Application of novel deuterium oxide tracer technique to determine the effects of ß-hydroxy ß-methylbutyrate (HMB) supplementation and exercise in ageing: a randomized placebo controlled trial

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Thesis Abstract

Background:

Skeletal muscles are crucial in our interaction with the environment considering the fact that they play vital role in our movement, maintenance of posture and regulation of metabolic milieu of our body. Almost 40 % of the body is comprised of skeletal muscle. Adequate skeletal muscle functioning is commensurate upon a dynamic equilibrium in protein turnover whereby a balance in synthesis and breakdown maintains muscle mass. Old age is associated with an imbalance in this dynamic equilibrium and, hence, strategies are needed to mitigate it.

Sarcopenia is a progressive and multifactorial process resulting in age-related deterioration in skeletal muscle mass and strength; characterized by decreases of 1-2%/year in muscle mass beyond the age of 50. Exercise, resistance (weight) training in particular, especially when combined with optimal nutritional interventions, has been shown to be an effective counter measure, arresting decline and substantially improving the physical strength and function. Advanced sarcopenia is synonymous with physical frailty, leading previously asymptomatic individuals to experience injurious events and acute/temporary disability. Given the high clinical costs of this insidious process; an in-depth understanding of aging muscle and protein metabolism is acutely needed.

Considering the established anabolic role of leucine and its metabolites in the stimulation of protein synthesis and inhibition of protein degradation; we hypothesized that exercise coupled to supplementation of the leucine metabolite ß-hydroxy ß-methylbutyrate (HMB) would positively impact the skeletal muscle

protein synthesis (MPS) leading to increased muscle mass and better functional capabilities in older individuals.

Objectives:

To determine the chronic effects (6 weeks) of ß-hydroxy ß-methylbutyrate (HMB) supplementation and exercise on muscle protein metabolism in older men.

Design:

This study was conducted in 16 healthy volunteers: Older men were randomly assigned to two groups (Treatment group; n=8, Age 67.8±1.1y, Height 1.76±0.02 m, BMI 28±1.2 kg.m-2: Placebo group; n=8, Age 68.5±1.1y, Height 1.74±0.02 m, BMI 27±0.9 kg.m-2) and asked to perform unilateral resistance exercise training regime (RET) of 6x8 repetitions at 75% 1RM for a period of 6 weeks. A randomised double-blinded placebo controlled trial was carried out and one group received ß-hydroxy ß-methylbutyrate (HMB) to ascertain the effects of supplementation on muscle protein synthesis.

Results:

Over a period of 6 weeks, RET increased 1-RM strength in both groups i.e. from a baseline of 398±22N to 499±30N in treatment group and 396±29N to 510±43N in placebo group. MVC increased from a baseline of 185±10Nm to 217±11Nm in placebo group ($P<0.05$) and 179±12Nm to 203±12Nm ($P<0.05$) in treatment group. Thigh lean mass assessed by DXA increased significantly over 6 weeks only in treatment group to 5734±245g $p=0.015$ vs 5644±323g $p=0.06$ in placebo group. VL thickness measured by ultrasound showed significant increases over 6 weeks in exercise legs of both groups, Placebo group: 20.7±0.6mm to 22.4±0.7mm $P<0.0001$ vs Treatment group: 21.6±0.9mm to 23.3±1.1mm $P=0.0001$. However, there were no significant differences between

both the groups in any of these measures. The qRT PCR results showed changes in cMyc gene expression only. We observed that trained leg responded to acute bout of exercise at weeks 0, 2, 4 and 6. However, there is no added effect of training over time and rest legs in both the groups do not exhibit any significant change throughout the study.

Conclusions:

These data suggest that HMB supplementation in free living healthy older individuals in conjunction with a 6 weeks RET regimen did not render any additive improvement in muscle strength, function and mass.

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List of Abbreviations:

AA – Amino Acid

AP- Atoms Percent

APE- Atom Percent Excess

ASMM- Appendicular Skeletal Muscle Mass

ATP- Adenosine Triphosphate

D_2O/^2H_2O- Deuterium oxide

DXA- Dual Energy X-ray Absorptiometry

EAA- Essential Amino Acid

FSR- Fractional Synthesis Rate

GC-pyr-IRMS - Gas chromatography-Pyrolysis- Isotope Ratio Mass Spectrometry

HMB- ß-hydroxy ß-methylbutyrate

MPS- Muscle Protein Synthesis

MPB- Muscle Protein Breakdown

RE- Resistance exercise

RM- Repetition maximum

TC-EA- IRMS- Thermal Conversion-Elemental Analyzer-Isotope Ratio Mass Spectrometry

USG- Ultrasonography

MVC- Maximum voluntary contraction

MAFbx- Human muscle atrophy F-box

MURF1- Muscle RING-finger protein-1

Myf5- Myogenic factor 5

REDD1- Regulated in development and DNA damage responses 1

EIF3f- eukaryotic translational initiation factor 3, subunit F

PPIA3- Peptidyl prolyl isomerase A-3/ cyclophilinA

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1. Chapter One: General Introduction- Sarcopenia – Muscle Loss and Ageing:

1.1 Chapter Introduction

Given the role of skeletal muscle in voluntary movements, maintenance of normal body temperature and determining the shape of body; it is imperative to have a detailed understanding of mechanisms culminating in its decline with old age. All of our voluntary body movements from walking, dressing, chewing, to nodding our head are controlled by skeletal muscle contraction. So, a decline in muscle strength and function may result in increased prevalence of physical disability and disabling co-morbidities in the elderly (Metter et al. 2002). This leads to a significant economic burden posing it as one of the biggest public health problems in the ageing population (Janssen et al. 2004). Moreover, the decline in muscle mass and strength is itself considered as an independent predictor of disability and mortality in older individuals (Newman et al. 2006).

Human beings are affected by aging in varied ways ranging from a decline in the quality of life and impaired function to more serious sequelae advancing to disease, disability and death. The most crucial factors affecting aging processes are said to be chronic diseases (e.g., Diabetes, Alzheimer's and Chronic Obstructive Pulmonary diseases) (Harman 1991; Ito & Barnes 2009) and environmental factors or societal factors (affecting food choices and physical activity) (Santangelo et al. 2011; Weinert & Timiras 2003). Moreover, the aging process in humans is quite dissimilar from that of other species because different species age at different rates, making it more difficult to extrapolate from one species to another. It is generally believed that the aging process is itself a foremost risk

for the development of many debilitating diseases like cancer and neurodegenerative illnesses. Thereby, making the interventions to defer the process and mitigate it, a central focus for aging research.

With a growing insight into aging mechanisms, it is now believed that aging is a combined outcome of genetics and environmental factors that impacts all tissues. Although more than 300 theories of aging have been proposed, few have gained universal acceptance. One of them being the "free radical theory" which states that aging is an indirect consequence of aerobic metabolism which leads to the build-up of oxidative damage to tissues and cells of the body over time (Harman 2006; Harman 1981). Whilst recent work has shown that role of reactive oxygen species in aging is unclear (Hekimi et al. 2011), it is still one of the most widely accepted mechanisms for aging. There are other theories at molecular level e.g., The DNA damage theory (Garinis et al. 2008), trace element theory (Eichhorn 1979), protein aggregation (Grune et al. 2004), mitochondrial dysfunction (Mammucari & Rizzuto 2010), the telomere shortening theory (Olovnikov 2015) and non-enzymatic glycosylation theory (Masoro et al. 1989) as possible mediators of aging.

Ageing is an inevitable process associated with wasting of muscles (Baumgartner et al. 1998). With advancing age, a decline is also observed in joint mobility, skeletal size, strength, endurance, and coordination (Baker et al.). Additionally, changes in body composition also take place leading to significant decreases in lean body weight (Borkan et al. 1983). It is said that a deterioration in muscle mass and, hence, strength is chiefly due to a decreased myofibrillar protein content (Montero & Serra 2013). Some have also reported decreases in the production rate of other muscle proteins (e.g., mitochondrial protein component) with the passage of time (Nair 2005). Whilst collagen synthesis is lower (collagen in old >

collagen in young) as compared to myofibrillar and mitochondrial synthesis, and remains unaltered after the provision of nutrients (Babraj et al. 2005).

In this introduction, the basics of muscle physiology and the concept of age-associated changes in human skeletal muscle mass and strength will be described. The effects of exercise and nutritional supplementation as interventions to prevent early onset of sarcopenia will also be reviewed along with the benefits of using novel deuterium oxide tracer techniques over traditional amino acid tracer previously used for determining muscle protein metabolism.

1.2. Structural organization of skeletal muscle:

Skeletal muscle is a form of striated muscle tissue and regarded as the organ of the muscular system. Each individual muscle is composed of myocytes and connective tissue supplied by nerves and blood vessels. Skeletal muscles are under the voluntary control of the somatic nervous system. Skeletal muscles vary in their size, shape and arrangement of fibres. In terms of size, they may be as small as the stapedius muscle of middle ear or as large as the muscles of thigh. Similarly, in terms of arrangement, muscle fibres are oriented in parallel to the long axis of the muscle in some cases and obliquely in others.

Each muscle cell is long and spindle shaped. Each individual muscle fibre is made of thousands of muscle fibres packed together and wrapped in a covering of connective tissue. The sheath of connective tissue which conceals each muscle is called the epimysium. Fragments of the epimysium protrude inwards and divide the muscle into further compartments. Each compartment contains muscle fibres organized in individual bundles called fascicles which are surrounded by a layer of connective tissue called perimysium (Purslow 2002; Trotter & Purslow 1992). Inside the fasciculus, each

individual muscle fibre is bounded by a thin connective tissue layer of collagen and reticular fibres called the endomysium (Rowe, 1981) (Figure 1). These connective tissue coverings provide support and protection to skeletal muscles and enable them to endure the powerful muscle contractions. They provide a medium for oxygen and nutrients to diffuse from capillaries to muscle tissues. The sheaths of collagen fibres extend further than the belly of the muscle and are attached to the bones by assembling in a rope-like fashion; as tendons (Kjær 2004).

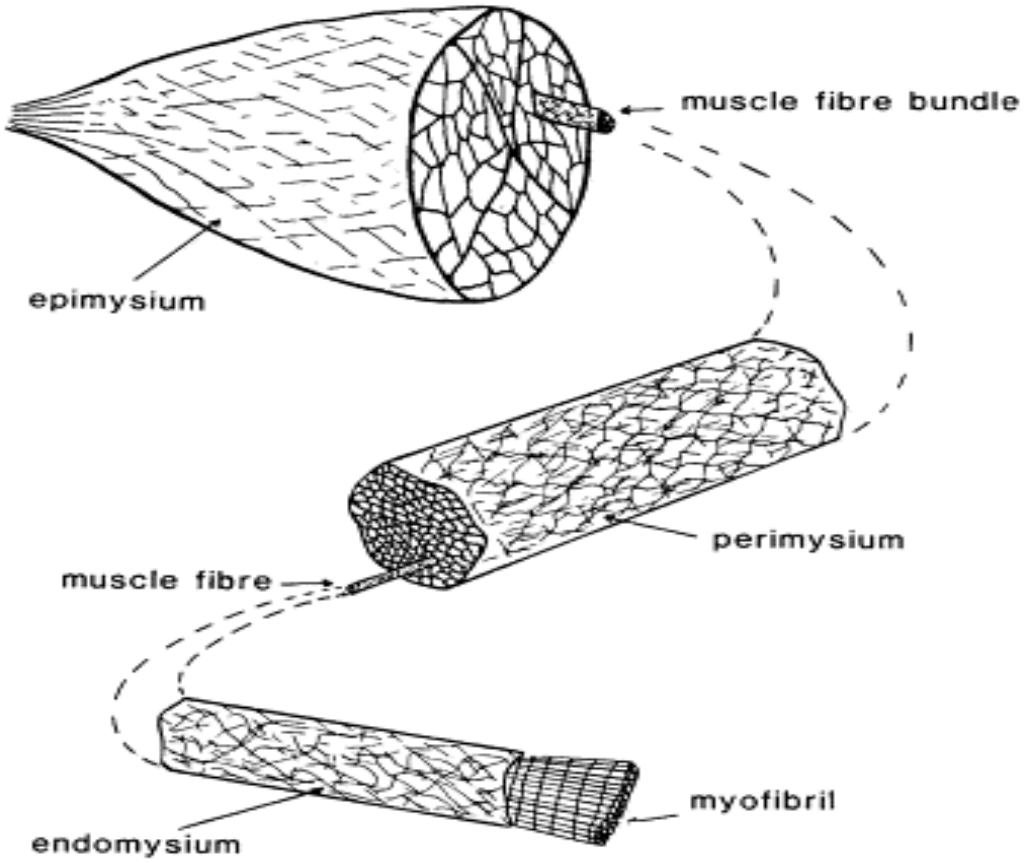


Figure 1 Schematic diagram to show the arrangement of the intramuscular connective tissue within skeletal muscle. Taken from (Purslow, 2002), with permission from Elsevier.

Skeletal muscle is composed of many fibres, ranging the whole length of muscle, between 10-100 μm in diameter and from a few millimetres to 3 cm or more in length (Rodrigues & Rodrigues 2010).

In addition, each of the muscle fibres is supplied by one nerve ending only. A thin plasma membrane called sarcolemma ensheathes the muscle fibres. The multi nucleated and cylindrical muscle fibres have nuclei located at the periphery of fibre and have numerous mitochondria which produce large amounts of adenosine triphosphate (ATP). A continuous supply of ATP is necessary for muscle contraction. Each muscle fibre is further composed of hundreds of myofibrils; a combination of specialized protein molecules called thick (myosin) and thin (actin) filaments arranged in series and in parallel; that are responsible for the contraction of muscle. The thin filament comprises of actin and two regulatory proteins, troponin and tropomyosin (McComas, 1996).

In order to bring about contraction, myosin and actin filaments partially overlap in a controlled manner forming units called sarcomeres (Figure 2); the functional units of a muscle fibre. Each individual sarcomere is formed of dark and light bands and separated from each other by dense material called Z-discs. Sarcomeres are arranged in a sequential order from one end of the muscle fibre to other (one Z-line to the next); giving the muscle a striated appearance. The light bands containing actin filaments are termed the I-band as it is isotropic to light. The dark bands, however, are a combination of myosin filaments along with the ends of actin filaments overlapping them forming A bands which are anisotropic to polarized light. The H-band is the region at the centre of A band where only thick filaments are present.

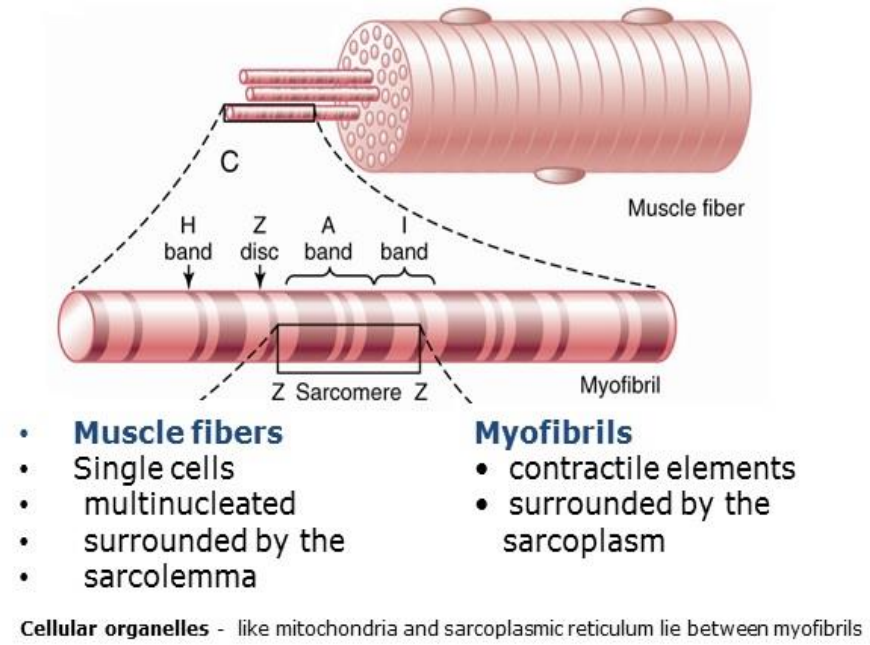


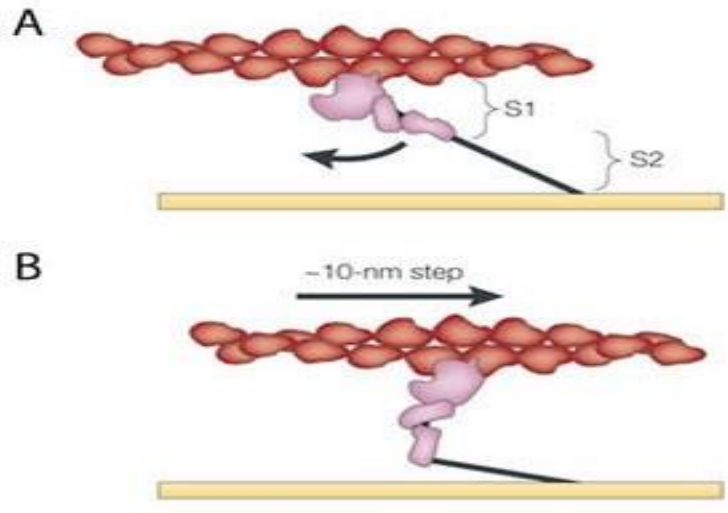
Figure 2 Cellular organization of sarcomere structure within skeletal muscle. Adapted from the "Textbook of Medical Physiology" by John. E Hall with permission from Elsevier.

Hundreds of myofibrils make up each muscle fibre. However, there are three basic types of protein that make up a myofibril. These are contractile, regulatory and structural. The two contractile proteins, myosin and actin, are responsible for generating force of muscle contraction by using energy from the ATP molecules. Hence, the myosin protein is also termed as motor protein.

1.2.1. Cross-bridge cycling:

Skeletal muscles contract by a mechanism known as cross-bridge cycling. It refers to the combination of myosin with actin in the presence of ATP within the muscle cell which brings about force of contraction. Myosin protein has a globular head close to actin

filament. Myosin heavy chain contains an ATP binding site and an enzyme (ATPase) which hydrolyses ATP to adenosine diphosphate (ADP) and inorganic phosphate; producing energy for muscle contraction. The bending of the myosin head explains the way myosin "walks" along actin. It has multiple hinged segments which can bend, in the presence of energy derived from ATP, and draws the actin filament closer to M-line bringing about muscle contraction (Spudich 2001). The myosin filament is regularly described as 'two golf clubs twisted together' which represent the myosin heads or cross bridges. The myosin cross bridge has attachment spot for both actin and ATP. The two regulatory proteins are troponin and tropomyosin which are responsible for turning muscle contraction "on and off". This repeated attachment and detachment of the myosin head (S1 region) with actin is known as myosin-actin cycling (Figure 3). As myosin binds and release actin, it forms cross bridges extending from the thick (myosin) filament to thin (actin) filament. The contraction of myosin head region is called the "power stroke" movement requiring hydrolysis of ATP. ATP is converted to ADP thereby providing energy to myosin to undergo contraction by binding actin. However, it stays bound to actin until another ATP molecule binds to the myosin, modifying its conformation and the same process is repeated again.



Actin (red) interacts with myosin, shown in globular form (pink) and a filament form (black line). The model shown is that of H. E. Huxley, modified to indicate bending (curved arrow) near the middle of the elongated cross bridge (subfragment 1, or S1) which provides the working stroke. This bending propels actin to the right approximately 10 nanometers (10 nm step). S2 tethers globular myosin to the thick filament (horizontal yellow line), which stays in place while the actin filament moves.

Figure 3 - The "power stroke" of swinging cross-bridge model. Taken from *(Krans, 2010)*, with permission from Nature Publishing Group.

1.2.2. Muscle fibre types:

Skeletal muscles are broadly classified as fast and slow fibres, on the basis of their shortening speed (McComas, 1996). This classification also correlates with the morphological difference as the fast muscle fibres appear white while the slow fibres appear red owing to a higher myoglobin (an iron-containing transport protein in red blood cells) content (Wittenberg 2003). However, on the basis of histochemical analysis (Engel 1962) skeletal muscle fibres are divided into three major types of fibres: type I (slow-twitch and fatigue-resistant), IIa (fast-twitch and moderate fatigue resistant) and IIx (fast twitch and non-fatigue resistant) (Ten Broek et al. 2010). There is considerable variation in the fibre-type composition in human muscle which explains their noteworthy variation in exercise performance. Interestingly, during 1970s, studies were

conducted to determine muscle fibre composition of successful athletes based on the belief that muscle fibre-type can predict the performance of athletes. It was found that endurance athletes have more slow-twitch fibres as compared to fast-twitch (Gollnick et al. 1972) while the musculature of sprinters is composed of largely fast-twitch fibres (Costill et al. 1976).

