Modulation of muscle protein metabolism through amino acid supplementation and exercise in older women

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THESIS ABSTRACT

Background:

Skeletal muscle plays an important role in our body, not just in terms of locomotion but also for metabolic purposes. It itself is dependent on two main factors: an adequate diet of essential amino acids as well as regular exercise to keep it in functioning condition. As humans age, the ability of the skeletal muscle to function adequately declines, largely due to age-related muscle loss. This muscle loss has been studied in great detail mostly in men aged 60 and above. It is also well-known that leucine is the main driver of anabolic response to protein supplementation.

Aim:

The aim of these studies was to quantify the anabolic response to two protein supplementations- Leucine enriched amino acids (LEAA) supplement at doses of 1.5 g, 3 g and 6 g, compared with "standard" whey protein 20 g & 40g, with and without resistance exercise in women aged 60-70 years. We also studied the effects of supplements and exercise on leg blood flow and muscle blood flow.

Design:

Forty-two acute studies were conducted, each lasting for just over 7 hours. Each study comprised of a fasting stage (with muscle biopsies) as well as

supplementation followed by unilateral knee extension exercise (to allow for nutrition only vs. nutrition + exercise comparisons), thereby marking the post supplementation stage. In each experiment, the supplement was either a leucine enriched supplement or a standard whey protein supplement (doses mentioned above). Regular blood samples as well as muscle biopsies were taken to assess responses. Blood flow was measured in femoral artery (to assess leg blood flow) as well as contrast enhanced ultrasound (CEUS) to assess microvascular blood flow through the muscle vastus lateralis. The main strength of this design was that each volunteer was her own control (i.e. one side was protein supplement only and the other side was supplement + resistance exercise).

Results:

Plasma insulinemia and essential amino acidaemia was higher in whey protein groups compared to LEAA in all doses ($P < 0.001$). All the supplements resulted in an increase in muscle protein synthesis (MPS).

The LEAA at the dose of 3g showed a similar anabolic response compared to whey protein 20g (both $P < 0.05$vs. basal). However, this effect returned to baseline (fasting) levels at 4 hours. In the exercised leg, MPS also increased at 0–2 h but remained elevated at 4 h (both $P < 0.05$ compared to basal).

Similarly, in the study which explored the anabolic responses to LEAA 1.5g, LEAA 6g vs. Whey Protein 40g, MPS increased similarly in response to LEAA_1.5 ($P < 0.05$) and WP ($P < 0.01$) over 0-2 h, with MPS significantly

greater than basal in the LEAA_6 and WP groups only over 0-4 h. However, in the FED-Ex leg, MPS significantly increased across all the groups from 0 to 4 h (LEAA-1.5P < 0.01; LEAA-6- P < 0.01 and WP- P < 0.001).

None of the supplements showed significant increases in leg blood flow or microvascular blood flow- only exercise groups showed an increase microvascular blood flow in LEAA 3g and WP 20g, reaching significance (P<0.05) Due to technical difficulties, only 6/group had their data used for LEAA 1.5g, LEAA6g and WP40, which didn't show any difference.

Conclusions:

The data generated from these studies suggest that smaller dose leucine enriched supplements may have a role to counteract sarcopenia, especially in elderly women, particularly when coupled with resistance exercise. Further studies in larger cohorts may be needed to confirm these results and if confirmed, these could form the basis for larger interventional trials in frail elderly patients suffering from mobility problems due to sarcopenia.

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ABBREVIATIONS

1RM	1 Repetition Maximum
α-KIC	α-Ketoisocaproic acid
AA	Amino Acids
AI	Acoustic Index
APS	Albumin Protein Synthesis
BCAA	Branched Chain Amino Acids
BFR	Blood Flow Restriction
BIA	Bio-electrical Impedance
BMI	Body Mass Index
CEUS	Contrast Enhanced Ultrasound
CI	Confidence Interval
CSA	Cross sectional area
CVA	Cerebrovascular Accident
DNA	Deoxyribonucleic acid
DXA	Dual Energy X-ray Absorptiometry
EAA	Essential Amino Acids
FSR	Fractional Synthetic Rate
HMB	β-hydroxy β-methylbutyrate

IV	Intravenous
LBF	Leg blood flow (mL/ min)
LEAA	Leucine Enriched Amino acid (supplement)
LEU	Leucine
MBF	Microvascular blood flow or perfusion
MPB	Muscle Protein Breakdown
MPS	Muscle/ myofibrillar Protein Synthesis
MS	Mass Spectrometry
mTOR(C)	Mammalian target of rapamycin (complex)
NEAA	Non- Essential Amino Acids
RAPTOR	Regulatory-associated protein of mTOR
RE(T)	Resistance exercise (training)
US	Ultrasound
WP	Whey Protein

CHAPTER 1: INTRODUCTION & LITERATURE REVIEW

1.1: CHAPTER SUMMARY

In this chapter the scene is set by first describing what sarcopenia is and what is known about this complex disease so far? The discussion then delves deep into skeletal muscle mass and looks at factors affecting or maintaining muscle mass in humans.

Two particular factors (nutritional supplementation and exercise) are then discussed in detail, including the interplay between these individual factors and other factors e.g. ageing and gender. Finally, the application of and principles behind the use of stable isotopes as a way to study muscle protein synthesis is discussed.

1.2: CHAPTER INTRODUCTION

Skeletal muscle tissue plays many important roles in human body, however providing help with locomotion and contribution to protein metabolism are two main key functions. Locomotion support includes providing strength to help with our activities of daily living, such as standing, walking as well as attending to our own needs via feeding (which itself is a breakdown of several complex tasks, such as chewing, swallowing, peristalsis etc.), respiratory manoeuvres (breathing, coughing and sneezing) and finally the ability to maintain personal hygiene in accordance with social norms. In addition, a rather less understood role of muscles is that they provide amino-acids support during times of fasting as well as stress (e.g. sepsis, massive injury and burns), to organs heavily

dependent of a constant supply of amino-acids (Felig *et al.*, 1969; Biolo, Zhang and Wolfe, 1995).

Any disturbance with locomotor functions results in individuals' reduced confidence as well as reduced mobility, accompanied by social isolation. According to Department of Health UK, falls are a major cause of morbidity and mortality, affecting annually 30% of those aged >65 years and increasing to 45% in those aged >80 years (Department of Health, 2009). Though not all of these result from muscle failure, it remains the main cause. Poor muscle mass and strength is an independent predictor of increased morbidity and mortality (Fielding *et al.*, 2011).

This chapter is divided into several parts:

- The first part (Section 1.3) discusses the basics of skeletal muscle metabolism, with a brief anatomy and exploration of determinants of muscle mass.
- Section 1.4 looks at nutritional modulation of muscle protein metabolism with effects of age and gender.
- The next section (1.5) explores the modulation of muscle protein synthesis via resistance exercise and again discusses several variables, concluding with effects of age and gender.
- Section 1.6 explores the interplay between nutrition and resistance exercise.
- Section 1.7 looks at the recent definition of sarcopenia and the shortcomings of this definition.
- Section 1.8 describes the aims and objectives of the studies described in this thesis.

1.3: BASICS OF SKELETAL MUSCLE METABOLISM

1.3.1: Introduction

Skeletal muscles are highly adaptable tissues, which help with not only with all aspects of movement and locomotion but also have important metabolic functions. This sub-section reviews the anatomy of skeletal muscles and then moves on to their role as protein reservoirs of body.

1.3.1.1: Anatomy of Skeletal Muscle

Skeletal muscle makes up about 40% of total body weight of an adult male human (though this is highly variable in states such as obesity, malnutrition and chronic disease states such as cancer), with protein contents making up about 20% of total skeletal muscle mass (Wackerhage and Rennie, 2006). Several factors such as age, gender, chronic disease states, adequate protein intake and exercise all affect the final skeletal muscle make of any human. These factors are studied in greater detail in this sub-section later.

The functional unit within each skeletal muscle is called a "muscle fibre" which is a multinucleated cylindrical contractile cell (see figure 1.1).

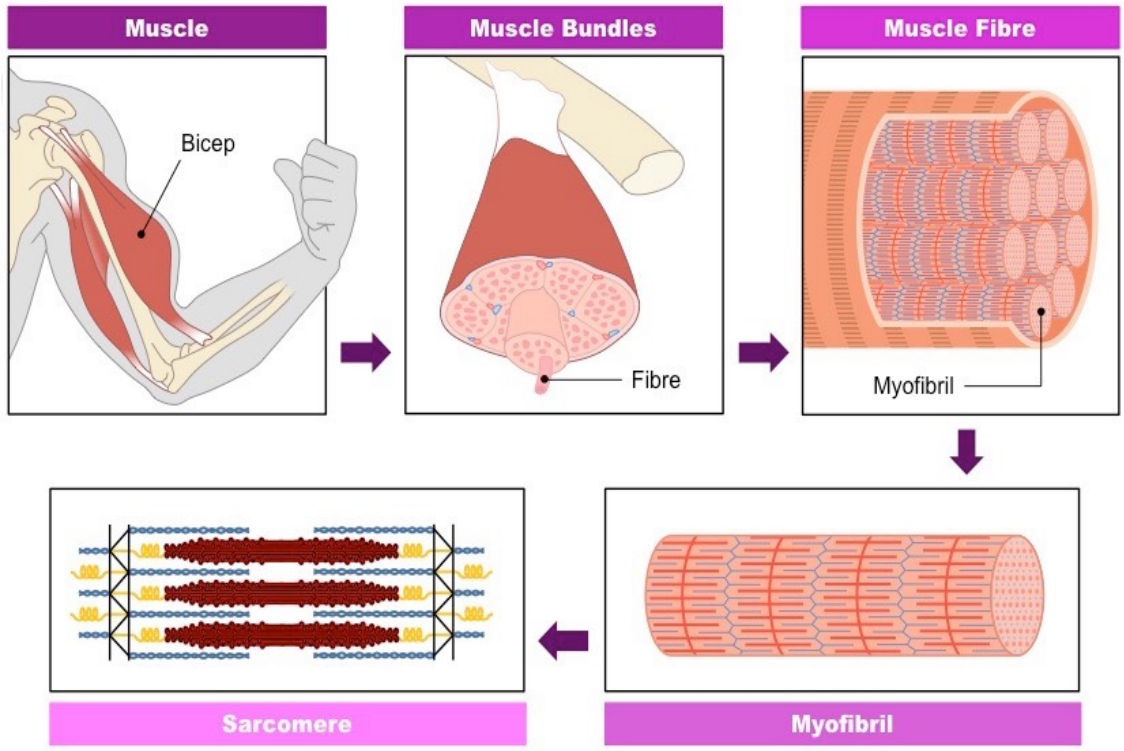


Figure 1.1 shows the anatomy of a muscle, muscle fibres and a myofibril.

The size and number of each muscle fibre determines the size and shape of each individual skeletal muscle and is a combination of both genetics and environment. The number of each muscle fibre in a muscle is determined via genetics however, the size of each muscle fibre is determined via a constant remodelling process (which is mainly determined due to several factors external to muscle such as protein intake, mechanical stress on muscle and any local/ generalised disease state) (Wackerhage and Rennie, 2006).

1.3.2: Factors affecting skeletal muscle mass

There are several factors affecting muscle mass, which are classified in two main groups, as mentioned in a table below:

Non-Modifiable factors	**Modifiable factors**
Age	Nutrition
Gender	Exercise
Chronic wasting diseases	Drugs (including insulin & steroids)

Table-1.1: Factors affecting skeletal muscle mass in human adults.

The non-modifiable risk factors are discussed briefly here. However, the effect of age and gender on modifiable risk factors in mentioned in their subsequent sections.

1.3.3: Impact of age on muscle mass

Human adults lose about 1-2% of muscle mass per year after the age of 50 years (Lauretani *et al.*, 2003). This is primarily due to loss of motor neurones with consequent progressive atrophy and loss of type 2 muscle fibres (Larsson, Sjödin and Karlsson, 1978). The term given to age related loss of muscle mass in "Sarcopenia", however is dependent on many other factors (Anton *et al.*, 2015). This is discussed in detail later in this chapter (see section 1.7).

Increasing age also affects the anabolic responses to several modifiable risk factors such as physical activity and nutrition. However, these are discussed in detail in their subsequent sub-sections within this chapter.

1.3.4: Impact of gender on muscle mass

It is generally observed that women have less muscle mass than men, a finding that has been corroborated in several studies (Miller *et al.*, 1993; Gallagher *et al.*, 1997; Janssen *et al.*, 2000; Merriwether, Host and Sinacore, 2012). The difference is also evident at a histological level, with women having smaller cross-sectional area and smaller muscles fibres (Miller *et al.*, 1993). The strength to cross-sectional area was found to be similar in both genders in this study, indicating that the increased strength seen in men (particularly in upper body) was due to larger muscle fibres. These differences are known to be due to different hormonal profile in both sexes. This conclusion comes from several studies looking at effect of testosterone supplementation on muscle mass and these studies are examined in section 1.3.8 later in this chapter.

A question that arises at this juncture is if men and women respond differently to the main anabolic stimuli (i.e. amino acids supplementation and resistance exercise)? Again, this requires extensive literature review and is documented below in relevant sections.

With advancing age, both sexes lose muscle mass, the decline is however greater in men than in women, as shown in figure below:

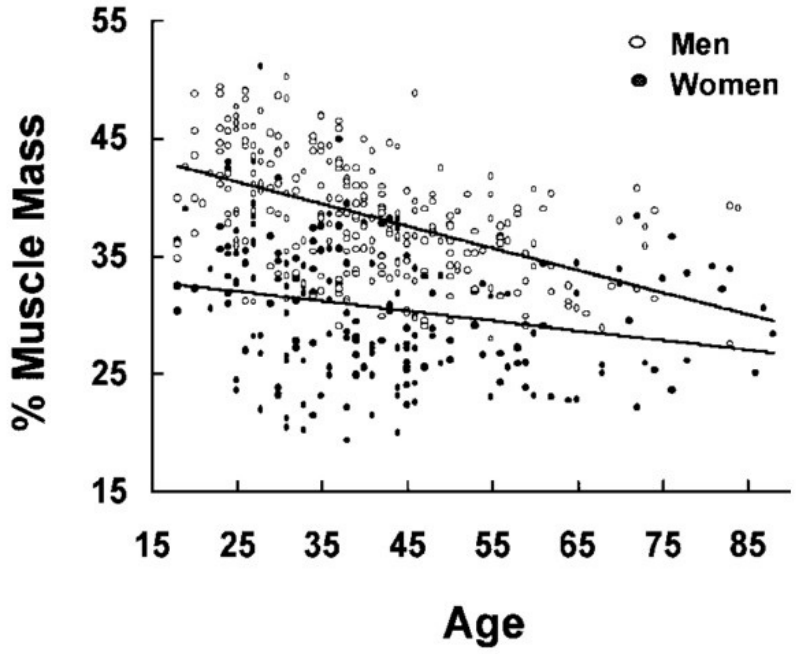


Figure 1.2: Relationship between age and whole-body skeletal muscle mass as percentage of body mass- Note the slope of regression line is greater in men than in women, indicating greater loss (Janssen et al., 2000)

1.3.5: Impact of chronic diseases on muscle mass & function

Several diseases, particularly in advanced stages, could cause or at least contribute to development of cachexia and sarcopenia (Kotler, 2000). These includes advanced cancers (Barber, Ross and Fearon, 1999), AIDS (Kotler, 2000), COPD (Debigaré, Côté and Maltais, 2001; Wüst and Degens, 2007), liver cirrhosis (Kalafateli *et al.*, 2015) and advanced heart failure (Fülster *et al.*, 2013). Any data to support this i.e. loss of mass and

or function?? Sarcopenia in these diseases frequently presents in advanced stages and is associated with increased poorer outcomes in these patients.

1.3.6: Impact of nutrition and amino acids on muscle mass and function

Nutrition is one of the two principle modifiable determinants of muscle mass, the other one being physical activity. It affects the two key processes affecting muscle mass:

1. Muscle protein synthesis (MPS)
2. Muscle protein breakdown (MPB)

The net effect or algebraic difference of these two processes either results in net muscle gain (also known as positive Net Protein Balance or NPB) or muscle loss (negative NPB)(Stokes *et al.*, 2018).

Due to relevance of this process with the data presented in this thesis, the effect of nutrition on muscle protein metabolism is discussed in a separate section (please see section 1.4).

1.3.7: Impact of exercise on muscle mass and function

Exercise is any physical activity performed to improve physical fitness. Depending upon the primary outcome of a given exercise, their main types are:

1. Endurance type exercise: These exercises cause a positive effect of cardio-vascular fitness and include exercises such as running, jogging or cycling.
2. Resistance exercise (also known as weight training): These exercises improve muscle mass and size. These include squats, pull-ups and other exercise that use barbells or dumbbells.
3. Balance: These include exercises that help develop core balance and include exercises such as Pilates and Zumba.
4. Hybrid exercise: While not a "type" of exercise per se, these exercises combine different exercises from categories above into a programme such as Cross-Fit® and kettle-bell.

As resistance exercise (RE) is the main type of exercise that induces muscle growth, it is the main focus of this literature review. There are several RE variables that affect the anabolic response, whether measured in form of molecular prosses such as muscle protein synthesis or as the ultimate end-point of muscle hypertrophy. The effect of RE of muscle mass and the variables affecting this are discussed in detail in its own sub-section below (please see section 1.5).

Endurance type exercise also causes a similar, albeit smaller anabolic response but only in older individuals (Konopka and Harber, 2014). This is, however beyond the scope of this thesis.

1.3.8: Impact of drugs and hormones on muscle mass and function

Among the hormones, testosterone has been known to induce a strong anabolic response. It increases muscle protein synthesis (Griggs *et al.*, 1989), and when used in supra-physiologic doses in men with and without RE, it increases muscle mass as well (Bhasin *et al.*, 1996). In this study, the participants were men aged 19-40 years divided into four groups:

1. No RE on placebo
2. RE with placebo
3. No RE with testosterone supplementation
4. RE with testosterone supplementation

The highest gains were understandably seen in men who combined both testosterone supplementation with RE (group 4). However, two interesting findings stand out from this study:

1. Firstly, that the group who had testosterone supplementation but no RE (group 3) had *HIGHER* muscle mass gain than compared to RE group who were on placebo (group 2).
2. Secondly, muscle *STRENGTH* also improved in those participants who were on testosterone but didn't participate in any RE (group 3).

These findings suggest higher potency of testosterone supplementation in terms of anabolic response than resistance exercise. The study however did not include a nutritional component- it would be interesting to see how availability (or lack of availability) of amino acids would affect these findings. However because of concerns of prostate cancers and other side effects such as cardiovascular problems (Basaria *et al.*, 2010) , the clinical use is limited. For now, testosterone therapy is being studied in chronic disease states such as end-stage renal disease and renal transplant, where most male patients suffer from hypogonadism (Snyder and Shoskes, 2016) Any Benefits?. In another study looking at effect of testosterone replacement in older men and women found that neither testosterone administration nor availability of free testosterone had any effect on age related declines in what measures? Be specific! seen in both genders (Henderson *et al.*, 2008). It also noted that women maintain higher MPS at baseline than men though the basal MPS rate declines with advanced ageing.

Because of lack of data on use of testosterone in general population, a set of co-ordinated trials undertaken in males aged 60 or above with low testosterone levels also show improved mood changes and self-reported improvement in physical function (along with improvements in sexual function as expected) (Snyder *et al.*, 2016). It's use is gaining acceptance under strictly controlled and monitored conditions (Akerman, Kovac and Lipshultz, 2017).

There are several other structurally similar analogues that exist, both in other animals and plants. For example: Ecdysteroids are steroids

hormones found in insects that help during moulting process. Phyto-ecdysteroids are structural analogues of these compounds extracted from plants. Both ecdysteroids and Phyto-ecdysteroids have shown anabolic responses in-vitro (Bathori *et al.*, 2008; Gorelick-Feldman *et al.*, 2008), however their use in humans have not been studied in detail so far.

1.4: NUTRITIONAL MODULATION OF SKELETAL MUSCLE PROTEIN METABOLISM

1.4.1: Historical perspective of nutritional modulation of muscle protein metabolism

Studies from 1980s showed that muscle protein synthesis or MPS increases in response to feeding (Rennie *et al.*, 1982). Subsequent work suggested the importance of amino-acid content of the meal, for example, studies showed that amino acids (even if used as infusion) without any energy substrates, contributed to increases in MPS; though did not affect the MPB (Bennett et al. 1990). Further work showed that it is infusion of *essential* amino acids which is entirely responsible for stimulating MPS (Bohé *et al.*, 2003), with leucine being the primary driver (Smith *et al.*, 1992, 1998).

Currently, proteins are considered essential part of a balanced diet and it is recommended that a recreationally active human consumes 0.8-1.0 g of

proteins per kg of body-weight (Richter *et al.*, 2019). However, for older people (those aged 65 years or above), the recommendations from the European Union Geriatric Medicine Society (EUGMS), in cooperation with other scientific organizations (part of PROT-AGE Study Group) recommend 1.0-1.2g of proteins per kg of body weight, increasing to 1.4g/kg if performing resistance exercise regularly (Bauer *et al.*, 2013). This is due to the data showing that higher protein content in one meal (>35 g) may help overcome reduced anabolic sensitivity seen in skeletal muscles of older people (Paddon-Jones and Rasmussen, 2009).

1.4.2: Physiology of Skeletal Muscle Protein Metabolism

In a normal healthy individual who is only recreationally active, the skeletal muscle protein turnover is about 0.05%/hour or ~1.2%/day (Atherton and Smith, 2012) though is highly variable due to a dynamic equilibrium between two main processes: Muscle Protein Synthesis (MPS) and Muscle Protein Breakdown (MPB). As the names suggest, MPS is a collection of sub-cellular processes that induces the production of either one or more of muscle protein fractions (myofibrillar, sarcoplasmic and mitochondrial). Conversely, MPB is the name given to cellular processes that cause muscle loss, releasing amino acids in blood stream. Either of these processes become dominant in particular states (usually associated with down regulation of counter processes) e.g. in fed state, MPS is the dominant

process with down regulation of MPB and vice versa in post-absorptive or fasting state. These changes are transient and have been measured using stable isotope infusion methods (Atherton and Smith, 2012)- see figure 1.3.

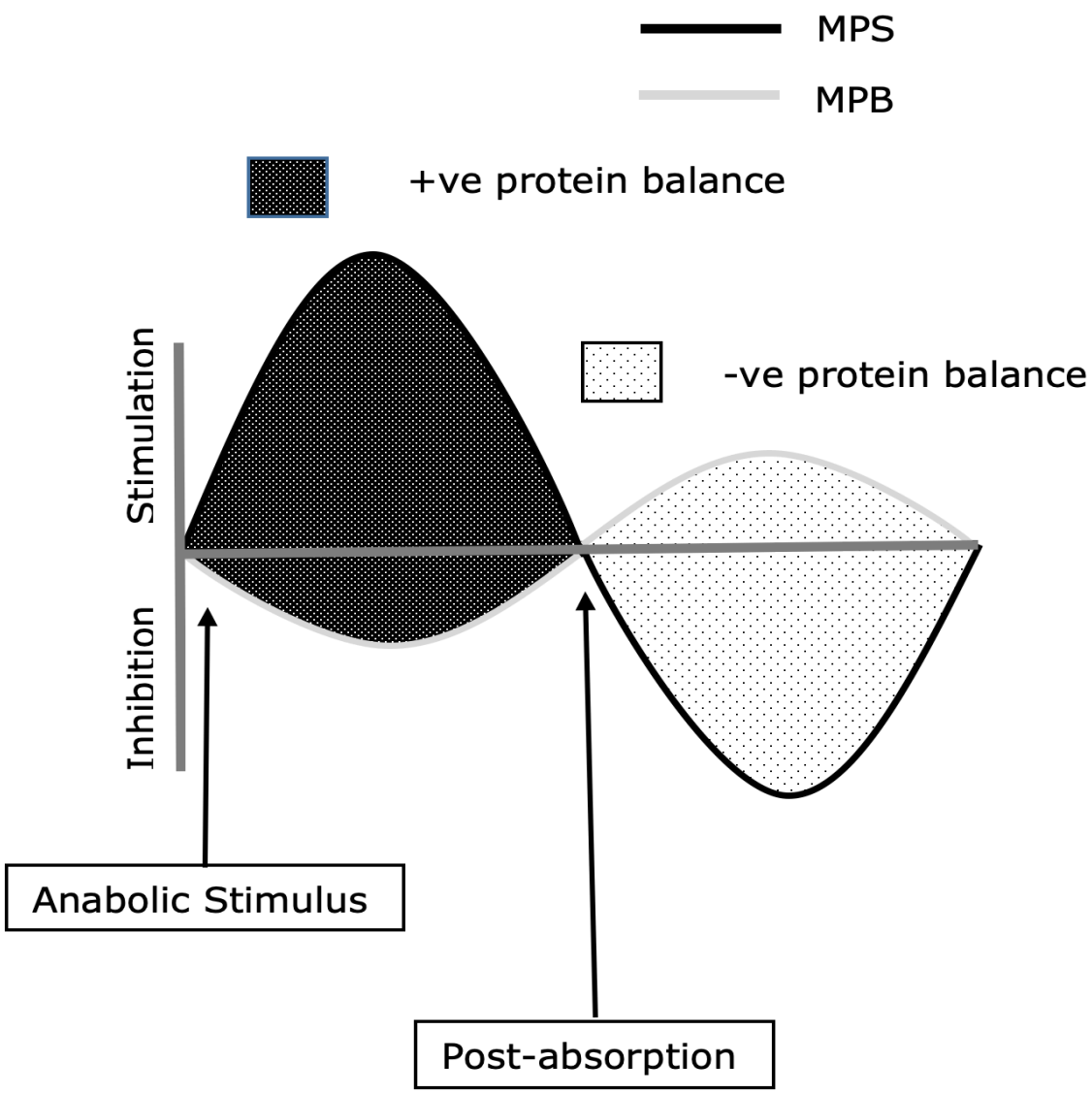


Figure 1.3 shows the anabolic response to nutritional stimuli, showing stimulation of muscle protein synthesis (MPS- black line) and inhibition of muscle protein breakdown (MPB- light grey line), resulting in net increase in muscle mass (area under curve- dark pattern for positive net protein balance).

The opposite happens in post-prandial or post absorptive state, resulting in net loss of muscle mass (area under curve- dotted) (Simplified diagram from multiple works referenced above)

These metabolic processes (MPS and MPB) are in turn dependent on several other factors e.g. fasted (more accurately called post-absorptive) or post-feeding state (Stokes *et al.*, 2018). For example, the breakdown of 20g milk-based protein is demonstrated below in figure 1.4:

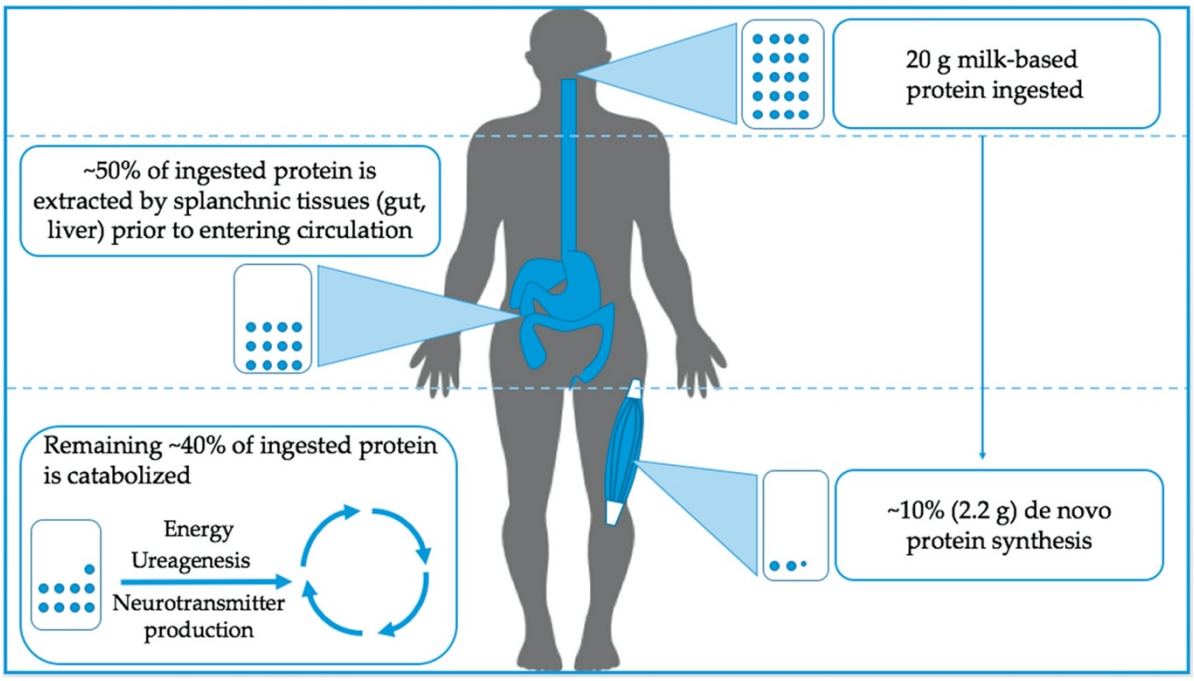


Figure 1.4 shows fate of 20g milk-based protein in human body- adapted from Stokes et al., 2018.

1.4.3: Modulation of MPS via oral amino-acids- start to finish

The metabolic effects of nutrient based stimulation start with digestion of a protein-based meal, which is discussed here. Intake of a protein rich meal induces muscle protein synthesis. The digestion of protein starts in stomach, aiming to breakdown large poly-peptides into single amino-acids or tri- and/or di-peptides (2 amino-acids) (see figure 1.5 below). The liver plays an important role in this, helping with not only the production of enzymes that help digest the protein content of the meal but also with regulation of amino-acids for muscle protein synthesis as well as for energy production and, when in excess, for oxidation (Charlton, 1996). This process ends in release of single amino acids in blood stream.

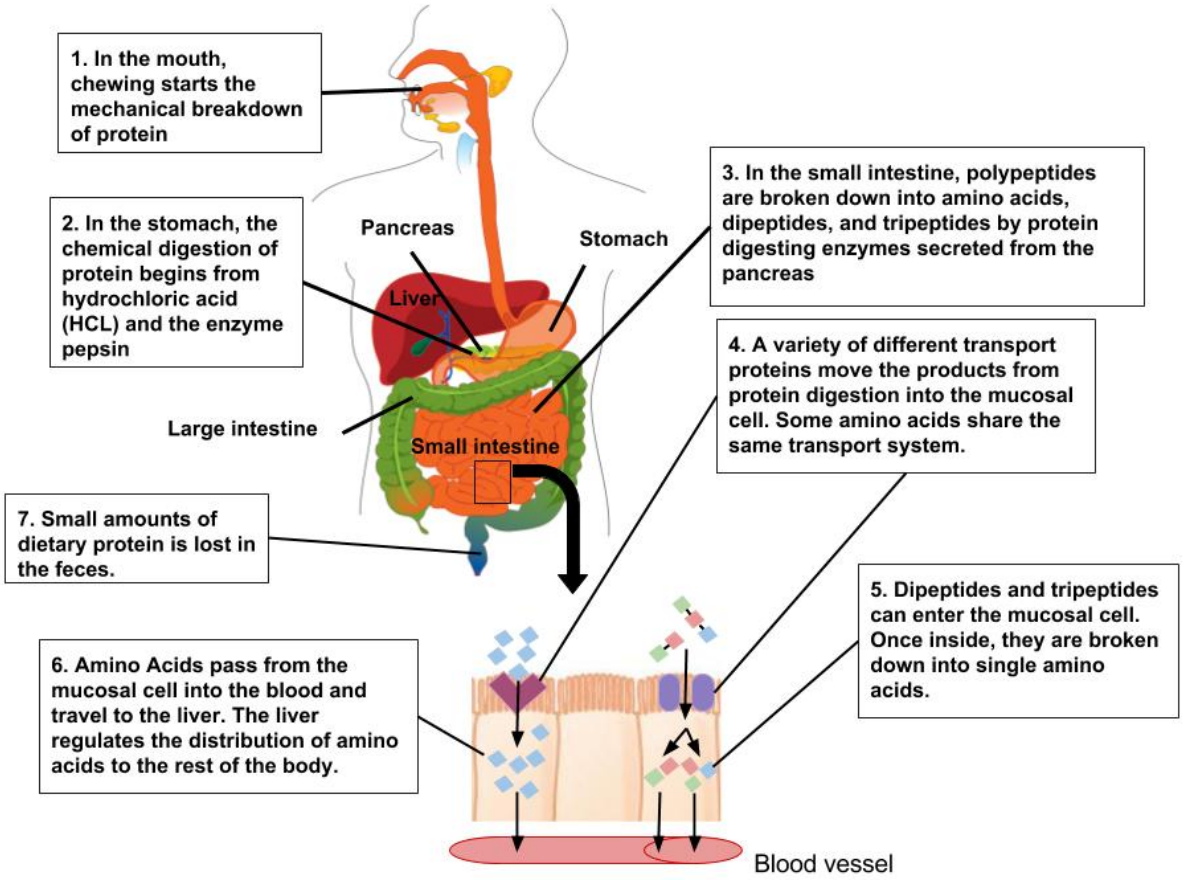


Figure 1.5 shows digestion of protein meal within the gastro-intestinal tract, till the amino-acids enter blood stream.

