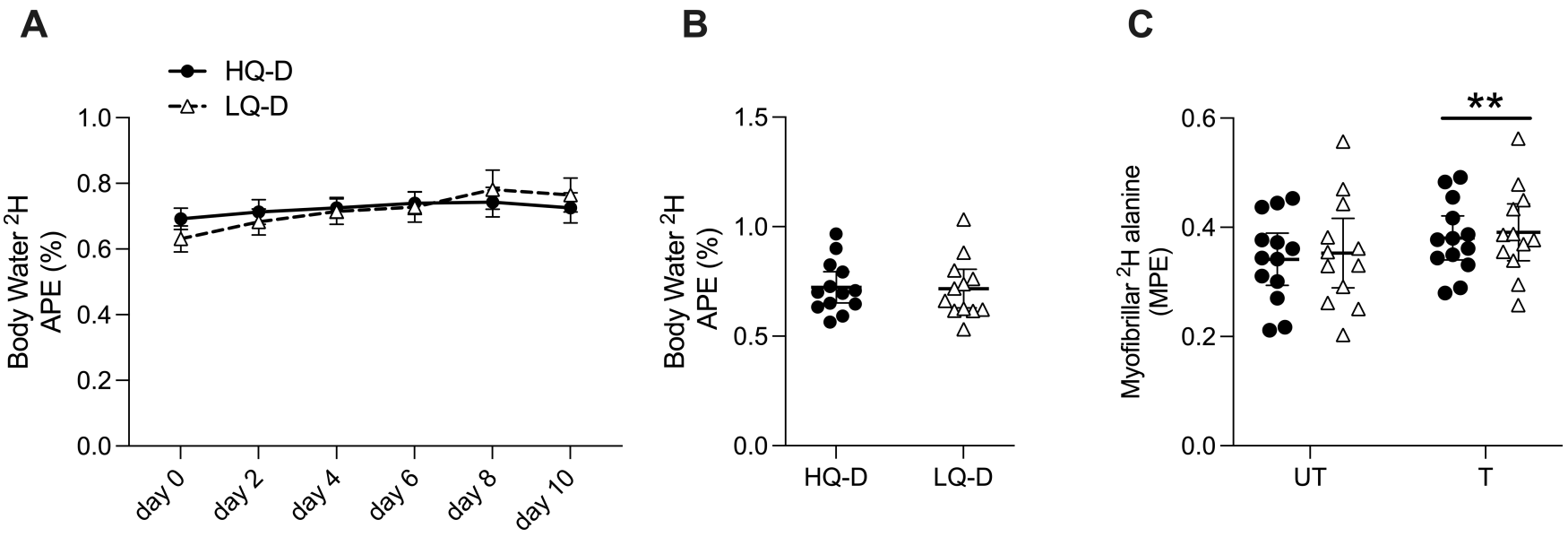



**Figure 5.3.** Deuterium enrichment in myofibrillar alanine and body water pool.

Time course of saliva enrichment over the 10-day intervention following $D_2O$ loading in atom percent excess (APE) (**A**) Two way ANOVA showed a significant effect of time ($P$<0.001), but no main effect for group ($P$=0.910) or interaction ($P$=0.214). Mean whole-body water deuterium ($^2$H) enrichment from saliva sampling during the intervention (day -1 to day 9 of intervention) following the loading phase (**B**). An independent samples t-test unveiled no significant difference in total body water enrichment between higher quality protein diet (HQ-D) or lower quality diet (LQ-D) groups ($P$=0.907). Myofibrillar bound alanine enrichment (in moles per cent excess; MPE) for UT (untrained) and T (trained) leg from day 0 to 10 (**C**) with no significant interaction ($P$=0.964) or group effect ($P$=0.733) but a main effect for training (**$P$<0.001). Significance was set at P≤0.05. Data is shown with individual data points for n=13 for HQ-D (black circles) and n=12 for LQ-D (white triangles) and plotted as the mean ± SEM.

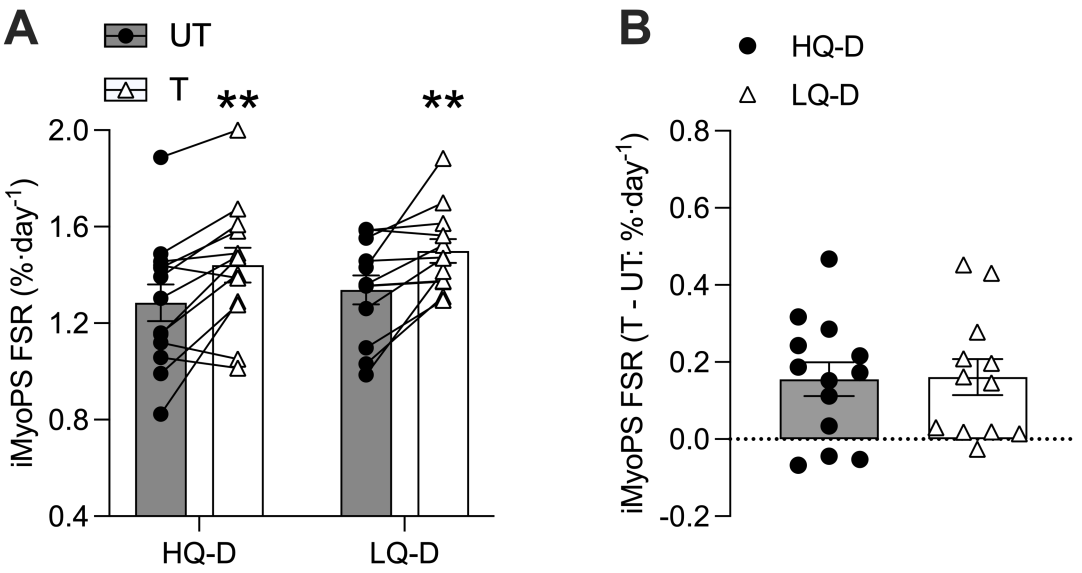



**Figure 5.4.** Integrated daily myofibrillar protein synthesis (iMyoPS) rates over 10-day intervention of divergent dietary protein quality with or without unilateral resistance exercise training.

Daily iMyoPS fractional synthesis rate (FSR) over a 10-day period untrained (UT: grey bar, black circles) and trained (T: white bar with white triangles) legs (**A**) in both higher quality protein diet (HQ-D) and lower quality protein diet (LQ-D). Connected black circles and white triangles denote individual responses following unilateral training. Two-way repeated-measures ANOVA showed no interaction (P=0.936), or main group effect (*P*=0.527) but a significant effect of training (**$P$<0.001). Delta change in iMyoPS rates for T relative to UT visu*a*lised in **B**. An independent samples t-test was used to compare the difference in iMyoPS between T and UT where there were no differences between groups ($P$>0.05). Significance was set at P ≤ 0.05. Data are shown with individual datapoints for n=13 for HQ-D (circles) and n=12 for LQ-D (triangles) and plotted as the mean ± SEM for **A** and **B.**

### 5.4.2 *Anabolic signalling phosphorylation*

There was no significant main interaction, group or training effect (all $P$>0.05) in phosphorylation over total protein expression for p-Akt$^{ser473}$/t-Akt, p-mTOR$^{ser2448}$/t-mTOR or p-eEF2$^{Thr56}$/t-eEF2 (**Figure 5.5A-C**) as determined by immunoblotting.

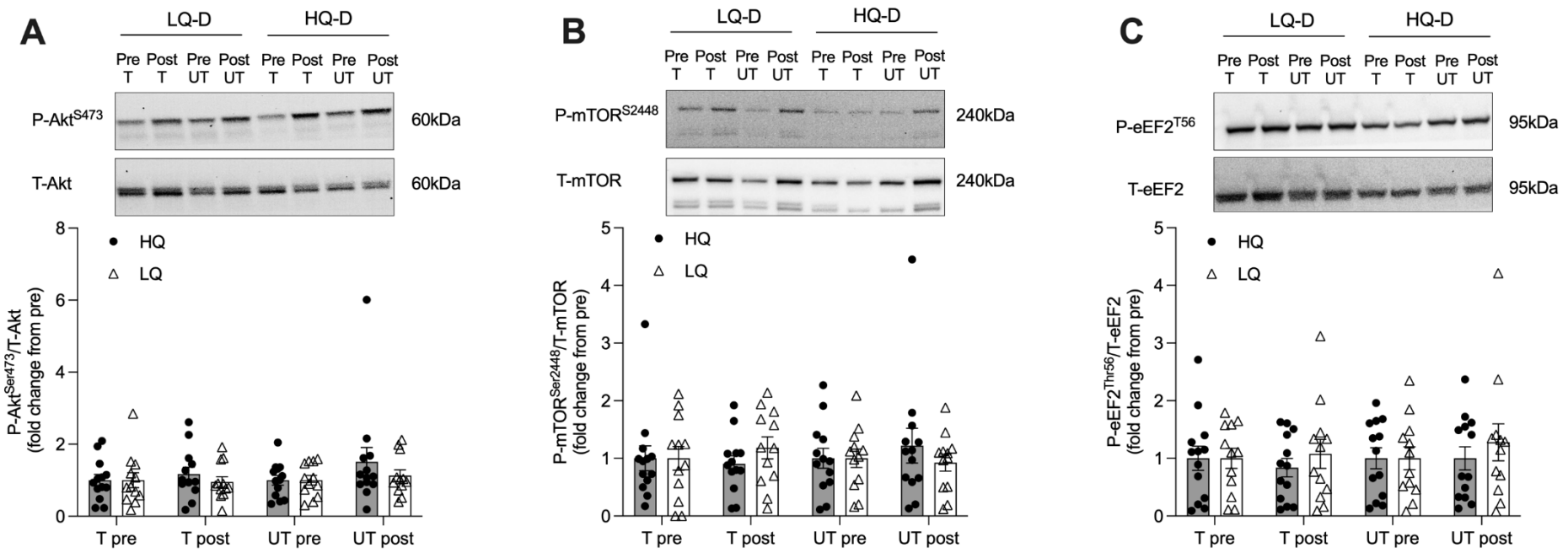



**Figure 5.5.** Immunoblots from trained and untrained leg pre- and post-intervention for anabolism-related protein targets.

