THE INFLUENCE OF DIETARY PROTEIN INTAKE ON THE RESPONSIVENESS OF SKELETAL MUSCLE TO RESISTANCE EXERCISE TRAINING IN OLDER ADULTS

By

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ABSTRACT

Sarcopenia, the loss of skeletal muscle mass and function, can have serious consequences for health and quality of life. With increasing life expectancy and an ageing population, the problem of sarcopenia as a public health issue continues to grow. Resistance exercise and protein ingestion are two of the main drivers of anabolism, and may potentially be targets for interventions to alleviate the effects of sarcopenia.

In Chapter 2 the existing literature was systematically reviewed to determine whether the effects of resistance exercise training (RET), which is known to improve muscle related outcomes in older adults, can be augmented by the addition of a protein supplement in adults aged ≥70 years. Contrary to previous reviews, evidence from 15 studies (n = 917 participants) indicated no evidence of an effect of supplementation. Chapter 3 examined the effects of timing and distribution of protein intake on older muscle, again using a systematic review. The identification of just six eligible studies (n = 135 participants) indicate a lack of data in this area, although evidence from two studies investigating daily protein distributions suggested a significant effect in favour of a skewed distribution.

There is a saturable dose-response relationship between protein intake and muscle protein synthesis (MPS) which is thought to plateau at a protein dose of 0.4 $g.kg^{-1}$ in older adults. It is suggested that the distribution of daily protein intake may be optimised by considering this threshold; a distribution in which the protein content of each of the three daily meals reaches this threshold would theoretically stimulate greater MPS than other distributions. The aim of Chapter 4 was to analysis habitual dietary protein intake in adults aged ≥70 years relative to this theory. Average daily

protein intake (1.14 $g.kg^{-1}.day^{-1}$) was adequate according to recommendations, however per meal intake data revealed that protein content failed to reach 0.4 $g.kg^{-1}.day^{-1}$ in at least two out of three daily meals for 79% of participants. Dietary protein distribution was identified as an area with potential for improvement, and therefore a target for intervention.

Chapter 5 reports the results of an intervention study, comparing the effects of even and uneven protein distribution diets alongside RET over two weeks, in women aged ≥65 years. Muscle biopsy and saliva samples were taken and participants consumed 150ml deuterated water, to measure myofibrillar muscle protein synthesis (MPS). Twelve participants (mean age 72.7 years) were recruited, and assigned to consume either an even or an uneven protein distribution for two weeks, and both groups completed unilateral RET throughout. There was no significant difference in MPS between the even and uneven diets in the trained leg (1.02 (0.30) $\%.day^{-1}$ vs 1.16 (0.26) $\%.day^{-1}$) or the untrained leg (1.05 (0.24) vs 1.17 (0.29) $\%.day^{-1}$). Knee-extension strength increased by 31 (14) % in the trained leg and 18 (18)% in the untrained leg, with no effect of distribution.

These results do not support the theory of an optimal protein distribution based on the maximal MPS threshold dose. However, it is suggested that there is scope for further research in this area, and a proposed trial design is presented in Chapter 6.

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PUBLICATIONS

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- Chapter 2
- Contributions: conducted literature searches, extracted data, conducted data analysis

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- Chapter 4
- Contributions: conceived the study, collected the data, completed food diary data entry, and completed data analysis

ABBREVIATIONS

1-RM	1-repetition maximum
4EBP	4E-binding protein
AA	amino acid
ADL	activities of daily living
AMDR	Acceptable Macronutrient Distribution Range
ANOVA	analysis of variance
ASMM	appendicular skeletal muscle mass
BCM	body cell mass
BIS	bioelectrical impedance spectroscopy
CSA	cross sectional area
CT	computed tomography
CV	coefficient of variation
D or ^{2}H	deuterium (2H or D)
D_2O	deuterium oxide
DXA	dual energy X-ray absorptiometry
EAA	essential amino acid
eIF4G/A/B	eukaryotic initiation factors 4 G/A/B
EWGSOP	European Working Group on Sarcopenia in Older People
FFM	fat free mass
FM	fat mass
FOPANU	Frail Older People - Activity and Nutrition Study in Umeå
FSR	fractional synthesis rate
$g.kg^{-1}.day^{-1}$	grams of protein per kilogram of body weight per day
IGF-1	insulin-like growth factor 1
LBM	lean body mass

LTM	lean tissue mass
MET	metabolic equivalent
MM	muscle mass
MMSE	Mini Mental State Examination
MPB	muscle protein breakdown
MPE	mole percent excess
MPS	muscle protein synthesis
mTOR	mammalian target of rapamycin
MVC	maximum voluntary contraction
NEAA	non-essential amino acids
NHANES	National Health and Nutrition Examination Survey
PEDro	Physiotherapy Evidence Database
POMA	Performance Oriented Mobility Assessment;
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
p70S6K1	ribosomal protein S6 kinase
RDA	recommended daily allowance
RE	resistance exercise
RET	resistance exercise training
RPS6	ribosomal protein S6
SD	standard deviation
SPPB	Short Physical Performance Battery
TUG	timed up and go
UHB	University Hospitals Birmingham

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1. GENERAL INTRODUCTION

1.1 Sarcopenia

1.1.1 Definition

Sarcopenia is defined as the age-related decline of skeletal muscle mass and function [1], recently recognised as a disease entity by the Centre for Disease Control and Prevention (ICD-10-CM (M62.84)). The rate of loss of muscle mass has been estimated as 0.47% and 0.37% per year in men and women respectively. Loss of muscle strength is more rapid at a rate of 2-4% per year [2].

As well as describing the process of muscle decline, individuals may also be diagnosed as sarcopenic using muscle mass cut-off points. Sarcopenia has previously been defined as having appendicular skeletal muscle mass which is more than two standard deviations below the average of a healthy young reference population [3]. More recently, the European Working Group on Sarcopenia in Older People (EWGSOP) has developed an algorithm to define sarcopenia in adults aged >65 years, which again includes low muscle mass, in combination with low muscle function identified as low gait speed and/or low grip strength [4].

1.1.2 Prevalence

As an ageing condition, the problem of sarcopenia is growing as the population ages. Data from the Office for National Statistics show an increase in the proportion of the UK population aged ≥65 years from 15.9% in 2006 to 18% in 2016 [5]; with the total UK population also growing, this translates into an increase of over 2 million individuals aged ≥65 years in 10 years. Population projections indicate further

increases in the coming years, predicting that the ≥65 age group will make up 24.7% of the population by 2046, and that the ≥85 age group will double in numbers [6].

These changes in population age structure are reflective of increases in life expectancy. There is a discrepancy between life expectancy and healthy life expectancy, with estimates of just 80% and 77% of total lifetime spent in 'good' health for men and women respectively, and changes in health life expectancy failing to match those of life expectancy [7]. Estimates of prevalence of sarcopenia vary by population and according the criteria used to define sarcopenia. Analysis of predicted appendicular skeletal muscle mass relative to younger reference data from the New Mexico Elder Health Survey estimated prevalence to be 13-24% in 50-70 year olds, increasing to more than 50% in the over 80s [3]. UK data from the Hertfordshire Sarcopenia Study (n = 103, mean age 73 years) and the Hertfordshire Cohort Study (n = 1787, mean age 67 years) analysed using the EWGSOP definition reported prevalence between 5-8% [8].

1.1.3 Consequences

Sarcopenia has a range of consequences, both on a personal and societal level. Decline in muscle function impacts upon overall functional ability, which can impair a person's ability to complete activities of daily living such as walking and carrying [9, 10], making it more difficult to maintain physical independence. Hence, sarcopenia increases the likelihood of developing disability in older age; a study of sarcopenia and disability incidence in a study of 5036 men and women aged >65 indicated a 79% greater likelihood of disability at baseline in those with severe sarcopenia compared with normal muscle mass, and 27% greater chance of developing disability over an eight year follow-up [11]. Those with sarcopenia may also an increased risk

of falls, as the likelihood of falls has been shown to be twice as likely in men with sarcopenia [12], and low muscle density and function increase the likelihood of being hospitalised by ,15% and 70% respectively [13]. Furthermore, once hospitalised, a study of infection following hospitalisation has shown twice the risk in patients who were sarcopenic upon admission [14], and hospital stays are typically longer, with one study of 432 patients indicating a mean stay of 13.4 days in sarcopenic patients compared with 9.4 days for those without sarcopenia [15]. It is clear that sarcopenia can have significant effects on daily living and general health, and may greatly decrease quality of life.

The impact on society of this spiral of declining physical function and health takes the form of a significant financial burden, as a result of increased living assistance and healthcare requirements. It was estimated that $18.5 billion of the US healthcare expenditure in 2000 could be attributed to sarcopenia and related conditions, accounting for 1.5% of total expenditure [16]. Hence, sarcopenia is considered a significant public health issue, and the development of interventions to reduce its effects is an important research goal.

1.1.4 Aetiology

The aetiology of sarcopenia is complex and multifactorial. It could be suggested that changes in lifestyle with increasing age, namely the reduction in physical activity, may be responsible for muscle loss. However, observations of performance in Masters weightlifting events indicate a progressive decline in performance with increasing age [17, 18], and data collected from Master weightlifters and untrained controls show a similar decline in both groups; in one study, the power results were comparable between an 85-year-old athlete and a 65-year-old control [19, 20]. These

results indicate a continuing effect of physical activity into older age, however the presence of a performance decline even in these highly active older adults indicate an underlying ageing process.

An overview of the aetiology of sarcopenia is represented in Figure 1.1, which illustrates the decreases in muscle fibre number (hypoplasia) and fibre size (atrophy) as key processes [21]. It has been shown that the greatest difference between younger and older muscle is a loss of motor units as a result of neuropathic process [22, 23]. Electromyography data indicate a decrease in motor units of 40-60% by age 70 [24, 25], which is not attenuated by exercise [26]; some abandoned muscle fibres are re-innervated, increasing the size of the remaining motor units, which may negatively affect fine motor control in older age [27]. The decrease in muscle fibre size which is also observed in older age shows greater atrophy of Type II fibres rather than Type I [28], and, combined with decreased numbers of motor unit and muscle fibres, causes a reduced muscle cross sectional area in older adults, While the causes of this decline are unclear, a number of factors have been implicated in these processes. A mechanism of many ageing process is the presence of chronic low-grade inflammation; a comparison of inflammatory cytokine production in older and younger adults indicated higher levels of inflammation in the older group (mean age 79 years) [29], and these inflammatory cytokines have the potential to interfere with the signalling pathways which lead to anabolism and catabolism [30, 31]. Also implicated are hormonal changes, specifically factors such as serum testosterone and insulin-like growth factor 1 (IGF-1) which are thought to influence body composition in older men [32, 33], and nutritional factors, for example reduced energy intake in older adults presents a higher risk of malnutrition [34] While the exact mechanisms of sarcopenia may not yet be fully understood, developments in

this area have helped to identify various risk factors for sarcopenia. Some of these risk factors are modifiable, and are therefore important as potential targets for intervention in combatting sarcopenia.

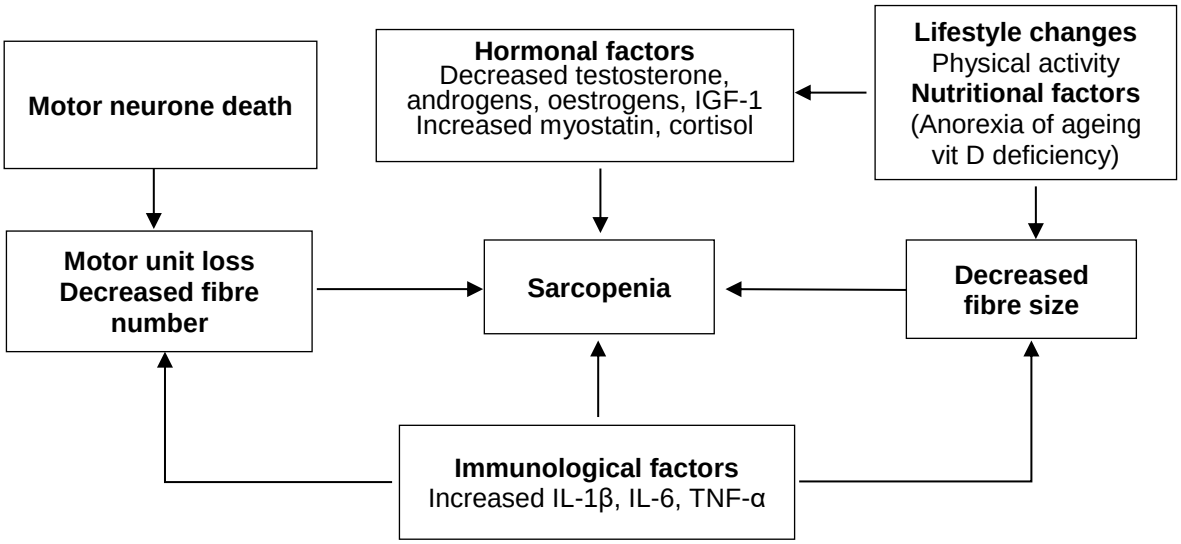


Figure 1.1: Schematic representation of the aetiology of sarcopenia (adapted from Narici and Maffulli [27]).

1.2 Responsiveness of older muscle to anabolic stimuli

1.2.1 Definition

Skeletal muscle proteins are in a constant state of flux, exhibiting a typical turnover rate of 1.2 %.day^{-1} [35]. Muscle protein balance is maintained by a combination of muscle protein synthesis (MPS) and muscle protein breakdown (MPB), which form a dynamic equilibrium. When MPB exceeds MPS, as occurs in the fasted or post-absorptive state, there is an overall net loss of muscle proteins, and the muscle is said to be in a catabolic state. As described in subsequent sections, this balance

shifts in response to stimuli such as feeding and exercise, so that MPS exceeds MPB, resulting in an overall net gain known as an anabolic state. Changes in this balance are responsible for plasticity of the muscle, and are the main drivers of adaptation.

1.2.2 Muscle protein synthetic response to resistance exercise and protein intake in younger adults

Muscle proteins lost to catabolism in the post-absorptive state must be replaced, and this occurs in response to feeding, specifically in response to the ingestion of protein. Following a protein meal there is an increase in amino acid (AA) availability in the blood, which stimulates a transient increase in MPS until approximately two hours after feeding [36]. Another effect of a protein meal is an increase in the production of insulin; this does not influence the rate of MPS, as demonstrated by a study using insulin clamps in healthy young men, which reported no changes in MPS in response to fixed plasma insulin between 5 and 167 mU/l while constant AA availability was maintained [37]. However, this study also measured leg protein breakdown (LPB), and reported an increase in LPB in response to increasing insulin concentration with fixed AA availability up to plasma insulin concentration of 30 mU/l, but no effect of AA dose on MPB when insulin was clamped at 5 mU/l. Hence, protein consumption can cause both increased MPS and reduced MPB. This combination of increased synthesis and decreased breakdown produces the net muscle protein gain seen in the anabolic state, thereby replacing the proteins lost when fasted. This loss-gain cycle between post-absorptive and post-prandial periods is illustrated in Figure 1.2 [38].

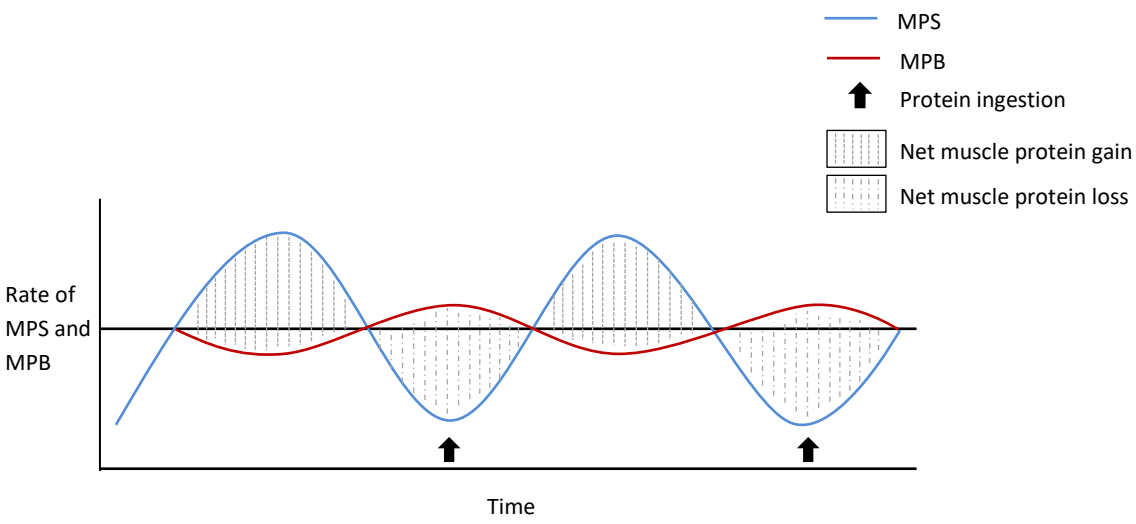


Figure 1.2: Cycle of muscle protein synthesis and breakdown in fed and fasted states [38].

Another stimulus which may increase MPS is activity, in particular resistance exercise (RE). During the period following a bout of RE, MPS has been shown to increase up to threefold compared with baseline [39-41], an effect which may persist for up to 48 hours after exercise. However, Phillips et al. (1997) also measured breakdown in response to RE in the fasted state, and identified an increase of 31% above resting three hours after RE which remained elevated at 18% above resting after 24 hours, although with increases with MPS at these time points, net muscle protein balance was also raised above resting [41]. While these results reflect responsiveness to RE in the fasted state, the combined effects of protein feeding and RE are also important, and shall be discussed in Section 1.5.

A key mechanism implicated in the anabolic response is the mammalian target of rapamycin (mTOR) pathway, specifically the mTORC1 complex. When this pathway is activated in response to an anabolic stimulus, a number of elements of translational machinery, namely initiation and elongation factors, are phosphorylated,

which promotes the translation of messenger RNA (mRNA). For example, initiation of mRNA translation requires the binding of three eukaryotic initiation factors, eIF4E, eIF4G, and eIF4A, which form the eukaryotic initiation factor complex. However, the formation of this complex in prevented by eIF4E binding protein 1 (4E-BP1), which binds to eIF4E, thereby preventing translation initiation. One of the mechanisms by which mTORC1 promotes translation when activated is to bring about the phosphorylation of 4E-BP1, which prevents it from binding to eIF4E and allows the complex of eIFs to form [42, 43]. Another role of mTORC1 is the regulation of elongation during translation; the activity of elongation factor 2 (eEF2) is inhibited when phosphorylated by elongation factor 2 kinase (eEF2k). P70 ribosomal protein S6 kinase 1 (P70S6K1) is activated as part of the mTOR signalling pathway, and can regulate translation elongation by phosphorylating eEF2k which prevents the inhibition of eEF2 [44]

There is evidence in the literature from in vivo studies which does indicate involvement of the mTOR pathway in hypertrophy. Bodine et al. (2001) applied rapamycin to a rodent model; rapamycin is a known inhibitor of mTOR activity, and indeed downstream components of the pathway, such as p70S6K1 phosphorylation, were blocked. Crucially, hypertrophy, as measured by muscle weight and cross-sectional area, did not occur with rapamycin [45]. In humans, Drummond et al. (2009) administered rapamycin to 15 healthy young men prior to exercise and compared MPS and downstream mTORC1 components with a non-rapamycin control group. Again, there were indications that the mTOR pathway had been inhibited as p70S6K phosphorylation increased and eEF2 phosphorylation decreased significantly in the control group only, and while MPS in the control group increased by 40% in the two hours after while there was no change in the rapamycin group [46]. However, in this

particular study, components of the MAPK/ERK pathway were also measured and were unexpectedly blocked by the rapamycin treatment; it is plausible that the blocking of this pathway contributed to the inhibition of MPS, and therefore this study cannot definitively isolate the effects of the mTOR pathway. In a similar study, Dickinson et al. (2011) investigated the effects of 10g essential amino acids (EAA) with and without rapamycin in a crossover design in young adults, and again reported elevated MPS and mTOR signalling in the control group with was blocker in the rapamycin group, although this study did not measure components of other pathways potentially influence by rapamycin [47]. Furthermore, studies of muscle protein synthetic response to anabolic stimuli have also measured components of the mTOR pathway and found similar change profiles [36, 39, 40]. However, Atherton et al. (2010) reported similar changes in MPS and signalling profiles until 90 minutes after exercise, before a decrease in MPS despite the persistence of elevated signalling levels, indicating some disparity between signalling and response [48].

While much of the existing evidence indicating the importance of mTOR in humans involves signalling as an indicator of pathway activity and MPS as the hypertrophic, Phillips et al. (2013) utilised genetic transcript profiles to identify associations between the expression of mTOR sensitive genes and gains in lean mass [49]. Forty-four participants aged 18-78 years completed 20 weeks of resistance exercise training (RET) and changes in lean mass were between -3% and 28%; conversely to much of the previous data, participants who exhibited the greatest gains exhibited suppressed expression of mTOR sensitive genes. It is suggested that baseline levels of rRNA may factor into this response, however the effects of the mTOR pathway and mechanisms of the anabolic response to yet to be fully elucidated.

1.2.3 Anabolic resistance in older age

Given that the balance between muscle protein synthesis and breakdown is responsible for the plasticity of skeletal muscle, it is logical to suggest that age-related muscle decline may be a result of long-term changes to this balance. Firstly, if the basal catabolic state is considered, it was initially thought that basal MPS was lower and MPB higher in older age [50-53]. In a comparison of eight young (<35 years) and eight older (>60 years), Welle et al. (1993) reported 44% lower rate of MPS in the older group [50]; Balagopal et al (1997) measured MPS in 24 individuals between the ages of 20 and 92 years, and results indicated a significant decline between young and middle age (52 years) [53]. However, these results are problematic, as the magnitude of the changes in muscle protein balance would cause the decline in muscle mass with age to exceed that which is actually observed [54]. More recently, Volpi et al. reported no significant difference in basal muscle protein turnover between younger and older men [55], and this result has been corroborated by baseline data in several studies measuring responses to anabolic stimuli [40, 56]. Protein losses in the fasted phase of the of the muscle protein loss-gain cycle do not appear to drive age-related muscle decline. Examination of the other side of this cycle, the anabolic fed state, has yielded different results. As first demonstrated by Dardevet et al. (2000) in muscle from young, adults, and old rats in vitro, the older muscle showed a diminished response to leucine [57]. In humans, Cuthbertson et al. (2005), measured MPS in a group of 20 young and 24 older men (mean ages 28 and 70 years respectively) in response to EAA doses of 0, 5, 10, and 20g, and also 40g for the older group, reporting significantly lower responses in the older men [56], demonstrating a blunting of the anabolic response to protein feeding. Known has anabolic resistance, this age-related blunting has been identified in a number of other

studies [58-61], however several studies by Symons et al., in which older and younger participants consumed equal servings of lean beef, found no such impairment of MPS [62, 63]. One potential explanation for this is the use of a whole food as the protein source rather than isolated EAA; this demonstrates the complexity of the subject of anabolic resistance, and its application to whole foods.

A similar pattern of anabolic resistance has been reported in response to resistance exercise. In a study by Kumar et al. (2009), two groups of 25 young and older men (mean age 24 and 70 years) performed resistance exercise at one of five intensities between 20% and 90% of their 1-repetition maximum (1-RM) for leg extensions and flexions, with the numbers of sets and repetitions set to equalise the volume of work across each intensity, and MPS was measured at 1, 2 and 4 hours after exercise [40]. Results indicated a sigmoidal dose-response relationship between exercise intensity and MPS, with the greatest increase between 40% and 60% 1-RM, and crucially MPS was significantly lower in the older group. Basal MPS was identical in the younger and older groups, the 1-2 hour timepoint represented both the peak MPS for both groups and the only point where the groups were statistically significantly different, and the return to basal values by the 2-4 hour time indicates that this measurement period was sufficient to detect the MPS response to the exercise. These results indicate that responsiveness to resistance exercise is also subject to anabolic resistance. This phenomenon of anabolic resistance has been implicated as a potential cause of sarcopenia. It is suggested that the optimisation of anabolic responsiveness to ‘rescue’ this effect may be achieved using modifiable lifestyle factors, thereby presenting an effective target for intervention.

References to anabolic resistance generally focus on MPS, and as described in the following section, one of the reasons for the dearth of research into the MPB

response is the difficulty of its measurement. There is some evidence of anabolic blunting of MPB in older adults, Wilkes et al. (2009) measured MBP in younger and older adults (mean ages 24.5 and 65.0 years), basally and in response to a plasma insulin concentration of 15 μIU/ml [64]. While there was no significant difference between the groups in terms of basal MPB, in response to insulin, the younger group exhibited suppression of MPB of 47% from baseline, but only 12% in the older group which was not statistically significant. Hence, anabolic resistance appears to affect both elements of muscle protein balance, however it is generally considered the MPS is the predominant driver of changes in muscle mass [65-69], and in terms of anabolic resistance the main focus of this thesis is therefore MPS.

1.2.4 MPS measurement

A vital component of understanding the influence of external factors on MPS is the ability to measure MPS, which is achieved using stable isotope technology. Isotopes are elements which contain the same number of protons and electrons, but differ in the number of neutrons, so share chemical properties but have a different atomic mass. The “heavier” isotopes are usually rarer, for example ^{12}C is the most abundant carbon isotope on Earth, while ^{13}C is a stable isotope which only account for 1.1% of the Earth’s carbon [70]. Stable isotopes are safe for use in humans, as opposed to radioactive isotopes, such as ^{14}C, which are subject to radioactive disintegration.

The basis of MPS measurement is to use these stable isotopes as a tracer, which is introduced to the subject within a precursor, and incorporated into the muscle as new muscle proteins are synthesised. Enrichment of the samples can be measured using isotope ratio mass spectrometry (IRMS), which distinguishes different isotopes based on the difference in atomic mass [71]. Measurement of the precursor/product

labelling ratio provides a basis for the calculation of the fractional synthesis rate (FSR) of muscle proteins.

The most well-established method of measuring MPS involves the provision of a combination of labelled and unlabelled amino acids as the tracer and tracee respectively. Amino acids are provided as either a primed constant infusion or as a flooding dose, and are incorporated into the endogenous supply where they are available for the synthesis of new muscle proteins [71]. One of the more commonly used tracers is leucine labelled with ^{13}C ([1, 2-$^{13}C_2$]leucine), as used in several of the studies described previously in this section; Kumar et al. (2009) supplied a primed continuous tracer infusion, obtained blood samples to measure precursor enrichment and muscle biopsies for product enrichment [40]. In this case, samples were taken before and after a bout of resistance exercise, to provide data for baseline and exercise response; an outline of the experimental methodology is shown in Figure 1.3. Another commonly used amino acid is phenylalanine, which may be labelled using ^{13}C or ^{2}H [72, 73].However, a significant limitation of this technique is the need for continued invasive procedures, in particular cannulation for tracer infusion and repeated blood samples. Hence, studies are restricted to controlled laboratory conditions. Furthermore, this method can only be used for relatively short studies (less than 24 hours).

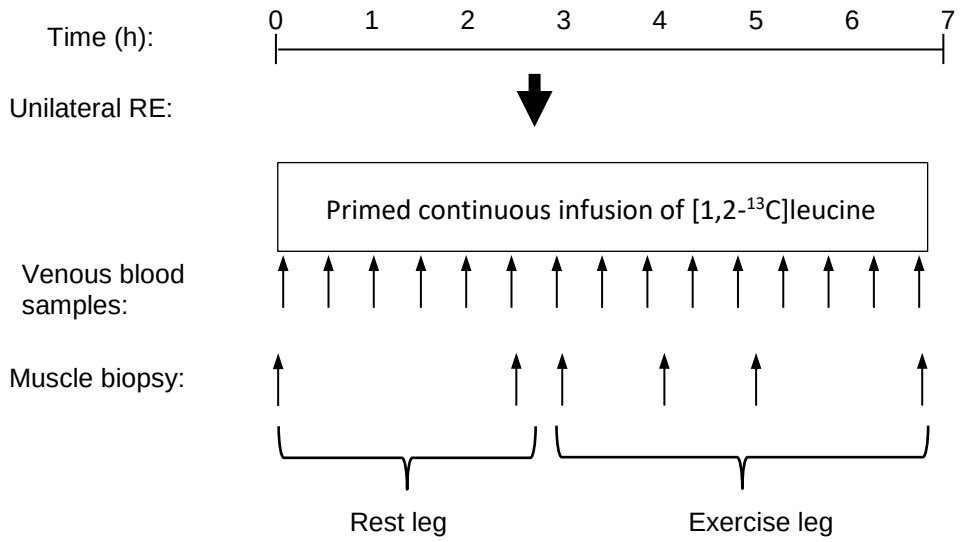


Figure 1.3: Example of amino acid tracer infusion protocol for measurement of MPS [40]; primed infusion of labelled amino acids throughout the protocol, with regular blood samples to measure precursor labelling, and muscle biopsies to measure product labelling and the start of the protocol, and prior to and following a stimulus (i.e. resistance exercise).