With regards to old age, it has been shown that muscle wasting or atrophy occurs due to perturbation of dynamic balance between fibre biosynthesis and degradation (Wang & Pessin 2013). A number of pathophysiologic conditions such as aging, cancer cachexia, sepsis, diabetes, heart failure and kidney dysfunction are characterised by reduction in muscle mass. Owing to their distinct physiological character and metabolic activity, different skeletal muscle fibre types respond differently to these atrophy signals. For example, it has been demonstrated that type II fibres predominate in aging, cancer cachexia and diabetes as compared to type I fibres (Schiaffino & Reggiani 2011). In fact, selective atrophy of type II muscle fibres is considered a possible mechanism for loss of muscle mass and strength in older age (Lexell 1989). Therefore, the knowledge of muscle fibre type distribution is important for our understanding of stimuli influencing the skeletal muscle mass and the potentially useful effects of promoting muscle fibre type switching in the prevention and treatment of metabolic diseases. Resistance exercise is one of the interventions that might encourage fibre type switching.

1.3. Overview of Sarcopenia

The term sarcopenia was first coined by Rosenberg in 1989 (Rosenberg 1989) and it was derived from Greek word “sarx” meaning flesh and “penia” referring to poverty or deficit of

(Rosenberg 1997). It is a progressive degenerative age-related phenomenon of declines in skeletal muscle mass, quality and strength (Lindle et al. 1997). Additionally, there is also a decrease in functional efficiency (strength per unit of muscle mass) with aging (Moller et al., 1995). The purpose of identifying a term for this phenomenon was to generate scientific interest in this area. Now, the literature is awash with thousands of peer-reviewed articles, establishing it as a keyword and identifying evidence-based principles suggesting further avenues of research. One of the reasons for increased interest in establishing a pathogenesis of sarcopenia is that it is a leading cause of disability, which is subsequently responsible for hefty economic costs owing to special care needed at home, nursing home institutionalization and hospitalization of elderly adults (Schneider et al., 1990).

Sarcopenia is considered a geriatric syndrome along with cachexia, frailty, delirium, falls and sarcopenic obesity (Cruz-Jentoft et al. 2010) all of which are complex and poorly defined clinical terms and that could not be assigned to any specific disease category (Visser 2009). Geriatric syndromes are poorly defined, complex and costly conditions reflective of compromised health in elderly (Cruz-Jentoft et al. 2010; Inouye et al. 2007)

1.3.1. Definition and classification of Sarcopenia

Owing to a growing interest in aging research, a number of definitions have been developed for sarcopenia but none of them have gained universal acceptance (Visser 2009). When first described by Rosenberg, the term signified the loss of lean mass in healthy aging individuals (Rosenberg 1989).

Sarcopenia is now considered as a degenerative syndrome leading to a generalized deficit of muscle mass and strength leading to possible detrimental consequences such as impaired mobility, physical

disability and increased mortality (Delmonico et al. 2007; Goodpaster et al. 2006) with advancing age. Baumgartner and colleagues were the first to define sarcopenia in 1998 based on studies presenting that mass of the arms and legs (free of fat and bone) estimated by dual-energy x-ray absorptiometry (DXA). DXA can be used to estimate the appendicular skeletal muscle mass (ASM). ASM weight (kg)/ height^2 (m^2) was calculated as an index of relative skeletal muscle mass. It is vital to measure relative muscle mass to define sarcopenia as absolute muscle mass is associated strongly with height. And according to Baumgartner and colleagues, the term sarcopenia refers to 'a deficiency in relative muscle mass'. (Baumgartner et al. 1998).

A practical definition and classification for age-related sarcopenia was recently proposed by the European Working Group on Sarcopenia in Older People (EWGSOP) (Cruz-Jentoft et al. 2010). It comprises an international working group of scientists from four European scientific organisations who defined it as "age-associated loss of skeletal mass and function" consequently leading to a risk of detrimental effects such as physical debility, disease and ultimately death (Cruz-Jentoft et al. 2010).

Sarcopenia is a multifactorial syndrome owing to a wide variety of factors influencing its development and progression. This includes age-related sarcopenia (which remains the primary form) and neuro-degenerative, nutritive, activity-related and endocrine related sarcopenia (secondary forms) among the most common factors (Cruz-Jentoft et al. 2010).

Primary form of sarcopenia has no other identifiable cause except aging, which is inevitable (Morley et al. 2001). The secondary form of sarcopenia, however, has a number of modifiable risk factors as it can be a consequence of a sedentary lifestyle (Evans & Suppl 1997),

malnutrition and gastrointestinal (Prado et al. 2008), inflammatory (Visser et al. 2002; Schaap et al. 2009) or endocrine diseases (Feldman et al. 2002).

For aiding in the practical clinical management of the condition, The European Working Group on Sarcopenia in Older People (EWGSOP, came up with the staging of sarcopenia as "1. Pre sarcopenia", "2. Sarcopenia" and "3. Severe sarcopenia". The 'pre sarcopenia stage' is representative of a decline in lean muscle mass without any effect on physical well-being. The 'sarcopenia stage' is reflective of a decline in muscle strength or performance in addition to a diminishing lean mass. 'Severe sarcopenia' occurs when all three factors (mass, strength and performance) are deteriorating simultaneously (Cruz-Jentoft et al. 2010).

There can be multiple complex mechanisms mediating sarcopenia but neuropathy (pathological involvement of both central and peripheral nervous systems) remains the most important of them (Narici & Maffulli 2010). Other considerable factors include dysregulation of anabolic hormones (insulin, growth and sex hormones) with advancing age (Feldman et al. 2002; Walrand et al. 2011), ectopic deposition of fat and adipose tissue content (Goodpaster et al. 2001), genetics (Phillips et al. 2013) , increased fatty infiltration of muscle and declines in neuromuscular junctions (Walston 2012). It is, hence, a multifactorial but poorly understood phenomenon.

Although the exact mechanisms responsible for sarcopenia remain unresolved yet; however, it is thought to be a result of chronic imbalance between muscle protein anabolism and muscle protein catabolism over years. Furthermore, the musculoskeletal decline, marked by the atrophy of muscle fibres [type II fibres in particular (Lexell et al. 1988)], is also discussed in the background of satellite

cells (SCs). The satellite cells are mononuclear undifferentiated cells that are found in skeletal muscle fibres and promote muscle growth, repair and regeneration. Whereas a few scientists report that numbers of satellite cells decline with old age (Kadi et al. 2004); others have shown them to remain unaltered (Dreyer et al. 2006). The performance of a satellite cell may also decline with aging which can be a likely contributing factor to sarcopenia (Yablonka-Reuveni 2011).

So, the role of muscle protein turnover and satellite cells (SCs) is probably important in the backdrop to aging. Another major limitation to study this phenomenon is the fact that it is a slow and progressive process, in contrast to the rapid muscle declines in disease (like cancer & cardiac cachexia) (Williams et al. 2012; Tisdale 2009), with metabolic dysregulation occurring over a period of years, instead of weeks. So, the establishment of novel sensitive techniques to determine muscle protein metabolism accurately remain crucial to understand the regulation of sarcopenia and develop or assess the efficacy of interventions to slow down its progression.

1.4. Normal Regulation of Muscle Protein Turnover

Studies have shown that sarcopenia has a negative impact on the rate of contractile protein synthesis as human beings lose lean protein tissue with advancing age (Cohn et al. 1980) and is replaced by adipose tissue. MPS is positively regulated by nutrition (Volpi, Kobayashi, Sheffield-moore, et al. 2003) and exercise (Schulte & Yarasheski 2001) and older adults retain the ability to respond to these interventions (Parise & Yarasheski 2000). Muscle protein turnover is tightly regulated in normal circumstances in healthy, active individuals with a turnover rate of 1-2% day-1 (Atherton &

Smith 2012) and a dynamic equilibrium exists between MPS and MPB (Phillips et al. 2012). In the following sections, the effects of nutrition and exercise on muscle protein homeostasis in older adults are discussed in detail.

1.4.1. Effects of nutrition on muscle protein homeostasis in old age:

In healthy human beings, muscle protein mass is efficiently regulated by maintenance of a strict balance between MPS and MPB. In the post-absorptive state, the rates of MPB are greater when compared to MPS. As a result, there is a negative net protein balance leading to loss of muscle protein. However, after intake of food (subject to sufficient protein or amino acid in diet), this negative balance is briefly reversed (i.e., MPS is increased and MPB is inhibited such that MPS>MPB and a positive balance exists in muscle) resulting in the neutral net protein balance every day (M. S. Brook, Wilkinson, Phillips, et al. 2015). The mechanisms responsible for anabolic effects of nutrition include the stimulation of MPS (Volpi, Kobayashi, Sheffield-moore, et al. 2003) and the suppression of MPB (Wilkes et al. 2009). The most important nutrients to stimulate protein synthesis are essential amino acids (EAAs) with the branched chain amino acids particularly leucine having the most effective anabolic effect (Dickinson et al. 2014). However, these nutrient-driven effects are found to be of short-lived nature (1.5-2 h) turning off afterwards regardless of the continually elevated amino acid supply (Atherton & Smith 2012). Rennie and colleagues reported that MPS is markedly increased (~2.8 fold) 1.5 hours after a primed constant infusion of amino acids before falling to a basal value in the succeeding 4 hours despite continued AA availability (Bohe et al. 2001). This is termed as the 'muscle full' effect (Atherton et al.

2010) although the exact mechanisms regulating this effect are poorly understood.

It has been stated that insulin promotes the net muscle protein anabolism and it exerts this effect by inhibition of muscle protein breakdown rather than by increasing protein synthesis (Gelfand & Barrett 1987). Greenhaff and colleagues established that increasing amino acid and insulin delivery can improve muscle protein anabolism via a stimulation in MPS (at all insulin concentrations i.e., post-absorptive 5mU/l, postprandial 30 and 70mU/l and physiologically high 170mU/l) but inhibition of MPB was observed only by increasing insulin significantly above post absorptive levels (i.e., 30mU/l) (Greenhaff et al. 2008). It has also been reported that in the presence of moderate insulin availability (15mU/l), the effect of insulin on MPB is blunted in older individuals with only a 12% fall in MPB as compared to a 47% fall in younger subjects (Wilkes et al. 2009).

Although metabolic responses to nutrients are tightly regulated, a fall is observed in muscle mass after 50 years of age. This demonstrates that the intricate balance between MBS and MPB is dysregulated in older age. Initially it was believed to be due to reductions in the rates of post-absorptive MPS in older subjects (Welle et al. 1995) but others (Kumar, Selby, et al. 2009a; Kumar et al. 2012) have established that the post-absorptive rate of MPS between younger and older adults is identical. This has led to the quest for an alternative explanation of the mechanisms affecting MPS in older age. The phenomenon of anabolic resistance has been described in this context which shows that the rise in MPS after food intake is less in older adults as compared to younger ones (Symonsi et al. 2011; Moore et al. 2014). Therefore, greater protein dosages may prove to be beneficial in older people to acquire comparable

anabolic responses as in younger adults. Some studies have also established that in older subjects, there is considerable decrease in sensitivity and responsiveness of MPS to EAA due to decreased (-30-50%) activation of the components of the amino acid signalling anabolic pathways (mTOR, p70s6 kinase, eIF4BP-1) and an increase in EAA intake to 40g does not increase MPS any further(Cuthbertson et al. 2005). Thus, in older people, this blunting of response to anabolic stimuli of nutrients (EAAs) may contribute to the development of sarcopenia in older age.

1.4.2. Effects of resistance-type exercise on muscle protein homeostasis:

As resistance exercise is an effective intervention to tackle muscle loss (Aagaard et al. 2010), there is a wealth of literature exploring the outcome of exercise on protein turnover. Indeed a single session of resistance exercise can lead to a two to three-fold increase acutely in muscle protein synthesis (Phillips et al. 1997; Kumar, Selby, et al. 2009b; Holm et al. 2010). Furthermore, this effect of exercise is also enhanced by protein intake along with RE (West et al. 2011).

However, it has been shown that the degree of acute response of muscle to RE is dependent upon workload and intensity with a 2-3 fold increase in MPS at intensities greater than 60% 1RM as compared to a reduced response at intensities of ≤40 1RM (Kumar, Selby, et al. 2009a). Some studies have also shown that lower intensity exercise can yield similar or even greater MPS response by increasing the volume of work (Kumar, Atherton, et al. 2009). It is thought that increased recruitment of type II muscle fibre (fast twitch fibre) due to fatiguing contractions leads to maximal fibre recruitment and MPS responses. This can be used as a means to induce muscle hypertrophy without performing heavy weight

exercise (Burd et al. 2010). It also shows the potential significance of fibre recruitment patterns for promoting muscle hypertrophy.

Immediately following high intensity exercise (to exhaustion) there is a latent period (up to 3 hours) where MPS is unchanged or even reduced and precedes a rapid rise in MPS (~3 fold), which is believed to be related to mechanical stress accompanying exercise (Cuthbertson et al. 2006). In a murine study, MPS was reduced during forceful muscle contraction in work-dependant manner (Atherton & Rennie 2006). After this period of latency is over, MPS rises sharply with a sustained increase up to 4h in fasted state (Kumar, Selby, et al. 2009a). However, this stimulation can be prolonged and may exceed to beyond 24 h period with a concurrent availability of amino acids (Cuthbertson et al. 2006).

There is also evidence of development of anabolic resistance to exercise with progression of age. For example, it has been reported that MPS was reduced in the aged individuals over a spectrum of intensities (20-90% 1RM) of resistance exercise, when compared to young (Kumar, Selby, et al. 2009a). Another study showed that aging blunts contraction-induced human skeletal muscle mTORc1 signalling and protein synthesis (Fry et al. 2011) providing further support of this view. Furthermore, these declines in MPS with ageing are not limited to resistance type exercise with some studies showing diminished MPS after endurance type exercise (walking) (Durham et al. 2010). Nonetheless, some studies have shown no difference in MPS responses in young and older people when resistance exercise is combined with protein intake (Symonsi et al. 2011) casting some doubt over the nature of anabolic resistance. However, some studies, which showed no blunting did not match data from young and aged individuals clearly as they did not use the same muscle fractions for comparison (Dreyer et al. 2008; Yang et

al. 2012; Witard et al. 2014; Dickinson et al. 2014). Whilst those who actually compared young and older individuals showing equal responses had primarily measured mixed muscle (Symonsi et al. 2011; Drummond et al. 2008). It is, however, established that when measuring myofibrillar protein synthesis; clear blunting is apparent (Kumar, Selby, et al. 2009b; Moore et al. 2014).

1.5. Emerging anabolic role of leucine and its metabolites on skeletal muscle metabolism:

The vital role of skeletal muscles in whole body protein synthesis can be ascertained from the fact that it is responsible for more than half of the total protein synthesis in a fed individual (Rennie et al. 1982). Additionally, evidence shows that increasing ingestion of amino acids is directly related to increased skeletal muscle protein anabolism (Wolfe 2002). There are nine essential amino acids important for humans and they must be included in diet as they cannot be synthesized endogenously. There are 20 amino acids which are incorporated into protein but the remainder are nonessential AAs and can be made endogenously. There are principally six amino acids (glutamate, aspartate, asparagine, valine, leucine and isoleucine) stimulating protein synthesis in human skeletal muscles (Norton & Layman 2006) with leucine being the most notable amongst all (Rieu et al. 2006; Layman 2002; Garlick 2005).

Substantial work has been carried out over the last two decades to demonstrate that supplementation with leucine and its metabolite, ß-hydroxy ß-methylbutyrate (HMB) is responsible for improvement in lean body mass and increase in muscle strength (Katsanos et al., 2006; Nissen et al., 1996; Volpi et al, 2003).

Buse et al. suggested in 1975, for the first time, that leucine has stimulatory effects on protein synthesis (Buse & Reid 1975). In early

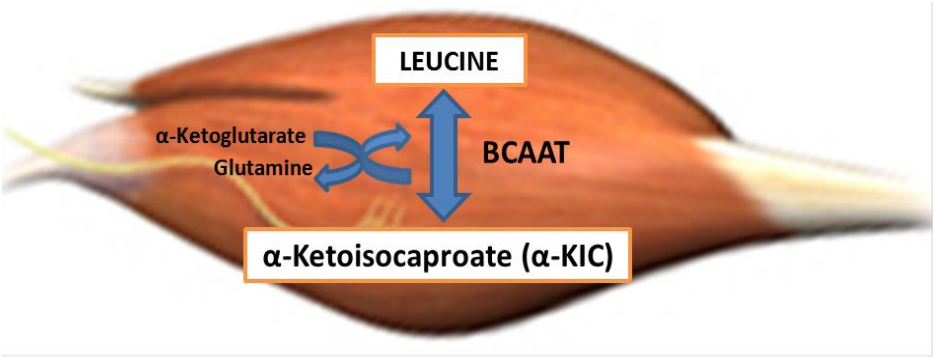
1980s, Tischler et al. (Tischler et al. 1982) put forward that leucine is a regulator of protein synthesis at lower concentrations (0.1mM) and it also inhibits proteolysis significantly at higher concentrations(0.2-0.5mM). Data from animal experimentation supports the human data that high levels of leucine stimulate protein synthesis and inhibit their degradation in rat skeletal muscles (Anthony, Anthony, et al. 2000; Crozier et al. 2005; Garlick 2005).

1.5.1. Metabolism of Leucine:

Leucine is a branched chain amino acid which must be taken in daily diet as it is not synthesized in humans. It is of particular interest among all BCAAs owing to its dynamic physiological roles in function of mitochondria, ketogenesis and insulin action (Sun & Zemel 2007; Teegarden 2003). It also acts as a muscle energy substrate and plays a significant role in the control of protein synthesis and regulation of cellular metabolism (Norton & Layman 2006).

Once ingested, leucine is metabolized in mitochondria of muscles as liver has no BCAAT enzyme, hence leucine ends up in the periphery at muscle. It is metabolized to α-Ketoisocaproate (KIC) by transamination in the presence of enzyme branched-chain amino acid transaminase (BCAAT) in the muscle. Further metabolism occurs in the mitochondria of liver whereby KIC is oxidized to isovaleryl-CoA in the presence of enzyme branched-chain keto acid dehydrogenase (BCKAD). Isovaleryl CoA is further metabolized to HMG-CoA which generates acetoacetate and acetyl-CoA inside liver mitochondria (Nissen & Abumrad 1997).

Another pathway of leucine metabolism shows that α-Ketoisocaproate (KIC) is oxidized in liver to ß-hydroxy ß-methylbutyrate (HMB) by the enzyme KIC dioxygenase (Sabourin & Bieber 2000).Thus, HMB is almost entirely synthesized endogenously from the catabolism of leucine (Figure-4).



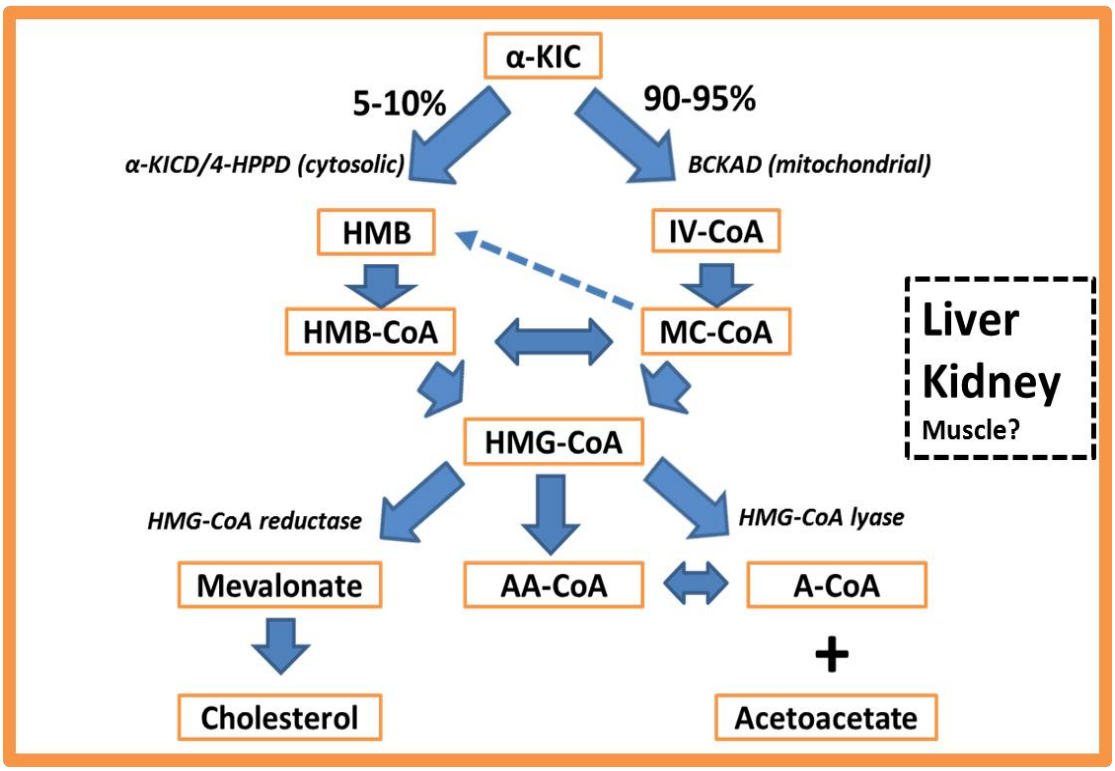


Figure 4 Pathways of Leucine Metabolism: Transamination of Leucine to α-Ketoisocaproate in mitochondria of skeletal muscles. And oxidation of α-KIC in liver to ß-hydroxy ß-methylbutyrate (HMB). Taken from (Wilkinson DJ, 2013).