These amino acids in blood stream then are sensed via specific proteins on skeletal muscle membrane that specially transport amino-acids from blood stream to skeletal muscle intracellular space- these proteins are called amino-acid transporters or AA-transporters. Two such transporters exist: System A transporter and System L transporter- both of these are ATP dependent (Dodd and Tee, 2012; Moro *et al.*, 2016), as shown in figure 1.6 below:

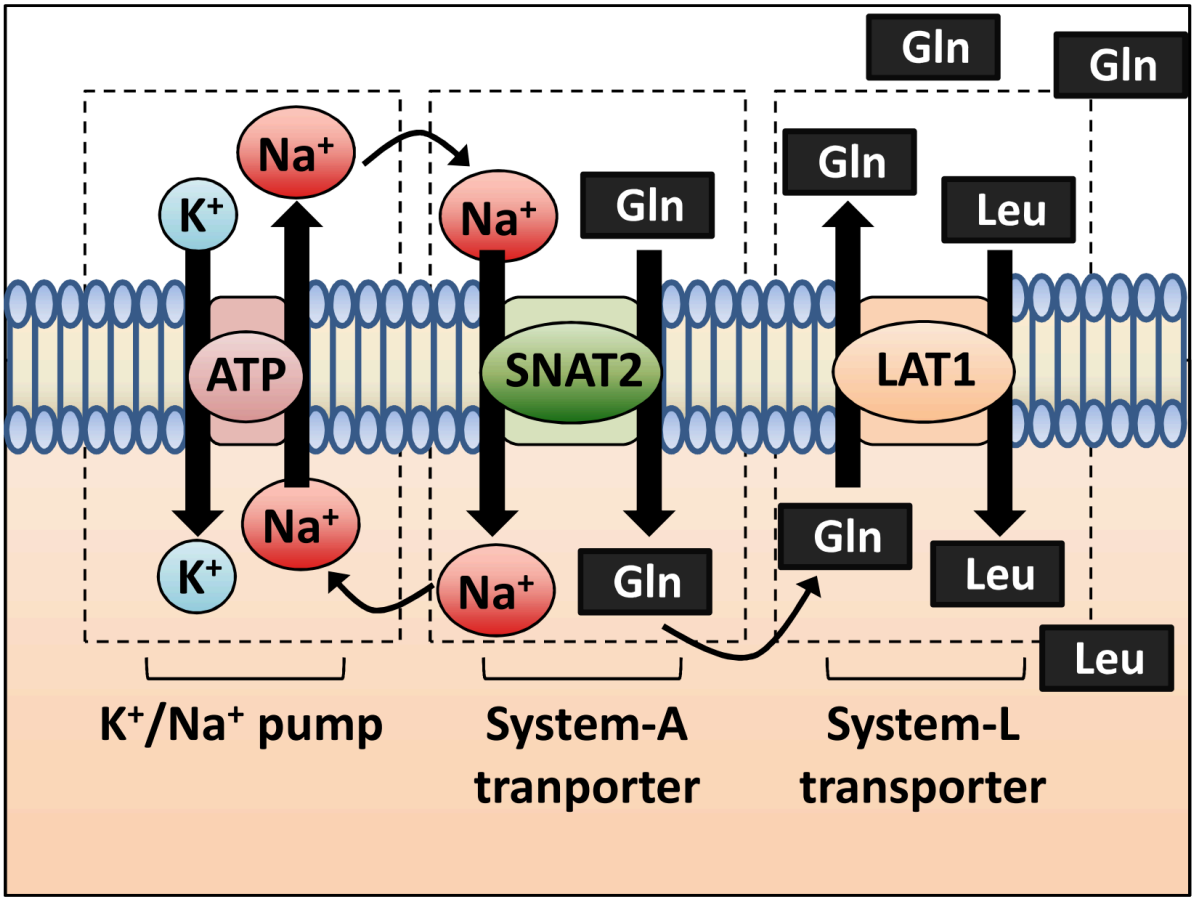


Figure 1.6 shows 2 amino-acid transporters on skeletal muscle membrane (System-A transporter and System-L transporter)- adapted from Dodd & Tee, 2012

Once the amino-acids are in skeletal muscle intra-cellular space, they stimulate muscle protein synthesis (MPS) via mTORC1 pathway as shown in diagram below:

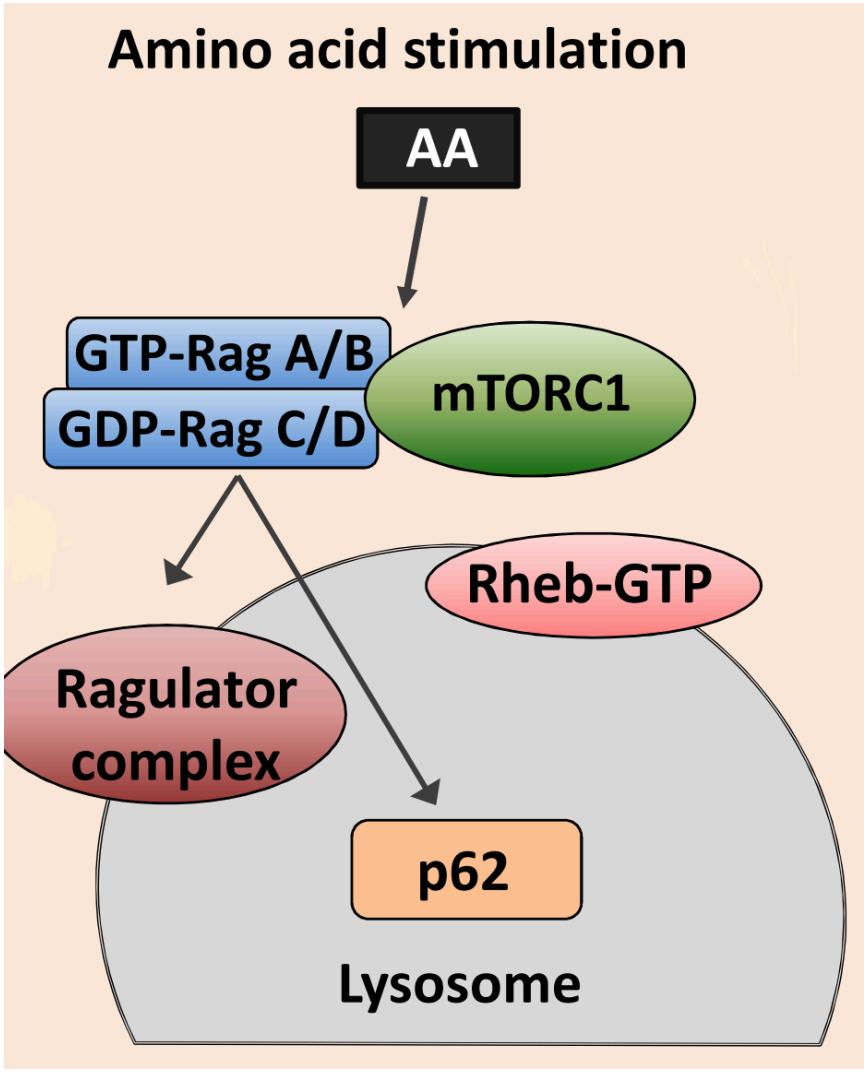


Figure 1.7 shows amino-acid (AA) stimulation of mTORC1 via Rag pathways- adapted from Dodd & Tee, 2012

Once activated, the mTORC1 pathway the starts the muscle protein synthesis via DNA transcription to from messenger-RNA or mRNA in nucleus, which is then transported to sarcoplasm to undergo translation process to form specific protein fractions. Figure 1.8 below shows the rate of synthesis of four main protein fractions in skeletal muscle after infusion of amino acids (Bohé *et al.*, 2001).

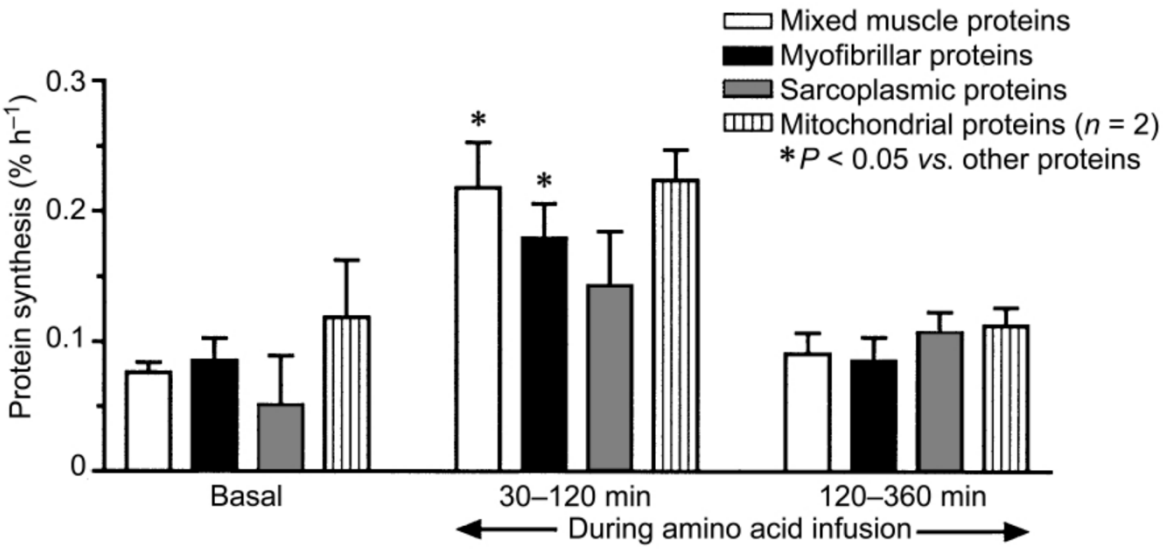


Figure 1.8 shows increase in protein synthesis of several skeletal muscle protein fractions in response to amino-acid infusion (adapted from Bohe et al., 2001)

The specific components of this pathway are reviewed in detail in subsequent subsections.

1.4.4: Chronology of nutrient stimulation

Previous studies show that there is a predictable time course that usually follows oral delivery of standardised amino acid meals such a 20g whey protein. After a lag of about 30 minutes, there is an increase of up to 3 fold in MPS reaching peak by 90-120 minutes, returning to basal levels at about 2 hours (Philip J Atherton *et al.*, 2010)- see figure 1.9.

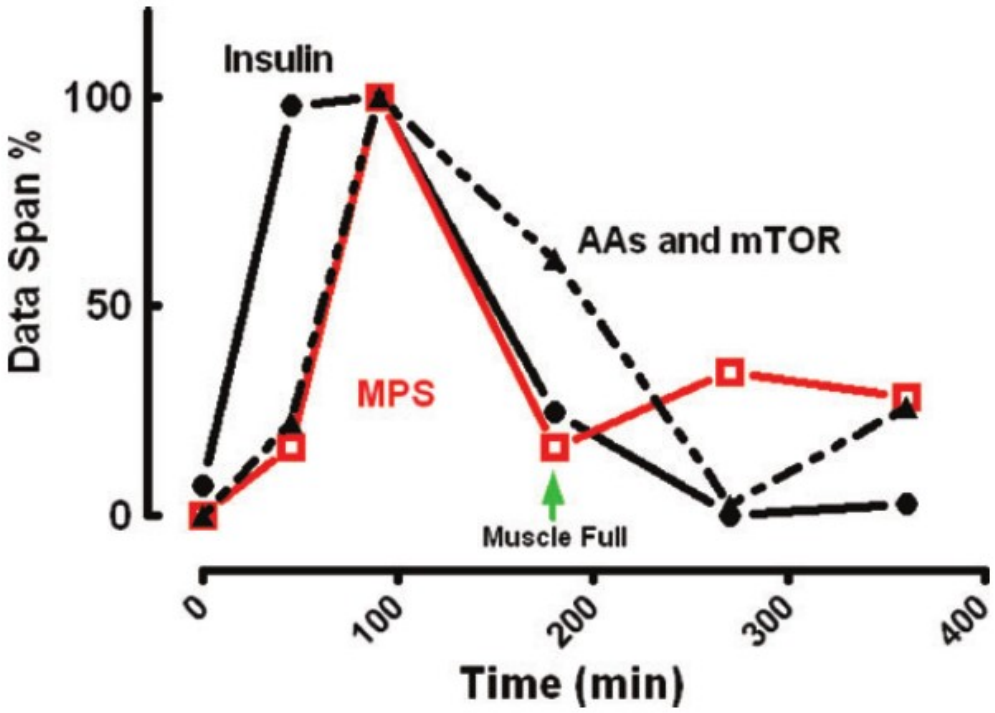


Figure 1.9 shows time course of serum concentrations of amino-acids, insulin and mTOR with muscle-full point- adapted from Atherton & Smith, 2010.

This happens even in the face of sustained availability of amino acids and "intracellular signalling", hence indicating a time point when muscle becomes refractory to further anabolic stimuli (the "muscle-full" point in figure above). A possible explanation for this may be inelasticity of collagen in endomysium surrounding each muscle fibre, thereby physically restricting protein synthesis and accretion (Millward *et al.*, 1994).

1.4.5: Role of mTORC1 in nutritional regulation of MPS

The mammalian target of rapamycin complex-1 (mTORC1) has received a lot of attention as a key regulator of muscle protein synthesis in humans. It is regulated by Regulatory-Associated Protein of mTOR (RAPTOR) and is an adapter protein that is encoded in humans by the RPTOR gene, which in turn is located on chromosome 17 (Hara *et al.*, 2002). An increase in activity of mTORC1 is seen with availability of amino acids in blood stream (Bodine *et al.*, 2001). Activation of mTORC1 leads to phosphorylation of 2 downstream effectors: Ribosomal protein S6 kinase-1 (p70S6K1) and eukaryotic initiation factor 4E binding protein-1 (4E-BP1)-please see figure 1.10 These subsequently lead to decreased phosphorylation activity of eukaryotic elongation factor-2 (eEF2). Activation of these effectors promote MPS (Laplante and Sabatini, 2009).

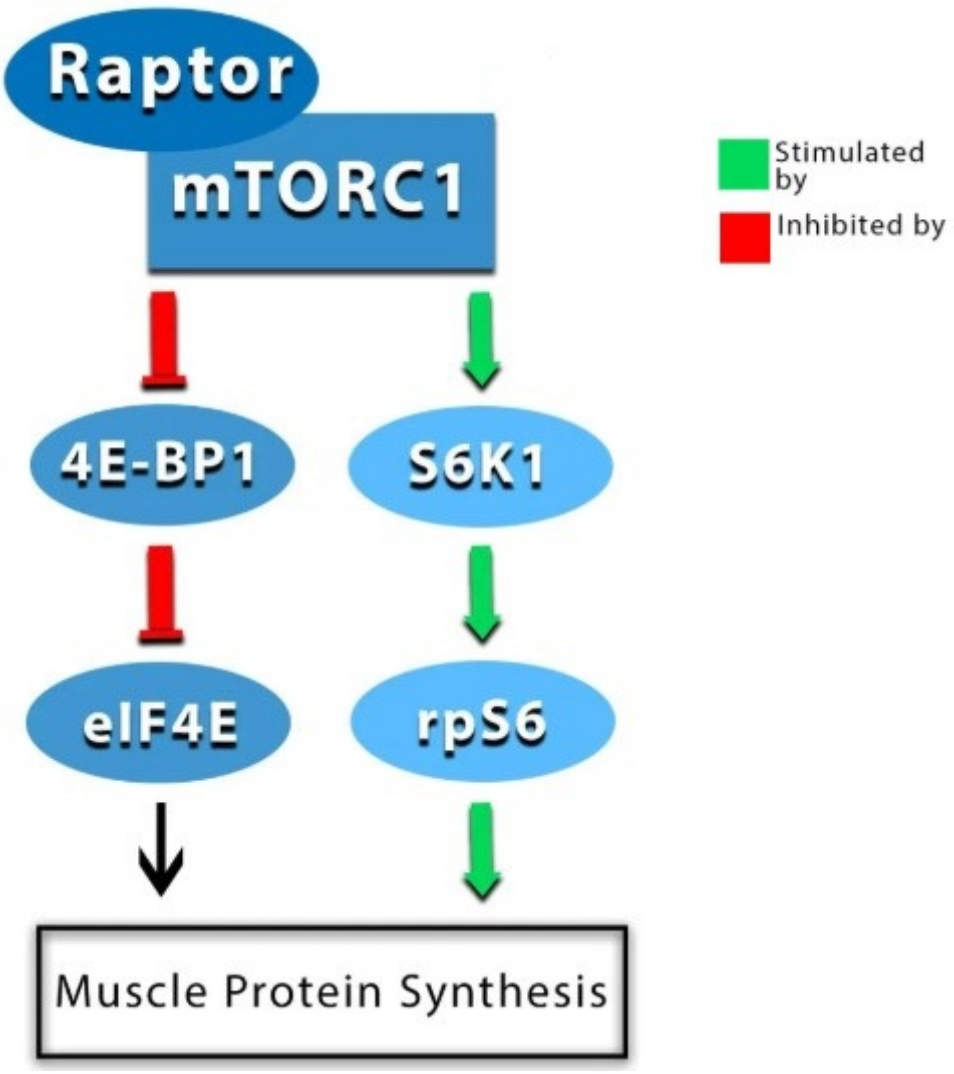


Figure 1.10: A simplified diagram of mTORC-1 pathway and its downstream effectors

The increase in mTORC1 is also seen with availability of amino acids is also closely associated with upstream increase in MPS (Philip J Atherton *et al.*, 2010). This association however is lost after 90 minutes, where the MPS starts to decline however mTORC1 activity remains elevated (figure 1.3-B).

Also, blockage of mTORC1 with rapamycin prior to EAA administration completely blocked the mTORC1 substrate phosphorylation and hence no

increase in MPS was seen (Dickinson *et al.*, 2011). These data support the importance of mTORC1 in regulation of MPS, at least during the initiation phase.

1.4.6: Role of Insulin:

As seen above, the serum insulin concentrations increase in response to amino-acid meal (see figure 1.9 above). Further research is now focussed on understanding the molecular mechanisms behind MPS and how to exploit them to obtain similar MPS results with lower doses of proteins/ essential amino acids (EAAs). Insulin has received some attention as well, partly due to rise in levels of insulin after amino acid infusion or ingestion of protein or EAA (Fryburg *et al.*, 1995). However when EAAs are infused with insulin clamped at post-absorptive levels using a Beta-cell inhibitor octreotide, the stimulation of MPS remains the same (at around 3 fold) however increasing insulin levels while keeping the AA infusion at the same levels reduced the MPB with no effect on MPS (Greenhaff *et al.*, 2008). The greatest reduction of MPB was seen at insulin levels 30 mU/l, wherein MPB was halved. Hence while EAAs drive the anabolic response to MPS, insulin on the other hand has an anti-catabolic response.

1.4.7: Effect of composition of proteins ingested

The anabolic response to natural proteins with different compositions (e.g. whey vs. soy vs. casein) has also been investigated. When compared with iso-caloric soy protein beverage, liquid skim milk resulted in greater fractional synthetic rates (FSR) of muscle proteins after a bout of RE in young untrained men; though both groups showed an increase (Wilkinson *et al.*, 2007). In a follow up study from same group, the comparison between iso-caloric fat-free milk vs. soy beverage vs. carbohydrate solution showed greater increase in muscle mass (particularly type 2 fibres) in the group consuming fat-free milk. Both milk and soy groups showed increase in type 1 muscle fibres. Finally Although there were no differences in muscle strength between the 3 groups (Hartman *et al.*, 2007). When three groups (providing whey/milk, soy and casein) were compared in a study where each delivered a dose of 10g EAAs to young untrained young men who performed a bout of unilateral leg extension exercise, whey hydrolysate resulted in higher circulating EAA concentrations (Tang *et al.*, 2009). The mixed MPS response was greater in whey protein group in both rest and exercise conditions, when compared to soy or casein groups, and soy was better than casein in stimulating MPS.

1.4.8: Role of Leucine and its metabolites

Leucine was discovered as the EAA with the greatest anabolic potency as a result of a series of co-ordinated research efforts, which were initiated after demonstrating anabolic activity from a mixed macronutrient intake. Further studies then determined that the anabolic response was dependent on protein derived amino acids, particularly EAAs (see 1st paragraph of section 1.4.06 for references).

At this juncture, it is important to differentiate between traditional whey protein supplementation and leucine supplementation. Traditional whey protein has ~10% leucine content- adding extra leucine to any protein supplement (usually bringing the leucine content to $\geq$ 25%) would be considered as leucine enrichment (though no universal definition exists) and may help with overcoming anabolic resistance (Katsanos *et al.*, 2006; Churchward-Venne, Breen, *et al.*, 2014).

Finally work done in this field showed that Leucine is that main driver of anabolic response seen with AA/EAA administration (Smith *et al.*, 1992, 1998). Subsequent studies, in C2C12 cells, looking at effect of each EAA on mTORC1 signalling found that phosphorylation of mammalian Target of rapamycin (mTOR) and 4E binding protein-1 (4EBP-1) were increased significantly with leucine but not with any other EAA (Philip J. Atherton *et al.*, 2010). The phosphorylation of ribosomal s6 kinase (p70S6K1) was increased by stimulation with all EAA but not with isoleucine and valine. However, the increase with leucine was significantly greater when compared to other essential amino acids (EAAs). These amino acids are

called essential as they cannot be synthesized by the human body and hence must be obtained by food- these are (in alphabetical order): histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine.

Leucine however can be metabolised within the skeletal muscle. This raises a possibility that it may be metabolites of Leucine that may exert some of all of the effects seen with leucine. Though 80% of leucine contributes to muscle protein synthesis, the rest is converted to two main metabolites which are α-ketoisocaproate (α-KIC) and β-hydroxy-β-methylbutyrate (HMB) (Duan *et al.*, 2016).

HMB shows promise as it has been shown to reduce muscle protein breakdown (MPB) (Eley, Russell and Tisdale, 2008) and increase muscle protein synthesis (MPS) in catabolic states where it is depressed normally (Eley *et al.*, 2007). It has also been shown previously to enhance muscle strength and lean body mass at doses of 3g, with or without higher protein intake, in young untrained men who performed RE for 3 weeks (Nissen *et al.*, 1996). The same study found HMB particularly beneficial for reducing post-RE proteolysis. Studies of HMB supplementation in older individuals show that in non-exercising older men and women, calcium β-hydroxy-β-methyl butyrate (Ca-HMB) in doses of 3g twice daily showed an improvement in lean body mass as well as muscle strength and functional measures such as "get up and go" test (Stout *et al.*, 2013). In phase two of same study, no significant differences in these parameters were seen when both groups performed 3 sessions of RE per week. In another study where the participants were older females, supplementation of 2g HMB

with arginine and lysine for 12 weeks showed 17% improvement in functional tests such as get-up-and-go test (Flakoll *et al.*, 2004).

The question that arises now is which is better in terms of anabolic effects: leucine or HMB? Is there any difference in their anabolic response profile? This was investigated in a study where, when compared to leucine itself, HMB showed reduced MPS stimulation (70% vs. 110% with leucine) although this was not statistically significant (Wilkinson *et al.*, 2013). Inhibition of MPB breakdown was seen with HMB in an insulin-independent manner, unlike Leucine.

Given the efficacy of anabolic responses of leucine, there have been studies looking at chronic leucine supplementation. Normal whey protein has about 10% leucine- hence leucine supplementation is usually adding extra leucine to a protein supplement (usually in range of >25%). This may help particularly in elderly who are at risk of muscle loss due to old age (sarcopenia) to see if chronic leucine supplementation results in increased muscle growth with or without any changes on muscle loss. Two recent meta-analyses have reported mixed results. The first one published by Xu and colleagues included 9 randomised controlled trials and showed that while the leucine group had higher MPS (measured via fractional synthetic rate- see chapter 2) however, the lean body mass and lean leg mass were similar in both groups (Xu *et al.*, 2015). The authors concluded that while chronic leucine supplementation results in increased muscle protein synthesis at molecular level, these results do not translate into higher muscle mass. The second meta-analysis from Komar & colleagues included 16 studies with 999 participants (Komar, Schwingshackl and

Hoffmann, 2015). The data showed increased body weight and lean body mass (particularly in those who had established sarcopenia) but no difference in strength was observed. These results suggest that there may be a sub-group of elderly who may benefit more from chronic leucine supplementation, though more studies are needed to identify those.

Lastly, is there an "optimal" quantity of leucine that human adults need to consume? While there is a recommendation of 39 mg/kg pf body weight per day (by WHO in their joint report published in 2007- see (WHO/FAO/UNU, 2007)), unfortunately this recommendation is not based on any data but based on a factorial model for growth and maintenance (Millward, 2012). The small scales studies have suggested a daily leucine intake of 40 mg/kg per day (Kurpad *et al.*, 2001) to 55 mg/kg of body weight per day (Riazi *et al.*, 2004). Similarly, there is a limited data about toxicity of leucine, though levels of >500mg/kg per day resulted in over-production on ammonia in one study (Pencharz, Elango and Ball, 2012). While this may cause several neurologic consequences, none were reported in this study.

Figure 1.11 below summarises the nutritional modulation of muscle protein synthesis (Dickinson and Rasmussen, 2013):

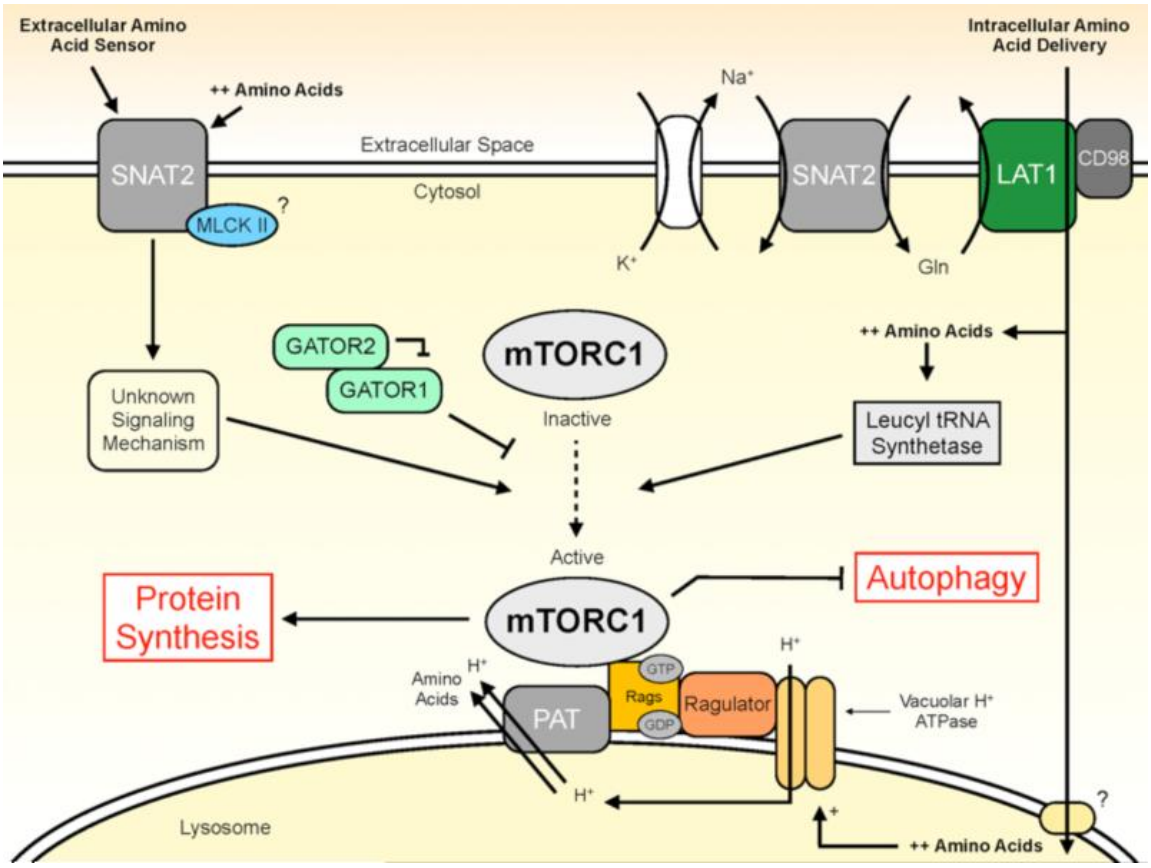


Figure 1.11 Schematic of the mechanisms regulating protein metabolism in response to increased amino acid availability and the role of amino acid transporters

Abbreviations: GATOR, GTPase activating protein [GAP] activity toward Rags; Gln, glutamine; LAT1, system L amino acid transporter; MLCK II, myosin light-chain kinase II; mTORC1, mechanistic target of rapamycin complex 1; PAT1, proton-assisted amino acid transporter 1; RAGs, Ras-related GTPases; SNAT2, system A amino acid transporter

Adapted from Dickinson and Rasmussen, 2013

1.4.9: Effect of ageing on anabolic response to nutrition

These responses have not only been studied in younger population but also has been compared to older population (Volpi *et al.*, 2000). Data from Cuthbertson and colleagues shows that compared to young males, older males show a reduced anabolic response to increasing doses of EAA (Cuthbertson *et al.*, 2005), the phenomenon termed **"anabolic blunting"**. Studies from other labs also confirmed this (Wall *et al.*, 2015) despite the fact that basal fractional synthetic rates are similar in both young and old (see figure 1.12 below).

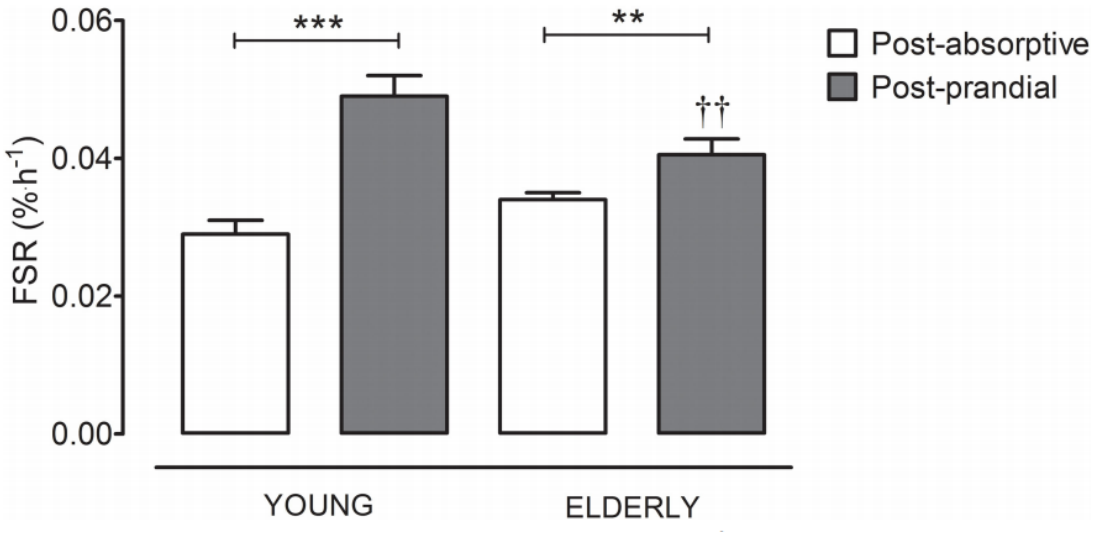


Figure 1.12 shows fractional synthetic rate in young and old men basal/ post-absorptive state as well as post-prandial state (adapted from Wall et.al. 2015)

Further work showed that the anti-catabolic effect of insulin is also blunted in older individuals (Wilkes *et al.*, 2009). However, in older individuals,

HMB has shown particular promise due to its ability to inhibit MPB and stimulate synthesis. There are some other studies however, whose findings contradict these e.g. in a study looking at MPS response to a 113 gm lean beef (containing 30 gm protein) in both young (mean age 34 $\pm$ 3 years) and older individuals (mean age 70 $\pm$ 2 years) found that the MPS responses were similar on both groups (Symons *et al.*, 2009). Although this study found that increasing the protein content by 3-fold didn't offer any benefits, this may have been due to the lower dose maximally stimulating MPS.

It may be noted that all of these studies however have predominantly been done in men. Two questions arise here:

1. Are women similar to men in their anabolic response to nutritional stimuli? This is addressed in section 1.4.10 below
2. Is there anything that can be done to stop or reverse this? This is discussed in section 1.7

1.4.10: Effect of gender on anabolic response

There are few studies that have compared anabolic responses in men and women. One study which compared anabolic response to nutritional stimuli (a hyperinsulinemic-hyperaminoacidemic-euglycemic clamped state after an overnight fast) and found no differences in synthetic responses between young men and young women (Smith *et al.*, 2009). This has led to the hypothesis that the difference seen between the muscle mass in both

sexes is NOT due to any differences in rates of protein metabolism (Markofski and Volpi, 2011).

What is the effect of age and gender on basal FSR and does a protein meal alter muscle protein metabolism in any way? This question was answered by our lab, in collaboration with the Mittendorf group (Smith *et al.*, 2008) which looked at MPS at post absorptive state as well as in fed state in both older men and women and found that women had higher basal MPS than men (figure 1.13) however only men showed a significant increase in feeding related MPS (which was also associated with an increase in several intra-cellular signalling elements e.g. phosphorylation of $eIF4E^{Ser209}$ and $eIF4E\text{-}BP1^{Thr37/46}$.

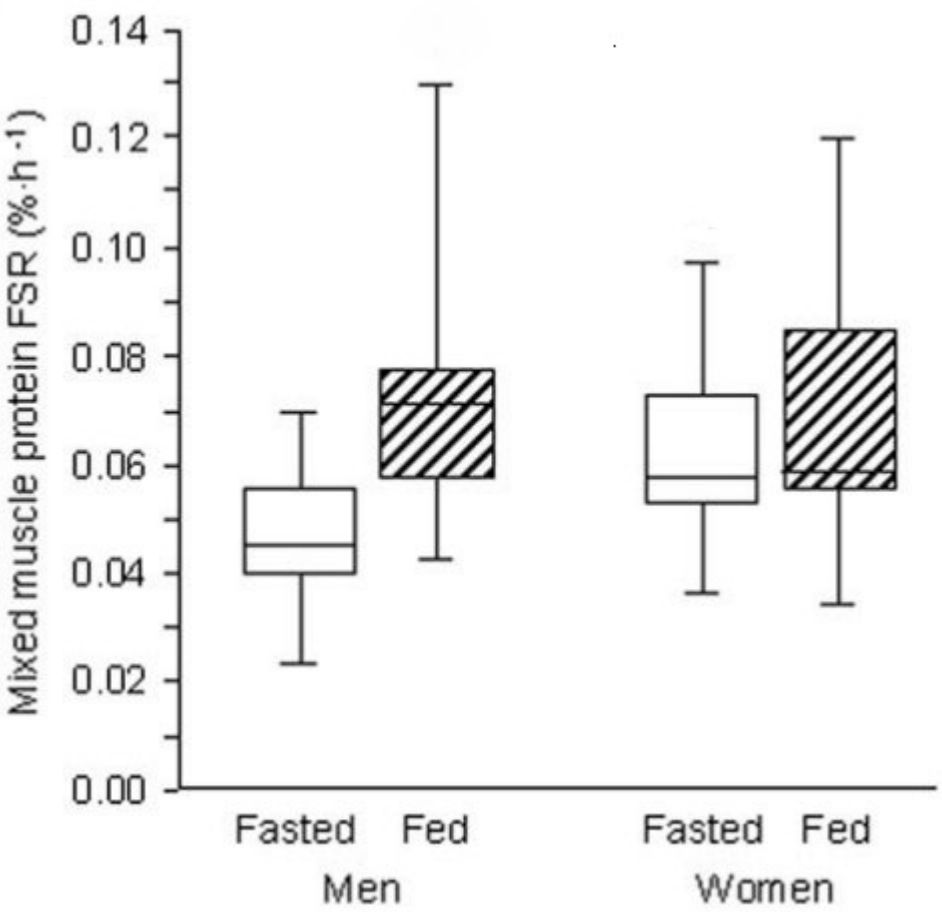


Figure 1.13 shows mixed muscle protein FSR in older men and women in fasted (post absorptive) and fed state

(Adapted from Smith et. Al. 2008)

Apart from subtle differences seen in muscle protein metabolism, differences between hormonal profiles between the sexes are also likely to be the cause behind the increased muscle mass in men when compared to women as noted above.

1.5: MODULATION OF SKELETAL MUSCLE METABOLISM VIA RESISTANCE EXERCISE

Muscle protein Synthesis (MPS) also increases after physical activity, particularly after a bout of resistance exercise i.e. weight training (Toigo and Boutellier, 2006). In this sub-section, this phenomenon is further explored.

Resistance exercise is defined as a physical activity that uses external force against a muscle (or a muscle group) in order to improve strength and/or size (Yang *et al.*, 2014). The external force could be either in form of weights, resistance bands or even gravity. Each weight has to be moved several times (called "repetitions"). Most exercises recommend that a muscle (or a muscle group) is exercised several times with a period of rest

in between (each "time under tension" is called a set- a set may have any number of repetitions such as 5, 10 or even 20 etc.) (Yang *et al.*, 2014).

As noted in this definition, there are several variables associated with resistance exercise (RE) e.g. sets, repetitions (Fry, 2004). Some sets do not have any firm number of repetitions and may have to be continued till the point of muscle failure (defined as a point where the muscle or the muscle group is unable to sustain a particular movement with proper form).

