THE INFLUENCE OF OPTIMAL DIETARY PROTEIN INTAKE FOR MUSCLE METABOLISM ACROSS THE LIFESPAN

By

Sophie L Mathewson, BSc., MSc.

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ABSTRACT

Research has demonstrated a deterioration of skeletal muscle mass and function with increasing age (termed ‘sarcopenia’), and protein nutrition, in particular amount and source of protein, has been highlighted as a key factor regulating its progression. This thesis endeavours to explore the role of protein dose and source on muscle metabolism across the lifespan; from younger adults to frail older adults in care homes. Chapter 2 of this thesis explores the causes of protein-energy malnutrition in the residential care setting and evaluates the interventional literature targeting this. A review of the literature establishes that oral nutritional supplementation and protein-fortified foods are most commonly used to increase daily protein intake in care home residents. However, the effectiveness of these on musculoskeletal health outcomes and their compliance rates are unclear and conflicting due to the complexity in carrying out interventional work in this setting. Chapter 3 of this thesis investigates the role of habitual dietary protein intake on physiological muscle parameters in healthy older adults, with results suggesting that there are no differences between the habitual protein intakes of these two populations. However, whole-body lean mass and knee extensor strength were significantly higher in younger adults, suggesting that in recreationally active, healthy older adults, other factors aside from habitual protein intake are playing a role in regulation of muscle metabolism. To further explore this, Chapter 4 aims to develop a human serum *ex vivo* C2C12 co-culture model to explore the use of fasted *vs.* fed serum to investigate muscle responsiveness to feeding, with results demonstrating negligible differences between serum conditions. Further optimisation of this model is needed, with no clear protocol for AA starvation period, serum treatment concentration, nor media glucose content yet identified. The final data chapter of this thesis explores the amino acid (AA) kinetics in young and older adults following ingestion of a vegan-blend and whey protein, and highlights a lower circulating concentration of several AAs, including leucine, following consumption of a vegan-blend protein in comparison to a whey protein, with little effect of age. Further investigation confirmed the muscle protein synthesis (MPS) response of C2C12 myotubes to AA treatment mimicking vegan and whey protein compositions. However, when ‘preconditioning’ C2C12 myotubes with young and older adult fasted serum prior to AA treatment, there is negligible age nor protein source differences in the myotube MPS response.

This thesis establishes that despite declines in muscle mass and strength in healthy older adults, there are no differences in habitual protein intake in comparison to younger adults, suggestive that a higher daily protein intake may be needed to fully stimulate MPS and prevent development of sarcopenia. In older adults residing in residential care, the need to increase habitual protein intake is even more important, however implementing protein supplementation strategies is increasingly complex in this setting. When considering protein quality, evidence suggests that the AA composition and resulting circulating serum AAs favour a higher anabolic potential of animal-based in comparison to plant-based proteins, however, further research is needed to establish the role of age. The use of an *ex vivo* co-culture model throughout this thesis highlights its potential role to investigate anabolic response to nutritional status and disease state, however, further optimisation is necessary to increase the reproducibility of the presented results.

Keywords: Sarcopenia, Protein Nutrition, Muscle Protein Synthesis, Ageing

COVID IMPACT STATEMENT

The COVID-19 pandemic considerably impacted the planned research in this PhD. Initially, this PhD aimed to investigate barriers to protein intake and protein responsiveness in frail older adults in nursing homes. Although the initial ~6-months of this PhD involved designing and obtaining ethical approval for this study, the COVID-19 pandemic meant that face-to-face research was not permitted in UK care homes from March 2020, with no indication of when regulations would change. Whilst unable to complete any human research trials, the work presented in Chapter 2 (narrative review) and Chapter 3 (secondary data analysis) of this thesis were completed. Due to slow reintroduction of human research trials in the care home setting following the COVID-19 pandemic, a decision was made to change the experimental work of this PhD. Still investigating the role of protein intake on musculoskeletal health outcomes, the decision was made to progress with a wet-lab based project, of which the results are presented in Chapters 4 and 5 of this thesis. This was to ensure a sufficient amount of time to complete data collection and analysis to complete this PhD thesis.

DECLARATION

I declare that the research presented in this thesis is the author's original work (Sophie L. Mathewson) other than that stated below. This thesis has not previously been submitted to this or any other university for a degree and does not incorporate any material already submitted for a degree.

Chapter 2: Overcoming protein-energy malnutrition in older adults in the residential care setting: A narrative review of causes and interventions

Mathewson SL, Azevedo PS, Gordon AL, Phillips BE, Greig CA. Overcoming protein-energy malnutrition in older adults in the residential care setting: A narrative review of causes and interventions. Ageing Res Rev. 2021 Sep;70:101401. doi: 10.1016/j.arr.2021.101401. Epub 2021 Jul 5. PMID: 34237434.

- I conducted the primary literature search, writing, reviewing, and editing of the manuscript.
- Paula S. Azevedo assisted with the literature search and manuscript writing.

Chapter 3: Determining the influence of habitual dietary protein intake on physiological muscle parameters in youth and older age

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- This work is a secondary data analysis of previously published data (1). All data collection was carried out by Prof. Beth Phillips.
- I completed all data analysis and interpretation, and reviewed and edited the manuscript.

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No tables included in this chapter.

ABBREVIATIONS

α-KIC	α-ketoisocaproate
1-RM	1 repetition maximum
4E-BP1	eIF4E- binding protein 1
AA	Amino acid
ACN	Aceonitrile
ADL	Activities of daily living
Akt	Protein kinase B
AMPK	Adenosine monophosphate protein kinase
ANOVA	Analysis of variance
AS160	Akt substrate-160
AUC	Area under the curve
BAPEN	British Association for Parenteral and Enteral Nutrition
BCAA	Branched chain amino acid
BMI	Body mass index
BSA	Bovine serum albumin
CG	Control group
DA	Dietary advice
DC	Detergent compatible
DHEA	Dehydroepiandrosterone
DIAAS	Digestible indispensable amino acid score
DMEM	Dulbecco's modified eagle medium
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DRM	Disease-related malnutrition
DXA	Dual-energy X-ray absorptiometry

EAA	Essential amino acid
EAR	Estimated average requirement
eIF4E	Eukaryotic translation initiation factor 4E
ERK 1/2	Extracellular signal regulated kinase 1/2
ESPEN	The European Society for Parenteral and Enteral Nutrition
EWGSOP	The European Working Group on Sarcopenia in Older People
FBS	Fetal bovine serum
FSR	Fractional synthetic rate
GC-MS	Gas chromatography mass spectrometry
GSK3-ß	Glycogen synthase kinase 3ß
HG	High glucose
IKKß	IKB kinase ß
IL-1	Interleukin-1
IL-1ß	Interleukin-1ß
IL-6	Interleukin-6
IRS1	Insulin receptor substrate 1
KES	Knee-extensor strength
LG	Low glucose
MNA	Mini nutritional assessment
MNA-SF	Mini nutritional assessment- short form
MPB	Muscle protein breakdown
MPS	Muscle protein synthesis
mRNA	Messenger ribonucleic acid
MTBSTFA	N-methyl-tert-butyl dimethylsilyl-trifluoroacetamide
mTORC1	Mammalian target of rapamycin 1
mTORC2	Mammalian target of rapamycin 2
MUST	Malnutrition universal screening tool

NEAA	Non-essential amino acid
NF-κB	Nuclear factor-kappa B
NPB	Net protein balance
NRS-2002	Nutritional risk screening
ONS	Oral nutritional supplementation
P70S6K	Ribosomal protein S6 kinase beta-1
PBS	Phosphate buffered saline
PDCAAS	Protein digestibility-corrected amino acid score
PEM	Protein-energy malnutrition
PFF	Protein fortified-foods
PI3K	Phosphoinositide 3-kinases
PKC-α	Protein kinase C-alpha
PRAS40	Protein-rich Akt substrate-40
QoL	Quality of life
RDA	Recommended daily allowance
REDD1	Transcriptional regulation of DNA damage response 1
RET	Resistance exercise training
Rheb	Ras homolog enriched in brain
RSK1	Ribosomal S6 kinase
SEM	Standard error of the mean
SGA	Subjective goal assessment
SUnSET	Surface sensing of translation
TNF-α	Tumour necrosis factor- alpha
TSC1	Tuberous sclerosis 1
TSC2	Tuberous sclerosis 2
ULLM	Upper leg lean mass
WBLM	Whole-body lean mass

WBS Whole-body strength

LIST OF PUBLICATIONS AND CONFERENCE PROCEEDINGS

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Mathewson SL, Gordon AL, Smith K, Atherton PJ, Greig CA, Phillips BE. Determining the Influence of Habitual Dietary Protein Intake on Physiological Muscle Parameters in Youth and Older Age. Nutrients. 2021 Oct 12;13(10):3560. doi: 10.3390/nu13103560. PMID: 34684561; PMCID: PMC8539198.

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1 General Introduction

1.1 The Ageing Demographic

The current phenomenon of the ageing population, a term used to describe the concurrent rising life expectancy and declining birth rates, resulting in an increase in the proportion of the population as older people (2), has highlighted the importance of healthy ageing. The number of adults aged over 65 is expected to increase from ~524 million in 2010 to nearly 1.5 billion in 2050, which will account for approximately 16% of the global population (3). This is in line with the increase in life expectancy observed from 75.6 y in 2000 to 79.4 y in 2017 in UK males (4), and is attributable to higher living standards and healthcare advances (5). However, despite population demographics demonstrating clear increases in life-span, this is not accompanied by increases in 'healthspan', the years in which an individual is in optimal health (6). Healthy life expectancy is significantly lower than total life expectancy, meaning that in the UK men can expect to live with disability for 17.1 years and women 22.1 years (7). This is a key time period in which individuals may demonstrate diminished optimal functioning in activities of daily living (5). We need to understand and define the mechanisms interacting with the ageing process to improve the healthspan of the ageing population.

1.2 Sarcopenia and musculoskeletal health

1.2.1 Defining and diagnosing sarcopenia

The term sarcopenia was first introduced in 1989 by Irwin H. Rosenberg, meaning 'loss of flesh' (*sarx:* flesh, *penia:* loss) (8). Sarcopenia is a multi-faceted condition of later life, resulting in decreased strength and functionality and negatively impacting individual quality of life (9). Since its origin, there have been multiple definitions of this term. The European Working Group on Sarcopenia in Older People (EWGSOP) initially published a worldwide definition of sarcopenia in 2010, aiding the identification and treatment of

sarcopenia in the older population (9). Sarcopenia is characterised by a progressive decline in muscle mass and function, along with increased risk of adverse outcomes (10,11). Thereby, the EWGSOP first recommended definition of sarcopenia as presence of: criterion 1, low muscle mass; with evidence of criterion 2, low muscle strength; or criterion 3, low physical performance; or both criterion 2 and 3 (9).

Since EWGSOP first published a definition of sarcopenia, there have been further developments in sarcopenia research. These include the discovery that sarcopenia begins earlier in life (it is now known that the process of sarcopenia begins around the 4th decade of life (12)), and that there may be a greater impact of other age-related factors than initially considered. Furthermore, sarcopenia is considered to be a muscle disease, characterised predominantly by low muscle strength as opposed to low muscle mass, partially due to the difficulties that have arisen in accurately measuring muscle mass and quality (13,14). Due to uncertainty in the most effective variables to determine sarcopenia, and how to measure these, sarcopenia has been overlooked and undertreated in mainstream clinical practice. These developments have led to EWGSOP2 updating its definition, and diagnostic strategies of sarcopenia to further enhance sarcopenic care and awareness worldwide (15).

The EWGSOP2 operational definition of sarcopenia states that it is a progressive and generalised skeletal muscle disorder, associated with increased likelihood of development of various adverse outcomes including decreases in muscular strength, include falls, fractures, physical disability, and mortality (15). Due to the strong association of muscle strength with the listed adverse outcomes, this factor is a key primary parameter in EWGSOP2's updated 2018 definition of sarcopenia. The updated recommended sarcopenia definition states that probable sarcopenia is defined by low muscle strength, and diagnosis is confirmed with additional documentation of low muscle quantity or quality. The level of sarcopenia is

considered as severe if there is an additional low physical performance demonstrated (15). Clinically, sarcopenia has been defined as a loss of muscle mass associated with adverse outcomes such as loss of independence, an increase in frailty, and increased incidence of falls (10). The association of sarcopenia with various comorbidities and clinical whole-body outcomes demonstrates the need to fully understand the processes contributing to the onset and progression of sarcopenia in older adults.

1.2.2 Sarcopenia prevalence, causes, and consequences

Sarcopenia prevalence is increasing worldwide due to the ageing population, however, discrepancies in methodology for sarcopenia diagnosis have resulted in large variation in its reported prevalence. A systematic review and meta-analysis aimed to estimate sarcopenia prevalence and assess contributors to reported sarcopenia heterogeneity (16). This analysis reported a sarcopenia prevalence of 10% in healthy adults aged >60 years, increasing to over 50% in men over the age of 80 years, and 43.8% in women over 80 years when using the same sarcopenia diagnosis guidelines (17–19). These differences are suggested to be due to hormonal changes, with men often exhibiting declines in testosterone levels following the eighth decade of life. Furthermore, this meta-analysis uncovered a higher reported prevalence of sarcopenia in institutionalised individuals, and in non-Asian individuals in comparison to Asian individuals. This is attributed to cultural differences including body size, dietary habits, and physical activity levels (16,20). Despite the highlighted differences, results demonstrate that a large proportion of the older population have sarcopenia and early diagnosis is needed to best minimise adverse outcomes (16).

Sarcopenia is a multifactorial disease with a complex aetiology. The aetiology of sarcopenia is in part, due to age-related changes such as alterations in hormone and circulating cytokine levels. However, lifestyle factors such as physical activity levels, diet, and smoking

play a large part in the progression of sarcopenia (21). In particular, physical inactivity has been shown to be associated with a loss of muscle mass and strength irrespective of age (22,23), with forced inactivity, such as bed-rest, studies mimicking an 'accelerated ageing' process (24–26). Research in master athletes has demonstrated the influence of physical activity on magnitude of muscle function decline in older adults (27–29), with sedentary individuals exhibiting accelerated muscle loss, thereby increasing their comorbidity risk (30) in comparison to master athletes.

The mechanisms underpinning the inherent progression of sarcopenia with increasing age are yet to be fully elucidated, however, there are several cellular mechanisms associated with sarcopenia development. These include, but are not limited to: a negative net protein balance (NPB), resulting from blunted muscle protein synthesis (MPS) levels (31) as opposed to an increase in muscle protein breakdown (MPB) (32); anabolic resistance of older muscle to anabolic stimuli such as mechanical loading (33) and protein ingestion (32); loss of motoneurons (34), infiltration of fat and connective tissue into the muscle (35); anabolic hormone changes (36); and increased release of inflammatory cytokines (37).

1.2.3 Burden on healthcare

Due to the complex nature of sarcopenia and its association with several negative health outcomes, sarcopenia presents a significant societal impact. As previously mentioned, sarcopenia has several negative associations including a decrease in functionality and independence which can lead to decreases in quality of life (15). However, when considering the burden sarcopenia places on the healthcare setting, the associated decrease in functionality and physical independence can lead to an increased risk of disability development. It is commonly reported in cross-sectional research that individuals with severe sarcopenia are at increased risk of developing disability, and may be 2-5 times more likely to develop

functional impairments compared to older adults with a normal muscle mass (17,38–41). Furthermore, adults with sarcopenia have an increased risk of hospitalisation and increased costs associated with their hospitalisation regardless of whether they were older or younger than 65-years (42). Research reported that individuals diagnosed with sarcopenia upon admission had costs approximately 5-fold higher than those without sarcopenia (42). However, heightened costs are also present in individuals in the community. Research investigating the association between muscle parameters, activities of daily living, and healthcare costs in individuals in adults aged over 65 living in an assisted living facility uncovered a strong correlation between muscle strength and physical performance with activities of daily living. Specifically, a low gait speed and slower chair stand were associated with increased probability of activities of daily living disability, and these were associated with a lower quality of life and increased healthcare costs (42). Estimates suggest that in 2000, $18.5 billion, which equates to 1.5% of total US health expenditure was as a result of sarcopenia and related conditions (43). This highlights the significant financial burden or sarcopenia on the healthcare settings, placing increasing interest on research combatting development of sarcopenia and its associated conditions.

1.3 Anabolic resistance

1.3.1 The role of anabolic resistance in ageing skeletal muscle

As previously described, older age is associated with a progressive loss of skeletal muscle mass and function (sarcopenia). The underlying pathology of sarcopenia is yet to be fully understood, however the loss of muscle protein is known to be caused by an imbalance between MPS and MPB (44), with many factors contributing to this imbalance. Research investigating whether this imbalance results from alterations in MPS or MPB, or a combination of the two, report conflicting findings. Early work reported that this imbalance

may be as a result of blunted basal MPS (45) and elevated basal MPB (46) in older adults, however this has since been disproven, with later research demonstrating no differences in basal MPS nor MPB in healthy older compared to younger adults (32,47). Cuthbertson and colleagues compared MPS responses in young and older adults to ingestion of essential amino acids (EAA) (2.5-40g), and showed that above 5g EAA, there were greater MPS increases in young compared to older men (44). To support this, if alterations in basal NPB were the primary reason for muscle protein loss, a faster rate of muscle loss would be observed in older adults than that widely reported (~1-1.5% muscle loss per decade) (48). Therefore, another factor must be contributing to NPB and resulting sarcopenia progression in older adults. Following research suggested that this factor is the inability of ageing skeletal muscle to respond to anabolic stimuli such as protein provision (and resulting hyperaminoacidemia (49,50)) or muscle contraction (i.e. resistance exercise (33)) to the same magnitude as young skeletal muscle (44). This phenomenon has been termed 'anabolic resistance', and its concept is illustrated in Figure 1.1.

While an abundance of studies support the notion of anabolic resistance and a blunted MPS response to anabolic stimuli (44,51,52), some research has conflicted this, reporting no difference in the ability of older muscle to respond to anabolic stimuli in comparison to younger muscle (47,53,54). For example, Symons and colleagues reported a ~50% increase in MPS in healthy young and older adults following consumption of 113g lean beef (equating to ~30g total protein) (54). These contrasting results are likely due to methodological differences in measuring MPS between studies. There are many other factors which may contribute to MPS responses other than total protein content, for example the source and amino acid (AA) composition of the provided protein source, the exercise load and volume, the timeframe of MPS measurement, and the subfraction of muscle protein being measured. This is well reported and discussed in a recent systematic review from Shad and colleagues (55). Despite

these conflicting results and assorted methodological differences, there is a consensus that anabolic resistance is a key contributing factor to onset and development of sarcopenia in older age.

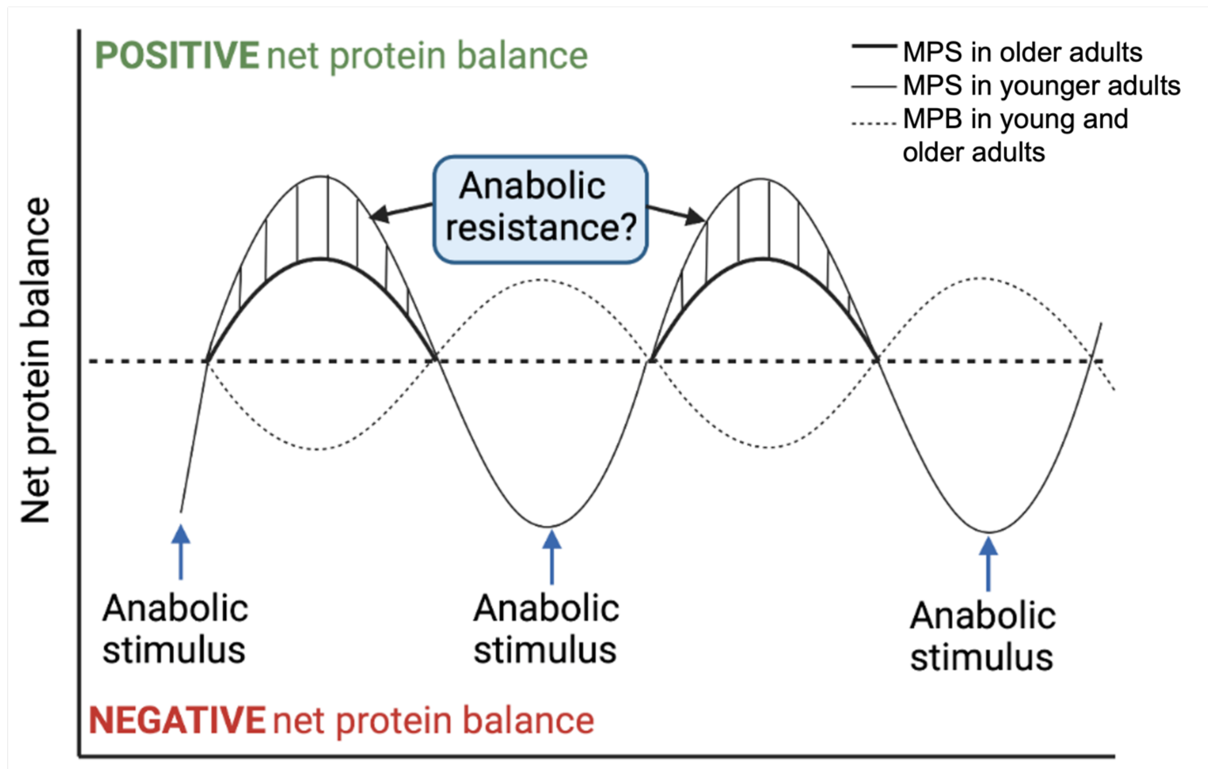

Figure 1.1- Representation of the balance between muscle protein synthesis and muscle protein breakdown and the influence of anabolic stimuli on net protein balance. Figure re-drawn from Breen and Phillips (2011)

1.3.2 Potential mechanisms of anabolic resistance development

Due to the prominence and adverse outcomes associated with sarcopenia, understanding the mechanisms of how anabolic resistance develops is important. The full mechanisms underpinning anabolic resistance are yet to be elucidated, but most are factors associated with the ageing process and a decrease in habitual physical activity. A summary of the known factors contributing to the development of anabolic resistance is illustrated in Figure 1.2.

A common factor between sarcopenia and many other age-related diseases is an increase in chronic low-grade inflammation, a condition which is often referred to as 'inflammageing' (37,56). Inflammageing is hallmark of ageing, and has been demonstrated to be an independent risk factor for mortality and morbidity (57). Inflammageing is thought to be a result of activation of pathways including the ubiquitin-proteasome and nuclear factor-kappa B (NF-kB) pathways, resulting in an increase in release of proinflammatory cytokines such as tumour necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) (58–60). There are several possible sites of release of cytokines, including but not limited to sites of infection, endothelial cells in the vasculature, and adipose tissue. Systemic inflammation in older adults does not necessarily have a direct effect on postabsorptive NPB, however, it may encourage anabolic resistance of ageing muscle to hyperaminoacidemia, thereby accelerating sarcopenia development (61). Animal research has demonstrated that administration of IL-6 or TNF-α increases skeletal muscle catabolism, and in older adults it has been reported that higher levels of inflammatory cytokines IL-6 and C-reactive protein are associated with a 2 to-3 fold risk of losing greater than 40% handgrip strength over 3-years (62). Although not the focus of this thesis, the interaction between systemic inflammation, anabolic resistance, and obesity should be noted. It is widely accepted that advancing age is associated with increases in body fat, and a key hallmark of sarcopenia is an increase in skeletal muscle fat infiltration (35,63), as demonstrated via immunohistochemistry. This is associated with increases in intramyocellular lipid content which may cause a reduction in anabolic sensitivity of skeletal muscle (64,65) through the associated increases in release of pro-inflammatory cytokines (66).

Another factor thought to be a key contributor in development of anabolic resistance in older age is insulin resistance. Insulin resistance is a term used to describe the inability of skeletal muscle glucose metabolism to appropriately respond to insulin signalling (67), occurring as a result of increased fat and macrophage infiltration into skeletal muscle. In

healthy muscle, increases in circulating insulin in turn increase muscle mass through the phosphoinositide 3-kinases (PI3K) protein kinase B (Akt) pathway, stimulating mammalian target of rapamycin-1 (mTORC1) (68). Ageing is associated with insulin resistance (1,58), however this is thought to be a result of a decrease in habitual physical activity and increases in body fat with increasing age as opposed to the inherent ageing process (59,60,69). An abundance of research has reported the role of inactivity on insulin resistance, with as little as 7 – 14 days of inactivity promoting insulin resistance (59,60,70). Insulin signalling has been demonstrated to be integral to the response of skeletal muscle to AA nutrition, with MPS responses to AA nutrition negatively associated with whole-body fat mass and insulin sensitivity (71). This is likely due to the aforementioned interaction between insulin signalling and the mTOR pathway (72,73).

A major contributor to regulation of MPS and MPB are sex hormones (36), with various hormones including cortisol (74), dehydroepiandrosterone (DHEA) (75,76), estrogen (77), and growth hormone (78) associated with ageing and alterations in muscle mass. There is an optimal hormonal milieu needed to combat sarcopenia progression in older age. However, ageing and associated increases in whole-body fat mass and inactivity have negative impacts on this milieu, with declines in hormones associated with anabolism (i.e. growth hormone, insulin-like growth factor-1, testosterone) and elevations in hormones associated with catabolism (i.e. inflammatory cytokines IL-6, IL-1, TNF-α) thereby favouring a blunted NPB. Ageing is associated with small increases in cortisol, a hormone which inhibits AKT, a key anabolic signal, leading to increases in protein degradation as a result of atrogene activation (74). Declines in DHEA levels have also been reported in older age, however supplementing DHEA in older adults resulted in increases in bone density, testosterone, and oestradiol levels, without effect on muscle size, strength, and function

(79,80). Thereby, the significance of DHEA changes on sarcopenia and anabolic resistance is unclear.

Testosterone has, though, been associated with increases in MPS and beneficial effects on musculoskeletal health. Testosterone levels in males decrease by ~1% per year, along with decreases in free testosterone and albumin-bound testosterone of ~2% per year (81). Testosterone plays a role in increasing MPS, with research reporting that supraphysiological exogenous testosterone supplementation can increase muscle mass and strength in both young and older men (82,83). Despite the reported benefits of testosterone supplementation, the risks may outweigh the benefits, with reports of sleep apnoea, thrombotic complications, and increased prostate cancer risk (83).

In women, increasing age is associated with declines in oestrogen (primarily occurring during menopause) which is thought to play a role in muscle mass loss. A positive association has been reported between oestrogen levels and lean body mass (84), highlighting the potential role oestrogen may play in sarcopenia and anabolic resistance development, likely through its interaction with proinflammatory cytokines such as IL-6 and TNF-α (83).

Satellite cells are the stem cells of skeletal muscle, and were first discovered in 1961 by Alexander Mauro when he viewed the quiescent mononucleated cells on the periphery or muscle fibres via electron microscopy (85). Satellite cells have the ability to quickly revert from a mitotically quiescent state to entering the cell cycle (86), thereby supporting the regeneration and building of muscle protein following damage. Ageing and inactivity have been associated with a decrease in number of satellite cells (87–90), highlighting their potential role in sarcopenia and anabolic resistance. Furthermore, skeletal muscle microvasculature (i.e. capillary density around skeletal muscle) has also been shown to decrease with ageing and inactivity (88–90). Yet, their role is not fully established as of yet.

Despite decreases in satellite cell number and microvasculature reported with increasing age, these factors have been shown to have no effect on postabsorptive muscle protein turnover, nor has a direct effect of these factors been associated with anabolic resistance to hyperaminoacidemia (61).

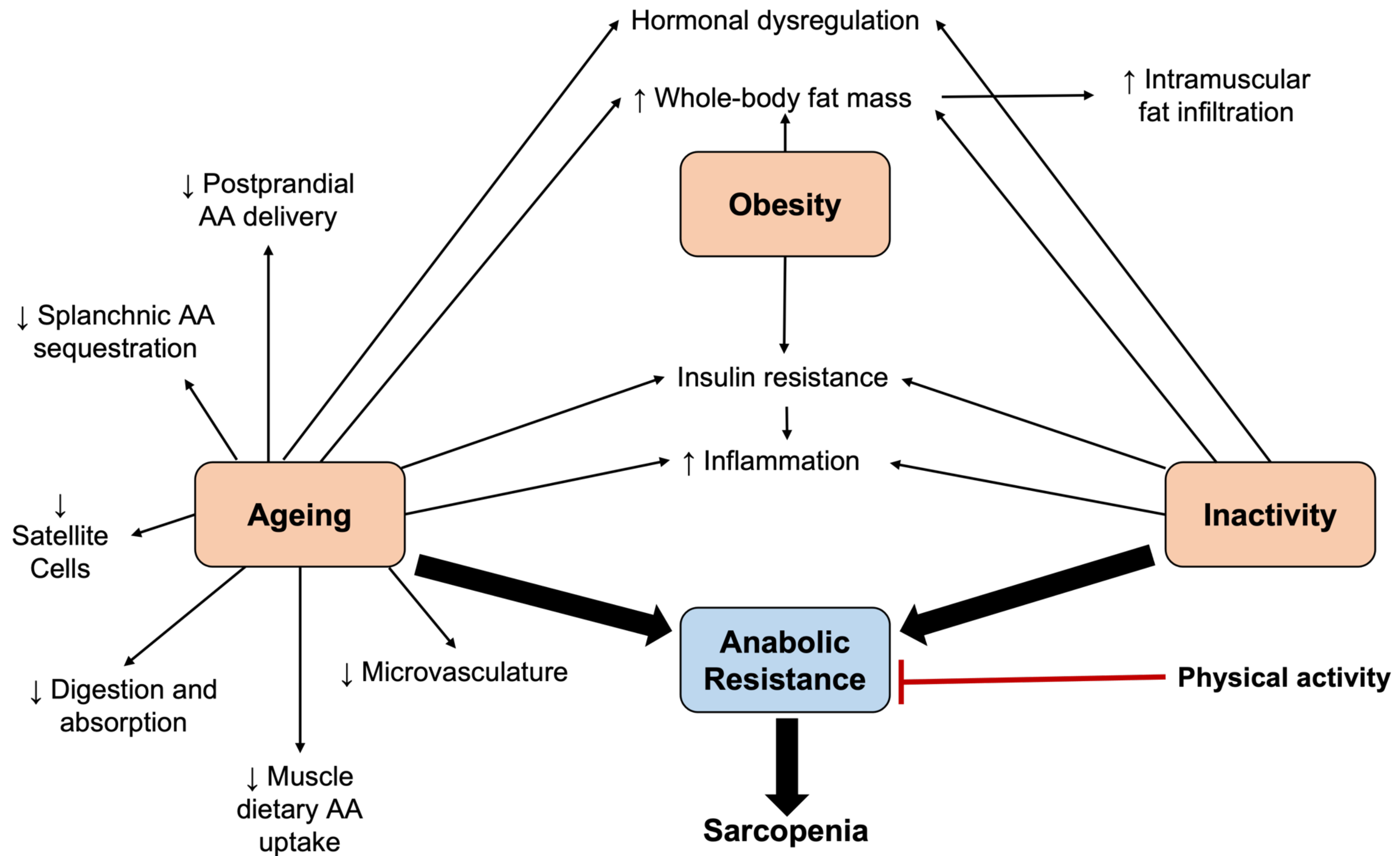

Figure 1.2- Graphical representation of the contributory processes of anabolic resistance. Anabolic resistance is a key contributor to progression of sarcopenia in older age. The main contributors to anabolic resistance development are graphically summarised here.

1.4 Frailty in older adults

1.4.1 Defining and identifying frailty

With increasing age, older adults are at a heightened risk of developing frailty, a condition which is associated with increased risk of adverse health outcomes, including mortality, institutionalisation, falls, and hospitalisation (91–93). Frailty is often defined as a biological syndrome, associated with a decreased reserve and inability to effectively respond to stressors as a result of loss of homeostatic balance and cumulative decline in physiologic systems (94–96). However, early work from Fried and colleagues (2001) developed a definition of frailty based on a 'frailty phenotype', allowing diagnosis and monitoring of frailty development (97). The frailty phenotype states that an individual is classed as frail if they present 3 or more of the following components of the frailty cycle:

1. Shrinking: unintentional weight loss of >5% body weight in one prior year.
2. Weakness: Grip strength in the lowest 20%, adjusted for sex and BMI (based on baseline values taken from general population).
3. Poor endurance and energy: determined via self-report of exhaustion
4. Slowness: Time to walk 15 feet in lowest 20%, adjusted for sex and BMI (based on baseline values taken from general population).
5. Low Physical Activity level: Low physical activity score (in the lowest quintile) based on MET's, adjusted for age and sex (based on baseline values taken from general population).

The frailty phenotype was developed by Fried and colleagues based on the 'cycle of frailty'. The cycle of frailty, as displayed in Figure 1.3, summarises the main factors contributing to frailty onset and development. This cycle, along with the 5 proposed components of the 'frailty phenotype' can be used to identify someone as 'pre-frail', in which

one or two criteria are present. In this state an individual is at high risk of progressing to frail, which is defined using this criterion as an individual displaying three or more of the proposed 'frailty phenotype' components (97,98). The frailty phenotype is arguably the most commonly used method of identifying an individual as frail, with clinical observations demonstrating the common development of frailty in this naturally progressing 'cycle' (98).

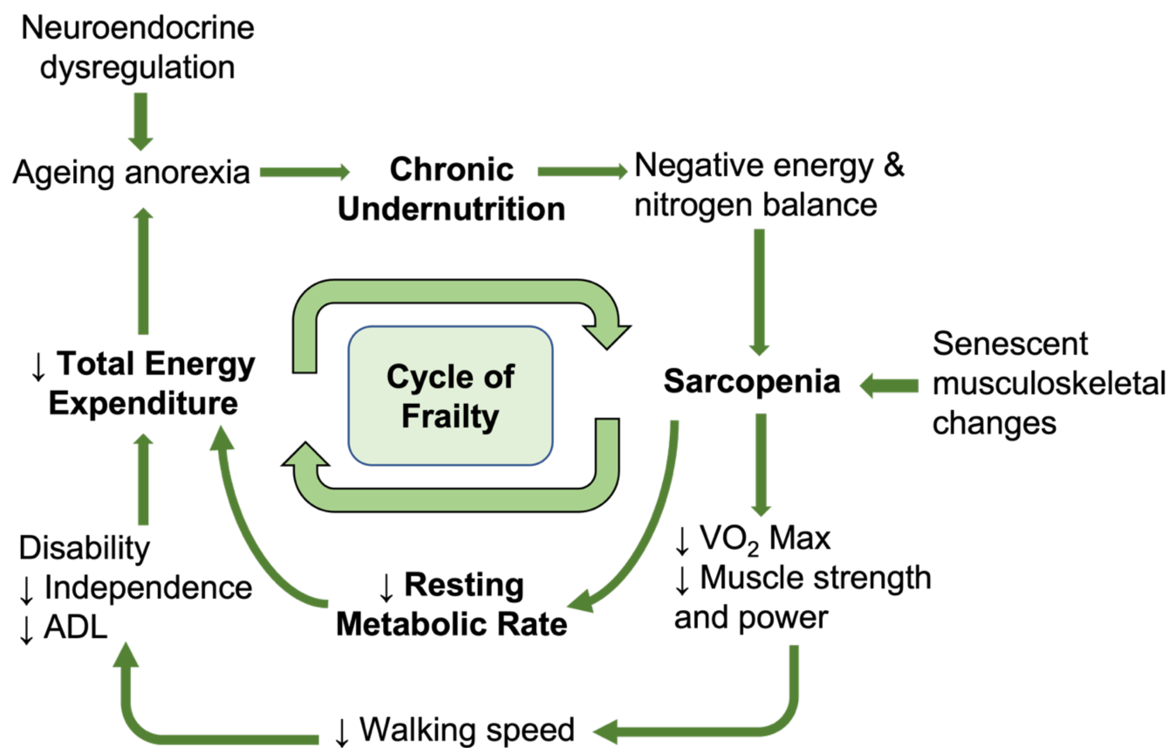

Figure 1.3- The Cycle of Frailty. Adapted from Fried *et al.,* 2001. Abbreviation: ADL, Activities of Daily Living

1.4.2 Frailty and musculoskeletal health

As referenced in the cycle of frailty, a key contributor and outcome of frailty is sarcopenia (97). As previously mentioned, sarcopenia is a term used to describe the age-related loss of skeletal muscle mass and function (8). There is great overlap between the development of sarcopenia and frailty, with many overlapping factors and adverse outcomes associated with both conditions. For example, sarcopenia has been reported as an independent

predictor of physical disability and development of mobility disorders (99,100), two key contributors and outcomes of frailty as identified in the cycle of frailty (Figure 1.3).

There is growing evidence to suggest that functional decline and mobility problems are one of the first markers of frailty (101). Declines in functional ability with increasing age (102) are associated with a loss of independence, and an inability for an individual to complete activities of daily living (ADLs) required for independent living such as washing, dressing, feeding, and toileting (103). Musculoskeletal decline can be a causative factor of functional impairment and disability, with statistics reporting musculoskeletal disorders to be one of the ten leading worldwide causes of disability (104). Furthermore, a decrease in functionality has been highlighted as a potential contributor to incidence of falls (105), with sarcopenic men reported to be twice as likely to experience falls (106) in comparison to non-sarcopenic counterparts.

1.4.3 Nutrition and frailty

When considering the pathophysiology of frailty, poor nutritional status is a key factor, with the potential to influence all 5 of Fried's proposed components of the 'frailty phenotype' (97). Undernutrition, the insufficient consumption of energy and nutrients to meet an individual's needs (107), is closely related with frailty. Both conditions are associated with increased risk of hospital admission, length of hospital stay, hospital readmission, institutionalisation, prescriptions, general practitioner visits, and dependency of others (107). Obesity has also been associated with increased frailty risk, with low muscle mass being the common factor between frail underweight and frail overweight individuals (107). The role of nutrition in frailty onset and development is of increasing interest as it is a modifiable risk factor, and thereby a possible interventional target.

Several factors relating to diet and nutrition have been investigated in regard to their association with frailty. The first is diet quality, with a ‘healthier’ diet being one containing a higher proportion of nutritious foods such as fruits, vegetables, and wholegrains (107). Several studies have investigated the association between diet quality and frailty, demonstrating a lower risk of frailty in individuals containing a ‘healthier’ diet (108), a higher risk of development from non-frail and pre-frail to frail after a 4-year follow up in those with poor diet quality (109), and a lower likelihood of frailty development in a 3-year follow up in older men consuming a ‘healthy’ diet pattern (110). The latter results remained present after adjustment of confounding factors including age, BMI, social class, smoking, cardiovascular disease, alcohol consumption, and energy intake.

Several other dietary factors have been associated with frailty. An abundance of research has reported the benefits of the Mediterranean diet, which is characterised by low consumption of animal-based foods such as meats and dairy, high consumption of plant-based foods such as fruits and vegetables, and use of foods high in antioxidants and polyphenols such as dark chocolate, olive oil, and red wine (111). Both longitudinal (112,113) and prospective (114,115) studies have reported benefits of the Mediterranean diet on reducing frailty risk and incidence. Similar to the Mediterranean diet, research has investigated the role that pro-inflammatory diets have played in frailty-associated outcomes. Particular dietary components, such as omega-3 fatty acids which are commonly found in foods such as fish and vegetable oils, have been demonstrated to have a protective effect through various mechanisms such as a decrease in release of inflammatory cytokines IL-6 and IL-1ß from blood mononuclear leukocytes (116). Systematic reviews further demonstrated this relationship, reporting an increased risk of frailty in individuals with a higher dietary inflammatory index (107).

As previously discussed, there is a prominent role of dietary protein intake as a contributor to maintenance of musculoskeletal health in older age, particularly in delaying the onset and progression of sarcopenia (117), a key factor of the frailty phenotype (97) and contributor to frailty development. On average, older adults consume less protein than younger adults, despite older adults needing more protein than younger adults to combat the disruption in balance between MPS and MPB in older age (117). The relationship between a low protein intake and frailty has been investigated, with a meta-analysis demonstrating a lower prevalence of frailty in older adults with a high (compared to low) protein intake across cross-sectional studies (118). However, longitudinal research demonstrated that in older men aged >65 years, there was no association between baseline protein intake and development of pre-frail or frail status after a 4.6-year follow-up (119). Yet, longitudinal research in older Finnish women aged 65-72 years, a higher protein intake was associated with a lower likelihood of pre-frailty or frailty at 3-years follow-up. This study found that women in the higher tertile of animal, but not plant-based protein intake, had a lower prevalence of frailty (120). This highlights the possible role of not only protein intake, but protein source, in mediating musculoskeletal health and associated outcomes such as frailty in older age.

The role of protein source will be discussed later in this chapter. Overall, the research consensus suggests that a higher protein intake is linked to a lower frailty incidence, indicating that protein supplementation may be an effective method of improving frailty status. However, research investigating the role of protein supplementation on frailty prevalence and development is unclear. Oktaviana and colleagues conducted a systematic review and meta-analysis investigating the effect of protein supplementation on muscle mass, strength, and function - key components of the frailty phenotype - in frail older adults (121). Results demonstrated no significant effects of protein supplementation (up to 32g per day) on lean body mass, handgrip strength, leg extension, leg press, short physical performance

battery, or gait velocity. These results suggest that alone, protein supplementation does not benefit musculoskeletal or functional outcomes in pre-frail or frail older adults (121).

1.5 The role of protein source and composition in optimising muscle metabolism

1.5.1 The cellular mechanisms of stimulating MPS

As previously described, sarcopenia and frailty are both conditions associated with ageing which are characterised by a decrease in muscle mass and strength, and functional decline. It is therefore important to understand the cellular mechanisms of building skeletal muscle protein, with particular interest in this thesis on protein nutrition as a method of inducing skeletal muscle anabolism.

The protein kinase mTOR (often referred to as “mammalian TOR” or “mechanistic TOR”) is involved in many bodily processes regulating the use of energy or nutrients (122). In the context of this thesis, the importance of mTOR will be discussed in relation to regulation of skeletal muscle net protein turnover. mTOR is a target of rapamycin, a macrolide with broad antiproliferative properties. mTOR belongs to the PI3K family as an atypical serine/threonine protein kinase, and has 2 complexes: mTORC1 and mTORC2 (122). Structurally, mTORC1 is a large protein and contains the sub-units Raptor (123) and PRAS40 (124), and responds to both intracellular and extracellular cues. These include AAs, stress, oxygen, energy, and growth factors (122).

There are a large number of upstream cues mTORC1 senses and responds to. Directly upstream of mTORC1 are tuberous sclerosis 1 (TSC1) and TSC2, key GTPase activating proteins for Ras homolog enriched in brain (Rheb) GTPase (122). When GTP is bound to Rheb, this directly stimulates mTORC1 kinase activity, with GDP-bound Rheb (negatively regulated by TSC1/2) negatively regulating mTORC1 (125). The role of TSC1/2 is key in

transmitting upstream signals including growth factors and insulin to mTORC1, and is directly phosphorylated by upstream effector kinases such as protein kinase B (Akt/PKB), extracellular-signal-regulated kinase 1/2 (ERK1/2), and ribosomal S6 kinase (RSK1), resulting in mTOR activation (125–129). Additional upstream regulators interacting with TSC1/2 include IkB kinase ß (IKKß- inflammatory pathway) (130), glycogen synthase kinase 3ß (GSK3-ß- Wnt ligands pathway) (125), and transcriptional regulation of DNA damage response 1 (REDD1- hypoxia pathway) (122). Akt and Adenosine monophosphate protein kinase (AMPK) both act directly on mTORC1 in addition to through TSC1/2 (124), with Akt invoking a dissociation of PRAS40 from mTORC1, subsequently activating it, and AMPK directly downregulating mTORC1. Despite it being known that AAs act on mTORC1 independently of TSC1/2 (131), the molecular mechanism used by mTORC1 to sense rises in intracellular AA levels is not fully understood. It is now known that Rag GTPases are needed for AAs to stimulate mTORC1, which allows translocation of mTORC1 to the lysosomal surface (132,133). However, its full mechanisms are not yet uncovered.

As previously mentioned, mTORC1 is a key regulator of many cellular processes, and when active, can influence the processes of protein synthesis, lipid synthesis, lysosome biogenesis, autophagy, and energy metabolism (122). When considering its role in protein synthesis, activation of mTORC1 results in phosphorylation of downstream targets eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) and ribosomal protein S6 kinase (p70S6K) (126). 4EBP1 phosphorylation prevents its binding to eIF4E, subsequently allowing formation of the eIF4E complex and promoting cap dependent translation. Secondly, when activated, mTORC1 phosphorylates p70S6K subsequently stimulating ribosomal biogenesis and mRNA translation initiation and elongation (126). This

results in stimulation of MPS. The upstream and downstream processes of mTORC1 regulation are illustrated in Figure 1.4.

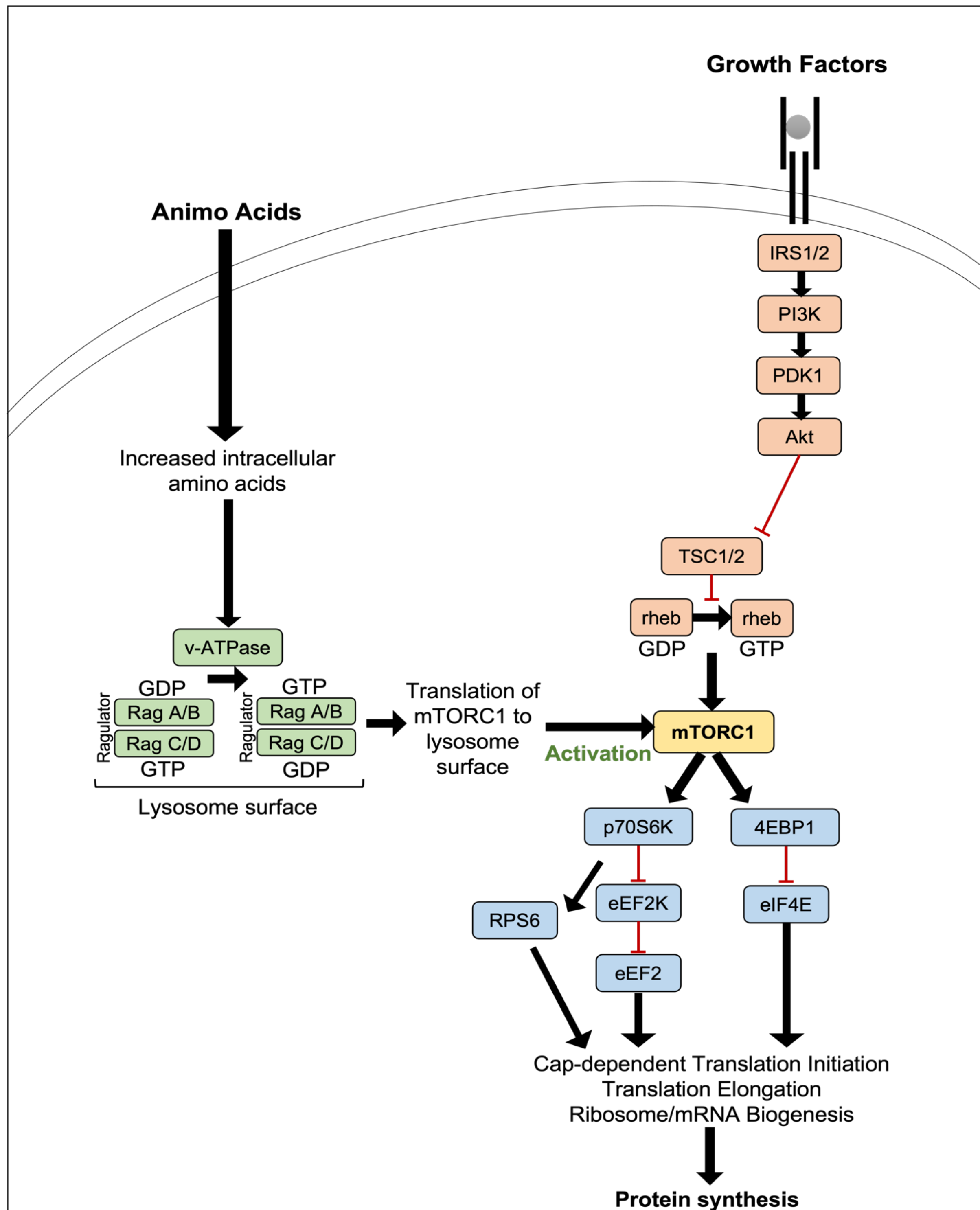

Figure 1.4- Signalling mechanisms of skeletal muscle protein synthesis. Graphical schematic reflecting a simplified version of the primary signalling pathways interacting with mTORC1 to contribute to MPS. Figure generated using Biorender. Adapted figure from Laplante and Sabatini (2012)

1.5.2 The role of protein quality in musculoskeletal health maintenance

The role of protein nutrition in maintenance of musculoskeletal health, particularly in older adults, is well reported. Protein nutrition is key in delaying the onset and development of sarcopenia and frailty in older adults. However, emerging research suggests that the 'quality' of a protein source may play a key role in predicting the anabolic potential of that source (134). There are several factors contributing to the quality of a protein source, notably including the protein source AA composition, the digestibility of the protein, and its digestion and absorption kinetics (135). There are proposed methods of quantifying the quality of a protein source, with the most recent and widely used being the digestible indispensable AA score (DIAAS). Briefly, the DIAAS is a method of calculating the AA quality of a protein source based on its ileal digestibility, with calculations used for each AA (135). When interpreting DIAAS scores, 100 or more is considered as an excellent score and 75-99 as good. It has been recommended that no nutrition claim be allowed for source or high protein for proteins with a DIAAS score less than 75 (136).

Before considering the AA composition of a protein, the different types of AA and their role in protein synthesis needs to be introduced. An AA is an organic molecule consisting of a basic amino nitrogen group ($-NH_2$), an acidic carboxyl group (-COOH), and an organic R group, all linked through a central carbon (137). There are 20 AAs which make up a protein, of which 9 can be classed as 'essential' AAs (EAAs), and 11 as 'non-essential' AAs (NEAAs). EAAs cannot be synthesised in the body so must be consumed in diet, whereas NEAAs can be made in the body from essential AAs or glucose (137). This will be described in further detail later in this thesis (Chapter 3), however, to note, the EAAs have been reported to be the only AAs needed to stimulate MPS (138,139).

When investigating the AA composition of a protein source and how this reflects on the 'quality' of the protein, leucine content has been highlighted to be of particular importance. An abundance of research has demonstrated that leucine is the key AA stimulating MPS (140–143). Recent research has demonstrated that the mechanism of this is through a protein named Sestrin2 (144). Following ingestion of protein and subsequent rises in intracellular leucine concentration, leucine binds with Sestrin2. This results in a dissociation of Sestrin2 from GATOR2 (a GTPase-activating protein), resulting in activation of mTORC1 and downstream signalling targets (134). Thus, it has been proposed that the leucine 'threshold', a term used to describe the action of rapid postprandial leucinemia and subsequent increase in intracellular leucine concentration, is the primary stimulator of MPS, suggesting that the leucine content of a protein may be a better predictor of postprandial anabolic response compared to total protein content of a food source (135). Additional EAAs which have been noted to play an important role, along with leucine, in stimulating MPS are lysine and methionine (145).

Another factor to consider when assessing the 'quality' of a given protein is its digestibility. Following ingestion of a protein source, it is digested and broken down into AAs and peptides. The body cannot absorb intact proteins; therefore, if a protein is not easily digestible this can hinder the ability of this protein to stimulate MPS (146). There are several factors which can influence how digestible a protein source is. These include the anti-nutritional factors in a protein source, such as presence of protease inhibitors, phytic acid, tannins, and lectins. These factors can reduce the digestive enzyme activity, reducing nutrient absorption (146).

