Effects of Leucine Supplementation in Combination with Resistance Training and Optimal Dietary Protein Intake in Pre-Frail and Frail Older Women

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ABSTRACT

Background: Frailty is a clinical condition associated with loss of muscle mass and strength, whose loss is termed sarcopenia, of which age-related resistance to anabolic stimuli is considered a key factor. Age-related anabolic resistance can be mitigated by a higher protein intake than presently recommended for the general population and has lower insulin sensitivity (IS) as a contributing factor, while mitochondria are centrally implicated in frailty and sarcopenia. Resistance training (RT) is the strongest stimuli to counteract sarcopenia via increasing muscle protein synthesis in the presence of sufficient amino acids, and may also stimulate mitochondrial biogenesis, as well as improve IS in frail older women. Leucine is a unique amino acid that acutely increases muscle protein synthesis independent of insulin. Leucine has paradoxically been shown to have both potential therapeutic value while also being implicated in the pathological development of insulin resistance. Furthermore, leucine has recently been shown to increase mitochondrial biogenesis in myocytes. The effects of chronic leucine supplementation in conjunction with RT on these factors within the context of frailty are currently unknown.

Objective: The purpose of this double-blinded placebo-controlled study is to determine the effects of leucine supplementation and RT on indices of anabolism, insulin sensitivity, and mitochondria function in pre-frail and frail older women consuming optimal protein intake.

Methodology: Nineteen pre-frail and frail elderly women (77.5 ± 1.3 y), based on the Frailty Phenotype, underwent 3-months of RT 3x/week with protein-optimized diet (~1.2 g·kg BW^{-1}·d^{-1}) and were randomized to 7.5 g/d of leucine supplementation or an isonitrogenous quantity of placebo (alanine). Indices of 1) insulin sensitivity during a metabolic test meal; 2) mitochondrial content (VDAC protein), function (respiration and reactive oxygen species production), and

sensitivity to apoptosis (calcium retention capacity and time to mitochondria permeability transition pore opening) in permeabilized muscle fibers; and 3) muscle protein synthesis determined using L-[ring-$^{2}H_5$]phenylalanine infusion in the postabsorptive and postprandial states, muscle fiber profile by immunohistochemistry, body composition by Dual X-ray Absorptiometry, physical function tests and maximal isometric strength were measured pre- and post-intervention.

Results: Our main findings were that compared to 12 weeks of RT in pre-frail and frail older women consuming an optimal protein intake, additional leucine supplementation had: 1) no detrimental nor beneficial effect on insulin sensitivity; 2) increased mitochondrial content without affecting mitochondria function, capacity, or sensitivity to apoptosis; and 3) no added benefit on improvements in muscle protein synthesis, muscle function, and Frailty Phenotype.

Conclusion: Leucine supplementation does not appear to influence insulin sensitivity, and although leucine increased mitochondrial content, this occurred without associated improvements in mitochondria function in older women consuming an optimal protein intake and undertaking RT in the context of frailty. The RT and dietary protein intake as described in this study appear to effectively and meaningfully stimulate basal (postabsorptive) muscle protein synthesis translating into measurable gains in muscle protein accretion, strength, and function resulting in marked improvements in the Frailty Phenotype.

RESUME

Introduction: La fragilité est une problématique qui apparaît lors du vieillissement et qui est associée à une perte de la masse et de la force musculaire connu sous le terme de la sarcopénie. La résistance anabolique associée à l'âge est considérée comme une des causes majeures qui contribue à la diminution de la masse musculaire. Cette résistance pourrait être amoindrie en partie par des apports en protéines plus élevés que ceux recommandés pour la population en générale et a comme facteur contributif une diminution de la sensibilité à l'insuline (SI), alors que la mitochondrie a un rôle primordial dans la fragilité et la sarcopénie. L'entraînement en résistance (ER) est le plus grand stimulus pour contrer les effets de la sarcopénie en augmentant la synthèse protéique musculaire lorsque suffisamment d'acides aminés sont présents, mais peut aussi stimuler la biogenèse mitochondriale et améliorer la SI chez les femmes âgées fragiles. La leucine est un acide aminé singulier qui augmente de façon intense la synthèse protéique musculaire et cela indépendamment de l'insuline. Pourtant, il fut démontré que la leucine a un effet paradoxal par sa valeur thérapeutique potentielle alors qu'elle est impliquée dans le développement néfaste de la résistance à l'insuline. De plus, il a récemment été démontré que la leucine augmentait la biogenèse mitochondriale dans les myocytes. Les effets d'une supplémentation prolongée en leucine combiné à un ER chez les personnes âgées fragiles sur les facteurs susmentionnés demeurent inconnus.

Objectif: Le but de cette étude est de déterminer les effets d'une supplémentation en leucine combiné à un ER sur les facteurs anaboliques, la sensibilité à l'insuline ainsi que les fonctions mitochondriales chez les femmes âgées pré-fragiles et fragiles recevant des apports protéiques optimaux.

Méthodologie: Dix-neuf femmes âgées (77,5 ± 1,3 ans) pré-fragiles et fragiles selon les critères du phénotype de fragilité, ont été recrutées et divisées en deux groupes aléatoirement. Toutes les participantes ont suivi un ER d'une durée de 3 mois (3/semaine) combiné à un régime riche en protéine (~ 1,2 g·kg BW^{-1}·d^{-1}). Un groupe ingérait 7.5g de leucine par jour tandis que le groupe placébo ingérait une quantité iso calorique d'alanine. Les indices de 1) sensibilité à l'insuline pendant un test de repas métabolique, 2) le contenu (la protéine VDAC et la fonction mitochondriale (respiration et production d'espèces réactives de l'oxygène (ROS)) dans des fibres musculaires perméabilisées, 3) la synthèse protéique musculaire évaluée par l'infusion de L-[anneau-$^{2}H_{5}$]phenylalanine en l'état postabsorptif et postprandial, le profil des fibres musculaires par immunohistochimie, ainsi que la composition corporelle par absorptiomètrie à double rayon-X (DXA), des tests fonctionnels et de forces isométriques maximales ont été mesurés avant et après l'intervention.

Résultats: Nos trouvailles principales ont été que, par rapport à 12 semaines de RT chez des femmes âgées pré-fragiles et fragiles consommant un apport en protéines optimal, une supplémentation en leucine n'avait: 1) aucun effet néfaste ni bénéfique sur la sensibilité à l'insuline; 2) augmentation du contenu mitochondrial sans affecter la fonction ou la capacité de la mitochondrie; et 3) aucun avantage supplémentaire sur les améliorations de la synthèse protéique musculaire, de la fonction musculaire et du phénotype de fragilité.

Conclusion: La supplémentation en leucine ne semble pas influencer la sensibilité à l'insuline et, bien que la leucine ait augmenté le contenu mitochondrial, cela s'est produit sans amélioration associée de la fonction mitochondriale chez les femmes âgées consommant un apport optimal en protéines et entreprenant un ER dans un contexte de fragilité. L'ER et l'apport en protéines

alimentaires décrits dans cette étude semblent stimuler efficacement et de manière significative la synthèse des protéines musculaires basales (état postabsorptif), ce qui se traduit par des gains mesurables en termes d'accumulation des protéines musculaires, de force et de fonction, ce qui entraîne une nette amélioration du phénotype de fragilité.

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DEDICATION

For Andrew, for always.

"Experience: that most brutal of teachers. But you learn, my God do you learn." – C. S. Lewis

CONTRIBUTION OF AUTHORS

For *Manuscript 1*, the candidate designed the study with Dr. José Morais and Dr. Stéphanie Chevalier. The candidate recruited participants and screened participants with Marie Lamarche. The candidate was responsible for the exercise and dietary interventions. The candidate, Marie Lamarche, Drs. Chevalier and Morais conducted the metabolic meal tests. The candidate performed all exercise testing. The candidate performed all statistical analysis, and major data interpretation was made by the candidate with guidance from Drs. Morais and Chevalier. The candidate was the primary author and wrote the manuscript. Drs. Morais and Chevalier provided revisions for the manuscript. The candidate was a recipient of the FRQ-S Bourse de formation en recherche. This study received funds from the MUHC-Montreal General Hospital Foundation through a grant from the Helen McCall Hutchison Family Foundation.

For *Manuscript 2*, the candidate designed the study with Dr. José Morais and Dr. Russell Hepple. The candidate recruited participants and screened participants with Marie Lamarche. The candidate was responsible for the exercise and dietary interventions, and the exercise testing. The candidate, Vita Sonjak, Anna Perez, and Dr. Sally Spendiff conducted the mitochondrial measurements. The candidate performed all statistical analysis, and major data interpretation was made by the candidate with guidance from Drs. Morais and Hepple, and Vita Sonjak. The candidate was the primary author and wrote the manuscript. Drs. Morais, Spendiff, Chevalier, and Hepple provided revisions for the manuscript. The candidate was a recipient of the FRQ-S Bourse de formation en recherche. This study received funds from the MUHC-

Montreal General Hospital Foundation through a grant from the Helen McCall Hutchison Family Foundation.

For *Manuscript 3*, the candidate designed the study with Dr. José Morais and Dr. Stéphanie Chevalier. The candidate recruited participants and screened participants with Marie Lamarche and Carole Spake. The candidate was responsible for the exercise and dietary interventions, and for the work of Carole Spake (Master's student), Jarred Slimovitch (summer student), Anita Hsieh (summer student), and Guy Hajj. The candidate, Marie Lamarche, Dr. Stéphanie Chevalier, and Dr. José Morais conducted the metabolic meal tests. The candidate and Carole Spake performed all exercise testing. The candidate and Vita Sonjak stained the muscle fibers and the candidate performed subsequent analysis. The candidate, Marie Lamarche, and Guy Hajj prepared the tissue for FSR measurement. Marie Lamarche and Guy Hajj obtained the FSR data. Guy Hajj and Dr. Stéphanie Chevalier analyzed the raw FSR data. Anita Hsieh analyzed all food recalls. Jarred Slimovitch analyzed all raw accelerometer data. The candidate analyzed the DEXA data. The candidate performed all statistical analysis, and major data interpretation was made by the candidate with guidance from Drs. Morais and Chevalier. The candidate was the primary author and wrote the manuscript. Dr. Morais provided revisions for the manuscript. The candidate was a recipient of the FRQ-S Bourse de formation en recherche. This study received funds from the MUHC-Montreal General Hospital Foundation through a grant from the Helen McCall Hutchison Family Foundation.

ADVANCE OF SCHOLARLY KNOWLEDGE

Statement of originality

This doctoral dissertation provides, for the first time, an analysis on the effect of 3 months of progressive resistance training with optimal dietary protein intake, with and without daily leucine supplementation in pre-frail and frail elderly women, on body composition, physical performance, multiple anabolic and metabolic parameters, as well as multiple characteristics of muscle fiber and mitochondria functioning.

Manuscript 1: Although resistance training has been shown to improve insulin sensitivity in late-middle-aged individuals, the combined effect of resistance training and leucine supplementation on insulin sensitivity has never been assessed in older women in the context of frailty, a condition associated with impaired glucose tolerance. Furthermore, leucine has conflicting implications on insulin sensitivity, being associated with both improving upon and contributing to the pathology of insulin resistance. This is the first study to assess the effects of 3 months of progressive resistance training with or without daily leucine supplementation in pre-frail and frail older women with well controlled and optimized dietary intake on measurements of insulin sensitivity including: 1) *in vivo* plasma glucose and 2) serum insulin responses to a standard mixed liquid formula meal, and 3) fasting glucose and HOMA-IR.

Manuscript 2: The mitochondria function profile of *ex vivo* measurements (respiration, reactive oxygen species production, sensitivity to mitochondria permeability transition pore opening, and quantity of mitochondria) has never been assessed in older women in the context of frailty. Furthermore, the effect of resistance training and the combined effect of resistance training and

daily leucine supplementation has never been investigated in this cohort. Although some studies exist in measuring mitochondria respiration in a variety of older persons and after resistance training, fewer studies have assessed reactive oxygen species production, and none have measured sensitivity to mitochondria permeability transition pore opening with frailty. Thus, this study is the first to assess mitochondria: 1) respiration; 2) reactive oxygen species production; 3) sensitivity to mitochondria permeability transition pore opening; and 4) quantity of mitochondria in a well-controlled cohort of pre-frail and frail older women with well controlled and optimized dietary intake, before and after 3 months of progressive resistance training with or without daily leucine supplementation.

Manuscript 3: The anabolic combination of strict leucine supplementation and resistance training has seldom been investigated in older persons, and never in the context of frailty and women. The few previously published studies have suffered from either a lack of strict leucine supplementation with appropriate control/placebo, uncontrolled habitual dietary intake (especially protein), heterogenous cohort of sexes, and/or a lack of robust measurements for defined outcomes. In addition, although these few studies have attempted to provide indications of muscle growth, none have measured the rate of muscle protein synthesis *in vivo*. This is the first study to assess: 1) *in vivo* postabsorptive and postprandial rates of myofibrillar protein synthesis; 2) frailty profile; 2) a comprehensive battery of physical performance tests; and 3) fiber type profiles in a well-controlled cohort of pre-frail and frail older women with well controlled and optimized dietary intake, before and after 3 months of progressive resistance training with or without daily leucine supplementation.

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%fat	percent body fat
1RM	1-repetition maximum
^{31}P-MRS	^{31}P-magnetic resonance spectroscopy
6MWT	six minute walk test
A1c	hemaglobin A1c
AA	amino acid
ACR	acceptor control ratio
ADP	adenosine diphosphate
Akt	protein kinase B
Ala	alanine
AMMI	appendicular muscle mass index
AMPK 5	' adenosine monophosphate-activated protein kinase
ANOVA	analysis of variance
ATP	adenosine triphosphate
BCAA	branched chain amino acids
BM	body mass
BMI	body mass index
BSA	bovine serum albumin
Ca^{2+}	calcium
CSHA	Canadian Study of Health and Aging
CHAMPS	Community Healthy Activities Model Program for Seniors
CHO	carbohydrate
CHS	Cardiovascular Health Study
CIM	Centre for Innovative Medicine
C_{max}	maximum concentration
CO2	carbon dioxide
CRC	calcium retention capacity
CRP	c-reactive protein
CSA	cross sectional area
D_2O	deuterated water
DTT	dithiothreitol
DXA	dual energy x-ray absorptiometry
EAA	essential amino acid
ECG	electrocardiogram
eEF2	eukaryotic elongation factor-2
eEF2K	eukaryotic elongation factor-2 kinase
E_{FAA}	enrichment of the precursor plasma free amino acid pool
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
eIF2B	eukaryotic initiation factor 2B
eIF4E	eukaryotic initiation factor 4E
eIF4E-BP1	eukaryotic translation initiation factor 4E-binding protein 1
E_{pb1}	enrichment of protein-bound phenylalanine at postabsorptive timepoint

E_{pb2}	enrichment of protein-bound phenylalanine at 2 h postprandial timepoint
EWGOP	European Working Group on Sarcopenia in Older Persons
FFM	fat free mass
FI	Frailty Index
FOXO3a	Forkhead box O3
FP	Frailty Phenotype
FSR	fractional synthesis rate
G+M	glutamate and malate
GAP	GTPase-activating protein
GATOR1	GAP activity towards Rags complex 1
GATOR2	GAP activity towards Rags complex 2
GDP	guanine diphosphate
GDS	geriatric depression score
GEF	guanine nucleotide exchange factor
GLUT4	glucose transporter type 4
GSK3	glycogen synthase kinase 3
GTP	guanosine-5'-triphosphate
Hb	hemoglobin
HIF	Hypoxia-inducible factor
HIRT	high intensity resistance training
HIV	human immunodeficiency virus
HMB	β-hydroxy-β-methyl butyrate
HOMA-IR	homeostatic model assessment of insulin resistance
HRP	horseradish peroxidase
IAAO	indicator amino acid oxidation
IGF-1	insulin-like growth factor 1
IRS1	insulin receptor substrate 1
IS	insulin sensitivity
KMES	2-(N-Morpholino)ethanesulfonic acid potassium salt
LBM	lean body mass
LC/MS	liquid chromatography tandem mass spectrometry
Leu	leucine
LRS	leucyl-tRNA synthetase
Mfn2	Mitofusin-2
MHCI	myosin heavy chain type 1
MHCIIa	myosin heavy chain type 2a
MHCIIx	myosin heavy chain type 2x
mLST8	mammalian Lethal with SEC13 protein 8
MMI	muscle mass index
MMSE	mini mental state examination
MNB	muscle net balance
MPB	muscle protein breakdown
MPS	muscle protein synthesis
mPTP	mitochondrial permeability transition pore

mRNA	messenger RNA
mTOR	mammalian target of rapamycin
mTORC1	mammalian target of rapamycin complex 1
MUAMA	mid-upper arm muscle area
MUHC	McGill University Health Centre
MyoFSR	myofibrillar fractional synthesis rate
N-balance	nitrogen balance
OGTT	oral glucose tolerance test
OMM	outer mitochondrial membrane
p70S6K1	ribosomal protein S6 kinase beta-1
PBS	phosphate buffered saline
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI3K	phosphatidylinositol 3-kinase
PPAR	Peroxisome proliferator-activated receptor
PRAS40	Proline rich Akt substrate of 40 kDa
Rags	Ras-related GTPases
Raptor	regulatory associated protein of mTOR
RDA	recommended dietary allowance
RE	resistance exercise
REE	resting energy expenditure
Rheb	Ras homolog enriched in brain
RIPA	Radioimmunoprecipitation assay
ROS	reactive oxygen species
rpS6	ribosomal protein S6
RT	resistance training
SEM	standard error of the mean
SFT	seniors fitness test
SIRT	sirtuin
SMI	skeletal muscle mass index
SPPB	short physical performance battery
Succ	succinate
TBC	Tre-2/Bub2/Cdc16
TBC1D7	TBC1 Domain Family Member 7
TSC1	Tuberous sclerosis complex 1
TSC2	Tuberous sclerosis complex 2
TSH	thyroid stimulating hormone
TUG	timed up and go
TUT	time under tension
v-ATPase	vacuolar H+-ATPase
VDAC	voltage-dependent anion channel
VO_{2peak}	peak rate of oxygen uptake
WHO	World Health Organization
WHR	waist to hip ratio
YA	young adult

CHAPTER 1: INTRODUCTION

1.1. Rationale

The World Health Organisation (WHO) has predicted that globally by 2050 those over 60 years old will double from about 11% to 22% of the population, making this cohort the world's fastest growing age bracket. One of the many resulting ramifications is a consequent increase in the incidence of chronic health conditions such as frailty. Frailty is a measurable clinical entity that encompasses states of vulnerability, the consequences include, but are not limited to, increased risk of falls [1], loss of independence [2], disability, depression & social isolation, increased risk of morbidity [3] and mortality [4-6]. The loss in muscle strength, mass, and performance (sarcopenia) prevalent in aging [7] can be both a precursor to, and the physical manifestation of, frailty. Reduced muscle mass means that there are fewer contractile elements and therefore diminished capacity to generate force [8]. Therefore, the ability to retain or regain muscle mass and strength is of paramount importance in aging individuals.

The physiological response to anabolic stimuli such as feeding and resistance exercise is blunted with aging [9, 10]. The blunted anabolic response to feeding can be overcome with sufficiently large intakes of protein per meal [11]. In fact, older persons have been shown to require approximately double the amount of protein per meal than their younger counterparts (~0.24 versus ~0.40 g protein·kg body mass^{-1}·meal^{-1}, in older versus younger persons) [12]. To illustrate, a 75 kg older woman would be required to ingest 30 g of high-quality protein per

meal. However, many older persons might find consuming high amounts of protein per meal unmanageable [13]. Thus, other solutions to ameliorate anabolic resistance are needed.

The essential amino acid, leucine, is not only a substrate for protein synthesis but is also unique in its ability to stimulate muscle protein synthesis, independent of insulin [14]. Acutely, it has been shown that the blunted anabolic response to feeding with aging can be overcome with co-ingestion of higher amounts of leucine [15-20]. These acute studies have highlighted the potential for leucine, given in sufficient quantities, to overcome the anabolic resistance to protein feeding without the need to ingest large quantities of protein [12]. Chronic leucine supplementation studies have yielded conflicting results on improving muscle mass and strength in older persons [21], however, evidence suggests that frail and/or sarcopenic persons are candidates better-suited to benefit from chronic leucine supplementation [22].

Older adults are able to increase their muscle strength through resistance training (RT), however the muscle hypertrophic (anabolic) response is not as straightforward [23, 24]. Most studies have shown a blunted hypertrophic response to RT [23, 25-30], while others have not [31, 32]. Importantly, women have been shown to have a reduced hypertrophic response to RT than men [33, 34].

Given that the anabolic effectiveness of both RT and feeding suffers with aging, it stands to reason that the anabolic response to resistance training could be augmented in combination with leucine supplementation, given sufficient habitual dietary protein intake. Interventions

have commonly not controlled for dietary protein intake, thus the nature of the amino acid/protein supplementation is an important confounding factor. There is compelling evidence that older persons should be consuming greater amounts of protein to mitigate sarcopenia and maintain health (~1.2 $g \cdot kg^{-1} \cdot d^{-1}$) [35, 36] than the current recommended dietary allowance (0.8 $g \cdot kg^{-1} \cdot d^{-1}$) [37]. Furthermore, the existing studies that have implemented a combined intervention (leucine or essential amino acids (EAA) high in leucine content) and RT have neglected to include a nitrogen-equivalent placebo control group [38, 39], which is an additional and unnecessary source of variation. The effect of RT in combination with leucine supplementation on the rate of (MPS) has never been investigated in the context of frailty in women.

A blunted protein anabolic response has been postulated to also be due in part to a decrease in insulin sensitivity (IS) [40]. Insulin resistance is a significant risk factor for type 2 diabetes, cardiovascular disease, and frailty [41, 42]. Because skeletal muscle accounts for the majority of the insulin-stimulated glucose uptake, it is an attractive target for interventions aimed at increasing IS [43]. Although RT has been shown to improve IS in late-middle-aged individuals [44], there are little data on how IS can be modulated by RT in frail older persons [45]. Leucine supplementation is an intervention that could simultaneously improve upon frailty, sarcopenia, and insulin resistance. Leucine supplementation has, however, been shown to have both beneficial [46-49], and detrimental [50, 51] implications on IS. The effect of RT in combination with leucine supplementation on IS has never been investigated in the context of frailty in women.

A decline in skeletal muscle mitochondria function has been observed with aging [52] and experimental evidence suggests that mitochondria are heavily implicated in the aetiology of sarcopenia [53]. Mitochondria produce reactive oxygen species (ROS) that can potentially cause damage to themselves [54]. Damaged mitochondria not only have a hindered respiratory capacity but can also produce elevated levels of ROS, resulting in further damage to mitochondria [55, 56]. They can also present with increased susceptibility to apoptosis via decreased calcium retention capacity (CRC) and/or time to mitochondrial permeability transition pore (mPTP) opening, leading to fiber atrophy and hampered muscle performance [57-60]. RT has been shown to significantly increase mitochondrial content in elderly older adults [61], and specifically women [62].

The stimulation of protein synthesis by leucine is an energy-consuming process, necessitating mitochondrial function to replenish consumed ATP [63]. It has recently been shown that leucine has the capability to stimulate mitochondrial biogenesis in cultured myocytes [64, 65]. Previous studies investigating the effects of RT on mitochondria in the elderly have not comprehensively assessed multiple aspects of mitochondrial function simultaneously [62, 66-69], and never in the context of frailty in women.

1.2. Thesis Objectives and Hypothesis

Three objectives and hypotheses are at the core of the work contained in this thesis, delineated in three manuscripts.

1.2.1. Manuscript 1

Objective 1: to determine the effects of leucine supplementation on insulin sensitivity in pre-frail and frail older women undergoing resistance training with optimal dietary protein in a randomized double-blinded placebo-controlled study design.

Hypothesis 1: we hypothesized that 12 weeks of resistance training would improve insulin sensitivity, with an added benefit when supplemented with leucine compared to placebo, in pre-frail and frail older women habitually consuming an optimal amount of dietary protein.

1.2.2. Manuscript 2

Objective 2: to examine the effects of resistance training and leucine supplementation in pre-frail and frail older women maintaining optimal dietary protein on mitochondrial content and functioning in a randomized double-blinded placebo-controlled study design.

Hypothesis 2: we hypothesized that 12 weeks of resistance training would improve inherent mitochondrial function (respiration, ROS production, and sensitivity to apoptosis) and/or increase the quantity of mitochondria to improve net mitochondrial function in pre-frail and

frail older women habitually consuming an optimal amount of dietary protein, with an added benefit when supplemented with leucine compared to placebo. In addition, these improvements would occur in conjunction with functional improvements in skeletal muscle such as maximal strength and aerobic performance.

1.2.3. Manuscript 3

Objective 3: to examine the effects of resistance training and leucine supplementation in pre-frail and frail older women habitually consuming an optimal amount of dietary protein on muscle protein synthesis, frailty status, muscle performance, body composition, and myofiber type profile in a randomized double-blinded placebo-controlled study design.

Hypothesis 3: we hypothesized that 12 weeks of resistance training would improve the primary outcome of myofibrillar fractional synthesis rate (fasted and/or fed states); and secondary outcomes of increasing maximal muscle strength and improving physical performance, increasing muscle mass and fiber cross-sectional area. There would be an added benefit to these measurements when supplemented with leucine compared to an isonitrogenous placebo, in pre-frail and frail older women habitually consuming an optimal amount of dietary protein.

CHAPTER 2: REVIEW OF THE LITERATURE

2.1. Aging Population

Persons greater than 65 years of age in Canada currently represent 16.5% of the population with this percentage projected to double in the next 40 years, with those greater than 80 years expected to triple [70, 71]. This trend is not limited to Canada but is manifesting on a world-wide level. The WHO has predicted that globally by 2050 those over 60 years old will double from about 11% to 22% of the population. Numerically, this corresponds to approximately 2 billion people aged 60 or older, with 400 million of those being 80 years old or more [72]. This profound change in aging population dynamics has vast and interconnected ramifications in society. Of particular interest to health care providers is the resulting increase in the incidence of chronic health conditions, physical impairment, and disability. The WHO has estimated the number of elderly people requiring long-term care due to the aforementioned conditions will quadruple by 2050 [72].

2.2. Sarcopenia & Frailty

One of the plethora of physiological age-related changes is sarcopenia. The term "sarcopenia" was coined by Dr. Irwin Rosenberg in 1989 (Greek "sarx" for flesh, and "penia" for loss) to describe the observed age-associated loss in muscle mass and strength. In 2009-2010, a practical working definition of sarcopenia was developed by the European Working Group on Sarcopenia in Older Persons (EWGOP) as "a syndrome characterized by progressive and generalized loss of skeletal muscle mass and strength with a risk of adverse outcomes such as

physical disability, poor quality of life and death" [7]. Of note, the EWGOP met again very recently and published a revised definition using low muscle strength as the primary indicator of sarcopenia, confirmed when low muscle quantity, quality, or physical performance is detected [7]. Approximately 30-40% of the young adult human body is muscle [73]. In addition to the locomotive function of muscle, it is also a major means of blood disposal of glucose and fat, contributor to resting (basal) metabolic rate, and reservoir of proteins that can be broken down into amino acids and released into the bloodstream during fasting and stressful events (e.g., starvation, inflammation, etc.) [74]. Thus, any changes to muscle are of large physiological consequence. The magnitude of sarcopenia is still growing with current population projections forecasting that by the year 2050 approximately 200 million people will be sarcopenic [75]. The consequences of sarcopenia are severe: the likelihood of functional impairment and disability has been estimated to be 2-4 times greater in those with sarcopenia than without [2, 76, 77]. To illustrate, elders with low muscle strength are ~2.6 times more likely to develop severe mobility limitations, ~4.3 times more likely to have slow gait speed, and are at a ~2.1-fold greater risk of mortality compared to older adults with high muscle strength [78]. Besides hindering locomotion, aging leads to difficulties in performing daily activities, increases the incidence of falls and the length of recovery, loss of autonomy, and when advanced, muscle wasting is correlated to morbidity and increased mortality [79]. In fact, in the year 2004, [80] calculated that a 10.5% reduction in sarcopenia could annually save the United States economy a staggering 1.1 billion USD in health care costs. If left unchecked, sarcopenia can further deteriorate to frailty and disability [81], cumulating in enormous economic burdens for healthcare systems on a global level [82, 83]. In fact, sarcopenia can be both a precursor to and

the physical manifestation of frailty. Frailty is a measurable clinical entity that encompasses states of vulnerability due to decreased functional physiological reserves across multiple physiological systems. The consequences of frailty include, but are not limited to, increased risk of falls [1], loss of independence [2], disability, depression & social isolation, increased risk of morbidity [3], and mortality [4-6].

2.2.1. Methods of Assessing Frailty

A multitude of tools (up to 67) to assess frailty have been developed [84, 85]. While there is no accepted gold-standard for measuring frailty, two of the most widely-used indices are Rockwood's Frailty Index (FI), and the Frailty Phenotype (FP) based on Fried Criteria. Rockwood's Frailty Index (FI) is the sum of the number of diseases and physical and psychosocial afflictions present in an older person divided by the total number of indexed conditions [86]. This index has been shown to have predictive value for poor outcomes, but due to its inherent inability to separate frailty from underlying causal comorbidities and also its inclusion of resultant disabilities it is much more a co/multimorbidity index than a true frailty measure. The FP [87] has been tested the most extensively for its validity, and is the most widely implemented frailty scale in research [85].

2.2.1.1. The Frailty Phenotype

The FP was developed from data collected in the Cardiovascular Health Study (CHS) in the USA of men and women (Caucasian and African-American), 65 y and older, with a broad

range of socioeconomic, functional, and health statuses. It was based upon a hypothesized cycle of frailty in [88] and demonstrates predictive validity for adverse geriatric outcomes (falls, hospitalizations, disability, and death) with a 3-y and 7-y follow-up period [87]. The Fried Criteria consists of the following: 1) unintentional weight loss/sarcopenia; 2) self-reported exhaustion; 3) muscle weakness; 4) slow walking speed, and; 5) low physical activity. Its main drawback lies in that different scales utilize different classification of the individual components (e.g., unintentionally losing more than 10 lbs, *or* 5% of body weight in one year). A person is considered frail if they meet three or more of the criteria. If they meet 1-2 criteria they are considered prefrail, and healthy if no criteria are met [87]. The use of the Fried Criteria to measure frailty results in a lower estimation of prevalence than other broader definitions such as the FI, but allows for relatively easier comparisons between studies [89]. In a recent systematic review by Choi, Ahn [89], six published journal articles were identified that assessed the prevalence of the FP in community-dwelling individuals representative of the national population. They reported that the prevalence of frailty increases with aging from ~5% in those 65-75 years to ~27% in those 85 years and older. Prefrailty also increases with aging from ~35% to ~51% in the same respective age groups [89]. The authors speculated that the variability seen between the six studies could be due to the subjective components of some of the Fried Criteria and the use of sample-specific cut points for other components. Using data from the Canadian Study of Health and Aging (CSHA), the prevalence of frailty using the Geriatric Status Scale [90] in community dwelling individuals ranged from ~7% (aged 65-74 years) to ~18% (aged 75-84 years) to ~37% (aged 85 years and older) [91]. In a longitudinal study over 7.5 years using the FP on 268 community-dwelling women who were non-frail at baseline, 66% became

prefrail; while of the 152 women who were prefrail at baseline, 23% became frail [92]. Additionally, the original Cardiovascular Health Study found that the prevalence of frailty was up to twofold higher for women than men by age group up until 90 y and older [87].