1.5.1: Chronology of MPS following RE:

This section starts by looking at the time course of MPS following resistance exercise (RE). As seen with nutritional interventions, there is a latency period after which the MPS starts to rise and stays elevated for a period of time before declining back to baseline levels. However, unlike EAA availability, these times can be changed with several interventions. The latency period is closely associated with magnitude of mechanical stress of exercise. It is well known that MPS is initially suppressed during an acute bout of RE (Atherton and Rennie, 2006; Rose *et al.*, 2009). The latency period is probably shorter for lower intensity exercise e.g. about 1 hour in RE comprising of 6×8 repetitions at 75% 1-RM (Kumar *et al.*, 2009) and longer for more intense RE sessions e.g. 3 hours as seen in another study where participants stepped up and down a knee high box, exercising for 12 minutes, and reported being fatigued at the end of session, unable to continue any further and had to be helped to a chair (Cuthbertson *et*

al., 2006). The dose response relationship was demonstrated in another study where a "dose" of resistance exercise (measured as percentage of 1RM- either 20% to 90%) was closely related to anabolic signalling and MPS, in young subjects and to a lesser extent, older individuals as well (Kumar *et al.*, 2009).

After a latency period, MPS starts to increase rapidly in 1-2 hours before falling back to baseline levels in 2-4 hours (Kumar *et al.*, 2009). Similar changes are seen in collagen in tendons and muscle (Miller *et al.*, 2005), lending support to the concept of co-ordinated adaptations in muscle and tendons when exposed to resistance exercise (RE). This duration can be manipulated by nutrition e.g. protein or essential amino acids (EAAs) as well as workload and intensity. This manipulation works both ways- adding EAA enhances MPS anabolic response to exercise and lack of protein supplements results in negative net balance (increasing MBP more than MPS) as previous data has shown (Biolo *et al.*, 1995).

1.5.2: Role of Workload and intensity:

The MPS response secondary to exercise depends on work load and intensity (Atherton and Smith, 2012). For example, while resistance exercise (RE) at >60% of 1-Repetition Maximum (both with 3 and 6 sets) caused 2-3 fold increase in MPS, RE at <40% 1RM (again both at 3 and 6 sets) didn't cause any significant increases in MPS (Kumar *et al.*, 2012). However, in another study, comparison of MPS responses as well as myogenic gene expression among one group performing RE with higher

loads continued till failure with another group performing RE with lower load till failure showed that while both groups were similar in their anabolic responses, though the myogenic responses were slightly higher in low-load group (Burd *et al.*, 2010). This suggests that low-load RE performed till the point of volitional failure, may be as good (and in some ways even better than) high-load RE, and may be preferable to those with poor muscle mass and or joint problems. While the reason behind this is not known, it has been proposed that exercising to failure causes maximum fibre recruitment which may be a key factor in maximising the anabolic response to RE.

1.5.3: Role of contractions:

The next question that arises looks at the two modes of contraction (concentric vs. eccentric) and determines whether one is better than the other. Concentric contractions are isotonic contractions where the muscle shortens in response to the load, whereas eccentric contractions are isotonic contractions where muscle lengthens. The studies however do not show any difference between the types of contractions and magnitude of the increase in MPS, although both types increase the MPS compared to baseline the difference between contraction modes was not significant (Cuthbertson *et al.*, 2006). However when it comes to muscle hypertrophy, eccentric RE, particularly when performed at higher intensities, may offer an advantage over concentric RE, as it is associated with increased muscle mass and strength gains (Roig *et al.*, 2009).

1.5.4: Effect of age on anabolic response to exercise:

Just as anabolic blunting to nutritional stimuli is seen with advanced ageing (see section 1.4.6.6), similar attenuated response to RE is seen in ageing e.g. in a study comparing MPS response to post-absorptive resistance exercise (in young men vs. older men) found that at varying intensities (20-90% 1-RM), MPS responses and signalling were both blunted in older men (Kumar *et al.*, 2009). This has been seen in yet another study which found that muscle contraction induced intracellular signalling of mTORC was impaired in older individuals (mean age 70 years) compared to younger men (mean age 27 years) (Fry *et al.*, 2011) when muscles were sampled at regular intervals post RE up to 24 hours. This anabolic resistance is not only seen with resistance type exercise but also with endurance type training for instance it has been shown that walking also reduced MPS response by up to 40% in older individuals (Durham *et al.*, 2010). This study also identified that while microcirculatory blood flow was reduced by up to 40% in older individuals, the intra-vascular and interstitial amino acid availability was similar (or higher) to that of younger men. These data refute the hypothesis that reduced micro-circulation in muscle somehow affects the amino-acid availability that contributes to reduced muscle mass secondary to ageing. On the other hand, there are studies that suggest that the anabolic response is not blunted in older individuals (Drummond *et al.*, 2008) as well as those which suggest that the response is just delayed (but similar in magnitude) when compared to

younger individuals (Symons *et al.*, 2011). These studies suggest that unlike anabolic response to essential amino acids (EAA), response to resistance exercise (RE) may require longer term studies rather than those looking at acute effects (Phillips, Hill and Atherton, 2012).

1.5.5: Effect of gender on anabolic response to exercise:

There are few studies looking at effect of RE in women. Previous work has shown however that 12 weeks of RE training is safe and well tolerated in older women and that it results in an increase of muscle strength as well as a significant increase cross sectional area of type II (type I fibre increase didn't reach significance) muscle fibres when compared to controls (Charette *et al.*, 1991). A more recent retrospective study of a large cohort (n=195 with 88 women and 107 men) looking at adaptive response to RE (for either 12 weeks or 24 weeks) in older men and women found that there were subtle sex related differences (Markofski *et al.*, 2015), though there was large heterogeneity with in the study. The study looked at several characteristics which are discussed below:

- **Changes in lean body mass:** Women had slightly higher gain (1.0 + 0.2 kg at 12 weeks and 1.2 $\pm$ 0.3 kg at 24 weeks) compared to men (0.8 $\pm$ 0.1 kg in 12 weeks and 1.0 $\pm$ 0.2 kg at 24 weeks). The difference was not statistically significant.

- **Type I and II fibre size measured in cross sectional area (CSA):** The study didn't find a statistically significant difference in type I fibre size between women and men post RE training however type II fibre size was significantly higher in men at 12 weeks. Men showed an increase of 451 $\pm$ 164 μm^2 and 1034 $\pm$ 172 μm^2 in type I and II fibre size, respectively, whereas women showed an increase of 97 $\pm$ 244 μm^2 and 108 $\pm$ 191 μm^2. At 24 weeks however, there were no differences between men and women in the change in type I or type II fibre size. Interestingly at baseline, men had higher cross-sectional area of both fibre types (type I fibre CSA of 6136 ± 174 μm^2 and type II fibre CSA of 5264 ± 166 μm^2) compared to women (mean type I fibre CSA of 5063 ± 227 μm^2 and a type II fibre CSA of 3298 ± 209 μm^2). One must note that, like the study mentioned above, while both the fibre types increased in women, only type II fibres reached statistical significance at 12 weeks (type I baseline CSA 5063 $\pm$ 227 μm^2, 12-week increase + 97 $\pm$ 224 μm^2- Type II fibres baseline 3298 $\pm$ 209 μm^2 and 12 weeks increase 108 $\pm$191 μm^2) At 24 weeks both fibre types increase significantly (increase in type I 473 + 236 μm^2 and type II 589 + 167 μm^2).
- **Muscle strength:** This was measured using leg extension and leg press and found that while both groups improved, men had higher gains in leg extension vs women (36 $\pm$ 2 kg for men vs 22 $\pm$ 2 kg for women). The difference in leg press didn't meet statistical significance though showed similar trend (men 53 $\pm$ 3 kg and

women 48 $\pm$ 4kg). It has to be noted however that at baseline, men had higher values at both leg press (men had a mean of 183 ± 5 kg vs. women whose mean 1RM was 126 ± 4 kg) and leg extension (mean 1RM for men 86 ± 2 kg and women 58 ± 2 kg).

- **Physical function**: This was measured using chair rise test- with no difference between men and women at baseline. There was no significant difference between men and women after 12 weeks of RE (with men showing a reduction of −0.6 ± 0.6 seconds and women −1.9 ± 0.5 seconds). This trend *did* become statistically significant after 24 weeks of training (men showing a reduction of −1.2 ± 0.4 seconds and women demonstrating a reduction of−3.2 ± 0.7 seconds- *P=0.01*).

Where does this leave us? The study confirmed that men had higher muscle power and strength to start with, however the increases seen in women were comparable to men. One interesting finding was that despite the increase in muscle strength i.e. leg extension being significantly higher in men, the functional attribute of chair rise was shorter in women. This study however didn't look into any mechanisms underlying the changes.

This was the question answered in another study from the group (Markofski *et al.*, 2015) comparing the effects of age and gender on basal muscle protein synthesis (measured via FSR) and mammalian target of rapamycin complex (mTORC) signalling. The study didn't find any significant differences in FSR between young and old subjects (see fig 1.14 adapted from the study) though mTORC phosphorylation was significantly higher in older men and women.

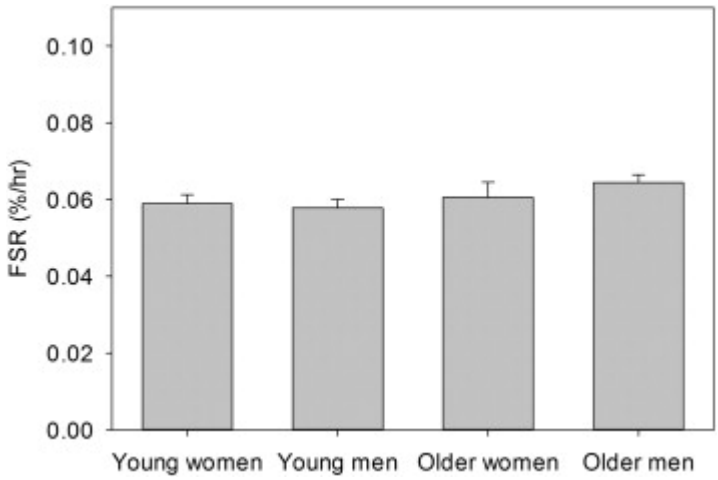


Figure 1.14: Graph showing effect of age and gender on Fractional Synthetic Rate (FSR in %/hour) - adapted from Markofski et. Al. 2015.

While these studies do make important contributions, it is difficult to determine the cause(s) of higher muscle mass in men and how muscle protein metabolism differs in women. While bone changes and changes after puberty related to testosterone may account for some of these changes, they don't provide all the answers.

1.5.6: Training with Blood Flow Restriction (BFR)

Blood flow restriction (BFR) involves training (usually but not always RE) with a cuff proximal to the muscle group where the intended benefits are desired. The cuff is inflated to a pressure high enough to stop venous out-flow but low enough to allow arterial inflow. The exercises are then

performed with the cuff inflated. This has been studied extensively, looking at different cuff pressures, the cuff size and types of exercises.

CUFF PRESSURE: The pressure of the cuff has a hermetic relationship with the anabolic response: When the pressure is too low, the anabolic response may not be significant. Then follows a range of pressure where the response is optimal and after that, higher pressures could negatively impact the anabolic response (Loenneke *et al.*, 2014). The research so far has determined that there is no one "universal" pressure or range, and that cuff pressures need to be tailored according to each individual (Scott *et al.*, 2015).

CUFF SIZE: One other variable that has to be considered with cuff pressures is cuff width- With a higher width (12-18 cm depending on use in upper limbs or lower limbs), it is easier to apply lower pressures to fine tune the anabolic response however the proper form of resistance exercise may be compromised. A lower width doesn't restrict proper form however higher pressures may be needed to achieve the same results, which tends to be more uncomfortable.

TYPE OF EXERCISE: The third variable in BFR is the type of exercise. While it is known to accentuate the anabolic response to traditional RE, there *are* some studies that have shown anabolic response secondary to traditional endurance type exercises such as walking and cycling. One such study looked at effects of low intensity walking (<5 kph- 5 sessions per week- each session was 20 minutes) in active elderly men & women (aged 60-78 years) with blood flow restriction and found that 6 weeks of training

showed significant improvements in muscle strength as well as mass, measures at muscle –bone cross-sectional area (CSA) and ultrasound (US) estimated muscle mass (Abe *et al.*, 2010). Interestingly however there were no improvements noted in cardiovascular endurance measures. This study, however only compared the exercising individuals with non-exercising (but otherwise active) controls. This limitation was overcome by another study which compared muscle mass (measured via MRI) and strength (in terms of maximal knee strength) in elderly patients (aged over 60 years) with 10 weeks of walking at 45% heart rate reserve (With BFR, n=13, without BFR n=10) (Ozaki *et al.*, 2011). They found significant improvements in muscle strength (~15%) and muscle cross-sectional area (3%) in participants training with BFR compared with those without.

In summary, training with blood flow restriction may offer some additional benefits to exercise, particularly to older individuals.

1.6: MODULATION OF SKELETAL MUSCLE METABOLISM- THE INTERPLAY BETWEEN NUTRITION AND EXERCISE:

The next question that arises in that how can these two factors, which have been studied in great detail, be manipulated to increase either the magnitude or the duration of the increases in muscle protein synthesis

(MPS). Given the data discussed above, it only follows that putting these two together may enhance the anabolic response. The earliest suggestion of this comes from a study which looked at muscle protein turnover in healthy volunteers using stable isotope techniques. They found that post-exercise, there was an increase in MPS but also MPB, and that overall net turnover was negative (Biolo et al. 1995). This demonstrates that in the post-absorptive state the net effect of exercise is loss of muscle mass and in order to maximise the anabolic potential of exercise, protein or amino acid is required to provide substrate for protein synthesis. This notion is further supported by the data that shows increased magnitude and duration of MPS when EAA supplementation in given immediately post-exercise (Pennings *et al.*, 2011). The effect was seen in both younger and older men. This was noted in another study looking at effect of RE on differential protein synthesis (myofibrillar vs sarcoplasmic) stimulated by protein ingestion (Daniel R. Moore *et al.*, 2009). The investigators found that RE stimulated myofibrillar protein synthesis and that it remained elevated up to 5 hours after protein ingestion in participants who had performed RE compared to those who hadn't (the effect was visible 1-hour post ingestion but returned to fasting levels at 3 and 5 hours).

Also noted in another study was effect of addition of carbohydrate to protein supplementation (i.e. study anabolic response from protein alone and protein + carbohydrate in both rest and exercise) which found that addition of carbohydrates did not have any further impact on anabolic response either at rest or at exercise (Staples *et al.*, 2011).

Another study looked at dose response relation of post-absorptive myofibrillar MPS to whey protein (WP) at 0, 10g, 20g and 40g in RE trained young men (age range 20-22 years) at rest and after a bout of RE (Witard *et al.*, 2014). The study found an increase in myofibrillar MPS by 49% and 56% respectively in 20g and 40g WP groups (when compared to 0g WP group), with increased ureagenesis and AA oxidation noted at doses >20g.

Hence the protein supplementation after RE creates an extra "anabolic" stimulus that results in enhanced MPS response compared to exercise alone, as shown in figure 1.15:

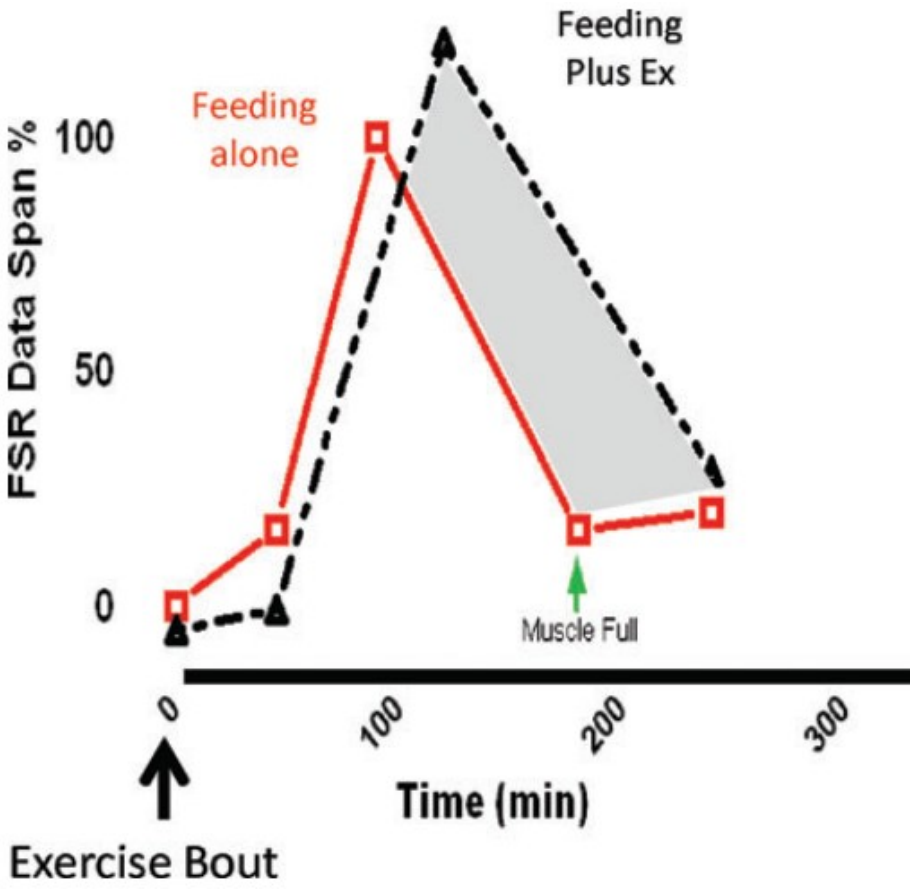


Figure 1.15: Relationship between magnitude of anabolic response to feeding alone (red/continuous) and feeding + RE (black/dotted) - Adapted from Atherton & Smith et al 2012.

The next question that arises from this is how long does this effect last? This was looked at in a study which looked at anabolic responses (mixed muscle, myofibrillar & sarcoplasmic protein synthesis, mTOR phosphorylation and Protein Kinase B phosphorylation), in young men divided into three groups: those who did maximal intensity RE till failure (4 sets at 90% max strength- 90FAIL), those who did low intensity RE till failure (30 FAIL) and finally those who performed low intensity RE work matched to high intensity group (30 WM). All participants had protein supplementation (and anabolic responses measured) a week before (Burd *et al.*, 2011). The study found that, as expected, the protein ingestion and RE both stimulated MPS above the fasting levels. However, RE enhanced the anabolic response to protein ingestion 24 hours later (only the myofibrillar MPS), but only in the two groups training to failure. The cell signalling responses were a bit different however, as mTOR responses were increased in exercise + protein group only in 30FAIL group (compared to protein only) while Protein Kinase B responses were higher in 90FAIL group only. The significance of this finding was unclear. What was also not clear is why the enhanced anabolic response was limited to groups training to failure and warrants further investigation.

1.7: MUSCLE LOSS IN OLD AGE- SARCOPENIA

1.7.1: Normal physiological loss

Though loss of body mass associated with old age has been described by Greeks as well as English literature, first *scientific* mention of the physiological loss of skeletal muscle is from Critchley in 1931 (Critchley, 1931). Since then, there have been several descriptive as well as analytical attempts at studying muscle loss with increasing age (Balagopal *et al.*, 1997; Forbes, 1999). It is, however, difficult to accurately *quantify* total body muscle loss as it is dependent on several factors (Forbes, 1999). These factors could include genetic make-up, life-style choices, medications and diseases. Also, diseases such as stroke which affect only a part of body can cause asymmetric loss of muscle mass, further complicating the study of muscle loss.

1.7.2: Sarcopenia- Terminology and definition

In 1989, Irwin Rosenberg first described the phenomenon of loss of lean body mass associated with ageing. He gave it two names: Sarcopenia and Sarcomalacia- The former appeared to have held on since (Rosenberg, 1997). The main reason why the term was coined was to consolidate research efforts into one specific direction: To prevent loss of muscle mass associated with ageing and to preserve function.

In an attempt to define Sarcopenia, Richard Baumgartner conducted a population-based survey of 883 elderly subjects living in the New Mexico. Of these, 199 underwent a Dual-energy X-Ray Absorptiometry (DXA) scanning to measure body composition. From these, an anthropometric equation was developed to *estimate* appendicular muscle mass. From this, a measure called "Appendicular Skeletal Muscle Mass index" was developed by dividing appendicular muscle mass (in kg) by height (in meters) squared. Sarcopenia was defined if this measure was less than 2 standard deviations of mean of young reference group (Baumgartner *et al.*, 1998). This classification, however, did not take into account any functional limitations or muscle strength. It was also based only on Hispanic/ white population based in New Mexico and did not attempt to sub-classify sarcopenia based on severity.

Another attempt in 2002 came from Ian Janssen (Janssen, Heymsfield and Ross, 2002) who attempted to sub-classify sarcopenia, by using data from 3rd National Health and Nutritional Examination Survey (NHANES III). Skeletal muscle mass was determined using Bio-electrical Impedance analysis (BIA) and from that data a measure called Skeletal Muscle Index (SMI) was developed, which was calculated as follows:

$$SMI = \frac{Skeletal\ Muscle\ Mass\ (in\ kg)}{Total\ Body\ Mass\ (in\ kg)}\ x\ 100$$

Subjects were classified as normal if their SMI was greater than 1 standard deviation above the mean of gender-specific adults aged 18-39 years. Class-1 sarcopenia was defined as SMI between 1-2 standard deviations below young adults' means, and class-2 sarcopenia was defined at SMI

below 2 standard deviations. While not part of classification, he also reported prevalence of objective functional impairments (e.g. walking, standing up from chairs and climbing stairs etc.) as well as subjective reporting of physical disabilities in study population, comparing normal individuals with those classified as having class-1 or 2 sarcopenia. The limitations of the study were generally due to the limitations of data collected in NHANES-III. Muscle strength was not a variable studied in the survey, so it was not available. Also, the survey could only include ethnic populations where BIA was validated to measure muscle mass (only subjects from non-Hispanic white & black and Mexican American ethnicities). Finally, it being a cross-sectional survey, could only comment on an association between sarcopenia and physical disability and could not imply causation.

Finally a more coherent effort to define sarcopenia came in 2010 by European Working Group on Sarcopenia in Older People (EWGSOP), who defined Sarcopenia as (Cruz-Jentoft *et al.*, 2010):

… a syndrome characterised by 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.

Thus, for the first time, the elements of definition included low muscle mass, associated with either low muscle strength or low physical performance. The group also classified sarcopenia into three broad categories: Pre-sarcopenia when only low muscle mass is present,

sarcopenia when 2 of the 3 elements are present (one of which has to be low muscle mass) and finally severe sarcopenia when all 3 elements are present.

1.7.3: Limitations of 2010 definition:

While there is support for this definition, some propose that the term "dynapenia" should be reserved for age related reduction in muscle *function*, arguing while age related decline in muscle mass is one of the causes, there are separate neurological as well as hormonal causes that also contribute to functional loss (Clark and Manini, 2008). The 2010 definition also doesn't address that sarcopenia may start earlier in life, which hampers efforts to identify those who are at risk of developing this condition.

1.7.4: EWGSOP-2 2018 definition:

In 2018, there have been renewed attempts by the European Working Group on Sarcopenia in Older People- 2nd meeting (EWGSOP-2) to revise the definition of sarcopenia (Cruz-Jentoft *et al.*, 2018). Sarcopenia now is defined as low muscle strength and low muscle quality/ quantity. When this accompanied with functional impairments, this become severe sarcopenia (Table 1.2)

2018 Operational definition of Sarcopenia
1. Low muscle strength 2. Low muscle quality or quantity 3. Low physical performance
Probable sarcopenia: Criterion 1 only Confirmed sarcopenia: Criteria 1 & 2 Severe sarcopenia: Criteria 1-3

Table 1.2: 2018 EWGSOP-2 definition of sarcopenia (adapted from Cruz-Jentoft et al. 2018)

The group also recognised following new insights based on research on sarcopenia so far:

1. While Sarcopenia can be seen in elderly, it originates much earlier. This is important as identifying at risk groups early may mean more targeted interventions.
2. Sarcopenia was now considered as a "muscle failure" disease, something also reflected in definition.
3. The meeting recognised that while sarcopenia has traditionally been defined as low muscle quality and quantity, it is much easier to apply these definitions in research than everyday clinical practice.
4. Building on comment no. 3, the meeting recognised that sarcopenia is overlooked and under-treated in routine clinical practice and that there needs to be an increase in awareness in clinical staff treating older people.

1.7.5: Epidemiology and clinical Impact of Sarcopenia:

The exact prevalence of sarcopenia varies from region to region though a study from US in community dwelling elderly population aged 64 and above had a prevalence of 22.6% in women and 26.8% in men (Iannuzzi-Sucich, Prestwood and Kenny, 2002). The study defined sarcopenia as appendicular skeletal muscle mass of below 2 standard deviation from general population (there was no operational definition in 2002!). Another study from Japan looked at prevalence of sarcopenia using a cross-sectional study (and its association with osteopenia and osteoporosis) in women only and reported 9% (with normal bone mineral density), increasing up to 29.7% in those with osteoporosis at hip (according to WHO classification) (Miyakoshi *et al.*, 2013).

The impairment of physical abilities associated with sarcopenia contribute to reduced functional abilities (Janssen, Heymsfield and Ross, 2002). Because of this association with this disability, sarcopenia has become a major healthcare concern (Anton *et al.*, 2015). This functional disability alone may not only result in increasing institutionalised care costs, but also costs associated with treatment of accompanying psychological problems including depression and physical ailments due to metabolic abnormalities associated with sarcopenia. Delayed recovery from hospitalisations, including surgeries also adds to this cost (Anton *et al.*, 2015). While it is difficult to put an exact number on it, in 2000, the cost of Sarcopenia

related healthcare costs were estimated to be 18.5 billion (with a sensitivity analysis indicating it to be between 11.8 to 26.2 billion US dollars) (Janssen *et al.*, 2004).

While the functional impairments associated with sarcopenia may define it, the syndrome is also associated with dysregulation of glucose metabolism, hormone production and intra-cellular processes resulting in diseases such as type 2 diabetes and metabolic syndrome e.g. in a Korean study (The Korea National Health and Nutrition Examination Survey (KNHANES)) which defined sarcopenia as appendicular skeletal muscle mass divided by weight (%) >2SD below gender specific young adults, sarcopenia was associated with insulin resistance, development of diabetes as well as metabolic syndrome, the effect more pronounced in non-obese elderly population (Moon, 2014). However, in order to understand a disorder characterised by "muscle failure", it is imperative to look at determinants of skeletal muscle mass and its regulation in health and disease.

1.7.5: Factors affecting sarcopenia & anabolic resistance:

While sarcopenia is an age related disorder, there are similarities with several disease processes seen in relatively younger population, such as insulin resistance seen in obesity- sarcopenia is associated with insulin resistance in a similar fashion to (and independent to) obesity as seen in

National Health and Nutrition Examination Survey (NHANES) III (Srikanthan, Hevener and Karlamangla, 2016). Other factors that may affect sarcopenia include immobility and chronic wasting diseases such as cancer (Morley, Anker and von Haehling, 2014).

Another key factor that may be associated with development of sarcopenia is Anabolic resistance, defined as a blunted response to anabolic stimuli (such as protein supplementation) seen in elderly when compared to younger population (Burd, Gorissen and Van Loon, 2013). This was first noted in a study by Cuthbertson and colleagues who noted compared the anabolic response to protein supplementation with insulin clamped at a constant rate (Cuthbertson *et al.*, 2005) in both young and older males. This study showed that the basal MPS rates were similar in both young and old however the skeletal muscles in older subjects demonstrated reduced anabolic sensitivity to essential amino acids, which was independent of insulin levels. This has then led to several possible reasons as to why the skeletal muscles in older people demonstrate reduced sensitivity to protein supplementation, as seen in figure 1.16:

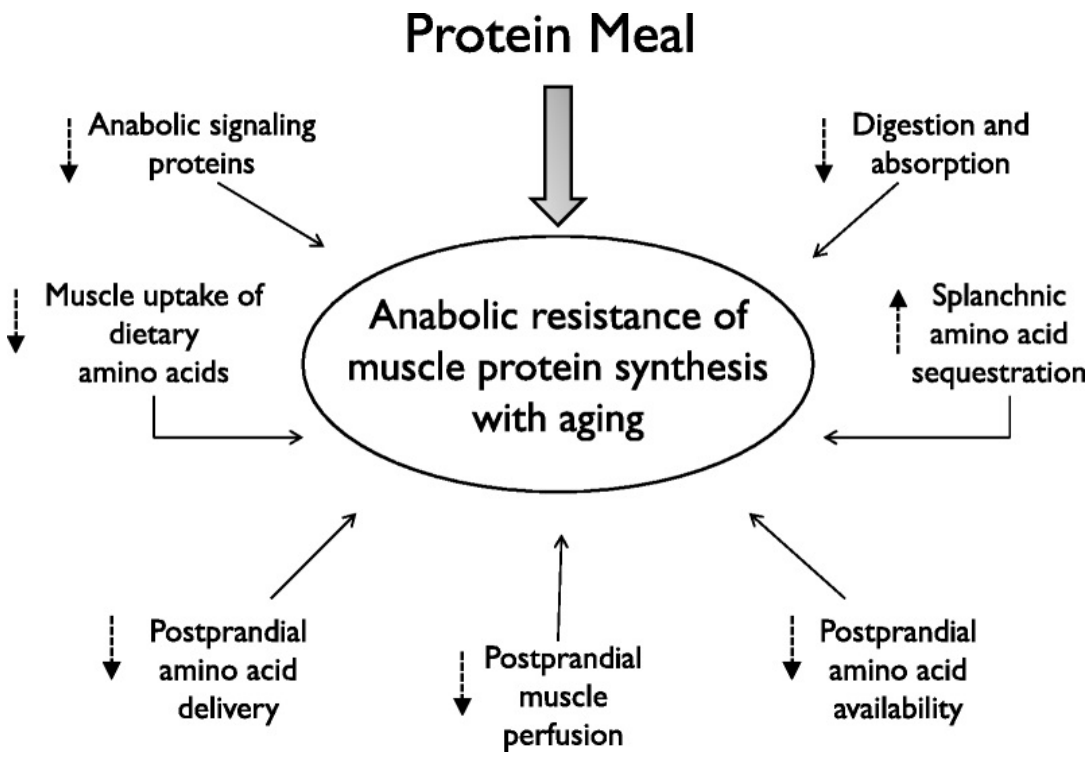


Figure 1.16: Shows factors associated with a protein meal that may cause reduced anabolic sensitivity of skeletal muscles in older people (adapted from Burd, Gorissen and Van Loon, 2013)

So, if the elderly skeletal muscle has reduced sensitivity to dietary protein supplementation, could it be that increasing the dose of dietary supplementation may overcome this resistance? This was the research question behind a study looking at MPS responses following several doses of whey protein (10, 20 and 35g) (Pennings *et al.*, 2012). All the doses showed an anabolic response (as would be expected) however, the accretion of amino-acids from 35g whey protein was much higher compared to 10g. This study did not have any young male controls, though previous studies have shown that 20g whey protein is sufficient to

maximally induce MPS response in younger males (Daniel R Moore *et al.*, 2009). Hence, increasing the dose of protein supplementation may help overcome the anabolic resistance- see figure 1.17 below:

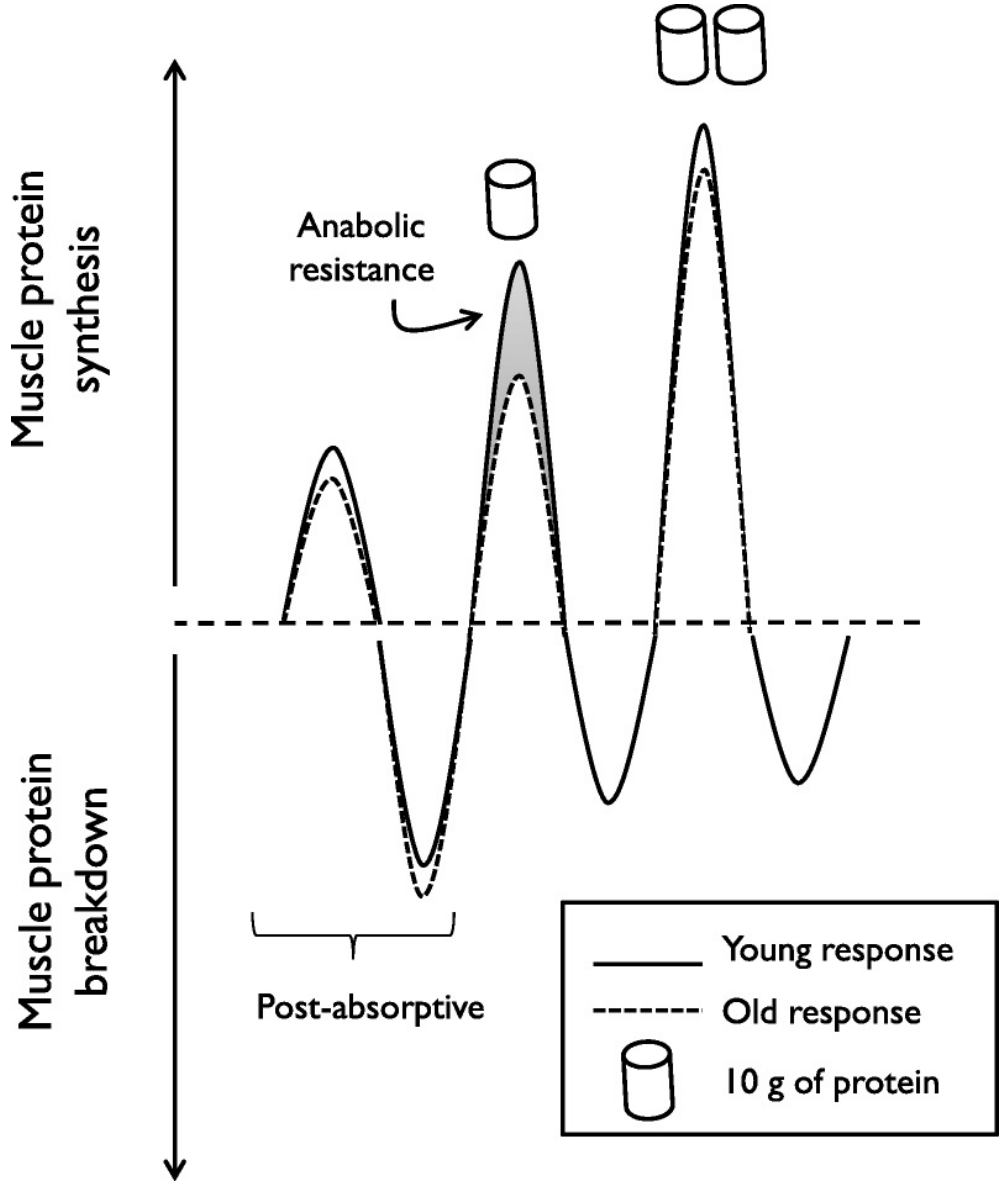


Figure 1.17: Shows increasing dose of protein supplementation may overcome reduced anabolic sensitivity of skeletal muscles in older people (adapted from Burd, Gorissen and Van Loon, 2013)

There may be a role for leucine to play here as well. There is some evidence to show that improving the leucine (LEU) content improves anabolic response to an oral essential amino-acid (EAA) supplement though the study only had 4 elderly participants (Katsanos *et al.*, 2006). Similar data from cancer patients (a population exhibiting muscle loss with anabolic resistance similar to sarcopenia) also showed that adding leucine to conventional food supplements may stimulate muscle protein synthesis better than conventional food supplements alone (Deutz *et al.*, 2011).