An abundance of evidence demonstrates differences in postprandial regional metabolism of dietary AAs between protein sources (147–151). There are several factors

which can influence this, including the rate of appearance from the gut. Research has demonstrated that following soy protein ingestion there is a large, rapid increase in AA levels. Resulting from this, these AAs were catabolised and transferred to urea at a quicker rate compared to that following ingestion of a milk protein (147,150). This was attributed to differing absorption rates of the proteins and variations in gastric emptying (152,153), and can result in a lower peripheral AA delivery (150).

1.5.3 The role of protein source in musculoskeletal health maintenance

There have been worldwide increases in consumption of a vegan and vegetarian diet for an abundance of reasons. Many individuals are consuming a greater amount of plant-based protein due to sustainability factors, with plant-based proteins having lower greenhouse gas emissions in comparison to animal-based proteins (154). Furthermore, observational research has demonstrated the health-related benefits of plant-based proteins, exhibiting a strong association between plant-based protein consumption and decreased risk of several chronic diseases including type 2 diabetes, cancer, and ischemic heart disease (155,156). However, it has been suggested that plant proteins are generally of a lower protein quality in comparison to animal proteins, resulting from their amino acid composition and content, and protein digestibility (155,157). Some evidence has shown that this may result in a reduced ability to stimulate MPS (155,157).

As aforementioned, some research suggests that animal-based proteins are of a higher quality in comparison to plant-based proteins (155,157), partly due to a lower digestibility of plant-based proteins. The lower digestibility of plant-based proteins is likely due to their structure, including a high content in ß-sheet conformation which contributes to proteolysis resistance in the gastrointestinal tract (158), the presence of non-starch polysaccharides and fibres (159), and several antinutritional factors such as protease inhibitors (160). These all

contribute to reduced digestibility of the protein source. When assessing protein quality using the DIAAS method, the highest quality proteins (having a DIAAS score greater than 100) included pork, casein, egg, whey isolated soy, and potato proteins (161). To overcome this, it has been suggested that combining various different plant protein sources may be a feasible method of optimising their EAA content and digestibility of plant-based proteins. When measuring protein quality using the DIAAS score, a score of 100 was achieved when combining potato protein with fava bean and corn or pea and wheat (161), which would be considered as an excellent quality protein source (136). However, it must be considered that the DIAAS method of quantifying the quality of a protein was designed for a single protein source, not a blend of protein sources, meaning the true reliability of this has not yet been proven (155).

Further, when considering daily total energy intake in healthy adults, it is recommended that 10-35% of the total daily caloric intake should be from protein consumption (162). Among European and North American ovolactovegetarian, vegan, and omnivorous populations, vegetarians and vegans consumed less protein in comparison to omnivores (163). However, this was not below the daily recommended amount of 1.2-2.0g/kg/day (164–166). Yet, due to the reported lower quality of plant-based proteins, it is recommended that vegan athletes consume towards the higher end of the daily recommended protein intake (1.4-2.0g/kg/day) (155). It should be noted that total protein relative to total energy content tends to be lower in plant compared to animal proteins, meaning that in general, a greater amount of a plant protein source is needed to be consumed to match the total protein content of an animal protein source (155).

As previously discussed, a key factor influencing the quality of a protein source is its EAA content and composition, with a dose-response relationship previously reported

between ingested EAAs and postprandial MPS response (44). Plant protein sources contain all EAAs, but this tends to be substantially lower than animal protein EAA content, depending on the source (155). Specifically, the lesser anabolic properties of plant-based proteins has been alluded to reduced levels of EAAs leucine, lysine, and methionine (167), resulting in a dampened stimulation of the mTORC1 pathway and lesser MPS response. Furthermore, when there is an EAA deficiency in a protein source, this can result in the AAs present in the protein source not being effectively utilised for protein synthesis, resulting in their deamination and oxidation (157). The differences in EAA, and specifically leucine content (% total protein) between plant and animal-based protein sources, is displayed in Figure 1.5. It has been suggested that per meal, 700-3500mg of leucine is adequate to stimulate MPS, with higher intakes of >2.5g/meal for 3 meals a day suggested for older adults (168). Gorissen and colleagues reported that a 25g dose of whey protein provides an adequate amount of EAA and leucine to stimulate MPS, whereas to match that leucine content in a plant-based protein a much greater bolus dose would be needed (169). Yet, there is much variability between plant-based protein sources in their EAA and leucine content, with certain plant-based proteins including soy, brown rice, pea, corn, and potato protein meeting the WHO/FAO/UNU requirements (170). Due to the large variability between plant-based protein sources, this highlights the potential for use of a combination of different plant-based proteins to provide a higher quality protein blend without consuming an excess protein dose (167).

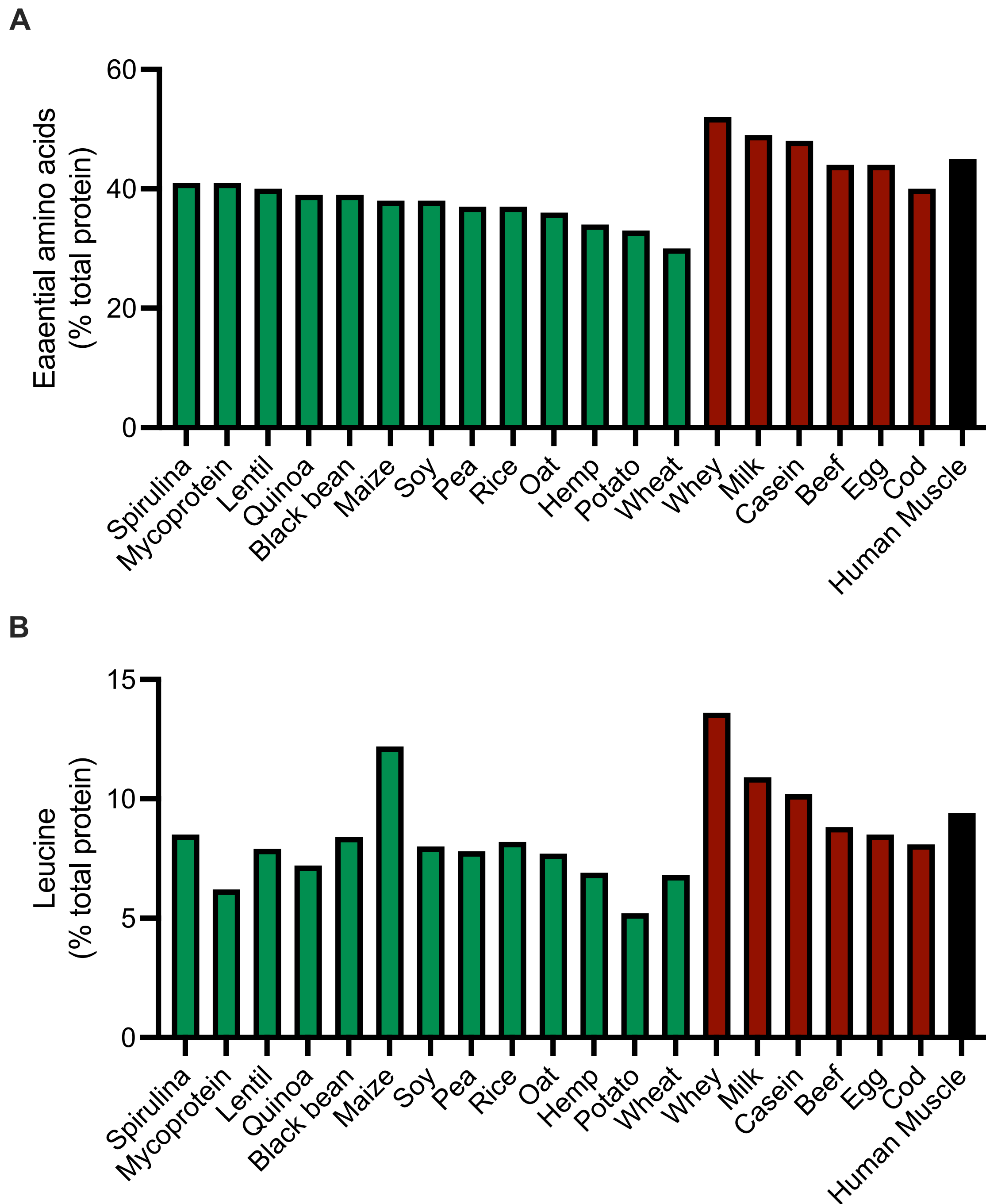

Figure 1.5- Essential Amino Acid and Leucine content (% total protein) of plant- and animal-based protein sources. Green bars show plant-based protein sources, red bars show animal-based protein sources. Figure redrawn from van Vliet et al., 2015

1.5.4 How different are the recommended protein requirements in older compared to younger adults?

Protein intake has been highlighted throughout this chapter as a key factor contributing to the growth and maintenance of musculoskeletal health, with particular importance placed on protein consumption in older adults. The current recommended daily allowance (RDA) for protein in older adults is no different to that in younger adults, with recommendations that 0.8 – 1.2g.kg^{-1}.day^{-1} is adequate to maintain muscle mass and function (170). However, these requirements are based on nitrogen balance studies, which come with several limitations including inaccuracy in measuring all nitrogen input and outputs, difficulties in determining short-term nitrogen turnover in muscle, and the inability to quantify the redistribution of nitrogen to tissues across the body (171). When considering how much protein older adults are consuming, wide variations have been reported. Longitudinal research has shown that in 1793 older men and women aged 68-82 years, protein intake on average was 1.0 ± 0.3g.kg^{-1}.day^{-1}, meaning half of this cohort were consuming less than 1g.kg^{-1}.day^{-1} protein (172).

An abundance of research has previously demonstrated the anabolic resistance of older muscle to anabolic stimuli, particularly protein provision (44,173,174), drawing into question why national guidelines do not explicitly highlight the recommendation for a higher protein intake in older adults. Early research demonstrated that after consuming the US protein RDA (0.8g.kg^{-1}.day^{-1}) for 10-days, older adults were in a negative nitrogen balance (175). Further, it has been reported that in sarcopenic and non-sarcopenic older women aged over 65 years, dietary protein was an independent predictor of muscle mass, with further reports that women consuming >1.2g.kg^{-1}.day^{-1} protein had a significantly higher muscle mass (176). Research-based recommendations now suggest higher protein intake for older

adults, with an average daily intake of 1.0-1.2g.kg^{-1}.day^{-1} recommended for sedentary adults aged 65+, and higher intakes recommended for active adults (177,178). If older adults do not consume adequate amounts of protein, the body will adapt to this by breaking down lean muscle tissue as a means of maintaining nitrogen balance, resulting in progression of frailty, sarcopenia, reduced quality of life (177).

1.6 Musculoskeletal ageing in the UK care home setting

The profound effects of sarcopenia and frailty on individual quality of life, functional capacity, and associated health outcomes in older adults have been introduced in the chapter. However, a population of individuals which has not yet been discussed is older adults living in long-term care homes. There are currently 425,000 older adults in the UK living in care homes (179); three times more people than are in hospital at any one time (180), with this number constantly increasing. In the UK, care homes provide accommodation for individuals who may be unwell, have a mental disorder, be disabled, or have been dependent on alcohol or drugs (181). Yet, the subset of this population which will be discussed in this thesis are specifically older adults aged > 65 years. Older adults make up the majority of the UK care home setting, with 91% of residents aged over 70, and a further 76% of individuals requiring mobility assistance (182). Due to the profound differences between community-dwelling older adults and older adults residing in residential care, many measurable research outcomes in healthy older adults cannot be directly compared to older adults in this setting.

As previously described, older adults are at increased risk of developing sarcopenia and frailty which can lead to several adverse outcomes including loss of independence, increased comorbidity risk, and loss of functional capacity, to name a few (15). Sarcopenia has been reported to be highly prevalent in older adults residing in residential care, in fact up to 6-times greater in comparison to community dwelling older adults (183,184). Associated

with sarcopenia, frailty has also been shown to be highly prevalent in the residential care setting, and is associated with increased risk of falls, fractures, and hospitalisation (185,186). In care home residents, individuals who likely already possess more comorbidities in comparison to community-dwelling older adults, incidence of a fall, fracture, or period of hospitalisation can be significantly more detrimental. A key factor which has been highlighted in this population to contribute to both development of sarcopenia and frailty is malnutrition (187).

Malnutrition can be defined as "a state of energy, protein, or nutrient excess or deficiency, resulting in adverse effects on body composition and function, and clinical outcomes" (188). Of particular interest in this thesis, protein-energy malnutrition (PEM), an insufficient protein and/or energy intake to meet the body's needs (189), can exacerbate declines in musculoskeletal health in older adults (190). Yet, difficulties arise in overcoming sarcopenia, frailty, and PEM in this setting as commonly used interventions and prevention strategies such as resistance exercise training and protein supplementations cannot be easily implemented in older adults residing in residential care (191). Several factors can contribute to the development of PEM in older adults, including a reduced appetite (192), presence of dementia (193,194), dysphagia (195), or dentition issues (196), and several psychosocial factors such as depression (197), education and income, race, and living situation (198). The burden of these contributing factors are often heightened in older adults in the care home setting compared to community-dwelling individuals, exacerbating the development of PEM. Yet, despite research reporting the long-list of adverse outcomes of PEM and the primary factors influencing its onset and development, recognising, diagnosing, and treating malnutrition in this setting can be a challenge (198).

Factors contributing to PEM, and possible nutritional strategies to overcome this will be further discussed in Chapter 2 of this thesis. Overall, research demonstrates that older adults residing in care homes are at a significant risk of developing musculoskeletal health disorders such as sarcopenia and frailty much higher than their age-matched community-dwelling counterparts. This highlights the need to fully understand and explore the factors contributing to progression of these conditions, the possible interventional strategies to prevent progression and onset of adverse outcomes, and the feasibility of implementing these in the care home setting.

1.7 Aims and Objectives

This thesis aims to use existing literature and primary data exploring the use of a novel cell culture technique to investigate responses and musculoskeletal health outcomes to protein ingestion across the lifespan in younger adults, older adults, and older adults residing in care homes, and potential methods of improving these outcomes.

The objectives of this thesis are as follows:

1. Review the existing literature exploring PEM in older adults in care homes, identifying possible causes, and the efficacy of different interventional strategies to improve musculoskeletal health outcomes.
2. Assess the pattern of habitual dietary protein intake of healthy young and older adults and how this influences cell-based and whole-body skeletal muscle outcomes.
3. Further the development of a novel human serum *ex vivo* co-culture cell culture technique to explore the muscle anabolic responses to fasted and fed human serum obtained following ingestion of plant-based vs. animal-based protein sources.
4. Explore the differences in quality of animal-based vs. plant-based protein sources, and how this may impact skeletal muscle metabolism in young and older adults.

2 Overcoming protein-energy malnutrition in older adults in the residential care setting: A narrative review of causes and interventions

Mathewson SL, Azevedo PS, Gordon AL, Phillips BE, Greig CA. Overcoming protein-energy malnutrition in older adults in the residential care setting: A narrative review of causes and interventions. Ageing Res Rev. 2021 Sep;70:101401. doi: 10.1016/j.arr.2021.101401. Epub 2021 Jul 5. PMID: 34237434.

2.1 Abstract

Malnutrition, in particular protein-energy malnutrition, is a highly prevalent condition in older adults, and has been associated with low muscle mass and function, and increased prevalence of physical frailty. Malnutrition is often exacerbated in the residential care setting due to factors including lack of dentition and appetite, and increased prevalence of dementia and dysphagia. This review aims to provide an overview of the available literature in older adults in the residential care setting regarding the following: links between sarcopenia, frailty, and malnutrition (in particular, PEM), recognition and diagnosis of malnutrition, factors contributing to PEM, and the effectiveness of different forms of protein supplementation (in particular, oral nutritional supplementation (ONS) and protein-fortified foods (PFF)) to target PEM. A lack of consensus of effective malnutrition diagnostic tools and lack of universal requirement for malnutrition screening in the residential care setting was uncovered, making identifying and treating malnutrition in this population a challenge. When assessing the use of protein supplementation in the residential care setting, the two primary forms of supplementation were ONS and PFF. There is evidence that these forms of protein supplementation can increase protein and energy intakes in residential care setting, yet their compliance and impact on functional status is unclear and conflicting. Further research comparing the use of ONS and PFF is needed to fully determine feasibility and efficacy of protein supplementation in the residential care setting.

Keywords: sarcopenia; frailty; protein-energy malnutrition; supplementation; residential care setting

2.2 Introduction

Malnutrition is an increasing problem in older adults, particularly those over 65 years of age (199) and residing in residential living facilities (200). Despite the increasing prevalence of malnutrition and the known detrimental effects on health, the effectiveness of malnutrition screening procedures are unclear, resulting in it remaining undetected and untreated (199). Individuals with malnutrition often suffer from muscle weakness, altered immune function, decreased functionality, and increased risk of infection (201), which can lead to increased mortality risk (202). In particular, protein-energy malnutrition (PEM), resulting from a decrease in protein and energy intake, has been associated with a number of health outcomes, disease states, and illnesses (203,204). It has been reported that under-nutrition is a prominent issue in older adults in the residential care setting (205–208). This highlights the need to assess the most effective methods of increasing protein and energy intake in this setting in order to decrease malnutrition prevalence, in particular PEM. Therefore, this review aims to provide an overview of the available literature in older adults in the residential care setting regarding the following: links between sarcopenia, frailty, and malnutrition (in particular, PEM), recognition and diagnosis of malnutrition, factors contributing to PEM, and the effectiveness of different forms of protein supplementation (in particular, oral nutritional supplementation (ONS) and protein-fortified foods (PFF)) to target PEM.

2.3 Sarcopenia, Frailty, and Malnutrition

The current phenomenon of an ageing population has placed considerable interest in geriatric syndromes such as sarcopenia. Sarcopenia, the established multifactorial condition associated with a progressive loss of skeletal muscle mass, quality, and physical function (15,209,210), has been shown to be highly prevalent in the residential care setting (184).

Indeed, in this setting the prevalence of sarcopenia has been reported to be up to 6 times greater than community dwelling older individuals (183,184). The process of sarcopenia begins around the 4th decade of life (12), proceeding at a rate of approximately 0.7-1.2% muscle mass lost per year, with up to 50% of muscle mass being lost by the 8th decade of life (211,212).

Sarcopenia is most commonly defined as an age-associated decrease in muscle mass and function, following The European Working Group on Sarcopenia in Older People (EWGSOP) guidelines for definition and diagnosis (15). However, sarcopenia is not an inevitable condition in all older adults, evident from the disparity of its progression rates and prevalence across the population (213). Using the EWGSOP definition of sarcopenia, a recent meta-analyses indicated the prevalence of sarcopenia in community-dwelling older adults to be approximately 13% (183). However, it can reach a significantly higher prevalence of up to 73% in long-term care homes and between 22-87% in assisted-living facilities (184). Diet and physical activity are key modifiable behavioural factors associated with sarcopenia development (214) and may be the largest contributors to the reported differences in sarcopenia prevalence between community-dwelling and older adults in the residential care setting.

Frailty is a whole-body geriatric syndrome associated with impairments in physiological systems and decreased homeostatic reserve with increasing age (97). Frailty results in an increased risk of negative health-related outcomes such as falls, fractures, and hospitalisation (186,215). Despite the organ-specific nature of sarcopenia and the whole-body nature of frailty, there is overlap with respect to their definitions and diagnosis. For example, sarcopenia is reported to be associated with muscle loss (15), whilst frailty is more commonly reported to be associated with weight loss (97); which may consist of muscle and/or fat loss.

However, both sarcopenia and frailty include characterisation of loss of functional mobility and strength, often measured by gait speed and handgrip strength when identifying a frail or sarcopenic individual (216). Importantly, malnutrition is considered to be a key contributor development of both of these conditions (187).

There are a number of definitions of malnutrition, with one of the most universally accepted definitions describing malnutrition as “a state of energy, protein, or nutrient excess or deficiency, resulting in adverse effects on body composition and function, and clinical outcomes” (188). Malnutrition is one of the most commonly unrecognised and under-treated health conditions worldwide, and an abundance of reports and research have recently identified under-nutrition as a prominent issue in care homes (205–208). It has been reported by The British Association for Parenteral and Enteral Nutrition (BAPEN) that 34% of older care home residents experience undernutrition (214), and results from a systematic review identified that 12-54% of care home residents experience under-nutrition (208). Furthermore, a recent policy statement published by The British Dietetic Association acknowledged the issue of increasing prevalence of under-nutrition in older adults living in residential care (200).

The European Society for Parenteral and Enteral Nutrition (ESPEN) have proposed terminologies to support a better understanding of malnutrition. In this attempt to afford enhanced understanding, ESPEN categorise undernutrition as i) disease-related malnutrition (DRM) with or without inflammation, and ii) non-DRM. Furthermore, ESPEN identify sarcopenia, frailty, micronutrient deficiency, and obesity as different forms of malnutrition (217). Unsurprisingly, due to the overlapping nature of differing types of malnutrition, ageing contributes to many aspects of these conditions. For instance, undernutrition has been identified as a risk factor for both sarcopenia and frailty (117,218) in older individuals,

increasing the risk of comorbidity development and adverse outcomes, and impairing quality of life (219). The different forms of malnutrition, as proposed by ESPEN, are summarised in Figure 2.1.

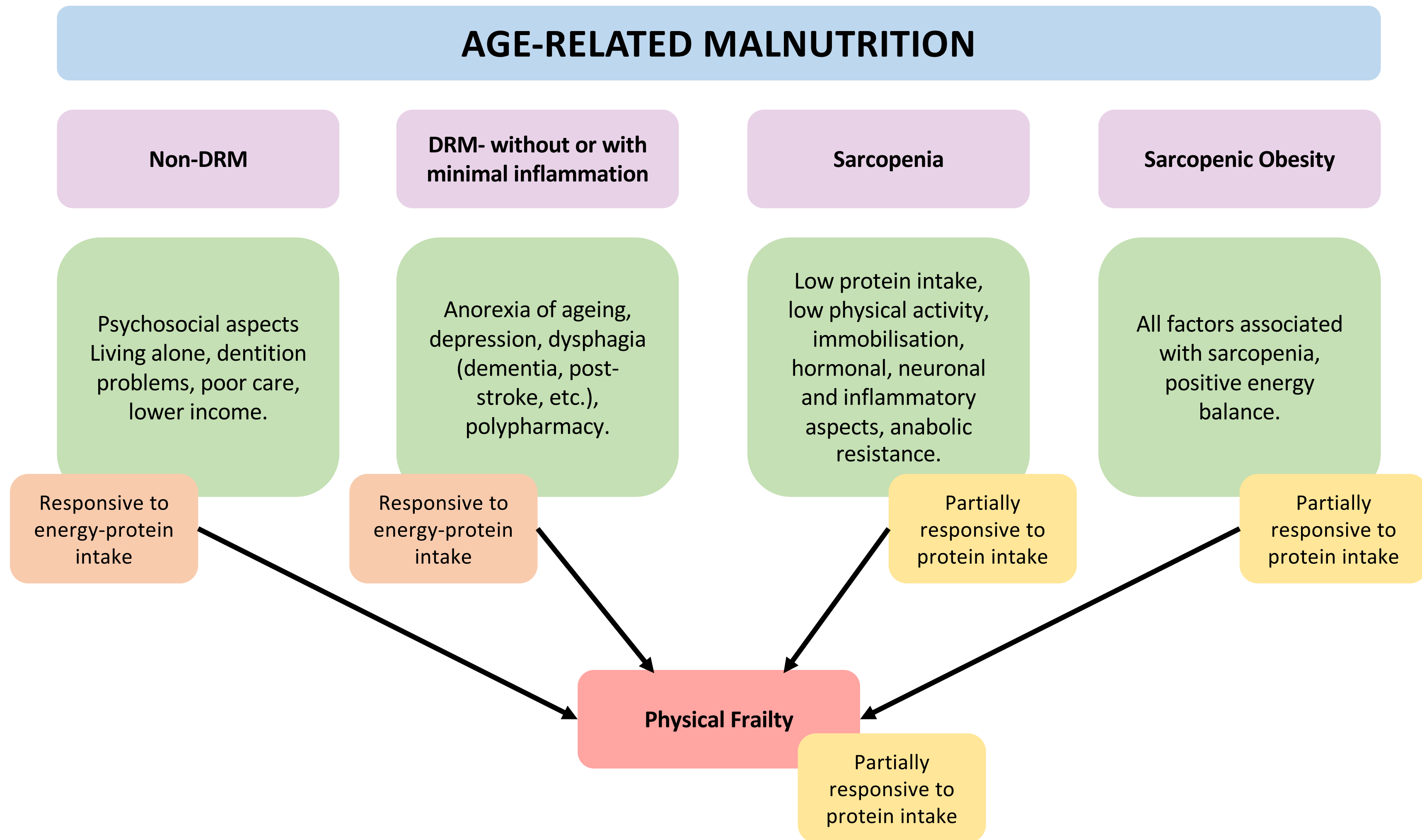

Figure 2.1- Summary of age-related malnutrition. DRM: disease-related malnutrition. Illustrated is the definition of distinct age-related nutritional diagnosis, the causes of each condition, and their responses to nutritional therapy. All malnutrition conditions have the potential to contribute to the progression of physical frailty. Conditions in which the central mechanism is the low intake of nutrients (Non-DRM, DRM without or with minimal inflammation) are well responsive to nutritional therapy. However, if other metabolic components are involved, as in sarcopenia and sarcopenic obesity, energy and or protein intake alone are not enough. This figure is adapted from Cederholm et al., (2017).

PEM, which is classified as non-DRM, is one of the most common forms of malnutrition in older adults, resulting when protein and/or energy intake fails to meet the nutritional requirements of the body (189). This impairs the ability to recover from illness and predisposes individuals to disease-related outcomes (220). Furthermore, PEM has a significant negative impact on mental and physical well-being of older adults in the residential care settings (221). For example, PEM has been shown to decrease quality of life, increase risk of depression, infection and pressure ulcer development, and adversely impact clinical outcome development (222–224). Clear evidence demonstrates the need for an increase in protein intake in older individuals due to the pivotal role PEM has been shown to play in sarcopenia and frailty onset and development (190). Older adults in the residential care setting are often the frailest older individuals most vulnerable to comorbidity and sarcopenia development, however, malnourishment is a common issue in care facilities (225).

A wealth of evidence based on the assessment of diet quality has established a relationship between a ‘healthier’ diet and an increase in muscle mass, along with a lower risk of decline of physical performance (226). To exemplify, observational research in community-dwelling older adults has demonstrated a significant relationship between a greater protein intake and a reduced percentage of lean muscle mass loss over a 3-year period (227). Similarly, interventional research has demonstrated that increasing protein intake from 0.8 to 1.6 $g.kg^{-1}.day^{-1}$ over a 10-week period can produce significant improvements in lean body mass and knee-extensor peak power (228). These findings highlight the option of using protein supplementation to maintain skeletal muscle protein anabolism and function in older adults. Older adults living with frailty are often in the residential care setting, presenting with the most extreme cases of sarcopenia and thus most in need of interventions to limit the decline of muscle mass and function. However, despite evidence presenting the effectiveness of physical activity and exercise training as anabolic stimuli for skeletal muscle (229), the

high prevalence of frailty in the residential care setting with limited functional mobility may limit its widespread use in this 'at-risk' group (191).

There is currently no difference in the RDA or estimated average requirement (EAR) of protein intake for young and older adults. The current RDA and EAR for protein is 0.8 $g.kg^{-1}.day^{-1}$ and 0.66 $g.kg^{-1}.day^{-1}$ respectively. However, it has been suggested that to maintain optimal health in older adults, higher dietary protein intakes may be necessary (178). The current recommendation for protein from the PROT-AGE group is 1.0-1.2 $g.kg^{-1}.day^{-1}$ for healthy older individuals, 1.2-1.5 $g.kg^{-1}.day^{-1}$ for individuals with chronic disease or acute injury, and up to 2.0 $g.kg^{-1}.day^{-1}$ for individuals experiencing severe malnutrition (178). Evidence has demonstrated an association between low protein intake and frailty in older adults (118), highlighting the need to increase protein intake in older adults in the residential care setting who are at increasing risk of malnutrition. Data obtained from care homes has demonstrated that up to 35% of residents do not consume an adequate amount of protein, markedly below the EAR (117,230). It must however be noted that beyond protein provision, several factors contribute to a decrease in protein consumption with advancing age, particularly in the residential care setting, including a decrease in appetite, and increased prevalence of dementia, dysphagia, and dentition issues (195,231,232). Related to these additional challenges of achieving optimal protein intake in older adults, there is a growing interest in protein supplementation for these individuals, and in particular in the residential care setting, where older adults are more susceptible to the adverse outcomes associated with low protein consumption, including sarcopenia and frailty.

This susceptibility is evident in the high, yet variable, reported prevalence of sarcopenia, frailty, and PEM in the residential care setting, with the reported prevalence of sarcopenia, for example, in nursing home residents ranging between 14-85% (233–236).

Differences in reported prevalence are likely explained by the variety of diagnostic tools used and their cut-off points for muscle mass, and the age and clinical status of the participants. Similarly, despite the prevalence of frailty being identified as higher in those in the residential care setting compared to community-dwelling individuals at 15-84% (97,237–239), the reported presence of frailty is also largely determined by the diagnostic tool used. When considering the prevalence of PEM in the residential care setting, a number cannot be presented reflecting a lack of knowledge on the prevalence of specifically PEM in this population (as the prevalence of general malnutrition is more commonly reported) (240). These figures are demonstrative that despite the growing interest, there is limited available research in this topic area, and the available research highlights the need to develop universal methods of determining sarcopenia, frailty, and PEM in this vulnerable population.

Currently 425,000 older people in the UK live in residential care homes (179). This is three times more people than are in hospital at any one time, and this number is set to increase further (180). Care home residents experience greater multi-morbidity and polypharmacy than age-matched community dwellers (179), and malnutrition is a prevalent contributory factor. As such, there is a clear need to explore and establish how much protein older adults in the residential care setting are currently receiving and consuming, which factors are influencing protein consumption in these individuals, and the effectiveness of protein supplementation to slow the rate of sarcopenia onset and development.

2.4 Recognition and Diagnosis of Malnutrition

Early recognition and diagnosis of malnutrition has been identified as one of the most pivotal factors to prevent or slow the malnutrition trajectory (241), yet there is no universal malnutrition screening or assessment in place in UK care homes. This has proven to be a problem in determining how many individuals in the residential care setting are at risk of, or

are diagnosed with malnutrition, in particular PEM. This is evident in the disparity of reported malnutrition prevalence when using different diagnostic tools (242,243). The Department of Health's Minimum Standards for Care Homes for Older People state that on admission to care homes, individuals must be weighed and that their diet preferences should be noted, however there is no universally accepted malnutrition assessment tool required for use in this setting. Established malnutrition assessment tools used in the healthcare setting include the Mini Nutritional Assessment (MNA) (244), Malnutrition Universal Screening Tool (MUST), Nutritional Risk Screening (NRS-2002), and Subjective Global Assessment (SGA) (245). Specific to the residential care setting, the MNA short-form (MNA-SF), which includes an assessment of functional, psychological, and cognitive parameters, has shown good predictive value for survival in nursing homes (246).

The discrepancies between tools used to evaluate malnutrition and PEM do not allow the precise identification of PEM in the residential care setting. These discrepancies often lie in the design of malnutrition screening tools, and their specificity to different populations. For example, the MUST is a broader malnutrition screening tool, designed for use in hospitals, community and other care settings, whereas the SGA is primarily a clinical malnutrition screening tool, designed for adult surgical populations to predict surgical outcome (247). Due to differences in the criteria used to assess malnutrition across screening methods, the reported prevalence of malnutrition in older adults in the residential care setting is broad at 5% to 70% (242,248). Diekmann and colleagues investigated these discrepancies in a comparative analysis of the MNA, NRS-2002, and MUST in nursing home residents (249). Their participants included individuals with cognitive impairment to ensure a true reflection of the care home population, however the complexity of the MNA as a malnutrition screening tool highlighted a low applicability rate in this population compared with the MUST and NRS-2002 (249). Similarly, low applicability of the MNA in comparison to the

SGA and NRS-2002 has also been previously reported in hospital in-patients on geriatric wards (250). Furthermore, one of the key differences in the design of these malnutrition tools is the body mass index (BMI) and weight-loss cut-offs. The NRS-2002 and MUST do not adjust their BMI cut-off values for older people and use the World Health Organisation recommendations, which are based on research in young and middle-aged, as opposed to older individuals (249). In contrast, the MNA uses BMI cut-offs adapted for older people, which often results in more sensitive, lower scores, indicating a higher malnutrition risk in comparison to the MUST and NRS-2002 (249).

The breadth of research comparing methods of malnutrition assessment lacks a clear consensus. However, upon evaluating the available literature, it may be perceived that the present problem is not just the form of malnutrition assessment being used, but the lack of universal requirement for malnutrition screening to take place in older adults on admission to care homes and nursing homes. It is recommended that care homes screen individuals for malnutrition every 3 months, yet there is no requirement for this or policy-based implication if this is not carried out (247,251). These discrepancies can make identifying those at risk of malnutrition, in particular PEM, challenging. In the case of malnourishment not being diagnosed or treated in a malnourished individual, this may result in exacerbation of existing, or development of new comorbidities.

2.5 Factors affecting protein consumption in older individuals

PEM is a complex process of grave concern in older age due to the associated negative consequences related to quality of life, physical functioning, and overall health (252). However, PEM is not simply a result of increasing age. There are many contributing physiological and psychosocial factors to PEM in older adults, some of which may explain

the higher proportion of individuals in the residential care setting with PEM compared to their community-dwelling counterparts. These factors are summarised in Figure 2.2.

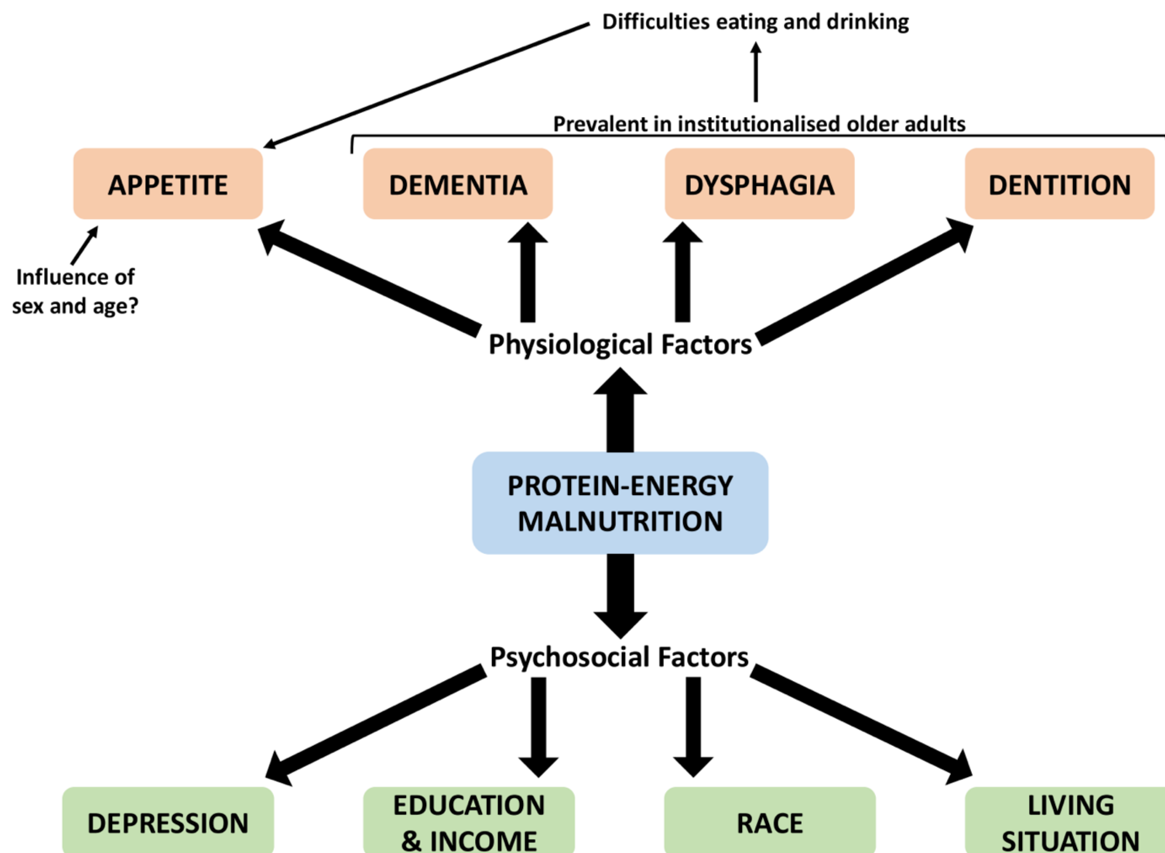

Figure 2.2- Summary of factors influencing protein-energy malnutrition in older adults. Several physiological and psychosocial factors influence protein-energy malnutrition in older adults. The primary physiological factor influencing not only protein intake, but also total energy intake, is decreased appetite (254,255), with research on the influence of sex and chronological age on appetite yielding conflicting data (195,262–264). Other physiological factors contributing to protein energy malnutrition, common in older adults in the residential care setting, are dementia, dysphagia, and dentition (191,257,258), which can also contribute to decreased appetite. Several psychosocial factors are also known to influence protein energy malnutrition in older individuals, including those in the residential care setting. These include depression, education and income, ethnicity, and living situation (231,266,267).

Appetite influences not only protein intake, but also total energy intake in older individuals. This has resulted in appetite often being studied in parallel to PEM (201,225,243). An association has been previously reported between low protein intake and appetite in community-dwelling older adults (253), however, this association has been reported as more common in nursing home residents (192). In older adults' poor appetite is

associated with low total energy intake (254,255), lower diet quality (255), and decreased diet variety (256). A key factor in older adults in the residential care setting which may impact upon appetite is the presence of dementia. It is widely reported that individuals suffering from dementia may experience increased difficulty eating and drinking, thereby requiring more assistance with these everyday tasks. Dementia is a known predictor of entry to residential care facilities (193,194), and it is reported that up to 75% of older individuals in the residential care setting suffer from dementia (257,258). This population group are therefore a high-risk group for PEM due to decreases in eating and drinking and associated declines in appetite.

Dysphagia, the difficulty in swallowing, is a prevalent factor contributing towards a decline in appetite and energy and protein (195,259) intake in older adults, and has been reported in 13-57% of older adults suffering from dementia (260). It has been reported that up to 60% of nursing home residents in the United States suffer from dysphagia (261), highlighting the prevalence of this problem in the residential care setting as well as in individuals suffering from dementia. Furthermore, research investigating appetite and protein intake has identified a relationship between declining oral health, or tooth loss, with increasing age and decreased protein consumption (252). Often occurring hand-in-hand with dysphagia, many older adults in the residential care setting experience dentition issues and poor oral hygiene (196). This largely impacts individual protein and energy intake and can lead to PEM due to individuals struggling to chew and swallow whole foods (232).

To date, the effects of sex and age on appetite and protein consumption are unclear. Recent research investigating the factors associated with protein consumption in older adults reported a significant difference in protein consumption (g, g/kg) between male and female participants (195). However, this contradicts previously published data, which reported no

difference in protein intake between males and females when expressed in g/kg as opposed to g (262–264). The well-reported decline in appetite in older individuals was first termed the 'anorexia of ageing' (265), however evidence shows no difference between older men and women in appetite nor protein consumption (195,230,262,263), with these results suggestive that other factors are present, such as comorbidities and declining oral health, that have a greater influence on protein intake in older individuals.

Recent evidence highlights the role of psychosocial factors affecting appetite and protein consumption in older adults, due to the strong influence of environment and mood on appetite regulation (231). Depression is a known psychological factor related to an impairment of appetite (197) and has been reported to be present in 27% of individuals living in care homes in the UK (266). Research investigating protein consumption and its relationship with several psychosocial factors in 90,000 women aged 50-79 demonstrated an association between low depression scores and a higher protein consumption (267). This study also identified several psychosocial factors associated with a greater protein intake. These include a higher level of education and income, not smoking, being younger and white ethnicity and co-habitation. Together, a combination of the described physiological and psychosocial factors can result in PEM, particularly in older adults in the residential care setting at greater risk of adverse health outcomes and comorbidity development.

2.6 Evidence of protein supplementation in older adults in the residential care setting

Literature investigating protein supplementation techniques in older adults in the residential care setting is sparse. Despite often being the individuals most vulnerable to malnutrition, in particular PEM, there is a lack of consensus of the feasibility and efficacy of protein supplementation in this population. Often due to increasing participant frailty and decreased mobility, existing methods used to assess outcome measures including

musculoskeletal ‘health’, functional ability, and basic anthropometric measures, are not feasible for use in the residential care setting. Furthermore, complications may lie in commonly used methods of supplementation due to a breadth of factors affecting protein consumption, as previously described. Making outcome measure adaptations and determining what interventions are feasible for use in the residential care setting is vital to progress research in this sector.

The most commonly used methods of supplementing protein are via ONS and PFF. An ONS is a source of energy and protein, often in liquid form, commonly used in malnourished individuals in the residential care setting when experiencing difficulty obtaining nutrients from whole foods (268). Previous research has demonstrated the benefits of ONS prescription, with individuals prescribed ONS for 6-months following hospitalisation showing a marked improvement in functional capacity and increased independence (269). However, evidence investigating ONS compliance and effectiveness is conflicting. Alternatively, PFF is a form of supplementing protein in the form of energy- and protein-dense meals or snacks; increasing protein intake without any dramatic change to the quantity of food an individual is consuming (270). Again, as with ONS, data on the compliance and effectiveness of PFF is largely equivocal.

2.6.1 Methods

This literature review aimed to identify articles supplementing ONS or PFF in residential care settings and provide a scoping overview of the outcomes of these studies, in particular those impacting malnutrition state. A scoping review was chosen as opposed to other review formats, such as a systematic review, due to the review aim of providing an overview of the current knowledge base regarding protein supplementation in older adults in the residential care setting based on published guidelines (271). This meant that extensive

inclusion criteria were not necessary, and studies of different protocols and study designs having a wide range of primary outcome measures were included. This is necessary in effectively summarising the evidence available regarding protein supplementation in the residential care setting. Scoping literature searches were performed by two researchers independently (S.L.M, P.A.) on PubMed and Google Scholar databases, and abstracts of relevant articles were examined. Further, a literature search using the PubMed MeSH only tool was used including the search terms “Protein” AND “Nursing Homes” or “Care Homes” or “Housing for the elderly” or “Geriatric nursing” or “Homes for the Aged”. 553 manuscripts were identified and screened for those mentioning protein supplementation, protein enriched diet (liquids, solids), dietary supplementation, or any other form of protein supplementation. Articles deemed to fit the aim of the present review were included. Inclusion criteria included articles available in English with full text available investigating the effects of ONS supplementation and/or PFF on older adults living in residential care settings. Following this literature search, 17 relevant protein supplementation studies in older adults based in residential care facilities were identified. Of these, 11 used ONS and 6 used PFF. Following identification of these studies, relevant information regarding study setting, design, participant number, participant age, percentage of female participants, intervention duration, intervention protein content goal ($g.day^{-1}$), and intervention energy content goal ($kcal.day^{-1}$) was extracted for comparison. These variables are displayed in Table 2.1. A summary of all study outcomes is displayed in Table 2.2. Further comparisons were made to assess the effectiveness of protein supplementation, the differences in outcome measures used, and the implications these may have had on supplement efficacy. These comparisons were made to provide a scoping overview of the available research in this topic area and identify gaps in the knowledge base to be further addressed.

Table 2.1 Studies investigating the effects of protein supplementation in older adults in the residential care setting

First author, year	Setting (Country)	Study design	Study participants	Age (yrs) Mean (SD)	Percent female	Intervention duration	Intervention protein content goal (g/d)	Intervention energy content goal (kcal/d)
Oral Nutritional Supplementation								
de Luis, 2018	Nursing homes and community-based (Spain)	Prospective Observational	N= 148 IG 1 (CD): 97 IG 2 (RE): 51	80 (8)	66	12-weeks	NS	NS
Parsons, 2017	Care homes (Hampshire, England, UK)	RCT	N= 104 IG: 53 CG: 51	89 (8)	86	12-weeks	16	600
Jobse, 2015 Stange, 2013	Nursing homes (Nurnberg and Furth, Germany)	RCT	N= 87 IG: 45 CG: 42	86 (6)	91	12-weeks	24	600
Allen, 2013	Hospital and nursing home environments (UK)	Crossover design study	N= 26 (n=18 nursing home residents, n=8 hospitalised patients)	84 (8)	69	1-week (ONS given 3xday on alternate days)	~ 42	~ 849

Carlsson, 2011	Residential care facilities (Umeå, Sweden)	RCT	N= 177 IG 1 (ENS): 42 IG 2 (EX+PL): 41 IG 3 (ONS): 47 IG 4 (PL): 47	85 (6)	74	3-months	7.4	~ 816
Manders, 2009	Institutes for chronic care (The Netherlands)	RCT	N= 176 IG: 119 CG: 57	81	74	24-weeks	8.75	250
Cruz-Jentoft, 2008	Nursing homes (Spain)	Prospective observational	N/ IG= 358 No control group	84 (7)	71	12-weeks	NS. State 200ml ONS, 20% protein.	NS
Wouters-Wesseling, 2006	Nursing homes (Wageningen, The Netherlands)	RCT	N= 34 IG: 18 CG: 16	83 (7)	85	5-weeks	11.2g	309
Lauque, 2000	Nursing homes (Toulouse)	RCT	N= 88 CG 1: 19 CG 2: 22 IG 1: 19 IG 2: 28	CG 1: 84 (8) CG 2: 85 (6) IG 1: 85 (6) IG 2: 88 (4)	84	60-days	NS	300-500

Johnson, 1993	Nursing homes (USA)	Retrospective case control	N= 109 IG: 56 CG: 53	IG: 88 (6) CG: 85 (8)	IG: 86 CG: 85	Up to 72-months	NS	NS
				Protein-fortified foods				
Beelen, 2017	Care facilities (The Netherlands)	Interventional	N/ IG= 22 No control group	83 (9)	59	10-days	Varying- aim to increase overall protein intake to 1.2 g/kg/d	NS
Van Wymelbeke , 2016	Nursing homes (Burgundy, France)	RCT	N= 68 IG 1 (PFF): 29 IG 2 (ONS): 17 CG: 22	IG 1: 84 (8) IG 2: 90 (7) CG: 87 (8)	79	12-weeks	IG 1: 12.8 IG 2: 14	IG 1: 180 IG 2: 200
Pouyssegur, 2015	Nursing homes (Nice, France)	RCT	N= 175 IG: 88 CG: 87	86 (8)	80	6-weeks	11.5	244
Iuliano, 2013	Low-level aged care facilities (Melbourne, Australia)	Prospective intervention	N= 130 IG: 62 CG: 68	87 (6)	78	4-weeks	Varying- modified diets to contain at least 2 additional serves of dairy food/day	NS

Leslie, 2013	Care homes (UK)	RCT	N= 41 IG: 22 CG: 19	91 (7)	88	12-weeks	NS	Maximum potential increase of ~400
Smoliner, 2008	Nursing homes (Germany)	RCT	N= 65 IG: 22 CG: 30	IG: 82.2 (9.5) CG: 84 (9.5)	IG: 77 CG: 75	12-weeks	NS. Protein added per meal on top of standard diet.	NS

Abbreviations: ONS, Oral Nutritional Supplement; PFF, Protein-fortified food; RCT, Randomised Controlled Trial; IG, Intervention Group; CG, Control Group; CD, Community-dwelling; RE, Residential; NS, Not Stated; Ex, Exercise; ENS, Exercise and Nutritional Supplement; Pl, Placebo.

Table 2.2. Oral nutritional supplementation and protein-fortified food study outcomes.

First author, year	Protocol Overview	Study Outcomes
		Oral Nutritional Supplementation
De Luis, 2018	12-weeks ONS supplementation, Nursing homes and community-based	• Significant increases observed in weight and BMI in comparison to baseline. • Decrease in NRS-2002 scores in ONS group (decreased malnutrition score) • Significant improvements in functionality measures (Katz ADL scores) and quality of life (EQ-5D-3L scores) in comparison to baseline.
Parsons, 2017	12-weeks ONS supplementation vs dietary advice, Care homes.	• Higher QoL in ONS group compared to dietary advice at 6- and 12-weeks. • Significant body weight increases in ONS group (at 12-weeks, not seen in dietary advice group). No significant differences between groups. • ONS group demonstrated increased total energy and protein intakes (not accounted for by discrepancies in voluntary food intakes). • Majority of macronutrient intakes were higher in ONS compared to dietary advice group at 6- and 12-weeks.
Jobse, 2015	12- weeks ONS supplementation, Nursing homes	• ONS compliance high in 35.7% and low in 28.6% of participants. • Those with higher compliance showed positive associations with body weight increases, BMI, upper-arm circumference, and MNA-SF scores. • Higher compliance in malnourished individuals with chewing difficulties. • Low compliance in those who were immobile, depressed, had gastrointestinal complaints, or consumed more than 4 daily drugs.
Stange, 2013		• Several outcome measures could not be assessed in a number of participants due to mobility issues and cognitive impairments (geriatric depression scale, 46%; handgrip strength, 38%, gait speed, 49%). • In those that could complete all assessments, there were no changes in functionality in the CG or IG, excluding ADL scores, which decreased in both groups. • When assessing QoL, there was a trend of "positive self-perception" to decrease in the CG and increase in IG. There was a significant drop of "being busy" in the CG.

Allen, 2013	1-week ONS supplementation (given 3x on alternate days), Hospital and nursing home environments	• When ONS was consumed, individuals were more likely to achieve their daily energy and protein requirements. This did not impact their habitual food consumption.
Manders, 2009	24-weeks ONS supplementation, Chronic care institutes	• ONS supplementation increased energy, macronutrient, vitamin, and mineral intake (excluding Vitamin A). • Body weight increased in the ONS group and decreased in the control group, yet this was not significant. • Several blood biomarkers demonstrated significant changes in favour of the IG (albumin, pre-albumin, Vitamin D, Hcy, folate, Vitamin B_{12}, and Vitamin B_6).
Cruz-Jentoft, 2008	12-weeks ONS supplementation, Nursing homes	• High compliance of ONS supplementation (97.46% at 6-weeks, 96% at 12-weeks). • No significant changes in BMI. • Significant improvements observed in MNA score and body weight.
Wouters-Wesseling, 2006	5-weeks ONS supplementation, Nursing homes	• Significant differences in weight changes were observed between the standard (-0.4kg) and supplementation (+0.8kg) groups. • No differences in anthropometric measurements. • No differences were observed in energy intakes nor macronutrient intakes between groups.
Lauque, 2000	60-days ONS supplementation, Nursing homes	• Higher energy intake after 60-days ONS supplementation. • Improvements in MNA score and body weight in the most malnourished and those at risk of malnutrition receiving ONS. • No improvements in MNA score nor weight in those at risk of malnutrition receiving ONS.
Johnson, 1993	Varying supplementation period (Up to 72-months), Nursing homes	• Many patients receiving ONS supplementation were below the age-adjusted body weight values upon admission, and until ONS supplementation began, an average decrease in body weight was observed. • Participants consuming ONS demonstrated an average gain of weight over 9-10 months to approximate admission weight. • Average age of ONS participants higher than control patients. More likely to be wheelchair- or bed-bound, require assistance eating, and eat a modified diet.

