

**Nutritional, pharmacological and hormonal manipulation of muscle  
metabolism**

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## **Declaration of Authorship**

I confirm that the production of this thesis and all work presented within it is of my own doing unless otherwise stated in the Section commencing on page 21.

Signed

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## **Abstract**

Sarcopenia and cachexia occur when there is a deficit between the rates of muscle protein synthesis and breakdown, such that a negative balance exists. In this thesis, the current literature regarding prevalence, pathophysiology and implications of muscle wasting is reviewed, and the following hypotheses are tested and discussed: 1) thalidomide, taken for 6 weeks, is superior to placebo in terms of weight gain in patients with oesophageal cancer and cachexia; 2) administration of high doses of insulin overcomes anabolic blunting in older human beings; and 3) the effect of a modest increase in blood insulin concentration upon suppression of leg protein breakdown is diminished in with ageing.

The data generated from three separate studies reveals that only the last of these hypotheses is likely to be true; nevertheless data obtained from the other two studies also adds to the existing literature. Specifically, patients with end-stage oesophageal cancer do not tolerate 200 mg per day of thalidomide nor does it carry any benefit in terms of acquisition of lean tissue or other control of disease-related symptoms, whereas aggressive medical and dietary support results in weight gain, including lean body mass, in the same patient group. Healthy volunteer studies using high dose insulin to overcome anabolic blunting in ageing were hampered by insufficient plasma AA concentrations; however blunted signalling responses remained despite equivocal rates of muscle protein turnover. Finally, the last study demonstrates a difference between young and older healthy volunteers in proteolytic responses to administration of modest doses of insulin.

This thesis reveals that deficits in protein turnover which occur with ageing are two-fold: both blunted anabolism and accelerated insulin-driven catabolism. Whether these defects are due to “insulin resistance” of muscle metabolism or other age related phenomena is unclear, although attempts to overcome anabolic blunting with supraphysiological insulin availability were unsuccessful.

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## **Involvement in work**

### *Protocol design*

The original version of Protocol 1 was designed entirely by Professor M.J. Rennie (MJR) and Doctor K. Smith (KS). Protocol 2 was developed with experimental work by the author on the basis of an original idea belonging to MJR. The RCT resulted from pilot work conducted by Doctor Z. Khan, who was also principally responsible for the protocol design.

### *Clinical studies*

The set up of new clinical facilities and conduct of all screening visits and acute studies were carried out by the author. Laboratory and/or clinical technical staff provided assistance during metabolic studies and some DEXA scans. REE measurements were performed by technical staff at Queen's Medical Centre, Nottingham. All clinical data collection, collation and analysis was performed by the author, including DEXA data analysis.

### *Laboratory work*

Glucose, insulin and cytokine measurements were carried out predominantly by the author; whereas plasma and muscle preparation and analysis for AA concentrations and labelling were conducted, for the most part, by laboratory scientists. Extraction of RNA and protein, gene analysis and western blotting were mostly carried out by the author, with support from laboratory post-doctoral staff.

## **Dissemination of Findings**

The data presented in this thesis has been presented at local, national and international meetings in both oral and poster format by the author. A book Chapter concerning skeletal muscle changes with ageing and nutrition was co-authored with MJR for a British Nutrition Foundation taskforce report (Wilkes & Rennie 2009). Three full manuscripts have been submitted to peer-reviewed journals, two of which have been published (appendices 1 and 2) and the other two are currently under consideration.

#### *Full manuscripts*

Wilkes EA & Rennie MJ (2009) Healthy Ageing: Skeletal Muscle. In: *Healthy Ageing: The Role of Nutrition and Lifestyle; The Report of a British Nutrition Foundation Task Force* (eds. Stanner S, Thompson R, & JL Buttriss), 1 edn Blackwells, London.

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*Abstracts*

Wilkes EA, Atherton PJ, Selby AL, Smith K, Rennie MJ, Cuthbertson DJ, Keller P, Pedersen BK (2006) Increased plasma TNF $\alpha$  together with higher cellular capacity for NF $\kappa$ B activation is associated with blunted eIF2 $\alpha$  dephosphorylation in response to amino acids (AA) in old people FASEB J. 20: A556

Wilkes EA, Khan Z, Freeman JG, Cole AT (2006) Does thalidomide prevent cachexia in oesophageal cancer? A randomized placebo controlled trial Gut; 55 (suppl V) A2

Wilkes EA, Atherton PJ, Patel R, Smith K, Rennie MJ (2007) Translational signalling in human muscle after hyperinsulinaemic hyperaminoacidaemic clamp in young and old men Life Sciences 2007 Proc Life Sciences, PC248

Wilkes EA, Selby A, Patel R, Rankin D, Smith K and Rennie MJ (2008) Blunting of insulin-mediated proteolysis in leg muscle of elderly subjects may contribute to age-related sarcopenia Proceedings of the Nutrition Society, 67 (OCE5) E153

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Finally, I would like to thank my family: my parents, for giving me the tenacity to finish; my husband, Neil, who supported me throughout the process and my daughter, Holly, who lost many mummy hours to the cause.

## **Chapter 1**

### **Introduction and literature review**

Skeletal muscle has a wide spectrum of functions ranging between the control of body position to blood glucose homeostasis. Ill-health and ageing deplete muscle mass, most noticeably leading to loss of strength, increased fatigueability and a decline in body stability, particularly at ankles, knees and hips, and thereby to an increased risk of falling and subsequent injuries (Roubenoff 2000; Wolfson *et al.* 1995). Severe sarcopenia increases the likelihood of developing a disability by 2-fold in men and 3-fold in women (Janssen, Heymsfield, & Ross 2002) and doubles the odds of dying (Cawthon *et al.* 2007). The subsequent costs of age-related wasting alone have been calculated to be of the order of \$18.5 billion in the USA (Janssen *et al.* 2004b), i.e. an excess cost of \$900 per individual (at 2003 prices). The true cost is likely to be greater still as these figures do not take into account the added healthcare costs associated with non-musculo-skeletal diseases in wasted individuals. Post-operative hospitalisation and complications, including pneumonia, are increased in patients with evidence of muscle wasting (Windsor & Hill 1988; Warnold & Lundholm 1984); diminished grip strength, which correlates with loss of lean tissue loss (Heimbürger *et al.* 2000), is associated with increased mortality due to heart disease, stroke, pneumonia and cancer (Gale *et al.* 2007; Sasaki *et al.* 2007). Thus preservation of muscle tissue is both an important preventative health measure and economic measure affecting the individual and society as a whole. A review of published data pertaining to muscle wasting, specifically exploring the underlying physiological processes by which muscle is maintained and lost, is presented in this thesis. The effects of amino acids (AA), and insulin upon muscle turnover in young and older human beings are explored, in addition to an evaluation of the role of thalidomide in cancer cachexia.

## 1.1 The role of muscle in health

The principal functions of skeletal muscle are to enable the body to maintain posture and to move, both of which depend upon an adequate mass of appropriately composed muscle and a properly functioning motor-control system. However, muscle also has many metabolic functions.

Skeletal muscle is probably the most important tissue for insulin-dependent glucose disposal in the body (Shulman 2000). The processes of glucose transport, glycogen synthesis and glucose oxidation all appear to be vulnerable to deterioration with ageing (Krishnan, Evans, & Kirwan 2003; Houmard *et al.* 1995; Gumbiner *et al.* 1992) and disease (Shulman 2000), resulting in hyperglycaemia and insulin resistance or insensitivity. Together with the heart, skeletal muscle accounts for most of the fatty acid oxidation in the body which, if diminished or inefficient, is associated with glucose intolerance and insulin resistance (Guo 2007). Thus loss of muscle has significant implications upon glucose homeostasis.

Muscle also contains 35 % of all total body protein (Cohn *et al.* 1980), which is maintained by a continual process of protein synthesis and breakdown accounting for between a quarter and a half of all protein turnover in the body, depending on the state of feeding (Tessari *et al.* 1996; Wagenmakers 1999). Amino acids released during protein turnover are used elsewhere in the body for wound healing or, in the cases of the amino acid glutamine, (which is mainly synthesized in muscle from glutamate) as a fuel for rapidly turning-over cells, including those of the immune system. Glutamine also provides a substrate for nucleic acid base synthesis and is a source of ammonia for acid-base regulation (Taylor & Curthoys 2004). Branched chain amino acids

(BCAA) also function as fuels for cardiac and skeletal muscle (although contributing no more than ~15% to the total fuel requirements during exercise (Rennie *et al.* 2006)). Glycogen is also stored in muscle and used locally as an energy source or, after glycolysis and conversion to lactate, exported for use by the heart or brain directly and for gluconeogenesis to produce glucose. Muscle is also important in the metabolism of BCAA (which are toxic if accumulated in the blood) to produce keto acids which are themselves used as fuel in a number of tissues and as a substrate for glutamine and alanine synthesis, both of which are gluconeogenic precursors.

Finally there is emerging evidence that muscle may have a humeral role, expressing interleukin-6 (IL-6), interleukin-8 (IL-8) and interleukin-15 (IL-15) (Pedersen *et al.* 2007). These cytokines act both locally and systemically and could plausibly be associated with the reduction in cancer risk associated with increased exercise and muscle mass. Thus, maintenance of an adequate skeletal mass is not only vitally important for locomotion but also blood sugar control, fat and protein homeostasis and immune functioning, to enable longevity and good health.

## **1.2 Means of measuring muscle mass**

Ideally, the diagnostic criteria for muscle wasting should be based upon knowledge of the normal distribution across all relevant ethnic populations of actual muscle mass, measured using techniques which are accurate, precise and reproducible – and if possible cheap and easily clinically applicable. A major problem is that no existent method measures the mass of skeletal muscle cells *per se*; all use model based quantification of a structural component of muscle, or a product of muscle metabolism, such as phosphocreatine. The degree of accuracy is model dependent although the

best method, magnetic resonance imaging (MRI), is validated against anatomical analysis of cadavers and is associated with errors of as little as 2 % (Engstrom *et al.* 1991; Mitsiopoulos *et al.* 1998). The inherent errors of each technique are likely to increase with greater degrees of wasting, and to a greater extent in methods validated indirectly e.g. impedance.

A further difficulty is that mass of muscle, determined by whatever means, does not necessarily predict *functional* muscle mass. Alterations in muscle fibre components (e.g. actin and myosin amounts and isoforms) and myofibre function, or metabolic characteristics (e.g. creatine phosphate content or mitochondrial oxidative capacity) are not given by any of the imaging modalities in current use. For a true composite picture, serial muscle biopsies, metabolic studies and muscle mass estimations are required; however recurrent invasive procedures are both costly and deter otherwise enthusiastic volunteers, limiting widespread applicability and limiting most studies to a single modality.

Most workers use lean body mass (LBM) (or fat free mass – terms often used interchangeably as they both reflect all mass which is not bone mineral or non-essential fat mass (Wang 1992)) as a proxy measure to quantify muscle mass. LBM is calculated by subtracting fat and bone mineral mass from total body mass (except when measured directly by whole body <sup>40</sup>potassium counting) and includes both intracellular and extracellular water. In health extracellular water remains relatively constant; however in disease states the proportion of interstitial water increases and limits the interpretation of changes in LBM. Nevertheless techniques that measure



muscle mass directly, i.e. MRI and CT, are limited by their expense or radiation exposure.

Notwithstanding financial constraints, MRI is currently the optimal technique, providing accurate, sensitive and reproducible values. The technique is safe for both investigators and participants and can yield muscle fibre type composition data if required (Houmard, Smith, & Jendrasiak 1995). Patient tolerability remains a limitation and increasingly individuals cannot be scanned due to implanted devices or clips. Conventional MRI incurs a cost of ~£300 per investigation in most NHS establishments; however the development of fixed magnet low field (0.2 Tesla) scanners means that MRI is becoming accessible to research units (Morse *et al.* 2005).

A slightly less expensive alternative to collect near gold-standard data, incurring errors of up to 6 % (Ross & Janssen 2005), is computerized tomography (CT). Radiation risk precludes serial measurements in healthy volunteers; but CT is used to stage and monitor disease progression in clinical practice and therefore a wealth of body composition data exists that has not been fully exploited. A major limitation is the absence of body composition software, so data manipulation remains a manual process.

Dual energy X-ray absorptiometry (DEXA) has been developed as an operator friendly tool for the assessment of body composition, with very simple and easy to use software. It is much cheaper than MRI (~ £50 per investigation), but it is less useful in imaging muscle tissue partly because muscle is relatively X-ray transparent and

complicated calculations need to be used to “subtract” for bone and fat masses; fluid retention also increases LBM but not fat (Humphries *et al.* 2000) which is of relevance when studying disease or treatment related changes in body composition. DEXA has a further disadvantage that it cannot be used tomographically i.e. no cross sectional images can be produced; only integrated plan views of density of a volume. Furthermore the errors for muscle or appendicular lean tissue are greater than for MRI.

Since the 1960s a limited number of specialist research units (approximately 30 worldwide) have used whole body <sup>40</sup>potassium (<sup>40</sup>K) counting as a measure of LBM (Forbes & Hursh 1961). In the human body potassium is an intracellular cation existing almost entirely within fat free tissue, thus estimates of total body potassium (TBK) correlate directly LBM. Potassium abundance in tissue is readily determined due to the presence of its radioactive isotope, <sup>40</sup>K, (~0.0118 % of TBK), which is detectable by gamma-ray emissions. At one time this technique was considered a “gold-standard” (Pierson, Lin, & Phillips 1974), providing a direct measure of LBM without the need to fast or exposure to radiation, in a patient-friendly environment. The availability of specialized facilities remains a major limitation of TBK counting, as is the lack of regional body composition data.

An alternative, older, technique (which also lacks regional body composition data) is the use of “heavy” water to quantify total body water (Schloerb *et al.* 1950; Lifson N, Gordon GB, & McClintock R 1955). Either deuterium oxide (D<sub>2</sub>O) or 18-oxygen water (H<sub>2</sub><sup>18</sup>O) is used as a tracer and administered after a baseline fluid sample is collected. Isotope dilution is measured in saliva, blood or urine after an equilibration period, as

water is distributed throughout all parts of the body except adipose tissue. Total body water can be used to estimate LBM using the simple equation:

$$\text{LBM} = \text{Total Body Water} / 0.72$$

Both tracers overestimate total body water as a result of binding to non-exchangeable sites, such as acidic amino acids, thus a correction factor is applied to minimise error. Albeit safe, easy to perform, and relatively accurate, use of this technique is limited due to its expense - both the isotope and analysis costs are high.

An inexpensive alternative is bioelectrical impedance analysis (BIA). This technique uses the differential electrical impedance of fat and lean tissue to estimate LBM. Development of multiple frequency algorithms have improved the validity of BIA (Rubiano, Nunez, & Heymsfield 2000), but substantially greater imprecision and inaccuracy persist with this method, particularly in the presence of electrolyte or fluid depletion, in comparison with other methods. Nevertheless BIA is cheap, portable and easily employed in epidemiological studies by relatively untrained staff.

These advantages are also shared by techniques measuring muscle metabolites in urine. Here muscle mass is estimated based on the excretion of creatinine or 3-methyl histidine (on the assumption that they are derived from muscle). A high degree of subject compliance is needed for both adherence to a strict meat free diet and complete 24 h urine collection. Accuracy of 3-methyl histidine as a marker of muscle

mass is a problem when total body mass is low, because of the proportionally greater contribution of 3-methyl histidine produced from the skin and smooth muscle in the gut (Afting *et al.* 1981; Rennie & Millward 1983). Furthermore, creatinine, when used as a measure of muscle mass in older adults, gives higher values than DEXA or deuterated water dilution (Proctor *et al.* 1999). Novel data demonstrates a tighter correlation between plasma myoglobin and the cross-sectional area of the quadriceps or body cell mass, as quantified by MRI and BIA respectively, when compared to creatinine values (Weber *et al.* 2007). The ease of measurement and reproducibility of this latest method suggest it may be utilized more widely in the future.

Despite the variety of techniques available to measure body composition, only relatively small numbers of the population (especially of sex and ethnic specific subgroups) have been examined by any method and even fewer have been measured using “near gold standard” methods such as MRI or CT. It has been calculated that in order to construct percentile charts of muscle mass at 5 y intervals that would be accurate and clinically useful, about 300 - 400 people of any particular group at each 5 y point would be needed (Guo *et al.* 2000). The biggest sample size of healthy adults examined so far with a reliable method is of the order of 1800 older adults with a mean age of 74 years (Goodpaster *et al.* 2006); very little data is published regarding the body composition changes associated with disease.

Table 1.1 Comparison of body composition measurement techniques

	<b>Benefits</b>	<b>Limitations</b>
<b>MRI</b> <b>Magnetic Resonance Imaging</b>	<ul style="list-style-type: none"> <li>• Gold-standard (cadaveric anatomical validation) for accuracy and precision</li> <li>• No radiation exposure</li> <li>• Direct measurement of skeletal muscle mass is possible</li> <li>• Low field MRI (for limbs only) is near perfect, much less expensive and easy to operate.</li> </ul>	<ul style="list-style-type: none"> <li>• Expensive (~£300 per scan) due to high initial capital costs (~£200,000), maintenance and service costs</li> <li>• Highly trained technical staff required and no standard software for body composition analysis</li> <li>• Subject acceptability - noise and claustrophobia.</li> <li>• Excludes subjects with implanted metal work</li> </ul>
<b>DEXA</b> <b>Dual Emission X-Ray Absorptiometry</b>	<ul style="list-style-type: none"> <li>• Reasonable precision and accuracy (within 1.5 and 4 % respectively for LBM)</li> <li>• Particularly useful for longitudinal change</li> <li>• Less expensive than MRI and CT (~£50 per scan)</li> <li>• Standard software for body composition analysis available</li> <li>• Minimal radiation exposure</li> <li>• Minimal operator training required.</li> </ul>	<ul style="list-style-type: none"> <li>• High initial capital costs (£60 000)</li> <li>• LBM is determined indirectly based on adipose and bone mass and thus is affected by hydration status and intramyocellular fat.</li> <li>• Skeletal muscle mass is not determined</li> <li>• No tomographic (i.e. cross sectional) information</li> </ul>
<b><sup>40</sup>K -whole body potassium counting</b>	<ul style="list-style-type: none"> <li>• Good precision and accuracy for LBM and skeletal mass, validated against MRI and cadaveric analysis</li> <li>• Direct quantification of LBM</li> <li>• No radiation exposure (<sup>40</sup>K occurs naturally within the body)</li> <li>• Subject friendly - fasting is not required</li> </ul>	<ul style="list-style-type: none"> <li>• Dedicated facilities required</li> <li>• Skeletal muscle mass is estimated using the ratio of total body potassium content to that in skeletal muscle - a relatively stable index in healthy adults, but not fully validated in wasting disorders</li> </ul>

<b>CT</b>  <b>Computer Tomography</b>	<ul style="list-style-type: none"> <li>• Good precision and accuracy</li> <li>• Direct measurement of anatomical skeletal muscle mass is possible</li> <li>• Routinely used in clinical practice.</li> </ul>	<ul style="list-style-type: none"> <li>• Substantial radiation exposure</li> <li>• Expensive but running costs less than MRI</li> <li>• Highly trained technical staff required and no standard software for body composition analysis</li> </ul>
<b>Urinary Excretion of 3-Methylhistidine (3-MH)</b>	<ul style="list-style-type: none"> <li>• Inexpensive</li> <li>• Home urine collections enable recruitment of housebound subjects</li> <li>• In healthy subjects, predominantly measures muscle mass</li> </ul>	<ul style="list-style-type: none"> <li>• Relies upon subject compliance to meat-free diet for <math>\geq 72</math> h and complete 24 h urine collection</li> <li>• Insensitive measure (CV <math>\sim 5\%</math> with good compliance)</li> <li>• Inaccuracies amplified when body mass low (greater contribution of 3-MH from gut and skin)</li> </ul>
<b>BIA Bioelectrical impedance analysis</b>	<ul style="list-style-type: none"> <li>• No radiation exposure</li> <li>• Portable</li> <li>• Inexpensive equipment</li> <li>• Minimal operator training required</li> </ul>	<ul style="list-style-type: none"> <li>• Precision and accuracy is poor</li> <li>• Very dependent upon hydration status</li> <li>• Pre-test exercise affects measurements</li> <li>• LBM overestimation in obesity</li> <li>• Few validations in ageing or disease populations</li> </ul>
<b>Total body water by isotope dilution - deuterium oxide (D<sub>2</sub>O) or heavy water (H<sub>2</sub><sup>18</sup>O)</b>	<ul style="list-style-type: none"> <li>• Accuracy and precision of total body water estimates are reasonable (<math>&lt; 1\%</math> and <math>&lt; 4\%</math> respectively).</li> <li>• No radiation</li> </ul>	<ul style="list-style-type: none"> <li>• Expensive (isotope and analysis costs)</li> <li>• LBM is an estimate based on a constant distribution of water in LBM</li> <li>• H<sub>2</sub><sup>18</sup>O overestimates total body water by <math>\sim 1\%</math>, as it can bind to non-exchangeable sites such as acidic amino acids, and D<sub>2</sub>O by <math>5\%</math> as take up into lipid.</li> <li>• Typical protocols take up to 6 h for complete equilibrium.</li> </ul>

## 1.3 Maintenance of muscle mass

### 1.3.1 Control of muscle protein turnover

Although the pathophysiology of muscle wasting, whether due to ageing or disease, or a combination of both processes, is complex and only partly understood, it is clear that changes in muscle protein turnover are required for alterations in muscle mass to occur. Many hormonal, metabolic and physical stimuli influence muscle protein synthesis (MPS) and muscle protein breakdown (MPB) to generate a net balance; a positive net balance results in muscle growth whereas muscle is lost if the balance is negative. For the most part MPS governs gradual changes in muscle mass (facilitative role) and MPB adapts in response to preserve tissue (adaptive role) (Rennie *et al.* 1983). The reverse is true of conditions resulting in rapid muscle loss, such as burns, trauma and sepsis; in these situations net muscle loss occurs due to accelerated MPB albeit with increased MPS, but to a lesser degree (Rennie 1985) (Figure 1.1).

In health, there is a diurnal change in muscle turnover. In the postabsorptive state (PA; i.e. following a 12 h fast) MPB exceeds MPS. During absorptive periods both rates increase, albeit MPS increases greater than MPB. Typical rates of muscle synthesis, after an overnight fast, expressed as tissue fractional synthetic rate (FSR; i.e. rate of protein increase proportional to the total muscle protein mass) vary between 0.04 and 0.08  $\% \cdot h^{-1}$  for mixed muscle protein depending upon the stable isotope tracer, the precursor estimate, analysis techniques and theoretical model used (Smith, Villareal, & Mittendorfer 2007); measurements taken with leucine are ~20 % greater than that using phenylalanine, regardless of the position of the labelled element. Typical values for mixed muscle, sarcoplasmic and myofibrillar protein synthesis in the PA state,

quantified using [ $^{13}\text{C}$ ]leucine and methodology consistent with that presented in this thesis, are 0.05, 0.06 and 0.03  $\% \cdot \text{h}^{-1}$ , respectively (Cuthbertson *et al.* 2005).

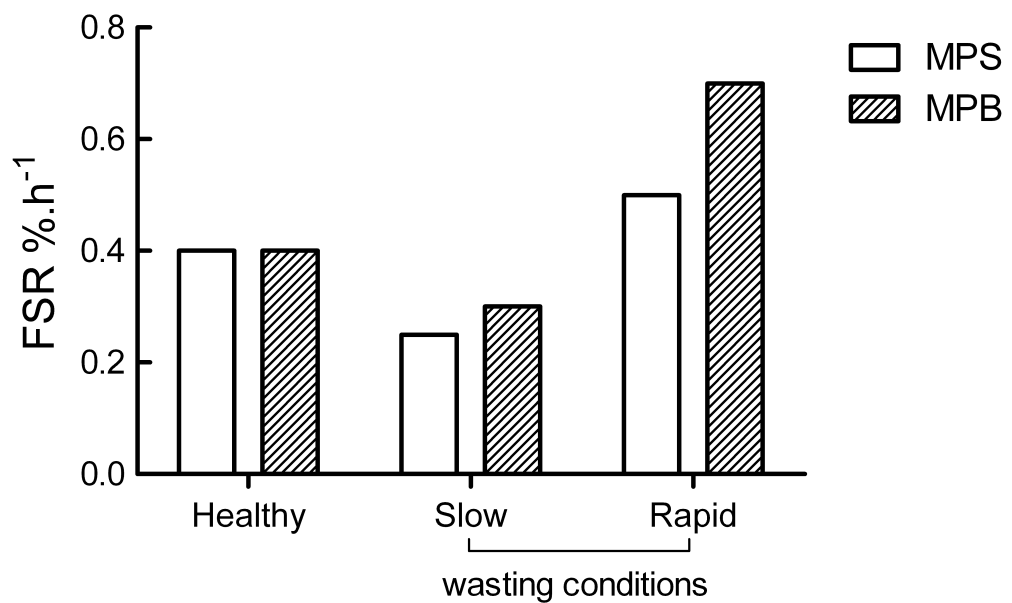


Figure 1.1 Representative values of skeletal muscle fractional synthetic rate (FSR) averaged over a 24 h period, assuming normal dietary intake, in healthy individuals and those with slow and rapid wasting conditions.



*Influence of amino acids upon muscle turnover*

In health and disease the balance of muscle protein turnover is controlled by a host of anabolic and catabolic stimuli, of which the most powerful anabolic agents are AA (Bennet *et al.* 1989; Rennie *et al.* 1982), specifically the essential AA (EAA) (Smith *et al.* 1992; Tipton *et al.* 1999; Volpi *et al.* 2003). Indeed there is some evidence suggesting that BCAA, particularly leucine, are sufficient to stimulate MPS independently, regardless of the availability of other AA (Smith *et al.* 1992; Smith *et al.* 1998; Kimball *et al.* 1999). Nevertheless all EAA are required to sustain muscle growth: intracellular EAA fall by as much as 50 % over a 90 minute period in the absence of continued provision.

The anabolic effect of EAA upon FSR correlates with extracellular availability (Bohé *et al.* 2003), although there appears to be a latency period of ~30 min before synthesis is increased (Bohé *et al.* 2001). A 2-3 fold increase in MPS results from a 50 % rise in EAA availability (Smith & Rennie 1996), but MPS returns to basal levels after ~2.5 h despite continued EAA availability (Bohé *et al.* 2001). The length of the ensuing refractory period, during which EAA administration does not result in accelerated MPS, is unknown. However this 2.5 h anabolic window is in keeping with the physiological changes in AA availability following a meal, in which AA typically return to basal levels by 3 h (Bergström, Furst, & Vinnars 1990).

The anabolic effect of AA appears restricted to synthesis; increased AA availability has no effect upon MPB (Biolo *et al.* 1997; Volpi *et al.* 1999; Volpi *et al.* 2003; Bennet *et al.* 1990b). Reports of increased MPB following AA administration have been published but the authors were unable to demonstrate a concomitant increase in MPS suggesting

flaws in the data (Nair, Schwartz, & Welle 1992; Louard, Barrett, & Gelfand 1990). Other workers have suggested that AA can suppress proteolysis but their data was generated by administering both AA and glucose without insulin clamping and thus the observed effect upon proteolysis is better explained by the accompanying insulin rise (Volpi *et al.* 2000). The effects of insulin upon proteolysis are discussed subsequently.

#### *Influence of exercise upon muscle turnover*

After AA, exercise is probably the most important anabolic stimulus for skeletal MPS. High-force contractions (resistance exercise) increase MPS by 50 – 100 % of basal rates, albeit with a simultaneous increase (~50 %) in MPB. Less is known about the effects of endurance exercise upon muscle turnover; walking (Carraro *et al.* 1990) and swimming (Tipton *et al.* 1996) increase MPS by 100 and 60 % respectively but there are no data on MPB. The amplitude of response to a bout of exercise appears to be influenced by duration, intensity and whether the muscle has been trained in that particular type of exercise. Indeed conflicting time course data is best explained by the variable study conditions and differences between human beings. Heavily trained muscle differs from that which is untrained in its response to resistance exercise (but not endurance exercise) in that FSR does not appear to increase (Tipton *et al.* 1996; Roy *et al.* 1997). Furthermore, the rate of anabolism is profoundly influenced by the availability of AA during and after exercise, although the time course of maximal anabolic responses with feeding is not fully understood. In the postabsorptive state maximal synthesis appears to occur between 1 and 2 h after resistance exercise (Kumar *et al.* 2009); however this may not hold true when exercise is accompanied by feeding.

FSR appears to be reduced during muscle contractions (Carraro *et al.* 1990; Dreyer *et al.* 2006) in keeping with whole body (Rennie *et al.* 1981) and rodent (Bylund-Fellenius *et al.* 1984) data, at least in the postabsorptive state. Recent data (Fujita *et al.* 2009a) suggests that feeding with a mix of AA and carbohydrates 1 h before RE attenuates the reduction in FSR; i.e. synthesis increased in the first hour with feeding but dropped during the exercise bout to rates similar to unexercised fasted muscle, whereas FSR fell below this rate during exercise in unfed control subjects. Signalling work also confirms that the phosphorylation of the eukaryotic elongation factor 2 (eEF2) at Thr56 (which slows protein synthesis) is increased by 5 – 7 fold within 1 min of exercise commencing (Rose *et al.* 2005). Conflicting data, collected during short bouts of exercise (~45 min) suggest either a rise (Sheffield-Moore *et al.* 2004) or no change in FSR (Durham *et al.* 2004); however, the analytical technique used in both studies, gas chromatography-mass spectrometry (GC-MS), is not sufficiently sensitive to accurately detect small changes in tracer enrichment within the muscle as occur over periods of less than 90 min.

Immediately after an exercise bout there is a “latency period” of variable duration, before accelerated synthesis is switched on and MPS rates increase by ~100 % (Chesley *et al.* 1992; Biolo *et al.* 1995). As the basal rate of MPB is greater than that of MPS when studied under PA conditions, and a simultaneous increase in MPB occurs (~50 %) (Biolo *et al.* 1995), net balance remains neutral (Figure 1.2). Thus, absorptive conditions are required to maximise the anabolic benefits of exercise. Amino acids and exercise synergistically increase MPS by up to ~2-3 times basal values (Louis *et al.* 2003; Cuthbertson *et al.* 2006) without a concurrent increase in MPB (Biolo *et al.* 1997) (possibly due to the rise in blood insulin concentrations), resulting in a positive net balance and accretion of muscle.

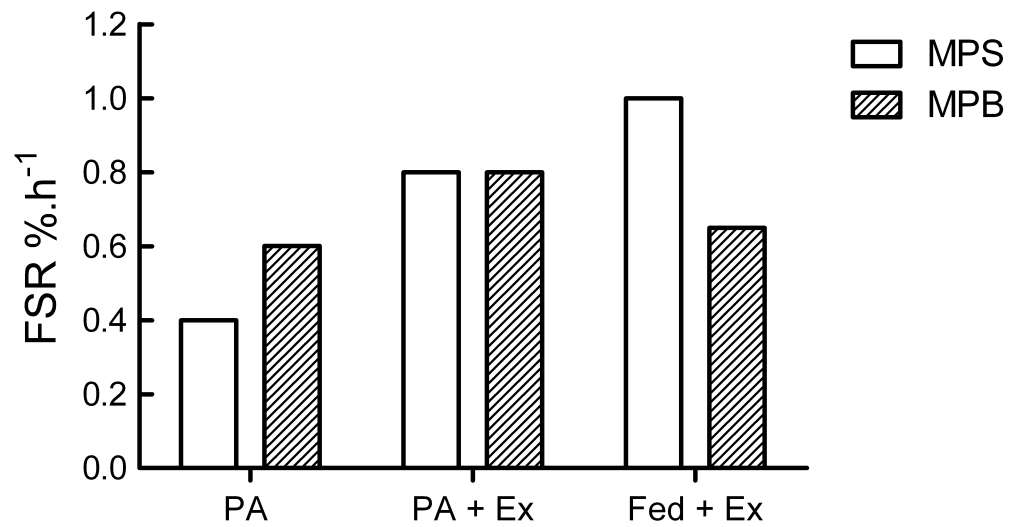


Figure 1.2 Muscle protein synthesis (MPS) and breakdown (MPB) rates during post-absorptive (PA) unexercised conditions, and in response to exercise (Ex) with and without feeding. Data are approximate and generated from the literature.

The state of enhanced anabolism following exercise lasts in the order of ~48 h (Phillips *et al.* 1997); however the latency period is less well defined. Some authors report maximal anabolic responses within 2 (Kumar *et al.* 2009), 3 (Louis *et al.* 2003) or 4 h (Biolo *et al.* 1997; Chesley *et al.* 1992; Tang *et al.* 2008), whereas others describe no synthetic response 3 h after extremely strenuous exercise, but maximal responses at 6 and 24 h (Cuthbertson *et al.* 2006), suggesting that the duration of the latency state may be related to the intensity of the exercise or availability of AA. Indeed data from habitually trained individuals, referred to earlier, indicated no change in FSR over 5 (Tipton *et al.* 1996) or 10 h (Roy *et al.* 1997) after a significant bout of resistance exercise (65 % and 85 % of 1 repetition maximum respectively), possibly due to a prolonged latency period; measurements were not made at latter time points to confirm this hypothesis.

Data regarding the optimum timing of AA feeding after exercise has been inconsistent, possibly due to marked differences between exercise protocols, the route and choice of nutrients, the degree of habitual activity of participants and activity levels immediately preceding the study. Nevertheless, early feeding after exercise appears to increase the maximal rate of protein synthesis (Levenhagen *et al.* 2001) and recently published data demonstrates a clear dose-response effect (up to a maximum dose of ~20 g of protein) (Moore *et al.* 2009). Robust DEXA data from two studies, each collected over a 12 w training programme, confirm that immediate protein supplementation after exercise results in greater accretion of LBM than feeding delayed by 2h or more (Hartman *et al.* 2007; Esmarck *et al.* 2001).

*Influence of insulin upon muscle turnover*

Insulin is a powerful anabolic stimulus, either accompanying nutrients, in isolation under experimental clamp conditions or after resistance exercise. Insulin promotes muscle “growth” primarily by inhibition of proteolysis and does so in a dose-dependent fashion (Chow *et al.* 2006; Moller-Loswick *et al.* 1994; Petrides, Luzi, & DeFronzo 1994; Louard *et al.* 1992; Arfvidsson *et al.* 1991; Fryburg *et al.* 1990; Flakoll *et al.* 1989; Gelfand & Barrett 1987; Tessari *et al.* 1986); although the maximal inhibitory dose is less clear. Gelfand and colleagues propose that maximal inhibition of proteolysis occurs at modest plasma insulin concentrations ( $\sim 30 \mu\text{IU}\cdot\text{ml}^{-1}$ ) (Louard *et al.* 1992; Gelfand & Barrett 1987; Pozefsky *et al.* 1969), whereas other workers suggest maximal inhibition occurs at concentrations exceeding the physiological range (upwards of  $400 \mu\text{IU}\cdot\text{ml}^{-1}$ ) providing that AA availability is maintained at postabsorptive concentrations (Flakoll *et al.* 1989; Tessari *et al.* 1986; Fukagawa *et al.* 1985).

Whether increased insulin concentrations stimulate MPS is controversial. Biolo and colleagues report unequivocally that MPS, as determined by both tracer incorporation and A-V balance, is stimulated by insulin even in the presence of relative hypoaminoacidaemia (Biolo, Fleming, & Wolfe 1995; Biolo *et al.* 1999). Other workers report a positive net balance when studying leucine and phenylalanine kinetics across the arm in response to hyperinsulinaemia; however phenylalanine kinetics indicate that anabolism is the result of reduced MPB, whereas leucine kinetics point towards enhanced MPS (Newman *et al.* 1994). More robust data demonstrates that human beings are almost insensitive to insulin in terms of stimulation of protein synthesis at PA concentrations of AA (Chow *et al.* 2006; Moller-Loswick *et al.* 1994; Gelfand & Barrett 1987) and that insulin has no summative effect upon EAA driven MPS

(Greenhaff *et al.* 2008). Nevertheless, insulin reduces leucine oxidation (Ang *et al.* 2000), increases blood flow to the tissue (Fujita *et al.* 2006; Biolo *et al.* 1999; Arvidsson *et al.* 1991) and may have a direct effect upon AA transport into the cell (Biolo, Fleming, & Wolfe 1995). Thus MPS may be enhanced under certain experimental conditions and not others due to variations in intracellular AA availability in response to hyperinsulinaemia, rather than a direct effect of insulin. Furthermore, some authors have suggested that low dose insulin may be a requirement for stimulation of MPS in response to hyperaminoacidaemia, a so called “permissive” role (Rennie 2005). Testing this hypothesis would require measurement of MPS in response to feeding with a full pancreatic clamp without exogenous replacement of insulin. No such study has been conducted to date.

### 1.3.2 Cellular control of protein synthesis

The rate of MPS is determined at the molecular level by the production and phosphorylation of eukaryotic initiation factors (eIF) which control the rate of protein translation. Translation, the process by which messenger ribonucleic acid (mRNA) is used to generate polypeptides, occurs within the cytoplasm in 3 stages: initiation, elongation and termination. The binding of eIF2·GTP·Met-tRNA<sub>i</sub><sup>met</sup> to the 40S ribosome forms the first step of initiation and is governed by the availability of eIF2·GTP (GTP: Guanosine triphosphate) complex. eIF2·GDP (GDP: Guanosine diphosphate) is in abundant supply; however phosphorylation to eIF2·GTP is catalyzed by eIF2B, an enzyme which is inhibited by eIF2 $\alpha$  and glycogen synthase kinase (GSK)  $\beta$ . Low AA concentrations and Protein Kinase B (PKB; also called Akt) inactivation promote eIF2 $\alpha$  and GSK3 $\beta$  activity. When AA supply is adequate eIF2·GTP·Met-tRNA<sub>i</sub><sup>met</sup> binding to the 40S ribosome creates the 43S pre-initiation complex.

mRNA binding to the 43S pre-initiation complex requires an assembly of 3 initiation factors, eIF4E, eIF4G and eIF4A, the assembly of which is termed eIF4F. Each component of eIF4F has an essential role: eIF4E binds to the mRNA cap structure, eIF4A unwinds secondary structures of the mRNA and eIF4G serves as a scaffold protein binding eIF4A, eIF4E and mRNA to the ribosome (Pain 1996). Here eIF4E is the rate limiting factor (Lang *et al.* 2003) and is regulated by eIF4E-binding protein 1 (4E-BP1). Increased EAA availability enhances 4E-BP1 hyperphosphorylation, rendering it inactive as an inhibitory binding protein and enabling eIF4E to enter the eIF4F complex. mRNA binding to the ribosome precipitates release of eIF4F and eIF2·GDP for recycling, leaving the active 80S initiation complex.

Once mRNA binds to the 80S ribosome, AA-charged transfer RNA (tRNA) are able to commence peptide formation. Transfer RNA are short nucleotide chains which selectively bind free AA to an anticodon at the 3' end. In the same way that three nucleotide bases form a codon within mRNA, forming a template for AA linkage, the anticodon consists of the opposite bases and dictates the AA specificity of each tRNA molecule. During elongation tRNA bound AA (aminoacyl-tRNA) are aligned and bonded together, in accordance with the mRNA code, to form a peptide. This elongation process requires a significant amount of metabolic energy and is aided by eukaryotic elongation factors (eEF) -1 and -2. eEF-1 facilitates aminoacyl-tRNA binding to the ribosome, whereas eEF2 is involved with movement of the peptidyl-tRNA through the ribosome (Proud 1994). eEF2 kinase, which phosphorylates and inactivates eEF2 appears to be negatively regulated by ribosomal protein S6 kinases (P70S6K), thus increased intracellular AA may also enhance elongation through mammalian target of rapamycin (mTOR) stimulation of P70S6K (Wang *et al.* 2001).



Stop codons within mRNA (UAA, UAG and UGA) are not recognized by tRNA and prevent further elongation, resulting in termination. Once this has occurred releasing factors detach the polypeptide chain from the ribosome whereupon post-translational modifications occur, including folding of the polypeptide into its tertiary protein structure.

### **1.3.3 Amino acid and insulin effects upon protein synthesis**

The intracellular signalling pathways by which AA control protein synthesis and breakdown are predominantly centred around mTOR and general control non-depressing kinase 2 (GCN2); whereas insulin control of protein metabolism - both facilitation of AA signalling and inhibition of breakdown – is governed by the activation of the PKB pathway (Figure 1.3).

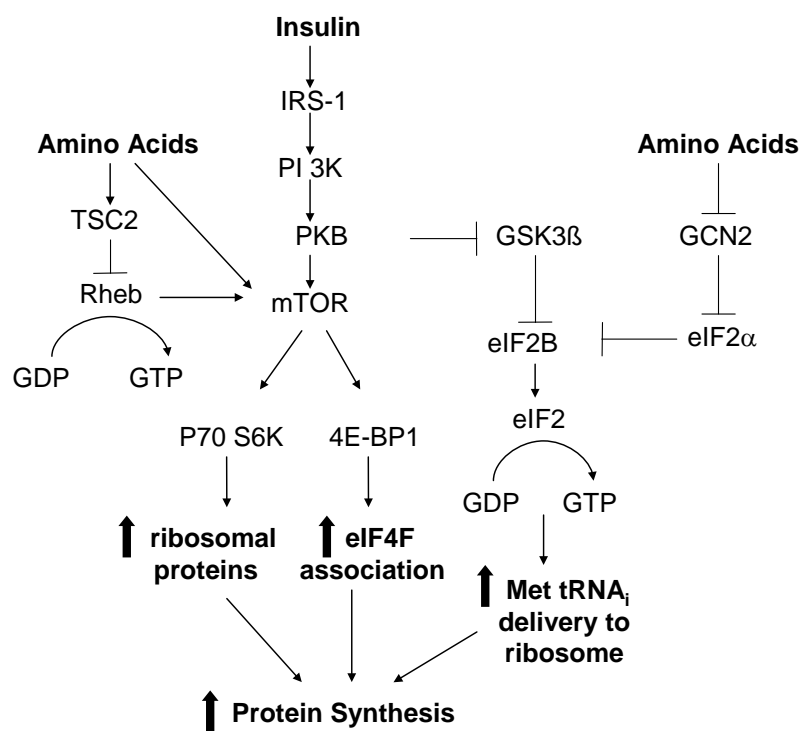


Figure 1.3 Interplay of AA and insulin signalling through protein kinase B and mammalian target of rapamycin. Inhibition is indicated by blunt ended lines.

*Mammalian target of rapamycin (mTOR)*

mTOR is a high molecular weight protein kinase which is particularly sensitive to the availability of AA, glucose and ATP (adenosine triphosphate) (Kim *et al.* 2003). It includes a 20 tandem HEAT (Huntingtin, eIF3, a subunit of protein phosphatase 2A, TOR) repeat sequence at the N terminus and a kinase domain at the C terminus. This kinase domain has structural similarity to the catalytic domain of phosphatidylinositol 3-kinase (PI3K) (which is involved in the production of phosphatidylinositol (3,4,5)-trisphosphate (PIP<sub>3</sub>) and PKB activation) and is responsible for the phosphorylation of 4E-BP1 and P70S6K (Hay & Sonenberg 2004).

Two stabilizing proteins facilitate the kinase activity of mTOR. The first, Raptor (Regulatory associated protein of TOR), binds avidly to the HEAT domains on mTOR and more weakly to the kinase domain; Raptor also binds directly to 4E-BP1 and P70S6K, facilitating phosphorylation (Kim *et al.* 2002a). The second stabilizing protein, G protein β-subunit-like protein (GβL), associates primarily with the kinase domain on mTOR, both enhancing its kinase activity and its ability to sense nutrients and recruit P70S6K and 4E-BP1 (Kim *et al.* 2003). Further AA sensing and signalling capabilities of the Raptor-mTOR-GβL complex result through its interaction with ras homologue enriched in brain (Rheb) and the tuberous sclerosis complexes (TSC-1 and TSC-2) (Bolster, Jefferson, & Kimball 2004; Gao *et al.* 2002; Pan *et al.* 2004; Saucedo *et al.* 2003).

The Raptor-mTOR-GβL complex, when fully activated by AA enhances protein translation by phosphorylation of p70S6K and 4E-BP1. Phosphorylation of 4E-BP1 at Thr37 and Thr46 by mTOR appears to have a priming function, allowing an additional

unknown protein kinase to phosphorylate 4E-BP1 at Ser65 and Thr70. In this hyperphosphorylated state 4E-BP1 dissociates from eIF4E, allowing eIF4E-eIF4G binding. P70S6K is also hyperphosphorylated in its active state; mTOR phosphorylation at Thr389 appears to prime this process for subsequent phosphorylation by 3-Phosphoinositide-dependent kinase-1 and-2 (PDK) amongst other kinases. Hyperphosphorylation of P70S6K enhances translation of a specific class of mRNAs that contain a 5'-terminal oligopyrimidine structure. Proteins encoded by these mRNAs include ribosomal proteins and translational elongation factors, hence cellular protein synthetic capacity is enhanced as a result of AA signalling through the Raptor-mTOR-GβL complex (Kimball, Farrell, & Jefferson 2002).

#### *General control non-depressing kinase 2 (GCN2) signalling*

In contrast to mTOR, which is activated in the presence AA, GCN2 senses and signals in response to AA deprivation. Under these conditions, uncharged tRNA (i.e. tRNA without bound amino acid) activates GCN2 resulting in phosphorylation of eIF-2 $\alpha$  at Ser51 (Dong *et al.* 2000; Sood *et al.* 2000). In the phosphorylated state, eIF-2 $\alpha$  inhibits eIF2B and subsequent formation of eIF2·GTP·Met-tRNA<sub>i</sub><sup>met</sup> which is required for first stage of translation initiation; furthermore, eIF-2 $\alpha$  phosphorylation enhances translation of genes encoding for GCN4, a transcriptional activator of over 40 genes which code for AA biosynthetic enzymes. Thus the AA-deprived cell is able to down-grade protein synthesis, and expresses genes which encode for novel AA synthesis (Hinnebusch 1997).

### 1.3.4 Role of proteolytic systems

The degradation of proteins is tightly controlled by at least 5 different proteolytic systems. These systems include lysosomal protein degradation (cathepsins), calpains, apoptotic proteases (caspases), matrix metalloproteinases and the ubiquitin proteasome pathway (Ventadour & Attaix 2006). The specific role of each of these systems in muscle protein breakdown is not completely understood, nor is it known whether up-regulation of different systems plays a role in muscle atrophy of different aetiologies. However, workers suggest that a sequential combination of two proteolytic pathways occur in atrophy states, involving either calpains or caspase-3 prior to ubiquitin degradation (Lecker, Goldberg, & Mitch 2006; Hasselgren & Fischer 2001; Jackman & Kandarian 2004; Bartoli & Richard 2005).

Three different calpains are in abundant supply within the muscle tissue, calpain-1 ( $\mu$  calpain), calpain-2 (m calpain) and calpain-3 (p94); their activity is governed by intracellular calcium concentrations, the abundance calpastatin (an inhibitory protein) and removal of the N-terminus (whether by autolysis, phosphorylation or association with the plasma membrane). Thus measurement of calpain activity is not a straightforward process. Nevertheless calpain-1 and -2 are thought to be involved in dissociation of the myofilaments from the z disc to enable subsequent ubiquitination. Calpain-1 and-2 may also impair regeneration of the myofibril, by amongst other mechanisms, inhibiting satellite cell regeneration. The role of calpain-3 is less clear, indeed there is some suggestion that calpain-3 inhibits muscle proteolysis (Bartoli & Richard 2005).

