Milk protein supplementation and the regulation of lean tissue mass in healthy community dwelling 50-70y old women and men

Milk protein supplementation and the regulation of lean tissue mass in healthy community dwelling 50-70y old women and men.

by

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Authors Declaration

I hereby declare that the work contained within this thesis is my own work, and was completed without collaboration or assistance from others apart from the counsel received from my supervisors, Professor Phil Jakeman of the Physical Education & Sports Sciences Department, University of Limerick and Professor William O'Connor of the Graduate Entry Medical School, University of Limerick. This work has also not been submitted to any other University of higher education institution, or for any other academic award within this University.

William McCormack

April 2014

Professor Phil Jakeman

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Mylo and Janet, thank you for your love and support. I’m very proud of my brother and sister.

And finally to Mam & Dad, the sacrifices you made provided this opportunity. I may never be able to repay you, but that won’t stop me from trying.

Abstract

Population demographics demonstrate that the number of older individuals will triple to approximately 1.5 billion by 2050. Age-related sarcopenia is associated with a progressive loss of lean tissue mass (LTM), strength and function. Based on population specific data from the University of Limerick Body Composition (ULBC) study, the rate of decline in LTM is approximately 0.35-0.45% (0.14-0.26 kg) per annum after the age of 50 years. Interest has developed in the retention of LTM with advancing age to maintain functional and metabolic benefits that an appropriate LTM afford. Older populations display a blunted muscle protein synthetic response to the main anabolic stimuli, namely nutrient ingestion and mechanical loading. In the absence of mechanical loading, a potential dietary strategy to overcome anabolic resistance is the distribution of daily protein intake to equal amounts (25-30 g) over the three main eating occasions of the day. A 20-30 g, quality protein intake is deemed to provide an extracellular essential amino acid (EAA) concentration to stimulate muscle protein synthesis (MPS). Specific to the EAAs, leucine commands greatest importance acting as both a substrate and intracellular regulator of MPS. Augmentation of the nutrient effect is provided by mechanical loading, i.e. resistance exercise, an interaction that needs to be evaluated in the elderly in situ with optimal timing and quality of nutrient intake.

Following in vitro evaluation of a milk-based protein nutrient formulation this thesis investigated the bioactive properties of oral ingestion of the nutrient formulation in vivo in elderly subjects. The nutrient formulation invoked a postprandial increase in circulating peak plasma EAA (1,848 ± 83 μM) and leucine (321 ± 22 μM) concentration. Parallel studies by in vivo skeletal muscle microdialysis (MD) confirmed that the extracellular tissue concentration of these key amino acids was similarly advanced. A 24 week RCT in a group of healthy 50-70y old community dwelling women and men was then conducted to explore the nutrient effect on the maintenance of LTM, measured by dual energy x-ray absorptiometry (DXA). For this RCT, isocaloric amounts of milk-based protein formulation (FORM) (0.33 $g.kg^{-1}.d^{-1}$) or maltodextrin (MALT) was added to the two smaller main meals of the day i.e. breakfast and lunch. Potential augmentation of the nutrient supplement was investigated by engagement in whole body, resistance-band exercise adapted specifically for this population (FORM + Progressive Resistance exercise Training (PRT)).

The main outcome of the RCT was a differential effect of the composition of the dietary supplement on whole body lean tissue mass. A statistically significant between treatment, net effect amounting to +0.60 kg LTM was observed (-0.16 ± 0.87 kg in MALT vs. 0.44 ± 1.06 kg in FORM; P = 0.004) after 24 weeks. In a separate group, the treatment effect attributed to supplementation with milk-based protein augmented the net change in LTM by engagement in PRT (0.80 ± 1.02 kg), but not to a statistically significant extent (P = 0.60).

In conclusion, a milk-based dietary supplement designed to correct habitual, sub-optimal, meal based protein intake in healthy, protein sufficient 50–70y old adults resulted in a positive effect on whole body lean tissue mass. The mechanism of action of this effect is informed by the investigation of the in vivo bioactivity of the milk-protein based supplement reported in this thesis.

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Abbreviations

WHO	World Health Organisation
LTM	Lean tissue mass
RCT	Randomised controlled trial
RE	Resistance exercise
ALTM	Appendicular lean tissue mass
ALTMI	Appendicular lean tissue mass index
SD	Standard deviation
EWGSOP	European working group on sarcopenia in older people
CSA	Cross sectional area
ULBC	University of Limerick Body Composition Study
MPS	Muscle protein synthesis
FSR	Fractional synthetic rate
DRI	Dietary reference intakes
NANS	National adult nutrition survey
IUNA	Irish universities nutrition alliance
NHANES	National Health and Nutrition Examination Survey
EAA	Essential amino acids
AA	Amino acid
MPB	Muscle protein breakdown
AMPK	Adenine monophosphate activated protein kinase
CEU	Contrast enhanced ultrasound
TOR	Target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1

eIF4EBP1	Eukaryotic translation initiation factor 4E binding protein 1
S6K	Ribosomal protein S6 kinase
1 RM	One repetition maximum
WP	Whey protein
PRT	Progressive resistance exercise training
NO	Nitric oxide
SNP	Sodium nitro-prusside
m. *VL*	Muscle *vastus lateralis*
AAT	Amino acid transporter
RT-PCR	Real time polymerase chain reaction
SNAT	Sodium-linked neutral amino acid transporter
LAT	L-type amino acid transporter
PAT	Proton assisted amino acid transporter
CAT	Cationic amino acid transporter
EAAT	Excitatory amino acid transporter
DXA	Dual-energy X-ray absorptiometry
PA	Physical activity
IV	Intravenous
AUC	Area under the curve
BCAA	Branch chain amino acid
MPC	Milk protein concentrate
DH	Degree of hydrolysis
RPHPLC	Reverse phase high performance liquid chromatography
OPA	o-phthalaldehyde

MPA	Mercaptopropionic acid
ANOVA	Analysis of variance
MD	Microdialysis
MWCO	Molecular weight cut-off
LC	Liquid chromatography
LC/MS	Liquid chromatography/Mass spectrometry
CE	Capillary electrophoresis
MeSH	Medical Library Subject Heading
AV	Arteriovenous
SLC	Solute linked carrier
WPI	Whey protein isolate
eFIR	Estimated food intake record
USDA	United States Department of Agriculture
IOM	Institute of Medicine
RPE	Rate of perceived exertion
SD	Standard deviation
IQR	Inter-quartile range
DOMS	Delayed onset muscle soreness

Chapter 1 Literature review

1.1 Context

In 2010, an estimated 524 million people were aged 65 or older, equating to 8% of the world's population. By 2050, this number is predicted to triple to approximately 1.5 billion, representing 16% of the world's population (World Health Organisation (WHO) (2011)). The term sarcopenia was coined in 1989 to describe the progressive aged related loss of lean tissue mass (LTM) (Rosenberg 1989). This definition has been updated to include not only a measurement of muscle mass but also the measurement of muscle strength and functional performance (Cruz-Jentoft *et al* 2010). The loss of LTM below a critical threshold compromises an individual's ability to complete activities of daily living and disability can ensue (Figure 1.1). The estimated direct health care cost of sarcopenia in the US in the year 2000 was $18.5 billion or approximately 1.5% of total healthcare expenditure for that year (Janssen *et al* 2004). Interest has therefore grown in strategies designed to preserve and/or increase LTM with advancing age (Figure 1.2), thus maintaining or improving the functional and metabolic benefits that an appropriate LTM affords. Two strategies that have been reported to be effective in maintaining and/or increasing LTM with advancing age are dietary and exercise interventions, either in isolation (Malafarina *et al* 2013, Peterson *et al* 2011) or in combination (Candow *et al* 2012, Dickinson *et al* 2013b).

Despite significant advances in the knowledge base with regard to the regulation of LTM, discrepancies exist within the literature. Of particular interest with regard to ageing are;

- the differing opinions on the definition of sarcopenia and its prevalence.
- the contribution of anabolic resistance to the process of muscle loss/wasting and;
- the effectiveness of dietary and exercise interventions designed to attenuate the age-related loss of skeletal muscle mass.

Specific to the dietary aspect of the paradigm, the proposal to increase the quality and quantity of protein intake and optimisation of the timing of protein ingestion in support of LTM preservation remains a contentious issue. Randomised controlled trials (RCT) investigating the impact of optimised distribution and improved quality of dietary protein intake on skeletal muscle mass, strength and function are required (Volpi *et al* 2013). This thesis will focus on the concept of 'keeping healthy people healthy' and as such the participants under investigation not overtly sarcopenic, functionality limited or energy/protein deficient. The research question is set against the effect of evenly distributing the daily protein load between the three main meals of the day, via

supplementation of the two smaller meals with milk protein. Change in LTM after 24-weeks is the main outcome variable.

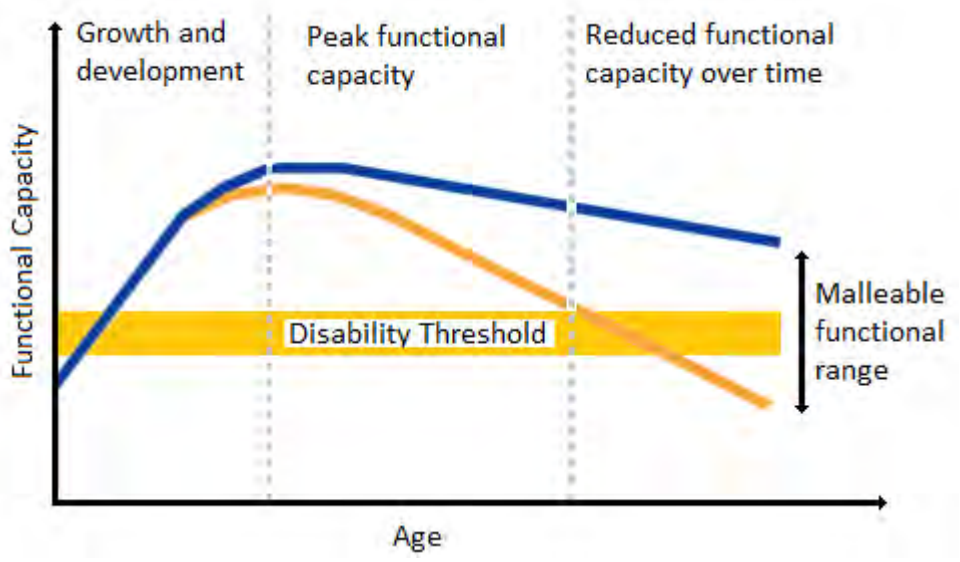

Figure 1.1 – Change in functional capacity over the life-course (adapted with permission from Kalache and Kickbusch (1997).

1.2 Sarcopenia

Data pertaining to the prevalence of sarcopenia depend on the criteria used to evaluate the condition. Baumgartner *et al* (1998) in the New Mexico Elder Survey defined sarcopenia as losses in skeletal muscle mass that resulted in an appendicular lean tissue mass (ALTM) greater than 2 standard deviations (SD) below the mean for young healthy controls. Iannuzzi-Sucich *et al* (2002) later defined sarcopenia as ALTM 2 SD below the ALTM normalised for stature relative to healthy young controls (appendicular lean tissue mass index, ALTMI). Patel *et al* (2013) using the European Working Group on Sarcopenia in Older People (EWGSOP) consensus definition (which includes measures of muscle mass, muscle strength and functional performance) reported a prevalence of sarcopenia of 4.6% and 7.9% for men and women, respectively, from a cohort of 765 male and 1,022 female participants (average age 67y) from the Hertfordshire Cohort Study. Within the literature, cross-sectional and longitudinal data describing the rates of change in skeletal muscle mass over time are equivocal depending on the age range observed and the variable(s) reported (total body LTM, upper leg (thigh) LTM, thigh cross sectional area (CSA)). Data from Hughes *et al* (2002) illustrate that total body LTM decreases at a rate of 2.0% per decade in men, with no change in women during a nine year period (initial age ~ 60 y). The authors state that the average change in LTM masked wider inter-individual variation.

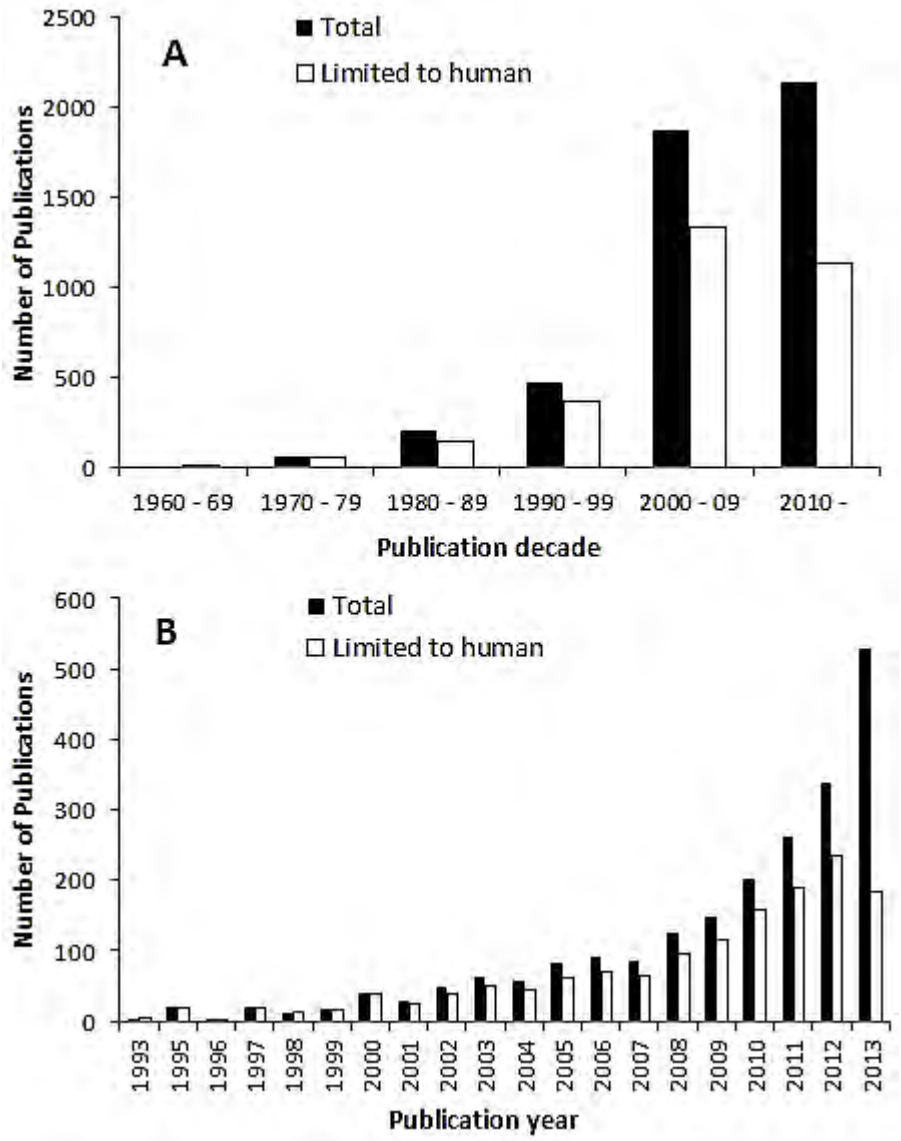

Figure 1.2 – Publications per decade (A) and per year (B) based on a bibliographical search on 7 February 2014 of the online database National Library of Medicines PubMed Central Medline (1950 to present) using the Medical Library Subject Heading (MeSH) terms "Muscle Wasting" AND sarcopenia (A) (1960 – 2013) and Sarcopenia (B) (1993 – 2013). Closed bar – total number of publications, open bar – limited to humans.

Goodpaster *et al* (2006) reporting data from the Health, Ageing and Body Composition (Health ABC) study report that the rate of decline ranges from 1 to 2 % of lower body LTM per-annum in populations aged 65+ and that this value does not vary between men and women. Mitchell *et al* (2012) summarising data from ~ 50 y of research examining the rate of decline in muscle mass in humans, calculated percentage loss per decade for each study included in their analysis. The median value for decline of total body LTM was 4.7 % per decade for men and 3.7% per decade for women when comparing populations at their peak LTM to those in their 7th, 8th and 9th decade. Cross-sectional data from the University of Limerick Body Composition Study (ULBC) (431

female and 121 male participants aged 50 and over) demonstrates that assuming a linear rate of decline the average loss is 1.39 kg per decade (3.5%) and 2.58 kg per decade (4.3%) for total body LTM and 0.66 kg per decade (3.7%) and 1.70 kg per decade (6.0%) for ALTM for women and men respectively (Figure 1.3, unpublished observations).

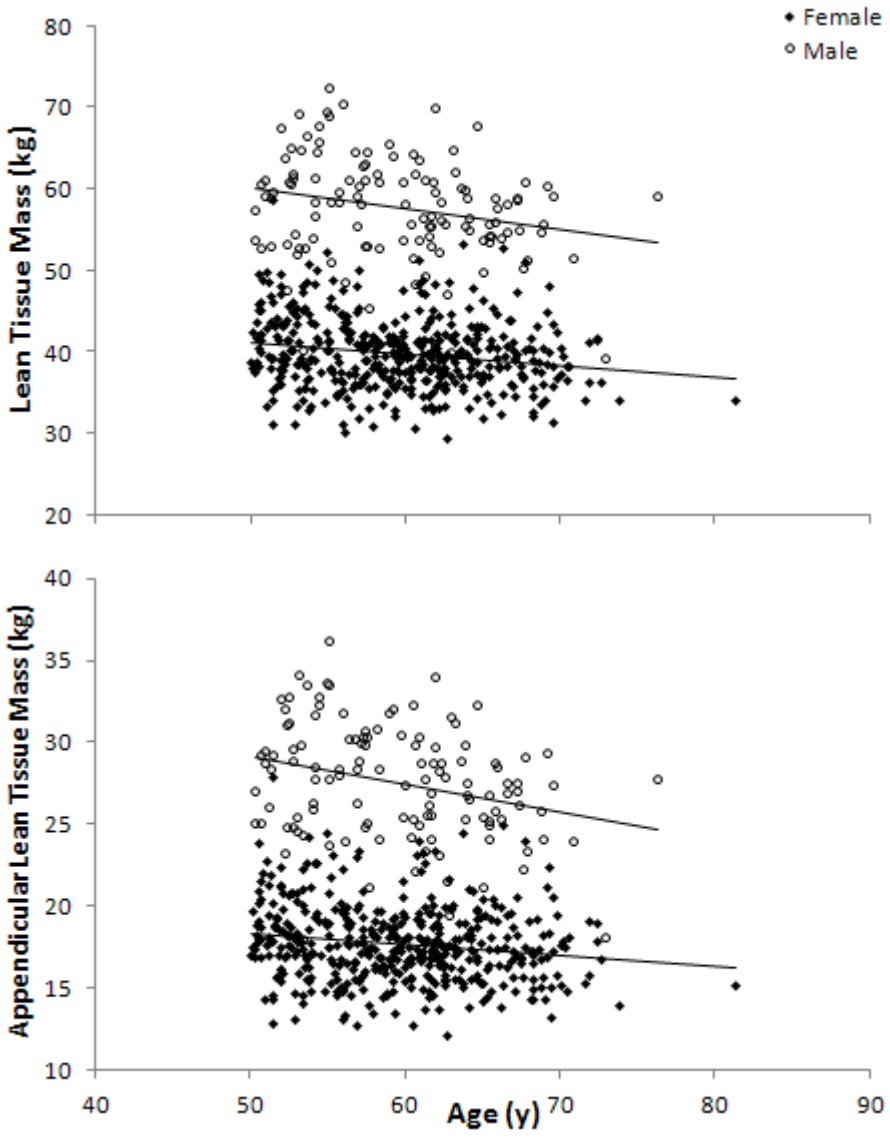

Figure 1.3 – Cross sectional change in lean tissue mass (A) and appendicular lean tissue mass (B) with age for 431 female and 121 male participants aged 50+ y from the University of Limerick Body Composition Study. Line represents liner regression line (line of best fit).

1.3 Dietary protein

The World Health Organisation (WHO) (Protein and amino acid requirements in human nutrition (World-Health-Organisation 2007)) states that "none of the published studies provides convincing evidence that protein requirement of elderly people differs from the protein requirement for younger adults (i.e. 0.85 $g.kg^{-1}$)". This report also advises that

"the main determinant of sarcopenia appears to be the decline in resistance-type physical activities with no evidence yet identified for any nutritional component". This view is supported by some senior researchers in the field of human muscle protein metabolism. Rennie (2009) commenting on supplementing the diet of elderly populations with additional protein states that "we are very sceptical of claims that supplementation of meals with extra amino acids (AA) or feeding more protein will restore the rate of muscle protein synthesis (MPS) in older people, relative to those found in the young". Millward (2008) comments that, rather than increased protein intakes, "the key to health and active longevity may be sufficient appropriate exercise and healthy eating to ensure adequate intakes of protein and most other key nutrients to maintain muscle and bone strength and mobility". In opposition to this Wolfe *et al* (2008) state that an intake of almost double the RDA (1.5 $g.kg^{-1}.d^{-1}$, 15-20% of total caloric intake) may be optimal in terms of maintaining health and function with advancing age. Houston *et al* (2008) reporting data from two thousand and sixty-six 70-79y old participants, again in the Health ABC study, demonstrated that energy adjusted protein intake was associated with three year changes in LTM. Participants in the highest quintile of protein intake lost approximately 40% less LTM than those in the lowest quintile of protein intake (−0.50 ± 0.11 kg vs. −0.88 ± 0.10 kg respectively).

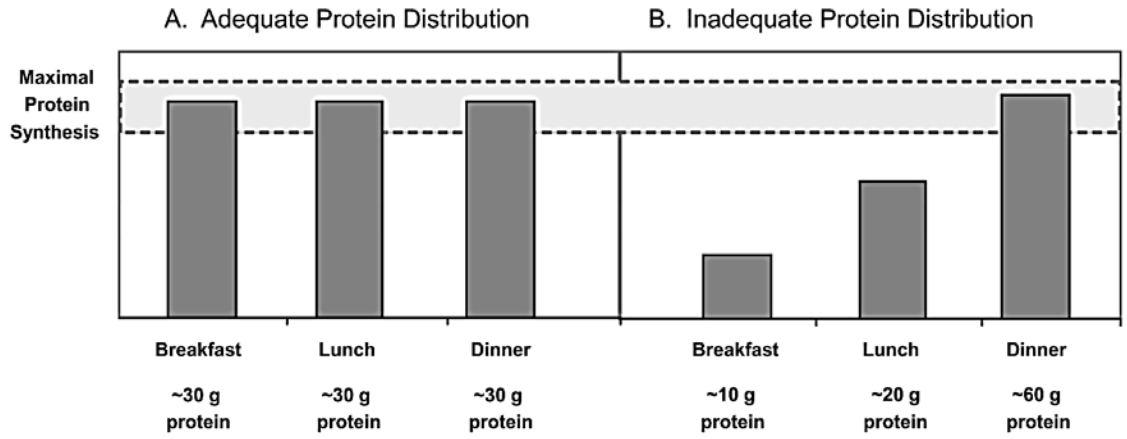

Figure 1.4 – Relationship between the amount of protein ingested per meal and the resultant anabolic response. (a) Ingestion of 90 g of protein, distributed evenly over 3 meals. (b) Ingestion of 90 g of protein unevenly distributed throughout the day (reproduced with permission from Paddon-Jones and Rasmussen 2009).

Paddon-Jones and Rasmussen (2009) recommend optimising the distribution of ingested protein over the course of the day to maximise MPS during each of the main eating occasions (Figure 1.4). The authors state that "rather than recommending a large global increase in the recommended dietary allowance for protein for all elderly populations, clinicians should stress the importance of ingesting a sufficient amount of

protein with each meal". They recommend ingesting 25-30 g of high quality protein per meal in order to maximally stimulate MPS at each eating occasion. This view is reinforced by the recent position paper from the PROT-AGE study group (Bauer *et al* 2013).

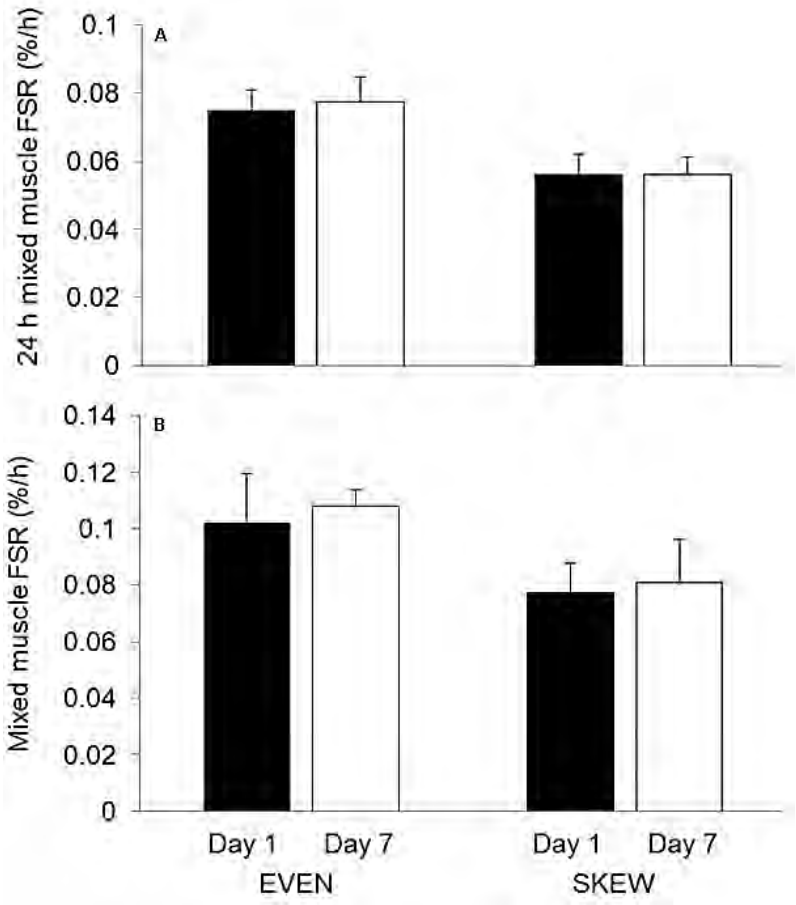

Figure 1.5 – 24 h mixed muscle protein fractional synthetic rate on day 1 and 7 after the ingestion of diets with EVEN or SKEW protein distributed (A) and mixed muscle protein fractional synthetic rate after the ingestion of a breakfast meal containing 30 g (EVEN) or 10 g (SKEW) of protein (B) (adapted with permission from Mamerow et al 2014).

Data from the Paddon-Jones group (Mamerow *et al* (2014)) has provided empirical support for the theory of distributing the protein load throughout the day in order to effectively stimulate MPS at each of the three main eating occasions. The effect of even (EVEN) versus skewed (SKEW) daily protein distribution on 24 h skeletal muscle protein fractional synthetic rate (FSR) was investigated in healthy adult men (n = 5) and women (n = 3) (36.9 ± 3.1 y). Utilising a 7-day crossover design with a 30 day washout period, the authors reported the change in the rate of MPS in response to diets with dietary protein intake distributed evenly or skewed between breakfast, lunch and evening (31.5 ± 1.3 g, 29.9 ± 1.6 g and 32.7 ± 1.6 g or 1.7 ± 0.8 g, 16.0 ± 0.5 g and

63.4 ± 3.7 g protein respectively). Over a period of 24h on day 1 and 7 of both the EVEN and SKEW diets muscle protein FSR was determined via a primed constant infusion of L-[ring-$^{13}C_6$]phenylalanine. Muscle biopsies were taken at 09:00h, 12:30h and at 24h after the initial biopsy (09:00) while meals were consumed at 09:30h, 13:00h and 17:00h. Daily energy intake and macronutrient content were not different between EVEN and SKEW (2400 kcal, 94.1 g protein, 312 g carbohydrate and 85.4 g fat vs. 2320 kcal, 90.1 g protein, 307 g carbohydrate and 81.7 g fat respectively). On days 1 and 7 FSR in response to EVEN was ~ 30% higher than SKEW (Figure 1.5 (A)). 24 h FSR was ~ 25% higher after breakfast in response to even distribution of protein intake relative to skewed (Figure 1.5 (B)). These data provide evidence that the distribution of the daily protein load to an even distribution between the main meals of the day offers a more favourable outcome for the retention and/or deposition of muscle protein than an intake skewed towards a large protein bolus ingested with the main meal of the day. This hypothesis ultimately needs to be tested in RCT to determine if acute changes in the FSR translate into long-term changes in skeletal muscle mass.

For a reference 65 kg female and 80 kg male the current dietary reference intake (DRI) (0.85 $g.kg^{-1}.d^{-1}$) represents an intake of 52 $g.d^{-1}$ and 68 $g.d^{-1}$ protein respectively. Harrington *et al* (2001) reported that a convenience sample of 51-64 year old Irish women (n = 162) and men (n = 173) consume a mean of 71 ± 17 $g.d^{-1}$ (~ 1.1 $g.kg^{-1}.d^{-1}$) and 96 ± 22 $g.d^{-1}$ (~ 1.2 $g.kg^{-1}.d^{-1}$) respectively. The National Adult Nutrition Survey (NANS) (2011) conducted by the Irish University Nutrition Alliance (IUNA) reported that in a convenience sample of women and men aged 65y and older, the average protein intake was 69.4 ± 17.8 $g.d^{-1}$ (1.02 $g.kg^{-1}.d^{-1}$) and 85.2 ± 24.1 $g.d^{-1}$ (1.03 $g.kg^{-1}.d^{-1}$). These data are comparable with the daily protein intake of 69.6 g and 66.8 g for women and 99.8 g and 89.3 g for men reported for 50-59y and 60-69y olds respectively in the National Health and Nutrition Examination Survey (NHANES) 2009-2010 (Rhodes *et al* 2012). Tieland *et al* (2012a) assessed daily protein intake, its distribution and source in 707 man and women aged between 65y and 97y who were participants in the Dutch National Food Consumption Survey (DNFCS 1998). Participants recorded their dietary intake over a two day period. Records were coded and energy and protein intakes were calculated with a computerised Dutch food consumption table (Dutch food consumption table, 1996). The following variables were reported; protein intake ($g.d^{-1}$), protein intake per kilogram body mass ($g.kg^{-1}.d^{-1}$), percentage of energy from protein (EN %), protein take (g) per mealtime moment (breakfast, lunch, evening and between meal snacks) and finally the protein source was reported. Participants were stratified into two age groups; 65-74 y and 75-97 y.

In the 65-74y old group (which most closely represents the population under investigation in this thesis) energy intake was 7.5 ± 1.9 $MJ.d^{-1}$. Protein intake was 72.9 ± 18.2 $g.d^{-1}$ or 1.03 ± 0.35 $g.kg^{-1}.d^{-1}$, corresponding to 16.9 ± 3.9% of energy intake. On average protein intake was 10 g, 27 g and 32 g during breakfast, lunch and evening meal respectively. Considerable inter-individual variability was evident in protein intake. The main contributors to daily protein intake were meat (29%), dairy (18%), bread (14%), cheese (9%), fish (5%) and other (25%). 10% of the population had a protein intake below the estimated average requirement (EAR) of 0.7 $g.kg^{-1}.d^{-1}$. These data inform as to the distribution of the daily total protein load (notwithstanding the inherent limitations of self-reported dietary food records) and are relevant in light of the arguments presented by amongst others Bauer *et al* (2013) and Paddon-Jones and Rasmussen (2009) with regard to the need to evenly distribute the daily protein load in order to stimulate MPS at each of the main eating occasions of the day. Breakfast in particular appears to provide an opportunity to increase the quantity of dietary protein ingested in support of the stimulation of net protein synthesis. These data do not however describe the protein quality (essential amino acid (EAA) profile for example) and this information is desirable at a meal level analysis as the EAA and in particular leucine are among the main drivers of the muscle protein synthetic response.

1.4 Regulation of skeletal muscle mass

The body of a 70 kg individual contains approximately 11 kg of protein (dry mass (DM)). Skeletal muscle accounts for approximately 43% of that protein and somewhere between one third and half of total protein turnover in the body (Rennie and Tipton 2000). Muscle is also the major store of free AA in the body, accounting for in excess of 90% of the total pool (Bergstrom *et al* 1974). Muscle is therefore a key regulator of whole body protein metabolism. The continuous cycling of AA through MPS and muscle protein breakdown (MPB) results in the maintenance of muscle protein net balance subject to appropriate energy intake and expenditure. Wackerhage and Rennie (2006) describe the process as;

"The normal temporal sequence of feeding and fasting throughout the diurnal cycle enables muscle protein synthesis during relatively short, fed periods to replace protein broken down during periods between meals and hence accomplish the regular renewal of structural and functional components of the muscle mass at least".

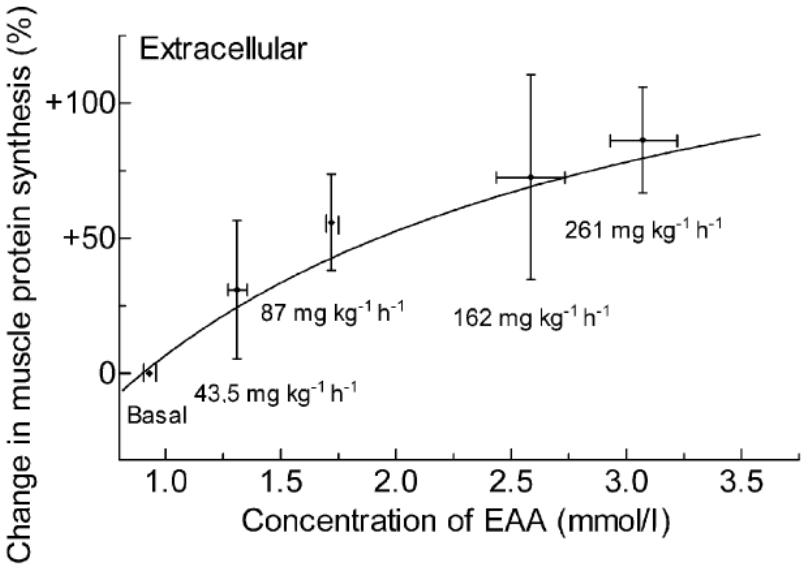

Figure 1.6 – The effect of mixed muscle protein synthesis (measured as incorporation of D_5-phenylalanine or D_3-leucine) in human skeletal muscle of extracellular essential amino acid concentration achieved by infusion of a mixed amino acid solution at increasing rates (adapted with permission from Bohe et al 2003)

Regulation of human skeletal muscle mass remains a relatively new branch of human science. The first measurements of human MPS via stable isotope tracer technologies were made less than forty years ago (Halliday and McKeran 1975). The effects of feeding (Rennie *et al* 1982) and resistance exercise (RE) (Chesley *et al* 1992) on MPS were reported in the years that followed. The age-related loss of muscle protein is attributable to an imbalance between MPS and MPB, resulting in a negative muscle protein net balance over time and a decline in skeletal muscle mass (Burd *et al* 2013). At rest, in the fasting post absorptive state, MPB is in excess of the rate of MPS and the body is in a catabolic state. Both infusion and ingestion of AA have been shown to stimulate MPS at rest such that muscle protein net balance becomes positive (i.e. synthesis is in excess of breakdown). The extent of the stimulation is dependent on the AA dose, the profile of the AA (essential vs. non-essential), the pattern of ingestion (constant dose vs. bolus ingestion), the age of the individual and their hormonal profile (Wolfe 2006). Bohe *et al* (2003) demonstrated that the rate of MPS is regulated by the extracellular EAA concentration over their normal diurnal range as oppose to the intramuscular EAA concentration and that this relationship is curvilinear (Figure 1.6). Following RE in the fasted state, rates of MPS and MPB are elevated but muscle protein net balance remains negative in the absence of AA feeding. There is a synergistic effect between RE and AA on MPS such that the anabolic response to AA following RE is greater than the sum of these stimuli in isolation (Figure 1.7).

1.4.1 Synergistic effect of resistance exercise and protein/amino acid feeding

Protein synthesis is temporarily inhibited following an acute bout of RE though increased adenine monophosphate activated protein kinase (AMPK) activation and reduced phosphorylation of key signaling proteins of translation initiation (Dreyer *et al* 2006). The capacity for MPS is restored approximately 60 min post exercise and ultimately enhanced above baseline via the activation of components of the mammalian target of rapamyacin complex 1 (mTORC1) signaling cascade. The signaling pathways stimulated by nutrient ingestion and contraction converge at mTORC1. mTORC1 regulates MPS through phosphorylating downstream protein effectors such as eukaryotic initiation factor 4EBP1 (eIF4EBP1) and ribosomal protein S6 kinase (S6K). AA provision post RE and the subsequent increase in MPS are associated with enhanced phosphorylation of components of the mTORC1 pathway over and above levels observed post exercise in the absence of feeding. Symons and colleagues (Symons *et al* 2011, Symons *et al* 2009, Symons *et al* 2007) demonstrated that a combination of high quality protein (~ 30 g fed as lean ground beef) and RE (6 sets of 8 repetitions of leg extension exercise at 80% of the individuals one repetition max (1 RM)) resulted in a 40% increase in FSR compared to ingestion of the meal only ($0.152 \pm 0.017.h^{-1}$ vs. $0.108 \pm 0.006.h^{-1}$ respectively).

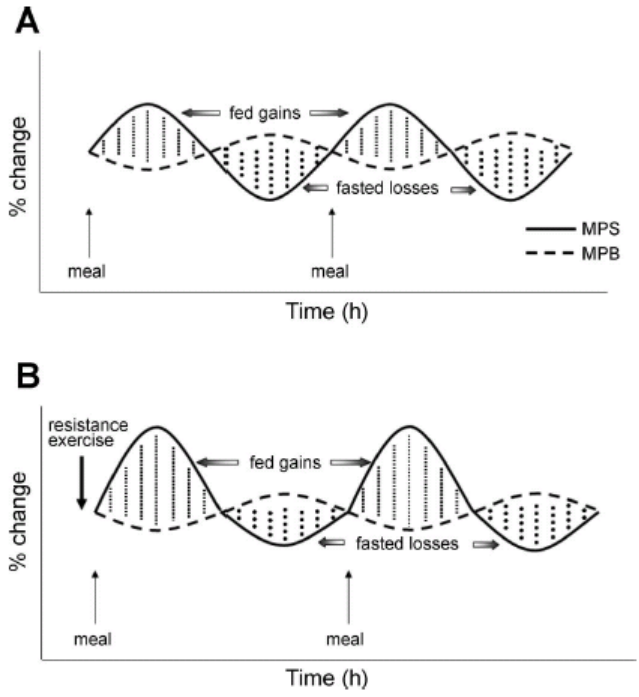

Figure 1.7 – Changes in muscle protein synthesis and muscle protein breakdown in response to feeding (A) (i.e. amino acids) and changes in muscle protein synthesis and muscle protein breakdown in response to feeding plus resistance exercise (B) (reproduced with permission from Burd et al (2009)).

Further evidence of the synergistic effect of protein ingestion and RE is provided by Yang *et al* (2012) who investigated the dose response of MPS to ingestion of graded amounts of whey protein (WP), both with and without prior RE. Thirty-seven elderly men (71 ± 4y) completed a bout of unilateral lower leg RE prior to ingesting 0, 10, 20 or 40 g of WP. Whole body leucine oxidation and post-prandial MPS were measured via infusion of L-[1-^{13}C]leucine and L-[ring-$^{13}C_6$]phenylalanine and subsequent bilateral muscle biopsy. The authors reported that ingestion of 20 g of WP, corresponding to ~ 2 g of ingested leucine, is necessary to increase myofibrillar MPS above basal rates in older adults at rest and this response is not enhanced when the dose is increased to 40 g of WP (Figure 1.8). Myofibrillar FSR was however greater in the exercised leg relative to the resting leg for all whey doses (13, 44 and 91% greater with post exercise ingestion of 10, 20 and 40 g of WP respectively). These data support the theory that the provision of a nutrient solution designed to support the maintenance of LTM in older populations will be enhanced through the concurrent adoption of a program of progressive resistance exercise training (PRT).

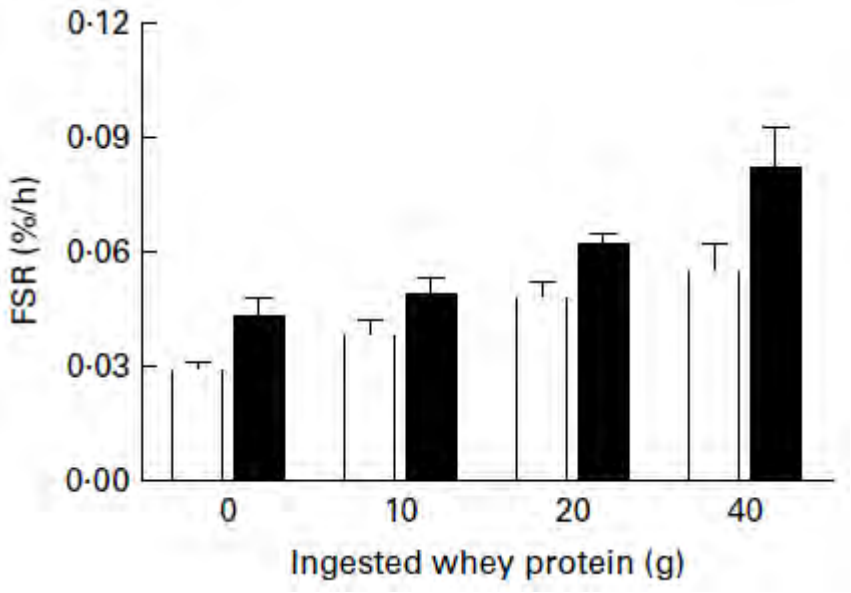

Figure 1.8 – Myofibrillar protein fractional synthetic rate (%.h^{-1}) based on gram amount of protein ingested per group. Data are mean with standard error bars. Open bar – resting leg, closed bar – exercised leg (adapted with permission from Yang et al 2012).

1.5 Anabolic resistance

Ageing is not thought to be associated with altered fasting post-absorptive rates of MPB or MPS, although some authors have reported impairments in elderly participants relative to young (Short *et al* 2004, Guillet *et al* 2004). The focus is currently on describing what appears to be a blunted muscle protein synthetic response to the main anabolic stimuli, muscle contraction (Chesley *et al* 1992) and EAA (Volpi *et al* 2003) in

older populations relative to healthy young adults. Contraction has been shown to elevate the rate of MPS for a period of up to 72h post the initial exercise bout (Phillips *et al* 1997) while EAA show a saturable and transient effect in stimulating MPS (Bohe *et al* 2001). This blunted response in the elderly has been termed anabolic resistance and can be defined as the impaired rate of cellular anabolism in response to anabolic stimuli. According to Burd *et al* (2013) contributing factors to anabolic resistance of MPS with ageing include impairments in; protein digestion and AA absorption, insulin mediated muscle tissue perfusion, AA uptake in muscle and the amount or activation status of key signalling proteins.