Similarly, lack of physical activity has also been implicated in development of anabolic resistance in younger subjects, for example Glover & colleagues have demonstrated that unilateral knee immobilisation in younger subjects for 10 days resulted in 27% reduction of post-absorptive MPS, with significant reduction in MPS following amino-acid infusion at two different doses (when compared to non-immobilized leg). Similar results were seen with older subjects with a 7-day bed-rest, with reduced mTORC1 signalling (Drummond *et al.*, 2012). So, does this mean that increasing physical activity may increase use of amino-acids derived from nutritional supplementation? While there aren't many studies answering this question, there is some data in older men to show that it might e.g. in a study involving 24 young and 24 older males, use of resistance exercise allowed for greater use of amino-acids derived from dietary protein supplement in both young and older participants (Pennings *et al.*, 2011). Similar results are seen with endurance exercise such as treadmill walking (Fujita *et al.*, 2007). Hence a component of physical activity may also enhance anabolic response to nutritional stimuli in older people, as seen

in figure 1.18 below:

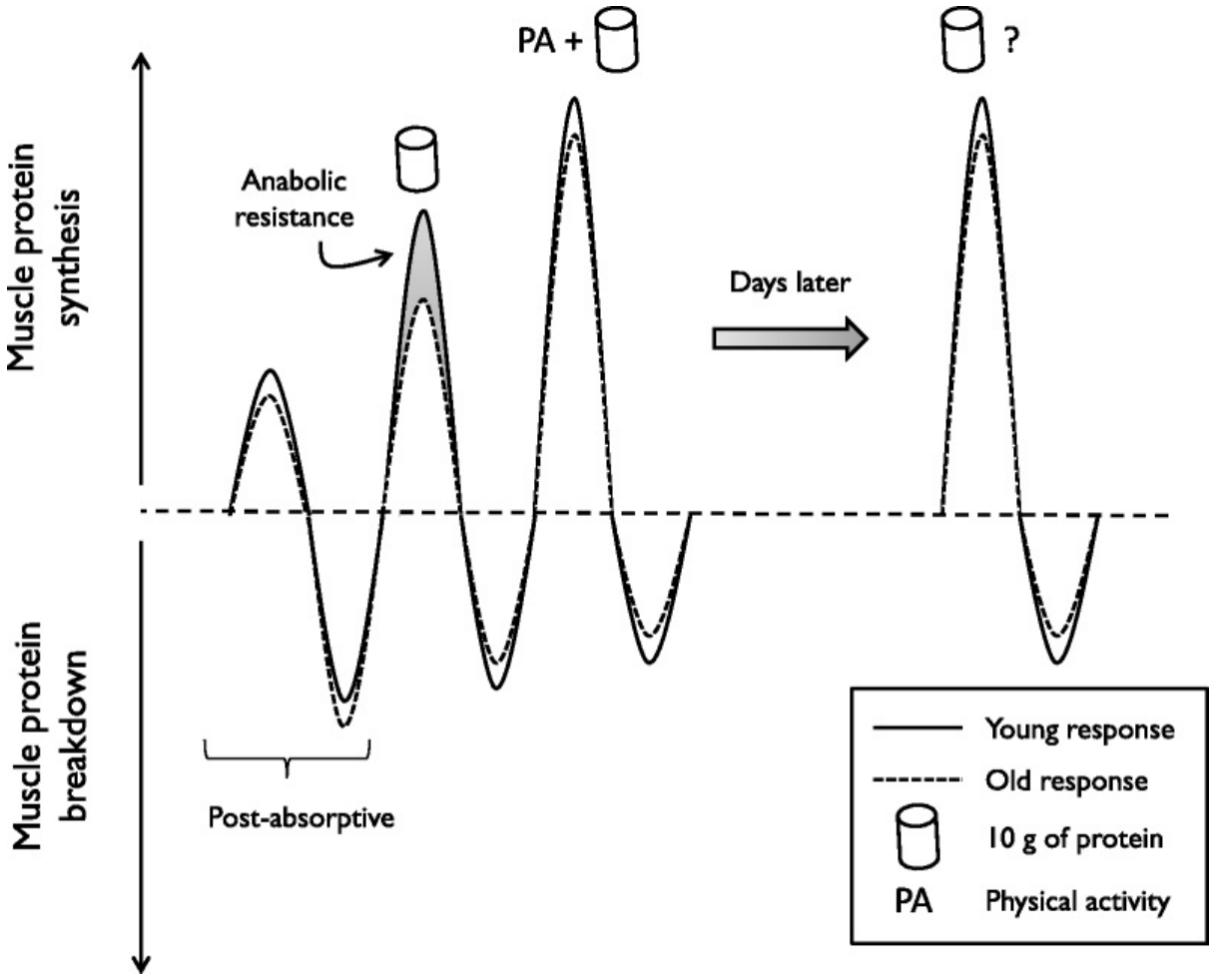


Figure 1.18 Shows physical activity in addition to protein supplementation may overcome reduced anabolic sensitivity of skeletal muscles in older people (adapted from Burd, Gorissen and Van Loon, 2013)

1.7.6: Sarcopenia- Gaps in current knowledge & future horizons

So far, the strategies that have been shown to overcome anabolic resistance (in studies performed in males only) are:

- Increasing the dose of whey protein to 40g.
- Adding leucine to nutritional supplementation ($\geq$ 35%)- though it remains to be seen if low dose leucine enriched supplement may be as effective as higher dose ones.
- Physical activity particularly resistance exercise activity in addition to supplementation
- Poor circulation may have a role to play however; it has been noted that RE improves the blood flow in elderly subjects as noted in a study involving, resistance exercise training (for 20 weeks) participants showed improvement in leg blood flow and microvascular blood flow (Phillips *et al.*, 2012).

While these strategies may be helpful, these have been studied in different populations and all the studies have been done in males only. There is paucity of data looking at effect of nutrition and resistance exercise in elderly females- this remains an under-represented population in research looking at anabolic responses in human skeletal muscles. It is not well known how do they respond to traditional whey protein supplementation and/or to leucine enriched amino acid supplementation with or without resistance exercise. Females aged 60-70 years may well be the best population to study the muscle protein metabolism as, they are less likely

to have any chronic wasting diseases or be on any medications that may affect MPS. Females aged over 70 are more likely to have developed such diseases, be on medications or have reduced mobility. It is also plausible that they may not want to volunteer in a study involving resistance exercise. Finally, due to concerns about osteoporosis, it was decided to restrict the inclusion criteria till aged 70 (see chapter 2 for inclusion and exclusion criteria).

1.8: AIM & OBJECTIVES OF THIS THESIS

1.8.1: Aim

The aim of this thesis is to study anabolic response to protein supplementation with Whey protein as well as Leucine enriched (40%) essential amino acid (LEAA) supplements (at various doses) with and without unilateral resistance exercise, in older women aged 60 years and above, using stable isotope methodologies.

1.8.2: Objectives

The specific objectives of this thesis are:

- To study the anabolic response to LEAA 3 g and to Whey protein 20 gm with and without acute resistance exercise training in older women (Chapter 3).
- To study the anabolic response to LEAA 1.5 g, LEAA 6 g and to Whey protein 40 g with and without acute resistance exercise training in older women (Chapter 4).
- To look at the effects of the nutritional supplements (mentioned above) with ***and*** without acute resistance exercise on leg blood flow (as measured by Doppler ultrasound) as well as microvascular blood flow (as measured by contrast enhanced ultrasound or CEUS) in older women (Chapter 5).

1.8.3: Hypothesis

The specific hypotheses tested in this thesis are:

1. Whether 40% leucine enriched amino acid supplement is as effective as whey protein? If so, then at what dose?
2. Whether there is an incremental anabolic response to increasing doses of LEAA?
3. Whether whey protein offer any additional anabolic benefit at 40g?

CHAPTER 2: MATERIALS AND METHODS

2.1 INTRODUCTION

This section starts by reviewing use of stable isotopes in muscle protein metabolism studies and then discusses the important details about the standard operating procedures for the studies described in this thesis.

2.2: USE OF STABLE ISOTOPE TRACERS TO STUDY SKELETAL MUSCLE PROTEIN METABOLISM IN HUMANS

2.2.1: Introduction

The knowledge of human protein metabolism and its regulation largely depends on the physiological data of direct and indirect measurements of protein synthesis, breakdown and exchange of amino acids between tissues. Many studies have been done in different physiological states, e.g. starvation, feeding, and in young and old age etc. These measurements have been possible because of use of amino acid tracers, using stable isotopically labelled amino acids to trace protein metabolism (Wagenmakers, 1999).

A "tracer" is a special element/molecule that is used to study metabolic dynamics of another, naturally occurring compound (called "tracee") (Vella and Rizza, 2009). An ideal tracer should be similar in chemical properties

to tracee except in one key aspect and should be detectable in minute quantities. Hence most tracers used in metabolic research in humans are similar to a naturally occurring compound of interest, except for one atom (which is either a radio-active or a non-radioactive isotope) (Vella and Rizza, 2009).

2.2.2: Types of Isotopes

Since early 1980s, the rate of protein metabolism in human skeletal muscles has been studied via the use of isotopes (Rennie *et al.*, 1982). There are two main types of isotopes:

1. Radioactive isotopes (which have a radioactive atom within its chemical structure) As radioactive isotopes are constantly in a state of decay, they are, by definition, "unstable" isotopes.
2. Stable isotopes are nonradioactive heavy atoms, meaning that they have same number of protons (and thus occupy same place on periodic table) but have different number of neutrons in their nuclei e.g. ^{13}C, ^{15}N and ^{2}H. This gives them similar chemical properties however different atomic weight. These atoms are then incorporated in amino acids (which makes them "heavier" than naturally occurring amino acids) which are infused to test subjects at a pre-determined rate. The incorporation of these isotope based amino acids are then detected in human skeletal muscle samples obtained via biopsy through mass spectrometry. Only the non-radioactive

isotopes are used as they are stable and do not involve exposure to radiation, hence the techniques have been used in children and infants (Rennie, 1999). Their use requires IV access as well as a primed, constant infusion to reach steady state.

2.2.3: History of use of stable isotopes

The earliest attempts to study human protein metabolism at a whole body levels was circa 1978 using [^{15}N]glycine (Waterlow, 1995). It was used either orally or as a continuous intra-gastric infusion and also required collection of urine samples to determine excretion. One critical assumption underlying this method (that was later proved to be incorrect) was that the whole body was treated as one nitrogenous pool (Wagenmakers, 1999). The later studies initially used leucine tracer (e.g. L-[1-13C] leucine) and later phenylalanine tracers (e.g. L-[2H5] phenylalanine). Both tracers were used as a primed infusion until a steady state has been reached in arterial blood. While initially there was a favour towards the leucine tracers, however when these were compared together, there were some discrepancies seen e.g. reduced breakdown and increased synthetic rates with leucine tracers (providing some early clues to its anabolic properties) (Wagenmakers, 1999).

A number of studies have compared continuous infusion vs. flooding approaches. Flooding (Garlick *et al.*, 1989) involves given a large bolus of a mixture containing both labelled and unlabelled amino acid (~20 APE)

over a short period of time to flood all the amino acid pools to the same extent. While this method is fast and can be useful in certain situations where longer studies may not be feasible, concerns have been raised over the validity of this approach, particularly as synthetic rates measured using constant infusions, were higher during the flooding dose, suggesting the flooding dose was stimulating MPS (Smith *et al.*, 1992).

2.2.4: Use of stable isotope tracers for metabolic studies

Most studies looking at human metabolic dynamics use stable isotopes due to chemical stability and lack of radiation exposure. As noted above, the tracers are composed of stable isotopes which are amino-acids with a heavy atom (but otherwise similar chemical structure, thereby affecting only the mass/charge ratio- which is then detectable by mass spectrometry) (Vella and Rizza, 2009). Also, a tracer is usually present in trace amounts in natural states.

The tracers are usually given an a constant infusion after a "bolus" as this usually means that a steady state (in form of square wave) is maintained (Smith and Rennie, 1996), hence the infusion continues till the end of the study.

Regular sampling of arterial blood throughout the procedure is required to ensure that a steady state is achieved. Also, regular muscle biopsies are required, the first one being shortly after the infusion is started and other

to look for incorporation of tracer into the muscle protein fractions- also known as muscle enrichment (Smith and Rennie, 1996). The need for first biopsy to be before or shortly after the start of tracer infusion may be avoided given certain conditions are met e.g. if small amounts of tracer are used and an easily accessible protein (such as serum albumin) is available to determine background labelling (Smith and Rennie, 1996). The concept is depicted in figure 2.1 below- adapted (Kim *et al.*, 2016):

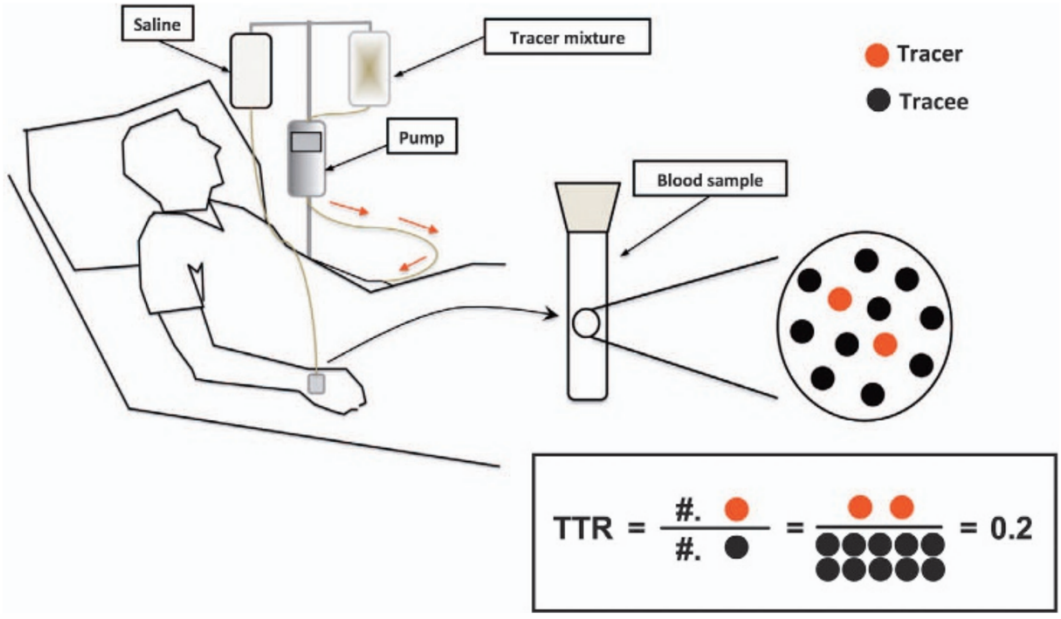


Figure 2.1: Shows schematic diagram of stable isotope study in humans- Note that tracer enrichment (tracer-to-tracee ratio or TTR) may be determined from blood or muscle- Adapted from Kim et al., 2016.

2.2.5: Determination of myofibrillar Fractional Synthetic Rate (FSR)

The fractional synthetic rate (FSR) is the rate of synthesis of any specific protein within the tissues- in this case the main protein of interest is myofibrillar protein within the skeletal muscle. The FSR of myofibrillar protein fraction is calculated from incorporation of tracer (i.e. L-[ring-$^{13}C_6$]-phenylalanine) within the myofibrillar protein between two subsequent biopsies. The FSR was calculated using the standard precursor-product equation:

$$FSR\ \left(\%/_{hour}\right) = \left[\frac{E2 - E1}{Em.T}\right] \times 60\ \times 100$$

Where:

- FSR: fractional synthetic rate of myofibrillar protein fraction
- E1 & E2: Myofibrillar enrichment of L-[ring-$^{13}C_6$]-phenylalanine at sequential biopsies
- Em: Mean L-[ring-$^{13}C_6$]-phenylalanine enrichment in the intramuscular pool
- T: Time interval between 2 biopsies

The increase in tracer enrichment in muscle proteins over a given specific duration is divided by steady state tracer precursor enrichment in blood or muscle, measured by Gas Chromatography Mass Spectrometry (GC-MS).

This gives us the fractional synthetic rate (FSR) of muscle proteins, i.e. a percentage of existing muscle pool synthesized over that specific time period compared to the precursor labelling (Wagenmakers, 1999).

2.2.6: Mass Spectrometry- the basics

Mass spectrometry is a technique that separates electrically charged ions/ molecules in time and space, on the basis of their mass to charge ratio (Urban, 2016). It quantifies the amount of a specific mass of an ion in a fragmented and (in this case) a labelled compound of interest (Kim *et al.*, 2016). After passing through the ionization source, the fragmented ions of interest pass through a mass analyser for a set time and hit the photomultiplier (see figure 2.2 for a simplified diagram), generating a quantitative graph on the basis of mass-to-charge ratio.

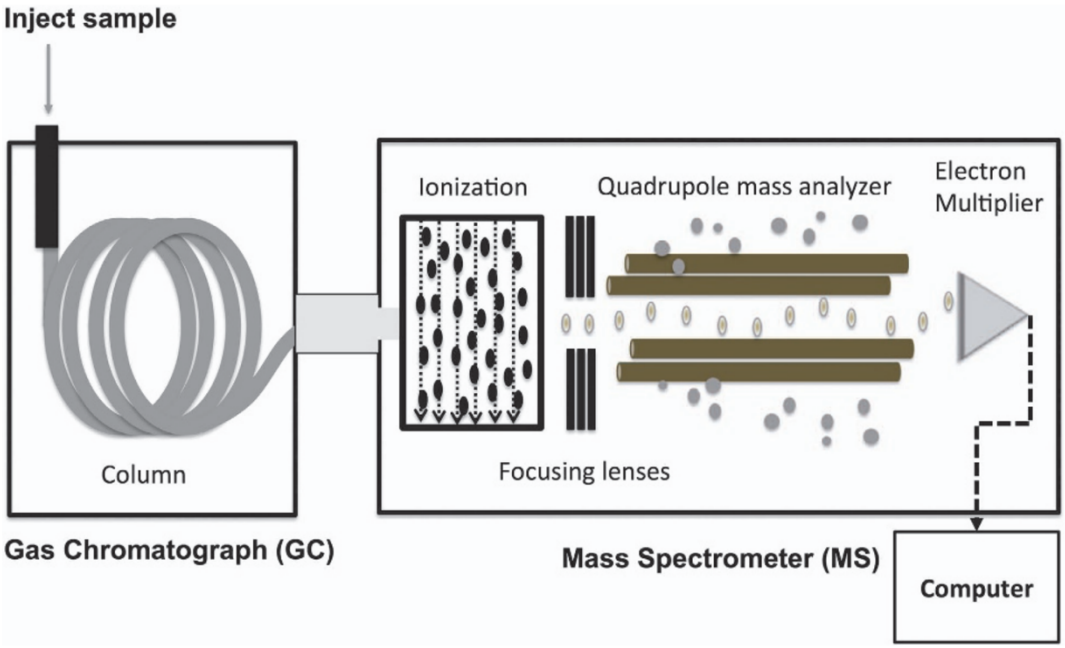


Figure 2.2: Shows a simplified Gas Chromatography-Mass spectrometry (GC-MS)- adapted from Kim et al., 2016).

2.2.7: Choice of tracers and precursor

Ideal tracer should be present in large quantities in the myofibrillar protein but not in the free pool (Smith and Rennie, 1996). While many tracers are available e.g. phenylalanine, leucine, valine and glycine to measure MPS (Smith and Rennie, 1996), we chose phenylalanine with ^{13}C labelling as it is the most common and cost-effective label available. Another advantage is that it is not subject to intermediary metabolism within the intra-muscular free amino-acid pool (Smith and Rennie, 1996), hence we used intra-muscular pool as a precursor. Also, similar studies from our lab (done in older men) have used L-[ring-$^{13}C_6$]-phenylalanine tracer- in order to generate comparable data in females, it is important that we use similar tracer.

2.2.8: The Future of Stable Isotopes

While amino acid stable isotopes provide a robust and accurate method to measure muscle protein metabolism, they have several drawbacks. In particular, they require IV access, multiple blood sampling (to ensure an equilibrium has been reached) and have significant preparation costs as they require sterile pharmacy settings. They can only study muscle protein metabolism in controlled lab settings over several hours. The data obtained from acute laboratory settings, may not be representative of "real world" situations.

D2O (Deuterium dioxide) provides an alternative approach that can study muscle protein synthesis over days to weeks. This involves an oral dose of D2O used as a tracer, to study muscle protein responses to diet and exercise over extended periods of time i.e. weeks (Robinson *et al.*, 2011), as well as on day to day (24h) basis (Wilkinson *et al.*, 2014). These have been compared to more "traditional" tracers such as L-[ring-13C6] phenylalanine and found to give identical rates and responses to feeding, and be less invasive and more cost-effective way to study MPS (Wilkinson *et al.*, 2015). However, as the aim of this project was to study anabolic responses in older women (possibly generating data comparable to older men), the research protocol and design as kept similar to the similar studies done by our lab in older people. Due to this reason, the tracer was kept identical as well.

2.3: SUBJECTS AND EXPERIMENTAL PROTOCOL:

Ethical Approval was obtained from the University of Nottingham Medical School Ethics Committee (United Kingdom), with all studies conducted in compliance with the Declaration of Helsinki and pre-registered at (clinicaltrials.gov registration no. NCT02053441).

2.3.1: Recruitment and screening visit

All subjects were recruited by postal invitation letters sent to an area local to the research centre. All invitation letters also included the "Participant information letter" which was also approved by the ethics committee. Those who expressed their interest were invited to a screening visit where they underwent a medical interview and examination programme using a structured proforma (see appendix-1) to ensure compliance with inclusion and exclusion criteria (See table 2.1). Exclusion criteria included any chronic diseases that may interfere with anabolic responses (as noted in previous chapter) as well as disorders such as clotting problems which could make taking biopsies risky. Finally the participants were advised not to take any medications that may interfere with muscle protein synthesis such as steroids (see chapter-1), paracetamol or non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen (Trappe *et al.*, 2002). They were also excluded if they had recently participated in a similar research study within the preceding 6 months- this is to bias and confounding factors (participants more likely to involve in research studies may be different from general population).

They had consent taken at the screening visit and after satisfactory interview and examination, also had blood tests to look for any abnormalities in renal, liver and thyroid functions as well as haematological profile.

INCLUSION CRITERIA Healthy women (60-69 y) who are generally healthy and not on any exercise regime or programme. .
EXCLUSION CRITERIA: Subjects will be excluded if they have evidence of: • Active cardiovascular disease • Cerebrovascular disease including previous stroke or aneurysm • Respiratory disease including pulmonary hypertension or COPD • Hyper/ hypo parathyroid, hyper/ hypothyroidism, Cushing's disease, diabetes • Active inflammatory bowel disease • Renal disease • Malignancy • Recent steroid treatment (within 6 mo), or hormone replacement therapy • Clotting dysfunction • Musculoskeletal or neurological disorders • Any disease requiring long-term drug prescriptions, including statins • Taking regular paracetamol or non-steroidal anti-inflammatory drugs (NSAIDs)

Table 2.1: Shows the inclusion and exclusion criteria

2.3.2: Exercise assessment

For research studies including a resistance exercise component, participants also underwent an exercise assessment to determine 1-repitition maximum (1RM) weight (in kilograms or kg) on their knee extensors, using a seated leg extension machine (Technogym, Gambettola, Italy). The procedure was always supervised by a member of the research team (usually Syed Bukhari). The procedure was first demonstrated to the participant, so that they can see how the leg extension machine works and that they would have to follow a tempo (2

secs up, 1 sec rest, 2 secs down and 1 sec rest). After taking their position, the research team member supervising made sure that the back rest and pad were adjusted according to participant's height. The subjects were then asked to lift a starting weight 5 times (2 seconds going up, 1 sec rest and 2 seconds to come down) and if able to do it, weights were increased steadily (after 2-minute break) until they reached a point where they were unable to do 5 reps or did so with poor form (e.g. lower back extension, incomplete extension of leg). The number of sets allowed on a particular day were 5 (i.e. if 1RM was not determined within 5 sets then the assessment would need to be repeated another day, though this was never needed as most participants reached their 1RM in 2-4 sets). The number of reps performed in last set and the weights (in kilograms/ kg) were used to calculate 1-repitition maximum or 1RM available online at http://www.exrx.net/Calculators/OneRepMax.html. In most cases, both legs were assessed (however all participants were given a choice to have both or either one leg assessed). In cases where both legs were assessed, it was up to the participant to decide which leg would be used for exercise (decision usually made on study day). In some cases participants requested only one leg to be assessed due to knee or hip problems in the other leg and their request was granted.

2.4: STUDY VISITS & PROTOCOLS

All were studied after an overnight fast and were asked to refrain from heavy exercise for 48h prior to study. All studies started between 0800 hrs and 0900 hrs.

2.4.1: Intravenous access

Subjects had either 20 or 22-g intravenous (IV) cannula inserted into a hand or antecubital fossa for a primed (0.4 $mg \cdot kg^{-1}$), constant infusion (0.6 $mg \cdot kg^{-1} \cdot h^{-1}$) of L-[ring-$^{13}C_6$]-phenylalanine (Isotec, Sigma Aldrich) tracer. A retrograde cannula was placed at the back of the hand for arterialised venous blood using a 20g or 22g cannula, with hand warmed using a custom made "hot-box". The temperature was kept stabilised at 50-60 C. This was done as this is a technique that allows access to arterialised blood with venous cannulation and is well described in literature to study glucose (McGuire *et al.*, 1976) and protein kinetics (Abumrad *et al.*, 1981).

They also had blood samples and muscle biopsies were taken as outlined in figure 2.3. Muscle biopsies were taken periodically from m. *vastus lateralis* using the conchotome technique (Dietrichson *et al.*, 1987) after infiltration of 5ml 1% lignocaine. This muscle was used as it is a large muscle, is easily accessible for biopsy and also easily isolated for exercise purposes. It does not have any major blood vessels or nerves lying over it. Also, as there is one on each side, it allowed us to use it on both exercise and non-exercise side.

Once obtained, the muscle sample was washed in ice-cold saline and visible fat and connective tissue removed before being frozen in liquid N_2 and stored at -80ºC until analysis. Biopsies were taken 1 h and 3 h after commencement of tracer to permit assessment of basal (post-absorptive) MPS. Subjects then performed a bout of unilateral resistance exercise (6×8 repetitions at 75% of pre-determined 1-RM) before consuming, randomized, either 20 g of Whey protein or 3 g LEAA ("Amino L40"; Ajinomoto Inc.,) in an aqueous solution (250 ml). For composition of both of these, please see table 2.2 on next page.

	LEAA (1.5 g), g	LEAA (3 g), g	LEAA (6 g), g	WP (20 g), g	WP (40 g), g
L-Leucine	0.6	1.2	2.4	2	4
L-Isoleucine	0.16	0.32	0.64	1.4	2.8
L-Valine	0.165	0.33	0.66	1.2	2.4
L-Threonine	0.14	0.28	0.56	1.4	2.8
L-Lysine	0.25	0.5	1.0	1.8	3.6
L-Methionine	0.05	0.1	0.2	0.4	0.8
L-Histidine	0.025	0.05	0.1	0.4	0.8
L-Phenylalanine	0.1	0.2	0.4	0.6	1.2
L-Tryptophan	0.01	0.02	0.04	0.4	0.8

Table 2.2: Shows the composition of both Whey Protein- WP (20g & 40g) and Leucine Enriched Amino-Acid (LEAA) supplement LEAA (1.5g, 3g & 6g).

Each solution was made and mixed on the study day. After study, all cannulas were removed and subjects fed and monitored for 30 min (protocol is in Figure 2.3 below):

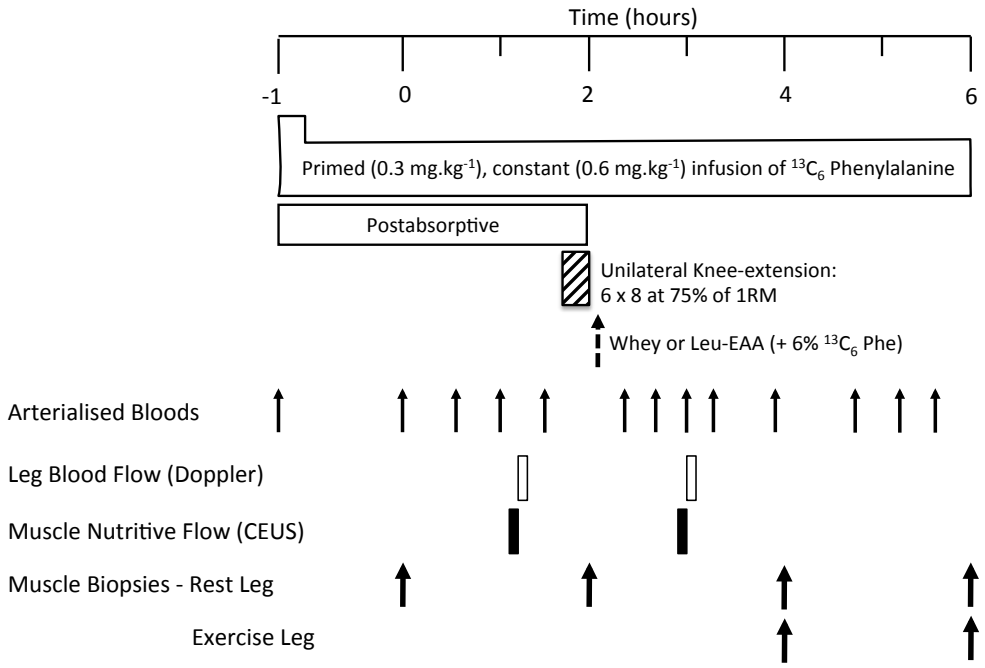


Figure 2.3: Study protocol for anabolic response to Whey Protein (WP) supplement and Leucine Enriched Amino Acid (LEAA) supplement.

The main strength of this study design meant each participant were their own rest/exercise control. The rested leg was exposed to feeding alone, while the exercised leg to the combination of nutrition and exercise (hereafter 'FED' or 'FED-EX', respectively). The major drawback was that the protocol did not allow us to study muscle protein breakdown. Adding this could have added further procedures and hence was avoided.

2.5: MEASUREMENT OF PLASMA INSULIN AND AA CONCENTRATIONS

Arterialized venous plasma insulin concentrations were measured using a high-sensitivity human insulin enzyme-linked immunosorbent (ELISA) assay (DRG Instruments GmbH, Marburg, Germany). For Amino Acid (AA) analyses, equal volumes of arterialized plasma and 10% sulfosalicylic acid were mixed and cooled to 4ºC for 30 min. Samples were centrifuged at 8000 *g* to pellet the precipitated protein and the supernatant fluid was passed through a 0.22 µm filter before analysis with a dedicated AA analyser (Biochrom 30, Biochrom, Cambridge, United Kingdom) using lithium buffers. All 20 AA concentrations were measured by comparison to a standard AA mix with nor-leucine as an internal standard.

2.6: MEASUREMENT OF LEG BLOOD FLOW (LBF) AND MUSCLE MICROVASCULAR BLOOD FLOW (MBF)

Leg blood flow (LBF) was measured using Doppler ultrasound, with a 9-3 MHz probe positioned over the origin of the common femoral artery. Flow was estimated as the product of vessel cross-sectional area and mean velocity over six cardiac cycles. Contrast enhanced ultrasound (CEUS) was

used to measure microvascular blood flow (MBF) as previously described (Phillips *et al.*, 2014). Briefly, Sonovue™ microbubbles (Bracco, Milan, Italy) were infused via an intravenous cannula in an antecubital fossa vein. An iU22 ultrasound scanner (Phillips Healthcare, Reigate, United Kingdom) with two 40-mm linear 9-3 MHz probes (Phillips L9-3) firmly secured on the both anterior thighs (one on left and another on right) using custom made probe grips (to measure individually both for the FED and FED-EX legs); were used to register microbubble appearance within the quadriceps muscle at rest and in response to FED and FED-EX. Intermittent high mechanical index (MI) "flashes" disrupted microbubbles whilst continuous low MI recording measured the rate of microbubble replenishment. Infusion was at $2ml \cdot min^{-1}$ for 1 min and $1ml \cdot min^{-1}$ for 3 subsequent min (1.5 min for each leg). During the last 90 s of this measurement protocol, 3×30 s flash/ replenishment recordings were made. Off line region-of-interest analysis using Q-Lab software (Phillips Healthcare, Surrey UK) was used to measure the plateau ("A" value) linear acoustic intensity and also the rate constant ("β" value); the product of which is proportional to volume blood · volume tissue^{-1} · second^{-1} and thus skeletal muscle tissue microvascular blood flow (MBF) (Phillips *et al.*, 2014).

2.7: MEASUREMENT OF APS

Plasma (200 µl) was precipitated with ice-cold 10% trichloroacetic acid (TCA). Following centrifugation (13,000 rpm, 4°C) to pellet proteins, the

albumin was extracted with ethanol to separate it from the non-alcohol soluble proteins. The ethanol extract was dried under nitrogen and solubilized in 0.3 M NaOH at 37°C for 60 min and then precipitated with 1M Perchloric acid. Isolated albumin bound AA were released by acid hydrolysis with 0.1 M Hydrochloric acid and Dowex H+ ion exchange resin (50W-X8-200, Sigma Aldrich, Poole, UK) heated overnight at 110°C, before purification by ion exchange chromatography; AA were derivatized as their n-acetyl-N-propyl esters and incorporation of [ring-$^{13}C_6$] phenylalanine into albumin assayed by gas chromatography-combustion-isotope ratio mass spectrometry (Delta Plus XP, Thermofisher Scientific, Hemel Hempstead, UK). Fractional synthesis rates of albumin (ASR) were determined using a standard precursor-product model:

$$\text{ASR } (\%/\text{h}) = \left[\frac{\Delta Ea}{E_P . t}\right] \text{ x } 100$$

Where ΔEa is the change in labelling of albumin phenylalanine between 2 blood samples, E_P is the mean enrichment over time of the precursor (taken as arterialized plasma Phenylalanine [ring-$^{13}C_6$] labelling) and t is the time in hours between blood samples.

2.8: MEASUREMENT OF MPS

Myofibrillar proteins were isolated, hydrolysed and derivatized using our standard techniques (Wilkinson *et al.*, 2013). Briefly, ~25 mg of muscle biopsy tissue was homogenized in ice-cold homogenization buffer (50 mm

Tris-HCl (pH 7.4), 50 mm NaF, 10 mm β-glycerophosphate disodium salt, 1 mm EDTA, 1 mm EGTA, 1 mm activated Na3VO4 (all Sigma-Aldrich, Poole, UK)) and a complete protease inhibitor cocktail tablet (Roche, West Sussex, UK) at 10 µl·µg^{-1} of tissue. Homogenates were rotated for 10 min and the supernatant collected by centrifugation at 13,000 × g for 5 min at 4°C. The resulting pellet was washed three times with homogenization buffer and 0.3M NaOH was added in order to facilitate the separation of the soluble myofibrillar fraction from the insoluble collagen fraction by subsequent centrifugation. The myofibrillar fraction was then removed and precipitated using 1 M perchloric acid (PCA) and pelleted by centrifugation. The myofibrillar pellet was then washed twice with 70% ethanol and the protein-bound AA were released by acid hydrolysis using 0.1 M HCl and 1 ml of Dowex ion-exchange resin (50W-X8-200) heated overnight at 110°C. Released AA were purified, derivatized and analysed as in the *Measurement of APS* section above with the fractional synthesis rates (FSR) of the myofibrillar proteins calculated using the same precursor-product method:

$$\text{FSR}\ (\%/\text{h}) = \left[\frac{\Delta Em}{E_p . t}\right] \text{x}\ 100$$

Where ΔE_m is the change in enrichment of bound L-[ring-$^{13}C_6$] phenylalanine in two sequential biopsies, t is the time interval between two biopsies in hours, and E_p is the mean free L-[ring-$^{13}C_6$] Phenylalanine enrichment in the intramuscular pool.

