MECHANISMS OF AND COUNTERMEASURES TO AGE-RELATED
MUSCLE ANABOLIC RESISTANCE AND SARCOPENIA

By

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ABSTRACT

Age-related skeletal muscle wasting, or sarcopenia, negatively impacts physical function and health. The mechanisms relating to sarcopenia are complex and unclear, but are thought to be underpinned by a blunted response of skeletal muscle to anabolic stimuli. Accordingly, Chapter 2 explored how dose, timing, distribution and source of dietary protein intake differed between healthy young (23.6 ± 4.2 yrs) and community dwelling older (77.2 ± 6.8 yrs) individuals. We showed that, whilst the recommended dietary allowance for protein of 0.8 g·kg⁻¹·day⁻¹ was met by most individuals, total protein intake was lower and skewed across meals in old. The observation of suboptimal protein intakes in older individuals has important implications for skeletal muscle maintenance. Therefore, in Chapter 3 we investigated the muscle anabolic response in young lean (25.5 ± 4 yrs), old lean (71.7 ± 6 yrs) and obese old (69.1 ± 2 yrs) individuals to a suboptimal protein dose of 15 g. Whilst young lean and, to a lesser extent, old lean significantly increased postprandial myofibrillar protein synthesis (MyoPS), this response was blunted in obese old. Furthermore, MyoPS correlated positively with step count and negatively with leg fat mass in old. Based on the observed age-related decrease in MyoPS, Chapter 4 assessed the purported anabolic properties of the nutraceutical phosphatidic acid (PA) to increase MyoPS in healthy older individuals. Upon acute PA consumption, MyoPS was unexpectedly blunted in the late phase of resistance exercise recovery, and was accompanied by impaired intramuscular anabolic signaling. In conclusion, this thesis enhanced our understanding on the mechanisms underpinning sarcopenia by investigating protein intake and pattern in young and older individuals, demonstrating the detrimental effects of obesity and low physical activity on the muscle anabolic response to a suboptimal protein feed, and establishing the muscle anabolic effects upon acute PA consumption.
“Age has no reality except in the physical world. The essence of a human being is resistant to the passage of time. Our inner lives are eternal, which is to say that our spirits remain as youthful and vigorous as when we were in full bloom.”

Gabriel García Márquez, novelist (1927 – 2014)
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Canadian Society for Exercise Physiology, Toronto 2105 – Poster communication. Short inter-set rest blunts resistance exercise-induced increases in myofibrillar protein synthesis and intracellular signalling in young males.


European Sports Science Conference (ECSS), Ruhr, July 2017 – Oral communication. Age-related anabolic resistance of Myofibrillar protein synthesis to moderate-dose protein ingestion is exacerbated in obese inactive individuals.
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*Under review*

Smeuninx B, McKendry J, Wilson D, Martin U, and Breen L. Age-related anabolic resistance of myofibrillar protein synthesis is exacerbated in obsess inactive individuals. Submitted to *Journal of Clinical Endocrinology and Metabolism*. 
LIST OF ABBREVIATIONS

1RM  one repetition maximum
4E-BP1  eukaryotic initiation factor 4E binding protein 1
µL  microliter
µmol  micromolar
AA  amino acids
Akt  protein kinase B
BMI  body mass index
CHO  carbohydrate
CRP  c-reactive protein
DAG  diacylglycerol
DAGK  diacylglycerol kinase
DGKζ  diacylglycerol kinase ζ
D₂O  deuterium oxide
DXA  dual energy x-ray
EAA  essential amino acids
EDTA  ethylenediaminetetraacetic
eEF2  eukaryotic initiation factor 2
eIF  eukaryotic initiation factor
ERK  extra-cellular regulated pathway
EWGSOP  European Working Group on Sarcopenia in Older People
FFM  fat free mass
FIPI  5-fluoro-2-indolyl des-chlorohalopemide
FKBP12: FK506-binding protein
FKBP36: FK506-binding protein 36
FM: fat mass
FRB: FK506-binding protein rapamycin binding
g: gram
GCMS: gas chromatography mass spectrometry
G3P: glycerol-3-phosphate
GDP: guanosine diphosphate
GTP: guanosine triphosphate
h: hour
HbA1C: glycated haemoglobin
IL-β: interleukin β
IL-6: interleukin 6
IMCL: intramyocellular lipid
IRMS: isotope ratio mass spectrometry
kcal: kilocalorie
kDa: kilo Dalton
kg: kilogram
L: litre
LPA: lysophosphatidic acid
LPAAT: lysophosphatidic acid acyl transferase
Leu: leucine
m: meter
MEK 1/2: mitogen-activated kinase ½
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>mg</td>
<td>milligram</td>
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<tr>
<td>min</td>
<td>minute</td>
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<tr>
<td>mL</td>
<td>millilitre</td>
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<tr>
<td>mmol</td>
<td>millimolar</td>
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<tr>
<td>MPB</td>
<td>muscle protein breakdown</td>
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<td>MPS</td>
<td>muscle protein synthesis</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>mTORC1</td>
<td>mechanistic target of rapamycin complex 1</td>
</tr>
<tr>
<td>mTORC2</td>
<td>mechanistic target of rapamycin complex 2</td>
</tr>
<tr>
<td>MuRF1</td>
<td>Muscle Ring Finger 1</td>
</tr>
<tr>
<td>MyoPS</td>
<td>myofibrillar protein synthesis</td>
</tr>
<tr>
<td>NEAA</td>
<td>non-essential amino acids</td>
</tr>
<tr>
<td>NPB</td>
<td>net protein balance</td>
</tr>
<tr>
<td>NSAID</td>
<td>non-steroidal anti-inflammatory drug</td>
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<tr>
<td>p70S6K1</td>
<td>ribosomal protein S6 kinase 1</td>
</tr>
<tr>
<td>PA</td>
<td>phosphatidic acid</td>
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<tr>
<td>Phe</td>
<td>phenylalanine</td>
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<td>PL</td>
<td>placebo</td>
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<tr>
<td>PLD</td>
<td>phospholipase D</td>
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<tr>
<td>PRAS40</td>
<td>proline-rich Akt substrate 40 kDA</td>
</tr>
<tr>
<td>RAG</td>
<td>recombination-activating genes</td>
</tr>
<tr>
<td>Raptor</td>
<td>regulatory-associated protein of mTOR</td>
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<tr>
<td>RDA</td>
<td>recommended dietary allowance</td>
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<tr>
<td>RE</td>
<td>resistance exercise</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>------------------------------------------------</td>
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<tr>
<td>Rheb</td>
<td>ras homolog enriched in brain</td>
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<td>RMR</td>
<td>resting metabolic rate</td>
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<td>RSMI</td>
<td>relative skeletal muscle mass index</td>
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<tr>
<td>SPPB</td>
<td>short physical performance battery</td>
</tr>
<tr>
<td>TEI</td>
<td>total energy intake</td>
</tr>
<tr>
<td>Thr</td>
<td>Threonine</td>
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<tr>
<td>TNF-α</td>
<td>tumour necrosis factor alfa</td>
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<tr>
<td>TSC1/2</td>
<td>tuberous sclerosis ½</td>
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<tr>
<td>VO₂</td>
<td>oxygen uptake</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
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CHAPTER 1

GENERAL INTRODUCTION
1.1 Musculoskeletal Ageing

Skeletal muscle mass accounts for approximately 40% of total body mass and is indispensable for gait, posture, metabolic health and independent living (65, 183, 206). Furthermore, skeletal muscle mass is the primary storage site for amino acids (AA), which can be released and used as a fuel or substrate for other tissues (142). With a constant turnover rate of 1-2% per day, skeletal muscle mass is continuously built and broken down (40). This tightly regulated and complex process results in an overall skeletal muscle mass accretion throughout adolescence to peak in early adulthood, and decline from the 4th-5th decade of life onwards at an annual rate of ~0.6% (111). The decrease in skeletal muscle mass is associated with myriad negative health outcomes, ranging from an increased susceptibility to fractures (101), insulin resistance (94), lower resting and basal metabolic rate (239), cardiovascular and metabolic disease (172) and, in severe cases, death (126). However, it is important to recognize that the gradual decline in skeletal muscle mass over time is interspersed with various acute health-related events such as immobilization and bed rest (during illness or hospitalization) (58, 85, 151). These acute events are thought to punctuate the ‘normal’ decline in muscle mass, accelerating the progression towards functional impairment and disability, beyond which, individuals are not able to perform activities of daily living without assistance (Figure 1.1) (24).

Decreases in skeletal muscle mass are often accompanied by reductions in muscle strength. Disturbingly, age-related decline in strength occurs at significantly greater rate than muscle mass, with annual reductions commonly reported to range from 3-4% in males and 2.5-3% in females (47, 133). This disproportionate loss of muscle strength to muscle mass could be indicative of a loss of muscle quality. Therefore, maintaining or increasing skeletal muscle mass is key to prevent age-related declines in muscle strength (65, 89, 135) and preserve physical function. To date, we are yet to fully understand the mechanisms underpinning age-related
musculoskeletal deterioration. Gaining insight into the nature of these mechanisms is of paramount importance to develop new therapeutic interventions to prevent or even treat sarcopenia.

Figure 1.1: Scenarios of age-associated decline in skeletal muscle mass. Each respective dotted grey line indicates the hypothetical chronological decline in muscle mass before or after an illness/injury if no other major disuse related events were to happen. The red line is the decline in muscle mass observed with periods of disuse. The solid black line represents the actual rate of decline. Redrawn from Witard et al. (2016) (227).

1.1.1 The Ageing Demographic

Worldwide, both more developed and least developed nations are growing older. Life-expectancy in the U.K. at birth rose from 73.4 y in 1991 to 78.9 y in 2012. Individuals who were aged 65 in the year 2000 were likely to live another 12 y, whilst individuals aged 65 in
the year 2012 were expected to live for 16 more years (149). This increased longevity is mainly attributable to better health care and increased living standards (150), which in combination with the post-World War II ‘baby-boom’ has contributed to an ageing population (168). In the U.K., the number of people aged 75 y and over is expected to rise by nearly 90% by 2039, reaching 9.9 million individuals. Importantly, the fastest growing sub-population of older individuals are the centenarians, whose rise is projected to increase six-fold from 14,000 in the year 2014 to 83,000 in 2039 (149).

Increased lifespan might, at first, appear to be a positive outcome. However, ageing demographics only tell one side of the story, as the gap between lifespan (i.e. number of years lived) and health-span (i.e. number of years live in good health) is expanding, which is reflected by a disproportionate number of older individuals using health services (150, 181). Therefore, healthy ageing should be prioritized over an increase in lifespan *per se*. It is clear that our society requires clear strategies to deal with the ever-expanding ageing population through better and more affordable healthcare. Influential governing bodies, such as the World Health Organisation (WHO), will undoubtedly play a pivotal role in fulfilling these shortcomings.

1.1.2 Sarcopenia: Definitions, Prevalence and Healthcare Burden

The term sarcopenia, derived from the Greek words “sarco” and “penia” meaning “flesh” and “poverty”, respectively, was first introduced by Irwin Rosenberg in 1989 to describe the age-related skeletal muscle mass loss (165). The complex and multifactorial aetiology of sarcopenia has led to numerous definitions. In 1998, the relative skeletal muscle mass index (RSMI) was introduced. The RMSI is obtained by dividing appendicular skeletal muscle mass (kg) by height (m²). An individual is classed as sarcopenic if the obtained RMSI is two or more standard deviations below that of either a young male or female reference group (14). In 2002, Janssen
and colleagues expanded this definition by using total, instead of appendicular skeletal muscle mass, and by differentiating between Class 1 (RSMI one to two standard deviations below that of a reference group) and Class 2 (RSMI over two standard deviations below that of a reference group) sarcopenia (110). However, it wasn’t until 2010 that the European Working Group on Sarcopenia in Older People (EWGSOP) developed a widely accepted and more practical clinical definition of sarcopenia based on set diagnostic criteria. The EWGSOP consensus describes sarcopenia as “a syndrome that is characterised by a progressive and generalised loss of skeletal muscle mass and strength with a risk of adverse outcomes such as physical disability, poor quality of life and death” (51). Based on muscle mass, muscle strength and physical performance, three stages of sarcopenia were identified by the EWGSOP. Stage 1, termed ‘pre-sarcopenia’ is characterized by a decreased muscle mass only. Stage 2, ‘sarcopenia’ is low muscle mass accompanied by either decreased muscle strength or performance. Stage 3, ‘severe sarcopenia’ is defined by a decrease in all three parameters (51). Furthermore, the EWGSOP attempted to clarify discrepancies between various definitions of sarcopenia by providing clear guidelines on measurement methods to assess each of the above diagnostic criteria. Skeletal muscle mass is typically quantified through computed tomography (CT), dual energy x-ray absorptiometry (DXA) or bioelectrical impedance or other anthropometrics (i.e. mid-arm circumference or skin-fold assessment). However, the latter is prone to erratic outcomes and is not recommended. Muscle strength should be determined using handgrip strength, knee flexion and/or extension power/strength, or peak expiratory flow. Finally, physical performance assessment is recommended using the Short Physical Performance Battery (SPPB) (96). However, limiting methods to assess muscle mass to modern, expensive and often exclusive machinery, poses a challenge to assess these diagnostic criteria in epidemiologic studies (108).
The prevalence of sarcopenia is difficult to determine with any accuracy as projections are often somewhat conflicting. Based on the specific population studied and definition of sarcopenia used, many studies have likely under- or over-estimated the actual prevalence of this condition. Generally, it is believed that the prevalence of sarcopenia ranges from 5-24% in persons aged under 80 y and increases to >50% in those aged 80 y and over (12, 14). With advancing age, the sarcopenic prevalence is thought to be higher in females due to lower initial levels of muscle mass and energy intake (110). Interestingly, sarcopenia is not exclusively limited to what one might consider to be ‘older’ individuals; with investigators observing sarcopenia even in middle-aged (45-54 yrs) community dwelling individuals (43). Considering the myriad negative health outcomes associated with sarcopenia, this condition places a significant strain on healthcare services and costs. Research investigating the socio-economic burden of sarcopenia is scarce. However, a study conducted in the U.S. in 2004 estimated the sarcopenic cost at $18.5 billion per annum (~1.5% of healthcare expenditure) (112). These data were based on individuals who expressed a loss of muscle mass, and is therefore most likely to be an underestimation. More recently, a study attributed a 58.5 % and 34 % increase in hospitalisation costs in those aged under 65 y and over 65 y, respectively, to sarcopenia (181).

1.1.3 Sarcopenic-Obesity: The Confluence of Two Epidemics

Besides sarcopenia, obesity is one the greatest public health challenges of the 21st century (188). The prevalence of obesity has tripled in several countries in the last few decades according to the WHO European Region, and has risen globally to over 600 million adults (224). Obesity itself is associated with a plethora of negative health outcomes, such as cancer (214), cardiovascular disease (106), diabetes (84) and is thought to contribute to the progression of
sarcopenia (118). On the contrary, a loss of muscle mass might lead to insulin resistance and facilitate fat mass gain (166, 182). Therefore, these two seemingly independent conditions are likely inter-related and, in co-existence, are referred to as ‘sarcopenic-obesity’ (150). The concurrence of obesity and sarcopenia seemingly act as a ‘double-edged sword’, in which the rate of muscle loss and fat mass gain are exacerbated, thus amplifying the risk of frailty, metabolic disease and mortality (219). Therefore, priority should be given to the development of affordable treatments to counteract the deleterious health consequences of these two combined conditions.

Sarcopenic-obesity is most commonly defined as having sarcopenia (according to the EWGSOP guidelines) in combination with being obese (body mass index ≥ 30 kg·m²) (42, 51). Besides the above-mentioned problems associated with characterising sarcopenia, obesity, although considered a disease, has not been clearly defined. Furthermore, complexities such as the age-associated ‘obesity paradox’, in which excess adiposity is thought to offer protective effects against skeletal muscle loss and other related health conditions, obstruct the pathway to a clear definition (45, 97). Nonetheless, obesity has most commonly been defined as having a body mass index (BMI) ≥30 kg·m² (42). Based on this definition, it is estimated that ~20% of the older population suffers from sarcopenic-obesity (12). The pathology of sarcopenic-obesity is complex and yet to be fully elucidated, mainly due to i) a vast range of implicated molecules and pathways (145, 195), ii) an overlap in the causes that might be involved in both conditions (184) and iii) age-related comorbidities that could act as confounding factors (48). Taken together, the global rise in sarcopenia, obesity, and sarcopenic-obesity, alongside the associated negative socio-economic impact, requires research to fill the hiatuses in our knowledge and understanding of how these conditions modulate skeletal muscle mass, strength and quality.
Furthermore, the search for effective non-pharmacological preventative and treatment countermeasures is essential.

1.2 Translational Regulation of Muscle Protein Turnover

Since the introduction of stable isotope amino acid tracer techniques, insights into the mechanisms regulating skeletal muscle mass have substantially improved (117). The continuous and complex interplay between muscle protein synthesis (MPS) and muscle protein breakdown (MPB) determine whether protein accretion or loss presides (159). In the postabsorptive state MPB exceeds MPS resulting in an overall negative net protein balance (NPB) and, ultimately, net muscle loss (17). Food ingestion, particularly protein-based nutrition, and skeletal muscle contraction (through physical activity/exercise) have the potential to promote net muscle protein accretion (19). Alterations in skeletal muscle mass are mainly caused by changes in MPS and, to a lesser extent, MPB (19, 155, 228). To date, the use of stable isotope tracers has provided us with invaluable information on how protein feeding and resistance exercise acutely alter MPS and MPB. Underlying these changes, are intramuscular signaling cascades that ultimately lead to increased or decreased translational efficiency. Over time changes in the net protein balance will lead to muscle mass accrual or loss (18, 155, 228). Developing novel methods to accurately measure these long-term changes in MPS and MPB remains an ongoing quest. Furthermore, unravelling the intramuscular mechanisms leading to alterations in skeletal muscle mass is of crucial importance to understand how anabolic stimuli, but also ageing, might influence the translational regulation of muscle protein turnover (52, 71, 164). In healthy young individuals not undertaking strenuous exercise training, diurnal alterations in MPS and MPB result in a neutral NPB or proteostasis, in which skeletal muscle mass is stable. From an evolutionary perspective, the energy demanding process of MPS will
only occur during favourable conditions. Therefore, it is safe to assume that this process is under strict regulation. The evolutionary intact serine/threonine protein kinase mTOR (mechanistic target of rapamycin) plays a crucial role in this complex process and consists of two functional, but distinct multiprotein complexes; mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (116). Both complexes are similar in that they both consist of the catalytic component mTOR and G protein β-subunit like protein, but are different in that mTORC1 additionally consists of raptor (regulatory-associated protein of mTOR) and PRAS40 (proline-rich Akt substrate 40 kDa), an insulin regulated mTORC1 inhibitor. Several signals, including those from growth factors, nutrients and mechanical stress, converge on mTORC1 to exert an influence on cellular growth. One of the most important activators of mTORC1 is Rheb (ras homolog enriched in brain). When in the GTP state, Rheb binds directly onto mTORC1 activating the complex (131). However, tuberous sclerosis 2 (TSC2) initiates GTP hydrolysis and is therefore a Rheb, and consequently mTORC1 activation, inhibitor (196). Several factors can converge on the TSC1/2 complex to modulate its GAP activity and relieve its inhibitory effects on Rheb (113).

Activation of mTORC1 leads to translation initiation, a process characterized by the binding of a ribosomal subunit to a messenger RNA strand (mRNA) starting its translation and formation of a polypeptide chain. The assembly of a ribosomal subunit at the mRNA strand requires the formation of the eukaryotic initiation factor complex, consisting of 3 eukaryotic initiation factors; eIF4E, eIF4G and eIF4a (132). The binding of eIF4E at the 5’ cap end attracts the two other eukaryotic initiation factors to form the eIF4F complex. However, when 4E-BP1 (eIF4E binding protein 1) is in a hypophosphorylated state, it binds eIF4E preventing the formation of the eukaryotic initiation complex (86). A well-described mechanism through which mTORC1
promotes translation initiation is through 4E-BP1 phosphorylation. When phosphorylated, 4E-BP1 will dissociate from eIF4E, allowing the eukaryotic initiation complex to be formed and promote translation initiation (35). Another well-studied target of mTORC1 is the ribosomal protein S6 kinase 1 (p70S6K1). As a protein kinase, it phosphorylates several downstream proteins all of which are involved in cell growth (132). One of the mechanisms exerted by p70S6K1 is the promotion of mRNA translation initiation through increasing the helicase activity of the eukaryotic initiation complex. Besides altering helicase activity, p70S6K1 activation can also increase translation elongation efficiency. When p70S6K1 becomes activated it phosphorylates eEF2k (eukaryotic elongation factor 2 kinase), which in turn becomes inactivated and hence alleviates its inhibitory effect on eEF2 (eukaryotic initiation factor 2) (218). Most commonly, p70S6K1 phosphorylation is measured at the Thr\textsuperscript{389} mTORC1-specific site. Although additional phosphorylation is required for full p70S6K1 activation, the Thr\textsuperscript{389} site correlates reasonably well to p70S6K1 activation and is hence often used in in vivo studies as a marker for translational efficiency (222).

1.2.1 Exercise Contraction and Muscle Protein Turnover

Both acute and chronic exercise acts as a potent modulator of muscle protein turnover (18). Studies investigating alterations in MPS and MPB during exercise contractile activity (i.e. mid-exercise) are scarce due to difficulties in achieving and maintaining a stable isotopic steady-state during conditions of exercise, or due to a lack of stable isotope incorporation into muscle tissue over such short periods of time (162). However, based on a study by Carraro and colleagues it is generally accepted that MPB rates are elevated and MPS rates attenuated during prolonged and vigorous exercise, resulting in an overall negative NPB (40). The majority of studies have investigated how exercise modulates muscle protein turnover in the recovery
period following exercise cessation. Generally, a 50-200% increase in MPS above basal rates has been reported over several hours after resistance exercise (RE) completion (18, 159). Without the intake of AA, the rise in myofibrillar protein synthesis upon RE returns to baseline after approximately 4 h (125), whilst mixed MPS stays upregulated for up to 48 h (159). A sigmoidal dose-response relationship has been observed in MPS stimulation upon RE exercise, reaching near maximal levels around 60-90% of 1 repetition maximum (29). Nevertheless, several RE-related factors such as load, volume and rest interval in-between exercising sets can influence the extent to which MPS is stimulated. A study by Burd and colleagues revealed that low-load RE was as effective as high-load RE in stimulating mixed and myofibrillar protein synthesis when performed to fatigue (33). Whereas McKendry et al have shown superior MPS rates in young men upon resting 5 min between RE sets compared with 1 min rest (134). Besides an upregulation in MPS, a concomitant increase in MPB rates of around ~30-50% are observed after RE completion (159). This post-exercise divergence in MPS and MPB responses are thought to improve overall NPB (159, 160). Assuming RE in the postabsorptive state would increase MPS rates to the extent that a positive NPB is achieved, would be premature. In order to achieve a positive NPB, amino acids (AA) are required as substrate to synergistically enhance MPS and further attenuate MPB rates (152). Importantly, one cannot necessarily extrapolate acute exercise-induced changes in MPS rates to quantitatively predict long-term skeletal muscle mass remodelling responses. Mitchell et al. reported that acute changes in MPS to RE plus protein ingestion did not correlate with the change in muscle mass seen after a 16-week RE training protocol (139). How MPS rates are altered over the course of a RE training regimen needs to be further defined, but one hypothesis is that alterations in MPS at the onset of RE training may be directed towards repair of muscle damage (53). Furthermore, the MPS response to a bout of RE changes as one progresses to a more trained state. It is possible that responders
and non-responders exhibit similar changes in MPS to an acute, first bout of RE but might show a different muscle anabolic response at some point during the training period (194). Measuring the MPS response to a bout of RE in the middle and at the end of a training regimen in the above-mentioned study performed by Mitchell et al (139) would have provided us with more insight on how the muscle anabolic response would change over time, and might be more predictive of a subject’s ability to gain muscle mass. Although a potent anabolic stimulus, RE is only a small part of an individual’s daily routine or life. Other factors such as nutrition (129) and daily physical activity outside structured exercise settings might have a significant impact on muscle mass gain or loss over time, and might therefore dilute the acute benefits of RE (31).

Therefore, it is important to measure MPS, and if possible MPB, over the period of several months to further elucidate how RE influences the ability to gain skeletal muscle mass.

The recent revival of deuterium oxide (D$_2$O) could potentially facilitate this quest, as it permits the assessment of MPS and MPB rates over chronic periods of time (weeks-to-months) under free-living conditions. Using D$_2$O in conjunction with acute stable isotope tracer infusions could resolve longitudinal and more acute MPS responses to RE training (27, 53, 176).

Mechanistically, RE has a profound impact on a number of proteins at a molecular level that regulate muscle protein turnover. Baar and Esser (7) were the first to observe how increases in skeletal muscle mass after a 6-week electrical stimulation protocol in rats positively correlated with acute changes in p70S6K1 phosphorylation. Since then, several studies have reported robust increases in p70S6K1 phosphorylation upon stimulation of MPS in animals (100, 220). One of the first pioneering in vivo studies validating the importance of mTORC1 on skeletal muscle growth was performed by Bodine et al (20). In this elegantly designed study, an mTORC1 inhibitor, rapamycin, prevented the occurrence of compensatory muscle hypertrophy
following synergist ablation in animals. When translating these observations into humans, similar results have been reported (60, 69). Administrating an acute dose of rapamycin (12mg) was able to block RE-induced increases in mTORC1 and MPS, confirming the critical role of mTORC1 in skeletal muscle plasticity (69). Furthermore, RE has been shown to downregulate eEF2, which, in a hypophosphorylated state, becomes active and improves ribosomal translocation along the mRNA strand (39), and upregulate protein kinase B (Akt) and 4E-BP1 (36, 134). Taken together, an acute bout of RE robustly increases MPS rates and, to a lesser extent, MPB rates through molecular networks that improve mRNA translation and regulate cell size.

1.2.2 The Role of Amino Acids

Amino acids (AA) fulfil several important bodily functions, especially as constituents for proteins and polypeptides. Of the 20 amino acids, 11 are endogenously synthesised, whilst the other 9 cannot be synthesised and are required through dietary consumption. These AA groups are respectively termed non-essential (NEAA) and essential amino acids (EAA). Protein nutrition is a potent stimulus for skeletal muscle accretion through stimulation of MPS and, to a lesser extent, attenuation of MPB (59). Of the AA, the EAA are thought to be predominantly responsible for feeding-induced alterations in MPS and MPB, with little-to-no stimulatory effect found for NEAA (179, 180, 201). A major muscle anabolic role has been attributed to the branched chain amino acid, leucine. In 1999, oral leucine supplementation was shown, for the first time, to stimulate MPS in rats (3). These observations have since been translated into in vivo mammalian and human studies, showing a similar capacity for leucine-induced stimulation of MPS (5, 73). In an elegantly designed study by Churchward-Venne and colleagues (46) the addition of leucine (3g) to low-dose protein ingestion (6.25g of whey) was
able to elicit a comparable MPS response to that observed with high-dose high-quality protein (25g of whey). Although many protein sources are capable of increasing postprandial MPS rates, the extent to which they do is largely dependent on the AA profile of the protein source ingested, the digestion and absorption kinetics, and the timing of protein intake in relation to exercise (23, 90, 202). Based on the absorption and digestive kinetics, proteins have been broadly classed as “fast” or “slow” proteins. Fast proteins, such as whey and soy, cause a rapid but transient rise in plasma AA, whilst slow proteins, such as casein, are more slowly released into circulation, causing a pattern of aminoacidemia that is lower but more sustained than so-called fast proteins. These different protein digestion and absorption kinetics modulate MPS each in different ways, with fast proteins causing a more pronounced rise in MPS rates than slow proteins (23, 193). Generally, MPS rates respond quite rapidly to an increased plasma AA availability, rising up to ~2-3 fold from basal levels at between 90-120 min post-feeding, returning to basal levels thereafter, even in conditions where plasma hyperaminoacidemia persist, a phenomenon referred to as the “muscle full” effect where MPS is thought to become refractory to the continued appearance of AA’s (137). Amino acids in se stimulate insulin secretion causing hyperinsulinemia. The exact role of AA-induced insulin release on MPS and MPB is much debated. Generally, it is accepted that a rise in insulin, up to a certain level and in the absence of sufficient AA’s, will stimulate MPS rates by upregulating the insulin receptor substrate (IRS)-Akt-mTORC1 pathway, and may play an important anti-proteolytic role when insulin levels are further elevated (92, 157).