Changes in protein expression for phosphor(p)-Akt$^{Ser473}$/total (t)-Akt (**A**), p-mTOR$^{Ser2448}$/t-mTOR (**B**), and p-eEF2$^{Thr56}$/t-eEF2 (**C**) in the postabsorptive state from pre to post 10-day dietary intervention in trained (T) and untrained (UT) leg. Data are expressed relative to the respective relative total protein, after accounting for loading control and shown as fold change from own leg at pre, as an internal control which was normalised to 1. Two-way ANOVA was used to compare between time points and legs within higher quality protein diet (HQ-D) and lower quality protein diet (LQ-D) groups. No significant differences between any time point or leg or interaction was observed (all $P>0.05$). Significance was set at $P \leq 0.05$. Data is shown with individual data points and SEM with individual datapoints for n=13 for HQ-D and n=12 for LQ-D for **A**, **B** and **C**.

### 5.4.3 *Resistance exercise training (RET)*

Unilateral knee extensor RET sessions were performed at a load equivalent to the estimated target of 75% 1RM (HQ-D; +7.2% than estimated 1RM, *P*=0.193; LQ-D: +4.8% than estimated 1RM, *P*=0.270) (**Table 5.5**). LQ-D had a significantly greater average RPE across all RET sets compared with HQ-D (*P*=0.026). There were no between-group differences in average training load, volume and repetition number. Following 5 days of unilateral RET, MVC and VA for trained and untrained legs did not differ from pre-intervention between groups (all interaction P>0.05), with no main effect of time or group for any training parameter (**Table 5.6**).

**Table 5.5.** Resistance exercise training characteristics

| | **HQ-D** | **LQ-D** | ***P* Value** |
|---|---|---|---|
| 1RM (kg) | 45.7 ± 16.6 | 56.5 ± 30.0 | 0.232 |
| Target training load (kg) | 34.3 ± 12.5 | 42.3 ± 20.2 | 0.179 |
| Actual training load (kg) | 32.0 ± 10.1 | 40.4 ± 19.5 | 0.232 |
| CR-10 RPE (0-10) | 6.5 ± 1.1 | 7.4 ± 1.0 | **0.026*** |
| Total Repetitions (n) | 417 ± 22 | 412 ± 18 | 0.487 |
| Total Volume (kg) | 13355 ± 4182 | 16735 ±8314 | 0.203 |

1RM: 1 repetition maximum; RPE: rating of perceived exertion (Category Ratio 10 scale). Significance was set at $P \leq 0.05$. Values are means ± SD for n = 14 participants for HQ-D and 13 participants for LQ-D during unilateral knee extension resistance exercise training (RET). Total volume was calculated as actual load lifted and number reps for all sessions. Independent samples t-test was run to find any statistical differences in each training variable between groups. * Denotes significant difference between LQ-D and HQ-D (* $P<0.05$).

**Table 5.6.** Skeletal muscle strength, function and architecture

| | HQ-D | | LQ-D | | *P* value | | |
|---|---|---|---|---|---|---|---|
| | Pre intervention | Post intervention | Pre intervention | Post intervention | *Interaction* | *Time* | *Group* |
| Trained leg MVC (N) | 224.1 ± 62.2 | 217.4 ± 87.2 | 262.5 ± 108.6 | 242.5 ± 127.0 | 0.521 | 0.176 | 0.402 |
| Untrained leg MVC (N) | 227.2 ± 81.4 | 220.4 ± 92.2 | 257.8 ± 111.1 | 254.3 ± 137.3 | 0.919 | 0.704 | 0.479 |
| Trained leg VA (%) | 79.8 ± 8.1 | 81.1 ± 8.1 | 80.9 ± 15.7 | 79.9 ±14.1 | 0.237 | 0.742 | 0.104 |
| Untrained leg VA (%) | 74.5 ± 15.0 | 71.5 ±13.6 | 82.6 ± 10.3 | 84.0 ± 11.0 | 0.395 | 0.919 | 0.992 |
| Trained leg PA (°) | 14.9 ± 2.4 | 13.9 ± 2.4 | 16.0 ± 3.5 | 15.5 ± 4.9 | 0.752 | 0.283 | 0.318 |
| Untrained leg PA (°) | 14.2 ± 1.4 | 14.5 ± 2.2 | 15.1 ± 4.3 | 16.2 ± 3.4 | 0.478 | 0.210 | 0.281 |
| Trained leg MT (cm) | 2.14 ± 0.29 | 2.10 ± 0.30 | 2.27 ± 0.38 | 2.27 ± 0.44 | 0.690 | 0.688 | 0.287 |
| Untrained leg MT (cm) | 2.02 ± 0.27 | 2.08 ± 0.36 | 2.31 ± 0.34 | 2.36 ± 0.42 | 0.746 | 0.137 | 0.057 |

MVC: maximum voluntary contraction; VA: voluntary activation; PA: pennation angle; MT: muscle thickness of *vastus lateralis*. Significance was set at $P \leq 0.05$. Values are means ± SD for n = 14 participants for HQ-D and 13 participants for LQ-D for PA and MT. In the trained leg MVC and VA were n=13 for HQ-D and 12 for LQ-D and untrained leg n=9 in HQ-D and n=12 in LQ-D. Two-way ANOVA was run test for significant differences between groups and timepoints.

### *5.4.4 Renal function and serum lipid status*

Markers of renal function and lipid status are presented in **Table 5.7**. Throughout the intervention, there were no changes in markers of renal function for either group as determined by serum urea, creatinine, sodium, potassium, HDL-c, triglycerides, cholesterol LDL-c, or eGFR (interaction all $P>0.05$). For non-HDL-c, there was a significant group x time interaction ($P=0.027$), and time effect ($P=0.047$), where Bonferroni post-hoc tests revealed non-HDL-c (-12.8%; $P=0.011$) were significantly lower following the intervention for LQ-D only. The ANOVA also revealed a main effect of time for total cholesterol ($P=0.026$), irrespective of group as well as a significant main group difference in eGFR ($P=0.049$), irrespective of time.

**Table 5.7.** Postabsorptive urinary and serum concentrations of renal and lipid markers before and after 10-days of higher and lower quality protein diet

| | **HQ-D** | | **LQ-D** | | ***P* value** | | |
|---|---|---|---|---|---|---|---|
| | Pre intervention | Post intervention | Pre intervention | Post intervention | *Interaction* | *Time* | *Group* |
| Urea ($mmol \cdot L^{-1}$) | 5.23 ± 1.55 | 4.69 ± 0.88 | 4.96 ± 1.30 | 4.83 ± 1.07 | 0.466 | 0.242 | 0.887 |
| Creatinine ($\mu mol \cdot L^{-1}$) | 67.3 ± 14.2 | 66.9 ± 13.3 | 74.1 ± 15.7 | 73.7 ± 18.2 | 0.991 | 0.673 | 0.297 |
| Sodium ($mmol \cdot L^{-1}$) | 140.2 ± 3.4 | 139.5 ± 1.5 | 140.7 ± 5.6 | 142.4 ± 10.7 | 0.225 | 0.610 | 0.483 |
| Potassium ($mmol \cdot L^{-1}$) | 4.87 ± 0.45 | 4.82 ± 0.32 | 4.87 ± 0.45 | 4.92 ± 0.67 | 0.632 | 0.967 | 0.763 |
| HDL-C ($mmol \cdot L^{-1}$) | 1.77 ±0.25 | 1.69 ± 0.26 | 1.57 ± 0.46 | 1.54 ± 0.49 | 0.455 | 0.053 | 0.268 |
| Triglycerides ($mmol \cdot L^{-1}$) | 0.82 ± 0.33 | 0.81 ± 0.35 | 0.81 ± 0.36 | 0.69 ± 0.32 | 0.184 | 0.093 | 0.649 |
| Cholesterol ($mmol \cdot L^{-1}$) | 5.46 ± 0.95 | 5.41 ± 0.95 | 5.47 ± 0.82 | 4.92 ± 0.78 | 0.058 | **0.026*** | 0.500 |
| eGFR ($mL \cdot min^{-1}/1.73m^2$) | 79.5 ± 4.6 | 80.6 ± 6.3 | 87.1 ± 7.5 | 84.7 ± 9.5 | 0.073 | 0.491 | **0.049*** |
| LDL-C ($mmol \cdot L^{-1}$) | 2.87 ± 0.74 | 2.92 ± 0.73 | 3.08 ± 0.89 | 2.69 ± 065 | 0.077 | 0.153 | 0.971 |
| Non-HDL ($mmol \cdot L^{-1}$) | 3.69 ± 0.91 | 3.73 ± 0.84 | 3.89 ± 0.95 | 3.38 ± 0.78$^{\#}$ | **0.027*** | **0.047*** | 0.833 |

HDL-C: high-density lipoprotein cholesterol; eGFR: estimated glomerular filtration rate; LDL-C: low-density lipoprotein cholesterol. Significance was set at $P \leq 0.05$. Values are means ± SD for n = 13 participants for HQ-D and n = 10 participants for LQ-D. 2-way ANOVA was run test for significant differences between timepoints (pre to post), groups (LQ-D vs HQ-D) and interaction (group x time) * Denotes significant differences in main ANOVA results ($^{*}P < 0.05$) and hash symbols ($^{\#}$) indicate significant difference within groups between timepoints when there is an interaction effect ($^{\#}P < 0.05$).

