Insulin Resistance in Health and Disease: Exploring the Role of Disuse Skeletal Muscle Atrophy, Testosterone and Beta-Hydroxy-Beta-Methylbutyrate (HMB) in young and older men

Supreeth Shanthaveerappa Rudrappa

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Abbreviations:

1-RM: One repetition maximum

3MH: 3-Methylhistidine

AA- Amino acids

ABR: Absolute breakdown rate

Akt: Protein Kinase B

AMPK: Adenosine monophosphate-activated protein kinase

AR: Androgen Receptor

AS160: Akt substrate of 160 kDa

ASP: alkaline soluble protein

ASR: Absolute synthetic rate

AUC; Area under curve

CaMK: Ca2+/calmodulin-dependent protein kinase.

CS: Citrate synthase

CSA: Cross sectional area

d – day or days

D_2O: Deuterium Oxide

DXA: Dual-energy X-ray absorptiometry

EAA: Essential Amino Acids

ERK ½: extracellular signal-regulated kinase ½

FBR: Fractional breakdown rate

FFM: Fat free mass

FGR: Fractional Growth Rate

FSH: Follicle-Stimulating Hormone

FSR: Fractional Synthetic Rate

GIR: Glucose infusion rate

GLUT: Glucose Transporter

h – Hour or Hours

HMB: Beta-Hydroxy-Beta-Methylbutyrate

HOMA: Homeostatic model assessment

IGF-1: Insulin like Growth Factor-1

IR: Insulin Resistance

LBM: Lean Body Mass

LH: Luteinising Hormone

LM: Legs fat free mass

min: Minute or Minutes

MPB: Muscle protein breakdown

MPS: Muscle protein synthesis

MRI- Magnetic Resonance Imaging

MRF: Myogenic regulatory factor

MT: Muscle thickness

mtDNA: mitochondrial DNA

mTOR: Mammalian target of rapamycin

MVC: Maximal voluntary contraction

OGTT: Oral glucose tolerance rate

P: Placebo

p70S6K: Ribosomal protein S6 kinase

PA: Pennation angle

PI3K: Phosphatidylinositol-3-kinase

RET: Resistance Exercise Training

ROS- Reactive Oxygen Species

T: Testosterone

TFM: Total fat mass

TFP: Total fat percentage

VL: Vastus lateralis

WHO: World Health Organisation

Chapter 1: Introduction- Setting the scene

It is now widely recognised that prevalence of type 2 diabetes is on the rise to a pandemic proportion. The World Health Organisation (WHO) estimates that there is 422 million people with diabetes (WHO, 2016a) and the global prevalence of diabetes was 8.5% in 2014 (mainly due to rise in type 2 diabetes) and reduced physical activity and poor diet is implicated as the most likely explanation (WHO, 2016b). Traditionally, physical activity and physical fitness has been linked with health; earliest evidence of exercise for health promotion stems from ancient China in 2'500 BC (Hardman and Stensel, 2003). Conversely, the widely available evidence also supports the conclusion that physical inactivity is one of the most important public health menace facing the 21st century and, may even be the most significant (Blair, 2008).

Furthermore, the worldwide demographics has changed; with increased life expectancy and ageing population, comes its own chronic health issues and economic implications (Phillips, 2012). Supporting this, Knoops et al., reported that 580 million people worldwide are over 60 years and this figure is expected to increase to 1000 million by 2020 (Knoops *et al.*, 2004) and to 2.1 billion by 2050 (United Nation, 2017). Worryingly, one of the significant and most visually apparent changes with ageing is decreased muscle mass and function loss and historically, this concept has been supported by various researchers (Evans and Lexell, 1995; Narici and Maffulli, 2010). This undesirable loss of

muscle mass due to ageing is termed ‘Sarcopenia’ (Rosenberg, 1997)(see section 1.1). Various theories (including some putative) have been put forward to explain the cause of sarcopenia: 1) Genetic predisposition; 2) Alteration in muscle protein turnover; 3) changes in hormones and cytokines (such as androgens, insulin, growth hormone, insulin like growth factor-1 (IGF-1) and interleukin-6 (IL)-6); 4) Loss of innervation to the muscle; 5) Impaired vascularisation of the muscles; 6) Increased oxidative stress; 7) Poor nutrition and 8) Physical inactivity (Phillips, 2012). Irrespective of operational definitions and causative theories, what is clear however is that, there is high prevalence of low muscle mass and poor muscle strength with ageing (Kalyani, Corriere and Ferrucci, 2014). It is now clear that with ageing, the prevalence of chronic diseases such as diabetes, obesity and cardiovascular diseases increase and further, approximately one-third of adults aged over 65 years have diabetes (46% undiagnosed)(Kalyani and Saudek, 2010). Moreover, up to 70% of adults with diabetes have difficulties in carrying out activities of daily life, more evidently lack of lower extremity mobility limitations, and diabetes is highlighted as a significant risk factor for most geriatric syndromes (Kalyani and Saudek, 2010). Consequently, to improve public health, it is paramount to understand the factors responsible for the functional disability (and mechanisms of sarcopenia) in older population, especially with diabetes (Kalyani and Saudek, 2010).

Continuing with the earlier theme, insulin resistance (IR) is noted to be more prevalent in older compared to younger individuals and is further associated

with frailty (a geriatric condition of physiological vulnerability to stressors) leading to disability and mortality (Fried *et al.*, 2001; Bandeen-Roche *et al.*, 2006). Moreover, hyperglycaemia (noted in IR state) is strongly linked to the development of frailty and incident mobility limitations, potentially mediated by loss of muscle mass (Kalyani *et al.*, 2012). Evidence from large cross-sectional and longitudinal studies report accelerated loss of muscle mass in individuals with persistent IR (i.e. people with diabetes)(Park *et al.*, 2007, 2009; Kuo *et al.*, 2009; Volpato *et al.*, 2012; Kalyani *et al.*, 2013; Leenders, Verdijk, van der Hoeven, *et al.*, 2013). Emerging evidence suggests type 2 diabetes accelerates skeletal muscle loss, possibly due to mechanisms specific to diabetes per se. Crucially, muscle specific insulin resistance due to disuse atrophy (common in old age and frailty) appears to drive further muscle loss independent of other mechanisms known to cause muscle insulin resistances. However, molecular mechanism explaining this observed complex relationship is still poorly understood. So, further mechanistic research is needed to evaluate these molecular processes and pathways (see section 1.3 for details).

On a separate note, the paradigm that testosterone increases skeletal muscle mass is well established (Bhasin *et al.*, 1996a) and on the contrary, its deficiency causes decreased appendicular skeletal muscle, decreased lean tissue and increased body fat (Boxer *et al.*, 2005). Furthermore, testosterone deficiency is common in diabetes and is partly explained by insulin resistance (Grossmann *et al.*, 2008). Conversely, insulin resistance in men is associated

with reduced Leydig cell testosterone production (Pitteloud, Hardin, *et al.*, 2005). Yet, the causality of this relationship between low testosterone and metabolic disease (type 2 diabetes and metabolic syndrome) is complex, with hypogonadism-induced obesity and obesity-induced hypogonadism acting bi-directionally effecting the disease pathology (Daniel M Kelly and Jones, 2013). As with disuse muscle atrophy and insulin resistance, the molecular mechanisms explaining the interplay between hypogonadism (low testosterone) and insulin resistance is still poorly understood. Hence, further research investigating these complex networking is warranted.

1.1 Sarcopenia: its current burden and effects on ageing population:

Ever since Rosenberg first coined the term 'sarcopenia' in 1989, it has been the topic of much interest to researchers and also one among the largest public health issues facing the ageing population world-wide today (Rosenberg, 1989). The European Sarcopenia Working Group (ESWG) defines sarcopenia as a "syndrome characterised by progressive loss of muscle mass and strength with a risk of adverse outcomes" (Cederholm, 2009). Prevalence of sarcopenia is estimated at 30% in population over 60 years and rises to more than 50% in those over 80-year-old (Gweon, Sung and Lee, 2010).

Furthermore, it is estimated that about 1 in 4 elderly people will have a serious fall each year due to sarcopenia (Doherty, 2003). Moreover, skeletal muscle acts as a major organ for glucose uptake (Kraegen *et al.*, 1990) and contributes significantly to basal energy expenditure (Radegran, 1999). It is

not surprising that sarcopenia is defined as a geriatric syndrome (i.e., a complex condition arising in ageing population which is very common and costly to manage) (Cederholm, 2009). Hence to reduce the risk of falls in elderly and to preserve the strength of the muscle necessary for daily activities, it is paramount that we aim to improve the incidence of sarcopenia (Doherty, 2003).

1.1.1 Ageing and Protein metabolism:

1.1.2 Aetiology and mechanism of skeletal muscle loss in ageing:

The aetiology of skeletal muscle loss due to ageing is a complex phenomenon and involves changes in central and peripheral nervous system and also alterations in hormonal, nutritional, immunological and physical activity (Narici and Maffulli, 2010). It has been reported that changes both in systemic and cellular properties plays a vital role in onset of sarcopenia. These systemic changes include increase in oxidative stresses, production of pro-inflammatory cytokines and decreased production in anabolic hormones and eventually these leads to cellular changes such as myocyte apoptotic signalling, altered protein synthesis and/ or turnover and impaired satellite cell function (Buford *et al.*, 2010). So mechanistically, sarcopenia can be attributed to 1) decreased muscle protein synthesis (MPS) and 2) increased muscle protein breakdown (MPB) (Altun *et al.*, 2010).

1.1.3 Nutrition intervention and skeletal muscle metabolism in aging population:

1.1.3.1 Signalling pathways involved in muscle protein metabolism:

Mammalian target of rapamycin (mTOR) is the major system for stimulation of MPS. This system senses four important stimuli or pathways responsible for the regulation of MPS, which are: 1. Insulin and insulin-like growth factor-1 (IGF-1) through IRS (Insulin receptor substrate) and PI3K (Phosphatidylinositol-3) pathway; 2. Amino acids through PI3K pathway, 3. Energy metabolism through AMPK (AMP activated protein kinase) pathway and 4. Resistance exercise through PLD (Phospholipase D) pathway (Wackerhage and Ratkevicius, 2008). Conversely, ubiquitin proteasome pathway (UPP) plays a major role in the regulation of MPB (Lecker, Goldberg and Mitch, 2006), supplemented by other system such as calcium activated (ATP–independent) proteolytic process and caspase-3 enzyme responsible for the initial cleavage of muscle myofibrils and actomycin (Glover, Yasuda, M. A. Tarnopolsky, *et al.*, 2010).

1.1.4 Nutrition and muscle protein metabolism:

Oral nutrition supplement remains a mainstay strategy to oppose the muscle loss in elderly (Milne, Avenell and Potter, 2006). Milne et al., concludes following his meta-analysis of 55 trials that showed positive weight change can be attained by giving oral protein and energy supplements to older people in the setting of hospitalised patients, long-term care patients and for elderly

living at home (Milne, Avenell and Potter, 2006). Furthermore, Gweon et al., reported that protein consumption of 0.08-0.8 g/kg body weight was associated with increased muscle protein fractional synthesis rate (FSR) in elderly and healthy young subjects (Gweon, Sung and Lee, 2010). It has been demonstrated that consumption of 1.2 -1.6g of protein per kg of body weight – or protein ingestion in excess of 12% of total calorie intake-can reduce the loss of muscle mass and older adults who consumed highest amount of protein lost least amount of muscle weight compared to adults consuming lowest amount of protein during a 3-year study (Houston *et al.*, 2008).

1.2 Human skeletal muscle disuse atrophy: effects on muscle protein synthesis, breakdown and insulin resistance - a qualitative review

1.2.1 Background:

The ever-increasing burden of an ageing population and pandemic of metabolic syndrome worldwide demands further understanding of the modifiable risk factors in reducing disability and morbidity associated with these conditions. Disuse skeletal muscle atrophy (sometimes referred to as “simple” atrophy) and insulin resistance are ‘non-pathological’ events resulting from sedentary behaviour and periods of enforced immobilisation e.g., due to fractures or elective orthopaedic surgery. Yet, the processes and drivers regulating disuse atrophy and insulin resistance and the associated molecular events remain unclear – especially in humans. The aim of this review is to present current knowledge of relationships between muscle protein turnover, insulin resistance and muscle atrophy during disuse, principally in humans. Immobilisation lowers fasted state muscle protein synthesis (MPS) and induces fed-state ‘anabolic resistance’. While a lack of dynamic measurements of muscle protein breakdown (MPB) precludes defining a definitive role for MPB in disuse atrophy, some proteolytic “marker” studies (e.g., MPB genes) suggest a potential early elevation. Immobilisation also induces muscle insulin resistance (IR). Moreover, the trajectory of muscle atrophy appears to be accelerated in persistent IR states (e.g., Type II diabetes), suggesting IR may contribute to muscle disuse atrophy under these

conditions. Nonetheless, the role of differences in insulin sensitivity across distinct muscle groups and its effects on rates of atrophy remains unclear. Multifaceted time-course studies into the collective role of insulin resistance and muscle protein turnover in the setting of disuse muscle atrophy, in humans, are needed to facilitate the development of appropriate countermeasures and efficacious rehabilitation protocols.

1.2.2 Human Skeletal Muscle Tissue:

Skeletal muscle tissue represents the largest protein/ amino acid (AA) reservoir in the human body (Bonaldo and Sandri, 2013). Skeletal muscles are not only crucial for locomotion but also represent the body's largest metabolically active tissue, glucose disposal site and fuel reservoir for other organs in fasting and pathological conditions (i.e., hepatic supply of amino acids for gluconeogenesis). Loss of muscle mass occurs with many common illnesses (Evans, 2010) including cancers (Stephens *et al.*, 2010), renal/heart failure (Gordon, Kelleher and Kimball, 2013), sepsis (Gordon, Kelleher and Kimball, 2013), muscle genetic diseases (Sandri, 2010) and neurodegenerative disorders (Verdijk *et al.*, 2012). Muscle atrophy also occurs in otherwise healthy individuals under situations of reduced neural input - such as during immobilisation e.g. due to leg casting for fractures (Phillips, Glover and Rennie, 2009a), bed-rest, spinal cord injury (Castro *et al.*, 1999) and during 'ageing' (i.e. sarcopenia) (Evans and Lexell, 1995). The main environmental determinants of muscle mass in adulthood are exogenous

essential amino acids (AA) (needing to be acquired through dietary protein intake), Newton's gravity and locomotion (DeFronzo and Tripathy, 2009). Indicative of this, lack of energy intake during starvation (Rennie *et al.*, 2010), inactivity (Wall, Dirks and Van Loon, 2013), spaceflight (Vandenburgh *et al.*, 1999) or limb immobilisation (Phillips, Glover and Rennie, 2009b) all lead to a reduction in muscle cross sectional area, an associated loss of function, and muscle insulin resistance. Crucially, loss of muscle mass is associated with greater morbidity and mortality (Sasaki *et al.*, 2007), reduced independence, especially in older populations (Leenders, Verdijk, Van Der Hoeven, *et al.*, 2013) and this is accelerated in type 2 diabetes (Leenders, Verdijk, van der Hoeven, *et al.*, 2013)

1.2.3 Overview of disuse atrophy, countermeasures and muscle metabolism:

Disuse atrophy is often referred to as "simple atrophy" in that atrophy is intrinsic to the muscle(s) specifically exposed to disuse; that is, disuse atrophy is not a systemic condition. Countermeasures for disuse atrophy are limited but include forms of mechanical loading (Wilkinson *et al.*, 2008) such as exercise/ electrical stimulation (Wall *et al.*, 2012), passive physiotherapy (Fowles *et al.*, 2000) and harnessing the adjunct anabolic effects of protein nutrition (T. A. Churchward-Venne *et al.*, 2012). Ascertaining an understanding of the mechanisms of disuse atrophy - particularly in relation to the regulation of muscle protein synthesis (MPS) and breakdown (MPB) - is important for designing countermeasures or rehabilitation protocols (Reggiani,

2015). Furthermore, despite accumulating evidence that physical inactivity plays a causative role in development of non-communicable diseases such as obesity, insulin resistance, type 2 diabetes and dyslipidaemia (Atherton *et al.*, 2016), the mechanistic role of muscle insulin resistance (IR) in driving muscle atrophy in the context of 'simple disuse' remains unclear (Atherton *et al.*, 2016). This review will focus on identifying different models of human disuse atrophy, the degree of muscle and strength loss and the regulation of muscle protein turnover and muscle IR. Future translational studies to mitigate disuse atrophy will rely upon robust evidence being present in humans of active mechanisms (often of putative mechanisms that have been pre-identified in animals). As such, this review will focus mainly on current evidence from clinical studies.

1.2.4 The impacts of experimental disuse on muscle mass and strength loss in humans:

A plethora of clinical studies have investigated the degree of muscle loss in humans exposed to disuse. The most frequent employed models to study disuse atrophy in humans are unilateral limb suspension (ULLS) using a knee brace or cast, and bed rest; other scenarios include spinal cord injury and spaceflight. In terms of muscle mass, the observed rate of decline in muscle size (cross sectional area (CSA)) for each day of ULLS in knee extensors was ~0.40% and ~0.36% for plantar flexors following 42 d of unloading (Hackney and Ploutz-Snyder, 2012). Other studies have demonstrated losses of muscle

strength and mass early on in disuse, i.e. 5 d of cast immobilisation lead to ~3.5% reductions in quadriceps CSA and ~9% in strength (Dirks *et al.*, 2014). This had progressed to ~8% reductions in CSA and ~23% reductions in strength by 14 d (Wall, Dirks and Van Loon, 2013). Additionally, Suetta et al. reported ~10% reductions in myofibre area and ~13% decreases in strength after just 4 d progressing to ~20% reductions in myofibre area and strength after 14 d of ULLS (Suetta *et al.*, 2012, 2013). A further study reported decreases in mid-thigh CSA of 11% following 28 d of bed rest (Brooks *et al.*, 2008). Lastly, a study by Castro et al., showed muscle CSA to be ~45% less compared to able-bodied controls 6-weeks after complete spinal cord injury (Castro *et al.*, 1999). Adding to the above constellation, Gibson et al., studied men who were immobilised following tibial fracture (thus having 6- weeks of casting) and reported reductions in quadriceps CSA of ~17% (Gibson *et al.*, 1987). Furthermore, Alkner et al., reported that 90 d bed rest led to ~10% and ~16% reductions in quadriceps and triceps surae mass after 29 d, with rates of weekly loss slowing during the last 2 months to roughly half that observed during the first month (Alkner and Tesch, 2004). Finally, muscle CSA decreased by ~5% (de Boer *et al.*, 2007; Glover, Yasuda, M. A. Tarnopolsky, *et al.*, 2010) at 14 d and 10%, at 23 days (23 d), i.e. 0.5% a day following ULLS (de Boer *et al.*, 2007). Collectively, these studies indicate a varying degree of rates of disuse muscle atrophy, depending on the duration and nature of immobilisation but also measurement techniques i.e. MRI/DXA/ultrasound/ myofibre CSA; however, it appears atrophy occurs more rapidly in first 3-14 d

of unloading and eventually reaching a nadir where further loss of muscle occurs at a slower rate despite continued unloading of muscle (Bodine, 2013).

Differences in the rate of muscle atrophy have also been observed according to different muscle and fibre types as well as the mode of immobilisation. For example, after prolonged disuse (~180 d of space flight), loss of fibre size and force was reported in the soleus and gastrocnemius muscles with the order of atrophy (greatest-least) being: soleus type I> soleus type II > gastrocnemius type I > gastrocnemius type II (Fitts *et al.*, 2010). Similar effects of disuse on fibre type following 35 d of bed rest was reported in the vastus lateralis (VL) muscle, i.e. the loss of fibre CSA was greater in type 1 than type II fibres (Brocca *et al.*, 2012). Conversely, muscle fibre type specificity has not been observed in other studies (Bamman *et al.*, 1998; Trappe *et al.*, 2008; Hvid *et al.*, 2010) where duration of immobilisation was shorter (<14 d). It is notable that; these studies have mainly focused on a single muscle with muscle biopsy taken from a single site in the periphery of the muscle. This is relevant because muscles do not atrophy uniformly across the entire length of a single muscle (Miokovic *et al.*, 2012), with differential atrophy across different muscles being observed following 27-60 d head-down-tilt bed rest. The investigators also reported that the posterior calf muscles atrophied faster than the knee extensor muscles (Vastus Lateralis) and ankle flexors (Tibialis anterior). In another study where multiple muscles were examined using MRI over 43 d disuse in the form of ankle immobilisation, the greatest rate of muscle loss was observed in soleus and medial gastrocnemius muscle followed by lateral

gastrocnemius and tibialis anterior (Psatha *et al.*, 2012). The aetiology driving distinct fibre type, and individual muscle atrophy susceptibility is poorly defined.

1.2.5 Regulation of skeletal muscle mass in ambulated and unloaded human muscle:

1.2.5.1 Ambulated regulation of MPS and MPB:

Skeletal muscle mass is regulated by the balance between MPS and MPB. Nutrients (i.e., AA) and nutrient derived hormones (i.e., insulin) play a crucial role in regulating the balance between MPS and MPB. In ambulated humans, intake of dietary protein stimulates MPS due to the essential amino acids (EAA) components of proteins (Atherton and Smith, 2012). These anabolic responses are dose-dependent and saturable; at a maximal stimulus, rates of MPS increase ~200-300% for a period of approximately 2 h following ~20 g protein (Cuthbertson *et al.*, 2005; Atherton and Smith, 2012). In contrast, insulin released following intake of dietary protein and/or CHO, is neither necessary nor sufficient to stimulate MPS (Greenhaff *et al.*, 2008). Reflecting this, the anti-catabolic effects of insulin upon MPB was not recapitulated via AA infusions when insulin concentrations were clamped at 5 $\mu U.ml^{-1}$ (post absorptive) (Greenhaff *et al.*, 2008). Instead, insulin concentrations of just 15 IU/ml (3× post absorptive) are sufficient to maximally suppress MPB (Wilkes *et al.*, 2009). This anti-catabolic effect of insulin acting on MPB was confirmed in a recent systematic review and meta-analysis of 44 human studies, which concluded insulin did not significantly affect MPS but has a crucial role in

reducing MPB (Abdulla, Smith, Philip J. Atherton, *et al.*, 2015). Therefore, while EAA's stimulate MPS, insulin suppresses MPB (and stimulates muscle glucose uptake). On the basis, EAA and insulin are so vital in maintaining muscle metabolic homeostasis, failure of these mechanisms inevitably leads to skeletal muscle atrophy and IR.

1.2.5.2 Impact of Disuse on MPS and anabolic pathways:

Disuse of human skeletal muscle alters muscle metabolism dynamics (Mallinson and Murton, 2013). For instance, early work by Gibson et al., showed that young men exposed to ULLS exhibited ~30% slower rates of fasted state MPS compared to the contralateral non-immobilised limb (Gibson *et al.*, 1987). Subsequent studies confirmed reductions in MPS; for instance, ~50% reductions in MPS following 2- weeks of bed rest (Ferrando *et al.*, 1996; Paddon-Jones *et al.*, 2004) and ULLS with braces/casting (Elisa I Glover *et al.*, 2008). In further agreement with this, Kortebein et al., reported ~30% reductions in post-absorptive MPS during a 24 h period in older adults after 10 d of bed rest (Kortebein *et al.*, 2007). Crucially, blunting of MPS in response to muscle unloading is not restricted to fasted periods. Glover et al., reported that ULLS led to ~27% reductions in postprandial rates of MPS at both low and high doses of AA infusions (Elisa I Glover *et al.*, 2008). Similarly, Drummond et al., reported that 7 d of bed rest blunted fed state MPS following EAA ingestion (Drummond *et al.*, 2012). Similarly, 14 d of ULLS led to a ~30% reduction in MPS after ingestion of 20 g dietary protein (Wall *et al.*, 2013). On

this basis, available evidence strongly supports the notion that skeletal muscle atrophy in humans during a period of disuse is driven by blunting of both postabsorptive and postprandial MPS (Rennie *et al.*, 2010; Wall *et al.*, 2013).

The mTOR (mechanistic target of rapamycin) pathway is the major signal transduction network “hub” involved in the regulation of mRNA translation. Cell and rodent based research suggests this system senses important stimuli responsible for the regulation of MPS i.e. 1) insulin and insulin-like growth factor-1 (IGF-1) through IRS (insulin receptor substrate) and PI3K (Phosphatidylinositol-3) pathways (Wackerhage and Ratkevicius, 2008); 2) AA through leucyl-tRNA/Rag-mTORc1 pathways (Wackerhage and Ratkevicius, 2008), 3) Energy stress through AMPK-eukaryotic elongation factor (AMP activated protein kinase/ eEF2) pathway (Wackerhage and Ratkevicius, 2008), and 4) mechanical stress e.g. through mechano-sensory pathways (Wackerhage and Ratkevicius, 2008). However, the impact of these factors in the regulation of MPS in human remains unclear. For example, under ambulated conditions, one of the mTOR upstream effector, the class III PI3K hVps34 (human vacuolar protein sorting-34) was shown to be inhibited in amino acid starved state (basal condition) and increased activity of this protein appears to be concomitant with increased S6K1, suggesting it to be key player in mTOR signalling pathway (Dickinson and Rasmussen, 2011). Furthermore, intake of dietary protein typically leads to increase in phosphorylation status of class III PI3K hVps34 (Dickinson and Rasmussen, 2011), mTOR and its downstream substrates regulating mRNA translation, such as p70S6K

(Drummond *et al.*, 2012). However, in response to disuse, blunted phosphorylation of mTOR and p70S6K was shown after 2- weeks of immobilisation while, no significant increases in phosphorylation of mTOR and p70S6K were noted after immobilisation (Wall *et al.*, 2013). In contrast, others have reported no decreases in Akt/mTOR signalling despite reductions in MPS (de Boer *et al.*, 2007; Wackerhage and Ratkevicius, 2008). It is worthwhile noting that peak stimulation of signalling proteins (mTOR and p70S6K) occurs 1 to 2 h after protein intake while many studies have muscle biopsies taken 3 to 6 h after protein intake when the response would be attenuated or perhaps absent (Glover, Yasuda, M. A. Tarnopolsky, *et al.*, 2010; West *et al.*, 2011; Drummond *et al.*, 2012; T. a Churchward-Venne *et al.*, 2012). Further studies are needed with frequent biopsy sampling to fully determine a role for deactivation in mTORC1-related signalling networks (or indeed other putative mechanisms) regulating depressions in post-absorptive and post-prandial MPS.

1.2.5.3 Impact of Disuse on MPB and catabolic pathways:

In contrast to the recognised deficits in MPS during immobilisation, the role of MPB in disuse atrophy is less clear. This is partly confounded by the technical and clinical challenges in measuring in vivo rates of MPB (e.g. arterio-venous balance methods to measure rate of appearance i.e. breakdown and pulse chase stable isotope approaches to measure fractional breakdown rates) (Kumar *et al.*, 2009; Atherton and Smith, 2012; Atherton *et al.*, 2016). In the

only study to our knowledge to measure this, Symons et al., demonstrated no increase in MPB in his study of healthy young volunteers exposed to 21 d of microgravity setting using a bed rest model (Symons *et al.*, 2009). Although not directly quantifying MPB, Wall et al., reported that muscle free tracer enrichment over 4-hour (4 h) post prandial period was >3 fold higher after immobilisation (Wall *et al.*, 2013). Since MPS has consistently been shown to be reduced with immobilisation, a likely explanation is that MPB is actually reduced (rather than increased) and hence the less unlabelled phenylalanine efflux was diluting the muscle free labelled tracer (L-(ring-2H5)) phenylalanine pool (Wall *et al.*, 2013). Alternatively, an accumulation of free tracer could simply be explained by established “anabolic resistance” i.e. where a failure of AA incorporation into the muscle lead to its accumulation (Elisa I Glover *et al.*, 2008; Phillips, Glover and Rennie, 2009b; Rennie *et al.*, 2010; Wall *et al.*, 2013). Nevertheless, regardless of the driving force behind muscle atrophy (i.e., disuse, ageing, cancer, organ failure), blunted postabsorptive and postprandial MPS (Figure 1-1) (anabolic resistance) seem to be the major drivers of disuse atrophy – rather than increases in MPB. Nonetheless, more work is needed across the time-course of unloading to verify this.

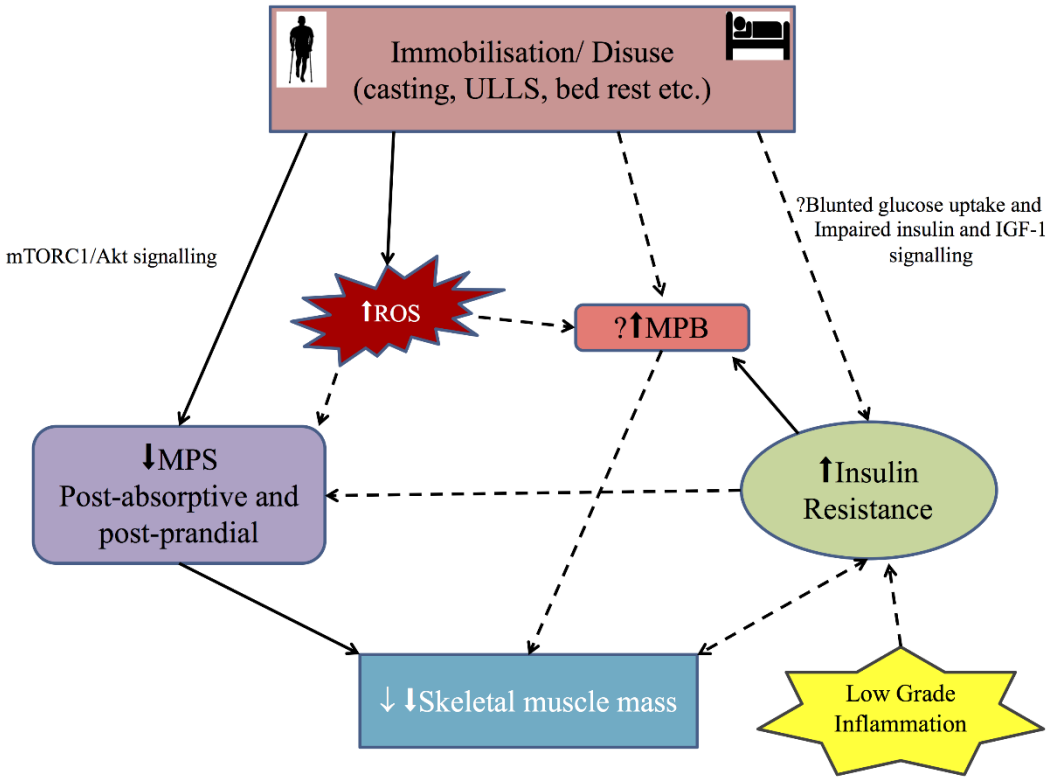

Figure 1-1- Diagrammatic representation of the main mechanisms involved in disuse skeletal muscle atrophy in humans. Immobilisation/ disuse reduces both postabsorptive and post prandial muscle protein synthesis (MPS) via the mammalian target of rapamycin (mTORC1) and Akt signalling. The role of MPS, muscle protein breakdown (MPB) and insulin resistance (IR) in simple disuse atrophy remain poorly defined in humans. So, the role of insulin resistance and MPB in the setting of disuse atrophy needs further evaluation. Inflammation probably leads to IR. Recently, reactive oxygen species (ROS) has been implicated in development of muscle atrophy in disuse setting, but the mechanism in human remains putative. Solid arrow shows positive association and broken arrow shows putative association. See text for more details.

In terms of the molecular regulation of MPB, the ubiquitin proteasome system (UPS) (Lecker, Goldberg and Mitch, 2006) supplemented by lysosomal and calcium activated calpain (ATP–independent) and caspase dependent cleavage of actinomyosin complexes (Glover, Yasuda, M. a Tarnopolsky, *et al.*, 2010) are the major catabolic pathways in muscle. The identification of the "atrogenes" as genes that are uniformly upregulated irrespective of the atrophy stimulus (e.g. denervation, disuse, thermal injury) has received much attention as key drivers of atrophy programming (Bodine, Latres, *et al.*, 2001; Jones *et*

al., 2004; Milan *et al.*, 2015). This led to members of the Forkhead Box (Fox) O family (Fox1, 3 and 4) being identified as downstream targets of Akt pathway and as the main transcription factors regulating MAFbx/atrogin-1 expression (Sandri *et al.*, 2004). In terms of disuse atrophy, mRNA expression of two E3 ubiquitin ligases was initially found to be crucial in immobilisation, unloading and denervation induced muscle atrophy (Bodine, Stitt, *et al.*, 2001). These genes, MuRF-1 (Muscle Ring Finger-1) and MAFbx/atrogin-1 (Muscle Atrophy F-box), are expressed in skeletal muscle at low levels but rapidly induced in response to unloading (Bodine, Stitt, *et al.*, 2001). In humans, after 5 d (Dirks *et al.*, 2014) and 2 weeks (Jones *et al.*, 2004) of immobilisation, MAFbx and MuRF1 mRNA were reported to be elevated. Nonetheless, while their expression is thought to be regulated by transcription factors such as FOXO1, FOXO3a (Sandri *et al.*, 2004) and NF-kB (p50 and Bcl-3)(Wu, Kandarian and Jackman, 2011), no increase in mRNA expression in FOXO's were noted after 4 or 14 d of immobilisation (Suetta *et al.*, 2012). De Boer et al., reported the expression of MuRF-1 but not MAFbx mRNA was increased after 10 d of ULLS (Jones *et al.*, 2004; de Boer *et al.*, 2007), while both had decreased by 21 d (de Boer *et al.*, 2007). Furthermore, increases in UPP components (particularly UBE2E) were up-regulated 48 h following instigation of ULLS (Urso *et al.*, 2006). In contrast, a recent study by Brocca et al., found that muscle atrophy following ULLS found no change in mRNA expression of ubiquitin-proteasome and autophagy systems (Brocca *et al.*, 2015). Some work has been done in relation to autophagy (and calpain-signalling) in relation to human disuse

(Jones *et al.*, 2004). Autophagy is responsible for removing unfolded, damaged and dysfunctional proteins and organelles via the formation of autophagosomes for degradation by lysosomes (Sandri, 2010). Interestingly, up regulation of autophagy markers such as Beclin-1 suggested increased autophagosome formation and hence a higher activity of the macro-autophagy by 24 d of bed rest; nonetheless, other autophagy markers such as P62, LC3II/I ratio and cathepsin-L were not up-regulated (Brocca *et al.*, 2012). While details of the pathways discussed above are outside the scope of this review, the readers are referred to reviews by Bonaldo (Bonaldo and Sandri, 2013) and Sandri (Sandri, 2010). What is clear however is that without more clinical studies with time-course acquisition, including dynamic measures of MPB in tandem with molecular markers spanning different proteolytic systems, no firm conclusions can be made surrounding the mixed results regarding whether existing molecular data suggest MPB is increased, decreased or unchanged in response to disuse in humans.