2.3. Muscle Changes with Aging

2.3.1. Changes in Muscle Strength with Aging

The decline in muscle mass and strength with aging are predominant features of both interrelated conditions of frailty and sarcopenia. The loss of strength with age is sometimes referred to as dynapenia. Longitudinal studies have reported an annual rate of muscle strength decline of 1-3% of the knee extensors beginning in a person's mid-40's [93-95] which greater in magnitude than the loss of muscle mass [96]. Other groups have reported a reduction of ~10%-15% per decade up to the age of 70 y, when the loss accelerates to ~25%-40% per decade [95, 97]. In fact, both cross-sectional and longitudinal studies have consistently noted an acceleration in the decline of muscle mass and force during the eighth decade of life [98]. Consistent with muscle strength and mass, physical function also decreases with aging. In women, declines in physical function are detected in conjunction with menopause and continue to decline throughout the remainder of lifespan [99-101]. The mechanism of physical functional decline is multifactorial and can largely be attributed to interrelated factors of body composition, physical activity, and muscle physical function [102]. A recent study [8] in young (~22 y) and old (~72 y) men & women with a 5-year follow-up in the older group concluded that muscle weakness was largely attributed to loss in muscle mass and lesser extent specific force

(muscle quality), and is the primary factor in progressive muscle weakness late into the seventh decade of life [8]. Current evidence indicates that the loss of muscle mass is a consequence of a decrease in size of cross-sectional area (CSA) of single myofibers as well as a decline in fiber number [8]. However, to what degree the functional decline in whole muscle is attributable to the decrease in the single myofibers' ability to develop force and power remains to be determined. Interestingly, single-fiber function remains relatively well-preserved into advanced ages [94, 103-106]. To illustrate, one longitudinal study of 5 elderly men (~71 y) tested after a 9 year follow-up reporting that although mass and strength significantly decreased, single-fiber maximal force production and maximum unloaded shortening velocity did not significantly differ at follow-up [94]. However, some cross-sectional studies have observed declines in various parameters of contractile functioning [107-109]. Although beyond the scope of this thesis, recent evidence suggests that the loss of whole muscle mass and strength is at least partially due to denervation of muscle fibers and reduced capacity for reinnervation [110, 111]. It is also worth noting that the decline in muscle quality (strength divided by area) with aging is postulated to be due in part to fat infiltration, primarily affecting muscle strength [112-114].

2.3.2. Changes in Muscle Mass with Aging

Concurrent with dynapenia is a loss of muscle mass. In a 2012 quantitative review, the median loss of muscle mass per decade from peak mass across studies was ~5% and ~4% for men and women, respectively [115]. Additionally, after the age of 70 y decreases in muscle mass has been estimated to be ~0.5-1% per year [115]. At the level of the muscle fiber,

although some studies [116, 117] did not identify any age-dependent atrophy, most studies agree that fast type 2 myofibers do undergo significant atrophy [8, 118-122]. Changes in type 1 myofibers with aging are more complex. A marked heterogeneity in fiber size consisting of extremely large and extremely small myofibers may mask age-related atrophy of type 1 fibers [122-125]. It is postulated that compensatory hypertrophy of some type 1 fibers is due to those fibers attempt to negate the functional deficits of atrophic fibers as well as possibly also compensating for hypoplasia [94].

2.3.3. Sexual Dimorphism in Muscle Changes with Aging

Although not a universally observed change, in general it has been found that men lose more muscle mass and strength than women do with aging, especially in regard to the lower limbs [117, 126]. Additionally, upper limb strength is better preserved in older women than older men [95]. A recent comprehensive study on sexual dimorphism in muscle composition of fiber type across multiple age categories for men and women revealed that women have a significantly higher proportion of type 1 fibers, and muscle atrophy in women may predominantly be driven by type 2 myofiber atrophy [123]. Compared to aging men, aging women had greater type 2 myofiber atrophy and greater CSA heterogeneity, but may also retain more fibers than males (i.e., less hypoplasia) [123]. There are currently no studies on fiber type profiles and changes with hypertrophic interventions in older women who have conditions related to heightened atrophy such as frailty.

2.4. Anabolic Resistance with Aging

Net muscle mass is determined by the balance between muscle protein synthesis (MPS) and breakdown (MPB). A negative (catabolic) net muscle protein balance occurs in the fasting state (postabsorptive) when MPB>MPS but becomes positive (anabolic) when MPS>MPB upon the increased circulating levels of insulin and amino acids (AAs) after feeding (postprandial) [127]. In weight-stable individuals, over the course of a day the postprandial net positive muscle protein balance and the postabsorptive net negative muscle protein balance abrogate each other resulting in the overall maintenance of muscle mass. Sarcopenia must therefore be the result of an incessant negative net protein balance in which MPS fails to match MPB over time. Aging appears to have no effect on rates of MPB in the basal postabsorptive state [79, 128-130] though some studies show otherwise [131, 132], while in the postprandial (hyperaminoacidemic and/or hyperinsulemic) state evidence remains inconclusive [129, 133, 134]. The effect of aging on basal MPS rate is more ambiguous, with some [131, 132, 135] but not all [20, 130, 134, 136-140] studies showing suppression with age. Studies on postprandial MPB with aging are lacking, which is mostly attributed to methodological limitations. Additionally, the anabolic effects of postprandial insulin secretion are also blunted with aging at the whole-body level [40, 129].

2.4.1. Anabolic Resistance to Muscle Protein Synthesis with Aging

The literature regarding the effect of aging on postprandial MPS is more plentiful and remarkably varied due in part to the different compositions and modalities of feeding (see [9]

for a detailed review), with studies showing suppression [11, 20, 134, 135, 141, 142], delayed [143] or no suppression of MPS in response to feeding between young and old participants [11, 20, 130, 138, 141, 144-149]. To illustrate the importance of protein quantity, MPS response has been shown to be ameliorated in the elderly with increased protein dose [11]. While 10 g of EAA was sufficient to illicit a maximal MPS response in young persons, 40 g of EAA was necessary to illicit a similar maximal MPS response in the elderly [11]. Indeed, a dose-dependent MPS response to protein exists whereby the response plateaus at a given dose. At quantities beyond the plateau point, the excess amino acids are oxidized rather than synthesized into muscle [150, 151]. A recent study [12] found that the relative amount of protein required per meal to illicit a maximal MPS response is considerably greater in older persons (~0.4 g·kg BW^{-1}) compared to their younger counterparts (~0.24 g·kg BW^{-1}). It is therefore apparent that aging impairs the postprandial anabolic response so that more protein is needed to elicit the physiological switch to positive net protein balance, the point at which this occurs is termed the anabolic threshold [79]. This adverse effect of aging on MPS is known as anabolic resistance. Unless compensated for, the normal equilibrium between muscle anabolism and catabolism is lost with the overall net muscle protein balance becoming negative, and over time the net amount of muscle protein declines with age contributing to sarcopenia and frailty.

2.4.2. Anabolic Resistance to Resistance Exercise with Aging

Resistance training is a potent stimulus to improve muscle mass, strength, and function in older adults [152]. However, resistance to anabolic stimuli other than feeding, such as RT, also occurs during aging in part due to low habitual physical activity [10]. Although older adults are able to increase their muscle strength through RT, the muscle hypertrophic (anabolic) response is not as straightforward [23, 24]. Older persons consistently show a blunted anabolic response to a single bout of resistance exercise (RE) in men [32, 139, 153-155] and women [29], with a concurrent blunting of the activation of the major regulator of protein synthesis, mTORC1 (mammalian target of rapamycin complex, see section 2.6 "Cellular Regulation of Muscle Protein Synthesis") [32, 155], while in young individuals mTORC1 activation remains elevated for ≥24 h post-exercise [155]. Downstream of mTORC1, p70S6K1 (ribosomal protein S6 kinase beta-1) signaling has also been shown to be blunted after acute resistance exercise in older individuals [139, 155, 156], while rpS6 (ribosomal protein S6) phosphorylation occurred in both young and older men [156]. Chronically, most RT studies have shown a blunted hypertrophic response to RT [23, 26-30, 156], while others have not [31, 32]. Women have been shown to have a reduced hypertrophic response to training in comparison to men [33, 34]. Additionally, despite the wide variety of training protocols, a meta-analysis of persons ≥50 y undergoing a wide-variety of RT (duration of 10 to 52 weeks) noted that gains in lean body mass (LBM) were modest (~1 kg), lending support to a blunted anabolic response to RT with aging [28]. Most of these previous studies did not investigate mechanisms of the blunted anabolic response to RT. Recent studies have postulated that blunted ribosomal biogenesis (reduced translation efficiency) is a potential explanatory mechanism [156, 157]. To the best of

our knowledge, only one study has attempted to investigate the effect of exercise training that included RT on the rate of MPS in a context that included frailty [158]. The authors reported that postabsorptive but not postprandial mixed muscle FSR was improved post-intervention. However, frailty was defined as scoring low on several functional indices and not using the FP, the participants were obese, included both sexes, dietary intake was not measured, the exercise intervention was multimodal (endurance, balance, and resistance training), and FSR was measured 12-14 h after the last bout of exercise. Thus, this study was not able to capture a true basal (postabsorptive) mixed muscle FSR, as it was measured well-within the timeframe for exercise-induced heightened sensitization to anabolic stimuli [159, 160] as well as elevation of mixed muscle MPS [161].

2.5. Determining Dietary Protein Requirements

2.5.1. Nitrogen Balance

A person's dietary protein requirement is reflected in their state of nitrogen balance. A positive balance exists when one consumes excess protein than that needed by the body (the excess is oxidized and excreted). A negative balance exists when one consumes insufficient amounts of protein to meet the body's needs. The balance is calculated on dietary nitrogen intake (primarily protein) and nitrogen losses through urine, feces, skin (primarily sweat), and miscellaneous (e.g., hair, nails, exhaled ammonia) [162]. The minimum amount of dietary protein that maintains the body protein mass (completely offsets nitrogen losses) in those at energy balance with modest levels of physical activity defines the dietary protein requirement

for healthy adults [163]. Determining nitrogen balance is an extremely lengthy and meticulous process involving participants consuming multiple different nitrogen intakes, each for a period of 10-14 days, with balance being measured for the last 3-5 days of each period [162]. Linear regression on the resulting data is then used to calculate a nitrogen balance of zero (intake = losses).

The currently accepted recommended dietary allowance (RDA) for protein intake from 2005 for North America (Canada and the United States) is 0.8 $g \cdot kg^{-1} \cdot d^{-1}$ [37], which was calculated based off of a meta-analysis of available individual nitrogen balance data in adults [162]. Theoretically, this is the amount of dietary protein that would meet the requirements of 97.5% of the healthy adult population. However, due to the nature of the experiments required, very few nitrogen balance studies exist in older persons [162, 164], disallowing for age-specific recommendations (see [165] for a detailed review).

2.5.2. Indicator Amino Acid Oxidation

A second methodology to determine dietary protein requirements is the indicator amino acid oxidation (IAAO) technique. This is a technique independent from nitrogen balance that uses a labelled amino acid (the "indicator") in excess quantities, while participants receive increasing intakes of dietary protein. If an intake of dietary protein is below the requirement, then the indicator will not be fully utilized by the body for protein synthesis and will be oxidized (measured as $^{13}CO_2$ in expired breath). As dietary protein intakes are increased, indicator

oxidation will decrease until at a given protein intake oxidation plateaus (the breakpoint), which is taken as the estimated average dietary protein requirement [163, 166]. The RDA protein requirements derived from IAAO studies are markedly greater than the current RDA derived from nitrogen balance studies. In fact, nitrogen balance studies may underestimate protein needs by up to 30% [167, 168]. Using the IAAO technique, protein requirement and safe intake amount (used where there is insufficient evidence to set an RDA) has been measured as 1.24 $g \cdot kg^{-1} \cdot d^{-1}$ in older men [167], 1.29 $g \cdot kg^{-1} \cdot d^{-1}$ in older women [169], and 1.15 $g \cdot kg^{-1} \cdot d^{-1}$ in octogenarian women [168]. However, there is still no consensus in the literature as to whether or not official dietary guidelines should be modified [165, 170-172].

Studies using stable isotope tracers to measure MPS have provided further support with a recent retrospective analysis [12] of 6 studies measuring MPS over a postprandial (0-40 g protein) period of 3-4 hours determining that the amount of protein relative to body weight required to maximally stimulate MPS was 67% higher in older versus younger men. Taken together with stable isotope tracer studies [9, 11, 12, 150, 151], there is compelling evidence that older persons should be habitually consuming higher amounts of protein (~1.2 $g \cdot kg^{-1} \cdot d^{-1}$) [35, 36]. Indeed, some recent studies suggest even greater amounts of dietary protein be consumed in older persons per day [173, 174].

2.5.3. Sexual Dimorphism in Protein Requirements and Muscle Protein Metabolism with Aging

It is well known that healthy women typically have less LBM and more fat mass than men [175, 176], and therefore exert a lesser maximal force than men [177, 178]. However, maximal strength normalized to muscle CSA or mass is comparable between sexes [179, 180]. As mentioned prior, the rate of age-associated muscle atrophy is higher in men than women [175, 181-183]. MPS and MPB appear to be similar between young men and women when muscle mass is stable, either at rest or after exercise [184-187]. To date, few studies have investigated the effect of age on sexual dimorphism in muscle metabolism and existing studies have focused only on MPS. Sexual dimorphism becomes apparent in more advanced ages [187, 188], with older women having a significantly higher rate of basal postabsorptive MPS compared to old and young men as well as young women [187]. Additionally, there is evidence that older women may have a blunted postprandial anabolic response compared to older men in obese persons, in response to high-leucine-containing proteins, and and exercise training compared to men [15, 17, 187-189]. Concurrently, it has been suggested that compared to older men, older women may also have elevated protein requirements [168, 169].

2.5.4. Frail Protein Metabolism at Whole-Body

Pertinently, we have found reduced muscle mass, but higher rates of whole-body protein turnover in 8 frail elderly women compared with healthy elderly controls [190]. Frail women had a higher contribution of muscle proteolysis (N-tau-methylhistidine) to whole-body

protein breakdown, possibly sparing visceral proteins, at low protein intakes (0.8 g·kg BW^{-1}·d^{-1}). However, they retained the capacity to conserve nitrogen when provided with an increased amount of protein (1.2 g·kg BW^{-1}·d^{-1}), for 12 days. If the higher protein intake is sustained for long enough periods of time, this could translate into increased muscle mass and thus be of benefit to frail elderly women [190].

2.6. Cellular Regulation of Muscle Protein Synthesis

The origin of anabolic resistance in the muscle remains to be elucidated. Current possible reasons include sedentariness [10], impaired protein digestion and amino acid absorption, reduced blood flow and thus delivery of amino acids to the muscle, impaired amino acid transport into the myofibers, and impaired intracellular anabolic signaling [191]. For the purposes of this thesis, only anabolic signaling will be discussed. At the cellular level, the protein synthesis signaling pathway is activated by two main stimuli upon feeding, being insulin-dependent and insulin-independent (e.g., leucine). Both pathways converge on mTORC1 activation.

2.6.1. Insulin-Dependent Anabolic Signaling

mTORC1 is formed by mammalian target of rapamycin (mTOR), regulatory associated protein of mTOR (Raptor), and mammalian Lethal with SEC13 protein 8 (mLST8). mTORC1 mainly acts on translation initiation of protein synthesis. The insulin-dependent pathway begins with insulin being secreted from the pancreas into the blood upon feeding, whereby it binds to

its cellular insulin receptor which then phosphorylates insulin receptor substrate 1 (IRS1). This activates the phosphatidylinositol 3-kinase – protein kinase B (PI3K/Akt) signaling cascade which both induces glucose transporter type 4 (GLUT4) translocation to the cell membrane, and also acts on several key inhibitory proteins upstream of mTORC1. These proteins include glycogen synthase kinase 3 (GSK3), Tuberous sclerosis complex 2 (TSC2), and Proline rich Akt substrate of 40 kDa (PRAS40). GSK3 inhibits mTORC1 by permissive phosphorylation of TSC2, as well as inhibiting eukaryotic initiation factor 2B (eIF2B) by phosphorylation, which is required for formation of a functional complex for translation initiation. Akt acts on GSK3 to inhibit it by phosphorylation. TSC2 forms the TSC-TBC complex with TSC1 and TBC1D7 (TBC1 Domain Family Member 7), which inhibits mTORC1 by regulating the amount of GTP bound Rheb (Ras homolog enriched in brain) proteins (Rheb-GTP), which are needed to activate mTORC1, through the TSC-TBC GTPase activating protein (GAP) domain activity. Akt phosphorylates TSC2 at multiple sites to inhibit the TSC-TBC GAP activity. PRAS40 is a negative regulator of mTORC1 by binding to the Raptor subunit of mTORC1, thereby inhibiting its association with substrates. Akt phosphorylates PRAS40 allowing substrate interaction with Raptor, thereby preventing its inhibitory effect on mTORC1. Akt is also well known to also inhibit FOXO3a, key regulator of protein breakdown, but this is beyond the scope of this thesis and will not be further discussed. Once inhibition mechanisms are removed by Akt, mTORC1 can be activated. Rheb is tethered at the lysosome membrane and exists in an inactive (Rheb-GDP) and active (Rheb-GTP) state, the latter of which activates mTORC1 at the lysosome membrane. The mTORC1 subunit Ragulator acts as a guanine nucleotide exchange factor (GEF), regulated by vacuolar H+-ATPase (v-ATPase). Upon feeding, amino acids induce a conformational change to v-ATPase which

subsequently activates Ragulator's GEF activity. Once active, at the lysosomal surface Ragulator associates with Ras-related GTPases (Rags) to activate mTORC1. Rags exist in heterodimeric pairs, RagA/B bound to RagC/D. In the inactive state RagA/B is bound to GTP (RagA/B-GTP) and RagC/D to GDP (Rag-GDP), and they recruit TSC2, leading to Rheb-GDP inhibition and mTORC1 disassociation from the lysosome. Active Ragulator's GEF activity induces the change from the Rags inactive to active state by switching from RagA/B-GTP to RagA/B-GDP and RagC/D-GDP to RagC/D-GTP, enabling mTORC1 translocation to the lysosome surface where it can interact with Rheb-GTP. Subsequent to the activation of mTORC1, phosphorylation of eIF4E-BP1 (eukaryotic translation initiation factor 4E-binding protein 1) and p70S6K occurs, which is a major regulation step in translation initiation of protein synthesis. The phosphorylation of eIF4E-BP1 releases eIF4E (eukaryotic initiation factor 4E), which when free displays a cap-binding site and alongside several other eIF4 proteins, assembles the pre-initiation complex. This pre-initiation complex is important in facilitating initial ribosomal subunit recruitment to the 5' end of messenger RNA (mRNA) to begin elongation. Phosphorylated p70S6K subsequently phosphorylates rpS6, which results in the modulation of translation initiation factors. Additionally, p70S6K is thought to contribute in the promotion of ribosome biogenesis, thus increasing the translational capacity of the cell. p70S6K also inhibits IRS1, creating a negative feedback loop to the action of insulin. mTORC1 also causes the inhibition of eEF2K (eukaryotic elongation factor-2 kinase) via its phosphorylation, allowing for the phosphorylation of eEF2 (eukaryotic elongation factor-2) contributing to elongation of proteins being synthesized [192, 193]. Thus, the downstream effects of mTORC1 are translation initiation and elongation.

2.6.2. Insulin-Independent Anabolic Signaling: Leucine

Insulin-independent anabolic signaling is primarily achieved by the EAA leucine [194]. Leucyl-tRNA synthetase (LRS) functions as a leucine sensor for mTORC1 by its activity as a GAP for RagD [195]. Leucine has recently been shown in cancer cells to activate mTORC1 through LRS GTP hydrolysis of RagD, thereby effectively "turning on" the Rags to their active states [196]. Sestrin2 has also been proposed as a leucine sensor for the mTORC1 pathway [197], though recent evidence in cancer cells suggests that Sestrin2 functions as an "OFF" switch by the GAP activity towards Rags complex 1 (GATOR1). GATOR1 switches the Rags into their inactive state, thereby inhibiting mTORC1, while GATOR2 is responsible for inhibiting GATOR1. Sestrin2 has been shown to inhibit GATOR2 [196], thereby allowing for the Rags to exist in their inactive states. It remains to be determined if this action of Sestrin2 occurs in normal, healthy cells and tissues.

2.7. Anabolic Effects of Leucine

2.7.1. Muscle Protein Synthesis, Fractional Synthesis Rate

2.7.1.1. Acute Effects of Leucine Ingestion on Fractional Synthesis Rate

The EAA, leucine, is a branched chain amino acid (BCAA) that is not only a substrate for MPS but is also unique in its ability to stimulate MPS, as described above [14]. In 2000 it was discovered that the muscle protein fractional synthesis rate (FSR) was blunted in excised epitrochlearis of aged (~20 mo) compared to adult (~7 mo) Wistar rats following leucine incubation [198]. The maximal FSR of adult rats was achieved in old rats but it took twice as

much leucine to achieve this level of FSR. Subsequently, in humans it was found that 6.7 g of EAAs containing 1.7 g leucine (26%) was sufficient to stimulate MPS in younger, but not older persons [20]. However, when the 6.7 g of EAAs contained a higher proportion of leucine (2.8 g, or 41%), the MPS response was similar to that of young persons (younger persons did not further benefit from 2.8 g of leucine). These results revealed a blunted response to the anabolic stimuli of leucine with aging (young adult vs old), which could be overcome with higher amounts of ingested leucine. These results were further supported by those of [19] studying the effect of leucine co-ingestion (2x the normal postprandial plasma leucine concentration for an equivalent meal) in a complete liquid meal given continuously over a 5 h period in elderly (~70 y) men, resulting in significantly greater postprandial myofibrillar FSR compared to control (alanine co-ingestion). Moreover, a subsequent study on the acute intake of a meal-like amount of protein (20 g bolus of casein) with or without 2.5 g leucine showed an elevated 6 h postprandial de-novo protein synthesis using intrinsically labelled protein in the leucine co-ingesting group compared to 20 g casein alone [18]. Studies in women are less abundant. A 2015 study in older women (~66 y) [17] showed that a 3 g mixture of EAAs containing 1.2 g of leucine was sufficient to induce MPS to the same extent as a 20 g bolus of whey. A more recent study in older women (~69 y) found that myofibrillar FSR significantly increased from baseline with the ingestion of 25 g of whey containing 3 g leucine or 10 g milk protein containing the same amount of leucine (3 g), with no difference in FSR between the two protein intakes [16]. A second study from this same group showed that consuming 15 g of milk protein containing 4.2 g leucine was able to elevate postprandial myofibrillar FSR to a greater extent than the same mixture containing 1.3 g leucine [15]. These latter studies highlight the potential of leucine,

given in sufficient quantities, to overcome the anabolic resistance to protein feeding without the need to ingest large quantities of protein [12]. This is important as many older persons might find consuming 30-40 g of protein per meal (equivalent to ~0.4 g protein·kg^{-1}·meal^{-1} [12]) unmanageable [13]. Although acute co-ingestion of sufficient amounts of leucine were able to induce robust elevations in MPS, even with suboptimal doses of protein, the question remained as to what are the implications of chronic leucine supplementation.

2.7.1.2. Chronic Effects of Leucine Ingestion on Fractional Synthesis Rate

In 2012 Casperson et al. demonstrated that two weeks of 4 g of leucine supplementation with each main meal (3x·d^{-1}) resulted in both a significantly greater postabsorptive and postprandial (7 g EAA & 10 g carbohydrate liquid meal) mixed muscle FSR in elderly (~68 y) men and women who consumed the current RDA of dietary protein (~0.8 g·kg BW^{-1}·d^{-1}) [199]. Subsequently, a study of 3 days of chronic leucine supplementation (5 g 3x·d^{-1}) in older men (~72 y, between 65-85 y) consuming either the current protein RDA, or a higher amount of 1.2 g·kg BW^{-1}·d^{-1} prior to study found that the addition of leucine was equally effective in increasing integrative myofibrillar FSR in both diets [174]. A recent study [16] in older women (~69 y, between 65-75 y) consuming 1.0 g protein·kg BW^{-1}·d^{-1} investigated the effects of 6 days of twice-daily consumption of an isocaloric beverage of either 25 g whey or 10 g milk protein, both containing 3 g leucine, on myofibrillar FSR response to the same respective isocaloric beverage [16]. Although they did not compare to basal state pre-intervention, they found no difference in myofibrillar FSR between the groups in the basal state post-intervention.

Using deuterated water (D_2O), the integrated (6 days supplementation plus supplement as test meal) postprandial myofibrillar FSR response was significantly elevated in the whey, but not milk protein group. Thus although 3 g of leucine added to 10 g milk protein was able to acutely elevate myofibrillar FSR after consumption of either 25 g whey or 10 g milk protein (both containing 3 g leucine, see above), 6 days of chronic supplementation of the whey, but not the milk protein, mixture was able to significantly elevate integrated postprandial myofibrillar FSR [16] in older women. Using similar methodology and older (65-75 y) women participant profile, this same group showed that 6 days of twice-daily supplementation of 15 g milk proteins containing either 1.3 or 4.2 g leucine elevated integrated postprandial myofibrillar FSR in the 4.2 g leucine beverage only, whilst maintaining the same amount of dietary protein throughout (1.0 g·kg BW^{-1}·d^{-1}) [15]. These results suggest a dose and protein-type depended response to leucine, highlighting the importance of selecting the appropriate leucine dose in intervention studies. A six-year longitudinal study [200] observed that those >65 y who were in the highest quartile of habitual leucine intake (7.1 +/- 0.6 g·d^{-1}) maintained LBM, whereas lower habitual leucine intakes were associated with LBM loss over 6 years. This effect was not observed in those younger than 65 y. Importantly, although the quartiles were separated based on leucine intake, the highest quartile of leucine intake was also the highest associated intake of dietary protein (1.26 g·kg BW^{-1}·d^{-1}), making it impossible to draw firm conclusions about whether high dietary protein or high leucine intake *per se* is associated with maintained LBM in elderly persons. Nevertheless, there is strong evidence that chronic leucine supplementation is

beneficial in elevating myofibrillar FSR, though additional studies are warranted to determine the effects of longer periods of leucine supplementation.

2.7.2. Chronic Leucine Supplementation Effects on Muscle Mass and Strength

2.7.2.1. Results of Meta-analysis

Additional studies on chronic leucine supplementation have reported outcomes on changes in muscle mass and strength, but not FSR, and have yielded conflicting results. A 2015 meta-analysis attempted to clarify the effect of chronic leucine supplementation by assessing 16 randomized control trials on supplements containing $\geq$ 2 g per day with a duration of 10 days to 2 years in elderly participants ($\geq$65 y) with no chronic diseases other than type 2 diabetes mellitus [21]. Their primary outcome was body composition and/or strength, with a secondary outcome of comparing healthy to sarcopenic persons. Considering the profound variation in study design, participants, supplement composition and protocol, etc., the authors found that supplementation containing $\geq$ 2 g per day significantly increased body weight, LBM, and body mass index (BMI). Additionally, leucine was more effective in increasing body weight and LBM in sarcopenic compared to healthy persons. However, because the supplementation composition and protocol widely varied, it is possible that the results reported are due to confounding factors such as co-ingestion of other amino acids and synergistic effects of macronutrient intake [21].

2.7.2.2. Results of Longitudinal Studies

Three examples of longitudinal studies (4-24 weeks) that had more clearly defined leucine supplementation published equivocal results on muscle mass and strength, but did not include measurements of FSR [201-203]. A 2009 study in healthy older men (~71 y) consuming ~1.0 g·kg BW^{-1}·d^{-1} who underwent 3 months of leucine supplementation (2.5 g leucine thrice daily) vs placebo (wheat flour) produced no gains in muscle mass or strength [201]. A 2011 study in older men (~71 y) with type 2 diabetes mellitus consuming ~1.0 g·kg BW^{-1}·d^{-1} who underwent 6 months of leucine supplementation (2.5 g leucine thrice daily) vs placebo (wheat flour) also reported no gains in muscle mass or strength [202]. A more recent study in 2016 [203] in older men and women (~71 y) consuming ~1.2 g·kg BW^{-1}·d^{-1} dietary protein who underwent 3 months of twice daily consumption of EAAs relative to body weight (0.21 g EAA·kg^{-1}·d^{-1}) with 40% leucine content (0.08 g leucine·kg^{-1}·d^{-1}, therefore ~6 g leucine per day) significantly increased LBM, whereas EAAs with 20% leucine content (~3 g leucine per day) or placebo (lactose) did not significantly increase LBM in older people. However, some measures of physical function were improved in the 20% leucine group and 40% leucine group. Of note, the results of this study must be carefully considered as adherence to supplements was suboptimal (~75%), and there was a marked attrition rate (~30%).

2.7.2.3. Results in Frail Persons

Of interest, the only study [22] that investigated older men and women (~65 y) identified as frail using the FP [87] consuming ~1.0 g·kg BW^{-1}·d^{-1} who underwent 6 months of

milk protein supplementation (15 g protein containing 1.4 g leucine, twice daily) reported no change in muscle mass. The authors did report a time effect for both 1RM knee extension and leg press, with a trend for interaction effect in leg press such that the protein group was borderline higher than the placebo group post-intervention. Thus, the optimal protocol for leucine supplementation (dose, co-ingestion, timing, frequency, etc.) and identifying the population in which supplementation would be most effective, remains to be determined. It appears as though healthy or diabetic elderly men consuming ~1.0 g·kg BW^{-1}·d^{-1} dietary protein typically do not significantly benefit from up to 6 months of thrice daily supplementation of 2.5 g leucine [202], while evidence suggests that frail and/or sarcopenic persons are better candidates to benefit from chronic leucine supplementation [21].

2.7.3. Combined Interventions

2.7.3.1. Acute Resistance Exercise and Leucine-Enriched Protein Supplementation (Fractional Synthesis Rate)

Recent meta-analyses have concluded that exercise in conjunction with amino acid/protein intake is able to overcome anabolic resistance of MPS [9, 204]. However, both the exercise modality and the amino acid/protein intake dose and composition varied. Therefore, a selection of relevant studies on the effect of acute RE in conjunction with leucine intake on FSR will be reviewed specifically. A 2008 study investigated healthy elderly men (~73 y) who performed acute RE on a leg press and underwent immediate subsequent feeding [205]. Participants consumed repeated boluses of a carbohydrate (CHO) and protein mixture with or

without additional leucine, relative to body weight so that they consumed 0.011 vs 0.041 $g \cdot kg^{-1} \cdot h^{-1}$ leucine in their respective groups. The mixtures were consumed every 30 min for 5.5 hours and the 6-hour mixed muscle FSR response was measured, with no difference observed between the two groups. To investigate the effects of a single-bolus, healthy elderly men (~72 y) performed bilateral knee extension RE and consumed a subsequent bolus of 10 g EAA containing either 1.85 or 3.5 g leucine 1 h post-RE [206]. Myofibrillar FSR was measured at 2, 5, and 24 h post-RE. Although myofibrillar FSR was significantly elevated in both groups at 2 and 5 h post-RE with no difference between the groups, it remained significantly elevated only in the higher-leucine group at 24 h post-RE [206]. In agreement with this, a subsequent study investigated healthy older women (~66 y), matched for indexes of muscle mass, who performed unilateral knee extensions and subsequently consumed 20 g whey (containing 2 g leucine) or 3 g EAA (containing 1.2 g leucine) [17]. The 4-hour myofibrillar FSR response was measured. They observed that both groups equally significantly increased myofibrillar FSR in both the rested and exercised legs. Interestingly, the combination of exercise and feeding resulted in the elevated FSR response remaining elevated for 4 h as opposed to returning back to baseline. Thus, in this cohort of elderly women, an acute intake 3 g of EAA containing 1.2 g leucine was able to elicit the same MPS response as 20 g whey, with the combination of RE sustaining the FSR for longer than without RE [17]. Thus, an acute bolus of leucine-enriched protein/AA appears to prolong the MPS response in healthy elderly men and women. However, these aforementioned studies did not address the chronic effects of RT and leucine supplementation on the rate of myofibrillar protein synthesis *in vivo*.

2.7.3.2. Resistance Training and Chronic Protein Supplementation (Muscle Mass and Strength)

The chronic effects of protein, and not specifically leucine supplementation and RT on muscle mass and strength have yielded equivocal results. Several meta-analyses have reported conflicting conclusions that protein supplementation augments muscle hypertrophy and strength gains with RT in both younger and older persons. An earlier meta-analysis [207], found improved fat free mass (FFM) but others confirmed that it was not specifically muscle mass or strength [208], or improved muscle strength or size, body composition, or functional ability [209]. However, all meta-analyses emphasize the severe limitations of both lack of studies in older persons, and the marked heterogeneity in study design including duration of intervention, participant profiles, RT protocols, supplementation (dose, composition, timing), and outcome measurements. These meta-analyses have concluded that prescriptive recommendations regarding RT and protein supplementation cannot be made at the current time and it is vital that more high-quality comprehensive studies be conducted in older adults. In addition, it is worth mentioning that other factors related to protein intake play a significant role in determining the degree of MPS following RT. Although not the focus of the current study, as eluded to above, factors such as timing [210, 211], quality [212-214], diurnal distribution [215] and importantly quantity of protein administered, all contribute to the effect of protein supplementation as comprehensively reviewed in [10, 216].