		Protein-fortified foods
Beelen, 2017	10-days PFF supplementation, Care facilities	• Consuming PFF increased protein intake but did not result in any changes in energy, fat, or carbohydrate intake. • All subjects reached a protein intake of 0.8 g/kg/day, and a higher proportion of individuals reached the recommended protein intake of 1.2 g/kg/d (n=9/22) as opposed to baseline (n=4/22). • PFF supplementation resulted in a significant increase in protein intake at breakfast and during the evening.
Van Wymelbeke, 2016	12-weeks PFF vs ONS supplementation, Nursing homes	• Higher total energy intakes in PFF group compared to CG and ONS at day 30 and 90. • Following intervention, a larger number of the PFF group participants (72%) reached the recommended protein intake of 0.8 g/kg/day in comparison to the ONS (53%) and CG (36%). • Blood biomarker assessment demonstrated an increase in blood levels of Vitamins B_9, B_2, D, B_6, B_{12} following PFF intervention. Decreases were observed in plasma homocysteine. • High reported PFF compliance (83% consuming all of brioche). This was lower for the ONS group (74% consuming all ONS). • No significant BMI, handgrip strength, or MNA score differences between or within groups.
Pouyssegur, 2015	6-weeks PFF supplementation, Nursing homes	• Increased average weight in PFF compared to IG. This persisted for 1-months and 3-months post- PFF supplementation. • Diarrhoea reduction was observed in PFF group in comparison to the IG post- nutritional intervention. • PFF demonstrated a steady increase in mean appetite throughout the intervention period.
Iuliano, 2013	4- weeks PFF supplementation, Low-level aged care facilities	• Increases in mean energy and protein intake, proportion of energy from protein, and proportion of estimated energy requirements in PFF group. Decrease in proportion of energy from fat in PFF group. • Increases in nutrients including calcium, Vitamin D, phosphorus, and zinc in PFF group only. • Prior to intervention, mean phosphorus and zinc intakes were below recommended levels. With PFF intervention, the recommended levels were attained.
Leslie, 2013	12-weeks PFF supplementation, Care homes	• Increase in mean energy intake and weight gain in PFF group only. • A small number of PFF participants (n=6/16) demonstrated an increase in BMI >18.5 kg m^{-2}.

Smoliner, 2008	12-weeks PFF supplementation, Nursing homes	• Higher protein intake in PFF group compared to CG. No differences in total energy intake. • Improvements in nutrition and body composition parameters (including BMI, MNA score, fat free mass) throughout intervention in PFF group and IG. • No improvements were observed in muscle function following nutritional intervention. • Decline in the Barthel Index and the physical functioning component of the Short Form 36 questionnaire observed in all participants following the intervention period.

Abbreviations: QoL, Quality of Life; ONS, Oral Nutritional Supplement; BMI, Body Mass Index; MNA-SF, Mini Nutritional Assessment- Short Form; CG, Control Group; IG, Intervention Group; ADL, Activities of Daily Living; PFF, Protein Fortified Food.

2.7 Protein supplementation in older adults in the residential care setting: A summary of findings

2.7.1 Oral Nutritional Supplementation

The use of dietary advice (DA) has been suggested to aid protein intake, yet its use in older adults in the residential care setting is not commonly reported. However, an identified study from Parsons *et al*., (2017) aimed to compare the effects of DA vs. ONS supplementation in n=104 malnourished care home residents (identified using MUST, mean age 88.5(7.9)yrs) without obvious dementia (no direct measure, determined via medical notes and care home staff input) (272)). Following 12-weeks of ONS supplementation (goal of 16g protein, 600kcal per day), results demonstrated a significantly higher QoL rating (measured using EuroQol 5 Domain Health Questionnaire) in the ONS group compared to the DA group. ONS increased total energy, protein, and majority of micronutrient intakes. However, no differences were found in appetite sensations (following resident interview) between the two groups, excluding fullness, which was identified to be significantly lower in ONS group compared to DA group. However, this study did not include individuals with dementia and therefore the results may not be generalisable to all older adults in the residential care setting

as it has previously been reported that up to 75% of individuals in the residential care setting suffer with dementia (257,258). This was the only identified ONS study comparing ONS with DA but does show that in this particular group of care home residents ONS may be more effective than DA at improving energy and protein intake, and that protein supplementation in the form of ONS may benefit quality of life and dietary intake.

A previous systematic review identified a positive effect of ONS containing at least 20% of calories from protein on handgrip strength (273). However, limited ONS studies investigate the effect of protein supplementation on functional status in older adults in the residential care setting. This is often due to the difficulties associated with taking these measurements in this patient population. However, Stange *et al*., (2013) investigated the effects of a low-volume, nutrient- and energy-dense ONS (24g protein, 600 kcal) on functional status, along with nutritional status and QoL in n=77 nursing homes residents (mean age 87(6)yrs) (274). Results demonstrated significant differences in several nutritional parameters and QoL in favour of the intervention group (see Table 2.1 for details). Regarding physical functioning, Stange *et al.,* (2013) also reported that hand grip strength and gait speed could not be assessed in 38% and 49% of participants, respectively, primarily due to mobility issues and cognitive impairment. Stange *et al.,* (2013) further identified a lack of compliance of functional testing in the residential care population, highlighting a knowledge gap that needs to be explored further. These results are suggestive that ONS may be used to increase energy and protein intake and improve nutritional parameters in older adults in the residential care setting. However, the true effects of ONS on functional status remain unknown due to problems identified in conducting functional tests in this population.

Several studies were identified that assessed the compliance and acceptability of ONS in the residential care setting to increase protein and energy intake (275–277). Specifically,

the most recent of these studies assessed compliance with an ONS intervention, and identified the specific characteristics associated with low compliance in n=87 nursing home residents (mean age 87(6)yrs) (275) (data taken as part of previously reported randomised controlled trial (RCT) (274)). Following 12-weeks ONS supplementation, with the goal to provide 24g protein (600kcal) per day, a wide range of compliance (determined by estimates of ONS consumption from nursing home staff) was observed (high compliance in 36% and low compliance in 29%). However, it was observed that positive changes in body weight, BMI, upper-arm circumference, and MNA-SF score were significantly higher in participants with a higher compliance. Similarly, an earlier study aimed to determine the acceptability of ONS in n=88 nursing home residents (age range 84-88 yrs), and its effect on the nutritional status of malnourished residents (277). Average daily ONS intake in individuals malnourished or at risk of malnourishment averaged at ~400kcal, with good observed compliance (determined via a survey). Despite using a different method of measuring compliance, a similar relationship was observed between compliance, MNA score, and weight. Most participants identified as malnourished or at risk of malnourishment demonstrated weight gain and improvement in MNA score, with no reported improvements in participants not receiving ONS supplementation. A comparable study published had similar study aims: to evaluate adherence to 12-weeks ONS in nursing home residents identified as suffering from or at risk of malnourishment and to study changes in defecation following ONS supplementation (276). High ONS compliance was observed (determined by number and quantity of ONS consumed) and resulted in significant changes in nutritional status, mirroring previously reported findings (Lauque *et al*., 2000; see Table 2 for more information). Despite not stating the full composition of the ONS, Cruz-Jentoft *et al.,* (2008) stated that the ONS contained 20% protein, which resulted in positive outcome measures (276). Cruz-Jentoft *et al.,* (2008) reported the highest compliance levels of all 3 studies, with Lauque *et al.,* (2000) reporting

good compliance (ONS protein content not stated, 300-500kcal), and Jobse *et al.,* (2015) reporting a wide range of compliance levels (24g protein, 600kcal). Due to the lack of information regarding the ONS composition and other factors affecting compliance in these studies, the relationship cannot be attributed solely to number of calories as protein. However, compliance with ONS was reported as high, suggesting that ONS may be a feasible method of increasing protein and energy intake in this setting. Further in-depth analysis and research is needed to fully explore ONS compliance and the factors which may influence this.

The final group of selected ONS studies for review investigated whether consumption of ONS influenced energy and protein intake from participants' regular diet. One of the earliest studies investigating the use of ONS in malnourished older adults examined the effect of ONS in older adults in nursing homes and investigated the nutritional assessment, if any, received by nursing home residents (278). This study provided an early perspective of malnutrition in the residential care setting, noting an average decrease in body weight in residents upon nursing home admission followed by an average weight gain over an average of 9-10 months back to admission weight. Interestingly, it was reported that ONS residents were older than control residents, and more likely to be wheelchair- or bed-bound, require assistance eating, and eat a modified diet. Incidentally, many of these factors, such as an increase in dependency and dentition issues are now known factors contributing to progression and diagnosis of malnutrition (201). Furthermore, Johnson and colleagues identified weight loss and poor appetite as the most common reasons for starting ONS supplementation, key factors which are now part of malnutrition screening tools such as the MNA. Despite not providing the total protein and energy content of the provided ONS or the average duration of ONS supplementation, this study reports beneficial effects of a high-protein oral supplement on factors such as body weight, which are now well-known factors associated with PEM. However, without information regarding the composition of the

supplemented ONS, the true effect of ONS on outcome measures cannot be determined. Similarly, Wouters-Wesseling *et al.,* (2006) investigated the effect of ONS on preventing weight loss in psychogeriatric nursing home residents following acute illness from infection (279). Specifically, this study included 2 groups: residents receiving early ONS supplementation (200ml ONS daily; 209kcal, 11.2g protein) prior to acute illness onset, and residents receiving standard treatment (dietary intervention occurring once a physician had observed weight loss, loss of appetite and/or low intake). Results demonstrated significant differences in weight changes between the standard (−0.4kg) and supplementation (+0.8kg) groups, yet these differences were not reciprocated in anthropometric measurements. As reported by Johnson and colleagues, the reported results state that ONS may facilitate weight gain in nursing home residents. However, no malnutrition screening tool was used. Despite weight loss influencing malnutrition development and assessment, using a screening tool such as the MNA which takes into consideration body weight and BMI among other factors such as appetite, mobility, protein intake, and prescription medication, may provide a more reliable assessment of malnutrition in a nursing home setting.

Later published work from Manders *et al.,* (2009) completed a RCT with a 24-week supplementation period in n=176 individuals in chronic care institutes (280). They aimed to determine whether ONS had a positive effect on dietary intake and nutritional status and had a secondary aim of investigating the effect ONS supplementation would have on their habitual food consumption. Favourable effects of ONS were seen, with a higher number of individuals reaching their energy and protein requirements and beneficial effects observed on body weight in the ONS group (see Table 2.2). Interestingly, both ONS and placebo groups displayed small decreases in energy intake from food (−0.5 MJ/day), suggesting that this is not a compensatory effect of ONS supplementation. Similarly, a study from de Luis *et al.,* (2018) investigated the effects of supplementing a high calorie and protein, β-hydroxy-β-

methylbutyrate (HP-HMB-ONS) containing ONS on nutritional status, ADL, and QoL in older malnourished adults (community-dwelling outpatients and older adults in residential care settings) (281). Beneficial effects were observed in malnutrition and nutritional status and measures of QoL and functionality (see Table 2.2). However, this study did not report the amount of protein or energy in the supplementing ONS, and no statistical comparisons were made between the adults in the community and those in residential living facilities. The lack of statistical comparison in this study is a challenge to determining the efficacy to of the HP-HMB-ONS in adults in the residential care setting.

Another study also looking at the addition of ONS to participants regular diet, albeit over a much shorter time period is Allen *et al.,* (2013). Specifically, they investigated whether in older adults in the residential care setting with dementia, a 1-week ONS supplementation would decrease energy and protein intake from their regular diet (282). Their secondary aim was to determine whether irrespective of changes in their regular diet, whether ONS can aid achievement of adequate protein and energy intakes (see Table 2.1 for full details). As previously reported (280,281), ONS supplementation provided favourable results. Results demonstrated a higher number of individuals met their energy and protein requirements, a weak but significant correlation between ONS consumption and body weight, and no changes in protein consumption (g) consumed in food on intervention compared to control days (see Table 2.2). As previously mentioned, the energy and protein content of the ONS supplement has been suggested to be a key factor to consider when investigating the effectiveness of ONS in the residential care setting. When comparing the above studies, the composition of the provided ONS was unclear in 2/5 studies (278,281), despite de Luis *et al.,* (2018) reporting the use of a high-protein ONS. In the remaining 3/5 studies protein content ranged from 8.75g protein (250 kcal) to 42g protein (849kcal) (279,280,282). All the above studies found a positive effect of ONS on outcome measures; strikingly, Allen and colleagues

reported a statistically significant correlation between ONS consumption and body weight after only 1-week of supplementation. Thus, this high protein and energy content (42g protein, 849kcal) ONS may not be realistic as a long-term ONS supplementation in the residential care setting due to factors such as appetite (254,255), yet the importance of high ONS protein content has been demonstrated.

Declining muscle mass in older age is a key contributor to sarcopenia development, with exercise often prescribed to older individuals as an adjuvant to protein nutrition to aid muscle mass maintenance. Yet, this is not always feasible in the residential care setting due to declining physical function. The effect of nutritional supplementation on muscle mass and function specifically in older adults in the residential care setting is not widely reported in the literature, yet, a study was identified that investigated the effects of an exercise programme and ONS on muscle mass in residential care facilities. Despite the focus of this review not being on exercise interventions in the residential care setting, this study has been included due to the presence of an ONS only supplementation group (n= 47, mean age 83(6)yrs) and a placebo group (placebo drink, no exercise intervention; n= 47, mean age 85(7)yrs) (283). Carlsson *et al.,* (2011) used ONS containing 7.4g protein and 10.8g carbohydrate, and placebo containing 0.2g protein and 10.8g carbohydrate, ensuring that differences between groups could not be attributed to differences in carbohydrate content. No differences were observed in muscle mass or body weight between the ONS and CG. This study provides contrasting results to the majority of other ONS studies, possibly as protein supplementation was not the primary focus of this study, meaning protein content (g) in ONS supplements were markedly lower compared with the other reported ONS supplementation studies. Of note, although out with the scope of this review to be discussed in detail, ONS combined with 3-months high intensity exercise did not result in any differences in muscle mass or body weight, with no beneficial effect of ONS supplementation.

2.7.2 Protein Fortification

An alternative approach to increase protein consumption in older individuals is the use of PFF. In the present review, 6 PFF studies in older adults in the residential care setting were identified. The majority of these studies aimed to investigate the effect of different forms of PFF on the nutritional status of older individuals in residential care facilities. However, Iuliano *et al*., (2013) conducted a 4-week feasibility intervention study in n=130 low-level aged-care residents (older individuals in the residential care setting requiring some assistance with daily activities, age range 87-88 yrs) to investigate compliance with consumption of 2 additional serves of dairy (specific protein and energy intakes not stated) to increase protein and calcium intake to recommended levels (284). This proved effective in increasing mean energy and protein intake, and proportion of energy from protein as well as several nutritional parameters. An earlier study conducted by Leslie *et al*., (2013) used PFF to increase protein intake of food without increasing meal sizes over 12-weeks (285). Their method involved adding dairy-based foods, such as double cream and butter, to resident's usual meals. This study was conducted in n=41 (mean (SD) age 91(7)yrs) UK care home residents, and demonstrated an increase in mean energy intake and body weight in the intervention group, supporting previously reported results (284) and further highlighting the potential for use of dairy-based products to increase protein and energy intake in older adults in the residential care setting.

A common method of PFF supplementation to increase protein and energy intake involves using regular diet items, such as bread and soup, to increase palatability of the supplemented food, and to keep the supplementation in line with the regular diet of the individual. Of the 6 PFF studies identified in this review, 2 used this form of PFF in a residential care setting (286,287). Beelen *et al.,* (2017) developed various protein-enriched

foods such as bread, soups, and mashed potatoes to substitute into the residents normal diet, with protein-enriched fruit juices offered as additional choices (286). Similarly, Smoliner *et al.,* (2008) used energy-enriched soups and sauces, with the addition of 2 snacks high in protein and energy served between meals (287). Both studies aimed to determine the effect the supplementation would have on individual nutritional status, with Beelen *et al*., (2017) specifically aiming to increase protein intake to 1.2g.kg^{-1}.day^{-1}; the recommended protein intake for older adults from the PROT-AGE group (178). Both studies observed an increase in protein intake in the PFF group compared to CGs, with no changes observed in energy intake. Furthermore, Beelen *et al*., (2017) observed a higher proportion of individuals reaching the recommended protein intake of 1.2g.kg^{-1}.day^{-1} following PFF intervention. However, Smoliner *et al*., (2008) also investigated the effect of PFF on muscle function (determined using hand grip strength) and observed no significant differences between the PFF and the CG. The above studies demonstrate the effectiveness of using foods familiar to the individual to increase protein intake, however, the role of PFF on muscle function cannot be concluded and is yet to be fully elucidated.

The final 2 of the 6 identified PFF studies selected for the current scoping review include supplementation of protein-fortified snacks, specifically brioche and cookies, to increase protein intake in the residential care setting (288,289). Specifically, Van Wymelbeke *et al*. (2016) investigated the effect of providing protein-enriched brioche compared to ONS or usual breakfast to n=68 nursing home residents over a 12-week period on various measures of nutritional intake and status and blood biochemistry measures (see Table 2.2 for full outcome measures) (288). Per portion, the brioche (65g) contained 180kcal energy and 12.8g protein, and the ONS (200ml) contained 200kcal energy and 14g protein. Results reported a higher total energy intake in the brioche group at day 30 and 90 in comparison to the CG, and a higher proportion of individuals reaching the protein RDA intake of 0.8g.kg^{-}

^{1}.day^{-1} (72%) compared to the ONS (53%) and CGs (36%). Furthermore, blood biochemical analysis demonstrated an increase in blood levels of vitamins B9, B2, D, B6, and B12, and a decrease in plasma homocysteine in the brioche group. Pouyssegur *et al*., (2015) focussed on the effect of 6 weeks protein-fortified cookie supplementation on weight gain in n=175 malnourished older adults in the residential care setting (289). Results reported an increase in average weight in the PFF group compared to control, which was maintained 1-month and 3-months after the supplementation period. Further analysis identified a positive impact of protein fortified cookie supplementation on several secondary outcome measures, including body weight, appetite, and presence of pressure ulcers (see Table 2.2 for full outcome measures). Together, these studies imply beneficial effects of providing protein fortified snacks with which older adults are familiar as a means to increase protein and energy intake, yet, due to the nature of the current review, further research and statistical analysis is needed to determine this.

6.3. ONS vs PFF: Which is more effective in older adults in the residential care setting?

To our knowledge, this is the first scoping review of the effects of ONS and PFF supplementation in the residential care setting. The selected studies used a wide range of outcome measures; however, it is important to compare the efficacy of these supplements in increasing protein and energy consumption in older adults in this setting. Several of the included studies did not explicitly report the change in protein (g) and energy (kcal) intake from baseline to post-supplementation period (275,276,278,281,283,287,289). This may be due to the primary aim being the assessment of compliance with the supplementation protocol as opposed to other outcome measures in some studies (275,276). However, of the studies reporting protein (g) and energy (kcal) intake, there appears to be a negligible difference with respect to increasing protein and energy intake between ONS and PFF supplementation. Despite many PFF studies not stating an explicit amount of protein

supplemented in the prescribed foods (285,287,288,290), observationally there appears to be a higher protein and energy content in ONS compared to PFF diets and supplements. However, this does not deem ONS as more effective than PFF, and all outcome measures need to be considered prior to advising what is more effective in older adults in the residential care setting.

2.7.3 Limitations and Future Research

This narrative review is the first of its kind highlighting the importance of dietary protein and the benefits of protein supplementation in the residential care setting, however, it is not without limitations. A systematic review or meta-analysis would provide more information regarding the effectiveness of the different methods of protein supplementation in this setting, however, this is not yet feasible. There is limited research on protein supplementation specifically in the residential care setting, although there is lack of harmonisation between study aims and outcome measures. As previously mentioned, there are also problems in evaluating the research. These problems include a lack of information across all studies regarding the energy and protein content of the supplemented protein, lack of universal nutritional assessment tool use, lack of compliance of testing functional status in this population, and a lack of true representation of this population (many studies do not include individuals living with dementia, despite up to 75% of individuals in the residential care setting being diagnosed with cognitive deficits). Further research in this area needs to tackle these problems to offer robust evidence of protein supplementation in the residential care setting. Despite this review not including studies not in the English language, not being of a systematic nature and not performing meta-analyses, it has clearly highlighted the problems that need to be addressed so further research can fully determine the true influence of protein supplementation in this population, and the most effective method of doing so.

2.8 Conclusions

It is widely reported in the literature that the prevalence of malnutrition is higher in older adults in the residential care setting compared with community dwelling adults. In particular, PEM is highly prevalent in the residential care setting and can result in the development and exacerbation of adverse health conditions. Despite this, the exact prevalence of different types of malnutrition in residential care settings cannot be fully determined due to discrepancies between malnutrition assessment tools and a lack of universal requirement for malnutrition screening. The most effective methods of targeting PEM in older adults are undoubtedly through increasing energy and protein intake, yet, due to factors such as appetite and dentition, interventions are not straightforward to develop or deliver. The most common forms of protein supplementation to target PEM in the residential care setting include ONS and PFF, yet studies yield differing results, such that the most effective method cannot yet be identified. Regarding sarcopenia prevention and treatment, there is limited evidence of how much protein or energy should be prescribed in this unique population. Further research comparing the use of ONS and PFF is needed to fully determine feasibility and efficacy in the residential care setting.

3 Determining the influence of habitual dietary protein intake on physiological muscle parameters in youth and older age

Mathewson SL, Gordon AL, Smith K, Atherton PJ, Greig CA, Phillips BE. Determining the Influence of Habitual Dietary Protein Intake on Physiological Muscle Parameters in Youth and Older Age. Nutrients. 2021 Oct 12;13(10):3560. doi: 10.3390/nu13103560. PMID: 34684561; PMCID: PMC8539198.

3.1 Abstract

Protein ingestion is a potent stimulator of skeletal muscle protein synthesis (MPS). However, older adults demonstrate resistance to anabolic stimuli. As a consequence, a larger acute protein dose is required in older compared to younger adults to elicit the same synthetic response, suggesting that older adults should be consuming higher habitual dietary protein to optimise muscle mass. However, limited research has explored dietary habits in different age groups or the relationship between habitual dietary intake and mechanistic physiological parameters associated with muscle mass and function. This work investigated the effect of habitual dietary intake in young (*n* = 10, 25.9 (3.2)y) and older (*n* = 16, 70.2 (3.2)y) community-dwelling adults (16:10 male: female) on physiological muscle parameters. Dietary intake was assessed using four-day diet diaries. Post-absorptive MPS and MPS responses to feeding (4.25x basal metabolic rate; 16% protein) were determined in muscle biopsies of the *m. vastus lateralis* via stable isotope tracer ([1, 2-$^{13}C_2$]-leucine) infusions with mass-spectrometric analyses. Body composition (whole-body lean mass WBLM) was measured by dual-energy x-ray absorptiometry. Whole body strength was assessed via 1-repetition maximum assessments. Expression of anabolic signalling targets (PKC-α, IRS-1, PRAS-40, AS160, AKT-substrate) were assessed using western blotting. No significant differences in habitual dietary intake (protein, fat, carbohydrate and leucine as $g.kgWBLM^{-1}.day^{-1}$) were observed between age groups. WBLM (61.8 ± 9.9 vs. 49.8 ± 11.9 kg, $p = 0.01$) and knee-extensor strength (87.7 ± 28.3 vs. 56.8 ± 16.4 kg, $p = 0.002$) were significantly higher in young adults. Habitual protein intake ($g.kg^{-1}.day^{-1}$) was not associated with WBLM, upper-leg lean mass, whole-body strength, knee-extensor strength, basal MPS or fed-state MPS across both age groups. Significant positive correlations were observed between PRAS40 and relative protein intake ($g.kgBW^{-1}.day^{-1}$) in all adults ($R^2 = 0.202$, $P = 0.02$), and between IRS-1 and relative leucine intake ($mg.kgWBLM^{-1}.day^{-1}$) in younger adults

only (R^2= 0.444, P= 0.04). However, these results were not consistent across signalling targets. These findings suggest that differences in muscle mass and strength parameters between youth and older age are not explained by differences in habitual dietary protein intake. Further research with a larger sample size is needed to fully explore these relationships and inform on interventions to mitigate sarcopenia development.

3.2 Introduction

We are currently living with an ageing population, with the number of older adults aged over 65 expected to accelerate from ~542 million in 2010 to nearly 1.5 billion in 2050 [1]. As described in the introduction to this thesis, increasing age is associated with a decline in muscle mass and function which is often striking, (Figure 3.1) (291). This decline is termed sarcopenia, and is a known multifactorial condition associated with loss of independence [2], increased risk of falls and fractures [3], and increased morbidity and mortality [4]. Skeletal muscle is in a constant state of turnover, meaning that in order for changes in muscle mass to occur, there must be alterations in this equilibrium. Net protein balance (NPB) is determined by the balance between muscle protein synthesis (MPS) and muscle protein breakdown (MPB), with MPS being elevated following an influx of amino acids (AAs) (following feeding) and MPB being elevated during a period of fasting, resulting in an efflux of AAs. Ageing is a key factor which may alter the ability of skeletal muscle to use circulating AAs to stimulate MPS.

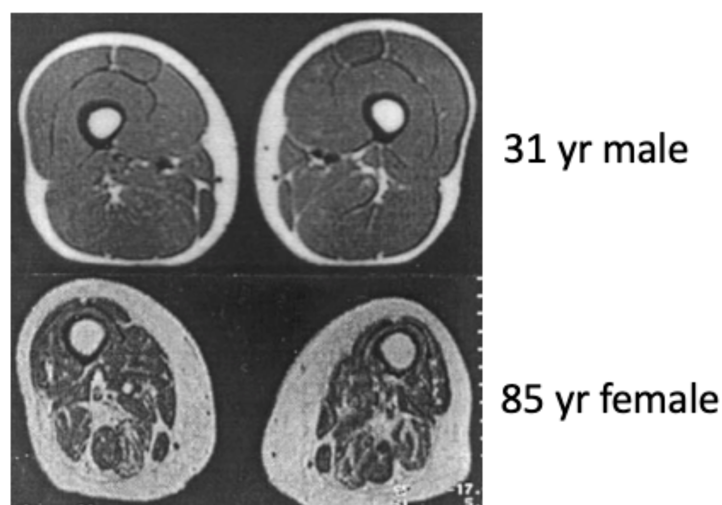

Figure 3.1- Representative cross-sectional MRI images of the upper thigh in a 31 yr. old male and 85. Yr female. Images taken from Parise & Yarasheski (2000).

In addition to losses of muscle mass and function, and recognised in the latest consensus on sarcopenia definition and diagnosis from the European Working Group on Sarcopenia in Older People (EWGSOP) [5], declines in muscle quality are also implicated in the development of sarcopenia, particularly in influencing muscle function [6]. Muscle quality can be defined as muscle strength per unit muscle mass, and is influenced by many factors including intramuscular fat infiltration, neural activation, and muscle composition (muscle fibre type, cross-sectional area, and muscle thickness) [7]. Given that contractile activity (i.e., exercise, and particularly resistance exercise [8]) and essential amino acids (EAAs; [8,9]) are accepted as the two most potent anabolic stimuli, sarcopenia is likely aggravated by reductions in physical activity and/or lower dietary protein intake, both of which are reported with advancing age [10], and each of which have been shown to be associated with low muscle mass in older adults [11,12]. In addition, it has been demonstrated that the muscle of older adults is not able to robustly increase MPS in response to these key anabolic stimuli (i.e., resistance exercise [13] and hyperaminoacidemia [6,14]) when compared to younger individuals (50).This phenomenon has been termed anabolic resistance [6] and has been postulated to be a significant contributor to the development and progression of sarcopenia [15].

Acute protein ingestion has been shown to result in acute increases in MPS, resulting in a positive NPB and promoting muscle mass maintenance and growth [8]. Despite the importance of protein ingestion as a robust acute stimulator of MPS via the provision of EAAs [16], increasing evidence demonstrates that older individuals consume less dietary protein compared to younger adults [17]. Indeed, despite observational and interventional evidence showing that higher protein intake elicits increases in muscle mass and strength [18–21] and quality of life [22], many older adults do not consume the daily recommended allowance (RDA) of protein (0.8 $g.kg^{-1}.day^{-1}$) [23]. There are many factors influencing

protein intake in older adults, including but not limited to appetite suppression (253), increased dementia (198) and dysphagia risk (195), sex, and age (195,198). Furthermore, there are several psychosocial factors including depression (197), education and income, race, and living situation (198). Despite evidence supporting the beneficial effect of habitual protein intake on muscle size and strength, its impact on mechanistic parameters underlying these benefits (i.e., MPS), is not widely reported. For example, higher daily protein intake has been reported to be associated with a preservation of muscle mass and increased quality of life in community-dwelling older adults [24,25], and increasing daily protein intake (from 1.2 to 1.6 g g.kg^{-1}.day^{-1}) has been shown to improve both whole-body [20] and appendicular lean mass [21] in older adults. However, these studies are interventional in nature and as such investigate the influence of protein supplementation over a period of time, as opposed to investigating the impact of true habitual protein intake.

Despite protein intake being a proven key factor in muscle mass preservation in older adults , another less-documented contributor is protein quality, which has been shown to have an important influence on MPS (135,292,293). There are several contributors to the quality of a given protein, including the AA, in particular leucine, content of the protein, the digestibility of the protein, determined by the AA bioavailability, and the digestion kinetics of the protein source (134). Evidence has demonstrated that when consuming a bolus dose (20g) of casein, casein hydrolysate, and whey protein, higher MPS rates were observed in whey protein compared to casein and casein hydrolysate despite comparable total protein content. Pennings *et al.,* (2011) concluded that the observed results were attributed to the faster digestion rate and absorption kinetics, and a greater leucine content in whey protein compared to casein and casein hydrolysate (294). Protein quality was previously most commonly measured using the protein digestibility-corrected amino acid score (PDCAAS)

(295); however, a superior method was more recently proposed- the digestible indispensable amino acid score (DIAAS) (296). DIAAS is defined as:

$$\textbf{DIAAS \%} = 100 \times \frac{\textbf{(mg of digestible dietary indispensable amino acid in 1g of the dietary protein)}}{\textbf{(mg of the same dietary indispensable amino acid in 1g of the reference protein)}}$$

This new method of measuring protein quality was developed due to several issues that were raised in the PDCAAS. It has been highlighted that EAAs should be regarded as individual nutrients rather than grouped as protein, PDCAAS scoring did not highlight the differences between ileal and faecal protein digestibility (296), creating further room for discrepancies in scoring, and recent research has highlighted that certain AAs may be reduced in terms of their bioavailability due to chemical modifications reducing the digestible AA value (135,296,297). The DIAAs was designed to address these issues.

As discussed in the introduction to this thesis, there are 20 AAs which combine to form proteins. These are grouped into essential AAs (EAA) and non-essential AAs (NEAA). Details of these are provided in Table 3.1.An AA of particular interest to the investigation of protein quality is leucine. Research has demonstrated the importance of leucine in increasing MPS, an idea which is often termed as the leucine ‘threshold’ (298–301). This is a term describing rapid postprandial leucinemia, increasing intracellular leucine (through binding to Sestrin2 (144)) and resulting in elevated MPS after consumption of a high quality, easily digestible protein. Thereby, it is important to consider the leucine content of a given protein when investigating the relationship between protein consumption and physiological muscle parameters such as muscle mass and strength, and MPS, as in addition to being a substrate, leucine is acting as a signal.

Table 3.1- Amino Acid Classifications

Essential amino acids	**Non-essential amino acids**
Histidine	Alanine
Isoleucine	Arginine
Leucine	Asparagine
Lysine	Aspartic acid
Methionine	Cysteine
Phenylalanine	Glutamic acid
Threonine	Glutamine
Tryptophan	Glycine
Valine	Proline
	Serine
	Tyrosine

Based on the concept of anabolic resistance [15], evidence indicates that older individuals need to consume a higher acute protein dose, or a protein of a higher quality, to elicit the same MPS feeding response as younger adults [26]. This suggests that in order to minimise sarcopenia development, older adults should be consuming more protein in their habitual daily diet to maximise their cumulative MPS responses. However, this suggestion may be limited, in part, by the "muscle full" phenomenon. This phenomenon describes the point at which in skeletal muscle, MPS stimulation becomes resistant to ongoing hyperaminoacidemia (302). This may be overcome by using a pulsed rather than bolus protein feeding technique, with research in older men (aged 70.0 (0.9yrs); bolus group, 70.3 (1.1yrs); pulsed group) demonstrating that a pulsed feeding technique resulted in sustained MPS superior to bolus feeding, appearing to delay the "muscle full" effect (303). Despite this, the relationship between habitual protein intake and MPS has not been fully investigated, and therefore it is unclear whether higher habitual protein intake will impact

fasted MPS rates, and/or MPS responses to feeding in older adults. Therefore, this study aimed to investigate differences in the habitual diets of independent community-dwelling, healthy young and older adults who were studied prior to participation in an exercise training intervention [27], and explore the relationship between habitual protein and leucine intake and muscle mass, function and protein metabolism (i.e., MPS), and expression of several anabolic signalling targets.

3.3 Materials and Methods

3.3.1 Participants and study overview

This is a secondary analysis of data obtained from a previously published study reporting on the impact of age on adaptive responses to RET (1). In brief, potential young and older participants (Table 3.2) were recruited through the local community, and underwent health screening via a medical questionnaire, physical examination, and ECG to confirm eligibility (Table 3.2).

Table 3.2- Participant Characteristics

	Young	**Old**
n	11	16
Age (years)	25 (4)	70 (3)
Body Mass Index (kg/m^2)	23.3 (2.5)	26.8 (2.0)

Table 3.3- Study Inclusion/Exclusion Criteria (1)

Inclusion:
Aged 18-35 or 65-75 years
Able to complete prescribed physical activity regimen
Able to provide informed consent
Have normal blood chemistry and are normotensive (BP <140/90)
Exclusion:
Participation in regular (>2 sessions/week) structured physical activity
Taking any form of nutritional supplementation
Overt muscle wasting (>1 SD below age norms)
Metabolic, respiratory, or cardiovascular disorders

All participants provided informed written consent, and all screening and study procedures took place at the University of Nottingham Medical School at the Royal Derby Hospital Centre. All procedures were conducted in accordance with the Declaration of Helsinki and were given favourable opinion by the University of Nottingham Faculty of Medicine and Health Sciences Research Ethics Committee (D/2/2006).

3.3.2 Muscle function and body composition assessments

Knee-extensor strength (KES; 1 repetition maximum (1-RM)) was assessed using a free-standing resistance exercise training machine (Leisure Lines, Hinckley, Leicestershire UK), with whole-body strength (WBS) determined as the sum of 1-RMs produced by 3 lower- and 3 upper-body exercises:

Lower Body	Upper Body
Leg extension	Latissimus pull-down
Leg Curl	Seated row
Leg Press	Seated chest press

Following confirmation of eligibility, participants attended the research facility after an overnight fast (water *ad libitum*) for an acute study visit. At this visit, body composition was measured using dual-energy X-ray absorptiometry (DXA; Lunar Prodigy II, GE Medical Systems). Analysis of the DXA scan included analysis of a custom upper-leg region of interest (ROI) given the functional importance of the muscle groups in this region [28]. The ROI was defined as the lowest visible point of the coccyx to the mid-patella (Figure 3.2). Although not the gold-standard assessment of body composition, DXA is a non-invasive method of providing regional body composition values, delivering only a small radiation dose (~0.5 μSv) meaning that it is suitable for multiple assessments (304). However, there are downfalls to using DXA as a body composition assessment. DXA scanning requires expensive equipment and trained technicians, and some evidence suggests that DXA may under-estimate hypertrophy, thereby may not be able to detect subtle differences in muscle mass changes (1).

After the DXA scan, a primed, constant infusion of [1, 2-$^{13}C_2$] leucine tracer (0.66 mg/kg, 1 mg/kg/h, 99 atoms percent; Cambridge Isotopes Ltd.) was continually infused throughout the duration of the study (250 min), with the tracer dose increased (to 1.2 mg/kg/h) upon nutrition provision (at 130 min) to prevent tracer dilution.

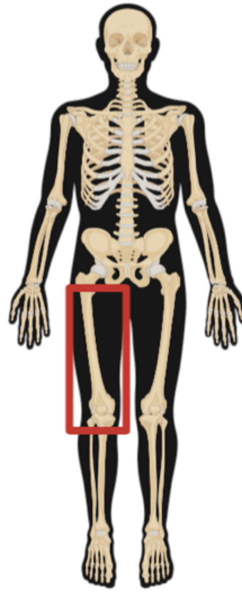

Figure 3.2- DXA upper leg region of interest

In order to determine the fractional synthetic rate of muscle, *m. vastus lateralis* muscle biopsies were collected using the conchotome biopsy technique [29] at 0, 120, and 250 min, using 1% lidocaine (B. Braun Melsungen) as local anaesthetic. Muscle samples were prepared accordingly and stored at −80 °C until further analysis. After the second biopsy (~130 min) participants received an oral feed (Fortisip, Nutricia Clinical Care) with composition similar to that of a normal mixed meal (16% protein, 49% carbohydrate, 35% fat). This feed was provided as an initial bolus, followed by four further doses at 30-min intervals thereafter. Doses were between 61 and 96 mL based on body weight to provide 6.5 kJ/kgBW/30 min [27]. Figure 3.3 provides a schematic representation of this acute study day.

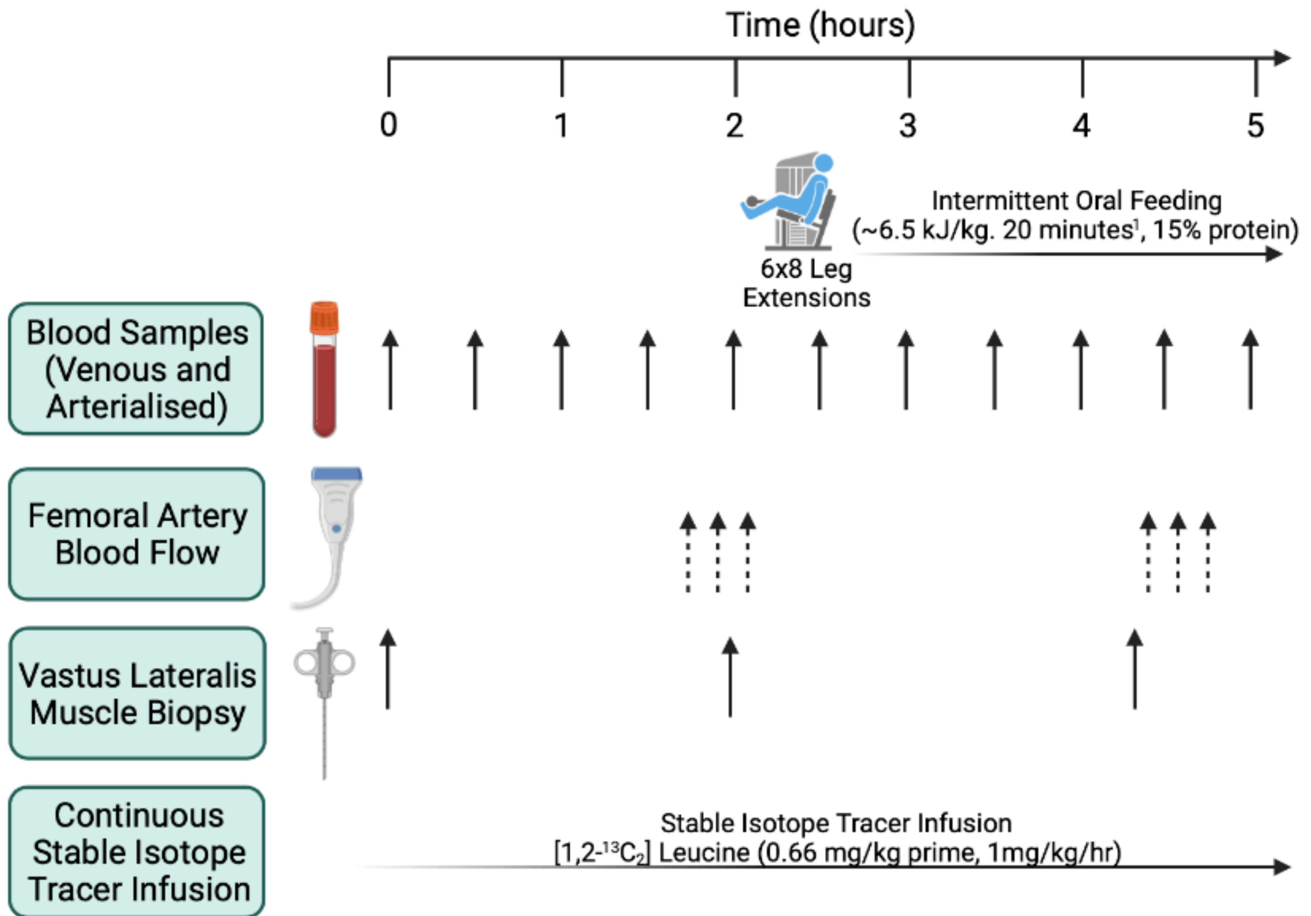

Figure 3.3- Schematic of acute study day. This study day was conducted before and after a 20-week whole-body resistance exercise training plan (3x each week, 70% 1-repetition maximum). Figure generated using Biorender. Figure redrawn from Phillips et al., (2017).

3.3.3 Dietary Assessment

Prior to completing any laboratory testing, all participants were required to complete a 4-day diet diary (adapted from the Royal Derby Hospital dietetic department) of all food and drink consumed. Diet diaries were analysed using Microdiet version 5 (Downlee Systems Ltd.).

3.3.4 Laboratory Analysis

3.3.4.1 Western Blotting

Protein expression of anabolic signalling targets were determined using western blotting (305). Western blotting (also referred to as immunoblotting) is a physiological technique

which can be used to investigate the presence of particular proteins, and the abundance of these proteins. Briefly, western blotting is a process involving 8 key steps. These steps are as follows:

1. Sample preparation- cellular proteins are extracted from a sample.
2. Gel electrophoresis- this step is also referred to as 'gel running', in which samples are loaded and run through a polyacrylamide gel to split the proteins in the sample according to their molecular weight in the gel matrix.
3. Membrane transfer- transferring the separated proteins from the polyacrylamide gel onto a nitrocellulose or PVDF membrane.
4. Membrane blocking- the membrane is submerged in a bovine serum albumin (BSA) or milk solution in order to 'block' the membrane, meaning that when you stain your membrane for a specific antibody, you minimise the risk of unspecific binding.
5. Primary antibody incubation- membranes are incubated in primary antibody for the target protein overnight.
6. Secondary antibody incubation- membranes are incubated in the relevant secondary antibody specific to the primary antibody used.
7. Detection- membranes are imaged (chemiluminescent or fluorescent) to determine the density of the target protein on the membrane, proportional to degree of antibody binding.
8. Analysis- the images are analysed using densitometry software to quantify the protein.

Western blotting is an essential method commonly used in physiological and biochemical research, and has many strengths. These include the sensitivity and specificity of the technique. Western blots can be used to detect as little as 0.1ng protein in a sample, and the ability to look at specific proteins using different antibodies. Furthermore, western blots are a

relatively cost-effective way of looking at protein abundance, and are often conducted prior to further, more in-depth testing. However, despite their convenience, there are several limitations which need to be considered when interpreting western blot data. There are several steps of western blots in which methods differ, including denaturation method during sample preparation, gel composition, effectiveness of gel transfer which influences probing of targets with primary and secondary antibodies and the specificity of the target protein antibodies, and the differences between individuals on sample detection and protein quantification methods (305). However, western blots are an invaluable method of investigating many regulatory processes. This secondary data analysis includes western blot results displaying the abundance of several anabolic signalling targets; Protein kinase C-alpha (PKC-α), Insulin receptor substrate 1 (IRS-1), Proline-rich Akt substrate-40 (PRAS-40), Akt substrate 160 (AS160), and AKT-substrate. These signalling targets interact with the mTOR-MPS pathway. However, PKC-α works downstream of mTORC2 and mainly influences actin

cytoskeletal remodelling. The association of these signalling targets with the mTOR-MPS pathway is graphically demonstrated in Figure 3.4.

3.3.4.2 Determination of muscle protein synthesis

Analysis and determination of MPS was conducted prior to this secondary data analysis. Briefly, a primed constant tracer infusion containing [1, $2^{13}C_2$]- Leucine was infused through the forearm antecubital vein to determine AA kinetics. Tracer infusion rate was calculated using subject body weight, and was administered with a prime dose of 0.6 $mg.kg^{-1}$ and constant infusion rate of $1mg.kg^{-1}.hr^{-1}$. This was increased to $1.2mg.kg^{-1}.hr^{-1}$ during the fed stage of the acute study day to prevent tracer dilution. Muscle protein synthesis was then

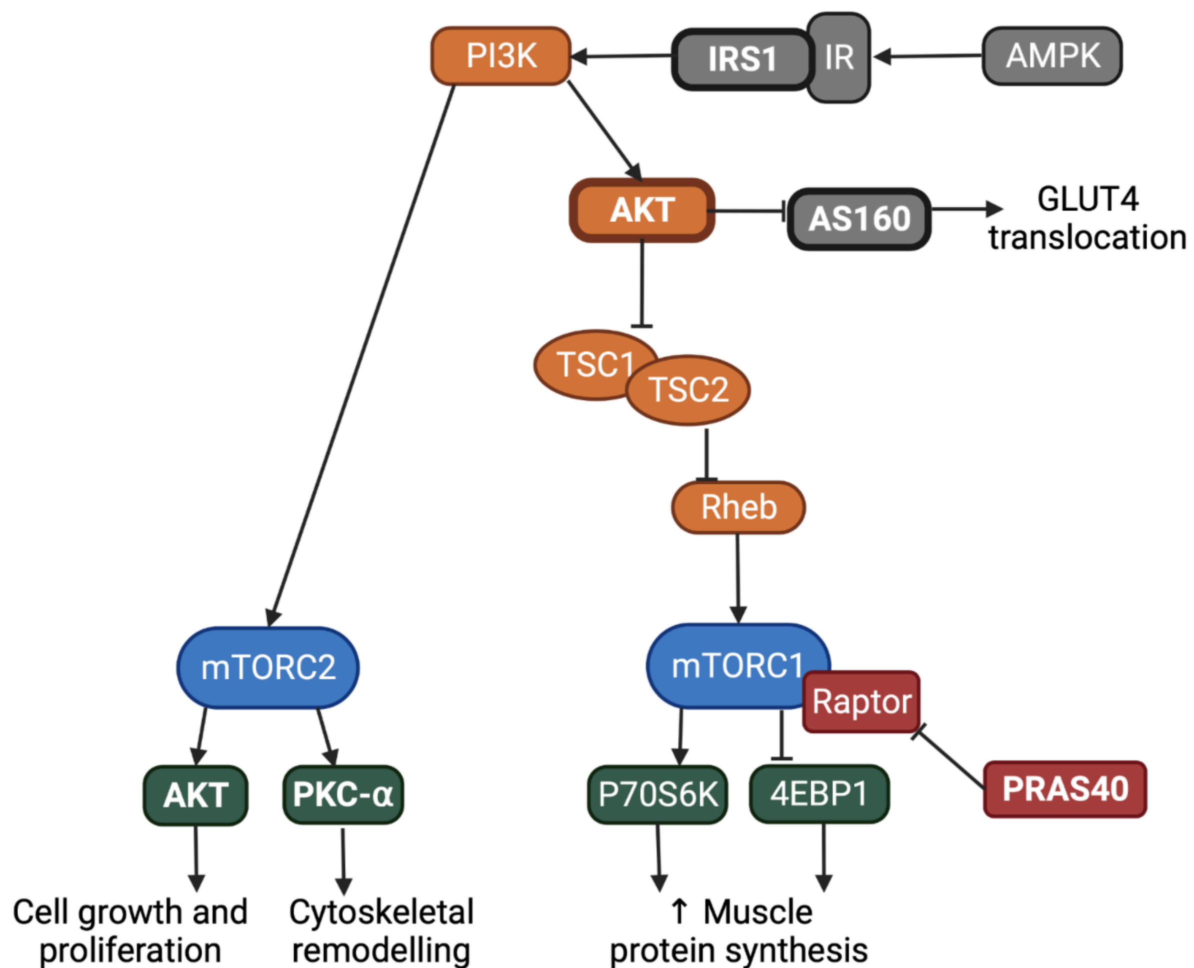

Figure 3.4- Graphical representation of association between measured anabolic targets and the mTOR-MPS pathway. Abbreviations: IRS1, Insulin receptor substrate 1; AS160, Akt substrate 160, PKC-α, protein kinase C- alpha; PRAS40, Proline-rich Akt substrate- 40.

determined using the tracer infusion, muscle biopsy data, and venous blood sampling, indicating the incorporation of the tracer into the muscle tissue protein.

In order to calculate MPS levels, the labelling of α-ketoisocaproate (α-KIC) needed to be measured as the primed constant isotope infusion method used relies on the precursor labelling of plasma α-KIC. Enrichment was determined using gas-chromatography-mass spectrometry, and subsequent rate of MPS between muscle biopsies was determined using a standard equation:

$$\mathbf{FSR\ (\%\cdot h^{-1}) = (\Delta E_m/E_p \times 1/t) \times 100}$$

ΔE_m= change in leucine muscle protein labelling between biopsy samples

E_p= mean enrichment of α-KIC $^{13}C_2$ labelling over time (protein synthesis precursor)

t = time between muscle biopsies

3.3.5 Statistical analysis

Statistical analysis was performed using GraphPad Prism v.9.0.0. All data were reported as mean (SEM), with significance set at $p < 0.05$. Unpaired t-tests were used to determine between group differences in habitual dietary intake and muscle-centric parameters between young and older adults. Linear regression and Pearson's correlation analysis were used to explore relationships between relative protein and leucine intake and muscle mass, function, and protein synthesis, and relationships between relative protein and leucine intake and expression of anabolic signalling targets.

3.4 Results

There were no significant difference in relative protein (Figure 3.5A), fat (Figure 3.5B), carbohydrate (Figure 3.5C) or leucine (Figure 3.5D) intake between young and older adults when expressed relative to whole body lean mass (WBLM) (g or mg.kgWBLM^{-1}.day^{-1}), nor when expressed relative to body weight (g or mg.kgBW^{-1}.day^{-1}). There was however a significant difference in relative fat intake between young and older adults ($p = 0.01$) when expressed relative to BW (Table 3.4).

Table 3.4- Differences in dietary intake between young and older adults

	Young per kg WBLM	Older per kg WBLM	Young per kg BW	Older per kg BW
Protein (g)	1.46 (0.15)	1.76 (0.10)	1.21 (0.10)	1.13 (0.06)
Fat (g)	1.37 (0.15)	1.30 (0.11)	1.14 (0.10)	0.83 (0.06*)
Carbohydrate (g)	3.71 (0.43)	4.08 (0.29)	3.04 (0.22)	2.62 (0.17)
Leucine (mg)	40.84 (8.09)	36.77 (4.90)	33.33 (5.30)	23.57 (11.89)

Abbreviations: WBLM, whole-body lean mass; BW, body weight. N = 11 young and N = 16 older adults. * represents a statistically significant difference compared to younger adults (P < .05). All data presented as mean ± SEM.