2.9: IMMUNOBLOTTING FOR AKT-MTORC1 SIGNALLING PATHWAY ACTIVITY (I.E. PHOSPHORYLATION)

Immunoblotting was performed as previously described (Philip J. Atherton *et al.*, 2010) using the sarcoplasmic fraction collected during MPS preparation described above. Sarcoplasmic protein concentrations were determined using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE-US) and adjusted to 2 $\mu l \cdot \mu g^{-1}$ in 3× Laemmli. Each sample was loaded onto pre-cast 12% Bis-Tris Criterion XT gels (BioRad, Hemel Hempstead, UK) at 20 µg/ lane and separated electrophoretically at 200 V for 1 h. Proteins were wet transferred at 100 V, 45 min and subsequently blocked in 2.5% non-fat milk in 1× Tris buffered saline/Tween 20 (TBST). Membranes were incubated in primary antibodies (1:2000 dilution in 2.5% BSA in TBS-T) rocking overnight at 4°C, AKT^{Ser473}, $p70S6K1^{Thr389,}$ $4EBP1^{Ser65/70}$, $eEF2^{Thr56}$ (New England Biolabs, Hertfordshire, UK). Membranes were subsequently washed 3×5 min in TBS-T, incubated in HRP-conjugated secondary antibody (New England Biolabs, Hertfordshire, UK; 1:2000 in 2.5% BSA in TBS-T) at ambient temperature for 1 h, followed by 3×5 min washes in TBS-T. Membranes were exposed to Chemiluminescent

HRP Substrate (Millipore Corporation, Billerica, MA-US) for 5 min and bands quantified by Chemidoc XRS (BioRad, Hertfordshire, UK). All signals were within the linear range of detection; loading anomalies corrected to coomassie (Welinder and Ekblad, 2011). Blotting data were captured using peak density analysis.

2.10: STATISTICAL ANALYSES:

Data are presented as means ± SEM, except if indicated. Demographic, anthropometric and CEUS comparisons were analysed within or between groups by unpaired or paired *t*-tests (all as two-tailed), as appropriate. All other data sets were analysed using two-way repeated measures ANOVA (feed type × time) with differences located via Holm Sidak post-tests using GraphPad Prism Version 5 with *P* values calculated using GraphPad Quickcalcs (GraphPad Software Inc, San Diego, CA); *P* values of <0.05 were considered significant.

CHAPTER 3: 3G LEAA VS. 20G WHEY PROTEIN

3.1 CHAPTER SYNOPSIS

Ageing is associated with blunted anabolic responses in skeletal muscle to both nutrition and exercise, which may contribute to sarcopenia. However, these effects have mostly been studied in men and there is paucity of data in women.

This study looks at the effects of two-feeding regimes in older women (66±2.5y N=8/group): bolus-whey protein (WP-20 g) or novel low-dose leucine-enriched essential amino acids (EAA) (LEAA-3g [40% leucine]). Using $^{13}C_6$-Phenylalanine infusions, muscle (MPS) and albumin (APS) protein synthesis were quantified at baseline (i.e. fasted state) and both in response to feeding (FED) and feeding-plus-exercise (FED-EX; RE: 6×8 knee-extensions at 75%-1RM). Plasma insulin/AA concentrations were measured via ELISA. Muscle anabolic signalling was determined by phospho-immunoblotting. Whole-leg blood flow was measured via Doppler ultrasound and muscle microvascular blood-flow (MBF) was quantified using CEUS. Blood flow results are presented and discussed in Chapter-5.

Plasma insulinemia and EAA-aemia were markedly greater after WP than LEAA (P<0.001). In response to FED, both WP and LEAA equally stimulated MPS 0-2h (P<0.05), abating thereafter (0-4h; P>0.05). In contrast, after FED-EX, MPS increased 0-2h and remained elevated 0-4h (P<0.05) with both WP and LEAA. No anabolic signals quantifiably increased after FED, but p70S6K1^{Thr389} increased after FED-EX (2h; P<0.05). APS increased similarly after WP and LEAA. Older women remain subtly responsive to

nutrition ± exercise. Intriguingly though, bolus-WP offers no trophic advantage over LEAA.

3.2 CHAPTER INTRODUCTION

In the UK as well as the rest of the developed world, the numbers of aged are increasing, so much so that in the UK, there are separate departments in hospital looking after those who are aged 65 and above. Though these patients seek medical help for a variety of reasons, musculoskeletal (MSK) problems form a significant proportion of these reasons. Under this umbrella term, most of the problems are either directly or indirectly related to sarcopenia, defined as age-associated loss of muscle mass. It is important to note that there established links between low muscle mass and all-cause mortality *per se* (Arango-Lopera *et al.*, 2013) but also, lower skeletal muscle mass associated with sarcopenia leads to increased frailty, risk of falls, sedentarism, poorer quality-of-life and prevalence of metabolic co-morbidities (Fielding *et al.*, 2011; Srikanthan, Hevener and Karlamangla, 2016).

There are many factors affecting muscle mass in humans however the 'modifiable' ones include a physical activity and a balanced diet.

For example, lack of physical activity has been shown to cause muscle loss by making it less responsive to EAAs (Glover *et al.*, 2008; Breen *et al.*, 2013). Conversely physical activity/ exercise has been shown (in numerous studies) to potentiate the trophic effects of nutrition i.e. anabolic

responses are greater when exercise and nutrient-intake are combined to facilitate adaptation and repair (Atherton and Smith, 2012). Thus, investigations into the effects of nutrition and the combination of nutrition and exercise are key to determining the [dis]-regulation of muscle protein metabolism in older age.

With regards to nutrition, oral consumption of proteins based foods (especially those containing plenty of essential amino acids or EAAs) stimulate muscle growth in young men, measured in form of muscle protein synthesis (MPS) (Philip J Atherton *et al.*, 2010), which though lasts for 2-3 hours. This brief increase in MPS above fasting rates serves not only to replace muscle mass lost during starvation but also replenish amino-acid stores in human body. Though the anabolic effects of EEAs on human skeletal muscle are not new, more recently, it has been shown that one of the EAAs, Leucine (LEU), is the main driver of MPS (Sancak *et al.*, 2008; Han *et al.*, 2012). It has been shown that LEU, even in absences of other AAs, robustly stimulates MPS (Wilkinson *et al.*, 2013). This suggests the possibility that smaller quantities of EAAs (enriched with LEU) may provide anabolic responses comparable to bolus doses of whey protein (WP). This would have the advantage of having less calories, and thus be less satiating.

However, most of these studies have been conducted in men, and therefore women, especially older women are grossly under-represented. There are significant differences in MPS between older men and older women (Smith *et al.*, 2012). Hence, one cannot extrapolate the results of studies conducted in older men to older women.

Hence there is a need for studies to determine effects of "traditional" bolus WP and compare it with low dose leucine enriched EAA supplement, in both resting conditions as well as exercise. Therefore, this study determines the effects of two distinct nutritional interventions under rested conditions and in tandem with exercise in older women. Adopting a unilateral acute resistance exercise (RE) protocol allowed us to quantify the effect of: i) low-dose leucine enriched (LEAA, 3 g EAA [40% LEU]) nutrition or, ii) a large whey protein bolus (20 g), at rest and after an acute bout of RE in older women.

3.3 SUBJECTS AND METHODS

This project involved healthy older women (N=8/group, 66±2.5y, BMI 29±1.2 [means ± SEM]) who were recruited as discussed in the previous chapter (see chapter 2 please). As the study involved an exercise session at 75% of their 1-RM, they also underwent an exercise assessment to determine their 1-RM (as mentioned in chapter 2). The subject characteristics are mentioned in table 3.1. The composition of the two different supplements is shown in table 3.2.

	LEAA (3 g)	WP (20 g)
Age, yr	66 ± 1	66 ± 1
BMI, kg/m^2	29 ± 1	29 ± 2
LBM, kg	41 ± 2	40 ± 2
EAA dose, g/kg LBM	0.075 ± 0.003	0.25 ± 0.01*
Appendicular lean mass, kg	17 ± 1	17 ± 1
SMI	25 ± 1	26 ± 2

Table 3.1: Shows the baseline characteristics of subjects in 2 groups.

	LEAA (3 g), g	WP (20 g), g
L-Leucine	1.2	2
L-Isoleucine	0.32	1.4
L-Valine	0.33	1.2
L-Threonine	0.28	1.4
L-Lysine	0.5	1.8
L-Methionine	0.1	0.4
L-Histidine	0.05	0.4
L-Phenylalanine	0.2	0.6
L-Tryptophan	0.02	0.4

Table 3.2: Shows the composition of both Whey Protein- WP (20g) and Leucine Enriched Amino-Acid (LEAA) supplement LEAA (3g).

3.4 RESULTS

3.4.1 EAA-consumption per lean mass

Groups were matched for appendicular lean mass (LEAA: 17±1 vs. WP 17±1 kg) and skeletal muscle index (LEAA: 25±1 vs. WP 26±2) but received markedly different (P<0.01) quantities of EAA i.e. LEAA: 0.075±0.003 vs. WP 0.25±0.01 g/kg lean body mass.

3.4.2: Plasma AA and insulin concentrations (Figure 3.1)

Plasma amino acidaemia occurred rapidly in response to LEAA and WP and peaked at ~40 min (Figure 2.1-A-E; P<0.05). Plasma availability of AA, EAA, BCAA and LEU was significantly greater and more sustained in response to WP than LEAA (i.e. returning to baseline concentrations 60-80 min after LEAA vs. 115-160 min after WP; P<0.001).

Plasma insulin concentrations increased over post-absorptive values (both groups ~5 $mU \cdot l^{-1}$) by 20 min both in response to LEAA and WP (to 14±3 vs. 20±3 $mU \cdot l^{-1}$, respectively; P<0.05). However, while plasma insulin returned to basal concentrations 40 min after LEAA intake, (Figure 2.1-F), WP led to sustained insulinemia up to [and beyond] 60 min (18±3 $mU \cdot l^{-1}$).

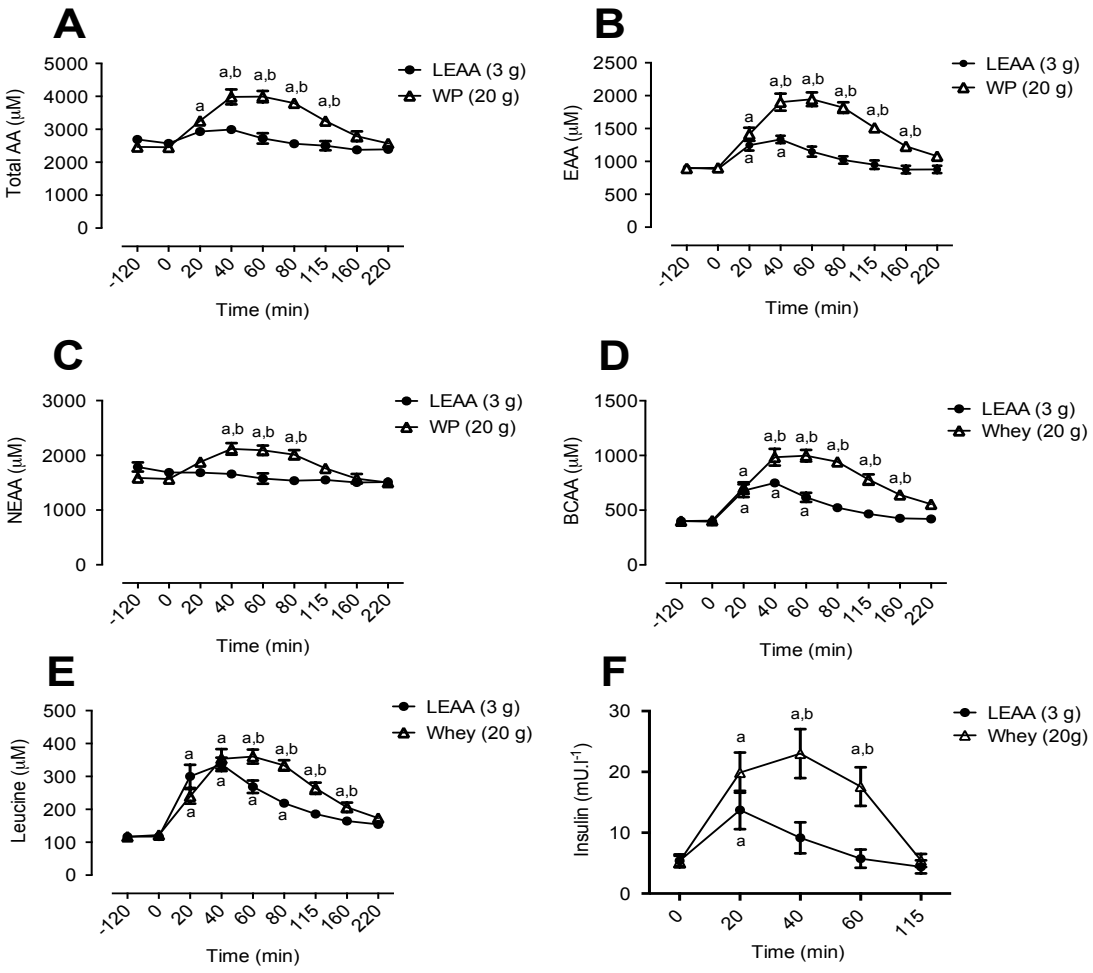


Figure 3.1 shows the concentrations of total amino acids or AA (3.1A), Essential AA (3.1B), non-essential AA (3.1C), Branched chain AA (3.1D), Leucine (3.1E) and Insulin (3.1F)

3.4.3: Effects of WP and LEAA on APS and MPS (Figures 3.2 & 3.3)

Increases in plasma APS were observed in response to WP while only a trend existed for LEAA 0-2h (LEAA BASAL-FED 0.19±0.02 to

0.23±0.03%·h^{-1}; WP BASAL-FED 0.17±0.01 to 0.26±0.04%·h^{-1}; *P*<0.1 and *P*<0.05 respectively, Figure 3.2). APS remained elevated in both groups 0-4h (LEAA BASAL-FED 0.19±0.02 to 0.25±0.02%·h^{-1}; WP BASAL-FED 0.17±0.01 to 0.29±0.02%·h^{-1}; *P*<0.05). In response to WP and LEAA, MPS was increased 0-2h (LEAA BASAL-FED 0.071±0.004 to 0.089±0.008%·h^{-1}; WP BASAL-FED 0.066±0.006 to 0.082±0.009%·h^{-1}; *P*<0.05); this anabolic effect abated rapidly (0-4h; *P*>0.05, Figure 3.3-A). In the exercised leg, MPS also increased 0-2h (LEAA BASAL-FED 0.071±0.004 to 0.085±0.014%·h^{-1}; WP BASAL-FED 0.066±0.006 to 0.095±0.013%·h^{-1}; *P*<0.05), but unlike with FED, MPS remained elevated over the entire 4h study period (LEAA BASAL-FED 0.071±0.004 to 0.089±0.005%·h^{-1}; WP BASAL-FED 0.066±0.006, 0.093±0.007%·h^{-1}; *P*<0.05, Figure 3.3B). Increases in MPS in response to both FED and FED-EX were indistinguishable between WP and LEAA groups (*P*>0.05).

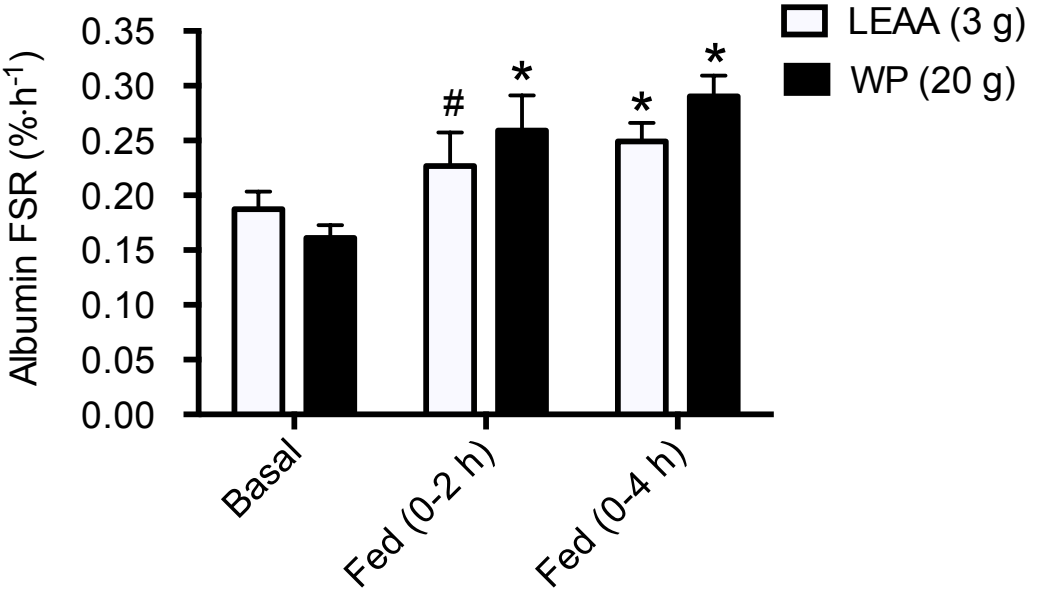


FIGURE 3.2: Albumin Protein Synthesis rates among the 2 groups at 0, 2 and 4 hours (#:P<0.1, *: P<0.05)

FIGURE 3.3:

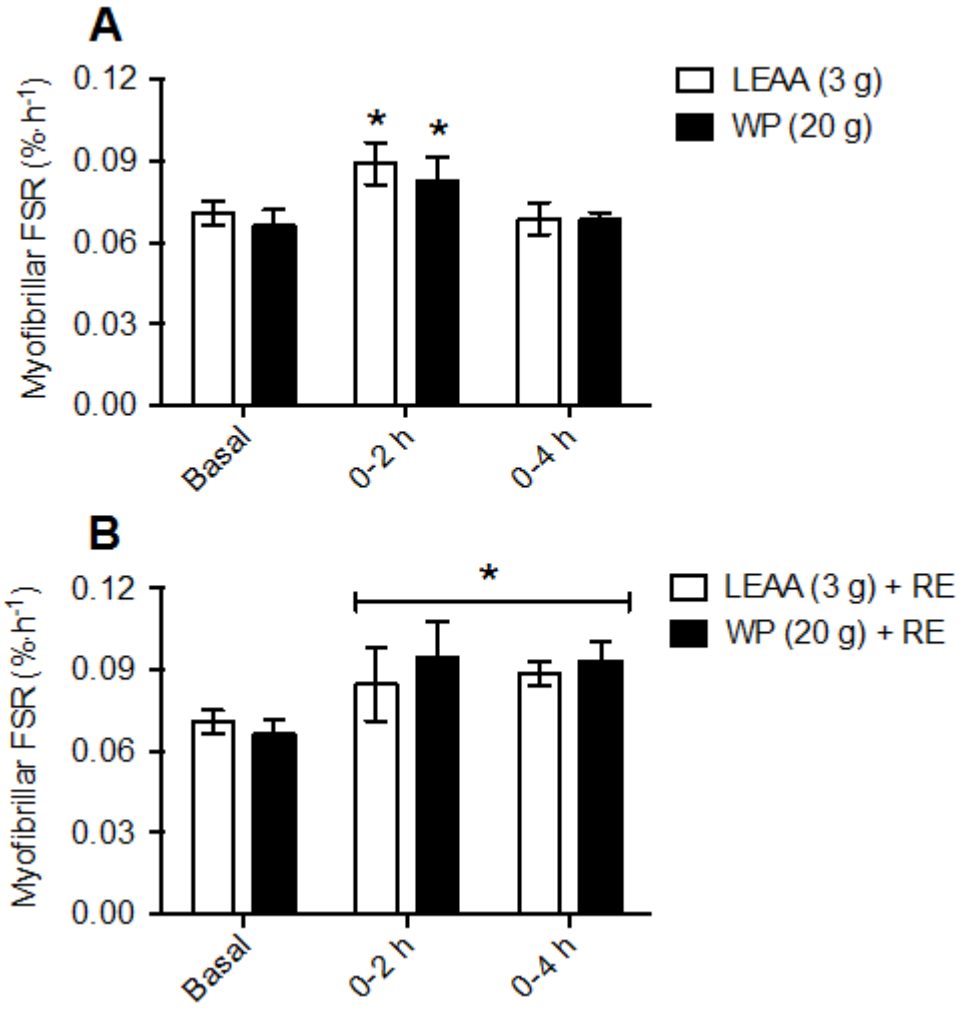


Figure 3.3 above shows myofibrillar Fractional Synthetic Rate (FSR) in both LEAA 3g vs. Whey protein 20g groups-

FED data: 3.3A FED-Ex data: 3.3B

3.4.4: Effects on muscle anabolic signalling (Figure 3.4)

Muscle anabolic signals as determined by measuring the phosphorylation of protein kinase B (AKT), the mechanistic target of rapamycin complex 1 (mTORc1) translational initiation substrates: ribosomal protein S6 kinase (p70S6K1) and 4E-binding protein 1 (4EBP1) and the elongation factor eukaryotic initiation factor 2 (eEF2) (Figure 2.6 A-F) were not detectably increased with FED. The FED-EX group exhibited significant increases (Figure 2.6D: +133±22%; $P<0.01$) in phospho-p70S6K1 but only after WP and not LEAA.

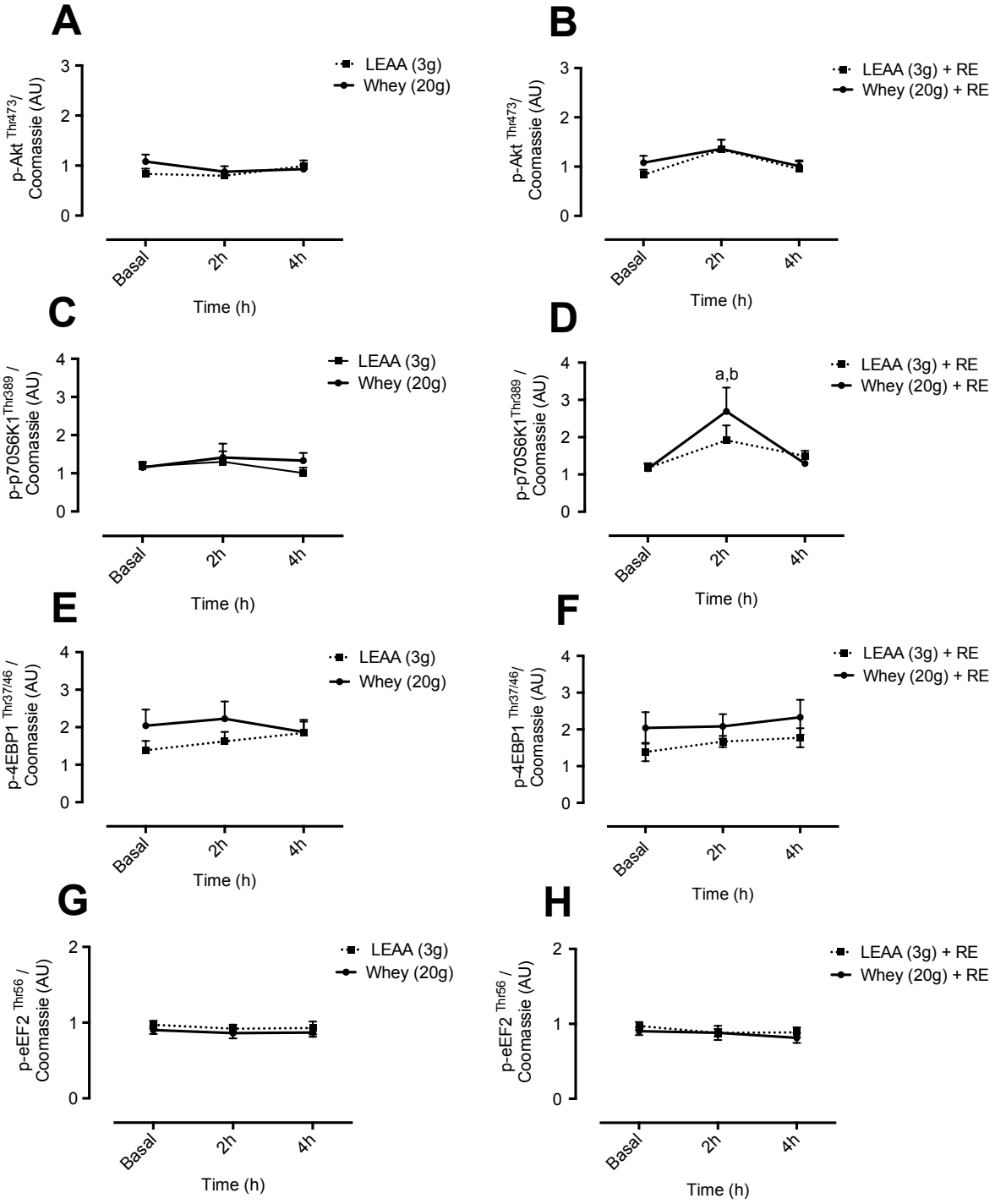


Figure 3.4 (A-H) above shows various anabolic signals in both LEAA and WP groups.

3.5: DISCUSSION

This study determines the effects of two different supplements- namely whey protein (WP- 20g) and leucine enriched essential amino acid supplement (LEAA- 3g) both at rest and with exercise, in healthy older women. The key findings are:

1) At rest, both supplements stimulate MPS, though the effect is transient (≤2 hrs).

2) With resistance type exercise (RE), the MPS continues beyond 4 hours with consumption of either supplement.

3) Albumin protein synthesis (APS) is stimulated similarly with both supplements.

It is shown here that increasing the quantity of EAA consumed does not augment MPS over and above ingesting ~1.2 g LEU (*plus* 1.8 g mixed-EAA) in older women. Therefore, providing greater quantities of AA as meals or supplements to older women is unlikely to bolster muscle maintenance beyond e.g. LEAA. Nonetheless, since anabolic resistance may be *overcome* by providing larger doses of WP to older men (~40 g) (Pennings *et al.*, 2012), it remains plausible that neither 20 g WP nor 3 g LEAA maximally stimulated MPS in skeletal muscles of older women; hence further studies with higher doses of WP as well as LEAA would help to confirm this.

While the stimulation of MPS by EAA has been long defined, it is becoming increasingly clear that protein or AA dose-response studies fail to identify

the importance of certain *highly* anabolic EAA. For example, LEU is a major driver of MPS (Wilkinson *et al.*, 2013) to the extent that LEU enrichment of 'sub-optimal' doses of protein or EAA robustly stimulates MPS (Churchward-Venne, Breen, *et al.*, 2014) as does provision of just ~3 g of LEU to ~70 kg younger men, entirely in the absence of other exogenous EAA (Wilkinson *et al.*, 2013). This study extends these findings by demonstrating that LEAA stimulates MPS in older women with equal efficacy to a large (20 g) bolus of WP, despite the latter providing >3 times more EAA per kg of LBM. Arguably, LEU was instrumental in this response given its unique potent anabolic properties (Casperson *et al.*, 2012; Churchward-Venne *et al.*, 2012; Wilkinson *et al.*, 2013). On this basis, research is needed to define the effects of LEU in the context of AA requirements for muscle health in old age (Verhoeven *et al.*, 2009; Dillon *et al.*, 2011; Rondanelli *et al.*, 2011; Symons *et al.*, 2011; Casperson *et al.*, 2012; Chalé *et al.*, 2013; Fukagawa, 2013).

Physical activity remains the best-known countermeasure against sarcopenia, either in the form of RE (Ivey *et al.*, 2000; Parise and Yarasheski, 2000; Häkkinen *et al.*, 2001; Kumar *et al.*, 2009; Churchward-Venne, Burd and Phillips, 2012) or even maintaining habitual movement (Timmerman *et al.*, 2012). However, ageing is associated with impaired anabolic responses to exercise (as it is for nutrition) meaning it is essential to study how exercise and nutrition can be best combined to yield maximal MPS in old age. For example, increases in MPS in response to exercise (Kumar *et al.*, 2009, 2012; Fry *et al.*, 2011) and the combination of exercise and nutrition (Fry *et al.*, 2011) are blunted in older men, as is

muscle hypertrophy in both men (Kosek *et al.*, 2006) and women (Greig *et al.*, 2011). In the present study, the data demonstrates that older women exhibit prolonged MPS responses to FED-EX (vs. FED) and that responses are equal between LEAA and WP. Therefore, intake of low doses of EAA enriched with LEU robustly stimulates MPS following exercise in older women, similar to what has been shown in younger men at rest (Churchward-Venne, Breen, *et al.*, 2014) and after exercise (Churchward-Venne *et al.*, 2012). It is noteworthy in the latter study that WP bolus, but not LEAA led to *sustainment* of the anabolic effects of the nutrient/ exercise combination. In contrast, the data showed similarly prolonged anabolic responses to FED-EX after both LEAA and WP. The lack of difference in MPS between WP and LEAA reflects blunted hypertrophy in older vs. young individuals (Welle, Totterman and Thornton, 1996; Martel *et al.*, 2006; Greig *et al.*, 2011) i.e. manifesting as diminished uptake of excess AA substrate into muscle proteins. This is also substantiated by the lack of robust increases in anabolic signalling (as previously reported in older men (Kumar *et al.*, 2012) and women (Smith *et al.*, 2008)) and leg/ muscle blood flow, facets which may underlie the moderate anabolic response to raised AA availability after FED and FED-EX in older women. Nonetheless, superimposed onto a metabolic background of prior RE, our observed increases in MBF with FED-EX may facilitate sustaining anabolic responses beyond >2 h.

An important discussion point is to what extent, and under what conditions, such WP or LEAA supplements yield efficacy e.g. on muscle mass/ function. Since protein supplements have significant effects to

positively influence muscle hypertrophy in response to RET (Hulmi, Lockwood and Stout, 2010; Yang *et al.*, 2012), it can be argued that low dose LEAA would be a similarly effective supplement (at least for older women) without the need for more satiating higher protein doses that may act as meal replacements, perhaps negatively impacting overall energy/ protein intake. Moreover, most studies focus on the effects of supplements on muscle *gains*; however, given the incipient nature of sarcopenia, longer-term studies are needed to determine the efficacy of supplements in offsetting *losses* in muscle mass/ function. Though robust trials are still awaited, one small study has indeed shown promise (Kim *et al.*, 2012), concluding that exercise with amino-acid supplementation was associated with increased muscle mass and strength compared to either AA supplementation only or exercise only groups in elderly sarcopenic women.. Additionally, while caution should be applied in terms of the extrapolation of one-off metabolic studies to the chronic scenario, one can argue that these acute metabolic readouts of protein anabolism form a solid platform for identifying candidate interventions for testing in longer-term supplement studies.

Potential study limitations warrant a comment here. Firstly, the data is unable to define the specific importance of LEU in mediating the efficacy of LEAA. Previous work by one of the current authors using I.V flooding dose techniques revealed anabolic effects of other EAA in our LEAA-feed (i.e. valine, phenylalanine) in man (Smith *et al.*, 1998). Nonetheless, given potent mTORc1 signalling actions attributed to LEU (Philip J. Atherton *et al.*, 2010; Gran and Cameron-Smith, 2011; Haegens *et al.*, 2012) it is

speculated from this data LEU was central. This has also been demonstrated in other studies, looking at effects of LEU alone on MPS (Wilkinson *et al.*, 2013). Secondly, the study protocol neither measured muscle protein breakdown nor whole-body protein turnover i.e. age-related differences in splanchnic metabolism (Moreau, Walrand and Boirie, 2013) and insulin handling may also impact muscle and whole-body responses to feeding/exercise. To conclude, our findings place low dose leucine enriched EAA-supplements at the fore of nutritional/exercise-interventions in older women.

CHAPTER 4: 1.5G LEAA VS. 6G LEAA VS. 40G WHEY PROTEIN

4.1 CHAPTER SYNOPSIS

This study determines the anabolic response to lower dose (1.5 g) LEAA as well as higher dose (6g) LEAA and compare that to 40g of bolus whey protein.

All studies were performed on 3 groups of older women (64±0.7 y N=24, 8/group): bolus-whey protein (WP-40 g) or novel leucine-enriched essential amino acids (EAA [40% leucine]) in two doses- 1.5 g and 6 g. Using $^{13}C_6$-Phenylalanine infusions, muscle protein synthesis (MPS) and albumin protein synthesis (APS) at baseline (i.e. fasted state) and both in response to feeding (FED) and feeding-plus-exercise (FED-EX; RE: 6×8 knee-extensions at 75%-1RM) were quantified. Plasma insulin/AA concentrations, whole-leg (LBF)/muscle microvascular blood-flow (MBF), and muscle anabolic signaling by phospho-immunoblotting were also measured. Blood flow results are presented in chapter-5. Plasma insulinemia and EAA-aemia were markedly greater after WP than LEAA (P<0.001). In response to FED, both WP and LEAA equally stimulated MPS 0-2h (P<0.05), abating thereafter (0-4h; P>0.05). In contrast, after FED-EX, MPS increased 0-2h and remained elevated 0-4h (P<0.05) with both WP and LEAA. No anabolic signals quantifiably increased after FED, but p70S6K1^{Thr389} increased after FED-EX (2h; P<0.05). APS increased similarly after WP and LEAA. Older women remain subtly responsive to nutrition $\pm$ exercise. Intriguingly though, bolus-WP offers no trophic advantage over LEAA.

4.2 CHAPTER INTRODUCTION

In the previous chapter, the discussion focussed on how protein intake as well as exercise contributed to maintaining muscle mass by increasing muscle protein synthesis (MPS). The role of leucine (LEU) as main driver of MPS was also discussed. The previous study explored and compared the anabolic responses from two different protein supplements (WP 20g vs. LEAA 3g) and determined that they were both equally effective as anabolic stimuli.

4.2.1: Rationale for different dose regimes

There are several reasons why it is important to study anabolic responses to higher dose whey protein (WP in dose of 40g), along with higher and lower doses of LEAA (1.5g and 6g)- these reasons are discussed below:

- Previous studies in men however have also shown that where they can consume higher quantities of whey protein e.g. 40g, it may confer additional benefits (Yang *et al.*, 2012), especially when combined with resistance training. However, these studies have been done in men and no studies have been done in older women so far. It is possible that none of the supplements studied maximally stimulated the MPS in older women.
- Many older people, however, are unable to consume larger doses of proteins. This may happen due to various reasons e.g. functional

decline, social isolation, depression, reduced appetite (due to diseases such as cancers or medications), and problems with swallowing (Ahmed, 2010). This means that many may find it hard to take higher doses of whey proteins or LEAA supplements.

- In addition to the point above, the lowest quantity of LEAA (1.5g) may fit in a capsule or a tablet, unlike any other supplements that have been studied. This may make this easier to take for elderly population.
- Finally, in small doses, they may not add to additional caloric burden so much so that they suppress appetite, which may be desirable in certain patient populations.

Hence, a second set of studies were conducted where the aim was:

- To evaluate the anabolic response to lower as well as higher dose of LEAA (lower dose at 1.5g and higher at 6 g, both with 40% LEU) and
- Compare that to the anabolic response to whey protein 40g.

4.3 SUBJECTS AND METHODS

This project involved 24 healthy older women (N=8/group, 64±0.7 y, BMI 29±1.2 [means ± SEM]) who were recruited as discussed in the previous chapter (please see table 4.1 for demographics).

