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Muscle architectural and physiological responses to immobilisation: impact of age and obesity

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Thesis Abstract

Clinical scenarios of recovery from illness or injury both require acute, mandatory periods of bed rest or immobilisation. However, this inevitably leads to; muscle loss, muscle weakness and a number of negative health problems which may be exacerbated in the elderly and in the obese. Both ageing and obesity are accompanied by reduced habitual physical activity levels and impaired muscle metabolic health which may pose an additive burden on musculoskeletal health in these patients. Therefore, understanding musculoskeletal deterioration associated with immobilisation is a major public health issue and embodies a vital area for scientific investigation.

This thesis describes a series of human volunteer studies performed to elucidate the short-term (3 days) and longer-term (14 days) impact of muscle unloading on muscle metabolic and physiological health in healthy young and older, normal weight and obese volunteers

The major findings were: 3 days of unilateral lower limb immobilisation in healthy normal weight young and older volunteers supressed myofibrillar protein FSR by ~25% ($p<0.01$) and ~22% ($P<0.05$) respectively when compared to the non-immobilised limb at baseline, whilst this was not apparent in healthy young obese volunteers. In addition, ultrasonography-determined medial gastrocnemius muscle volume declined during immobilisation in the young normal weight group and the young obese group by ~7% ($p<0.05$) and ~15% ($p<0.01$) respectively, but not the older normal weight group. A significant association was observed between ultrasonography and MRI determined baseline muscle

volume (medial gastrocnemius and vastus lateralis) and the absolute decline in muscle volume during unloading across all age groups and BMI ranges. Finally, 14 days of bed rest initiated similar changes in targeted mRNA expression during bed rest from which IPA analysis predicted similar changes in cellular functions in young and older humans, whilst 14 days of exercise rehabilitation in older humans resulted in an attenuated response in mRNA expression from which IPA analysis predicted a differential response in cellular functions relating to muscle differentiation and lipid metabolism.

This thesis has provided novel insight regarding changes in muscle architecture and protein metabolism in response to short-term muscle unloading in humans and further impacts of aging and obesity on these parameters. In addition, this thesis has provided novel insight regarding mRNA gene expression changes during longer-term bed rest in young and older volunteers and during rehabilitation and has offered insight of metabolic functions most affected by these changes. These findings have important implications for the development of nutritional and exercise strategies to attenuate muscle atrophy during muscle unloading.

Declaration

All of the experimental data presented in this thesis were generated by myself with the assistance of academic and technical staff at the David Greenfield Human Physiology Unit and the Stable Isotope Mass Spectrometry Facility, University of Nottingham

In Chapter 3, 4 and 6 the muscle biopsies were performed mainly by Dr Tariq Taylor and the ultrasound scans were performed and analysed by Dr Joanne Mallinson. Muscle myofibrillar protein FSR and muscle protein expression analysis was performed by myself with the assistance of colleagues from the stable isotope mass spectrometry facility, University of Nottingham (Ms Jessica Cegielski, Ms Debbie Rankin, Dr Matthew Brook, Dr Daniel Wilkinson, Professor Kenneth Smith and Professor Phillip Atherton).

In Chapter 5, muscle biopsy samples and MRI data were obtained from the Institute for Kinesiology Research, Science and Research Centre, University of Priorska, Koper, Slovenia. The MRI data has previously been published (Biolo et al., 2017; Pisot et al., 2016; Rejc et al., 2018) however, muscle RNA extraction and mRNA analysis was performed at the University of Nottingham by myself with assistance from Dr Tim Constantin. Informatics analysis (Ingenuity Pathway Analysis) was performed by Dr Despina Constantin.

I hereby declare that the work presented in this thesis, has not been submitted for any degree or diploma, at this, or any other university, and

that all of the experiments, unless otherwise stated, were performed by me.

Signed:_____ Arfan M Ali

Date:_____

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Abbreviations

4E-BP1	Eukaryotic translation initiation factor 4E-binding protein 1
AKT	Protein kinase B
ATF4	Activating Transcription Factor 4
CHOP	C/EBP homologous protein
CSA	Cross sectional area
CT	Computed tomography
D2O	Deuterium oxide
DXA	Dual-energy X-ray absorptiometry
ER	Endoplasmic reticulum
FAK	Focal adhesion kinase
FSR	Fractional synthetic rate
FoXO1	Forkhead box protein O1
FoXO3	Forkhead box protein O3
GLUT	Glucose transporters
GSK-3b	Glycogen synthase kinase 3 beta
IGF-1	Insulin growth factor -1
IMAT	Intramuscular adipose tissue
IRMS	Isotope ratio mass spectrometry
MPB	Muscle protein breakdown
MPS	Muscle protein synthesis
MRI	Magnetic resonance imaging
PAL	Physical activity level
PCR	Polymerase chain reaction
PDC	Pyruvate dehydrogenase complex
PDK2	Pyruvate Dehydrogenase Kinase 2
PDK4	Pyruvate Dehydrogenase Kinase 4

PI3K	Phosphoinositide 3-kinase
PKB	Protein kinase B
UPS	Ubiquitin proteasome system
eIF4E	Eukaryotic translation initiation factor 4E
mSIN1	Mammalian stress-activated protein kinase interacting protein 1
mTOR	Mammalian target of rapamycin
p70S6K	Ribosomal protein S6 kinase beta-1

Chapter 1

Literature Review

1.1 Models of muscle disuse

Musculoskeletal deterioration following hospital admissions is a major public health and sporting problem in both young and older adults. Clinical scenarios of recovery from illness or injury both require acute, mandatory periods of bed rest or immobilisation. However, this inevitably leads to; muscle loss, muscle weakness and a number of negative health problems which may be exacerbated in the elderly and in the obese. Both ageing and obesity is accompanied by reduced habitual physical activity levels and impaired muscle metabolic health which may pose an additive burden on musculoskeletal health in these patients. Therefore, understanding the musculoskeletal deterioration associated with immobilisation is a major public health issue and embodies a vital area for scientific investigation. Various clinical models exist to study the negative impact of muscle disuse induced atrophy on musculoskeletal health ranging from immobilisation to complete bed rest.

1.1.1 Spaceflight

It is well established that space flight results in loss of skeletal muscle mass and strength (Fitts et al., 2010; Tesch et al., 2005). In normal gravity environments, the muscles work against gravity to maintain an upright posture and locomotion. However, in a space flight with a microgravity environment, the weight of an object such as the human body is negligible and the requirements for the muscle's antigravity function is imperceptible. Therefore, spaceflight leads to whole body muscle disuse induced atrophy through reduced muscle load bearing.

However, studying the physiological changes during spaceflight is difficult due to the complexity of the study and cost. Therefore, there is only a limited number of spaceflight studies and interpretation of data is difficult due to the small sample size and variations in space flight.

1.1.2 Bed rest and dry immersion

Numerous variants of the bed rest model exist to study muscle disuse induced atrophy. These include head-down tilt model (de Boer et al., 2008; Kortebein et al., 2007; LeBlanc et al., 1992) and dry immersion (Demangel et al., 2017) both of which are used to mimic weightlessness and reduced muscle loading during spaceflight. Similar to spaceflight, bed rest head down tilt and bed rest head up dry immersion both lead to whole body muscle disuse induced atrophy and are commonly employed during research studies.

1.1.3 Unilateral lower limb suspension and unilateral lower limb immobilisation

The unilateral lower limb suspension (ULLS) model was first developed in 1991 as a human inactivity model to understand the effects of muscle unloading during spaceflight (Berg et al., 1991). During the ULLS model the participant wears a shoe with a raised platform and the contralateral limb is flexed with the support of a shoulder strap. Support crutches are provided to the participant so they are able to continue with their daily living activities. The elevated limb prevents ground contact for the contralateral limb thereby inducing muscle unloading. Further revised

ULLS models of this technique removed the need for a shoulder strap and the unloaded leg was fully flexed.

The unilateral lower limb immobilisation is a more extreme model of muscle disuse when compared to the ULLS method. Joint restriction in a targeted limb prevents joint movement and therefore reduces muscle contraction. Limb restriction is achieved through either the use of a full leg plaster cast or a fixed knee brace. Crutches are provided to the participant to aid them in their daily living activities. The unilateral lower limb immobilisation can be viewed as an intermediate approach between bed-rest and reduced physical activity and induces local muscle atrophy as well as allowing the contralateral limb to act as a control. It is also of high clinical relevance and more likely to be undertaken by experimental volunteers than sustained bed-rest.

1.1.4 Reduced physical activity

The inception of reduced physical activity to understand skeletal muscle disuse atrophy has been fairly recent and is the lowest in terms of intensity on muscle disuse induced atrophy. The participant's daily step-count is reduced to ~1500 to study the effects of reduced physical inactivity of musculoskeletal health. Therefore, the participant is still allowed to contract their muscle and keep mobile and this provides a more a realistic approach to understanding the effects of sedentariness of muscle health.

1.2 Human muscle disuse on muscle mass

Muscle unloading in the form of bed rest and immobilisation leads to many negative physiological adaptations. The decline in muscle contractile activity associated with physical inactivity leads to muscle weakness and reduced functional capacity, muscle mass loss, insulin resistance and ultimately chronic non-communicable disease progression. Therefore, maintenance of muscle mass is important for health and well-being.

1.2.1 Quantification of muscle mass

Muscle mass depletion is an important physiological adaptation to muscle unloading and a characteristic of sarcopenia. Magnetic resonance imaging (MRI) and computed tomography (CT) are considered the gold standards for quantifying muscle volume (Mitsiopoulos et al., 1998). However, both MRI and CT are not feasible for the assessment of muscle mass in daily practice. MRI has limited availability and is very expensive to perform whilst CT uses ionising radiation and therefore is not performed on a routine basis. Dual-energy X-ray absorptiometry (DXA) uses a much lower ionising radiation dose and is also a widely used technique to determine muscle mass in a research setting. However, DXA also has limited availability. Therefore, the use of ultrasound is potentially a good alternative to CT, MRI, and DXA in a clinical research setting as it is a non-ionizing imaging technique that facilitates dynamic assessment of soft tissue structures. In addition, ultrasonography is relatively cheaper than MRI and CT, is portable and highly accessible. Furthermore,

ultrasound derived muscle thickness has been validation against CT and MRI and demonstrated a good correlation (Arbeille et al., 2009; Thomaes et al., 2012) and ultrasound derived muscle cross sectional area has been validated against MRI derived muscle cross section area showing good correlation (Takai et al., 2011). Therefore, the use of ultrasound is a valid and reliable method to assess muscle thickness, muscle cross sectional area and muscle volume in a clinical setting.

1.2.2 Human muscle unloading on muscle mass

It is well established muscle that unloading in young humans leads to muscle atrophy. Five weeks of horizontal bed rest in young males results in a decrease in ultrasonography determined muscle thickness of ~12% in the gastrocnemius muscle and 8% in the vastus lateralis muscle (de Boer et al., 2008). Furthermore, a short period of 5 days in a unilateral full leg cast young males results in a decrease in quadriceps muscle volume of ~4% when measured using MRI (Wall et al., 2013). Jones and colleagues demonstrated 2 weeks of unilateral full leg immobilisation in young volunteers results in a reduction in quadriceps lean mass quantified by DXA of ~5% (Jones et al., 2004). De Boer and colleagues demonstrated following 14 days of unilateral lower limb suspension, MRI determined quadriceps muscle cross sectional area (CSA) had decreased by 5.2% (i.e. at −0.37 ± 0.05% per day) and at 23 days by 10.0% (i.e. −0.51 ± 0.21% per day). Therefore, muscle unloading leads to a decline in muscle mass indices which are greater at the onset of muscle unloading.

When measuring muscle volume as a whole, the pattern continues. For example two weeks of unilateral full leg immobilisation with a lightweight fiber cast results in a decrease of quadriceps muscle volume by ~9% in young participants when measured using MRI (Suetta et al., 2009). Similarly, 14 days of bed rest resulted in a ~6% decline in quadriceps muscle volume in young participants when measured using MRI (Pisot et al., 2016). However, the magnitude of muscle atrophy during immobilisation appears to vary across muscle groups, even in the weight bearing lower limbs. For example, De Boer and colleagues using ultrasonography showed medial gastrocnemius and vastus lateralis muscle underwent greater atrophy following 5 weeks of bed-rest in young volunteers when compared to the tibialis anterior over the same time period (-12% and -8% vs. no change) (de Boer et al., 2008). Similarly, following 90 days of bed rest the medial gastrocnemius muscle was found to have undergone a 28% decline in muscle volume in comparison to a decline of 15% in the tibialis anterior in the same limb using MRI (Belavy et al., 2017). Therefore, it seems the medial gastrocnemius muscle is most affected to disuse induced atrophy.

In addition to the magnitude of muscle loss varying across muscle groups during immobilisation, individual differences in pre-immobilisation habitual physical activity levels may impact upon the rate of muscle loss during subsequent disuse. For example, a randomised controlled trial involving untrained, young, healthy volunteers reported that 8 weeks resistance exercise training protected forearm flexor muscle volume (MRI determined) during 21 days of subsequent arm suspension when

compared to a control group that had no pre-immobilisation training (Miles et al., 2005). However, given the authors failed to determine muscle volume following 8 weeks resistance exercise training gains in muscle volume during the exercise training were not taken into consideration. The authors did observe muscle volume loss to be less in individuals with relatively lower forearm flexor muscle volume at baseline (Miles et al., 2005), which has also been observed when comparing the response to immobilisation in older vs younger volunteers (Suetta et al., 2009). Therefore, it seems plausible to suggest that initial muscle mass is a determinant of the magnitude of muscle mass loss during subsequent immobilisation, with the former being dictated by habitual physical activity modulating muscle protein turnover.

Whether older people are more or less susceptible to immobilisation induced muscle mass loss is unclear, but clearly immobilisation will accentuate sarcopenia if muscle mass is not restored upon remobilisation, which has negative health implications. Fourteen days of unilateral full leg immobilisation in a light weight fibre cast resulted in a decline in quadriceps muscle volume of ~9% in young males which was significant when compared to ~5% in the elderly males when measured using MRI (Suetta et al., 2009). On the other hand, Pisot et al demonstrated quadriceps muscle volume following 14 days of bed rest had declined by ~6% in young males which was significantly different to the ~8% in older males when measured using MRI (Pisot et al., 2016). Furthermore, following 5 days of bed rest, leg lean mass was found to have declined by 4% in older volunteers which was significantly different from young

volunteers which remained unchanged measured using DXA (Tanner et al., 2015). Therefore, there appears to be no clear consensus as to whether older people are more or less susceptible to muscle disuse related atrophy than younger people, or indeed whether they are no different.

1.3 Muscle protein metabolism

1.3.1 Muscle protein FSR

The regulation of muscle mass in adults is dependent on the balance between MPS and MPB and both processes are responsive to external factors i.e. physical inactivity (Breen et al., 2013; de Boer et al., 2007; Glover et al., 2008; Wall et al., 2016), protein supplementation (Cuthbertson et al., 2005; Glover et al., 2008; Smith et al., 1992), exercise (Brook et al., 2015) and infection, trauma and inflammation (Puthucheary et al., 2013).

1.3.1.1 Methodological techniques to measure muscle protein FSR

Traditionally, *in vivo* measurements of myofibrillar protein FSR has been made using stable isotope tracers of amino acids with either an isotope of carbon, hydrogen or nitrogen (^{13}C, ^{2}H or ^{15}N) and are generally accepted for providing reliable measurements (Rennie et al., 1982). Principally, by administrating a known amount of labelled (tracer) and unlabelled amino acid (tracee) the tracer and tracee will mix in the endogenous pool and become incorporated into protein over time. Two methods exist by which the tracer and tracee are administered. The first being a primed-constant infusion were a bolus priming dose of tracer and tracee are administered and continuously provided throughout the experiment at low concentration to maintain enrichment. However, the primed-constant infusion approach requires isotopic steady-state conditions, which takes around one hour to achieve, depending on the tracer. Furthermore, another 2 or 3 hours will be required to achieve adequate incorporation of

the tracer in to the protein-bound pool to allow for accurate measurements. The second method by which stable isotope tracers are administered is the flooding dose were a supraphysiological bolus of known tracer is provided over a short period of time (seconds to minutes). The flooding dose method does not require isotopic steady-state conditions as the dose exceeds the endogenous free amino acid level by several-fold (Rennie et al., 1994). However, the flooding dose can stimulate MPS when an essential amino acid is used as a tracer (Smith et al., 1998). In addition to the methodological limitations stated above, stable isotope tracers of amino acids (^{13}C, ^{2}H or ^{15}N) only permit measurements to be performed over a short duration (typically <8–12 h). Furthermore, the experimental setup often requires costly sterile infusions, venous/arterial cannulation, and multiple biopsy collection, all within a controlled laboratory environment.

An alternative method to measure myofibrillar protein FSR, but less familiar and can circumvent these potential limitations is the use of deuterium oxide ($^{2}H_2O$). The use of deuterium oxide (D_2O) to measure myofibrillar protein FSR was first described by 1941 by Hans Ussing, (Ussing, 1941). Ussing administered D_2O to mice and rats which and showed through the incorporation of ^{2}H atoms into the body water pool, it was possible to measure the newly synthesised protein in skeletal muscle (as ^{2}H-labeled amino acids), liver and kidneys. One limitation of using D_2O tracer to quantify muscle myofibrillar protein FSR was the large doses of D_2O required to determine muscle myofibrillar protein FSR when using gas chromatography-mass spectrometry (GC-MS) as an analytical

tool due to low sensitivity issues, which resulted in administering large doses of D_2O in humans which increased possibility of side effects (vertigo and nausea) (1967; Landau et al., 1996). Due to recent technological advancements in mass spectrometry, particular the development of pyrolysis-isotope ratio mass spectrometry (IRMS) systems which facilitates high precision quantification of hydrogen and oxygen stable isotopes (at low doses), this had led to the re-emergence of D_2O in metabolic research (Brook et al., 2015; Wilkinson et al., 2014). The application of D_2O tracer in a metabolic research provides distinct methodological advantages as researchers are able to quantify accumulative changes in muscle myofibrillar protein FSR over days as opposed to hours in a clinical setting thereby providing a more accurate reflection of the dynamic process of muscle myofibrillar protein synthesis in a 'free-living' environment. In addition, the use of D_2O negates the need for costly sterile infusions and therefore venous/arterial cannulation and reduces the number of biopsies required in a clinical study

D_2O has successfully been employed in previous human metabolism studies to quantify muscle myofibrillar protein FSR. Wilkinson and colleagues validated the application of D_2O in measuring muscle protein versus an amino acid stable isotope (L-[ring-$^{13}C_6$]-phenylalanine) and found the results to be comparable (Wilkinson et al., 2015). In addition, Gasier and colleagues demonstrated the stimulation of myofibrillar protein FSR using D_2O following 24 h from a single exercise bout (Gasier et al., 2012). Furthermore, Brook and colleagues have demonstrated the long-term application of D_2O during a resistance exercise programme of 6

weeks in young males in free-living conditions, highlighting its unique suitability for determining longer-term MPS. Therefore, the use of D_2O provides a less invasive, cost-effective, and flexible means by which to quantify MPS acutely over several hours.

1.3.1.2 Effect of muscle unloading on muscle protein FSR

Whenever human MPS has been measured after a period of muscle disuse induced atrophy during a non-disease state, a marked decline has been observed in the post-absorptive and postprandial state in MPS (Table 1.1). For example, two-weeks of bed rest in young volunteers has shown to reduce vastus lateralis myofibrillar protein FSR by 50% in the post absorptive-state (Ferrando et al., 1996) when measured using an amino acid stable isotope tracer (L- [ring-$^{13}C_6$]phenylalanine). Similarly, de Boer and colleagues demonstrated a reduction in myofibrillar protein FSR of 52% following 10 days and 57% following 21 of unilateral lower limb suspension in young males using an amino acid stable isotope tracer ([^{15}N]proline) (de Boer et al., 2007). Therefore, it would seem post-absorptive myofibrillar protein FSR is depressed at the onset of immobilisation and remains depressed throughout muscle unloading. Furthermore, two-weeks of unilateral lower limb immobilisation in young humans has shown to induce a 27% reduction in post-absorptive quadriceps myofibrillar FSR in the immobilised limb when compared to the non-immobilised limb when using an amino acid stable isotope tracer (L- [ring-$^{13}C_6$]phenylalanine) (Glover et al., 2008). In addition, Glover and colleagues, observed in response to a low dose of amino acid infusion, an attenuation of 54% myofibrillar protein FSR and an

attenuation of 68% in myofibrillar protein FSR in response to a high dose of amino acid in the immobilised limb when compared to the non-immobilised limb. Therefore, muscle unloading leads to an attenuation in myofibrillar protein FSR in the post-absorptive and postprandial state (Glover et al., 2008; Wall et al., 2016). In addition to using bed rest and immobilisation as techniques to reduce muscle contractile activity, reduced habitual physical inactivity in itself can be utilised as a method reduce muscle mass and myofibrillar protein FSR. Two weeks of reduced (~80%) ambulatory activity is sufficient to induce lower limb muscle atrophy in young participants when measured using DXA (Krogh-Madsen et al., 2010), which appears to be underpinned by reductions in postprandial myofibrillar protein FSR (Breen et al., 2013). Breen and colleagues demonstrated two weeks of reduced physical activity (~1500 daily step) in older men resulted in an attenuation of 26% in postprandial rates of myofibrillar protein FSR (Breen et al., 2013).

To date, only one study has documented chronic myofibrillar protein FSR in response to immobilisation, which was documented in middle aged (45-60 years old) men (Mitchell et al., 2018). Rather surprisingly, over 14 days of unilateral lower limb immobilisation, that induced a significant 4.1% reduction in thigh muscle CSA (peripheral quantitative CT), the average myofibrillar protein FSR (%/day) was reported to be no different from the basal non-immobilised state (Mitchell et al., 2018). The authors did not quantify any temporal response of myofibrillar protein FSR over the 14 days and therefore any fluctuation in myofibrillar protein FSR over this time (particularly at the onset of immobilisation where the decline in

myofibrillar protein FSR is thought to be greatest [see above]) could have been missed.

Table. 1.1. *Studies investigating the impact of muscle unloading in healthy humans on muscle protein synthesis using a stable amino acid isotope tracer methodology*

Author Date	n	Sex	Age (years)	BMI (kg m^{-2})	Muscle Group	Imm. Period (days)	Imm. Method	Tracer	Tracer Method	Feeding state	Baseline MPS (% h^{-1})	Post imm. MPS (% h^{-1})	% Change MPS
(Ferrando et al., 1996)	6	M	30 ± 6		VL	14	Bed rest	L-[*ring*-$^{13}C_6$]phenylalanine	Constant infusion	Post-absorptive	0.085 ± 0.089	0.075 ± 0.089	-12%
(Symons et al., 2009)	7	M	28 ± 1	26.5 ± 0.8	VL	21	Bed rest	l-[ring-13C6]phenylalanine, & l-[ring-15N]phenylalanine	Flooding dose	Post-absorptive	0.081 ± 0.000	0.042 ± 0.000	-49%
(de Boer et al., 2007)	5	M	19 ± 1	22.5 ± 2.5	VL	10	Leg suspension	[1-13C]proline [15N]proline	Flooding dose	Post-absorptive	0.047 ± 0.004	0.022 ±0.007	-53%
(de Boer et al., 2007)	4	M	19 ± 1	22.5 ± 2.5	VL	21	leg suspension	[1-13C]proline [15N]proline	Flooding dose	Post-absorptive	0.047 ± 0.004	0.020 ± 0.009	-57%
(Glover et al., 2008)	12	10M + 2F	21 ± 1	24.5 ± 1.3	VL	14	Knee-brace immobilisation	l-[ring-13C6]phenylalanine	Constant infusion	Post-absorptive	NI: 0.037 ± 0.003	I: 0.027 ± 0.003	-27%
(Glover et al., 2008)	12	10M + 2F	21 ± 1	24.5 ± 1.3	VL	14	Knee-brace immobilisation	l-[ring-13C6]phenylalanine	Constant infusion	Postprandial			-54%
(Wall et al., 2013)	12	M	24 ± 1	25.0 ± 1.1	VL	5	Full leg cast	l-[ring-(2)H5]phenylalanine and l-[1-(13)C]leucine	Constant infusion	Post-absorptive	0.032 ± 0.005	0.015 ± 0.002	-41%
(Wall et al., 2013)	12	M	24 ± 1	25.0 ± 1.1	VL	5	Full leg cast	l-[ring-(2)H5]phenylalanine and l-[1-(13)C]leucine	Constant infusion	Postprandial	0.044 ± 0.003	0.020 ± 0.002	-53%

M = Males, F = Females, VL = Vastus lateralis, Imm. = immobilised, MPS = Muscle protein synthesis, NI = non-immobilised limb, I = immobilised limb.