### 5.4.5 *Whole-body nitrogen balance (WBNB)*

WBNB was determined as the difference between excreted nitrogen in urea and creatinine from 24h urine collection over day 0-1 and day 9-10 of the intervention and the level of dietary protein ingestion on these days. There were no significant within or between-group differences in absolute WBNB (interaction, group and time effects all *P*>0.05) **Figure 5.6A** or the relative change in WBNB over the 10-day intervention (*P*>0.05) **Figure 5.6B.**

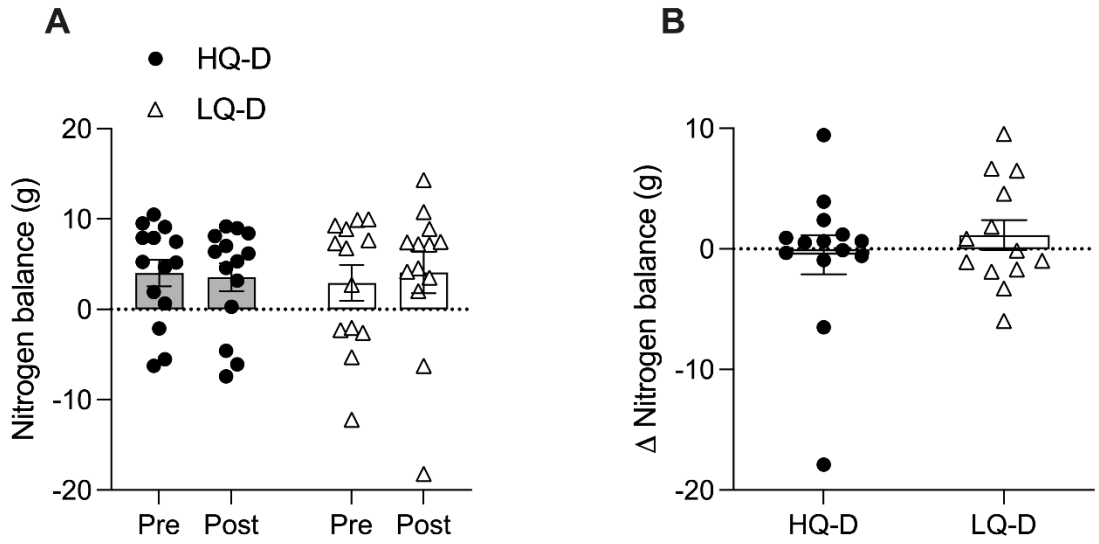



**Figure 5.6.** Whole body nitrogen balance (WBNB) determined from urinary nitrogen excretion and ingested nitrogen (from dietary protein).

WBNB prior-to (pre) and following (post) a 10-day dietary intervention containing higher-quality (HQ-D) or lower-quality (LQ-D) protein (**A**). A 2-way ANOVA was used to test for significant differences between HQ-D and LQ-D, pre- and post-intervention with no main interaction (*P*=0.436), group (*P*=0.928) or time effect (*P*=0.746). Delta change in WBNB from pre- to post-intervention (**B**). An independent samples t-test was run to test differences between HQ-D and LQ-D in WBNB change from baseline, with no significant difference between groups (*P*>0.05). Significance was set at P ≤ 0.05. Data is shown with individual datapoints for n=14 for HQ-D (circles) and n=13 for LQ-D (triangles) with bars showing the mean ± SEM for **A** and **B**.

## 5.5 Discussion

To date, the influence of dietary protein quality to support muscle anabolism in the context of ageing has largely been investigated under acute controlled experimental conditions using isolated supplements. Studies of the longer-term muscle anabolic effects of whole-food diets differing in protein quality have generally incorporated a diet including ample protein supplementation [38, 40]. As the impact of protein quality on stimulating post-prandial protein synthesis can be compensated for by ingesting a greater quantity of a lower quality protein source [56], it is apparent that consuming a diet with a higher protein intake can preclude us from detecting the impact of protein source and quality on muscle protein synthesis rates when consuming a normal diet providing 1.0-1.1 g·kg $BM^{-1}$·$day^{-1}$ [42]. In the present study, we provided middle-to-older aged adults a 10-day controlled diet containing 1.0 g·kg $BM^{-1}$·$day^{-1}$ protein from higher-quality animal-derived (HQ-D) or lower-quality plant-derived (LQ-D) sources, with unilateral leg RET performed every other day. Our novel findings show that daily muscle protein synthesis rates do not differ when consuming either a diet providing 1.0 g·kg $BM^{-1}$·$day^{-1}$ of more animal (HQ-D) or plant-derived (LQ-D) These data demonstrate that the quality of ingested protein in a typical diet of middle-to-older aged adults has no additive influence on skeletal muscle anabolism. Irrespective of the quality of ingested protein in the typical diet of middle-to-older aged adults, RET is a potent stimulus to enhance muscle anabolism with implications for muscle maintenance with advancing age.

In the present study, it was hypothesised that the addition of WPC to low-to-moderate protein-containing meals and snacks in HQ-D would support greater rates of daily iMyoPS, compared with the addition of PPI to meals and snacks in LQ-D. In contrast to our hypothesis, we did not observe any differences in rested or RET-

induced rates of daily iMyoPS between HQ-D and LQ-D. The absence of any difference in iMyoPS between diets could be due to the lack of robust differences in postprandial EAA availability in response to the mixed meals and supplements that were provided. Firstly, the EAA profile and postprandial availability of PPI is relatively high compared with other isolated plant-derived proteins [34]. Thus, the postprandial EAA availability of PPI may have been sufficient to elicit a comparable muscle anabolic response to WPC in middle-to-older aged adults, particularly when consumed as part of a mixed meal that would address EAA deficiencies [57, 58]. Secondly, PPI and WPC were ingested in smoothie and were consumed alongside a whole-food mixed meal or snack, which would also have altered/slowed EAA digestion and absorption kinetics [59] and potentially influenced acute postprandial muscle anabolism [60]. However, as part of a separate sub-study, we observed significantly greater temporal and net postprandial plasma leucine availability over 2 h following the HQ-D breakfast meal and WPC smoothie, compared with the LQ-D breakfast meal and PPI smoothie (Korzepa et al., unpublished data, **Chapter 4**). It is likely that the divergent postprandial EAA and particular leucine availability between HQ-D and LQ-D meals/smoothies was not as apparent as would be seen if isolated animal- and plant-derived proteins were ingested in water outside of a mixed meal [61]. Nonetheless, although the greater postprandial leucine availability would likely persist across the WPC- and PPI-supplemented lunch and snack events, this did not provide the basis for greater daily iMyoPS rates with HQ-D in middle-to-older aged adults in the present study. This observation is congruent with recent data from McKendry and colleagues [62] demonstrating that supplemental whey and pea protein provided at breakfast and lunch as part of a higher protein diet, enhanced rested rates of daily iMyoPS to a

similar extent in older males despite greater postprandial EAA availability in whey supplemented meals.

Habituating to a new (lower) level of dietary protein intake has been reported to alter WBNB, muscle protein turnover, body weight and RMR [63, 64], which could have obscured any potential differences in outcomes between HQ-D and LQ-D. However, whilst dietary protein intake was controlled at ~1 g·kg $BM^{-1}day^{-1}$ for the 10-day intervention, and protein intake was theoretically sub-optimal for maximal postprandial muscle anabolism at breakfast, lunch and snack [15], participants were consuming similar levels of daily and per/meal protein habitually. Based on the absence of any group differences in WBNB, RER, or body mass, we suggest the controlled diet intervention did not induce any major metabolic stress that would obfuscate any potential impact of dietary protein quality on the outcomes measured.

Despite the RET-induced increase in rates of daily iMyoPS for HQ-D and LQ-D, there were no discernible effects of RET in intramuscular anabolic signalling. However, post-intervention muscle biopsies were obtained ~24 h following the final bout of RET to ensure that iMyoPS measures captured the cumulative anabolic response to all RET bouts [20]. Given evidence that the intramuscular anabolic signalling response to RET returns to rested pre-exercise values acutely (~4-5 hours) following a combined stimulus of RET and protein ingestion [8, 65, 66], it is perhaps unsurprising that we did not capture any effect of RET on these outcomes. Whilst older adults may experience a delayed and/or blunted acute intramuscular signalling response to anabolic stimuli [9], differences in anabolic signalling ~24 h following RET in the present study is consistent with previous findings [67]. Undoubtedly, alterations in intramuscular anabolic signalling would have been apparent in the acute post-RET phase and underscore the enhanced rates of daily iMyoPS in the trained leg.

The completion of five unilateral RET sessions over the 10-day intervention elicited greater rates of daily iMyoPS compared with the untrained leg, to a similar extent in HQ-D and LQ-D. This observation is consistent with data demonstrating that RET of similar volume to the present study, enhanced acutely measured muscle protein synthesis [68] for at least 24 h afterwards [20, 69]. Specifically, RET is thought to overcome age-related muscle anabolic resistance by increasing the utilization of dietary-derived amino acids for protein synthesis in older adults [70]. Hence, the RET-induced increase in daily iMyoPS observed herein would be regarded as the basis for muscle conditioning and remodelling [71]. Whereas others have failed to detect any increase in iMyoPS over 3-weeks of unilateral RET in older adults [10], the greater frequency of RET bouts over a shorter 10-day period herein, may explain how we were able to capture enhanced rates of daily iMyoPS in the trained leg [72]. Despite greater rates of daily iMyoPS in the trained leg for HQ-D and LQ-D, no changes in strength, voluntary activation (VA), muscle morphology or architectural properties were detected, likely due to the short timeframe of RET. For example, RET-induced changes in these parameters in middle-to-older aged adults are apparent after 8 weeks [73–75], but not in response to shorter-term interventions [10]. The health status of the middle-to-older aged adults in the present study may also explain the absence of neuromuscular adaptation, as short-term strength improvements are more profound when pre-training strength is lower [76]. Nonetheless, the present data highlight high-volume RET as a potent muscle anabolic stimulus in middle-to-older aged adults consuming a typical protein-containing diet, with the potential to support longer-term muscle adaptive remodelling and offset sarcopenia progression [73, 77, 78].