The caspases, specifically caspase-3, provide an alternative system for initiation of myofibril degradation prior to ubiquitination. Caspase-3 cleaves actinomyosin and in doing so produces a characteristic 14 kDa fragment of actin, which can be quantified as a marker of caspase-3 activity (Du *et al.* 2004).

The fragments of contractile proteins generated by either calpains or caspases are further degraded by the 26 S proteasome after tagging by ubiquitin proteins; it is this multi-step tagging process that ensures selectivity. Before tagging can occur, ubiquitin, a small protein co-factor, is activated by an ATP-dependent enzyme, E1. Upon activation, ubiquitin is transferred by E1 to a carrier protein, E2, which enables one of a family of ubiquitin-protein ligases (E3 ligases) to couple ubiquitin to the target protein. This process is repeated so that a chain of five or more ubiquitins attach to the substrate protein, targeting it for proteolysis by the proteasome. Specificity in this system is derived from the E2 carrier proteins and E3 ligases: specific E2-E3 pairings are required for ubiquitination of each target protein (Mitch & Goldberg 1996).

Animal work has identified two E3 ligases which are up-regulated in muscle atrophy states: Muscle RING Finger 1 (MuRF1) and Muscle Atrophy F-box (MAFbx; also known as atrogin-1). MuRF1 and MAFbx are ordinarily expressed in human cardiac and skeletal muscle, but expression is enhanced in a variety of rat atrophy models, including denervation, immobilization, unweighting, and glucocorticoid induced muscle wasting (Bodine *et al.* 2001). Observations in human beings with wasting disorders confirm increased proteasome expression following head injury (Mansoor *et al.* 1996), sepsis (Tiao *et al.* 1997), cancer (Williams *et al.* 1999) and AIDS (Llovera *et al.* 1998). Indeed, in cancer patients, expression of ubiquitin mRNA and 20 S proteasome

subunits were increased 2-3 fold even prior to the onset of overt muscle wasting (Williams *et al.* 1999).

Control of MuRF1 and MAFbx expression is governed by the Forkhead box Other (FOXO) family of transcription factors. Four mammalian isoforms have been identified to date: FOXO-1, FOXO-3a, FOXO-4 and FOXO-6 (Nader 2005). All four isoforms are involved in metabolic signalling, but specific functions of each are not clearly defined. Unphosphorylated, and thus active, FOXOs are located predominantly within the nucleus, bound to DNA, whereas phosphorylation by PKB results in nuclear exclusion. Re-activation of FOXOs by dephosphorylation and nuclear localization results from inactivation of PKB, thus providing a second theoretical mechanism by which insulin suppresses proteolysis. Nevertheless, the inhibitor of nuclear factor kappa B kinase (IKK), c-Jun-N-terminal kinase (JNK) and proteins associated with glucose metabolism have also been shown to alter FOXO activity (Barthel, Schmoll, & Unterman 2005).

Lysosomal proteolysis is driven by the cathepsins, of which cathepsin L is involved in breakdown of most myofibrillar proteins. Rat models of atrophy due to sepsis, cancer, glucocorticoids and disuse have been associated with over expression of cathepsin L (Deval *et al.* 2001; Taillandier *et al.* 1996); however its role in atrophy is not entirely clear. Cathepsin inhibition studies have failed to show a reduction in protein breakdown (Bechet *et al.* 2005) and as with other proteolytic enzymes, quantification of cathepsin mRNA does not directly correlate with lysosomal activity.

Understanding of intracellular signalling pathways which control muscle proteolysis is extremely limited as data is scarce, particular that collected in human beings. Both anabolic and catabolic stimuli are believed to regulate the ubiquitin-proteasome system, but the signalling pathways which control initiation of MPB, by either calpain or caspase-3 degradation of the myofibrillar apparatus, are unknown (Ventadour & Attaix 2006; Glass 2005).

#### **1.4 Determinants of muscle loss**

Involuntary loss of muscle tissue results from three separate processes: starvation, sarcopenia and cachexia. Starvation occurs in health, is reversed by the re-introduction of a normal diet and is caused entirely by nutrient deprivation (Thomas 2007). Sarcopenia and cachexia, however, do not readily respond to feeding and are more difficult to define. The term sarcopenia was originally coined by Rosenberg to describe age-related loss of muscle tissue (Rosenberg 1997), although it has been largely used as a generic term for the involuntary loss of muscle with relative preservation of body weight, regardless of the underlying aetiology. In the context of this thesis, the term sarcopenia will refer purely to loss of muscle tissue due to ageing against a background of normal health. In contrast, cachexia is disease-related, is classically associated with other symptoms and appears to be a more rapid process. Sarcopenia and cachexia are likely to be related entities existing at two different ends of the same spectrum; with severe cachexia at one end and early sarcopenia at the other; intermittent illnesses accelerate sarcopenia, and cachexia is more common in the elderly.



## 1.5 Starvation

There are no specialized cells that store protein for times of nutrient deprivation but muscle protein fulfils the requirement to supply AA during the diurnal cycle and longer periods of fasting. In short-term starvation (less than three days), proteolysis is up-regulated in all tissues (except the brain and reproductive organs) which results in mobilization of AA for glucose production and protein synthesis. Proteolysis provides the substrate for gluconeogenesis maintaining adequate cerebral blood glucose concentrations after hepatic glycogen stores are depleted but before the brain has fully adapted to using ketone bodies for fuel. During long-term starvation lipolysis of adipose tissue becomes the predominant source of energy and proteolysis is suppressed, thus reducing the loss of muscle mass during prolonged nutrient depletion. It is thought that the high concentrations of ketone bodies – acetone, acetoacetate and  $\beta$ -hydroxybutyrate, produced as a result of acetyl-CoA accumulation within mitochondria – are responsible for the suppression of proteolysis (Finn & Dice 2006).

## 1.6 Sarcopenia

Sarcopenia seems to be an inevitable consequence of ageing and affects both sexes and all races although not necessarily to the same extent. Like most multifactorial physiological and pathophysiological processes, it is the result of both genetic and environmental influences.

### 1.6.1 Defining sarcopenia

All of the current definitions of sarcopenia require a comparison of LBM or skeletal muscle mass of the subject under consideration with those of height-and-weight

matched young adults. Some workers take two standard deviations below the mean as defining significant sarcopenia (Gallagher *et al.* 1997; Baumgartner *et al.* 1998). This results in estimates of prevalence of 13 - 24 % for men and women aged 65 - 70 y and greater than 50 % in those aged more than 80 years. Others (Janssen *et al.* 2004a) use assigned thresholds, for both sexes, in relation to the so called skeletal mass index ratio i.e. LBM per height squared, a modification of body mass index (BMI). Values of 5.75 kg.m<sup>-2</sup> for women and 8.5 kg.m<sup>-2</sup> for men were identified as thresholds for increased risk of disability using epidemiological data. More recently the muscle mass of the limbs, i.e. appendicular lean tissue mass (ALBM) (Kim *et al.* 2002b), has been used in relation to height squared (or more rationally to height alone, since this effectively removes height as a variable in the final quotient) to produce an index which is clinically useful.

### **1.6.2 Rate of muscle loss with ageing**

There is broad agreement between authors who have conducted cross-sectional and longitudinal studies that diminution of muscle mass and function has already started in the third decade of life, with the process accelerating markedly by the fifth decade (Larsson, Grimby, & Karlsson 1979; Melton, III *et al.* 2000; Marcell 2003; Janssen *et al.* 2004a). However, the rate of decline is not clearly defined; a variety of authors interpreting cross-sectional data have obtained rates varying by an order of magnitude. There is some consensus that the period of the fastest decline is from 60 y onwards at ~0.5 - 2 % per year, depending upon the anatomical location and fibre type composition of the muscle (Frontera *et al.* 2000; Sehl & Yates 2001; Hughes *et al.* 2002; Visser *et al.* 2003; Goodpaster *et al.* 2006).

Losses of strength and power are reportedly up to three-fold greater than that of mass (Goodpaster *et al.* 2006), although there remains great variability between data. Large longitudinal cohorts, including one of over 8,000 participants studied over an average of 27 y, suggest an average decline of 1 - 2 % per annum in isometric strength (Jubrias *et al.* 1997; Rantanen *et al.* 1998; Hughes *et al.* 2001). Racial differences were observed in the Health ABC cohort (Goodpaster *et al.* 2006) in which over a third of participants were black; African Americans lost 28 % more strength than their Caucasian counterparts. These participants were assessed identically confirming inconsistency in the rates of loss of function (and mass), due to innate biological variability and differing degrees of habitual physical activity, nutritional state and independence in activities of daily living. Indeed in some older human beings strength is maintained over a number of decades (Rantanen *et al.* 1998; Greig, Botella, & Young 1993).

### **1.6.3 Changes in protein turnover with ageing**

There has been some debate whether basal rates of protein synthesis change with age, but it is now widely accepted that this is not the case. Opponents (Welle, Thornton, & Statt 1995; Hasten *et al.* 2000; Balagopal *et al.* 1997) suggest that, with ageing, individual muscle proteins respond in an aberrant fashion (i.e. myosin heavy chain or sarcoplasmic proteins demonstrate diminished synthesis rates with ageing but other proteins respond as per young muscle), but inconsistencies between reports places great doubt over their validity. Workers reporting no difference in basal synthesis rates have done so consistently for all muscle fractions (Katsanos *et al.* 2006; Volpi *et al.* 2000; Yarasheski *et al.* 1999; Cuthbertson *et al.* 2005; Volpi *et al.* 2001). Most authors agree that MPB does not differ with senescence in the PA state

(Hasten *et al.* 2000; Welle, Thornton, & Statt 1995; Volpi *et al.* 2000; Volpi *et al.* 2001; Yarasheski, Zachwieja, & Bier 1993).

### *Response to amino acids*

When studied under absorptive conditions, older human beings do not demonstrate the same amplitude of response to protein feeding as seen in the young (Katsanos *et al.* 2006; Paddon-Jones *et al.* 2004; Cuthbertson *et al.* 2005; Volpi *et al.* 2000). This “anabolic blunting” appears to be underpinned by dampened phosphorylation responses within the AA driven anabolic signalling pathways, specifically involving mTOR, (Cuthbertson *et al.* 2005) and suggest that, in older subjects, the ability to acquire muscle protein is likely to be less at a given availability of amino acids than in the young.

Nevertheless some workers report no anabolic blunting with ageing. One such report, where each volunteer was fed an identical minced-beef patty, demonstrated no difference in FSR response to feeding between the groups aged  $41 \pm 8$  y and  $70 \pm 5$  y, over a 5 h period (Symons *et al.* 2007). However the participants in the older group were significantly lighter (70 kg, compared with the mean weight of young participants of 88 kg) and thus had a smaller volume of distribution, as demonstrated by plasma EAA and leucine concentrations which, at their peak, were nearly double that of the young. Paddon-Jones *et al.* also reported no difference in anabolic responses with ageing following a set dose of EAA (15 g), albeit their young and old volunteers were of similar BMI (Paddon-Jones *et al.* 2004). Change in plasma insulin concentration in response to feeding was significantly lower in the older group, whereas the intracellular concentrations of phenylalanine were higher (old:  $150.2 \pm 19.4$  vs. young:  $115.6 \pm 5.4$

mg Phe. $\text{leg}^{-1}$ ). Leucine values were not published but it is fair to assume they parallel that of phenylalanine. Thus plausibly differences in intracellular availability of phenylalanine (and possibly leucine) may account for comparable rates of MPS in young and older participants. Indeed in a recent review, the same authors promote the role of anabolic blunting in the progression of sarcopenia (Paddon-Jones *et al.* 2008).

Very high leucine concentrations may overcome anabolic blunting providing a further explanation of discrepant data. Two separate studies from the same research group confirmed no difference in anabolic responses with ageing when plasma leucine concentrations were raised by 3-fold (Volpi *et al.* 1999; Drummond *et al.* 2008). In the more recent study plasma leucine concentrations were higher in the older participants but intracellular concentrations did not differ; whereas in the preceding work, plasma concentrations did not differ between the groups suggesting that anabolic blunting may be overcome with a high degree of stimulation. This hypothesis is supported by data from old rats fed chow supplemented with leucine; anabolic responses were in keeping with that of adult rats, whereas data from old rats fed control chow remained blunted (Combaret *et al.* 2005). Unfortunately a 3 month period of leucine supplementation (7.5  $\text{g}\cdot\text{d}^{-1}$ ) has no effect upon body composition or muscle strength in older men, although its effect upon protein metabolism has not been measured (Verhoeven *et al.* 2009).

### *Response to exercise*

Anabolic responses to exercise also appear blunted in older human beings. Fit active older adults fail to elicit the doubling of FSR seen with exercise in their younger counterparts, although some data suggests that responses may be normalized after a relatively short training programme (Yarasheski, Zachwieja, & Bier 1993; Hasten *et al.*

2000) or that the latency period may differ with senescence (Drummond *et al.* 2008). Despite this, investigators have consistently reported anabolic blunting at 3, 6 and 24 h after exercise over a wide spectrum of exercise intensities, to the order of ~30 % (Kumar *et al.* 2009). Even data collected after participants had undergone a 3 month intensive resistance exercise programme confirmed anabolic blunting. Here myofibrillar FSR measurements taken 24 h post exercise bout were markedly diminished in older volunteers ( $0.062 \pm 0.005$  vs.  $0.045 \pm 0.005$   $\% \cdot h^{-1}$ ) (Welle, Thornton, & Statt 1995). These findings are supported by signalling data in rats: GSK 3 $\alpha$  and 4E-BP1 phosphorylation measured 6 h after high frequency electrical stimulation (a surrogate for exercise) were markedly diminished with older rats (Funai *et al.* 2006).

The two groups which oppose the concept of senescent anabolic blunting following exercise took measurements following a 2 w exercise programme. Hasten *et al.* report a greater anabolic response in older participants than the young, (measured 3 h after exercise at the end of a 2 w exercise programme) with changes in FSR of 166 % and 102 % increase over basal values respectively (Hasten *et al.* 2000). Whereas Yarasheski *et al.* report no difference in anabolic responses to exercise, with both young and older participants exhibiting a rise of ~50 % over basal values (Yarasheski, Zachwieja, & Bier 1993). Nevertheless data from both groups requires careful interpretation as exercise performed in the previous few days (which is not clearly stated) will have influenced the rate of anabolism. If time course changes are ignored, these data may also be explained by acceleration of anabolic responses in muscle which was untrained just 2 w earlier, particularly so in the older volunteers. The issue of anabolic blunting in response to either AA or exercise in trained older healthy volunteers is currently being addressed in an RCT.

### *Response to insulin*

Suppression of whole-body protein breakdown by insulin also appears blunted with senescence. At equivalent insulin and amino acid concentrations, rates of whole body protein breakdown are increased when compared with the young, although less so at higher insulin concentrations (Guillet *et al.* 2004b; Boirie *et al.* 2001). Measurements of leg balance and leg protein breakdown (LPB), assumed to be due mainly to muscle, are not different in the basal state in young and older subjects (Volpi *et al.* 2001). However it is plausible that during the fed state, when insulin secretion is stimulated, normal inhibition of proteolysis by insulin may occur to a lesser degree with ageing. This hypothesis has not been tested, but will be addressed in Chapter 5 of this thesis.

## **1.7 Cachexia**

Cachexia presents with the same spectrum of symptoms (mostly attributed to disease driven cytokine production) in a wide variety of different diseases, which may be as diverse as cancer and renal failure. Nevertheless, cachexia carries a poorer prognosis than the stage of disease alone would confer, regardless of the aetiology.

### **1.7.1 Defining Cachexia**

The spectrum of symptoms collectively termed cachexia, or the cachexia-anorexia syndrome by some authors, underpin the problems in accurately defining the condition. Rather than change in one easily measurable variable such as LBM, as occurs in sarcopenia, other features must also be taken into account. These may include anorexia, nausea, early satiety, fatigue, excessive sweating, asthenia and dyspnoea in addition to muscle wasting; although distinguishing between symptoms due to cachexia

and that of the underlying pathology, can be difficult. Indeed the lack of diagnostic criteria is in part due to the fact prognostic implications of each component of cachexia are unknown. Is the outcome worse if more symptoms are present, or if the degree of weight loss is greater?

Weight loss is often chosen as a marker of cachexia as it is readily quantified and, in health, remains static for many years providing a bench mark for the pre-morbid condition. Some investigators have defined cachexia as unintentional weight loss of 7.5 % healthy body weight over a 6 month period (Anker *et al.* 1997) while others have used 5 % (Kondrup *et al.* 2002) or 10 % (Fearon, Voss, & Hustead 2006) change over the same duration. These values are in essence arbitrary as the cachectic process begins before loss of total body weight (TBW) is evident, as demonstrated by metabolic changes (Pisters & Pearlstone 1993). In addition changes in total body water may disguise weight loss: fluid retention alone may account for 10 – 20 % of body weight (Hall I, Pollard BJ, & Campbell IT 1992). A further definition based upon weight uses a BMI threshold of  $<18.5 \text{ kg.m}^{-2}$  associated with unintentional weight loss, in keeping with the World Health Organisation definition of undernutrition (Shetty & James 1994). This definition selects out those individuals with severe cachexia or whose pre-morbid weight was at the lower end of the normal spectrum, but excludes the cachectic obese and those with early cachexia.

Fearon *et al.* confirmed the short-comings of using TBW in isolation (Fearon, Voss, & Hustead 2006). Investigators assessed the prognostic and functional impact of three separate variables, weight loss, food intake and C-reactive protein (CRP), in patients with pancreatic cancer cachexia. No difference was demonstrated in terms of LBM,



quality of life (QOL) or inflammatory status when individuals were assigned to groups based upon the degree of weight loss alone. However, abnormal values for all three variables were associated with a significant decrease in QOL, LBM and health status. In terms of prognosis, Karnofsky performance scores (KPS; see Appendix 2), LBM, health status, food intake and CRP were all indicative of a worse outcome whereas degree of weight loss alone was not; thus supporting the hypothesis that weight loss alone is not sufficient to diagnose cachexia.

Experts within the field of cachexia research agree that a definition is required and work is underway (Springer, von Haehling, & Anker 2006). For the purposes of work described in this thesis, “cachectic” participants have been selected providing there is evidence of body composition changes, although an arbitrary threshold has not been used for the reasons given above.

### **1.7.2 Prevalence and rates of muscle loss in cachexia**

Conservative estimates of cachexia prevalence, using reported unintentional weight loss to identify cases, lie between 10 and 40 % for patients with renal, respiratory, cardiac, rheumatological and malignant diseases (Table 1.2) (Morley, Thomas, & Wilson 2006). The prevalence of cachexia in patients with critical illnesses, such as sepsis, burns, multi-organ failure and trauma, is more difficult to ascertain as rapid muscle loss occurs over a shorter duration in these patients, usually from a healthy baseline weight.

In a critical care environment, ultrasound measurements taken from 50 patients at mid-arm, fore-arm and thigh revealed the median rate of muscle loss was 1.6 %. $d^{-1}$  (range 0.2 - 5.8 %. $d^{-1}$ ) with more muscular patients losing muscle at a greater rate than their less muscular counterparts (Reid, Campbell, & Little 2004). This rate of wasting is in keeping with earlier reports of 1 - 9 %. $d^{-1}$  in critically ill patients (Campbell *et al.* 1995; Newsome, Mason, & Pruitt 1973; Streat, Beddoe, & Hill 1987). Compare this with the rate of 0.5 - 2 % *per annum* seen in ageing.

Table 1.2 Prevalence rates of cachexia

Disease process	Patients affected by cachexia
Chronic renal failure	40 %
Malignancy	30 %
Chronic respiratory disease	20 %
Cardiac disease	20 %
Rheumatoid disease	10 %

(Morley, Thomas, & Wilson 2006)

Chronic inflammatory disorders including inflammatory bowel disease, rheumatoid arthritis, fibrosing and obstructive lung diseases, cirrhotic liver disease and congestive heart failure are associated with a more insidious loss of lean tissue. In many cases the rate of muscle wasting follows the relapsing-remitting course of the underlying inflammatory process, with periods of relative recovery during times of disease quiescence. Thus there is a paucity of longitudinal data and cross-sectional studies are flawed by very small sample sizes.

A good quality cross-sectional study was conducted in children and young adults with Crohn's disease (Burnham *et al.* 2005); body composition data from 104 patients was compared with that from 233 healthy age-matched controls. Fat mass did not differ between the groups whereas the lean mass of Crohn's patients was 6 % less than healthy controls, regardless of height, age, race and gender. Furthermore an inverse correlation existed between activity of disease and lean mass.

### **1.7.3 Effects of cachexia upon protein turnover**

Only a small amount of muscle metabolism work has been carried out in patients with wasting due to disease, rather than ageing. The existent data are predominantly drawn from patients with cancer and involve small numbers of subjects, a lack of homogeneity in those studied, and techniques that have largely been superseded. Nevertheless, whole-body protein turnover (WBPT) in patients with wasting due to cancer demonstrates, for the most part, no difference when compared with healthy controls in both fasted (Dworzak *et al.* 1998; Richards *et al.* 1993; Heslin *et al.* 1992) and fed (Emery *et al.* 1984; Heslin *et al.* 1992) states, although data suggesting that turnover is increased is also reported (Shaw *et al.* 1991; Melville *et al.* 1990; Jeevanandam *et al.* 1984; O'Keefe *et al.* 1990; McMillan *et al.* 1994). Studies of patients with congestive cardiac failure (Toth & Matthews 2006; Norrelund *et al.* 2006), cirrhotic liver disease (O'Keefe *et al.* 1990) and Crohn's disease (Motil *et al.* 1982) also show no difference, in terms of WBPT data, from healthy controls. However, whole body synthesis and breakdown data, in patients with inflammatory bowel disease, is increased in proportion to the degree of inflammation (Powell-Tuck *et al.* 1984).

Muscle specific protein turnover is even less well studied, but observations are consistent. Measured under PA conditions in malignant disease, Dworzak *et al.* (Dworzak *et al.* 1998) reported a 60 % reduction in MPS in four patients with advanced gastric cancer compared with control data. These findings are supported by work in cachectic MAC 16 (Smith & Tisdale 1993) and XK1 (Emery, Lovell, & Rennie 1984) tumour-bearing mice models, in which PA MPS is suppressed by ~60 %. Recent data from eleven weight stable human beings with colon cancer concur with these findings in that there is ~40 % reduction in MPS in the PA state (Rennie *et al.* 2007). The anabolic response to feeding is completely suppressed in these cancer patients, in keeping with older data (Emery *et al.* 1984), but is restored to normal values upon resection of the tumour. MPB in cancer patients does not appear to differ from normal values whether measured by D<sub>5</sub> phenylalanine kinetics (Rennie *et al.* 2007) or 3-methyl-histidine flux (Lundholm *et al.* 1982). This pattern of depressed MPS with preservation of MPB extends to non-malignant disease. Three-methylhistidine efflux (a marker of MPB) across the leg in patients with emphysema does not differ to that of healthy control subjects, although PA MPS is depressed (Morrison *et al.* 1990).

### **1.8 Pathophysiology of sarcopenia and cachexia**

Loss of muscle tissue, whether due to ageing or disease, results from changes in muscle protein turnover as already described; the influences which manipulate the rates of protein synthesis and breakdown are more complex and less well understood. The degree of inflammation, provision of nutrients, habitual level of physical activity performed, prescribed medications and hormonal disturbances are key factors in the maintenance of muscle tissue. However, insulin resistance, whether a direct result of the underlying disease or its consequences (e.g. the result of inflammation or

glucocorticoid administration), appears pivotal in the control of muscle protein turnover (Figure 1.4).

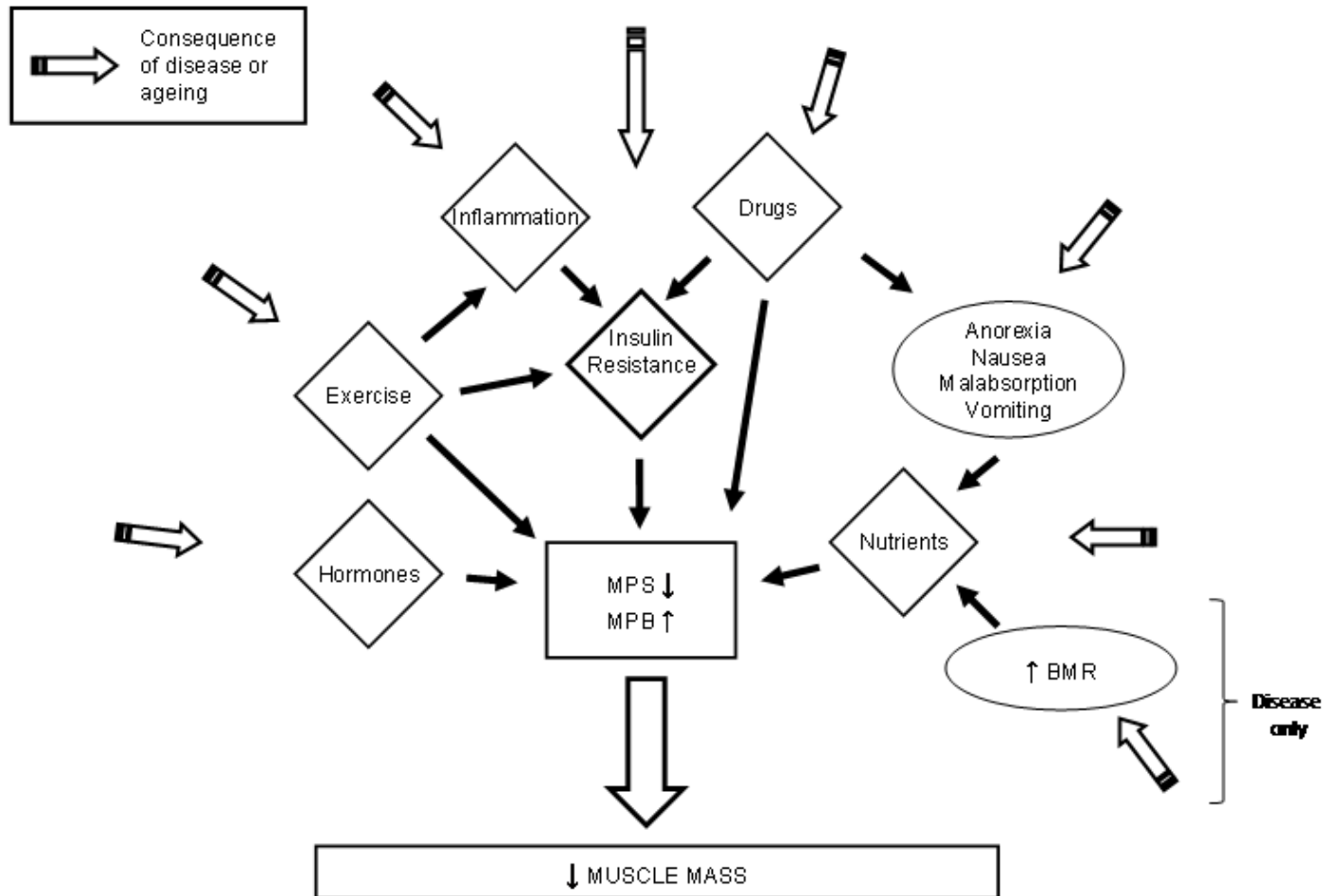


Figure 1.4 Factors involved in ageing and disease related loss of muscle tissue. (MPS muscle protein synthesis; MPB muscle protein breakdown; BMR Basal metabolic rate)

### 1.8.1 Insulin resistance

Insulin sensitivity decreases with ageing (Boirie *et al.* 2001; Pagano *et al.* 1996; Dardevet *et al.* 1994; Elahi *et al.* 1993; Broughton *et al.* 1991; Jackson *et al.* 1988; Rowe *et al.* 1983; DeFronzo 1979), albeit in many cases with few objective signs of glucose intolerance (i.e. fasting hyperglycaemia and elevated insulin:glucose ratios). Reduced muscle mass, increased adiposity, inactivity and intercurrent illnesses are partly responsible by enhancing chronic low-grade inflammation in healthy older populations (Grimble 2002; Bruunsgaard & Pedersen 2003). This inflammation is believed to underpin insulin resistance (Yudkin *et al.* 1999; Festa *et al.* 2000).

To varying degrees, insulin resistance is also universally recognized in acute and chronic disease (Kahn 1978). Nearly all patients with established cirrhotic liver disease have evidence of insulin resistance (Petrides *et al.* 1998) as do approximately 50 % of non-diabetic patients with hypertension (Ginsberg 2000). To an extent disease activity correlates with inflammation and consequent propensity towards insulin resistance; although in some diseases inflammation may be the underlying cause rather than effect, such as chronic kidney disease or atherosclerosis (Howard *et al.* 1996; Ritz 2008). Nevertheless, the presence of inflammatory mediators, including TNF $\alpha$ , IL-6 and CRP, appear to underpin an insulin resistant state.

To date investigators have been unable to clearly localise the site of insulin resistance within cellular signalling pathways; although it is believed to be contained within the PI3K pathway, rather than Grb2-Sos-Ras. Insulin resistance of protein metabolism differs from that of carbohydrate metabolism and it is plausible that different defects may be responsible. Lack of a clear signalling defect in carbohydrate metabolism has

led to suggestions that a global reduction in kinase activities (perhaps at the lower end of the normal spectrum) accompanied by environmental triggers, such as ill-health or high insulin concentrations, explain poor signal transduction without evidence of specific defects (Pessin & Saltiel 2000). Other proposals include a reduction in the number of insulin receptors (IR), down-regulation of IR kinase activity through serine/threonine phosphorylation, diminished IRS tyrosine phosphorylation or defects in the association of IRS-1 with PI3K (Cusi *et al.* 2000). Nevertheless, TNF $\alpha$  has been shown to inhibit insulin signalling by enhanced serine phosphorylation of IRS-1 (thus reducing its kinase activity) (Hotamisligil *et al.* 1996); thus providing one mechanism by which inflammation impairs insulin sensitivity. Much less is known about insulin resistance of protein metabolism.

Researchers have clearly demonstrated that insulin availability has no additional effect upon MPS in healthy young volunteers (Greenhaff *et al.* 2008); however the effects of insulin upon anabolic blunting have not been properly examined. Volpi *et al.* used a 50:50 oral preparation of glucose and amino acids in order to produce an endogenous hyperinsulinaemic state in young and elderly individuals (Volpi *et al.* 2000). Under these conditions endogenous hyperinsulinaemia failed to elicit comparable rates of synthesis in young and older adults, indeed FSR did not exceed basal values in the older group. This is at odds with data from other groups, where a blunted, rather than absent, synthetic response to AA feeding has been reported (Cuthbertson *et al.* 2005; Welle *et al.* 1993; Balagopal *et al.* 1997). Further experiments are required to elucidate whether anabolic blunting may be overcome by the administration of high dose insulin and will be addressed in Chapter 4 of this thesis.

### 1.8.2 Nutrient provision

Muscle wasting, regardless of other aetiological factors, is accelerated by inadequate provision of nutrients. However, excessive provision of AA results in energy surplus and subsequent increases fat mass rather than lean tissue, as demonstrated by aggressive parenteral nutritional support (Streat, Beddoe, & Hill 1987). To identify patients at risk of inadequate nutrition, two screening tools are recommended for hospitalized patients – Malnutrition Universal Screening Tool ([www.bapen.org.uk/pdfs/must/must\\_full.pdf](http://www.bapen.org.uk/pdfs/must/must_full.pdf)) and Nutrition Risk Screening 2002 (Kondrup *et al.* 2003) - and a third is appropriate for screening older people in the community – Mini Nutritional Assessment (Guigoz, Vellas, & Garry 1996). These tools provide a simple means for nursing staff to assess nutritional risk and identify patients who require referral to dietetic services for a comprehensive nutritional assessment. Nevertheless, individuals with isolated deficiencies in vitamins and minerals, which include up to a third of over 65 y olds living independently (Stroud, Duncan, & Nightingale 2003), are not identified using these tools.

### 1.8.3 Exercise

Inadequate physical activity exacerbates muscle loss regardless of the underlying cause. However, even individuals who continue to be physically active throughout life demonstrate decreases in functional ability and muscle mass (Roth, Ferrell, & Hurley 2000), which emphasizes the importance of maintaining, or even increasing, activity levels with senescence. Unfortunately social isolation, lack of confidence and intercurrent illnesses often reduce physical activity in high risk individuals.



Many patients with chronic diseases are reluctant to participate in exercise programs although the potential benefit is greatest in these individuals. For example, patients with chronic renal insufficiency and low protein dietary restrictions not only benefit from muscle mass accretion from resistance exercise training, but also improved metabolic function. These patients were capable of achieving a work rate of up to 80 % 1RM during a 12 w supervised exercise programme, which improved protein utilization and enabled maintenance of baseline weight (not seen in the sham group) and resulted in hypertrophy of muscle fibre cross sectional area (Castaneda *et al.* 2001).

Exercise may also modify the underlying disease process. Insulin resistance, atheromatous disease and inflammation are reduced directly by exercise. A single bout of resistance exercise improves insulin sensitivity by  $13 \pm 5$  % (Koopman *et al.* 2005). Risk and risk of recurrence of malignancy is also reduced. Colon cancer risk falls by more than 20 % in both men and women that are physically active (Samad *et al.* 2005) and the reduction in mortality and disease recurrence following the diagnosis of malignancy approaches 50 %, regardless pre-morbid activity levels (Meyerhardt *et al.* 2006a; Meyerhardt *et al.* 2006b). Furthermore, intensive exercise programmes improve self-esteem, muscle strength and tolerance of chemotherapy regimes in patients with malignancy (Courneya *et al.* 2007).

#### **1.8.4 Anabolic hormones**

Changes to circulating hormone concentrations with ageing and disease influence muscle maintenance. In critical illness the anabolic hormones testosterone and growth hormone are suppressed whilst the catabolic hormone cortisol is markedly increased (Vanhorebeek & Van den 2004). With healthy ageing, the production of testosterone

diminishes in men (Harman *et al.* 2001) and hypogonadism and low testosterone production are associated with a greater risk of sarcopenia (Szulc *et al.* 2004). In women, the decline of muscle mass is greatest after the menopause possibly because oestradiol conversion to testosterone is diminished, accounting for the relative preservation of muscle mass with the use of hormone replacement therapy (Cauley *et al.* 1987; Greeves *et al.* 1997). Although, older men lose appendicular (i.e. limb) muscle mass more rapidly than women (Zamboni *et al.* 2003; Gallagher *et al.* 1997; Hughes *et al.* 2002), despite 10-fold greater concentrations of free testosterone. A difference in the basal rates of MPS (~30 % higher in women than men), may account for this sexual dimorphism (Smith *et al.* 2008).

### **1.8.5 Pre-morbid body composition**

Those at greatest risk of morbidity due to muscle loss are those with the lowest pre-morbid muscle mass. The prevalence of sarcopenia is two-fold greater in women than men (Janssen *et al.* 2004a) yet loss of appendicular muscle mass with ageing is greater in men, reflecting the smaller peak life-time muscle mass in women. This principle extends to veteran weightlifters who lose muscle protein mass at about the same rate as sedentary individuals, but retain muscle strength for longer and suffer less disability (Pearson *et al.* 2002).

Obesity is also protective against sarcopenia, both in terms of age and disease related muscle loss, perhaps reflecting the effect of excessive nutrient provision over many years, although data does not tease out the effect of peak muscle mass rather than obesity *per se*. Nevertheless mortality from congestive cardiac failure following hospitalization is profoundly reduced with increasing BMI – including in individuals who

are morbidly obese - such that, in a study of over 100,000 patients, mortality was reduced by 10 % with every 5 kg.m<sup>-2</sup> rise in BMI (Fonarow *et al.* 2007). Fewer patients with chronic obstructive pulmonary disease (COPD) have been studied but again lower mortality is seen with greater BMI (Hallin *et al.* 2007).

There is emerging evidence that intrauterine influences affect muscle mass in later life. A positive correlation between birth weight and LBM has been found in men and women living in geographically distinct areas within the UK (Gale *et al.* 2001; Sayer *et al.* 2004). Adult grip strength also shows a strong positive correlation with birth weight (Sayer *et al.* 2004; Kuh *et al.* 2002). Aside from genetic constraints, maternal age (less than 17 and above 35 years), poor nutrition (especially a low protein diet), smoking, alcohol consumption and parity all reduce birth weight in otherwise healthy pregnancies, and hence may play a role in modelling gestational muscle development, and thus setting future risk of sarcopenia.

#### **1.8.6 Medications and other toxins**

Alcohol consumption, cigarette smoking and administration of some prescribed medications appear to increase sarcopenia risk, either through direct myocyte toxicity or through influences upon muscle protein turnover.

A positive association has been described between smoking and low muscle mass (Szulc *et al.* 2004), in part due to the effects of smoking upon TBW. On a cellular level, smoking decreases oxidative capacity and increases type IIx fibre proportion in muscle (Orlander, Kiessling, & Larsson 1979). Protein turnover data from apparently healthy

volunteers who smoked heavily (more than 20 pack years) demonstrate a reduced rate of MPS in the PA state (Petersen *et al.* 2007); however when compared to control data, the smokers had significantly lower forced expiratory volumes and greater residual volumes, suggesting that they were developing pulmonary changes consistent with early COPD. Thus it is unclear whether smoking increases the risk of sarcopenia through low grade inflammatory changes related to the onset of COPD or due to direct myocyte toxicity.

In contrast, alcohol appears directly toxic to skeletal muscle. Prolonged alcohol exposure leads to catabolism of muscle, through both enhanced MPB (Martin *et al.* 1985; Preedy *et al.* 1989) and a reduction of MPS responses to AA feeding (Lang *et al.* 2003; Lang *et al.* 1999). Acute alcohol exposure increases the expression of E3 ligases but does not appear to induce proteolysis (Vary, Frost, & Lang 2008); thus moderate alcohol intake is unlikely to have a significant impact on the rate of muscle decline, although there is no data to confirm this suggestion.

Endogenous steroid hormones are, for the most part, anabolic; however corticosteroids, specifically glucocorticoids, when present in excessive concentrations are profoundly catabolic. This is well demonstrated in patients who demonstrate proximal limb weakness and muscle wasting with Cushing's syndrome, or with chronic exogenous glucocorticoid use. Enhanced MPB (Lofberg *et al.* 2002) appears primarily responsible although inhibition of MPS is also thought to occur (Odedra & Millward 1982). This inhibition of MPS may reflect increased insulin resistance as a secondary complication of glucocorticoids rather than a primary defect. Glucocorticoid administration also expedites muscle loss and weakness due to immobility alone by as

much as 3-fold. This is predominantly due to changes in MPB rather than synthesis (Ferrando *et al.* 1999).

Glucocorticoids modulate cell physiology by interacting with transcription factor control of gene expression. In skeletal muscle, glucocorticoids are known to interfere with the transcription factors Myogenin, ATF4, FOXO and REDD1. Myogenin, which is involved in the differentiation of satellite cells, and ATF4, which facilitates AA transport into the cell and synthesis of non-essential AA and aminoacetyl tRNA, are both suppressed by glucocorticoids, whereas REDD1, a repressor of mTOR, and FOXO are activated (Schakman, Gilson, & Thissen 2008). As only FOXO is involved in MPB, this data supports suggestions by Odedra *et al.* that diminished MPS also plays a role in glucocorticoid associated muscle loss.

Other drugs used in immunosuppressant therapy may also impede anabolism. Theoretically rapamycin (also termed sirolimus) should impair muscle anabolism through the mTOR pathway. Current data supports this notion, but is limited to signalling work in rats: pre-treatment with rapamycin reduces nutrient stimulated phosphorylation of mTOR, eIF4G and P70S6K (Vary *et al.* 2007). Nevertheless, rapamycin is not used routinely in transplant patients due to poor tolerability: ~30 % of liver transplant recipients discontinue therapy due to side-effects (Rowe, Gunson, & Thorburn 2009). The more commonly used calcineurin inhibitors, ciclosporin and tacrolimus, also appear detrimental to muscle preservation as they inhibit the NF-AT (nuclear factor of activated T cells) pathway which controls the differentiation of slow-twitch muscle fibres (Naya *et al.* 2000). This has been shown in transplant patients immunosuppressed with ciclosporin, in the form of skeletal myopathy (Goy *et al.* 1989). Furthermore, mice administered ciclosporin prior to hind limb loading experiments fail

to demonstrate muscle hypertrophy as seen in controls (Rumpf & Henning 1990). Nevertheless, no protein balance data is available for these or other immunosuppressant agents.

Beta ( $\beta$ ) adrenoreceptor antagonists, also known as  $\beta$  blockers, may also be catabolic and are far more commonly used than immunosuppressants, particularly in patients with multiple co-morbidities. Animal studies suggest  $\beta$ 2 agonists enhance LBM by suppression of MPB (Costelli *et al.* 1995); thus  $\beta$  antagonism may be detrimental to the preservation of muscle tissue. This possibility has not been tested in human beings.

### **1.9 Interventions to preserve muscle mass**

Avoidance of muscle-specific toxins, obtaining significant peak muscle bulk and avoidance of disease are key to the preservation of muscle mass. However, approximately a quarter of the UK population smoke (<http://www.statistics.gov.uk/>) and nearly 50 % of over 65 y olds have three or more chronic medical conditions (20 % have five or more) (Boyd *et al.* 2005). Therefore, interventions will be required for high risk individuals.

At present the only proven, safe interventions to ameliorate loss of muscle tissue with ageing and disease are adequate protein intake and regular exercise (preferable resistance exercise). Much of the at-risk population will have been given this advice as part of cardiovascular disease and diabetes management, yet uptake remains poor. Pharmaceutical options, as described in the remainder of this section, are mostly experimental and not advocated for use outside of clinical trials.

### 1.9.1 Hormonal therapies

Androgen replacement therapy has been explored using testosterone and synthetic analogues such as oxandrolone. In sarcopenia, testosterone replacement therapy appears to have only a small effect in significantly hypogonadal men (Wittert *et al.* 2003). However, when used in patients with 50 – 90 % body surface area (BSA) burns, studied 2 and 4 w post injury, testosterone significantly reduced MPB and had no effect upon MPS, returning net balance to zero. There were no control data for this study but weight loss continues in patients with burns of this size for approximately 8 w (Newsome, Mason, & Pruitt 1973), implying that testosterone is markedly anabolic under these conditions. In health, testosterone administration appears to increase muscle mass through enhancing the rate of PA MPS, although feeding responses and breakdown data have not been collected (Griggs *et al.* 1989).

Oxandrolone also increases PA rates of MPS in healthy volunteers, with no change in MPB (Sheffield-Moore *et al.* 1999). In both adults and children recovering from burn injuries, lean weight gain and muscle strength on oxandrolone is approximately double that of controls, resulting in reduced hospitalization (Jeschke *et al.* 2007; Demling & DeSanti 1997). However, a Cochrane review of anabolic steroid use in patients with HIV-associated wasting was less impressive (Johns, Beddall, & Corrin 2005). This meta-analysis of 13 randomized placebo controlled trials, involving over 1000 patients, reported inconsistent results across the studies and a mean increase of just 1.1 kg TBW, (1.3 kg LBM) on active treatment. Taking into account the potential side-effects of androgen use, which include virilization (i.e. deepening of the voice, hirsutism, acne and clitoromegaly), hepatotoxicity (including peliosis hepatitis, cholestatic hepatitis and tumours), fluid retention, prostatic hypertrophy and carcinoma, hypercalcaemia and

bleeding diatheses (due to suppression of clotting factors II, V, VII, and X) wide-spread use is not advocated. Hepatotoxicity and virilization are said to be less prevalent with oxandrolone yet risks still out-weigh benefits for use in sarcopenia (Orr & Fiatarone 2004).

The progesterones, medroxyprogesterone and megestrol acetate, have been used for their appetite stimulating properties which are thought to be the result of cytokine suppression (Mantovani *et al.* 2001; Inui 2002). Nevertheless, benefits are limited to weight gain rather than restitution of LBM or strength as confirmed by placebo controlled trials in cachexia due to COPD (Weisberg *et al.* 2002), end-stage malignancy (Feliu *et al.* 1992; Bruera *et al.* 1990) and HIV (Oster *et al.* 1994; Von Roenn *et al.* 1994). In addition, the side-effect profile (fluid retention, mood changes, hyperglycaemia, adrenal insufficiency and venous thrombo-embolism (VTE)) limits wide-spread use.

Stimulation of anabolism with growth hormone (GH) has been trialled by a number of investigators, initially with promising results. In healthy volunteers and post-operative patients, GH appears to increase insulin-like growth factor 1 and nitrogen balance (Dahn, Lange, & Jacobs 1988; Sevette *et al.* 2005; Lehmann *et al.* 1990); however a large study of critically ill patients revealed that GH increased mortality (Takala *et al.* 1999), possibly the result of increased insulin resistance and hyperglycaemia.



### 1.9.2 Tumour necrosis factor alpha antagonism

Direct tumour necrosis factor alpha (TNF $\alpha$ ) inhibition has been investigated in muscle wasting with pentoxifylline, infliximab and thalidomide. Both pentoxifylline (Biolo *et al.* 2002) and infliximab (a monoclonal antibody against TNF $\alpha$ ) (Steiner *et al.* 2007) suppress proteolysis; however infliximab also suppressed MPS therein leaving net balance unchanged. Neither weight gain nor appetite improvements were seen with pentoxifylline use in patients with solid tumours when compared with placebo over an 8 w period (Goldberg *et al.* 1995).

More promising data exists for thalidomide use in cancer cachexia. An open-labelled study of 11 cachectic oesophageal cancer patients reported an increase in LBM of 1.75 kg after 2 w of thalidomide compared with 1.11 kg lost in the preceding 2 w on normal diet alone (Khan *et al.* 2003). Further data from a randomized controlled trial involving 50 cachectic pancreatic cancer patients, confirmed that thalidomide significantly attenuated loss of total and lean weight when compared with placebo over a 4 w period (Gordon *et al.* 2005). However, neither group measured circulating blood TNF $\alpha$  concentrations or changes in protein turnover.

Thalidomide is known to reduce blood TNF $\alpha$  concentrations by accelerating the degradation of TNF $\alpha$  mRNA transcript (Moreira *et al.* 1993) and prevent activation of nuclear factor kappa B (NF $\kappa$ B) through phosphorylation of its inhibitory protein I $\kappa$ B $\alpha$  (Keifer *et al.* 2001). Nuclear factor kappa B is a ubiquitous transcription factor which has multiple genomic binding sites and is involved in the control of cell differentiation, proliferation, and survival in addition to immune regulation through enhanced cytokine generation. Thus changes in body composition resulting from thalidomide use may

stem from its influences upon NF $\kappa$ B activity, rather than TNF $\alpha$  *per se*. Nevertheless further work is required to confirm the efficacy of thalidomide and elucidate the mechanism by which anabolism occurs.

### 1.9.3 Other agents

Three-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) reductase inhibitors, otherwise known as statins, are widely advocated in primary and secondary prevention of heart disease and have been shown to reduce the progression to diabetes by as much as 30 % (Freeman *et al.* 2001). A possible mechanism by which insulin sensitivity is maintained is the reduction of IL-6 and TNF $\alpha$  production (Rosenson, Tangney, & Casey 1999). To date there are no metabolic studies quantifying the effect of statins upon muscle anabolism, but a recent abstract indicates benefit in terms of acquisition of LBM following resistance exercise (Riechman *et al.* 2007) and identification of individuals at higher risk of myopathy, a rare side-effect of statin use, is now possible by identifying a genetic polymorphism on Chromosome 12 (Link *et al.* 2008).