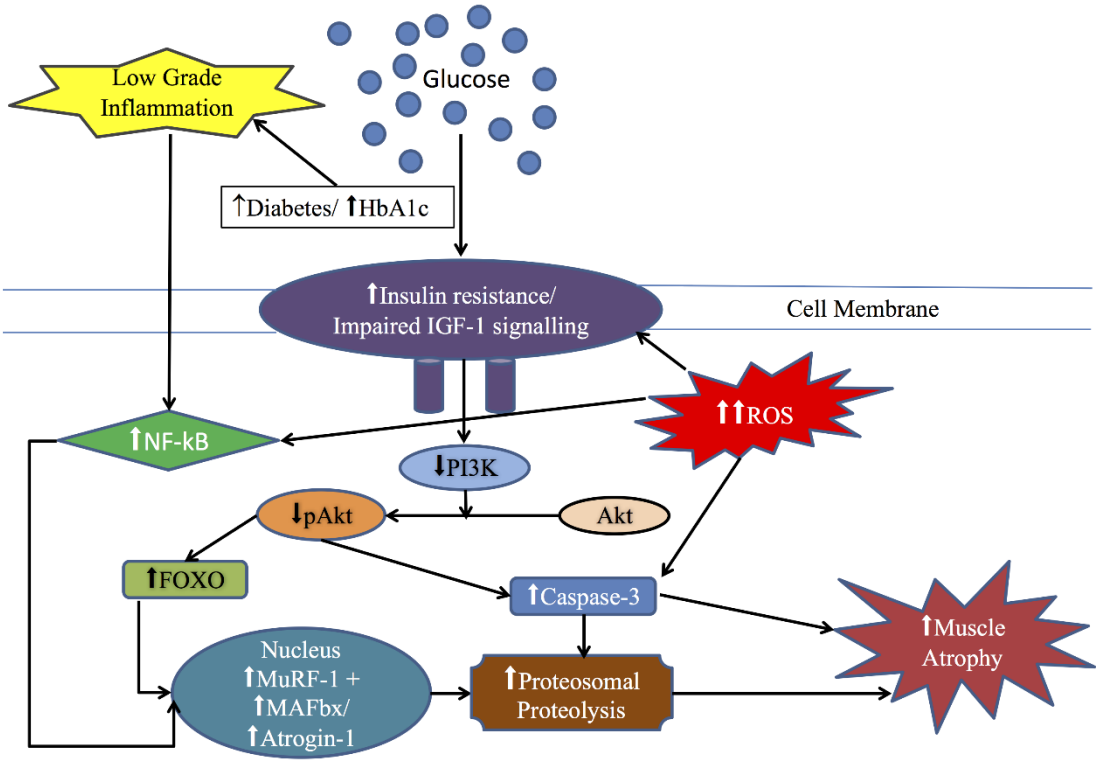

Figure 1-2- Diagrammatic representation of the overlap between insulin signalling pathway, reactive oxygen species (ROS), inflammatory cytokine such as NF-kB and ubiquitin-proteasome system in insulin resistant (IR) states particularly diabetes: In IR state, PI3K activity is decreased, leading to decreased activity of Akt, which in turn release the inhibition of FOXO and caspase-3 resulting in elevation of muscle ring finger-1 (MuRF-1) and muscle atrophy F-box (MAFbx/atrogin-1) finally leading to increased proteolytic activity. Also, ROS and low-grade inflammation via NF-kB pathway led to muscle atrophy. See text for more details.

In addition to the anabolic and catabolic pathways described above, emerging evidence indicates that disturbed redox signalling may also be an important regulator of MPS and MPB in muscle disuse atrophy (Powers, Smuder and Judge, 2012; Zuo and Pannell, 2015). Oxidative injury has been shown to occur in muscle fibres during periods of disuse in locomotor skeletal muscles (Min *et al.*, 2011) and in non-load bearing muscle such as the diaphragm during prolonged mechanical ventilation (Kavazis *et al.*, 2009). After 35 d of

bed rest, vastus lateralis muscle showed ~18% muscle fibre atrophy and increased protein carbonylation (Dalla Libera *et al.*, 2009). Furthermore, an inverse linear relationship was observed between normalised levels of protein oxidation and muscle fibre CSA (Dalla Libera *et al.*, 2009). An analysis of gene expression showed upregulation of pathways involved in the oxidative stress response (increase in mRNA for stress response gene heme oxygenase-1) following 48 h of unilateral lower leg suspension (ULLS) (Reich *et al.*, 2010). Conversely, in a limb immobilisation human model with ~5.7% muscle and 11.8% muscle fibre loss after 14 d of immobilisation, no increase in lipid peroxidation and protein oxidation in vastus lateralis was observed (Glover, Yasuda, M. a Tarnopolsky, *et al.*, 2010). Although information on oxidative stress and potential mechanisms explaining proteolysis in disuse human muscle is still sparse (Pellegrino *et al.*, 2011), these findings support the extensive evidence available from animal studies that oxidative stress inhibits MPS (Powers, Smuder and Criswell, 2011) and increases muscle MPB (via increased gene expression of key proteins involved in the proteolytic pathways such as autophagy, calpains and proteosomes; activation of both calpain and caspase-3 and possibly by modification of myofibrillar proteins which enhances their susceptibility to proteolytic processing)(Powers, Smuder and Criswell, 2011). Interaction between ROS and insulin signalling pathway has also been described, i.e. ROS may regulate Insulin growth factor-1 (IGF-1) signalling either positively or negatively depending on the amount of ROS produced (Papaconstantinou, 2010). Low levels of endogenous ROS due to

their reversible oxidative inhibition of protein tyrosine phosphatases induces phosphorylation of tyrosine residue on the insulin receptor and its substrates triggering IGF-1 signalling (Bashan, Kovsan and Kachko, 2009). In contrast, the IGF-1 signalling pathway is inhibited by higher levels of ROS and recent evidence suggests ROS down regulates the IGF-1 cascade and induces insulin resistance (Bashan, Kovsan and Kachko, 2009) (Figure 1-2). For detailed discussion of the signalling pathways linking ROS and muscle atrophy, the interested reader is referred to recent reviews on oxidative stress and disuse muscle atrophy (Pellegrino *et al.*, 2011; Powers, Talbert and Adhihetty, 2011; Powers *et al.*, 2014; Zuo and Pannell, 2015).

1.2.5.4 Impact of disuse on muscle IR and links to muscle mass in persistent IR states

Insulin-mediated glucose uptake is also blunted with muscle disuse (Mikines *et al.*, 1991; Biensø *et al.*, 2012); that is, unloaded muscle becomes IR. This IR can be observed at a whole-body level following bed-rest, but is most apparent at the muscle level across the physiological range of insulin concentrations under clamp conditions (Mikines *et al.*, 1991). Recently, a one week bed-rest study in young males by Dirks et al, revealed reduced muscle mass (~1.4 kg lean tissue and ~3% quadriceps CSA) and whole-body insulin sensitivity (~29%) (Dirks *et al.*, 2016). Thus, disuse lowers MPS, induces anabolic resistance to nutrients and impairs insulin-mediated muscle glucose uptake - even in healthy adults (Fink, 1983).

The role of IR in driving muscle atrophy however is poorly defined. Evidence from large cross-sectional and longitudinal studies report accelerated loss of muscle mass in individuals with persistent IR (i.e., people with diabetes), perhaps pointing to mechanistic links. For instance, declines in muscle mass were inversely related to duration of diabetes or HbA1c (Park *et al.*, 2007, 2009; Kalyani *et al.*, 2013) and attenuated with insulin sensitisers (Kuo *et al.*, 2009). Human muscle tissue accounts for 80% of glucose uptake after food ingestion and insulin resistance (HOMA-IR) is associated with reduced quadriceps muscle strength (Kalyani *et al.*, 2013; Leenders, Verdijk, van der Hoeven, *et al.*, 2013), power (Kalyani *et al.*, 2013) and muscle mass (Leenders, Verdijk, van der Hoeven, *et al.*, 2013) in humans. Approximately a 50% more rapid decline in knee extensor strength has been observed in older patients with type 2 diabetes compared with patients without diabetes over a 3 year period, suggesting that decreased muscle strength may be accelerated in type 2 diabetes (Park *et al.*, 2007). In a further study (Volpato *et al.*, 2012) it is reported that the differences in walking speed, muscle strength, power and muscle quality between individuals with and without diabetes were independent of co-existing peripheral motor neuropathy or peripheral vascular disease, suggesting a direct effect of diabetes per se on muscle performance. These findings are important because in catabolic conditions such as diabetes, atrophy in combination with reduced activity decrease quality of life and increase mortality (Zinna and Yarasheski, 2003). Yet, despite clear evidence linking accelerated muscle loss in diabetes compared to non-

diabetes, studies investigating the direct effect of immobilisation on muscle protein turnover in patients with diabetes compared to those without diabetes are scant. Furthermore, clear distinction between Type 1 and Type 2 diabetes needs to be made when investigating patients with diabetes. This is because Type 1 diabetes is a condition with severe depletion of energy stores and reduced mitochondrial function resulting in accelerated muscle protein loss (Hebert, Clinic and Sw, 2010), which can be reversed by insulin replacement (Workeneh and Bajaj, 2013). In contrast, muscle loss, whilst accelerated in type 2 diabetes, is unaffected by insulin treatment (Workeneh and Bajaj, 2013), possibly due to IR. Hence, skeletal muscle mass loss, whilst common, appears to occur less predictably and to varying degree in Type 2 diabetes compared with Type 1 diabetes (Workeneh and Bajaj, 2013). Collectively, these data are consistent with the notion that diabetes causes muscle mass loss possibly due to mechanisms driving muscle IR, however there is lack of data regarding the effects of immobilisation or disuse on muscle mass in individuals with diabetes.

The mechanistic regulation of muscle IR in driving muscle atrophy in the setting of ‘simple disuse’ remains vague (Atherton *et al.*, 2016). Early human studies by Shulman et al, showed that, under steady state plasma concentration of both glucose and insulin, mimicking postprandial conditions, the mean rate of muscle glycogen synthesis accounted for most of the whole body glucose uptake and virtually all of non-oxidative glucose metabolism in both healthy and diabetic subjects (Shulman, 2000), with defects in muscle

glycogen synthesis playing a major role in causing insulin resistance in type 2 diabetes (Shulman, 2000). This may be explained by defects in the insulin receptor substrate (IRS)-1/ phosphatidylinositol (PI) 3-kinase pathway, leading to reduced glucose uptake and utilisation in insulin target tissues (Draznin, 2006). Free fatty acids induce muscle IR by inhibiting glucose transport/phosphorylation and reductions in both the rate of muscle glycogen synthesis and glucose oxidation (Roden *et al.*, 1996). Additionally, many other mechanisms have been postulated to explain free fatty acid-induced muscle IR, including the Randle cycle, oxidative stress, inflammation and mitochondrial dysfunction (Martins *et al.*, 2012). Full details regarding above mechanisms escape the scope of this article and readers are referred to a review by Martins et al., (Martins *et al.*, 2012). With regard to disuse induced muscle atrophy, following (7 d) bed-rest healthy volunteers showed reduced glucose infusion rate and leg glucose extraction (after bed rest) along with reduced muscle GLUT4, hexokinase II, protein kinase B/Akt1, and Akt2 protein levels, and a tendency for reduced 3-hydroxyacyl-CoA dehydrogenase activity (Biensø *et al.*, 2012). Further in the same study, the ability of insulin to phosphorylate Akt and activate glycogen synthase was reduced post bed-rest (Biensø *et al.*, 2012); but whether this observation is causative or a consequence of immobilisation is not clear. However, a substantial decline in glucose uptake within 24 h of immobilisation would argue against a causative effect (Atherton *et al.*, 2016). Recently, Vigelsø et al. (Vigelsø *et al.*, 2016), showed an inverse association between the increase in muscle pyruvate

dehydrogenase complex (PDC) activation and leg lactate release during contraction after 2 weeks unilateral lower limb immobilisation, suggesting PDC as a potential key regulator of immobilisation induced muscle IR. Overall, the above data suggests that muscle disuse results in development of whole body and muscle specific IR, fuelling the argument that lack of muscle contraction per se may be the main physiological driver for this dysregulation, however a mechanistic explanation for this still remains unclear (Atherton *et al.*, 2016).

1.2.5.5 Summary:

Disuse muscle atrophy causes many undesirable complications. There seems to be complex interplay of numerous mechanisms contributing to the aetiology of disuse muscle atrophy. During muscle disuse, both post-absorptive and post-prandial MPS is suppressed, with little evidence to support there being an increase in 'bulk' protein MPB. Moreover, animal models show increased (2.5 times) rate of muscle protein turnover and are also very sensitive to disuse, while exhibiting marked fibre-type-dependant differences in rates of muscle protein turnover (type I fibres being twice as great as type II fibres) when compared to humans. Due to these inherent species-specific differences, pre-clinical findings cannot easily be reconciled with nor extrapolated to humans. So, further research quantifying MPS and MPB and their temporal relationship during disuse in humans is warranted. There is strong evidence that type 2 diabetes accelerates muscle loss, possibly due to mechanisms innate to diabetes. Crucially, muscle IR secondary to disuse

appears to drive the procession of disuse muscle atrophy independent of other mechanisms known to cause muscle IR. Nonetheless, the mechanistic role of muscle IR driving this atrophic response is poorly defined. Because, common proteolytic mechanisms may exist across ‘simple muscle atrophy’ and other catabolic conditions (e.g. type 2 diabetes, inflammation, cachexia etc.), these two process can rarely be seen as being mutually exclusive (Atherton *et al.*, 2016). Further, many questions remain unanswered especially the molecular regulation of MPS and MPB and muscle insulin resistance. This whole area of research has potential implications for the wider clinical community as similar metabolic processes occur during cancer cachexia, metabolic syndromes including type 2 diabetes, ageing (i.e., sarcopenia), sepsis and many neurodegenerative disorders. Henceforth, further translational research is necessary before this knowledge can be effectively applied in developing targeted strategies to prevent this in the setting of disuse muscle atrophy.

1.3 Testosterone: Effects on skeletal muscle protein and Glucose metabolism:

1.3.1 Physiology of testosterone:

Although, testosterone has been a focus of scientific society for almost 150 years due to its distinct biological effects, particularly sexual and physical effects. However, it still remains inspiration to new research, particularly its effects on human skeletal muscle physiology (Freeman, Bloom and McGuire, 2001). Testosterone (17β-hydroxy-4-androstene-3-one) is a 0.288kD C_{19} steroid hormone synthesised from cholesterol via a series of enzymatic process catalysed by specific enzymes (Mendelson, Dufau and Catt, 1975). Biologically synthesised testosterone has a powerful androgenic and anabolic effects (Kadi, 2008) and is mainly produced in Leydig cells of testes and small amounts is also produced in adrenal glands and other peripheral tissues (Freeman, Bloom and McGuire, 2001). The later, site of testosterone production is mainly spillover from the process of other hormones synthesis such as cortisol and aldosterone in the adrenal glands and estradiol in the ovaries respectively (Conley and Bird, 1997). The above two process explains how the adrenal gland and the ovaries can produce testosterone despite the absence of Leydig cells in these tissues (Vingren *et al.*, 2010).

1.3.2 Hypothalamic-Pituitary-Gonadal Axis:

The signal for gonadal testosterone synthesis and release originates from specialised neurons in hypothalamus, which produce and release

gonadotrophin releasing hormone (GnRH) (Kim, 2007). GnRH released directly in to the hypothalamic-hypophyseal portal vein, travels to anterior pituitary gland and stimulates the production and release of luteinising hormone (LH) and follicle-stimulating hormone (FSH) from the gonadotrophs (Kim, 2007). LH and FSH then enters the systemic circulation and are transported to the gonads. In the gonads, LH stimulates testosterone production in the Leydig cells of men and the theca cells of women. Binding of LH to a G-protein-coupled membrane receptor activates cyclic adenosine monophosphate-dependent protein kinases (protein kinase A), which in turn stimulates the rate limiting step in the testosterone production (Miller, 1988). As with any steroid hormones, testosterone cannot be stored in the cells they are produced and are released immediately in the blood stream following the their synthesis (Vingren *et al.*, 2010). However, in women testosterone is further processed in to estradiol in the granulosa cells adjacent to the theca cells (Vingren *et al.*, 2010). Interestingly, FSH does not appear to have direct effects on testosterone synthesis in men, however it is important in stimulation of steroid binding protein synthesis in the liver. Similarly, in women, FSH stimulates the synthesis of pregnenolone in the granulosa cells and steroid binding protein synthesis in the liver (Vingren *et al.*, 2010). The signaling pathway from FSH is similar to that induced by LH in the theca and Leydig cells and the pregnenolone synthesised in the granulosa cells travels to theca cells, where it is further processed to testosterone (Vingren *et al.*, 2010). Eventually, p450 aromatase produced due to FSH stimulation is responsible

for conversion of testosterone to estradiol in the granulosa cell. This complex signaling cascade from hypothalamus to the gonads leading to production and secretion of testosterone (and estradiol) is coined as the 'hypothalamic-pituitary-gonadal axis' (Figure 1-3) (Harris, 1951).

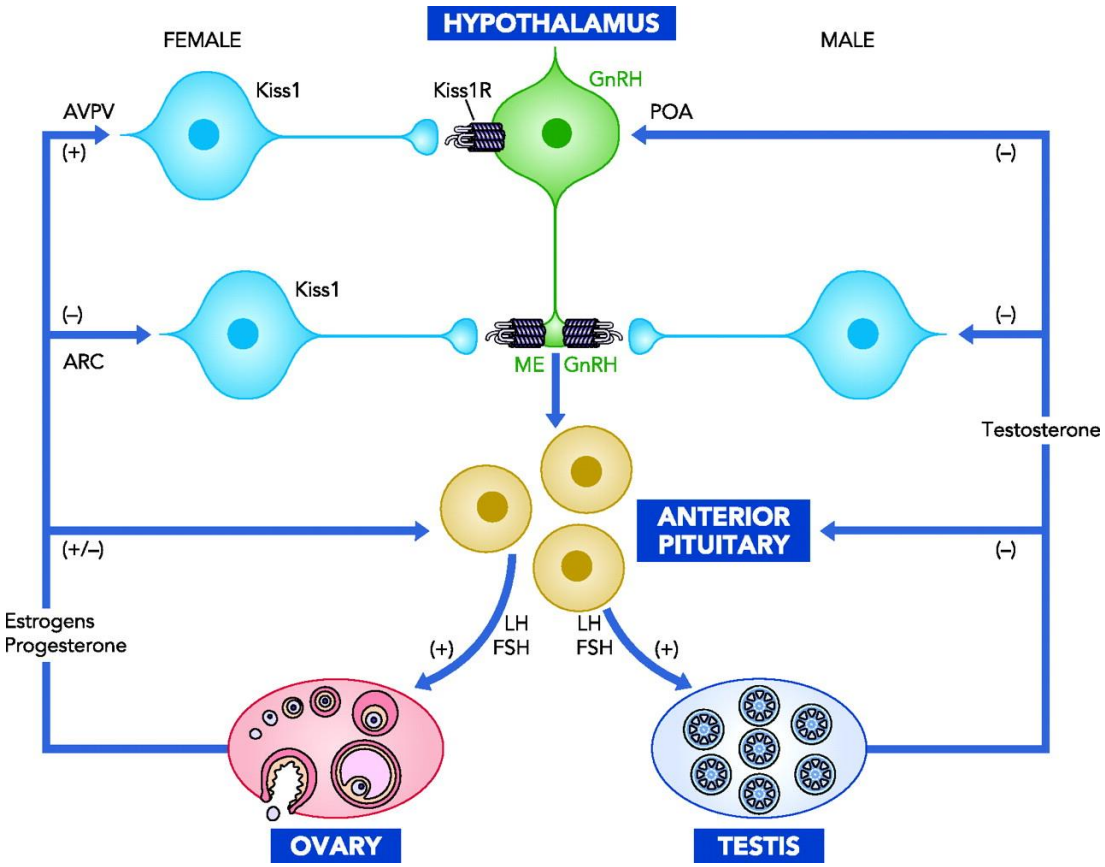

Figure 1-3- The mammalian hypothalamic pituitary gonadal axis. At puberty, pulsatile secretion of gonadotrophic releasing hormone (GnRH) stimulates the anterior pituitary to release the gonadotrophic hormones, luteinising hormone (LH), and follicle-stimulating hormone (FSH). These acts on the gonads to promote gamete formation and the production of gonadal steroid hormones, which form feedback loops to regulate GnRH, LH, and FSH release. Kisspeptin (Kiss1) neurons act as a principal relay for steroid feedback on GnRH secretion. In females, high levels of estrogens and progesterone stimulate kisspeptin neurons of the AVPV to induce the preovulatory surge of GnRH/LH, whereas they inhibit KISS1 expression in the arcuate nucleus (ARC). In the male, GnRH and gonadotrophic hormone release are negatively regulated by circulating testosterone, partly through the activity of kisspeptin neurons of the ARC. POA, preoptic area; AVPV, anteroventral periventricular nucleus; ME, median eminence. (adapted from (d'Anglemont de Tassigny and Colledge, 2010)

1.3.3 Effects of testosterone on skeletal muscle protein turnover:

Accumulating evidence suggests that testosterone produces muscle hypertrophy by enhancing fractional muscle protein synthesis (Urban *et al.*, 1995; Brodsky, Balagopal-F and Sreekuhl4ran, 1996). Furthermore, Urban *et al* (1995) states that testosterone stimulates the expression of IGF-1 and downregulates IGF binding protein-4 in the muscle. So, this reciprocal changes caused by testosterone between IGF-1 and its binding protein provide a potential mechanism for enhanced anabolic signal (Bhasin, Woodhouse and Storer, 2001). Conversely, suppression of endogenous testosterone production by administration of gonadotropin-releasing hormone (GnRH) analogue in young men resulted in marked decrease in the rate of whole-body muscle protein synthesis, in muscle strength and in fat oxidation together with increase in muscle adiposity (Mauras *et al.*, 1998). In addition, manipulation of the circulating testosterone level in addition to administering GnRH analogue showed existence of positive relationship between testosterone concentration and fat-free mass, muscle strength and size (Bhasin, Woodhouse and Storer, 2001). Moreover, attenuated physiological response to a period of 12 week strength training was noted in volunteers receiving GnRH analogue in a randomised double blind placebo controlled study (Kvorning *et al.*, 2006). Overall, the above studies support the prevailing notion that testosterone has profound anabolic effects on the human skeletal muscle protein synthesis.

1.3.3.1 Mechanisms of testosterone action on skeletal muscle:

The evidence exploring molecular mechanisms of testosterone action on skeletal muscle is still poorly understood (Mudali and Dobs, 2004). However, an early study (Griggs *et al.*, 1986) reported that muscle RNA was significantly elevated suggesting the possibility of testosterone acting via hormone receptor situated on muscle nuclei. Further, it is postulated that, testosterone appears to stimulate IGF-1 expression both directly and indirectly; resulting in muscle protein synthesis and growth, and also may counter the inhibitory effects of myostatin, cytokines and glucocorticoids (Mudali and Dobs, 2004).

1.3.3.2 Effects of testosterone on lean body mass (LBM) and muscle protein synthesis (MPS):

Early animals (increase in nitrogen retention in castrated animals) (Kochakian, Tilloston and Endahl, 1956) and human studies has shown that anabolic steroids, particularly testosterone administration increases lean body mass (LBM) and has a features of typical dose response curve (Forbes, 1985). Furthermore, in a study of healthy men receiving pharmacological dose (3mg/kg/week) of testosterone enanthate over 12 weeks showed ~20% mean increase in muscle mass estimated by creatinine excretion and MPS rate estimated by measuring (^{13}C) Leucine incorporation increased by ~27% mean in all 9 subjects (Griggs *et al.*, 1989). Supporting this, significant increase in MPS (estimated by rate of incorporation of stable isotope in to quadriceps muscle during primed infusion of L-[1-13C]leucine) following administration of

similar dose (3mg/kg/week) of testosterone was reported in 6 male patients with myotonic dystrophy (Griggs *et al.*, 1986). In a further study, 6 healthy old men receiving testosterone to increase their testosterone concentration equal to that of healthy young men reported significant increase in quadriceps and hamstring muscle mass and also in fractional synthetic rate of muscle protein (stable isotope infusion technique) (Urban *et al.*, 1995). Since then, few other studies in healthy hypogonadal men have reported increase in fat free mass ~5kg (~9.9%) (by underwater weight and D_2O) (Bhasin *et al.*, 1996a); ~7% (by bioelectrical impedance) (Katznelson *et al.*, 1996); ~15% (by DEXA scan) (Brodsky, Balagopal-F and Sreekuhl4ran, 1996); ~2% (by DEXA scan) (Wang *et al.*, 1996); ~3kg (by DEXA scan) (Snyder *et al.*, 2000) and ~2.7kg (by DEXA scan) (Wang *et al.*, 2000) following administration of various preparation of testosterone. Interestingly, Borst et al., reported ~4 kg increase in fat free mass in 60 older hypogonadal men receiving testosterone (125mg/week) in combination with finasteride (type II 5α-reductase inhibitor) highlighting the musculoskeletal effects of testosterone without the side effects of prostatic enlargement (in finasteride group) (Borst *et al.*, 2014). Collectively, these studies support the prevailing notion that, testosterone has significant anabolic effects both on skeletal muscle mass (lean body mass or fat free mass) and MPS both at physiological and pharmacological doses.

1.3.3.3 Effects of testosterone on muscle protein breakdown (MPB):

Despite a multitude of studies conducted over last few decades to look at effects of testosterone on muscle mass and MPS (as summarised in 1.3.3.2) there is still a lack of understanding of influence of testosterone on muscle protein breakdown. None of the above studies (as summarised in 1.3.3.2) measure muscle protein breakdown. The observed increase in muscle mass and protein synthesis in aforementioned studies may indicate that muscle protein breakdown was not increased (Rooyackers and Nair, 1997). In a recently concluded study involving 57 hemodialysis patients (35% patients having low testosterone concentrations), the testosterone concentration was independently and positively correlated with lean body mass (measured by bioimpedance method) suggesting that hypogonadism (low testosterone concentration) may be associated with increased muscle breakdown (Cobo *et al.*, 2017). However, further studies with direct measurement of MPB are needed to clarify this issue.

1.3.4 Effects of testosterone on skeletal muscle glucose disposal and insulin resistance:

There is now plethora of evidence to support the concept that testosterone plays a paramount role in regulating lean muscle mass and body fat composition, especially in men (Daniel M Kelly and Jones, 2013). Furthermore, testosterone deficiency is associated with increased fat mass (particularly truncal), decreased insulin sensitivity, impaired glucose tolerance,

elevated triglycerides and cholesterol and low HDL-cholesterol. Aforementioned factors are extremely prevalent in metabolic syndrome (MetS) and type 2 diabetes, leading to increased cardiovascular risk (Daniel M Kelly and Jones, 2013). Moreover, testosterone deficiency is associated with decreased lean body mass, and relative muscle mass is inversely related to insulin resistance and development of pre-diabetes (Srikanthan and Karlamangla, 2011). Given the knowledge that, up to 70% of body's insulin sensitivity is accounted by muscle tissue, testosterone deficiency may lead to insulin resistance via anabolic and/ or metabolic processes in the muscle (Pitteloud, Hardin, *et al.*, 2005). Worryingly, little is known about the influence of testosterone on insulin action (and molecular mechanisms involved) in skeletal muscle and subsequently, the effects on carbohydrate metabolism and risk of developing type 2 diabetes (Daniel M Kelly and Jones, 2013).

1.3.5 Molecular mechanisms of testosterone action on skeletal muscle glucose metabolism:

Early animal studies, has long established that castrated rats muscle has decreased glycogen storage levels in levator ani and perineal muscles and testosterone replacement induces significant increase in glycogen content (Leonard, 1952; Apostolakis, Matzelet and Voigt, 1963; Bergamini, Bombara and Pellegrino, 1969). Similarly, notable rise in muscle glycogen synthesis (due to increased testosterone-induced glycogen synthase activity) is observed following testosterone replacement in castrated rats compared to

their untreated controls leading to reduced blood glucose (Ramamani, Aruldhas and Govindarajulu, 1999). Indeed, testosterone replacement normalised glycogen phosphorylase activity in castrated rats resulting in reduced breakdown of muscle glycogen avoiding elevation in blood glucose (Bergamini, 1975). Conversely, the rate of glycogenesis was shown to be reduced in rat skeletal muscle following testosterone administration and increased glycogen content is postulated as possible reason than decreased glycogenolysis (Bergamini, 1975). In addition to aforementioned role of testosterone in skeletal muscle glucose uptake, action on few key enzymes involved in glycolysis is also postulated (Daniel M. Kelly and Jones, 2013). Elevated activity of phosphofructokinase and hexokinase is also reported in cultured rat skeletal muscle cells (Sato *et al.*, 2008). Supporting this, Bergamini et al., reported a maximal 40% increase in hexokinase activity in castrated rats 12 h after administration of testosterone compared to their untreated controls (Bergamini, Bombara and Pellegrino, 1969). Indeed, in a recent study of testicular feminised (TfM) mouse (wherein affected animal has low endogenous testosterone levels and non-functional androgen receptor (AR)) showed significantly reduced expression of hexokinase-2 in the skeletal muscle compared to their wild type (McLaren *et al.*, 2012). Interestingly, in the same study, testosterone replacement did not alter hexokinase-2 expression postulating involvement of AR-mediated mechanisms (McLaren *et al.*, 2012). Collectively, these animal studies support the argument that testosterone plays a very important role in glucose uptake (via insulin independent

mechanisms) especially in skeletal muscle and may explain the possible mechanism via which glucose disposal is impaired in testosterone deficient states.

1.3.6 Insulin action on skeletal muscle glucose homeostasis- in health and persistent insulin resistance state (Type 2 diabetes):

Skeletal muscle and adipose tissue glucose uptake via insulin are mainly mediated by GLUT4 glucose transporter isoform. In normal resting state, majority of GLUT4 molecules reside inside the membrane vesicles in the cell, following stimulation by insulin (via insulin receptors, activation of cascades involving insulin receptor substrate-1 (IRS1) and other signaling pathways) they migrate to cell membranes (Bryant, Govers and James, 2002). So, diminished GLUT4 expression correlates with reduced insulin sensitivity (Daniel M. Kelly and Jones, 2013) and in patients with type 2 diabetes, defects at the level of insulin receptor, IRS-1 and GLUT4 expression is observed (Pessin and Saltiel, 2000).

1.3.7 Effect of testosterone on skeletal muscle insulin sensitivity and resistance:

In relation to androgen (testosterone) action on glucose control, both GLUT4 and IRS-1 were up-regulated in cultured skeletal muscle cells and adipocytes following testosterone treatment at low dose and short incubation periods (Chen *et al.*, 2006). Likewise, IRS-2 mRNA expression was elevated in cells isolated from male human skeletal muscle biopsies following testosterone

treatment (Salehzadeh *et al.*, 2011). Furthermore, GLUT4 expression and translocation was elevated in cultured skeletal muscle cells of neonatal rats after treatment with testosterone (Sato *et al.*, 2008). Additionally, testosterone increased the phosphorylation of Akt and protein kinase C (PKC), a pivotal step in the insulin signaling cascade for regulation of GLUT4 translocation (Sato *et al.*, 2008). Crucially, these effects were opposed by dihydrotestosterone (DHT) inhibitors, implying that local conversion of testosterone to DHT and activation of AR is an important step in skeletal muscle glucose uptake (Sato *et al.*, 2008). Interestingly, patients with Kennedy disease (a neuromuscular disorder encompassing a genetic polymorphism with excessive CAG repeat in exon 1 of AR) leading to relative insensitivity of AR (Stanworth *et al.*, 2008, 2011). Furthermore, validating AR-mediated mechanisms, men with Kennedy disease have an increased risk of developing type 2 diabetes, which indeed strongly supports the link between impaired androgen status and type 2 diabetes (Stanworth *et al.*, 2008, 2011; Zitzmann, 2009). Crucially, elevated GLUT4 expression in AR-deficient (TfM) mouse following testosterone treatment suggested an AR-independent mechanism in action (McLaren *et al.*, 2012). Collectively, these studies reinforce the notion that testosterone via various (insulin dependent) mechanisms improves glucose handling by skeletal muscles. Lack or failure of these beneficial actions (pathways) may promote muscle specific insulin resistant state, contributing to development of Type 2 diabetes.