These mechanisms of action are complex physiological phenomenon and this fact has led to some conjecture within the literature as to the actual existence of age associated anabolic resistance. Several studies have failed to measure differences in muscle protein metabolism between young and old populations in response to a number of different paradigms including AA infusion (Volpi *et al* 1998), ingestion of crystalline AA (Paddon-Jones *et al* 2004) and intact protein in isolation (Symons *et al* 2007) and when taken in conjunction with RE (Symons *et al* 2011). Resolving the mechanisms underpinning anabolic resistance is therefore quite difficult if the phenomenon cannot be repeatedly demonstrated or reproduced. Some of the variance observed within the literature can be accounted for by the fact that different research groups have adopted varying approaches to the measurement of the rate of MPS *in vivo* in human skeletal muscle (e.g. primed constant tracer AA infusion vs. flooding dose tracer AA administration to measure FSR, choice of tracer (L-[1-^{13}C] Leucine vs. L-[1^{13}C] phenylalanine), choice of precursor/product (ketoisocaproate (KIC), muscle free leucine/phenylalanine, plasma free leucine/phenylalanine), bound protein analysis (GC-C-IRMS vs. GCMS), biopsy timing and the relatively small sample numbers reported (<10 participants in many instances) (Smith *et al* 2011). According to Smith *et al* (2011) “The reported values vary by as much as four-to-five fold even when the comparison is limited to a single experimental approach applied to well-defined study populations and conditions”. The fact that participants are often in the fasting post-absorptive state prior to commencing the assessment is also a methodological limitation. Finally differences in the amount, quality and timing of protein ingestion coupled with variation in the volume, duration and intensity of exercise undertaken has also contributed to inconsistent findings within the extant literature with regard to anabolic resistance in ageing/aged populations. Notwithstanding the discrepancies within the literature, the consensus appears to be that anabolic resistance is a reality as a contributing factor to the age-related decline in LTM.

1.6 Mechanisms underlying anabolic resistance

1.6.1 Protein digestion and absorption

Boirie *et al* (1997) compared the postprandial response of leucine kinetics to a standardised meal in six young and six elderly male participants (mean age 22.7 y and 68.2 y respectively). Whole body and splanchnic protein metabolism were determined before and after consumption of a standardised mixed meal (41.8 $kJ.kg^{-1}$, 50% EN carbohydrate, 35% EN fat, 15% protein (whey protein concentrate)) by employing a combination of intravenous L-[1-^{13}C] leucine and oral L-[5,5,5-$^{2}H_3$] leucine tracers. The authors reported that splanchnic extraction (gut + liver) of dietary leucine increases with age in the fed state irrespective of its expression as a function of total body mass or fat free mass (Table 1.1), although splanchnic extraction was more variable in the older participants than the younger (CV; 48% and 21% respectively). The authors postulate that this increased splanchnic extraction observed in the older participants could be as a result of increased leucine oxidation in the gut and/or liver, increased utilisation of leucine for hepatic protein synthesis and increased transamination of leucine in the gut or liver (or a combination of all three). The result however and its relevance to anabolic resistance is the decreased availability of dietary leucine to the peripheral tissues that could contribute to decreased rates of MPS during feeding. Leucine not only acts as a substrate for MPS, but also acts as a nutrient signal that regulates the mRNA translational machinery through direct activation of the mTORC1/S6K pathway (Drummond and Rasmussen 2008).

Table 1.1 – Splanchnic extraction of dietary leucine in response to a mixed meal (adapted from Boirie et al (1997)).

Leucine Kinetics (Splanchnic Extraction)		
	Young (n=6)	**Old (n=6)**
μmol.kgBM^{-1}.min^{-1}	0.30 ± 0.03	0.66 ±0.15
μmol.kgFFM^{-1}.min^{-1}	0.35 ± 0.03	0.99 ± 0.25

Data are mean ± SE

Volpi *et al* (1999) measured MPS, MPB and AA transport in 7 young (30 ± 2y) and 8 elderly (71 ± 2y) participants in the post absorptive state and in response to ingestion of an AA solution (40 g AA dissolved in 540 mL water, 7.4% (w/v)). The solution was consumed in eighteen 30 mL bolus, every 10 min for 180 min. Muscle protein FSR was measured as the rate of incorporation of L-[ring-$^{2}H_5$] phenylalanine while L-[ring-$^{13}C_6$] phenylalanine was added to the ingested AA solution to measure first pass splanchnic extraction. The authors reported that first pass splanchnic extraction of oral phenylalanine was greater in the elderly relative to the young (47 ± 3% vs. 29 ± 5% respectively). These data are consistent with those reported by Boirie *et al* (1997).

Muscle protein FSR was similar between young and elderly in the fasted state (young 0.0435 ± 0.0037 %.h^{-1}, elderly 0.0495 ± 0.0088 %.h^{-1}). Ingestion of MAA resulted in an increase in mixed muscle protein FSR with no statistically significant difference between the two groups (young 0.0793 ± 0.0093 %.h^{-1}, elderly 0.0953 ± 0.0144 %.h^{-1}).

Table 1.2 – Arterial phenylalanine (phe) concentration and delivery of phenylalanine to the leg at rest and in response to oral amino acid ingestion (adapted from Boirie at al (1997)).

	Young		Elderly	
	Basal	**AA Ingestion**	**Basal**	**AA Ingestion**
Arterial phe [ ] (μmol.l^{-1})	55 ± 3	113 ± 10	59 ± 2	120 ± 8
Delivery to the leg (nmol.min^{-1}.100mL leg^{-1})	178 ± 25	355 ± 50	174 ± 28	396 ± 75

Data are man ± SEM.

Boirie *et al* (1997) did not report an increase in above basal levels in the rate of MPS in response to a mixed meal and there are several methodological differences between these studies that could explain the difference in outcomes in relation to the rate of MPS (timing, dose, quality of protein ingested for example). Based on these data, Volpi *et al* (1999) state that "it is unlikely that the loss of muscle mass with age is due to an age-related impairment of the intestinal absorption of AA or to an increased net splanchnic utilisation of the ingested EAA". Despite the high splanchnic extraction both arterial phenylalanine concentration and phenylalanine delivery to the muscle increased to a similar extent in the young and the elderly in response to the ingestion of the same gram amount of protein (Table 1.2).

Dangin *et al* (2003) investigated the rate of digestion of dietary protein on whole body post-prandial protein metabolism in healthy elderly (n = 9, 72 ± 1 y) and young (n = 6, 24 ± 2 y) men. Protein metabolism was assessed via a combination of oral (L-[5,5,5-$^{2}H_3$]leucine) and intravenous (L-[1-^{13}C]leucine) stable isotope measurements. Participants were studied on three separate occasions. On each occasion they consumed a mixed meal (0.75 g.kg^{-1} CHO and 0.13 g.kg^{-1} fat) with the addition of either one of casein (CAS) (0.48 g.kg^{-1}, ~ 34 g protein, 296 μM leucine.kg^{-1}), WP with the same amount of leucine (WPiL) (0.31 g.kb^{-1}, ~ 22 g protein, 296 μM leucine.kg^{-1}) or WP with the same amount of nitrogen (WPiN) (0.48 g.kg^{-1}, 34 g protein, 449 μM leucine.kg^{-1}). The percentage of ingested leucine extracted by the splanchnic tissues was 31 ± 4 %, 34 ± 1% and 36 ± 3% in young and 25 ± 4%, 31 ± 2% and 32 ± 1% in elderly for WP-iN, WP-iL and CAS respectively. The rate of leucine splanchnic extraction was not higher in the elderly relative to the young and was lower than rates reported elsewhere (Boirie *et al* 1997). Rates of MPS and MPB were not different between age groups in response to any of the three conditions.

Koopman *et al* (2009) investigated whether dietary protein digestion and absorption kinetics and postprandial MPS is impaired in healthy elderly man (n = 10, 64 ± 1y) relative to healthy young controls (n = 10, 23 ± 1y). The authors measured exogenous and endogenous rate of appearance (R_a) of phenylalanine, splanchnic phenylalanine extraction and mixed muscle protein FSR via stable isotope tracer techniques prior to and for a 6h period following ingestion of ~ 35 g of intrinsically L-[1-^{13}C]phenylalanine labelled intact CAS. Exogenous phenylalanine appearance rates were 38 ± 2 μM.6h^{-1}.kg^{-1} in elderly man and 39 ± 3 μM.6h^{-1}.kg^{-1} in young men. Splanchnic extraction did not differ between elderly (73 ± 1%) and young (72 ± 2%) participants. Rates of MPS did not differ between elderly and young men when measured as either the rate of incorporation of intrinsically labelled phenylalanine (0.063 ± 0.006 %.h^{-1} vs. 0.054 ± 0.004 %.h^{-1}) or labelled leucine from tracer infusion (0.064 ± 0.007 %.h^{-1} vs. 0.052 ± 0.005 %.h^{-1}). The authors conclude that in response to ingestion of a large bolus of intact CAS protein, rates of digestion, absorption and MPS are not impaired in healthy elderly men relative to young controls. Koopman and colleagues comment that "the percentage of the AA extracted within the splanchnic area seems to vary among different AA and depends on the amount, quality and digestibility of the dietary protein source". These characteristics may help explain discrepancies observed within the literature with regard to differing rates of splanchnic extraction observed between young and elderly participants (Volpi *et al* (1999) and Boirie *et al* (1997) both reported elevated rates of splanchnic extraction in elderly participants relative to young while Dangin *et al* (2003) and Koopman *et al* (2009) reported similar rate of splanchnic extraction between young and elderly participants). Differences in these characteristics may also explain conflicting findings within the literature with regard to rates of MPS between young and elderly participants in response to protein and AA feeding.

Pennings *et al* (2012) investigated digestion and absorption kinetics in response to ingestion of graded amounts of WP and its subsequent incorporation into newly synthesised muscle protein in older men (73 ± 2y). Thirty-three healthy older men were randomly assigned to one of three treatments to receive 10 g, 20 g or 35g of intrinsically L-[1-^{13}C]phenylalanine labelled WP. Digestion and absorption kinetics (Figure 1.9), splanchnic extraction, whole body protein metabolism and postprandial MPS were measured via stable isotope tracer techniques. The authors reported that 61 ± 1%, 63 ± 3% and 59 ± 2% of the ingested dietary phenylalanine appeared in the circulation in response to ingestion of 10 g, 20 g and 35 g of WP, respectively. These rates of appearance did not differ between treatments. Expressed as absolute amounts, these values correspond to the retention by the gut of 4 ± 1 g, 8 ± 1 g and 14

± 1 g of the ingested dietary WP that therefore did not appear in the circulation during the 4h postprandial period that participants were observed. Basal FSR were not different between treatments (10 g 0.035 ± 0.002 %.h^{-1}, 20 g 0.037 ± 0.003 %.h^{-1}, 35 g 0.039 ± 0.003 %.h^{-1}). Ingestion of 10 g of WP did not increase FSR (0.029 ± 0.004 %.h^{-1} (-1 ± 14 % from basal)) while ingestion of 20 g and 35 g of WP increased FSR (0.041 ± 0.004 %.h^{-1} (16 ± 13 % from basal), 0.052 ± 0.004 %.h^{-1} (44 ± 16 % from basal) respectively). These data demonstrate that ingestion of 35 g of WP results in greater AA absorption and subsequent MPS relative to 10 g or 20 g of equivalent protein in elderly men.

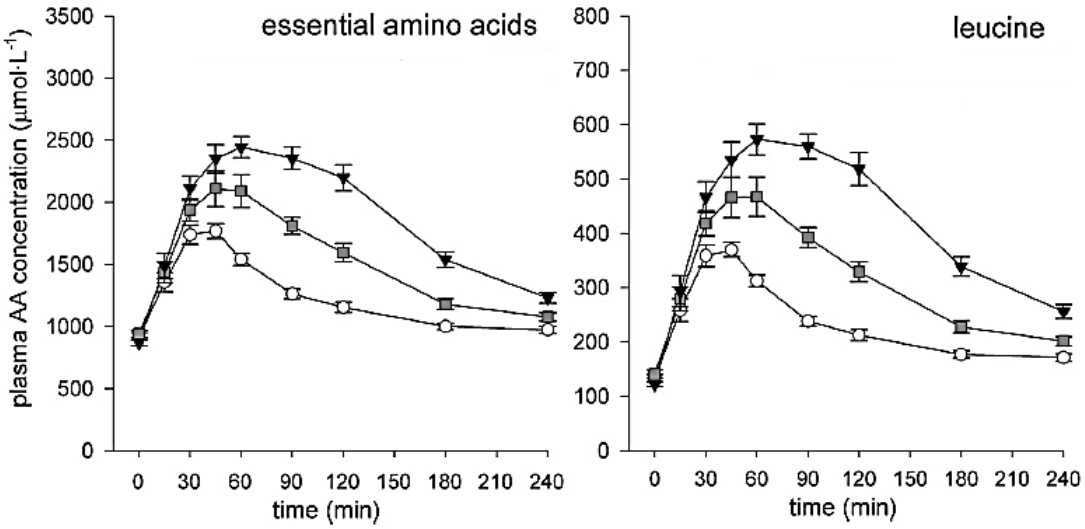

Figure 1.9 – Plasma leucine and essential amino acid concentration following ingestion of 10, 20 and 35 g of intrinsically labelled whey protein (reproduced with permission from Pennings et al 2012).

1.6.2 Age-related muscle protein insulin resistance

Insulin is deemed permissive as opposed to modulatory in terms of regulation of MPS and plasma insulin levels of ~ 70-100 pM.L^{-1} are sufficient to allow a maximal muscle protein synthetic response to meal ingestion (Koopman and van Loon 2009). However MPS is resistant to the anabolic action of insulin specifically in the elderly. Fujita *et al* (2009) investigated if the age-related defect in muscle protein anabolism is related to insulin resistance in the elderly and could be overridden by supra-physiological hyperinsulinemia. 14 healthy elderly participants were randomly assigned to either one of two conditions, postprandial dose (PD) (0.15 mU.min^{-1}.100 mL^{-1}, n = 8) or high dose (HD) (0.30 mU.min^{-1}.100 mL^{-1}, n = 6) insulin infusion. Leg blood flow (indocyanine green (ICG) dye dilution), mixed muscle protein FSR (rate of incorporation of L-[ring $^{13}C_6$] phenylalanine) and Akt/mTORC1 signalling (immunoblotting) were measured over an 8h period in the post-absorptive state. Leg blood flow, muscle protein FSR and

Akt/mTORC1 signalling were not different between groups at baseline. In response to hyperinsulinemia, blood flow and MPS increased in the HD group only (PD; 0.063 ± 0.006 %.h^{-1} to 0.060 ± 0.005 %.h^{-1} vs. HD; 0.061 ± 0.007 %.h^{-1} to 0.098 ± 0.007 %.h^{-1}). Muscle Akt phosphorylation increased in both groups while S6K1 phosphorylation increased in the HD group only. mTORC1, eIF4EBP1 and eIF2 phosphorylation did not change in either group in response to insulin infusion with no differences between treatments. The authors conclude that skeletal muscle protein metabolism is insulin resistant in healthy non-diabetic older persons, as protein anabolism can be elicited only by supra-physiological hyperinsulinemia. This phenomenon appears to be related to endothelial dysfunction and blunted anabolic signalling. These data are however from a relatively small number of participants who are investigated in an atypical state (post-absorptive at baseline with a further period of fasting over the course of the 8h investigation). It is unclear as to the effect that the extended fasting condition may have influenced the response to the insulin infusion.

Table 1.3 – Leg blood flow and phenylalanine (phe) kinetics in two groups of healthy older subjects in the basal state and during local insulin infusion in one leg with sodium nitroprusside (SNP) or without (Control) (data from Timmerman et al 2010).

	Control		SNP	
Variable	**Basal**	**Insulin**	**Basal**	**Insulin**
Leg blood flow (mL.min^{-1}.100 mL leg^{-1})	2.6 ± 0.5	3.4 ± 0.6	2.4 ± 0.5	5.6 ± 0.7
Delivery to the leg (nmol.min^{-1}.100 mL leg^{-1})	162 ± 31	196 ± 28	151 ± 31	346 ± 39
Phe Net balance (nmol.min^{-1}.100 mL leg^{-1})	-17 ± 3	-2 ± 3	-15 ± 3	26 ± 12

Data are mean ± SE.

Timmerman *et al* (2010) investigated whether a restoration of nitric oxide (NO) dependent vasodilation during hyperinsulinemia would increase MPS and net protein balance in older adults. Twelve healthy, non-diabetic, older participants (71 ± 2y) were randomised to one of two groups, control (insulin infusion only, infusion rate 0.15 mU.min^{-1}.100 mL of leg^{-1}, n = 6) or insulin plus sodium nitroprusside (SNP) infusion (average SNP dose 0.06 ± 0.01 µg.min^{-1}.100 mL of leg^{-1}, n = 6). Leg blood flow (dye dilution), muscle microvascular perfusion (contrast enhanced ultrasound), Akt/mTORC1 signalling (immunoblotting), and MPS (incorporation of L-[ring$^{13}C_6$] phenylalanine,) were measured over a seven hour period. There were no differences between groups at baseline. Leg blood flow, muscle perfusion, phenylalanine delivery to the leg and intracellular availability of phenylalanine increased considerably in the SNP group only (Table 1.3). MPS and protein net balance increased in the SNP group but not in the control group. The authors conclude that enhancement of muscle

perfusion and AA availability during hyperinsulinemia as a result of SNP infusion improves the muscle protein anabolic effect of insulin in older adults.

Rasmussen *et al* (2006) examined the effects of hyperinsulinemia on muscle protein metabolism in 7 healthy young volunteers (4 women, 3 men, age 25 ± 2y) and 6 healthy older volunteers (1 female, 5 male, age 68 ± 1y). Femoral artery blood flow (ICG dye dilution) phenylalanine kinetics and MPS (rate of incorporation of a primed continuous infusion of L-[ring-$^{2}H_5$]phenylalanine or L-[ring-$^{13}C_6$]phenylalanine tracer into muscle protein) were measured in the fasted post-absorptive state and in response to the infusion of insulin (0.15 μU.min^{-1}.100 mL leg^{-1}) into the femoral artery. Muscle biopsies were taken from the muscle *vastus lateralis* (m. *VL)* at 2h, 5h and 8h after the commencement of labelled phenylalanine infusion. Blood flow was lower in the older participants relative to the younger at rest (young; 4.0 ± 0.4 mL.min^{-1}.100 mL leg^{-1}, older; 2.6 ± 0.2 mL.min^{-1}.100 mL leg^{-1}) and during hyperinsulinemia (young: 6.7 ± 1.3 mL.min^{-1}.100 mL leg^{-1}, older 2.7 ± 0.2 mL.min^{-1}.100 mL leg^{-1}). Phenylalanine disappearance from the blood (Rd) and utilisation for protein synthesis (F_{om}) were not different between groups at baseline (Table 1.4). However phenylalanine net balance and muscle protein FSR became positive only in the young, reflecting that a switch from net protein breakdown to net protein synthesis occurred only in the young in response to insulin infusion. The authors conclude that MPS does not increase when older skeletal muscle is subjected to local hyperinsulinemia when compared to younger populations. There was no effect of the change in AA concentration on the change in protein synthesis but there was a positive relationship with the change in blood flow and AA availability. A reduced vasodilatory response of older muscle therefore plays an important role in the reduced anabolic action of insulin in ageing by decreasing nutrient flow and substrate bioavailability.

Table 1.4 – Phenylalanine kinetics across the leg in young and older adults in the basal state and during insulin infusion in the femoral artery of one leg (data from Rasmussen et al 2006).

	Young		Old	
	Basal	**Insulin**	**Basal**	**Insulin**
F_{OM}	40 ± 8	93 ± 21	58 ± 8	57 ± 12
Rd	35 ± 8	71 ± 14	48 ± 9	40 ± 7
Phenylalanine NB	-22 ± 5	15 ± 4	-15 ± 2	-4 ± 2

Data are mean ± SE

Dillon *et al* (2011) investigated whether age-related differences exist in the muscle protein anabolic response to AA between young and older adults when macro and micro-vascular leg blood flow were increased through infusion of the NO donor, SNP.

Participants (young; n = 7, 5 female, 2 male, 30 ± 2y and older; n = 7, 4 female, 3 male, 67 ± 2y) were studied in the fasted post-absorptive state and in response to infusion of SNP and AA. Femoral artery blood flow (ICG dye dilution) and micro-vascular blood flow (contrast enhanced ultrasound (CEU)), mixed muscle FSR (rate of incorporation of L-[ring-$^{13}C_6$] phenylalanine into protein), phenylalanine kinetics (three-pool model), and key regulators of NO synthesis, cellular energy metabolism and skeletal muscle anabolism (immuno-blotting) were measured over a 7h period (4h basal measurements, 3h SNP + AA infusion).The primed continuous infusion of L-[ring-$^{13}C_6$]-phenylalanine was started at time 0h and maintained for 7h. After 4h infusion of SNP (0.114 μg.kg^{-1}.min^{-1}) and a primed constant infusion of AA began. A total of four biopsies were taken at 2, 4, 5 and 7h. There were no differences between young and older participants in macro or micro-vascular blood flow, phenylalanine net balance, muscle protein FSR and model-derived MPS at baseline. Irrespective of age, infusion of SNP and AA induced increases in macro-vascular flow (young: 4.3 vs. old: 4.4 mL.min^{-1}.100 mL leg^{-1}), micro-vascular flow (young: 1.4 vs. old: 0.8 video intensity.s^{-1}), phenylalanine net balance (young: 59 vs. old: 68 nM.min^{-1}.100 mL leg^{-1}), muscle protein FSR (young: 0.02 vs. old: 0.02 %.h^{-1}) and model derived MPS (young: 62 vs. old: 49 nM.min^{-1}.100 mL leg^{-1}). These data support the concept that healthy ageing vasculature is responsive to exogenous NO and that there is no age-related difference as such in AA induced anabolism under hyperemic conditions.

1.6.3 Age-related decrements in anabolic signalling

RE and AA stimulate protein synthesis via diverse signaling pathways that converge at the level of target of rapamycin (TOR). TOR is purported to be the master regulator of cellular processes such as transcription, translation, ribosomal biogenesis and autophagy. TOR is an evolutionary conserved, 289kDa Serine/Threonine kinase composed of 2,459 AA and is present in mammalian cells as two structurally and functionally distinct complexes mTORC1 and mTORC2. mTORC1 is composed of the proteins mTOR, mLST8 and raptor (regulatory associated protein of TOR) and is considered the nutrient responsive mediator of cell growth regulation. mTORC2 contains mTOR, mLST8, rictor (rapamycin-insensitive companion of TOR), mSIN1 and PRR5 and is involved in the regulation of the actin cytoskeleton and certain AGC kinase sub-families such as Akt/PKBs (Avruch *et al* 2009). mTORC1 is thought to control cell growth through two mechanisms; (a) regulating efficiency of the protein translation process through inducing phosphorylation of its substrates, S6k1 and eIF4EBP1 and (b) increasing translational capacity through regulating ribosomal RNA (rRNA) production within the nucleolus (Iadevaia *et al* 2012) (Figure 1.10).

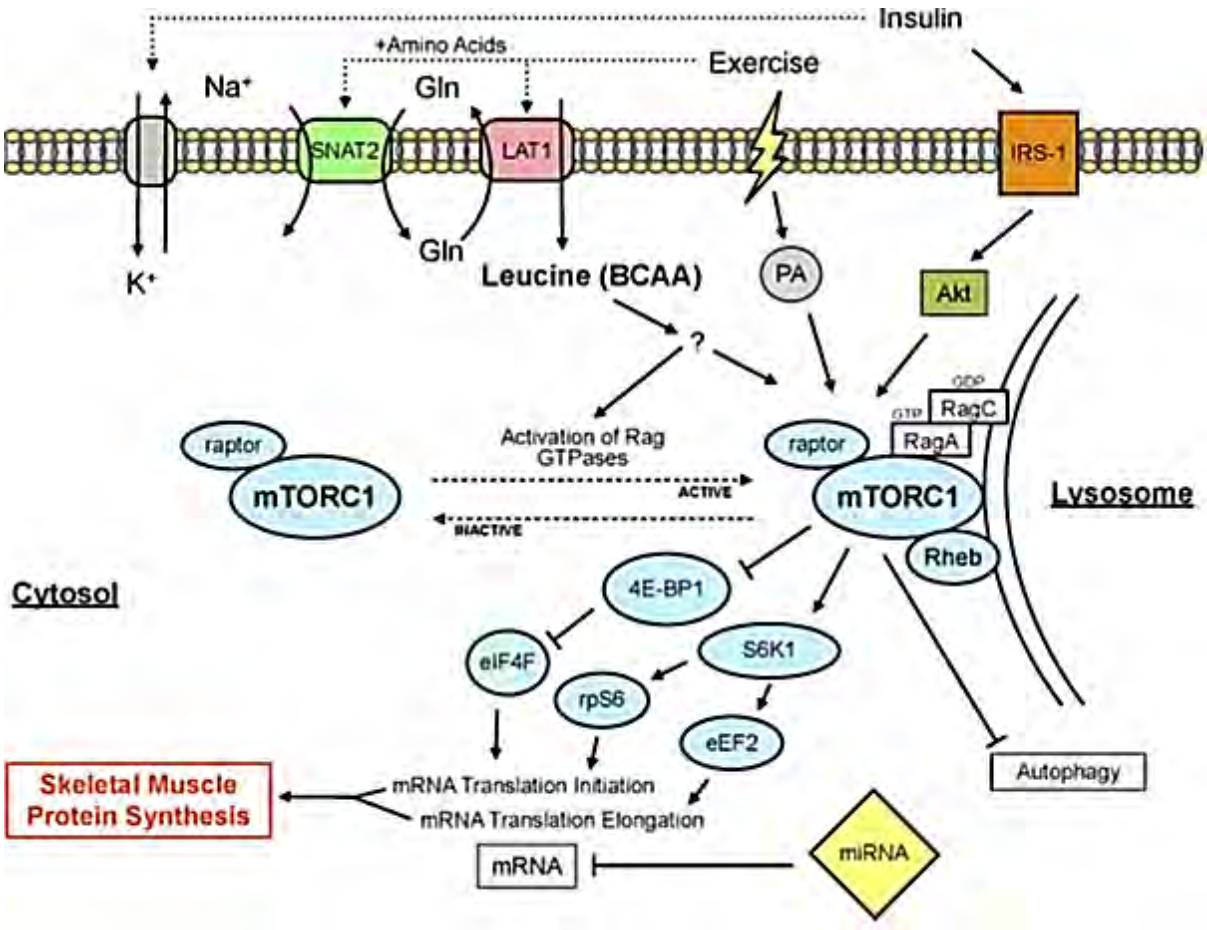

Figure 1.10 – Cellular mechanisms regulating skeletal muscle protein synthesis in response to amino acids, insulin, and exercise. 4E-BP1, 4E binding protein 1; Akt, protein kinase B; BCAA, branch chain amino acids; eEF2, eukaryotic elongation factor 2; eIF4F, eukaryotic initiation factor 4F; Gln, glutamine; IRS-1, insulin receptor substrate 1; LAT1, L-type amino acid transporter 1; mTORC1, mammalian target of rapamycin complex 1; PA, phosphatidic acid; Rag, Ras-related GTPase; rpS6, ribosomal protein S6; S6K1, ribosomal S6 kinase 1; SNAT2, sodium coupled neutral amino acid transporter 2 (reproduced with permission from Dickinson et al 2013).

Under basal conditions S6k1 and eIF4EBP1 are bound to a scaffolding protein, the eukaryotic initiation factor 3 (eIF3) translation initiation complex. Upon stimulation, mTORC1 phosphorylates and activates S6k1 and eIF4EBP1, causing them to dissociate from eIF3. Activation of eIF4EBP1 inhibits 5'-cap-dependent mRNA translation by binding and inactivating eukaryotic initiation factor 4E (eIF4E). Translation initiation results from the release of eIF4E following phosphorylation of eIF4EBP1 (Gingras *et al* 2004). S6k1 and S6k2 regulate the functions of translation initiation factors during protein synthesis (Ma and Blenis 2009). S6k1 phosphorylation also facilitates recruitment and translation of a specific mRNA subset that contains a 5' polypyrimide tract (5'-TOP). 5'-TOP transcripts encode several ribosomal proteins and translation elongation factors and as such facilitates ribosomal biogenesis and translation elongation. mTORC1 is inhibited by the immunosuppressant drug

rapamycin making it functionally easier to investigate its action relative to that of the rapamycin insensitive mTORC2. mTORC2 is less well understood than mTORC1 but it is considered part of the PI3k-Akt pathway as it directly phosphorylates Akt on one of two sites necessary for Akt activation in response to growth factor signaling (Lian *et al* 2008). mTORC2 activation is thought to be independent of AA (Polak and Hall 2009).

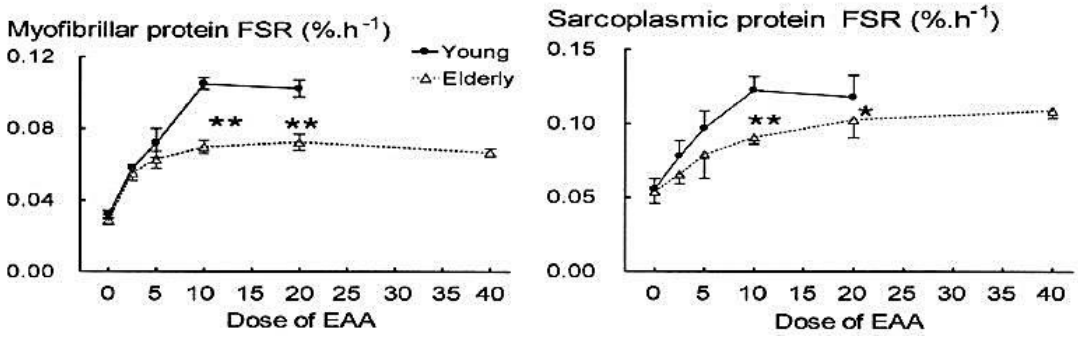

Figure 1.11 – Relationship between rates of myofibrillar and sarcoplasmic protein synthesis and dose of oral EAA (adapted with permission from Cuthbertson et al 2005).

Cuthbertson *et al* (2005) measured the protein synthetic response and components of the anabolic signaling pathway in response to mixed oral EAA ingestion in 20 young (28 ± 1y) and 24 elderly (70 ± 1y) individuals. Participants were divided into groups of four and consumed 500 mL of water containing 0 g, 2.5 g, 5 g, 10 g, 20 g and (for the elderly group only) 40 g of EAA. Blood glucose and insulin concentration were clamped at basal levels. [1-^{13}C]leucine was added to the oral EAA solution and along with a primed, constant infusion of [1-^{13}C]ketoisocaporic acid (KIC) facilitated the measurement of muscle protein FSR. Muscle biopsies were taken prior to ingestion of the EAA solution and again three hours post ingestion for the determination of key signaling proteins (relative abundance and phosphorylation state). Basal rates of MPS were similar in elderly and young participants (0.054 ± 0.008 %.h^{-1} and 0.056 ± 0.007 %.h^{-1} for sarcoplasmic proteins respectively). 2.5-10 g of EAA stimulated MPS in a dose dependent manner with 20 g EAA failing to elicit any further increase in young participants. Elderly participants also demonstrated a dose-dependent increase in the rate of MPS in response to EAA ingestion, however relative to their younger counterparts the elderly dose-response curve was shifted downward and to the right (Figure 1.11). The amount and phosphorylation state of mTORC1 and S6K1 was also reduced in the elderly relative to the young. The authors conclude from these data that "the decreased sensitivity and responsiveness of MPS to EAA associated with decrements in the expression and activation of components of anabolic signaling

pathways are probably major contributors to the failure of muscle maintenance in the elderly".

Fry *et al* (2011) demonstrated that mTORC1 and its associated downstream targets and muscle protein FSR were stimulated to a greater degree in a group of young participants relative to a group of older individuals in response to an acute bout of RE. Sixteen young (8 male and 8 female, 27 ± 2y) and sixteen older (8 male and 8 female, 70 ± 2y) completed a bout of leg extension exercise (8 sets of 10 repetitions at 70% of their individual 1 RM). Serial muscle biopsies and a primed constant infusion of L-[ring-$^{13}C_6$]phenylalanine facilitated measurement of components of the mTOR signaling pathway and muscle protein FSR pre, 3h, 6h and 24h post exercise. mTORC1, S6k1 and eIF4EBP1 phosphorylation status were increased in the young group only. There was no change in protein content in either group during the 24h post exercise recovery period. FSR was not different between young and old prior to exercise and while it increased in both groups in response to exercise, the increase in the young was more pronounced and sustained for longer. These data demonstrate that in response to an acute bout of RE, ageing impairs activation of components of the mTOR signaling pathway and there is an attenuated muscle protein synthetic response.

1.6.4 Amino acid signaling

EAA stimulate MPS by two mechanisms; (1) supplying substrate for protein synthesis and (2) acting as a regulatory factor for the activation of intracellular signaling pathways that regulate mRNA translation. The mechanism of AA stimulation of mTORC1 is emerging as evidenced by studies by Sancak, et al (2008) and Kim (2008) (Figure 1.11). These reports identified the Rag GTPases as regulators of mTORC1 in response to AA. The Rag proteins are a family of 4 related small GTPases and AA are said to induce GTP loading of the Rag proteins that then bind raptor and transport mTORC1 to lysosome that contains the mTORC1 activator Rheb (Ras homolog enriched in brain). The mechanism by which Rheb activates mTORC1 remains to be clarified but is thought to involve the interaction of Rheb and a negative regulator of mTORC1, tuberous sclerosis complex 1 and 2 (TSC1 and 2).

Breen and Phillips (2011) propose the existence of a 'leucine threshold' on the dose response curve between the gram amount of ingested protein and the change in MPS from baseline. This threshold must be exceeded in the postprandial state in order to effectively stimulate MPS above resting levels. This threshold is shifted to the right in elderly populations relative to young (Figure 1.12) due to a lack of sensitivity in the elderly to the anabolic actions of leucine. Data from Katsanos *et al* (2006) supports the

theory of the existence of a leucine threshold for stimulation of MPS. This study attempted to evaluate the effects of enriching an EAA supplement with leucine on MPS in elderly populations. Participants were divided into four groups (elderly 26% leucine, n = 10, 66.7 ± 2.0y; elderly 41% leucine, n = 10, 66.5 ± 2.0y; young 26% leucine, n = 8, 30.6 ± 2.0y; young 41% leucine, n = 8, 28.8 ± 2.6y. Participants consumed 6.7 g of EAA with a leucine content of either 26% of 41% of the total EAA content. FSR was determined by muscle biopsy of the *m. VL* in combination with a primed continuous infusion of L-[ring-$^{2}H_{5}$]phenylalanine. In elderly participants there was a small but not statistically significant increase in FSR when 24% leucine supplement was ingested while there was a greater increase in FSR when the 41% leucine supplement was ingested. FSR was stimulated to a similar degree in the young participants when 26% or 41% leucine was consumed. The acute MPS response to ingestion of an EAA supplement can therefore be enhanced through increasing the leucine content of the supplement.

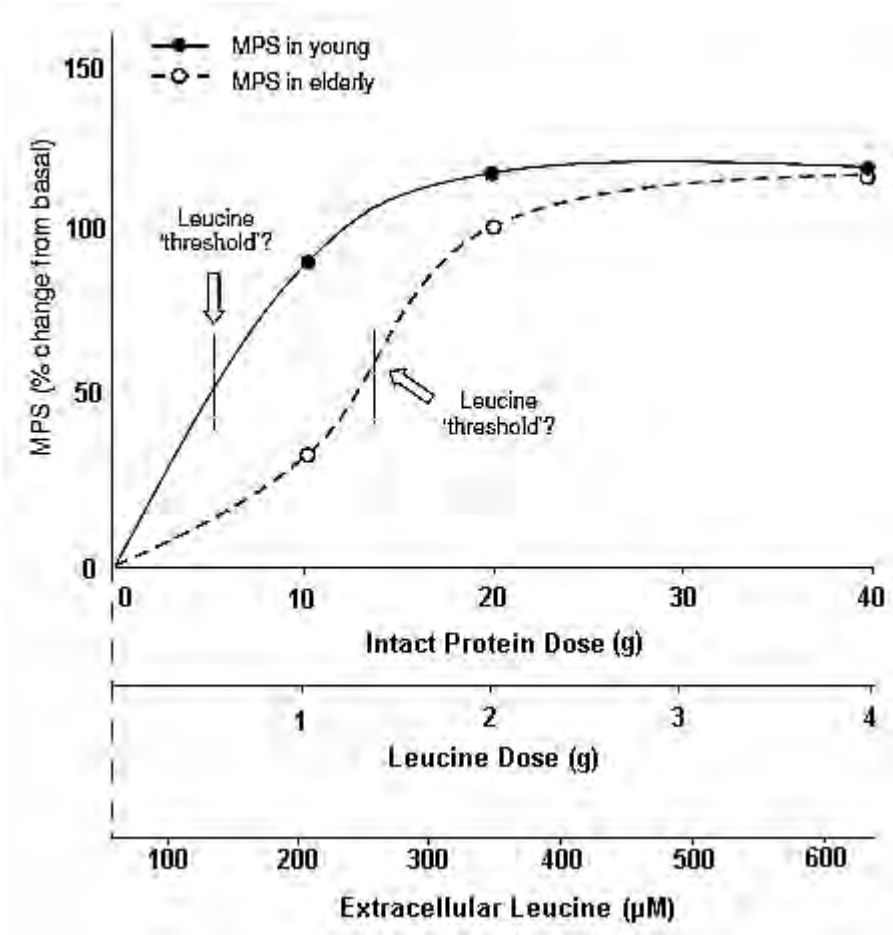

Figure 1.12 – Dose response curve of protein ingestion at rest and muscle protein synthetic response in young and elderly muscle (adapted with permission from Breen and Phillips 2011).

Wall *et al* (2013) examined the effect of leucine co-ingestion with a single bolus of dietary protein on MPS in elderly men. Twenty-four participants were randomly assigned to receive 20 g intrinsically L-[1-^{13}C]phenylalanine labeled casein protein with (n = 12, 74.2 ± 0.7y) or without (n = 12, 74.3 ± 1.2y) the addition of 2.5 g of crystalline leucine. Over the 6h post-prandial period ingestion of protein plus leucine resulted in a greater MPS synthetic response (measured as the rate of incorporation on labeled AA into muscle protein sampled by biopsy) than casein protein consumed in isolation (0.049 ± 0.003 vs. 0.040 ± 0.003 %.h^{-1} respectively). The authors concluded that "fortifying meals with free leucine may represent an effective strategy to improve postprandial muscle protein accretion".

Casperson *et al* (2012) explored the effect of supplementing meals of eight older individuals (5 male, 3 female, 68 ± 2y) with leucine (4 g.meal^{-1}, 3 meals.d^{-1}) for a two week period. Muscle protein FSR (measured as the rate of incorporation of L-[ring-$^{13}C_6$]phenylalanine), body composition and phosphorylation status of key signaling proteins associated with MPS were measured before and after ingestion of a protein/carbohydrate simulated meal prior to the two week supplementation period and again at the end of the supplementation period. The authors reported increased fasting post-absorptive rates of MPS (day 1 - 0.063 ± 0.0045 %.h^{-1}, day 15 - 0.074 ± 0.007 %.h^{-1}) as well as increased FSR in response to the simulated meal (day 1 - 0.075 ± 0.006 %.h^{-1}, day 15 - 0.01 ± 0.007 %.h^{-1}). Increases in the phosphorylation state of mTORC1 (day 1 - 1.03 ± 0.07 AU, day 15 - 1.23 ± 0.11 AU), eIF4EBP1 (day 1 - 0.30 ± 0.02 AU, day 15 - 0.37 ± 0.03 AU) and S6K1 (day 1 - 0.89 ± 0.06 AU, day 15 - 1.01 ± 0.07 AU) were also reported. No statistically significant changes in total protein were observed in response to simulated meal ingestion or two weeks of leucine supplementation. Unsurprisingly no change in LTM was observed as the intervention period was too short to achieve measurable changes in body composition.

Rieu *et al* (2006) investigated the effect of adding leucine to a mixed meal (10.2 kcal.kg^{-1}, 0.4 g.kg^{-1} protein, 1.3 g.kg^{-1} carbohydrate and 0.36 g.kg^{-1} fat) on whole body protein kinetics and myofibrillar FSR measured as the rate of incorporation of L-[1-^{13}C]phenylalanine into muscle. Twenty healthy elderly men were assigned to receive a control meal or control meal plus 0.052 g.kg^{-1} leucine (~3.9 g leucine for a 75 kg individual). The control feed was supplemented with 0.071 g.kg^{-1} alanine to ensure that the same amount of total nitrogen was fed in each instance. The meal were ingested as 15 small 50 mL aliquots every 20 min over a 5h period. Leucine supplementation resulted in increased rates of myofibrillar muscle protein FSR relative to control after 5h (leucine; 0.083 ± 0.008%.h^{-1}, con; 0.053 ± 0.009%.h^{-1}). The authors attributed this

effect to increased plasma leucine as plasma free leucine concentration was the only variable (other than increased plasma insulin concentration that was deemed permissive to MPS) that differed between the control and leucine group. Rieu *et al* (2006) demonstrated a measureable difference in MPS in response to a mixed meal with supplemented leucine versus mixed meal alone. The feeding paradigm employed in this instance (15 x 50 mL bolus over a 5h period) is however of little relevance in a practical setting. Studies that examine the chronic effect of supplementing the diet with leucine or leucine rich AA supplements on muscle mass, strength and function are however lacking.