As the study involved an exercise session at 75% of their 1-RM, they also underwent an exercise assessment to determine their 1-RM (as mentioned

in chapter 2). All subjects gave their written, informed consent to participate after all procedures and risks were explained.

	LEAA_1.5	LEAA_6	WP
Age	65 ± 1 y	63 ± 1 y	66 ± 1 y
Height (m)	1.62 ± 0.02	1.64 ± 0.02	1.60 ± 0.02
Weight (kg)	65.4 ± 3.5	68.4 ± 5.4	65.0 ± 4.6
BMI (kg m^{-2})	25.1 ± 1.3	25.1 ± 1.3	25.0 ± 1.5
SMI	6.32 ± 0.11	6.18 ± 0.13	6.13 ± 0.35

FIGURE 4.1: Demographic data for participants in this study

Following screening subjects were randomly assigned, on the day of the acute study, to one of three groups receiving either:

1. 40g Whey Protein (WP, N=8),
2. 1.5g Leucine enriched essential amino acids (LEAA_1.5, N=8) or
3. 6g LEAA (LEAA_6, N=8).

The amino acid composition of each supplement is given in table 4.2. The rest of the details of recruitment, study day (including exercise and biopsies) and sample analyses can be found in chapter 2 (Materials and methods).

Amino-acids	LEAA (1.5g), g	LEAA (6g), g	WP (40g), g
L- Leucine	0.6	2.4	4
L-Isoleucine	0.16	0.64	2.8
L-Valine	0.165	0.66	2.4
L-Threonine	0.14	0.56	2.8
L-Lysine	0.25	1.0	3.6
L-Methionine	0.05	0.2	0.8
L-Histidine	0.025	0.1	0.8
L-Phenylalanine	0.1	0.4	1.2
L-Tryptophan	0.01	0.04	0.8

Table 4.2: Amino acid composition for the supplements used in this study

4.4 RESULTS

4.4.1: Plasma AA concentrations (Figure 4.1-4.4)

Plasma EAA, BCAA and leucine concentrations increased rapidly in both the 6g LEAA and 40g WP groups peaking at ~40 – 60 mins. In the 1.5g LEAA group there was only a significant increase in plasma leucine concentrations (Fib 4.1), with BCAA and EAA showing no significant increase above baseline.

As expected WP showed greater plasma AA availability than both the LEAA groups with EAA, BCAA and leucine concentrations remaining significantly elevated above baseline up to 220mins (Fig 4.2-4.3 A&B), whereas concentrations in the LEAA groups returned to baseline within 100 mins.

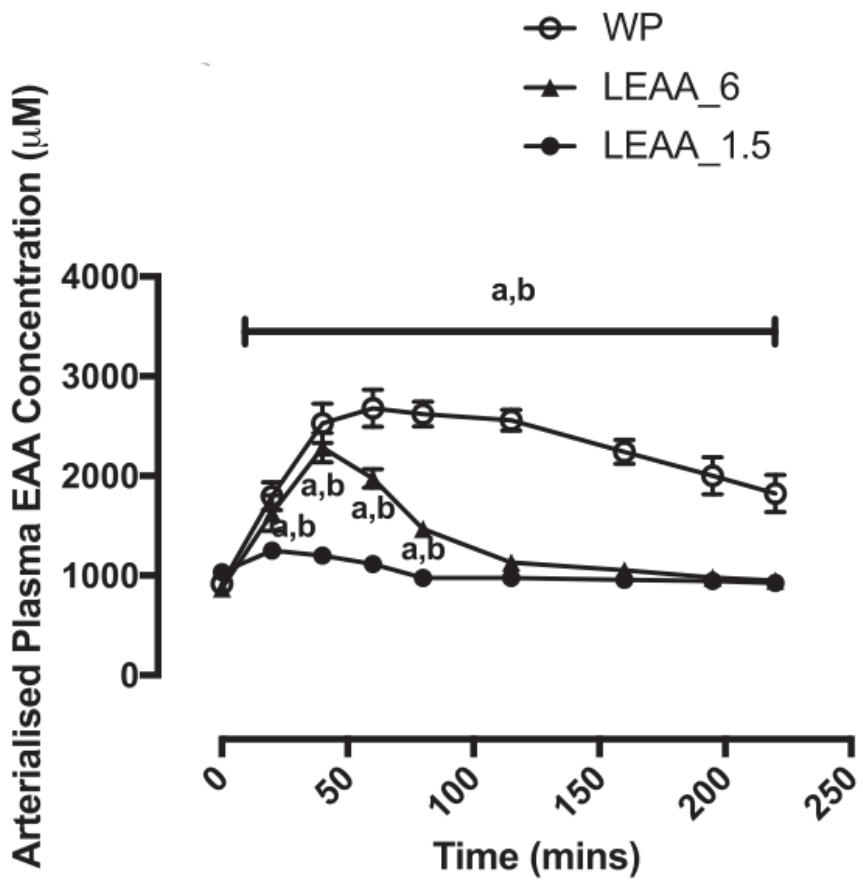


Fig 4.1- Arterialised Plasma EAA concentration

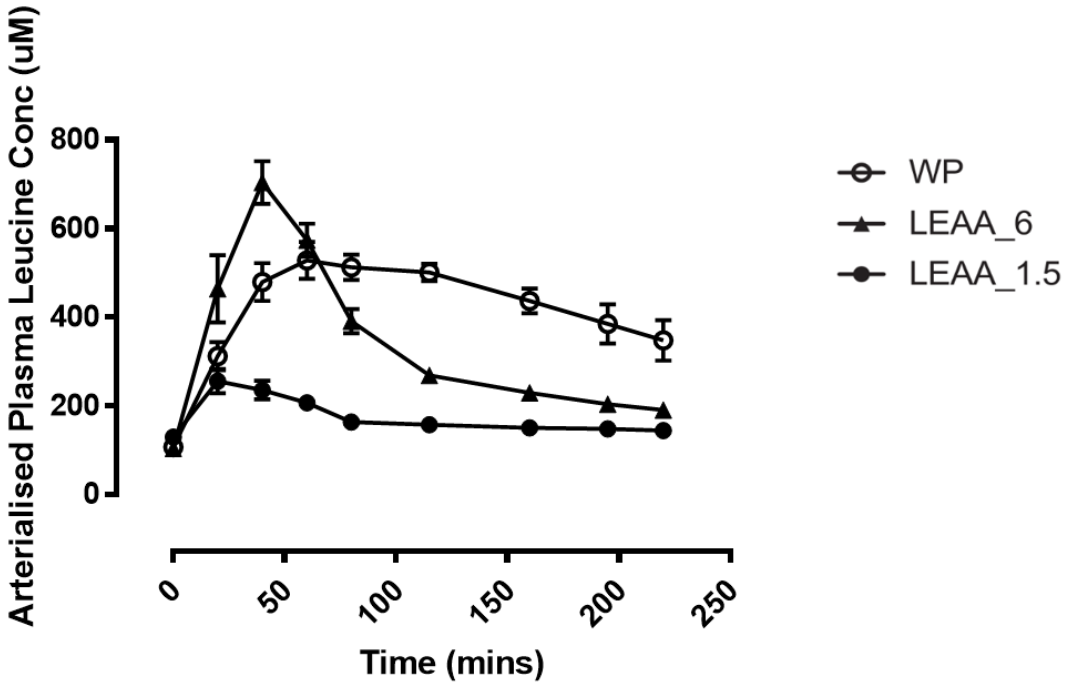


Fig 4.2- Arterialised Plasma LEU concentration

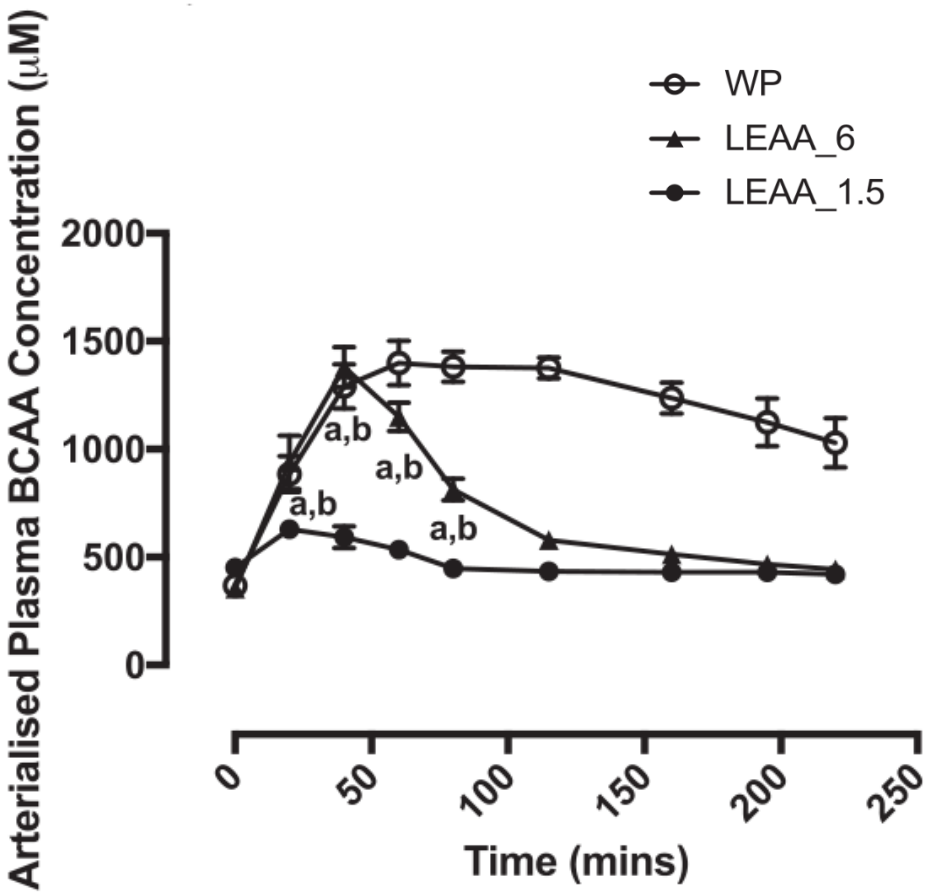


Fig 4.3- Arterialised BCAA concentration- WP 40g, LEAA 6 g and LEAA 1.5g

a: significantly different vs. basal (P < 0.05).

b: significantly different between groups (P < 0.05)

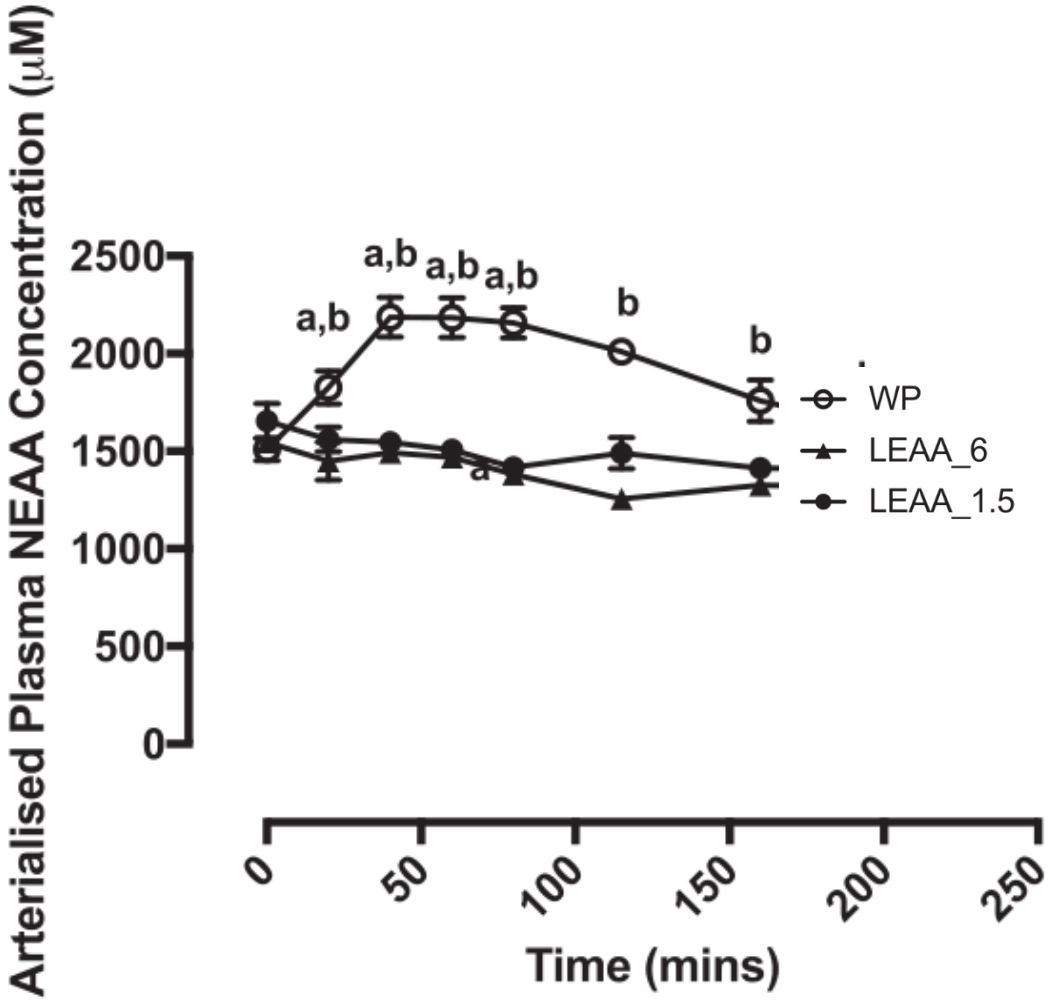


Fig 4.4 Arterialised Plasma NEAA concentration in different groups (WP 40g, LEAA 6g & LEAA 1.5g

a: significantly different vs. basal (P < 0.05).

b: significantly different between groups (P < 0.05)

4.4.2: Plasma insulin concentrations (Figure 4.5)

Plasma insulin concentration increased over post-absorptive values in all groups, peaking at 11.50±1.83 uU/mL, 12.76±1.61 uU/mL and 21.03±3.62 uU/mL, for LEAA 1.5g, LEAA 6g and WP 40g respectively (figure 4.5). However, while plasma insulin returned to near basal concentration 40 min after both 1.5g and 6g LEAA intake, insulin concentrations in response to 40g of WP were significantly elevated up to [and beyond] 60 min, with significantly higher insulin concentrations at 20, 40 and 60mins compared to the LEAA.

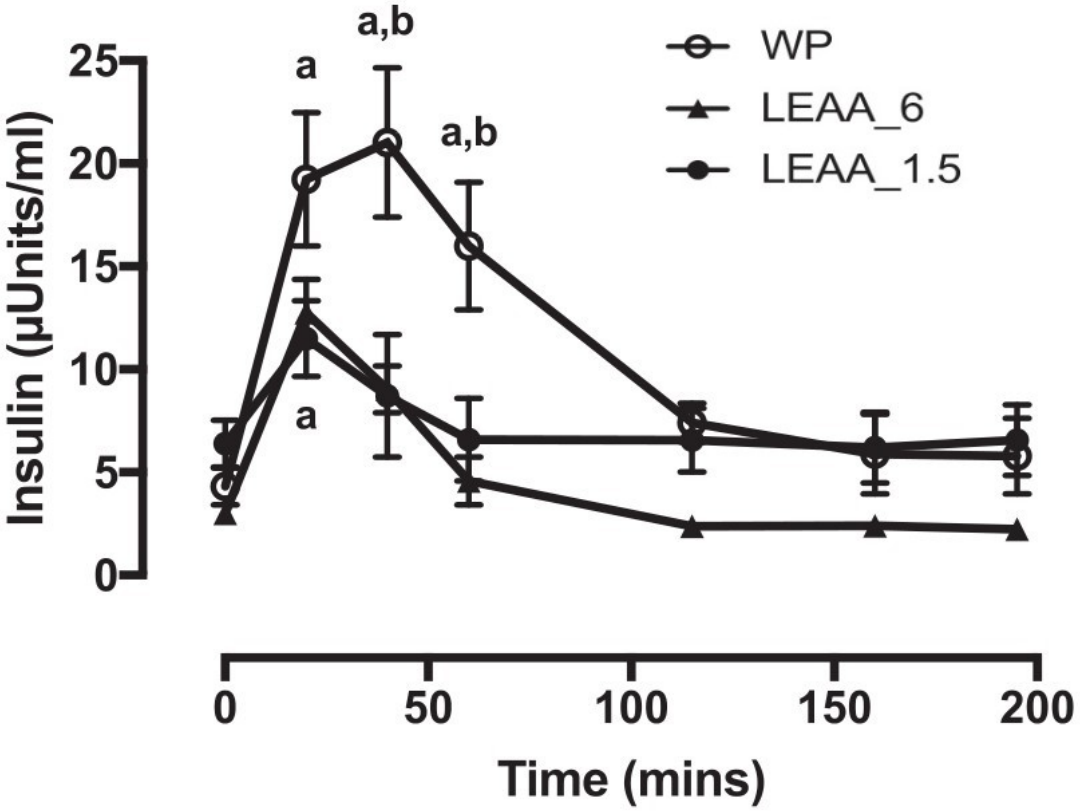


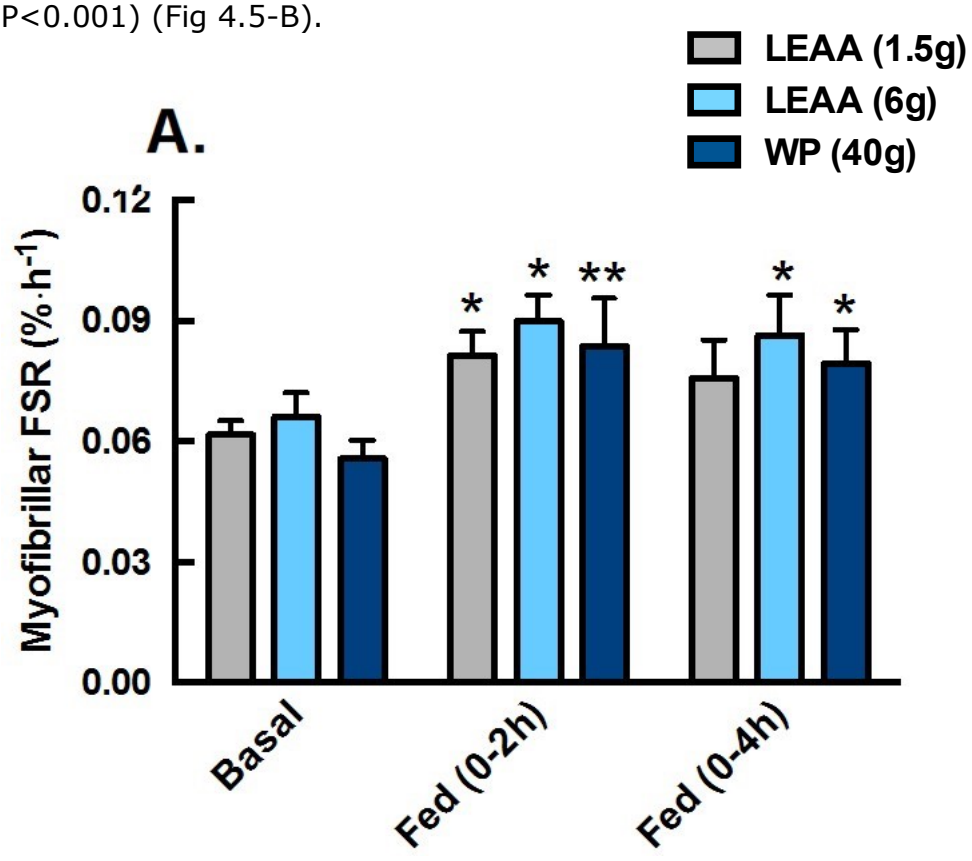
Fig 4.5 above shows insulin levels in response to LEAA 6 g, LEAA 1.5 g and WP 40g

a: significantly different vs. basal (P < 0.05)

b: significantly different between groups (P < 0.05)

4.4.3: Effects of WP and Dose Response LEAA on MPS (Figure 4.6 A & B)

In the FED leg MPS increased similarly in response to 1.5 g LEAA (0.061±0.003 vs. 0.086±0.005 %/h, P<0.05), 6 g LEAA (0.066±0.006 vs. 0.090±0.007 %/h, P<0.05) and WP (0.056±0.004 vs. 0.084±0.011 %/h, P<0.01) over 0-2 h, with MPS significantly higher than basal in the 6g LEAA and WP groups only over 0-4h (Fig 4.5-A). However, in the FED-Ex leg, MPS was significantly increased across all the groups from 0-4h (1.5 g, 0.061±0.003 vs. 0.091±0.007 %/h, P<0.01; 6 g, 0.066±0.006 vs. 0.097±0.007 %/h, P<0.01 and 40 g WP, 0.56±0.004 vs. 0.104±0.011 %/h, P<0.001) (Fig 4.5-B).



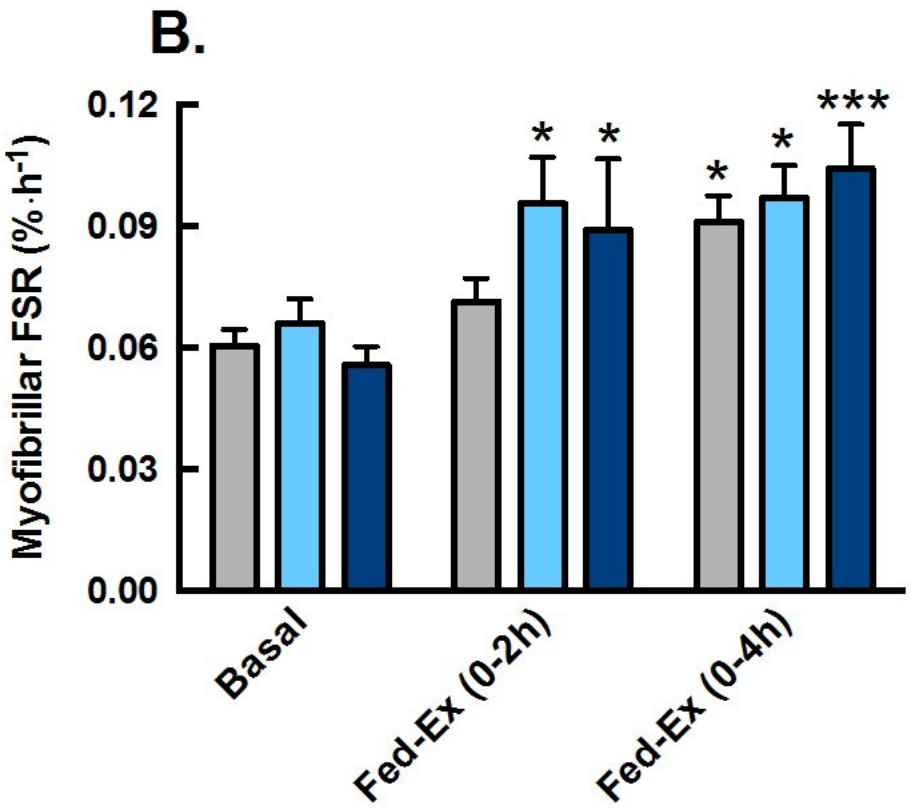


Figure 4.6 A&B: Myofibrillar fractional synthetic rate (FSR) in response to feeding (A.) and feeding-plus-exercise (B.) when given different feeding regimes. *=P<0.05 vs. basal.

4.4.4: Effect on mTORC & effectors

Anabolic signalling relating to the control of MPS was assessed by measuring the mechanistic target of rapamycin complex 1 (mTORC1) translational initiation substrates; p70S6K1 and 4E-BP1, and the elongation factor eEF2. In the FED state, only p-p70S6K1 was significantly elevated in the WP group at 2 h and 4 h, however, with FED-EX phosphorylation of p70S6K1 was elevated in both the LEAA_6 and WP

groups at 2 h, with phosphorylation remaining elevated at 4 h in the WP group only (Figure 4.7).

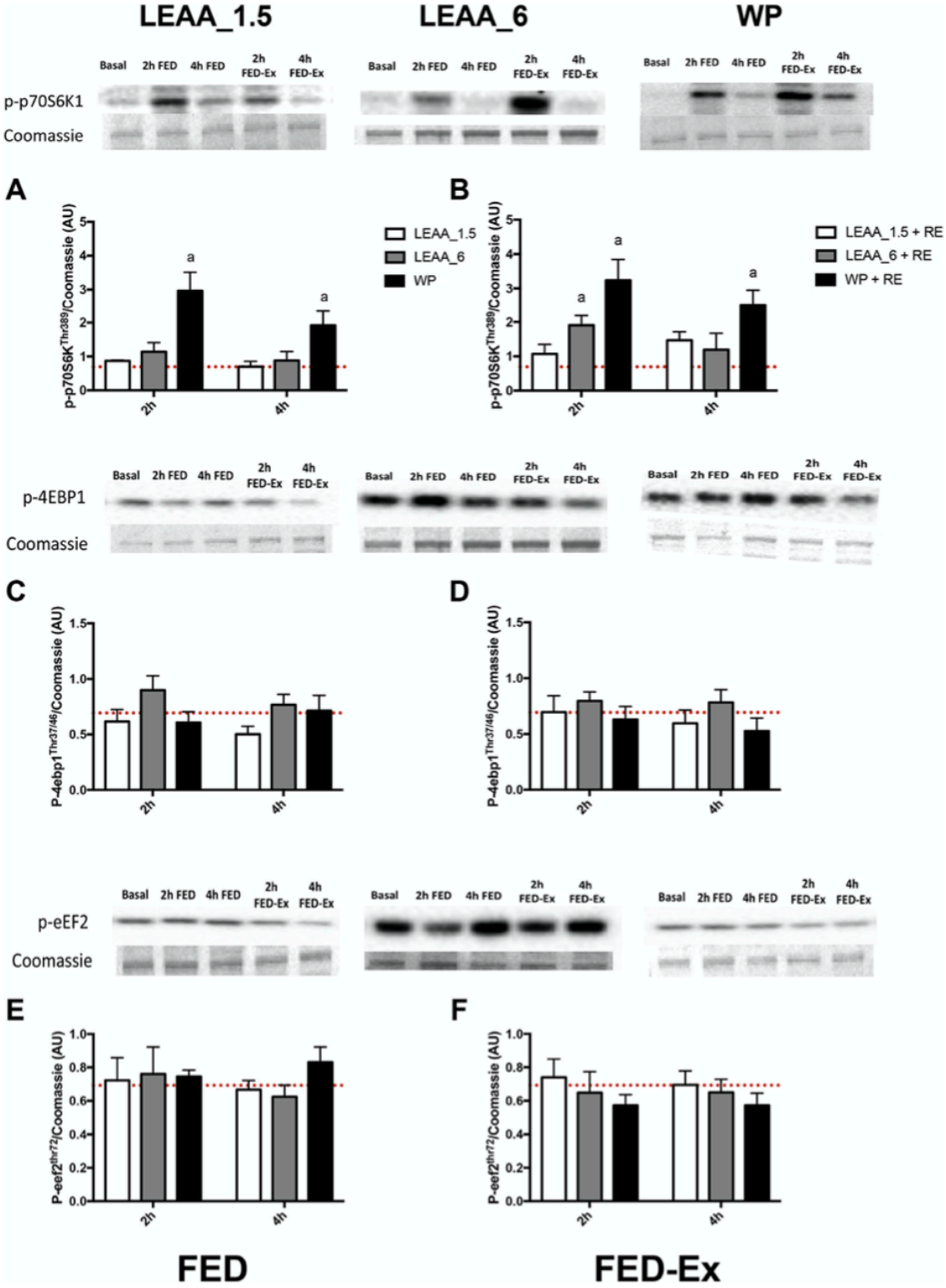


Figure 4.7 (A-F): The effects of 1.5 g, 6 g of LEAA or 40 g WP on muscle signalling responses to FED and FED-EX. Responses were log

transformed and normalized to the basal-fasted biopsy (represented by dotted line). a: significantly different vs. basal (P < 0.05).

4.5: DISCUSSION

Anabolic resistance has been studied well in recent years, e.g. compared to young men, MPS responses are blunted in older men in response to anabolic stimuli like diet; particularly EAAs (Cuthbertson *et al.*, 2005), exercise (Kumar *et al.*, 2009) as well as anti-catabolic effects on insulin (Wilkes *et al.*, 2009). The effects of anabolic resistance are seen as reduced skeletal hypertrophic response to resistance exercise (RE) in both men (Kosek *et al.*, 2006) and women (Greig *et al.*, 2011). Attempts have been made to understand the phenomenon at molecular level (Fry *et al.*, 2011) however more recent attempts are focused on increasing the effectiveness of exercise in form of RE (Kumar *et al.*, 2012) as well as diet, particularly increased doses of whey protein (in magnitude of 35-40g) have been shown to overcome anabolic resistance (Pennings *et al.*, 2012; Yang *et al.*, 2012). However, it isn't clear whether the effect seen was due to the increased dose of *all* amino acids or just EAAs (LEU in particular).

It has been known for over 25 years that EAAs are the primary driver to MPS responses. Recent studies have identified LEU as the key EAA which drives the MPS responses. Leucine supplementation in young males have been shown to increase MPS responses despite lower AA content (Churchward-Venne, Breen, *et al.*, 2014) and they have also been shown

to improve post-absorptive MPS rates when use chronically in older adults, despite maintaining lower protein intakes (Casperson *et al.*, 2012), however it did not result in increased muscle mass or strength when used for 3 months in older men (Verhoeven *et al.*, 2009). Similarly, for resistance exercise (RE), studies have shown increased MPS in older males when exercise intensity was doubled (Kumar *et al.*, 2012) However, none of these studies look combine the diet with RE and almost all were done in older men.

Previous data from our lab has shown that 3g LEAA stimulates MPS in skeletal muscle of older women to a similar extent to a 20g WP bolus (Bukhari *et al.*, 2015). However, it remained entirely plausible that neither regime maximally stimulated the MPS. The aim of this project was to experiment with higher dose of LEAA (6g), along with lower dose (1.5g LEAA) and compared these to WP 40g bolus. The research data showed that in FED state, all three supplements increased MPS over 2 hours however only LEAA 6g and WP 40g reached significance in 0-4 hours. In FED-EX all the three supplements increased MPS in 0-2 hours which remained elevated (and statistically significant) over 0-4 hours. Hence; a bona fide LEU/ EAA dose-response to MPS in older women could not be shown. In addition, WP in doses of 40g showed greater aminoacidemia (essential, non-essential and branched chain) which was expected as the supplement had greater amount of amino acids as well as greater plasma insulin response. It is to be noted that amount of leucine in whey protein (4g) was *greater* than either LEAA 6g (2.4g) and LEAA 1.5g (0.6g), though the proportion was lower (10% of total amino acid content in WP was

leucine, compared to 40% in LEAA). Hence there does not appear to be a bona fide dose-response to essential amino acid in form of MPS (with or without exercise) in older women. The importance of this data, being different from response seen in older men, re-confirms our initial suspicions that older women need to be studied separately as there are differences between anabolic responses to diet (in form of nutritional supplements) as well as resistance exercise when compared to men.

It has been proposed that the delivery profile of amino acids may influence the anabolic response to nutritional supplements and rapid absorbing proteins such as WP in larger boluses may help maximise MPS (Wilkinson *et al.*, 2007; West *et al.*, 2011). Here we see that MPS response was similar between WP 40g and LEAA 6g, despite showing a three-fold rapid peak in aminoacidemia (including leucine and BCAA) as well as longer aminoacidemia with WP 40g. This is consistent with similar data from our lab in which we showed that a bolus feeding of 15g of EAA (which provides rapid aminoacidemia) does not offer any additional anabolic response when compared to spread out/ pulsed EAA in similar total dose (Mitchell *et al.*, 2014). These data suggest that it is the composition of the feeding (and quantity of leucine) rather than the dose or feeding pattern being the primary driver of anabolic response.

Our present findings support the ever-growing body of evidence in the literature for the potent in vivo anabolic role of leucine (Bukhari *et al.*, 2015; Atherton *et al.*, 2017), with in vitro data showing that leucine provides the most robust stimulation of mTORc1 of all EAA (Philip J. Atherton *et al.*, 2010). In the present study the phosphorylation of

p70S6K1 was the only anabolic target increased in the FED or FED-EX state, with 40 g WP providing the most robust and sustained increase in phosphorylation, although 6 g LEAA also increased phosphorylation at 2 h in the FED-EX state. Despite this lack of increase in anabolic signalling, there was a robust stimulation of MPS for all groups. Discordance between MPS and anabolic signalling has been previously reported (Greenhaff *et al.*, 2008; Philip J Atherton *et al.*, 2010) again showing that anabolic signal measurements using standard immunoblotting approaches are not sensitive quantitative biomarkers of MPS in response to nutrition and exercise, and in this instance probably reflect between group differences in serum insulin concentrations. This, in addition to the observation of blunting of anabolic signalling pathways in older age (Guillet *et al.*, 2004; Cuthbertson *et al.*, 2005) may explain the present findings. Nonetheless, it is possible that peak signalling events may differ between such distinct feeding regimes such that the key points of stimulation of anabolic signalling in response to each feed may not have been captured by our biopsy timings – this is an inherent limitation of such studies.

How can such a low dose of EAA lead to such a robust stimulation of MPS and where do the EAA's required to support increased MPS derive, if not provided in the diet? The increase in MPS in the absence of exogenous EAA is not an unusual phenomenon over acute periods of measurement i.e. 90–120 min. Indeed, flooding dose studies, performed more than 20 years ago, showed clearly that it was possible to stimulate MPS acutely through the provision of a single bolus of EAA (~3 g Leu or Phe) without the addition of other EAA (Smith *et al.*, 1992, 1998). For this to occur, EAA must be

derived from other pools, i.e. plasma, intracellular or protein (via whole-body or tissue protein breakdown). We and others have demonstrated, that acutely, there is clearly sufficient EAA availability to sustain short-term increases in MPS. In these studies, we have shown decreases in other EAA i.e. intramuscular valine fell by 30% (Smith *et al.*, 1992), threonine by 20% (Smith *et al.*, 1998), and indeed upon provision of a low dose whey protein (6.25 g – equivalent to only 3 g EAA) supplement blood valine, isoleucine and total EAA concentrations drop (muscle levels were not measured), yet MPS is still stimulated (Churchward-Venne, Breen, *et al.*, 2014). Clearly these falls are the result of increased demand due to elevated MPS. Furthermore, a metabolite of leucine: β-hydroxy-β-methyl-butyrate (HMB: which provides no additional nutritional value), has been shown to stimulate MPS (whilst also suppressing MPB) acutely in young males despite providing no additional EAA (Wilkinson *et al.*, 2013). This acute stimulated MPS response has also been demonstrated during the provision of insulin (whereby MPB will also be significantly suppressed) in the absence of additional exogenous EAA (Timmerman *et al.*, 2010). Ergo, it is clear from the literature that in the short term, the absence of additional EAA, does not acutely limit MPS. This study was conducted acutely over 4 h, as part of a larger study investigating the dose response of MPS to EAA (enriched with leucine) as a potentially less satiating (and less calorific) alternative to consuming the larger quantities of protein suggested to be needed in older age to maximize MPS (Baum, Kim and Wolfe, 2016). Indeed, the findings from the present study support recent evidence for the positive benefits of similar low dose AA supplementation

(in conjunction with exercise) in supporting anabolism in older adults (Kim *et al.*, 2012).