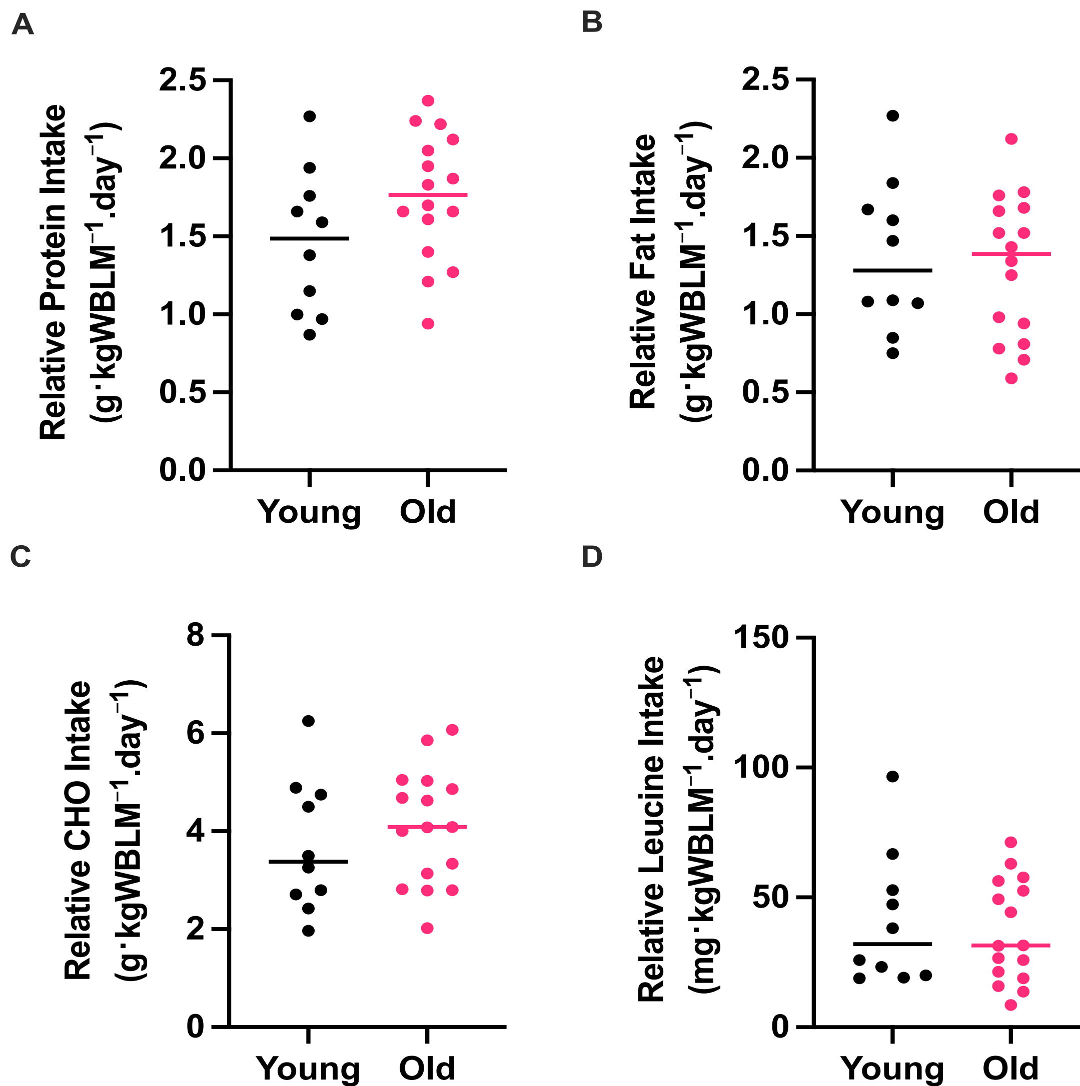

Figure 3.5- Habitual dietary intake parameters in young (n = 10) and older (n = 16) adults. (**A**) Protein intake (p = 0.62), (**B**) fat intake (p = 0.81), (**C**) carbohydrate (CHO) intake (p = 0.62), and (**D**) leucine intake (p = 0.35). Analysis via unpaired t-tests.

WBLM was significantly higher in young compared to older adults (Figure 3.6A, 61.8 (9.9) vs. 49.8 (11.9) kg, p = 0.01), although there was no significant difference in upper-lean leg mass (ULLM) (Figure 3.6B; 4.99 (1.28) vs. 4.55 (1.25) kg, p = 0.40). Despite the lack of difference in ULLM, young adults demonstrated significantly greater knee-extensor strength (KES) compared to older adults (Figure 3.6D, 87.7 (28.3) vs. 56.8 (16.4) kg, p = 0.002), although whole-body strength (WBS) was not significantly different (Figure 3.6C; 79.9 (21.9) vs. 65.3 (19.4) kg, p = 0.08) between the age groups.

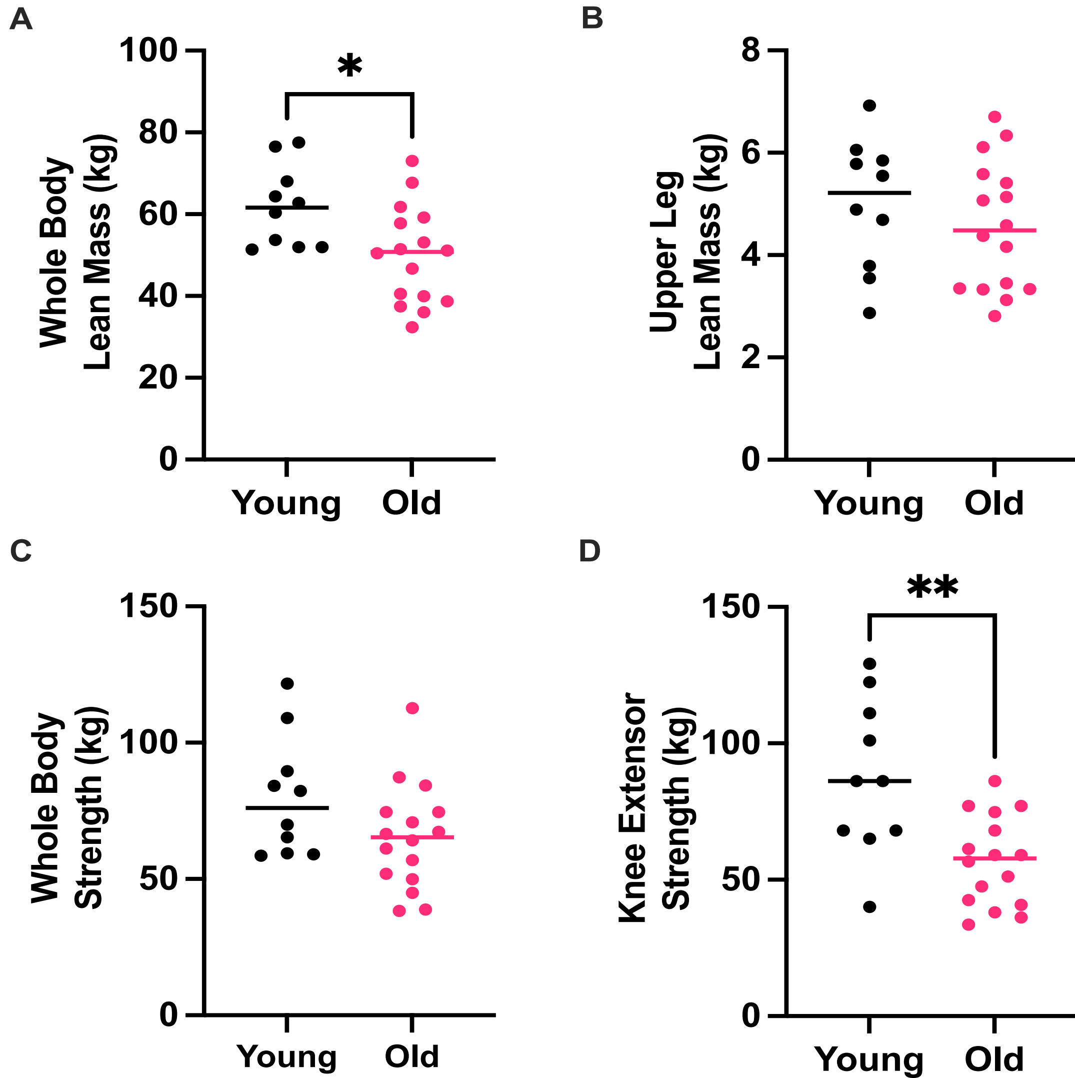

Figure 3.6- Muscle mass and strength parameters in young (N = 10) and older (N = 16) adults. (A) Whole body lean mass (p = 0.01), (B) upper leg lean mass (p = 0.40), (C) whole body strength (p = 0.08), and (D) knee extensor strength (p = 0.002). Analysis via unpaired t-tests, * denotes p <0.05, ** denotes p < 0.01.

No significant differences were observed in Fasted MPS (FSR %/h) between younger and older adults (Figure 3.7A, 0.044 (0.015) vs. 0.042 (0.013), p = 0.65). Furthermore, there was also no significant difference observed in Fed MPS (FSR %/h) between younger and older adults (Figure 3.7B, 0.086 (0.031) vs. 0.081 (0.024), p = 0.64).

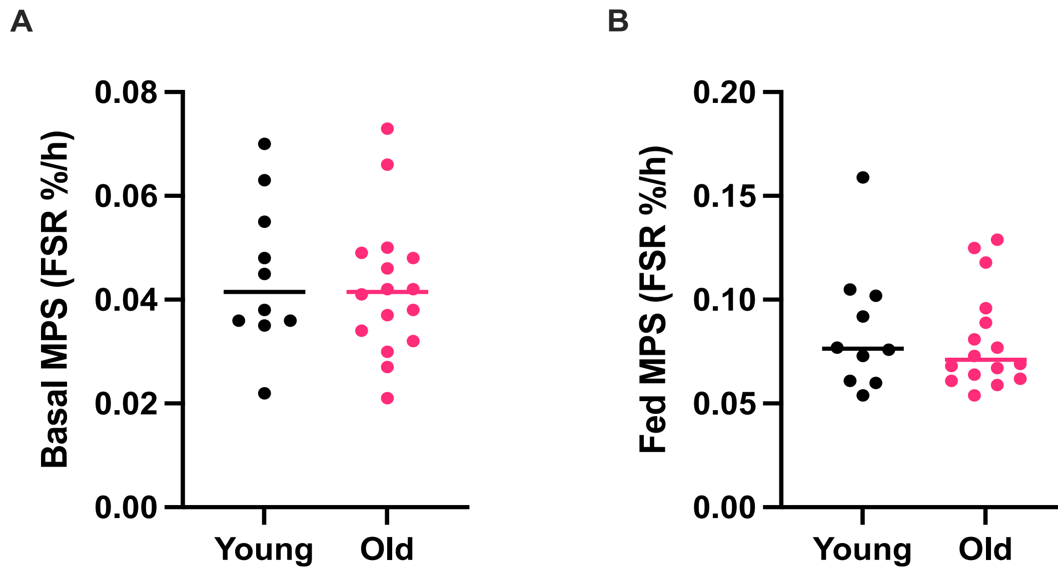

Figure 3.7- Fasted (basal) and Fed MPS (FSR %/h) values in young (n = 10) and older (n = 16) adults. (**A**) Basal MPS (p = 0.65), (**B**) Fed MPS (p = 0.64). Analysis via unpaired t-tests.

As there was no significant difference in relative protein intake (in $g.kgWBLM^{-1}.day^{-1}$ or $g.kgBW^{-1}.day^{-1}$) between young and older adults, data from both age groups were pooled to increase the statistical power to explore the relationship between facets of habitual protein intake and muscle mass, and metabolic and functional parameters. There was no significant relationship between habitual protein intake (in $g.kg^{-1}.day^{-1}$) and MPS, either in the basal state ($R^2 = 0.003$, $p = 0.79$) nor in response to feeding ($R^2 = 0.043$, $p = 0.31$) (Figure 3.9A,B). There were no significant relationships between habitual protein intake (in $g.kg^{-1}.day^{-1}$) and WBLM ($R^2 = 0.037$, $p = 0.35$) or ULLM ($R^2 = 0.002$, $p = 0.81$) (Figure 3.8A,B). There were also no relationships with whole-body muscle strength (WBS ($R^2 = 0.034$, $p = 0.37$) or KES ($R^2 = 0.001$, $p = 0.86$) (Figure 3.8C,D).

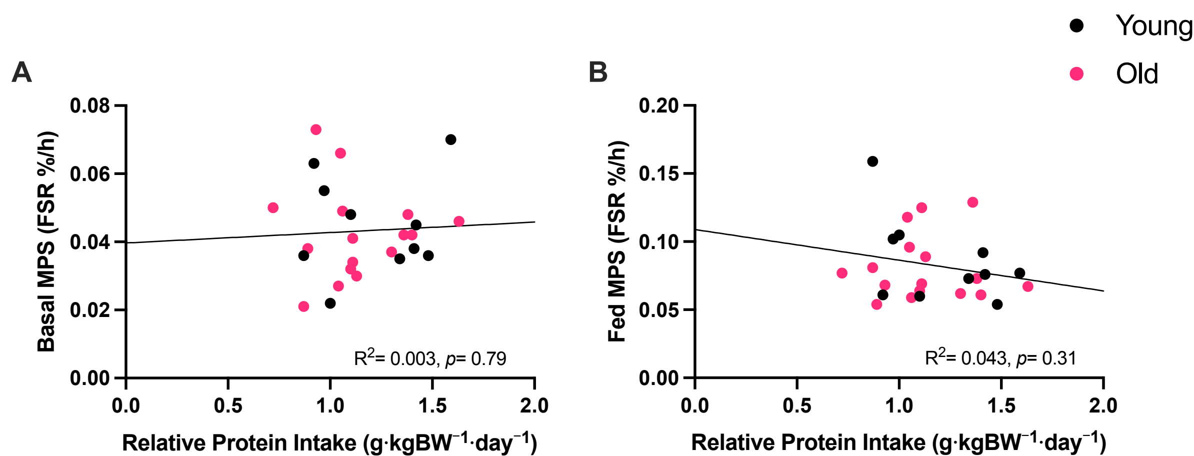

Figure 3.9- The relationship between relative protein intake expressed relative to body weight and muscle protein synthesis in young (N = 10) and older (N = 16) adults. A) basal MPS, B) fed state MPS. Analysis via linear regression.

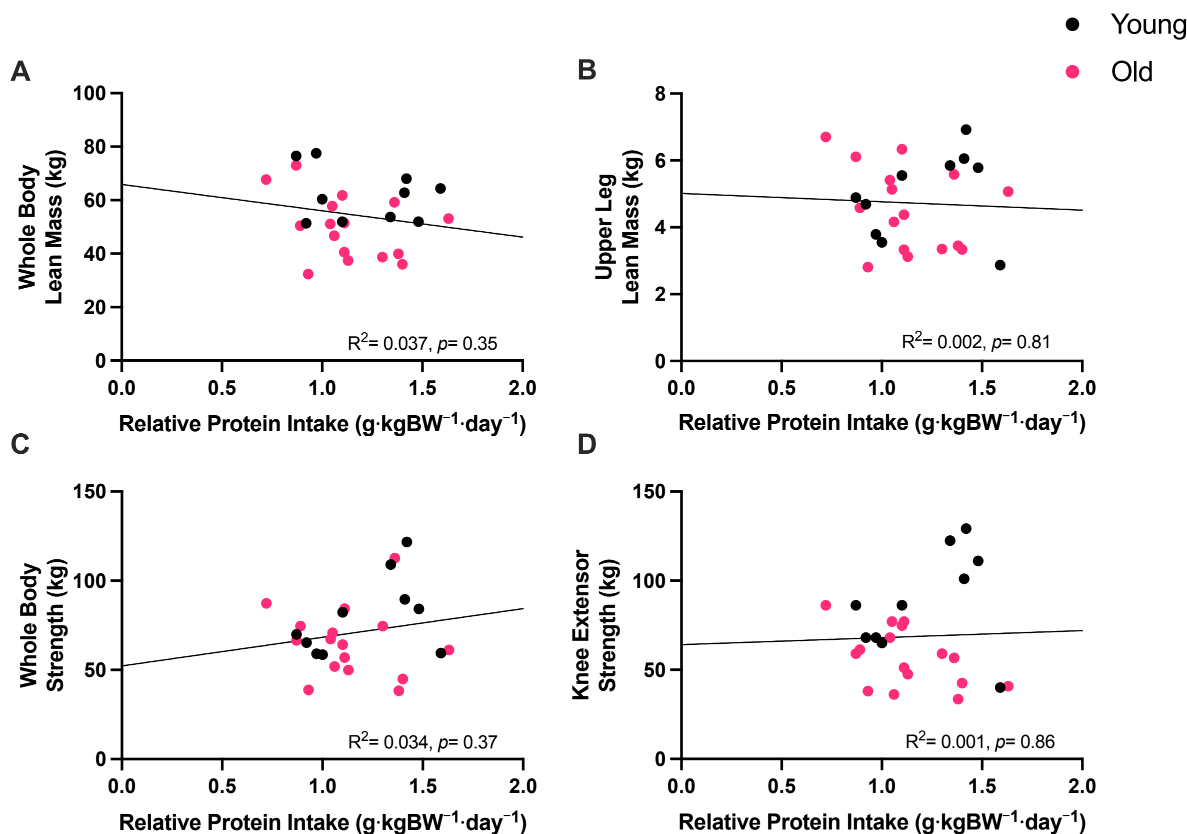

Figure 3.8- The relationship between relative protein intake expressed relative to body weight and physiological muscle parameters in young (N = 10) and older (N = 16) adults. A) whole body lean mass, B) upper leg lean mass, C) whole body strength, D) knee extensor strength. Analysis via linear regression.

Akin to that seen when habitual protein intake was expressed relative to body weight (Figure 3.8, Figure 3.8), there was also no relationship between habitual protein intake and any aspect of muscle mass, function, or metabolism when protein intake was expressed relative to WBLM (Table 3.5) excluding the relationship with WBLM (*p*<.001), however, this is expected when expressing protein intake relative to WBLM.

Table 3.5- Relationship between habitual protein intake relative to whole-body lean mass and muscle-centric parameters.

Parameter	R^2	P-value
Whole-body lean mass	0.3907	<0.001*
Upper-leg lean mass	0.029	0.40
Whole-body strength	<0.01	0.91
Knee-extensor strength	0.022	0.47
Post-absorptive muscle protein synthesis	<0.001	0.98
Postprandial muscle protein synthesis	0.116	0.09

Analysis via simple linear regression. A statistically significant relationship was observed between relative protein intake ($g.kgWBLM^{-1}.day^{-1}$) and whole-body lean mass, as expected. No other significant relationships were observed.

As for leucine intake, there was no significant difference in relative leucine intake (when expressed as $mg.kgBW^{-1}.day^{-1}$) between young and older adults. As such, data from both age groups were pooled to increase the statistical power and explore the relationship between habitual leucine intake and muscle mass, functional and metabolic parameters.

There was no significant relationship between habitual leucine intake and WBLM ($R^2 = 0.013$, $p = 0.57$) or ULLM ($R^2 = 0.024$, $p = 0.45$) (Figure 3.10A,B), and no relationship with WBS ($R^2 = 0.014$, $p = 0.57$) or KES ($R^2 = 0.002$, $p = 0.83$)) (Figure 3.10C,D). Neither postabsorptive ($R^2 = 0.029$, $p = 0.40$) nor postprandial ($R^2 = 0.006$, $p = 0.71$) MPS was associated with habitual leucine intake (Figure 3.11A,B).

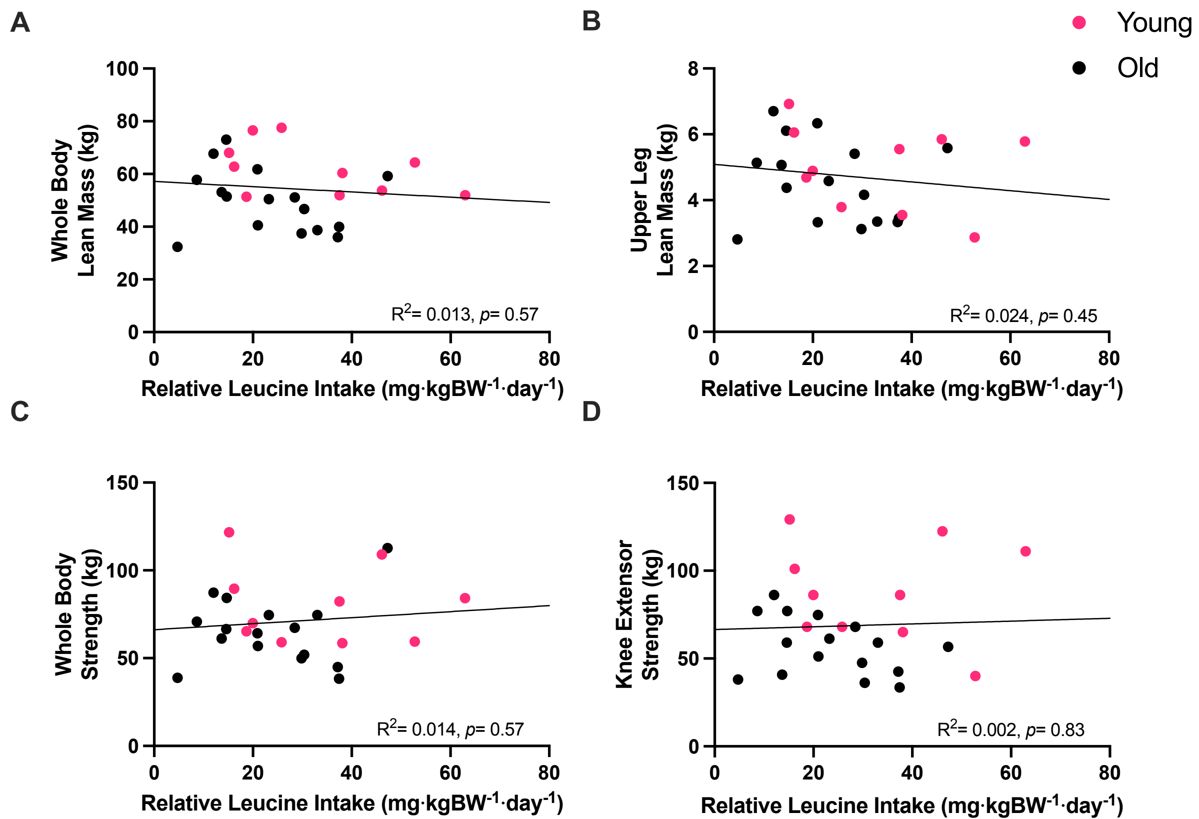

Figure 3.10- The relationship between relative leucine intake expressed relative to body weight and physiological muscle parameters in young (N = 10) and older (N = 16) adults. A) whole body lean mass, B) upper leg lean mass, C) whole body strength, D) knee extensor strength. Analysis via linear regression.

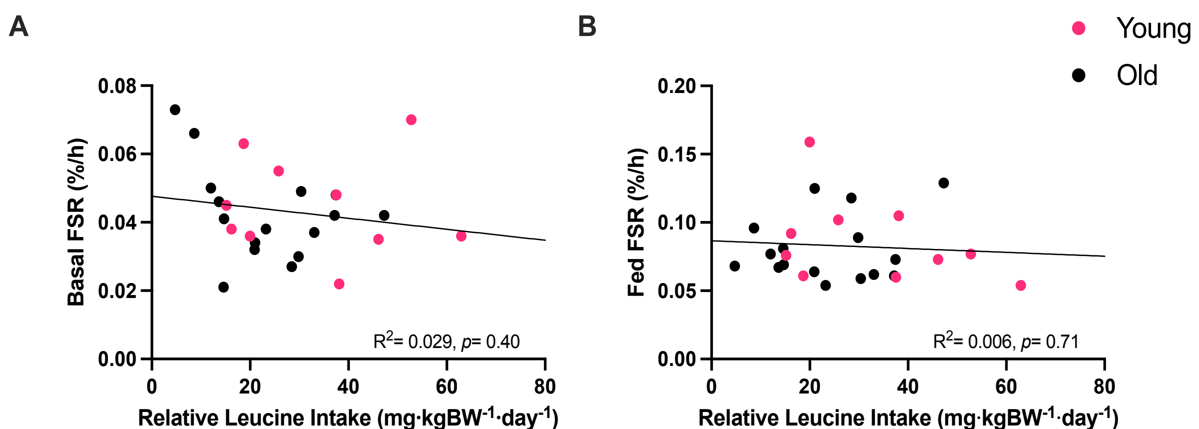

Figure 3.11- The relationship between relative leucine intake expressed relative to body weight and muscle protein synthesis in young (N = 10) and older (N = 16) adults. A) basal MPS, B) fed state MPS. Analysis via linear regression.

When habitual leucine intake was expressed relative to WBLM, there was also no relationship between habitual leucine intake and any aspect of muscle mass, function, or metabolism when leucine intake was expressed relative to WBLM (Table 3.6), excluding a significant relationship with WBLM (p = .01), however, this is expected considering leucine intake is being expressed relative to that parameter.

Table 3.6- Correlation coefficients of relationship between habitual leucine intake relative to whole-body lean mass and muscle-centric parameters.

Parameter	R^2	P-value
Whole-body lean mass	0.232	0.01*
Upper-leg lean mass	0.019	0.50
Whole-body strength	0.011	0.62
Knee-extensor strength	<0.01	0.99
Post-absorptive muscle protein synthesis	0.055	0.25
Postprandial muscle protein synthesis	0.027	0.42

Analysis via simple linear regression. A statistically significant relationship was observed between relative leucine intake ($mg.kgWBLM^{-1}.day^{-1}$) and whole-body lean mass, as expected. No other significant relationships were observed.

In addition to exploring the relationships between habitual protein and leucine intake and structural (mass), functional (strength) and metabolic (MPS) muscle-centric parameters, linear regression analysis was used to investigate the relationship between relative protein and leucine intake expressed relative to body weight (BW) and WBLM, and protein expression of anabolic signalling proteins.

No relationship was observed between any anabolic signalling target and habitual protein intake ($g.kgBW^{-1}.day^{-1}$) in young or older adults, nor when the two age-groups were combined, excluding PRAS-40. A significant positive relationship was observed between PRAS-40 and habitual protein intake when combining results from both age groups (Figure

3.12, Table 3.7). Furthermore, no significant relationship was observed between relative leucine intake (g.kgWBLM^{-1}.day^{-1}) and anabolic cell signals in young or older adults, nor when the two age-groups were combined (Figure 3.13, Table 3.8).

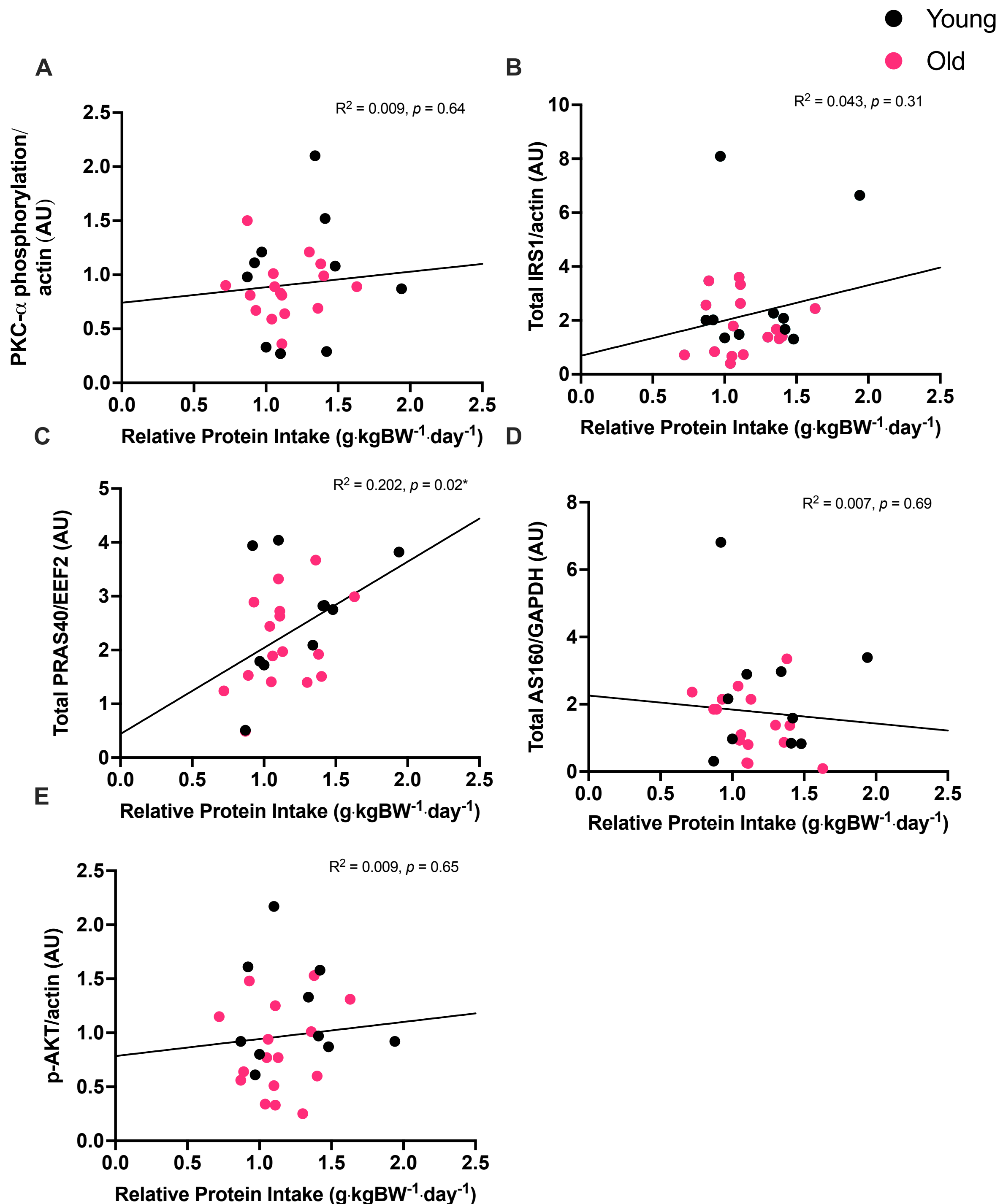

Figure 3.12- Relationship between habitual protein intake relative to body weight and protein expression of anabolic cell signals in young and older individuals and in both age-groups combined. Analysis via simple linear regression. Abbreviations: PKC-α, protein kinase C- alpha; IRS-1, insulin receptor substrate-1; PRAS-40, proline-risk AKT substrate 40; AS160, AKT substrate 160; AKT.

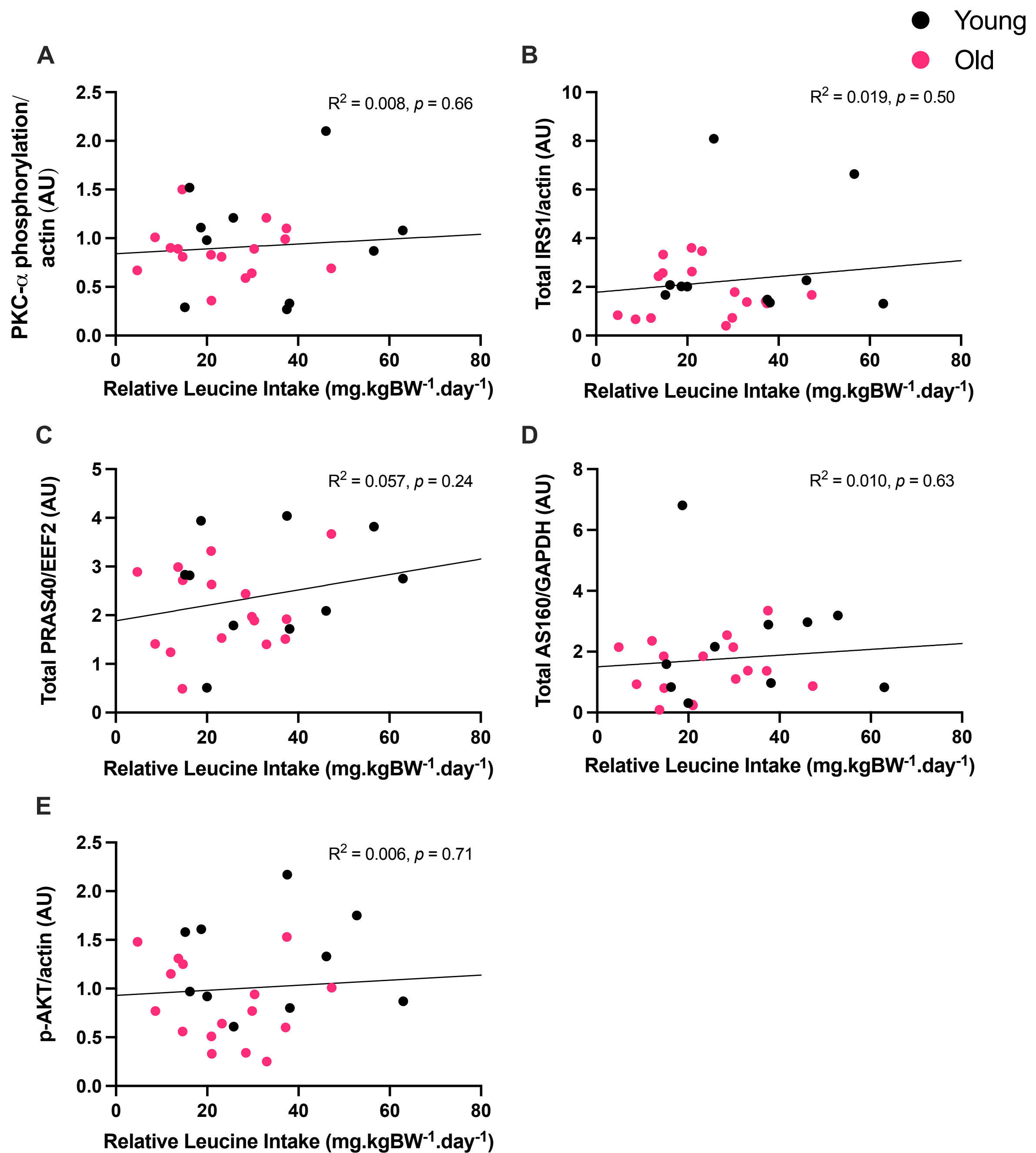

Figure 3.13- Relationship between habitual leucine intake relative to body weight and protein expression of anabolic cell signals in young and older individuals and in both age-groups combined. Analysis via simple linear regression. Abbreviations: PKC-α, protein kinase C- alpha; IRS-1, insulin receptor substrate-1; PRAS-40, proline-risk AKT substrate 40; AS160, AKT substrate 160; AKT.

Table 3.7- Correlation coefficients of relationship between habitual protein intake relative to body weight and protein expression of anabolic cell signals in young and older individuals and in both age-groups combined.

Target	Young		Old		Combined	
	R^2	P-value	R^2	P-value	R^2	P-value
PKC-α	0.010	0.78	<0.001	0.93	0.009	0.64
IRS-1	0.048	0.54	0.001	0.89	0.043	0.31
PRAS-40	0.191	0.21	0.160	0.13	0.202	0.02*
AS160	0.003	0.88	0.104	0.22	0.007	0.69
AKT-substrate	0.009	0.79	0.020	0.61	0.009	0.65

Analysis via simple linear regression. Abbreviations: PKC-α, protein kinase C- alpha; IRS-1, insulin receptor substrate-1; PRAS-40, proline-risk AKT substrate 40; AS160, AKT substrate 160; AKT.

Table 3.8- Correlation coefficients of relationship between habitual leucine intake relative to body weight and protein expression of anabolic cell signals in young and older individuals and in both age-groups combined

Target	Young		Old		Combined	
	R^2	P-value	R^2	P-value	R^2	P-value
PKC-α	0.008	0.80	<0.001	0.91	0.008	0.66
IRS-1	0.012	0.77	0.011	0.70	0.019	0.50
PRAS-40	0.038	0.59	0.018	0.62	0.057	0.24
AS160	0.006	0.83	0.021	0.59	0.010	0.63
AKT-substrate	0.003	0.89	0.045	0.43	0.006	0.71

Analysis via simple linear regression. Abbreviations: PKC-α, protein kinase C- alpha; IRS-1, insulin receptor substrate-1; PRAS-40, proline-risk AKT substrate 40; AS160, AKT substrate 160; AKT.

Furthermore, no significant relationships were observed between any anabolic signalling target and habitual protein (Figure 3.14, Table 3.9) intake when expressed relative to WBLM ($g.kgWBLM^{-1}.day^{-1}$).

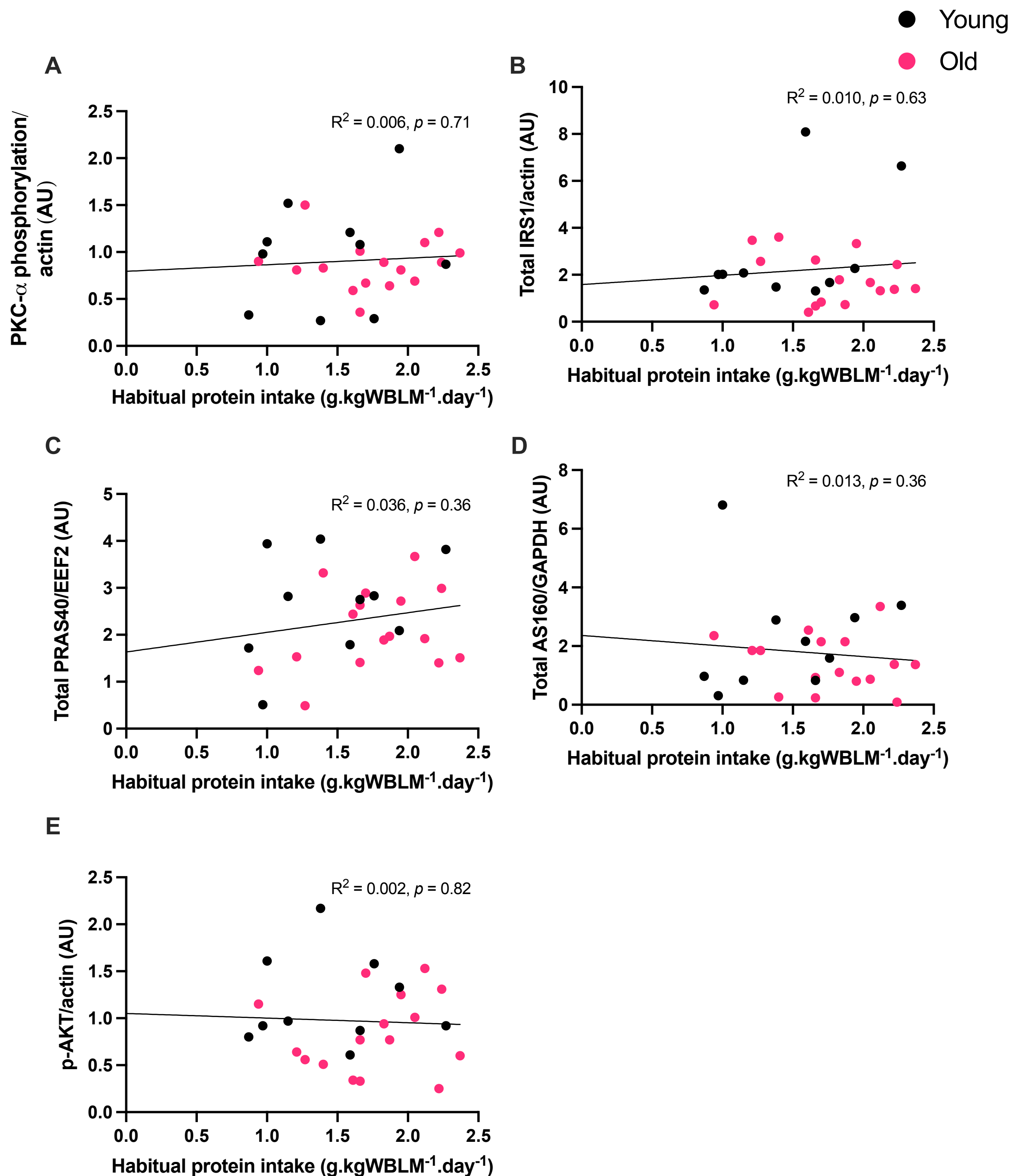

Figure 3.14- Relationship between habitual protein intake relative to whole body lean mass and protein expression of anabolic cell signals in young and older individuals and in both age-groups combined. Analysis via simple linear regression. Abbreviations: PKC-α, protein kinase C- alpha; IRS-1, insulin receptor substrate-1; PRAS-40, proline-risk AKT substrate 40; AS160, AKT substrate 160; AKT.

Table 3.9- Correlation coefficients of relationship between habitual protein intake relative to whole body lean mass and protein expression of anabolic cell signals in young and older individuals and in both age-groups combined

Target	Young		Old		Combined	
	R^2	P-value	R^2	P-value	R^2	P-value
PKC-α	0.043	0.56	<0.001	0.93	0.006	0.71
IRS-1	0.232	0.16	0.020	0.60	0.010	0.63
PRAS-40	0.098	0.38	0.083	0.28	0.036	0.36
AS160	0.009	0.79	0.035	0.49	0.013	0.58
AKT-substrate	<0.001	0.98	0.024	0.57	0.002	0.82

Analysis via simple linear regression. Abbreviations: PKC-α, protein kinase C- alpha; IRS-1, insulin receptor substrate-1; PRAS-40, proline-risk AKT substrate 40; AS160, AKT substrate 160; AKT.

For the most part, there were no significant relationships between habitual leucine intake expressed relative to WBLM ($mg.kgWBLM^{-1}.day^{-1}$) and anabolic cell signalling. One significant positive correlation was observed between habitual leucine intake and IRS-1 protein expression in young adults only (Figure 3.15, Table 3.10).

Table 3.10- Correlation coefficients of relationship between habitual leucine intake relative to whole body lean mass and protein expression of anabolic cell signals in young and older individuals and in both age-groups combined

Target	Young		Old		Combined	
	R^2	P-value	R^2	P-value	R^2	P-value
PKC-α	0.080	0.43	<0.001	0.98	0.03	0.38
IRS-1	0.444	0.04*	0.028	0.53	0.131	0.07
PRAS-40	0.115	0.34	0.007	0.77	0.052	0.26
AS160	0.204	0.19	0.024	0.56	0.117	0.09
AKT-substrate	<0.001	0.98	0.042	0.45	0.004	0.77

Analysis via simple linear regression. Abbreviations: PKC-α, protein kinase C- alpha; IRS-1, insulin receptor substrate-1; PRAS-40, proline-risk AKT substrate 40; AS160, AKT substrate 160; AKT.

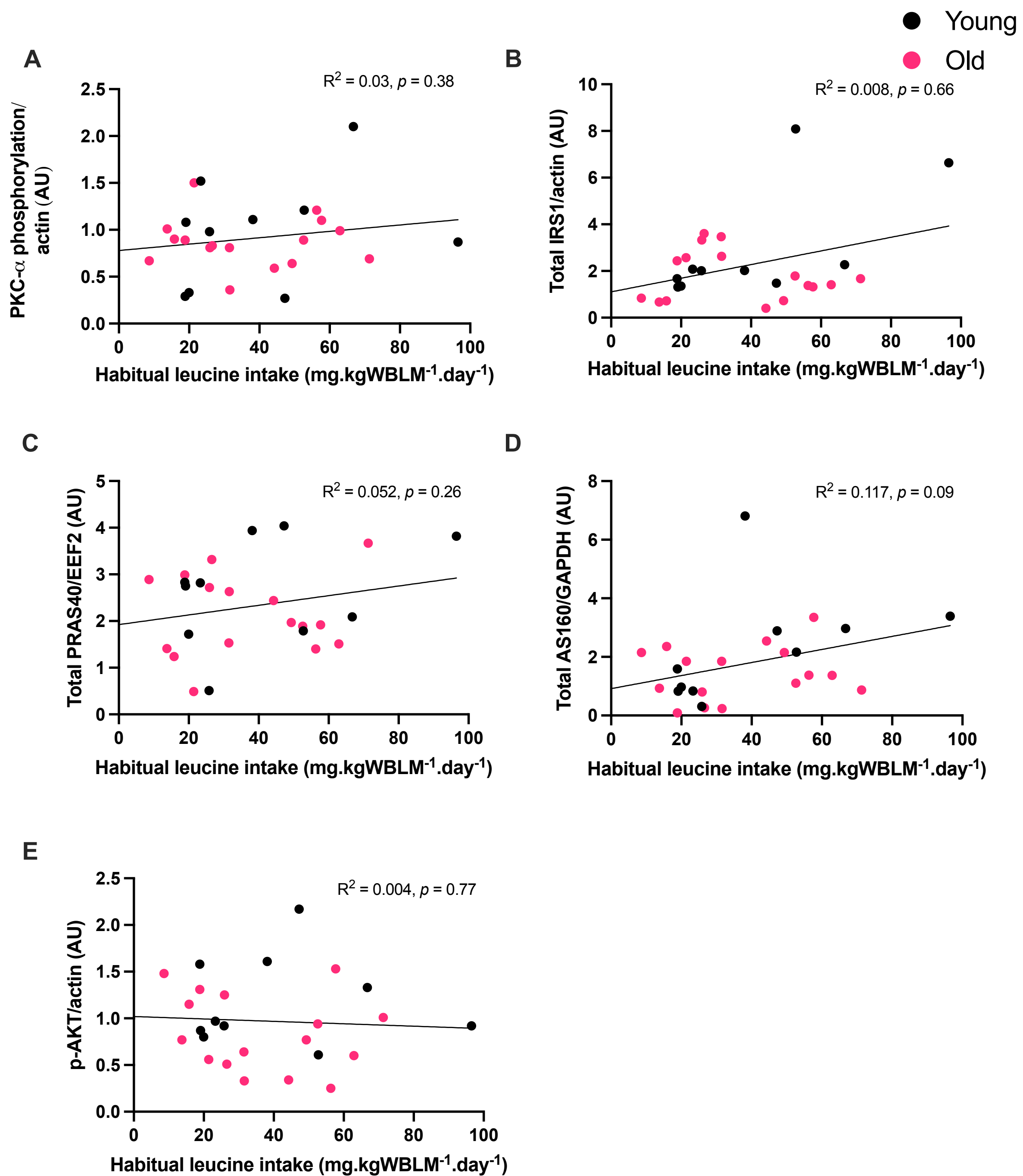

Figure 3.15- Relationship between habitual leucine intake relative to whole body lean mass and protein expression of anabolic cell signals in young and older individuals and in both age-groups combined. Analysis via simple linear regression. Abbreviations: PKC-α, protein kinase C- alpha; IRS-1, insulin receptor substrate-1; PRAS-40, proline-risk AKT substrate 40; AS160, AKT substrate 160; AKT.

3.5 Discussion

This secondary data analysis reports significant differences in habitual dietary intake expressed relative to either WBLM or BW between young and older adults, and no relationship between indices of habitual protein intake (including leucine intake) and measures of muscle mass, function or MPS. Further, expected reductions in whole-body, but not upper limb, muscle mass was observed in older adults, with significantly lower knee-extensor, but not whole-body, strength. For the main part there were no significant associations observed between relative protein or leucine intake, excluding PRAS40 and relative protein intake ($g.kgBW^{-1}.day^{-1}$) across all ages, and IRS-1 and relative leucine intake ($g.kgWBLM^{-1}.day^{-1}$) in younger adults only.

The current RDA for protein intake in both young and older adults is $0.8g.g^{-1}.day^{-1}$ (306), however, there are suggestions that this should be higher in older adults (251), especially in individuals with chronic disease or injury, or experiencing severe malnutrition (178). When considering these guidelines, all the young adults, and all the older adults in this study except one (94%) achieved this threshold. Further, even when considering the suggestion of alternative guidelines for healthy older adults (as proposed by the PROT-AGE group) of 1.0-1.2 $g.kg^{-1}.day^{-1}$ (178), only 25% of the older adults in this study did not achieve this. These results highlight that, based on the individuals recruited to this study, independent, community-dwelling, healthy older adults are consuming the recommended amount of protein to aid skeletal muscle maintenance and growth. Despite this, our older adults did present with lower WBLM and reduced knee-extensor (a muscle group crucial for activities of daily living (307)) strength. This finding of sufficient protein intake in the older age group is in contrast to previously published results investigating the dietary habits of young, middle-aged, and older adults, which reported a significant difference in relative protein intake

between young and older adults (308), and also reported that 65% of older adults did not reach a protein intake of 1.0g.kg^{-1}.day^{-1}. However, this study by Smeuninx *et al*., reported on a larger sample (n = 40 young and n = 40 older adults) compared with those in the present study, and importantly recruited an older age-group that was on average 7 years older than that here. These results suggests that insufficient protein intake is unlikely to explain the reductions in muscle mass and function that we observed.

Aside from protein intake, another important dietary factor to consider in influencing skeletal muscle health is EAA intake (303), particularly leucine (52). There is a growing body of evidence supporting the positive role of protein quality on skeletal muscle health, with affirmation that the capacity of a protein source to stimulate MPS is due, at least in part, to the leucine content of the protein (52,300,301). Leucine is a key building block of protein and is a potent activator of the mammalian target of rapamycin complex-1 (mTORC1) signalling cascade, subsequently stimulating MPS (141–143,309). Leucine has been shown to enhance the acute MPS response following a low protein dose in older adults (52) and when integrated into daily meals, has been shown to enhance integrated MPS rates in older men (310). It has been suggested that older adults have a higher leucine threshold, thereby requiring a higher dose of leucine to maximally stimulate MPS (52,311). No differences in habitual leucine intake between young and older adults were found in this study, and no associations between relative leucine intake and any aspect of muscle composition, function, or metabolism (i.e., MPS). This suggests that there are other factors contributing to the observed reductions in muscle mass in older compared with young adults. Physical activity is a key factor which can contribute to muscle mass losses with older age. A clear association between regular physical activity and improved muscle function has been previously demonstrated in master athletes (27–29), with an accelerated magnitude of muscle function decline in sedentary individuals, resulting in lifestyle- and age-associated disease risk (30). Furthermore, there are several

cellular mechanisms which may contribute to muscle mass losses with increasing age, including but not limited to blunted skeletal muscle MPS (31), motoneuron losses (34), 'inflammageing' (increase in chronic low-grade inflammation with increasing age) (37), anabolic hormone changes (36), and an inability of satellite cells (muscle precursor cells) to repair and regenerate damaged tissue (312). These have been demonstrated in human-based research, alongside mechanistic studies.

Beyond muscle mass losses, there were observed reductions in KES in our older adults. Considering sarcopenia as an umbrella term used to describe whole-body declines in muscle mass and function with advancing age (15), it is interesting that recent research has demonstrated specific early losses of lower limb function with advancing age in both men and women (313–316); results consistent with the finding in this study of age-associated reductions in KES but not WBS. These results are similar to that of a previous longitudinal study demonstrating losses of KES over 3-years, 9-years, and 10-years (11,48,316) in older adults. However, these studies also report early losses of lower limb mass, while the presented findings display no significant differences in ULLM between young and older adults. The decreases in lower limb muscle strength observed in older adults, even in the absence of reductions in lean mass, may be due, in part, to decreases in muscle quality such as muscle architecture (317), myosteatosis (318) and/or neuromuscular function (319), each of which have been shown to impact muscle function (11,317). It must also be considered that these results may reflect inherent limitations of DXA not being sufficiently sensitive to detect subtle differences in body composition (320,321), especially given the relatively small sample size and physiological heterogeneity (e.g., males and females) of the participants. Considering this proposition, and the observation of a reduction in whole-body lean mass, it may be that the smaller size of the custom upper-leg ROI may have not allowed for a true measure of ULLM to be recorded.