An alternative technique has been developed which overcomes these limitations. A stable isotope of hydrogen (H) is deuterium (D or ^{2}H), which has a natural abundance of 0.02% [70]. Deuterium oxide (D_2O), also known as heavy water, was first proposed for use in measurement of protein synthesis in 1941 [74], and the technique has been developed in recent years. The tracer, D_2O, can be ingested orally, and is rapidly incorporated into the body water pool. Via the process of transamination, amino acids such as alanine are labelled, as D atoms equilibrate with H atoms in free amino acids [75, 76]. Significantly, this labelling can occur intracellularly, unlike amino acids tracer methods in which the labelled amino acids must be transported into the cells.

When using stable isotope techniques, MPS is presented as a fractional synthesis rate (FSR), which refers to the rate of precursor incorporation of the project per unit of product. For D_2O measurement of MPS, FSR can be calculated using the following equation [71]:

$$FSR = [(MPE_{Ala})] \times (n \times MPE_{BW} \times t) \times 100$$

MPE_{Ala} and MPE_{BW} (mole percent excess) refer to D labelling of protein-bound alanine and of body water respectively. The former is measured in muscle biopsy samples, and the latter from saliva samples. *n* is the mean number of D molecules incorporated per alanine (3.7 in mammals) and *t* is the time between biopsies.

Given that the tracer can be given orally rather than as an infusion, and precursor incorporation measured from saliva rather than blood samples, this method is significantly less invasive than previous protocols and is therefore more suitable for measurement of MPS in free-living participants. The technique also allows the measurement of MPS over a longer duration not feasible with infusion protocol methods, although this may also be considered a limitation in terms of loss of resolution and sensitivity; measurement over a time course of days rather than hours eliminates the ability to measure the response to specific stimuli.

There are a number of examples in the literature of the implementation of this technique in humans [77-83]. For example, Wilkinson et al. (2014) measured MPS in young men completing an 8-day unilateral RET programme [84]. Participants consumed a 150ml D_2O bolus on Day 0. Muscle biopsies were taken on Days 0, 2, 4, and 8, and saliva was sampled daily; results indicated significantly greater MPS in the exercised legs across the 8-day protocol ($p<.05$). A longer study was undertaken by Brook et al. (2015); participants completed six weeks of unilateral RE, during

which time they consumed a 150ml D_2O bolus on Day 0 with weekly 50ml top-up doses, and provided regular saliva samples [81]. Muscle biopsies were taken on Day 0, and after 3 and 6 weeks. MPS in the trained leg was 1.6 %.day^{-1} between Weeks 0-3, which was significantly greater than the untrained leg (p<.05), however this fell to 1.29 %.day^{-1} in Weeks 3-6 which was the same as the untrained leg. This pattern was also reflected in the mTOR signalling response. Hence, D_2O has been shown to be effective in the measurement of MPS over an intermediate time scale.

Data obtained using these techniques will be referred to throughout the thesis. However, it should be noted that while muscle protein breakdown is also an important component of the anabolic response, it is less frequently used as an outcome measure, as many technical difficulties are associated with its measurement. For example, indirect methods such as the measurement of urinary 3-methlyhistidine (3-MH), a product of protein breakdown, has the benefit of being non-invasive, however requires the assumption that skeletal muscle is the sole source of excreted 3-MH, which doesn't consider smooth and cardiac muscle or metabolism of ingested meat [85]. Measurements involving stable isotope tracers are highly invasive, requiring catheterisation to obtain venous and arterial blood samples as well muscle intracellular samples, and require an assumption of a physiological steady state, which creates difficulties in measuring changes in response to stimuli [86]. Also, the separation of rates of MPS into different subfractions (myofibrillar, sarcoplasmic, etc.) is a useful tool which is more problematic when measuring MPB. Recent advances include the development of a method for MPB measurement using D_2O, firstly in rodents before being applied to humans, in which the rate of disappearance of labelled alanine following deuterium incorporation can be used to measure the breakdown of proteins with a slow turnover rate [87]. This technique

allows greater specificity, in that the breakdown of a particular protein can be selected, and is less invasive so can obtain measurements from free-living participants. However, as with measurement of MPS using D_2O, the resolution of the measurement is such that acute responses to stimuli cannot be measured, and the MPS and MPB techniques cannot be employed simultaneously to allow calculation of muscle protein balance [86]. Hence, advancements in the measurement of MPB continue to develop, but they have not matched those of MPS measurement [35].

1.3 Resistance exercise training

1.3.1 Definition

Section 1.2.2 identifies RE as one of the factors which may stimulate an increase in MPS, and may therefore have a role in alleviating the effects of sarcopenia. However, thus far in this chapter only the acute effects have been described, i.e. the response in the hours immediately after a bout of exercise. To assess its potential as an intervention which may influence chronic muscle loss or gain, the long-term effects of repeated bouts of RE, or resistance exercise training (RET), must be considered.

The relationship between acute anabolic responsiveness and chronic adaption to RET is still unclear; it is suggested that adaptation may occur through the accumulation of mRNA and the encoded protein as a result of repeated increases in anabolic signalling in response to RE. Associations have been found in rodents and humans between activity of elements of the anabolic signalling pathway following an initial bout of RE, such as p70S6K1 phosphorylation [88, 89] and IGF-1 mRNA expression [90, 91], and subsequent muscle hypertrophy after a period of training. [91]. As noted in Section 1.2.2, there may be disparity between changes in signalling

and MPS, therefore these measures of signalling alone are not sufficient to deduce a connection between acute increases in MPS and longer-term hypertrophy. Mitchell et al. (2014) measured MPS at rest and for 1-3 and 3-6 hours after the first session of a 16 week RET programme; while there was evidence of hypertrophy in that quadriceps volume increased by 7.9%, this did not correlate with acute MPS rates following the first exercise session [92](Ref Mitchell). However, these studies only consider anabolic signalling at the start of a training programme, allowing no distinction to be made between initial and subsequent responses to RET, which may be particularly pertinent if participants are previously untrained. Damas et al. (2016) addressed this limitation by considering different phases of a 10-week training programme, by taking measurements at baseline (T1) and weeks 3 (T2) and 10 (T3) [93]. Muscle biopsies were collected 24 hours and immediately before exercise at these timepoints, as well as 24 and 48 hours after, and D_2O techniques were used to measured MPS, while muscle damage was also measured from 0 and 48 hour samples, and fibre cross sectional area (fCSA) was measured as an indicator of hypertrophy. Both muscle damage and post-exercise MPS were highest at T1 but not correlated with fCSA, while MPS was the same at T2 and T3, and both were correlated with hypertrophy. These results indicate different influence of acute MPS on hypertrophy at different points in the training programme, and it is suggested that this may be a result of greater muscle damage after the initial exercise session. While these findings address the issue of immediate and longer-term MPS responses to RET, it could be argued that some elements of the response were lost by the use of D_2O rather than a tracer infusion protocol, as the immediate changes in MPS in the hours after exercise could not be distinguished from the full 24 hours post exercise.

1.3.2 RET as an intervention improve muscle strength and function in older adults

It is widely accepted that RET can lead to improvements in skeletal muscle strength, size, and body composition [94]. As demonstrated by Kumar et al. (2009), resistance exercise is capable of eliciting an increase in MPS from baseline levels of up to 75% in older adults, and, as may be expected, this is reflected in the chronic responsiveness to RET [40]. A systematic review by Liu and Latham (2009), included 121 trials (n = 6700 participants) assessing the effects of RET in adults with a minimum mean age of 60 years [95]. Significant improvements were reported in measures of muscle strength and functional ability in response to RET, with standardised mean differences of 0.84 for lower limb strength (73 trials, n = 3059, “large” effect), and 0.94 chair rise time (11 trials, n = 384, “moderate to large” effect), and a mean difference of 0.08 m/s for gait speed (24 trial, n = 1179, “modest” effect). This result also holds true in the older old; Stewart et al., 2013, identified four studies which implemented physical training in adults aged ≥75 years, reporting improvements in muscle mass of 1.5-15.6% in three trials (with a decrease of 3% in the fourth), a mean difference of 2.31cm^2 for thigh muscle cross-sectional area, and a standardised mean difference of 1.04 for muscle strength from a total of n = 143 participants [96]. This result indicates a greater magnitude of improvement than in the previous systematic review by Liu and Latham, however with only thee trials included in the meta-analysis, results may have been influenced by variability in the trial durations, which ranged from 10 weeks to a year. An additional systematic review indicates that this can also be applied to frail older adults, with significant improvements reported in muscle mass, strength and power, as well as physical capacity and risk of falls [97].

In fact, despite 30 years of research into effective interventions to protect against sarcopenia, RET remains the only intervention which has shown any real impact in terms of improving muscle-related outcomes in older adults.

1.3.3 Blunted chronic responsiveness to RET in older adults

Although the effects of RET on older muscle are well documented, the results are not equivalent to those observed in younger adults. Kumar et al. (2009) demonstrated the phenomenon of anabolic resistance in terms of the acute response to RE, and it has been shown that this translates into blunting of the chronic responsiveness to RET in older adults compared with younger adults [40]. For example, measurements of muscle mass and strength in older and younger women (median ages 26 and 80 years) in response to a 12-week RET programme indicated significantly lower gains in the older group [98]. Analysis of gene expression from muscle biopsies also indicated lesser ability to upregulate anabolic mTOR signalling in response to resistance exercise in the older group. Similarly, Brook et al. (2016) reported blunted hypertrophic responses following six weeks of RET in older adults, and an increase in leg-extension strength of 25% in older men which was significantly lower than the 35% reported in younger adults ($p < .01$) [82]. MPS was also measured using D_2O throughout the study, and was found to increase in the younger participants only, between Weeks 0-3.

Hence, RET can be used as an effective intervention to reduce the effects of sarcopenia, but the improvements do not match those which can be achieved by younger adults. An important research goal is therefore the optimisation of responsiveness to RET to lessen this deficit.

1.4 Protein intake

1.4.1 Acute response

A number of studies have investigated the time course of the anabolic response to protein. During a 6-hour infusion of amino acids in younger adults, Bohe et al. (2001) observed a 30-minute latent period, followed by a rapid increase in MPS which peaked at approximately 2.8 times the basal rate after 2 hours, followed by a rapid decline to basal values [99]. This final decline was in spite of continued AA availability. A similar time course has been reported in the anabolic response to a bolus of whey protein, in a study which also measured phosphorylation of p70S6K1 and 4EBP1 as components of the mTOR signalling system [36]. Again, the decline in MPS back to the basal value was in spite of plasma EAA availability, as well as continued signalling via the mTOR pathway. This limit of EAA utilisation for MPS has been termed the “muscle full” effect, meaning that the anabolic effect of EAA is transient, and in the presence of continued availability muscle will not continue with elevated MPS indefinitely.

The relationship between the size of protein meal and the magnitude of anabolic response is also important. Studies such as that of Cuthbertson et al., (2005) which measured the acute MPS response to a range of EAA doses between 0 and 40g, showed that there is a dose-response relationship between intake and response (Figure 1.4) [56]. This dose-response relationship was also present in older adults, however the authors note a depressed dose response curve which is shifted to the right; this indicates a blunting in the magnitude of the response, and higher doses needed to produce similar levels MPS, all of which is indicative of anabolic blunting [56]. It has also been noted that this dose-response relationship continues only to a threshold protein dose, beyond which additional protein does not elicit any further response [56, 100], and that this threshold dose is higher in older than younger

muscle [56, 61]. Yang et al. (2012) identified the threshold beyond which there was no additional response to be 20g at rest in older adults, based on the responses of older men to 10, 20, and 40g whey protein; this cut-off was based on statistically significant differences in MPS between 0g and 20g but not between 20g and 40g [60]. However, caution should be used when using this threshold to generalise, as it refers specifically to the responses of older men, while there are indications of sexual dimorphism in muscle protein turnover in older age [101]; also, as discussed subsequently in Section 1.4.3, responsiveness may be influenced by the source of protein, which may also affect this threshold. Symons et al. (2009) estimate a threshold of 30g based in the responses of young and older men and women to portions of lean beef containing 30g and 90g protein (equivalent to 10g and 30g EAA). MPS did not differ between doses, however with such a large difference between these doses, further research with a range of dose closer to 30g would be needed to identify a more specific threshold [60, 63]. An alternative analysis pooled data from a number of studies measuring MPS in response to various protein doses to better define the dose-response relationship. Breakpoint analysis was used to identify the plateau threshold, but with protein intake expressed relative to body weight [100]. Again, the threshold was found to be higher in older adults; threshold doses were reported as 0.24 and 0.40 $g.kg^{-1}$ for younger and older men respectively.

Hence, the anabolic response to protein is limited both in duration, i.e., MPS returns to baseline after two hours in spite of continued stimulus, and in magnitude, as increasing the amount of protein does not continue to increase MPS indefinitely.

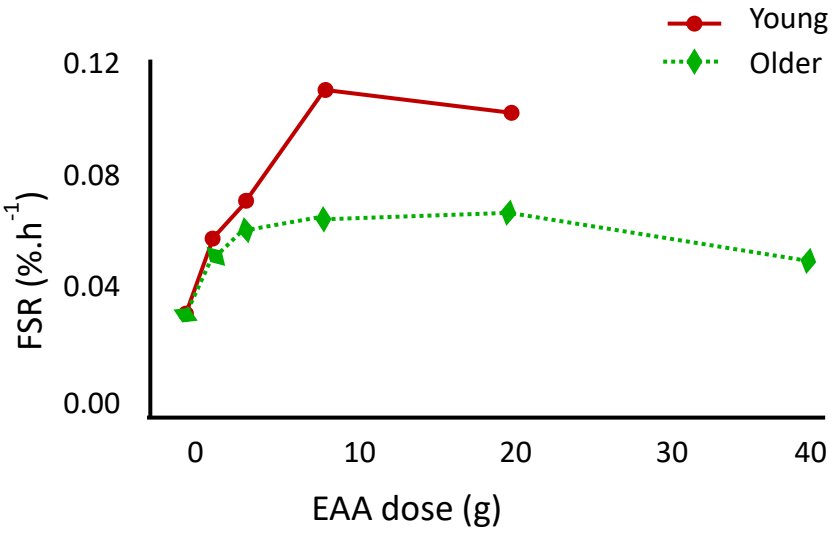


Figure 1.4: The relationship between protein dose and MPS is response is both dose-dependent and saturable, for example Cuthbertson et al. compared responses to a range of EAA doses in older and men, and found the curve to be blunted in older men. [56].

1.4.2 Factors affecting response

Protein is comprised of amino acids (AA), which can be categorised as either essential (EAA) or non-essential (NEAA), and it is thought that it is EAA which are primarily responsible for the anabolic response to protein feeding [55, 102-105]. Tipton et al. reported increased nitrogen uptake in response to 13.4g EAA, indicating that EAA alone are sufficient to stimulate MPS and NEAA are not necessary [103], although not that they were unable to; Smith et al. provided flooding doses of individual AA, and reported indications of MPS with EAA phenylalanine and threonine but not with NEAA glycine or serine, although the EAA arginine also failed to produce a response [102], but again this does not show that a greater response could not be reached with combined EAA and NEAA. In older adults, Volpi et al. (2003) compared the MPS response to an 18g dose of EAA, or the same EAA dose combined with an

additional 22g NEAA [105]. The addition of the NEAA elicited no additional response, which would appear to indicate that they do not have any effect on the anabolic response, however the dose size of the EAA given to both groups should be considered; previously, doses 20g of EAA [56] or protein [106] have been found to be the threshold doses for maximal response, thus with a dose of 18g EAA given to both groups there was little potential for improvement. Hence, it is still generally considered that EAAs are the primary drivers of the anabolic response, however the evidence less than clear cut.

As well as providing substrate for MPS, AA act as a trigger to initiate the MPS process, and leucine specifically is thought to be the most potent initiator. A number of animal studies have shown the ability of leucine to signal the mTOR pathway which is crucial to MPS [107-109]. Atherton et al. (2010) stimulated myocyte cells with each EAA individually and observed the effects of phosphorylation of the mTOR pathway; only leucine increased phosphorylation of mTOR and 4EBP1, and the phosphorylation of p70S6K1 and ribosomal protein S6 (RPS6) increased 5.9-fold and 3.8-fold respectively with leucine, both of which were significantly greater than with other EAA [48]. Recently, MPS was measured in young and older men following a bout of RE and a 10g protein drink supplemented with 4.2g of either leucine or alanine. Greater MPS and p70S6K1 phosphorylation were reported with the leucine drink in both age groups [110]. However, another recent study in post-menopausal women has compared anabolic signalling and MPS in response to either a whey protein drink containing 0.45 $g.kg^{-1}$ FFM of protein including 0.0513 $g.kg^{-1}$ of leucine, the same amount of leucine only, or a control drink during a hyperinsulinaemic-euglycaemic clamp to eliminate the different effects of the drink on insulin production [111]. Contrary to previous evidence described above, both the protein and leucine

drinks stimulated anabolic signalling, but only the protein drink decreased eIF2 phosphorylation by 35% and stimulated a significant increase in MPS of 100%. Hence, again while there does appear to be of particular role of leucine in the anabolic response, the importance of other AA should not be discounted.

1.4.3 Effect of protein quality on response

Protein quality may be described as the ability of an ingested protein to stimulate MPS [112], and is dependent upon the digestibility of the protein, as well as the amino acid profile as indicated in the previous section. Proteins can be referred to as 'fast' or 'slow' depending on how quickly they are digested, and a number of studies have compared the metabolic response of proteins with different digestibility. For example, Dangin et al. (2001) analysed leucine kinetics following one of four protein doses; doses were identical in terms of AA composition but differed in rate of digestion [113]. The 'fast' doses (whey, free AA mimicking casein composition) produced more rapid aminoacidemia and leucine flux than the 'slow' doses (casein, whey divided into 13 smaller doses to imitate a slower digestion), while leucine balance over the 7-hour observation period was higher with the 'slow' doses. Similarly, Koopman et al. (2009) compared the responses in older men to casein and hydrolysed casein, which is more rapidly digested; plasma AA concentrations were significantly greater following the hydrolysed casein ($p < .01$) [114].

The effect of the amino acid profile has been demonstrated by a study comparing responsiveness to ingestion of whey and soy protein in older men, both of which are rapidly digested proteins, but whey has a higher leucine content [106]. Myofibrillar MPS over the four hours after ingestion was significantly greater with whey protein compared to soy at both 20g and 40g doses.

This has implications for dietary protein, as the quality of protein varies depending on its source. There is evidence of higher quality protein content in animal proteins compared with plant-based proteins [106, 115, 116]. Hence, a diet with contains a greater proportion of protein derived from animals may be considered beneficial.

1.4.4 Increased requirements in older adults?

The question of dietary protein requirements of older adults is a controversial one, which shall be referred to in several chapters of the thesis. Just as resistance exercise stimulates an acute anabolic response which translates to responsiveness to RET in chronic muscle related outcomes, it has been suggested that increasing protein intake in older adults may increase anabolism and therefore have similar effects. Hence, increased protein intake could be used to help overcome the issues of sarcopenia.

Currently, the recommended daily allowance (RDA) for protein for older adults does not reflect this premise; the RDA of 0.8-1.2 $g.kg^{-1}.day^{-1}$ is the same as that of younger adults [117]. However, this is based on nitrogen balance studies [118], and it due to the limitations of this method, such as the difficulties in monitoring all possible routes of nitrogen intake and output, the inability to identify redistribution of nitrogen across difference tissues, and the problems with detecting turnover on muscle over shorter durations, it is suggested that this may produce inaccuracies in determining optimal intake in older adults [119]. There is a growing evidence base which indicates the benefits of higher protein intake in older adults, although much of this evidence come from the identification of associations between factors, so is not able to demonstrate a causal link. For example, when protein intake data from the Health Ageing and Body Composition Study were divided into quintiles, there was an

association with loss of lean mass over the three-year follow-up, although. [120]. Less mass was lost with higher protein, with a difference of 40% between the highest and lowest quintiles. Furthermore, Genaro et al. (2015) evaluated dietary protein intake and body composition in 35 sarcopenic and 165 non-sarcopenic women aged over 65 years, and reported dietary protein to be a predictor of muscle mass, with significantly higher muscle mass in those consuming >1.2 $g.kg^{-1}.day^{-1}$ [121]. Consequently, an evidence-based recommendation has been produced which suggests that an increase in the lower end of the recommended intake range to 1.0-1.2 $g.kg^{-1}.day^{-1}$, would be advisable for older adults [119]. However this recommendation remains controversial, as there is also evidence which does not indicate a need for higher protein intake in older adults. The recommendation suggests that older adults' requirements are higher than those of younger adults, however comparisons of nitrogen balance in young (age 21-46 years) and healthy older participants (63-81 years) in response to three 18-day trials, during which they consumed 0.50, 0.75, and 1.00 $g.kg^{-1}.day^{-1}$ protein, indicate the same requirements for both groups [122]. Participants (age 50-80 years) in a study by Iglay et al. (2009) consumed diets containing either 0.9 or 1.2 $g.kg^{-1}.day^{-1}$ for 12 week alongside an RET programme, and there was no effect of diet on body composition, again indicating no benefit of increased protein intake [123]. A recent meta-analysis investigated the effects of protein or AA supplementation in older adults, and from eight trials (n = 557) the analysis reported no significant effect of supplementation on LBM or strength, despite the inclusion of supplemented doses up to 30g whole protein or 15g EAA [124].

The question of actual dietary protein intake in older adults, relative to recommendations, will be addressed in Chapter 4 within a sample of community-dwelling adults aged 70 years and older.

Regarding the question of protein supplementation as an intervention to protect against the effects of sarcopenia, this has been addressed in several systematic reviews. Xu et al. (2015) included 9 randomised controlled trials of participants aged ≥65 years (n = 267 participants), which compared efficacy of protein/EAA supplementation with that of a placebo, ranging from 9 days to 6 months in duration [125]. Results indicated no effect of supplementation on measures of lean body mass and muscle strength. The effects of protein supplements have also been considered in older adults who are at risk of malnutrition; a Cochrane review of 62 studies (n = 10,187 participants) concluded that protein intake did not influence measures of functional status, which included muscle functioning (i.e., strength and power), mobility, and activities of daily living [126].

1.4.5 Effect of protein distribution on responsiveness to protein intake

While there is continued debate over the importance of total protein intake in preventing sarcopenia, there may also be a role for the pattern of protein intake, i.e., protein distribution. Given the transient and saturable nature the of anabolic response to protein described in previous sections, the way in which protein meals are divided into doses may influence the MPS response they elicit, and therefore impact upon other aspects of musculoskeletal health.

Evidence for significant effects of protein distribution on MPS in younger adults is mixed. A study in which participants were provided with 1.0 $g.kg^{-1}.day^{-1}$ egg protein conducted a comparison of this diet divided either into three protein meals consumed

over 24 hours, or to 10 smaller hourly protein meals over the same period, demonstrated greater protein retention with the former [127, 128]. MPS can be similarly influenced; this was demonstrated during a 12-hour recovery period from a bout of resistance exercise, in which young men consumed 80g protein as either 8x10g doses every 1.5 hours, 4x20g every 3 hours, or 2x40g separated by 6 hours [129]. There were significant differences in MPS over the recovery period, with the intermediate distribution, consisting of 4 x 20g doses, eliciting the greatest response. Over a slightly longer period, Mamerow et al. (2014) used a 7-day crossover design to compare the effects of 3 daily protein meals of ~30g each (even), with 3 meals containing 10g, 15g and 65g of protein (skew), using a 24-hour stable isotope tracer infusion protocol on Day 7 of each to measure MPS [130]. They reported 25% greater 24-hour MPS with the even distribution. On the other hand, a 14-day trial of similar distributions in young women identified no difference in protein retention [131]. Mitchell et al. (2015) also reported no effect of protein distribution, when measuring the MPS response in participants fed 15g EAA as either a single bolus or 4 fractions [132]. However, in this case the doses were separated by only 45 minutes, and given the time course of the anabolic response shows a return to baseline after approximately 120 minutes [36, 99], these doses would have been too closely spaced to elicit distinct responses.

With respect to older adults, the evidence base is even more limited, but the results of several cross-sectional studies indicate that the distribution of dietary protein across the day may affect chronic outcomes. In a dietary survey of volunteers aged ≥75 years, protein intake was determined, along with frailty status assessed according to Fried's frailty phenotype, which consists of five criteria including low grip strength and low walking speed [133]. Frailty was not associated with total protein

intake, but with unevenness of intake, i.e., the amount of protein in each meal was more similar in non-frail participants, akin to the ‘even’ distribution used in studies described above [130, 131]. Loenneke et al. (2016) also found an association between per meal protein intake, leg lean mass, and knee-extensor strength in older adults; both outcomes were greater in participants who more frequently consumed meals containing more than 30g protein (i.e., the proposed threshold for maximal MPS) [134].

Experimental data relating to the chronic effects of protein distribution in older adults is relatively scarce. Evaluation of the existing evidence in this area is the subject of a systematic review in Chapter 3, and an intervention study assessing the effects of protein distribution in older adults is reported in Chapter 5.

1.5 Effect of combining RET and protein supplementation on anabolic responsiveness

1.5.1 Acute

As well as the individual influence of resistance exercise and protein supplementation, a combination of the two can have additive effects on muscle protein balance, by the increased stimulation of MPS, and by the suppression MPB with is otherwise elevated following RE [65]. RE sensitises muscle to the effects of protein feeding, enhancing subsequent MPS elevation. Moore et al. (2009) measured MPS from bilateral muscle biopsies in young men in the fasted state, and then 1, 3 and 5 hours after a bout of unilateral RE and ingestion of 25g whey protein [135]. The non-exercised leg showed elevated synthesis of 163% above fasted levels at only the 3-hour timepoint, whereas elevated synthesis was maintained at all time points in the exercised leg at 100, 216 and 229% above baseline at 1, 3 and 5 hours; RE

increased both the duration and magnitude of the MPS response to feeding. This effect has been reported elsewhere [66, 136, 137], and Moore et al. (2011) also reported increased mTOR signalling reflective of the differences in MPS [138]. Furthermore, Burd et al. (2011) determined that sensitisation of the muscle persists for 24 hours after RE [72].

This acute sensitising effect has also been reported in older adults [106, 136, 137]. In fact, Pennings et al. (2011) measured the effect in both older and younger participants (mean ages 74 and 21 years) and reported no age effect on the efficacy of exercise-induced increases in MPS [137]. Drummond et al. (2008) also compared older and younger adults (mean ages 70 and 30 years), and found similar MPS increases in both age groups, although the response was delayed in the older group [136]. A recent systematic review of age-related anabolic resistance included comparisons of young and old MPS in response to exercise, nutrition, and a combination of both, and concluded that the acute MPS response to both was comparable between the age groups [139].

1.5.2 Chronic effects of RET and protein supplementation in younger adults

Given the additive effects of protein and resistance exercise in an acute setting, it may be considered that an RET intervention which also integrates protein supplementation may yield greater results. The evidence suggests this to be the case in younger adults. A systematic review of studies involving at least six weeks of RET combined with a protein supplement in at least one group concluded that supplementation augmented the effects of RET in terms of fat free mass (FFM) and 1-RM leg press strength [140]. A larger, more recent systematic review supports this

conclusion. Morton et al. (2017) reviewed 49 studies (n = 1,863 participants) comparing the effects of RET with and without protein supplementation [141]. Average augmentation of the effects of RET of 9%, 27%, 38% and 14% were reported for 1-RM, FFM, fibre cross sectional area (CSA) and mid-femur CSA respectively. The analysis also included meta-regression to allow for continuous covariates, which included total daily protein intake, and a breakpoint analysis similar to that used by Moore et al. [100] to determine the threshold protein dose for maximal MPS; these results were consistent with a protein intake threshold for changes in FFM, showing no increased gains beyond a total intake of 1.6 $g.kg^{-1}.day^{-1}$.

1.5.3 Older adults

The responsiveness of older muscle differs from younger muscle in a number of ways, including blunted acute anabolic responsiveness, blunting of chronic adaptation, and a higher threshold for maximal MPS. Hence, despite comparable responses to combined exercise and protein when the intensity and dose are sufficient [139], it is plausible that the effects of supplementing RET with additional protein may also differ in older adults. Indeed, the meta-regression analysis in the systematic review of Morton et al. (2017) included age as a covariate, and indicated a reduction in the influence of supplementation with increasing age [141].

While the evidence base is more limited for older adults, the combination of RET and protein has been the subject of several previous systematic reviews [140, 142]. However, the definition of “elderly” used in these reviews may be questioned, and this question forms the basis of a systematic review in a Chapter 2.

1.6 Aims and objectives

Using existing literature, as well as data from current dietary habits of older adults, to the overall aim of this thesis is to identify areas for improved efficacy of protein delivery on outcomes relating to muscle health.

