The protein metabolic response to ingestion of a complete meal in sarcopenic women and the potential anabolic effect of a leucine-rich meal: results from a pilot study

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December 2011

A thesis submitted to McGill University in partial fulfillment of the requirements of the Degree of Master of Science

Abstract:

Sarcopenia – muscle loss that occurs with aging – is a growing concern affecting physical strength and function. This thesis assessed whether 1) the anabolic response of protein metabolism to a meal is impaired in older sarcopenic women – a phenomenon we believe to be largely responsible for the daily imbalance between protein synthesis (S) and breakdown that ultimately causes muscle loss, 2) meal supplementation with leucine (LEU), an essential amino acid which has been shown to stimulate protein S, improves the protein anabolic response to a meal, and 3) a LEU-rich meal affects meal thermogenesis or satiety. Using tracer methodology, whole-body LEU balance was measured, as well as energy expenditure, blood hormone, nutrient and appetite profiles, and activity of muscle intracellular pathways of protein S, in both healthy control (n = 4; body mass index: 24.9 ± 1.5 kg/m^2; lean body mass (LBM): 39.4 ± 2.1 kg) and sarcopenic (n = 5; 22.6 ± 1 kg/m^2; 34.7 ± 0.4 kg) older women (≥ 61y) after ingestion of a LEU-rich and non-LEU-rich meal in random crossover design. Preliminary results by kg LBM reveal no difference in whole-body LEU balance between groups. The LEU-rich meal achieved greater net balance in both groups without affecting thermogenesis and appetite. Muscle immunoblot data (N = 6) do not suggest blunted activation of mTORC1 and its downstream substrates PRAS40, 4E-BP1, S6K1 and rpS6 in older sarcopenic women, however additional subjects are necessary to draw any conclusions. Future longitudinal supplementation studies assessing postprandial rates of muscle protein synthesis (MPS) in the sarcopenic population will also be necessary to confirm any immunoblot results.

Résumé:

La sarcopénie – perte musculaire associée au vieillissement – est une source de préoccupation, puisqu'elle affecte la force physique et le fonctionnement. Cette thèse a évalué si 1) la réponse anabolique du métabolisme protéique au repas est déficiente chez la femme âgée sarcopénique – un phénomène que nous estimons être largement responsable du déséquilibre entre la synthèse (S) et le catabolisme protéique qui résulte ultérieurement en la perte musculaire, 2) un repas enrichi en leucine (LEU), un acide aminé essentiel avec lequel il fut démontré qu'il stimule la S protéique, améliore la réponse anabolique au repas et 3) un repas enrichi-LEU affecte la thermogénèse et l'appétit. Nous avons procédé à la mesure du bilan en LEU corporel à l'aide d'un traceur isotopique, de même qu'à la dépense énergétique, aux profils des hormones sanguines, nutriments et appétit, et à l'activité intracellulaire de la voie de transduction de la S protéique musculaire, chez des femmes âgées > 61 ans, témoins en santé [n = 4; indice de masse corporelle : 24.9 ± 1.5 kg/m^2; masse maigre (MM) : 39.4 ± 2.1 kg] et chez des femmes sarcopéniques (n = 5; 22.6 ± 1 kg/m^2; 34.7 ± 0.4 kg), après ingestion d'un repas enrichi-LEU et non-enrichi-LEU dans un devis aléatoire, pairé et croisé. Les résultats préliminaires en kg MMC ne démontrent aucune différence entre les groupes dans le bilan en LEU corporel pour l'un ou l'autre des repas. Cependant le repas enrichi-LEU a stimulé d'avantage le bilan net sans affecter la thermogénèse et l'appétit. Les données musculaires obtenues par immunoblots (n = 6) ne démontrent pas d'activité réduite du mTORC1 et de ses substrat en aval PRAS40, 4E-BP1, S6K1 et rpS6 chez les femmes sarcopéniques. Il est par contre nécessaire d'obtenir plus de sujets avant de pouvoir formuler des conclusions. Des études longitudinales avec des suppléments LEU évaluant les taux de S protéique musculaire (SPM) postprandiaux chez les femmes sarcopéniques seront aussi nécessaires pour confirmer ces résultats.

Acknowledgments:

There are so many people without whom this work would not have been possible. From the first day I walked into the Crabtree Labs, I have seen and experienced the incredible teamwork that goes on here, and feel lucky to have been part of such a wonderful group. I could not have asked for a better work and learning environment these past two years.

Thank you Dr. Morais for being so supportive of me throughout my studies, both academically and financially; for being a great teacher and mentor, who also gave me the independence to mature academically and personally; and for somehow always managing to create time in your busy schedule for our meetings. Thank you for patiently listening to and thoroughly answering my questions as well as working with me to come up with solutions to some research hurdles along the way. I will always be impressed by your unwavering dedication to both your patients and your students.

Thank you Dr. Chevalier for your continued guidance from the start and for acting as interim supervisor at times. I appreciated and learned so much from your helpful feedback, never leaving your office without a new understanding or new questions to consider.

Thank you Dr. Errol Marliss, Director of the McGill Nutrition Centre, and Dr. Régeanne Gougeon, without whom none of this important research in nutrition and metabolism would happen. Your expertise advice and feedback on various aspects of the study, as well as your informal teaching in journal clubs and meetings were essential for my continued learning and progression of my work.

I also thank all of the truly amazing lab technicians. Thank you Connie Nardollilo for training and helping me with subject recruitment, preparation and data collection throughout the study, never missing one detail – something so important when carrying out such complex studies. Thank you Marie Lamarche, Donato Brunetti, Daniel White, Ginette Sabourin and Alexandre Morais for performing and teaching me to perform some of the blood and muscle analyses, tasks that are so simply said but take so much work to complete. I am so grateful for your hard work, staying late and even coming in on weekends to finish up analyses for my project. Students in the lab are so privileged to work with and learn from each of you. To my fellow students in the lab I wish you all the best in your future endeavours. Thank you Josie Plesia and Isabelle Lalonde for taking care of all administrative issues and making them a non-issue. Your work made my time here run so smoothly.

Thank you to all the wonderful women who participated in this study for your generosity and genuine interest in taking part in clinical research. Each one of you has touched me with your open minded, adventurous nature and many stories and experiences. I can only hope to be so full of life and wisdom at your age.

I'm also so grateful to my family for always encouraging and believing in me; thank you Lionel for graciously and tirelessly listening and participating with interest in many a sarcopenia and leucine conversation, and for being a calming presence when I needed it most. I love and appreciate you more than you know.

Finally, this is dedicated to my sister and granny whose memory has given me the determination and inspiration to complete this thesis.

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List of Abbreviations

AA	amino acid(s)
ALA	alanine
AUC	area under the curve
B	rate of protein breakdown
BIA	bioelectrical impedance analysis
BCAA	branched-chain amino acid
CIU	Clinical Investigation Unit
CRP	C-reactive protein
DXA	dual X-ray absorptiometry
EAA	essential amino acid
ECG	electrocardiogram
eIF4E	eukaryotic initiation factor 4E
FFM	fat-free mass
GDH	glutamate dehydrogenase
GTP	guanosine tri-phosphate
IL-6	interleukin-6
IRMS	isotope ratio mass spectrometry
IRS-1	insulin receptor substrate-1
LBM	lean body mass
LEU	leucine
mLST8/GβL	mammalian LST8/G-protein β-subunit like protein

MPB	muscle protein breakdown
MPS	muscle protein synthesis
MRI	magnetic resonance imaging
mTOR	mammalian target of rapamycin
MUHC	McGill University Health Centre
NEAA	non-essential amino acid
PHE	phenylalanine
PI3K	phophatidylinositol 3 kinase
PKB/AKT	protein kinase B
PRAS40	proline-rich Akt substrate of 40 kilodaltons
$p70^{s6k1}$ or S6K1	ribosomal protein 70S6 kinase
raptor	regulatory associated protein of mTOR
REE	resting energy expenditure
Rheb	Ras homolog enriched in brain
rpS6	ribosomal protein S6
RVH	Royal Victoria Hospital
S	rate of protein synthesis
SEM	standard error of the mean
SD	standard deviations
TEF	thermic effect of food
TNF-α	tumor necrosis factor alpha
TSC1/2	tuberous sclerosis complex 1 and 2 heterodimer
4E-BP1	eukaryotic initiation factor 4E-binding protein

INTRODUCTION

With modern medicine and lifestyles, we are living longer. In fact, Canadian census data report life expectancy for current births to be 81 years, and that a 65 year-old man can expect to see another 18 years, more than 2 years above the estimate from the 90's. The result of increased life expectancies combined with the large "baby boom" population entering their senior years is a rapidly expanding senior population (≥65 years of age) which is expected to reach up to 10.9 million by 2036 - more than double the 4.7 million in 2009, and will comprise 25% of the general population [1]. This accelerated increase in the number of elderly persons makes maintenance of physical function and health in our aging population mandatory, if they are to enjoy their extended years and live as independent seniors. Unfortunately, it is well known that with aging comes changes that can affect physical function and independence, including the gradual involuntary loss of muscle mass, called sarcopenia [2]. Based on observational data, it has been estimated that between 20 and 80 years of age, total lean body mass (LBM) decreases by 18% in men and 27% in women [3], and muscle area up to 50% [4]. In other words, skeletal muscle mass reaches its peak around age 20 – 35 [5] and decreases by 1 - 2% per year after the 4^{th} decade of life [3, 6].

Not surprisingly, loss of muscle can have consequences on strength and mobility, affecting physical function and ultimately, quality of life [7-9]. Low muscle mass increases risk of sudden falls and fractures and may lead to loss of functional independence and increased risk for physical disability [10-12]. It has also been associated with osteoporosis, another risk factor for bone fractures [13]. Amino acids (AA) must be available when elevated rates of protein synthesis (S) are required, for example during the immune response during illness. Because it is the largest compartment of protein in the body, it is mostly the breakdown of muscle which supplies the necessary AA (i.e. protein building blocks) [14]. Sarcopenic individuals with low muscle mass may therefore be more vulnerable to and recover more slowly from illness or injury [15]. Due to all of these factors, sarcopenia is associated with a higher mortality [9]. The impact of sarcopenia from a public health perspective is clear - in 2000, sarcopenia was estimated to

account for approximately $18.5 billion in health care costs in the United States [16]. With the growing aged population, these costs are no doubt increasing dramatically. Because of its function, relative size and the fact that it is such a metabolically active tissue, muscle mass is crucial in determining whole body functioning and metabolism and therefore loss of muscle mass may also predispose to or exacerbate other aging-related metabolic diseases and syndromes, including type 2 diabetes, cachexia and obesity [17-18]. For all these reasons, preserving muscle mass into the senior years is of great importance for maintenance of health and quality of life with aging.

Despite the clinical significance of skeletal muscle loss, the mechanisms leading to the onset and progression of sarcopenia are still relatively unknown, and potential strategies to counteract or reverse it are limited. The mechanism of sarcopenia is complex and may be influenced by several environmental, behavioural and physiological factors, however this gradual loss of muscle mass is ultimately the result of an ongoing daily imbalance between protein synthesis (S) and protein breakdown (B). S exceeds B in response to anabolic stimuli such as mechanical stimulation from physical activity or nutrient stimuli from feeding, achieving net anabolism (i.e. protein deposition). Consequently, alterations in the metabolic response to these stimuli result in muscle loss. Resistance exercise is a well-known anabolic stimulus to increase skeletal muscle size [19]. However this may not always be feasible in an elderly, possibly frail population, and evidence shows that aging sarcopenia prevails even in top athletes who maintain their body weight and high activity levels [20].

This thesis therefore focuses on understanding the anabolic resistance of protein metabolism to the stimulus of feeding, which we believe to be largely responsible for this daily imbalance of S and B in sarcopenia, by assessing the protein metabolic response to a mixed meal. Secondly, we evaluated meal supplementation with leucine (LEU), an essential amino acid (EAA) which has been shown to have unique signalling properties to stimulate S, as a possible nutritional intervention to recover the protein anabolic response to a meal, counteracting the progression of sarcopenia.

1 BACKGROUND LITERATURE REVIEW

1.1 Sarcopenia of aging

The term sarcopenia, first coined by Rosenberg in 1989, is the loss of muscle mass that is associated with aging - "sarx" denoting "flesh" and "penia" signifying "loss." It is characterized by progressive atrophy, and cross-sectional histological data indicate that it involves a decrease in both the number (hypoplasia) and size (hypotrophy) of myocytes (ie. muscle cells/fibres)[4]. Myofibrillar contractile proteins, including myosin heavy chain (MHC), make up the majority of muscle protein (~66%) [21] and are responsible for force generation. Myocytes can be classified into two types based on which isoform of MHC they contain - type I (slow twitch) and type 2 (fast twitch) fibers [22]. Type 2 fibres are able to generate greater force more rapidly but for a shorter duration, and are utilized during high-intensity anaerobic movements, while type 1 fibres show a slower development of force when activated and are used during slow sustained movements, since they are able to maintain tension for longer periods [22-23]. Hypotrophy involves loss of myofibrillar proteins, mainly MHC, and biopsy studies in both animals [24] and humans demonstrate either preferential loss or reduction of type 2 fibres with advancing age with no major changes in slow type 1 fibres [25-27], or equal loss of both type 2 and type 1 fibres and a decrease in size of type 2 fibres only [4]. Because they are capable of up to 4 times greater force production than type 1 fibres [28], the preferential loss of or decreased size of type 2 fibres with age might explain reduced muscle power with aging [29-30]. Either scenario results in a progressive loss of muscle mass, beginning around the third decade of life [31].

Of course, body compositional changes that occur with aging are not limited to muscle. Both cross-sectional and longitudinal studies show that while muscle mass decreases with age, adiposity increases in absolute and/or relative proportion [3, 32-35]. An average adult will gain 1 kg of fat per year from age 30 to 60, while losing around ½ kg of muscle in that same time period, however how much fat-free mass (FFM) is lost, maintained or gained is highly dependent on the

direction and magnitude of body weight change, indicating a clear relationship between changes in body composition and total body weight [34, 36-37], an association important to consider when studying aging sarcopenia. The aging-related loss of muscle mass and increase in fat mass may not only affect physical strength and function, but these body compositional changes have been related to decreased rates of whole-body protein turnover in aging, when rates are expressed per kg of body weight [38]. These effects on whole-body metabolism may be linked to other metabolic diseases and syndromes associated with aging, such as type 2 diabetes, obesity and muscle wasting seen in cancer cachexia [17-18, 39]. Thus, counteracting muscle loss with age may also carry beneficial effects for their prevention or treatment.

1.1.1 Diagnosis and prevalence

Since the coining of the term "sarcopenia," much research has been focused on understanding and preventing it, due to its association with functional decline and mortality and its relevancy in the context of our aging population. However, one clear consensus definition of sarcopenia is lacking, primarily because the mechanism likely involves the interplay between many internal and external factors and the nature of these relationships are not yet fully understood [31]. When does it become clinically relevant and how are we to identify pre-clinical non-pathological sarcopenia? The most commonly used definition to diagnose sarcopenia is a cut-off value based on a definition of low skeletal muscle mass [6]. This is determined by an appendicular (ie. limb) skeletal muscle mass index (AMMI), which is obtained by dividing total kilograms of appendicular skeletal muscle mass (ASM), as evaluated by DXA [40], by body height squared (m^2):

$$\textbf{AMMI} = \textbf{ASM/height}^2\ \textbf{(kg/m}^2\textbf{)} \qquad [6]$$

Individuals with an AMMI more than 2 SD below the mean of a reference group of young healthy control subjects are classified as sarcopenic. Based on this criterion, the prevalence of sarcopenia has been reported from 6-15% in individuals 65 years and older [31] to over 50% in people 80 years and older [6, 41], with sex differences showing greater loss in men [41]. In a UK population,

sarcopenia was prevalent in 20% of women aged 60 - 70 years, and 33% of women 70 years and older [42]. While sarcopenia may be a normal consequence of aging, not everyone becomes sarcopenic.

1.2 Other muscle changes that occur with aging

Aside from progressive loss of its mass, aging muscle declines in quality [43], which may lead to impaired muscle strength and function [7-8], and could also be related to quantitative decreases. Along with a decrease in muscle mass, there is simultaneous infiltration of fat (ie. myosteatosis) and other non-contractile material (ie. connective tissue), which may compromise the quality of the existing muscle tissue and has been associated with both poorer lower extremity performance in older adults [44] and muscle insulin resistance in obesity [45]. Other morphological abnormalities include disarrangement of myofilaments and z-lines, and accumulation of ragged muscle fibres [46]. Oxidative damage acquired across the lifespan leads to damaged and dysfunctional cellular proteins, lipids and DNA [47-50]. In particular, mitochondrial DNA mutation results in a decrease in the amount of functional and total mitochondria, compromising muscle oxidative capacity [51-52]. Muscle tissue is especially vulnerable to oxidative stress due to its high oxygen use and large number of postmitotic cells, which are more prone to accumulating free radical oxygen species [53]. The possibility that oxidative damage to protein cell components might contribute to an impaired stimulation of muscle protein synthesis (MPS) with age has been proposed, and antioxidant treatment in old rats was shown to improve impaired muscle protein anabolic response to the amino acid LEU [54]. However the effects in humans and exact mechanisms involved remain to be confirmed. Aging muscle is also characterized by changes in the neuromuscular system leading to α-motor neuron degeneration and subsequent denervation of muscle fibres, resulting in a loss of functional and total motor units [55-56]. A motor unit consists of an α-motor neuron and the muscle fibres it innervates [57]. When number of motor units was estimated by counting in humans, healthy adults aged 60 years and older had half the amount of their young counterparts [58].

1.3 What is causing muscle mass loss?

The causes of sarcopenia are complex and multifactorial, with both internal (ie. physiological) and external (ie. lifestyle) contributing and interrelated factors. However, the loss of muscle mass is ultimately due to an imbalance between daily rates of MPS and muscle protein breakdown (MPB) and therefore, assessment of how either of these processes may be altered with aging should reveal important mechanisms in the progression of sarcopenia. It is first necessary to realize how daily turnover of all body protein can affect muscle mass.

1.4 Daily protein turnover: relation to muscle mass loss in sarcopenia

Proteins are made up of a combination of any or all of the 20 AAs. In the adult human, 12 of these are considered non-essential amino acids (NEAAs), since they can be synthesized by the body. The remaining 8 are called EAAs, since humans are unable to synthesize them and must attain them from protein sources in the diet [59]. AAs in the body can be considered to be in either one of two "pools": (1) unbound as free AAs in the blood plasma, interstitial fluid, and intracellular compartment or (2) bound within body proteins. Because we do not store protein reserves as we do fat, each body protein we have at any given moment is serving a function, with excess AAs recycled to make new proteins, oxidized for energy or stored as fat, and excess nitrogen excreted as urea or ammonia in the urine [60]. To maintain their integrity and function, body proteins are continually undergoing cycles of degradation and re-synthesis (i.e. turnover), though different proteins have different rates of turnover depending on their structure and function [61-62].

In the weight-maintaining situation in healthy young adults, daily protein gains are equal and opposite to daily protein losses, resulting in a net zero balance (no change) of body protein [61, 63]. Net balance, whether positive or negative, is determined by the two components of protein turnover; S and B. Rates of S exceed those of B in response to anabolic stimuli such as physical activity and during the postprandial period following meals, resulting in a positive protein balance (ie anabolism). The body protein gained during anabolic periods (S > B)

is matched by the body protein losses that occur in the absence of anabolic signals such as inactivity and fasting or post absorptive periods (i.e. overnight and in between meals, B > S), when catabolic breakdown of body protein is necessary to maintain basal levels of plasma AAs required for homeostatic protein turnover and normal body processes (i.e. cell replication, growth and obligatory loss of nitrogen through nails, hair, and other body excretions) [64]. Some, but not all of the AAs released from protein degradation are re-integrated into new proteins. Consequently, the lost EAAs that are not recycled but rather are excreted or used for energy must always be replaced by AAs from dietary protein intake, thus highlighting the importance of dietary AAs as both the anabolic signal and building blocks for body protein S [60].

In the absence of dietary protein, it is degradation of primarily muscle protein that provides the necessary AAs in the postabsorptive state, since muscle mass is the largest compartment of protein and source of AAs in the body, comprising approximately 45% of total body protein and accounting for roughly half of the total cell mass in humans [64-65]. Therefore, a decrease in muscle mass as seen in sarcopenia is essentially due to the inability to replace during feeding, the muscle protein losses that have occurred to maintain whole-body protein turnover and obligatory protein losses during fasting. This could be due to either greater losses during fasting or lesser gains in response to feeding, however significant evidence supports the latter [66]. Regardless, the increase or decrease could be attributed to changes in S, B or both.

1.5 Causes and mechanisms of a negative daily protein balance

Alterations in rates of MPS and MPB can result from an increase in catabolic stimuli, or a reduction of or resistance to anabolic signals; however it is likely that a combination of these mechanisms work in parallel. All of these alterations, alone or together, will lead to an imbalance in daily protein turnover such that net muscle protein mass is lost over time.

1.5.1 Increase in catabolic and decrease in anabolic stimuli

Evidence for immunological aberrations with aging has led to the hypothesis that chronic low-grade inflammation may be a significant factor in aging comorbidities, including insulin resistance and sarcopenia [67-71]. Many [72-74], but not all [75] studies indicate an elevation in circulating systemic inflammatory markers with aging, including TNF-α, CRP and in particular IL-6. These cytokines are naturally part of the stress response, able to stimulate muscle proteolysis [76-77] and have been linked to insulin resistance in obesity [78]. Mild elevation of IL-6 and TNF-α inflammatory markers has been correlated with lower muscle mass in healthy elderly [68], however a recent study found that elevation of TNF-α, a cytokine particularly associated with skeletal muscle wasting, did not affect whole-body or muscle protein turnover in healthy adults [79] and clarifying studies are needed.

While inflammation-associated muscle catabolism may increase with aging, there is a concurrent decline in anabolic stimuli including: secretion of anabolic hormones, mechanical stimulation from physical activity and optimal food and protein intake. After a person reaches age 30 there is a steady decline in growth hormone (GH), insulin-like growth factor 1 and anabolic sex steroids, such as testosterone [80-81]. However, declining GH secretion does not appear to be an important factor in the progression of sarcopenia [73], and supplementation studies in humans do not support GH administration in older men and women as a means to stimulate MPS and substantially augment skeletal muscle mass.

Recent data from the Centers for Disease Control reveal that less than half of Americans aged 60-69 years perform any kind of physical activity and this decreases with increasing age to only a third of individuals aged 70 years and older [82]. In the absence of muscle contraction from physical activity, there is a decrease in muscle fibre size referred to as unloading-induced or disuse atrophy [83], as seen in states of muscle disuse such as bed rest [84]. An increasingly sedentary, less active lifestyle that often occurs with aging may play an important role in sarcopenia, which could be reversed by increasing activity levels. The anabolic effect of exercise has been demonstrated in young [19] and older adults

[85-86]. However, in the fasting state following exercise, net muscle protein balance is still negative, indicating that any improvement in net protein balance depends on provision of exogenous AAs [87]. While both aerobic and strength training are accessible strategies to induce muscle hypertrophy, this may not always be feasible in older, possibly frail adults [88]. Moreover, even athletes who maintain their fitness and body weight through regular exercise are unable to evade loss of FFM at rates similar to their sedentary peers - approximately 2 kg per decade [3, 20, 89]. Unfortunately, it would appear that exercise alone is not enough to maintain muscle mass with age.