1.5.2. Role of leucine in protein synthesis:

Although it is established that leucine promotes protein synthesis (Suryawan et al. 2012); its inhibitory effects on muscle catabolism during intervals of caloric deprivation and long-term endurance exercise training are also documented (Mortimore & Pösö 1987; Vazquez et al. 1985; B.K. et al. 2007).

In early 90s, Yarasheski et al demonstrated the effects of leucine on MPS by prime constant infusion method and concluded that the skeletal muscle fractional protein synthesis rates calculated by this method were comparable with published values determined using the conventional analytical approach (Yarasheski et al. 1992). Garlick et al. showed the stimulatory effects of leucine on MPS by using leucine as flooding dose which resulted in MPS values twice as high as prime constant infusion method (Garlick et al. 1997). In early 90s, Smith et al. showed the stimulatory effects of leucine on muscle protein synthesis by combining both the flooding dose and primed constant infusion methods (Smith et al. 1992).

The stimulatory effect of leucine on protein synthesis is believed to be mediated by activation of mammalian target of rapamycin (mTOR) pathway in skeletal muscle. It leads to the activation of S6 ribosomal protein kinase (S6K1) and eukaryotic initiation factor 4E (eIF4E) (Fumarola et al. 2005; Dann et al. 2007). Leucine can mediate protein translation by initiating signal transduction pathways in an insulin-dependent and rapamycin-sensitive manner (Xu et al. 2001). However, the exact mechanism for this activation remains unclear yet.

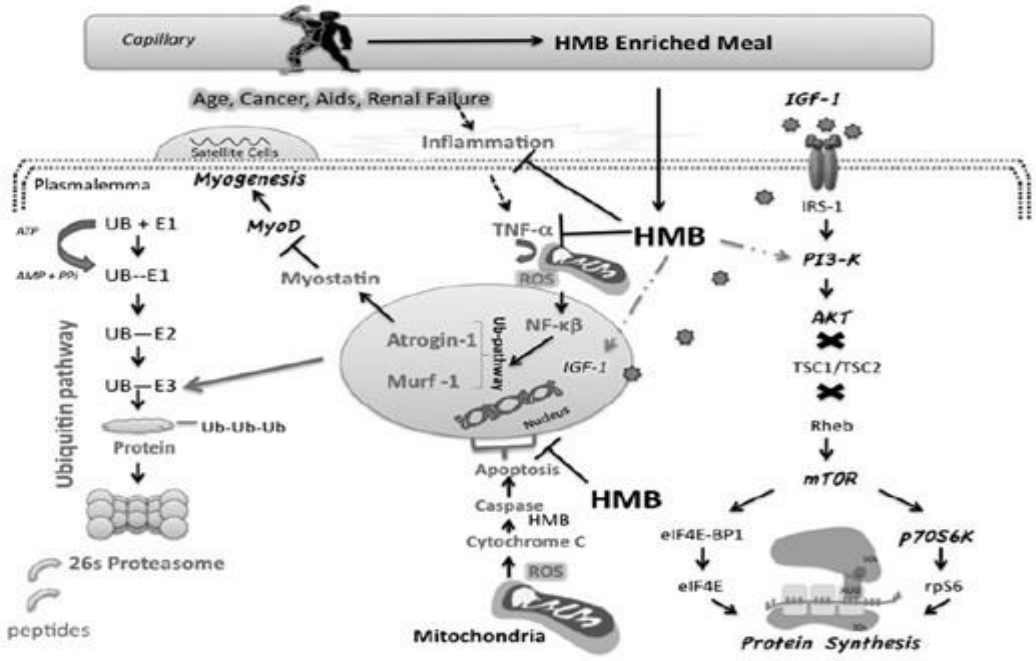
1.5.3. Metabolism of HMB:

HMB is synthesized by oxidation of α-Ketoisocaproate (KIC) by KIC dioxygenase in the liver. It is further metabolized in liver to HMG-CoA by either direct carboxylation or dehydration to MC-CoA first. The enzyme HMG-CoA reductase then acts upon it to transform it into mevalonate and eventually cholesterol. It is well established that HMB carbon is ultimately incorporated into cholesterol (Bloch et al. 1961; Stancliffe & Zemel 2012).

As a result of leucine metabolism, only a small amount of HMB (0.3-0.4g/d is produced endogenously (Fitschen et al. 2013). In addition

to being a metabolite of leucine, HMB is also naturally present in many foods like asparagus, catfish, cauliflower, avocado and grapefruit (Qiao et al. 2013). It is also commercially available as Calcium-HMB monohydrate salt. Another free acid form of HMB (HMB-FA) has been developed and it is increasingly used in studies owing to its improved absorption rate, better bioavailability and hence efficacy (Fuller et al. 2011; Fuller et al. 2015). We used free acid form of HMB in this study. HMB metabolism is summarized in Figure 5 taken from Fitschen and colleagues (Fitschen et al. 2013).

There is considerable evidence which confirms HMB is a cholesterol precursor with its carbon skeleton integrated into cholesterol (Nissen et al. 2000). However, the exact contribution of HMB carbon in formation of cholesterol is still not known. An alternative fate of HMB is excretion in urine. However, there is a difference in the clearance rates of Ca-HMB and the free acid form. We used the free acid form of HMB as it results in faster and greater plasma concentration and enhanced clearance (+25%) of HMB from plasma as compared to the Ca-HMB salt (Fuller et al. 2011).



Potential mechanisms by which HMB increases anabolic and decreases catabolic pathways in skeletal muscle. Italicized text refers to signalling pathways that are decreased in skeletal muscle cells of elderly and clinical populations as compared to healthy populations, while bold text refers to signalling pathways that are increased. HMB may increase protein synthesis by decreasing inflammation, increasing IGF-1 and protein translation through activation of mTOR. Additionally HMB may decrease protein catabolism through downregulation of ubiquitin proteasome pathway and reductions in caspase activity.

Figure 5 Effects of HMB on skeletal muscle metabolism taken from Fitschen et al (Fitschen et al. 2013). Reproduced with permission from Elsevier.

1.5.4. HMB consumption and dosage in humans:

HMB supplementation is known to bolster muscle health in a variety of human populations including athletes (Tenley & Stacy), the elderly (Fitschen et al. 2013) and clinical populations (e.g. cancer) (Zanchi et al. 2011). HMB is considered to be safe for consumption in humans wherein the high dosage of 6g/day for 1 month demonstrated no effect on hepatic or renal function, haemoglobin, cholesterol or lymphocytes (Gallagher et al. 2000b). However, some people, elderly women in particular, have reported experiencing mild symptoms at higher doses (4-6g/d) like nausea and dizziness immediately after intake. In most studies, an HMB dosage of 3g/d has been used (Wilson et al. 2008). Most researchers agree that this dosage is optimal e.g. Nissen (Nissen et al. 1996) reported that HMB supplementation of 3g/d in healthy individuals undergoing strength-training; improved strength in a dose-dependent manner. This view was not supported by Gallagher et al. (Gallagher et al. 2000a) who demonstrated a significant increase in body strength in strength-trained individuals who received 3g/d of HMB and did not report any additional improvement in strength in individuals receiving 6g/d of HMB supplementation.

Although these studies demonstrated no side effects in clinical and elderly populations following a short-term intake (Clark & Singh), its long term effects are still not known.

1.5.5. Role of HMB in protein synthesis:

HMB is known to modulate protein synthesis by augmenting phosphorylation of mTOR which is crucial to initiate translation (Eley et al. 2007). It also diminishes the induction of ubiquitin-proteasome proteolytic pathway leading to a decrease in protein breakdown (Smith et al. 2004). A number of clinical trials purport the ability of HMB consumption in increasing lean mass, reducing

muscle damage and improving muscle strength and function (Slater et al. 2015; Jówko et al. 2001; Flakoll et al. 2004). Lean body mass is calculated by subtracting fat weight from total body weight and constitutes about 75% of it (Demling 2009). The skeletal muscle makes up most of the lean mass and its conservation is essential in maintaining metabolic function, strength and mass (Wolfe 2006).

Considering the increased susceptibility of the elderly population to loss of muscle mass; studies have been conducted to monitor the effects of HMB supplementation in this population. But limited data is available on HMB supplementation in healthy older subjects. Hseih et al. conducted a study to determine the effect of HMB on body composition and protein metabolism in bed ridden elderly subjects receiving tube feeding. The subject were given 2g/d HMB supplementation for 28 days. It was observed that HMB supplementation increased weight and body mass index (Hsieh et al. 2010). Since, these changes were observed without any exercise training of the subjects; it is suggestive that HMB alone may increase lean muscle mass. Vukovich et al. carried out an 8-week study on elderly subjects with a mean age of 70 years. A randomized double blinded control trial was undertaken and subjects in HMB group received 3g/d of calcium HMB. All the subjects took part in exercise training 5 days a week. Lean body mass (LBM) of the participants was measured by skinfold calipers and DXA. An increase in fat free mass was observed in HMB group ($P=0.08$) compared to the control group (Vukovich et al. 2001a). Another study conducted by Flakoll and colleagues; investigated the effects of 12 weeks of HMB supplementation in elderly women living in nursing homes. They also reported a near significant increase in lean muscle mass ($P=0.08$) and significant improvement in functionality ($P=0.002$) (Flakoll et al. 2004). However, the subjects

in this study received supplementation of arginine (5g/d) and lysine (1.5g/d) in addition to HMB (2g/d). So, the sole effect of HMB cannot be ascertained by the results of this study. A year-long supplementation of HMB/arginine/lysine has been reported to improve lean mass but not muscle strength or function (Baier et al. 2009).

Recently, a randomized double-blinded control trial was conducted by Stout and colleagues in two phases, where HMB (3g/d) was supplemented in elderly men and women either alone (phase I) or in combination with resistance exercise training (phase II) for 24 weeks. They reported significant increases in HMB supplemented group in total weight, total lean mass, muscle strength and function in phase I of study. However, the RE training protocol in phase II of study resulted in increased lean mass and strength both with and without HMB. This data advocates that HMB supplementation can enhance muscle strength, function and body composition with or without resistance exercise training (Stout et al. 2013) however, there is no additive effect of HMB supplementation in the treatment group as both the groups provided same end result.

1.6. Application of stable isotope tracers - In quantification of muscle protein turnover:

Isotopes are atoms of elements having the same number of electrons and protons but differ with regards to number of neutrons in the nucleus. There are two main types of isotopes i.e. stable and radioactive ones. Stable isotopes do not decay into other elements while the radioactive ones do.

The less abundant stable isotopes of any element have additional neutrons inside their nucleus making them heavier and, hence, they can be easily distinguished using mass spectrometric techniques.

The stable isotope tracer approach is indispensable for the study of muscle protein turnover as it provides a measure of the rate of turnover and numerous amino acid tracers can be used concurrently to assess protein synthesis.

The utility of stable isotope lies in the fact that any process which involves protein, carbohydrate or lipid (Guan et al. 2014; Reichardt et al. 2011) metabolism can be studied by incorporation of heavy isotope motifs of carbon (^{13}C), hydrogen (^{2}H), nitrogen (^{15}N), or oxygen (^{18}O) into biological compounds (Patterson 1997).

The labelling of stable isotopes is, hence, an effective tool for studying metabolism in various model systems e.g. bacterial, fungal, animal and human studies (Lechene et al. 2006; Lane et al. 2011; Castro-Perez et al. 2011; Kluger et al. 2013; Reichardt et al. 2011).

Calculation of the muscle protein synthesis is traditionally accomplished by measuring the incorporation of labelled AA into the protein fraction and thereby assessing the fractional synthesis rate (FSR). Conventionally, direct incorporation of tracers like [^{13}C] leucine or [^{13}C], [^{15}N]-, or [^{2}H] phenylalanine has been used to quantify human skeletal muscle protein synthesis (MPS) by calculation of fractional synthesis rate (FSR) (Smith et al. 2007). A number of other isotope labelled amino acids (e.g., glycine, valine and proline) have also been used for the same purpose. This approach involves the amino acid being administered intravenously either as a primed constant infusion (i.e. a priming bolus of a known amount of labelled (tracer) is given and a constant infusion maintained to generate plateau enrichment) or as a flooding dose (i.e. a large bolus is provided over few minutes) (Garlick et al. 1980; Smith et al. 1998; Smith et al. 1992). Whilst these AA tracer methods are considered 'gold standard' approaches for the

quantification of MPS; they have various limitations as well (MacDonald et al. 2013). These include invasive blood sampling, employing venous (and arterial) cannulations, sustained bed rest (4-8 hours), preparation of sterile IV tracer infusions, pharmacy costs and associated multiple tissue biopsies. This makes them difficult to apply in some clinical or frail populations affected by chronic diseases or aging e.g. elderly individuals, patients in critical care, CKD, cancer cachexia, younger children and adolescents.

A significant drawback is the requirement to be infused intravenously which makes them inconvenient and unsuitable for long term i.e. >8h use in chronically ill patients or even healthy volunteers. Additionally, arteriovenous cannulations (which might be needed in some cases) incur high clinical costs and must be carried out in controlled study environment. This confines the natural activity levels of the subjects as well. Thus, the possibility of conducting study in subjects under free living conditions over protracted periods of time is not practical with this AA tracer procedure (Gasier et al. 2010). Furthermore, these infusion protocols are performed over short time periods (usually 8-12 hours) to measure acute responses but their utility is limited when it comes to long-term and chronic measurement of the consequence of diseases such as chronic kidney disease or cachexia associated with cancer and/or quantifying responses to resistance exercise training and nutritional supplementation which are employed over periods of several weeks to months. All of these factors make their use restricted and hence provide the impetus to look for other potentially more suitable means of labelling amino acids. Hence, the recent re-introduction of deuterium oxide (D_2O, or "heavy water") to circumvent these underlying limitations of traditional tracer

approaches (Wilkinson et al. 2014) although we still need biopsy with D_2O.

The stable isotope of deuterium was first discovered by Harold Urey in 1931 (Urey et al. 1932; Urey 1931). In the following decade, Schoenheimer and Rittenberg pioneered the development of approaches to study metabolism of organic compounds using deuterium labelled tracers (Schoenheimer & Rittenberg 1935; Schoenheimer & Rittenberg 1940). These isotope approaches have been extensively used recently due to advances in mass spectrometric instrumentation and to develop a detailed understanding of metabolic pathways involved in lipid, carbohydrate DNA or protein metabolism. Figure 6 (Matthew S. Brook et al. 2015).

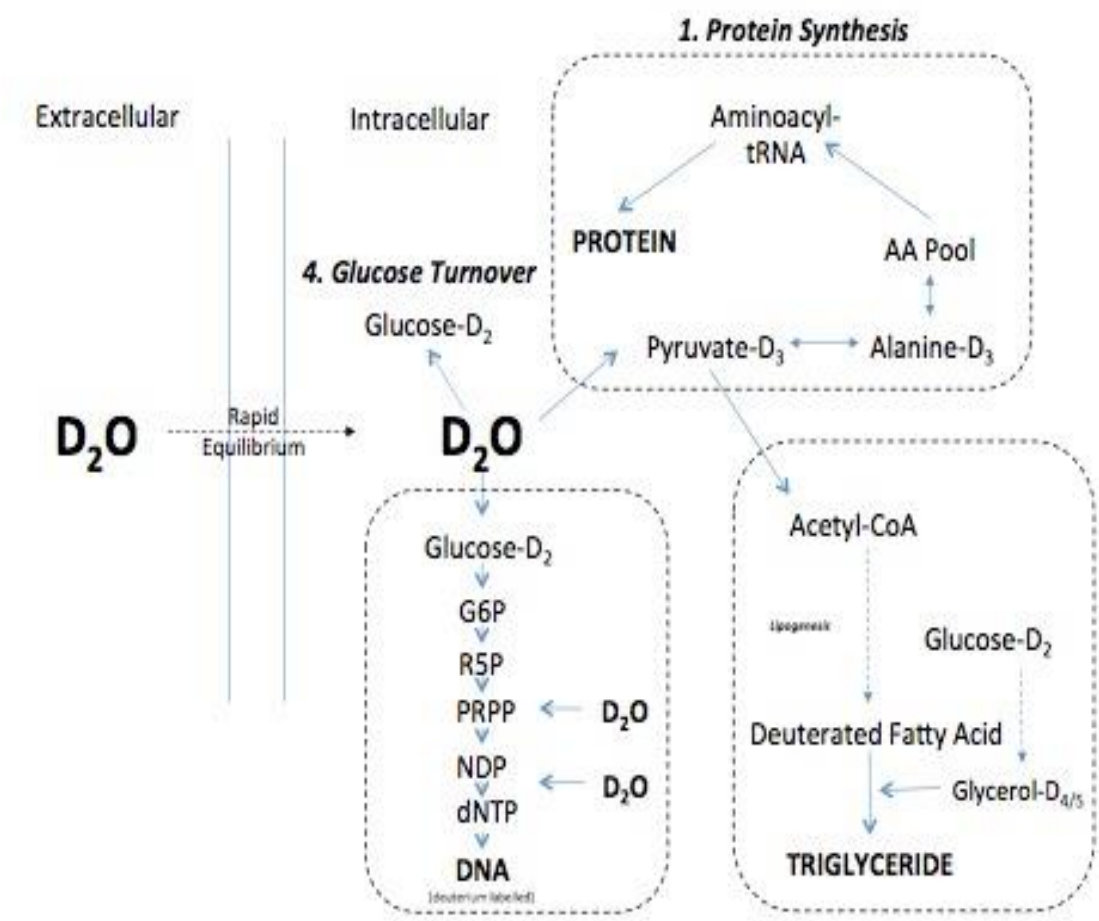


Figure 6 - Illustration of pathways for the incorporation of deuterium from D_2O into multiple tissue pools. Intracellular cycling of alanine to pyruvate results in deuterium incorporation from body water at α and β hydrogen's, with an enrichment ~3.7x body water. This labelled alanine will mix with the alanyl-tRNA pool and be incorporated into new muscle proteins during protein synthesis. As D_2O is incorporated into multiple pools, nucleotides and triglycerides, multiple aspects of muscle metabolism can be measured. G6P Glucose 6 Phosphate, R5P Ribose 5 Phosphate, PRPP Phosphoribosyl Pyrophosphate, NDP Nucleotide Diphosphate, dNTP Deoxy Nucleotide Triphosphate and D_2O (Deuterium Oxide). Taken from (Matthew S. Brook et al. 2015). Reused with permission from Taylor & Francis.

Over the past two decades, a number of researchers have employed these techniques to quantify protein turnover (synthesis-breakdown) responses in human skeletal muscle in response to resistance exercise and amino acid consumption (Gasier et al. 2010; Robinson et al. 2011; MacDonald et al. 2015a; MacDonald et al. 2013; Wilkinson et al. 2014; M. S. Brook et al. 2015) These methodologies are fundamental to extend our knowledge of the regulatory pathways governing, specifically, skeletal muscle mass (Atherton & Smith 2012) and for providing insight into mechanisms of sarcopenia generally.

Recent work by Wilkinson and colleagues established that deuterium oxide tracer can be successfully used to robustly measure MPS and, hence, assess day to day changes (Wilkinson et al. 2014) as well as acute (~3-6 h) changes (Wilkinson et al. 2015) in synthesis of human muscle protein. Previously, researchers have employed the same technique to determine MPS in humans over 1 to 2 days (Gasier et al. 2012) and longer term over 8 weeks (Robinson et al. 2011). D_2O is more practical method for measurement of long term MPS, as it is consumed orally, making it far less invasive and more economical, obviating the need for repeated intravenous tracer infusions rendering it suitable for elderly subjects (Robinson et al. 2011) and patients with chronic metabolic disease (e.g. Chronic kidney disease and cancer) (Previs et al. 2004; MacDonald et al. 2015b). Although we need to perform muscle biopsies with D_2O, it is still a practically useful alternative to the traditional AA tracer approaches.