1.3.1.3 Other factors negatively affecting muscle protein FSR

1.3.1.3.1 Aging on muscle protein FSR

In addition to muscle unloading negatively affecting muscle protein FSR, other factors negatively impact on muscle protein FSR. In the postabsorptive state MPS rates between young and older humans is similar (Cuthbertson et al., 2005; Tanner et al., 2015). However, Cuthbertson and colleagues utilising an euglycemic insulin clamp to separate the dose-response effects of amino acid ingestion from the associated hormonal response of insulin on the subsequent stimulation of MPS rates in the young and old volunteers (Cuthbertson et al., 2005) and demonstrated, 40 g of essential amino acids stimulated MPS rates in the young, but this response was blunted in the elderly (Cuthbertson et al., 2005). In addition, Kumar and colleagues demonstrated in young participants a sigmoidal dose–response relationship between MPS at 1–2 h post-exercise and exercise intensity, whilst this response was blunted in the elderly (Kumar et al., 2009). Therefore, aging is associated with a diminished muscle protein synthetic response to protein ingestion and in response to exercise (across all intensities) termed anabolic resistance and may likely contribute to the age-related loss of muscle mass beyond the age of 50. In addition, Cuthbertson and colleagues demonstrated following amino acid ingestion, activation of anabolic signaling (phosphorylation state of; mTOR, p70s6 kinase, eIF4BP-1) in the elderly was blunted when compared to the young. The mechanisms responsible for anabolic resistance in aging is currently unknown. However, two weeks of unilateral lower limb immobilisation has shown to induce

anabolic resistance in young humans (Glover et al., 2008) and aging is associated with habitually reduced physical activity (Frisard et al., 2007), such that the reduced sensitivity of older muscle to a given protein dose as compared with the young may be due to reduced habitually physical activity. Indeed, physical activity has shown to make the muscle more sensitive to amino acids feeding in young males (Burd et al., 2011).

1.3.1.3.2 Obesity on muscle protein FSR

Obesity is defined as excessive fat accumulation that presents a risk to health and is a major risk factors for a number of chronic diseases, including diabetes, cardiovascular diseases and cancer (Pi-Sunyer, 1999). In the postabsorptive state MPS rates between young normal weight and young obese humans has shown to be lower (Guillet et al., 2009) and elevated (Nair et al., 1983) whilst in middle aged men, MPS rates in the postabsorptive state have shown to be similar (Murton et al., 2015). However, whilst Guillet and colleagues demonstrated a similar increase in MPS from the postabsorptive state to the postprandial state in young normal weight and young obese humans (Guillet et al., 2009), Murton and colleagues demonstrated in older normal weight men a doubling of myofibrillar protein FSR in response to mixed amino acids which was did not occur in the obese older aged men (Murton et al., 2015). However, unlike in aging were the activation of anabolic protein signalling is also blunted in response to feeding, with obesity induced anabolic resistance in response to feeding, this occurred without any differential response in the activation of anabolic protein signalling between older normal weight and older obese humans (Murton et al., 2015). In addition, Beals and

colleagues have demonstrated the synergistic stimulatory effect of resistance exercise and protein feeding on myofibrillar protein FSR is blunted in young obese humans when compared to normal weight counterparts (Beals et al., 2018). Therefore, obesity is associated with altered protein metabolism which may accelerate muscle atrophy during muscle unloading. The mechanism responsible for obesity induced anabolic resistance is currently unknown. However, obesity is associated with elevated basal free fatty acid and Stephens and colleagues have demonstrated infusing intralipid suppresses post-prandial myofibrillar protein FSR in young obese individuals when compared to young normal weight individuals (Stephens et al., 2015).

1.3.1.3.3 Smoking and alcohol on muscle protein FSR

In addition to aging and obesity, smoking negatively impacts on muscle protein FSR. Petersen and colleagues have shown in 8 young smokers and 8 young non-smokers, the post-absorptive muscle protein FSR rates are depressed by 37% in the smokers (Petersen et al., 2007). Furthermore, alcohol consumption has shown to supress the anabolic response of exercise and protein ingestion in young males (Parr et al., 2014).

1.3.1.4 Gender effects

In comparison to men, women have less muscle mass and more body fat (Forbes and Reina, 1970) which is usually attributed to differences in sex hormones. Testosterone is an anabolic stimulus in muscle (Isidori et al., 2005) stimulating MPS and inhibiting MPB (Ferrando et al., 2003). On the contrary, female sex hormones have shown to inhibit MPS in rodents

(Toth et al., 2001). Notwithstanding, basal myofibrillar FSR between young males and females has been reported to be similar (Smith et al., 2009). In addition, in response to intravenous amino acid feeding under a hyperinsulinemic-hyperaminoacidemic-euglycemic clamp the increase in muscle FSR between young males and females was similar (Smith et al., 2009). Similarly, in response to resistance exercise, there does not seem to be any gender differences in the stimulation of MPS (West et al., 2012). However, Smith and colleagues demonstrated post-menopausal women to have increased basal MPS rates when compared to older men (Smith et al., 2008) and post-menopausal women to have an attenuated response to feeding induced stimulation of MPS when compared to young women (Smith et al., 2012). Therefore, gender differences in muscle protein metabolism may appear with age as a result of changes in hormones. However, more importantly, differences in physical activity between gender in older humans do exist (Li et al., 2017) and the recruitment of active older males vs. inactive older females or active young females vs. inactive older females may be having an impact. Indeed, it is well known immobilisation and reduced physical activity can attenuate the MPS response to protein feeding (Breen et al., 2013; Glover et al., 2008). Conversely, physical activity has shown to make the muscle more sensitive to amino acids feeding in young males (Burd et al., 2011).

1.3.1.5 Molecular mechanism regulating muscle protein synthesis

The AKT/mTOR/p70S6K signalling axis by regulating protein translation initiation is thought to be the dominant pathway controlling MPS in

response to exercise and feeding. Protein kinase B (PKB) also known as AKT, plays a diverse cellular role regulating many cellular processes including; cell proliferation, cell growth, cellular metabolism and cell survival (reviewed by Manning and Cantley, 2007). AKT is activated by numerous stimuli, including growth factors, mechanical stimulation, and in response to insulin secretion (reviewed by Nader, 2005). AKT is a downstream target of the (insulin growth factor -1) IGF-1 / Phosphoinositide 3-kinase (PI3K) pathway, and animal models reveal AKT signalling to be crucial in inducing muscle hypertrophy (Lai et al., 2004). Downstream targets of AKT include Glycogen synthase kinase 3 beta (GSK-3β) and mammalian target of rapamycin (mTOR) (*Figure. 1.1.*).

First discovered as the target for Rapamycin and thus named the mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase which regulates a diverse array of cellular processes (reviewed by Laplante and Sabatini, 2009). mTOR is a held in a two-form complex state comprising of mTOR/regulatory-associated protein of mTOR (Raptor)/mammalian lethal with SEC13 protein 8 (MLST8) also known as GβL/PRAS40 and DEPTOR to form mTOR complex 1 (mTORC1). Alternatively, mTOR can be held in a separate complex comprising mTOR/rapamycin-insensitive companion of mTOR (RICTOR), GβL, and mammalian stress-activated protein kinase interacting protein 1 (mSIN1).

The predominate mechanism by which activated AKT upregulates skeletal MPS occurs via the mTORC1 complex which promotes translational

initiation and ribosomal biogenesis promoting protein synthesis, leading to increased cell mass. AKT activates mTORC1 through the disinhibition of TSC2 which in held in complex with TSC1 and promotes the formation of Rheb-GTP thereby activating mTORC1 (Inoki et al., 2002). Downstream effector proteins of mTORC1 include Ribosomal protein S6 kinase beta-1 (p70S6K) and 4E-BP1 both of which are involved in translational initiation.

Eukaryotic initiation factor 4E (elF4E) binds to mRNA at the 5' end and induces translational initiation. However, the action of elF4E is prevented by 4E-BP1. Phosphorylation of 4E-BP1 by mTORC1 prevents the binding of 4E-BP1 to elF4E promoting cap-dependent translation (reviewed by Richter and Sonenberg, 2005). Likewise, activation of p70S6K by mTORC1 results in; ribosomal biogenesis, cap-dependant translation initiation (reviewed by Ma and Blenis, 2009). Although the primary role of mTORC2 is not involved in protein synthesis, mTORC2 does provide a stimulating feedback loop activating AKT at Ser^{473} (Sarbassov et al., 2005).

Activated AKT phosphorylates and thus inhibits GSK-3β which results in dephosphorylation (and thus activation) of elF2B (Welsh and Proud, 1993) therefore propagating the preinitiation translation complex (Cohen and Frame, 2001).

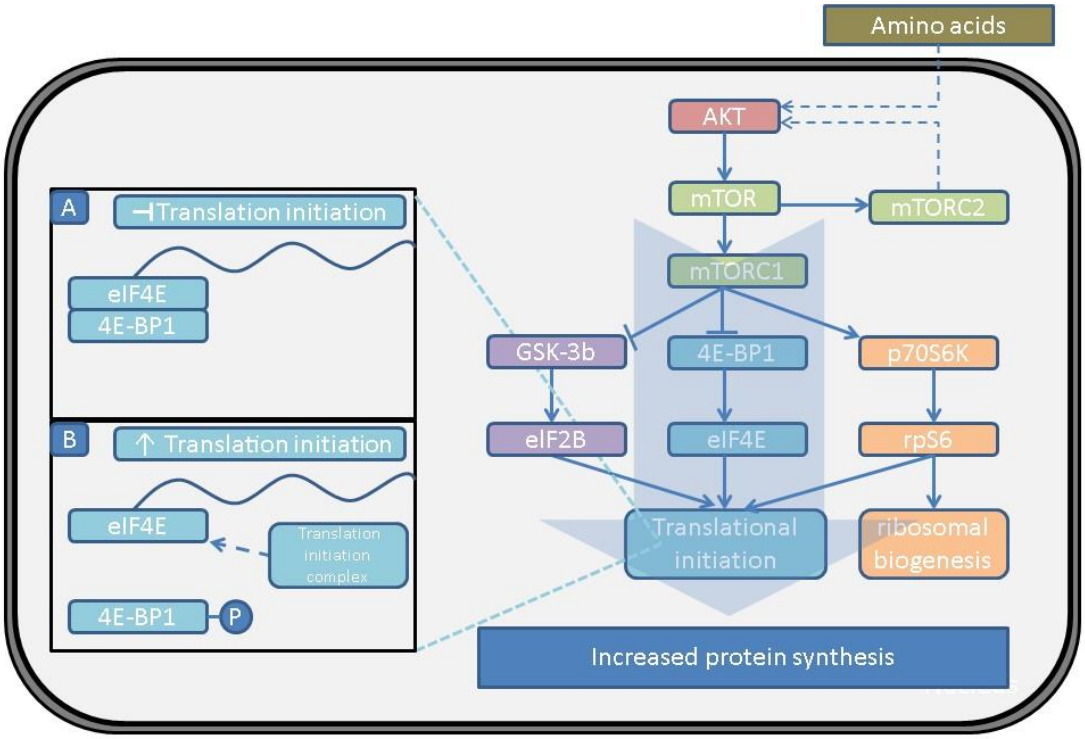

Figure 1.1 *– An overview of protein signalling involved in translational initiation leading to increased muscle protein synthesis. During* ***A*** *4E-BP1 binds eIF4E preventing binding of further proteins involved in translational initiation thereby inhibiting the process. During* ***B*** *Phosphorylation and thus disinhibition of 4E-BP1 by the mTORC1 facilitates the recruitment of eIF4G and eIF4A to eIF4E thus propagating the translational initiation signal.*

1.3.2 Molecular mechanism regulating muscle protein breakdown

Proteolysis is the process by which protein are broken down into smaller polypeptides and amino acids. The process is important for degrading proteins which are worn out. However, dysregulation can lead to muscle loss and weakness. In skeletal muscle four proteolytic pathways have

been identified for the degradation of proteins. These are; lysosomal proteolysis, calcium-mediated proteolysis, caspase-mediated proteolysis and ubiquitin proteasome system (UPS). The latter, UPS has been the most extensively studied pathway and is thought to be the principal regulator of skeletal muscle atrophy at the cytoplasmic level. Importantly, the UPS is unable to degrade intact myofibrillar proteins and therefore a co-ordinated response between pathways must exist to degrade myofibrillar proteins.

1.3.2.1 Ubiquitin Proteasome System

Identified in 1975 (Goldstein et al., 1975) and almost universally expressed, the primary role of ubiquitin is proteasomal degradation through tagging of proteins by ubiquitin and degradation via the 26s proteasome. However the role of ubiquitin is far more diverse and may play a role in; DNA repair, protein kinase activation and cell growth (reviewed by Chen and Sun, 2009).

Ubiquitin (Ub) is a 76 linked amino acid protein which is highly conserved. Ubiquitination is a post-translational modification process whereby the Gly^{76} residue on ubiquitin is attached to a lysine residue present on the substrate protein. This process consists of three reactionary phases in which ubiquitin is attached to a target protein for degradation (reviewed by Pickart, 2001).

Firstly, ubiquitin is activated by the ubiquitin activating enzyme (E1) by adenylating the C-terminal glycine of ubiquitin in an ATP-dependant manner. Subsequently, ubiquitin is transferred to the active site of the E1

ligase forming a high energy thiol ester bond with a cysteine residue and thus liberating free AMP. Secondly, ubiquitin is transferred from E1 to the ubiquitin conjugating enzyme (E2). Ubiquitin is transferred to the active site of the E2 cysteine residue forming a thiol ester bond. In the human proteome ~40 E2 enzymes have been identified with varying functions. Finally, ubiquitin is transferred from E2 to a specific ubiquitin ligase (E3) in complex with a protein substrate via the formation of an isopeptide bond between a ξ-amino group of a lysine residue present on substrate protein and the C-terminal glycine present on ubiquitin. In the human proteome ~600 E3 enzymes have been identified with varying functions and tissue expression (reviewed in Metzger et al., 2012; Pickart, 2001). The ubiquitylation process is repeated until 4 or more chains of ubiquitin are covalently attached. The 26s proteasome detects tagged proteins for degradation through the formation of a multiply linked Lys^{48} polyubiquitin chain.

The 26s proteasome comprises of two sub-units, the 19s regulatory cap and the 20s catalytic core (reviewed by Smalle and Vierstra, 2004). The 19s cap recognises the polyubiquitin chain present on the target protein and facilities entry into the 20s catalytic core in an ATP-dependent manner. The 20s catalytic core is comprised of four heptameric rings composed of two different subunits; the α-subunits provide structural support for complex and the β-subunits provide the proteolytic activity for cleaving of proteins into small polypeptides and recycling the ubiquitin into the cytosol.

In skeletal muscle, two specific E3 ligases are expressed known as MAFbx and MuRF-1 which have been shown to increase in muscular atrophy condition such as disuse and inflammation (Dirks et al., 2014; Doucet et al., 2007). Additionally, animal models utilising knockouts of MAFbx or MuRF-1 demonstrate a sparring effect to denervation induced muscular atrophy (Bodine et al., 2001). However, a recent report has questioned our understanding of MuRF-1 in muscle atrophy conditions (Baehr et al., 2011). MuRF-1 null mice demonstrated a muscle atrophy sparring effect when treated with dexamethasone whereas MAFbx null mice resulted in no protection on muscle atrophy sparring (Baehr et al., 2011). Although MuRF-1 remains acceptable as a marker for muscle atrophy in animal models, its role in protein degradation remains elusive. Surprisingly, and contrary to initial observations, MuRF-1 may play a role in regulating protein synthesis (Baehr et al., 2011).

1.3.2.2 Calcium mediated

Calpains are a family of cysteine proteases which are activated by calcium. At least 15 members of the calpain family have been identified, some of which are expressed ubiquitously or tissue specific (reviewed by Sorimachi and Ono, 2012). Skeletal muscle expresses two ubiquitous calpains, the micromolar calcium activating protease (μ-calpain), and the millimolar calcium activating protease (m-calpain) along with a muscle specific calpain-3. A variety of muscle atrophy disorders exhibit an upregulation of calpain activity. Furthermore, studies have demonstrated calpains to degrade certain myofibrillar proteins including; desmin, nebulin, titin, and troponin albeit at a slow rate (Delgado et al., 2001).

Conversely, upregulation of an endogenous calpain inhibitor, calpastatin results in profound muscle hypertrophy in animals (Jackson et al., 1997). Therefore, calpains must play an important role in regulating muscle atrophy.

Recent studies suggest calpains proteolysis is thought to function upstream of the UPS. Calpains are unable to degrade myofibrils to amino acids while the 26s proteasome is unable to degrade intact myofibrils. Therefore initial cleaving of the myofibrils by calpains is thought to provide protein substrates to the UPS (Smith and Dodd, 2007). It should be highlighted *in-vitro* analysis of calpain activity requires supraphysiological concentrations of calcium to activate the protein. Therefore, the full extent of the role of calpain in skeletal muscle atrophy remains ambiguous.

1.3.2.3 Caspase mediated proteolysis

Caspases are a family of cysteine proteases which hold an integral role in protein degradation during apoptosis. Unlike calpains which are a part of the cysteine proteases family, caspase activity is not dependent upon calcium activation. 14 members of the caspase family have been identified (see review by Fan et al., 2005) with caspase-3 being linked to muscle atrophy. Du et al demonstrated caspase-3 to degrade actomyosin to a 14kDa actin fragment *in vitro* with a subsequent increase in UPS mediated proteolysis (Du et al., 2004). Accordingly, it was hypothesised caspase-3 acts upstream of UPS mediated proteolysis in the turnover of myofibrillar proteins. Furthermore, in 49 patients with various catabolic

conditions, measurement of the 14kDa actin fragment level correlated with fractional protein breakdown rate (Workeneh et al., 2006).

1.3.2.4 Lysosomal degradation

Lysosomes are membrane bound vesicles which contain a highly acidic lumen (pH 4-5). The lumen comprises of various acid hydrolyses including proteases, glycosidases, lipases, nucleases and phosphatases. With their intracellular properties, lysosomes are able to degrade a variety of macromolecules with the help of the endopeptidase activity of cathepsins through autophagic delivery of proteins (reviewed in Bechet et al., 2005; Sandri, 2011). Cathepsins are proteases which are ubiquitously expressed albeit at low levels in skeletal muscle. Nonetheless, *in vitro* models have demonstrated cathepsins to degrade certain myofibrillar proteins (reviewed by Bechet et al., 2005). Furthermore, up-regulation of cathepsins mRNA is observed in skeletal muscle in muscle atrophy conditions (Lecker et al., 2004). However the overall contribution of lysosomal degradation to skeletal muscle under conditions of atrophy is relatively low, as inhibition of lysosomal activity does not supress protein breakdown in septic rodent models (Voisin et al., 1996) .

1.3.3 Muscle unloading on molecular muscle mass regulators

1.3.3.1 Muscle protein synthesis signalling

The AKT/mTOR/p70S6K signalling axis by regulating protein translation initiation is thought to be the dominant pathway controlling MPS in response to exercise and feeding. However, the role of this signalling

cascade during human immobilisation remains unclear. Following 10 days of unilateral lower limb suspension in young volunteers, de Boer and colleagues documented a decline in post-absorptive myofibrillar protein FSR of 52%, which occurred in the absence of any change in total abundance or phosphorylation status of AKT, mTOR and p70S6K protein compared to the pre-immobilised state (de Boer et al., 2007). Additionally, although myofibrillar protein synthetic response to the low and high dose protein feeding was markedly reduced in healthy, young volunteers following 14 days of unilateral lower limb immobilisation when compared with the contralateral non-immobilised limb (so called anabolic resistance), this had no impact on the phosphorylation status of AKT, mTOR and p70S6K protein when comparing limbs (Glover et al., 2008). It is not unreasonable to assume that static measurements of protein expression or phosphorylation state of AKT, mTOR and p70S6K signalling proteins may not give a true indication of the dynamic process of MPS (i.e. turnover rate). Indeed, a non-linear relationship between the phosphorylation state of AKT and P70S6K and MPS has been observed in humans in response to increased amino acid provision (Greenhaff et al., 2008). Similarly, a dissociation between MPS and mTOR signalling in response to feeding in the immobilised state has been reported (Glover et al., 2008) Nevertheless, downstream targets of the AKT/mTOR/P70S6K pathway, such as phospho-4E-BP1, may play a regulatory role in modulating muscle myofibrillar protein FSR (Greig et al., 2011). However, De Boer and colleagues demonstrated following 10 days of unilateral lower limb suspension, the total expression and phosphorylation level of 4E-BP1 remained unchanged (de Boer et al.,

2007) whilst Jespersen confirmed this finding following 8 days of a military field exercise were the subjects remained in the prone position (Jespersen et al., 2014). Therefore, the molecular mechanisms responsible for the attenuation of myofibrillar protein FSR during immobilisation do not seem to be the reverse of exercise and feeding induced stimulation of myofibrillar protein FSR.

1.3.3.2 MPB-related pathways

Similar to the response of MPS related proteins to muscle unloading, not a lot is known about the contribution of MPB during human immobilisation induced muscle atrophy as quantification of MPB is difficult to quantify and requires invasive a-v balance measurements. Nedergaard and colleagues demonstrated following 2 weeks lower limb immobilisation in young males, the mRNA levels of FOXO1 and MAFbx were decreased whilst there was no change in the protein expression of MuRF1 and ubiquitin (Nedergaard et al., 2012). However, Jespersen and colleagues demonstrated following 8 days of a military field exercise were the subjects remained in the prone position, Messenger RNA (mRNA) levels of the FoxO3, MAFbx and MuRF1 increased by 36%, 53%, and 71% respectfully whilst MuRF1 protein expression increased by 51%. In addition, following 48 h of unilateral lower limb immobilisation, an increase in ubiquitin protein conjugates has been observed (Urso et al., 2006) and following 72 h of unilateral lower limb suspension, an increase in 3-methylhistidine has been documented (Tesch et al., 2008). Therefore, it seems increased MPB may play an early and possibly transient contribution to muscle unloading induced atrophy in humans.

Nevertheless, elevation of MPB markers over the time course of muscle immobilisation in human volunteers, in the absence of firm evidence of increased MPB based on the application of tracer methodologies, makes the exact contribution of MPB to the aetiology of immobilisation induced muscle atrophy in humans speculative.

1.3.3.3 ER stress-related pathway regulating muscle mass

Endoplasmic reticulum (ER) stress signalling has been suggested to play a role in regulating MPS and MPB in muscle disuse atrophy (Powers et al., 2012). Activating Transcription Factor 4 (ATF4) is a transcription factor which promotes the expression of oxidative stress responsive genes, and is thought to inhibit myofibrillar protein FSR by upregulating 4E-BP1 (Ebert et al., 2015), which is a negative regulator of cap-dependent translation. Furthermore, ATF4 downstream signalling targets include Gadd45a, which is thought to stimulate MPB via the MEKK4 pathway (Bullard et al., 2016). In addition, ATF4 has shown to be increased during denervation induced muscle atrophy in rodents (Sacheck et al., 2007), and ATF4 knock-out mice demonstrate a 50% reduction in muscle loss during unilateral lower limb immobilisation (Fox et al., 2014). These animal model data suggest that ATF4 could play an important role in the regulation of muscle mass, a suggestion yet to be corroborated in humans.

1.3.4 Animal models of muscle disuse

The nature and methodologies used to impose hind limb immobilisation in animals is profoundly more severe than experimented on humans such

as severing the motor nerve or hind limb suspension. In addition, less invasive techniques such as cast immobilisation in animal models can also lead to confounding results. Traditional cast immobilisation can account for up to 40% of a rodent's total body weight with some animals showing skin ulceration and urine retention. Thus, the muscle loss may not be reflective of human immobilisation induced muscle loss. Therefore, translation of animal immobilisation induced muscle loss to human studies should be taken with caution.

Following on from this point, there also lies a disparity between the results obtained from animal and human immobilisation studies. Animal studies exhibit a profound decrease in MPS and MPB while human models reveal a decrease in MPS to a lesser degree in MPB. Three reasons could be attributed to the inconsistency between translating results from animals of immobilisation to human studies. Firstly, rodents which are used experimentally are in their infancy and still growing. Secondly and continuing on from the first point, the metabolism of rodents is seven times higher than that of humans. Finally, the methodologies imposed on animal models are far more severe than that of which are imposed on in humans.

1.4 Impact of human muscle unloading on carbohydrate and lipid metabolism

Skeletal muscle is by far the most energy demanding tissue in the body and accounts for ~40% of the basal metabolic rate at rest. Metabolism can be defined as the chemical processes essential for maintenance of a living organism. The processes can be anabolic resulting in the generation of larger molecules i.e. glucose → glycogen which requires energy. Likewise, the cellular processes can be catabolic producing smaller molecules i.e. ATP → ADP + Pi which produce energy. Human muscle unloading has been associated with insulin resistance (Richter et al., 1989).