1.3.8 Effects of testosterone and RET on skeletal muscle and glucose metabolism

Emerging evidence shows that prevalence of type 2 diabetes is on the rise to a pandemic proportion (WHO, 2016a). Insulin resistance (IR) is noted to be more prevalent in older compared to younger individuals and is further associated with frailty leading to disability and mortality (Fried *et al.*, 2001; Bandeen-Roche *et al.*, 2006). Ageing and exercise training related changes in glucose handling and insulin resistance involve several tissues, but skeletal muscle metabolic changes leads to particularly large impact on whole body glucose metabolism (Tsintzas *et al.*, 2013). Further, age related decline in skeletal muscle mass and strength (sarcopenia)(Mitchell *et al.*, 2012) is associated with increases muscle insulin resistance (Cleasby, Jamieson and Atherton, 2016).

On a different note, aging is associated with insulin resistance and a decline in testosterone secretion (Morley *et al.*, 1997; Harman *et al.*, 2001; Feldman *et al.*, 2002). Recent meta-analysis showed that testosterone replacement therapy increases muscle mass in elderly men by increasing insulin-like growth factor-1 (IGF-1) expression, regulating forkhead box O (FOXO1), peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1), and p38 mitogen-activated protein kinases (MAPK), and also stimulates hypertrophy via intracellular androgen receptor signaling cascade dependent upon Erk and mammalian target of rapamycin (mTOR) (Neto *et al.*, 2015). Interestingly, Pitteloud et al. (Pitteloud, Mootha, *et al.*, 2005), reported that

men with hypogonadal testosterone levels are twice as insulin resistant as their eugonadal counterparts, confirming a positive co-relation between Testosterone level and insulin sensitivity across full spectrum of glucose tolerance. So, testosterone therapy in men with persistent insulin resistant state (T2DM) and hypogonadism increases insulin sensitivity, increases lean mass, and decreases subcutaneous fat (Dhindsa *et al.*, 2016).

Furthermore, accumulating evidence shows that Resistance Exercise Training (RET) improved glycemic control and insulin sensitivity in adults with T2DM (Gordon *et al.*, 2009). Moreover, RET increases insulin sensitivity of muscle by a coordinated increase in insulin-stimulated microvascular perfusion and molecular signaling at the level of TBC1D4 and glycogen synthase in muscle and in addition securing improved glucose delivery and uptake in to the muscle (Sjøberg *et al.*, 2017). Remarkably, RET has also been indicated in inducing significant endogenous hormonal (testosterone, GH, IGF-1) release (Fink, Schoenfeld and Nakazato, 2018).

There is still a paucity of evidence showing cumulative anabolic effect of Testosterone therapy and RET on muscle mass and strength (Bhasin *et al.*, 1996b) and glucose metabolism (Jensen *et al.*, 2018).

1.4 Beta-Hydroxy-Beta-Methylbutyrate (HMB) and Glucose metabolism:

1.4.1 Leucine:

In relation to HMB and protein metabolism, it is not only the amount of protein intake which is important but also the quality of protein for positive effect on muscle protein synthesis. To support this argument Tang et al, reported that whey protein was superior to soy and casein in young healthy males in his study (Tang *et al.*, 2009). Furthermore, different protein types have various profiles of essential amino acids (EAA), branched-chain amino acids (BCAA) and leucine. Accumulating evidence shows that leucine is not only a substrate for protein synthesis but also stimulate mTOR signalling pathway involved in protein anabolism (Katsanos *et al.*, 2006). Supporting the above argument in a separate study Paddon-Jones et al reported that iso-caloric ingestion of 15g EAA with 15g whey protein against 15g whey protein alone showed more stimulation of MPS in the former group compared to later (Paddon-Jones *et al.*, 2006).

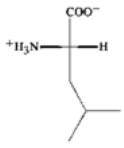

Figure 1-4: Structure of amino acid Leucine (Aristoy and Toldrá, 2004)

1.4.2 Beta-Hydroxy-Beta-Methylbutyrate (HMB):

Beta-Hydroxy-Beta-Methylbutyrate (HMB) is a metabolite of leucine (Figure 1-4) one of the essential branched chain amino acids (Vukovich *et al.*, 2001b). The benefits of dietary supplement of HMB to boost anabolic processes has been at the realm of scientific society for long time. Accumulating literature suggest that, HMB particularly enhances protein metabolism by stimulating MPS and attenuating MPB (Eley, Russell and Tisdale, 2008). HMB also plays a vital role in skeletal muscle myogenesis by expressing myoD and stimulate muscle anabolism by activating Akt pathway, which is critical in initiating MPS signalling (Kornasio *et al.*, 2009). Furthermore, a recent study by Wilkinson et al., showed that oral consumption of 3.42g of free-acid HMB in healthy young male stimulated MPS and attenuated MPB (-57%) measured by Arterio-Venous (A-V) balance (Wilkinson *et al.*, 2013). This conceivable idea that, HMB can be a potent anabolic nutraceutical has interested commercial arena as well.

1.4.3 HMB and carbohydrate metabolism:

Accumulating evidence has shown that Beta-Hydroxy-Beta-Methylbutyrate (HMB) has positive impact on skeletal muscle protein metabolism such as enhancing muscle protein synthesis and suppressing muscle protein breakdown (Wilkinson *et al.*, 2013, 2018; USU *et al.*, 2019). However, published data, to date on carbohydrate metabolism and HMB supplementation is controversial, suggesting either null, negative or positive

effects. Further, these studies were limited to younger individuals (Vukovich *et al.*, 2001a) and were pre-clinical or animal studies (Kreider *et al.*, 2000). Interestingly, related research in this area used calcium form of HMB which is deemed to have poor bioavailability and systemic clearance compared to free acid gel form (Fuller *et al.*, 2011). Given the paucity of evidence regarding the effects of HMB supplementation on glycaemic parameters and insulin kinetics in humans (young and old), this study will attempt to explore this unchartered territory.

So, combined exercise and nutraceutical interventions seems to have the potential to reverse some of the catabolic state associated with human skeletal muscle disuse atrophy and to positively influence glucose metabolism, however mechanisms underlying these processes are poorly defined and needs further exploration.

1.5 Aim of the thesis:

As summarised in the introduction section, disuse of human skeletal muscle (due to immobility, cancer, ageing etc.,) causes lot of undesirable effects leading to derangement of metabolic processes. Furthermore, ageing is a very complex process leading to loss of skeletal muscle mass and strength (sarcopenia) and inversely related to testosterone levels which together may have more detrimental effects of these metabolic process leading to morbidity and mortality. Further, available evidence to date on carbohydrate metabolism and HMB supplementation is controversial. So, molecular mechanism underlying the above process (involving ageing and muscle loss) and the counter measure (exercise, diet and rehabilitation) to abate this processes is largely unknown, hence:

1. Firstly, we aim to investigate the effects of single leg immobilisation on muscle mass, muscle protein turnover and glucose uptake in healthy young men. We hypothesize hypothesise that in healthy young men, single leg immobilisation impairs skeletal muscle insulin resistance and causes muscle mass loss.
2. Secondly, we aim to investigate the role of endogenous and exogenous testosterone (T) therapy in regulating resistance exercise-induced modulation of glycaemia control in young and older men, respectively. We hypothesise that testosterone suppression in young men blunts and supplementation in old men improves glycaemic control, respectively.

3. Finally, we aim to investigate the effects of Beta-Hydroxy-Beta-Methylbutyrate (HMB) on carbohydrate metabolism in young and old healthy men. We hypothesise that HMB improves glycaemic control.

Chapter 2: General Methods

This chapter will briefly discuss various methods and procedures commonly used across all the studies discussed below. Individual methodology relevant to specific chapters will be described in detail separately.

2.1 Muscle Biopsies:

Muscle biopsies are performed using conchotome biopsy technique as described by Dietrichson and colleagues (Dietrichson *et al.*, 1987). After sterilising the skin over vastus lateralis of the quadriceps muscle, the skin and deep tissue is anaesthetised using 5ml of 1% Lidocaine. After 2-10 minutes a 0.5 to 1cm skin incision will be made through the skin, subcutaneous tissue and muscle fascia. Using the conchotome about 200-300mg of muscle tissue will be taken. This will be immediately washed in ice cold 0.9% saline before blood, visible fat and connective tissue are removed. Immediately after this the muscle tissue will be frozen in liquid nitrogen before stored in -80 °C until further analysis is conducted on the samples.

2.2 DXA- Derived muscle mass:

To analyse the total body muscle and fat mass before and after the study, DXA (64752, GE Medical Systems-Lunar Prodigy, US) was used to determine total FFM, legs LM, total fat percentage (TFP), total fat mass (TFM), FFM index (FFM divided by height squared (FFM·height-2), and appendicular FFM (fat

free mass of arms and legs in kilograms divided by square of height in metres). Participants were asked to attend overnight fasted having not performed any heavy physical activity 24 h prior to scanning. For the DXA scan, participants wore loose comfortable clothing with no metal or plastic zippers, buttons or snaps. Prior to use on participants, a QA block phantom is used to calibrate the system, to ensure optimal measurement. In addition, spine phantoms are run bi-monthly to assess the reproducibility and accuracy of the system over time.

2.3 Oral Glucose tolerance test (OGTT) and Glucose Assay:

All participants completed a 2 h OGTT using 75 g unflavoured dextrose dissolved in water during the study. Venous blood samples were obtained at before and every 15 min (0, 15, 30, 60, 75, 90, 105, and 120 min) after dextrose ingestion to be assayed for glucose and insulin levels.

2.4 Glucose, Insulin and c-peptide analysis:

Blood glucose was analysed using glucose analyser machine (YSI 2300 STAT PLUS, UK) and plasma insulin and C-peptide concentrations were measured using high-sensitivity insulin enzyme linked immunosorbent assays (Insulin: DRG Instruments GmbH, Marburg, Germany; C-peptide: EZHCP-20K, Millipore, US) according to the manufacturer's protocol.

Chapter 3: The effects of single leg immobilisation on muscle mass, muscle protein turnover and glucose uptake in healthy young men:

3.1 Overview:

Background and Aims: To determine the short term (4 d) impacts of unilateral knee brace- mediated immobilisation model, in relation to muscle mass, protein turnover and glucose uptake.

Methods: 9 Healthy young men (22±2 y, BMI 24±1 kg/m^2) underwent 4 d immobilisation wherein one leg was immobilised and other leg remained ambulant (control). Muscle mass via ultrasound derived vastus lateralis (VL), muscle thickness (MT) and maximal voluntary contraction (MVC) via dynamometer and thigh lean mass (TLM) via DEXA were collected in both legs at baseline and after 4 d of immobilisation. After a baseline blood and saliva sample, subjects consumed 3 mL/kg of 70% deuterium oxide (D_2O) with additional saliva samples collected throughout the study. Bilateral VL muscle biopsies were collected on day 4 (at t=-120, 0, 90, and 180 min) to determine 4 d integrated MPS, estimated MPB, and acute fasted/fed MPS using a primed constant infusion of L-[ring-$^{13}C_6$]-phenylalanine, with cellular muscle anabolic and catabolic as well as glucose signaling quantified via immuno-blotting.

Results: After 4 d, the immobilised leg showed a decrease in TLM (Pre: 7477 ± 423g, Post: 7352 ± 427g (P <0.01)), MT (Pre: 2.67 ± 0.17cm, Post: 2.56 ± 0.17cm (P <0.05)) and strength (Pre: 264 ± 23 N.m, Post: 229 ± 12 N.m (P <0.05)) with no change in control. 4d integrated MPS was decreased in immobilised vs control legs (Control: 1.55 ± 0.07 $\%.d^{-1}$, Immobilised: 1.29 ± 0.06 $\%.d^{-1}$ (P<0.01), whilst FBR remained unchanged (Control: 1.44 ± 0.18 $\%.d^{-1}$, Immobilised: 1.73 ± 0.12 $\%.d^{-1}$)). Only the control leg increased acute MPS in response to feeding (Fasted: 0.043 ± 0.004 $\%.h^{-1}$, Fed: 0.065 ± 0.006 $\%.h^{-1}$ (P<0.01)) with no change in immobilised (Fasted: 0.034 ± 0.005 $\%.h^{-1}$, Fed: 0.049 ± 0.005 $\%.h^{-1}$). There was no change in markers of anabolic and catabolic signalling with immobilisation. GLUT4 levels significantly reduced in immobilised group (p<0.05) compared to control group. Plasma insulin concentration during IV EAA/ Dextrose clamp showed significantly higher (p<0.05) compared to basal values.

Conclusion: Disuse atrophy acutely is driven by decline in MPS rather than increase in MPB and seems to induce insulin resistance. Pro-anabolic strategies may circumvent these catabolic effects.

3.2 Background:

From a health perspective, skeletal muscle is a critical, but somewhat underappreciated, tissue. Beyond its role in generating contractile force for movement, skeletal muscle is the primary site of postprandial glucose disposal, contributes largely to circulating lipid oxidation, accounts for a large portion of resting metabolic rate, and is vitally important with advancing age. Reduced physical activity or muscle disuse results in muscle atrophy, which can lead to impaired functional capacity, the onset of insulin resistance, as well as an increased risk for morbidity and mortality. Periods of muscle disuse are unavoidable due to a variety of situations such as hospitalisation, homebound sickness, or orthopedic injuries. In fact, people will likely experience multiple bouts of skeletal muscle disuse during their lifetime from which some might fail to fully recover. Thus, muscle disuse is a significant and continuing problem resulting loss of muscle mass, strength/function, and metabolic health (particularly insulin-induced glucose disposal). Understanding the etiology of muscle disuse atrophy is essential if we are to develop effective strategies to combat this process, reduce healthcare costs, as well as improve human health and independence. The mechanism(s) causing muscle atrophy remain a subject of debate, which has been recently discussed in a Crosstalk article (Phillips and Mcglory, 2014; Reid, Judge and Bodine, 2014). The major question that remains to be addressed is which process – muscle protein synthesis (MPS) or muscle protein breakdown (MPB) – is predominantly affected with skeletal muscle disuse leading to atrophy in humans? The

current study will assess both MPS and MPB but will also discuss muscle glucose uptake following single leg immobilisation.

3.3 Aim of the study:

Our overall aim is to define the molecular mechanisms causing disuse atrophy in young men and the effects of single leg immobilisation on muscle mass, protein turnover and glucose uptake.

3.4 Materials and Methods:

3.4.1 Inclusion Criteria:

1) Healthy males, between the ages of 18 and 30 y
2) BMI between 18.5 and 30.0 kg/m2
3) Able and willing to provide informed consent

3.4.2 Exclusion Criteria:

1) The use of anti-inflammatory and/or analgesic medication, whether it is prescription or not
2) A history of neuromuscular disorders or muscle/bone wasting diseases
3) Any acute or chronic illness, cardiac, pulmonary, liver, or kidney abnormalities, uncontrolled hypertension, insulin-dependent or insulin-independent diabetes, or the presence of any other metabolic disease – all of which will be determined via a medical history screening questionnaire

4) The use of any medications known to affect protein metabolism (glucocorticoids, nonsteroidal anti-inflammatory medication, or prescription strength acne medication, etc.)
5) A (family) history of thrombosis
6) The use of anticoagulant medications
7) Consumption of tobacco-containing products
8) Excessive alcohol consumption (>21 units/week)
9) History of bleeding diathesis, platelet or coagulation disorders, or antiplatelet/anticoagulation therapy

3.4.3 Sample Size Calculations:

The sample size calculation is based on data from Wall et al.(Wall *et al.*, 2016), who used a similar study design to assess rates of muscle protein synthesis in the postprandial state following lower limb immobilisation. They observed lower postprandial muscle protein synthesis rates in the immobilised when compared with non-immobilised leg (0.020±0.007 vs. 0.044±0.010 %/h, respectively). Based on this observed difference, we will require 5 participants in the current study to ensure a power of 95% and a significance level of 5% (see Figure 3-1 below for details).To account for subject dropouts, we recruited 9 participants for the study.

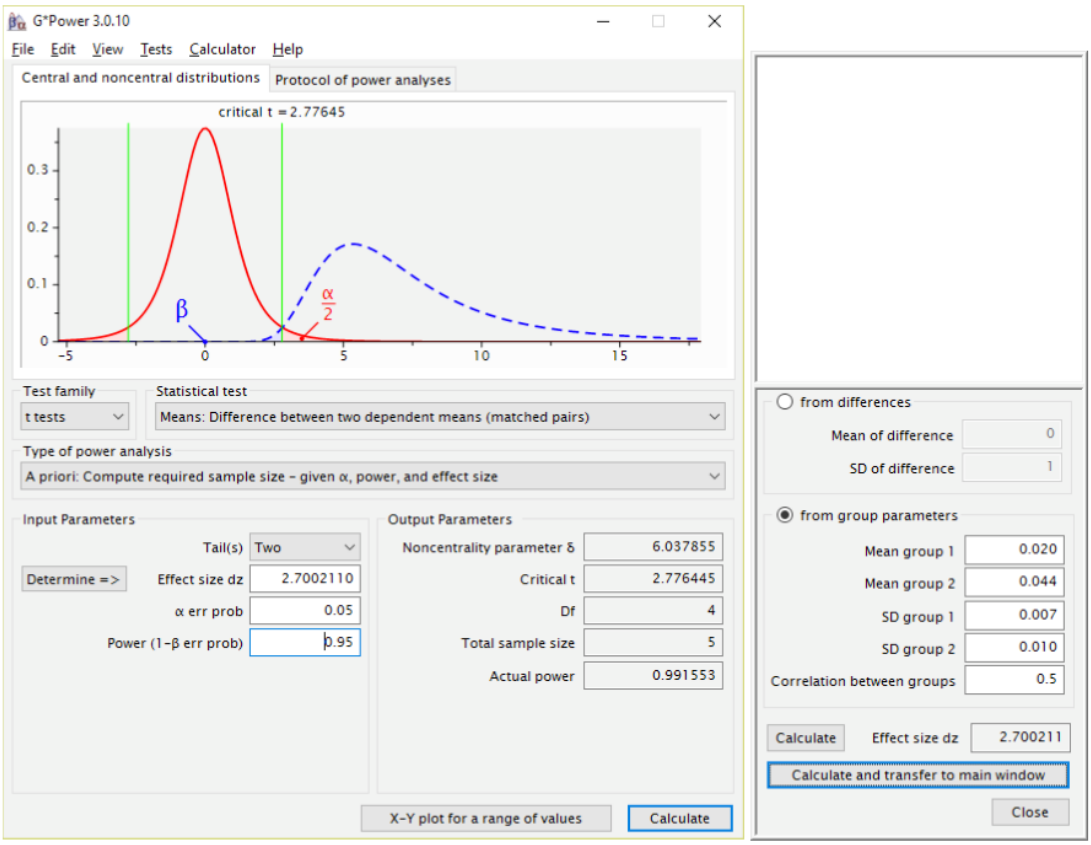

Figure 3-1: Sample Size Calculation

3.4.4 Conduct of the studies:

A schematic representation of the experimental design can be seen in Figure 3-2. Nine young, healthy males (mean ± SEM: age, 22 ± 1 y; body mass index, 24 ± 1 $kg.m^{-2}$) volunteered to take part in this study. Volunteers were initially screened by medical questionnaire, with exclusions for history of any neuromuscular disorder or muscle/bone wasting disease, acute or chronic metabolic, respiratory or cardiovascular disorder, or any other signs of ill health. All participants were performing activities of daily living or recreation upon entry to the study, but were not routinely undertaking heavy, structured

exercise. Participants did not use tobacco-containing products or consume excessive alcohol (> 21 units per week). The experimental procedures were approved by the Hamilton Integrated Research Ethics Board (HiREB #2192) and conformed to the Declaration of Helsinki. Written informed consent was obtained from all participants prior to their participation.

This study involved a bilateral leg protocol, with one leg randomly assigned to be immobilised and the other consequently used as a co-temporal contralateral control. Upon inclusion, participants were asked to visit the laboratory on two separate occasions, both of which followed an overnight fast. On the first visit, participants had the thigh lean mass of both legs measured using dual-energy X-ray absorptiometry (Lunar iDXA; GE Medical Systems, Mississauga, ON), before ingesting a bolus dose of D_2O (3 $mL.kg^{-1}$) for the measurement of integrated MPS rates. A saliva and blood sample was obtained prior to and 2 h following D_2O consumption for measurement of deuterium enrichment in body water. Mid-thigh vastus lateralis CSA was assessed using B-mode ultrasonography (Vivid Q, GE Medical Systems, Horten, Norway). During the first assessment, a mark was made with permanent marker 50% of the distance between the greater trochanter of the femur and the lateral epicondyle of the knee, identified by palpation. A thick layer of acoustic gel was applied on the leg at the area of measurement and care was taken to avoid depressing the muscle belly during image acquisition. Maximal voluntary isometric contraction torque (MVC) of each leg was assessed using Biodex dynamometer (Biodex System 3; Biodex Medical

Systems, Shirley, New York) at an angle of 60°. Thereafter, a knee brace (X-Act ROM; DonJoy, Dallas, TX, USA) was fitted to the immobilisation randomised leg and fixed at an angle of 60° of knee flexion. Participants continued to wear the knee brace as described for a duration of 3 full days, collecting a saliva sample each day, before returning to the laboratory for their second visit exactly 4 d after the first visit. Upon arrival for visit 2, participants had their knee brace removed, DXA thigh lean mass measured, Ultrasound scan and a further saliva sample was obtained. Participants were transported between stations in a wheelchair to prevent weight bearing in the immobilised leg prior to undergoing the infusion trial described below.

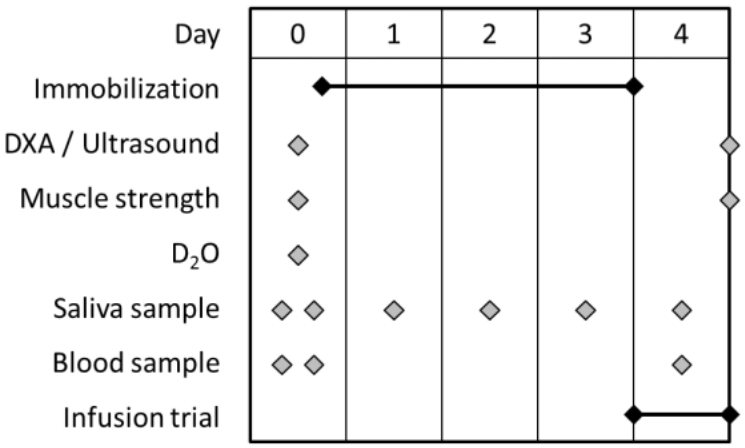

Figure 3-2: Schematic representation of study Design

Participants reported to the laboratory in the overnight fasting state (at least 10 h fasted). The knee brace was removed, and participants remained in a supine position on a laboratory bed for the entire duration of the infusion trial. A catheter was placed in an antecubital vein for amino acid and dextrose

infusions. A second catheter was inserted into an antecubital vein of the contralateral arm and placed under a heated blanket for arterialised blood sampling. After obtaining a baseline blood sample (t = -210 min), a primed constant infusion of L-[ring-13C6]-phenylalanine (0.4 mg/kg prime; 0.6 mg/kg/h infusion) was initiated for the assessment of MPS. Additional blood samples were collected at multiple time points: t = -180, -120, -100, -80, -70, -60, -50, -40, -30, -20, -10, 0, 20, 40, 60, 80, 100, 110, 120, 130, 140, 150, 160, 170 and 180 mins. At t = -120 min, a saliva sample was collected, and the first muscle biopsies were taken from both the immobilised and control leg for measurement of integrated MPS. Further muscle biopsies were collected from both legs at t = 0 min to determine basal MPS based on L-[ring-13C6]-phenylalanine incorporation. After collecting the muscle biopsies, primed constant infusions of amino acids (34 mg/kg prime; 102 mg/kg/h infusion; PRIMENE 10%, Baxter Corporation, Mississauga, Ontario, Canada) and dextrose (blood glucose clamped at 7.0-7.5 mM) was started to mimic a postprandial state for the remainder of the infusion trial. During the postprandial state, the L-[ring-13C6]-phenylalanine infusion rate was increased to 0.86 mg/kg/h to account for unlabelled phenylalanine entering the free amino acid pool and prevent dilution of the precursor pool. At t = 90 and t = 180 min, muscle biopsies were collected from the immobilised and control legs. After the final biopsies, participants were provided with juice and a snack, the dextrose infusion was gradually decreased, and other infusions were stopped. Plasma glucose was monitored for another 30 min in order to prevent

hypoglycaemia. Blood samples were collected in EDTA containing tubes and centrifuged at 1800 g for 10 min at 4°C. Aliquots of plasma were frozen in liquid nitrogen and stored at -20°C. Biopsies were collected from the middle region of the m. vastus lateralis, approximately 15 cm above the patella and 3 cm below entry through the fascia, using the percutaneous needle biopsy technique. Muscle samples were dissected carefully, freed from any visible non-muscle material, immediately frozen in liquid nitrogen, and stored at -80°C until further analysis. A schematic representation of the infusion trial can be seen Figure 3-3

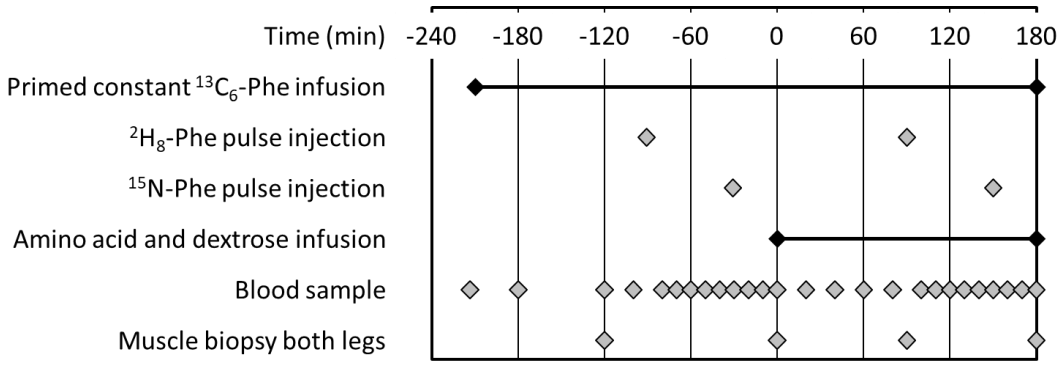

Figure 3-3 Schematic representation of Infusion trial

3.4.5 Measurement and measurements instruments:

During the screening visit, body height (m) and mass (kg) were measured to calculate BMI (kg/m^2). Systolic and diastolic blood pressure (mmHg) were assessed using an automated blood pressure monitoring device. On day 0 and at the end of the study, body composition (i.e., fat (%), appendicular lean

mass (kg), and lean body mass (kg)) were determined by dual-energy X-ray absorptiometry (DXA). Ultrasound was used to assess thigh muscle thickness and CSA, and a Biodex dynamometer was used to quantify maximal isometric strength in both the immobilised and control legs.

Baseline saliva and blood samples were collected on day 0 immediately before and 2 h after ingesting D_2O to determine deuterium enrichments in the body water and protein-bound (albumin) pool, respectively. Additional blood samples were taken every 15 min during the 2h amino acid infusion. One saliva sample was collected every morning afterwards (day 1- 3) to measure the decay of deuterium enrichment in the body water. On the last day of the study (day 4) participants collected a final saliva sample and had their muscle biopsies taken from both the immobilised and control legs (muscle biopsies from the infusion trial will be used). This method allows for the determination of muscle protein synthesis rates over the entire duration of the immobilisation trial by measuring the incremental incorporation of deuterium-labelled alanine into the muscle protein-bound pool and assesses whether daily MPS rates differ between the control and immobilised leg. Comparisons can then be made between the chronic measurements of muscle protein synthesis (using the D_2O method) and the acute measurements of muscle protein synthesis (using 13C6-Phe method) in response to a transient period of immobilisation. During the infusion trial (day 4), a primed constant infusion of 13C6-Phe in combination with repeated plasma sampling and muscle biopsy collection to

determine both basal and postprandial MPS rates using the precursor-product method were used.

All muscle biopsy samples were collected under sterile conditions, using the conchotome biopsy technique (Dietrichson *et al.*, 1987) with 1% $w \cdot v^{-1}$ lidocaine as local anaesthetic. Any fat tissue and connective tissue was rapidly dissected out and muscle was washed in ice-cold phosphate-buffered saline (PBS) and frozen in liquid nitrogen or liquid-nitrogen cooled isopentane, before storage at -80°C.

Dextrose was infused according to a protocol adapted from DeFronzo et al. (DeFronzo, Tobin and Andres, 1979) until a steady-state blood glucose concentration of 7-7.5 mM was obtained. To measure glucose concentrations over the course of the dextrose infusion, serial blood sampling was performed every 5-minutes and analysed with the YSI STAT 2300.

Muscle biopsies were collected (as described above) from the immobilised and non-immobilised leg on day 4 during the infusion trial at t=-120, 0, 90, and 180 min. Myofibrillar protein were extracted to determine myofibrillar protein-bound 2H-alanine (only t=-120), 13C6-Phe, 2H8-Phe, and 15N-Phe enrichments by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). The saliva samples were analysed for 2H enrichments by cavity ringdown spectroscopy. Plasma samples were analysed for 13C6-Phe, 2H8-Phe, and 15N-Phe enrichments. Fractional synthetic rates of muscle protein synthesis were calculated by dividing the increment in muscle protein-bound

2H-alanine or 13C6-Phe enrichment between two muscle biopsies (or plasma albumin) over time by the average enrichment in total body water or plasma. Myofibrillar protein breakdown rates were determined by measuring the dilution of the muscle intracellular amino acid pool upon cessation of tracer incorporation.

3.4.6 Body water and protein bound alanine muscle fraction enrichment

Body water enrichment was measured as previously described (Daniel J. Wilkinson *et al.*, 2014). Saliva was heated in a vial at 100°C, then cooled rapidly on ice and the condensate transferred to a clean vial ready for analysis. Deuterium enrichment was measured on a high-temperature conversion elemental analyser connected to an isotope ratio mass spectrometer (TC/EA-IRMS Thermo Finnigan, Hemel Hempstead, UK). For isolation of myofibrillar, sarcoplasmic, and collagen fractions, 30–50 mg of muscle was used. The muscle was homogenised in ice-cold homogenisation buffer [50mM Tris·HCl (pH 7.4), 50mM NaF, 10mM ␣-glycerophosphate disodium salt, 1mM EDTA, 1 mM EGTA, and 1mM activated Na3VO4 (allfrom Sigma-Aldrich)] and a complete protease inhibitor cocktail tablet (Roche, West Sussex, UK) at 10 µl/µg tissue. Homogenates were rotated for 10 min, and the supernatant was collected by centrifugation at 1000 g for 15 min at 4°C. The pellet was re-suspended in 500 µL mitochondrial extraction buffer (MEB), dounce homogenised and the supernatant removed after centrifugation at 1000g for 5 min at 4 °C. The myofibrillar pellet was solubilised in 0.3 M NaOH and

separated from the insoluble collagen by centrifugation, and the myofibrillar protein was precipitated with 1 M perchloric acid. The protein was then precipitated, acid hydrolysed, and the free amino acids were purified and derivatised as their n-methoxycarbonyl methyl esters (MCME). Incorporation of deuterium into protein bound alanine was determined by gas chromatography-pyrolysis-isotope ratio mass spectrometry (GC-pyrolysis-IRMS, Delta V Advantage; Thermo Finnigan, Hemel Hempstead, UK) alongside a standard curve of known dl-Alanine-2,3,3,3-d4 enrichment to validate measurement accuracy. MPS, FGR and FBR were calculated as previously described (Gharahdaghi *et al.*, 2019). The incorporation of L-[ring-13C6]-phenylalanine was determined by gas chromatography–combustion-isotope ratio mass spectrometry (Delta plus XP; Thermofisher Scientific, Hemel Hempstead, United Kingdom) with Muscle intracellular phenylalanine enrichment was measured by gas chromatography-mass spectrometry (Trace DSQ; Thermofisher Scientific) following precipitation of the sarcoplasmic fraction and purification of the aqueous supernatant using Dowex H+ resin as described above, with AAs converted to their tert-butyldimethylsilyl derivatives. The fractional synthesis rates (FSR) of the myofibrillar proteins was calculated using the precursor-product relationship as previously described (Bukhari *et al.*, 2015).