The objectives are:

1. Systematically review the literature to determine the influence of supplementing with protein in older adults (mean age ≥70 years) on the effectiveness of RET

2. Systematically review to evaluate the existing evidence relating to the timing and/or distribution of protein intake in older adults (mean age ≥65 years)

3. Assessment of the current dietary habits of the intervention target population, relative to recommendations for protein intake

4. Conduct an intervention study to determine the influence of manipulating protein distribution on the responsiveness of older muscle to resistance exercise training, in terms of (a) muscle protein synthesis, measured using D_2O tracer techniques; (b) muscle strength; (c) feasibility of the intervention.

2. SYSTEMATIC REVIEW: COMBINING RESISTANCE EXERCISE TRAINING WITH PROTEIN SUPPLEMENTATION IN OLDER ADULTS

Objective: Systematically review the literature to determine whether regular dietary supplementation with protein/EAA during a RET regimen augments the effects of RET on skeletal muscle in older adults.

Methods: A literature search was conducted in August 2015 using MEDLINE, EMBASE, SPORTDiscus and CINAHL Plus to identify all controlled trials using a RET regimen with and without protein/EAA supplementation. Primary outcome was muscle strength, and secondary outcomes were muscle size, functional ability and body composition.

Results: Fifteen studies fulfilled the eligibility criteria, including 917 participants with a mean age of 77.4 years. Studies involving both healthy participants and those described as frail or sarcopenic were included. Overall, results indicated that protein supplementation did not significantly augment the effects of RET on any of the specified outcomes. Exceptions included some measures of muscle strength (three studies) and body composition (two studies). Meta-analyses were conducted but were limited due to methodological differences between studies, and results were inconclusive.

Conclusions: Systematic review and meta-analysis of controlled trials reveal that protein/EAA supplementation does not significantly augment the effects of progressive RET in older adults.

2.1 Background

2.1.1 Rationale

Resistance exercise and protein ingestion have received a great deal of attention as potential components of interventions to protect against sarcopenia, due to their ability to acutely stimulate (MPS). However, as noted in the previous chapter, only resistance exercise training (RET) has demonstrated significant improvements in muscle related outcomes in older adults, and its efficacy is still less than that of younger adults. It has been proposed that a combination of RET and protein supplementation may elicit greater effects than either intervention alone.

As discussed in Chapter 1, resistance exercise and protein ingestion have acute additive effects on the rate of MPS, which has been observed in both younger and older adults. In younger adults this appears to translate into chronic improvements in muscle related outcomes; a systematic review has demonstrated greater improvements in FFM (mean difference 0.69kg) and muscle strength (1-RM leg press, mean difference 13.5kg) with RET and protein supplementation compared with RET alone [140]. However, given that older muscle exhibits a blunted response to both resistance exercise and protein ingestion as individual stimuli, the effectiveness of a combined intervention in older adults requires separate consideration.

This issue has been addressed in two previous systematic reviews, which have reported some additive effects of RET and protein/ EAA supplementation, in terms of fat free mass (FFM) [140, 142] and muscle strength [140]. However, there are issues with these reviews. Both used a relatively young minimum age limit to define ‘older’ adults; one, which included data from adults of all ages, used a cut-off of 50 years for

subgroup analysis of ‘older’ adults, and the oldest participant included was 72 years [140]. In the other, the lower age limit was an average of 60 years and included studies in which some participants were as young as 50 years [142]. These age categories are not necessarily representative of older adults; longitudinal evidence shows that muscle strength and power continue to decline into advanced older age [143], and a dramatic increase in the prevalence of sarcopenia has been observed in the eighth decade of life [3]. Hence, the performance of the muscle of a 50-year-old differs compared to that of someone aged 70 years or even older, meaning that the responsiveness to anabolic stimuli is also likely to differ. The inclusion of such relatively younger participants may therefore mask any difference in the effects of the intervention on truly older adults; an older age limit would be more appropriate. Furthermore, the most important outcomes for older adults in terms of a practical impact of an intervention are those related to functional ability. Such outcomes are highly relevant to quality of life and the maintenance of an independent lifestyle, which are key priorities when setting lifestyle recommendations, but were not addressed within previous systematic reviews.

2.1.2 Aims

The aim of this systematic review was to determine whether protein or EAA supplementation can augment the effects of RET in older adults, i.e., studies with a mean age of 70 years or older. These effects include changes from baseline in muscle strength as the primary outcome, and secondary outcomes of muscle size, body composition and indicators of functional ability, where functional ability was defined as the ability to perform everyday tasks and activities important for the maintenance of physical independence.

2.2 Methods

The systematic review was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) report [144]. Although this protocol has not been previously published, all procedures were determined in advance.

2.2.1 Information sources and search

An electronic search of online databases was conducted in August 2015, using selected key words, 'free text' terms, indexed terms, and Boolean operators. A search strategy was constructed for each database, composed of search terms to identify papers including older participants, involving supplementation with protein or amino acids, a resistance exercise component, and outcomes relating to muscle. A search filter was also applied to limit retrievals to studies in humans. An example search strategy is included in Table 2.1. The search strategies were applied to MEDLINE (1946 to August 2015); EMBASE (1980 to August 2015); CINAHL Plus (1937 to August 2015); SPORTDiscus (1949 to August 2015). Additional studies were identified by recursive searching of the bibliographies of eligible studies and relevant reviews.

2.2.2 Eligibility criteria

Studies were screened for eligibility according to the following inclusion criteria: (i) Controlled trials in humans; (ii) Trials which implemented a progressive RET regimen alongside supplementation with protein or EAA; (iii) Inclusion of a comparison group combining RET with either a placebo/non-protein supplement or no supplement at all. Studies comparing higher versus lower protein diets were accepted providing the low protein diet was equivalent to the US recommended daily allowance (RDA) for

protein (0.8 $g.kg^{-1}.day^{-1}$) [117]; (iv) Studies including participants with a mean age of 70 years or over, both healthy and frail; (v) Studies within any publication category and all languages; (vi) Outcome measures including muscle strength (primary), muscle size, functional ability (defined as the ability to perform everyday tasks and activities important for the maintenance of physical independence) and body composition (secondary).

Studies were excluded if the intervention was administered with an agent previously shown to result in muscle gains (with the exception of vitamins and minerals). Studies involving a specific patient group, or with the aim of treating a clinical condition other than frailty or sarcopenia, were also excluded.

Table 2.1 Example search strategy*

1	Aged/ or "aged, 80 and over"/ or frail elderly/
2	Aging/ or longevity/
3	(old* adj (adult* or age* or people or person* or population*)).tw.
4	(elder* or old* or ?enarian or aged or ag?ing or senior* or geriatric* or frail).mp.
5	1 or 2 or 3 or 4
6	Muscles/ or muscle, skeletal/
7	Exp Muscle Strength/
8	Muscle Weakness/
9	Muscular atrophy/ or sarcopenia/
10	(musc* adj2 (mass or strength or size or cross sectional area or CSA or thick* or power or growth or enlarge* or area or volume or hypertrophy)).tw.
11	Muscle Development/
12	Exercise therapy/ or resistance training/
13	(weigh* OR streng* OR resis*) adj2 (train* OR exerc* OR therap*).mp.
14	Hypertrophy/
15	6 or 7 or 8 or 9 or 10 or 11 or 12 or 13 or 14
16	Exp Dietary Supplements/
17	Food, Fortified/
18	((protein* OR amino acid*) adj3 supplement*).tw.
19	Proteins/
20	Exp Amino Acids/
21	Exp Dietary Proteins/
22	16 or 17 or 18 or 19 or 20 or 21
23	5 and 15 and 22
24	Exp animals/ not humans.sh
25	23 not 24

*Ovid MEDLINE (R) search, adapted for other databases

2.2.3 Study selection

Titles were screened for relevance by one reviewer (DKC) and irrelevant titles removed, following which abstracts were screened by the same reviewer. For the remaining articles, full-texts were obtained via a combination of online databases and direct contact with the authors, and these were evaluated to determine whether they met the inclusion criteria. Two reviewers (DKC and CAG) independently assessed full-texts for eligibility, with a third reviewer (DHS) moderating if necessary. Studies deemed eligible were included in the systematic review.

2.2.4 Data extraction

Data were extracted from each of the included papers using a standardised data extraction form, including the following details of interest;

(i) Participants: number (total and per group), age, gender, frailty/sarcopenic status, baseline protein intake

(ii) RET regimen: duration, number of weekly sessions, exercise type, exercise intensity

(iii) Protein supplementation: protein type, frequency (daily or with training), dose, timing, control treatment, addition of vitamin D

(iv) Outcome measures: for measures of muscle strength, muscle size, functional ability and body composition; where necessary, the required data were interpolated from figures or calculated from the reported data.

Corresponding authors were contacted if this information could not be obtained from the paper, and if data could not be obtained, the study (or outcome measure) was excluded from meta-analysis.

2.2.5 Summary measures and synthesis of results

Extracted data were collated and a review was conducted for the primary outcome, i.e., muscle strength, as well as the secondary outcomes of muscle size, functional ability and body composition. This included description of studies and tabulation of data (presented as mean (SD) unless stated otherwise).

Meta-analysis was conducted on comparable outcomes reported in a minimum of two studies. Studies were required to be sufficiently similar to each other in certain aspects of study design in order to be included in the same meta-analysis; despite all included studies addressing the questions posed by this review, fundamental differences in their protocols meant that a number of study combinations were unsuitable for meta-analysis. Key criteria for determining study similarity included the frequency of protein supplementation (i.e. daily or only on training days), the timing of supplementation (including number of doses), the amount of protein supplemented, and additional supplementation with vitamin D (which may also influence muscle related outcomes [145], with consideration also given to the type of protein supplemented and the duration of the study. Where meta-analysis was appropriate, effect sizes were calculated (mean differences) with 95% confidence intervals using random effects models; forest plots were generated. All calculations were performed using RevMan Version 5.2. Risk of publication bias was not assessed using a funnel plot when fewer than ten studies were included in each meta-analysis.

Methodological quality of included studies was assessed using the Physiotherapy Evidence Database (PEDro) scale [146]. A score of six or higher indicated moderate to high quality.

2.3 Results

2.3.1 Study selection

A total of 11770 articles were identified by the literature search, of which 16 articles including 15 studies met all of the inclusion criteria and were included in the systematic review [147-162] (Figure 2.1). Two articles [150, 153] reported results from the Frail Older People - Activity and Nutrition Study in Umeå (FOPANU), and therefore were considered here as one study [150].

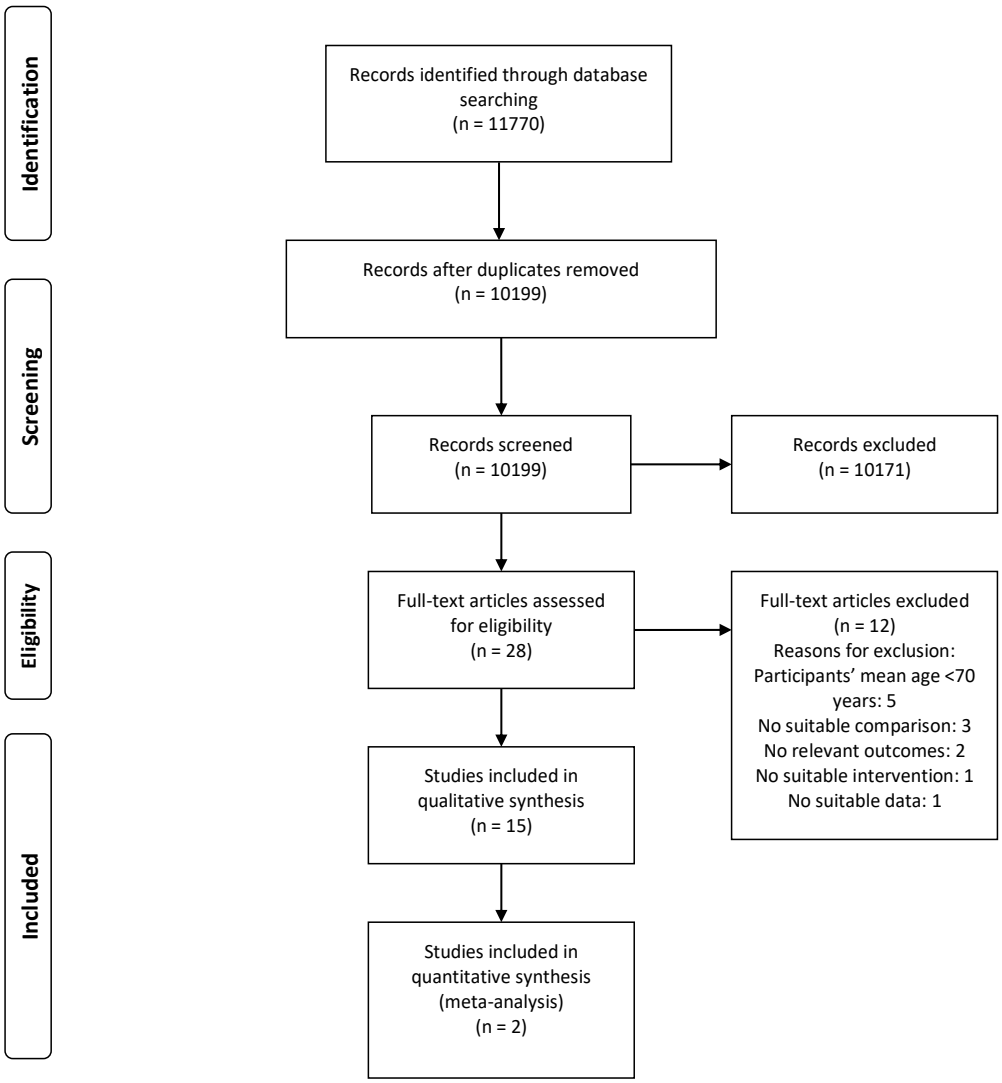


Figure 2.1 Flow of studies through the review process and reasons for exclusion at full text level

2.3.2 Participant characteristics

The 15 eligible studies included 917 participants with a mean age of 77.4 years (range 60-100 years) (Table 2.2). Six studies including 400 participants were conducted in older adults described as frail, sarcopenic, or mobility limited [147, 150, 152, 154, 155, 157]. Individuals in the remaining nine studies were categorized as healthy [148, 149, 151, 156, 159-163]. Three studies included only male populations [148, 151, 161], two included female only populations [154, 159], and the remaining 10 were mixed populations [147, 149, 150, 152, 155-158, 160, 162]. Of the total participants, 32% were male and 68% were female. All studies were published in English.

2.3.3 Interventions

Resistance exercise regimens varied in frequency from two to five occasions per week, with a mean (SD) of 3 (1) per week. Programs lasted between seven weeks and one year, with a mean (SD) of 18 (11) weeks. All studies reported a progressive exercise regimen; six involved training of the lower limbs alone [147, 148, 150-152, 162], and the remaining nine comprised both upper and lower limb training [149, 154-161]. In addition to resistance exercise, participants of five studies undertook co-interventions including functional and/or balance exercises [150, 152, 154, 159, 162] (Table 2.2).

Table 2.2 Study characteristics[1]

	Participant details				Training details				Protein/EAA and placebo supplement details				
Author, year	**N**	**Mean age**	**Frail/mobility limited/ sarcopenic**	**Baseline protein intake ($g.kg^{-1}.day^{-1}$)†**	**Study length and RT frequency**	**Type of RT**	**RT intensity**	**Functional/ balance training**	**Type of protein**	**Frequency (daily/with training)**	**Timing of ingestion**	**Amount**	**Control treatment**
Fiatarone et al, 1994 (122)	50	86.7	Y	NA	3 d/wk x 10 wk	LL	80% 1-RM	N	Soy-based	Daily	Evening	15g approx.	Water
Godard et al, 2002 (123)	17	71.5	N	1.14	3 d/wk x 12 wk	LL	80% 1-RM	N	EAA (1.86g L-lysine, 2.24g L-leucine, 1.40g L-valine, 1.86g L-phenylalanine, 1.76g L-threonine, 1.30g L-histidine, 1.2g L-isoleucine, 0.38g L-methionine)	Daily	After training	12g	Exercise only
Bunout et al, 2004 (124)	47	74.1	N	NA	2 d/wk x 1 year	LL + UL	Light	N	Undisclosed protein	Daily	Between meals	15g	Exercise only
Rosendahl et al, 2006 (125)[2]	91	85.2	Y	NA	5 d/2wk x 13 wk	LL	8-12 RM	Y	Milk-based	With training	After training	7.4g	CHO
Verdijk et al, 2009 (126)	28	72.0	N	1.10	3 d/wk x 12 wk	LL	60-80% 1-RM	N	Casein	With training	10g before, 10g after training	20g	Water
Zak et al, 2009 (127)	40	78.7	Y	NA	5 d/wk x 7 wk	LL	80% 1-RM	Y	Undisclosed protein	With training	Before training	12g approx.	Water
Kim et al, 2012 (129)	77	79.2	Y	NA	2 d/wk x 3 months	LL + UL	Moderate	Y	EAA	Daily	Twice daily	6g	Exercise only

Tieland et al, 2012 (130)	62	78.5	Y	1.00	2 d/wk x 24 wk	LL + UL	50%-75% 1-RM	N	Milk-based	Daily	15g after breakfast, 15g after lunch	30g	CHO
Arnarson et al, 2013 (131)	161	73.9	N	1.00	3 d/wk x 12 wk	LL + UL	75-80% 1-RM	N	Whey	With training	After training	20g	CHO
Chalé et al, 2013 (132)	80	77.7	Y	0.97	3 d/wk x 6 months	LL + UL	80% 1-RM	N	Whey	Daily	20g after breakfast, 20g after dinner	40g	CHO
Leenders et al, 2013 (133)	60	70.0	N	1.15	3 d/wk x 24 wk	LL + UL	60-80% 1-RM	N	Milk-based (80% Casein, 20% Whey)	Daily	After breakfast	15g	CHO
Daly et al., 2014 (134)	100	72.8	N	1.08	2 d/week x 4 months	LL + UL	Moderate	Y	Red meat	6 d/week	Meals, after training	45g[3]	CHO
Franzke et al., 2015 (135)	64	82.7	N	NA	2 d/wk x 6 months	LL + UL	Light to heavy	N	Whey	Daily	Morning and after training	20.7g	Exercise only
Mitchell et al., 2015 (136)	16	74.4	N	NA	3 d/wk x 12 weeks	LL + UL	75-85% 1-RM	N	Chocolate milk	Daily	After breakfast or after training	14g	Placebo drink
Trabal et al., 2015 (137)	24	84.5	N	1.20	3 d/wk x 12 weeks	LL	65% 1-RM	Y	Leucine	Daily	5g after lunch, 5g after dinner	10g	CHO

[1]1-RM, 1-repetition maximum; CHO, carbohydrate; EAA, essential amino acid; LL, lower limb; N, no; RT, resistance training; UL, upper limb; Y, yes. †NA indicates studies did not report baseline protein intake. [2]Rosendahl et al. 2006 (26) includes Carlsson et al. 2011 (29). [3]Approximately 220g (raw weight) or 160g (cooked weight) lean red meat equated to 45g protein

A total of 10 studies included daily protein supplementation [147-149, 154, 155, 157, 158, 160-162], one included supplementation on six days per week [159], and in the remaining four, participants received supplements only on the day of training [150-152]. Baseline daily protein intake was reported in eight studies [148, 151, 155-159] (Table 2.2), giving a mean (SD) of 1.08 (0.07) $g.kg^{-1}.d^{-1}$ (range 0.97-1.24 $g.kg^{-1}.d^{-1}$). The amount of protein supplemented varied from 6g per day to 45g per day with a mean (SD) of 19 (11) g (Table 2.2). Eight supplemented groups [150, 151, 155-158, 160, 161] received protein derived from milk (casein, whey, chocolate milk), one group received soy-based protein [147], one group was supplemented with lean red meat [159], two studies did not disclose the nature of the protein supplement [149, 152], two groups received EAA [148, 154] and one group received only leucine [162]. Timing of ingestion was inconsistent; in three studies the supplement was administered immediately after training [148, 150, 156]; in one study administration was immediately before training [152]; in one study half of the supplement was administered before training and half after [151]; eight studies administered supplements at a consistent time relative to meals [147, 149, 154, 155, 157-159, 162]; the two remaining studies used a combination of supplementation after meals and after training [160, 161]. In addition to protein, six studies also supplemented participants with vitamin D [147, 149, 152, 159-161], with reported doses ranging from 2-25 μg, and two doses given as approximate proportions of recommendations.

The studies were highly variable in terms of both study characteristics and outcome measures. As a consequence only two studies [155, 157] were sufficiently similar to be included in a meta-analysis according to the requirements detailed in section 2.2.5.

2.3.4 Study quality

The median overall quality score derived using the PEDro scale was 7/10 (range 4-10) and the median score for internal validity was 5/8 (range 2-8) (Table 2.3). All studies scored 2/2 for statistical reporting.

Table 2.3 PEDro scale for assessment of study quality[1]

Author, year	Fiatarone et al, 1994 (122)	Godard et al, 2002 (123)	Bunout et al, 2004 (124)	Rosendahl et al, 2006 (125)[2]	Verdijk et al, 2009 (126)	Zak et al, 2009 (127)	Kim et al, 2012 (129)	Tieland et al, 2012 (130)	Arnarson et al, 2013 (131)	Chale et al, 2013 (132)	Leenders et al, 2013 (133)	Daly et al., 2014 (134)	Franzke et al., 2015 (135)	Mitchell et al., 2015 (136)	Trabal et al., 2015 (137)
1. Eligibility criteria were specified	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
2. Subjects were randomly allocated to groups	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
3. Allocation was concealed	N	N	N	Y	N	N	Y	Y	Y	Y	Y	Y	N	N	N
4. The groups were similar at baseline regarding the most important prognostic indicators	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	Y	Y	Y
5. There was blinding of all subjects	N	N	N	N	Y	N	N	Y	Y	Y	Y	N	N	Y	Y
6. There was blinding of all therapists who administered the therapy	N	N	N	N	N	Y	N	Y	Y	Y	Y	N	Y	N	Y
7. There was blinding of all assessors who measured at least one key outcome	Y	N	N	Y	Y	Y	Y	N	Y	Y	Y	N	Y	N	N
8. Measures of at least one key outcome were obtained from more than 85% of the subjects	Y	N	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	N

initially allocated to groups															
9. All subjects for whom outcomes were available received the treatment or control condition as allocated or, where this was not the case, data for at least one key outcome was analysed by "intention to treat"	**Y**	**N**	**N**	**Y**	**Y**	**N**	**N**	**Y**	**Y**	**Y**	**N**	**Y**	**N**	**N**	**N**
Internal Validity	**5**	**2**	**2**	**6**	**6**	**5**	**5**	**7**	**8**	**8**	**6**	**5**	**4**	**4**	**4**
10. Results of between-group statistical comparisons are reported for at least one key outcome	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**
11. The study provides both point measures of variability for at least one key outcome	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**
Statistical Reporting	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**
Score/10 (Criterion 1 is not used to calculate PEDro score)	**7**	**4**	**4**	**8**	**8**	**7**	**7**	**9**	**10**	**10**	**8**	**7**	**6**	**6**	**6**

[1]N, criterion satisfied; Y, criterion not satisfied. [2]Rosendahl et al. 2006 [125] includes Carlsson et al. 2011 [128].

2.3.5 Effect of intervention on outcome measures

2.3.5.1 Muscle strength

All 15 studies included in the systematic review included a measurement of muscle strength, although a number of different muscle groups were studied (Table 2.4). Eleven out of 15 studies demonstrated significant improvements from baseline in every measure of muscle strength in all groups undertaking RET with protein/ EAA supplementation. Of the remaining studies, one demonstrated significant improvements in six of the eight strength measurements included [152], two reported significant increases in all measurements except handgrip strength [155, 158], and one measured only handgrip strength and reported no change [160]. Three of the 15 studies reported significant differences between control and supplemented groups, with greater improvements in the supplemented groups for measures of knee-extension strength [154, 159] and hand grip strength [149], and one study reported a trend for greater improvement in leg flexion strength [162]. A meta-analysis of data obtained from 130 participants across the two comparable studies measuring leg press strength showed no statistically significant difference in leg press strength between groups (mean difference: 4.9 kg; 95% CI: -10.8, 20.6; P = .54) (Figure 2.2a).

2.3.5.2 Muscle size

Eight studies investigated the effect of supplementation on muscle size (Table 2.4). Six studies measured thigh muscle cross sectional area (CSA) using computed tomography (CT), another measured mid-arm, calf and hip circumference and the other measured mid upper arm muscle area, triceps skinfold and calf circumference. All but one of the studies which used CT to measure CSA reported significant increases in both supplemented and non-supplement groups, however there were no

significant differences between the groups. No changes were reported in any other measure of muscle size. No measures of muscle size were suitable for meta-analysis.

Table 2.4 Summary of outcome measures and significant results[1]

Author, year	Outcome measures		Significant protein effect
Fiatarone et al., 1994 (122)	**Muscle strength** **Muscle size** **Functional ability**	1-RM leg strength (sum of L/R knee and hip extensors) Thigh muscle CSA Self-paced gait velocity	NS
Godard et al., 2002 (123)	**Muscle strength** **Muscle size**	Knee-extensor isometric and isokinetic MVC; 1-RM bilateral knee-extension Right thigh whole muscle CSA	NS
Bunout et al., 2004 (124)	**Muscle strength** **Muscle size** **Functional ability** **Body composition**	L/R bicep isometric strength; L/R knee-extensor isometric strength; L/R handgrip strength Mid arm, hip and calf circumference 12-minute walk capacity FFM; FM	RH grip strength (P = .031)
Rosendahl et al., 2006 (125)[2]	**Muscle strength** **Functional ability**	1-RM leg press Balance test; self-paced and maximum gait velocity; chair-stand test	NS
Verdijk et al., 2009 (126)	**Muscle strength** **Muscle size** **Body composition**	1-RM leg press; 1-RM leg-extension Quadriceps muscle CSA LBM; FM; % FM; leg LTM; leg % FM	NS
Zak et al., 2009 (127)	**Muscle strength** **Functional ability**	L/R 1-RM knee-extension; L/R 1-RM knee flexion; L/R 1-RM hip extension; L/R 1-RM hip knee flexion 6-minute walk capacity; POMA	NS
Carlsson et al., 2011 (128)[2]	**Body composition**	MM (intra cellular water proxy)	NS
Kim et al., 2012 (128)	**Muscle strength** **Functional ability** **Body composition**	Knee-extension Self-paced and maximum gait velocity Total MM; appendicular MM; leg MM	Knee-extension strength (P = .01)

Tieland et al., 2012 (130)	**Muscle strength** **Functional ability** **Body composition**	1-RM leg press; 1-RM leg-extension; handgrip strength SPPB LBM; FM; appendicular LTM	LBM (P = .006); appendicular LTM (P < .001); FM (P = .001)
Arnarson et al., 2013 (131)	**Muscle strength** **Functional ability** **Body composition**	Knee-extensor isometric MVC Timed up-and-go; 6-minute walk capacity LBM; appendicular LTM	NS
Chalé et al., 2013 (132)	**Muscle strength** **Muscle size** **Functional ability** **Body composition**	1-RM leg press; L/R 1-RM knee-extension Total muscle CSA of non-dominant thigh SPPB; stair climb speed; 10x chair rise time; gait velocity LBM; FM	NS
Leenders et al., 2013 (133)	**Muscle strength** **Muscle size** **Functional ability** **Body composition**	1-RM leg press; 1-RM leg-extension; handgrip strength Quadriceps muscle CSA 5x chair rise time LBM; FM; % FM; leg LTM; leg FM	NS
Daly et al., 2014 (134)	**Muscle strength** **Muscle size** **Functional ability** **Body composition**	1-RM leg-extension Femur muscle CSA 4-square step test; timed up-and-go; 30-s chair rise test Total body FM; body fat percentage; LBM; arm LTM, leg LTM	Leg extension strength (P = .010); LBM (P = .007); leg LTM (P < .05)
Franzke et al., 2015 (135)	**Muscle strength** **Functional ability**	Handgrip strength 6-minute walk capacity; 30-s chair rise test	NS
Mitchell et al., 2015 (136)	**Muscle strength**	1-RM leg press; 1-RM leg-extension; 1-RM chest press; Knee-extension isometric MVC	NS
Trabal et al., 2015 (137)	**Muscle strength** **Muscle size** **Functional ability**	Leg flexion overcoming isometric strength Mid upper arm muscle area; triceps skinfold; calf circumference Balance test; TUG; 5x chair rise time; 4m walk time	Leg flexion (P = .056)

[1]NS indicates no significant differences between the RET and protein supplement and RET only groups. For significant results, the outcome measure and *P*-value, where available, are reported. 1-RM, 1-repetition maximum; CSA, cross-sectional area; FFM, fat free mass; FM, fat mass; L/R, right and left; LBM, lean body mass; LTM, lean tissue mass; MM, muscle mass; MVC, maximum voluntary contraction; RH, right hand; POMA, Performance Oriented Mobility Assessment; SPPB, Short Physical Performance Battery. [2]Rosendahl et al. 2006 [125] and Carlsson et al. 2011 [128] report results from the same study, presented separately as report different outcome measures.