The anabolic effect of acute protein ingestion on skeletal muscle is well-reported, however, the impact of habitual protein intake on physiological muscle parameters supporting muscle mass and function, in particular MPS, has not been widely investigated. Previous research has reported that a lower acute protein dose is needed to maximally stimulate MPS in younger compared to older adults (0.24g/kg vs. 0.40g/kg) (173). However, it is unclear whether these findings also apply when considering the habitual protein intake. A retrospective analysis of previously published work (59,174,301,322–324) suggested that healthy older men may have attenuated sensitivity to low protein intake (173), thereby requiring a higher protein intake relative to BW to maximally stimulate MPS. Moore and colleagues (2015) demonstrated that in order to maximally stimulate MPS in older men, ~68% and ~140% greater protein intake (relative to body mass and lean body mass respectively) were required, in comparison to younger adults (173). There is however a proven limitation to the concept that older adults should simply consume more protein to increase their rates of MPS, commonly presented as the "muscle-full" phenomena, describing the point at which in skeletal muscle, MPS stimulation becomes resistant to ongoing hyperaminoacidemia (302). Although the role of habitual protein intake on MPS is unclear, 2-weeks habituation to a low or high protein diet (0.7g/kg vs. 1.5g/kg) did impact AA availability in healthy older men. This augmentation is likely due to reductions in splanchnic AA retention in the low protein group (325). However, despite this, there were no differences in MPS responses to an acute dose of whey protein (25 g), possibly due to a redistribution of protein-derived AAs to other tissues (325), and/or the aforementioned intrinsic "switch-off" of MPS when "full".

An important point to consider in relation to protein intake and muscle-centric parameters beyond habitual, or even daily protein, intake is the distribution pattern of protein feeding. Recent and emerging evidence suggests that a pivotal factor in maximising the

anabolic response of skeletal muscle to protein ingestion is an even distribution of protein intake across the day as opposed to high habitual protein intake via a skewed distribution (308). Although there is evidence for an equivalent effect of bolus compared to pulse feeding in older men, pulse feeding was shown to be associated with a delayed onset of the "muscle full" phenomenon (302,303,326), highlighting the potential of pulsed feeding exhibiting clinical benefits. However, the temporal nature of protein intake was not assessed in this study.

When interpreting the results of this study, it is important to consider the influence of pre-sleep protein and fasting on MPS levels following an acute protein dose. Based on the dynamic equilibrium of muscle protein metabolism in which both MPS and MPB levels rise and fall across a diurnal cycle, MPS levels are suppressed overnight (and MPB increases) due to limited AA availability, with some suggesting that a larger acute protein bolus may be needed to maximise MPS levels following an overnight fast (327). However, previous research suggests that ingestion of pre-sleep protein (~40 g) results in elevated MPS overnight, particularly following exercise training (328), and as such the activity and pre-study day dietary intake of the participants may have impacted the findings. In the present study, participants attended the laboratory following an overnight fast of ~10 h, and although their dietary intake in the preceding days was not provided or monitored, they were instructed to consume an evening meal representative of their habitual dietary intake the evening before the study day, and to not perform strenuous physical activity in the 72-hours before. Considering this methodological limitation, the relatively small sample size, and significant developments in methodology to determine longer-term "free-living" rates of MPS (e.g., via doubly labelled water (D_2O) (329)), further research is needed to fully determine the influence of habitual protein intake on cumulative MPS responses to feeding and activity.

Exploring the relationship between anabolic signalling targets known to interact with the mTOR signalling pathway and aspects of habitual protein intake revealed for the main part, no relationship. A significant positive relationship was observed between PRAS-40 and relative protein intake ($g.kgBW^{-1}.day^{-1}$) when young and older adults were grouped together. PRAS-40 has been previously identified as a binding partner for mTORC1, a key factor in the MPS pathway (330). This relationship suggests that a higher relative protein intake, may in part, activate a key factor of this MPS pathway via PRAS-40, indicating that greater protein intake does result in greater gains related to muscle mass maintenance and growth. Furthermore, a significant positive relationship was observed between IRS-1 expression and relative leucine intake ($mg.kgWBLM^{-1}.day^{-1}$). IRS-1 works with IRS-2 to stimulate the Akt-mTOR pathway following feeding, increasing AA uptake and promoting MPS (331). This relationship was observed only in younger adults, suggesting that older adults may not be able to readily activate this pathway. Although a relationship between the rapid aminoacidemia observed following acute protein feeding and intramuscular anabolic signalling has been previously reported on numerous occasions (332), so too has the dissociation between physiological and anabolic responses (333). Greenhaff *et al.,* (2008) demonstrated increases in expression of anabolic signalling targets but no change in MPS or leg protein breakdown following increasing intravenous infusion of AAs in muscles of healthy young men. As such, an increased expression of anabolic signalling targets may not necessitate an increase in MPS responses.

This work is not without limitations. The older adult participants were community-dwelling older volunteers, and as such, were relatively healthy and high functioning, and were not classed as sarcopenic. Given the prevalence of sarcopenia in those over the age of 65 years (and higher still in those over the age of 80 years), the participants in this study are not wholly representative of sarcopenic prevalence in older adults, nor of the high multi-

morbidity and associated polypharmacy (179) reported in this age group. Future research needs to include a wider range of older adults, including those with sarcopenia, and potentially those in residential care facilities who represent a growing proportion of older adults (179), and who are known to present with distinct characteristics (e.g., dysphagia (195)) with potential to impact nutrient intake and absorption (334). Studying protein metabolism in such populations is not without challenges, but the development of new oral tracer techniques such as those using D_2O which can measure MPS in longer-term 'free-living' scenarios may provide additional insight into the impact of habitual protein intake on muscle mass and function in these 'at-risk' populations (335). Further, new novel combined tracer-methodologies (e.g., COSIAM, Combined Oral Stable Isotope Assessment of Muscle (336)) may provide a minimally invasive way to determine relationships between muscle mass, synthesis and breakdown to better understand declines in muscle health in hard-to-study populations. The need to better understand the physiology of older adults living in supported settings has been recognised, with a particular focus on nutrition for the older adult championed by forums such as the British Dietetic Association special interest group (337). In addition, NIHR-funded national initiatives such as ENRICH (ENabling Research In Care Homes) provide support for studies in care homes to be run effectively and collaboratively. It must be noted that two primary outcome measures in this data analysis are self-reported diet diaries and western blotting (measured in arbitrary units) which may be subject to bias and human error. Therefore, it must be considered that the presented data may not be precise enough to detect small differences and relationships in the presented dataset. Finally, given inter-individual variation in dietary habits due to multiple lifestyle constraints (i.e., cost, preparation time) and food preferences across all ages, a larger, more diverse (i.e., including middle-aged adults and the oldest old) sample size is needed to confirm the findings of this work.

3.6 Conclusion

In conclusion, the findings of this work suggest that high functioning, community-dwelling, healthy older adults are consuming the recommended daily amount of protein, and that their dietary intake (including protein and leucine intake) is not different to that of younger adults. As such, these findings suggest that the age-associated reductions in muscle mass and function observed in this study are not attributable to low protein intake or low protein quality. The lack of relationship between habitual protein intake and MPS/anabolic cell signals highlights the role that other factors, such as physical activity and intrinsic cellular processes, likely play in maintaining skeletal muscle mass and function. Further research, with a larger sample size and more diverse ageing populations (i.e., older adults including those with comorbidities and/or supported living environments) is needed to fully investigate this relationship.

4 Characterising the time course of muscle protein synthesis in a C2C12 model following 25g of plant vs. animal-based protein in younger and older adults

4.1 Abstract

Introduction: With advancing age, the preservation of muscle mass is pivotal to maintain physical function and quality of life, with nutritional sufficiency, especially protein, a key component of this. The proportion of adults consuming vegan and vegetarian diets is rapidly increasing as a result of economic, ethical, sustainability, and health reasons. However, some research suggests that plant-based protein sources may not be as effective at maintaining and building muscle mass compared to animal-based protein sources, primarily due to differences in protein quality. Therefore, the impact high-quality protein consumption, a pivotal factor in provision of muscle mass and function in older adults, is a growing area of interest. The aim of the experiments reported in this Chapter was to utilise an *ex vivo* co-culture C2C12 human serum treatment model to assess differences in muscle protein synthesis (MPS) and anabolic cell-signalling responses between young and older adults following consumption of an animal- or plant-based protein source. **Methods:** To investigate this, serum samples were obtained from 8 young (21 ± 1 year) and 8 older (74 ± 5 years) adults at 2 laboratory visits when fasted and incrementally for 2-hours following consumption of a whey and vegan-blend protein bolus (matched for 25g total protein). C2C12 myotubes were serum and amino acid starved and treated with 10% younger adult serum obtained 0, 40, 60, and 90-minutes following protein consumption for 4-hours. MPS was determined through the surface sensing of translation (SUnSET technique) and regulatory signalling pathways measured via Western Blot. Following initial experiments, further experiments were conducted to investigate serum treatment percentage and glucose concentration of cell culture medium, utilising the same methodology. Final experiments compared the MPS and anabolic signalling target response of C2C12 myotubes to pooled younger and older adult serum obtained 0, 40, 60, and 90-minutes following protein consumption. **Results:** 2-way ANOVA analysis revealed that initial experiments treating C2C12 myotubes with 10% human serum from younger adults at

0, 40, 60, and 90-minutes following whey and vegan-blend protein consumption showed no significant time, condition, or time x condition effects on protein expression of p-Akt, p-mTOR, or puromycin incorporation (all expressed as fold change relative to 0 timepoint). However, a significant time effect was observed in p-RPS6 protein expression ($p = 0.0070$). Subsequent experiments comparing a 10% to 20% serum treatment and C2C12 myotubes cultured in a low glucose media compared to a high glucose media revealed no consistent trends with respect to differences in puromycin incorporation not protein expression of anabolic signalling targets between treatment conditions (no statistical analysis performed). 2-way ANOVA analysis revealed no significant time, treatment condition, or time x treatment condition effect in puromycin incorporation nor p-mTOR expression in the final set of experiments. However, a significant treatment effect was observed on p-Akt ($p = 0.0029$) and p-RPS6 ($p = 0.0388$) protein expression. **Conclusion:** Results presented here are inconsistent with previously published literature utilising this serum treatment model, suggesting that this model needs further optimisation to be able to detect differences between fasted and fed serum before investigating the differences, if any, between protein sources.

4.2 Introduction

The preservation of muscle mass in older age is pivotal to maintain physical function and quality of life (15). Skeletal muscle protein turnover is determined by the balance between skeletal muscle protein synthesis (MPS) and breakdown (MPB), which is primarily influenced by physical activity and diet. Dietary intake results in hyperaminoacidemia, causing increases in MPS and decreases in MPB, resulting in a positive net protein balance (NPB). However, research has demonstrated the presence of anabolic resistance to MPS stimuli with increasing age (50), meaning that older adults are at increased risk of sarcopenia, the age-associated loss of muscle mass and function (9).

Previous research (see recent literature review (134)) has demonstrated the importance of consumption of high-quality protein in older adults (134). However, an increasing number of adults are consuming plant-based diets, highlighting the need to explore the anabolic properties of plant-based protein sources. Numerous potential explanations have been given to explain the rise in plant-based protein consumption, including the environmental impact of animal-based protein sources and potential health benefits of consuming a plant-based diet (155). Human research exploring the anabolic potential of plant vs. animal-based protein sources report that animal protein sources may have a greater ability to stimulate MPS in comparison to plant-based proteins (145,157,338) due to several factors including a higher digestibility and a more complete amino acid (AA) profile from animal-based sources (339,340). Further research has confirmed the relationship between increasing circulating AAs and increases in MPS rate *in vitro* (341–344) and *in vivo* (345–348), in particular highlighting the importance of leucine (344) in stimulating MPS. However, older adults often exhibit a blunted appetite for protein-rich foods (198), which often translates to increased consumption of plant-based protein sources due to their lower protein and energy content in

comparison to animal-based protein sources (155). Therefore, the true influence of an animal-based protein source vs. a plant-based protein source to optimise skeletal muscle protein turnover in older age is unclear.

MPS is commonly measured in human studies using *in vivo* stable isotope tracer techniques (335), based on the use of stable isotope tracers of certain AAs (for example, ^{18}O, ^{15}N, ^{13}C, and ^{2}H (335)) and investigating the incorporation of these labelled AAs into muscle protein to determine MPS (349). However, there are limitations to this method. Stable isotope tracer techniques cannot be performed over periods >8 - 12-hours, and due to requirements of sterile infusions, cannulation, and muscle biopsy collection, require measurement in a laboratory environment (329). Recent developments have highlighted the use of D_2O, a non-substrate-specific tracer, to provide a measure of MPS in 'free living' situations (329). This allows temporal fractional synthetic rate measurement within multiple muscle protein subfractions, and has proven to be effective in measuring responses to anabolic stimuli (329). However, despite these advancements, both of these measurements are invasive in nature, requiring multiple muscle biopsies and/or saliva/blood samples, which may not be possible in all populations such as those living with frailty and/or residing in residential care (350). Therefore, using an *in vitro* cell-based model can provide a plausible and effective alternative method to investigate MPS and responses to various treatments (351).

Previous research has used an *ex vitro in vitro* cell based model to investigate MPS responsiveness to human serum treatment (352–355). These models demonstrated that conditioning culture medium with human serum in fully differentiated C2C12 myotubes influenced MPS. Furthermore, Carson and colleagues (2018) demonstrated that when using this cell culture serum treatment model, they were able to detect differences in MPS (measured via puromycin incorporation) and protein expression of anabolic signalling targets

(mTOR, P70S6K, and 4EBP1) between C2C12 myotubes treated with media conditioned with fasted vs. fed serum from younger adults (355). Furthermore, research from Allen and colleagues (2021) used a comparable cell culture serum treatment model and reported a significant reduction in MPS fold change in myotubes treated with media conditioned in older adult fasted serum compared to younger adult fasted serum, with further differences uncovered in how these conditioned myotubes responded to a 5mM leucine treatment (352). Collectively, this recent research highlighted a gap in this research area investigating the response of C2C12 myotubes to media conditioned in fasted and fed serum obtained from both younger and older adults following plant versus animal-protein consumption.

Initially, this project aimed to investigate the acute muscle protein synthetic and regulatory signalling dose response to animal versus plant-based protein in young and older adults in C2C12 muscle cells using an *ex vivo* co-culture serum treatment model. However, after initial experiments, it became clear that further optimisation of this model was needed prior to its use to make comparisons between conditions. Therefore, the secondary aim of this chapter was to optimise the treatment and culture conditions of this *ex vivo* co-culture serum treatment model before repeating initial experiments to investigate differences between protein source and age.

4.3 Materials and Methods

4.3.1 Participants

Eight young participants (18-35 years, BMI 19-29.9 kg/m^2) and eight older participants (65-80 years, BMI 19-29.9 kg/m^2, ~4 male: 4 female for each participant group) (Table 4.1) were recruited through the research team's networks, including the University of Birmingham 1000 Elders Group and the NIHR Birmingham BRC Muscle Health Public Involvement in Research group. Participants underwent remote (telephone) health screening

via a general health questionnaire prior to their first visit to the lab. Information from the general health questionnaire was compared to inclusion and exclusion criteria (Table 4.2) to confirm eligibility to participate.

Table 4.1- Participant Characteristics

	Young		Old	
	Male	***Female***	***Male***	***Female***
n	4	4	4	4
Age (years)	21.75 (0.50)	19.75 (0.50)	71.75 (6.18)	76.75 (3.30)
Body Mass Index (kg/m^2)	24.02 (2.33)	22.93 (0.38)	24.76 (2.75)	24.45 (1.74)

Note: Data displayed as Mean (SEM)

Table 4.2- Participant inclusion and exclusion criteria

Inclusion/Exclusion Criteria
Inclusion:
18-35 years old OR 65-80 years old
Body mass index (BMI) between 19-29.9 kg/m^2
Healthy (as assessed using the general health questionnaire)
Non-smoker and non-diabetic
No history of gut disease (i.e., Crohn's disease, IBD, Whipple's disease, coeliac disease or colitis) or abdominal surgery
Exclusion:
History of neuromuscular disorders
Current or recent smoker (previous 3-months)
Allergy to milk/ lactose intolerant

All participants provided informed written consent, and all study procedures took place at the University of Birmingham School of Sport, Exercise, and Rehabilitation Sciences. All procedures were conducted in accordance with the declaration of Helsinki and were approved by the University of Birmingham Research Ethics Committee (ERN_21-1544).

4.3.2 Experimental Procedures

All participants completed 2 visits to the laboratory, a minimum of 1-week apart. Participants were asked to refrain from consuming caffeine or alcohol 24-hours prior to each visit, and to complete a diet diary recording everything they consumed from 6pm onwards the evening before each visit; with a request to consume their evening meal between 7-9pm. Participants then remained fasted (water *ad libitum*) from 9pm the evening before until their visit to the laboratory the following morning. Each participants visits remained identical with the exception of the protein source consumed. Each participant consumed either a whey protein (unflavoured impact whey protein, Myprotein, Warrington, UK) or vegan protein (unflavoured vegan protein blend, Myprotein, Warrington, UK) at each visit, with the order in which these were provided randomised prior to their first visit.

4.3.3 Laboratory visits

Participants arrived at the laboratory at ~0800 h following an overnight fast. Upon arrival at the laboratory, participants provided informed consent. Body mass was recorded to the nearest 0.1 kg in loose clothing without shoes (Electronic balance scale, Ohaus Champ II) and height was measured to the nearest 0.1cm (Seca 231 stadiometer). Following recording of baseline anthropometric data, a cannula (BD Venflon Pro 20G) was inserted into the forearm (antecubital) vein of the subject and a resting pre-trial blood sample (7ml) was drawn into a serum separator 8.5ml EDTA tube (BD Vacutainer SST II Advance). Following this

sample, participants were asked to consume either a whey or vegan blend protein shake containing 25g total protein. Nutritional information including the essential amino acid (EAA) content of the protein shakes is shown in Table 4.3 and Table 4.4. Further blood samples were taken from participants at 20, 40, 60, 90, and 120-minutes post protein ingestion. Once all samples were taken, the cannula was removed from the vein and pressure was applied to forearm. Participants were offered refreshments before being free to leave the lab. The visit layout is illustrated in Figure 4.1.

Table 4.3- Protein source nutritional information when matched for 25g total protein

	Whey protein	**Vegan blend protein**
Serving size	29.7 g	31.25 g
Total protein	25 g	25 g
Energy	122.6 kcal	106.1 kcal
Fat	2.26 g	0.31 g
Of which saturates	1.55 g	0 g
Carbohydrate	1.19 g	0.52 g
Of which sugars	1.19 g	0 g
Fibre	0 g	0.52 g
Salt	0.15 g	0.48 g

Table 4.4- Protein source essential amino acid profile per 100g

Amino Acid	Whey protein	Vegan-blend protein
Histidine	1.7 g	2.19 g
Isoleucine	6.4 g	4.18 g
Leucine	10.6 g	7.77 g
Lysine	9.6 g	6.33 g
Methionine	2.2 g	1.47 g
Phenylalanine	3 g	7.6 g
Threonine	6.7 g	3.12 g
Tryptophan	1.4 g	0.80 g
Valine	5.9 g	4.52 g
Total g EAA	47.5 g	37.98 g

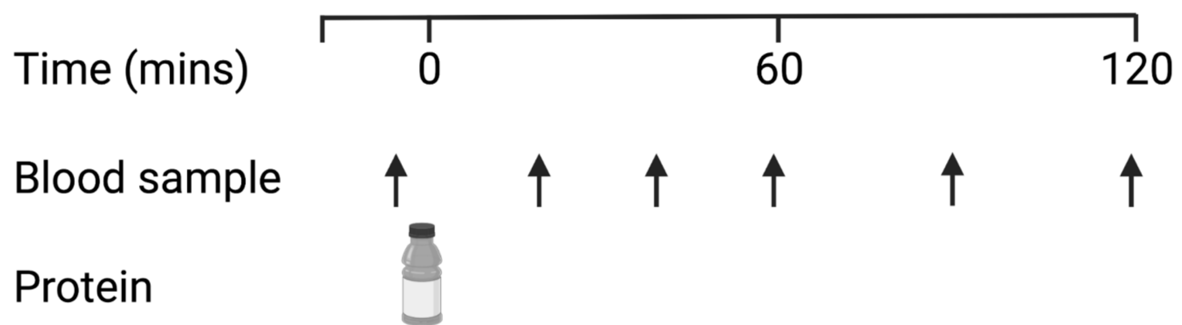

Figure 4.1- Summary of participant laboratory visit

4.3.4 Blood sample preparation

Blood samples were left at room temperature for 30-mins following collection and were placed on ice until centrifugation. Samples were centrifuged (Heraeus Multifuge X1R Centrifuge, Thermofisher Scientific) for 10 minutes (3000g, 4ºC) to isolate the serum. The obtained serum was frozen in microcentrifuge tubes until needed for cell culture use.

4.3.5 Cell Culture

4.3.5.1 C2C12 Cell Line

Immortalised mouse skeletal muscle C2C12 myoblasts were obtained (American Type Culture Collection, Manassas, VA, USA), and the vial of cells were proliferated (Section 4.3.5.3- *Proliferating Myoblast Expansion*) and frozen down (Section 4.3.5.4- *Freezing Cells*) at serial passages for future use. Throughout this thesis experiments were conducted with cells < passage 10.

4.3.5.2 Thawing Cells

C2C12 cell cryovials were removed from liquid nitrogen storage and placed upon ice to thaw. 9ml of pre-heated growth media was added to a T25cm^2 flask (Sigma-Aldrich, St Louis, USA), and the cell suspension was added to this immediately once thawed. Cell confluency was monitored the following day, and cells were passaged, or growth media was changed if needed as described in Section 4.3.5.3 (*Proliferating Myoblast Expansion)*.

4.3.5.3 Proliferating Myoblast Expansion

C2C12 skeletal muscle myoblasts were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco) when proliferating in standard conditions (5% CO_2, 100% humidity, 37°C). DMEM was supplemented with the following, resulting in a 5mM total growth media glucose concentration:

- 10% fetal bovine serum (FBS, F9665, Sigma-Aldrich, St. Louis, MO, USA)
- 1% penicillin/streptomycin (P/S, 15070-063, Gibco)
- 0.5% 1M glucose stock (5mM glucose, G7021, Sigma-Aldrich)
- 1mM sodium pyruvate (113600-070, Gibco)
- 1mM GlutaMAX^{TM} Supplement (35050-038, Gibco)

Confluency of cells were monitored, and growth media was changed every 48-hours. Upon reaching ~70-75% confluency, cells were passaged. To passage cells, they were initially washed in 10ml phosphate-buffered saline (PBS, pH 7.4, 10010-015, Gibco). After washing, 3ml TrypLE™ Express (1X) (12605-010, Gibco) was added to the tissue culture flask. Cells were incubated for 5-minutes to trypsinise the cells and allow them to detach from the tissue culture flask. 7ml growth media was then added to the flask and all contents were transferred to a 15ml falcon tube and centrifuged for 5-minutes (10,000g). Once centrifuged, the supernatant was discarded leaving the cell pellet. The cell pellet was resuspended in 1ml growth media to fully mix the cells. Once mixed, a further 9ml growth media was added to the cell mix and mixed. This cell mix was then transferred to the relevant number of T75 or T125 flasks to passage, or plated for experiments. If plating for experiments, cells were counted (Section 4.3.5.5- *Counting Cells*) and seeded into 6-well tissue culture treated plates (CC7672-7506, CytoOne, USA Scientific) at a density of $1.0x10^5.ml^{-1}$ (200,000 cells per well).

4.3.5.4 Freezing Cells

To collect a store of C2C12 myoblasts for future experiments, excess myoblasts were frozen and stored in liquid nitrogen when not needed for further passaging or plating for experiments. As previously described in Section 4.3.5.3 (*Proliferating myoblast expansion*), when passaging C2C12 myoblasts, they were washed, trypsinised, and centrifuged to obtain a cell pellet. The supernatant was removed, and cells were resuspended in a freezing mixture made up of 900µl growth media and 100µl dimethyl sulfoxide (DMSO). This mixture was transferred to a cryovial and stored for 24-hours in a NALGENE™ freezing container containing 500ml isopentane. When placed in this container in -80°C storage, cells are frozen at a rate of 1°C a minute. After 24-hours, the vial was transferred to liquid nitrogen storage.

4.3.5.5 Counting Cells

When plating for cell experiments, a manual cell count was performed to ensure cells were plated at the correct seeding density. After resuspending cells in 10ml growth media, a mixture of 10µl cell suspension and 10µl 0.4% trypan blue (15250-061. Gibco) was pipetted into a 1.5ml microcentrifuge tube (1:1 dilution). A glass coverslip was placed on a Neubauer haemocytometer (Hawksley, Lancing; ruling 1/400mm^2, cell depth: 0.1mm ± 1%), and 10µl of the mixed solution was pipetted underneath each chamber of the glass coverslip. The haemocytometer was placed underneath a microscope (Evos XL Core, Thermofisher Scientific) and the number of cells in the 4 corner grids were counted. Cells were only counted if the membrane was intact, and thereby the cell was not stained blue. Once the cell counts were completed, the following equation was used to calculate the number of cells in the population:

$$\text{Cells/ml}^{-1} = \frac{\text{Mean cell number}}{\text{Number of grids (4)}} \times \text{ml} \times \text{Dilution factor} \times 10^{4}\,\text{ml}^{-1}$$

This equation was used to calculate the cell population and seed cells into 6-well plates for future experiments. The calculated cell suspension volume was added to growth media (total 2ml cell/growth media solution) and thoroughly mixed to ensure even cell distribution across the 6-well plates. The process of cell counting is illustrated in Figure 4.2.

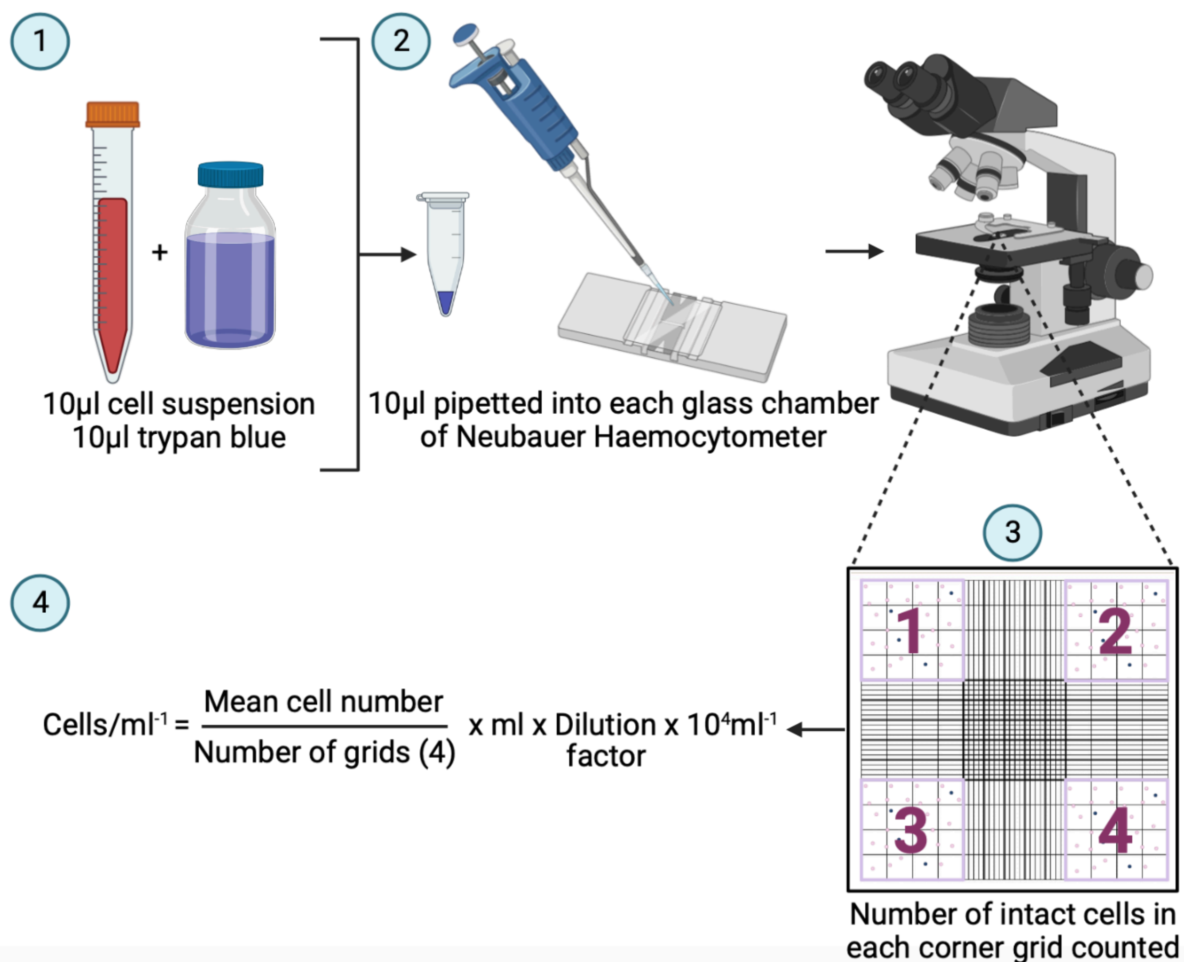

Figure 4.2- Graphical representation of the cell counting process. Figure generated using Biorender.

4.3.5.6 Differentiating Cells

After plating for experiments, cells were kept in growth media and confluency was monitored. Once reaching ~90% confluency, growth media was removed, and all 6-well plates were washed with PBS (2ml). Cells were then induced to differentiate, with differentiation media being changed every 24-hours over a period of 5-7 days. Differentiation media was made up of DMEM supplemented with the following:

- 2% (v/v) horse serum (16050-122, Gibco)
- 1% penicillin/streptomycin (P/S, 15070-063, Gibco)
- 0.5% 1M glucose stock (5mM glucose, G7021, Sigma-Aldrich)
- 1mM sodium pyruvate (113600-070, Gibco)

- 1mM GlutaMAX^{TM} Supplement (35050-038, Gibco)

4.3.6 Cell Culture Treatments

As previously described, C2C12 myoblasts were maintained in growth media (formulation described in Section 4.3.5.3- *Proliferating myoblast expansion*), and were seeded into 6-well plates at a density of $1.0x10^5$. ml^{-1} (200,000 cells per well). Once C2C12 myoblasts reached ~90% confluency, cells were maintained in 2ml differentiation media, with media changes every 24-hours. Myotube growth was monitored and deemed ready for experiments between day 6-7 differentiation, depending on rate of growth. Representative images of myoblasts on day 0 myotubes ready to treat on day 7 of differentiation are shown in Figure 4.3.

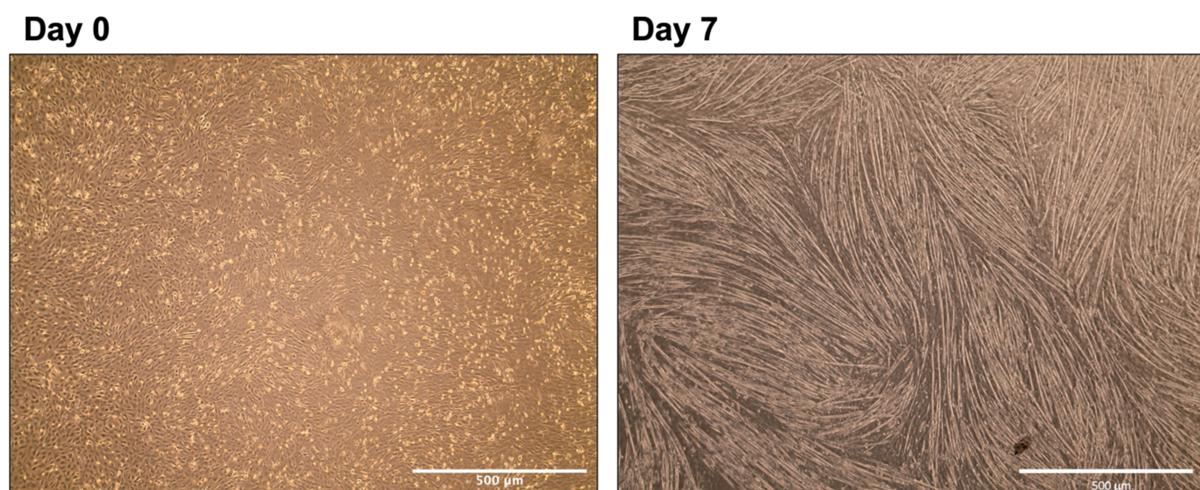

Figure 4.3- Representative cell culture images of C2C12 myoblasts at Day 0 differentiation and C2C12 myotubes at Day 7 differentiation

Once ready to treat, cells were washed with PBS, and then changed to a serum and AA-free media (1ml, pH 7.3, D9800-13, US Biological, Salem, MA, USA). C2C12 myotubes were then left to incubate for 1-hour to undergo an AA and serum starve. Previous research has demonstrated the contribution of growth media in upregulating MPS and expression of anabolic signalling targets (355,356). Specifically, evidence shows a reduction in growth medium induced MPS with 30-60 min serum and AA starvation, indicated in downregulation

of mTOR pathway signalling proteins. This means a 1-hour "preconditioning" serum and amino acid starve is necessary before adding any treatment to the cells, ensuring that any changes displayed in MPS, and expression of anabolic signalling targets can be attributed to the treatment itself, rather than the media in which the myotubes are cultured. Following the 1-hour starve, C2C12 myotubes were given the appropriate treatment, as described in further sections of this chapter. In all experiments, puromycin was added to myotubes for the final 30-minutes of incubation (1µmol/L, P8833, Sigma-Aldrich). Puromycin was added to myotubes as a means of determining acute MPS via the surface sensing of translation (SUnSET) technique. Goodman *et al.,* (2011) first utilised and developed the SUnSET technique and confirmed its effectiveness in quantifying MPS in various *ex vivo* and *in vivo* conditions (357). The SUnSET technique is based on the use of puromycin, a tyrosyl-tRNA analog antibiotic, and measurement of its incorporation into nascent peptide chains (357).

4.3.7 Protein Analysis

4.3.7.1 Cell Lysis

Following cell culture treatments, all culture media was removed from myotubes, and they were washed with PBS 3 times. After washing, PBS was removed and 150µl ice cold 1X RIPA lysis buffer (Merck Millipore, Watford, UK) was added to each well. This was left on ice for 15-minutes to allow lysis of the myotubes. Subsequently, a rubber cell scraper (Cyto One 220mm Cell Scraper) was used to collect all contents of the well, and the resultant solution was transferred to a 1.5ml microcentrifuge tube. Cell lysates were then centrifuged at 13,000g for 15-minutes at 4°C.

4.3.7.2 Sample Preparation

The concentration of protein in the samples was determined using a detergent compatible (DC) protein assay (Bio-Rad, Hercules, California, USA). Briefly, the DC protein

assay uses a colorimetric method to determine protein concentration following detergent solubilisation. Absorbance was read at 750nm using a FLUOstar Omega plate reader (BMG Labtech, Aylesbury, UK). Following this, equal amounts of protein from each sample were added to 1 x Laemmli sample buffer (LSB, 161-0747, Biorad), and samples were boiled at 99°C for 5-minutes to denature the protein. Samples were then stored in -80°C until needed for further experiments.

4.3.7.3 Gel Preparation

A combination of commercial and homemade gels were used for western blots throughout this thesis. Commercial gels used were Criterion™ TGX™ 4-20% graded pre-cast gels (Biorad, US). When making homemade gels, the following combination was used to make the relevant percentage resolving gel:

Table 4.5- Western blotting resolving gel formulation

Final acrylamide %	**8%**	**10%**	**12.5%**	**15%**
H_2O (ml)	4.73	4	3.2	2.4
4X Tris-SDS pH 8.8 (ml)	2.5	2.5	2.5	2.5
30% Acrylamide solution	2.5	3.33	4.2	5
10% APS (µl)	100	100	100	100
TEMED (µl)	12	12	12	12

The following combination was used to make the stacking gel (5% final acrylamide), irrelevant of resolving gel acrylamide percentage:

- 4.67ml H_2O
- 2ml 4X Tris-SDS pH 6.8
- 1.33ml 30% acrylamide solution

- 65 µl 10% APS
- 12 µl TEMED

Gels were left to polymerise and were stored at 4°C if not immediately being used for electrophoresis. Samples were thawed on ice prior to use. Once thawed, 15-30µg of protein was loaded into the relevant gel, along with 5µL Precision Plus Protein™ Dual Color Standards (1610394, Biorad). Gel electrophoresis was run on a PowerPac™ HC (Biorad) at 100V for 10-minutes for samples to run through the stacking gel. Voltage was then increased to 150V for 1-hour to allow full separation of samples.

4.3.7.4 Gel Transfer and Blocking

Once electrophoresis was completed, proteins were transferred from a gel to a PVDF membrane at 100V for 1-hour. Once transfer was completed, the membrane was stained with Ponceau S solution and imaged to provide a loading control (358). After imaging, gels were blocked in 3% bovine serum albumin (BSA) (Thermo Fisher Scientific), or 5% low-fat milk diluted in Tris-buffered saline and 0.1% Tween-20 (TBS-T) for 1-hour.

4.3.7.5 Antibodies

After blocking, membranes were washed in TBS-T (3 x 5 min), and primary antibodies (all prepared in BSA) were added to the membrane. Membranes were left to incubate at 4°C in primary antibody overnight. Following overnight incubation, the primary antibody was removed, and membranes were washed in TBS-T (3 x 5 min). The relevant secondary antibody was then added to the membrane and incubated for 1-hour at room temperature. Antibody details are listed in Table 4.6.

Table 4.6- Primary and secondary antibodies

Antibody	Product Code	Company	Host	Dilution
Primary Antibodies				
IgG2a monoclonal anti-puromycin	Clone 12D10	Merck Millipore	Mouse	1:1000
Total-mTOR	#2972	CST	Rabbit	1:1000
Phospho-mTOR Ser2448	#2971	CST	Rabbit	1:1000
Total-AKT	#9272	CST	Rabbit	1:1000
Phospho-AKT Ser473	#3787	CST	Rabbit	1:1000
Total-RPS6	#2217	CST	Rabbit	1:1000
Phospho-RPS6 Ser 240/244	#5364	CST	Rabbit	1:1000
Secondary Antibodies				
Anti-rabbit IgG horseradish peroxidase (HRP)- linked antibody	#7074	CST	Rabbit	1:10000
Anti-mouse IgG, HRP-linked antibody	#7076	CST	Mouse	1:10000
CST, Cell Signalling Technology				

4.3.7.6 Membrane Imaging and Protein Quantification

Upon completion of the secondary antibody incubation, membranes were washed in TBS-T (3 x 5-min). Before imaging, each membrane was incubated for 2-min at room temperature in Immobilon Western chemiluminescent HRP substrate (Merck Millipore). Membranes were imaged on GeneSys capture software (Syngene, Cambridge, UK) using a G:BOX Chemi XT4 imager. Protein expression bands were quantified using ImageJ software (US National Institutes of Health, Bethesda, MD, USA). In order to accurately measure MPS,

the whole lane was quantified when analysing puromycin blots (357). All protein expression values were expressed relative to a protein band stained with ponceau S solution (Section 4.3.7.4- *Gel transfer and blocking*) .

4.4 Experimental Process

4.4.1 Conditioning C2C12 cells with *ex vivo* young serum obtained following plant- and animal- protein ingestion

4.4.1.1 Methods

Initial cell culture experiments involved treating C2C12 myotubes with serum and AA-free media containing 10% human serum (1800µl serum and AA-free media, 200µl human serum) for 4-hours. Serum samples were obtained from younger adults pre- and post-animal and plant protein consumption, as previously described (Section 4.3.3- *Laboratory Visits*). For each participant, 12 serum samples were obtained and used to treat cells. These included a fasted sample, and samples obtained 20, 40, 60, 90, and 120-minutes following whey protein consumption, and the same timepoints following plant-based protein consumption. For this set of experiments, C2C12 myotubes were treated with individual serum samples as opposed to pooled participant serum samples for each timepoint. This was done to allow investigation of any biological variation between the participant serum samples. A 10% serum treatment was chosen as this has previously been demonstrated to have a high cell viability over a 4-hour treatment period (355), and has been used to detect differences in MPS and expression of anabolic signalling targets across different serum 'conditions' (352,353).

Previous work used a comparable method of treatment to investigate differences in MPS and expression of several anabolic signalling, and reported good cell adherence and

viability when utilising a 10% serum treatment model (352,355). Furthermore, results from this study demonstrated significant increases in muscle protein synthesis, p-P70S6K, p-mTOR, and p-4EBP1 expression in myotubes treated with *ex vivo* human serum for 4-hours compared to a control (355), highlighting the potential use of this model to investigate myotube response to different protein sources. It must be noted that for cell culture serum treatment experiments, not all serum time points were used due to cell culture resource availability. Research has shown large increases in MPS around 45-minutes following protein ingestion, with levels remaining elevated 45 to 90-minutes post protein ingestion. Therefore, in all cell culture experiments serum samples obtained 0, 40-, 60-, and 90-minutes post protein ingestion were utilised.

4.4.1.2 Statistics

Statistical analysis was performed using GraphPad Prism v.9.4.1. All data were reported as mean (SEM), with significance set at $p < 0.05$. Two-way ANOVAs (time x treatment) were used to assess differences in puromycin incorporation and anabolic signalling. When results of the 2-way ANOVA revealed a positive interaction effect, further comparisons were completed using Tukey's HSD. Statistical significance was set at $p < .05$.

4.4.1.3 Results

There were no significant time, condition, or time x condition effects on protein expression of p-Akt, p-mTOR, or puromycin incorporation (all expressed as fold change relative to 0 timepoint) (Figure 4.4A, B, D). However, when investigating protein expression of p-RPS6, a significant time effect was observed ($p = 0.0070$). Further comparisons revealed that expression of p-RPS6 was significantly higher in C2C12 myotubes treated with serum taken 90-minutes post plant protein ingestion compared to 40-minutes post plant protein ingestion ($p = .0038$) (Figure 4.4C). All p values are shown in Table 4.7.

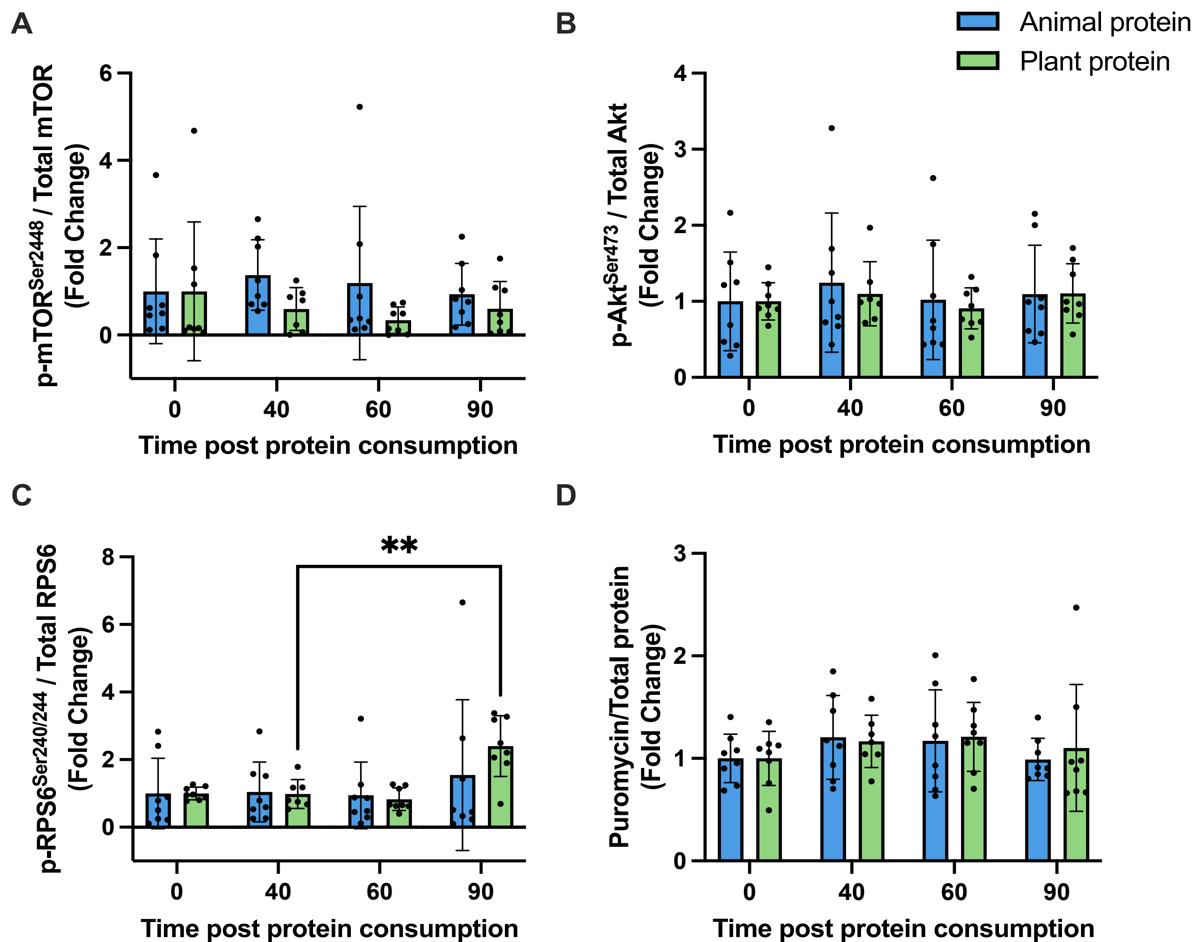

Figure 4.4- Muscle protein synthesis (measured by puromycin incorporation) and protein expression of anabolic signalling targets following a 4-hour younger adult serum treatment (fasted serum, 40, 60, 90-minutes post- protein consumption). (A) phosphor-mTOR (Ser2448)/total-mTOR, (B) phosphor-Akt (Ser473)/total Akt, (C) phospho-RPS6 (Ser 240/244)/total-RPS6, (D) puromycin incorporation. Data are expressed as Mean ± SD, with n = 8 per group. Each individual point corresponds to an individual serum treatment. Note: ** represents p<.01

Table 4.7- MPS and anabolic signalling target protein expression analysis

Target	Time	Condition	Time x Condition
p-RPS6	0.0070**	0.7366	0.1926
p-Akt	0.4277	0.7274	0.7504
p-mTOR	0.6878	0.2844	0.3732
Puromycin	0.0921	0.7679	0.9480

p values from 2-way ANOVA analysis of protein expression of puromycin and anabolic signalling targets in C2C12 myotubes treated with young adult serum obtained following plant- and animal-protein ingestion. No significant effects were observed, excluding a time effect of p-RPS6.

4.4.2 Investigating the role of media glucose concentration and young human serum treatment percentage on MPS and expression of anabolic signalling targets

4.4.2.1 Methods

Previous work using a comparable C2C12 cell culture model used a high-glucose (HG) media and 20% serum treatment (355), however, previous work in our department has demonstrated that a low-glucose (LG) media and 10% serum treatment produced comparable results (352). Therefore, to establish the role of media glucose concentration and serum treatment percentage in this cell culture model, experiments were conducted comparing a HG (25mM) vs. LG (5mM) glucose media and a 10% vs. 20% serum treatment.

As previously described, C2C12 cells were expanded, seeded into 6-well plates, and differentiated for 7-days (Section 4.3.5- *Cell Culture*). Two batches of cells were grown and plated for these experiments. One batch was grown and differentiated in a low glucose (5mM) media, and the other in a high glucose (25mM) media. On day 7 of differentiation, cells underwent a 1-hr serum and AA-free starve, and were then treated with either 10% or 20% serum for 4-hrs (using pooled fasted serum from younger adults). Puromycin was added to the myotubes 30-minutes prior to the end of this treatment. As previously described, upon completion of this treatment myotubes were washed in PBS, lysed, and a DC protein assay was used to determine the protein content of each condition (Sections 4.3.6- *Cell Culture Treatments*, 4.3.7- *Protein Analysis*). Samples were then prepped accordingly, and western blotting was used to determine protein expression of puromycin and anabolic signalling targets.

4.4.2.2 Results

No statistical analysis was performed on the above set of data as these were preliminary experiments to optimise serum treatment conditions and thereby were not

repeated at different passage numbers. This set of experiments revealed no consistent trends with respect to differences in puromycin incorporation nor protein expression of anabolic signalling targets between treatment conditions (media glucose concentration, serum percentage treatment) (Figure 4.5, Figure 4.6).

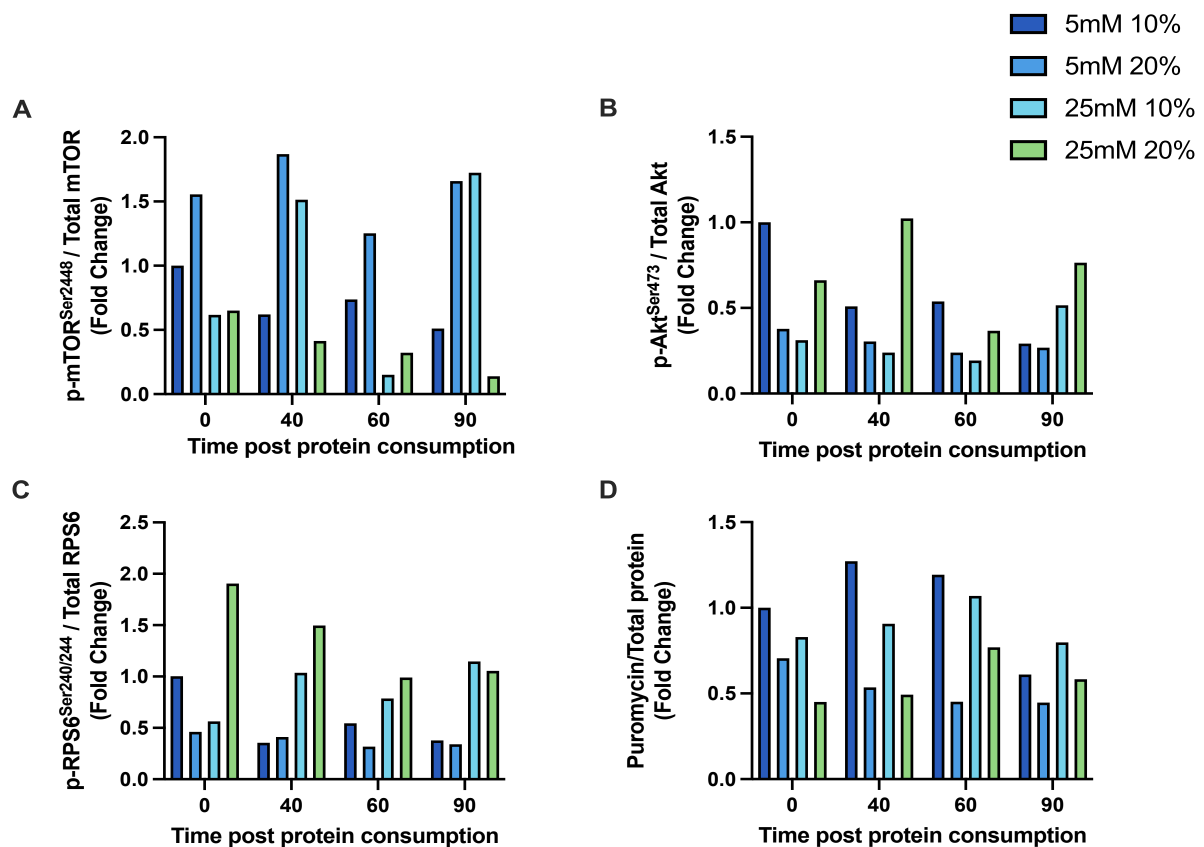

Figure 4.5- Muscle protein synthesis (measured by puromycin incorporation) and protein expression of anabolic signalling targets following a 4-hour younger adult pooled serum treatment (fasted serum, 40, 60, 90-minutes post- animal protein consumption). (**A**) phospho-mTOR (Ser2448)/total-mTOR, (**B**) phospho-Akt (Ser473)/total Akt, (**C**) phospho-RPS6 (Ser 240/244)/total-RPS6, (**D**) puromycin incorporation.