3.4.7 Immunoblotting for muscle signalling pathway activity

Immunoblotting was performed as previously described (Atherton *et al.*, 2010) using the sarcoplasmic fraction isolated from MPS preparation. Sarcoplasmic protein concentrations were analysed using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE-US) and sample concentrations adjusted to 1µg/µl in 3x Laemmli buffer to ensure equivalent protein loading onto a Criterion XT Bis-Tris 12 % SDS-PAGE gel (Bio-Rad, Hemel Hempstead, UK) for electrophoresis for 1h at 200 V. Separated proteins were transferred onto a PVDF membrane for 45 min at 100 V, then blocked in 5 % low-fat milk in Tris-buffered saline and 0.1 % Tween-20 (TBST) for 1h at room temperature. Membranes were then incubated at 4OC over-night in 5 % milk diluted in TBST primary antibody solutions. Afterwards membranes were washed 3 x 5 min with TBST and incubated for 1h at room temperature in their respective HRP conjugated secondary antibody, anti-rabbit (Cell Signalling Technologies) 1:2000 5 % low-fat milk in TBST, anti-mouse (Cell Signalling Technologies) 1:2000 5 % low-fat milk in TBST. Membranes were washed 3 x 5 min in TBST and incubated for 5 min in enhanced chemiluminescence reagent (Millipore, Watford, UK) and visualized using a Chemidoc XRS. Bands were quantified using ImageLab software and normalised to total loaded protein visualised by Coomassie brilliant blue staining [29]. Primary antibodies against p-AKT Ser473 (1:2000, #4060), Pan-AKT (1:2000, #4685), p-TSC2 Thr1462 (1:2000, #3611), TSC2 (1:2000, #4308), p-mTOR Ser2448 (1:2000, #2976), mTOR (1:2000, #2972), p-p70S6K1 Thr389 (1:2000, #9234), p70S6K1 (1:2000, #2708), p-S6RP

Ser235/236 (1:2000, #2211), S6RP (1:2000, #2217), p-4e-BP1 Thr37/46 (1:2000, #2855), 4e-BP1 (1:2000, #9644), p-eIF4E Ser209 (1:2000, #9741), eIF4E (1:2000, #9742) were from Cell Signalling Technologies. Fed (90 min) data was normalised to control mean and transformed using Y= (log(1+Y)).

3.5 Results:

Mainly, muscle mass, MPS, MPB, some biomarkers related to protein metabolism and glycemic control will be discussed below (in relation to this thesis).

3.5.1 Muscle mass, VL Thickness and Strength:

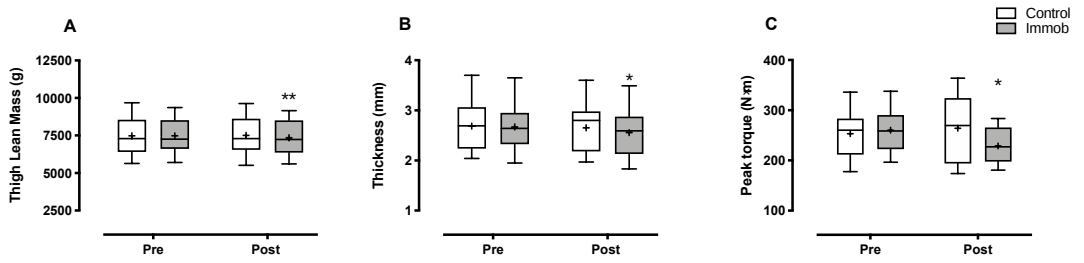

*Figure 3-4: Pre and post measures in control and immobilised legs of A) Thigh Lean Mass B) VL muscle thickness C) Peak torque. ** P<0.01, * p<0.05*

At baseline there was no significant difference in DXA derived TLM, VL MT or strength, between legs. Over the 4 d study period the control leg showed no change in TLM (Pre: 7476 ± 475 g, Post: 7501 ± 477 g), MT (Pre: 2.69 ± 0.18 cm, Post: 2.65 ± 0.17 g) or strength (Pre: 253 ± 16 N.m, Post: 260 ± 15 N.m). The immobilised leg showed a decrease from baseline in TLM (Pre: 7477 ± 423 g, Post: 7352 ± 427 g (P <0.01)) (Figure 3-4A), MT (Pre: 2.67 ± 0.17 cm, Post: 2.56 ± 0.17 g (P <0.05)) (Figure 3-4B), and strength (Pre: 264 ± 23 N.m, Post: 229 ± 12 N.m (P <0.05)) (Figure 3-4C).

3.5.2 Integrated MPS, Fractional growth and breakdown rates:

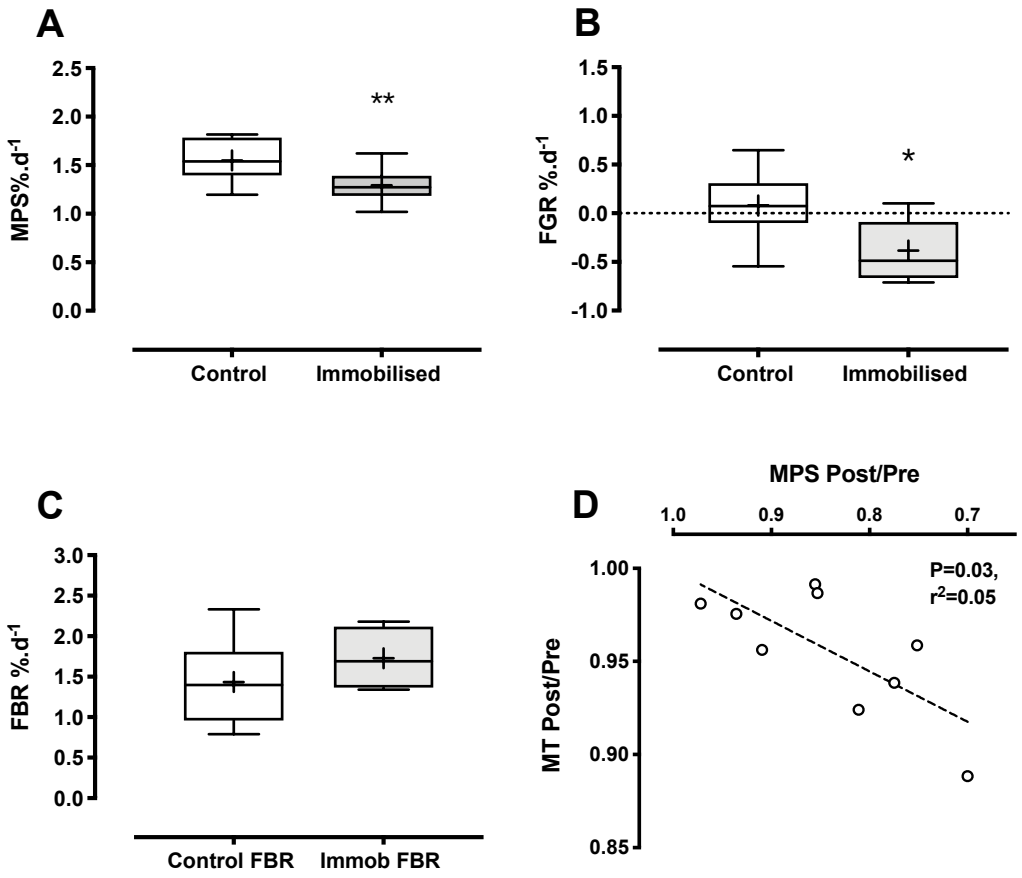

*Figure 3-5: Over the 4 d study period in control and immobilisation legs A) muscle protein synthesis %.d^{-1} B) fractional growth rate %.d^{-1} C) fractional breakdown rate %.d^{-1} D) correlation between the change in MPS vs change in VL MT. ** P<0.01, * P <0.05.*

Post 4 d study period integrated MPS was decreased in the immobilised vs the control leg (Control: 1.55 ± 0.07 %.d^{-1}, Immob: 1.29 ± 0.06 %.d^{-1} P<0.01) (Figure 3-5A). Likewise, the FGR was decreased in the immobilisation leg (Control: 0.08 ± 0.12 %.d^{-1}, Immob: -0.38 ± 0.11 %.d^{-1} P<0.05) (Figure 3-5B), whilst the FBR remained unchanged (Control: 1.44 ± 0.18 %.d^{-1}, Immob: 1.73 ± 0.12 %.d^{-1}) (Figure 3-5C). The change in MT was correlated with the change

in MPS (P=0.03, r^2 = 0.05)(Figure 3-5D) and was not correlated with the change in FBR (P=0.17, r^2 = 0.29).

3.5.3 Acute muscle protein synthesis:

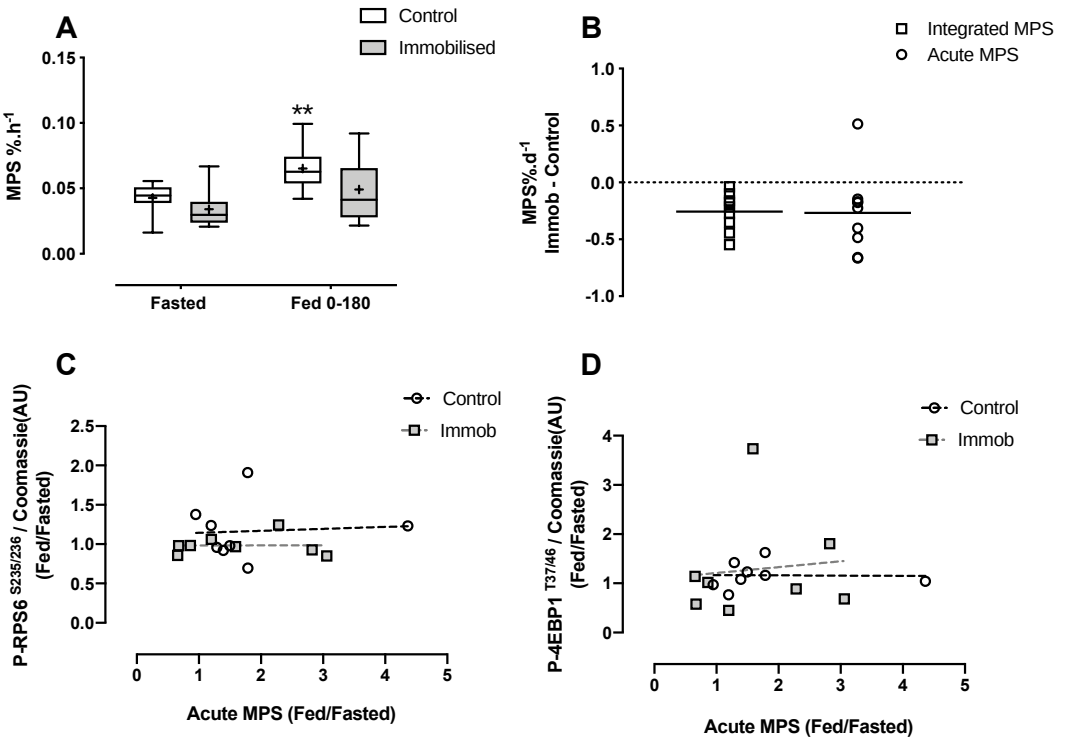

*Figure 3-6: A) acute fasted and fed MPS rates in control and immobilised legs B) the absolute decrease in integrated or acute muscle protein synthesis %.d^{-1} assuming 2/3 of the day is spent fasted and 1/3 fed). Correlation between change in fasted to fed muscle protein synthesis vs C) change in fasted to fed P-RPS6 and D) change in fasted to fed P-4EBP1 **P<0.01*

After 4 d of immobilisation, there was an overall trend for decreased MPS (effect of immobilisation P=0.07) yet fasted and fed rates of acute MPS remained unchanged (Figure 3-6A). However, only the control leg significantly

increased MPS in response to feeding (Fasted: 0.043 ± 0.004 %.h^{-1}, Fed: 0.065 ± 0.006 %.h^{-1} (P<0.01)) with no change in immobilisation (Fasted: 0.034 ± 0.005 %.h^{-1}, Fed: 0.049 ± 0.005 %.h^{-1}) (Figure 3-6A). The absolute decrease in MPS %.d^{-1} from integrated or acute MPS were similar (Integrated: -0.26 ± 0.05 %.d^{-1}, Acute: -0.27 ± 0.12 %.d^{-1} (assuming 2/3 of the day is spent fasted and 1/3 fed) (Figure 3-6B). The change in MPS was not correlated with either the change in RPS6 or 4EBP1 phosphorylation from fasted to fed states (Figure 3-6C&D). Overall, markers of both anabolic and catabolic markers were similar between control and immobilised leg, with no effect of immobilisation on markers of MPB (Table 3-1).

*Table 3-1: Muscle anabolic and catabolic signalling in control and immobilisation legs. *Significantly different to fasted, * P <0.05*

		Fasted	Fed
P-mTOR ^{S2448}	C	0.60 ± 0.16	0.61 ± 0.09
	I	0.56 ± 0.17	0.52 ± 0.09
P-4EBP1 $^{T37/46}$	C	0.67 ± 0.08	0.72 ± 0.06
	I	0.84 ± 0.10	0.84 ± 0.09
P-AKT ^{S473}	C	0.67 ± 0.08	1.02 ± 0.17
	I	0.60 ± 0.07	1.12 ± 0.21*
P-RPS6 $^{S235/236}$	C	0.52 ± 0.21	1.78 ± 0.19*
	I	0.57 ± 0.17	1.62 ± 0.34*
P-eIF4E ^{S209}	C	0.66 ± 0.09	0.66 ± 0.10
	I	0.78 ± 0.10	0.59 ± 0.07
P-eIF4B ^{S422}	C	0.69 ±0.05	0.88 ± 0.15
	I	0.59 ± 0.10	0.77 ± 0.13
P-eEF2 ^{T56}	C	0.68 ± 0.06	0.67 ± 0.04
	I	0.65 ± 0.06	0.71 ± 0.04
Calpain 1	C	0.69 ± 0.04	0.74 ±0.04
	I	0.78 ± 0.04	0.77 ± 0.03
MAFbx	C	0.63 ± 0.13	0.74 ± 0.15
	I	0.76 ± 0.08	0.72 ± 0.11
Ubiquitin	C	0.69 ± 0.04	0.70 ± 0.03
	I	0.76 ±0.06	0.64 ± 0.03

3.5.4 Glucose related biomarker and Insulin levels:

Levels of GLUT4 were significantly reduced in immobilised group ($p<0.05$) compared to control group. No changes in the levels of AS160 and PGC1b were noted compared to control and immobilised group (Figure 3-7). Plasma insulin concentration during IV EAA/ Dextrose clamp showed significantly ($p<0.05$) higher compared to basal values (Figure 3-8).

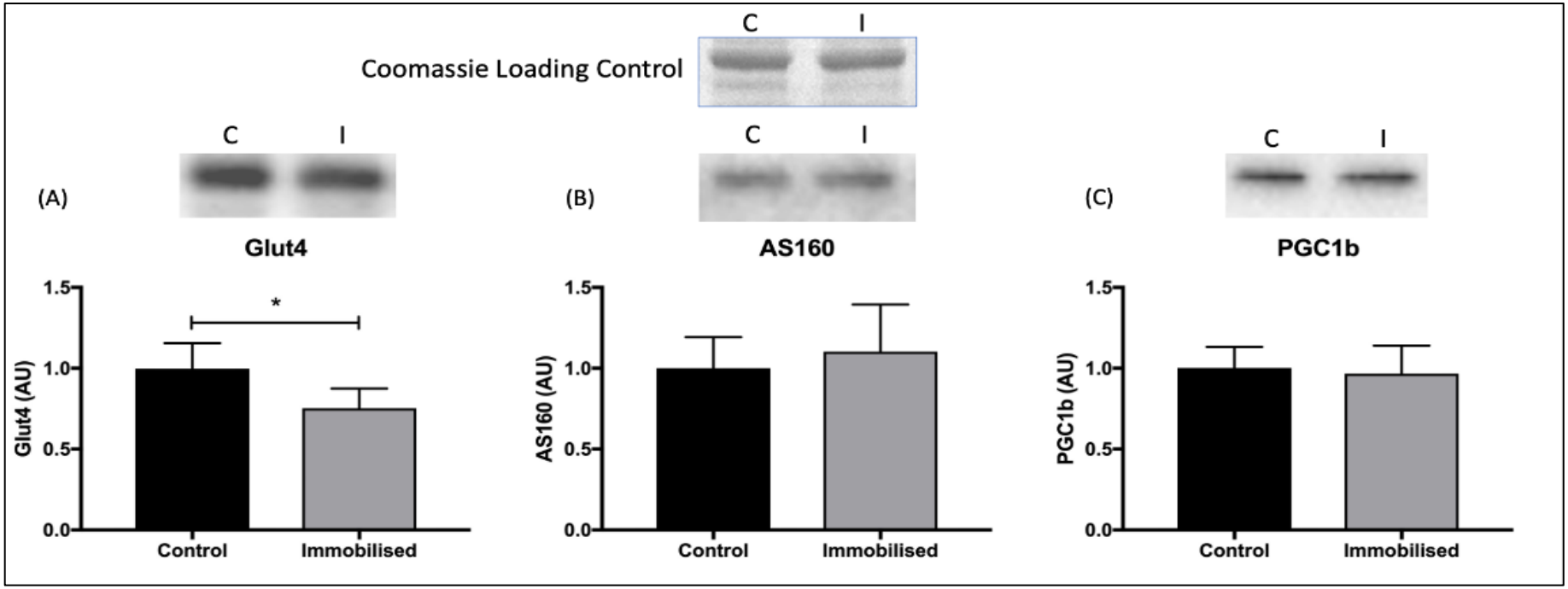

*Figure 3-7: Baseline Control (C) vs Immobilised (I)– Quantification of western blots of (A) GLUT4, (B) AS160 & (C) PGC1-β. * significant different from control ($p<0.05$). Measured by paired t test. n=8*

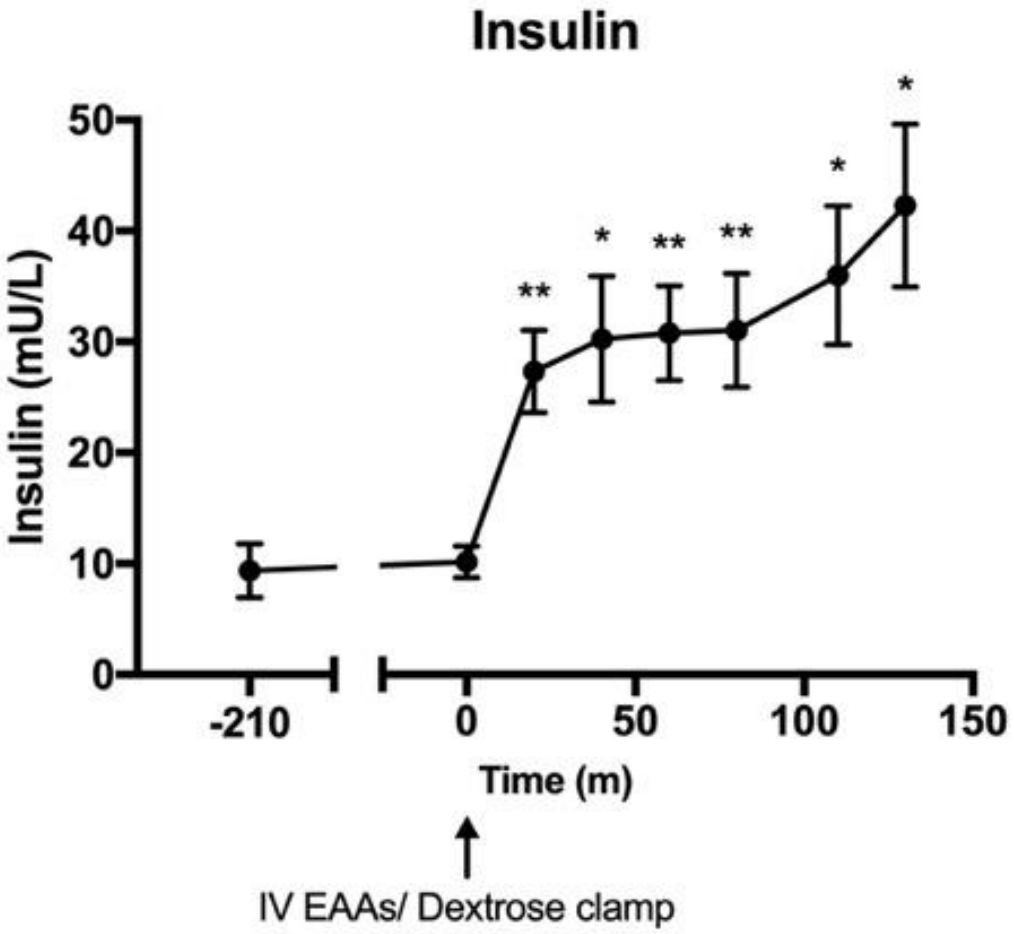

*Figure 3-8: Insulin response to IV EAAs/ Dextrose – Plasma insulin concentrations. *Greater than basal p<0.05, ** p<0.01.*

3.6 Discussion:

Burgeoning problem of persistent state of insulin resistance, such as Type 2 diabetes and ageing, and its association with sarcopenia is a growing concern (Nilwik *et al.*, 2013; Perry *et al.*, 2016). Hence, there is a great need amongst scientific field to understand the effects of human skeletal muscle disuse atrophy and its effects on MPB, MPS and glycemic control. Many situations such as hospitalisation, recovery from illness, injury etc., results in a period of

physical inactivity that is associated with the accelerated loss of muscle mass and muscle strength (Kouw *et al.*, 2019). This muscle atrophy is clearly noticeable in prolonged period of immobilisation (Trappe *et al.*, 2008; Fitts *et al.*, 2010). But emerging evidence suggests these process happening at much earlier in the periods of skeletal muscle disuse (Wall *et al.*, 2014; Kilroe *et al.*, 2020). Furthermore, short successive periods of immobility called as “catabolic crisis” have been proposed to contribute to the persistent age-related skeletal muscle loss and strength (Alley *et al.*, 2010; Wall, Dirks and Van Loon, 2013; Kouw *et al.*, 2019).

There is an overabundance of clinical studies, using different immobilisation models, that have investigated the degree of muscle loss in humans exposed to disuse (Rudrappa *et al.*, 2016). The most frequent employed models to study disuse atrophy in humans are unilateral limb suspension (ULLS) using a knee brace or cast, and bed rest; other scenarios include spinal cord injury and spaceflight. In terms of muscle mass, the observed rate of decline in muscle size (cross sectional area (CSA)) for each day of ULLS in knee extensors was ~0.40% and ~0.36% for plantar flexors following 42 d of unloading (Hackney and Ploutz-Snyder, 2012). Other studies have demonstrated losses of muscle strength and mass early on in disuse, i.e. 5 d of cast immobilisation lead to ~3.5% reductions in quadriceps CSA and ~9% in strength (Dirks *et al.*, 2014). This had progressed to ~8% reductions in CSA and ~23% reductions in strength by 14 d (Wall, Dirks and Van Loon, 2013). Supporting the above findings, we herein, report loses of ~11% in as little as

4 d of unilateral immobilisation using knee braces. Additionally, Suetta et al. reported ~10% reductions in myofibre cross sectional area and ~13% decreases in strength after just 4 d progressing to ~20% reductions in myofibre area and strength after 14 d of ULLS (Suetta *et al.*, 2012, 2013). Likewise, we report a significant decline in DXA derived muscle loss of 1.7%; correlated with significant decline in MT of 4%. Hence, our data adds to current literature suggesting that muscle loss occurs rapidly at onset of immobilisation.

It is reported that, disuse skeletal muscle atrophy results from both decreased protein synthesis and increased protein breakdown, resulting in net skeletal muscle mass loss (Huang and Zhu, 2016; Rudrappa *et al.*, 2016). Early studies using amino acid tracers revealed that immobilisation results in a decline in both fast and fed-state MPS likely inducing a state of anabolic resistance (de Boer *et al.*, 2007; Elisa I. Glover *et al.*, 2008; Wall *et al.*, 2013). But, these studies possibly showed only a 'snap shot' of protein balance (Atherton *et al.*, 2015). Hence, measures to evaluate integrated MPS is important, so D_2O provides an effective tool at quantifying this bye encapsulating anabolic and catabolic responses over the entire measurement periods (Daniel J Wilkinson *et al.*, 2014; Brook *et al.*, 2015; Mcglory *et al.*, 2019). Herein, we combined integrated D_2O MPS measures along with acute AA tracer to capture not only early integrative changes in MPS but also acute feeding responses. We show that VL MPS is significantly depressed over 4 d of ULLS and that this was correlated with loss of VL MT, providing further evidence that declines in MPS are predominant in muscle mass loss. Interestingly, we show that by day 4 this

is primarily a result of impaired feeding responses and a tendency for overall reduced muscle protein synthesis in fasted/fed states; the earliest observation of immobilisation induced anabolic resistance. Additionally, not only we showed similar declines in daily integrative MPS (i.e., from our D_2O measures) but also in daily acute MPS (i.e., from our acute measures). These findings further support the argument that MPS can alone explain declines in muscle mass, in the absence of bulk rise in MPB.

Furthermore, the role of MPB remain unclear in setting of disuse muscle atrophy. Moreover, increased markers of MPB during early stages of disuse has been indicated in a possible role in causation (Urso *et al.*, 2006; Dirks *et al.*, 2014). Emerging evidence suggests that calpains plays an important role in dysregulation of proteolysis seen in muscle atrophy and further, abnormally activated calpain results in muscle atrophy via its downstream effects on ubiquitin-proteasome pathway (UPP) and Akt phosphorylation (Huang and Zhu, 2016). In contrast, our study showed no changes to calpain mediated pathway in setting of skeletal muscle disuse. Interestingly, increased calpain-dependent protein degradation seen in calcium-treated skeletal muscle could be associated with reduced HSP90 and hence Akt activation (Huang and Zhu, 2016). But, in our study no increase in Akt was noted. Further, we showed rates of MPS combined with the net change in muscle mass per day (FGR) to calculate FBR. Studies has shown that, using these validated equations fairly accurate estimation of FBR can be obtained (Hector *et al.*, 2018; Gharahdaghi *et al.*, 2019). Herein, we calculated no overall increase in FBR with single leg

immobilisation. Interestingly, changes in FBR were not correlated with declines in muscle mass and no change in key markers of MPB. Above finding suggests the notion that reduced MPS rather than increased MPB may be a major contributor in muscle mass loss during immobilisation.

Insulin-mediated glucose uptake is also blunted with muscle disuse (Mikines *et al.*, 1991; Biensø *et al.*, 2012); that is, unloaded muscle becomes IR. This IR can be observed at a whole-body level following bed-rest, but is most apparent at the muscle level across the physiological range of insulin concentrations under clamp conditions (Mikines *et al.*, 1991). Recently, a one week bed-rest study in young males by Dirks et al, revealed reduced muscle mass (~1.4 kg lean tissue and ~3% quadriceps CSA) and whole-body insulin sensitivity (~29%) (Dirks *et al.*, 2016). Thus, disuse lowers MPS, induces anabolic resistance to nutrients and impairs insulin-mediated muscle glucose uptake - even in healthy adults (Fink, 1983). Furthermore, it is accepted that the GLUT4 glucose transporter is a major mediator of glucose removal from the circulation and a key regulator of whole-body glucose homeostasis and mainly expressed on adipose tissue and skeletal muscles (Huang and Czech, 2007). In addition, Blakemore et al. reported that, the concentration of GLUT4 protein were significantly reduced (50%) in immobilised limb after 1 week and it persisted for 6 weeks (Blakemore *et al.*, 1996). In support of this observation our study showed significantly ($p<0.05$) reduced GLUT4 in immobilised leg compared to control (non-immobilised leg). Accumulating evidence has shown that peripheral insulin resistance (hyperinsulinemia) develops immediately

after immobility (such as post-surgery immobilisation) and might be associated with perturbations in insulin stimulated GLUT4 pathway (Thorell *et al.*, 1999). Likewise, in our study, we noted significant increase in plasma insulin level post immobilisation suggesting development of peripheral insulin resistance.

In conclusion, 4 d single leg immobilisation in young men induces significant skeletal muscle atrophy driven by sustained reduction in MPS. MPB does not seem to play any major role in muscle atrophy, as evidenced by lack of changes in catabolic markers and no increase in FBR, albeit a calculated measure with its own limitations. That being said, we showed decreased in integrated MPS accompanied by anabolic resistance possibly driven by development of peripheral insulin resistance (supported by reduced GLUT4 activity). So, development of effective counter measure is warranted to overcome this cascade of catabolic processes triggered by muscle disuse atrophy.

Chapter 4: The role of endogenous and exogenous testosterone (T) therapy in regulating resistance exercise-induced modulation of glycaemia control in young and older men, respectively:

4.1 Overview:

Insulin resistance has assumed paramount importance as a risk factor for many metabolic diseases, coincident with the established pandemic of obesity, worldwide. The main behavioral strategies to overcome insulin resistance remain diet and exercise. However, the “andropause”, so-called due to age-related testosterone (T) depletion has been associated with impaired glycaemia control – leading to investigations of the role of T in regulating insulin sensitivity. Recently, T therapies have been evaluated in humans with variable effects, such that the role and efficacy of T therapy in relation to glycaemia control across age remains unclear. The aim of this study was therefore to investigate the mechanistic and therapeutic role of T therapy during 6- weeks of supervised resistance exercise training (RET), in relation to insulin sensitivity in groups of healthy younger and older men. We hypothesised T is a key factor in regulating exercise-induced improvements in glycaemia control.

Methods: Healthy non-hypogonadal men were recruited (n=34; 'younger group'~18-30y and 'older group' ~60-75y; all BMI≤30kg.m^{-2}, serum T>230 ng·dl^{-1}) with the aim of determining the role of: i) endogenous suppression of T in relation to RET-induced improvements in glycaemia control, and ii) provision of exogenous T therapy adjuvant to short-term RET in older men. Thus, young men were assigned in a randomised double-blinded fashion to receive injection of either: placebo (YP; saline, n=8) or the GnRH analogue, Goserelin acetate 3.6mg (Zoladex) (YZ; n=8). Instead, older men received placebo (OP; saline, n=9) or Testosterone esters 250 mg (OT; n=9). All subjects underwent 6- weeks whole body RET (3-sets, 8-10 reps at 80% 1-RM) with 75g oral glucose tolerance testing (OGTT baseline, after injection and after RET)

Results: Provision of Goserelin significantly reduced serum T in YZ (vs. YP), while the OT group elicited marked enhanced serum T (both P<0.05) that were sustained throughout RET. Over 6-weeks of RET, both the OT group (1367±572 mU-1.2h^{-1}, P=0.03) and YP group (2381±1013 mU-1.2h^{-1}, P=0.009) showed decreased insulin area under curve (AUC), respectively. Further, measures of C-Peptide highlighted decreases in OT compared to OP (OT: 601±46 ng.ml^{-1} vs. OP: 830±77 ng.ml^{-1}, P=0.02), with a decreasing trend in YP compared to YZ (551±57 ng.ml^{-1} vs. 789±116 ng.ml^{-1}, P=0.08). Additionally, Cederholm index of peripheral insulin sensitivity in YP showed an increase from OGTT before and after RET (61.7±4.7 vs. 74.5±3.7 mg·L2·mmol·L^{-1}·mU−1·min^{-1}, P<0.05), and in OT, showed a significant

increase from baseline to OGTT after RET (57.3±5.1 vs 68.6±5.3 $mg{\cdot}L^{2}{\cdot}mmol{\cdot}L^{-1}{\cdot}mU{-}1{\cdot}min^{-1}$, P<0.05). Finally, Matsuda index of insulin sensitivity in YP yielded significant increases based on fold-change from baseline OGTT to OGTT after RET (9.9±2.4 vs 12.8±3.2, P<0.05). **Conclusions:** T therapy (OT) and non-suppressed endogenous T (YP), enhanced insulin sensitivity (supported by reduced c-peptide) in older and young men, respectively, coupled with RET. We conclude: i) endogenous T is an important mediator of exercise-induced improvements in insulin sensitivity, and ii) T therapy adjuvant to pre/re-habilitation exercise may be of value to overcome the burgeoning trend of worsening insulin resistance with aging.

4.2 Aim of the study:

To investigate the mechanistic and therapeutic role of T therapy during 6-weeks of supervised resistance exercise training (RET), in relation to insulin sensitivity in groups of healthy younger and older men.

4.3 Materials and Methods:

4.3.1 Ethics and study participants:

This study was approved by The University of Nottingham Faculty of Medicine and Health Sciences Research Ethics Committee (G11082015 SoMS MSGEM), was conducted according to the declaration of Helsinki, and was pre-registered at clinicaltrials.gov (NCT02152839). Before entry into the study, participants provided written informed consent to participate after all

procedures and risks were explained to them. All participants performed activities of daily living and were recreationally active but had not partaken in RET within the previous 12 months. Participants were screened by medical questionnaire, physical examination, routine blood chemistry and a resting electrocardiogram. Participants who presented with metabolic, respiratory or cardiovascular disorders or who were prescribed medication (e.g., beta-adrenergic blocking agents, statins, anti-inflammatory drugs) and any other medications which could influence T metabolism were excluded. Of the pre-screened volunteers, eighteen older (O) and sixteen younger (Y) non-hypogonadal healthy normotensive (<140/90) volunteers males with morning normal serum T concentrations were assigned in a random double-blinded fashion to receive bi-weekly either: Old placebo, OP (saline, n=9) or Old Testosterone, OT (Sustanon 250-mg, n=9) injections in older groups and one off either: Young Placebo, YP (n=8) or young GnRH analogous (Goserelin, so called Zoladex, YZ) (3.6-mg, n=8) injections in younger groups over 6-weeks of whole-body supervised RET. All participants involved in the study were monitored throughout the study for any negative side effects of T injections. No adverse events were reported during or after completion of the study.