As outlined above, molecular regulation of MPS and cell size is mainly modulated by mTORC1 activity, which can be modified by protein nutrition (through constituent EAA’s). Although our understanding of how AA modulate mTORC1 activity is not entirely clear, we do know that
AA are a crucial signal for mTORC1 activity, since growth factors are incapable of efficiently activating mTORC1 when AA are lacking (113). Put simply, AA promote the formation of the active RAG GTPase complex, causing mTORC1 to bind to this complex at the lysosome where it becomes activated (113). However, the first step in sensing AA availability for mTORC1 signaling is currently unknown. Recently, evidence has emerged supporting the importance of AA transporters in the sensing of AA levels and the subsequent activation of intracellular signaling cascades. Specific amino acid transporters could act as nutrient sensors and subsequently initiate cellular signals, or the binding of AA to the nutrient sensor is enough to evoke intracellular signaling events (107). Increased mTORC1 phosphorylation through AA provision is further linked to the phosphorylation of downstream factors p70S6K and 4EBP1, resulting in increased protein translation efficiency (4, 82). In summary, protein/AA provision in healthy young individuals increases MPS and attenuates MPB, through phosphorylation of mTORC1-mediated signaling pathway and enhanced capacity for mRNA translation efficiency and ribosome biogenesis.

1.3 Age-Related Alterations in Muscle Protein Turnover

As highlighted above, skeletal muscle mass declines from the 4th to 5th decade of life at a rate of ~0.6 % per annum (111). Intuitively, sarcopenia must be driven by alterations in muscle protein turnover that contribute to an overall net protein loss (24). In contrast to proteostasis in healthy young individuals, imbalances in muscle protein turnover in older individuals lead to greater periods of net protein loss, which are thought to primarily underpin the progression of sarcopenia (24). Initially, Trappe et al (203) attributed age-related skeletal muscle loss to an increase in basal-state rates of MPB. However, this study measured 3-methylhistidine (3-MH), a breakdown product from myosin and actin, as a surrogate marker for MPB in blood. This
experimental approach is liable to a few assumptions, such as accepting that the amount of methylated myosin and the amount of muscle sampled by the microdialysis is equal among young and old. Further to this work, Yarasheski et al (233) reported decreased basal-state rates of MPS in older individuals compared with the young. In line with these results, Balagopal and co-workers found a decline in mixed MPS and whole-body protein turnover from young to middle age (10), and Welle et al observed reduced myofibrillar MPS in older compared with younger individuals (221). Collectively, these studies infer that basal-state rates of MPS are reduced by 20-40% and MPB rates elevated by 30%. However, inferring the NPB from these values would result in a rate of skeletal muscle mass loss far exceeding the commonly reported 0.6% muscle loss per annum in older age. Since, studies have refuted the observed differences in postabsorptive MPS and MPB between young and older individuals, showing no differences in basal MPS and MPB (81, 213). Given the importance of protein-based nutrition and physical activity in the regulation of muscle protein turnover, researchers have turned their attention to understanding how ageing modifies skeletal muscle anabolic and anti-catabolic responses to these stimuli (24).

1.3.1 Age-Related Impairments in Exercise-Induced Muscle Protein Turnover

Despite recent advances in pharmaceutical interventions to enhance skeletal muscle mass, exercise is still the most potent stimulus for muscle protein accretion (124, 233). In young individuals, resistance exercise (RE) robustly increases MPS (134, 225) and, when repeated frequently over time, promotes muscle hypertrophy and strength increases (53). An important discovery is that the net anabolic response to RE is diminished in older age. A seminal study from Kumar et al (125) showed the MPS response to unilateral leg extension, across a range of intensities, was lower in older compared with younger individuals. Accompanying this blunted
MPS response to RE was a dampened p70S6K and 4E-BP1 signaling response (80). The phosphorylation status of these proteins was associated with MPS rates over 1-2 h post-exercise in young, but not older individuals (125). This sensitivity ‘shift’ in aged muscle might be alleviated by increasing exercise training volume. Specifically, follow-up work by Kumar et al (124) showed that doubling RE volume (i.e. from 3 to 6 sets of moderate-load training) restored a ‘youthful’ muscle anabolic response in older individuals, which was matched by a greater phosphorylation of p70S6K. The necessity in older individuals to double RE volume could be the results of a lower strength-related performance, i.e. older individuals lifted significantly less weight per unit muscle cross-sectional area, and, consequently, had to double RE volume to elicit a similar MPS response compared with younger individuals. An additional explanation could be found in Henneman’s size principle where slow twitch fibres are primarily recruited during the onset of RE, whilst increasing muscular force/fatigue will lead to an upregulation in the recruitment of type II fibres (99). Doubling RE volume in older individuals at 40% of 1RM would have led to greater muscle fatigue and hence increased recruitment of Type II muscle fibres, resulting in elevated MPS rates. On the contrary, doubling RE volume at 40% 1RM in younger individuals did not significantly alter MPS, as skeletal muscle in younger individuals is more fatigue resistant, and, hence, would have led to a lower recruitment of Type II fibres (208). Even though evidence supporting the age-related blunting in the anabolic response to acute exercise is compelling, others have been unable to reproduce these findings (68, 233). This might partially be due to the protein fraction isolated for MPS interrogation. The majority of studies that have assessed protein synthesis in a mixed protein fraction instead of an isolated myofibrillar fraction, have been unable to detect the presence of age-related anabolic resistance to feeding or exercise (189, 190, 212). Although myofibrillar proteins constitute ~60% of the mixed muscle protein pool, the residual 40% contains proteins with differing turnover rates that
could potentially alter/mask any differences in MPS between young and older individuals (28). Although limited, studies measuring myofibrillar protein fractional synthesis rates often do detect the evidence of age-related muscle anabolic resistance (8, 52, 95, 125).

Furthermore, some of the alterations observed in muscle protein turnover in old age might be caused by sexual dimorphisms. Although, no differences in postabsorptive or postprandial MPS rates are commonly observed between young-to-middle aged males and females (66, 83, 177), sex has been reported to affect MPS in older individuals. A study by smith et al (178) measured basal and postprandial MPS in young and old men and women. Compared with young and old men and young women, basal MPS was elevated in older women. Upon infusion of combined glucose, insulin and AA, MPS increased in young and to a lesser extent in old men, but did not increase in old women. It might be argued that the lack of postprandial MPS in older women is a consequence of higher basal MPS rates, or due to the relatively small amount of protein provided; 21g and 13 g in males and females, respectively, spread over the course of three hours. Furthermore, older women had a significantly greater relative fat mass compared with older men. Hence, it is questionable if the observed changes in MPS are due to gender dimorphism or are the consequence of differences in body composition or relative amount of ingested protein. To date, it appears that body composition rather than gender per se, plays a greater role in influencing muscle protein turnover in old age.

Resistance exercise training, is arguably the most effective measure to increase muscle mass and counteract the onset and progression of sarcopenia (78). In older individuals, the muscle remodelling response to RE training is somewhat impaired (93, 122). This might partially be due to deficits in cumulative MPS, lower ribosomal biogenesis, and inferior translational efficiency as measured by p70S6K phosphorylation (26). Nevertheless, RE training is an
effective method to increase muscle mass and strength (154) even in frail nonagenarians (78). Future studies should investigate the underlying mechanism for the blunted RE training-induced MPS response in older individuals in order to modify physical activity recommendations for this population.

1.3.2 Age-Related Impairments in Amino Acid-Induced Muscle Protein Turnover

Protein-based nutrition (via constituent EAA’s), is a potent stimulator of MPS in younger individuals (200). As extracellular EAA availability is of primary importance to stimulate MPS (21), and AA delivery to skeletal muscle does not appear impaired in older individuals despite a higher first-pass capture of AA in splanchnic tissues (212), anabolic resistance can be directly attributed to a muscle-specific defect. Early research, in which AA were administered to older individuals, suggested elevated MPS rates compared with younger individuals. However, in the absence of a young control group it was impossible to compare the influence of age per se on postprandial MPS rates (211). One of the first studies to show a blunted response to food ingestion, specifically protein nutrition, was conducted by Dardevet et al (54). Young and old rat muscles were treated in vitro with different concentrations of leucine, with a weakened MPS response evident in older muscles. Subsequent to this study, Cuthbertson et al. (52) provided young and older individuals with graded doses of crystalline EAA and showed a decreased responsiveness of MPS in older individuals. Since these pioneering studies, several others have explored the relationship between protein provision and MPS rates in younger vs. older individuals, with the general consensus being that older individuals are less responsive to low-moderate dose protein provision and may require larger protein-doses to overcome this muscle anabolic resistance (8, 95, 231). Recently, Moore et al. (143) provided strong evidence that the relative amount of protein required to maximally stimulate MPS is considerably greater in older
adults (0.4 g·kg⁻¹·body mass⁻¹) than in the young (0.24 g·kg⁻¹·body mass⁻¹). Put into context, for an average 75- to 80-kg older individual, this equates to 30 g of high-quality protein to maximally stimulate MPS. In support of these data, Wall et al. (216) have demonstrated that the MPS response to 20 g of casein protein ingestion is 16% lower in older than in young individuals. The precise mechanisms underlying this age-related anabolic resistance to protein provision are still unclear, but deficits in mTORC1-mediated signaling might partially explain this phenomenon. Specifically, p70S6K phosphorylation was reported to be less sensitive to in vitro stimulation by leucine in older compared with younger rat muscle, and was maximally stimulated only when higher leucine concentrations were used (54). In older humans, the relative protein content of mTOR, p70S6K and eIF2B was reported to be significantly lower compared with younger individuals and, when stimulated by EAA, phosphorylation of mTOR and p70S6K was significantly lower in older individuals (52).

Research translating the concept of age-related muscle anabolic resistance to protein provision from acute experimental models to chronic long-term observational studies is scarce and yields conflicting results. Whilst some studies fail to report any beneficial effects on skeletal muscle mass or strength following long-term protein supplementation in older individuals (127, 209), others have reported positive outcomes on muscle function and acute postprandial MPS rates (41, 62, 198). Methodological differences, such as the specific older population studied and dose/source of ingested protein, most likely underlie these conflicting findings between the aforementioned studies. Supplementing large amounts of high quality proteins or EAA over longer periods of time will most likely yield in muscle mass gains (62). However, the feasibility of merely increasing protein intake in older individuals is questionable as their appetite is downregulated (161). Furthermore, executing long-term supplementation studies over several
months are expensive and very demanding for both researcher and participant, especially of stable isotope tracers are used for the precise determination of muscle protein kinetics. Experimenting with protein fortified foods might be the way forward. These foods would allow an increase in protein intake without a concomitant increase in total energy intake. Most studies investigating the effectiveness of protein supplementation have been undertaken in a Caucasian study population. However, cultural and ethnical differences affect food choice and consequently protein intake (175). Furthermore, failure to include an exercise regimen alongside a nutritional intervention will most likely result in a sub-optimal muscle anabolic stimulus (199). Addressing these methodological difficulties might shed light on the potential therapeutic benefits of high protein diets or protein supplementation in healthy and frail older individuals.

In conclusion, the current consensus regarding changes in muscle protein turnover in older individuals that underlie sarcopenia iterates that both postabsorptive rates of MPS and MPB are unchanged with advancing age, and that any imbalance in NPB likely stems from a blunted MPS response to normally anabolic stimuli (i.e. exercise and protein nutrition). However, in stark contrast are the numerous studies that have been unable to detect the presence of muscle anabolic resistance to exercise and protein nutrition, alone or combined, in older individuals (5, 70, 119, 152, 212). Methodological discrepancies between studies might underlie these inconsistent findings. For example, the period of tracer incorporation over which MPS is measured varies greatly between studies. This has important implications on the interpretation of MPS data in young and older individuals. When MPS is measured over longer periods (≥ 6 h), it is likely that any peak transient MPS response is diluted by lower postprandial MPS rates during the later phase of protein ingestion. Given that MPS rates return back to basal
postabsorptive levels ~3 h post-protein ingestion, it is unlikely that muscle anabolic resistance will be detected in older individuals when MPS is measured over longer periods (174). However, it is important to realize that by measuring MPS, only one side of the story is told. Omitting MPB from the NPB equation makes it difficult to elucidate the effectiveness of an anabolic stimulus on skeletal muscle remodelling (176). Furthermore, age-related muscle anabolic resistance is unlikely to be observed when studies provide a robust enough anabolic stimulus to healthy older individuals with high levels of physical activity (174). For example, when older individuals ingest a very high protein dose in the context of an acute exercise setting, muscle anabolism is likely to be maximally stimulated. Considering the above information, anabolic resistance is most likely to be observed when older individuals are provided with a sub-optimal anabolic stimulus of physiological relevance to their habitual lifestyles (i.e. low-moderate dose protein ingestion).

1.4 Potential Mechanisms of Muscle Anabolic Resistance in Sarcopenia

Age-associated loss of skeletal muscle mass, strength and function is undoubtedly an inevitable part of chronological ageing, and is even apparent in master athletes whose exercise/activity levels far exceed those observed in the general ageing population (98). The mechanisms that induce age-related muscle anabolic resistance to exercise and/or protein nutrition are yet to be fully understood, but are essential to understand in order to treat or prevent the progression of sarcopenia. Factors contributing to muscle anabolic resistance in sarcopenia are thought to include, myosteatosis (i.e. excess subcutaneous and intramuscular fat accumulation) (191) and elevated levels of systemic and local pro-inflammatory cytokines, including tumour necrosis factor α (TNF-α), interleukin 6 (IL-6), c-reactive protein (CRP) and interleukin β (IL-β) (167). In addition to obesity and inflammatory-mediated mechanisms, reductions in sex hormones
(110) and neuromuscular apoptosis (238) may contribute the progression of sarcopenia, although it is not entirely clear how these parameters negatively impact muscle protein turnover in the old. Importantly, physical inactivity and dietary protein malnourishment may play an important role in modulating the extent to which these factors drive sarcopenia. Ultimately, these myriad contributing elements are not mutually exclusive, but can aggravate one another, hasten age-related skeletal muscle deterioration, and result in disability, morbidity and death (64, 110, 126). Figure 1.2 provides an overview of the impact of anabolic resistance on the muscle anabolic response.
Figure 1.2: Representation of the relationship between the optimal protein intake and postprandial muscle protein synthesis. Optimal protein intake is the amount needed to maximally stimulate MPS as defined by Moore et al via breakpoint analysis (143). Factors that affect the optimal protein intake can shift the protein intake curve to the left (positive) or right (negative). Anabolic resistance is represented as an increased protein intake to elicit maximal postprandial muscle protein synthesis. Redrawn with permission from English and Paddon-Jones (2010) (72).
As outlined above, protein nutrition is essential for skeletal muscle maintenance and function (37). The current recommended daily allowance (RDA) for protein intake in adults is 0.8 g·kg⁻¹·day⁻¹, which is believed to be adequate to fulfil bodily metabolic demands of this macronutrient. However, studies assessing dietary intake have reported an inability of older individuals to consume this amount of protein (15). This might be problematic as older individuals require a greater absolute and relative protein dose compared to younger individuals to maximally stimulate MPS (0.40 g·kg⁻¹·body mass⁻¹ per meal in older individuals vs. 0.24 g·kg⁻¹·body mass⁻¹ in the young) (143, 231). Recently, Cardon-Thomas and co-workers (38) studied dietary protein intakes in a local cohort of Birmingham-based older individuals to understand if these individuals reached the required protein intake ‘threshold’ of 0.40 g·kg⁻¹·body mass⁻¹ per meal set forth by Moore et al (143). Surprisingly, only 3, 42 and 68% of older individuals reached this threshold for protein intake at breakfast, lunch and dinner, respectively, even though total daily protein intakes were well in excess of the current RDA (38). Similarly, dietary protein consumption patterns in a Northern-American (15) and Dutch (197) individuals aged over 50 y and 75 y, respectively, revealed that the majority of protein was consumed during the evening meal. These results require researchers and health institutions to embrace a more pragmatic approach to dietary protein intakes in older individuals, possibly expressing protein intake on a meal-to-meal basis. Indeed, a case is being put forward by recent studies to increase dietary protein recommendations and promote equal protein distribution across meals in older individuals to enhance overall health outcomes (104, 141, 210). Besides dose and pattern of dietary protein intake, protein source has an important impact on the ability of younger and older individuals to stimulate MPS (232). The extent to which MPS
is stimulated upon protein intake is largely dependent on the AA profile, absorption and digestive properties of the protein source consumed (23). Protein sources that are rapidly digested and absorbed will robustly increase blood aminoacidemia/leucinemia and hence evoke elevations in MPS to a greater extent than those observed after the ingestion of slower digestive proteins with inferior AA or leucine profiles (21, 23). Based on this information, it is generally accepted that most animal-derived proteins instigate greater increases in MPS compared with plant-derived proteins (232). The suggestion that protein quality, besides protein quantity, is an important factor in regulating the extent to which MPS changes, led to the development of the Protein Digestibility-Corrected Amino Acid Score (PDCAAS). The PDCAAS gives common protein sources a score based on their absorption-digestion kinetics compared with a reference protein (i.e. egg, with a PDCAAS of 1.00). Protein sources with a lower PDCAAS are hence considered to be of inferior quality compared with those given a higher score. However, more recently concerns regarding the PDCAAS have been raised, as the scoring system was based on faecal rather than ileal protein digestibility, and the inability of distinguishing protein sources that are superior to the reference protein (156). These shortcomings are some of the reasons why the Digestible Indispensable Amino Acid Score (DIAAS) has been introduced and has been suggested a better protein quality ranking system (1). The DIAAS uses a theoretical reference protein that covers all the requirements for the indispensable amino acids, and can be calculated by dividing the amount (in mg) of digestible dietary IAA (as determined by a dietary test) by the amount (in mg) of the same IAA in the reference protein. The DIAAS reflects the minimal required intake for each individual AA and allows protein sources to score higher than the theoretical reference protein (1).

Meals in a typical Western diet are composed of both animal- and plant-derived proteins, with the majority of proteins originating from animals (90). These animal-derived protein sources
have the ability to robustly increase MPS, as they have a high leucine content and an AA profile that closely matches the bodily needs. The superiority of animal-derived proteins is, hence, generally reflected by a higher DIAAS (32). On the other hand, plant-based proteins often lack, or contain less of, one or more specific EAA, resulting in an inferior AA profile which consequently renders them inferior to most animal-derived proteins (207). Nevertheless, the total amount proteins derived from a mixed meal should provide all AA needed to fulfil bodily functions if consumed in large enough quantities. However, with advancing age, a decrease in appetite and chewing efficiency is often observed (138, 161), making it more difficult for older individuals to consume sufficient amounts of dietary proteins. Recommendations on dietary protein intake in older individuals should therefore be more pragmatic and promote guidelines ensuring a sufficient quantity of high-quality proteins are consumed with every meal.

1.4.2 The role of Inactivity and Disuse in Age-Related Muscle Anabolic Resistance

Maintaining appropriate physical activity levels is pivotal for healthy musculoskeletal ageing. UK physical activity guidelines propose older individuals perform 150 min of moderate-intensity physical activity per week. Furthermore, exercises to improve muscle strength should at least be performed twice weekly in combination with exercises to improve balance and prevent falls (91). Unfortunately, the current guidelines are somewhat vague and require refinement. Accurate assessment of physical activity levels and intensities in our ageing population is therefore of crucial importance. Unfortunately, physical activity is often assessed by means of retrospective questionnaires, activity diaries or interviews, and is hence prone to subjectivity and over- or under-reporting (128). With the introduction of accelerometry, these potential problems can be circumvented. Accelerometers have been used successfully as a valid and objective measurement tool to assess physical activity in a variety of different populations.
As accelerometry is fairly new and quite costly, only a small number of studies have utilised this tool in older individuals, reporting decreased physical activity levels with advancing age, leading into deleterious effects on muscle metabolism (130, 229). For example, merely reducing daily step-count in older individuals for 14 days induced muscle atrophy, blunted postprandial MPS rates and impaired insulin sensitivity (25). Besides decreased physical activity levels, older individuals often undergo protracted periods of disuse and immobilization due to hospitalization, falls or illness. These more drastic disuse events are associated, both in young and older individuals, with rapid muscle atrophy driven by alterations in muscle protein turnover (58, 63, 76, 120, 121). This was first demonstrated by Gibson et al (85), who reported a 30% reduction in postabsorptive MPS rates in a limb that had been immobilized for ~6 weeks compared with the non-immobilized limb. Since then, a number of studies have demonstrated a decline in postabsorptive MPS rates (76) and a blunted MPS response to AA provision (87, 215, 217) in response to disuse/immobilization events. The precise mechanisms underlying disuse-induced alterations in muscle protein turnover are unknown, but could partially be attributed to impairments in AA transport capacity and intramuscular signaling (67). Interventions to minimize or counteract disuse-induced muscle atrophy have included additional protein provision (77) and RE (147). However, it appears that older individuals require aggressive, tailored RE training compared with younger individuals to restore disuse-induced skeletal muscle losses (186, 187). The role of MPB in “healthy” models of disuse is dissimilar to the role in “diseased” disuse events. The latter is marked by a significantly elevated inflammatory state that elicits a pronounced rise in MPB, whilst in the former, systemic levels of inflammation are not drastically elevated and MPB is thought to play a lesser role (144, 158, 170).
As physical activity is a potent stimulator of MPS, the majority of studies advise older individuals to refrain from vigorous activity/exercise prior to a stable isotope infusion visit. However, only one study sought to directly monitor physical activity levels via accelerometry in the days prior to an infusion trial (44). Given the fact that the muscle anabolic response to protein nutrition is enhanced for up to several hours after exercise cessation (34), it is of utmost importance to rigorously control physical activity levels prior to the assessment of MPS. Physical (in)activity is a potent regulator of MPS in young and older individuals. However, our knowledge on the complex interactions between age-related muscle loss and physical activity levels is still in its infancy, and is to be further elucidated to help the development of successful anti-sarcopenic strategies.

1.4.3 The Role of Obesity and Inflammation in Age-related Muscle Anabolic Resistance

Undoubtedly, preserving muscle quality is of utmost importance to maintain robust anabolic sensitivity with advancing age (50, 57). Defining muscle quality is dependent on the physiologic perspective and methodology used to characterize the tissue parameters and assess performance. Although this has led to myriad definitions, the term ‘muscle quality’ allows us to look beyond the constructs of age-related muscle loss and explore how different facets influence this aspect (50). An important factor thought to influence muscle quality is adipose tissue and intramyocellular lipid (IMCL) infiltration, collectively termed myosteatosis (136). Ageing is, in most cases, associated with a decrease in whole-body fat-free mass and concomitant increase in adipose tissue mass (13). This ‘shift’ in body composition has various negative health outcomes and has been associated with mobility limitations (55) and all-cause mortality (16). Nevertheless, some studies advocate the potential protective effects obesity might exert in old age, especially during the recovery from adverse health events (97). Studies
supporting this apparent “obesity paradox” (where excess adipose tissue may protect ageing muscles from the wasting process) have most likely defined obesity based on BMI; a poor discriminator of fat mass. In comparison, studies using more accurate methods to determine whole-body fat mass have failed to support the protective effects of obesity on skeletal muscle in old age (45). Besides whole-body and regional changes in fat and fat-free mass, increases in IMCL content are apparent as one ages, irrespective of changes in body mass (57). Increased IMCL content has been associated with an increased risk for insulin resistance in healthy and young and older individuals and Type II diabetics (2, 88). The mechanisms through which IMCL may induce insulin resistance might be dependent on the lipid oxidative capacity of the muscle cell (88) and impairments in the insulin receptor substrate 1 (IRS1)-phosphatidylinositol 3-kinase (PI3K)-Akt pathway (153). More recently, lipid-induced insulin resistance has been suggested to be associated with muscle anabolic resistance to AA ingestion in healthy young individuals. Concomitantly with this impaired response to AA feeding, a suppression in the phosphorylation of 4E-BP1 was suggested as a possible explanatory mechanism (185). A study by Kouw et al. (123) demonstrated no difference in the postabsorptive and postprandial MPS rates in older normal-weight diabetic individuals. Collectively, these data indicate that myosteatosis, but not necessarily insulin resistance per se, may induce muscle anabolic resistance.

Skeletal muscle adipose infiltration is exacerbated by obesity and is linked to increased concentrations of systemic inflammatory markers (148). This increase in inflammatory markers is partially caused by a dysregulation in the secretion of both pro-inflammatory or anti-inflammatory ‘adipokines’ that will, over time, lead to chronic low-grade inflammation (11). Therefore, increased systemic concentrations of pro-inflammatory cytokines such as interleukin-6 and c-reactive protein are commonly found in obese individuals. This state of
chronic low-grade inflammation can result in insulin resistance, Type 2 diabetes and other metabolic diseases (205). Furthermore, low-grade inflammation has been shown to blunt the postprandial muscle anabolic response in old rats, providing evidence for the contribution of a low-grade inflammatory state towards sarcopenia (9). On the other hand, reducing low-grade inflammation through provision of non-steroidal anti-inflammatory drugs (NSAID; i.e. Ibuprofen) restored postprandial muscle anabolic sensitivity in old rats, allowing for greater muscle mass retention in these animals (163). In line with these results, Trappe and colleagues (204) observed greater increases in muscle volume and strength in older individuals upon consuming ibuprofen or acetaminophen in combination with resistance exercise. These results seem to support the idea that daily consumption of NSAID in older individuals will decrease systemic inflammation and consequentially support superior muscle remodelling. On the contrary, a study by Buffière et al showed no impairment in the mixed muscle FSR response to protein provision in older individuals suffering from low-grade inflammation (30). These conflicting findings could have been the consequence of a continuous, rather than bolus, administration of protein, which would likely result in a lower FSR response (223). This lower FSR response was probably further diluted by measuring MPS over a 5-hour period, as MPS peaks around 1h post protein ingestion (22). A study by Dideriksen et al. tried to overcome these problems by feeding their older subjects a bolus of whey protein and measuring MPS over a 3-hour period (61). Furthermore, a subgroup of older individuals with low-grade inflammation were treated with ibuprofen to investigate the effects of NSAID on the muscle anabolic response to protein feeding. Surprisingly, no differences between the control, placebo and ibuprofen group were found for postprandial MPS, indicating no beneficial effect of NSAID on the muscle anabolic response to protein feeding and exercise. To date, the long-term effects of NSAID consumption are somewhat unclear and warrant further examination.
Although research has highlighted the negative effects of obesity on muscle metabolic quality, investigations examining the complex interactions between ageing, physical activity, obesity and myosteatosis are lacking. Given the evidence outlined above, it is tempting to propose a model where obesity might be protective for muscle mass in old age up to a certain point, beyond which it becomes detrimental and elicits a precipitous decline in skeletal muscle mass and metabolic health. This hypothetical threshold for the protective effects of obesity on ageing skeletal muscle mass may be influenced by physical activity levels and myosteatosis, particularly IMCL infiltration. Exploring this hypothesis further could potentially shed light on these complex interactions that underpin age-related muscle anabolic resistance in sarcopenia and inform researchers on how best to tackle this problem.

1.5 Nutrient Interventions to Alleviate Age-related Muscle Anabolic Resistance and Sarcopenia

So far two main interventions to counteract sarcopenia have been discussed; exercise and protein nutrition. These two anabolic stimuli have been proven to alleviate and potentially treat/reverse sarcopenia. Nonetheless, attention has recently turned to uncovering novel therapeutic interventions with the potential to enhance the potency of exercise and protein-based nutrition in aged skeletal muscle and, thus, abolish age-related muscle anabolic resistance. Specifically, others have tried to identify various other nutritional compounds that might beneficially influence metabolic health. Such potentially ergogenic nutrients have been termed ‘nutraceuticals’ and have potentially important health benefits for a variety of individuals, ranging from athletes (79), frail older individuals (114) and those suffering from chronic diseases (171). Due to the novelty of the term, there is no set definition for nutraceuticals. In a recent review article, we defined nutraceuticals as a nutrient-derived
compound “with added extra health benefits” (173). More recently, Deane et al. (56) termed nutraceuticals as “a compound that alone or in tandem with exercise, impacts major physiological end-points”. At present, many of these potentially ergogenic nutrients have no solid scientific underbuilt and require further investigation.

1.5.1 Phosphatidic Acid: A Potential ‘Nutraceutical’?

The never-ending quest for nutritional compounds that might potentially promote skeletal muscle protein accretion has led to the recent discovery of phosphatidic acid (PA) (230). PA is a phospholipid consisting of a glycerol backbone with two fatty acids and a phosphate attached to it. The fatty acids are connected to neighbouring C-atoms at position sn-1 and sn-2, whereas the phosphate group is attached to the sn-3 position of the C-atom. Whether the fatty acid is saturated or unsaturated determines the ability for PA to activate mTORC1 and potentially upregulate MPS. However, when one or both fatty acids are unsaturated PA is able to increase mTORC1 activity (234). Most forms of PA have a saturated fatty acid at the sn-1 position, rendering the structure of the fatty acid at the sn-2 position determinative of PA’s ability to stimulate mTORC1. Soy-derived PA contains a higher concentration of unsaturated fatty acids, and is therefore probably more potent in upregulating mTORC1 activity, as derived from p70S6K1 phosphorylation, compared with egg-derived PA (115).