1.4.1 Insulin-mediated muscle glucose uptake

In response to ingestion of nutrition (carbohydrates and protein), insulin is secreted from the β-cells of the pancreas. The primary role of insulin is to reduce blood glucose levels. This is achieved by facilitating glucose transport across skeletal muscle which accounts for the main reservoir of insulin stimulated glucose uptake (Ferrannini et al., 1988). To a lesser extent glucose transport is also stimulated in adipose and liver tissue. A family of transmembrane proteins essential for transport of glucose from the extracellular matrix into the cell have been identified as glucose transporters (GLUT) (Mueckler et al., 1985). Twelve isoforms of the GLUT have been identified with varying tissue distribution and affinity. Of interest and highly studied is the GLUT4 isoform which is responsible for insulin stimulated glucose uptake in skeletal muscle and adipose tissue.

Insulin signalling is initiated by the binding of insulin to its extra-cellular, α2-β2-heterotetrameric receptor (reviewed by Leto and Saltiel, 2012). Activation of the insulin receptor results in trans-autophosphorylation of the intracellular component, resulting in intracellular tyrosine phosphorylation. The intracellular component of the insulin receptor now acts as a docking site for Insulin Receptor Substrate-1 (IRS-1), thus phosphorylating tyrosine residues and activating IRS-1. This results in the recruitment of Phosphoinositide-3-kinase (PI3K) to the cell membrane. PI3K catalyses the reaction of lipid substrates located in the plasma membrane from PIP_2 to PIP_3. The formation of PIP_3 on the cell surface facilitates the recruitment of plekstrin homology (PH) domain containing proteins including the serine/threonine protein kinase, AKT *(Figure. 1.2).* Three isoforms of AKT have been identified with Akt2 being largely constituted to insulin mediated GLUT4 translocation. On the other hand, Akt1 has been shown to contribute to cell growth and Akt3 highly expressed in the brain. The protein AKT plays a diverse role in cellular processes including; glucose metabolism, apoptosis, cell proliferation and transcription (see review Manning and Cantley, 2007). Recruitment of AKT to the plasma membrane results in full activation of the protein after fulfilment of two phosphorylation sites. Firstly, Thr^{308} is phosphorylated by Phosphoinositide-dependent kinase-1 (PDK1) via the PH domain and Ser^{473} possibly through Phosphoinositide-dependent kinase-2 (PDK2) in complex with mTORC2 (Sarbassov et al., 2005). The exact mechanism by which activated AKT results in the translocation of GLUT4 remains unknown. However, a downstream target of AKT, AS160 which is phosphorylated at many sites by AKT, is believed to play a crucial role

(Sano et al., 2003). AS160 contains a GAP homology domain which is involved in the regulation of the GTP/GDP cycle. During phosphorylation and thus activation of AS160, the GAP homology domain is suppressed thereby increasing active Rab-GTP which is believed to facilitate GLUT4 vesicle translocation (Miinea et al., 2005).

Conversely, there lies a mechanism by which insulin mediates GLUT4 translocation independently of PI3K (reviewed by Leto and Saltiel, 2012). Activation of the insulin receptor results in the binding of APS to the insulin receptor. APS recruits a complex comprising c-Cbl and c-Cbl-associated protein (CAP) (Liu et al., 2002). Phosphorylation and thus activation of c-Cbl facilitates recruitment of an additional complex containing CRK and C3G. The latter protein, C3G catalyses the activation of an effector protein identified as TC10 which is a member of the Rho-family of GTPases and regulates GLUT4 translocation *(Figure. 1.2).* In addition, exercise too stimulates GLUT4 translocation into the cell membrane. This occurs independently of insulin and is mediated through AMPK (Musi et al., 2001). Interestingly, two pools of GLUT4 have been identified which respond to each stimuli accordingly, i.e. insulin and exercise (Coderre et al., 1995).

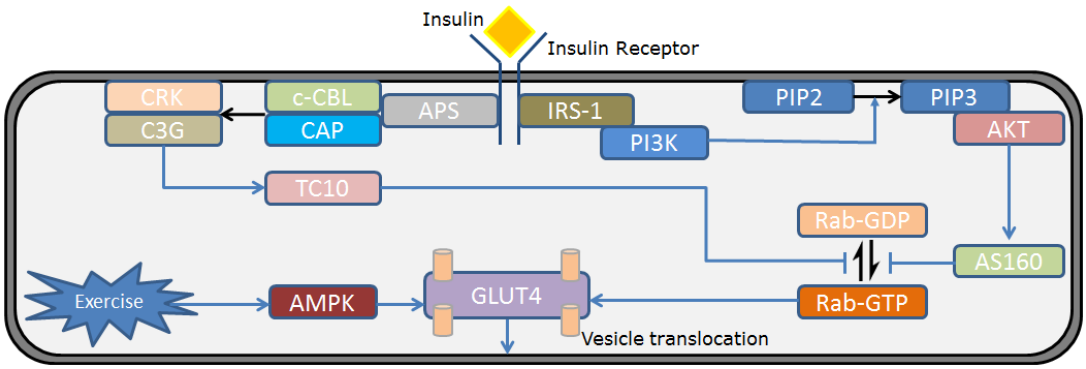

Figure. 1.2 *The signalling cascade responsible for muscle GLUT4 translocation.*

AMPK = AMP-activated protein kinase, APS = Adaptor protein substrate C3G = Cyanidin 3-glucoside, CAP = Cbl associated protein, IRS-1 = Insulin receptor substrate 1, PI3K = Phosphoinositide 3-kinase, PIP2 = Phosphatidylinositol 4,5-bisphosphate, PIP3 = Phosphatidylinositol (3,4,5)-trisphosphate.

1.4.2 Impact of muscle unloading on carbohydrate and lipid metabolism

Following entry into the muscle, the rate limiting step in carbohydrate oxidation is the entry of glucose into the mitochondria via the pyruvate dehydrogenase complex (PDC) which facilities the entry of pyruvate to form acetyl-CoA in the mitochondria, thus allowing for carbohydrate oxidation. However, inhibition of AKT leads to the disinhibition of FOXO which up-regulates PDK2 and PDK4 in the nucleus. PDK is an enzyme which inhibits the PDC complex, thus reducing oxidative glycolysis in the mitochondria, limiting the entry of glucose into the mitochondria and thereby causing insulin resistance.

After a period of immobilisation adaptive changes in skeletal muscle result in deleterious metabolic effects. Seven days of a unilateral limb immobilisation in young males resulted in a blunting of local muscle glucose uptake following a euglycemic hyperinsulinemic clamp, whilst utilising the contralateral non-immobilised limb as a control (Richter et al., 1989). Similarly, bed rest induces whole body insulin resistance in human, as Mikines and colleagues demonstrated following seven days of bed rest the action of insulin was depressed in the whole body with reduced glucose uptake measured using a euglycemic clamp. It would seem the time course of muscle insulin resistance occurs fairly early as following 3 days of bed-rest whole body muscle glucose uptake following an oral glucose tolerance test was depressed (Smorawinski et al., 2000). In addition, it has been shown peripheral insulin resistance develops following 2 weeks of reduced physical activity measured using a hyperinsulinemic-euglycemic clamps (Krogh-Madsen et al., 2010). Therefore, taken together the decline in muscle contractile activity drives the attenuation in postprandial muscle glucose uptake. However, the full mechanism by which this occurs has not been elucidated.

From a molecular signalling perspective, seven days of bed-rest has been shown to attenuate insulin stimulated AKT phosphorylation in young humans (Kiilerich et al., 2011). Furthermore, 7 days of bed-rest are been shown to reduce GLUT4 protein content, reduce muscle hexokinase activity and decrease glycogen synthase activity (Bienso et al., 2012). However, whether responses to muscle unloading a drivers of muscle insulin resistance or occur as a consequence remain unknown.

It is well documented that obesity leads to muscle insulin resistance (DeFronzo and Tripathy, 2009). One possible mechanism for the development of muscle insulin resistance during muscle unloading could be due to the increase in IMAT following muscle unloading. Manini and colleagues demonstrated following 4 weeks of unilateral lower limb suspension in young subject, IMAT increased by 20% in the calf and 14% in the thigh when quantified using MRI (Manini et al., 2007). Furthermore, Boettcher and colleagues demonstrated increased levels of IMAT quantified using MRI was associated with insulin resistance quantified using a glucose clamp (Boettcher et al., 2009). Therefore, it seems logical that increased IMAT content may play a role in the aetiology of skeletal muscle insulin resistance during muscle unloading. Furthermore, the increase in IMAT will increase intracellular fatty acid metabolites, such as long chain fatty acyl CoAs, long chain acylcarnitines, diacylglycerols and ceramides, which are known to inhibit insulin signalling and pyruvate dehydrogenase flux thereby reducing muscle glucose utilisation.

1.5 Muscle rehabilitation following muscle unloading

1.5.1 Muscle rehabilitation on muscle architecture

Skeletal muscle has high plasticity and during muscle disuse, atrophies. However, during a programme of increased contractile activity, skeletal muscle mass can be restored. Franchi and colleagues demonstrated following 12 weeks of isokinetic resistance exercise in young male quadriceps muscle thickness increased by 8%, CSA by 5% and volume by 5%, measured using ultrasonography. Jones and colleagues demonstrated 2 weeks of full limb immobilisation resulted in a reduction in quadriceps lean mass ~5% as determined by DXA analysis in young volunteers. Furthermore, exercise training in the form of 5 bouts of 30 maximal intensity isokinetic knee extensions on a Cybex, 3 times a week, leg lean mass remained lower than basal values at 1 week and was restored after six weeks of exercise training (Jones et al., 2004). Furthermore, Suetta and colleagues demonstrated following 2 weeks of unilateral limb immobilisation and subsequent rehabilitation in the form of 3 sessions per week for 4 weeks of mixed strengthening exercise, quadriceps muscle volume determined through MRI increased by 8% in the young whilst 4% in the elderly. However, there are mixed reports concerning whether the muscle of older individuals is as responsive as younger muscle to resistance exercise, with reports of attenuated (Welle et al., 1996), similar (Ivey et al., 2000) and greater (Kosek and Bamman, 2008) muscle mass recovery in the elderly. The discordance in the literature may be attributable to the difference in the exercises,

intensity, gender and previous habitual physical activity of the volunteers.

1.5.2 Molecular mechanism leading to muscle hypertrophy

The molecular mechanism by which resistance exercise leads to an increase in skeletal muscle mass after a program of atrophy (i.e. immobilisation) or even in healthy individuals remains to be fully understood. Brook and colleagues demonstrated following 6 weeks of unilateral resistance exercise [6×8 repetitions, 75% 1 repetition maximum (1-RM) 3/wk], D_2O-derived MPS increased by ~18% following 3 weeks of training, when compared to the contralateral non-trained leg, a finding which has been confirmed previously using an stable amino acid isotope approach (Biolo et al., 1995; Cuthbertson et al., 2006; Wilkinson et al., 2008). Therefore, resistance exercise leads to a sustained increase in MPS and to a lesser extent in MPB (Biolo et al., 1995) leading to a net protein balance, thereby inducing muscle hypertrophy. However, with aging it has been shown the response to stimulate MPS post exercise is blunted. For example, Kumar and colleagues demonstrated in young participants a sigmoidal dose–response relationship between MPS at 1–2 h post-exercise and exercise intensity, whilst this response was blunted in the elderly across a range of intensities (Kumar et al., 2009). Furthermore, it has been shown dietary protein supplementation during resistance exercise training (>6 weeks) results in greater gains in muscle mass than resistance exercise alone in younger and older adults (Cermak et al., 2012). In addition, 4 months of aerobic exercises in the form of biking at 80% peak heart rate for 45 min, 3-4 days/week has shown to

increase mixed MPS by 22% in young and older humans (Short et al., 2004).

Unlike human muscle unloading where there is no clear consensus regarding the molecular drivers of muscle atrophy (See section 1.3.1.4), the AKT/mTOR/p70S6K signalling axis is thought to be the major driver of promoting MPS in response to resistance exercise. Following muscle shortening and lengthening exercises to fatigue in young males, the phosphorylation levels of AKT and p70S6K were reported to increase 3-fold after 3 hours and remain elevated for 24 hours (Cuthbertson et al., 2006) and has been reported to increase following resistance training as early as 30 minutes following contraction (Terzis et al., 2008). However, an increase in myofibrillar and sarcoplasmic protein FSR was only detected following 6 hours from the muscle exercises (Cuthbertson et al., 2006).

In addition to a sustained period of net protein gain leading to muscle hypertrophy, myogenesis is thought to play an important role by providing myonuclei to enlarging myofibers leading to muscle hypertrophy. Located between the basal lamina and sarcolemma of muscle fibers, satellite cells are muscle specific stem cells which are normally quiescent. However, upon activation satellite cells migrate to the site of injury proliferating and differentiating to fuse with existing muscle fibers augmenting and forming new muscle fibers. (see review by Wang and Rudnicki, 2012).

Satellite cell myogenesis is regulated by a family of transcription factors termed muscle regulatory factors (MRFs) (see review by Yablonka-Reuveni et al., 2008). This family includes myogenic determination factor 1 (MyoD), myogenic factor 5 (Myf5), myogenin and myogenic regulatory factor 4 (MRF4). Further regulation occurs via inhibition of MyoD, MRF4 and Myf5 by myostatin which is a negative regulator of muscle differentiation. Furthermore, treatment of human skeletal muscle cells *in vitro* with myostatin results in a 50% decrease in AKT phosphorylation (Trendelenburg et al., 2009) thus a reduction in anabolic signalling. Conversely, muscle contraction results in a decrease in the mRNA levels of myostatin (Jones et al., 2004) therefore propagating muscle differentiation. Furthermore, a single bout of resistance exercise in conjunction with neuromuscular electrical stimulations to fully activate skeletal muscle in humans leads to an increase in MyoD and myogenin 12 hours post-exercise (Bickel et al., 2005) which mediates muscle growth/repair.

1.6 **Thesis aims**

The principle aim of this thesis was to investigate the impact of short-term (3 days) unilateral lower limb immobilisation on muscle architecture (volume, thickness, fascicle length and pennation angle), D_2O-derived myofibrillar protein FSR, physical activity levels and the putative regulators of MPS, MPB and ER-stress related pathways during 3 days unilateral lower limb immobilisation in young, normal weight. In addition, to determine whether ageing and obesity further impacted on muscle architecture, protein metabolism and physical activity responses to unilateral lower limb immobilisation. In addition, limited changes in muscle protein expression in response to short-term immobilisation have been observed. Therefore, this thesis aimed to quantify muscle architecture and mRNA expression of 96 gene targets known to regulate muscle mass and structure, and muscle carbohydrate and lipid metabolism in young and older volunteers utilising a RT-PCR microfluidic, low-density array cards approach and use IPA bioinformatics analysis to unravel novel insights into gene responses to muscle disuse in bed-rest and subsequent rehabilitation in the context of aging.

Chapter 2

General Methods

2 General Methods

2.1 Three-day immobilisation study protocol

In chapters 3, 4 and 6, volunteers were screened by a Clinical Research Fellow that involved completion of a medical questionnaire (Appendix 1.1), physical examination, resting 12 lead electrocardiogram, and a blood sample taken for clinical chemistry and clotting factors. Volunteers were excluded from the study if evidence of metabolic disease, cardiovascular disease, overt muscle wasting, cerebrovascular disease, respiratory disease, inflammatory bowel disease, renal disease and/or clotting dysfunction was present. In addition, participants were non-smokers and did not consume more than >21 units of alcohol per week, as smoking reduces basal myofibrillar protein FSR (Petersen et al., 2007) and alcohol consumption suppresses muscle anabolic responses (Parr et al., 2014).

Study protocol

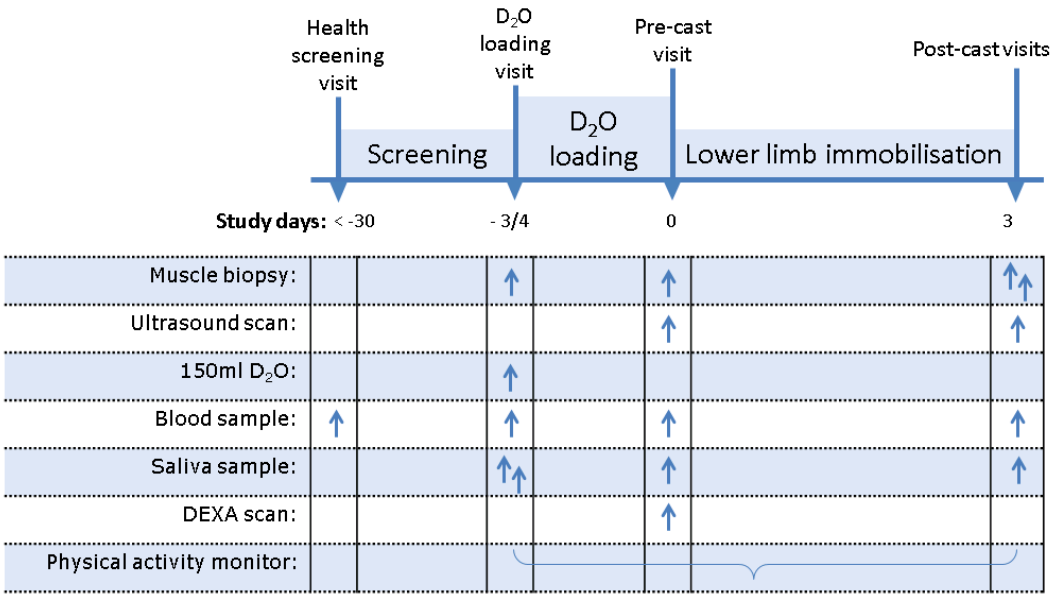

Figure 2.1. A schematic overview of the study protocol.

A schematic overview of the study is depicted in Fig. 2.1. Following recruitment into the study, participants were studied over a 3 to 4 day pre-immobilisation period during which they were asked to perform their normal habitual activities and over 3 days of unilateral lower limb immobilisation. On the first day of study, subjects arrived in the morning ~09:00 h following an overnight fast. A unilateral, medial gastrocnemius muscle biopsy (described in detailed in section 2.3) was performed using ultrasound guidance and a minimally invasive percutaneous micro-needle technique under sterile conditions, with a maximum of 6 passes through the same entry point (Hayot et al., 2005). Following removal from the limb, muscle tissue was rapidly dissected free of fat and connective tissue, dabbed down using a non-woven gauze to absorb any blood and then rapidly frozen and stored in liquid nitrogen until further analysis. Following the muscle biopsy, a venous blood sample was collected from

the superficial hand or a forearm vein into a lithium heparin tube and immediately centrifuged at 4,400 g at 4oC, plasma was aliquoted and frozen at –80°C until analysis. A saliva sample was collected into a gallipot, immediately centrifuged at 13,500 rpm at 4°C, and the saliva supernatant was aliquoted and frozen at –80°C until analysis. Participants then consumed a 150 ml bolus of D2O (70 atom%; Sigma-Aldrich, Poole, UK) in two portions of 75ml, 45 minutes apart, and a saliva sample was obtained 2 hours after consumption of the second D2O bolus.

For reasons highlighted in the introduction, this study was designed to take into consideration participant's physical activity levels pre-immobilisation whilst 'free living' and during the 3 days of unilateral lower limb immobilisation (described in detailed in section 2.4). This was achieved using activity monitors (Actiheart, CamMtech Ltd, Cambridge, UK). During this period of 'free living' participants carried on with their routine daily living, after which they returned to the laboratory following an overnight fast. Volunteers then underwent assessment of body composition by dual energy X-ray absorptiometry (DXA; Lunar Prodigy II, GE Medical Systems) and bilateral medial gastrocnemius muscle architecture (muscle volume, muscle thickness, muscle pennation angle and muscle fascicle length) using ultrasonography (described in detailed in section 2.2; Mylab 70; Esaote Biomedica, Italy), after which a second medial gastrocnemius muscle biopsy was obtained from the same limb as the first biopsy. Following this, a venous blood sample and saliva sample

were collected and centrifuged with the supernatant fractions collected and frozen.

Immobilisation

We chose to study the impact of immobilisation in the medial gastrocnemius muscle as previous studies have shown it to be susceptible to disuse atrophy (Belavy et al., 2017; de Boer et al., 2008). To do this, we utilised a unilateral, below knee leg cast model (Fig. 2.2). The limb to be immobilised was chosen at random (using a coin toss on the first study visit day). A Stockinette and 2 layers of Webril were applied over the limb to be immobilised and with the fibre glass cast roll immersed into water and applied to the limb from approximately the metatarsophalangeal joints to below the knee with the fibre glass roll hardening through an exothermic reaction. Participants were informed on how to use elbow crutches and to non-weight bear on the immobilised limb whilst resting at home. A taxi service was provided for participants to get home and be collected for the final study visit

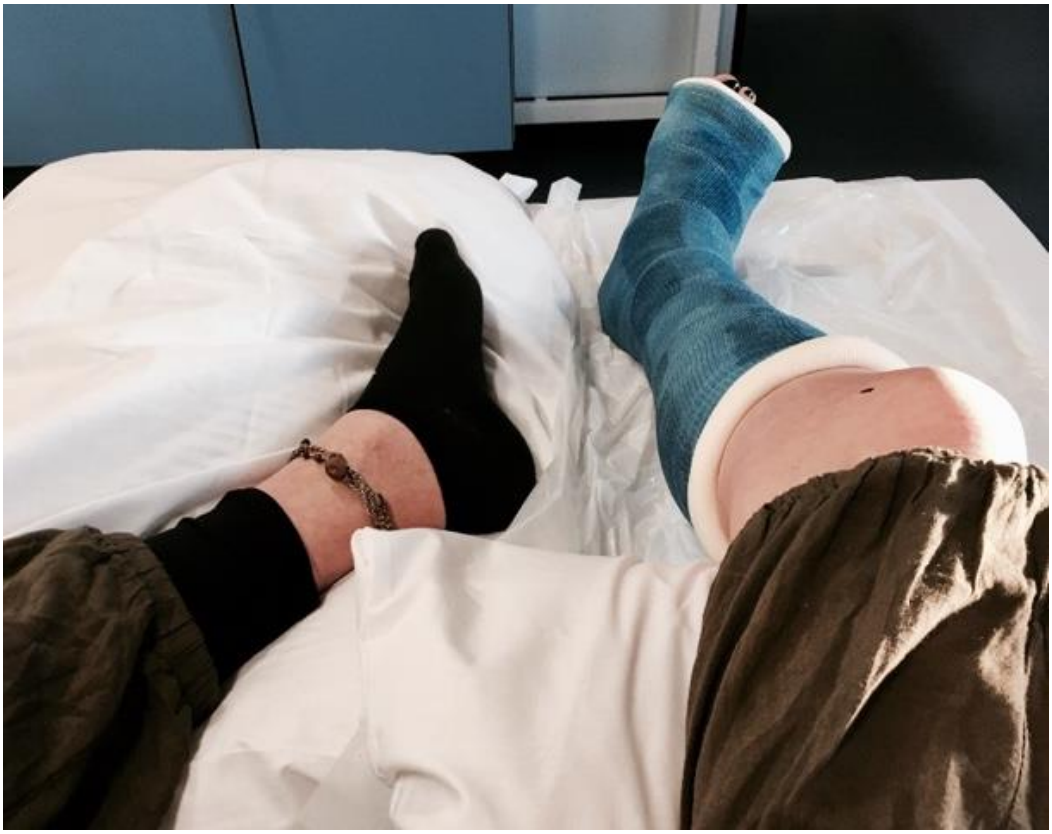

Figure 2.2. An image of a study participant with a below knee leg cast applied.

A research team member was in daily contact with participants throughout the immobilisation period. Participants returned to the laboratory the morning after 3 days of immobilisation following an overnight fast. Following assessment of medial gastrocnemius muscle architecture in both limbs by ultrasonography (Mylab 70; Esaote Biomedica, Italy), participants underwent a biopsy of the medial gastrocnemius muscle in both limbs, with a maximum of 6 passes through the same entry point. Following this, venous blood and saliva samples were collected, centrifuged and the supernatant fraction was frozen at -70oC until analysis.