Further promise exists for melatonin use in maintenance of muscle mass. Melatonin, principally involved in the control of circadian rhythm, controls cell differentiation and proliferation, enhances immune function and has anti-oxidant effects (Brzezinski 1997). When given to patients with solid tumours, TNF $\alpha$  concentrations halve from baseline values and body weight is maintained over a 12 w period (Lissoni *et al.* 1996). A more recent study, which was flawed by very small numbers, confirmed melatonin's potential for use as an anabolic agent, albeit with less impressive effects upon cytokine suppression (Persson *et al.* 2005). Melatonin may be of use in the management of

sarcopenia and cachexia as it is very well tolerated and side-effects are limited to hypersomnolence and ovulatory dysregulation.

### **1.10 Research questions and hypotheses**

Despite important functions to maintain health and physical independence, muscle tissue is depleted during healthy ageing and many diseases by common mechanisms. Many of these pathophysiological processes are not fully understood; however anabolic blunting in response to AA appears to play critical role, particularly in slow wasting conditions. Enhanced proteolysis features more commonly in rapid wasting conditions, such as sepsis, burn injuries and advanced cancer, and is believed to be mediated through pro-inflammatory cytokines and catabolic hormones. Loss of LBM, whether due to blunted anabolism or proteolytic disease states, is compounded by inactivity and poor nutritional intake, which are common in unwell or older human beings. Many unexplored avenues exist in this area of physiology, in part due to the difficulties conducting physiological research in human beings, particularly those with overt muscle wasting. Data from animal studies, particularly intracellular signalling from mouse and rat work, is not consistently applicable to human beings. Therefore a plan of studies was developed involving both patients with cachexia due to oesophageal cancer and healthy volunteers aged 18 to 33 y or 65 to 75 y.

The first objective was to validate the use of thalidomide in the treatment of cachexia due to oesophageal cancer; specifically to ensure that the anabolic effect of thalidomide is superior to placebo, is sustained over a longer duration and is well tolerated by patients with end-stage disease. A randomized, parallel arm, placebo controlled trial (RCT) was designed to test the hypothesis that 200 mg of thalidomide

taken orally for 6 w would prevent loss of TBW, including lean tissue, in patients with weight loss due to oesophageal cancer.

Subsequently, a mechanistic study was planned concerning the mechanisms by which thalidomide exerts its apparent anabolic effect, to address the following questions: 1) is there attenuation of MPS rates either in the basal state or in response to feeding, suppression of proteolysis or a combination of both processes? And 2) is the effect orchestrated through a reduction in pro-inflammatory cytokines concentrations, or a direct effect of NF $\kappa$ B antagonism? A subsequent, stable isotope study was designed, ethically approved and funded, to specifically address these questions. Recruitment was due to commence upon completion of the RCT; however negative findings (Chapter 3) cast doubt over the efficacy and tolerability of thalidomide and it was deemed unethical to proceed.

A striking feature of anabolic blunting in sarcopenia is the defect in signalling responses to AA feeding, specifically mTOR and the upstream PI-3K pathway. As the PI-3K pathway is implicated in insulin resistance pathophysiology, it is plausible that increasing insulin availability above normal post-prandial concentrations would overcome anabolic blunting by restoring the activation of these signalling enzymes in older human beings. Thus MPS measurements in young and older healthy volunteers under fasted and hyperaminoacidaemic hyperinsulinaemic conditions were conducted to test the following hypotheses: First, administration of insulin at supraphysiological doses to older human beings in the presence of AA feeding restores the rate of MPS to that of their younger counterparts; and secondly, that phosphorylation of signalling proteins within the mTOR pathway would be restored.

The final objective, to investigate the effects of modest insulin concentrations upon MPB in young and older healthy volunteers, aimed to test the hypothesis that insulin-dependent suppression of muscle specific proteolysis is blunted with ageing. As most of the effects of insulin upon skeletal muscle appear to have occurred at concentrations of  $< 30 \mu\text{IU}\cdot\text{ml}^{-1}$ , target insulin concentrations of 5 and  $15 \mu\text{IU}\cdot\text{ml}^{-1}$  were sought to achieve a degree of insulinemia comparable with that which follows a low glycemic index meal, such as a light breakfast. A two phase metabolic study was conducted for this purpose.

## **Chapter 2**

### **Methods**

## **2.1 Clinical methods**

### **2.1.1 Ethical approval**

All protocols were prospectively approved by the appropriate research ethics committee and conducted in accordance with International Conference on Harmonisation (ICH) / World Health Organisation (WHO) Good Clinical Practice standards. Protocols of healthy volunteer studies were submitted to the University of Nottingham Medical School Research Ethics Committee for approval, whereas studies involving patients were sanctioned by Derbyshire Research Ethics Committee.

### **2.1.2 Recruitment and screening**

Healthy volunteers were recruited from the local community by poster advertisements in libraries, shops, museums and leisure centres, and radio and newspaper coverage facilitated through the University of Nottingham press office. Patient recruitment is detailed in Section 2.1.11. Participant information sheets and a basic health questionnaire were sent out in response to healthy volunteer enquiries with an invitation to return the questionnaire if interest was retained.

Volunteers were invited to attend the research facility before entry into the studies. At this visit a detailed discussion of the proposed study took place to ensure the participants were able to give full informed consent. Each participant underwent a full medical history and examination including blood pressure measurement and an electrocardiogram. Blood was drawn and analyzed by the hospital laboratory for the following screening tests: fasting glucose, full blood count, plasma electrolytes, liver

function tests, thyroid function tests and, for studies involving muscle biopsies, a coagulopathy screen.

Study specific inclusion and exclusion criteria were checked at the primary visit and in the case of the patient study, also validated from hospital records. Healthy volunteers were excluded if they had evidence of heart disease, chronic pulmonary diseases, diabetes mellitus, malignancy or glucocorticosteroid use (within the preceding year). Women of child bearing age were specifically screened for pregnancy (Clearview HCG II, Inverness medical UK).

### **2.1.3 Body composition measurements**

#### *Anthropometric measurements*

All healthy volunteers and patients were studied in the postabsorptive state and wore a hospital gown and their underclothes for all body composition measurements. Height was recorded in meters using the Bedford rule; a single set of electronic scales were used for weight measurements.

Mid-arm muscle circumference (MAMC) was calculated from triceps skin fold thickness (TSF) and mid-arm circumference (MAC) using the following equation:

$$\text{MAMC} = \text{MAC} - \pi (\text{TSF})$$

(Bishop, Bowen, & Ritchey 1981)



MAC and TSF measurements were taken at a point equidistant from the acromion process and olecranon. TSF measurements were made vertically along the posterior midline using skin fold callipers (Holtain, Pembrokeshire, UK). The technique for these measurements was practiced over 50 times before the study to optimize reproducibility. All measurements were taken by the author to avoid inter-operator error.

#### *Dual energy X-ray absorptiometry (DEXA)*

Fan beam DEXA equipment, Lunar Prodigy II (GE Medical Systems, Buckinghamshire, UK), was used for all healthy volunteer studies. Subjects were positioned to ensure separate body compartments could be analyzed i.e. where possible a space was left between the legs and between arms and torso.

The delineation between leg and torso, for the purpose of determining the individual leg volumes or appendicular lean body mass (ALBM), was defined as tissue distal to a horizontal line passing through the symphysis pubis (Figure 2.1). Arms were classified as all tissue lateral to a vertical line passing through the glenohumeral joint, where possible excluding breast tissue. Leg volumes included all tissue types whereas appendicular lean body mass (ALBM) values were calculated from the sum of lean tissue within all leg and arm compartments.

A pencil-beam DEXA, Lunar DPX IQ™ (GE Medical Systems, Buckinghamshire, UK) was used for the first 30 patient studies. This model was up-graded before completion of the trial hence the Lunar Prodigy II was used for the last 4 participants. All patients who underwent repeat studies were scanned on the same equipment.

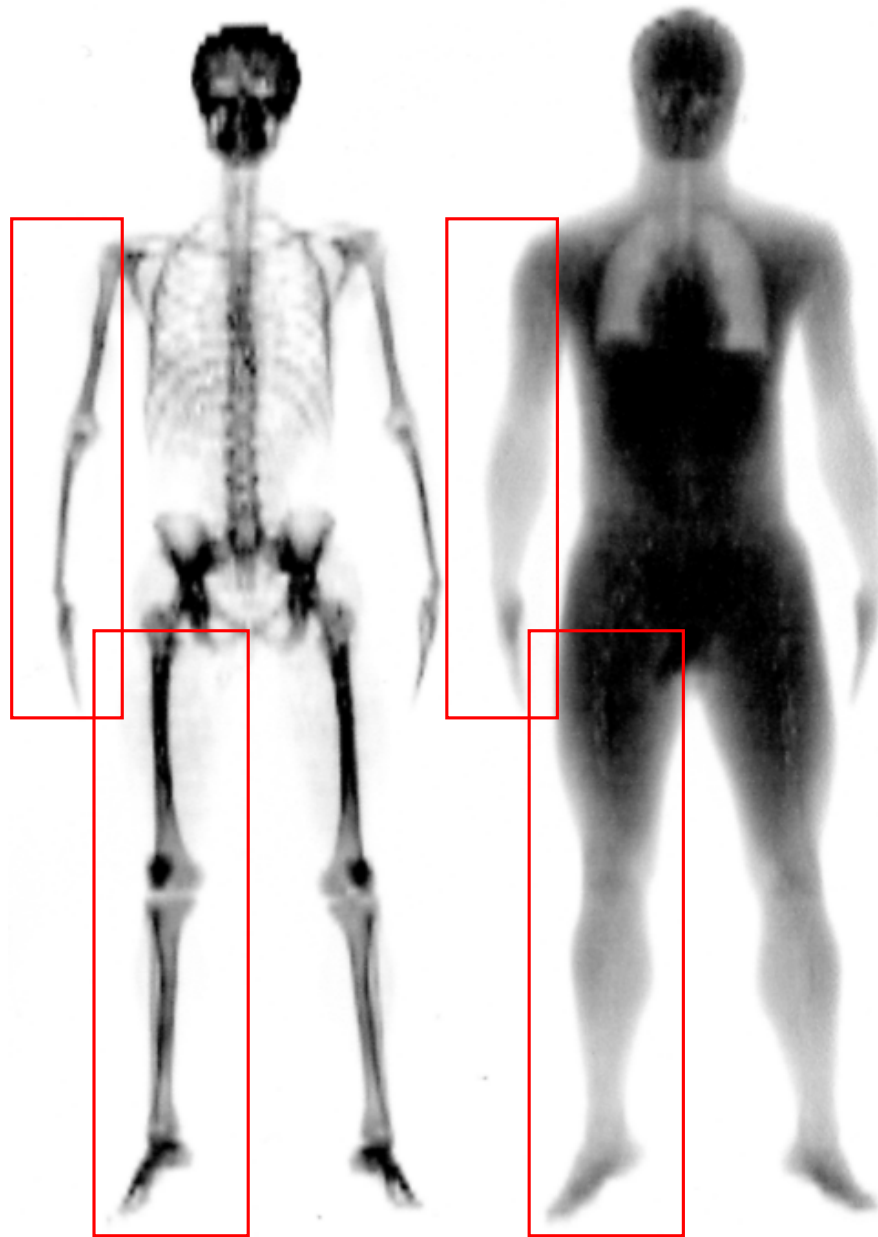


Figure 2.1 DEXA images of bone (left) and soft tissue (right) with unilateral arm and leg regions of interest marked (rectangles). Image taken from Lunar Prodigy II (GE Medical Systems, Buckinghamshire, UK).

### 2.1.4 Measurement of insulin resistance

Fasting whole blood glucose and plasma insulin concentrations were quantified (Section 2.2) for all healthy volunteer studies. Insulin resistance was calculated using both the homeostatic model assessment for assessment of insulin resistance (HOMA-IR) and the quantitative insulin sensitivity check index (QUICKI).

$$\text{HOMA-IR} = [\text{fasting insulin } (\mu\text{U}\cdot\text{ml}^{-1}) \times \text{fasting glucose } (\text{mmol}\cdot\text{l}^{-1})] / 22.5$$

(Matthews et al. 1985)

$$\text{QUICKI} = 1 / [\log \text{fasting insulin } (\mu\text{U}\cdot\text{ml}^{-1}) + \log \text{fasting glucose } (\text{mg}\cdot\text{dl}^{-1})]$$

(Katz et al. 2000)

Using the following conversion for glucose:

$$X \text{ mg}\cdot\text{dl}^{-1} \times 0.055 = Y \text{ mmol}\cdot\text{l}^{-1}$$

### 2.1.5 Stable isotope tracers

In order to determine AA kinetics, trace quantities of stable (non-radioactive) isotope labelled AA (the “tracer”; Figure 2.2) were administered intravenously. [1,2 <sup>13</sup>C<sub>2</sub>]leucine (99% <sup>13</sup>C) and D<sub>5</sub> phenylalanine (98% <sup>2</sup>H) were both manufactured by three different companies, dependent upon cost and lead time (Isotec, Sigma-aldrich, UK; CK Gas Products Ltd, Hampshire UK; Cambridge Isotopes Limited, Massachusetts, USA). Enriched leucine and phenylalanine were assessed to purity and sterility quality standards, prior to the manufacture of infusions. The required dose of each tracer was aseptically dissolved in 250 ml of 0.9 % saline, either individually or in combination

according to the specific protocol. The desired infusion rate for each tracer (Table 2.1) was determined by subject weight in order to obtain a constant level of precursor labelling at ~6 %; during AA feeding the tracer infusion rate increased pro rata to ensure a constant level of labelling. Appropriately certified hospital pharmacy departments carried out quality control and tracer manufacture.

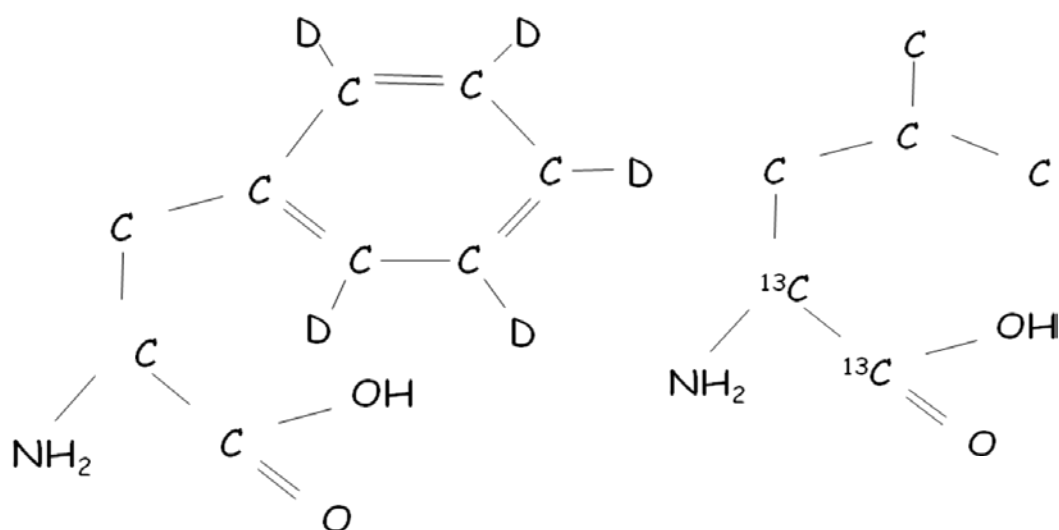


Figure 2.2 Molecular structure of [D<sub>5</sub>] Phenylalanine (left) and [1,2-<sup>13</sup>C<sub>2</sub>] Leucine (right)

Table 2.1 Tracer infusion rates

Tracer	Prime	Continuous infusion
[1,2- <sup>13</sup> C <sub>2</sub> ]leucine	0.75 mg.kg <sup>-1</sup>	1 mg.kg <sup>-1</sup> .h <sup>-1</sup>
D <sub>5</sub> phenylalanine	0.3 mg.kg <sup>-1</sup>	0.6 mg.kg <sup>-1</sup> .h <sup>-1</sup>

### 2.1.6 Sample collection

#### *Blood sampling*

The heated hand technique was used to obtain arterialized-venous blood samples as a safer alternative to direct arterial sampling. This technique, from the early 80's, involves placing the hand inside a heated box (~55 °C) to create an arterio-venous shunt (by vasodilation of veins in the fingers) enabling arterialized-venous blood sampling from a retrograde catheter in the dorsum of the hand (Abumrad *et al.* 1981).

Either a peripheral or femoral vein catheter was used to collect venous blood samples. The femoral vein catheter was inserted by the Seldinger technique (Seldinger 1953), using ultrasound imaging, for direct sampling of blood draining the leg. All venous and arterialized-venous blood samples were collected into pre-filled vacutainer tubes containing either ethylenediaminetetraacetic acid (EDTA), lithium heparin or clot activator. Samples were immediately stored on ice until centrifuged at 2000 x *g* for 20 min; plasma was stored in aliquots at -80°C for later analysis.

#### *Muscle sampling*

All 3 techniques for percutaneous muscle biopsy (needle (Bergström 1975), conchotome (Dietrichson *et al.* 1987) and Bard gun (Cote *et al.* 1992)) were observed by the author. Tissue samples obtained from the conchotome technique remained intact and were larger (~50 mg per sample) than those obtained by the other methods and volunteers appeared to tolerate the procedure better. Thus all biopsies were taken by the author using the Conchotome technique, following hands-on training (Figures 2.3 and 2.4).

Muscle biopsies were all taken from vastus lateralis, ~15 cm above the distal insertion, under local anaesthetic (0.5 – 1 % lignocaine), with subsequent biopsies taken at least 2 cm proximal to the last. At each time point three ~50 mg tissue samples were collected from the same biopsy site, at 120° from each other; samples collected for tracer and protein extraction were washed in ice-cold phosphate buffered saline (PBS) before snap frozen in liquid nitrogen. The sample collected for RNA extraction was immediately snap frozen, without washing. The biopsy sites were closed with 3.0 synthetic monofilament sutures (Ethilon, Johnson & Johnson) and covered with shower-proof dressing for at least 72 h. For the first 24 h, wounds were also dressed with a compression bandage (Coban, 3M, Berkshire, UK) to minimize bruising. Sutures were removed at 7 d.

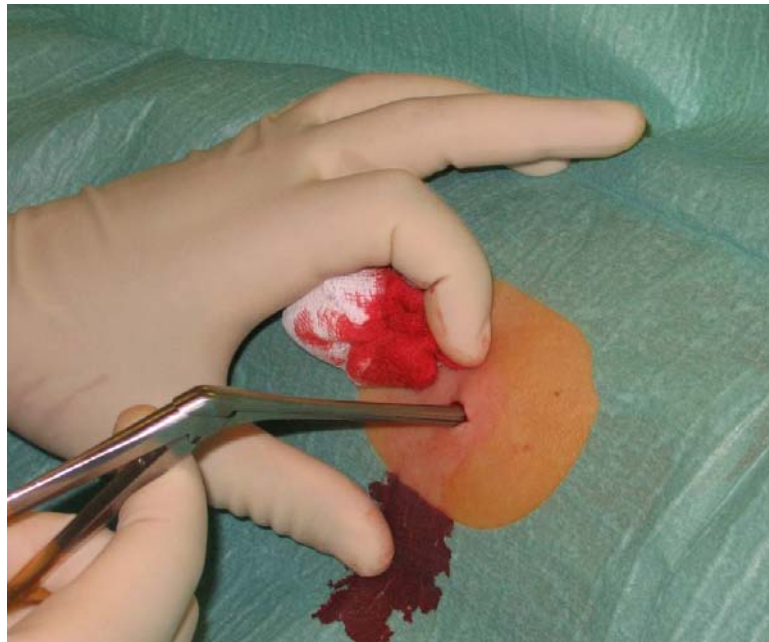


Figure 2.3 Muscle sampling from quadriceps muscle taken using the Conchotome technique.



Figure 2.4 Removal of biopsy tissue from forceps by an assistant.

### 2.1.7 Blood flow measurements

Pulsed wave doppler ultrasound (Nemio 17, Toshiba, Crawley, UK) was used to quantify blood flow through the common femoral artery. The angle of insonation was maintained at 60° or less and gate adjusted to ensure optimal sampling. Training for blood flow measurements was obtained from ultrasonographers within the clinical environment and adapted to maximize accuracy and reproducibility for the purposes of flow measurements.

### 2.1.8 Pancreatic clamp

To assess the effects of supraphysiological plasma insulin concentrations upon MPS in young and older individuals, the hyperinsulinaemic euglycaemic pancreatic clamp (DeFronzo, Tobin, & Andres 1979) was modified to achieve target insulin concentration of  $\sim 100 \mu\text{IU}\cdot\text{ml}^{-1}$ . Endogenous insulin production was suppressed by the continuous infusion of octreotide (Sandostatin, Novartis, Camberley, UK;  $30 \text{ ng}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ) and replaced with short acting insulin (Humulin S, Lilly, Basingstoke, UK;  $4.3 \text{ IU}\cdot\text{m}^2 \text{BSA}^{-1}\cdot\text{h}^{-1}$ ). The pancreatic clamp technique was modified for a second time to assess the effects of low postprandial concentrations of insulin upon MPB. Target plasma concentrations for this protocol were  $\sim 5$  and  $\sim 15 \mu\text{IU}\cdot\text{ml}^{-1}$  and achieved by administering exogenous insulin at  $2$  and  $10 \mu\text{IU}\cdot\text{m}^2 \text{BSA}^{-1}\cdot\text{min}^{-1}$  respectively.

For all insulin clamps  $\sim 5$  ml of the patients' blood was added to the diluent (50 ml 0.9 % saline, Baxter, Thetford UK) to minimize insulin adherence to the syringe and administration set. Arterialized-venous blood sampling was conducted at 5 min intervals to monitor blood glucose and enable titration of 20 % dextrose solution (Baxter, Thetford, UK) to maintain a glucose concentration of  $4.5 - 5.0 \text{ mmol}\cdot\text{l}^{-1}$ . After



the study, participants were given a high energy meal containing a mixture of simple and complex carbohydrates and warned of the signs and appropriate treatment of hypoglycaemia.

### 2.1.9 Amino acid feeding regimes

A mixed intravenous AA preparation (Glamin, Kabi Fresenius, Homburg, Germany) was used for both protocols and infused as per Table 2.2. Composition data is given in Appendix 3.

Table 2.2 Glamin infusion regimes

Protocol	Prime dose	Infusion rate	Aim
1	0.25 ml.kg	0.75 ml.kg <sup>-1</sup> .h <sup>-1</sup>	Double plasma [AA]
2	none	0.19 ml.kg <sup>-1</sup> .h <sup>-1</sup>	Maintain plasma [AA]

Ideally continuous AA concentration monitoring would have been carried out during each study, particularly where tight control of PA plasma AA concentrations were required. The AA analyser (Biochrom 30, Biochrom, Cambridge, UK) was not suited for this purpose as its run time per sample is ~20 min. Other workers have used a fluorometric assay to obtain results within 5 min (Chevalier *et al.* 2005); however, despite extensive experimentation, it was not possible to reproduce this technique with any accuracy. Therefore low dose insulin clamp studies were conducted on volunteers, to experiment with the timing and doses of an AA supplementation, based upon subject weight and glucose requirements, in order to maintain PA plasma AA concentrations.

### 2.1.10 Protocols for randomized placebo controlled trial of thalidomide use in patients with cachexia due to oesophageal cancer

*Study Design*

Change in TBW and LBM over a 6 w treatment period were sought as primary end-points and assessed using a randomized parallel arm double-blind placebo control trial. Secondary end-points, selected to demonstrate a mechanistic explanation to underpin expected changes in body composition and to assess drug tolerability were resting energy expenditure (REE), Karnofsky Performance Scale (KPS), Piper Fatigue Score (PFS), routine biochemistry, blood counts and symptoms profiles. Both investigator and participants were blinded to the allocated treatment arm throughout the study period and laboratory analysis (Figure 2.5).

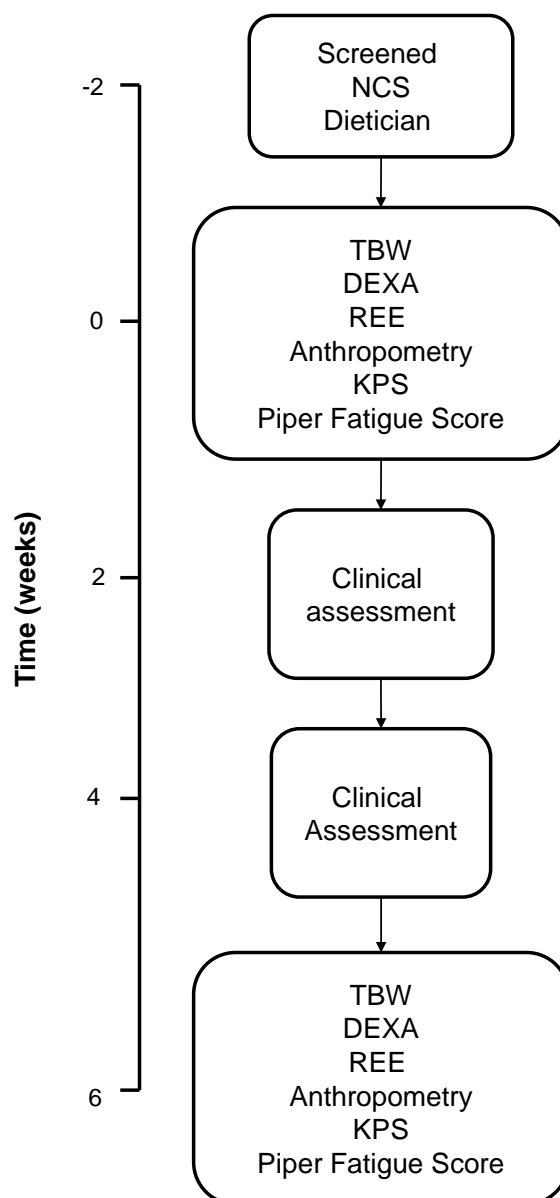


Figure 2.5 Protocol to investigate the effects of thalidomide upon body composition in patients with oesophageal cancer cachexia. (NCS nerve conduction studies; KPS Karnofsky performance scale; TBW total body weight; REE resting energy expenditure).

*Patients, inclusion and exclusion criteria*

Male and female adult patients with incurable oesophageal cancer and evidence of wasting were identified at a weekly multi-disciplinary meeting attended by an oncologist, gastroenterologist, gastrointestinal surgeon, radiologist and palliative care physician. Evidence of wasting was sought from weights recorded in the case notes and from patient recollection. All histological types were eligible, as were type 3 gastro-oesophageal junction tumours. Suitability for study participation was considered after case discussion and consideration of established oncological and surgical interventions. It was not deemed ethical to delay potentially curative surgery for 6 w for the study to complete pre-operatively and therefore individuals scheduled for surgery where not included. Patients offered palliative endoscopic treatments for moderate to severe dysphagia were selected for screening.

Contra-indications to thalidomide prescribing and factors preventing an adequate dietary intake underpinned the exclusion criteria. To eliminate the possibility of teratogenic sequelae pre-menopausal women were excluded and men were instructed to wear condoms during intercourse (thalidomide is excreted in seminal fluid). Thalidomide causes a progressive sensory neuropathy with prolonged use, thus all subjects were screened for existing peripheral nerve dysfunction. Subjects who demonstrated clinical evidence of neuropathy or had previous chemotherapy exposure underwent formal nerve conduction studies before entering the study. Sensory nerve action potential amplitudes of median, radial and sural nerves were recorded on two separate days to screen for axonal neuropathies and established a baseline for later comparison should patients develop neurological symptoms, as per thalidomide prescribing guidelines (Powell & Gardner-Medwin 1994).

In order to ensure participants were able to maintain adequate nutrition and complete the study significant untreated dysphagia (inability to swallow), on-going chemotherapy or radiotherapy and extreme frailty were exclusion factors. Dysphagia was assessed as per the Bown dysphagia score (Table 2.3) (Bown *et al.* 1987); patients with a score of 2 or less were included, as were those receiving enteral feeds (i.e. via a naso-gastric tube or percutaneous gastrostomy), whereas patients with grade 3 and 4 dysphagia attended for endoscopic thermoablative treatment or oesophageal stenting before randomization. Side-effects, such as nausea and anorexia, precluded patients with on-going chemotherapy and radiotherapy. A washout period of 4 w was required after oncological treatments to ensure metabolic effects, such as alterations in energy expenditure, had returned to baseline. Finally patients were assessed in terms of mobility and estimated life-expectancy to maximise the number able to attend the hospital for visits at baseline and 6 w for body composition measurements. Domiciliary visits at 2 w intervals were offered to patients to minimise the inconvenience of participation.

Table 2.3 Bown dysphagia score

0	Normal swallow
1	Difficulties with dry solids
2	Semi-solid diet
3	Liquid diet
4	Complete dysphagia

(Bown *et al.* 1987)

### *Study Drug*

Capsules of thalidomide and identical placebo were obtained as a gift from Pharmion Ltd, (Wales, UK) and stored by Derby hospitals NHS Foundation Trust Pharmacy. Male pharmacy staff were responsible for packaging and labelling both active drug and placebo and dispensed the appropriate trial medication according to the randomization protocol.

Subjects were told they were given sufficient capsules for the study duration and asked to bring their trial medications to each consultation to enable assessment of compliance. Four capsules provided 200 mg d<sup>-1</sup> of thalidomide or identical placebo. Subjects were advised to take their capsules at night to minimize the somnolence effects of treatment. At completion of the trial period all subjects were offered open-labelled thalidomide which was prescribed in accordance with Pharmion's risk management programme.

### *Dietetics*

Prior to randomization, the dietary intake of each subject was reviewed by a dietician, after which they were established on an isocaloric diet over a 10 d run in period, based on an estimation of the total daily energy content using the Schofield equation for REE (Schofield 1985) with a standard increment above the baseline to allow for activity. Dietary intake was monitored using a 4 d diet diary immediately before baseline testing and at 4 w.

*Resting energy expenditure*

Indirect calorimetry measured using a ventilated hood apparatus (Figure 2.6; GEM, NutrEN technologies Ltd, Burnley, UK) was used to determine REE in 29 patients (equipment was unavailable for the remaining 5 patients). Recordings of expired  $pO_2$  and  $pCO_2$  were collected over 20 min at rest. Values for REE were extrapolated over a 24 h period and reported per kg FFM, as determined by DEXA.



Figure 2.6. Volunteer demonstrating ventilated hood apparatus for measurement of resting energy expenditure and heated box for arterialized-venous blood sampling.

*Functional measurements and symptom profiles*

At each visit patients were directly questioned about symptoms of disease progression and adverse events. Patients were asked about appetite, ability to swallow, nausea, vomiting, constipation and excessive tiredness. A full examination was conducted at each visit, including neurological assessment, to document disease progression, identify concurrent illnesses, and detect adverse effects of the trial drug. New symptoms were classified as disease progression or drug side-effect and dealt with accordingly. Where symptoms were attributed to the study drug, the dose was reduced in the first instance (where possible) and only discontinued if symptoms persisted.

The KPS (Appendix 2) (Karnofsky 1950), a graded index of physical function ranging between 100 % (full health) and 0 % (death), was recorded at each visit by the author. Whereas the PFS questionnaire (Appendix 4) (Piper *et al.* 1998) validated to measure subjective fatigue patterns, was completed independently by the subject. This research instrument comprises of 22 questions each scaled from 0 to 10 which subdivide into 4 dimensions: behaviour/severity, affective meaning, sensory and cognitive/mood. Data collation was conducted in a blind fashion at the end of the study.



### 2.1.11 Protocols for healthy volunteer studies

#### *Protocol 1 Is anabolic blunting overcome in hyperinsulinaemic states?*

This study was designed to compare the anabolic response of skeletal muscle to mixed AA feeding under hyperinsulinaemic conditions in young and older healthy volunteers. Male and female healthy volunteers aged between 20 and 30 y (young) or 60 - 75 y (older) were included in this study. See Figure 2.7 for sampling protocol.

#### *Protocol 2 Does age affect the degree of suppression of proteolysis by insulin?*

For this protocol, target insulin concentrations of  $\sim 5 \mu\text{IU}\cdot\text{ml}^{-1}$  and  $\sim 15 \mu\text{IU}\cdot\text{ml}^{-1}$  were sought, each for 1.5 h period, while AA were maintained at PA concentrations (Figure 2.8). To ensure sub-clinical diabetes mellitus was not present, fasting insulin and glucose concentrations were measured as a screening tool. The age ranges for young and older healthy volunteers were 18 - 33 y and 60 - 75 y, respectively.

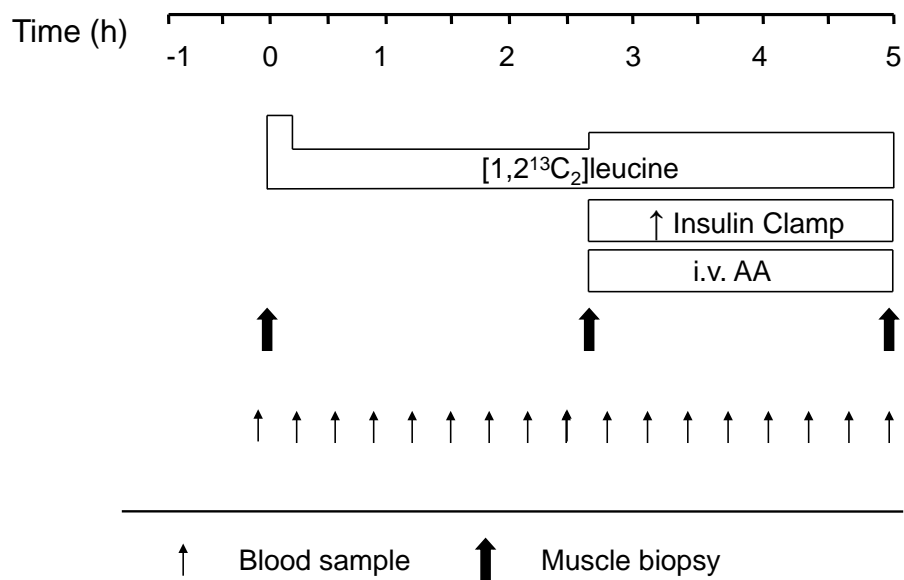


Figure 2.7 Protocol to assess the effects of high dose insulin and intravenous AA feeding upon muscle protein synthesis, in young and older healthy volunteers (Protocol 1).

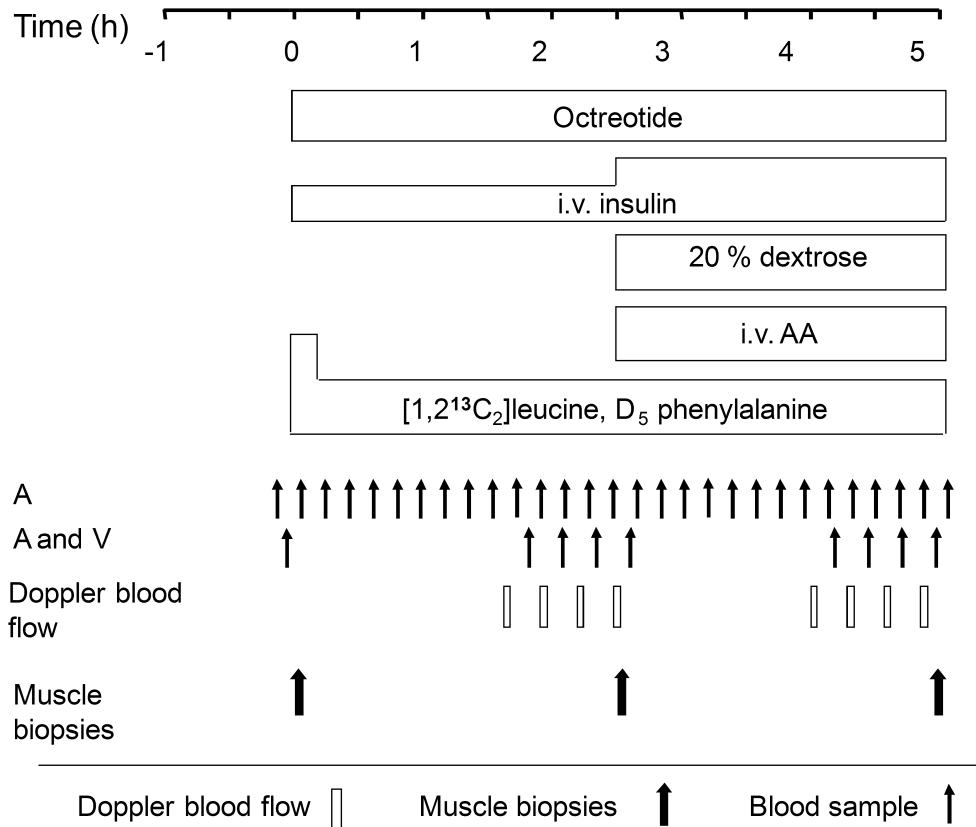


Figure 2.8 Protocol to assess leg proteolysis at plasma insulin concentrations of  $\sim 5$  and  $\sim 15 \mu\text{U}\cdot\text{ml}^{-1}$  in young and older healthy volunteers (Protocol 2). Arterialized-venous blood (A) was collected at  $\sim 5$  min intervals to monitor blood glucose concentrations infusions. Paired samples of arterialized-venous and femoral vein blood (A and V) were collected at baseline and 20 minute intervals through the last hour of each clamp period (small arrows), preceded by doppler blood flow measurements (rectangles). Muscle biopsies are indicated by large arrows.

## 2.2 Laboratory methods

### 2.2.1 General chemicals

All chemicals, unless otherwise specified, were sourced from Sigma-Aldrich (Poole, UK).

### 2.2.2 Analytical methods for blood

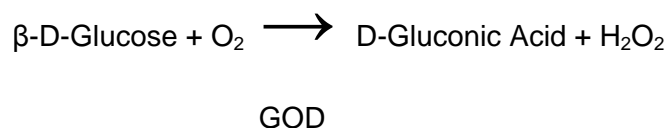
#### *Hormone and cytokine assays*

Concentrations of plasma insulin (BioSource Europe S.A.), C-reactive protein (CRP) (BioSource Europe S.A), TNF $\alpha$  (Bender MedSystems) and IL-6 (DRG instruments GmbH, Germany), were determined by enzyme-linked immunosorbant assays (ELISA). Each assay was processed according to the manufacturers' protocol.

In brief, monoclonal (capture) antibodies, directed against distinct epitopes on the test hormone, were pre-bound to a 96-well plate, to which the calibrators and samples were added prior to a secondary antibody labelled with horseradish peroxidase (HRP). The TNF $\alpha$  ELISA required an amplification step at this stage in order to detect low serum levels, but otherwise all protocols were not dissimilar. Any unbound antibody was washed off the plate before a chromogenic solution, which reacts with HRP, was added. The reaction was stopped with hydrochloric acid and the optical density read at 450 nm against a reference filter set at 650 nm (Multiskan Ascent plate reader with Ascent software v.2.6, Thermo Scientific, UK) to generate a 4 parameter logistic standard curve from which the sample values could then be read.

*Whole blood glucose concentration*

Whole blood samples were used for real-time glucose analysis during clamp studies. Approximately 200  $\mu\text{l}$  of blood was mixed for  $\sim 2$  min with fluoride, heparin and nitrite, in pre-prepared tubes (GMRD-034, Analox instruments, London, UK) until oxidized as indicated by a chocolate-brown discolouration. A sample of 10  $\mu\text{l}$  was injected into the GM7 Stat Analyzer (Analox Instruments, UK) for measurement of oxygen consumption as per the following reaction:



GOD = glucose oxidase

Both 5 and 8 mM standards were used as calibrants.

*Free fatty acid measurement*

Plasma free fatty acids were measured using a colorimetric assay (Roche Applied Science, Indianapolis, USA).

*Plasma amino acid labelling*

To determine labelling (atoms % excess; APE) and concentration of arterialised-venous and venous leucine, phenylalanine and  $\alpha$ -ketoisocaproate ( $\alpha$ -KIC), plasma was deproteinised with 100 % ethanol, dried and, for amino acid analysis, re-suspended in 0.5 M HCl. Lipids were removed by ethyl acetate extraction and then converted to their *tert*-butyldimethylsilyl (t-BDMS) derivatives.  $\alpha$ -KIC was converted to its quinoxalinol

derivative and separated by extraction into ethyl acetate, which was then evaporated and the t-BDMS derivative prepared. Concentrations and enrichments were determined by gas chromatography-mass spectrometry (GC-MS) using a Trace DSQ GC-MS (Thermo Fisher Scientific, Hemel Hempstead) using appropriate internal standards.

#### *Amino acid analysis*

Equal volumes of plasma and 10% sulphosalicylic acid were mixed and cooled at 4°C for 30 min. The samples were spun to remove the precipitated protein and passed through a 0.22 µm filter prior to analysis on an amino acid analyser (Biochrom 30, Biochrom, Cambridge, UK) using a lithium buffer separation. All 20 AA concentrations were determined by comparison to a standardized sample, using norleucine as an internal standard.

### **2.2.3 Analytical methods for muscle**

#### *Myofibrillar protein isolation*

Approximately 30 mg of muscle was minced finely using pointed scissors in ice-cold extraction buffer (0.02 M Tris, 0.15 M NaCl, 0.1 M EDTA, 0.1% Triton X). The homogenate was centrifuged at  $1,600 \times g$  for 20 min to remove the supernatant with subsequent resuspension of the myofibrillar/collagen pellet in 0.3 M NaOH. The soluble myofibrillar protein and the insoluble collagen were separated by centrifugation. The myofibrillar fraction was precipitated using 1M perchloric acid and the pellet washed twice with 70 % ethanol. Myofibrillar protein was hydrolysed in 0.05 M HCl/Dowex 50W-X8-200 at 110°C overnight (Balagopal, Nair, & Stirewalt 1994), and the liberated amino acids were purified then eluted in 2 M NH<sub>4</sub>OH. The amino acids were

subsequently derivatized as their *N*-acetyl-*n*-propyl (NAP) ester (Meier-Augenstein 1999). Leucine labelling was analysed by capillary gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS; Delta-plus XL, Thermo Fisher Scientific, Hemel Hempstead, UK); separation was achieved on a 25 m x 0.25 mm x 1.0  $\mu$  film DB 1701 capillary column (Agilent Technologies, West Lothian, UK).

#### *Extraction of protein for Western Blotting*

For protein extraction 30 - 40 mg of each muscle sample were minced then homogenized for 30 sec in ice cold buffer (10  $\mu$ l.mg<sup>-1</sup> muscle) containing 50 mM Tris HCl, 0.1 % Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.1 % 2-mercaptoethanol, 10 mM  $\beta$ -glycerophosphate, 0.5 mM sodium orthovanadate and complete protease inhibitor cocktail (Roche). A Bradford assay was used to quantify the protein concentration of the supernatant prior to denaturing with Laemmli buffer at 95°C for 5 min.

For PKB kinase activity, PKB was immunoprecipitated from 300  $\mu$ g protein before incubation with 1 mM ATP and GSK3 $\beta$  fusion protein substrate (New England Biolabs, Crawley, UK). Total and phosphorylated protein concentrations of PKB<sup>Ser473</sup>, eEF2<sup>Thr56</sup>, P70S6K<sup>Thr389</sup>, 4E-BP1<sup>Thr37/46</sup> and FOXO1a<sup>Ser256</sup> were quantified as follows. Fifty micrograms of each protein sample (or 15  $\mu$ l for kinase reactions) was loaded on to a 7-15 % SDS-PAGE gel (Criterion XT Bis-Tris; Bio-Rad, Hemel Hempstead, UK) for electrophoresis at 150 V for ~75 min. Following electroblotting to a PVDF membrane (Bio-Rad), proteins were incubated for 1 h with 5 % powdered milk in TBS-T (Tris buffered Saline and 0.1 % Tween-20) before incubation overnight with the primary antibody at 4°C (New England Biolabs, Herts, UK). The membranes were washed with TBS-T and incubated for a further 1 h at room temperature with the HRP-conjugated

anti-rabbit secondary antibody (New England Biolabs, Herts, UK), before further washing with TBS-T and incubation for 15 min with ECL reagents (enhanced chemiluminescence kit, Immunstar; Bio-Rad, Hemel Hempstead, UK). Blots were imaged and quantified using Chemidoc XRS system (BioRad, Hemel Hempstead, UK).

#### *Extraction of RNA*

RNA was extracted from ~20 mg of each muscle sample using 0.5 ml Tri-reagent as per the manufacturer's protocol. Integrity and purity of RNA was assessed by gel electrophoresis and luminescence. QuantiTect reverse transcription kit (Qiagen Ltd, Crawley, UK) was used for cDNA synthesis and genomic DNA elimination. Real-time PCR was performed using SYBR Green supermix (BioRad, Hemel Hempstead, UK) and primers (MWG, Ebersberg, Germany) as per Table 2.4. Reverse transcription and PCR were conducted using the ICyclerIQ (BioRad, Hemel Hempstead, UK). Primers were, in the most part, individually designed and tested previously.

Table 2.4 Gene primers used for real-time PCR. All 5'-3'

Gene	Sense	Anti-sense
MuRF1	AGTGACCAAGGAGAA CAGTCA	CACCAGCTTTGTGGACTTGT
MAFBx	TCCTGGAAGGGCACTGAC	CCATATTCCCAGCTCTCCAG
C2 subunit	CATTGAAAAGGGCGCAATC	GCCATATCGTTGTGTTGGTA
GLUT4	CAGTATGTTGCGGAGGCTAT	CCTCGAGTTTCAGGTA CTCTT
Cathepsin L	TGAAGAATCAGGGTCAGTGTGG	TGAGGCCCCAGAGCAGTCTAC
Ubiquitin	GATCTGCCGCAAGTGCTA	CAGTCAATGAAA GGGACACT
GAPDH	CTGACCTGCCGTCTAGAAAA	GCCAAATTCGTTGTCATACC



### 2.2.5 Calculations

#### *Fractional synthetic rate of muscle*

The rate of MPS was calculated using the following equation:

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

(Nair, Halliday, & Griggs 1988)

where  $\Delta E_m$  is the change in labelling of muscle protein leucine between two biopsy samples,  $E_p$  is the mean enrichment over time of the precursor for protein synthesis (taken as venous  $\alpha$ -KIC  $^{13}\text{C}_2$  labelling), and  $t$  is the time between biopsies. Venous  $\alpha$ -KIC was chosen to represent the immediate precursor for protein synthesis, i.e. leucyl-tRNA (Watt *et al.* 1991).

#### *Arterio-venous AA flux*

Leg protein flux (i.e. breakdown; LPB) was calculated from the arterio-venous dilution of  $\text{D}_5$  phenylalanine and  $[1,2 \text{ } ^{13}\text{C}_2]$ leucine tracers using the following equation:

$$\text{LPB} = [(E_a / E_v) - 1] \times C_a \times \text{blood flow}$$

(Bennet *et al.* 1990a)

where  $E_a$  and  $E_v$  are the values of amino acid labelling at steady state in arterial and femoral venous plasma,  $C_a$  is the mean concentration in the arterial blood with blood flow in  $\text{ml} \cdot 100 \text{ ml leg}^{-1}$ , adjusted for the haematocrit and leg volume (assessed by DEXA). The net amino acid balance was calculated as the difference in arterial and venous concentrations multiplied by the flow.