1.6.5 Amino acid transport

Amino acid transporters (AAT) are plasma membrane proteins that mediate the transport of AA across the plasma membrane (Kanai and Endou 2003). Muscle is said to have a full complement of AAT. The operation of AAT has been described in general terms as being directly proportional to the prevailing AA concentration inside and outside the sarcoplasmic membrane due to the low substrate affinity of the transporters. Transport rates are sufficient however such that the metabolic processes of protein synthesis and AA catabolism are thought not to be limited by AAT in the presence of sufficient substrate (Rennie and Tipton 2000). Transporters can be divided into passive and active transporters (Figure 1.13). Passive or facilitated transporters allow passage of solute across membranes down their electrochemical gradients. Active transporters create ion/solute gradients across membranes utilising energy-coupling mechanisms. Active transporters can be classified as either primary or secondary active transporters according to the directness coupling to cellular energy. Primary-active, ATP dependent transporters generate and maintain electrochemical ion gradients across membranes while secondary-active ion-coupled transporters utilise these ion gradients to drive the uphill transport of nutrients (e.g. AA) across biological membranes.

Recent years has seen more focus on AAT and their role in the regulation of human skeletal muscle protein metabolism in response to protein feeding and exercise. Drummond *et al* (2010) investigated the effect of ingestion of an EAA solution on the levels of mRNA and protein expression of a number of AAT including L-type, A-type and members of the general AA control pathway (general control non-repressed (GCN_2), activating transcription factor (ATF_4), and eukaryotic initiation factor (eIF_2) α-subunit). Seven participants (3 male, 4 female, (29 ± 2y)) took part in the trial. Following an overnight fast, two baseline muscle biopsies were taken. Participants then ingested 10 g of EAA mixed in 500 mL of a non-caloric, non-caffeinated carbonated

beverage. Further biopsies were taken 1h, 2h and 3h post ingestion. MPS was measured as the rate of incorporation of L-[1-^{13}C]leucine into muscle protein. mRNA expression was determined by real time polymerase chain reaction (RT-PCR). Relative change in protein expression was measured by immunoblotting. EAA ingestion resulted in a 2.4 fold increase in MPS above baseline (baseline - 0.28 ± 0.0413 μM.min^{-1}.100 mL leg^{-1}, 1h post - 0.67 ± 0.13 μM.min^{-1}.100 mL leg^{-1}) before returning to baseline levels 2h post ingestion. L-type amino acid transporter (LAT) 1 mRNA expression was elevated at 1h post ingestion while LAT1 protein increased by 25% 3h post ingestion. Sodium-linked neutral amino acid transporter (SNAT) 2 followed a similar trend with mRNA expression elevated at 1h post and elevations in protein expression of ~ 25% 2h and 3h post EAA ingestion. Proton-assisted amino acid transporters (PAT) 1 mRNA expression was elevated 1h post while PAT2, cationic amino acid transporter (CAT) 2 and excitatory amino acid transporter (EAAT) 1 mRNA expression were unchanged over the course of the trial. Finally, while ATF4 mRNA expression remained stable, ATF4 protein expression increased by ~ 25% 2h post EAA ingestion. GCN2 mRNA and protein expression were unchanged as was the phosphorylation state of eIF2α. Several AAT are therefore sensitive to the ingestion of EAA and are up-regulated rapidly and transiently to improve AA transport in response to AA feeding.

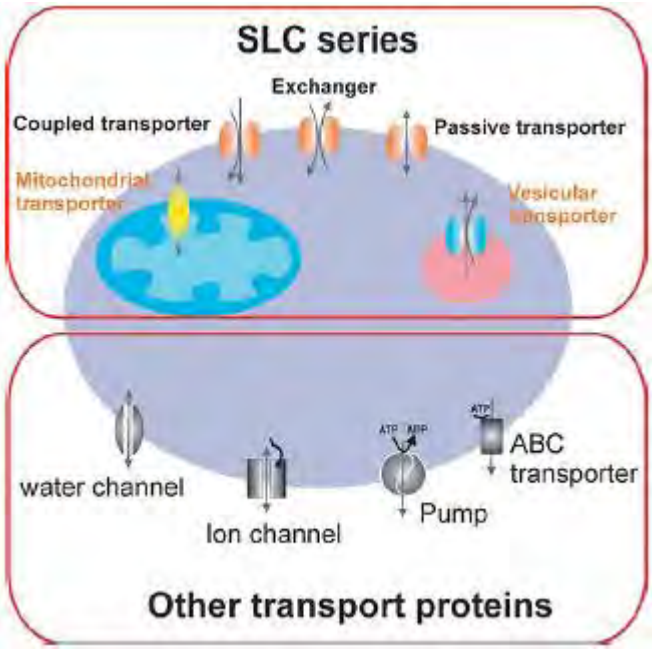

Figure 1.13 – Cell with solute-linked-carrier (SLC) and non-SLC transporters expressed in the plasma membrane or in intracellular compartment membranes (reproduced with permission from Hediger et al (2013)).

In a follow up study, Drummond *et al* (2011) examined skeletal muscle AAT RNA and protein expression in response to an acute bout of RE in young and older individuals. Thirteen young (8 male, 5 female (28 ± 2y)) and older individuals (8 male, 5 female (68 ± 2y)) completed a bout of bi-lateral leg-extension exercise (8 sets, 10 reps @ 70% of their individual 1 RM) following an overnight fast and basal muscle biopsies. Further biopsies were taken at 3h, 6h and 24h post exercise. Muscle samples were analysed for AAT mRNA, protein expression, regulators of AAT transcription utilising RT-PCR and Western-blotting. Basal AAT expression was not different between young and older individuals. The authors reported that the RE bout increased mRNA expression of LAT1, SNAT2, PAT1 and CAT1 in both young and older populations with no difference between the two groups. Ribosomal protein S6 (RPS6) phosphorylation and protein expression of ATF4 were increased in the young only in response to RE while protein expression of signal transducer and activator of transcription (STAT) 3 was increased to a greater extent in older adults. These data indicate that control of AA transporter expression following RE may be differentially regulated between young and elderly populations. The AA transporter response to an acute bout of RE involves integrative signals from mTORC1, ATF4, eIF_2 and STAT3. This may serve to sensitise muscle to the anabolic actions AA post RE in both young and older populations.

A more recent study from the same group in Galveston, Texas (Dickinson *et al* 2013a) measured AA transporter expression in skeletal muscle from young and older individuals in response to an acute RE bout in conjunction with the ingestion of EAA. Muscle biopsies were obtained from 7 young men (30 ± 2y) and 6 older men (70 ± 2y) at rest and 3h and 6h post exercise and EAA ingestion. Participants ingested a 500 mL solution containing 20 g EAA 1h post a bout of bilateral leg extension exercise (8 sets, 10 repetitions at 70% of 1 RM). Dickinson *et al* (2013) reported that the combination of RE and EAA ingestion increased LAT1, SNAT2 and CAT1 mRNA independent of age group. Protein expression of SNAT2 was higher in young men whereas older individuals exhibited higher protein expression of LAT1 post exercise and EAA ingestion. The authors also report that in young man the combination of RE plus EAA augmented AAT expression relative to that observed through RE alone. Specifically elevation of LAT1/SLC7A5 mRNA expression, increased SNAT2/SLC38A2 mRNA expression and increased SNAT2 protein expression were noted. The relevance of this finding is substantial as these transporters are thought to facilitate the accumulation of intracellular leucine, and as such enhance the activation of mTORC1 and *de facto* MPS. This augmented response was not evident in older populations as EAA in addition to RE failed to enhance the effect observed through RE in isolation.

1.7 Intervention studies

Studies of the acute muscle protein synthetic response to protein ingestion in elderly populations demonstrate a beneficial effect, in terms of enhancing the rate of MPS, resulting from optimisation of the quality, quantity and timing of protein ingestion. The translation of these acute increases in FSR into measureable changes in LTM during long term RCTs (12 weeks or greater duration) is difficult due to the myriad of factors that may affect LTM over the period of observation. Several recent meta-analyses have provided important overviews of the existing body of literature while also highlighting gaps that need to be addressed in the extant literature.

1.7.1 Nutritional interventions

Malafarina *et al* (2013) conducted a meta-analysis in accordance with the Preferred Reporting Items for Systematic Reviews and Meta Analyses (PRISMA) guideline examining research conducted between 1991 and 2012 on the effect of orally administered nutritional interventions (controlled clinical trials, clinical trials, RCTs) lasting 8 weeks or longer in populations with an average age of 65 y and over on body composition as measured by bioelectrical impedance or Dual-energy X-ray absorptiometry (DXA). Seventeen studies met the inclusion criteria and of those seventeen, four were undertaken with a healthy population (Cornish and Chilibeck 2009, Dillon *et al* 2009, Vukovich *et al* 2001, Verhoeven *et al* 2009). Cornish and Chilibeck (2009) supplemented with alpha-linolenic acid and as such the outcome is not related to the focus of this thesis. Vukovich *et al* (2001) supplemented participants with the leucine metabolite β-hydroxy-β-methylbutyrate (HMB) (1 g three times per day). Supplementation was undertaken in conjunction with a program of PRT. A supplement only group was not included in the trial and as such it is not possible to determine the impact of HMB supplement in isolation from this study. However as measured by DXA, participants in the HMB group experienced a non-significant 0.3 ± 0.4 kg increase in fat free mass versus a 0.1 ± 0.4 kg increase in LTM in the control group.

Dillon *et al* (2009) examined the effect of an EAA supplement ingested twice daily, between meals, (7.5 g x 2, total 15 $g.d^{-1}$) for 12-weeks on muscle protein FSR and body composition. Fourteen elderly women (68 ± 2 y) were randomly assigned to receive either the EAA supplement (n = 7) or and energy matched control (lactose) (n = 7). Muscle protein FSR in response to an acute dose of oral EAA (7.5 g) was assessed before and after the 12-week supplementation period. MPS was measured as the rate of incorporation of L-[ring-$^{2}H_{5}$]phenylalanine into muscle. Participants also completed DXA scans for the analysis of body composition at baseline and again at the end of the

supplementation period. Similar FSR responses were observed between groups to acute ingestion of 7.5 g of EAA at baseline. Basal rates of MPS were increased at 12-weeks in the EAA supplement group only, while the magnitude of the increase in FSR at 12-weeks in response to acute EAA ingestion was similar between groups. In the EAA supplement group there was an average increase in LTM of 1.7 kg (baseline; 43.5 ± 2.8 kg, 12-week; 45.2 ± 3.0 kg, $p < 0.05$) while the control group experienced an average increase of 0.3 kg (baseline; 40.7 ± 2.4 kg; 12-week; 41.0 ± 2.8 kg). Bicep curl, triceps extension, leg extension and leg curl 1 RM along with the amount and phosphorylation status of Akt, mTORC1, S6k1 and eIF4EBP1 were not different between groups at baseline or 12-weeks and did not change over time as a result of EAA supplementation.

Verhoeven *et al* (2009) assessed the effect of 12-weeks of leucine supplementation of muscle mass and strength in healthy elderly men. Participants were randomly assigned to receive either 7.5 $g.d^{-1}$ leucine distributed evenly (2.5 g per main meal) over the day (n = 15) or placebo (wheat flour) (n = 14) in capsule form. Strength was assessed by 1 RM strength tests on leg press and leg extension machines. CSA of the quadriceps muscle was measured by a CT scanner. Body composition was measured by DXA. Blood samples were drawn for measurement of basal plasma glucose and insulin concentration, plasma AA and lipid profiles, serum creatinine and blood glycated hemoglobin content. Muscle biopsies were taken from the *m. VL* and analysed for the expression of myosin heavy chain isoforms (MHC I, IIa, IIX). Leucine supplementation did not increase whole body LTM, leg LTM or quadriceps CSA (Table 1.5).

Table 1.5 – Change in lean tissue mass (LTM), leg lean tissue mass, quadriceps (quad) cross sectional area (CSA)and myosin heavy chain (MHC) isoforms resulting from 12 weeks of either leucine supplementation (7.5 $g.d^{-1}$) or placebo (data from Verhoeven et al, 2009).

	Placebo		Leucine	
	Pre	**Post**	**Pre**	**Post**
LTM (kg)	55.8 ± 0.9	56.2 ± 1.1	54.6 ± 1.0	55.0 ± 1.5
Leg LTM (kg)	17.6 ± 0.4	18.0 ± 0.4	17.1 ± 0.5	17.6 ± 0.4
Quad CSA (cm^2)	71 ± 3	71 ± 3	71 ± 3	71 ± 3
MHC I	59 ± 3%	65 ± 4%	64 ± 2%	65 ± 3%
MHC IIa	31 ± 2%	28 ± 3%	30 ± 2%	30 ± 3%
MHC IIx	10 ± 1%	7 ± 1%	6 ± 1%	6 ± 1%

There was also no change in muscle MHC composition, glycemic control or blood lipid profiles over time. Verhoeven and colleagues state that “the present study clearly shows that previous reports on the acute stimulating properties of an increased leucine

content of a meal on the post-prandial muscle protein synthetic response do not translate into an effective long-term interventional strategy to augment skeletal muscle mass in healthy elderly men". There is a lack of high quality empirical data to support the hypothesis that long-term supplementation with additional dietary protein in healthy, community dwelling, energy and protein sufficient populations will offset the age-related decline in LTM.

1.7.2 Nutrition and exercise interventions

Cermak *et al* (2012) reported a meta-analysis conducted according to the PRISMA statement, examining RCTs that combined PRT (≥ 6 weeks in duration, minimum 2 sessions per week) and at least one subject group that was supplemented with protein or consuming a high protein diet (> 1.2 $g.kg^{-1}.d^{-1}$). Inclusion for outcome improvements in body composition was limited to three discrete measurements, hydrodensitometery, whole body air plethysmography and DXA. Six studies (12 groups) in older populations met the inclusion criteria (Holm *et al* 2008, Iglay *et al* 2009, Kukuljan *et al* 2009, Verdijk *et al* 2009, Bemben *et al* 2010, Campbell *et al* 1995). Campbell *et al* (1995) conducted a trial whereby eight men and four women (56-80y) were randomly assigned to groups that consumed diets providing either the RDA for protein (0.8 ± 0.02 g $protein.kg^{-1}.d^{-1}$) or twice the RDA for protein (1.62 ± 0.02 g $protein.kg^{-1}.d^{-1}$) in conjunction with 12-weeks of RT. All 12 participants consumed a basal diet consisting of repeated cycles of 3 daily menus of lactovovegetarian foods providing 0.6 $g.kg^{-1}.d^{-1}$ protein. The additional 0.2 and 1.0 $g.kg^{-1}.d^{-1}$ protein fed to the lower and higher groups respectively were provided as two milk based beverages. The RE protocol consisted of three sets of chest press, latissimus dorsi pull down, knee flexion and extension at 80% of 1 RM on three non-consecutive days of the week for twelve weeks. Participants completed 8 reps during the first two sets and a third set to 12 reps of voluntary fatigue. Body composition was measured from body density and total body water measurements using a three compartment model. Body density was measured by hydrostatic weighing, lung residual volume by nitrogen dilution while total body water was determined using the deuterium oxide-dilution technique. After twelve weeks the group consuming the RDA for protein experienced an average increase in fat free mass of 1.0 kg while consumption of the higher protein diet resulted in an average increase of 1.8 kg. This difference was not statistically significant between treatments.

In an RCT conducted by Holm *et al* (2008) 29 healthy community dwelling women (55 ± 1 y) were randomly assigned to a nutrient group (n = 13) or control group (n = 16). The nutrient group was supplemented with 730 kJ comprising of 10 g WP, 31 g CHO, 1 g fat, 5.0 μg vitamin D and 250 mg calcium. The control group received 102 kJ

consisting of 6 g CHO and 12 mg calcium. Both nutrient and control group completed 24-weeks of PRT, 2 - 3 times per week consisting of leg press (3-5 sets, 8-15 reps at 8-20 reps of the individuals rep max (RM), leg extension (3-4 sets, 10-15 reps at 10-20 reps of the individuals RM, lat pull-down (1 set, 10-15 reps at 20 RM), sit-ups and back extension (10-20 reps). Participants consumed the supplement immediately post training. Body composition was assessed by DXA pre and post completion of the 24-week intervention. The nutrient group experienced an increase of 0.8 kg in LTM (within group effect $p < 0.05$) however there was no difference between groups with respect to change in LTM in response to 24 weeks of supplementation in combination with PRT. Participants were supplemented on training days only and the dose (10 g) was quite low. The training program was also predominantly focused on the lower body (and in particular the quadriceps) and may have benefited from the inclusion of hamstring/glut and additional upper-body exercises. The limitations with respect to the supplement/supplementation and the narrow focus of the exercise program may have limited the scope to measure changes between experimental treatments.

Kukuljan *et al* (2009) assessed whether a milk-based nutritional supplement could enhance the effects of RT on muscle mass, size, strength and function in middle aged an older men. The authors employed an 18-month factorial (RCT) design in which 180 healthy men aged 50-79y were allocated to the following treataments; exercise (n = 46), exercise plus fortified milk (n = 45), fortified milk only (n = 45) and control (n = 44). Participants assigned to milk consumed 400 $mL.d^{-1}$ of low-fat milk, providing an additional 836 kJ, 1000 mg calcium, 800 IU vitamin D3 and 13.2 g $protein.d^{-1}$. Participants were encouraged to consume one 200 mL tetra-pack in the morning and one in the afternoon or evening but the timing of supplementation ingestion was not necessarily optimised with respect to the PRT program. RE consisted of supervised exercise performed on three non-consecutive days per week for 18 months. The primary exercises used throughout the program included squats/leg presses, lunges, hip abduction and adduction latissimus pull-downs/seated row, back extensions and abdominal and core stability exercises.

Body composition was measured by DXA, mid-femur muscle CSA was measured using quantitative computed tomography. Muscle strength was measured by 1 RM tests of leg press, lat pull-down and bench press. Nutrient intake were measured using a 3-day food diary (Goldberg cutoff was applied to identify and exclude under-reporters). PA levels were assessed using the Community Healthy Activities Model Program for Seniors PA questionnaire. There were no statistically significant differences between groups at baseline (other than greater muscle strength in the control group relative to

the exercise, milk or exercise plus milk group). Average daily dietary protein intakes for all participants were above or equivalent to the current RDI's. Following 18 months of exercise, exercise plus milk or milk only there was no statistically significant difference between treatments for total body LTM. This was despite the fact that gains in LTM and mid-femur muscle CSA were two to three-fold greater in the exercise plus milk group relative to the exercise alone, milk alone or control group (Table 1.6). Supplementation with fortified milk resulted in a 16% increase in dietary protein intake at 12 months but not at 18 months. PA levels did not differ between the groups over the course of the intervention.

Table 1.6 – Within group changes (mean (95% confidence interval)) in lean tissue mass (LTM) (kg) and mid-femur cross sectional area (CSA) (cm^2) relative to baseline (data from Kukuljan et al 2009).

	Δ LTM (kg)		**Δ CSA (cm^2)**
	12 month	**18 month**	**18 month**
Exercise + Milk	1.0 (0.4, 1.5)	0.9 (0.3, 1.6)	1.4 (0.1, 2.7)
Exercise	0.7 (0.2, 1.1)	0.3 (-0.2, 0.9)	0.7 (-0.5, 1.8)
Milk	0.4 (-0.1, 0.8)	0.2 (-0.4, 0.8)	-0.1 (-1.0, 0.8)
Control	0.1 (-0.4, 0.5)	-0.2 (-0.5, 0.1)	-1.5 (-2.5, -0.5)

Limitations associated with this study include the use of a slow digesting protein source (milk protein is predominantly casein based) while timing of supplement intake was not optimised with respect to training to take advantage of the sensitising effect of RE on the muscle protein synthetic machinery. The participants under investigation were not protein deficient according to WHO/IOM guideline and were not overtly sarcopenic. As such the scope for gains in LTM may have been limited. This study also lacked an isoenergetic control arm. As such the gains observed, although not statistically significant could be attributable to an increase in total energy intake in the supplemented groups as oppose to active components of the formulation. Finally, the PRT program was not optimised for hypertrophy throughout the study as there was an increased focus after 12 months on exercises designed to promote increases in bone mineral density.

Verdijk *et al* (2009) assessed the effect of optimisation of the timing of protein intake relative to RT in healthy elderly men. Participants were randomly assigned to protein (n = 13, 72 ± 2y) or placebo (n = 13, 72 ± 2y) groups. Body composition was measured by DXA while CSA of the *m. quadriceps femoris* was assessed by CT. Muscle strength was assessed by 1 RM test of the leg press and leg extension. Muscle biopsy were taken for the *m. VL* for the determination of MCH I content, total number of muscle

fibres, mean fibre CSA and percentage of area occupied per fibre type for type I and type II muscle fibres. During the warm-up and cool-down period, participants consumed either water or 10g casein hydrolysate (20 g in total, 10 g before and 10 g after RT). Participants performed supervised RT 3 times per week for 12 weeks. RT consisted of 4 sets of leg extension and leg press exercise. Intensity and volume of the exercise was progressively increased based on on-going assessment of 1 RM scores. In response to 12 weeks RT increases in leg LTM were observed via DXA (6 ± 1%) and fibre CSA (9 ± 1%) with no differences between treatments. Type II fibres experienced greater hypertrophy than type I fibres (placebo - 28 ± 6% and 5 ± 4% versus protein – 29 ± 4% and 13 ± 6% respectively). While there were consistent improvements in all variables reported in response to RT, no statistically significant differences were observed between supplement groups.

Bemben *et al* (2010) randomly assigned forty-two 48-72 y old men to one of four groups (A. 480 mL Gatorade, B. 480 mL Gatorade + 5g creatine, C. 480 mL Gatorade + 35 g WPC or D. 480 mL Gatorade + 5g creatine + 35g WPC) to examine the effect of supplementation in combination with 14 weeks of PRT undertaken 3 times per week. Exercises included; knee extension, knee flexion, bicep curls, tricep extensions, military press, lat pull down, seated leg press and bench press. Participants performed three sets of eight repetitions at 80% of 1 RM. Drinks were consumed at the end on each training session and on training days only. Changes in body composition were assessed by DXA and BIA. Training effects were observed for total body LTM but no significant group x training interaction was observed for any of the variables reported. The placebo group experienced an average change in LTM of -0.4 kg while the Gatorade + WP experienced an average increase in LTM of 0.7 kg (net difference 1.1 kg). Inadequate numbers per experimental group, and the fact that participants consumed supplements on training days only and the duration of the intervention may have limited the opportunity to measure differences between treatments in this instance.

Cermak *et al* (2012) concluded that compared to placebo, protein supplementation significantly augmented gains in LTM during prolonged PRT (pooled estimate = 0.48 kg; 95% C.I. - 0.01, 0.85 kg, P = 0.01). This was in spite of the fact that each of the individual trials included in the analysis reported non-significant differences in the protein compared to the placebo condition. The error terms reported in trials investigating changes in LTM in response to intervention, highlight the fact that there is considerable variability among individuals in the response to the same stimulus (be it nutritional, exercise or a combination of both). This phenomenon is eloquently

illustrated in a review by Drummond *et al* (2012) (Figure 1.14). The underlying mechanisms contributing to this variance in response are not fully elucidated. It does however complicate the establishment of statistical difference between intervention treatments utilising nutrition and exercise paradigms.

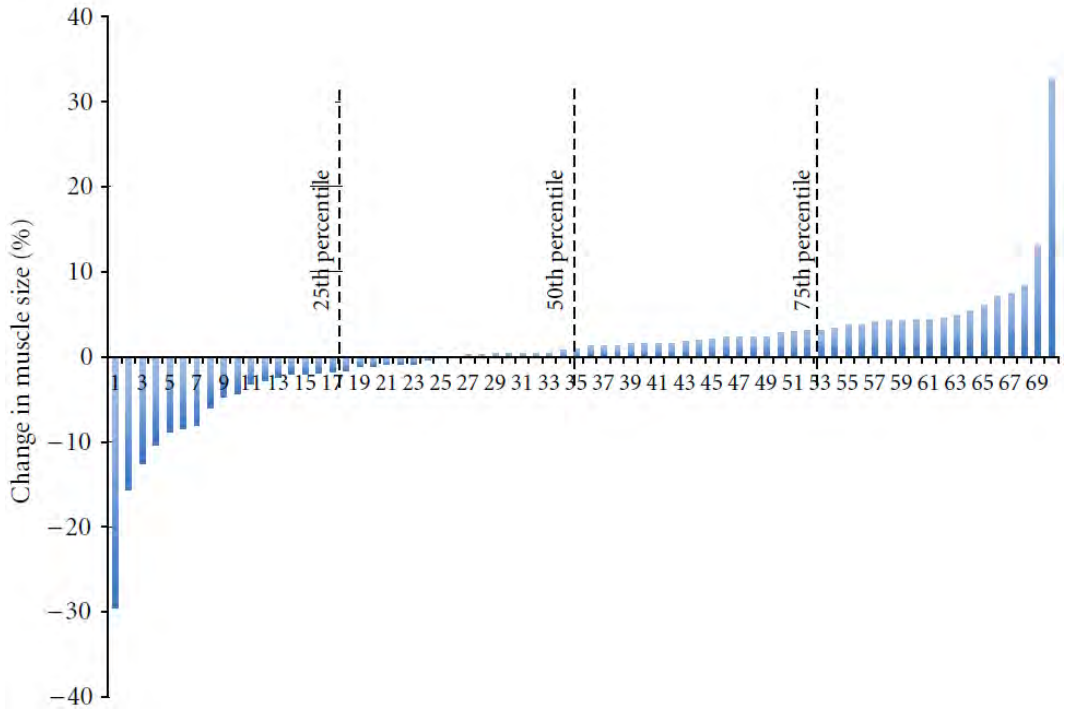

Figure 1.14 – Individual percentage and quartiles of change in muscle size following 12-weeks of a multicomponent resistance exercise program in mobility impaired older adults.

1.8 Microdialysis

Microdialysis (MD) is a membrane catheter-based sampling technique. It can provide near continuous sampling of interstitial fluid compounds with alleged minimal perturbation to the tissue of interest depending of the experimental paradigm and sampling parameters. Tissue can be dialysed for a continuous period of hours and even days (Haus *et al* 2007) and when coupled to an on-line analysis method can provide near real time concentration-time profiles. Passive diffusion allows for bi-directional exchange of solute across the dialysis membrane that is dependent on the solute concentration gradient. Thus the probe can be used as a volume-neutral local delivery and sampling vehicle of key regulatory metabolites. In this regard the MD catheter has been described as an artificial blood vessel introduced into the tissue. Depending on the molecular weight cut-off (MWCO) of the dialysis membrane, which generally ranges from 20 kDa to 100kDa, the dialysate can be relatively, or totally, free of large protein molecules. The MWCO is a solute rejection term defined as the molecular weight that is 90% rejected by the membrane (Lee *et al* 2002). Larger

molecules and associated binding proteins not blocked from crossing the capillary endothelium, into the interstitium are “filtered” by the dialysis membrane depending on the MWCO. The lack of binding proteins and other large protein molecules support the assertion that only the non-protein bound substrates are recovered in the dialysate. As most enzyme proteins are of high molecular weight, a low MWCO membrane acts as an effective enzyme inhibitor as it excludes enzymes that could cause degradation of the solute(s) for example, the 24 kDa serine protease trypsin (Davies *et al* 2000). Using low MWCO membranes (20 kDa or less) affords the opportunity to compile a metabolomic profile of the interstitium.

In MD experimentation a trade-off exists between perfusion flow-rate, analyte relative recovery and temporal resolution. It is desirable to perfuse at a relatively low flow rate (1 $\mu L.min^{-1}$ or less) in order to optimise relative recovery but at low flow rates the temporal resolution of the experiment may be compromised without access to an appropriately sensitive (pico and nano molar concentration range) and reproducible analytical technique that can accommodate small sample sizes (2 µL or less). Since its inception MD has presented an extensive challenge to analytical scientists. Conventional analytical systems such as enzyme-linked immunosorbent assay (ELISA) have limited application in MD due to relatively large sample requirements and limited sensitivity. The continued development and integration of powerful analytical methodologies such as liquid chromatography (LC), liquid chromatography/mass spectrometry (LC/MS) and capillary electrophoresis (CE) have provided justification for the use of MD as an investigative tool. These methods of analysis in combination with the MD technique itself are costly and this can prove prohibitive to research and therefore must be considered as a limitation of the system. Extensive pre-trial planning is required in order to optimise flow-rate, recovery and temporal resolution to provide the most favourable conditions to effectively answer the experimental question(s).

1.8.1 Microdialysis of amino acids in human skeletal muscle

A bibliographical search was performed on 30th November 2013 of the online databases : OVID SP MEDLINE, OVID EBSCO and National Library of Medicine’s PubMed Central Medline using the Medical Library Subject Heading (MeSH) term ‘microdialysis’, combined with the set operator ‘AND’ plus each of ‘amino acid*’ and ‘skeletal muscle’. Articles were limited to those published in English, between the year’s 1989 and present. A total of twenty-eight articles were returned, six of which reported AA concentration in the interstitium of human skeletal muscle as sampled via MD. Investigators examining whole body and skeletal muscle protein metabolism have utilised techniques such as serial venous blood sampling (Power *et al* 2009),

arteriovenous (AV) difference across a limb (Tipton *et al* 2001) and muscle biopsy (Filho *et al* 1997) in isolation and in combination to describe the muscle protein response associated with among others, nutrient ingestion/infusion, mechanical loading and disease states. A-V differences contain substrate contributions from tissues other than muscle that are present in the limb (adipose, bone), while serial muscle biopsy are undesirable due to the trauma associated with repetitive sampling necessary to monitor a dynamic process. The sample site for skeletal muscle biopsy is also quite small and variable in terms of depth-related changes in fibre type.

Table 1.7 – Basal skeletal muscle interstitial amino acids concentration reported by Maggs et al (1995), Gutierrez et al (1999) and Durham et al 2010).

EAA (μM)	Maggs	Gutierrez	Durham	Non-EAA (μM)	Maggs	Gutierrez	Durham
Phenylalanine	72±7	59±4	66±6	**Alanine**	333±35	533±66	410±41
Tryptophan$^{\alpha}$	-	22±2	-	**Asparagine$^{\alpha}$**	56±4	71±7	-
Methionine	14±2	34±3	28±3	**Citrulline**	48±11	42±7	57±13
Threonine	185±17	170±25	126±16	**Glutamine**	672±80	1581±228	511±60
Lysine$^{\alpha}$	-	167±21	176±19	**Glycine**	400±48	313±30	294±26
Isoleucine	70± 10	67±8	46±4	**Serine**	125±9	140±15	88±10
Leucine	165±19	131±15	172±18	**Taurine**	165+19	1135±268	23±3
Valine	257±25	233±26	191±21	**Tyrosine**	62± 15	69±5	68±5
Total EAA	763	694	629	**Total NEAA**	1805	1722	1451
BCAA	492	431	409	**Total AA**	2568	4507	2080

Data are mean ± SEM *expressed in μM.* **α –** *Not included in value for Total AA.*

Maggs *et al* (1995) were the first to report AA concentrations in human skeletal muscle. Utilising 5kDa 'homemade' concentric MD probes the authors employed a zero flow rate calibration protocol with flow rates between 0.25 to 2.5 μL.min^{-1}, analysed by non-linear regression to extrapolate to zero flow rate and thus the point at which the interstitium is in compete equilibrium with the dialysate. Dialysate AA concentration was measured by ion exchange chromatography in ten healthy participants at rest in the post absorptive state. There was a trend towards higher dialysate amino acid concentrations relative to arterialised plasma with the highest interstitial/arterial concentration gradient reported for aspartate, glycine, taurine and citrulline.

Gutierrez *et al* (1999) investigated interstitial AA concentrations in eight healthy participants. The authors employed a 20 kDa MD (CMA60, CMA Microdialysis, Stockholm, Sweden) probe perfused at a flow rate of 0.3 μL.min^{-1} to sample skeletal muscle interstitial fluid during five seventy-five minute collection periods after an initial thirty minute rest period post insertion. The authors did not employ a conventional MD probe calibration procedure, instead relying on the assumption that "100% equilibrium can be obtained with a very slow rate of perfusion". Plasma and dialysate AA

concentrations were measured by reverse RPHPLC with fluorimetric detection. In agreement with Maggs *et al* (1995) the interstitial AA concentration was slightly higher than the corresponding venous plasma concentration with the largest concentration gradients reported for glutamate, glutamine, taurine and citrulline in this instance.

Gutierrez *et al* (1999) reported the dialysate concentration of carnosine as a possible marker of cellular trauma resulting from probe insertion (Figure 1.15). Carnosine is a naturally occurring histidine containing di-peptide (Begum *et al* 2005). It is predominately stored in muscle and nerve cells with an average concentration in the *m. quadriceps femoris* of 20 $\mu M.g.dry wt^{-1}$ (range 7.2–31 $\mu M.g.dry wt^{-1}$) (Mannion *et al* 1992). Carnosine is not detectable in plasma (Gardner *et al* 1991) as it is hydrolysed by serum carnosinease and is only found in the intracellular environment making it a suitable bio-marker of cellular trauma resulting from the insertion of a MD probe. Gutierrez *et al* reported elevated dialysate concentration of carnosine (471 ± 200 $\mu mol.L^{-1}$) during the first measurement period (0-75 min) although considerable inter-individual variability was noted. The carnosine concentration decreased markedly during the second sampling period (90 ± 41 $\mu mol.L^{-1}$) but was still measurable in the final sample (301–375 min) from all six participants (23 ± 7 $\mu mol.L^{-1}$) (Figure 1.15). Gutierrez *et al* (1999) conclude that reliable baseline AA values cannot be obtained until approximately 2-2.5h post probe insertion. This time is required to allow for clearance from the leakage of intracellular AA associated with probe insertion. The authors deemed carnosine a viable biomarker of cellular damage resulting from MD probe insertion.

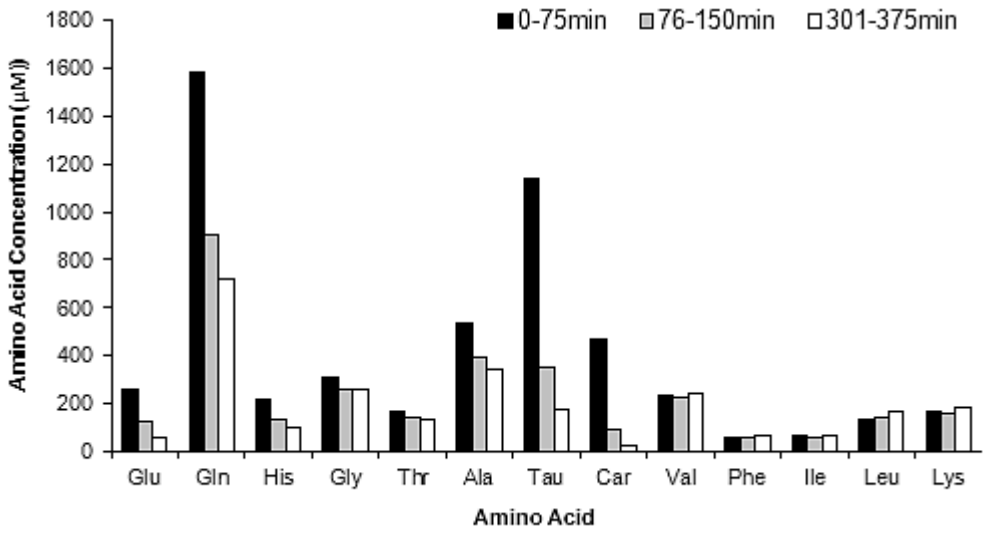

Figure 1.15 – Interstitial skeletal muscle amino acid concentration reported by Gutierrez et al (1999) during three collection periods of 75 minutes. Sampling begun 30 minutes post catheter insertion.

Nordsborg *et al* (2003) investigated the degree of sarcolemmal rupture caused by MD catheter insertion into *m. VL* and the effect of subsequent exercise with the probes *in situ* in three healthy participants. Dialysate carnosine concentration from two 20kDa probes (CMA 60, CMA Microdialysis, Stockholm, Sweden) in each subject (6 probes in total) were determined by RP HPLC during collection periods of 10 to 20 min (Insertion) and 60 to 70 min (Rest 1) post insertion at a flow rate of 2.0 $\mu L.min^{-1}$. In addition dialysate was also collected during sub maximal one legged kicking at 20 W (20 watt sample) and 80 to 90 min after a second bout of sub maximal one legged kicking (Rest 2) and finally during intense exhaustive single-leg kicking lasting no longer than 5 min (Intense). The dialysate carnosine concentration was elevated post insertion and during sub maximal and intense exercise undertaken with the MD catheter *in situ*. During exercise with MD catheters *in situ*, additional cellular trauma occurs (Figure 1.16).

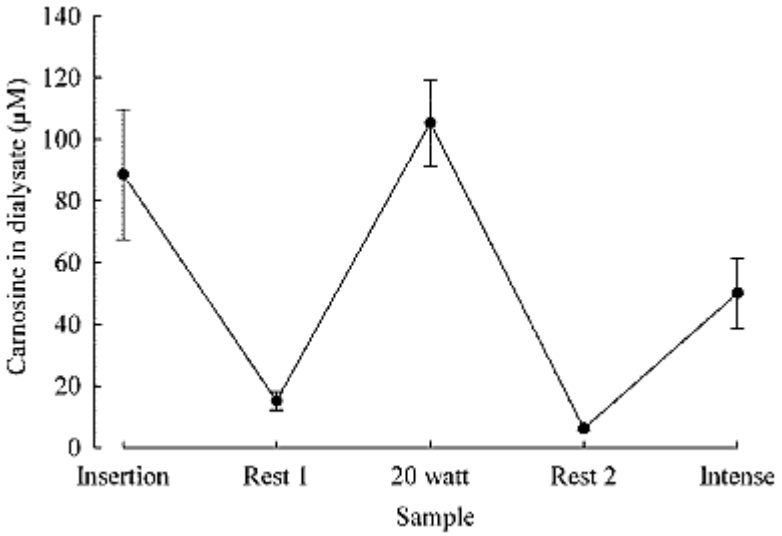

Figure 1.16 – Carnosine concentration in the dialysate obtained from 6 probes (3 participants). Reproduced with permission from Nordsborg et al (2003).

Durham *et al* (2010) and Dillon *et al* (2011) reported dialysate AA concentration from human skeletal muscle in response to the infusion of AA. Durham *et al* (2010) investigated the response of muscle protein metabolism to endurance exercise (treadmill walking for 45 min @ ~40% $VO_{2\ peak}$) via contrast-enhanced ultrasound, MD sampling of skeletal muscle interstitium and stable isotope methodology. Seventeen healthy men, 9 young (30 ± 1.7y) and 8 older (67 ± 1.6y) were studied prior to and after completing the exercise bout. The authors hypothesised that older populations would exhibit reduced microcirculatory blood flow, interstitial AA concentration and AA transport when compared to younger controls. Three CMA 60 MD catheters (MWCO

20 KDa) were inserted into the *m. VL* and perfused at a rate of 5.0 μL.min^{-1} with 5mM ethanol and 0.108 μCi.mL^{-1} of D-[^{3}H]phenylalanine and 0.108 μCi.mL^{-1} of D-[^{14}C]leucine added to the perfusate to measure skeletal muscle nutritive blood flow in the area of the MD probe and MD probe recovery via the internal reference method respectively. Upon insertion, catheters were perfused for 45 min to reach equilibrium after which dialysate samples were collected in 30 min aliquots. Durham et al (2010) reported that circulating and interstitial AA concentration and phenylalanine transport into skeletal muscle were all equal or higher in older individuals than in the young in response to AA infusion (Figure 1.17). This was in spite of evidence of reduced nutritive blood flow in the elderly relative to the young by two independent measures (MD ethanol washout and contrast-enhanced ultrasound). The authors reported that MPS was increased over time with no difference between groups. The authors also described sensitivity of MPS to AA by calculating fractional outward transport and synthetic efficiency through 4-pool modelling of phenylalanine kinetics. Older populations were reported to have increased fractional outward transport and decreased synthetic efficiency relative to younger populations. The authors concluded that aged related anabolic resistance in elderly populations is not mediated via reduced availability of AA when a large dose of amino acids are infused over a three hour period.

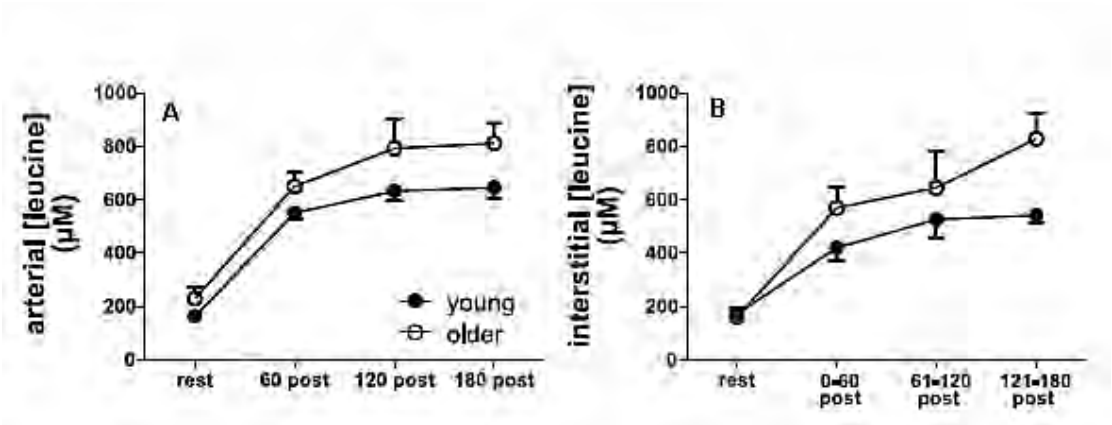

Figure 1.17 – Arterial (A) and interstitial (B) leucine concentration in young and older individuals in response to amino acid infusion (data are mean with SEM bars). Reproduced with permission from Durham et al 2010.

Dillon *et al* (2011) hypothesised that relative to young, older people would exhibit a lower anabolic response to the infusion of AA when the NO donor sodium nitro prusside (SNP) was concomitantly administered in an attempt to stimulate micro-vascular blood flow. Seven young (5 women, 2 male, 30 ± 2y) and 7 older (4 women, 3 male, 67 ± 2y) were studied at rest in the fasting post-absorptive state and in response to infusion of AA in combination with SNP. Macro-vascular blood flow was measured

by IGD dilution while micro-vascular blood flow was measured by contrast-enhanced ultrasound and the MD ethanol washout technique. FSR was measured as the rate of incorporation of L-[ring-$^{13}C_6$]phenylalanine into muscle protein. Plasma and interstitial AA concentration were measured by ion exchange HPLC. Arterial leucine concentration increased by 288% and 353% 60 min after the commencement of infusion in younger and older participants respectively. Dialysate leucine concentration increased 170% and 126% in young and older individuals respectively over the same time period (although the absolute dialysate concentrations reported do not appear to be corrected for the reduced recovery resulting from the perfusion flow rate of 5.0 $\mu L.min^{-1}$). Macro and micro-vascular blood flow increased in response to AA and SNP infusion with no differences between groups. Similar responses were observed for phenylalanine net balance, FSR and model derived MPS with increases from baseline independent of group. Contrary to the author's original hypothesis, younger and older individuals responded similarly to AA infusion during concomitant SNP infusion with no evidence of anabolic resistance to AA in older participants under hyperemic conditions. One criticism of both the Durham *et al* and Dillon *et al* studies is that due to the invasive nature of the investigations only healthy, non-frail older individuals were included for investigation. This may have impacted on the ability to detect differences in muscle protein metabolism between the young and older populations.