PA can be synthesised through three main pathways. The first major pathway is able to synthesise PA de novo via glycerol-3-phosphate (G3P), a by-product formed during glycolysis. When glucose is converted into fructose 1,6-biphosphate, it is cleaved into two units; glyceraldehyde-3-phosphate (not to be confused with G3P) and dihydroxyacetone phosphate (DHAP). The latter is catalysed by glycerol-3-phosphate dehydrogenase into G3P which is
subsequently acylated into lysophosphatidic acid (LPA) catalysed by glycerol-3-phosphate acyltransferase, and finally acylated by lysophosphatidic acid acyltransferase (LPAAT) into PA. It should be noted that this de novo PA production favours the production of PA species containing two saturated fatty acids. A second pathway forms PA from phosphatidylcholine (PC), a reaction catalysed by phospholipase D (PLD). PLD consists of two isoforms PLD1 and PLD2 and have been deemed important for mTORC1 activation as PLD inhibition by 1-butanol prevented any increase in mTORC1. However, recent research has condemned the use of 1-butanol to inhibit PLD activity as it does not always successfully reduce PA accumulation and might result in independent deleterious events. With the more specific PLD inhibitor 5-fluoro-2-indolyl des-chlorohalopemide (FIPI) advances in the field have been made and have shown a weak correlation between PLD activity and cellular PA concentration. Finally, PA can be formed through deacylation of triglycerides to diacylglycerol (DAG) and phosphorylation through diacylglycerol kinase (DGK) (Figure 1.3).

PA, a major constituent of the cell membrane, plays a key role in regulating various metabolic processes in the cell. The endogenous or exogenous upregulation of PA concentration could therefore pose a viable solution in the battle against sarcopenia (173).
Figure 1.3: Proposed model of endogenous (mechanical load) and exogenous phosphatidic (supplementation) acid-induced activation of mTOR leading to increased muscle protein synthesis. Redrawn from Shad et al (2015) (173).
1.5.2 Exercise-Induced Endogenous Phosphatidic Acid

As outlined above, RE is a potent stimulator for muscle hypertrophy, stimulating mTORC1 and downstream factors.Whilst the effects of exercise on mTORC1’s downstream factors are well-defined, less is known on how exercise affects factors upstream of mTORC1. In an attempt to unravel these upstream factors, Fang and co-workers stimulated HEK293 cells and observed an increase in PA concentration (75). They suggested a direct binding of PA to the FK506-binding protein (FKBP12)-rapamycin-binding (FRB) domain on mTORC1, which would be in direct competition with rapamycin. This led them to attribute a key role for mechanical load induced PA production in the process of cell growth (103, 146, 237).

However, the fundamental question of what mechanism was responsible for the mechanical load increased intracellular PA concentration was left unanswered. At first, hydrolysis of PC through PLD was believed to be the primary mechanism through which mechanical loading increased intracellular PA concentration. This notion was supported by a handful of studies successfully using 1-butanol to inhibit mechanical induced rises in mTORC1 activation and PA concentration. However, when the more specific PLD inhibitor FIPI was used, mechanical load induced increases in PA leading to mTOR phophorylation could not be prevented. More recently, research has focussed on PA synthesising enzymes of the DGK family, as earlier research showed a 2-fold increase in DAG and PA in rats after electrical stimulation of the sciatic nerve (49), and an upregulation in DAG content when rat cardiac myocytes were mechanically stretched ex vivo (169). A crucial role for the DGKζ isoform was set aside, as this specific isoform had been shown to induce mTORC1 dependent phosphorylation of p70S6K1(6). The importance of the DGKζ isoform was later confirmed by You et al., who in an elegantly designed study showed an impaired response in mechanically induced PA concentration and mTORC1 signalling in DGKζ/- mice (236). In summary, mechanical load
induced increases in intracellular PA are obtained through the conversion of DAG into PA and is catalysed by DGKζ. PA will then bind directly onto the FRB domain of mTORC1, enhancing its protein translation efficiency.

1.5.3 Exogenous Phosphatidic Acid Provision

Besides endogenous increases in PA concentration, PA can be administered exogenously through supplementation. Foods naturally rich in PA content are cabbage, tomato and cucumber (~0.55, 0.25 and 0.2 mg·g wet weight⁻¹). However, the way in which these foods are prepared might alter PA content, as for example boiling will lead to the inactivation of PA synthesising enzymes (192). Recently, PA has become commercially available as a supplement with claims as a potent stimulus for enhancing skeletal muscle mass. Beyond these claims, research is still required to fully elucidate the molecular pathways through which exogenous PA provision acts, and leads to muscle protein accretion. Cell culture and animal-based studies have proven to be an effective platform to begin unravelling these mechanistic pathways. Increasing PA availability in cell culture media of mouse wild-type C2C12 myoblasts (146) and feeding PA to rats was able to increase mTORC1-mediated p70S6K signaling, but did not alter Akt phosphorylation (140). Together, these results suggest an alternative pathway from insulin and growth factors through which exogenous PA provision stimulates mTORC1 phosphorylation. At first it was proposed that exogenous PA, similar to endogenous PA, competes with the mTOR inhibitor FK506 binding protein 38 (FKBP38) to bind the mTORC1 FRB domain. However, exogenous PA provision was not able to reverse FKBP38’s inhibitory effects and failed to increase mTORC1 stimulation when FKBP38 was knocked out (235). Alternatively, it has been proposed for PA to stimulate mTORC1 through the extracellular regulated kinase (ERK) pathway. In order for PA to exert its stimulatory effect through the ERK pathway, it
needs to be converted into LPA. Once converted, LPA will bind its receptor ‘endothelial differentiation gene’ (EDG2) and subsequently activate G-protein, Ras, mitogen-activated kinase (MEK1/2) and ERK. ERK activation then leads to the phosphorylation of TSC1/2, which in turn places Rheb in its GTP bound state, allowing it to bind and activate mTORC1 (173). Evidence supporting this pathway was provided by Winter et al (226) showing that MEK1/2 inhibition negated PA and LPA induced ERK and p70S6K phosphorylation. Besides stimulating MPS, PA has been touted an inhibitor of MPB via attenuation of atrophy related genes (109). More recently, acute PA supplementation has been shown to increase MPS in rodent skeletal muscle compared to a placebo. However, when PA and whey protein were given together MPS was attenuated compared to either condition alone (140). Adding PA to whey protein might alter the pathways leading to mTORC1 activation and thus shift peak MPS (56). Taken together, more research, and especially human research, is needed to fully elucidate how PA supplementation might lead to enhanced MPS (Figure 1.3).

1.5.4 Phosphatidic Acid and Human Skeletal Muscle Hypertrophy

Ex vivo and animal studies have provided us with promising results regarding PA’s potency to increase MPS and, potentially, decrease MPB. Based on the premise that endogenous PA acts through an ERK-independent (direct binding onto the mTOR FRB domain) pathway, and exogenous PA through an ERK-dependent pathway, it is tempting to speculate that activation of both pathways through feeding and exercise might heighten mTORC1 phosphorylation/activity leading to increased MPS. To date, evidence supporting this notion is scarce. The first study investigating the effects of an 8-week resistance training programme with or without daily ingestion of 750 mg soy-derived PA revealed a likely but non-significant benefit on increases in lean tissue mass, and strength improvements, assessed through 1
repetition maximum (RM) bench press squat (102). Although the study slightly supports the use of PA supplementation to increase muscle mass and improve body composition, some limitations need to be addressed. These limitations include lack of control over the timing of PA ingestion, no supervised resistance exercise training, and a small sample population potentially masking any differences between the treatment or placebo group. A study by Joy et al addressed these limitations and subjected 28 persons to an 8-week supervised resistance training programme. Fourteen subjects consumed a daily dose of 750 mg soy-derived PA, either split up in 450 and 300 mg to, respectively, consume 30 min prior and immediately after resistance exercise. On non-exercising days participants were asked to consume 450 mg PA with breakfast and the remaining 300 mg with dinner. Participants in the PA group significantly increased their lean body mass, rectus femoris cross sectional area and leg press strength over the placebo group. Even though the evidence of the two studies discussed above seem to support a likely positive effect of PA supplementation on increasing skeletal muscle mass, more participants, and a longer supplementation and training period might have been necessary to detect the possible anabolic benefits of PA over a placebo treatment. The necessity of subjecting ample participants to an extensive RE training regimen has been reiterated in a study by Hubal et al., revealing a wide range of muscular responses in both men and women to 12 weeks of progressive dynamic RE training (105). More research is needed to establish what mechanisms potentially underlie the PA-derived anabolic effects before this novel ‘nutraceutical’ compound might be applied as a potential intervention to counteract sarcopenia. Research should further explore the metabolic pathways through which PA might act in human skeletal muscle, through application of stable isotope tracer and muscle biopsy techniques to accurately measure the acute effects of PA on muscle protein turnover. Furthermore, research should establish what
the optimal dose and dosing strategy would be for PA to elicit its maximal skeletal muscle anabolic potential.

1.6 Specific Thesis Objectives

Based on the current gaps in our understanding of i) how dose and intake pattern of daily dietary protein differ between young and older individuals and contribute towards sarcopenia, ii) the mechanisms of age-related muscle anabolic resistance in sarcopenia, and iii) burgeoning interest in potential therapeutic strategies to counteract these deleterious age-related musculoskeletal phenomena, the studies presented in this thesis aimed to enhance our understanding on the mechanisms of and potential countermeasures to age-related muscle anabolic resistance to protein nutrition and exercise. Chapter 2 describes a study in which we determined dietary protein intake and patterns of consumption in healthy young and community-dwelling older individuals living in the Birmingham area. Three-day weighed food diaries were analysed to quantify daily and meal-specific protein intake in both younger and older individuals. Furthermore, given the importance of protein source on the ensuing muscle protein turnover response, the main protein sources consumed at each meal were assessed. We hypothesised that daily- and meal-specific relative protein intakes would be higher in younger compared with older individuals. Furthermore, we hypothesised that younger individuals would reach the threshold for maximal MPS stimulation more often than older individuals. Chapter 3 describes a study in which myofibrillar protein synthesis (MyoPS) rates were determined in the postabsorptive state and in response to moderate-dose protein provision in young lean, older lean and older obese individuals. Furthermore, physical activity, whole-body/muscle metabolic health and inflammatory characteristics of these cohorts were established to see if these parameters were associated with MyoPS rates. We hypothesised that postprandial MyoPS rates
would be impaired in old lean, but to a greater extent in old obese, compared with young lean individuals, and therefore associated with levels of physical activity, adiposity and inflammation. Chapter 4 describes a study in which the acute effects of oral phosphatidic acid ingestion on MyoPS rates and intramuscular signaling at rest and after exercise were determined in healthy older individuals. We hypothesised that phosphatidic acid ingestion alone would not stimulate MyoPS, but would modulate RE-induced intramuscular signaling and MyoPS rates in older individuals compared with a placebo treatment. Chapter 5 discusses the main findings of the studies described in the previous chapters and provides an overview of the main conclusions. Practical implications and recommendations for further research are provided.
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CHAPTER 2

PROTEIN NUTRITION AND AGEING
Lower Total Daily and Meal-Specific Dietary Protein Intakes in Community-Dwelling Older Compared with Younger Individuals.

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2.1 Abstract

Background: Sub-optimal dietary protein consumption may partially underlie age-related muscle loss (sarcopenia). Specifically, dose, timing, and distribution of dietary protein across the day might play an important role in regulating muscle anabolism in older individuals.

Purpose: The aim of the present study was to characterise daily and meal-specific dose and source of protein intake in young and older individuals. Methods: 30 healthy young (23.6 ± 4.2 yrs) and 40 healthy community dwelling older (77.2 ± 6.8 yrs) individuals were recruited. Dietary intake was recorded via the completion of 3-day weighed food diaries. Absolute and relative daily and meal-specific protein intakes were compared against the current recommended daily allowance (RDA) of 0.80g·kg⁻¹·day⁻¹, and the recently outlined threshold for maximal muscle protein synthesis stimulation (0.24 g·kg⁻¹ and 0.4 g·kg⁻¹ in young and old respectively). Results: Average daily dietary protein intake was higher in young compared with older (1.52 ± 0.5 vs. 1.14 ± 0.4 g·kg⁻¹·day⁻¹; P = 0.002), and correlated with total energy intake in young only (r² = 0.32, P < 0.001). Ninety-eight percent and 83% of young and older, respectively, met the RDA for protein of 0.8 g·kg⁻¹·day⁻¹, whilst 18% of young and 10% of older reported 3 daily meals with a protein content ≥0.24 and 0.4 g·kg⁻¹, respectively. Protein intake was uneven with 55% of young and older consuming the largest amount of protein at dinner. Main ingested protein source only differed between young and older at lunch (poultry/eggs and bread, respectively). Conclusion: Although the protein RDA was met by the majority of individuals, total protein intake and distribution across meals was lower in the older compared with young individuals, with important implications for maintenance of skeletal muscle mass.
2.2 Introduction

Dietary protein provision is essential to maintain skeletal muscle mass and overall health (23, 53). In young individuals, protein provision robustly stimulates muscle protein synthesis (MPS) and, to a lesser extent, decreases muscle protein breakdown (MPB), resulting in an overall net muscle protein accretion (7). Constituent essential amino acids (EAA) in protein, particularly leucine, drive net muscle protein accretion through potent stimulation of MPS, insulin secretion and upregulation of intramuscular signaling events that enhance mRNA translational efficiency (1). Taken together, it is clear that dietary protein nutrition is vital for maintenance of skeletal muscle mass.

Age-associated loss of skeletal muscle mass, strength and performance (sarcopenia; (46)) may be underpinned primarily by a blunted MPS response to protein-based nutrition (18, 49) that is exacerbated by illness (45) and lifestyle factors, such as inactivity (12) and bed rest (6). The current recommended daily allowance (RDA) for protein suggests 0.8 g·kg\(^{-1}\)·day\(^{-1}\) to be “adequate” and to meet the metabolic demand of this macronutrient. However, recent studies suggest protein intakes above the current RDA, in the range of 1.0-1.5 g·kg\(^{-1}\)·day\(^{-1}\), are associated with myriad health benefits for older individuals (4, 57). In addition to daily dietary protein intake, distribution of protein intake throughout the day is suggested to have a profound impact on MPS stimulation and overall net protein balance. It has been demonstrated that older individuals generally consume dietary protein in an uneven manner, with the majority of protein consumed in the evening meal (16). On a meal-by-meal basis, older individuals require a considerably greater protein dose (0.4 g·kg body mass\(^{-1}\)) to maximally stimulate MPS compared with the young (0.24 g·kg body mass\(^{-1}\)) (38). Thus, a more evenly distributed pattern of protein
intake through the day would, in theory, provide a greater muscle anabolic stimulus (43), although experimental evidence to support this notion is equivocal (31, 35).

Alongside the dose and pattern of consumption, the source of dietary protein is an important determinant of the acute MPS response in young and older individuals (61). Specifically, the AA profile, absorption and digestive properties of ingested protein may determine the extent of MPS stimulation (10). Proteins that elicit a rapid increase in blood aminoacidemia/leucinemia, generally stimulate MPS to a greater extent than proteins with slower digestive properties or an inferior AA/leucine profile (9, 10). Therefore, it is generally accepted that most animal-derived proteins evoke greater MPS stimulation compared with plant-derived proteins (51, 61). In a Western diet, protein consumption primarily originates from meat and dairy products (25). These protein dense products, in contrast to plant-derived proteins, have an AA profile that closely matches the bodily requirements, and are hence capable of robustly stimulating MPS (13, 56). As most mixed meals consist of both animal- and plant-derived proteins, all EAA should be provided if meals are consumed in large enough quantities. However, older individuals experience a loss of appetite and chewing efficiency (36, 44), making it challenging to consume sufficient meal protein. Furthermore, cultural and/or geographical differences influence dietary intake, making it difficult to compare findings across studies where different populations and/or age-groups are being investigated (50). Therefore, a more pragmatic approach is required in outlining protein intake guidelines for older individuals, that should be formulated on a meal-by-meal basis.

To date, information on alterations in dietary protein intakes across the life-span is scarce, and this may be hampering the development of tailored dietary advice for older individuals. The
majority of studies investigating dietary habits of older individuals; i) omit a young control group, making direct comparisons across age within the same cultural population difficult, ii) use 24 h dietary recall questionnaires to assess food intake, which may give inaccurate representation of habitual diet (55), and iii) is country- or culture-specific (16, 30, 52). Therefore, the aim of the present study was to directly compare the habitual dietary intake of healthy young and community dwelling older individuals living in the UK, with a focus on dietary protein intake. Further to this, we assessed meal-specific protein intake and source of intake at breakfast, lunch, dinner and snacks. We hypothesised that overall daily and meal-specific protein intakes would be greater in younger compared with older individuals, resulting in a higher proportion of younger individuals reaching the theoretical threshold for maximal MPS stimulation compared with their older counterparts. Furthermore, we hypothesised consumption of animal-derived protein to be more prominent in younger compared with older individuals.

2.3 Methods

2.3.1 Participants

Healthy young and healthy community dwelling older individuals were recruited from the Birmingham area (West Midlands, UK) to participate in the present study. Participants were aged between 18-35 yrs, or ≥ 70 yrs, and were eligible if deemed healthy as assessed by the outcome of a general health questionnaire, living independent, and ambulatory. All participants were informed on the study procedures and provided written consent prior to study commencement. Ethical approval was obtained through the University of Birmingham Research Ethics Committee (#13-1475A). The study conformed to the latest guidelines set by the Declaration of Helsinki (7th edition).
2.3.2 Dietary Intake Recording

Participant height and body mass were assessed to the nearest 0.1 cm and 0.1 kg, respectively, using a digital scale and stadiometer. Participants were provided with a 3-day weighed food diary, to be completed over 2 week-days and 1 weekend-day. All participants received written and verbal instructions on how to accurately complete the weighed food diary, which required participants to provide information on time, cooking method, brand, and weighed amount of all foods and drinks consumed during the 3-day period. Participants were provided with a set of kitchen scales (Wuwangni, WeiHeng, Hong Kong) to accurately determine the weight of foods and drinks ingested.

2.3.3 Nutritional Data Analyses

Weighed food diaries were analysed using Dietplan 7 software (Forestfield Ltd, West Sussex, UK, V7.00.46). Daily total energy intake, macronutrient and micronutrient composition data were generated. Dietary protein values were calculated relative to participant total body mass (g·kg⁻¹) and also as a relative percentage of total energy intake. Daily relative protein intake was compared with the current RDA for protein consumption (0.8 g·kg⁻¹·day⁻¹), and with an alternative recommendation (1.0 g·kg⁻¹·day⁻¹) (4), to determine the percentage of participants that met these recommendations. Daily dietary intake was divided into 4 time points; T1, T2, T3, and T4, respectively corresponding to breakfast (6.00h – 10.00h), lunch (11.30h – 14.30h), dinner (17.30 – 22.00h), and snacks (remainder of the day). Average protein intake was calculated for each specific time point, as was main source of protein consumed. Per meal protein intakes were compared against the 0.24 g·kg⁻¹ and 0.40 g·kg⁻¹ threshold for maximal MPS stimulation in young and older individuals (38), respectively, to assess the proportion of
individuals that managed or failed to reach this threshold. The most commonly consumed protein sources at each time point were determined for each individual. This was accomplished by ranking all dietary foods consumed over the 3-day period according to the total amount of protein they provided. The top five protein sources per meal were consequently determined and presented as a percentage of total protein intake for the respective meal.

### 2.3.4 Statistical Analysis

Data were analysed using Graphpad Prism V7.0 (Graphpad Software, CA, USA). Within group differences for meal-specific protein intake were identified using a 1-way repeated measures ANOVA, between group differences for meal-specific protein intake were analysed using a 2-way ANOVA. Differences between groups for total intakes were assessed using *t*-tests. Pearson’s correlations were calculated to determine relationships between protein intake and total energy intake (TEI). Sidak post hoc analyses were utilised to determine differences within and between group differences. A test of two proportions was used to determine differences in proportions between both groups. Significance was set at *P* < 0.05. All values are expressed as mean ± SD or SEM.

### 2.4 Results

#### 2.4.1 Participant Characteristics

Participant characteristics are presented in Table 2.1. In total, complete data for 70 participants, 40 young and 30 older, was analysed. Data from males and females was pooled for analysis due to no observed differences in average dietary protein intake, sources of protein intake or distribution pattern between males and females in young and older cohorts. No differences were
found between height and weight. However, older individuals had a significantly higher BMI compared with the young (P = 0.042).

Table 2.1. Participant characteristics

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<th>Characteristic</th>
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<tr>
<td>Sample size (N)</td>
<td>40</td>
<td>30</td>
</tr>
<tr>
<td>Male/Female (N/N)</td>
<td>26/14</td>
<td>13/17</td>
</tr>
<tr>
<td>Age (y)</td>
<td>23.6 ± 4.2</td>
<td>77.2 ± 6.8**</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>70.7 ± 11.9</td>
<td>71.7 ± 12.2</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>173.2 ± 8.4</td>
<td>169.7 ± 8.3</td>
</tr>
<tr>
<td>BMI (kg·m⁻¹)</td>
<td>23.4 ± 2.6</td>
<td>24.8 ± 2.9*</td>
</tr>
</tbody>
</table>

* indicates a significant difference from young (* P < 0.05, **P < 0.01). Values are presented as means ± SD.

2.4.2 Dietary Energy and Macronutrient Intake

No differences were found between young and older individuals for daily total energy intake (TEI) or carbohydrate intake. Dietary fat intake (as a proportion of TEI) was significantly greater in older compared with young individuals (P = 0.006). Significant differences were observed for absolute and relative daily protein intake, which were ∼35% (P = 0.002) and 32% (P < 0.001) greater in young compared with older individuals. In the young, both absolute and relative daily protein intake significantly correlated with TEI (r² = 0.32, P < 0.001 for both), whilst no correlation was found in older individuals. The current RDA for protein intake of 0.8 g·kg⁻¹·day⁻¹ was met by ∼98% in young and 83% in older individuals (Figure 2.1). However, the alternative recommendation of 1.0 g·kg⁻¹·day⁻¹ (27) were met by ∼83% and 60% in young and older, respectively. Examining relative protein intake in young and old on a meal-by-meal basis against the proposed threshold for maximal MPS stimulation (38) (0.24 g·kg⁻¹ in young
and 0.40 g·kg⁻¹ in older), resulted in a ~38%, 73% and 86% accomplishment in young, and 12%, 28% and 58% accomplishment in older for T1, T2 and T3, respectively. This was indicative of a significantly higher proportion in young compared with older to meet this threshold at each time point (P < 0.001 for all). An overview of average daily energy and macronutrient intakes is provided in Table 2.2.

![Figure 2.1: Average daily protein intake in healthy young and healthy community dwelling older individuals, expressed relative to body mass. Dashed line indicates current recommended daily allowance for dietary protein allowance of 0.8 g·kg⁻¹·day⁻¹. * indicates significantly different from old, P < 0.05. Values are presented as individuals and means ± SEM.](image_url)
### Table 2.2. Average daily energy and macronutrient intakes in young and old

<table>
<thead>
<tr>
<th></th>
<th>YOUNG</th>
<th>OLD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Energy Intake (kcal)</strong></td>
<td>2223 ± 454</td>
<td>2212 ± 482</td>
</tr>
<tr>
<td><strong>Total Protein Intake (g)</strong></td>
<td>109 ± 44</td>
<td>81 ± 25*</td>
</tr>
<tr>
<td><strong>Protein Intake (g·kg⁻¹)</strong></td>
<td>1.52 ± 0.5</td>
<td>1.14 ± 0.4**</td>
</tr>
<tr>
<td><strong>Protein (% TEI)</strong></td>
<td>19 ± 7</td>
<td>15 ± 3**</td>
</tr>
<tr>
<td><strong>Total CHO Intake (g)</strong></td>
<td>241 ± 76</td>
<td>238 ± 66</td>
</tr>
<tr>
<td><strong>CHO intake (g·kg⁻¹)</strong></td>
<td>3.56 ± 1.2</td>
<td>3.42 ± 1.2</td>
</tr>
<tr>
<td><strong>CHO (% TEI)</strong></td>
<td>43 ± 8</td>
<td>44 ± 6</td>
</tr>
<tr>
<td><strong>Total Fat Intake (g)</strong></td>
<td>86 ± 22</td>
<td>91 ± 24</td>
</tr>
<tr>
<td><strong>Fat Intake (g·kg⁻¹)</strong></td>
<td>1.25 ± 0.3</td>
<td>1.30 ± 0.4</td>
</tr>
<tr>
<td><strong>Fat (% TEI)</strong></td>
<td>33 ± 7</td>
<td>38 ± 7**</td>
</tr>
<tr>
<td><strong>Total alcohol intake (g)</strong></td>
<td>17 ± 22</td>
<td>9 ± 8</td>
</tr>
<tr>
<td><strong>Alcohol (g·kg⁻¹)</strong></td>
<td>0.25 ± 0.34</td>
<td>0.13 ± 0.12</td>
</tr>
<tr>
<td><strong>Alcohol (% TEI)</strong></td>
<td>5 ± 6</td>
<td>3 ± 3</td>
</tr>
</tbody>
</table>

TEI; total energy intake, CHO; carbohydrate. * indicates a significant difference from young (* P < 0.05, **P < 0.01). Values are presented as means ± SD.

#### 2.4.3 Dietary Protein Distribution

Daily dietary protein intake was distributed unevenly across the day with ~15, 29, 43, and 13% of protein in young and 20, 29, 41, and 10% of protein in older individuals being consumed at T1, T2, T3 and T4 respectively. In young, both absolute and relative protein intake were greater at T2 (P < 0.001 for both) and T3 (P < 0.001 for both) compared with T1, and were greater at T3 compared with T2 (absolute P = 0.01; relative P = 0.006). Furthermore, absolute and relative protein intake in the young were lower at T4 compared with T2 (P < 0.001 for both) and T3 (P < 0.001 for both). In older individuals, absolute and relative protein intake were greater at T3 compared with T1 (P < 0.001 for both). Protein ingested at T4 in older individuals was significantly lower compared with T1 (P = 0.004), T2 (P < 0.001) and T3 (P < 0.001). Significant differences between young and older individuals were only observed at T2 for
relative protein intake \((P = 0.049)\), and at T3 for absolute \((P = 0.009)\) and relative \((P = 0.023)\) protein consumption. Meal-specific protein intakes are presented in Figure 2.2.

**Figure 2.2:** Meal-specific average protein intakes in healthy young and healthy community dwelling older individuals. Dashed lines represents threshold protein intakes of \(0.24 \text{ g·kg}^{-1}\) and \(0.4 \text{ g·kg}^{-1}\), suggested for maximal stimulation of MPS in young and older, respectively (38). * indicates significantly different from T1, † indicates significantly different from T2, \(\zeta\) indicates significantly different from T3, # indicates significantly different from young. Values are presented as means ± SD. Significance was set at \(P < 0.05\).
2.4.4 Patterns of Dietary Protein Intake

The uneven daily protein distribution in both young and older resulted in 9 observed protein intake patterns (Figure 2.3). Approximately 93% and 90% in young and older, respectively, distributed their daily protein intake according to one of three most frequently observed intake patterns. In young, ~55% and 28% of individuals consumed the largest portion of protein at T3 and T2, respectively, resulting in following daily protein distributions; 16%, 26% 58% and 19%, 48%, 33%. Similar findings were observed in older individuals with ~60% and 20% of individuals consuming the largest proportion of protein at T3 and T2, respectively, resulting in daily protein distributions of 21%, 27%, 52% and 14%, 52%, 34%.

2.4.5 Common Sources of Dietary Protein Intake

Table 2.3 represents the 5 most commonly consumed protein sources at each meal for young and older individuals for each meal. At T1, young and older, primarily consumed animal-derived proteins in the form of milk. At T2, young mainly consumed animal-derived proteins (poultry/eggs), whereas older individuals predominantly ingested plant-derived proteins (bread). No major differences were observed at T3, with animal-derived proteins being the commonly ingested protein source. Protein intake at T4 in young was mainly derived from protein supplements (liquid and solid-form), whereas milk was the predominant protein source in older individuals.
Table 2.3. Top 5 most commonly consumed protein sources during breakfast (T1), lunch (T2), dinner (T3) and snacks (T4) in young and old.