2.7.3.3. Resistance Training and Chronic Leucine Supplementation

To the best of my knowledge, no studies exist investigating the effects of RT and chronic leucine supplementation versus an isonitrogenous control, and/or where all participants maintained sufficient protein intake (~1.2 g·kg BW^{-1}·d^{-1}), and did not differ in habitual dietary macronutrient or leucine intake, separate from supplement. The two studies in which leucine is the predominant or only AA supplemented (and thus the anabolic effects of leucine are less likely to be confounded by receiving excess quantities of protein in the given supplement) compared with control groups receiving no supplements have yielded some positive results. The first study was conducted in elderly sarcopenic women (~78 y) who underwent 12 weeks of a multi-modal exercise program with a predominant progressive RT component, twice a week [38]. Participants in the supplement group consumed 3 g of AAs containing 42% leucine (1.2 g leucine) twice-daily, while the control group received no supplementation. Protein dietary intake was not assessed at any point in the study. No differences were seen in total body muscle mass, appendicular muscle mass index (AMMI), or BMI with training or combined with supplementation. Although leg muscle mass significantly improved post-RT with or without supplementation, knee extension strength measured with a dynamometer significantly improved only in the combined group. The second study was conducted in older adults (~84 y) who underwent a 3-month long RT program three times a week and assessed at 4 and 12 weeks [39]. Participants in the supplement group consumed 5 g leucine twice-daily, while the control group received no supplementation. Notably, of the 24 participants assessed at 4 weeks, only eleven completed the study and were assessed again at 12 weeks (*n*=7 for supplement, and *n*=4 for control). Post-intervention, although statistically borderline significant

(p=0.056), the supplemented group improved leg flexion strength (measured using a dynamometer) from baseline, while the control group did not. In addition, only the supplemented group had an increase in estimated mid-upper arm muscle area (MUAMA), and borderline significant increase in calf circumference (p=0.071) post-intervention. Protein dietary intake was not monitored through the duration of the intervention, but was assessed at baseline, 4 and 12 weeks. At baseline participants were consuming ~1.2 g·kg BW^{-1}·d^{-1}, and no significant changes were noted from baseline at 4 or 12 weeks in either group.

Thus, there are currently no studies on the effects of leucine supplementation and progressive RT on robust markers of protein synthesis, fiber type composition, muscle mass, muscle strength and function compared to an isonitrogenous control in a population most suited to benefit from such an intervention who habitually consume an optimal amount of dietary protein.

2.8. Insulin Sensitivity

2.8.1. Frailty & Insulin Sensitivity

Aging is associated with a decrease in IS and higher circulating levels of glucose [217, 218]. However, impaired IS is not inevitable, as some centenarians have preserved insulin functioning [219]. Thus, older persons with impaired IS may be those who are vulnerable to adverse health outcomes. Indeed, the prevalence of diabetes is associated with frailty [220] and increases with pre-frail and frail status [221], while hyperglycemia is associated with increased

risk of frailty [222]. Furthermore, a study of non-diabetic octa- and nonagenarian women observed a more exaggerated and prolonged insulin and glucose response to a 75 g bolus of glucose in those who were frail versus non-frail [223].

2.8.2. Resistance Training and Insulin Sensitivity

RT is still able to increase muscle mass in aged individuals, also resulting in more muscle to take up glucose. Twelve weeks of whole-body RT in a wide range of ages including older persons of 55-82 y, resulted in improved fasting glucose, fasting insulin, and insulin action (M-value), concurrent with a significant increase in lean body mass. Furthermore, these findings were positively related with AS160 phosphorylation, the Akt substrate regulating GLUT4 translocation to plasma membrane during a 120 min euglycemic-hyperinsulinemic clamp. Although RT has been shown to improve IS in late-middle-aged individuals [44], there are little data on how IS can be modulated by RT in frail elderly persons. One recent study in frail elderly women (~70 y) showed that 4 months of RT improved upon the action of insulin, by enhancing insulin-induced suppression of endogenous glucose production [45].

2.8.3. Anabolic Resistance and Insulin Sensitivity

A blunted protein anabolic response has been postulated to also be due in part to a decrease in IS [40, 224, 225]. Insulin resistance is a significant risk factor for type 2 diabetes, cardiovascular disease, and frailty [41, 42]. Skeletal muscle is responsible for almost 75% of the insulin-stimulated glucose uptake and is therefore an attractive target for interventions aimed

at increasing IS [43]. However, muscle mass is not the only tissue that influences IS. The age-related accumulation of abdominal and ectopic fat is associated with insulin resistance, with declines in muscle mass also contributing to metabolic dysfunction [42].

2.8.4. Leucine Supplementation and Insulin Sensitivity

Leucine supplementation is one such intervention that could simultaneously improve upon frailty, sarcopenia, and insulin resistance. Not only can leucine directly stimulate MPS independent of insulin [193], but is also one of the three BCAAs that induces the release of insulin from the pancreas [226]. Leucine acutely elevates plasma insulin levels in young healthy humans, the duration of which is dose-dependent [227]. Indeed, [174] observed that ingesting 5 g of leucine mid-meal elevated insulin C_{max} (maximum insulin concentration) in those consuming dietary protein of both 0.8 g·kg BW^{-1}·d^{-1} or 1.2 g·kg BW^{-1}·d^{-1}. In normal, non-pathological conditions, leucine supplementation has been shown to directly stimulate protein synthesis pathways with a concurrent increase in IS [46]. Leucine has also been utilized as a nutritional therapy for conditions related to reduced IS such as sarcopenia and immobilization-induced atrophy [47-49].

Conversely and paradoxically, leucine has also been implicated in the pathology of insulin resistance. While a causal role has not been established, circulating plasma levels of BCAA and, in particular, leucine have been observed to be increased in obese individuals and positively correlate with HOMA-IR ($r = 0.26$-0.58, $p < 0.03$) [51]. Additionally, leucine and the

other BCAA's have demonstrated predictive qualities for type 2 diabetes [50, 51]. However, the results of a recent study using the more robust methodology of hyperinsulinemic-euglycemic clamp showed that although there were correlations between many AAs and insulin resistance, the relationships were almost entirely explained by central adiposity [228]. A recent study showed that treatment with an insulin sensitizer improved IS along with reduced plasma BCAA [229]. Therefore, BCAAs including leucine may not only be indicative of, but also contribute to the pathogenesis of insulin resistant conditions. Mechanistically, leucine can directly activate mTORC1 which activates p70S6K, thereby impairing glucose metabolism in skeletal muscle by increasing serine phosphorylation of IRS1 via a negative feedback loop [230], and decreasing PI3K activity with reduced whole body glucose uptake [231]. However, a recent study using hyperinsulinemic-euglycemic clamp in post-menopausal women demonstrated that the impairment of glucose disposal following protein ingestion is independent of leucine-mediated mTOR-p70S6K and Akt signaling [232].

2.8.5. Combined Resistance Training and Chronic Leucine Supplementation and Insulin Sensitivity

To the best of our knowledge, the only two studies in which leucine is the predominant or only AA supplemented compared with control groups receiving no supplements, have not measured any indices of IS [38, 39]. Given the conflicting dual nature of leucine and IS, it is extremely pertinent to investigate the effects of chronic leucine supplementation with RT on IS in a population vulnerable to insulin resistance, such as pre/frail elderly women.

2.9. Mitochondria and Aging

2.9.1. General Concepts

Mitochondria are unique cellular organelles responsible for the oxygen-consuming production of ATP. Skeletal muscle is rich in mitochondria, which accounts for approximately 4-7% of muscle volume [233]. Mitochondria also heavily influence cellular homeostasis through their modulation of energy supply, ROS production and consequent signaling, and are crucial in the instigation of regulated cell death (apoptosis) [234]. A decline in skeletal muscle function is a characteristic of aging and experimental evidence suggests that mitochondria are heavily implicated in the aetiology of age related muscle atrophy [52, 53, 57, 235].

A confusing large body of literature spanning multiple decades exists on mitochondria function with aging, due to varying methodology (notably, mitochondrial isolation versus permeabilization techniques), rodent models (confounding pathologies of in-bred strains), normalization to mitochondria content, etc. Historically, mitochondrial based investigations did not take mitochondria content into account, and/or the investigative techniques used did not keep the mitochondrial structure/network intact. Currently developed techniques, such as using saponin-permeabilized myofibers, allow the structure and content of mitochondria to be preserved in bundles of myofibers while mitochondrial function is measured *ex vivo* [236]. Importantly, this technique also allows for the aging milieu to be preserved. Three intriguing

aspects of study concerning mitochondria implicated in aging are mitochondrial respiration, ROS production, and sensitivity to mPTP opening.

2.9.2. Mitochondria Function and Aging

Some [237-242] but not all [243-248] studies have observed a decline in mitochondrial respiratory capacity with aging. However, not only do many of these studies use unideal methodologies, the majority did not account for other potential confounders such as body composition and fitness characteristics of participants, as well as mitochondria quantity. Indeed, a recent study observed that maximal ATP synthesis of muscle is impacted by physical activity level to a greater degree than chronological age [249]. In addition, it has been recently shown that mitochondria have a reduced sensitivity to ADP with aging in that older men had lower respiration compared to younger men at physiological (submaximal) concentrations of ADP [52]. Therefore, future studies using appropriate participant cohorts and optimal methodologies in aged persons are clearly warranted.

There appears to be no difference in the mitochondria capacity for ROS production with aging. In a recent study, older sedentary persons tended to have greater maximal ROS emission than their active and younger counterparts [249], while a second recent study lends further support that the capacity for ROS production is not altered with aging [52]. However, ROS emission at submaximal ADP concentrations was elevated in older vs younger men resulting in augmented markers of oxidative stress [52]. This is an important finding given that

mitochondria-generated ROS can affect muscle mass by depressing protein [250-252] and impairing postprandial Akt anabolic signaling [253]. Mitochondria-generated ROS could also further damage the mitochondrion [55, 56]. ROS production can also induce mPTP opening [254]. The mPTP is a large conductance pore in the inner mitochondrial membrane, whose gating action is tightly regulated under normal physiological conditions [255]. Mitochondrial uptake of disproportionate amounts of Ca^{2+} results in mitochondrial swelling, outer mitochondrial membrane rupture, and mPTP opening which releases the mitochondrial contents to the cytosol to initiate apoptosis [255-258]. The voltage-dependent anion channel (VDAC) is a channel on the outer mitochondrial membrane (OMM) and is postulated to be a key component of the mPTP [259, 260], and also has been widely used as a marker of mitochondria content [247, 261-265]. The role of VDAC includes the passage of mitochondrial metabolites across the OMM depending on the trans-membrane voltage, Ca^{2+} homeostasis [266], and mitophagy [267]. VDAC has been implicated in apoptosis [266], along with mPTP it is proposed to facilitate the release of cytochrome C from the mitochondria into the cytoplasm to initiate apoptosis [268]. Oxidative damage from ROS [269] and mitochondrial dysfunction (depolarization) can also cause the mPTP to open [255]. A greater sensitization to mPTP opening with aging has been revealed [235, 255, 270], potentially increasing susceptibility to apoptosis contributing to age-related muscle atrophy, and thus weakness [57-60], as well as resulting conditions such as frailty.

Of note, many of the aforementioned declines are thought to be attributed to reduced abundance of mitochondria in older persons. Rates of mitochondrial protein synthesis have

been shown to decline with aging [271]. However, it has been proposed that the differences seen in older persons quantity of mitochondria can be attributed to increased sedentariness (as opposed to an inherent change with aging) [272-275], although this has not always been seen [270].

2.9.3. Mitochondrial Function and Frailty

Impaired mitochondria function has been associated with physical functional status, particularly in hospitalized and frail older subjects [276]. A recent study [273] reported an association between pre-frailty and declining *in vivo* and *ex vivo* mitochondrial function, though it is likely that the decline was due to a concomitant decline in content of mitochondria. One of the FP criteria is slow walking speed [87]. Skeletal muscle mitochondria capacity has been shown to be associated with preferred walking speed in older men and women [59], further establishing a link between mitochondria function and frailty. A study investigating transcriptome profiles of young, old healthy, and old pre-frail and frail observed that pathways related to mitochondria function were lowest in the pre-frail and frail older subjects, although the fold changes for individual genes were modest (1.1-1.2) [277]. However, following 6 months of RT no changes were observed in mitochondria-related genes. Thus, furthering our understanding of how interventions aimed at ameliorating age-related conditions such as frailty affect (or is affected by) mitochondria function is of upmost importance.

2.9.4. Sexual Dimorphism in Mitochondrial Function

Although not addressed in this study, it is worth noting that sexual dimorphism is also apparent in mitochondria function in young men and women. Both sexes have similar rates of maximal respiration, while women have lower respiration rates at physiological concentrations of ADP compared to men [278]. A second recent study also found no differences in respiratory capacity between the sexes when normalized for mitochondrial content [279]. In this same study, young women were found to utilize a greater proportion of fat as a substrate for energy during aerobic exercise [279]. As well, sexual dimorphism in ROS production and mPTP opening in various tissues in young rodents has been widely observed [280]. These studies highlight the value of separately studying mitochondria function in each sex. Very little is known about mitochondria functioning in aging women as opposed to men. Future studies are clearly needed to assess sexual dimorphism on multiple aspects of mitochondria function with aging.

2.9.5. Resistance Training and Mitochondrial Function with Aging

In addition to increasing muscular mass and strength [152], RT has been shown to significantly increase mitochondrial volume density in older men and women after 24 weeks of RT [69], and after 6 and 12 mo RT in postmenopausal women [62]. Furthermore, a recent study in elderly men and women showed that 8 weeks of RT significantly increased mitochondria content in skeletal muscle [61]. Studies investigating alterations in mitochondria function following RT interventions in older persons are less abundant.

Mitochondrial oxidative capacity was increased in older men and women after 24 weeks of RT measured by ^{31}P-MRS [69], and after 12 weeks of RT measured by respirometry in permeabilized fiber bundles [52]. Conversely, no change in maximal respiration was seen in recent studies using respirometry in isolated mitochondria [66]. Results of the effects of 12 weeks of RT on ROS production are varied, with one study resulting in an increased capacity for ROS production without affecting oxidative state of muscle in older men [52], while no change in ROS capacity or markers of oxidative stress were found in older adults [67]. Although not measured in the current thesis proposal, markers of oxidative stress were reduced following 12 weeks of RT in older women [68]. Currently, no literature exists regarding the effects of RT on mPTP sensitivity in older women.

2.9.6. Mitochondria Function and Leucine

As described prior (section 2.6 “Cellular Regulation of Muscle Protein Synthesis”), leucine stimulates muscle protein synthesis by activating mTORC1 [194]. mTORC1 signaling stimulates ATP-consuming processes (protein synthesis), necessitating mitochondria function to replenish consumed ATP. Consequently, mTORC1 activity has been shown to correlate with mitochondrial activity [63]. It has recently been shown that leucine has the capability to stimulate mitochondrial biogenesis, although the precise mechanisms remain unclear [64, 65]. Additionally, in a recent study [65] leucine treatment of cultured myoblasts resulted in heightened oxygen consumption which was attributed to a proportional increase in

mitochondria content. The consequent effects of leucine on mitochondria function have yet to be determined in tissue from human skeletal muscle.

2.9.7. Combined Resistance Training and Chronic Leucine Supplementation Mitochondria Function

To the best of our knowledge, it is currently unknown if mitochondria function can be improved upon in a cohort of pre/frail elderly women who are sedentary at baseline and undergo a 12-week resistance training program with or without leucine supplementation, which mandates on this specific population.

CONNECTING STATEMENT I

The following sections (Chapter 3-5) of this thesis are based on three manuscripts:

Chapter 3 (Manuscript 1):

Kathryn J. Jacob, Stéphanie Chevalier, Marie Lamarche, José A. Morais (2018). *Leucine supplementation does not alter insulin sensitivity in pre-frail and frail older women following a resistance training protocol*. The Journal of Nutrition (resubmitted).

Chapter 4 (Manuscript 2):

Kathryn J. Jacob, Vita Sonjak, Sally Spendiff, Russell T. Hepple, Stéphanie Chevalier, Anna Perez, José A. Morais (2019). *Leucine increases mitochondrial quantity in conjunction with resistance training and adequate protein intake in pre/frail women*. Ready to be submitted to the Journal of Physiology.

Chapter 5 (Manuscript 3):

Kathryn J. Jacob, Marie Lamarche, Vita Sonjak, Carole Spake, Guy Hajj, Jarred Slimovitch, Anita Hsieh, Vivian Lau, Frances Comte, Stéphanie Chevalier, José A. Morais (2019). *Leucine is not required in conjunction with resistance training and optimized protein intake to reverse frailty by increasing basal muscle protein synthesis in older women*. To be submitted to the Journal of Cachexia, Sarcopenia and Muscle.

CHAPTER 3: MANUSCRIPT 1

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Leucine supplementation does not alter insulin sensitivity in pre-frail and frail older women following a resistance training protocol

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3.1. Abstract

Background: Frailty is a clinical condition associated with loss of muscle mass and strength (sarcopenia). Although sarcopenia has multifactorial causes, it might be partly attributed to a blunted response to anabolic stimuli. Leucine acutely increases muscle protein synthesis and resistance training (RT) is the strongest stimuli to counteract sarcopenia that was recently shown to improve insulin sensitivity (IS) in frail older women. Discrepancies exist whether chronic supplementation of leucine in conjunction with RT can improve muscle mass and IS.

Objective: The purpose of this double-blinded placebo-controlled study is to determine the effects of leucine supplementation and RT on IS in pre-frail and frail older women.

Methods: Using the Fried Criteria (2001), 19 non-diabetic pre-frail (1-2 criteria) and frail (≥3 criteria) older women (77.5 ± 1.3 y, BMI: 25.1 ± 0.9 kg/m^2) underwent a 3-month intervention of RT 3x/week with protein optimized diet of 1.2 g·kg^{-1}·d^{-1} and 7.5 g·d^{-1} of L-leucine (Leu) supplementation vs. placebo L-alanine (Ala). Pre/post-intervention primary outcomes were fasting plasma glucose, serum insulin and 4-hour responses to a standard meal of complete liquid formula. Secondary outcomes of resting energy expenditure (REE) using indirect calorimetry, and body composition using Dual-energy X-ray Absorptiometry were obtained. Paired t-tests analyzed pooled data and two-factor Repeated Measures ANOVA determined supplementation, training, and interaction effects.

Results: No significant time, group, or interaction effects were observed for postprandial areas under the curve of serum insulin or plasma glucose, or REE in Leu vs Ala. Total lean body mass

increased and percent body fat decreased significantly for both groups post-intervention (0.76 ± 0.13 kg, and -0.92 ± 0.33 kg respectively, time effect: $p<0.01$).

Conclusion: IS was not affected by RT and leucine supplementation in non-diabetic pre-frail and frail older women. Therefore, leucine supplementation does not appear to influence IS under these conditions. (ClinicalTrials.gov ID: NCT01922167).

Keywords:

Frailty, older women, leucine supplementation, insulin sensitivity, glucose metabolism, clinical trial, resistance exercise

3.2. Introduction

Dietary protein ingestion is one of the most potent anabolic stimuli, largely driven by the quantity of ingested protein and the essential amino acid content of the protein (in particular, leucine) under the regulation of insulin upon feeding (1, 2). Although the cause of muscle loss with aging is multifactorial, one factor is the development of anabolic resistance, whereby a stronger stimulus (e.g., protein intake, muscle contraction) is required to elicit the same given increase in protein synthesis of that of younger persons (3). It is believed that older persons are required to ingest more or higher quality protein (higher amount of essential amino acids, in particular leucine) than their younger counterparts in order to maximally stimulate muscle protein synthesis (MPS) (4, 5). Consequently, it has been proposed that older persons consume higher amounts of dietary protein ($\geq$1.2 g·kg^{-1}·d^{-1}) than the current recommended dietary allowance of 0.8 g·kg^{-1}·d^{-1}, (6, 7).

Another potent anabolic stimulus is resistance training (RT). Although muscle protein breakdown (MPB) increases post-RT, a simultaneous increase in MPS is several times greater (8), and this effect persists for 24-48 h. Some (9-12) but not all (13, 14) studies have shown a blunted anabolic response to resistance exercise with aging, even when circulating and muscle amino acid concentrations are controlled for (15, 16). Despite this, RT is still able to increase muscle mass in aged individuals, also resulting in more muscle to take up glucose. Twelve weeks of whole-body RT in a wide range of ages including older persons of 55-82 y, resulted in improved fasting glucose, fasting insulin, and insulin action (M-value), concurrent with a

significant increase in lean body mass (LBM). Although RT has been shown to improve insulin sensitivity (IS) in late-middle-aged individuals (17), there are little data on how IS can be modulated by RT in frail older persons. One recent study in frail older women (~70 y) showed that 4 months of RT improved upon the action of insulin, by enhancing insulin-induced suppression of endogenous glucose production (18).

A blunted protein anabolic response has been postulated to also be due in part to a decrease in IS (19). Insulin resistance is a significant risk factor for type 2 diabetes, cardiovascular disease, and frailty (20, 21). Skeletal muscle is responsible for almost 75% of the insulin-stimulated glucose uptake and is therefore an attractive target for interventions aimed at increasing IS (22). However, muscle mass is not the only tissue that influences IS. The age-related accumulation of abdominal and ectopic fat is associated with insulin resistance, with declines in muscle mass also contributing to metabolic dysfunction (21).

Leucine supplementation is one such intervention that could simultaneously improve upon frailty, sarcopenia, and insulin resistance. Leucine is an essential amino acid that is unique in its ability to directly stimulate MPS independent of insulin via activation of amino acid sensing pathway mammalian target of rapamycin complex 1 (mTORC1) (23). Leucine is also one of the three branched chain amino acids (BCAA) that induces the release of insulin from the pancreas (24). Leucine acutely elevates plasma insulin levels in young healthy humans, the

duration of which is dose-dependent (25). In normal, non-pathological conditions, leucine supplementation has been shown to directly stimulate protein synthesis pathways with a concurrent increase in IS (26). Leucine has also been utilized as a nutritional therapy for conditions related to reduced IS such as sarcopenia and immobilization-induced atrophy (27-29).

Conversely and paradoxically, leucine has also been implicated in the pathology of insulin resistance. While a causal role has not been established, circulating plasma levels of BCAA and, in particular, leucine have been observed to be increased in obese individuals and positively correlate with HOMA-IR ($r = 0.26\text{-}0.58$, $p < 0.03$) (30). Additionally, higher circulating levels of leucine and the other BCAA's have demonstrated to have predictive value for increased risk of developing type 2 diabetes (30, 31). Therefore, BCAA's including leucine may not only be indicative of, but also contribute to the pathogenesis of insulin resistant conditions

With the above context in mind, our research objective was to determine the effects of leucine supplementation on IS in pre/frail older women undergoing RT. We hypothesized that 12 weeks of RT would improve IS, with an added benefit when supplemented with leucine compared to placebo, in pre/frail older women.

3.3. Participants and Methods

3.3.1. Study Design

The study was part of a larger randomized double-blinded placebo-controlled clinical study (ClinicalTrials.gov ID: NCT01922167) designed to examine the effect of leucine combined with RT on fractional synthesis rate (FSR). All participants underwent a 12-week high-intensity progressive resistance exercise training program (HIRT) and followed a protein optimized diet (~1.2 $g \cdot kg^{-1} \cdot d^{-1}$). Half were randomized to receive L-leucine (Leu, 2.5 g 3x/d) supplementation and the other half an isonitrogenous amount of L-alanine (Ala, 1.7 g 3x/d). All tests were performed before and after the intervention. The study was approved and monitored by the McGill University Health Centre (MUHC) Human Research Ethics Board. All participants read and signed an informed consent form before participating in the study and the screening visits.

3.3.2. Participant Recruitment and Screening

Frail or Pre-Frail, but not disabled, non-diabetic, sedentary, community-dwelling older women (>65 y) according to a modified Fried (32) criteria specific for older women (see below) were invited to participate in the study. Participants were recruited from the Geriatrics outpatient clinic of the MUHC and advertisements placed in the local seniors' newspaper. After an initial telephone call, older women were invited to a first initial screening visit at the Clinical Innovative Medicine (CIM) research area of the MUHC-Royal Victoria Hospital (Montreal, QC). Inclusion criteria: cognitively intact with a Mini Mental State Examination score (MMSE) $\geq$24, no acute disease, Body Mass Index (BMI) of 18.5-35 kg/m^2, normal complete blood count,

biochemistry, A1C, TSH, urine analysis, negative serology for hepatitis and HIV, and normal chest X-ray and ECG results. Exclusion criteria included dependence on walking aids, 15-item Geriatric Depression Score (GDS) >5, substance abuse, eating disorders, active medical conditions including diabetes and cancer other than skin within 5 years, serum creatinine >110 μmol/L, hemoglobin (Hb) <110 g/L, and medications known to interfere with metabolic endpoint measurements (e.g., beta-blockers).

The participants underwent a venous blood sampling by a phlebotomist. Body weight was measured to the nearest 0.1 kg (Scale-Tronix, Welch Allyn, NY, USA) and height to the nearest 0.5 cm using a stadiometer. Participants completed a 3-d food diary, with instructions to estimate portion sizes provided by a nutritionist and analyzed using the Food Processor SQL software (Version 10.11.0, ESHA Research, Salem OR). If needed, adjustments were made to their dietary protein intake to obtain an isoenergetic diet with a protein intake of 1.2 $g \cdot kg^{-1} \cdot d^{-1}$ for at least 24 h prior to meal tests.

Frailty status was determined using the modified Fried criteria as follows: (1) Slowness identified as a 4 m gait speed of $\leq$ 1 m/s (33); (2) Weakness identified as a handgrip strength $\leq$ 20 kg using a Jamar hydraulic hand dynamometer (Sammons Preston, Inc., IL, USA) (34); (3) Sedentariness identified by a CHAMPS Physical Activity Questionnaire score $\leq$ 125 (35); (4) Sarcopenia identified by a muscle mass index (MMI) < 6.76 kg/m^2 via BIA (RJL Systems Inc., MI, USA) (36) using the Roubenoff, et al. (37) equation validated for older women; and (5)

Exhaustion identified by at least one positive response to either of the 2 following questions: "How often do you feel like 'I just could not get going'; and 'Everything I did was an effort'" (32). To qualify, participants were deemed to be pre-frail if they met 1-2 criteria (Slowness, Weakness, or Sarcopenia had to be one of the criteria), and frail if they met 3 or more criteria.

On a second visit to the CIM, participants underwent a standard oral glucose tolerance test (OGTT) after an overnight fast and physical examination by the study physician. An intravenous catheter was inserted into an anticubital vein and after baseline blood sampling, consumed an oral dose of 75 g dextrose, administered in the form of a sweet orange-flavoured drink (Glucodex 300 mL, Rougier Pharma, Ratiopharm Inc., Canada). Blood samples were taken at 30, 60, 90, 120 and 180 minutes post-ingestion, and plasma was analyzed for glucose concentration. Only participants with normal glucose tolerance (plasma glucose <7.8 mmol/L) or glucose intolerance (plasma glucose between 7.8-11.0 mmol/L) at the 120 min mark were included (38). After the OGTT, a medical history and physical examination was performed by the study physician.

3.3.3. Meal Tests, before and after intervention

The day before the Meal Tests, participants had measurements of total weight, BMI, LBM, and percent body fat (%Fat) obtained using a 3-compartment model via dual-energy x-ray absorptiometry (DXA) (GE Lunar iDXA). Appendicular muscle mass index (AMMI) was calculated by taking the sum of LBM of arms and legs, then dividing by height (in meters) squared. Regions

of interest (Android and Gynoid) were defined according to standards (39) and percentage of fat was determined (Android %Fat and Gynoid %Fat) in each region. Waist-to-hip ratio (WHR) was measured using standard techniques (40).

Participants arrived at the CIM in the morning following an overnight fast. A catheter was inserted into a posterior hand vein with the hand in a warming box at 65°C for arterialized blood sampling. Samples were drawn at postabsorptive baseline, then 30, 45, 60, 90, 120, 150, 180, 210, and 240 min following oral ingestion of Ensure® Plus Calories, with corresponding L-leucine or L-alanine supplement as per randomization consumed within 20 minutes. Doses were calculated to deliver 0.60 g protein·kg LBM^{-1}·$meal^{-1}$ with additional 0.07 g L-leucine or 0.0476 g L-alanine per kg LBM (average macronutrient intake: 82.8 $\pm$ 1.5 g carbohydrate; 22.2 $\pm$ 0.4 g protein; 16.9 $\pm$ 0.4 g fat). Resting Energy Expenditure (REE) was measured in the postabsorptive state by indirect calorimetry (Vmax, Summit Technologies, Princeton, N.J.) for 20 min, using the average of the last 15 min of values to estimate REE. Nitrogen balance was estimated as described previously based on a 24-hour urinary collection (measured urea, and estimated fecal, skin and nitrogen excretion) with corresponding nitrogen intake from dietary analysis (41).

3.3.4. Intervention: Diet & Supplementation

Participants were randomized via random-number generator by an administrator not involved in the study. Oddly generated numbers entered one arm of the study, and even

numbers entered the other arm. Powdered supplements of L-leucine and isonitrogenous amounts of L-alanine were provided in sterile sealed screw-top 100 mL containers, of individual doses (2.5 g L-leucine [ProteinCo. QC, CA] and 1.7 g L-alanine [PureBulk® OR, USA]). Participants were instructed to consume one complete dose of supplement at the onset of each main meal (breakfast, lunch, dinner) for the duration of the intervention. Log sheets were provided to track compliance and were collected every 2 weeks. All participants were given instruction and guidance by a study nutritionist on how to maintain their protein dietary intake of 1.2 $g \cdot kg^{-1} \cdot d^{-1}$ by making minor adjustments to their normal food intake. Food recalls (24 h) were obtained from participants throughout the study duration to verify dietary intake.

3.3.5. Intervention: Exercise Training

Participants trained three times per week on non-consecutive days, for ~1 h per session under supervision. Each session consisted of a 10-minute warm-up walk on the treadmill at a self-selected speed, 5 minutes of range of motion and breathing exercises, 45 minutes of RT, and 5 minutes of cool down stretching and breathing exercises. The four resistance exercises targeting the major muscle groups of the upper and lower limbs were: Horizontal Leg Press (C-403, Atlantis Inc. Laval, QC) in the seated position, Chest Press (PE-140, Atlantis Inc.), Knee Extension (C-230, Atlantis Inc.), and Lat Pulldown (D-123, Atlantis Inc.). Participants performed 3 sets of 15 repetitions for each exercise. Between each set, participants engaged in active recovery through superset exercises: shoulder press (Leg Press), calf raises (Chest Press), torso flexion/extension (Knee Extension), and torso lateral rotation (Lat Pulldown).

Resistance for the four main exercises on the machines was increased by 1-5 lbs when the participant could perform up to 15 repetitions with proper technique. If they could not perform at least 8 repetitions, then the resistance was decreased. A visual Borg Scale (6-20) was used by the subject to indicate their rating of perceived exertion at the end of each set. The duration of each set (Time Under Tension) was obtained (>35 seconds) in order to ensure that the participants were not using momentum to complete the motions. Training weights were determined to consistently be 60-80% of their 1-repetition maximum (1RM). 1RMs for each exercise were obtained pre and post intervention, and changes in muscle strength reported as percent change from baseline.

3.3.6. Laboratory Analysis

Serum insulin values were determined by ELISA (Mercodia Inc, NC, USA; 3.2 %CV) and plasma glucose by the oxidase method (GM9 Fast Plasma Glucose Analyzer, Analox Technologies, ON; 0.59 %CV). Serum insulin and plasma glucose fasting values, peak values, and AUC's were obtained from the results. HOMA-IR was calculated as [(fasting insulin (pmol/L) x fasting glucose (mmol/L)) / 22.5] (42, 43). Insulin-like growth factor-1 (IGF-1) was determined by ELISA (R&D Systems, Inc. MN, USA). Fasting high-sensitivity C-reactive protein (CRP) was measured at the MUHC-Central Biochemistry Laboratory using the Beckman Coulter AU System CRP Latex reagent (Beckman Coulter Canada, LP, ON, Canada).

3.3.7. Statistical Analysis

Results are presented as means ± SEM. Normality was determined using the D'Agostino-Pearson test. Independent t-tests were used to determine differences between the two groups at baseline. Two-factor Repeated Measures ANOVA was used to determine the supplementation (group) and training (time) effect on metabolic responses with multiple time points. When no differences were found between groups with only two time points (pre- and post-intervention), Ala and Leu groups were pooled and analyzed using paired t-tests to determine pre- vs post-intervention differences. The primary outcome under study (insulin sensitivity) is a secondary outcome of a larger study powered to detect differences in myofibrillar FSR. The sample size estimation was based on a difference of 20% in FSR between Leu versus Ala placebo groups, with a standard deviation of ~15% (44). Therefore, with an effect size of 1.25, 9 subjects per group were required (α=0.05; β=0.80). Significance was set at $p \leq 0.05$. Statistical analyses were performed using Prism 7.0a (GraphPad Software, Inc. CA, USA).