1.5.2 Appetite and its hormonal control

Suboptimal dietary intakes could also lead to decreased AA availability for postprandial anabolism, and a subsequent increase in MPB to compensate. Indeed, longitudinal evidence reveals a decrease in both energy and protein intake with increasing age [90-91]. This could be due to a number of different social, psychological, or functional factors (ie. income, education, convenience, etc.), however it may also involve an apparent internal "physiologic anorexia of aging" [92]. This progressive loss of appetite with age has been well documented by both longitudinal and observational studies [93]. Proposed physiologic mechanisms include an altered sense of taste and smell, and age-related changes in digestion. In elderly subjects, slower rate of gastric emptying of solid and liquid meal components was related with decreased post-meal hunger, suggesting a causative relationship [93]. The satiating effect of delayed gastric emptying may be related to the secretion of incretins - gastrointestinal hormones secreted in the absence of or in response to meal ingestion - that regulate feelings of appetite and satiety (ie. fullness) in the appetite center of the brain hypothalamus [94-95]. Incretins may be orexigenic (ie. stimulate appetite), such as ghrelin, or anorexigenic (ie. suppress appetite), such as Peptide YY (PYY) [96]. PYY, a satiety signal, is present at low levels between meals, increases upon nutrient ingestion, and slowly declines back to baseline [97]. As a hunger signal, plasma ghrelin follows the opposite trend, peaking just prior to a meal and steeply declining just after meal ingestion to nadir levels in the first hour [98]. Postabsorptive ghrelin

concentration and its decrease with feeding have both been found to be lower with aging [99], frailty [100], obesity and insulin resistance [101]. However, whether changes in incretin levels play a significant role in sarcopenia, or if levels or responses to a meal are different in sarcopenic versus healthy individuals is not known.

1.5.3 Resistance of protein metabolism to food intake

More and more evidence points to another possibility - that AA intake and availability with feeding is not decreased with age due to behavioural or physiologic factors but rather aging involves an anabolic resistance to feeding stimuli, whereby elderly individuals are unable to efficiently use their dietary protein to replace postabsorptive protein losses. Indeed, studies in humans do not support differences between young and elderly (matched for physical activity level) in rates of either protein S [66, 102-103] or protein B [104] during the postabsorptive state between meals, again suggesting that the metabolic failure occurs in the response to feeding during the postprandial period just after eating a meal. Consistent with this, evidence does support differences between protein anabolism of young and elderly in response to meal stimuli.

1.5.3.1 Effects of feeding on muscle protein metabolism

Insulin is a potent anabolic hormone secreted by the beta cells of the pancreas in response to rising blood glucose and AA levels following a meal [105]. It is most known for its regulation of glucose metabolism, but it is also a principal regulator of lipid and protein turnover. Insulin's main effect on protein metabolism is to inhibit B of both whole-body [106-107] and muscle [108-110] protein. However it has also been shown to stimulate protein S itself [111] and in the presence of basal plasma AA levels [106, 112]. Studies in animals and humans reveal a permissive role of insulin for the increase in MPS caused by hyperaminoacidemia, since raising insulin past a certain basal level will not further stimulate MPS [113-114]. Insulin and AAs thus work synergistically and additively to increase MPS [112], however AAs have been proposed to be the main protein anabolic stimulus following meal ingestion [103, 115]. Studies in

humans suggest peak rates of MPS stimulated by feeding occur within 60-90 minutes [116-117].

It is relatively recent that AAs have come to be appreciated as not only the precursor building blocks for protein, but also as regulators of protein anabolism [103, 118-119]. AAs primary anabolic effect is to stimulate protein S, regardless of an increase in insulin [114, 120-121]. This corresponds to increased phosphorylation of protein anabolic signalling substrates protein kinase B (PKB/AKT), mammalian target of rapamycin (mTOR), S6 kinase 1 (S6K1) and 4E-binding protein 1 (4E-BP1) in muscle biopsies [122]. This insulin-independent, stimulatory effect on MPS by AA has been demonstrated with EAA alone as well [103, 123] and appears to be dose-dependent with maximal effects occurring around a 50 - 80% rise in blood plasma EAAs [103, 115-116]. NEAA are not necessary for this effect [124], emphasizing the importance of EAAs alone for protein anabolism.

1.5.3.2 Intracellular signalling mechanisms

1.5.3.2.1 mTOR pathway of protein synthesis

Insulin and AAs stimulate mRNA translation initiation and protein elongation, both processes of protein S, by independent mechanisms that converge at the level of the intracellular kinase mTOR. The mTOR pathway coordinates nutrient availability with cell growth and proliferation processes by acting as a sensor of available nutrients and regulator of downstream substrates involved in protein synthesis (ie. mRNA translation) [125-126]. Therefore, mTOR keeps nutrient availability and cell growth processes in communication and in balance with one another. It can exist as a part of two functional complexes; mTORC1 or mTORC2 [127-128]. mTORC1 is the mTOR complex responsible for regulation of protein translation initiation, containing mTOR; raptor, a scaffold protein that helps associate mTOR with its downstream targets; mLST8/GβL; and PRAS40, a substrate of both mTOR and PKB/AKT [129-131]. PRAS40 acts as a negative regulator of mTORC1 when bound to it, and dissociates from mTORC1 upon insulin stimulation [132].

There are many downstream substrates of mTORC1, however two of the most well characterized are 4E-BP1 and S6K1 [133-134], both of which have been shown to play a role in mTOR's capability to increase cell size [135] (Fig. 1). The eIF4E initiation factor is part of an active initiation complex involved in the initial steps of mRNA translation. In the absence of anabolic stimulation, 4E-BP1 associates with eIF4E, inhibiting it from forming the active complex. Upon mTORC1 phosphorylation of 4E-BP1, eIF4E is released and translation initiation may proceed. S6K1, when phosphorylated by mTOR, goes on to phosphorylate rpS6, which is involved in translation of proteins necessary for cell growth [136]. Increased translation initiation together with increased activation and translation of ribosomal and other translational proteins leads to enhanced protein synthesis and cell growth [137]. S6K1 is also able to phosphorylate IRS-1 at inhibitory sites, providing negative feedback regulation of insulin simulation (see Fig. 1) [138].

Upstream of mTOR, insulin binding to its receptor tyrosine kinase causes subsequent receptor dimerization and activation by autophosphorylation. Once activated, the insulin receptor initiates a phosphorylation cascade of intracellular substrates, including insulin receptor substrate 1 (IRS-1), phosphoinositol-3-kinase (PI3K) and PKB/AKT [139]. Downstream of PKB/AKT, tuberous sclerosis complex 1 and 2 heterodimer (TSC1/2) inhibits the mTOR activator Ras homolog enriched in brain (Rheb), a small GTPase [140]. When phosphorylated by PKB/AKT, TSC1/2 allows GTP-loading of Rheb [141-142], which is thought to stimulate mTORC1 activity by enhancing its association with downstream targets [143-145].

AAs are also able to stimulate protein S by augmenting mTOR activity [146] independent of insulin activation of the PI3K/AKT pathway [147]. However it is only recently that significant progress has been made in uncovering the precise mechanism. AA activation of mTOR works downstream of insulin, as TSC1/2 is not necessary for their action [147-148]. Rather, the AA mechanism depends on small GTPases called Rag proteins [149-151]. These Rag GTPases exist as a heterodimer and are activated (ie. charged with GTP) in an amino acid-

dependent manner [152]. Once GTP-charged, the Rag GTPase heterodimer directly or indirectly causes translocation of mTORC1 to a membrane compartment containing its activator, Rheb [153].

1.5.3.3 Aging effect on the protein metabolic response to feeding

Like the age-acquired insulin resistance of glucose metabolism [154], there is evidence of a similar age-related change of insulin's effects on protein metabolism. The suppression of whole-body protein B in response to mildly elevated insulin levels was less in elderly compared to young but similar at higher levels [155]. On the other hand, at the muscle level, administration of an AA and glucose mixture, which elevated insulin to postprandial levels, showed normal suppression of MPB but a failure of increased MPS in elderly subject when compared to young [102]. Similar results were found at the whole-body level under steady-state conditions of elevated insulin and basal glucose and AA levels, and were associated with both a reduction in FFM and an increase in fat mass [156]. Decreased responsiveness of MPS to EAA by bolus ingestion [103] also find the defect to be a result of a failure of protein S rather than an accelerated rate of protein B, and correlated with a lower total amount and phosphorylation of mTOR and its downstream substrates. This blunted response of protein S has also been demonstrated with combined increased insulin and AAs. In response to a state of hyperaminoacidemia (elevated plasma AA) and hyperinsulinemia (elevated insulin), induced by an AA and glucose infusion, leg MPS was potently stimulated and MPB suppressed in young subjects. Older subjects maintained suppression of MPB but did not show the increase in MPS, resulting in a lower net protein balance across the leg muscles [102]. Studies in which basal glucose levels are maintained (euglycemia) in the presence of hyperinsulinemia and hyperaminoacidemia support this phenomenon as well [157-158]. Some evidence has suggested an impaired stimulation of S6K1 as a possible mechanistic explanation for the blunted response of MPS with aging [113, 157].

1.6 Hyperaminoacidemia improves response of protein synthesis

Despite the age-acquired anabolic resistance of protein metabolism, this impaired response may be improved in response to higher levels of AAs [159-160]. When given small boluses of 7 g EAA, elderly subjects showed lower rates of MPS and lower muscle protein deposition compared to young subject [161]. However, when a larger EAA bolus (15 g) or a high EAA infusion was administered, whole-body and MPS responses to increased EAA availability responded normally in older adults [66, 160, 162-163]. In these studies, plasma EAAs were brought to higher levels than when giving smaller boluses.

Not all studies find an altered anabolic response in aging to a meal-like situation [164]. Under hyper-3 clamp conditions (ie. stably elevated levels of insulin, glucose and AA), results revealed no differences between young and elderly healthy active women in either the whole body or muscle protein anabolic responses. Although the older women exhibited a slower protein turnover, as demonstrated by lower rates of whole-body B, AA uptake, and AA oxidation, there were no differences in net balance after adjusting for a their lower insulin secretion. There was also no effect of aging on muscle protein FSR, whole-body S, or activity of intracellular mTOR substrates [164]. These results indicate that healthy aging is not necessarily associated with a decline in the anabolic response to meal-like levels of glucose, AA and insulin. The discrepancy may be that these were healthy, active and non-sarcopenic women. Our previous results in elderly frail women (many of whom were sarcopenic) revealed indications of insulin resistance in response to ingestion of a mixed meal [165]. Furthermore, in frail mildly-malnourished elderly women, rates of myofibrillary protein B based on 3-methylhistidine urinary excretion were higher than their healthy counterparts, suggesting alterations in protein metabolism in this subset of the population [166]. Together, these findings indicate that insulin resistance of protein metabolism may be present only in older individuals who already show sarcopenic body compositional changes, and not in their healthy age-matched counterparts who may have maintained their protein anabolic sensitivity.

In summary, strong evidence points to a blunted response of protein metabolism to feeding as a contributing factor in aging sarcopenia, and that an impaired stimulation of protein S may be key [167]. Additionally, increasing plasma AAs to higher levels has been shown to recover rates of protein S to levels comparable to young healthy individuals. Studies evaluating the effects of "fast" vs. "slow" proteins on MPS in both young and elderly support this hypothesis. Fast proteins are those that are quickly digested and absorbed and slow proteins the opposite. As a consequence of these different digestion kinetics, fast proteins increase plasma concentrations of AA more rapidly and to more elevated levels than slow proteins [168-169]. The milk proteins whey and casein are fast and slow proteins, respectively [170]. In young adults, who are sensitive to AA availability, a casein meal induced a higher net postprandial AA balance over 7 hours when compared with whey [171]. In contrast, studies in older men showed that whey was more efficient at stimulating postprandial muscle protein deposition and this effect was attributed to faster digestion and absorption of whey, and consequent higher plasma AA levels [168-169]. These results support the hypothesis that daily body protein loss in sarcopenia may be due to a resistance of protein S in response to postprandial AA availability in aging, and that threshold levels of plasma AAs necessary to efficiently stimulate postprandial MPS and muscle deposition may be higher in the elderly. Interestingly, whey stimulated muscle protein synthesis to a greater extent than a casein hydrolysate, which differed from whey only in LEU content [169], supporting the hypothesis that this essential branched-chain amino acid (BCAA) plays a crucial role in eliciting the anabolic response of MPS to feeding.

1.7 Leucine meal supplementation as an anabolic stimulus

While studies suggest that the current World Health Organization dietary reference intake of 0.8 g protein/kg/day [172] is not sufficient for maintenance of muscle protein balance in older adults [173-174], findings do not support increasing protein intake in the elderly above that of the average of a North American population, which is approximately 1.5 g protein/kg FFM/d (US

population) [175-177]. A 10-day high protein diet (3.0 g/kg FFM/d) did not increase whole-body protein S or muscle mitochondrial function in either young or older adults above that in response to meals of usual protein content [177]. These results suggest that an increase in all the AA building blocks may not be the key to restoring the protein anabolic response, but rather an increase in the primary anabolic signals, one of which may be the BCAA LEU [178].

1.7.1 A stimulus for protein synthesis

Accumulating evidence has long emphasized the importance of the single AA LEU as a regulator of protein turnover [179], and more specifically a potent anabolic stimulus for protein S [180-181]. Like the effects of a mixture of AAs or EAAs, studies evaluating rates of MPS in myocytes [182] as well as in animals [181] and humans, indicate that LEU alone is able to stimulate MPS and this corresponds to increased phosphorylation of mTORC1 downstream substrates, including 4E-BP1, eIF4E and rpS6 [147, 159, 181-184].

The recovery of the blunted MPS response in older animals and humans to supraphysiological AA levels has also been replicated by high levels of LEU alone [159, 185]. In an important study conducted in healthy young and elderly men, Katsanos et al. [186] showed that when ingesting a bolus of EAA (6.7g) with a LEU content typical of high quality proteins (27%; ie. 1.72g), MPS is not stimulated above baseline in elderly men, whereas it increases in young men. However, doubling the LEU content of the bolus to 41% (2.79g) is able to overcome the anabolic resistance in elderly men, recovering their MPS rates to values comparable to young subjects [186]. Interestingly, the higher dose of LEU did not further stimulate MPS in the young. This robust effect was reproduced in a similar study where a complete balanced meal (rather than a bolus of EAA alone), with or without added LEU was continuously ingested in small amounts [187]. This study confirmed LEU's stimulatory effect in the elderly in the context of a normal meal. In both studies the only difference between the two treatments was plasma LEU levels, indicating that LEU's anabolic effect is independent of both a rise in other AAs and insulin. However, basal levels of other AAs are still

necessary [184, 188] - while LEU may be the primary signal, protein S is still dependent on availability of the building blocks.

1.7.2 An insulin secretagogue

Aside from its ability to stimulate protein S, LEU may also exert its protein anabolic effect by acting as an insulin secretagogue. Although it has been shown *in vitro* [189], *in situ* [190] and *in vivo* [191] to stimulate insulin secretion from the pancreatic beta cell, the exact mechanisms are not yet known. Proposed mechanisms include LEU allosteric activation of GDH, an enzyme that catalyzes the transamination reaction of the NEAA glutamate to α-ketoglutarate (α-KG) and vice versa [190, 192], and the importance of α-KG formation and its energy substrate by-products, such as NADPH [190].

1.7.3 Relevancy in the sarcopenic population

These studies overwhelmingly support the acute anabolic effect of LEU and its potential as an easily accessible, non-invasive nutritional strategy against muscle loss. In contradiction to this, results from 2 recently published long-term (3 and 6 months duration) LEU supplementation studies found no benefit on LBM [193-194]. However, we do not view these as definitive results for a few reasons. These studies were performed in elderly adults who were either healthy or had type 2 diabetes, but in both cases were not reported as being sarcopenic or having a low muscle mass. As our recent results suggest, healthy individuals who do not have a low muscle mass exhibit an intact anabolic response to postprandial levels of nutrients [164]. In previous studies, elevated plasma LEU did not further increase the MPS response in healthy young persons, who do not show anabolic resistance to typical meal levels [186, 195], whereas it reversed the blunted response in elderly persons [186]. These findings together lead us to propose that the main effect of elevated postprandial LEU may be to restore a blunted response of protein anabolism in individuals who are already identified as sarcopenic, and who we hypothesize to show anabolic resistance to meals when compared to healthy age-matched counterparts.

Additionally, both Katsanos [186] and Rieu [187] showed that a doubling of plasma LEU concentration was necessary for its anabolic effect in the elderly.

In both long-term studies, 2.5 g of LEU was given with each meal, however postprandial plasma LEU levels were not measured in either study and it is possible that they did not reach threshold levels. Finally, in the Leenders et al. [194] study, all except a few of the patients were on blood-glucose lowering medication, primarily metformin. Metformin, which increases insulin sensitivity in muscle [196], may already improve protein metabolic responses in these patients, and could mask any additional effect of LEU. Long-term LEU supplementation may only benefit already-sarcopenic individuals who are otherwise healthy, and studies of LEU supplementation directly in this population are necessary to investigate this possibility.

1.7.4 Potential effects on thermogenesis and satiety

In addition to measuring the anabolic effect of LEU, it is also important to evaluate whether a LEU-rich may also carry unwanted effects, such as inducing a greater thermic effect of food (TEF) and early or enhanced satiety (ie. fullness). As a macronutrient, protein is known to induce satiety to a greater extent than carbohydrate or fat, and an increased TEF has been proposed as a potential explanation, since higher TEF has been associated with hunger suppression [197]. TEF is the energy required for digestion, absorption and disposal of ingested nutrients and is influenced by meal composition. How a greater TEF might induce greater satiety remains unknown but could possibly be due to greater stimulation of protein turnover.

Moreover, there is evidence that these effects on satiety and thermogenesis may depend on the protein source [198-199]. Since LEU appears to be one of the primary anabolic components of protein, increasing its levels alone could also have appetite suppressing effects by augmenting TEF. Additionally, different proteins have been shown to modulate responses of appetite-regulating incretins. Greater satiety associated with whey vs. casein protein intake, as assessed by subjective ratings and lower *ad libitum* food intake 90 minutes after protein ingestion, has been linked with higher glucagon-like peptide and PYY release, with less of an effect on ghrelin response [200]. Therefore it is also possible that LEU induces greater satiety by altering post-meal plasma levels of incretins.

Finally, central LEU administration in rats results in hypothalamic mTOR signalling as well as decreased food intake and body weight, suggesting a direct appetite suppressing effect [201]. Thus, it is possible that meal supplementation with LEU may impede hunger, decreasing energy and protein intake – presumably unwanted effects in sarcopenia since an accompanying decrease in postprandial protein anabolism would be expected. Therefore, this must also be investigated directly in a sarcopenic population.

2 SUMMARY AND RATIONALE

Strong evidence suggests that there is no significant aging effect on protein metabolism in the fasting state. Because body protein lost during fasting, including muscle, is replaced during the fed state and studies in aged animals and humans show a blunted protein anabolic response to feeding when AA supply is less than optimal, studies of protein kinetics in the fed state are mandatory to identify possible altered responses that could explain the sarcopenia of aging.

Our recent results revealed that protein metabolism in healthy elderly women responds normally to a hyper-3 fed-state clamp [164], however clamp conditions are non-physiological for many reasons – the clamp does not address physiological factors present after oral consumption of a meal, including digestion and absorption kinetics and subsequent dynamic fluctuations in hormones and nutrients. Moreover, exposure to such a prolonged period of stably elevated postprandial levels of hormones and nutrients is not physiologically accurate and thus could alter the normal meal responses. Additionally, these women were not sarcopenic, in whom we have shown indications of insulin resistance and altered protein metabolism. This mandates testing the protein metabolic response to normal ingestion of a complete meal, directly in sarcopenic women. While much research is focused on identifying causes of sarcopenia, assessment of the responses to feeding directly in sarcopenic persons is unavailable yet necessary to identify differences from healthy aging.

In addition to the need to characterize the mechanism of sarcopenia, interventions must be tested directly in the older sarcopenic population. Since nutritional interventions are among the most accessible and non-invasive potential strategies to counteract muscle loss, and AAs are a primary stimulus for protein anabolism, meal supplementation with the EAA LEU should be tested, as it has been shown to have anabolic properties specifically in aging. Results could provide evidence on which to base longitudinal clinical trials of LEU supplementation with the goal of attenuating muscle loss in older sarcopenic persons.

3 OBJECTIVES AND HYPOTHESES

Our primary **objectives** were to study older sarcopenic and non-sarcopenic healthy women, to 1) measure the effects of sarcopenia on whole-body and muscle protein anabolic responses to a complete meal, with the goal of uncovering intracellular mechanism, 2) determine whether a meal with added LEU increases postprandial protein anabolism in older sarcopenic women, and (3) quantify the effect, if any, of LEU supplementation on the thermic effect of food, appetite and its hormonal regulation during meal.

We **hypothesized** that in older sarcopenic women 1) whole-body protein anabolism is blunted in response to a complete liquid mixed meal, 2) this blunted response is associated with defects in intracellular mTOR signalling of translation initiation at the level of the muscle, 3) this anabolic resistance of protein metabolism is corrected by the anabolic effect of the AA LEU, when it is added as a meal supplement, and 4) LEU added to the meal should not affect thermic effect of food, appetite and its hormonal regulation.

4 SUBJECTS AND METHODS

4.1 Study Design

The study was a randomized paired crossover design. Older women were classified into two groups, sarcopenic and healthy. Each subject underwent two meal studies and each meal study consisted of 4 days of controlled diet followed by a meal test, during which postabsorptive and 5-hour postprandial whole-body protein kinetics were assessed as well as phosphorylation status (activation or inactivation) of substrates implicated in MPS pathways, to elucidate the underlying mechanisms involved in the regulation of muscle protein metabolism following a meal with or without added LEU. Blood hormones and nutrient substrates were also measured to look for differences in the metabolic response to each meal. Finally, REE was measured to assess the thermic effect of the meal. One meal test measured these responses to a LEU-rich meal and the other measured these responses to a non-LEU-rich meal equivalent for total energy, macronutrient, and nitrogen content. Each subject received the two meal studies in random order.

4.2 Recruitment and screening of healthy control and sarcopenic subjects

The study protocol was approved by the MUHC Human Research Ethics Board. Healthy and sarcopenic community-dwelling subjects (n = 4 and 5, older post-menopausal women, ≥61 years) were recruited through ads published in local newspapers for seniors as well as flyers distributed throughout the MUHC-Royal Victoria Hospital (Appendix 1). Only women were studied primarily to compare results from previous metabolic studies performed in our lab, since there are sex-dependent differences in protein metabolism [38]. Also, although age-related muscle loss affects both genders, women are at higher risk for loss of functional independence due to sarcopenia because: (1) they tend to live longer [202], and (2) at any given age of adulthood women have a proportionally smaller muscle mass than men due to sex specific differences in body composition [3].

All potential participants who called to inquire about the study were given a detailed overview of the protocol by a member of the research team. A pre-screening questionnaire (Appendix 2) was conducted over the phone to assure subjects met basic criteria for age, BMI ($\geq$ 22 kg/m^2 and $\leq$ 29 kg/m^2), and healthy status (non-diabetic, non-smoker, no current health conditions or use of medications interfering with metabolism or that increase risk for complications during the study).

4.2.1 Screening visit 1

After passing the pre-screening questionnaire, eligible potential participants were invited to the RVH-CIU for an initial screening. Subjects came in after an overnight fast. After giving informed, written consent (MUHC Ethics Committee-approved study consent form; Appendix 3), body weight was measured to the nearest 100g (Scale-Tronix digital scale; Ingrim & Bell-Meditron, Le Groupe, Don Mills, Canada), and standing height measured to the nearest 0.1 cm using a wall-mounted stadiometer. A urine sample was collected to screen for proteinurea and glycosuria, and venous bloods were drawn to assess thyroid hormones (TSH, T_3 and T_4), fasting plasma glucose, complete blood count with differential, lipid profile, biochemistry, and serology (Hepatitis A, B, C, HIV). Positive results or values not within an acceptable range for good health, as determined by cut-off values and by the discretion of the study physician, excluded potential participants from the study. A standard chest X-ray and ECG were performed at the RVH cardiology and radiology clinics to rule out abnormalities.