<table>
<thead>
<tr>
<th></th>
<th>YOUNG</th>
<th></th>
<th>OLD</th>
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<tr>
<td></td>
<td>T1</td>
<td>T2</td>
<td>T3</td>
<td>T4</td>
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<tr>
<td></td>
<td>T1</td>
<td>T2</td>
<td>T3</td>
<td>T4</td>
</tr>
<tr>
<td>Milk</td>
<td>(30%)</td>
<td>Milk</td>
<td>(67%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(30%)</td>
<td>Bread</td>
<td>(30%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Poultry/eggs</td>
<td>Poultry/Eggs</td>
<td>(41%)</td>
<td>Poultry/Eggs</td>
</tr>
<tr>
<td></td>
<td>(30%)</td>
<td>Milk</td>
<td>(22%)</td>
<td></td>
</tr>
<tr>
<td>Milk</td>
<td>(67%)</td>
<td>Bread</td>
<td>(30%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(19%)</td>
<td>Fish</td>
<td>(22%)</td>
<td></td>
</tr>
<tr>
<td>Bread</td>
<td>(19%)</td>
<td>Red meat/Pork</td>
<td>(32%)</td>
<td>Red Meat/Pork</td>
</tr>
<tr>
<td></td>
<td>(13%)</td>
<td>Fish</td>
<td>(27%)</td>
<td></td>
</tr>
<tr>
<td>Poultry/Eggs</td>
<td>(11%)</td>
<td>Bread</td>
<td>(19%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(8%)</td>
<td>Pizza</td>
<td>(8%)</td>
<td></td>
</tr>
<tr>
<td>Yogurt</td>
<td>(19%)</td>
<td>Fish</td>
<td>(5%)</td>
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<tr>
<td>Yogurt</td>
<td>(8%)</td>
<td>Oats/Cereal</td>
<td>(5%)</td>
<td>Oats/Cereal</td>
</tr>
<tr>
<td></td>
<td>(17%)</td>
<td>Yogurt</td>
<td>(5%)</td>
<td></td>
</tr>
<tr>
<td>Cheese</td>
<td>(5%)</td>
<td>Vegetarian Meat Substitutes</td>
<td>(3%)</td>
<td>Vegetarian Meat Substitutes</td>
</tr>
<tr>
<td></td>
<td>(3%)</td>
<td>Yogurt</td>
<td>(5%)</td>
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<tr>
<td>Cheese</td>
<td>(5%)</td>
<td>Nuts</td>
<td>(5%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(5%)</td>
<td>Cheese</td>
<td>(5%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(3%)</td>
<td>Bread</td>
<td>(3%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(3%)</td>
<td>Cheese</td>
<td>(3%)</td>
<td></td>
</tr>
</tbody>
</table>
2.4.5 Common Sources of Dietary Protein Intake

Table 2.3 represents the 5 most commonly consumed protein sources at each meal for young and older individuals. At T1, young and older, primarily consumed animal-derived proteins in the form of milk. At T2, young mainly consumed animal-derived proteins (poultry/eggs), whereas older individuals predominantly ingested plant-derived proteins (bread). No major differences were observed at T3, with animal-derived proteins being the commonly ingested protein source. Protein intake at T4 in young was mainly derived from protein supplements (liquid and solid-form), whereas milk was the predominant protein source in older individuals.
Figure 2.3. Percentage of healthy young and community dwelling older individuals in each category of protein intake. Patterns depicted above each bar represent the relationship between breakfast, lunch and dinner, respectively, in each category.
2.5 Discussion

In the present study, dietary intake in young and older individuals was assessed over a 3-day period by means of weighed food diaries. Whilst no difference was found between groups for TEI, young consumed more protein on a daily basis than older individuals. Evaluation of dietary protein intake revealed that 98% and 83% of young and older individuals, respectively, met the protein RDA of 0.8 g·kg⁻¹·day⁻¹. However, the pattern of daily protein intake was uneven in young and older, and indicative of a sub-optimal protein distribution for maximal stimulation of MPS in older individuals at breakfast and lunch (i.e. ≤0.4 g·kg⁻¹). Protein sources consumed were similar between groups at all time points except for T2, at which young consumed larger amounts of animal-derived protein compared with older individuals.

Adequate protein nutrition is key to maintaining skeletal muscle mass in older individuals (29). Our data demonstrate that both absolute and relative daily protein intake was higher in young compared with older individuals, with no difference in TEI. Previously, others have reported lower relative protein intakes in older compared with younger individuals (47). However, in this latter study information on TEI and other macronutrients was omitted, making it difficult to deduct whether older individuals might have compensated for the reduced protein intake through consumption of different macronutrients. Herein, we demonstrate that older individuals negate the energy deficit instigated by a decreased protein intake through increased fat intake. In addition, protein intake and TEI were positively correlated in young but not in older individuals. This might suggest that merely increasing TEI in older individuals would not be a viable strategy to augment protein consumption. Instead, prioritising changes in the dietary composition (i.e. substituting nutrients high in fat for protein-dense foods) could be a more practical approach to increase total protein intake in older individuals. To date, an adequate
protein consumption is defined as a protein intake that meets, or exceeds, the RDA of 0.8 g·kg⁻¹·day⁻¹. In the present study ~98% and 83% of younger and older individuals, respectively, met this guideline, and were therefore considered to have adequate protein intake. However, in recent years, mounting evidence has emerged advocating improved musculoskeletal adaptations with higher protein intakes (29). In light of these findings, we assessed daily relative protein intake to an alternative RDA of 1.0 g·kg⁻¹·day⁻¹ (27), and observed that ~15% and 23% fewer younger and older individuals, respectively, met this alternative guideline. Put differently, ~60% of older individuals met the alternative guideline for protein intake, clearly emphasizing the need for strategies to promote and encourage an increase in dietary protein intakes in a large proportion of older individuals. Increasing dietary protein intakes up to, or beyond, the suggested alternative RDA of 1.0 g·kg⁻¹·day⁻¹, might be a viable approach to attenuate the sarcopenic progression.

The accurate assessment of dietary intake is key in the development of future dietary guidelines. In the present study, participants were provided with a set of weighing scales and asked to record the weight of all foods and drinks consumed over a three-day period. Although this method allowed for a meticulous assessment of dietary intake, some disadvantages need to be addressed (5). Individuals who are not used to cooking on a regular basis, and are hence not familiar with weighing out foods, might have found this a complex and time-consuming method to assess dietary intake. The demanding nature of recording the weight of all foods and drinks consumed might have impacted (un)intentionally one’s dietary behaviour (15, 41). Finally, the onerous task of keeping a weighed food diary, limits the period over which food intake can be assessed, as it needs to be balanced against the likelihood of poor compliance (41). Based on the Harris-Benerdict equation modified for light physical activity, the younger and older
individuals in the present study would have had a daily energy expenditure of approximately 2520 and 1959 kcal, respectively (28). These values are in line with the energy intakes of 2223 and 2212 kcal presently reported in young and older individuals, respectively. Furthermore, similar dietary intake values have been previously reported in a study by Cardon-Thomas (16) et al. and in a study by Tieland et al (52). Based on the above information, it is safe to assume dietary intake has been reported accurately in the present study.

Beyond total daily protein intake, per-meal protein intake has a significant impact on acute muscle protein turnover and, thus, skeletal muscle mass gains (2, 35, 37). The idea that per-meal protein intake in older individuals is an important determinant of muscle protein accretion is supported by a study revealing a positive association between leg lean mass and the number of meals ingested containing a protein dose of at least 30g (34). Whereas this latter study employed an absolute protein dose of 30g as cut-off point, we decided to utilise a threshold value based on a relative protein-dose for maximal MPS stimulation. This threshold value is expressed relative to body mass, and thus takes into account the possibility that heavier individuals might require a higher protein dose to meet the metabolic demand for this macronutrient. In the present study, protein threshold values were 0.24 and 0.4 g·kg⁻¹ for young and older individuals, respectively, as outlined by Moore et al. (38). The ability to meet, or exceed, this threshold 3 times on one of the measured days was only achieved by 18% of young and 10% of older individuals. Alarmingly, only four young and one older individual(s) met their respective threshold for maximal MPS stimulation with every main meal over the entire 3-day measurement period. Comparing average protein intakes per meal revealed that older individuals attained their respective threshold at dinner (T3) only, whereas young individual reached their threshold at lunch (T2) and dinner (T3). These observations reiterate the need for
meal-specific guidelines in older individuals. Promoting a more even distribution of dietary protein across the day might be of importance to maintain an elevated anabolic response in older individuals.

In the present study, daily protein consumption in younger and older individuals was uneven across the day, with the majority of protein consumed at T3. These data are consistent with recent observations in British (16), Dutch (52), and American (42, 43) cohorts of older individuals of varying health status. However, two longitudinal studies have challenged the superiority of an evenly distributed protein intake across the day, and have revealed no benefit of an even compared with uneven daily protein distribution in older individuals (3, 11). These conflicting results could be explained by the fact that meals in the evenly spread protein group were <0.4 g·kg⁻¹, and hence not fully saturated MPS. Furthermore, Kim et al. (33) recently investigated the effects of uneven vs. even protein distribution over 8 weeks in older individuals on muscle mass accretion, strength and function. Even though the per meal protein dose in the evenly spread condition in this study was sufficient to theoretically maximize MPS in older individuals, no differences were observed between groups in muscle mass, strength or function. These results aligned with earlier acute work from the same group, demonstrating no differences in the MPS response to even and uneven protein ingestion patterns (32). The authors argued that higher protein intakes might be required in the context of a mixed meal to further enhance the muscle anabolic response in older individuals and that an 8-week intervention period might not have been long enough to detect any significant changes between both the even and uneven protein intake group. Indeed, a study by Dillon and colleagues where older females were supplemented over a 12-week period with EAA did find increases of 1.7 kg in lean body mass (20). As sarcopenia is characterized by a loss of muscle mass over several years,
it is important to consume protein intake doses capable of saturating acute MPS responses with every meal throughout the life-span.

The premise underpinning the superiority of an evenly spread protein consumption throughout the day is based on a cut-off, or threshold, value sufficient to elicit a maximal MPS stimulation (in this case 0.24 and 0.4 g·kg⁻¹ in young and older individuals). That this amount of protein would elicit a maximal muscle anabolic response is somewhat presumptuous, as postprandial muscle protein accretion may also be dictated by rates of MPB (8). Protein ingestion provides a stimulus that increases MPS and, to a lesser extent, decreases MPB (59). The anti-proteolytic effect upon protein ingestion is thought to be mediated by insulin secretion, of which a small amount may also contribute toward postprandial MPS rates (26). However, the role of postprandial insulin secretion in the regulation of muscle protein turnover may be impaired with ageing (24, 58). Therefore, one could assume that increasing protein consumption beyond the point of MPS saturation in older individuals might promote an insulinemic response that would attenuate MPB (19). To date, it is yet to be elucidated if protein intakes beyond the threshold of 0.4 g·kg⁻¹ in older individuals have the potential to instigate a greater positive net protein balance leading to long-term muscle mass accretion/maintenance.

Not only are protein dose and timing of major importance to augment MPS, the specific protein source ingested will significantly influence the extent to which MPS is stimulated. High-quality proteins, able to robustly stimulate MPS, are defined by rapid absorption/digestion kinetics, and a high proportion of EAA and leucine (10, 21). Based on these characteristics, most animal-derived proteins are considered a “high-quality” protein, whilst plant-based proteins are in most cases considered a “lower-quality” proteins (40). In addition, the proportion of AA required by
the human body corresponds more closely to the AA profile observed in animal-derived proteins, whilst plant-based proteins might contain insufficient amounts of certain AA to support bodily functioning (56). Identifying the most commonly consumed dietary protein sources in older and younger individuals could be an important step forward in the search for novel strategies to increase or maintain muscle mass with age. In the current study, most meals consisted of both animal- and plant-based protein sources, theoretically providing all the EAA needed (56). Furthermore, no differences were observed in the predominant protein sources consumed at T1 and T3 between young and older individuals. However, at T2, more plant-based proteins (mainly in the form of bread) were consumed by older compared with younger individuals. Bread and most grain-based products can be considered low-quality proteins due to their low protein-digestibility-corrected amino acid score (48). Therefore, substituting these low-quality proteins for higher quality proteins might enhance skeletal muscle anabolism in older individuals. Protein sources contributing to snacks were generally animal-derived in both groups, with 70% originating from milk in older and 22% from whey protein supplements in younger individuals. Both these protein sources are considered high-quality proteins, and are able to robustly stimulate MPS in young and older individuals (14, 22). However, maximal stimulation of MPS requires greater protein intakes in older compared with young individuals (60). In light of this, and taking into account that the majority of milk products consumed as a snack in older individuals were additives to tea or coffee, it is unlikely for this dose to evoke a robust increase in MPS. In contrast, whey protein supplementation in younger individuals would have provided a high-quality protein dose of ~15-25g per serving, sufficient to fully saturate MPS (39). In summary, our results suggest that protein source only differed at lunch between young and older individuals. Therefore, it is most likely the reduced amount of protein consumed with each meal rather than the protein source that contributes to the observed age-
related muscle loss. Future protein intake recommendations for older individuals should hence prioritise total per-meal protein rather than protein source.

The notion that there may be sex-specific differences in dietary protein intake and pattern of consumption between young and older individuals was not reinforced by our findings. Previously, others have reported lower relative protein intakes in older compared with younger males, but no difference between young and older females (47). In contrast, our data showed similar dietary protein intakes, sources, and patterns of distribution between males and females in each cohort, hence why we chose to pool data from males and females for analysis. The reason for this discrepancy may relate to potential differences in participant physical activity and training status between studies. Although we did not assess physical activity or training status of cohorts in the present study, it is likely that a greater proportion of young were engaged in higher intensity activity/exercise compared with older individuals. This may explain the observation that 18% of younger individuals consumed supplemental protein (predominantly whey-based) on at least one of three measured days at T4, with the aim of supporting training-induced muscle remodelling (17). Nonetheless, dietary protein intakes remained significantly greater in young compared with older individuals when supplemental protein was removed from dietary analysis (1.49 vs 1.14 g·kg⁻¹·day⁻¹ in young and older, respectively, P = 0.002).

In addition to the recommended increase in the absolute amount and distribution pattern of dietary protein intake, it is important to consider lifestyle strategies to maximise the muscle anabolic response to sub-optimal protein ingestion in older individuals, that may improve or maintain long term musculoskeletal health and function. In an acute setting, it has previously been demonstrated that 45 min of moderate-intensity treadmill walking enhanced the muscle
protein synthetic response to a subsequent mixed protein-carbohydrate feed in older individuals (54). More recently, it was demonstrated that the combination of resistance training with protein supplementation was required to increase muscle mass in older individuals (53), which is supported by a recent meta-analysis by Cermak and colleagues (17). Taken together, these results indicate the potential benefits of adding resistance or endurance exercise to a nutritional intervention to maximise muscle accretion in older individuals. Embracing the synergism between protein nutrition and exercise offers the most potent means of maintaining musculoskeletal mass, strength and function in old age.

In conclusion, protein intake in the majority of young and older individuals generally met or exceeded current protein recommendations, even though relative and absolute protein intakes were greater in the young. Whilst TEI did not differ between groups, a positive correlation with total protein intake was found in young but not older individuals. Protein distribution throughout the day was uneven in both groups, with the majority of protein consumed at dinner and lunch (T1<T2<T3). Although the predominant protein sources ingested at each main meal were generally similar, except for lunch, older individuals ate considerably less protein at lunch and dinner. Increasing and ensuring an even distribution of protein intake across meals might be an effective strategy to maximally stimulate MPS in older individuals and attenuate age-related muscle loss. Future research should further elucidate if increasing protein intake to levels that exceed MPS saturation would result in an augmented net protein balance, and hence lead to better maintenance of muscle mass over time in older individuals. Furthermore, research should explore the potential additive effects of exercise (endurance and resistance) when combined with a nutritional intervention.
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2.7 Additional Information

Competing interests
The authors have no competing interests to disclose.

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Contributing Statement
All authors gave their final approval of the version of the article to be published. BS, CG and LB designed the study. BS and LB organized and carried out data collection. BS and LB performed the statistical analysis of the data and wrote the manuscript together. BS and LB are the guarantors of this work and take responsibility for the integrity and accuracy of the data analysis.
CHAPTER 3

MECHANISMS OF AGE-RELATED MUSCLE LOSS
Age-Related Anabolic Resistance of Myofibrillar Protein Synthesis is Exacerbated in Obese Inactive Individuals

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Running title: Mechanisms of Age-Related Muscle Loss

KEYWORDS: Sarcopenia; obesity; nutrition; physical activity

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3.1 Abstract

Background: Age-related muscle loss (sarcopenia) may be partly underpinned by a diminished muscle anabolic response to protein nutrition, the cause of which is unclear. Purpose: The study aim was to determine the influence of old age and obesity on myofibrillar protein synthesis (MyoPS). Methods: Ten old lean (OL: 71.7 ± 6 yrs), 7 old obese individuals (OO: 69.1 ± 2 yrs) were 18 young lean controls (YL: 25.5 ± 4 yrs) were recruited. Anthropometrics, insulin resistance systemic inflammation, habitual diet and physical activity were determined. Skeletal muscle biopsies were obtained prior-to and 4h after ingestion of 15g of milk protein during a primed-continuous infusion of L-[ring-13C6]-phenylalanine. Results: Whole-body and regional fat mass, insulin resistance and systemic inflammation were greater in OO compared with YL and OL. Relative fat-free mass and Type I and II fibre CSA was lower in OL and OO compared with YL (P < 0.01) and Type II fibre intramyocellular lipid coverage was significantly greater in OO compared with YL and OL (P < 0.01). Daily step-count was lower in OO compared with YL and OL (P < 0.01). Postprandial MyoPS rates increased above postabsorptive values by 81% in YL and 37 % in OL, respectively (P < 0.01), with no increase in OO. Postprandial MyoPS correlated positively with step-count ($r^2 = 0.26$, $P = 0.036$) and negatively with leg fat mass ($r^2 = 0.4$, $P < 0.01$) in OO and OL. Conclusion: Age-related muscle ‘anabolic resistance’ to protein nutrition may be exacerbated in obese inactive individuals.
3.2 Introduction

Age-related muscle loss (sarcopenia (37)) leads to functional impairment, increases the risk of falls/fractures and metabolic disease and is associated with mortality (21, 23, 29, 30). As the number of older individuals globally dramatically over the next 30 years, so too will the prevalence of sarcopenia, placing a significant strain on healthcare resources (16, 24). Skeletal muscle proteostasis is a tightly coupled interplay between muscle protein synthesis (MPS) and muscle protein breakdown (MPB). Although there is no detectable change in postabsorptive rates of MPS or MPB in old age (49), recent studies demonstrate that a diminished MPS response to protein/amino acid provision in the old may underlie the progression of sarcopenia (11, 25). This age-related muscle ‘anabolic resistance’ is more apparent in response to low-to-moderate dose protein intake, commonly consumed in the diet of older individuals (33, 40, 46). The underlying mechanisms of age-related muscle anabolic resistance are unclear, but are essential to understand in order to develop targeted therapeutic strategies to improve age-related musculoskeletal health.

Although the aetiology of sarcopenia is undoubtedly multifactorial, there is contention as to the role of chronological and biological ageing (i.e. inactivity, malnourishment, disease) in this process (35). It is well accepted that chronological ageing is accompanied by declining physical activity levels interspersed by protracted periods of disuse (i.e. during illness/hospitalization) (17, 39). This is noteworthy, as it has been demonstrated that bed rest (43), limb immobilization (18) and reduced daily activity (6) induce muscle anabolic resistance and atrophy in older individuals, whereas acute treadmill walking restores postprandial muscle anabolic responsiveness (47). Thus, physical activity represents an important locus of control in age-related MPS. To date, very few studies have assessed physical activity levels in conjunction with MPS in older individuals and, as such, any association between the two is lacking.
Sarcopenia is often accompanied by a concomitant increase in adiposity (3) and intramuscular fat infiltration (myosteatosis) (19), the confluence of which exacerbates the progression towards frailty and metabolic disease (27). Recently, Murton and colleagues (34) demonstrated a blunted MPS response to amino acid administration in obese vs. normal-weight older males. Furthermore, high-fat feeding, lipid administration and ectopic fat deposition induce muscle anabolic resistance in rodents and humans (1, 5, 42, 44), although postprandial MPS rates are not impaired in non-obese Type II diabetics (12, 28). Collectively, these data suggest that obesity, but not insulin resistance per se, may impair MPS, potentially through compromised muscle quality or elevated levels of adipose-derived inflammatory cytokines (‘adipokines’) (38). Currently, the interactive effects of myosteatosis, insulin resistance and inflammation on age-related muscle anabolic resistance are unclear and warrants further investigation.

Therefore, the aim of the present study was to comprehensively assess the physical activity, whole-body/muscle metabolic health and inflammatory characteristics of i) young lean (YL), ii) older lean (OL) and iii) older obese (OO) individuals to establish for the first time whether, and to what extent, these parameters were associated with MyoPS rates in the postabsorptive state and in response to moderate-dose protein provision. We hypothesized that postprandial MyoPS rates would be impaired in OL, but more so in OO compared with YL, and would therefore be closely associated with levels of physical activity, adiposity and inflammatory markers.
3.3 Materials and Methods

3.3.1 Participants

In the present study, 10 lean older (OL) and 10 obese older (OO) individuals were compared against 18 young lean individuals (YL) (characteristics are provided in Table 3.1). Participants completed a general health questionnaire and were excluded from study participation if they had Type II Diabetes, uncontrolled hypertension, neuromuscular/cardiovascular/metabolic disease, or if they were smokers, losing weight or taking non-steroidal anti-inflammatory drugs. Participants were informed of the purpose and methodology of the study prior to providing written consent. Ethical approval was obtained through the NHS Black Country Research Ethics Committee (13/WM/0429). The study conformed to standards set by the Declaration of Helsinki (7th edition). Three OO participants were omitted from the study due the detection of one or more exclusion criteria during preliminary assessments (n=2), and non-collection of muscle biopsy tissue (n=1).

3.3.2 Experimental Design

Following an initial screening visit, participants reported to the Clinical Research Facility (CRF) of the Queen Elizabeth Hospital, Birmingham, on three separate occasions. During the initial visit, participants were given a weighed food diary, pedometer and accelerometer to record habitual food intake and physical activity/intensity over a four-day period. Five days following this initial visit, participants returned to the CRF for assessments of body composition, resting metabolic rate (RMR) and fasting plasma glucose and HbA1-C. On the third visit, participants underwent an experimental trial, comprising of a stable amino acid isotope tracer infusion with serial muscle biopsies and blood samples to determine basal
postabsorptive and postprandial MyoPS rates, intracellular signaling and muscle fibre/IMCL properties.

### 3.3.3 Preliminary Assessments

**Body composition:** Participants reported to the CRF at ~0800 h, following a 10 h overnight fast, where body mass was first determined in loose clothing and without shoes to the nearest 0.1 kg on an electronic balance scale. Height was measured to the nearest 0.1 cm using a stadiometer. Whole-body and regional fat mass, fat-free mass and visceral adipose tissue (VAT) was determined using dual energy x-ray absorptiometry (DXA) scanning (GE iDXA, GE Healthcare, Chicago, IL, U.S.). VAT was obtained by identifying the android/gynoid region and the horizontal plane by the area between the pelvis and the rib cage, with its vertical limits being the inner abdominal muscle wall. VAT is computed by subtracting subcutaneous fat from the total android fat mass in the android region. Briefly, participants were placed in a supine position on the DXA scanner with their feet at shoulder width, secured with tape to avoid any discomfort. DXA scan duration was 7 min and all images were analysed by a trained radiographer. Obesity was defined by Body Mass Index of $\geq 30$ kg·m$^{-2}$.

**Resting metabolic rate:** Participants rested in a supine position for 30 min whilst expired gases were collected via a ventilated plastic hood and analysed for O$_2$ and CO$_2$ concentrations using a Moxus metabolic cart (AEI Technologies, Naperville, IL, U.S.), equipped with electrochemical gas analysers (AME-TEK model S-3A/1 and CD-3A). Breath-by-breath measurements were analysed for VO$_2$ and VCO$_2$ and values averaged over 30 sec periods using Moxus software (version 2.8.02). During RMR measurement, participants were instructed to lay as still as possible, breath normally, not to speak and stay awake. RMR was predicted using
the Weir equation ($RMR = (3.94 \times VO_2) + (1.11 \times VCO_2)$) and was based upon a five-minute measurement period where VO$_2$ measurements showed the lowest coefficient of variation (51).

**Fasting plasma blood glucose and HbA1-C:** Following RMR assessment, a 10mL venous blood sample was obtained to measure fasting plasma glucose and HbA1-C concentrations. Participants were excluded from study participation if fasting plasma glucose and HbA1-C values exceeded 7 mmol/L and 48 mmol/mol, respectively, indicating high-risk or presence of Type II diabetes (2).

**Physical activity/intensity levels:** Participants were fitted with a wrist-worn accelerometer with a triaxial sensor (GENEActiv, ActivInsights, U.K.) for four consecutive days, including at least one weekend day. Accelerometers were initialized to sample data at a 10 Hz frequency. Data were downloaded onto a laboratory computer, converted into 60 second epochs and analysed using the GENEActiv software (version 2.2, ActivInsights). Activities were split into 4 categories based upon the following metabolic equivalent (MET) values; i) sedentary activity (<1.5 METs) ii) light activity (1.5 – 3.99 METs) iii) moderate activity (4.0 – 6.99 METs) and iv) vigorous activity (>7 METs) (15). Furthermore, participants recorded their daily step count over four consecutive days using a hip-worn pedometer (Vital Steps, Omron Healthcare UK Ltd., Milton Keynes, U.K.).

**Dietary intake:** Habitual dietary intake was assessed over a 4-day period. Participants were given a food diary and digital balance scales to record and weigh out their dietary and fluid intake. Food diaries were analysed using Dietplan software (Dietplan 7, Forestfield software Ltd, U.K).
3.3.4 Experimental trial

The evening prior to the experimental trial, participants consumed a standardised evening meal (787 kcal) composed of ~19% protein (~37.5 g), ~46% carbohydrate (~90.7 g) and ~35% fat (~30.8 g). After a ~10-12 h overnight fast and having refrained from any strenuous activity for the previous 48 h, participants reported to the CRF at 0730 h having driven or taken public transport to ensure that resting MyoPS rates were maintained. Upon arrival, a 21G cannula was inserted into an antecubital vein of both forearms. One cannula was used for serial blood sampling throughout the day, whilst the other was used to administer a primed continuous infusion of L-[ring-\textsuperscript{13}C\textsubscript{6}] phenylalanine (prime dose 2 \(\mu\text{mol}\cdot\text{kg}^{-1}\); continuous infusion 0.05 \(\mu\text{mol}\cdot\text{kg}^{-1} \cdot \text{min}^{-1}\); Cambridge Isotope Laboratories, Andover, MA, USA), which was initiated after obtaining a fasted resting blood sample. Further blood samples were drawn at -160, -95, -35, -5 min prior to consumption of a milk protein beverage (described below) and 20, 40, 60, 90, 120, 180 and 240 min post-beverage consumption. A total of 10 mL of blood was sampled at each time point and arterialized by heating the forearm to ~60°C. Blood samples were collected in serum separator and ethylenediaminetetraacetic acid (EDTA) treated vacutainers (BD, Oxford, U.K.), centrifuged at 3000 rpm for 10 min at 4°C, and plasma and serum aliquots were stored at -80°C until further analyses. At 150 min into the infusion a muscle biopsy was obtained from quadriceps vastus lateralis of a randomly selected leg under local anaesthesia (1% lidocaine) using the Bergström technique. Muscle biopsy samples were freed from any visible blood, adipose and connective using ice-cold saline and snap-frozen in liquid nitrogen before being stored at -80°C. A separate piece of muscle tissue (~20mg) was mounted in O.C.T compound (VWR Chemicals, Leuven, Belgium) and frozen in 2-methylbutane (Sigma-Aldrich) before being stored at -80°C for immunohistochemical analyses. Immediately after biopsy obtainment, participants consumed 15 g of milk protein isolate (MyProtein, Cheshire, U.K.)
dissolved in 300 mL of water. The amino acid content of the milk protein isolate was (as percentage content) Ala, 3.0; Arg, 3.7; Asp, 7.4; Cys, 0.9; Gln, 9.1; Gly, 1.7; His, 2.3; Ile, 5.6; Leu, 9.8; Lys, 8.7; Met, 2.6; Phe, 4.7; Pro, 9.9; Ser, 5.3; Thr, 3.8; Trp, 1.7; Tyr, 4.6; and Val, 6.0, providing 13.6g of amino acids in total. A small amount of L-[ring-\textsuperscript{13}C\textsubscript{6}] phenylalanine tracer (28.2mg to enrich to 4%) was added to the drink to minimize changes in \textsuperscript{13}C\textsubscript{6} phenylalanine enrichment. Following drink consumption, participants rested in a supine position for the remainder of the trial. Approximately 240 min after drink consumption, a second biopsy was obtained ~3 cm proximal to the first biopsy and indicated the end of the experimental trial.