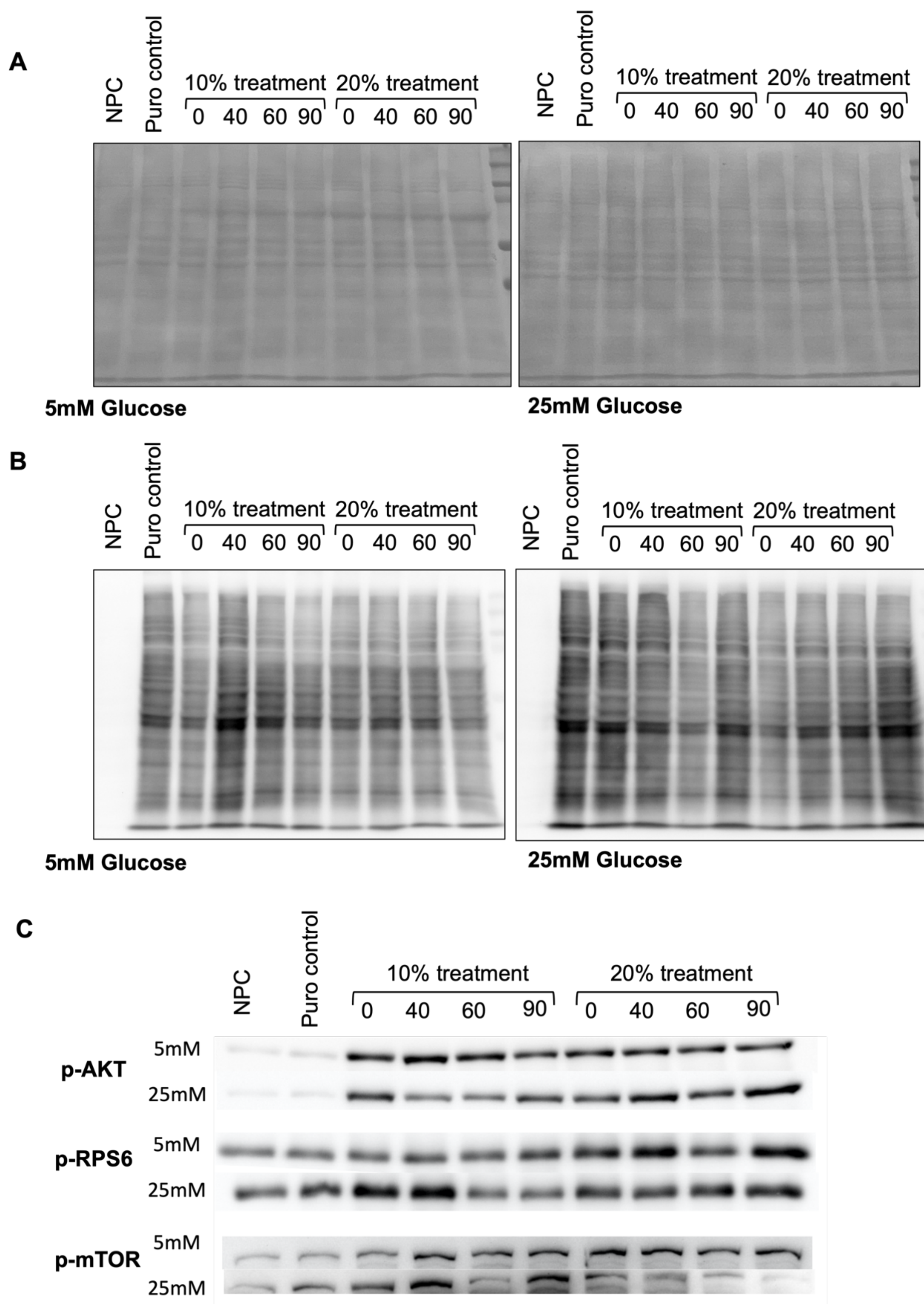

Figure 4.6- Representative western blot images of total protein (**A**), puromycin incorporation (**B**), and protein expression of anabolic signalling targets phospho-Akt (Ser473), phospho-RPS6 (Ser240/244), and phospho-mTOR (Ser2448) (**C**) following a 4-hour C2C12 myotube human serum treatment. Images are displayed for both myotubes incubated in a low glucose (5mM) media and a high glucose (25mM) media treated with 10% and 20% human serum. Abbreviations: NPC, No-puromycin control; Puro control, Puromycin treatment control.

4.4.3 Conditioning C2C12 myotubes with *ex vivo* human serum obtained from both young and older adults following plant- and animal- protein ingestion

4.4.3.1 Methods

After conducting experiments to determine the optimal treatment conditions (media glucose concentration and serum treatment percentage), a final set of experiments were run to investigate the MPS and anabolic signalling response of C2C12 myotubes to serum obtained from young and older adults following plant and animal protein ingestion. As no significant differences were observed in previous experiments, initial methodology of LG media and 10% serum treatment was used.

C2C12 cells were expanded, seeded into 6-well plates, and differentiated for 7-days (Section 4.3.5- *Cell Culture*). Cells were grown and differentiated in a low glucose (5mM) media. Once ready to treat, cells underwent a 1-hr AA- and serum- free starve. C2C12 myotubes were then treated with 10% serum obtained from young and older adults following plant- and animal-based protein ingestion for 4-hours. For these experiments, participant serum was pooled for each condition and timepoint (younger adult plant, younger adult animal, older adult plant, older adult animal). This was done to allow easy direct comparison of conditions between each time point. For each condition, C2C12 myotubes were treated with samples from the following timepoints: 0 (fasted), 20, 40, 60, 90, and 120-minutes post protein ingestion. Puromycin was added to myotubes 30-minutes before the end of the treatment period. As previously described, upon completion of this treatment myotubes were washed in PBS, lysed, and a DC protein assay was used to determine the protein content of each condition (Sections 4.3.6- *Cell Culture Treatments*, 4.3.7- *Protein Analysis*). Samples were then prepped accordingly, and western blotting was used to determine protein

expression of puromycin and anabolic signalling targets. This set of experiments were repeated 3 times (Passages 9, 10, and 11) to provide technical replicates for data analysis.

4.4.3.2 Statistics

Statistical analysis was performed using GraphPad Prism v.9.4.1. All data were reported as mean (SEM), with significance set at $p < 0.05$. Two-way ANOVAs (time x treatment) were used to assess differences in puromycin incorporation and anabolic signalling. When results of the 2-way ANOVA revealed a positive interaction effect, further comparisons were completed using Tukey's HSD. Statistical significance was set at $p < .05$.

4.4.3.3 Results

A 2-way ANOVA was used to determine any significant differences between treatment conditions or time points on puromycin incorporation and protein expression of anabolic signalling targets. No significant time, treatment condition, or time x treatment condition effect was observed in puromycin incorporation, or expression of p-mTOR (Figure 4.7A, D). However, a significant treatment effect was observed on p-Akt ($p = 0.0029$, Figure 4.7B), and a significant time effect was observed on expression of p-RPS6 ($p = 0.0388$, Figure 4.7C). Further post-hoc analysis highlighted several significant differences between conditions in p-AKT and p-RPS6 expression. All significant differences and their *p* values are displayed in Table 4.8 and Table 4.9. Representative western blot images (all taken from passage 9 repeat) are shown in Figure 4.8.

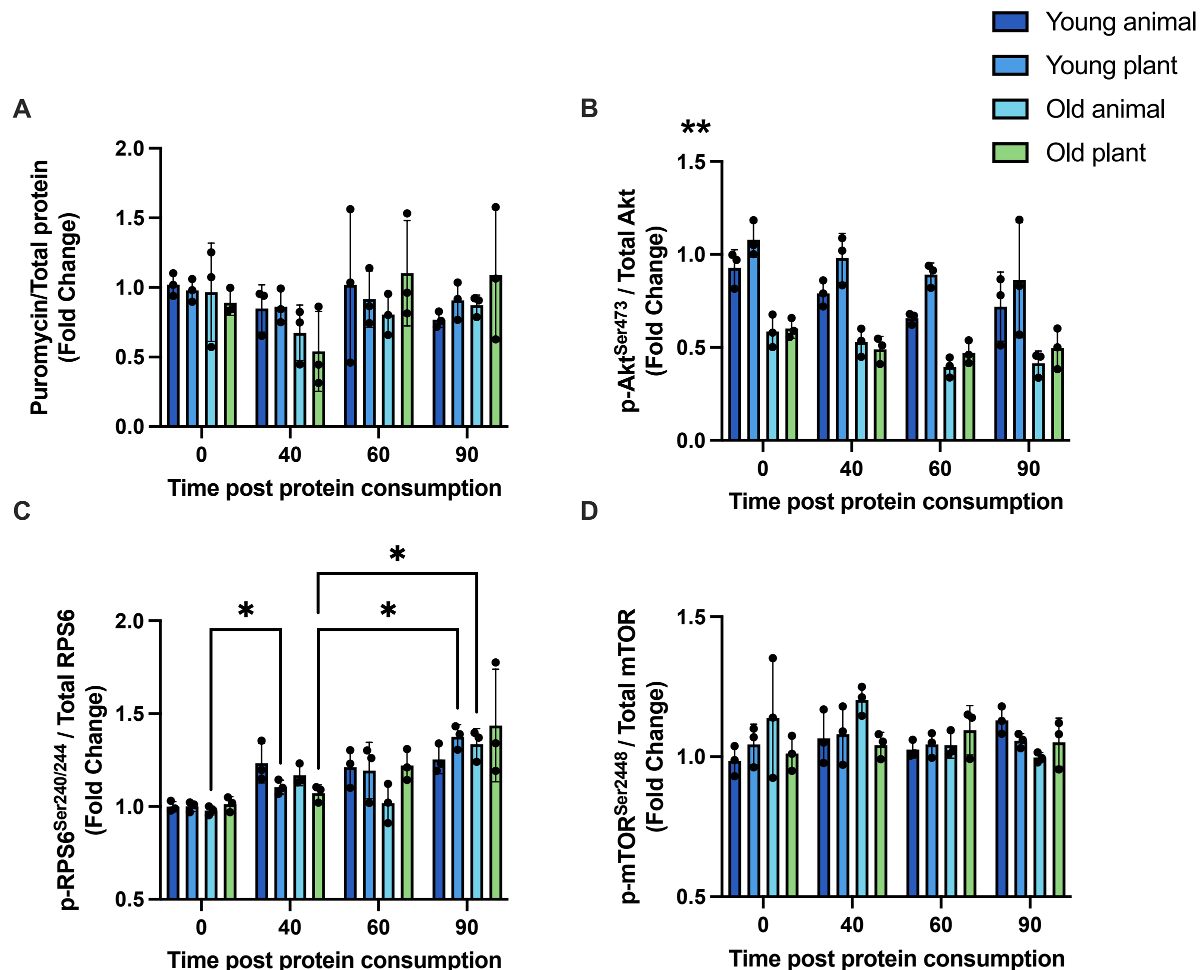

Figure 4.7- Muscle protein synthesis (measured by puromycin incorporation) and protein expression of anabolic signalling targets following a 4-hour younger and older adult serum treatment (fasted serum, 40, 60, 90-minutes post- protein consumption). (**A**) phospho-mTOR (Ser2448)/total-mTOR, (**B**) phospho-Akt (Ser473)/total Akt, (**C**) phospho-RPS6 (Ser 240/244)/total-RPS6, (**D**) puromycin incorporation. Data are expressed as Mean (SEM), with n = 3 per group. Each individual point corresponds to a passage repeat. Note: * represents p<.05, ** represents p<.01

Table 4.8- 2-way ANOVA analysis of protein expression of puromycin and anabolic signalling target expression in C2C12 myotubes treated with young and older pooled serum obtained following plant- and animal- protein ingestion

Target	Time	Condition	Time x Condition
p-RPS6	0.0388*	0.4202	0.2559
p-Akt	0.1937	0.0029**	0.6222
p-mTOR	0.3206	0.2592	0.3213
Puromycin	0.2377	0.6521	0.4009

Table 4.9- Multiple comparisons analysis of protein expression of puromycin and anabolic signalling target expression in C2C12 myotubes treated with young and older pooled serum obtained following plant- and animal- protein ingestion

Comparison	*p* value
p-Akt	
Young plant (0) vs. Old animal (0)	0.0017**
Young plant (0) vs. Old plant (0)	0.0160*
Young plant (0) vs. Old plant (40)	0.0145*
Young plant (0) vs. Old animal (60)	0.0124*
Young plant (0) vs. Old plant (60)	0.0086**
Young plant (0) vs. Old animal (90)	0.0187*
Young plant (0) vs. Old plant (90)	0.0101*
Old plant (0) vs. Old animal (60)	0.0125*
Old plant (0) vs. Old plant (60)	0.0132*
Young animal (60) vs. Old animal (60)	0.0200*
Young animal (60) vs. Old animal (90)	0.0476*
p-RPS6	
Old animal (0) vs. Young plant (40)	0.0293*
Old plant (40) vs. Young plant (90)	0.0127*
Old plant (40) vs. Old animal (90)	0.0463*

Note: * represents $p < .05$, ** represents $p < .01$

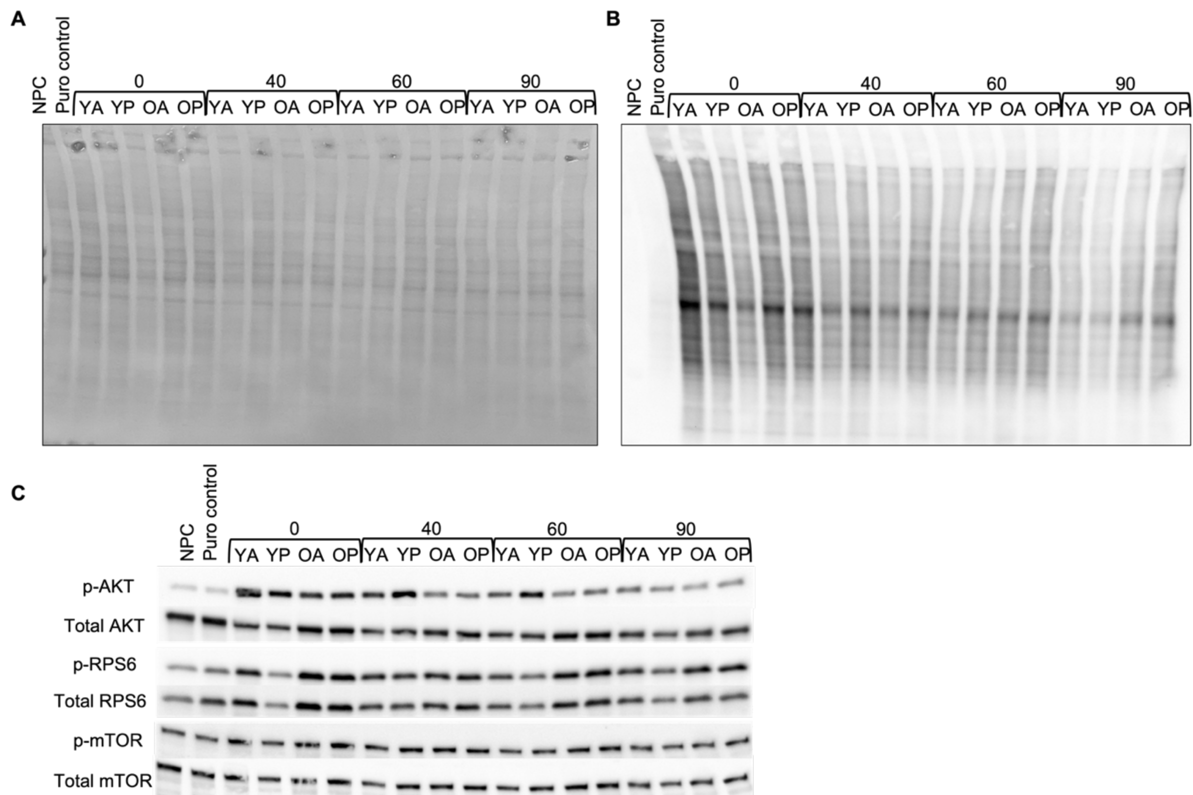

Figure 4.8- Representative western blot images of total protein (**A**), puromycin incorporation (**B**), and protein expression of anabolic signalling targets phospho & total-Akt (Ser473), phospho & total-RPS6 (Ser240/244), and phospho & total-mTOR (Ser2448) (**C**) following a 4-hour C2C12 myotube human serum treatment. Western blots taken from passage 9 experiments. Abbreviations: NPC, no-puromycin control; Puro control, Puromycin treated control, YA, Young animal protein; YP, Young plant protein; OA, Old animal protein; OP, Old plant protein

Further analysis demonstrated that when grouping younger adult and older adult data, a 2-way ANOVA revealed a significant age effect ($p = 0.0001$), and a significant time effect ($p = 0.029$) on p-Akt protein expression (Figure 4.9). Furthermore, the previously established significant time effect of p-RPS6 was re-established ($p < .0001$). No significant differences were observed in MPS or p-mTOR protein expression between time or age. Further post-hoc analysis highlighted several significant differences between conditions in p-AKT and p-RPS6 expression. All significant differences and their *p* values are shown in Table 4.10 and Table 4.11.

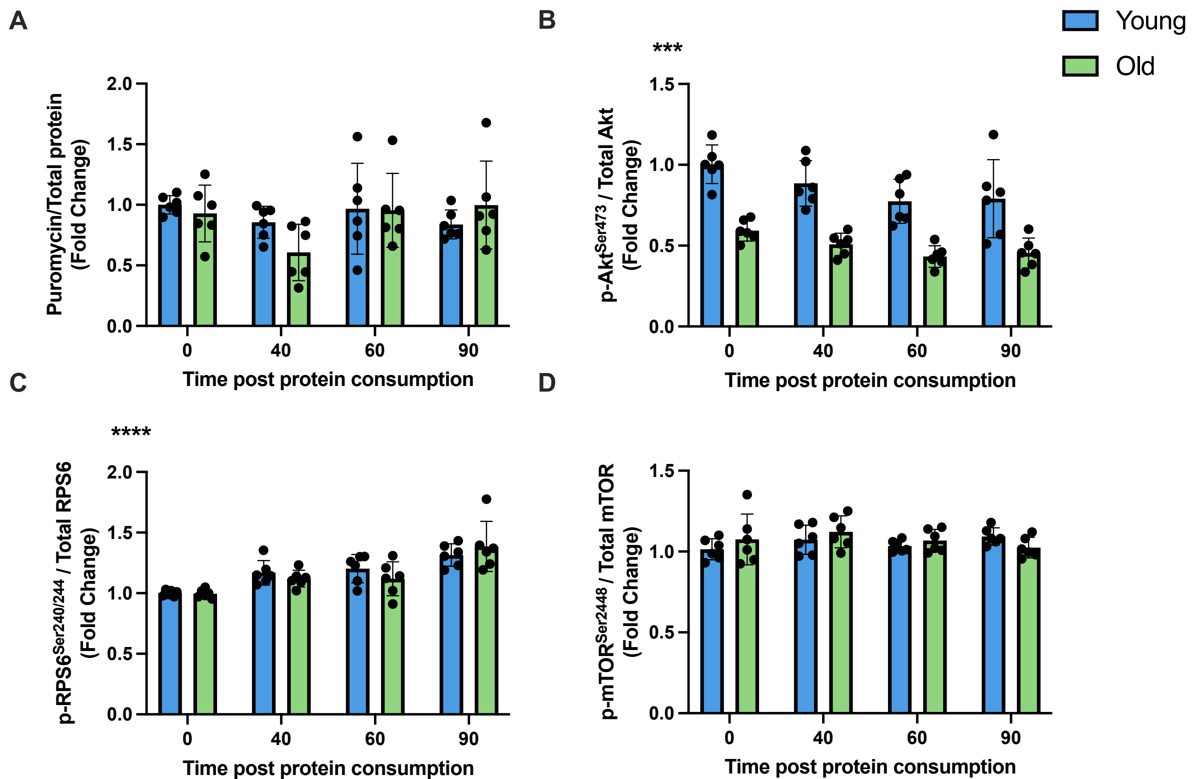

Figure 4.9- Muscle protein synthesis (measured by puromycin incorporation) and protein expression of anabolic signalling targets following a 4-hour young and older adult serum treatment (fasted serum, 40, 60, 90-minutes post- protein consumption) when results are grouped according to age. (**A**) phosphor-mTOR (Ser2448)/total-mTOR, (**B**) phospho-Akt (Ser473)/total Akt, (**C**) phospho-RPS6 (Ser 240/244)/total-RPS6, (**D**) puromycin incorporation. Data are expressed as Mean (SEM), with n = 6 per group (3 passage repeats, grouped plant and animal protein data). Note: *** represents p<.001, **** represents p<.0001

Table 4.10- 2-way ANOVA analysis of protein expression of puromycin and anabolic signalling targets in C2C12 myotubes treated with young and older adult pooled serum obtained following plant- and animal-protein ingestion. Pooled age data

Target	Time	Age	Time x Age
p-RPS6	<.0001****	0.5885	0.2020
p-Akt	0.0292*	0.0001***	0.6700
p-mTOR	0.2820	0.2431	0.3991
Puromycin	0.0570	0.5502	0.2504

Note: * represents $p < .05$, ** represents $p < .01$, *** represents $p < .001$, **** represents $p < .0001$

Table 4.11- Multiple comparisons analysis of protein expression of puromycin and anabolic signalling target expression in C2C12 myotubes treated with young and older pooled serum obtained following plant- and animal- protein ingestion. Pooled age data

Comparison	***p* value**
p-Akt	
0 Young vs. 0 Old	0.0229*
0 Young vs. 40 Old	0.0091**
0 Young vs. 60 Old	0.0012**
0 Young vs. 90 Old	0.0014**
0 Old vs. 60 Old	0.0054**
40 Young. vs 60 Old	0.0252*
60 Young vs. 60 Old	0.0225*
p-RPS6	
0 Young vs. 60 Old	0.0192*
0 Young vs. 90 Young	0.0003***
0 Young vs. 90 Old	<.0001****
0 Old vs. 60 Young	0.0161*
0 Old vs. 90 Young	0.0003***
0 Old vs. 90 Old	<.0001****
40 Young vs. 90 Old	0.0112*
40 Old vs. 90 Young	0.0253*
40 Old vs. 90 Old	0.0018**
60 Old vs. 90 Old	0.0391*
60 Old vs. 90 Young	0.0247*
60 Old vs. 90 Old	0.0018**

Note: * represents $p < .05$, ** represents $p < .01$, *** represents $p <.001$, **** represents $p < .0001$

4.5 Data Interpretation & Discussion

The initial conducted experiments were based on previously published work from Carson and colleagues (2018), who reported that when conditioning C2C12 myotube medium with *ex vivo* fasted serum for 4-hours, MPS (measured via puromycin incorporation) and protein expression of anabolic signalling targets mTOR, P70S6K, and 4EBP1 were significantly elevated compared to a control. They conducted experiments utilising serum obtained from young men (25 ± 3.6 yrs) 60-minutes post protein ingestion (0.33 g/kg body mass^{-1} whey protein), and demonstrated significantly increased mTOR activation in fed compared to fasted serum, and a significantly higher P70S6K and 4EBP1 activation in C2C12 myotubes treated with fed compared to fasted serum (355). Therefore, the initial experiments reported in this chapter aimed to explore this work further, investigating the response of C2C12 myotubes to fasted and fed serum (following 25g protein) from younger adults after consuming an animal-based (whey) protein, or plant-based (vegan blend) protein. A secondary aim was to investigate the time-course of these effects, thereby treating the C2C12 myotubes with serum obtained 40, 60, and 90-minutes post-protein ingestion, as opposed to a single treatment time point (60-mins post protein ingestion (355)).

Results of the initial serum-treatment experiments demonstrated no significant difference between fasted and fed serum in MPS (determined via puromycin incorporation), along with no significant differences in p-AKT or p-mTOR protein expression. A significant time difference was observed in p-RPS6 protein expression, with expression being significantly higher 90-mins post- plant-based protein ingestion compared to 40-minutes post- plant-based protein ingestion. However, as previously stated, this difference was not observed across any other anabolic signalling target or in MPS.

There are several differences in the methodology performed by Carson and colleagues compared to the initial experiments reported here, which were initially suspected to be the cause of the different results. The two primary differences were the serum treatment percentage, and the media glucose concentration. Carson and colleagues utilised a 20% serum treatment model and observed the previously discussed differences between fasted and fed serum-treated cells, however, investigations into this *in ex vivo* co-culture treatment model have utilised a range of plasma and serum treatment doses ranging from 5-20% (352–355,359). Specifically, previous investigations completed in our department investigating the role of the serum treatment percentage, observed comparable effects across dilutions (352). Thereby, to conserve serum stocks, a 10% treatment was used initially, as opposed to a 20% treatment. Furthermore, a primary difference between the initial experiments reported herein and those from Carson and colleagues was the media glucose concentration. Carson and colleagues grew and differentiated C2C12 myotubes and myoblasts in HG media (25mM glucose) and supplemented the AA and serum-free media with 6mM D-glucose. However, in the experiments reported here, a LG (5mM) media was used to proliferate myoblasts and differentiate C2C12 myotubes in. C2C12 cells are known to highly express GLUT4, the insulin-responsive glucose transporter which facilitates insulin-simulated glucose uptake (360). It has been shown that in C2C12 cells, the glucose concentration of growth medium may interfere with GLUT4 translocation, thereby influencing the insulin-dependent signalling pathway (360,361). Investigations into use of a HG (25mM) growth medium in C2C12 cells has demonstrated a desensitisation of the insulin-dependent signalling pathway, resulting in a reduction in glucose uptake. Furthermore, expression of several anabolic signalling targets related to the mTOR signalling pathway, including AMPK, AKT, IRS1, and PKC (362,363), have been shown to interact with the insulin-dependent signalling pathway, resulting in a dysregulation of the mTOR pathway (364), mimicking states of ageing, diabetes, obesity, and

cancer (365). These states can have profound effects on cell metabolism and growth. This is why the initial serum treatment experiments here were conducted using a LG rather than a HG media. Further experiments investigating the role of serum treatment percentage and media glucose concentration were subsequently conducted (Section 4.3.2.1), to establish whether these factors may have caused the lack of differences observed in MPS and anabolic signalling target expression following fasted and fed serum treatment.

C2C12 cells were cultured in a LG (5mM) or HG (25mM) medium and their responsiveness to pooled fasted and fed serum at different treatment percentages (10% or 20%) was measured. As this experiment was repeated only once for clarification of the role, if any, of these factors, no statistical analysis could be performed. There were no consistent trends of MPS or anabolic target protein expression across different conditions. However, there was a notable difference in puromycin expression in cells cultured in HG medium compared to those cultured in LG medium, with a greater expression in the HG condition. However, this was across all conditions including the puromycin control, and when values were expressed relative to their LG or HG puromycin control, there were no observable differences between conditions across all values and treatment percentages. It was concluded that the lack of differences observed in initial serum treatments were not due to methodological differences between the present experiments and those of Carson and colleagues (355). The definite reason for these lack of differences cannot be determined; however, it may be due to differing AA compositions of the serum samples, which will be further explored in Chapter 5. Another factor which may be contributing to the differing reported results between here and those from Carson and colleagues may be that this methodology is very novel, and all possible contributing factors have not yet been explored. Following on from this, for the final serum treatments, a 10% treatment in C2C12 cells cultured in a low glucose (5mM) media was utilised. This was because it was deemed that

differences in presented results were not as a result of the media glucose concentration, nor the serum treatment percentage. Therefore, a lower serum treatment percentage was maintained to conserve serum stocks, and cells continued to be proliferated and differentiated in a LG media to avoid any interference with GLUT4 translocation which may occur when using a HG media (361).

Experiments investigating the effect of a 4-hour pooled serum treatment (young vs older adults, fasted vs. fed serum) in C2C12 myotubes showed no significant differences in MPS in myotubes conditioned in younger adult fasted serum compared to older adult fasted serum, which has been previously reported when using this *ex vivo* co-culture serum treatment model (353). These results contradict those previously reported from Allen and colleagues (2021), however, previous studies have shown that older age does not influence basal MPS rates (32). Volpi and colleagues reported that kinetic indicators of MPS showed a trend of greater MPS in older men compared to younger men, and concluded that differences in MPS across age are not determinants of muscle loss with increasing age (32). These findings may explain the lack of difference in MPS between myotubes treated with younger compared to older adult fasted serum. Furthermore, there was no difference in MPS in myotubes conditioned in young adult fed serum compared to older adult fed serum (at any time point or condition). Due to the lack of differences between young and older adults fasted serum, and the lack of reported differences between fasted and fed serum treatment MPS in previous experiments, this was an unsurprising finding. Furthermore, a lack of difference in MPS between treatment of fasted and fed serum has previously been reported (355). The lack of differences here are likely due to the AA profile of the serum samples, which will be explored in Chapter 5 of this thesis. Previous work has demonstrated that a greater increase in extracellular leucine (~80μmol^{-1}) is needed to result in MPS changes, with smaller increases needed to elicit change in MPS signalling targets (326,366), highlighting the possibility that

the AA profiles, particularly leucine concentration, of the young and old fasted serum may not be sufficiently different to result in MPS differences.

Despite no differences being observed in MPS, several significant differences were observed in protein expression of anabolic signalling targets. 2-way ANOVA analysis demonstrated no significant effects on expression of p-mTOR, however, a significant treatment effect was observed in p-AKT expression ($p = 0.0029$), and a significant time effect was observed in p-RPS6 expression ($p = 0.0388$). Interestingly, p-Akt data demonstrated a trend of a reduction in protein expression with an increase in the postprandial serum timepoint. Post-hoc comparisons revealed several significant differences, to note a significantly higher p-Akt expression in young animal (60) compared to old animal (60), and in young animal (60) compared to old animal (90). Further analysis revealed that when grouping the young and older adult data, there was a significant age effect of p-Akt protein expression ($p < .0001$), with expression being consistently higher in younger compared to older adults. These data suggests that despite the lack of age-related differences in MPS, myotubes conditioned in older adult serum elicited a diminished MPS signalling response across all time points. Interestingly, this finding differs from previously published results from Allen and colleagues (2021), who reported no differences in p-Akt in C2C12 myotubes treated with young and old fasted serum.

When considering p-RPS6 protein expression, a significant time effect was observed ($p = 0.0388$), with a graded increase in p-RPS6 expression in C2C12 myotubes treated with serum obtained a greater time after protein ingestion (i.e., expression was lowest in fasted state, with graded increases at 40-, 60-, and 90-mins post protein ingestion). This relationship was stronger when data were pooled according to age ($p < .0001$). It has been shown that circulating AA levels increase following a bolus protein ingestion in both young and older

adults (53), however, the progression of sarcopenia, the age-related loss of muscle mass and function, has been associated with anabolic resistance. Anabolic resistance describes the phenomenon that in older age, skeletal muscle cannot as effectively respond to anabolic stimuli such as exercise or amino acid-based nutrition (55), a concept which has been highlighted in both *in vivo* human and animal experimental studies (44,367–369). These results of a diminished p-Akt response in C2C12 myotubes treated with older compared to younger adult serum aligns with the concept of anabolic resistance, suggesting that this serum treatment model may be effective in investigating this phenomenon.

The significant time-course effect observed in p-RPS6 protein expression may reflect the serum AA profile analysis which is reported in Chapter 5. Research demonstrates that following a bolus ingestion of a milk-based and plant-blend protein, plasma EAA concentrations remain elevated for 300 and 240-minutes respectively, with peak EAA concentrations being reported at 36±10 mins following milk protein, and 75±26 minutes following ingestion of a plant-blend protein (338). When considering this, it may be expected that MPS and anabolic target protein expression may peak earlier in the animal protein condition compared to plant-based protein, however, the composition and EAA content of a protein is not the only factor influencing its resultant impact on MPS and MPS signalling targets. Other factors include the protein digestion and absorption kinetics (53,153,339). Furthermore, these factors may influence MPS and anabolic signalling target expression differently in younger compared to older adults (53).

The inconsistency in results across MPS and the anabolic signalling targets measures suggests that this model may not be optimal to investigate the *in vitro* response of C2C12 myotubes to *ex vivo* fasted and fed human serum. Despite this model providing a possible alternative method of investigating the effect of the integrated systemic environment on

protein synthesis in myotubes, further work needs to be done before the model can be applied to different treatments and conditions. This work is not without limitations, primarily the serum treatment percentage used. Using this model, the response of C2C12 myotubes to both a 10% and 20% serum showed no significant differences between the conditions observed, however, this model may not be sensitive enough to detect differences in such small AA concentration changes. Using a higher serum treatment percentage would provide a better representation of the human muscle systemic environment following protein ingestion. However, it has been previously shown that treating myotubes with a serum percentage greater than 20% resulted in a reduction in cell viability (355). Previous work from Allen and colleagues has highlighted the possible use of an additional AA treatment following a fasted serum 'preconditioning' period, resulting in observed differences in how C2C12 myotubes conditioned in younger compared to older adult medium respond to this additional anabolic stimuli (352). This model of using a 4-hour fasted serum treatment as a 'preconditioning' of cells followed by an anabolic stimuli treatment may be a more effective use of this model as opposed to comparing fasted and fed serum treatment. Previous research has demonstrated the ability of a 24-hour 10% serum incubation period to induce changes in muscle morphology, with differences observed in myotube diameter between myotubes treated in older vs younger adult fasted serum, and a significantly higher nuclear fusion index observed in myotubes treated with younger adult fasted serum in comparison to a control (352). Furthermore, this treatment period has been able to detect myotube diameter differences between disease states, with myotubes treated in fasted serum from non-cirrhotic non-alcoholic fatty liver disease and end-stage liver disease patients demonstrating lower myotube diameter in comparison to fasted serum from healthy age-matched controls (353). This suggests that this model may be better used to investigate morphological muscle differences

following a longer incubation period as opposed to acute MPS and anabolic signalling target responses to an acute (4-hour) serum treatment.

To conclude, this chapter describes the development of an *ex vivo* co-culture serum treatment model to investigate MPS and associated signalling targets. Results are inconsistent between outcome measures, suggesting that treating C2C12 myotubes with fasted and fed serum (following animal- or plant-protein ingestion) from young and older adults results in no differences in MPS responses, or expression of anabolic signalling targets associated with the mTOR signalling pathway. Further work is needed to optimise this model, primarily investigating the use of a fasted serum ‘preconditioning’ treatment followed by an external AA anabolic stimulus. Using this model may allow investigation of the differences in response of myotubes conditioned in older adult compared to younger adult serum to plant-based compared to animal-based protein sources.

5 Investigating the influence of protein quality on amino acid kinetics in young and older adults using an *ex vivo* co-culture C2C12 model

5.1 Abstract

Introduction: With increasing numbers of adults consuming vegan and vegetarian diets worldwide, there has been increasing interest placed on the anabolic potential of plant-based protein in comparison to animal-based sources. Emerging research suggests that plant-based protein sources may be of a lower protein quality, primarily attributed to their lower digestibility and essential amino acid (EAA) content. This difference may impact maintenance of musculoskeletal health in older adults, an area of increasing interest and importance, with some studies suggesting that due to their lower quality, plant-based protein sources are not as effective at overcoming the anabolic resistance of older skeletal muscle and as such may not be as effective at mitigating the onset and progression of sarcopenia. The aims of this chapter were to investigate the amino acid (AA) kinetics in young and older adults following consumption of a whey and vegan-blend protein, and to utilise an *in vitro* co-culture human serum C2C12 model to investigate age-related and protein source differences in muscle protein synthesis (MPS) and anabolic signalling target protein expression. **Methods:** Human serum samples were obtained from 8 younger (21 ± 1 year) and 8 older (74 ± 5 years) adults at 2 laboratory visits when fasted and 20, 40, 60, 90, and 120-minutes following consumption of a whey and vegan-blend protein bolus (matched for 25g total protein). Blood serum samples were run through gas chromatography-mass spectrometry to determine AA enrichment and concentration. Subsequent cell culture experiments investigated the MPS and anabolic signalling target protein expression response of C2C12 myotubes to whey and vegan-blend protein AA treatments. MPS was determined through the surface sensing of translation (SUnSET) technique and expression of anabolic signalling targets was determined via Western Blot. The effect of various doses (0.5mM, 1mM, 2mM, 5mM, 10mM, and 15mM) and treatment times (30-inutes, 1-hour, 2-hours, 4-hours) was investigated to determine the optimal treatment dose and time for following

experiments. Once this was determined, C2C12 myotubes were serum and AA starved for 1-hour and conditioned with fasted young and older adult serum (10%) for 4-hours. After 4-hours C2C12 cells were treated with 10mM whey or vegan-blend AA mix for 1-hour. **Results:** 2-way ANOVA analysis (protein source x age) revealed significantly elevated alanine ($p = 0.03$), leucine ($p = 0.005$), proline ($p = 0.04$), methionine ($p < 0.0001$), threonine ($p = 0.0003$), lysine ($p = 0.05$), and tryptophan ($p = 0.0007$) concentrations (area under curve, AUC) following consumption of a whey protein compared to a vegan-blend protein, irrespective of age. Serum concentrations of cysteine ($p = 0.0008$) and glutamine ($p < 0.0001$) were significantly higher in older adults following protein ingestion, whereas serum tryptophan concentrations were elevated in younger adults following protein ingestion ($p < 0.0001$). No statistical analysis was performed on the vegan vs. whey AA dose-response experiments, however, from the dataset a 10mM 1-hour treatment was determined as optimal. Final cell culture experiments revealed no significant age, treatment condition, or age x treatment condition for puromycin incorporation or for phosphorylation of Akt, RPS6, or mTOR. When pooling age groups, results of a one-way ANOVA revealed a significant p-RPS6 treatment effect ($p = 0.01$). **Conclusions:** Mass spectrometry data highlights a clear influence of protein source on circulating AA concentrations, with limited influence of age. This suggests that the quality of a protein source, in particular the EAA content and digestibility of the protein, plays a key role in determining circulating AAs. Despite the potential for an *ex vivo* co-culture human serum treatment model to investigate muscle anabolic responses to different disease states and nutritional status, the developmental work on this model reiterates its need for further optimisation.

5.2 Introduction

As highlighted throughout this thesis, the maintenance of muscle mass and strength is of great importance in older adults to maintain physical function, independence, and quality of life (15). In both young and older adults protein ingestion and physical activity are the two key stimuli for maintaining musculoskeletal health, however, older adults exhibit resistance to these anabolic stimuli, meaning that aged skeletal muscle cannot respond as effectively (49,50) (33) as young skeletal muscle (44). Based on this, it has been suggested that older adults should consume a greater amount of protein to maximise stimulation of muscle protein synthesis (MPS) and aid maintenance of musculoskeletal 'health' (i.e., mass, structure and associated function) (178). However, recent evidence has highlighted the role of protein quality in maintaining musculoskeletal health, with some research suggesting that plant-based proteins may be of a lower protein quality, and thereby have a lower anabolic potential, in comparison to animal-based proteins, which may be considered a higher quality protein source (134,155). Several factors contribute to the quality of a protein source. These include the AA composition and content of protein source, and the digestibility of the protein source, determined by the AA bioavailability and digestion kinetics of the protein source (155).

With worldwide increases in veganism and vegetarianism (155), and plant-based proteins now contributing more than animal-based proteins to overall protein intake worldwide (370) there is growing interest in the use of plant-based proteins as an alternative to animal-based proteins for stimulating MPS. This rise in plant-based protein consumption may be attributed, in part, to the reduced environmental cost of producing different protein sources. Animal-based protein food production emits significantly more greenhouse gas emissions in comparison to plant-based foods (371). However, despite the environmental impact of animal-based protein production, recent research suggests that plant-based proteins

may not stimulate MPS to the same extent as animal-based proteins (134) due to reduced protein quality, which if proven may have numerous health-based implications associated with reduced muscle mass maintenance (155,157). Several factors contribute to the quality of a protein source including the AA composition and content of protein source and the digestibility of the protein source, determined by the AA bioavailability and digestion kinetics (155).

When considering the AA composition of a protein source, leucine is of particular importance (372). Leucine has been demonstrated to be a key AA in stimulating MPS (141,373), and the leucine content of a protein is an independent predictor of its ability to stimulate MPS (292,293,372). This is in part due to the ability of leucine to enhance activation of anabolic signalling proteins related to mTOR (374–376). Leucine concentrations are consistently lower in plant-based proteins (7.1% ± 0.8%) compared to animal-based proteins (8.8% ± 0.7%) (145,167),with the highest leucine content (% total protein) reported in whey protein (13.6%), while the leucine content of plant-based protein sources ranges from ~6-8% (167). However, the AA composition of a protein source (in particular, the leucine content), is not the only factor contributing to protein quality. The digestibility of a protein source also plays an important role. Previous research in young men highlighted the role of protein digestibility when investigating acute MPS responses to whey hydrolysate, casein, or soy protein isolate at rest and after a single bout of resistance exercise (377). Unsurprisingly, the greatest increases in blood total AA, branched chain AA (BCAA), and leucine concentrations occurred after ingesting whey protein, compared to soy and casein. Leucine appeared more rapidly in the blood following whey and soy protein ingestion compared to casein, despite soy being a plant-based protein and casein being an animal-based protein source. Casein protein has been labelled as a ‘slow’ protein source, meaning that it is a slow-digestible protein (153), and as such may play a greater role in inhibiting whole-body

protein breakdown as opposed to increasing intramuscular protein synthesis levels (51,152,153). This is because casein is not as soluble as whey protein, resulting in it clotting into the stomach, thereby delaying gastric emptying and resulting in a slower AA release (153). Further, despite comparable rises in EAAs with each protein source following resistance exercise, whey protein ingestion resulted in a significantly higher MPS response compared to soy protein (377). This study serves to highlight the importance of multiple factors such as AA digestibility and absorption when considering the anabolic potential of a protein source.

The rate of AA appearance in blood is not only influenced by protein source, but by the age of an individual consuming the protein. Despite observing comparable basal MPS rates in younger and older men, healthy older men show a need for a higher relative protein intake in a single meal compared to healthy younger men to maximally stimulate postprandial MPS (173). This highlights the role of other factors influencing MPS stimulation following protein ingestion in older compared to younger adults other than total protein intake. When comparing the impact of dietary protein digestion rate of a 'slow' protein (whey) and a 'fast protein' (casein) on protein metabolism between young (n = 6) and older men (n = 9), results demonstrated that when matched for leucine content, postprandial protein utilisation in younger adults was comparable between conditions. However, postprandial protein utilisation was different between protein sources in older adults (greater in whey compared to casein protein), demonstrating an age-related effect of protein digestibility on postprandial protein gain (51). Furthermore, significant differences in protein digestion and phenylalanine absorption kinetics have been reported in older compared to younger adults (results from a recent literature review) (378). Increased splanchnic AA retention in older adults is a likely contributory factor (153,379). Furthermore, hormonal changes associated with older age may influence the anabolic response to protein ingestion, such as a decreased insulin sensitivity

which can result in reduced muscle AA uptake (380) resulting from a decrease in postprandial microvascular muscle tissue perfusion (381,382).

The gold standard method of measuring MPS responses to protein ingestion is via stable isotope AA tracer methodology which allows a direct measurement of postprandial MPS following ingestion of a protein. However, this is expensive, and cannot always give detailed information about what is happening at a cellular level without use of invasive muscle biopsies. Therefore, cell culture methodology can be utilised to investigate the effect of AA provision on 'myotubes' in culture. As used throughout this thesis, the C2C12 cell line is often used to investigate responsiveness of 'myotubes' to different treatments. This cell line is often used as C2C12 myoblasts rapidly differentiate to form multinucleated myotubes comparable to those produced when culturing primary muscle tissue. Previous research from Atherton and colleagues investigated the anabolic signalling responses to 2mM AA treatments in C2C12 myotubes and demonstrated a significant elevation in expression of anabolic signalling targets mTOR and 4EBP1 in response to leucine treatment, but not in response to any other EAA (344). Further immunoblotting from this study suggested that EAAs modulate anabolic signalling via translational initiation via mTORC1, p70S6K1, and RPS6 (344). Although Atherton and colleagues demonstrated the effective use of treating C2C12 myotubes with AAs to investigate anabolic signalling responses, no published research has sought to investigate the response of C2C12 myotubes to a mixed AA composition. Treatment of C2C12 myotubes with a mixed AA composition mimicking a 'whey' versus a 'vegan' protein composition may provide information on the direct anabolic response and rate of MPS following a mixed AA treatment. Furthermore, identifying the optimal dose of these AA mixes would allow for further exploration of the human serum *ex vivo* co-culture treatment (described and used in Chapter 4).

Therefore, the primary aims of this chapter were:

1. To investigate the time-course and concentration of AAs in serum obtained from young and older healthy adults fasted and following ingestion of a whey protein and a plant-based protein blend,
2. To determine the optimal dose and treatment time of AA profiles mimicking the composition of a whey and plant-blend protein in C2C12 myotubes,
3. To optimise the previously used *ex vivo* co-culture human serum treatment model (described and used throughout Chapter 4) to investigate the MPS and anabolic signalling response of C2C12 myotubes preconditioned in fasted young and older adult serum to mixed AA treatments, mimicking provision of a whey or vegan-blend protein.

5.3 Materials and Methods

5.3.1 Gas Chromatography- Mass Spectrometry

Blood serum samples were run through gas chromatography-mass spectrometry (GC-MS) to determine AA enrichment and concentration. GC-MS is a method of gas chromatography followed by mass spectrometry analysis. In brief, gas chromatography is a separation technique in which a small amount of the sample of interest is injected into the sample port where it is vaporised. The vaporised samples are then carried by an inert gas through a glass column where the different substances within the sample are separated. Following separation, these samples then enter a mass spectrometer, which identifies the compound and separate them by their molecular mass. A chromatogram is then produced which shows peaks which represents different compounds. The serum samples used here were obtained from n = 8 young and n = 8 older adults over 2 visits to the laboratory (as

previously described in Chapter 4). The collection protocol of these samples are summarised in Figure 5.1.

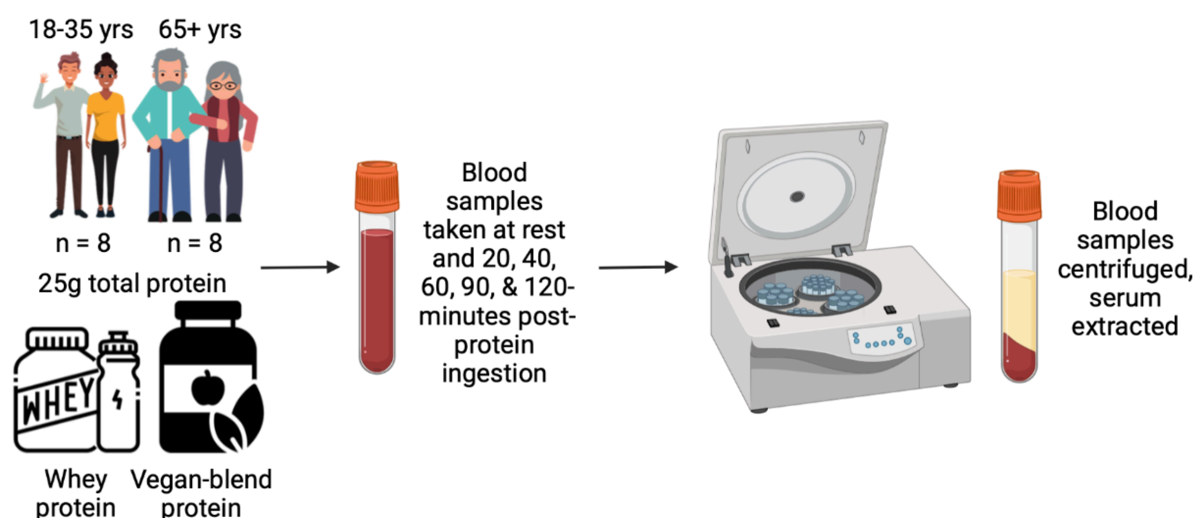

Figure 5.1- Graphical representation of blood sample collection protocol. Figure generated using Biorender.

100µl serum aliquots were stored at -80ºC prior to being defrosted on ice when needed. Once defrosted, aliquots were spun for 3-minutes at 9,600g in a microcentrifuge. 10µl internal standard mix (pre-made stable isotopically labelled mixture of AAs) and 10µl urease solution (7mg Urease (Sigma Aldrich U1500) was added to 1ml 70% ethanol. This was pulse centrifuged for 20-seconds to pellet urease protein. The supernatant was then removed and discarded, and the remaining pellet washed 3-times with distilled water. 1ml DDH_2O was then added to the pellet, and vortexed until the pellet was fully dissolved, leaving the urease solution to add to the serum samples and standards) This was left for 20-minutes at room temperature to incubate. Following this period, 0.5ml ice-cold EtOH was added to the microcentrifuge tube and vortexed. This was then left in the fridge (4ºC) for a further 20-minutes to cool. All samples and standards were then centrifuged for 5-minutes at 12,000g in a microcentrifuge. The remaining supernatant was added to glass Turbovap tubes (Chromatography Direct) and placed in a Techne block under N_2 at 90ºC for 15-minutes (or until all EtOH is fully evaporated). Once fully dried down, 0.5ml 0.5M HCl was added to the

Turbovap tubes and vortexed. 2ml Ethyl Acetate was then added to the tubes and vortexed. The lipids were then extracted, and the upper layer discarded. These samples were fully dried down in a Techne Block under N_2 at 90ºC. before being derivatised with N-methyl-tert-butyl dimethylsilyl-trifluoroacetamide (MTBSTFA, Sigma Aldrich, M-108). 70µl MTBSTFA and 70µl Aceonitrile:Dimethylformamide (ACN:DMF, 1:1) was added to the Turbovap tubes, and these were placed at 90ºC for 60-minutes to incubate. Following incubation, 50-100µl was transferred to auto-sampler vials, and these were run through GC-MS.

5.3.2 Cell Culture and Protein Analysis

Immortalised mouse muscle C2C12 myoblasts were obtained and proliferated for use in cell culture experiments. The processes of thawing cells, proliferating myoblast expansion, freezing cells, counting cells, differentiating cells, and preparing cells for treatment are identical to that previously described in Chapter 4- Sections 4.3.5 and 4.3.6.

Similarly, the process of protein analysis, including cell lysis, sample preparation, gel preparation, gel transfer and blocking, antibody use, and membrane imaging and protein quantification are identical to that described in Chapter 4- Section 4.3.7.

5.3.3 Dose-response cell culture experiments

Previous research has demonstrated the effective utilisation of an additional leucine treatment following 4h serum treatment in a C2C12 model to investigate differences in the anabolic response to treatment between serum conditions (352). Therefore, a similar protocol was used to explore the impact of a 'vegan' and 'whey' protein treatment (AA mix) following 4h fasted serum treatment (in young and older adults). Previous work has utilised the method of treating C2C12 myotubes with single AAs for 30-minutes to investigate the anabolic response to these treatments (344), however, no research has investigated treatment

of a 'mixed' AA composition in C2C12 myotubes. Therefore, initial dose-response cell culture experiments needed to be conducted to determine the optimal 'physiological' dose of these AA mixes, and the optimal treatment time before conducting further serum treatment experiments.

The AA mixes were made to mimic the composition of the Myprotein Impact Whey protein and the Vegan protein blend used in the human trial described in Chapter 4. The composition of these sources are reported in Chapter 4 (Table 4.3.3) and the EAA content of each protein dose (when matched for 25g total protein) is displayed in Table 5.1. To prepare the treatment mixes, the molecular weight of each AA powder was used to make up a 200mM mix of each individual EAA in DDH_2O. Once all AA mixes were made up, the percentage of total EAA content for each individual AA was calculated and used to create a 200mM 'master mix' of both vegan and whey treatments.