3.4. Results

Participant recruitment to completion is described in **Figure 3-1**. Of the five participants who left the study, two fell ill with conditions unrelated to the study, one sustained an injury unrelated to the study, one moved out of province, and one was unable to maintain adherence to the protocol. The remaining 19 participants were all included in the analysis as all maintained an adherence of at least 80%, with no difference between the two groups. Adherence to

exercise was 90.8 ± 1.1% for all participants. Adherence to supplements in the Ala group was 94.1 ± 1.8% and in the Leu group was 97.3 ± 0.7%. No adverse events related to the supplements were reported during the study.

Participants did not differ at baseline for any characteristic, body composition, macronutrient intake, or number of frailty criteria (**Table 3-1**). No significant group, or interaction effects were observed on primary outcomes of serum insulin and plasma glucose AUC's (serum insulin, Ala: 69.9 ± 12.4 vs 71.9 ± 12.4 nmol/L·min; serum insulin, Leu: 81.9 ± 7.83 vs 72.5 ± 9.00 nmol/L·min; plasma glucose, Ala: 752 ± 68 vs 744 ± 99 mmol/L·min; plasma glucose, Leu: 814 ± 80.0 vs 753 ± 70.0 mmol/L·min; all pre vs post respectively), fasting, or peak values (**Figures 3-2** & **3-3**). Similarly, no significant group, time, or interaction effects were observed on secondary outcomes of HOMA-IR, IGF-1, N-balance, nor REE (kcal/d or kcal/(LBM·d)), and therefore results of both groups were pooled and presented as one group, pre- and post-intervention (**Table 3-2**). Pre-intervention, both groups were slightly in positive nitrogen balance (Ala, p=0.002; Leu, p=0.04). Usual protein intakes before dietary adjustments were made did not differ between the Leu (1.34 $g \cdot kg^{-1} \cdot d^{-1}$) and Ala (1.17 $g \cdot kg^{-1} \cdot d^{-1}$) groups. Post-intervention, both groups were in nitrogen balance. The Leu group had higher CRP concentrations than the Ala group before and after the study (Ala: 1.30 ± 0.27 vs 0.99 ± 0.20 mg/L; Leu: 2.69 ± 0.65 vs 2.88 ± 0.62 mg/L; pre vs post respectively; group effect p=0.03), with concentrations within the normal range (45) for both groups and time points. There was no time or interaction effect observed on these variables.

Because there were no differences between Leu and Ala in any of the parameters, body composition, protein intake, and muscle strength results were pooled (**Table 3-3**). There were no differences pre versus post in weight, BMI, WHR, AMMI, and Android %Fat. %Fat significantly decreased by 0.92 $\pm$ 0.33% and Gynoid %Fat significantly decreased by 0.79 $\pm$ 0.30%, post-intervention. LBM significantly increased by 762 $\pm$ 125 g post-intervention. Dietary protein intake was maintained during the intervention and remained unchanged from those presented in Table 3-3. 1RMs significantly increased post-intervention from baseline for all exercises: Leg Press increased by 37%, Knee Extension by 34%, Chest Press by 20%, and Lat Pulldown by 18% ($p<0.001$, all exercises).

Table 3-1 Characteristics of pre-frail and frail elderly women by Ala or Leu supplement group at baseline[1]

Characteristic	Ala			Leu			p-value
n	9			10			
Age (y)	76.2	±	1.8	78.7	±	2.1	0.39
Weight (kg)	61.8	±	2.5	62.9	±	2.9	0.77
BMI (kg/m^2)	23.8	±	1.0	26.2	±	1.3	0.18
WHR	0.76	±	0.01	0.80	±	0.01	0.04
Frailty criteria, *n*	2.7	±	0.3	2.6	±	0.3	0.89
Energy intake, kcal/(kg · d)	27.4	±	2.1	25.6	±	2.1	0.30
Protein intake, g/(kg · d)	1.24	±	0.06	1.22	±	0.07	0.28
%Fat	36.0	±	2.2	41.3	±	1.5	0.06
LBM (kg)	38.1	±	1.3	35.2	±	1.4	0.21
AMMI (kg/m^2)	6.36	±	0.16	6.46	±	0.29	0.78
Android region, %Fat	34.9	±	4.0	41.4	±	2.3	0.17
Gynoid region, %Fat	41.3	±	1.7	45.7	±	1.3	0.051

[1]Values are means ± SEM. Ala: alanine supplemented group (control); AMMI: appendicular muscle mass index; BMI: body mass index; LBM: lean body mass; Leu: leucine supplemented group; WHR: waist-to-hip ratio. %Fat: percent body fat.

Table 3-2 Metabolic characteristics in the fasting state and during the metabolic meal test in the Leu and Ala groups combined before and after resistance exercise training in pre-frail and frail elderly women[1]

Characteristic	Pre	Post	p-value
n	19	19	
Meal Response Measurements			
Peak serum insulin (pmol/L)	638 ± 69.3	595 ± 67.4	0.23
Peak plasma glucose (mmol/L)	10.7 ± 0.28	10.5 ± 0.29	0.46
Fasting Measurements			
Serum insulin (pmol/L)	18.9 ± 1.98	17.0 ± 1.42	0.20
Plasma glucose (mmol/L)	5.12 ± 0.07	5.10 ± 0.08	0.43
HOMA-IR	0.72 ± 0.08	0.64 ± 0.05	0.18
Serum IGF-1 (ng/mL)	84.8 ± 4.79	84.2 ± 5.03	0.85
N-Balance (g·N/d)	1.43 ± 0.34 [2]	0.66 ± 0.50	0.10
REE (kcal/d)	1103 ± 28	1095 ± 31	0.65
REE (kcal/(LBM·d))	30 ± 1	30 ± 1	0.07

[1]Values are means ± SEM. Ala: alanine supplemented group; HOMA-IR: Homeostatic Model Assessment - Insulin Resistance; IGF-1: insulin-like growth factor-1; Leu: leucine supplemented group; N-Balance: nitrogen balance; REE: resting energy expenditure.

[2]Different from zero, $p < 0.05$

Table 3-3 protein intake & body composition of pre-frail and frail elderly women in the Leu and Ala groups combined before and after resistance exercise training[1]

Characteristic	Pre	Post	p-value
n	19	19	
Weight (kg)	62.0 ± 1.8	62.4 ± 1.7	0.27
BMI (kg/m^2)	25.1 ± 0.8	25.2 ± 0.8	0.24
WHR	0.79 ± 0.01	0.79 ± 0.01	0.69
%Fat	38.8 ± 1.4	37.9 ± 1.3	0.01
LBM (kg)	36.6 ± 1.0	37.4 ± 1.0	< 0.001
AMMI (kg/m^2)	6.41 ± 0.16	6.52 ± 0.16	0.09
Android region, %Fat	38.3 ± 2.3	37.4 ± 2.1	0.13
Gynoid region, %Fat	43.6 ± 1.1	42.8 ± 1.1	0.02
Protein Intake (g/kg BW)	1.23 ± 0.05	1.22 ± 0.06	0.80

[1]Values are means ± SEM. Ala: alanine supplemented group; AMMI: appendicular muscle mass index; BMI: body mass index; BW: body weight; LBM: lean body mass; Leu: leucine supplemented group; WHR: waist-to-hip ratio. %Fat: percent body fat.

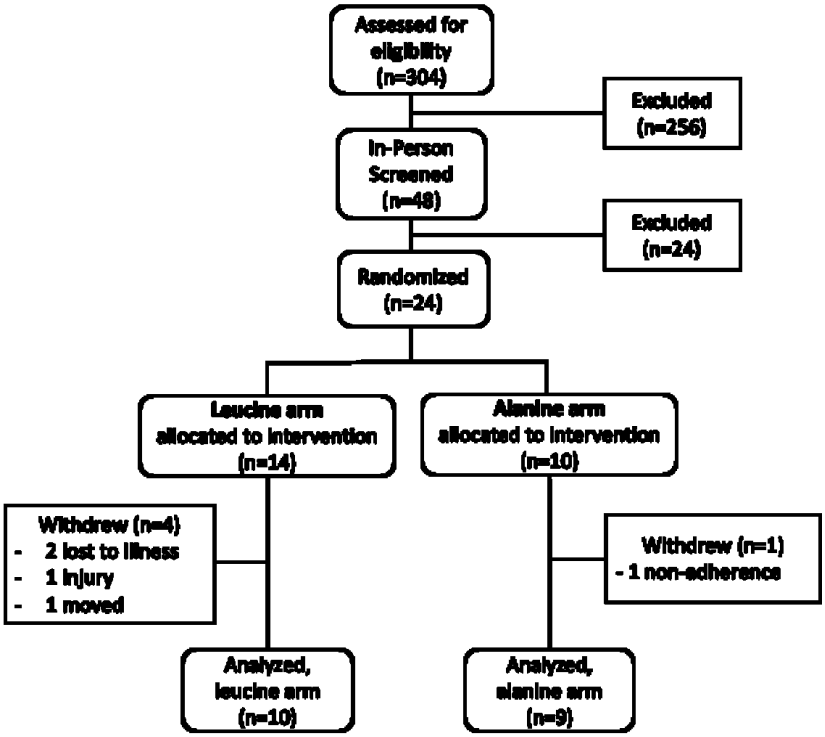

Figure 3-1 Flow diagram depicting participant recruitment to study completion.

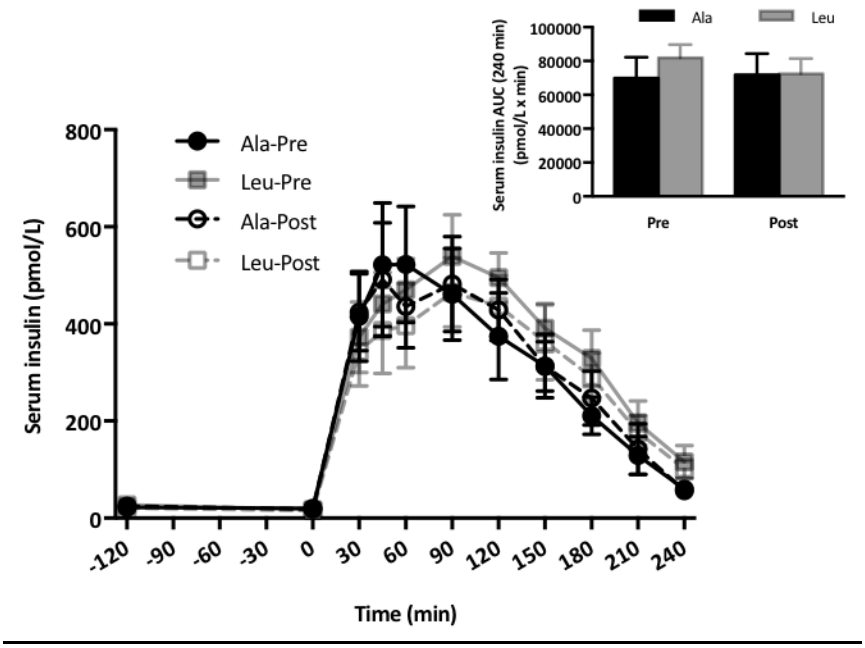

Figure 3-2 Serum insulin responses to a complete standard liquid meal before and after resistance exercise training in pre-frail and frail elderly women supplemented with Leu or Ala. Error bars depict SEM. Leu: *n*=10, Ala: *n*=9. No group, time, or interaction effects in AUC and peak responses were observed. Ala: alanine supplemented group (control); Leu: leucine supplemented group.

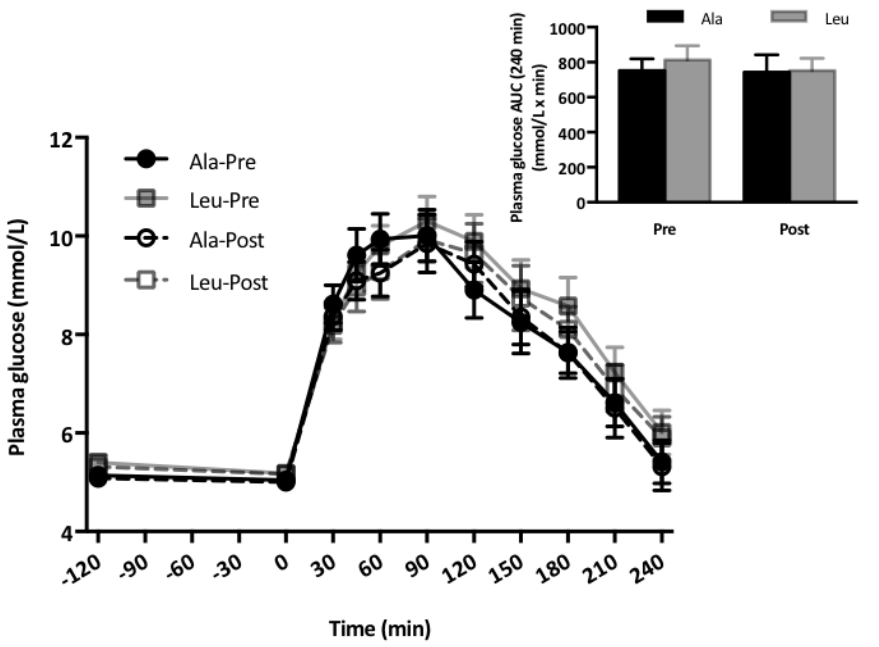

Figure 3-3 Plasma glucose responses to a complete standard liquid meal before and after resistance exercise training in pre-frail and frail elderly women supplemented with Leu or Ala. Error bars depict SEM. Leu: *n*=10, Ala: *n*=9. No group, time, or interaction effects in AUC and peak responses were observed. Ala: alanine supplemented group (control); Leu: leucine supplemented group.

3.5. Discussion

The purpose of this study was to determine if leucine supplementation has an effect on IS in pre/frail older women undergoing RT. We hypothesized that 12 weeks of RT would improve IS, with an added benefit when supplemented with leucine compared to placebo, in pre/frail older women. Our results showed that leucine had no beneficial nor, importantly, no detrimental effects on fasted and post-meal IS in pre/frail older women, a population at risk of developing insulin resistance. To the best of our knowledge, this is the first such study to be conducted in pre/frail older women.

Our participants significantly improved their muscle strength and LBM after 12 weeks of progressive RT. In the current study, RT had no measurable effect on IS in pre/frail older women. Resistance exercise remains the most potent stimulus to gain muscle mass, which theoretically could lead to improved IS. For example, in a study by Bucci, et al. (46), older (~70 y) daughters of either lean or obese mothers (offspring are predisposed to insulin resistance) underwent 4 months of resistance exercise three times per week. At study onset, skeletal muscle, but not whole-body IS, was impaired in older offspring of obese mothers compared to those of lean mothers. Following completion of the intervention, quadriceps muscle mass significantly increased in both groups, but IS measured by glucose uptake was improved in offspring of obese mothers only (18). Our study did not measure glucose uptake directly and was performed in the context of a mixed formula meal but results contrast with these findings as an overall measure of glucose homeostasis measured as plasma glucose AUC did not change

with RT in either group. One possible reason why is that the hereditary predisposition to diabetes was not established in our protocol. Another consideration would be a potential lack of power. Using our results of the glucose and insulin AUC responses to the meal for all participants pre and post-intervention, we estimated that it would take *n*=147 (insulin AUC) or *n*=168 (glucose AUC) participants to detect a difference. The interpretation of such a high number of participants is that for all purposes there is no significant differences pre and post in our study. Furthermore, multiple indices of IS agree with this interpretation (fasting plasma glucose, HOMA-IR, plasma glucose and serum insulin AUCs) and were not altered in our study, which renders it unlikely that our intervention had a significant impact on IS measured via other indices.

In the current study, participants gained muscle mass without a corresponding change in IS. Srikanthan and Karlamangla (47) clearly described an inverse relationship between MMI (muscle mass divided by height) measured by BIA and insulin resistance based on HOMA-IR across the whole range of MMI. After excluding those with diabetes and adjusting for age, sex, ethnicity, continuous BMI, generalized obesity, overweight status and central obesity, the authors found that each 1 kg/m^2 increase in MMI was associated with a 4% relative reduction in HOMA-IR, 0.3% relative reduction in HbA1C, and 9% relative reduction in prediabetes prevalence, indicating improved IS (47). Conversely, Matta et al. (48) using a path analysis modelling data from the Quebec Longitudinal Study on Nutrition and Aging (NuAge) showed a positive relationship between MMI and HOMA-IR, suggesting that the greater the muscle mass,

the greater the insulin resistance. However, a previous study determined that the effect of muscle loss on glucose tolerance was only a minor contribution to the development of glucose intolerance, and that visceral fat was primarily implicated in the risk of developing insulin resistance with aging (49). Indeed, even persons with a high MMI who also have a high fat mass, have more intermuscular fat and lower IS (50, 51), while those with less muscle (malnourished) who also have less fat, possess a greater IS (52). Furthermore, in frail persons, it is concurrent obesity which appears to trigger insulin resistance rather than low muscle mass alone (53). Thus, the relationship between muscle mass and impaired glucose homeostasis is far from clear, with many other studies reporting conflicting results, independent of the methodology employed (see (54) for an in-depth review of the topic). Noteworthily, results drastically change depending on whether fat-free mass is expressed as a percentage of body weight or normalized to height, and care must be taken when interpreting and comparing such results (54).

Sarcopenia is a key component of frailty and, as mentioned above, a consequence of reduced muscle mass is limited muscle availability for insulin-stimulated glucose uptake. Interestingly, although frail persons who are sarcopenic have less muscle available for glucose disposal, many are also malnourished, which increases IS (55). This compensatory mechanism has been termed “burn-out diabetes”. Although our participants were not malnourished, it is of interest to note that muscle retains its ability to re-sensitize to insulin.

Contrary to the above previous findings, we observed no effect of 12 weeks of RT on IS. A possible reason for this discrepancy could be that our participants were insulin-sensitive at baseline as evidenced by their HOMA-IR score and normal fasting plasma glucose (see Table 2). Ergo, there were no significant deficits in insulin function to improve upon. Even though CRP levels were slightly elevated in the Leu group, indicating a relatively higher presence of systemic inflammation, the levels were within normal reference ranges (0.00-3.00 mg/L) at both time points (45). Usually CRP confers cardiovascular risk when values are above 3.0 mg/L (56). This provides further support that both groups were relatively free from metabolic risks at the onset of the study. A growing number of studies suggests that any positive or negative effects of leucine supplementation on glucose metabolism occur only in states of abnormal glucose homeostasis. Thus, it appears that RT benefits those who are either at-risk or have impaired insulin action and exhibits no further improvements in those who have normal IS, which is consistent with some (18, 57-59), but not all previous recent findings (46, 60, 61).

A large body of literature exists regarding insulin and leucine signalling (62). Aging is associated with reduced insulin-induced inhibition of catabolism (63) as well as impaired insulin signalling (64, 65). Thus, in populations at risk of insulin resistance, such as aging populations, it is important to explore interventions to ameliorate said risk.

Although leucine has been attributed a potential negative impact on IS, we did not observe any effect acutely, i.e., during the postprandial period pre-intervention, nor after 12 weeks of supplementation. Potential rationales for our findings include that the threshold of leucine supplementation needed to span from a physiological anabolic signal to an insulin-resistant one may be high, and our doses were well below the tolerable upper limit (27, 66). Furthermore, the timing of leucine supplementation appears to play a role in the impact of leucine on IS. Rats who consumed leucine in their drinking water (non-pulsatile) had a significantly higher fasting glycemia compared to rats who were given the same amount of leucine in a twice-daily bolus (pulsatile feeding) (67). This indicates that tissues required time for the leucine signal to dissipate, and the continual presence of leucine could be metabolically aggravating. In the current study, the participants consumed leucine in a pulsatile fashion. Of note, leucine was supplied as a free amino acid added to a formula mixed meal which, in and of itself, implies the simultaneous interaction of complex metabolic pathways related to the different macronutrients. It has been shown, with an intravenous meal mimicking the fed state, that glucose metabolism was not further compromised by leucine (68). Finally, two previous studies consisting of non-frail older men with (69) and without (70) type 2 diabetes mellitus undergoing leucine supplementation (without exercise training) similar to our study for a duration of 3-6 months resulted in no positive or negative effects on indices of IS compared to control. These previous studies are consistent with the novel results of the current study in pre/frail older women.

Possible limitations of the current study include that we used a model of mixed meals based on a liquid formula diet to maximally control quantities of macronutrients to test our hypothesis. There remains a possibility that leucine could still affect glucose metabolism in a regular solid mixed meal situation with further nutrient interactions. Our results were obtained in frail but without intercurrent diseases, relatively homogenous non-obese participants (only *n*=1 was glucose intolerant), and with a healthy average BMI (see Table 1), therefore testing sarcopenic-obese subjects who have by definition more glucose intolerance, may lead to different results. Finally, the state-of-the-art technique for assessing insulin resistance remains the hyperinsulinemic, euglycemic clamp test which could be applied to answer any remaining contention on the effect of leucine on glucose metabolism in frail persons.

In conclusion, twelve weeks of RT in women consuming adequate dietary protein and consuming 7.5 g of leucine per day had no beneficial nor importantly, no detrimental effects on IS in pre/frail older women. These women were insulin sensitive at study onset and experienced no consequences on glucose metabolism with prolonged leucine supplementation. Thus, leucine supplementation remains a viable and safe method of supplementation in this population. Future studies should include frail participants who are insulin resistant.

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Statement of authors' contributions to manuscript

K.J.J., S.C., and J.A.M. designed research, K.J.J., M.L., S.C., and J.A.M. conducted research, K.J.J., M.L., S.C., and J.A.M. analyzed data, K.J.J., S.C, and J.A.M. wrote the paper, and J.A.M. had primary responsibility for final content. All authors have read and approved the final manuscript.

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CONNECTING STATEMENT II

The previous manuscript showed that chronic leucine supplementation had no detrimental (or beneficial) effect on indices of insulin sensitivity in pre-frail and frail older women after 12 weeks of progressive resistance training. It is recognized that leucine exposure has been shown to increase mitochondrial biogenesis while simultaneously enhancing lipid oxidation in cultured myocytes [281]. Furthermore, chronic essential amino acid supplementation containing leucine has improved insulin sensitivity along with mitochondrial biogenesis and genes related to ATP production (mitochondrial function) in adult mice with hyperglycemia [282]. Thus, insulin sensitivity has been linked with mitochondrial quantity and function. Mitochondria have been heavily implicated in the aetiology of sarcopenia [53]. A decline in skeletal muscle in *ex vivo* mitochondria function mimicking physiological conditions has recently been observed with aging [52]. Declines seen in reductions of mitochondria function capacity have been proposed to be attributed at least in part to a reduction in mitochondrial content concomitant with reduced levels of physical activity [274], although this is not universally noted [270]. Leucine has also been shown to increase mitochondrial biogenesis in cultured myocytes [64, 65]. RT is the strongest stimulus to counteract sarcopenia and may enhance mitochondrial biogenesis [61, 62]. Previous studies investigating the effects of RT on mitochondria in the elderly have not comprehensively assessed multiple aspects of mitochondrial function simultaneously [62, 66-69], and never in pre-frail and frail women. Therefore, we measured the respiratory capacity, ROS production capacity, calcium retention capacity, and time to pore opening (latter two measurements are indices of sensitivity of the mitochondria to initiate apoptosis) in our participants mitochondria, *ex vivo*, before and after

our intervention. We hypothesized that 12 weeks of resistance training would improve inherent mitochondrial function and/or increase the quantity of mitochondria to improve net function in pre-frail and frail older women habitually consuming an optimal amount of dietary protein, with an added benefit when supplemented with leucine compared to placebo. In addition, these improvements would occur in conjunction with functional improvements in skeletal muscle such as maximal strength and aerobic performance.

CHAPTER 4: MANUSCRIPT 2

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Leucine increases mitochondrial quantity in conjunction with resistance training and adequate protein intake in pre/frail women

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4.1. Abstract

Background: Frailty is a clinical condition associated with loss of muscle mass and strength (sarcopenia). Mitochondria are centrally implicated in frailty and sarcopenia. Leucine (Leu) can increase mitochondrial biogenesis in myocytes, while resistance training (RT) is the strongest stimulus to counteract sarcopenia and may enhance mitochondrial biogenesis.

Objective: We determined the effects of Leu supplementation and RT on mitochondrial content and function in pre/frail elderly women in a randomized double-blinded placebo-controlled study.

Methods: Nineteen pre/frail elderly women (77.5 ± 1.3 y, BMI: 25.1 ± 0.9 kg/m^2), based on the Frailty Phenotype, underwent 3-months of RT 3x/week with protein-optimized diet and were randomized to 7.5 g/d of Leu supplementation or placebo alanine (Ala). Pre/post-intervention mitochondrial respiration, reactive oxygen species (ROS) production, calcium retention capacity (CRC), time to permeability transition pore (mPTP) opening, mitochondrial VDAC protein content, leg press 1-repetition maximum (1RM), and 6-minute walk test (6MWT) were measured.

Results: No time, supplementation, or interaction effects were observed for respiration, ROS, time to mPTP opening, and CRC. VDAC levels significantly increased in the Leu group post-intervention ($p=0.012$). Both groups significantly increased leg press 1RM and 6MWT, with no effect of supplementation.

Discussion: Leu supplementation with 3 months of RT increased mitochondrial content. Future studies should investigate if there is an increase in mitochondrial turnover or a shift in quality control (mitophagy) in leucine supplemented pre/frail elderly women who undergo 12 weeks of RT. (ClinicalTrials.gov ID: NCT01922167).

4.2. Introduction

The World Health Organization has predicted that globally, by 2050 those over 60 years old will double from about 11% to 22% of the population. Numerically, this corresponds with approximately 2 billion people aged 60 or older, and 400 million of those being 80 years old or more [1]. Coinciding with this increase will be a surge in the occurrence of people with low strength and muscle mass (sarcopenia). If left unchecked, the development of sarcopenia can further deteriorate to frailty and disability [2], cumulating in enormous economic burdens for healthcare systems on a global level [3, 4]. The gain in life expectancy of women is in part offset by increased frailty and years with disability [5].

A commonly-used clinical tool for diagnosing frailty is to use the Frailty Phenotype [6], which is comprised of five criteria: slowness, weakness, unintentional weight loss, exhaustion, and sedentariness [6]. Individuals meeting 1-2 criteria are deemed pre-frail, and those with $\geq$3 are frail. Sarcopenia is defined as muscular weakness and unintentional weight loss (atrophy) and is considered severe when muscle functioning is also compromised (e.g., slowness) [7]. A decline in skeletal muscle function is also characteristic of aging and experimental evidence suggests that mitochondria are heavily implicated in the aetiology of sarcopenia [8]. A decline in *ex vivo* mitochondria function (respiration and ROS production) has been observed in our group [9], and another study mimicking physiological conditions has recently also observed a decline with aging [10]. Damaged mitochondria not only have a hindered respiratory capacity but can also produce elevated ROS, resulting in further damage to mitochondria [11, 12]. They can also present with increased susceptibility to apoptosis via decreased calcium retention capacity (CRC) and/or time to mitochondrial permeability transition pore (mPTP) opening, which may ultimately lead to fiber atrophy and hampered muscle performance [13-16]. Thus, mitochondria appear to be central to many detrimental age-related physiological changes. Indeed, both mitochondrial quality control (mitophagy & biogenesis), abundance, and function [17-23] have been shown to decline with aging. However, this is not always observed [20, 23-

26] and similarly to respiratory capacity in our aforementioned study [9], many of these declines are thought to be attributed to lower mitochondrial abundance in older persons, likely attributed to increased sedentariness (as opposed to an inherent change with aging) [17, 27, 28]. A recent study by our group demonstrated that mitochondria content was significantly lower in frail elderly women compared to young inactive controls [9]. Furthermore, very little is known about mitochondrial functioning in aging women as opposed to men, though recent studies have suggested sex-dependent differences in mitochondrial physiology [29].

Leucine stimulates muscle protein synthesis by activating mammalian target of rapamycin complex 1 (mTORC1) [30]. mTORC1 signaling stimulates ATP-consuming processes (e.g., protein synthesis), necessitating mitochondrial function to replenish consumed ATP. Consequently, mTORC1 activity has been shown to correlate with mitochondrial activity [31]. It has recently been shown that leucine has the capability to stimulate mitochondrial biogenesis, although the precise mechanisms remain unclear [32, 33]. Additionally, in a recent study by Schnuck et al. [33] leucine treatment of cultured myoblasts resulted in heightened oxygen consumption which was attributed to a proportional increase in mitochondrial content. Therefore, leucine has received attention as a potential nutraceutical that may have benefits on mitochondrial function and/or content.

It is well known that resistance training (RT) increases muscular mass and strength [34]. RT has been shown to significantly increase mitochondrial content in elderly women [35] after 6 months of training. Furthermore, a recent study in elderly men and women showed that 8 weeks of RT significantly increased mitochondrial content in skeletal muscle [36]. Finally, recent evidence in muscle of both healthy and pre/frail older adults at the transcriptome level has provided further insight regarding the plasticity of aging muscle to adapt to chronic resistance training [37].

Differences have been found in older persons who are active versus sedentary in mitochondrial function (possibly due to reduced mitochondrial content with sedentariness) [38], although this is not always seen [28]. Additionally, previous studies investigating the effects of RT on mitochondria in the elderly have not comprehensively assessed multiple aspects of mitochondrial function simultaneously (e.g., respiration, ROS production, mPTP sensitivity and mitochondria content) [35, 39-42]. To the best of our knowledge, it is currently unknown if mitochondrial function can be improved upon in a cohort of pre/frail elderly women who are sedentary at baseline and undergo a 12-week resistance training program with or without leucine supplementation. With the above context in mind, our research question was to determine the effects of leucine supplementation on mitochondrial function in pre/frail elderly women undergoing resistance training. We hypothesized that 12 weeks of resistance training would improve inherent mitochondrial function and/or increase the quantity of mitochondria, with an added benefit when supplemented with leucine compared to placebo. In addition, these improvements would occur in conjunction with functional improvements in skeletal muscle such as maximal strength and aerobic performance.

4.3. Participants and Methods

4.3.1. Study Design

The study was conducted as a registered randomized double-blinded placebo-controlled trial (ClinicalTrials.gov ID: NCT01922167). All participants underwent a 12-week high-intensity progressive resistance exercise training program and followed a protein-optimized diet (~1.2 g/kg/d). Half were randomized to receive leucine (2.5 g 3x/d) supplementation and the other half an isonitrogenous amount of alanine, an amino acid known not to stimulate muscle protein synthesis (1.7 g 3x/d)[43]. All tests were performed before and after the intervention.

4.3.2. Participant Recruitment and Screening

Frail or Pre-Frail community-dwelling elderly women (>65 y) according to the Fried [6] criteria were recruited from the Geriatrics outpatient clinic of the McGill University Health Centre (MUHC) and advertisements posted in the local seniors' newspaper. Three hundred and four women were screened via telephone, 24 entered the study, and 19 completed the study. Of the five participants who left the study, two became ill with conditions unrelated to the study, one sustained an injury unrelated to the study, one moved out of province, and one was unable to maintain adherence to the protocol. The remaining 19 participants maintained adherence of at least 80% to both exercise program and supplement intake. Inclusion criteria consisted of non-disabled women who were cognitively intact with a Mini Mental State Examination score (MMSE) $\geq$24, body mass index (BMI) of 18.5-35 kg/m^2, normal complete blood count, biochemistry, A1C, TSH, urine analysis, no diabetes determined by a 75-g oral

glucose tolerance test (OGTT), negative serology for hepatitis and HIV, and normal chest X-ray and ECG results. Exclusion criteria were: dependence on walking aids, Geriatric Depression Score (GDS) short form <6 [44], substance abuse, eating disorders, active medical conditions other than skin cancer within 5 years, serum creatinine >110 µmol/L, hemoglobin (Hb) <110 g/L, and medications known to interfere with metabolic endpoint measurements (e.g., beta-blockers). The study was approved and monitored by the MUHC Human Research Ethics Board (REB code: 13-211-BMB). All participants read and signed an informed consent form before participation and screening visits. All outcome measurements were performed pre- and post-intervention.