\textbf{3.3.5 Blood Analyses}

\textit{Blood analyte and hormone concentrations}

Fasting plasma glucose was analysed by enzymatic colorimetric assay (Glucose Oxidase kit, Instrumentation Laboratories, Cheshire, U.K.) using an ILAB 650 Clinical Chemistry Analyzer (Instrumentation Laboratory, Warrington, U.K.). Plasma HbA1-C concentration was analysed via a Tosoh G8 HPLC semi-automated analyser (Tosoh Bioscience, Pennsylvania, U.S.). Serum interleukin-6 (IL-6), C-reactive protein (CRP) and insulin concentrations were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (IL-6: R&D systems Europe, Abingdon, U.K.; CRP: RayBiotech, Norcross, GA, U.S.; Insulin: IBL International, Hamburg, Germany) according to the manufacturer’s instructions.

\textit{Plasma amino acid enrichment and concentrations}

Plasma \textsuperscript{13}C\textsubscript{6} phenylalanine enrichment was determined by gas chromatography-mass spectrometry (GCMS; model 5973; Hewlett Packard, Palo Alto, CA, USA) by monitoring ions 234/240. Briefly, 300 \textmu L of plasma was diluted 1:1 with acetic acid before being purified
through cation-exchange columns and dried down overnight under nitrogen. The purified amino acids were then converted to their N-tert-butyldimethyl-silyl-N-methyltrifluoracetamide (MTBSTFA) derivative. Simultaneously, leucine and phenylalanine concentrations were measured by GCMS using the internal standard method based on the known volume of plasma (300 μL) and internal standard (6 μL) added and the known amino acid concentration of the internal standard. The internal standards were U-[13C₆] leucine (ions 302/308) and U-[13C₉–15N] phenylalanine (ions 336/346) added in a ratio of 100 μL·mL⁻¹ of blood.

3.3.6 Muscle Tissue Analyses

Myofibrillar protein enrichment

Approximately 30mg of muscle tissue was minced in 10μL/mg of homogenization buffer (50mM Tris-HCl, 1mM EDTA, 1mM EGTA, 10mM β-glycerophosphate, 50mM sodium fluoride, 1 x protease inhibitor tablet, 0.5mM sodium orthovanadate, pH 7.5) on ice using small scissors and a Teflon pestle before being shaken for 10 min at 1500rpm at room temperature, and finally centrifuged at 11,000rpm at 4°C for 5 minutes. The supernatant containing the sarcoplasmic proteins was transferred to a 2 mL Eppendorf for intracellular signaling analysis (described below). A 500μL volume of doubly distilled water (ddH₂O) was added to the pellet, which was vortexed and centrifuged at 4500rpm at 4°C for 5 min and the supernatant discarded. The myofibrillar fraction was separated from collagen by adding 1mL of 0.3M NaOH and heating for 30 min at 37°C. The sample was then spun at 13,000 rpm at 4°C for 20 min. This process was repeated twice and both resultant supernatants were transferred to a 4mL glass collection tube. Myofibrillar proteins were precipitated by adding 1mL of 1M PCA to the supernatant, which was vortexed and centrifuged at 3200 rpm at 4°C for 20 min. The pellet was then washed with 2mL of 70 % EtOH, centrifuges at 3200 rpm at 4°C for 20 min. The remaining
myofibrillar protein pellet was hydrolysed overnight at 110°C in 0.1N HCL and 1mL of activated Dowex 50W-X8 100-200 resin (Bio-Rad laboratories INC, USA). Constituent amino acids in the myofibrillar fraction were purified on cation-exchange columns (Bio-Rad laboratories INC, USA). Amino acids in the myofibrillar fraction were then converted to their N-acetyl-n-propyl ester derivative and phenylalanine labeling determined by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS, Delta-plus XP, Thermofinnigan, Hemel Hempstead, UK). Ions 44/45 were monitored for unlabeled and labeled CO$_2$, respectively.

**Fibre-type intramyocellular lipid**

Lyophilised bodipy (#D3922, Thermo Fisher, Leicestershire, U.K.) and Wheat Germ Agglutinin (WGA, Alexa Fluor 350, Thermo Fisher, Leicestershire, U.K.) were diluted in ethanol and ddH$_2$O, respectively (1 mg/mL), and stored at -20°C. Muscle cross sections were fixed for 60 min in 3.7% formaldehyde and washed 3 times in ddH$_2$O. Muscle cross sections were then incubated for 5 min in 0.5% Triton X-100, and 2 h in myosin heavy chain type I antibody (M IgM; DHSB, Iowa, U.S [1:25 diluted in 5% normal goat serum and 1x PBS]). Subsequently, muscle cross sections were incubated for 30 min in goat anti mouse IgM 594 (#A21044, Thermo Fisher, Leicestershire, U.K. [1:50 GAM IgM diluted in 1x PBS]), washed 3 times 5 min in 1x PBS, incubated for 30 min in WGA stock solution (1:100 diluted in 1x PBS), washed as before, and incubated for 20 min in bodipy (1:50 stock solution diluted in 1x PBS). Coverslips (No1, VWR International, Leicestershire, U.K.) were mounted with 20µl of mowiol; 6g glycerol, 2.4 g mowiol 4-88 and 0.026g 1,4 diazobicyclo-[2,2,2]-octane (DABCO) dissolved in 18 mL 0.2 mol/L Tris-buffer (pH 8.5) (Sigma-Aldrich, Dorset, U.K.). Images were taken with a Nikon Eclipse E600 (Nikon, Badhoevedorp, The Netherlands) at 40x zoom. Image Pro Suite version 5.1.2.59 was used to quantify fibre type, size and lipid droplet content.
**Intramuscular signaling**

Western blotting was performed on the sarcoplasmic fraction obtained during isolation of the myofibrillar protein fraction (described above) with the protein concentration determined by a DC protein assay. Equal amounts of protein (30 μg) were boiled for 5 min in 1 x Laemmli sample buffer and separated on 7.5–10% gels by SDS-PAGE for 1 h. Following electrophoresis, proteins were transferred to a Protran nitrocellulose membrane (Whatman, Dassel, Germany) at 100 V for 1 h. Membranes were incubated overnight at 4°C in following primary antibodies: phospho-S6K1 Thr389 (#9205), total 70 kDa S6 protein kinase (p70S6K1; #9202), phospho-eukaryotic initiation factor 4E binding protein (4E-BP1) Thr37/46 (#9459), total 4E-BP1 (#9452), phospho-eukaryotic elongation factor 2 (eEF2) Thr56 (#2331), total eEF2 (#2332), phospho-protein kinase B (Akt) Ser473 (#3787), total Akt (#9272) each purchased from Cell Signaling Technology [New England Biolabs (UK) Ltd, Hitchin, UK]. Membranes were then washed 3 times in TBST for 5 min, incubated for 60 min in their respective secondary antibody and washed 3 times 5 min again in TBST. Immobilon Western chemiluminescent HRP substrate (Merck Millipore, Watford, UK) was used to quantify protein content following IgG binding, visualized on a G:BOX Chemi XT4 imager using GeneSys capture software (Syngene, Cambridge, U.K.). A Chemi Genius Bioimaging Gel Doc System (Syngene) was used to image and quantify bands.

**3.3.7 Calculations**

The standard precursor-product method was used to calculate MyoPS rates from $^{13}\text{C}_6$ phenylalanine incorporation:

$$FSR (\% \text{h}^{-1}) = \Delta E_b / E_p x 1 / t x 100$$
Where \( \Delta E_b \) in the above equation is the algebraic difference in the amount of bound \(^{13}\)C\(_6\) phenylalanine between two biopsy samples, \( E_p \) is the precursor enrichment, and \( t \) is the time between muscle biopsies samples expressed in minutes. Mean plasma \(^{13}\)C\(_6\) phenylalanine enrichment from arterialised blood samples was used as the precursor enrichment. Pre-infusion plasma \(^{13}\)C\(_6\) phenylalanine enrichment was used as a proxy for basal muscle protein enrichment, an approach that has been previously validated in older individuals (8).

### 3.3.8 Statistics

Data analysis was performed using SPSS version 22 (IBM, Chicago, IL, USA) and Graphpad Prism (Graphpad software Inc. La Jolla, CA, USA). A Shapiro-Wilks was used to test the normality of the data. Subsequently, non-normally distributed variables were logarithmically transformed. Anthropometric, dietary, physical activity, metabolic health/inflammatory and muscle fibre characteristics were analysed using a one-way ANOVA. MyoPS rates and intracellular signaling data were analysed using a two-way, repeated measures ANOVA with one within (two levels; ‘basal’ and ‘postprandial’ state) and one between factor (three levels; group). Insulin, plasma amino acid and \(^{13}\)C\(_6\) phenylalanine enrichment were analysed using a two-way, repeated-measures ANOVA (time x group). Additionally, \(^{13}\)C\(_6\) phenylalanine enrichment were analysed using linear regression to assess the existence of any deviation in enrichment. Tukey’s HSD post hoc analysis was performed whenever a significant F ratio was found to isolate specific differences. Correlational analysis was performed using a tow-tailed Pearson Correlation Coefficient test. Significance for all analyses was set at \( P < 0.05 \). All values are presented as means ± SD or SEM.
3.4 Results

3.4.1 Anthropometric and Metabolic Health Characteristics

Body mass, BMI, fat mass (relative and absolute) and visceral adipose tissue were significantly greater in OO compared with OL and YL (P < 0.05), with no differences between YL and OL. Fat-free mass (FFM), expressed relative to total body mass, was significantly lower in OO compared to YL and OL (P < 0.001 and P = 0.001, respectively). Leg FFM, expressed relative to total body mass, was lower in OO and OL compared with YL (P < 0.001 and P = 0.038, respectively) and was lower in OO compared with OL (P = 0.023). Plasma IL-6, CRP, glucose, serum insulin and HOMA-IR were significantly greater in OO compared with OL and YL (P < 0.01). Resting metabolic rate (RMR) was significantly lower in OL compared with YL (P = 0.015). Anthropometric and metabolic health characteristics are presented in Table 3.1.
<table>
<thead>
<tr>
<th></th>
<th>YL</th>
<th>OL</th>
<th>OO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Participants (M/F)</td>
<td>10/8</td>
<td>4/6</td>
<td>4/3</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>25.5 ± 3.7</td>
<td>71.7 ± 6.2**</td>
<td>69.1 ± 2.6**</td>
</tr>
<tr>
<td>Body mass (kg)</td>
<td>70.1 ± 12.9</td>
<td>64.5 ± 10.9</td>
<td>91.6 ± 15***††</td>
</tr>
<tr>
<td>BMI (kg·m⁻²)</td>
<td>23.3 ± 2.4</td>
<td>22.7 ± 2.5</td>
<td>32.9 ± 4***††</td>
</tr>
<tr>
<td>Fat mass (% of body mass)</td>
<td>24.3 ± 6.5</td>
<td>28.3 ± 7.1</td>
<td>41 ± 2***††</td>
</tr>
<tr>
<td>Fat mass (kg)</td>
<td>16.8 ± 5.6</td>
<td>17.7 ± 4.4</td>
<td>36.7 ± 6.4***††</td>
</tr>
<tr>
<td>Leg fat mass (kg)</td>
<td>6.6 ± 2.5</td>
<td>5.8 ± 1.3</td>
<td>9.9 ± 2.1***††</td>
</tr>
<tr>
<td>Visceral adipose tissue (kg)</td>
<td>0.23 ± 0.15</td>
<td>0.58 ± 0.48</td>
<td>2.76 ± 1.44***††</td>
</tr>
<tr>
<td>Fat-free mass (kg)</td>
<td>49.7 ± 10</td>
<td>43 ± 9.6</td>
<td>50.3 ± 8.8</td>
</tr>
<tr>
<td>Fat-free mass (% of body mass)</td>
<td>71 ± 6.3</td>
<td>66.5 ± 7.3</td>
<td>54.8 ± 2.1***††</td>
</tr>
<tr>
<td>Leg fat-free mass (kg)</td>
<td>16.7 ± 3.4</td>
<td>14 ± 3.6</td>
<td>17.1 ± 3.4</td>
</tr>
<tr>
<td>Leg fat-free mass (% of body mass)</td>
<td>23.8 ± 2.14</td>
<td>21.6 ± 2.6*</td>
<td>18.6 ± 1.3***††</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>2.28 ± 0.91</td>
<td>2.16 ± 0.98</td>
<td>6.10 ± 3.02**††</td>
</tr>
<tr>
<td>Fasting plasma glucose (mmol/L)</td>
<td>5.3 ± 0.5</td>
<td>5.9 ± 0.5**</td>
<td>6.5 ± 0.3**</td>
</tr>
<tr>
<td>Fasting serum Insulin (µIU/mL)</td>
<td>9.7 ± 3.7</td>
<td>8.2 ± 3.5</td>
<td>21.1 ±***††</td>
</tr>
<tr>
<td>Plasma IL-6 (pg/mL)</td>
<td>0.43 ± 0.29</td>
<td>1.1 ± 0.51**</td>
<td>1.49 ± 0.71††</td>
</tr>
<tr>
<td>Plasma CRP (mg/L)</td>
<td>0.37 ± 0.14</td>
<td>0.92 ± 0.34</td>
<td>2.34 ± 0.98***††</td>
</tr>
<tr>
<td>Plasma HbA1-C (mmol/mol)</td>
<td>-</td>
<td>37.3 ± 3.9</td>
<td>38.3 ± 3.4</td>
</tr>
<tr>
<td>RMR (kcal)</td>
<td>1681 ± 296</td>
<td>1276 ± 353*</td>
<td>1515 ± 449</td>
</tr>
</tbody>
</table>

Values presented as mean ± SD. BMI; Body Mass Index, IL-6; interleukin-6, CRP; C-reactive protein, RMR; Resting Metabolic Rate. * is significantly different from YL, † is significantly different from OL. * indicates P<0.05, ** P<0.01, † P<0.05, †† P<0.01.

### 3.4.2 Physical Activity and Dietary Characteristics

Average daily step-count was significantly lower in OO compared with OL and YL (P < 0.01). Relative and absolute time spent in ‘light’ intensity activity was significantly greater in OL com
pared with YL ($P = 0.003$), but not different from OO. Dietary protein intake was significantly lower in OO compared with YL ($P = 0.009$), but was not different from OL. No other differences in physical activity or dietary intake were found between groups. Physical activity and dietary characteristics are detailed in Table 3.2.

**Table 3.2.** Activity and diet characteristics.

<table>
<thead>
<tr>
<th></th>
<th>YL</th>
<th>OL</th>
<th>OO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daily step count</td>
<td>11870 ± 3905</td>
<td>11102 ± 3271</td>
<td>5399 ± 2079**††</td>
</tr>
<tr>
<td>Sedentary Activity (%)</td>
<td>70.8 ± 6.6</td>
<td>65.5 ± 13.8</td>
<td>74.9 ± 7.4</td>
</tr>
<tr>
<td>Light Activity (%)</td>
<td>10.2 ± 2.1</td>
<td>15 ± 5.2*</td>
<td>11 ± 2.4</td>
</tr>
<tr>
<td>Moderate Activity (%)</td>
<td>16.8 ± 5.2</td>
<td>18.4 ± 9.7</td>
<td>13.9 ± 5.9</td>
</tr>
<tr>
<td>Vigorous Activity (%)</td>
<td>2.3 ± 2.8</td>
<td>0.8 ± 1.5</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>Sedentary Activity (min)</td>
<td>643 ± 56</td>
<td>594 ± 115</td>
<td>684 ± 104</td>
</tr>
<tr>
<td>Light Activity (min)</td>
<td>93 ± 19</td>
<td>140 ± 50*</td>
<td>101 ± 24</td>
</tr>
<tr>
<td>Moderate Activity (min)</td>
<td>153 ± 53</td>
<td>169 ± 89</td>
<td>126 ± 48</td>
</tr>
<tr>
<td>Vigorous Activity (min)</td>
<td>21 ± 26</td>
<td>7 ± 14</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Daily Energy Intake (kcal)</td>
<td>2162 ± 473</td>
<td>2091 ± 451</td>
<td>2412 ± 628</td>
</tr>
<tr>
<td>Daily Protein (g·kg$^{-1}$)</td>
<td>1.51 ± 0.46</td>
<td>1.36 ± 0.37</td>
<td>0.95 ± 0.20**</td>
</tr>
<tr>
<td>Daily CHO (g·kg$^{-1}$)</td>
<td>3.20 ± 1.22</td>
<td>3.38 ± 0.91</td>
<td>2.65 ± 1.25</td>
</tr>
<tr>
<td>Daily Fat (g·kg$^{-1}$)</td>
<td>1.17 ± 0.33</td>
<td>1.29 ± 0.46</td>
<td>1.07 ± 0.40</td>
</tr>
<tr>
<td>Daily Alcohol (g·kg$^{-1}$)</td>
<td>0.22 ± 0.22</td>
<td>0.22 ± 0.25</td>
<td>0.33 ± 0.25</td>
</tr>
<tr>
<td>Daily Fibre (g·kg$^{-1}$)</td>
<td>0.30 ± 0.14</td>
<td>0.34 ± 0.10</td>
<td>0.24 ± 0.12</td>
</tr>
</tbody>
</table>

Values presented as mean ± SD. * is significantly different from YL, † is significantly different from OL. * indicates $P<0.05$, ** $P<0.01$, † $P<0.05$, †† $P<0.01$.

**3.4.3 Muscle Fibre Characteristics and Lipid Content**

Type I and II muscle fibre cross-sectional area (CSA) was greater in YL compared with OO ($P < 0.001$) and OL ($P < 0.001$) and was significantly different between OO and OL ($P < 0.001$).
Type I fibre lipid droplet number was significantly greater in YL compared with OL (P < 0.001) and OO (P = 0.021), and was different between OL and OO (P < 0.001). Type II fibre lipid droplet number was significantly greater in YL and OO compared with OL (P < 0.001 for both) with no difference between YL and OO. When normalized to fibre CSA, Type I lipid droplet area was lower in OL compared to YL (P = 0.014) but not OO, whereas Type II lipid droplet area was significantly greater in OO compared with YL (P < 0.001) and OL (P = 0.042). Muscle fibre CSA and IMCL characteristics are presented in Table 3.3. Representative IMCL staining images are presented in Figure 3.1A-C.
### Table 3.3. Muscle fibre and IMCL characteristics

<table>
<thead>
<tr>
<th></th>
<th>YL</th>
<th>OL</th>
<th>OO</th>
<th>Values</th>
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</thead>
<tbody>
<tr>
<td>Type I fibre (%)</td>
<td>48 ± 16</td>
<td>49 ± 18</td>
<td>36 ± 15</td>
<td></td>
</tr>
<tr>
<td>Type II fibre (%)</td>
<td>52 ± 16</td>
<td>51 ± 18</td>
<td>65 ± 16</td>
<td></td>
</tr>
<tr>
<td>Type I area (μm²)</td>
<td>4031 ± 1978</td>
<td>3009 ± 1251**</td>
<td>3421 ± 1528**††</td>
<td></td>
</tr>
<tr>
<td>Type I lipid droplet number</td>
<td>616 ± 458</td>
<td>412 ± 298**</td>
<td>533 ± 505*††</td>
<td></td>
</tr>
<tr>
<td>Type I lipid droplet area (μm²)</td>
<td>287 ± 238</td>
<td>175 ± 159**</td>
<td>244 ± 267**††</td>
<td></td>
</tr>
<tr>
<td>Type I lipid droplet area (% fibre area)</td>
<td>7.7 ± 6</td>
<td>6.4 ± 6.3**</td>
<td>6.9 ± 6.5</td>
<td></td>
</tr>
<tr>
<td>Type II area (μm²)</td>
<td>4009 ± 1733</td>
<td>2170 ± 1243**</td>
<td>3390 ± 1199**††</td>
<td></td>
</tr>
<tr>
<td>Type II lipid droplet number</td>
<td>377 ± 399</td>
<td>242 ± 312**</td>
<td>415 ± 407††</td>
<td></td>
</tr>
<tr>
<td>Type II lipid droplet area (μm²)</td>
<td>133 ± 157</td>
<td>76 ± 99**</td>
<td>201 ± 207**††</td>
<td></td>
</tr>
<tr>
<td>Type II lipid droplet area (% fibre area)</td>
<td>3.1 ± 2.8</td>
<td>3.2 ± 3.4</td>
<td>5.8 ± 5.4**††</td>
<td></td>
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</tbody>
</table>

Values presented as mean ± SD. * is significantly different from YL, † is significantly different from OL, * indicates P<0.05, ** P<0.01, † P<0.05, †† P<0.01.
Figure 3.1: Intramyocellular lipid infiltration in YL (A), OL (B), and OO (C). The top panel represents the bodipy 493/503 stain. The second panel represents the wheat germ agglutinin alexa fluor 350 stain for the cell border. The third panel shows myosin heavy chain I stain. Bottom panel is a composite picture. Scale bars are 50μm.
3.4.4 Plasma Amino Acid and Insulin Concentrations

Plasma leucine and phenylalanine concentrations increased above basal fasted values after ingestion of 15 g of milk protein isolate (P < 0.001; Fig. 3.2A and 3.2B), peaking between 20-40 min post-ingestion, with no difference between groups. Plasma leucine and phenylalanine concentrations had returned to basal fasted values by 180 and 90 min post-ingestion, respectively, with no difference between groups. Basal fasted serum insulin concentration was significantly elevated in OO compared with YL (P < 0.001) and OL (P < 0.001). Serum insulin levels increased above basal fasted values at 20 min post-ingestion (P < 0.01 for all groups), returning to basal fasted values by 60 min post-ingestion (Figure 3.2C). Absolute serum insulin values at 20 and 40 min post-protein ingestion were greater in OO compared with YL (P = 0.004 and 0.016, respectively) and OL (P = 0.001 and 0.007, respectively). Plasma $^{13}$C$_6$ phenylalanine enrichment had increased above basal (-180 min) values 60 min after the initiation of the stable isotope tracer infusion (P < 0.001) and remained stable for the entire trial duration (Figure 3.2D). Linear regression analysis identified that the slopes of the plasma $^{13}$C$_6$ phenylalanine enrichment was not significantly different from zero, indicating the presence of an isotopic steady state (P = 0.3).
Figure 3.2: Plasma leucine concentration (A), phenylalanine concentration (B), serum insulin concentration (C) and plasma $^{13}$C$_6$ phenylalanine enrichment (D) in experimental trials. At $t = 0$, a 15g bolus of milk protein isolate was consumed. Values are means ± SEM. Significance was set at $P < 0.05$. * is significantly greater than fasting values (-180 min), † is significantly greater in OO compared with other groups.

3.4.5 Myofibrillar Protein Synthesis and Intramuscular Signaling

Postabsorptive MyoPS rates did not differ between groups. Following ingestion of 15 g of milk protein isolate, MyoPS rates increased by ~81% in YL (0.049 ± 0.011 %·h$^{-1}$; $P < 0.001$) and ~38% in OL (0.042 ± 0.01 %·h$^{-1}$; $P = 0.002$), respectively, with no significant increase found
in OO (0.036 ± 0.006 %·h⁻¹; P = 0.11). Postprandial MyoPS rates were significantly greater in YL compared with OO (P = 0.02), but were not different between YL and OL (P = 0.21) or between OL and OO (P = 0.11; Figure 3.3A). Postprandial MyoPS, expressed as the delta change from postabsorptive rates, was significantly greater in YL compared with OL (P = 0.032) and OO (P < 0.001), with no significant difference found between OL and OO (P = 0.173; Figure 3.3B). Phosphorylation of p70S6K¹ Thr389, Akt Ser473, eEF2² Thr56 and 4EBP1 Thr37/46 was not significantly different from basal fasted values at 4 h post-protein consumption, and was not different between groups at any time point (Figure 3.4 and 3.5). The lack of any significant differences in signaling between groups is most likely due to the biopsy sampling time-points, which were primarily chosen to assess MyoPS.
Figure 3.3: Myofibrillar fractional synthesis rate (FSR) in rested postabsorptive and 4 h postprandial state (A) following ingestion of 15 g of milk protein (dashed line at 0 min) with values are presented as means ± SEM. Postprandial change in myofibrillar FSR (B), showing the median (central horizontal line), 25th and 75th percentiles (box), minimum and maximum values (vertical lines) and mean (cross). Significance was set at $P < 0.05$. * indicates significantly greater than corresponding postabsorptive values, † indicates significantly greater than OO, ‡ indicates significantly greater than OL.
**Figure 3.4:** Intracellular signaling phosphorylation of p70S6K\(^{\text{Thr389}}\) (A), eEF2\(^{\text{Thr56}}\) (B), Akt\(^{\text{Ser473}}\) (C) and 4E-BP1\(^{\text{Thr37/46}}\) (D) in rested postabsorptive and postprandial state. Data are expressed relative to total protein. Values are presented as means ± SEM. Significance was set at P < 0.05.
3.4.6 Correlations

The delta change in MyoPS from postabsorptive values correlated negatively with leg fat mass for OL and OO combined ($r^2 = 0.4$, $P = 0.006$, Figure 3.6 A) and positively with average daily step count ($r^2 = 0.26$, $P = 0.036$, Figure 3.6 B). HOMA-IR correlated positively with Type I fibre lipid droplet number ($r^2 = 0.41$, $P = 0.018$), Type I fibre lipid droplet area ($r^2 = 0.47$, $P = 0.009$), leg fat mass ($r^2 = 0.4$, $P = 0.007$) and average daily step count ($r^2 = 0.26$, $P = 0.036$) for OL and OO combined. Although not significant, there was a trend for a negative correlation between the delta change in MyoPS and serum CRP ($r^2 = 0.23$, $P = 0.050$).
Figure 3.6: Correlations between the 4 h postprandial change in myofibrillar FSR and leg fat mass (A) and average daily step-count (B) in OL and OO combined.
3.5 Discussion

Age-related impairment in the muscle protein synthetic response to protein-based nutrition is thought to be the primary metabolic defect underpinning the progression of sarcopenia (40). To what extent this age-related muscle ‘anabolic resistance’ is a consequence of chronological and/or aspects of biological ageing is unknown. The present data demonstrate that rates of myofibrillar protein synthesis (MyoPS) are increased above postabsorptive values following moderate-dose milk protein ingestion in young lean (YL) and older lean (OL), but not older obese (OO) individuals. Although there was no difference in absolute postprandial MyoPS rates between YL and OL, the net postprandial rise in MyoPS (delta change from postabsorptive rates) was greater in YL compared with both OL and OO. Postprandial MyoPS rates (absolute and delta change from postabsorptive) were associated with leg fat mass and average daily step count in older individuals. Collectively, these data demonstrate muscle anabolic resistance is apparent in healthy old age, but is exacerbated in obese individuals with low physical activity.

In the present study, we provided participants with an oral 15g bolus of milk protein isolate ((~0.3, 0.35 and 0.3g·kg⁻¹·lean mass⁻¹ for YL, OL and OO, respectively), based on evidence that age-related muscle anabolic resistance may be most apparent with low-to-moderate protein doses typically consumed in the diet (10, 40). This feeding strategy produced postprandial MyoPS responses that were ~81% and 38% greater than postabsorptive values in YL and OL, respectively. Although not statistically different, absolute postprandial MyoPS rates in were 13% lower in OL vs. YL. Furthermore, we observed that the net postprandial MyoPS response above postabsorptive values was significantly lower in OL vs. YL. We acknowledge that the differences in net postprandial MyoPS response between OL and YL we observe here may differ with higher-dose protein intake (≥30g) or in the context of a
mixed-macronutrient meal. In line with our findings, others have reported that ingestion of 20g of casein protein increases muscle protein synthesis by ~75% and 21% in healthy lean young and older males, respectively, with absolute postprandial protein synthesis rates being ~16% lower in old vs. young (50). Collectively, these findings provide evidence of age-related muscle anabolic resistance to protein ingestion. We also observed that postprandial MyoPS rates increased by only ~9% above postabsorptive values in OO. Absolute postprandial and net change in postprandial MyoPS rates were not statistically lower in OO vs. OL, likely due to the relatively small sample size. However, Murton and colleagues (34) recently demonstrated an impaired postprandial MyoPS in obese vs. normal-weight older males. Furthermore, the associations between absolute and net postprandial MyoPS responses and leg fat mass/step count reported here, suggest that age-related muscle anabolic resistance is exacerbated in obese inactive older individuals.