4.6 CONCLUSION:

In summary, our present data expands upon the findings from our previous work that a leucine enriched low dose amino acid supplement provides acute robust (perhaps maximal) stimulation of MPS in older women, with our present data showing as little as 1.5 g of LEAA stimulates MPS to levels equivalent to 40 g WP (a dose known to maximally stimulate MPS in adult males) both at rest and after exercise. Based on these findings, it would be prudent to investigate the efficacy in larger cohorts to see if these acute responses to low dose LEAA are translatable and reproducible over more chronic supplementation periods.

CHAPTER 5: BLOOD FLOW STUDIES

5.1 CHAPTER SYNOPSIS

This chapter explores whether any of the supplements studied so far (LEAA at the doses of 1.5 g, 3 g and 6 g, and whey protein at doses of 20 g and 40 g) affect leg blood flow or muscle microvascular blood flow within the leg. In summary, the findings of this chapter report that none of the supplements altered either leg or muscle microvascular blood flow, exercise however did increase muscle microvascular blood flow.

5.2 CHAPTER INTRODUCTION

As discussed in previous chapters, the loss of muscle mass in sarcopenia is due to an imbalance in muscle protein synthesis (MPS) and muscle protein breakdown (MPB), with a negative net balance favouring muscle loss (Dalle, Rossmeislova & Koppo, 2017). Factors such as anabolic resistance (see chapter-1) may play a role in this, yet the underlying mechanisms behind anabolic resistance are not yet fully elucidated. Poor blood flow to limbs and consequently (perhaps) to the skeletal muscles may contribute to anabolic resistance via impaired delivery of several nutrients, in particular amino acids (Biolo *et al.*, 1997; Fujita *et al.*, 2006). Hence the potential importance of blood flow in the study of ageing skeletal muscles.

It is of note that this chapter will discusses *blood flow impairment* in the context of this being a long-term physiological maladaptation and is not to

be confused with *blood flow restriction* (acute, and instigated via physical vessel occlusion) as discussed in chapter 1.

5.2.1: Effect of ageing on blood flow

Blood flow to the limbs is reduced in older compared to younger men when assessed via Doppler ultrasound (Dinenno *et al.*, 1999) with this reduction associated with increased sympathetic vasoconstrictor activity. Muscle microvascular blood flow has also been shown to be reduced with advancing age (Socha and Segal, 2018), as have poor responses to anabolic stimuli such as amino acid feed (Mitchell *et al.*, 2013) and exercise (Hildebrandt *et al.*, 2017).

The histological assessment of young human skeletal muscle vs. older muscle may offer some explanation for this reduction, demonstrating that although the proportion of muscle fibre types remains the same with advancing age, the number of capillaries (as well as the mitochondrial activity) are approximately 25% lower in older individuals (Coggan *et al.*, 1992). In addition, advancing age is also associated with endothelial dysfunction which impairs vasodilatory responses to certain stimuli (Seals *et al.*, 2006), such as exercise (Donato *et al.*, 2006) and nutrition (Skilton, 2004). Although apparent in both males and females (Phillips *et al.*, 2017), these reductions in vascular function are reported by some to be most pronounced in females, particularly after menopause (Celermajer *et al.*, 1994), suggesting that there may be a hormonal basis to this impairment, and that blood flow as a component of 'the nutrient delivery system' may

be at least in part be responsible for anabolic resistance seen during old age.

5.2.2: Are these effects irreversible?

The next question that arises in relation to these age-associated reductions in vascular function (macro and microvascular) is whether these age-related declines are "fixed" or modifiable? A 2005 review article suggests that these age-related effects may be modifiable by both environmental and pharmacological interventions (Najjar, Scuteri and Lakatta, 2005). However, this review concludes with a section outlining the need for further investigation into strategies aimed at preventing or retarding arterial ageing. Although several attempts to use specific amino acids with vasodilatory properties have been made since the time of the aforementioned review (e.g. Arginine (Tang *et al.*, 2011) or Citrulline (Churchward-Venne *et al*>, 2014)), these approaches have not been successful so far. Contrary to these somewhat disappointing findings in relation to amino acids as a strategy to improve vascular function/ responsiveness, resistance exercise training (RET) has been shown to hold some promise. For example, a 2012 intervention study in young, middle-aged and older adults (males and females) showed that RET for 20-weeks is able to elicit improved leg blood flow (LBF) responses to mixed macro-nutrient nutrition in older adults, effectively restoring the vascular responsiveness of older adults (Phillips *et al.*, 2012). A follow-up study from the same research team subsequently showed that the same RET

regime also positively impacted muscle microvascular blood flow (MBF) in response to nutrition when measured in the vastus lateralis via contrast enhanced ultrasound (CEUS) (Phillips *et al.*, 2015). It must however be noted that in this latter study of MBF, the increases in MBF were not associated with concomitant increases in MPS, although this study was in males only and a control (no exercise) group were compared to those who had undertaken RET, and as such individuals did not act as their own controls.

5.3: STUDYING BLOOD FLOW IN MUSCLES

When assessing the impact of age and/ or any intervention on 'muscle blood flow' two distinct assessments can be made, these being blood flow TOWARDS the muscles, and blood flow THROUGH the muscles.

The blood flowing through major blood vessels (e.g. femoral artery) into a limb and TOWARDS the muscle is referred to in this chapter as macrovascular or leg blood flow (LBF) and is assessed via Doppler ultrasound. The blood flow THROUGH the microvasculature of muscle (e.g. *the m. vastus lateralis*) is termed microvascular blood flow or MBF and is assessed via contrast-enhanced ultrasound (CEUS). This microvascular blood flow is further sub-divided into nutritive blood flow (i.e. blood flow through the sarcolemmal AA transporters) and non-nutritive blood flow (mainly through the adipose and connective tissues, resulting in "marbling" of the skeletal muscle (Clark *et al.*, 2000)). These have been illustrated in the figure below:

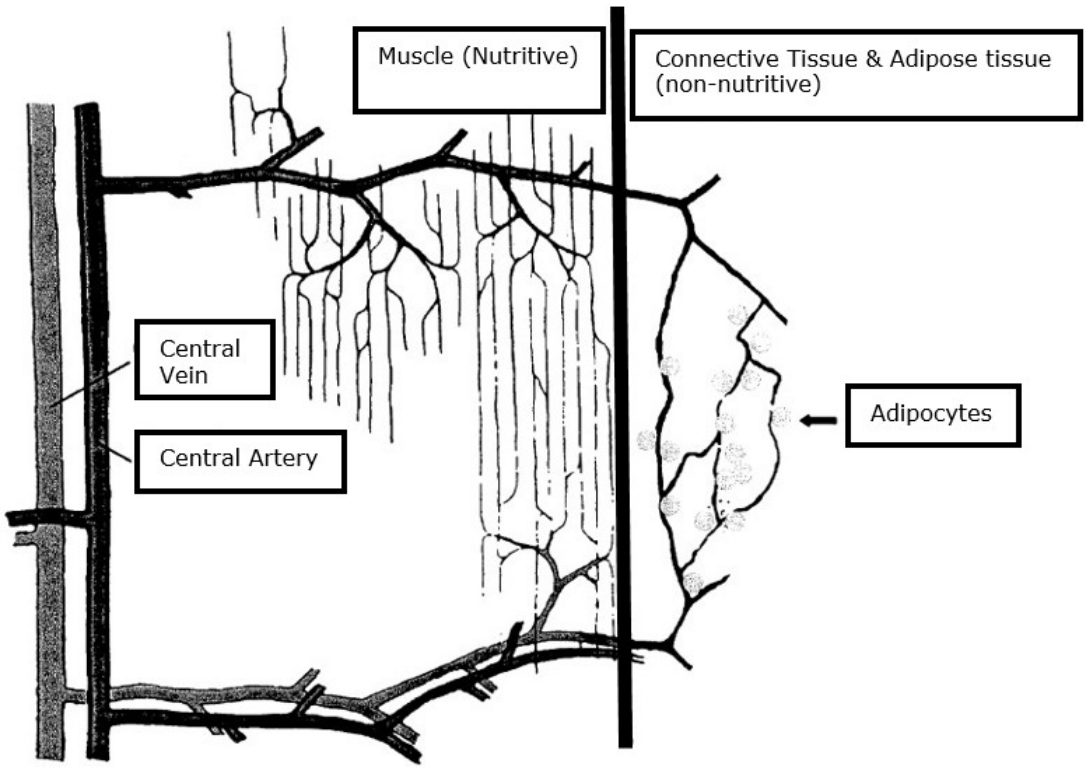


Figure 5.1: A diagrammatic representation of the concept of nutritive vs. non-nutritive microvasculature blood flows through skeletal muscle (adapted from Clarke et al., 2000)

Nutritive blood flow has been a major area of research interest in relatively recent years as this blood flow is highly responsive to essential amino acid (EAA) intake as well as exercise (Mitchell *et al.*, 2016), in an insulin dependent as well as insulin independent manner. As mentioned above, Contrast enhanced ultrasound (CEUS) allows measurement of muscle microvascular blood volume indicating capillary recruitment, as well as microvascular flow velocity signalling proximal arteriolar dilation (Mitchell *et al.*, 2016).

Both of these techniques (Doppler ultrasound for LBF and CEUS for MBF) are discussed in more detail below.

5.3.1: Macrovascular Blood Flow Measurements

Macrovascular blood flow looks at measuring arterial blood flow through a major blood vessel (in our case, femoral artery). There are many techniques that can be used with an overview below.

5.3.1.1: Introduction- Techniques to assess macrovascular blood flow

There are a number of ways to measure blood flow through major blood vessels, that are broadly divided into:

1. Non-invasive methods such as strain gauge plethysmography (Mortensson, Hallböök and Lundström, 1975), Doppler ultrasound (discussed in detail below) and Magnetic resonance velocity imaging (Rebergen *et al.*, 1993) to
2. Invasive methods such as dye dilution tests (Rådegran, 1999).

Although only a few of these techniques have been directly compared against each other, Doppler ultrasound has been shown to outperform strain gauge plethysmography in terms of accuracy, although strain gauge plethysmography was found to be more reproducible (Pallarés

et al., 1994). Perhaps based on the relative ease of use (once trained) and the ease of accessibility of ultrasound machines, Doppler is the most commonly reported method in the literature, and appears to be the modality of choice for studying macrovascular blood flow not just in healthy volunteers for research purposes (Hoskins, 1990), but also in patients with critical illness (Hussain, 1997; Hoskins, 2010).

5.3.1.2: Introducing Doppler ultrasound

The variables used for the classical calculation of blood flow through a blood vessel using Doppler ultrasound (Hoskins, 1990) were:

i) vessel diameter,

ii) beam-vessel angle, and

iii) mean Doppler frequency,

whereby a change in any of these parameters could affect the result obtained.

Now days, modern ultrasound machines tend to automatically calculate the blood flow velocity as well as vessel diameter (to determine radius) via manual or automated vessel edge calliper placement to provide a calculation of blood flow through a vessel using the formula:

$$\textbf{BF (l/min)} = \frac{\textbf{[BV (cm/sec}^2\textbf{) x Pi x femoral radius}^2\textbf{ (mm}^2\textbf{)] x60}}{\textbf{1000}}$$

5.3.1.3: Local Protocol for Doppler ultrasound

For the purpose of these studies, leg blood flow from common femoral arteries was recorded. The common femoral artery is an extension of the external iliac artery, which itself originates from the descending aorta and is responsible for blood flow to the leg. It is, however, deep in the pelvis and hence cannot be visualised in good detail in most subjects. The common femoral artery however, bifurcates into two branches: superficial and deep femoral arteries.

Leg blood flow in the common femoral artery was measured using a Phillips iU22 ultrasound scanner (Philips Healthcare, Santa Ana, CA) with a 9-3 MHz linear array transducer (Philips Healthcare) on the pre-set vascular arterial mode.

The femoral arteries were first identified in their anatomical position in the femoral triangle using a transverse view first and then visualised in longitudinal mode as is the standard practice in vascular Doppler ultrasound (Hwang, 2017). Once the position of the transducer was secure, volume flow callipers were used to select the whole cross section of the blood vessel. Using spectral Doppler mode, several (a minimum of six) pulsed wave Doppler waveforms were obtained (see Figure 5.2), each corresponding to one complete cardiac cycle, with mean analysis of 6 waveforms constituting a single measurement and a mean of three consecutive measurements at each time being used to generate the data presented in this chapter as per published protocols (Mitchell *et al.*, 2013, 2014; Phillips *et al.*, 2014).

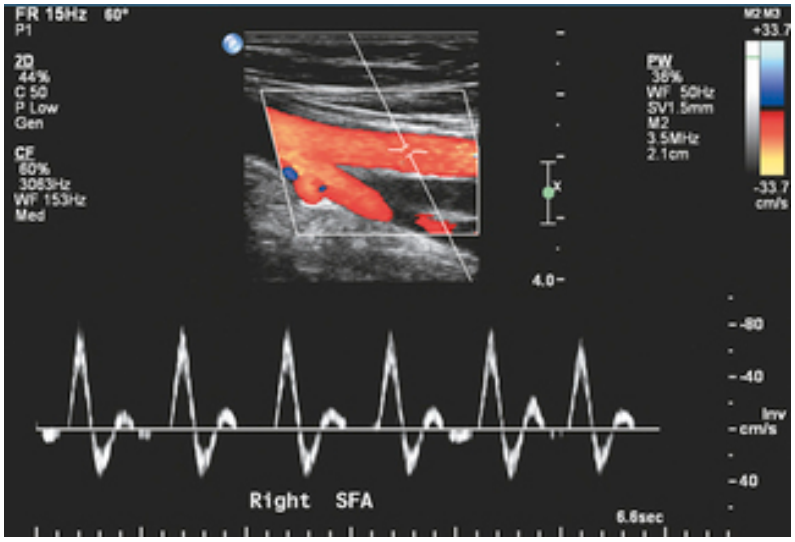


Figure 5.2: Doppler Ultrasound image of the common femoral artery with 6 waveforms.

5.3.2: Techniques to assess muscle microvascular blood flow

As with macrovascular blood flow, several techniques have been employed to study microvascular blood flow through skeletal muscles, including:

1. Laser Doppler Fluxmetry (LDF): This is one of the older techniques that can be used to study microvascular blood flow through skeletal muscle (Clark *et al.*, 2001). This method can show vasoconstriction/vasodilation in muscle capillaries.
2. Nuclear Magnetic Resonance (NMR) Spin labelling: This non-invasive technique can show temporal and spatial resolution (Raynaud *et al.*, 2001) that can help with the assessment of muscle

oxygenation and energy consumption when combined with spectroscopy (Carlier, 2011).

3. Positron Emission Tomography (PET): This technique uses radioisotopes with a short half-life to provide an image of blood flow across several organs and has previously been used to measure oxygen consumption and recruitment of capillaries during exercise (Heinonen *et al.*, 2010).
4. Near-Infra Red Spectroscopy (NIRS) is another non-invasive method that can detect tissue oxygen availability, and has been used in both health and disease (Boushel *et al.*, 2001; Van Beekvelt *et al.*, 2001).
5. Contrast Enhanced ultrasound (CEUS): This technique has been used to study tissue perfusion in several organs such as skeletal muscles (Mitchell *et al.*, 2013), kidneys (Wang and Mohan, 2016), liver (Pang *et al.*, 2018) and brain (Vinke *et al.*, 2017), and can detect deficits in the microcirculation such as those seen in type-2 diabetes (Amarteifio *et al.*, 2013). As CEUS was used as the method of choice in the studies described in this thesis, it is discussed in more detail below.

5.3.3: Contrast enhanced ultrasound:

Before discussing contrast enhanced ultrasound (CEUS), it is important to discuss the basics of ultrasound and how does it work.

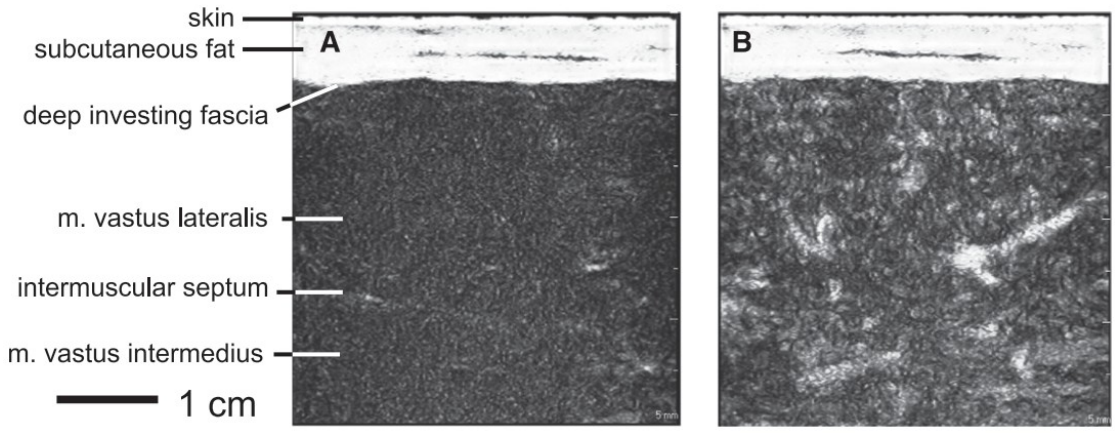
5.3.3.1: The basic concepts of Ultrasound:

Ultrasound uses sound waves of specific frequencies to generate echoes that can then be used to generate black and white (or greyscale spectrum) images, with white denoting return of maximum echoes and black denoting a lack of any echoes.

The frequency employed by a transducer to produce an image is above the human auditory limit (of 20 KHz) and is called the *fundamental frequency*. When sound waves pass through different tissues at the fundamental frequency, many are reflected back to the transducer at different frequencies due to different densities of tissues. These reflection frequencies are often multiples of the fundamental frequency and are known as *harmonic frequencies*. Ultrasound technology employs these harmonic frequencies in a method known as harmonic imaging to generate high-quality tissue images. This theory is fundamental to all forms of ultrasound, including Doppler ultrasound and CEUS as presented in this chapter.

Another aspect of ultrasound, and one of particular importance for CEUS, is the *mechanical index (MI)*- the power of the ultrasound beam. This is of particular significance for CEUS as microbubble behaviour differs at different MI's, giving different image quality at specific MI levels. For example, at an MI of 0.5, the microbubble oscillation gives the best

harmonic imaging to visualise the contrast agent, however increasing the MI to 1.0 renders the microbubbles unstable and causes them to burst (Mitchell *et al.*, 2013). It is this varied response of contrast agent microbubbles to different MI's that allows us to capture muscle MBF using ultrasound. Under the right (low) MI conditions microbubbles oscillate and give out harmonic frequencies that are detected by the ultrasound transducer and as such can be used to image microvascular blood vessels in a region of interest (ROI). Once a steady state of microbubbles is achieved, a high MI "flash" can then be used to destroy all the microbubbles so that the rate and volume of microbubbles reappearing in the ROI can be determined as microvascular flow rate (beta) and microvascular volume (A value). These two parameters can then be multiplied to calculate microvascular blood flow (Mitchell *et al.*, 2013). The figure below (figure 5.3) shows the view on ultrasound screen immediately after the high MI flash (1-A) and at the end of a subsequent 30-second recording (1-B). The replenishment curves (raw and mean) generated via Q-Lab CEUS analysis software (Philips Healthcare) and Graphpad Prism are also presented in this figure (1-C and 1-D, respectively).



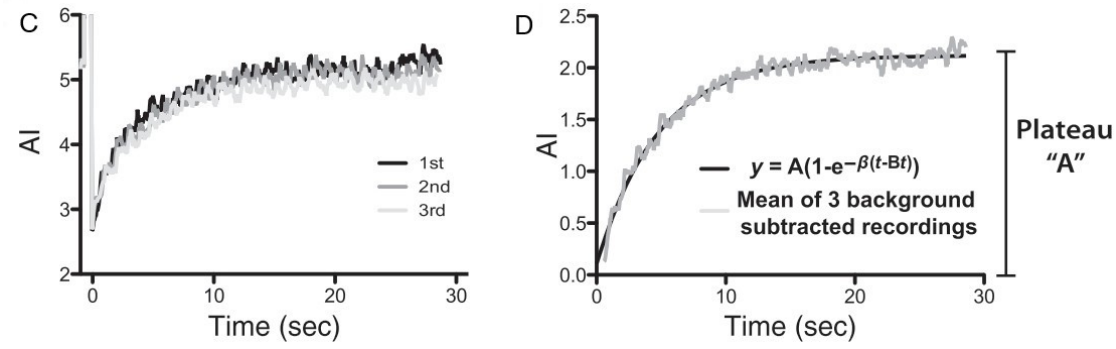


Figure 5.3: A. An ultrasound image immediately after a high mechanical index (MI) flash (destroying all SF6 microbubbles); B. an ultrasound image of SF6 microbubbles after 30 seconds of low MI reperfusion; C. 3 consecutive destruction-replenishment cycles; D. a mean replenishment curve to be used for numerical analysis. (Mitchell et al., 2013).

In this study, once recordings were obtained (for three 30-second destruction-replenishment cycles at each time-point) these video files were then exported to CEUS quantification software (Q-Lab, Phillips). This software facilitated the definition of a ROI which was free from large rapid-filling vessels and any other structural artefacts. The software was then able to present the echogenicity within this ROI as an acoustic index (AI) value against time, with these values exported to Microsoft Excel to generate mean values and then Graphpad Prism for further data handling and statistical analysis.

5.3.3.2: The Contrast: Sonovue®

Although other contrast agents are available for CEUS, and have been used in both clinical (Amarteifio *et al.*, 2013; Vinke *et al.*, 2017) and physiological research (Phillips *et al.*, 2014), the contrast agent of choice for this research was Sonovue® (Bracco, Milan); an agent that has been successfully used by our lab before (Mitchell *et al.*, 2013). Sonovue® is a contrast agent that was first introduced in late 1990's (Schneider, 1999) and is available as a cryodesiccated powdered vials (25 mg) of Sulphur Hexafluoride SF6 microbubbles which is mixed with 5ml normal saline (0.9% solution of NaCl) to form a solution with 8µL SF6 microbubbles/ml.

Sonovue® is safe and well tolerated in healthy individuals, with some research study protocols administering up to 10 vials per day with no reported adverse events (Mitchell *et al.*, 2013). Sonovue® also rapidly achieves systemic steady state (~2.5-min compared with ~9-min for Definity™ – an alternative contrast agent), and is eliminated rapidly; 40-50% of the drug is eliminated via a pulmonary route in first minute and up to 90% eliminated within 11-minutes of infusion cessation, with no differences observed between men and women (Morel *et al.*, 2000). It has also been shown that Sonovue® provides a linear relationship between concentration and signal intensity facilitating the assessment of microvascular blood flow in a unit known as acoustic index or AI (Wei *et al.*, 1998).

5.3.2.3: Local protocol for CEUS:

The local research protocol (with the time points designated for leg blood flow measurements and CEUS) are shown in figure 5.4

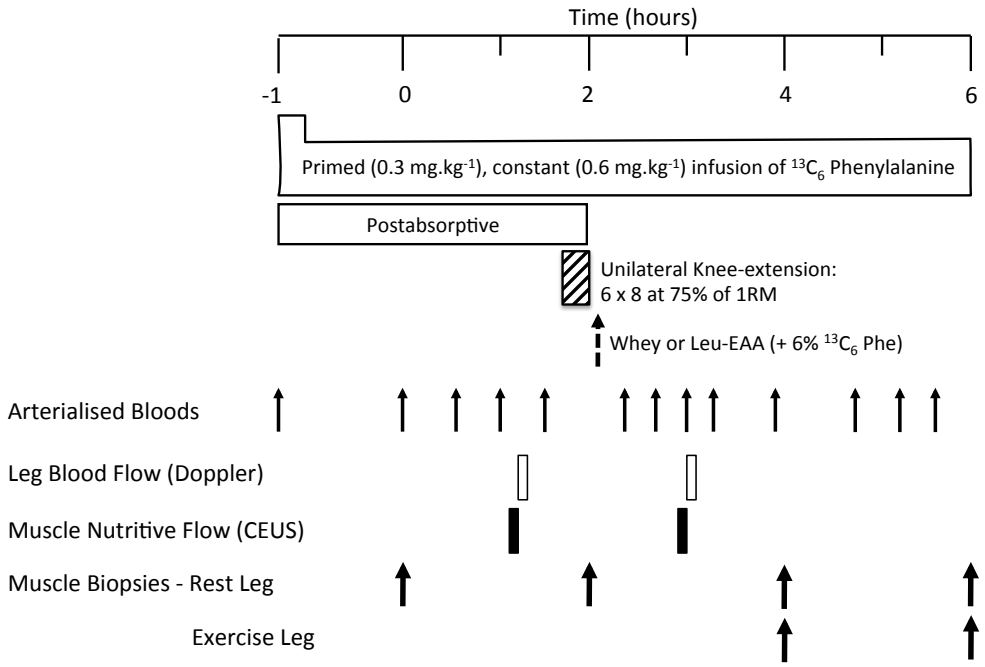


Figure 5.4: Study protocol for anabolic response to Whey Protein (WP) supplement and Leucine Enriched Amino Acid (LEAA) supplement showing time points for Leg Blood Flow and CEUS

In order to study muscle MBF in a consistent ROI throughout a whole study visit, ultrasound transducers were secured onto the thigh of each subject using a locally manufactured polyethylene unit, which was then fastened around with circumferential Velcro straps. At first application, the site was marked on the skin with a marker pen to allow the position of the probe

to be checked and maintained after the acute exercise bout which took place as part of the study visit.

The polyethylene units were fixed and remained on anterior aspect of the thigh for the duration of each study visit to allow assessment of muscle MBF in the *m. vastus lateralis (*and occasionally a section of the *m. vastus intermedius*).

Due to the research design assessing muscle MBF at multiple times in both legs over the course of a steady day, multiple Sonovue® 25 mg vials were used. As outlined above, each vial was in powdered form and was mixed with 5 ml normal saline. The solution of each reconstituted vial was then placed in a Vueject continuously oscillating injection pump (Bracco, Italy) which oscillated for the duration of the study visit to keep the SF6 microbubbles in suspension. The contrast agent was infused from the Vueject infusion device using an 18G or 20G cannula in the antecubital fossa. The infusion and analysis protocol was based on that previously published (Mitchell *et al.*, 2013). The initial infusion was at a rate of 2ml/min for 1 minute (as an initial priming) followed by 1ml/min for 2-minutes to achieve systemic steady state. With the ultrasound machine set to record 30 second video captures, high MI "flashes" were delivered every 30-seconds such that each video contained a destruction and replenishment phase. Three consecutive 30 seconds cycles were recorded totalling 90-seconds for each measurement.

5.4 RESULTS

5.4.1: Leg blood flow (LBF) and microvascular blood flow (MBF) for 3g LEAA & 20g WP

LBF was not different between the groups at baseline (LEAA: 0.27±0.03 vs. WP: 0.34±0.04 $l \cdot min^{-1}$,) and was unaltered in response to FED or FED-EX at 60-120 min post feed ($P>0.05$) in both groups (Figure 5.4: A, C). Conversely, MBF increased following FED-EX in both the LEAA (+91±22%, $P<0.05$) and WP groups (WP: +229±23%; $P<0.05$) (Figure 5.4: B, D).

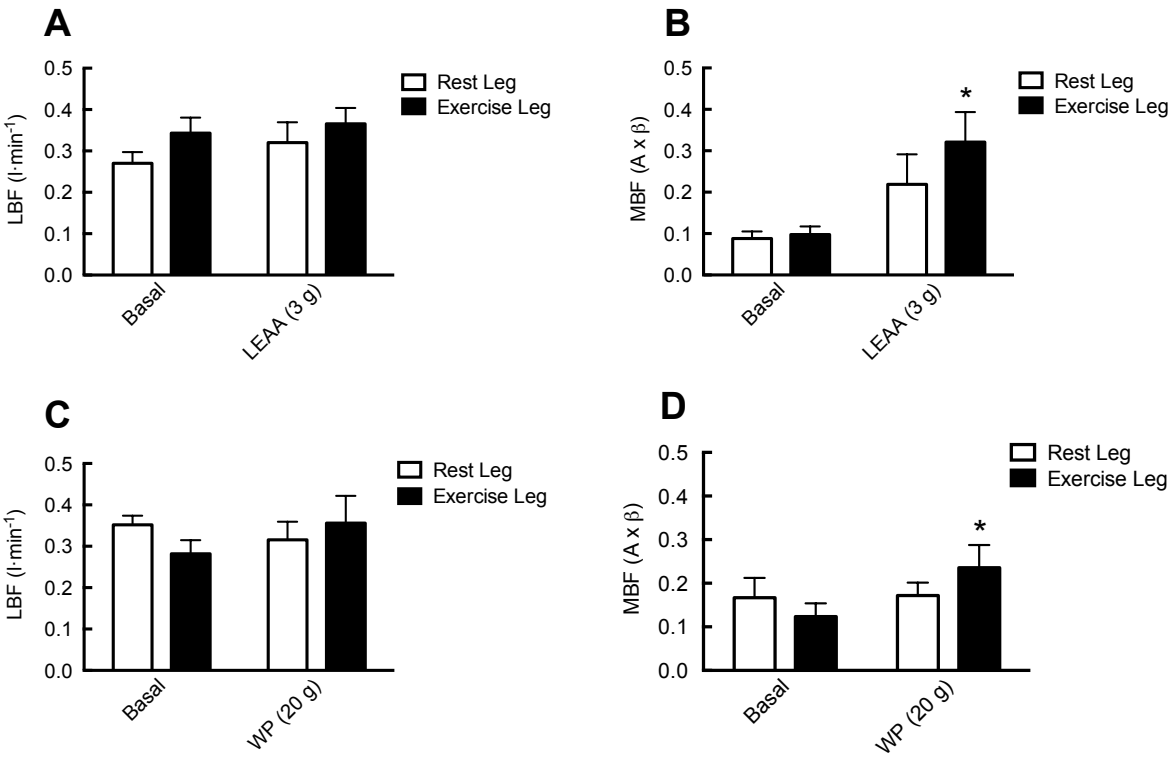


Figure 5.4: Leg blood flow (LBF) (A & C) and muscle microvascular blood flow (B & D) in rested and exercised legs of older women consuming 3g leucine enriched amino acids (LEAA) or 20g whey protein (WP). Data is mean +/-SEM for N=8 per group. *=P<0.05 vs. basal.

5.4.2: Leg blood flow (LBF) and microvascular blood flow (MBF) for 1.5g & 6g LEAA & 40g WP

Due to technical issues with the collection of these measurements, MBF and LBF data was only collected for 6 subjects per group. In keeping with the findings for low-dose LEAA and WP, no significant increases in LBF were evident in response to FED or FED-EX (Figure 5.5: A, C, E). Contrary to the results observed in the low dose groups there were also no significant increases in MBF observed in response to FED or FED-EX (Figure 5.5.: B, D, F). It must however be considered that this may be due to insufficient statistical power to detect changes in these groups with reduced numbers, especially as numerical increases are apparent in response to FED-EX for all groups.

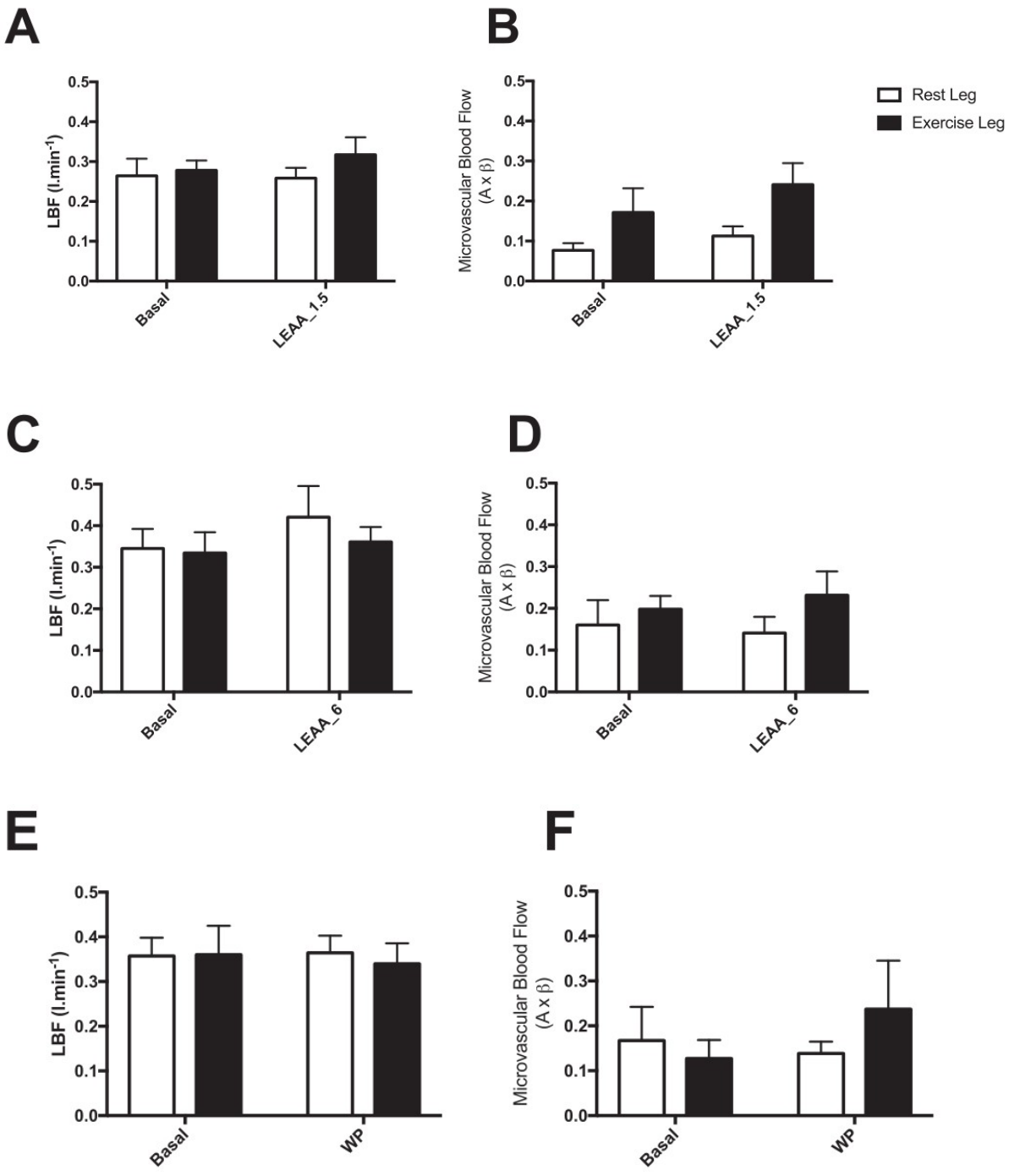


Figure 5.5: Leg blood flow (LBF) (A, C & E) and muscle microvascular blood flow (B, D & F) in rested and exercised legs of older women consuming 1.5g or 6g leucine enriched amino acids (LEAA) or 40g whey protein (WP). Data is mean +/- SEM for N=6 per group.

5.4.3: Insulin responses in different supplement doses:

The role of insulin in human skeletal muscle protein metabolism has been discussed in detail in chapter-1 (please see section 1.4.6.2). Similarly, the analysis of plasma insulin via ELISA is detailed in chapter-2 (please see section 2.3).

In the first comparison of 3g LEAA versus 20g WP, plasma insulin concentration increased over post-absorptive values (~5 mU/l, in both groups) by 20-min post-feeding in both groups (to 14 $\pm$ 3 (LEAA) vs. 20 $\pm$ 3 mU/l (WP), both $P< 0.05$), with no significant difference between the insulin concentrations of the two groups at that time-point. However, whereas plasma insulin returned to values not different from basal concentrations by 40-min after LEAA intake (Fig. 5.6), insulin concentrations remained elevated up to (and likely beyond) 60-min after WP consumption (18 $\pm$ 3 mU/l, $P<0.05$ vs. basal).