4.3.3. Intervention: Supplementation

Participants were randomized into receiving either leucine or alanine supplementation by an independent source based on random generated numbers. Powdered supplements of leucine and isonitrogenous amounts of alanine were provided in sterile sealed screw-top 100 mL containers, of individual doses (2.5 g leucine [ProteinCo. QC, CA] and 1.7 g alanine [PureBulk® OR, USA]). Participants were instructed to consume one complete dose of supplement at the onset of each main meal (breakfast, lunch, dinner) for the duration of the intervention. Log sheets were provided to track compliance and were collected every 2 weeks.

4.3.4. Intervention: Exercise Training

Participants trained three times per week on non-consecutive days, for ~1 h per session under supervision, as previously described [45]. Participants performed resistance exercises targeting the major muscle groups of the upper and lower limbs: horizontal leg press, chest press, knee extension, and lateral pulldown. Participants performed 3 sets of 15 repetitions for each exercise and resistance was increased by 1-5 lbs (0.45-2.27 kg) when the participant could perform up to 15 repetitions with the proper technique. Training weights were determined to consistently be 60-80% of their 1-repetition maximum (1RM).

4.3.5. Physical Testing Outcome Measures

Physical testing was done at least 48 hours before biopsy. Participants were instructed to refrain from vigorous physical activity 48 hours prior to testing. Leg press 1RM was performed on a horizontal leg press (C-403, Atlantis Inc. Laval, QC) by trained kinesiologists according to standard protocol [46]. The 6-minute walk test (6MWT) was performed over a 30-m hallway (60-m course) according to standard protocol [47].

4.3.6. Mitochondrial Function Outcome Measures

Methodology outlined below has been previously published [9, 28].

4.3.6.1. Sample Collection:

Participants presented to the clinical unit after an overnight fast, and at least 48 h after the last bout of physical activity. Skeletal muscle samples were obtained from the lateral

portion of the *vastus lateralis* ~20 cm above the knee, 4-5 cm apart using the Bergström needle biopsy technique [48]. After fat removal, samples were separated into aliquots. Due to limited muscle tissue from a few participants, some measurements were only performed on a subset of muscle biopsy specimens. The sample size used for each measurement is indicated in the results section of each technique used in this study.

4.3.6.2. Preparation of permeabilized fiber bundles

About 70-100 mg of fresh muscle tissue was put in ice-cold buffer A (2.77 mM CaK_2EGTA, 7.23 mM K_2EGTA, 6.56 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 50 mM KMES, 20 mM imidazol, 20 mM taurine, 5.3 mM Na_2ATP, 15 mM phosphocreatine, pH 7.3). Manual dissection under a stereomicroscope served to identify 2-6 mg fiber bundles that were subsequently chemically permeabilized for 30 min in buffer A with 50 µg mL^{-1} of saponin at 4°C with gentle agitation.

4.3.6.3. High Resolution Respirometry:

Saponin-permeabilized fiber bundles were washed 3 × 10 min in buffer B (2.77 mM CaK_2EGTA, 7.23 mM K_2EGTA, 1.38 mM $MgCl_2$, 3 mM K_2HPO_4, 0.5 mM DTT, 20 mM imidazole, 100 mM KMES, 20 mM taurine and 2 mg/mL BSA, pH 7.3). Respiration of 3–5 mg of wet weight fibers was then measured at 37°C with continuous stirring in 2 mL of buffer B in an Oxygraph-2 K (Oroboros, Innsbruck, Austria), under hyperoxygenated conditions to prevent oxygen diffusion limitation. Respiration was recorded after addition of the following substrates:

glutamate (10 mM) + malate (5 mM) (state 2); ADP (2 mM) (maximal state 3 respiration driven by complex I substrates); succinate (10 mM) (state 3 respiration driven by complex I & II substrates); Cytochrome c (5 mM) (assess mitochondrial outer membrane integrity); antimycin A (10 µM) (inhibit complex III). The acceptor control ratio (ACR) was calculated as respiration driven by the addition of ADP (state 3) divided by respiration driven by glutamate and malate (state 2) as an indication of mitochondrial coupling.

4.3.6.4. Mitochondrial ROS Emission:

Permeabilized fiber bundles for ROS emission were washed 3 × 10 min in buffer Z (110 mM KMES, 35 mM KCl, 1 mM EGTA, 3 mM $MgCl_2$, 10 mM K_2HPO_4 and 0.5 mg/mL BSA, pH 7.3). ROS emission was determined by recording the rate of generation of the fluorescent compound resorufin (product of the oxidation of Amplex red by H_2O_2 and fatty acid hydroperoxides released from the mitochondria). Fluorescence was measured using an F-2500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) at an excitation/emission wavelength of 563/587 nm. Following baseline measurements, permeabilized fiber bundles (3-6 mg) were added to 600 µl of solution Z, with 5.5 µM Amplex red and 1 U per mL horseradish peroxidase (HRP) in a magnetically stirred quartz cuvette at 37°C. The substrates were then added sequentially: glutamate (10 mM) + malate (2 mM), succinate (10 mM), ADP (10 µM), ADP (100 µM) and Antimycin A (10 µM). ROS emission was determined using a standard curve constructed on the same day of the experiment using known concentrations of H_2O_2. Fiber bundles were retrieved and stored at -80°C until western blot analysis.

4.3.6.5. Time to mPTP opening and CRC:

Permeabilized fiber bundles for mPTP function were washed 3 × 10 min in solution C (80 mM KMES, 50 mM Hepes, 20 mM taurine, 0.5 mM DTT, 10 mM $MgCl_2$ and 10 mM ATP, pH 7.3) and then had myosin extracted using solution D (800 mM KCl, 50 mM Hepes, 20 mM taurine, 0.5 mM DTT, 10 mM $MgCl_2$ and 10 mM ATP, pH 7.3) without agitation. Myofibers were then washed 3 × 10 min in CRC solution (250 mM sucrose, 5 µM EGTA-Tris Base and 10 mM Tris-MOPS, pH 7.4). The bundles (4-6 mg) were then added to 600 µL of CRC solution containing glutamate (5 mM), malate (2.5 mM), phosphate (10 mM), oligomycin (0.5 nm) and Calcium Green™-5 N, Hexapotassium Salt (0.001 mM) (Life Technologies, USA). The Ca^{2+} uptake by the mitochondria was measured by monitoring the decrease in fluorescence observed corresponding to reduction in free Ca^{2+} in the solution when Ca^{2+} was taken up into the mitochondria. The time point at which the fluorescence began to increase was taken as the time to pore opening, while the amount of Ca^{2+} up taken by the mitochondria prior to pore opening as the CRC. Fluorescence was detected using an F-2500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) at an excitation/emission wavelength of 505/535 nm. The Ca^{2+} concentration in the solution was determined using a calibration curve of known Ca^{2+} concentrations performed on the day of the experiment. Fiber bundles were retrieved and stored at -80°C until western blot analysis.

Analysis of all mitochondrial function experiments was performed using bespoke software created in-house using Igor Pro Software (Wavemetrics; https://www. wavemetrics.com)[23].

4.3.6.6. Mitochondrial Protein Content:

Western blotting for voltage-dependent anion channel (VDAC, an outer membrane protein) was used as a marker of mitochondrial content in all retrieved fiber bundles. Five to 36 mg of muscle tissue was homogenized 2x45 s with a robot homogenizer (Minibead-beater, Biospec Products, USA) with 1.4 mm ceramic beads and a 10x volume of RIPA extraction buffer (50 mM Tris base, 150mM NaCl, 1% Triton X-100, 0.5% sodium deoxycolate, 0.1% sodium dodecyl sulphate, and protease inhibitor cocktail tablet [1 tablet/10 µl of RIPA buffer; ROCHE]). Samples were then incubated at 4°C for 2 h and then centrifuged at 12,000g for 20 min at 4°C. The supernatant was collected and protein content was assessed via Bradford assay. Immunoblotting was performed with 15 µg of tissue protein diluted in 4x Laemli buffer and extraction buffer and boiled at 95°C for 5 min. 24 µl of sample was then loaded onto a 12% acrylamide gel, electrophoresed by SDS-PAGE and transferred at 4°C to polyvinylidene fluoride membranes (Amersham Hybond ECL, GE Healthcare Life Sciences). The membranes were blocked in 5% semi-skimmed milk for 1 h at room temperature and incubated over night at 4°C with a primary mouse monoclonal anti-VDAC antibody (ab14734, dilution 1:1000; Abcam) diluted in 5% BSA. Total protein loading (Mini-PROTEAN® TGX Stain-Free™ Precast Gels, Bio-Rad Laboratories, Inc. PA, USA) was used to normalize protein loading [9, 49]. Membranes were incubated with rabbit anti-mouse HRP-conjugated secondary antibody diluted in 5% milk (ab6728, diluted 1:2000; Abcam) for 1 h at room temperature. Protein bands were detected with SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Scientific, Waltham, MA, USA) and imaged with a BIO-RAD image system (Bio-Rad ChemiDoc™MP Imaging System). Identification and quantification of protein bands was performed using Image Lab™ Software, Version 6.0.1 (Bio-Rad Laboratories, Inc. PA, USA), where both bands at the approximate molecular mass of VDAC in a given blot were combined to obtain an index of the quantity of VDAC protein [49].

All mitochondrial function values obtained in the study were expressed relative to VDAC to normalize the respiratory capacity per mitochondrion (i.e., the intrinsic organelle function), except time to mPTP opening which is independent of mitochondrial content [50]. VDAC protein content was chosen to normalize mitochondrial function data over mitochondrial enzyme function because changes in physical activity can alter enzymatic function [51-54].

4.3.7. Statistical Analysis

Numerical results and bar graphs are presented as means $\pm$ SEM. Normality was determined using the Shapiro-Wilk test. Independent t-tests were used to determine differences between the two groups at baseline. Two-factor repeated measures ANOVA was used to determine the leucine supplementation (group) and exercise training (time) effects. When significant interaction effects were observed, post-hoc comparisons were performed using the Sidak test. Significance was set at $p \leq 0.05$. Statistical analyses were performed using Prism 7.0a (GraphPad Software, Inc. CA, USA).

4.4. Results

Study participant profiles have been published [45]. Respectively, at baseline Ala (n=9) and Leu (n=10) groups were 76.2 $\pm$ 1.8 and 78.7 $\pm$ 2.1 y; weighed 61.8 $\pm$ 2.5 and 62.9 $\pm$ 2.9 kg; BMI was 23.8 $\pm$1.0 and 26.2 $\pm$ 1.3 kg/m^2 and had 2.7 $\pm$ 0.3 and 2.6 $\pm$ 0.3 Frailty Criteria. Participants did not differ at baseline for any characteristic (Table 4-1).

VDAC: The VDAC protein expression, a marker for mitochondrial content (Figure 4-1) did not differ pre-training and increased only in the Leu group (*n*=7) with exercise training (interaction p=0.034; time effect, p=0.020). Ala (*n*=8) remained unchanged from baseline.

Respiration: Respiratory capacity did not change over time or differ between groups, and no interaction effects were observed for any substrate, normalized to bundle wet weight (Ala: *n*=8; Leu *n*=7) (Figure 4-2A) or mitochondrial content (VDAC, Ala: *n*=8; Leu *n*=6) (Figure 4-2B). ACR did not differ over time or between groups, and no interaction effects were observed (Ala: *n*=8; Leu *n*=5) (Figure 4-3).

ROS: ROS production did not differ over time or between groups, and no interaction effects were observed for any substrate, normalized to bundle wet weight (Ala: *n*=8; Leu *n*=7) (Figure 4-4A) or mitochondrial content (VDAC, Ala: *n*=7; Leu *n*=6) (Figure 4-4B).

CRC & mPTP: CRC did not differ over time or between groups, and no interaction effects were observed normalized to bundle wet weight (Ala: *n*=6; Leu *n*=5) (Figure 4-5A) or mitochondrial content (VDAC, Ala: *n*=5; Leu *n*=4) (Figure 4-5B). Time to pore opening did not differ over time or between groups, and no interaction effects were observed (Ala: *n*=6; Leu *n*=5) (Figure 4-6).

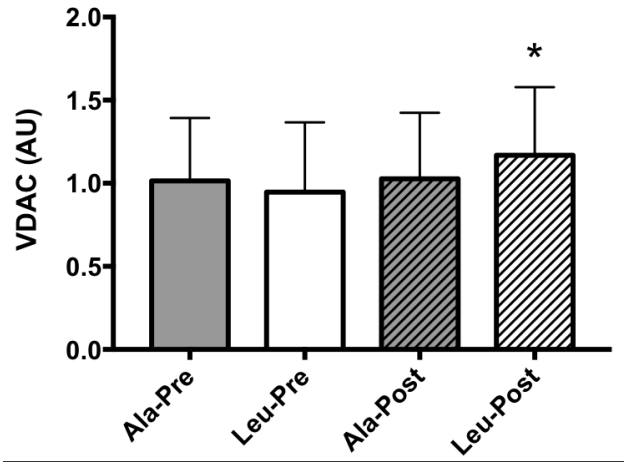
Leg Press & 6MWT: Maximal anaerobic muscular function, tested via a Leg Press 1RM, significantly increased from pre to post intervention (time effect, $p<0.0001$), with no group or interaction effect (Ala: 76.0 ± 8.1 vs. 99.6 ± 9.2 kg; Leu: 71.3 ± 5.9 vs. 96.4 ± 5.9 kg, pre vs post, respectively). Muscular aerobic function was measured via the 6MWT. Distance walked during the 6MWT significantly increased from pre to post intervention (time effect, $p=0.039$), with no group or interaction effect (Ala: 508 ± 28.8 vs. 538.0 ± 20.1 m; Leu: 474.4 ± 27.3 vs. 497.0 ± 26.6 m, respectively).

Table 4-1 Participant Characteristics of Pre/Frail Women by Supplement Group at Baseline

Characteristic	Ala	Leu
n	9	10
Age (y)	76.2 ± 1.8	78.7 ± 2.1
Weight (kg)	61.8 ± 2.5	62.9 ± 2.9
BMI (kg/m^2)	23.8 ± 1.0	26.2 ± 1.3
Number of Frailty Criteria	2.7 ± 0.3	2.6 ± 0.3
%fat	36.0 ± 2.2	41.3 ± 1.5
LBM (kg)	38.1 ± 1.3	35.2 ± 1.4

BMI: body mass index; LBM: lean body mass; %fat: percent body fat

A



B

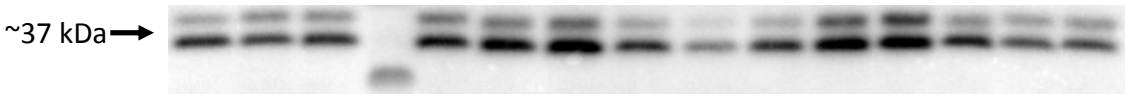


Figure 4-1 VDAC protein expression determined by Western Blot in pre/frail women with and without leucine supplementation before and after 12 weeks of resistance exercise training (A). Representative example of Western Blot (B).

Error bars depict ± SEM. Ala: *n*=8, Leu: *n*=7. No significant group or time effects were observed. *denotes interaction effect, $p<0.05$. Ala: alanine supplemented group (control); Leu: leucine supplemented group.

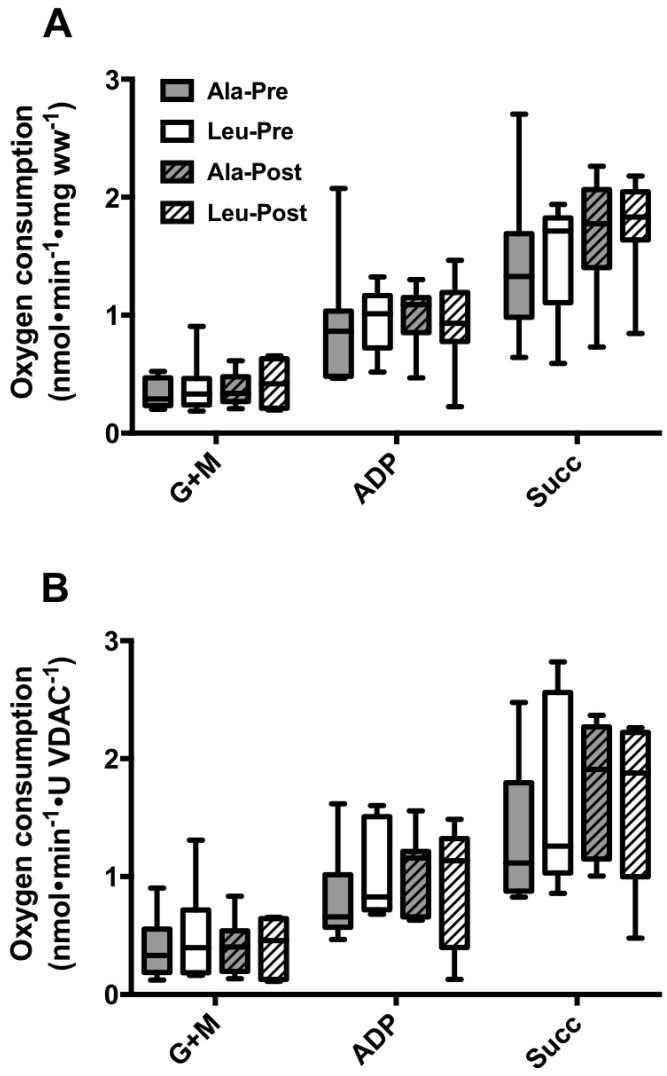


Figure 4-2 Mitochondrial respiration in permeabilized fiber bundles of *vastus lateralis* normalized to (A) fiber wet weight, and (B) VDAC abundance as a marker of mitochondrial content in pre/frail women with and without leucine supplementation before and after 12 weeks of resistance exercise training.

Error bars depict ± SEM. (A) Ala: *n*=8, Leu: *n*=7; (B) Ala: *n*=8, Leu: *n*=6. No significant group, time, or interaction effects in respiration in any state were observed. ADP: adenosine

triphosphate; Ala: alanine supplemented group (control); G+M: glutamate + malate; Leu: leucine supplemented group; Succ: succinate.

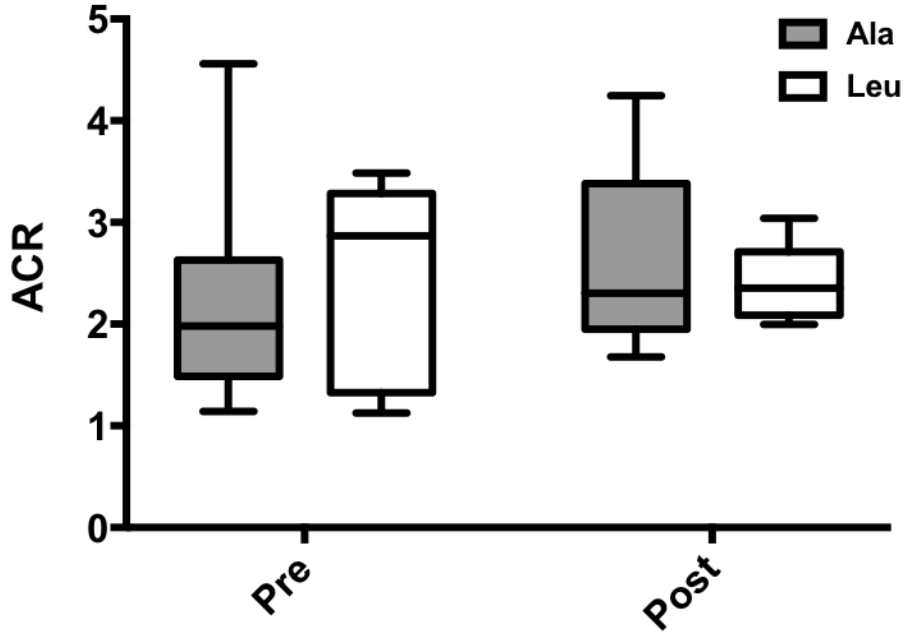


Figure 4-3 Acceptor control ratio obtained by the division of ADP by glutamate+malate respiration.

Error bars depict ± SEM. Ala: *n*=8, Leu: *n*=5. No significant group, time, or interaction effects were observed. ACR: acceptor control ratio; Ala: alanine supplemented group (control); Leu: leucine supplemented group.

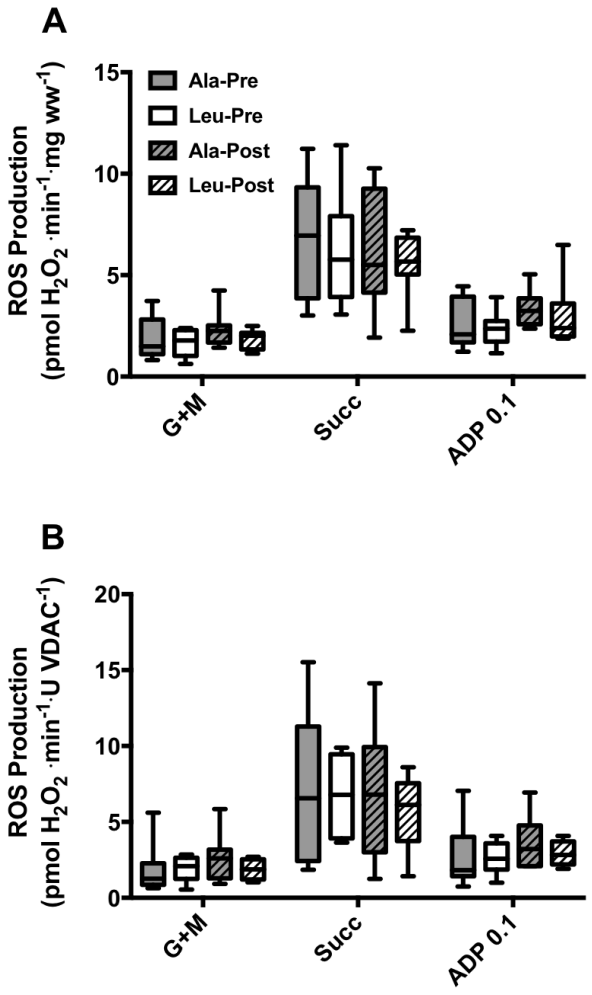


Figure 4-4 Mitochondrial ROS production in permeabilized fiber bundles of *vastus lateralis* normalized to (A) fiber wet weight, and (B) VDAC abundance as a marker of mitochondrial content in pre/frail women with and without leucine supplementation before and after 12 weeks of resistance exercise training. Error bars depict ± SEM. (A) Ala: *n*=8, Leu: *n*=7; (B) Ala: *n*=7, Leu: *n*=6. No significant group, time, or interaction effects in ROS production in any state were observed. ADP: adenosine triphosphate; Ala: alanine supplemented group (control); G+M: glutamate + malate; Leu: leucine supplemented group; Succ: succinate.

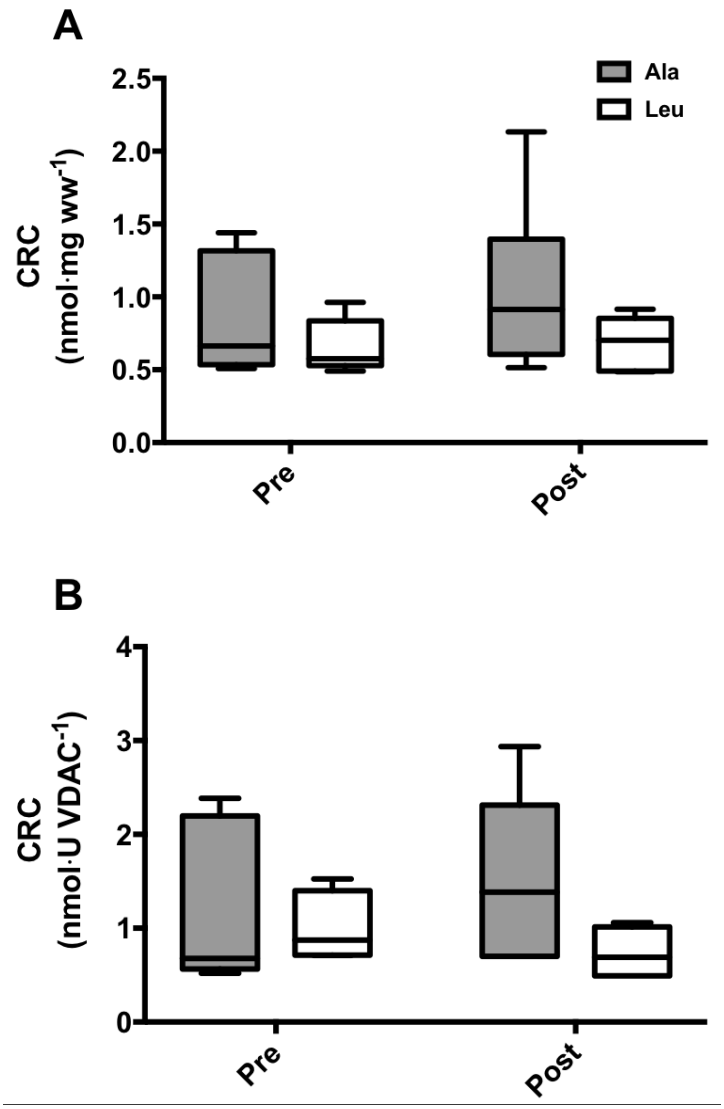


Figure 4-5 Calcium retention capacity (CRC) in permeabilized fiber bundles of *vastus lateralis* normalized to (A) fiber wet weight, and (B) VDAC abundance as a marker of mitochondrial content in pre/frail women with and without leucine supplementation before and after 12 weeks of resistance exercise training. Error bars depict ± SEM. (A) Ala: *n*=6, Leu: *n*=5; (B) Ala: *n*=5, Leu: *n*=4. No significant group, time, or interaction effects in CRC were observed. Ala: alanine supplemented group (control); CRC: calcium retention capacity; Leu: leucine supplemented group.

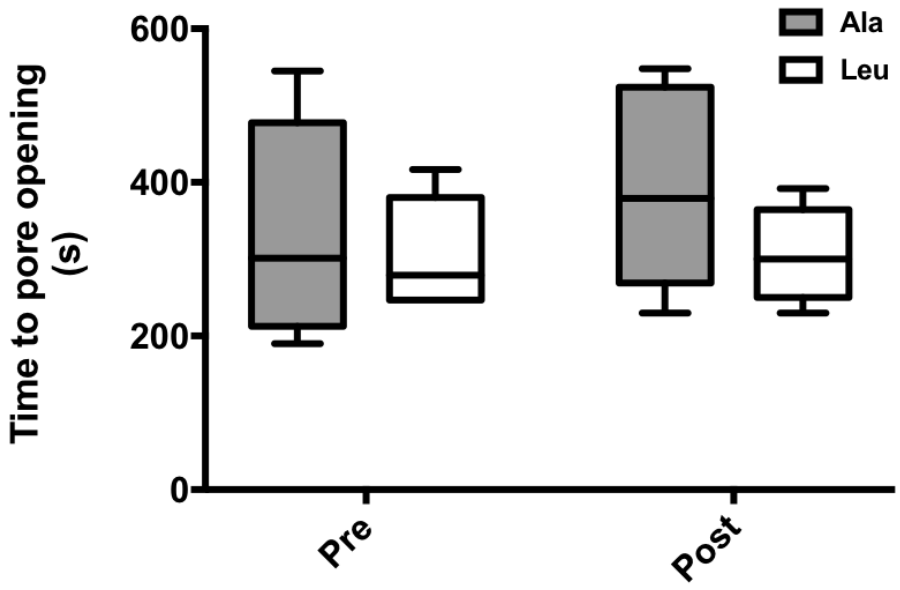


Figure 4-6 Time to mitochondrial permeability transition pore (mPTP) opening in permeabilized fiber bundles of *vastus lateralis* in pre/frail women with and without leucine supplementation before and after 12 weeks of resistance exercise training.

Error bars depict ± SEM. Leu: *n*=6, Ala: *n*=5. No significant group, time, or interaction effects in time mPTP opening were observed. Ala: alanine supplemented group (control); Leu: leucine supplemented group; mPTP: mitochondrial permeability transition pore.

4.5. Discussion

It is currently unknown if mitochondrial functioning can be improved by resistance training in combination with leucine supplementation in women experiencing the clinical entity of frailty or pre-frailty. We measured several states of substrate-supported mitochondrial respiration and ROS, as well as CRC and time to mPTP opening as indices of sensitivity to undergo mitochondrial permeability transition, an event associated with release of proteins that cause death in mononucleated cells in frail and pre-frail elderly women who consumed adequate dietary protein and underwent 12-weeks of RT with or without leucine supplementation. We included two indices of muscular function, aerobic (6MWT) and anaerobic (Leg Press 1RM), measured pre and post intervention. Our main findings were that 12 weeks of RT: 1) significantly increased VDAC protein levels in Leu supplement group; 2) had no effect on mitochondrial respiration capacity, ROS production, or CRC regardless of leucine supplementation; and 3) significantly increased both Leg Press 1RM and 6MWT, with no added effect of leucine supplementation.

In our recent study we showed that VDAC was reduced in vastus lateralis muscle of a similar cohort of pre-frail and frail elderly women, suggesting reduced mitochondrial content [9]. We observed an increase in mitochondrial content in the Leu, and not Ala, group post-intervention as evidenced by VDAC protein expression. This suggests an increased level of mitochondrial biogenesis (and/or suppression of mitophagy) in the Leu group but not the Ala placebo group. Therefore, we observed an inconsistent effect of exercise training between the supplement groups. Although RT has been associated with increased mitochondrial biogenesis in young healthy men [27], elderly men and women [36], and postmenopausal women [35], it is interesting that in the current study the placebo (Ala) supplementation group did not increase mitochondrial content following exercise. It is unknown if alanine has inhibitory effects on exercise-stimulated mitochondrial biogenesis. We are aware of only one study alluding to this question in 20-month-old rats that underwent 8 weeks of voluntary wheel running with or without HMB (beta-hydroxy-beta-methylbutyrate, a leucine metabolite) plus alanine

supplementation [55]. Unexpectedly, in that prior study the supplemented group had reduced amounts of PGC-1α in the medial gastrocnemius as well as reduced markers of autophagy in comparison to the HMB group post-intervention. The mechanism underlying these results was not determined in that study. Leucine has been previously shown to stimulate PGC-1α expression via activation of SIRT1's action on AMPK, resulting in mitochondrial biogenesis in cultured myotubes [32, 56, 57]. However, this increase in mitochondrial content has not been previously demonstrated in human muscle tissue *ex vivo*. Leucine can also increase peroxisome proliferator-activated receptor alpha (PPARα) and PPARβ/δ expression, leading to PPARβ/δ-dependent increases in mitochondrial content and oxygen consumption in cultured myotubes [33]. Another potential explanation is that in pre/frail elderly women, RT alone is not able to increase mitochondrial biogenesis, which is in agreement with a recent study where VDAC content was not altered in older men who underwent 12 weeks of RT [10]. Thus, to the best of our knowledge, this is the first study demonstrating that leucine supplementation in conjunction with RT increases mitochondrial biogenesis in elderly women. Future studies should also investigate how leucine supplementation impacts the mitochondrial biogenesis response with exercise interventions in frail vs sarcopenic vs healthy-elderly persons to determine if the observed effect is unique to frailty.

We observed no changes in mitochondrial respiration with the intervention. However, the intensity of the training was sufficient to improve muscle performance based upon increases in 1RM & 6MWT, with no difference between supplement groups. Accounting for mitochondrial quantity, it has been demonstrated that the respiratory capacity of skeletal muscle appears to be preserved with aging in healthy active older men [23], while a mild impairment in mitochondrial respiratory capacity was observed in older inactive men [28]. Furthermore, mitochondrial respiration (state 4) normalized per dry weight (and not mitochondrial content) was reduced in old versus young persons, and further reduced in low versus highly functioning elderly persons [22]. Importantly, these previous studies have investigated either men only or combined groups of sexes, while women remained

understudied. Our group is the first to report mitochondrial respiration in an all-female cohort. We have recently shown that when normalized to wet weight, state 3 driven respiratory capacity was reduced in inactive pre-frail and frail elderly women (a cohort comparable to the current study participants at pre-intervention) [9], but these differences disappeared when normalized to mitochondria content. A recent study [24] showed no differences in mitochondrial respiration across a wide range of ages and cardiorespiratory fitness when normalized for mitochondrial content. A subsequent study by the same group concluded that mitochondrial respiration is affected more by chronic physical activity status rather than chronological age [20]. The nature of the chronic physical activity in their study, being aerobic, could account for the greater mitochondrial content in their active participants, and thus account for superior mitochondrial respiration. Our results are in agreement with these and other groups which have attributed any decline in mitochondrial respiration to mitochondrial quantity, rather than an intrinsic impairment of mitochondrial respiratory functioning [23, 27], although this is not a uniform finding [28]. It was recently shown using a novel methodology, that although maximal levels of respiration were not different between young and older men with similar VO_{2peaks}, reduced respiration was seen in older men compared to young over a range of biologically relevant ADP levels suggesting a reduced sensitivity to ADP with aging [10]. Furthermore, 12 weeks of RT increased both maximal (state 3) mitochondrial respiration (normalized to wet weight) as well as respiration at submaximal ADP concentrations [10]. The aforementioned study observed an improvement in maximal respiration per unit of muscle following RT, and thus, perhaps we did not see improvements in the current study because maximal capacity of mitochondrial respiration was measured with saturating levels of ADP, and not assessed under physiological levels of ADP as done in this recent study.