Age-related muscle loss is commonly accompanied by a concomitant increase in whole-body adiposity and ectopic fat deposition within skeletal muscle (4, 19), which has been associated with accelerated progression of sarcopenia and metabolic disease risk (31). It has been postulated that obesity-induced blunting of postprandial MyoPS might be due to IMCL accumulation (34), or adipose tissue-derived inflammatory cytokines (adipokines) (36). Alongside the negative association between the net postprandial MyoPS response and leg fat mass, we observed a trend for a similar negative association with fasting serum CRP, but were unable to demonstrate any association between with fibre-specific IMCL content, despite two-fold higher Type II fibre IMCL in OO compared with OL and YL. This was somewhat surprising given previous reports of associations between IMCL and muscle anabolic resistance in old rodents (45). In our hands, Type I fibre IMCL content was equivalent between YL and
OO. However, Type I fibre IMCL content was negatively associated with insulin sensitivity for OL and OO, but not YL, reinforcing the notion that the capacity for IMCL oxidation would likely have been greater in YL compared with OL and OO. Thus, although we did not observe any association of fibre-type IMCL content with the postprandial change in MyoPS, the subcellular location of IMCL and/or specific class of lipid intermediates (i.e. ceramides and diacylglycerol) may associate more closely with age-related anabolic resistance.

Despite the near-completely diminished postprandial MyoPS response in OO, there was no significant differences in whole-body or regional lean mass compared with OL and YL. In fact, the lower RMR in OL vs. YL and OO may be explained by marginally lower whole-body lean mass. Surprisingly, muscle fibre cross-sectional area was preserved to a greater extent in OO than OL, although still reduced compared with YL. Similarly, others have reported greater lean mass in obese compared with normal-weight older and younger individuals (22, 34). These data refute the mechanistic role of muscle anabolic resistance in sarcopenia and support the existence of an age-associated ‘obesity paradox’, in which the presence of obesity may protect against sarcopenia (20). Collectively, these data beg the question; when (if at all) does obesity-induced muscle anabolic resistance manifest in an accelerated loss of skeletal muscle in older individuals? We postulate that the extra work performed during locomotion and daily living in OO might offer a certain degree of protection against sarcopenia up to a point, beyond which the decline in muscle mass may be precipitous. This hypothetical threshold point would, therefore, most likely be influenced by muscle quality (i.e. inter- and perhaps intra-muscular adiposity) rather than quantity. Furthermore, the greater insulin resistance in OO compared with OL implies that the quality of muscle tissue retained in old age, and not necessarily the quantity, may play a more relevant role in whole-body metabolic health.
Physical activity is an important locus of control in the regulation of age-related muscle protein turnover (6, 47). Herein, we demonstrate that YL and OL took ~122 and 106% more daily steps compared with OO, respectively. Interestingly, absolute postprandial MyoPS and the extent to which postprandial MyoPS increased above basal values correlated significantly with average daily step-count in older individuals, which aligns with evidence that reduced physical inactivity elicits muscle anabolic resistance in older individuals (6). However, the net postprandial MyoPS response was still lower in OL compared with YL despite equivalent daily physical activity levels, suggesting that increasing average daily step count in OO to a level observed in OL may, at best, only partially restore postprandial MyoPS responses. Indeed, others have demonstrated that 45 min of moderate-intensity treadmill walking enhanced the muscle protein synthetic response to amino acid provision in older males (47). The addition of activity-matched OO and OL individuals would have allowed us to reconcile this important point, but was unfortunately beyond the scope of the present study. In addition to average daily step count, we assessed the relative time spent in different physical activity intensities. Despite the absence of any difference between groups in the relative time spent at different activity intensities, combined time spent in light plus moderate plus vigorous intensity activity revealed a deficit of ~38 and 87 min for OO compared with YL and OL, respectively, which might explain the lower average daily step count in OO, assuming an average of ~100 steps-min⁻¹ and slower preferred walking speed (26, 48). Taken together, our findings suggest that a minimum number of daily steps (or time spent active) might offer partial protection against age-related muscle anabolic resistance. However, this notion requires further attention in future work.
In the present study, arterialized venous blood samples were used as a surrogate measurement for the true precursor enrichment and were obtained by heating the forearm vein to ~60°C (32). In contrast, sampling from a non-heated vein would yield lower enrichment values. Some studies advocate the use of venous plasma samples to determine precursor enrichment as the enrichment values are closer to those found in the intracellular pool, and might therefore give a better representation of the true precursor pool (7, 13, 14). However, a concern using venous blood samples during prolonged tracer infusions (several days for skeletal muscle) might cause tracer reappearance due to breakdown. This violates one of the main stable isotope tracer assumptions, i.e. tracer recycling (52). Furthermore, baseline MyoPS was calculated using the single biopsy approach. To date this approach is widely used and has been validated for the measurement of MyoPS in both young and older individuals (8, 9). Isolating protein from plasma for baseline enrichments, coupled with a carbon-based tracer and GC-C-IRMS, serves as a reliable alternative to the two-biopsy method for the calculation of MyoPS, provided the tracer incorporation is long enough (9). If the tracer incorporation time would be too short, MyoPS would show inflated rates as changes in 1.009 δ 13C PDB would occur (9). Taken together, the above information supports the use of arterialized venous blood sampling and the 1-biopsy approach to determine plasma 13C₆ Phenylalanine enrichment and basal MyoPS rates, respectively.

In conclusion, we have demonstrated that old age per se, reduces the net postprandial MyoPS response to moderate-dose protein ingestion, whilst obesity and inactivity exacerbate, and are associated with, this muscle anabolic resistance. Despite the negative association between leg fat mass and the net postprandial MyoPS response in older individuals, we were unable to demonstrate a similar association with fibre-type IMCL content. Interestingly, despite evidence
of muscle anabolic resistance in OO, absolute lean tissue mass was equivalent and muscle fibre area greater than OL, which questions the mechanistic role of muscle anabolic resistance in sarcopenia. As such, further work is required to delineate whether, and to what extent, obesity and inactivity exacerbates sarcopenia (i.e. protective or harmful?), particularly in the very old individuals (>80 y). There is also a need to understand whether the sub-cellular location of IMCL is associated with age-related muscle anabolic resistance. Although we did not directly address potential differences in MyoPS rates between older men and women, there was no discernible pattern of difference. Nonetheless, others have demonstrated sexual dimorphism in muscle protein synthesis in older individuals (41), and this issue warrants further clarification. Finally, our data suggest that physical activity may partially protect against muscle anabolic resistance in older individuals, although further work is required to determine whether chronic physical activity interventions can restore muscle protein anabolism in normal-weight and obese older individuals.
3.6 References


3.7 Additional Information

Competing interests
The authors have no competing interests to disclose.

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Contributing Statement
All authors gave their final approval of the version of the article to be published. BS and LB designed the study. BS and LB organized and carried out the clinical experiments with the assistance of JM, DW and UM. BS, JM and LB performed the data analyses. BS and LB performed the statistical analysis of the data and wrote the manuscript together. BS and LB are the guarantors of this work and take responsibility for the integrity and accuracy of the data analysis.
CHAPTER 4

MUSCLE ANABOLIC EFFECTS OF

PHOSPHATIDIC ACID
Oral Phosphatidic Acid Ingestion Attenuates Resistance Exercise-Induced Myofibrillar Protein Synthesis Rates and Intramuscular Signaling in Older Males

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Running title: Muscle Anabolic effects of Phosphatidic Acid

KEYWORDS: Ageing, skeletal muscle, nutraceuticals, exercise

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4.1 Abstract

Background: Age-related muscle loss (sarcopenia) has myriad adverse health consequences, and may be driven by a diminished myofibrillar protein synthesis (MyoPS) response to anabolic stimuli (i.e. exercise and feeding). Phosphatidic acid (PA) has been reported to stimulate resting MyoPS in rodents, and enhance resistance training-induced muscle remodelling in young humans. Purpose: This study examined the acute effects of oral PA ingestion on resting and exercise-induced MyoPS rates and intracellular signaling in older healthy male individuals.

Methods: Sixteen older males performed 6 sets of unilateral leg resistance exercise with their dominant leg at 75% of 1 repetition maximum, followed by oral ingestion of 1.5 g of soy-derived PA (n = 8; 68.4 ± 1.8 yrs) or a rice-flour placebo (PL) (n = 8; 69.4 ± 3.6 yrs) ingested 0 min (750 mg) and 60 min (750 mg) post-exercise. A primed-continuous infusion of L-[ring-13C6]-phenylalanine with serial muscle biopsies was used to obtain MyoPS at rest and between 0-150 and 150-300 min post-exercise. Intracellular anabolic signaling was determined at rest and 150 and 300 min post exercise cessation. Results: MyoPS in the rested leg did not significantly differ within and between groups at baseline, between 0-150 min and 150-300 min, and over the aggregate period. A trend for a reduction MyoPS rates in the rested leg between 150-300 min in PA was observed (P = 0.053). Exercise increased MyoPS rates above basal values only between 150-300 min post-exercise in PL (P = 0.001), whilst no effects of exercise on MyoPS were observed in PA (P = 0.83). Phosphorylation of p70S6K and 4E-BP1 was elevated above basal levels in the exercised leg at 300 min post-exercise for PL only (P = 0.018 and P = 0.011, respectively), and was significantly greater than PA (P = 0.002 and P = 0.01, respectively). Conclusions: Oral phosphatidic acid ingestion does not augment intramuscular signaling and the MyoPS response to resistance exercise in older males and might not be a viable treatment to counteract sarcopenia.
4.2 Introduction

Sarcopenia, or age-related skeletal muscle loss, leads to myriad adverse health consequences, ranging from reductions in strength (39), impaired functional capacity (32), increased risk of fractures (25), metabolic disease (33) and is associated with premature mortality (36). Whilst the incidence of age-related muscle wasting has been observed in people as young as 45 y, its prevalence increases with advancing age (14). The healthcare costs associated with sarcopenia are extensive and, in the context of a rapidly expanding global ageing population, are expected to increase considerably in the coming decades (46).

Skeletal muscle proteostasis is dependent on the equilibrium between muscle protein synthesis (MPS) and muscle protein breakdown (MPB). One of the most effective strategies to attenuate the progression of sarcopenia is resistance exercise (RE) training (20, 48, 53). In young individuals, RE robustly increases MPS rates by 2 to 3-fold (37), thereby enhancing overall net protein balance for muscle hypertrophy (8, 42). However, the acute MPS response to RE is blunted in older compared with younger individuals (7, 35), which may explain the impaired muscle remodelling response to prolonged training in old age (7, 23). The age-related blunting of the muscle anabolic response to RE may be underpinned by impairments in ribosomal biogenesis and/or translational efficiency in the mechanistic target of rapamycin complex 1 (mTORC1) signaling pathway (7, 24, 35). Collectively, these data have galvanized the search for strategies to abolish the impaired muscle anabolic response to RE in the old, in order to maximize the therapeutic benefits of this non-pharmacological intervention.

The lipid second messenger phosphatidic acid (PA), has been touted as an important factor in the regulation and activation of mTORC1 for MPS. Specifically, mechanical contraction
increases the activity of diacylglycerol kinase \( \zeta \) to convert diacylglycerol into PA, which in turn binds mTORC1 directly at the FKB12 rapamycin binding (FRB) domain (19, 28), increasing its activity and ultimately leading to an enhanced protein translational efficiency (27). In addition to its endogenous synthesis, exogenous PA administration in cell culture and isolated animal muscles is converted to lysophosphatidic acid (1), thereby inactivating the tuberous sclerosis complex (TSC1/2) through the extracellular regulated kinase (ERK) pathway to bolster mTORC1-mediated signaling (51). Thus, mechanical load-induced PA and exogenous PA appear to converge on mTORC1 through distinct proximal pathways. As such, we have postulated that exogenous PA via oral administration, may modulate the intramuscular anabolic signaling and MPS response to RE in older individual (44). Indeed, studies in healthy young humans report additive benefits of oral PA supplementation on increases in strength and lean body mass during prolonged RE training compared with a placebo (26, 34). However, before PA can be applied as a potential means to modulate RE-induced muscle anabolic signaling and MPS in older individuals, there is a need to understand the specific intramuscular mechanisms through which this purported ‘nutraceutical’ compound acts (16).

Therefore, the primary aim of the present study was to establish the acute effects of oral PA ingestion on rates of myofibrillar protein synthesis (MyoPS) and intramuscular signaling at rest and in the ‘early’ (0-150 min) and ‘late’ (150-300 min) phase of recovery from a bout of RE in older individuals. We hypothesised that PA ingestion alone would not stimulate MyoPS, but would modulate RE-induced intramuscular signaling and MyoPS rates in older individuals compared with a placebo.
4.3 Methods

4.3.1 Participants

Sixteen older males were recruited for the present study. All participants were recreationally active and deemed healthy based on their responses to a general health questionnaire. Participants suffering from uncontrolled hypertension, Type 2 diabetes or generalised neuromuscular or cardiovascular diseases were excluded from the study. Furthermore, smoking and consuming non-steroidal anti-inflammatory drugs or any medication that might interfere with muscle metabolism rendered the participant ineligible to participate. Prior to obtaining written consent, participants were informed of the purpose and methodology of the study. Ethical approval was obtained through the Solihull Research Ethics Committee (15/WM/0228). The study conformed to the latest guidelines set by the Declaration of Helsinki (7th edition).

4.3.2 Experimental design

Following an initial screening, participants visited the laboratories of the School of Sport, Exercise and Rehabilitation Sciences (SportExR) on two separate occasions. During the initial visit, anthropometric characteristics and isotonic leg strength were determined. Approximately one week after this initial visit, participants underwent an experimental trial to determine the muscle anabolic properties of supplemental PA. The experimental trial consisted of a stable isotope amino acid infusion combined with serial muscle biopsies to determine the MyoPS response to PA or placebo consumption alone or in combination with resistance exercise. The study was parallel-designed and single-blinded, with participants randomized to receive either the placebo control (PL; n = 8) or phosphatidic acid treatment (PA, n = 8).
4.3.3 Preliminary Assessments

**Body mass and height:** Participant body mass was recorded in loose clothing and without shoes to the nearest 0.1kg using a digital balance scale. Height was determined to the nearest 0.1cm using a stadiometer.

**Body composition:** Following determination of body mass and height, participants underwent a dual energy x-ray absorptiometry scan (DXA) (Discovery DXA Systems, Hologic Inc., Bedford, MA) to determine whole-body and regional bone mineral density, fat- and fat-free mass. DXA scans were performed after a ~10 h overnight fast. Participants rested supine on the scanner in loose clothing with their feet positioned at shoulder width apart and held in place with micropore tape. Each scan took 7 min and was analysed by a trained DXA operator.

**Isotonic leg strength:** Participant knee extensor one repetition maximum (1RM) strength was estimated in the dominant leg using a leg extension machine (VR-3, Medway, MA, USA) in order to determine the appropriate load of 75% 1RM for the subsequent experimental trial. Briefly, prior to exercise commencement, a baseline blood pressure measurement was obtained to ensure participants were normotensive (diastolic blood pressure 60-90 mmHg, systolic blood pressure 120-140 mmHg) to reduce the risk of adverse events during heavy-load, fatiguing RE. Following blood pressure assessment, participants initiated a self-selected 1-set warm-up after which the exercising load was gradually increased over subsequent sets until participants were unable to perform >10 repetitions. This final load was used to estimate knee extensor 1RM strength via the Brzycki equation (9). The increment in loading was based on subjective ratings of exercise intensity, which were monitored immediately after each lifting attempt using a modified Borg category-ratio scale (CR-10) (10). Each exercise set was separated by 2 min of passive rest. Strength assessments were led by a trained strength and conditioning specialist.
**Dietary control:** Participants were provided with a standardised meal the evening prior to the experimental trial. The meal contained ~787 kcal, comprised of ~19% protein (~37.5 g), ~46% carbohydrate (~90.7 g) and 35% fat (30.8 g). Participants were asked to abstain from alcohol and caffeine for 24 h prior to the experimental trial. Furthermore, participants were asked to refrain from strenuous exercise for 48 h prior to the experimental trial.

### 4.3.4 Experimental Trial

Following an overnight fast, participants returned to the SportExR laboratory at 0630 h following a ~10 h overnight fast. Upon arrival, a 21G cannula was inserted in an antecubital vein of both forearms. One cannula was used for frequent blood sampling, whilst the other was used to administer a stable isotope amino acid infusion. After obtaining a baseline blood sample, a primed continuous infusion of L-[ring-$^{13}$C$_6$] phenylalanine was initiated (prime: 2 $\mu$mol·kg$^{-1}$; infusion: 0.05 $\mu$mol·kg$^{-1}$, Cambridge Isotope Laboratories, Andover, MA, USA). The contralateral arm was warmed to ~60°C using an electric heating blanket to obtain arterialized blood samples at -180, -120, -60 and immediately prior to exercise, and at 20, 40, 60, 90 ,120, 180, 240 and 300 min of post-exercise recovery. A total of 10 mL of arterialized blood was sampled at each time point and separated into ethylenediaminetetraacetic (EDTA) and serum separator vacutainers (BD, Oxford, UK). Blood samples were centrifuged for 10 min at 3000G and 4°C. Plasma and serum were aliquotted and stored at -80°C until further analyses. After ~150 min of stable isotope infusion, a muscle biopsy was obtained under local anaesthesia (1% lidocaine) from the quadriceps *vastus lateralis* of the non-dominant, non-exercised leg using the Bergström technique (4). Muscle biopsy tissue was quickly rinsed in ice-cold saline and freed from any visible blood, connective tissue and fat before being snap-frozen in liquid nitrogen and stored at -80°C. Immediately after biopsy obtainment, participants completed a
unilateral leg extension RE bout of the dominant leg consisting of two warm-up sets followed by 6 sets of 12 repetitions at 75% of their previously estimated 1RM, designed to elicit a rating of ~8-9 on the Borg CR-10 scale. Exercise sets were interspersed by 2 min of passive rest during which participants remained seated on the machine. Participants completed the exercise with a lifting-lowering cadence of ~1 sec without pause. Time-under-tension and Borg CR-10 rating were recorded after each exercise set. Following RE completion, participants consumed their respective PA or placebo control treatment over 1 h post-RE (described in detail below) and remained in a supine position for the remainder of the experimental trial. At 150 and 300 mins after starting treatment consumption, muscle biopsies were obtained from the non-exercised and exercised legs. Muscle biopsies were sampled ~2-3 cm from one another in a distal-to-proximal orientation. An overview of the experimental trial is provided in Figure 4.1.

![Figure 4.1: Schematic overview of the experimental trial. PA; 750mg of phosphatidic acid-enriched soybean phospholipid supplement, PL; 750mg of rice-flour placebo.](image-url)
4.3.5 Treatment Administration

Immediately and 1 h after RE completion participants consumed two 375 mg gelatine capsules (totalling 1500 mg over 1 h post-exercise) of either a rice-flour placebo control (PL) or phosphatidic acid-enriched soybean phospholipid supplement (PA; Mediator® 50P, ChemiNutra, Austin, US). Treatment capsules were visually identical and ingested with water. The phospholipid composition of PA was; 50-60% phosphatidic acid, 5-15% phosphatidylcholine, 5-15% phosphatidylethanolamine, 1-5% phosphatidylinositol, 1-5% lysophosphatidylcholine, 1-5% N-acyl phosphatidyl ethanolamine. The PA source, dosing and ingestion pattern chosen herein was based on the recent work of others demonstrating that the same soy-derived PA product; i) robustly increases p70S6K signaling in cultured cells (34), ii) elevates muscle protein synthesis in rats when consumed acutely in a dose equivalent to 1500mg for humans (38), iii) elevates circulating PA and lysophosphatidic acid concentrations in humans (43), and iv) enhances RE-induced lean body mass and strength increases in young males (26, 34).

4.3.6 Blood Analyses

Plasma amino acid enrichment and concentration

Plasma \([^{13}\text{C}_6]\) phenylalanine enrichment was determined by gas chromatography-mass spectrometry (GCMS; model 5973; Hewlett Packard, Palo Alto, CA, USA) by monitoring ions 234/240. Briefly, 300 \(\mu\)L of plasma was diluted 1:1 with acetic acid before being purified through cation-exchange columns and dried down overnight under nitrogen. The purified amino acids were then converted to their N-tert-butyldimethyl-silyl-N-methyltrifluoracetamide (MTBSTFA) derivative. Simultaneously, leucine and phenylalanine concentrations were measured by GCMS using the internal standard method. By adding a known amount of internal
standard (6 μL) to 300 μL of plasma, the amount of tracee was calculated. The internal standards were U-[\(^{13}\)C\(_6\)] leucine (ions 302/308) and U-[\(^{13}\)C\(_2\)–\(^{15}\)N\(_2\)] phenylalanine (ions 336/346) added in a ratio of 100 μL·ml\(^{-1}\) of blood.

4.3.7 Muscle Tissue Analyses

*Myofibrillar and muscle-free amino acid extraction*

The myofibrillar protein fraction and muscle free pool were extracted for the analysis of \(^{13}\)C\(_6\) phenylalanine enrichment from ~30 mg of muscle tissue as previously described (2). Briefly, muscle tissue samples were homogenised in a 2 mL Eppendorf using clean sharp scissors. Homogenised samples were placed on a shaker (IKA, Vibrax, Germany) for 10 min at room temperature at 1500 rpm and subsequently centrifuged at 11000 g for 15 min at 4°C. The supernatant containing the amino acid free pool (IC) was transferred to a clean Eppendorf and stored at -80°C for further Western blot analyses, whilst the myofibrillar pellet washed twice through centrifugation at 11000 g for 15 min at 4°C with 500 μL homogenisation buffer. To separate the collagen fraction from the myofibrillar pellet, the pellet was incubated in 750 μL of 0.3 M NaOH for 30 min at 30°C, giving the sample a vortex mix at 15 and 30 min. The sample was then spun at 13000 rpm for 10 min at 4°C and the supernatant transferred to a 4 mL glass collection tube. A 750 μL volume of 0.3 M NaOH was then added to the pellet, centrifuged at 13000 rpm for 10 min at 4°C and the supernatants combined. Myofibrillar proteins were then precipitated by adding 1 mL of 1 M PCA, which was centrifuged at 3200 rpm for 20 min at 4°C. The pellet was washed twice with 2 mL of 70% ethanol. The remaining myofibrillar pellet was hydrolysed at 110°C overnight in 1 mL of 0.05 M HCl and 1 mL of activated Dowex 50W-X8 100-200 resin (Bio-Rad laboratories INC, USA). Constituent amino
acids of the myofibrillar fraction and IC free-pool were purified on cation-exchange columns by eluting with 2 M NH₄OH and evaporating to dryness. Amino acids were then derivatised as their n-acetyl-n-propylester, and phenylalanine labelling determined by gas chromatography-combustion-isotope mass spectrometry (Delta Plus XP, ThermoFisher Scientific, Hemel Hempstead, UK). Due to limited sample availability, tracer enrichment in the intracellular pool was only analysed in a subset of participants (n=11) and yielded a tracer to tracee ratio of 4.37 ± 0.9%.

**Intramuscular signaling**

Western blot analyses were performed on the sarcoplasmic protein fraction obtained during myofibrillar protein isolation (described above). Sarcoplasmic protein content was determined by a DC protein assay before western blot aliquots of 2 μg protein per 1 μL were prepared in 4x Laemmli sample buffer and sucrose lysis buffer and subsequently boiled for 5 min. Equal amounts of protein (30 μg) were loaded onto 10–15 % gels and separated by SDS-PAGE for 1 h. Following electrophoresis, proteins were transferred onto a biotrace nitrocellulose membrane (Pall Laboratory, Portsmouth, U.K.) for 1 h at 100 V. Membranes were subsequently blocked in 5 % milk for 1 h and washed 3 times for 5 min in TBST before being incubated overnight at 4°C in following primary antibodies: Muscle Ring Finger protein 1 (MuRF1, sc-398608) purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Dallas, Texas, U.S.), phospho-p70S6K1 Thr389 (#9205), total 70 kDa S6 protein kinase (p70S6K1; #9202), phospho-eukaryotic initiation factor 4E binding protein (4E-BP1) Thr37/46 (#9459), total 4E-BP1 (#9452), phospho-eukaryotic elongation factor 2 (eEF2) Thr56 (#2331), total eEF2 (#2332), phospho-protein kinase B (Akt) Ser473 (#3787), total Akt (#9272) each purchased from Cell Signaling Technology (New England Biolabs (UK) Ltd, Hitchin, U.K.). Membranes were then washed 3 times for 5 min in TBST and incubated for 1 h in their respective secondary
antibody and washed again 3 times for 5 min in TBST. Protein quantification was achieved by incubating the membranes for 5 min in Immobilon Western chemiluminescent HRP substrate (Merck Millipore, Watford, UK) before being imaged using a G:BOX Chemi XT4 imager using GeneSys capture software (Syngene, Cambridge, U.K.). Bands were quantified using Image Studio Lite (Li-Cor, Lincoln, Nebraska, U.S.).

4.3.8 Calculations

MyoPS rates were calculated from $^{13}$C$_6$ phenylalanine incorporation by using the standard precursor-product model:

$$FSR (\% h^{-1}) = \Delta E_b / E_p x 1/t x 100$$

Where $\Delta E_b$ is the difference in bound $^{13}$C$_6$ phenylalanine enrichment between two biopsy samples, $E_p$ is the mean plasma precursor enrichment and $t$ is the time in min between muscle biopsy samples. Resting fasted MyoPS were calculated using the pre-infusion plasma $^{13}$C$_6$ phenylalanine enrichment as a proxy for basal muscle protein enrichment, a technique that has previously been validated in tracer naïve older individuals (12).

Plasma leucine and phenylalanine concentrations were calculated using the internal standard method:

$$Concentration (mmol/mL) = (Tracer \ amount / TTR) / Volume$$

Where Tracer amount is the amount of internal tracer in mmol, TTR is the tracer to tracee ratio in the sample, and Volume is the amount of plasma sample in mL.

4.3.9 Statistics

Data analysis was performed using SPSS version 22 (IBM, Chicago, IL, USA). Anthropometric and exercise parameters were analysed using a one-way ANOVA. MyoPS rates, intracellular
signaling and insulin were analysed using a two-way, repeated measures ANOVA with one within (three levels; basal, early and late MyoPS) and one between factor (two levels; group). A Tukey’s HSD post hoc test was performed whenever a significant F ratio was found to determine specific differences. Significance was set at p < 0.05 for all analyses. All values are presented as means ± SD or SEM.
4.4 Results

4.4.1 Anthropometric and Exercise Parameters

There was no significant difference between groups for body mass, BMI, whole-body or regional FM and FFM or knee extension 1RM strength (Table 4.1). There was no significant difference in resistance exercise variables or perceived intensity (Borg CR-10) between PL and PA during the experimental trial (Table 4.1).

Table 4.1. Participant anthropometric, strength and resistance training characteristics.

<table>
<thead>
<tr>
<th></th>
<th>PL (n = 8)</th>
<th>PA (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>69.4 ± 3.6</td>
<td>68.4 ± 1.8</td>
</tr>
<tr>
<td>Body mass (kg)</td>
<td>73.9 ± 7.4</td>
<td>73.7 ± 8.9</td>
</tr>
<tr>
<td>BMI (kg·m⁻²)</td>
<td>23.1 ± 1.6</td>
<td>24.4 ± 2.1</td>
</tr>
<tr>
<td>Whole-body FM (kg)</td>
<td>16.4 ± 2.9</td>
<td>19.4 ± 4.0</td>
</tr>
<tr>
<td>Leg FM (kg)</td>
<td>5.2 ± 1.0</td>
<td>5.9 ± 1.5</td>
</tr>
<tr>
<td>Whole-body FFM (kg)</td>
<td>53.6 ± 5.7</td>
<td>50.6 ± 5.6</td>
</tr>
<tr>
<td>Leg FFM (kg)</td>
<td>17.1 ± 1.4</td>
<td>16.1 ± 1.9</td>
</tr>
<tr>
<td>Knee extension 1RM (kg)</td>
<td>87.4 ± 18.3</td>
<td>79.4 ± 20.7</td>
</tr>
<tr>
<td>Training volume (kg)</td>
<td>1918 ± 377</td>
<td>1725 ± 602</td>
</tr>
<tr>
<td>Volume per set (kg)</td>
<td>320 ± 63</td>
<td>288 ± 100</td>
</tr>
<tr>
<td>Time-under-tension total (sec)</td>
<td>183 ± 37</td>
<td>170 ± 66</td>
</tr>
<tr>
<td>Time-under-tension per set (sec)</td>
<td>31 ± 6</td>
<td>28 ± 11</td>
</tr>
<tr>
<td>Average load per set (kg)</td>
<td>26.9 ± 4.3</td>
<td>24.3 ± 8.2</td>
</tr>
<tr>
<td>Average Borg CR-10</td>
<td>8.4 ± 1.4</td>
<td>7.1 ± 1.5</td>
</tr>
</tbody>
</table>

Values are presented as means ± SD. BMI; body mass index, FM; fat-mass, FFM; fat-free mass, 1RM; one-repetition maximum, Borg CR-10; Borg category-ratio scale.