2.2 Determination of medial gastrocnemius muscle thickness, muscle volume, muscle fascicle length and muscle pennation angle using Doppler ultrasound

In the experiments described in Chapters 3, 4 and 6, Doppler ultrasound scans were performed to measure medial gastrocnemius muscle thickness, muscle volume, muscle pennation angle, and muscle fascicle length on both legs before, and subsequently after 3 days of unilateral lower limb immobilisation. The participant lay in the supine position with the knee of the scanned leg flexed outwards exposing the medial gastrocnemius muscle *(Figure 2.1)*. The ultrasound probe (7.5 MHz linear-array probe) was connected to a Mylab 25 (Esaote Biomedica, Italy) Doppler ultrasound machine and the participants were asked to keep their legs relaxed during the scans. The scanning probe was placed on the middle plane of the medial gastrocnemius muscle with transmission gel applied to the limb to improve acoustic coupling and minimal pressure applied to the limb to prevent muscle compression. The proximal and distal regions of the medial gastrocnemius muscles were identified and referenced as the myotendinous and osteotendinous junctions. The reference points were marked on the skin and the distance between reference points calculated as the medial gastrocnemius muscle length. The midpoint between the proximal and distal region were

marked and the lateral and medial muscle border were scanned along the midpoint axis and marked *(Figure 2.3).*

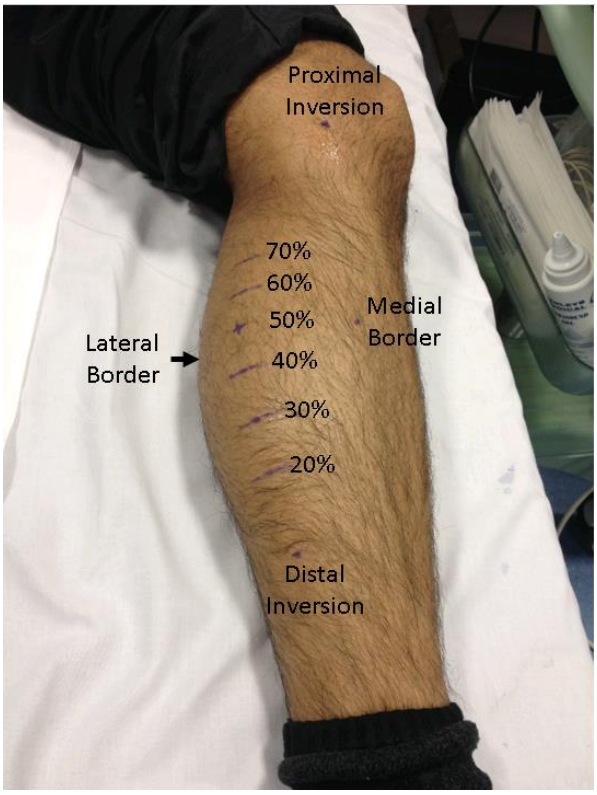

Figure 2.3. *Ultrasound reference point marking on the medial gastrocnemius muscle.*

The scanning probe was placed longitudinally to the medial gastrocnemius muscle along the middle plain axis of the medial gastrocnemius muscle (Figure 2.3; 50% marking). Three digitised images were taken at the medial gastrocnemius midpoint to measure muscle thickness, muscle fascicle length and muscle pennation angle using image analysis software. Image analysis was performed using ImageJ (NIH Image, National Institute of Health, Bethesda, USA). Muscle thickness was calculated as the average distance between the superficial and deep aponeuroses in the distal and proximal regions of the image

(Figure 2.4) and an average of the 3 readings was taken with the image being scaled using the ultrasound reference markings.

Muscle pennation angle was measured as the intersection between fascicles and the aponeurosis of the muscle (Figure 2.4) and an average of the 3 readings taken**.** Muscle fascicle length was measured as the length between a muscle fibre between the superficial and deep aponeurosis (Figure 2.4) and an average of the 3 readings taken.

Ultrasound determined muscle architectural measures have been validated against MRI and CT scans. Ultrasound determined rectus femoris muscle thickness has been validated against CT derived rectus femoris muscle thickness and shown to have a correlation coefficient of variance (CV) of 0.92% with a reliability correlation coefficient of 0.97% in test-retest (Thomaes et al., 2012). The CV values for ultrasound determined medial gastrocnemius muscle thickness from our method was 1.67%. Ultrasound determined muscle pennation have been validated in young children and shown to have a CV value of 4.1% and the CV values for ultrasound determined muscle pennation angle from our method were similar at 2.0%. Ultrasound determined muscle fascicle length has been validated in young humans and shown to have a CV of 1.0%. The CV values for ultrasound determined muscle fascicle length from our method was 2.0%. Therefore, the use of ultrasound is a valid and reliable method to assess muscle thickness, muscle pennation angle and fascial length

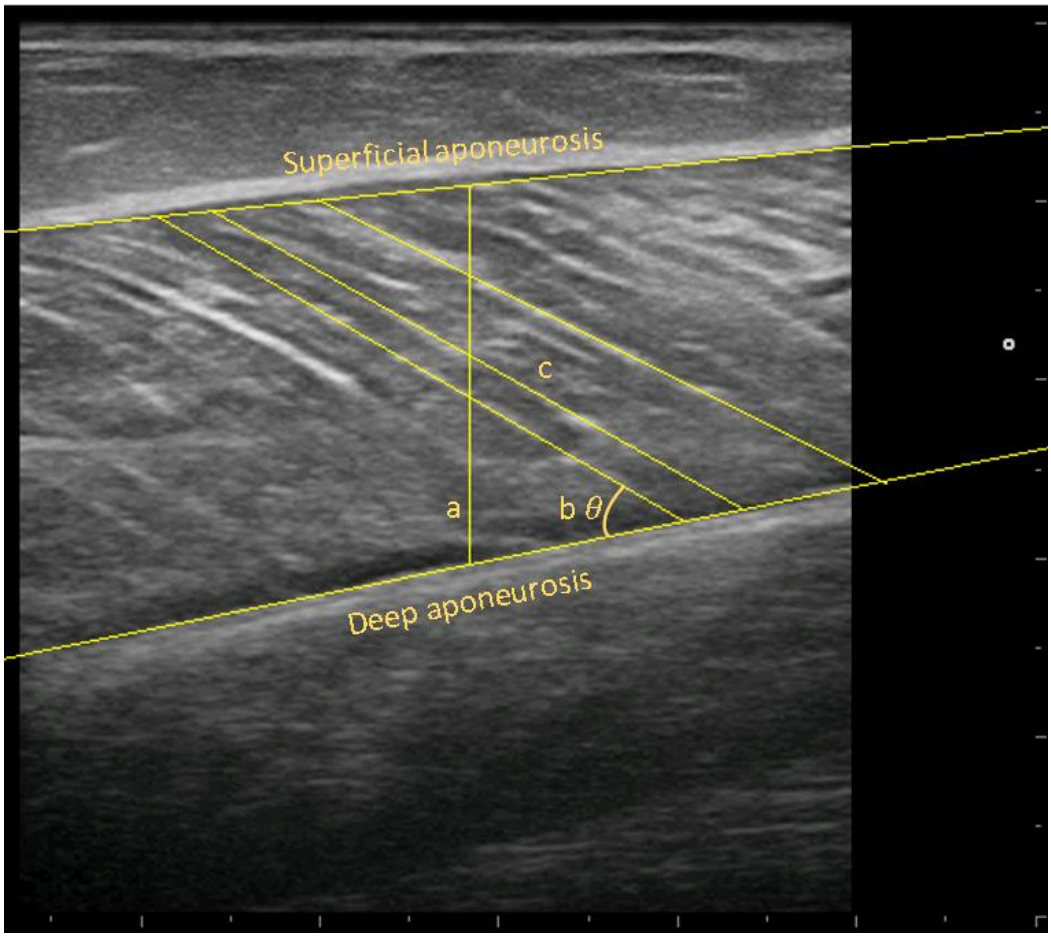

Figure 2.4. *An ultrasound image of the medial gastrocnemius muscle at midpoint (50% of length).* ***a:*** *The distance between the superficial and deep aponeurosis is calculated as muscle thickness.* ***b:*** *The angle of the muscle fibres is calculated as the pennation angle.* ***c:*** *The length between an observed muscle fibre between the superficial and deep aponeurosis. The image has been analysed using ImageJ software analysis (NIH Image, National Institute of Health, Bethesda, USA) and ultrasound guidance used as a reference scale.*

Eight axial-plane scans using the ultrasound machine in its panoramic view were taken along the length of the medial gastrocnemius muscle at

0% (myotendinous), 20%, 30%, 40%, 50%, 60% and 70% and 100% (osteotendinous) length of the medial gastrocnemius muscle to determine muscle CSA. The panoramic images were automatically digitised by the ultrasound machine and analysed using ImageJ software. Using ImageJ software, the epimysium of medial gastrocnemius muscle was plotted and the anatomical cross-sectional area (aCSA) calculated using the ultrasound reference guideline (Figure 2.5).

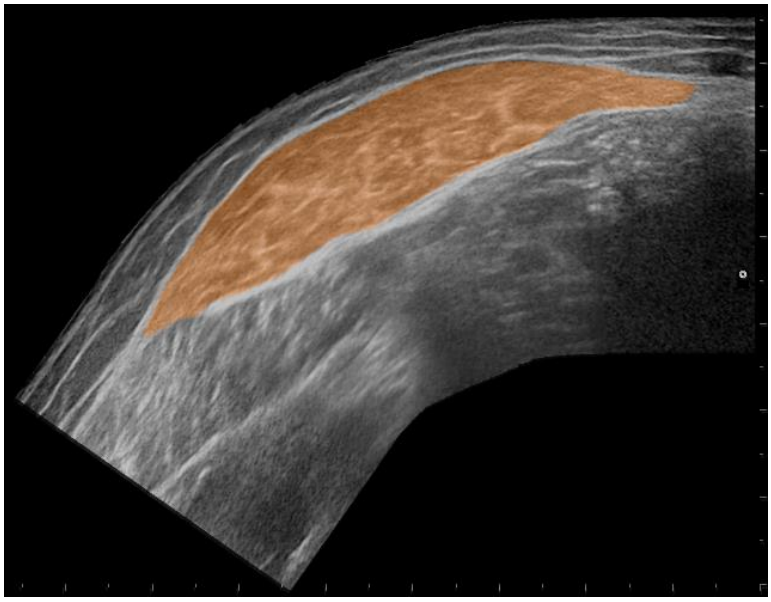

Figure 2.5. *An ultrasound image of the medial gastrocnemius muscle taken horizontally using panoramic view at 30% length. Using ImageJ software analysis (NIH Image, National Institute of Health, Bethesda, USA) the anatomical cross-sectional area (aCSA) has been highligted and calculated using ImageJ software and ultrasound guidance as a reference scale.*

Ultrasound scans were performed in triplicates and the average values of muscle cross sectional area used in the calculation of muscle volume using the trapezoid equation.

$$V = \frac{1}{3}\, x\, h\, \left(a + \sqrt{(ab) + b}\right)$$

where h is the distance between the two scans and a and b are the aCSAs of the muscle in the two scans. The entire medial gastrocnemius muscle volume was calculated by summing up the inter-scan muscular portions. The same operator performed all ultrasound scans for participants on both visits. Furthermore, Arbeille and colleagues validated the use of ultrasonography against MRI to measure changes in vastus intermedius and the vastus medialis muscle volume during a 60d bed rest study in young female volunteers and calculated the correlation coefficient of muscle volume change determined by MRI and ultrasonography to be 0.78% (Arbeille et al., 2009). Therefore, ultrasonography is a valid method for the assessment of muscle volume.

2.3 **Muscle sampling**

In the experiments described in Chapters 3, 4 and 6, muscle biopsies were obtained from the medial gastrocnemius muscle using the minimally invasive percutaneous micro-needle technique as described by (Hayot et al., 2005) with a few adaptions. Previous applications of this method have biopsied the vastus lateralis which provides a risk-averse anatomical region for study. However, studies have demonstrated the medial gastrocnemius muscle to be most affected by skeletal muscle disuse during bed-rest (Belavy et al., 2017) and following lower limb injury (Psatha et al., 2012).

The medial gastrocnemius is smaller in size when compared to the vastus lateralis. Taking these points into consideration an ultrasound guided approach was taken to minimise the risk of the micro-needle cutting through an intramuscular vein or passing through into the Soleus muscle. A safe biopsy region was considered when no veins where seen passing through the muscle on the ultrasound view. The area was cleaned using iodine solution and a surgical drape was applied around the limb to be biopsied. Local anaesthesia was applied to the skin and deeper into the surrounding tissue (Lidocaine; 1 %). Following local anaesthesia, a small incision (<1 cm) was made through the fascia using a sterile scalpel blade. The biopsy was performed using a spring-loaded Bard® Magnum® reusable core biopsy system (MG1522) with a 12-gauge needle (MN1210) (Figure 2.6).

Figure 2.6. *The Bard® Magnum® core biopsy system (MG1522) with a 12 gauge needle (MN1210) used to perform muscle biopsies.*

A maximum of 6 passes were performed through the same incision point to yield 30-60mg (wet weight) of muscle tissue which was dapped down in a non-woven gauze to absorb any blood and then rapidly frozen in liquid nitrogen.

In the experiment described in Chapter 5, muscle biopsies were performed on the mid-region of the left vastus lateralis muscle. Local anaesthesia (lidocaine (2 ml, 2%) was applied to the skin and surrounding tissue before a small incision was made to the fascia of the muscle allowing the passage of a purpose-built rongeur (Zepf Instruments, Tuttlingen, Germany) to harvest muscle. The muscle sample was frozen in isopentane cooled with liquid nitrogen.

2.4 **Physical activity monitoring**

In chapters 3, 4 and 6, physical activity levels were determined using tri-axial accelerometery (ActiHeart, CamNtech, Cambridge, UK). The ActiHeart is a small chest worn device which records heart rate and activity levels through an inbuilt 3-axis accelerometer. Participant anthropomorphic measurements (gender, height and weight) were entered into the device and data were transformed to an estimated energy expenditure using the branched equation parameters of "Group Cal JAP2007" which is incorporated into the Actiheart software. The ActiHeart was setup to record in long term mode with and a sample rate of 30 seconds to measure and record heart rate and activity. Participants were asked to wear a chest belt which allowed the ActiHeart to be attached (Figure 2.7) to record physical activity throughout the duration of the study (6-7 days). The ActiHeart has previously been used in various research settings (Brage et al., 2004; Takken et al., 2010). The ActiHeart has been validated against Polar measurements of heart rate and an ECG in resting and during physical activity (treadmill running) and shown to be a reliable method to access heart rate and physical activity (Brage et al., 2005).

Figure 2.7. *An ActiHeart chest belt with an Actiheart monitor attached to measure heart rate and physical activity.*

2.5 Myofibrillar protein fractional synthesis rates

In chapters 3, 4 and 6, myofibrillar protein FSR were determined through the use of a recently validated deuterated "heavy" water approach (Wilkinson et al., 2014).

2.6 Body water deuterium enrichment

In chapter 3, 4 and 6, saliva samples were defrosted and aliquots of 100μl were pipetted into inverted auto-sampler vials which were then placed on a heating block at 90°C for 2 hours. The vials were then quickly cooled by placing on ice for 10 minutes and the water distillate transferred to a fresh vial. Saliva sample enrichment levels were determined through direct liquid injection of saliva samples into a high-temperature conversion elemental analyzer (TC/EA; Thermo Finnigan, Thermo Scientific, Hemel Hempstead, UK) which was connected to an isotope ratio mass spectrometer (IRMS; Delta V Advantage, Thermo). Saliva samples were passed through the TC/EA were the samples were converted to H_2 gas and passed through to the IRMS where the ^2H /^1H ratio was determined. Total body water enrichment was calculated using the following equation.

$$Body\ water\ enrichment\ (\%) = \frac{100\ x\ AR\ x\ (\delta^2H\ x\ 0.01 + 1)}{1 + AR\ (\delta^2H\ x\ 0.001 + 1)}$$

Where AR represents the absolute ratio constant for deuterium based on the Vienna Standard Mean Ocean Water (VSMOW) standard and equates to 0.00015595 and δ^2H is the deuterium isotopic enrichment which is normalised by subtracting the baseline pre-enrichment sample. Samples

were run in triplicates and further validation was provided by running a standard curve of known D_2O enrichment alongside the saliva samples for calculation of body water deuterium enrichment.

2.7 Myofibrillar protein and collagen fractional synthetic rates

Approximately 20-25mg of frozen wet muscle obtained from the medial gastrocnemius muscle biopsies was used to determine muscle protein FSR using GC-pyrolysis mass spectrometry. Ice-cold homogenisation buffer [50 mM Tris·HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerophosphate disodium salt, 50 mM NaF, and 1 mM activated Na3VO4)], all of which were obtained from Sigma-Aldrich, and a complete protease inhibitor cocktail tablet (Roche, West Sussex, UK) were dissolved and pipetted into an Eppendorf tube at 10 µl/µg of muscle tissue, which was then homogenised using scissors. The resulting homogenate was then placed on a Vibrax shaker at 1000-1500rpm for 10 minutes at room temperature. Following mixing, the homogenate was centrifuged at 11,000*g* for 15 min at 4°C forming a muscle sarcoplasmic supernatant and myofibrillar/collagen pellet. The muscle sarcoplasmic supernatant was transferred into a clean Eppendorf tube and used for protein expression analysis **(See section 2.8).**

The myofibrillar and collagen pellet was washed in homogenisation buffer (same as previous step) and centrifuged at 11,000*g* for 15 min at 4°C with the supernatant discarded. NaOH (750µl, 0.3M) was added to the myofibrillar and collagen pellet in a warm water bath at 37°C for 30 minutes to solubilise the myofibrillar fraction and separate from the insoluble collagen fraction. Following fraction separation, the samples were centrifuged at 13,000rpm for 10 min at 4°C and the solubilised

myofibrillar fraction were transferred to a boiling tube. NaOH (750µl, 0.3M) was added to the insoluble collagen fraction and centrifuged at 13,000rpm for 10 min at 4°C with supernatant added to the previous myofibrillar fraction. The myofibrillar fraction protein was precipitated with perchloric acid (1ml, 1M) for 10 min at 4°C and subsequently centrifuged at 3000g for 20 min at 4°C with the supernatant discarded. Both the collagen pellet and myofibrillar pellet were washed twice in ethanol (75%, 1.5ml) and centrifuged at 3200rpm with the supernatant discarded. Both the collagen and myofibrillar pellets were re-suspended and hydrolysed in HCl (0.1M, 1ml) in Dowex H^+ resin slurry (1ml) overnight. Protein-bound AAs hydrolysates from myofibrillar and collagen fractions were released by eluting from the Dowex H+ resin with NH_4OH (2M, 4ml) and evaporated to dryness in a Techne heating block with flow of nitrogen gas. Distilled water (60 µl) was added to the dried myofibrillar and collagen samples and briefly vortexed to re-suspend the pellet. Following vortex, methanol (32 µl), pyridine (10 µl) and methylchloroformate (10 µl) were added to the samples and vortexed for 30 seconds and allowed to react for 5 minutes at room temperature to form methyl carbonyl methyl ester (MCME) amino acids. The MCME amino acids were extracted into chloroform (100 µl) and $NaHCO_3$ (100ul, 0.001M) and the aqueous layer removed by pipetting and further removed with the addition of a molecular sieve. Newly formed muscle and collagen protein were determined through incorporation of deuterium into protein bound alanine which was detected by gas chromatography-pyrolysis-isotope ratio mass spectrometry (Delta V Advantage; Thermo

Scientific). The FSR of myofibrillar protein and collagen were determined using the following equation.

$$Fractional\ synthetic\ rate\ (\%) = -\ln\left(\frac{-1\left[\frac{(APE_{ala})}{(APE_p)}\right]}{t}\right)$$

Where APE_{ala} equals the enrichment of deuterium-labelled alanine into the metabolic compartment being analysed (i.e. myofibrillar or collagen) and APE_P equals the mean enrichment of deuterium into the body's water pool over the specific time period. APE_P was adjusted taking into account the mean number of deuterium moieties incorporated per alanine (3.7). t is the time (hours) between biopsies. Samples were run in triplicates and Further validation was provided by running a standard curve of known L-Alanine-2,3,3,3-d_4 enrichment alongside the samples (Figure 2.8).

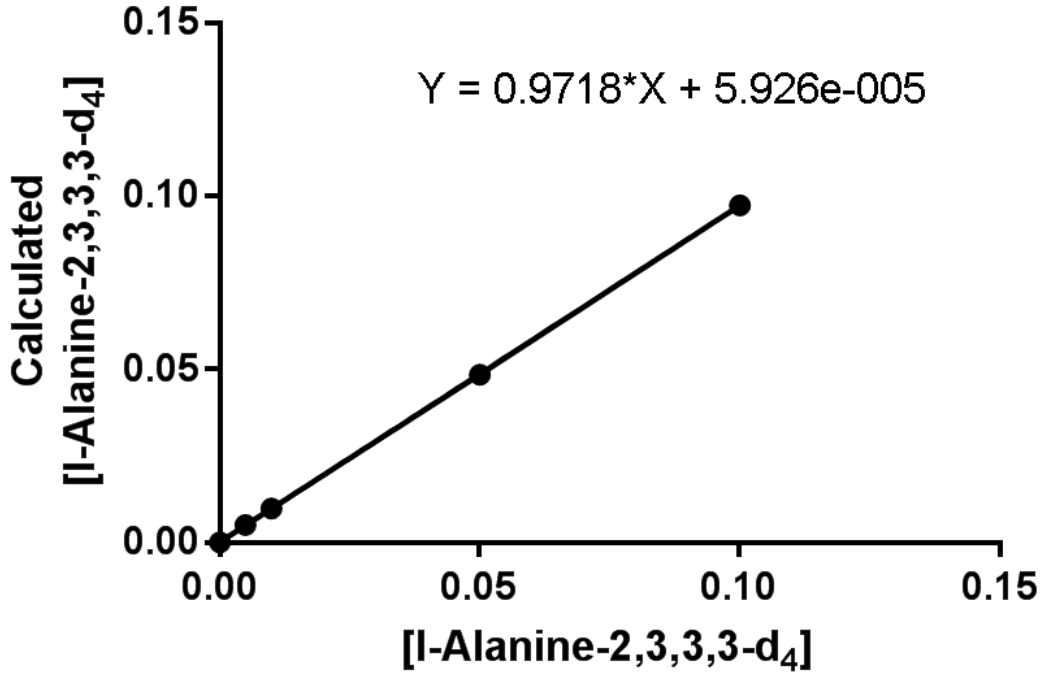

Figure 2.8. *A standard curve for a known concentration of l-Alanine-2,3,3,3-d_4 and calculated (mole %excess) concentration of l-Alanine-2,3,3,3-d_4*

2.8 **Muscle protein expression (Western blotting)**

In chapters 3, 4 and 6, western blotting was used to quantify target protein expression. Western blotting, also known as immunoblotting, is a well-established and routinely used analytical technique used for the detection of specific proteins and phosphorylation status of proteins in a sample of tissue extract. The principles of Western blotting relay on forming a protein to antibody complex through specific binding of a primary antibody to a membrane-bound target protein separated by its molecular weight. Detection of the primary antibody is generally achieved through a primary antibody specific secondary antibody.

Western blotting was performed on sarcoplasmic protein extracts from the fractional muscle protein and collagen synthesis extraction protocol **(See section 2.7)**. Protein concentration was quantified using spectrophotometry (NanoDrop™ Lite) and samples were prepared that equated to 1.5 μg/μl using homogeniser buffer [50 mM Tris·HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerophosphate disodium salt, 50 mM NaF, and 1 mM activated Na3VO4)] all of which were obtained from Sigma-Aldrich, a complete protease inhibitor cocktail tablet (Roche, West Sussex, UK) and 3x Leammli SDS sample buffer (1M Tris-Cl pH 6.8, 20% SDS, Glycerol (100%), B-mercaptoethanol 1.6 ml and Bromophenol blue 0.006g). Samples were heated to 95°C on a heating block for 5 minutes to denature protein to their primary structure. Precast Criterion XT Bis–Tris–12% SDS-PAGE gels (Bio-Rad) were loaded using 15 μg of proteins following which electrophoresis occurred at 200 volts for 1 hour. Following electrophoresis, samples were transferred onto polyvinylidene

difluoride membranes (PVDF) for 45 minutes at 100 volts. Following transfer, the PVDF membrane was blocked in 2.5% low-fat milk (diluted in Tris-buffered saline and 0.1% Tween-20 (TBS-T)) for 1 hour at room temperature whilst on a rocker. Following blocking, the membrane was incubated in the presence of the primary antibody overnight whilst in a rocker at 4°C. The following morning, the membrane was washed three times in 1xTBST for 5 minutes each with gentle agitation. The PVDF membrane was then incubated in 2.5% low-fat milk which was dissolved in 1XTBST containing HRP-conjugated secondary antibody (which was primary antibody specific). Following incubation with the secondary antibody, the PVDF membrane was washed three times in 1xTBST for 5 minutes each. Protein expression was detected using chemiluminescent HRP Substrate using and analysed using ChemiDoc XRS Systems. Following detection, protein loading was quantified using coomassie.