There is reasonable agreement between data from Maggs and Gutierrez and Durham (Table 1.7) describing the resting, post-absorptive AA concentration in dialysate from human skeletal muscle other than the AA with high intra-cellular to extra-cellular concentration gradients. This is encouraging considering that each study employed a different calibration technique (extrapolation to zero-flow, low flow and retro-dialysis calibration methods respectively). Overall the AA concentration reported for the interstitium was slightly higher than those of plasma. To the best of author's knowledge, no published data currently exists describing the skeletal muscle interstitial AA response, as sampled by MD, to an orally administered AA solution at rest or post RE.

1.9 Summary

Research in relation to the mechanisms underlying age-related sarcopenia, and treatment modalities including dietary and exercise interventions are rapidly emerging. This is evidenced by the number of review/meta-analysis published within this field of investigation in the very recent past (Sayer *et al* 2013, Bauer *et al* 2013, Wall and van Loon 2013, Malafarina *et al* 2013, Dickinson *et al* 2013b, Cermak *et al* 2013, Robinson

et al 2012, Cermak *et al* 2012, Breen and Phillips 2011, Peterson *et al* 2011, Sayer 2010). Applying the theory of 'prevention is better than cure' this thesis seeks to examine if a milk protein supplement can offset the annual loss of LTM in healthy community dwelling women and men who are already consuming dietary protein in excess, based on WHO/IOM recommendations and who are not suffering from sarcopenia based on the EWGSOP definition. A secondary aim is to determine if the synergistic effect of RE and milk protein supplementation results in measurable differences in LTM over and above those observed through supplementation alone.

Digestion of protein/amino acids is not impaired with ageing provided sufficient protein is ingested at the mealtime. Older populations are resistant to the anabolic actions of insulin, particularly with relation to stimulation of nutritive blood flow (this may explain the higher circulating concentrations of AA observed in older populations relative to young in response to feeding). Signaling proteins related to MPS (mTORC1, S6K1, eIF4EBP1) and muscle protein FSR are reduced in the elderly relative to the young in response to feeding and/or RE, although this finding is not universal. The threshold for stimulation of MPS is higher in the elderly and therefore requires greater amounts and quality of protein at each meal in order to surpass this threshold. AA transporter signaling and action is a relatively new area of exploration but initial data demonstrates differential regulation between young and elderly populations.

A rapidly digested essential AA supplement, rich in leucine, which can be easily incorporated into the normal dietary intake, such that the distribution of protein ingestion over the course of the entire day is roughly even between the three main meals of the day should prove beneficial in terms of building or at least maintaining lean body mass in healthy elderly populations. A PRT intervention designed to promote the accretion of muscle protein, undertaken in combination with optimally timed consumption of a milk protein supplement should also result in an augmented response relative to milk protein supplementation is isolation. It remains to be confirmed if measureable differences in muscle mass can be observed in long term (12-24 weeks in duration) intervention studies in response to PRT and/or optimally timed protein intake in populations. Previous studies employing a RCT design have proven effects over time of supplementation, PRT and supplementation plus PRT however between group changes have not been repeatedly demonstrated.

1.10 Thesis objectives

The primary aim was to investigate the effect of adding a daily, milk-based protein supplement to the low protein meals (i.e. breakfast and lunch) on LTM in healthy

community dwelling 50-70y old women and men. A secondary aim was to access if a program of progressive, whole-body resistance exercise could further enhance this nutrient effect. Human studies were undertaken to examine (a) the bioavailability of key regulators of MPS provided by ingestion of milk protein and milk protein based derivatives and (b) measured by DXA, the effect of a six-month period of supplementation with milk protein on LTM.

1.11 Thesis construct

Chapter 1 introduces the concept of age-related decline in LTM and explores the physiological mechanisms underlying this phenomenon. Nutritional and exercise interventions designed to offset this decline are reviewed. Chapter 2 describes the change in post-prandial plasma concentration of key regulators of MPS in response to the ingestion of a milk protein beverage (FORM) compared to an isocaloric maltodextrin control beverage (MALT). These data are discussed with reference to the stimulation of acute rates of MPS based on the extant literature and their potential impact on LTM in the longer term. Chapter 3 describes the application of *in vivo* human microdialysis to assess the bioavailability of amino acid substrate in skeletal muscle tissue in response to the ingestion of milk protein and milk protein based derivatives For comparison, change in the extracellular substrate in response to the ingestion of milk protein and milk protein based derivatives when preceded by unilateral knee extensor exercise is also reported. Chapter 4 reports the change in total body and appendicular lean tissue mass resulting from a 24 week RCT of ninety, 50-70y old community dwelling women and men assigned to one of three treatments; (i) isocaloric maltodextrin (MALT), (ii) milk protein formulation (FORM) and (iii) milk protein formulation in addition to a program of progressive resistance exercise training (FORM + PRT). Chapter 5 outlines the overall conclusions for the data presented in this thesis. Recommendations for future investigation and possibilities to develop the current research theme are also expanded upon.

Chapter 2 The effect of ingestion of milk protein on the post-prandial concentration of plasma amino acids, glucose and insulin in healthy 50-70 year old women.[1]

[1] The study described herein is currently in preparation for publication. McCormack W, Power O., Jakeman, P 2014. The effect of ingestion of milk protein on the post-prandial concentration of plasma amino acids, glucose, insulin and incretin hormones in healthy 18-25 and 50-70 year old women.

2.1 Introduction

Sarcopenia is the progressive decline in skeletal muscle mass, strength and function associated with ageing (Fielding *et al* 2011, Cruz-Jentoft *et al* 2010). Age-related sarcopenia is a multifaceted condition with possible interrelated mechanisms including increased oxidative stress, persistent low grade inflammation, dysregulation of endogenous growth hormone, estrogen and androgen production, suboptimal protein intake and reduced levels of physical activity (PA), (Doherty 2003). Projections illustrating a 3-fold increase in the number of people aged 65y and older over the next 40-50y have resulted in prioritisation of the research and development of strategies designed to maintain lean tissue mass (LTM) and the functional and metabolic advantages that an appropriate lean body mass affords with advancing age. The progressive loss of lean body mass over time is indicative of an alteration in the rate of muscle protein synthesis (MPS) and/or muscle protein breakdown (MPB) with advancing age. Research has demonstrated that fasting post-absorptive rates of MPS and MPB are unaltered in elderly populations relative to young (Volpi *et al* 2001). However, others have reported depressed rates of MPS (Rooyackers *et al* 1996) and elevated rates of MPB in fasting post-absorptive older populations (Trappe *et al* 2004). These acute rates of MPS and MPB seem unlikely as if these rates were correct, the degree of muscle wasting observed with ageing would be much greater than that reported to date in cross-sectional and longitudinal studies of body composition (Baumgartner *et al* 1998, Hughes *et al* 2002). Anabolic resistance, the blunted response to nutrient ingestion and physical activity (the main anabolic stimuli to MPS) with advancing age reviewed by Burd *et al* (2013) has therefore become the research focus in this field.

One of the mechanisms implicated in the development of anabolic resistance is impaired digestion and/or absorption of dietary protein in older populations relative to young. There is evidence within the literature of elevated rates of splanchnic extraction of amino acids (AA) in elderly populations (Boirie *et al* 1997) although this finding is not universal (Koopman *et al* 2009). Direct comparison of the data from these studies is hindered by differences in the study design (the amount and type of protein (casein, whey, soy) fed in each individual trial along with the pattern of ingestion (bolus vs. spread feeding)). Despite the apparent elevation in rates of splanchnic extraction in elderly populations a common feature of trials measuring plasma AA concentration between young and old in response to AA ingestion and infusion has been elevated plasma AA concentration and area under the curve (AUC) in the elderly populations relative to the young (Koopman *et al* 2009, Durham *et al* 2010). Digestion therefore

does not appear to limit the protein synthetic response as a comparable amount of AA are available in the circulation in elderly populations as a result of equivalent protein ingestion relative to young. Reduced nutritive blood flow, owing to a decreased sensitivity to the nitric oxide dependent vasodilatory action of insulin on skeletal muscle (Rasmussen *et al* 2006) and reduced amount and/or functioning of amino acid transporters on the cell membrane (Dickinson *et al* 2013a, Drummond *et al* 2011) are two possibilities that may in part explain this defect at the extracellular level. There is a reduced amount and activation status of key signalling proteins such as mammalian target of rapamycin complex 1 (mTORC1) involved in regulating MPS in elderly populations relative to young in response to protein feeding (Cuthbertson *et al* 2005, Guillet *et al* 2004). mTORC1 activity is up regulated during AA sufficiency and both *in vitro* (Atherton *et al* 2010) and *in vivo* (Dreyer *et al* 2008) data reveal that leucine is particularly potent in its stimulation of mTORC1. It remains to be determined if a reduction in the amount and activation status of key signalling proteins involved in regulating MPS is a consequence of, or a contributing factor to, a reduced ability to traffic AA into the cell in support of MPS in elderly populations.

A hypothesis that has gained significant attention recently has been the concept of a leucine stimulatory threshold for mTORC1 and thus MPS (Breen and Phillips 2011, Dardevet *et al* 2012). This leucine threshold must be exceeded in order for a robust increase in the rate of MPS and thus net protein synthesis to take place. This leucine threshold is shifted to the right in elderly populations relative to young indicating that a greater amount of dietary leucine is required per meal to stimulate MPS in older individuals relative to young (Figure 1.11, Chapter 1, pg. 24). Pennings *et al* (2012) and Yang *et al* (2012) reported a consistent increase in rates of mixed muscle and myofibrillar protein synthesis in response to the ingestion of 20 g of whey protein (WP), (~ 2 g leucine). This is despite plasma leucine concentration peaking at ~ 450 μM during the Pennings *et al* trial with a corresponding peak concentration of ~ 250 μM reported during the Yang *et al* trial in response to an equivalent dose of WP. In practical terms if the 'leucine threshold' premise is true, the ingestion of sufficient amounts of high quality protein must be evenly distributed throughout the day such that the leucine threshold for stimulating MPS is achieved at the three main eating occasions (breakfast, lunch and evening meal) in order for older populations to be able to repeatedly produce an anabolic response to protein ingestion and thus maintain lean body mass over time.

While leucine is seen as a key regulator of mTORC1, three months of free leucine feeding (7.5 $g.d^{-1}$, distributed evenly as 2.5 g co-ingested with each of the three main meals of the day) in an elderly population by Verhoeven *et al* (2009) failed to demonstrate an effect of supplementation on lean body mass and strength. One possible explanation for this observation is that while leucine may upregulate mTORC1 activation, concurrent elevation in the bioavailability of the remaining essential amino acids (EAA) is required to support the deposition of muscle protein. Therefore the purpose of this study was to evaluate the change in post-prandial plasma EAA and insulin concentration following ingestion of a novel milk protein formulation. In addition we wished to investigate if leucine reaches a proposed extracellular threshold for the stimulation of MPS. Milk protein is a suitable candidate for evaluation as a dietary supplement to offset the decline in lean body mass with ageing due to the high proportion of both EAA and leucine relative to other protein sources.

2.2 Aims and objectives

This study seeks to investigate the change in post-prandial plasma concentration of key AA regulators of MPS, glucose and insulin in response to milk protein formulation (FORM) or carbohydrate (MALT) feeding. The research challenges the hypothesis that;

> H_0: Compared to MALT there will be no difference over time in the post-prandial plasma concentration of AA, glucose or insulin in response to FORM ingestion.

These data will inform as to the bioavailability of AA and in particular the EAA and leucine, in support of MPS during acute ingestion of milk protein and serve to substantiate the observations from a study of the effect of chronic ingestion of milk protein over a 24 week period (described in detail in Chapter 4 of this thesis).

2.3 Materials and methods

2.3.1 Materials

The commercial milk protein substrates used in this study were provided by Carbery Ingredients and Glanbia Nutritionals. Milk protein concentrate (MPC) (Glanbia Nutritionals, Kilkenny, Ireland), whey protein hydrolysate (degree of hydrolysis (DH) 45%) (Glanbia Nutritionals), calcium (TruCal) (Glanbia Nutritionals), vitamin D (cholecalciferol) (Glanbia Nutritionals), whey protein hydrolysate (DH 32%) (Carbery Ingredients, Ballineen, Ireland). Maltodextrin (MALT) (Corcoran Chemicals Ltd, Dublin Ireland) was used as the carbohydrate control. Phosphate buffered saline (P4417), sodium phosphate diabasic (S0876), sodium tetraborate decahydrate (S9640), sodium azide (S2002), phosphoric acid (S09617), tryptophan (Trp; T0254), norvaline (N7627), glutamine (G8540) were all obtained from Sigma-Aldrich (Dublin, Ireland). Borate buffer (5061-3339), o-phthalaldehyde (OPA; 5061-3335), amino acid standard mix (1 nM in 0.1 M HCl; 5061-3330) were all obtained from Agilent Technologies Inc. (Wilmingtom, USA). High performance liquid chromatography (HPLC) grade acetonitrile (ACN) and methanol were obtained from Lennox Laboratory Supplies (Dublin, Ireland). The Elecsys insulin kit was from Roche Diagnostics (Mannheim, Germany). The UniCel DxC 800 glucose kit was from Beckman Coulter Inc. (CA, USA).

Table 2.1 – Participants' characteristics

Age (y)	59.4 ± 2.6
Body Mass (kg)	61.1 ± 3.5
Height (cm)	162 ± 2.5
BMI (kg.m^{-2})	22.3 ± 1.1
Body fat %	31 ± 1.2
Lean body mass (kg)	38 ± 1.7
Basal plasma glucose (mM)	5.0 ± 0.2
Basal plasma insulin (pM)	46.1 ± 6.0
[1]HOMA-IR	1.48 ± 0.2
Creatinine (µmol.L^{-1})	67.5 ± 5.0
Albumen (g.L^{-1})	43.7 ± 1.0

Values are mean ± SEM, (n = 8). [1] Homeostasis model assessment insulin resistance

2.3.2 Participants

Eight 50-70y old women were recruited to partake in this investigation (participant characteristics are described in Table 2.1). Participants were in good health at the time of the investigation and were instructed to maintain their normal diet and to refrain from vigorous physical activity (PA) in the 24h preceding each experimental trial. All

experimental protocols were approved by the University of Limerick Research Ethics Committee (EHSREC Approval 2013_01_13). Participants were informed of the risks associated with participating in the study prior to completing written informed consent.

2.3.3 Experimental procedures

Participants completed two trials in a randomised, single blind order separated by a period of no less than 7 days. On test days, participants reported to the PESS Department Project Laboratory, University of Limerick at 8am on the morning of the experimental trial having fasted since 10pm the previous evening. Upon arrival participants were placed in the supine position (sitting reclined with their knees slightly bent, on a physiotherapy table) and an intravenous (IV) indwelling 20 gauge cannula (B Braun, Melsungen, DE) was placed in an antecubital vein of the arm. Patency of the catheter was maintained by saline infusion. Participants were then seated at a workstation and allowed to read/study/browse the internet while pre and postprandial blood samples were drawn as per the time series depicted in Figure 2.1. All blood samples were decanted into pre-chilled lithium heparin tubes (Sarstedt, Numbrect, DE) centrifuged (2000 relative centrifugal force (*rcf*), 4°C for 10 min (Eppendorf 5417R centrifuge, Eppendorf AG, Hamburg, DE) with the resultant supernatant aliquot and stored at -80°C until analysis. On a separate morning, following an overnight fast, participants reported to the DXA laboratory, PESS building, University of Limerick for measurement of height, mass and body composition assessment via DXA (Lunar iDXA™, GE Healthcare, Chalfont St Giles, Bucks., UK).

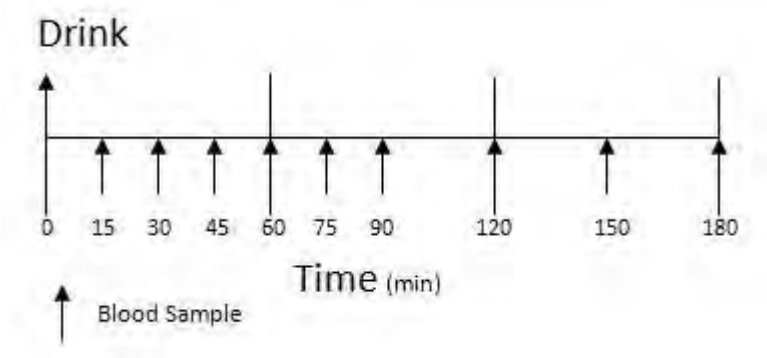

Figure 2.1 – Experimental protocol including blood sampling timeline.

2.3.2.1 Test solutions

Post cannulation, one of two test beverages of 400 mL were delivered at room temperature and consumed within 5 minutes of presentation. FORM consisted of a proprietary blend of milk protein concentrate (MPC), two whey proteins hydrolysates (WPH) (degree of hydrolysis (DH) 32% and DH 45%), calcium and vitamin D (Table 2.2). The *in vitro* antioxidant and insulinotropic properties of the WPHs have been

described elsewhere (Appendix A) and form the basis for their inclusion in the formulation. An isocaloric carbohydrate (MALT) solution acted as the control beverage.

Table 2.2 – Composition of test solutions (based on the average weight of the participants - 61.1 kg)

	FORM	MALT
Volume (mL)	400	400
Powder (g)	32	29
% Solution (w/v)	7.1	7.3
Energy (kcal)	110	110
CHO (g)	3.0	27
Protein (g)	20	-
EAA (g)	9.3	-
Leucine (g)	1.9	-
Calcium (mg)	1250	-
Vitamin D3 (IU)	720	-

2.3.3.1 Analysis

2.3.3.1.1 Plasma amino acid analysis

Perchloric acid plasma extracts (1.7:1, 0.4 M $HClO_4$:plasma) of AAs were measured by pre-column derivatisation with o-phthalaldehyde (OPA) and quantified by reverse phase high performance liquid chromatography (RPHPLC, Agilent 1200 RRLC, Agilent Technologies, CA, USA) using a modification of the procedures described by Turnell and Cooper (1982) and R. C. Dorresteijn *et al* (1996) . The system consisted of an online degasser (G1322A), binary pump (G1212B), temperature controlled auto-sampler (G1367D), thermostat compartment (G1330B), temperature controlled column compartment (G1316B), ultraviolet visible absorbance detector (G1315C) and a scanning fluorescence detector (G1321A). Peak integration software (Chemstation™, Revision B.04.03 [16]) was used to evaluate peak area and retention time of each AA. Briefly, plasma AAs were derivatised in line with OPA/2-mercaptopropionic acid (MPA) and separated by gradient elution RPHPLC (temperature controlled at 40° C). A C18-bonded silica column (Zorbax Eclipse Plus C18, 4.6 x 50 mm x 1.8 µm, Agilent Technologies) and were eluted by binary solvent gradient comprising; (A) 10 mM Na_2HPO_4:10 mM $Na_2B_4O_7$:5 mM NaN_3, pH 8.2 and (B) acetonitrile:methanol:water (45:45:10, v:v:v). OPA derivatives were detected by fluorescence (Ex 330, Em 450 nm). Norvaline was employed as the internal standard. AA derivatives were identified by retention time relative to the reference peak and quantified as area under the curve (AUC) by reference to standards of known concentration based on a five-point calibration curve. Analytical performance was established by repeated assay of pooled perchloric acid plasma extracts spiked with AA of known concentration. Inter and intra

assay CV's were less than 3% for all AA except the low abundance AAs methionine and cysteine.

2.3.3.1.2 Plasma insulin analysis

Plasma insulin was measured by electrochemiluminescence on a Meso Scale Discovery (MSD) QUICKPLEX SQ120 (Meso Scale Discovery, MD, USA). The intra-assay CV was less than 5%.

2.3.3.1.3 Plasma glucose analysis

Plasma glucose concentration was measured by a glucose oxidase method on a UniCel DxC 800 instrument (Beckman Coulter, Inc. CA, USA). The intra-assay CV was less than 5%.

2.3.4 Statistical analysis

All data was tested for normality using Shapiro-Wilk test. Data are presented as the mean ± SEM (n = 8). The EAA and leucine were selected for analysis above other AAs due to their recognised role in the stimulation of and support to MPS. A two-factor (treatment X time) analysis of variance (ANOVA) was used with repeated measures on the factor time to examine differences over time and between treatments (Feed; MALT and FORM). Non-time dependent variables were examined by univariate ANOVA. AUC above baseline for each analyte was calculated by trapezoidal integration and difference in AUC between treatments was analysed by univariate ANOVA. Statistical significance was set at $P < 0.05$. All analyses were performed using SPSS (v.20, SPSS Inc, Chicago, IL, USA).

2.4 Results

2.4.1 Essential amino acids

Basal plasma EAA concentration did not differ prior to MALT and FORM ingestion [Table 2.3, Fig 2.2, Feed ANOVA; $F_{(1,14)} = 0.02$; $P = 0.885$, 1-ß = 0.05 , $\eta_p^2 = 0.00$]. Plasma EAA concentration decreased following ingestion of MALT and remained below baseline concentration throughout the 3h postprandial period whilst plasma EAA concentration increased following ingestion of FORM and remained elevated above baseline throughout the 3h postprandial period [Table 2.3, Figure 2.2, Time ANOVA; $F_{(1.80,25.17)} = 22.26$; $P < 0.01$, 1-ß = 1.0 , $\eta_p^2 = 0.61$, Time x Feed ANOVA; $F_{(1.80,25.17)} = 27.68$; $P < 0.01$, 1-ß = 1.0 , $\eta_p^2 = 0.66$]. Minimum plasma EAA concentration (C_{MIN}) was 721 μM in response to MALT ingestion whilst maximal plasma EAA concentration (C_{MAX}) was 1922 μM in response to FORM ingestion [Feed ANOVA; $F_{(1,14)} = 182.06$; P

< 0.01, 1-ß = 1.0 , η_p^2 = 0.93]. Time to minimum plasma EAA concentration (T_{MIN}) occurred at 92 ± 9.6 min in response to MALT ingestion whilst time to maximum plasma EAA concentration (tC_{MAX}) occurred at 41 ± 5.5 min in response to FORM ingestion. EAA AUC above baseline was -24 ± 5.9 mM.3h^{-1} in response to MALT ingestion whilst EAA AUC above baseline was 94 ± 6.0 mM.3h^{-1} in response to FORM ingestion [Feed ANOVA; $F_{(1,14)}$ = 195.80; P < 0.01, 1-ß = 1.0 , η_p^2 = 0.93].

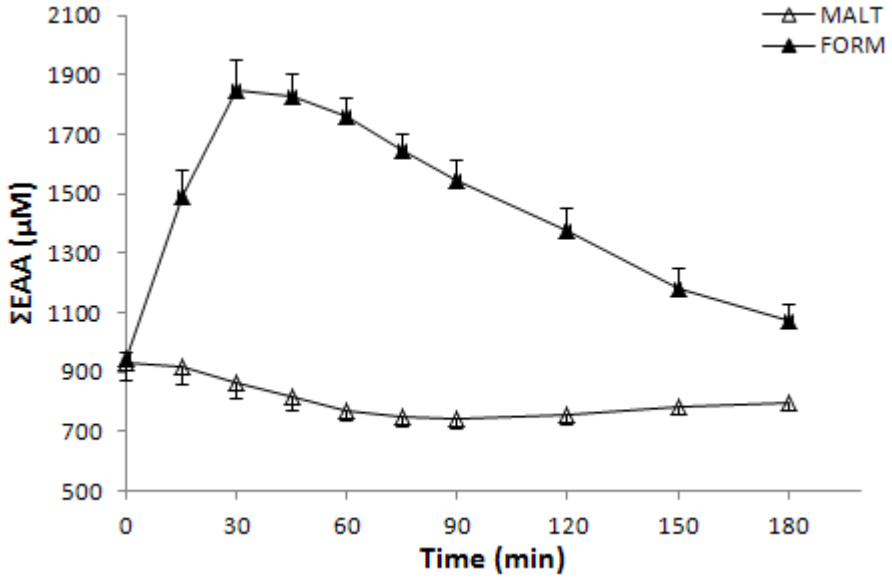

Figure 2.2 – Plasma essential amino acid concentration in response to ingestion of MALT or FORM in healthy 50-70y old women. Data are mean ± SEM (n = 8).

2.4.2 Leucine

Basal plasma leucine concentration did not differ prior to MALT and FORM ingestion [Table 2.3, Fig 2.3, Feed ANOVA; $F_{(1,14)}$ = 0.77; P = 0.396, 1-ß = 0.13 , η_p^2 = 0.05]. Plasma leucine concentration decreased following ingestion of MALT and remained below baseline throughout the 3h postprandial period whilst plasma leucine concentration increased in following ingestion of FORM and remained elevated above baseline throughout the 3h postprandial period [Table 2.3, Fig 2.3, Time ANOVA; $F_{(1.82,25.54)}$ = 20.95; P < 0.01, 1-ß = 1.0 , η_p^2 = 0.60, Time x Feed ANOVA; $F_{(1.82,25.54)}$ = 27.94; P < 0.01, 1-ß = 1.0 , η_p^2 = 0.67]. C_{MIN} was 61 µM in response to MALT ingestion whilst C_{MAX} was 335 µM in response to FORM ingestion [$F_{(1,14)}$ = 147.84; P < 0.01, 1-ß = 1.0, η_p^2 = 0.91]. T_{MIN} occurred at 84 ± 6.3 min in response to MALT ingestion whilst tC_{MAX} occurred at 39 ± 4.9 min in response to FORM ingestion. Leucine AUC above baseline was -4.2 ± 0.8 mM.3h^{-1} in response to MALT ingestion whilst leucine AUC above baseline was 23 ± 1.5 mM.3h^{-1} in response to FORM ingestion [Feed ANOVA $F_{(1,14)}$ = 264.29; P < 0.01, 1-ß = 1.0 , η_p^2 = 0.95].

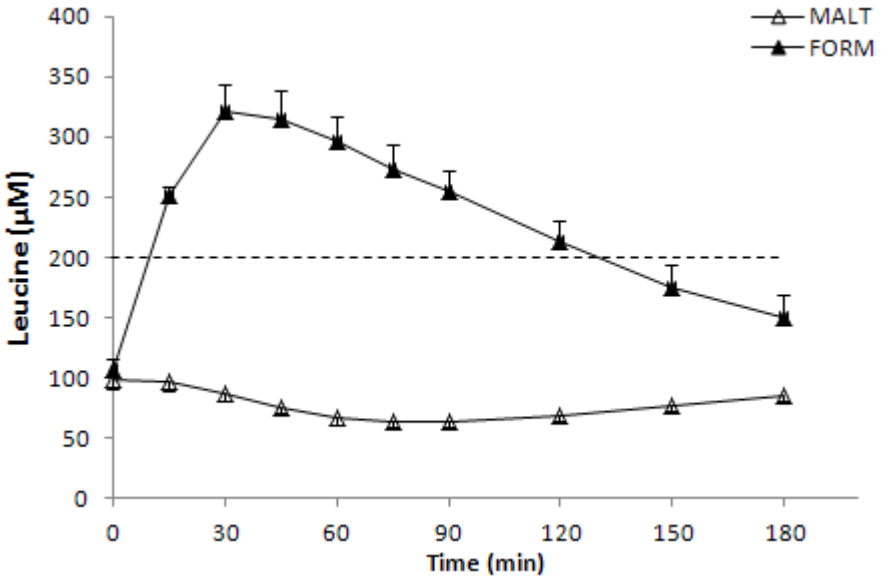

Figure 2.3 – Plasma leucine concentration in response to ingestion of MALT or FORM in healthy 50-70y old women. Data are mean ± SEM (n = 8). Dotted line indicates a proposed leucine threshold for stimulation of muscle protein synthesis.

2.4.3 Glucose

Basal plasma glucose concentration did not differ prior to MALT and FORM ingestion [Figure 2.4, ANOVA; $F_{(1,14)} = 0.631$; $P = 0.44$, 1-ß = 0.115 , $\eta_p^2 = 0.04$]. Plasma glucose concentration increased following ingestion of MALT, peaking between 15 min and 30 min post ingestion before decreasing to values below baseline at 60 min and remaining below baseline for the remainder of the trial. Following ingestion of FORM plasma glucose concentration increased slightly at 15 min post ingestion before decreasing to 45 min. At 45 min post ingestion glucose concentration began to recover to values similar to baseline for the remainder of the trial [Figure 2.4, Time ANOVA; $F_{(2.62,36.67)} = 13.05$; $P < 0.01$, 1-ß = 1.0 , $\eta_p^2 = 0.48$, Time x Feed ANOVA; $F_{(2.62,36.67)} = 17.09$; $P < 0.01$, 1-ß = 1.0 , $\eta_p^2 = 0.55$]. C_{MAX} was 7.3 mM in response to MALT ingestion whilst C_{MIN} was 4.0 mM in response to FORM ingestion [$F_{(1,14)} = 51.36$; $P < 0.01$, 1-ß = 1.0, $\eta_p^2 = 0.79$]. tC_{MAX} occurred at 39 ± 6.3 min in response to MALT ingestion whilst T_{MIN} occurred at 26 ± 3.8 min in response to FORM ingestion. Glucose AUC above baseline was -31 ± 32 mM.3h^{-1} in response to MALT ingestion whilst glucose AUC above baseline was -5.4 ± 20 mM.3h^{-1} in response to FORM ingestion [$F_{(1,14)} = 0.46$; $P = 0.508$, 1-ß = 0.01 , $\eta_p^2 = 0.03$].

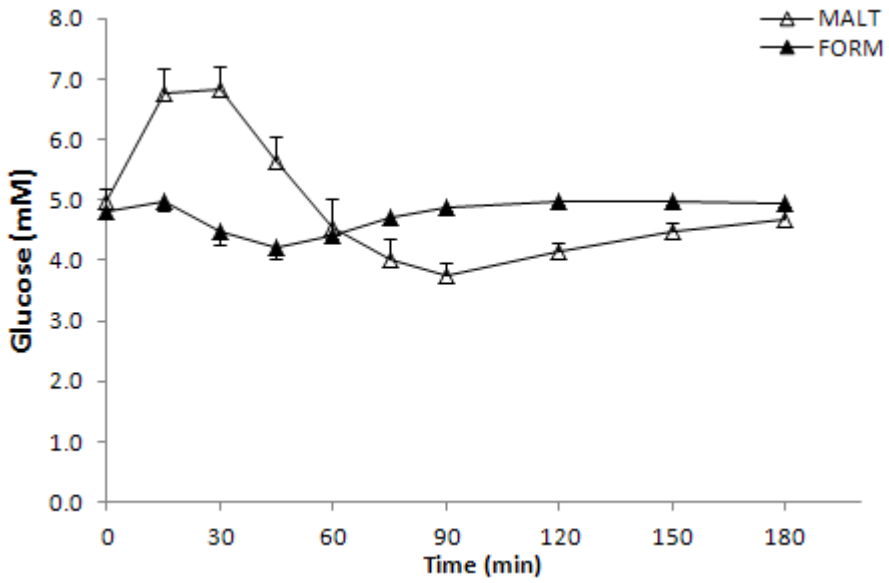

Figure 2.4 – Plasma glucose concentration in response to ingestion of MALT or FORM in healthy 50– 70y old women. Data are mean ± SEM (n = 8).

2.4.4 Insulin

Basal plasma insulin concentration did not differ prior to MALT and FORM ingestion [Figure 2.5, ANOVA; $F_{(1,14)}$ = 0.47; P = 0.560, 1-ß = 0.1 , η_p^2 = 0.03]. Plasma insulin concentration increased following ingestion of MALT and FORM and remained elevated above baseline for approximately 90 min post ingestion, thereafter returning to values similar to baseline for the remainder of the trial [Figure 2.5, Time ANOVA; $F_{(2.01,28.19)}$ = 15.39; P < 0.001, 1-ß = 0.99 , η_p^2 = 0.52, Time x Feed ANOVA; $F_{(2.01,28.19)}$ = 2.31; P = 0.118, 1-ß = 0.43, η_p^2 = 0.14]. C_{MAX} was 465 pM and 342 pM in response to MALT and FORM ingestion respectively [$F_{(1,14)}$ = 0.86; P = 0.369, 1-ß = 0.14, η_p^2 = 0.06]. tC_{MAX} was 34 ± 4.7 min and 26 ± 3.8 min in response to MALT and FORM ingestion respectively. Insulin AUC above baseline was 14.9 ± 4.4 nM.3h^{-1} and 10.9 ± 1.6 nM.3h^{-1} in response to MALT and FORM ingestion respectively [$F_{(1,14)}$ = 0.76; P = 0.398, 1-ß = 0.13, η_p^2 = 0.05].

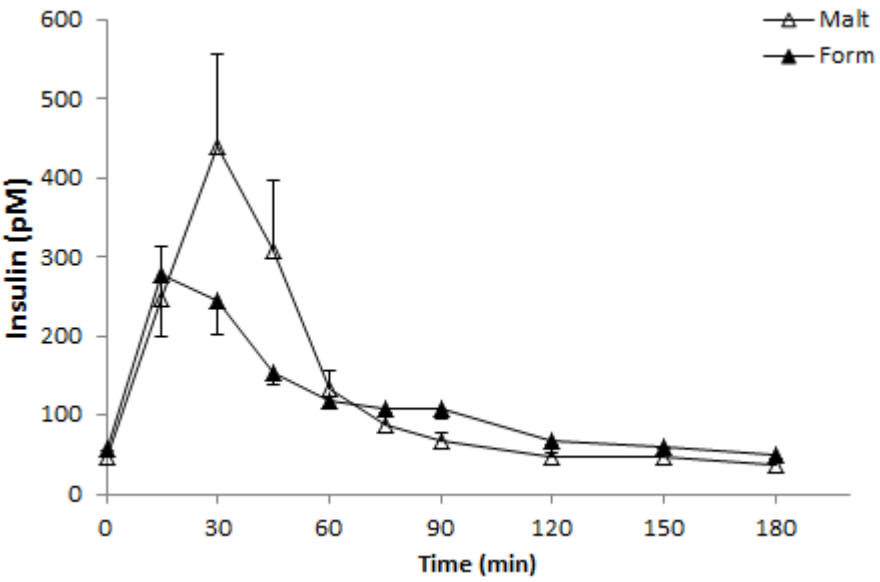

Figure 2.5 – Plasma insulin concentration in response to ingestion of MALT or FORM in healthy 50-70y old women. Data are mean ± SEM (n = 8).

Table 2.3 – Plasma amino acid concentration at baseline, minimum (min)/maximum (max) concentration, % change from baseline to minimum/maximum concentration and area under the curve above baseline in response to ingestion of a maltodextrin or milk protein solution in healthy 50-70 year old women. Data are mean ± SEM (n = 8).

	MALT				FORM			
AA (μM)	**Baseline**	**Min**	**Δ%**	**AUC ($mM.3h^{-1}$)**	**Baseline**	**Max**	**Δ%**	**AUC ($mM.3h^{-1}$)**
Glu	32 ± 5	23 ± 2	-27%	-0.9 ± 0.7	28 ± 3	45 ± 5	63%	1.5 ± 0.5
Ser	99 ± 6	81 ± 3	-18%	-2.3 ± 0.9	104 ± 4	162 ± 8	56%	3.4 ± 0.6
Gln	628 ± 29	565 ± 24	-10%	-6.3 ± 2.9	663 ± 18	775 ± 37	17%	3.9 ± 3.9
His	80 ± 5	69 ± 4	-13%	-1.1 ± 0.8	89 ± 2	113 ± 7	27%	0.8 ± 0.6
Gly	274 ± 20	243 ± 16	-11%	-4.0 ± 1.4	281 ± 23	303 ± 26	8%	-6.0 ± 1.4
Thr	127 ± 18	103 ± 14	-19%	-3.2 ± 0.8	118 ± 4	203 ± 13	72%	8.8 ± 0.9
Arg	88 ± 16	72 ± 12	-19%	-1.6 ± 1.5	92 ± 6	155 ± 11	69%	5.3 ± 1.1
Ala	421 ± 36	361 ± 19	-14%	-3.8 ± 2.5	426 ± 29	581 ± 45	36%	10 ± 2.0
Tau	13 ± 1	10 ± 1	-26%	-0.3 ± 0.2	19 ± 2	21 ± 4	11%	-0.6 ± 0.4
Tyr	55 ± 6	39 ± 4	-29%	-2.0 ± 0.7	53 ± 2	120 ± 11	125%	8.4 ± 1.1
Cyst	15 ± 6	13 ± 5	-11%	-0.2 ± 0.2	20 ± 6	23 ± 7	15%	-0.3 ± 0.4
Val	203 ± 16	160 ± 10	-21%	-5.5 ± 1.2	200 ± 7	364 ± 17	83%	20.5 ± 1.4
Met	13 ± 3	7 ± 1	-43%	-0.7 ± 0.4	14 ± 2	49 ± 4	262%	3.6 ± 0.3
Trp	67 ± 4	58 ± 2	-13%	-0.7 ± 0.5	71 ± 2	112 ± 4	56%	3.8 ± 0.4
Phe	50 ± 5	36 ± 2	-27%	-1.8 ± 0.4	50 ± 3	82 ± 6	64%	3.1 ± 0.4
Iso	43 ± 5	23 ± 2	-47%	-2.6 ± 0.5	43 ± 3	154 ± 10	261%	11 ± 0.7
Leu	99 ± 8	63 ± 4	-36%	-4.2 ± 0.8	107 ± 7	321 ± 22	199%	23 ± 1.5
Lys	253 ± 15	217 ± 7	-14%	-4.4 ± 1.5	252 ± 8	461 ± 23	83%	19 ± 1.4
ΣAA	2558 ± 147	2213 ± 76	-13%	-46 ± 15	2629 ± 42	4008 ± 160	52%	120 ± 13
ΣEAA	934 ± 62	743 ± 31	-20%	-24 ± 5.9	943 ± 20	1848 ± 83	96%	94 ± 6.0
ΣBCAA	344 ± 27	246 ± 14	-29%	-12 ± 2.4	349 ± 15	834 ± 50	139%	55 ± 3.5

2.5 Discussion

The aim of this study was to describe the post-prandial change in plasma AA, glucose and insulin in response to the ingestion of FORM (derived to support the maintenance and/or accrual of lean body mass in ageing populations) and an isocaloric MALT solution in healthy 50-70y old women. Ingestion of FORM resulted in a rapid and transient increase in plasma AA concentration. The EAA concentration increased two-fold above baseline during the post-prandial period. The leucine concentration increased by almost three-fold 30 minutes post FORM ingestion. The decrease in plasma AA concentration following MALT ingestion is likely due to a combination of insulin mediated suppression of proteolysis (Gelfand and Barrett 1987, Louard *et al* 1992), and stimulation of AA uptake into tissue cells (Biolo *et al* 1999) with the nadir at 90 minutes post ingestion. There was no direct measurement of anabolic signaling (mTORC1 phosphorylation) or MPS (incorporation of labeled phenylalanine into muscle protein) in response to feeding in this study. However, based on the extant literature (Paddon-Jones *et al* 2004, Yang *et al* 2012) and the plasma AA concentration reported here it is reasonable to assume the leucine concentration achieved was sufficient to surpass the proposed leucine threshold (Figure 1.12) while the provision of EAA was also sufficient to sustain an increase in the acute rate of MPS in response to FORM ingestion. Bohe *et al* (2003) describe the curvilinear relationship between the extracellular EAA concentration and its effect on mixed MPS in repose to AA infusion in human skeletal muscle (Figure 1.6). In this study the EAA concentration exceeds 1.5 mM and peaks at 1.8 mM in response to FORM ingestion. Based on the curvilinear response of MPS to increasing concentrations of EAA reported by Bohe and coworkers (2003), the EAA concentration achieved in response to feeding during the current trial should be sufficient to support an increase of ~ 50% in the acute rate of MPS.

It is difficult to pronounce definitively that any particular extracellular leucine concentration has activated mTORC1 and thus facilitated stimulation of MPS *in vivo* without a direct measurement of the some component of the signaling pathway and/or muscle protein fractional synthetic rate. There is also some variance in the plasma leucine response reported in various trials, despite similar participant phenotypes, feeding paradigms and analytical methods. In this instance we have proposed a plasma leucine concentration of ~ 200 μM (i.e. a doubling of the baseline concentration) as a threshold value for stimulation of MPS. Despite the variance in the plasma leucine concentration reported, a consistent finding with regard to measurement of MPS in elderly populations has been the increase in the rate of MPS in response to the ingestion of two grams or more of leucine as either a constituent of

WP or as the free AA. These data support the assertion that in this instance, the ingestion of FORM resulted in an acute increase in the rate of MPS. The stimulation of MPS in combination with the insulin mediated suppression of MPB (the AA induced secretion of insulin observed in this trial is comparable with data within the extant literature that demonstrates a suppression of proteolysis) should contribute to an overall positive shift in net protein balance. Caution must be advised when extrapolating observations from acute trials into longer term intervention studies. For example, during a 3-month intervention study, Verhoeven *et al* (2009) were unable to demonstrate the increase in LTM over time predicted based on acute MPS data from Katsanos *et al* (2006). Katsanos and co-workers reported that the acute rate of MPS was increased in response to feeding additional leucine with an EAA bolus. Mitchell *et al* (2014) recently reported that acute changes in FSR of muscle protein following an initial bout of resistance exercise (RE) in naïve individuals are not correlated with muscle hypertrophy following a period of chronic progressive resistance exercise training (PRT) in healthy young men.

It has been previously shown in our lab that both intact and hydrolysed WP act as insulin secretagogues (Power *et al* 2009). The present study demonstrates the FORM stimulates insulin secretion with a relative potency of 74% compared to MALT. The insulinotropic response is independent of plasma glucose. The change in the concentration of circulating EAA and the secretion of incretin hormones (gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) have been proposed as the primary regulators of protein mediated insulin secretion (Floyd *et al* 1970, Holst and Gromada 2004). These data inform as to the bioavailability of AA and in particular leucine and insulin in support of MPS post ingestion of FORM. These observations suggest that chronic ingestion of FORM over a six-month period may lead to an increase in net MPS and in turn and increase in LTM in 50-70y old individuals. The intervention study described in Chapter 4 will challenge this hypothesis.