Mitochondria are a significant source of ROS production within cells and therefore an impairment in their metabolism has been suggested to play a role in the development of frailty. Indeed, it has been shown that skeletal muscles of elderly persons with reduced physical function are more susceptible to oxidative damage [22]. However, there is great discrepancy in

the literature about whether or not ROS production increases with aging [23, 25, 58, 59]. It has recently been shown that ROS emission in *vastus lateralis* muscle is markedly higher in pre-frail and frail compared to young inactive women after normalization to mitochondrial content [9]. If ROS emission is greatest in sedentary muscle, then increased muscular contraction could reduce ROS emission [11]. However, we observed no changes in mitochondrial ROS production after 3 months of RT in any of the supplemental groups in our pre-frail and frail women. This is consistent with findings of a recent study in healthy older men ($\geq$ 60 y) who underwent a 12-week RT program (without supplementation) showing no changes in ROS production post-intervention [40]. In addition, ours is the first study performed in pre-frail and frail older women to show that ROS emission was not ameliorated with leucine supplementation. To the best of our knowledge, only one study has investigated if leucine impacts ROS emission. It has been found that 5 days of a leucine-rich diet resulted in decreased ROS production in both *in vivo* and cultured epithelial cells of piglets [60]. This was attributed to leucine causing a metabolic shift from oxidative phosphorylation to glycolysis by activation of the mTOR-HIF-1α pathway. However, no changes in ROS production were seen in the current study, possibly due to the different tissues, ages, and species studied. The aforementioned study in older men who underwent 12 weeks of RT [10] yielded results differing from our own. Following 12 weeks of RT maximal ROS production increased at saturating levels of ADP, while ROS emission was attenuated at submaximal levels of ADP. Thus, our results highlight a possible difference between sexes, as to our knowledge no studies exist investigating sexual dimorphism and ROS production in saponin-permeabilized myofibers with aging.

Leucine can directly activate SIRT1 which subsequently phosphorylates AMPK, and downstream of that, PGC-1α (a major modulator of mitochondrial biogenesis) and SIRT3 [32]. SIRT1 has been shown to deacetylate Mfn2 resulting in increased mitophagy [61]. SIRT3 can deacetylate a component of the mPTP resulting in the inhibition of mPTP-mediated apoptosis and increased mitophagy in cardiomyocytes [62]. However, in a recent study in obese adolescent males, the authors determined that aerobic, and not resistance training, is

necessary to induce SIRT3 in skeletal muscle [63]. To our knowledge this is the first study to investigate the impact of any type of exercise training on CRC and mPTP opening. It remains to be determined if endurance training would have a different effect on mPTP sensitivity with or without leucine supplementation. Future studies should investigate this unanswered question, as well as including measurements of markers of mitochondrial quality control (mitophagy, fusion/fission) and PGC-1α.

A potential limitation of the current study is that changes in fiber type were not considered. A fiber-type profile with predominantly slow-twitch muscle would be more oxidative, while predominantly fast-twitch fibers could produce more ROS [13, 50]. Additionally, depending on the type of physical activity i.e., endurance vs. resistance, there are divergent effects on the different fiber types [13]. A prominent future direction to the current study is to examine the effects of our intervention on muscle denervation. Our group has recently shown that inactive pre-frail and frail elderly women have a greater intrinsic mitochondrial ROS emission compared to young inactive women, possibly due to an increase in denervation-induced mitochondrial ROS production [9]. It remains to be determined if an exercise intervention such as that of the current study would have the capacity to improve myofiber reinnervation as well as ameliorate denervation-induced mitochondrial ROS production.

In conclusion, 12-weeks of RT in pre/frail elderly women with and without leucine supplementation increased leg strength and walking distance; while mitochondrial content was increased with RT only in combination with leucine supplementation. A more comprehensive understanding of the functioning of the mitochondria for different sexes, ages, and disease states is critical for mitochondria to be a viable therapeutic target for age-related conditions including sarcopenia and the development of frailty, therefore contributing the health span.

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CONNECTING STATEMENT III

The previous two manuscripts showed that leucine has no effects on insulin sensitivity or mitochondrial function but did have an effect on increasing mitochondrial content. Both insulin sensitivity and mitochondria functioning have repercussions on muscle and are implicated in muscle atrophy [283]. Chronic leucine supplementation studies have yielded conflicting results on improving muscle mass and strength in older persons [21]. The actual anabolic effect of chronic leucine supplementation and in combination with resistance training on direct measurements of muscle protein synthesis *in vivo*, myofiber cross sectional area and type, and a comprehensive assessment of muscle strength and performance had yet to be determined in our cohort of pre-frail and frail older women. Apriori literature has suggested that frail and/or sarcopenic persons would stand to benefit the most from chronic leucine supplementation [22, 284]. Preceding studies [38, 39] have suffered from a multitude of limitations including lack of appropriate placebo-control, unaccounted-for and uncontrolled protein dietary intake, and a lack of robust methodological measurements for desired outcomes. Thus, we sought to determine the *in vivo* postabsorptive and postprandial rates of myofibrillar protein synthesis in conjunction with a comprehensive battery of physical performance tests, fiber type profiles, and effects on frailty profile in pre-frail and frail older women with well controlled and optimized dietary intake, before and after 3 months of progressive resistance training with or without daily leucine supplementation in a randomized clinical trial. We hypothesized that 12 weeks of resistance training would improve the primary outcome of myofibrillar fractional synthesis rate; and secondary outcomes of increasing maximal muscle strength and improving physical performance, increasing muscle mass and

fiber cross-sectional area. There would be an added benefit when supplemented with leucine compared to an isonitrogenous placebo, in pre-frail and frail older women habitually consuming an optimal amount of dietary protein.

CHAPTER 5: MANUSCRIPT 3

To be submitted to the *Journal of Cachexia, Sarcopenia and Muscle*.

Resistance training but not leucine can reverse frailty by increasing basal muscle protein synthesis in older women consuming optimized protein intake

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5.1. Abstract

Background: Frailty is a clinical condition associated with loss of muscle strength and mass (sarcopenia). Although sarcopenia has multifactorial causes, it might be partly attributed to a blunted response to anabolic stimuli. Leucine acutely increases muscle protein synthesis and resistance training (RT) is a strong anabolic stimulus to counteract sarcopenia. The effects of chronic leucine supplementation in conjunction with RT are unknown.

Objective: The purpose of this double-blinded placebo-controlled study is to determine the effects of leucine supplementation and RT on muscle anabolism in pre/frail older women consuming optimal amounts of dietary protein.

Methodology: Pre/frail elderly women (n=19, 77.5 ± 1.3 y, BMI: 25.1 ± 0.9 kg/m^2), based on the Frailty Phenotype, underwent 12 weeks of progressive RT with protein-optimized diet (1.2 g·kg BW^{-1}·d^{-1}) and were randomized to 7.5 g/d of leucine (Leu) supplementation or placebo alanine (Ala). The primary outcome was myofibrillar fractional synthesis rate (MyoFSR), determined using L-[ring-$^2H_5$]phenylalanine infusion in the postabsorptive and postprandial states. Secondary outcomes were muscle function, 1-repetition maximum (1RM), body composition (DXA), and myofiber profiles.

Results: Leucine had no added anabolic benefit to the intervention. Basal MyoFSR increased by 66%, which occurred in conjunction with an increase in type 1 and 2a myofiber cross sectional area (CSA) (16% and 28%, respectively), and whole lean body mass (2%). The number of Frailty Criteria was reduced by 64%, which occurred in conjunction with significant improvements in physical function and strength.

Conclusion: RT with optimal protein intake significantly improved upon the Frailty Phenotype with associated improvements in physical function, strength, and increased basal MyoFSR along with type 1 and 2a myofiber CSA and whole lean body mass, with no added benefit of leucine supplementation.

5.2. Introduction

Persons greater than 65 years of age in Canada currently represent 16.5% of the population with this percentage projected to double in the next 40 years in addition to tripling in those greater than 80 years [1, 2]. With the aging population continually on the rise, there is a consequent increase in the incidence of age-related conditions such as frailty and sarcopenia. Sarcopenia's recently revised operational definition requires low muscle strength and is confirmed by coexisting low muscle mass, quality, or physical performance [3]. Sarcopenia can be both a precursor to and the physical manifestation of frailty [4]. On average women outlive men [5], therefore there is a greater prevalence of frailty in older women [6]. Frailty is a measurable clinical entity that encompasses states of vulnerability due to decreased functional physiological reserves across multiple physiological systems. The consequences of frailty include, but are not limited to, increased risk of falls, loss of independence, disability, depression & social isolation, increased risk of morbidity and mortality [7].

Muscle mass is determined by the net balance (NB) between rates of muscle protein synthesis (MPS) and breakdown (MPB). Anabolism (muscle accretion) occurs when MPS>MPB with catabolism the reverse (i.e. MPB>MPS). Over the course of a day, if the postprandial positive muscle NB counterbalances the postabsorptive negative muscle NB then there is an overall maintenance of muscle mass. Evidence to date strongly suggests that MPS is the more highly regulated variable being more susceptible to changes with aging and sensitivity to anabolic stimuli [8], and thus can have a substantial impact on NB. Aging appears to create a state of protein anabolic resistance that has its greatest effect on the diurnal cycle via a blunted

postprandial anabolic response [9-15], which can be overcome with higher quantities of ingested protein [12, 16]. Accordingly, there is mounting evidence that the dietary protein requirements of older persons are higher than the current recommended dietary allowance (RDA) (0.8 g·kg BW^{-1}·d^{-1} [17]), and may be as high as 1.2 g·kg BW^{-1}·d^{-1} [18-20]. The essential amino acid (EAA), leucine, is a branched chain amino acid (BCAA) that is not only a substrate for MPS but is also unique in its ability to stimulate MPS by the activation of mammalian target of rapamycin complex 1 (mTORC1), independent of insulin [21]. Acutely, it has been shown that the blunted anabolic response to feeding with aging can be overcome with co-ingestion of higher amounts of leucine [11, 22-26]. These acute studies have highlighted the potential of leucine, given in sufficient quantities, to overcome the anabolic resistance to protein feeding without the need to ingest large quantities of protein [16]. There is strong evidence that chronic leucine supplementation is beneficial in elevating myofibrillar MPS [22, 23, 27, 28], but outcomes on changes in muscle mass and strength have yielded conflicting results [29].

Resistance to other anabolic stimuli such as resistance training (RT) may also occur during aging, in part due to low habitual physical activity [30]. Older adults are able to increase their muscle strength through RT, however the muscle hypertrophic (anabolic) response is not as straightforward [31, 32]. Although RT has been shown to increase the rate of MPS in older adults [33], older have been reported to have a blunted anabolic response to a single bout of resistance exercise in men [34] and women [35]. Most studies have shown a blunted hypertrophic response to RT [32, 35-40], while others have not [41, 42]. Women have been shown to have a reduced hypertrophic response to training than men [43, 44]. Other aspects of

sexual dimorphism in regard to muscle fiber profiles have been observed, such as greater type 2 myofiber atrophy with aging in women [45]. Hence, it is important to study the sexes independently to unmask the uniqueness of each.

To the best of our knowledge, no studies exist investigating anabolic effects of RT and chronic leucine supplementation versus an isonitrogenous control, while maintaining sufficient protein intake (~1.2 g·kg BW^{-1}·d^{-1}) in older women. In the two studies in which leucine is the predominant amino acid [46, 47], there was no nitrogen-equivalent placebo control group, protein dietary intake was not monitored through the duration of the intervention, nor were measures of rates of MPS obtained. Thus, our objective was to study in a randomized double-blinded placebo-controlled clinical trial the effects of leucine supplementation while consuming an optimal amount of dietary protein during progressive RT on rates of protein synthesis, fiber type composition, muscle mass, muscle strength and function compared to an isonitrogenous control in pre-frail and frail older women, a group most suited to benefit from such an intervention. We hypothesized that 12 weeks of RT with leucine supplementation would result in superior gains in postprandial muscle protein synthesis, reflected in an increase in lean body mass, myofiber cross sectional area, and gains in muscle strength and function than RT alone in pre-frail and frail elderly women habitually consuming an optimal amount of dietary protein.

5.3. Methods

The study was conducted as a registered randomized double-blinded placebo-controlled trial (ClinicalTrials.gov ID: NCT01922167) and was approved and monitored by the McGill University Health Centre (MUHC) Human Research Ethics Board (REB code: 13-211-BMB). Study design and participant profiles have been previously published [48]. All participants read and signed an informed consent form before participating in the study and screening. All participants underwent a 12-week high-intensity progressive resistance exercise training program (RT) and followed a protein-optimized diet (~1.2 g·kg BW^{-1}·d^{-1}). Half were randomized to receive leucine (2.5 g 3x·d^{-1}) supplementation and the other half an isonitrogenous amount of alanine, an amino acid known not to stimulate muscle protein synthesis (1.7 g 3x·d^{-1})[49]. All tests were performed before and after the intervention. The muscle protein kinetic studies were performed at the Centre for Innovative Medicine (CIM) of the MUHC in the postabsorptive (fasting) and postprandial (standard meal) states.

5.3.1. Participants & Recruitment

Frail or Pre-Frail community-dwelling elderly women (>65 y) according to a modified Fried [6] criteria were recruited from the Geriatrics outpatient clinic of the MUHC and advertisements posted in the local seniors' newspaper. Three hundred and four women were screened via telephone, 24 entered the study, and 19 completed the study. Of the five participants who left the study, two became ill with conditions unrelated to the study, one sustained an injury unrelated to the study, one moved out of province, and one was unable to

maintain adherence to the protocol. The remaining 19 participants maintained adherence of at least 80% to both exercise program and supplement intake. Inclusion criteria consisted of non-disabled women who were cognitively intact with a Mini Mental State Examination score (MMSE) ≥24, body mass index (BMI) of 18.5-35 kg/m^2, normal complete blood count, biochemistry, A1C, TSH, urine analysis, no diabetes determined by a 75-g oral glucose tolerance test (OGTT), negative serology for hepatitis and HIV, and normal chest X-ray and ECG results. Exclusion criteria were: dependence on walking aids, Geriatric Depression Score (GDS) short form <6 [50], substance abuse, eating disorders, active medical conditions other than skin cancer within 5 years, serum creatinine >110 µmol/L, hemoglobin (Hb) <110 g/L, and medications known to interfere with metabolic endpoint measurements (e.g., beta-blockers).

5.3.2. Intervention – Resistance Training

Participants performed resistance exercise three times per week on non-consecutive days, as previously described (Jacob 2018 *J Nutr*, submitted). Exercises were horizontal leg press, chest press, knee extension, and lateral pulldown. Participants performed 3 sets of 8-15 repetitions for each exercise and resistance was increased by 1-5 lbs (0.45-2.27 kg) when the participant could perform up to 15 repetitions with proper technique. A visual Borg Scale (6-20) was used for the subject to point to at the end of each set to obtain their rating of perceived exertion, with a target of 14-16. The duration of each set (Time Under Tension, TUT) was obtained (>35 seconds) in order to ensure that the participants were not using momentum to complete the motions. Resistance was determined to consistently be 60-80% of their 1-

repetition maximum (1RM). Participants were given an accelerometer (ActiGraph GT3X+, ActiGraph, LLC, USA) worn on the waist for 3 consecutive days (one weekend day and two weekdays) before the intervention, and again mid-intervention to assess if the RT was influencing their habitual physical activity levels (average kcal, percentage of time spend sedentary, light, moderate, or vigorous activity).

5.3.3. Intervention – Supplementation

Participants were randomized into supplement groups by an independent source based on random generated numbers. Individual doses of powdered supplements of L-leucine (2.5 g, ProteinCo. QC, CA) or isonitrogenous amounts of L-alanine (1.7 g, PureBulk® OR, USA) were provided in sterile sealed screw-top 100 mL identical containers. Participants were instructed to consume one complete dose of supplement in 80-100 mL of water or sugarless juice at the onset of each main meal (breakfast, lunch, dinner) for the duration of the intervention. Log sheets were collected every 2 weeks to track compliance.

5.3.4. Intervention – Dietary Protein Intake

Dietary caloric and protein intake was assessed by an initial screening 24 h food recall and subsequent pre-intervention 3-d food diaries. Dietary intake was analyzed using the Food Processor SQL software (Version 10.11.0, ESHA Research, Salem OR). Participants were given instruction and guidance by a study nutritionist on how to maintain an isoenergetic protein dietary intake of 1.2 g·kg BW^{-1}·d^{-1} by making minor adjustments to their normal food intake.

Food recalls (24 h) were obtained from participants pre- post- and at least once mid-intervention to verify maintenance of dietary intake.

5.3.5. Outcome Measures

5.3.5.1. Frailty Phenotype:

Frailty status was assessed using the modified Fried Criteria [6] at baseline (mandatory for participating) and after intervention. Participants meeting 1-2 of the criteria were diagnosed as pre-frail, whereas those ≥3 diagnosed as frail. The five criteria are summed as follows: (1) slowness identified as a 4-m gait speed of ≤ 1 m/s [51]; (2) weakness identified as a handgrip strength ≤ 20 kg using a Jamar hydraulic hand dynamometer (Sammons Preston, Inc., IL, USA) [52]; (3) sedentariness identified by a CHAMPS Physical Activity Questionnaire score ≤ 125 [53]; (4) sarcopenia identified by a muscle mass index (MMI) < 6.76 kg/m^2 via BIA (RJL Systems Inc., MI, USA) [54] using the Roubenoff, Baumgartner [55] equation validated for older women; and (5) exhaustion identified by at least one positive response to either of the 2 following questions: “How often do you feel like ‘I just could not get going’; and ‘Everything I did was an effort’” [6].

5.3.5.2. Body Composition:

Total weight, BMI, lean body mass (LBM), percent body fat (%fat), and appendicular muscle mass index (AMMI) were obtained using a 3-compartment model via dual-energy x-ray absorptiometry (DXA) (GE Lunar iDXA). AMMI was calculated by taking the sum of lean mass of

arms and legs, then dividing by participant height (in meters) squared. DXA was performed at least 24 h after the physical function testing (pre- and post-intervention) and at least 48 h after the final training session.

5.3.5.3. Physical Function & Strength Tests:

All physical function and strength tests were performed on-site at the gym by trained kinesiologists, blinded to the supplement intervention using standard methods. Tests were 1) Short Physical Performance Battery (SPPB) [56], 2) Timed up-and-go test (TUG) [57], 3) handgrip strength with the Jamar® dynamometer (Model PC5030J1) [52], 4) Senior Fitness Test (SFT) [58], and 5) 1RM of the four major exercises (leg press, chest press, knee extension, lat pull-down) [59]. The above tests were selected for their validity, prognostic value and broad use in the aging literature. Post-intervention testing occurred on the next non-consecutive day after the last bout of training.

5.3.5.4. Muscle tracer studies

The muscle tracer studies occurred at least 48 h after completion of the physical function tests pre-intervention, and ~48 h post-intervention. Participants arrived at the CIM in the fasting state. After catheter insertions for tracer infusion and arterialized blood sampling [60], the rate of MPS was measured in both the postabsorptive and postprandial states using primed (2 $\mu mol \cdot kg^{-1}$), continuous (0.05 $\mu mol \cdot kg^{-1} \cdot min^{-1}$) infusion of L-[ring-$^{2}H_5$]phenylalanine (Cambridge Isotope Laboratories, Inc., Andover, MA) tracer (Figure 1). Because participants

were naïve to the tracer pre-intervention, a single muscle biopsy was obtained at 3 h after beginning the infusion in the postabsorptive state. Conversely, the first biopsy for baseline enrichment of the tracer was performed 1.5 h into infusion for post-intervention, followed by a second biopsy at 3 h. Immediately after and within 20 min, subjects consumed a complete formula Ensure® (inherent leucine content of 0.52 g/100 mL), calculated to cover 1/3 of energy and protein (0.67 g protein·kg LBM^{-1}) with added leucine or alanine in powder as per group assignment, as well as oral tracer (in % molar enrichment) to maintain postprandial serum and muscle isotopic enrichment. The postprandial biopsy was taken 2 h after the meal. Human muscle samples were obtained from the lateral portion of the vastus lateralis ~20 cm above the knee, 4-5 cm apart using a UHC needle biopsy technique [60]. After removal of fat tissue, the biopsy sample was cut in sections for the different analysis. For MPS, ~30 mg of tissue was stored at -80°C. For fiber typing, transverse muscle portions of ~30 mg were mounted on cork, snap frozen in liquid nitrogen cooled isopentane, and stored at -80°C. Due to technical difficulties, some measurements were only performed on a subset of muscle specimens. The sample size used for each measurement is indicated in the results section of each technique used in this study.

5.3.5.5. Measurements of MPS

MPS was measured based on the myofibrillar fractional synthesis rate (MyoFSR), calculated according to a precursor-product relationship [61]. Approximately 30 mg of muscle was homogenized and myofibrillar proteins were separated by centrifugation, then the

myofibrillar fraction was precipitated and amino acids liberated (modified from [62]). The tracer/tracee ratio of phenylalanine was determined by liquid chromatography-tandem mass spectrometry (LC/MS/MS)(Agilent 1290 LC system with 6460 Triple Quadrupole MS). Detection and quantification of phenylalanine was performed using multiple reaction monitoring in positive ion mode with the following transitions: m/z 166 > 120 (phenylalanine); m/z 171 > 125 ([$^2H_5$]phenylalanine). 3-nitro-L-tyrosine served as an internal standard used to monitor data quality and reproducibility for LC/MS/MS analysis. FSR was calculated according to the precursor-product relationship from rates of [$^2H_5$]phenylalanine incorporation over time: FSR = $(E_{pb2} – E_{pb1})/ (E_{FAA} \cdot \Delta t)$. E_{pb1} and E_{pb2} are enrichment of protein-bound phenylalanine at postabsorptive and 2 h postprandial, respectively; E_{FAA} is the enrichment in the precursor plasma free amino acid pool, and Δt is the length of time between E_{pb1} and E_{pb2}. Postabsorptive FSR was calculated from the single biopsy approach in tracer naive participants, pre-intervention [63].

5.3.5.6. Fiber Type Profiles:

Transverse muscle sections were cut on a HM505E cryostat (Microm, Walldorf, Germany) at -24°C, mounted on frosted glass slides, and stored at -80°C. Prior to staining, slides were left at room temperature to thaw and dry for 1 hour. Sections were washed in PBS and then blocked using 10% normal goat serum in PBS. Sections were then incubated for 1 h at room temperature with the following antibodies (purchased from Developmental Studies Hybridoma Bank, University of Iowa): MHCI (BA-F8 mouse monoclonal IgG2b, dilution 1:25),

MHCIIa (Sc71, mouse monoclonal IgG1, dilution 1:200), MHCIIx (6H1 mouse monoclonal IgGM, dilution 1:25), and rabbit polyclonal IgG anti-laminin (L9393, dilution 1:750; Sigma, St Louis, MO, USA). Following primary incubation, sections were washed (3 × 5 min in PBS) and then underwent a secondary incubation with secondary antibodies Alexa Fluor 350 IgG2b goat anti-mouse, Alexa Fluor 594 IgG1 goat anti-mouse, Alexa Fluor 488 IgM goat anti-mouse, and Alexa Fluor 488 IgG goat anti-rabbit (Life Technologies, Grand Island, NY, USA). Following secondary incubation, sections were washed (3 × 5 min in PBS) and then slides were mounted using Prolong Gold Hard Set Mounting Medium (Invitrogen, Carlsbad, CA, USA). Images were captured using an Axio Imager M2 fluorescence microscope (Carl Zeiss, Oberkochen, Germany) and subsequently analysed using ImageJ software (National Institutes of Health, Bethesda, MD, USA) for myofiber cross sectional area (CSA, μm^2). Fibers were then measured and fiber-typed by hand based on their colour, with an average of 215 fibers assessed per muscle section.

5.3.6. Statistical Analysis

Unless otherwise indicated, data are presented as means $\pm$ SEM. Normality was determined using the Shapiro-Wilk test. Outliers were determined using the ROUT method. Independent t-tests were used to determine differences between the two groups at baseline. Paired t-tests were used to determine changes in plasma enrichment. A 2-way ANOVA with Tukey's *post hoc* was used to compare changes of fiber area for each fiber type between the supplement (group) or training (time) effects. A two-factor repeated measures ANOVA was used to determine the leucine supplementation (group) and exercise training (time) effects for

all other outcomes except MyoFSR. When significant interaction effects were observed, *post hoc* comparisons were performed using the Sidak test. Differences in MyoFSR were determined using a 2x2x2 3-way mixed ANOVA with meal (basal vs. fed) and time (pre- vs. post-RT) as within-subject factors and group (Leu vs. Ala) as the between-subject factor. Differences in between-subject interaction effects were determined using a one-way repeated measure ANOVA with Tukey's *post hoc*. The sample size estimation was based on a difference of 20% in MyoFSR between leucine versus alanine placebo groups, with a standard deviation of ~15% [28]. Therefore, with an effect size of 1.25, 9 subjects per group were required (α=0.05; β=0.80). Significance was set at $\alpha \geq 0.05$. The 3-way mixed ANOVA was analyzed using IBM SPSS Statistics 24.0 (International Machines Business Corp., Armonk, NY, USA). All other analyses were performed using Prism, version 7.0a (GraphPad Software, Inc., La Jolla, CA, USA).

5.4. Results

Baseline Characteristics

Subject characteristics (age, weight, BMI) did not differ at baseline (Table 5-1), nor did any frailty parameter (Table 5-2), dietary intake parameter (Table 5-3), body composition parameter (Table 5-4), MyoFSR (Table 5-5), muscle strength, or physical function parameter (Table 5-6). Groups did not differ at baseline for average kcal, percentage of time spent in sedentary, light, or moderate activity (Table 5-7). Ala had a statistically higher percentage of time spent in vigorous activity at baseline, but the difference was 0.12% which is clinically negligible. At baseline, fiber type 1, 2a, and 2x CSA were greater in the Leu group than Ala

(p=0.024, <0.001, and 0.011, respectively) (Figure 5-2A). No baseline differences were observed for any other measured fiber parameter.

Myofibrillar Fractional Synthesis Rate

Plasma enrichment remained stable throughout the postprandial and postabsorptive states (average ± 10% with SD < 1%, data not shown. Ala: *n*=8, Leu: *n*=10). A meal effect on MyoFSR was observed (p<0.001) (Table 5-5). When ignoring group and time, feeding significantly increased MyoFSR. A trend for time effect was also noted (p=0.007) (Table 5-5). When ignoring group and feeding status, the training intervention significantly increased MyoFSR. Consequently, a meal x time interaction effect was also observed (p<0.05) (Figure 5-3). Analysis of the interaction revealed that the basal MyoFSR was significantly increased post-intervention (+47%). No group effect and no other interaction effects were observed.

Frailty Criteria

Both groups significantly decreased their number of frailty criteria (time effect, p=0.001) (Table 5-2), but no group or interaction effects were observed. The percentage of participants who reduced their Frailty score by 0, 1, 2, or 3 criteria was, respectively: 5.3%, 5.3%, 21.1%, and 15.8% (Ala); 5.3%, 26.3%, 15.8%, and 15.8% (Leu). Both groups significantly increased their CHAMPS score (time effect, p=0.001) (Table 5-2), but no supplement or interaction effects were observed. Both groups significantly increased their SMI (time effect, p=0.001) (Table 5-2), but no supplement or interaction effects were observed.

Dietary Intake

No significant effects were observed between any timepoint (pre-, mid-, or post-intervention) for caloric intake, absolute or relative protein intake (Table 5-3). The Ala group had a lower dietary leucine intake at the mid-intervention time point only (interaction effect, $p=0.013$, one outlier was removed at mid-intervention in the Ala group), but no time or group effects were observed (Table 5-3). However, a t-test comparing Ala vs Leu at this time point showed no difference between the two groups ($p=0.15$).

Body Composition

No significant effects were observed for total body mass (Table 5-4). Both groups increased their LBM (time effect, $p<0.0001$) (Table 5-4), but no group or interaction effects were observed. No significant effects were observed in AMMI (Table 5-4). Both groups decreased their %fat (time effect, $p<0.015$) (Table 5-6), but no group or interaction effects were observed.

Physical Function & Strength Tests

Both groups significantly increased their strength for each of the four 1RM exercises (leg press, chest press, knee extension, lateral pull-down: time effect, $p<0.0001$) (Table 5-6). No group or interaction effects were observed for any exercise. Both groups significantly increased their 4-m gait speed (time effect, $p<0.0001$), SPPB score (time effect, $p=0.002$), number of chair stands completed in 30s (SFT: time effect, $p=0.0006$), number of arm curls completed in 30s (SFT: time effect, $p<0.0001$), upper body flexibility (SFT, back scratch: time effect, $p=0.042$), and

lower body flexibility (SFT, sit-and-reach: time effect, p<0.0001), with no group or interaction effects observed (Table 5-6). Both groups significantly decreased their TUG time (time effect, p=0.010), time to complete 5 chair stands (SPPB: time effect, p=0.0001), and 8' up-and-go time (SFT: time effect, p=0.001), with no group or interaction effects observed (Table 5-6). The Ala group increased their handgrip strength (p=0.010) post-intervention (interaction effect, p=0.01) (Table 5-6), but no group or time effects were observed. Pre- vs post-intervention, 22.2% and 11.1% of participants in the Ala group, while 60.0% and 20.0% of those in the Leu group were not able to complete all balance tests of the SPPB.

Accelerometer

No significant effects were observed for average kcal, percentage of time spent in sedentary, light, or vigorous activity (Table 5-7, Ala: *n*=7, Leu: *n*=8). A group effect was observed for percentage of time spent in moderate activity (p=0.019), with Ala higher than Leu at both time points. No other time or interaction effects were observed for percentage of time spent in moderate activity.

Myofiber Profiles

Myofiber type 1 area was greater in the Leu compared to Ala group at both time points (group effect, p=0.004). Both groups significantly increased type 1 CSA (time effect, p<0.0001), but no interaction effect was observed (Figure 5-2A). Fiber type 2a area was greater in the Leu compared to Ala group at both time points (group effect, p<0.0001). Both groups significantly increased type 2a CSA (time effect, p<0.0001), but no interaction effect was observed (Figure 5-

2A). Effects in fiber type 2x CSA were impossible to calculate due to their absence in the Leu group post-intervention. No significant change in type 2x CSA in the Ala group was observed post-intervention (Figure 5-2A). No significant effects were observed for type 1/2a and 2a/2x CSA (Figure 5-2A). No difference at baseline and no significant effects were observed for fiber type 1, 2a, 2x, or 1/2a distribution (Figure 5-2B). Both groups significantly decreased the distribution of type 2a/2x fibers post-intervention (time effect, $p=0.021$) (Figure 5-2B), but no group or interaction effects were observed.

Table 5-1 Participant characteristics of pre/frail women by supplement group at baseline

Characteristic	Ala	Leu	p-value
n	9	10	
Age (y)	76.2 ± 1.8	78.7 ± 2.1	>0.05
Weight (kg)	61.8 ± 2.5	62.9 ± 2.9	>0.05
BMI (kg/m^2)	23.8 ± 1	26.2 ± 1.3	>0.05

Data are means ± SEM. BMI: body mass index.