Notwithstanding, the intervention diet for LQ-D provided ~44% more fibre than HQ-D, largely driven by differences in the whole food dinner meal, which was devoid of any protein supplementation. Given that plant-derived proteins are rich in fibre [79], this may explain the decrease in non-HDL-c observed in LQ-D [80, 81], which could be interpreted as favourable for long-term cardiometabolic health [82]. However, it is important to highlight that despite time-course changes in certain blood lipids, there were no significant interaction effects in blood lipid changes between HQ-D and LQ-D. Similarly, neither HQ-D nor LQ-D had any influence on markers of renal function, suggesting that the source of dietary protein in a typical diet of middle-to-older aged adults has no detrimental effects on renal function.

In the present study, we chose to use milk-derived and pea-derived supplements as the predominant method of manipulating the quality of dietary protein intake over the 10-day intervention, based on their well characterised and divergent EAA profiles and postprandial aminoacidemia [34, 62, 83]. Supplements were delivered a moderate dose of protein in a palatable nutrient-rich smoothie as part of a low-to-moderate protein containing meal or snack. Indeed, this is not typical in a plant-based vegan diet where animal proteins are typically substituted for plant-based whole foods. We believe the approach in the present study of modulating supplemental protein source holds strong ecological validity, particularly for scenarios where dietary energy, micronutrient and protein intake may be compromised (i.e., appetite dysregulation, hospitalisation). In this regard, the importance of dietary protein quality for muscle anabolism and maintenance in clinical scenarios, or in those at an advanced older age warrants further attention.

In conclusion, consuming the majority of dietary protein from predominantly higher-quality animal-derived or lower-quality plant-derived sources did not modulate

rates of daily iMyoPS, WBNB or intramuscular anabolic signalling in middle-to-older aged adults over a 10-day period. Short-term RET increased daily rates of daily iMyoPS in middle-to-older aged adults, with no influence of the quality of protein consumed in the diet. Therefore, in middle-to-older aged adults consuming a typical protein-containing diet for 10-days, an increased proportion of lower quality plant-derived protein does not compromise daily muscle protein synthesis. The potential for higher quality animal-derived protein to influence muscle anabolism and maintenance/accretion with advancing age may be more apparent in very low protein-containing diets, particularly in clinical scenarios or at a more advanced older age.

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## 5.7 Supplementary Material

**Supplementary Material 5.1.** Example representative diet plan for the intervention

**Meal rotation 1**

Breakfast (Whole food and protein smoothie)

Muesli, Greek Yoghurt, Blueberries

Whey or Pea protein containing smoothie drink

Lunch (Whole food and protein smoothie)

Mixed salad bowl with creaser salad dressing, Pretzels, Apple or dried tropical fruit pieces

Whey or Pea protein containing smoothie drink

Dinner (Whole food only)

Jacket potato (heated), mixed vegetables, tuna in sunflower oil, butter.

Greek Yoghurt or Alpro Yoghurt (depending on consditon)

Snack (Whole food and protein smoothie)

Rice Cakes, Philadelphia Cheese, fruit pieces, nuts, crisps.

Whey or Pea protein containing smoothie drink

**Meal rotation 2**

Breakfast (Whole food and protein smoothie)

Porridge oats (made with water or used part of daily semi-skimmed milk allocation), mango chunks.

Whey or Pea protein containing smoothie drink

Lunch (Whole food and protein smoothie)

Chicken and Mushroom or Tomato Soup, Wholemeal bread, butter, dried tropical fruit pieces

Whey or Pea protein containing smoothie drink

Dinner (Whole food only)

Pasta, meatballs (meat free or beef depending on condition), garlic bread slice, ricotta cheese, sweetcorn and mayonnaise.

Rice pudding, raisins and dried cranberries.

Snack (Whole food and protein smoothie)

Cream crackers, Philadelphia Cheese, orange, cranberries and raisins.

Whey or Pea protein containing smoothie drink

For both rotations, semi-skimmed Milk and fruit juice measured and provided to consume throughout each day. Quantities of foods varied according to protein requirements and energetic needs for each participant. Food sources and quantities at dinner time were changed according to the condition to be primarily plant-derived or animal-derived protein.

# Chapter 6 General Discussion

## 6.1 Overview

Protein nutrition and resistance exercise are important prerequisites to stimulate muscle protein synthesis (MPS). With advancing age, the ability for MPS to be stimulated by such anabolic stimuli decreases [1, 2], with greater dietary protein intake and exercise volume stimuli required to support MPS and mitigate muscle mass and strength loss (sarcopenia). Sarcopenia is associated with reduced quality of life [3, 4] and increased all-cause mortality [5, 6], which is precipitated by defects in cellular signalling and underscored by skeletal muscle type II fibre atrophy. Unfortunately, fibre type and fibre area-specific investigations are lacking, preventing an understanding of fibre type-specific cellular regulation differences, especially in the context of ageing. Sarcopenia onset commences at 40 years of age and appears to accelerate beyond 60 years of age [7, 8]. Current research generally focuses on older adults where sarcopenia diagnosis (and/or associated consequences) is more prevalent, although greater attention in middle-aged adults is likely necessary to devise strategies to minimise early skeletal muscle decline. Considering food and protein consumption generally decrease with age [9], attention to the type and source of protein consumed may be of particular importance for maximising MPS and anabolism, whereby a summary of contemporary literature on the situational importance of protein source/quality currently lacking. Current research has mainly evaluated postprandial responses to very high doses of protein in older adults (>40g, i.e., the equivalent of 1100mL of milk) provided in isolated supplements [10, 11], and whole food mixed meals [12]. However, studies evaluating the role of protein quality at lower doses as part of whole food mixed meals are currently lacking where combining plant versus animal protein sources with low protein mixed meals may be a strategy to increase protein intake, with unknown effect on anabolism. Understanding the postprandial

plasma amino acid and appetite responses and longer-term muscle adaptive response to typically dosed protein-supplemented mixed meals of divergent protein sources in middle-to-older aged adults is of great relevance to advance our understanding of how postprandial handling of mixed foods may affect future feeding opportunities and muscle anabolism. Considering the effectiveness of resistance exercise for muscle anabolism, it is of interest to compare different source of protein on their anabolic potential when paired with high-load resistance exercise training (RET). As such the main aims of this thesis are fourfold:

i) Evaluate if abundance and spatial occupancy proteins regulating muscle protein synthesis differ between young and old are conserved between type I and type II skeletal muscle fibres.
ii) Summarise literature to evaluate reasoning for greater protein requirements with age and the situational importance of dietary protein quality for older adults.
iii) Determine the acute postprandial plasma aminoacidemia and indices of appetite regulation from provision of whole food mixed meal combined with a small bolus of plant-based (pea) or animal-based (whey) protein in middle-to-older age adults.
iv) Determine the impact of modulating dietary protein source for 10 days on integrated rates of daily muscle protein synthesis, with and without exercise in middle-to-older age adults.

## 6.2 Thesis novelty

This thesis presents novel investigations and findings regarding the underlying intracellular mechanisms of sarcopenia and the importance of dietary protein quality and RET as strategies to support age-related muscle health. Data from **Chapter 2** shows alterations in reduction in the number and size of type II muscle fibres with advancing age is not explained by different regulatory proteins visualised between different fibre types when determined by immunofluorescence microscopy, at least in the healthy younger and older populations observed. Indeed, age-related differences in regulatory proteins which govern MPS between young and old were found to be divergent at rest, perhaps underpinning the age-related blunted response to RET and dietary protein anabolic stimuli.

**Chapter 3** presents the first in-depth summary of contemporary literature on the importance of protein source and quality for older adults. In **Chapter 4,** and **Chapter 5** enlisted middle-aged and older male and female adults undertaking acute and 10-day interventions where protein source was manipulated, adding to the small pool of literature on protein handling and muscle anabolism in this middle-aged population. Considering much of the literature explores the use of supplements at high doses and in isolation, **Chapter 4** compared a typical low protein mixed food breakfast alongside an EAA-rich whey protein supplement or comparatively less EAA diverse pea supplement. For the first time, I show that a lower protein-containing mixed meal breakfast in combination with small bolus whey or pea protein supplement smoothie were shown to elicit similar postprandial appetite responses and overall plasma aminoacidemia; although whey protein elicited greater plasma leucine responses which may be important for greater MPS stimulation.

In **Chapter 5**, a tightly controlled diet with thrice-daily protein smoothie drinks was provided as part of a typical protein-containing diet (1 g·kg $BM^{-1}$·$day^{-1}$) over 10 days in 50–70-year-old males and females, where protein was provided primarily (~75%) from either animal-based (high-quality diet; HQ-D) or plant-based sources (lower-quality diet; LQ-D). Additionally, single-leg RET was undertaken every other day throughout the 10-day intervention phase. Muscle biopsies and saliva samples were combined with deuterium oxide stable isotope tracer consumption to explore the 10-day integrated rates of daily muscle protein synthesis (iMyoPS) in rested and exercised legs with both dietary interventions. We observed that the source of dietary protein intake did not differentially influence rested or exercised rates of daily iMyoPS. RET unequivocally augmented rates of daily iMyoPS over the intervention phase, representing a potent strategy to support muscle health with advancing age.