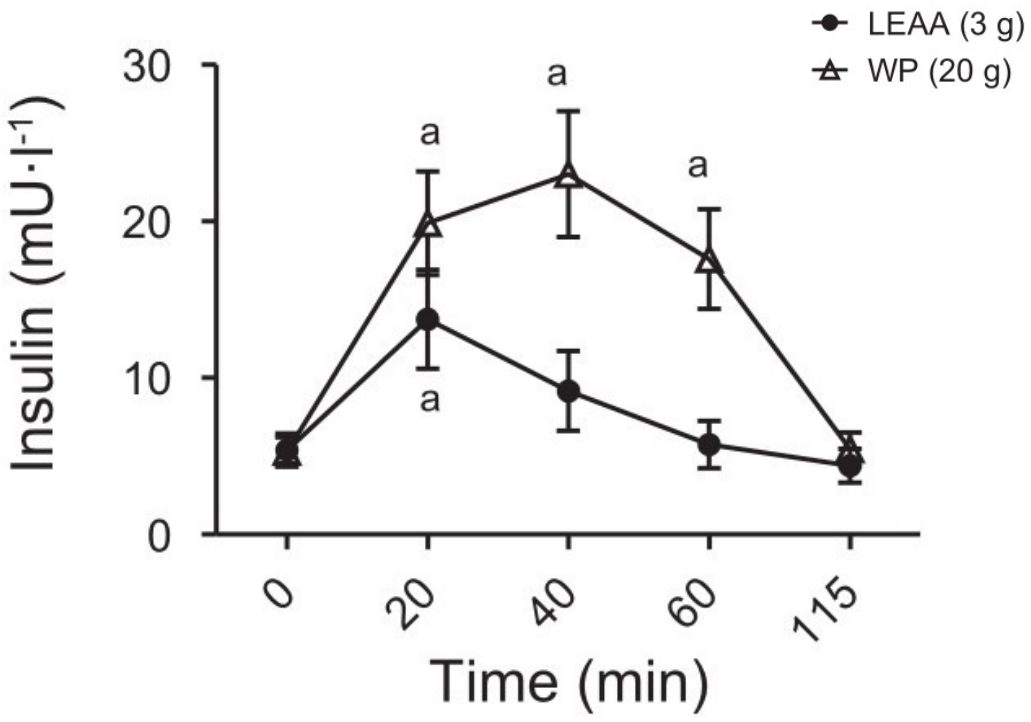


Figure 5.6: Plasma insulin concentrations of older women after 3g leucine enriched amino acids (LEAA) or 20g whey protein (WP). Data is mean +/- SEM for N=8 per group.

[a] greater than basal (P < 0.05)

[b] greater than other group at the specific time point indicated (P <0.05).

Comparing the insulin responses of older women consuming low (1.5g) or high doses (6g) of LEAA, or high dose (40g) WP, showed that all of these feeding strategies increased plasma insulin concentrations above postabsorptive values peaking at 11.5 ± 1.8 uU/mL, 12.8 ± 1.6 uU/mL and 21.0 ± 3.6 uU/mL, respectively (all *P*<0.05 vs. basal) (Fig. 5.7). However, while plasma insulin returned to values not significantly different

from basal concentrations by 40-min after both 1.5g and 6g LEAA intake, insulin concentrations in response to 40g of WP were significantly elevated for up to 60-min, with significantly higher insulin concentrations at 40 and 60 min compared to both of the LEAA groups.

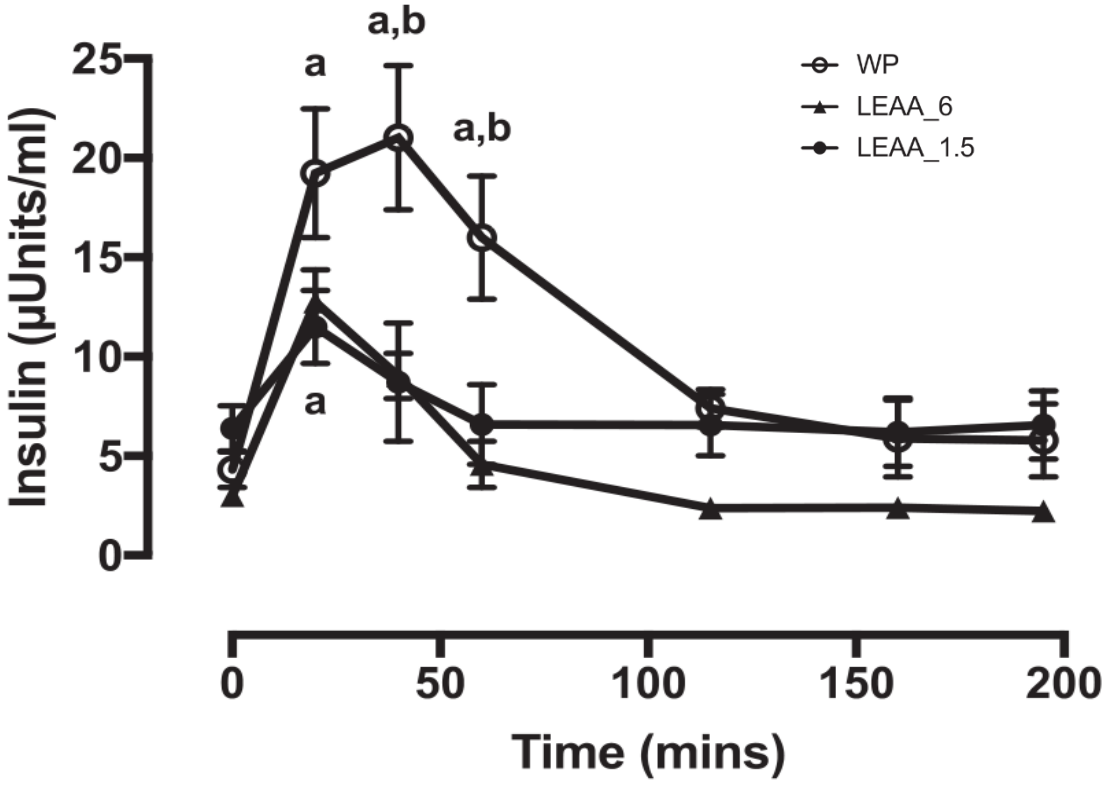


Figure 5.7: Plasma insulin concentrations of older women after consuming 1.5g or 6g leucine enriched amino acids (LEAA) or 40g whey protein (WP). Data is mean +/- SEM for N=8 per group.

a: significantly different vs. basal (P < 0.05). b: significantly different between groups (P < 0.05).

5.5: DISCUSSION

Given the higher total amino acid concentration in our WP feed, our *a priori* hypothesis was that WP would result in increased insulin secretion, and that due to an increased amount of the purported 'vasodilatory amino acid' arginine, leg blood flow would be increased to a greater extent in the WP groups when compared to the LEAA groups. As expected, higher arginine concentrations were seen in the WP groups (reported in chapter 3), as well as heightened and prolonged insulin responses. However, in both of our comparison sets, LBF and MBF responses were not different between WP and LEAA. Although this finding does share some similarities with previous work from our group and others, the literature body is not entirely consistent.

In a previous study from our research group where LBF was studied in 3 mixed sex groups across the life-course (young (18-28 years), middle age (45-55 years) and old age (65-75 years), it was reported that LBF responses to both feeding (mixed meal) and feeding-plus-exercise were only apparent in young and middle aged but not older adults (Phillips *et al.*, 2012) supporting our current findings of no responses in older adults, with CEUS not measured in this particular study. Another point of interest is that in this particular study the lack of vascular responsiveness with advancing age was attenuated by resistance exercise training (RET). As all of the females recruited to this study were independent and community-dwelling but did not participate in regular structured exercise, it may be

that RET could offer a strategy to improve the vascular function of this specific cohort.

Our current results do however differ from another study looking at the effects of oral amino acid supplementation in older adults with a mean age of 62 years. In this study, oral amino acid supplementation did improve leg blood flow, in both patients with intermittent claudication (an disease of chronic arterial narrowing causing insufficiency of calf muscles on walking) and healthy controls (Killewich *et al.*, 2007). If these findings had only been apparent in the patient population it may have been that improvements were only able to be made in those with pre-existent vascular compromise (beyond ageing *per se*), however data from the age-matched healthy controls in this study does not support this proposition. There are however, two notable differences between this study and ours. Firstly, they used strain gauge plethysmography (vs. Doppler ultrasound) to assess leg blood flow, and secondly their measures were all based in the lower limb (due to its clinical relevance in intermittent claudication).

A more recent study from our research group with more similar methodology to those used herein is supportive of our findings showing no increase in the muscle MBF of older females in response to feeding alone. Using an identical CEUS protocol to the one performed in this study, Mitchell *et al.,* determined microvascular flow parameters in both young (18-28 years) and older participants (65-75 years) after amino acid feeding (Mitchell *et al.*, 2013). Mitchell and colleagues reported that in younger participants, there was an initial increase in microvascular blood volume, with a later increase in microvascular flow velocity, with no such

response in either parameter in older adults. In this paper, Mitchell *et al.,* suggested that the early volume response was due to vasodilation which temporally correlated with an insulin response, adding further support to our initial hypothesis. This study was however only in males.

Given the focus of this thesis being the importance of muscle mass maintenance for whole-body health, and the possibility of nutritional strategies to alleviate age-associated anabolic resistance in older females, the relationship between both macro and microvascular blood flow parameters and anabolic responses was of great interest. Although previous work by our research group has shown that both nutrition (acute cocoa flavanols) (Phillips *et al.*, 2016) and exercise-based interventions (Phillips *et al.*, 2012, 2015) can improve the vascular responses of older males, in these studies (and indeed via pharmacological manipulation of blood flow parameters in young males- (Phillips *et al.*, 2014)) improvements in leg and MBF have not been associated with improved MPS. This is echoed to some extent in this work whereby no LBF response to feeding or feeding-plus-exercise is observed, despite increases in MPS in response to these stimuli, although the MBF increase to the dual stimuli only does present with heightened MPS.

To conclude, this study reports that in older women muscle MBF is enhanced after a bout of acute resistance exercise, demonstrating some degree of vascular responsiveness in this largely understudied population. Heightened MPS responses to the combination of feeding-plus-exercise were seen when compared to feeding alone, which is not wholly surprising given that contractions are a known key anabolic stimulus. However,

whether there is a mechanistic link of causality here remains to be seen, and future work is required to determine this.

CHAPTER 6: OVERVIEW, FUTURE DIRECTIONS AND CONCLUSIONS

6.1 OVERVIEW OF FINDINGS

This thesis started by looking at understanding of sarcopenia on the basis of studies conducted so far. The main determinants of skeletal muscle mass in humans (protein/amino acid intake and resistance exercise) and subsequently the effect of ageing and gender on these factors were examined. It was identified that females have not been studied as extensively as males, but that there were data showing differential responses of females to nutrition and exercise interventions.

All of the studies were conducted in community dwelling females aged 60-70 years who were recruited via a postal survey. They all underwent a screening interview as well as medical examination as well as blood tests to ensure that they met the inclusion criteria and that no exclusion criteria were found. All of them also underwent a 1-repetition maximum (1RM) assessment of knee extension. All studies were conducted after an overnight fast and commenced with a DXA scan to determine whole body as well as leg muscle mass.

The first study (described in chapter 3) noted the effect of whey protein (WP) 20 g and leucine enriched essential amino acid supplementation or LEAA at the dose of 3 g (with 40% Leucine i.e. 1.2 g) in elderly women aged 60 and above, with and without resistance exercise. The results showed that both WP and 3 g LEAA (with 40% LEU) stimulated MPS equally at 2 hours and abated afterwards (at 4 hours). The anabolic response however continued over 4 hours when feeding was combined with

unilateral resistance exercise (6x8 knee extension at 75% 1RM. Albumin protein synthesis (APS) also increased in both groups similarly in response to feeding. These effects were noted despite greater insulinemia and essential amino-acid-aemia noted with WP.

The second set of studies considered the anabolic effects of different doses of LEAA (1.5g and 6g) as well as very high dose of WP (40g) in elderly females using the similar study design. Again, insulinemia and Essential aminoacidemia was noted to be higher with WP 40g, though LEAA 6g peaked similarly to WP in the early stages, which was not sustained. However, when it comes to anabolic response measured via muscle protein synthesis (MPS), it was increased to the same extent (compared to baseline) in all 3 groups at 2 hours interval in the fed-only leg. At 4 hours, only WP 40g and LEAA 6g sustained a higher MPS. As seen in previous study, resistance exercise (using similar protocol- 6x8 at 75% of pre-determined 1RM) prolonged the MPS response to 4 hours in all groups.

The fifth chapter explores the influence of leg blood flow as well microvascular blood flow on muscle protein metabolism in these subjects. It was noted that none of the supplements at any doses altered either the leg blood flow or the microvascular blood flow, whereas RET increased the microvascular blood flow, though it reached statistical significance only in first set of studies (comparing LEAA 6g with WP20g).

In summary, LEAA even at lower doses i.e. 1.5g (with 0.6g LEU) stimulates MPS to same extent as higher doses of LEAA (3g and 6 g) as well as WP (20g and 40), in elderly community dwelling women. This anabolic effect

can be prolonged by resistance exercise (RE). These effects are summarised in Figure 6.1 below.

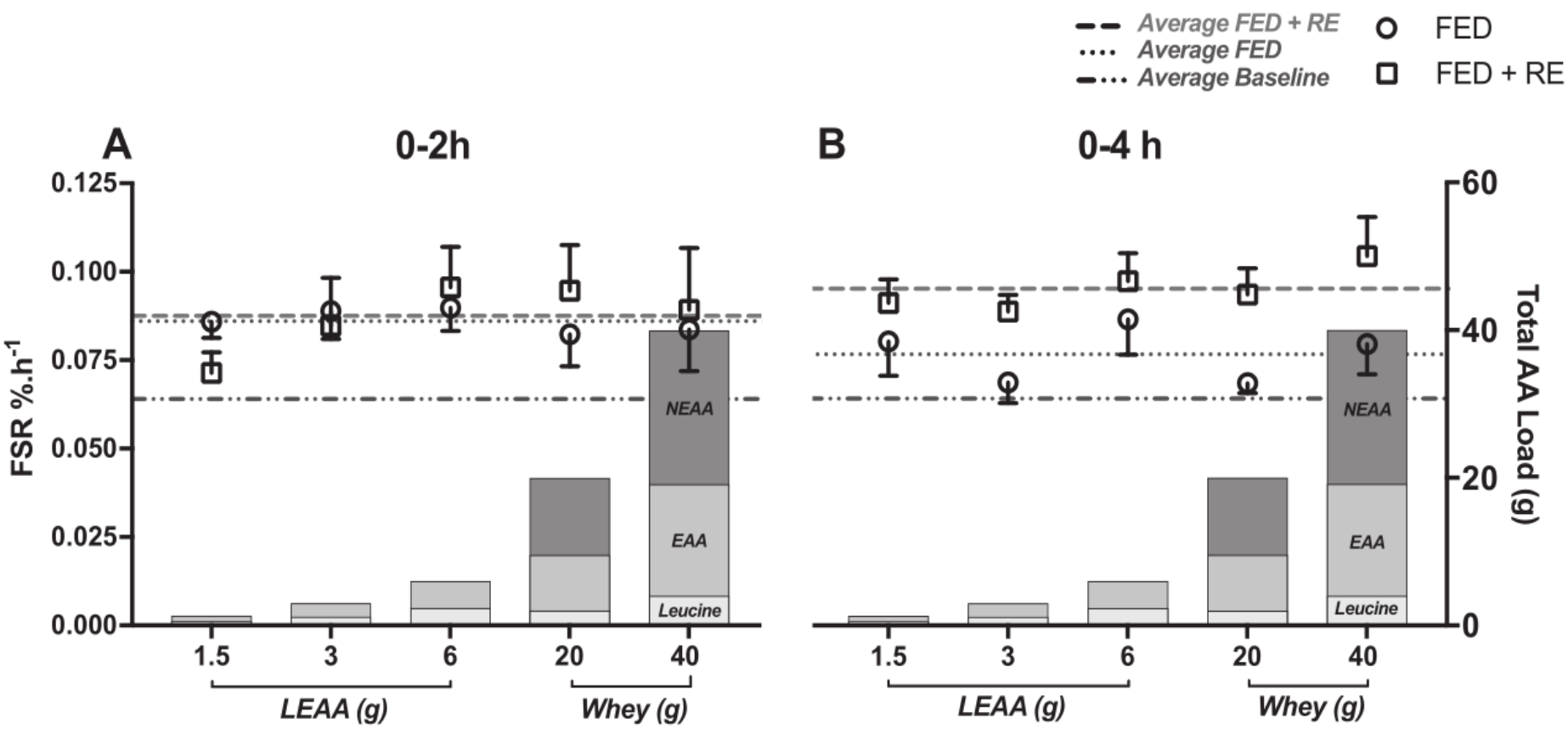

Average FED + RE
Average FED
Average Baseline
FED
FED + RE
A
0-2h
B
0-4 h
FSR %.h-1
Total AA Load (g)
0.125
0.100
0.075
0.050
0.025
0.000
60
40
20
0
NEAA
EAA
Leucine
1.5
3
6
20
40
LEAA (g)
Whey (g)

Figure 6.1: Summary dose-response relationship between MPS and AA feeding (±RE) in older women.

Over 0-2 h, feeding increased MPS with little enhancement with RE (A). Over the entire 4 h (B) postprandial measurement period, MPS was elevated although diminished with feeding, whilst RE sustained elevated MPS. All groups displayed similar MPS in response to feeding (±resistance exercise) suggesting only a small dose of leucine and/or EAA are required in combination with exercise to maintain elevations in MPS post nutrition, at least over the first 4 h. Left Y axis shows FSR for each feed (open circles FED, open squares FED þ RE) whilst dotted lines show average FSR of all groups for baseline, FED and FED + RE. Right Y axis shows total AA load and content of leucine, EAA and NEAA.

None of the supplements on their own affected leg blood flow (LBF) or muscle blood flow (MBF) as measured using CEUS. RE however did affect the muscle (microvascular) blood flow (MBF) but to varying degrees e.g. RE increased the MBF in LEAA 3g and WP 20g groups (reaching statistical significance). Similar trend was seen in other groups however it failed to reach statistical significance (please see figures 5.2 and 5.3).

6.2: LIMITATIONS OF THESE STUDIES

A potential limitation of these studies is that they were all acute studies, and limited to studying MPS responses to feeding and exercise over 4 hours (total study duration 8-9 hours) and one cannot extrapolate findings beyond this period. The results obtained in acute studies in lab setting may or may not be applicable to community dwelling and free-living conditions. This is a limitation due to study design, when using IV $^{13}C_6$- phenylalanine (or for that matter, any IV) tracer due to costs and the need to control the environment when infusing tracers. Longer terms studies are now possible especially with the use of D2O (Wilkinson *et al.*, 2014) these have been validated to show day-to-day quantification of MPS, when compared to IV tracer methods, in both fasting and post-prandial (after consuming 20g EAA drink) states (Wilkinson *et al.*, 2015).

Another limitation with acute studies looking at MPS responses is the discordance between MPS and anabolic signalling which has previously been reported (Greenhaff *et al.*, 2008; Philip J Atherton *et al.*, 2010). The

data for these previous bodies of work suggests that anabolic signal measurements using standard immunoblotting approaches are not sensitive quantitative biomarkers of MPS in response to nutrition and exercise, and in this instance probably reflect between group differences in serum insulin concentrations. This, in addition to the observation of blunting of anabolic signalling pathways in older age (Guillet *et al.*, 2004; Cuthbertson *et al.*, 2005) may explain the present findings. Nonetheless, it is possible that peak signalling events may differ between such distinct feeding regimes such that the key points of stimulation of anabolic signalling in response to each feed may not have been captured by our biopsy timings – this is an inherent limitation of such studies.

A third limitation with these studies was that there was no direct comparison with men in terms of response to LEAA. Previous research studies from our lab have been conducted in older men (Kumar et al. 2009- among many others) which included RE component as well, but there was no direct comparison. Similar studies in men (younger vs. older) from our lab have shown a submaximal protein feed (EAA 10g with 24g carbohydrate) enriched with LEU shows enhanced anabolic response to RE when compared to iso-nitrogenous ALA enhanced feed (Atherton *et al.*, 2017). While these results support our findings, this was a separate set of studies with a different feed and hence unsuitable for direct comparison.

Furthermore, the participants of these studies were healthy, community dwelling females aged 60-70. They were recruited via postal survey. It is plausible that the groups of females taking part in this study were "healthier" that many other in the community and may not be

representative of the whole community. Many other women may not have volunteered, simply because of mobility and transport related problems. It remains to be seen whether these findings would be replicated in sedentary individuals, or those with limited mobility (inactive), frailty or sarcopenia. Another question that arises is the impact of an acute medical illnesses (that might limit a person's mobility) such as a chest infection or pneumonia, Urinary tract infection (UTI) or cellulitis i.e. how short periods of inactivity impact the responses to protein feeding on MPS? Though there are no studies looking at patients suffering from short-term illness, a study performed in healthy older volunteers shows that reduced physical activity (in form of reduced step count, for example) results in several metabolic changes (increased insulin resistance, reduced insulin sensitivity), increased inflammatory markers (such as CRP and TNF-alpha) and reduced post-prandial MPS (Breen *et al.*, 2013). This indicates that activity may play a role in regulation of maintaining "metabolic haemostasis" as well as regulation of muscle anabolism.

Then there are patients suffering from stroke or a fracture (with a cast) who are unable to mobilise for longer periods - previous studies have shown that artificial methods such as trans-cutaneous electrical stimulation of muscles may prevent muscle loss seen in orthopaedic patients (Gibson, Smith and Rennie, 1988) as well as critically ill patients (Gerovasili *et al.*, 2009).

6.3: CLINICAL IMPLICATIONS OF THESE FINDINGS

The scientific literature from previous studies uncovered the subtle intricacies involved in science of muscle physiology, such as the concepts of muscle-full phenomenon, anabolic resistance and, now from the studies described in this thesis, the lack of any dose-related effects of amino acid supplements such as WP and LEAA on muscle protein metabolism. Does this data have any clinical relevance? Could this data help the sarcopenic patients who are being admitted to medical wards on daily basis in hospitals across the UK?

Many elderly patients are usually admitted with several manifestations of sarcopenia (e.g. falls, inability to maintain balance, reducing or deteriorating mobility and finally loss of swallowing and aspiration related symptoms) with or without an acute illness. In the NHS hospitals of the UK, these patients are usually assessed by "Frailty" teams in hospital and are usually subjected to physical therapy with help from social services.

6.3.1: The case for chronic supplementation

Does this data warrant further clinical studies with chronic supplementation? The efficacy of LEAA supplementation with meals, for maintaining muscle mass and function chronically, remains to be fully tested. Moreover, contradictory findings for the beneficial effects of chronic leucine supplementation in ageing have been reported in the past, with

2.5 g of leucine administered alongside each main meal a day showing no improvements in muscle mass or strength in healthy older men (Verhoeven *et al.*, 2009). However, it should be considered that there are distinct design and methodological differences between the studies of Verhoeven (Verhoeven *et al.*, 2009)and Kim (Kim *et al.*, 2012) which make comparison difficult. Firstly, the chronic leucine supplementation in the work of Verhoeven & colleagues was performed in healthy, non-sarcopenic males, whereas the work of Kim & colleagues was performed specifically in pre-defined sarcopenic older adults, where supplementation may be most beneficial. Secondly, the leucine supplementation of Verhoeven & colleagues was provided alongside main meals, where MPS may already be near maximal and any beneficial effect could have been negligible. Finally and most importantly, as highlighted in our main aims, the work of Verhoeven (Verhoeven *et al.*, 2009) was performed in a group of older males, here and in the work of Kim (Kim *et al.*, 2012) we are specifically investigating interventions in females, where known sexual dimorphism particularly in response to nutrition are well defined (Smith *et al.*, 2012), thereby potentially explaining why differences are observed in the outcomes of the two studies.

On the other hand, a double blind placebo controlled trial of Leucine enriched amino acid mixtures has recently been published shows different results (Ispoglou *et al.*, 2016). The trial studied small numbers - 25 participants who were otherwise healthy, community dwelling older adults (14 female, 11 male- aged 65-75 years). They were divided into 3 groups, LEAA 20%, LEAA 40% total EAA and a placebo without any LEU content.

There was **no** RE or a physical therapy component. The primary outcomes include difference in lean-tissue m-mass (LTM) and fat mass (FM) as well as differences in functional parameters (such as 30-s arm-curl test; 30-s chair-stand test; 6-min walk test and handgrip strength) in the subjects studied. The data showed an improvement in LTM of 40% LEU group (group B: 1.1 $\pm$ 1.1% kg, $p<0.05$ compared to baseline) and in 6-min walk test (5.8 ± 6.6 m $p<0.05$). While there were improvements in many other aspects, they failed to reach statistical significance. The authors concluded that larger studies are needed to confirm the effect. As noted above however, the study didn't include any physical therapy or RE sessions. A recent review article (Witard *et al.*, 2016) recommends incorporating protein rich diet (150-190% of RDA of protein) and resistance exercise into life-style interventions aimed at preserving healthy muscle mass and function in ageing.

6.3.2: The case for chronic supplementation with exercise or physical therapy

It is plausible that the elderly patients admitted due to mobility problems due to sarcopenia may have a better response if they were supplemented with LEAA during their physical therapy sessions or the supplementation was combined with a session of resistance exercise.…

The next logical step would be to try LEAA supplements- preferably lower dose such as 1.5g (so that they can be taken as capsules or in syrup form- as many older adults are unable to take high volume satiating drinks) in patients admitted with sarcopenia related problems with controls who are given placebo (while they all have standard care with physical therapy with calorie-matched diet) to see if this would make any difference to their outcome. The primary outcomes in such studies is usually increase in lean muscle mass though functional improvements may also be regarded as a measure of success.

Indeed, a study along these lines has been conducted in Japan, where they looked at effect of an amino acid supplement in combination with exercise in community dwelling and sarcopenic women aged 75 and above (Kim *et al.*, 2012). In this randomised controlled trial, 175 women were divided into 4 groups:

- Group-1: HE: Those receiving health education (acting as controls)
- Group-2: EX: This group performed regular 60-minute exercise (5-minute warm-up, 30 minutes strengthening exercise, 20 minutes with gait and balance related exercises and 5-minute cool down) twice weekly
- Group-3: AAS: a group having amino-acid supplement only
- Group-4: EX + AAS: Final group had the supplement as well as performing exercise similar to group 2.

They found increased walking speed in all the 3 intervention groups, increased muscle mass (in both exercise groups) and increased knee

extension strength in in only exercise + amino-acid supplementation group (9.3% increase- p<0.01). Please see the figure 6.2 below.

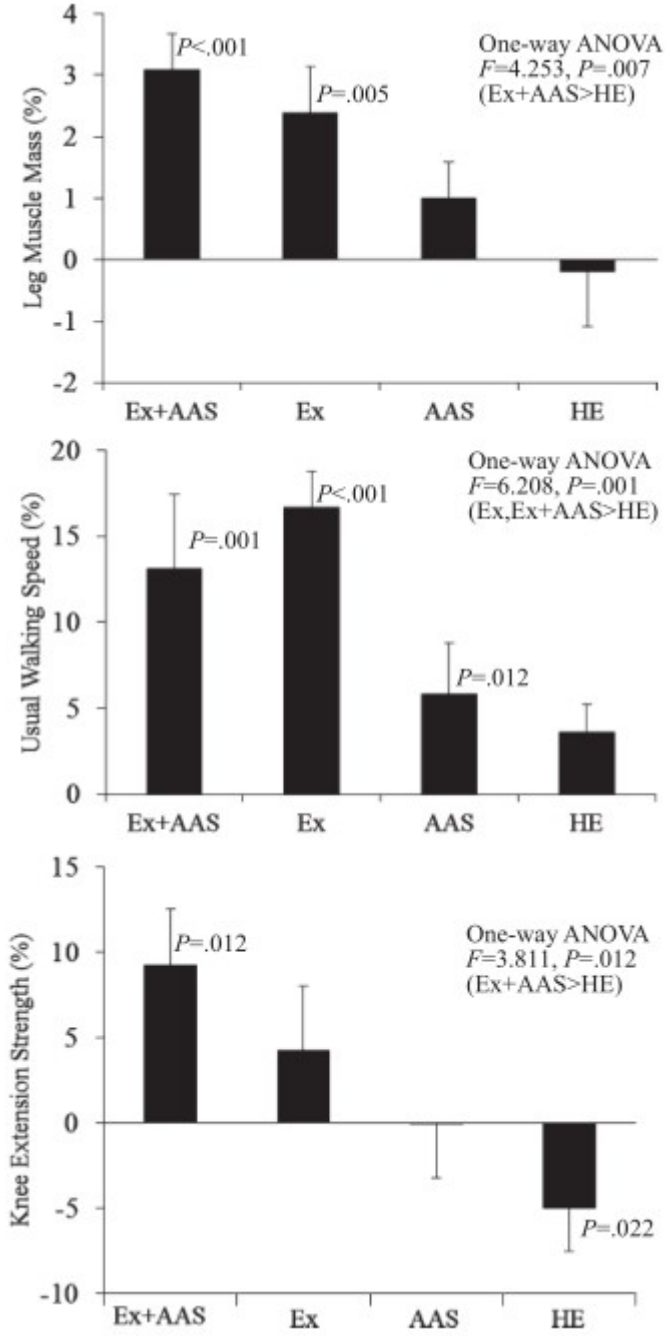


Figure 6.2: Shows effects of amino-acid supplementation (AAS) and exercise on body composition and functional improvements in older Japanese women (Kim *et al.*, 2012)- see texts for details.

The authors conclude that a combination of supplement with standard exercise would be beneficial in community dwelling sarcopenic females.

For maintaining tissue perfusion, good blood flow is also important and diseases such as claudication due to peripheral vascular disease are associated with reduced muscle mass (McCully, Halber and Posner, 1994). In spite of this, studies for our lab (Phillips *et al.*, 2014) as well as others (Tang *et al.*, 2011), note that improving blood flow does not improve muscle protein synthesis and indeed with impaired blood flow as well, the interstitial amino acid concentration remains the same (Durham *et al.*, 2010). Nonetheless one cannot rule out other benefits of improved blood flow such as better oxygen and nutrient delivery. Further studies are needed in this area as there may be a select group of patients who may benefit from strategies that improve muscle blood flow e.g. smokers with narrow blood vessels or those with advance peripheral vascular disease etc.

6.4: FUTURE DIRECTIONS

As noted in a recent review, sarcopenia remains a complex and multi-faceted disease (McCormick and Vasilaki, 2018). And probably because of its complexity (as well as a lack of uniform definition until recently), there are no known strategies so far that have conclusively been shown to counteract sarcopenia. The most promising factors so far shown are nutrient (essential amino acid) supplementation with or without any other

macronutrients, exercise particularly (but not exclusively) resistance type and drugs such as testosterone (Gharahdaghi *et al.*, 2019) and other related compounds e.g. 17β-Hydroxyestra-4,9,11-trien-3-one or trenbolone (a new class of drugs called Selective Androgen Receptor Modulators or SARMs) (Yarrow *et al.*, 2011).

So far there have been attempts at European level (EWGSOP-2) to modify the definition of sarcopenia as recently as 2018 (Cruz-Jentoft *et al.*, 2018) to improve upon the 2010 definition. However, the current definition is yet to achieve wider acceptance. It is also prudent to identify those who are at risk of sarcopenia early to try and direct efforts towards prevention and *avoid* sarcopenia. The current definition accepts this and recognises that sarcopenia may develop much earlier than old age. Hence the interventions noted above could be employed to help avoid it those at risk and reverse it those who have developed sarcopenia- This is one of the future directions the work in this field may take place. But for this to work, there needs to be a clear consensus and accepted definition to identify those who are most at risk.

Another potential future direction involves improving our understanding of the mechanisms regulating muscle protein metabolism and the differences that exist between men and women. As discussed extensively within this thesis, most studies looking at anabolic response in human skeletal muscle to several stimuli have studied men only and there is paucity of similar data on women, particularly older women. This thesis is an attempt to raise awareness about the regulation of protein metabolism in this group and perhaps bridge this gap. It is highly probable that improving the

understanding of muscle metabolism in both sexes will also enhance our overall understanding of sarcopenia and its progression.

Also, there is a need for clinical studies in patients (both community dwelling and those admitted to hospitals) with several consequences of sarcopenia such as poor mobility & falls. Many of these patients may benefit from LEAA supplementation (in addition to continued physical therapy). There should also be more controlled trials looking at clinical efficacy of potential strategies against sarcopenia (LEU enriched amino acid supplement and RE) in patients with advanced diseases such as cancers, chronic obstructive pulmonary disease (COPD), diabetes, obesity, rheumatoid arthritis and also those who are affected by osteoarthritis.

Finally, there needs to be an increased awareness of the existence of sarcopenia amongst clinical staff as well as general practitioners or family physicians, when treating older patients. They need to be equipped with the tools and knowledge to identify those who are sarcopenic as well as those who are at risk i.e. pre-sarcopenic. They should also be armed with knowledge on how to prescribe and motivate individuals to improve their lifestyle to help slow down the development of sarcopenia using intervention strategies including amino acid supplementation and regular exercise/ increasing activity levels. In absence of any proven alternatives e.g. pharmaceutical interventions that reverse or delay sarcopenia, these are our best options currently available to limit muscle loss, maintain muscle mass and function- thereby improving health and quality of life. There is also a need for increasing public awareness as well as informing public health policy makers about improving "metabolic health" of the

population (Witard and Ball, 2018). This can include measures such as improved education about diet in general educational curricula, labelling of food products for sale (which is already in place) and use of protein supplements for patients being treated for problems associated with sarcopenia.

It is only when the data from the studies described in this thesis, as well as other published data, are translated into clinical practice, will allow it to make a significant difference to the lives of our ever-increasing ageing population.

APPENDICES

Appendix-1: Screening proforma (2 pages)

The University of Nottingham

Title of Project:
The modulation of muscle metabolism by muscle protein metabolism by essential amino acids
Dr Phil Atherton, Dr Ken Smith, Dr John Williams, Dr. William Mitchell & Dr Syed Bukhari

Date			
ID	AJ2-		
Name			
Contact Phone Number:			
Contact Email:			
Age		**Address**	
DOB			
Ethnicity			
Study Date			
Anatomical Screen	Clear vessels for line insertion?		
Height	m		
Weight	kg		
BMI	$kg.m^2$		
Examination	J | A | C | C | O | L		
Heart Sounds	**BP: _____ mm Hg**		
Chest			
Abdomen	**HR: _____ bpm**		
ECG	Peak Flow:		
PMH	**Drug Hx**		
Known Allergies (including asthma)	Smoking status:		
Clear Bloods	Notes...		
	Notes...		

CHECKLIST:

Event	Date when Done	Notes
Screening		
Consent		
Acute Study booked		Date Booked: _____ Participant Informed ☐
Tracer Ordered		
Acute Study Done		
Suture Removal		
Any Problems:		

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PUBLICATIONS

PAPER-1

Intake of low-dose leucine-rich essential amino acids stimulates muscle anabolism equivalently to bolus whey protein in older women at rest and after exercise

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PAPER-2

Effects of leucine-enriched essential amino acid and whey protein bolus dosing upon skeletal muscle protein synthesis at rest and after exercise in older women

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