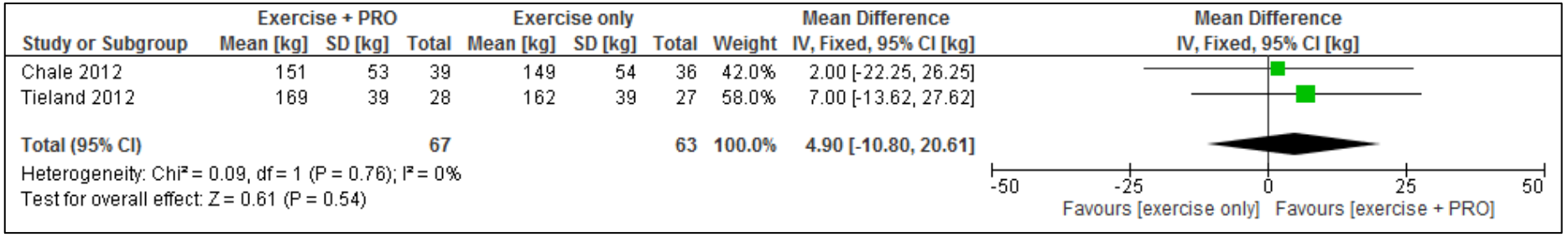
2.4.5.3 Functional ability

At least one functional ability outcome was assessed in 12 out of the 15 included studies, with 27 outcomes assessed in total (Table 2.4). There was much heterogeneity among outcome measures, which included chair rise ability (six studies), gait velocity (five studies), walking capacity (four studies), the Timed Up and Go (TUG) test (three studies), balance tests (two studies), stair climb speed (one study) and the 4-square step test (one study). Three studies included a 'composite' of some of these physical performance indicators; two used the Short Physical Performance Battery (SPPB) which combines balance, gait speed and chair rise ability [164], and one used Tinetti's Performance Oriented Mobility Assessment (POMA) test combining balance and gait [165]. Of the 27 functional ability measurements, 21 were significantly improved after the intervention period, although none of these improvements were significantly different with protein/ EAA supplementation compared with non-supplemented controls. A meta-analysis of SPPB data from two studies indicated no difference between groups (mean difference: 0.3; 95% CI: -0.3, 0.9; P = .36) (Figure 2.2b).

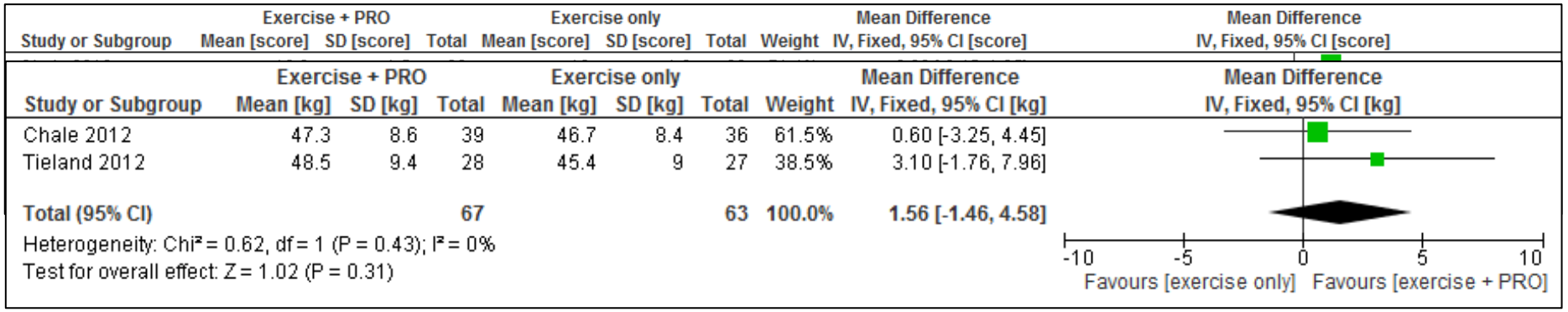
2.4.5.3 Body composition

Body composition was assessed in nine studies (Table 2.4). Body composition was assessed using dual energy X-ray absorptiometry (DXA) in all studies except two; one of which used a segmental multifrequency bioelectrical impedance analysis (BIA) technique, and the other used bioelectrical impedance spectroscopy (BIS). Measurements included total lean body mass (LBM) in six studies; total body fat mass (FM) in six studies; percentage FM in three studies; estimated total muscle mass (MM) in two studies; fat free mass (FFM) in one study. A number of studies also included regional measurements of body composition: leg lean tissue mass

(LTM) in three studies; appendicular LTM in two studies; estimated appendicular MM in one study; estimated leg MM in one study; leg FM and percentage FM in one study; arm LTM in one study. Of the 28 measurements of body composition, six demonstrated no significant change during the studies. Improvement in body composition with no difference between treatment groups was shown in 17 measurements, although within-group analysis of two of these measurements within one study indicated a significant decrease in total FM and body fat percentage in the protein supplemented group but not the control group [159]. Five measurements from two studies indicated significant differences between groups [155, 159], with greater increases in LBM, leg LTM, appendicular LTM and FM in the supplemented groups compared to the exercise-only controls. Meta-analyses of two studies indicated no difference between groups for measures of LBM (mean difference: 1.6 kg; 95% CI: -1.46, 4.6; P = .31) or FM (mean difference: 0.2 kg; 95% CI: -2.3, 2.9; P = .86) (Figure 2.2c and 2.2d).

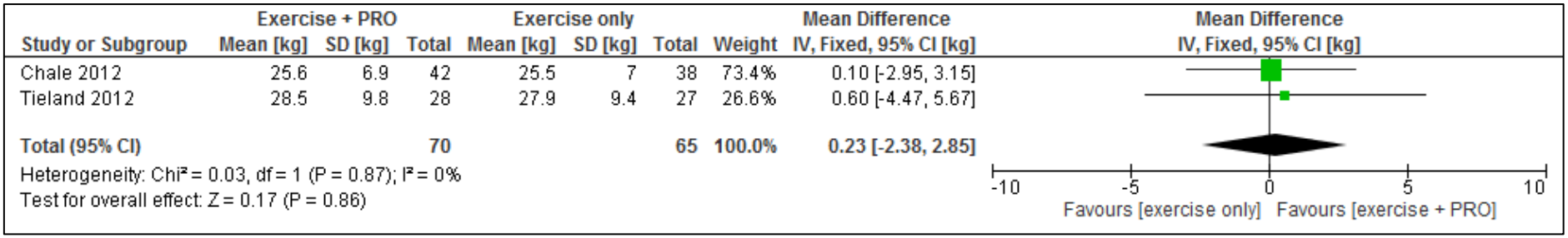


(a) leg press strength (mean difference: 4.9 kg; 95% CI: -10.8, 20.6; *p* = .54)



(b) SPPB (mean difference: 0.3; 95% CI: -0.3, 0.9; *p* = .36)

(c) LBM (mean difference: 1.6 kg; 95% CI: -1.46, 4.6; *p* = .31)



(d) FM (mean difference: 0.2 kg; 95% CI: -2.3, 2.9; *p* = .86).

Figure 2.2 Forest plot of random effects meta-analysis on (a) leg press strength; (b) SPPB; (c) LBM; (d) FM. Mean difference between intervention and control groups is represented by the shaded squares, with horizontal lines indicating 95% confidence intervals. The size of each square is indicative of the weight given to that study in the meta-analysis. The pooled mean difference is represented by the diamond.

2.4 Discussion

This systematic review presents evidence from 15 studies investigating the additive effects of RET and protein supplementation on skeletal muscle strength and size, body composition and functional ability in older adults. Studies reported overall improvement from baseline for the majority of outcomes, indicating a positive effect of RET. However, across the 15 studies these improvements were not significantly different in groups receiving protein/ EAA supplements and undergoing RET compared with RET alone, indicating no additional effects of supplementing with protein. Although limited, the meta-analyses of comparable outcomes found no statistically significant differences, supporting this conclusion.

A previous systematic review has shown that older muscle demonstrates an adaptive response to RET across a range of outcomes [166], hence RET alone is considered an effective strategy for combatting sarcopenia. Given the anabolic properties of both resistance exercise and protein/EAA ingestion, it is plausible that the combination of these factors in a chronic intervention may have an additive effect, and so enhance the responses shown with RET alone. Certainly, this has been shown to be the case in younger adults [140]. Although individual significant results in strength and body composition outcomes are reported in the present review, the overall results indicate that this does not hold true for older adults.

This overall absence of an additive effect, in contrast to that of younger adults, may be a result of the mechanisms of anabolic resistance in older muscle. For example, the expression and activation of proteins responsible for EAA sensing and signalling are reduced in older people [56], meaning the extent to which the subsequent cascades can be activated is limited, causing a blunted anabolic response compared with younger adults. Thus, if the limit for activation has been reached, any increase in

the upstream signal (i.e. more amino acids) will not result in any additional response. Where reported, all baseline protein intakes were within the RDA, and with lower sensitivity to higher protein intakes, this may have been sufficient to elicit a maximal protein synthetic response in combination with RET, prior to any supplementation. Certainly, there is evidence to suggest that there is no benefit for older adults in consuming more than the RDA for protein; in a 12-week trial of adults aged 50-80 years daily protein intake was increased from 0.9 $g.kg^{-1}.day^{-1}$ to 1.3 $g.kg^{-1}.day^{-1}$ with no additional response to RET [123]. Furthermore, when the effects of consuming the RDA were compared with a higher protein dose in older adults performing RET, the metabolic adaptations to increased protein intake actually reduced the utilization of protein [167]. However, a recent study in older men measured phosphorylation of the anabolic signalling molecule p70S6K as an indicator of mTOR pathway activation, in response to a range whey protein doses between 0-40g following a bout of resistance exercise [168]. Phosphorylation was correlated with protein dose, which indicates a dose-response relationship in the acute setting. As this relationship does not appear to translate to a chronic setting, this suggests that the mechanisms of this anabolic effect require further investigation.

The alternative protein intake recommended for older adults by the PROT-AGE study group offers a slightly different perspective [119]. If the findings of this review are considered in terms of this range of 1.0-1.2 $g.kg.^{-1}day^{-1}$, most included studies were above the lower limit of this recommendation, and so the idea that baseline intakes were sufficient to maximally stimulate MPS would still apply. However, baseline intakes in two studies, while meeting the RDA, were at or below the alternative recommendation [155, 157], and the supplemented groups in these two studies provided the greatest increase in protein intake relative to baseline. One of these

studies also fell into the small number which gave a significant difference in LBM between supplemented and control groups [155], and the other reported a significant difference in leg press peak power [157]. Although not included as an outcome of the review, muscle power is highly relevant in this context, as it is dependent upon muscle mass and also declines with ageing, impacting upon functional ability [27, 169]. This suggests that, under circumstances of lower baseline protein intake, there may be potential for an additive effect of RET and protein supplementation. This is of particular relevance to older adults who are frail or in institutionalised care, as protein intakes for these groups have been found to be lower than community dwelling older people, at 1.0 and 0.8 $g.kg^{-1}.day^{-1}$ respectively [170].

In addition to the total daily protein intake, the influence of protein supplementation with respect to the size of an individual dose of supplement should be considered. Acute studies have demonstrated that older adults require a bolus of at least 20-40g of protein after resistance exercise to stimulate the MPS level above that of an exercised, unfed state [60, 106]. The studies included in this review used a range of protein doses, some of which were at or above thresholds previously found to be effective in an acute setting to increase MPS. However, there were no consistent differences between the results of these studies compared with those utilising lower protein doses. Again, acute effects do not appear to translate to a chronic response. This also has implications for total protein intake recommendations for older adults conducting regular exercise, which are partially based on this acute evidence. It is recommended that older adults in this category require more protein than their inactive counterparts, and that they should consume at least $1.2g.kg^{-1}.day^{-1}$ including a 20g supplement after exercise [119]. However, the results from this review indicate

that, from the perspective of improvements in muscle mass and function, there may not necessarily be any benefit from the increased protein intake.

The efficacy of protein supplementation in addition to RET has been addressed by previous systematic reviews, with contrasting results. Cermak et al. (2012) found in favour of an additive effect in terms of FFM and muscle strength, concluding that protein/EAA supplementation augmented responsiveness to RET in both older and younger participants, a discrepancy most likely due to the different criteria used to define older populations [140]. A mean age of at least 70 years was required for studies to be included in the present review, giving an overall mean age of 77.4 years and a range of participant ages between 60 and 100 years, whereas the older cohort included in the previous review was aged between 48 and 72 years. The previous review was also restricted to only healthy participants, while the present review included participants defined as frail or sarcopenic, and in fact only one study was common to both reviews [151]. More recently, Finger et al. (2015) considered the effects of RET and protein supplementation in older adults in terms of FFM and muscle mass and strength [142]. Again, the lower age limit was younger than that of the current review at 60 years, and included studies with participants as young as 50 years. Of the nine included studies five were also included in the present review, with the remaining four excluded at either the abstract or full text screening stages due to the age criterion. Meta-analysis indicated a significant effect on FFM, which may again be a result of inclusion of younger participants. The meta-analysis is an area in which the methodology differed from the current review; previously, all studies with comparable outcomes were included in meta-analyses, however in this review any prior decision to include studies in a meta-analysis was on the basis of similarity of study protocols based on a number of characteristics. As a consequence, very few of

the studies were truly comparable. Further methodological differences also allowed the current review to provide a more comprehensive view of the subject; a more extensive list of outcome measures includes measures of functional ability, which are highly relevant when considering the practical effects of an intervention, as well as a greater range of body composition outcomes. Furthermore, the eligibility criteria for the current review were less restrictive, as exclusions were not on the basis of other macronutrients in the supplement, allowing a greater number of studies to be included.

In general, the overall quality of the included studies was moderate to high, although several studies scored poorly for internal validity. In particular, approximately half of studies did not report blinding of all participants, and four failed to use any placebo in the control groups, meaning these studies may have been susceptible to performance bias.

2.5.1 Update

One of the studies included in the review reported results from the Vienna Active Ageing Study [160], and since completion of the review, additional results from relevant outcomes have been published in a separate article [171]. Additional outcomes were isokinetic knee-extensor and flexor strength, an arm lifting test and a functional reach test. All measurements increased significantly over time with RET ($p<.05$), however there was no significant difference between supplemented and non-supplemented groups.

Recently, an additional systematic review of the influence of protein supplementation on RET efficacy has also been published [141]. Data from 49 studies were included (n=1863 participants), encompassing both younger and older adults, with younger

and older subgroups defined as below and above 40 years. Outcome measures were muscle strength, FFM and muscle size. Consistent with previous reviews, improvements with RET were reported for all outcomes, which were significantly enhanced by the addition of supplementation with mean differences of 2.49 kg for 1-RM strength, 0.30kg for FFM, 310 μm2 for muscle fibre CSA, and 7.2 mm^2 for mid-femur CSA ($p<.05$). As well as meta-analysis, data analysis included a meta-regression using several variables as covariates, including age of participants. Results indicated decreasing efficacy of protein supplementation on FFM with increasing age (k=.002), which is consistent with the lack of effect reported here. The authors suggest that this age effect may be due to the amount of protein consumed; while older adults require higher protein doses to stimulate MPS [100], the average supplemental dose was lower in the studies of older adults compared with young (20 g vs 42 g). Similarly, mean supplemental dose in the current review was 19 g, and so this explanation may also be applied to the results presented here, although the possible influence of low baseline protein intake suggested here was not supported by this new review.

As with previous reviews in this area, when conducting subgroup analysis for older participants a relatively young cut-off of 45 years was used. Hence, while the meta-regression provided insights into the effect of ageing on the efficacy of protein supplementation, arguably the results of the subgroup analysis do not truly reflect that of older adults. Again, no measures of functional ability were included.

2.5.2 Limitations

The greatest limitation of this review is the lack of meta-analysis data. Outcome measures showed a high degree of heterogeneity and data were not presented uniformly. Methodological diversity was high, with variation in protein/ EAA

supplementation, training protocols and duration of intervention. As a result, comparable outcome measures were limited, and differences in methodology meant that comparisons between most studies would not have been valid. Ideally, subgroup analysis would have been completed, for example for frail/sarcopenic and healthy groups, and for different distributions and timings of protein intake as the number of doses and proximity to exercise may have affected the response, but methodological differences did not allow this. However, the vast majority of results indicate no additional effect of protein supplementation, and this did not appear to differ according to population or protocol characteristics, other than that of baseline protein intake discussed above.

The review may also be limited by the sample sizes of the included studies, some of which were relatively low and therefore may have lacked sufficient power to identify small differences between groups. Eight of the studies [150, 152, 154-159] reported a power calculation which deemed the sample size to be adequate, and there was no difference in terms of significant results between these and the studies which did not report a power calculation. However, the majority of these were powered for only selected outcome measures, usually body composition, meaning the sample sizes may not have been sufficient for other outcome measures; this is particularly important to consider with respect to more complex outcomes, such as measures of functional ability, which may require larger sample sizes to detect significant differences.

2.5.3 Conclusions

Protein/ EAA supplementation does not significantly augment the effects of progressive RET in older adults in terms of muscle strength, muscle size, body composition or functional ability. The review does, however, support the prescription

of RET regimens to maintain and increase muscle mass and strength in older populations, which may help to combat sarcopenia and frailty.

The findings also suggest that there may be an additional benefit of protein supplementation and RET programs in frail older adults who do not regularly consume sufficient protein, particularly those in institutionalized care. Thus, future research may consider exploring this by conducting trials placing greater emphasis on the baseline protein intake of participants. Likewise, little discussion has been given here to the protein supplement characteristics, such as the amount, timing and distribution of ingestion, and further research may investigate these areas to fully determine whether protein supplementation could be protective against sarcopenia.

3. SYSTEMATIC REVIEW: RESPONSIVENESS OF SKELETAL MUSCLE TO PATTERN OF DAILY PROTEIN INTAKE IN OLDER ADULTS

Objective: Systematically review the literature to investigate the influence of the pattern (timing and/or distribution) of daily protein intake on skeletal muscle in older adults.

Methods: Systematic search strategies were applied to MEDLINE, EMBASE, SPORTDiscus, and CINAHL Plus in December 2013, to include studies in adults aged ≥ 65 years, comparing at least two patterns of protein intake differing in timing and/or distribution. Data were extracted for measures of body composition, muscle strength and size, functional ability, nitrogen balance and protein turnover. Quantitative synthesis and meta-analysis were conducted on comparable outcomes.

Results: Six studies including 135 participants were identified. Two compared even and uneven distributions of daily protein intake, and four compared timing of a protein supplement relative to an exercise intervention. Changes in body composition, nitrogen balance and protein turnover were significantly greater with the uneven distribution. Comparison of timing studies was problematic due to variable protocols; meta-analysis was possible of two studies measuring nitrogen balance but there was not significant difference treatments (mean difference: 0.52; 95% CI -0.32, 1.35; p = .23).

Conclusions: Evidence relating to the influence of timing and distribution of protein intake in older muscle is limited and requires further investigation.

3.1 Background

3.1.1 Rationale

Despite its anabolic properties, the role of dietary protein in interventions to protect against sarcopenia is unclear. Evidence of the effects of increased protein intake is mixed, and as indicated by the systematic review in Chapter 2, there do not appear to be any additional benefits of protein supplementation in older adults undertaking RET. Due to the response profile of muscle protein synthesis (MPS) to protein ingestion, it has been proposed that the delivery of dietary protein, rather than the total daily amount, may influence the response.

The acute anabolic response to a dose of protein is transient, persisting for approximately two hours, and demonstrates a dose-dependent relationship which is saturable beyond a certain protein dose. Hence, the total anabolic response over the course of a day may be affected by both the timing of intake, and the distribution of protein into doses. As discussed in Section 1.4.4, there is some evidence of an effect of protein distribution; younger adults have exhibited changes in the rate of MPS with different protein distributions, for example Mamerow et al. (2014) reported 25% greater 24-hour MPS following seven days of consuming 90 $g.day^{-1}$ protein as an evenly spread distribution compared with a uneven intake [130]. Furthermore, associations have been found between habitual protein distribution and indicators of muscle health in older adults; more even distribution of protein across the day was associated with greater muscle strength and lean mass, and lower frailty scores [133, 134]. However, experimental evidence of the effects of protein distribution on muscle health is very limited.

In terms of the timing of a dose of protein, again the effects are unclear. There has been a particular interest in the timing of protein supplementation relative to a bout of exercise, however despite a number of studies there is no consensus even in younger adults [172]. For example, greater MPS has been demonstrated with EAA and carbohydrate supplementation before, rather than after, resistance exercise [173], however no such effect was found when this was repeated with whey protein [174]. Several studies have considered the chronic effects of proximity of protein supplements to resistance exercise in terms of training-induced changes in muscle strength and body composition, again with mixed results [175, 176]. This indicates that the timing of supplementation may be important in younger adults, and so also requires consideration in older adults.

3.1.2 Aims

Currently, there is limited evidence to suggest an effect of protein intake pattern on skeletal muscle size and function in older adults, and no consensus as to how this may be used to combat sarcopenia. The aim of this systematic review was to evaluate the influence of the pattern of protein intake in older adults on measures of muscle strength and size, body composition, nitrogen retention, protein turnover and functional measures.

3.2 Methods

This systematic review was conducted according to a pre-defined protocol informed by the Cochrane Handbook for Systematic Reviews of Interventions [177] and is reported in line with the Preferred Reporting Items for Systematic Reviews and Meta-analysis (PRISMA) statement [144].

3.2.1 Information sources and search

Search strategies were designed to include components relating to participant age, dietary protein, relevant outcome measures, and timing/distribution. Table 3.1 includes an example search strategy.

Searches were conducted by one reviewer (DKC) in December 2013 using the following electronic databases (to 11 December 2013): MEDLINE (from 1946); EMBASE (from 1980); CINAHL Plus (from 1937); SPORTDiscus (from 1949). Recursive bibliography searching of included articles was also performed.

3.2.2 Eligibility criteria

Articles were assessed for eligibility according to the following criteria; (i) conducted in adults aged ≥65 years (or mean age ≥65 years); (ii) a chronic intervention (i.e., more than one discrete bout) related to the timing and/or distribution pattern of protein/EAA ingestion; (iii) comparison with at least one alternative protein/EAA intake pattern differing in the timing and/or distribution of doses, or normal care (i.e. no treatment); (iv) measurement of at least one of muscle strength, muscle size, body composition, nitrogen balance/retention, protein turnover/synthesis/breakdown and functional ability. Articles were excluded if they were (i) administered to a specific patient group or (ii) used with the intention of treating a specific health condition other than frailty or sarcopenia. Unlike the systematic review in Chapter 2, an exercise component was not required for eligibility as this was primarily a study of protein intake, but studies containing an exercise component were not excluded.

Table 3.1: Example Ovid MEDLINE (R) search strategy[1]

1	Aging/
2	Exp aged/
3	(65 adj2 (years or age* or old*))
4	(old* adj (adult* or people or person* or population* or men or women))
5	(elder* or senior* or geriatric* or ?enarian or ag?ing)
6	(age* or aging or old* or elder*) adj1 (musc*))
7	1 or 2 or 3 or 4 or 5 or 6
8	Proteins/
9	Exp Amino Acids/
10	Exp Dietary proteins/
11	((protein* or amino acid*) adj3 (intake or requirement))
12	((protein* or amino acid*) adj3 (supplement* or feeding or ingest*)
13	8 or 9 or 10 or 11 or 12
14	Muscle Development/
15	Muscle, Skeletal/
16	Muscle Strength/
17	(musc* adj2 (function* or power or strength))
18	(musc* adj2 (grow* or hypertrophy or size or mass or csa or cross sectional area or volume))
19	Body Composition/
20	(lean adj3 mass)
21	(protein adj2 (turnover or synthesis or breakdown))
22	(nitrogen adj2 (balance or turnover or synthesis or breakdown or retention or loss or retain*))
23	Sarcopenia/
24	14 or 15 or 16 or 17 or 18 or 19 or 20 or 21 or 22 or 23
25	Meals/
26	((pulse or bolus or spread) adj2 (feeding or ingest* or intake or pattern))
27	((ingest* or feeding or meal* or intake or provision or administration) adj2 (pattern or timing* or time* or distribution*))
28	((distribution* or timing*) adj3 (protein* or amino acid*))
29	25 or 26 or 27 or 28
30	7 and 13 and 24 and 29
31	Filter 30 – humans
32	Remove duplicates from 31

[1]Ovid MEDLINE (R) search, adapted for other databases

3.2.3 Study selection

Following duplicate removal, search results were screened by one reviewer (DKC) to determine eligibility according to the criteria, firstly on the basis of Title and then Abstract. Full texts were obtained for all articles which could not be excluded on the basis of Title and Abstract, and two reviewers (DKC and CAG) assessed these full texts independently. A study was included when both reviewers determined it was

eligible on the basis of the full text. Any disagreements were resolved upon further discussion.

3.2.4 Data extraction

Data were extracted by one reviewer (DKC) using a standardised data extraction sheet. Data relating to the following items were extracted;

(i) Study design and duration

(ii) Participant: number (total and group), age, gender, health status

(iii) Intervention details: protein/EAA doses and timings, exercise type, intensity and frequency (when applicable)

(iv) Outcome measures: a definition of the outcome, the method of measurement, results from each study group and the presence or absence of a statistically significant difference between groups. These data were extracted for measures of muscle strength, muscle size, body composition, nitrogen balance/retention, protein turnover/synthesis/breakdown and functional ability

3.2.5 Summary measures and synthesis of results

Extracted data were summarised using qualitative methods, including description of studies and tabulation of results. Meta-analysis was conducted on comparable outcome measures from studies using similar interventions (RevMan Version 5.2). A fixed effect meta-analysis model was used for data from functionally similar studies, and for continuous data measured using the same scale the effect size was expressed as a mean difference with 95% confidence intervals [178]. Analyses were conducted from summary data given, and, where necessary, means and standard

deviations were estimated from statistics or figures presented in the published articles [179].

3.2.6 Risk of bias

Two reviewers (DKC and CAG) independently assessed internal validity of the studies with moderation by a third (AR) when necessary. The Cochrane 'risk of bias' assessment tool [180] was used, as recommended by the Cochrane Handbook for Systematic Reviews of Interventions. It was acknowledged that blinding of participants and personnel would not be feasible for certain interventions, such as those which altered the distribution of total daily protein/EAA intake, but that it was unlikely to affect physiological measures such as nitrogen balance. Such judgements are permitted within the Cochrane tool, as assessment includes evaluating the likely effect of a lack of blinding.

Risk of bias across studies could not be addressed using a funnel plot to identify publication bias since fewer than 10 studies were included in the review [181]. Selective reporting was assessed within the Cochrane 'risk of bias' tool [180].

3.3 Results

3.3.1 Study selection

The literature search identified 159 articles. Following the removal of duplicates and ineligible articles, six studies were included in the review [182-187] (Figure 3.1). Within one of the articles, trials were carried out in two cohorts of participants, under hypocaloric and hypercaloric conditions [186]. As the trials were performed in different cohorts they were considered separately; only the hypercaloric trial was

included in the review as the hypocaloric group did not fulfil the age criterion required for eligibility.