The aim of preserving muscle mass and physiological functions in elderly is a well-acknowledged goal and is crucial to maintaining healthy lifespan in an ever ageing demographic population. However, the multifarious metabolic role of skeletal muscle during

the life course and its impact in precluding common and progressively predominant clinical conditions like diabetes, cancer cachexia and obesity are less well documented. Therefore, it is imperative we conduct studies to gain insight into the physiological role of skeletal muscle and the effects of potential interventions like resistance exercise training (RET) and nutritional supplementation on maintaining muscle mass, function and metabolic health. Resistance exercise training is the most practicable means for improving and conserving muscle mass in elderly (Häkkinen et al. 1998; Venturelli et al. 2012) and clinical populations (Pollock et al. 2000). Similarly, sufficient protein consumption is considered indispensable for healthy aging (Houston et al. 2008; Volpi, Kobayashi, Sheffield-Moore, et al. 2003). To understand changes in muscle mass, the quantification of muscle protein synthesis is considered vital; the unique ability of D_2O tracer for quantifying MPS chronically in free living humans has been objectively documented and can provide previously unavailable insight into the regulation of muscle protein turnover in aging and disease.

1.7. Aims and Hypothesis:

The aim of this study is to determine the effect of HMB supplementation and exercise on MPS in elderly males. We aimed to evaluate the effects of these interventions by:

1- Determining myofibrillar protein synthesis using stable deuterium oxide (D_2O) labelled amino acids incorporated in muscle protein with Gas Chromatography-Pyrolysis-IRMS.
2- Assessing muscle thickness (MT) of vastus lateralis (VL) using Ultrasonography (USG) and regional body composition using Dual-energy X-ray absorptiometry (DXA).
3- Determining muscle function by measuring maximal voluntary contraction (MVC) using an isokinetic dynamometer.
4- Ascertaining the alterations in expression of genes implicated in catabolic and anabolic signalling pathways regulating skeletal MPS, as a result of these interventions.

Considering the known anabolic effects of HMB on skeletal muscle metabolism, we hypothesized that provision of HMB along with resistance exercise will lead to an increase in muscle protein synthesis leading to muscle hypertrophy in elderly human subjects. Thereby, improving the functional capabilities of muscle and provide us with an effective tool to combat age-related muscle loss, chronic metabolic diseases and disability.

Chapter Two - Material and Methods:

2.1. Study Design: Subject Characteristics and Ethics:

Ethical approval was obtained from the University of Nottingham Medical School Ethics Committee (United Kingdom), with the study following guidelines set forth by the Declaration of Helsinki. 16 healthy older men were recruited and randomly assigned (8 per group- Treatment Group; n=8, Age 68±1.1y, Height 1.76±0.02 m, BMI 28±1.2 $kg.m^{-2}$: Placebo Group; n=8, Age 68.5±1.6y, Height 1.74±0.02 m, BMI 27±0.9 $kg.m^{-2}$) to conduct a randomised placebo controlled trial via letters of invitation (sent to addressees nearby our study centre). All volunteers were screened by means of a medical questionnaire, resting ECG, physical examination, with the exclusions for metabolic, respiratory, and cardiovascular disorders or other symptoms of ill health. Subjects had normal blood chemistry, were normotensive (BP <140/90) and were not prescribed any medications; all subjects performed activities of daily living and recreation. They were not involved in regular resistance exercise training. They gave their written, informed consent to participate after all procedures and risks were duly explained.

2.2. Study Procedures:

All older subjects were randomly assigned (8 per group) to either a placebo (calcium carbonate) or supplement group [3.4g/ of free-acid ß-hydroxy ß-methylbutyrate (HMB) solution]. This is designed as a pilot study to determine the effects of supplementation in

muscle protein synthesis using the novel deuterium oxide tracer technique. Both groups underwent unilateral RET for 6 weeks.

A week before the start of the intervention, a pre-study visit was conducted in which strength was assessed for the training leg using an isokinetic dynamometer along with the assessment of their one-repetition maximum (1-RM). The training leg was randomly selected among the subjects. Additionally, muscle architecture of both legs was also assessed by ultrasound. The same measurements were also repeated at 3 weeks and 6 weeks. Subjects were asked to maintain their normal nutritional intake and record a 4-day diet diary. This was then repeated at week 5 to monitor dietary intake during the study. All volunteers were asked to attend our lab 3 times per week for 6 weeks to perform a unilateral resistance exercise training regime (RET) involving 6x8 repetitions at 75% 1 repetition maximum (1RM). On day 0, subjects underwent DXA and ultrasound scans of both legs to determine baseline body composition and muscle architecture (muscle thickness). A biopsy from both legs and saliva samples were taken on day 0 to establish basal isotope labelling of protein fractions and body water. In addition, venous blood samples were collected into lithium heparin coated tubes, immediately cold centrifuged at 1,750 g for 20 mins, with plasma fractions aliquoted and frozen at -80 ºC until analysis. All subjects were then asked to attend our lab 3 times/week to undertake a 6-week unilateral RET program with 1-RM assessments of the trained leg every ~10 days to ensure progressive intensity. They consumed an initial priming dose of D_2O on day 0 (200 ml), with the aim to label body water pool to 0.2% APE and enrichment maintained in a pseudo-steady state by a top up after 4 weeks with another bolus of 200ml D_2O (70 atom%; Sigma-Aldrich, Poole, UK). Post RET, subjects were asked to collect another small sample of

saliva in provided sealable tubes, ~4 h post first dose then thrice weekly (collected at midday ≥30 min after the last meal or drink) thereafter to monitor body water enrichment throughout the study. An extra sample was taken ~3h after second 200ml D_2O bolus in week 4 to ensure body water enrichment was accurately represented. The samples were collected in sterile plastic tubes and kept refrigerated, and participants were asked to bring these to each training session. Upon receipt, they were immediately cold centrifuged at 16,000g to remove any debris that might have been present; they were then aliquoted into 2ml glass vials and frozen at -20ºC until further analysis.

Further DXA, ultrasound scans and venous blood samples were taken from volunteers at weeks 3 and 6 in order to determine temporal changes in leg muscle mass and architecture. Bilateral muscle biopsies (exercised and non-exercised) legs were taken at weeks 2, 4 and 6 under aseptic conditions from vastus lateralis muscle 60-90 mins (75 ±2 min) after unilateral exercise by using the conchotome biopsy (Dietrichson et al. 1987) technique with 1% lidocaine (B. Braun Melsungen) as local anaesthetic. Muscle was dissected free of fat and connective tissue rapidly, washed in ice-cold saline (PBS), frozen in liquid N_2 and then stored at -80 ºC until further analysis. Training progression was monitored by an assessment of MVC strength performed every ~10 days. A detailed representative schematic of the study protocol is provided in Figure-7.

Figure 7 Study protocol

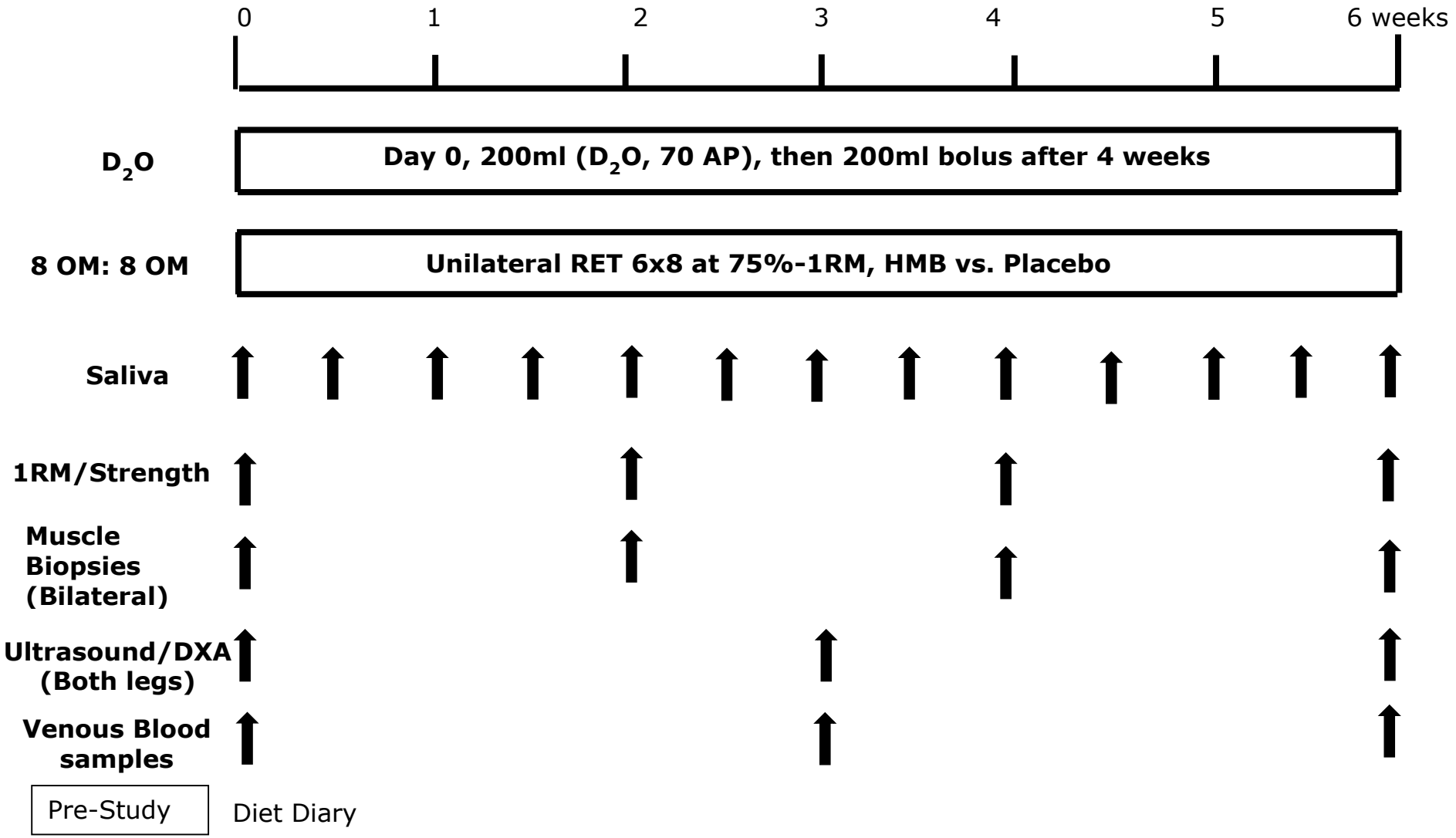


Figure 7. Schematic of measurement of muscle protein synthesis by dosing with D_2O in free living older individuals: Effect of Exercise and Nutritional Supplementation in older men.

2.3. Body water enrichment (TC-EA-IRMS):

Saliva samples were processed prior to analysis to isolate the water as follows. Samples were thawed at room temperature before pipetting 50µl into the inside of an upturned vial cap. An upturned vial was carefully positioned over the sample and then capped. All upturned vials were then placed on a metal heating block (90 ºC) for 4 hours. Immediately after heating, vials were turned upright and placed in ice for 10 mins to condense out the evaporated water. The cap was then removed, along with any debris within the cap, and the water distillate from the sample pipetted into a fresh auto sampler vial and capped. The samples were then ready for direct liquid injection of water (0.1µl volume) into a high-temperature conversion elemental analyser (TC/EA; Thermo Finnigan, Thermo Scientific, Hemel Hempstead, UK) connected to an isotope ratio mass spectrometer (IRMS; Delta V Advantage, Thermo). This TC-EA-IRMS converts the bulk of solid and liquid sample into H_2, CO and N_2 using high temperature (1400 ºC). These gases are then separated by GC column prior to entering the IRMS with the carrier gas (helium).

Each sample was injected a minimum of four times to minimize the effect of carry over amongst samples. A standard curve of known D_2O enrichment was run along with the saliva samples for checking the performance of the machine

2.4. Isolation and derivatization of myofibrillar protein fraction:

Approximately 30–40 mg of muscle was used for the separation of myofibrillar, sarcoplasmic, and collagen fractions. The muscle was homogenized in ice-cold homogenization buffer [50 mM Tris·HCl (pH 7.5), 50mM NaF, 10mM ß-glycerophosphate disodium salt, 1 mM

EDTA, 1 mM EGTA, and 1 mM activated Na_3VO_4 (all from Sigma-Aldrich)] and a complete protease inhibitor cocktail tablet (Roche, West Sussex, UK) at 10 µl/µg tissue. Homogenates were vortexed for 10 min, and the supernatant was collected by centrifugation at 13,000 g for 5 min at 4°C. The myofibrillar pellet was solubilized in 0.3 M NaOH (750µl) vortex mixed and separated from the insoluble collagen by centrifugation (13000rpm, 4°C for 10 mins). This step was repeated twice and then myofibrillar protein was further precipitated with 1 M perchloric acid (PCA). It was then vortexed and centrifuged at 3000g at 4°C for 20 minutes to obtain the myofibrillar pellet. The pellet was then washed with 70% ethanol (2ml) and centrifuged again at 3000g at 4°C for 20 minutes. And the supernatant was discarded thereafter. Protein-bound AAs from myofibrillar protein were released using acid hydrolysis by incubating in 0.1 M HCl in Dowex H+ resin slurry at 110°C overnight (for 16 hours approx.) and further purified on Dowex resin before being eluted with 2M NH_4OH and evaporated to dryness.

The sample to be analysed using gas chromatography should be volatile to permit chromatographic separation. The amino acids are highly polar compounds and they need to undergo "derivatisation" to make them more volatile and reduce their polarity.

The AAs were derivatized as their n-methoxycarbonyl methyl esters (MCME) according to the protocol of Husek (Hušek 1995) with slight modification. The dried samples were resuspended in 60 µl of distilled water and 32 µl of methanol, and following a brief vortex, 10 µl of pyridine and 8 µl of methylchloroformate (MCF) were added. Samples were then vortexed for 30 s and left to react at room temperature for 5 min. The newly formed MCME AAs were then extracted into 100 µl of chloroform; any remaining water was

removed from the sample with the addition of a molecular sieve. Incorporation of deuterium into protein bound alanine was determined by gas chromatography-pyrolysis-isotope ratio mass spectrometry (Delta V Advantage; Thermo Scientific) alongside a standard curve of known L-Alanine-2, 3, 3, 3-d4 enrichment to validate measurement accuracy of machine.

2.5. GC-pyrolysis IRMS Deuterium Analyses:

Mass spectrometric techniques and the use of stable isotope tracers permit the precise measurement of muscle protein turnover. There are many techniques used but the three main ones are Gas Chromatography-Mass Spectrometry (GC-MS), Isotope Ratio Mass Spectrometry (IRMS) and Gas Chromatography-Pyrolysis-Isotope ratio Mass Spectrometry (GC-Pyr-IRMS). Only the third technique has been applied to this study.

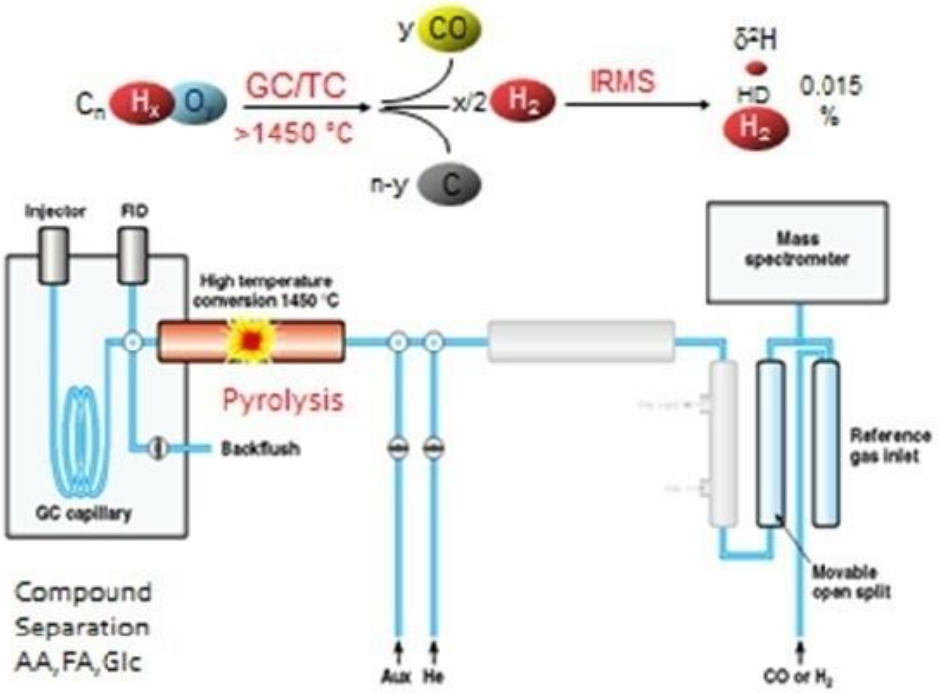


Figure 8 Schematic of a GC-pyrolysis-IRMS system used in this work. Taken from (ThermoScientific, Hemel Hempstead)

The technique of Gas Chromatography-Pyrolysis-Isotope ratio Mass Spectrometry (GC-Pyrolysis-IRMS) was first developed in 2006 (Sessions 2006) for the analysis of deuterium and hydrogen ($^{2}H/^{1}H$). In this technique, a complex mixture is separated by gas chromatography (in this case AA), eluted from the column and enters the reaction chamber. The AA then undergoes incomplete combustion resulting in the formation of by-products of H^{2} and CO gases which are then passed on to the isotope ratio mass spectrometer for isotope analysis. The hydrogen ions of different masses are then detected and their intensities are measured e.g., H:D (hydrogen:deuterium) ratio is determined from the relative intensity of mass 2 and 3 i.e., ^{1}H and ^{2}H. A correction for H^{3+} should be made as it can interfere in $^{1}H/^{2}H$ detection.

Before each batch analysis, the IRMS system was calibrated and tested for measurement accuracy to match the manufacturer's technical specifications.

For GC-pyrolysis-IRMS analysis of MCME alanine, samples were separated on a DB-wax column (30 m x 0.32 mm x 0.25 µm; AgilentJ&W) following split less injection. The oven temperature program for acetone was start at 50°C and hold for 2 min and then ramp at 30°C/min to 240°C and hold for 2 min, and for alanine it was started at 70°C and was held for 3 mins and then ramped at 10°C/min to 240°C and held for 15 min. The separated MCME AA derivatives were then passed through a high temperature (1,420°C) H_2-CO conversion reactor, where the analytes were converted to H_2 gas before being directed to the IRMS, where the $^{2}H/^{1}H$ ratio was determined.

For TC/EA analysis of body water enrichment, following direct liquid injection into the TC/EA, where samples were immediately converted to H_2 gas, sample gases were then directed to the IRMS,

where the $^{2}H/^{1}H$ ratio was determined. The deuterium isotopic enrichment is provided as $\delta^{2}H$.

If any sample fell outside the dynamic range of the instrument, these were re-injected or re-prepared. Figure 9 shows a typical chromatogram from the GC-pyrolysis-IRMS.