4.2.1.1 Mini-mental state Exam, Timed-up-and-go, and gait speed

Tests to evaluate cognition (MMSE; Appendix 4), mobility and physical function (TUG; gait speed) were performed to ensure competence to consent and to rule out obvious frailty or disability. For the TUG test, subjects sat in an armed chair. A stop-watch was used to time how long it took them to stand up from the chair, walk 3 metres (marked by tape on the floor) at their usual pace, turn around and sit down with their back resting on the chair [203]. Gait speed was evaluated by marking two lines, 4 meters apart on the floor. Subjects were instructed to

stand with their toes behind one line and they were timed on how long it took them to walk at their normal pace and cross the other line. The examiner instructed the subject that they were to start on the count of 3. The gait speed in m/s was then calculated. Cut off values used were a score ≥24 out of 30 on the MMSE for normal cognitive abilities [204], <12 sec. for normal elderly TUG and ≥1.0 m/s for normal gait speed [203, 205].

4.2.1.2 Bioelectrical Impedance Analysis and Dual X-ray Absorptiometry

Bioelectrical Impedance Analysis (BIA) was performed using the RJL-101A Systems instrument (Detroit, MI). BIA is a fast, simple and non-invasive tool commonly used to estimate body composition. By sending a small electrical current through the body, it measures the impedance to the current resulting from the resistance (R) and reactance (X_c) of body tissues, and is based on the premise that FFM is a better conductor of electrical current than fat mass. Therefore, these two tissue types will differentially affect R and X_c values [206]. Population-specific equations using the R and X_c values are then applied to estimate FFM (all non-fat tissue), which includes appendicular (skeletal) and smooth muscle mass, bones, viscera, skin, connective tissue and body water. Fat mass is approximated by subtracting FFM from total body weight. In our group of older women, the validated Roubenoff [207] equation was applied:

$FFM\ (kg) = 7.7435 + 0.4542 \times [Height(cm)^2/R] + 0.1190 \times [Weight(kg)] + 0.0455 \times X_c$

Subjects rested lying flat on a bed for 5 minutes, after which electrodes were placed on the hand and foot of the dominant side and BIA performed. The average of three consecutive R and X_c measurements was used as the final measurement.

Sarcopenia was first pre-screened for by BIA results, using the skeletal muscle mass index (SMI; as a %) cut-off proposed by Janssen et al. [208] and skeletal muscle index as a pre-screening tool for sarcopenia. Skeletal muscle mass was estimated as:

$$[height\ (cm)^2/R] \times 0.401 + [(age \times -0.071) + 5.102]$$

and the skeletal muscle mass index (SMI; %) calculated as:

$$skeletal\ muscle\ mass\ (kg)/body\ mass\ (kg) \times 100$$

Class 1 and class 2 sarcopenia were defined as SMI within 1-2 SD or greater than 2 SD of the mean of healthy young subjects, respectively [208]. BIA results for all subjects were then validated using DXA (Lunar Prodigy Advance; GE Healthcare, Madison, WI), performed at the McGill Nutrition and Performance Laboratory.

DXA is a method used to assess body composition, allowing quantification of body fat, bone and LBM (ie. non-bone and non-fat) [209]. It works by directing X-rays of two different energy levels into the body. Because diminution of the energy intensity of the X-ray depends on the composition, density, and components of the different tissue types, this allows discrimination between and quantification of these tissues. Since radiation levels are kept low and reproducibility has been shown to be 99%, DXA is both safe and precise [209], and is commonly used in the research setting to evaluate bone mineral density and body composition [35, 210].

Goulet et al. [211] defined two degrees of clinical sarcopenia (class 1 and class 2) using a similar methodology to Baumgartner et al. [6]. Instead of using AMMI, the ASM derived from DXA (which measures the lean mass of the limbs, which is primarily muscle) was used to estimate total skeletal muscle mass and total skeletal muscle mass index (MMI), as per equations derived from validation studies comparing DXA and MRI results [210]. Individuals with an MMI between 1 and 2 SD below the gender-specific mean value of a group of young Québec control subjects are classified as having class I sarcopenia, and those with an MMI > 2 SD below the mean are class II sarcopenic. Because young Québec women were used as the reference group, we found this definition appropriate for our older Québec women. For the current study, we used the class II cut-off by Goulet et al. [211] as the sole value to define sarcopenia (similar to the Baumgartner criteria of an AMMI > 2 SD below healthy young subjects), as we decided this cut-off would effectively identify significant sarcopenia with high sensitivity in our population.

4.2.1.3 Resting Metabolic Rate (RMR)

Resting metabolic rate (RMR; ie. resting energy expenditure; REE; kcal/d), the energy required to maintain body homeostasis (ie. respiration,

maintenance of body temperature, etc.) under basal conditions [212], was measured using a ventilated open circuit indirect calorimeter (Parvomedics TrueOne 2400, Sandy, UT) [213]. Body energy is derived from substrate oxidation, whereby carbon fuels are converted into carbon dioxide (CO_2), water (H_2O), and heat in the presence of oxygen (O_2). Indirect calorimetry indirectly assesses the amount of heat generated based on the amount and pattern of O_2 use and CO_2 production during respiration [212]. By measuring the volume of O_2 inspired (VO_2) and CO_2 expired (VCO_2) under a ventilated hood, the Weir [214] equation may then be applied to convert these volumes to REE expressed in kilocalories per day (kcal/d) and by including a known amount of urinary N (UN) to account for protein oxidation:

REE (kcal/d) = [5.616 x VO_2 (ml/min)] + [1.584 x VCO_2 (ml/min)] - [2.17 x UN (g/d)]

Indirect calorimetry also allows for determination of substrate (ie. carbohydrate, protein or fat) utilization, by calculating the respiratory quotient (RQ) - the ratio between VCO_2 and VO_2. RQ values of 1.0 and 0.7 are obtained for complete oxidation of glucose, and fat, respectively, with protein oxidation lying between these values at approximately 0.8 [212]. Just prior to the measurement, the instrument was calibrated according to the manufacturer's instructions. Subjects were required to rest lying in supine position for 30 minutes under thermal neutral conditions, after which a ventilated plastic canopy, with an outflow tube and a valve to allow inflow of oxygen, was placed over their head. Subjects were asked to breathe normally and refrain from moving, sleeping or speaking during the measurement period of 20 minutes. Data from the last 15 minutes of the measurement was used, as gas equilibration and flow rate adjustment occurred during the first 5 minutes.

4.2.1.4 Nutritional assessment

A 24-hour food recall and Food Frequency Questionnaire were taken to obtain information on usual daily dietary intake and eating habits. This was later used to create the isocaloric isoproteic diet during the study. Specific information on the types of foods typically eaten, portion size and mode of food preparation

were recorded and analyzed for macro and micronutrient content by The Food Processor SQL software (Version 10.8.0, ESHA Research, Salem OR).

4.2.2 Screening visit 2: Oral glucose tolerance test (OGTT) and physical exam

If potential participants passed the first screening visit, they returned to the Crabtree Labs to undergo an oral glucose tolerance test (OGTT) to rule out the presence or onset of diabetes, and a complete physical exam performed by the study physician. Subjects arrived in the morning after an overnight fast. An intravenous catheter was inserted in an anticubital vein for blood sampling. After baseline blood samples were taken at -15 and 0 minutes, an oral dose of 75 g dextrose, given in the form of a sweet orange-flavoured drink (Glucodex 300 mL, Rougier Pharma, Ratiopharm Inc., Canada), was ingested within 5 minutes. Blood sampling was then performed to measure plasma glucose at 30, 60, 90, 120 and 180 minutes after ingestion of the drink. Samples were collected in heparinised tubes to prevent coagulation, vortexed and centrifuged at 5^0C and 3000 rpm for 15 minutes. Plasma was then analyzed for glucose concentration by the glucose oxidase method (GM7 Micro-Stat; Analox Instruments USA, Lunenberg, MA). Subjects were deemed to have normal glucose tolerance and pre-diabetes was ruled out if their plasma glucose was <7.8 mmol/l at the 120 minute reading [215]. Sarcopenic subjects were not required to meet the criteria of normal glucose tolerance response to the OGTT. This was decided based on our previous observation that sarcopenic elderly women exhibit might insulin insensitivity [165], and our hypothesis that insulin resistance of protein and possibly glucose metabolism could play an important role in the process of muscle loss that occurs with aging.

4.3 5-Day Meal Study Protocol

4.3.1 Overview

The randomized crossover design involved paired meal studies - each subject underwent two meal studies in random order, one with and one without added LEU on the meal test day. Both the research team (except the technician

who prepared the meals) and subjects were blinded to whether the LEU-supplemented meal was given at study 1 or study 2. Each meal study lasted 5 days, the first 2 days were spent at home and the remaining three days at the Royal Victoria Hospital Clinical Investigation unit (RVH-CIU). On day 5, the day long meal test was performed, which included 3 hours in the postabsorptive state and the 5 hour postprandial metabolic responses to a complete liquid meal.

4.3.2 Days 1 and 2: Diet at home

During days 1 and 2 of each meal study, subjects followed a special diet consisting of a mixture of their typical foods along with bottles of a meal replacement liquid formula (Ensure®, Abbott Laboratory, St. Laurent, QC, Canada). The special diet was based on their 24-hr dietary recall taken at screening, and devised to maintain their usual dietary habits and current energy and macronutrient (protein, fat, carbohydrate) intake. While following the isocaloric isoproteic diet, subjects were asked to record in detail what they ate and daily dietary intakes were later analyzed by The Food Processor SQL software (Version 10.8.0, ESHA Research, Salem OR). The special diet was given to assure that food intake during the 4 days prior to each meal test was approximately equal in energy and macronutrients and followed their habitual intake, maintaining subjects at their current weight.

4.3.3 Days 3 and 4: Stay at the CIU

During day 3 and 4, the special diet was continued, with a bran cereal and milk breakfast and meals provided from the hospital cafeteria. All food and drink consumed were recorded and weighed to the nearest 0.1 gram, and daily macronutrient intake estimated (The Food Processor SQL software, Version 10.8.0, ESHA Research, Salem, OR). Complete 24-hour urine was collected each day and analyzed for glucose, urea, creatinine, electrolytes, and total nitrogen excretion (aliquots frozen at -20^0C). Anthropomorphic measurements (skinfold thicknesses and body circumferences) were taken with a Lange caliper [216] for comparison with BIA and DXA measurements.

4.3.4 Day 5: The Meal Test

The meal test on day 5 was performed at the Crabtree Labs of the MUHC-RVH. This included the overnight postabsorptive state followed by a liquid formula mixed-meal (Ensure®) with or without added LEU and measurements taken for 5 hours after the meal (postprandial). A timeline of the meal test on day 5 is shown in Fig. 2.

At 5:30 am after an overnight fast, subjects were weighed and completed their 24-hr urine collection, after which they were escorted from the CIU to the Crabtree Labs. At 8 am, a contralateral hand dorsal vein catheter was placed for blood sampling and a baseline blood sample was collected with their hand placed in a 65 ºC warming box to arterialize venous blood [217]. At 9:00 am, a primed (0.5 mg/kg) continuous infusion (0.008 $mg.kg^{-1}.min^{-1}$) of L-[1-^{13}C] LEU (^{13}C-LEU) was started through the same catheter. Infusion of the stable isotope ^{13}C-leu allowed for measurement of whole-body protein turnover and net protein balance.

The liquid mixed meal (Ensure®, Abbott Laboratory, St. Laurent, QC, Canada) consisted of 15% energy from protein, 24% from fat and 61% from carbohydrate. For all subjects the energy content of the meal on day 5 was kept constant for kg LBM (16 kcal/kg LBM), as measured by DXA. The meal had added to it either LEU (0.07 g/kg LBM) or ALA (0.0476 g/kg LBM), and was ingested 3 hours following initiation of ^{13}C-LEU infusion, at approximately 12:00 pm. ALA, a NEAA with no effect on protein metabolism was added to the non-LEU-rich meal to assure the two meals were isonitrogenous [187]. A bolus of ^{13}C-LEU (based on 4.1% ^{13}C-KIC mole percent enrichment) was given with the meal to maintain the ratio of labelled to unlabelled isotope stable based on previous pilot data, so that any change in enrichment of the isotope was due to meal induced changes in protein kinetics. Both AA powders were FDA-approved pharmaceutical grade (AjiPure™, Ajinomoto Amino Science LLC). Subjects were allowed to listen to music and watch movies throughout the day. At the end of the study, the catheters were removed and subjects were provided with dinner and acetaminophen to prevent any discomfort from the biopsies.

4.3.5 Data collection during the meal test

Blood samples were taken through the hand catheter every ½ hour throughout the day (see Fig. 2). Plasma and serum samples were stored at -80 ^{0}C for later measurement of serum insulin, plasma glucose, BCAA and incretins. REE was measured by indirect calorimetry for 20 minute periods; once just prior to the liquid meal (baseline measurement) and at the end of each of the 5 hours following the meal. Assuming that REE would not change if the meal was not ingested, TEF was calculated as the elevation of the REE measurements made after the meal over the baseline measurement [165, 218]. Appetite profile was evaluated using the validated 10 cm visual analog scale (VAS; Appendix 5) [219] four times throughout the day; once just before the meal and at 40 minutes, 1.5 and 5 hours after the meal. Breath samples were collected into a balloon, transferred to 10 mL Vacutainer tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) and stored at room temperature for later analysis of $\%^{13}CO_2$ enrichment.

4.3.6 Muscle biopsies (N = 6)

Two muscle biopsies were performed during day 5 and used to determine phosphorylation status of cell signalling components involved in protein translation pathways. The first baseline biopsy was taken just prior to the meal and the second one taken two hours after the meal (Fig. 2), since previous studies in humans suggest peak rates of MPS stimulated by feeding occur within 60-90 minutes [116-117]. Each time, a small tissue sample was taken from the *vastus lateralis* muscle of the thigh. After subcutaneous anaesthesia with 2 % lidocaine, the biopsy was performed (approximately 120 - 150 mg wet weight muscle tissue) using a 6 mm Bergström muscle biopsy needle (Popper & Sons, USA) under sterile conditions [220]. Visible fat and connective tissue was removed and the muscle sample immediately frozen in liquid nitrogen and stored at – 80 ^{0}C for later analysis [221]. Adhesive strips and a waterproof pressure bandage were used to close the incision and pressure applied by hand for 10 minutes to prevent bleeding and facilitate proper closure of the incision. In all, 6 of the 9 subjects studied (3 control and 3 sarcopenic) were able to provide muscle biopsies.

Additionally, one sarcopenic subject was unable to provide the postprandial biopsy during the LEU meal test, resulting in an n = 2 for the sarcopenic group during the LEU meal for immunoblot data.

4.4 Between the two meal studies

A 1 – 3 week washout period was taken between study 1 and study 2 to allow for blood cell replenishment and for all isotope tracer to be cleared from the body. When leaving the CIU after meal study 1, subjects were provided with a worksheet on which they were asked to keep a basic record of their activities throughout 5 consecutive days (3 weekdays and 2 weekend days). They were also provided with a triaxial accelerometer (RT3; Triaxial Research Tracker; Stay Healthy Inc., Monrovia, CA) which they were asked to wear on the hip at all times for these 5 days, except when bathing or sleeping, while going about their usual daily activities. This pager-like device measures movement on three axes to estimate physical activity by application of a formula to estimate the total daily energy expenditure, broken down into REE and energy expenditure from physical activity, derived from non-linear prediction models based on whole-room indirect calorimetry measurements [222]. The average physical activity energy expenditure over the 5 days was used to estimate and compare levels of physical activity between subjects.

4.5 Second Meal Study

After the washout period, participants returned for the second meal study, which was identical to the first study with the following exceptions. If the meal was enriched with LEU for study 1, ALA was given during the second meal study and vice versa. Because they were already measured, DXA and anthropomorphic measurements were not taken. To ensure that no major body compositional changes had occurred during the washout period, a repeat BIA was performed.

4.6 Background ^{13}C enrichment study

The Carbon-13 (^{13}C) isotope is present in nature and approximately 1.1% of all atmospheric CO_2 is $^{13}CO_2$ [223]. While we take this into account when calculating $^{13}CO_2$ enrichment in the breath, we do not know whether the meal affects background breath enrichment of $^{13}CO_2$ during the postprandial period of the study. The ^{13}C content of different energy sources varies and metabolic changes, such as those that occur after eating a meal, may also significantly alter background enrichment [224]. To test whether the Ensure® meal on the study day affected background $^{13}CO_2$ recovered in the breath, a study was performed to determine if the ingested diet was "carbon-13 neutral" [225]. One subject came to complete one meal study, following the usual special Ensure®-supplemented diet for days 1 - 4. During the meal test on day 5, blood and breath was collected as usual, to determine whether there was significant ^{13}C enrichment in plasma KIC and expired CO_2, despite the absence of ^{13}C-leu infusion. No ^{13}C-leu tracer was added to the meal, and there were no muscle biopsies or infusion of ^{13}C-leu tracer.

4.7 Data analyses

4.7.1 Twenty-four hour urine and blood parameters

Daily 24 hour N balance for days 3 and 4 of each meal study was calculated as: daily N intake (calculated by dietary g protein/6.25) minus daily N losses:

N balance = N intake – urinary N – [fecal + misc. N excretion] [38]

Urinary N included N content of urea and creatinine, which were measured by automated methods at the RVH biochemistry labs, while fecal and miscellaneous losses were estimated based on factors derived from previously measured subjects [38]. Urine collection was deemed complete and the special diet followed if daily urine creatinine was within 2 SD of the subject's mean value for days 3 and 4 of the study and N excretion stable [38]. Serum insulin and concentrations of plasma incretins (PYY_{3-36}, ghrelin) were detected by radioimmunoassay (RIA kits; Millipore, St. Charles, MO, USA). Plasma glucose was measured by the glucose oxidase method (GM7 Micro-Stat; Analox Instruments USA, Lunenberg, MA),

and total plasma BCAAs by an enzymatic fluorometric assay (spectrofluorometer; Turner model 430 equipped with a xenon lamp, Sequoia-Turner, Mountain View, CA), a method that has been validated against high-performance liquid chromatography in our lab [106].

4.7.2 Whole-body leucine kinetics using ^{13}C-Leu tracer

Rate of LEU oxidation (*O*), which is not dependent on a physiologic steady state, was calculated from $^{13}CO_2$ enrichment in the breath:

LEU *O* rate (μmol/kg·min) = F$^{13}CO_2$ · [1/^{13}C-KIC MPE - 1/99.1] · 100 [226-227]

Transamination is the first step in LEU oxidation, yielding α-ketoisocaproic acid (KIC). For postabsorptive kinetics, ^{13}C-KIC rather than ^{13}C-LEU itself is measured as the precursor LEU pool enrichment because LEU is only converted to KIC within the cell, and therefore plasma KIC-enrichment has been shown in validation studies to better indication intracellular LEU available for protein S [227-228].

Total *O* was calculated as the net AUC of LEU oxidation rate for the 5 hours following the meal. Since total LEU intake (*I*) was known (total LEU given in meal + total amount of ^{13}C-LEU infused), total net balance of LEU over the 5-hour postprandial period was calculated by:

LEU net balance (*g LEU*5 hrs*$^{-1}$) = Total *I* – Total *O* [229]

All ingested LEU was assumed to be absorbed into the free amino acid pool. ^{13}C-enrichment in plasma KIC, as measured in a t-butyldimethylsilyl derivative of α-hydroxyisocaproate, was measured in all blood samples from the meal test day by negative ion chemical ionization gas chromatography mass spectrometry (GC-MS), from the ratio of m+1:m_0 [230-231]. Collected breath samples were analyzed for $^{13}CO_2$-enrichment by isotope ratio mass spectrometry (IRMS; Micromass 903D; Vacuum Generators, Winsforce, UK). Retention factors were applied to correct for the fraction of $^{13}CO_2$ produced by ^{13}C-LEU oxidation that was not present in expired air. Factors of 0.67 during pre-meal fasting conditions and 0.80 for the two hours following the meal, decreasing in increments back down to 0.67 by 5 hours following the meal, were applied. These numbers were based on recovery studies performed during the fasting and fed

states in our lab [106] as well as studies from other groups under similar conditions of ingestion of a complete meal [225].

4.7.3 Immunoblot analysis for substrate activation

Western blot analysis was performed to measure total and phosphorylated amounts of AKT^{Ser473}, 4E-$BP1^{Ser65}$, $S6K1^{Thr389}$, $rpS6^{Ser235\text{-}6,\ Ser240\text{-}44}$, $PRAS40^{Thr246}$, and total RagC, all signalling molecules involved in translation initiation or elongation during protein synthesis [122] (Fig. 1). Primary antibodies were from Cell Signaling Technology (Beverly, MA). Substrate activity was measured at basal conditions and at 2 hours postprandial. Muscle tissue was homogenized, centrifuged and equal amounts of supernatant proteins (5 µg, measured by Bradford assay) extracted and loaded onto 8% or 12% gels. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transfer to polyvinulidene fluoride membrane (GE Healthcare, Baie D'Urfé, QC, Canada) was performed. Membranes were then incubated overnight at 4°C with phospho-specific primary antibodies, then washed in Tris buffered saline with Tween, followed by incubation with secondary antibody (GE Healthcare) in Tris buffered saline with Tween containing 5% nonfat dried milk for 1 hour at room temperature. Signals were detected by enhanced chemiluminescence reagents (GE Healthcare) and analyzed by Chemi-DOC XRS system (Bio-Rad, Mississauga, ON, Canada). After detection of the phospho-specific signal, the antibodies were stripped off the membrane. The membranes were washed, blocked, and re-probed with primary antibodies that recognized the proteins irrespective of their phosphorylation state. The densities of phosphorylated signals were normalized to total protein levels.

4.7.4 Statistical analyses

Baseline subject characteristics were compared between sarcopenic and control groups by two-tailed independent t-tests. Pearson's correlation coefficient (r) was used to determine the relationship between variables. Peak values and AUC of the postprandial responses between meal studies were compared by two-tailed paired t-tests for the effect of LEU (ie. differences between ALA and LEU meals), and two-tailed independent t-tests for the effect of sarcopenia (ie. differences between sarcopenic and control groups). To determine the response of

variables to the mixed meal, two way repeated measures ANOVA was performed, with LEU (ie. ALA vs. LEU meal) and meal (ie. time) as main factors. When ANOVA revealed significant effects, pairwise differences were determined using paired and independent t-tests. Values are expressed as mean ± SEM and differences considered significant when $p < 0.05$. All analyses were performed using PASW version 18 software (SPSS Inc., Chicago, IL).

5 RESULTS

5.1 Subject characteristics

Subjects' body compositional characteristics are shown in Table 1. Groups did not differ in age, height, fat mass (total or percentage) or REE when normalized to LBM. Sarcopenic subjects did have a lower weight, and therefore a lower absolute but not %LBM (Table 1 and Fig. 3). Sarcopenic subjects had a lower AMMI and thus lower MMI (calculated from ASM). This appeared to be due to their lower leg lean mass and smaller thigh circumference (which translated into a lower leg lean/ht^2 ratio; $p < 0.01$, results not shown), since groups did not differ in arm or trunk lean mass (Table 1). When LBM was correlated with measures of muscle strength and physical function, hand-grip strength, but not TUG or gait speed, was positively correlated with LBM ($r = 0.702$; $p < 0.05$, data not shown). Accelerometer data indicated the 5-day-average of physical activity-related energy expenditure (ie. PAEE = Total daily EE – REE – TEF) in the sarcopenic group was nearly half that of the controls.

Clinical and dietary subject characteristics are shown in Table 2. Both groups showed similar fasting and 120-min. OGTT plasma glucose. Average energy and protein intake per kilogram LBM per day was the same for both groups during the two days of weight-maintaining diet. As well, nitrogen balance and creatinine excretion (data not shown) were constant, indicating complete urine collections and adherence to the special diet, and both groups were in equivalent nitrogen balance (Table 2) during both studies. Baseline albumin, CRP, and growth hormone did not differ between groups.

5.2 Background study

Results from the background enrichment study showed a nominal effect of the Ensure diet on background ^{13}C enrichment in exhaled CO_2, equivalent to approximately 16% of the total $^{13}CO_2$ enrichment during each test meal. This minimal amount of enrichment did have a small effect on calculations of leucine kinetics, however because both the ALA and LEU meal studies were subject to

the same increase in enrichment, it did not affect any comparison in leucine kinetics between the two meals (data not shown).