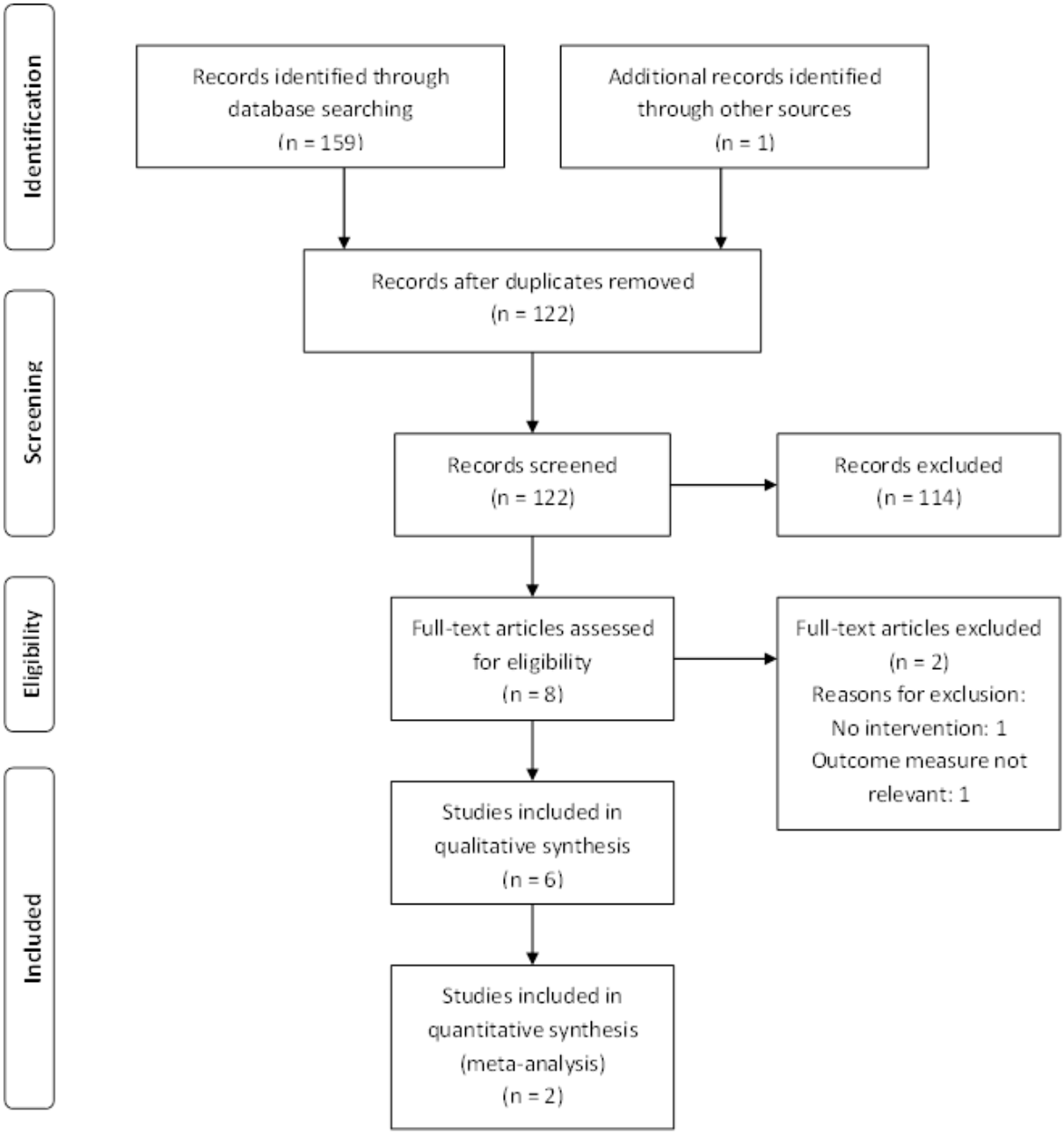


Figure 3.1 Flow chart showing the number of articles included at each stage of the review process (from [144])

3.3.2 Participant characteristics

A total of 135 participants with a mean age of 75.5 years were included in the analysis (Table 3.2), and the mean (SD) sample size was 23 (21). Two studies were conducted in only male participants [183, 184], one in female participants [182], and the remaining three used mixed cohorts, giving 47% male and 53% female participants. Five studies investigated healthy participants, however in one study (n=63) participants were hospitalised and judged to be malnourished or at risk of malnourishment (i.e. scored <23.5 on the Mini Nutritional Assessment) [187]. Two used a crossover design [185, 186] and the remaining studies were of a parallel group design.

3.3.3 Interventions

There was a clear division in terms of the interventions used, and the studies were therefore considered in two separate groups (Table 3.2).

In the first group, the distribution of total daily protein intake was manipulated [182, 187]. Protein was given in an even, or ‘Spread’, diet, as four equal or similar doses, compared with an uneven, or ‘Pulse’, diet in which approximately 80% of protein was consumed in a single dose at noon and the remainder spread over a further two or three meals. The studies differed in duration, from 14 days [182] to six weeks [187]. Both studies based the experimental diets on participants’ usual dietary habits, however one also included supplementation with milk powder to enhance protein content [187].

The four studies in the second group compared different timings of protein supplements relative to exercise. Two studies included 12-week resistance exercise

training (RET) programmes; Esmarck et al. (2001) supplemented either immediately or two hours after exercise [183], while Candow et al. (2006) compared supplementation immediately before and after exercise, as well as a placebo group [184]. In the remaining two studies participants completed aerobic exercise, and were given 2 x 3-day protein timing interventions in a crossover design [185, 186]. Supplements were given either as a carbohydrate drink in the morning and a protein drink immediately after exercise in the afternoon, or in the comparison intervention these drinks were reversed (i.e., protein in the morning and carbohydrate in the afternoon). In all exercise studies supplements were given in liquid form; 15g doses of a milk and whey protein combination were given in both aerobic exercise studies [185, 186], Esmarck et al. (2001) gave 10g of a commercial protein supplement containing milk and soy proteins [183], and Candow et al. (2006) gave 0.3g.kg^{-1} body weight of a different commercial supplement containing whey protein [184].

Table 3.2: Study characteristics[1]

Distribution studies

Author, year	N	Mean age	Study design	Daily protein distributions		Duration
Arnal et al., 1999 (157)	15	68	Parallel	PULSE: 7, 79, 14%	SPREAD: 25, 25, 25, 25%	14 days
Bouillanne et al., 2013 (162)	63	85	Parallel	PULSE: 6, 78, 2, 13%	SPREAD: 20, 30, 20, 30%	6 weeks

Exercise studies

Author, year	N	Mean age	Study design	Exercise protocol	Protein dose	Protein timings		Duration
Candow et al., 2006[2] (159)	19	65	Parallel	RET 3 x 60 min per week	0.3g.kg^{-1} body weight	PRO-B: immediately before training	PRO-A: immediately after training	12 weeks
Esmarck et al., 2001 (158)	13	74	Parallel	RET 3 x 30 min per week	10g	P0: immediately after training	P2: 2 hours after training	12 weeks
Jordan et al., 2010 (160)	9	65	Crossover	Aerobic cycling 1 hour per day	15 g	PRO: after training (CHO drink in morning)	CHO: in morning (CHO drink after training)	3 days
Minor et al., 2012 (161) (hypercaloric cohort) [3]	6	65	Crossover	Aerobic cycling 1 hour per day	15g	PRO: after training (CHO drink in morning)	CHO: in morning (CHO drink after training)	3 days

[1]RET, resistance exercise training

[2]Study also included non- protein supplemented control group, given placebo drinks

[3]Article reports two separate study in different cohorts – only the 'hypercaloric' cohort was included in the review due to age criteria

3.3.4 Outcome measures

No outcome measures were common to both intervention studies (Table 3.3). Arnal et al. (1999) measured fat mass (FM) and fat free mass (FFM) using $H_2{}^{18}O$, as well as nitrogen balance and protein turnover. Bouillanne et al. (2013) included three different components of body composition; lean body mass (LBM), appendicular skeletal muscle mass (ASMM) and body cell mass (BCM) indices, all using dual-energy X-ray absorptiometry (DXA) and/or bioelectrical impedance analysis (BIA), as well as hand grip strength, and activities of daily living (ADL) as a measure of functional ability [187].

Of the four timing and exercise studies, both RET studies included measures of body composition, muscle strength and muscle size, however each measured different components using different techniques (Table 3.3) [183, 184]. Additionally, one study included urinary 3-methylhistidine excretion as an indicator of protein breakdown [184]. The two aerobic exercise studies measured nitrogen balance [185, 186].

Table 3.3: Summary of outcome measures and significant results[1]

Author, year		Outcome measures	Significant results
Arnal et al., 1999 (157)	**Body composition** **Nitrogen balance** **Protein turnover**	FM, FFM (isotopic dilution measurement) 5-day intake and output 3-day average and fed-state synthesis and breakdown (isotopic nitrogen measurement)	FFM change: PULSE > SPREAD ($p < .05$) Nitrogen balance: PULSE > SPREAD ($p < .05$) Protein turnover: PULSE > SPREAD ($p < .05$)
Bouillanne et al., 2013 (162)	**Strength** **Body composition** **Functional ability**	Handgrip strength LBM, ASMM, BCM indices (DXA measurement) ADL	LBM change: PULSE > SPREAD ($p = .01$) ASMM: PULSE > SPREAD ($p = .01$) BCM: PULSE > SPREAD ($p = .002$)
Candow et al., 2006 (159)	**Strength** **Body composition** **Muscle size** **Protein turnover**	Leg press strength, bench press strength LBM (air displacement plethysmography measurement) Elbow, knee and ankle flexor thickness Elbow, knee and ankle extensor thickness Urine 3-methylhistidine levels	NS
Esmarck et al., 2001 (158)	**Strength** **Body composition** **Muscle size**	Knee-extension strength LBM (DXA measurement) Quadriceps femoris CSA	CSA change: P0 > P2 ($p < .01$) LBM: P0 > P2 ($p < .05$)
Jordan et al., 2010 (160)	**Nitrogen balance**	3-day intake and output	Nitrogen balance: PRO > CHO ($p < .05$)
Minor et al., 2012 (161)	**Nitrogen balance**	3-day intake and output	Nitrogen balance: Trend for PRO > CHO ($p = .09$)

[1]FM, fat mass; FFM, fat free mass; LBM, lean body mass; ASMM, appendicular skeletal muscle mass; BCM, body cell mass; DXA, dual-energy X-ray absorptiometry; ADL, activities of daily living; CSA, cross-sectional area

3.3.5 Effect of interventions on outcome measures

The results relating to the two groups of studies are reported separately. Within each group there were few comparable outcome measures and the majority of the analysis is quantitative. A summary of results and significant p-values is presented in Table 3.3. One outcome measure of nitrogen balance was suitable for meta-analysis in two studies of timing of protein relative to exercise, both including low participant numbers [185, 186].

3.3.5.1 Distribution studies

Both distribution studies reported significant effects of distribution on body composition [182, 187]. Arnal et al. (1999) reported within group results showing a decrease in FFM over two weeks with the Spread diet but no change with the Pulse diet, and a significant difference between groups in terms of change in FFM in favour of the Pulse diet [182]. The two treatment groups did not differ with respect to change in FM. The results of Bouillanne et al. (2013) showed significantly greater increases in LBM, ASMM and BCM indices with the Pulse diet compared with the Spread diet [187].

Only Arnal et al. (1999) measured nitrogen balance and protein turnover [182]. Nitrogen balance was significantly greater with the Pulse diet compared with Spread. Twenty-four-hour protein turnover was also greater with the Pulse diet, and measurements of postprandial protein turnover indicated greater protein gains with the Pulse diet compared with Spread.

There were no significant changes in handgrip strength and ADL measures [187].

3.3.5.2 Timing with exercise-supplement studies

One study reported greater LBM increases with protein supplementation immediately after exercise (P0) compared with supplementation two hours after exercise (P2), and a significantly greater increase of 3.7 cm^2 in quadriceps femoris cross-sectional area (CSA) with the P0 intervention compared to P2 [183]. The change in knee-extension strength did not differ between treatment groups for the majority of measurements, although there was a trend (p = .07) for greater gains in isokinetic strength at 60deg.s^{-1} in the P0 group compared to P2. Another study reported a greater increase in muscle thickness of elbow, knee and ankle flexors in one supplemented group compared with the placebo group [184]. However, there were no significant differences between protein supplemented groups in any of the included measures of strength, muscle thickness, LBM or protein breakdown.

Of the two studies measuring nitrogen balance with aerobic exercise, one reported significantly greater results when the protein supplement was ingested immediately after exercise compared with supplementation in the morning [185], while the other reported a trend in the same direction (p = .09) [186]. However, the meta-analysis (fixed effects; mean difference: 0.52; 95% CI: -0.32 to 1.35) did not show a significant effect of intervention on nitrogen balance (p= .23) (Figure 3.2)

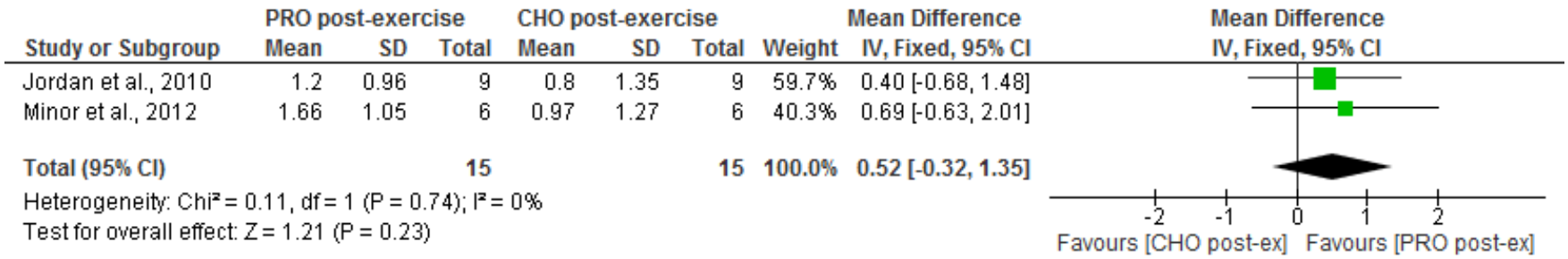


Figure 3.2: Fixed effects model on 3-day nitrogen balance (mean difference: 0.52; 95% CI: - 0.32 to 1.35, *p* = 0.23). Forest plot illustrating the mean difference between the PRO and CHO groups (squares), 95% confidence intervals (horizontal lines) and mean difference (diamond). The size of each square is indicative of the weight given to that study in the meta-analysis.

3.3.6 Risk of bias

For all studies the majority of components could only be judged as unclear due to a lack of reported information (Table 3.4), however all studies had at least one high risk component and therefore were evaluated to be at high risk of bias. The most common reason for this judgement was a lack of usual care/control group, and, for the crossover studies, the absence of a washout period between interventions. All studies were included in the analysis to provide an overview of results, as no low risk studies were available.

Selective reporting was identified in one study. The outcome measurements of Bouillanne et al. (2013), as listed under a clinical trials registry, included plasma amino acid levels as a secondary measure, however these results were not presented in the published article [187]. This was not a key outcome of interest for this review.

Table 3.4: Summary assessment of overall risk of bias for each study[1]

Author, year	Components of risk of bias							Summary	Comments on high risk components
	1	2	3	4	5	6	7		
Arnal et al., 1999 (157)	U	U	L	U	U	U	H	**High (1)** Unclear (5) Low (1)	One high risk component: 7 No primary outcome specified No usual care control group
Bouillanne et al., 2013 (162)	U	U	U	U	L	H	H	**High (2)** Unclear (4) Low (1)	Two high risk components: 6, 7 Protocol available at http://clinicaltrials.gov, plasma amino acids listed as secondary measure but results not reported No usual care control group
Candow et al., 2006 (159)	U	U	L	L	U	U	H	**High (1)** Unclear (4) Low (2)	One high risk component: 7 Difference in baseline protein intake between groups, possibility of effect on results not addressed
Esmarck et al., 2001 (158)	U	U	U	U	L	U	H	**High (1)** Unclear (5) Low (1)	One high risk component: 7 No primary outcome specified No non-protein control group
Jordan et al., 2010 (160)	U	U	L	U	L	U	H	**High (1)** Unclear (4) Low (2)	One high risk component: 7 No washout period No non-protein/protein only control group
Minor et al., 2012 (161)	U	U	L	U	U	U	H	**High (1)** Unclear (5) Low (1)	One high risk component: 7 No washout period No non-protein/protein only control group

[1]Components of risk of bias criteria: 1, sequence generation; 2, allocation concealment; 3, blinding of participants/personnel; 4, blinding of outcome assessment; 5, Incomplete outcome data; 6, selective outcome reporting; 7, other potential threats to validity. Levels of risk of bias: H, high risk of bias; U, unclear risk of bias; L, low risk of bias

3.4 Discussion

The aim of this review was to establish whether the responsiveness of older muscle to protein ingestion is affected by the timing and/or distribution pattern of protein intake. Data from only six studies including 135 participants were eligible for inclusion, indicating a lack of research in this area. Studies were categorised into one of two groups according to a clear distinction in the type of intervention used; two studies compared different distributions of total daily protein intake, and four compared timings of a protein supplement relative to exercise. Meta-analysis was only possible for one outcome measure included in two studies. All other data analyses were qualitative. Some individual results from these studies indicate that the responsiveness of older muscle to protein may indeed be affected by the timing and distribution of intake, although more specific conclusions cannot be drawn due to the limited study comparability.

All included studies were evaluated as being at high risk of bias, which must be considered when interpreting these conclusions. Some issues contributing to this risk of bias related to reporting issues, such as the failure to specify a primary outcome or report all secondary measures listed in the study protocol. However, a greater number of risk of bias components indicate design issues, as only one of the six studies included a usual care/control group, and the studies using a crossover design did not include a washout period.

3.4.1 Studies of daily protein distribution

Both distribution studies implemented a Pulse protein distribution, characterised by one very large combined with smaller protein meals, and a Spread distribution consisting of more even protein meals [182, 187]. Individual results from the two

studies indicate a possible influence on body composition, nitrogen balance and protein turnover, and it is noteworthy that the more beneficial results were consistently observed in the Pulse intervention groups. This is perhaps surprising given other evidence in this area; following 7 days of similar distribution interventions, Mamerow et al. (2014) reported greater 24-hour MPS with the even intervention, although this was a study of younger adults (37 ± 3 years) [130]. Furthermore, the association between frailty and the daily unevenness of habitual protein intake reported by Bollwein et al. (2013) would suggest better results with the even distribution [133]. The apparent benefits of the Pulse distribution compared with a Spread distribution may be explained by considering the amount of protein in each individual dose. It is suggested that postprandial MPS is restricted in older age due to greater splanchnic extraction of EAA, resulting in lower bioavailability of EAA for MPS [188]. Larger protein doses may saturate this splanchnic utilisation, thus enabling higher plasma concentrations of EAA to be used in muscle protein synthesis. In the case of the protein intake distribution patterns used in the studies by Arnal et al. and Bouillanne et al., it is plausible that only the large doses in the Pulse distributions, and none of the Spread doses, were sufficient to reach this saturation.

However, previous research has shown that, rather than continuing to increase with greater protein/EAA doses, a plateau is reached with respect to MPS [56, 63], which may have implications for these patterns of protein intake. As described above, in a study of even and uneven protein distribution patterns in younger adults, Mamerow et al. (2014) reported greater MPS following 10 days of the even diet [130]. Crucially, in this case the total daily protein intake was ~90g per day, and was divided into three meals rather than four, meaning all even doses were approximately 30g. If the dose required to maximally stimulate MPS is taken to be 30g [60, 63], the even distribution

would have stimulated this maximal rate three times a day, and the uneven distribution only once.

When considered in terms of this threshold, the distribution studies included in the review give mixed results. As absolute protein doses, for both studies only the large Pulse dose reached the 30g threshold, and all of the Spread doses fell below. When intakes were standardised for body weight and considered relative to the proposed threshold of 0.4 $g.kg^{-1}$ [100], in one study again only the large Pulse dose of 0.83 $g.kg^{-1}$ exceeded the threshold [182]. However, the other study reported two Spread doses which approached the threshold at 0.38 and 0.39 $g.kg^{-1}$ [187]. Given that both studies showed greater improvements with the Pulse diet, the influence of this maximal MPS protein dose is unclear from these studies. Furthermore, caution must be applied with this interpretation of the data, as the concept of a protein dose threshold is a controversial one. Studies reporting this threshold effect have generally measured only MPS, and less is known about the relationship between protein dose and muscle protein breakdown (MPB) due to the greater methodological advancements in the measurement of MPS. Through the suppression of MPB, net protein gains may continue to increase at greater protein intakes, impacting on total muscle accretion [189]. The trajectory of this increase would be important in determining the optimal protein distribution. Future research is required to further investigate the effect of more frequent maximal stimulation within an even protein distribution diet.

The question of the impact of protein distribution is further complicated by a more recent study, published since the searches for this review were completed, and which would have been eligible for inclusion. Kim et al. (2015) compared the effects of high (1.5 $g.kg^{-1}.day^{-1}$) and low (0.8 $g.kg^{-1}.day^{-1}$) total protein intake diets, which were

divided into either even (33, 33, 33%) or uneven (15, 20, 65%) distributions across the day, in 20 healthy older adults (aged 52-75 years) [190]. MPS and whole-body protein turnover were measured following three days of these interventions. Findings indicated that whole-body protein synthesis and net balance, as well as MPS, were significantly different between the high and lower protein diets; this is consistent with the recommendation that older adults require more protein than the minimum RDA of 0.8 $g.kg^{-1}.day^{-1}$ [119]. However, the results also revealed no effect of protein distribution at either level of protein intake, despite the higher protein diet being such that all even doses would have exceeded the 0.4 $g.kg^{-1}$ threshold. The authors proposed several possible explanations for the discrepancy between these and previous results, such as those of Mamerow et al. (2014) described above [130]; the increased threshold for older adults may be compounded by varying protein quality within the mixed meals of the interventions, meaning the protein doses consumed may still have been insufficient to stimulate maximal MPS. There is also a potential gender effect on the response to the interventions due to the gender dimorphism in MPS which has been demonstrated previously [101] which may have affected interpretation of the results, particularly given the relatively small sample sizes. The findings were corroborated by a more recently published study, using the same distributions of 1.1 $g.kg^{-1}.day^{-1}$ protein, but this time over eight weeks with outcome measures of lean mass, muscle strength and functional outcomes, with a 24-hour stable isotope infusion trial to measure MPS before and after the intervention [191]. Again, no difference was between distribution groups for any outcome measure. These findings would also appear to contradict the findings of this review, further demonstrating that the existing evidence is insufficient to determine what role, if any, dietary protein distribution may play in the prevention of sarcopenia.

3.4.2 Studies of protein supplement timing relative to exercise

The timing of protein supplementation appeared to influence body composition and muscle size, with no effect on strength. The individual study results indicated a possible effect on nitrogen balance, however the meta-analysis did not confirm this. There was disparity in the interventions used within this group, in terms of the type of the exercise component, the dose, source and timing of the protein supplements, and general characteristics such as the study duration.

In a recent meta-analysis, Schoenfeld et al. (2013) considered the influence of protein ingestion timing in combination with RET in both older and younger adults [192]. Included studies implemented a RET programme of at least six weeks, and compared the effects of a protein supplement (≥6g EAA) given within two hours of exercise, with interventions in which no protein was consumed within two hours. No effects of protein timing on muscle strength or hypertrophy were reported. The study differs from the current review, as the lower age limit for classification as ‘older’ was just 50 years. Furthermore, rather than comparing alternative timing of a protein supplement, as in the current review, the comparison criterion in the Schoenfeld et al. review was the presence and absence of protein within two hours of exercise only, meaning not all interventions included any protein supplement. In fact, of the included studies involving older adults, all but one compared a protein supplemented group with a non-supplemented group, and in the remaining study participants had type II diabetes so would not have been eligible for inclusion in the current review. In the studies reported by Schoenfeld et al. it is feasible that the protein supplement itself was responsible for the observed results, rather than any effect of timing.

3.4.3 Limitations

Few studies were eligible for inclusion in the review, and the methodologies of the included studies were highly variable, giving few opportunities for comparison. Further to this, even within similar studies there were very few comparable outcome measures. As a result, a meta-analysis was only possible on one outcome from two studies. Furthermore, all included studies were categorised as high risk of bias. Thus, the conclusions drawn from this review must be tentative, and be used to identify knowledge gaps to be addressed by future research.

3.4.4 Conclusions

This review has identified very low quality evidence for an influence of timing and distribution of protein intake on selected outcomes of skeletal muscle function in older people [193]. However, no further conclusions or consensus can currently be drawn as to how to manipulate these factors to optimise the maintenance of muscle mass and function with age.

It would appear from the distribution studies that some benefit to body composition, nitrogen balance and protein turnover may result from a daily Pulse distribution of protein, however there is reason to believe that further investigation with increased protein doses may produce a different result. Furthermore, the results require corroboration by additional intervention studies as these studies have no comparable outcome measures, and the addition of a control group to continue with their usual diet would determine whether these alternative distributions are superior to usual behaviour. In terms of the timing of a protein supplement relative to exercise, the current evidence is not sufficient to support any specific recommendation, and as such further studies are needed with direct comparison of different timing patterns.

4. OBSERVATIONAL STUDY: DIETARY PROTEIN INTAKE IN OLDER ADULTS; ADEQUATE DAILY INTAKE BUT POTENTIAL FOR IMPROVED DISTRIBUTION

Aims: The development of sarcopenia may be influenced by the distribution of dietary protein across the day, and more specifically by the amount of protein per meal. The aims of this study were to characterise total protein intake in older adults, and to determine how it was distributed across the day. Secondary aims were to collect physical activity and sedentary behaviour data.

Methods: 3-day food diaries recorded protein intake in 38 participants aged ≥70 years. The quantity of protein in the diet was calculated, along with the pattern of distribution, the coefficient of variation (CV) for protein intake, and per meal protein content. Accelerometry was used to collect physical activity data, as well as the volume of sedentary time and patterns by which it was accumulated.

Results: Average protein intake was 1.14 $g \cdot kg^{-1} \cdot day^{-1}$. Distribution was uneven (CV = 0.67), and 79% of participants reported <0.4 $g \cdot kg^{-1}$ protein content in at least two of their three daily meals. Protein intake was significantly correlated with step count (r = 0.439, p = 0.007) and negatively correlated with sedentary time (r = −0.456, p = 0.005) and Gini index G, which describes the pattern of accumulation of sedentary time (r = −0.421, p = 0.011).

Conclusions: Total daily protein intake was sufficient; however, distribution did not align with the proposed 'optimal' intake pattern, nor was average total intake sufficient to reach the recommended per meal amounts; increasing protein intake may help to facilitate optimization of distribution. Associations between protein and other risk factors for sarcopenia may also inform protective strategies.

4.1 Background

4.1.1 Rationale

There are a number of factors which contribute to the development of sarcopenia, and among these are several aspects of lifestyle which have been identified as potential causes. These risk factors are modifiable, meaning they may be targets for intervention, and the characterisation of usual habits within the target population may inform the development of such interventions.

As one of the main regulators of muscle protein synthesis, intake of dietary protein is one such lifestyle factor. While a great deal of attention has been given to the amount of protein required by older adults, there is also potential for the distribution of protein across the day to be manipulated to improve muscle health. An optimal protein distribution has been proposed, based on the observation that muscle protein synthesis (MPS) increases with protein dose up to a threshold intake, beyond which it plateaus. As discussed in Section 1.4.1, a relative value for this threshold has been calculated based on previous protein dose-response data; Moore et al. (2015) reported a threshold of 0.4 $g.kg^{-1}$ for older adults, which is greater than the 0.24 $g.kg^{-1}$ threshold reported for younger adults [100]. It is recommended that protein should be spread evenly across the daily meals such that the protein content of each meal is sufficient to stimulate MPS to a maximal extent [54, 194]. As well as informing the specific pattern of protein intake, this would have consequences for total daily intake recommendations; in order to provide sufficient protein to reach 0.4 $g.kg^{-1}$ per meal, total intake would need to be at least 1.2 $g.kg^{-1}.day^{-1}$. This would support the conclusion that older adults' protein requirements are greater compared with those of younger adults [119], and place older adults' requirements at the upper end of both

the recommended daily allowance (RDA) of 0.8-1.2 $g.kg^{-1}.day^{-1}$ [117] and the PROT-AGE recommendation of 1.0-1.2 $g.kg^{-1}.day^{-1}$ [119].

While experimental evidence of an effect of protein distribution is limited (Chapter 3), previous observations of typical dietary intake in older adults do suggest some effect. A recent analysis of data from the 1999-2002 National Health and Nutrition Examination Survey (NHANES), from men and women aged 50–85 years, reported greater lean mass and muscle strength in participants habitually consuming a higher frequency of meals containing at least 30 g of protein [134]. This cut-off of 30g respresents the threshold for maximal MPS when defined as an absolute amount; the relative threshold of 0.4 $g.kg^{-1}$ has yet to be considered in an analysis of dietary intake.

In addition to dietary protein, other modifiable lifestyle factors may influence muscle health. An association has been shown between low physical activity and low skeletal muscle mass index, which identifies low physical activity as a risk factor for declining muscle function and therefore a factor which may increase the progression of sarcopenia [195, 196]. Participation in physical activity is particularly low among older adults [197], which presents a strong potential target for intervention. Also associated with this is sedentary behaviour, which has often been presented as part of the problem of low physical activity, but is actually an independent issue. Defined as low energy activity (≤1.5 METS energy expenditure) in a seated or lying position, sedentary time is a risk factor for a range of health issues including sarcopenia [198]. The risks associated with sedentary behaviour are reflected in public health guidelines, which recommend minimising time spent sitting [199]. However, as with protein intake, it is not sufficient to consider only the amount of sedentary time; the

pattern of accumulation is also an important factor, and there appear to be health benefits to breaking up bouts of sedentary time [200]. With one study reporting total daily sedentary time of 17-18 hours in older adults [200], again this represents a substantial target for intervention. Along with dietary protein, physical activity and sedentary behaviour are modifiable lifestyle factors which can influence skeletal muscle in older age, and the identification of associations between these factors may be informative.