Table 5-2 Frailty profiles of pre/frail women with and without leucine supplementation before and after 12 weeks of resistance exercise training

	Ala		Leu			
Criteria	**Pre**	**Post**	**Pre**	**Post**	**p-value**	**Effect**
Number of Criteria met	2.7 ± 0.3	0.7 ± 0.3	2.6 ± 0.3	1.2 ± 0.2	<0.0001	Time
Walking speed (m/s)	1.02 ± 0.04	1.20 ± 0.03	0.99 ± 0.05	1.17 ± 0.06	<0.0001	Time
Handgrip strength (kg)	19.2 ± 1.6	22.6 ± 1.9	22.7 ± 2.0	21.7 ± 1.9	0.01	Interaction
SMI (kg/m^2) (BIA)	6.96 ± 0.23	9.15 ± 0.24	6.32 ± 0.27	8.89 ± 0.20	<0.0001	Time
CHAMPS score	138 ± 52	379 ± 59	121 ± 41	442 ± 73	<0.0001	Time

Data are means ± SEM. Ala: *n* =9, Leu: *n* =10. SMI: skeletal muscle mass index; CHAMPS: community healthy activities model program for seniors questionnaire.

Table 5-3 Caloric, protein and leucine habitual dietary intake in pre/frail women with and without leucine supplementation before and after 12 weeks of resistance exercise training

	Ala			Leu				
Criteria	**Pre**	**Mid**	**Post**	**Pre**	**Mid**	**Post**	**p-value**	**Effect**
Caloric Intake (kcal/d)	1680 ± 99	1513 ± 81	1574 ± 86	1555 ± 89	1546 ± 115	1696 ± 89	>0.05	-
Protein Intake (g/d)	76.6 ± 2.5	76.9 ± 6.5	76.4 ± 4.2	74.6 ± 3.0	76.6 ± 3.3	74.1 ± 4.4	>0.05	-
Protein Intake (g/kg/d)	1.24 ± 0.06	-	1.23 ± 0.09	1.22 ± 0.07	-	1.21 ± 0.09	>0.05	-
Dietary Leucine Intake (g/d)	5.77 ± 0.20	4.65 ± 0.22	5.52 ± 0.32	5.10 ± 0.30	5.16 ± 0.24	5.39 ± 0.37	0.013	Interaction

Note: protein and leucine values do not include supplement intake
Data are means ± SEM. Ala: *n* =9, Leu: *n* =10

Table 5-4 Body composition measurements by DEXA in pre/frail women with and without leucine supplementation before and after 12 weeks of resistance exercise training

	Ala		Leu			
Criteria	**Pre**	**Post**	**Pre**	**Post**	**p-value**	**Effect**
Total body mass (kg)	61.8 ± 2.5	62.7 ± 2.3	62.9 ± 2.9	62.2 ± 2.8	>0.05	-
LBM (kg)	38.1 ± 1.3	38.9 ± 1.4	35.2 ± 1.4	35.9 ± 1.5	<0.0001	Time
AMMI (kg/m^2)	6.4 ± 0.2	6.6 ± 2	6.5 ± 0.3	6.5 ± 2.6	>0.05	-
%Fat	36 ± 2.2	35.5 ± 2.1	41.3 ± 1.5	40 ± 1.7	0.015	Time

Data are means ± SEM. Ala: *n* =9, Leu: *n* =10. AMMI: appendicular muscle mass index; BMI: body mass index; LBM: lean body mass; %fat: percent body fat.

Table 5-5 Basal and fed MyoFSR in pre/frail women with and without leucine supplementation before and after 12 weeks of resistance exercise training

	Pre		Post		Training effect	Feeding effect	Supplement
Group	**Basal**	**Fed**	**Basal**	**Fed**	**(p-value)**	**(p-value)**	**effect (p-value)**
Ala	0.025 ± 0.003	0.053 ± 0.004	0.042 ± 0.007 #	0.045 ± 0.005	0.065	<0.001	0.983
Leu	0.028 ± 0.002	0.051 ± 0.003	0.037 ± 0.005 #	0.050 ± 0.005			

Data are means ± SEM. Ala: *n* =8, Leu: *n* =10. # depicts a meal x time interaction (p<0.05).

Table 5-6 Physical performance testing results in pre/frail women with and without leucine supplementation before and after 12 weeks of resistance exercise training

	Ala		Leu			
Test	**Pre**	**Post**	**Pre**	**Post**	**p-value**	**Effect**
4-m gait speed (m/s)	1.02 ± 0.04	1.20 ± 0.03	0.99 ± 0.05	1.17 ± 0.06	<0.0001	Time
TUG (s)	10.3 ± 0.6	9.1 ± 0.3	10.6 ± 0.6	8.9 ± 0.4	0.0095	Time
SPPB						
Total score	10.0 ± 0.6	11.4 ± 0.2	9.9 ± 0.3	11.2 ± 0.5	0.0018	Time
5 chair stands (s)	13.2 ± 1.0	11.1 ± 0.7	13.1 ± 0.5	10.6 ± 0.6	0.0001	Time
8' walk (s)	2.6 ± 0.2	2.5 ± 0.1	2.4 ± 0.1	2.5 ± 0.2	>0.05	Time
Balance % not perfect	22.2	11.1	60.0	20.0	-	-
SFT						
30 chair stands (#)	11.1 ± 0.7	13.6 ± 1.1	11.8 ± 0.4	14.0 ± 0.5	0.0006	Time
Arm curls (#)	16.0 ± 0.9	19.7 ± 1.4	15.8 ± 1.0	20.0 ± 0.6	<0.0001	Time
Sit-and-reach (in)	0.4 ± 2.0	3.8 ± 1.7	-1.3 ± 1.9	1.0 ± 1.3	<0.0001	Time
Back scratch (in)	1.5 ± 0.7	2.5 ± 0.7	-0.8 ± 1.2	0.1 ± 0.9	0.0415	Time
8' up-and-go (s)	7.4 ± 0.9	6.0 ± 0.4	7.9 ± 0.6	7.0 ± 0.5	0.0014	Time
1RMs						
Leg press (kg)	75.4 ± 8.6	99.8 ± 9.8	71.2 ± 6.4	96.6 ± 6.4	<0.0001	Time
Chest press (kg)	24.0 ± 1.4	29.5 ± 2.3	23.1 ± 2.3	28.6 ± 1.8	<0.0001	Time
Knee extension (kg)	40.4 ± 2.3	54.0 ± 4.1	49.9 ± 7.7	59.4 ± 7.3	<0.0001	Time
Lat pull-down (kg)	32.7 ± 1.4	38.1 ± 1.4	33.1 ± 1.8	39.0 ± 2.3	<0.0001	Time

Data are means ± SEM. Ala: *n* =9, Leu: *n* =10. SFT: seniors fitness test; SPPB: short physical performance battery; TUG: timed up and go; 1RM: 1-repetition maximum

Table 5-7 habitual physical activity profile measured by accelerometry in pre/frail women with and without leucine supplementation before and after 12 weeks of resistance exercise training

	Ala		Leu			
	Pre	**Mid**	**Pre**	**Mid**	**p-value**	**Effect**
Average kcal	341 ± 58	363 ± 52	290 ± 40	272 ± 37	> 0.05	-
%Sedentary	87.4 ± 1.7	87.9 ± 1.1	88.4 ± 1.3	89.6 ± 1.2	> 0.05	-
%Light	10.7 ± 1.5	9.6 ± 0.9	10.4 ± 1.0	9.0 ± 1.0	> 0.05	-
%Moderate	1.8 ± 0.4	2.3 ± 0.3	1.0 ± 0.3	1.2 ± 0.3	0.019	Group
%Vigorous	0.2 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.040	Baseline

Data are means ± SEM. Ala: *n* =7, Leu: *n* =8.

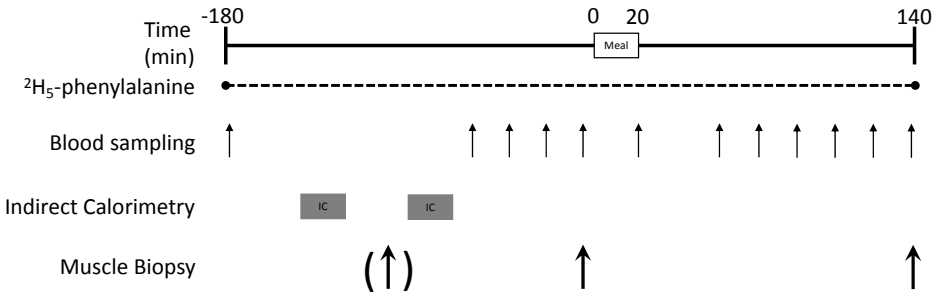
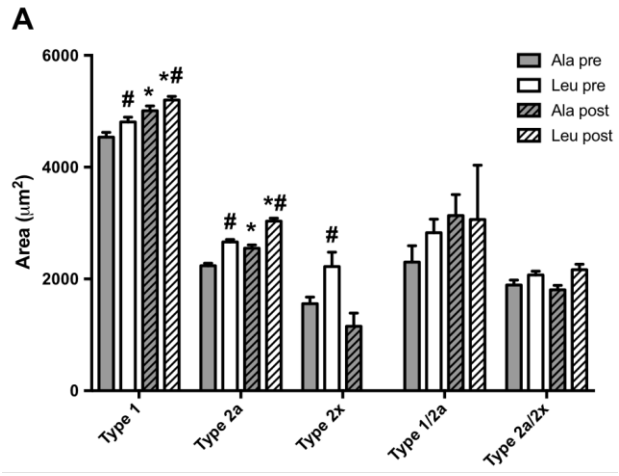
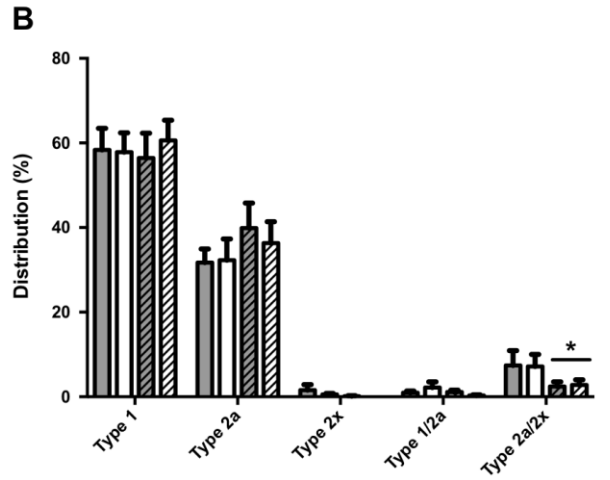


Figure 5-1 Schematic of the infusion (tracer) study design.


A
6000
4000
2000
0
Area (μm²)
Ala pre
Leu pre
Ala post
Leu post
#
*
*#
Type 1
Type 2a
Type 2x
Type 1/2a
Type 2a/2x


B
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60
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Distribution (%)
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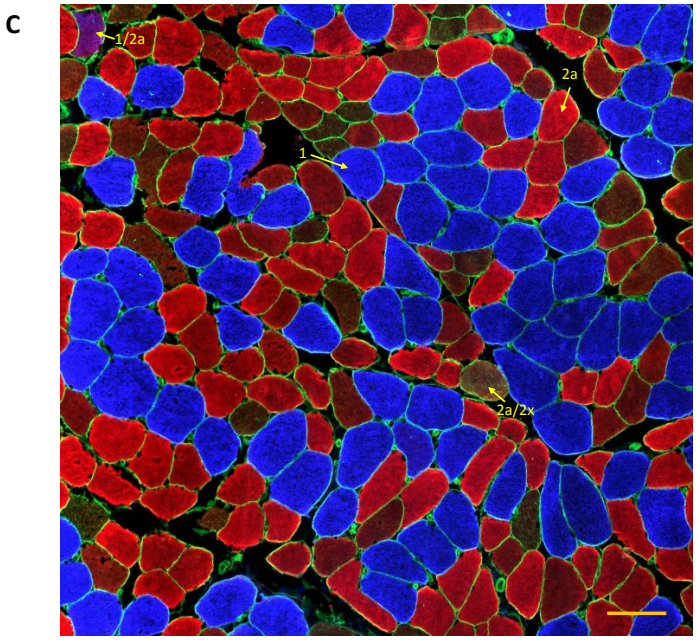
C
1/2a
2a
1
2a/2x

Figure 5-2 Myofiber cross sectional area (CSA) (A), distribution (B), and example of immunohistochemically stained section (bar is 100 μm)(C) in pre/frail women with and without leucine supplementation before and after 12 weeks of resistance exercise training.
Data are means ± SEM. Ala: *n*=8, Leu: *n*=9. * depicts time effect, # depicts Leu different from Ala at same timepoint ($p<0.05$).

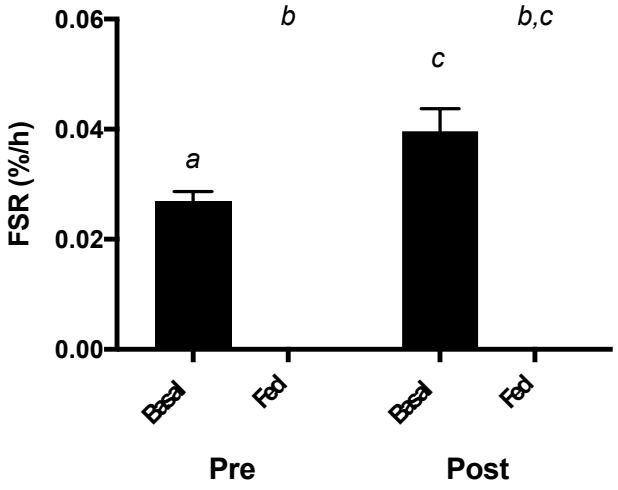

Figure 5-3 Myofibrillar fractional synthesis rate in pre/frail women in the postabsorptive (basal) and postprandial (fed) state, before and after 12 weeks of resistance exercise training.
Data are means ± SEM. Ala: *n*=8, Leu: *n*=10. Bars not sharing the same letter are significantly different (meal x time interaction effect, $p<0.05$).

5.5. Discussion

It is currently unknown if leucine supplementation has additional benefits on muscle anabolism in aging persons who are also physiologically vulnerable to muscle loss and its subsequent consequences. The purpose of the study was to investigate the effects of leucine supplementation in conjunction with RT, given sufficient habitual dietary protein intake in women within the context of frailty. We hypothesized that 12 weeks of RT would result in greater gains in postprandial MPS, which in turn would also translate into a greater increase in LBM, myofiber CSA, and gains in muscle strength and function than RT alone in pre-frail and frail elderly women habitually consuming an optimal amount of dietary protein.

The main findings of the study were: 1) Leucine did not have any additional effect on RT in any parameter (other than handgrip strength) in pre/frail women consuming an optimized amount of dietary protein. Because there was no other effect of leucine supplementation, we will henceforth discuss the results as the effects of RT with leucine and alanine supplemented participants presented as a combined group. Therefore, 12 weeks of RT in pre-frail and frail older women habitually consuming an optimized amount dietary protein: 2) improved their Frailty Phenotype along with a concurrent improvement in physical function and strength; 3) increased basal myofibrillar MPS, and; 4) increased type 1 and 2a myofiber CSA along with a concurrent increase in whole body LBM.

The current study is the first to investigate postabsorptive and postprandial MyoFSR before and after 12 weeks of RT in older women with an optimized dietary protein intake. We

observed that basal (postabsorptive) MPS was significantly higher (47%) post-intervention, with no change in postprandial MyoFSR. A recent study in healthy older adults (~71 y) reported that basal mixed protein FSR significantly increased by ~30% after 12 weeks of RT without further increasing the MPS response to feeding [64]. Our results are in agreement, with the caveat that our training-induced increase in basal MyoFSR was ~1.5-fold that of the aforementioned study. A possible reason why we observed a dramatically higher response is that we investigated the myofibrillar fraction, while the former study investigated mixed protein FSR. Mixed protein FSR in a previous study has shown a smaller change in basal MPS post-RT in young men [65]. Thus, the fraction analyzed may be important in determining the effect of RT on basal MPS. To the best of our knowledge, this is the first study to report the change in basal MPS in the myofibrillar fraction in an aged cohort. A 2001 study [66] supports our findings, in which FSR was measured in isolated myosin heavy chains (MHC) before and after 3 mo of RT in 46-79 y men and women. They found that the MHC FSR increased by 47% post-RT in the exercise group only. However, our results consist of a better-defined cohort more pertinent to aging (older individuals only), where we showed that even in the context of frailty, older women can respond to RT. Because we studied women only, there is no potential confounding effect of sex [67]. We investigated MyoFSR over the two nutritional states (postabsorptive and postprandial) in a fraction consisting of both major functional contractile proteins (myosin and actin), and thus we report a more comprehensive analysis than the aforementioned study. A previous 2011 study [68] attempted to investigate basal mixed protein FSR after 3 mo of a multi-modal exercise training program in 9 obese low-functioning older participants. Although they reported a robust increase in postabsorptive mixed muscle FSR (~50%), this measurement was captured

12-14 h after the last bout of exercise, and thus was well-within the timeframe for exercise-induced heightened sensitization to anabolic stimuli [69, 70] as well as elevation of mixed muscle MPS [71], and therefore cannot be considered a true basal rate of MPS. However, when taken in consideration with our results it is interesting that the effect of RT-increased MPS persists well after the last bout of exercise (~48 h), providing further evidence that the heighted rate of basal MPS consequent to RT could be capable of having a substantial positive impact on net muscle protein balance. The implications of this finding could help explain the muscle hypertrophy seen in our participants. Because humans typically spend the majority of their time in the postabsorptive state [70, 72], an increase in basal MPS could result in more muscle accretion (and thereby limiting muscle loss if MPB remains unaltered) than that which occurs as a consequence of increased postprandial MPS. In support of this, a recent study observed a significant increase in basal mixed protein FSR, net mixed protein balance, and *vastus lateralis* thickness in healthy young men [65]. The change in basal mixed protein FSR was significantly and positively associated with the change in *vastus lateralis* muscle thickness (r=0.56). Indeed, in the current study the increase in basal MyoFSR occurred in concert with a significant increase in type 1 and 2a myofiber CSA (16% and 28%, respectively), and on a larger scale, whole LBM (2%).

To the best of our knowledge, this is the first study to report the change in Frailty Phenotype after 12 weeks of RT in older women with an optimized dietary protein intake. We showed that the number of met Fried Criteria significantly decreased post-intervention. Indeed,

9 participants improved from frail to pre-frail, 4 from pre-frail to healthy, and 2 from frail to healthy. Only 3 pre-frail participants did not improve upon their category. Thus, the RT intervention appears to have a particularly robust effect on those who are classified as frail. The significant improvements in all physical function and strength outcomes provide further support that the improvement in Frailty Phenotype was meaningful for all participants. Four of the five Frailty Criteria had significant improvements post-intervention. The only anomaly was handgrip strength, where the only difference between the supplement groups lay. An interaction effect was observed in that only the Ala group significantly improved handgrip strength. Although handgrip strength has been shown to be useful in cross-sectional studies to assess physical performance capacity [73, 74], it has been shown to be an ineffective measurement of changes longitudinal studies unless the intervention includes a specific handgrip exercise [75], which the current study did not. Additionally, and more importantly, the Leu group improved to the same extent as the Ala group in all other strength tests, including other upper body tests (1 RM chest press, 1RM lat-pull down, 30s arm curls). Thus, we consider our result in handgrip strength to have no meaningful impact on functional improvements between the supplement groups.

We observed no change in myofiber distribution, other than a significant decrease in type 2a/2x distribution post-intervention. We speculate that this decrease could be attributed to the well-known 2x to 2a shift observed with RT [32]. Due to the initial small proportion of type 2a/2x, and large proportion of 2a myofibers, the potential resulting increase in type 2a fibers would likely not have been statistically detectable. Our results are in agreement with

others that RT does not affect the distribution of type 1 fibers in older adults [75-77]and specifically older women [32, 43]. Some [43, 77] but not all [32] studies have seen an increase in the distribution of type 2a fibers with RT in older adults and specifically old women. Our results are in agreement with the latter, as we saw no change in type 2a distribution in either group post-intervention. The two studies that have measured type 2x fiber distribution in older women [32, 43] observed a significant reduction with RT, while a study in older adults noted a trend in reduction [77]. We did not observe a significant reduction in type 2x distribution in either group post-intervention, though we observed a much smaller distribution of type 2x fibers at both timepoints (~ 3% and 1%, pre- vs post-intervention) than previous studies, which have generally observed ~10-20% [32, 43, 45, 77, 78]. Although we do not know why our participants had a much smaller type 2x distribution than others, we can speculate that some of the 2x fibers from previous publications [32, 43] may have been misidentified as type 2a/2x co-expressors, as we identified far fewer type 2x than type 2a/2x fibers. Finally, to the best of our knowledge, this is the first study to report type 1/2a distribution in older women, which was not affected by the intervention.

Our participants significantly increased their type 1 and 2a fiber CSA. To the best of our knowledge, this is the first study to show an increase in type 1 myofiber CSA with 3 months of RT, in a cohort of frail and pre-frail older women. Previous RT interventions (8-34 weeks duration) in older adults [32, 75, 79-81], men only [82], and women only [32, 43, 75] all reported no significant gain in type 1 myofiber CSA. In contrast, our participants increased their type 1 CSA by approximately 16%. To the best of our knowledge, our results are in agreement

and expand upon previous work on type 2a myofiber CSA with RT in older adults. Previous studies have reported type 2a CSA percent gains in men of ~23% [82], and in women only of ~7% [43] and 21% [32] (both not significant) and 28% [75] (significant), which was the same magnitude as reported in the current study. Additionally, type 2a percent changes in older adults have varied with reports of gains of ~18% [80] and no change in adults >80 y [81] with classical RT, and large gains (~41%) in 2 mo of power training [79]. Our results are in agreement with others that RT does not affect the CSA of type 2x fibers in older adults [76, 77] and specifically older women [32, 43]. To the best of our knowledge, only one study has reported changes in type 1/2a (+~60%) and 2a/2x (+~40%) CSA in older adults [64]. Our results do not agree with the aforementioned study, as we saw no changes in either co-expressor post-RT. The discrepancy in findings could potentially be due to the difference in participants (healthy older men and women, versus pre-frail and frail older women only). Future studies are clearly warranted to clarify RT adaptations in co-expressing myofibers. Thus, our results enhance and expand upon previous results in myofiber CSA and distribution, especially in regard to type 1 CSA and hybrid myofiber profile changes with RT in frail and pre-frail older women. Future studies should incorporate co-expressing fibers into their methodologies in determining changes in myofiber profiles with anabolic interventions in aging. Additionally, the significant increases in type 1 and 2a myofiber CSA occurred in conjunction with a significant increase in whole body LBM, providing further support that the changes at the fiber-level impacted changes at the level of the whole body.

An obvious question arising from our results is why did leucine supplementation have no added anabolic benefit to the intervention? One possible reason is that perhaps the 2.5 g dose of leucine per meal was not high enough [22]. For example, the peak plasma leucine concentration resulting from 2.8 g leucine to an EAA-containing beverage was ~700 μM [11], while a more recent study providing 5 g leucine to a mixed-macronutrient meal produced a much lower peak plasma leucine concentration (~450 μM) [27]. Therefore, it is possible that significantly higher doses of leucine are required when given in mixed-macronutrient meals in order to enhance MPS in older adults. Future studies are required to determine the optimal dose of leucine in the context of a mixed-macronutrient meal needed to effectively enhance MPS in older persons. Another possible reason why we did not observe an enhanced effect of leucine supplementation in our study is that perhaps the RT in combination with sufficient dietary protein intake was an optimal anabolic stimulus, resulting in no anabolic deficits for leucine to improve upon in this cohort. It is known that the anabolic effect of RT is elevated when combined with protein [70, 83], and a recent systematic review reported compelling findings that anabolic resistance seen with aging can be overcome with RT and sufficient provision of protein [8].

Strengths of the current study include that our cohort was well-controlled in their diet (through multiple food-recalls) and physical activity (by accelerometry). Previous studies have usually neglected to control for these potent confounding factors. In the same regard, our study also utilized an appropriate placebo (alanine), which provided nitrogen balance. We utilized current methodology for assessing fiber typing which allowed for detection and measurement

of co-expressing myofibers. Our tracer methodology enabled us to measure MPS in the postabsorptive and postprandial states in the myofibrillar fraction, which is the fraction most representative of the functional contractile proteins in muscle. Thus, we investigated the fraction that would be most affected by the intervention. An important limitation of our study is that no measurements of MPB were taken, which is necessary to obtain a true measure of net protein balance. Future studies should include institutionalized/hospitalized frail and also sarcopenic persons. Because one can be frail without significant muscle atrophy, studying truly sarcopenic persons would provide information relating directly to states of muscle atrophy.

In conclusion, leucine supplementation had no added benefit to 12 weeks of RT in pre-frail and frail older women habitually consuming an optimal amount of dietary protein. The RT intervention significantly improved upon the Frailty Phenotype with associated improvements in physical function and strength, as well as increasing basal MyoFSR with associated increases in type 1 and 2a myofiber CSA and whole LBM. Thus, RT and optimized protein intake are robust and effective anabolic stimuli in frail and pre-frail older women.

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CHAPTER 6: GENERAL DISCUSSION AND FUTURE DIRECTIONS

6.1. Main Findings

This was a randomized double-blinded placebo-controlled study consisting of 12 weeks of RT with and without leucine supplementation in pre-frail and frail older women consuming an optimal intake of dietary protein. The main findings of this study were as follows. 1) Leucine had no detrimental nor beneficial effect on insulin sensitivity. 2) Leucine increased mitochondrial content as evidenced by increased VDAC protein but had no effect on the capacity of mitochondria function or its measured sensitivity to apoptosis. 3) Leucine had no added benefit on improvements in muscle protein synthesis, strength, function, and Frailty Phenotype. Finally, 4) the exercise intervention resulted in an increase in LBM, supported by increased basal (postabsorptive) MyoFSR, which occurred in conjunction with an increase in type 1 and 2a myofiber CSA, the former two effects not previously known in this older cohort.

6.2. Manuscript 1

We strived to determine the effects of leucine supplementation on important aspects of frailty. We commenced by determining if leucine would have any impact on insulin sensitivity, as there is contention in the literature on deterioration or improvement of insulin action with leucine supplementation. Although RT in conjunction with optimal protein intake had a robust hypertrophic effect as evidenced by the increase in basal MyoFSR, type 1 and 2a CSA, and LBM observed in the study in its entirety, no effects on any indices of insulin sensitivity were observed. An increase in muscle protein results in more muscle to take up glucose out of the bloodstream and thus can improve glucose homeostasis [43, 285]. However, muscle is not the only body tissue of note, and excess body fat heavily influences insulin sensitivity [286-288].

Even though our participants also significantly decreased their percentage of body fat (~2%) post-intervention, we did not observe any improvements in insulin sensitivity. Despite the fact that frail persons are vulnerable to develop insulin resistance [289], perhaps because our participants were relatively insulin-sensitive and non-obese at baseline, no significant deficits existed upon which to improve. Therefore, the effects of leucine supplementation on glucose metabolism may occur only in states of abnormal glucose homeostasis. Conversely, no negative effects of leucine supplementation were observed on any indices of insulin sensitivity. Leucine was given in a pulsatile fashion with each main meal. Using this method of supplementation, our results are in agreement with others in rats [290] and humans [201, 202] that leucine has no detrimental effects on insulin sensitivity. We therefore report that leucine is a safe and viable method of supplementation in our study population.

6.2.1. Manuscript 1: Future directions

In this Manuscript we measured the serum insulin and plasma glucose responses during a metabolic meal test. Although this technique has been successfully utilized by our group [291, 292] and others [293], the gold standard remains the hyperinsulinemic/euglycemic clamp. Although we were not at liberty to clamp the values of glucose due to the overall study design (primary outcome of MyoFSR using a stable isotope tracer in response to a meal), future studies could utilize the hyperinsulinemic/euglycemic clamp to aid in determining metabolic differences in similar interventions. Additionally, recruiting persons with known abnormalities in glucose homeostasis would ensure that there are deficits upon which for leucine to improve.

6.3. Manuscript 2

Another aspect addressed in this thesis was the potential role of leucine in regard to mitochondria quantity and function as mitochondria has been implicated in sarcopenia and frailty. We observed no effect on any measured indices of mitochondria function, even when normalized to mitochondrial content, despite a significant improvement in an aerobic physical function test (6MWT). We observed an increase in content of mitochondria with the addition of leucine supplementation, as evidenced by increased VDAC protein content. RT has been shown to increase mitochondria biogenesis in elderly persons [61, 62]. It is therefore curious that the placebo Ala group did not also increase their mitochondrial content, especially given that the magnitude of all training effects that were observed were the same between the two groups (e.g., improvements in strength, function, hypertrophy etc. See Manuscript 3). There remains a remote possibility to be determined that alanine has an inhibitory effect on RT-stimulated mitogenesis [294]. Conversely, perhaps RT alone is insufficient to increase mitochondria biogenesis in older or frailer elderly [52], and the addition of leucine is required to elicit this response. It has recently been shown that cultured myotubes exposed to leucine increase mitochondria biogenesis [64, 65, 281, 295], but this is the first study to report the same result in human muscle tissue. An increased sensitivity to mPTP opening has been observed with aging [235, 270]. Our study suggests that mPTP sensitivity cannot be improved upon with RT. However, a different exercise modality (i.e., aerobic) may be necessary to elicit changes in mPTP [296, 297]. Furthermore, leucine has been implicated in the signaling pathway involving SIRT3 inhibition of mPTP-mediated apoptosis [64, 296]. Therefore, perhaps our participants would have a different response in mPTP sensitivity post-intervention with endurance training,

an exercise modality shown to induce SIRT3 [297], in conjunction with leucine supplementation. The lack of change in capacity of mitochondrial respiration seen in the current study is in agreement with groups which have attributed any decline in mitochondrial respiration to mitochondrial quantity, rather than an intrinsic impairment of mitochondrial respiratory functioning [235, 248, 272], although this is not a uniform finding [270]. The lack of change in capacity of mitochondrial ROS production is consistent with findings of a recent study in healthy older men (≥60 y) who underwent a 12-week RT program (without supplementation) showing no changes in ROS production post-intervention [67]. In addition, ours is the first study performed in pre-frail and frail older women to show that ROS emission was not ameliorated with leucine supplementation.

6.3.1. Manuscript 2: Future directions

The study of mitochondria and aging remains an extremely varied and prolific area of research, and thus there are many different aspects of mitochondria to be studied in the future. Pertinent to our results, it has recently been shown that age-related changes in mitochondria function (respiration and ROS production) become more readily apparent at physiological and not saturating levels of ADP [52], the latter of which was conducted in the current study. Therefore, future studies should modify their methodology to include mitochondrial respiration and ROS production driven by physiological levels of ADP. Our finding of increased mitochondrial content should also be investigated in future studies to determine the mechanism (biogenesis versus reduced degradation). Future studies using a similar intervention could also employ electron microscopy to obtain more in-depth analysis of changes in

mitochondrial content, including detailed analysis of mitochondrial population affected (intermyofibrillar and subsarcolemmal) [275]. Another potential future area of research concerning the mitochondria is mitochondrial quality control. Because leucine exerts its anabolic effects through activation of mTORC1, an unexplored potential consequence of increased mTORC1 activation could be suppression of catabolic processes such as autophagy [193], or mitophagy when directed towards the degradation of dysfunctional mitochondria [298, 299]. Furthermore, along with mitophagy, mitochondrial dynamics (fusion and fission) play an integral role in mitochondrial quality control [299, 300]. The implications of mitochondrial quality control during the aging process and how it can be affected by different modalities of exercise training is a current and relevant area of research, and studies are lacking in our cohort which could yield further insights into the role of mitochondria in muscle aging and frailty.

6.4. Manuscript 3

Finally, we addressed the role of leucine in a diversity of endpoints ranging from physical performance to body composition, mechanistic aspects in muscle protein synthesis as well as potential changes in myofiber profiles. We covered an array of expected effects of leucine, and report that leucine had no added benefit to 12 weeks of RT given optimal protein intake. We based our dosage of leucine on two previous studies in older persons which showed a robust positive MPS response acutely [20, 201], but no gains in muscle mass or strength after 3 months of supplementation, however the participants were healthy and not frail or sarcopenic. One possible reason that could account for the lack of effects of leucine in the

current study is that perhaps the given 2.5 g dose of leucine was inadequate when administered in the context of a mixed-macronutrient meal [15, 174]. For example, the peak plasma leucine concentration resulting from the addition of 2.8 g leucine to an EAA-containing beverage was ~700 µM [20], and similarly, 2.5 g leucine to a 20 g bolus of casein was ~650 µM [18]. Conversely, a more recent study providing 5 g leucine to a mixed-macronutrient meal produced a much lower peak plasma leucine concentration (~450 µM) [174]. Thus the 2.5 g dose of leucine given in the current study, as well as others [201, 301], may have been insufficient to elicit an enhanced anabolic stimulus in the context of a mixed-macronutrient meal.