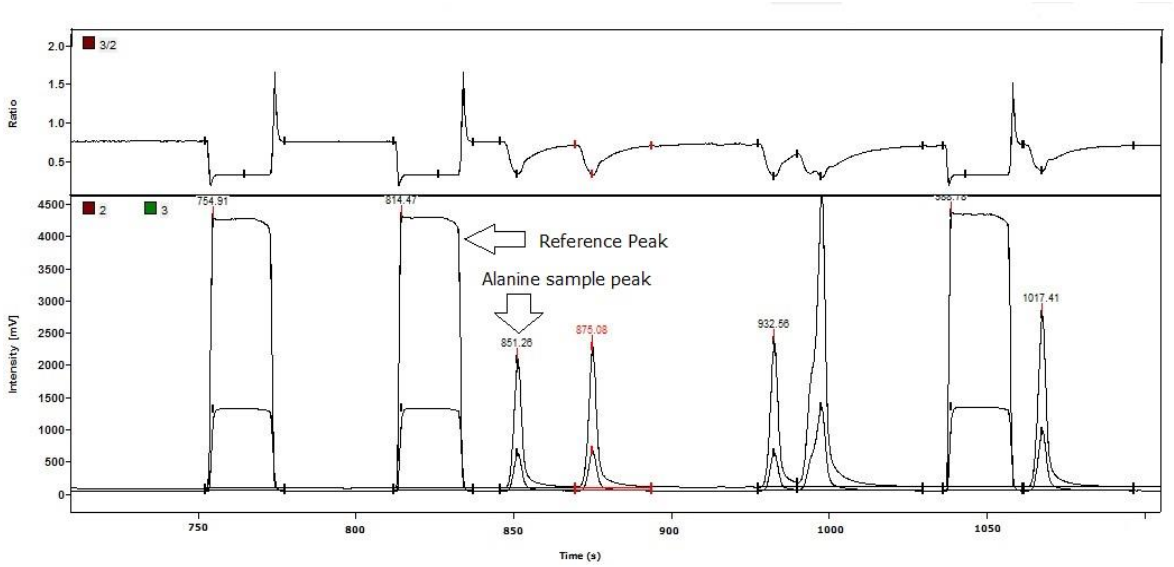


Figure 9- An example of chromatogram from GC-pyrolysis-IRMS displaying the hydrogen isotope mass charge ratio (m/z) of 2 and 3 and the 3/2 delta ratio for reference and sample peaks.

2.6. Assessment of Muscle Architecture, DXA derived mass and muscle strength:

The changes in the muscle architecture were assessed in both legs via ultra-sonographic technique to detect changes in muscle architecture (muscle thickness and pennation angle) after 3-6 weeks of resistance exercise training and HMB/placebo supplementation.

Ultra-sonographic images of Vastus lateralis muscle were taken from both legs at rest via B-mode ultrasonography (Mylab 70; Esaote Biomedica) with a 100mm, 10-15 MHz, Linear array probe. Images were taken at full knee extension (180°) and the transducer

was aligned in the fascicle plane in order to visualize and optimal portion of the fascicle while the participants were lying in a supine position. The parameters of muscle architecture (fascicle length and pennation angle) were determined from the ultrasound scans using Image J Software 1.42q (National Institute of Health). The visible part of fascicle length was directly assessed using this software. Pennation angle was measured as the juncture between fascicles and the deep tendon aponeurosis. An ultrasound image of Vastus Lateralis (VL) muscle is shown in Figure 10. Adapted from (Franchi et al. 2014). DXA derived thigh mass was determined from the lowest point of the Ischium to the knee space.

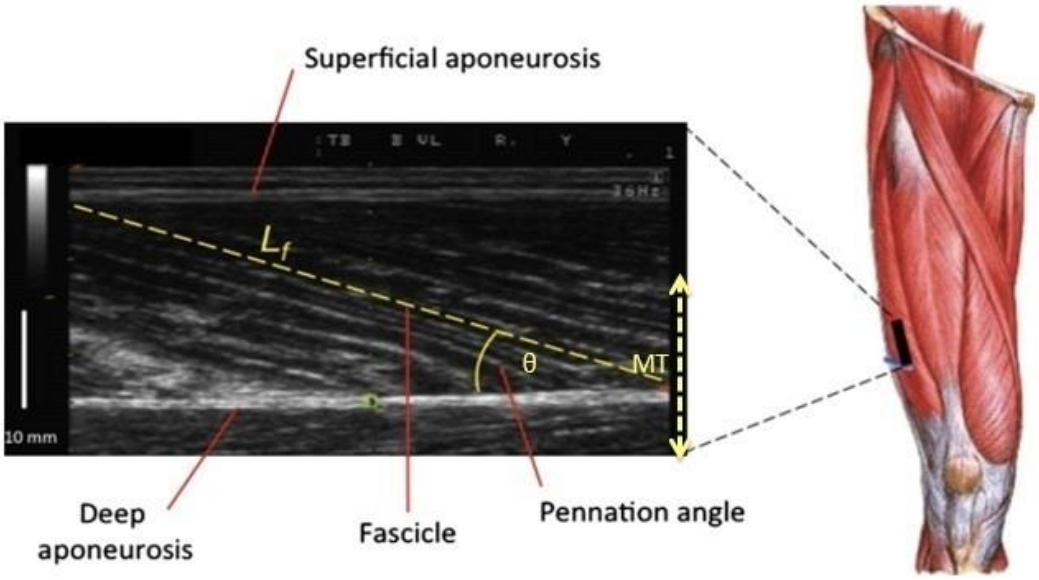


Figure 10 Anultra-sonographic image of Vastus Lateralis muscle showing muscle thickness (MT), pennation angle (θ) and length of the fascicle (L_f).

Figure 10 - An ultrasonographic image of vastus lateralis muscle showing muscle thickness.

Basal measures of maximal voluntary contraction (MVC) and one repetition maximum (1-RM) were assessed in the week prior to the first study day. MVC was measured with isometric contractions

conducted in a sitting position using an isokinetic dynamometer (Isocom; Isokinetic Technologies, Eurokinetics) throughout a range of four knee joint angles from 90° to 60°, with full extension corresponding to 0°. Subjects were seated in the dynamometer chair and secured into position using straps across the chest. The lower leg was strapped to a pad situated at the end of the Cybex lever arm and the knee joint centre of rotation was aligned with the dynamometer fulcrum. The torque produced on the Cybex dynamometer was sampled into an analogue to digital acquisition system (Biopac System, Inc., Aero Camino Goleta, CA, USA) at a frequency of 200 Hz and displayed on the screen of an Apple computer (MAC, Apple, Cupertino, CA, USA). Contractions lasted for 4s, with a rest period of 30s between contractions and a rest period of 90s between knee joint angle changes. The maximum voluntary contraction (MVC) at optimal joint angle was chosen for statistical analysis. It was evaluated by participants performing an isometric maximum voluntary contraction (MVC) at every 10° from 90° to 60° (of knee joint angle (180°= full extension). Two MVCs were recorded at each joint angle with 2-min separating each contraction, and the highest torque produced was used to assess MVC changes from pre- to post-training.

Unilateral leg extension 1-RM was assessed on the training leg, which was selected randomly among subjects (Technogym, Gambettola, Italy). After the procedure was explained, subjects performed a light warm up to avoid injury and ensure familiarity whilst avoiding fatigue; 1-RM was then achieved in as few repetitions as possible with a maximum of 3-4 repetitions. The first repetition was estimated at 50% 1-RM and then increased throughout the range of motion, with 3 minutes rest in between attempts. Functional tests were repeated throughout the study

(every 10 days), with each set of tests performed on a non-biopsy day to avoid influencing subsequent measures.

2.7. Plasma HMB concentrations:

Plasma HMB was analysed by gas chromatography-mass spectrometry (GC-MS) as previously described by Nissen (Steven Nissen, 1990). The plasma was transferred to a 25 x 150mm glass culture tube and extracted with ethyl ether. The HMB was then back-extracted into 0.1 M phosphate buffer, dried and analysed by GC-MS as for plasma.

2.8. RNA Extraction:

Muscle biopsies were taken from both rest and exercise legs of the subjects at 0, 2, 4 and 6 week time points. Approximately 5-10mg of muscle tissue was chipped and placed in 2ml RNase free tube. Each muscle sample was homogenized in a total of 200µl of TRIzol Reagent using a Tissue Lyser (TissueLyserII, QIAGEN). Homogenized samples were incubated at room temperature for 10 minutes followed by the addition of 80µl of chloroform. Samples are then vortexed vigorously for 15 seconds. After another 10 minutes of incubation at room temperature, samples were centrifuged at 12,000rpm for 15 minutes at 4ºC. The samples were separated into three layers after centrifugation with the RNA being in the upper aqueous phase. The aqueous phase was then transferred to a fresh nuclease free tube and the volume was measured using a pipette. An equal volume of 100% isopropanol was then added and mixed thoroughly. The tubes were then left at room temperature for 10 minutes to allow the precipitation of RNA. The samples were then centrifuged at 7,000rpm for 10 minutes at 4ºC. The RNA pellet thus obtained was then washed twice with 1ml 80% ethanol per ml of tri-reagent and centrifuged again at 7,000rpm for 15minute at 4ºC.

Pellets were then left to air dry for a few minutes till they became translucent. The dried pellets were then re-suspended in 22µl of RNase-free water.

2.9. RNA quantification and quality check:

To check the quantity and quality of the RNA, the spectrophotometer (NanoDrop Lite, Thermo Scientific) was blanked with RNase free water. The absorbance at 260nm and 280nm was measured. The measure of A260 gave the quantity of total RNA in µg. The ratio A260/280 gave an estimation of the purity of the RNA. A ratio of 1.7-2.0 is commonly accepted as 'pure' for RNA. If the ratio is noticeably lower, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280nm.

2.10. Complementary DNA synthesis through reverse transcription (RT):

For qRT PCR, 500ng of total RNA per 20µl reaction was used as input amount and reverse transcribed using a commercially available kit (High Capacity cDNA Reverse Transcription Kit; Life Technologies) according to the manufacturer's instructions. The final RT product was stored at −20°C until qRT-PCR was performed.

Table 1: Master Mix made for the reverse transcription reaction (Adapted from *(Biosystem, 2006)*)

Components	Kit with RNase Inhibitor (Volume/ Reaction)µl	(Volume/ Reaction)µl
Master mix	1 reaction	e.g. 400 reactions
10x RT buffer	2.0	800
25x dNTP Mix	0.8	320
10x RT Random Primers	2.0	800
MultiScribe Reverse Transcriptase	1.0	400
RNase Inhibitor	1.0	400
Nuclease free water	3.2	1280

2.11. Quantitative real-time polymerase chain reaction (qRT PCR):

2.11.1. Primer design:

Real-time RT-PCR exon specific primers were designed for human muscle atrophy F-box (MAFbx), myostatin, human caplain, Ubiquitin, MyoD, myogenin, cMyc, Muscle RING-finger protein-1 (MURF1), Myogenic factor 5 (MYF5), REDD1 (regulated in development and DNA damage responses 1), eIF3f (eukaryotic translational initiation factor 3, subunit F) and Peptidylprolyl isomerase A (PPIA3) gene using Primer express software. Finally, all

sequences were submitted to the Primer blast software, available on the National Centre for Biotechnology Information website (www.ncbi.nlm.nih.gov), in order to evaluate gene specificity. All mRNA-specific primers were validated before use. Primer sequences are displayed in Table-2.

Table 2 qRT-PCR Primer Sequences

Gene Name	Forward Sequence	Reverse Sequence
MAFbx	5'-CCTTCACTGACCTGCCTTT-3'	5'-CCTTCACTGACCTGCCTTT-3'
MuRF1	5'-AGTGACCAAGGAGAACAGTCA-3'	5'-CACCAGCTTTGTGGACTTGT-3'
Myostatin	5’-GCTGCGCCTGGAAACAGCTC-3’	3’-ATCAGTTCCCGGAGTGGAGGC-5’
MyoD	5’-CCg CCT GAG CAA AGT AAA TG-3’	5’-GCC CTC GAT ATA GCG GAT-3’
Myogenin	5'-ACC CTA CAG ATG CCC ACA AC -3'	5'-GGAAGGCCACAGACACATCT-3'
REDD1	5'-AAGACACGGCTTACCTGGA-3'	5'-CAGTAGTTCTTTGCCCACCT-3'
Calpain	5’-GCC AAG CAG GTG AAC-3’	5’-TGA AGT CTC GGA ATG ACA TC-3’

Ubiquitin	5’-GAT CTG CCG CAA GTG CTA-3’	5’-CAG TCA ATG AAA GGG ACA CT-3’
eIF3f	5'-GTT GGG AAC TGT CGA CAA AC -3'	5'-ATT CCA TGT CAA CAG CCA CT -3'
Myf5	5'-ATGCCCGAATGTAACAGT CCT-3'	5'-GGTTGCTCTGAGGAGGTGA T-3'
PPIA3	5’-CATGCCTAGCTGGATTGC AGA-3’	5’-ACATTGACACTTCCTGGGAC T-3’
cMyc	5’-CCCAGCAAGGACACTGA GCAAG-3’	5’-AGGCCCCTCCTGTTATTATG GGG-3’

2.11.2. Optimizing the PCR Reaction:

All experiments were carried out in triplicates and the values of amplification were normalized to the “housekeeping gene” Peptidylprolyl isomerase A (PPIA3). The SYBR® Green qPCR Master Mix was purchased from Applied Biosystems. For each gene, the real-time RT PCR was conducted in triplicate and final volume of each reaction was 7µl containing 3.31 µl of 1x SYBR Green Master mix, 0.04µl of each forward and reverse primer, 2.7 µl of Nuclease free water and 1µl of 1:5 diluted cDNA templates (Table 3). The PPIA3 was used as ‘housekeeping gene’ as its expression remains unaltered due to exercise and supplementation. And relative gene expression was assessed using a standard curve of known concentrations of mRNA and then normalized to CyclophilinA (PPIA) gene expression. Sybr-Green real-time RT-PCR analyses were carried out on the iQ5 real-time PCR detection system (Bio-Rad) using the following cycle conditions: 1 cycle of denaturation for

10min at 95°C, followed by 40 cycles of denaturation 1min at 60°C, and 15 s at 95°C.

A melting curve analysis was generated at the end of each qRT-PCR assay. A single melt peak was observed for each sample, validating that only one product was present. The data for qPCR were analysed by using the standard curve method. For each qRT-PCR experiment, a standard curve was generated for each target gene. The relative gene expression were calculated using the $2^{-\Delta CT}$ method(Livak & Schmittgen 2001). This method generates a value in arbitrary units (AU) of the gene of interest (GOI) expression normalized to the housekeeping gene (HKG) expression ($\Delta CT = C_{TGOI} - C_{THKG}$).

Table 3- qPCR reaction components

	1 reaction	e.g. 100 reactions
SYBR Green qPCR Master Mix	3.31 µl	331 µl
Forward primer	0.04 µl (250nM)	4 µl
Reverse Primer	0.04 µl (250nM)	4 µl
cDNA template	1 µl	100 µl
RNase free water	2.7 µl	270 µl

2.12. Statistical Analysis

All data are presented as means ± SE. Differences between and within groups were tested using repeated-measures two-way ANOVA with a Bonferroni correction using GraphPad Prism (La Jolla, CA) Software Version 6. For qRT PCR, data were analysed using $2^{-\Delta C_T}$ method. It was log transformed as it did not pass normality test and analysed with two way repeated measures ANOVA using GraphPad Prism (La Jolla, CA). At each time point the differences from the pre value for each group and between the groups were tested with a Bonferroni post hoc t-test. The α-level of significance was set at $P<0.05$ until otherwise indicated.

Chapter Three - Results:

3.1. Subject Characteristics:

Table 4: Subject characteristics - Older Males

	Placebo Group	Treatment Group	Significance
Age (y)	69 ± 1.6	68 ± 1.1	
Height (m)	1.74 ± 0.02	1.76 ± 0.02	0.66
Weight (kg)	83 ± 2.9	86 ± 4.1	0.538
BMI ($kg.m^{-2}$)	27 ± 0.9	28 ± 1.2	0.67
ASM (kg)	24.8 ± 1.06	25.3 ± 1.31	0.76
ASMI ($ASMkg.m^{-2}$)	7.8 ± 0.33	8.5 ± 0.43	0.23
Thigh lean mass (kg)	5.5 ± 0.31	5.6 ± 0.26	0.81

16 healthy older men were randomly assigned to two groups (8 per group). The table shows subject demographics were equally matched in both the groups.

3.2. 1-Repetition maximum (1-RM):

Figure 11- 1-RM

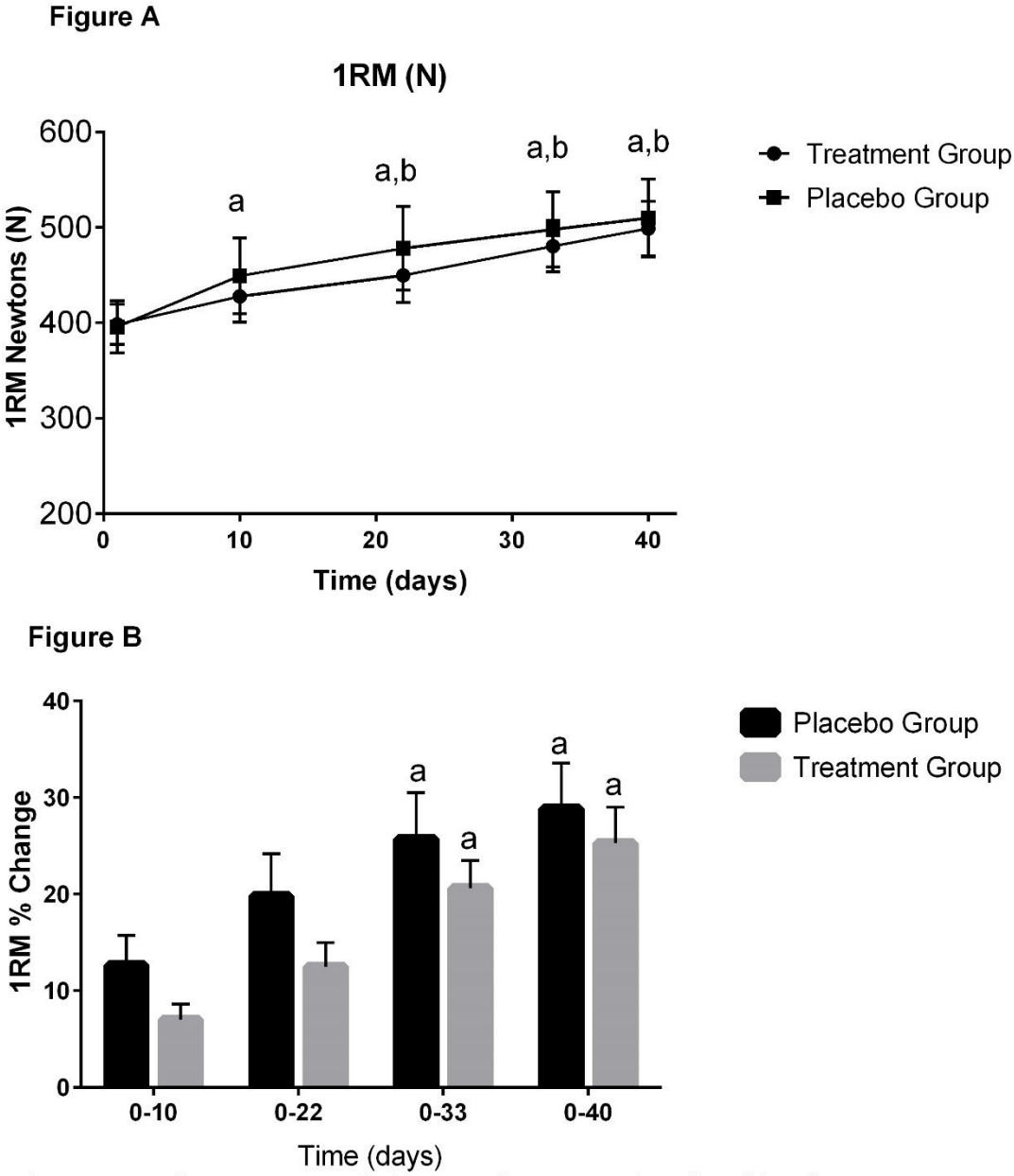


Figure 11. Time course of changes in 1-RM of trained leg in treatment and placebo groups (Figure A)
and percent (%) change in trained leg from baseline (B).
Figure A. a- $p<0.05$ significantly different from baseline in placebo group, b- $p<0.05$ significantly different from baseline in treatment group. Figure B. a: $p<0.05$ significantly different from 0-10.
No significant differences between the groups at any time-point.

Over a period of 6 weeks, RET increased 1 RM in both groups. After 6 weeks, 1-RM had increased from a baseline of 398±22.63N to 499±30.84N in treatment group and 396±29.45N to 510±43.82N in placebo group. So, there is main effect of training over time but not effect of treatment as there was no significant difference in 1RM between the groups at any time-point ($P>0.05$). The % change in 1-RM after 6 weeks in treatment group (25±3.98%) and placebo group (29±5.09%) was not significantly different between the groups either.