## 2.3 Statistical analysis

### 2.3.1 Sample size calculations

For healthy volunteer studies a sample size of 8 participants in each group was calculated from a formal power calculation, with  $\alpha$  set at 0.05 and power at 80 %. The data for this calculation was based upon a substantial number of muscle metabolism studies of normal healthy young and older men and women conducted by the research group. For repeated measures of muscle protein labelling in the same sample, taken through the entire extraction and analysis process, the coefficient of variation (CV) is ~3.8 %. The population CV is ~ 10 %, for young men and women and 12 % for older men, thus a common value of 15 % was adopted. The CV of mRNA expression is approximately 30 %, and therefore well below the changes in mRNA expression seen after a physiological intervention in muscle of up to 4 fold. For western analysis CV are approximately 20 % in healthy young and older adults.

A power calculation was again used for the patient study to determine an adequate sample size ( $n = 17$ ). Power was set at 90 % to detect a significant change in TBW and LBM as small as 2 % change, with significance set at 5 %. This assumed a standard deviation for the measurement of LBM of each patient of at most 1.8 % of body weight, although, in practice, it was expected that the standard deviation would be less than 1.8 %, giving more credence to the estimation of power as being more than 90 %. Participants were randomized to receive active drug or placebo after screening using a randomization protocol generated by an independent statistician using block randomization (blocks length of 4). Drug allocation was concealed until all participants had completed the study and data points had been recorded.

### 2.3.2 Data analyses

Data was analyzed using Prism version 5 (Graphpad, San Diego, CA) and SPSS version 14.0, (SPSS Inc.). Tests for normality (Shapiro-Wilk, Kolmogorov-Smirnov & Q-Q plots) were used to assess whether or not data was distributed in a normal pattern. A Gaussian distribution was found for much of the healthy volunteer data and as such means  $\pm$  standard error of the mean (SEM) and parametric tests, specifically t-tests and ANOVA with Bonferroni post hoc test, are presented. Significance was assigned at  $P < 0.05$  for all data and two-tailed tests have been used throughout, unless specifically stated.

For the randomized controlled trial an intention to treat analysis was performed albeit limited by missing data where patients were unable to complete the protocol. Tests for normality confirmed the data was not distributed in a normal pattern; therefore medians, interquartile ranges (IQR) and non-parametric tests were used, specifically Mann-Whitney test, Wilcoxon signed ranks test and Pearson Chi-Square test. Survival comparisons were made between groups using Kaplan-Meier plots and log rank tests.

## **Chapter 3**

**Randomized placebo controlled trial of thalidomide use in patients with cachexia due to oesophageal cancer**

Over 7,500 individuals are diagnosed with oesophageal cancer in the UK each year and only 10 % survive to 5 years beyond diagnosis despite surgical and oncological treatments. Profound skeletal muscle wasting is a common feature of the disease (despite effective endoscopic management of dysphagia and nutritional supplementation) and results in a poorer prognosis (DeWys 1982; Di *et al.* 2006). Established treatments for wasting are limited to supplements, megestrol acetate, and corticosteroids. At best these agents may increase adipose tissue, but fail to stimulate skeletal muscle synthesis or slow muscle protein breakdown; hence novel treatment strategies are required.

Preliminary clinical data suggests thalidomide is an effective treatment in cachexia; however, to date there is insufficient evidence to advocate widespread use in oesophageal cancer cachexia. This study was designed to test the hypothesis that thalidomide increases total body weight and lean body mass in patients with oesophageal cancer, over and above body changes seen in patients taking placebo.

## **3.1 Results**

### **3.1.1 Patient Flow**

Of 126 patients which were identified as eligible 84 declined entry into the study, 6 were excluded due to neuropathy or a clinical deterioration, and a further 2 died prior to randomization. Thirty-four patients were randomized to receive thalidomide or placebo. Eight patients in the thalidomide arm were able to attend for repeat studies at week six, 2 of whom had discontinued treatment due to toxicity; a further 2 patients were able to tolerate treatment but were too unwell to attend for repeat studies. The remaining

patients withdrew participation due to drug toxicity or disease progression. Sixteen patients who received placebo were able to complete the protocol; one patient had died unexpectedly in his sleep one week after entry.

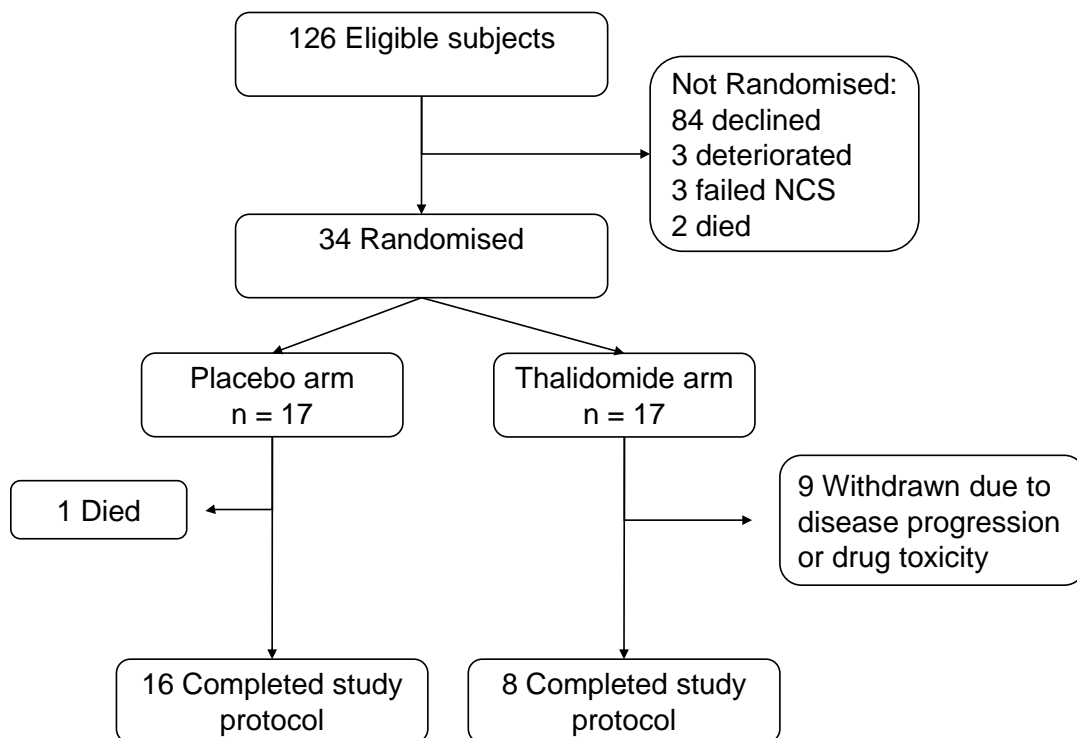


Figure 3.1 Patient flow

### 3.1.2 Baseline demographics

The groups were well matched in all areas except body composition; the thalidomide group was significantly heavier in terms of TBW, LBM and MAMC (Table 3.1).

Table 3.1 Group demographics at baseline. Values are median (IQR) unless count (%) denoted by \*

	Thalidomide n = 17	Placebo n = 17	<i>P</i>
Sex (M:F)*	15:2	13:4	ns
Age (y)	68.0 (57.7,80.2)	65.9 (58.9,79.9)	ns
Histology *			
Adenocarcinoma	14 (82.4)	11(64.7)	ns
Squamous Cell	2 (11.8)	4 (23.5)	ns
Undifferentiated	1 (5.9)	2 (11.8 )	ns
PreviousTreatments *			
Oesophagectomy	4	4	ns
Radiotherapy	4	3	ns
Chemotherapy	5	8	ns
Karnofsky score	70 (60,80)	80 (70,90)	ns
Piper fatigue score	6.0 (3.9,7.8)	4.5 (3.1,5.4)	ns
C-Reactive Protein	31.3 (11.1,46.8)	23.7 (2.8,39.3)	ns
TNF $\alpha$	3.59 (2.30,5.34)	2.23 (1.83,4.02)	ns
Haemoglobin (g.dl <sup>-1</sup> )	11.3 (10.2,12.0)	10.1 (9.5,12.5)	ns
Albumin (g.l <sup>-1</sup> )	32.0 (28.0,36.8)	32.0 (27.5,36.0)	ns
BMI (kg.m <sup>-2</sup> )	23.1 (19.8,25.2)	19.1 (17.2,20.4)	<0.01
TBW (kg)	64.0 (60.1,72.0)	50.7 (47.0,59.9)	<0.01
LBM (kg)	50.2 (44.7,51.8)	42.2 (39.0,45.8)	<0.01
MAMC (cm)	23.5 (22.4,26.0)	20.1 (19.5,22.1)	<0.01

### 3.1.3 Body composition changes

Over the 6 week study period TBW and LBM did not differ significantly from baseline in either study group (Figure 3.2), median change in TBW 0 kg (-0.85, 1.75) and -1 kg (-3.85, 0.30) for placebo and thalidomide arms respectively. Availability of additional MAMC data points failed to show muscle mass changes at 2 or 4 weeks of treatment (Figure 3.3). Furthermore there was no difference in TBW or LBM between the treatment groups (all  $P > 0.05$ ).



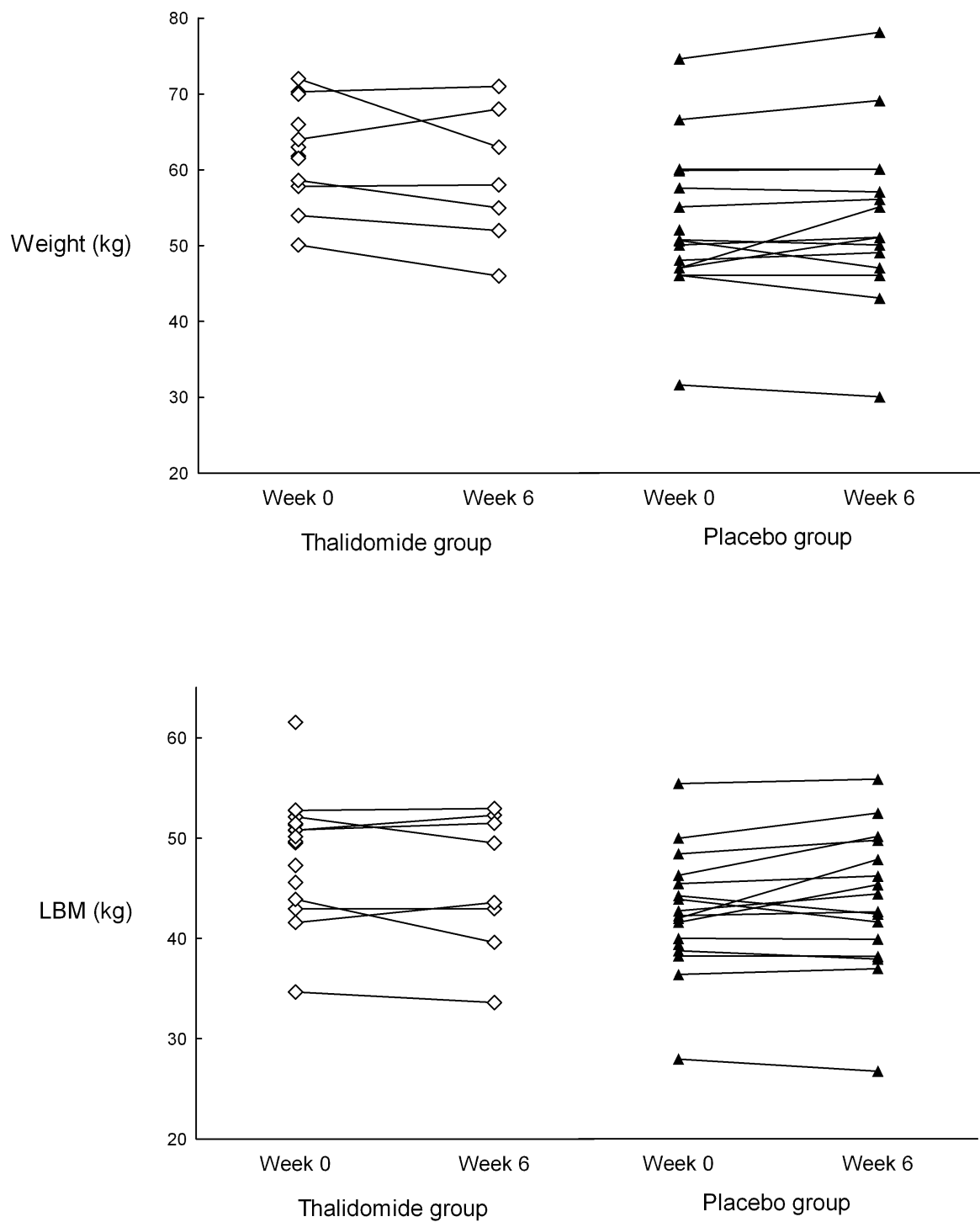


Figure 3.2 Total body weight and lean body mass (LBM) at baseline and after treatment with either thalidomide (open diamonds) or placebo (closed triangles) for 6 weeks.

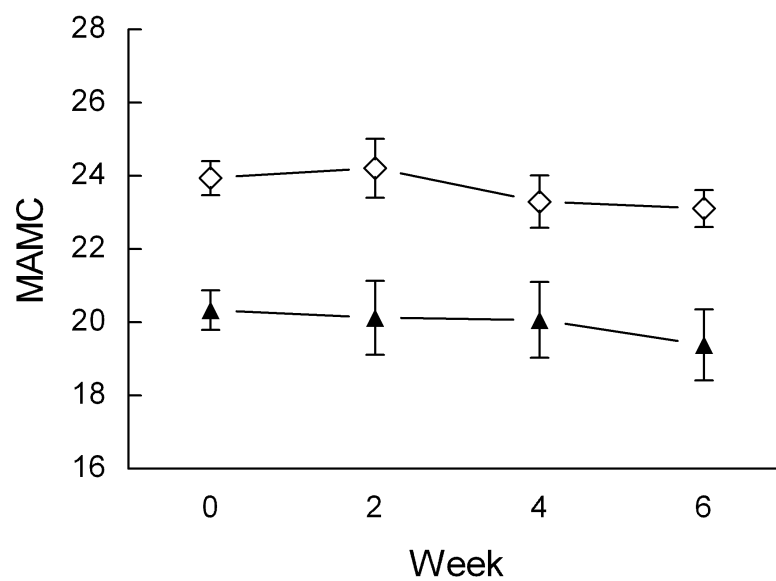


Figure 3.3 Change in mid-arm muscle circumference (MAMC) in patients taking thalidomide (open diamonds) or placebo (closed triangles).

### 3.1.4 Metabolic studies

Resting energy expenditure per kg LBM did not differ significantly between the study groups at baseline: Median REE in kcal per Kg LBM per day was 32.8 (28.3, 36.7) and 33.6 (32.0,37.3) kcal.kg LBM<sup>-1</sup>.d<sup>-1</sup> for patients taking placebo and thalidomide respectively (Figure 3.4). Follow up data was available for 13 patients on placebo and only 6 patients on thalidomide; the placebo group demonstrated a statistically (but not clinically) significant increase from baseline readings ( $P = 0.02$ ). There was no difference from baseline in the thalidomide group.

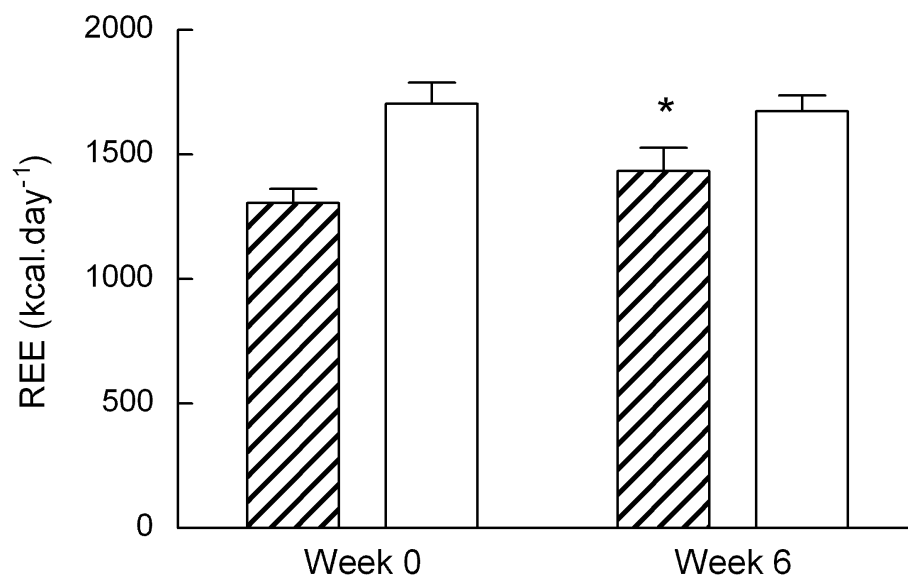


Figure 3.4 Change in resting energy expenditure (REE) in patients taking thalidomide (open bars) or placebo (hashed bars). Asterisk denotes significant change compared to week 0 values ( $P < 0.05$ ).

### 3.1.5 Survival

The mean time to death of all patients was 109 (43.3,174.7) days from the date of enrolment. Survival was not affected by group allocation, or whether the patient was able to complete the protocol (Figure 3.5).

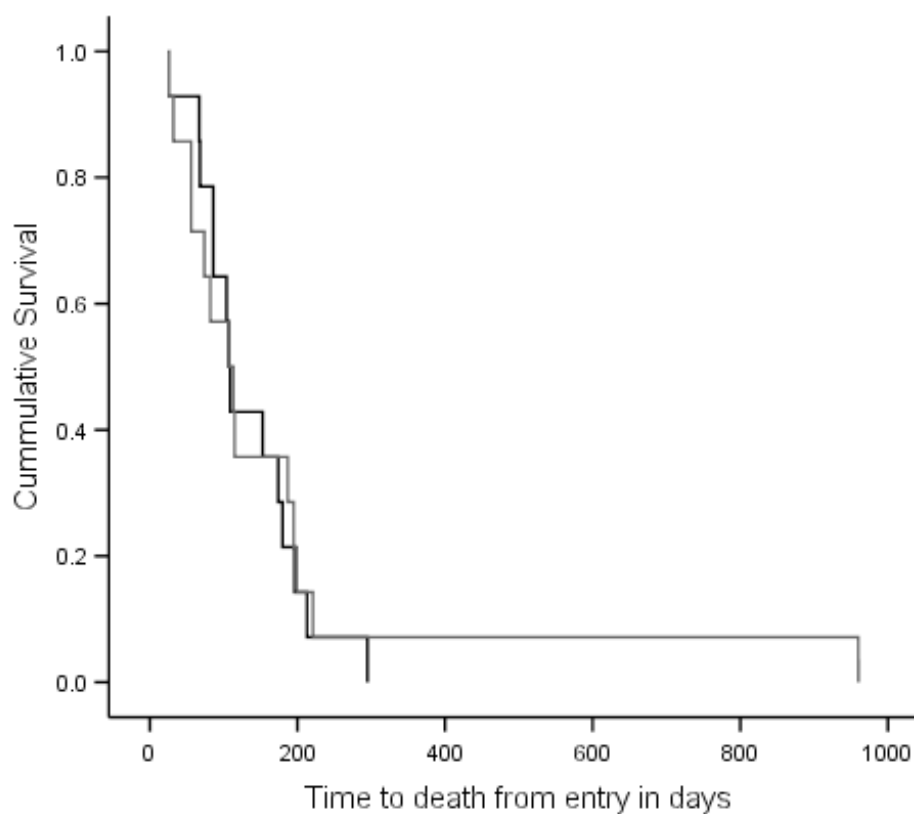


Figure 3.5 Kaplan-Meier survival curves for patients taking either thalidomide (grey line) or placebo (black line).

### 3.1.6 Performance indices

The median Karnofsky score at baseline was 10 points higher in the group given placebo (Table 3.2), albeit not reaching statistical significance and scores remained relatively constant over the study period. Individual components of the Piper fatigue scores did not differ between the groups at baseline. Limited complete data sets were available (4 for thalidomide recipients and 10 for placebo recipients) for repeated measures analysis. Of those available, only the severity component increased significantly in the placebo arm ( $P = 0.03$ ), whereas affect and sensory components increased in the thalidomide arm ( $P = 0.02$  and  $0.05$  respectively; Figure 3.6). There was no significant difference between the groups.

Table 3.2 Karnofsky Scores at baseline and two weekly intervals. Median and IQR given.

	Placebo	Thalidomide
Baseline	80 (70,90)	70 (60,80)
Week 2	80 (70,90)	70 (65,90)
Week 4	80 (70,90)	70 (50,70)
Week 6	80 (65,80)	60 (45,75)

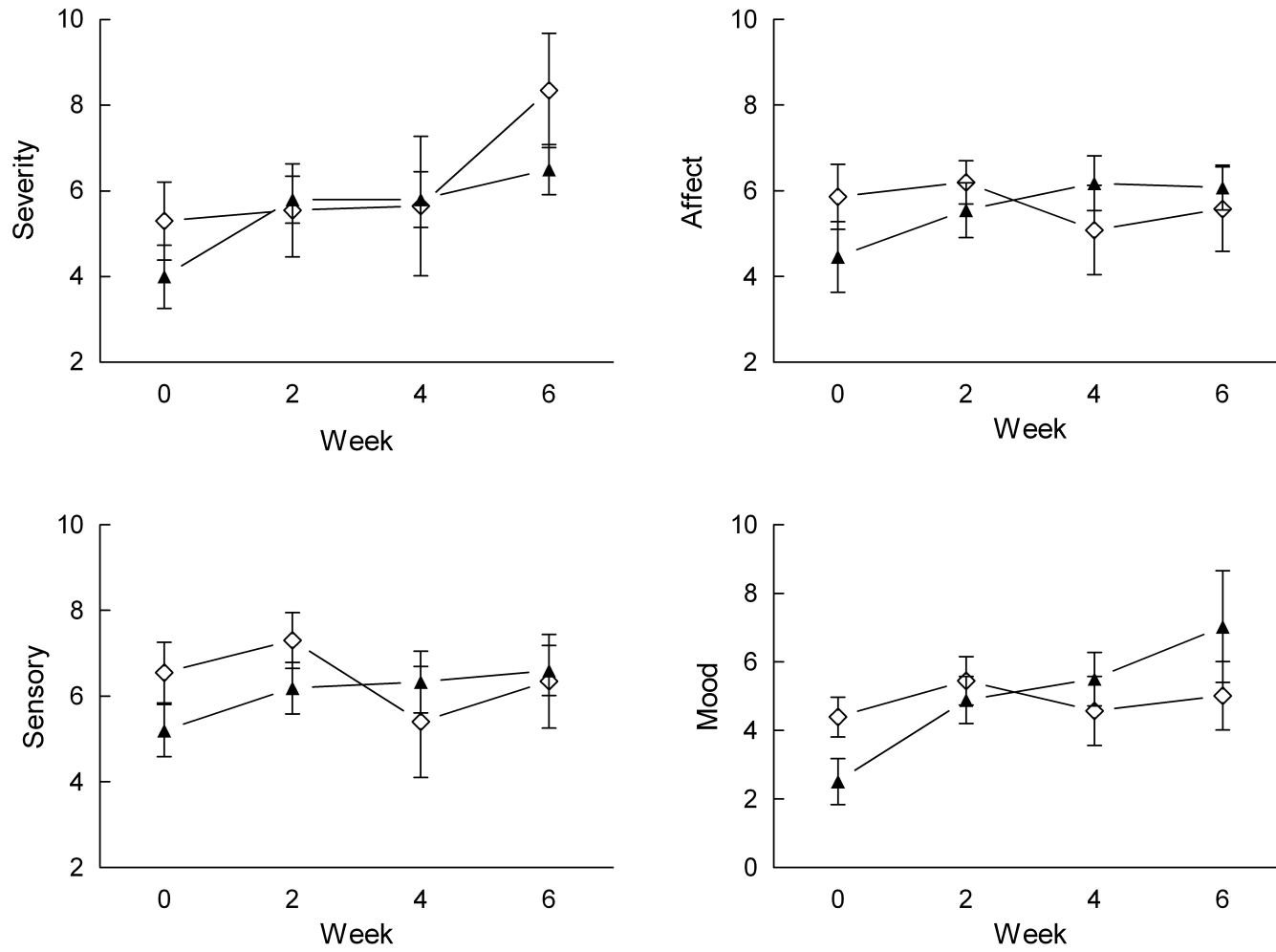


Figure 3.6 Change in piper fatigue scores over time presented as individual components. Mean  $\pm$  SEM given; all  $P > 0.05$  when groups compared.

### 3.1.7 Biochemistry

The concentration of circulating TNF $\alpha$  and CRP did not differ between the groups at baseline, nor did they change with treatment, whereas albumin levels dropped in both groups. The reduction was greatest in patients taking thalidomide (35.3 to 31.6 g.l<sup>-1</sup>,  $\Delta$  3.8 g/L;  $P = 0.01$ ) and was not significant in the placebo group (31.3 to 29.8 g.l<sup>-1</sup>,  $\Delta$  1.5 g.l<sup>-1</sup>;  $P = 0.12$ ); differences between the groups approached statistical significance ( $P = 0.058$ ).

### 3.1.8 Drug toxicity

Drug toxicity was experienced by 6 patients (some with multiple adverse events) and, in all but one case, resolved upon cessation of treatment (summarized in Table 3.3).

Table 3.3 Drug toxicities

Adverse event	Number of cases
Cutaneous reactions	3
Headache with vomiting	1
Neutropenia	1
Hypersomnolence	1
Venous thrombo-embolic events	2
Parasthesia	3

Three patients experienced cutaneous reactions within 1 week of enrolment; 1 patient developed a pruritic maculo-papular rash involving the limbs and trunk; a second giddiness and a macular non-pruritic rash (Figure 3.7); a third patient developed an

eczematous reaction with peri-orbital and limb dermatitis which did not resolve on cessation of thalidomide but required oral prednisolone for 8 weeks (Figure 3.8).

One patient experienced severe headaches, nausea and vomiting at 3 weeks, resembling raised intracranial pressure but brain CT was normal and symptoms resolved on drug withdrawal. Hypersomnolence was reported by one patient who required dose reduction to 100 mg nocte. In a further case neutropenia occurred after 4 weeks of treatment (absolute neutrophil count  $0.47 \times 10^9 \cdot l^{-1}$ ) and resolved within 7 days of stopping thalidomide.

Confirmed venous thrombo-embolic events (VTE) occurred in two patients receiving the active drug. Bilateral pulmonary emboli were diagnosed on CT pulmonary angiography 15 days after commencing therapy in the first patient and a deep vein thrombosis was diagnosed 7 days after trial completion in the second.

Three patients developed parasthesia on treatment. One patient developed symptoms after 2 weeks of thalidomide, but chose to continue on the background of normal nerve conduction studies. On trial completion he elected to take open-labelled thalidomide for a further 6 months without evidence of progressive nerve toxicity. Two patients who had also elected to take open-labelled thalidomide developed parasthesia after study completion. In both cases the patients discontinued thalidomide and experienced complete resolution of symptoms.





Figure 3.7 Macular rash presenting on the lower limb within 4 days of thalidomide use



Figure 3.8 Eczematous reaction with peri-orbital dermatitis following thalidomide use.

## **3.2 Discussion**

### **3.2.1 Body Composition changes**

The high attrition rate limited availability of paired body composition data preventing conclusions from being drawn as to the efficacy of thalidomide in oesophageal cancer cachexia. Although the participants who received placebo did not demonstrate loss of TBW and LBM, as anticipated, indeed 7 patients taking placebo gained weight during the study period, compared with 2 patients taking thalidomide. Furthermore, 9 patients gained or maintained LBM on placebo. As the thalidomide prescribed cohort had a greater BMI (23 vs.19 kg.m<sup>-2</sup>) and LBM, difference between the groups are unlikely to be responsible. It is plausible that the placebo group gained lean tissue due to an increase in physical activity (perhaps reflected by the increase in REE), combined with an adequate dietary intake, both facilitated by the positive psychological effects of study participation and regular dietetic support. In contrast patients taking thalidomide were less able to experience this psychological effect or indeed drug benefit due to drug related toxicity. Alternatively, these findings may simply reflect a type 1 error in a small sample.

### **3.2.2 Functional indices**

Despite a greater LBM, participants randomized to the thalidomide arm had a lower Karnofsky score at baseline (median 70 versus 80), which may have contributed to the higher morbidity in patients receiving thalidomide. During the course of the study performances indices fell in the thalidomide group but it is not possible to dissect out how much of this decline in well-being is due to disease progression compared with that due to thalidomide related side-effects, bar commenting on the relative stability of scores within the placebo arm. The number of participants recruited was determined by

the primary end-points rather than functional outcome measures *per se* and so the sample is likely to be too small to accurately comment, particularly as so few participants were able to complete the protocol in the thalidomide group.

Piper fatigue questionnaire data demonstrated an increase in fatigue in both groups over the 6 week study period, reflecting the declining health of participants. Scores for severity indicate the interference of fatigue upon normal activities, such as ability to work or socialise; affect describes the degree to which the patient finds fatigue unpleasant and abnormal; energy levels are reflected by the sensory component and cognitive functions are indicated by mood scores. The increase in fatigue severity in both groups suggests the symptom is disease related rather than treatment, whereas affect and sensory components appear to be specific adverse effects of thalidomide treatment. Entry into the study is a confounding factor upon the mood component and likely to give falsely low values at baseline for all participants; as the study progressed mood also deteriorated in the placebo arm but not in the thalidomide arm.

### **3.2.3 Drug tolerability**

Differences between the groups could account for the excess of adverse events seen on treatment; however survival beyond randomization and serological indices did not differ between the groups at baseline. Over the treatment period, serological parameters deteriorated in patients taking thalidomide reflecting either the poor drug tolerance or general deterioration due to disease. Nevertheless, the drug toxicity profile experienced by trial participants is widely reported; adverse events of thalidomide are multi-systemic extending beyond its notorious teratogenicity (Table 3.4).

Hypersomnolence is the most common adverse effect of thalidomide therapy. The dose-responsive effect is mediated through the diencephalic sleep centers within the brain and results in increased REM sleep without respiratory depression (Kanbayashi *et al.* 1999). Severe somnolence requiring drug reduction was only experienced in one study patient, possibly due to night time dosing or lack of patient reporting, as many patients with end-stage malignancy experience sleep disturbances and insomnia and find drugs with sedative side-effects particularly helpful.

Dermatological adverse effects of thalidomide were not so well tolerated and required drug withdrawal in 3 patients. Pruritis and rashes are common, affecting up to 50 % of thalidomide users (Dimopoulos & Eleutherakis-Papaiakovou 2004). Most skin rashes are vesicular, morbilliform, maculopapular or non-specific dermatitis and resolve on discontinuation of the drug, allowing re-introduction at a lower dose if necessary. Rarely more serious dermatological reactions can occur; erythroderma (Hall *et al.* 2003), toxic epidermal necrolysis (Rajkumar, Gertz, & Witzig 2000) and Steven-Johnson reactions (Clark *et al.* 2001) have been reported. High-dose thalidomide combined with dexamethasone results in a greater incidence of dermatological toxicity, and may increase the risk of serious reactions occurring (Hall *et al.* 2003).

Co-administration of dexamethasone and thalidomide is also associated with a higher incidence of VTE (Dimopoulos & Eleutherakis-Papaiakovou 2004; Ikhlague *et al.* 2006), compared with thalidomide monotherapy which carries a 1 - 5 % risk (Bennett *et al.* 2006). Neither of the participants who had a diagnosed VTE were taking dexamethasone; however malignancy independently confers a 4-fold increase in VTE risk

(Heit *et al.* 2000). Thus it is not possible to directly attribute these events solely to thalidomide use.

Table 3.4 Recognized adverse events associated with thalidomide use.

Neuropathic	Headache
	Peripheral neuropathy
	Sedation/Somnolence
	Asthenia
	Depression/Mood Changes
	Postural tremors
	Fever
Dermatological	Rashes
	Pruritis
	Alopecia
	Dry skin
Endocrine	Hypothyroidism
Gastrointestinal	Nausea/Vomiting
	Constipation
	xerostomia
Cardiovascular	Thrombosis
	Hypotension
	Oedema
	Bradycardia
Haematological	Neutrapenia
	Thrombocytopenia
	Thromboembolic events
Genitourinary	Impotence
	Teratogenicity

Peripheral nerve toxicity caused by thalidomide was not seen during the study period as nerve damage is dose-dependent, requiring cumulative doses of ~50 g before onset of mild symptoms (Fullerton & O'Sullivan 1968), whereas the total cumulative dose at study completion was 8.4 g. Patients were offered open-labelled thalidomide at the end of the study, but none lived long enough to encounter dose-related side-effects. Nevertheless parasthesia was experienced below this 50 g threshold in patients with normal nerve conduction studies.

Other studies evaluating the efficacy of thalidomide in cachexia have shown more promising outcomes although risk/benefit ratios remain high. Gordon *et al.* demonstrated benefit over placebo in patients with pancreatic cancer cachexia in which 7 (28 %) patients were withdrawn due adverse events (Gordon *et al.* 2005). Similarly patients with HIV wasting disease who were given either 100 mg or 200 mg of thalidomide or placebo experienced adverse events leading to drug withdrawal in 22 %, 37 % and 14 % of participants respectively. Patients given the 100 mg dose did better in terms of total weight and fat free mass gain than those given 200 mg (Kaplan *et al.* 2000). Thus tolerability may be increased by commencing patients on low doses initially and escalating as tolerated.

#### **3.2.4 Future research questions**

Whether thalidomide would be better tolerated in patients with less advanced disease, at lower doses or as an adjunct to chemotherapy remains open for investigation. Thalidomide has been shown to delay the progression of some solid tumours (Singhal & Mehta 2002; Gordinier *et al.* 2007) and improve the tolerability of GI toxic

chemotherapy agents such as Irinotecan (Govindarajan *et al.* 2000). Therefore it is possible that thalidomide may enable preservation of lean body mass during chemotherapy, justifying further clinical trials of thalidomide in oesophageal cancer cachexia.

### **3.2.5 Conclusions**

In conclusion this study demonstrates that thalidomide confers no benefit over placebo to patients with end-stage oesophageal cancer, predominantly due to drug toxicity. Thirty-five percent of patients given active treatment were unable to continue taking the drug due to side-effects which, when combined with disease-related morbidity, lead to insufficient data for meaningful analysis of body composition end-points.



## **Chapter 4**

### **Overcoming anabolic blunting with supraphysiological insulin availability**

The blunted response of MPS to AA with ageing appears to contribute to the pathophysiology of sarcopenia, although the mechanism by which this occurs is not known. Insulin resistance, commonly present with ageing, affects the P-I-3K pathway and may influence activation of nutrient sensing proteins, including mTOR, thus may contribute to anabolic blunting. This chapter addresses the effects of administering insulin, with the aim of achieving supraphysiological plasma concentrations, upon AA driven MPS, in an attempt to overcome anabolic blunting.

#### **4.1 Study aims and hypotheses**

Taking into account the work of other investigators, a protocol was designed to address two specific aims; first, to determine whether anabolic blunting could be overcome by increasing the availability of insulin, and second, to determine the PKB-mTOR signalling responses under these conditions. Thus the following hypotheses could be tested: H<sub>1</sub> insulin resistance underpins age related anabolic blunting; H<sub>2</sub> Rates of protein synthesis in older adults can be restored to that of the young by the administration of insulin; H<sub>3</sub> Phosphorylation of anabolic signalling proteins, specifically mTOR, 4E-BP1 and P70S6K, in response to AA do not differ between young and older volunteers in whom supraphysiological insulin concentrations have been administered.

#### **4.2 Study protocol**

Figure 4.1, which is a modified version of Figure 2.7, duplicated here for ease of reference, describes the protocol. A full description of recruitment, screening and study protocols are given in Chapter 2.

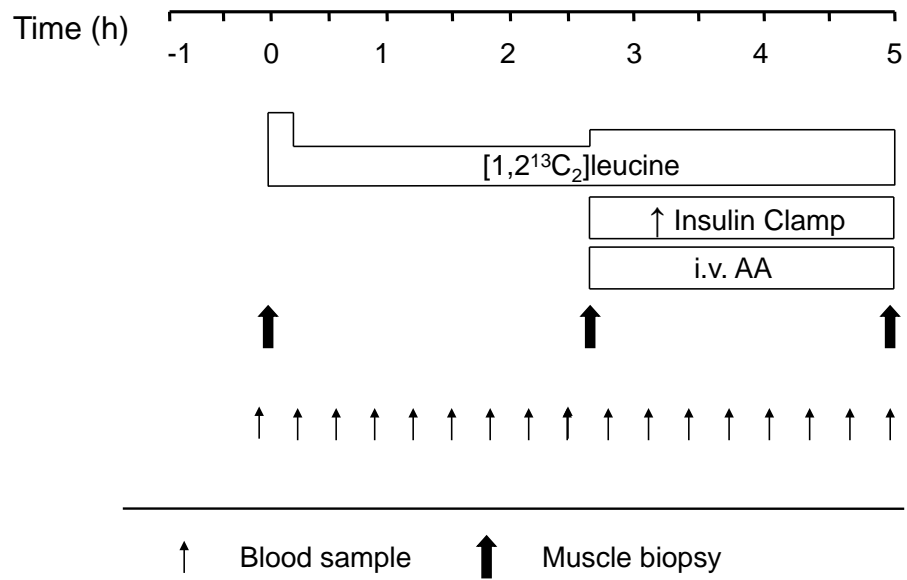


Figure 4.1. Protocol to assess the effects of high dose insulin and intravenous AA feeding upon muscle protein synthesis, in young and older healthy volunteers.

## 4.3 Results

### 4.3.1 Participants

Initially 8 young and 11 older healthy volunteers were recruited and completed the screening protocol: 15 completed the study protocol. All volunteers were deemed eligible to participate at screening and had no biochemical or haematological blood abnormalities. Of the two older volunteers who failed to complete the protocol, one failed to attend on the study day and one had an adverse event during the study; both young volunteers failed to attend on their study days. Data from these four individuals have been excluded from subsequent analysis.

The mean age of the young ( $n = 6$ ) and older ( $n = 9$ ) participants were  $22.3 \pm 1.7$  and  $66.0 \pm 1.7$  y respectively. In the young group there were equal numbers of male and female participants; there was one additional male participant in the older group. Participant characteristics are given in Table 4.1.

Body composition did not differ between the age groups, (for both groups mean BMI was  $\sim 24 \text{ kg}\cdot\text{m}^{-2}$  and LBM was  $\sim 48 \text{ kg}$ ). Each group demonstrated clustering of LBM values reflecting sexual dimorphism (Figure 4.2). The mean plasma concentrations of CRP at baseline appear to be higher in the older volunteers ( $6.4 \pm 2.6$  vs.  $2.2 \pm 0.3 \text{ }\mu\text{g}\cdot\text{ml}^{-1}$ ) but this does not reach statistical significance ( $P = 0.36$ ).

Table 4.1 Characteristics of participants. Mean  $\pm$  SEM.

	Young	Older	<i>P</i>
n	6	9	
Age (y)	22.3 $\pm$ 1.7	66.0 $\pm$ 1.7	<0.01
M:F	3:3	5:4	ns
Height (m)	1.7 $\pm$ 0.5	1.7 $\pm$ 0.0	ns
Weight (kg)	72.7 $\pm$ 7.8	67.7 $\pm$ 2.9	ns
BMI (kg.m <sup>-2</sup> )	24.6 $\pm$ 1.7	24.1 $\pm$ 0.6	ns
LBM (kg)	47.8 $\pm$ 6.8	47.5 $\pm$ 3.1	ns
ALBM (kg)	23.3 $\pm$ 3.6	22.8 $\pm$ 1.8	ns
RSMI (kg.m <sup>-2</sup> )	7.8 $\pm$ 0.8	8.0 $\pm$ 0.5	ns
CRP ( $\mu$ g.ml <sup>-1</sup> )	2.2 $\pm$ 0.3	6.4 $\pm$ 2.6	ns

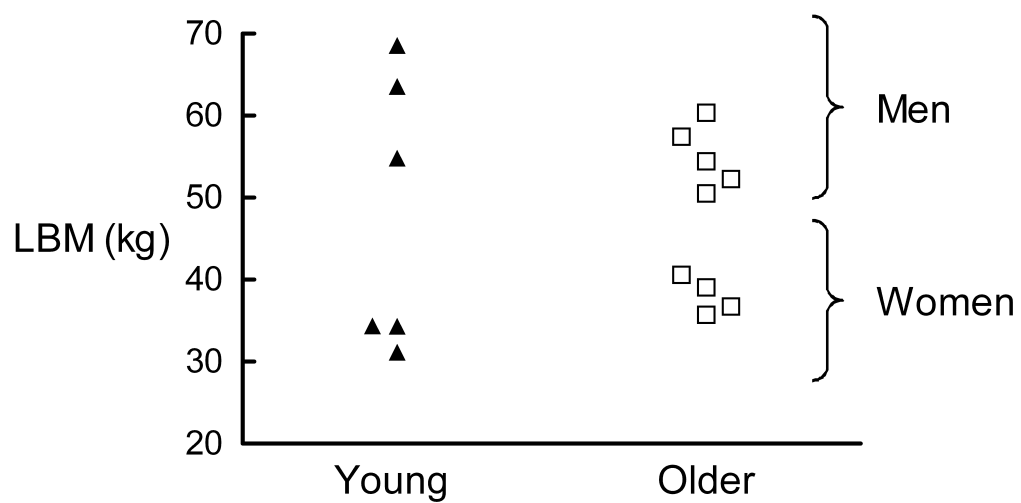


Figure 4.2 Scatter plot of lean body mass demonstrating sexual dimorphism.

### 4.3.2 Insulin sensitivity

There was no evidence of diabetes mellitus or insulin resistance in any of the participants at baseline. Basal insulin sensitivity (as determined by blood insulin concentration or HOMA-IR) did not differ between the groups (Table 4.2). Glucose requirements in order to maintain plasma glucose between 4.5 and 5.0 mmol.l<sup>-1</sup> during the hyperinsulinaemic euglycaemic clamp were also similar, although this data could not be used as a direct indicator for insulin sensitivity due to the accompanying AA infusion.

Table 4.2 Insulin sensitivity and glucose requirements; mean  $\pm$  SEM

	Young	Older	<i>P</i>
PA Insulin ( $\mu$ IU.ml <sup>-1</sup> )	4.4 $\pm$ 0.7	6.7 $\pm$ 1.6	ns
PA Glucose (mmol.l <sup>-1</sup> )	4.7 $\pm$ 0.1	5.1 $\pm$ 0.3	ns
HOMA-IR	1.0 $\pm$ 1.2	0.9 $\pm$ 0.1	ns
Glucose requirement (g)	750 $\pm$ 108	810 $\pm$ 114	ns

### 4.3.3 Amino acid concentrations

Total quantities of essential and BCAA concentrations did not differ significantly between the younger and older age groups in the postabsorptive state. Of the individual AA only histidine and isoleucine concentrations differed between the groups – both were significantly greater in the young (Table 4.3).

Table 4.3 Basal venous amino acid concentrations in young and older healthy volunteers. Mean  $\pm$  SEM given, all values in nmol.l<sup>-1</sup>.

	Young	Older	<i>P</i>
Alanine	289.8 $\pm$ 28.0	303.3 $\pm$ 11.9	ns
Arginine	80.4 $\pm$ 3.6	75.2 $\pm$ 6.9	ns
Asparagine	57.4 $\pm$ 3.7	48.9 $\pm$ 2.9	ns
Aspartic Acid	11.9 $\pm$ 2.0	17.8 $\pm$ 3.6	ns
Cysteine	20.0 $\pm$ 5.5	28.7 $\pm$ 8.1	ns
Glutamic acid	94.2 $\pm$ 14.7	112.3 $\pm$ 28.3	ns
Glutamine	332.2 $\pm$ 48.7	352.3 $\pm$ 44.6	ns
Glycine	206.6 $\pm$ 17.0	197.3 $\pm$ 14.3	ns
Histidine	81.2 $\pm$ 4.8	67.4 $\pm$ 3.7	<b>0.04</b>
Isoleucine	61.9 $\pm$ 5.4	45.5 $\pm$ 2.8	<b>0.01</b>
Leucine	140.6 $\pm$ 12.6	122.5 $\pm$ 7.5	ns
Lysine	165.6 $\pm$ 14.1	177.7 $\pm$ 11.0	ns
Methionine	20.1 $\pm$ 2.1	19.7 $\pm$ 2.9	ns
Phenylalanine	57.1 $\pm$ 4.3	52.4 $\pm$ 3.2	ns
Proline	191.9 $\pm$ 13.2	146.2 $\pm$ 18.9	ns
Serine	95.4 $\pm$ 9.1	89.8 $\pm$ 10.6	ns
Threonine	117.3 $\pm$ 11.3	106.3 $\pm$ 6.9	ns
Tryptophan	52.1 $\pm$ 5.1	35.8 $\pm$ 6.2	ns
Tyrosine	54.0 $\pm$ 4.7	59.5 $\pm$ 4.1	ns
Valine	211.2 $\pm$ 15.3	197.5 $\pm$ 14.1	ns
Total BCAA	413.7 $\pm$ 32.3	365.6 $\pm$ 23.9	ns
Total EAA	988.2 $\pm$ 63.5	899.9 $\pm$ 46.1	ns
Total AA	2131 $\pm$ 45.6	2121 $\pm$ 164.0	ns

#### 4.3.4 Hyperinsulinaemic euglycaemic clamp

During the clamped phase of the study the mean insulin concentration for both groups exceeded the target of  $100 \mu\text{IU}\cdot\text{ml}^{-1}$  and differed significantly from PA values (both  $P < 0.001$ ), although concentrations differed between the young and older participants (mean concentrations  $107.4 \pm 4.4$  and  $133.0 \pm 5.5 \mu\text{IU}\cdot\text{ml}^{-1}$  respectively;  $P = 0.01$ ). Plasma insulin availability was maintained in all groups throughout the 2.5 h feeding period (Figure 4.3) (ANOVA repeated measures, all  $P > 0.05$ ). Mean plasma glucose concentrations differed: the concentrations were lower in the young participants, albeit not to a clinically significant degree ( $4.6 \pm 0.1$  and  $5.1 \pm 0.1 \text{ mmol}\cdot\text{L}^{-1}$ ;  $P < 0.01$ ).

One older participant had an adverse reaction to the clamp which was consistent with octreotide intolerance and occurred within 4 min of commencing the clamp infusions. Facial flushing and disorientation developed with erythematous tracking overlying the infusion site. On stopping the infusion there were no further ill effects. Four volunteers reported diarrhoea within 12 to 24 h of the clamp which resolved spontaneously, in keeping with steatorrhoea due to octreotide induced fat malabsorption. In subsequent studies pancreatic enzymes were offered to participants to take prior to meals within 12 h of the clamp, which markedly reduced these symptoms.