4.3.2 Conduct of study:

Following baseline measurements of maximal voluntary contraction (MVC) and 1-repetition maximum (1-RM; on separate days); regardless of group assignment, all participants were further characterised at baseline. This

involved collection of fasting blood, oral glucose tolerance test (OGTT), muscle ultrasound (Mylab 70; Esaote Biomedica, Italy) of the m. vastus lateralis muscle (VL) and a dual-energy X-ray absorptiometry (DXA; Lunar Prodigy II, GE Medical Systems, Little Chalfont, UK) scan. Finally, injection of T or P in older groups and Z or P in younger groups was administered by an un-blinded research technician. The fully supervised RET protocol then commenced and continued for the next 6-weeks. A detailed schematic of the study protocol is depicted in Figure 4-1.

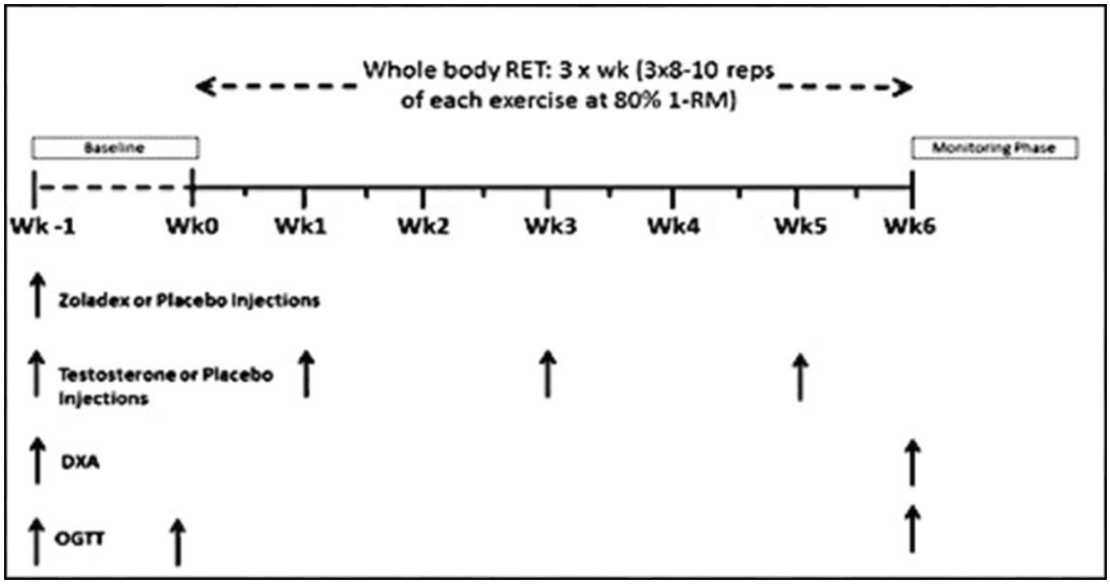

Figure 4-1: Schematic of study protocol

4.3.3 Resistance Exercise Training (RET) procedures and strength assessments:

Participants in both groups performed the same whole-body RET including leg-extension, leg-press, leg-curl, lat-pull-down, shoulder-press and bench-press (all 3-sets of 8-10 reps at 80% 1-RM)) (Sundell, 2011) 3-times· weeks ·

[1] non-consecutively for 6-weeks. Individuals' 1-RM (Dohoney *et al.*, 2002) was re-assessed every 2-weeks before the corresponding training session to maintain intensity with progression.

4.3.4 DXA-derived muscle mass:

To analyse the total body muscle and fat mass before and after the study, DXA (64752, GE Medical Systems-Lunar Prodigy, US) was used to determine total FFM, legs LM, total fat percentage (TFP), total fat mass (TFM), FFM index (FFM divided by height squared (FFM·height^{-2}), and appendicular FFM (fat free mass of arms and legs in kilograms divided by square of height in metres). Participants were asked to attend overnight fasted having not performed any heavy physical activity 24 h prior to scanning. For the DXA scan, participants wore loose comfortable clothing with no metal or plastic zippers, buttons or snaps. Prior to use on participants, a QA block phantom is used to calibrate the system, to ensure optimal measurement. In addition, spine phantoms are run bi-monthly to assess the reproducibility and accuracy of the system over time.

4.3.5 Metabolic and biochemical measurements:

4.3.5.1 Oral Glucose tolerance test (OGTT) and Glucose Assay:

All participants completed a 2 h OGTT using 75 g unflavoured dextrose dissolved in water 3 times (OGTT1: before injection; OGTT2: ~5 d after first injection; OGTT3: after 6-weeks RET) during the study. Venous blood samples

were obtained at before and every 15 min (0, 15, 30, 60, 75, 90, 105, and 120 min) after dextrose ingestion to be assayed for glucose and insulin levels.

4.3.5.2 Glucose, Insulin and C-peptide analysis:

Blood glucose was analysed using glucose analyser machine (YSI 2300 STAT PLUS, UK) and plasma insulin and C-peptide concentrations were measured using high-sensitivity insulin enzyme linked immunosorbent assays (Insulin: DRG Instruments GmbH, Marburg, Germany; C-peptide: EZHCP-20K, Millipore, US) according to the manufacturer's protocol.

4.3.5.3 Testosterone Assay (ELISA):

Venous blood samples were collected into EDTA-coated tubes intermittently during the study i.e., before injections and prior to individual RE sessions in the mornings to measure fluctuations in total T concentrations. Blood samples were immediately cold centrifuged at 1750 g, with resulting plasma fractions aliquoted and frozen at –80°C until further analysis. An enzyme-linked immunosorbent assay (ELISA; ab108666, Abcam, United Kingdom) competitive technique was used to assess the abundance of total T in the plasma of all participants the intra-assay coefficient of variation was <5%, and the assay sensitivity was 70 $ng \cdot dl^{-1}$; the detection range was 20-1600 $ng \cdot dl^{-1}$.

4.3.5.4 Calculation of insulin resistance/sensitivity:

$$\text{HOMA} - \text{IR} = \frac{G_0 \times I_0}{22.5}$$ (Matthews *et al.*, 1985)

$$\text{Matsuda} = \frac{1000}{\sqrt{G_0 \times I_0 \times G_{mean} \times I_{mean}}}$$ (Matsuda and DeFronzo, 1999)

$$\text{HOMA} - \beta = \frac{20 \times C - \text{Peptide}_0}{G_0 - 3.5}$$ (Wallace, Levy and Matthews, 2004)

$$\text{Cederholm} = \frac{75000 + (G_0 - G_{120}) \times 1.15 \times 180 \times 0.19 \times \text{Weight}}{120 \times \log(I_{mean}) \times G_{mean}}$$ (Cederholm and Wibell, 1990)

$$\text{GIR} - \text{Index} = \frac{20}{C - \text{Peptide}_0 \times G_0}$$ (Ohkura *et al.*, 2013)

Where. G_0 is fasting glucose level, I_0 is fasting insulin level, G mean or I mean are mean of glucose or insulin levels during the OGTT, C-peptide$_0$ is fasting C-peptide level, HOMA-IR is Homeostatic model assessment-insulin resistant, HOMA-β is Homeostatic model assessment-β cell function, Matsuda and Cederholm are indices of insulin sensitivity, GIR-index is glucose infusion rate-index. In HOMA-IR, insulin levels is used to determine the resistance to insulin action and in HOMA-β C-peptide is used to determine pancreatic insulin secretion (β-cell function) (Matsuda and DeFronzo, 1999).

4.4 Results:

Physiological characteristics of participants are shown in Table 4-1. In accordance, only the OT study group significantly increased weight (P=0.006) and BMI (P=0.006) after 6-weeks of RET; primarily through significant gains in FFM in the OT group vs. baseline (see body composition section below). Further, total T concentrations in the OT therapy group were significantly higher and in YZ group were significantly lower than in the corresponding P group at all time-points after baseline (P<0.05). Plasma T concentrations during the study are shown in Figure 4-2.

Table 4-1: Physiological characteristics of participants

	YZ(n=8)		*YP(n=8)*		*OT(n=9)*		*OP(n=9)*	
	Baseline	Week 6	Baseline	Week6	Baseline	Week6	Baseline	Week6
Age (y)	22.2(0.8)	—	21.1(1.2)	—	69.7 (0.8)	—	69.5 (1.3)	—
Height (m)	1.7(0.1)	—	1.8(0.1)	—	1.75 (0.02)	—	1.75 (0.02)	—
Weight (kg)	72.9(4.1)	74.1(4.3)	75.7(4.4)	76.7(4.3)	78.6 (3.1)	80.4 (3.2)[a]	81.8 (4.3)	82.4 (4.1)
BMI ($kg.m^{-2}$)	23.1(1.1)	23.4(1.1)	22.9(1.4)	23.2(1.4)	25.7 (1)	26.2 (1)[a]	26.5(1.1)	26.7 (1.1)

Values are means (SEM).

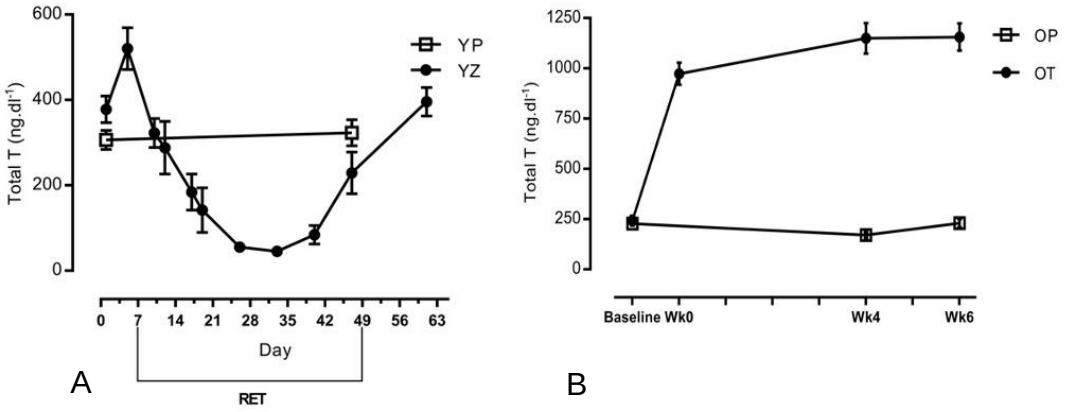

Figure 4-2: Time course of changes in total T in young (A) and older (B) groups

4.4.1 Body Composition:

Total FFM increased significantly following 6-weeks RET in OT (53.0±1.7 to 56.0±5.2 kg, 5.8%, P<0.0001, Figure 4-3 C), but not in OP (54.1±6.3 kg to 54.8±5.8 kg, 1.46%). Further, TFM (22.5±2.0 kg to 21.3±1.8kg vs. 24.7±2.8kg to 24.5±2.7kg, P=0.01) decreased significantly in OT, but not in OP (Figure 4-3 D). Additionally, FFM increased significantly to a greater extent following the 6-week RET in YP (55.9±1.7kg to 57.4±1.7kg, 2.7%, P=0.006, Figure 4-3 A) than YZ (55.4±2.8kg to 55.8±3.1kg, 0.65%). However, YP study group showed a trend in decrease in TFM (19.1±3.4kg to 18.5±3.3kg, P=0.4) vs. YZ (16.7±1.9 kg to 17.4±2.1 kg, P=0.2, Figure 4-3 B).

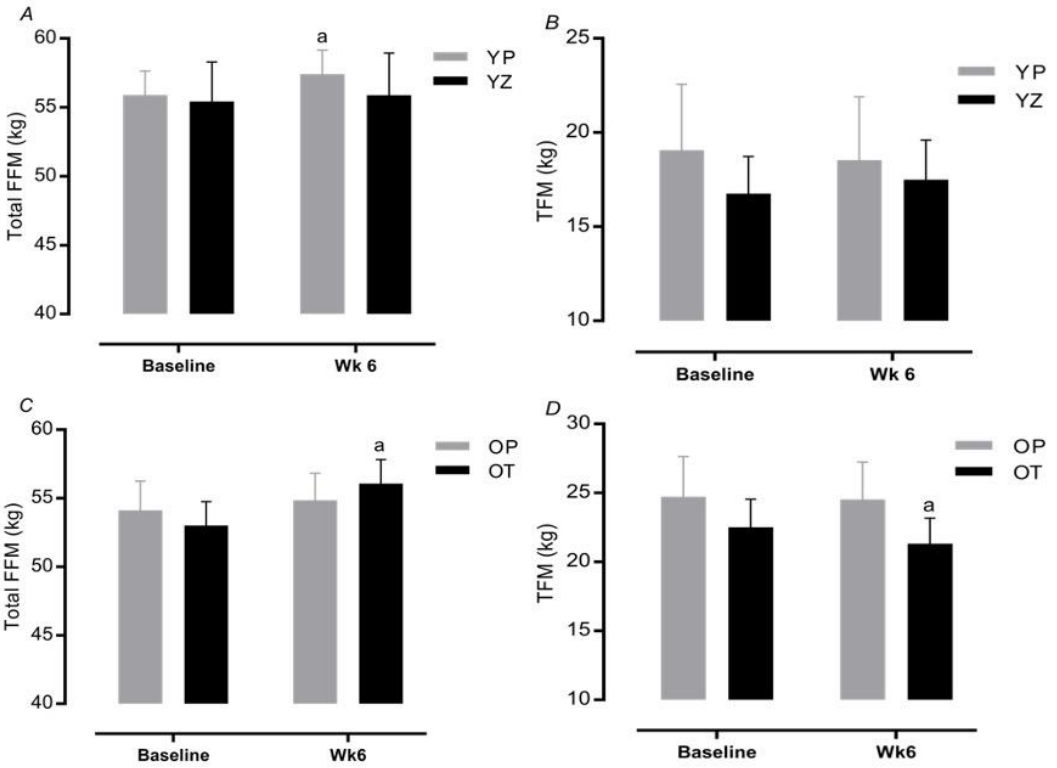

Figure 4-3: changes in fat free mass (FFM) and total fat mass (TFM) in younger and older groups. Values are means ± SEM. a- Significantly different from baseline

4.4.2 Glucose, insulin and C-peptide levels:

Over the RET, both the YP and OT subjects decreased AUC insulin, YP decreasing 2381 ± 1013 $mU^{-1}.2h^{-1}$, P = 0.009 and OT 1367 ± 572 $mU^{-1}.2h^{-1}$, P =0.03. Further, measures of C-Peptide showed decreases in OT compared to OP (OT: 601±46 $ng.ml^{-1}.2h^{-1}$vs. OP: 830±77 $ng.ml^{-1}.2h^{-1}$, P=0.02), with a trend in YP compared to YZ (551±57 vs. 789±116, P=0.08). However, by 6 weeks of RET there was no changes in glucose AUC of all groups (Figure 4-4).

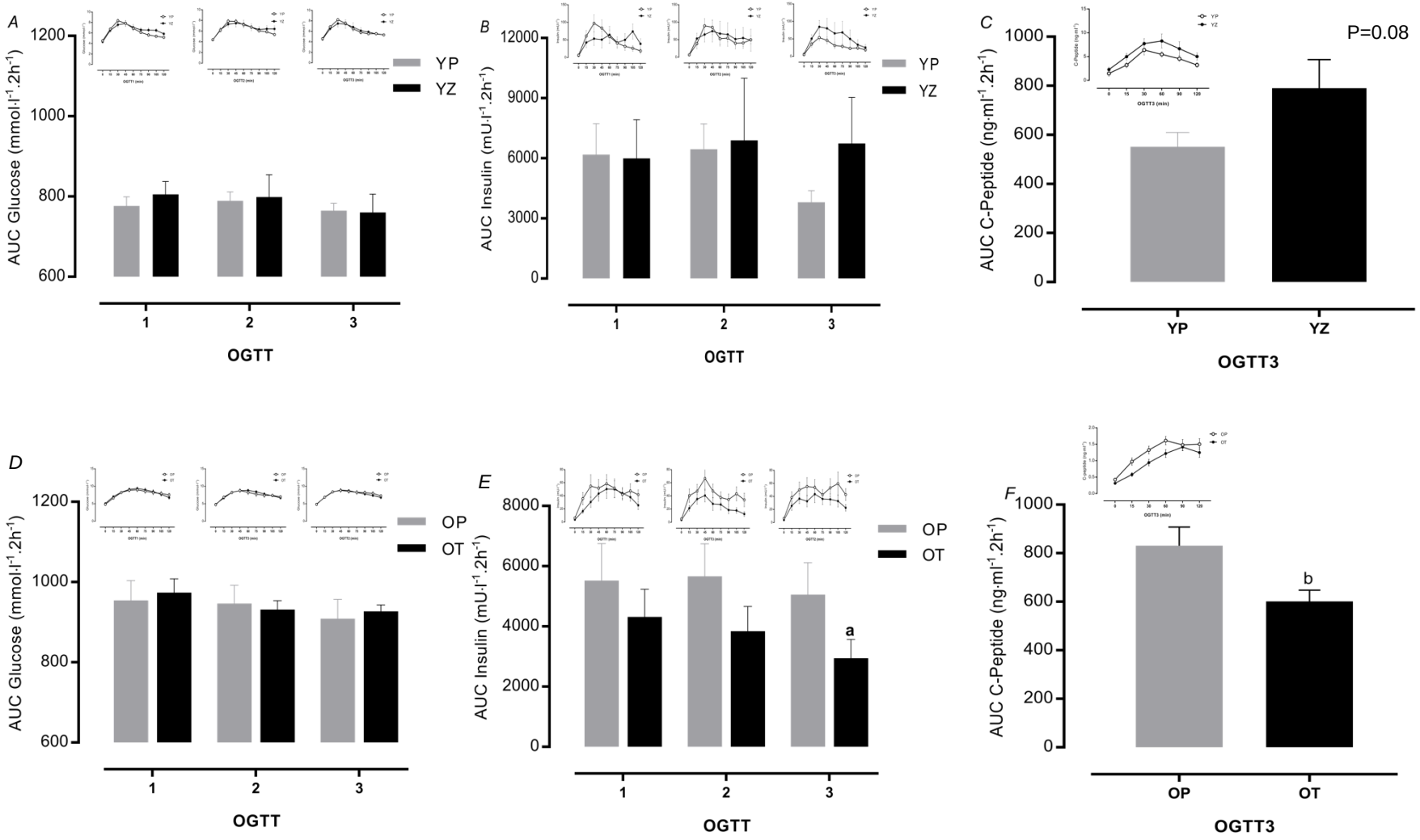

Figure 4-4 changes in insulin, glucose and C-peptide (A-F). Values are means ± SEM. a Significantly different from baseline, P<0.05; b significantly different between two groups, P<0.05. AUC: area under curve.

4.4.3 Insulin resistance and sensitivity:

Over the RET, HOMA-β and HOMA-IR did not change significantly (P<0.05). However, GIR-Index and Matsuda increased significantly in YP (P<0.05) and Cederholm index increased significantly in both YP and OT groups (P<0.05, Table 4-2)

Table 4-2 Glycaemic control-related calculations.

Index	Group	OGTT1	OGTT2	OGTT3	Group	OGTT1	OGTT2	OGTT3
HOMA-β	YP(n=8)	----------	----------	27.8±10.5	OP(n=9)	----------	----------	36.7±5.1
	YZ(n=8)	----------	----------	48.9±8.5	OT(n=9)	----------	----------	27.1±4.8
HOMA-IR	YP(n=8)	1.28±0.3	1.27±0.2	0.99±0.1	OP(n=9)	1.33±0.2	1.37±0.2	1.03±0.2
	YZ(n=8)	0.91±0.2	1.36±0.4	1.36±0.3	OT(n=9)	0.89±0.1	0.88±0.2	0.78±0.1
GIR-Index	YP(n=8)	----------	----------	8.2±2.5[d]	OP(n=9)	----------	----------	2.4±0.6
	YZ(n=8)	----------	----------	2.5±0.4	OT(n=9)	----------	----------	2.7±0.3
Matsuda	YP(n=8)	9.9±2.4	7.6±1.2	12.8±3.2[C]	OP(n=9)	8.4±2.4	8.1±2.1	10.1±2.6
	YZ(n=8)	12.4±2.6	10.1±1.7	8.4±1.1	OT(n=9)	12.2±2.8	13.4±2.5	15.3±2.6
Cederholm	YP(n=8)	68.1±5.1	61.7±4.7	74.5±3.7[b]	OP(n=9)	49.1±3.7	49.8±3.7	57.1±5.1
	YZ(n=8)	65.7±6.1	66.1±6.9	68.1±5.3	OT(n=9)	57.3±5.1	60.1±3.5	68.6±5.3[a]

a significantly different from OGTT1, b significantly different from OGTT2, c significantly different based on fold changes, d significantly different between groups. HOMA-IR: homeostasis model assessment for insulin resistance; HOMA-B: homeostasis model assessment for beta cell function; GIR-index: Glucose infusion rate index.

4.4.4 Correlations and ratios:

Increase in FFM was correlated with increase in glucose levels in YZ and OP groups with lower T levels (Figure 4-6A); however, increase in FFM was negatively correlated with increase in insulin levels in OT group with higher T levels (Figure 4-6.B). Moreover, insulin levels were negatively correlated with T levels in OT (Figure 4-6.D). Nevertheless, there was no correlation between insulin and TFM (Figure 4-6.C). In addition, in line with moderately further decrease in glucose levels per unit of FFM in OT group (Figure 4-5.A), insulin levels per unit of FFM decreased in YP and OT with higher T levels during 6-weeks RET, compared to YZ group (Figure 4-5.B). To support, insulin to glucose ratio decreased in YP and OT groups significantly in comparison with YZ (Figure 4-5.C).

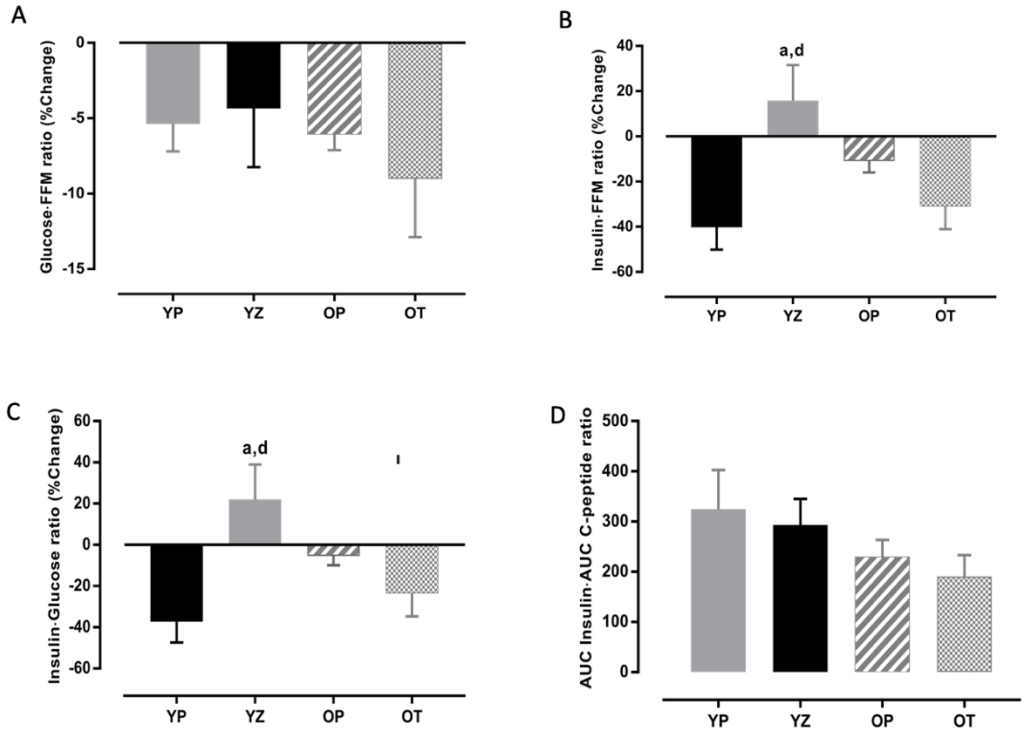

Figure 4-5: Ratios (A-D). Values are means +/- SEM. a Significantly different from YP, P<0.05; d Significantly different from OT, P<0.05. AUC: Area under curve

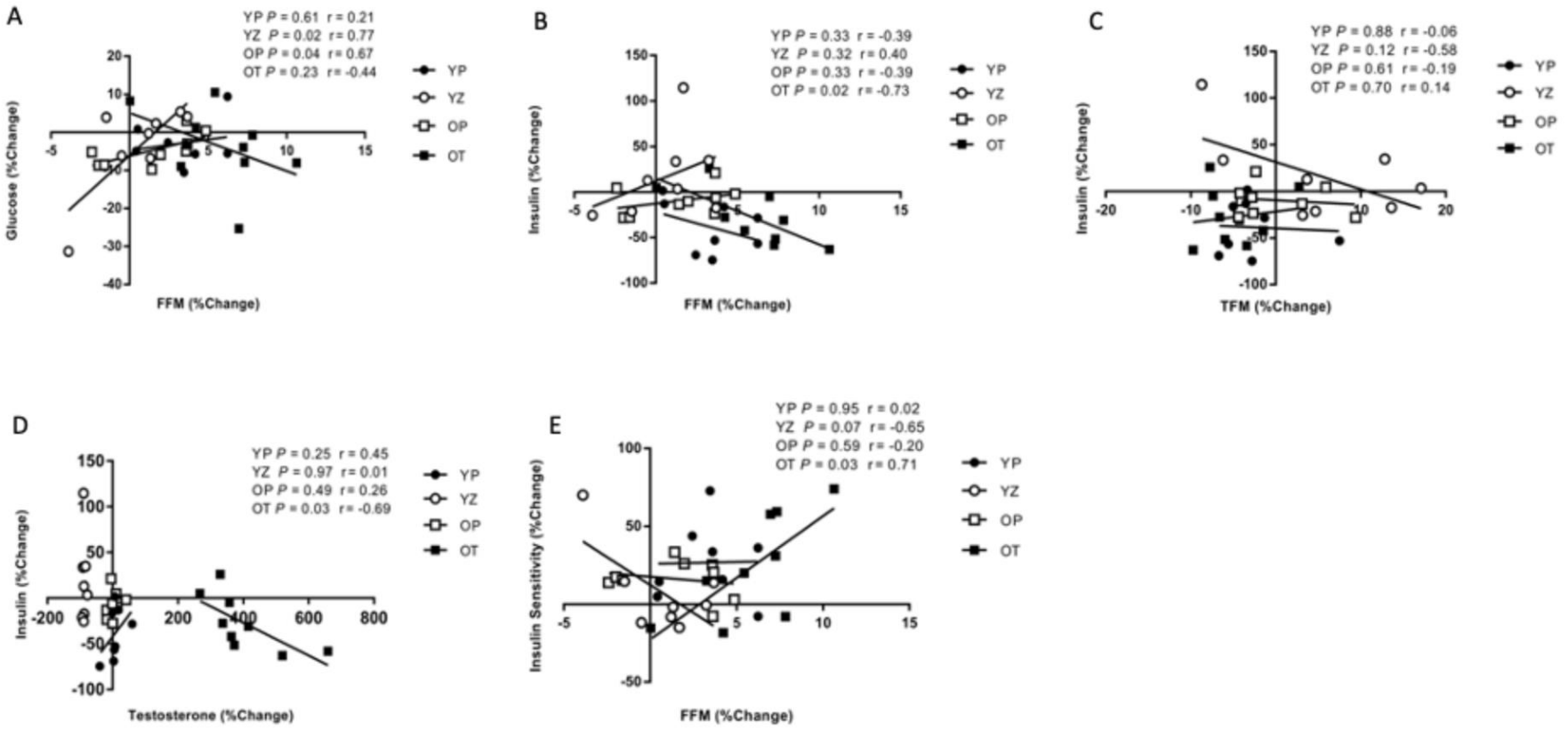

Figure 4-6: Correlations (A-E). Values are means ±SEM.

4.5 Discussion:

There is plethora of evidence suggesting low endogenous testosterone (T) levels are associated with development of insulin- resistant states and type 2 diabetes (Ottarsdottir *et al.*, 2018). However, there is still a paucity of evidence looking at the role of endogenous T and interventions that will alter glucose metabolism in a positive way with ageing (Muraleedharan and Jones, 2010) and anabolic hormonal therapy. Hence, in this study, in line with gaining FFM, with 6 weeks of RET coupled with the suppression of endogenous T (YZ) and provision of exogenous T (OT) showed significant effects particularly YZ was associated with blunted decrease in AUC insulin and on the other hand OT showed attenuated AUC insulin, when compared to baseline measures respectively.

The role of FFM changes in relation to RET and various metabolic diseases in altering glycemic control has been contentious recently (Lagacé, Brochu and Dionne, 2020). Further, a recent meta-analysis of studies investigating the effects of 8 weeks of RET in older Type 2 diabetics showed that, altogether improvement in glycaemia control assessed with HbA1c were independent of variation in FFM (Lee, Kim and Kim, 2017). Interestingly, Baldi et al., reported inverse relationship of HbA1c with variation of FFM with 10 weeks of RET in individuals with T2DM (Baldi and Snowling, 2003). In addition, Shaibi et al., reported 16 week of RET (twice per week) significantly insulin sensitivity in overweight Latino males independent of changes in body composition (Shaibi

et al., 2006). Supporting the above argument, it is reported that 20 weeks (Smutok *et al.*, 1993) and 3 months (Eriksson *et al.*, 1997) of RET augmented insulin sensitivity similar to those observed following endurance training. In line with majority of studies as discussed above, this study with 6 weeks of RET in YP and OT observed significant increase in FFM and positive effects on glycemic control. Indeed, increased FFM enhanced availability of glucose storage area in total resulting in efficient clearance of circulating glucose and therein facilitating normal glucose tolerance (Ibañez *et al.*, 2005; Misra *et al.*, 2008). Accordingly, augmented FFM seems to positively associated with improvement in glycaemia control in men after RET (Eriksson *et al.*, 1997) although it appears to be independent of alteration in intrinsic capacity of muscle to respond to insulin (Miller, Sherman and Ivy, 1984; ET *et al.*, 2000). In the present study, we showed that the groups that had augmented FFM gain (i.e., YP and OT) represented attenuated insulin levels and augmented insulin sensitivity after 6-weeks RET. However, in line with previous studies (Takala *et al.*, 1999), RET did not change insulin-stimulated glucose uptake per kilogram of FFM after 6-weeks RET, and as increased glycaemia control represents a true long-term adaptation to RET (Ebeling *et al.*, 1993); hence, it seems failure to show increase in insulin sensitivity in YZ and OP was probably due to short-term (6-weeks) RET (Takala *et al.*, 1999). Though, 12-weeks combined RET and aerobic training had no effect on glycaemia control (i.e. glucose and insulin levels) in men with prostate cancer undergoing androgen deprivation therapy (>2-months) (Galvão *et al.*, 2010). In addition, 16-weeks

heavy RET with/without growth hormone provision increased FFM but did not change glucose, insulin, and C-peptide levels in healthy older men (Zachwieja *et al.*, 1996). Consequently, considering a positive correlation between FFM gain (YZ: 0.6% vs. OP: 1.4%) and glucose levels in both YZ and OP groups, and negative correlation between T and insulin in OT group, the positive effects of RET on glycaemia control in YP and OT cannot be attributed to a mere increase in FFM (Andersen *et al.*, 2003) and likely via factors independent of changes in FFM (Shaibi *et al.*, 2006).