4.1.2 Aims

The RDA does not currently specify a recommendation for per meal protein amount. As data relating to dietary protein distribution and how dietary habits align with the literature are lacking, it is not clear whether interventions are required to optimize protein distribution, and the design of interventions and recommendations is therefore limited. The primary aim of this study was to determine the total and per meal protein intake in a sample of older adults. Secondary aims were to objectively measure physical activity and sedentary behaviour within the same sample.

4.2 Methods

4.2.1 Recruitment

Thirty-eight participants were recruited from July to November 2014. Recruitment took place through advertisements and meetings with local housing supported housing facilities and seniors' groups, and information sent to members of the Birmingham 1000 Elders database of volunteers. To be eligible for inclusion, participants were required to be ≥70 years of age, ambulatory, and living

independently. This study was conducted in accordance with the Declaration of Helsinki and was approved by the ethics committee of the University of Birmingham.

4.2.2 Data Collection

Data collection consisted of two appointments separated by at least seven days, which took place in participants' own homes. Instructions and materials were given as described below in the first appointment and collected in the follow-up appointment. Participant characteristics including age, sex and self-reported body weight were also collected during the first appointment; if body weight was unknown participants were asked to obtain a measurement in time for the follow-up meeting.

Dietary intake was reported using 3-day food diaries, during which time participants were asked to maintain their usual diet. In the first appointment participants were provided with a standardised recording sheet and given both verbal and written instructions. They were required to complete the diary over three consecutive days including at least one weekend day, and were instructed to record details of all food and drink consumed, including the specific variety or brand of each item and any cooking methods used. The recording sheet also included a field to note the amount consumed, and participants were instructed to use weights and household measures where appropriate, or when necessary estimated portion sizes based on the guidance sheet provided. Diaries were reviewed during the second meeting, at which point participants provided verbal clarification or additional details when needed.

Physical activity and sedentary behaviour were measured for seven days following the first appointment using ActivPAL3^{TM} accelerometers. These monitors are able to collect postural as well as acceleration data, and can therefore determine whether the wearer is upright or sitting/lying down. This allows behaviour in non-active time

periods to be classified as either standing, which is low-intensity but not defined as sedentary, or truly sedentary behaviour, as when sitting or lying down. Use of ActivPAL™ monitors been validated in terms of step count and active/sedentary time [201, 202].

Pre-programmed ActivPAL3™ monitors were attached to the anterior of the thigh during the first appointment using two 3M Tegaderm™ dressings to form a seal around the monitor. Participants were instructed to continue their normal daily routine, and to keep the monitor on throughout the monitoring period, including sleeping and showering, and to only remove it when bathing or swimming. Monitors were programmed to stop recording seven days after the first appointment, at which point they were removed.

4.2.3 Nutritional Data Analysis

Food diary data were entered into Dietplan6 software (Forestfield Ltd., West Sussex, UK, v6.70.73). Data were initially entered as whole days, and mean daily intakes of energy, macronutrients and micronutrients were calculated. Further data on protein intake were extracted from the software, including the percentage of total energy intake taken from protein, and the proportion of protein originating from plant and animal sources. Daily protein intake relative to body weight was also calculated ($g \cdot kg^{-1}.day^{-1}$).

The daily distribution of protein was determined according to the eating times recorded by participants. Each day was split into 30-minute timeslots between 05:00 and 23:59, the protein intake for each slot was calculated and expressed relative to body weight, and the values for each slot were averaged across the three days. From this, protein intake could be grouped into one of four time periods generated by

examining patterns within the data. The periods were 05:00–11:00, 11:00–15:00, 15:00-17:30, and 17:30–23:59, which encompassed all daily meals within the data (i.e. breakfast, lunch and dinner) and afternoon snack in the 15:00-17:30 period where applicable. By summing intake in each 30-minute timeslot within each period, per meal protein intake was calculated for each participant. Upon further examination it was clear that the 15:00-17:30 period containing afternoon snacks was not informative as protein content was negligible, and so the distribution was adjusted to three time periods to correspond to three daily meals; 05:00–11:00 (Period 1), 11:00–16:00 (Period 2), and 16:00–23:59 (Period 3). Within these three daily meals, the main meal of the day was defined as a meal with the highest protein content; the period containing this meal was also identified for each participant.

The per meal protein intakes were analysed relative to the proposed 0.4 $g \cdot kg^{-1}$ threshold for maximal MPS [8]. For each time period the proportion of meals containing less than 0.4 $g.kg^{-1}$ was calculated, and for each participant the average daily number of meals below the threshold was recorded (range 0-3).

The average protein intakes for each time period were also used to examine the distribution of protein intake across the day, to determine whether protein intake was skewed or evenly distributed. This was quantified by calculating the coefficient of variance (CV) of protein distribution, as utilised in a previous study of dietary protein intake [133]. This dimensionless measure of distribution indicates evenness of intake across meals (CV = standard deviation/mean); a CV of zero would indicate the same amount in each period [133].

Finally, protein distribution was defined according to the relative differences in protein intake between meals across each day. Each day was assigned to one of nine

categories, depending on whether there were differences in the amount of protein between meals, and which meals were larger. A threshold amount of 0.1 $g \cdot kg^{-1}$ protein (approximately 10% of total intake) was used to define a difference between meals; for differences smaller than this, meals were classed as containing the same amount of protein.

4.2.4 Physical Activity and Sedentary Data Analysis

Activity data were downloaded from monitors using ActivPAL™ Analysis software (PAL Technologies Ltd., Glasgow, UK, v7.2.29). Only data from fully recorded days were used, and no attempt was made to remove sleep time (day or night) from sedentary data. Average daily step count, and volume of sedentary, standing and stepping time were extracted.

Sedentary data from activity monitors were processed using MATLAB (The MathWorks Inc., Natick, MA, USA, vR2012b) [17] to calculate variables to describe the patterns of sedentary behaviour: (i) Weighted median sedentary bout length: when sedentary bouts were ordered from shortest to longest, the length of bout that contained the 50% point of accumulated sedentary time in each participant. Higher values indicate that sedentary time was accumulated predominantly in longer sedentary bouts [18]. (ii) Inter-sitting time: average difference between the start times of each two consecutive sedentary bouts, high values indicate either infrequent sitting down from standing or activity, or long periods of sitting with few transitions to standing. (iii) Fragmentation index: ratio of number of sedentary bouts to total sedentary time; high values indicate high fragmentation, suggesting accumulation of sedentary time in a large number of short bouts, as opposed to the longer, infrequent bouts indicated by a low fragmentation index [18]. (iv) Gini index G: a standardized

statistic used to measure inequality, with a range of 0–1, calculated by integration of the Lorentz curve [203]. A value of zero indicates that all sedentary bouts lengths contribute equally to sedentary time (i.e., a fragmented pattern), while a value close to one indicates a high level of inequality, meaning accumulation in a small proportion of long bouts.

4.2.5 Statistical Analysis

Sample size was determined by point estimate calculations based on measures of baseline protein intake from previous studies; a margin for error of 0.09 $g.kg^{-1}.day^{-1}$ was deemed acceptable in the context of daily intake recommendations and feasible, and level of significance was 95%. Data were assessed using SPSS Statistics (IBM, New York, NY, USA, v23). Data were reported as mean (standard deviation). *t*-tests were used to identify differences between men and women and between participants living in supported housing and those not. Pearson's correlation coefficients were calculated to assess associations between variables, and one-way ANOVAs were used to identify differences when participants were grouped by the number of daily meals containing less than 0.4 $g \cdot kg^{-1}$ of protein. Where assumptions of normality were not met, non-parametric tests were used. All tests were completed to a 95% significance level, and data are reported as mean (SD).

4.3 Results

Thirty-eight participants took part in the study (12 men, 26 women) and there was no dropout. Participant characteristics are displayed in Table 4.1. Of these, 36 provided a complete data set; one participant completed the food diary but opted not to wear

the activity monitor, and for another there were technical issues when extracting data from the monitor.

Table 4.1 also contains key variables for protein intake and activity data. All were normally distributed except percentage of energy from protein, step count, bout length and fragmentation index. Macronutrient intakes as a percentage of energy are in Figure 4.1. No significant differences were found between men and women for any variable.

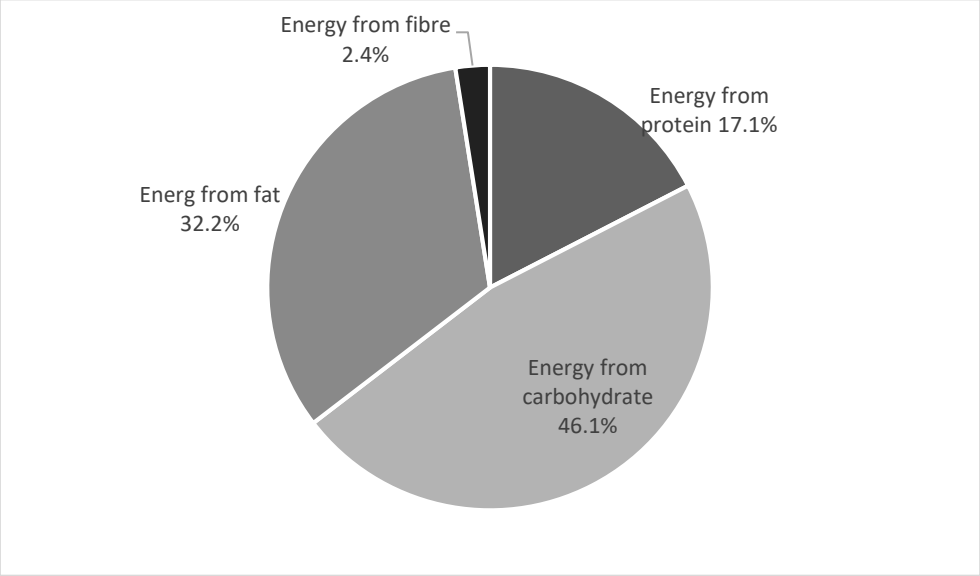


Figure 4.1 Macronutrient intakes as a percentage of energy

Table 4.1 Participant characteristics and key variable results from dietary analysis and physical activity/sedentary behaviour[1]

Participant Characteristics	
N	38
Male (*n* (%))	11 (30)
Age (years)	78 (5)
Body weight (kg)	68 (12)
Dietary Intake	
Energy intake (kcal·day^{-1})	1815 (363)
Protein intake (g·kg^{-1}·day^{-1})	1.14 (0.25)
Protein (energy %)	17.0 (3.4)
Physical Activity and Sedentary Behaviour	
Step count (steps·day^{-1})	7136 (3276)
Sedentary time (h·day^{-1})	18 (1.9)
Standing time (h·day^{-1})	4.5 (1.5)
Active time (h·day^{-1})	1.5 (0.6)
Sedentary bout length (h)	1.6 (0.7)
Inter-sitting time (h)	0.5 (0.1)
Fragmentation index	3.1 (1.0)
Gini index	0.75 (0.04)

[1]Data are presented as mean (SD) unless otherwise stated.

4.3.1 Protein Intake

Average daily protein intake was 75.4 (12.3) g.day^{-1}, or 1.14 (0.25) g·kg^{-1}·day^{-1} when standardised by body weight. The RDA lower limit for protein intake of 0.8 g·kg^{-1}·day^{-1} was met or exceeded by 92% of participants [117], and the alternative recommendation of 1.0 g·kg^{-1}·day^{-1} [119] was also reached by 76% of participants.

The contributions of plants, meat and fish, and dairy sources of protein were 37 (8)%, 42 (10)%, and 21 (7)% of total intake, respectively.

Protein intake was not correlated with energy intake. Energy intake decreased significantly with age ($r = -0.487$, $p = 0.002$), and the negative correlation between age and protein intake approached significance ($r = -0.304$, $p = 0.063$). There was no significant difference according to whether participants lived in supported housing for any dietary intake variable.

4.3.1.1 Protein distribution

The distribution of protein across the day was skewed; Periods 1, 2, and 3 contained 18%, 39%, and 44% of daily protein respectively, and mean CV of protein distribution was 0.67 (0.20). This skew was even more pronounced when participants were grouped according to the period in which they consumed their main meal. For 15 participants the main meal of the day was consumed during Period 2 (i.e. lunch), and their protein intake was distributed as 16%:55%:28% across the three time periods, and for the remaining 23 participants for whom Period 3 (i.e. dinner) contained the main meal, the distribution was 18%:27%:54%. There was no significant correlation between protein intake and CV of protein distribution.

Daily protein intake patterns were categorised according the relationship between the amounts in each time period, as illustrated in Figure 4.1. Four categories were highly prevalent (1, 2, 5, and 7), accounting for 86% of the 114 included days. Common to all four of these categories was a small amount of protein at breakfast followed by a relatively larger lunch and/or dinner. It is also noteworthy that category 9, which represents even distribution across the three time periods, contained less than 5% of days.

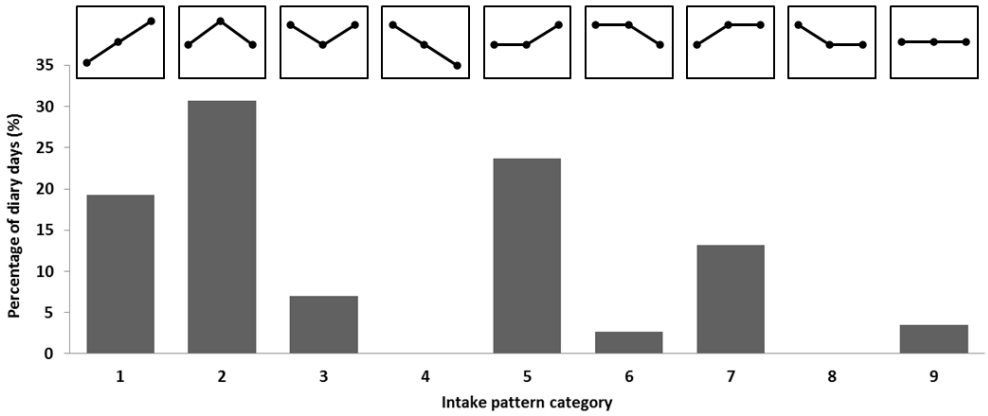


Figure 4.2 Percentage of days in each category of protein intake pattern. Patterns are depicted above each bar, showing the relationship between the amounts consumed in Periods 1 (05:00–11:00), 2 (11:00–16:00), and 3 (16:00–23:59) in each category. A difference of 0.1 g·kg^{-1} protein between periods was required for them to be considered as different amounts.

4.3.1.2 Per meal protein amounts

In relation to the 0.4 g·kg^{-1} threshold for maximal MPS, for Periods 1, 2, and 3 the proportions of participants meeting the threshold were 3%, 42%, and 68% respectively (Figure 4.2). On a participant level, no participant met the threshold in all three of their daily meals, 21% consumed two meals per day above the threshold, 71% consumed just one meal above, and 8% did not reach the threshold in any of their three daily meals. One-way ANOVA comparing total daily protein intake in participants consuming below-threshold amounts for one, two, and three meals a day indicated a significant difference between groups ($F(3, 35) = 6.112$, $p = 0.005$). Post hoc analysis showed differences between one vs. three meals below ($p = 0.004$) and two vs. three meals below ($p = 0.015$); in both cases, intake was lower in the group consuming three meals per day below the threshold.

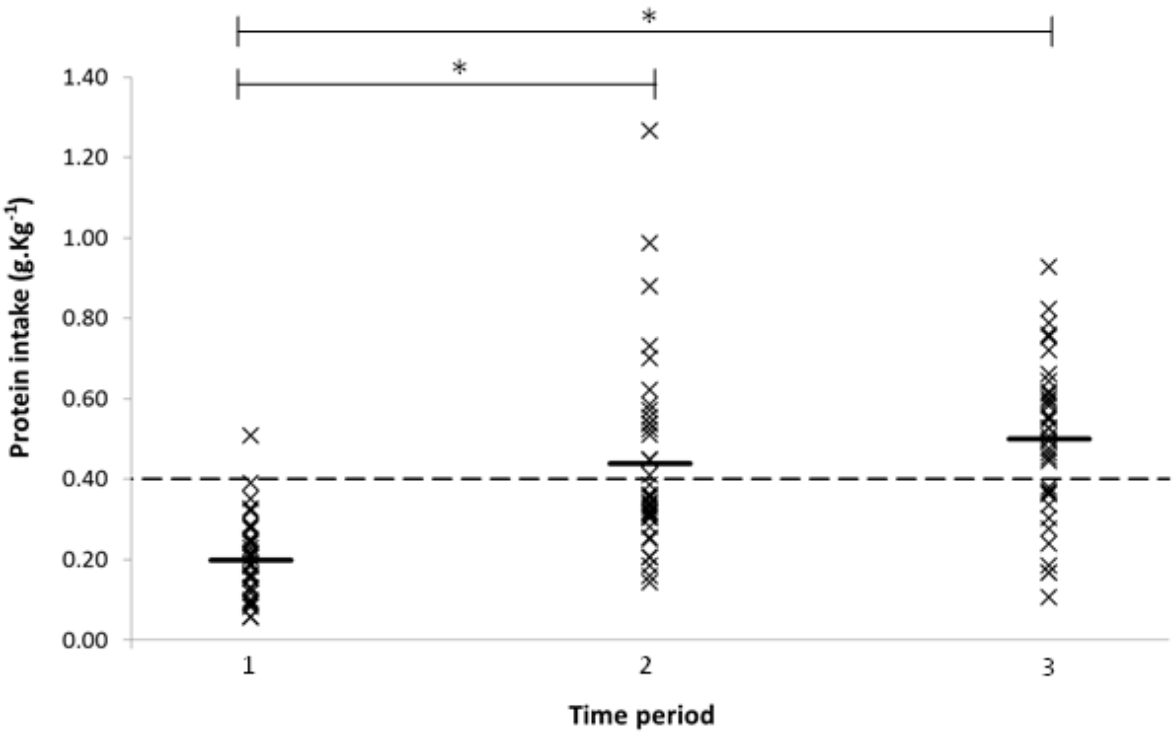


Figure 4.3 Mean protein intake for each participant by time period (05:00–11:00, 11:00–16:00, 16:00–23:59), bars represent mean for each period. Dashed line represents 0.4 g·kg^{-1} threshold. * indicates significant differences between periods ($p < 0.05$).

4.3.2 Physical Activity and Sedentary Behaviour

Mean daily step count was 7136 (3276) steps·day^{-1}. Daily sedentary time was 18.0 (1.9) h·day^{-1}, which equated to 75% of the time measured, with the remaining time divided into 19% standing and 6% stepping. For step count there was no significant difference according to gender or housing situation, and no correlation with body weight. There was a significant negative correlation between step count and age, (r = −0.502, p = 0.002) as well as correlation between step count and protein intake (r =

0.439, p = 0.007). There were significant differences between participants living in supported housing and those who did not, in terms of sedentary time (p = .033) and standing time (p = .039); mean daily sedentary time was 1.8 h.day^{-1} higher and standing time 1.3 h.day^{-1} lower in participants in supported housing.

Variables describing the accumulation of sedentary time are presented in Table 1. Protein intake was significantly negatively correlated with sedentary time (r = −0.456, p = 0.005), and with the Gini index (r = −0.421, p = 0.011), indicating an association between low protein intake and a high volume of sedentary time accumulated in long bouts.

4.4 Discussion

The primary aims of this study were to determine total daily protein intake in adults aged 70 years and older, and to investigate how this daily intake was broken down into per meal amounts. The average protein intake in this sample was 1.14 g·kg^{-1}·day^{-1}, which is a sufficient amount according to recommendations [117, 119]. However, per meal intake analysis showed that no participant consumed 0.4 g·kg^{-1} of protein in all three meals of the day, indicating suboptimal protein distribution according to current literature.

Data from the UK National Diet and Nutrition Survey (NDNS) reports a protein intake of 1.24 g·kg^{-1}·day^{-1} for older adults [204], although the analysis included trimming to allow for underreporting, which may account for the lower value in the current study. Analysis from similar surveys conducted in the Netherlands reported intakes of 1.1 g·kg^{-1}·day^{-1} and 0.9 g·kg^{-1}·day^{-1} in community dwelling adults aged over 65 years [170, 205]. For the majority of the participants in the current study protein intake was sufficient according to the RDA, and even with respect to the updated

recommendation of 1.0–1.2 g·kg^{-1}·day^{-1} [119], less than a quarter of participants had an intake which was below the lower limit. The proportion of energy obtained from protein was 17%, while the NDNS data indicated a value of 13.7% [204]; the current data were more similar to those of Tieland et al. (2015), which reported 16% of daily energy from protein in community-dwelling older adults [205]. This average value fell within the Acceptable Macronutrient Distribution Range (AMDR) recommended by the Institute of Medicine [206]. In addition to the quantity of protein, the proportions of intake derived from plant and animal sources were considered, and it was found that animal sources were the highest contributor to protein intake which is also consistent with NDNS data [207]. Protein source is an indicator of the quality of protein consumed; it has been demonstrated that the muscle protein synthetic response to soy protein is lower than that of various animal proteins [106, 115, 116], which is thought to be due to higher digestibility and a more favourable amino acid profile in animal proteins [112]. A dietary protein intake which tends towards a majority of animal-based proteins may therefore be considered beneficial. Hence, from the perspective of quantity and quality of dietary protein, it would appear that the dietary protein intake of this sample fell within the parameters which would promote muscle health.

However, there is a body of evidence which suggests that it is not sufficient to consider just the amount of protein consumed, but that the way in which it is distributed across the day is also important for muscle health. In this sample, an average distribution pattern of 18:39:44% was calculated, indicating a skewed distribution. This became even more pronounced when calculated separately depending on the timing of the main meal of the day, revealing that over half of the day's protein was consumed in the main meal. It was also clear that the least protein

was typically consumed at breakfast, both from the percentage split across the meals, and the intake pattern analysis illustrated in Figure 4.1. This pattern of particularly small amounts at breakfast is consistent with previous reports [170, 205].

This skewed nature of intake pattern was corroborated by a relatively high protein distribution CV of 0.67. Consistent with this, a previous study in community-dwelling adults aged ≥75 years reported a CV of 0.68 for non-frail participants [133]. A significant difference in CV between frail and non-frails participants was also reported, which is one indication of a potential effect of protein distribution on muscle health.

Behind this proposed effect is the suggestion that per meal protein amounts may influence muscle accretion; this may explain the mechanism by which protein distribution influences muscle health. It is suggested that optimal MPS could be achieved by consuming a sufficient amount to reach the threshold for maximal MPS in each meal. Cross-sectional data for 50–85 year-olds extracted from the 1999–2002 NHANES have shown that leg lean mass and strength are associated with the frequency of meals containing at least 30 g of protein [134]. This previous analysis was based on the estimates of the maximal MPS threshold dose given as absolute protein doses [60, 63], whereas the data in the present study were analysed in relation to the proposed threshold of 0.4 $g.kg^{-1}$, given as protein dose relative to body weight [100]. From the present analysis it was found that at least two of the three daily meals contained less than 0.4$g.kg^{-1}$ for 79% of participants, and no participant ate sufficient protein to reach the threshold in all three meals. Unsurprisingly, total protein intake was significantly lower in those participants who did not reach the threshold for any daily meals, although there was no difference in total protein

between those achieving the threshold for either one or two meals. This concept of recommended per meal protein dose also impacts upon daily intake recommendations; to achieve three doses of 0.4 g·kg^{-1} in a day the diet would need to contain at least 1.2 g.kg^{-1}.day^{-1}, which sits at the higher end for both sets of recommendations; in this respect, the average total intake for this sample fell slightly short. As per the theory of optimal protein distribution, there is clearly potential for improvement in terms of protein distribution, as well as a need to increase total protein intake, to facilitate optimal of protein doses in line with this theory.

As indicated by the findings of the systematic review in Chapter 3, existing experimental literature on protein distribution in older adults is scarce, however the results of the two chronic studies contradict this theory [182, 187]. Both studies reported greater improvements with a distribution skewed towards a lunchtime meal, rather than evenly spread protein doses. This may be explained by considering the amount of protein per meal as well as the overall distribution; in neither study did the doses in the Spread distribution reach 0.4 g·kg^{-1}. Interventions to optimize protein distribution should manipulate per meal protein amounts in relation to data on acute responses.

The basis of this distribution hypothesis is the saturable, dose-response relationship between protein dose and MPS response [60, 100]; however, the importance of this observation is contentious and not yet fully defined. The MPS response to a protein dose is only one part of the anabolic response, and the effect on muscle protein breakdown (MPB) also contributes to net muscle accretion. As well as stimulating MPS, the ingestion of protein also prompts a rise in insulin, which suppresses MPB [37]. There are indications that this does not plateau at higher protein intakes,

meaning increasing protein doses may continue to bring about greater net gains of muscle protein [189]. On the other hand, even with continued suppression of MPB at higher protein intakes, a plateau in the MPS responsiveness would still alter the relationship between protein dose and net balance at doses beyond the threshold, which would impact upon the effects of protein distribution on muscle gains. This would again indicate an optimal distribution of three daily threshold protein doses. Due to the challenges associated with MPB measurement, focus in the literature tends towards measuring only MPS when studying anabolic responsiveness [189], meaning the MPB response is less well defined, particularly in a chronic setting. As described in Chapter 3, Kim et al. [190] compared three days of Even and Uneven protein distributions in older adults aged 52–75 years, and included outcome measures of whole body protein breakdown and net balance. This study reported no effect of protein distribution on either of these outcomes, however there was also no difference reported in MPS, which is inconsistent with previous studies [130, 182]. The effects of protein distribution on MPS, MPB, and net balance are still unclear; without further research, this remains a limitation of the protein distribution theory.

A secondary objective of this study was to collect physical activity and sedentary behaviour data from the same sample. These are relevant measures to consider when looking at lifestyle factors in older adults, as both low physical activity and high sedentary time are additional risk factors for sarcopenia [195, 196, 198], and there are health benefits associated with fragmentation of sedentary time [208]. The average step count of 7136 step.day^{-1} was in line with results from a previous systematic review in older adults [209]. While public health recommendations for physical activity do not include step count targets, an analysis of existing data by Tudor-Locke et al. (2011) has translated the target of 150 minutes per week of

moderate to vigorous physical activity into an estimated 7000-10000 steps.day^{-1} [210]. As the average daily step count found in this study was just above the lower end of the range, it is not surprising that 58% of participants did not achieve 7000 steps.day^{-1}. Sedentary time accounted for 75% of the time studied, which is also similar to a previous study of physical activity and sedentary behaviour in older adults measured using ActivPAL^{TM} monitors [200]. Correlations with protein intake indicated that participants consuming less protein also had a lower step count and greater sedentary time, and that sedentary time was accumulated in longer bouts. Associations between these risk factors for sarcopenia are worthy of further investigation; the identification of clusters of unhealthy behaviour, or even causal relationships such as suppressed appetite caused by inactivity potentially resulting in low protein intake, may aid in the development of more effective interventions.

The sample size in this study was relatively low compared to previous studies of dietary intake, which may be considered a limitation. However, point estimate calculations indicated that this sample size was sufficient to detect differences in protein intake. With respect to the method of data collection, the use of food diaries for dietary assessment in older adults has been validated [211], and the accuracy of 3-day records is acceptable when compared with nine-day records [212]. Furthermore, the allocation of protein to just three meals in a day has previously been considered a limitation [133], however the data analysis in this study initially included an additional time period to allow for a snack, and the protein content of this period was negligible, indicating that it is valid to assess intake in three meals per day. This is the first study to assess per meal protein intake with respect to a threshold protein dose expressed relative to body weight rather than in absolute

terms, which is arguably more relevant to dietary recommendations, which are also given in this format.

In conclusion, total protein intake was generally aligned with recommendations; however, per meal protein intake relative to body weight was suboptimal according to current literature. Intervention targets may include a focus upon an even distribution to achieve maximal MPS across the day, as well as increased total intake to facilitate optimal distribution. Additionally, lower protein intake was associated with lower physical activity and higher sedentary time, which may be further explored and potentially combined in strategies to reduce the effects of sarcopenia in older adults.

5. INTERVENTION STUDY: INFLUENCE OF DIETARY PROTEIN DISTRIBUTION ON RESPONSIVENESS TO RESISTANCE EXERCISE TRAINING IN OLDER ADULTS

Aims: To compare the effects of Pulse and Spread distributions of dietary protein, in combination with resistance exercise training (RET), on muscle protein synthesis (MPS) and muscle strength in women aged 65 years and older.