The increase in basal (postabsorptive) MPS in the myofibrillar fraction in the current study was double that seen in the mixed-protein fraction of a previous study with a similar RT intervention in older adults [302]. We speculate that because the aforementioned study measured mixed-protein FSR, the potent hypertrophic effect of RT on the functional contractile proteins could be diluted in mixed-protein fractions containing proteins not effected as much by the training stimuli (i.e., non-contractile proteins). Furthermore, humans typically spend the majority of their time in the postabsorptive versus postprandial state [160, 303], the former of which is capitalized upon with the increase in basal MPS seen in the current study. Indeed, the increase in basal MyoFSR occurred in concert with a significant increase in type 1 and 2a myofiber CSA (16% and 28%, respectively), and on a larger scale, whole body LBM (2%). A recent study in healthy young men who underwent a similar RT intervention lends support to our findings. They reported that the increase in basal mixed-protein FSR was significantly and

positively associated with hypertrophy in *vastus lateralis* seen as increased muscle thickness (r=0.56) [304]. In addition, taken together with a 2011 study [158], the effect of RT-increased postabsorptive MPS persists well after the last bout of exercise (~48 h), providing further evidence that the heighted rate of basal MPS consequent to RT could be capable of having a substantial positive impact on net muscle protein balance over the long-term.

We used a current immunohistochemical methodology to determine myofiber profiles [305]. Our results highlight the importance of identifying and assessing co-expressing fibers when investigating myofiber profiles in interventions targeted at improving muscle characteristics. Not only are such analysis sparse in the literature, our results yielded type 2x distributions that are markedly lower (~1-3%) than those previously reported (~10-20%) [23, 33]. We speculate that some of the 2x fibers from previous publications [23, 33] may have been misidentified and were in fact type 2a/2x co-expressors, as we identified far fewer type 2x than type 2a/2x fibers. The fact that this is the first study to report type 1/2a distribution changes in an anabolic intervention in a cohort of older women (which was not affected by the intervention) also highlights the scarcity of such analysis, and the need to include fiber co-expression when investigating myofiber profiles.

6.4.1. Manuscript 3: Future directions

In this Manuscript, the role of leucine was investigated in the context of supplying optimal amounts of dietary protein during a RT program. However, many older persons are unable to perform such an intense RT program, thus leucine still remains an attractive modality

for its known role as a substrate and trigger for the machinery of protein synthesis. Future studies should directly compare the FSR in other protein fractions such as sarcoplasmic and mitochondrial, the latter being of particular interest given the increase in mitochondrial content by VDAC protein expression observed in Manuscript #2. Doing so would provide superior insight into where RT specifically effects MPS in aging muscle. Future studies could also utilize the D_2O methodology [306] to investigate changes in integrated MPS over a longer time-frame representative of daily living. Due to logistical restraints, the current study was only able to measure MPS. However, MPS is only half of the equation in determining net muscle protein balance, the true measure of anabolism, if positive. In order to delineate the true dynamics of anabolism, MBP must also be assessed. Historical methodologies for capturing sensitive measurements of MBP have been particularly invasive with the need for catheterisation of large vessels [307, 308] and therefore not easily amendable for physiologically vulnerable persons. However, newer methodologies are being developed which are promising in their applicability to the aging cohorts of interest (see [308]), and future studies should pursue this avenue of research. Because we found indications of a robust response to the RT intervention given habitually optimal protein intake in the frailer participants, it would be logical to conduct similar interventions in malnourished frail who represent ~30% of those who are frail [309], as this is a subgroup with additional complexity and in the trajectory of increased disability [310]. Any improvements in the Frailty Phenotype could have significantly meaningful impacts on the functioning and thus quality of life of frail older adults. Furthermore, because one can be frail without significant muscle atrophy, studying truly sarcopenic persons would provide information relating directly to muscle. Thus, investigating if the changes seen in the current

study regarding basal MyoFSR and consequent muscle protein accretion are also seen in those with significant age-related muscle atrophy would be of high relevance.

6.5. Strengths of Dissertation

There were many strengths of the studies conducted in this Dissertation. The overall study design accounted for several important variables that have historically been lacking in previous literature. These include the randomized clinical trial design utilizing an isonitrogenous placebo, optimizing and maintaining dietary protein intake throughout the intervention, and habitual physical activity assessed and confirmed as stable during the intervention. Our participants had a high adherence to the RT (90.8 $\pm$ 1.1%) and supplementation (94.1 $\pm$1.8% and 97.3 $\pm$ 0.7%, Ala and Leu, respectively). We included a comprehensive assessment of physical function and strength. We also utilized methodologies pertaining to the highest level of development at time of implementation. These included stable isotope tracer (*in vivo* MPS), the validated DXA method for body composition, permeabilized fiber bundles (*ex vivo* mitochondrial function), and the immunohistochemical assessment of myofiber co-expressors.

6.6. Limitations of Dissertation

Limitations of the studies conducted in this Dissertation include that we assume that what is measured in what is captured in the biopsy from the *vastus lateralis* is representative of the whole muscle, muscle group, and/or whole body (MPS, myofiber profile, mitochondria function), while it is known that variations exist within the same muscle [119]. Our chosen dosage of leucine per meal (2.5 g) was potentially insufficient to elevate plasma leucine levels

to induce an effective signal in a mixed-macronutrient context, although this exact threshold has yet to be determined. We also recruited both pre-frail and frail women instead of just frail, increasing the variability in our study cohort. We were also logistically constrained in the magnitude of the study and thus were not at liberty to include a group of men. At the same time, we did not want to contaminate our findings by mixing men and women into one group, and because women experience a higher prevalence of frailty [87] in our studied age range while also being the understudied sex, we chose to recruit only women. Because extremely few studies exist directly comparing men and women, and sexual dimorphism is known to exist in the mechanisms of detrimental age-related muscle changes (see sections 2.3.3; 2.5.3; 2.9.4), future studies are clearly required consisting of separate and comparable sex groups.

CHAPTER 7: CONCLUDING REMARKS

We undertook to determine the effects of leucine on a combined RT and optimal protein intake in pre-frail and frail older women in a randomized double-blinded placebo-controlled clinical trial. This subgroup of the elderly population would derive the most benefits from such an intervention. The study of leucine administration has generated a substantial amount of literature in support of its usefulness in mitigating the age-associated anabolic resistance through its effects in stimulating muscle protein synthesis. We hypothesized that leucine would exert benefits beyond and above those expected with RT, given the concurrent consumption of optimal dietary protein intake to provide adequate substrates for stimulated muscle protein synthesis. This Dissertation reveals for the first time that leucine supplementation has no added benefit to 12 weeks of RT in pre-frail and frail older women habitually consuming an optimal amount of dietary protein on any indices of insulin sensitivity, mitochondrial function or sensitivity to apoptosis, muscle protein synthesis or function. However, leucine supplementation may increase mitochondrial content although the impact of which was not detected on the aerobic clinical performance test. The RT intervention with a sufficient protein intake did significantly improve upon the Frailty Phenotype with associated improvements in physical function and strength. In addition, the RT increased basal postabsorptive MyoFSR that may have contributed to increases in type 1 and 2a myofiber CSA and LBM. Future studies should expand upon this work in populations with more apparent muscle atrophy and deficits in glucose homeostasis to improve upon, and importantly, determine the dosage of leucine required to effectively raise peak plasma leucine

concentrations in the context of a mixed macronutrient meal. Nevertheless, RT and optimized protein intake are safe, robust and effective anabolic stimuli in frail and pre-frail older women.

CHAPTER 8: APPENDIX

8.1. APPENDIX A: Ethics Approval

Centre universitaire de santé McGill | McGill University Health Centre

Bureau d'éthique de la recherche
Research Ethics Office

November 1, 2013

Dr. Jose Morais
MUHC – RVH
Room H6.61

Re: "The Impact of Exercise Training and Leucine Supplementation in Frail Elderly Women with an Exploration Into Mechanistic Explanations"

Dear Dr. Morais:

We are writing in response to your re-submission providing clarifications and revised documents required by the Biomedical B (BMB) Research Ethics Board (REB) for the study referenced above. The study was assigned MUHC Study Code 13-211-BMB as the MUHC reference when discussing the study. At the MUHC, sponsored research activities that require US federal assurance are conducted under Federal Wide Assurance (FWA) 00000840.

The proposal received Full Board review, and was found to meet the ethical standards for conduct at the MUHC. The information was entered accordingly in the minutes of the meeting.

We are pleased to inform you that the study was first reviewed at the convened meeting of the BMA REB on September 12, 2013 and re-reviewed at the convened meeting of the BMA REB on October 10, 2013.

Approval, for the study was provided on October 11, 2013 and includes the:

Research Protocol (September 24, 2013);
Revised Informed Consent Document (September 24, 2013) in French and English;
Telephone Questionnaire (September 2013) in French and English;
Study Advertisement (November 1, 2013) in English and French;
Muscle Biopsy Incision Care Instructions (undated) in French and English;
Training Program and Exercises (Undated);
Physical Performance Battery (Undated);
Study Questionnaires (Undated);
Supplement Logs (Undated);
How Are You Feeling Today (Undated) in English;
Rating of Perceived Exhaustion (Uncated) in French and English.

All research with human subjects requires ongoing REB oversight and the approval will be in effect until **October 10, 2014.** Prior to the expiration of ethics approval, it is your responsibility to submit to the REB either an "Application for Continuing Review" when the study is ongoing, or a "Study Completion Report" if the research has been completed.

The MUHC Research Ethics Boards (RERs) work under the published guidelines of the *"Tri-Council Policy Statement 2"*, and the *"Plan d'action ministériel en éthique de la recherche et en*

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37052/34923

intégrité scientifique", and in compliance with the *"Food and Drugs Act"*, including the *"Food and Drug Regulations"*, the *"Medical Devices Regulations"*, and the *Natural Health Products Regulations*, and act in conformity with standards set forth in the *(US) "Code of Federal Regulations"* governing human subjects research, and in a manner consistent with internationally accepted principles of good clinical practice.

You must report to the REB promptly without delay should a modification to the research be proposed, and without delay if an unanticipated problem occurs before the next required review. Regulations do not permit you to modify conduct of the study prior to ethics approval for a study amendment; except where urgent action is required to eliminate an apparent immediate hazard to a study subject or other person.

It is important to note you may initiate the study only after all required reviews have been completed and all decisions are favorable. At that time you will receive MUHC Authorization to conduct the study in correspondence issued by the Research Institute of the MUHC.

We trust this will prove satisfactory to you.

Sincerely,

Norbert Gilmore, MD, PhD
Chair
BMB REB

Cc: 13-211-BMB
Stephanie Lamarche

8.2. APPENDIX B: Informed Consent Form

Patient Information and Consent Form for Study Participants

McGill University Health Centre (MUHC)
Glen Site Hospital: Centre for Innovative Medicine, Department of Medicine

Title of the project:	**The Impact of Exercise Training and Leucine Supplementation in Frail Elderly Women with an Exploration into Mechanistic Explanations**
Sponsor:	**Geriatric Medicine**
Investigator:	**José A. Morais, MD; telephone: 514-934-1934 #34499 (8 am to 4 pm) or by pager: 514-406-0163 (any time)**

Co-investigators/Collaborators:

Stéphanie Chevalier, Ph.D.:	**telephone: 514-934-1934 #35019**
Russell Hepple, Ph.D.:	**telephone: 514-834-1934 #35509**
Louis Bherer, Ph.D.:	**telephone: 514-987-3000 #4779**
Kathryn Wright, M.Sc.:	**telephone: 514-934-1934 #35024**

INTRODUCTION:
You are being invited by Drs. José A. Morais and Stéphanie Chevalier from the MUHC Crabtree Nutrition Laboratories to take part in a study on exercise and protein metabolism in aging. Your eligibility to take part in this study has been determined following the telephone screening process.

Before deciding to participate in the study, you should clearly understand its requirements, risks, and benefits. This document provides information about the study, and it may contain words you do not fully understand. Please read it carefully and ask the study staff any questions you may have. They will discuss the study with you in detail. If you decide to participate, you will be asked to sign this form and a copy will be given to you.

BACKGROUND:
Frailty is a clinical condition associated with an increase in risk for disease and death and becomes more common as people age. Frailty has a strong relationship with the age-related loss of muscle and strength, termed sarcopenia. Sarcopenia and frailty are strongly associated with disability, especially in women. Adequate protein intake, the amino acid leucine (one of the building blocks of proteins), and resistance exercise training (lifting weights) have been individually shown to increase muscle mass to varying degrees. However, no studies have investigated how a longer-term resistance exercise training program with added leucine when protein intake is optimized could increase muscle mass in frail and pre-frail elderly women. In addition, this is the population that stands the most to gain from such an intervention.

PURPOSE OF THE STUDY:

The purpose of this study is to investigate the effects of the amino acid leucine added to resistance exercise training on muscle mass and physical performance in frail and pre-frail elderly women with adequate protein intake. In a random fashion, participants are assigned by chance to different groups that compare interventions; neither the researchers nor the participants can choose which group they will be in.

A total of 24 participants will take part in this study, conducted at the McGill University Health Centre (MUHC) Glen Site Hospital and the Institut Universitaire de Gériatrie de Montréal (IUGM). All participants will undergo adjustments to their diet to optimize protein intake and a resistance exercise training program. Half of the participants will receive a supplement of powdered leucine (an amino acid), and the other half of the participants will receive a placebo in the same powder form. A placebo is a substance that looks similar to leucine, but does not have any effect on the body. Neither the participants nor the study investigators will know which participants are receiving the leucine nor which are receiving the placebo. Please note that although you may receive the placebo, you will still benefit from the altered diet and the resistance exercise.

In addition to visits to the gym, your participation in this study will involve 4 total visits: 2 initial screening visits followed by 2 day long stays at the Centre for Innovative Medicine (CIM) of the MUHC Glen Site Hospital. These two stays will be spaced apart by 12 weeks of the intervention (dietary adjustments, resistance exercise training, and the powdered supplement). By participating, you would help us to better understand how the presence of aging affects the body's responses to resistance exercise and how leucine, may help build muscle. Information gained from this study could help improve diet and exercise programs that would help prevent muscle loss that is often seen with aging.

STUDY PROCEDURES:

After the initial over-the-phone screening, you will be invited to the research unit at the MUHC-Glen Site Hospital for the first visit (Visit 1). Please see the addendum table of study visits.

Visit 1) Information and Health Assessment Visit: For this first session you will come to the research unit after an overnight fast (no eating or drinking anything, except water starting at 11:00 p.m. the night before the visit). You will meet with the research staff to discuss the study and have your questions answered. If you agree to participate you will sign the consent form. You will have a health assessment, standard blood and urine tests, a chest X-ray and an electrocardiogram (ECG). Your cognitive status, quality of life, and weekly energy expenditure will be assessed by questionnaires, as described below. You will be given a form to fill out your diet for three days, to be completed before the next visit (Visit 2) to determine your usual food intake and dietary protein adjustments. Detailed instructions will be given to you about how to fill in this form. The first visit takes approximately 3 hours to complete and a meal will be provided to you.

Cognitive status assessment

Your cognitive status will be assessed using the Mini-Mental State Examination (MMSE) questionnaire. 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.

Quality of life assessment
Your quality of life will be assessed using the EQ-5D questionnaire and Geriatric Depression Scale (GDS). You will be asked several questions concerning mobility, self-care, usual activities, pain/discomfort, and anxiety/depression. These questionnaires take about 5 minutes to complete.

Weekly energy expenditure assessment
Your weekly caloric energy expenditure will be assessed using the CHAMPS Physical Activity Questionnaire for Older Adults. You will be asked questions whether you participate in various activities or daily tasks, and if so, for how long and how many times per week. This questionnaire takes about 15 minutes to complete.

Handgrip strength
For this test, you will be seated upright and squeeze as hard as you can using your dominant hand on a hand dynamometer (a small device with a handle) three times. The highest measurement taken will be used as an indicator of overall body strength.

Timed Up and Go
This test measures the time it takes you to stand from an armchair, walk a distance of 3 metres, turn around and sit back down in the chair.

Gait Speed
You will be asked to walk at your usual pace across a distance of 4 metres (13 feet). The time to perform this test will be recorded with a stopwatch.

Visit 2) <u>Oral Glucose Tolerance Test (OGTT) and Physical Examination and Functioning Visit</u>:

Oral Glucose Tolerance Test (OGTT)
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). This test is to determine your body's "blood sugar" response to a high sugar drink, over a period of 3 hours. This test requires that you come to the research unit 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 carbonated 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 catheter into a vein in your arm before you take the drink. This catheter will allow for multiple blood samples to be collected without any pain to you after the initial prick. Blood samples will be taken 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. This visit will take about 4 hours to complete.

Review of Food Diary
Research personnel will determine your usual food intake from your completed dietary forms given to you during Visit 1 and will discuss with you any adjustments to your dietary protein.

Physical examination
The study doctor (Dr. José Morais) will take a full medical history and perform a physical exam at this time.

2-Week Run-In
After Visit 2 and preceding Visit 3, there will be a period of 2 weeks (separate from the 12-week intervention) known as a "run-in" where you will begin and adhere to your protein-optimized diet. You will wear an accelerometer for three days once during this period of time. Once per week for these 2 weeks you will go to the Institut Universitaire de Gériatrie de Montréal (IUGM) to become familiarized with the gym facilities and training program. You will not be asked to lift weights at this time. During the first visit, you will have your physical functional capacity measured.

Physical functional capacity assessments
Physical function will be assessed using several simple physical performance tests: the timed up and go test, 6-minute walk test, handgrip strength, and Short Physical Performance Battery test as described below. All of these procedures are comfortable to do and explanations will be given to you in person while you are undergoing the tests.

6-Minute Walk
For this test, you will be asked to walk as far as possible for 6 minutes, back and forth along a 100 foot (30 metres) hallway. The total distance that you walk will be measured.

Short Physical Performance Battery
This test consists of the following five simple tests. 1) Repeated Chair Stands: you will be asked to try to stand up from a chair five times without using your arms, as quickly as you can. 2) Semi-tandem Stand: you will be asked to try to stand with the side of the heel of one foot touching the big toe of the other foot for 10 seconds. 3) Side-by-Side stand: you will be asked to try to stand with your feet together, side by side, for 10 seconds. 4) Tandem Stand: you will be asked to try to stand with the heel of one foot in front of and touching the toes of the other foot for 10 seconds. 5) 4 metre walk (13 feet): you will be asked to walk at your usual pace across a distance of 4 metres. The time to perform each of these tests will be recorded with a stopwatch.

Accelerometer
You will be asked to wear an accelerometer around your waist for three consecutive days 3 times during the study. The first time will be prior to the start of the intervention, during the 2 week run-in. The second time will be in the middle of the intervention. The third time will be at the end of the intervention. You will be given an instruction sheet on how to wear the accelerometer properly. The purpose of wearing the accelerometer is to measure your daily physical activity levels to see if there are any changes throughout the study.

Visit 3) Meal Test
After the 2-week run-in you will be asked to come to the Centre for Innovative Medicine (CIM) at the MUHC Glen Site Hospital for Visit 3.

Day 1: DXA
You will arrive at the Glen Site Hospital at 12:30pm. Your body composition will be measured by a scanning technique called dual X-ray absorptiometry (DXA). This test will take place at the Nuclear Medicine clinic of the MUHC Glen Site Hospital, where you will be accompanied by a member of the research team. For this test that lasts about 10 minutes, you will lie down and be 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.

Day 2: Meal test to assess metabolic responses
The meal test will be performed at the CIM, Glen Site Hospital and will last approximately 8 hours. All your activities during this period will be closely supervised by the research team.

You will arrive at the Glen Site Hospital at 7:00am. At 8:00 am on that morning, while you are fasting and resting comfortably in a bed, infusion of $^{2}H_{5}$-phenylalanine will be started through a catheter and continued for the rest of the study at a slow rate. Phenylalanine is an amino acid, a compound that we eat from dietary protein and that the body uses to make new proteins. This form of phenylalanine contains the stable isotope deuterium, meaning that it is not radioactive. We can detect it with very sensitive instruments because it is slightly heavier than normal phenylalanine. Stable isotopes occur naturally and are safe to use even in children and pregnant women.

At 9:00 am, a second catheter will be inserted in a vein on the back of your hand. Your hand will be placed in a warming box for the duration of the Meal test at 65°C to make the blood in the vein similar to that of an artery. This catheter will be used for taking repeated, painless blood samples every 30 minutes (about 20 in total). This is not a painful or uncomfortable procedure. You will also be asked to undergo a resting metabolic rate test. For this test, 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.

At 12:30 pm the first muscle biopsy will be performed, and the second one will be taken two hours after you consume the meal. The biopsy procedure is a standard one for research studies and diagnosis. It is even done in athletes who exercise immediately after the procedure. A sample will be taken from the muscle on the outside side of your thigh. The area 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 taken 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 procedure. You will be given at the end of the visit an information sheet containing instructions on the precautions to follow at home for the biopsy site.

At 1:00 pm, you will have a meal composed of 400 mL (about a cup and a half) of a complete liquid formula that you will have to consume within about 20 minutes.

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). The study will end 3 hours after the ingestion of the liquid meal. After the study ends, you will be offered a regular meal and will return home.

Following 12 weeks: study intervention

Diet & Supplementation
After Visit 3, you will begin to take the supplements. Neither you nor the study investigators will know if you are taking leucine or the placebo. You will take supplements three times a day and record your intake of them in daily supplement logs that will be collected every 2 weeks. You will also complete a 24-h food recall over the phone every 2 weeks.

Exercise
At the same time as beginning to take the supplements, you will also begin the 12-week training program. You will attend kinesiologist-supervised resistance exercise training sessions three times per week at the IUGM. Each session will last for 60 minutes and will occur with 2-3 other participants of the study. The exercises you will do are safe and will be tailored to your capabilities. Each training session will consist of a warm-up, posture adjustments, breathing, joint range of motion, resistance exercises and a cool-down with relaxation techniques. The trainers will complete training logs for you for each training session. You will be asked questions such as how you are feeling, your mood, and also how hard you feel you are working during a given exercise. Every few weeks the kinesiologist will find out how much weight you can safely lift one time (1-RM). At the conclusion of the 12-week intervention period, all physical functioning tests will be repeated as described above for the pre-intervention measurements.

Visit 4) Second stay at the MUHC Glen Site Hospital:
This second stay will occur at least 48 hours after the last training session or physical functioning testing. **This second stay is conducted similarly to the first:**

Day 1: Body composition measurements will be conducted.

Day 2: The meal study will take place as written above in "Visit 3", with an additional muscle biopsy before the tracer infusion begins (i.e., 3 muscle biopsies in total for this day), and your supplement added to the meal.

Analyses on Muscle Samples
We will perform an analysis called gas-chromatography mass-spectrometry in order to measure the rate of protein synthesis in your muscle. With this technique we will be able to measure the incorporation of the stable isotope $^{2}H_{5}$-phenylalanine into your muscle protein during the meal test. A higher amount of $^{2}H_{5}$-phenylalanine detected in your muscle protein is interpreted as a higher rate of protein synthesis. We will then perform an analysis called immunoblotting that allows us to label and then measure the relative quantities of different kinds of proteins. We wish to label proteins that tell the muscle to increase protein synthesis or not. We will also perform an analysis called immunohistochemistry on very thin slices of your muscle to stain them for different types of muscle fibers. We will measure the relative proportions of the types of muscle fibers seen and also measure their size, called cross-sectional area.

Safekeeping of Muscle Samples

Muscle samples will be immediately frozen in liquid nitrogen after being divided into several parts and then stored in a locked -80ºC freezer. The samples will be labeled in such a way as to maximally protect your confidentiality. In a first step, a code will be assigned that links your name to the sample. That code will be under the direct supervision of Dr. Morais and the study coordinators, and only they can authorize using the specific information about the results. Before being submitted for analysis by a third party, a different code will be the only identification for the material extracted from the sample. One of the co-investigators of this study will control this code. Those performing the analysis will have no way of tracing it to you.

Your samples will be stored for a period of 25 years from the date of the biopsies being taken. The freezers are at the Crabtree Nutrition Laboratories on H6 of the MUHC-Glen Site Hospital. The use of your sample or medical information is not intended to provide you or your physician with any results. The study doctor will not make any research results available to you, any insurance company, your employer, your family, or any other physician who treats you now or in the future.

You can change your mind at any time and withdraw your consent and your samples will be destroyed.

The sharing of the samples for future research is subject to approval by the Research Ethics Board (REB). An MUHC REB will review new research proposals that would like to use your samples. The REB is a committee that oversees medical research studies to protect participants' rights and welfare.

Potential Commercialization and Financial Benefits
Investigators will only use your samples for research. They will not be sold. Future research that uses your samples may lead to new products, but you will not receive payment for these products. Some future studies may need health information that we may not already have. If so, the study team will review your medical records for this information.

POTENTIAL BENEFITS:
Participants may benefit from taking part in a supervised and personalized exercise training program. This may lead to improved strength, flexibility, mobility, and could possibly lead to improvements in overall quality of life. In addition, because exercise will be performed in small groups, socialization may be a benefit to taking part in the study. Participants may benefit from receiving in-depth nutritional information and an individualized optimized diet as well as general health knowledge with the additional medical examinations that are required for the study. Although there are expected benefits from participating in the study, it may not be the case in every person. However, information learned from this study may help by adding to medical knowledge in this area and to better treatment for people in the future.

POTENTIAL RISKS:
Blood Draws: The risks involved in blood sampling are considered to be minimal. There may be slight pain or discomfort while doing blood tests with a slight risk of bruising. The amount of blood drawn during the entire study will not exceed that of an ordinary blood donation (80 mL during the screening process and 180 mL for each of the meal tests).

Muscle Biopsy: The risk of muscle biopsies is considered more than minimal, with 0.16% of participants experiencing some sort of complication (Tarnopolsky, Pearce et al. 2011), all of which are

considered minor in severity. The most common complication, although rare, is local skin infections (0.06%). Rare localized numbness or pain lasting more than 3 days occurred in 0.04% of adults. Lastly, larger bruising was also seen rarely in 0.01% of biopsy participants.

Exercise: Exercise may lead to some muscle fatigue during exercise and possibly soreness in the days following the exercise. This is generally transient and is not harmful. As with any type of strenuous physical activity there is a very slight risk of a serious event (e.g. heart attack) during the exercise that you will perform, but this risk is no different than if such exercise were performed at home or local gym. In fact, it is probably safer because you will be closely watched and exercise will be stopped immediately if there are any signs of too much strain.

Socio-Economic Risks: The risks associated with this research project relate to the impact that disclosure of your personal information to outside parties, such as employers or insurance companies, could have on your chances of acquiring insurance or certain types of employment. Such risks are minimal, however, since no identifying information will be included with your blood or muscle samples and study data. Furthermore, provincial and federal laws governing individual rights and privacy protection could protect you from invasive inquiries.

Please note that the comfort of the subject is of priority and sought during the meal test. Your study will be supervised by experienced kinesiologists, nurses, and doctors who will make every effort to keep you comfortable during the study. Pain control with Tylenol® (acetaminophen) will be offered after the meal test to prevent pain and personnel will also remain in contact with you.

INCIDENTAL FINDINGS:

Should any new findings develop during the course of this study that may affect your health, these findings will be communicated to you and your physician of choice.

CONFIDENTIALITY:

While you take part in this study, the study researcher and team will collect and take down information about you in a research study file. Only information necessary for the research study will be collected. The information in your study file could include your past and present medical history, information about your way of life and test results from exams and procedures done during this study. Your file could also contain other information, such as your name, sex, date of birth, and ethnic origin.

All the information collected about you during the study will remain confidential as the law demands. To protect your privacy, your information will be identified with numbers and/or letters. Only the researcher in charge of the study knows the numbers and/or letters that link them to you.

The study researcher will use the study information collected about you for research purposes, only to reach the study goals as they are explained in this information and Consent Form. Your study information will be kept by the researcher in charge for 25 years.

The study information could be printed in medical journals or shared with other people at scientific meetings, but it will be impossible to identify you.

To make sure that the study is being done properly, your study file as well as your medical file could be checked by a person authorized by the Research Ethics Board of the McGill University Centre (MUHC), or by the institution, by a person authorized by special people or groups. These people and groups are obliged to respect your privacy.

For your safety and to be able to reach you quickly, your family name, first name, coordinates and the date you started and ended the study will be kept for one year after the study ends in a separate list kept by a researcher in charge of the study or by the institution.

You have the right to look at your study file in order to check the information gathered about you and to correct it, if necessary, as long as the study researcher or institution keeps this information. However, you may only have access to certain information once the study has ended so that the quality of the research study is protected.

VOLUNTARY PARTICIPATION AND/OR WITHDRAWAL:

You may choose whether you would like to take part in this study. If you choose to take part now, you can change your mind later and stop at any time and for any reason. Tell the researcher in charge of the study or one of the members of the research team of your decision.

There is a chance that we may learn new information while you take part in the research study. This information may affect your health or well-being or change your decision to continue taking part in the study. You will be told any new information, at once, and it will also be given to you in writing.

The researcher in charge of the study or the Research Ethics Board of the MUHC may take you off the study without your consent at any time if: New information shows that taking part in the study is not right for you; You do not follow directions given to you by the researcher in charge of the study or by a member of the study staff; You experience a deterioration in your health.

INDEMNIFICATION AND COMPENSATION IN CASE OF INJURY:

If you should suffer any injury following your participation in the research project, you will receive the appropriate care and services for your medical condition without any charge to you.

By accepting to participate in this project, you are not waiving any of your legal rights nor discharging the researchers or the institution of their civil and professional responsibility.

COMPENSATION:

You will receive $30 for the Visit 1 screening and physical functioning testing and $30 for Visit 2, and $100 for each full meal study (two overnight stays) for costs and inconvenience because you took part in this study. If you choose to stop taking part in the study or are removed from it before the study is completed, you will be paid only part of this study depending on the time you took part.

CONTROL OF THE ETHICAL ASPECTS OF THE RESEARCH PROJECT:

The Research Ethics Board of the MUHC approved this research project and is responsible for its follow-up. In addition, it will first approve any review and amendment made to the information/consent form and to the study protocol.

QUALITY ASSURANCE PROGRAM:

The MUHC implemented a Quality Assurance Program that includes active continuing review of projects (on site visits) conducted within our establishment. Therefore, it must be noted that all human subject research conducted at the MUHC or elsewhere by its staff, is subject to MUHC Routine and Directed Quality Improvement Visits.

CONTACT PERSONS/ QUESTIONS:

If you have any questions about your participation in this study, you should contact the study doctor, Dr. José Morais at (514) 934-1934 #34499, from 8 am to 4 pm, or by pager (514) 406-0163 at any time. You may also contact members of the research team or research coordinators Marie Lamarche or Kathryn Jacob at (514) 934-1934 #35024, who are available to respond to your questions and concerns concerning this study.

If you have any questions about your rights as a study participant, you should contact the Hospital Ombudsperson at (514) 934-1934 local 35655.

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DECLARATION OF CONSENT:

I have read the contents of this consent form, and I agree to participate in this research study. I have had the opportunity to ask questions and all my questions have been answered to my satisfaction. I have been given sufficient time to consider the above information and to seek advice if I choose to do so. I will be given a signed copy of this consent form. By signing this consent form, I am not giving up any of my legal rights.

Subject's Name	Subject's Signature	Date

Person Conducting the Informed Consent Discussion

Name of Person Conducting the Informed Consent Discussion	Signature of Person Conducting the Informed Consent Discussion	Date

Visit	Length of Visit	Purpose of Visit	Content of Visit
Screening			
#1	3 hours	Information and Health Assessment	- Informed Consent - Health assessment - Standard blood & urine tests - Chest X-ray, electrocardiogram - Questionnaires for cognitive status, quality of life, weekly energy expenditure, receive food diary
#2	4 hours	Oral Glucose Tolerance Test and Physical Examination & Functioning	- Oral Glucose Tolerance Test - Review of completed food diary - Physical examination by physician - Gait speed & Handgrip strength tests
2-Week Run-In			
First Admission - Baseline Testing			
#3, Day 1	8h onwards	DEXA	- Dual X-ray absorptiometry - 24-hour urine collection
#3, Day 2	Until 17h	Meal Test	- Infusion of phenylalanine tracer - Blood draws - Muscle biopsies - Liquid meal
INTERVENTION			
Second Admission - Post Intervention			
#4, Day 1	8h onwards	DEXA	- Dual X-ray absorptiometry - 24-hour urine collection
#4, Day 2	Until 17h	Meal Test	- Infusion of phenylalanine tracer - Blood draws - Muscle biopsies - Liquid meal

CHAPTER 9: REFERENCES

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