Furthermore, the interplay between hypogonadism and the development of the metabolic syndrome is still unclear (Laaksonen *et al.*, 2004), low testosterone *per se* is associated with metabolic syndrome (i.e. decreased insulin sensitivity (Keating, O'Malley and Smith, 2006), increased insulin resistance (Grossmann, 2014; Wang *et al.*, 2016) and type 2 diabetes (Wang *et al.*, 2016), likely via decrease in FFM and increase in TFM (Wang *et al.*, 2016). In fact, to support this argument, our results showed higher T levels in OT group during RET was associated with attenuated insulin level, it is postulated that higher T levels and FFM were associated with up-regulated glucose metabolism in healthy men (Pitteloud, Mootha, *et al.*, 2005; Hou *et al.*, 2015). Interestingly, T therapy seems to counter act glycaemia control deficiency during ageing/ illnesses (Grossmann, Gianatti and Zajac, 2010); e.g. provision of exogenous T (200mg, every 2-weeks for 3-months (Kapoor *et al.*, 2006) or oral 160mg/d for 6-months (Emmelot-Vonk *et al.*, 2008)) has shown to reduce insulin resistance and improves glycaemia control in hypogonadal men with

type 2 diabetes and healthy older men, respectively. On the contrary, T therapy alone for 40-weeks (1000 mg injection, every ~2 months) did not improve insulin resistance and glycaemia control in hypogonadal men with type 2 diabetes, albeit increased in FFM and decrease in TFM (Gianatti *et al.*, 2014); suggesting the greater benefits of T therapy adjuvant to RET in our study. Moreover, short-term (12-weeks) (Smith, Lee and Nathan, 2006) and long term (24-months) (Haidar *et al.*, 2007) androgen deprivation therapy in men with prostatic cancer decreased insulin sensitivity and increased TFM which were associated with an increased risk of developing type 2 diabetes (Kintzel *et al.*, 2008). Furthermore, lower endogenous T levels were associated with increase C-peptide levels, in turn suggesting impaired pancreatic ß-cell function and increased insulin secretion in older men (Tsai *et al.*, 2000). In our study, we assessed C-peptide levels to measure endogenous insulin secretion, GIR and ß-cell function (Jones and Hattersley, 2013). Both C-peptide and insulin are secreted in equimolar amounts from the ß-cells as the reliable surrogate marker for assessing pancreatic ß-cell function and GIR (Chailurkit *et al.*, 2007; Ohkura *et al.*, 2013). Further, C-peptide is only minimally removed and metabolised by the liver and has longer half-life when compared to insulin 20-30 min vs. 3-5 min) (Jones and Hattersley, 2013). In our study, both younger group with higher endogenous T and older group with exogenous T provision had lower levels of C-peptide after 6-weeks of RET, demonstrating decreased secretion of endogenous T in both groups with higher peripheral insulin sensitivity and GIR. Henceforth, T had permissive role

in improvement of glycemic control during short-term RET and T provision coupled to RET seems to be an effective strategy to combat andropause-related metabolic disorders. Interestingly, some previous studies have reported debatable results about the effects of long term RET on glycemic control in older men with lower endogenous T; 16- weeks (Zachwieja *et al.*, 1996) RET showed no improvement in glycemic control (measuring c-peptide levels) in older men, nevertheless 12-weeks (Iglay *et al.*, 2007) and 4-months (Hansen *et al.*, 2012) of RET decreased fasting c-peptide but, those studies did not estimate insulin sensitivity and insulin levels in older and middle aged healthy men, respectively. In our study, we showed only 6-weeks of RET adjuvant to T therapy (vs. RET alone) augmented glycaemia control in older men; furthermore, T deprivation during RET blunted glycaemia control improvement in younger men (vs. RET with normal T levels). However, it appears RET eliminated worsened glycemic control induced T suppression (Smith, Lee and Nathan, 2006).

To conclude, T therapy (OT) and non-suppressed endogenous T (YP), enhanced insulin sensitivity (supported by reduced C-peptide) in older and young men, respectively, coupled with RET, suggesting endogenous T is a permissive mediator of RET-induced augmentation of insulin sensitivity; and T therapy adjuvant to pre/re-habilitation RET may be of value to overcome the burgeoning trend of worsening glycaemia control with ageing.

Chapter 5: Effects of Beta-Hydroxy-Beta-Methylbutyrate (HMB) on Carbohydrate Metabolism in young and old healthy men:

5.1 Overview:

Insulin resistance remains a principal component in development of various metabolic diseases. The main basis of treatment of insulin resistance until now remains diet and exercise but complicated by poor adherence to these measures. Beta-hydroxy beta-methylbutyrate (HMB) is a nutraceutical supplement with controversial effects on insulin sensitivity in animal models and poor understanding in humans. The aim of this study is to evaluate the impact of acute supplementation of HMB on insulin sensitivity in humans during an oral glucose tolerance test.

Methods: Ten young (18-35y) and 10 older (65-85y) males underwent two 75g oral glucose tolerance test.

Results: HMB significantly reduced the insulin area-under-the-curve (AUC) with no difference in glucose AUC, resulting in a trend towards an increase in the Cederholm index of insulin sensitivity in younger men. In older men, HMB had no effect on insulin or glucose responses.

Conclusions: Acute HMB supplementation may improve insulin sensitivity following a single oral glucose load in young men; however, this does not appear to be sustained into older age.

5.2 Aim of the study:

The aim of this study was to evaluate the acute impact of a novel nutraceutical, a metabolite of amino acid leucine termed HMB supplementation, on glycaemic control during an oral glucose tolerance test in young and older humans.

5.3 Materials and Methods:

Institutional research ethics approval (University of Nottingham Faculty of Medicine and Health Sciences Research Ethics Committee) was obtained (A12092016 SoM MS GEM) to recruit 10 healthy young (aged 18-35 y) and 10 healthy older (aged 65-85 y) male subjects by local advertising.

Exclusion criteria included extremes of BMI (<18 or >32 kg/m2) and diagnosis of metabolic disorders. The study was registered with clinicaltrials.gov (NCT03018496) and complied with the 1964 Declaration of Helsinki.

After providing written informed consent to participate in the study, all subjects underwent a clinical examination involving measurements of height, weight and resting blood pressure (OMRON M3, Omron Healthcare, UK) before being enrolled. Each subject performed two, 3 h 75g oral glucose tolerance tests (OGTT)(FM *et al.*, 2007; Wopereis *et al.*, 2009), spaced 2-weeks apart. During one OGTT, the glucose was administered with 3g HMB-FA (a dose previously

shown to produce a measurable effect on protein metabolism (Dj *et al.*, 2013)), with glucose alone administered during the alternate OGTT. The order in which the subjects completed the two studies was randomised via a computer-generated randomisation tool (sealedenvelope.com).

On each study day, subjects presented to the laboratory at 0900h fasted from 2100h the preceding evening. On arrival, a 22G retrograde intravenous cannula was inserted into a vein in the dorsum of a hand to facilitate blood sampling (using the hot-hand method (heated to 55 ^{o}C)(Liu *et al.*, 1992)).

Blood was drawn for real-time quantification of blood glucose concentrations with aliquoted plasma frozen for later quantification of insulin concentrations.

After baseline measures, participants drank 75g oral dextrose (Myprotein, Northwich, UK) dissolved in 200mL of water, with or without 3g HMB-FA gel (Metabolic Technologies Inc., Ames, Iowa, USA) provided immediately after the glucose. Blood samples were drawn at baseline then 15, 30, 45, 60, 107 80, 100, 120, 140, 160 and 180 minutes after the dextrose drink.

Leg blood flow was measured as a potential mechanism by which HMB could affect glucose and insulin kinetics. Measurements were made of the common femoral artery of the right leg at three time points during each study visit (baseline, 60 and 120 minutes after dextrose) using Doppler ultrasound (iU22, Phillips Healthcare, Guildford, UK) and a 9-3mHz probe. Blood flow was

calculated as the product of the artery cross sectional area and the mean blood velocity over 3 cardiac cycles (Phillips *et al.*, 2012).

Blood glucose concentrations were determined using a near patient glucose analyser (YSI Life sciences, Ohio, USA). Plasma insulin concentrations were determined using an ultrasensitive enzyme linked immunosorbent assay (ELISA) (Mercodia Ultrasensitive Insulin ELISA, Mercodia, Uppsala, Sweden).

Area-under-the-curve (AUC) analyses were performed for both glucose and insulin concentrations facilitating calculation of the Cederholm and Matsuda indices of insulin sensitivity, with the homeostatic model of insulin resistance (HOMA-IR) calculated from fasting values (Patarrão, Wayne Lautt and Paula Macedo, 2014).

β-hydroxy-β-methylbutyric acid (HMB-FA, CAS# 625-08-1; Formula: C5H10O3) was manufactured by TSI Co., Ltd (Shanghai, China) for Metabolic Technologies, Inc. (Ames, IA, USA) and purity were 98% as measured by high performance liquid chromatography (HPLC).

Area under curve (AUC) analyses were performed for both glucose and insulin concentrations enabling calculation of Cederholm (Cederholm and Wibell, 1990) and Matsuda indices (Matsuda and DeFronzo, 1999) of insulin sensitivity. Further, Homeostatic model of insulin resistance (HOMA-IR) was calculated form fasting values (Patarrão, Wayne Lautt and Paula Macedo, 2014)

HOMA-IR was calculated using formula:

$$\text{HOMA IR} = \frac{\text{Insulin assay (microunits/ml)} \times \text{Fasting Plasma Glucose (mmol/ml)}}{22.5}$$

All statistical analyses were performed using Graphpad Prism Version 7.02 (California, USA) with data presented as mean (SD). After conformation of normality, analysis was performed using 2-way ANOVA by age and treatment with Sidak's multiple comparison post-hoc testing. Significance was set at $p<0.05$.

5.4 Results:

Ten young and ten older subjects were recruited to study with all subjects completing both OGTT's. Definition of impaired fasting glucose (IFG- if fasting plasma glucose 6.1 to 6.9mmol/l and 2-h post ingestion of 75g oral glucose <7.8mmol/l) and Impaired glucose tolerance (IGT- if fasting plasma glucose <7.0mmol/l and 2-h post ingestion of 75g oral glucose ≥7.8 and <11.1mmol/l)(WHO, 2006). Subject characteristics are displayed in Table 5-1.

HMB did not alter leg blood flow responses to glucose in either the young or older participants at any time point, although there was a main effect of age at all three time points (Table 5-2).

There was a main effect of time of insulin and glucose in both young and older subjects. (all $P<0.0001$).

HMB significantly reduced the insulin AUC (4158 (2753) vs. 5836 (4451) mU/Lx180min; $p=0.02$) in younger subjects (Figure 5-1C), with no difference in glucose total AUC (1127 (147) vs. (1142 (122) mmol/Lx180min; $p=0.8$) (Figure 5-1D). This led to a trend towards an increase in the Cederholm index of insulin sensitivity (62.7 (13.9) vs. 55.1 (11.0); $p=0.08$))(Figure 5-1E). There was no change in the Matsuda index of insulin sensitivity (10.3 (4.9) vs. 8.9 (4.4); $p=0.19$) (Figure 5-1F, respectively).

Table 5-1- Subject characteristics (Mean (SD))

	Young participants (N=10)	Older participants (N=10)
Age (years)	22 (4)	72 (3)[1]
Height (cm)	179 (6)	176 (6)
Weight (Kg)	81 (10)	82 (7)
BMI (Kg/m2)	25.4 (2.5)	26.5 (2.7)
Systolic blood Pressure (mmhg)	129 (8)	143 (19)[2]
Diastolic blood pressure (mmhg)	76 (9)	83 (15)
HOMA-IR	1.2 (0.9)	1.3 (0.6)
Impaired fasting glucose (6.1-6.9 mmol/l) (n)	0	0
Impaired glucose tolerance 120min sample (≥ 7.8 and <11.1mmol/l (n)	0	7

[1]$p<0.001$ vs. young participants, [2]$p<0.05$ vs. young participants

Table 5-2- Leg blood flow (ml/min)

Time	Young			Older		
	HMB	No HMB	p	HMB	No HMB	p
Baseline	510 (148)	440 (147)	0.37	275 (97)	281 (121)	0.99
60 min	478 (168)	534 (180)	0.74	334 (134)	371 (150)	0.79
120 min	572 (183)	518 (148)	0.69	343 (119)	302 (113)	0.81

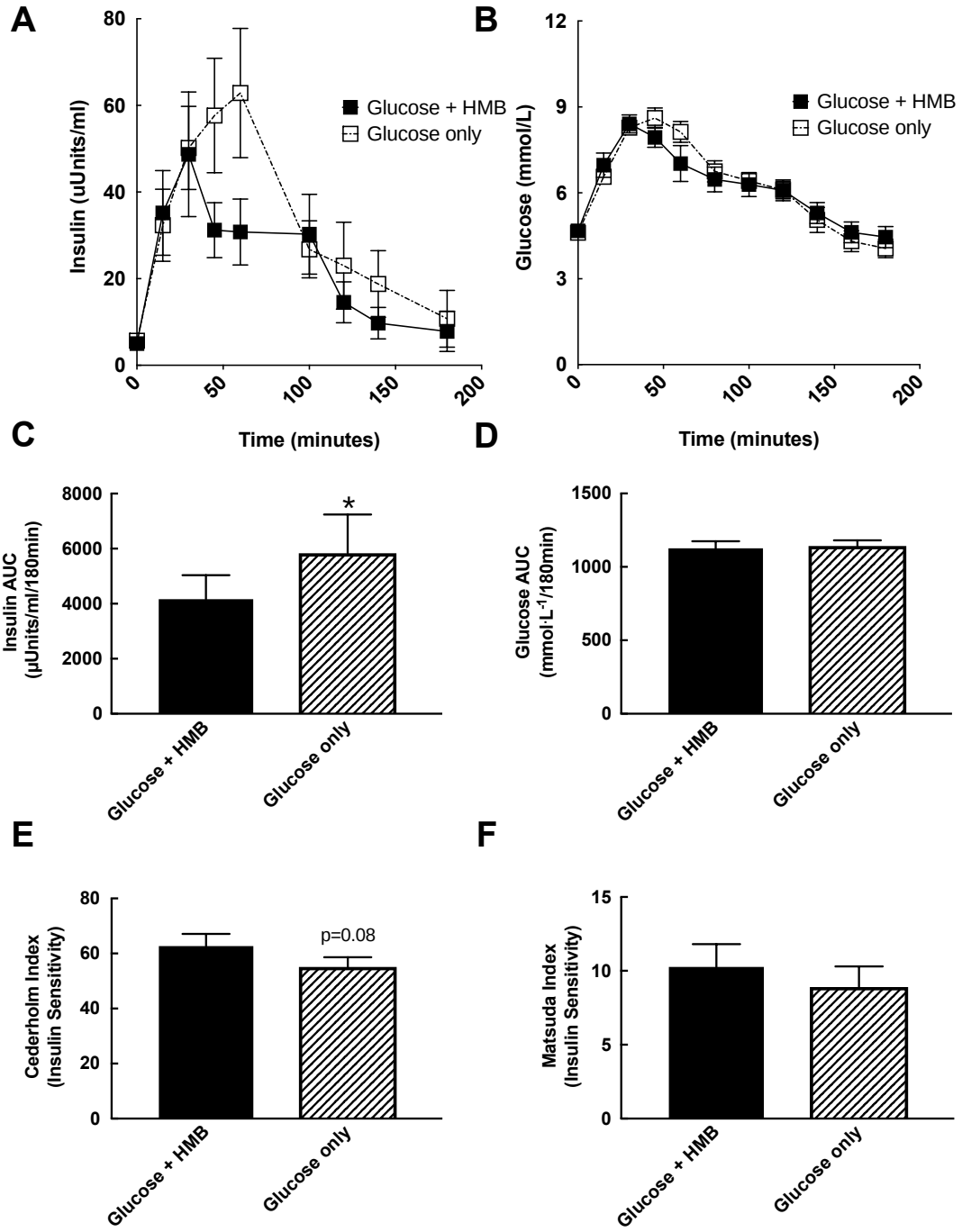

Figure 5-1- Insulin (A) and glucose (B) concentrations before (time 0) and after a 75g glucose challenge. Area-under-the-curve (AUC) for Glucose (C) and insulin (D), and Cederholm (E) and Matsuda indices (F) of insulin sensitivity for young participants

In older subjects, HMB had no effect on the insulin (5712 (2488) vs. 5980 (2854) mU/Lx180min; p=0.88) (Figure 5-2C) or total glucose AUC (1426 (194) vs. (1409 (180) mmol/Lx180min; p=0.84) (Figure 5-2D), with no resultant difference in either their Cederholm (57.9 (14.2) vs. 55.6 (10.7); p=0.72) or Matsuda index (9.2(4.3) vs. 8.8(5.2); p=0.88) (Figure 5-2E and Figure 5-2F, respectively). There was a main effect of age on total glucose AUC, which was not present for insulin AUC or either index of insulin sensitivity. All of the statistical findings reported for total glucose AUC were also apparent for incremental glucose AUC.

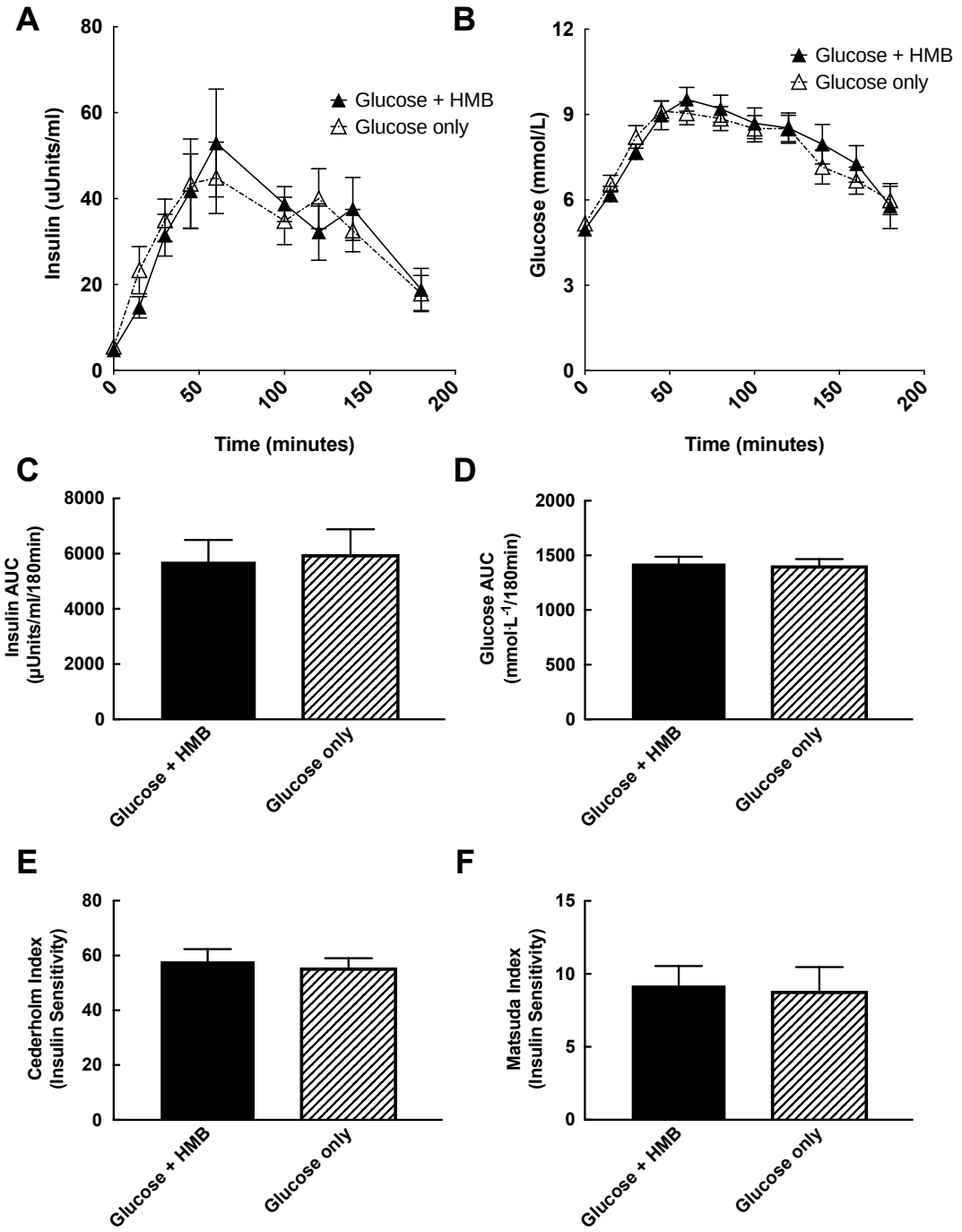

Figure 5-2- Insulin (A) and glucose (B) concentrations before (time 0) and after a 75g glucose challenge. Area-under-the-curve (AUC) for Glucose (C) and insulin (D), and Cederholm (E) and Matsuda indices (F) of insulin sensitivity for old participants (65-85y)

5.5 Discussion:

There is ongoing intensive effort amongst scientific community towards identifying novel nutraceutical approach to treat insulin resistance (IR) particularly with ageing, given IR is a major factor in Type 2 diabetes and also, associated with many maladies such as obesity, essential hypertension, dyslipidaemia, non-alcoholic fatty liver disease, obstructive sleep apnoea and cancer (Evans and Goldfine, 2013).

This study has demonstrated a single 3g dose of HMB can reduce acute insulin response to an oral glucose load in younger men indicating a possible role in improving insulin sensitivity, however same was not observed in older men suggesting this effect is not persistent as you get older. This observation dampens the potential of HMB to be used as a nutraceutical to improve insulin sensitivity, as insulin resistance is prevalent in old age (Ryan, 2012).

Given, emerging evidence suggests that HMB has been positively associated with acute anabolic responses (Wilkinson *et al.*, 2013, 2018) it can be postulated that, HMB may lead to improvement in insulin sensitivity allowing for the fact that insulin plays an important role in anabolic processes (Wilkes *et al.*, 2009; Abdulla, Smith, Philip J Atherton, *et al.*, 2015). Furthermore, despite clear evidence suggesting anabolic effect of HMB in older adults (Holeček, 2017) what is not clear is, why there is no improvement in insulin sensitivity in older adults.

Recently a review showed that, in healthy as well as in overweight and type 2 diabetic subjects, the short term energy balanced high protein diet only showed limited and minor effects on insulin sensitivity concluding the results were equivocal (Rietman *et al.*, 2014). However, Harber et al reported that acute (24 h average insulin concentration was 50% lower, $p<0.001$, after 2 and 7 d) low carbohydrate and high protein diet caused as immediate postprandial reduction in daily plasma insulin (Harber *et al.*, 2005). Conversely, Chen et al reported negative effect of high protein diet on insulin sensitivity (Chen *et al.*, 2020). So, our data herein suggests that specific amino acid metabolites (HMB and their precursor amino acids) may enhance insulin sensitivity under certain conditions. We postulate, one potential mechanism for this improvement in insulin resistance in our study could be improvement in leg blood flow leading to increase in peripheral glucose uptake, however this was not the case in this study. It may be noted that, given leg blood flow was only recorded at specific time points (baseline, 60 min and 120 min), an earlier or later improvement in leg blood flow may have been missed. Further in our study, there was main effects of age on leg blood flow at all 3 time points (baseline, 60min and 120min) further supporting the notion that basal whole-leg blood flow and vascular conductance decrease progressively with advancing ageing in healthy men (Dinenno *et al.*, 1999) and this may in turn explain the lack of improvement in insulin sensitivity in older men.

HMB predominantly available in calcium salts (CaHMB) and a study by Fuller et al reported that Free acid gel form of HMB resulted in quicker and greater

concentration (+185%) and improved clearance (+25%) of HMB form plasma, concluding that gel form of HMB could improve HMB availability and efficacy to tissues both in health and pathological state (Fuller *et al.*, 2011). Intriguingly, Vukovich et al reported that acute HMB supplementation in young men had not effect on plasma glucose and insulin concentrations (Vukovich *et al.*, 2001a). This was contrary to what is observed in this study and one possible explanation for above observation could be the gel form of HMB used in this study. Also, Vukovich et al, in their study used different method of quantification of insulin (Radioimmunoassay, RIA) compared to ELISA method used in this study. Further, a reduced blood sampling frequency was reported (large differences were reported at 45and 140 minutes in this study, time points which were not measured by Vukovich et al.,). So, HMB in gel form (used in this study) seems to enhance insulin sensitivity in young men contrary to what is reported in literature in certain condition.

In this study, HMB elicited a trend for increasing Cederholm index of insulin sensitivity (predominantly peripheral) in our young subjects, whilst having no effects on Matsuda index (both hepatic and peripheral) suggesting HMB is affecting predominantly peripheral rather than hepatic element of insulin sensitivity (Gutch *et al.*, 2015). This is conflicting to the observation which has previously been shown in a chronic study of HMB in rodents where an improvement in hepatic insulin sensitivity mediated via GLUT2 was noted (Sharawy *et al.*, 2015). A separate chronic HMB rodent study did however show a potential reduction in GLUT4 mediated peripheral insulin sensitivity

(Yonamine *et al.*, 2014), a finding which may be more in keeping with the results of this study. Per se, further investigation of the mechanistic basis behind the differential effects of HMB on acute glucose handling and changes in insulin sensitivity with advancing age should be concentrated on looking at peripheral mechanisms of insulin sensitivity, including a more detailed analysis of vascular responses. Furthermore, chronic supplementation studies measuring longer-term changes in insulin sensitivity will be required before HMB could be considered as a potential nutraceutical therapeutic agent for improving insulin sensitivity in humans. Finally, although the results herein suggest that the detrimental impact of HMB on insulin sensitivity seen in some rodent studies (Yonamine *et al.*, 2014) does not translate to humans, further clinical trials are required to confirm this.

Two limitations of this study were noted; firstly, no detailed food and physical activity diary were recorded between the study visits. However, to prevent any confounders, all subjects were advised to replicate their dietary and physical activity behaviours for 24 h period before each visit. Further, each subject fasted for 12 h preceding each study visit which is likely to negate the impact of this limitation. Secondly, only male subjects were recruited which limits the generalisability of the finding to wider population group, particularly female population.

In conclusion, acute HMB supplementation can significantly reduce plasma insulin concentrations in young men in response to glucose challenge without

changing blood glucose concentrations, leading towards a trend for improvement in insulin sensitivity. This is not the case in older men. Future trials particularly looking at acute (with detailed consideration to vascular responses) and chronic supplementation with HMB on insulin sensitivity with a through consideration of age (e.g., middle-aged adults) and gender is needed.

Chapter 6: Conclusions:

6.1 Overview of studies and general conclusion:

In this thesis, we demonstrated that human skeletal muscle disuse causes many undesirable complications with emphasis placed on development of peripheral insulin resistance. The underlying processes seem to be very complex and have numerous interplays between different mechanisms. We report that, during skeletal muscle disuse, both post absorptive and post prandial MPS is suppressed, with some evidence albeit controversial supporting increase in protein MPB. In our short term (4 d) single leg immobilisation study showed significant loss of skeletal muscle mainly driven by sustained reduction in MPS. Indirect measures of MPB using validated calculations to derive FBR showed no significant increase. Also, a lack of increase in markers of MPB point towards a minor role, if any, of MPB in short term disuse muscle atrophy. Further, we showed that integrated MPS driven by blunting of anabolic resistance to protein intake (both fasted and fed state) was reduced in short term (4 d) single leg immobilisation. Given, muscle disuse results in development of whole body and muscle specific IR and supporting this argument, our study showed reduced GLUT4 activity in immobilised leg possibly leading to significant elevation of plasma insulin levels. It is also suggested that muscle insulin resistance (IR) secondary to disuse appears to drive the procession of disuse muscle atrophy independent of other mechanisms known to cause muscle IR. Thus, our data suggests

findings consistent with widely reported notion that human skeletal muscle disuse causes peripheral (muscle IR) insulin resistance and hence the search for targeted strategies (to improve basal MPS and anabolic response to feeding) to prevent the setting of muscle atrophy is paramount.

Leading on from above discussion, in search for therapeutic intervention, we postulated that short-term testosterone suppression augments peripheral IR in young men coupled with 6 weeks of RET. In accordance, we also showed poor glycaemic control (blunted decrease in AUC insulin vs. baseline). Suggesting that endogenous testosterone plays a major role in maintaining glycaemic control (improve insulin resistance). On the contrary, in the same study, we also showed that 6 weeks of RET coupled with the exogenous provision of testosterone had significant positive effect on peripheral glycaemic control (attenuated insulin levels and augmented insulin sensitivity) via net protein accretion through anabolic pathways in older men. Collectively, we demonstrated that just 6 weeks of whole body RET coupled with unsuppressed endogenous testosterone (young men) and provision of exogenous testosterone (older men) had positive effects on peripheral glycaemic control via improving net protein accumulation through anabolic pathways. So, we demonstrated the potential of testosterone in reversing the ageing-induced muscle wasting and in turn having a positive effect on glycaemic control. Thus, we argue long-term testosterone supplementation may be an effective strategy to overcome sarcopenia, an unwanted accompaniment of ageing and the potential benefits in glucose metabolism

and potential reductions in the risks of developing type 2 diabetes. However further studies to establish its safety and efficacy over long term is warranted.

In addition, we also postulated Beta-hydroxy beta-methylbutrate (HMB) a nutraceutical supplement with contentious effects on insulin sensitivity in animal models has a positive effect on insulin sensitivity in humans during an oral glucose tolerance test. In accordance, we showed that the supplementation of 3g of HMB during a OGTT in young men significantly reduced the insulin area-under-the-curve (AUC) with no difference in glucose AUC, resulting in a trend towards an increase in the Cederholm index of insulin sensitivity. Interestingly, similar observation was not noted in older men when given HMB. Suggesting, acute HMB supplementation may improve insulin sensitivity following a single glucose load in young men; however, this does not appear to be sustained into older age.

Cumulatively, we can conclude that a multitudinous approach may be needed to overcome the burgeoning burden of worsening insulin resistance and development of persistent IR states such as Type 2 diabetes associated with loss of skeletal muscle: sarcopenia (disuse, disease, ageing etc.) and anabolic hormone resistance (ageing). So, our studies try to approach this complex area and it may be possible to address this by combining pre/re-habilitation (combined with RET) post muscle disuse, combined with anabolic hormone replacement (testosterone) and with nutraceuticals (HMB) supplementation.

6.2 Limitations of studies and future directions:

This thesis aims to draw conclusions and attempts to find intervention strategies (from 3 experimental studies) to address major conundrum the current scientific society is facing particularly sarcopenia and ever-increasing burden of metabolic syndrome. We recognise that there were some limitations with the studies, this and future directions are discussed here.

The immobilisation study looked at short-term (4 d disuse) effects on muscle mass, MPS, MPB and glycaemic control, so may not be capturing full picture of all muscle related metabolic and molecular adaptions as changes may be temporal. So, studies looking at long-term muscle disuse model may be needed. We initially planned to conduct both 3 d and 14 d immobilisation but due to disruption in supply of amino acid tracer we could only complete 4 d immobilisation study in time for this thesis. Also, further research in to establishing direct measure of MPB instead of indirect measure (as used in our study) may offer more in-depth knowledge of the actual catabolic processes.

We recognise that there were number of limitations in relation to our study into effects of testosterone in men coupled with RET. Whilst, in non-hypogonadal men, testosterone both unsuppressed endogenous in young men and exogenous supplementation in older men has shown to improve glycaemic control. But same cannot be assumed for women. The clinical relevance of testosterone in women over 65yrs is not clearly known and high testosterone

level may be associated with insulin resistance, metabolic syndrome and cardiovascular disease (Patel *et al.*, 2009). Further, in women, the effects of testosterone on all insulin-targeted tissues is tightly regulated by circulating hormone levels with a narrow window and accumulating evidence suggest any excess (mainly reported in setting of polycystic ovarian syndrome) contributes to insulin resistance (Diamanti-Kandarakis, Pappalou and Kandaraki, 2019). Given this concern regarding development of IR in women with testosterone supplementation our findings from this study cannot be generalised to entire population. Hence, further studies are needed to investigate the effects of testosterone therapy with or without RET in pre/ post-menopausal women to investigate this controversial area. Also, further studies are needed to investigate the effects of physiological vs. supra-physiological doses of testosterone on the aforementioned end points, including efficacy and safety.

Though our study showed acute HMB-FA can significantly reduce plasma insulin concentration in young men in response to oral glucose load and indicated a possible trend towards improved insulin sensitivity, further investigation of the mechanistic basis of this using glucose clamp techniques (deemed as a gold standard in studying insulin sensitivity) may be warranted. In addition, chronic supplementation studies measuring longer-term changes in insulin sensitivity may be required before HMB-FA could be considered as a therapeutic agent for improving insulin sensitivity.

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Chapter 8: Appendices:

8.1 Appendix 1

Exercise Metabolism Research Group

Department of Kinesiology, McMaster University

Participant Information Sheet and Consent Form

The Effects of Single Leg Immobilization on Muscle Protein Synthesis, Protein Breakdown, and Glucose Uptake in Healthy Young Men

Funding Source: Canadian Institutes of Health Research

INVESTIGATORS

Local Principal Investigator Professor Stuart Phillips Department of Kinesiology McMaster University Hamilton, Ontario, Canada (905) 525-9140 ext. 24465

E-mail: phillis@mcmaster.ca

Co-Investigators Tanner Stokes

Department of Kinesiology McMaster University Hamilton, Ontario, Canada (289)-680-1900

E-mail: stokest@mcmaster.ca

Dr. Stefan Gorissen Department of Kinesiology McMaster University Hamilton, Ontario, Canada (905) 730-5894

E-mail: gorisses@mcmaster.ca

Dr. Mark Tarnopolsky Department of Pediatrics McMaster University Hamilton, Ontario, Canada (905) 525-9140 ext. 75226

E-mail: tarnopol@mcmaster.ca

You are being invited to participate in this research study investigating the impact of single leg immobilization on skeletal muscle mass. Before deciding whether or not you would like to participate in this research study, you should be aware of what is involved as well as the possible health risks present. This form will provide you with a detailed explanation about the research study, which will also be discussed with you in person. Please take your time to make your decision. Feel free to discuss it with your friends and family. The decision to take part in the study is completely up to you. McMaster University and Professor Stuart Phillips have received a grant from the Canadian Institutes of Health Research (CIHR) to cover the costs of conducting the study.

PURPOSE OF THE STUDY

Periods of muscle disuse are commonly experienced in young and elderly individuals as a result of short-term hospitalization or leg casting after injury. Periods of immobilization result in a profound loss of muscle mass and strength. Aside from skeletal muscle's central role in movement, the muscle also plays a large role in general health. For example, skeletal muscle is the largest 'sink' into which blood sugar (glucose) is taken up following a meal. Leg immobilization results in an impairment in the body's ability to take up glucose into skeletal muscle tissue, which can eventually result in insulin resistance. In our study, we are interested in looking at the regulation of muscle mass during and after a short period of leg immobilization. We will measure the rate of muscle growth and breakdown as well as the rate of glucose uptake by the muscle. Understanding the underlying mechanisms causing muscle loss after immobilization will help us to create interventions to prevent the loss of muscle mass and function during periods of disuse.