4.4.2 Plasma Amino Acid and Serum Insulin Concentrations

Plasma leucine and phenylalanine concentrations did not differ between groups in the basal state or throughout the experimental trial (Figure 4.2A and 4.2B, respectively). Serum insulin
concentration decreased significantly below basal values by 90 min post-exercise cessation until 240 and 300 min in PL (P < 0.05) and PA (P < 0.05), respectively, with no differences between groups (Figure 4.2C). Plasma $^{13}$C$_6$ phenylalanine enrichment significantly increased above basal values (-180 min) 60 min after initiation of the stable isotope tracer infusion and remained elevated for the duration of the trial in PL and PA (P < 0.001). Linear regression analysis revealed that the $^{13}$C$_6$ phenylalanine enrichment slopes in both groups were not significantly different from zero, confirming the obtainment of an isotopic steady state (Figure 4.2D).

Figure 4.2: Plasma phenylalanine (A), leucine (B) and insulin concentration (C) and $^{13}$C$_6$ phenylalanine enrichment (D) in experimental trials. At $t = 0$ and 60 min, participants orally
ingested 750mg of PA or PL with water. * indicates significantly different from basal fasting values (-180 min) for PL and PA. † indicates significantly different from basal fasting values for PA only. Values are means ± SEM. Significance was set at P < 0.05.

4.4.3 Myofibrillar Protein Synthesis

Temporal MyoPS rates in rested and exercised legs are presented in Figure 4.3A and 4.3B, respectively. Aggregate MyoPS rates in rested and exercised legs are presented in Figure 4.3C and 4.3D, respectively. In the rested leg, no significant within or between group differences were found for MyoPS rates in the basal state or over 0-150 min, 150-300 min and aggregate 0-300 min. In the exercised leg, MyoPS rates over 150-300 min were significantly greater than basal values (P = 0.001) and 0-150 min (P = 0.019) in PL only, and were ~40% greater than basal values over the aggregate 0-300 min in PL only (P = 0.023). In the exercised leg, there was a strong trend for greater MyoPS rates over 150-300 min in PL compared with PA (P = 0.051). There was no significant temporal or aggregate change in MyoPS rates from basal values in rested or exercised legs in PA, although there was a trend for a reduction in MyoPS from basal values in the rested leg over 150-300 min in PA only (P = 0.053). There was a trend for MyoPS rates in the exercised leg between 150-300 min to be greater than corresponding values in the rested leg for PL only (P = 0.073).
Figure 4.3: Myofibrillar fractional synthesis rate (FSR) during basal postabsorptive conditions (prior to exercise and/or treatment administration) and over the 0-150 and 150-300 min post-exercise recovery period in the resting leg (REST; A) and exercised leg (EX; B), and over the aggregate 0-300 min post-exercise recovery period in the resting leg (C) and exercised leg (D) after PA or PL ingestion. * indicates significantly greater than basal state MyoPS rates in the same leg, † indicates significantly greater than MyoPS rates over 0-150 min in the same leg. Values are means ± SEM. Significance was set at P < 0.05.

4.4.4 Intramuscular Signaling

Phosphorylation of p70S6K\textsuperscript{Thr389} increased by 13.6-fold above basal levels in the exercised leg at 300 min in PL (P = 0.02), and was 19.4-fold greater than corresponding values in the rested
leg (P = 0.02; Figure 4.4A). Phosphorylation of 4EBP1\(^{Thr37/46}\) increased by 2.7-fold and 1.9-fold above basal (P = 0.01) and 150 min (P = 0.047) values, respectively, in the exercised leg at 300 min in PL. Phosphorylation of 4EBP1\(^{Thr37/46}\) at 300 min in the exercised leg for PL was greater than the rested leg at all time-points (P < 0.05; Figure 4.4B). Akt\(^{Ser473}\), MAPK\(^{Ser44/42}\), eEF2\(^{Thr56}\) phosphorylation and MuRF1 protein did not change over time in rested or exercised legs or differ between groups at any time-point (Figure 4.4C-F).
Figure 4.4: Intracellular signaling phosphorylation of p70S6K\textsuperscript{Thr389} (A), 4E-BP1\textsuperscript{Thr37/46} (B), Akt\textsuperscript{Ser473} (C), eEF2\textsuperscript{Thr56} (D), ERK1/2\textsuperscript{Thr202/Tyr204} (E) and MuRF1 protein (F) expressed as fold-change from basal values at 150 and 300 min post-exercise in rested and exercised legs with PA and PL ingestion. * indicates significantly greater than basal value, † indicates significantly greater than value at 150 min, # indicates significantly greater than PA at same time. Values are presented as means ± SEM. Significance was set at P < 0.05.
Figure 4.5: Representative western blot images
4.5 Discussion

The present study is, to our knowledge, the first *in vivo* investigation of the effect of oral phosphatidic acid (PA) or placebo (PL) ingestion on resting and exercise-induced intramuscular signaling and MyoPS rates in healthy older individuals. In the exercised leg, MyoPS rates increased above basal values in late phase (150-300 min) of RE recovery for PL, but not PA. Similarly, RE increased intracellular anabolic signaling (p70S6K and 4E-BP1) at 300 min for PL only. In the rested leg, there was no alteration in MyoPS for PL, but a trend for a reduction in MyoPS rates below basal values between 150-300 min in PA. Thus, in contrast to our initial hypotheses, oral PA ingestion attenuated RE-induced stimulation of MyoPS and intracellular anabolic signaling in older males. Taken together, our acute findings suggest that PA provision may not be an effective means of augmenting the muscle remodelling response to RE training in older individuals.

Resistance exercise (RE) provides a potent stimulus to increase MyoPS in both young (11) and older individuals (53). However, a blunted response to this anabolic stimulus has been observed in older individuals, that has been attributed to an inability to promptly activate mTORC1-mediated signaling compared with the young (21, 35). This muscle anabolic resistance to RE likely explains the diminished adaptive response to long-term RE training in older compared with younger individuals (3, 23). Thus, interventions to maximize the MyoPS response to RE in older individuals are of great importance in combatting sarcopenia. In this regard, PA has been touted as a ‘nutraceutical’ with purported muscle anabolic properties (16, 44).

Although studies in cell culture (34, 51) and animal models (38, 40, 55) have explored the mechanisms through which PA modulates protein translation, studies in human skeletal muscle
are scarce. Endogenous production and exogenous provision of PA phosphorylate mTORC1-mediated proteins (i.e. p70S6K and 4E-BP1) and protein synthesis through distinct proximal pathways (19). Whilst endogenous PA (i.e. mechanical load-induced) binds directly to the FKB12 rapamycin binding domain on mTORC1 (54), exogenous PA provision is thought to activate mTORC1 through the ERK pathway (51). Furthermore, others have reported that prolonged PA supplementation (identical source and dosing strategy to that used herein) enhances RE training-induced increases in lean mass and strength in younger individuals (26, 34). In a study by Hoffman et al, 16 resistance-trained men were either supplemented with 750mg of PA or a placebo whilst undergoing an 8-week resistance training program. A likely benefit on strength improvements and very likely benefit on lean tissue gains were found for the PA treatment group (26). Whilst these results might favour the use of PA, caution is warranted. The timing of PA ingestion was not controlled, which might have led to participants ingesting the supplement either in the morning or in the evening. Although, to date, research investigating the timing of PA ingestion is non-existent, this aspect might likely impact PA’s anabolic potential. Furthermore, RE sessions were not supervised. Although participants were asked to keep a training log, actual training volume and load could not be verified. Finally, 8 weeks of RE training is likely too short to reveal any significant differences between groups containing such small number of participants (placebo: n=9, PA: n=7) (29, 49). Research by Joy and colleagues (34) addressed some of the shortcomings in the above mentioned study (26) by supervising training sessions and controlling PA ingestion time. Joy et al. found superior increases in muscle cross sectional area and lean body mass when subjects were supplemented with PA compared with a placebo. These findings were supported by the upregulation of anabolic signaling in cell culture upon PA treatment. However, no in vivo signaling measurement were made. In order to investigate the separate and synergistic effects of distinct
PA-mediated signaling pathways on MyoPS in older individuals, we provided PA exogenously through oral ingestion, whilst mechanical contraction was used as a stimulus for endogenous PA production. Although we could not directly confirm that RE induced an increase in PA synthesis, others have demonstrated that ex vivo eccentric contractions elicit a sustained increase in PA in mouse EDL muscle (40). Surprisingly, exogenous oral PA ingestion alone tended to reduce MyoPS rates below basal values between 150-300 min, and completely blunted the RE-induced increase in intracellular anabolic signaling and MyoPS observed in PL between 150-300 min. Thus, given that inhibition of RE-induced mTORC1-mediated signaling via rapamycin impairs MPS in humans (17), the attenuated RE-induced p70S6K and 4E-BP1 signaling response in PA offers a likely explanation for the absence of any RE-induced increase in MyoPS.

The present findings of a potential interference effect of PA on RE-induced anabolic signaling and MyoPS are difficult to reconcile with previous in vitro evidence of the pro-anabolic effects of PA on mTORC1-mediated signaling (18, 34) and protein synthesis (38). However, indirect support for our findings can be gleaned from the recent work of Mobley and colleagues (38), who demonstrated that PA ingestion alone tended to stimulated MPS, but blunted the MPS response to whey protein ingestion. Despite the PA-induced attenuation of the MPS response to whey protein, the authors did not report an accompanying impairment in intramuscular anabolic signaling (38), indicating a degree of discordance between anabolic signaling and MPS responses in this experimental model compared with the present human study. Indeed, the capacity for maximal mTORC1-mediated signaling activity has been reported to exceed that required for peak MPS stimulation in rodents (15). Nonetheless, the specific mechanisms through which PA interferes with RE-induced MyoPS and potentially impairs basal-state
MyoPS in older individuals are intriguing. One possibility is that PA-derived production of diacylglycerol (DAG), catalysed by the enzyme phosphatidic acid phosphatase, may impair insulin signaling and blunt MyoPS rates in older individuals (13, 47). Although somewhat permissive for mechanical contraction-induced protein synthesis, distal components of the insulin signaling pathway (i.e. Akt) converge with mTORC1-mediated signaling (6). In the present study, we did not detect any change in Akt phosphorylation from basal values or difference between PL and PA, although we cannot rule out that insulin signaling proteins proximal to Akt may have been impaired in the presence of DAG production.

In addition to blunting the intramuscular signaling and MyoPS response to RE in older individuals, exogenous oral PA provision surprisingly had no detectable effect on ERK1/2 phosphorylation at rest or following RE in the present study. The ERK signaling pathway has been suggested as an important mechanistic link between exogenous PA provision and mTORC1-mediated signaling in vivo (51). However, PA-induced activation of ERK and thus mTORC1-mediated signaling hinges on the conversion of PA to lysophosphatidic acid (LPA) which may, in turn, depend on the presence of the converting enzyme, phospholipase A (PLA) (51). Thus, the absence of mTORC1-mediated signaling in response to exogenous oral PA ingestion suggests that this route of administration may not have altered LPA production. Unfortunately, we were unable to measure intramuscular PA, LPA, PLA and DAG concentrations due to technical limitations and tissue availability (40). However, the blunted intracellular anabolic signaling and MyoPS response to RE in older individuals following PA ingestion, suggests a degree of cross-talk between this nutrient compound and skeletal muscle.
It is well described that RE robustly increases MPS and, to a lesser extent, MPB (41, 45). Provision of amino acids in close proximity to RE further increases MPS and decreases MPB, enhancing overall net balance compared with fasted-state RE (5, 50). In order to delineate the specific effects of PA provision on MyoPS at rest and in response to RE, we chose to provide PA or PL alone, in the absence of amino acid provision. As expected, our results demonstrate a significant, albeit delayed, increase in MyoPS in PL between 150-300 min post-RE in older individuals. It is possible that the RE-induced increase in MyoPS in our experimental model might have been driven by concomitant elevations in MPB, in which the release of free amino acids from the muscle intracellular free-pool would be re-utilized for stimulation of MyoPS (52). Thus, we were interested to understand whether the blunted MyoPS response to RE in PA (and trend for a reduction in MyoPS rates in the rested leg) could be explained by an anti-proteolytic effect which, theoretically, would potentially decrease the availability of circulating amino acids required for MyoPS. This rationale was based on earlier work from Jaafar et al. (31), in which overexpression of the PA precursor, phospholipase D1, in myotubes, negatively regulated the expression of factors involved in muscle proteolysis, including MuRF1, and prevented atrophy in the presence of TNF-α and dexamethasone. Given that MuRF1 expression has been shown to be upregulated after RE in older individuals (22) and was therefore measured in the present study as a proxy marker for proteolysis in the absence of direct in vivo MPB rates. However, we found no difference in MuRF1 protein over the post-exercise recovery period compared with basal values for PL and PA. In addition, we did not detect any differences between PA and PL in circulating amino acid or insulin concentrations that could support a role of PA in suppressing proteolysis. Taken together, our data refute the notion that PA-mediated inhibition on RE-induced proteolysis may explain the impairment in MyoPS rates, and instead suggest that PA-mediated anabolic blunting may underlie this response. However, given the
complexity of factors involved in muscle proteolysis, the potential for PA to modulate different intramuscular proteolytic pathways in response to RE in older individuals requires further exploration.

In conclusion, we are the first to study the in vivo anabolic properties of acute oral PA ingestion in healthy older individuals. PA ingestion attenuated the RE-induced increase in MyoPS and tended to exert similar deleterious effects on resting MyoPS rates. Mechanistically, the attenuation of RE-induced MyoPS in PA was accompanied by impaired p70S6K and 4E-BP1 phosphorylation. The precise cause of the apparent interference effect of PA on RE-induced muscle anabolism in older individuals remains to be fully elucidated, but may partly be attributed to PA-mediated DAG synthesis and impaired insulin signaling. Future research should address whether oral PA provision results in endogenous DAG production in aged skeletal muscle. Furthermore, given the reported enhancement in muscle remodelling in young individuals supplemented with PA during long-term RE training, potential age-related differences in the acute and chronic muscle anabolic response to PA provision should be probed. Furthermore, it is yet to be elucidated if a PA-loading phase is desirable to enhance its anabolic effect. Similar to creatine, a PA-loading phase might be necessary to gradually increase intramuscular PA concentrations (30). If this is the case, future studies should investigate the optimal length of, and PA dose to be administered during, the loading period. Dose-response studies need to shed light over the question what the optimal PA dose is to be administered either acutely or during a loading phase to elicit a maximal muscle anabolic or anti-catabolic response. Although speculative, our acute mechanistic data hint that long-term PA supplementation would not enhance, and may potentially impede, RE training-induced muscle remodelling in older individuals.
4.6 References


4.7 Additional Information

Competing interests

The authors have no competing interests to declare.

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Contribution Statement

All authors gave their final approval of the version of the article to be published. BS, PJA and LB designed the study. BS and LB organized and carried out the clinical experiments with the assistance of JM. BS, JM, YN, ML, KS, PJA and LB performed the data analyses. BS, and LB performed the statistical analysis of the data. BS, PJA and LB wrote the manuscript together.
BS and LB are the guarantors of this work and take responsibility for the integrity and accuracy of the data analysis.
5.1 Introduction

Since the introduction of the term “sarcopenia” by Irwin Rosenberg (50), research aimed at unravelling the underlying mechanisms of and potential countermeasures to this phenomenon has been forthcoming. Although significant progression has been made, much work still needs to be done to have a meaningful clinical impact, particularly in the context of the rapidly expanding ageing population (48).

The mechanisms underlying sarcopenia are undoubtedly complex and multifactorial (46), but ultimately manifest in a negative net muscle protein balance, and hence muscle protein loss over time. This negative net protein balance appears to be primarily driven by a blunted response to pro-anabolic stimuli, such as protein nutrition (32) and exercise contraction (37). It has hence been suggested that older individuals would need to increase total exercise volume/workload and consume greater amounts of dietary protein throughout the day and on a meal-by-meal basis to restore the muscle anabolic response (36, 42). Furthermore, a shift in body composition towards relative fat mass gains is observed with ageing (14). Combined, muscle anabolic resistance in the face of increasing fat mass might lead to the development of sarcopenic-obesity (49); a condition leading to adverse metabolic health and frailty (38), posing monumental strains on health care costs (30). To date, we are yet to fully elucidate the mechanisms underpinning sarcopenia, obesity, and sarcopenic-obesity. These underlying factors are of utmost importance in order to develop successful strategies to counteract sarcopenia and sarcopenic-obesity. Therefore, the purpose of the present thesis was:

I. To determine intake, pattern and source of dietary protein in healthy young and community dwelling older individuals
II. To investigate how old age per se, and the addition of obesity and physical inactivity affect the muscle anabolic response to moderate-dose protein ingestion.

III. To determine the effectiveness of a novel nutraceutical, phosphatidic acid (PA), in enhancing rates of myofibrillar protein synthesis at rest and after exercise in healthy older individuals compared with a placebo.

The present chapter will provide a brief summary of the key findings from the studies described in previous chapters of this thesis. Based on Chapter 2 we will discuss the daily protein intake, distribution, and source in older individuals, provide some rationale for alternative guidelines, and formulate directions for future research. Based on the observations made in Chapter 3 we will discuss how ageing, physical activity and obesity might play a role in the anabolic response to moderate-dose protein ingestion, and formulate directions for future research. Finally, we will discuss the findings of Chapter 4, and formulate recommendations for future research to outline whether there are any the potential muscle anabolic properties in phosphatidic acid.

5.2 Protein Distribution in the Ageing Population

Muscle anabolic resistance to protein nutrition is thought to be one of the primary mechanisms leading to age-related muscle loss (12), and reiterates the need for greater dietary protein intakes in older individuals to attenuate this muscle loss (66). Besides the ingested protein amount, the pattern of intake (39) and source (67) of dietary protein can influence the extent to which muscle protein synthesis (MPS) is stimulated. Therefore, in Chapter 2 of the present thesis, we assessed intake, pattern, and source of dietary protein in both healthy young and community dwelling older individuals upon completion of a 3-day weighed food diary. Our data demonstrate that old compared with young individuals consumed less overall dietary protein,
although the RDA (0.8 g·kg\(^{-1}\)·day\(^{-1}\)) was met by the majority of individuals in both groups. Furthermore, dietary protein intake throughout the day was unevenly distributed in both young and older individuals, meaning older individuals failed to reach the reported threshold requirement for maximal stimulation of MPS at breakfast and lunch (0.4 g·kg\(^{-1}\)). Ingested dietary protein sources were equivalent between young and old, with the exception that older individuals consumed less animal-derived protein at lunch compared with the young.

5.2.1 The Importance of Protein Dose and Intake Pattern

Recently, debate has arisen as to whether total dietary protein intake is more important than pattern of protein consumption to stimulate MPS and increase whole-body lean mass in older individuals (2, 34, 39). Current guidelines recommend daily dietary protein intakes of 0.8 g·kg\(^{-1}\)·day\(^{-1}\) to be adequate and meet the metabolic demand of this nutrient. Nevertheless, several research studies conducted over the past few years have demonstrated beneficial effects on muscle mass accretion when daily protein consumption is increased in older individuals (26). The results presented in Chapter 2 concerning total daily protein intake are in line with previous studies (9, 56), showing that the majority of older individuals meet the current RDA for protein intake. However, we observed a drastic decrease in the number of individuals reaching an alternative guideline 1.0 g·kg\(^{-1}\)·day\(^{-1}\) (4). Furthermore, our data suggests that only 1 in 10 older people ingested the amount of protein, as proposed by Moore et al (42), to maximally stimulate MPS within a given meal (0.4 g·kg\(^{-1}\)) 3 times over the time course of a single day. Based on this observation, it would be safe to assume that older individuals likely spend a significant proportion of the day in a relative protein deficient state, which would consequently lead to suboptimal MPS rates and, potentially, to skeletal muscle loss over time (63). Our findings support the position that meal-specific protein intake in older individuals is
suboptimal and is characterised by an uneven protein distribution throughout the day. Increasing protein intake on a meal-by-meal basis might be pivotal in mounting repeated, robust skeletal muscle anabolic responses in older individuals, whereas solely assessing total daily protein intake would not indicate if the maximal anabolic response within each meal has been achieved.

It has been suggested recently that adult men and women consuming protein evenly across the day have superior 24 h MPS stimulation compared with those consuming uneven protein amounts throughout the day (39). The theory of an evenly distributed protein intake is based on the premise that a protein dose capable of maximally stimulating MPS is consumed at each meal. Therefore, studies that fail to provide this amount (0.24 and 0.4 g·kg\(^{-1}\) in young and old, respectively) are unlikely to find an advantage of even over uneven protein distribution (2, 6). More recently, complementary acute and longitudinal studies were published, in which the effects of even and uneven protein intake patterns were probed in older individuals (34, 35). Although the protein dose provided with each meal in the evenly distributed group was between 30-40g, this might not have been sufficient to fully stimulate MPS as no differences were observed in MPS between an even and uneven protein distribution (34). The threshold for maximal MPS stimulation, as demonstrated by Moore et al (42), is based on the ingestion of a “high-quality” protein. Since most meals in a Western diet consist of both animal- and plant-derived proteins, varying degrees of protein quality will be consumed within a meal. Therefore, the actual protein amount to be consumed within a mixed meal might be greater than that determined by Moore et al (42). Indeed, the plasma aminoacidemia and leucinemia upon consumption of a mixed meal is lower compared with that observed after the ingestion of pure AA/EAA (47, 64). Therefore, older individuals might need to consume protein amounts
>0.4g·kg\(^{-1}\)·meal\(^{-1}\), to maximally stimulate MPS. A further consideration is that increasing the levels of protein consumption beyond the RDA will result in greater insulinemia. Increased systemic insulin concentrations have been associated with anti-proteolytic effects. In young individuals, this insulin-mediated MPB inhibition has been shown to saturate (21), whilst older individuals show an impaired proteolytic response to increased insulin concentrations (60). These data reaffirm the need for higher protein intakes in older individuals with each main meal, especially in the context of mixed meal consumption. Although the study described in Chapter 2 did not measure MPS or MPB, we reaffirmed the observation of a suboptimal protein intake in older community dwelling individuals at breakfast and lunch. Future guidelines should emphasise the importance of consuming protein doses equal to, or exceeding, the threshold for maximal MPS stimulation proposed by Moore and co-workers (42) with each meal, regardless of the distribution pattern.

### 5.2.2 Future Research

To date, evidence supporting the superiority of evenly distributed protein intake across the day is inconclusive (34, 35, 39). Therefore, future investigations should explore the potential of consuming protein doses beyond those shown to saturate MPS, in order to elucidate when and whether a mixed meal is able to fully stimulate and inhibit MPS and MPB, respectively, in older individuals. Besides protein consumption, exercise has been deemed a potent driver of MPS via increasing the anabolic effect of nutrient intake (57). Implementing an exercise training regimen alongside a tailored protein nutrition intervention might therefore increase the muscle–specific and whole-body anabolic responses to protect against sarcopenia. Finally, optimizing protein intake in institutionalised and/or hospitalised populations is of pivotal importance, as malnutrition is often observed in these clinical settings (33, 40).
5.3 Anabolic Resistance in an Aged and Obese Population

Worldwide, obesity rates have reached an all-time high (59). Whilst being associated with plethora of comorbidities (45, 53, 59), less is known on how obesity might affect the progression of sarcopenia (44, 68). Physical activity is an important locus of control in the regulation of skeletal muscle mass in both young and older individuals, with increased physical activity levels stimulating MPS (36), and decreased physical activity levels blunting MPS (7). Therefore, in Chapter 3 of the present thesis, we aimed to broaden our knowledge on the complex relationship between ageing, physical activity and obesity with postabsorptive and postprandial MPS rates. Our results show that the postprandial MyoPS responses to ingestion of a moderate protein dose was robust in young lean (YL), more modest in old lean (OL) and non-existent in old obese (OO) individuals. No correlations were found between any of the intramyocellular lipid (IMCL) characteristics and MyoPS in younger and older individuals. However, average daily step count and leg fat mass were significantly correlated with MyoPS in older individuals.

5.3.1 Lipid Induced Anabolic Resistance

Ageing is associated with a shift in body composition towards an increase in relative fat mass and ectopic fat deposition (11, 14). Elevated IMCL content has been observed in insulin resistant obese Type II diabetic individuals (43) and has been associated with impaired myofibre contraction in older individuals (11). Furthermore, obesity has been associated with decreased postprandial MPS in older individuals (44) and ectopic fat deposition contributes to anabolic resistance in old sarcopenic-obese rats (54). These data seem to support an obesity-induced blunting of postprandial MyoPS due to IMCL accumulation. However, we were unable
to find associations between any of the fibre-specific IMCL properties and MyoPS rates in both young and older individuals, despite two-fold greater IMCL content in Type II fibres of OO compared with OL and YL. When examining Fibre Type I IMCL content similar results were found between YL and OL. These rather peculiar findings might be explained by a phenomenon referred to as the “athlete’s paradox”, or a state of increased IMCL content in endurance trained athletes (58). Although similar at first, the increased IMCL content in endurance trained athletes serves very distinct functions to that observed in obese individuals. Endurance trained individuals will most likely benefit from these intramuscular lipid droplets as a fuel substrate, and could hence be seen as an evident adaptation to endurance training (55). The intracellular localisation of these lipid droplets in trained individuals will consequentially lie in close proximity to mitochondria and will display high turnover rates (55). In contrast are the elevated IMCL levels in obese individuals. Due to increased levels of physical inactivity, the lipid droplets will likely not be readily used as a fuel and hence not co-localise closely with mitochondria. Therefore, the turnover rate of lipid droplets in obese inactive individuals will be slow compared with highly active individuals (58). This IMCL accumulation in obese individuals has been associated with insulin resistance and thought to have a negative impact on metabolic health (11, 20). Measurements of IMCL localisation and turnover rate were beyond the scope of the study presented in Chapter 3. Assessing these characteristics would shed light on potential mechanisms through which obesity and IMCL accumulation may adversely affect skeletal muscle proteostasis in older individuals. Furthermore, investigating the potentially adverse effects of different lipid specifies, such as diacylglycerol and ceramides, on muscle protein turnover might expand our knowledge on the mechanisms of obesity-induced muscle anabolic resistance. Although we were unable to detect any association between IMCL content and the observed decreases in MyoPS in OO, significant correlations between insulin
sensitivity and Fibre type I IMCL content, and between leg fat mas and MyoPS were found. These observations, although indirect, provide some support for the obesity-induced blunting of postprandial MyoPS rates in older individuals.