For this dose-response experiment the following doses of vegan and whey and vegan AAs were used: 0.5mM, 1mM, 2mM, 5mM, 10mM and 15mM. As previously described, leucine has been used as an anabolic stimulus following 4h fasted serum treatment in C2C12 cells. Allen and colleagues determined 5mM leucine to be an optimal treatment in terms of MPS response (352), however, previous research has demonstrated that treating C2C12 myotubes with 2mM leucine significantly increased expression of anabolic signalling targets, although this was the only EAA to produce this response (344). Therefore, it was anticipated dose greater than 5mM of a mixed AA treatment would be needed to elicit a significant response, allowing detection of any differences between AA mixes (vegan or whey) in the ability to stimulate MPS. In addition to exploring AA dose, the effect of time of treatment was also investigated for each dose with the following treatment times investigated: 30-minutes, 1-hour, 2-hours and 4-hours.

Table 5.1- Protein source essential amino acid profile per 25g total protein

Essential Amino Acid	AA content (g) per dose (25g total protein)	
	Vegan blend protein	Whey protein
Histidine	0.68	0.50
Isoleucine	1.31	1.90
Leucine	2.43	3.15
Lysine	1.98	2.85
Methionine	0.46	0.65
Phenylalanine	2.38	0.89
Threonine	0.98	1.99
Tryptophan	0.25	0.42
Valine	1.41	1.75
Total (g)	11.87	14.11

As previously described in Chapter 4 (Section 4.3.6), cells were ready to treat on day 6 to 7 of differentiation. Before adding any treatment to the cells, they underwent a 1-hour serum and AA starve. Following this, cells were washed in phosphate buffered saline (PBS), and the correct dose was added to each well. The following mix was then added to the correct well to make up each treatment dose:

Table 5.2 Dose-response experiment AA and media doses

Dose	µl serum- and AA-free media	µl AA master mix (200mM)
0.5mM	1995µl	5µl
1 mM	1990µl	10µl
2 mM	1980µl	20µl
5 mM	1950µl	50µl
10 mM	1900µl	100µl
15 mM	1850µl	150µl

Puromycin was added to myotubes for the final 30-minutes of incubation (1μmol/L, P8833, Sigma-Aldrich) as a means of determining acute MPS. Upon completion of the treatment, myotubes were washed 3-times with PBS, and lysed with RIPA lysis buffer. Cell lysates were then centrifuged, and prepped accordingly for western blotting (Chapter 4, Section 4.3.7). This set of experiment was only conducted at one cell passage (p10), meaning that no statistical analysis was performed on this data. C2C12 myoblasts continue to proliferate up to ~20-25 passages, and maintain the ability to differentiate well up to ~15 passages. Therefore, we ensured to use a cell passage lower than this, and monitored the myoblasts during the differentiation period to ensure that viable myotubes were being formed.

5.3.3.1 Serum and Amino Acid Treatment

Following dose-response experiments, the 'optimal' AA treatment dose and time was determined to inform this next set of experiments for which the same methodology as described in Chapter 4 (Section 4.4.3) was utilised. However, the only serum conditions which were added to myotubes were pooled young and older adult fasted serum samples. This is because these experiments utilised a previously published methodology reporting significant differences in MPS and anabolic signalling target protein expression to a 5mM leucine treatment in C2C12 myotubes 'preconditioned' in younger compared to older adult fasted serum (352). Based on this protocol, following the 4-hour serum 'preconditioning' treatment, 10mM vegan or whey amino acid treatment mix was added to each serum treatment well for 1-hour, with both the dose and time based on the first cell culture experiment reported in this chapter. Puromycin was added to myotubes for the final 30-minutes of incubation (1μmol/L, P8833, Sigma-Aldrich) to determine the acute MPS response. This set of experiments were repeated at three cell passages (p8, p9, p10).

5.3.3.2 *Statistics*

Statistical analysis was performed using GraphPad Prism v.9.4.1. All data were reported as mean (SEM), with significance set at $p < 0.05$, and values are expressed as fold change compared to a young, fasted average. To determine differences between AA serum concentrations, a two-way ANOVA (protein source x age) was used. Two-way ANOVAs (age x treatment) were also used to assess differences in puromycin incorporation and anabolic signalling. When results of the 2-way ANOVA revealed a position interaction effect, further comparisons were completed using Tukey's post-hoc testing. Further analysis was conducted with age values grouped by treatment. One-way ANOVAs were used to assess any differences between treatments when age values were grouped. When results of the one-way ANOVA revealed a positive effect, further comparisons were completed using Tukey's post-hoc testing.

5.4 Results

5.4.1 Mass Spectrometry

The area under the curve (AUC) was determined for each AA using GraphPad Prism v9.5.1 (528) for each of the following 4 conditions:

- Young adult plant protein
- Young adult animal protein
- Older adult plant protein
- Younger adult plant protein.

The time-course of AA appearance in serum is shown in Figure 5.2 (EAAs) and Figure 5.3 (NEAAs). It should be noted that not all AA data could be acquired as some AAs are highly liable and can spontaneously convert to other metabolites if samples are not

collected, stored, or analysed appropriately, and as such this dataset is missing Isoleucine, Asparagine and Histidine. Furthermore, full datasets were not possible for every AA displayed as some AAs were not detectable in all runs. The number of datapoints used in each group analysis is shown in Table 5.5, along with the peak AA serum concentration and time to peak concentration.

AUC data was used to determine any differences in AA concentrations between protein source or age. Table 5.3 shows the significance values for this analysis. Significant protein source effects were observed for alanine ($p = 0.03$), leucine ($p = 0.005$), proline ($p = 0.04$), methionine ($p < 0.0001$), threonine ($p = 0.0003$), lysine ($p = 0.05$), and tryptophan ($p = 0.0007$) serum concentration ($\mu mol.l^{-1}$), with concentration being higher following ingestion of whey protein in comparison to a vegan-blend protein. There were also significant age effects for cysteine ($p = 0.0008$), glutamine ($p < 0.0001$), and tryptophan ($p < 0.0001$) serum concentration ($\mu mol.l^{-1}$). Serum concentrations of cysteine and glutamine were higher in older adults compared to younger adults following protein ingestion, whereas serum tryptophan concentrations were elevated in younger adults in comparison to older adults following protein ingestion. No significant source x group interaction effects were observed. Where significant values were observed, multiple comparisons analysis was completed. This data is displayed in Table 5.4. Further analysis investigated the peak AA serum concentration, and time to peak concentration. This data are summarised in Table 5.5.

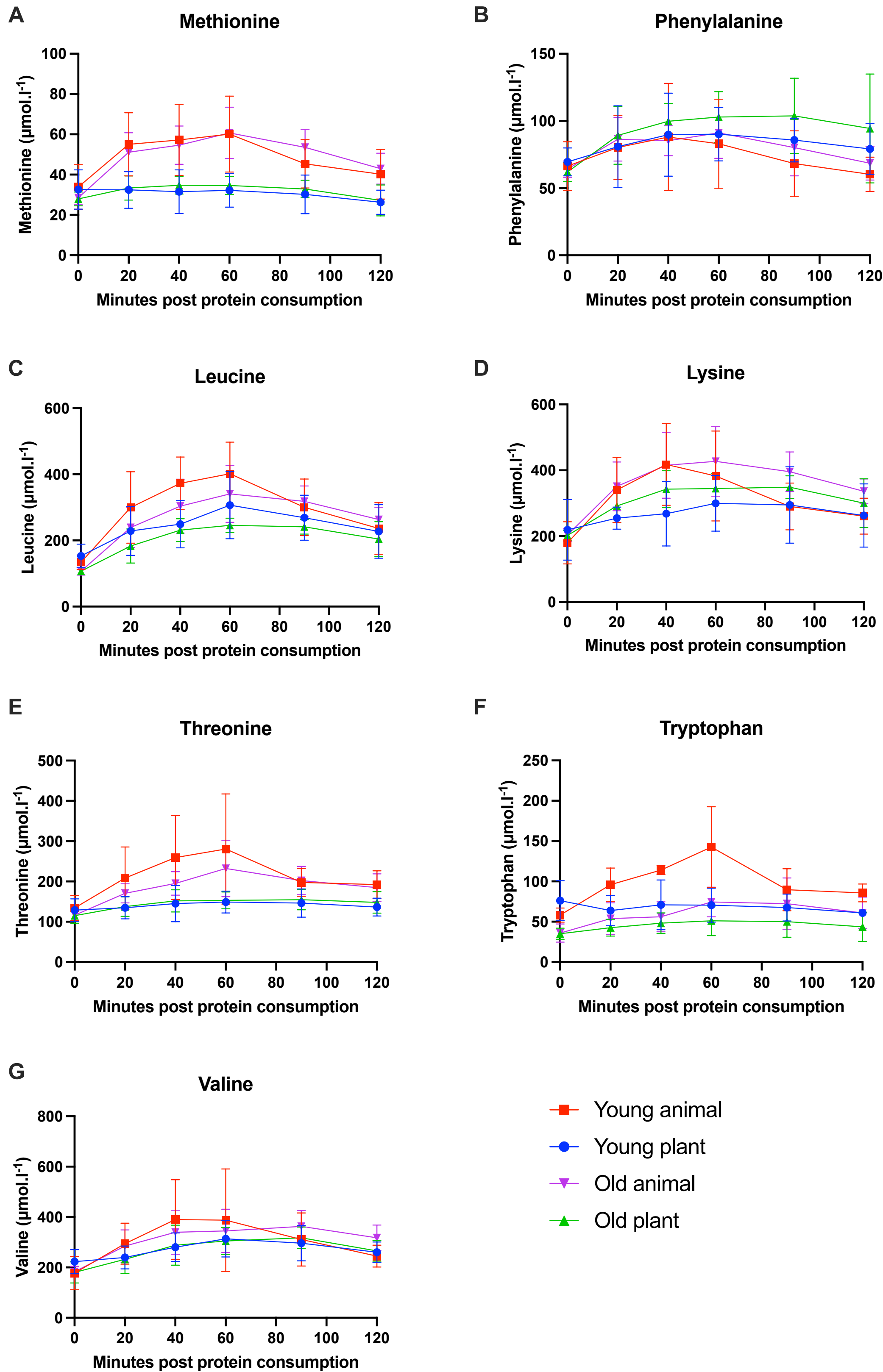

Figure 5.2- Time course of EAA serum appearance in young and older adults following plant- and animal-based protein consumption (**A**) Methionine, (**B**) Phenylalanine, (**C**) Leucine, (**D**) Lysine, (**E**) Threonine, (**F**) Tryptophan, (**G**) Valine, all values in µmol.l^{-1}. Displayed as Mean (SEM).

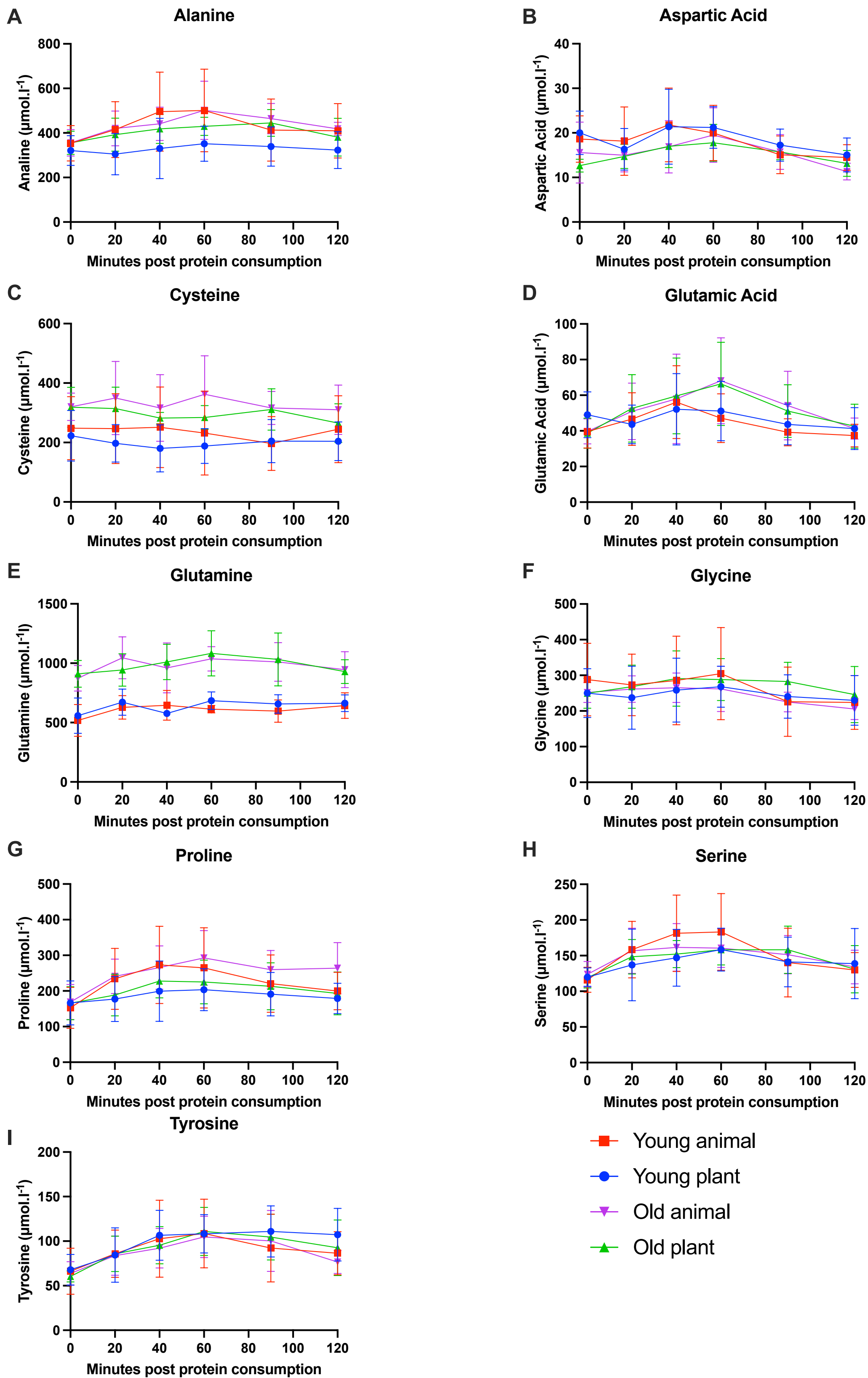

Figure 5.3- Time course of NEAA serum appearance in young and older adults following plant- and animal-based protein consumption (**A**) Alanine, (**B**) Aspartic Acid, (**C**) Cysteine, (**D**) Glutamic Acid, (**E**) Glutamine, (**F**) Glycine, (**G**) Proline, (**H**) Serine, (**I**) Tyrosine, all values in µmol.l^{-1}. Displayed as Mean (SEM).

Table 5.3- 2-way ANOVA results investigating any age, protein source, or interaction effects in serum AA concentration in young and older adults following consumption of plant- and animal-based protein sources.

Amino Acid	Age	Protein Source	Interaction
Alanine	0.1895	0.0343*	0.6976
Glycine	0.8808	0.7813	0.3292
Valine	0.9366	0.1300	0.9591
Leucine	0.1478	0.0052**	0.8556
Proline	0.3436	0.0417*	0.8868
Methionine	0.7072	<0.0001****	0.9330
Serine	0.9473	0.5069	0.6559
Threonine	0.4852	0.0003***	0.3264
Phenylalanine	0.2442	0.1251	0.7166
Aspartic Acid	0.1195	0.9447	0.7450
Cysteine	0.0008***	0.2324	0.9960
Glutamic Acid	0.1072	0.8798	0.8056
Lysine	0.0842	0.0477*	0.9437
Glutamine	<0.0001****	0.6440	0.7736
Tyrosine	0.7088	0.2659	0.8869
Tryptophan	<0.0001****	0.0007***	0.1974

Note: * represents $p < .05$, ** represents $p < .01$, *** represents $p < .001$, **** represents $p < .0001$

Table 5.4- Multiple comparisons results investigating any age, protein source, or interaction effects in serum AA concentration in young and older adults following consumption of plant- and animal-based protein sources.

Amino Acid	Comparison	Significance
Leucine	Young animal vs. Old plant	0.0149*
Methionine	Young plant vs. Young animal	0.0003***
	Young plant vs. Old animal	0.0002***
	Young animal vs. Old plant	0.0014**
	Old plant vs. Old animal	0.0007***
Threonine	Young plant vs. Young animal	0.0052**
	Young animal vs. Old plant	0.0119*
Cysteine	Young plant vs. Old animal	0.0079**
Glutamine	Young plant vs. Old plant	<0.0001****
	Young plant vs. Old animal	<0.0001****
	Young animal vs. Old plant	<0.0001****
	Young animal vs. Old animal	<0.0001****
Tryptophan	Young plant vs. Young animal	0.0092**
	Young animal vs. Old plant	<0.0001****
	Young animal vs. Old animal	0.0009***

Note: * represents $p < .05$, ** represents $p < .01$, *** represents $p < .001$, **** represents $p < .0001$

Table 5.5- Peak AA serum concentrations and time to peak concentration

Amino Acid	Young plant			Young animal			Old plant			Old animal		
	N	Peak	TTP	N	Peak	TTP	N	Peak	TTP	N	Peak	TTP
Alanine	46	351.4	60	47	501.3	60	45	445	90	46	501.3	60
Glycine	45	268.3	60	47	304.9	60	43	291.1	40	46	265.2	40
Valine	46	313.4	60	48	390.1	40	44	318	90	46	363.2	90
Leucine	46	306.7	60	48	401.9	60	43	245.7	60	45	340.8	60
Proline	46	203.6	60	47	273.3	40	44	227.8	40	40	292.6	60
Methionine	46	32.66	0	46	60.1	60	44	34.74	40	46	60.7	60
Serine	46	158.5	60	47	183.2	60	44	158.3	90	46	161.5	40
Threonine	46	149	60	47	280.5	60	43	154.6	90	46	232.5	60
Phenylalanine	46	90.24	60	47	88.12	40	44	103.8	90	46	91.14	60
Aspartic Acid	46	21.38	40	47	21.79	40	44	17.77	60	41	19.51	60
Cysteine	46	222.7	0	47	251.1	40	44	318.6	0	41	362.3	60
Glutamic Acid	46	52.09	40	47	56.11	40	43	66.34	60	46	68.11	60
Lysine	46	299.5	60	47	417.7	40	44	348.9	90	41	427.2	60
Glutamine	28	684.7	60	30	646.1	40	26	1083	60	23	1046	20
Tyrosine	46	110.9	90	47	108.6	60	30	110.9	60	30	104.8	60
Tryptophan	24	76.08	0	24	142.6	60	30	51.06	60	30	74.52	60

Peak AA serum concentration and time to peak concentration based on area under the curve data. N value shows number of points used to create area under the curve

Further calculations were made to retrospectively determine the total dose of AAs applied to C2C12 cells in the serum treatment experiments described in Chapter 4 and in the subsequent cell culture experiments reported in this chapter, based on GC-MS derived serum AA concentrations. Table 5.6 shows the estimated AA doses in young and older adult pooled serum samples at all time points (0, 20, 40, 60, 90, and 120-minutes post protein consumption) following animal and plant protein consumption. This shows the doses that were used throughout Chapter 4. Table 5.7 shows the estimated AA doses in fasted young and older adult pooled serum samples used in subsequent cell culture experiments described in a later section of this chapter (*Section 5.4.3- Conditioning C2C12 myotubes with ex vivo fasted human serum obtained from young and older adults with 'whey' and 'vegan' amino acid treatment)*. However, it must be noted that as previously described, not all AA data were available in this dataset, meaning that these dose values may be slightly lower (missing histidine, asparagine, and isoleucine AA values).

Table 5.6- Cell culture fasted and fed serum treatment AA concentrations

Treatment		**Young**		**Old**	
		µmol	**mM**	**µmol**	**mM**
100% serum treatment (2ml serum)					
Plant	**0**	5.348	0.00535	5.982	0.00598
	20	5.805	0.00580	6.875	0.00687
	40	6.090	0.00609	7.562	0.00756
	60	6.586	0.00659	7.836	0.00784
	90	6.266	0.00627	7.741	0.00774
	120	5.895	0.00590	6.802	0.00680
Animal	**0**	5.163	0.00516	5.905	0.00591
	20	6.937	0.00694	7.725	0.00772
	40	7.996	0.00800	8.110	0.00811
	60	7.990	0.00799	9.080	0.00908
	90	6.472	0.00647	8.166	0.00817
	120	6.246	0.00625	7.362	0.00736

10% serum treatment (200µl serum in 2ml media)					
Plant	**0**	0.535	0.00053	0.598	0.00060
	20	0.580	0.00058	0.687	0.00069
	40	0.609	0.00061	0.756	0.00076
	60	0.659	0.00066	0.784	0.00078
	90	0.627	0.00063	0.774	0.00077
	120	0.590	0.00059	0.680	0.00068
Animal	**0**	0.516	0.00052	0.591	0.00059
	20	0.694	0.00069	0.772	0.00077
	40	0.800	0.00080	0.811	0.00081
	60	0.799	0.00080	0.908	0.00091
	90	0.647	0.00065	0.817	0.00082
	120	0.625	0.00062	0.736	0.00074
20% serum treatment (400µl serum in 2ml media)					
Plant	**0**	1.070	0.00107	1.196	0.00120
	20	1.161	0.00116	1.375	0.00137
	40	1.218	0.00122	1.512	0.00151
	60	1.317	0.00132	1.567	0.00157
	90	1.253	0.00125	1.548	0.00155
	120	1.179	0.00118	1.360	0.00136
Animal	**0**	1.033	0.00103	1.181	0.00118
	20	1.387	0.00139	1.545	0.00154
	40	1.599	0.00160	1.622	0.00162
	60	1.598	0.00160	1.816	0.00182
	90	1.294	0.00129	1.633	0.00163
	120	1.249	0.00125	1.472	0.00147

Cell culture serum treatment AA doses in young and older adult pooled serum samples at all time points (0, 20, 40, 60, 90, 120-minutes post protein consumption) following animal and plant protein consumption. Values shown for 100% serum treatment, 10% serum treatment, and 20% serum treatment (µmol and mM).

Table 5.7- Cell culture fasted serum treatment doses

Treatment	Young fasted		Old fasted	
	µmol	**mM**	**µmol**	**mM**
100% serum treatment	5.247	0.00525	5.949	0.00595
10% serum treatment	0.525	0.00052	0.595	0.00059
20% serum treatment	1.049	0.00105	1.190	0.00119

Cell culture serum treatment AA doses in young and older adult pooled fasted serum samples. Values shown for 100% serum treatment, 10% serum treatment, and 20% serum treatment (µmol and mM).

5.4.2 Vegan vs. whey dose-response experiments

No statistical analysis was performed on the following data as these were preliminary experiments to determine the optimal AA dose and treatment time for the subsequent cell culture serum treatment experiments. All values are expressed as fold change compared to control (no AA treatment). For each time condition and AA dose, values are displayed for puromycin incorporation (Figure 5.4, Figure 5.5), p-mTOR (Figure 5.7, Figure 5.6), p-Akt (Figure 5.8, Figure 5.9), and p-RPS6 (Figure 5.10, Figure 5.11) protein expression.

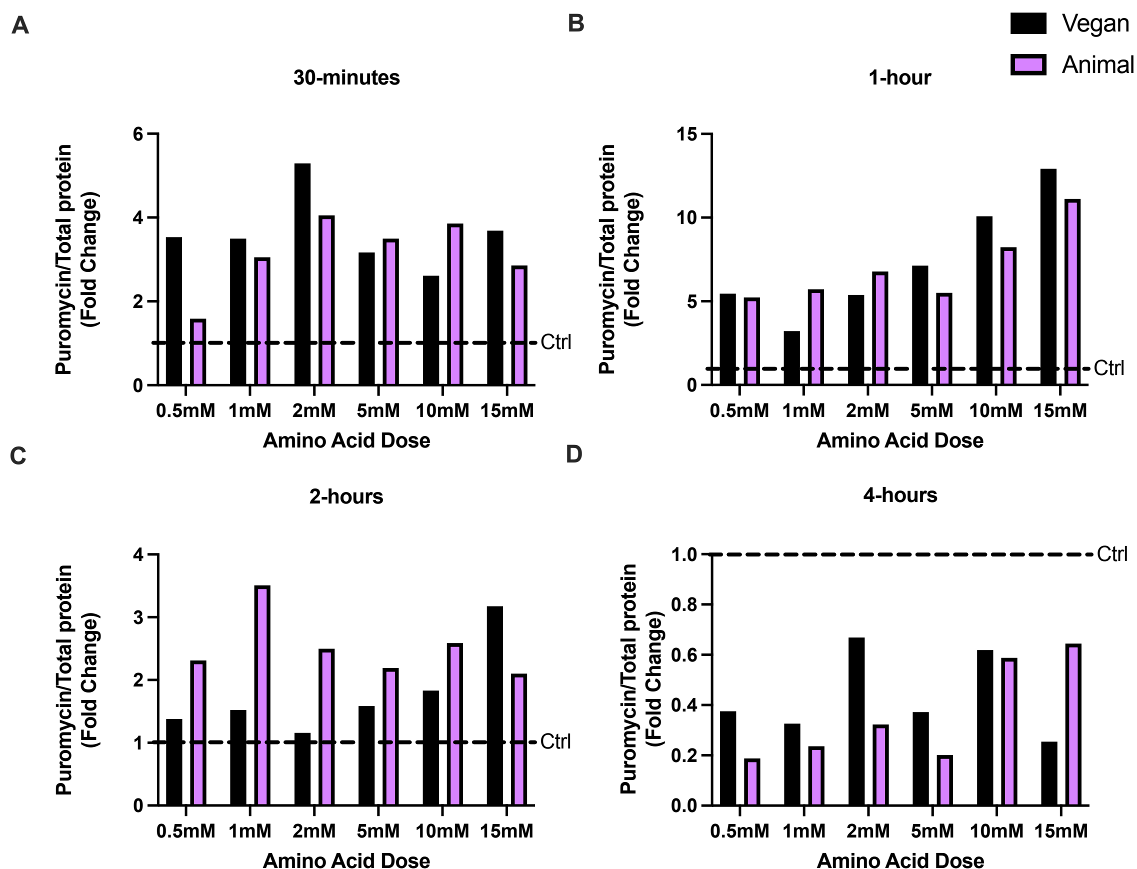

Figure 5.4- Muscle protein synthesis (measured by puromycin incorporation) in C2C12 myotubes treated with different doses of a whey and vegan amino acid mix for (**A**) 30-minutes, (**B**) 1-hour, (**C**) 2-hours, and (**D**) 4-hours. The dashed line on each graph demonstrates the control puromycin protein expression for that time treatment.

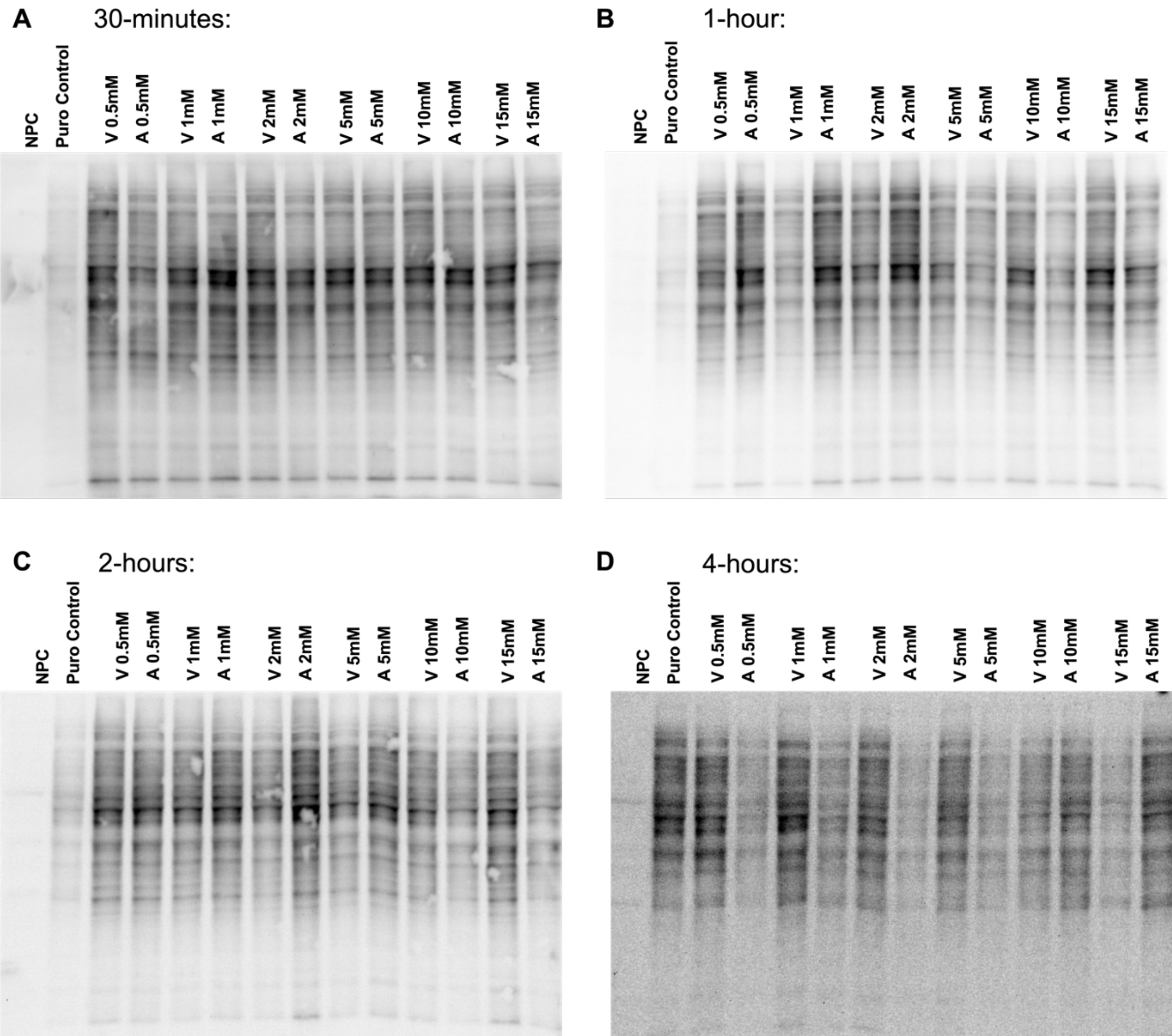

Figure 5.5- Representative western blot images of puromycin incorporation in C2C12 myotubes treated with different doses of a whey and vegan amino acid mix for (**A**) 30-minutes, (**B**) 1-hour, (**C**) 2-hours, and (**D**) 4-hours. Abbreviations: NPC, No-puromycin control; Puro control, Puromycin treatment control.

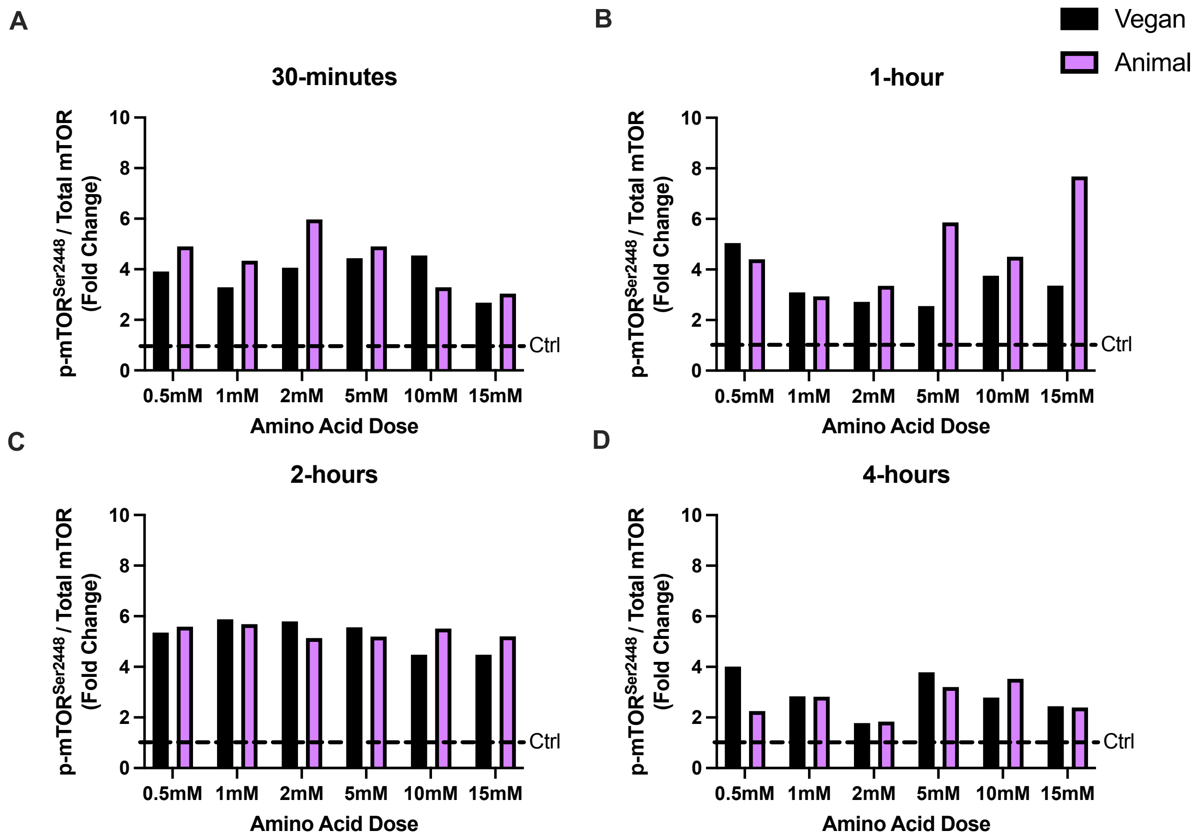

Figure 5.7- Protein expression of anabolic signalling target phospho-mTOR (Ser2448)/total-mTOR in C2C12 myotubes treated with different doses of a whey and vegan amino acid mix for (**A**) 30-minutes, (**B**) 1-hour, (**C**) 2-hours, and (**D**) 4-hours. The dashed line on each graph demonstrates the control phospho-mTOR (Ser2448)/total-mTOR protein expression for that time treatment.

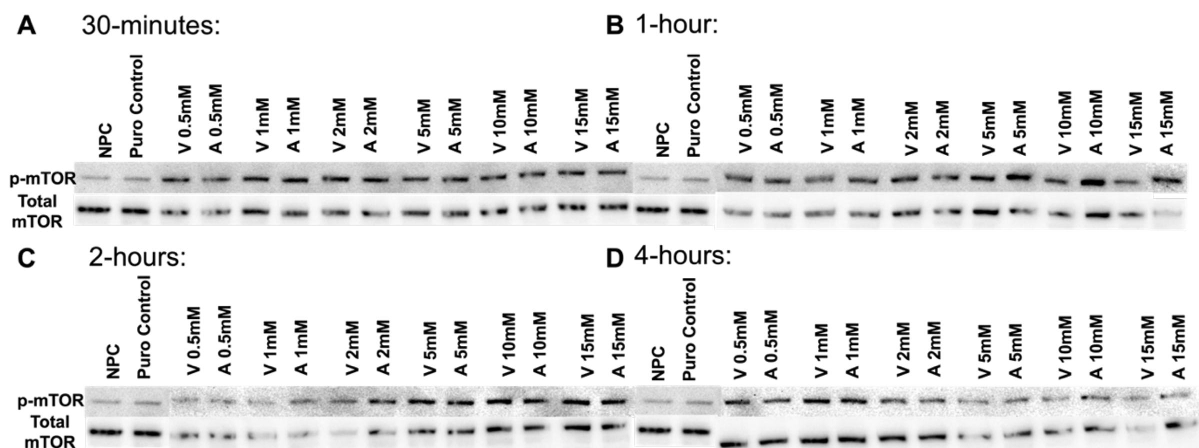

Figure 5.6- Representative western blot images of protein expression of anabolic signalling target phospho-mTOR (Ser2448)/total-mTOR in C2C12 myotubes treated with different doses of a whey and vegan amino acid mix for (**A**) 30-minutes, (**B**) 1-hour, (**C**) 2-hours, and (**D**) 4-hours. Abbreviations: NPC, No-puromycin control; Puro control, Puromycin treatment control.

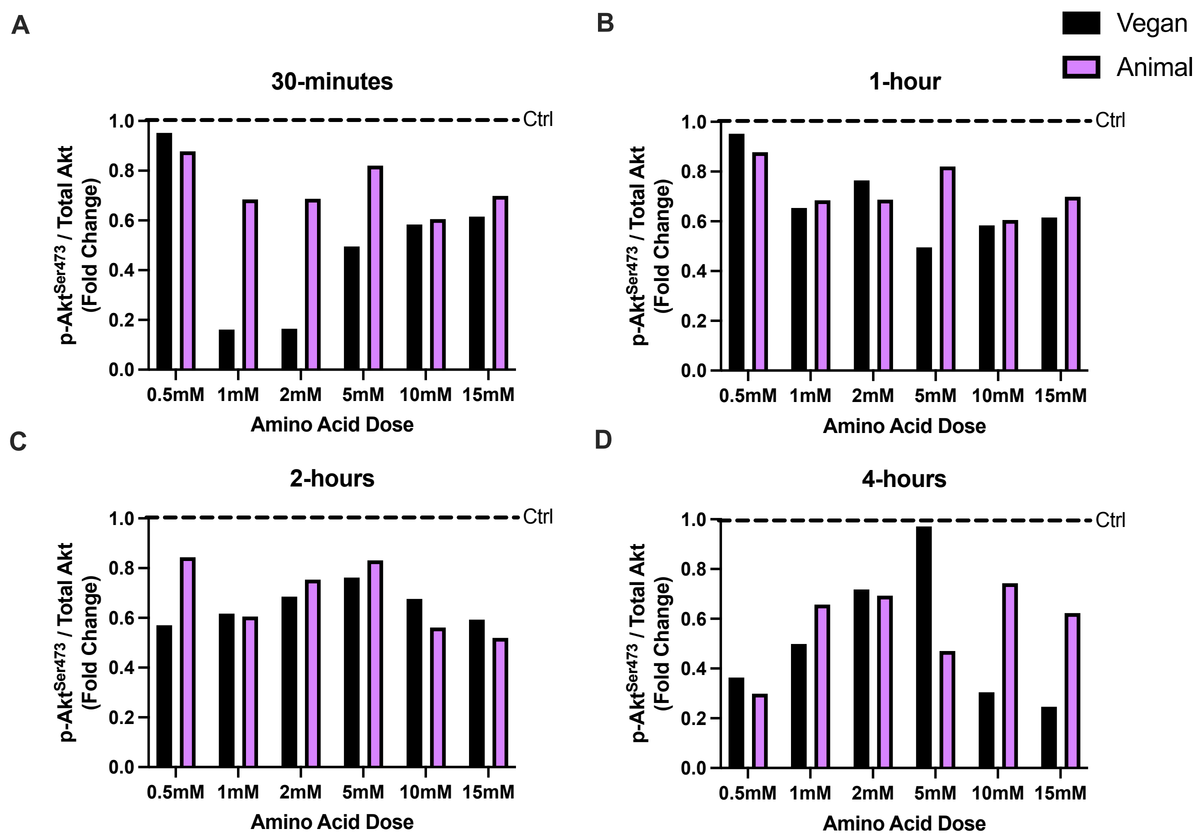

Figure 5.8- Protein expression of anabolic signalling target phospho-Akt (Ser473)/total-Akt in C2C12 myotubes treated with different doses of a whey and vegan amino acid mix for (**A**) 30-minutes, (**B**) 1-hour, (**C**) 2-hours, and (**D**) 4-hours. The dashed line on each graph demonstrates the control phospho-Akt (Ser473)/total-Akt protein expression for that time treatment.

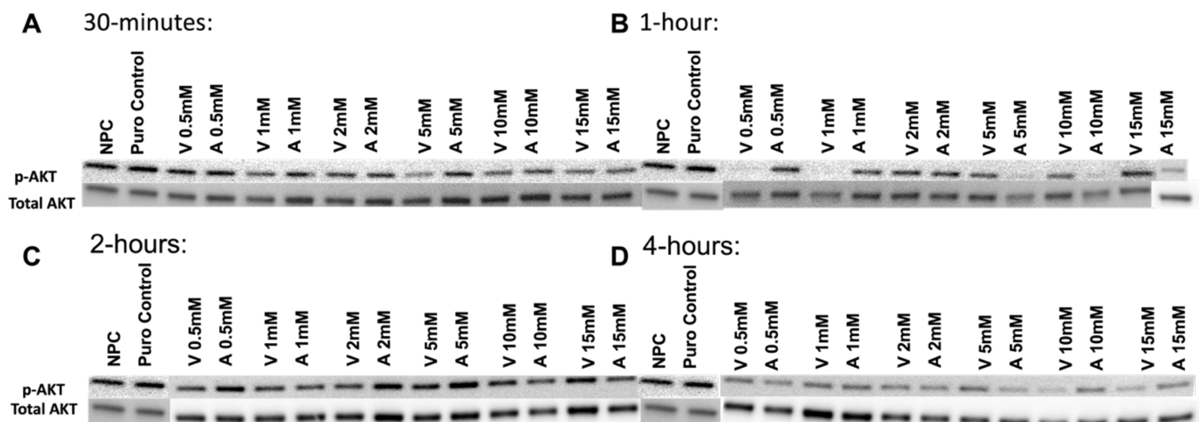

Figure 5.9- Representative western blot images of protein expression of anabolic signalling target phospho-Akt (Ser473)/total-Akt in C2C12 myotubes treated with different doses of a whey and vegan amino acid mix for (**A**) 30-minutes, (**B**) 1-hour, (**C**) 2-hours, and (**D**) 4-hours. Abbreviations: NPC, No-puromycin control; Puro control, Puromycin treatment control.

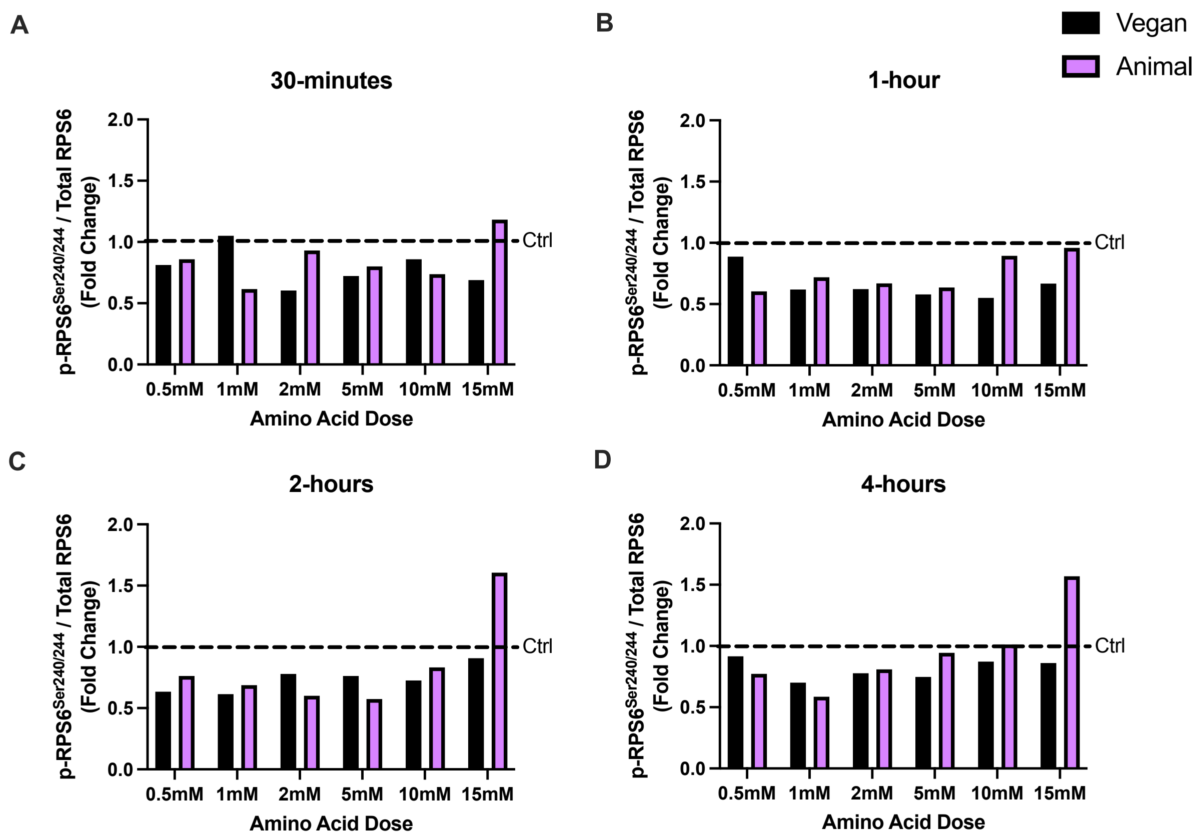

Figure 5.10- Protein expression of anabolic signalling target phospho-RPS6 (Ser240/244)/total-RPS6 in C2C12 myotubes treated with different doses of a whey and vegan amino acid mix for (**A**) 30-minutes, (**B**) 1-hour, (**C**) 2-hours, and (**D**) 4-hours. The dashed line on each graph demonstrates the control phospho-RPS6 (Ser240/244)/total-RPS6 protein expression for that time treatment.

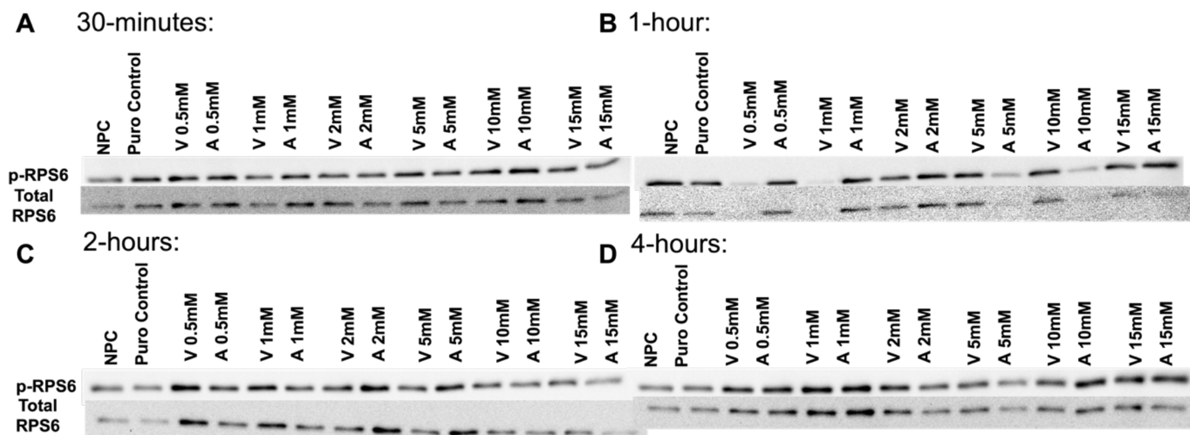

Figure 5.11- Representative western blot images of protein expression of anabolic signalling target phospho-Akt (Ser473)/total-Akt in C2C12 myotubes treated with different doses of a whey and vegan amino acid mix for (**A**) 30-minutes, (**B**) 1-hour, (**C**) 2-hours, and (**D**) 4-hours. Abbreviations: NPC, No-puromycin control; Puro control, Puromycin treatment control.

Based on the data presented above, primarily the puromycin incorporation results, a 10mM 1-hour AA treatment was deemed to be optimal, and was used in the following serum treatment experiments. Both 10mM and 15mM 1-hour treatments demonstrated visible differences in puromycin expression between the vegan and whey AA treatments. These differences were also visible in p-Akt and p-RPS6 protein expression. 10mM was chosen as opposed to 15mM as these conditions demonstrated comparable results, and 15mM may be considered as a more 'supraphysiological' amino acid dose.

5.4.3 Conditioning C2C12 myotubes with *ex vivo* fasted human serum obtained from young and older adults with 'whey' and 'vegan' amino acid treatment

No significant effect of age, treatment condition, or age x treatment condition was observed for puromycin incorporation (Figure 5.12A) or for phosphorylation of Akt (Figure 5.12B), RPS6 (Figure 5.12C) or mTOR (Figure 5.12D). Representative western blot images are shown in Figure 5.13.

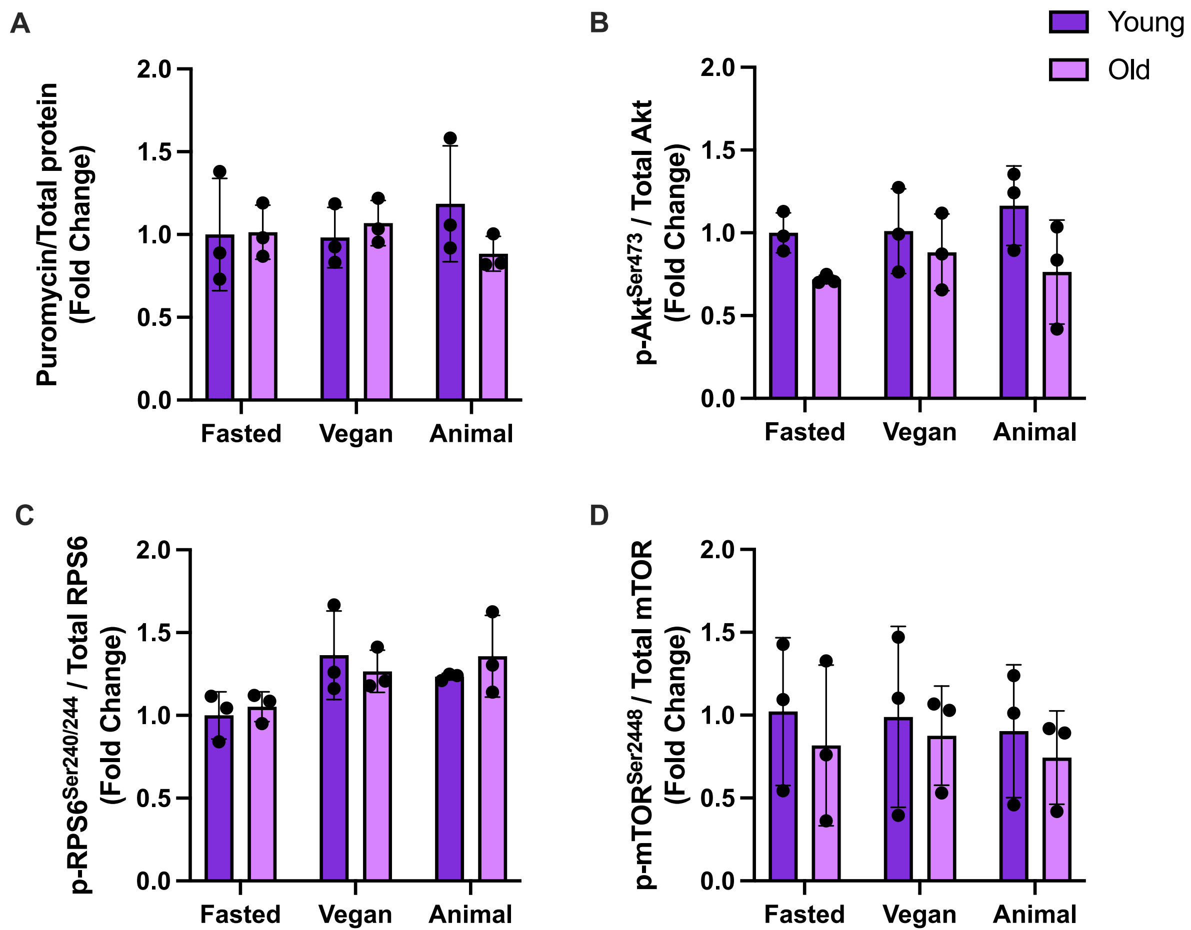

Figure 5.12- Muscle protein synthesis (measured by puromycin incorporation) and protein expression of anabolic signalling targets following a 4-hour fasted young and older adult serum treatment, with added vegan and whey amino acid treatments. (**A**) puromycin incorporation, (**B**) phospho-Akt (Ser473)/total Akt, (**C**) phospho-RPS6 (Ser 240/244)/total-RPS6, (**D**) phospho-mTOR (Ser2448)/total-mTOR. Data are expressed as Mean (SEM), with n = 3 per group (3 passage repeats).