5.3 Meal responses

5.3.1 Whole-body LEU kinetics (Figs. 4a – b)

Since amount of LEU added to the LEU-rich meal was based on LBM, by design, sarcopenic subjects' absolute LEU intake was lower then control subjects during the LEU-rich meal (data not shown), however when expressed as grams per kg LBM, LEU intake did not differ between groups (Fig. 4a). Repeated measures ANOVA yielded a significant meal by LEU interaction ($p < 0.0001$), but no effect of sarcopenia on LEU oxidation rate (Fig. 4b). Regardless of meal or group, LEU oxidation rate did not decrease back to baseline by 5-hours ($p < 0.01$; Fig. 4b). Postprandial oxidation rates over time of the meal (Fig. 4b), total 5-hour postprandial net AUC LEU oxidation (data not shown) and total net LEU balance (Fig. 4a) were greater after the LEU-rich meal, however there were no differences between groups in response to each meal. Largely because net LEU balance during the LEU meal was correlated with LBM ($r = 0.744$, $p < 0.05$) (results not shown), the same results were found when balance was expressed per kg/LBM (Fig. 4a).

5.3.2 Blood hormones and substrates (Figs. 5 – 9)

Responses of insulin and C-peptide are shown in Figs. 5 - 6. Repeated measures ANOVA revealed significant main effects of meal ($p < 0.0001$) and LEU ($p < 0.05$), but not sarcopenia, on serum insulin (Fig. 5a) and C-peptide ($p < 0.0001$ and $p < 0.05$) (Fig. 6a) levels, with no significant interactions. Insulin increased to peak levels between 45 - 90 minutes following the meal and returned to baseline values by 5 hours (Fig. 5a). Both insulin and C-peptide responses to the ALA-rich meal did not differ between control and sarcopenic groups, either at individual time points or in the total AUC response. Peak insulin values did not differ between meal or group (Fig. 5a), however peak C-peptide levels in the sarcopenic group, occurring at 120 min., were significantly higher than those of the control group, occurring at 90 min., during the LEU-rich meal (Fig. 6a). With

the LEU-rich meal, 5-hour AUC responses of insulin (Fig. 5b) and C-peptide (Fig. 6b) were greater than the ALA meal in the sarcopenic group.

Glucose response is shown in Fig. 7. There was a main effect of meal ($p < 0.0001$), but no effect of LEU or sarcopenia, and no significant interactions. Glucose increased with the meal, reaching peak values between 60 - 90 minutes postprandial. There were no differences between groups or between meals in the glucose net AUC 5-hour response (data not shown). By 5 hours postprandial, levels decreased back to baseline in the control group, and to below baseline in the sarcopenic group for both the LEU (5.0 ± 0.1 vs. 3.6 ± 0.2 mmol/L, $p < 0.01$) and ALA (4.9 ± 0.1 vs. 4.1 ± 0.3 mmol/L, $p < 0.05$) meal (Fig. 7).

Plasma BCAA is shown in Figs. 8a-b. ANOVA showed significant main effects of meal ($p < 0.0001$) and LEU ($p < 0.0001$), with a meal by LEU interaction ($p < 0.01$), but no effect of sarcopenia on plasma BCAAs, which increased to peak values between 30 – 45 minutes postprandial. Peak values in response to the LEU meal were significantly higher in both groups (Fig. 8a) and 5-hour net AUC (Fig. 8b) in response to the LEU meal was significantly greater in the sarcopenic group and close to significantly greater in the controls (AUC: 63.3 ± 13.4 vs. 38.9 ± 5.5 mmol/L, $p = 0.063$).

FFA levels ($n = 2$ for sarcopenic group), shown in Fig. 9, decreased upon ingestion of the meal reaching nadir values between 60 – 150 minutes postprandial and remained suppressed at 5 hours after meal in both groups. Serum triglycerides increased by 180 min. postprandial in both groups during both meals, however triglycerides were more elevated in the sarcopenic group, regardless of meal (data not shown). Total cholesterol, HDL and LDL levels attained a nadir by 180 min. postprandial and, though they showed a trend toward increasing, they remained significantly below baseline at 5 hours (data not shown).

5.3.3 Muscle intracellular signalling activity (Figs. 10a – 14b)

In the control group, phosphorylation status of AKT, expressed as p-AKT/total AKT ratio, increased from postabsorptive (PA) to posptprandial (PP) states during both the ALA and LEU meals, at the Ser473 (ALA: $p < 0.05$; LEU: $p < 0.01$) site, while in the sarcopenic group, there was no significant change in p-

AKT^{Ser473} in response to either meal. Phosphorylation status of AKT^{Thr308} did not significantly change in either group in response to either meal (NS). When the differences between the average PA to PP (ie. the changes induced by meal) were compared, there were no significant differences in phosphorylation status of AKT between any group-by-meal combination at either the Ser473 or Thr308 site (Fig. 10a and 10b).

Phosphorylation status of PRAS40 at the Thr246 site was significantly increased in response to both meals in the control group ($p < 0.05$ for both meals), while p-PRAS40^{Thr246} status did not significantly change in response to the ALA meal and showed a trend to increased in response to the LEU meal ($p = 0.06$) in the sarcopenic group (data not shown). When the average PA to PP changes in p-PRAS40^{Thr246}/total PRAS40 ratio were compared, the change in the sarcopenic group was significantly greater than and nearly 2-fold that of the control group in response to the LEU meal (0.89 ± 0.09 vs. 0.44 ± 0.07; $p < 0.05$) (Fig. 11a). p-PRAS40 at Ser183 was not significantly different between PA and PP states in either group during either meal, however in the sarcopenic group, the average increase in response to the LEU meal showed a trend to be greater than the increase in response to the ALA meal (0.97 ± 0.20 vs. 0.56 ± 0.50, $p = 0.056$; Fig. 11b).

Like p-PRAS40, in the control group only, both the LEU and ALA meal resulted in a significant increase in p-4E-BP1^{Ser65}/total 4E-BP1 ratio ($p < 0.05$), while p-4E-BP1^{Ser65} status did not significantly change in response to the ALA meal and showed a trend to increased in response to the LEU meal ($p = 0.077$), in the sarcopenic group (data not shown). When the average PA to PP changes in p-4E-BP1^{Ser65}/total 4E-BP1 ratio were compared, there were no significant differences between groups or between meals for the change in 4E-BP1 phosphorylation status (Fig. 12).

In the control group, neither the ALA nor LEU meal had an effect on phosphorylation of S6K1^{Thr389} (data not shown). The average increase in p-S6K1^{Thr389}/total S6K1 ratio for the sarcopenic group showed a trend to be greater

than that of the control group during the ALA meal (Sarc: 1.12 ± .024 vs. Cont: 0.43 ± 0.13; p = 0.064) (Fig. 13).

In the sarcopenic group, p-rpS6$^{Ser240-44}$/total rpS6 ratio significantly increased from PA to PP during the LEU meal (PA: 0.11 ± 0.08 vs. PP: 0.86 ± 0.15; p < 0.05) and showed a trend to increase during the ALA meal (PA: 0.11 ± 0.06 vs. PP: 0.47 ± 0.14; p = 0.057). In the control group, neither meal resulted in a significant change in p-rpS6$^{Ser240-44}$/total rpS6 ratio from PA to PP (data not shown). When the average PA to PP changes in p-rpS6$^{Ser24-044}$/total rpS6 ratio were compared, there was a trend for a greater change in the sarcopenic group during the ALA meal (Fig. 14a). At the Ser253-6 site, neither groups showed a significant PA to PP change in p-rpS6$^{Ser253-6}$/total rpS6 ratio during either meal and no significant differences between groups or meal in average change from PA to PP (Fig. 14b). Total PA levels of intracellular Rag C did not differ between groups, nor did they change in response to either the LEU or ALA meal (data not shown).

5.3.4 Thermic effect of food and respiratory quotient (Figs. 15 – 16)

TEF in response to both meals is shown in Fig. 15. Repeated measures ANOVA showed a main effect of meal (p < 0.05), but no effect of LEU or sarcopenia. Peak values occurred between 50 – 150 minutes after the meal. Total postprandial AUC did not differ regardless of group or meal (data not shown).

There was a main effect of meal (p < 0.0001), as well as a meal by sarcopenia (p < 0.01) and meal by LEU (p < 0.05) interaction on postprandial RQ, shown in Fig 16. From 40 – 160 minutes postprandial, the sarcopenic group displayed higher RQ's after both the LEU and ALA meals. Regardless of group or meal, RQ increased to peak values at 40 min. postprandial. RQ decreased back to baseline by 5 hours after the LEU meal in both groups, but remained elevated above baseline levels at 5 hours after the ALA meal in both groups (sarc: 0.82 ± 0.02 vs. 0.79 ± 0.01; p< 0.05; cont: 0.84 ± 0.01 vs. 0.76 ± 0.01; p < 0.01) (Fig. 16).

5.3.5 Appetite profiles (VAS) (Fig. 17)

Appetite profiles of hunger and fullness are shown in Fig. 17. A significant effect of meal on VAS scores of hunger ($p < 0.0001$) and fullness ($p < 0.0001$), with a meal by LEU interaction for scores of hunger ($p < 0.05$) was found. Feelings of hunger decreased following the meal and increased back to baseline, or above baseline in controls (ALA: 31.8 ± 10.5 vs. 63.9 ± 11.1mm; $p < 0.05$) by 5 hours, and feelings of fullness followed the opposite pattern. When data were expressed as the change in VAS score from baseline, significant effects of meal ($p < 0.01$) and LEU ($p < 0.05$) were found (data not shown). This was due to the fact that control subjects unexpectedly reported being less hungry before the ALA meal (31.8 ± 10.5 vs. 58.8 ± 8.4; $p < 0.01$), however the meal suppressed their appetite to the same extent in both meals (Fig. 17).

5.3.6 Incretins (Figs. 18 – 19)

There was an effect of meal ($p < 0.0001$) but not LEU or sarcopenia on active ghrelin levels, shown in Fig. 18. In both groups, active ghrelin decreased after both LEU and ALA meal ingestion, reaching nadir values at 60 – 120 minutes postprandial. Net 5-hour AUC response did not differ regardless of group or meal (data not shown). Like ghrelin, there was no effect of LEU or sarcopenia on active PYY ($n = 2$ for sarcopenic group) (Fig. 19). PYY_{3-36} increased with the meal and remained elevated above baseline ($p < 0.05$) 5-hours after all but the LEU-rich meal in the sarcopenic group (Fig. 19).

Table 1. Subject characteristics: body composition and function

Group	Sarcopenic	Control
N	**5**	**4**
Age (y)	70.2 ± 3.4	70.0 ± 4.1
Height (cm)	158.2 ± 1.1	160.6 ± 5.3
Weight (kg)	56.5 ± 2.5 *	63.9 ± 0.8
Body mass index (kg/m2)	22.6 ± 1.0	24.9 ± 1.5
Lean body mass (kg)	34.7 ± 0.4 *	39.4 ± 2.1
Lean body mass (%)	61.8 ± 2.5	61.7 ± 3.2
Total leg lean mass (kg)	10.9 ± 0.2 *	13.2 ± 0.8
Thigh circumference (cm)	53.6 ± 2.1 *	59.9 ± 0.6
Total arm lean mass (kg)	3.3 ± 0.1	3.9 ± 0.4
Trunk lean mass (kg)	17.6 ± 0.5	19.3 ± 1.1
Appendicular skeletal muscle mass (kg)	14.2 ± 0.2 *	17.1 ± 1.1
Appendicular muscle mass index (kg/m^2)	5.68 ± 0.13 **	6.60 ± 0.13
Total muscle mass index (kg/m^2)	6.35 ± 0.15 **	7.45 ± 0.16
Body fat (kg)	20.0 ± 2.4	23.0 ± 2.2
Body fat (%)	34.9 ± 2.8	36.1 ± 3.6
Resting energy expenditure (kcal/d)	1066 ± 21	1183 ± 52
Resting energy expenditure (kcal/kgLBM/d)	30.7 ± 0.2	30.1 ± 0.9
Physical activity energy expenditure (kcal/d)	248 ± 37 **	604 ± 73
Timed Up and Go (sec)	9.0 ± 1.3	8.3 ± 0.6
Gait speed (meters/sec)	1.2 ± 0.1	1.3 ± 0.3
Hand-grip strength (kg force)	19.3 ± 2.4	21.5 ± 5.3

Values are mean ± SE, body compositional data from DXA measurement, * $p < 0.05$ and ** $p < 0.01$ significant difference from control

Table 2. Subject characteristics: clinical and dietary

Group	Sarcopenic	Control
N	**5**	**4**
Fasting glucose (mmol/L)	4.8 ± 0.3	4.7 ± 0.3
OGTT (120 min. glucose, mmol/L)	7.6 ± 0.6	6.1 ± 0.8
Albumin (g/L)	40.8 ± 0.7	40.5 ± 0.9
Growth hormone (μg/L)	0.88 ± 0.56	2.80 ± 1.34
CRP (mg/L)	1.1 ± 0.4	0.6 ± 0.2
TSH (mIU/L)	2.34 ± 0.57	1.88 ± 0.46
FT4 (pmol/L)	9.7 ± 0.6 *	11.7 ± 0.6
Average daily Nitrogen balance (g/d)	2.21 ± 0.52	1.69 ± 0.48
Average intake during controlled diet		
Energy (kcal/kg LBM·d)	52.6 ± 3.2	48.8 ± 2.8
Protein (g/kg LBM·d)	1.98 ± 0.15	1.89 ± 0.08
Test meal intakes		
Energy (mean of LEU and ALA meal)		
Kcal	558.3 ± 2.5 *	628 ± 21.6
kcal/kg LBM	16.11 ± 0.11	15.93 ± 0.02
kcal/kg body weight	9.95 ± 0.29	9.83 ± 0.32
Protein (mean of LEU and ALA meal)		
G	21.0 ± 0.1	23.6 ± 0.8
g/kgLBM	0.61 ± 0.00	0.60 ± 0.00
g/kg body weight	0.37 ± 0.01	0.37 ± 0.01
Leucine		
Leucine-rich meal		
G	4.44 ± 0.03 *	4.99 ± 0.26
g/kg LBM	0.13 ± 0.00	0.13 ± 0.00
g/kg body weight	0.08 ± 0.00	0.08 ± 0.00
Alanine-rich meal		
G	1.98 ± 0.01 *	2.23 ± 0.12
g/kg LBM	0.06 ± 0.00	0.06 ± 0.00
g/kg body weight	0.04 ± 0.00	0.04 ± 0.00

Values are mean ± SE, * $p < 0.05$ significant difference from control

Figure 1. mTORC1 signalling pathway for protein synthesis

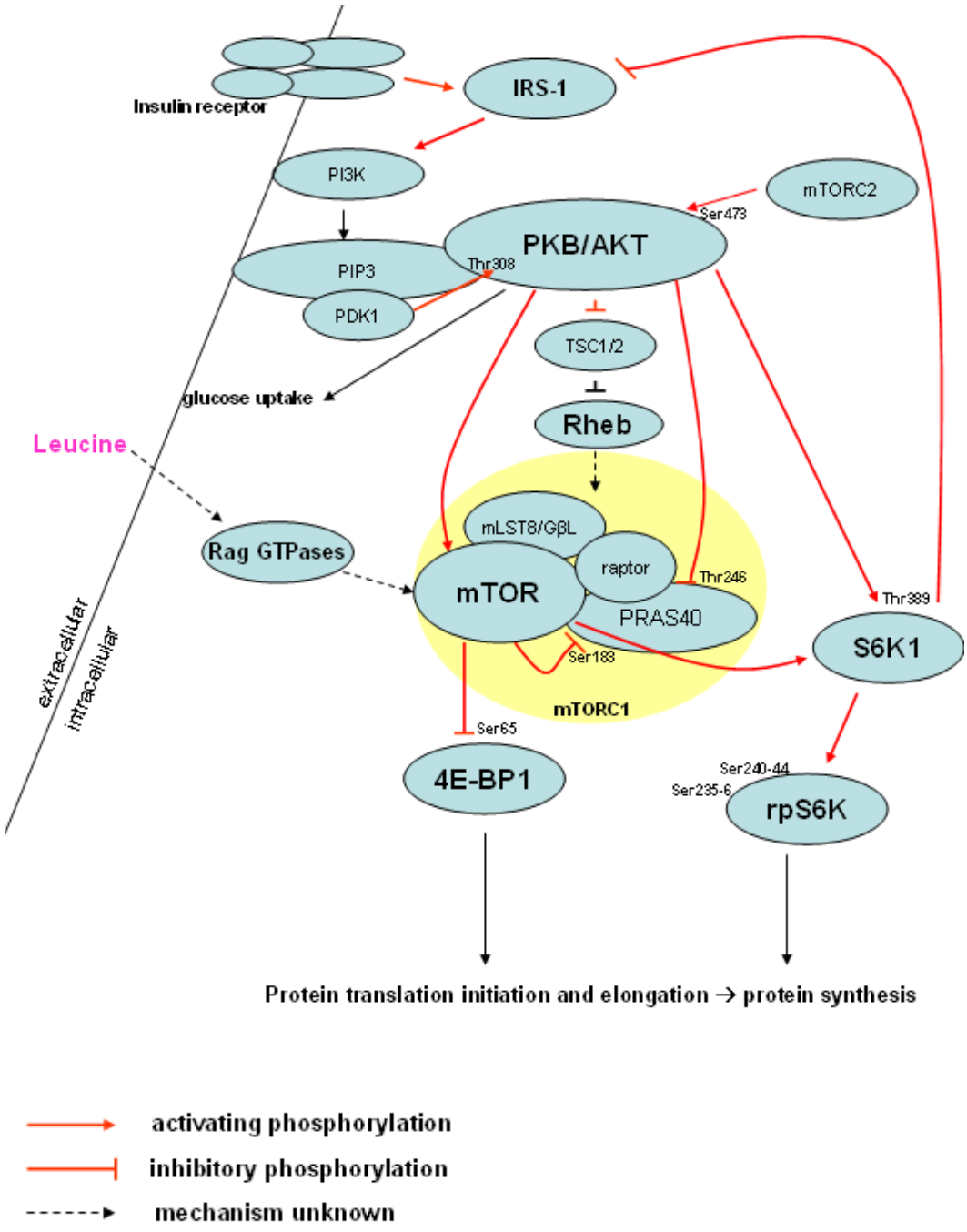


Figure 2. Meal test protocol

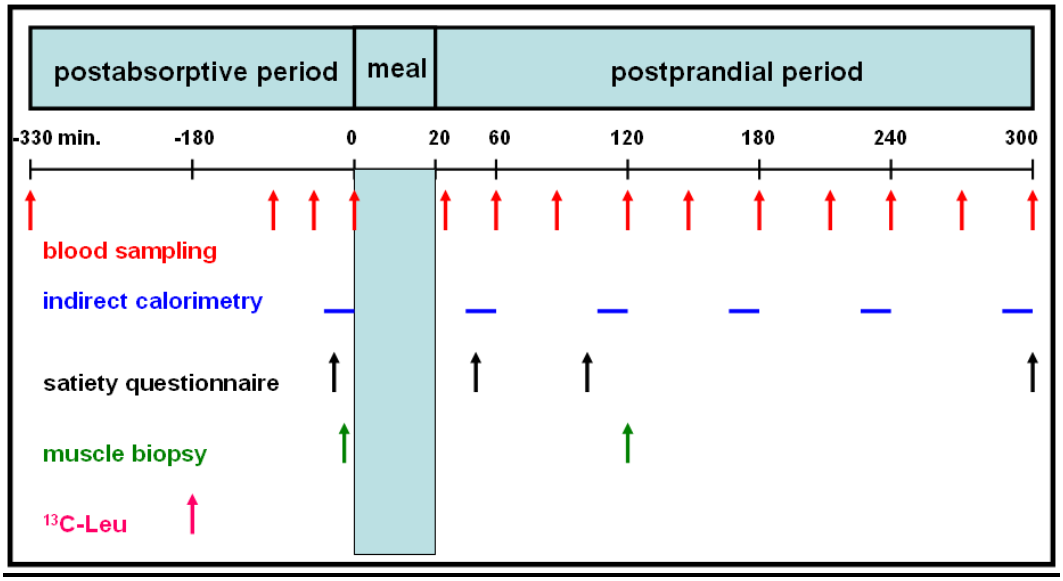
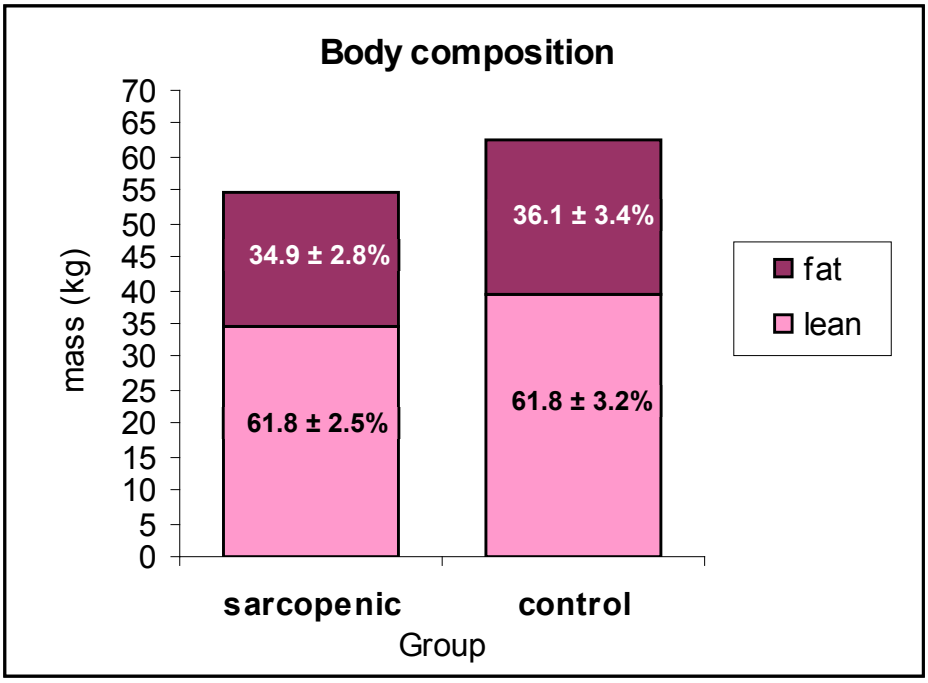
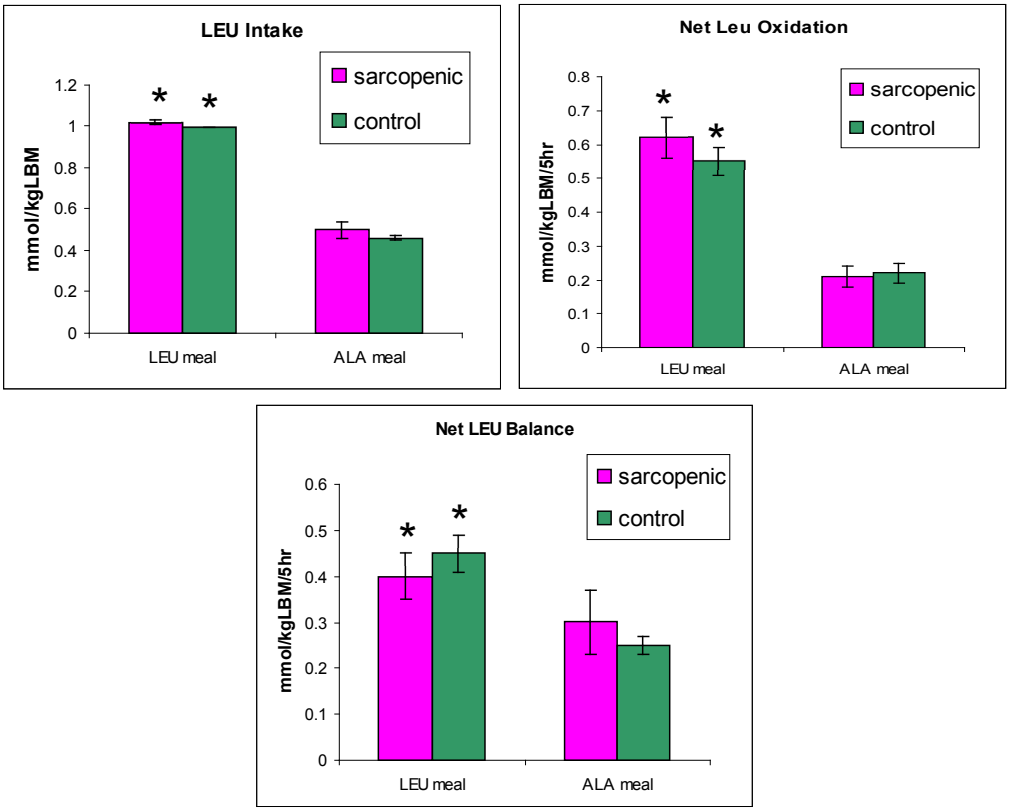


Figure 3. Subjects' body composition



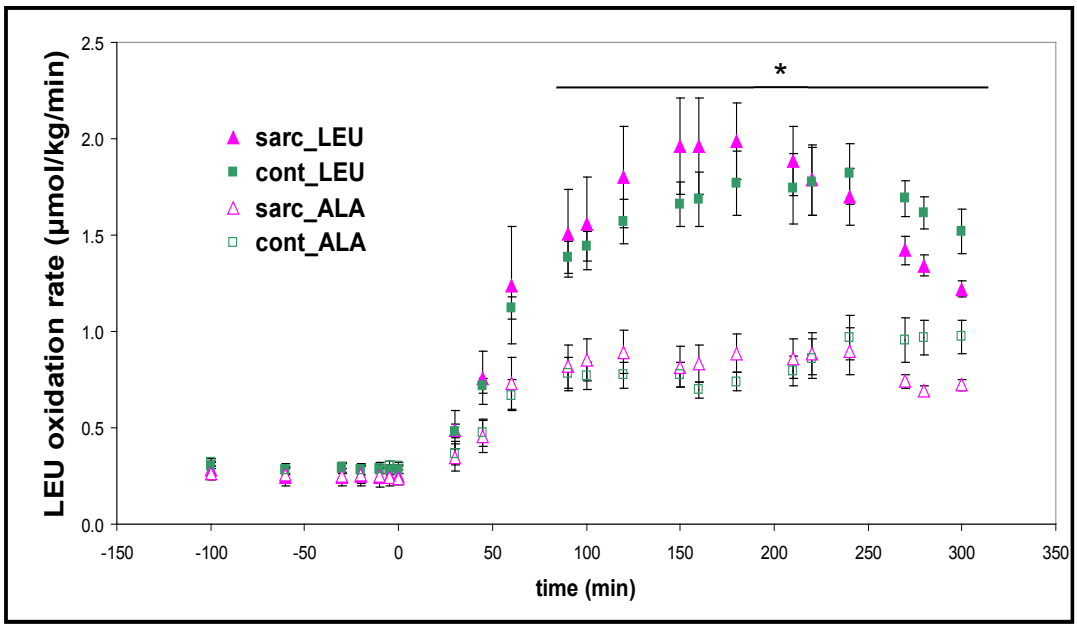
% fat and lean body masses expressed as mean ± SE. Absolute weight and LBM were different.