5.3.2 The Ageing-Obesity Paradox

Previous research has suggested a protective effect of obesity on skeletal muscle mass (28), and has controversially been suggested as potentially beneficial in the battle against sarcopenia. In Chapter 3 of this thesis, we found that skeletal muscle mass, and fibre Type I and Type II CSA were somewhat protected in OO compared with OL. These results are supportive of the existence of the “ageing-obesity paradox”. Nevertheless, we observed a blunted MyoPS upon consumption of moderate-dose protein ingestion in OO compared with OL. Taken together, these observations beg the question, is obesity detrimental to the skeletal muscle anabolism, and, more importantly, when any potential deleterious effect of obesity might occur? It has been widely accepted that, with increasing body mass, absolute skeletal muscle mass increases (29). Consequentially, obese individuals most likely have increased absolute skeletal muscle mass over normal weight individuals, and are therefore considered to have a head start when it comes to being protected against sarcopenia. On the other hand, obesity is associated with increased muscle adipose tissue infiltration (11), which will lead to insulin resistance (52) and an impaired MPS response to anabolic stimuli (44). Based on our observations and those of others, we postulated the existence of an ageing-obesity ‘threshold’ beyond which, obesity-induced muscle anabolic resistance results in a precipitous, rapid decline in muscle mass. This threshold is likely based on muscle quality rather than absolute levels of skeletal muscle mass. For example, this could be determined by the ratio between leg fat and fat-free mass. Even though in our study absolute skeletal muscle mass and fibre CSA were protected in OO, the muscle
anabolic response to a protein feed was severely blunted. This might indicate that the OO included in our study have just passed this muscle quality threshold value, and will suffer severe muscle loss (and incidence of metabolic disease) in the foreseeable future. Indeed, calculating the leg FM:FFM ratio results in 0.58, 0.44 and 0.42 in OO, OL and YL individuals, respectively, and showed a strong correlational trend with MyoPS in older individuals ($r^2 = 0.22$, $P = 0.056$). Taken together, our observations support the hypothesis of a “muscle quality threshold”, beyond which accelerated muscle loss occurs (Figure 5.1). Preserving muscle quality with ageing is therefore of utmost importance to conserve a healthy anabolic response.
Figure 5.1: Hypothetical relationship between muscle quality and rate of muscle accretion or loss with advancing age. The dotted line represents the muscle quality threshold. Arrows represent factors that might influence muscle quality. The red line represents a decreased muscle quality. The green line is representative of muscle quality maintenance. The yellow line depicts the normal chronological ageing process.
5.3.3 The Role of Physical Activity

Resistance and endurance exercise have the ability to robustly increase MyoPS (61). In Chapter 3 step count was significantly elevated in YL and OL compared with OO, and significantly correlated with MyoPS in older individuals. However, no correlations were found between any of the physical activity intensity levels and MyoPS. These results might advocate the importance of time spent being active rather than the actual physical activity intensity. This hypothesis might seem to contradict recent studies advocating the positive effects of high intensity interval training (HIIT) on metabolic health (19). However, in Chapter 3 we used accelerometry to determine physical activity levels based on metabolic equivalent (MET) cut-off values. A MET value of 1 is defined as the oxygen consumption rate at rest or sitting and equals 3.5 mL·kg body mass$^{-1}$·min$^{-1}$. However, i) relative maximal oxygen consumption ($\text{VO}_{2\text{max}}$) declines with advancing age (22), and ii) whilst absolute $\text{VO}_{2\text{max}}$ does not differ between sedentary non-obese and obese individuals, relative $\text{VO}_{2\text{max}}$ values will decrease significantly in obese individuals (8). Given the above information, the actual physical activity intensity at a given MET value will depend on age, $\text{VO}_{2\text{max}}$, and body mass. Therefore, the MET cut off value for vigorous exercise (a MET value of 7 in our study), will likely have been perceived as easier by YL and OL compared with OO. Based on this information, even though we did not find any differences in accelerometry-derived physical activity levels between groups, obese old individuals might have worked relatively harder at each physical activity intensity level. This would support our theory of advocating the importance of time spent being active rather than physical activity intensity per se. Therefore, obese older individuals, for whom intense physical activity might not even be feasible, could potentially benefit from merely increasing physical activity time rather than intensity.
5.3.4 Future Research

Future research should further investigate how physical activity interventions in (older) obese individuals might alter muscle quality and lead to an improved muscle anabolic response. Furthermore, investigating whether muscle quality is preserved in physically active (older) obese individuals might shed light on whether lifelong exercise would be protective against sarcopenia in normal weight and obese older individuals. Finally, further exploration of the role of IMCL in obesity-induced muscle anabolic resistance and sarcopenia is pivotal. Specifically, investigating the co-localisation of lipid droplets and mitochondria, IMCL turnover rate via isotopic measurement methods and the role of specific lipid species could expand our knowledge on the underlying mechanisms of sarcopenic-obesity.

5.4 Phosphatidic Acid as a Countermeasure to Age-Related Muscle Anabolic Resistance

Recently the search for novel nutraceutical compounds to enhance musculoskeletal health indices has increased exponentially (13). Phosphatidic acid (PA) has recently reported to increase intracellular anabolic signaling in cell culture and animal models (3, 16, 25), and enhance muscle mass and strength increases when supplemented over time in young individuals (23, 24, 31). However, in vivo human research investigating the mechanisms behind the potential beneficial effects of oral PA ingestion are lacking. Furthermore, it is unknown if the reported beneficial effects in younger individuals translate to an older population. Therefore, the study presented in Chapter 4 of the current thesis investigated the effects of acute oral PA or placebo (PL) ingestion on resting and exercise-induced intramuscular signaling and MyoPS rates. We found increased MyoPS in PL, but not PA, following resistance exercise. Furthermore, anabolic signaling was increased in PL only. These results suggest a potential
inhibitory effect of acute PA provision on the muscle anabolic response to RE in older individuals.

5.4.1 The Phosphatidic Acid Interference Effect

Increasing or maintaining muscle mass is of primordial importance with ageing. Resistance exercise (RE) is regarded as one of the most potent stimuli able to fulfil this objective. Whereas healthy young individuals show a robust increase in MPS upon resistance exercise, this anabolic response is somewhat negated in older individuals (37), and has been associated with impaired intramuscular anabolic signaling (17). Following investigations in cell culture and rodents, PA, shown to upregulate intracellular anabolic signaling, could pose a viable strategy to enhance the MyoPS response to RE. In Chapter 4, we examined the mechanistic effects of acute oral PA supplementation and found a blunted anabolic response to oral PA ingestion compared with PL. Reconciling these findings with previous in vitro studies demonstrating a pro-anabolic response upon PA treatment (15, 31) is difficult. To date research is still to elucidate how oral PA provision in humans might modulate intramuscular anabolic signaling. One possible explanation may be found in the intracellular production of diacylglycerol (DAG), catalysed via phosphatidic acid phosphatase (10). Increased intracellular concentrations of DAG might play a role in the insulin resistance of human muscle, and could hence impair MyoPS (1). However, we did not observe any differences between groups in plasma insulin concentrations and Akt phosphorylation. This could imply that increased DAG concentrations might impair insulin signaling proximal to Akt. However, we did not assess intracellular DAG and phosphatidic acid phosphatase content due to insufficient tissue availability. Measuring the concentration of these lipids and enzymes in future research might shed light on possible mechanisms through which PA might impair intracellular signaling. Moreover, no study to date
has reported intracellular lipid concentrations or availability upon PA supplementation. Designing methods to measure intracellular PA concentrations would inform us on its bioavailability (if any at all), and might give us valuable information on what an optimal dosage could be to provoke potential muscle anabolic effects. Additionally, methodological differences between previous studies and our work, might explain some of the discrepancies. Whereas previous research mainly investigated the effects of PA in cells or rodents (31, 41, 62), we investigated the mechanistic effects of PA provision in vivo in humans. Translating findings from ex vivo or animal studies into a human model is not always accurate and might yield different outcomes (51). Therefore, future research should further investigate the effects of acute or chronic PA supplementation in older individuals to expand our understanding on how PA could act as an anabolic stimulus.

5.4.2 Anti-Catabolic Properties of Phosphatidic Acid

Resistance exercise increases MPS and MPB, and in the absence of protein nutrition, results in a negative net protein balance (5). In order to examine the sole effects instigated by PA, we chose not to provide AA in close proximity to the RE bout. As expected, in PL, MyoPS increased in the late phase (150-300 min) of RE recovery. This stimulation could be explained by elevated MPB rates upon RE completion, resulting in increased free intracellular AA concentrations, which in turn could be re-utilised as building blocks for MPS (63). Based on the theorem of an increased MPB after RE, we hypothesised that the observed decrease in MyoPS with oral PA ingestion might have been the result of decreased MPB rates. This would cause free intracellular AA concentrations to decrease, and hence inhibit MPS rates. Support for this hypothesis was found in a study by Jaafar et al (27), allocating anti-proteolytic characteristics to phospholipase D1 (a PA precursor). In this study, PLD1 overexpression...
prevented myotube-atrophy in the presence of TNF-α, and was characterized by a decrease in MuRF1 expression. Based on these results, and evidence of increased MuRF1 expression after RE (18), we decided to examine MuRF1 concentration in our study. However, we failed to observe any difference in the expression of MuRF1 between PA and PL, and hence concluded that the blunted MyoPS upon PA ingestion was most likely due to decreased anabolic signaling. Nevertheless, we acknowledge the complexity of factors involved in the MPB process. Ideally, future studies should measure MPB upon PA supplementation via isotope tracer techniques (65). Furthermore, measuring additional ubiquitin proteins might shed more light on the possible anti-catabolic characteristics of PA. As we decided not to provide AA in conjunction with PA, future research should explore if AA feeding could rescue the observed decrease in MyoPS in older individuals, as AA appear to signal to mTORC1 through divergent proximal pathways to exogenous PA. Based on our findings, PA might not be a viable strategy to counteract sarcopenia as it potentially blunts intramuscular anabolic signaling in healthy older individuals.

5.4.3 Future Research

Despite our results suggesting an inhibited muscle anabolic response upon acute oral PA ingestion in healthy older individuals, it would be presumptuous to conclude PA supplementation is ominous for muscle accretion. Future research should investigate whether a PA loading phase might be necessary to elicit its full potential as an anabolic or anti-catabolic stimulus. Furthermore, studies should investigate the acute and longitudinal effects of combined PA and protein supplementation in both young and older individuals. Finally, our study implemented a PA dosing strategy based on previous research. However, to date no dose-response study has investigated what the optimal supplementation dosage of PA would be.
5.5 Conclusions

In this thesis, we have explored some of the potential causes, mechanisms of and countermeasures to age-related muscle anabolic resistance and sarcopenia. We observed a sub-optimal dietary protein intake pattern in healthy older community dwelling individuals compared with the young, even though the majority consumed sufficient amounts of daily dietary protein according to RDA guidelines. Furthermore, we did not find any difference in protein source consumption between young and old, except at lunch where older individuals consumed greater amounts of bread, a “lower-quality” plant-based protein.

In response to moderate-dose protein ingestion, we observed a blunted 4 h postprandial MyoPS response in obese older individuals compared with young lean and older lean individuals. Although we did not find an association between any of the IMCL characteristics and change in postprandial MyoPS rates, step count and leg fat mass did significantly correlate with postprandial MyoPS in older individuals.

Finally, we investigated if oral ingestion of the purported nutraceutical, PA, could acutely modulate MyoPS in healthy older individuals at rest and following resistance exercise. We observed a blunted MyoPS response, both at rest and after resistance exercise, with acute PA provision, which was supported by an impaired intramuscular anabolic signaling.

In the present thesis, we explored the mechanisms of and countermeasures to age-related sarcopenia. We observed a suboptimal pattern of protein intake in older individuals, and demonstrated the detrimental effects of obesity in combination with physical inactivity on the muscle anabolic response to protein feeding. Finally, we were the first to establish the mechanisms underlying the potential anabolic properties of PA as a countermeasure to sarcopenia.
5.6 References


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CHAPTER 6

APPENDICES
6.1 Methods for GCMS and GC-C-IRMS

Skeletal muscle mass is constantly being synthesized and degraded (5). Any observed changes in whole-body muscle mass are therefore the consequence of alterations in muscle protein synthesis (MPS) or muscle protein breakdown (MPB). In vivo measurement of alterations in MPS and MPB is best performed using stable, non-radioactive isotope tracers (4). These tracers are intravenously introduced into the circulation, from where they are taken up by muscle cells into the intracellular pool and subsequently integrated in de novo skeletal muscle (2). Using the Bergström needle biopsy technique, skeletal muscle is sampled at different time points. This technique results in the obtainment of muscle sample sizes ranging from ~50-150mg (1). Muscle biopsy samples are subsequently used to determine stable isotope tracer incorporation. Fractional or total muscle protein synthesis rates are then calculated by establishing the difference in isotope tracer incorporation over time (7).

In Chapter 3 of this thesis we investigated the myofibrillar protein synthesis response to a sub-optimal dose of protein in young lean, older lean and older obese individuals. Extraction of myofibrillar protein fractions was based on the University of Nottingham-Derby protocol. Although, minor alterations were made and are noted in bold, italic in parentheses. This method uses tissue homogenisation and centrifugation to separate the different protein fractions. Chapter 4 of the thesis investigated the myofibrillar protein at rest and after exercise in combination with the ingestion of a phosphatidic acid supplement or placebo. The procedure for myofibrillar protein extraction was processed according to the University of Nottingham-Derby protocol. Minor alterations between the specific isolation protocol in each of these chapters were implemented and In both chapters, plasma $^{13}$C$_6$ Phenylalanine enrichment and plasma Phenylalanine and Leucine concentration were measured using the internal standard
method (7). Specifically, by adding a known amount of stable isotope tracer to an unknown amount of tracee, one can calculate the concentration by determining the tracer to tracee ratio, resulting in following formula (7):

\[ \text{Concentration} = \frac{\text{Tracer amount} \cdot \text{TTR}}{\text{Volume}} \]

### 6.1.1 Method of muscle myofibrillar protein isolation.

**Method Preparation and Supplies**

- Skeletal muscle tissue required: 20-30mg
- 4mL black screw top glass vials
- Scissors to divide muscle on N\(_2\)
- Plastic weighing boats to weigh muscle
- Plastic 1.5mL eppendorfs
- Pipette tips (10µL, 200µL, 1000µL, 5mL)
- Plastic columns with filter disc (biorad)
- Auto-sampler glass vials with inserts and screw tops
- Polystyrene box to hold liquid N\(_2\)

**Reagent Preparation**

**Homogenisation buffer pH 7.5**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>FW</th>
<th>Final Concentration</th>
<th>100mls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl</td>
<td>157.6</td>
<td>50mM</td>
<td>0.788g</td>
</tr>
<tr>
<td>EDTA</td>
<td>372.2</td>
<td>1mM</td>
<td>0.0372g</td>
</tr>
<tr>
<td>EGTA</td>
<td>380.4</td>
<td>1mM</td>
<td>0.0380g</td>
</tr>
<tr>
<td>B-Glycerophosphate</td>
<td>216.0</td>
<td>10mM</td>
<td>0.216g</td>
</tr>
<tr>
<td>NaF</td>
<td>41.99</td>
<td>50mM</td>
<td>0.2g</td>
</tr>
</tbody>
</table>
This makes a stock solution, of which 10mL is extracted. A Protease Inhibitor Tablet EDTA-free (Roche No. 05892791001) and 25μL of a 200mL sodium orthovanadate stock solution is added.

**Preparation of Sodium Orthovanadate**

- Prepare Sodium orthovanadate to 200mM with ddH₂O
- Adjust pH to 10 with 1M NaOH or 1M HCl. At pH 10, solution will be yellow in colour.
- Boil solution until it turns colourless, this takes ~10 minutes.
- Allow solution to cool to room temp.
- Readjust pH to 10 and repeat steps 3 and 4 until solution remains colourless and pH stabilizes at 10.
- Store aliquots at -20°C.

**Sample Preparation Prior to Isolation**

- Fill polystyrene box with liquid N₂, weigh the weighing boat before placing it in N₂.
- Take muscle sample, which is store in a plastic screw top cryovial, out of the dewar and tip it onto the weighing boat.
- Weigh sample and adjust its weight on N₂ using scissors if it is not between 20-30mg. When not weighing the sample, keep muscle frozen on N₂.
- Store sample to be used for protein isolation in clean 1.5mL eppendorf with pierced lid.
- Store any remaining tissue in the old cryovial for later use.
- Add 10 uL of ice-cold homogenisation buffer per mg of tissue, rapidly homogenise tissue using clean sharp scissors (wash scissors in Decon 90, ddH₂O and then wipe with 100% ethanol). If a more dilute/concentrated protein extraction sample is required, then
the buffer volume can be altered accordingly. Keep samples on ice until all samples have been homogenised.

- Place samples on Vibrax shaker for 10 minutes at room temperature at speed 1000-1500 rpm (making sure the samples are well mixed, speeds towards 1500 rpm might be required).
- Centrifuge samples at 11,000 g at 4°C for 15 min to pellet the myofibrillar, collagen and mitochondrial fractions. Transfer the supernatant to a clean eppendorf for protein quantitation (keep on ice at all times). Keep myofibrillar pellet on ice!
- Wash myofibrillar pellet twice. For each wash use 500uL of homogenisation buffer and centrifuge at 11,000g at 4°C for 10 min. Discard after both washes. This step makes sure any excess sarcoplasmic fraction is removed.

**Separation of Myofibrillar Protein Fractions**

- Add 1000µL of 0.3N NaOH to the myofibrillar pellet and vortex to mix. Incubate in a water bath (or heating block) at 37°C for 30min. Vortex mix at 15 and 30 minutes. *(Heating block is set at 50°C and sample vortex mixed every 10 min)*
- Centrifuge samples at 13000 rpm at 4°C for 20 minutes. Transfer the solubilised myofibrillar supernatant to a clean boiling tube.
- To the pellet add 500µL of 0.3N NaOH and vortex to mix. Centrifuge at 13000 at 4°C for 20 minutes. Remove and combine supernatants.
- To the supernatant (which contains the myofibrillar fraction) add 1mL of 1M PCA to precipitate the protein and vortex to mix. Centrifuge at 3200 rpm at 4°C for 20 minutes. Discard the supernatant.
• Myofibrillar pellet: Wash the pellet from step 5 twice with 2mL of 70% ethanol, centrifuge at 3200 rpm at 4°C for 15 min. Discard supernatant using a fine tip Pasteur pipette.

• Protein hydrolysis of both fractions. Re-suspend pellets in 0.1N HCl and then add 1mL of Dowex slurry. Cap and incubate the resulting sample at 110°C overnight for approximately 16 hours. *(Protein hydrolysis is performed by adding 2mL of 0.5N HCl and 1mL Dowex slurry)*

• Remove the sample from the oven, if it has dried out add 2mL of 0.05N HCl and re-incubate the tube for 2 hours at 110°C. Allow sample to cool. To continue follow SOP for isolation of AA using Dowex H+ resin.

**Baseline Plasma Protein Bound \(^{13}C_6\) Phenylalanine**

• Spin plasma at 4°C for 5 min at 11.000g

• Aliquot 50µL in new eppendorf, add 200µL of 100% ethanol

• Vortex and put in fridge for 10 min.

• Spin plasma at 4°C for 5 min at 11.000g

• Discard supernatant, add 1mL of ethanol, spin at 4°C for 5 min at 11.000g → twice

• Hydrolyze pellet overnight in 1mL of 0.1N with 1mL of activated dowex resin.

• Hydrolyzed substrate can now be treated similar to myofibrillar fraction. Start at column procedure

**6.1.2 Intracellular \(^{13}C_6\) Phenylalanine Enrichment from Sarcoplasmic Fraction**

• Take 100µl of sarcoplasmic fraction from each muscle sample. For the basal value, take 50µl of basal plasma.
• Add 20μl of 1N PCA and 2μl of D2 Phenylalanine (20μg/mL internal standard).
• Vortex samples, place samples in fridge at 4°C for 10 min. Centrifuge for 10 min at 13,000rpm.
• Bring samples to a neutral pH. Use liquid universal indicator to check pH. KOH/PCA can be used to adjust pH.
• Add 10μl of urease for 20 min at room temperature.
• Fractions are ready to be treated as myofibrillar fractions. Start at column procedure.

Isolation of Amino Acids

• Columns with filter discs are prepared and 2mL of dowex slurry is added to the column or 1mL for plasma and intracellular AA isolation (volumes in brackets).
• Wash the column with 6mL (4mL for IC) of 2N NH₄OH. Flow may need to be initiated using a 5mL pipette to remove airlock. Use a repeater pipette.
• Neutralise the NH₄OH by adding 9mL (6mL) distilled water.
• Prime the columns with 6mL (4mL) of 1N HCl. The columns are then prepared for the sample addition.
• The samples can now be added to the columns (poor it). Add 3x 0.5mL of 1N HCl to rinse the sample tube out. Make sure to remove as much of the Dowex as possible.
• Add 1mL of 1N HCl to the columns and when the liquid has dripped through add a further 1mL.

N acetyl, n-propyl ester (NAP) Derivatisation.

• The AA form the dowex columns should have been eluted in 3mL of NH₄OH. These samples need reducing to at least 1mL by evaporation under nitrogen at 90°C.
• Aliquot the sample into auto-sampler vials labelled with permanent marker and cover with cellotape or printed label and completely dry at 70°C under nitrogen in turbovap.

• In the fume hood add 200µL propyl acetate and 100uL BF3:Propanol (14%). Cap and vortex to mix the samples and heat at 110°C for 30 mins (in oven). Place vials in an appropriate sample tray, i.e. heat resistant, and remove from oven using heat resistant gloves and allow samples to cool.

• De-cap the tubes and dry completely in the turbovap with nitrogen at 70°C (approx. 10-15 min).

• In the fume hood add 100uL BF3:Propanol (14%) and dry in the turbovap with nitrogen at 70°C (approx. 10-15min).

• In the fume hood add 50µL acetonitrile and 25µL 1,4 dioxane to the dried sample. (Vortex to mix).

• Add 37.5µL triethylamine then add 22.5µL acetic anhydride, cap and vortex. Heat in oven at 55°C for 15 min. Allow samples to cool.

• Uncap the tubes and transfer to 1.5mL eppendorf tubes. Add 50 µL chloroform Add 150 µL 0.001M NaHCO₃ and tap tubes to mix. Two layers should from quickly in the eppendorf.

• Remove the upper layer with a 200mL pipette and discard. Ensure that you have completely removed the top layer, including the small liquid bubble that remains on the top.

• Add a molecular sieve to each tube and leave for 2 min to absorb any remaining aqueous layer.

• Transfer to auto-sampler vials with insert and cap (top up with 50µL chloroform if the sample is likely to be too concentrated).

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6.1.3 Analysis of $^{13}$C$_6$ Phenylalanine Enrichment by Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry

**Establish:** $^{13}$C$_6$ Phenylalanine in myofibrillar fractions.

<table>
<thead>
<tr>
<th>Rate ($^\circ$C-min$^{-1}$)</th>
<th>10$^\circ$C-min$^{-1}$</th>
<th>1$^\circ$C-min$^{-1}$</th>
<th>20$^\circ$C-min$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature ($^\circ$C)</td>
<td>110$^\circ$C</td>
<td>235$^\circ$C</td>
<td>242$^\circ$C</td>
</tr>
<tr>
<td>Time (min)</td>
<td>1.0’</td>
<td>3.0’</td>
<td>2.0’</td>
</tr>
</tbody>
</table>

- **GC-C-IRMS:** Thermo DeltaplusX MS, Thermo Trace GC ultra, Thermo GC combustion III, Thermo AS2000 auto-sampler
- **Column:** Delta’s (column Agilent DB1701 30M x 0.25 mm x 1.0 μm).
- **Inlet method:** Temp 250°C injection volumes of 0.2-2.0μl ran split less at 1.4ml/min with constant purge

6.1.4 Extraction of Free Plasma Amino Acid

**Method Preparation and Supplies**

**Plasma needed:** 300μl

**Reagents needed:**
- 70% Ethanol (EtOH)
- 1N HCl
- 2N NH$_4$OH
- MTBSFTA (N-tert-butylidimethyl-silyl-N-methyltrifluoracetamide, 394882 ALDRICH)
• Acetonitrile (271004 SIGMA-ALDRICH)
• Activated AG 50W-X8 Resin, 100-200 mesh hydrogen form

**Supplies needed:**

• 1.5mL eppendorf tube
• 4mL screw top vial
• Glass auto-sampler vials, vial inserts, screw caps
• Heating block with N₂ to dry samples
• Plastic columns with filter disc (biorad)

*Plasma $^{13}$C₆ Phenylalanine Enrichment and AA Concentration from Plasma*

**Sample Preparation**

• Take samples and internal standard out of the freezer and defrost
• Label new eppendorfs and weigh eppendorfs
• Vortex samples and internal standard once defrosted
• Centrifuge samples (3000rpm, 5 min, 4°C)
• Transfer 300µl of sample to new eppendorf – re-weigh eppendorf – record weight
• Add 6µl of internal standard to sample, re-weigh eppendorf
• Add 300µl of acetic acid (1:1 dilution)

**Column procedure**

• Rinse columns with ddH₂O
• Fill columns with ~ 3.5mL activated resin (up to second line)
• Rinse the columns 2 x 3mL HCl (1N) (each time letting it drain through)
• Vortex samples
• Place prepared sample on the column with Pasteur pipette, add 1ml ddH₂O to sample tube to rinse
• Add 3ml HCl 1N to the column
• Rinse column 9 times with 3mL ddH₂O (or until neutral, check using pH paper)
• Put column on glass tube and add 3mL NH₄OH, collect and test pH
• If not alkaline add 1mL NH₄OH at a time
• Add 3mL ddH₂O
• Vortex screw top tube
• Place tubes with the liquid under N₂ until dry

*N-tert-butyldimethylsilyl- N-methyltrifluoroacetamide (MTBSTFA) Plasma Derivatisation*

• Dissolve the dried sample or standard in 50µL acetonitrile and 50µL MTBSTFA
• Place the tubes for 1 hour at 70°C in the heating block (with caps)
• Centrifuge (3000rpm, 5 mins, 25°C)
• Transfer into auto-sampler vials

6.1.5 Analysis of Free Plasma [¹³C₆] Phenylalanine Enrichment by Gas Chromatography

*Mass Spectrometry*

**Establish:** Free plasma [¹³C₆] Phenylalanine in myofibrillar fractions and AA concentrations.

<table>
<thead>
<tr>
<th>Rate (°C·min⁻¹)</th>
<th>10°C·min⁻¹</th>
<th>30°C·min⁻¹</th>
<th>50°C·min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>100°C------→ 170°C------→ 235°C------→ 250°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time (min)</td>
<td>0.5’</td>
<td>1.5’</td>
<td>1.0’</td>
</tr>
</tbody>
</table>
Column: Capillary column, Agilent 19091J-433, 30m x 250µm x 0.25µm

Inlet method:

- Mode: Split, with 25:1 split ratio
- Split flow: 62.4mL/min; Total flow: 67.7mL/min
- Gas type: Helium

6.2 Method for Lipid Droplet Staining Using Bodipy (493/503)

Triacylglycerol (TAG) is most often stored in white adipose tissue. Once stored within the organelle it is then referred to as a neutral lipid droplet (LD). However, under specific conditions, such as obesity, TAG can be stored in tissues other than white adipose tissue (3, 6). The accumulation of TAG in these non-adipose depots is referred to as ectopic fat. In Chapter 3 of the present study we examined LD accumulation within the muscle cells of young lean, old lean and old obese individuals. Two prominent measurement methods to quantify intramuscular LD have been used in previous literature; Oil Red O (ORO) and Bodipy (493/503). Whilst ORO is a powerful tool to quantify intramuscular LD, it has some disadvantages such as a short shelf-life and difficulties getting it into solution. Therefore, we decided to image intramuscular lipid droplets using Bodipy (493/503), which has a longer shelf life and dissolves easily into solution (6). The Bodipy (493/503) dye has previously been shown a successful method to quantify lipid droplets and allows the simultaneous use of other stains (e.g. myosin heavy chain and cell border) (6).

6.2.1 Method Preparation and Supplies

- Pipette tips (10µL, 200µL, 1000µL, 5mL)
- Glass/plastic holder for slides
- Plastic box with lid to incubate slides
- Make up 3.7% formaldehyde, dilute with dH$_2$O.
- Make up primary antibody: 25uL per section+ spare. You need a 1:25 solution using 5% NGS. For 1 section, you need 1uL primary antibody and 24uL of 5% NGS. Dilute with 1x PBS.
- Secondary antibody: 1:150 dilute, in 1x PBS. 25uL per section + spare is needed
- WGA solution 1:100 dilute 1uL of stock solution into 99uL of 1x PBS. 25uL per section + spare is needed
- Bodipy solution is 1:50 dilute 1uL of stock solution into 49uL of 1x PBS. 25uL per section + spare is needed

### 6.2.2 Staining protocol

Four to six muscle sections are put on a slide. With a glycerol containing pen, draw around the sections. This prevents any antibodies or solutions to drip of the glass slide containing the sections. Once the sections are on the slide, sections are fixed for 1h in 3.7% formaldehyde, whilst put on a rocker at low speeds (±50 rpm). Upon fixing the section, the slides are washed 3 times for 30 seconds in ddH$_2$O, incubated for 5 min in 0.5% triton-100 in (diluted in 1x PBS), and washed 3 times for 5 min in 1x PBS. Sections are consequently incubated for 2 h in the myosin heavy chain primary antibody (DSHB laboratories, University of Iowa, US). Primary antibody is diluted 1:25 in 5% normal goat serum (diluted in 1x PBS). Thereafter, sections are washed 3 times for 5 min in 1xPBS, and incubated for 30 min in the secondary antibody (diluted 1:150, goat anti mouse IgM 594, Thermofisher, A21044). Upon incubation sections are washed 3x for 5min in 1xPBS and incubated in WGA350 (Wheat Germ Agglutinin, Alexa Fluor 350 Conjugate, Thermofisher, W11263) for 30 min (1:100 diluted in 1x PBS). Sections are then
washed 2x for 2-3 min in 1x PBS, and incubated for 10 min in Bodipy493/503 (Thermofisher, D3922) diluted 1:50. The bodipy solution is light sensitive, extra care needs to be taken when incubating (keep away from light). Sections are then washes 3 times 3 min in 1x PBS. Finally cover slides are mounted in mowiol.
6.3 References