INCLUSION AND EXCLUSION REQUIREMENTS

How many people will be recruited for this study?

If you consent to participate in this study, you will be one of 8 participants. One of your legs will be immobilized for the 4-day duration of the study, with the other leg acting as a control. We will randomly select which leg will be immobilized, such that 4 participants will have their dominant leg immobilized and 4 participants will have their non-dominant leg immobilized.

Inclusion criteria

We will only include males in this study, who are between the age of 18 and 30 year. In order to participate in this study, you need to be healthy and not smoke.

Exclusion criteria

1) The use of anti-inflammatory and/or analgesic medication, whether it is prescription or not

2) A history of neuromuscular disorders or muscle/bone wasting diseases

3) Any acute or chronic illness, cardiac, pulmonary, liver, or kidney abnormalities, uncontrolled hypertension, insulin-dependent or insulin-independent diabetes, or the presence of any other metabolic disease – all of which will be determined via a medical history screening questionnaire

4) The use of any medications known to affect protein metabolism (glucocorticoids, non- steroidal anti-inflammatory medication, or prescription strength acne medication, etc.)

5) A (family) history of thrombosis

6) The use of anticoagulant medications

PROCEDURES INVOLVED IN THE RESEARCH

Time Commitment

Your time commitment during the study will be a continuous period of 5 days. Prior to the start of the study, we will plan an introductory meeting (screening). During this visit the investigators will determine if you match the criteria required for your participation and will answer any questions that you may have at that time. If you consent to participate in the study following this visit, we will schedule a date for you to start the trial. Over the course of the study duration, you will visit McMaster University two times for testing. The first testing day (day 0) will last roughly 3 hours, whereas the infusion day will last roughly 8 hours. While outside of the laboratory (day 1-3) you will be asked to leave the knee brace in position at all times, as applied by the investigators of the study.

Screening

During the screening you will be asked to complete a medical questionnaire. Moreover, body height, weight, and blood pressure will be taken by an investigator of the study. Finally, we will familiarize you to the strength testing machine that you will be asked to use during the study.

Study Days

Day 0

You will be asked to visit McMaster University for baseline testing. During this visit you will come to the laboratory in the fasted state (not having eaten anything for a period of at least 10 hour). Upon arrival to the laboratory, you

will undergo a body scan to assess your body composition including total body weight, lean mass, and fat mass, etc. We will then place a

catheter in both of your arms; one for blood sampling and one for infusion of amino acids. You will be asked to saturate a cotton swab with your saliva, and we will take a blood sample, then ingest the ‘heavy water’ that we will provide you with (explained below). Subsequently, we will start the amino acid infusion, which will continue for 2 hours. Every 15 minutes we will take another blood sample. Two hours after ingestion of the heavy water you will be asked to saturate another cotton swab. During the infusion you will undergo an ultrasound scan on your mid-thigh region of both of your legs for the assessment of muscle thickness. Following the infusion, you will also be asked to perform three leg extension contractions for the measurement of maximal leg strength. Before leaving McMaster University, one of the investigators will apply a knee brace to one of your legs to start the immobilization period. Once the knee brace is in place, we kindly ask that you do not tamper with the positioning of the knee brace at all. Following application of the knee brace you will be sent home.

Day 1 - 3

During this day you will not be visiting McMaster University. You must ensure that the knee brace is still in place and you will receive a phone call from one of the investigators to ensure no problems have arisen overnight or over the course of the day. You will be asked to obtain another saliva sample upon waking.

Day 4 (see Figure 2 for an overview of this day)

During this day you will visit McMaster University in the fasted state. Upon arrival an investigator will remove the knee brace and you will be asked to lie on your back on one of our laboratory beds for commencement of the infusion trial. A catheter will be placed in both arms to allow us to perform the infusion trial. Before beginning the infusion, we will obtain a baseline blood sample. Following the collection of baseline blood, we will begin the first infusion via one of the catheters, which will be continued for the entire duration of the trial. Periodically the investigators will collect blood samples from the other catheter. The catheter allows us to take multiple blood samples without having to use a needle every time. After 1.5 hours, muscle biopsies are collected under local anaesthesia from both legs. Another muscle biopsy will be taken from the immobilized and control leg 2 hours later, signifying the end of the fasted state measurements.

Next, to simulate conditions after a meal, we will start the second infusion containing a mixture of amino acids (building blocks of dietary protein) and

dextrose (sugar). Additional muscle biopsies will be collected from both legs at 90 and 180 minutes of the amino acid/dextrose infusion. The last muscle biopsy marks the end of the infusion trial. After ending the infusion trial, the investigators will measure your body composition, muscle thickness, muscle cross sectional area, and muscular strength as previously described. Following this testing the investigators will send you home.

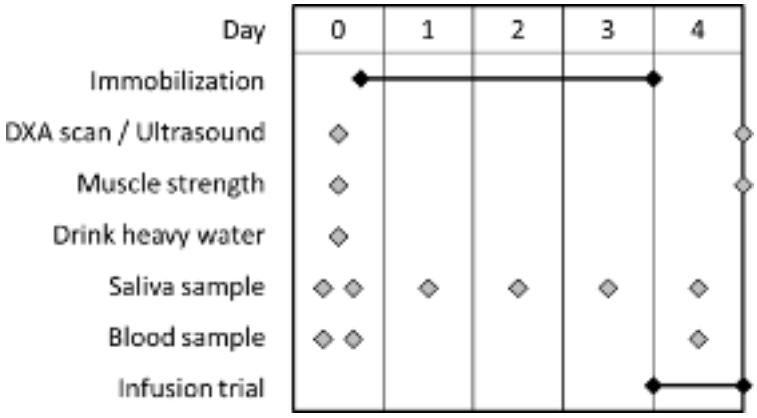


Figure 1. Study Schematic. Heavy water will be provided on day 0. You will consume the heavy water after you take the saliva sample while at the lab, under investigator supervision. Heavy water is similar chemically and behaves like regular drinking water.

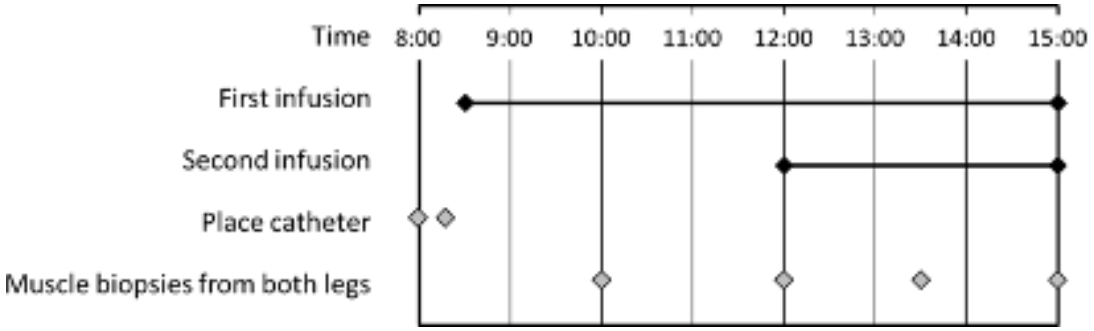


Figure 2. Schematic overview of the infusion trial (day 4). We will be infusing stable tracers to track the rate of muscle growth and breakdown and how this process may be impacted by the period of disuse. We will also infuse nutrients to mimic the state that your body would be in if you had just eaten a meal. At the beginning of the trial (8:00 am) we will place a catheter in each arm, one for the infusions and one to perform serial blood sampling every 10 minutes.

STUDY MEASURES

<u>Muscle mass and function</u>

Dual energy X-ray absorptiometry (DXA) scan

In this study we will perform two DXA scans, which will take place before (day 0) and following immobilization (day 4). A DXA scan is generally used to determine bone mineral density and diagnose osteoporosis, a condition in which the bones are extremely fragile. However, DXA scan also provides information on body fat percentage and lean body mass. The DXA procedure uses a very small dose of radiation which is not harmful for your body (see risks below). The procedure takes approximately 7 minutes and involves lying still on a bed while the scan arm passes over your body.

Ultrasound

Ultrasound will be used on 2 occasions (before and after immobilization) to measure the size of your thigh muscles. During the ultrasound test a probe with gel on the tip will be applied to the middle region of your thigh to allow for the measurement of muscle thickness. Ultrasound works by emitting sound waves through the skin. When the sound wave comes into contact with an object, such as an organ or bone, it is reflected back. The reflected signal is then picked up by the ultrasound probe which is able to produce an image of your skin, muscle and bone in your leg.

Muscle strength

Leg muscle strength will be measured on 2 occasions (before and after immobilization) using a Biodex dynamometer, which is a chair fitted with an arm to which your leg is strapped. In order to determine muscle strength, you will be asked to repeatedly kick your leg out against the arm of the machine. The force produced by your leg will be recorded by the force transducer in the arm of the machine.

Heavy water and saliva sampling

On one occasion (day 0) you will consume approximately 200 mL of heavy water. Heavy water contains deuterium, which is a form of hydrogen that is naturally occurring at a very low concentration of approximately 0.01% in the human body. We provide deuterium-labelled water that is 70% enriched, which allows us to detect and trace deuterium in your body. We will measure deuterium in the saliva samples as well as the muscle biopsies to be able to calculate the rate of muscle growth during the 3 days of immobilization.

Infusions and blood sampling

During the infusion trial we apply various infusions that allow us to measure muscle growth and breakdown as well as muscle glucose (sugar) uptake. We would like to measure these processes in both the fasted state as well as after meal intake. In this study, we will simulate meal intake by infusing nutrients directly into the bloodstream. All infusions will be applied through one catheter which will be placed in one of the veins in your arm. In the other arm, we will

also place a catheter for regular blood sampling. Placement of the 2 catheters is not painful and could be compared to the sting from a single blood draw.

Muscle biopsies

During this study, muscle biopsies will be obtained from both legs at 4 time points (all during day 4). The muscle biopsy procedure will be performed by trained and qualified personnel. Muscle biopsies are obtained from the middle region of the thigh muscle under local anaesthesia. First, the skin as well as the sheet surrounding the muscle (fascia) will be frozen (anesthetized) using an injection of lidocaine. Second, a small incision in the skin and fascia (approximately 5 mm) will be made using a scalpel. Through the incision in the skin and fascia, a muscle biopsy needle will be inserted into your muscle. Using this biopsy needle, a small piece of muscle tissue (50-100 mg; about the size of a match head) will be cut off. The biopsy needle will then immediately be removed from your leg. The incision will be stitched or glued and wrapped with a tensor bandage.

POTENTIAL HARMS, RISKS, AND DISCOMFORTS

DXA scan

The radiation dose from one DXA scan is approximately 10 microsievert (μSv), which is about the amount of radiation an average person receives every 24 hours from natural radiation in our environment (i.e. from the sun, television and computer screens, etc.). This procedure is painless and non-invasive.

Heavy water

Heavy water contains deuterium, which is a hydrogen isotope, yet non-radioactive and is in fact already present in small amounts in the water we drink daily (i.e., 0.01%). In very large amounts, deuterium-labelled water can have adverse effects. A very high dose (more than 40 cups consumed in a very short time period for a person weighing about 150 pounds) may cause a toxic response. In this study you will receive 60 times less than this amount. In previous research studies using heavy water, very few people who drank deuterium-labelled water reported temporary dizziness or vertigo. There is a 3.3% chance of encountering these side effects and only upon the first- or second-time heavy water is ingested. If it does occur, the side effects may last up to two hours and will go away on their own. If this occurs, we ask you that you rest quietly and refrain from driving or operating heavy machinery. About half the water in your body is replaced every week. Once you stop drinking heavy water, deuterium will return to its normal concentration in 20-30 days.

Single leg immobilization

One leg will be immobilized for 3.5 days using a knee brace. The knee brace will be placed in a fixed flexion position and sealed with a plastic strap. In case of emergency, you can break the

seal and remove the knee brace. The knee brace may cause some itching or discomfort. There is also a potential risk of deep vein thrombosis (DVT; blood clot). A DVT occurs in 1.2 per 1,000 patients per year. The incidence increases with age: from 0.2 (15 to 24 years) to 6.5 (over 75 years) per 1,000 patients per year. Immobilization is a risk factor for DVT, but the exact increased risk is unknown. As women have a higher risk of DVT, we will include men only. Moreover, the incidence strongly increases above the age of 75 years. To limit the risk of DVT we will exclude individuals with a (family) history DVT or blood clotting disorders. Symptoms are not always apparent but may include swelling of the calf, ankle, foot, or thigh; increased warmth of the leg; pain in the leg; and red or bluish discoloration of the skin on the leg, foot, or toes. If you experience one of these symptoms, please contact the study investigators (Dr. Stefan Gorissen, Tanner Stokes, or Dr. Stuart Phillips) immediately.

Muscle biopsy procedure

The muscle biopsy technique is routinely used in research, and complications are rare provided that proper precautions are taken. During the time that the biopsy sample is being taken (approximately 5 sec), you may feel the sensation of deep pressure in your thigh and, on some occasions, this is moderately painful. However, the discomfort very quickly passes, and you are immediately capable of performing exercise and daily activities. You should not do strenuous exercise for the rest of the day. However, low-intensity exercise such as walking, or cycling may help to accelerate the healing process. Once the anaesthetic wears off, your leg may feel tight and often there is the sensation of a deep bruise or "Charlie Horse". Analgesics (pain killers) such as Acetaminophen (Tylenol) or Ibuprofen (Motrin) can be taken if you experience pain associated with the biopsy. It is also beneficial to periodically apply an ice pack to the biopsy site the following day, as this will help to reduce any swelling and any residual soreness. The following day your leg may feel uncomfortable when going downstairs. The tightness in the muscle usually disappears within 2 days and subjects routinely begin exercising at normal capacity within a day.

There is a risk of internal bleeding at the site of the biopsy, which can result in bruising and temporary discoloration of the skin. On occasion a small lump may form under the site of the incision, but this normally disappears within 2-3 weeks. As with any incision there is also a risk of infection. However, this risk is virtually eliminated through proper cleansing of the area and daily changing of wound coverings. In order to allow the incisions to heal properly and minimize any risk of infection, you should avoid prolonged submersion in

water for 2-3 days. Daily showers are acceptable, but baths, swimming, saunas, etc. should be avoided for at least 4 days following the biopsy procedure. If the incision does not heal within a few days or you are in any way concerned about inflammation or infection (usually this means that the incision site is red, hot, swollen, and/or itchy), please clean the incision and contact us immediately. In very rare occasions, there can be damage to a superficial sensory nerve, which will result in temporary numbness in the area. There is also an extremely small chance that you will be allergic to the local anaesthetic (lidocaine); the real incidence of lidocaine allergy is unknown.

In past experience with healthy young subjects, 1 in 2,200 have experienced a local skin infection; 1 in 500 have experienced a small lump at the site of the biopsy (in all cases this disappeared within approximately 2-3 weeks by rubbing the area); 1 in 1,500 have experienced a temporary loss of sensation in the skin at the site of incision (an area of numbness about the size of a quarter which lasted up to 3-4 months), and 1 in 30 have experienced bruising around the site of incision which lasted for approximately 4-5 days. To the best of our knowledge, older subjects who have undergone the muscle biopsy procedure have reported no adverse reactions. While there is also a theoretical risk of damage to a small motor nerve branch (these allow your muscle to move) of the medial vastus lateralis muscle, this has never been seen in over 9,500 biopsies performed by the investigators at McMaster University and the risk of damaging a small motor nerve branch is considered small.

Venous blood sampling

A soft plastic catheter will be inserted into a forearm vein by a physician or a medically trained and certified member of the laboratory group, and a blood sample will be drawn (~5 mL or 1 teaspoons). The discomfort of this procedure is transient and is very similar to having an injection by a needle, e.g., when donating blood. Upon removal of the needle or catheter any discomfort should subside. During the course of the experiment, four separate catheters will be inserted into your arm (2 on each occasion). In this experiment the total blood taken is ~250 mL, which is half of the blood removed during a donation to a blood bank. This amount of blood loss is not enough to affect your physical performance in any way. After each blood sample has been taken pressure will be placed on the site in order to minimize bleeding and facilitate healing.

The insertion of a needle or catheter for blood sampling is a common medical practice and involves few risks if proper precautions are taken. The needles are inserted under completely sterile conditions; however, there is a theoretical risk of infection. There is a chance of internal bleeding if adequate pressure is not maintained upon removal of the needle. This may cause some minor discomfort and could result in bruising/skin discoloration, which could last up to a few weeks. In very rare occasions, trauma to the vessel wall could result

in the formation of a small blood clot, which could travel through the bloodstream and become lodged in a smaller vessel. However, we have never experienced such a complication after several thousand blood draws.

RESEARCH RELATED INJURY

If you are injured as a direct result of taking part in this study, all necessary medical treatment will be made available to you at no cost. Financial compensation for such things as lost wages, disability or discomfort due to this type of injury is not routinely available. However, if you sign this consent form it does not mean that you waive any legal rights you may have under the law, nor does it mean that you are releasing the investigator(s), institution(s), and/or sponsor(s) from their legal and professional responsibilities.

POTENTIAL BENEFITS

There are no direct benefits for you to participate in this study. However, the findings of this study may contribute to the development of nutritional interventions that prevent or attenuate the loss of skeletal muscle mass with disuse, aging, or in various clinical settings. Moreover, you will receive information about your own health as we perform a DXA scan.

PAYMENT OR REMUNERATION

If you participate in this study, you realize that there are no direct benefits to you. You will receive an honorarium of $400 upon the completion of the study to compensate you for your time commitment. Should you wish/be required to withdraw from the study at any point, we will reimburse you on a pro rata basis for the time you have spent on the study (i.e., half of the study completed = $200).

PLEASE NOTE: Remuneration will be in the form of a cheque issued by McMaster University. The cheque for the full amount will be mailed to you at the completion of the study. While we will attempt to have the remuneration forwarded to you as soon as possible, it may take at least four weeks following the completion of the study for the cheque to be processed and the remuneration received.

In addition to the remuneration, we will also cover your on-campus parking during the experimental phases.

CONFIDENTIALITY

Muscle biopsies will be stored for up to 15 years after completion of the study for analyses. All data collected during this study will remain confidential and stored in offices and on computers to which only the investigators have access. Any identifying information will be coded, with corresponding code-

identifiers to be kept stored in a locked filing cabinet under the supervision of Professor Stuart Phillips, Dr. Stefan Gorissen, and Tanner Stokes. Information kept on a computer will be protected by a password. All data will be stored in the locked filing cabinet under the supervision of Professor Stuart Phillips. Professor Phillips, Dr. Gorissen, and Tanner Stokes will have access to the data and will supervise access to other researchers within the group. Information about you will not be released to any other person for any reason. If the results of the study are published, your identity will remain confidential. The data for this research study will be retained for 15 years.

For the purposes of ensuring the proper monitoring of the research study, it is possible that a member of the Hamilton Integrated Research Ethics Board (HiREB) may consult your research data. However, no records which identify you by name or initials will be allowed to leave the university/hospital. By signing this consent form you authorize such access.

You should be aware that the results of this study will be made available to the scientific community through publication in a scientific journal, although neither your name nor any reference to you will be used in compiling or publishing these results. Upon completion of the study, we are able to provide a personalized report of our findings at your request, as well as direct you towards all publications resulting from this study.

PARTICIPATION AND WITHDRAWAL

At any time, you can choose whether to participate in this study or not. You may remove yourself or your data from the study at any time if you wish. You may also refuse to answer any questions posed to you during the study and still remain a subject in the study. The investigators reserve the right to withdraw you from the study if they believe that circumstances have arisen that warrant doing so.

You will receive a completed (i.e., signed) copy of this ethics form. You may withdraw your consent to participate in this study at any time, and you may also discontinue participation at any time without penalty. In signing this consent form or in participating in this study you are not waiving any legal claims or remedies.

QUESTIONS ABOUT THE STUDY

For any questions related to this study, please contact Tanner Stokes at 289-680-1900 or Dr. Stefan Gorissen at 905-730-5894. You can also contact them if you have concerns about your health or any of the procedures occurring during the study.

In the event of an emergency please contact Professor Stuart Phillips at 905-525-9140 ext. 24465 (office), Dr. Stefan Gorissen at 905-730-5894, or Tanner

Stokes at 289-680-1900. As with any medical emergency, you should proceed to the urgent care/emergency department of the closest hospital if an emergency should arise during the course of the study.

This study has been reviewed by the Hamilton Integrated Research Ethics Board (HiREB). The HiREB is responsible for ensuring that participants are informed of the risks associated with the research, and that participants are free to decide if participation is right for them. If you have any questions about your rights as a research participant, please call the HIREB office at 905- 521-2100 ext. 42013.

CONSENT STATEMENT

Signature of research participant

I have read the preceding information thoroughly. I have had the opportunity to ask questions, and all of my questions have been answered to my satisfaction. I agree to participate in this study. I understand that I will receive a signed copy of this form.

Name of Participant…….

Signature of Participant…… Date (mm/dd/yyyy)……..

Consent form administered and explained in person by:…….

Name and title……..

Signature……. Date (mm/dd/yyyy)……..

Signature of investigator…….

In my judgment, the participant is voluntarily and knowingly giving informed consent and possesses the legal capacity to give informed consent to participate in this study.

Signature of Investigator…….. Date (mm/dd/yyyy)……..

8.2 Appendix 2

Healthy Older Volunteer's Information Sheet

Clinical, Metabolic and Molecular Physiology Research Group

Title of Project: The regulation of skeletal muscle protein synthesis by systemic hormones and its influence on ageing and anabolic resistance

Names of Investigators:

Dr Daniel Wilkinson **Dr Iskandar Idris,**

Dr Phil Atherton **Dr Haitham Abdullah,**

Dr Ken Smith **Dr Supreeth Rudrappa**

Dr Bethan Phillips

You have been invited to take part in a research study. Before you decide whether to take part it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends and relatives if you wish to. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. If you decide to take part, you will be given a copy of your consent form and this leaflet.

Background

Muscles represent the largest organ in the body, making up over 50% of total body weight. Most people know that skeletal muscles are important for movement and to support the skeleton, but not everyone is aware of how important muscles are for whole-body health. For example, muscles represent a vast protein store containing amino acids (the building blocks of protein) which can be broken down in times of fasting, infection and disease in order to provide energy to help other vital organs. Hormones can play an important role in the regulation of muscle mass throughout adult life, with reductions in hormones levels due to illness or disease leading to a reduction in muscle mass and strength. It however still remains unclear how these

hormones regulate these changes in muscle mass. This knowledge could be key in developing future pharmaceuticals for treatment of muscle wasting disease. Further to this, most people will have noticed that with age people become frail. This is principally due to the wasting of skeletal muscle known as “sarcopenia”. Crucially, sarcopenia is more than just a symptom of weakness and poor functional capacity; it exposes people to an increased risk of falls and fractures, impacting quality of life, independence, health status and ultimately lifespan. It is because of these detrimental effects on health, and the associated health costs that sarcopenia is of grave concern. Interestingly, with ageing there is also an associated reduction in hormone levels and a lower stimulation of hormonal release in response to things like exercise. This association leads to the possibility that reductions in hormone levels with age could contribute to the frailty and sarcopenia associated with normal ageing, and therefore there is a significant clinical need to identify whether this is a significant contributing factor to this muscle loss so that it can be specifically targeted for intervention (i.e., hormonal therapies).

What does the study involve?

Screening Visit

Before you formally enter the study, you will be asked to attend our facility (the Medical School, Royal Derby Hospital), without having eaten anything (from 2000h the night before), a screening visit to ensure that you are eligible for participation in this study. During this visit (approximately 60 minutes), we will explain the study in full and answer any questions you may have, then (i) ask you to sign a consent form for the study, (ii) take a blood sample (10-20ml), and (iii) conduct a health assessment - including measuring your height and weight and a trace of your heart’s electrical activity (an ECG).

Main Study

Once we have received the results of your blood tests from screening, we will contact you to inform you of your results and confirm your inclusion into the study. If you are happy to proceed with the study, we will organise a mutually convenient day on which you will attend our facility to begin the study. The study period will last a total of 8 weeks in duration, and over this period you will be asked to attend our exercise suite 3 times a week for supervised exercise training and for collection of samples.

On the initial lab visit a DXA scan and ultrasound will be performed to measure your body composition, your leg muscle mass and muscle architecture (fibre length and thickness). The ultrasound is similar to the scan carried out to image babies during pregnancy and involves some jelly and a probe being placed on your thigh. A leaflet describing the DXA scan in more detail is attached. In brief, this is a whole-body low

dose X-ray scan that provides us with information about the tissue under your skin. The radiation exposure from this scan is very low, equivalent to ~3 days background exposure and significantly less than a flight to America. A test called an oral glucose tolerance test (OGTT) will also be performed. This test assesses your body's response to a single glucose drink and is commonly used by GPs to assess a person's health. This test involves a small 75g glucose drink followed by regular 15 min blood samples over the following two hours, this sampling will be performed via the placement of a small tube in a vein in your arm (the OGTT will be repeated again at the start and end of the exercise training period). Following this your strength will be assessed and you will be familiarised with the exercise protocol to be performed during the study period. Your 1 repetition maximum (1-RM) will then be determined for six muscles (this is the highest amount of weight you can lift once with one leg) so we can determine your workload during the exercise training. Following completion of the exercise test a muscle biopsy will be collected from the vastus lateralis muscle (i.e., a region in the front of the thigh muscle) after an injection of local anaesthetic to numb the area. After the local anaesthetic a trained clinician will make a small (0.5 cm) cut through the skin and underlying muscle sheath then take the biopsy (a small piece about the size of an orange pip) with a special set of forceps. With the local anaesthetic you may feel pushing during this procedure but no pain. A stitch is placed in the skin afterwards to aid healing. A tight bandage will be placed around the thigh after the biopsy and should remain in place for ~12h to speed healing. You will then provide a saliva sample and be provided with a drink of 'heavy water' (~250 ml) to consume. This water contains a stable isotope tracer (deuterium in place of hydrogen, but is not radioactive), which is slightly different from normal water, but it is perfectly safe. We will ask you to provide a small sample of saliva 3-4 hours later, (which you can store refrigerated in sealed tubes we will provide). We will also ask you to collect samples of saliva daily around midday and at least an hour after food or drink for the first week. You will be provided with a small volume (~10 ml) of additional heavy water to consume daily for the remainder of the study period. This tracer is what allows us to measure rates of muscle growth and metabolism. In addition to the heavy water, we will provide you with a small drink of labelled creatine and 3-methylhistidine tracers (as with the heavy water, these are not radioactive and are perfectly safe, both creatine and 3-methylhistidine occur naturally in your muscles), we will then ask you to collect urine samples over the next 72h, before returning at 72h for a series of small blood samples. These samples will provide us with a measure of your muscle weight and how quickly your muscle is breaking down and will be repeated at 3 and 6 weeks into the exercise period to assess for any changes related to the testing.

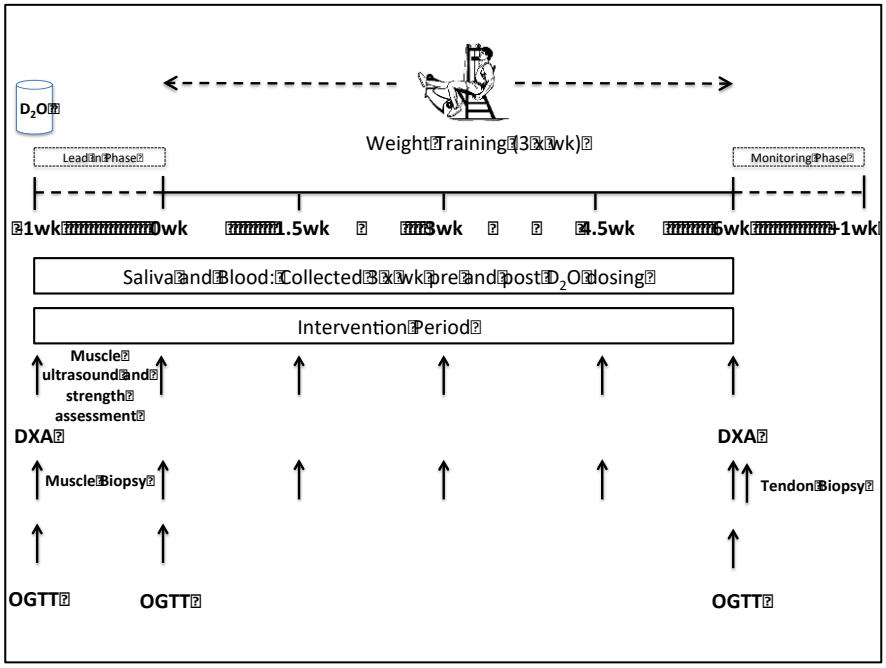
You will then be randomly assigned to one of two testing groups, either: 1) a placebo group, or 2) a testosterone supplemented group (Sustanon 250: 250mg intramuscular injection every 2-3wks). The testosterone intervention is aimed at increasing levels of testosterone in your blood, which are known to be lower in ageing populations and are anticipated to contribute to reduced muscle mass with ageing and in response to exercise training.

Following an initial lead in period of one week, where you will perform no exercise, you will return to the lab 3 x week for 6-weeks for a whole body progressive supervised resistance exercise training programme. You will be asked to perform 3 sets of 10 repetitions of both upper and lower body exercises at a weight equivalent

to 80% of your 1-RM. At the start of the exercise period (designated week 0 on the attached diagram), and every 1.5 weeks up to the end of the exercise period (at 1.5, 3, 4.5 and 6 weeks as highlighted in the diagram, you will be asked to return to the lab for muscle biopsies. As well as using the biopsies to assess changes in metabolism due to the interventions, biopsies will also be used for cell-culture work to explore important mechanisms regulating the numerous roles of muscle.

During the intervention period, you will also have repeat ultrasound measurements, muscle function and strength tests every 1.5 weeks. An additional small patellar tendon biopsy will also be obtained at 6 weeks – this is the tendon that attaches the bottom of your kneecap (patellar) to the top of your shinbone (tibia). This biopsy will be taken using a microneedle technique, guided by ultrasound. This procedure has been conducted regularly within our labs without any reports of discomfort (average discomfort reported during and following the procedure was 2 out of 10; with 1 being no discomfort and 10 being maximal discomfort). Prior to each muscle biopsy an ultrasound scan of the muscle will be performed (with a repeat DXA scan) to monitor any changes in muscle structure due to the training regime. Following the training regime the intervention will be stopped and you will be monitored for one additional week to ensure hormonal levels have returned to normal. Following this a final muscle biopsy will be obtained. A diagram of the study protocol is provided below.

Why have you been chosen?



Primarily because you have expressed an interest in taking part in the study and you have met with the entry criteria and do not have on-going medical problems. We will be recruiting healthy, recreationally active males aged 65-75y.

Do you have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part, you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part, you are still free to withdraw at any time and without giving a reason.

What do I have to do?

You are requested to fast on the night before and avoiding heavy exercise for 48h prior to your visits to the laboratory. We will provide you with a recommended dietary intake for the study period that we would like you to adhere to where possible. This will follow a standard balanced diet according to current recommended daily allowances for carbohydrates, fats and proteins, and will help us to limit the impact of dietary intake upon the outcomes of study.

What is the drug or procedure that is being tested?

One group of individuals will receive slow-release testosterone (the other a placebo), which provides increases in testosterone levels, it is safe to use in healthy humans and similar testosterone products have been previously used in studies within our research group and is regularly used as a treatment in humans. Testosterone (or placebo) will be administered via intramuscular injection every 2-3wks. You will be provided with all necessary information regarding this compound and any potential side effects (see below) and will be continually monitored by our researchers.

What are the side effects of any treatment or procedures received when taking part?

Research studies often involve some risks, not all of which may be currently known. In the light of our experience, muscle and tendon biopsies are well tolerated by all our volunteers. Scarring is hardly perceptible and fades with time. However, the procedure may cause mild pain in some volunteers, but with appropriate local analgesia this is minimal; indeed data from a previous audit of the procedure demonstrated that 83% of our participants experienced less than or the expected discomfort during the muscle biopsy and all respondents said that they would or may be willing to participate in a similar study in the future. Some discomfort (muscle tenderness and stiffness) may be felt for 2-3 days afterwards but mild painkillers will usually effectively abolish this. There is a slight risk of infection at biopsy sites, however all procedures are performed under sterile conditions to minimize any risk of infection. Damage to small nerves of the skin during the biopsy is possible; however these nerves grow back with restoration of normal sensation, without difficulty.

We will provide you with a short, anonymized questionnaire about your experience of the biopsy procedures during this study should you wish to respond.

The risks involved in venous blood sampling are very small. Occasionally, however there may be bruising due to leakage from a blood vessel on withdrawal of the needle, but this is uncommon with good practice. The loss of 60 ml of blood has no ill effects in healthy persons and is much less than if you donate blood.

As with any pharmacological intervention there may be some side effects associated with the administration of the Testosterone, however these are uncommon and are generally only associated with prolonged administration over periods longer than what is being performed in the current study. Those side effects which may be encountered are mild and include headache, increased hypertension, acne, increased blood count and skin irritation at the gel site (a full list of all potential side effects associated with testosterone are attached with this information sheet). If you are randomized into a testosterone (vs. placebo) group, you will be given the chance to discuss these with any of the clinicians if unsure.