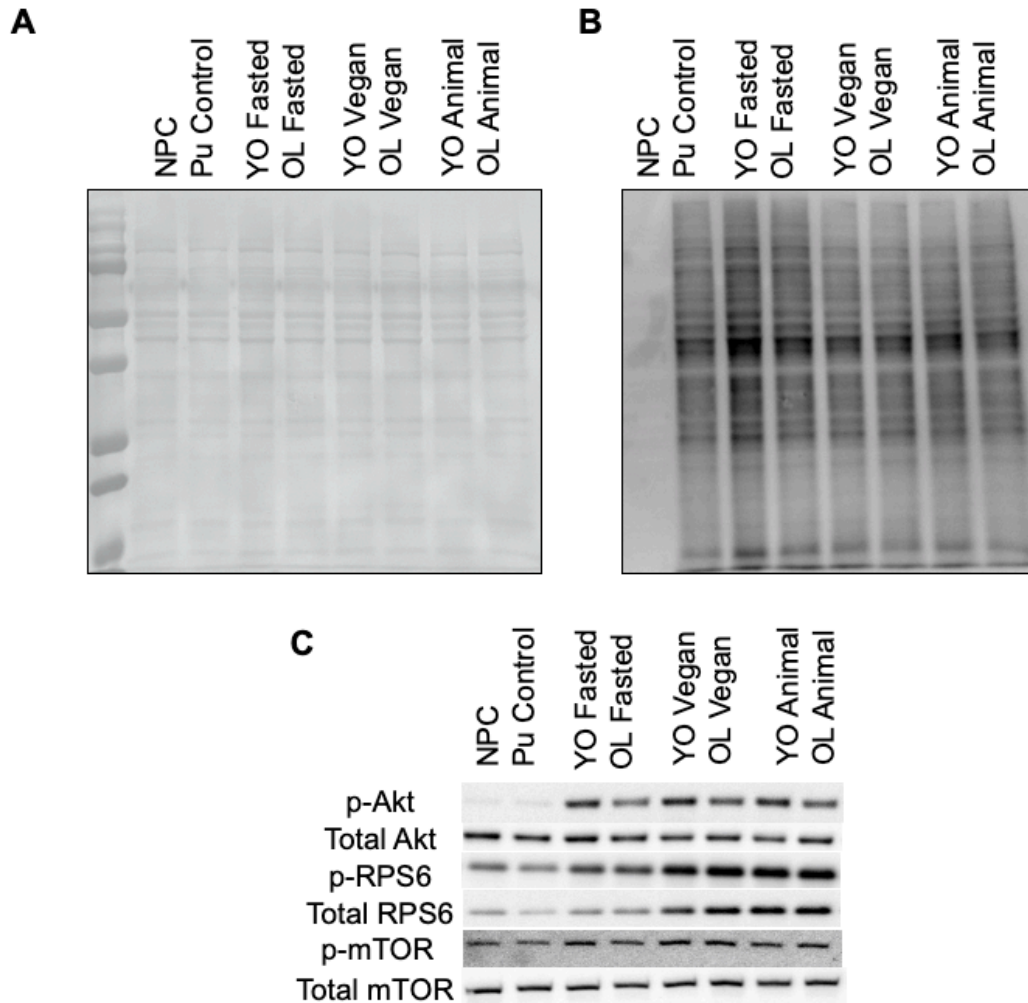

Figure 5.13- Representative western blot images of total protein (**A**), puromycin incorporation (**B**), and protein expression of anabolic signalling targets phospho & total-Akt (Ser473), phospho & total-RPS6 (Ser240/244), and phospho & total-mTOR (Ser2448) (**C**) following a 4-hour C2C12 myotube human serum treatment and 1-hour amino acid treatment. Western blots taken from passage 9 experiments. Abbreviations: NPC, no-puromycin control; Pu control, Puromycin treated control, YO, Younger adult, OL, Older adult

As we observed no significant differences in MPS or anabolic signalling target protein expression between young and older adults, age groups were pooled to compare the differences in puromycin incorporation and protein expression of anabolic signalling targets between fasted, vegan, and animal AA treatments independent of age. Results of a one-way ANOVA uncovered no significant differences between conditions for puromycin incorporation (Figure 5.14A, $p = 0.9772$) or phosphorylation of Akt (Figure 5.14B, $p = 0.75$) or mTOR (Figure 5.14D, $p = 0.86$). There was however a significant treatment effect for

phosphorylation of RPS6 (Figure 5.14C, $p = 0.01$) with lower RPS6 phosphorylation in the fasted state compared to both vegan ($p = 0.02$) and animal ($p = 0.03$) AA treatment.

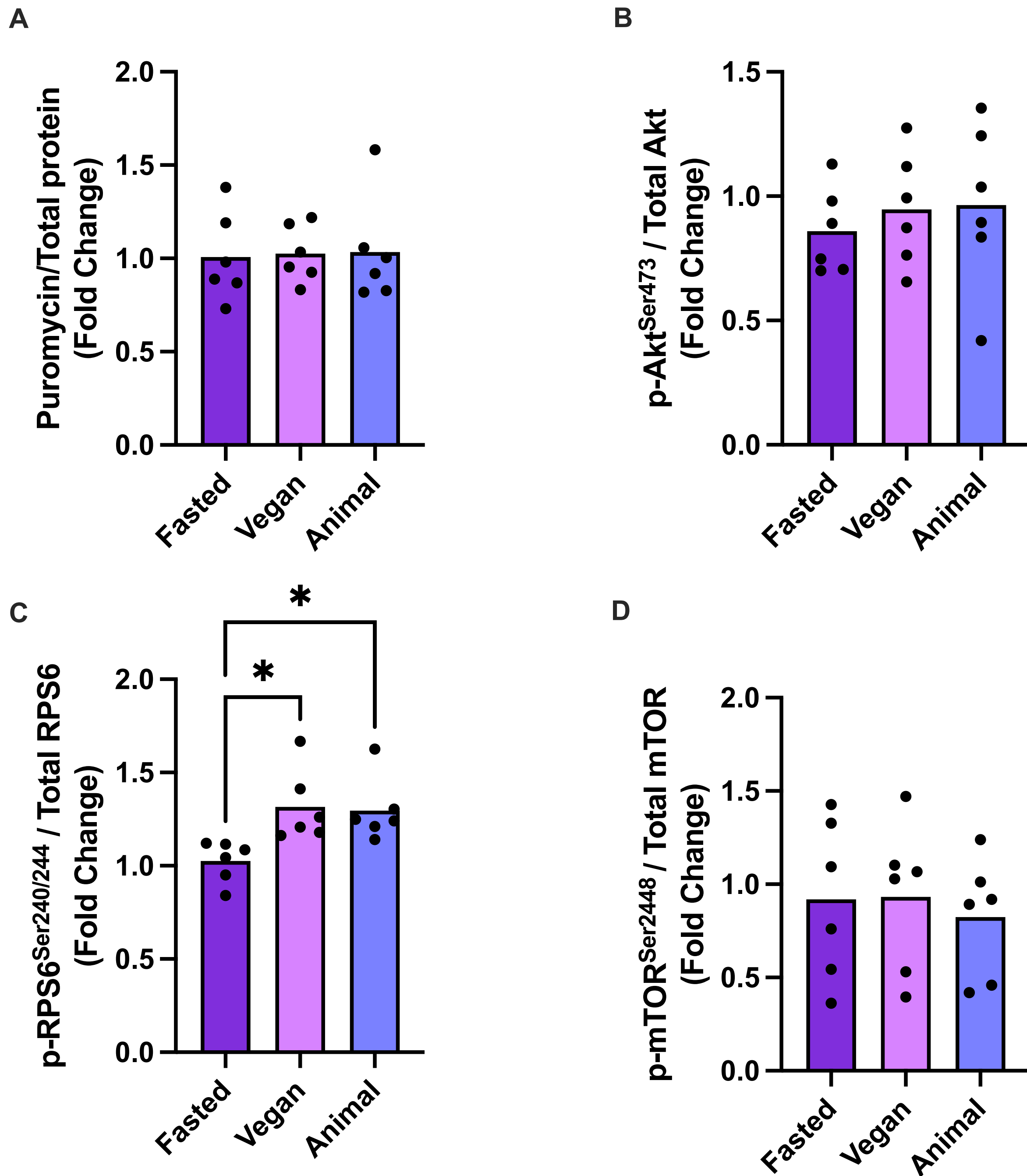

Figure 5.14- Muscle protein synthesis (measured by puromycin incorporation) and protein expression of anabolic signalling targets following a 4-hour fasted young and older adult serum treatment, with added vegan and whey amino acid treatments. (**A**) puromycin incorporation, (**B**) phospho-Akt (Ser473)/total Akt, (**C**) phospho-RPS6 (Ser 240/244)/total-RPS6, (**D**) phospho-mTOR (Ser2448)/total-mTOR. Data are expressed as Mean (SEM), with n = 6 per group (Young and old serum treatments grouped together, 3 passage repeats).

5.5 Discussion and Conclusions

The use of an *ex vivo* co-culture serum treatment model poses an exciting potential new methodology to provide mechanistic insights into different cellular responses to treatments and disease states. However, on the basis of the results presented in this chapter, further optimisation may be needed before results from this model can be accurately interpreted and translated to human studies. The mass spectrometry analysis of serum samples obtained before and after consumption of a whey or vegan-blend protein bolus highlights an interesting pattern of AA appearance in serum, and clear differences between protein sources, with little influence of age. When translating these results to a dose-response AA treatment experiment, it is clear that an *ex vivo* co-culture treatment model may not be optimal as when using a 10% treatment model, or even 20% as utilised by Carson and colleagues (355), the mM of the AA treatment is extremely low and therefore unlikely to produce a significant MPS or anabolic signalling response. For reference, Atherton and colleagues utilised a 2mM AA treatment in C2C12 myotubes, and despite MPS not being measured, this was sufficient to increase protein expression of anabolic signalling targets including p-p70S6K and p-RPS6 in the majority of EAAs (344). When putting this in context with AA concentrations in a 10% serum treatment, this is ~0.000525mM for fasted values, which is just 0.26% of the AA treatment dose used by Atherton and colleagues (2010).

The mass spectrometry results presented in this chapter provide an insight into AA kinetics in young and older adults following consumption of plant and animal protein sources. The importance of understanding AA kinetics is essential due to the established role of extracellular AA concentration in modulating MPS changes (343). When looking at the time-course of the appearance of AAs in serum and time to peak AA concentration, the majority of AAs display an increase in concentration from baseline (fasted) to postprandial

values. Exclusions to this include methionine, cysteine, and tryptophan after plant protein consumption by young individuals, and only cysteine after plant protein consumption by older individuals. Each of the AA demonstrate peak concentration when fasted. When comparing the EAA dose per serving (25g total protein) of plant vs. animal protein in this study, methionine and tryptophan concentration were markedly lower in the vegan protein blend (methionine: 0.46g vs. 0.65g; tryptophan: 0.25g vs. 0.42g), providing a possible explanation for the lack of postprandial increase in concentration of these AAs. However, for the most part, in all other AAs across both age groups and protein sources, peak concentrations were observed 40 to 60-minutes post-protein consumption. These results contrast with previously published results in which a more rapid increase in EAA appearance following whey protein consumption in comparison to a dampened response following soy protein was reported (377). The results reported in this chapter show differences in the magnitude of the peak, but no differences in time to peak between protein sources. This may be due to the differences between the plant protein sources utilised in these studies. The vast difference in quality of plant protein sources, in terms of their EAA content and digestibility, has been widely reported (145). This study used a vegan protein blend as opposed to a single plant protein source, which is likely to impact the AA digestion and absorption kinetics, and may explain lack of difference in time to peak between the protein sources utilised. Previous research by Moore and colleagues (2009) investigated acute MPS changes and plasma AA concentrations at rest following ingestion of a bolus dietary protein dose (25g whey protein) in healthy young males (174). EAAs, specifically leucine plasma concentrations, peaked ~1-hour post protein ingestion, with a gradual decrease back to ~baseline levels 120 – 180-minutes post-protein ingestion. This is consistent with the results reported here in, and with observations of increases in whole-body protein metabolism often observed from 40 to 60-minutes post consumption of a protein bolus or discrete meal. These rates often remain

elevated up to 3-hours post protein ingestion (152,153,174). However, Moore and colleagues did not compare circulating EAA concentrations following whey protein consumption to that following plant-based protein consumption.

The importance of protein quality has been frequently referred to throughout this thesis, with research suggesting that plant protein sources may be lower quality in comparison to animal protein sources (155,157). Many factors are suggested to influence the quality of a given protein, including the EAA content of a protein, and its digestibility (155,157). The results in this chapter demonstrate that the serum concentration of the AAs alanine, leucine, proline, methionine, threonine, lysine, and tryptophan were all significantly lower in the plant protein condition compared to animal protein. This may be reflective of the EAA composition of the protein source as in this case, when considering the EAA dose per serving (matched for 25g protein), leucine, methionine, threonine, lysine, and tryptophan were all lower in the vegan blend in comparison to the whey protein. Of particular interest, lysine, methionine, and leucine have each been highlighted as key EAAs for stimulating MPS and are predominantly lower in plant-based proteins. As such, this may be largely contributing to the reduced quality and anabolic properties of the respective protein source (167,383). It has previously been suggested that ingesting a combination of plant protein sources may be a useful means to counteract, in particular lysine and methionine, deficiencies in plant protein sources (145,167). However, the plant protein source consumed here was a vegan protein blend (pea protein isolate, fava bean isolate). This suggests that even when combining plant protein sources, there are still deficiencies present in the EAA content. Early research in this area from Bos and colleagues (2003) investigated postprandial serum AA concentrations and kinetics of appearance of individual ^{15}N-AA in healthy young adults following consumption of ^{15}N-soy or ^{15}N-milk proteins in a mixed meal. Despite reporting no differences in AA plasma concentrations, there was a direct effect of the dietary protein AA

composition on the circulating peripheral indispensable AAs available for metabolic utilisation and protein synthesis (147). This further reaffirms the importance of EAA content of the protein source in the resulting anabolic potential of a protein source.

Older adults display a blunted response to anabolic stimuli including protein provision, meaning that they are often recommended to consume a higher daily protein intake in comparison to younger individuals to support muscle maintenance (178). However, emerging research is investigating the potential role of protein source in contributing to anabolic resistance to determine whether in older adults, plant protein sources are as effective at stimulating MPS compared to animal protein sources (134). Here we did not investigate the MPS response to plant and animal protein sources in older adults, however, we did explore serum AA concentrations in young and older adults following plant and animal protein consumption, a factor which has been shown to highly correlate with MPS stimulation (147). Interestingly, there was a significantly higher expression of circulating cysteine and glutamine in older adults compared to younger adults. This is consistent with previously published literature reporting a ~10-15% higher peak plasma AA concentration in older compared to younger men following a 35g bolus dose of intrinsically L-[1-^{13}C]phenylalanine-labelled micellar casein protein (53), and it is suggested that this is indicative of a reduction in whole-body AA flux and/or a reduction in specific AA redistribution volume in older adults (53,384,385). However, it must be noted that different protein sources were used by Koopman *et al.* (2006) compared to this current study. Herein ingestion of a whey protein versus a vegan-blend protein was investigated, whereas Koopman and colleagues investigated the effects of a casein protein. Casein is regarded as a slowly digested protein, meaning that AA responses are often blunted, yet prolonged, in comparison to a ‘fast’ protein such as whey (164).

In the work presented in this chapter, tryptophan concentration is significantly higher in young compared to older adults, irrespective of protein source. EAA have been proven to be primarily responsible for postprandial MPS in older adults (138), highlighting the role tryptophan may play in MPS stimulation. It should however be recognised that, alone, tryptophan has not been highlighted as a key EAA, unlike leucine, valine, and isoleucine (BCAAs) (167) in influencing anabolic outcomes. Due to a lack of comparable evidence in this field of research, the reason for this reduced tryptophan concentration in older compared to younger adults cannot be fully determined. Overall, the results presented investigating serum AA concentrations in younger and older adults highlight a more prominent impact of protein source as opposed to age on postprandial AA kinetics.

A dose-response relationship between EAA administration and MPS responses has been previously reported (343), and in cell culture, anabolic responses to administration of individual AAs have been explored (344). However, to date, no work has investigated and determined the optimal dose-response relationship of vegan and whey AA formulations in cell culture. Herein, a set of experiments was conducted to determine an optimal physiological dose of whey and vegan 'AA mixes' in C2C12 cells due to its highlighted potential in the development of the previously reported human serum *ex vivo* co-culture cell treatment model (351,352). No statistical analyses were performed on the dose-response experiment as the primary aim of this experiment was to inform further serum and AA treatment experiments, therefore the experiment was only performed once. However, looking at trends of results from this experiment, there was increased expression of puromycin at 30-minutes, highlighting small increases in MPS, followed by peak expression at 1-hour, a small decrease at 2-hours with values comparable to 30-min treatment, and a large decrease at 4-hours, with control puromycin expression being higher than all treatment doses. Atherton *et al.* investigated MPS responses (via stable isotope tracer- primed continuous infusion of [1,2-

$^{13}C_2$] leucine with intermittent quadriceps muscle biopsies) in the fasted state and in response to a bolus 48g whey protein dose. They reported a large increase in MPS (~ 3-fold increase) around 45-minutes following ingestion of protein. This remained relatively stable 45 to 90-minutes post-protein consumption before rapidly decreasing to comparable postabsorptive rates of MPS (302). The observed decreases in MPS despite continuous increases in circulating AA availability is likely due to the 'muscle full' phenomenon, a term used to describe the point at which skeletal muscle becomes unresponsive to stimulation despite a continuous supply of AAs (hyperaminoacidemia) (302). When comparing this established pattern of increase in MPS following protein ingestion with puromycin expression in the dose-response experiment, it is unsurprising that MPS appears to peak at the 1-hour time point, decrease at 2-hours, and be lower than control values at 4-hours across treatment doses. However, it must be considered that in this experiment C2C12 myotubes were directly treated with an AA mix, whereas, when investigating MPS responses to protein ingestion in humans, other factors need to be considered which may delay the MPS response, such as protein digestibility.

A dose-response has previously been reported between EAA dose (2.5g, 5g, 10g, 20g) and MPS in healthy young and older men, with reports of an increase in MPS with increasing doses up to 10g, with higher EAA doses not resulting in greater MPS stimulation (44). Despite not being able to quantify AA treatments at a cellular level (measured in mM) to a dose of protein or EAA ingestion in humans, a similar dose-response pattern with increasing AA treatments was anticipated. Previous work using a C2C12 cell model investigated the effects of individual EAA treatment on protein expression of anabolic signalling targets, and utilised a 2mM treatment to investigate these differences (344). Results from this study demonstrated the ability of a 2mM treatment to uncover differences in the anabolic capacity of different EAA, specifically highlighting increased phosphorylation of anabolic signalling

targets mTOR and 4EBP1 with 2mM leucine treatment only (344). Therefore, for this dose-response experiment, a range of doses of whey and vegan-blend 'AA mixes' ranging from 0.5mM to 15mM were utilised, mimicking the composition of whey and vegan-blend protein powders used in the human study described in Chapter 4.

When looking at the trend of puromycin expression and phosphorylation of anabolic signalling targets p-mTOR, p-RPS6, and p-Akt, there is a varied response. When considering expression of p-RPS6 and p-Akt, there is a general lack of response to AA treatments irrespective of dose or treatment time, with expression being lower than a no treatment control. This is excluding a slightly increased expression of these targets with 15mM whey AA mix at 30-minutes, 2-hours, and 4-hours only. Previous AA treatment on C2C12 myotubes has demonstrated comparable results, with Atherton *et al.,* (2010) reporting no changes in Akt phosphorylation in response to AA treatment (344). It has been previously reported that despite being a potent upstream regulator of mTOR, EAA stimulates mTORC1 phosphorylation independently of Akt (344), possibly explaining the lack of response reported herein. However, Atherton and colleagues reported a significant increase in RPS6 phosphorylation following 2mM leucine treatment, which was not found here, despite the 10mM and 15mM amino acid mixes containing >2mM leucine. This may be due to these treatments being mixed as opposed to a single AA treatment, which may be stimulating other anabolic targets downstream of mTOR, such as eEF2.

When considering mTOR phosphorylation, there was comparable expression across 30-min, 1-hour, and 2-hour treatments, with a decrease at the 4-hour treatment. 1-hour AA treatment resulted in visible differences between dose and treatment (i.e., whey vs. vegan), with visibly higher p-mTOR expression in whey compared to vegan protein at 5mM, 10mM, and 15mM. This treatment method isolates the effect of the AA composition of the protein,

removing conflicting factors of protein digestibility and AA absorption. These results suggest that the whey protein AA mix may be of a higher 'quality' compared to the vegan AA mix due to the lower BCAA content as a percentage of total EAAs (vegan vs. whey, isoleucine: 11.01% vs. 13.47%; leucine: 20.46% vs. 22.32%; valine: 11.90% vs. 12.42%). This supports previous work highlighting the importance of BCAAs in stimulating MPS; despite leucine being highlighted as the key MPS stimulator, isoleucine and valine may play a key role in ensuring that their corresponding amino acyl-tRNAs are not rate limiting factors. Based on this dose-response experiment, 10mM AA treatments were utilised in the subsequent serum- and AA-treatment experiments, which when comparing the relative leucine content of these AA mixes is comparable to the 2mM leucine dose utilised by Atherton *et al.,* (2010) when investigating anabolic responses to individual AA treatments in C2C12 myotubes.

Building on the human serum treatment experiments described in Chapter 4, the final set of experiments reported in this thesis aimed to utilise AA treatments, of which the optimal dose was determined in the aforementioned dose-response experiments. Due to the lack of stimulation of MPS and associated anabolic signalling targets following treatment of C2C12 myotubes with fasted and fed human serum obtained following consumption of 25g animal- and plant-based protein (results in Chapter 4), this set of experiments utilised previously published methodology (352). Allen *et al.,* (2021) investigated the effect of 4h *ex vivo* fasted human serum from young and older adults' treatment with the addition of a 30-minute 5mM leucine treatment on stimulation of MPS and associated anabolic signalling targets. The current methodology utilised the 4h serum treatment as a 'preconditioning' period to investigate how C2C12 myotubes responded to an additional AA treatment. Previously conducted dose-response experiments confirmed 10mM as an 'optimal' dose of vegan and whey AA mixes, and these were used as the additional AA treatment in this set of serum treatment experiments. Allen and colleagues reported that irrespective of the additional

leucine treatment, MPS was reduced in myotubes treated with older adult serum compared to younger adult serum. Furthermore, in response to additional leucine treatment, they reported a significantly higher expression of puromycin, indicating increased MPS stimulation, and higher expression of anabolic signalling targets Akt, p70S6K, and eEF2 in myotubes 'preconditioned' with fasted serum from younger compared to older adults (352). In contrast to this, the results from the current experiments demonstrated no difference in puromycin expression or phosphorylation of Akt, RPS6 or mTOR expression between myotubes treated with young and old fasted serum only, or those 'preconditioned' in young or old fasted serum for 4-hours with 1-hour whey or vegan AA mix treatment.

The lack of differences between young and old fasted serum on stimulation of MPS and anabolic signalling targets is largely unsurprising, as this has been previously reported in the literature (353), and is comparable to findings reported in Chapter 4 of this thesis. It has been previously reported that basal i.e., postabsorptive, MPS rates are not different between young and older men (32), and when considering the circulating AA levels in the fasted serum used in this set of experiments, mass spectrometry analysis demonstrated no significant differences between young and older adults; in fact total circulating AAs numerically were higher in older compared to younger adults. Furthermore, when comparing C2C12 myotubes treated with young and old fasted serum only, there were no differences in phosphorylation of Akt, mTOR or RPS6. This is consistent with previous findings (352,353), and when translated into human trials is consistent with data reporting no age-related differences in basal anabolic signalling (33,44,386). However, when treating serum preconditioned cells with whey and vegan AA mixes (10mM total EAA), there were no significant differences in MPS or phosphorylation of anabolic signalling targets between myotubes treated with young and older adult fasted serum. This finding is in contrast to Allen *et al.,* and appears to contradict human studies demonstrating anabolic resistance of muscle to

AAs (following protein ingestion) in older adults (44,367,369). As such, how physiologically representative this cell model is of *in vivo* human muscle must be considered with some questioning the inconsistencies present and reproducibility of these results (351).

Due to the lack of differences observed between C2C12 myotubes treated with younger adult and older adult serum, the data were grouped to increase the power and investigate differences between fasted serum treatment, fasted serum and whey AA treatment, and fasted serum and vegan AA treatment, irrespective of age. There were no significant differences in expression of puromycin, p-Akt, or p-mTOR. However, a significant treatment effect was observed for p-RPS6 expression, with elevated p-RPS6 expression in C2C12 myotubes treated with whey or vegan AA mixes in comparison to the fasted control condition. There were no differences between the AA treatments. This suggests that this dose of AA is sufficient to stimulating anabolic pathways associated with mTOR, and is reflective of human trials demonstrating increased MPS and expression of anabolic signalling targets following EAA ingestion (343).

Treating cells with serum and/or plasma is a growing area of research in various cells lines, including C2C12, L6, LHCN-M2, and human primary skeletal muscle cells, and has been used as a mechanism of mimicking the *in vivo* skeletal muscle systemic environment (351). However, results in the literature and those reported here are inconsistent. A recent review of the current research utilising this *ex vivo* co-culture model highlighted several factors which may explain the results reported in this chapter (351). To date there is no established methodology for this co-culture model, meaning that differences between published research protocols exists, including in serum treatment percentage and cell culture media use (i.e., media glucose concentration and serum starvation period (351)). When investigating the total AA concentrations of the fasted serum samples used in this set of

experiments, these were markedly lower than any published C2C12 myotube AA treatment (344). When utilising a 10% serum treatment model, it was calculated that C2C12 myotubes were being treated with ~0.560µM (averaged young and older adult data, this equates to ~0.00056mM). When considering this, the lack of anabolic response was not surprising, as this dose of AAs is not high enough to stimulate MPS nor expression of anabolic signalling targets (344).

This work is not without limitations. The methodology of this novel co-culture model has been explored throughout this thesis, and despite attempts to optimise this model, these findings suggest that it is not yet a reliable method of conditioning media to regulate anabolic signalling responses and MPS. As previously reported by Carson *et al.,* C2C12 cell lines do not remain viable for extended periods (4-hours) in high serum concentrations (355), which when considering the mM of AAs in the serum treating the C2C12 myotubes, is a prominent limitation. Furthermore, despite work from Allen *et al.,* demonstrating the effectiveness of an additional AA treatment following a 4-hour fasted serum preconditioning period, it is not possible to directly compare an *in vitro* AA dose (in mM) to a bolus protein dose *in vivo*. Hence, one cannot directly determine whether an AA dose *in vitro* is supraphysiological. Future work developing the use of this model in primary human skeletal muscle cells may provide more insight into the *in vivo* skeletal muscle systemic environment and intrinsic factors which may interact with the systemic cellular environment. However, there is a greater risk of losing myogenic phenotype when using human primary skeletal muscle cells in comparison to the C2C12 cell line (387).

To conclude, mass spectrometry analysis of serum samples from young and older adults obtained following consumption of a protein-matched dose of animal and plant-based protein highlights a clear influence of protein source on circulating AA concentrations. There is a

limited influence of age, suggesting that the quality of the protein source, in particular the EAA content and digestibility of the protein, plays a key role in determining circulating AAs. Despite the potential for an *ex vivo* co-culture human serum treatment model to investigate muscle anabolic responses to different disease states and nutritional status, the developmental work on this model presented in this thesis, reiterates the need for further optimisation. We report minimal effects of using fasted human serum as a 'preconditioning' period to investigate the response of different 'conditions' (for example, here we were investigating the effect of age) to a vegan and whey protein AA dose, suggestive of no impact of protein quality, here modulated by AA composition, on MPS responses.

6 General discussion and conclusions

6.1 Introduction

The worldwide phenomenon of an increasing ageing population has societal, clinical, and financial implications. This is primarily as a result of healthspan, the years in which an individual is in optimal health, not increasing in sync with the increase in lifespan (6). This means a greater number of older adults may expect to live a substantial number of years in of ill health, often associated with loss of independence and physical function (10). Sarcopenia is a primary health outcome associated with older age, and is characterised by declines in muscle mass, strength, and functionality, and is associated with increased frailty incidence, decreased mobility, and increased risk of falls (15).

Due to the myriad of negative outcomes associated with the onset and progression of sarcopenia, increasing research effort is being directed at counteracting this syndrome, primarily through increasing protein intake and physical activity (21). This thesis primarily explores the influence of protein nutrition for muscle metabolism in older adults, with a focus on the role of protein quality. An increasing number of adults are consuming vegan and vegetarian diets (155), yet, evidence suggests that plant-based protein sources may be of a lower 'quality' (134), characterised by a lower essential amino acid (EAA) content and reduced digestibility (155), in comparison to animal-based protein sources. An abundance of research demonstrates anabolic resistance in older adults (44,50,51,388). When considering protein quality, this means that older adults consuming a high proportion of plant-based proteins may need to consume a greater amount of protein daily or greater variety of plant-based protein sources in comparison to those consuming predominantly animal-based proteins (135,155) to minimise the negative musculoskeletal health outcomes associated with increasing age. However, the *in vivo* evidence investigating differences between plant-based and animal-based protein supplementation strategies in older adults is still conflicting

(155,157), with the cellular mechanisms and factors such as the amino acid (AA) kinetics following plant-based protein ingestion yet to be fully understood in older adults.

A population of older adults which are largely under-researched due to ethical and methodological difficulties are those which are arguably most in need of intervention. Older adults residing in residential care are at increased risk of developing musculoskeletal syndromes such as sarcopenia and frailty (184), with the prevalence of sarcopenia reported to be 6-times greater in older adults in the residential care setting in comparison to their community-dwelling counterparts (183,184). Protein-energy malnutrition (PEM) is common in this setting (189,221), with evidence taken from care home residents reporting that up to 35% of residents consume markedly less than the estimated average requirement (EAR) ($0.66g.kg^{-1}.day^{-1}$) (117,230). However, implementing a protein supplementation trial is not simple in this setting, with several other factors contributing to this reduction in protein ingestion such as reduced appetite and increased prevalence of dementia, dysphagia, and dentition issues (195,231,232). Thereby, considering protein quality as a factor in possible supplementation studies makes this an increasingly complex research area.

The purpose of this thesis was:

1. To evaluate the existing literature exploring protein supplementation strategies to improve musculoskeletal health outcomes in older adults in the residential care setting,
2. Assess differences in habitual protein intake in young and older adults, and how these influence musculoskeletal health outcomes at a cellular and whole-body level,
3. Develop the use of an *ex vivo* human serum C2C12 co-culture model to explore cellular mechanisms regulating MPS and anabolic signalling differences between

young and older adults following consumption of plant- and animal-based protein sources,

4. To investigate serum AA kinetics following consumption of plant- and animal-based protein sources in young and older adults

This chapter will summarise the results presented in Chapters 2-5 of this thesis and discuss the interaction between key findings and the potential future research they inform.

6.2 The role of dietary protein intake in older adults and the effectiveness of protein supplementation strategies across the lifespan

Anabolic resistance of skeletal muscle to protein nutrition is proven to be associated with the onset and development of sarcopenia in older age (117), highlighting the importance of daily protein nutrition in older adults to attenuate sarcopenia. However, increasing evidence demonstrates that older adults consume less dietary protein in comparison to younger adults, and the recommended daily allowance (RDA) and EAR recommendations (0.8 $g.kg^{-1}.day^{-1}$ and 0.66 $g.kg^{-1}.day^{-1}$ respectively) are no different for young and older adults (178).Therefore, Chapter 3 of this thesis aimed to investigate the habitual protein (and leucine) intake in healthy young and older adults, and explore any relationships between these factors with muscle mass, function, and anabolic responses (muscle protein synthesis (MPS), protein expression of anabolic signalling targets). No significant differences in habitual dietary intakes (protein, fat, carbohydrate, leucine ($g/mg.kgWBLM^{-1}.day^{-1}$) were observed, and despite whole-body lean mass (WBLM) and knee-extensor strength (KES) being significantly higher in younger adults, no significant associations were observed between habitual protein intake and WBLM, upper-leg lean mass (ULLM), whole-body strength (WBS), KES, basal MPS, or fed MPS across both age groups. These results suggest that there are other factors contributing to the reductions observed in WBLM and KES in

older adults other than habitual protein intake, such as physical activity status, with older adults who have been physically active throughout their life exhibiting minimal muscle loss in comparison to sedentary older adults (27–29). Not only were these older adults consuming the RDA, but 75% of these older adults were consuming 1.0-1.2 $g.kg^{-1}.day^{-1}$, the alternative RDA proposed by the PROT-AGE group for older adults. This contrasts with previous results reporting that 65% of older adults in the UK (n = 40) did not reach a protein intake of 1.0 $g.kg^{-1}.day^{-1}$ (308). Despite the findings reporting older adults consuming above the RDA protein, it must be considered that this secondary data analysis was from a baseline dataset of a resistance exercise training study (1), and the participants were relatively healthy, high functioning, and non-sarcopenic older adults. When considering sarcopenic older adults, the role of protein intake is likely to have a more significant impact, with a recent systematic review indicating that despite sarcopenic older adults recommended to consume a higher daily protein intake to maintain musculoskeletal health (178), these individuals consumed a lower protein intake compared to their non-sarcopenic peers (389). Furthermore, an association has been previously established between incidence of frailty and low protein intake in older adults (118). Therefore, if a sarcopenic older adult dataset was compared to the data presented in Chapter 3, it is probable that these adults would demonstrate even heightened declines in WBLM and KES. This would likely be paired with a reduced daily protein intake, along with a lower responsiveness of frail muscle to an intended growth stimulus . It is also likely that due to associations between sarcopenia and reductions in physical function, reductions in ULLM would be observed in this population as sarcopenia is predominantly associated with loss of lower body muscle mass preceding upper body muscle mass declines (15). This is a prominent contributor to the declines in physical function and increased incidence of falls observed in sarcopenic individuals (15).

Protein supplementation is most beneficial to musculoskeletal health when combined with resistance exercise training (50,135,390). This is a feasible method of improving musculoskeletal health in healthy older adults, however, in older adults in care homes, implementing protein supplementation alone can be complex without the added stressor of resistance exercise training. The two primary forms of protein supplementation in the residential care setting, as discussed in Chapter 2, are oral nutritional supplements (ONS) and protein-fortified foods (PFF). ONS are often in liquid form, and are commonly used in this setting when individuals cannot consume whole foods, whereas PFF maintains the quantity of food an individual is consuming whilst increasing daily protein and energy intake. These forms of protein supplementation are commonly used due to the many factors which contribute to supplementation compliance in this setting which are not as commonly reported in community-dwelling older adults, such as decreases in appetite and dentition and dysphagia issues (195,231,232). When reviewing the available research in this area, results were inconclusive due to the nature of these studies often focussing on compliance of the supplementation strategies as opposed to musculoskeletal health outcomes. Compliance is difficult to assess and collecting reliable outcome measure data in this population is also challenging. Observationally, negligible differences were observed between the effectiveness of the two methods of protein supplementation in this setting, however, this may be best prescribed on an individual basis. For example, an individual struggling with appetite may be better suited to PFF, and an individual struggling with dentition and dysphagia issues may be better suited to ONS. When consumed, the protein supplementation strategies proved effective at increasing daily protein intake, however, this did not consistently result in increases in muscle mass, strength, or functional outcomes. This was contributed to by difficulties in accurately measuring muscle mass and strength, and functional status in this setting, and despite protein supplementation resulting in increases in daily protein intake, it

was unlikely that these individuals reached the recommended RDA of 1.4g.kg^{-1}.day^{-1} (178). The review of the available research in Chapter 2 highlights the complexity of carrying out interventional research in the residential care setting, and the need to consider individual needs to maximise musculoskeletal health benefits.

A primary aim of this thesis was to investigate the role of protein quality, specifically to compare the anabolic potential of plant-based and animal-based protein sources. This is an emerging research area, with preliminary research suggesting that plant-based protein sources are of a lower 'quality' in comparison to animal-based protein sources, primarily due to their lower EAA content and lower digestibility (155). In Chapter 5 of this thesis, serum AA concentrations were measured in young and older adults following consumption of both a vegan-blend protein and a whey protein. The results highlighted several significant differences in serum AA kinetics between protein sources in young and older adults, including significant elevations in concentration of alanine, leucine, proline, methionine, threonine, lysine, and tryptophan following consumption of whey protein in comparison to a vegan-blend protein. Despite being matched for total protein (25g per dose), the vegan-blend protein had a lower total EAA content. Furthermore, despite using a vegan-blend protein, which is often recommended in contrast to using a single plant-based protein source to combat the lower EAA content and digestibility (134), the lower serum AA concentrations reported here were likely also a consequence of the lower digestibility of the vegan-blend protein in comparison to the whey. However, it must be noted that a key limitation here is that subsequent MPS rates were not measured, as the primary aim of the human trial was to obtain serum samples for use in future cell culture experiments. Measuring resulting MPS would determine whether the serum AA differences were reflected in MPS changes and whether this reflects previous research in this area which reported lower postprandial MPS in

young and older adults following ingestion of a soy in comparison to whey protein both at rest and following resistance exercise training (340,377,391).

Despite research reporting differential anabolic capacities (resulting MPS) following consumption of whey protein compared to soy protein (340,377,391), when considering other plant-based protein sources, studies have reported no differences between acute MPS in healthy young and older adults following consumption of a plant-based protein (wheat, potato, corn) in comparison to a whey protein (392–395), with results suggesting that the total amount of protein intake is a more important factor to consider than the protein source. There is a magnitude of research reporting differences in the anabolic capacity of different plant-based protein sources, making it important to assess the quality of the specific plant protein source when interpreting and comparing data (167). For example, leucine has been highlighted throughout this thesis as a key AA in stimulating MPS, and when comparing commercially available plant protein isolates, leucine (% of total protein) varied from 5.1% in lupin protein to 13.5% in corn protein, highlighting the large variability between plant-protein sources. Furthermore, several plant-based proteins did not meet the WHO/FAO/UNU AA requirements (170) (oat, 21%; lupin, 21%; wheat, 22%; hemp, 23%; and microalgae, 23%), whereas all animal-based protein sources reached these requirements (whey, 43%; milk, 39%, calcium caseinate, 38%; casein, 34%; egg, 32%).

6.3 The effectiveness of an *ex vivo* human serum C2C12 co-culture model to investigate anabolic responses to physiological status and nutritional stimulation

In vitro models to investigate the cellular responses to different disease states and nutritional status enable investigation of mechanisms which may not be feasible or too complex to investigate *in vivo* (351). The *ex vivo* co-culture model is primarily based on models of parabiosis, with Conboy and colleagues (2005) demonstrating that when sharing

the circulatory system of young and old mice, thereby altering their systemic environment, exposure of older mice to the systemic environment to younger mice led to increases in satellite cell proliferative and regenerative capacity and improvements in notch signalling (396). The opposite effects were observed when exposing younger mice to the systemic environment of older mice (396). Following on from this, the *ex vivo* co-culture model (351) was developed as a method of utilising serum or plasma as a conditioning treatment, exposing cells *in vitro* to an *in vivo* systemic environment, and investigating the resulting cell proliferation and differentiation capacity (397–400), and myotube diameter regulation (359), MPS (352–355), and expression of anabolic and catabolic signalling targets (401,402).

To utilise this *ex vivo* co-culture model throughout this thesis, with a focus on the role of ageing and protein quality on MPS and anabolic signalling target protein expression, serum was obtained from young and older adults fasted and following consumption of a whey and vegan-blend protein (20, 40, 60, 90, 120-mins post protein consumption). Initial experiments reported in Chapter 4 utilised serum samples obtained from younger adults, with individual serum treatments finding no significant time or protein source effects on protein expression of p-Akt, p-mTOR, or MPS stimulation. In the context of investigating the role of protein quality, these results suggest that there are negligible differences in the ability of plant-based protein to stimulate MPS in comparison to animal-based proteins. However, the results also demonstrated no difference between fasted and fed serum treated myotube MPS and anabolic signalling target expression, contrasting with previously published work utilising comparable methodology (355). Further experiments were conducted to investigate the methodological differences between the original set of experiments and those by Carson *et al.,* (2018) reporting significant MPS and anabolic signalling target differences following treatment of fasted compared to fed serum (355). The key identified differences between the methodologies were percentage serum treatment (10% vs 20%) and media glucose

formulation (5mM vs 25mM), with Carson and colleagues utilising a 20% serum treatment and C2C12 myotubes proliferated and differentiated in a high glucose (HG) media. The fact that the experiments reported in Chapter 4 showed negligible differences between fasted and fed conditions, indicates that the treatment conditions do not explain the lack of differences in C2C12 myotube responsiveness (355). Throughout this thesis a low glucose (LG) media concentration (5mM) was used to avoid risk of mimicking a diabetic-like environment, as C2C12 cells have been shown to highly express GLUT 4 and when proliferated and differentiated in a HG environment, a desensitisation of the insulin-dependent signalling pathway has been reported (360,361). When considering the AA concentration of the serum samples used to treat the C2C12 myotubes (reported in Chapter 5), it was unsurprising to see a lack of anabolic response due to the extreme low concentration of AAs with which the myotubes were treated. The serum AA concentrations in these experiments were in fasted and fed states were comparable with those reported by Carson *et al.,* (2018), therefore this was not the determining factor causing discord in the presented results. Analysis revealed that even if myotubes were treated with 100% serum, which would not be feasible as previous studies have reported that above 20%, cells do not remain viable in serum for long periods of time (352,355), these cells would only be treated with ~0.0055mM total AAs. These experiments did not provide a clear explanation as to the null results here in comparison to other studies, but primarily highlighted that in its current form, this *ex vivo* co-culture model is inherently unworkable for investigating differences in fasted and fed human serum. This is due to the extreme low concentrations of AAs in these samples, making C2C12 myotubes unlikely to respond to any serum sample, let alone be able to detect differences in the AA content of fasted compared to fed serum.

Along with the overarching aim of investigating the role of protein quality throughout this thesis, was a secondary aim to investigate how protein quality may play a role in

musculoskeletal health in older adults. Chapter 4 reported the use of human serum obtained from younger adults only, however, previous work has identified different anabolic responses of C2C12 myotubes when conditioned in serum from young and older adults (352). This suggests that despite AA concentrations likely to be too low to result in differences in MPS responses, there may be other differences between human serum obtained from young and older adults contributing to MPS differences. Therefore, a further set of experiments using the *ex vivo* co-culture model were conducted to investigate anabolic responses of C2C12 cells to pooled fasted and fed serum from both younger and older adults following ingestion of a vegan blend and whey protein (reported in Chapter 4). Analysis investigating any time or treatment condition differences found no differences in puromycin or p-mTOR expression, however, a significant treatment condition effect was observed in p-Akt. Multiple comparisons analysis reported several significant differences between young and older adults in p-Akt expression across time points, with limited differences between protein sources in young and older adults. This was confirmed when grouping young and older adult data (eliminating any comparison of protein source), with analysis showing a highly significant difference between p-Akt protein expression between young and older adults. In Chapter 5 limited differences in serum concentrations of AAs were reported between young and older adults, with the majority of differences in concentrations observed between protein sources rather than between age groups. Results of this *ex vivo* co-culture experiment suggest that despite the minimal differences between young and older adults in circulating AA (particularly EAA) concentrations, C2C12 myotubes have an impaired anabolic signalling response when conditioned in older adult serum. With increasing age, skeletal muscle exhibits resistance to anabolic stimuli including protein nutrition, however, C2C12 myotubes do not reflect the intrinsic properties of skeletal muscle, suggesting that these results are due to differences present in the serum itself. Based on the results of this set of experiments, this

differential factor cannot be fully determined, however, it could be postulated to be a result of differences in inflammatory cytokine levels between young and older adult serum. This was not investigated in this study; however, ageing is associated with increases in chronic low-grade inflammation (62), and despite the role of this on postprandial MPS in humans being unclear (403,404), evidence in rodents demonstrates an interaction between low-grade inflammation and age-related anabolic resistance to nutrition (405,406). Furthermore, as previously mentioned, these experiments were based on previously published results from Allen *et al.,* (2021), who did report elevated plasma C-reactive protein and IL-6 in older adults compared to younger adults (352). It must be reiterated that these results were not reflected in puromycin expression nor p-mTOR or p-RPS6, and profound differences have been previously reported when using comparable methodology (355). When considering results here report significant differences in p-Akt with no differences observed in p-mTOR nor p-RPS6, this may be as Akt is upstream of both mTOR and RPS6, suggesting that this may primarily be activating other downstream factors, such as AS160 (associated with GLUT 4 translocation), as opposed to mTOR. The reliability of this model is not yet confirmed, and further optimisation of this model is needed before its use to detect differences between fasted and fed serum conditions is confirmed.

Due to the difficulties in confirming the reliability of the *ex vivo* co-culture model in detecting differences between fasted and fed serum, further work presented in Chapter 5 aimed to investigate the *in vitro* anabolic potential of plant-based compared to animal-based protein sources via a dose-response experiment. This experiment allowed determination of an ‘optimal’ AA dose reflecting the vegan-blend and whey protein sources used in the human trial described in Chapter 4. As previously mentioned, several factors contribute to the quality of a given protein, one of these being the EAA content of the protein, in particular BCAAs leucine, isoleucine, and valine. The results of this experiment demonstrated a clear time

course of MPS stimulation from 30-minutes to 4-hours, with peak MPS observed across all treatment doses (0.5mM – 15mM) at the 1-hour timepoint. This reflects *in vivo* human trials, with MPS rates reported to peak 45-90-minutes postprandial (302). When considering the optimal dose of these AA mixes, 10mM and 15mM doses showed observational differences between protein sources in MPS. Limited trends were observed in expression of anabolic signalling targets p-mTOR, p-Akt, and p-RPS6, despite increases in expression exhibited in comparison to controls. However, a discordance between MPS and mTORC1 signalling has been previously reported following protein ingestion in young men (302). Further experiments utilised a version of the previously discussed *ex vivo* co-culture model used by Allen *et al.,* (2021), which reported that when 'preconditioning' C2C12 myotubes with fasted serum from young and older adults, differences were observed in their ability to respond to a 5mM leucine treatment (352). This model was adapted to investigate protein quality, and identical experiments were conducted using the 'optimal' dose of vegan and whey AA mixes, as determined in the previous dose-response experiments. However, as in Chapter 4, no significant age or protein source differences in MPS, nor in expression of p-mTOR, p-Akt, or p-RPS6 were found. The lack of significant differences reported in this set of experiments are likely due to underpowering. Despite this experiment being repeated at 3 passages, there was large inter-group variation. Furthermore, pooled serum was used. The use of individual serum treatments would have increased the power of these results and allowed for identification of any individual outliers in the results. However, when considering the serum AA concentrations, it is unsurprising that this did not result in significant differences in MPS and anabolic signalling target protein expression when treating myotubes for 4-hours. In conclusion, the presented results in this thesis show that at present, this human serum *ex vivo* co-culture model is not effective in determining anabolic responses to physiological status or nutritional stimulation.

6.4 Translation to older adults in the residential care setting

Much of the research into nutritional status and musculoskeletal health reported throughout this thesis has had a focus on healthy, community-dwelling older adults, who are arguably not the population that require most intervention to prevent the onset and development of musculoskeletal disorders such as sarcopenia and frailty. PEM is a common issue in the residential care setting and can delay recovery from illness, increase risk of disease-related outcomes, and have a negative impact on physical and mental well-being. Chapter 2 discusses both the factors contributing to

PEM and supplementation strategies to overcome PEM in the residential care setting, highlighting the complexity of conducting research in this population. When considering habitual protein intake, analysis in Chapter 3 of this thesis reports no differences between young and older adults, and contrasts previous data suggesting that older adults do not consume the recommended protein intake guidelines as proposed by the PROT-AGE group for older adults (308). When considering older adults in the residential care setting, the PROT-AGE group recommends even higher protein RDAs, with 1.2 – 1.5 $g.kg^{-1}.day^{-1}$ recommended for older adults who have acute or chronic diseases (178), which is common in this setting. However, up to 35% of care home residents are reported to have a daily protein intake markedly below the EAR (117,230). Furthermore, the prevalence of sarcopenia is reported to be up to 73% in long-term care homes and between 22-87% in assisted-living facilities (184), deeming it probable that this high prevalence of sarcopenia is very likely to be due to PEM among older adults residing in residential care. Emerging research suggests that pattern of protein intake may play just as important a role in maximising MPS and musculoskeletal health outcomes as daily protein intake. Previous research has shown that older adults show an uneven distribution of protein intake throughout the day, and in order to

maximally stimulate MPS, 0.4g.kg^{-1}.day^{-1} should be consumed 3 times daily. This provides a rationale for future interventional work in the residential care setting investigating not only total protein intake, but pattern of protein intake, to minimise time spent in a protein deficient state and prevent musculoskeletal health declines in care home residents. Furthermore, future interventional work should seek to explore differences in the anabolic capacity of plant-based versus animal-proteins in residential care settings, and whether there are differences such as the palatability of protein sources which would make either a preferential source of protein in this setting.

Chapter 4 and 5 of this thesis predominantly utilise a novel *ex vivo* co-culture model to investigate the anabolic potential of a vegan-blend and whey protein, and determine any influence age may have on the anabolic properties of these protein sources. Despite previously published work reporting the ability of this model to detect differences in fasted and fed serum conditions (355), and serum conditions between young and older adults (352), negligible significant results were reported to support these. The results suggest that this model needs further optimisation before it can be used to investigate anabolic responses to disease states and nutritional status, with it not being effective in its current form primarily due to extreme low serum AA concentrations. However, once optimised, this model could be utilised to investigate anabolic responses to nutritional stimuli *in vitro*, and explore differences in the 'systemic environment' in older adults residing in residential care to community-dwelling counterparts. In comparison to community-dwelling older adults, those residing in residential care experience greater multi-morbidity and polypharmacy (179), and the use of this novel *ex vivo* co-culture model could provide better understanding of the cellular mechanisms underpinning this. This model has been previously used to study the systemic environments of different disease states, including those living with COPD (407) and liver disease (353), proving its potential use in investigating cellular protein synthesis and

growth. This could provide crucial insight into skeletal muscle metabolism as traditional methods of investigating this, such as through the use of muscle biopsies, are not possible in increasingly frail older adults such as those residing in residential care (350).

6.5 Conclusions

To conclude, this thesis has provided an insight into the role of protein nutrition in skeletal muscle metabolism across the lifespan, with a focus on the role of the anabolic properties of plant-based in comparison to animal-based proteins. This work highlights the clear differences between community-dwelling older adults and those residing in residential care, and the need to develop strategies to increase habitual protein intake in the residential care setting, in which older adults are at increased risk of onset and development of sarcopenia and frailty. Current research suggests that ONS and PFF can be effective in increasing daily protein intake, however, in the residential care setting, this may be better prescribed on an individual basis, as opposed to a 'one size fits all' approach. In addition, the role of protein quality has been explored, with results demonstrating differences in AA kinetics in both younger and older adults in response to ingestion of a vegan-blend and whey protein, suggesting that plant-based proteins may have a lower anabolic potential in comparison to animal-based proteins. Furthermore, the quality of plant- and animal-based proteins in relation to their ability to stimulate MPS in young and older adults was explored via the use of a human serum *ex vivo* co-culture model, with inconclusive results, indicating that this model needs to be further developed. However, once optimised, this is a promising model to investigate the influence of disease state and nutritional status on cellular mechanisms associated with MPS.

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