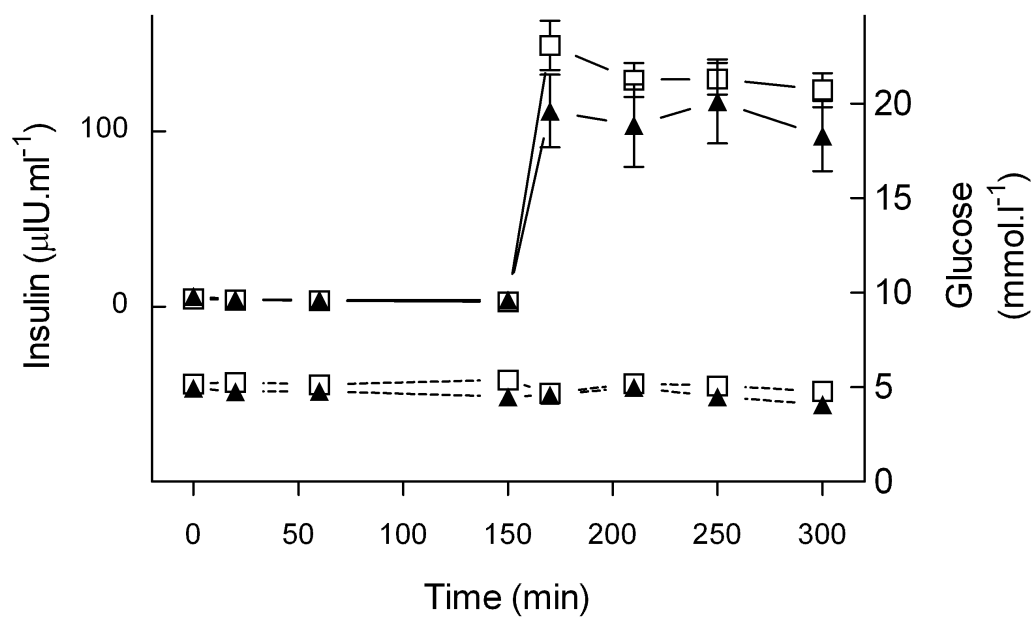


Figure 4.3 Plasma insulin (left axis; solid line) and whole blood glucose (right axis; dashed line) concentrations over time for young (filled triangles) and older (open squares) participants. All mean  $\pm$  SEM (error bars not seen are within symbols).

#### 4.3.5 Plasma AA concentrations

During the fed period, venous plasma AA concentrations were elevated in all groups, albeit substantially less than anticipated. Plasma BCAA (leucine, isoleucine and valine) and total EAA concentrations were significantly elevated over fasting concentrations and did not differ between young and older participants (all  $P < 0.05$ , Mann Whitney one-tailed test; Figure 4.4). Area under the curve data calculated above basal concentrations for leucine and EAA were also similar for all participants (Figure 4.5).

Plasma enrichment of  $[1,2^{13}\text{C}_2]$   $\alpha$ -keto-isocaproate was maintained at steady state throughout both periods and did not differ between participants. See Appendix 6.

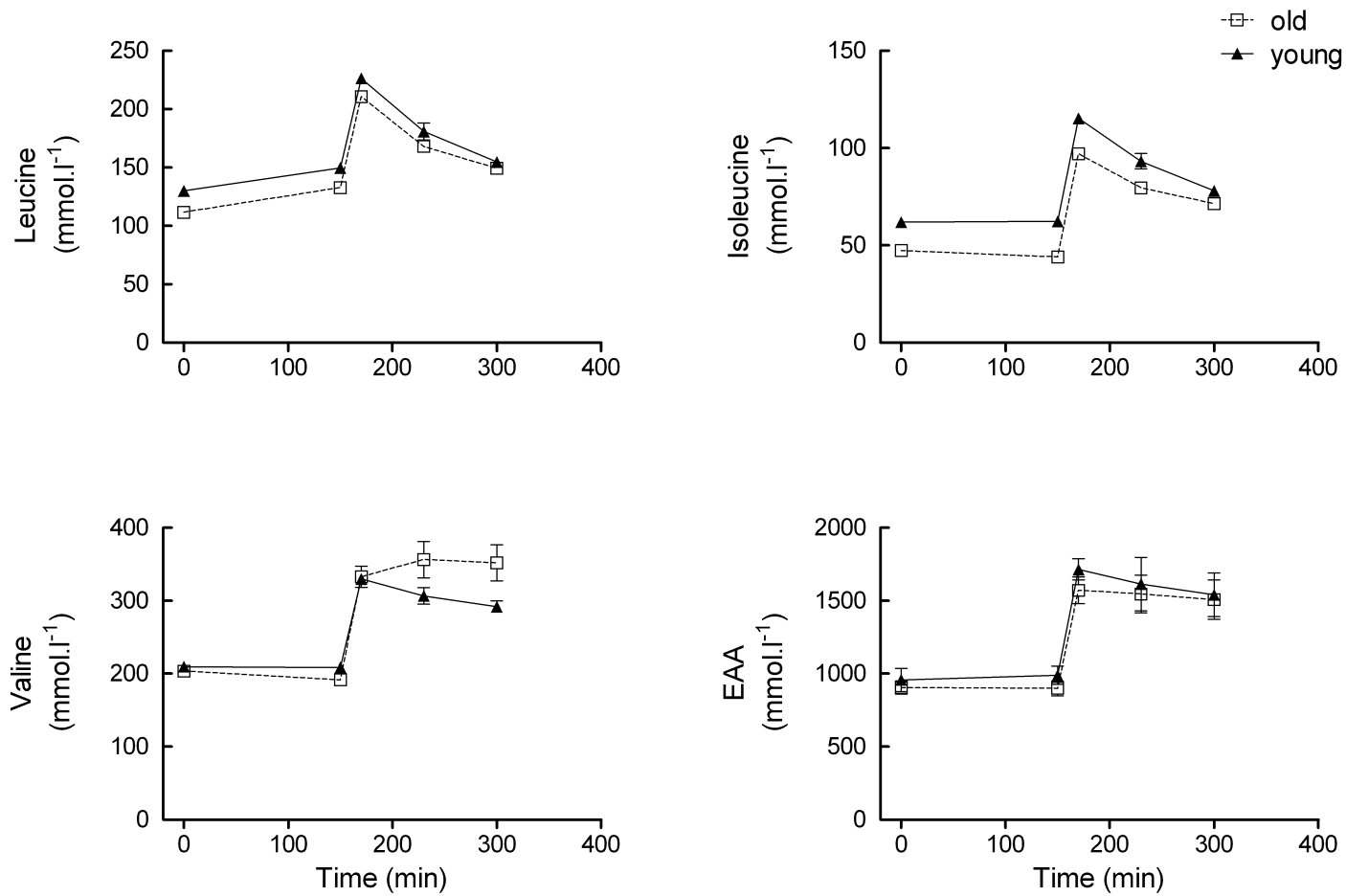


Figure 4.4 Plasma concentrations of individual branched chain amino acids and total essential amino acids (EAA) during postabsorptive and fed periods for young (solid line and filled triangles) and older (dashed line and open squares) healthy volunteers. Error bars not shown are hidden within symbols.

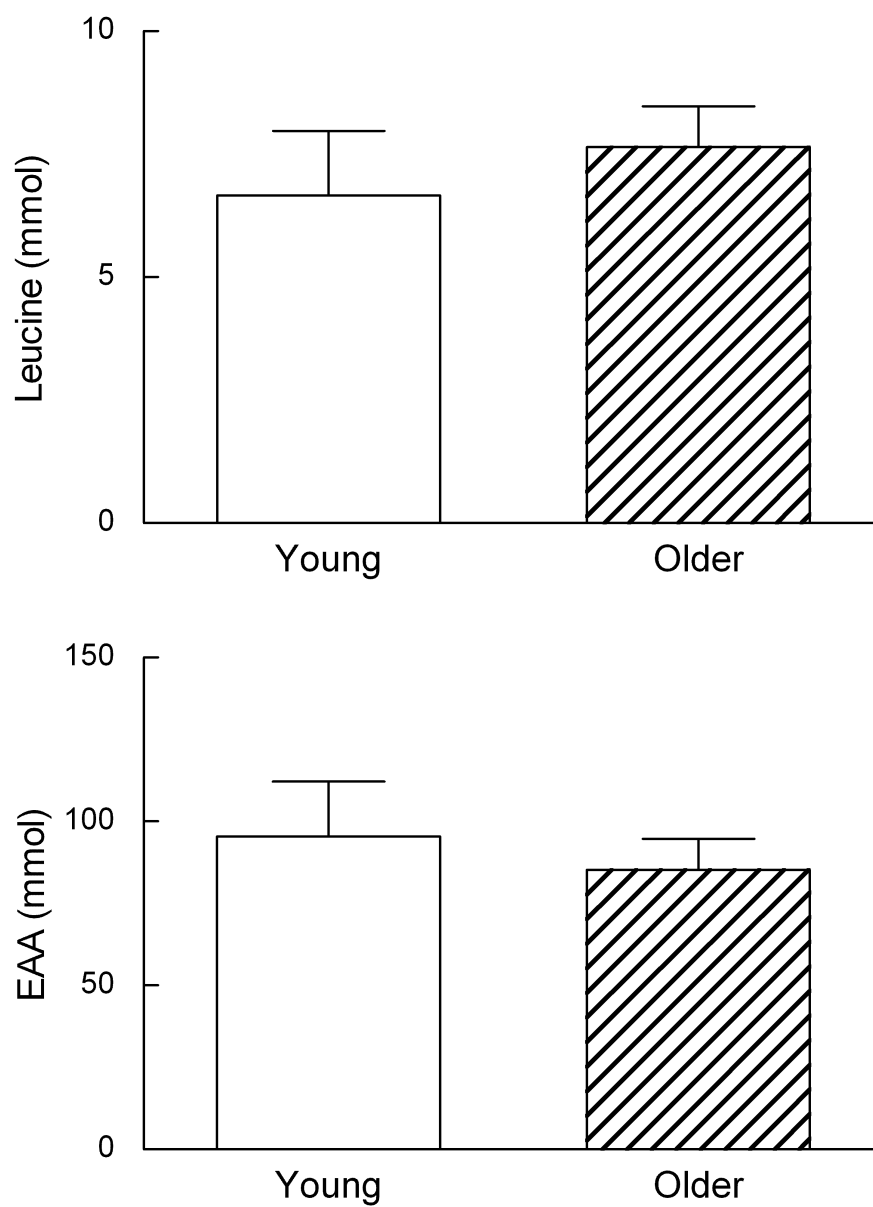


Figure 4.5 Area under the curve above postabsorptive concentrations for plasma leucine and essential amino acids (EAA) during fed periods.

### 4.3.6 Muscle Protein Synthesis

In the PA state FSR did not differ between young and older volunteers:  $0.040 \pm 0.008$  and  $0.047 \pm 0.004 \text{ \%}\cdot\text{h}^{-1}$ , respectively ( $P = 0.4$ ). FSR values increased significantly in both groups following insulin and AA administration but between group values did not differ (young:  $0.070 \pm 0.006$  and older:  $0.069 \pm 0.010 \text{ \%}\cdot\text{h}^{-1}$ ; both  $P < 0.05$  compared with respective PA values; Figure 4.6).

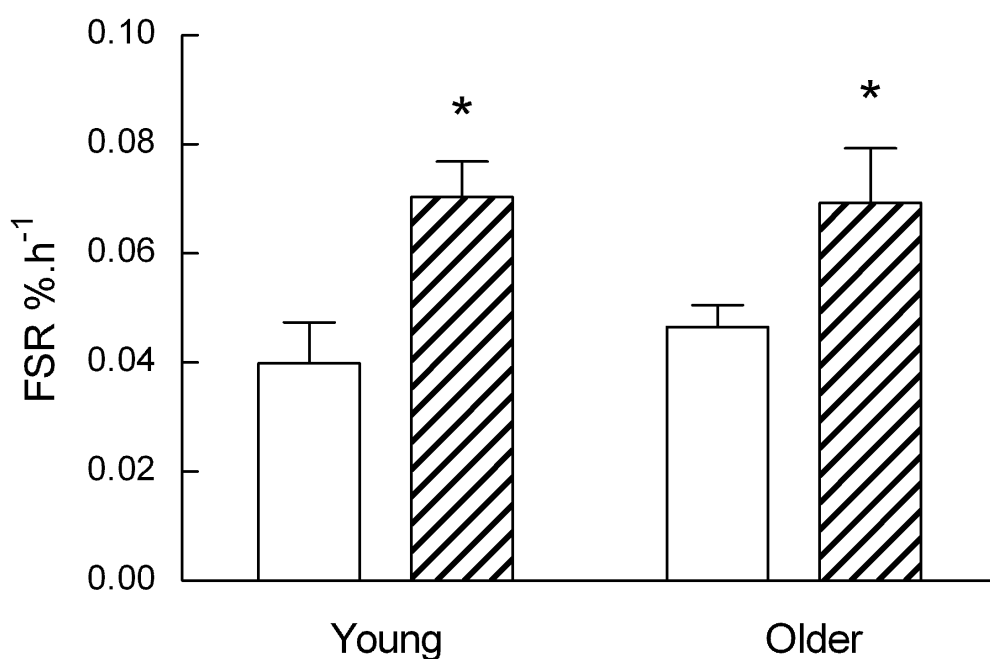


Figure 4.6 The effect of postabsorptive (empty bars) and fed conditions with supraphysiological insulin concentrations (hashed bars) upon FSR in young and older adults. Asterisks indicate a significant increase from PA values;  $P < 0.05$ .

### 4.3.7 Anabolic signalling pathways

Western Blotting of total and phosphorylated proteins involved in the PKB and mTOR pathways was performed on muscle samples taken before and after the simultaneous AA and insulin infusions.

Phosphorylation of PKB at Thr308 was increased in both young and older participants, although the response to AA infusion and hyperinsulinaemic clamp conditions was significantly greater in the young ( $P < 0.05$ ; Figure 4.7).

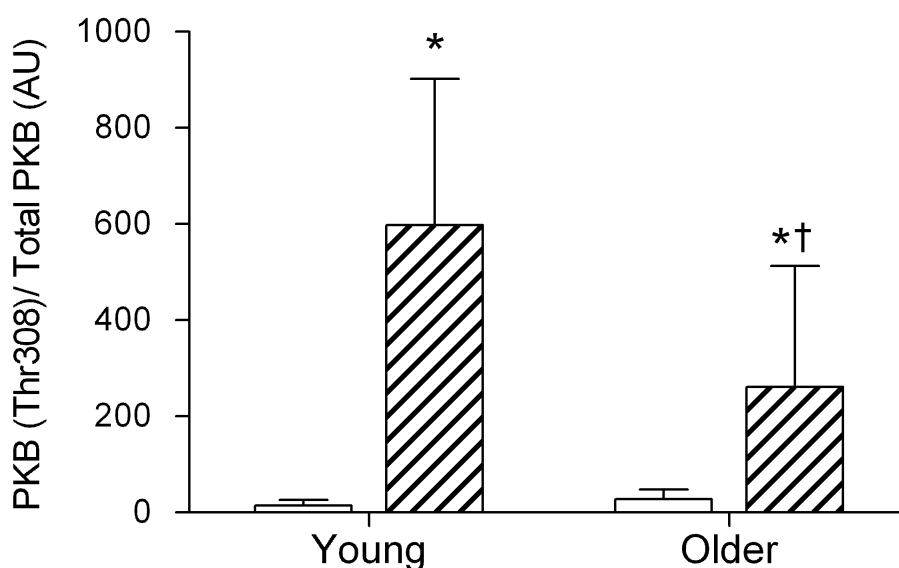


Figure 4.7 Phosphorylation status of PKB at Thr308 relative to total protein under postabsorptive (open bars) and fed (hashed bars) conditions. Asterisks denote significant difference from PA phosphorylation state; dagger indicates significant difference from young data;  $P < 0.05$ .

In both young and older groups GSK 3 $\beta$  inactivation, as determined by increased phosphorylation at Ser9, was increased by hyperinsulinaemic hyperaminoacidaemic conditions. Again, the increase was significantly greater in the young ( $P < 0.05$ ; Figure 4.8).

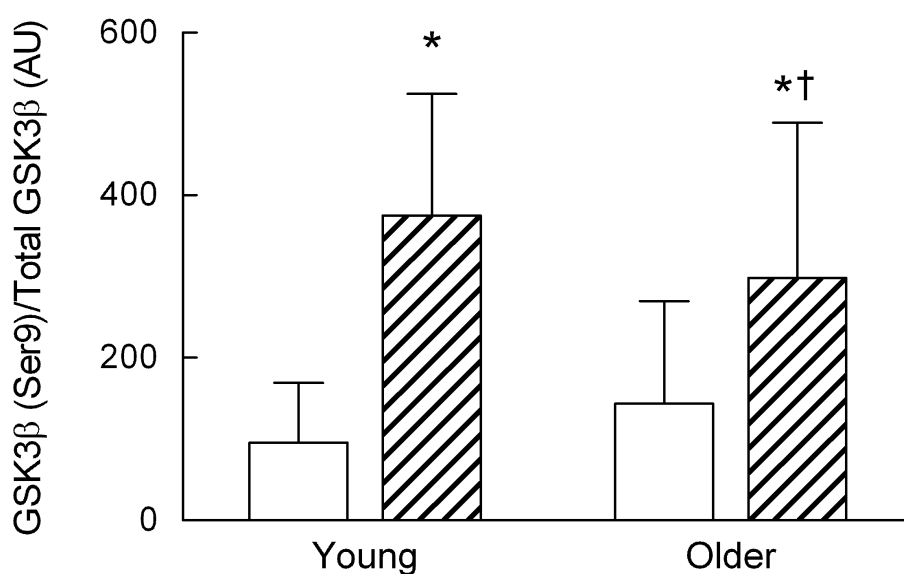


Figure 4.8 Phosphorylation of GSK3 $\beta$  at Ser9 under postabsorptive (open bars) and fed (hashed bars) conditions. Asterisks denote significant difference from PA phosphorylation state; dagger indicates significant difference from young data;  $P < 0.05$ .

The proportion of phosphorylated eEF2 at Thr56 did not differ with feeding or between young and older participants (Figure 4.9, all  $P > 0.05$ ).

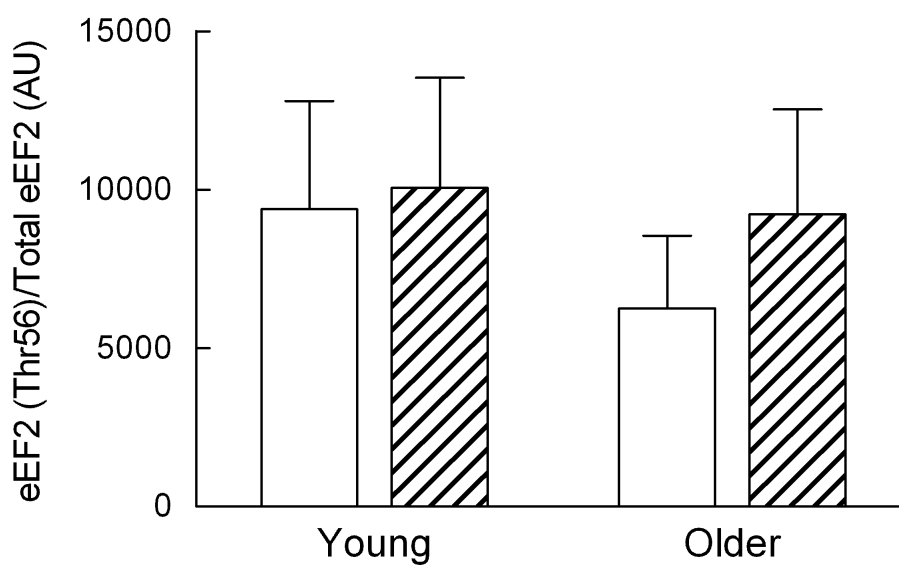


Figure 4.9 Phosphorylation of eEF2 at Thr56 relative to total eEF2 under postabsorptive (open bars) and fed (hashed bars) conditions for young and older participants.



mTOR phosphorylation at Ser2448 did not differ between the young and older adults when measured following either the PA or fed phase nor was there a difference between age groups (Figure 4.10).

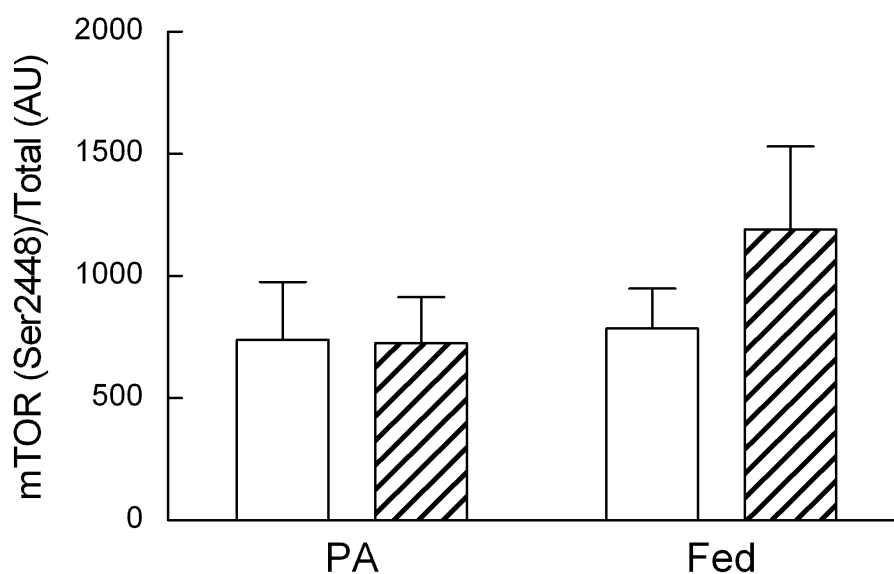


Figure 4.10 Phosphorylation status of mTOR at Ser2448 relative to total protein under postabsorptive (open bars) and fed (hashed bars) conditions.

Under PA conditions phosphorylation of downstream substrates of mTOR, namely P70S6K at Ser389 and 4E-BP1 at Thr36-46, were also similar regardless of age. Phosphorylation of both proteins increased significantly in response to fed conditions (Figures 4.11 and 4.12) but to a greater extent in the young ( $P < 0.05$ ). eIF4G – eIF4E association did not change in either group in response to hyperinsulinaemia and AA feeding (Figure 4.13).

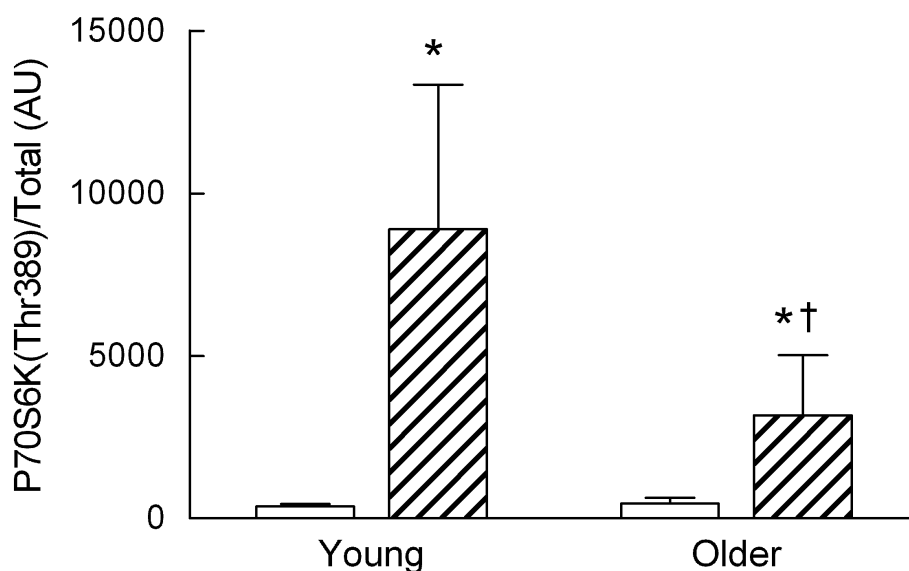


Figure 4.11 Phosphorylation status of P70S6K at Thr389 relative to total protein with postabsorptive (open bars) and fed (hashed bars) conditions. Significant change from PA given by asterisks; significant difference from young data indicated by dagger;  $P < 0.05$ .

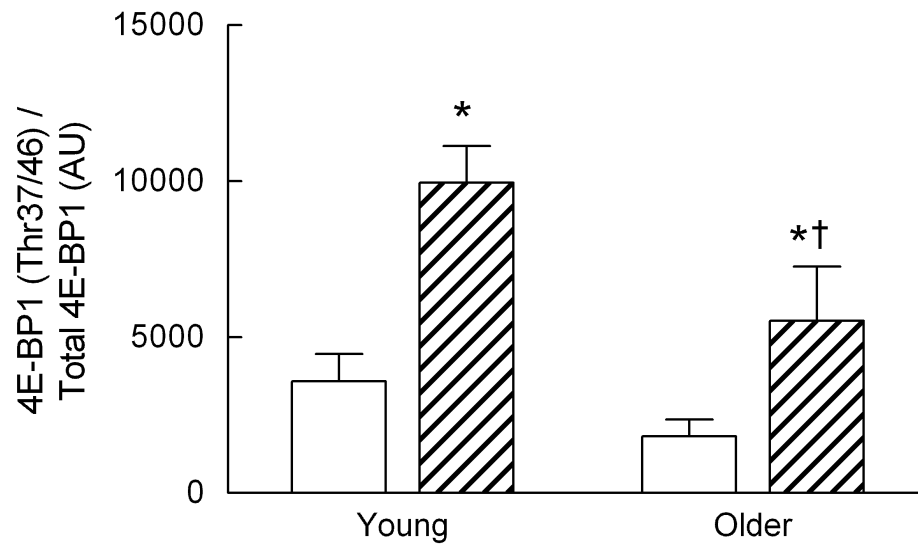


Figure 4.12 Phosphorylation of 4E-BP1 at Thr37/46 relative to total protein under postabsorptive (empty bars) and fed (hashed bars) conditions. Significant change from PA given by asterisks; significant difference from young data indicated by dagger;  $P < 0.05$ .

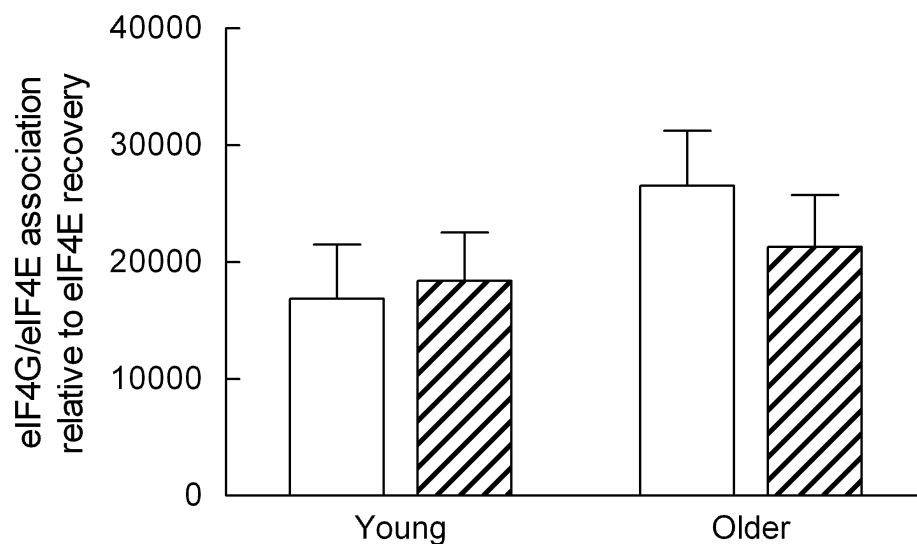


Figure 4.13 Association of eIF4G and eIF4E relative to eIF4E recovery before (open bars) and after hyperinsulinaemia and AA feeding (hashed bars) in young and older participants.

The proportion of phosphorylated eIF2 $\alpha$  protein at Ser51 was similar between young and older participants following the PA period ( $P = 0.15$ ). Phosphorylation decreased significantly in response to hyperinsulinaemia and AA feeding in the young ( $P < 0.05$ ), but not in the older group (Figure 4.14).

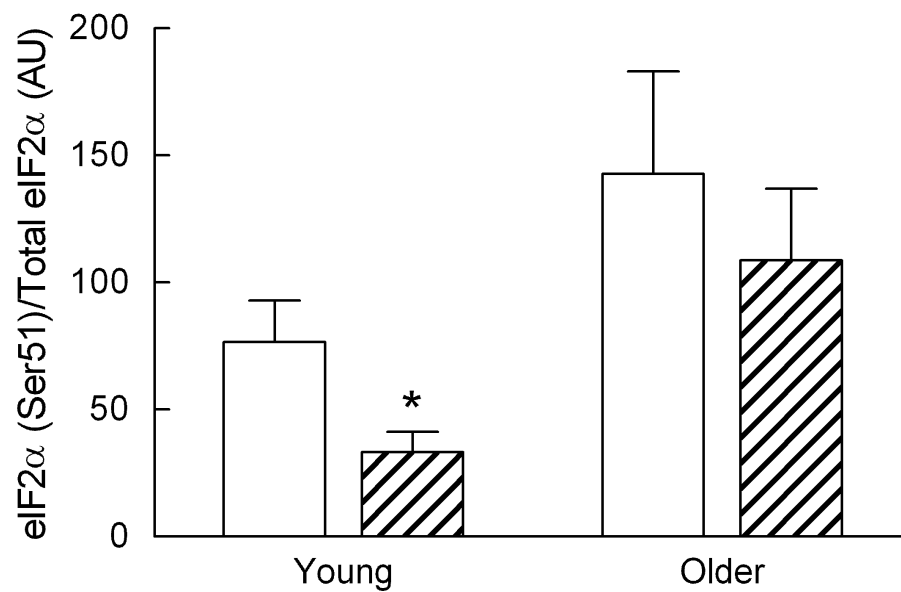


Figure 4.14 Proportion of phosphorylated eIF2 $\alpha$  following postabsorptive (open bars) and fed periods (hashed bars). Significant change with fed conditions indicated by asterisk;  $P < 0.05$ .

#### 4.3.8 Proteolytic markers

Administration of supraphysiological doses of insulin and AA reduced expression of MAFbx mRNA in both the young and older volunteers by ~25 % (Figure 4.15). The same conditions resulted in a similar reduction of MuRF-1 mRNA expression in muscle from older adults, but expression was increased by 25 % in the young (Figure 4.16). For older participants changes in MAFbx and MuRF-1 achieved significance (both  $P < 0.02$ ), although neither was significant in the young. When change in expression of MAFbx and MuRF-1 were compared between young and older participants, no significant difference was seen.

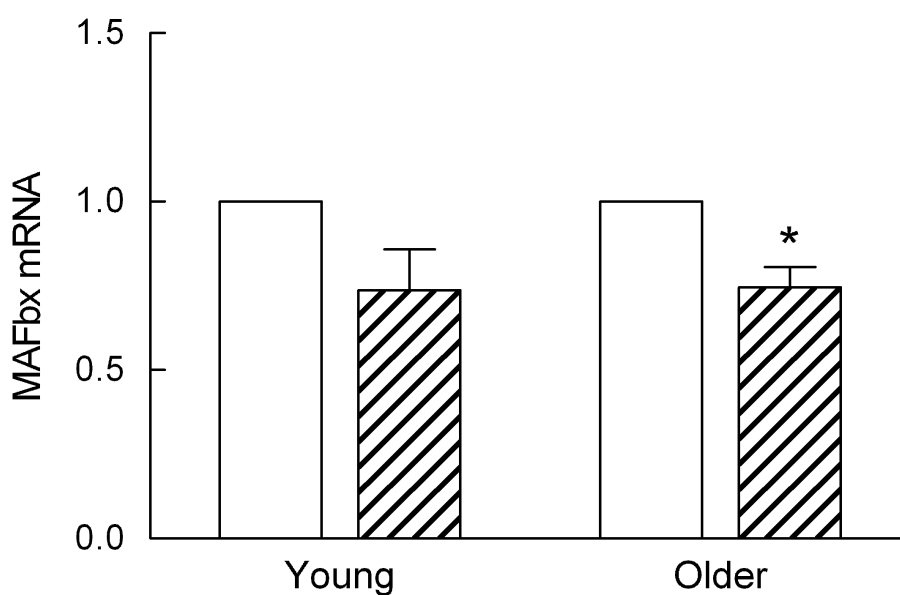


Figure 4.15 Quantification of MAFbx mRNA (normalized to PA values, arbitrarily given here as empty bars) in response to hyperinsulinaemia and AA (hashed bars) for young and older participants. Mean  $\pm$  SEM given; significant reduction from PA values indicated by asterisk ( $P < 0.02$ ).

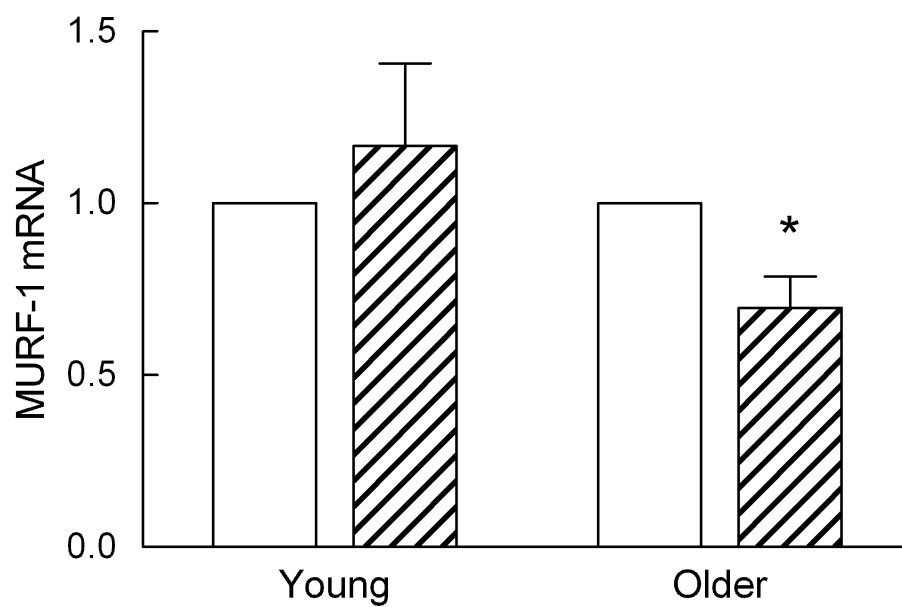


Figure 4.16 Quantification of MuRF-1 mRNA (normalized to PA values, arbitrarily given here as empty bars) in response to hyperinsulinaemia and AA (hashed bars) for young and older participants. Mean  $\pm$  SEM given; significant reduction from PA values indicated by asterisk ( $P < 0.02$ ).

## 4.4 Discussion

### 4.4.1 Participants

As volunteers were recruited in response to advertisements, there was no selection process *per se*, yet body composition data for young and older participants were well matched by random selection. This appears to contradict the concept of sarcopenia; however the data reflect a very sample size of active older adults rather than a cross section of the whole population. Nevertheless differences are seen in signalling responses to feeding which suggests that the underlying physiological changes associated with sarcopenia are still present.

### 4.4.2 Plasma branched chain amino acid availability

Dose-response data for plasma BCAA following a mixed AA intravenous infusion under hyperinsulinaemic conditions was not available at the outset of the study, therefore an estimate was used based upon data from Cuthbertson *et al.* (Cuthbertson *et al.* 2005). The dose chosen was comparable to a 40 g EAA oral feed. Lower plasma concentrations of AA were anticipated due to the high target insulin concentrations, as insulin enhances AA oxidation and gluconeogenesis and suppresses protein breakdown. The degree to which this was offset by the increased bioavailability of AA when given intravenously when compared with oral administration was unknown. Nevertheless the plasma BCAA concentrations observed during the study equated to a lower dose than expected, i.e. ~ 50 % increase in plasma AA. The area under the curve values above basal for leucine were equivalent to a 2.5 g oral dose of EAA.

A higher rate of AA infusion may have produced target plasma doses; although these may not have been sustained. This is demonstrated by the reduction of leucine and isoleucine concentrations to basal values over the 2.5 h administration period, despite a continuous rate of infusion. Total EAA concentrations remained constant which suggests that either leucine and isoleucine were preferentially metabolised or that the proportion of these BCAA within the AA feed were insufficiently matched to utilization. As the proportions of leucine and isoleucine in PA plasma and the AA infusion did not differ, preferential metabolism is likely to be responsible for the accelerated removal of these AA from the plasma, whether by oxidization, glyconeogenesis or muscle protein synthesis.

#### **4.4.3 Changes in FSR in response to AA feeding and high insulin availability**

FSR increased, as expected, following AA feeding but there was no difference between values for young and older participants; however this is not inconsistent with anabolic blunting data. Other workers have shown no difference with senescence following low dose feeds (e.g. 2.5 g EAA given orally) but blunting at higher plasma AA concentrations (Cuthbertson *et al.* 2005). FSR values in Cuthbertson's study were marginally lower than those seen here, despite employing the same tracer and analytical methodology, but this may be readily explained as measurements were made over a 3 h period, rather than 2.5 h. The maximal synthetic response to feeding occurs within the first 2.5 h after a meal (Bohe *et al.* 2001). Thus the average FSR taken over 3 h includes a period of PA synthesis and will therefore be lower than when measured over a 2.5 h absorptive period. It is unlikely that the differing values result from the addition of high dose insulin administration, as others have shown that insulin has no additive effect upon MPS (Greenhaff *et al.* 2008).



It is unknown whether the threshold for maximal stimulation of MPS occurs earlier in older adults than in the young or whether at higher AA doses the muscle reaches this threshold more rapidly (Rennie, Bohe, & Wolfe 2002). A shorter window of MPS in response to feeding would account for differences seen in the rates of muscle protein synthesis in the young and older participants by other investigators (Cuthbertson *et al.* 2005; Welle *et al.* 1993; Balagopal *et al.* 1997) i.e. absorptive FSR values from older individuals taken over 3 h or longer reflect a greater proportion of time points during which synthesis has reverted to postabsorptive rates. Although this concept is not new, first put forward by Price *et al.* (Price *et al.* 1994), it is unknown whether this theoretical tachyphylaxis is driven by chronological or dose-related thresholds.

#### **4.4.4 Blunted anabolic signalling protein responses to AA in older adults**

The phosphorylation responses of signalling proteins involved in the sensing and signalling of AA were diminished in the older group when compared to that of the young, even though no difference was seen in FSR between the groups. This is in keeping with other published data (Cuthbertson *et al.* 2005). The degree of phosphorylation of mTOR, p70S6K and 4E-BP1, relative to total concentration, following a 10 g EAA feed, was higher in young men when compared with their older counterparts. Leaving aside the mTOR data, this suggests that a blunted signalling response to AA is not overcome by supraphysiological insulin concentrations, as 4E-BP1 and p70S6K activation in response to AA was diminished in the older participants of the present study. Furthermore the high insulin availability did not elicit the same degree of PKB and GSK3 $\beta$  phosphorylation in the older adults: either there is a greater resistance to phosphorylation in older adults or that high circulating insulin concentrations in the blood are unable to overcome any up-stream signalling defects

due to insulin resistance. The differences seen in PKB and GSK3 $\beta$  activation between the two age groups support the suggestion that anabolic defects in older human beings stem from aberrant insulin signalling rather than a direct problem with AA signalling.

Another group, assessing the effects of hyperinsulinaemia and hyperaminoacidaemia upon protein synthesis and anabolic signalling, were unable to demonstrate significant differences in PKB, mTOR or 4E-BP1 phosphorylation between young and older adults, although FSR responses were blunted in the older participants (Guillet *et al.* 2004a). In the fed phase of this study plasma BCAA concentrations differed significantly between the young and older participants (~1.8 fold and ~2.7 fold increase respectively), potentially accounting for the similar signalling responses. The rise in insulin concentrations also differed between the groups (44 and 63  $\mu\text{IU}\cdot\text{ml}^{-1}$ ) but did not reach significance. However p70S6K phosphorylation rose less in the older group and PKB and mTOR phosphorylation changes with feeding were less pronounced than seen in the young (again failing to reach statistical significance) in concordance with the data presented here.

p70S6K is relevant to insulin resistance of glucose metabolism as it appears to suppress insulin signalling by negative feedback through IRS-1, such that AA have an inhibitory effect upon insulin signalling through PI-3K (Patti 1999). Animal data further supports this argument: p70S6K knock-out mice remain insulin sensitive when exposed to a high fat diet, unlike their wild-type controls, and demonstrate enhanced PKB phosphorylation in response to insulin administration (Um *et al.* 2004). Thus in older subjects, in whom p70S6K phosphorylation is reduced, PKB activation by insulin ought

to be enhanced; in the present study this was not the case, reflecting the likelihood that this is a gross simplification.

mTOR phosphorylation at Ser2448 did not differ with feeding or between the young and older groups, potentially explained by the timing of the biopsy. It is possible that mTOR phosphorylation is returning to basal levels 2.5 h after the onset of the feed but down-stream substrates of the kinase remain up-regulated. Alternatively the high insulin concentrations may have acted as a direct inhibitor of mTOR phosphorylation, or precipitated cellular re-distribution of mTOR to the nucleus preventing its phosphorylation by activated PKB.

The phosphorylation status of down-stream substrates fails to add any further light to the mTOR picture, as although p70S6K and 4E-BP1 phosphorylation were up-regulated, the phosphorylation of eEF2, an elongation factor which is ordinarily inhibited by mTOR, remained unchanged. Although it is possible that eEF2, like mTOR, may be temporally responsive and thus its phosphorylation status may have returned to PA values at the time point sampled.

Alternatively, it is plausible that kinase activity is not dependent upon phosphorylation status of mTOR at Ser2448. The phosphorylation site was Ser2448 was chosen for two reasons: i) PKB induces phosphorylation at this site and at Thr2446 which lies adjacent to it (Hay & Sonenberg 2004), and ii) insulin has also been shown to specifically induce phosphorylation at Ser2448 (Sekulic *et al.* 2000); nevertheless the significance of phosphorylation at these sites, in terms of mTOR activation, is unclear. Experiments

using rapamycin to inhibit activation of mTOR suggest that disruption of the mTOR-Raptor dephosphorylation *per se*. Other data supports this suggestion: replacement of Thr2446 and Ser2448 residues with alanine does not appear to alter mTOR activity (Sekulic *et al.* 2000).

Recently other workers (Adegoke *et al.* 2009; Fujita *et al.* 2009b) have shown this pattern of signalling responses to hyperinsulinaemic clamp conditions. In the young men studied by Adegoke *et al.*, phosphorylation of PKB, P70S6K1 and 4EBP1 were increased after 2h of hyperinsulinaemic hyperaminoacidaemic and hyperglycaemic clamp conditions, but phosphorylation of mTOR Ser 2448 did not differ significantly from basal values. These authors also suggest that Raptor binding may explain the apparent mTOR activation without expected changes in phosphorylation status. Whereas Fujita *et al.*, who measured responses to supraphysiological insulin availability at 3 h suggest phosphorylation of mTOR may have occurred at an earlier time point and returned to basal levels at the time sampled.

#### **4.4.5 Future research questions**

In order to fully address the aims of this study, i.e. whether hyperinsulinaemia overcomes sarcopenic anabolic blunting of protein synthesis, subjects would need to receive higher doses of AA to double plasma AA concentrations, ideally with real-time plasma AA analysis. A control arm, of feeding with insulin clamped at PA concentrations, would also be useful. However a modified study has not been carried out as the signalling data suggests that administration of supra-physiological insulin doses does not overcome anabolic blunting in older human beings.

Further important areas for research which need to be addressed are the time course of anabolic responses to AA and whether AA pharmacokinetics differ between young and older human beings. Such data would not only provide information about the optimum protein content and timings of meals for maximum anabolism, but also help to unravel the activation time course and role of mTOR, PKB, p70S6K and their substrates in the sensing and signalling of AA stimulated protein synthesis.

#### **4.4.6 Conclusions**

In conclusion, the specific aims of this study were not met as target plasma BCAA concentrations (i.e. doubling of PA values) were not achieved, probably due to the hyperinsulinaemia; however the data collected is novel and adds to the existing literature on anabolic blunting in older adults. The study confirms that AA feeding initiates an anabolic response in skeletal muscle and, following a low dose AA feed, the synthetic response does not differ between the young and older healthy volunteers, yet key anabolic signalling responses to AA are blunted in older adults.

## **Chapter 5**

### **The control of muscle protein breakdown by insulin in ageing**

The inhibitory activity of insulin upon both muscle and whole body protein breakdown has been demonstrated in young healthy volunteers (Flakoll *et al.* 1989; Denne *et al.* 1991). When the responses of whole body protein turnover are compared between young and older subjects, it appears that, at equivalent insulin and amino acid concentrations, rates of protein breakdown are higher in older subjects (Guillet *et al.* 2004b; Boirie *et al.* 2001). The proposition that this may also be true for muscle protein has never been tested. Thus, this chapter specifically examines the effects of insulin upon leg protein turnover, on the assumption that leg is mainly muscle, in young and older human beings, to test the hypothesis that the effects of insulin upon leg proteolysis are blunted with healthy senescence.

### **5.1 Study protocol**

The study protocol, approved by University of Nottingham Medical School Research Ethics Committee (Ref G/4/2004), is detailed in Figure 5.1 (this is a duplicate of Figure 2.8 for ease of reference). A detailed account of recruitment, screening and study protocols are given in Chapter 2.

As constant AA availability and tight insulin control was required for this protocol, clamp development work was carried out before commencing the tracer studies, with prior approval from the ethics committee. Eight volunteers participated in the development phase, which involved octreotide, insulin, AA and dextrose infusions over 3 h with arterialized-venous blood sampling at 5 min intervals. Insulin and AA infusion protocols were modified to consistently achieve target plasma insulin and AA concentrations.

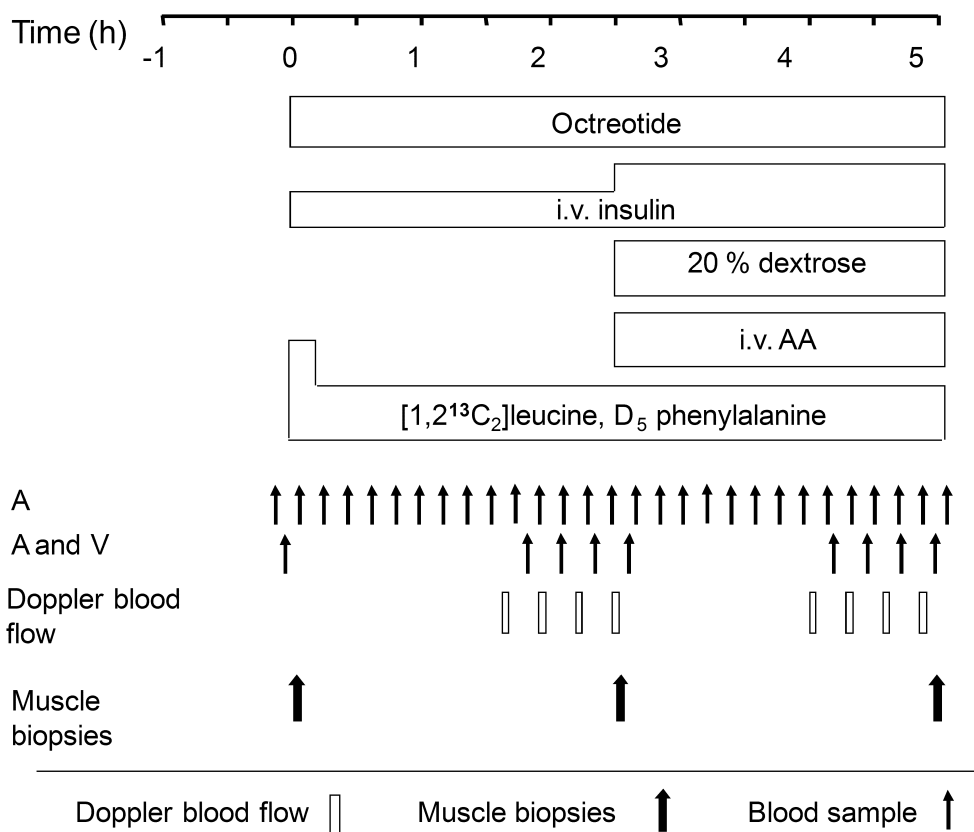


Figure 5.1 Protocol to assess leg proteolysis at plasma insulin concentrations of  $\sim 5$  and  $\sim 15 \mu\text{IU}\cdot\text{ml}^{-1}$  in young and older healthy volunteers. Arterialized-venous blood (A) was collected at  $\sim 5$  min intervals to monitor blood glucose concentrations infusions. Paired samples of arterialized-venous and femoral vein blood (A and V) were collected at baseline and 20 minute intervals through the last hour of each clamp period (small arrows), preceded by doppler blood flow measurements (rectangles). Muscle biopsies are indicated by large arrows.