2.9 **Muscle mRNA expression measurements**

In chapter 5 targeted mRNA expression was quantified using real-time polymerase chain reaction (PCR). This is one of the most widely used techniques in molecular biology for mRNA expression quantification because of its high sensitivity and higher sample throughput (when compared to traditional PCR). Building on the principle of PCR, real-time PCR combines the amplification and detection process thus allowing for a `real-time` determination of gene amplification after each thermal cycle. Target RNA is isolated and reversed transcribed into a cDNA template. The newly formed cDNA template subsequently binds to its complimentary DNA oligonucleotides in a 5` to 3` direction. The cDNA is heated to allow cDNA strand separation and cooled to allow annealing of probes and primers to the cDNA template in the presence of DNA polymerase and deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, dTTP). Subsequent heating and cooling steps thus allow for the potential exponential amplification of the target amplicon (Figure 2.9).

Quantification of DNA amplification after each thermal cycle is determined through a variety of different probes with different chemistries. We used Taqman® Gene Expression Array Cards which contained Taqman® probes. A custom designed low-density RT-PCR microfluidic array card approach was utilised to measure the relative abundance of multiple mRNA genes from the samples. In designing the microfluidic array card, candidate genes were selected based upon a literature search with the most significantly changed gene during skeletal muscle disuse studies being selected. In addition, previous data

generated from our laboratory and by Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA, USA) was taken into consideration. The final designed low-density RT-PCR microfluidic array card (384 format; Applied Biosystems, Foster City, CA, USA) consisted of 96 unique gene targets **(Appendix 1.2)** and was used to measure the relative mRNA abundance from multiple pathways involved in muscle mass regulation, glucose, protein and lipid metabolism and musculoskeletal architectural structure.

The Taqman probes comprises of a short oligonucleotide, 5' reporter dye (6-carboxyl-fluorescein; FAM™) and a 3' minor groove binder (MGB) non-fluorescent quencher (NFQ). The 5` reporter and 3` quencher is covalently attached to the short oligonucleotide. Excitation of the fluorescent 5`reporter dye in the Taqman probe is prevented in the presence of a 3`quencher probe when in close proximity as the excitation energy is transferred to 3` quencher probe via Fluorescent Resonance Energy Transfer (FRET). However, following thermal separation of cDNA, the Taqman® probe containing the 5` reporter and 3` quencher anneals to the cDNA strand. During polymerization, DNA polymerase cleaves the attached 5` reporter and 3` quencher through its exonuclease activity liberates the reporter and quencher probe from the oligonucleotide. The newly liberated reporter probe is able to be spectrophotometrically detected in response to excitation (via a light source) after each thermal cycle. This allows for the determination of DNA amplification after each cycle. During the early thermal cycles of rt-PCR, the intensity of the reporter dye is below background fluorescence levels such that the end

products cannot be quantified. Once the intensity of the reporter dye is above the background fluorescence (Ct threshold), the quantification of end products can occur till the plateau phase. When a particular sequence of DNA is highly expressed, amplification of the sequence is observed in earlier thermal cycles.

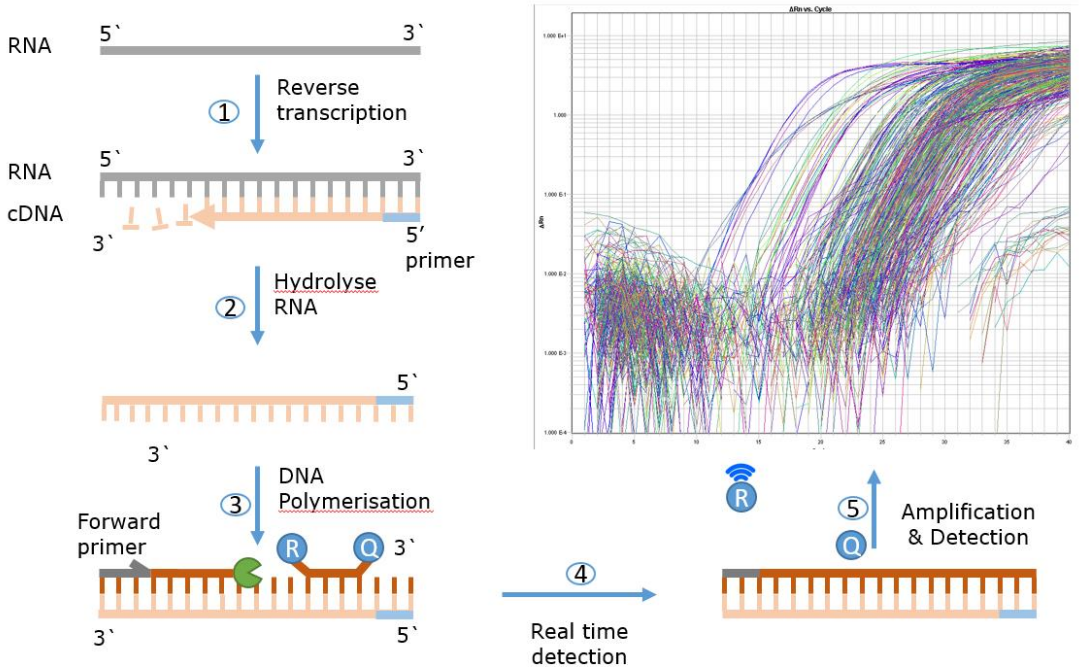

Figure 2.9. *A schematic overview of rt-PCR. Step 1: Target RNA is isolated and reversed transcribed into a cDNA template using reverse transcriptase. Step 2: RNA is hydrolysed using RNAse. Step 3: The newly formed cDNA template subsequently binds to its complimentary DNA oligonucleotides in a 5` to 3` direction in the presence of DNA polymerase and deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, dTTP) thus liberating the reporter dye. Step 4: After each thermal cycle the intensity of the reporter dye increases thus allowing for real time product quantification.*

Relative mRNA expression levels were quantified using the $2^{-\Delta\Delta CT}$ method with the participant's baseline sample as the calibrator and HMBS as the housekeeping gene (endogenous control) using the following formula.

$$2^{\Delta\Delta Ct} = \Delta Ct_{\text{target gene baseline}} - \Delta Ct_{\text{target gene post-intervention}}$$

$$\text{whereby: } \Delta Ct = Ct_{\text{target gene}} - Ct_{\text{housekeeping gene}}$$

The comparative Ct method quantifies relative mRNA expression in relation to the housekeeping gene. Therefore, selecting an appropriate housekeeping which does not alter with the intervention is vital for interpreting gene expression data. Housekeeping genes which are normally selected for studies are those which are universally expressed and transcribed at a relative constant rate e.g. (GAPDH (Glyceraldehyde 3-phosphate dehydrogenase), 18s and HMBS (hydroxymethylbilane synthase)). For this study we used HMBS which has shown to not change during muscle wasting conditions (Murton et al., 2009).

2.10 **Muscle RNA extraction**

Total RNA was extracted from approximately 20-25mg of frozen wet muscle by homogenising for ~30 seconds using a polytron homogenizer in TRIzol® (1ml, Invitrogen, Paisley, UK) and glycogen (10μg/μl; Sigma-Aldrich, Dorset, UK). The newly formed homogenates were incubated at room temperature for 5 minutes. Following incubation Chlorophorm (200μl) was added to the homogenates and briefly vortexed before additional incubation for 3 minutes at room temperature. Following incubation, the homogenates were centrifuged for 15 minutes at 12,000g for 15 minutes at 4°C. The newly centrifuged samples contained 3 layers, with the clear top aqueous layer containing RNA being transferred to a new Eppendorf tube. Isolated RNA was precipitated using iso-propanol (500 μl, Sigma-Aldrich, Dorset, UK) and incubated at 4°C for 20 minutes. Following incubation, the samples were centrifuged at 12,000g for 15 min @ 4°C to pellet the isolated RNA. The supernatant was removed and the isolated RNA pellet was air dried. The dried RNA pellet was washed in 1 ml of 75% Ethanol and briefly vortexed and subsequently centrifuged at 7,500 rpm for 10 minutes at 4°C. Following centrifugation, the supernatant was removed and the RNA pellet was air dried and subsequently dissolved in 50μl of RN-ase free water.

RNA concentration was determined spectrophotometrically using a NanoDrop® (NanoDrop® Technologies Inc, Wilmingtion, USA) by measuring nucleic acid absorbance at 260nm and 280nm and calculated using Beer-Lambert law (which predicts a linear change in absorbance

with concentration). RNA purity was determined by the 260:280 ratios, with a value ranging between 1.8-2.0 considered highly purified RNA.

2.11 **cDNA synthesis**

Following spectrophotometric quantification, 1μg of total RNA was incubated in 2μl random hexamers (Promega C1181) and 1μl dNTPs (Promega U1240) with a final volume made of 13μl using RNAse free water. The samples were heated at 65°C for 5 minutes in a PCR thermocycler to denature RNA secondary structure and immediately placed on ice. First strand cDNA was created from the RNA with the addition of 4 μl Enzyme buffer, 1 μl DTT, 1 μl RN-ase inhibitor (Promega N2511) and 1μl SuperScript™ III reverse transcriptase (Invitrogen Ltd, Paisley, UK) and subsequently processed in a PCR thermocycler with heating cycles set at 25°C for 5 minutes then 50°C for 60 minutes and finally 70°C for 15 minutes. Samples were then stored at -80°c until analysis.

2.12 mRNA expression quantification

mRNA expression was quantified using an ABI PRISM 7900T sequence detection system and SDS 2.1 software (Applied Biosystems, Foster City, CA, USA). Data was analysed using SDS RQ manager which automatically determined threshold cycle CT for each gene. Relative mRNA abundance was calculated using the ΔΔCT method with the participant's baseline sample as the calibrator and HMBS as the endogenous control.

2.13 Statistical analysis

Data are presented throughout the thesis as mean ± SEM, unless otherwise stated. For normally distributed of data, within-group comparison of multiple time points was be analysed using one-way analysis of variance (ANOVA) with the Fisher's least significant difference (LSD) post hoc test applied to determine any significant differences between treatments. When significant main effects were detected, data were further analysed using Student's t tests and a Bonferroni correction. Two-way ANOVA will be used determine across group significance (the impact of age and obesity). For non-normally distributed data, within-group comparison of more than two time points will be performed using Friedman's non-parametric analysis, and comparison of two independent groups will performed using Mann–Whitney's non-parametric test. Significance was set at $p<0.05$. Analysis was performed using GraphPad Prism 7 (GraphPad Software Inc., California, USA) and SPSS (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp)

Chapter 3

3 The effect of three-day unilateral lower limb immobilisation on chronic muscle protein synthesis, muscle thickness and volume and the expression of protein targets implicated in muscle mass regulation in healthy, young volunteers

3.1 Introduction

Physical inactivity and sedentary behaviour is the fourth leading cause of global mortality (Lee et al., 2012) and a major driver of ill health in today's society. The decline in muscle contractile activity associated with physical inactivity leads to muscle weakness and reduced functional capacity, muscle mass loss, insulin resistance and ultimately chronic non-communicable disease progression. Therefore, musculoskeletal deterioration associated with physical inactivity is a major public health problem and embodies a vital area for scientific investigation.

Various clinical models exist to study the negative impact of physical inactivity on musculoskeletal health ranging from reduced step count to complete bed rest, with the latter inducing whole body muscle wasting. Unilateral lower limb immobilisation can be viewed as an intermediate approach and induces local muscle atrophy as well as allowing the contralateral limb to act as a control. It is also of high clinical relevance and more likely to be undertaken by experimental volunteers than sustained bed-rest. Irrespective of this point, two weeks of bed rest in healthy, young volunteers induced an ~6% loss of quadriceps muscle

volume measured using MRI (Pisot et al., 2016), whilst two weeks of unilateral full leg cast immobilisation in healthy, young volunteers induced a 5% loss of knee-to-hip lean tissue mass determined using DXA (Jones et al., 2004), which demonstrates good comparability.

Five weeks of horizontal bed rest in young males results in a decrease in ultrasonography determined muscle thickness of ~12% in the gastrocnemius muscle and 8% in the vastus lateralis muscle (de Boer et al., 2008). Furthermore, a short period of 5 days in a unilateral full leg cast young males results in a decrease in quadriceps muscle volume of ~4% when measured using MRI (Wall et al., 2013). Jones and colleagues demonstrated 2 weeks of unilateral full leg immobilisation in young volunteers results in a reduction in quadriceps lean mass quantified by DXA of ~5% (Jones et al., 2004). De Boer and colleagues demonstrated following 14 days of unilateral lower limb suspension, MRI determined quadriceps muscle cross sectional area (CSA) had decreased by 5.2% (i.e. at −0.37 ± 0.05% per day) and at 23 days by 10.0% (i.e. −0.51 ± 0.21% per day). Therefore, muscle unloading leads to a decline in indices of muscle mass which are greater at the onset of muscle unloading. Furthermore, Demangel and colleagues (2017) demonstrated that 3 days of muscle unloading in healthy adult subjects was sufficient to significantly decrease muscle CSA (2.4%), tone and force, and to induce changes in function relating to a weakness in aerobic metabolism and muscle fibre denervation. This study therefore focused on a 3 day period to quantify changes in muscle architecture, protein synthesis and

anabolic signalling in both non-immobilised and immobilised limbs of healthy, normal-weight, young volunteers.

One complication with this aim is that the magnitude of muscle atrophy during immobilisation appears to vary across muscle groups, even in the weight bearing lower limbs. For example, De Boer and colleagues using ultrasonography showed medial gastrocnemius and vastus lateralis muscle underwent greater atrophy following 5 weeks of bed-rest in young volunteers when compared to the tibialis anterior over the same time period (-12.2% vs. no change) (de Boer et al., 2008). Similarly, following 90 days of bed rest the medial gastrocnemius muscle was found to have undergone a 28% decline in muscle volume in comparison to a decline of 15% in the tibialis anterior in the same limb using MRI (Belavy et al., 2017). Therefore, relating gross limb muscle volume changes to protein turnover and molecular measurements in muscle biopsy samples from a specific muscle must be viewed with at least some caution. This and subsequent chapters will therefore focus on muscle architecture and biopsy analyses being performed solely on the medial gastrocnemius muscle, which has been demonstrated to be highly affected by immobilisation. It is also acknowledged that age, body fat mass and chronic morbidities may further impact upon the magnitude of muscle mass loss during immobilisation, which will be the subject of **Chapter 4** (aging) and **Chapter 6** (obesity) of this thesis.

In addition to the magnitude of muscle loss varying across muscle groups during immobilisation, individual differences in pre-immobilisation habitual physical activity levels may impact upon the rate of muscle loss

during subsequent disuse. For example, a randomised controlled trial involving untrained, young, healthy volunteers reported that 8 weeks resistance exercise training protected forearm flexor muscle volume (MRI determined) during 21 days of subsequent arm suspension when compared to a control group that had no pre-immobilisation training (Miles et al., 2005). However, given the authors failed to determine muscle volume following 8 weeks resistance exercise training gains in muscle volume during the exercise training were not taken into consideration. The authors did observe muscle volume loss to be less in individuals with relatively lower forearm flexor muscle volume at baseline (Miles et al., 2005), which has also been observed when comparing the response to immobilisation in older vs younger volunteers (Suetta et al., 2009). It seems plausible to suggest therefore that initial muscle mass is a determinant of the magnitude of muscle mass loss during subsequent immobilisation, with the former being dictated by habitual physical activity modulating muscle protein turnover.

Muscle mass is constantly being turned over through the dynamic processes of MPS and MPB. In mature adults, MPS and MPB fluctuate throughout a diurnal cycle but remain in overall balance to maintain muscle mass (Atherton and Smith, 2012). Nevertheless, as outlined above sustained periods of muscle unloading, in the form of reduced step count (Breen et al., 2013), unilateral limb immobilisation (Jones et al., 2004) and bed-rest (Ferrando et al., 1996; LeBlanc et al., 1992; Symons et al., 2009) lead to muscle mass loss. In healthy volunteers this is thought to occur through a decline in post-absorptive myofibrillar protein

FSR (de Boer et al., 2007; Ferrando et al., 1996) and to a greater extent the blunting of post-prandial amino acid induced increases in myofibrillar protein FSR (Glover et al., 2008).

All of the above studies, in which myofibrillar protein FSR was quantified during reduced physical activity or immobilisation, employed traditional stable isotope amino acid tracer methodologies to quantify acute changes (hours) in myofibrillar protein FSR. These studies cannot however provide insight about myofibrillar protein FSR responses over sustained periods of unloading in `free living` conditions. It is assumed however that acute quantification of myofibrillar protein FSR translates to the chronic (days) scenario, which seems unrealistic given the likely non-linear decline in muscle protein FSR during limb immobilisation (de Boer et al., 2007) and the repeated finding that acute feeding induced increases in muscle protein FSR do not translate to chronic muscle mass gains. To date, only one study has documented chronic myofibrillar protein FSR in response to immobilisation, which was reported for middle aged (45-60 years old) men (Mitchell et al., 2018). Rather surprisingly, over 14 days of unilateral lower limb immobilisation, that induced a significant 4.1%reduction in thigh muscle cross-sectional area (peripheral quantitative computed tomography), the average myofibrillar protein FSR (%/day) was reported to be no different from the basal non-immobilised state (Mitchell et al., 2018). The authors quantified myofibrillar protein FSR using the same approach employed in this thesis, i.e. muscle incorporation of heavy water administered orally as 70% aliquots, but did not quantify any temporal response of myofibrillar protein FSR over the

14 days and therefore any fluctuation in myofibrillar protein FSR over this time (particularly at the onset of immobilisation where the decline in myofibrillar protein FSR is thought to be greatest [see above]) was missed.

The AKT/mTOR/p70S6K signalling axis by regulating protein translation initiation is thought to be the dominant pathway controlling MPS in response to exercise and feeding. However, the role of this signalling cascade during human immobilisation remains unclear. Following 10 days of unilateral lower limb suspension in young volunteers, de Boer and colleagues documented a decline in post-absorptive myofibrillar protein FSR of 52%, which occurred in the absence of any change in total abundance or phosphorylation status of AKT, mTOR and p70S6K protein compared to the pre-immobilised state (de Boer et al., 2007). Additionally, although myofibrillar protein synthetic response to the low and high dose protein feeding was seen markedly reduced in healthy, young volunteers following 14 days of unilateral lower limb immobilisation when compared with the contralateral non-immobilised limb (so called anabolic resistance), this had no impact on the phosphorylation status of AKT, mTOR and p70S6K protein when comparing limbs (Glover et al., 2008). Therefore, the molecular mechanisms responsible for the attenuation of myofibrillar protein FSR during immobilisation in humans remains unresolved.

Similarly, not a lot is known about the contribution of MPB during human immobilisation induced muscle atrophy as quantification of MPB is difficult to quantify and requires invasive a-v balance measurements.

However, increased expression of MPB related proteins, namely ubiquitin protein conjugates (Glover et al., 2008) and 3-methylhistidine (Drummond et al., 2012) documented during the early phase (a few days) of muscle immobilisation points towards an early and possibly transient contribution of MPB to muscle atrophy in humans. Nevertheless, elevation of MPB markers over the time course of muscle immobilisation in human volunteers, in the absence of firm evidence of increased MPB based on the application of tracer methodologies, makes the exact contribution of MPB to the aetiology of immobilisation induced muscle atrophy in humans speculative.

Finally, ER stress signalling has been suggested to play a role in regulating MPS and MPB in muscle disuse atrophy (Powers et al., 2012). ATF4 is a transcription factor which promotes the expression of oxidative stress responsive genes, and is thought to inhibit myofibrillar protein FSR by upregulating 4E-BP1 (Ebert et al., 2015), which is a negative regulator of cap-dependent translation. Furthermore, ATF4 downstream signalling targets include Gadd45a, which is thought to stimulate MPB via the MEKK4 pathway (Bullard et al., 2016). In addition, ATF4 has shown to be increased during denervation induced muscle atrophy in rodents (Sacheck et al., 2007), and ATF4 knock-out mice demonstrate a 50% reduction in muscle loss during unilateral lower limb immobilisation (Fox et al., 2014). These animal model data suggest that ATF4 could play an important role in the regulation of muscle mass, a suggestion yet to be corroborated in humans.

3.2 Aims and Hypothesis

The aim of this study to provide novel insight of the impact of unilateral, below knee, limb immobilisation on chronic myofibrillar FSR (%/day) in the medial gastrocnemius muscle of healthy, non-obese, young volunteers over a 3 day period, when compared to both the free living pre-immobilised state and the contralateral non-immobilised limb of the same individual. These FSR measurements were made in conjunction with the quantification of medial gastrocnemius muscle volume and protein expression levels of the putative regulators of anabolic, catabolic and ER stress related pathways. It was hypothesised that immobilisation would reduce chronic myofibrillar FSR in the immobilised limb compared to the basal state and contralateral limb, and would relate to immobilisation induced decrements in medial gastrocnemius muscle volume. Additionally, we hoped to provide novel insight of how changes (if any) in the putative regulators of muscle mass aligned with these muscle protein metabolic and architectural measurements.

3.3 Methods

Participants

Following informed consent, 15 healthy young (7 males and 8 female) volunteers (see **Table 3.1** for anthropometric parameters) were recruited to participate in a 3 day unilateral lower limb immobilisation study. See Chapter 2 section 18 for detailed protocol.

3.4 **Results**

Table. 3.1 Baseline anthropometric characteristics of participants (n=15; 7M, 8F)

Variables	Mean + SD
Age	23 ± 4
Height (m)	1.77 ± 0.11
Weight (kg)	72.10 ± 13.04
BMI (kg·m^{-2})	22 ± 2 kg·m-2
Body fat (%)	26.2 ± 9.9
Legs fat mass (g)	7304 ± 3292
Legs lean tissue mass (g)	17460 ± 4298.15

Physical activity levels

Fig. 3.3 shows the daily physical activity levels (PAL) during periods of free-living and lower limb immobilisation. The X axis indicates study day. There was no difference in the daily PAL score between days during the free-living period. Similarly, during unilateral lower limb immobilisation there was no difference in the PAL score between days. Daily physical activity tended to be similar when comparing the free-living state with the immobilised state, however PAL was less on day 2 of immobilisation compared to day 2 of the free-living state ($p < 0.05$).

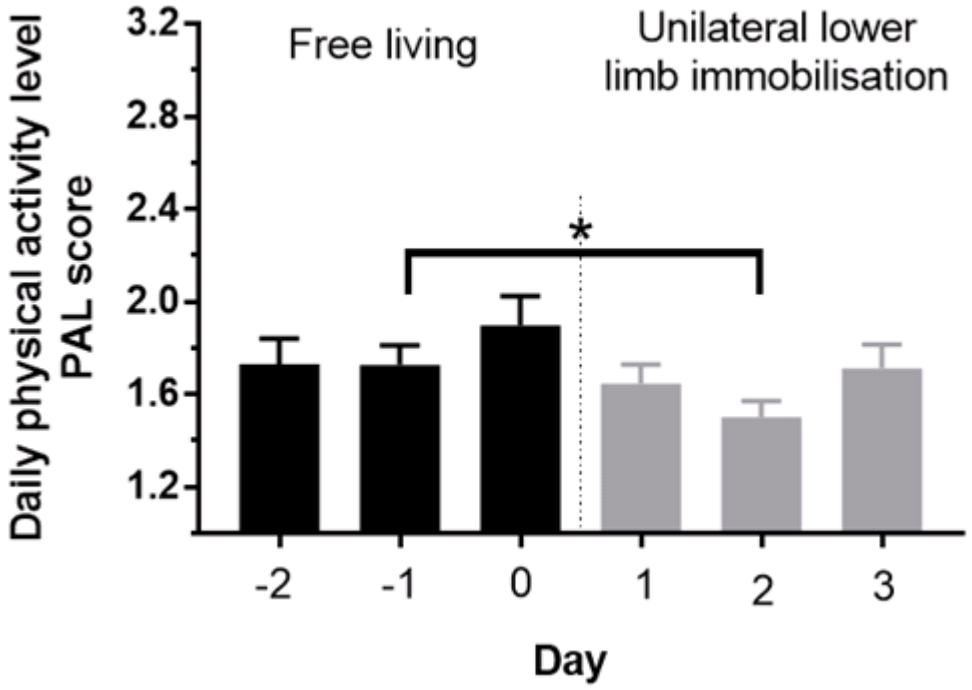

Figure 3.3. *Daily physical activity level (PAL) score measurements recorded using an ActiHeart monitor during 3 days in the free-living state and 3 days of unilateral lower limb immobilisation in young male and female participants. Values are mean + SEM. * signifies significant difference between days (*$p < 0.05$).

Fig. 3.4 shows the average daily PAL during 3 days of free-living and 3 days of unilateral lower limb immobilisation. Average daily PAL declined by 8% from the free-living state during immobilisation ($p < 0.05$).