Figure 4a. Total leucine intake, total oxidation and 5-hour net balance



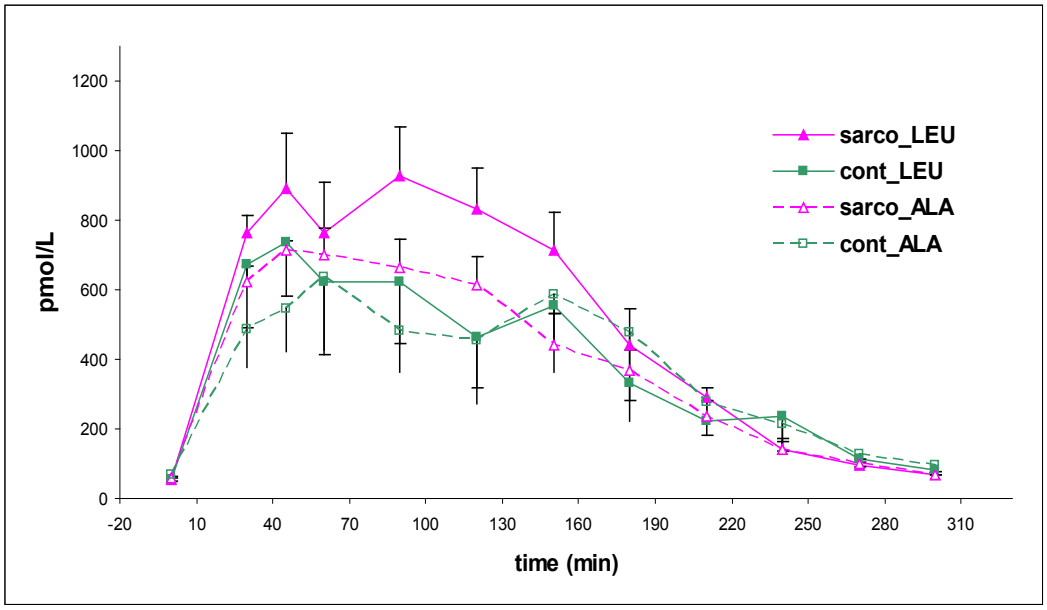
$p < 0.05$ difference from ALA meal

Figure 4b. Leucine oxidation rate in response to the meal



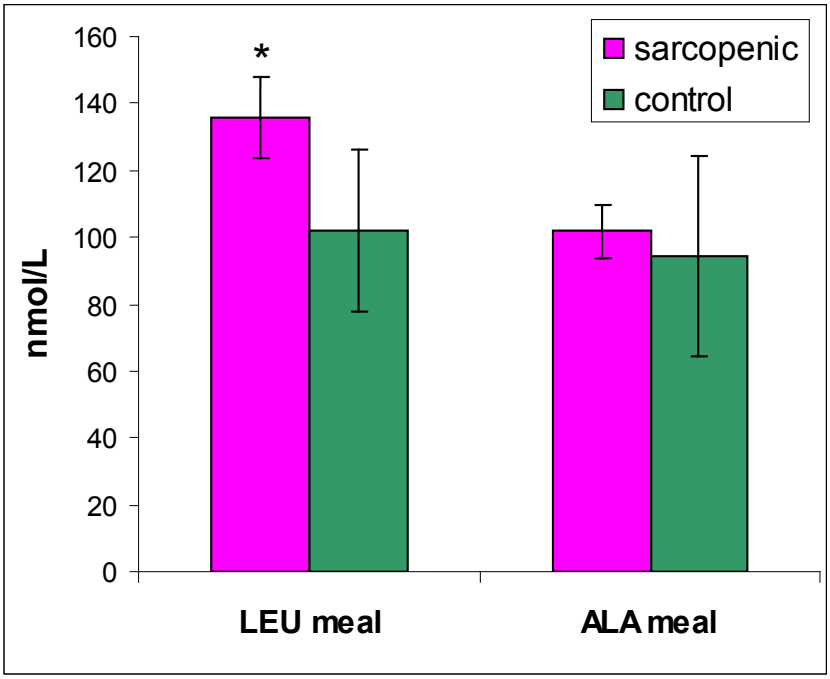
* $p < 0.05$ difference from ALA meal; ** $p < 0.01$ difference from baseline

Figure 5a. Serum insulin meal response



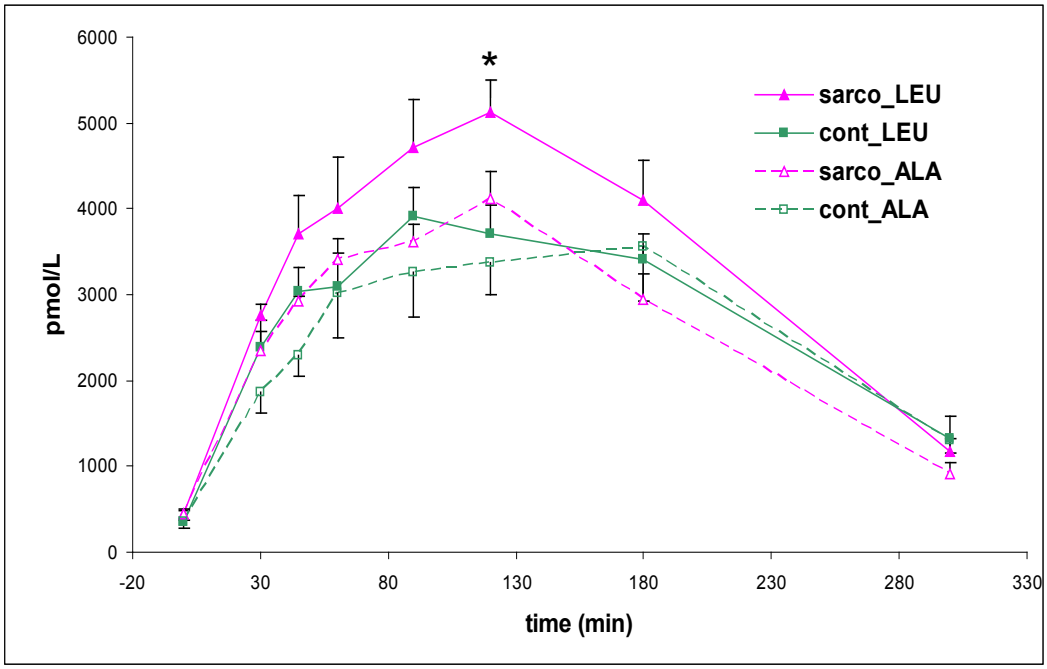
Repeated measures ANOVA showed significant main effects of meal ($p < 0.0001$) and LEU ($p < 0.05$)

Figure 5b. Net 5-hr AUC of insulin meal response



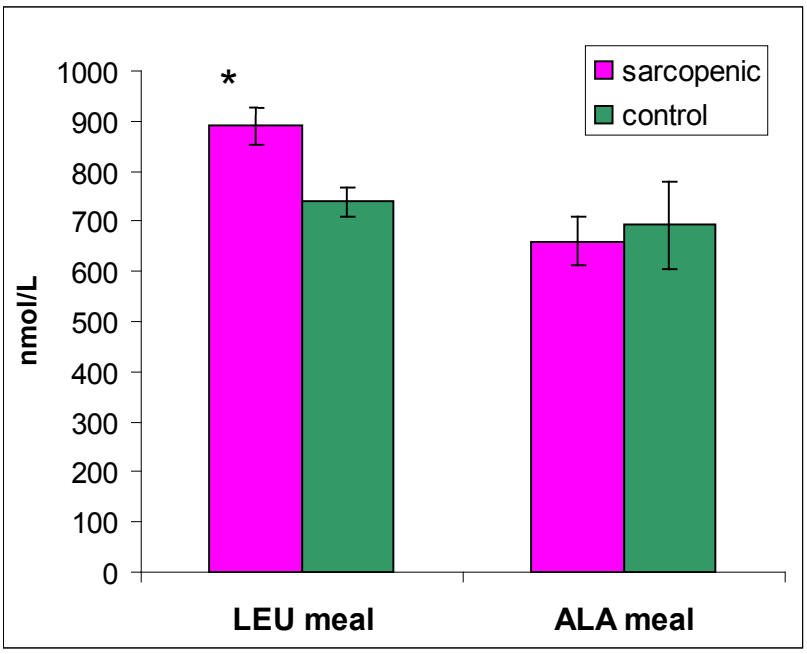
* $p < 0.05$ difference from ALA meal

Figure 6a. C-peptide meal response



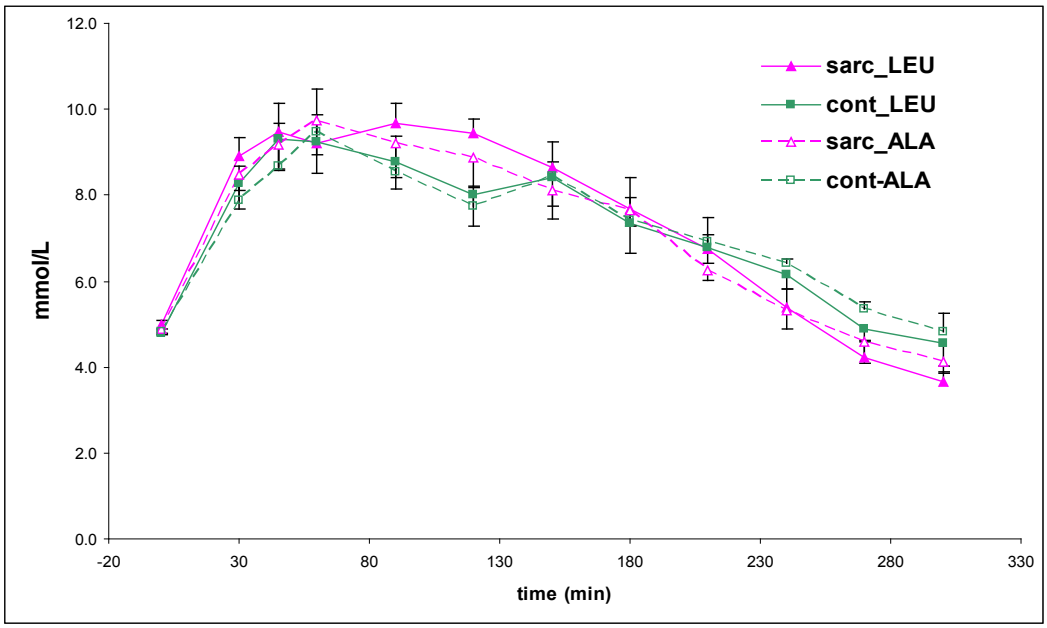
Repeated measures ANOVA showed significant main effects of meal ($p < 0.0001$) and LEU ($p < 0.05$); * $p < 0.05$ difference between peak values of sarco_LEU and cont_LEU

Figure 6b. Net 5-hour AUC of C-peptide meal response



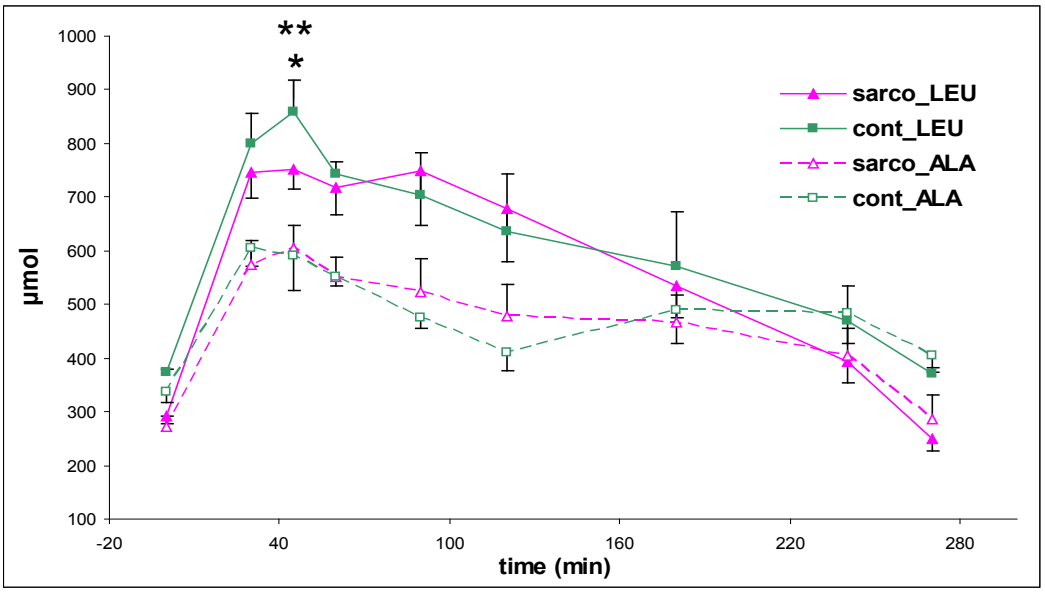
* $p < 0.05$ difference from ALA meal

Figure 7. Plasma glucose meal response



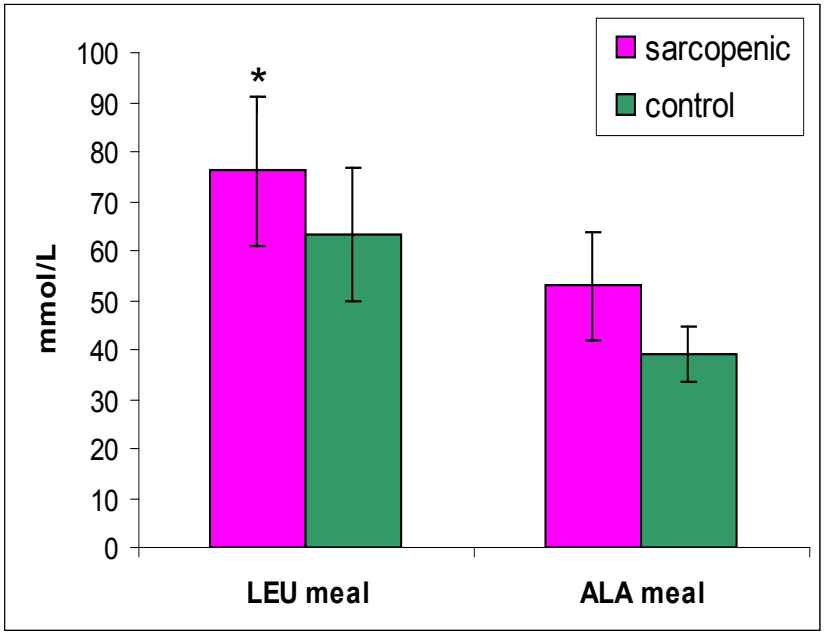
Repeated measures ANOVA showed a significant main effects of meal only ($p < 0.0001$)

Figure 8a. Plasma BCAA meal response



Repeated measures ANOVA showed significant main effects of meal ($p < 0.001$) and LEU ($p < 0.0001$) and a meal x LEU interaction ($p < 0.01$); * $p < 0.01$ difference between sarco_LEU and sarco_ALA peak values at 40 min. PP; **$p < 0.05$ difference between cont_LEU and cont_ALA peak values at 45 and 30 min. PP respectively

Figure 8b. Net 5-hour AUC of BCAA meal response



* $p < 0.05$ difference between sarco_LEU and sarco_ALA; trend ($p = 0.06$) for difference between cont_LEU and cont_ALA

Figure 9. Plasma free fatty acids meal response

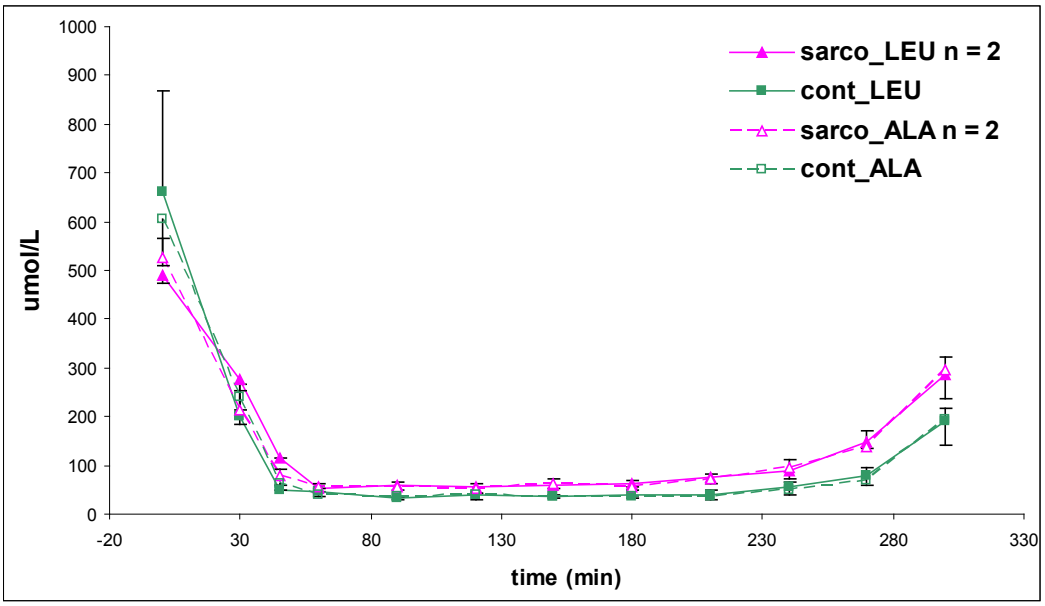
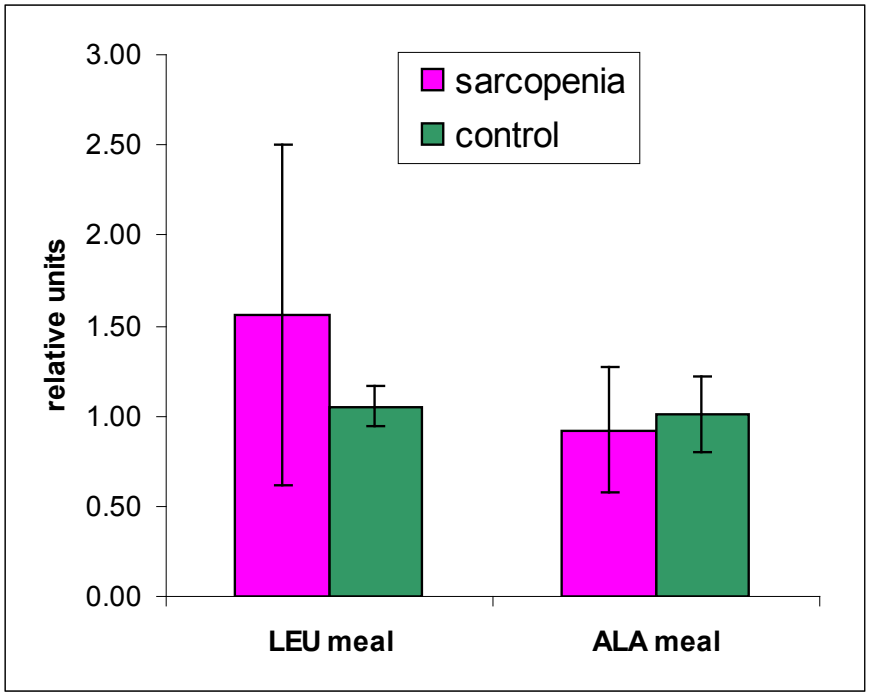
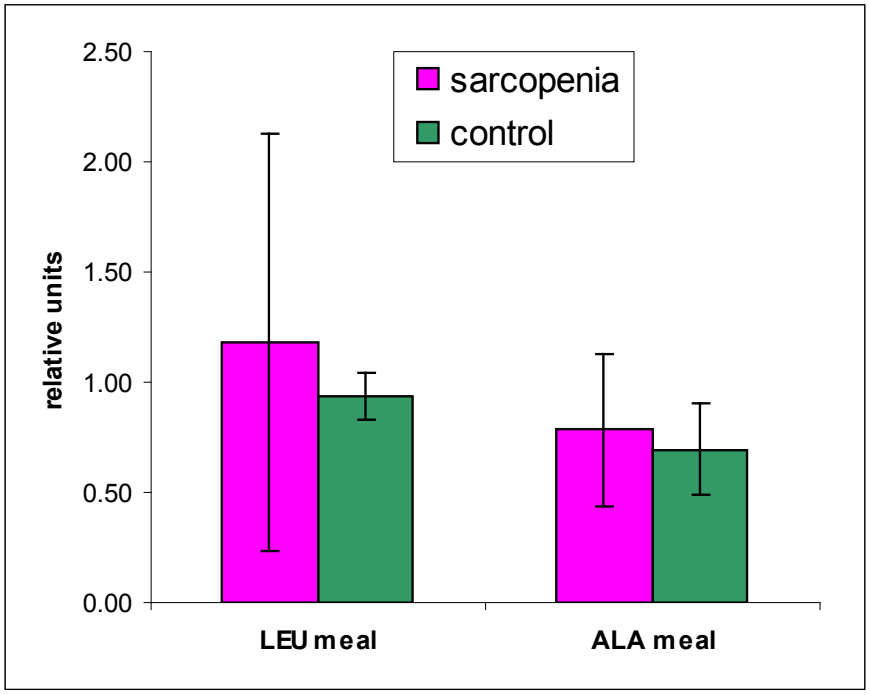