2.6 Conclusion

The provision of milk protein resulted in a rapid and transient increase in the plasma AA concentration, peaking approximately 30 minutes post ingestion and remaining elevated above baseline concentration for 180 minutes post ingestion in healthy 50-70y old women. The plasma EAA, leucine and insulin concentration increased to levels that are indicative of an acute stimulation of MPS and inhibition of MPB respectively. Evidence with the extant literature suggests that these changes will result in net protein synthesis. These data form the basis of a hypothesis that the habitual ingestion of

FORM as part of the normal diet over a 24 week period in a group of healthy 50-70y old individuals will result in the accrual of LTM. Ingestion of an isocaloric carbohydrate beverage resulted in a decrease in the plasma AA concentration with the nadir at approximately 90 min post ingestion. The relative contribution of insulin mediated AA uptake into tissue cells or inhibition of proteolysis to this observation remains to be elucidated. The data reported here are informative in terms of describing post-prandial changes in key regulatory components of muscle protein metabolism in 50-70y old women. However, in reality macronutrients are rarely, if ever, consumed in isolation. The argument with regard to effecting a quantitative and qualitative change in the distribution of the protein load could be further advanced through investigation of the effect of supplementation of the 'normal' breakfast and lunch with additional protein. This could form the basis for future work in this area.

Chapter 3 Extracellular amino acid bioavailability, as measured by in vivo microdialysis, in response to exercise and ingestion of milk protein.[2]

[2] The study described herein is currently in preparation for publication. McCormack W., Cooke J., O'Connor, W, Jakeman, P. (2014). Change in intercellular amino acid concentration in response to milk protein ingestion and resistance exercise measured by in vivo microdialysis (in preparation).

3.1 Introduction

The musculoskeletal system comprises approximately 75% of the body mass of a healthy individual (Forbes 1987). Skeletal muscle protein makes up more than 60% of whole body protein mass while protein metabolism in skeletal muscle accounts for approximately 25% of whole body protein turnover (Henriksson 1991) and between 10-20% of the basal metabolic rate (Rasmussen and Phillips 2003). Many questions regarding the regulation of lean tissue mass (LTM) in health, disease and aging remain unanswered. As such the mechanisms that regulate muscle mass during growth, development, maintenance and age-related loss of muscle mass (sarcopenia) continue to be worthy of scientific endeavor. Feeding (Volpi *et al* 2003) and resistance exercise (RE) (Chesley *et al* 1992) have been shown to transiently increase muscle protein fractional synthetic rate (FSR). In the feeding paradigm, human muscle protein synthesis (MPS) is regulated by the extracellular concentration of the essential amino acids (EAA) as opposed to the intramuscular concentration (Bohe *et al* 2003, Wolfe (2002), Fujita *et al* 2007). Leucine has been identified as having a particularly prominent role in the stimulation of MPS. The extracellular (venous plasma) amino acid (AA) concentration in response to the ingestion of milk protein has been described in Chapter 2 of this thesis. In order to develop this theme we undertook and exploratory study to investigate the suitability of microdialysis (MD) as a tool to quantify substrate bioavailability at the tissue level in response to perturbations such as nutrient ingestion and RE.

MD is a membrane based sampling technique purported to be a minimally invasive *in vivo* analytical tool. It can provide data on substrate bioavailability at the tissue level by directly sampling the extracellular space surrounding the tissue cells. Directly measuring tissue chemistry via MD is a more desirable way to provide biologically meaningful data in relation to perturbations to tissue homeostasis than, for example, blood sampling from a peripheral vein or repeated tissue excision. The MD technique provides the opportunity to directly quantify substrates related to the regulation of muscle protein metabolism at the tissue level in response to the main anabolic stimuli, nutrient ingestion and RE. However, consideration must be given to factors inherent to the MD technique such as the perturbation caused by the insertion of a MD catheter into the tissue of interest and the relative recovery of analytes of interest from the interstitial space.

3.2 Aims and objectives

This study seeks to investigate the bioavailability of AA substrates and key AA regulators of MPS, sampled from the interstitial fluid of human skeletal muscle in response to RE with and without milk protein feeding. The research challenges the hypothesis that, as sampled by MD;

> H_0: Compared to a resting contra-lateral limb there will be no difference in bioavailability of interstitial AA in skeletal muscle in response to RE as measured by area under the curve (AUC).
>
> H_0: Compared to a resting contra-lateral limb there will be no difference in bioavailability of interstitial AA in skeletal muscle in response to RE when fed a protein substrate three hours post RE as measured by AUC.

3.3 Materials and methods

3.3.1 Materials

The commercial milk protein substrate used in this study (whey protein isolate (WPI) (Isolac Instant, 3674) was provided by Carbery Ingredients, Ballineen, Ireland. The control material contained strawberry flavour 79.4%, sucralose (12.7% and carmine (7.9%) (Carbery Ingredients). Phosphate buffered saline (P4417), sodium phosphate diabasic (S0876), sodium hydroxide (S5881), sodium azide (S2002), phosphoric acid (S09617), tetrahydrofuran (34865), tryptophan (Trp; T0254), norvaline (N7627), glutamine (G8540), carnosine (C9625), taurine (T0625) were all obtained from Sigma-Aldrich (Dublin, Ireland). Borate buffer (5061-3339), o-phthalaldehyde (OPA; 5061-3335), amino acid standard mix (1 nM in 0.1 M HCl; 5061-3330) were all obtained from Agilent Technologies Inc. (Wilmingtom, USA). High performance liquid chromatography (HPLC) grade acetonitrile (ACN) and methanol were obtained from Lennox Laboratory Supplies (Dublin, Ireland).

3.3.2 Participants

Four healthy male participants took part in the study (subject characteristics presented in Table 3.1). Participants were recreationally active but not involved in a regular resistance or endurance training programme. Ethical approval was obtained from the University of Limerick Research Ethics Committee (ULREC Approval 09/07). Participants were fully apprised of experimental procedures prior to obtaining written informed consent. Participants completed a pre-test questionnaire on the morning of each experimental day that screened for contraindications to RE, blood and MD sampling and whey protein (WP) ingestion.

Table 3.1 – Physical characteristics of participants (n = 4)

Age (y)	26.2 ± 1.4
Height (cm)	181 ± 4.0
BM (kg)	83.2 ± 3.2
LTM (kg)	60.1 ± 5.0
FTM (kg)	20.8 ± 1.2
BF (%)	26 ± 2.5%

Data are mean ± SEM

3.3.3 Experimental procedures

All four participants completed the same pre-experimental protocol (Figure 3.1). Participants reported to the Metabolic Control Room at the Physical Education and Sports Sciences Department, University of Limerick at 7am on the morning of each trial. Participants had consumed a prescribed 2 MJ breakfast (Table 3.2) before arrival

following an overnight fast from 8pm the previous evening. The breakfast consisted of 30 g of breakfast cereal, 330 mL of whole milk and a 45 g cereal bar. During the 24h prior to the test day participants were advised to maintain their normal dietary and PA patterns whilst avoiding heavy exercise. Dietary intake and PA level was recorded for the 24 h prior to each subject's initial trial. Participants were instructed to repeat their recorded dietary intake and PA levels in the 24 h prior to their second experimental day.

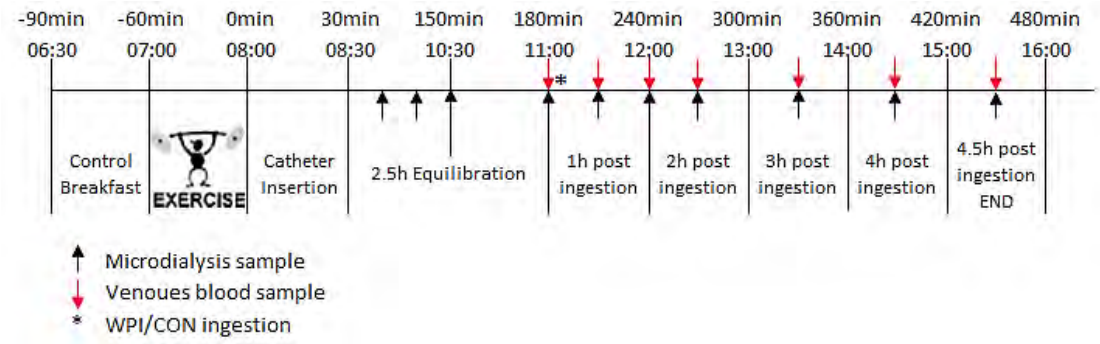

Figure 3.1 – Schematic representation of the experimental protocol. Time values are in minutes from the beginning of microdialysis catheter insertion.

Table 3.2 – Nutritional information for prescribed breakfast consumed on morning of test days.

	Prescribed Breakfast	Energy Contribution (kJ)	% of Total Energy
Energy			
- kJ	2055		
- kcal	487		
Protein (g)	15.2	256	13%
Carbohydrates (g)	71.1	1137	55%
- sugars (g)	33.9		
- starch (g)	35.0		
Fat (g)	15.9	587	29%
- saturates (g)	7.7		
Fibre (g)	2.5		
Sodium (g)	0.3		
Total		1983	97%

3.3.3.1 Anthropometric measures

On a separate day prior to the MD experimental days, height was measured to the nearest 0.1 cm using a stadiometer. Body mass was measured to the nearest 0.01 kg using an electronic scale (Tanita Body Composition Monitor MC-180, Tanita Corporation of America, Inc. Illinois, USA). Body composition (lean tissue mass (LTM), fat tissue mass (FTM) and percentage body fat (% BF) was measured by DXA (Lunar

iDXA; GE Healthcare, WI, USA). Participants were assessed in bare feet wearing light clothing.

3.3.3.2 Exercise protocol

Upon arriving at the laboratory on experimental days participants sat quietly, reclined on a physiotherapy plinth for 10 minutes. Participants then began the RE protocol. After a 5 minute warm-up on a cycle ergometer (~60 W) participants completed a session of unilateral (dominant leg) lower-limb exercise consisting of eight sets of ten repetitions of concentric isokinetic knee extensor exercise at 30° per second on an isokinetic dynamometer (ConTrex Multi Joint System, Zurich, CH) (Figure 3.2). Participants were instructed to contract maximally for the duration of each repetition. Participants were verbally encouraged and offered visual guidance to maintain effort. Rest time between sets was two minutes and the entire exercise protocol required approximately 35 minutes to complete. Similar RE protocols have been show to acutely increase post exercise MPS (Witard *et al* 2009).

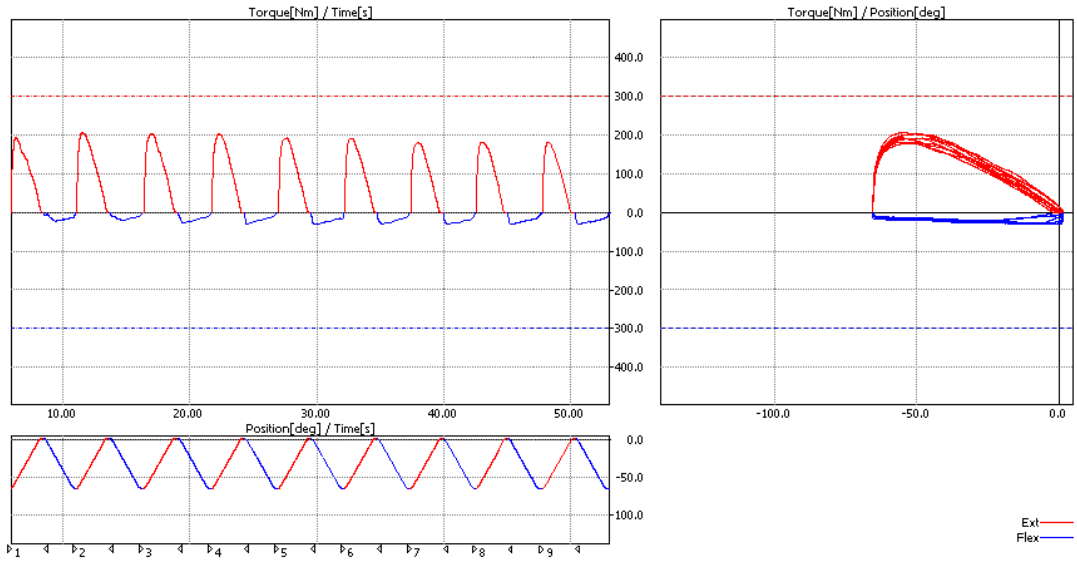

Figure 3.2 – Typical torque(Nm)/time(s), torque(Nm)/position(deg.) and position(deg.)/time(s) profile recorded during one set of the single leg exercise (knee extension) protocol participants completed prior to MD cannulation and CON or WPI ingestion.

3.3.3.3 Blood and microdialysis sampling

All MD catheters were purchased from CMA Microdialysis (Solna, Sweden). The area into which the MD catheters were to be inserted was shaved and local anaesthesia (2% Lidocaine, B-Braun, Melsungen, DE) was induced subcutaneously and above the muscle fascia. MD catheters (CMA 63, 20kDa MWCO) were inserted bilaterally into the quadriceps femoris muscles (*m. VL*). Briefly, a steel guide cannula (CMA SI-2) with guide tubing fitted on the outside is introduced in parallel to the muscle fibre. The guide

cannula is then removed leaving the guide tubing in place in the tissue. The pre-whetted MD catheter is then introduced through the guide tubing. The guide tubing is then removed by splicing upon retraction before the catheter is fixed in position using surgical tape (Figure 3.3 and Figure 3.4). The inlet tubing of the CMA 63 catheters were connected to CMA 107 portable pumps and perfused at a rate of 0.3 $\mu L.min^{-1}$. MD catheters were perfused with CMA Perfusion Fluid T1 (see Table 3.3 for composition). Prior to insertion the catheters were perfused at a rate of 10 $\mu L.min^{-1}$ for at least the minimum time required to fill the dead space of the catheter. The distal end of the outflow tubing was cut just before the micro-vial holder. Sample was collected by placing the end of the outflow tubing in 300 µL plastic vials (Milian, Geneva, CH) covered with Parafilm M to prevent evaporation of the perfusate.

Post sample collection vials were placed in a refrigerated centrifuge at 1,000 *rcf* for 10 seconds before the Parafilm was removed to ensure that all fluid was collected at the bottom of the vial. Samples were weighed immediately prior to and post sampling on a precision balance (AE200, Mettler-Toledo, Leicester, UK) to determine sample volume. The initial MD samples (60–150 min) from each catheter where analysed for glucose and lactate concentration on the CMA 600 analyser to confirm catheter operation. Subsequent to confirmation all further samples were immediately capped post centrifugation and frozen at -80^{o} C in order to conserve sample for AA analysis. Blood was drawn at regular intervals from an intravenous (IV) indwelling 20 gauge catheter (B Braun, Melsungen, DE) located in an antecubital vein of the right arm. Patency of the catheter was maintained by saline infusion. Approximately 5 mL of whole blood was drawn in each instance and divided between serum and plasma vials. Samples were centrifuged (2000 *rcf*, 4^{o}C for 10 minutes) and the resultant supernatant aliquot and stored at -80^{o}C.

Table 3.3 – Ionic Composition of CMA Perfusion Fluid T1

Element	Concentration ($mM.L^{-1}$)	Amount ($g.L^{-1}$)
Na^{+}	147	3.38
K^{+}	4	0.156
Ca^{2+}	2.3	0.092
Cl^{-}	156	5.59
Osmolality	290 mosm/kg	
pH	c. 6.0	

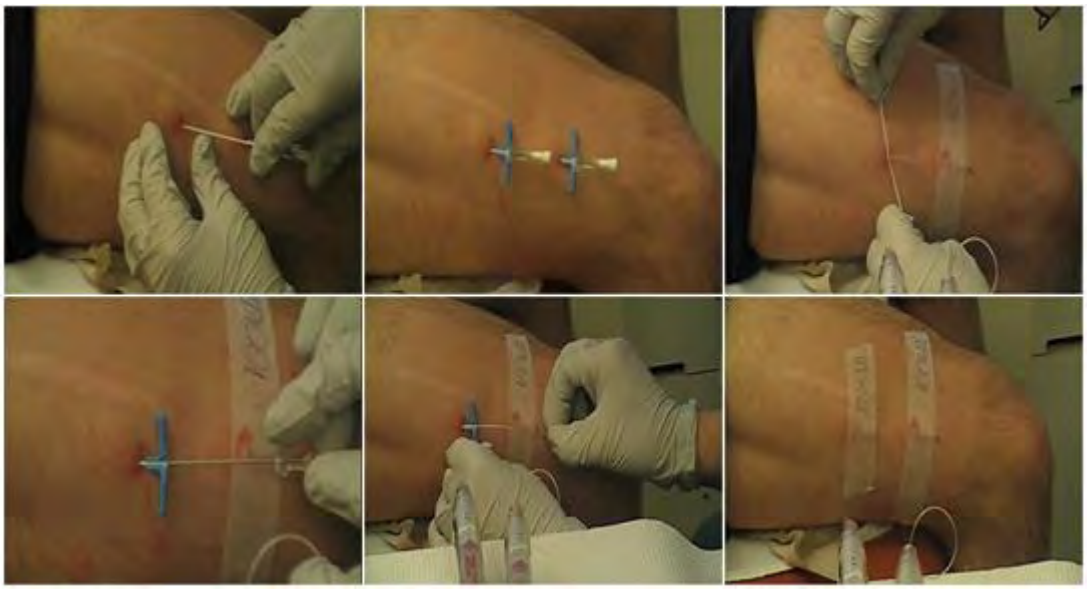

Figure 3.3 – Insertion of a microdialysis catheter into the muscle vastus lateralis.

Figure 3.4 – Ultrasound image illustrating the position of a microdialysis catheter inserted into the muscle vastus lateralis of a healthy male (microdialysis tip probe is circled in red).

3.3.3.4 Test solutions

Two test solutions were administered on alternate days during this study (Table 3.4). One solution acted as a control (CON) and contained strawberry flavour (79.4%), sucralose (12.7%) and carmine (7.9%) to match the protein solution for colour and flavour and to ensure that participants were blinded as to the nature of the solution they

were ingesting. The second solution contained whey protein isolate (WPI) (Isolac Instant, 3674 Carbery Food Ingredients, Cork, Ireland)). The dosage of the placebo beverage was 0.126% w/v. Participants were fed 0.55 g WPI per kg.BM^{-1} in 6 mL of water per kg.BM^{-1} (9% w/v solution) to give an equivalent protein, EAA and leucine dose of ~ 45 g, 20 g and 4 g respectively. The test solution was prepared on the morning of the trial and stored at 4^{o}C. It was thoroughly mixed prior to ingestion and was consumed at room temperature within 5 minutes of presentation.

Table 3.4 – Composition of test solutions

Composition	WPI	CON
Volume (mL)	480	480
Amount (g)	44	0.61
Protein (g.L^{-1})	89.8	-
Energy (kJ.L^{-1})	1398	-
EAA load (g.L^{-1})	40.8	-

Composition is based on an 80 kg individual (0.55 g.kg^{-1}, 6 ml.kg^{-1}).

Table 3.5 – Amino acid profile of Isolac Instant whey protein isolate.

Essential amino acids			Non-essential amino acids		
	% of Total AA	Grams in PRO Drink		% of Total AA	Grams in PRO Drink
Isoleucine	5.1	2.2	Alanine	5.4	2.3
Leucine	9.9	4.3	Arginine	1.9	0.8
Lysine	9.2	4.0	Aspartic Acid	11.7	5.1
Methionine	2.3	1.0	Cystine	2.5	1.1
Phenylalanine	3.1	1.3	Glutamic Acid	17.1	7.4
Theronine	7.4	3.2	Glycine	1.7	0.7
Tryptophan	2.0	0.9	Proline	6.0	2.6
Valine	5.0	2.2	Serine	5.3	2.3
Histidine	1.5	0.6	Tyrosine	2.9	1.2
Total	45	19.6		55	23.5

Composition is based on an 80 kg individual (0.55 g.kg^{-1}, 6 ml.kg^{-1}). *EAA – Essential amino acids, NEAA – non-essential amino acids.*

3.3.3.5 Feeding Protocol

Following the exercise bout participants returned to the metabolic control room and were seated reclined on a physiotherapy plinth to facilitate venous and MD cannulation. They would remain in this position for the remainder of the test day. Three hours post exercise (2.5h post catheter insertion) participants consumed one of the two test beverages within 5 minutes of presentation, either WPI (AA composition in Table 3.5) or CON. The test beverage was provided in a clear shaker type beaker.

3.3.3.6 Analytical Methods

3.3.3.6.1 Amino Acid Analysis

Plasma AA were analysed as per described in Chapter 2 of this thesis. Dialysate AAs were measured by pre-column derivatisation with o-phthalaldehyde (OPA) and quantified by reverse phase high performance liquid chromatography (RPHPLC) (Agilent 1200 RRLC, Agilent Technologies, CA, USA) using a modification of the procedures described by Turnell and Cooper (1982) and Dorresteijn *et al* (1996). Briefly, dialysate AAs were derivatised inline with OPA/2-mercaptopropionic acid (MPA) and separated by gradient elution RPHPLC (temperature controlled). A C18-bonded silica column (Zorbax Eclipse Plus C18, 4.6 x 100mm x 1.8 µm, Agilent Technologies) was eluted by 2-solvent gradient comprising (A) 12.5 mM phosphate buffer adjusted to pH 6.93 with NaOH, and tetrahydrofuran 99:1 (vol:vol) and (B) methanol, acetonitrile and deionized water 35:15:50 (vol:vol)]. OPA derivatives were detected by fluorescence (Ex330, Em450 nm). Norvaline was employed as the internal standard. Dialysate AA derivatives were identified by retention time relative to the reference peak and quantified as AUC by reference to standards of known concentration (Figure 3.5). Analytical performance was established by repeated assay of pooled perchloric extracts spiked with AAs of known concentration. Inter and intra-assay precision was determined by assaying a QC sample. A pooled plasma sample acted as QC across the plasma and MD AA analysis. Typically six QC samples were analysed per analytical run. Inter and intra assay CV's were less than 5% (Table 3.6) for all AA except the low abundance AA methionine and cysteine.

Table 3.6 – Inter and intra assay Cv's for the analysis of plasma and dialysate essential amino acids, branch chain amino acids and leucine.

		ΣEAA	ΣBCAA	Leucine
Plasma	Inter-assay CV	2.7%	2.0%	2.2%
	Intra-assay CV	2.9%	2.9%	2.8%
Microdialysis	Inter-assay CV	1.7%	0.7%	0.2%
	Intra-assay CV	2.8%	2.9%	3.3%

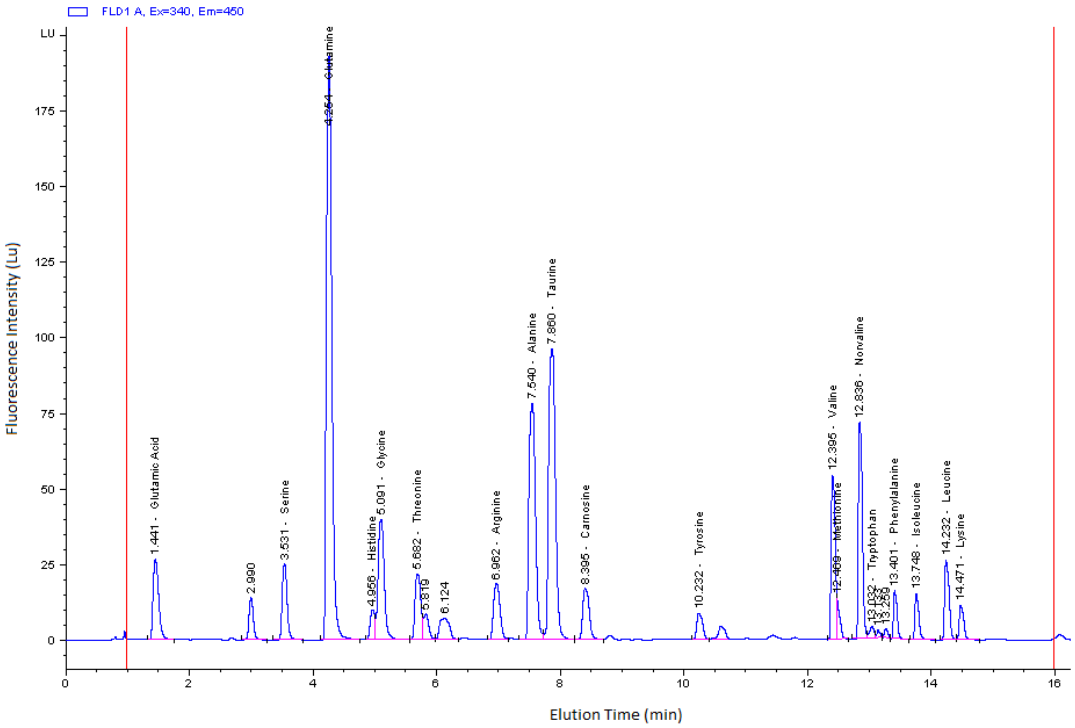

Figure 3.5 – Typical sample chromatogram illustrating the separation and quantification of 18 individual amino acids plus internal standard (norvaline) from a microdialysis sample.

3.3.4 Statistical Analysis

All data was tested for normality using Shapiro-Wilk test. All data are presented as the mean ± SEM (n = 4). AUC above baseline for each analyte was calculated by trapezoidal integration.

3.4 Results

3.4.1 Markers of insertion trauma

Dialysate carnosine concentration one hour post catheter insertion was 209 ± 66 μM (n = 16 catheters). Thereafter it decreased by 86% from 1 to 2.5h post insertion (Figure 3.6). Carnosine was still detectable in dialysate from skeletal muscle seven hours post catheter insertion. Dialysate glutamine and taurine followed a similar pattern to carnosine with elevated concentration one hour post insertion (Glutamine – 1319 ± 219 μM, Tau – 494 ± 71 μM, n = 16 catheters). Glutamine and taurine declined by 35% and 53% respectively from 1h to 2.5h post catheter insertion (Figure 3.6). Dialysate concentration of carnosine, glutamine and taurine continued to decrease 2.5h post insertion but the rate of decline was less severe than observed during the initial 2.5h post insertion period (27%, 5% and 1% decline from 2.5h to 3.5h post insertion for carnosine, glutamine and taurine respectively, n = 16 catheters for carnosine, n = 8 catheters for glutamine and taurine due to WPI feeing on one of the experimental days). Considerable variability between catheters in the dialysate concentration of carnosine, glutamine and taurine was evident during the initial sampling periods.

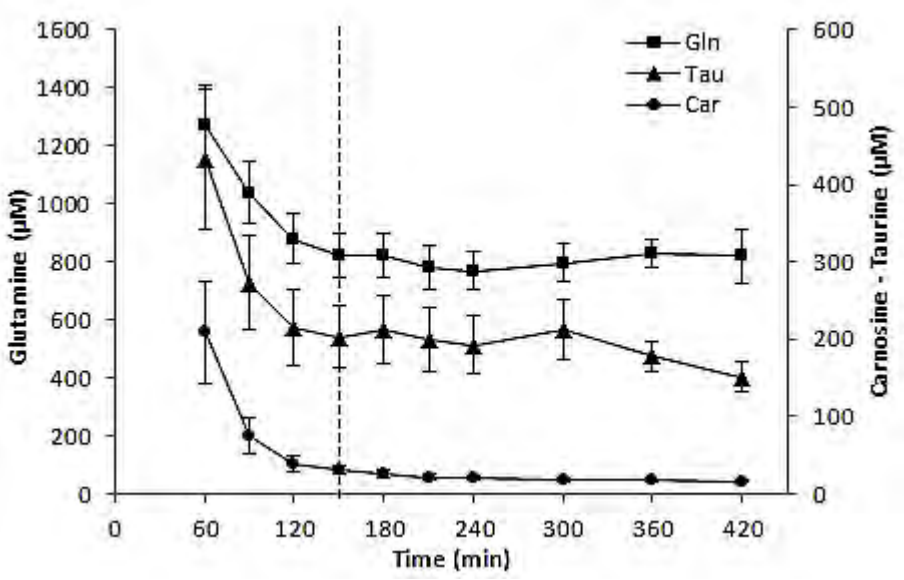

Figure 3.6 – Dialysate carnosine, glutamine and taurine concentration in response to insertion of a microdialysis catheter into the muscle vastus lateralis. Dotted line indicates the point at which participants consumed either WPI or control beverage. Values are mean ± SEM (n = 16 catheters to 150 min, n = 8 catheters for glutamine and taurine from 180 min onwards).

Dialysate leucine, isoleucine and valine concentration one hour post catheter insertion was 173 ± 15 μM, 72 ± 10 μM and 269 ± 22 μM respectively (n = 16 catheters). Thereafter they decreased by 20%, 19% and 15% respectively from 1h to 2.5h post insertion (Figure 3.7). Dialysate concentration of isoleucine and valine decreased

slightly (3% and 2% respectively) from 2.5h to 3.5h post insertion while dialysate leucine concentration increased by 3% (n = 8 catheters).

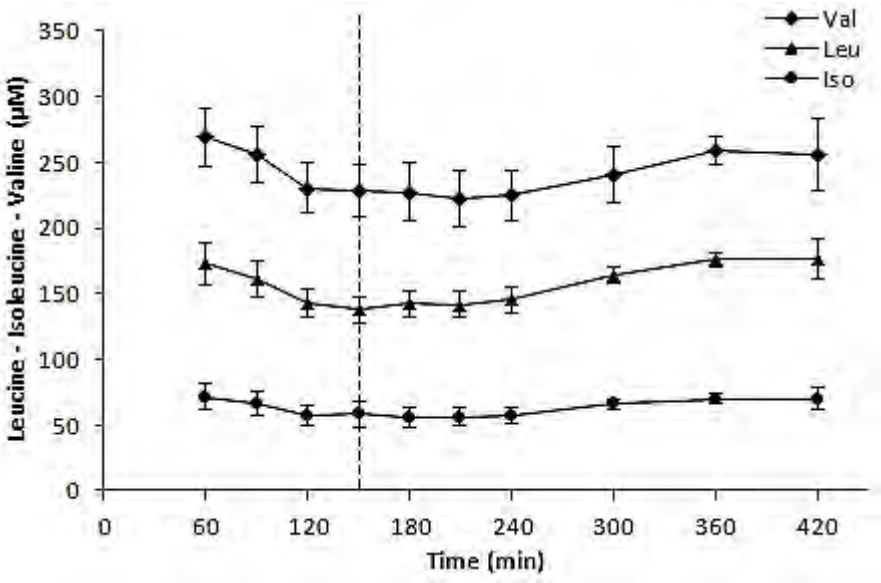

Figure 3.7 – Dialysate leucine, isoleucine and valine concentration in response to insertion of a microdialysis catheter into the m. vastus lateralis. Dotted line indicates the point at which participants consumed either WPI or control beverage. Values are mean ± SEM (n = 16 catheters to 150 min, n = 8 catheters from 180 min onwards).

3.4.2 Dialysate and plasma amino acid concentration

3.4.2.1 Essential Amino Acids

Basal dialysate and plasma EAA concentration were similar prior to CON or WPI ingestion (Table 3.7/3.8, Figure 3.8). Dialysate EAA concentration increased following ingestion of CON and WPI and remained elevated above baseline concentration for the duration of the 4.5h postprandial period (Figure 3.8 (A)). Peak EAA concentration was 15% and 19% higher in dialysate from resting and exercising legs respectively relative to peak plasma EAA concentration following CON ingestion. Peak EAA concentration was 23% and 22% lower in dialysate from resting and exercising legs respectively relative to peak plasma EAA concentration following WPI ingestion. EAA AUC above baseline was 1122% and 961% higher in dialysate from rest and exercise legs relative to plasma in response to CON ingestion [12 ± 10 mM.4.5h^{-1} and 10 ± 6.3 mM.4.5h^{-1} vs. 1.0 ± 9.5 mM.4.5h^{-1}]. EAA AUC above baseline was 7% and 6% higher in dialysate from rest and exercise legs relative to plasma in response to WPI ingestion [220 ± 29 mM.4.5h^{-1} and 218 ± 28 mM.4.5h^{-1} vs. 206 ± 7.9 mM.4.5h^{-1}].

3.4.2.2 Leucine

Basal dialysate and plasma leucine concentration was not different prior to CON and WPI ingestion (Table 3.7/3.8, Figure 3.9). Dialysate leucine concentration increased

following ingestion of CON and WPI and remained elevated above baseline concentration for the duration of the 4.5h postprandial period (Figure 3.9 (A)). Peak leucine concentration was 21% and 26% higher in dialysate from resting and exercising legs respectively relative to peak plasma leucine concentration following CON ingestion. Peak EAA concentration was 24% and 27% lower in dialysate from resting and exercising legs respectively relative to peak plasma leucine concentration following WPI ingestion. Leucine AUC above baseline was 271% and 349% higher in dialysate from rest and exercise legs respectively relative to plasma in response to CON ingestion (4.7 ± 2.3 mM.4.5h^{-1} and 5.7 ± 1.6 mM.4.5h^{-1} vs. 1.3 ± 1.4 mM.4.5h^{-1}). Leucine AUC above baseline was 3.0% and 1.2% higher in dialysate from rest and exercise legs relative to plasma in response to WPI ingestion (52 ± 6.5 mM.4.5h^{-1} and 51 ± 6.6 mM.4.5h^{-1} vs. 50 ± 1.9 mM.4.5h^{-1}).

3.4.2.3 Glutamine

Basal dialysate glutamine concentration was approximately 70% higher relative to plasma concentration prior to CON and WPI ingestion (Table 3.7/3.8, Figure 3.10). Dialysate and plasma glutamine concentration remained relatively stable to 90 min post ingestion followed by a gradual but progressive increase post ingestion of CON (Figure 3.10 (C)). Peak glutamine concentration was 41% higher in dialysate from both resting and exercising legs respectively relative to peak plasma glutamine concentration following CON ingestion. Peak glutamine concentration was 35% and 34% higher in dialysate from resting and exercising legs respectively relative to peak plasma EAA concentration following WPI ingestion. Glutamine AUC above baseline was 100% and 35% lower in dialysate from rest and exercise legs relative to plasma in response to CON ingestion (-9 ± 7.3 mM.4.5h^{-1} and -6 ± 4.1 mM.4.5h^{-1} vs. -4 ± 3.7 mM.4.5h^{-1}). Glutamine concentration increased in dialysate and plasma from baseline following ingestion of WPI (Figure 3.10 (B)). Glutamine AUC above baseline was 20% and 50% greater in dialysate from rest and exercise legs relative to plasma in response to WPI ingestion (10 ± 10.7 mM.4.5h^{-1} and 13 ± 6.5 mM.4.5h^{-1} vs. 8 ± 3.9 mM.4.5h^{-1} respectively).

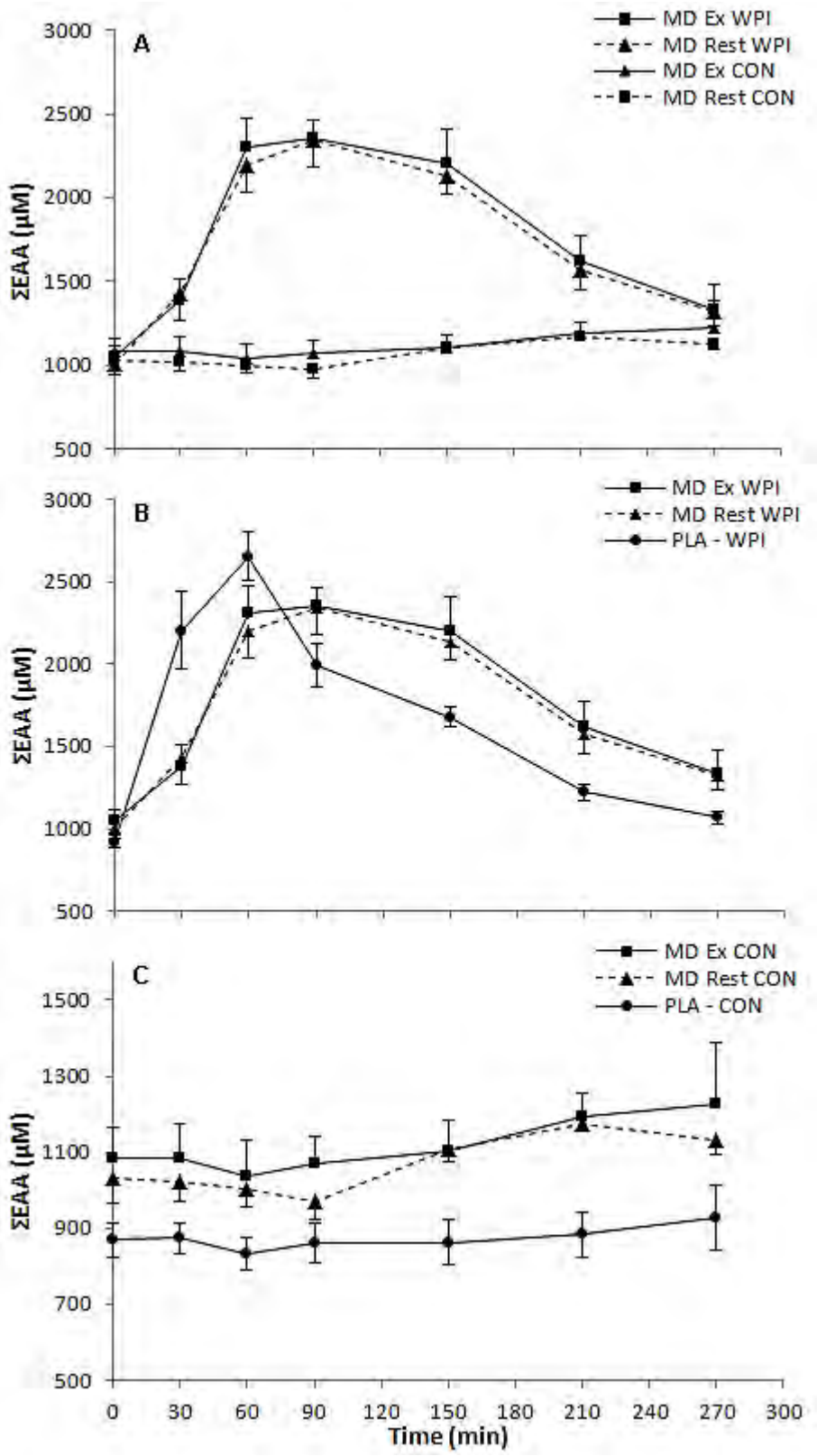

Figure 3.8 – (A) Dialysate EAA concentration in response to WPI and CON ingestion, (B) dialysate and plasma EAA concentration in response to WPI ingestion and (C) dialysate and plasma EAA concentration in response to CON ingestion. Data are mean ± SEM (n = 4).

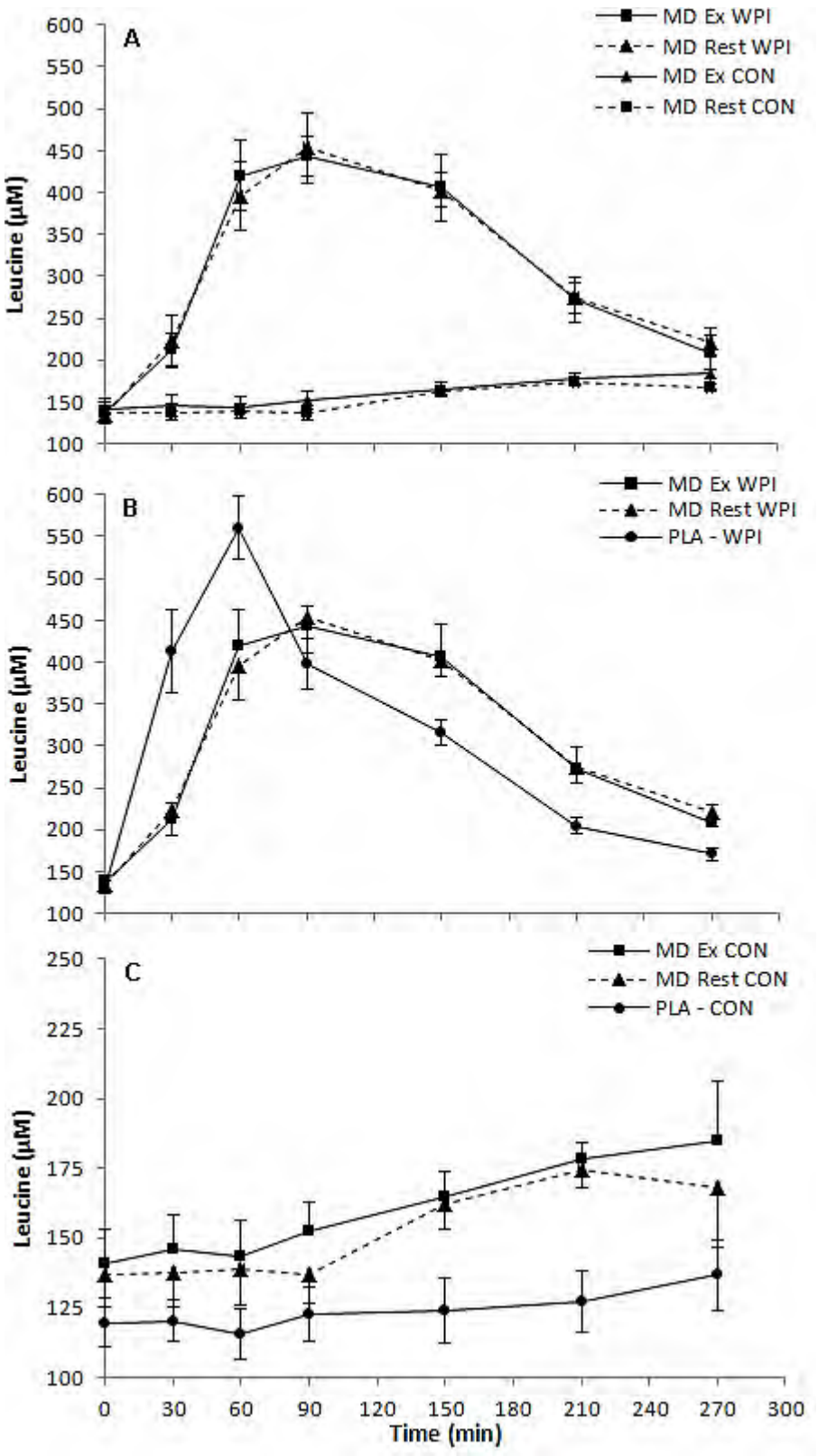

Figure 3.9 – (A) Dialysate leucine concentration in response to WPI and CON ingestion, (B) dialysate and plasma leucine concentration in response to WPI ingestion and (C) dialysate and plasma leucine concentration in response to CON ingestion. Data are mean ± SEM (n = 4).