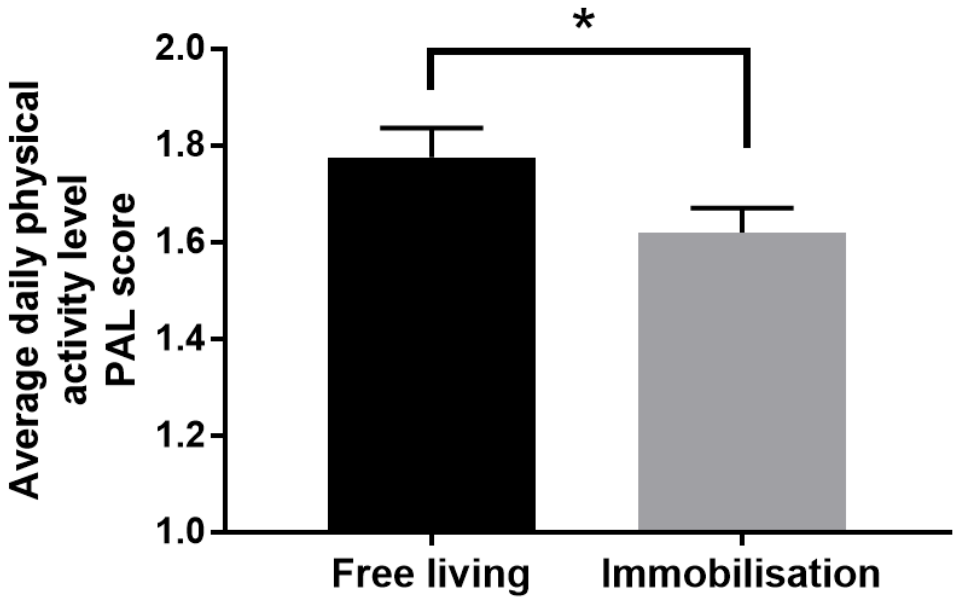

Figure 3.4. *Average daily physical activity level (PAL) score measurements recorded using an ActiHeart during free living and 3 days of unilateral lower limb immobilisation in young male and female participants. Values are mean + SEM. * signifies significant difference between days (*$p < 0.05$).

Muscle volume, thickness, fascicle length and pennation angle

Fig. 3.5A shows medial gastrocnemius muscle volume (cm^3) before and immediately following unilateral lower limb immobilisation and at the corresponding time points in the non-immobilised limb. Medial gastrocnemius muscle volume in the non-immobilised limb did not change during immobilisation of the contralateral limb. However, muscle volume in the immobilised limb declined by ~7% from the pre-immobilised state in the immobilised limb, and this decline in volume was discernible in 11 of the 15 volunteers.

Fig. 3.5B shows medial gastrocnemius muscle thickness (cm) before and immediately following unilateral lower limb immobilisation and at the corresponding time points in the non-immobilised limb. Muscle thickness of the non-immobilised was unchanged during immobilisation of the contralateral limb. Similarly, muscle thickness of the immobilised limb did not change during immobilisation.

Fig. 3.5C shows medial gastrocnemius muscle fascicle length (cm) before and immediately following unilateral lower limb immobilisation and at the corresponding time points in the non-immobilised limb. Muscle fascicle length of the non-immobilised was unchanged during immobilisation of the contralateral limb. Similarly, muscle fascicle length of the immobilised limb did not change during immobilisation

Fig. 3.5D shows medial gastrocnemius muscle pennation angle (°) before and immediately following unilateral lower limb immobilisation and at the corresponding time points in the non-immobilised limb. Muscle pennation angle of the non-immobilised was unchanged during immobilisation of the contralateral limb. Similarly, muscle fascicle length of the immobilised limb did not change during immobilisation

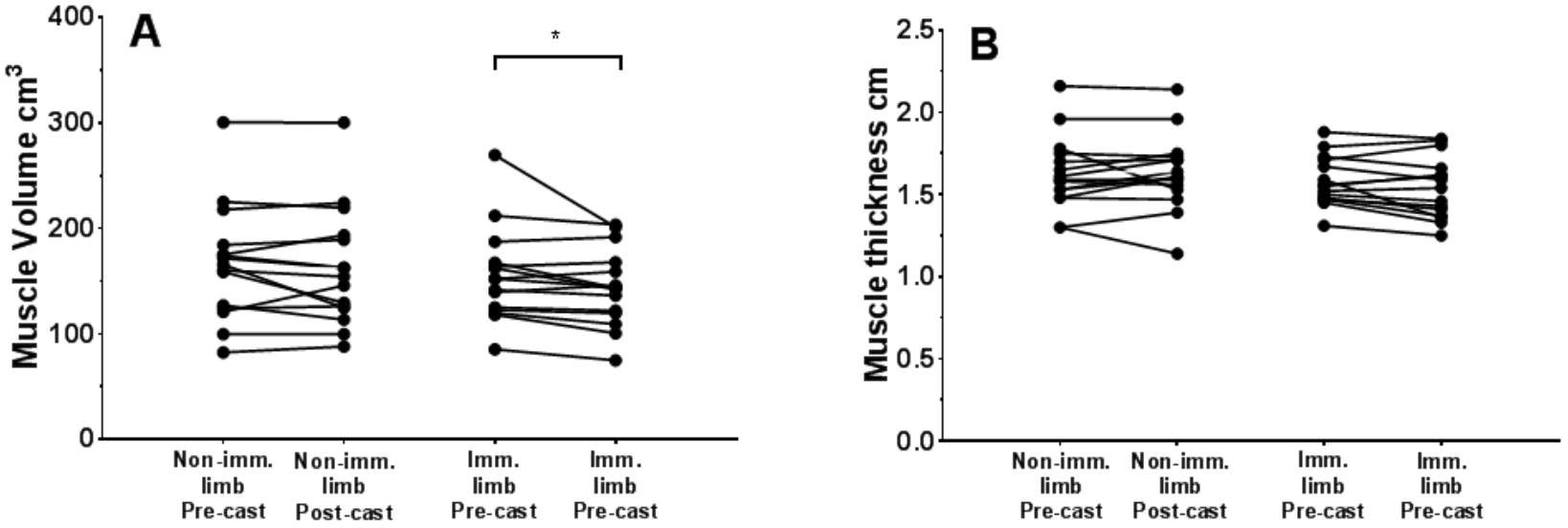

Figure 3.5A&B . *Medial gastrocnemius muscle volume (A) and muscle thickness (B) measured in young, healthy males and females using ultrasonography in a non-immobilised (non-imm.) limb and the contralateral immobilised (imm.) limb before 3 days of unilateral lower limb immobilisation (pre-cast) and following three days of unilateral lower limb immobilisation (post-cast) . * signifies significant difference within limb over time (P<0.05).*

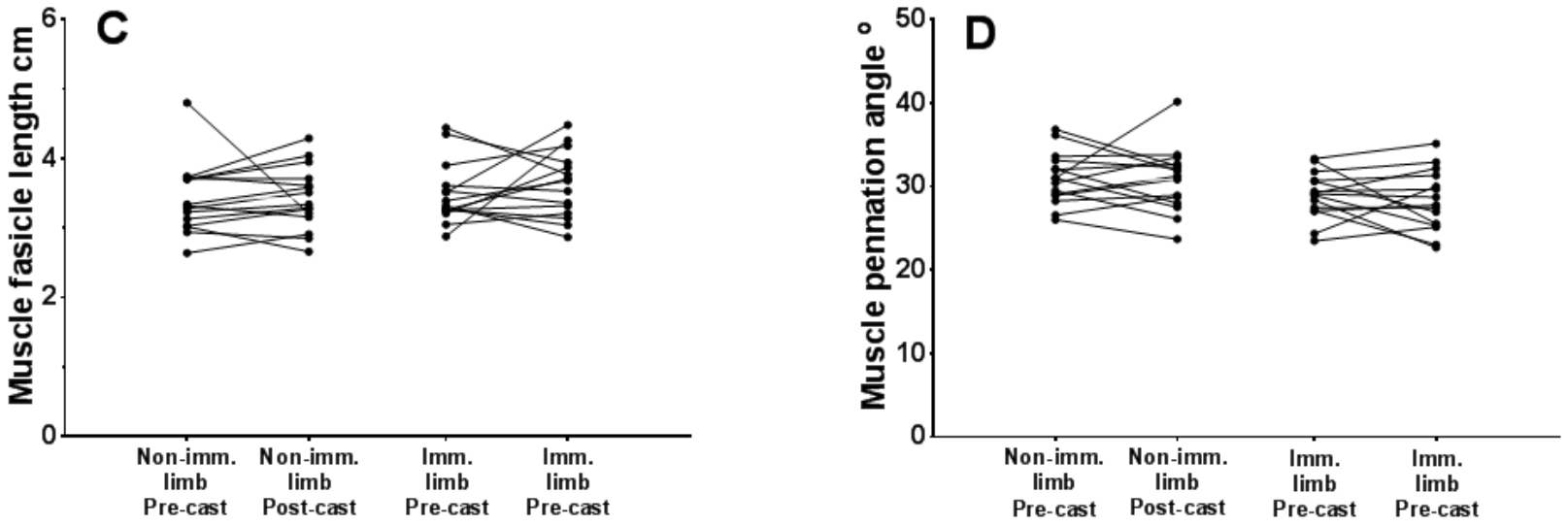

Figure 3.5C&D. *Medial gastrocnemius muscle fascicle length (C) and muscle pennation angle (D) measured in young, healthy males and females using ultrasonography in a non-immobilised (non-imm.) limb and the contralateral immobilised (imm.) limb before 3 days of unilateral lower limb immobilisation (pre-cast) and following three days of unilateral lower limb immobilisation (post-cast) . * signifies significant difference within limb over time (P<0.05).*

Chronic Myofibrillar FSR

Fig. 3.6 shows chronic myofibrillar protein FSR (%/d^{-1}) in the medial gastrocnemius muscle during free-living conditions and over 3 days in the immobilised limb and over the same period in the contralateral non-immobilised limb. Myofibrillar protein FSR during free living conditions was 1.62 ± 0.21%.d^{-1}, which was no different from that recorded in the non-immobilised limb (1.44 ± 0.30%.d^{-1}, *Figure 3.6*). However, after 3 days immobilisation myofibrillar FSR in the immobilised limb (1.22 ± 0.37%.d^{-1}) was 25% less than the free-living state ($p<0.01$) and 15% less than the non-immobilised contralateral limb ($p<0.05$, *Figure 3.6*). In 11 of the 15 volunteers, myofibrillar FSR in the immobilised limb was less compared to the contralateral non-immobilised limb. Furthermore, in 13 of the 15 volunteers myofibrillar FSR in the immobilised limb was less compared to the free-living state.

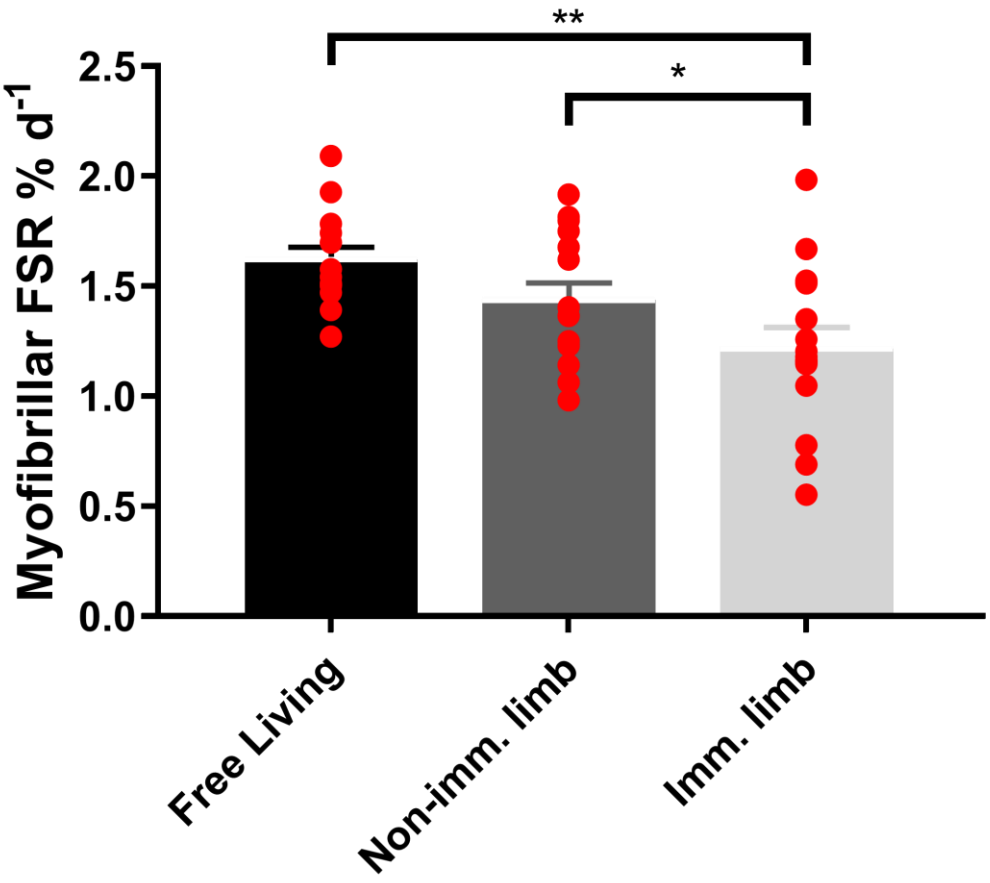

Figure 3.6. *Chronic myofibrillar protein FSR in the medial gastrocnemius muscle during free living conditions, following three days unilateral lower limb immobilisation (Imm.) and over the same time period in the non-immobilised limb (Non-imm.) contralateral immobilised limb in young, healthy volunteers. Values are mean + SEM. * (P<0.05) and ** (P<0.01) signify significant difference between conditions.*

Muscle protein expression

Fig. 3.7 shows muscle protein expression in the medial gastrocnemius muscle in the immobilised and contralateral non-immobilised limb following 3 days of unilateral lower limb immobilisation. The X axis depicts the target protein expression and phosphorylation (when appropriate) in each limb. No differences were observed when comparing target protein expression levels between limbs, with exception to phospho-4E-BP1 (Thr37/46) which was 52% ($P<0.05$) less in the immobilisation limb when compared to the contralateral non-immobilised limb (Figure 3.7).

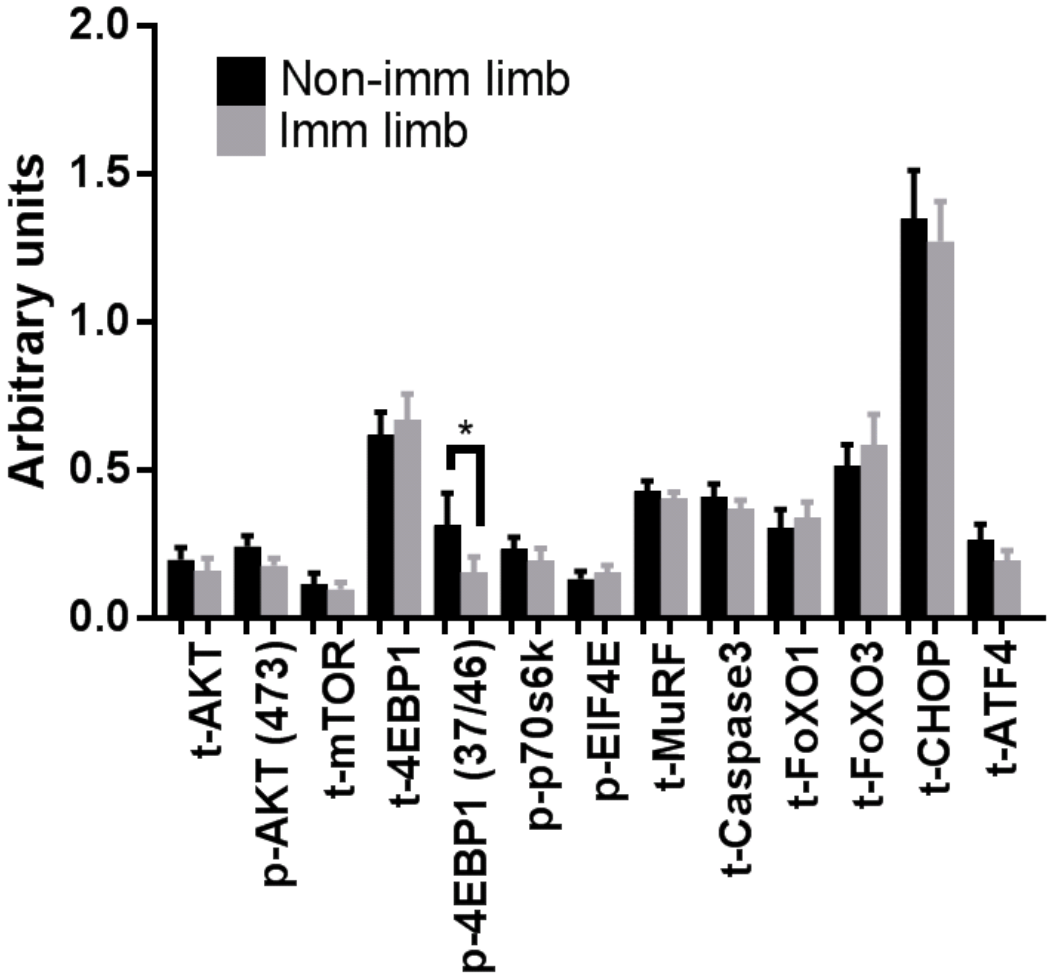

Figure 3.7. *Medial gastrocnemius muscle protein expression in the immobilised limb and the contralateral non-immobilised limb following 3 days of unilateral lower limb immobilisation in young males and females.* Values represent mean + SEM. * signifies significant difference between limbs ($p < 0.05$). AKT = Protein kinase B, mTOR = mammalian target of rapamycin, 4EBP1 = Eukaryotic translation initiation factor 4E-binding protein 1, P70S6K = Ribosomal protein S6 kinase beta-1, EIF4E = Eukaryotic translation initiation factor 4E, FoXO1 = Forkhead box protein O1, FoXO3 = Forkhead box protein O3, CHOP = C/EBP homologous protein and ATF4 = Activating transcription factor 4.

3.5 Discussion

The change in acute myofibrillar protein FSR from the pre to the post-immobilised state has been documented in humans (de Boer et al., 2007; Ferrando et al., 1996; Glover et al., 2008; Symons et al., 2009; Wall et al., 2016). However, this study is the first to quantify chronic myofibrillar protein FSR in a habitual free-living state and in response to subsequent immobilisation, including measurements in a contralateral non-immobilised limb. Medial gastrocnemius muscle myofibrillar FSR during free living conditions was 1.61 ± 0.20 $\%/d^{-1}$ which is similar to value recorded by Wilkinson and colleagues for the vastus lateralis (1.45 ± 0.10 $\%/d^{-1}$) under similar conditions (Wilkinson et al., 2014) quantified using the same tracer methodology applied during this study. Following 3 days of unilateral lower limb immobilisation, medial gastrocnemius muscle myofibrillar FSR was attenuated by 25% ($P<0.01$) in the immobilised limb when compared to the non-immobilised limb during free living conditions Figure 3.6. In addition, myofibrillar FSR values were lower following immobilisation in all but 2 volunteers in the immobilised limb when compared to free living myofibrillar FSR values (Figure 3.6). Furthermore, following immobilisation myofibrillar FSR in the immobilised limb was also less than the FSR rate measured in the contralateral non-immobilised limb over the same period, which was no different from the free-living state.

Previous studies of muscle disuse induced atrophy in humans have also demonstrated a reduction in acute myofibrillar protein synthesis following muscle unloading. Two-weeks of bed rest in young volunteers has been

shown to reduce vastus lateralis myofibrillar protein FSR by 50% (Ferrando et al., 1996) when measured using an acute tracer (L- [ring-$^{13}C_6$]phenylalanine) method. Furthermore, two-weeks of unilateral lower limb immobilisation in young humans using an acute tracer method (L-[ring-$^{13}C_6$]phenylalanine) induced a 27% reduction in post-absorptive quadriceps myofibrillar FSR in the immobilised limb when compared to the non-immobilised limb (Glover et al., 2008). Acutely measuring MPS using [^{15}N]proline tracer, de Boer and colleagues showed a reduction in myofibrillar protein FSR of 52% following 10 days of unilateral lower limb suspension (de Boer et al., 2007). This study is methodologically distinct from previous immobilisation studies which have employed acute tracer methodologies to measure rates of myofibrillar protein FSR over few hours on two separate days (i.e. on baseline and following muscle unloading). This approach fails to take into consideration the myofibrillar protein FSR response to the entire period of muscle unloading, i.e. throughout a diurnal cycle which can be influenced by nutrition (Aagaard et al., 2001) and physical activity (Breen et al., 2013). Here, utilising a D_2O tracer to quantify chronic myofibrillar protein FSR over a sustained 3 day period off unloading it is shown that medial gastrocnemius myofibrillar FSR was attenuated by 25% on average when compared to the contralateral non-immobilised limb and by 15% when compared to the free-living state Figure 3.6. In the only other comparable study available, Mitchell and colleagues quantified chronic myofibrillar protein FSR in middle-aged (45-60 years) men over 14 days of unilateral lower limb immobilisation (Mitchell et al., 2018), and contrary to the present findings reported it to be unchanged from baseline. There is no

immediate explanation for this disparity, particularly given Mitchell and colleagues reported mid-thigh muscle cross-sectional area declining significantly by ~4% when quantified using peripheral quantitative computed tomography.

Medial gastrocnemius muscle volume measured using ultrasonography declined by 7% following 3 days of unilateral lower limb immobilisation Figure 3.5. Two weeks of unilateral full leg immobilisation with a lightweight fiber cast results in a decrease of quadriceps muscle volume by ~9% in young participants when measured using MRI (Suetta et al., 2009). Similarly, 14 days of bed rest resulted in a ~6% decline in quadriceps muscle volume in young participants when measured using MRI (Pisot et al., 2016). Given the muscle unloading intervention mentioned in the above studies is of a longer duration, our reported medial gastrocnemius muscle volume loss of 7% following 3 days of immobilisation initially may seem high. However, the magnitude of muscle loss is greatest at the onset of immobilisation (de Boer et al., 2007) and the loss of muscle mass varies amongst muscle groups with the medial gastrocnemius being demonstrated to be most affected during muscle disuse induced atrophy. De Boer and colleagues using ultrasonography showed medial gastrocnemius underwent greater atrophy following 5 weeks of bed-rest in young volunteers when compared to the tibialis anterior over the same time period (-12.2% vs. no change) (de Boer et al., 2008). Similarly, following 90 days of bed rest the medial gastrocnemius muscle was found to have undergone a

28% decline in muscle volume in comparison to a decline of 15% in the tibialis anterior in the same limb using MRI (Belavy et al., 2017).

Although muscle volume did decline during immobilisation when measured using ultrasonography, muscle architectural parameters (muscle thickness, muscle fascicle length and pennation angle) remained unchanged. Muscle volume is a more representative measure of muscle mass as it takes into consideration the whole muscle, as opposed to muscle thickness measured at the midpoint of the muscle. This could provide a possible explanation as to why we did not observe any change in muscle thickness during immobilisation.

The overall decline in medial gastrocnemius myofibrillar protein FSR of ~25% from the free-living state in the immobilised limb and medial gastrocnemius muscle volume decline of ~7% in the same limb could not be explained at an individual level, i.e. there was no correlation between the magnitude of decline in myofibrillar protein FSR and the magnitude of decline in muscle volume. This disassociation in myofibrillar protein FSR decline and muscle volume loss during immobilisation observed cannot be obviously attributed precision of the ultrasonography technique which has high reliability (Narici and Cerretelli, 1998). Furthermore, Arbeille and colleagues validated the use of ultrasonography against MRI to measure changes in vastus intermedius and the vastus medialis muscle volume during a 60d bed rest study in young female volunteers and calculated the correlation coefficient of muscle volume change determined by MRI and ultrasonography to be 0.78% (Arbeille et al., 2009), therefore ultrasonography determined muscle volume provides an

accurate measure of muscle volume. Indeed, dehydration levels which are difficult to control for but can influence muscle volume measurements may have played a contribution (Turton et al., 2016). Furthermore, it has been demonstrated the medial gastrocnemius muscle does not atrophy uniformly across the length of the muscle following 60 days of bed-rest (Miokovic et al., 2012) such that extrapolating regional myofibrillar protein FSR rates across the muscle may not be appropriate.