3.3. Maximum voluntary contraction:

Figure 12- Maximum voluntary contraction (MVC)

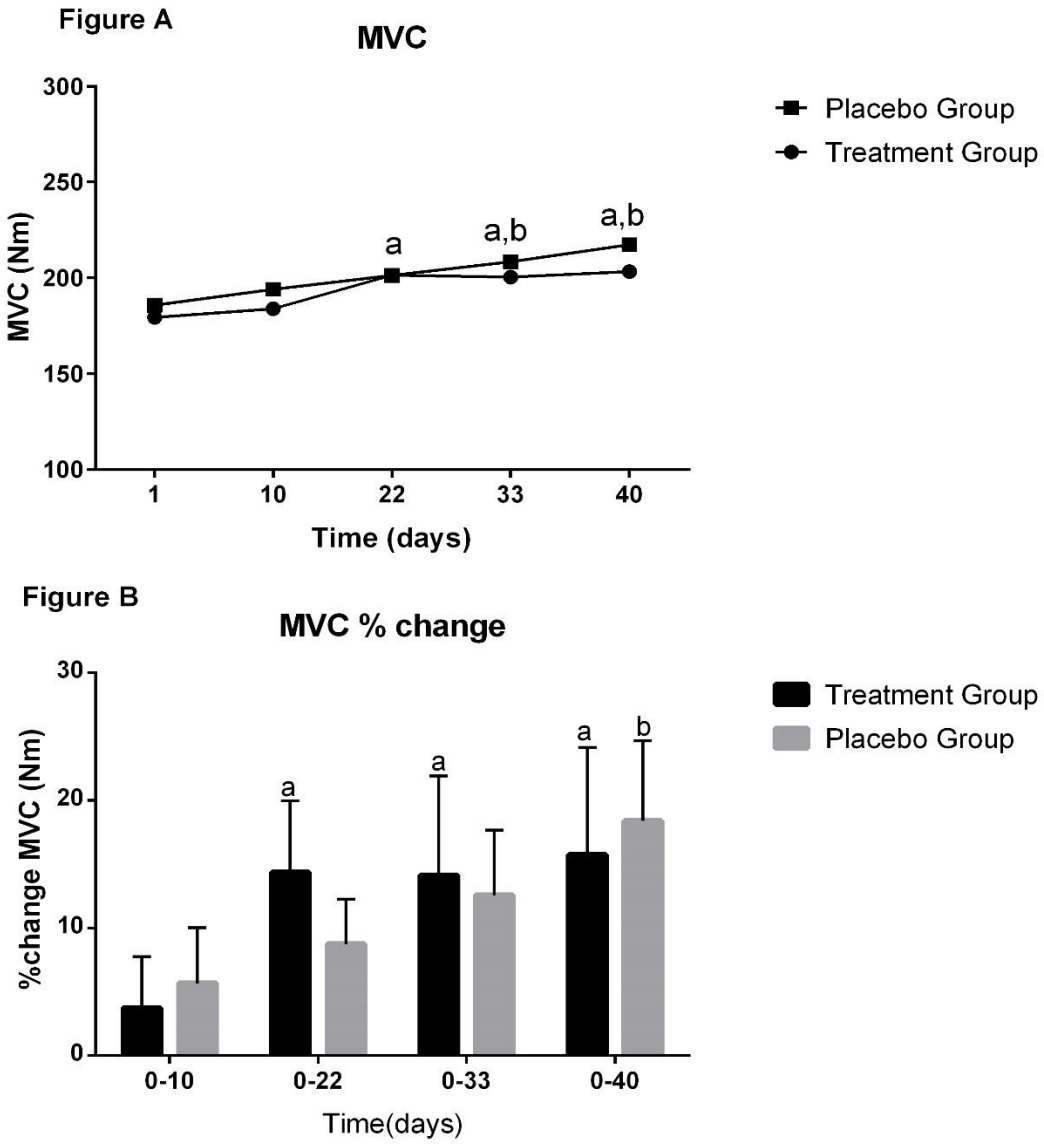


Figure 12. Maximum voluntary contraction (MVC) in trained legs of treatment and placebo groups (A) and percent (%) change in trained leg from baseline (B). Figure A and B. a- $p<0.05$ significantly different from baseline in treatment group, b- $p<0.05$ significantly different from baseline in placebo group. No significant differences between the groups at any time-point.

MVC increased from a baseline of 185±10Nm to 217±11Nm in Placebo group ($P<0.05$) and 179.4±12Nm to 203.3±12Nm ($P<0.05$) in treatment group. Hence, there is an effect of training over time but no effect of treatment was observed as there were no

significant differences between both the groups at any time-point. The % change in MVC 15.7±8.3% in treatment group and 18.3±6.3% in placebo group after 6 weeks was not significantly different between the groups either.

3.4. Thigh lean mass:

Figure 13 – Thigh Lean Mass

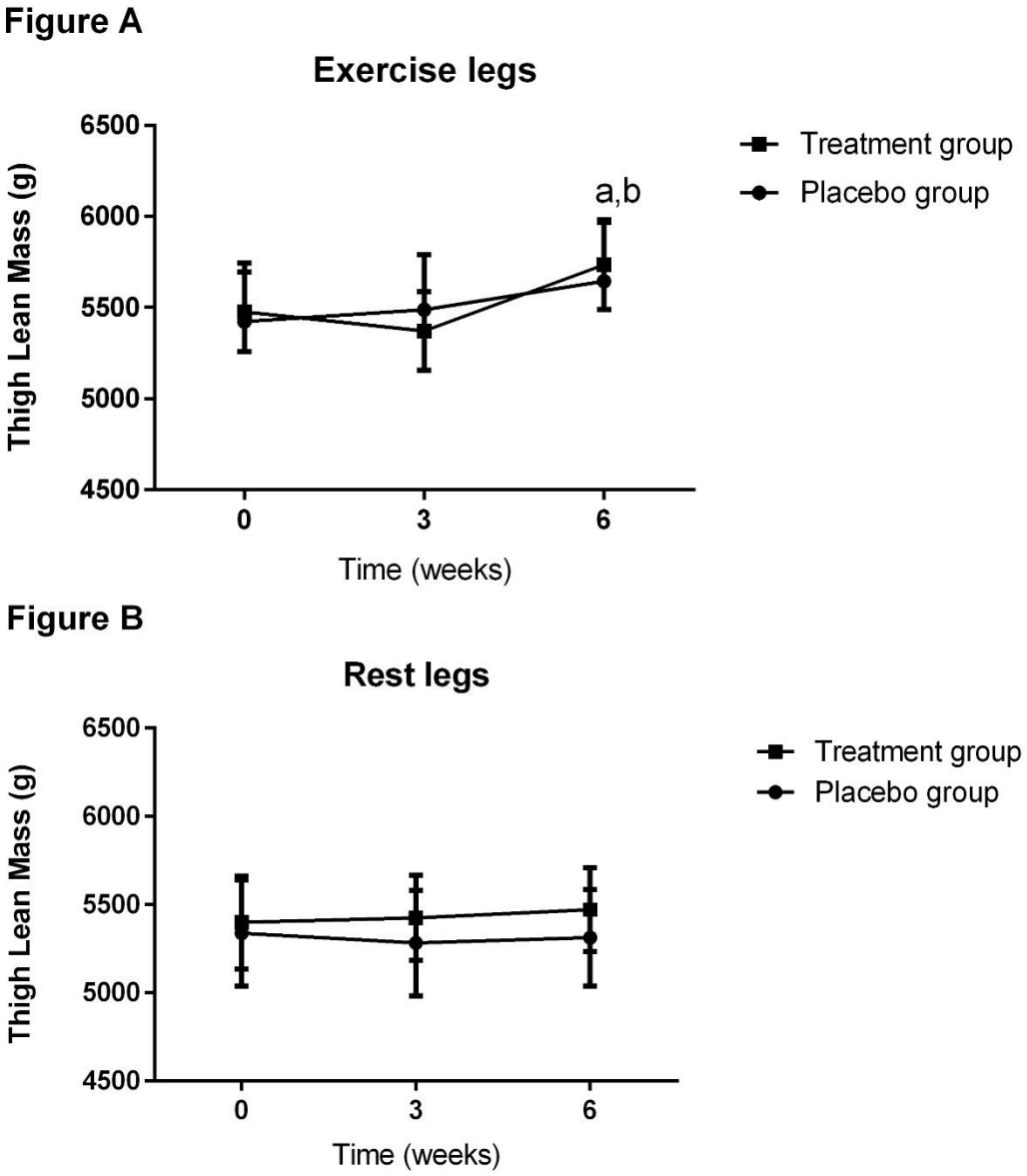


Figure 13. Thigh lean mass over the 6 week training period in rest and exercise legs of treatment and placebo groups.
Figure A. a- $p<0.05$ significantly different from baseline in treatment group, b- $p=0.06$ from baseline in placebo group.
No significant differences between the groups at any time-point.

Thigh lean mass assessed by DXA was not significantly different in the exercise leg between the groups at the start of study i.e.

baseline (5422.5±321g Placebo group vs 5476±220.5g Treatment group) with only treatment group increasing lean muscle mass over 6 weeks significantly to 5734±245g p=0.015 (<0.05) vs 5644±323g p=0.06 in placebo group. So, we did observe effect of training over time in both groups. Also, there was no significant increase in the thigh lean mass of rest leg over 6 weeks i.e. baseline 5338±299.8g in placebo group vs 5399±262.4g in treatment group and after 6 weeks 5311±274g in placebo group vs 5470±237g in treatment group (p>0.05). And there were no significant differences between the groups at any given time-point in terms of thigh lean mass.

3.5. Muscle architecture:

Figure 14- Muscle Architecture

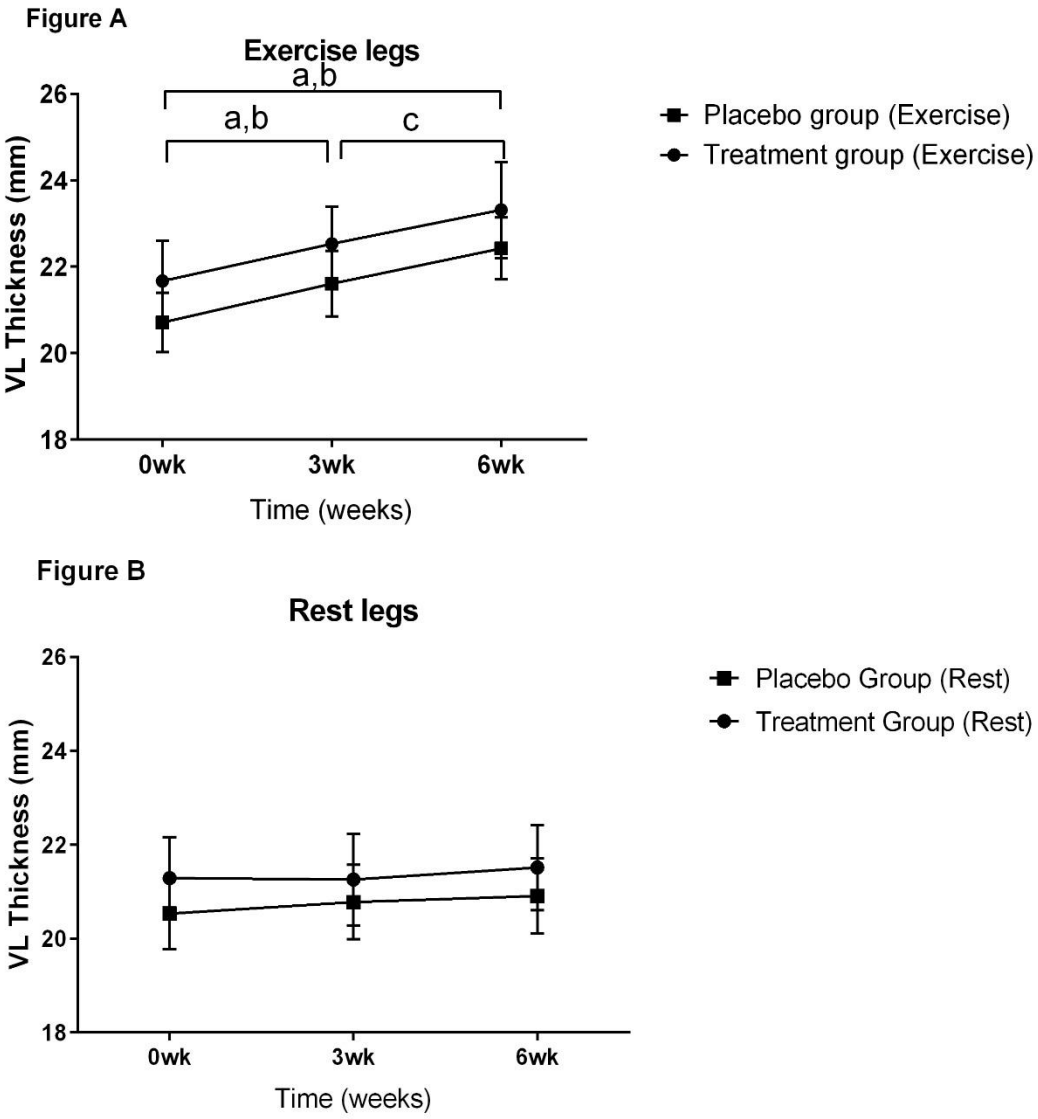


Figure 14. Time course of vastus lateralis (VL) muscle thickness in the exercise and rest legs of both groups at 0,3 and 6 weeks.
Figure A. a- $p<0.05$ significantly different from baseline in placebo group, b- $p<0.05$ significantly different from baseline in treatment group, c- $p<0.05$ significantly different from previous time point for placebo group.
No significant differences between the groups at any time-point.

Measures of Vastus Lateralis (VL) thickness showed significant increases over 6 weeks in exercise legs of both groups, Placebo

group: 20.7±0.6mm to 22.4±0.7mm P<0.0001 vs Treatment group: 21.6±0.9mm to 23.3±1.1mm P=0.0001. So, we did observe effect of training over time in both groups but no effect of treatment as there were no significant differences between both the groups at any time-point. And rest legs of both the groups remained consistent in thickness over 6 weeks, Placebo group: 20.5±0.7mm to 20.9±0.7mm vs Treatment group: 21.2±0.8mm to 21.5±0.9mm.

3.6. qRT PCR (cMyc gene expression):

Figure 15 – qRT PCR (cMyc) gene expression in treatment and placebo groups

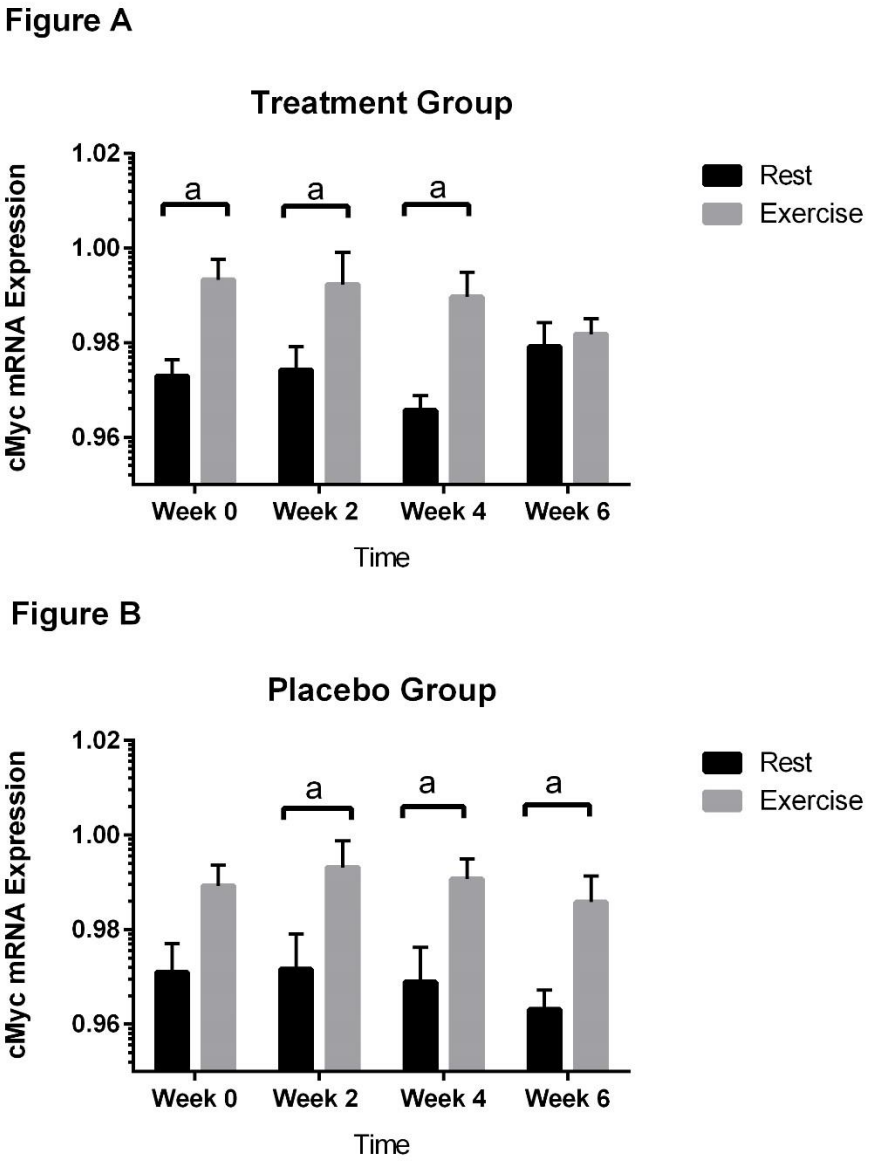


Figure 15. Quantitative real-time PCR to measure expression of cMyc gene in rest and exercise legs of treatment and placebo groups.
Figure A and B: a-p<0.05 significantly different from rest leg.

Real-time qPCR results for cMyc mRNA expression in rest and exercise legs of subjects in placebo and HMB groups at 0,2,4,6 weeks. Results are normalized to PPIA3 (cyclophilin) mRNA expression and values are presented as mean±SEM. Data is

presented as log-transformed values because it was not normally distributed. cMyc expression in trained leg responded to acute bout of exercise at weeks 0, 2, 4 and 6. We have also observed blunting in cMyc gene expression in response to exercise by 6 weeks in both groups. However, there is no main effect of treatment and training over time in both the groups. Also, the rest leg in both groups do not exhibit any significant change throughout the study. No changes in expression in response to exercise and supplementation were observed in any of the other genes measured. Graphical representation of various genes is given at the end in appendix.

Figure 16 qRT PCR-cMyc gene expression across groups

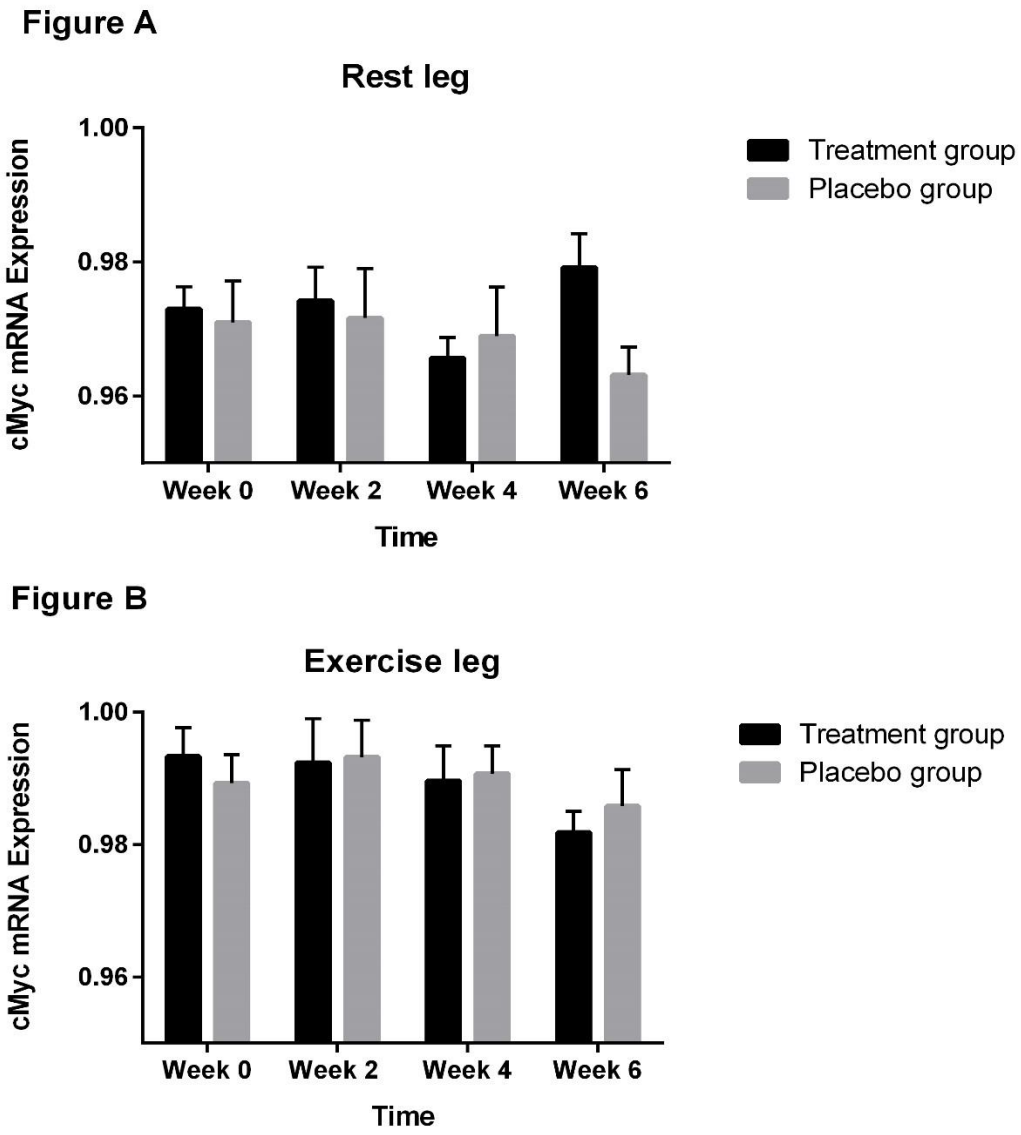


Figure 16. Quantitative real-time PCR for gene expression of cMyc: comparison between the rest and exercise legs of treatment and placebo groups.
No significant differences were found between the groups at any time-point.