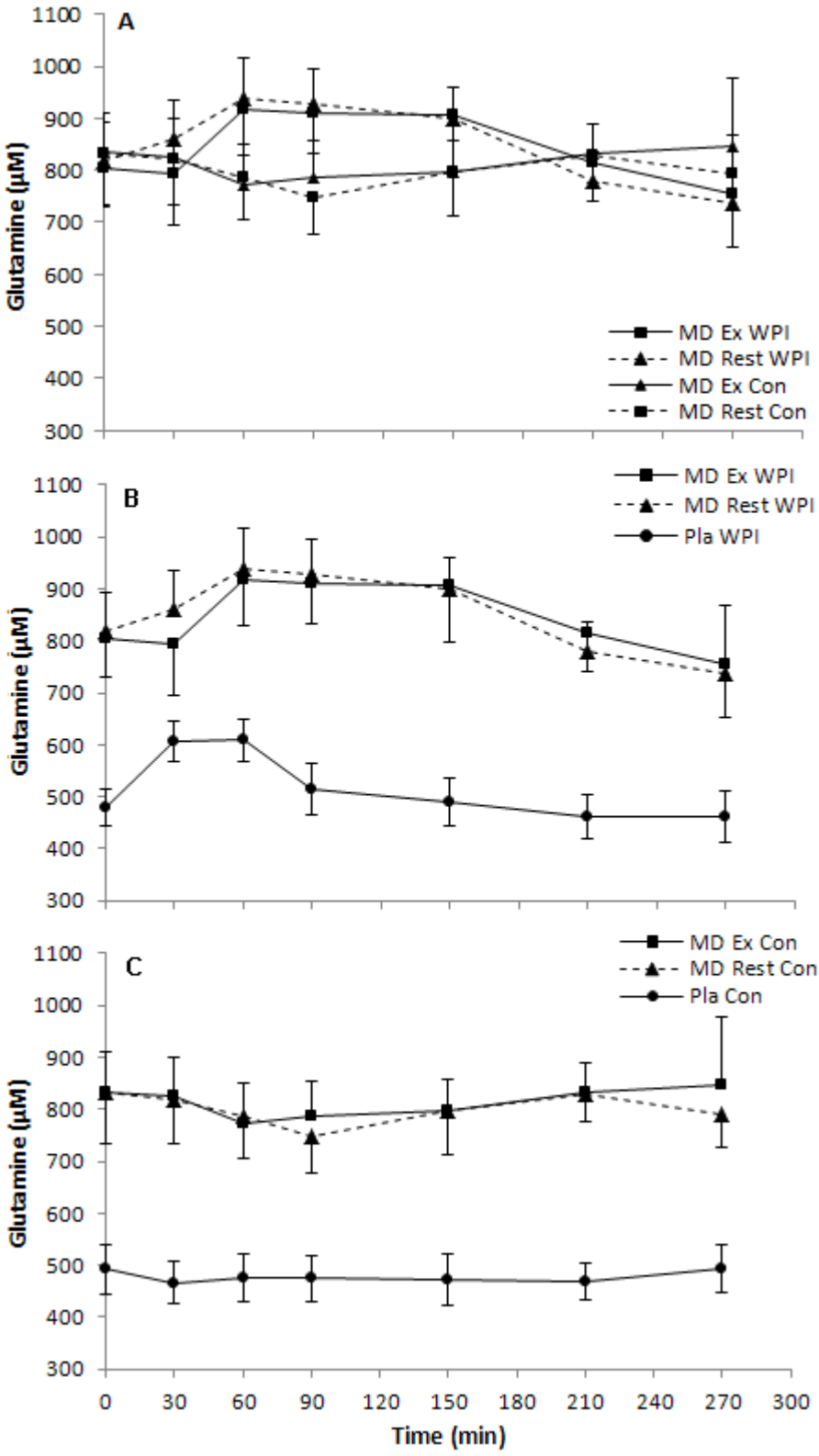

Figure 3.10 – (A) Dialysate glutamine concentration in response to WPI and CON ingestion, (B) dialysate and plasma glutamine concentration in response to WPI ingestion and (C) dialysate and plasma glutamine concentration in response to CON ingestion. Data are mean ± SEM (n = 4).

Table 3.7 – Dialysate & plasma AA concentration at baseline, peak concentration in response to CON ingestion & % change from baseline to peak concentration.

	MD Rest CON			MD Ex CON			Plasma CON		
AA (μM)	**Baseline**	**Peak**	**Δ%**	**Baseline**	**Peak**	**Δ%**	**Baseline**	**Peak**	**Δ%**
Glu	89 ± 14	*	*	124 ± 20	*	*	81 ± 7	100 ± 9	23%
Ser	110 ± 5	124 ± 6	13%	120 ± 8	132 ± 13	10%	87 ± 8	97 ± 4	12%
Gln	833 ± 99	*	*	834 ± 75	846 ± 111	1%	492 ± 46	493 ± 46	0.2%
His	97 ± 11	*	*	106 ± 9	*	*	77 ± 4	*	*
Gly	242 ± 16	254 ± 14	5%	259 ± 20	284 ± 43	9%	198 ± 14	*	*
Thr	151 ± 18	*	*	156 ± 19	*	*	106 ± 12	*	*
Arg	109 ± 10	*	*	111 ± 8	*	*	61 ± 7	85 ± 9	40%
Ala	452 ± 34	*	*	470 ± 28	*	*	356 ± 36	*	*
Tau	210 ± 57	*	*	249 ± 44	*	*	20 ± 5	27 ± 9	33%
Car	28 ± 5	*	*	31 ± 3	*	*	ND	ND	ND
Tyr	65 ± 8	73 ± 5	11%	68 ± 7	75 ± 8	10%	47 ± 4	*	*
Cyst	45 ± 1	60 ± 3	34%	42 ± 4	63 ± 7	51%	16 ± 8	22 ± 7	38%
Val	219 ± 20	259 ± 12	18%	225 ± 19	260 ± 32	16%	187 ± 13	199 ± 15	7%
Met	24 ± 8	*	*	26 ± 8	*	*	8 ± 2	14 ± 2	76%
Trp	13 ± 2	16 ± 1	17%	12 ± 2	17 ± 3	37%	65 ± 3	74 ± 5	14%
Phe	54 ± 4	71 ± 2	33%	58 ± 3	77 ± 7	32%	41 ± 3	47 ± 2	15%
Iso	54 ± 6	70 ± 2	30%	56 ± 7	74 ± 11	32%	46 ± 6	57 ± 7	23%
Leu	137 ± 13	174 ± 3	27%	141 ± 12	185 ± 18	31%	120 ± 9	137 ± 13	14%
Lys	211 ± 13	254 ± 15	20%	226 ± 24	268 ± 45	18%	219 ± 7	233 ± 12	7%
ΣAA	3084 ± 240	3307 ± 161	7%	3283 ± 208	3523 ± 388	7%	2223 ± 137	2370 ± 138	7%
ΣEAA	960 ± 69	1116 ± 21	16%	1006 ± 81	1169 ± 140	16%	869 ± 46	945 ± 73	9%
ΣBCAA	410 ± 45	503 ± 13	23%	422 ± 45	516 ± 61	23%	353 ± 28	393 ± 35	11%

*Data are mean ± SEM (n = 4). * Baseline concentration was equivalent to peak concentration during the 4.5h postprandial period. ND – not detectable*

Table 3.8 – Dialysate & plasma AA concentration at baseline, peak concentration in response to WPI ingestion & % change from baseline to peak concentration.

	MD Rest WPI			MD Ex WPI			Plasma WPI		
AA (μM)	**Baseline**	**Peak**	**Δ%**	**Baseline**	**Peak**	**Δ%**	**Baseline**	**Peak**	**Δ%**
Glu	100 ± 16	156 ± 7	55%	99 ± 13	156 ± 8	58%	98 ± 5	189 ± 27	93%
Ser	106 ± 6	182 ± 11	71%	111 ± 8	188 ± 17	68%	91 ± 8	172 ± 13	90%
Gln	817 ± 88	939 ± 79	15%	804 ± 73	918 ± 88	14%	478 ± 35	609 ± 37	28%
His	92 ± 14	*	*	96 ± 11	*	*	76 ± 1	98 ± 3	29%
Gly	237 ± 6	272 ± 10	15%	246 ± 5	275 ± 17	12%	199 ± 14	244 ± 18	22%
Thr	135 ± 14	280 ± 20	108%	138 ± 16	284 ± 23	106%	103 ± 9	281 ± 25	173%
Arg	113 ± 15	146 ± 8	29%	112 ± 12	147 ± 8	32%	69 ± 7	135 ± 14	95%
Ala	449 ± 69	643 ± 74	43%	445 ± 60	633 ± 89	42%	349 ± 41	551 ± 54	58%
Tau	167 ± 41	*	*	185 ± 13	*	*	22 ± 2	35 ± 6	61%
Car	27 ± 6	*	*	35 ± 7	*	*	ND	ND	ND
Tyr	64 ± 7	114 ± 3	79%	63 ± 6	118 ± 4	87%	49 ± 5	119 ± 10	145%
Cyst	38 ± 10	80 ± 6	110%	48 ± 5	76 ± 4	60%	17 ±	24 ± 10	40%
Val	227 ± 16	487 ± 33	115%	227 ± 13	483 ± 26	113%	199 ± 9	496 ± 20	149%
Met	14 ± 3	32 ± 7	127%	18 ± 3	38 ± 7	113%	11 ± 1	44 ± 11	304%
Trp	13 ± 1	27 ± 2	107%	15 ± 1	28 ± 2	83%	68 ± 1	154 ± 6	128%
Phe	51 ± 3	81 ± 4	58%	50 ± 4	83 ± 5	67%	42 ± 1	77 ± 2	86%
Iso	54 ± 5	232 ± 24	330%	55 ± 5	228 ± 22	315%	54 ± 6	322 ± 21	498%
Leu	135 ± 9	453 ± 41	235%	137 ± 8	442 ± 25	222%	131 ± 8	560 ± 37	328%
Lys	210 ± 17	473 ± 48	126%	242 ± 23	492 ± 53	104%	241 ± 10	625 ± 40	159%
ΣAA	3022 ±265	4856 ± 168	61%	3091 ± 206	4878 ± 203	58%	2297 ± 107	4735 ± 173	106%
ΣEAA	931 ± 65	2157 ± 158	132%	978 ± 60	2174 ± 126	122%	925 ± 39	2657 ± 152	187%
ΣBCAA	416 ± 35	1172 ± 1366	182%	419 ± 32	1153 ± 58	175%	384 ± 20	1378 ± 75	259%

*Data are mean ± SEM (n = 4). * Baseline concentration was equivalent to peak concentration during the 4.5h postprandial period. ND – not detectable*

3.5 Discussion

The aim of this study was to determine the bioavailability of AA substrates and key AA regulators of MPS, sampled from the interstitial fluid of human skeletal muscle in response to RE with and without milk protein feeding. Ingestion of CON did not overtly influence dialysate EAA or leucine concentration. There was a gradual increase in their concentration over time with peak concentration occurring between 4h and 4.5h post ingestion. This increase over time was mirrored in the venous plasma EAA and leucine concentration and has been reported previously in the literature (Gutierrez *et al* 1999). Single leg RE designed to stimulate MPS, undertaken three hours prior to CON ingestion did not influence the subsequent bio-availability of dialysate EAA, leucine and glutamine during the 4.5h post-prandial period relative to a resting contra-lateral control leg. The relative change from baseline to peak concentration was indistinguishable for the sum of the total AA, EAA and BCAA in response to CON ingestion.

The dialysate glutamine concentration continued to decrease immediately post CON ingestion. This may reflect continued clearance of glutamine from the trauma of catheter insertion up to 90min post CON ingestion (270 min post insertion). After 90 min the concentration of dialysate glutamine increased gradually over time and returned to values similar to those observed immediately prior to CON ingestion. The source of the increased dialysate AA concentration over time in response to CON ingestion is intriguing and could be reflective of net AA efflux at the cellular level (MPB in excess of MPS). AA may have been released from the muscle cell in support of gluconeogenesis in the fasted state. This is despite the fact that participants consumed a mixed macronutrient breakfast in advance of attending the laboratory on test days. The dialysate concentration of some but not all of the gluconeogenic AA increased over time. Further substantiation of these limited data is required in order to substantiate or refute this hypothesis. A shift in the distribution of total body water and thus a concentration of substrate in the interstitial fluid cannot be ruled out in this instance as plasma volume was not measured during the trial. Fryburg *et al* (1990) did however demonstrate accelerated rates of amino acid appearance in response to short term (12h) and long term (60h) fasting via measurement of A-V difference of labelled amino acids across the forearm in humans.

Dialysate AA demonstrated a rapid and transient increase in concentration in response to WPI ingestion that mirrored the change in the venous plasma, albeit with an approximately 20% lower peak concentration relative to plasma. The time to reach peak concentration was also longer in dialysate relative the plasma. Single leg RE

designed to stimulate MPS, undertaken three hours prior to WPI ingestion did not influence the subsequent bio-availability of dialysate EAA, leucine and glutamine during the 4.5h post-prandial period relative to a resting contra-lateral control leg. Biolo *et al* (1995) demonstrated that ~3h post a bout of heavy RE rates of muscle protein turnover and AA transport were increased in 5 healthy male volunteers studied in the fasting post-absorptive state. No change in femoral artery AA concentration was observed post exercise but the rate of inward transport of amino acids into muscle (presumably through the interstitial fluid) increased. Bohé *et al* (2003) demonstrated that at low levels of AA infusion, the intramuscular AA concentration decreases. The authors suggested that the rise in extracellular AA concentration due to infusion is sensed and leads to the increased deposition of AA into protein. This increased deposition may occur faster than can be accommodated by a stimulation of AA membrane transport and thus intramuscular AA concentrations fall, even though the flux through the intracellular compartment increases.

Phillips *et al* (1997) reported that the effects of an acute bout of RE on muscle protein FSR persisted for up to 48h. Rasmussen *et al* (1999) demonstrated that EAA and carbohydrate ingestion in combination with RE stimulated MPS to a comparable level when the solution is consumed 1h or 3h post-exercise. Rasmussen *et al* describe a theory whereby the cellular protein synthetic machinery is primed post RE such that when AA becomes available the rate of MPS is enhanced. In the present study we hypothesised that the single RE bout would acutely stimulate MPS and may influence the interstitial concentration of AA (particularly leucine) due to increased uptake into the cell in support of protein synthesis. Ingestion of WPI or CON was not undertaken until 3h post RE to allow sufficient time for the trauma response to insertion to subside and to complete baseline sampling. It was plausible to except that in this experimental paradigm the appearance of AA in the dialysate of a pre-exercised limb may be different to that of a non-exercised limb. This difference may manifest as an altered baseline concentration, peak concentration and/or AUC.

The EAA and leucine were highlighted for analysis over and above other candidate AA's due to their prominent role as both stimuli and substrate to the process of MPS (discussed in detail in Chapters 1 and 2 of this thesis). Glutamine has previously been classified as a non-essential AA. Based on more recent evidence it has been reclassified as a conditionally essential and/or functional AA (Newsholme 2001),(Wu 2013). Little of the dietary derived glutamine enters the blood stream under normal dietary conditions. Much of it is consumed by the epithelial cells of the intestine where it is utilised as a respiratory fuel. Nicklin *et al* (2009) reported that the ability of EAA (and

leucine in particular) to enter cells and activate mTORC1 signalling depends on their exchange for glutamine across the cell membrane. Glutamine is an important substrate in several processes related to tissue homeostasis such as protein translation, metabolic flux and nitrogen balance. As mTORC1 is a major regulator of cell size and tissue mass it is perhaps appropriate that its activity is sensitive to both intra and extracellular levels of glutamine. SLC7A5/SLC3A2 is a bi-directional transporter that regulates the simultaneous efflux of glutamine out of cells and transport of EAA (leucine) into cells. Thus leucine/glutamine flux across the cell membrane has been implicated in mTORC1 regulation. As such glutamine was identified as an important AA with respect to its concentration in skeletal muscle interstitial fluid. However, given (a) the leakage of glutamine from the intracellular to the extracellular space due to insertion of the MD catheter and (b) the variance observed in the glutamine response to WPI and CON ingestion and (c) the limited data set, it is difficult to make definitive assertions as to the relationship between the concentration of glutamine and leucine in the interstitial fluid as measured by MD in response to exercise during this trial.

There is good agreement with the data reported in this instance and the extant literature (allowing for lab to lab variation in the quantification of AA) in that the baseline concentration of dialysate AA was slightly higher than those in the venous plasma (with the exception of tryptophan, due to the fact that only free tryptophan is measured in the dialysate whereas total tryptophan is measured in the plasma due to the precipitation of albumin). These data demonstrate that in the absence of a marker of catheter recovery (infusion of a labelled amino acid for example) the utilisation of a low flow rate (0.3 $\mu L.min^{-1}$) can yield recoveries approximating 100% of the actual tissue concentration for small molecules such as AA. It is therefore possible to measure the magnitude and time course of the change in dialysate AA concentration at the tissue level in response to nutrient ingestion in a contralateral limb controlled exercise trial. The AA concentration can be accurately and reliably measured in a 9 µL sample collected over a 30 min period at a perfusate flow rate of 0.3 $\mu L.min^{-1}$. Indeed the HPLC method was refined to the extent that the AA concentration could be determined from a 2 µL sample which offers to improve the temporal resolution of future trials while maintaining the low flow rate to ensure appropriate recovery of substrate (e.g. 0.3 $\mu L.min^{-1}$ * 15 min = 4.5 µL sample).

AA that have a high intracellular/extracellular concentration gradient within skeletal muscle cells act as suitable biomarkers of tissue perturbation when cells are lysed as a result of mechanical trauma. Their return to stable levels over time is indicative of the appropriate stage to begin baseline sample collection post MD catheter insertion.

Consistent with previous observations within the literature (Gutierrez *et al* 1999) stabilisation, based on visual inspection of data, was observed in the concentration of carnosine, glutamine and taurine between 2h and 2.5h post catheter insertion. The concentration of these AA continued to decrease 2.5h post catheter insertion in the instance where participants consumed the control beverage. The rate of decline was, however, far more gradual then that observed during the initial 2h post insertion sampling period. Various research groups have reported timelines for tissue stabilisation post catheter insertion, typically ranging from 30 min to 90 min (Langberg *et al* 1999). Numerous bio-markers of stability have been reported in support of these claims. Glucose, lactate and glycerol for example appear to be stable 60 min post catheter insertion (Rosdahl *et al* 1998). If a 60 min timeframe was applied as the appropriate period for insertion related trauma to subside, even the AA's that do not exhibit a large concentration gradient across the cell membrane, for example the BCAA, demonstrate a measurable decrease in concentration (~18%) between 60 min and 150 min post insertion. This has obvious implications with respect to the introduction of an artefact into the data by way of a false baseline concentration.

There is an argument that can be made for the selection of specific bio-markers of trauma subsidence relative to the experimental aim of the trial. Stating that stable glucose and/or lactate concentration or carnosine, glutamine and taurine concentration are indicative of stability across all substrates could be problematic. Indeed there is some evidence of increasing concentration of cytokines over time in dialysate samples from human skeletal muscle (Rosendal *et al* 2005) and adipose tissue (Siklova-Vitkova *et al* 2009). Some intra and inter-individual variability was evident with regard to the trauma response to the catheter insertion, particularly during the first hour of sample collection. This may be due to the guide cannula causing more damage in some instances due to the angle of the guide cannula relative to the muscle fibre orientation on insertion. Every effort was made during the trial to standardise the insertion procedure both within and between participants. Another source of variability could be the concentration of carnosine in each individual's muscle which varies depending on training status and fibre type composition.

3.6 Conclusion

A bout of unilateral knee extension exercise undertaken 3h prior to WPI or CON ingestion does not influence subsequent dialysate AA bioavailability. Ingestion of CON did not result in an immediate alteration of plasma or dialysate AA concentrations but rather a slow but consistent increase in AA concentration consistent with the net release of AA from muscle. Ingestion of WPI resulted and a rapid and transient increase in the concentration of dialysate AA that remained elevated above baseline 4.5h post ingestion. The pattern of appearance is similar between plasma and dialysate AA when measured as the AUC. Peak dialysate EAA and leucine concentration was however approximately 20% lower than the peak venous plasma concentration. MD is a safe and tolerable method to sample substrate concentration at the tissue level. Sample collection at a low perfusion flow rate to ensure a high relative recovery in the absence of catheter calibration is feasible but challenging due to the small volumes recovered. Insertion of the MD catheter causes a perturbation to the muscle tissue that subsides after 2h to 2.5h when AA markers of trauma are measured. This is the minimum time necessary to establish a stable baseline prior to beginning any further investigation. MD offers a tool to investigate, at the tissue level, differences in the proteomic/metabolic response to an acute nutrient and/or exercise stimulus between different populations (young vs. old) or population subgroups (active vs. inactive, responder vs. non-responder).

Chapter 4 The effect of 24 weeks of milk protein supplementation on lean tissue mass in healthy 50-70 year individuals.[3]

[3] The study described herein is currently in preparation for publication. McCormack, W, Toomey, C, Norton, C, Francis P, Jakeman, P 2014. A randomised controlled trial investigating the effect of 24 weeks of milk protein supplementation on lean tissue mass in healthy 50-70 year olds.

4.1 Introduction

Whether older people require more protein than younger adults and, thereby, should ingest more than the current WHO/IOM dietary reference intake (DRI) of 0.85 $g.kg^{-1}.d^{-1}$ in an effort to offset the age-related decline in skeletal muscle mass termed sarcopenia is the topic of much debate currently. A 70 kg individual comprises approximately 11 kg of protein (dry matter), approximately 43% of which is present in skeletal muscle (Trumbo *et al* 2002). Almost half of the total protein content is present in four proteins, myosin, actin, collagen and hemoglobin with collagen (~ 25% of the total) the most stable. Depending on age and nutrition, skeletal muscle accounts for between 30 and 50% of whole body protein turnover. Protein turnover in the adult human is upward of 250 $g.d^{-1}$ (Waterlow 1984) resulting in the excretion of approximately 11 to 15 g of nitrogen in the urine, mostly in the form of urea, derived from ammonia and aspartate. The benefit of increasing dietary protein intakes above the current RDA in the elderly on muscle mass and muscle function and mass is, as yet, equivocal (Koopman and van Loon 2009). Consensus opinion indicates that a quantitative and qualitative modification of dietary protein intake is necessary to counteract progressive LTM loss in the elderly (Boirie 2009) with a recommendations to increase intake ranging from to 1.0 – 1.6 $g.kg^{-1}.d^{-1}$ in the elderly (Paddon-Jones *et al* 2008, Wolfe *et al* 2008).

This prescribed protein intake should be spread across the main meals of the day to yield maximum protein synthesis (Paddon-Jones and Rasmussen 2009). Proteins that are digested rapidly induce a better anabolic response in older people than proteins which are digested slowly (Dangin *et al* 2002). The post-prandial change in key AA regulators of MPS in response to milk protein ingestion has been reported in Chapter 2 of this thesis. Peak aminoacidemia occurred within 30 minutes of FORM ingestion with peak leucinemia almost threefold greater than the baseline concentration. These data support the hypothesis that the chronic ingestion of the milk protein will augment LTM over a 6 month intervention period in healthy 50-70y old women and men. This is based on the theory that there are approximately 550 main eating occasions over a 24-week period and assuming a skewed distribution of protein intake towards the main meal of the day, the inclusion of a milk protein supplement with the two smaller meals of the day will ensure that the leucine threshold for stimulation of MPS is achieved during all 550 eating occasions as oppose to as few as 180.

In older people, when protein is co-ingested with carbohydrate and/or the quantity of protein is less than 20 g per meal, muscle protein synthesis (MPS) is blunted (Paddon-Jones and Rasmussen 2009). There is a dose-dependent effect (i.e. 6 vs.12 g) of EAA

ingestion on MPS (Rasmussen and Phillips 2003). Additionally, non-essential amino acids are ineffective in driving the anabolic response (Tipton *et al* 1999). Emphasising the importance of EAAs to lean tissue anabolism it is pertinent to note that the actual requirement and current recommended dietary intake of essential (or indispensable) amino acids has doubled over the last ~30y. Specific to the EAAs, leucine acts as a substrate for AA oxidative deamination by skeletal muscle, a regulator of protein synthesis and an insulin secretagogue. Thus, the addition of leucine to a mixed nutrient meal has been shown to increase protein synthesis in older people (Katsanos *et al* 2006). However, leucine is deemed not to provide any additional benefit on muscle protein anabolism when a sufficient amount of dietary protein is ingested (Koopman *et al* 2008).

Harrington *et al* (2001) reported that a convenience sample of 51 – 64 year old Irish women (n = 162) and men (n = 173) consume a mean of 71 ± 17 $g.d^{-1}$ (1.1 $g.kg^{-1}$) and 96 ± 22 $g.d^{-1}$ (1.2 $g.kg^{-1}$) protein, respectively. The National Adult Nutrition Survey (NANS) (2011) conducted by the Irish University Nutrition Alliance (IUNA) reported that in a convenience sample of women and men aged 65y and older, the average protein intake was 69.4 ± 17.8 $g.d^{-1}$ (1.02 $g.kg^{-1}$) and 85.2 ± 24.1 $g.d^{-1}$ (1.03 $g.kg^{-1}$) respectively. For a reference 65 kg women and an 80 kg male, the current dietary reference intake (DRI) (0.8 $g.kg^{-1}.d^{-1}$) represents an intake of 52 $g.d^{-1}$ and 64 $g.d^{-1}$ protein respectively. Increasing the protein intake to ~ 1.3-1.5 $g.kg^{-1}.d^{-1}$ represents an intake of between 90 – 120 $g.d^{-1}$, an increase of ~ 40-60 $g.d^{-1}$ relative to the DRI. This relatively modest increase in protein intake, up to 20-35% of energy intake, is unlikely to result in issues such as renal dysfunction in healthy individuals. In this respect, the upper level of protein intake approximates to 250 $g.d^{-1}$ (45% of energy intake), a level considered to be the maximal rate for disposal of excess amino acids (hyperaminoacidaemia; defined by the maximal rate of urea synthesis of the liver ~ 65 mg urea $N.h^{-1}.kg^{-1}$). Tieland *et al* (2012a) reported that in a group of community dwelling 65 – 74 y old women and men, the daily protein intake averaged 72.9 ± 18.2 $g.d^{-1}$ (1.03 $g.kg^{-1}$) and 85.9 ± 23.9 $g.d^{-1}$ (1.11 $g.kg^{-1}.d^{-1}$) respectively. Protein intake was distributed between the three main meals of the day as follow; breakfast - 10 g, lunch - 27 g and evening - 32 g. These data provide a clear rationale, particularly with respect to breakfast, to alter the distribution of daily protein intake in support of the optimal stimulation of MPS at each of the three main daily eating occasions.

The average Irish 50-70 year old female and male has a LTM of 39.7 ± 4.4 kg and 57.7 ± 6.2 kg, respectively (ULBC, unpublished observation). Appendicular LTM (ALTM) is the sum of the LTM of the arms and legs. The average Irish 50-70 year old female and

male has an ALTM of 17.7 ± 2.3 kg and 27.6 ± 3.5 kg respectively (ULBC, unpublished observation). Age-related muscle loss from the appendages has the greatest capacity to impact on ambulation, mobility, activities of daily living and thus functional independence (Cruz-Jentoft *et al* 2010). Reduced functionality of the appendages is linked to the loss of mobility in elderly populations (Bassey *et al* 1992, Visser *et al* 2002). Assessment of the ALTM via DXA has therefore emerged as a key clinical and research metric for elderly groups (Hansen *et al* 2007). Nutrition and exercise strategies should focus on maintaining/enhancing ALTM in order to preserve functional independence with advancing age. Interventions designed to off-set the age-related decline in skeletal muscle mass should report this metric if available.

Resistance exercise (RE) increases the acute rate of MPS in older adults (Cuthbertson *et al* 2005, Drummond *et al* 2008). The synergistic effect of RE in combination with protein ingestion on the rate of MPS evident in the young (Dreyer *et al* 2008, Witard *et al* 2009, Witard *et al* 2014) is reproducible in the elderly (Sheffield-Moore *et al* 2004, Drummond *et al* 2008, Yang *et al* 2012). Recent reviews/meta-analysis support the use of dietary (Malafarina *et al* 2013) and exercise interventions (Peterson *et al* 2011) designed to facilitate the maintenance of lean body mass with advancing age either in isolation, or in combination (Candow *et al* 2012, Dickinson *et al* 2013b). Bauer et al, (2013) state that “additional clinical studies are needed to determine whether both feeding patterns (pulse and spread feeding) are effective or whether one is clearly favoured over the other. Such strategies should be tested in both long- and short-term clinical interventions”. This is the first attempt on our group’s part to quantify the benefit of spread protein feeding, in a group of healthy 50-70 year old women and men to identify if any benefit from a nutrient intervention is enhanced through the concurrent adoption of progressive resistance exercise training (PRT) over a 24 week period. For this study the timing of protein intake, particularly relative to exercise, is a principal independent variable that has not been optimised in longitudinal studies of protein supplementation and lifestyle intervention (Kukuljan *et al* 2009, Kemmler *et al* 2010).

4.2 Aims and objectives

The study seeks to investigate the change in total body and appendicular lean body mass in response to supplementation via isocaloric carbohydrate (MALT) or milk protein formulation, either in isolation (FORM) or in combination with a program of progressive resistance-exercise training (FORM + PRT) over a 24 week period. The research challenges the hypothesis that;

H_0: Compared to MALT there will be no difference over time in the change in total body or appendicular lean tissue mass as measured by DXA in response to FORM or FORM + PRT.

Assuming a mean change in total body LTM of ~2% (i.e. ~1-1.5 kg with the estimated SD of the treatment effect = 4.5 kg) over the period of the intervention, statistical power would be > 90% at $P < 0.05$ for a study of 30 participants.

4.3 Materials and methods

4.3.1 Participants

A convenience sample of 204 women and men aged 50-70y were recruited through email invitation and by word-of-mouth (Figure 4.1) . Participants were screened by a medical doctor and provided a full medical history. Those defined as healthy, i.e. disease-free based on Greig *et al* (1994), independent-living and willing to consume a nutrient supplement to their habitual diet, twice per day for 24-weeks (6 months) were invited to participate and to provide written, informed consent. On entry to the study participants were randomly assigned to receive food supplement containing a milk-protein based formulation (FORM), food supplement containing a milk-protein based formulation in conjunction with a prescribed program of PRT (FORM + PRT) or an isocaloric maltodextrin control (MALT) in a randomized, controlled single blind manner. Completion of a 4-day dietary intake record and body compositional analysis preceded the 24-week intervention. All procedures were in accordance with the Faculty of Education and Health Sciences Research Ethics Committee (EHSREC Approval 10/45), University of Limerick.

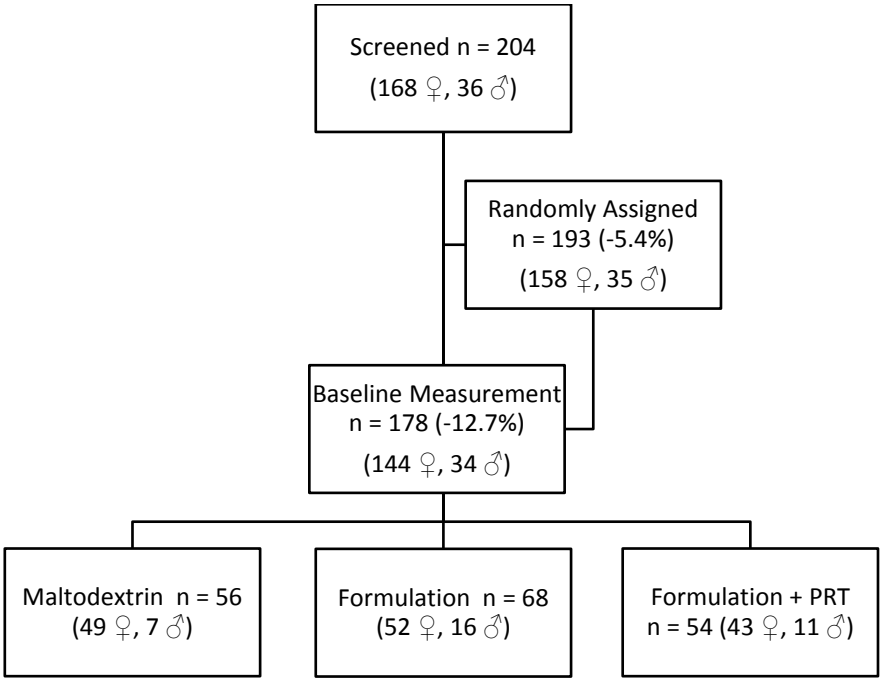

Figure 4.1 – Flow chat illustrating participant numbers through recruitment, screening and randomised group allocation.

4.3.2 Experimental procedures

4.3.2.1 Dietary analysis

A four-day estimated food intake record (eFIR) was used to collect dietary intake data. Participants were instructed to record all food and drink consumed for four consecutive days to include at least one weekend day. Participants were asked to record detailed information regarding the amount (using household measure and estimates of portion sizes) and types of all foods, beverages and nutritional supplements consumed over the recording period and where applicable, the cooking methods used, brand names of the foods consumed and details of recipes. Data were also collected on the time of each eating or drinking occasion, the respondent's definition of each eating or drinking occasion (e.g. morning snack, lunch) and the location of the preparation of the meal or snack consumed (e.g. home, takeaway). Food intake data were coded and subsequently analysed using WISP© (Tinuviel Software, Anglesey, UK). The accuracy of self-reported EI is calculated by expressing energy intake (EI)/basal metabolic rate (BMR). In the current study acceptable reporters were defined as having the EI/BMR ratio above 1.1 which is consistent with the Irish Universities Nutrition Alliance (IUNA) National Adult Nutrition Survey (NANS). BMR was calculated by the Schofield equation. Following exclusion of invalid eFIRs due to dietary misreporting, 59 participant records were analysed further. Analysis was carried out on mean daily protein intakes to allow comparison with recommended daily allowances (RDA) and meal level analysis to determine distribution of reported intake.

4.3.2.1.1 Development of the WISP© essential amino acid database

Tinuviel Software constructed a mapping database in WISP® Version 4 (WISPV4) which linked United States Department of Agriculture (USDA) codes to one or more UK food codes. Where foods were reasonably similar in nutritional makeup, it was deemed possible to map certain nutrients which were in the USDA database to equivalent foods within the UK databank. The AA values from the USDA, factored by the relative amounts of protein in the USDA and UK food (if different) were entered into the UK databank. Using the food database in WISPV4 it was identified that of the 7374 foods in the database, 7132 foods had a listed known protein content assigned (99% coverage of the total number of foods in the database). However investigation of the supplementary database which contains the newly introduced USDA AA values revealed an average coverage rate for EAAs of only 12% (n = 838 foods). This was not sufficient to quantify habitual EAA intakes. Using the WISPV4 contributions data, those foods which contributed significantly to group mean total protein intake in this study population were identified. Priority was given to those food items occurring on greater

than ten occasions or those foods with greater than 10 g protein per 100 g food. Further interrogations of both the WISPV4 and USDA databases were carried out to identify potential matches to expand the database. Using the code replacer function, foods without AA values were replaced in the dietary records for foods with matching descriptors but whose AA values were assigned. The resultant database contained 2057 individual food items, of which 69% (n = 1421) were assigned an AA value. In order to determine how much of the reported daily protein intake was accounted for with the revised database further analysis of reported intakes was necessary. Any foods in the database for which it was not possible to assign an AA value were deleted from duplicate food intake records to allow comparison with the original reported protein intake. This investigation revealed that the database can account for 96 ± 6% of reported protein intakes (range; 70 – 100%).

4.3.2.2 Body composition analysis

The following protocol was established in order to standardise test conditions and ensure adequate hydration levels for each participant and each scan. All procedures were in line with the official positions of the International Society for Clinical Densitometry (ISCD) for body composition DXA scanning (Shepherd *et al* 2013). All participants included in the study were instructed to refrain from strenuous exercise in the 12 hour period before testing and to attend after an overnight fast or fasting for a minimum of 3 hours before the scan. Participants were required to consume 500 mL of water one hour before testing and to empty their bladder immediately prior to measurement and defecate if required. Height was measured to the nearest 0.1 cm using a stadiometer (Seca, Birmingham, UK) and body mass to the nearest 0.1 kg (Tanita MC-180MA Body Composition Analyser, Tanita UK Ltd.). A Lunar iDXA™ scanner (GE Healthcare, Chalfont St Giles, Bucks., UK) with enCORE™ v.14.1 software was used to capture whole body scans. Calibration by tissue phantom was undertaken as per the manufacturer's instructions to confirm the reported accuracy of DXA instrument. Participants were asked to attend in light clothing without removable metal or jewellery and were positioned in supine on the scanner bed according to the manufacturer's recommendations. Arms were positioned at either side with hands in a mid-prone position facing towards the body and slightly apart from the trunk. Gentle longitudinal traction was applied by the operator at the feet and head to align the spine in neutral. Where participants were too wide to fit within the boundary of the scan, the right hand side of the body was scanned and the results were doubled for the missing region. This procedure has been shown to be closely comparable ($R^2 > 0.99$) to whole-body scanning by Rothney *et al* (2009). All scans were conducted and analysed by the

same operator. To accurately interpret serial measurements, knowledge of the least significant change (LSC) is necessary to govern confidence that true loss or gain in body composition parameters has occurred. LSC (95% confidence interval (CI)) was determined as 0.65 kg for whole body LTM and 0.61 kg for ALTM using a sub-group of 50-70y old women and male (n = 22) from the ULBC study.

Table 4.1 – Macro and micro-nutrient breakdown of milk protein formulation per 100 g of powder and per sachet in the individual weight categories.

	Per 100 g	A (45-60 kg) 12.6 g	B (61-75 kg) 16.1 g	C (76-90 kg) 19.7 g	D (91+ kg) 23.3 g
Protein (g)	70.3	8.9	11.3	13.8	16.4
Ash (g)	13.0	1.6	2.1	2.6	3.0
Carbohydrate (g)	9.4	1.2	1.5	1.9	2.2
Moisture (g)	5.1	0.6	0.8	1.0	1.2
Fat (g)	2.3	0.3	0.4	0.4	0.5
Energy (Kcal)	339	43	55	67	79
Calcium (mg)	3903	492	628	769	909
Sodium (mg)	427	54	69	84	99
Potassium (mg)	764	96	123	151	178
Magnesium (mg)	242	30	39	48	56
Phosphorus (mg)	2433	307	392	479	567
Vitamin D3 (ug)	56.3	7.1	9.1	11.1	13.1
Vitamin D3 (IU)	2250	284	362	443	524
T-ORAC (mM (TE))	25.2	3.2	4.1	5.0	5.9

T-ORAC – Total oxygen radical absorbance capacity, TE – trolox equivalents.

4.3.2.3 Supplementation

The control supplement was isocaloric carbohydrate (maltodextrin). The protein formulation consisted of a proprietary blend of MPC, two whey proteins hydrolysates (WPH), DH 32% and DH 45% in the ratio 9:2:1 (MPC:DH32:DH45). The relative contribution of the two main milk proteins are therefore as follows; 60% intact casein protein, 25% WPH and 15% intact WP. The formulation was also fortified with calcium and vitamin D (Table 4.1). MPC was chosen as the 'core protein' in the formulation due to its EAA composition (Table 4.2) in combination with the fact that the bitter aftertaste associated with protein hydrolysates could be masked more easily than some other protein sources (the importance of palatability is obvious as participants were asked to consume the formulation daily over a 24 week period during the intervention study). MPC is also more suitable than whey protein for incorporation into pH neutral ready to drink beverages (RTD), making the formulation a more commercially viable product, subject to the establishment of clinical efficacy. The WPHs were included due to their

potent antioxidant and insulinotropic bioactivity measured *in vitro* (Appendix A). Calcium and vitamin D were included in the formulation based on their positive association with the retention of skeletal integrity with advancing age (Dawson-Hughes *et al* 1997, Tang *et al* 2007) while vitamin D status also has an influence on regulation of muscle mass (Park *et al* 2013).

Table 4.2 – Amino acid composition of milk protein formulation per 100 g of powder and per sachet for the individual weight categories.

AA Content (*g*)	Per 100 g	A (45-60 kg) 12.6 g	B (61-75 kg) 16.1 g	C (76-90 kg) 19.7 g	D (91+ kg) 23.3 g
Alanine	2.4	0.3	0.4	0.5	0.6
Arginine	2.1	0.3	0.3	0.4	0.5
Aspartic acid	5.3	0.7	0.9	1.0	1.2
Cystine	1.0	0.1	0.2	0.2	0.2
Glutamic acid	13.2	1.7	2.1	2.6	3.1
Glycine	1.1	0.1	0.2	0.2	0.3
Histidine	1.9	0.2	0.3	0.4	0.4
Isoleucine	3.4	0.4	0.6	0.7	0.8
Leucine	6.3	0.8	1.0	1.2	1.5
Lysine	5.1	0.6	0.8	1.0	1.2
Methionine	2.0	0.3	0.3	0.4	0.5
Phenylalanine	2.9	0.4	0.5	0.6	0.7
Proline	5.6	0.7	0.9	1.1	1.3
Serine	3.4	0.4	0.5	0.7	0.8
Threonine	3.2	0.4	0.5	0.6	0.8
Tryptophan	0.0	0.0	0.0	0.0	0.0
Tyrosine	2.6	0.3	0.4	0.5	0.6
Valine	4.3	0.5	0.7	0.8	1.0
ΣAA	65.8	8.2	10.6	12.9	15.5
ΣEAA	29.1	3.6	4.7	5.7	6.9
ΣBCAA	14.0	1.7	2.3	2.7	3.3

ΣAA – Sum of amino acids, ΣEAA – sum of the essential amino acids, ΣBCAA – sum of the branch chain amino acids.