To our knowledge, this is the first study to measure physical activity prior and during an immobilisation study. Although the mean daily physical activity level was less during the immobilisation period when compared to the free-living state, this was significant only on day 2 of immobilisation when compared to day 2 of the free-living state (Figure 3.3; $p < 0.05$). However, when considering average daily physical activity levels across each 3 day period, physical activity declined significantly (8%) during the immobilisation from the free-living state ($p<0.05$, Figure 3.4). Reduced habitual physical inactivity in itself can be utilised as a method reduce muscle mass and myofibrillar protein FSR, although invariably of a much larger magnitude than that observed here. Two weeks of reduced (~80%) ambulatory activity was sufficient to induce lower limb muscle atrophy in young participants when measured using DXA (Krogh-Madsen et al., 2010), which appears to be underpinned by reductions in postprandial myofibrillar protein FSR (Breen et al., 2013). However, the present data demonstrate that an 8% reduction in physical activity was not sufficient to create a measurable change in muscle architecture or myofibrillar protein FSR from the free-

living state in the non-immobilised limb. It would be interesting to see the impact of a sustained (weeks) 8% reduction in physical activity has on muscle protein FRS and muscle volume, and indices of muscle health.

The AKT/mTOR/p70S6K signalling axis by regulating protein translation initiation is thought to be the dominant pathway controlling MPS in response to exercise and feeding. It is generally assumed this pathway is regulated via phosphorylation cascade, such that phosphorylation of AKT results in phosphorylation of downstream mTOR, which in turn inhibits 4E-BP1 and activates p70S6K stimulating protein translation initiation. However, declines in medial gastrocnemius myofibrillar protein FSR and volume during immobilisation in the present study occurred in the absence of robust differences in expression of putative regulators of MPS, MPB and ER stress, with exception to phospho-4E-BP1 (Thr37/46) which was 52% less in the immobilisation limb when compared to the non-immobilised limb (Figure 3.7). In keeping with this, the total expression and phosphorylation status of AKT/mTOR and p70S6K has been demonstrated to remain unchanged following 8 days of bed rest in young participants (Jespersen et al., 2014) and following 10 or 21 of unilateral lower limb suspension model in young participants (de Boer et al., 2007). It is not unreasonable to assume that static measurements of protein expression or phosphorylation state of AKT, mTOR and p70S6K signalling proteins may not give a true indication of the dynamic process of MPS. Indeed, a non-linear relationship between the phosphorylation state of AKT and P70S6K and MPS has been observed in humans in response to increased amino acid provision (Greenhaff et al., 2008). Similarly, a

dissociation between MPS and mTOR signalling in response to feeding in the immobilised state has been seen reported (Glover et al., 2008) Nevertheless, downstream targets of the AKT/mTOR/P70S6K pathway, such as phospho-4E-BP1, may play a regulatory role in modulating muscle myofibrillar protein FSR (Greig et al., 2011). Indeed, a 52% reduction in muscle phospho-4E-BP1 (Thr37/46) expression was observed in this study following 3 days immobilisation when compared to the contralateral non-immobilised limb. elF4E binds to mRNA at the 5' end and induces translational initiation. However, the action of elF4E is prevented by 4E-BP1. Phosphorylation of 4E-BP1 prevents the binding of 4E-BP1 to elF4E promoting cap-dependent translation (reviewed by Richter and Sonenberg, 2005). Therefore, the reduction in myofibrillar protein FSR following 3 days of unilateral lower limb immobilisation could be due to reduced phospho-4E-BP1 thereby attenuating cap dependent translational initiation and reducing myofibrillar protein FSR during immobilisation. However, our finding of reduced phospho-4E-BP1 (Thr37/46) goes against previous data from the literature (de Boer et al., 2007; Jespersen et al., 2014). The discordance in our results could be due to the difference in muscle group studied. We studied the medial gastrocnemius muscle which is most susceptible to disuse induced atrophy (Belavy et al., 2017; de Boer et al., 2008), whilst *de Boer et al* and *Jespersen et al* studied the medial vastus lateralis. Additionally, the immobilisation duration of 3 days is likely to represent a period when the decline in FSR is likely to be most obvious relative to the studies of *de Boer et al* (2007) (10 and 23 days) and *Jespersen et al (2014)* (8 days),

where any acute transient changes in protein phosphorylation could have been missed.

So, in the face of no obvious and robust change in the AKT/mTOR/p70S6K signalling axis, what might explain the reduction in chronic protein FSR observed in the present study? A reduction in myofibrillar protein FSR during muscle unloading could be modulated through the mechanotransduction sensing protein focal adhesion kinase (FAK). FAK resides on the extracellular matrix of the muscle and converts mechanical stimulus (muscle contraction) into electrochemical activity, and is thought to work upstream of the AKT/mTOR/P70S6K pathway in regulating myofibrillar protein FSR (Kosek and Bamman, 2008). Indeed FAK phosphorylation expression has been reported to be attenuated during immobilisation in humans by 30% following 14 days of unilateral lower limb suspension (de Boer et al., 2007).

The molecular regulation of MPB includes multiple pathways. A predominant pathway in muscle is the ubiquitin-proteasome pathway. In skeletal muscle, two muscle specific E3 ligases, muscle atrophy F-box (MAFbx; also known as atrogin-1) and muscle RING finger-1 (MuRF-1), are responsible for protein ubiquitination and degradation. Expression levels of MAFbx and MuRF-1 have been reported to be a prerequisite for muscle wasting, particularly in animal models and including immobilisation (Bodine et al., 2001; Lecker et al., 2004).The expression levels of MPB markers (MuRF1, FoXO1, FoXO3 and caspase-3) were no different between immobilised and non-immobilised limbs in this study Figure 3.6. Muscle proteolysis during immobilisation in humans remains a

controversial topic. This is in accordance with results observed from *Nedergaard et al* showing total MuRF and FoXO1 and FoXO3 protein expression levels remained unchanged following 2 weeks unilateral full leg cast with biopsies obtained from the medial vastus lateralis (Nedergaard et al., 2012). However, MuRF1 protein expression levels have been shown to be elevated following 60 days bed rest in the soleus muscle, but unchanged in the vastus lateralis muscle (Salanova et al., 2008). Furthermore, MuRF protein expression levels have shown to be increased following 8 day of bed rest in the vastus lateralis (Jespersen et al., 2014). The disparity in results observed in the literature could be due to differences in the muscle group studied, differences in time studied (i.e. short (1-7 days), medium (7-21 days) and long-term (21+ days) and differences in antibodies used during analysis. Furthermore, in the absence of dynamic measures of muscle or limb protein breakdown these purported markers of protein breakdown will provide limited insight. Irrespective of this point, following 10 days of limb suspension in healthy young volunteers, De Boer and colleagues detected a 50% decline in the rate of post-absorptive myofibrillar protein FSR when compared to pre-immobilisation (de Boer et al., 2007), which the authors concluded was of sufficient magnitude to fully account for the decline in muscle cross sectional area recorded in the same volunteers. Therefore, even if it could be measured, the contribution from MPB to total muscle mass loss during human immobilisation studies is probably small.

To our knowledge, this is the first study to measure protein expression of ATF4 and CHOP in humans following immobilisation, which was found to

be no different between immobilised and non-immobilised limbs Figure 3.6. ATF4 is a transcription factor which promotes the expression of oxidative stress responsive genes and is thought to inhibit myofibrillar protein FSR by upregulating 4E-BP1 (Ebert et al., 2015), a negative regulator of cap-dependent translation. Furthermore, ATF4 downstream signalling targets include Gadd45a, which is thought to stimulate MPB via MEKK4 pathway (Bullard et al., 2016). Although rodent models of disuse induced muscle atrophy show ATF4 to play a pivotal role in regulating muscle mass (Sacheck et al., 2007, Fox et al., 2014) our data suggest this is probably not the case in human muscle disuse induced atrophy in an acute (3 days) setting. Rodent models of muscle disuse related atrophy such as muscle denervation induced atrophy (Sacheck et al., 2007) and rodent limb immobilisation (Fox et al., 2014) probably elicit a higher stress related response to the study intervention and medical procedures than consenting humans would do.

In summary, this study for the first time demonstrates a reduction in chronic muscle myofibrillar protein FSR from the free-living state during immobilisation, which was paralleled by a decline in muscle volume in the same limb. Furthermore, chronic muscle protein FSR in the immobilised limb was 15% less than in the non-immobilised limb. Despite these observations, the expression levels of proteins thought to regulate MPS, MPB and ER stress was no different between immobilised and non-immobilised limbs, with exception to phospho-4E-BP1 (Thr37/46). This latter finding suggests suppression of cap-dependent translational

initiation may play a role in the decline of myofibrillar protein FSR during immobilisation.

Chapter 4

4 The effect of three-day unilateral lower limb immobilisation on chronic muscle protein synthesis, muscle thickness and volume and the expression of protein targets implicated in muscle mass regulation in healthy, normal weight, young and older volunteers

4.1 Introduction

The population of over 65s currently accounts for 18.6% of UK residents, which is expected to rise to 23.9% (nearly 1 in 4) by 2036 (Office for National Statistics, 2017). With an aging population, health expenditure in the later years of life increases. For example, health care costs for a 70 year old are 3 fold greater than for a 30 year old, which increases to 5 fold for an 80 year old (Office for Budgetary Responsibility, 2016). Aging is associated with a decline in skeletal muscle mass and impaired muscle performance termed sarcopenia which negatively impacts on quality of life (Beals et al., 2018) and even mortality rates (Constantin et al., 2011). Therefore, research into the ageing process, especially aimed at improving musculoskeletal health in later life is imperative for reducing the pressure on the ever increasingly strained social and healthcare sector.

Ageing is associated with reduced habitual physical inactivity (Frisard et al., 2007) and as discussed in **Chapter 3**, acute periods of muscle unloading in the form of 3 days unilateral lower limb immobilisation in young volunteers can have a detrimental effect on muscle metabolic and

physiological health, as would habitual reduced physical inactivity in older volunteers.

Skeletal muscle is one of the most dynamic and plastic tissues of the human body comprising approximately 40% of total body weight and is crucial for locomotion and metabolic health as the muscle plays a central role in whole body protein metabolism by serving as the principal reservoir for amino acids to maintain protein synthesis in vital tissues and organs. In addition, altered muscle metabolism plays a key role in the formation of multiple chronic disease states such as sarcopenia and diabetes, and therefore plays a key role in the prevention of these chronic disease states.

The regulation of muscle mass in adults is dependent on the balance between MPS and MPB and both processes are responsive to external factors i.e. physical in/activity (Breen et al., 2013; de Boer et al., 2007; Glover et al., 2008; Wall et al., 2016), protein supplementation (Cuthbertson et al., 2005; Glover et al., 2008; Smith et al., 1992), exercise (Brook et al., 2015) and infection, trauma and inflammation (Puthucheary et al., 2013). However, as mentioned above aging is associated with reduced habitual physical activity (Ivey et al., 2000) and a diminished muscle protein synthetic response to protein ingestion (Cuthbertson et al., 2005) and exercise across all intensities (Kumar et al., 2009), which likely contribute to the age related loss of muscle mass beyond the age of 50. Sarcopenia beyond the age of 50 is reported to occur at a rate of ~1% per annum in men and ~0.5% per annum in women (Mitchell et al., 2012).

Sarcopenia is a chronic phenomenon, but periods of complete inactivity during limb immobilisation or bed rest results in accelerated muscle mass loss irrespective of age. In adults, MPS and MPB fluctuate throughout a diurnal cycle but remain in overall balance to maintain muscle mass (Atherton and Smith, 2012). Nevertheless, sustained periods of muscle unloading, in the form of reduced step count (Breen et al., 2013), unilateral limb immobilisation (Jones et al., 2004) and bed-rest (Ferrando et al., 1996; LeBlanc et al., 1992; Symons et al., 2009) lead to muscle loss. This is thought to occur principally through a decline in post-absorptive myofibrillar protein FSR (de Boer et al., 2007; Ferrando et al., 1996) and a blunting of post-prandial amino acid induced increases in myofibrillar protein FSR (Glover et al., 2008) in both young and older humans (Tanner et al., 2015). Whether older people are more or less susceptible to immobilisation induced muscle mass loss is unclear, but clearly immobilisation will accentuate sarcopenia if muscle mass is not restored upon remobilisation, which has negative health implications. Fourteen days of unilateral full leg immobilisation in a light weight fibre cast resulted in a decline in quadriceps muscle volume of ~9% in young males which was significant when compared to ~5% in the elderly males when measured using MRI (Suetta et al., 2009). On the other hand, Pisot et al demonstrated quadriceps muscle volume following 14 days of bed rest had declined by ~6% in young males which was significantly different to the ~8% in older males when measured using MRI (Pisot et al., 2016). Furthermore, following 5 days bed rest, leg lean mass was found to have declined by 4% in older volunteers which was significantly different from young volunteers which remained unchanged measured

using DXA (Tanner et al., 2015). Therefore, there appears to be no clear consensus as to whether older people are more or less susceptible to muscle disuse related atrophy than younger people, or indeed whether they are no different. Furthermore, little is known about whether susceptibility to immobilisation induced atrophy is influenced by both habitual physical activity levels and age or simply reflects the former given aging is associated with physical inactivity (Frisard et al., 2007)

Numerous factors are likely to influence the rate of muscle atrophy during disuse. Importantly, studies have invariably failed to take into consideration that habitual physical activity levels of participants prior to immobilisation which may be important. Given physical activity is an accepted stimulus to MPS (Kumar et al., 2009), and immobilisation results in a rapid decline in MPS (de Boer et al., 2007) and induction of anabolic resistance to protein nutrition (Glover et al., 2008) it is not unreasonable to propose that recreationally active individuals with a larger muscle mass would experience greater muscle mass loss during immobilisation when compared to age-matched habitually inactive individuals with a lower initial muscle mass. Indeed, the discordance in the literature regarding the magnitude of muscle atrophy during immobilisation in younger vs older volunteers may be at least in part attributable to age related differences in muscle mass (Pisot et al., 2016) and/or habitual physical activity levels prior to immobilisation (Frisard et al., 2007). In addition to the above, little is known about the molecular mechanism regulating human muscle atrophy during muscle unloading, especially in context to aging.

4.2 Aims and Hypothesis

The aim of this study was to determine the impact of 3 days of unilateral, below knee limb immobilisation on chronic myofibrillar FSR in the medial gastrocnemius muscle compared to the free living non-immobilised state and the contralateral non-immobilised limb in healthy, normal-weight younger volunteers vs healthy, normal-weight older volunteers. These measurements were made in conjunction with ultrasound-based determination of medial gastrocnemius muscle volume and protein expression levels of putative regulators of anabolic, catabolic and ER stress related pathways. Finally, we aimed to provide novel insight regarding any impact that habitual physical activity, pre-immobilisation muscle volume and aging may have on these measures.

It was hypothesised that unilateral limb immobilisation would reduce chronic myofibrillar FSR in both young and older volunteers when compared to both the free-living state and the non-immobilised contralateral limb, which would be paralleled by immobilisation induced decrements in medial gastrocnemius muscle volume and changes in the putative molecular regulators of muscle mass, and that these responses may or may not differ between young and older volunteers.

4.3 Methods

Participants

Following informed consent, 15 healthy, normal-weight, young and 10 healthy, normal-weight, older volunteers (see **Table 4.1** for

anthropometric parameters) were recruited to participate in a 3 day unilateral lower limb immobilisation study (see Chapter 2 section 1 for detailed protocol). For the sake of clarity, the healthy, normal-weight, young data presented in Chapter 3 are used as a control comparator group in this chapter.

4.4 **Results**

Table. 4.1 Baseline anthropometric characteristics of participants (n.s = not significant; * signifies *p* value < 0.05)

Variables	Mean + SD	Mean + SD	*P* value
Group	Young	Older	-
Age	23 ± 4	70 ± 4	-
***n* (Male:Female)**	15 (7M:8F)	10 (4M:6F)	-
Height (m)	1.77 ± 0.11	1.68 ± 0.09	*
Weight (kg)	72.10 ± 13.04	64.31 ± 9.67	*n.s*
BMI (kg·m-2)	22 ± 2 kg·m-2	23 ± 2	*n.s*
Body fat (%)	26.2 ± 9.9	28.1 ± 8.5	*n.s*
Legs fat (g)	7304 ± 3292	6150 ± 2085	*n.s*
Legs lean tissue (g)	17460 ± 4298	14030 ± 2585	*

Physical activity levels

Fig. 4.3 shows the daily PAL during periods of free-living and lower limb immobilisation in healthy young and older normal-weight volunteers. The X axis indicates study day. There was no difference in the daily PAL score between days during the free-living period within the young group or older group. The mean daily PAL score was less in the older group when compared to the young group during the free living period, and this was significant on day 0 (25%, $P<0.05$). There was no difference in the daily PAL score between days during unilateral lower limb immobilisation in the young group. In the older group, the daily PAL score was 5% less ($P<0.05$) on day 2 when compared to day 1. Although mean daily physical activity was less during the immobilisation period when compared to the free living days, this was only significant on day 2 of immobilisation when compared to day -1 of free living, in both the young (13%, $P<0.01$) and older (13%, $P<0.05$) groups.

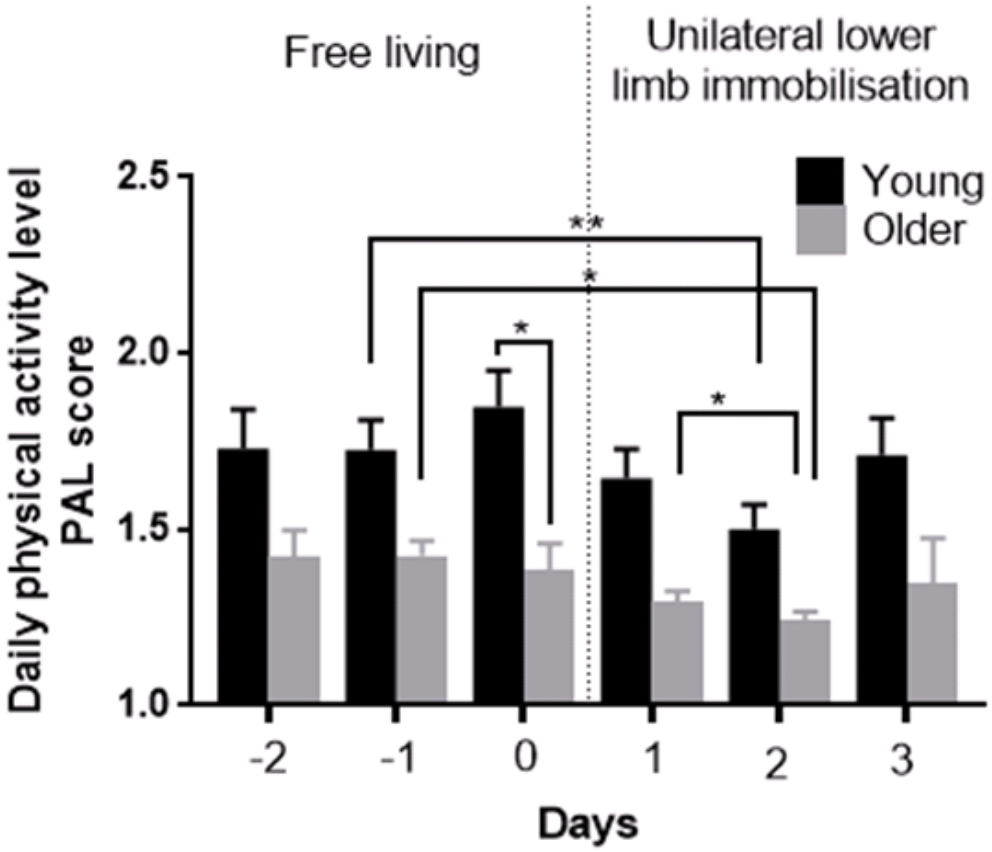

Figure 4.3. *Daily physical activity level (PAL) score measurements recorded using an ActiHeart during 3 days of free living and 3 days of unilateral lower limb immobilisation in healthy, normal-weight, young and older volunteers. * signifies P< 0·05 and ** signifies P<0·001.*

Fig. 4.4 shows the average daily PAL over 3 days of free living and 3 days of unilateral lower limb immobilisation in young and older volunteers. Average daily PAL during free living conditions was significantly less in the older group compared to the younger (22%, $P \leq 0.001$), which was also true of the immobilised state ($P \leq 0.001$). Average daily physical activity levels declined significantly by 8% ($P<0.05$) from the free living state during immobilisation in the young group, this was not evident in the older group. In short therefore older volunteers were less physically active than the younger volunteers in both the free living and immobilised state, and immobilisation slightly reduced physical activity levels in the younger volunteers.

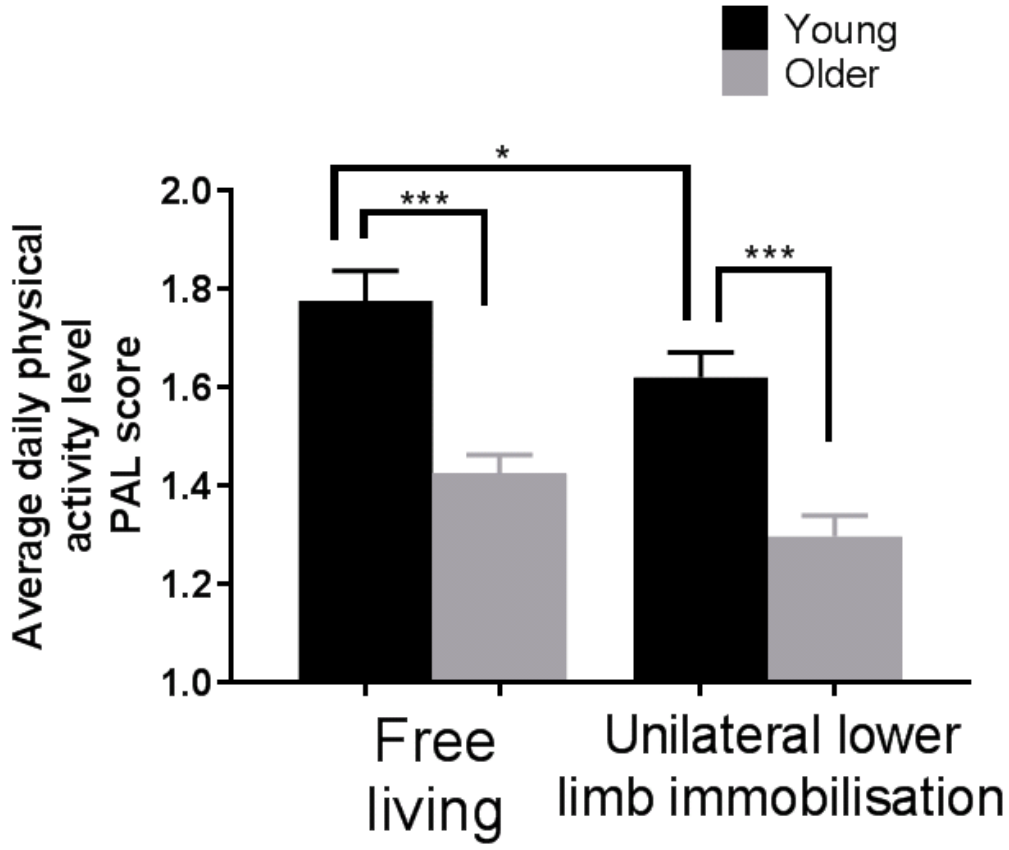

Figure 4.4. *Average daily physical activity level (PAL) score recorded using an ActiHeart during 3 or 4 days of free living and 3 days of unilateral lower limb immobilisation healthy, normal-weight, young and older volunteers. * signifies P< 0·05 and ** signifies P<0·001.*

Muscle volume, thickness, fascicle length and pennation angle

Fig. 4.5A shows medial gastrocnemius muscle volume before and immediately following unilateral lower limb immobilisation in young and older participants, and at the corresponding time points in the non-immobilised limb. Although mean medial gastrocnemius muscle volume was less at baseline in both limbs in the older volunteers when compared to the young volunteers, this was not significant. Muscle volume in the

non-immobilised limb did not change during immobilisation of the contralateral limb in both young and older volunteers. However, muscle volume in the immobilised limb declined by ~7% ($P<0.05$) from the pre-immobilised state in the young group, and this decline in volume was discernible in 11 of the 15 volunteers. On the other hand, muscle volume remained unchanged in the older group during immobilisation.

Fig. 4.5B shows medial gastrocnemius muscle thickness before and immediately following unilateral lower limb immobilisation in young and older participants and at the corresponding time points in the non-immobilised limb. Muscle thickness was no different between groups at baseline. Muscle thickness of the non-immobilised limb was unchanged during immobilisation of the contralateral limb in both the young and older volunteers. Similarly, muscle thickness of the immobilised limb did not change during immobilisation in the young and older volunteers.