Methods: Twelve older women (mean age 72.7 years) were recruited to this 2-week trial, and randomised and assigned to either Pulse or Spread groups. All participants followed a meal plan designed to provide 1.2 $g.kg^{-1}.day^{-1}$ protein, divided into either 33:33:33% in the Spread group or 10:80:10% in the Pulse group, and all completed unilateral RET (knee-extension, 6 x 8 repetitions at 75% 1-repetition maximum (1-RM), 3/week). Participants consumed 150ml D_2O (70 atom %) on Day 0 and 50ml on Day 7. Body water enrichment was measured from saliva samples, vastus lateralis muscle biopsies on Days 0 and 14 were used to calculate myofibrillar MPS. Knee-extension 1-RM tests were conducted at baseline and on Day 14 in trained (T) and untrained (UT) legs. Compliance with the study diets was monitored throughout.

Results: Trained leg MPS was 1.02 (0.30) $\%.day^{-1}$ in the Spread group (n = 3) and 1.16 (0.26) $\%.day^{-1}$ in the Pulse group (n=4). Corresponding values for the untrained leg were 1.05 (0.24) and 1.17 (0.29). There was no significant difference in MPS between distribution groups in either T (p = .75) or UT leg (p = .44), nor was there an overall difference between T and UT (p = .50). Strength increased by 28% in the T leg and 15% in the UT leg; change in strength was significantly greater with training (p = .02), but there was no effect of distribution in either leg (T p = .99, UT p = .69).

Mean protein intake during the study was 1.21 $g.kg^{-1}.day^{-1}$, and compliance was 90%.

Conclusion: Dietary protein distribution had no effect on MPS or knee-extension strength over two weeks.

5.1 Background

5.1.1 Rationale

It is suggested that an even distribution of dietary protein is optimal for muscle health [54, 194]; this is not supported by the results of the systematic review in Chapter 3, however the reasons for the apparent benefits of an uneven distribution identified in those studies are still unclear, and with manipulation of dose sizes relative to the optimal dose threshold it is plausible that an even distribution may be advantageous.. The protein content of each meal should be equal to the threshold dose to stimulate maximal MPS, whether that be defined as an absolute amount of 30g or a relative dose of 0.4 $g.kg^{-1}$. In this respect, the results of the observational study in Chapter 4 indicate room for improvement within older adults' dietary habits. While total daily intake was sufficient for most participants, intake was generally skewed rather than evenly distributed, and per meal protein content fell short of the threshold dose such that no participant reached the threshold in each daily meal. Hence, protein distribution is a prime target for intervention.

The lack of experimental evidence in this area is clear from the findings of the systematic review in Chapter 3, in which only two studies of protein distribution in older adults were identified [182, 187]. These findings contradict the even distribution theory, and the picture becomes even less clear with the addition of two studies from

Kim et al. (2015, 2018), in which protein distribution had no significant effect on protein turnover in a 3-day study [190], or lean body mass (LBM), strength or functional ability in an 8-week study [191]. Hence, the role of protein distribution, and the mechanisms of action are still unclear.

Furthermore, the effects of resistance exercise on muscle, and its blunted efficacy in older adults, are well known. In spite of this, there has been little research into the influence of dietary protein distribution on this response. Given that there is an interaction effect of protein and exercise in terms of acute responsiveness, it is plausible that this interaction may extend to exercise and protein distribution.

Therefore, a 2-week intervention study into the efficacy of protein distribution and resistance exercise training (RET) in older adults was proposed. The protein distributions would be achieved by dietary manipulation, and the evenly spread distribution was designed to provide three daily doses of $0.4 g.kg^{-1}.day^{-1}$ as per the proposed optimal distribution [100]. The uneven distribution was based on the findings of the systematic review in Chapter 3 which reported greater improvements when approximately 80% of daily protein was consumed at lunchtime [182, 187]. RET would be unilateral lower limb exercise, to allow for comparison between trained and untrained legs as well as between the two parallel-design protein distribution groups. The study was to be single-sex, to avoid masking of any significant results by the different responses of older men and women to exercise [101]; women were chosen due to the greater loss of function experienced by women in older age [169]. Selected outcome measures would relate to muscle protein metabolism and muscle strength, with a 2-week duration selected as changes in outcomes would be expected over a short number of weeks [81, 82]. Meal plans were to be used to achieve the required protein distributions, whereas previous studies have provided

the meals, and compliance was to be used as an indicator of feasibility to inform the design of future studies.

5.1.2 Objectives

The primary objective was to investigate the effects of dietary protein distribution on the rate of MPS, with and without RET, in older women over two weeks.

Secondary objectives were as follows:

(i) Determine whether the effect of RET on muscle strength is influenced by protein distribution

(ii) Investigate whether differences in MPS predict changes in muscle strength

(iii) Monitor participant compliance to meals plans, to consider the feasibility of this method of manipulating dietary protein distribution

5.2 Methods

5.2.1 Study design

Parallel study design comparing Pulse and Spread protein distributions with unilateral RET, with outcomes assessed at baseline and on Day 14 (Figure 5.1).

5.2.2 Participants

Participants were recruited between May 2016 and January 2018. Potential participants were identified from the Birmingham 1000 Elders database of volunteers, based on inclusion criteria; letters of invitation, along with a Participant Information Sheet, were sent to women aged ≥65 years. Upon expressing interest, potential

participants completed a telephone health questionnaire to determine eligibility, and if eligible were invited to attend a pre-trial visit.

Criteria for inclusion were; (i) Female; (ii) Aged ≥65 years; (iii) Ambulatory (with or without walking aids). Exclusion criteria were; (i) Already engaging in regular exercise (>1 per week); (ii) A selection of health criteria, listed in table 5.1, based on previously published criteria for exercise studies [213]; (iii) Treatment with anticoagulants or antiplatelets. This was a safety measure for muscle biopsies; an exception was made for participants taking a regular dose of aspirin, who were eligible to participate but were required to stop the doses 3 days prior to each biopsy.

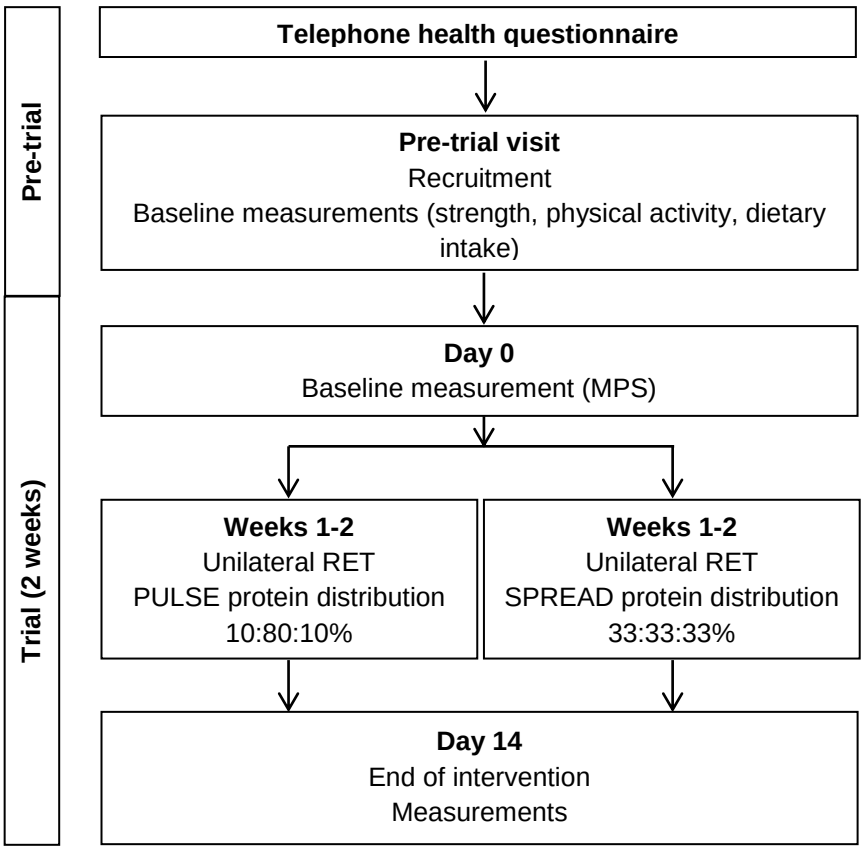


Figure 5.1: Summary of trial design

Table 5.1 Exclusion criteria[1]

History of myocardial infarction within previous 2 years
Cardiac illness: moderate/ severe aortic stenosis, acute pericarditis, acute myocarditis, aneurysm, severe angina,
Clinically significant valvular disease, uncontrolled dysrhythmia, claudication within the previous 10 years
Thrombophlebitis or pulmonary embolus within the previous 2 years
History of cerebrovascular disease (CVA or TIA) within the previous 2 years
Treatment with anticoagulants (Warfarin, rivaroxaban, apixaban, dabigatran) and antiplatelets (dipyridamole, clopidogrel, prasugrel, ticagrelor, glycoprotein IIb/IIIa antagonists). Nb. those regularly taking aspirin will be asked to stop for 3 days prior to biopsies and restart the day after
Acute febrile illness within the previous 3 months
Severe airflow obstruction
Uncontrolled metabolic disease (e.g., thyroid disease or cancer)
Significant emotional distress, psychotic illness or depression within the previous 2 years
Lower limb fracture sustained within the previous 2 years; upper limb fracture within the previous 6 months; non arthroscopic lower limb joint surgery within the previous 2 years
Any reason for loss of mobility for greater than 1 week in the previous 2 months or greater than 2 weeks in the previous 6 months
Resting systolic pressure >200 mmHg or resting diastolic pressure >100mmHg
Poorly controlled atrial fibrillation
Poor (chronic) pain control
Moderate/ severe cognitive impairment (MMSE <23)
Renal impairment (Stage 4 or 5)

.[1] Based on previously published criteria for exercise studies [213]

5.2.3 Pre-trial visit

All study visits took place within the NIHR / Wellcome Trust Clinical Research Facility in the Queen Elizabeth Hospital, Birmingham. During the pre-trial visit, participants repeated the health questionnaire, blood pressure was measured, and the Mini Mental State Examination (MMSE) was completed. The eligibility criteria were applied, and if eligible, participants were recruited to the study.

Height and body weight were measured, and baseline strength measurements were taken; participants completed one-repetition maximum (1-RM) tests of knee-extension on each leg. Participants were also given a food diary to complete as per the observational study in Chapter 4, and an ActivPAL^{TM} accelerometer to wear for seven days.

Baseline food diary data were entered into Dietplan6 software (Forestfield Ltd., West Sussex, UK, v6.70.73). Protein and energy intake were extracted, daily protein intake relative to body weight was calculated ($g \cdot kg^{-1}.day^{-1}$), and coefficient of variation (CV) for protein intake across the day was calculated as in Chapter 4.

5.2.4 Dietary intervention

Following the pre-trial visit, participants were assigned to either Pulse or Spread distribution groups; randomisation was completed by in advance by a University Hospitals Birmingham Statistician using a computer generated programme, and concealed in sequentially numbered opaque envelopes.

Total daily protein intake was calculated for each participant based on body weight, and diets were designed to provide 1.2 $g.kg^{-1}.day^{-1}$. This was divided equally across breakfast, lunch and dinner for the Spread group, and in a 10:80:10% distribution for the Pulse group. Individual meals plans were customised for each participant using Dietplan6 software, according to their protein requirements and food preferences. Participants were provided with a meal plan consisting of 30 options containing the appropriate amount of protein for each of the three daily meals. Participants were free to choose from the selection of meals, in order to facilitate compliance, and prepared them themselves. Meal plans were composed of common foods, with the addition of a protein supplement to increase protein intake where necessary. The

supplement was a commercial neutral tasting powder (Myprotein Milk Protein Smooth), derived from milk with a protein composition of 80% casein and 20% whey.

5.2.5 Exercise intervention

Participants completed three sessions per week of knee-extension exercise. Sessions consisted of 6 x 8 repetitions, with an intensity of 75% 1-RM, calculated from the test completed in the pre-trial visit. Training was unilateral, using only the dominant leg.

5.2.6 Outcome measures

5.2.6.1 MPS

The stable isotope tracer deuterated water (D_2O) was used to measure the fractional synthesis rate (FSR) of myofibrillar proteins over the study period. Muscle biopsies were taken from the quadriceps *vastus lateralis* on Days 0 and 14 under local anaesthesia (1% lidocaine), using a 5mm Bergström needle. A single biopsy was taken from the non-exercised leg on Day 0, and samples from both legs were collected on Day 14. Samples were freed from visible fat and connective tissue and immediately frozen in liquid nitrogen, before storing at -80°C until further processing took place.

Immediately following the biopsy on Day 0, participants provided a saliva sample, and drank 150ml D_2O, as three doses of 50ml separated by 45 minutes (70 atom%; Sigma-Aldrich, Poole, UK). This split dosage was an effort to avoid the side effects of nausea and vertigo sometimes associated with D_2O consumption. Three hours after the final dose of D_2O participants provided a second saliva sample; for practical reasons participants were no longer in the facility at this point, so were given a plastic

tube to take home along with instructions to record the time of the sample and to place it in the freezer until the next visit.

Participants consumed a top-up dose of D_2O on Day 7. Again, they provided an initial saliva sample, followed immediately by a single 50ml dose of D_2O, and another saliva sample was taken at home three hours later. A final saliva sample was taken following the biopsies on Day 14. Participants were observed to ensure the D_2O was consumed in its entirety. Saliva samples were centrifuged at 13000 RPM for 10 minutes at 4°C to remove any debris, and the supernatant was stored at -20°C until further processing.

A summary of the procedures conducted for the measurement of MPS is shown in Figure 5.2.

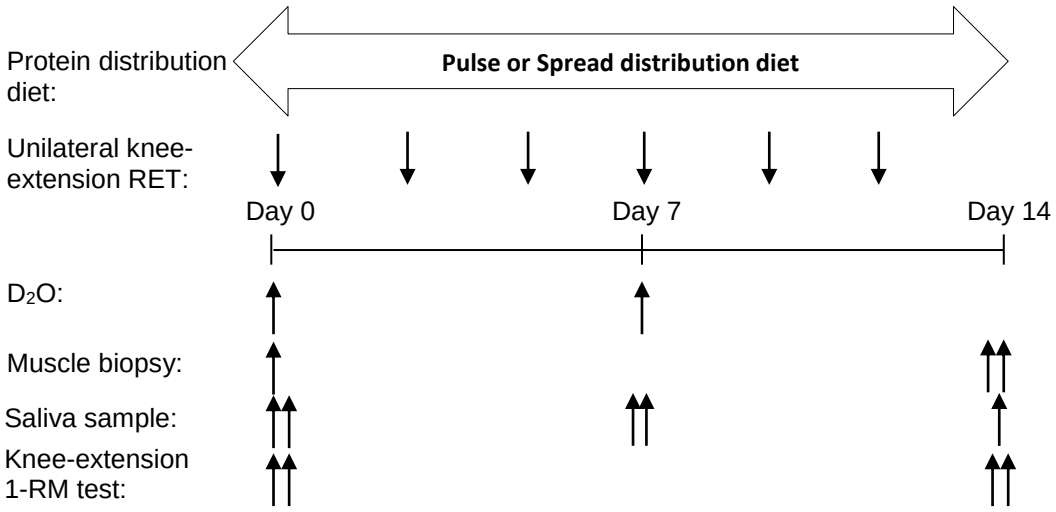


Figure 5.2: Schematic of interventions and measurements

5.2.6.2 Muscle strength

Knee-extension strength was measured using 1-RM. Baseline strength was measured during the pre-trial visit, and the second measurement on Day 14. Both trained and untrained legs were tested.

5.2.6.3 Protein intake and compliance

To monitor adherence to the study diets, participants were provided with a standard compliance recording sheet. For each day they were asked to indicate whether they had consumed three whole meals from the plan, and to provide details of any leftovers or other deviations. Participants were made aware of the importance of adherence to study diets, and of reporting instances of non-compliance. To obtain more specific information on protein intake during the study period, participants also repeated the 3-day food diary during the second week of the diet. Food diary data were entered into Dietplan6 software, and total and per meal protein and energy intake were extracted.

5.2.7 Body water enrichment analysis

Body water and muscle protein enrichment were measured according to published procedures [84]. Body water deuterium enrichment was determined from saliva samples. Samples were defrosted at room temperature, and 100µl placed in an autosampler cap before sealing with an inverted 2ml autosampler vial. Pure body water was extracted from the sample by heating at 100°C for 4h, and condensed by cooling upright in ice for 10 min, before transferring to clean autosampler vials.

Samples were injected into a high-temperature conversion elemental analyser (Thermo Finnigan, Thermo Scientific, UK) connected to an isotope ratio mass spectrometer (Delta V Advantage, Thermo Scientific).

5.2.8 Muscle protein enrichment analysis

In order to measure the incorporation of deuterium into protein-bound alanine in the myofibrillar muscle protein, samples were first separated into myofibrillar, sarcoplasmic and collagen fractions, and the amino acids released and derivatised as their n-methoxycarbonyl esters. Muscle samples were weighed and, where possible, 30-50mg muscle was taken for analysis, or when the sample weight was less than 30mg the whole sample was used. Sample were homogenised using scissors in 10µl.mg^{-1} ice-cold homogenisation buffer (50 mmol Tris-HCl, 50 mmol NaF, 10 mmol β-Glycerophosphate, 1 mmol EDTA, 1 mmol EGTA, 0.5 mmol activated Na_3VO_4 (Sigma-Aldrich, UK) with a complete protease inhibitor cocktail tablet (Roche, UK). The homogenate was rotated for 10 minutes before centrifuging for 5 minutes 13000g at 4°C, and the supernatant containing the sarcoplasmic fraction was removed. The pellet was solubilised in 0.3M NaOH and centrifuged, separating the solubilised myofibrillar fraction from the insoluble collagen, 1M perchloric acid was used to precipitate the myofibrillar protein fraction. The protein bound amino acids were released by cation exchange chromatography; firstly 0.1 HCl and Dowex H^+ were added and samples were incubated overnight. Amino acids were then eluted from the resin with NH_4OH and dried down. To derivatise the amino acids, dried samples were resuspended in 60µl distilled water and 32µl methanol, vortexed, and 10µl pyridine and 8µl methyl chloroformate added. Samples were vortexed and left for 5 minutes at room temperature to react, before extraction into 100µl chloroform, and a molecular sieve was added to each sample for ~20s to

remove remaining water. Samples were transferred to autosampler vials, now ready for gas chromatography-pyrolysis-isotope ratio mass spectrometry to determine alanine deuterium incorporation.

5.2.9 Calculation of FSR

The equation used was:

$$FSR = -Ln\left(\frac{1 - \left[\frac{(APEala)}{(APEp)}\right]}{t}\right)$$

where APEala = deuterium enrichment of protein-bound alanine, APEp = mean precursor enrichment over the time period, and t is the time between biopsies. Additionally, as protein synthesis tends to follow zero order kinetics, the fractional synthesis rate was also determined based on a single compartmental model by fitting a time course of net labelling to an exponential rise curve equation: $E(t) = Emax \times (1 - e^{-kt})$ where Emax is the number of hydrogens labeled (assuming 3.7) multiplied by the average body water labelling [78, 84].

5.2.10 Data analysis

Data were analysed using SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp. Data are presented as mean (SD). All data were checked for normality, transformations attempted where necessary, and non-parametric tests used if data did not meet assumptions of normality and equal variances. Descriptive and baseline statistics were generated and 2-sample *t*-tests were used to detect differences between Pulse and Spread groups. Baseline knee-extension was tested for correlation with protein intake and CV of protein intake. For post data, as a measure of compliance a 1-sample *t*-test was used to test for a significant difference in protein

intake from the target of 1.2g.kg^{-1}.day^{-1}. Any changes in protein or energy intake from pre-trial data were detected using paired *t*-tests, and differences between Pulse and Spread group using 2-sample *t*-tests. Paired *t*-tests detected differences between trained and untrained legs for FSR and change in knee-extension strength, as well as pre- to post- trial changes in strength, and 2-sample *t*-tests for differences between treatment groups. FSR and change in knee-extension strength were tested for correlation.

5.3 Results

5.3.1 Participants

Twelve participants were recruited to the study; two withdrew during the study for unrelated reasons, collection of MPS data for a further two was not possible due to failed muscle biopsies, and one participant completed the study and provided all samples but a signal could not be detected from muscle samples. Four participants experienced dizziness and nausea following D_2O ingestion on Day 0, which has previously been reported as a side effect of D_2O ingestion [71].

5.3.2 Baseline data

Baseline data are shown in Table 5.2. Overall mean age was 72.7 (4.6) years, and t-test indicated a significant difference between the ages of Pulse and Spread distribution groups (p = .04). Average protein intake was 1.12 g.kg^{-1}.day^{-1}, which is consistent with the intake reported in Chapter 4, and daily distribution followed the same pattern of a lower protein content in the breakfast meal (Table 5.3). Neither protein nor caloric intake differed between groups. Coefficient of variance (CV) for

protein intake was also calculated as in Chapter 4 to be 0.62 (0.18), with no significant difference between groups.

Baseline knee-extension strength (Table 5.3) was greater in the Spread group than the Pulse group in the training (i.e. dominant) leg (p = .04) but not the untrained leg. Neither the strength of the training leg nor or the untrained leg was correlated with protein intake (p = .38; p = .61) or protein intake CV (p = .80; p = .25).

	Total	Spread group	Pulse group
N	12	7	5
Age (years)	72.7 (4.6)	70.4 (3.7)	75.8 (4.1)[1]
Height (cm)	161 (6)	161 (6)	162 (8)
BW (kg)	64.5 (12.4)	69.1 (12.3)	58.1 (10.5)
BMI (kg/m^2)	24.9	26.8	22.2
Protein intake (g.kg^{-1}.day^{-1})	1.12 (0.31)	1.26 (0.20)	1.00 (0.31)
Energy intake (kcal)	1750 (362)	1858 (254)	1698 (381)
Protein CV	0.62 (0.18)	0.61 (0.26)	0.63 (0.12)
Step count	7504 (2856)	5754 (1110)	8904 (3142)

Table 5.2 Baseline data for all participants, means (SD). [1]Significant difference between Spread and Pulse groups.

5.3.3 Protein intake

Total daily protein and caloric intake did not change between the pre-trial and study period food diaries (p = .37; p = .37). Mean protein intake during the study period was 1.21 (0.11) g.kg^{-1}.day^{-1}, divided into 1.21 (0.12) in the Spread group and 1.20 (0.11) in the Pulse group. The two groups consumed the same amount of protein (p = .85) and energy (p = .32). Meal plans were well adhered to in terms of the amount of protein consumed; a t-test indicated that daily intake was not significantly different from the target intake of 1.2 g.kg^{-1}.day^{-1}. Compliance data indicated that meal plans

were adhered to on 80% of days and 90% of meals within the study period; compliance did not differ between Pulse and Spread groups.

The distribution of protein across the three daily meals is shown in Table 5.3. The Pulse group consumed the majority of their protein in the lunchtime meal as per the study diet, which accounted for a mean of 69 (16) % of their daily intakeThe Spread diet means were within 3% of the planned distribution percentages.

	Breakfast	**Lunch**	**Dinner**
Pre-trial (g.kg^{-1})	0.21 (0.09)	0.35 (0.17)	0.53 (0.16)
Pre-trial (%)	18 (7)	36 (10)	46 (12)
Trial period			
Spread group (g.kg^{-1})	0.37 (0.05)	0.46 (0.08)	0.38 (0.05)
Spread group (%)	31 (3)	36 (6)	33 (6)
Pulse group (g.kg^{-1})	0.17 (0.07)	0.82 (0.15)	0.21 (0.19)
Spread group (%)	15 (6)	69 (16)	17 (14)

Table 5.3 Per meal protein intake data from pre-trial and during trial 3-day food diaries, means (SD). Expressed as intake relative to body weight, and as percentage of total daily intake

5.3.4 MPS

MPS in the trained leg was 1.02 (0.30) %.day^{-1} in the Spread group (n = 3) and 1.16 (0.26) %.day^{-1} in the Pulse group (n = 4), and 1.05 (0.24) %.day^{-1} and 1.17 (0.29) %.day^{-1} in the untrained leg. A paired t-test indicated no effect of training on MPS (p = .50). For both the trained and untrained legs, there was no difference in MPS between treatment groups (p = .54, p = .59) (Figure 5.3).

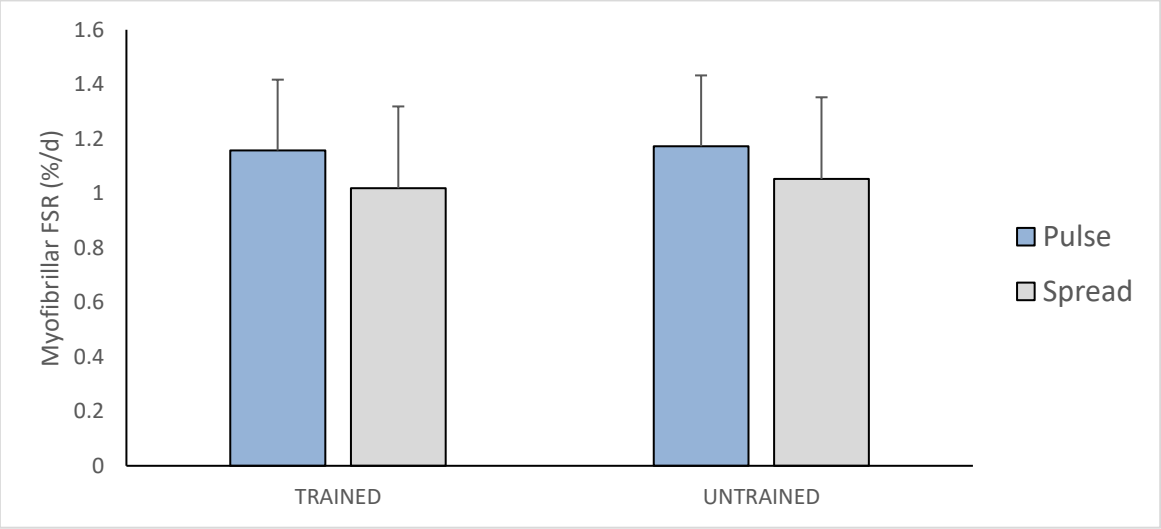


Figure 5.3: FSR of myofibrillar MPS by distribution group in trained and untrained legs, means ± SD. Pulse group n = 4, Spread group n = 3

5.3.5 Knee-extension strength

Strength data for the trained leg in the Pulse group was not normally distributed and could not be rectified by transformation, so non-parametric tests were used. Knee-extension strength increased from pre- to post- trial by 31 (14)% in the trained leg (p = .005), and by 18 (18)% in the untrained leg (p = .021) (Table 5.4). The change in strength was significantly greater in the trained leg (p = .019). Between the Pulse and Spread groups, there was no difference in either the post-trial knee-extension strength (trained p = .257; untrained p = .995), or in the pre- to post- trial change in strength (trained p = .999; untrained p = .862) (Figure 5.4).

There were no significant correlations between MPS and change in knee-extension strength (trained p = .807; untrained p = .633).

Table 5.4 Pre- and post-trial knee-extension 1-RM, means (SD)

	Total	**Spread group**	**Pulse group**
Pre-trial			
Trained leg 1RM (kg)	24.3 (6.7)	27.3 (7.5)	20.0 (0.9)[1,2]
Untrained leg (kg)	21.0 (4.0)	21.6 (4.7)	20.3 (3.1)
Post-trial			
Trained leg 1RM (kg)	30.4 (5.7)	32.5 (6.0)	27.2 (3.6)
Untrained leg 1RM (kg)	24.4 (3.6)	24.4 (4.2)	24.4 (3.2)

. [1]Significant difference between Spread and Pulse groups. [2]Assumption of equal variance not met, Welch's t-test used

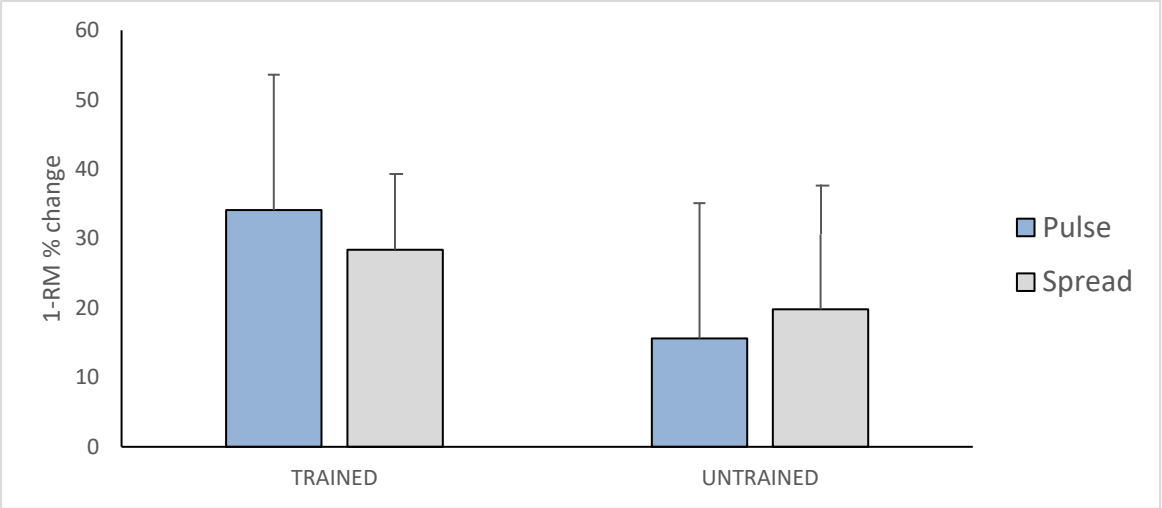


Figure 5.4: Knee-extension 1-RM strength as percentage change from baseline, by protein distribution group in trained and untrained legs, means ± SD. Pulse group n = 4, Spread group n = 6

5.4 Discussion

The primary aim of this study was to investigate the effects of dietary protein distribution on the muscle of older women in combination with unilateral RET, using D_2O tracer techniques to measure the fractional synthesis rate of MPS over the 2-week study duration. Secondary aims were to assess the effect of distribution on

muscle strength over the same period, and to determine whether any changes in muscle strength could be predicted by MPS. Finally, the feasibility of manipulating dietary protein distribution in free-living participants was assessed on the basis of compliance with the study diets.