Participants were instructed to consume two sachets of maltodextrin or formulation per day. In the FORM and FORM + PRT treatments this equated to an increase of ~ 0.33 $g.kg^{-1}.d^{-1}$ in their daily protein intake. The supplements were packaged in silver foil packets with a single number and letter on the outside to identify contents to the researchers. Participants were divided into one of four supplement categories (A, B, C, D) based on body mass (Table 4.1). The following flavour options were offered, 1. Strawberry, 2. Banana, 3. Orange and mango, 4. Pineapple and 5. Cream. Participants were provided with a shaker and instructions for making the shake. The suggested

volume to dissolve the powder in was 200 mL. Participants were also given a recipe booklet for incorporating the formulation into everyday meals (cereal, yogurt for example). Participants were instructed to take one sachet with breakfast and another with the smaller meal of the remaining two main meals of the day. Participants in the FORM + PRT group were instructed to take one sachet immediately post exercise on training days and the second with either of the two smaller meals of the day. Each participant was given a month's supply of formulation at a time and compliance was assessed through count-back at the end of each month.

4.3.2.4 Exercise program

PRT was performed on 3 non-consecutive days per week for 24 weeks. Participants were instructed to complete at least one session per week under the supervision of qualified exercise trainers at the host institution. Each session lasted 30-45 min and consisted of warm-up and cool-down activities and PRT. The supervised session provided on-going personal attention, tuition, and supervision to ensure that participants adhered to the principle of progressive overload. The remaining sessions each week were completed in the home as to qualify as a readily assimilated lifestyle intervention the programme had to be based primarily within the home/community domain and not within the host institution. The PRT included a combination of upper and lower body exercises designed around the use of Thera-bands® and the Reebox Step® with the main focus on inducing hypertrophy in the prime movers. The primary exercises used throughout the program included the following: squats, lunges, hip abduction, push-up, dips, latissimus dorsi pull-down (or standing row), and a combination of abdominal and core stability exercises. Additional exercises including calf raises, bicep curls, triceps extensions, deadlifts and lumbopelvic and spine stabilization exercises were also rotated throughout the program to ensure the development of muscle balance. The program was divided into six 4-week training cycles. The first phase involved the completion of four exercises (two upper-body, two lower-body). Thereafter each phase consisted of six exercises (three upper-body, three lower-body). During the first week of each training cycle where new exercises were introduced, participants completed 3 sets of 8-12 repetitions of each new exercise. Thereafter, the training volume increased to 3 sets of 12-14 repetitions (week 2), 4 sets of 10-12 repetitions (week 3) and finally 4 sets of 12-14 repetitions (week 4). Exercise intensity was modified by increasing/decreasing the strength of the Thera-band used for a particular exercise for each individual participant (Figure 4.2).

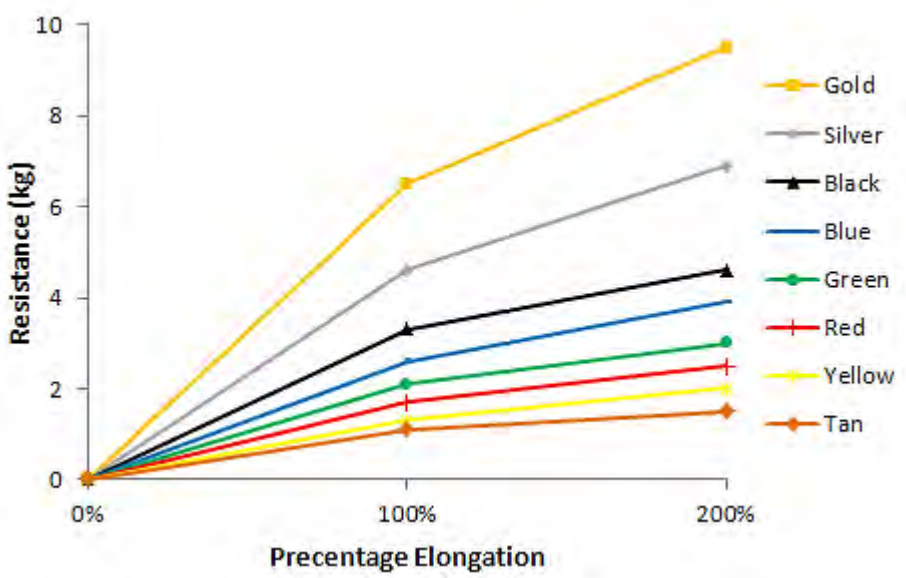

Figure 4.2 – Thera-Band resistance in kilograms based on percentage elongation.

Specific Thera-bands were prepared for each individual based on their shoulder height and breadth. The complexity of the primary exercises was advanced throughout the program as the participants became more proficient (e.g. squat was progressed from squat to/from a chair to free squat, to Thera-band squat to single leg Bulgarian Squat. Participants completed a rate of perceived exertion (RPE) scale for each exercise after each session and Thera-Band strength was modified by the trainers based on the RPE in conjunction with verbal feedback from the participant. Participants were instructed to perform each repetition in a slow, controlled manner to a tempo of 4:1:2 (eccentric:static:concentric) with a rest period of 30 seconds rest between sets. Alternative sets of upper and lower body exercises were performed to minimise fatigue while optimising training time. Exercise compliance was reviewed from training logs completed by the participants during sessions completed at home and in the host institution. Training records were also completed daily by the trainers at each of the sessions at the host institution. The personal trainers also recorded any adverse events or injuries associated with the program.

4.3.3 Statistical analysis

All data was tested for normality using a Shapiro-Wilk test. Mean values and standard deviations (SD), median and interquartile range (IQR) are reported for baseline descriptive statistics. Differences between groups at baseline were assessed by one-way ANOVA (normally distributed variables) or Kruskal-Wallis test (non-normally distributed variables). The dependent variables (24-week change in lean body mass (Δ LTM), 24-week change in appendicular lean body mass (Δ ALTM) were found to be

normally distributed and therefore analysed using a univariate ANOVA with treatment group and gender as fixed factors. To account for variance in baseline values of the dependant variables, the relative change from baseline was also analysed. Where significant interaction was evident a pairwise comparison with Bonferroni correction was employed to identify differences between treatments. Statistical analyses were performed using PASW Statistics 20.0 for Windows (SPSS, Inc., Chicago, IL.). Statistical significance (two-tailed) was set at $P < 0.05$ for all analyses.

4.4 Results

4.4.1 Participant recruitment

204 volunteers were recruited to participate in the study. Ten women and one male (6.5%) were excluded following medical screening (musculoskeletal issues; n = 6, coeliac disease; n = 2, impaired fasting blood glucose concentration, n = 1, cancer in the previous two years, n = 1, cardiorespiratory issues; n = 1). 193 participants were randomly assigned to MALT, FORM or FORM + PRT. Baseline measurements were completed on 178 participants. Fifteen participants (8.4%) chose not to participate after assignment, citing time commitment or dissatisfaction with allocation to a non-exercising group. Final group allocations were as follows; MALT (n = 56), FORM (n = 68) and FORM + PRT (n = 54) (Figure 1). A total of 90 participants (51% of those assigned) completed the study to 24 weeks (MALT n = 29 (24♀, 5♂) FORM n = 31 (22♀, 9♂) and FORM + PRT n = 30 (23♀, 7♂). Reasons for withdrawal included the following: intolerance to the supplement (n = 28), opted to drop-out after 3 months (n = 25), injury/illness unrelated to the study (n = 14), time constraints (n = 6), compliance (n = 8), change in personal circumstances (n = 4) and relocation (n = 2) and complications owing to an existing dietary intervention (n = 1).

Table 4.3 – Young adult mean lean tissue mass (LTM), lean tissue mass index (LTMI), appendicular lean tissue mass (ALTM) and appendicular lean tissue mass index (ALTMI) and European Working Group on Sarcopenia in Older People (EWGSOP) recommended cut-off for the diagnosis of age-related sarcopenia. Data from University of Limerick Body Composition (ULBC) study.

	Female (n = 332, 22.8y)		**Male (n = 431, 22.6y)**	
	Mean	**Cut-off**	**Mean**	**Cut-off**
LTM (kg)	42.0 ± 4.9	32.2	62.0 ± 7.0	48.0
LTMI (g.cm^{-2})	15.1 ± 1.4	12.3	19.1 ± 1.6	15.9
ALTM (kg)	19.4 ± 2.6	14.2	30.5 ± 3.9	22.7
ALTMI (g.cm^{-2})	7.0 ± 0.8	5.4	9.4 ± 0.9	7.6

4.4.2 Baseline characteristics

Baseline characteristics are presented in Table 4.4 (body composition), Table 4.5 (haematological profile) and Table 4.6 and Figure 4.3 (dietary intake). There were no significant differences between groups for any of the baseline characteristics except for total cholesterol. Mean LTM and ALTM was 39.7 kg and 17.6 kg for female participants and 57.9 kg and 27.4 kg for male participants, respectively. No participant was classified as sarcopenic (class II sarcopenia) based on the European Working Group on Sarcopenia in Older People (EWGSOP) definition utilising LTM, LTMI, ALTM or ALTMI as the criterion measure (Table 4.3). EWGSOP recommends the use of normative healthy young adult (YA) rather than other predictive reference populations, with cut-off point two standard deviations below the mean reference value. In this

instance the normative group were healthy 18-29 y old women and men who participated in the ULBC study.

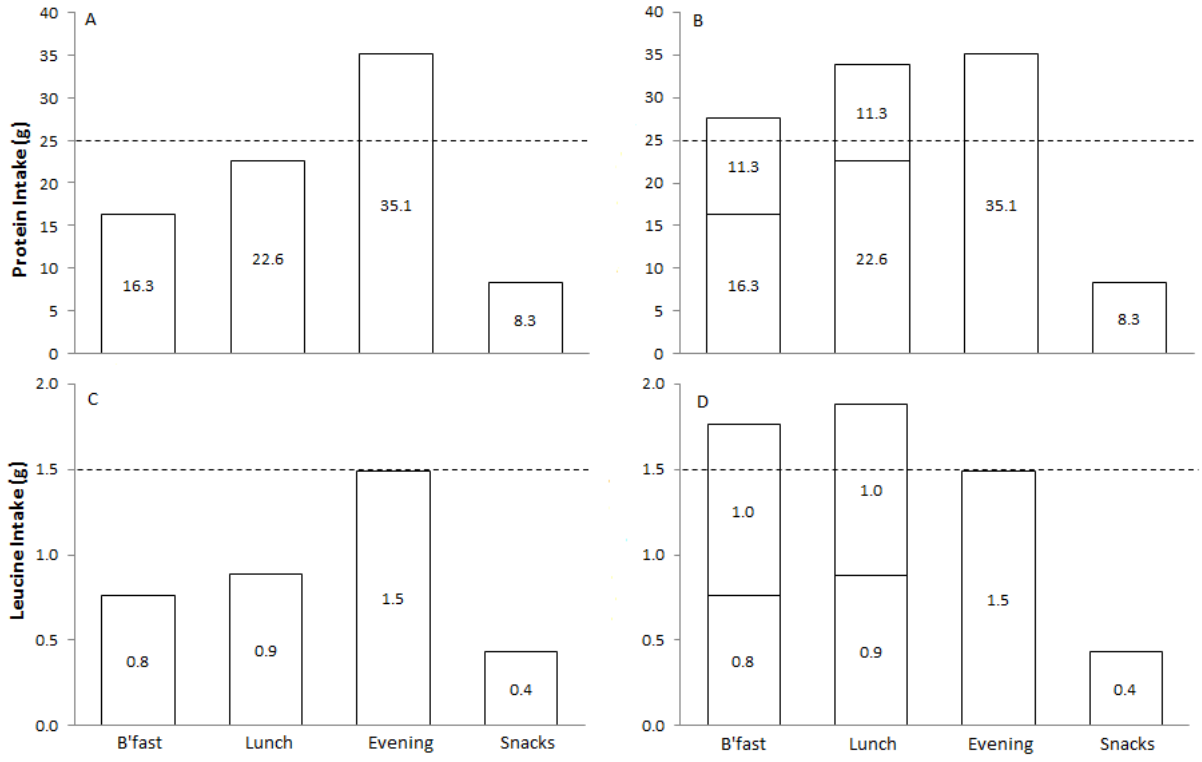

Figure 4.3 – Protein and leucine intake per meal time moment at baseline (A and C) and theoretical correction of distribution following milk protein supplementation (B and D). Dashed line represents the proposed threshold for protein and leucine intake required to overcome "anabolic resistance" in older individuals (Paddon-Jones and Rasmussen 2009, Figure 1.4, Chapter 1 pg.6).

4.4.3 Compliance

The average supplement compliance in the MALT group was 86 ± 13% and was not different to the FORM (91% ± 9%) and the FORM + PRT (86 ± 12%) (group $P = 0.150$). Exercise compliance was assessed through a combination of training logs completed by the participants, attendance of sessions at the host institution and subjective assessment of participant progression during the program by the trainers. Two participants completed the 24 week intervention and reassessment but were excluded on the basis that they were not compliant with the PRT program. All other participants were deemed to have complied based on the criteria outlined.

4.4.4 Body composition

The change in body composition for participants who completed the 24 week intervention is presented in Tables 4.7 and 4.8 and Figures 4.4 and 4.5.

Table 4.4 – Baseline body composition profile of participants (n = 90) who completed the 24 week dietary and training intervention.

	MALT (n = 29)			FORM (n = 31)			FORM + PRT (n = 30)			*P*
	Mean ± SD	**Median (IQR)**	**Range**	**Mean ± SD**	**Median (IQR)**	**Range**	**Mean ± SD**	**Median (IQR)**	**Range**	
Age (y)	60 ± 5.8	60 (10.3)	51 - 70	62 ± 4.7	63 (6.6)	53 - 70	59 ± 5.6	58 (7.0)	51 - 70	0.069
Height (cm)	167 ± 5.9	166 (5.3)	158 - 180	165 ± 7.7	163 (9.8)	153 - 181	166 ± 8.5	165 (14)	155 - 186	0.471
Body Mass (kg)	72 ± 12.4	70 (17.8)	52 - 104	71 ± 11.7	68.4 (18.0)	50 - 92	72 ± 14	68 (23)	52 - 107	0.908
BMI (g.cm^{-2})	25.9 ± 4.1	24.9 (3.5)	19 - 37	25.7 ± 3.1	25.6 (4.2)	20 - 34	26.1 ± 4.3	25.6 (4.5)	19 - 36	0.911
[1]FTM (kg)	25.7 ± 8.5	22.8 (10.2)	10 - 43	24.2 ± 7.3	25 (10.3)	7 - 37	25.7 ± 6.8	23.6 (8.1)	14 - 41	0.985
LTM (kg)	43.8 ± 8.0	40.2 (7.0)	37 - 66	44 ± 9.1	40 (16.6)	33 - 61	44.2 ± 8.9	42.0 (10.8)	34 - 67	0.784
LTMI (g.cm^{-2})	15.7 ± 2.0	15.1 (1.9)	13 - 22	15.9 ± 2.2	14.9 (3.3)	13 - 21	15.9 ± 2.1	15.3 (2.6)	12 - 22	0.872
ALTM	19.6 ± 4.1	17.6 (3.2)	16 - 31	19.9 ± 4.7	17.7 (8.5)	14 - 30	20.2 ± 5.1	18.4 (6.1)	15 - 33	0.831
ALTMI (g.cm^{-2})	7.0 ± 1.1	6.6 (1.0)	5.8 - 10.3	7.2 ± 1.2	7.0 (1.7)	5.8 - 9.6	7.2 ± 1.3	6.9 (1.8)	5.4 - 10.9	0.920
[1]Body Fat%	35.2 ± 8.3	35.0 (8.3)	17 - 49	34.2 ± 8.1	35.1 (11.9)	11 - 46	35.3 ± 5.1	34.4 (5.8)	27 - 46	0.913

[1] Normally distributed (Shapiro-Wilk p > 0.05). BMI, Body mass index; FTM, Fat tissue mass; LTM, Lean tissue mass; LTMI, Lean tissue mass index; ALTM, Appendicular lean tissue mass; ALTMI, Appendicular lean tissue mass index; SD, standard deviation; IQR, interquartile range. Between group differences at baseline examined by One-way ANOVA (normally distributed variables) or Kruskal Wallis test (non-normally distributed variables).

Table 4.5 – Baseline haematological profile of participants (n = 90) who completed the 24 week dietary and exercise intervention.

	MALT (n = 29)			FORM (n = 31)			FORM + PRT (n = 30)			
	Mean ± SD	**Median (IQR)**	**Range**	**Mean ± SD**	**Median (IQR)**	**Range**	**Mean ± SD**	**Median (IQR)**	**Range**	***P***
Glucose (mM)	4.9 ± 0.3	4.9 (0.3)	4.2 - 5.7	4.9 ± 0.5	4.9 (0.7)	4.0 - 6.3	5.2 ± 0.7	5.1 (0.7)	4.2 - 7.3	0.115
HOMA IR	1.5 ± 1.3	1.0 (1.2)	0.4 - 6.5	1.4 ± 1.0	1.2 (1.0)	0.3 - 4.6	1.6 ± 1.0	1.3 (1.2)	0.3 - 4.5	0.544
[1]Cholesterol (mM)	5.7 ± 0.7	5.5 (1.1)	4.6 - 7.6	5.2 ± 0.8	5.3 (1.3)	3.3 - 6.3	5.3 ± 0.7	5.2 (0.9)	4.1 - 6.5	**0.029**
Albumin (g/L)	42 ± 2.5	42 (3.0)	37 - 47	41 ± 3.4	41 (5.2)	35 - 51	42 ± 2.7	42 (2.0)	39 - 50	0.663
Creatinine (μM)	71 ± 15	72 (15)	44 - 102	70 ± 16	65 (17)	45 - 103	70 ± 11	71 (17)	50 - 96	0.715
Urea (mM)	5.0 ± 1.6	4.5 (1.5)	3.3 - 11.2	4.9 ± 1.5	4.7 (2.0)	2.1 - 8.0	5.1 ± 1.2	5.0 (1.6)	3.0 - 8.1	0.745
[1]Calcium (mM)	2.32 ± 0.1	2.33 (0.1)	2.24 - 2.45	2.28 ± 0.1	2.27 (0.1)	2.04 - 2.50	2.33 ± 0.1	2.32 (0.1)	2.18 - 2.56	0.048
[1]Vitamin D (nM)	65 ± 20	67 (26)	23 - 119	65 ± 24	68 (30)	25 - 123	68 ± 29	61 (41)	15 - 143	0.851
eGFR	86 ± 19	83 (23)	51 - 134	90 ± 16	89 (26)	66 - 132	86 ± 14	85 (20)	67 - 116	0.667

[1] Normally distributed (Shapiro-Wilk $p > 0.05$). HOMA IR; Homeostasis model assessment of insulin resistance, eGFR; estimated glomerular filtration rate. Between group differences at baseline examined by One-way ANOVA (normally distributed variables) or Kruskal-Wallis test (non-normally distributed variables).

Table 4.6 – Baseline macronutrient, essential amino acid (EAA) and leucine intake of participants (n = 59) who completed the 24 week dietary and exercise intervention.

	MALT (n = 26)			FORM (n = 15)			FORM + PRT (n = 18)			
	Mean ± SD	**Median (IQR)**	**Range**	**Mean ± SD**	**Median (IQR)**	**Range**	**Mean ± SD**	**Median (IQR)**	**Range**	***P***
Energy (kcal)	1890 ± 380	1777 (593)	1335 - 2636	2079 ± 576	2149 (752)	1373 - 3170	2027 ± 385	1975 (656)	1408 - 2594	0.482
[1]Protein ($g.d^{-1}$)	88 ± 18	87 (26)	59 - 110	87 ± 24	85 (32)	59 - 140	84 ± 16	89 (21)	52 - 111	0.842
[1]Fat ($g.d^{-1}$)	73 ± 20	72 (22)	32 - 121	71 ± 18	73 (23)	43 - 99	80 ± 18	78 (23)	55 - 121	0.279
Carbohydrate ($g.d^{-1}$)	206 ± 54	199 (67)	104 - 348	249 ± 95	227 (47)	113 - 492	223 (63)	211 (85)	112 - 335	0.322
Protein ($g.kg^{-1}$)	1.25 ± 0.3	1.21 (0.4)	0.86 - 1.97	1.29 ± 0.3	1.27 (0.4)	0.78 - 2.07	1.26 ± 0.25	1.34 (0.28)	0.79 - 1.63	0.854
[1]EAA ($g.d^{-1}$)	17.0 ± 7.2	16.8 (10.3)	5.3 - 31.6	16.5 ± 8.0	13.3 (10.3)	9.0 - 36.2	16.9 ± 5.2	16.7 (4.9)	6.5 - 26.1	0.972
Leucine ($g.d^{-1}$)	4.0 ± 1.6	3.9 (2.3)	1.3 - 7.4	3.8 ± 1.8	3.1 (2.3)	2.0 - 8.2	4.0 ± 1.1	3.9 (1.2)	1.5 - 6.1	0.708

[1] Normally distributed (Shapiro-Wilk $p > 0.05$). Between group differences at baseline examined by One-way ANOVA (normally distributed variables) or Kruskal Wallis test (non-normally distributed variables).

Table 4.7 – Change in body composition from baseline (24 week – 0 week) of participants (n = 90) who completed the 24 week dietary and exercise intervention.

	MALT (n = 29)			FORM (n = 31)			FORM + RE (n = 30)			*P*
	Mean Δ ± SD	**Median (IQR)**	**Δ Range**	**Mean Δ ± SD**	**Median (IQR)**	**Δ Range**	**Mean Δ ± SD**	**Median (IQR)**	**Δ Range**	
[1]**Body Mass (kg)**	-0.22 ± 1.50	-0.30 (1.84)	-2.7 - 3.5	0.70 ± 1.86	0.74 (2.08)	-5.3 - 4.4	-0.07 ± 1.99	0.26 (1.84)	-5.2 - 3.5	0.112
FTM (kg)	-0.06 ± 1.39	-0.03 (1.57)	-2.5 - 3.7	0.24 ± 1.39	0.41 (1.38)	-4.0 - 2.7	-0.87 ± 1.99	-0.20 (1.76)	-6.9 - 1.9	**0.035**
[1]**LTM (kg)**	-0.16 ± 0.87	-0.22 (1.16)	-1.9 -1.4	0.44 ± 1.06	0.37 (1.61)	-1.2 - 2.5	0.80 ± 1.02	0.70 (1.10)	-1.4 - 2.8	**<0.001**
[1]**ALTM (kg)**	0.01 ± 0.41	0.06 (0.65)	-0.9 - 0.7	0.27 ± 0.58	0.16 (0.85)	-0.5 - 1.5	0.33 ± 0.50	0.40 (0.58)	-0.9 - 1.5	**0.004**
Body Fat %	0.08 ± 1.55	-0.17 (2.35)	-0.2 - 2.4	0.06 ± 1.25	0.15 (1.22)	-2.8 - 2.3	-1.26 ± 2.14	-0.88 (2.18)	-9.5 - 1.8	**0.003**

[1] *Normally distributed (Shapiro-Wilk p > 0.05). FTM, Fat tissue mass; LTM, Lean tissue* mass; ALTM, Appendicular lean tissue mass. *P* – univariate ANOVA (normal distribution) or Kruskal-Wallis test (non-normal distribution) examining changes over time between groups.

Table 4.8 – Relative change from baseline in body composition (24 week – 0 week) of participants (n = 90) who completed the 24 week dietary and exercise intervention.

	MALT (n = 29)			FORM (n = 31)			FORM + RE (n = 30)			*P*
	Mean Δ ± SD	**Median (IQR)**	**Δ Range**	**Mean Δ ± SD**	**Median (IQR)**	**Δ Range**	**Mean Δ ± SD**	**Median (IQR)**	**Δ Range**	
[1]**Body Mass (%)**	-0.17 ± 2.1	-0.45 (2.3)	-3.8 - 5.1	0.95 ± 2.5	1.1 (2.7)	-6.5 - 5.3	-0.04 ± 2.8	0.37 (2.7)	-6.9 - 5.5	0.089
FTM (%)	1.0 ± 6.5	-0.11 (6.9)	-8.0 - 17.9	1.69 ± 5.7	1.9 (5.7)	-10.9 - 16.8	-3.3 ± 8.7	-0.97 (6.4)	-36.8 - 8.8	**0.037**
[1]**LTM (%)**	-0.30 ± 2.1	-0.51 (2.8)	-4.4 - 3.6	0.91 ± 2.4	0.9 (3.5)	-3.0 - 6.2	1.77 ± 2.3	1.80 (2.8)	-4.1 - 6.7	**0.010**
[1]**ALTM (%)**	0.06 ± 2.2	0.25 (3.5)	-4.1 - 4.1	1.11 ± 2.7	0.84 (3.6)	-2.9 - 6.0	1.72 ± 2.8	2.09 (2.4)	-5.9 - 8.3	**0.042**
Body Fat (%)	0.94 ± 5.5	-0.39 (6.4)	-6.0 - 15.3	0.56 ± 4.0	0.54 (3.9)	-7.8 - 11.4	-3.7 ± 6.9	-2.87 (5.3)	-32.2 - 6.8	**0.016**

FTM, Fat tissue mass; LTM, Lean tissue mass; ALTM, Appendicular lean tissue mass. *P* - univariate ANOVA (normal distribution) or Kruskal-Wallis test (non-normal distribution) examining changes over time between groups.

Table 4.9 – Change in haematological profile from baseline (24 week – 0 week) of participants (n = 90) who completed the 24 week dietary and exercise intervention.

	MALT (n = 29)			FORM (n = 31)			FORM + PRT (n = 30)			
	Mean Δ ± SD	***Median (IQR)***	**Range**	**Mean Δ ± SD**	***Median (IQR)***	**Range**	**Mean Δ ± SD**	***Median (IQR)***	**Range**	***P***
[1]**Glucose (mM)**	0.17 ± 0.46	0.10 (0.40)	-0.4 - 1.8	0.14 ± 0.39	0.2 (0.5)	-0.5 - 1.2	0.09 ± 0.51	0.1 (0.6)	-1.0 - 0.9	0.784
HOMA IR	-0.20 ± 1.26	-0.11 (0.8)	-5.2 - 1.6	0.07 ± 0.51	0.06 (0.4)	-1.1 - 1.7	0.01 ± 1.08	0.04 (1.1)	-2.4 - 2.7	0.628
Cholesterol (mM)	-0.02 ± 0.63	-0.01 (0.7)	-1.5 - 1.6	0.11 ± 0.49	0.17 (0.7)	-0.8 - 1.1	-0.02 ± 0.63	-0.06 (0.8)	-1.1 - 1.5	0.451
[1]**Albumin (g/L)**	0.96 ± 2.48	0.90 (3.0)	-4.9 - 5.6	0.45 ± 3.57	0.0 (1.4)	-1.7 - 3.9	0.40 ± 2.63	1.1 (3.1)	-6.0 - 4.8	0.727
Creatinine (μM)	-0.69 ± 8.86	-1.0 (9.0)	-26 - 27	-3.58 ± 6.02	-3.0 (7.0)	-15 - 11	-0.59 ± 9.27	-2.0 (11.0)	-30 - 23	0.183
Urea (mM)	-0.44 ± 2.02	-0.1 (2.0)	-5.9 - 2.7	0.30 ± 1.41	0.1 (1.4)	-1.7 - 3.9	-0.19 ± 1.07	-0.2 (1.4)	-2.7 - 2.2	0.442
Calcium (mM)	-0.05 ± 0.43	0.02 (0.1)	-2.3 - 0.1	0.04 ± 0.11	0.04 (0.1)	-0.12 - 0.4	0.03 ± 0.08	0.01 (0.1)	-0.1 - 0.2	0.936
Vitamin D (nM)	-1.13 ± 19.3	-4.3 (15.0)	-33 - 55	8.31 ± 15.7	7.8 (18.2)	-17 - 59	8.46 ± 17.5	7.0 (20)	-35 - 41	0.061
eGFR	-4.4 ± 16.7	-1.4 (17.6)	-57 - 21	-0.85 ± 15.8	1.8 (20.6)	-39 - 32	-0.53 ± 20.5	-0.17 (18)	-51 - 57	0.768

[1] Normally distributed (Shapiro-Wilk p > 0.05). HOMA IR; Homeostasis model assessment of insulin resistance, eGFR; estimated glomerular filtration rate. *P* – univariate ANOVA (normal distribution) or Kruskal-Wallis test (non-normal distribution) examining changes over time between groups.

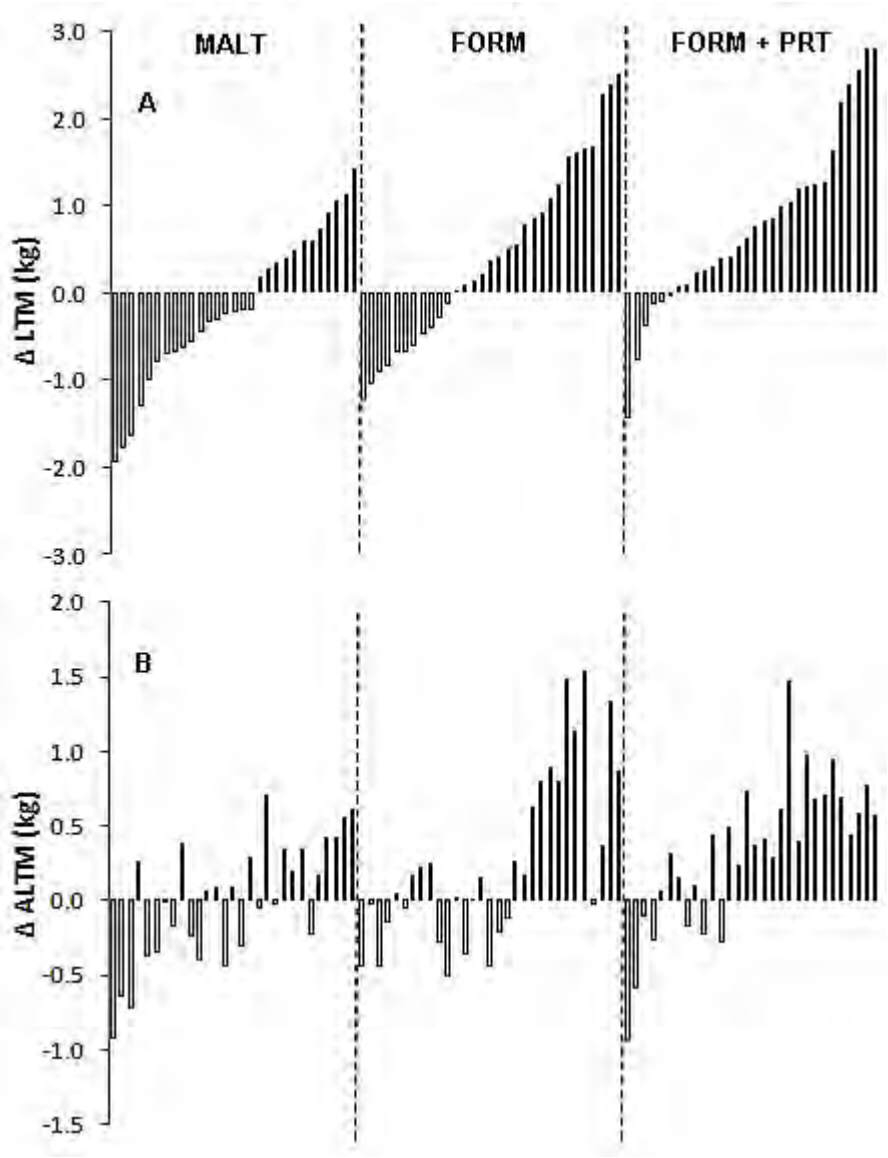

Figure 4.4 – Individual change in lean tissue mass (LTM, A) sorted from low to high response and corresponding change in appendicular lean tissue mass (ALTM, B) for the maltodextrin (MALT), formulation (FORM) and formulation plus progressive resistance exercise training (FORM + PRT) groups.

4.4.4.1 Lean tissue mass

At 24 weeks the mean change in LTM resulted in a net difference of 0.6 kg between MALT and FORM and 0.96 kg between MALT and FORM + PRT treatments [ANOVA; $F_{(5,84)}$ = 5.08, P < 0.001, 1-ß = 0.98, η_p^2 = 0.23]. Post-hoc analysis revealed a statistically significant difference between the MALT and FORM (P = 0.004) and MALT and FORM + PRT treatments (P < 0.001). Expressed as a percentage, the change in LTM at 24 weeks resulted in a significant group interaction [ANOVA; $F_{(5,84)}$ = 3.23, P = 0.01, 1-ß = 0.87, η_p^2 = 0.16]. Post-hoc analysis revealed a statistically significant difference between the MALT and FORM (P = 0.037) and MALT and FORM + PRT treatments (P = 0.002).

4.4.4.2 Appendicular lean tissue mass

At 24 weeks the mean change in ALTM resulted in a net difference of 0.26 kg between MALT and FORM and 0.34 kg between MALT and FORM + PRT treatments [ANOVA; $F_{(5,84)}$ = 3.75, P = 0.004, 1-ß = 0.92, η_p^2 = 0.18]. Post-hoc analysis revealed a statistically significant difference between the MALT and FORM group (P = 0.023) and a trend towards significance in the MALT and FORM + PRT group (P = 0.078). Expressed as a percentage, the change in ALTM at 24 weeks resulted in a significant group interaction [ANOVA; $F_{(5,84)}$ = 2.43, P = 0.042, 1-ß = 0.74, η_p^2 = 0.13]. Pairwise comparison did not however reveal any statistically significant differences between experimental treatments.

4.4.5 Adverse events

Over the course of the PRT program one participant suffered a partially dislocated shoulder which reset itself immediately upon cessation of the exercise. A second participant fainted at the end of an exercise class during the cool-down period. Both participants were reviewed onsite by a physiotherapist and recovered sufficiently by the end of the session such that further medical attention was not warranted. There were no reports of significant events occurring during the completion of the program in the home. Several participants suffered mild, acute bouts, of delayed onset muscle soreness (DOMS) due to unaccustomed exercise. These may have required modification of exercises to reduce the intensity during a subsequent session but had resolved by the next training occasion.

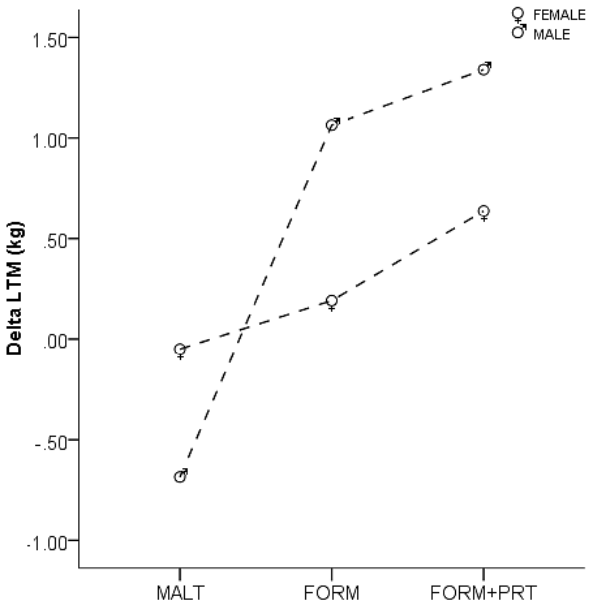

Figure 4.5 – Change in lean tissue mass per experimental group (MALT - maltodextrin, FORM - formulation and FORM + PRT - formulation plus progressive resistance exercise training) according to gender.

4.5 Discussion

The main outcome from this 24-week RCT is that evenly distributing daily protein intake through addition of 0.33 $g.kg^{-1}.d^{-1}$ milk protein to the diet is an effective strategy to offset age-related decline in LTM. The inclusion of a program of PRT, to compliment supplementation with milk protein, augmented gains relative to the FORM group, but not to a statistically significant degree. Considerable inter-individual variability was evident in the LTM response to supplementation and RE (individual losses and gains of greater than ± 1 kg were evident in each of the three experimental treatments). The most consistent response was apparent in the FORM + PRT group. Extrapolating this 24-week observation to 52-weeks (assuming a linear trend over time without accommodation to the increased protein load) would result in an increase in LTM of 0.8 kg. If combined with a predicted annual loss of 0.1 kg as observed in the MALT group during the present study, the net change in LTM would be ~ 0.9 kg as a result of milk protein supplementation and the consequent even distribution of protein intake over the main meals of the day.

Dillon *et al* (2009) reported an average increase in LTM of 1.7 kg in response to ingestion of 7.5 g of EAA twice daily over a 12-week period in seven healthy older women. This is ~ 1.2 kg in excess of the average increase observed in the current study in the FORM group in half the time. The reason for this discrepancy is not obvious but the EAA and leucine doses were lower in the present study than during the Dillon *et al* trial (~ 4.7 g vs. 7.5 g EAA and ~ 1.0 g vs. 1.4 g leucine respectively). This raises the possibility that the quantity and quality of protein fed was not sufficient to maximally stimulate MPS even when ingested in conjunction with either a breakfast or afternoon meal. The quantity and quality of protein utilised in the present study was however deemed to be the best balance achievable between delivering a commercially viable (based on cost per effective dose) and efficacious supplement in terms of retaining/augmenting LTM with advancing age.

The mean change in LTM observed is consistent with the response reported by Verhoeven *et al* 2009 to the addition of 2.5 g leucine to each of the main meals of the day (i.e. 0.4 kg). Taken together these data suggest that daily supplementation with either ~ 9.4 g of EAA containing ~ 2 g of leucine or 7.5 g of leucine in isolation is not as effective as supplementation with 15 g of EAA containing 2.8 g of leucine in terms of promoting LTM accrual in older populations. During discussion on the derivation of the milk protein formulation, MPC was chosen as the core protein, fortified with two WPH. It was believed that the AA composition of the MPC and the WPH negated the

requirement for supplementation with individual AA (i.e. additional leucine). The main drawback with the 'reverse engineered' approach to the derivation of the nutrient formulation is the difficulty in identifying the relative contribution of each component in the formulation to the observed outcome. To overcome this issue would require separate branches within the longitudinal study to investigate each component in isolation and in combination (seven or more branches including an isocaloric control). While this was beyond the capacity of the current work program it is desirable as part of good scientific practice.

Consistent with the total body LTM, there was a significant between group effect on the change in ALTM. Post-hoc testing revealed that the change in ALTM was significant between the MALT and FORM treatments with a trend towards significance between the MALT and FORM + PRT treatments. This is somewhat counter-institutive as one would expect the change in ALTM to be superior to that observed for the nutrient effect due to the optimised timing of nutrient ingestion relative to the training session. One dataset in the FORM + PRT group may be influencing this outcome as this individual experienced a loss of almost 1 kg from the appendages over the 24-week intervention period. When sorted according to the change in total body LTM, the change in ALTM followed a similar pattern (Figure 4.4, A and B). However there were instances where the change in total body LTM was not reflected in the ALTM. Caution is therefore advised in interpreting changes in total body LTM in response to nutrient interventions as the change may occur in a region that may not benefit the individual in terms of functional outcome (i.e. the trunk). The most consistent increase in ALTM was also evident in the FORM + PRT group.

In terms of the potential augmentation of the nutrient effect through adoption of a program of PRT, consistent with our findings, Kukuljan et al. (2009) reported in an 18-month RCT, despite a greater than two-fold increase in total body LTM in an RE + milk group relative to an exercise only and milk only group, this change was not statistically significant. The authors attributed this to the fact that participants in the study were healthy community dwelling men with adequate energy and protein intakes based on DRI's and had adequate circulating 25-hydroxyvitamin D levels. An alternative explanation was that the timing of milk consumption was not optimised during the Kukuljan trial. In the present study, participants were instructed to consume the supplement with the two smaller meals of the day. On training days, participants in the FORM + PRT were instructed to consume one sachet immediately post exercise and the second sachet with one of the remaining two smaller meals of the day. This would have provided approximately 10 g of protein (~ 4.7 g EAA, ~ 1 g leucine) dependent on

body mass immediately post exercise. This amount of protein may not have been sufficient to maximally stimulate MPS despite the sensitising effect of RE on MPS. However the effect of an acute bout of RE on muscle protein FSR has been demonstrated to persist for up to 48 h in younger individuals (Phillips *et al* 1997). It is therefore probable that some residual effect of the exercise session of FSR was evident when participants ingested their next protein containing meal.

The data from the present study are very similar to those reported by Holm *et al* (2008). During a 24-week supplement plus RE intervention a 0.8 kg increase in LTM was observed. Despite the LTM of a control plus PRT group remaining relatively constant the change was not statistically significantly different. Campbell et al, (1995) reported a gain in LTM, 1 kg in excess of that observed in the present study in response to 12-weeks of high protein intake and PRT. Both the Holm *et al* and Cambell *et al* study employed classical gym based RE protocols (sets and reps of exercise at intensity's based on individual one repetition maximum (1 RM). These regimented protocols may offer more scope for increases in LTM but their application to the wider public is limited. In the present study the PRT was designed around a program that could be completed in the home/community setting using minimal equipment. This was deemed more applicable to the general population who may not have access to, or wish to engage in, a structured gym based program. This may however have impacted on the potential of the program to induce hypertrophy but may in the long term be a more appropriate method to promote exercise paradigms designed to offset the age-related decline in skeletal muscle mass. While two adverse events occurred over the course of the trial both of these instances had extenuating circumstances (a participant who dislocated their shoulder had a history of doing so while a participant who fainted had come to a morning session in a fasted state).Therefore the program is a safe and effective means to augment LTM in a home or community environment.

Participants were openly recruited for this RCT. This resulted in a considerable gender bias in the final numbers that completed the intervention (69 women and 21 men). This gender bias influenced the final outcome and was accounted for through inclusion of gender as a fixed factor in the statistical analysis. In essence the male participants responded to a greater degree in each of the three experimental treatments (on average men lost more LTM than women in the MALT group and gained more LTM in the FORM and FORM + PRT treatments (Figure 4.5)). It may have been more simplistic to recruit an exclusively female or male cohort to potentially reduce the variability of the response as per the Top Institute Food and Nutrition (TIFN) and Maastricht University research groups in The Netherlands (Koopman *et al* 2008,

Koopman *et al* 2009, Verdijk *et al* 2009, Verhoeven *et al* 2009, Pennings *et al* 2012, Tieland *et al* 2012b). The study was however designed to be applicable to as wide a target audience as possible. It could not be foreseen at the outset that the recruitment would be so biased towards women. The decision was taken not to redress the gender balance during recruitment and target men exclusively as to do so would have violated the open recruitment principle of the trial designated from the outset.