Figure 10a. Change in p-AKT^{Ser473}/Tot AKT ratio from PA to PP



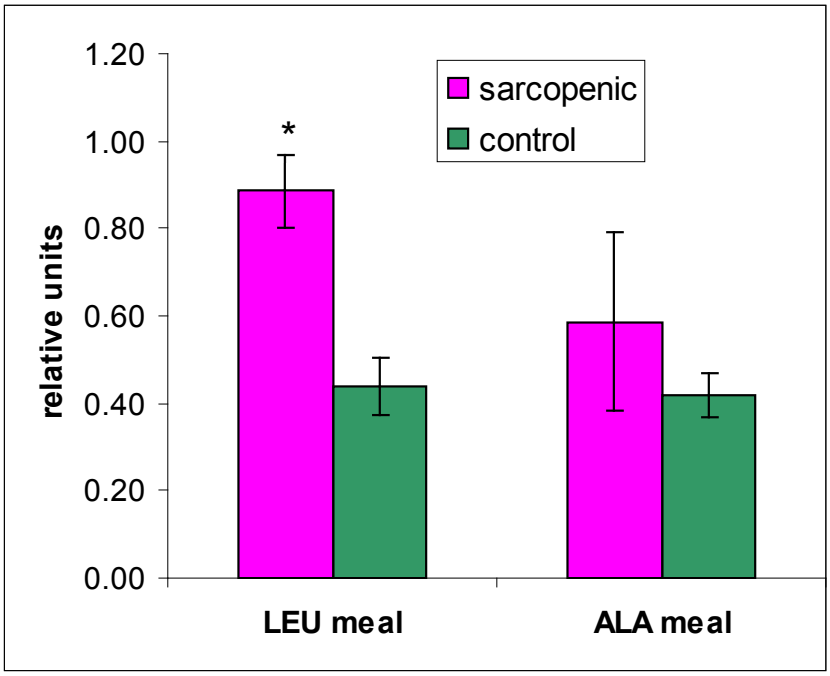
n = 3 for cont_LEU, cont_ALA and sarco_ALA; n = 2 for sarco_LEU group

Figure 10b. Change in p-AKT^{Thr308}/Tot AKT ratio from PA to PP



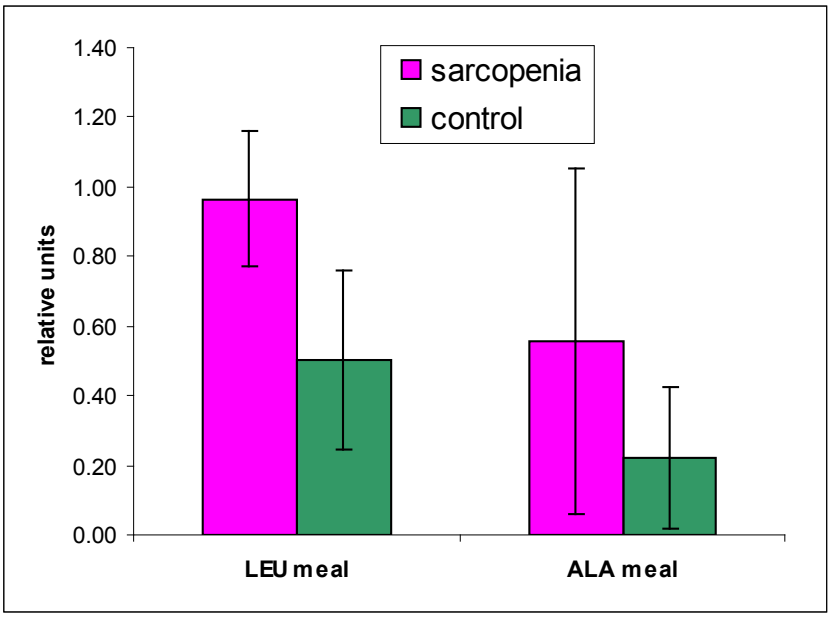
n = 3 for cont_LEU, cont_ALA and sarco_ALA; n = 2 for sarco_LEU group

Figure 11a. Change in p-PRAS40^{Thr246}/Tot PRAS40 ratio from PA to PP



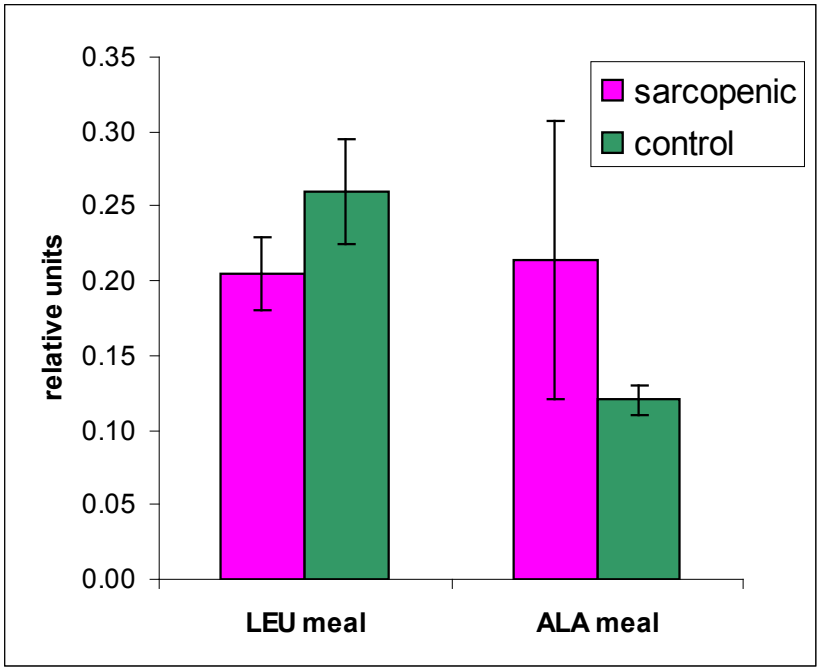
* p < 0.05 difference from control LEU; n = 3 for cont_LEU, cont_ALA and sarco_ALA; n = 2 for sarco_LEU group

Figure 11b. Change in p-PRAS40^{Ser183}/Tot PRAS40 ratio from PA to PP



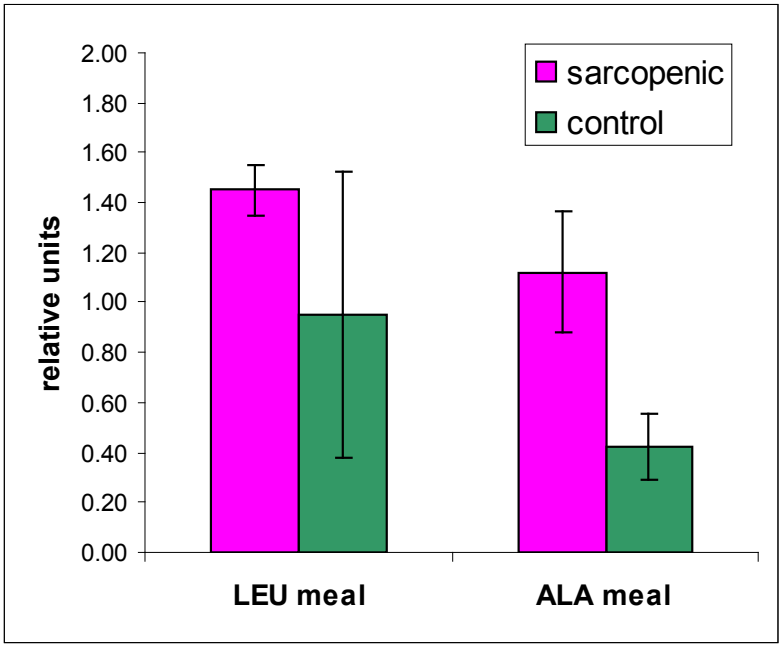
trend (p = 0.056) for difference between LEU and ALA in sarcopenic group; n = 3 for cont_LEU, cont_ALA and sarco_ALA; n = 2 for sarco_LEU group

Figure 12. Change in p-4E-BP1^{Ser65}/Total 4E-BP1 ratio from PA to PP



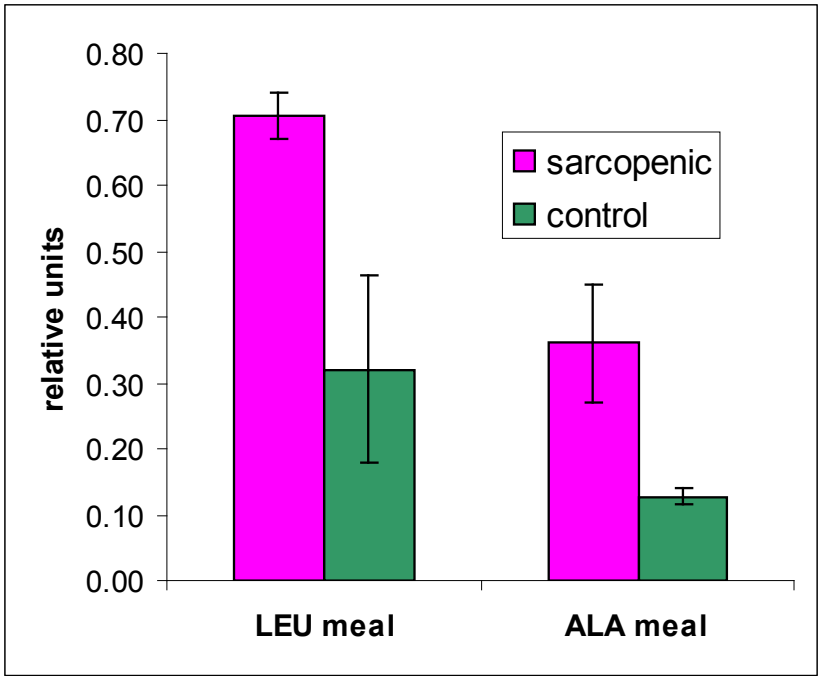
n = 3 for cont_LEU, cont_ALA and sarco_ALA; n = 2 for sarco_LEU group

Figure 13. Change in p-S6K1^{Thr389}/Total S6K1 ratio from PA to PP



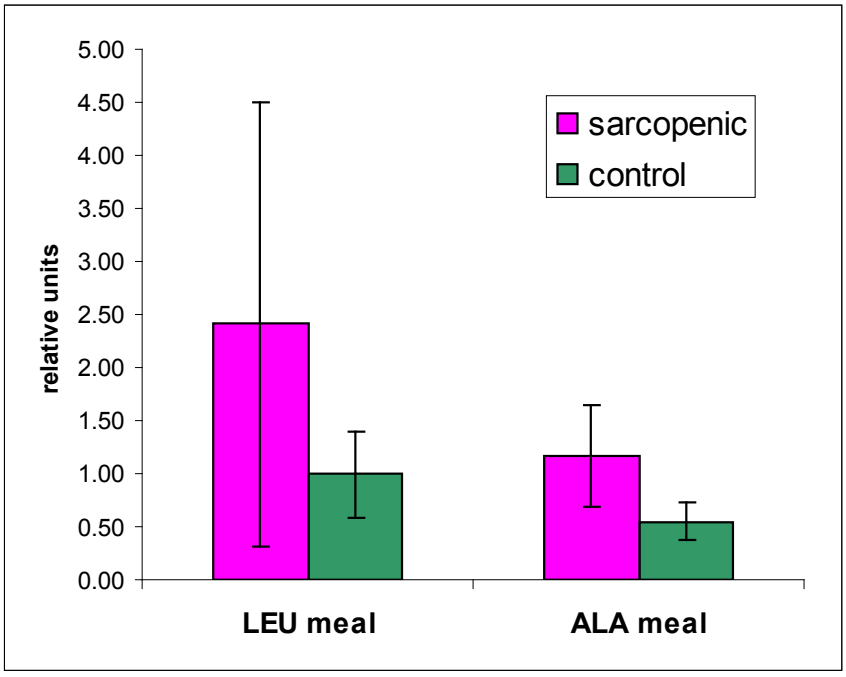
trend (p = 0.064) for difference between sarcopenic and control groups during ALA meal;
n = 3 for cont_LEU, cont_ALA and sarco_ALA; n = 2 for sarco_LEU group

Figure 14a. Change in p-rpS6$^{Ser240-44}$/Total rpS6 ratio from PA to PP



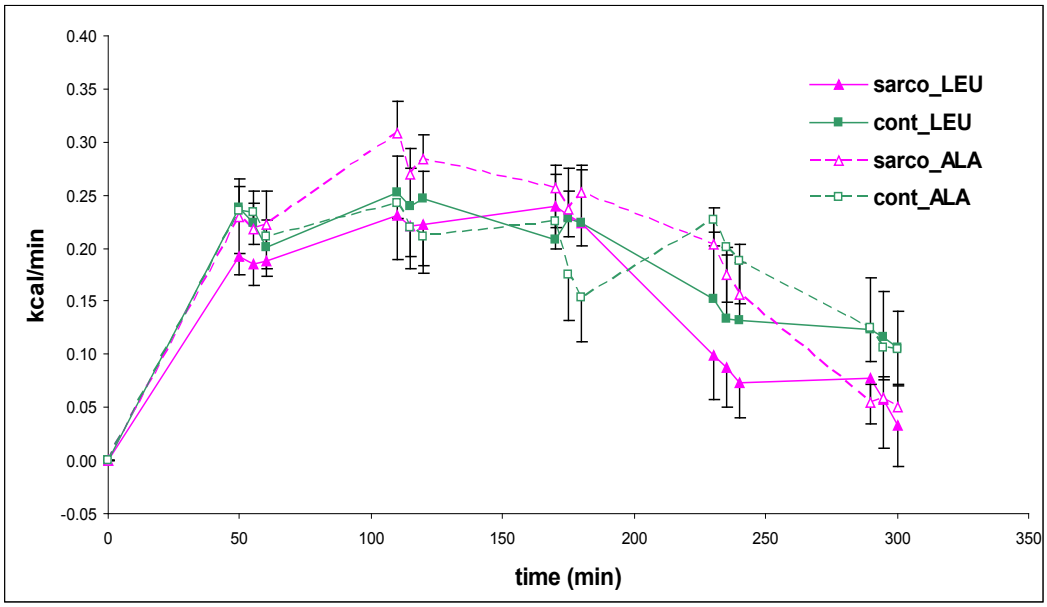
trend (p = 0.061) for difference between sarcopenic and control during ALA meal; n = 3 for cont_LEU, cont_ALA and sarco_ALA; n = 2 for sarco_LEU group

Figure 14b. Change in p-rpS6$^{Ser235-6}$/Total rpS6 ratio from PA to PP



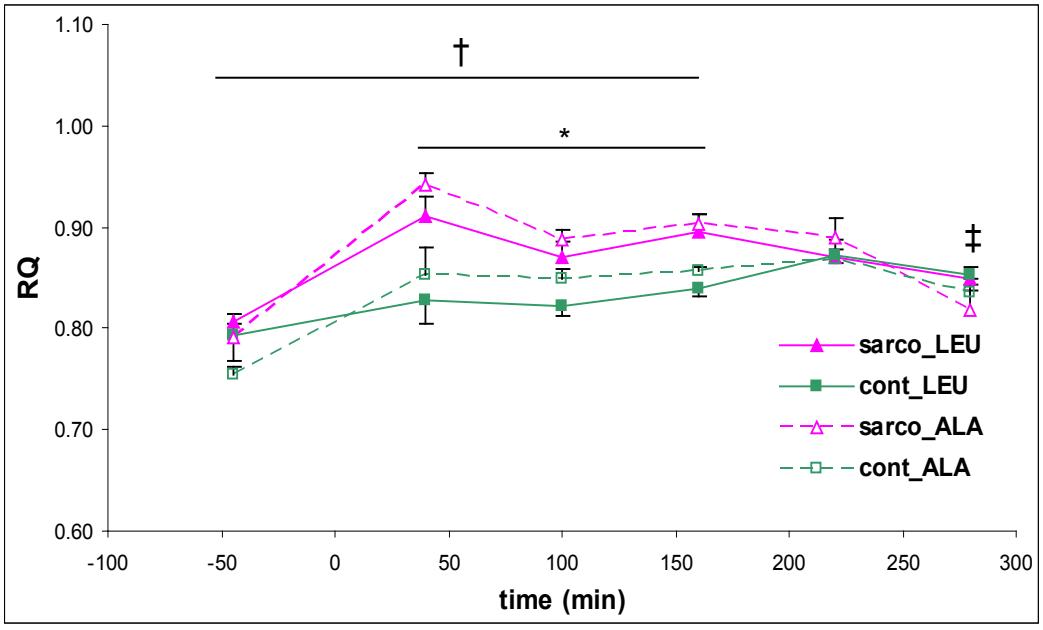
n = 3 for cont_LEU, cont_ALA and sarco_ALA; n = 2 for sarco_LEU group

Figure 15. Thermic effect of the meal



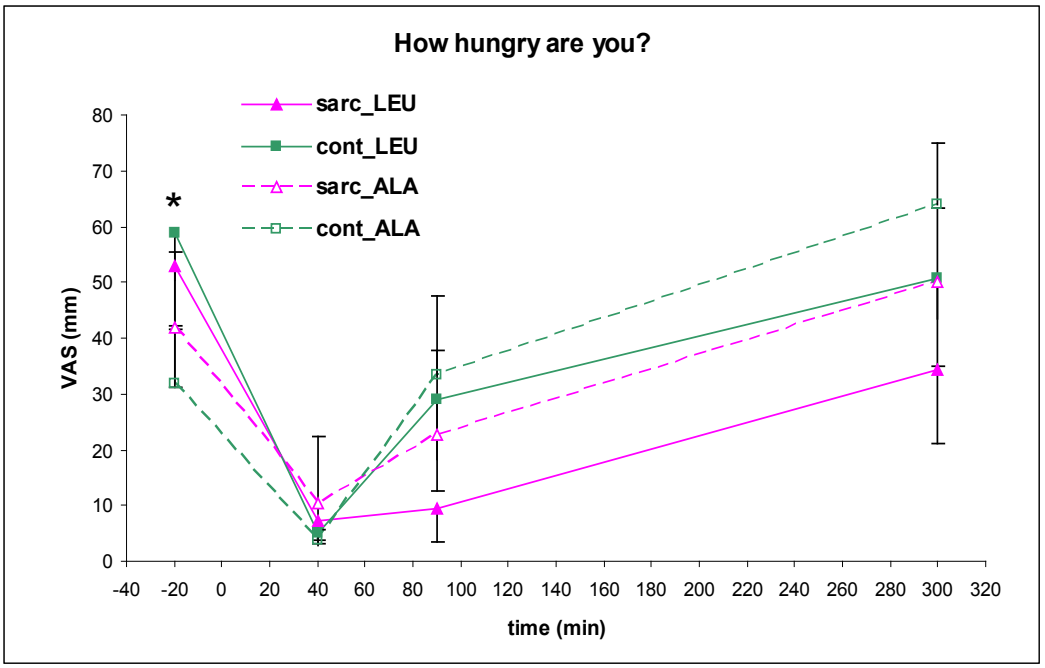
Repeated measures ANOVA showed significant main effect of meal only ($p < 0.0001$)

Figure 16. Respiratory quotient in response to meal

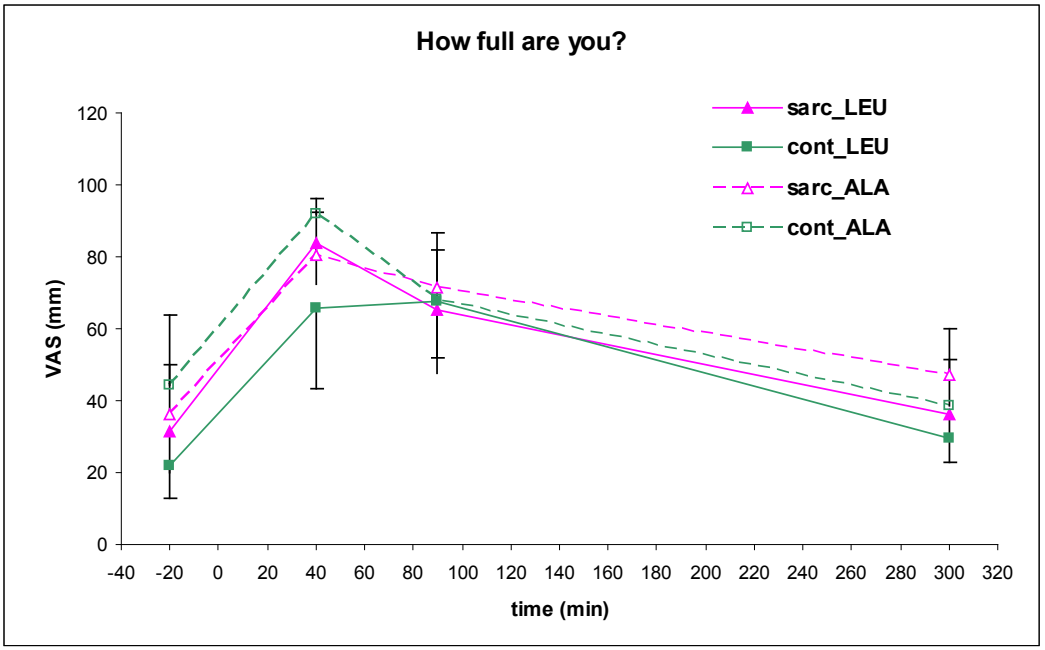


Repeated measures ANOVA showed a significant main effect of meal ($p < 0.0001$), as well as meal x sarcopenia ($p < 0.01$) and meal x LEU ($p < 0.05$) interactions; * $p < 0.05$ difference from control during LEU meal; † $p < 0.05$ difference from control during ALA meal; ‡ $p < 0.05$ difference from baseline during ALA meal

Figure 17. VAS ratings of hunger and fullness in response to meal

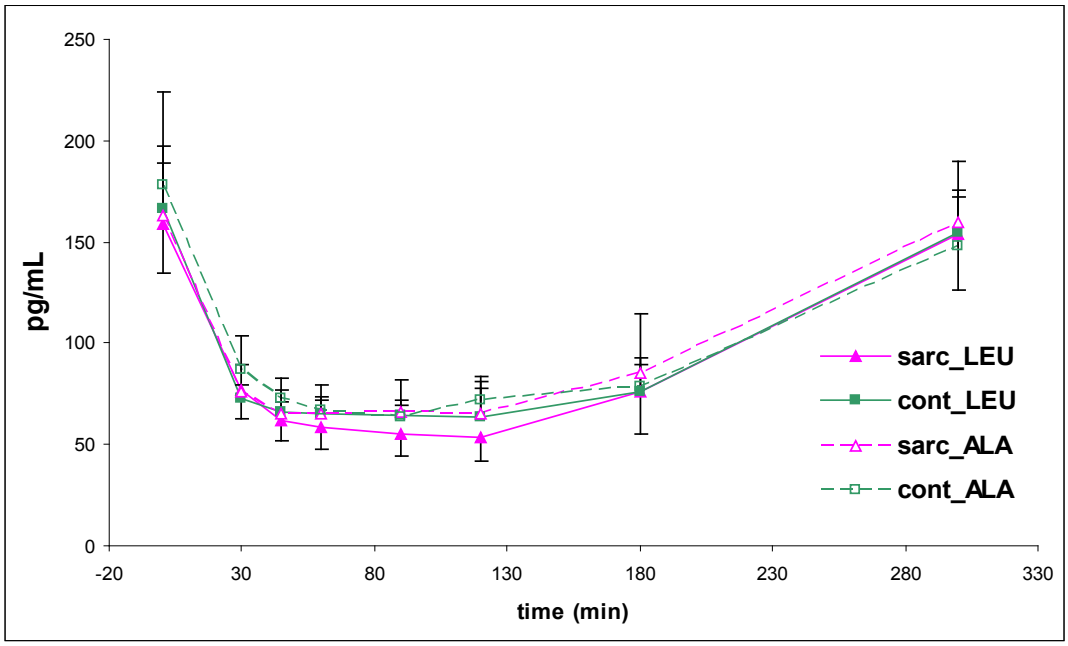


Repeated measures ANOVA showed a significant main effect of meal ($p < 0.0001$) and a meal x LEU interaction ($p < 0.05$); * $p < 0.05$ difference between cont_LEU and cont_ALA