### 6.3 Intracellular signalling within muscle fibres, where do the differences lie?

The age-related decline in muscle mass is often attributed primarily to type II muscle fibre atrophy, which combined with a potential decline in fibre number can result in decreased strength, power and thus an increased risk of falls in advancing age due to an inability to rapidly produce force. The mechanisms for type II fibres to preferentially atrophy over type I fibres is much debated [13, 14], with cellular underpinnings yet to be fully elucidated. Despite this, in **Chapter 2**, I was able to uncover differences in the abundance and localisation of key anabolic targets between fibres via immunofluorescence microscopy (IF). To date, my thesis offers the first visualisation of regulatory proteins within the mechanistic target of rapamycin (mTOR) pathway on a fibre type-specific basis and further, I compare this between the skeletal muscle of both younger and older adults. My findings show that there are no

differences in localisation or abundance between fibre types in many regulatory protein markers (Ras homolog enriched in brain (Rheb), Sestrin2 and tuberous sclerosis complex 2 (TSC2) at rest. However, the regulatory markers identified in the present study are not exhaustive, and other regulatory proteins or transporters (e.g., ribosomal proteins, amino acid transporters) may still differ between fibres. Furthermore, to fully evaluate dysregulation at rest, phosphorylation sites should be visualised to confirm whether the spatial occupancy of targets (i.e., more peripheral) is truly indicative of dysfunctional activation [15]. The use of IF allowed comparison between fibre types in some key regulatory proteins in both young and old skeletal muscle, taking into context differences in muscle fibre area. However, to confirm the absence of differences in mTOR-related protein abundance and localisation between fibre types, more regulatory protein targets need to be visualised at rest and following anabolic to confirm any defects which may be more prone in type II fibres of older adults.

### *6.3.1 Considerations of immunofluorescence microscopy*

In **Chapter 2**, I used IF to stain for type I fibres (using MHC1) alongside TSC2 and Rheb. On serially cut sections (i.e., the next 7μm sections) WGA, mTOR and Sesrin2 were co-stained. The benefit of serially cutting cryosections allowed for manual alignment of type I positive fibres, even on WGA/mTOR/Sestrin2 stained slides, which were devoid of a fibre type stain. Such efforts ensured fibre type-specific analysis could occur, but multiple different mTOR-related regulators could still be visualised, facilitating greater insight into *in situ* basal mTOR regulation in younger and older men. Alternating cryosections were collected on separate glass slides, with at least 4 sections (per participant) on each to ensure the best chance of analysing high-quality

serially cut cryosections for MHC1/TSc2/Rheb and WGA/mTOR/Sestrin2 stained slides, respectively. Ideally, fibre type staining protocol would typically be done in multiple configurations i.e., type I fibres co-stained with Sestrin2 and Rheb, and WGA contained with TSC2 and mTOR. This approach would have ensured that findings were due to the way targets have been co-stained. Indeed, serial dilution tests were carried out to select the dilution of each antibody which would not cause 'bleed-through' into other channels which could artificially elevate the fluorophore intensity in other channels. If carrying out such fibre type-specific analysis again, where only one cryosection is stained with MHC1 stain and the subsequent section is aligned to ascertain fibre type, samples should be collected on additional slides to facilitate other staining configurations.

The conceptualisation of **Chapter 2** emerged from a desire to understand if there was differential mTOR-related regulators abundance and localisation between type I and type II fibres, and further if there were any discernible differences with age. In the present study, only MHC1 was used as a positive stain for type I fibres, whereby it was assumed that all fibres devoid or not aligning to an MHC1 stain were grouped as type II fibres, where hybrid fibres (i.e., type IIA, type IIX) were not identified. It would have been interesting to account for hybrid fibres and compare between type I and specific type II fibre isoforms, where specifically type IIx fibres are the first to atrophy in ageing and disease [16] and thus potentially experience more aberrant cellular defects. Indeed, my ability to capture hybrid fibres was impaired in **Chapter 2**, owning to methodological considerations and sample availability. Firstly, there were only three channels meaning a choice had to be made as to which targets were co-stained, where sections on the same slide had identical antibody treatments. This was to prevent any inadvertent cross interference (due to antibody running/wash steps),

which could have been introduced if other targets were stained on other sections of the same slide. Additionally, from a logistical standpoint, manually aligning fibres from MHC1 stained slide to identify the fibre type on the MHC1 devoid slides was highly time-consuming. It is easy to reconcile why fibre type-specific analysis of skeletal muscle cross sections is scarce in the literature, as it requires manually aligning serial cut sections or to co-stain fibre type stain with each target, limiting the coverage and amount of protein targets that can be analysed with fibres.

To summarise, in **Chapter 2**, most proteins visualised had a similar localisation at rest, irrespective of fibre type and age group, except for Rheb. However, the use of phospho-immunofluorescence would not have been suitable for Rheb as it does not undergo such post-translational modifications. Instead using IF to identify targets which do undergo phosphorylation may provide insight into dysregulation at rest. Furthermore, IF is well suited to inform spatial occupancy of targets, but immunoblotting is better suited to confirm protein target abundance. Concurrent use of fibre type-specific IF to gain spatial proximity data of proteins alongside fraction and fibre-type specific immunoblotting techniques (i.e., type I and II sarcoplasmic or myofibrillar) [17] to confidently ascertain abundance would ultimately helping research to move away from mixed muscle analysis and associated generalisations that all fibres will behave the same.

### 6.4 Acute appetite responses to protein meals, is there really no role for protein source?

Due to the supplemental nature of protein provision and matched constituent elements of the whole food breakfast used in **Chapter 4**, groups were described as consuming divergent protein sources from either plant-based pea protein isolate (PPI) or animal-derived whey protein concentrate (WPC). As such, whilst the focus was on

protein source, there was an implied investigation of protein quality due to the differing EAA profiles between WPC and PPI. It is also worth noting comparable digestibility was presumed (the other factor which underscores protein quality) due to the isolated powder form and ultimately identical beverage preparation for both PPI and WPC.

In **Chapter 4,** I showed there was no difference between groups consuming low-protein mixed breakfast with WPC or PPI in postprandial plasma appetite hormone response. Whilst these data corroborate the findings of others where high dose (40g) of pea or whey protein supplementation elicited comparable increases in appetite responses [18], it adds that the addition of WPC of PPI with a low protein mixed meal, does not differentially affect appetite. Indeed, other have shown long-term emphasis of higher protein meals has superiority over lower protein meals for supressing perceived and hormonal derived appetite responses [19]. Henceforth, protein source *per se* is likely not the main driver of appetite regulation. Indeed, this could largely be anticipated as total AAs provided from WPC or PPI were similar between groups, thereby any appetite differences which may ordinarily emerge with different AA provision would not be present. Furthermore, provision of protein on a relative basis (i.e., per body mass) likely minimised variation in appetite response, which may have otherwise been seen with the 52-112kg participant body weight range in consideration of hormone secretion relates to body weight [20]. Habitually, people rarely consume food according to body weight and instead eat according to behaviors [21] and appetite response [22, 23]. Nonetheless, the comparable appetite response seen in **Chapter 4** suggests that supplementation with either PPI or WPC on a low protein meal will induce similar transient responses and neither appear to perturb future eating capacity as perceived and hormonal appetite responses return to baseline following 180 minutes. However, the appetite response from mixed breakfast alone is unknown due

to the absence of a control group. Implementing only whole foods of divergent quality (i.e., primarily plant-based vs animal-based meals) was not undertaken in **Chapter 4,** as the additional variation in non-protein components would make any differential response to whole food breakfast with supplementation from WPC or PPI difficult to ascertain. Importantly, ~65% of total protein was obtained through supplemental PPI or WPC, whereby increased protein intake from liquid may accelerate gastric emptying [24] and hunger sensations [25, 26] compared to whole foods alone. However, appetite responses to low-protein mixed meals of differing protein sources (i.e., plant and/or animal) need to be elucidated to add meaning to the findings of **Chapter 4** and finalise if protein source is obsolete in potentiating postprandial appetite responses.

### 6.5 What does postprandial aminoacidemia imply about muscle anabolic potential?

The AA profile for PPI and WPC were considered divergent and thus different qualities in terms of EAA profile, but not their digestibility. Postprandially, plasma EAA appearance (**Figure 4.2 and Figure S4.4**) generally matched the EAA profile of both supplements (**Table 4.2**). In addition, components of the mixed whole food breakfast were matched between conditions and thus any differences in aminoacidemia were attributed to the supplement. It was important to identify the excursion of each EAA, as pooling all plasma total EAA may have masked any individual differences. For example, EAA appearance was similar between groups, but this was created from disparaging amounts and subsequent plasma appearance of methionine, phenylalanine and leucine. Henceforth, studies should present all individual EAA responses to capture overall EAA availability, as depicted in **Chapter 4**.

The transient appearance of plasma EAA and leucine appeared similar between groups in **Chapter 4**, suggesting that the uptake of EAAs and leucine to tissues was

likely not different between groups. However, the incremental area under the curve (iAUC) from leucine was significantly greater in MB+WPC compared with MB+PPI, which was subsequently theorised to potentiate longer term integrated myofibrillar protein synthesis (iMyoPS) rates. Surprisingly though, despite the superior leucinemia in MB+WPC (**Chapter 4**) a low protein diet supplemented with WPC did not augment actual 10-day iMyoPS over a low protein diet supplemented with PPI (**Chapter 5**). This discordance may be reconciled by the fact the postprandial AA response does not consider non-protein components which may bolster anabolism, such as carbohydrates and minerals [27, 28]. Indeed, increased carbohydrate bioavailability is known to enhance insulin-stimulated IRS1 signalling and minimise AMPK activation, augmenting mTOR-mediated MPS stimulation [29, 30]. Additionally, it has been theorised that with complex foods, these non-protein components are more influential on MPS than the bioavailability of leucine, at least in younger adults [28, 31]. Importantly, the acute plasma aminoacidemia in **Chapter 4** and the 10-day anabolic response in **Chapter 5** do not have groups which are without supplemental protein. As such, the lack of a comparator control group consisting of whole food only consuming either primarily plant- or animal-derived proteins prevented this thesis from truly disentangling any anti-nutritive effect on skeletal muscle remodelling.