Real-time qPCR results for cMyc mRNA expression in rest and exercise legs of subjects in placebo and HMB groups at 0, 2, 4, 6 weeks showing no significant differences between the groups.

Results are normalized to PPIA3 (cyclophilin) mRNA expression and values are presented as mean±SEM.

3.7. Plasma HMB concentration:

Figure 17 Plasma HMB concentration in response to oral HMB consumption

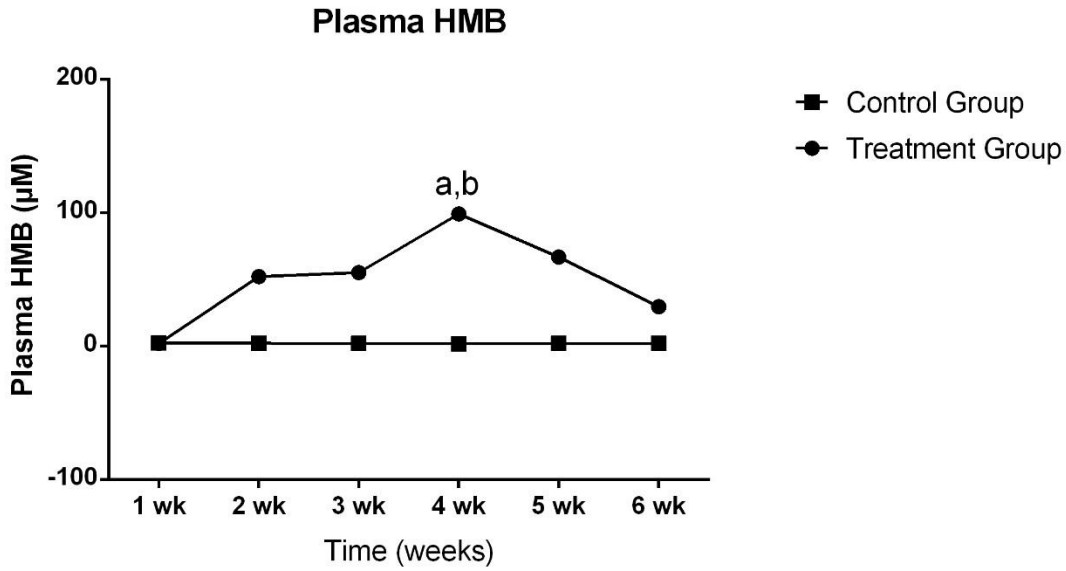


Figure 17. Plasma HMB concentration in the treatment (filled circle) and placebo (filled square) groups following oral HMB consumption in treatment group only.
Letters indicate statistical significance (P<0.05): a= different from respective basal; b= different between groups at equivalent time-point.

With ingestion of HMB, fasted plasma HMB concentration of 2.1±0.2µM increased significantly to 99.2±57µM (P<0.05) by 4 weeks of study and remained elevated over the basal value by the end of study in the treatment group only (29.8±25 µM; 6weeks post consumption). While the plasma HMB concentration remained consistently same in the control group throughout the study (2.4±0.2 µM at the start of study to 2.2±0.4 µM at 6 weeks). Data are presented as mean ± SEM.

3.8. Muscle protein enrichments:

Figure 18 Deuterium isotopic enrichment in muscle provided as δ^2H.

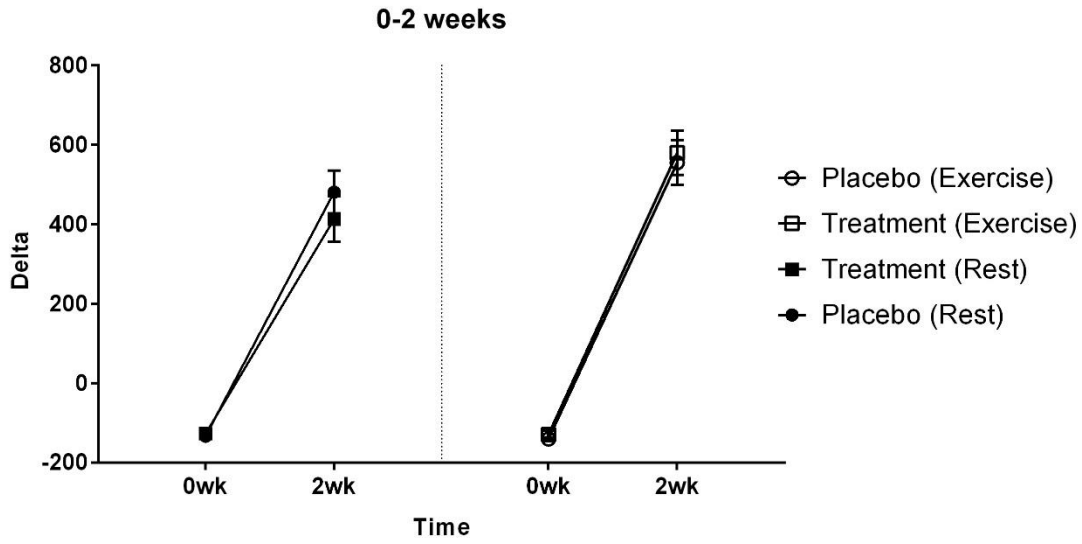


Figure 18. The deuterium isotopic enrichment provided as δ^2H in rest and exercise legs of treatment and placebo groups over 0-2 weeks. No significant differences were observed between the groups at any time-point.

Baseline enrichments in rest legs of placebo and treatment groups increased from -133.1±6.6 δ^2H to 481.4289±53.9 δ^2H and from -127.2±9.3 δ^2H to 413.3±57.0 δ^2H respectively after 2 weeks. Similarly, enrichment increased over time in exercise legs from a baseline of -140.4±0.4 δ^2H to 555.8±56.4 δ^2H by 2 weeks in placebo group and from 129.2±9.8 δ^2H to 580.1±55.3 δ^2H by 2 weeks in treatment groups. But, there is no effect of training over time. Also, there are no significant differences in rest and exercise legs between the groups over time course of 0-2 weeks suggesting that there is no effect of treatment.

Data are presented as mean ± SEM.

Chapter Four - Discussion:

Although there is substantial research on the effects of physiological adaptations to resistance exercise and amino acid supplementation but the chronic impact of leucine metabolite, beta-hydroxy-beta-methylbutyrate (HMB) supplementation in conjunction with resistance exercise training in the promotion of skeletal muscle hypertrophy remains poorly understood. We have used novel deuterium oxide tracer techniques to determine the effects of HMB supplementation and RET on skeletal muscle mass and function in elderly men.

A number of studies have reported that HMB supplementation may result in several ergogenic advantages like anabolic (Jówko et al. 2001; Nissen et al. 1996) and anti-catabolic (Knitter et al. 2000; Hsieh et al. 2006) effects. It is also known that RET prompts muscle hypertrophy but addition of nutritional supplements may be deemed essential along with RET for maximally amplified gains in muscle mass and strength. Our aim was to discern if HMB supplementation can enhance the effects of exercise on skeletal muscle mass and functions in elderly male subjects.

We have used ultrasonography for assessing changes in muscle mass as it is considered a sensitive approach to determine early changes in muscle mass (Ismail et al. 2015) and architecture (Aagaard et al. 2001). We found a significant increase in the thickness of vastus lateralis muscle in the exercise legs of both groups after only 3 weeks of RET in contrast to some studies reporting a delay in muscle hypertrophy (Häkkinen et al. 1998). Other studies from our lab, also support this notion of early hypertrophic response with RET i.e. after 3 weeks (Brook et al. 2015; Seynnes et al. 2007). Moreover, further significant increase was also observed between 3-6 weeks of RET for placebo group

only. Also, we did not observe any changes in the rest legs in either group over the study period of 6 weeks. This is in accordance with previous observation that increases in exercise legs were in response to resistance exercise training as there were no significant differences between the placebo and treatment groups. HMB supplementation did not afford any further benefit suggesting that it does not alter chronic changes at all or the length of our study (6 weeks) might not be adequate to establish the effects of HMB in elderly population.

Likewise, we have also observed improvements in functional measures like 1RM and MVC after just 3 weeks of training which is in agreement with some of the studies previously done (Seynnes et al. 2007; Rabita et al. 2000) showing an increase in muscle strength coupled with muscle hypertrophy. This suggests an earlier response to exercise training than previously reported (Fatouros et al. 2006; Granacher et al. 2009). We observed an increase in 1-RM strength after 6 weeks of training in both groups i.e. ~25% for treatment group and 29% for placebo group. Also, there were no significant differences between the groups at the end of 6 week study period in contrast to some studies reporting a significant gain in strength and functionality in the HMB group compared to the placebo (Vukovich et al. 2001; Flakoll et al. 2004). However, it has to be taken into account that these studies were conducted over much longer duration (8 and 12 weeks respectively) and one study (Flakoll et al. 2004) included supplementation of lysine and arginine along with HMB, therefore, the improvements in strength may not be attributed to the effect of HMB alone. Also, Vukovich and colleagues measured lower body strength using three different exercises and found significant increase by only leg curl exercise

suggesting that the type of exercise undertaken may also influence the response (Vukovich et al. 2001).

For measuring skeletal muscle mass, clinical imaging methods like MRI, CT and dual-energy X-ray absorptiometry(DXA) have been used since the early 1970s (Heymsfield et al. 2014). We employed DXA to determine thigh fat free mass; DXA derived measures have been validated for use in elderly subjects (aged 51-84 years) (Lustgarten & Fielding 2011). In addition, there are noteworthy benefits of using DXA which includes low clinical costs as compared to MRI and CT scans, minimal radiation exposure, rapid (whole body scan in <20 minutes), easier to perform, highly reproducible and commonly available (Albanese et al. 2003). Moreover, DXA-derived lean soft tissue mass was found to be correlated with skeletal muscle mass for whole body ($r=0.94$, $P<0.001$) and leg region ($r=0.91$, $P<0.001$) measured by more well-known techniques like MRI (Chen et al. 2007). We have observed a significant increase in thigh lean mass in the exercise leg of treatment group only ($P=0.015$) over 6 weeks. Although there is a trend for increase in the exercise leg of the placebo group ($P=0.06$), there were no significant differences between groups at any time-point. The rest leg mass also remained unchanged during the 6 week training period. This is potentially an interesting observation as it is suggestive that there might be an effect of treatment. Nonetheless, under these conditions and with a small sample size, we were unable to observe any differences in muscle mass measured by ultrasound or muscle strength by MVC and 1-RM between the groups. It is indicative that even if there is an effect of treatment observed on DXA derived measures; it didn't translate into a functional change. Therefore, the results of muscle mass measured by DXA and USG do not quite agree but these are two independent

measures. Even though the architectural measurement by USG has not been validated against the other gold standard techniques; it is also reported that DXA tends to over-estimate the muscle mass (Lustgarten & Fielding 2011; Bredella et al. 2011). Consequently, although the effect of treatment on muscle mass measured by DXA might be suggestive of an effect of HMB, there were no functional improvements compared to placebo in this study. One limitation of this study is it is not a cross over study and subjects in our study were free-living individuals. And individuals may respond differently to treatment. However, it merits further investigation and doesn't preclude this being pursued as an avenue for research in larger groups and over much longer periods. Future studies conducted for investigation of the effect of HMB supplementation over longer periods along with an increase in number of subjects are, therefore, required.

A randomized double blinded placebo controlled trial carried out in two phases reported that body composition, muscle strength and function in elderly individuals (men and women) is enhanced by Calcium-HMB supplementation with or without RET (Stout et al. 2013). This study was conducted over a period of 24 weeks and in two phases where placebo or 3g/d HMB supplementation was given to elderly men and women either alone (Phase I) or in combination with resistance exercise (Phase II). Subjects recruited for phase I and II of the study were different. The findings of phase I presented by Stout and colleagues were interesting where they reported Ca-HMB enhances muscle strength and quality in elderly men and women without RE. Although the results of phase II of study, which is quite similar to our study design, where effects of Ca-HMB was seen in combination with RET, total, arm and leg lean mass, muscle strength and function increased significantly in both placebo and

HMB groups with no differences between the groups. These results are consistent with our findings as we have also observed that 6 weeks of RET improved lean mass, strength and function in elderly men and there is no additive effect of HMB as both placebo and HMB groups delivered the same end result. However, this study was conducted over a much longer period (24 weeks vs 6weeks) as compared to our study and they used slightly higher intensity for exercise (80% 1RM vs 75% 1RM).

We also measured myofibrillar protein synthesis using stable deuterium oxide (D_2O) tracer; deuterium labelled amino acids are incorporated into muscle protein and the labelling determined by GC-pyrolysis-IRMS. The data are presented in terms of deuterium isotopic enrichment as δ^2H. We did not observe any significant differences in rest and exercise legs of both groups from 0-2 week of study suggesting that HMB supplementation had no positive additive impact on MPS in older males. This data is in agreement with the other measures of muscle mass and function. We have been unable to fully resolve the muscle enrichments for 4-6 weeks and fractional synthesis rate (FSR) data due to an instrument failure and time constraints but hope to finalize it soon for publication. Additionally, if there were any changes, we would expect to have observed them in the 0-2 week's period as MPS is reported to robustly increase in this active period of muscle growth (Brook et al. 2015) following RET. However, since we did not observe any additive effect of HMB on muscle mass and functional measures, we can perhaps assume that any impact on MPS in this study is likely limited.

HMB is thought to increase lean muscle mass via a net increase in protein balance brought about by the inhibition of protein degradation and multiple catabolic pathways may be involved in this

process (Smith et al. 2005); so it is also considered as an anti-catabolic agent. We chose to determine the alterations in expression of genes implicated in catabolic and anabolic pathways regulating skeletal MPS as a result of HMB supplementation and exercise interventions. It is known that mammalian target of rapamycin (mTOR) is a key pathway for regulation of protein synthesis and hence muscle growth (Bodine et al. 2001). It is a protein kinase up regulated by insulin (Avruch et al. 2006), growth factors and amino acids, particularly by leucine (Anthony et al. 2000). It stimulates cell growth by phosphorylating p70 ribosomal S6 kinase-1 (S6K1), eukaryotic initiation factor 4E-binding protein-1 (4E-BP1)(Guertin et al. 2006) and activating the transcription factor cMyc. In addition, REDD1 is recognized as negative regulator of mTOR. REDD1 is affected by diverse stresses such as hypoxia (Brugarolas et al. 2004), dexamethasone (Wang et al. 2006) and energy depletion (Sofer et al. 2005). It is known to modulate the function of tuberous sclerosis 1 (TSC1) and 2 (TSC2) complex and inhibits mTOR function to regulate cell growth. Unlike a study done by Drummond and colleagues, reporting a decrease in REDD1, 6 hours after potent anabolic stimulus (RET+EAA ingestion) in old subjects (Drummond et al. 2009), we did not observe any significant changes in REDD1 mRNA. It is important to note that their study was limited to measuring gene expression that did not extend beyond 6 h after exercise while we measured it 45-60min post exercise. It is also recognized that changes in mRNA expression are governed by the daily variations in metabolic status, timings of biopsies and the technique, mode and intensity of exercise in addition to the muscle and species being studied (Vissing et al. 2005; Yang et al. 2005). So, it is likely the difference in REDD1 expression is due to timing of the biopsy post exercise.

We also measured the transcription factor and downstream target of mTOR, cMyc which has also been identified as a regulator of ribosomal biogenesis. It is known to be up regulated by insulin and downregulated by rapamycin (Teleman et al. 2008). It is involved in upregulation of the translation initiation complex eIF4F (Lin et al. 2008). We noted significant elevation in cMyc mRNA expression in the exercise leg of subjects in both groups after an acute bout of exercise and it continued to respond to training throughout the study period although we did observe a small drop in gene expression after 6 weeks. This is in accordance with previous studies reporting an upregulation in cMyc mRNA expression within hours after exercise stimulus and EAA intake (Trenerry et al. 2007; Drummond et al. 2009). It has been suggested that training adaptations on the metabolic and cellular level are the outcome of combined effects of transitory variations in gene expression after each exercise bout. Our result endorses that resistance exercise is involved in the alteration of cMyc expression which is linked to cell growth, synthesis of new ribosomes, cellular differentiation and also regulates translation initiation complexes in humans.

We have also measured the markers expressed by activated satellite cells during the different stages of regeneration and involved in the specification of myogenic program like Myf5, MyoD, myogenin and myostatin. These myogenic genes are important regulators of muscle cell growth and differentiation. We also assessed the expression of muscle specific E3 ligases [muscle ring finger-1 (MURF1), MAFbx/Atrogin-1 (muscle atrophy F-box)] involved in the ubiquitin proteasome pathway, following exercise and HMB supplementation. The UPP pathway is associated with muscle atrophy and is found to be upregulated with exercise. To our knowledge, this is the first study to determine the effects of both

HMB supplementation and RET on the expression of these genes and there exists very sparse human data studying the effects of exercise and amino acid supplementation on proteolytic pathways in the human muscle. The genes involved in ubiquitin proteasome pathway (UPP) are responsible for protein degradation by tagging of proteins with ubiquitin molecules and consequent degradation by 26S proteasome, hence, leading to loss of muscle mass. Both MURF1 and MAFbx (muscle atrophy F-box) are upregulated by the same transcription factor FOX3A (forkhead box 3A) and are involved in muscle atrophy (Jones et al. 2004). We also measured the expression of myostatin, a member of TGF-β superfamily and a negative regulator of muscle growth. Its suppression has been found to induce hypertrophy in rats (Thomas et al. 2000) and some human studies suggest an attenuation in myostatin expression after resistance training (Kim et al. 2005; Louis et al. 2007)**.** We did not observe any changes in mRNA expression of any of these genes in response to exercise or HMB supplementation. We also measured calpain-1 (calcium activated cysteine proteases), which plays crucial role in cytoskeletal protein assembly in the muscle and its mRNA displayed no change in expression in response to RE. This is in accordance with previously published data on calpain-1 (Yang et al. 2006; Louis et al. 2007).

In summary, although both groups improved in 1RM/MVC strength, VL thickness and lean body mass during the 6 weeks RET program, no significant differences were observed between the groups. Also there were no significant changes in expression of various catabolic and anabolic genes measured following RET and HMB interventions in 6 weeks study period. This suggests that HMB supplementation may not favor additional improvement in strength and functional measures in a 6 week RET program. This is in agreement with the

results of a previous study done by Gallagher and colleagues (Gallagher et al. 2000) although the age of our participants (~68yr vs ~22yr), doses of HMB supplementation (3g.d^{-1} vs 0,1.5g.d^{-1} & 3g.d$^{-1)}$, study period (6-wk vs 8-wk) and protocols used to assess these measures were different than our study.