## 5.2 Results

### 5.2.1 Participant characteristics

Eight young and 8 older participants, of which 4 of each group were women, were successfully screened and completed the study protocol. Participants were ages  $24.5 \pm 1.8$  and  $65.0 \pm 1.3$  y respectively (all data presented are mean  $\pm$  SEM, unless specified).

C-reactive protein was within the normal range for all healthy volunteers and did not differ between the young and older participants. Basal plasma AA concentrations were also similar and are given in Table 5.1.

Both groups were of similar total body weight (young:  $71.1 \pm 5.0$  and older:  $73.1 \pm 3.3$  kg) but the older participants had a greater BMI ( $26.3 \pm 0.4$  compared with  $23.9 \pm 0.9$ ;  $P = 0.03$ ) with equal amounts of lean tissue when normalized for height (Table 5.2).

Table 5.1 Basal AA concentrations for young and older participants. All values mean  $\pm$  SEM expressed in mmol.l<sup>-1</sup>; intergroup t-test analysis for each AA, all  $P > 0.05$ .

	Young	Older
Alanine	264.7 $\pm$ 22.8	283.8 $\pm$ 47.8
Arginine	74.2 $\pm$ 5.0	110.9 $\pm$ 33.2
Asparagine	72.2 $\pm$ 3.7	76.2 $\pm$ 4.8
Aspartic Acid	5.4 $\pm$ 0.8	13.8 $\pm$ 7.5
Cysteine	36.7 $\pm$ 8.0	48.9 $\pm$ 5.5
Glutamine	441.9 $\pm$ 35.3	433.7 $\pm$ 27.9
Glutamic acid	21.8 $\pm$ 4.2	33.3 $\pm$ 13.6
Glycine	236.1 $\pm$ 50.6	223.0 $\pm$ 15.6
Histidine	55.0 $\pm$ 12.1	75.5 $\pm$ 4.1
Isoleucine	50.0 $\pm$ 4.0	53.0 $\pm$ 3.5
Leucine	113.4 $\pm$ 11.5	114.2 $\pm$ 8.2
Lysine	135.5 $\pm$ 17.1	177.8 $\pm$ 11.4
Methionine	27.4 $\pm$ 3.1	28.9 $\pm$ 3.3
Phenylalanine	51.5 $\pm$ 4.4	56.2 $\pm$ 2.8
Proline	168.1 $\pm$ 27.5	139.7 $\pm$ 14.0
Serine	102.4 $\pm$ 14.5	87.3 $\pm$ 6.0
Threonine	116.8 $\pm$ 12.1	113.6 $\pm$ 4.6
Tryptophan	66.5 $\pm$ 18.7	52.4 $\pm$ 8.3
Tyrosine	58.5 $\pm$ 10.9	62.3 $\pm$ 4.0
Valine	199.8 $\pm$ 11.7	208.7 $\pm$ 13.1
Total BCAA	414.8 $\pm$ 28.8	432.1 $\pm$ 26.4
Total EAA	890.2 $\pm$ 46.0	991.3 $\pm$ 51.2
Total AA	2286.7 $\pm$ 176.4	2425.6 $\pm$ 70.0

### 5.2.2 Insulin sensitivity

Basal plasma insulin concentrations were significantly higher in the young participants ( $6.0 \pm 0.5 \mu\text{IU}\cdot\text{ml}^{-1}$  compared with  $4.2 \pm 0.5 \mu\text{IU}\cdot\text{ml}^{-1}$ ;  $P = 0.04$ ) although glucose concentrations were similar. Calculated indices of insulin resistance were also marginally higher in the young but did not achieve statistical significance whether assessed using QUICKI ( $0.38 \pm 0.01$  vs.  $0.40 \pm 0.01$ ; young vs. older;  $P = 0.13$ ) or HOMA-IR ( $1.18 \pm 0.11$  vs.  $0.93 \pm 0.16$ ; young vs. older;  $P = 0.21$ ).

Table 5.2 Characteristics of subjects. All values mean  $\pm$  SEM; no significant difference (ns) between young and older participants when  $P > 0.05$ .

	Young	Older	<i>P</i>
n	8	8	
Age (y)	$24.5 \pm 1.8$	$65.0 \pm 1.3$	<0.0001
M:F	4:4	4:4	ns
Weight (kg)	$71.1 \pm 5.0$	$73.1 \pm 3.3$	ns
Height (m)	$1.7 \pm 0.0$	$1.7 \pm 0.0$	ns
CRP	$2.1 \pm 0.8$	$3.5 \pm 1.3$	ns
BMI ( $\text{kg}\cdot\text{m}^{-2}$ )	$23.9 \pm 0.9$	$26.3 \pm 0.4$	0.03
LBM (kg)	$47.3 \pm 4.4$	$45.8 \pm 4.1$	ns
LBM. $\text{ht}^{-1}$ ( $\text{kg}\cdot\text{m}$ )	$27.2 \pm 1.8$	$27.3 \pm 2.0$	ns
PA insulin ( $\mu\text{IU}\cdot\text{ml}^{-1}$ )	$6.0 \pm 0.5$	$4.3 \pm 0.5$	0.04
PA glucose ( $\text{g}\cdot\text{dl}^{-1}$ )	$4.4 \pm 0.1$	$4.8 \pm 0.2$	ns
PA leucine ( $\mu\text{M}\cdot\text{l}^{-1}$ )	$113.2 \pm 11.5$	$114.2 \pm 8.2$	ns

### 5.2.3 Clamp conditions

Target conditions for the euglycaemic isoaminoacidaemic clamp studies were achieved for all participants. Insulin concentrations for the two study periods were  $4.4 \pm 0.6$  and  $15.0 \pm 0.7 \mu\text{IU}\cdot\text{ml}^{-1}$  in the young and  $3.3 \pm 0.2$  and  $14.9 \pm 0.4 \mu\text{IU}\cdot\text{ml}^{-1}$  in older participants. Mean whole blood glucose across both periods was maintained at  $4.7 \pm 0.1$  and  $4.8 \pm 0.1 \text{ mmol}\cdot\text{l}^{-1}$  for young and older volunteers, respectively. Plasma insulin and glucose concentrations did not differ between the groups throughout the study (Figure 5.2).

During the clamps, venous plasma leucine and total EAA concentrations rose above basal values, albeit not achieving statistical significance (Table 5.3). At each time point venous plasma concentrations were consistently higher in the older group than the young for leucine and EAA (both  $P < 0.01$ ). Arterialized-venous leucine concentrations were also lower in the young ( $P < 0.02$ ) but EAA did not differ significantly (Figures 5.3 and 5.4). The CV for insulin, leucine and EA were all within 15 % of the mean.

Table 5.3 Venous plasma concentrations of essential amino acid in young (Y) and older (O) healthy volunteers. All values in  $\mu\text{mol.l}^{-1} \pm \text{SEM}$ ; compared by student's t-test (paired for within group and unpaired between group measurements). None of the differences between groups were significant.

	Young: basal and at 5 and 15 $\mu\text{U.ml}^{-1}$ insulin				Older: basal and at 5 and 15 $\mu\text{U.ml}^{-1}$ insulin				Y vs. O	Y vs.O	Y vs.O
	Basal	5	15	5 vs. 15 <i>P</i>	Basal	5	15	5 vs. 15 <i>P</i>	Basal <i>P</i>	5 <i>P</i>	15 <i>P</i>
Arg	74.2±5.0	104.6±7.4	123.7±10.8	0.26	110.9±33.2	130.4±31.5	152.9±31.1	<b>0.02</b>	0.30	0.41	0.37
His	55.0±12.1	76.4±9.2	92.1±15.2	0.17	75.5±4.1	86.8±4.3	106.0±9.1	<b>0.04</b>	0.13	0.34	0.46
Iso	50.3±4.0	58.4±4.2	57.1±5.4	0.86	53.0±3.5	64.3±2.8	68.2±5.7	0.47	0.59	0.27	0.18
Leu	113.4±11.5	144.4±5.4	138.8±7.9	0.54	114.2±8.2	161.2±7.3	168.8±16.1	0.53	0.95	0.08	0.11
Lys	135.5±17.1	170.5±17.2	201.2±26.0	0.16	177.8±13.4	208.7±13.4	243.4±24.6	0.05	0.06	0.11	0.26
Met	27.4±3.1	29.6±2.9	46.6±7.4	<b>0.04</b>	28.9±3.3	35.0±3.4	49.6±5.2	<b>0.01</b>	0.74	0.25	0.75
Phe	51.5±4.3	65.3±3.9	72.6±5.8	0.22	56.2±2.8	68.2±4.0	82.2±5.5	<b>0.04</b>	0.39	0.61	0.25
Thr	116.8±12.1	130.4±9.9	140.9±10.7	0.30	113.6±4.6	128.8±7.0	139.5±6.4	0.20	0.81	0.91	0.91
Try	58.5±10.9	52.1±2.7	52.7±3.5	0.83	62.34±4.0	60.42±4.0	64.4±4.9	0.35	0.75	0.10	0.07
Val	199.8±11.7	210.4±7.1	219.6±13.7	0.51	208.7±13.1	234.0±11.4	248.7±22.5	0.40	0.62	0.09	0.28

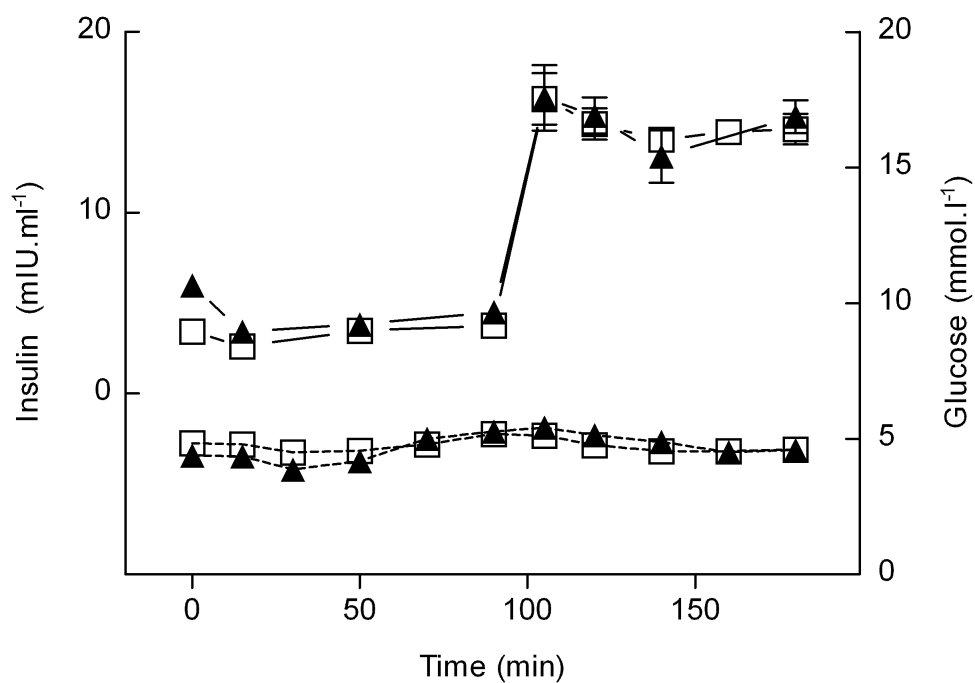


Figure 5.2 Venous plasma insulin (left axis, solid line) and arterialized-venous whole blood glucose (right axis, dashed line) concentrations at baseline and during both pancreatic clamp periods for young (solid triangles) and older (open squares) participants. Mean  $\pm$  SEM given, error bars not seen lie within symbols.

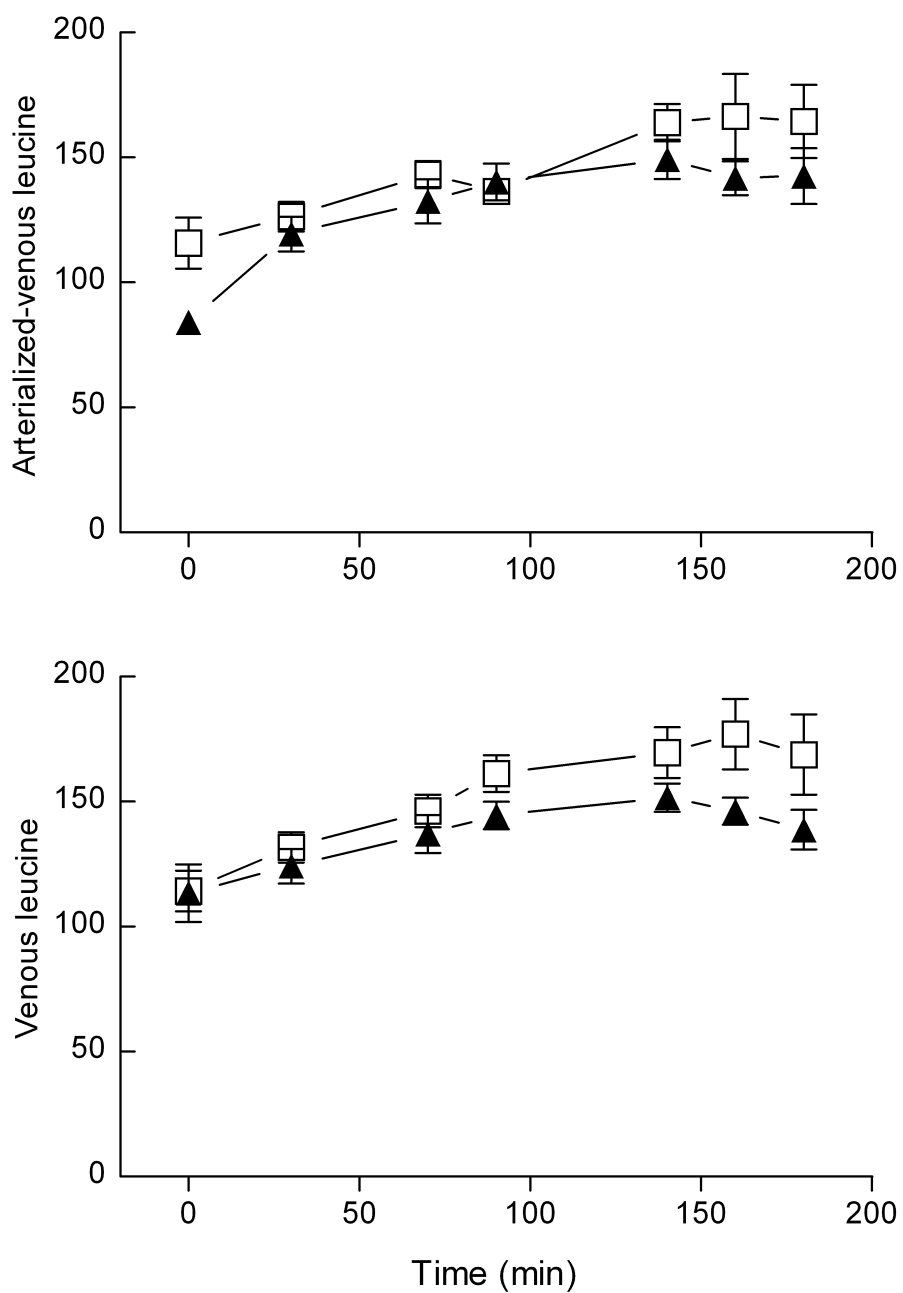


Figure 5.3 Plasma concentrations of venous and arterialized-venous leucine at baseline and during pancreatic clamping of insulin for young (closed triangles) and older (open squares) participants. All values mean  $\pm$  SEM (error bars not seen lie within symbols), expressed in mmol.l<sup>-1</sup>.

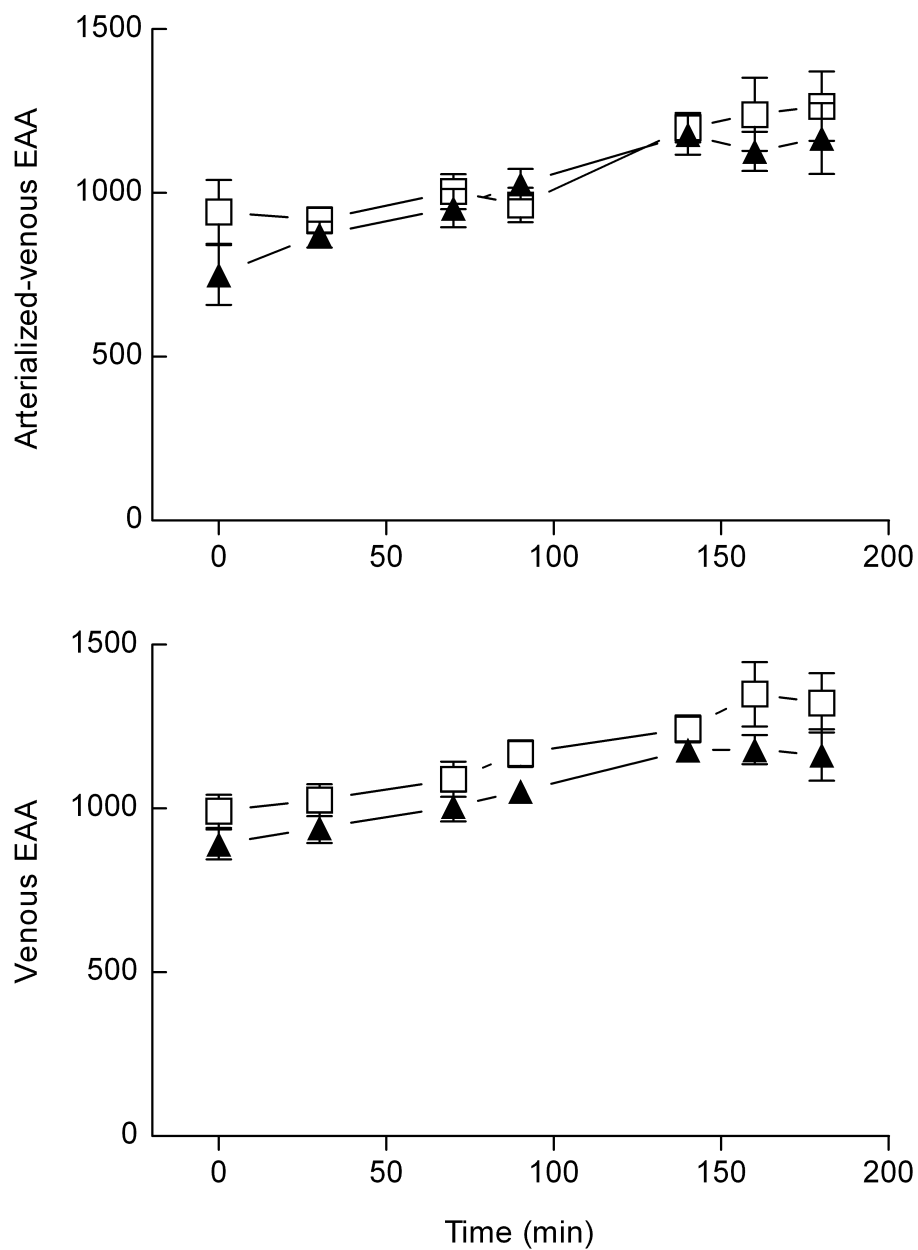


Figure 5.4 Plasma concentrations of venous and arterialized-venous essential amino acids (EAA), at baseline and during pancreatic clamping of insulin for young (closed triangles) and older (open squares) participants. All values are mean  $\pm$  SEM (error bars not seen lie within symbols), expressed in  $\text{mmol.l}^{-1}$ .



### 5.2.4 Isotope enrichment

Plasma enrichment of  $[1,2^{13}\text{C}_2]\alpha$ -ketoisocaproate (Figure 5.5) and  $\text{D}_5$  phenylalanine (Figure 5.6) were maintained at steady state throughout both periods for all participants.

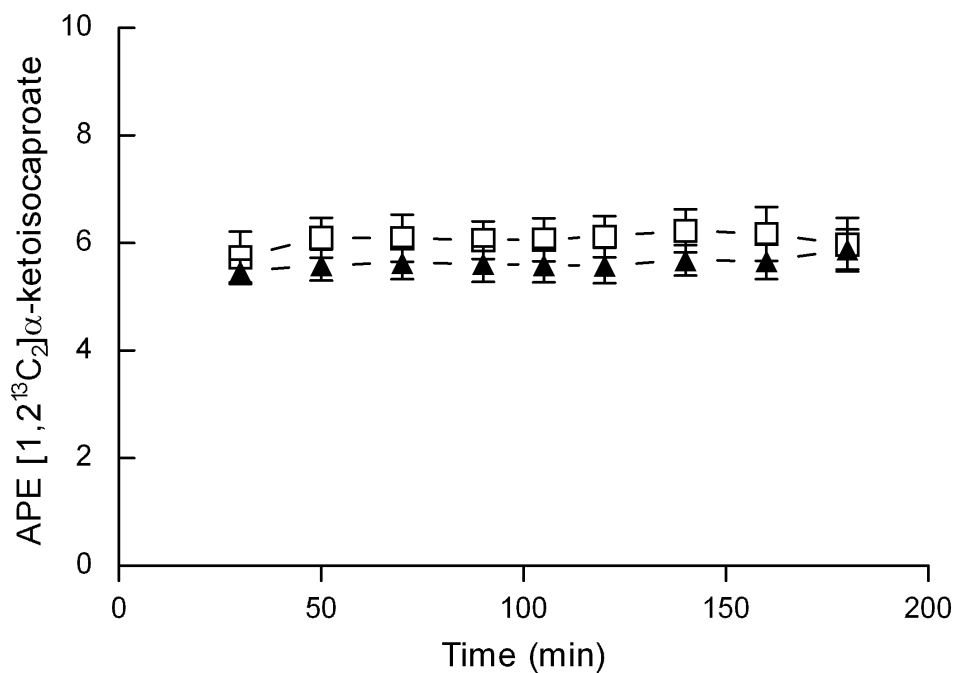


Figure 5.5 Atoms percent excess (APE) of  $[1,2^{13}\text{C}_2]$   $\alpha$ -ketoisocaproate in plasma taken from venous blood of young (closed triangles) and older (open squares) participants, throughout the study. Mean  $\pm$  SEM presented; error bars not seen lie within symbols.  $P > 0.05$  ANOVA repeated measures within groups;  $P < 0.001$  young vs. older.

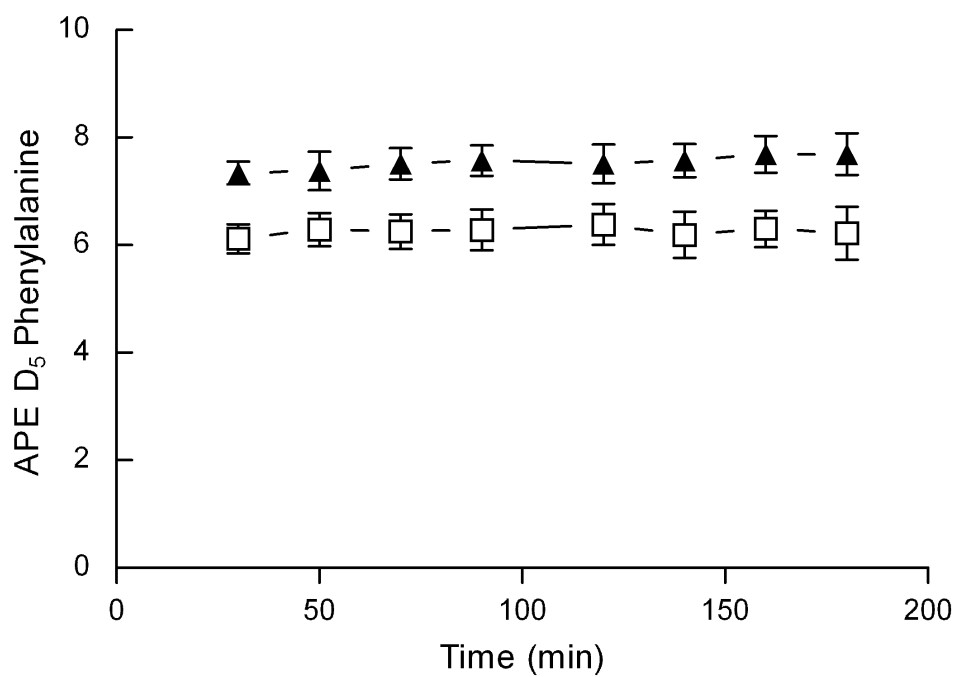


Figure 5.6 Atoms percent excess (APE) of D<sub>5</sub> Phenylalanine in plasma taken from venous blood of young (closed triangles) and older (open squares) participants, throughout the study. Mean  $\pm$  SEM presented; error bars not seen lie within symbols.  $P > 0.05$  ANOVA repeated measures;  $P < 0.001$  young vs. older.

### 5.2.5 Femoral artery blood flow

Femoral artery blood flow was greater in the young participants during both clamp periods. Increased insulin availability did not significantly alter blood flow in either group (Table 5.4).

Table 5.4 Mean femoral artery blood flow. Given as ml per 100ml leg per min; mean  $\pm$  SEM; young vs. older  $P < 0.05$ , within group analysis comparing insulin availability  $P > 0.05$ ; student's t-test.

	Young	Older	<i>P</i>
~ 5 $\mu\text{IU}\cdot\text{ml}^{-1}$ insulin	3.64 $\pm$ 0.30	2.39 $\pm$ 0.20	0.01
~ 15 $\mu\text{IU}\cdot\text{ml}^{-1}$ insulin	3.49 $\pm$ 0.33	2.56 $\pm$ 0.26	0.02
<i>P</i>	0.15	0.25	

### 5.2.6 Phenylalanine and leucine kinetics

#### *Phenylalanine flux across the leg*

The phenylalanine balance differed with insulin availability significantly in the young but not the old (Figure 5.7). In the young, at  $5 \mu\text{U}\cdot\text{ml}^{-1}$  insulin, phenylalanine balance was negative and significantly different from zero (or neutral balance) but at  $15 \mu\text{U}\cdot\text{ml}^{-1}$  insulin there was no difference from neutral phenylalanine balance. The older group did not differ from neutral balance at either insulin availability. An alternate version of this figure, accounting for the infused phenylalanine, is given in Appendix 7.

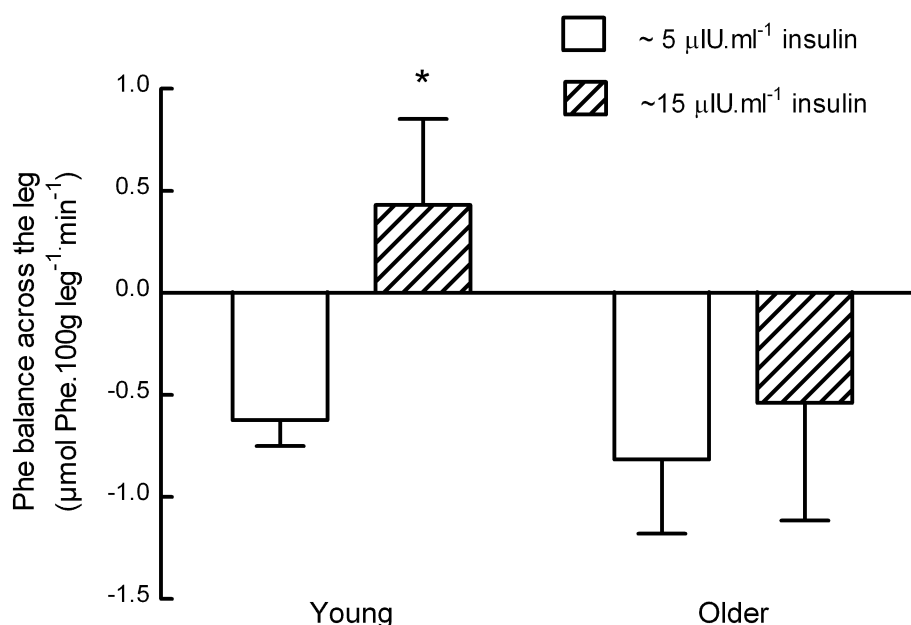


Figure 5.7 Balance across the leg of unlabelled phenylalanine after pancreatic clamping at  $\sim 5$  (open bars) and  $\sim 15 \mu\text{U}\cdot\text{ml}^{-1}$  insulin (hashed bars) for young and older healthy volunteers. Values are mean  $\pm$  SEM;  $n = 8$  both groups; the change in phenylalanine balance occurring with change in insulin availability was significant in the young (as indicated by the asterisk,  $P < 0.05$  two-way ANOVA) but not the older participants.

*Whole body leucine flux*

Whole body leucine flux did not differ between participants of different ages or when studied under different insulin availability (Figure 5.8).

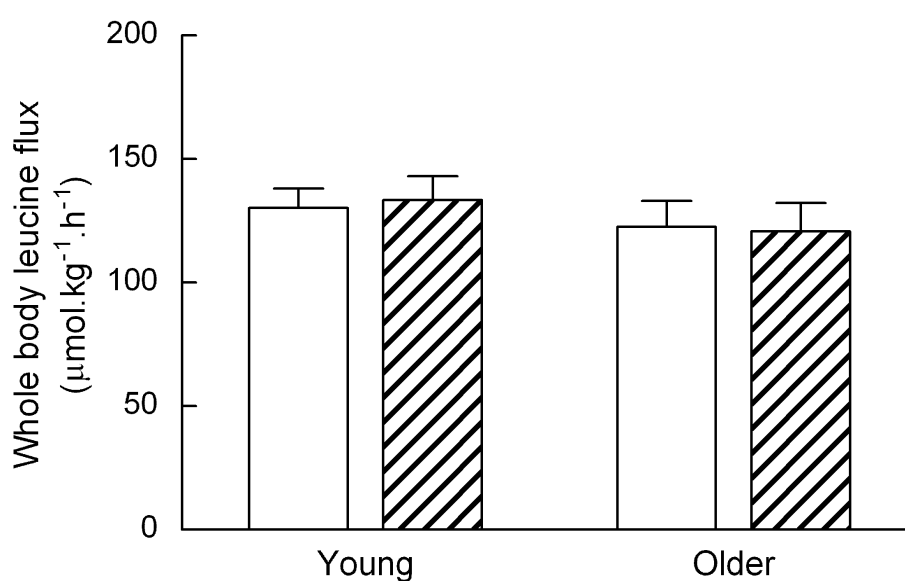


Figure 5.8 Whole body leucine flux for young and older participants measured following pancreatic clamping at ~ 5 (open bars) and ~ 15  $\mu\text{IU.ml}^{-1}$  insulin (hashed bars). Mean  $\pm$  SEM; all  $P > 0.05$  two-way ANOVA.

*Leg protein breakdown*

Leg protein breakdown (LPB), whether determined by leucine or phenylalanine tracer dilution in whole blood, did not differ between the groups at PA insulin concentrations. At higher insulin availability, LPB was suppressed in the young participants by ~ 60 % ( $P < 0.05$ ), whether measured using leucine or phenylalanine kinetics. In the older participants LPB was not significantly reduced with increased insulin (Figures 5.9 and 5.10).

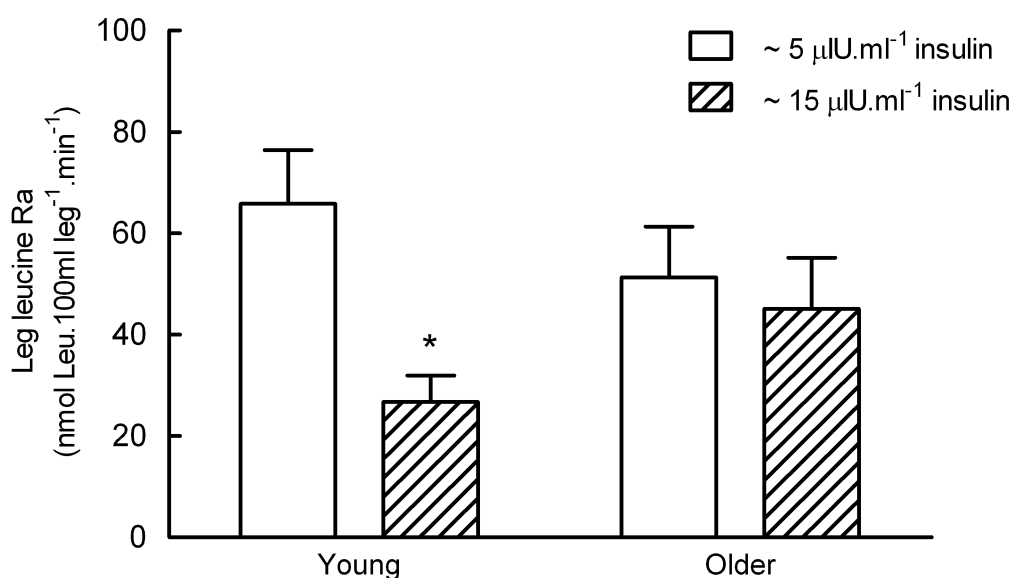


Figure 5.9 Leg protein breakdown as determined by leucine flux across the leg at ~ 5 (open bars) and ~ 15 μU.ml<sup>-1</sup> insulin (hashed bars) for young and older healthy volunteers. All values mean ± SEM. Significant change in the rate of leg protein breakdown, seen with higher insulin availability in the young, is marked by an asterisk ( $P < 0.05$ ; two-way ANOVA).

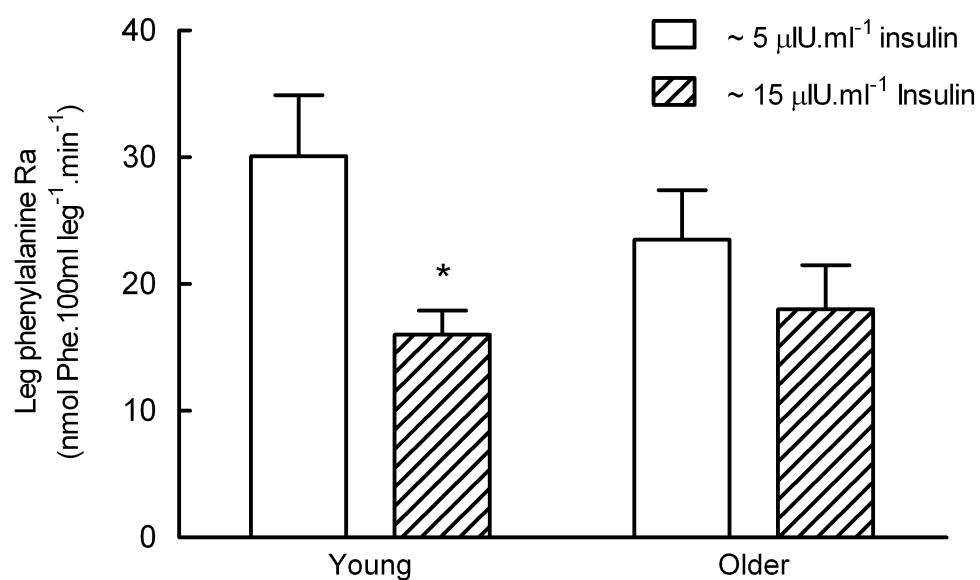


Figure 5.10 Leg protein breakdown as determined by phenylalanine flux across the leg at ~ 5 (open bars) and ~15  $\mu\text{U}.\text{ml}^{-1}$  insulin (hashed bars) for young and older healthy volunteers. All values mean  $\pm$  SEM. Significant change in rate of leg protein breakdown seen with higher insulin availability in the young is identified by the asterisk ( $P < 0.05$ ; two-way ANOVA).

### 5.2.7 Muscle protein synthesis

The rate of muscle protein synthesis, as determined by the incorporation of [1,2  $^{13}\text{C}_2$ ] leucine (Figure 5.11), did not change throughout the study for either group, nor was there a difference between data from young and older participants. Calculated rates of synthesis from phenylalanine flux did not differ from incorporation data (Figure 5.12).

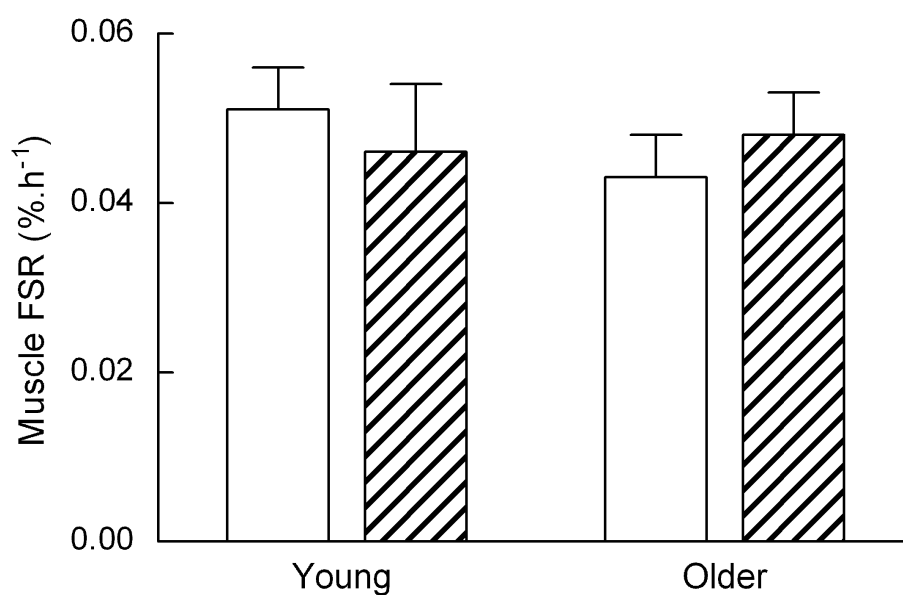


Figure 5.11 Fractional synthetic rate (FSR) of mixed muscle measured over 90 min at plasma insulin concentrations of ~ 5 (open bars) and ~ 15  $\mu\text{IU}\cdot\text{ml}^{-1}$  (hashed bars) for young and older healthy volunteers. All values mean  $\pm$  SEM; all  $P > 0.05$  two-way ANOVA.



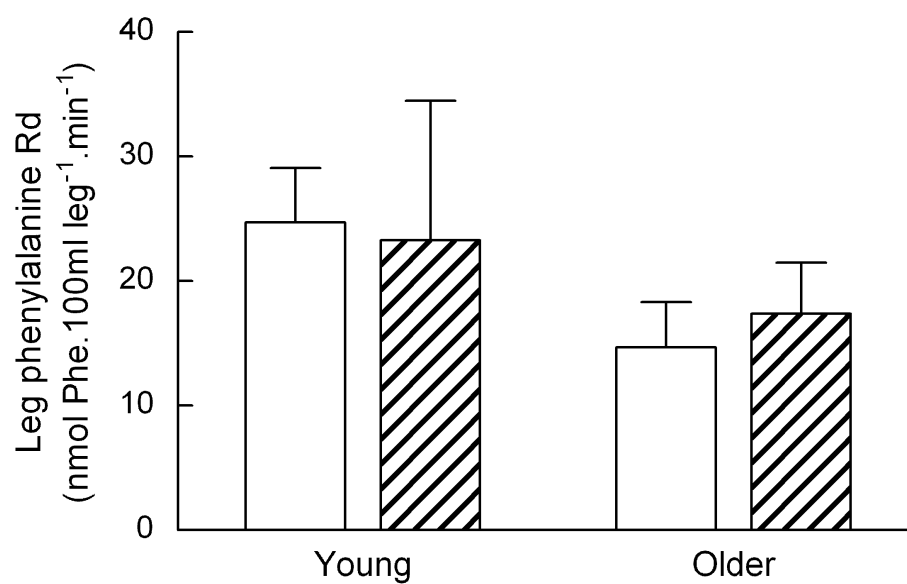


Figure 5.12 Leg protein synthesis as determined by phenylalanine flux (rate of disappearance, Rd) across the leg at ~5 (open bars) and ~15  $\mu\text{U}.\text{ml}^{-1}$  insulin (hashed bars) for young and older healthy volunteers. All values mean  $\pm$  SEM; all  $P > 0.05$  two-way ANOVA.

### 5.2.8 Activity of muscle signalling proteins

In both young and older subjects, PKB activity fell with increased insulin availability (both  $P < 0.03$ ; Figure 5.13). Phosphorylation status of eEF2, p70S6K, 4E-BP1 and FOXO1a did not change in either group at the time points sampled (Figures 5.14 – 5.17).

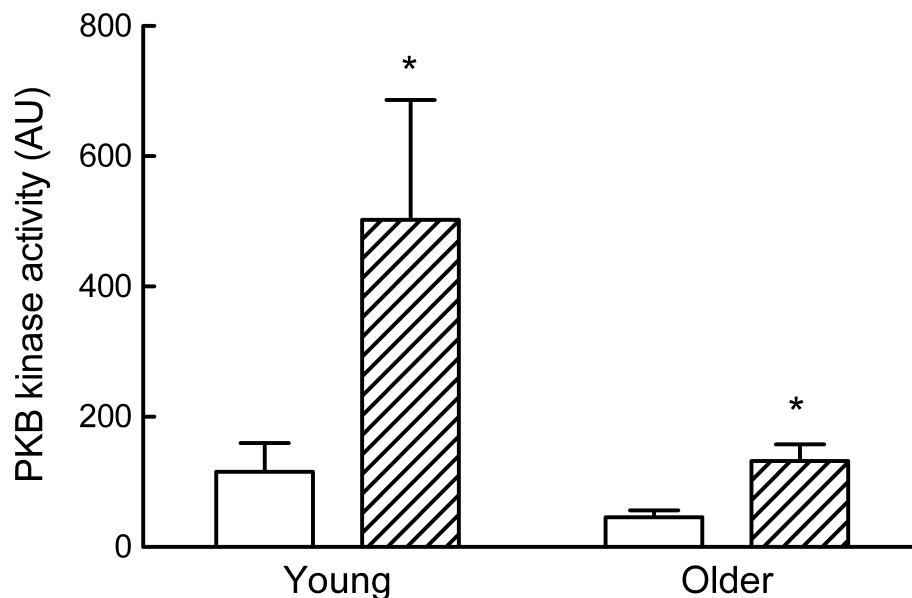


Figure 5.13 Protein kinase B activity relative to PKB recovery from immunoprecipitates for young and older healthy volunteers after 90 min pancreatic clamping at insulin concentrations of  $\sim 5$  (open bars) and  $\sim 15$  (hashed bars)  $\mu\text{IU}\cdot\text{ml}^{-1}$ . All arbitrary units (AU), with mean  $\pm$  SEM given; asterisk indicates the significant effect of insulin availability within each age group ( $P < 0.05$ ; two-way ANOVA). No interaction exists between age and insulin availability.

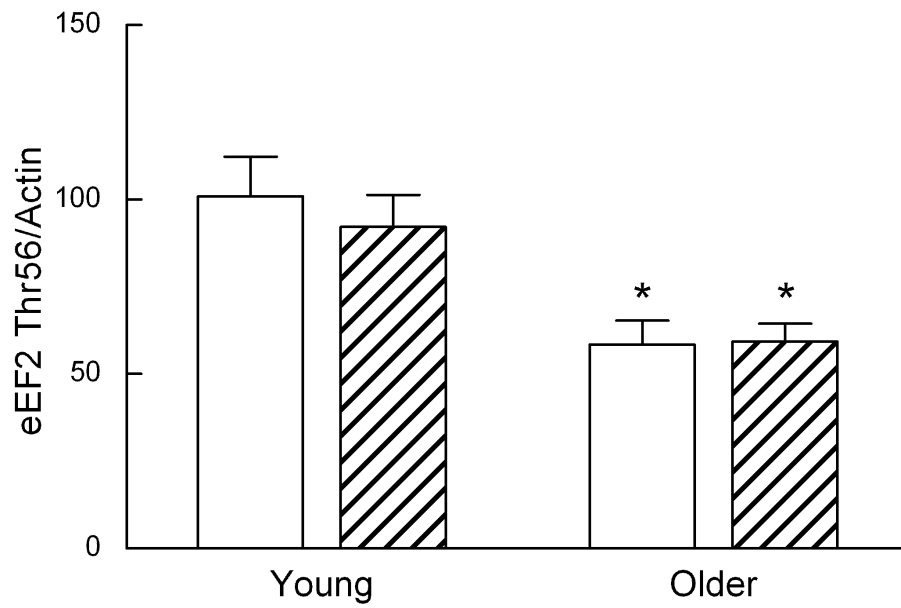


Figure 5.14 Change in phosphorylation status of eEF2 relative to actin for young and older healthy volunteers following 90 min pancreatic clamping at insulin concentrations of ~ 5 (open bars) and ~ 15 (hashed bars)  $\mu\text{IU}\cdot\text{ml}^{-1}$ . All arbitrary units, mean  $\pm$  SEM; the effect of age, but not insulin availability, was significant ( $P < 0.05$ , two-way ANOVA). No interaction exists between age and insulin availability. Asterisk denotes significant difference from young data.

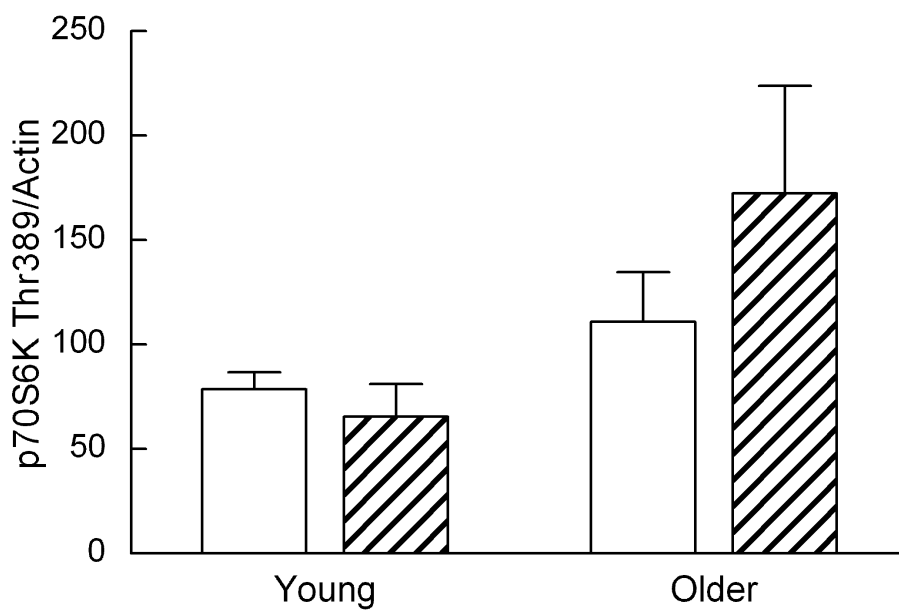


Figure 5.15 Change in phosphorylation status of p70S6K relative to actin for young and older healthy volunteers following 90 min pancreatic clamping at insulin concentrations of ~ 5 (open bars) and ~ 15 (hashed bars)  $\mu\text{U}\cdot\text{ml}^{-1}$ . All arbitrary units, mean  $\pm$  SEM; all differences, including interaction  $P > 0.05$ , two-way ANOVA.