### 6.6 Evaluating sex-specific iMyoPS responses

Studies detailed in **Chapter 4** and **Chapter 5** included both male and female participants to allow for the greatest translatability of findings for the general population. Males and females have divergent sex hormones which contribute to differential retention of lean mass in younger men compared with women [32]. Whilst sex hormone levels were not measured in the present study, it was anticipated age-related reduction in sex hormones (such as hypogonadism and menopause) would mean

responses would be similar between middle-to-older aged men and women. Additionally, groups were balanced according to sex therefore it would not have impacted comparisons between MB+WPC or MB+PPI in **Chapter 4** or LQ-D and HQ-D in **Chapter 5**. Whilst controlling for sex hormone differences between males and females, one component that was anticipated to differ was fat mass, which in **Chapter 5**, was significantly greater in females than males (females: 33.1 ± 4.8%, males: 21.0 ± 4.1%; $P<0.001$), consistent with previous literature [33]. This increase in fat mass coincides with lower total body water (TBW %) in females than males as seen in the cohort in **Chapter 5** (males: 41.4 ± 7.7 TBW%; females: 30.0 ± 5.1 TBW%, $P<0.001$) which is consistent with others [34]. Subsequently, this increased body fat and lower TBW in females compared with males, likely explains the higher deuterium body water enrichments in females than males (**Figure 6.1A-B)**, with data obtained from the study in **Chapter 5**. Nonetheless, whilst this is an interesting finding, such differences did not translate to any difference in untrained or trained iMyoPS rates when grouping for sex instead of protein quality condition as seen in **Figure 6.2C**. Indeed, sexual dimorphism with age has been seen with basal MPS rates at rest being higher in older women, and subsequently older women having inferior anabolic response to acute RET [35, 36]. Reasonings as to why no sex differences were observed in the present study may be due to the absence of basal MPS delineation, where the magnitude expressed as iMyoPS from day 0 to 10 (opposed to acute mixed-muscle hourly change as seen in [35]) was comparable between males and females, and thus accounts for variation in basal muscle protein synthesis rates. In the present thesis, 5 sessions of high-intensity RET had the capacity to increase 10-day iMyoPS rates, irrespective of protein quality in both middle-to-older aged males and females; suggesting hormonal decline with age makes for comparable responses between sexes longer term.

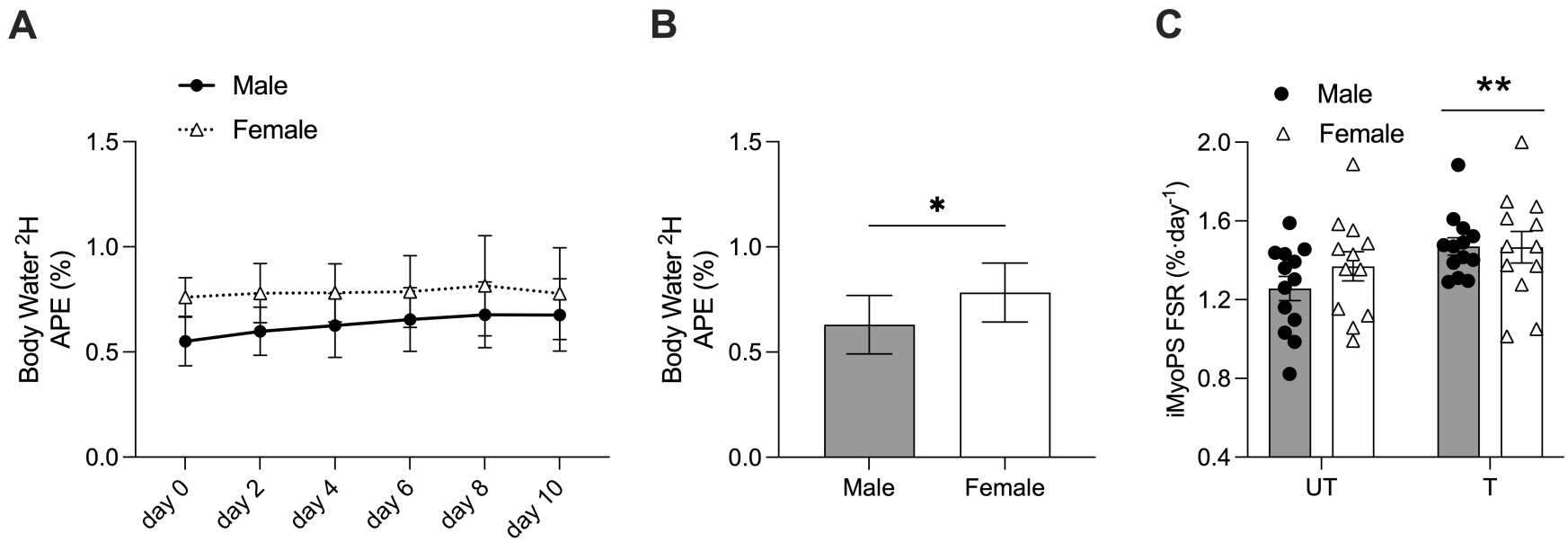


**Figure 6.1.** Sex based comparison of deuterium enrichment and iMyoPS

Comparison in saliva deuterium enrichment between males and females following loading phase from day 0 to day 10 of the intervention (**A**). Two-way ANOVA unveiled no interaction (*P*=0.232) but a main effect for group (*P*=0.012) and time (*P*=0.004). Mean deuterium saliva enrichment over all intervention days (day 0-10) (**B**) Independent samples t-test unveiled significant difference (*P*=0.012) between males and females. iMyoPS from day 0 to 10 in trained and untrained leg males and females (**C**). Two-way ANOVA was run to unveil a significant main effect leg (P<0.001) but no main effect for group (*P*=0.541) or interaction (*P*=0.062). iMyoPS for males, differences between T and UT is +0.21%day (P<0.001), and for females difference between T and UT was 0.10%day (*P*=0.032). Data presented as mean and SEM with individual data points for males n= 13 (black circles) and females n=12 (white traingles) * *P*<0.05, ** *P*<0.01

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## 6.7 Comparing middle age to older age iMyoPS responses

Another important emphasis within **Chapter 4** and **Chapter 5** is the inclusion of both middle age and older adults, with inclusion criteria from 50 to 70 years of age. Indeed, it was thought to be challenging to include middle-aged adults given the time commitment of the study and the fact that many people under 65 years of age are still actively working, being under retirement age. Despite this, we successfully recruited people ranging from ages 51-70 years of age, where there was no difference in daily time spent completing moderate and vigorous physical activity (MVPA %) (≥65y: 17.2± 8.8%; <65y: 21.2 ± 9.4%, P=0.314). Indeed, when dichotomising the data, there were 15 participants (male n=8; female n=7) 65 years or younger and 10 participants (females n=5; female n=5) over 65 years of age. Considering muscle decline commences following the age of 40, we were not expecting disparaging differences in any muscle-related outcomes between older (≥65y) and middle-aged adults. Indeed, when comparing participants aged 65 and under to participants over the age of 65, changes in iMyoPS were the same as seen when comparing protein quality groups (**Figure 5.4** in **Chapter 5**) and sex **Figure 6.1**. In all 3 scenarios, it prevails that training consistently elicits a greater iMyoPS rate. Together this thesis adds to the knowledge that irrespective of protein source, biological sex and even age beyond 50 years, the completion of RET is likely the best defence against sarcopenia, at least when protein intake is at the RDA or above. Although this does not refute the possibility that precluded protein intake, closer the RDA may unveil an emphasised importance on the source of ingested protein.

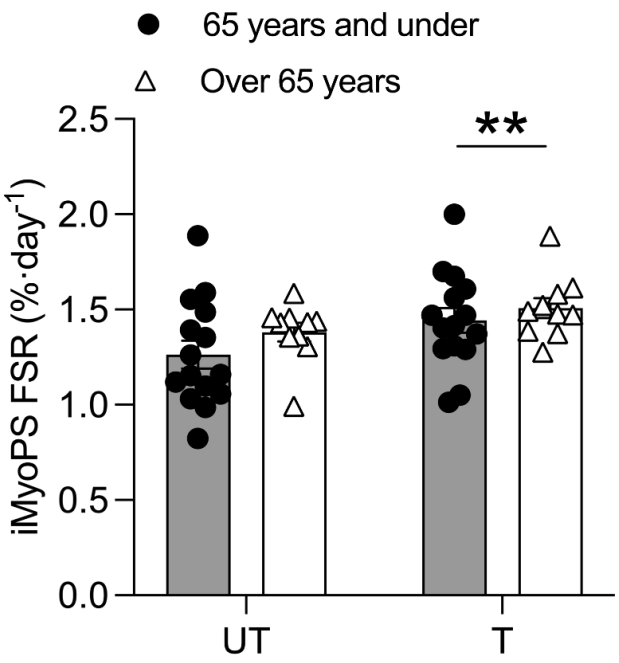


**Figure 6.2** Middle to old age comparison of iMyoPS in trained and untrained leg

Data presented as mean ± SEM. Over 65 years (n=10) and 65 years and under (n= 15). Significant main effect for leg ($P$<0.001) but not group ($P$=0.311) or interaction ($P$=0.429). iMyoPS differences between UT and T for over participants 65 years +0.13 %·day$^{-1}$ ($P$=0.019) and participants 65 and under +0.09 %·day$^{-1}$ ($P$<0.001).