5.4.1 Effect of protein distribution

FSR in the exercised leg was 1.16 and 1.02 %.day^{-1} in the Pulse and Spread groups respectively, with no effect of protein distribution. These are slightly lower than previous measurements; Brook et al. (2016) reported trained leg MPS of 1.49 %.day^{-1} in older adults over three weeks [82], and Murphy et al. (2018) measured MPS in older adults undergoing RET with caloric restriction, reporting 1.52 %.day^{-1} with an uneven protein distribution and 1.64 %.day^{-1} with an even distribution [214]. Knee-extension strength increased significantly over time, with greater increases in the exercise leg, however again there was also no difference with distribution.

The basis of the hypothesised effect of protein distribution is repeated saturation of the dose-response relationship between protein and MPS [54, 194]. The experimental diets in this study were designed such that the Spread group were consuming 3 daily doses of 0.4 g.kg^{-1}.day^{-1}, the threshold dose for maximal MPS in older adults [100]. Hence, a higher FSR may have been expected in the Spread group; these findings do not support the hypothesis.

Section 1.4.5 details acute evidence which indicates an effect of protein distribution on responsiveness of the muscle. In terms of results from comparable longer-term studies, as demonstrated by the systematic review in Chapter 3, evidence is limited with contradictory results to date. Similar findings to this study have been reported in two studies comparing evenly spread doses with a 15:20:65% distribution; following

3-day habituation to the study diets, Kim et al. (2015) reported no difference in MPS or whole-body protein kinetics from a 24-hour stable isotope tracer infusion trial [190]. When the same distributions were applied as an 8-week intervention, results were the same for protein metabolism, and similarly there was no effect on body composition, muscle strength or functional measures [191]. However there is also evidence which does support the spread distribution hypothesis. Mamerow et al. (2014) reported a greater MPS response with evenly distributed doses compared with a skewed intake (11:17:72%), although this was in younger adults [130]. In older adults, a 4-meal spread distribution of 1.3g.kg^{-1}.day^{-1} has been shown to an increase MPS to a greater extent than a skewed distribution, an effect which increased with exercise [215]. However, this was under conditions of dietary energy restriction, and a 13-hour infusion trial conducted during energy balance within the same study found no effect. This was attributed to the energy restriction, although an increase in protein intake from 1.0 g.kg^{-1}.day^{-1} to 1.3 g.kg^{-1}.day^{-1} between the energy balance and restriction trials may also have been a factor. Finally, there is also evidence which suggests greater benefits of a skewed distribution. The two distribution studies included in the systematic review in Chapter 3 reported greater improvements in body composition, protein retention, and nitrogen balance, with distributions skewed towards the lunchtime meal which contained approximately 80% of daily protein [182, 187].

Evidence of an influence of protein distribution on MPS and longer-term indicators of muscle health is contradictory. The findings of this study support a lack of influence of distribution on responsiveness to protein intake, in spite of the target doses for the Spread group being equal to the threshold dose of 0.4g.kg^{-1}, and only a small amount of deviation in terms of actual intakes. This may be due to the protein doses used.

These were determined according to previous studies of acute responsiveness to isolated doses; Moore et al. (2015) included data from 6 studies, all of which used supplements of high quality animal protein [100]. Protein sources in the study diets were more varied, and given the influence of protein source on the muscle protein synthetic response [106, 115, 116], the inclusion of lower quality proteins in the study diet would increase the protein dose required to reach maximal MPS. The use of isolated protein in acute studies is also significant, as in the context of dietary intake it is more likely that protein would be consumed as part of a mixed meal. Other nutrients consumed in proximity to protein ingestion may interact with the effects of protein. In particular, carbohydrate stimulates the release of insulin, which influences net muscle protein balance by suppressing muscle protein breakdown [37]. Some of the existing literature is consistent with this notion that higher protein doses than the identified maximal MPS threshold may be required to overcome the effects of a mixed meal intake. For example, the even distribution used by Mamerow et al. (2014) provided approximately 0.41g.kg^{-1} in each meal, however this was a study in younger adults [130]. At 70% above the threshold of 0.24g.kg^{-1} calculated in younger adults [100], this dose is more likely to be sufficient to stimulate maximal MPS even with the potential effects of lower protein quality and co-ingestion with other nutrients. Furthermore, as described above a previous study of energy restriction has shown greater MPS with an even compared with a skewed distribution [215]. A recently published study implemented the same distribution interventions over 2-week periods using D_2O to measure MPS throughout, and reported a conflicting result suggesting no effect of distribution [214]. One key difference between the studies was that, during the infusion protocols used to measure MPS in the prior study, protein doses were given as isolated whey protein drinks, whereas in the latter protein was

consumed as mixed meals. This provides a potential explanation for the disparity in responsiveness between the two results.

The discrepancy between the trials included in the Chapter 3 systematic review, and the lack of effect found here, is difficult to explain. It may be noteworthy that at approximately 80% of daily protein, the large Pulse dose in these studies was greater than in any of the other distribution studies mentioned previously [182, 187]. A proposed explanation for the apparent benefits of the Pulse distribution in these studies is the influence of protein distribution on EAA bioavailability. One of the potential causes of anabolic resistance to protein ingestion is differences in splanchnic sequestration of essential amino acids (EAAs) in response to feeding, as greater retention of EAAs by the gut and liver can reduce the increase in plasma EAA concentrations, and therefore bioavailability for MPS. Splanchnic sequestration of EAAs increases with ageing; specifically leucine extraction was found to be twice as high in older men as younger men, and was inversely related to plasma leucine concentrations following feeding [188]. Older adults have also shown greater phenylalanine sequestration [216]. Leucine bioavailability is of a particular concern, given its ability to stimulate MPS above that of other EAAs [217, 218], and its signalling role in the mTOR pathway [48, 108]. It has also been shown that this process of splanchnic sequestration is saturable in response to high protein doses [219]. Bouillanne et al. (2013) suggest that the Pulse dose reached this saturation point; a separate paper reporting plasma EAA concentrations in response to the two protein distributions noted an increase in leucine concentration of 101% above baseline in the Pulse group, compared with 51% in the Spread group [220]. This effect persisted after 6 weeks of the intervention, and is proposed as the mechanism of the greater improvements in the Pulse group. If this is indeed the case, this may

explain the conflict in the results of the current study; total daily protein intake was 1.5g.kg^{-1}.day^{-1} compared with 1.2g.kg^{-1}.day^{-1} in the current study. Therefore the large Pulse dose would have been considerably higher in comparison to the current study, and the Pulse dose used here may not have been sufficient to have the same effect.

5.4.2 Effect of exercise

This study employed a unilateral RET model, meaning that the influence of diet could be assessed both with and without exercise for measures of MPS and knee-extension strength. This is the first study of dietary protein distribution in older adults to introduce an exercise component without accompanying energy restriction; the lack of significant effects in both trained and untrained legs indicates the same influence of protein distribution with and without exercise. An increase in 1-RM knee-extension strength of 28% in the trained leg was significantly greater than that which measured in the untrained leg, which was to be expected given the known effects of RET on muscle strength [95, 96]. However, this was not accompanied by any difference in MPS in response to training. It has previously been shown that the acute responsiveness to resistance exercise is subject to anabolic resistance in older muscle [56], and it appears that longer term MPS is also blunted in older age. Brook et al. (2016) conducted a study with a similar training protocol in both younger and older adults, with measurements at 3 and 6 weeks [82]. As in the current study, the D_2O tracer was used to measure MPS over the study duration, and strength was measured as knee-extension 1-RM. While both groups displayed increases in 1-RM with training, an increase in MPS over that of the untrained leg was observed only in the younger group. This was accompanied by blunted anabolic signalling in the older group. This apparent anabolic resistance is consistent with the lack of effect of training on MPS reported here. One potential explanation for this strength increase in

the absence of any difference in MPS, is that the observed increase in strength was actually a result of improved coordination as a result of training. Rutherford and Jones (1986) concluded that improvements in performance in strength tests were, in part, due to improved ability to coordinate other muscle groups [221]. Given that the improvements in the present study were observed over a relatively short period of time, this may be a plausible explanation for these results.

5.4.3 Feasibility of intervention

Another aim of the study was to assess the feasibility of manipulating dietary protein distribution in free-living participants using personalised meal plans. Participants prepared their own food at home in accordance with the plans, while in most previous distribution studies participants been provided with pre-prepared meals to be consumed either on-site or at home [130, 182, 190, 191, 215], or were hospital inpatients and so received meals from the hospital kitchen [187]. Distribution studies have so far been of a relatively short duration, however with a view to longer-term studies, meal plans may present a more practical method. The meal plans in this study were designed to promote participant compliance, by including a variety of foods, allowing certain amount of choice, and by asking participants to record adherence. This was informed by previous studies of high protein/carbohydrate and heart-healthy diets, which included these elements to increase compliance [222, 223]. A high compliance of 90% of individual meals indicates that, over a 2-week period, this method of manipulating protein distribution is feasible, and this may be considered in the design of future studies. The greatest deviation in protein intake from the planed distribution was seen in the large lunchtime Pulse dose. The timing of the large dose was based on previous studies [182, 187], however in other studies the large protein dose has been given in the evening meal. Both baseline data from

this and the observational data in Chapter 4 indicate a tendency for the evening meal to contain the most protein, hence a distribution study may see higher compliance by moving the large Pulse accordingly.

One existing study has used D_2O methodology to measure MPS throughout a protein distribution study in older adults [214]. However, this previous study involved obese older men under conditions of energy restriction, which is thought to potentially accelerate loss of muscle as well as adiposity during weight reduction [224]. This is the first study to implement this technique when examining the effect of protein distribution in the absence of a deliberate weight loss element. MPS is included as an outcome measure in a number of distribution studies described above, but these have generally used stable isotope tracer infusion protocols to measure acute responsiveness over a 12-24-hour period at the start and end of a of the distribution intervention [130, 190, 191, 215]. Insights gained from such methods are limited, in that they can inform how the intervention may affect acute responsiveness, but not how protein metabolism may be altered during the intervention. Given that the theory in favour of a Spread distribution is based upon generating greater MPS across each day compared to the Pulse distribution, the daily rate of MPS during the study period is an important outcome in understanding the effects of protein distribution. Unlike the more invasive tracer infusion protocol, the D_2O method allows measurement of MPS in a free-living situation. While this does introduce an uncontrolled behavioural element to the study as a potential source of variation, it does make the results more applicable to daily life.

5.4.4 Limitations

The conclusions of this study are limited by the small sample size and increased chance of Type II error, and the lack of significant distribution effects may therefore be a product of sample size. This was largely due to delays and time constraints, and the relatively small initial sample size was compounded by a high rate of attrition. Attrition consisted of participants who withdrew for unrelated reasons, and those for whom there were difficulties obtaining viable muscle samples, potentially due to age-related changes in muscle composition, such as increased fatty infiltration [1, 225].

5.4.5 Conclusions

In conclusion, in a comparison of Pulse and Spread distributions in women aged 65 years and older, there was no significant effect of distribution pattern on MPS over two weeks, nor was there a difference in strength between the distribution groups. Compliance to the study diets was high, indicating individual meal plans are feasible as a method of manipulating protein distribution.

6. SUMMARY AND GENERAL DISCUSSION

The development of interventions to protect against sarcopenia in older age is an important research goal, however the role which dietary protein may play in such interventions is unclear. Data from acute studies is promising with regards to increasing MPS, however this has yet to translate into any definitive conclusion on a protein-based intervention which consistently influences older muscle in a chronic setting, i.e. across more than a single day. The aim of the studies reported in this thesis was to contribute to this ongoing goal by identifying areas for improved efficacy of dietary protein; this was to be achieved by reviewing several aspects of the existing literature, assessing typical protein intake in a sample of older adults, and with the development and implementation of an intervention study based on these findings.

6.1 Summary of findings

6.1.1 Chapter 2: Protein supplementation and RET

Systematic literature searches identified 15 studies in adults aged 70 years and older comparing the effects of RET with and without protein supplementation on muscle strength and size, functional ability, or body composition. The main finding of the review was that, while improvements were seen with exercise in in each of these categories in the majority of studies, there were no additional effects with supplementation. This is a significant departure from the conclusions of previous systematic reviews evaluating these interventions; Cermak et al. (2012) and Finger et al. (2015) both identified significant additive effects of RET and protein supplementation in older adults, with regard to body composition and muscle strength [140, 142]. One key difference between the review reported in Chapter 3

and previous reviews is the age of participants in the included studies, which may be the cause of this discrepancy. With mean participant age in each study set at a minimum of 70 years, the youngest participants included in this review were 60 years old; this is higher than the previous reviews, and a plausible reason for the differing results. Hence, these findings can be said to be more truly representative of older adults than any previous reviews. In contrast to the effects seen in younger adults [140], it would appear that supplementing RET with protein is not likely to be an effective intervention to combat protect against sarcopenia, assuming total daily intake meets minimum requirements.

6.1.2 Chapter 3: Protein intake pattern

This second systematic review demonstrated that chronic studies investigating the effects of protein timing and distribution in older adults are relatively scarce. A dearth of data was anticipated when designing the search strategy, and indeed only six relevant studies were identified. Of these, two provided a comparison of different distributions of dietary protein across the day [182, 187]. Both studies compared a relatively even spread of protein over the daily meals (Spread), with distributions containing one large bolus of protein in the lunchtime meal and small amounts in other meals (Pulse). Both studies reported some significant differences between the groups (body composition, nitrogen balance, protein turnover), and interestingly it was consistently the Pulse distribution which produced the superior results. This is a significant finding, as it contradicts suggestions based on acute responsiveness data that an even protein distribution may have more beneficial results [54, 194]. This review indicated the need for future research into this area. Furthermore, as these results contradict hypotheses generated from the acute data, further investigation was required to either corroborate these findings, or to explain the deviation of these

results from the current thinking. The methodologies of these studies also provided the basis of the protein distributions to be used in a new intervention study reported in Chapter 5, for the purposes of comparability.

6.1.3 Chapter 4: Typical dietary habits of older adults

Food diary and physical activity data were collected from 38 participants aged 70 years and above. Total daily protein intake was 1.14 $g.kg^{-1}.day^{-1}$, and 92% of participants were consuming sufficient protein to exceed the RDA lower limit of 0.8 $g.kg^{-1}.day^{-1}$, while 76% were meeting the higher recommendation of 1.0 $g.kg^{-1}.day^{-1}$ [117, 119]. Distribution across the day was uneven, with a split of 18:39:44% across breakfast, lunch and dinner, and per meal protein intake reached the maximal MPS threshold of 0.4 $g.kg^{-1}$ [100] by 3%, 42% and 68% of participants in each of these meals. Few studies have previously recorded typical per meal protein amounts as well as the total intake in older adults, and this is the first to analyse these data relative to the 0.4 $g.kg^{-1}$ threshold. Although total protein intake was sufficient for most participants according to recommendations, with regard to the theory of optimal MPS stimulation by reaching the threshold dose in every meal, there is clear room for improvement as no participant consumed amounts above the threshold in all three daily meals, and the threshold was rarely reached in breakfast meals. These results informed the design of a protein distribution intervention study, identifying a potential target for intervention in the form of per meal protein amounts, and also by providing information used in the design of future meal plans.

6.1.4 Chapter 5: dietary protein distribution and RET intervention study

A protocol was developed based on the findings of the systematic review and observational data in previous chapters, to investigate the influence of protein

distribution on responsiveness to RET in women aged 65 years and older. For the primary outcome of MPS, measured over the two-week intervention period, there was no difference between Pulse and Spread protein distribution groups. This was also the case for 1-RM knee-extension strength, which increased by the same amount in both distribution groups. This is the first study to design the per meal protein doses around the proposed threshold of 0.4 $g.kg^{-1}.day^{-1}$, in order to stimulate maximal MPS three times daily in the Spread group. The distributions were also chosen to be consistent with those used by the studies included in the systematic review to allow comparability, albeit slightly altered with respect to the threshold. In spite of this, however, the results neither supported the spread protein distribution theory, nor were consistent with previous results of a superior Pulse distribution as per Chapter 3, indicating no effect of protein distribution. The results are, however, similar to a very recent study of protein distribution conducted using D_2O to measure MPS, this time in older men under conditions of energy restriction [214]. During two weeks of protein distribution intervention only, followed by two weeks of distribution with RET intervention with RET, Murphy et al. (2018) found no difference in myofibrillar MPS between distribution groups [214]. A previous study from the same group implemented the same interventions, but measuring MPS before and after intervention using an acute tracer infusion protocol, reported a contrasting result with greater MPS in the Spread group [215]. One potential explanation for this discrepancy is the meal composition; in the earlier study isolated protein was given during the infusion trials, whereas measurement with D_2O throughout the study meant that protein was consumed as mixed-nutrient meals as part of the diet, which may alter anabolic responsiveness. This is also proposed as an explanation for the lack of effect found in the current study, as the Spread distribution was designed to

stimulate maximal MPS based on a threshold calculated using isolated, high quality protein. The threshold may be altered by an altered anabolic response due to co-ingestion with other nutrients, hence it is plausible that doses were too low to maximally stimulate MPS in this context. While the mechanism remains unclear, a key finding of this study is that three daily meals containing 0.4 $g.kg^{-1}$ protein do not significantly increase MPS over the response to a Pulse distribution, although with the caveat that the study was possibly underpowered due to a small sample size.

6.2 Implications of the findings

This research has implications for dietary recommendations for older adults. Based on previous evidence, older adults undertaking RET may have been recommended a protein supplement to increase the improvements in body composition and muscle size and function, when in fact there appears that this would not have any effect when total protein intake is otherwise sufficient. There has also been some suggestion that, as well as consuming sufficient protein to meet the RDA, older adults should consider spreading their protein intake evenly across daily meals. The evidence for this is mixed at best, and while the intervention study found no effect of distribution, the systematic review actually found in favour of skewing intake towards one large meal. Hence, a particular daily distribution should not be recommended at present.

Observational data indicate an average daily protein intake which meets recommendations, however this was not universal to all participants, especially with regard to the higher recommended minimum intake of 1.0 $g.kg^{-1}.day^{-1}$ which 24% of participants failed to reach. This indicates that total protein intake is still a problem area for some, and several potential targets for intervention may be considered

based on these data. Firstly, intake patterns show that breakfast typically included the lowest protein content of the day, which is consistent with previous observations [170, 205]. Hence, a focus upon increasing breakfast protein content specifically may be an effective strategy for increasing daily intake. The data also indicate an association between lower protein intake and higher sedentary time accumulated over a few long bouts. The identification of this cluster of behaviours likely to impact muscle health may be useful in the design of interventions to combat sarcopenia; for example, interventions to reduce sedentary time, and therefore increase energy expenditure, may help to overcome the reduced appetite which often leads to protein deficiency in older age [226].

6.3 Future research

The effects of dietary protein distribution on older muscle remain unclear. The results reported in this thesis indicate no effect, although results have been reported in favour of both Spread and Pulse style distributions. Further research is required to determine whether distribution may be manipulated to counter sarcopenia, and to explain the range of results reported by previous studies.

In terms of preliminary work, the 0.4 $g.kg^{-1}$ threshold was used here as the basis of the Spread distribution design, however this was calculated from data collected from older men [100]. Given the differences in protein metabolism between men and women [101, 227], it may be that this value differs in older women; thresholds have yet to be calculated using female data.

When investigating the influence of protein distribution on older muscle, there are two types of outcome to consider; the mechanistic measures such as MPS, and the more functional outcomes such as strength and power, which are more relevant when

addressing the problems of sarcopenia which are faced by older adults. Integration of these outcomes within the same study is difficult, as changes in the fractional synthesis rate of myofibrillar muscle proteins tend to occur over a relatively short duration [82], whereas a longer period of intervention is required to detect clinically relevant changes in other outcomes such as body composition and muscle function. A proposed study design to address this problem is shown in Figure 6.1. The protocol is divided into two parts; Part 1 is essentially similar to the study protocol of the Chapter 5 study, using D_2O to measure muscle protein synthesis with unilateral RET over two weeks. Part 2 is of a longer duration, suggested 10 weeks, and is designed to measure those outcomes which would respond to a chronic intervention. The same study diets would continue throughout both parts of the trial, but in Part 2 unilateral RET would be replaced with whole-body resistance exercises. The two parts are designed to run consecutively without interruption rather than as standalone studies, providing the opportunity to investigate any associations between short- and long-term differences, and therefore whether changes in MPS may influence the chronic outcomes.

As shown in Chapter 5, dietary compliance results indicate that meal plans implemented by participants are an effective method of manipulating protein distribution over two weeks. They may therefore be considered for use in future studies, again with compliance monitoring to assess the feasibility in a longer-term intervention.

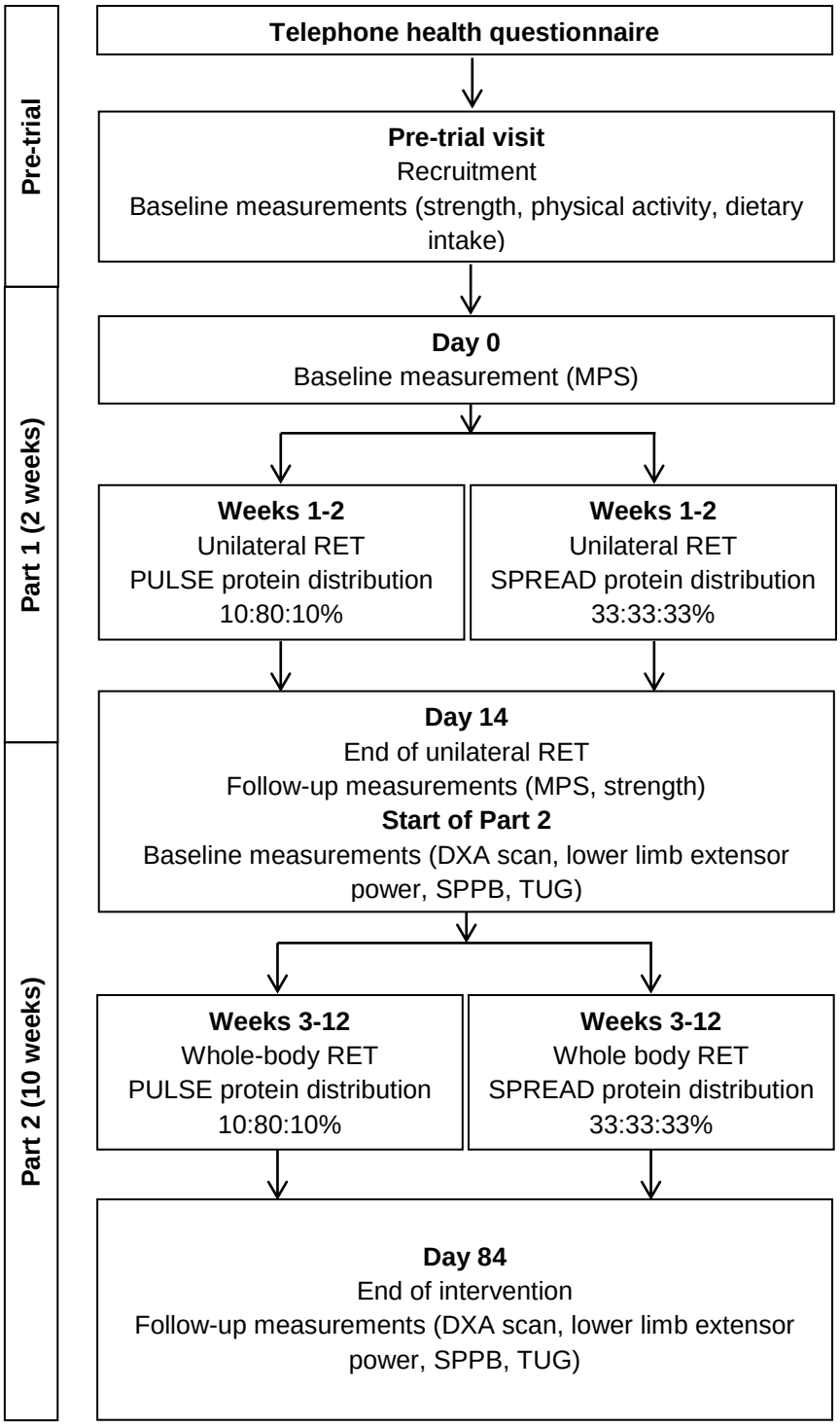


Figure 6.1: Summary of proposed trial design for integrated measurement of mechanistic and functional outcomes in response to different protein distributions combined with RET

It is likely that the responsiveness to a distribution is determined by the actual amount of protein in each dose. As has been alluded to in previous sections, it is possible that the protein doses used in distribution research so far have been insufficient in size. Bearing in mind the maximal MPS threshold dose of 0.4 $g.kg^{-1}$, the theory that a distribution in which every meal reaches this threshold has not been tested in many of the existing distribution studies, due to the tendency to split even distributions into four meals [182, 187, 214, 215]. Results from Chapter 5 indicate no effect even with the Spread diet designed around this threshold; one potential explanation is that the dose sizes were still insufficient as a result of altered responsiveness due to variable protein quality and co-ingestion with other nutrients. Hence, when designing future studies a higher total protein intake may be considered, although defining a specific threshold is more complex in the context of meals rather than isolated protein due to the number of different variables.

Chronic outcome measures may be similar to those identified in the systematic review in Chapter 2. Body composition measures are relevant, as loss of lean body mass is one of the key characteristics of sarcopenia, as well as muscle power, which declines with ageing and affects functionality [27, 169]. When considering the impact of this intervention on the lives of older adults, arguably the most important outcomes are measures of functional ability. For example, the Short Physical Performance Battery (SPPB) consists of three tests (balance, walking speed, and chair rise ability); scores have been shown to correspond to self-reported levels of disability in older participants [164] and it is an outcome measure recommended by the EWGSOP for use in studies involving older adults [228]. The timed up and go (TUG) test has been similarly validated in older participants [229].

Additionally, there may be benefits to the inclusion of plasma AA concentration as an outcome measure. One of the studies which did report an effect of protein distribution, in favour of the Pulse pattern, attributes this to saturation of splanchnic sequestration by the large protein dose only, leading to greater EAA bioavailability as indicated by plasma concentrations [187, 220]. Measurement of plasma AA concentrations during a future distribution would enable further comparisons with previous results, and may help to reconcile the wide variety of responses protein distribution reported in older adults.

The most appropriate measure of MPS in this context is using D_2O tracer methodology over a period of multiple days [84]. However, in the Chapter 5 study this technique was not without its problems, namely the nausea and vertigo experienced by some participants which led to substantial delays. This is a known side effect which can usually be mitigated by dividing into smaller, spread-out doses [71]. This incidence of reactions in this study is difficult to explain, as doses were appropriately space to minimise the chance of side effects, and anecdotal reports and published papers from studies using the technique indicate that reactions are not common. Robinson et al. (2011) reported a slight dizziness in the participant with the lowest body weight [77], however here there was no distinguishing characteristic of the participants who experienced a reaction. This would need to be a consideration when designing future studies, particularly when planning recruitment and timelines, to account for the consequent withdrawals.

6.4 Conclusions

Loss of muscle mass and function is a significant problem in older age, and dietary protein is one lifestyle factor which may be manipulated in order to promote muscle

health and attenuate this decline. However, there appears to be no augmentation of RET benefits with protein supplementation, and evidence of an effect of daily dietary protein distribution is mixed at best. Even when distributions are designed for optimal MPS according to an acute data theory, MPS was not influenced by protein distribution. There is currently no basis to alter dietary protein recommendations to include a suggested distribution or per meal dose size, however there is still scope for future research to fully investigation the potential influence of dietary protein distribution.

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