What are the exclusion criteria for this study?

We are recruiting healthy men between the ages of 65 to 75 years, who are of normal weight or slightly overweight. If you have been a subject in any other research study in the last three months which involved: taking a drug, being paid a disturbance allowance or having an invasive procedure (e.g. blood sample >50ml) you would not be eligible to take part.

You would also be unsuitable if you have particular medical conditions or are taking certain medications. If you are interested in this study, please discuss these further with the study doctor.

What are the possible disadvantages and risks of taking part?

Healthy volunteers have been chosen for this study; however, it is possible that the routine tests could detect unknown health problems. Should this be the case you will be informed and advised to attend your GP practice for further management. Your GP will be notified. Be advised, however, that future insurance status e.g. for life insurance or private medical insurance, could be affected by these diagnoses.

What if something goes wrong?

In the unlikely event of a complication following the study or that you should wish to voice grievances, in the first instance please contact the clinician involved in the execution of the study or the lead investigator, Dr Phil Atherton (T: 01332 724725; E: philip.atherton@nottingham.ac.uk). If you remain unhappy and wish to complain formally, you should then contact the FMHS Research Ethics Committee Administrator, c/o The University of Nottingham, School of Medicine Education Centre, B Floor, Medical School, Queen's Medical Centre Campus, Nottingham University Hospitals, Nottingham, NG7 2UH. E-mail: louise.sabir@nottingham.ac.uk.

Will my taking part in this study be kept confidential?

We respect your right to privacy, and we will take measures to safeguard confidentiality. A single form, on which you are asked to sign to give consent for involvement will carry details of your name and address, but no health-related details. This is kept securely in a locked cabinet within the Medical School. Access to this

cabinet is restricted to personnel directly involved in the study and to University staff with direct responsibility for ensuring the study is conducted appropriately.

We will follow current ethical and legal practice and all information about you will be handled in confidence. If you join the study, some parts of the data collected about you will be looked at by authorised persons from the University of Nottingham who are organising the research. They may also be looked at by people authorised to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and we will do our best to meet this duty.

All information which is collected about you during the course of the research will be kept strictly confidential, stored in a secure and locked office, and on a password protected database. Any information about you that leaves the University will have your name and address removed (anonymised) and a unique code will be used so that you cannot be recognised from it, with the exception of a letter sent to your GP advising of your participation in this study.

Your personal data (address, telephone number) will be kept for 5 years after the end of the study so that we are able to contact you about the findings of the study and possible follow-up studies. All other data (research data) will be kept securely for 7 years. After this time your data will be disposed of securely. During this time all precautions will be taken by all those involved to maintain your confidentiality and only direct members of the research team will have access to your personal data.

What will happen to any samples I give?

We will use the tendon and biopsy samples to see how your muscle and tendons respond to exercise and/ or the effect of age or hormones. We may also use your muscle samples to generate cell-culture samples so that we can further investigate mechanisms involved in muscle health and disease outside of the human body. We will also seek your consent for any samples remaining after analysis for this study has been completed, to be stored and used in future research. This is optional and you will be asked to give separate consent for this. The samples will be securely stored with a code unique to you at the University of Nottingham under the Universities Human Tissue Research Licence (No. 12265).

Some of these future studies may be carried out by researchers other than the current research team. This may include researchers working for commercial companies. Any samples or data used will be anonymised so that you could not be identified in

any way. If you do not agree to this, any remaining samples will be disposed of in accordance with the Human Tissue Authorities code of practice.

Will any genetic tests be done?

In the current study we will collect a blood sample that can be used to obtain information about your genes (RNA and DNA), however this analysis will not give us information on disease prognosis and samples will not be subject to genetic manipulation.

Who is funding the research?

This research is funded by The University of Nottingham.

Who has reviewed the study?

This study has been reviewed and approved by the University of Nottingham Medical School Ethics Committee.

Contact for Further Information

Thank you for taking part in the study. For further information you can contact Mr Nima Gharahdaghi on 01332 724685 or at mzxng@nottingham.ac.uk, or Dr Daniel Wilkinson (Research Fellow) on 01332 724850 or at d.wilkinson@nottingham.ac.uk, both are alternatively contactable at Clinical, Metabolic and Molecular Physiology, University of Nottingham, School of Medicine, Division of Graduate Entry Medicine and Health, Royal Derby Hospital, Uttoxeter Road, Derby, DE22 3DT.

Testosterone Reported Adverse Reactions

(taken from http://www.medicines.org.uk/emc/medicine/22159)

Adverse Drug Reactions terminology used for the classification of incidence: Very common ≥10% - Common ≥1% to <10% - Uncommon ≥0.1% to <1% - Rare ≥0.01% to <0.1% - Very rare <0.01% - Not known (can not be estimated from the available data).

The cumulative safety experience of testosterone is derived from Phase I to Phase III clinical trials and post-marketing experience.

The adverse reactions listed in the table below have been observed in clinical studies with testosterone and/or post-marketing experience.

System Organ Class	**Adverse Reaction**	**Frequency**
Nervous disorders	Headache	Common
Vascular disorders	Hypertension worsened	Common
	Hot flushes/flushing	Uncommon
Skin and subcutaneous tissue disorders	Acne	Common
	Pruritus	Uncommon
Reproductive system and breast disorders	Azoospermia	Very rare
General disorders and administration site disorders	Application site reaction (including erythema, rash and pruritus)	Common
	Peripheral oedema	Uncommon

Investigations	PSA increased, haematocrit increased, haemoglobin increased, red blood cell count increased	Common

Uncommonly, gynaecomastia may develop and persist in patients being treated for hypogonadism with testosterone.

8.3 Appendix 3

Healthy Younger Volunteer's Information Sheet

Clinical, Metabolic and Molecular Physiology Research Group

Title of Project: The regulation of skeletal muscle protein synthesis by systemic hormones and its influence on ageing and anabolic resistance

Names of Investigators:

Dr Daniel Wilkinson **Dr Iskandar Idris,**

Dr Phil Atherton **Dr Haitham Abdullah,**

Dr Ken Smith **Dr Supreeth Rudrappa**

Dr Bethan Phillips

You have been invited to take part in a research study. Before you decide whether to take part it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends and relatives if you wish to. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. If you decide to take part, you will be given a copy of your consent form and this leaflet.

Background

Muscles represent the largest organ in the body, making up over 50% of total body weight. Most people know that skeletal muscles are important for movement and to support the skeleton, but not everyone is aware of how important muscles are for whole-body health. For example, muscles represent a vast protein store containing amino acids (the building blocks of protein) which can be broken down in times of fasting, infection and disease in order to provide energy to help other vital organs. Hormones can play an important role in the regulation of muscle mass throughout adult life, with reductions in hormones levels due to illness or disease leading to a reduction in muscle mass and strength. It however still remains unclear how these hormones regulate these changes in muscle mass. This knowledge could be key in developing future pharmaceuticals for treatment of muscle wasting disease. Further

to this, most people will have noticed that with age people become frail. This is principally due to the wasting of skeletal muscle known as “sarcopenia”. Crucially, sarcopenia is more than just a symptom of weakness and poor functional capacity; it exposes people to an increased risk of falls and fractures, impacting quality of life, independence, health status and ultimately lifespan. It is because of these detrimental effects on health, and the associated health costs that sarcopenia is of grave concern. Interestingly, with ageing there is also an associated reduction in hormone levels and a lower stimulation of hormonal release in response to things like exercise. This association leads to the possibility that reductions in hormone levels with age could contribute to the frailty and sarcopenia associated with normal ageing, and therefore there is a significant clinical need to identify whether this is a significant contributing factor to this muscle loss so that it can be specifically targeted for intervention (i.e., hormonal therapies).

What does the study involve?

Screening Visit

Before you formally enter the study, you will be asked to attend our facility (the Medical School, Royal Derby Hospital), without having eaten anything (from 2000h the night before), a screening visit to ensure that you are eligible for participation in this study. During this visit (approximately 60 minutes), we will explain the study in full and answer any questions you may have, then (i) ask you to sign a consent form for the study, (ii) take a blood sample (10-20ml), and (iii) conduct a health assessment - including measuring your height and weight and a trace of your heart’s electrical activity (an ECG).

Main Study

Once we have received the results of your blood tests from screening, we will contact you to inform you of your results and confirm your inclusion into the study. If you are happy to proceed with the study, we will organise a mutually convenient day on which you will attend our facility to begin the study. The study period will last a total of 8 weeks in duration, and over this period you will be asked to attend our exercise suite 3 times a week for supervised exercise training and for collection of samples.

On the initial lab visit a DXA scan and ultrasound will be performed to measure your body composition, your leg muscle mass and muscle architecture (fibre length and thickness). The ultrasound is similar to the scan carried out to image babies during pregnancy and involves some jelly and a probe being placed on your thigh. A leaflet describing the DXA scan in more detail is attached. In brief, this is a whole-body low dose X-ray scan that provides us with information about the tissue under your skin. The radiation exposure from this scan is very low, equivalent to ~3 days background exposure and significantly less than a flight to America. A test called an oral glucose

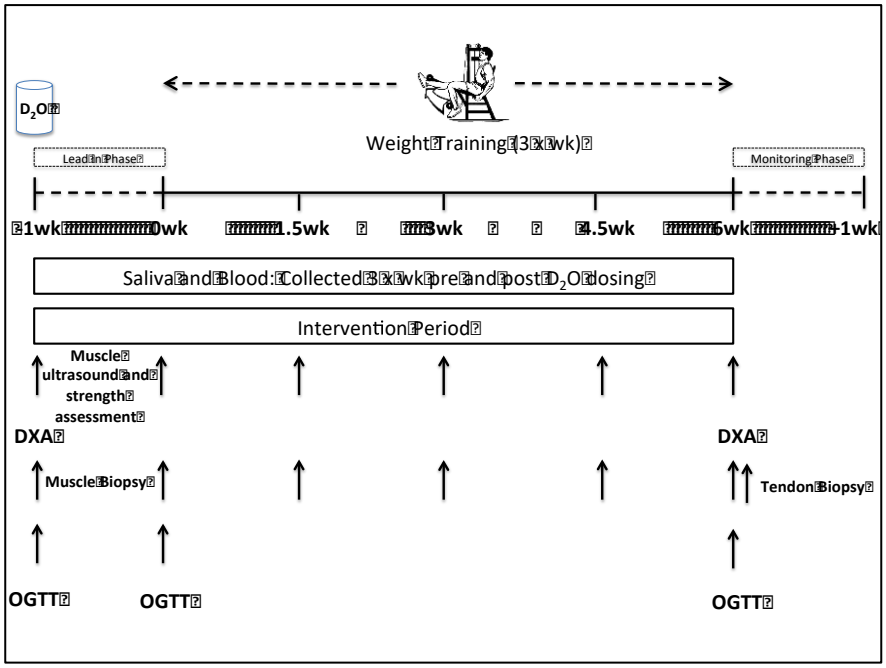
tolerance test (OGTT) will also be performed. This test assesses your body's response to a single glucose drink and is commonly used by GPs to assess a person's health. This test involves a small 75g glucose drink followed by regular 15 min blood samples over the following two hours, this sampling will be performed via the placement of a small tube in a vein in your arm (the OGTT will be repeated again at the start and end of the exercise training period). Following this, your strength will be assessed, and you will be familiarised with the exercise protocol to be performed during the study period. Your 1 repetition maximum (1-RM) will then be determined for six muscles (this is the highest amount of weight you can lift once) so we can determine your workload during the exercise training. Following completion of the exercise test a muscle biopsy will be collected from the vastus lateralis muscle (i.e., a region in the front of the thigh muscle) after an injection of local anaesthetic to numb the area. After the local anaesthetic a trained clinician will make a small (0.5 cm) cut through the skin and underlying muscle sheath then take the biopsy (a small piece about the size of an orange pip) with a special set of forceps. With the local anaesthetic you may feel pushing during this procedure but no pain. A stitch is placed in the skin afterwards to aid healing. A tight bandage will be placed around the thigh after the biopsy and should remain in place for ~12h to speed healing. You will then provide a saliva sample and be provided with a drink of 'heavy water' (~250 ml) to consume. This water contains a stable isotope tracer (deuterium in place of hydrogen, but is not radioactive), which is slightly different from normal water, but it is perfectly safe. We will ask you to provide a small sample of saliva 3-4 hours later, (which you can store refrigerated in sealed tubes we will provide). We will also ask you to collect samples of saliva daily around midday and at least an hour after food or drink for the first week. You will be provided with a small volume (~10 ml) of additional heavy water to consume daily for the remainder of the study period. This tracer is what allows us to measure rates of muscle growth and metabolism. In addition to the heavy water, we will provide you with a small drink of labelled creatine and 3-methylhistidine tracers (as with the heavy water, these are not radioactive and are perfectly safe, both creatine and 3-methylhistidine occur naturally in your muscles), we will then ask you to collect urine samples over the next 72h, before returning at 72h for a series of small blood samples. These samples will provide us with a measure of your muscle weight and how quickly your muscle is breaking down and will be repeated at 3 and 6 weeks into the exercise period to assess for any changes related to the testing.

You will then be assigned to one of two testing groups, either: 1) a placebo group or 2) a testosterone lowering group (Zoladex: 3.6mg/4weeks). Each of these interventions will be provided via an injection just under your skin. The testosterone lowering intervention is aimed at determining the influence of testosterone levels in your blood on muscle mass gains due to exercise training.

Following an initial lead in period of one week, where you will perform no exercise, you will return to the lab 3 x wk for 6-weeks for a whole body progressive supervised resistance exercise training programme. You will be asked to perform 4 sets of 8 repetitions of both upper and lower body exercises at a weight equivalent to 80% of your 1-RM. At the start of the exercise period (designated week 0 on the attached diagram), and every 1.5 weeks up to the end of the exercise period (at 1.5, 3, 4.5 and 6 weeks as highlighted in the diagram), you will be asked to return to the lab for additional muscle biopsies. As well as using the biopsies to assess changes in

metabolism due to the interventions, biopsies will also be used for cell-culture work to explore important mechanisms regulating the numerous roles of muscle.

During the intervention period, you will also have repeat ultrasound measurements,



muscle function and strength tests every 1.5 weeks. An additional small patellar tendon biopsy will also be obtained at 6 weeks – this is the tendon that attaches the bottom of your kneecap (patellar) to the top of your shinbone (tibia). This biopsy will be taken using a microneedle technique, guided by ultrasound. This procedure has been conducted regularly within our labs without any reports of discomfort (average discomfort reported during and following the procedure was 2 out of 10; with 1 being no discomfort and 10 being maximal discomfort). Prior to each muscle biopsy an ultrasound scan of the muscle will be performed (with a repeat DXA scan) to monitor any changes in muscle structure due to the training regime. Following the training regime the intervention will be stopped and you will be monitored for one additional week to ensure hormonal levels have returned to normal. Following this a final muscle biopsy will be obtained. A diagram of the study protocol is provided below.

Why have you been chosen?

Primarily because you have expressed an interest in taking part in the study and you have met with the entry criteria and do not have on-going medical problems. We will be recruiting healthy, recreationally active males aged 18-30y.

Do you have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part, you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part, you are still free to withdraw at any time and without giving a reason.

What do I have to do?

You are requested to fast on the night before and avoiding heavy exercise for 48h prior to your visits to the laboratory. We will provide you with a recommended dietary intake for the study period that we would like you to adhere to where possible. This will follow a standard balanced diet according to current recommended daily allowances for carbohydrates, fats and proteins, and will help us to limit the impact of dietary intake upon the outcomes of study.

What is the drug or procedure that is being tested?

One group of individuals will receive a drug named Zoladex, a gonadotropin releasing hormone analogue. This is a licensed drug that reduces the production of testosterone within the body. It is safe to use in healthy humans and has been previously used in a number of similar studies to assess testosterone metabolism in humans. This will be administered by a small injection into soft tissue of the abdomen every 4 weeks. You will be provided with all necessary information regarding this drug and any potential side effects (see below) and will be continually monitored by our researchers.

What are the side effects of any treatment or procedures received when taking part?

Research studies often involve some risks, not all of which may be currently known. In the light of our experience, muscle and tendon biopsies are well tolerated by all our volunteers. Scarring is hardly perceptible and fades with time. However, the procedure may cause mild pain in some volunteers, but with appropriate local analgesia this is minimal; indeed data from a previous audit of the procedure demonstrated that 83% of our participants experienced less than or the expected discomfort during the muscle biopsy and all respondents said that they would or may be willing to participate in a similar study in the future. Some discomfort (muscle tenderness and stiffness) may be felt for 2-3 days afterwards but mild painkillers will usually effectively abolish this. There is a slight risk of infection at biopsy sites, however all procedures are performed under sterile conditions to minimize any risk of infection. Damage to small nerves of the skin during the biopsy is possible; however, these nerves grow back with restoration of normal sensation, without difficulty.

We will provide you with a short, anonymized questionnaire about your experience of the biopsy procedures during this study should you wish to respond.

The risks involved in venous blood sampling are very small. Occasionally, however there may be bruising due to leakage from a blood vessel on withdrawal of the needle, but this is uncommon with good practice. The loss of 60 ml of blood has no ill effects in healthy persons and is much less than if you donate blood.

As with any pharmacological intervention there may be some side effects associated with Zoladex administration, however these are uncommon and are generally only associated with prolonged administration over periods longer than what is being performed in the current study. Those side effects which may be encountered over the proposed study period are generally mild and include Acne, hot flushes, erectile dysfunction, altered blood sugar, mood changes and irritation at injection site (a full list of all potential side effects associated with testosterone are attached with this information sheet). If you are randomized into a Zoladex (vs. placebo) group, you will be given the chance to discuss these with any of the clinicians if unsure.

What are the exclusion criteria for this study?

We are recruiting healthy men between the ages of 18 to 30 years, who are of normal weight or slightly overweight. If you have been a subject in any other research study in the last three months which involved: taking a drug, being paid a disturbance allowance or having an invasive procedure (e.g., blood sample >50ml) you would not be eligible to take part.

You would also be unsuitable if you have particular medical conditions or are taking certain medications. If you are interested in this study, please discuss these further with the study doctor.

What are the possible disadvantages and risks of taking part?

Healthy volunteers have been chosen for this study; however, it is possible that the routine tests could detect unknown health problems. Should this be the case you will be informed and advised to attend your GP practice for further management. Your GP will be notified. Be advised, however, that future insurance status e.g., for life insurance or private medical insurance, could be affected by these diagnoses.

What if something goes wrong?

In the unlikely event of a complication following the study or that you should wish to voice grievances, in the first instance please contact the clinician involved in the execution of the study or the lead investigator, Dr Phil Atherton (T: 01332 724725; E: philip.atherton@nottingham.ac.uk). If you remain unhappy and wish to complain formally, you should then contact the FMHS Research Ethics Committee Administrator, c/o The University of Nottingham, School of Medicine Education Centre, B Floor, Medical School, Queen's Medical Centre Campus, Nottingham University Hospitals, Nottingham, NG7 2UH. E-mail: louise.sabir@nottingham.ac.uk.

Will my taking part in this study be kept confidential?

We respect your right to privacy, and we will take measures to safeguard confidentiality. A single form, on which you are asked to sign to give consent for involvement will carry details of your name and address, but no health-related details. This is kept securely in a locked cabinet within the Medical School. Access to this cabinet is restricted to personnel directly involved in the study and to University staff with direct responsibility for ensuring the study is conducted appropriately.

We will follow current ethical and legal practice and all information about you will be handled in confidence. If you join the study, some parts of the data collected about you will be looked at by authorised persons from the University of Nottingham who are organising the research. They may also be looked at by people authorised to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and we will do our best to meet this duty.

All information which is collected about you during the course of the research will be kept strictly confidential, stored in a secure and locked office, and on a password protected database. Any information about you that leaves the University will have your name and address removed (anonymised) and a unique code will be used so that you cannot be recognised from it, with the exception of a letter sent to your GP advising of your participation in this study.

Your personal data (address, telephone number) will be kept for 5 years after the end of the study so that we are able to contact you about the findings of the study and possible follow-up studies. All other data (research data) will be kept securely for 7 years. After this time your data will be disposed of securely. During this time all precautions will be taken by all those involved to maintain your confidentiality and only direct members of the research team will have access to your personal data.

What will happen to any samples I give?

We will use the tendon and biopsy samples to see how your muscle and tendons respond to exercise and/ or the effect of age or hormones. We may also use your muscle samples to generate cell-culture samples so that we can further investigate mechanisms involved in muscle health and disease outside of the human body. We will also seek your consent for any samples remaining after analysis for this study has been completed, to be stored and used in future research. This is optional and you will be asked to give separate consent for this. The samples will be securely stored with a code unique to you at the University of Nottingham under the Universities Human Tissue Research Licence (No. 12265).

Some of these future studies may be carried out by researchers other than the current research team. This may include researchers working for commercial companies. Any samples or data used will be anonymised so that you could not be identified in any way. If you do not agree to this, any remaining samples will be disposed of in accordance with the Human Tissue Authorities code of practice.

Will any genetic tests be done?

In the current study we will collect a blood sample that can be used to obtain information about your genes (RNA and DNA), however this analysis will not give us information on disease prognosis and samples will not be subject to genetic manipulation.

Who is funding the research?

This research is funded by The University of Nottingham.

Who has reviewed the study?

This study has been reviewed and approved by the University of Nottingham Medical School Ethics Committee.

Contact for Further Information

Thank you for taking part in the study. For further information you can contact Mr Nima Gharahdaghi on 01332 724685 or at mzxng@nottingham.ac.uk, or Dr Daniel Wilkinson (Research Fellow) on 01332 724850 or at d.wilkinson@nottingham.ac.uk, both are alternatively contactable at Clinical, Metabolic and Molecular Physiology,

University of Nottingham, School of Medicine, Division of Graduate Entry Medicine and Health, Royal Derby Hospital, Uttoxeter Road, Derby, DE22 3DT.

Zoladex reported Undesirable effects (taken from http://www.medicines.org.uk/emc/document.aspx?documentid=7855)

Very common (≥1/10), Common (≥1/100 to <1/10), Uncommon (≥1/1,000 to <1/100), Rare (≥1/10,000 to <1/1,000), Very rare (<1/10,000) and Not known (cannot be estimated from the available data). – These are associated with chronic administration

8.4 Appendix 4:

Division of Medical Sciences and Graduate Entry Medicine

School of Medicine

The University of Nottingham
UNITED KINGDOM · CHINA · MALAYSIA

Healthy Volunteers Information Sheet

Study Title: Exploring time-efficient strategies to improve fitness for surgery in older adults

Name of Investigators:

Mr Philip Herrod

Dr Beth Phillips

Mr Jon Lund

You have been invited to take part in a research study. Before you decide whether to take part it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends and relatives if you wish to. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. If you decide to take part, you will be given a copy of your consent form and this leaflet.

Background:

One change associated with human aging is a reduction in "fitness", both in terms of how far or quickly a person can run/cycle/swim and also in how well their blood vessels work. Additionally, a person's metabolic fitness decreases; this is how well the body copes with nutrition and is why, for example, older people have a higher risk of diseases like type 2 diabetes. Previous studies have shown that as people

become less fit, they are at increased risk of suffering a complication whilst undergoing surgery. We also know that measuring how fit someone is, is better at predicting the risk of a surgical complication than purely using a person's age alone. Lots of research has shown that exercise can potentially reverse some of the age-related declines in fitness, however, most has used long sessions of continuous exercise over a long period of time. We have recently shown that high-intensity interval training (HIT), whereby people cycle on an exercise bike very hard for a minute, followed by a short rest, repeated over the course of 15 minutes can rapidly improve a person's fitness. However not everyone will be able to, or indeed want to participate in exercise this strenuous; therefore, we would like to explore if other options, such as dietary supplements can improve aspects of fitness

We intend to examine the effect of giving a dietary supplement on the way a person's body handles the glucose (from carbohydrates/ sugars) in a meal. Previous work has shown that taking a beta-hydroxy beta-methyl butyrate (HMB) supplement may improve a patient's response to glucose; however, the evidence is not clear. HMB is a commercially available nutritional supplement (sold in, for example Holland and Barrett) providing a compound that your body produces each time you eat protein and is most commonly used by bodybuilders to help gain muscle.

We aim to recruit 8 younger (18-35) and 8 older (age 65-85) male volunteers to study the effect of 1 dose of HMB on the body's response to a sugary drink over 3 hours. Volunteers will be asked to return 1 week later to undergo the same procedure but with a placebo in place of HMB. The order in which the two tests are carried out will be at random, so you will not know on which day you have been given the placebo and which day you are given the HMB.

Why have you been chosen?

In order for this study to be successful, volunteers have been chosen who fit the entry criteria and do not have significant on-going medical problems. We are inviting 8 younger participants and 8 older participants to take part.

Do you have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part, you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part, you are still free to withdraw at any time and without giving a reason.

Would an inconvenience allowance be paid?

No inconvenience allowances are available for this study.

What does the study involve?

The location for this study is The Medical School at the Royal Derby Hospital. The study consists of a screening visit (~1 hour) and 2 acute study visits (one week apart) each of which should last approximately 4 hours.

Screening Visit

Prior to being enrolled onto the study you will be asked to attend the Medical School at the Royal Derby Hospital for a screening visit to assess your suitability for the study, give you the opportunity to ask questions about the study and ask you to provide written consent for your participation in the study. This visit will last approx. 60 min and we will ask you to arrive in the morning (~9am) fasted (free to drink water) from (~9pm) the night before. During this visit we will:

i. take a small amount of blood to measure health parameters, including fasting glucose, liver and kidney function

ii. measure your height and weight

iii. conduct a health assessment, including an ECG to monitor your heart, a blood pressure check and conducts an assessment of previous medical history

Study Day 1

Once we have the received the results of your blood tests and on the premise that all of your results are within normal range, we will enrol you on to the study.

On your first study day we will ask you to attend at ~9am, again fasted from ~9pm the night before. At the start of this visit we will take measures to estimate your body composition using a specialist set of scales that give us an estimate of your muscle and fat mass. You will then take part in an oral glucose tolerance test (OGTT). This test will give us information about how your body produces insulin and manages the sugar within your body. Firstly, a small cannula (a fine, flexible plastic tube) will be inserted into a vein in your hand or forearm to allow us to take blood samples. This cannula will then be connected to a drip of saline (sterile water) in order to keep it working. Your hand will be placed in a warm box and will remain here for the duration of the test. After an initial blood sample is taken from the cannula, you will then be asked to consume a sugary drink plus either the HMB or the placebo. Over the following 3 hours small samples of blood will be taken regularly (~every 15 minutes) from the cannula. In addition, before the drink and 3 points after (15 min, 60 min and 120 min) the blood flow in a large vein at the top of your leg will be measured. This will be done using ultrasound. Similar to how babies are viewed during pregnancy cool ultrasound gel will be placed in your groin region and an ultrasound probe will be placed on the gel to measure blood flow. At the end of the test, we will provide you with a drink and light snack and monitor your blood sugar for a further 30 minutes.

Study Day 2

We will ask you to attend the following week to undergo the same procedures as outlined above but with the alternate drink (HMB or placebo).

What do I have to do?

We are studying the effects of a single dose of a dietary supplement on the way your body processes a sugary drink. We are studying these aspects in healthy young and older adults, so we need volunteers who are eating and exercising as they would normally. We ask you to fast on the nights before your screening and study day visits, and we also request that you try and eat the same foods on the day before each study day visit. With these exceptions please do not modify your usual diet or activity levels for this study.

What is the drug or procedure that is being tested?

The study is testing the effects of beta-hydroxy beta-methyl butyrate, a commercially available nutritional supplement on glucose handling in older adults.

What are the side effects or risk of any treatment or procedures received when taking part?

Research studies often involve some risks, not all of which may be currently known. Blood tests and the insertion of cannula can result in bruising around site, however with good practice this bruising should be minimal. Pressure will be applied to the cannula site at the end of each study day visit to minimise the risk of bruising.

What are the possible benefits of taking part?

We cannot promise the study will help you directly, but you will receive information on your blood test results and blood pressure check. In addition, the information we get from this study will give us a better understanding of the effects of HMB supplementation which may improve glucose handling in older people and potentially help improve a patient's fitness to undergo surgery. Finally, if you would like a copy of any findings arising from this research please let a member of the research team know.

What are the possible disadvantages and risks of taking part?

Healthy volunteers have been chosen for this study; however, it is possible that the routine tests could detect unknown health problems. Examples of these include diabetes and high blood pressure, as they are common and often undiagnosed. Should this be the case, you will be informed and advised to attend your GP practice for further management. Your GP will also be notified.

What are the exclusion criteria for this study?

We are recruiting healthy volunteers between the ages of 18- 35 and 65 to 85 years, who are of normal weight or slightly overweight. If you have been a subject in any other research study in the last three months which involved: taking a drug; being paid a disturbance allowance or having an invasive procedure (e.g., blood sample >50ml) you would not be eligible to take part.

You would also be unsuitable if you have particular medical conditions or are taking certain medications. If you are interested in this study, please discuss these further with the research team.

What if something goes wrong?

If you suffer any symptoms or side effects, you should report them immediately to the research team (see end of sheet for contact details). If you have a concern about any aspect of this study, you should ask to speak to the research team who will do their best to answer your questions. The researchers contact details are given at the end of this information sheet. If you have a complaint on your treatment by a member of staff or anything to do with the study, you can initially approach the lead investigator Dr B Phillips. If this achieves no satisfactory outcome, you should then contact the Ethics Committee Secretary, Mrs Louise Sabir, University of Nottingham, Faculty of Medicine & Health Sciences Research Ethics Committee c/o School of Medicine Education Centre, B Floor, Medical School, QMC Campus, Nottingham University Hospitals, NG7 2UH. E-mail: louise.sabir@nottingham.ac.uk.

Will my taking part in this study be kept confidential?

We respect your right to privacy, and we will take measures to safeguard confidentiality. A single form, on which you are asked to sign to give consent for involvement will carry details of your name and address, but no health-related details. This is kept securely in a locked cabinet within the Medical School. Access to this cabinet is restricted to personnel directly involved in the study and to University staff with direct responsibility for ensuring the study is conducted appropriately.

We will follow current ethical and legal practice and all information about you will be handled in confidence. If you join the study, some parts of the data collected about you will be looked at by authorised persons from the University of Nottingham who are organising the research. They may also be looked at by people authorised to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and we will do our best to meet this duty.

All information which is collected about you during the course of the research will be kept strictly confidential, stored in a secure and locked office, and on a password protected database. Any information about you which leaves the hospital will have your name and address removed (anonymised) and a unique code will be used so that you cannot be recognised from it, with the exception of a letter sent to your GP advising of your participation in this study (if applicable).

Your personal data (address, telephone number) will be kept for 5 years after the end of the study so that we are able to contact you about the findings of the study and possible follow-up studies. All other data (research data) will be kept securely for 7 years. After this time your data will be disposed of securely. During this time all precautions will be taken by all those involved to maintain your confidentiality and only direct members of the research team will have access to your personal data.

What will happen to any samples I give?

We would also like to seek your consent for any samples remaining after analysis for this study has been completed, to be stored and used in future research. This is optional and you will be asked to give separate consent for this. The samples will be securely stored with a code unique to you at the University of Nottingham under the Universities Human Tissue Research Licence (No. 12265).

Some of these future studies may be carried out by researchers other than the current research team. This may include researchers working for commercial companies. Any samples or data used will be anonymised so that you could not be identified in any way. If you do not agree to this, any remaining samples will be disposed of in accordance with the Human Tissue Authorities code of practice.

Will any genetic tests be done?

In the current study we will not be using your samples for genetic testing.

What will happen to the results of the research study?

Data collected during the study will be published in the scientific literature enabling other health professionals and scientists to use the information. You will not be

identified in any publication. There is usually a delay of at least one year from the study completion before this occurs. Should you wish to be informed of publications resulting from this study, please inform the study team.

Who is funding the research?

This study is funded by the Royal College of Surgeons of England and the Dunhill Medical Trust.

Who has reviewed the study?

This study has been reviewed and approved by the University of Nottingham Medical School Ethics Committee.

Contact for Further Information

Thank you for your interest in this study. For further information please contact Dr Phil Herrod on 01332 724731/ pherrod@nhs.net or the principal investigator, Dr Beth Phillips. Both of these researchers are alternatively contactable at The University of Nottingham, Division of Medical Sciences and Graduate Entry Medicine, School of Medicine, Royal Derby Hospital, Derby, DE22 3DT.

8.5 Publications and abstracts:

Publications:

Supreeth S. Rudrappa, Daniel J. Wilkinson, Paul L. Greenhaff, Kenneth Smith, Iskandar Idris*† and Philip J. Atherton*†

Human Skeletal Muscle Disuse Atrophy: Effects on Muscle Protein Synthesis, Breakdown, and Insulin Resistance—A Qualitative Review. ***Frontiers in Physiology, Frontiers in Physiology (2016).***

Poster and Presentation:

55th annual meeting of The European Association for the Study of Diabetes (EASD)- September 2019, Barcelona, Spain.

The role of endogenous and exogenous testosterone (T) therapy in regulating resistance exercise-induced modulation of glycaemic control in young and older men, respectively. ePoster 525