## 6.8 Considerations for food provision and consumption reporting in human research

### *6.8.1 Tackling underreporting*

Within any human research involving food provision, it is prudent to consider whether habitual diet diary reporting was carried out to the same accuracy for all participants, as dietary underreporting is known to be a common phenomenon in human research [37]. It is possible to quantify the amount of underreporting by calculating the ratio between energy intake (EI) and indirect calorimetry-derived respiratory exchange ratio (RER), (EI/RER); where this value correlates with body composition changes [38]. In **Chapter 5**, both the HQ-D and LQ-D groups reported baseline habitual EI which was lower than the intervention provision and predicted

requirements. Given that RER did not change between pre- and post-intervention, despite changes in body mass for HQ-D, it suggests that underreporting in habitual diet likely occurred. Importantly though, it is challenging to isolate if all macronutrients are affected equally during underreporting. For instance, it is common for people to omit details from food diaries they may feel judgement for consuming (i.e., often confectionary items) [39]. As such, if fewer confectionary items are declared within food diaries, this may translate to lower carbohydrate intake, as seen in the case of habitual intakes in **Chapter 4** and **Chapter 5.**

### *6.8.2 Logistical challenges with providing a controlled diet*

In **Chapter 5** the provision of 10-days of dietary provision which is individualised to body weight whilst matching for protein dose, source and estimated calorie requirements was logistically challenging. Specifically, when trying to control for more than 2 variables within a dietary intervention, it was noted that is often at the detriment of another variable. Considering protein diets were divergent in terms of their primary protein source being of animal or plant origin, it is unsurprising that fibre content was higher in LQ-D, which was likely driven by the higher fibre content in the whole food only main meals (4.6 ± 0.9g higher in LQ-D than HQ-D ($P$<0.001)). Further, in achieving the target protein intake (1 g·kg $BM^{-1}$·$day^{-1}$), without affecting protein source or protein dose meant that higher calorie carbohydrate-dense foods were used. These included pretzels, dried fruits and pasta, subsequently offering further explanation towards the higher carbohydrate intake during the intervention phase, compared with habitual intake in both groups (**Table 5.2**). Finally, the constituent elements of the breakfast had to be identical for both groups to complete the acute feeding outcomes detailed in **Chapter 4**, consequentially I had to ensure protein and calorie requirements were accounted for in the remaining 3 meals (in meal option 1) following

the acute mixed breakfast trial. For added complexity, protein provision needed to not differ between meal option days 1 and 2 for either condition, particularly since option 2 was where RET was undertaken. Any differential protein provision between groups or training days would have compromised any ability to effectively compare the role of protein source, with and without RET, but in **Chapter 5** meal options were closely matched for protein dose and target protein source provision.

### *6.8.3 Monitoring and maintaining adherence*

In providing the intervention diet, foods were provided on each visit and given 2 days in advance. This meant that food needed to be prepped fresh, weighed, labelled and sealed individually so that participants could consume the correct foods easily at home. Anecdotally, this promoted adherence with participants reporting that instructions were easy to follow for at home meal preparation. Adherence was also promoted with regular check-ins regarding tolerability of the intervention diet, whereby participants could share any aspect of the diet they did not enjoy. In this situation, I was able to offer substitutes to promote adherence and avoid boredom. Indeed, the only evidence of adherence in the present study, as is common for most provided dietary interventions, is trust in participants and dietary records. Participants were encouraged to log the times of meals and if any additional food and drink were consumed and/or if assigned food was not eaten. This self-reporting approach acts as a reminder of the importance of the dietary intervention and provides a sense of heightened control of when they eat, even if there is limited control over their food choices. Alternative approaches include using photographs to evidence how much participants have consumed in an attempt to minimise underreporting and maximise adherence [40]; although these methods have limited accuracy and were deemed

unsuitable in the present population of 50–70-year-olds. In consideration of the invasive nature of the intervention (e.g., skeletal muscle biopsies, cannulations etc) and length of the intervention it was paramount to minimise any additional burden for participants to minimise attrition. Despite this, some confidence can be given to adherence from physiological measures given subtle plasma-derived lipid marker changes (**Table 5.5**) which could be explained by dietary alterations.

In **Chapter 5,** trials operated in single blind fashion to tightly control protein source, content and overall calorie provision, which was highly cumbersome. Indeed, outsourcing the provision of food to an external company could have been adopted but would have removed confidence in protein source provision with discrete macronutrient requirements for each participant according to the intervention and body mass. Further, this would have removed any form of personability and the capacity to offer alternative foods to maintain participant satisfaction and ultimately adherence. Despite the single blind nature, few participants were able to identify which condition they were under, with 36% in HQ-D, and 15% in LQ-D guessing correctly. It is possible that the higher detection in HQ-D was due to the greater proportion of animal-derived foods i.e., in lunch meal option 2, HQ-D were provided with chicken and mushroom soup and LQ-D had tomato soup. In this situation, the obvious presence of animal-based products may have unveiled the condition; although considering it was unknown what was in the other condition, this remains speculative.

### *6.8.4 Issues and assumption with weighing and packaging of prepared food*

All food and supplemental drinks were weighed and provided to participants during the 10-day dietary intervention in **Chapter 5** using food containers. As mentioned, these allowed for easy transportation for participants and made weighing and accurate labelling possible. However, placing food in these containers meant residual food was left, where likely the amount varied between participants (due to differing diligence) and according to the type of foodstuff. This is also a consideration for the breakfast food provision in **Chapter 4** since the smoothie ingredients were blended and whole food breakfast components pre weighed in plastic containers. As demonstrated in **Figure 6.3** residual foodstuff in containers was near unavoidable and varied according to the viscosity of the substance. The same is also true for protein smoothies where each time there was transfer of the ingredients from blender to pot and then to be drank there was some loss or residual substance left **Figure 6.4**. Hence, the less than 100% efficiency of the transfer of foods from container to consumption means that the values reported for intervention dietary intake are a 'best case' scenario. In reality, less foodstuff, including whole food-derived and smoothie-derived protein was consumed than provided. Given this loss was inconsistent across different food stuff and even likely between blends of the protein drink, it is challenging to account for accurately. It could be considered that more food should be provided (i.e., provide 10% more of each to account for typical losses) however this would dampen the rigor taken to control for protein dose and source.

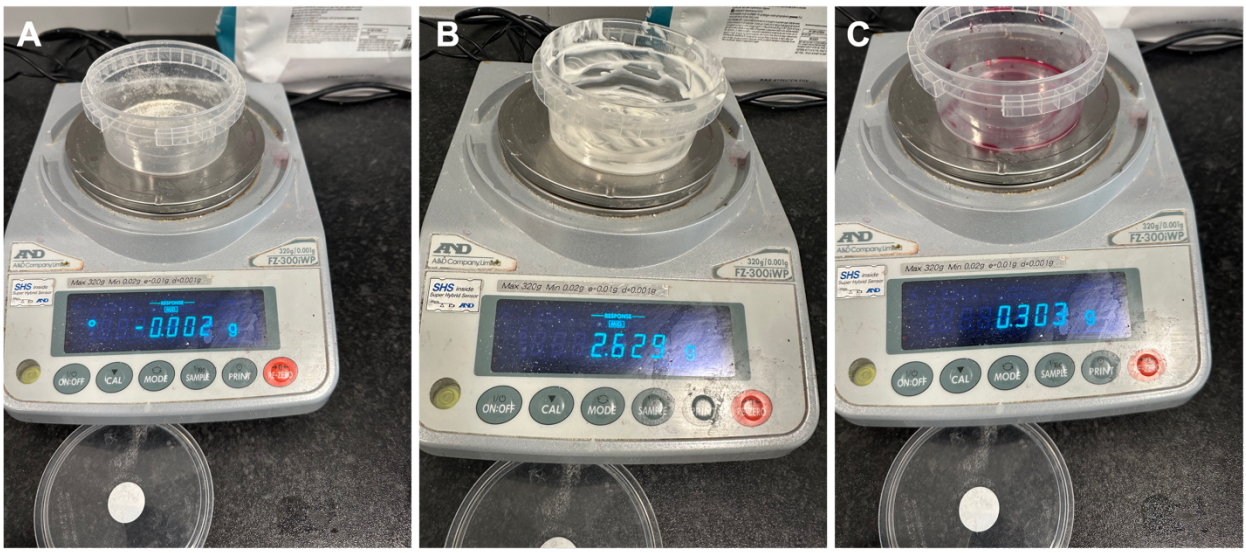


**Figure 6.3.** Depiction of transfer efficiency from storage receptacles during breakfast meal

Net food stuff weight following decanting from container after being weighed (relative to body mass requirements) for muesli (**A**) with a negligible net loss, Greek yoghurt (**B**) with 2.6g net residual loss and blueberries (**C**) with 0.3g net residual loss in containers.