However, we acknowledge a few study limitations. Firstly, our study was limited to four metabolic/molecular time points (0, 2, 4 and 6 weeks). Secondly, we may have been unable to ascertain any synergistic or additive effects of HMB because ours was a 6-week study and studies conducted over a much longer period of time may be required to uncover any effect of HMB supplementation. Thirdly, we had only 8 individuals per group and a larger sample size may be needed to observe effects of HMB. Fourthly, when we used sarcopenic index (appendicular lean mass) to classify subjects recruited for our study, 7 out of 16 turned out to be sarcopenic. Future studies should distinguish between sarcopenic and non-sarcopenic individuals to reduce the variability within the subjects based on their muscle mass. Lastly, we have been unable to fully resolve the FSR data for myofibrillar protein synthesis within the time constraints of MRes due to machine failure. This is a potential limitation as we have enrichment data which depicts the actual incorporation of deuterium labelled alanine into muscle protein and not the fractional synthesis rate (FSR). Nevertheless, we did not observe additive effect of HMB treatment on any of the functional and strength measures. It is also reflected by the muscle enrichment data as there is lack of any additive effect of HMB under rest or exercise conditions demonstrating that any effect of MPS in this study is superfluous. Even though, we observed a small but significant increase in the thigh lean mass measured by DXA in the treatment group only which might suggest an effect of HMB and

warrants further investigation, though there were no significant differences between both the groups in DXA derived measures. Additionally, muscle thickness measured by ultrasound did not differ between the groups and this observation is in accordance with the measures of muscle strength and function obtained by other methods. To conclude, HMB supplementation provided no added advantage for muscle hypertrophy, enhanced strength and function in elderly male individuals.

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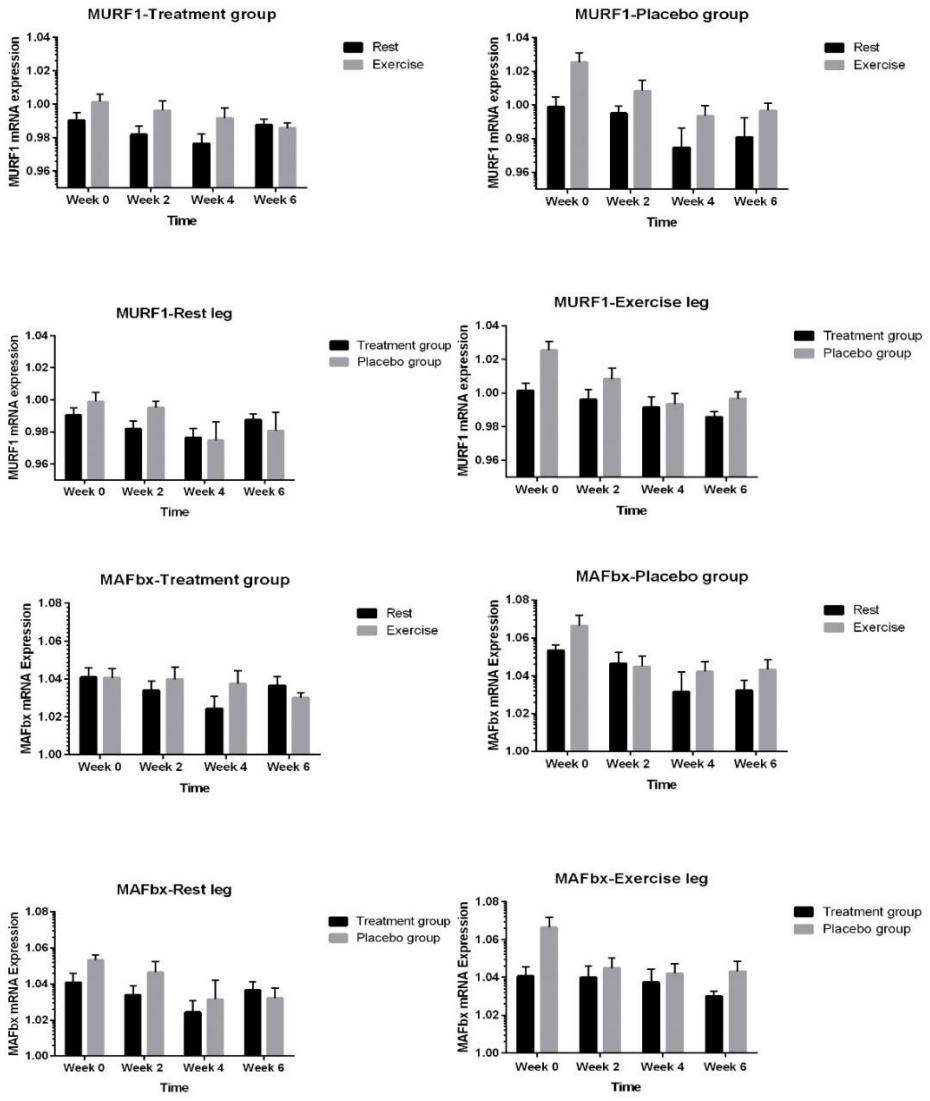
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Appendix:

qRT PCR (MURF1 and MAFbx):

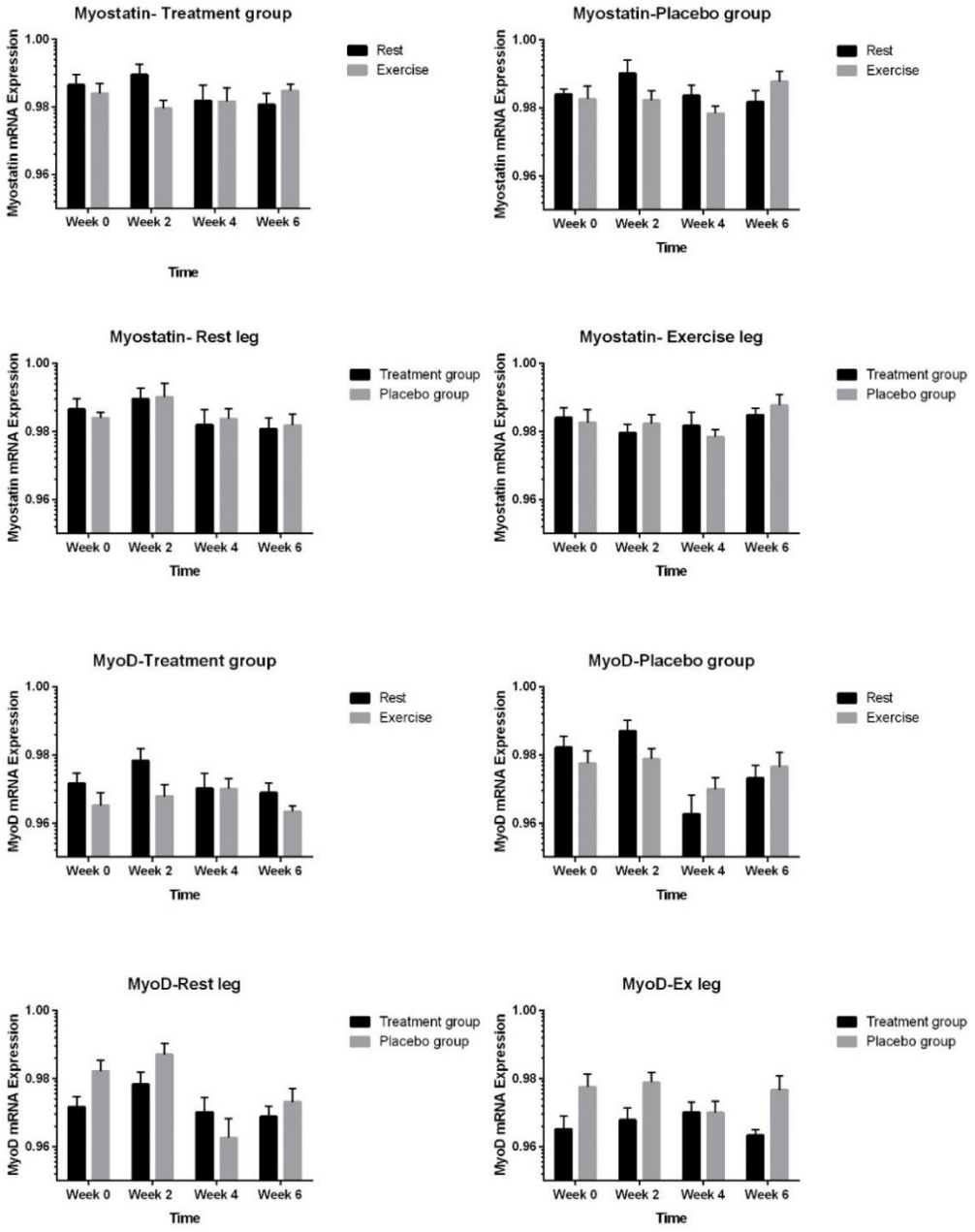
Figure 19 – qRT PCR (MURF1 and MAFbx)



Murf1 and MAFbx mRNA expression following resistance exercise in rest and exercise legs of placebo and treatment groups. No significant changes for exercise and supplementation were observed for the genes measured.

qRT PCR (Myostatin and MyoD):

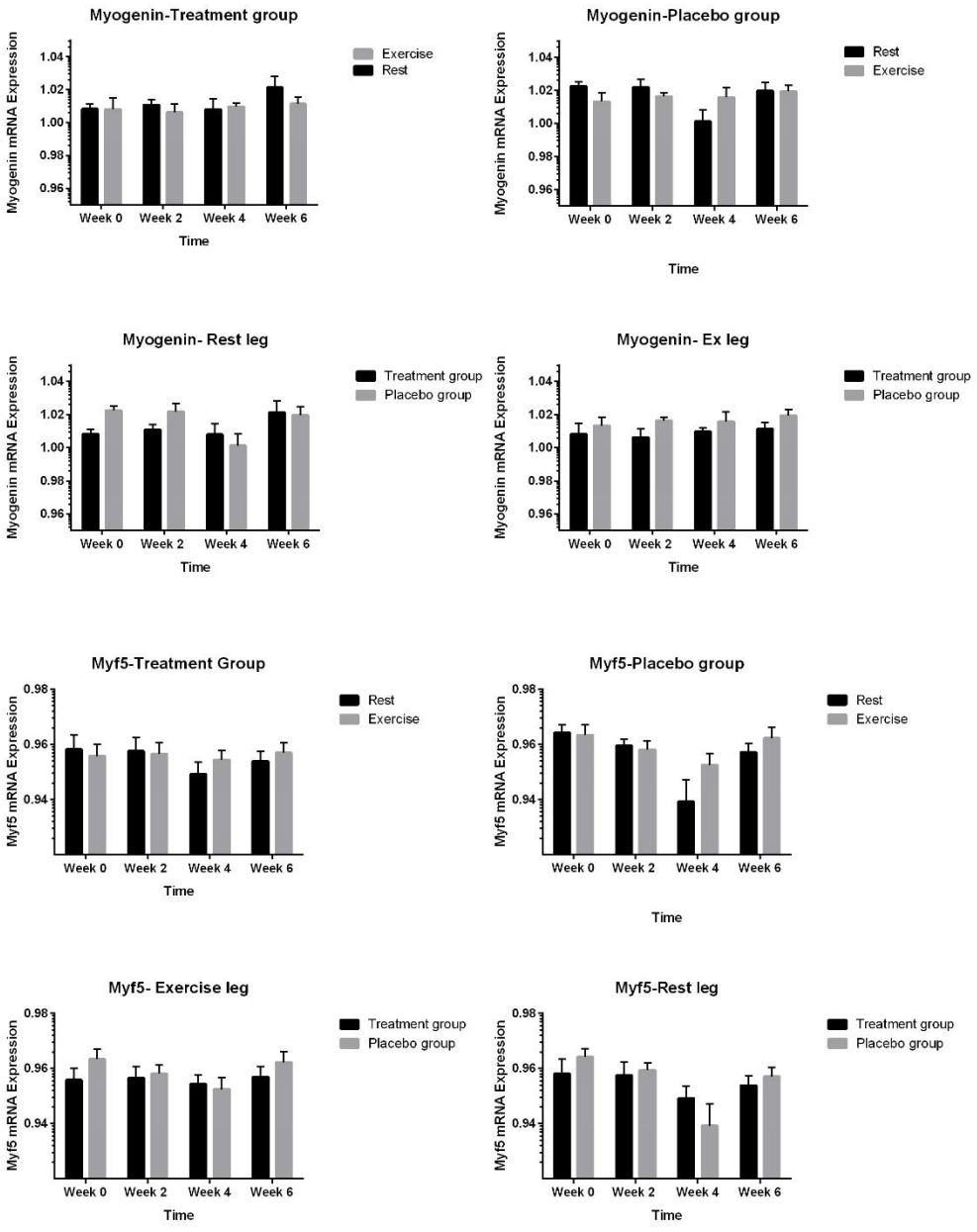
Figure 20 - qRT PCR (Myostatin & MyoD)



Myostatin and MyoD mRNA expression following resistance exercise in rest and exercise legs of treatment and placebo groups. No significant changes for exercise and supplementation were observed for the genes measured.

qRT PCR (Myogenin and Myf5):

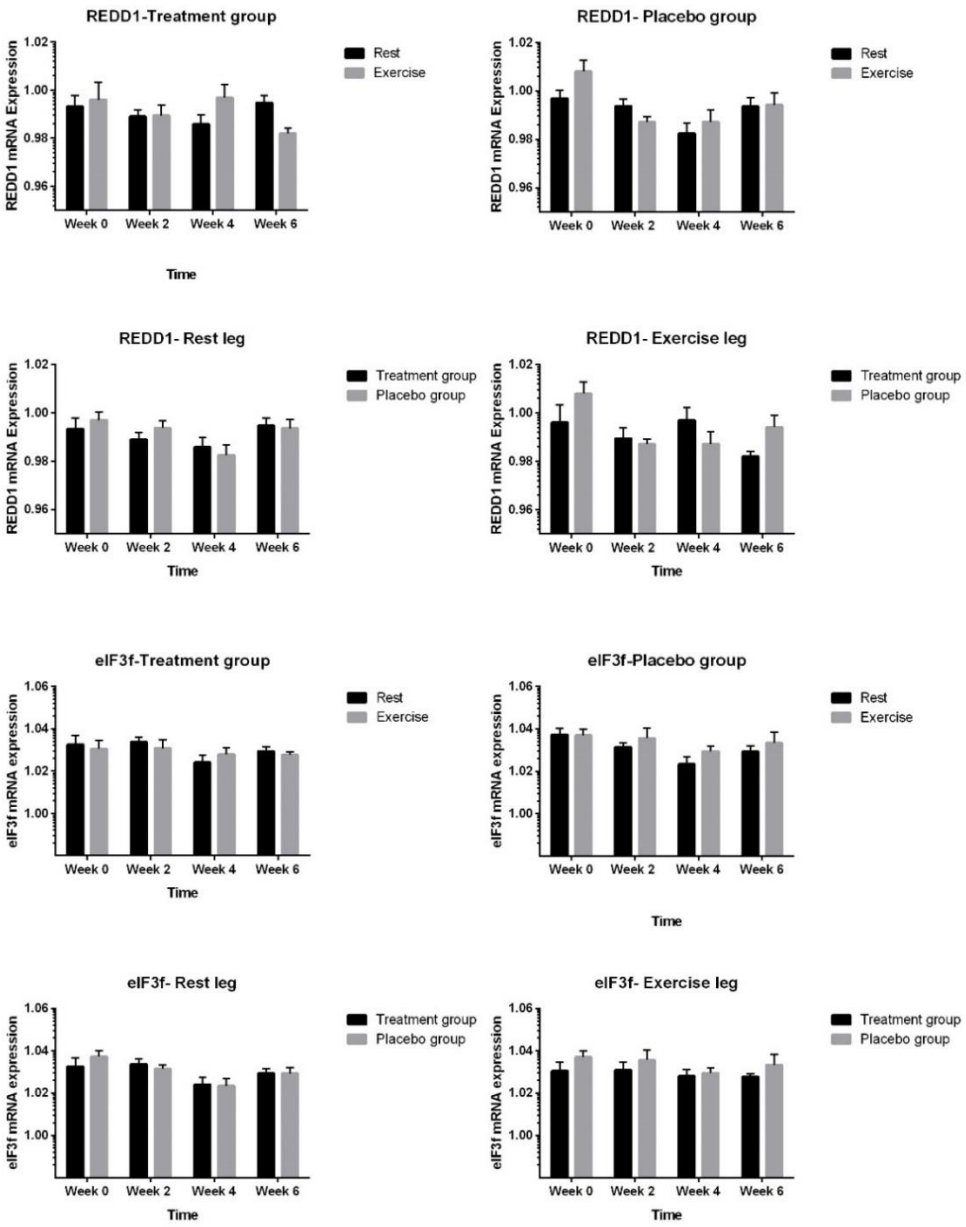
Figure 21 – qRT PCR (Myogenin and Myf5)



Myogenic regulatory factors (myogenin and Myf5) mRNA expression. No significant changes were observed.

qRT PCR (REDD1 and eIF3f):

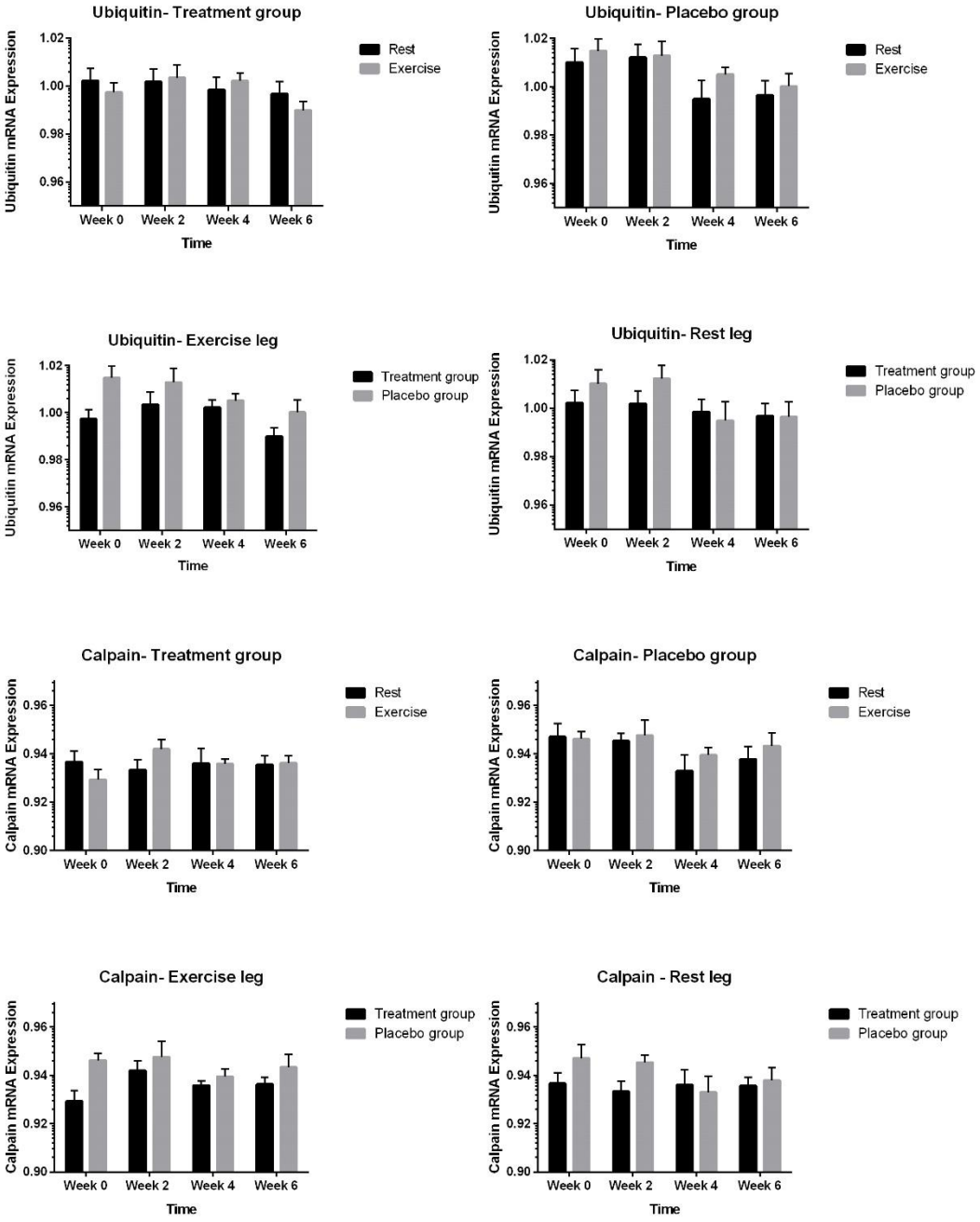
Figure 22 – qRT PCR (REDD1 & eIF3f)



REDD1 (negative regulator of mTOR) and eIF3f mRNA expression following resistance exercise in rest and exercise legs of placebo and treatment groups. No significant changes for exercise and supplementation were observed for the genes measured.

qRT PCR (Ubiquitin and calpain-1):

Figure 23 - qRT PCR (Ubiquitin and Calpain-1)



Ubiquitin and calpain mRNA expression following resistance exercise in rest and exercise legs of treatment and placebo groups. No significant changes for exercise and supplementation were observed for the genes measured.