Based on the ULBC 50-70y old data set (itself a convenience sample population) we can say that these results are applicable to the general population as the LTM and ALTM were identical between both study cohorts. The rate of attrition was slightly higher than the 50% drop-out rate predicted prior to recruitment. Approximately one third of participants who dropped out of the study cited intolerance to the supplement. It is pertinent to note that a group of 50-70 year old women and men are not habituated to the consumption of powdered formulas. The participants were also blinded as to the contests of the sachets. This may have impacted on their willingness to consume the supplement over the duration of the study. Packaging the formulation into a ready to drink (RTD) format is one option to improve tolerance but the expense of this operation was prohibitive in this instance. The success of an RTD format is evident from the Kukuljan study as the attrition rate owing to the supplement was much lower with a total number of drop-outs of just 8 of 180 (4.4%). A post-hoc power calculation based on the current observations suggests that n = 90 would be required to observe a difference at $\alpha = 0.05$ level between FORM and FORM + PRT treatments with 90% power. This would suggest that the study was underpowered at the outset but the primary investigation involved examining the nutrient effect against the isocaloric maltodextrin control. The study would have benefited from the inclusion of an MALT + PRT training group to quantify the effect of the training programme independent of milk protein supplementation. Limitations in funding, curtailing the resources available, combined with time constraints enforced by the grant provider precluded additional branches to the trial. It was deemed appropriate with the resource available to conduct three branches where the primary focus should be on investigating the nutrient effect.

The baseline dietary intakes recorded in this instance are also similar to those reported by Harrington *et al* (2001) again indicating that the study population are representative of the general Irish population. Participants completed two eFIR at baseline and an additional record in their final week of supplementation. It was envisaged that the eFIR would serve to confirm that a true dietary supplementation (and not a substitution) had taken place. However, upon analysis it was deemed that 4-day eFIR were too blunt an instrument to detect changes in protein and amino acid intake even in the range of 20 g

per day. As such it cannot be confirmed that participants did not alter their dietary habits in response to the additional protein (possibly due to the satiating effect of protein). It can be argued that the most beneficial time frame to supplement with additional protein is the breakfast meal due to the lower protein intakes at this time (Tieland *et al* 2012a) and the beneficial effect that supplementation of the breakfast meal has on FSR (Mamerow *et al* (2014)). If a substitution were to have taken place it would likely happen during the afternoon and/or evening meals in the day. Protein intake is closer to the required 25-30 g during these meals without supplementing with additional protein and as such even with the potential for substitution the threshold for protein intake is likely to be exceeded. There was a significant within group increase in body mass for the FORM group which would suggest that the additional supplemented protein and carbohydrate did not negatively influence total energy intake over the 24 week period. It is interesting to note that lack of change in body mass in the FORM + PRT group masks a 0.8 kg increase in LTM and a 0.8 kg decrease in FTM.

25-OH Vitamin D concentration was considerably higher than previously reported for a group of older community dwelling women in Ireland. Anderson *et al* (2005) reported that the average 25OH vitamin D concentration for a sample of forty-three women with an average age of 72 years was 44 nM (95% C.I. 17, 89 nM). This value is 33% lower than the average baseline concentration measured in the present study. However, the analytical platform used to measure vitamin D concentration was different between the two investigations. Another possible explanation for the discrepancy is the time of year that vitamin D was measured (during the winter months in the Anderson *et al* (2005) report while baseline vitamin D concentration was measured during the summer months during this trial). Substantial inter-individual variability was observed in the serum vitamin D concentration and a limitation of the study was that exposure to sunlight (measurement via electronic dosimeter badges or polysulfone film) was not undertaken in this instance. Participants classified as vitamin D deficient, inadequate, adequate or optimal based on the IOM recommendations (Ross *et al* 2011) were largely unaffected by supplementation with either MALT or FORM.

The appropriateness of the population chosen for investigation is contentious. No participant in the study cohort was suffering from age-related sarcopenia based on the EWGSOP definition. No participant was energy or protein deficient at baseline. Considering these facts it is pertinent to ask if these participants could benefit from increased protein intakes. Several distinguished researchers within the field of human muscle protein metabolism are forthright in their views that the consumption of additional protein above the current RDI (i.e. 0.85 $g.kg^{-1}$) is unnecessary in healthy

populations, irrespective of age (Rennie 2009, Millward 2008, Campbell *et al* 2008). However the fact that a positive outcome was observed in terms of the change in LTM in this instance would suggest that the prescription of a recommended quantity of protein to maintain nitrogen balance in the short term may be too simplistic an argument with respect to dietary protein requirements. Recent human data describing the 24h FSR response to protein consumed evenly over the three main meals of the day, supports the theory that protein intakes in excess of the current DRI, but distributed evenly throughout the day are beneficial for the retention and/or augmentation of LTM (Mamerow *et al* (2014)). Data from the present study is, to the best of the author's knowledge, the first to report the effect of 24-weeks of even distribution of daily protein intake through supplementation with milk protein on LTM in a healthy 50-70y old population.

4.6 Conclusion

An unprecedented proportion of the global population will be aged greater than 60y by the year 2050. In combination with increased longevity, this will place considerable demand on health service professionals and budgets to care appropriately for this challenging sub-population. Strategies that encourage and prolong functional independence in older populations have become important research themes, particularly over the last decade. Two such strategies are dietary and exercise interventions designed to offset the age-related decline in LTM. Recent data informs as to the quantity and quality of dietary protein required to stimulate acute muscle protein FSR in older populations. These data, when considered alongside records of dietary intake analysed at the per meal level, provide a rationale to expand the argument with regard to protein intake beyond universal requirements in gram per kilogram body mass. Supplementing the two smaller meals of the day with additional protein could help offset the age-related decline in LTM termed sarcopenia. Long-term intervention studies designed to test this hypothesis are lacking.

Utilising an open recruitment RCT we have demonstrated that the even distribution of daily protein intake through addition of 0.33 $g.kg^{-1}$ milk protein to the daily diet, is effective in off-setting the age-related decline in LTM. A program of PRT proved effective in augmenting this nutrient effect. The absence of an exercise plus maltodextrin group to quantify the magnitude of the exercise stimulus is however a weakness in the design. Issues with the delivery matrix need to be addressed to make the formulation more palatable and incorporation into a RTD beverage is necessary. While open recruitment is a desirable attribute in an RCT, imbalances in the number of women or men recruited can lead to complications in the analysis of outcome variables due to variation in the response between the sexes.

Chapter 5 General Discussion and Future Work

5.1 General discussion

The role of dietary protein in the regulation of growth and development has long been recognised (Millward 1978, Fielding 2013). The age-related decline in skeletal muscle mass has received increasing attention from the scientific community in recent years as evidenced by the exponential growth in publications illustrated in Chapter 1 of this thesis. The knowledge base with regard to the underlying mechanisms contributing to loss of lean tissue mass (LTM) with age is expanding. Older populations display a blunted muscle protein synthetic response to the ingestion of protein and/or amino acids (AA) relative to the younger population (Cuthbertson *et al* 2005, Guillet *et al* 2004, Katsanos *et al* 2005). This is termed anabolic resistance and may reside at the level of protein digestion, AA absorption, plasma AA availability, hormonal response, postprandial tissue perfusion and/or intracellular signalling proteins (Koopman and van Loon 2009). Evidence demonstrating the optimal source, timing, amount and composition of dietary protein for older populations has challenged current World Health Organisation (WHO) recommendations for adults, irrespective of age (i.e. 0.8 $g.kg^{-1}.d^{-1}$) (Bauer *et al* 2013). Healthy older populations can benefit from strategies designed to maintain/augment LTM thus slowing the decline in functional capacity and maintaining independent living for longer. Specifically the even distribution of daily protein intake over the three main meals of the day has been proposed as a strategy to counteract the age-related decline in LTM (Paddon-Jones *et al* 2008). Long term studies examining the effects of evenly distributed protein intakes are currently lacking.

The main findings of this thesis were:

- **Following invention, a study of the 3h post-prandial bioavailability and bioactivity of a milk based protein formulation in healthy 50-70y old population was conducted. Based on the extant literature, the time course and magnitude of post-prandial EAA, leucine and insulin response was shown to be consistent with that required to affect a positive net increase in muscle protein FSR. *In vivo* human microdialysis (MD) can be applied as a tool to further inform as to substrate bioavailability at the skeletal muscle tissue level.**
- **Following dietary analysis of the protein intake of 50-70y old healthy, community dwelling, men and women, a 24 week RCT examined the effect of adding the milk-based formulation as a supplement to the two smaller protein containing meals of the day, i.e breakfast and lunch. Compared to an isocaloric, non-protein control, subjects supplemented with milk**

protein showed a positive mean change in LTM. The mean net effect was +0.60 kg of LTM suggesting that the strategy adopted in this RCT could be effective in the management of the age-related decline in LTM. Following invention, a progressive, home based programme of PRT, was similarly studied within the RCT. The aim of this study was to investigate whether the nutrient effect of the milk-based protein supplement could be further enhanced by PRT. The resultant net mean change in LTM of +0.96 kg (FORM+PRT – MALT) was indicative of an enhanced effect, but the study was underpowered to detect a difference between the FORM and FORM + PRT treatment effect.

- **Open recruitment for the RCT resulted in an uneven distribution of men and women within the study cohort. Though the gender effect was factored into the analysis it must be recognised that the male participants responded to a greater degree in each of the study treatments (i.e. men in the control treatment lost more LTM than women and men in the experimental treatment gained more LTM than women).**

Milk protein is a potential option as a dietary supplement to offset age-related declines in LTM. Its AA composition, digestion kinetics, insulinotropic and antioxidant properties may counteract many of the mechanisms that influence age-related declines in LTM. Delivering an appropriate amount of milk protein in a suitable matrix to the ageing population is however challenging. The current program of work within our institution and others set about this task in conjunction with the leading dairy companies in Ireland through the Food for Health Ireland research program. Chapter 2 of this thesis describes the post-prandial plasma AA, glucose and insulin response to the ingestion of a milk protein formulation in healthy community dwelling 50-70y olds. Ingestion of 0.33 $g.kg^{-1}$ milk protein resulted in a doubling of the plasma EAA concentration and a trebling of the plasma leucine concentration during a three hour postprandial period. This study lacked a measurement of muscle protein FSR but based on knowledge from the extant literature it is reasonable to assume that the ingestion of milk protein resulted in the stimulation of FSR (Yang *et al* 2012). The fact that recent data suggest that there is no direct correlation between acute changes in FSR and increases in muscular hypertrophy lessen the impact of the absence of this measurement in this instance (Verhoeven *et al* 2009, Mitchell *et al* 2014). The inclusion of protein hydrolysates in the formulation based on their *in vitro* insulinotropic capacity was justified as the formulation has a high relative potency compared to isocaloric maltodextrin. This has important implications in light of the proposed age related

blunting in insulin mediated tissue perfusion. The rationale for inclusion of similar components in the formulation based on their *in vitro* antioxidant capacity is less clear due to the difficulties in translating *in vitro* observations into the *in vivo* setting in terms of measuring plasma anti-oxidant capacity (Power 2013).

The milk protein formulation and the isocaloric control maltodextrin were fed in isolation during this study. During the subsequent human intervention study the milk protein and control formulations were fed in conjunction with the two smaller meals of the day. Addition of other macro and micronutrients could influence the digestion and absorption kinetics of the milk protein formulation. We are however more knowledgeable of the 'normal' per meal protein intake (in conjunction with other macronutrients) as a result of the dietary record data collected and analysed during the 24-week intervention study. Future work could involve utilising these data to investigate the postprandial change in plasma AA concentration in response to supplementation of the normal breakfast, afternoon and/or evening meal with additional protein.

Chapter 3 of this thesis described the application of *in vivo* microdialysis to the study of muscle protein metabolism in response to the resistance exercise and milk protein ingestion. Chapter 2 described the response to milk protein ingestion based on the concentration in venous plasma. These data did not however inform as to the bioavailability of substrate at the tissue level. MD was employed to assess its suitability as a technique that could provide concentration time profiles in response to perturbations such as nutrient ingestion and mechanical loading at the tissue level. MD has been applied to the study of muscle protein metabolism and ageing in Melinda Sheffield Moore's group in Galveston, Texas. Within this group they have applied MD in an AA infusion model in combination with endurance exercise (Durham *et al* 2010) and SNP infusion (Dillon *et al* 2011) focusing on micro-vascular blood flow and delivery of substrate to the muscle. As the technique was being established in our laboratory for the first time it was decided to utilise healthy male participants in this instance (greater muscle mass and reduced fat mass relative to older participants ensured that the catheters were easier to place in muscle during these initial experiments). Based on previous reports (Gutierrez *et al* 1999, Nordsborg *et al* 2003) it was decided to inset the MD catheters post-exercise. The 3h equilibration period facilitated subsidence of the damage response owing to catheter insertion and collection of baseline samples prior to ingestion of milk protein. It is pertinent to note that numerous reports in the literature state that the trauma response has reduced sufficiently to begin baseline sampling ~ 60 minutes post catheter insertion. Our albeit limited dataset suggest that this is too short a time period for the insertion related trauma to fully resolve based on the AA

biomarkers measured. A bout of unilateral RE undertaken prior to milk protein ingestion did not influence the dialysate AA concentration. The peak dialysate AA concentration was lower relative to plasma but the dialysate concentration remained higher for a longer period of time ensuring that the dialysate and plasma AUC were almost identical over the 4.5h post-prandial period.

The intervention study reported in Chapter 4 of this thesis challenged the hypothesis that summating the acute postprandial AA response to milk protein ingestion observed in Chapter 2 over a 24 week period, would result in at least the retention and possibly the augmentation of LTM in a group of healthy, community dwelling, 50-70y olds. Over a 24-week period there are approximately five hundred core eating occasions. Supplementation of the smaller of the two core meals of the day with milk protein increases the likelihood of surpassing the required thresholds for key signalling molecules, leucine for example, and stimulating net muscle protein synthesis at each of the 500 odd eating instances as oppose to 336 instances if two meals meet the proposed threshold signalling requirements and 168 instances if only one daily eating occasion meets these requirements. Participants were enrolled in the study through a policy of open recruitment. This led to a considerable gender bias in the study population with more than three times the number of women than men completing the 24-week intervention. A considerable number of participants failed to complete the intervention with many citing intolerance to the supplement as the reason for withdrawal. An important point to note with regard to this matter is that participants were blinded as to the group they had been assigned to. A fuller understanding of the nature of the intervention on behalf of the participants may have encouraged more to adhere to the program of supplementation for the full duration of the study.

While the average LTM response to milk protein supplementation was positive relative to the isocaloric maltodextrin control, considerable inter-individual variability was evident. This variability is consistent with previous intervention studies examining the change in LTM in response to protein and/or AA supplementation (Kukuljan *et al* 2009, Verdijk *et al* 2009, Verhoeven *et al* 2009, Dillon *et al* 2009). There are several reasons why the individual response varies to such a degree, including but not limited to, training status, habitual exercise training and physical activity levels, dietary macro/micro nutrient intake, general health and wellbeing, age and gender. Notwithstanding the variability in response the overall outcome was the offsetting of the age-related decline in LTM resulting from milk protein supplementation. This nutrient effect was complimented by a program of PRT, designed to be incorporated into normal daily activities of the participants without the requirement of a large amount of

equipment or a specialised space. The variability in terms of the change in LTM measured in response to milk protein supplementation was also evident in the supplementation + exercise group. One of the main limitations of this study is the lack of an exercise only branch to quantify the effect of PRT in isolation on LTM. Future work should involve the completion of this branch of the study. Due to the inherent limitations in dietary recording, the intervention effect could not be confirmed (i.e. that habitual protein intakes increased from ~ 1.2 $g.kg^{-1}.day^{-1}$ to 1.5 $g.kg^{-1}.day^{-1}$ in the protein supplemented treatments and that this intake was evenly distributed throughout the day). eFIR were deemed not to be appropriately sensitive to detect a change in protein intake as a result of the intervention.

5.2 Recommendations for future work

Based on the outcomes of this thesis there are several possibilities for future investigations in this area. Within the acute setting, many of the studies reporting changes in circulating concentrations of key regulators of MPS have been undertaken using food supplements as opposed to whole foods. Data investigating changes in postprandial AA concentrations in response to ingestion of representative breakfast, afternoon and evening meals would further inform as to the level of supplementation required to meet proposed thresholds for the stimulation of MPS. The influence of the typical macronutrient content of a mixed meal when ingested with a supplement is also worthy of further investigation. If, for example, the rate of gastric emptying is altered due to the fat content of a mixed meal, a supplement with sufficient leucine to meet the required threshold for stimulating MPS when fed in isolation may never reach that extracellular concentration when fed in conjunction with other macronutrients.

In vivo MD can be applied in a similar paradigm to investigate, at the tissue level, the change in postprandial AA concentration in response to ingestion of a mixed meal either in isolation or when supplemented with additional protein. MD also offers an intriguing proposition to explore an *ex vivo* model that has already been employed in other research groups (Hoffner *et al* 2003). Incubation of cells lines in muscle tissue dialysate facilitates examination of the effect of different nutrient and/or exercise interventions on outcomes such as cell proliferation and phosphorylation states of key signalling proteins such as the mTORC1 complex and its associated downstream targets. Use of the contralateral limb design employed in this thesis ensures that the nutrient and nutrient + exercise effect can be examined within the same participant during a single test day. As the cells would be incubated in dialysate from muscle

tissue they would be exposed to the physiological concentration of the bioactive(s) making the model relevant to the *in vivo* condition.

In the longer-term intervention trials, specific to the work that we have undertaken to date, the priority would be to establish the magnitude of the exercise stimulus in isolation and in combination with MALT supplementation. Application of the paradigm to specific subpopulations, older, frail and/or sarcopenic elderly for example is also desirable. Continuing the theme of the contralateral limb design from the acute studies, a future intervention study of considerable potential would involve a single leg training study combined with optimally timed nutrient ingestion. This could involve replicating the six-month intervention study reported in Chapter 4 of this thesis. However, in this instance, all participants undertake single-leg resistance exercise in addition to supplementation designed to evenly distribute daily protein intake. In this model, the resting contralateral limb responds to the nutrient stimulus while the exercising limb responds to the nutrient + exercise stimulus. The issue of variability in response to the intervention is also addressed in such a model as each individual acts as their own control.

This thesis marks a preliminary effort by our research group to investigate the effect of evenly distributing the daily protein load through supplementation with milk protein in offsetting the age-related decline in LTM. The proposed research outlined briefly above is attainable within our research group and should serve to advance the knowledgebase further in terms of establishing effective strategies to combat the age-related decline in LTM.

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Appendix A

Poster Presentations

Proceedings of the Nutrition Society (2011), **70** (OCE6), E357 doi:10.1017/S0029665111004423

Winter Meeting, 6–7 December 2011, 70th Anniversary: Body weight regulation – food, gut and brain signalling

A comparison of the insulinotropic and enterogastric response to ingestion of an equivalent quantity of maltodextran and whey protein

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In the absence of carbohydrate, the insulinotropic effect of whey protein ingestion induces a rise in plasma insulin and lowering of blood glucose during the first 30 min after ingestion (Power *et al.* 2009) [1]. It was hypothesized that the insulinotropic action of whey protein may, in part, be mediated via the secretion of the enterogastric hormones, represented by glucagon-like peptide-1 (GLP-1).

The present paper reports on the post-prandial response of plasma glucose, insulin and GLP-1 to feeding maltodextran and an equivalent quantity of whey protein. With ethical approval and informed consent four young, healthy subjects ($n = 4$, age 25(2.8) y, BMI 21.8(1.1) kg/m^2) undertook a randomized trial of two treatments, either a protein (WHEY 0.33 g/kg, 10% w/v water) or maltodextran (MALT; 0.33 g/kg, 10% w/v water), each treatment separated by 5 days. Subjects fasted overnight (10 h) prior to participation. Serial blood samples were withdrawn prior to and post ingestion for a period of 2 h and batch analysed for glucose, insulin and total GLP-1. Data are presented as mean (SEM; $n = 4$). Area under the curve for plasma insulin or total GLP-1 (AUC $_{0-120}$) was calculated by trapezoidal integration. Difference in the mean response was analysed by paired Student's t-test.

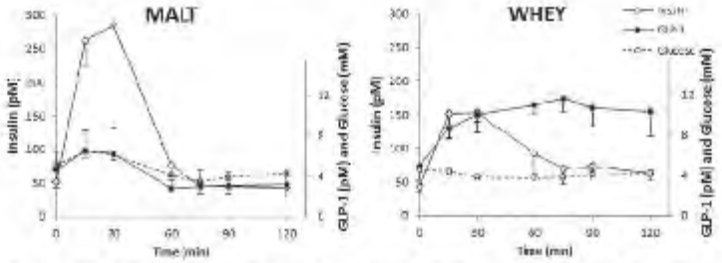

Figure 1. Mean plasma insulin, glucose and total GLP-1 for maltodextran (MALT) and whey protein concentrate (WHEY) during the post-prandial period. Values are mean (SEM; $n = 4$).

Following ingestion of the MALT, plasma insulin increased rapidly in the first 30 min and reached a maximal concentration (C_{max}) of 299(48) pM which directly correlated with the change in plasma glucose (Fig. 1). Ingestion of WHEY resulted in a C_{max} for plasma insulin of 182(23) pM within 30 min and was independent of a change in plasma glucose (Fig. 1). The differing insulin responses for MALT and WHEY were reflected in the AUC, 8506(2059) *vs.* 6512(1368) pM.2 h ($P = 0.29$). In contrast, ingestion of MALT gave a transient increase in total GLP-1 in the first 30 min and a C_{max} of 7.4(3) pM but ingestion of WHEY gave a prolonged increase with a C_{max} of 11.4(2) pM at 75 min. Total GLP-1 AUC was −64(40) vs. 536(102) pM.2 h ($P = 0.013$) for MALT and WHEY, respectively.

The enterogastric response, GLP-1, was greater following ingestion of an equivalent protein (WHEY) versus carbohydrate (MALT) load. This contrasts with previous reports that GLP-1 secretion is glucose dependent [2]. There was a shift in the time course of maximal plasma insulin and GLP-1 response and this data does not support the hypothesis that GLP-1 directly mediates the insulinotropic action of protein. The magnitude of the protein mediated insulin response can be augmented by hydrolysis of the protein [3]; therefore future studies are warranted to examine the effect of protein hydrolysis and those of peptide components upon GLP-1 secretion.

All protein ingredients were kindly supplied by Carbery Ingredients. Work described herin was supported by Enterprise Ireland under grant number CC20080001.

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A reference base for age-related decrease in lean tissue mass: whole body and segmental lean tissue mass of 1616 Irish men and women 18 to 76y

Ref No: ***H&WB-A.120:110511***

INTRODUCTION

Ageing is accompanied by a progressive loss of lean tissue mass. Sarcopenia defines the age-related loss in skeletal muscle mass estimated to affect ~30% of people older than 60y and >50% of those older than 80y. The magnitude of change in lean tissue mass (LTM) with ageing is referenced to race and gender specific healthy young adults[1]. The UL Body Composition Study[2] is a prospective study that seeks to establish age- and gender-specific reference ranges for whole-body and segmental body composition based on a convenience sample of the Irish population residing within the UL community. The present report provides a reference base for whole body and segmental lean tissue mass established from an analysis of 889 men and women aged 18 to 29y.

AIMS

- ***To describe whole body and segmental lean tissue mass in healthy young Irish adults aged 18-29 years***
- ***Using criteria derived from the young adult population, to investigate the prevalence of sarcopenia in a convenience sample of older adults, aged 50-76 years.***

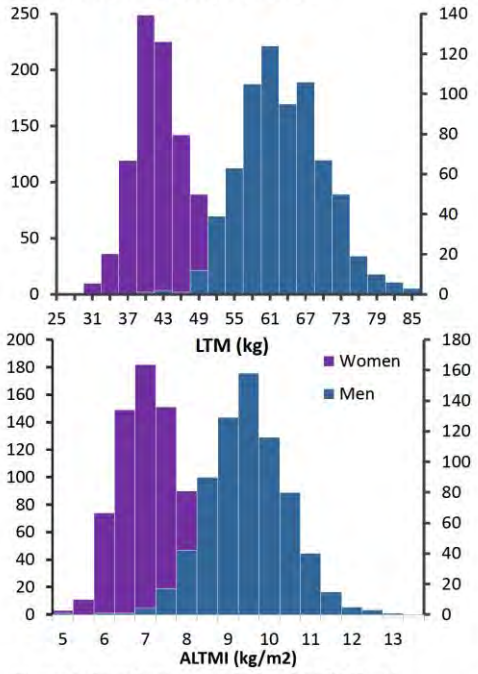

Figure 1. Distribution of LTM and ALTMI and prevalence of sarcopenia in men and women.

METHODS

With ethical approval (ULREC 08/07) and informed, written consent height, weight and body composition was measured by dual energy X-ray absorptiometry (DXA; iDXA™; GE Healthcare, Chalfont St Giles, Bucks., UK) in 497 men and 392 women aged 18-30y and an additional 194 men and 512 women aged 30-76y. Total body lean tissue mass (LTM) was segmented into appendicular (legs + arms) lean tissue mass and expressed relative to stature squared generating an appendicular lean tissue mass index (ALTMI; Table 1). Two criteria for defining sarcopenia were investigated; total body LTM and ALTMI.

Table 1. Descriptive Statistics of young adults [Mean (SD)]

Subjects	Age (y)	Height (m)	Mass (kg)	BMI (kg/m²)	LTM (kg)	ALTM (kg)	ALTMI (kg/m²)
Men (n=497)	22.3 (2.8)	1.80 (0.07)	80.6 (11.7)	24.8 (3.2)	62.4 (6.9)	30.3 (4.0)	9.3 (1.0)
Women (n=392)	22.4 (3.1)	1.67 (0.06)	64.6 (9.5)	23.2 (3.3)	41.6 (4.5)	19.0 (2.4)	6.8 (0.74)

RESULTS

The data revealed predicted sex specific differences in the distribution of total body (LTM) and appendicular lean tissue mass index (ALTMI; Figure 1).

Class I sarcopenia, defined as LTM or ALTMI < 1SD below the young adult mean, corresponded to an **LTM < 55.5kg or ALTMI < 8.3 kg/m² in men;**
an **LTM < 37.1kg or ALTMI < 6.1 kg/m² in women.**

Class II sarcopenia, defined as LTM or ALTMI > 2SD below the young adult mean, corresponded to an **LTM < 48.6kg or ALTMI < 7.3 kg/m² in men**;
an **LTM < 32.6kg or ALTMI < 5.4 kg/m² in women.**

Figure 1 and Table 2 demonstrate the prevalence of sarcopenia in older adults according to these criteria.

Table 2. Prevalence of sarcopenia in older adults aged 50-76 years

		Men (n=106)		Women (n=359)	
Criteria		50-60y	60-76y	50-60y	60-76y
LTM	Class I	29%	51%	23%	3%
	Class II	5%	6%	3%	4%
ALTMI	Class I	27%	32%	18%	18%
	Class II	2%	9%	1%	2%

CONCLUSION

The data from the young adult population establish reference criteria for sarcopenia in Irish men and women. These criteria can be applied to the general population to estimate the prevalence of age-related sarcopenia and to monitor the efficacy of strategies for prevention and/or treatment.

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2. Faculty of Education and Health Sciences (2009) University of Limerick – Body Composition Study

CARDI Conference, Dublin, 3-5th November 2011.

WP1 and WP8

Antioxidant Properties of a Nutrient Solution for Healthy Ageing

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Introduction

Peptides generated from digests of food proteins have been shown to possess AO activity[1]. Enzymatic hydrolysis has proven an effective means to release AO peptides from dairy proteins[2, 3]. The structure-function relationship and the mechanism of peptide induced antioxidant activity are yet to be fully elucidated. Peptides derived through hydrolysis have been shown to act as radical scavengers, reducing agents and metal chelators [4]. Preliminary work in this area suggests that the antioxidative activity of peptides is related to the amino acid composition[5] and the sequence of the peptide [6]. Ageing is associated with an increase in oxidative stress [7]. A nutrient solution rich in antioxidative peptides may prove beneficial to the ageing population.

Objectives:

- Establish a methodology to quantify *in vitro* antioxidant activity.
- Evaluate the *in vitro* antioxidant activity of dairy proteins.
- Select key ingredients and examine their *in vivo* antioxidant activity.
- Determine if a relationship exists between the *in vitro* and *in vivo* antioxidant response.

Expected outcome

Identification of key protein ingredients and their associated hydrolysates for inclusion in a nutrient solution for healthy ageing.

Methodology

In vitro antioxidant activity of whey protein ingredients was evaluated using the oxygen radical absorbance capacity assay[8] (ORAC; Figure 1). Test compounds were compared to a trolox reference standard and values expressed µmol.TE.100g of protein. All data are presented as mean (SEM; n=4) on two separate occasions.

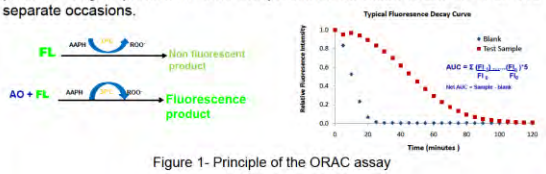

Figure 1- Principle of the ORAC assay

With ethical approval (EHSREC10- 34) and informed consent 5 young, healthy, adults (2 males, 3 females; Mean (SD) 25(3) yr, 1.72(6) m, 67(9) kg, 22.4(2) kg.m^{-2}) participated in a randomised intervention, each trial separated by a period of 3 d. Following an overnight fast, subjects ingested 0.33 g per kg BW of protein (intact/hydrolysed) or maltodextran (Table 1). Serial blood samples were withdrawn prior to and post ingestion for a period of 3 hr (Figure 2).

Table 1 – Composition of the test solutions

Composition	Intact	Formulation	Hydrolysate	Hydrolysate	Maltodextran
Test Protein	WPC 80	MPC 80 WPC DH 32 WPI DH 45	WPI DH 45	WPC DH 32	------
Flavour	Vanilla	Banana	Banana	Toffee Vanilla	Mango
Mean Protein (g)	22	22	22	22	------
Mean Energy (Kcal)	111	107	145	112	89
Mean EAA load (g dose)	10.9	10.2	8.8	11.3	------
ORAC Dose (umol.TE.mean dose)	2889	7935	16439	8360	0

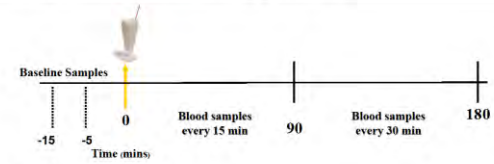

Figure 2 – Test day protocol

Results

Both intact and hydrolysed proteins have antioxidant activity *in vitro*. The nature of the protein alters the response. An intact whey protein had a lower activity than an intact milk protein concentrate. Enzymatic hydrolysis increased the *in vitro* antioxidant activity, between 2 and 7 fold (Figure 3).

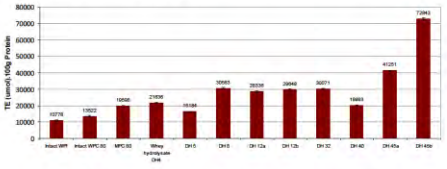

Figure 3 – *In vitro* antioxidant activity of dairy proteins and hydrolysates.

Increasing the degree of hydrolysis increases the antioxidant activity, but in a non linear manner (R^2 = 0.37; Figure 4). Therefore, the degree of hydrolysis is not the primary determinant of antioxidant activity; enzyme specificity, the peptides released and their amino acid composition are also important variables. Blending intact and hydrolysed proteins increases the antioxidative potency above that of the intact protein. The final WP8 formulation having and *in vitro* antioxidant activity 36,069 (665) µmol.TE.100g of protein (Figure 5) balances taste/palatability with bioactive potency making it suitable for a chronic human intervention study.

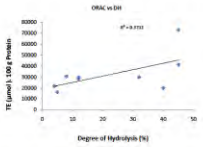

Figure 4 – ORAC score vs degree of hydrolysis

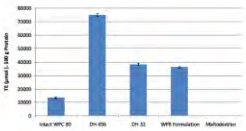

Figure 5 – Antioxidant activity of key ingredients and formulation

Future developments:

Additional analysis needs to be performed including:

- measurement of plasma antioxidant capacity using a range of *in vitro* assays.
- measurement of plasma amino acids

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WP8 and WP1

Insulinotrophic Properties of a Nutrient Solution for Healthy Ageing

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Introduction

Essential amino acids (EAA) are the primary regulators of proteins synthesis[1]. Thus making them an essential component of a nutrient formulation designed to limit sarcopenic losses. EAA's are also potent insulin secreatagogues [2].

Addition of protein to a carbohydrate load can synergistically increase the plasma insulin response[3]. The insulinotrophic actions of protein independent of carbohydrate are an important consideration. The insulinotrophic properties of protein are dependent on the nature of the protein [4] and the degree of hydrolysis of the protein [5]. A milk protein concentrate fortified with insulinotrophic hydrolysates has been specifically designed to meet the nutritional needs of the active ageing population.

Objectives:

- To design a dairy protein-based nutrient compound for a human intervention study.
- To select key ingredients based on their *in vitro* insulinotrophic properties.
- *In vivo* evaluation of the insulinotrophic properties of the key ingredients and final nutrient compound.

Expected outcome

Identification of key protein ingredients and their associated hydrolysates for inclusion in a nutrient solution for healthy ageing

Research strategy and methodology

Pancreatic BRIN BD11 beta cells were used to measure acute insulin secretion (Figure 1). Cells received hydrolysates at 1mg/ml in Krebs ringer buffer containing 16.7mM glucose for 20 minutes and insulin level was measured by ELISA (Mercodia). Alanine at 10mM was used as a positive control.

Figure 1 – *In Vitro* measurement of acute insulin secretion.

With ethical approval (EHSREC10- 34) and informed consent 5 young, healthy, adults (2 males, 3 females; Mean (SD) 25(3)yr, 1.72(6) m, 67(9) kg, 22.4(2) kg.m^{-2}) participated in a randomised intervention, each trial separated by a period of 3 d. Following an overnight fast, subjects ingested 0.33 g per kg BW of protein (intact/hydrolysed) or maltodextran (Table 1). Serial blood samples, were withdrawn prior to and post ingestion, for a period of 3 hr (Figure 2). Samples were batch analysed for glucose (EIA) and insulin (EIA). All data are presented as mean (SEM; n=4). The effect of the treatment on each variable was examined, relative to the intact protein and maltodextran control.

Table 1 – Composition of the test solutions

Composition	Intact	Formulation	Hydrolysate	Hydrolysate	Maltodextran
Test Protein	WPC 80	MPC 80 WPC DH 32 WPI DH 45	WPI DH 45	WPC DH 32	------
Flavour	Vanilla	Banana	Banana	Toffee Vanilla	Mango
Mean Protein (g)	22	22	22	22	------
Mean Energy (Kcal)	111	107	145	112	89
Mean EAA load (g.dose)	10.9	10.2	8.8	11.3	------

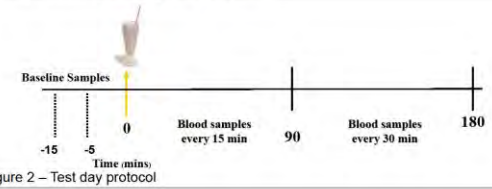

Figure 2 – Test day protocol

Results

In Vitro insulintrophic properties of hydrolysed whey protein (Figure 3) were evaluated to identify the most potent hydrolysates to be included in the final nutrient compound.

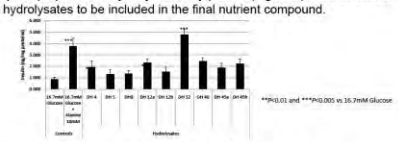

Figure 3 – *In vitro* insulinotrophic properties of hydrolysed whey protein.

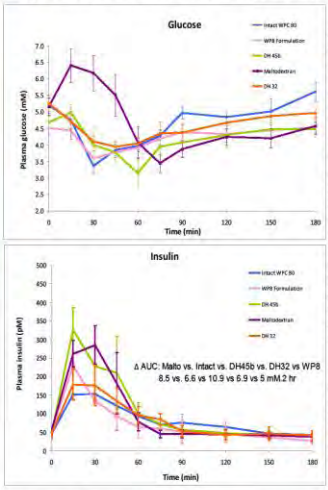

Figure 4– Plasma glucose and insulin responses.

The insulinotrophic properties of the nutrient compound, potent hydrolysates, and maltodextran (control) were evaluated *in vivo*. On ingestion of maltodextran the plasma glucose concentration increased rapidly, peaking within 15 min (Figure 4). Correspondingly, there was a large increase in plasma insulin over the first 60 min of the post-prandial period (Figure 4).

Following protein ingestion there was a decrease in the plasma glucose concentration over the first 60 min. All responses reached their lowest concentration at 60 min (in some subjects these were within the hypoglycaemic range) and returned after 120 min to near basal values. As plasma glucose concentrations decreased, plasma insulin increased peaking within 15-30 min. This insulinotrophic action following protein ingestion was not modulated by glucose. The magnitude and duration of the response was dependent on the nature of the protein substrate. In comparison to the intact protein, both hydrolysate (DH 32 and DH 45b) had a greater insulinotrophic action displaying ΔAUC per 120 min which were 5 and 65% higher, respectively. Compared to maltodextran (positive control) only the DH 45b had a greater insulinotrophic potency, ie. 37% greater Δ AUC per 120 min (Figure 4).

Future developments:

Additional analysis needs to be performed including:

- measurement of plasma amino acids.
- measurement of the incretin hormones, e.g., GIP and GLP-1.

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Appendix B

Papers under review.

An in vivo microdialysis characterization of the transient changes in the interstitial dialysate concentration of metabolites and cytokines in human skeletal muscle in response to insertion of a microdialysis probe (in review (Cytokine)).

William McCormack[1], Brian P. Carson[1], Clare Conway[1], John Cooke[2], Jean Saunders[3], William T. O'Connor[2] and Philip M. Jakeman[1].

1. Human Science Research Unit, Physical Education and Sport Sciences and 2. Graduate Entry Medical School (GEMS), Faculty of Education and Health Sciences and 3. Statistical Consulting Unit, University of Limerick, Limerick, Ireland.

Abstract

Skeletal muscle has recently been described as an endocrine organ, capable of releasing cytokines and regulators of metabolism. Microdialysis of the interstitial space of skeletal muscle enables analysis of the release of such cytokines. The purpose of this study was to determine the transient changes in concentration of metabolites and cytokines in human skeletal muscle in a 7h period following the insertion of a microdialysis probe. In total, sixteen microdialysis catheters were inserted into the vastus lateralis of male participants (Age 26.2 ± 1.35 y, Height 180.8 ± 3.89 cm, Mass 83.9 ± 3.86 kg, BMI 25.7 ± 0.87 $kg.m^{-2}$, Body fat 26.1 ± 3.0 %). Serial samples were analysed by micro-enzymatic and multiplexed immunoassay. Muscle interstitial glucose and lactate levels remained stable throughout, amino acid concentrations stabilized after 2.5h, however, insertion of a microdialysis catheter induced a 29-fold increase in peak IL-6 ($p<0.001$) and 35-fold increase in peak IL-8 concentrations ($p<0.001$) above basal levels 6h post insertion. In contrast to stable amino acid, glucose and lactate concentrations after 2h, commonly reported markers of tissue homeostasis in *in vivo* microdialysis, the multi-fold increase in IL-6 and IL-8 following insertion of a microdialysis catheter is indicative of a sustained disturbance of tissue homeostasis.

Key Words: Microdialysis Muscle Cytokine Interstitium

Appendix C

Papers in preparation.

Two site comparison of a liquid chromatography mass spectrometry, and a competitive binding assay for measurement of 25OH-Vitamin D.

Liam Blake[1], Paula O'Shea[1], William McCormack[2], Catherine Norton[2], Phil Jakeman[2]

1. Department of Clinical Biochemistry, Galway Roscommon University Hospitals and 2. Human Science Research Unit, Physical Education and Sport Sciences Department, Faculty of Education and Health Sciences, University of Limerick.

Abstract

Our two laboratories compared the results of 25OH Vitamin D measurements on serum samples using two analytical methods. Our objective was to compare these two methods in routine settings. Liquid chromatography/mass spectrometry (LC/MS) is used as the method of measuring 25OH Vitamin D in the hospital setting. The MassChrom 25-OH-Vitamin D3/D2 kit (Chromsystems Munich) is used with an Agilent 1290 UPLC and 6440 Triple Quad LC/MS to measure 25 OH Vitamin D3 and 25 OH Vitamin D2 in patients serum samples. The sum of these parameters is reported as 25 OH Vitamin D. On-line solid phase extraction after protein precipitation is employed. The Elecsys Vitamin D Total (Roche Diagnostics, Mannheim Germany) for measurement of 25 OH Vitamin D is used in the research laboratory. This assay employs a competitive test principle using recombinant Vitamin D Binding Protein (VDBP) as a capture protein to bind 25OH Vitamin D3 and 25OH Vitamin D2.

98 patient, and 97 study participant, serum samples were assayed at both sites. For all samples (n=195) the correlation coefficient was 0.94 between the assays. Passing Bablock regression analysis yielded a slope of 1.045; 95% confidence interval (CI) 1.00 – 1.03; intercept, 6.2 nmol/L (95% CI 9.38, 3.00). For samples with 25 OH Vitamin D2 and 25 OH Vitamin D3 detected (n=32) by the LC/MS method, the correlation coefficient was 0.92 between the assays. Passing Bablock regression analysis yielded a slope of 0.964 (95% CI 0.750 – 1.180); intercept, 7.5 nmol/L (95% CI 18.1, 3.3). For samples with only 25 OH Vitamin D3 detected by the LC/MS method (n=163), the correlation coefficient was 0.94 between the assays. Passing Bablock regression analysis yielded a slope of 1.043 (95% CI 1.000 – 1.103); intercept, 5.3 (95% CI 8.7, 3.0). When compared across two sites, the Roche Elecsys Vitamin D Total assay correlates well with the MassChrom 25-OH-Vitamin D3/D2 kit on the Agilent platform.

McCormack W., Power O., Jakeman, P. The effect of ingestion of milk protein on the post-prandial concentration of plasma amino acids, glucose, insulin and incretin hormones in healthy 18-25 and 50-70 year old women (in preparation).

McCormack, W, Toomey, C, Norton, C, Francis P, Jakeman, P. A randomised controlled trial investigating the effect of 24 weeks of milk protein supplementation on lean tissue mass in healthy 50-70 year olds (in preparation).

McCormack W., Cooke J., O'Connor, W, Jakeman, P. Change in intercellular amino acid concentration in response to milk protein ingestion and resistance exercise measured by in vivo microdialysis (in preparation).