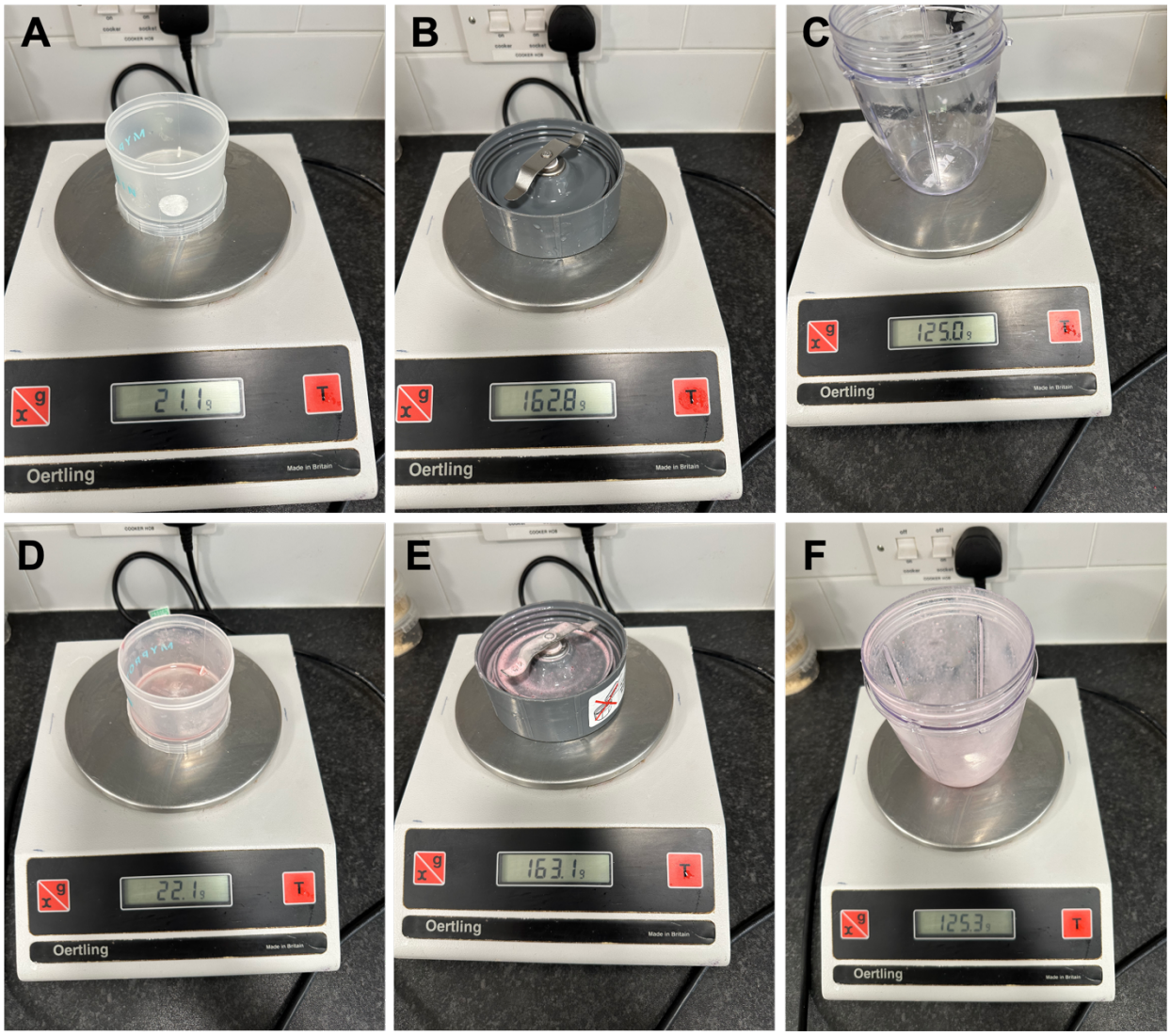


**Figure 6.4.** Depiction of product lost during protein smoothie formulation

Ingredients were weighed out individualised to body weight (for protein powder to be 0.13 g·kg $BM^{-1}$) and placed in tubs (**A**) with no residual for protein powder or frozen fruit, although cranberry juice left a residual ~1g (**D**). When blended and decanted into containers, there was a residual ~0.3g of production on Lid (B and E) and 0.3g in the container (**C** and **F**)

## 6.9 Mitigations for bias

Participants were randomised upon entry into the trial, according to sex, with potential participants provided a study code ahead of screening which was already preassigned to a condition (relevant for **Chapter 4** and **Chapter 5**).In doing this, the risk of bias as to how someone may respond to the trial was diminished, and it was encouraged for there to be a representative and balanced pool of males and females in both groups. Additionally, participants who were following an exclusively omnivorous or plant-based diet were excluded from the study, not only did this minimise any potential bias of habituation for either dietary intervention, but further meant that dietary considerations could not influence the randomisation. Despite best endeavours, there was a significant age difference between LQ-D and HQ-D (**Table 5.1**). Whilst undesirable, it is an inherent consequence of the randomisation, as splitting the cohort in other ways e.g., for sex would have meant there was no significant difference in age between groups (males 61 ± 7 years, females 62 ± 6 years, *P*=0.53). It is possible that the younger age of participants in LQ-D may have contributed to the greater total workload completed during RET (**Table 5.6**), especially since LQ-D were typically more active as shown by habitual MVPA (**Table 5.1**). Despite this consideration, participants were encouraged equally and the resultant trained iMyoPS did not differ between LQ-D and HQ-D.

## 6.10 Real world applicability

### *6.10.1 Accounting for variations in physical activity*

Each apparently healthy individual has a varied physical activity pattern, be it different activities of daily living, time of activity or type of activity. In **Chapter 5,** participants had to travel to undertake metabolic trial visits and RET sessions and as such the activity type was thought to vary from non-exercise days due to the act of

travelling. However, there was no significant difference according to wrist worn accelerometery in physical activity between visit days and non-visit days. However, the wrist worn accelerometer did not account for the additional activity completed on the knee extension machine. Indeed, on these days due to the substantial training volume (**Table 5.1)** we estimate a greater energetic cost compared with rest days. Given this consideration, there was not a compensatory increase in calorie provision on the days (Meal rotation 2; **Table 5.4**), due to prioritising matching meal options 1 and 2 and ensuring protein provision was matched between HQ-D and LQ-D. Considering the day-to-day variation, the provision of a stagnant and individualised amount of food for each meal rotation may have encouraged negative energy balance in some participants. Indeed, during the intervention, HQ-D were provided with an average of -29kcal and for LQ-D +55kcal compared to total energy estimations from Harris Benedict (**Table 5.1**). Whilst calorie provision was remarkably close to estimations, the estimations were static for each person and do not reflect daily activity alterations. Indeed, an increase in activity does not always necessitate greater ad libitum food intake [41], although in the present study increasing food intake was discouraged as calories, protein dose and protein source were all tightly controlled to enable causality to be drawn from any differential effects. Reports of hunger during the intervention were not uncommon, likely due to the limited food selection and potential interplay of heightened hunger from decreased whole food intake due to supplemental protein increase [25, 26]. Henceforth, allowing participants to consume additional calories may have ameliorated any energy deficit, and would offer greater efficacy for maximising RET-induced anabolism, particularly over extended periods. Indeed, it was necessary to tightly control diet whilst monitoring uncontrolled variables such as physical activity. to allow myself to draw conclusions about the effectiveness

of the 10-day intervention in Chapter 5. However, the act of controlling diet or any parameter of typical daily living partly confounds the real-life translatability. Nonetheless, the investigations were necessary to better understand the role of primarily plant vs primarily animal sources for muscle anabolism, adaptation and overall metabolism.

### *6.10.2 Future directions for attention to protein source with advancing age*

When protein intake is very low i.e., ~0.6 g·kg $BM^{-1}$·$day^{-1}$ and provided from diet alone, i.e., akin to that of **Chapter 5**, the source of protein may be particularly important, especially if rapidly digested supplemental proteins are not used. Likewise, compromised intakes below the RDA are commonly seen in acute illness and hospitalisation [42–44]. In both scenarios whereby all protein intake is from whole foods, and not combined with protein supplements, the reliance on exclusively plant or lesser quality protein sources may exacerbate negative nitrogen balance and eventual muscle mass loss. Whilst this concept remains to be elucidated, the lower digestibility commonly seen in plant-based whole foods likely delays the delivery of AAs [45]. Further, the bioavailability of EAAs is lower following consumption of plant-based whole foods [46] compared to better digested animal sources [47]. Hence while protein values displayed on food labels show absolute protein content, it is a theoretical maximum as the proportion of this protein to be effectively used by the body and skeletal muscle likely differs between plant and animal-based whole foods owning to differences in food matrix and digestibility, although this remains to be elucidated. It would therefore be of interest to identify if manipulating the source of protein exclusively via whole foods when protein intake is lower than the maximum (i.e., ~1.0 g·kg $BM^{-1}$·$day^{-1}$) differentially affects nitrogen balance and MPS. Indeed, manipulating

protein content with whole food alone at even lower doses such as those seen in hospilatisation would also be of interest; although such restriction of dietary protein may perturb muscle anabolism and thus require post intervention rehabilitation to compensate any skeletal muscle loss.

### 6.11 Conclusions

This thesis compiles evidence of age-related dysregulation at the cellular level within myofibers, with no apparent differences between type I and type II fibres using immunofluorescence microscopy (**Chapter 2).**Herein, an extensive review of literature in **Chapter 3** suggests resistance exercise and high protein intake are needed to maximise muscle mass maintenance with advancing age, however, there are barriers to undertaking exercise and consuming large protein doses. As such, protein nutrition is an easily modified factor whereby attention to the source of protein (i.e., consuming higher quality, more EAA-rich sources) may be important when protein intake is below the threshold to maximise anabolism in older adults. For the first time, I show that despite similar EAA profiles, whole food breakfast alongside either an EAA-rich whey protein smoothie drink or lesser EAA-containing pea protein smoothie drink evoke similar postprandial appetite responses (**Chapter 4**). Nonetheless, divergent leucinemia was observed and due to the poignant role of leucine in MPS stimulation, it was theorised that a low-moderate protein diet supplemented with whey would augment the anabolic response more so than supplementing with pea. In **Chapter 5**, novel investigations into integrated rates of muscle protein synthesis following 10-days of primarily plant-derived or animal-derived protein diet, with unilateral RET were evaluated. The findings of this chapter are the first to show that even at low-to-moderate protein doses, which are typical of middle to older aged adults, the source

of protein when provided as supplements alongside whole foods does not offer differential rested or resistance exercise induced MPS in middle to older aged adults.

The data within this thesis capitalise on the shortcomings of previous studies and offer great translatability for several reasons including i) inclusion of male and female middle-aged as well as older adults, ii) implementation of feasible protein doses commonly consumed by the target population and iii) determination of the effects of whole food consumption alongside supplements, rather than supplements alone. These findings do not negate a role of protein source at very low protein intakes and invite future studies to continue to investigate the role of protein source when purely manipulated by whole foods alone. Overall, when protein dose is moderate, protein from either EAA rich whey protein or lesser EAA rich pea protein offers comparable appetite responses, overall aminoacidemia and supports similar stimulation of MPS over 10-days. Whilst interventions of this length are too short to see substantial metabolic and musculoskeletal remodelling it provides the scientific basis for future endeavours in this field.

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