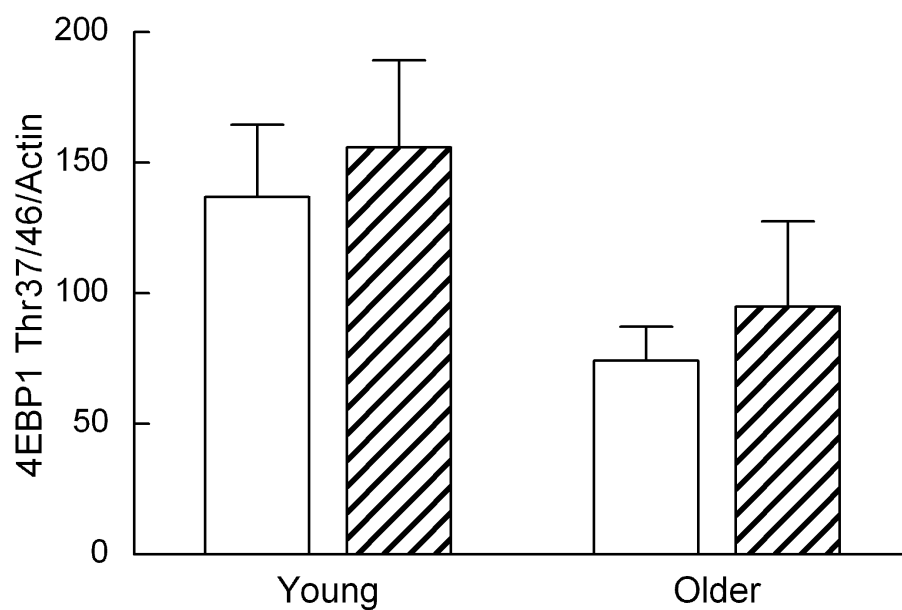


Figure 5.16 Change in phosphorylation status 4E-BP1 relative to actin for young and older healthy volunteers following 90 min pancreatic clamping at insulin concentrations of  $\sim 5$  (open bars) and  $\sim 15$  (hashed bars)  $\mu\text{U}\cdot\text{ml}^{-1}$ . All arbitrary units, mean  $\pm$  SEM; all differences, including interaction of age and insulin,  $P > 0.05$ , two-way ANOVA.

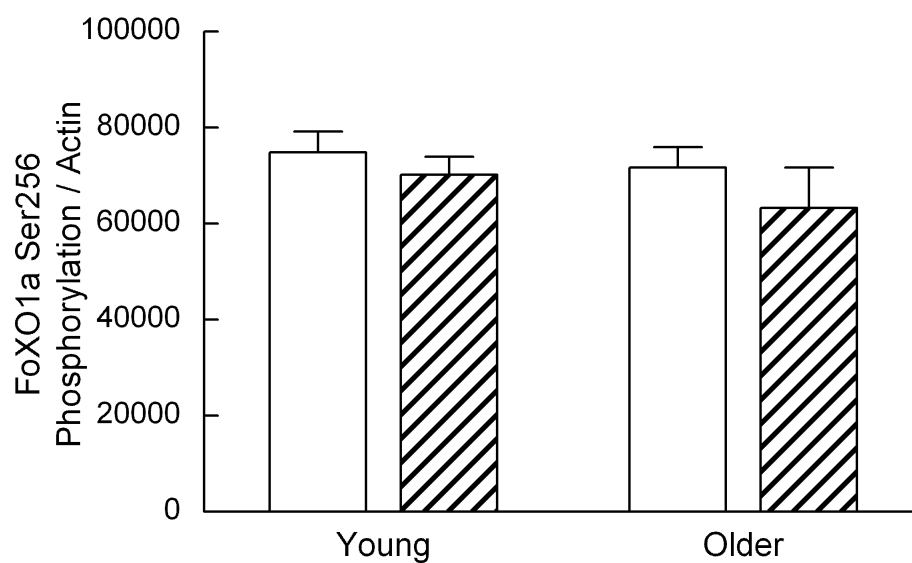


Figure 5.17 Change in phosphorylation status of FoxO1a relative to actin for young and older healthy volunteers following 90 min pancreatic clamping at insulin concentrations of ~ 5 (open bars) and ~ 15 (hashed bars)  $\mu\text{IU}\cdot\text{ml}^{-1}$ . All arbitrary units, mean  $\pm$  SEM; all differences  $P > 0.05$ , including interaction,  $P < 0.05$ , two-way ANOVA.

### 5.2.9 Change in gene expression

The change in expression of C2 proteosomal subunit, MuRF1, MAFbx, Cathepsin L and Ubiquitin mRNA in muscle tissue taken after 90 min exposure to plasma insulin concentrations of  $\sim 5 \mu\text{IU}\cdot\text{ml}^{-1}$  insulin did not differ to that measured after exposure to  $\sim 15 \mu\text{IU}\cdot\text{ml}^{-1}$  insulin (Figures 5.18 and 5.19). GLUT4 mRNA expression increased with the higher concentration of insulin in both groups, albeit only reaching significance in the older group (Figure 5.20).

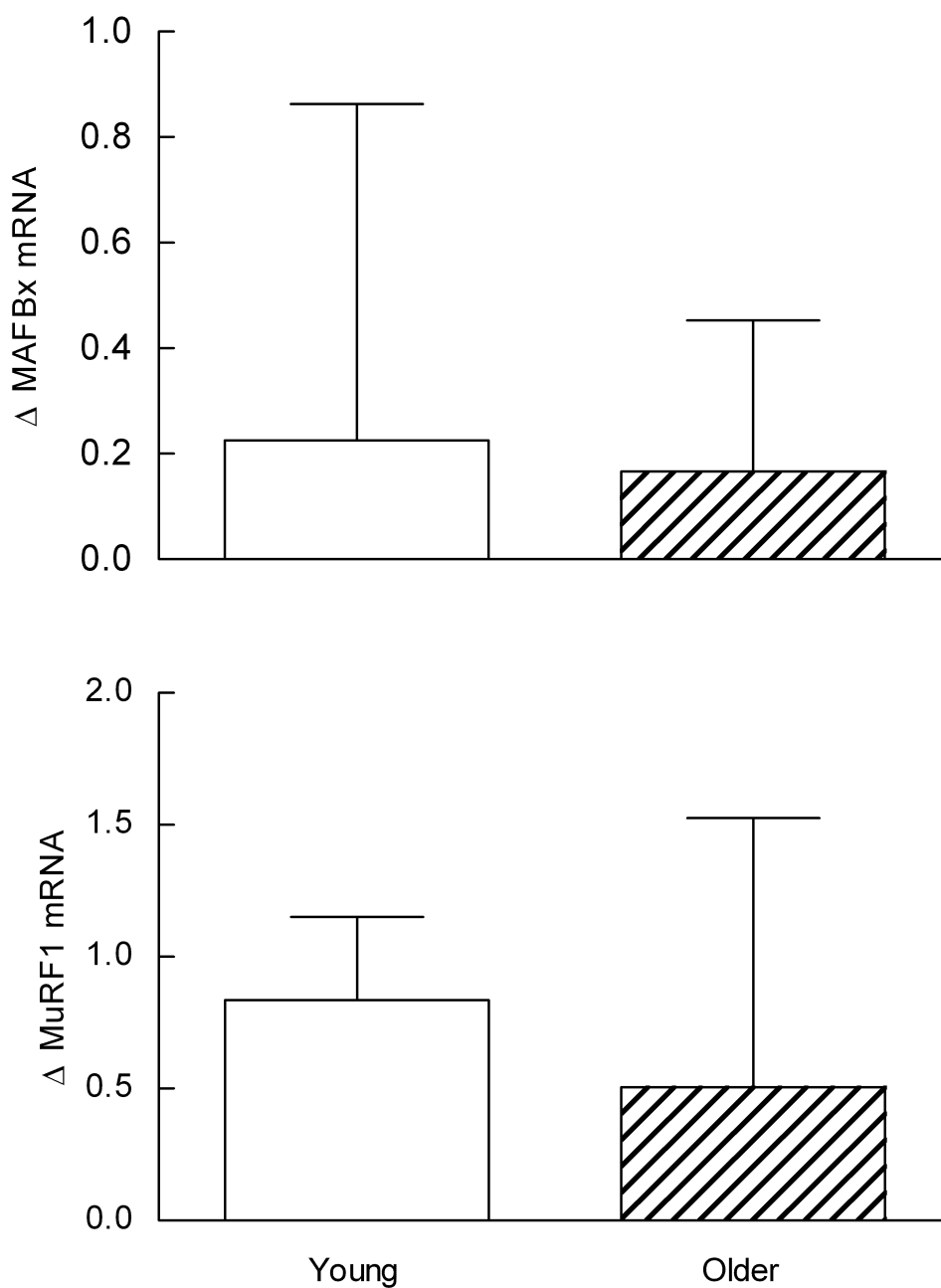


Figure 5.18 Fold change in expression of atrogene mRNA, specifically MAFBx and MuRF1, in young (open bars) and older (hashed bars) healthy volunteers after 90 min pancreatic clamped at insulin concentrations of  $\sim 15$  compared with  $\sim 5 \mu\text{IU}\cdot\text{ml}^{-1}$ . Bars represent mean  $\pm$  SEM; all  $P > 0.05$  young vs. older Student's *t* test.



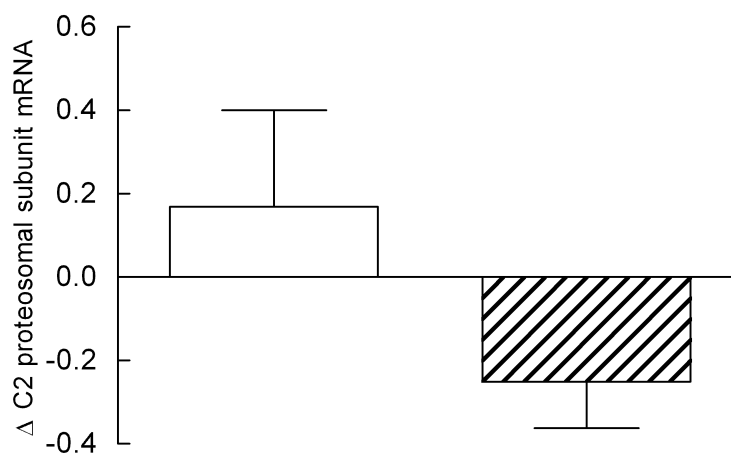
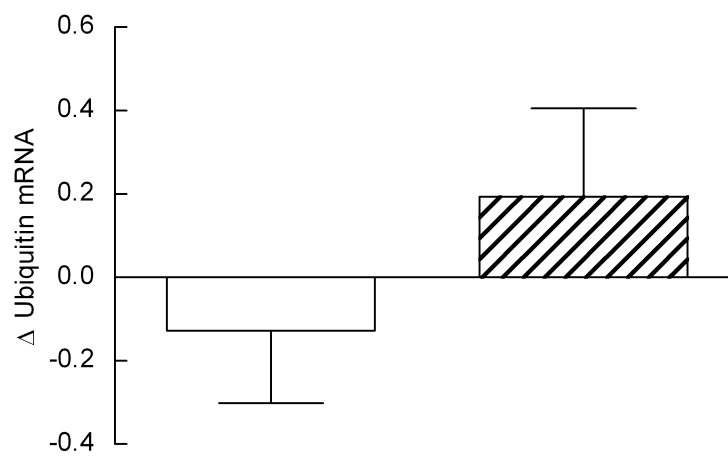
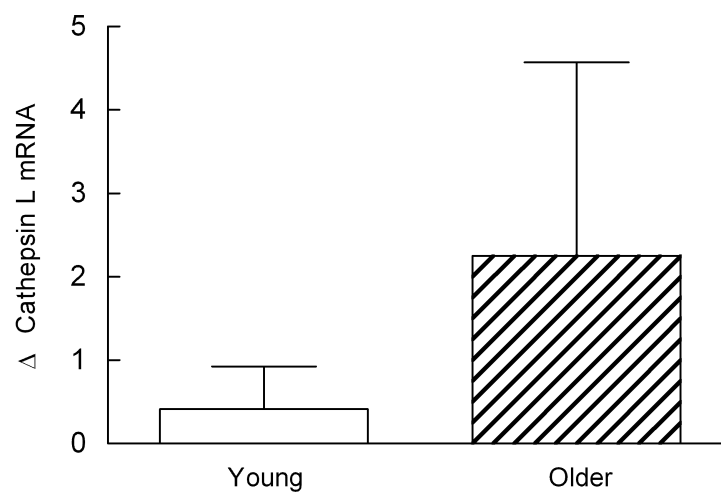


Figure 5.19 Fold change in expression of Ubiquitin, C2 proteosomal subunit and Cathepsin L mRNA in young (open bars) and older (hashed bars) healthy volunteers following 90 min pancreatic clamped at insulin concentrations of ~15 compared with ~5  $\mu\text{IU}\cdot\text{ml}^{-1}$ . Bars represent mean  $\pm$  SEM; all  $P > 0.05$  young vs. older, Student's t test.

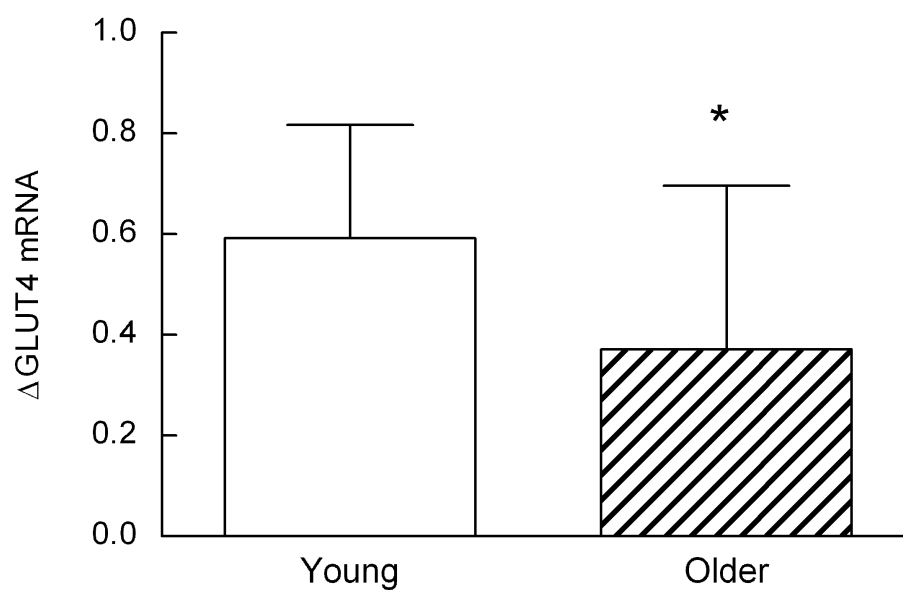


Figure 5.20 Reduction in expression GLUT4 mRNA in young (open bars) and older (hashed bars) healthy volunteers after a 90 min period of higher insulin availability, where mRNA expression during the lower insulin clamp is normalized to 1.0. Bars represent mean  $\pm$  SEM; a significant reduction from  $\sim 5 \mu\text{IU}\cdot\text{ml}^{-1}$  clamp values occurs in the older group indicated by asterisk ( $P < 0.05$  Student's *t* test). No difference was seen between the groups.

### 5.3 Discussion

These data demonstrate that ageing interferes with the normal suppression of LPB in response to a moderate rise in plasma insulin concentration. Under conditions of PA insulin availability, i.e.  $\sim 5 \mu\text{IU}\cdot\text{ml}^{-1}$ , there is no age differential in the control of leg protein turnover; whereas elevation of plasma insulin concentrations to  $\sim 15 \mu\text{IU}\cdot\text{ml}^{-1}$  (into the postprandial range) suppresses leg proteolysis in the young to a greater degree than in older healthy volunteers.

#### 5.3.1 Clamp conditions

The data presented in this chapter cannot simply be explained by differences in body composition or insulin sensitivity as young and older healthy volunteers were not dissimilar. As in Chapter 4, the older participants had no evidence of pathological sarcopenia, although 2 of the older subjects had lost up to 1.5 kg LBM since participating in a previous program  $\sim 2$  y earlier. Both groups had normal calculated values for insulin sensitivity ruling out the possibility of incipient diabetes contributing to impaired muscle metabolism. Indeed basal insulin concentrations, HOMA-IR and QUICKI indices all suggest a greater degree of insulin sensitivity in the older group. Also, availability of free fatty acids, which have been shown to induce insulin resistance in skeletal muscle (Dresner *et al.* 1999), did not differ at any time point.

Elevated plasma AA concentrations were seen in both the young and older volunteers over the 3 h study period, suggesting that infused AA were given excessively. Ideally real-time BCAA analysis would have been used alongside the glucose measurements in order to titrate the AA infusion to plasma concentrations; however development of a

real-time fluorometric assay, as used by others (Chevalier *et al.* 2004; Beckett 2000), was hampered by analytical problems. Thus best guess estimates were used in the development phase to establish a consistent protocol for later use. The main aim, to prevent hypoaminoacidaemia, was achieved for all participants and plasma AA concentrations did not increase sufficiently to elicit muscle protein synthesis.

Venous plasma concentrations of individual EAA did not differ between young and older participants throughout the clamps; nevertheless half of the individual EAA concentrations rose in the second period in the older group, but not in the young. Impaired inhibition of MPB may be responsible, although it is feasible to suggest that hepatic deamination, which is diminished with ageing, is also culpable (Odeh-Ramadan & Remington 2002). Indeed other workers have also reported similar differences in plasma AA profiles with ageing (Rudman *et al.* 1989). Nevertheless increased AA availability is not thought to alter rates of MPB (Bennet *et al.* 1990b; Biolo *et al.* 1997; Volpi *et al.* 1999; Volpi *et al.* 2003).

A second characteristic which differed between the groups was blood flow. The phenomenon of vasodilator blunting in response to insulin as a result of ageing has been recognized previously (Rasmussen *et al.* 2006; Meneilly *et al.* 1995) and may account for significantly lower blood flow seen in our older healthy volunteers. Other cardiovascular factors affecting blood flow, such as cardiac output and peripheral resistance, were not measured but are also likely to contribute. A dose dependent effect of insulin upon blood flow to the limb has been reported at insulin concentrations which exceed  $45 \mu\text{IU}\cdot\text{ml}^{-1}$  (Fujita *et al.* 2006; Biolo *et al.* 1999; Arfvidsson *et al.* 1991),

but not with moderate changes in insulin (Fujita *et al.* 2006) in keeping with the data presented here.

### 5.3 2 Whole body and leg protein kinetics

Other workers (Guillet *et al.* 2004b; Boirie *et al.* 2001) have measured whole body, but not muscle, leucine flux in response to different availabilities of insulin (up to 30  $\mu\text{IU}\cdot\text{ml}^{-1}$ ). They have shown that protein breakdown is suppressed when plasma insulin is increased to absorptive concentrations in volunteers of all ages, but the degree of suppression is reduced in older human beings. However the whole body leucine flux data presented in this chapter did not confirm their findings: in the present data, whole body leucine flux did not differ with increasing insulin availability or between participants of different ages.

These disparate findings may be accounted for by differences in body composition, as this differed markedly between studies, particularly for the younger participants. Mean values for BMI and proportion of fat mass were substantially lower in the work by Boirie and colleagues when compared with this data; thus the relative contribution of muscle protein turnover (rather than splanchnic protein turnover) to whole body flux is greater. The converse is true for less lean subjects. Consequently, suppression of MPB by insulin is offset by enhanced splanchnic proteolysis and whole body net balance remains unchanged. This differential effect of insulin on splanchnic and muscle tissue has been described previously, albeit not under isoaminoacidaemic conditions (Nygren & Nair 2003; Meek *et al.* 1998). Thus, if the whole body work of Boirie and colleagues (Guillet *et al.* 2004b; Boirie *et al.* 2001) predominantly reflects changes in muscle

proteolysis in response to insulin, then their data corresponds well with that presented here.

There are currently no other studies which assess the effect of postprandial insulin on the rate of leg or muscle proteolysis under isoaminoacidaemic conditions with ageing. Estimates of MPB in the PA state do not appear to differ with age (Hasten *et al.* 2000; Volpi *et al.* 2000; Volpi *et al.* 2001; Yarasheski, Zachwieja, & Bier 1993) in keeping with the data presented here. In young adults, suppression of MPB appears dependent upon the dose of insulin, provided that isoaminoacidemia is maintained (Chow *et al.* 2006; Moller-Loswick *et al.* 1994; Petrides, Luzi, & DeFronzo 1994; Louard *et al.* 1994; Arfvidsson *et al.* 1991; Fryburg *et al.* 1990; Flakoll *et al.* 1989; Gelfand & Barrett 1987; Tessari *et al.* 1986). Thus postabsorptive plasma AA concentrations were maintained throughout both phases of the study using a proprietary AA preparation, such that arterial EAA concentrations crept up slightly above basal values for both age groups towards the end of the study. Nevertheless, the rise in EAA availability was not sufficient to stimulate MPS, nor anabolic signalling protein phosphorylation, both of which remained at post absorptive values for both phases of the study. Furthermore, age did not affect rates of basal mixed MPS, in keeping with other published results (Volpi *et al.* 2001; Volpi *et al.* 2000; Katsanos *et al.* 2006; Yarasheski *et al.* 1999; Cuthbertson *et al.* 2005). Also, insulin has no effect upon MPS, beyond that of altering AA delivery to the tissues, as show by other workers (Greenhaff *et al.* 2008; Gelfand & Barrett 1987; Chow *et al.* 2006; Moller-Loswick *et al.* 1994) and confirmed by the current results.

Contradictory data published by Rasmussen *et al.* and Fujita *et al.* separately suggest that insulin can be infused directly into the femoral artery without eliciting a change in the rate of local MPB (Rasmussen *et al.* 2006; Fujita *et al.* 2006). Rasmussen *et al.* compared responses to local insulin infusions between young and older healthy volunteers in whom venous amino acid concentrations were maintained within 10 % of basal; however insulin concentrations within the femoral vein were doubled in the older group ( $40.3 \pm 1.0$  vs.  $83.4 \pm 6.2$   $\mu\text{IU}\cdot\text{ml}^{-1}$ ; young vs. older,  $P < 0.05$ ). When results from young volunteers with comparably high insulin concentrations are compared with the older group data there is a clear trend towards inhibition of proteolysis. Fujita *et al.* present data which include a drop in the rate of appearance of phenylalanine at both low (~24%) and high insulin concentrations (~35%) albeit only reaching significance at the higher concentration, again confirming the presence of MPB suppression.

### 5.3.3 Changes in phosphorylation of signalling proteins

In terms of signalling proteins, PKB activity (not just phosphorylation status) which is frequently used as a proxy of insulin action (Adams *et al.* 2004), rose as expected with increased insulin availability. However, the presence of activated PKB should ordinarily inactivate FoxO1a (involved in muscle atrophy programmes) through phosphorylation and nuclear exclusion (Brunet *et al.* 1999), but this was not seen at the higher insulin availability. The combination of no change in FoxO1a phosphorylation status and failure to detect down-regulation of atrogene mRNA expression reduces the likelihood of inhibition of MPB by the PKB-FoxO1a-atrogene axis.

Both the anabolic sensing-signalling protein mTOR and the elongation factor eEF2, appear, at least *in vitro*, to be activated by insulin eEF2 (Redpath, Foulstone, & Proud



1996; Proud 2006) and are strongly implicated in the regulation of protein translation. However these data demonstrate that at low physiological insulin concentrations, despite PKB signalling, down-stream phosphorylation targets of mTOR and eEF2 signalling are unaffected which may explain the lack of protein synthetic response to insulin.

#### **5.3.4 Significance and future research**

In real terms, the degree of insulinaemia achieved during the second period of this study ( $\sim 15 \mu\text{U}\cdot\text{ml}^{-1}$ ) is comparable with that expected after a “healthy” low glycemic index meal, such as breakfast or a light lunch (Juntunen *et al.* 2002; Brand-Miller *et al.* 2005), yet it would appear from these results that the normal suppression of proteolysis by insulin at this concentration is impaired with ageing. Whether this blunted sensitivity to insulin is also present at higher insulin concentrations, as may occur with meals containing refined carbohydrates, remains to be investigated. Nevertheless it appears that loss of muscle with ageing may be accelerated not only by a blunted anabolic response to AA but also relative insensitivity to the anti-proteolytic effects of insulin.

## **Chapter 6**

### **General Discussion**

## 6.1 Discussion

This thesis aimed to address a number of different hypotheses. Specifically:

1. That daily administration of 200 mg thalidomide taken orally for 6 weeks is superior to placebo in terms of weight gain (both TBW and LBM) in patients with cachexia caused by oesophageal cancer and that it is well tolerated.
2. That administration of insulin to achieve supraphysiological plasma concentrations overcomes anabolic blunting in older human beings when compared to their younger counterparts, both in terms of rates of muscle protein synthesis and phosphorylation of key intracellular signalling proteins in the mTOR pathway.
3. That the effect of a modest increase in blood insulin concentration upon suppression of leg protein breakdown (and key signalling proteins) is diminished in older healthy volunteers, when compared with young controls.

The previous 3 chapters have presented data from a series of experiments conducted in vivo on healthy volunteers and patients with advanced oesophageal cancer, which aimed to test these hypotheses. Unfortunately it was not possible to reject the null hypothesis in the study assessing thalidomide use in the oesophageal cancer (Chapter 3), as too many participants were unable to complete the protocol due to ill-health and/or side-effects of the study drug. Nevertheless the trial demonstrated a high degree of toxicity in the patients studied, which was not expected from pilot work (Khan et al. 2003) which suggested 200 mg was both well tolerated and efficacious over a 2 w period. Whether the same adverse event profile would have been seen with a smaller dose, or indeed whether a smaller dose would have a beneficial effect upon body

composition is unclear. However, patients allocated to receive placebo maintained their body weight, including lean tissue, against expectation, presumably as they maintained adequate nutrition. Aggressive dietary and medical advice alone appears sufficient, or in the very least superior to thalidomide, to maintain body mass in end-stage oesophageal cancer.

In sarcopenia, high dose insulin administration fails to overcome blunted intracellular anabolic signalling pathways in response to intravenous AA feeding in older healthy volunteers (Chapter 4). Blood AA concentrations were only modestly elevated, such that no difference could be detected between the FSR of young and older quadriceps muscle; however the phosphorylation of key intracellular proteins, specifically 4E-BP1, p70S6K, PKB and GSK3 $\beta$  in response to AA feeding was blunted in the young. This blunted signalling response suggests that excessive insulin availability is not sufficient to overcome anabolic blunting to AA in ageing. Nevertheless it does not disprove the idea that insulin resistance of protein metabolism underpins anabolic blunting. Indeed this may explain the lack of suppression of leg protein breakdown in older healthy volunteers induced by administering modest doses of insulin to simulate a light meal (Chapter 5); although no difference was seen between rates of leg protein breakdown in young and older healthy volunteers at basal insulin availability.

## 6.2 Further work

It appears from the available literature and work presented in this thesis that physiological changes occur in skeletal muscle with healthy ageing and that the deficits are two-fold: both blunted anabolism and accelerated insulin-driven catabolism. A vast amount of work is still required to further elucidate the mechanism by which these processes occur, such that effective therapeutic agents can be developed. Specifically:

1. Determining the time course of anabolic responses to AA and whether AA pharmacokinetics differ with ageing.
2. Elucidating whether blunted suppression of protein breakdown with ageing occurs at higher insulin availabilities, such that occur following a high glycaemic index meal. It is plausible that “healthy” eating may be counterproductive in older human beings as higher insulin concentrations may be required to adequately suppress muscle catabolism.
3. Exploring other pharmaceutical agents such as statins or melatonin.

At present, the simple, safe and inexpensive intervention of adequate, regular dietary intake remains germane to both sarcopenia and cachexia.

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## **Appendix 1**

**Rennie MJ and Wilkes EA (2005) Maintenance of Musculoskeletal Mass by Control of Turnover: The Concept of Anabolic Resistance and its Relevance to the Transplant Recipient. Annals of Transplantation. 10, 32-35**

# Maintenance of the Musculoskeletal Mass by Control of Protein Turnover: The Concept of Anabolic Resistance and its Relevance to the Transplant Recipient

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ANNALS OF TRANSPLANTATION, Vol. 10, No. 4, 2005, pp. 32–35

## Abstract:

Although the overall size of the musculoskeletal mass is constrained by genetic limitations, both the day to day maintenance and muscle wasting and rehabilitation are regulated by protein synthesis (particularly the initiation and elongation stages of translation) and by protein breakdown. These are directly influenced by the nutritional state (size and composition of meals) and type, mode and duration of exercise. In the context of food-related changes, recent work has demonstrated that human muscle protein synthesis is almost entirely controlled by the availability of essential amino acids and protein breakdown by availability of insulin. Muscle protein synthesis is also markedly stimulated by preceding exercise in a manner independent of but additive to any effect of food. The sensing and signalling pathways within muscle are activated by food and exercise in normal healthy subjects to elevate net muscle balance for many hours after strenuous exercise.

In many circumstances such as immobilisation, ageing and many chronic diseases of the lung, kidney, heart, etc (such as those often suffered by pre-transplant patients), the general debilitation includes muscle wasting. In these subjects there appears to be a general failure to respond adequately to food – so called „anabolic resistance“. It seems highly likely that this circumstance will also apply to transplant recipients. It is also likely that anabolic resistance can be, to some extent, reversed by regular physical activity which may „tune up“ the anabolic pathways to act in a more normal fashion. Nevertheless, the extent of re-growth and adaptation of composition of muscle in transplant patients could be hindered by drug treatment including the use of rapamycin (sirolimus) cyclosporine and corticosteroids. These predictions should be tested by examining longitudinal effects of different modes of exercise and nutritional regimens on rehabilitation of muscle in transplant patients.

## Acknowledgements:

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**Key words:** Protein Synthesis; Protein Breakdown; Muscle; Bone; Tendon; Signalling

## Introduction

Musculoskeletal mass i.e. muscle, tendon, ligament and bone account for about 75% of the body mass and contain about 85% of the total body protein. Muscle alone accounts for about half of all of the whole body protein turnover during the diurnal period in the human body because although the turnover in muscle is only of the order of 1-2% per day it accounts for 40% of the total body mass. Although collagen comprises a large part of the total protein in our body, possibly as much as 3 kg compared to say 12kg of non-collagen protein in muscle, its nutritional physiology is poorly understood although with the use of new and better techniques a substantial amount of new data is currently accruing.

Genetic influences are undoubtedly important but it is remarkable how much the environment influences body mass and composition. Even identical twins who follow completely different exercise and diet regimes are able to substantially modify the appearance and size of their bodies (Fig. 1). Diet and exercise are the most important of the physiological variables altering the extent of the turnover of protein in the musculoskeletal mass and they appear to have an

influence over remarkably short periods of time – for example within half an hour of taking a meal or finishing exercise, anabolic processes are often accelerated at least two fold. These changes by and large come about as the result of manipulation of pre-existing protein and mRNA within the cells of the musculoskeletal system, probably occurring too quickly to be the result of the production of new mRNA by transcription in the nucleus (setting aside examples of the so-called early response genes, such as *c-fos* and *c-jun*). In this article we will describe some of the changes which occur as a result of feeding and exercise in healthy muscle, tendon and bone and describe the concept of anabolic resistance and its possible importance for understanding chronic wasting in a number of conditions, such as ageing and many chronic diseases leading to sarcopenia such as failure of kidneys, heart, liver and lungs. We predict that similar circumstances will occur in transplant candidates and recipients; we

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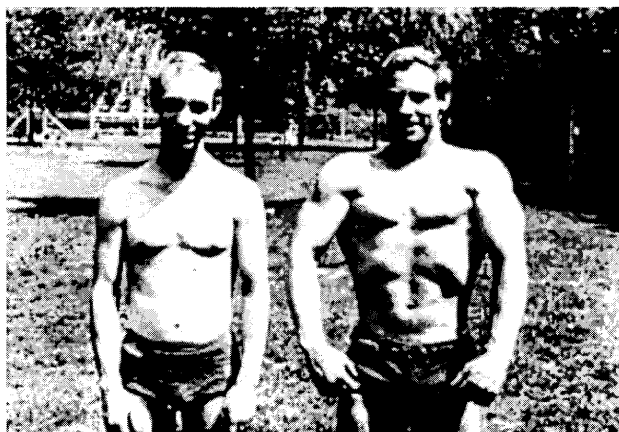


Figure 1. Otto and Ewald- identical twins with different bodies due to different nutrition and training.

discuss specific circumstances of the care of patients after transplant which may influence their ability to rehabilitate their muscles in terms of mass and fibre type composition.

***Human muscle is not necessarily like animal muscle***

Many workers have relied upon the help of surgeons who have been able to provide, with suitable consent, samples of for example rectus abdominus, intercostal muscles or limb muscles, adventitiously. However, the major problem has been knowing whether these muscles behave, in terms of protein turnover, like their counterparts in animals. In rodents, lagomorphs and birds there are quite distinctly different rates of protein metabolism according to whether the muscles are dark or pale with the dark, slow-twitch muscles often turning over protein twice as fast as the fast-twitch pale muscles. We have recently published results [1] showing that this is unlikely to be the case for human beings since the rates of muscle protein synthesis in soleus, quadriceps and triceps muscle (which are respectively mostly dark type I, a 50: 50 mixture and mostly pale type II muscles) all have very similar rates of muscle protein synthesis in the basal post absorptive state and after feeding. What this means is that for the purposes of studies of muscle protein turnover with feeding or comparisons of patients in a particular metabolic state, one kind of muscle is as good as another. This is probably likely not to be the case for muscles which have different exposures to physical activity, although we are not yet certain of this. A further difference between animals and human beings in terms of responses to feeding is the relative importance of amino acids and insulin. In rodents (which are nearly always studied as relatively immature animals) insulin markedly stimulates muscle protein synthesis but as will be described below, it has little influence on protein synthesis in human beings [2-4].

***Effects of feeding***

In our experience, muscle protein synthesis can be doubled by feeding a mixed meal given either orally or intravenously and most of the effect in stimulating muscle protein synthesis appears to be due to the amino acids, especially the essential amino acids and probably in particular leucine [5-7].

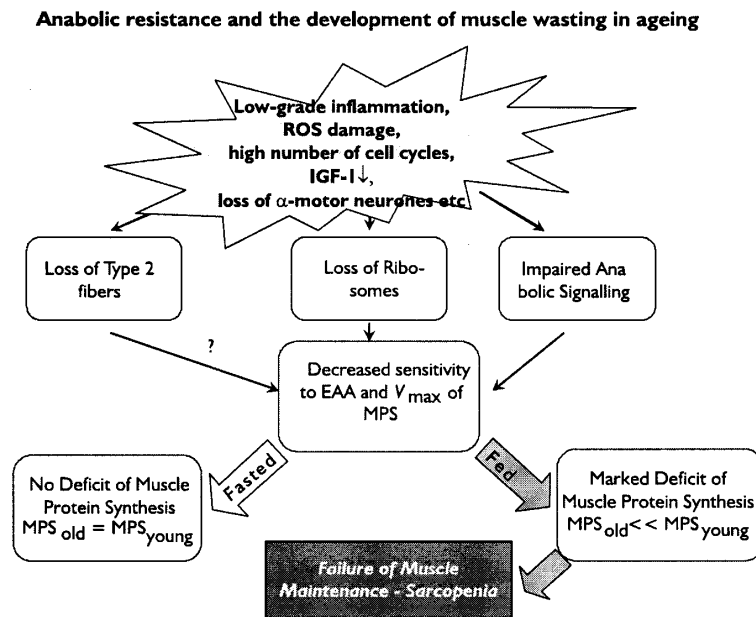
Although there is still some doubt about the importance of the value of the availability of basal concentrations of insulin in the post absorptive state, our results suggest very strongly that insulin has no effect in modulating the increase in protein synthesis due to amino acids. The findings of others and ourselves strongly indicates that instead insulin has a major role in inhibiting muscle protein breakdown whereas amino acids in human beings at least have very little effect on this [2-4,7].

***The effect of nutrition on collagen containing tissues***

Unlike myofibrillar and sarcoplasmic proteins of the myofibre it appears that collagen present in muscle (made by inter myofibre fibroblasts) is insensitive to nutrition as is the collagen made by tenocytes [8]. However, collagen made by human bone is exquisitely sensitive to nutrition showing almost as much of an increase with mixed feeding [9] as does human muscle cell protein (i.e. not muscle collagen).

***Responses to physical activity***

Strenuous exercise markedly increases muscle protein synthesis and although it was previously thought that these increases occurred only with resistance type exercise [10], we now have evidence that strenuous dynamic exercise either one legged kicking or stepping up and down onto a block carrying a weight can stimulate muscle protein synthesis as much as resistance type exercise [11,12]. The mechanisms involved in stimulation of muscle protein synthesis by exercise and by feeding appear to be additive, suggesting that the final effect occurs by different,



**Figure 2.** Scheme showing underlying changes leading to anabolic resistance in old muscle- and possibly transplant recipients

separate pathways in each case. It is now known that there are a number of anabolic sensing and signalling pathways within muscle involving intracellular proteins which may be phosphorylated or dephosphorylated, consequently changing their activity to alter rates of the initiation and elongation phases of muscle protein synthesis. We and others have shown the involvement of PKB, mTOR, S6 kinase, eIF2B, 4E-BP1, etc. [13]. However, it is not yet known what the initial sensors detecting the increased availability of amino acids or increased mechanical activity are.

#### **Anabolic resistance**

In a number of circumstances of chronic muscle wasting such as ageing, immobilization, chronic obstructive pulmonary disease, liver cirrhosis, kidney failure, etc, there appears to be a depression of muscle protein synthesis without a marked elevation of muscle protein breakdown [14]. A close examination of the circumstances in ageing suggests that in relatively healthy older people who have yet to show marked loss of muscle there are no decrements in the resting post absorptive (i.e. pre-breakfast) state but that the ability of such individuals to respond adequately to amino acids supplied either orally or intravenously is diminished; we have called this condition "anabolic resistance" [7]. We would predict that in patients who have had failing organs requiring a transplant and then suffer the insult of a major operation to transplant a new graft organ, this condition of anabolic resistance will manifest itself.

We would predict that in such patients there will be a loss of total capacity for protein synthesis indicated

by a fall in the RNA/DNA ratio or in the RNA/protein ratio (i.e. representing fewer ribosomes per cell) and that in addition, as we find in elderly people, there will be decrements in the total amounts of signalling proteins and in their ability to respond appropriately to increased availability of nutrition and possibly also of insulin. These circumstances would make it very much harder to rehabilitate individuals who have lost skeletal muscle mass as a result of their operation and underlying disease state. This situation would be worsened by the treatment of such patients with immunomodulatory drugs such as rapamycin (sirolimus) or cyclosporin which have major inhibitory effects on the anabolic signalling pathways in muscle and other tissues in the case of sirolimus and in the case of The calcineurin inhibitors cyclosporin and tacrolimus are prime examples, inhibiting the NFAT pathway which controls so-called slow muscle gene programme. Thus, it would be very difficult to adapt muscle fibre types to increase their oxidative capacity switching from type II to type I in the presence of cyclosporin or tacrolimus and very difficult to build muscle in the presence of sirolimus. Other immunosuppressive drugs such as methotrexate and dactinomycin both inhibit DNA synthesis, which is likely only to affect satellite cell activity in muscle (otherwise a post mitotic tissue) although effects on bone and tendon and on the fibroblasts producing muscle collagen are all likely to be adversely affected. In addition overuse of corticosteroids such as methyl prednisolone can have a devastating effect in inhibiting muscle protein synthesis and accelerating breakdown.

**Exercise as a way of re-tuning the system**

It is our strong suspicion that exercise could help re-set a system affected by anabolic resistance so that it became more normally responsive to the anabolic effects of food and we are in the process of testing this for a number of clinical conditions. It seems likely that if dynamic exercise can overcome some of the deficits of anabolic resistance in the elderly this mechanism is likely to be of general significance and could be used to overcome anabolic resistance in other chronic

conditions involving muscle wasting, such as in transplant recipients.

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## **Appendix 2**

**WILKES EA, SELBY AL, ATHERTON PJ et al. (2009) Blunting of Insulin Inhibition of Proteolysis in Legs of Older Subjects May Contribute to Age-Related Sarcopenia. Am.J Clin Nutr. 90, 1343-1350.**



## Blunting of insulin inhibition of proteolysis in legs of older subjects may contribute to age-related sarcopenia<sup>1-3</sup>

Emilie A Wilkes, Anna L Selby, Philip J Atherton, Rekha Patel, Debbie Rankin, Ken Smith, and Michael J Rennie

### ABSTRACT

**Background:** Reduced postprandial muscle proteolysis is mainly due to increased insulin availability. Whether rates of proteolysis in response to low physiologic doses of insulin are affected by aging is unknown.

**Objectives:** We tested the hypothesis that suppression of leg protein breakdown (LPB) by insulin is blunted in older subjects, together with blunted activation of Akt-protein kinase B (PKB).

**Design:** Groups of 8 young [mean ( $\pm$ SD) age: 24.5  $\pm$  1.8 y] and older (65.0  $\pm$  1.3 y) participants were studied during euglycemic (5 mmol/L), isoaminoacidemic (blood leucine  $\approx$  120  $\mu$ mol/L) clamp procedures at plasma insulin concentrations of  $\approx$  5 and  $\approx$  15  $\mu$ IU/mL for 1.5 h. Leg amino acid balance, whole-leg protein turnover (as dilution of amino acid tracers), and muscle protein synthesis were measured with D<sub>5</sub>-phenylalanine and [1,2-<sup>13</sup>C<sub>2</sub>]leucine. The kinase activity of muscle Akt-PKB and the extent of phosphorylation of signaling proteins associated with the mTOR (mammalian target of rapamycin) pathway were measured before and after the clamp procedures.

**Results:** Basal LPB rates were not different between groups (66  $\pm$  11 compared with 51  $\pm$  10 nmol leucine  $\cdot$  100 mL leg<sup>-1</sup>  $\cdot$  min<sup>-1</sup> and 30  $\pm$  5 compared with 24  $\pm$  4 nmol phenylalanine  $\cdot$  100 mL leg<sup>-1</sup>  $\cdot$  min<sup>-1</sup> in young and older groups, respectively). However, although insulin at  $\approx$  15  $\mu$ IU/mL lowered LPB by 47% in the young subjects ( $P < 0.05$ ) and abolished the negative leg amino acid balance, this caused only a 12% fall ( $P > 0.05$ ) in the older group. Akt-PKB activity mirrored decreases in LPB. No differences were seen in muscle protein synthesis or associated anabolic signaling phosphoproteins.

**Conclusions:** At moderate availability, the effect of insulin on LPB is diminished in older human beings, and this effect may be mediated through blunted Akt-PKB activation. *Am J Clin Nutr* 2009;90:1343-50.

### INTRODUCTION

Sarcopenia appears to be an inevitable consequence of aging and affects both sexes and all races. The prevalence of disabling sarcopenia is estimated as between 13% and 24% for men and women aged 65-70 y and is  $>50\%$  in those aged  $>80$  y (1, 2). This progressive loss of lean tissue occurs as a result of a diurnal net imbalance between the rates of muscle protein breakdown (MPB) and muscle protein synthesis (MPS), but the exact nature of the failure of muscle maintenance is currently unknown.

Insulin promotes muscle accretion by inhibition of proteolysis, apparently in a dose-dependent fashion (3-11). The physiologic

inhibitory dose of insulin is less clear. Louard et al (6), Gelfand and Barrett (10), and Pozefsky et al (12) have proposed that maximal inhibition of proteolysis occurs at modest plasma insulin concentrations ( $\approx 30$   $\mu$ IU/mL), whereas other investigators have suggested maximal inhibition occurs at concentrations exceeding the physiologic range ( $>400$   $\mu$ IU/mL), providing that amino acid (AA) availability is maintained at postabsorptive concentrations (9, 11, 13). Two reports from the same laboratory have concluded, against the accepted paradigm, that insulin, infused directly into the femoral artery, does not suppress rates of proteolysis (14, 15), but the data in these studies may be susceptible to a type I error because of insufficient participants and high variability.

When the responses of whole-body protein turnover are compared in young and older subjects, it appears that at insulin and AA concentrations elevated above basal, postabsorptive values, rates of protein breakdown are higher in older subjects (16, 17), although this differential appears to be lost at higher insulin concentrations. Measurements of leg balance and leg protein breakdown (LPB), assumed to be due mainly to muscle, are not different in the basal state in young and older subjects (18). However, a possibility exists that, during feeding when insulin secretion is stimulated, there may, in addition to a decreased rate of MPS, also be blunted suppression of MPB. Furthermore, most of the effect of insulin in suppressing LPB appears to have occurred in full by  $\approx 30$   $\mu$ IU/mL and may occur at even lower concentrations (19). Thus, we hypothesized that LPB would be suppressed in the young subjects at insulin availability as low as 15  $\mu$ IU/mL but to a lesser extent in older subjects. [We aimed to achieve a degree of insulinemia comparable with that which follows a low-glycemic index meal such as wholemeal pasta (20, 21).] This hypothesis has never, to our knowledge, been tested directly in a systemic fashion. The aims of this work were 1) to fill this gap and 2) to determine, in

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addition, whether alterations of activation by phosphorylation of components of muscle cell signaling pathways, particularly activation of Akt-protein kinase B (PKB), and short-term changes in gene expression of proteolytic enzymes might provide clues to the mechanisms involved.

#### SUBJECTS AND METHODS

The protocol was approved by the University of Nottingham Medical School Research Ethics Committee, conducted in accordance with International Conference on Harmonization, World Health Organization Good Clinical Practice standards, and began recruitment in 2007. Written informed consent was obtained from all participants. Two groups of 8 young [age (mean  $\pm$  SEM): 24.5  $\pm$  1.8 y] and older (65.0  $\pm$  1.3 y) healthy volunteers, 4 men and 4 women per group (Table 1) participated after completion of medical screening, which included measurement of postabsorptive blood glucose and insulin concentrations to exclude individuals with insulin resistance or diabetes as defined as a value of  $>4.65$  with the homeostasis model assessment of insulin resistance (22).

#### Study protocol

All participants attended the clinical investigation suite at 0800 after a 12-h fast, during which they were permitted water ad libitum. Participants were asked to eat their usual diet and to refrain from strenuous exercise in the preceding 72 h. Body composition data were acquired by dual-energy X-ray absorptiometry (Lunar Prodigy II; General Electric, Amersham, United Kingdom) on the morning of the study. Cannulae were inserted in the antecubital fossa, the dorsum of the contralateral hand, and the common femoral vein. The first was used to infuse tracer, insulin, octreotide, glucose, and unlabeled AAs, whereas the other 2 were sampling ports for arterialized venous [using the heated hand technique (23)] and venous blood, respectively. Arterialized whole-blood glucose concentrations were measured in real time at 5-min intervals (GM7 Micro-Stat; Analox Instruments Ltd, London, United Kingdom).