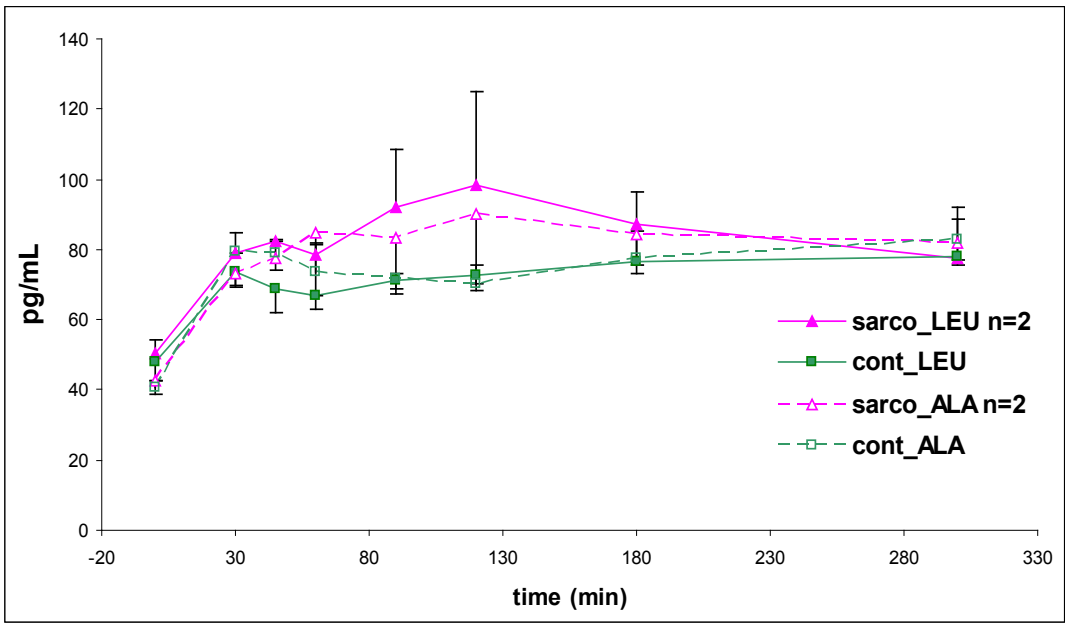
Repeated measures ANOVA showed a significant main effect of meal ($p < 0.0001$)

Figure 18. Acylated (active) ghrelin meal response



Repeated measures ANOVA showed a significant main effect of meal only ($p < 0.0001$)

Figure 19. PYY_{3-36} (active) meal response



6 DISCUSSION

The objectives of this study were first, to determine whether the protein metabolic response to a complete meal differs between healthy and sarcopenic older women and second, to measure the effects of a LEU-rich meal on whole-body protein balance as well as blood hormones, nutrients, intracellular substrates of MPS and any potential effects on TEF or satiety. Though these are preliminary results, to our surprise, whole-body protein balance, in response to ingestion of a complete meal was not blunted in our group of older sarcopenic women who have a lower muscle mass but otherwise similar body composition to age-matched non-sarcopenic controls. A LEU-rich meal stimulated a greater postprandial net protein balance equally in both sarcopenic and non-sarcopenic healthy older women, without affecting TEF or satiety. Curiously, at the muscle level, phosphorylation status of AKT, 4E-BP1, S6K1 and rpS6 in response to either meal,was not different between sarcopenic and control groups. Conversely, the increase in p-PRAS40 at Thr246, the site at which AKT phosphorylates PRAS40, was greater in response to the LEU meal in the sarcopenic group, suggesting that PRAS40, an inhibitor of mTOR activity, may have been more inhibited by the LEU meal. However, because of the preliminary nature of these findings, additional subjects will be necessary to gain a full understanding of the muscle intracellular differences.

6.1 Leucine oxidation rate and net balance

Although whole-body LEU net balance was greater in response to the LEU-rich meal, our finding of no difference between groups was unexpected. The lack of differences in total and % body fat (Fig. 3) may explain why whole-body net protein metabolism in response to meal-induced insulin secretion did not differ between groups. Previous studies have found that decreased insulin sensitivity of protein metabolism is related to adiposity rather than aging per se [156]. However, final determination of whether differences in whole-body protein metabolism exist will depend on subsequent measurement of the processes of protein S and B, each of which may show differences between groups. We

recently demonstrated in our lab that even in insulin resistant states such as diabetes, when subjects are infused with abundant AAs, they overcome their insulin resistance of protein metabolism, exhibiting similar anabolism to healthy controls [232]. Such a situation may have taken place in our study and potential differences between groups masked, since an adequate amount of dietary energy and generous amount of high quality dietary protein was provided to all subjects; ~2 g/kg LBM/d (1.2 g/kg/d) (Table 2), a level of protein intake above recommendations but well within usual intake observed in population studies [175]. This was also true during the actual meal test. Although BCAA levels and total response were highest after the LEU meal, they were still doubled in response to the ALA meal, achieving levels shown previously to stimulate MPS and overcome defective responses in elderly subjects [186-187]. Therefore, this effect of the ALA meal may have masked differences that may have been seen with lower plasma AA levels.

While group similarities in adiposity and adequate protein intake might explain a lack of difference in LEU balance (an indication of protein balance) between groups at the whole-body level does not necessarily imply the same result in the metabolism of muscle protein as its turnover is slower than other non-muscle tissue [233]. Indicative that the muscle compartment may have had a different response, our muscle immunoblot data show effects of a LEU-rich meal in the sarcopenic group.

6.2 Phosphorylation status of muscle intracellular substrates

Although we do not have direct measurements of MPS, phosphorylation state of intracellular substrates can shed light on the activity of MPS pathways and provide insight on what is happening in the muscle tissue. As mentioned before, it is important to note that only 6 of the 9 subjects studied (3 control and 3 sarcopenic) were able to provide muscle biopsies and one sarcopenic subject was unable to provide the postprandial biopsy during the LEU meal test, resulting in a sample size of 2 for the sarcopenic group for the intracellular responses to the LEU-rich meal. Therefore, any conclusions drawn from the immunoblot data are

very preliminary, since there is always the possibility that some or all the current results will change with a larger sample size. On the other hand, trends that were seen but did not reach significance (for example, a greater increase in mTOR phosphorylation of PRAS40 at the Ser183 site in the sarcopenic group in response to the LEU-rich meal; $p = 0.056$) could also be confirmed with a larger sample size. Thus a larger sample size should resolve these discrepancies.

The change in p-AKT in response to feeding did not differ between the LEU-rich and ALA-rich meals in either group, although the sarcopenic group during the LEU-rich meal, consisting of only 2 subjects, showed a large variability. Regardless, this is consistent with the literature and our hypothesis that AAs, including LEU, stimulate intracellular protein synthesis pathways downstream of AKT. Indeed, studies performed in hepatocytes have demonstrated that AAs stimulate phosphorylation of 4E-BP1 but not upstream AKT [234] (Fig. 1). Inconsistent with this however, we found no effect of LEU on the phosphorylation status of 4E-BP1 in either group. Additionally, we found that the LEU-rich meal further augmented the meal-induced phosphorylation of PRAS40 at the Thr246 site (the site of phosphorylation by AKT) in the sarcopenic women, which seems to be at odds with the hypothesis that LEU would stimulate the mTOR pathway downstream of AKT. While these results are puzzling, it's important to remember that firm conclusions cannot be drawn based on a sample size of 2.

At the same time, in response to the LEU-rich meal there was a trend for increased phosphorylation of PRAS40 at Ser183, an mTOR phosphorylation site, in the sarcopenic group only ($p = 0.056$; Figure 11b). This would support our hypothesis that a LEU-rich meal does not carry further benefit to healthy, active, non-sarcopenic individuals, in whom we have shown a normal postprandial response of MPS [164]. It is reasonable to postulate that MPS increased in response to the LEU-rich meal in the sarcopenic subjects only; however this may only be determined upon accumulation of a larger sample size as well as later determination of muscle protein fractional synthesis rate.

Prod'homme et al. [113] and Guillet et al. [157] recently suggested impaired stimulation of S6K1 as a mechanistic explanation for the anabolic failure of MPS with aging based on rat studies, so we may have expected to see a blunted activation of S6K1 in the sarcopenic group. The observed trend for a greater activation of p-S6K1 in the sarcopenic subjects compared to the controls after the ALA-rich meal is inconsistent with this (Figure 13). Rapamycin is known to selectively inhibit mTOR kinase function, and has been shown to inhibit over 98% of S6K1 activity in mammalian cells [235]. As such, S6K1 phosphorylation at Thr^{389} is often used as an indication of mTOR kinase activity [236]. p-S6K1 in the present study thus suggests that in sarcopenic subjects, mTORC1 activation in response to a complete meal may be greater than in age-matched controls and this is not further stimulated by a LEU-rich meal. However, if this was so, it would also be expected to see a greater increase in 4E-BP1 downstream of mTOR, which was not the case (Fig. 12).

Aside from the limitations of a small sample size and large variability, these discordant and unexpected intracellular signalling results could also be related to the timing of when the posprandial biopsy was taken. Because subjects were receiving a complete meal accompanied with increases in blood nutrients and insulin, AKT and 4E-BP1 phosphorylation must have been augmented, however it is possible that these events took place earlier than when the muscle biopsy was performed. It is possible that by taking the postprandial muscle biopsy at 2 hours after the meal, we may have missed the period of peak stimulation of intracellular substrates. Studies in humans suggest that the window of AAs anabolic effects on MPS begins at 30 minutes, after which the rate of increase quickly peaks from 60 – 90 minutes and then declines, despite elevated plasma AA levels [115, 117]. These time intervals are estimates based on studies in young adults, where AAs were constantly infused peripherally. Therefore, they do not take into account time course differences that may be present in older adults during normal meal ingestion, where digestion and absorption dynamics may be influencing factors. Nonetheless, using these values as a reference point, it remains a possibility that our results of no change in substrate phosphorylation

may have been due to the timing of the biopsy. Due to the invasive nature of the biopsy procedure and the older study population, taking additional biopsies, while ideal, is not realistic. However, future consideration for taking the biopsies slightly earlier may reveal differences that were missed in the present study.

6.3 Insulin secretion, BCAAs and plasma glucose

The LEU-rich meal induced greater insulin secretion in the sarcopenic group only, apparent from significantly higher insulin and C-peptide peak levels and AUC responses. Plasma BCAAs paralleled this, with a greater AUC response to the LEU-rich meal in the sarcopenic group. Because the only difference between the two meals was a doubling of the LEU content, the increase of the BCAA response during the LEU-rich meal over that during the ALA-rich meal is assumed to be due to LEU. LEU has been shown to be an insulin secretagogue [189-191] and has been shown in humans, when ingested with carbohydrate, to induce a 100% greater insulin response within the second hour after ingestion compared to carbohydrate alone [237]. Therefore, the higher insulin secretory response in the sarcopenic group could have been due to greater stimulation by higher plasma LEU concentrations.

At the same time, LEU meal enrichment was proportionally equal in all subjects, as the dose given was based on kg of LBM. Therefore, higher plasma BCAA levels in the sarcopenic group could be due to 1) blunted stimulation of protein S and subsequent decreased BCAA uptake or 2) a failure of insulin, despite high levels, to suppress rates of protein B, leading to greater influx of AAs from body protein into the plasma free pool. Either case would support a decreased rate of net AA uptake for protein accretion, which would translate into muscle protein lost if sustained for prolonged periods. If one considers the same degree of carbohydrate oxidation, the differences found in postprandial non-protein RQ with higher RQ values in the sarcopenic group (Fig. 16) could suggest they were oxidizing more protein, as opposed to fat substrate for energy. If the protein being oxidized was coming from the muscle, this would support the second possibility above, which would explain similar TEF between groups if the lower protein S was compensated for by higher protein B. The fact that we

previously found increased daily rates of MPB in frail elderly women despite no apparent differences in net protein balance when compared to controls further supports the same possibility in sarcopenic women [166]. The other side of the coin of course is that the sarcopenic women were oxidizing more of the dietary protein since, having less muscle mass, they needed less exogenous protein for muscle protein turnover, which is supported by our correlation of LBM with LEU balance ($r = 0.744$). Hence, these discrepancies will only be solved upon determination of both whole-body and muscle protein rates of S and B.

Interestingly, despite the greater insulin response in the sarcopenic group during the LEU-rich meal, peak plasma glucose levels and total postprandial response did not differ between groups. Sarcopenic subjects were tolerant to glucose, evident from their normal OGTT results (Table 1). Therefore, the absence of a more rapid decrease in plasma glucose in response to the higher insulin secretion could suggest that the sarcopenic women were exhibiting acute insulin resistance of glucose in response to the LEU-rich meal. In support of this, nutrient-mediated inhibition of insulin signalling has been proposed as a mechanism of insulin resistance [238]. In the insulin signalling pathway the insulin receptor phosphorylates IRS-1, initiating a phosphorylation cascade that eventually leads to translocation of glucose transporters to the plasma membrane and subsequent glucose uptake at the cell plasma membrane [239]. This same phosphorylation cascade, branching off at the level of AKT, leads to increased activation of downstream mTOR and protein synthesis (Figure 1). As previously mentioned, the action of AAs on protein synthesis is thought to join up with the insulin pathway at the level of mTOR (Figure 1). Recent studies reveal a negative feedback loop, whereby mTOR-activated S6K1 directly phosphorylates IRS-1 at inhibitory sites, suggesting that increased activation of mTOR by AAs could also increase inhibitory phosphorylation of IRS-1, preventing IRS-1 from assuming its role in glucose uptake [138, 240-241]. Similarly, studies in liver cells indicate that while AAs (and specifically LEU) stimulate protein S by activating S6K1, they may simultaneously inhibit insulin's effects on glucose metabolism through greater S6K1 phosphorylation of IRS-1 and subsequent downregulation of PI3-K,

an important component in pathways leading to glucose uptake [234] (see Fig. 1). In the current study, p-S6K1 did not significantly increase after the LEU-rich meal to a greater extent than after the ALA-rich meal in the sarcopenic women, however a larger sample size might be expected to reveal such a result, which could mechanistically explain the possible acute insulin resistance of glucose metabolism observed. Due to technical difficulties, we were unable to quantify p-IRS-1, and future studies including this data as well as a larger sample size and longer supplementation period will give more definitive results.

6.4 Thermic effect, satiety and incretins

Protein is known to induce a greater TEF and to be more satiating than carbohydrate or fat [242]. This knowledge has been applied in high-protein diets designed to reduce energy intake, causing weight loss [242-243]. Because LEU is a primary anabolic component of protein, we evaluated whether a LEU-rich meal might also induce a higher TEF and/or greater postprandial satiety, as well as altered incretin responses, potentially unwanted effects for sarcopenia. Increased plasma AAs has also been proposed as a mechanism for protein-induced satiety [200]. However, despite inducing higher plasma BCAA levels, our results do not indicate that a LEU-rich meal induces greater meal thermogenesis nor is it more satiating than a non-LEU-rich meal (Figs. 15 and 17). Control subjects did unexpectedly report as being less hungry before the ALA meal than before the LEU meal, however feeding suppressed their appetite to the same extent during both meals suggesting that the LEU meal was more satiating (Fig. 17). However, additional subjects will be necessary to determine whether this is a real effect, or simply an artefact generated by low sample size and high variability of the measure. Regardless, this initial data does not suggest that a LEU-rich meal would dampen hunger or decrease food intake during longer supplementation in sarcopenic subjects. Plasma acylated ghrelin and PYY_{3-36} data also support this in both groups (Figs. 18-19). Mean ghrelin postabsorptive and postprandial levels were the same in both groups. Both postabsorptive levels and postprandial suppression were greater than that of obese postmenopausal women [101],

suggesting that altered ghrelin levels may not play a significant role in healthy aging or sarcopenia. In response to both meals, plasma active ghrelin – an orexigenic (ie. appetite-stimulating) hormone - was elevated just prior to the meal, sharply declined following the meal, and slowly increased back to postabsorptive levels by 5 hours. The mirror image trend for PYY was seen, as it is an anorexigenic (appetite suppressing) hormone, however in both groups in response to both meals, PYY levels did not return back to baseline by 5 hours.

6.5 Sarcopenia: definition considerations

Definition and diagnosis of sarcopenia are challenging and ever evolving, since the process and contributing factors are not fully understood [39]. For practical reasons, a single cut-off of appendicular or total muscle mass index is often applied [6, 41-42, 211]. However, defining sarcopenia by one static measure comes with the risk of misdiagnosing individuals who may not have undergone significant loss of muscle mass with aging but have had lifelong low muscle mass or are simply overall smaller. The fact that lighter individuals require less skeletal muscle for movement is both observed and intuitive [3]. The sarcopenic subjects in our study had a lower muscle mass (as indexed to height) but they also weighed less. When LBM was correlated with measures of muscle strength and physical function, hand-grip strength was positively correlated with LBM, confirming previous findings supporting hand-grip strength as an easily accessible and accurate marker for low LBM [244]. The lower muscle mass appeared to be due to loss of leg muscle, since leg but not arm or trunk lean mass was significantly lower in the sarcopenic group (Table 1). This phenomenon of a preferential loss of lower extremity muscle with age has previously been found from DXA data in both sexes [3], and is a logical link to explain the observed association between sarcopenia and functional impairment [208], giving us confidence in our sarcopenia diagnosis.

It is possible that one nutritional cause leading to sarcopenia is lack of adequate calorie and protein intake. Curiously, although the sarcopenic subjects were on average lighter and had lower muscle mass (as indexed over height),

groups did not differ in daily energy and macronutrient intake, as reported from food recalls and measured during the 4-day controlled diet prior to each meal test (Table 2), suggesting that lower dietary energy and protein intake was not a main factor. Recent results revealing undisturbed protein metabolic responses to postprandial levels of nutrients and insulin in healthy, highly active elderly women reiterated the importance of inactivity in the mechanism of sarcopenic muscle atrophy [164]. Our sarcopenic subjects on average expended half the amount of energy on physical activity as controls (Table 1), supporting low physical activity as a prominent factor causing atrophy and anabolic resistance in sarcopenia.

In the ongoing endeavour to efficiently diagnose sarcopenia, emerging definitions tend to include measures of muscle strength or function suggesting that sarcopenia, identified strictly by a low muscle mass, is not enough of a diagnostic to distinguish those individuals at risk for functional decline and loss of mobility [39, 245]. As has been pointed out, there is significant longitudinal evidence that the loss of muscle mass and strength, while both have been shown with aging, are not necessarily dependent on one another and when they are, the relationship is usually not linear [246]. This is because there are many other aging-related factors that can contribute to loss of muscle strength (ie. dynapenia) – including arthritic stiffness of the joints and neurological changes – and even more factors that may lead to disability, including adiposity and number of chronic diseases [247]. Our sarcopenic subjects, though they had lower muscle mass, did not show signs of compromised strength or mobility. Indeed, despite having a lower leg lean mass and smaller thigh circumference, sarcopenic subjects performed equally well as controls on TUG and gait speed tests, as well as on hand-grip strength. Nonetheless, sarcopenia is one component of the aging process that may eventually affect physical strength and we may view its early diagnosis from low muscle mass as a risk factor and regard its treatment as a preventative measure against disability. Sarcopenia also increases vulnerability to situations that require metabolic resilience. Muscle serves as a metabolic reserve, mobilized during disease, illness or injury, when there is greater need for rapid protein synthesis

and turnover. In such situations, individuals with lower muscle mass, especially elderly, are more vulnerable to further catabolism and poor outcomes [15]. Thus, maintenance of muscle mass regardless of obvious loss of strength remains fundamental.

6.6 Limitations and Future Directions

6.6.1 Immunoblot data

There are important limitations that must be acknowledged and considered when interpreting the immunoblot data. Aside from the lack of power due to low samples size, especially in the sarcopenic group during the LEU meal mentioned earlier (n = 2), and the issues of timing of the postprandial biopsy, it must also be mentioned that the immunoblot quality was not always optimal. Due to the presence of more fat and connective tissue in the muscle samples of these older subjects, a larger pellet of non-protein content was obtained after protein extraction and only 5 μL (compared to the usual 25 μL from younger healthy subjects) of muscle intracellular protein was available for loading. Longer exposure times were necessary to achieve signals dense enough to quantify, which also resulted in more background noise. Increasing the amount of tissue sample taken in studies with older subjects may be a future consideration.

6.6.2 Measurement of rates of protein synthesis and breakdown

Aside from the need to increase sample size to gain significant power, it will be essential to measure rates of both protein S and B – the two components of protein turnover that together determine net balance – at the whole-body and muscle levels, in order to understand more completely the dynamics of the anabolic response to the meal, and perhaps uncover differences that were not apparent from the whole-body net LEU balance and muscle intracellular data. Indeed during a previous study, although frail elderly women appeared to be in similar whole-body daily net protein balance as healthy controls, they exhibited elevated rates of whole-protein S and B as well as MPB [166].

Whole-body rates of protein S may be assessed upon GC-MS analysis of plasma ^{13}C-LEU enrichment, HPLC analysis of plasma LEU concentrations, and

application of non-steady state modelling [248]. Rates of whole-body B will require use of an oral tracer distinguishable from ^{13}C-LEU [248]. To ensure the same metabolism of the tracer and use of the same mathematical models, it should be LEU as well. Therefore, a LEU tracer labelled in a different position, which can be distinguished by GC-MS, will be necessary for such measurements.

At the muscle level, fractional synthesis rate of mixed myofibrillary proteins as assessed by incorporation of D5-Phenylalanine (D5-Phe), another stable isotope tracer, into the muscle tissue, will corroborate intracellular signal transduction results, confirming whether or not sarcopenic subjects exhibit a blunted MPS meal response and whether a LEU-rich meal augments postprandial MPS. D5-Phe – phenylalanine with deuterium replacing the 5 hydrogens of the phenyl ring – is distinguished from unlabelled Phe by GC-MS. Though harder to measure and quantify in human studies, the rates of MPB could be estimated in the future by performing ^{3}H-methylhistidine studies [249]. There is evidence supporting LEU-mediated suppression of protein B in the whole-body [250], and in myocyte cultures [179, 251]. Its effect is thought to work via the ubiquitin - proteasome pathway [252-253] and recently a LEU-supplemented meal was shown to restore the defective inhibition of MPB in old rats through suppression of this pathway [252], however, the exact molecular mechanisms by which LEU might regulate skeletal MPB and whether this suppression is relevant for preserving muscle mass are still unknown.

6.6.3 Measurement of extra and intracellular leucine

Another future area to be addressed is how the cell senses elevated plasma LEU and what mechanisms regulate its transmembrane transport [254]. While some human studies find that it is extracellular, rather than intracellular AA availability that correlates with rates of MPS [115], other studies in animal cells suggest that it is intracellular AA availability that is important for inducing gene expression associated with protein synthesis and that flux of AAs through their cell membrane transporters may initiate their intracellular signalling [254]. It would thus be of interest in the future to measure both extra- and intracellular LEU concentrations in response to the meal. Mechanisms involved in AA sensing

are at the moment poorly understood, however characterization of these aspects will be important to fully explain the anabolic action of LEU and AAs.