TABLE 1  
Characteristics of subjects<sup>1</sup>

	Young group	Older group	P value
Age (y)	24.5 $\pm$ 1.8 <sup>2</sup>	65.0 $\pm$ 1.3	<0.0001
M:F	4:4	4:4	NS
Weight (kg)	71.1 $\pm$ 5.0	73.1 $\pm$ 3.3	NS
Height (m)	1.7 $\pm$ 0.0	1.7 $\pm$ 0.0	NS
CRP ( $\mu$ g/mL)	2.1 $\pm$ 0.8	3.5 $\pm$ 1.3	NS
BMI (kg/m <sup>2</sup> )	23.9 $\pm$ 0.9	26.3 $\pm$ 0.4	0.03
LBM (kg)	47.3 $\pm$ 4.4	45.8 $\pm$ 4.1	NS
Fat mass (kg)	19.6 $\pm$ 2.8	22.3 $\pm$ 1.2	NS
LBM/ht (kg/m)	27.2 $\pm$ 1.8	27.3 $\pm$ 2.0	NS
PA insulin ( $\mu$ U/mL)	6.0 $\pm$ 0.5	4.3 $\pm$ 0.5	0.04
PA cortisol (ng/mL)	36.3 $\pm$ 4.4	34.3 $\pm$ 4.2	NS
PA glucose (g/dL)	4.4 $\pm$ 0.1	4.8 $\pm$ 0.2	NS
PA leucine ( $\mu$ mol/L)	113.2 $\pm$ 11.5	114.2 $\pm$ 8.2	NS

<sup>1</sup>  $n = 8$  for both groups. CRP, C-reactive protein; ht, height; LBM, lean body mass; PA, postabsorptive. No significant differences were observed between young and older healthy volunteers,  $P > 0.05$  (Student's  $t$  test).

<sup>2</sup> Mean  $\pm$  SEM (all such values).

To achieve target plasma insulin concentrations of  $\approx 5$  and  $\approx 15$   $\mu$ U/mL, each for 1.5 h consecutively, insulin (Humulin S; Lilly UK, Basingstoke, United Kingdom) was infused at 2 and 10  $\mu$ U  $\cdot$  m<sup>2</sup> body surface area<sup>-1</sup>  $\cdot$  h<sup>-1</sup> accompanied by a constant infusion of octreotide (Sandostatin; Novartis UK, Camberley, United Kingdom; 30 ng  $\cdot$  kg<sup>-1</sup>  $\cdot$  min<sup>-1</sup>). Glucagon was not given because it has been shown to have no effect on AA metabolism (24). Euglycemic isoaminoacidemic conditions were maintained with the use of 20% dextrose (infused at rates determined by the extent of deviation of arterialized blood glucose from 5 mmol/L) and, for the later part of the study, a mixed AA preparation (25.5 mg  $\cdot$  kg<sup>-1</sup>  $\cdot$  h<sup>-1</sup>; Glamin; Fresenius Kabi, Bad Homburg, Germany). The stable isotope-labeled AA tracers, [1,2-<sup>13</sup>C<sub>2</sub>]leucine (99% <sup>13</sup>C) and D<sub>5</sub>-phenylalanine (98% <sup>2</sup>H) (both from Cambridge Isotopes Ltd, Cambridge, MA), were administered in primed, constant infusions ([1,2-<sup>13</sup>C<sub>2</sub>]leucine: 0.75 mg/kg prime and 1 mg  $\cdot$  kg<sup>-1</sup>  $\cdot$  h<sup>-1</sup>; D<sub>5</sub>-phenylalanine: 0.3 mg/kg prime and 0.6 mg  $\cdot$  kg<sup>-1</sup>  $\cdot$  h<sup>-1</sup>).

Doppler ultrasound (Nemio 17; Toshiba Medical Systems Ltd, Crawley, United Kingdom) measurements of blood flow through the common femoral artery were taken just distal to the inguinal ligament at frequent intervals. Muscle biopsies from vastus lateralis were obtained under local anesthetic with the use of the conchotome technique (25).

Blood samples were collected at each time point into pre-treated tubes containing lithium heparin and immediately stored on ice before separation at 2000  $\times g$  for 20 min; plasma was kept for later analysis at  $-80^{\circ}\text{C}$ . AA fluxes were calculated from plasma AA concentrations after correction for hematocrit. Muscle tissue, other than that stored for RNA extraction, was rapidly washed in ice-cold phosphate-buffered saline on collection and blotted before being frozen in liquid nitrogen. Muscle samples for RNA extraction were directly frozen. All reagents used were purchased from Sigma-Aldrich (Poole, United Kingdom) unless otherwise specified (Figure 1).

#### Insulin, C-reactive protein, cortisol, and free fatty acids

The concentrations of plasma insulin, C-reactive protein, and cortisol were determined by enzyme-linked immunosorbent assays, according to manufacturer instructions (DRG Instruments GmbH, Marburg, Germany). Plasma free fatty acids were measured with the use of a colorimetric method (Roche Applied Science, Indianapolis, IN).

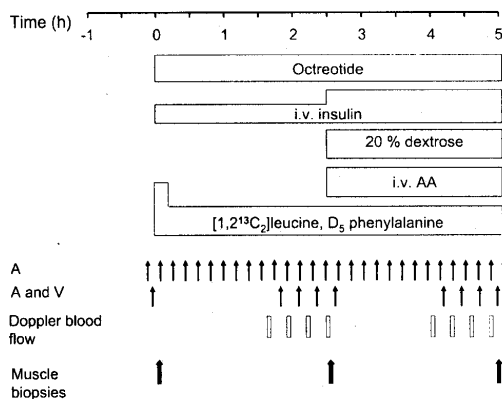
#### AA analysis

Equal volumes of plasma and 10% sulfosalicylic acid were mixed and cooled at 4°C for 30 min. The samples were spun at 1000  $\times g$  to remove the precipitated protein and passed through a 0.22- $\mu$ m filter before analysis by a dedicated AA analyzer (Biochrom 30; Biochrom, Cambridge, United Kingdom) with the use of a lithium buffer separation. All 20 AA concentrations were determined by comparison to a standardized sample, with the use of norleucine as an internal standard.

#### Myofibrillar protein isolation

Muscle (30 mg) was minced with the use of fine scissors in ice-cold extraction buffer (0.02 mol Tris/L, 0.15 mol NaCl/L, 0.1 mol EDTA/L, 0.1% Triton X). The homogenate was centrifuged at





**FIGURE 1.** Study protocol. Arterialized venous blood (A) was collected at  $\approx 5$ -min intervals to monitor infusions of blood glucose concentrations. Paired samples of arterialized venous and femoral vein blood (A and V) were collected at baseline and at 20-min intervals through the last hour of each clamp period (small arrows), preceded by Doppler blood flow measurements (rectangles). Muscle biopsies are indicated by large arrows. AA, amino acid; i.v., intravenous.

1600  $\times$  g for 20 min, the supernatant fluid was removed, and the myofibrillar-collagen pellet was resuspended in 0.3 mol NaOH/L. The soluble myofibrillar protein and the insoluble collagen were separated by centrifugation. The myofibrillar fraction was precipitated with 1 mol perchloric acid/L, and the pellet was washed twice with 70% ethanol. Myofibrillar protein was hydrolyzed in 0.05 mol HCl/Dowex 50W-X8-200/L at 110°C overnight (26), and the liberated AAs were purified then eluted in 2 mol  $\text{NH}_4\text{OH}$ /L. The AAs were subsequently derivatized as their *N*-acetyl-*n*-propyl ester (27). Leucine labeling was analyzed by capillary gas chromatography combustion isotope ratio mass spectrometry (Delta-plus XL; Thermo Fisher Scientific, Hemel Hempstead, United Kingdom); separation was achieved on a 25 m  $\times$  0.25 mm  $\times$  1.0  $\mu$ -film DB 1701 capillary column (Agilent Technologies, West Lothian, United Kingdom).

#### Plasma AA labeling

To determine labeling (atom % excess; APE) and concentrations of arterialized venous and venous leucine, phenylalanine, and  $\alpha$ -ketoisocaproate ( $\alpha$ -KIC), plasma was deproteinized with 100% ethanol, dried, and, for AA analysis, resuspended in 0.5 mol HCl/L. Lipids were removed by ethyl acetate extraction, and the AAs were converted to their *tert*-butyldimethylsilyl derivatives.  $\alpha$ -KIC was converted to its quinoxalinol derivative and separated by extraction into ethyl acetate, which was then evaporated, and the *tert*-butyldimethylsilyl derivative was prepared. Concentrations and enrichments were determined by gas chromatography-mass spectrometry (GC-MS) with the use of a Trace DSQ GC-MS (Thermo Fisher Scientific, Hemel Hempstead, United Kingdom) with the use of appropriate internal standards (28).

#### Calculations

The rate of MPS between the biopsies was calculated with the use of standard equations, fractional protein synthesis [in %/h;

$(\Delta E_m/E_p \times 1/t) \times 100$  (29)], where  $\Delta E_m$  is the change in labeling of muscle protein leucine between 2 biopsy samples,  $E_p$  is the mean enrichment over time of the precursor for protein synthesis (taken as venous  $\alpha$ -KIC  $^{13}\text{C}_2$  labeling), and  $t$  is the time in hours between biopsies. Venous  $\alpha$ -KIC was chosen to represent the immediate precursor for protein synthesis, ie, leucyl-*t*-RNA (30).

Whole-body leucine flux was calculated with the use of the reciprocal pool model,  $\text{Flux} = i \times (E_i/E_p) - 1$ , where  $i$  is the rate of tracer infusion (in  $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ),  $E_i$  is the enrichment of the infusate, and  $E_p$  is the enrichment in the pool chosen to represent the precursor for protein synthesis, ie, venous KIC (31).

Leg protein flux (ie, breakdown) was calculated from the arteriovenous dilution of  $[\text{D}_5]$ phenylalanine and  $[1,2-^{13}\text{C}_2]$ leucine tracers with the following equation:  $[(E_a/E_v) - 1] \times C_a \times \text{blood flow}$  (32), where  $E_a$  and  $E_v$  are the values of AA labeling at steady state in arterial and femoral venous plasma,  $C_a$  is the mean concentration in the arterial blood with blood flow in mL/100 mL leg, adjusted for leg volume (assessed by dual-energy X-ray absorptiometry). For each 1.5-h period the values for enrichment and concentration were obtained from the mean of 4 separately analyzed samples, collected over the last hour. The net AA balance was calculated as the difference in arterial and venous concentrations multiplied by the plasma flow, which was also the average of 4 blood flow readings taken immediately before each of the blood samples and adjusted with each subject's hematocrit.

#### Western analysis of signaling proteins

Phosphorylated protein concentrations of eukaryotic elongation factor 2 ( $\text{eEF}2^{\text{Thr}256}$ ), p70 ribosomal S6 kinase ( $\text{Thr}389$ ), eukaryotic initiation factor 4 binding protein ( $\text{Thr}37/46$ ), and Forkhead box O $\alpha$  ( $\text{FoxO}1^{\text{Ser}256}$ ) and Akt-PKB kinase activity (through phosphorylation of pseudosubstrate glycogen synthase kinase 3  $\beta^{\text{Ser}9}$ ) were determined by Western blotting. Proteins were extracted from  $\approx 30$  mg of crudely minced muscle in ice-cold buffer (10  $\mu\text{L}$ /mg muscle) containing 50 mmol Tris HCl/L, 0.1% Triton X-100, 1 mmol EDTA/L, 1 mmol EGTA/L, 0.1% 2-mercaptoethanol, 10 mmol  $\beta$ -glycerophosphate/L, 0.5 mmol sodium orthovanadate/L, and complete protease inhibitor cocktail (Roche, West Sussex, United Kingdom). A Bradford assay was used to quantify protein concentrations of 10,000 g supernatant fluids. For PKB kinase activity, Akt-PKB was immunoprecipitated from 300  $\mu\text{g}$  protein before incubation with 1 mmol ATP/L and glycogen synthase kinase 3  $\beta$  fusion protein substrate (New England Biolabs, Hitchin, Hertfordshire, United Kingdom). Fifty micrograms of each protein sample (or 15  $\mu\text{L}$  for kinase reactions) was loaded onto 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel (Criterion XT Bis-Tris; Bio-Rad, Hemel Hempstead, United Kingdom) for electrophoresis at 150 V for  $\approx 75$  min and electroblotted to polyvinylidene difluoride membranes (Bio-Rad). After incubation for 1 h with 5% bovine serum albumin in TBS-T (Tris-buffered saline and 0.1% Tween-20), membranes were incubated overnight with primary antibody against the aforementioned targets at 4°C (New England Biolabs). Membranes were then washed with TBS-T and incubated for 1 h at room temperature with the horseradish peroxidase-conjugated antirabbit secondary



antibody (New England Biolabs), before further washing with TBS-T and incubation for 5 min with enhanced chemiluminescence reagents (enhanced chemiluminescence kit; Immunstar; Bio-Rad). Blots were imaged and quantified with the use of the Chemidoc XRS system (Bio-Rad). Immunoblots were not repeated because the laboratory CV between sample-to-sample ratios on repeated gels is low ( $\approx 10\%$ ).

#### Gene expression

Total RNA was extracted from  $\approx 20$  mg of each muscle sample with 0.5 mL Tri-reagent as per the manufacturer's protocol. Integrity and quantity of RNA was assessed by gel electrophoresis and luminescence. QuantiTect reverse transcription kit (Qiagen Ltd, Crawley, United Kingdom) was used for cDNA synthesis and genomic DNA elimination. Real-time polymerase chain reaction was performed with SYBR Green supermix (Bio-Rad) and individually designed and tested primers (MWG, Ebersberg, Germany) as follows (5'-3'): MuRF1 sense, AGT-GACCAAGGAGAA CAGTCA, and antisense, CACCAGCTT-TGTGGACTTGT; MAFBx sense, TCCTGGAAGGGCACTGAC, and antisense, CCATATCCCAGCTCTCCAG; C2 proteasomal subunit sense, CATTGAAAAGGGCGCAATC, and antisense, GCCATATCGTTGTGTGGTA; GLUT4 sense, CAGTATGTTG-CGGAGGCTAT, and antisense, CCTCGAGTTTCAGGTACTC-TT; cathepsin L sense, TGAAGAATCAGGGTCAGTGTGG, and antisense, TGAGGCCAGAGCAGTCTAC; and Ubiquitin sense, GATCTGCCGCAAGTGCTA, and antisense, CAGTCAATGAA-AGGGACACT. GAPDH sense, CTGACCTGCCGTCTAGAAAA, and antisense, GCCAAATTCGTTGTCATACC, were used as reference gene. Reverse transcription and polymerase chain reaction were conducted with the ICyclerIQ (Bio-Rad).

#### Power calculation and statistical analyses

The sample size was determined prospectively with a power calculation and taking a population variance of 15% (based on previous laboratory data) and CV of laboratory techniques also of 15%, to detect (with 80% confidence at the 5% significance level) change in the primary endpoint, ie, a difference between rates of LPB between the 2 age groups at each insulin concentration. A post hoc sample size calculation was performed with the actual data. Given the large effect of insulin, and that the groups were of the same size with 14 df and with variance set for both groups at 33% (an overestimate for some data sets) then with  $\alpha$  at 0.05, the power would be 97% for leucine rate of appearance and 75% for phenylalanine.

Results are presented as means  $\pm$  SEMs. The data were analyzed with the use of Prism 5 (Graph Pad, San Diego, CA). All the data were normally distributed, as confirmed by the Kolmogorov-Smirnov test. Analysis of variance with Bonferroni post hoc test and Student's *t* test were used to identify statistically important differences. Two-tailed tests have been used throughout.

Propagated errors were estimated with the binomial theorem taking into account blood flow, phenylalanine-leucine enrichment, AA concentrations, and leg volume, which have respective CVs of  $\approx 20\%$ , 5%, 5%, and 3%. Thus, the maximal estimate of mean propagated errors is  $\approx 21\%$ .

#### RESULTS

The participants were of similar body weight, but the older participants had a greater body mass index ( $26.3 \pm 0.4$  compared with  $23.9 \pm 0.9$  kg/m<sup>2</sup>;  $P = 0.03$ ). Fasting glucose concentrations and calculated values of the quantitative insulin sensitivity check index (QUICKI) (33) (young group,  $0.38 \pm 0.01$ ; older group,  $0.40 \pm 0.01$ ;  $P = 0.13$ ) did not differ significantly, although plasma insulin concentrations were higher in the young group at baseline. Basal AA profiles and concentrations of C-reactive protein and cortisol were similar (Table 1).

Target clamp conditions were achieved for all participants, including adequate provision of AA throughout both clamp periods; CV for insulin, leucine, and essential AAs (EAAs) were within 15% of the mean. Plasma enrichment of  $[1,2-^{13}\text{C}_2]\alpha\text{-KIC}$  and  $[\text{D}_5]\text{phenylalanine}$  were maintained at  $\approx 6$  APE in the young group and  $\approx 7$  APE in the older group (CV  $< 8\%$  for all;  $< 5\%$  for phenylalanine in the older group) across both study periods. For clamp data, see Figures S1, S2, S3, and S4 and Table S1 under "Supplemental data" in the online issue.

Plasma concentrations of free fatty acids were not different between the groups at any time (basal at time zero: young group,  $0.47 \pm 0.12$  mmol/L, and older group,  $0.54 \pm 0.15$  mmol/L; older group,  $0.54 \pm 0.14$  mmol/L; after  $\approx 15$   $\mu\text{IU/mL}$  insulin clamp: young group,  $0.31 \pm 0.19$  mmol/L, and older group,  $0.13 \pm 0.15$  mmol/L). In both groups there was a marked suppression of plasma concentrations of free fatty acids, which were not different in extent between groups.

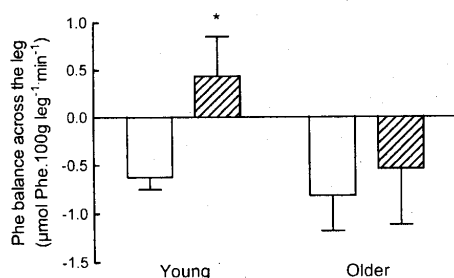
Femoral artery blood flow was greater in the young participants during both clamp periods but did not change with higher insulin availability ( $3.64 \pm 0.30$  compared with  $2.39 \pm 0.20$  mL  $\cdot$  100 mL leg<sup>-1</sup>  $\cdot$  min<sup>-1</sup> at  $\approx 5$   $\mu\text{IU/mL}$ ,  $P < 0.01$ , and  $3.49 \pm 0.33$  compared with  $2.56 \pm 0.26$  mL  $\cdot$  100 mL leg<sup>-1</sup>  $\cdot$  min<sup>-1</sup> at  $\approx 15$   $\mu\text{IU/mL}$ ,  $P < 0.02$ , in young compared with older groups, respectively).

The phenylalanine balance differed with insulin availability significantly in the young group but not the old group (Figure 2). In the young group, at 5  $\mu\text{IU/mL}$  insulin balance was also significantly different from zero (or neutral balance), but at 15  $\mu\text{IU/mL}$  insulin there was no difference from neutral phenylalanine balance. The older group did not differ from neutral balance at either insulin availability. Whole-body leucine flux did not differ between participants of different ages or when studied under different insulin availability, with values of  $130.3 \pm 7.7$  and  $133.4 \pm 9.7$   $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$  for young participants and  $122.6 \pm 10.4$  and  $120.8 \pm 11.5$   $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$  for older participants (each  $\approx 5$  and  $\approx 15$   $\mu\text{IU/mL}$ , respectively).

LPB, whether determined by leucine or phenylalanine tracer dilution, did not differ between the groups at insulin concentrations of  $\approx 5$   $\mu\text{IU/mL}$  (Figure 3). At the higher insulin concentration, LPB was substantially suppressed in young participants (59% with phenylalanine and 47% with leucine tracer; both  $P < 0.05$ ), whereas LPB was reduced in the older group to a lesser extent, achieving significance when calculated with phenylalanine but not leucine (23% and 12% reduction from basal values, respectively).

No differences were observed between groups in the rates of MPS as determined by the incorporation of  $[1,2-^{13}\text{C}_2]\text{leucine}$ , and they did not change with increased insulin availability (fractional protein synthesis of quadriceps muscle in young compared with





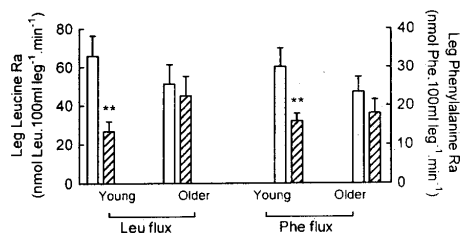
**FIGURE 2.** Mean ( $\pm$ SEM) balance of unlabeled phenylalanine (Phe) across the leg after clamping at  $\approx 5$   $\mu$ U/mL (open bars) and  $\approx 15$   $\mu$ U/mL (hatched bars) insulin for young and older healthy volunteers;  $n = 8$  for both groups. The change in phenylalanine balance occurring with change in insulin availability was significant in the young (as indicated by the asterisk;  $P < 0.05$ , 2-factor ANOVA) but not the older participants.

older participants:  $0.051 \pm 0.005$  compared with  $0.043 \pm 0.005\%/h$  at  $\approx 5$   $\mu$ U/mL and  $0.046 \pm 0.005$  compared with  $0.048 \pm 0.005\%/h$  at  $\approx 15$   $\mu$ U/mL). The values for leg protein synthesis showed a similar pattern (data not shown).

In both young and older subjects, Akt-PKB activity rose with the increase of insulin to  $\approx 15$   $\mu$ U/mL (Figure 4). Phosphorylation status of eEF2, p70 ribosomal S6 kinase, eukaryotic initiation factor 4 binding protein 1, and FoxO1a did not change in either group at either insulin concentration (Figure 5). The gene expression of the ubiquitin ligases MuRF1 and MAFBx, the C2 proteosomal subunit, cathepsin L, and ubiquitin also remained unchanged (Figure 6).

#### DISCUSSION

To our knowledge this is the first study reporting a comparison of the effects of low physiologic amounts of insulin on the rate of leg proteolysis at postabsorptive AA concentrations in young and older human beings. It appears that aging interferes with the normal suppression effect of LPB in response to a moderate rise in plasma insulin concentration, despite there being no differences in the basal state in leg (probably mainly muscle) proteolysis.



**FIGURE 3.** Mean ( $\pm$ SEM) leg protein breakdown as determined by the rate of appearance (Ra) of leucine (Leu; left axis) and phenylalanine (Phe; right axis) across the leg at  $\approx 5$   $\mu$ U/mL (open bars) and  $\approx 15$   $\mu$ U/mL (hatched bars) insulin for young and older healthy volunteers;  $n = 8$  for both groups. Leg protein breakdown was reduced significantly at higher insulin availability whether determined by leucine or phenylalanine kinetics ( $P < 0.001$ , 2-factor ANOVA) in the younger subjects, highlighted by asterisks, but not in the older subjects; age had no independent effect on basal tracer fluxes regardless of which tracer was used.



**FIGURE 4.** Mean ( $\pm$ SEM) protein kinase B (Akt-PKB) kinase activity relative to Akt-PKB recovery from immunoprecipitates for young and older healthy volunteers after 90-min pancreatic clamping at insulin concentrations of  $\approx 5$   $\mu$ U/mL (open bars) and  $\approx 15$   $\mu$ U/mL (hatched bars);  $n = 8$  for both groups. Asterisk indicates the significant effect of insulin availability within each age group ( $P < 0.05$ , 2-factor ANOVA). No interaction exists between age and insulin availability. AU, arbitrary units.

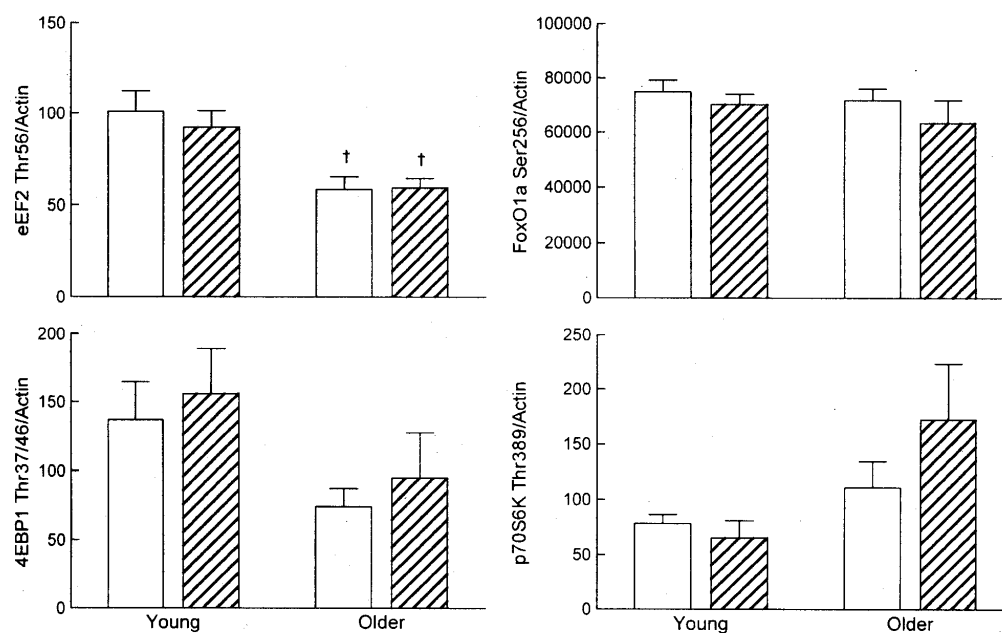
These findings cannot simply be explained by differences in body composition or conventionally described insulin sensitivity in respect of glucose metabolism because in all respects the young and older healthy volunteers were well matched. The older participants had no evidence of pathologic sarcopenia, and all participants had normal calculated values for insulin sensitivity, ruling out the possibility of incipient diabetes contributing to impaired muscle metabolism. Furthermore, availability of free fatty acids did not differ at any time point. Blood flow was significantly lower in the older participants, which is not a novel finding, and may be explained by the phenomenon of endothelium-dependent vasodilator blunting in response to insulin with aging (14, 34).

The reported dose-dependent effect of insulin on blood flow to the limb (7, 15, 35) occurs at higher insulin concentrations than present during the second period of this study. Thus, it is likely that the delivery of insulin was too low to elicit an effect on blood flow. Indeed, no change in bulk femoral artery blood flow with small changes to insulin concentrations has been reported previously (15).

Other researchers have measured whole-body leucine flux under similar conditions and have shown that protein breakdown is suppressed when plasma insulin is increased to absorptive concentrations ( $\leq 30$   $\mu$ U/mL) with the degree of suppression being reduced in older subjects (16, 17). However, the whole-body data we present here did not confirm those findings; we found no differences in whole-body leucine flux with a moderate increase in insulin availability or between participants of different ages.

The differences in these findings from those of the French workers may be accounted for by differences in body composition. The body composition of participants, particularly young participants, differed markedly between studies. Mean values for body mass index and proportion of fat mass were substantially lower in the subjects studied by Boirie et al (17) than in our volunteers, so that the relative contribution of muscle (rather than splanchnic protein turnover) to whole-body flux would be greater. The converse would hold for less lean subjects. Consequently, suppression of MPB by insulin might be offset by enhanced and





**FIGURE 5.** Mean ( $\pm$ SEM) change (in arbitrary units) in phosphorylation status of eukaryotic elongation factor 2 (eEF2), Forkhead box Other 1a (FoxO1a), eukaryotic initiation factor 4E binding protein 1 (4E-BP1), and p70 ribosomal S6 kinase (p70S6K) relative to actin for young and older healthy volunteers after 90-min pancreatic clamping at insulin concentrations of  $\approx 5 \mu\text{IU/mL}$  (open bars) and  $\approx 15 \mu\text{IU/mL}$  (hatched bars);  $n = 8$  for both groups. All differences are  $P > 0.05$  except for the effect of age on eEF2 phosphorylation at both insulin concentrations as marked by daggers ( $P < 0.05$ , 2-factor ANOVA). No interaction exists between age and insulin availability.

insulin-resistant splanchnic proteolysis with whole-body net balance possibly remaining unchanged. This differential effect of insulin on splanchnic and muscle tissue has been described previously, albeit not under isoaminoacidemic conditions (36, 37).

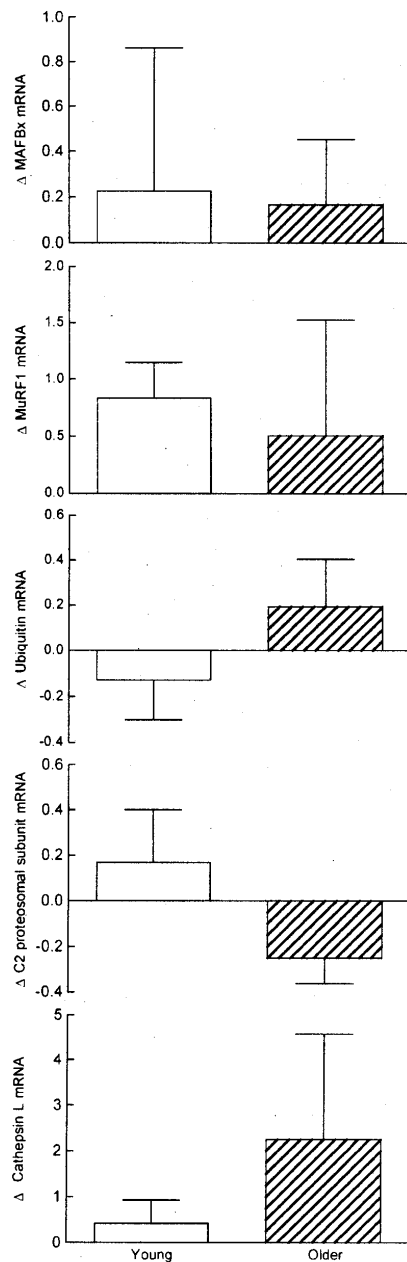
Most previous estimates of MPB in the fasted postabsorptive state do not differ with age (14, 18, 38–40), in accordance with data presented here for both flux of unlabeled phenylalanine and dilution of stable isotope-labeled AA across the leg. In young adults, suppression of MPB appears dependent on the dose of insulin, during isoaminoacidemia (3–11). Thus, postabsorptive plasma AA concentrations were maintained throughout both phases of the study with the use of a proprietary AA preparation, such that arterial EAA concentrations crept up slightly above basal values for both age groups toward the end of the study, albeit to the same degree. Nevertheless, the rise in EAA availability was not sufficient to stimulate MPS or anabolic signaling protein phosphorylation, both of which remained at post-absorptive values for both phases of the study. Furthermore, age did not affect rates of basal mixed MPS, in keeping with other published results (18, 39, 41–43). We and others have previously shown that, regardless of AA concentrations, insulin has no effect on MPS, beyond that of altering AA delivery to the tissues (3, 4, 10, 19), and this is also confirmed by the current results.

As mentioned earlier, reports suggest that insulin can be infused directly into the femoral artery without eliciting a change in the rate of local MPB (14, 15). The investigators report that

venous AA concentrations are maintained within 10% of basal when local infusion of insulin is used, although they also report that phenylalanine concentration within the tissue falls significantly in both young and older participants, especially in the older group (reduction  $> 25\%$ ), suggesting that MPB did fall. Furthermore, Fujita et al (15) did actually report falls in the rate of appearance of phenylalanine at both low ( $-24\%$ ) and high ( $-35\%$ ) insulin concentrations, albeit only reaching significance at the higher concentration. We have no explanation for their results except the existence of a possible type 1 error because of insufficient numbers of subjects given the variability experienced.

Rasmussen et al (14) compared responses to local insulin infusions between young and older healthy volunteers, although insulin concentrations within the femoral vein were doubled in the older group (young group:  $40.3 \pm 1.0 \mu\text{IU/mL}$ ; older group:  $83.4 \pm 6.2 \mu\text{IU/mL}$ ;  $P < 0.05$ ). When results from young volunteers with comparably high insulin concentrations are compared with the older group data, there is a clear trend toward inhibition of proteolysis.

To summarize, in our study we maintained plasma leucine concentrations well and found that MPB was markedly suppressed in young subjects with moderately increased insulin availability infusion in accord with most of the published literature, irrespective of systemic or close arterial infusion of insulin, including that by Gelfand et al (10), who also used close arterial infusion of insulin.



**FIGURE 6.** Mean ( $\pm$ SEM) fold change in expression of Muscle Atrophy F Box (MAFBx), Muscle RING Finger 1 (MuRF1), ubiquitin, C2 proteasomal subunit, and cathepsin L mRNA in young (open bars) and older (hatched bars) healthy volunteers after 90-min pancreatic clamping at insulin concentrations of  $\approx 15 \mu\text{IU/mL}$  and  $\approx 5 \mu\text{IU/mL}$ ;  $n = 8$  for both groups. All  $P > 0.05$  for young compared with older volunteers (Student's  $t$  test).

In terms of signaling proteins, Akt-PKB kinase activity (not simply phosphorylation status), which is frequently used as a proxy of "insulin action" (44), rose with increased insulin availability, as expected. However, in the present study increased Akt-PKB activity had no effect on FoxO1a phosphorylation status, and this coupled to the failure to detect down-regulation of "atrogene" (ie, MAFBx, MuRF1; Figure 6) mRNA expression (45) reduces the likelihood of inhibition of MPB by the Akt-PKB-FoxO1a-atrogene axis.

Both the anabolic sensing-signaling protein mammalian target of rapamycin (mTOR) and the elongation factor eEF2 appear, at least in vitro, to be activated by insulin (46) and are strongly implicated in the regulation of protein translation. However, our data show that at low physiologic insulin concentrations, despite Akt-PKB signaling, mTOR and eEF2 signaling are unaffected, which may explain the lack of protein synthetic response to insulin we report.

What is the broader significance of our work? In real terms, the degree of insulinemia achieved during the second period of this study ( $\approx 15 \mu\text{IU/mL}$ ) is comparable with that expected after a "healthy" low-glycemic index meal, such as breakfast or a light lunch, yet it would appear from these results that the normal suppression of proteolysis by insulin at this concentration is impaired with aging. Whether this blunted sensitivity to insulin is also present at higher insulin concentrations, as may occur with meals containing refined carbohydrates, remains to be investigated. Nevertheless, it appears that loss of muscle with aging may be accelerated not only by a blunted anabolic response to AAs but also relative insensitivity to the anti-proteolytic effects of insulin.

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## **Appendix 3**

### **Karnofsky Performance Scale**

Taken from: KARNOFSKY DA (1950) Nitrogen Mustards in the Treatment of Neoplastic Disease. *Adv.Intern.Med.* 4, 1-75.

- 100 % Normal no complaints; no evidence of disease
- 90 % Able to carry on normal activity; minor signs or symptoms of disease
- 80 % Normal activity with effort; some signs or symptoms of disease.
- 70 % Cares for self; unable to carry on normal activity or to do active work
- 60 % Requires occasional assistance, but is able to care for most of his personal needs
- 50 % Requires considerable assistance and frequent medical care.
- 40 % Disabled; requires special care and assistance
- 30 % Severely disabled; hospital admission is indicated although death not imminent.
- 20 % Very sick; hospital admission necessary; active supportive treatment necessary.
- 10 % Moribund; fatal processes progressing rapidly
- 0 % Dead

## **Appendix 4**

### **Amino Acid constituents of Glamin**

	Concentration (mg/ml)	Dose infused over 2.5 h (mg/kg)
Alanine	16.0	34.0
Arginine	11.3	24.0
Asparagine	0	0
Aspartic Acid	3.4	7.2
Cysteine	0	0
Glutamic acid	5.6	11.9
Glutamine	20.0	42.5
Glycine	11.21	23.8
Histidine	6.8	14.5
Isoleucine	5.6	11.9
Leucine	7.9	16.8
Lysine	9.0	19.1
Methionine	5.6	11.9
Phenylalanine	5.85	12.4
Proline	6.8	14.5
Serine	4.5	9.6
Threonine	5.6	11.9
Tryptophan	1.9	4.0
Tyrosine	2.28	4.8
Valine	7.3	15.5
Total AA	136.6	290.3
Total EAA	66.9	142.2

## **Appendix 5**

### **Revised Piper Fatigue Scale**

Taken from: PIPER BF, DIBBLE SL, DODD MJ *et al.* (1998) The Revised Piper Fatigue Scale: Psychometric Evaluation in Women With Breast Cancer. *Oncol.Nurs.Forum* 25, 677-684.

Directions: For each of the following questions, circle the number that best describes the fatigue you are experiencing now. Please make every effort to answer each question to the best of your ability. Thank you very much.

1. How long have you been feeling fatigue? (*check one response only*)

- a. Minutes \_\_\_\_\_
- b. Hours \_\_\_\_\_
- c. Days \_\_\_\_\_
- d. Weeks \_\_\_\_\_
- e. Months \_\_\_\_\_
- f. Other (*please describe*): \_\_\_\_\_

2. To what degree is the fatigue you are feeling now causing you distress?

**No distress** **A great deal of distress**  
 0    1    2    3    4    5    6    7    8    9    10

3. To what degree is the fatigue you are feeling now interfering with your ability to complete your work or school activities?

**None** **A great deal**  
 0    1    2    3    4    5    6    7    8    9    10

4. To what degree is the fatigue you are feeling now interfering with your ability to visit or socialize with your friends?

**None** **A great deal**  
 0    1    2    3    4    5    6    7    8    9    10

5. To what degree is the fatigue you are feeling now interfering with your ability to engage in sexual activity?

**None** **A great deal**  
 0    1    2    3    4    5    6    7    8    9    10

6. Overall how much is the fatigue you are feeling now interfering with your ability to engage in the kind of activities you enjoy doing?

**None** **A great deal**  
 0    1    2    3    4    5    6    7    8    9    10

7. How would you describe the degree of intensity or severity of the fatigue which you are experiencing now?

**Mild** **Severe**  
 0    1    2    3    4    5    6    7    8    9    10

To what degree would you describe the fatigue which you are experiencing now as being:

8. **Pleasant** **Unpleasant**  
 0    1    2    3    4    5    6    7    8    9    10

9. **Agreeable** **Disagreeable**  
0 1 2 3 4 5 6 7 8 9 10
10. **Protective** **Destructive**  
0 1 2 3 4 5 6 7 8 9 10
11. **Positive** **Negative**  
0 1 2 3 4 5 6 7 8 9 10
12. **Normal** **Abnormal**  
0 1 2 3 4 5 6 7 8 9 10
13. To what degree are you now feeling:  
**Strong** **Weak**  
0 1 2 3 4 5 6 7 8 9 10
14. To what degree are you now feeling:  
**Awake** **Asleep**  
0 1 2 3 4 5 6 7 8 9 10
15. To what degree are you now feeling:  
**Lively** **Listless**  
0 1 2 3 4 5 6 7 8 9 10
16. To what degree are you now feeling:  
**Refreshed** **Tired**  
0 1 2 3 4 5 6 7 8 9 10
17. To what degree are you now feeling:  
**Energetic** **Unenergetic**  
0 1 2 3 4 5 6 7 8 9 10
18. To what degree are you now feeling:  
**Patient** **Impatient**  
0 1 2 3 4 5 6 7 8 9 10
19. To what degree are you now feeling:  
**Relaxed** **Tense**  
0 1 2 3 4 5 6 7 8 9 10
20. To what degree are you now feeling:  
**Exhilarated** **Depressed**  
0 1 2 3 4 5 6 7 8 9 10
21. To what degree are you now feeling:  
**Able to concentrate** **Unable to concentrate**  
0 1 2 3 4 5 6 7 8 9 10
22. To what degree are you now feeling:



<b>Able to remember</b>									<b>Unable to remember</b>	
0	1	2	3	4	5	6	7	8	9	10

23. To what degree are you now feeling:

<b>Able to think clearly</b>								<b>Unable to think clearly</b>		
0	1	2	3	4	5	6	7	8	9	10

24. Overall, what do you believe is most directly contributing to or causing your fatigue?

---

25. Overall, the best thing you have found to relieve your fatigue

is: \_\_\_\_\_

26. Is there anything else you would like to add that would describe your fatigue better to us?

---

27. Are you experiencing any other symptoms right now?

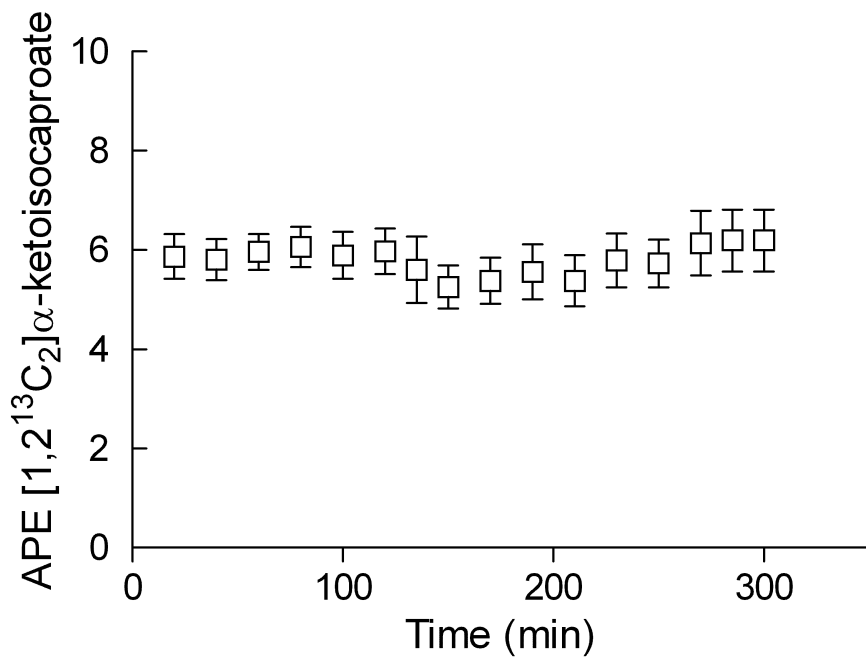
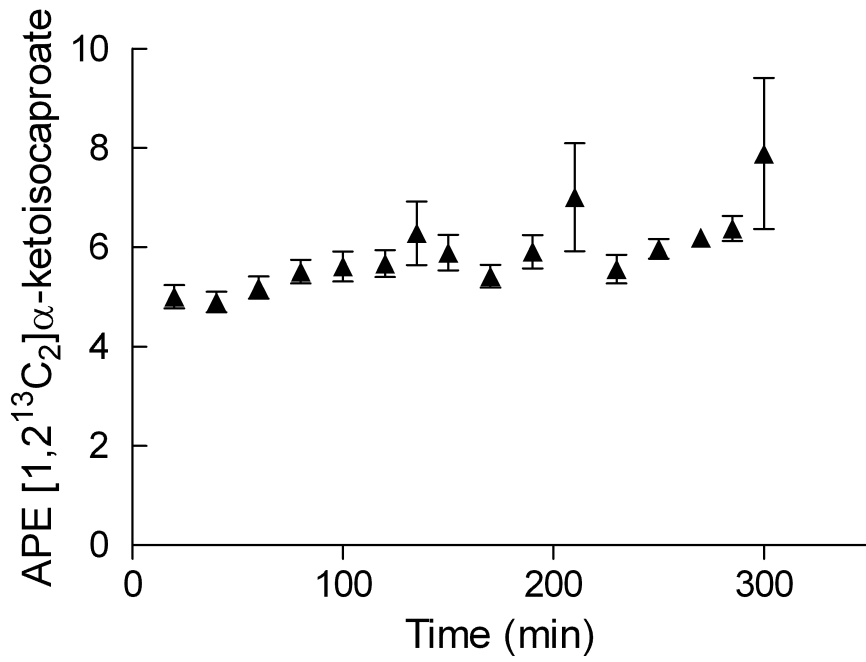
No

Yes Please describe \_\_\_\_\_

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## Appendix 6

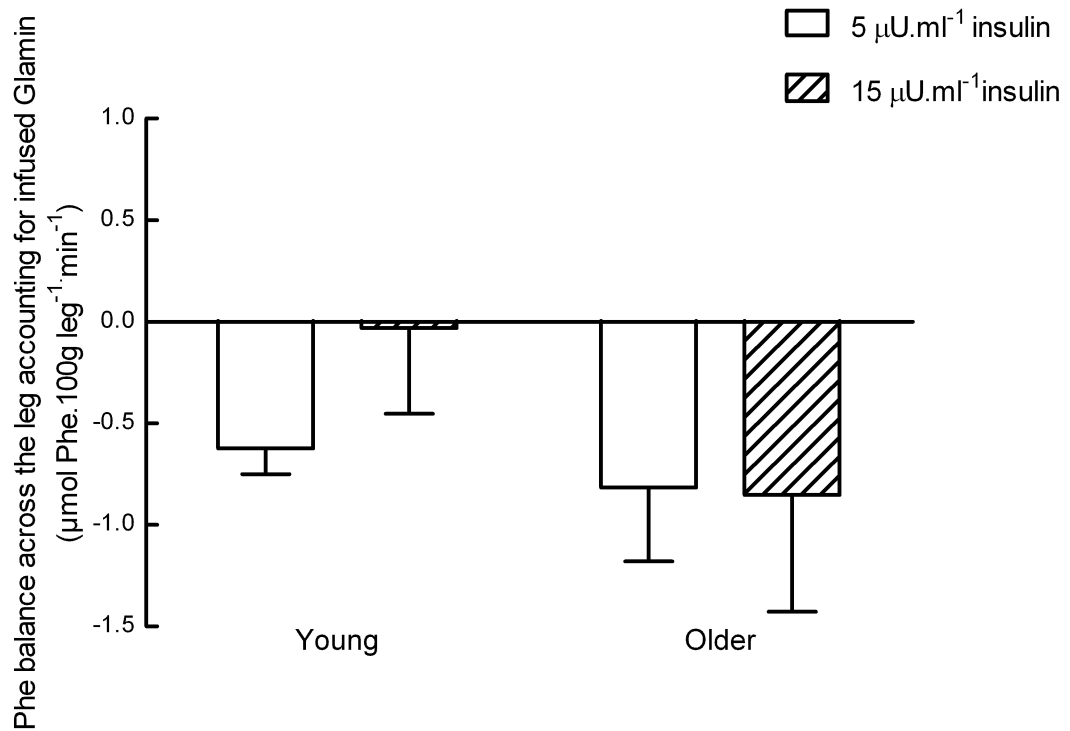
### APE of [1,2<sup>13</sup>C<sub>2</sub>] α-KIC during Protocol 1



Atoms percent excess (APE) of [1,2-<sup>13</sup>C<sub>2</sub>] α-ketoisocaproate in plasma taken from venous blood of young (upper graph, closed triangles) and older (lower graph, open squares) subjects undergoing Protocol 1. Mean ± SEM presented.

## **Appendix 7**

**Figure 5.7 modified to account for infused phenylalanine**



Balance across the leg of unlabelled phenylalanine after pancreatic clamping at ~ 5 (open bars) and ~15  $\mu\text{U}.\text{ml}^{-1}$  insulin (hashed bars) for young and older healthy volunteers, taking into account infused phenylalanine. Values are mean  $\pm$  SEM;  $n = 8$  both groups; change in phenylalanine balance occurring with change in insulin availability did not differ significantly between insulin concentrations or age of participants (all  $P > 0.05$  two-way ANOVA).