7 Conclusion

This study is the first to evaluate the protein anabolic response to an ingested meal as well as the stimulatory effect of a LEU-rich meal directly in older sarcopenic subjects. Our results suggest that a LEU meal supplement of 0.07 g/kg LBM (~2.6 g in older women) is sufficient to increase whole-body net protein balance above that of an equivalent meal without added LEU, without altering postprandial appetite profiles or augmenting meal thermogenesis in older women with or without sarcopenia. Preliminary muscle intracellular signal transduction data does not suggest blunted activation of mTORC1 and its downstream substrates 4E-BP1, S6K1 and rpS6 in older sarcopenic women compared to age-matched healthy controls during a meal, regardless of whether or not it was enriched with LEU. However, due to the preliminary nature of this study, additional subjects will be necessary in order to draw more definitive conclusions. Future longitudinal supplementation studies assessing postprandial rates of MPS in the sarcopenic population will also be necessary to directly assess whether a LEU-rich meal augments postprandial MPS in older sarcopenic women.

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Appendix 1.

Participant recruitment flyer (English)

Centre universitaire de santé McGill
McGill University Health Centre

Recruitment of Healthy women

For a study of metabolic responses

CANDIDATE PROFILE

- women of 65 years of age and older
- normal weight
- non-diabetic (Type I or II)
- non-vegetarian

Length of study: Two 3-5 day stays

INVESTIGATORS:
José A. Morais, MD and Stéphanie Chevalier, PhD
McGill Nutrition and Food Science Centre,
MUHC-Royal Victoria Hospital

For more information
Please contact:
The Research Investigators above or Research Coordinators
Connie Nardolillo and Emily Redmond at 514-843-1665

Appendix 2.

Phone pre-screening questionnaire (English)

Telephone questionnaire for potential subjects

Date:_____ Protocol: _____

Name:_____ Phone #: _____

Age:_____ Weight:_____ Height:_____
BMI:_____

1. Currently in another study? **Yes - End date? _____**
No

2. Previous participation? **Yes** **No**

3. Student? **Yes University/Faculty_____**
No

5. Stable weight for 6 months? **Yes** **No** **Don't know**

6. Allergies (Food/ Medications)? **Yes** **No**

7. Smoker or ex-smoker? **Yes** **No**

8. Vegetarian (red meat, eggs, cheese, milk, tofu, vegetables, fish, poultry)?
Yes
No - How much red meat? _____

9. Drink alcohol (wine, beer)? **Yes – Less than 3 drinks/day? _____**
No

10. Medicare card? **Yes** **No**

11. RVH card? **Yes** **No**

12. Any diseases within the family? **Yes _____**
No

13. Type 2 DM? **Yes** **- For how many years? _____**
- Medication (dosage, frequency)? _____
_____
_____
No

14. Post-menopausal? **Yes - Last period? _____**
No - Regular periods? _____

15. Surgery/Hospitalization/ Accidents? **No** **Yes – Why? When**
_____
_____
_____
_____

16. Blood transfusions? **Yes – Why? When?** **No**
_____
_____
_____

17. Other Medical Conditions & Medications

Medical condition	**How many yrs?**	**Current medications** **(Dosage, frequency)**
Diabetes complications		
Heart/High blood pressure		
Thyroid		
Anemia/Fe deficiency		
Kidney		
Liver		
Respiratory		
Cancer		
Cholesterol/Lipids		
Psychiatric		
Other: (including HIV, Hep)		

18. Herbs & Vitamins? Over the Counter Pills? Yes – Which ones?
No

_____
_____
_____

19. Activities/Exercise:

_____
_____
_____
_____

20. Where did you hear about the study?

Poster - Where? _____
Newspaper - Which one?_____
Other _____

Comments:

_____
_____
_____
_____
_____
_____
_____

Participation in study: **Yes**
No - Why was subject excluded?

_____
_____
_____

Questionnaire done by:

_____

Treating Physician: _____

Appendix 3.

Meal study consent form
(English)

Patient Information and Consent Form for Study Participants

McGill University Health Centre

Royal Victoria Hospital: Clinical Investigation Unit, Department of Medicine

Title of the project:	**Muscle protein metabolism in sarcopenic elderly – meal study**
Sponsor:	**Canadian Institutes of Health Research (CIHR)**
Investigators:	**José A. Morais, MD; telephone: 514-843-1665 (9 am to 5 pm)**
	or by pager: 514-406-0163 (any time)
	Stéphanie Chevalier, PhD; telephone: 514-843-1665 (9 am to 5 pm)
	Linda Wykes, PhD; telephone: 514-398-7739 (9 am to 5 pm)

Before you give your consent to be a research volunteer, please take time to read carefully and consider the following information which describes the purpose and procedure, the possible risks and benefits and other information about the proposed research study. Please ask the study doctor or the research staff to explain any words or information that you do not clearly understand.

Reason for the study

You have been invited by Drs. José A. Morais and Stéphanie Chevalier from the McGill Nutrition and Food Science Centre to take part in a study on protein metabolism in aging. Your participation in this study will involve 4 total visits: 2 initial screening visits followed by two 3 or 5-day stays at the Clinical Investigation Unit of the MUHC-Royal Victoria Hospital, spaced by 2 weeks of normal activities at home. By participating, you would help us to better understand how the presence of aging affects the body's responses to a meal and how leucine, one of the amino acids that make up proteins, may help retain more proteins. This in turn, could lead to defining diet strategies to prevent aging-associated muscle loss.

Visit 1) <u>Information and Health Assessment Visit</u>: For this first session you will come to the research unit to meet with the research staff for an interview, to

discuss the study and have your questions answered. If you agree to enter the study you will sign the consent form. You will have a nursing health assessment, standard blood and urine tests, a chest X-ray, and an electrocardiogram (ECG). A research technician will assess your body composition using bioelectrical impedance analysis (BIA) and measure your resting metabolic rate (RMR) by indirect calorimetry. An assessment of your mental capacities using the mini-mental state examination (MMSE) and physical function capacity using the "timed up and go' test (TUG) will be carried out. All of these procedures are comfortable to do and are described in detail in the following sections of this consent. Explanations will also be given to you, in person, while you are undergoing the tests. The first visit takes approximately three hours to complete.

Mental capacity assessment

Your mental capacity will be assessed using the mini-mental state examination (MMSE) test. You will be asked several questions to test your memory and to perform a few simple tasks. This is a screening test for memory and takes about 5 minutes to complete.

Physical functional capacity assessment

Your physical function capacity will be assessed with a simple physical performance test called the "Time up and go" test (TUG). The TUG measures the time it takes you to stand from an armchair and walk a distance of 3 meters, turn around and sit back down in the chair. The time to perform this test is recorded with a stopwatch.

Visit 2) <u>Oral Glucose Tolerance Test (OGTT) Visit</u>: If all the tests performed at the first session indicate that you are eligible to participate, you will be asked to undergo an oral glucose tolerance test (OGTT), unless you are already known to have diabetes, to determine your body's "blood sugar" response to a high sugar drink, over a period of three (3) hours. This test requires that you come to the research clinic after an overnight fast (not eating or drinking anything except water from 11:00 pm the night before the visit). You will be asked to drink 75 g of dextrose (sugar), given in the form of a pleasant-tasting, orange-flavored drink. You will be asked to remain in the research unit, resting quietly for 3 hours following the glucose drink. The research nurse will insert an intravenous (IV) catheter into a vein in your arm before you take the drink, in order to take 2 blood samples before and at 30, 60, 90, 120 and 180 minutes after the glucose drink. At each sample time, 8 mL (1½ teaspoon) of blood is taken. At the end of the test, the catheter will be removed and you will be offered juice and lunch. The study doctor (Dr. Morais) will take a full medical history and perform a physical exam.

Physical activity evaluation: If you are found eligible to participate, you will be asked to maintain your usual diet and physical activities until the beginning of the 5-day metabolic study. You will also be asked to wear a small portable device (similar to a pager) on your belt to measure your physical activity, during 5 consecutive days of normal activities.

Visit 3) <u>First stay (5 days) Metabolic Study at the MUHC-Royal Victoria Hospital Clinical Investigation Unit (CIU:</u>

If you chose to spend the first 2 days of the 5-day metabolic study at home, for these days you will follow a special diet designed by our nutritionists to maintain your current body weight. This diet, which you will prepare at home, will be composed of a mix of your normal foods supplemented with a liquid formula called Ensure®, which will be provided to you. If you chose to spend all 5 days of the metabolic study with us at the CIU, you will receive the special diet there, where all of your meals will be provided to you by the hospital.

Days 1 – 4: At the CIU

You have the option of performing the first two days of this portion of the study at home. This involves eating the special diet, and no other food or drink except water, as well as collecting all of your urine in 24-hour urine bottles over the two days. Depending on your choice, you will arrive on day 1 or day 3 of following the controlled diet around 8:00 am, after an overnight fast. You will be asked to breathe under a plastic canopy for 20 minutes, while lying on a bed. This test is to calculate what your body is using as a "fuel" for energy. You will need to lie as still as you can on the bed, relaxed but not sleeping, for the test to be accurate. Your body fat and lean tissues will be measured by a safe, painless test, using a very low electric current that lasts for a few seconds only. Your body composition will also be measured by a scanning technique (dual X-ray absorptiometry, DXA). This test will take place at the McGill Nutrition and Performance Lab, where you will be accompanied by a member of the research team. For this test that lasts for about 10 minutes you will lie down still on a mattress under the scanner. The amount of radiation received from this test is less than that received from exposure to a chest x-ray.

During all days in the CIU, you will be offered bran cereal and milk for breakfast. The rest of your diet will be made of normal foods and a complete liquid formula. You will sleep in a private room at the CIU. Your morning weight and temperature will be recorded daily and you will collect all your urine in a special container. Regardless of whether you decide to spend the first 2 days at home or at the CIU, the research staff will explain how you can carefully collect complete daily 24-hour urine, from day 1 to day 4 of the study. At the CIU, this also involves having the staff help you to (a) weigh yourself each morning in sleep-wear after voiding (emptying your bladder) (b) take your temperature each morning (c) measure your daily water intake. The staff will provide you with a notebook and assist you to record all of your information, and answer any questions you may have. If you are a person with diabetes, you will perform capillary glucose monitoring (finger pricks) four times per day and will be kept on your usual medications. If on occasion, the capillary glucose goes too high, a subcutaneous dose of insulin will be administered.

During your CIU stay, a fasting blood sample of 5 mL (1 teaspoon) will be taken. We will assess your fasting levels of gluconeogenesis, a process by which the

body uses amino acid or glycogen substrates to produce glucose. At 10:30 pm on the night of day 4, you will then drink four small portions of water that is isotopically labeled to allow its detection, within a period of 1½ hours. At 10:30 am on day 5, a 30ml blood sample will be taken from the already inserted catheter in your hand. Measures of your body circumferences and skinfold thickness will be made around the chest, waist, hip, arm and leg. A magnetic resonance image (MRI) of your chest and thigh will be also taken to assess your body composition. There is no radiation or danger involved in the MRI and the only slight discomfort will be that it requires you to lie very still for some time. If you feel uncomfortable in confined spaces, please notify one of the research personnel. Your handgrip strength, walking speed and time required to get up from a chair will be measured. You will be asked to complete a questionnaire called CHAMPS: Community Healthy Activities Model Program for Seniors, to evaluate your level of physical activity. The research assistant will offer any help that you may require. It will take approximately 15 minutes to complete this questionnaire.

In the afternoon of day 4, the research nurse will insert a catheter (small flexible plastic tube) in your arm vein, which will be kept in place overnight and until the end of the next day (24 h). A standard intravenous saline solution will be infused through the catheter during this time.

Day 5: Meal test to assess metabolic responses
The metabolic study will be performed and will last approximately 11 hours. Your study doctor and nurse will remain with you at the bedside in order to closely supervise your care during all the study activities of this day.

- At 6:00 am on that morning, while you are fasting and resting comfortably in a bed, infusion of ^{2}H-phenylalanine will be started through the catheter already in place and continued for the rest of the study at a slow rate. Phenylalanine is an amino acid, a compound which the body uses to make protein. This form of phenylalanine is not radioactive. We can detect them with very sensitive instruments; because they are slightly heavier than normal (they contain the stable isotopes carbon-13 and deuterium). Stable isotopes occur naturally and are safe even in children and pregnant women.
- At 9:00 am, you will receive infusions of very small amounts of radioactive sugar (tritiated glucose) and another stable isotope of the amino acid, Leucine. The small amount of radiation from the tritiated glucose is considered negligible. A second catheter will be inserted in a vein on the back of your hand. Your hand will be placed in a warming box at 65^{o}C to make the blood in the vein similar to that of an artery. This catheter will be used for repeated, painless blood samples to be taken. This is not a painful or uncomfortable procedure.
- At 11:30 am the 1st muscle biopsy will be performed, and the 2nd one will be taken two hours after you ingest the meal. The biopsy procedure is a standard one for studies and diagnosis. It is even done in athletes, who

exercise immediately after the procedure. A sample will be taken from a muscle on either side of your thigh. The skin and tissue under it will be anesthetized (like a dentist's "freezing") before a cut in your skin of 0.7 cm (about the width of a pencil) is made. A needle (hollow cylinder of 6 mm diameter) will be inserted into your muscle to remove a piece of about one tenth of a gram (the size of a small pill). Once the biopsy is obtained the skin will be held together with sterile strips of adhesive tape and a protective dressing will be applied on top of this. Firm pressure will be applied to the area for 10 minutes to prevent bruising. Because of the local anesthetic, you should feel no pain during the study.

- At noon, you will have a meal composed solely of the liquid formula that you will have to consume within 20 minutes. The formula will contain an extra amount of leucine or another amino acid, alanine, but you will not be informed which one of the two you will drink.
- Blood samples of 15-30 mL (1-2 tablespoons) will be taken periodically before and after the meal, for a maximum of 180 mL (6 ounces).
- Six times during the day, you will be asked to breathe under the plastic canopy for 20 minutes.
- Samples of expired air will be taken, about 20 times throughout the study by simply blowing into a special bag.
- Three times during the day, you will be asked to rate your feelings of hunger and desire to eat.
- At the end of the study, you will be offered a complete meal and will return home.

Following two-weeks: At home

This two week-period allows your body to eliminate the stable isotopes, through the urine. During this period, you will return to and maintain your usual diet, physical activities and occupations.

Visit 4) Second stay (3 or 5 days) at the MUHC-Royal Victoria Hospital:

For day 1 – 4 of the second stay, you will follow the same special diet, composed of foods from your usual diet, and again supplemented with the liquid Ensure® formula.

This second stay is conducted similarly to the first, with the following exceptions:

Days 1 – 4 (or 3 - 4): at the CIU. No body composition, breathing of function tests, or assessment of gluconeogenesis will be performed; only the controlled diet, urine collection and one fasting blood sample will be repeated.

Day 5: the test meal of the metabolic study will contain the other amino acid, leucine or alanine, not tested at visit 3. There will an additional muscle biopsy taken at approximately 9:00 am so that there will be 3 muscle biopsies on this day. The rest of the meal study will take place as written above.

Risks and Benefits: The risks involved in consuming the diet and in blood sampling are considered to be minimal. You may feel warm when breathing under the plastic canopy, but air conditioning will be on in the room to minimize this effect. There may be slight pain or discomfort while doing blood tests with a slight risk for bruising. The amount of blood drawn over the entire study will not exceed that in an ordinary blood donation. This amount will not cause symptoms after the study. The risks of muscle biopsies are considered more than minimal. You may feel some pain in the thigh following the study and for a few days, from the muscle biopsies. This should be controlled by acetaminophen (Tylenol®). The rare complications of muscle biopsy include infection, denervation and bleeding. There is a chance that the small scars on your skin will not disappear completely. The radiation from the DXA and the labeled glucose and water is considered negligible. The amount of radioactive material received represents a radiation exposure similar to that received from a standard X-ray of the chest. The dose is minimal and disappears totally from the body (mainly urine) within 2 1/2 weeks of administration.

Your study will be supervised by experienced nurses and doctors who will make every effort to keep you comfortable during the study. They will also remain in contact with you until your return to your usual state.

Your participation in this study is voluntary. You may withdraw from the study at any time without affecting your usual medical care. Although this protocol is not expected to provide you any direct benefit, it is hoped that the information obtained will lead to the advancement of scientific knowledge in the field of nutrition and aging. You will be offered dietary counselling by a registered dietician during your stay, if you wish to learn about and improve your nutritional status.

Confidentiality of Records: The results of this study will be treated in complete confidence. The urine, blood and expired air samples will be coded and safely stored for a maximum of 5 years, for analyses at the McGill Nutrition Centre. All records obtained from your sample analysis as well as related hospital and office documents will be kept in a secure and private research office. The results of this research may be presented at meetings or in publications but your identity will not be disclosed. Your name will not appear in any publication or report produced from this study. As part of normal research practice it is important that information related to the study is checked for accuracy. It may be necessary for regulatory agencies such as Health Canada, the Canadian Institutes of Health Research or members of the McGill University Health Centre Research Institute, or Research Ethics Board to review the information obtained from your medical records. In such circumstances, confidentiality will be maintained at all times.

Contact persons: Any questions you may have about the study procedures, or the study results will be answered promptly by contacting the study doctors, Dr. José A. Morais or Dr. Stéphanie Chevalier at 514-843-1665, from 9 am to 5 pm., or by pager 514-406-0163 at any time.

Should you have any question regarding your rights as a research subject, and wish to discuss them with someone not associated with the study, you may contact the Ombudsman of the McGill University Health Centre at (514) 934-1934 local 35655.

Compensation: You will receive $35 for the morning screening and $50.00 for the OGTT visit, and $500 for the full meal study (two 5 day stays). Funds you will receive for participating in this research study will compensate for losses and/or inconveniences that are related to your participation. Receipt of funds is not the reason why you volunteered.

Liability: By signing this consent you do not waive any of your legal rights.

Study Title: Muscle protein metabolism in sarcopenic elderly – meal study

Subject's Declaration of Consent

I,_____, have read the above description with a member (s)

of the research team, _____

_____.

- I fully understand the procedures, advantages, and disadvantages of the study which has been explained to me.
- I freely and voluntarily consent to participate in this project.
- I understand that I may seek more information and that I am free to withdraw at any time if I desire, and that it will not compromise my medical care.
- I understand that my personal information will be kept confidential.

Dated at Montreal: (month) _____ (day) _____ (year) 20 _____
mm dd yy

PARTICIPANT: _____
Signature

INVESTIGATOR: _____
Signature

Appendix 4.

Mini-mental state examination (English)

ESEMM / SMMSE (FOLSTEIN)

Points Score ▼

1. • Quelle année sommes-nous?
What year is this?
• Quelle saison sommes-nous?
What season is this?
• Quel mois sommes-nous?
What month of the year is this?
• Quelle date sommes-nous aujourd'hui?
What is today's date?
• Quel jour de la semaine sommes-nous?
What day of the week is this? /5

2. • Dans quel pays sommes-nous?
What country are we in?
• Dans quelle province sommes-nous?
What province are we in?
• Dans quelle ville sommes-nous?
What city are we in?
• Quel est le nom de cet hôpital?
What is the name of this hospital?
• À quel étage sommes-nous?
What floor of the building are we on? /5

3. • Répétez les trois mots suivants : _____, _____, _____.
Repeat the following three words: _____, _____, _____. /3

4. • Épelez le mot «monde» à l'envers ou soustrayez 7 de 100 et continuez à soustraire 7 du résultat, et ainsi de suite jusqu'à ce que l'on vous arrête.
Spell the word «world» backwards or subtract 7 from 100 and keep subtracting seven from what's left until I tell you to stop. /5

5. • Vous souvenez-vous des trois mots que vous avez répétés tout à l'heure?
What were the three words that I asked you to remember? /3

6. • Nommez cet objet. (montrez une montre)
What is this called? (show a watch) /1

7. • Nommez cet objet. (montrez un crayon)
What is this called? (show a pencil) /1

8. • Répétez la phrase suivante: «Pas de si, de et, ni de mais».
Repeat the following phrase: «No ifs, ands or buts». /1

9. • Prenez cette feuille de papier avec la main droite/gauche, pliez-la en deux et posez-la par terre.
Take this paper in your right/left hand, fold the paper in half and put it on the floor. /3

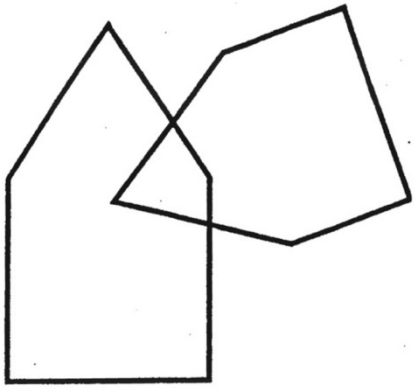
10. • Lisez ce qui est écrit et faites ce qu'on vous demande : «Fermez vos yeux».
Read the words on this paper and do what it says: «Close your eyes». /1

11. • Copiez ce dessin.
Copy this design. /1

12. • Écrivez une phrase complète sur cette feuille.
Write a complete sentence on this piece of paper. /1

FERMEZ LES YEUX

CLOSE YOUR EYES



DESSIN / DRAWING

PHRASE / SENTENCE

DATE: SIGNATURE: TOTAL: / 30

106

Appendix 5.

Visual Analog Scale Appetite Questionnaire (English)

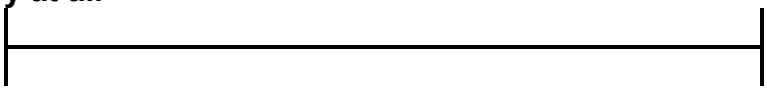
Subject ID: _____

Date: _____Time:_____

Please use a vertical line to indicate the position on the line below which best corresponds to your feelings at the moment.

1. **How <u>hungry</u> do you feel?**

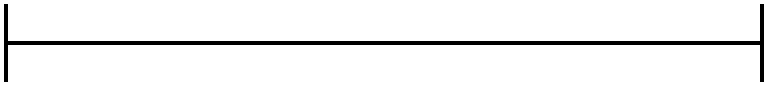
I am not hungry at all



I have never been more hungry

2. **How <u>full</u> do you feel?**

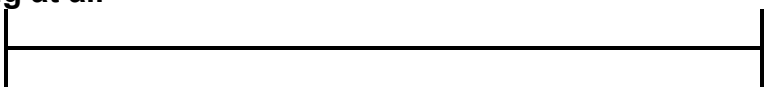
Not at all full



Totally full

3. **How <u>much</u> do you think you can eat?**

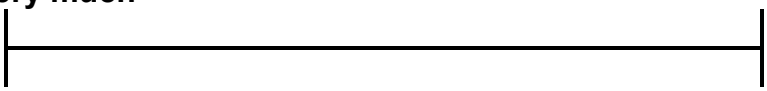
Nothing at all



A lot

4. **Would you like to eat something <u>sweet</u>?**

Yes, very much

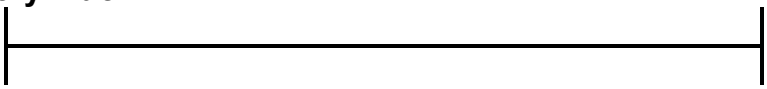


No, not at all

Subject ID: _____ (continued)

5. **Would you like to eat something fatty?**

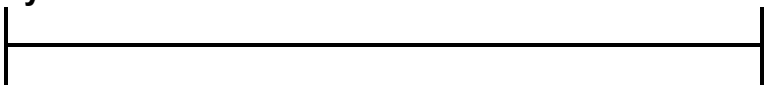
Yes, very much



No, not at all

6. **Would you like to eat something salty?**

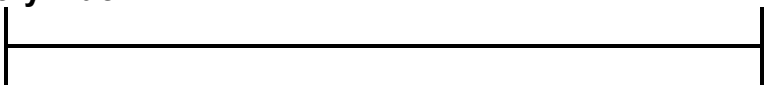
Yes, very much



No, not at all

7. **Would you like to eat some meat or fish?**

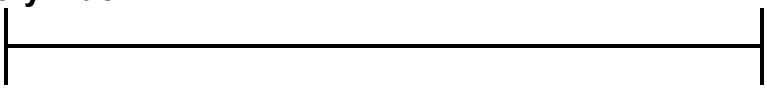
Yes, very much



No, not at all

8. **Would you like to eat a meal?**

Yes, very much



No, not at all