Fig. 4.5C shows medial gastrocnemius muscle fascicle length before and immediately following unilateral lower limb immobilisation in young and older participants, and at the corresponding time points in the non-immobilised limb. Muscle fascial length was no different between groups at baseline. Muscle fascicle length of the non-immobilised limb was unchanged during immobilisation of the contralateral limb in both groups. Similarly, muscle fascicle length of the immobilised limb did not change during immobilisation in either group.

Fig. 4.5D shows medial gastrocnemius muscle pennation angle before and immediately following unilateral lower limb immobilisation in young

and older participants, and at the corresponding time points in the non-immobilised limb. Muscle pennation angle was significantly lower in the non-immobilised limb in the older volunteers when compared to the young at baseline (17%, $P<0.05$). Muscle pennation angle of the non-immobilised limb was unchanged during immobilisation of the contralateral limb in both groups. Muscle fascicle length of the immobilised limb did not change during immobilisation in the young group, but declined by 9% ($P<0.05$) in the older group.

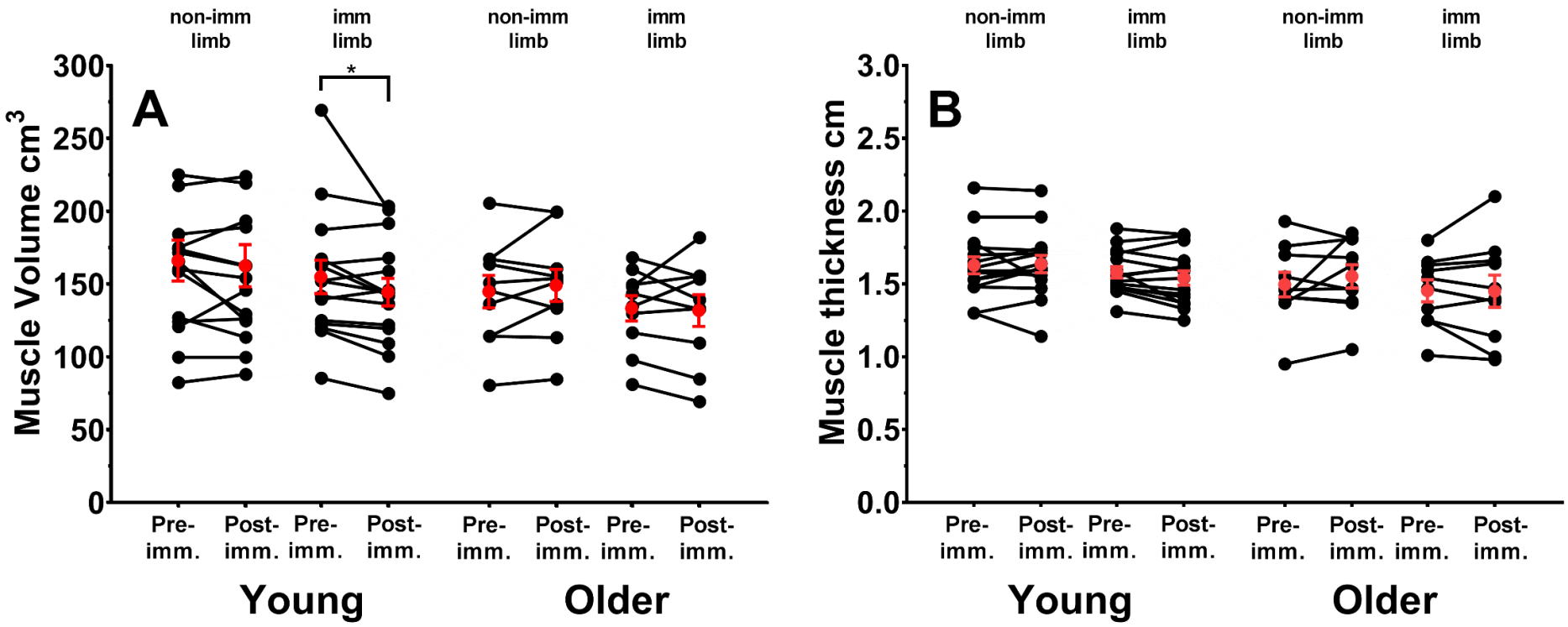

***Figure 4.5A&B**. Medial gastrocnemius muscle volume (A), thickness (B) measured using ultrasonography before unilateral lower limb immobilisation (Pre) and following three days of unilateral lower limb immobilisation (Post) in a non-immobilised (non-imm) and immobilised (imm) limb in healthy, normal-weight, young and older volunteers. Black spots are individual values and red spots are Mean ± SEM. * signifies P<0.05*

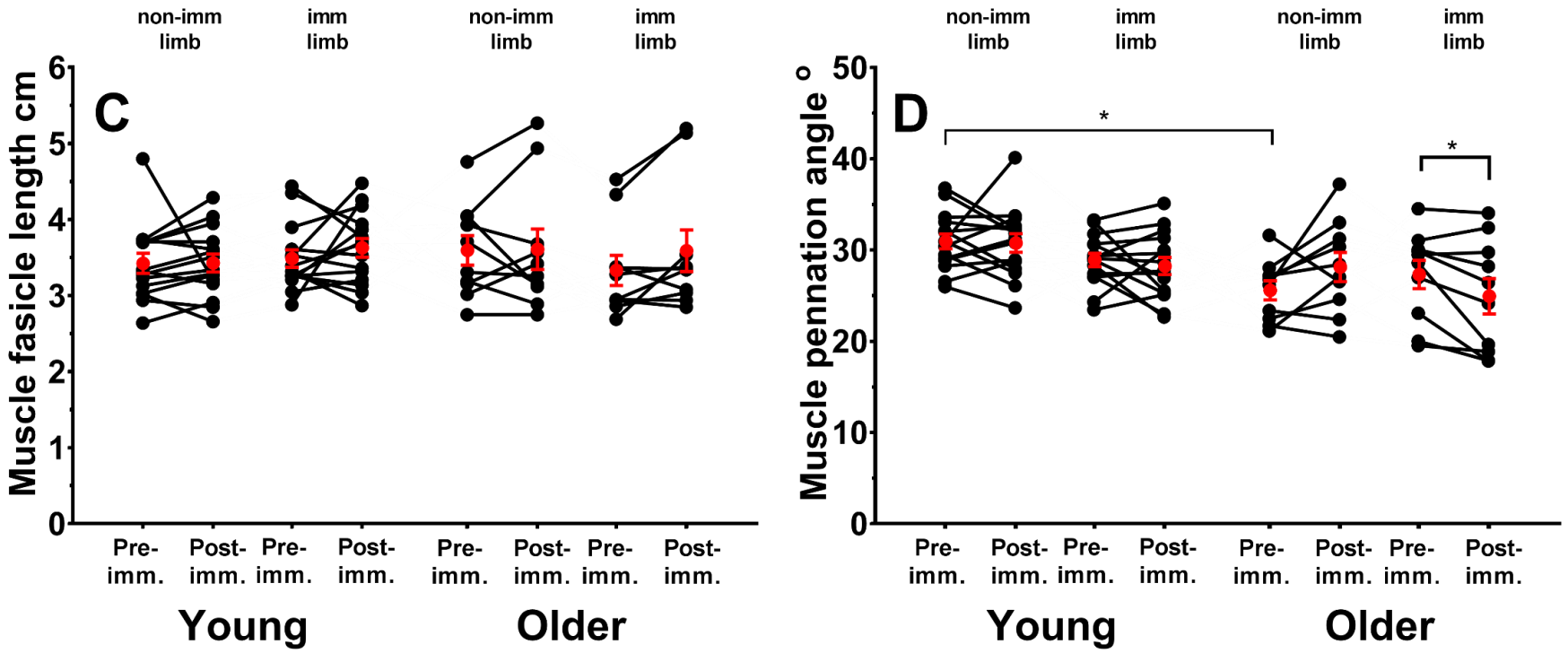

***Figure 4.5C&D**. Medial gastrocnemius muscle fascicle length (C) and pennation angle (D) measured using ultrasonography before unilateral lower limb immobilisation (Pre) and following three days of unilateral lower limb immobilisation (Post) in a non-immobilised (non-imm) and immobilised (imm) limb in healthy, normal-weight, young and older volunteers. Black spots are individual values and red spots are Mean* ± SEM. ** signifies P<0.05*

Fig. 4.6 illustrates the association between baseline medial gastrocnemius muscle volume and the absolute change in medial gastrocnemius muscle volume during 3d unilateral lower limb immobilisation in young and older volunteers. There was no association between baseline muscle volume and the decline in muscle volume during immobilisation in the older volunteers. However, there was a significant correlation (R^2= 0.33, P<0.05) between baseline muscle volume and the decline in muscle volume during 3d immobilisation in young volunteers, which remained significant with the data combined (P<0.05, R^2= 0.19).

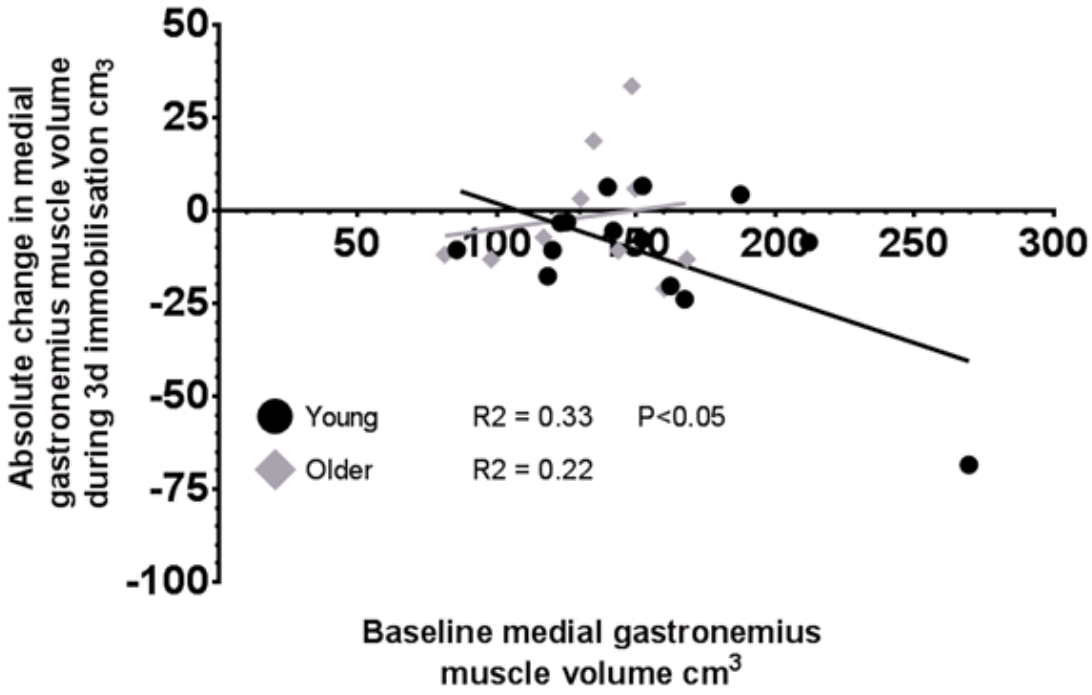

Figure 4.6. *The correlation between baseline medial gastrocnemius muscle volume and the absolute change in muscle volume during 3d unilateral lower limb immobilisation in young (black) and older (grey) volunteers.*

Chronic Myofibrillar FSR

Fig. 4.7 shows chronic myofibrillar protein FSR in the medial gastrocnemius muscle during free living conditions and following immobilisation in the immobilised and contralateral non-immobilised limb in young and older volunteers. Data values are individual (red spots) and mean ± SEM. Chronic myofibrillar protein FSR in the medial gastrocnemius muscle during free living conditions was similar when comparing young and older volunteers, 1.62 ± 0.21%.d^{-1} vs. 1.62 ± 0.21%.d^{-1}, respectively. Following 3 days of unilateral lower limb immobilisation in the young

volunteers, myofibrillar protein FSR in the non-immobilised limb (1.44 ± 0.30%.d^{-1}) was no different from the free living state (1.62 ± 0.21%.d^{-1}, Figure 4.7). However, in the immobilised limb myofibrillar protein FSR (1.22 ± 0.37%.d^{-1}) was 25% less than in the free living state ($p<0.01$) and 15% less than in the contra-lateral non-immobilised limb ($p<0.05$; Figure 4.7). Furthermore, myofibrillar FSR in the immobilised limb was less in 13 of the 15 volunteers when compared to the free living state, and less in 11 of the 15 volunteers when compared to the contralateral non-immobilised limb.

In older volunteers following 3 days of unilateral lower limb immobilisation, myofibrillar protein FSR in the non-immobilised limb (1.26 ± 0.25%.d-1) was 22% less than in the free living state (1.62 ± 0.21%.d^{-1} , $p<0.05$), and the same was true for the immobilised limb (1.24 ± 0.45%.d^{-1}, $p<0.05$). Furthermore, there was no difference in myofibrillar protein FSR between limbs (Figure 4.7).

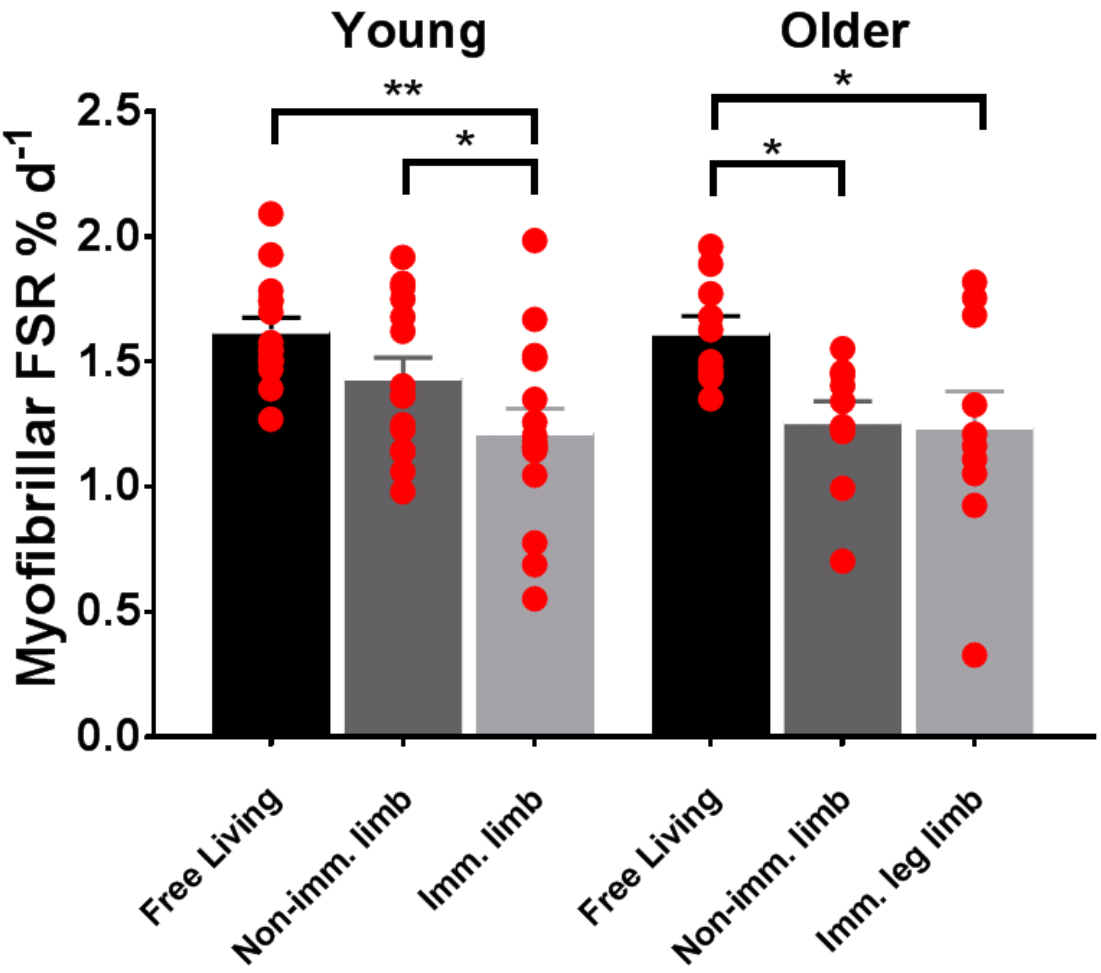

Figure 4.7. *Chronic medial gastrocnemius muscle myofibrillar protein FSR during free living conditions, prior to immobilisation in the non-immobilised limb (non-imm) and following three days of unilateral lower limb immobilisation in the non-immobilised limb (non-imm limb) and the contralateral immobilised limb (Imm. limb) in young and older humans. Values are individual (red dots) and Mean ± SEM.*

Muscle targeted protein expression

Fig. 4.8 shows muscle protein expression levels of targeted anabolic signalling proteins (A) and proteins thought to regulate MPB (B). No differences were observed when comparing target protein expression

levels between limbs following 3 days of unilateral lower immobilisation in the young and older volunteers (Figure 4.8 A and B), with exception to phospho-4E-BP1 (Thr37/46) expression which was less in the immobilised limb in young and older volunteers compared to the contralateral non-immobilised limb (51% $p<0.05$ and 52% $p<0.01$ respectively; Figure 4.8A). In addition, the magnitude of decline in protein expression of phospho-4E-BP1 (Thr37/46) 3x greater in older volunteers when compared to the young ($P<0.05$).

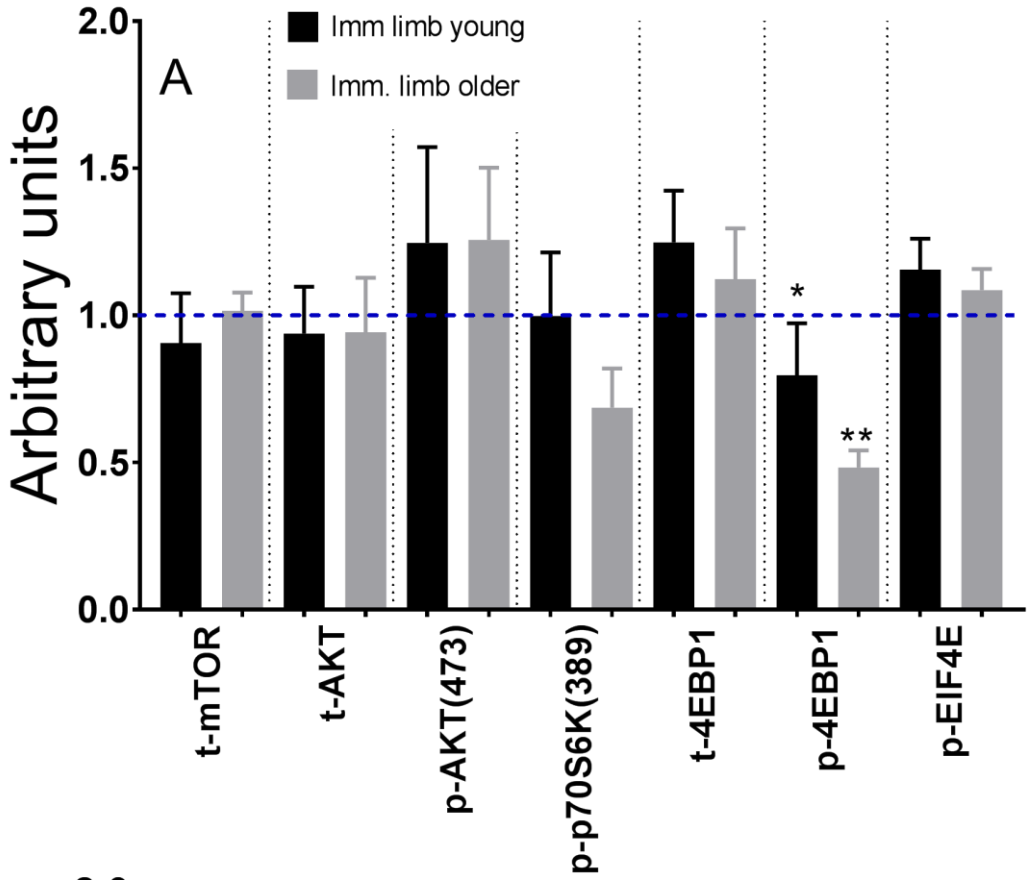

Figure 4.8A. *Medial gastrocnemius muscle protein expression in the immobilised limb presented as a fraction of the contralateral non-immobilised limb (blue line) following 3 days of unilateral lower limb immobilisation in young and older volunteers.* Values represent mean + SEM. * *and* ** signifies significant difference between limbs ($p < 0.05$ and $p < 0.01$ respectfully) AKT = Protein kinase B, mTOR = mammalian target of rapamycin, 4EBP1 = Eukaryotic translation initiation factor 4E-binding protein 1, P70S6K = Ribosomal protein S6 kinase beta-1 and EIF4E = Eukaryotic translation initiation factor 4E.

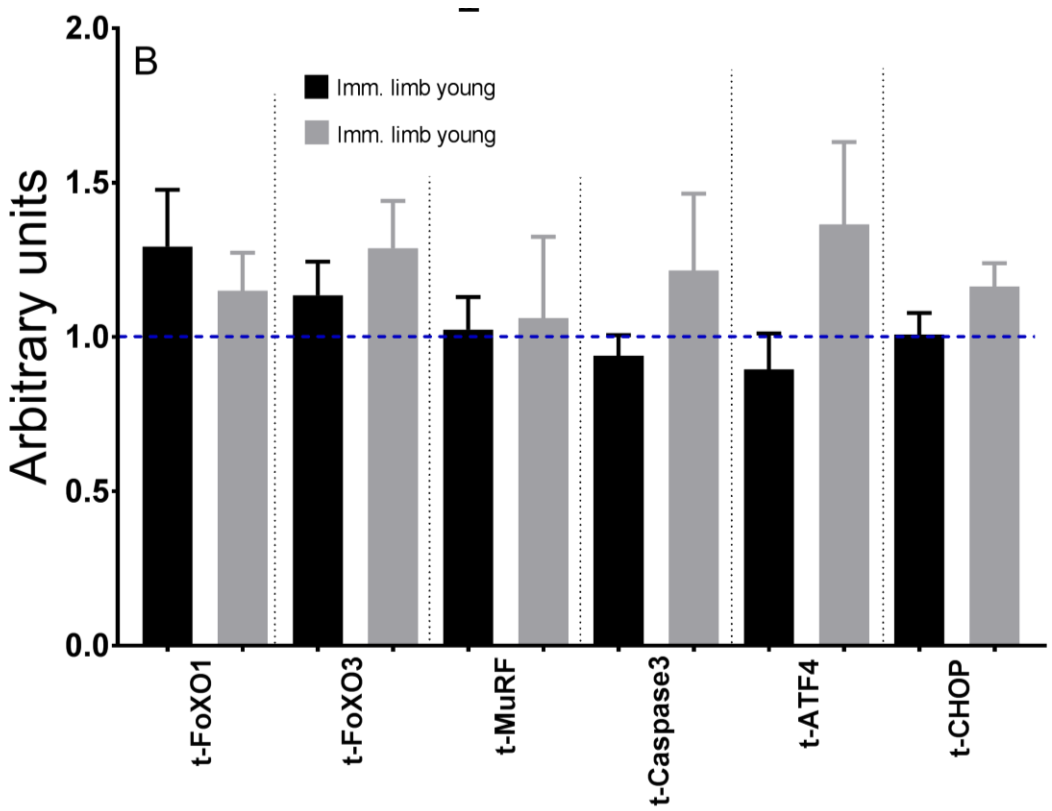

Figure 4.8B. *Medial gastrocnemius muscle protein expression in the immobilised limb presented as a fraction from the contralateral non-immobilised limb (blue line) following 3 days of unilateral lower limb immobilisation in young and older volunteers.* Values represent mean + SEM. FoXO1 = Forkhead box protein O1, FoXO3 = Forkhead box protein O3, CHOP = C/EBP homologous protein and ATF4 = Activating transcription factor 4.

4.5 **Discussion**

One aim of this study was to determine chronic myofibrillar FSR in the free living state and the impact of 3 days unilateral lower limb immobilisation on myofibrillar FSR in both the immobilised limb and the contralateral non-immobilised limb of young and older volunteers. Myofibrillar FSR in the medial gastrocnemius during free living conditions was similar between young (1.61 ± 0.20%d^{-1}) and older (1.61 ± 0.21 %d^{-1}) volunteers, and these values are similar to those recorded by Wilkinson and colleagues (1.45 ± 0.10 %d^{-1}) in young volunteers using the same methodology employed in the present study (Wilkinson et al., 2014). Major novel insight from this study reveals myofibrillar protein FSR over 3 days of unilateral lower limb immobilisation was significantly attenuated in both young (25%, $P<0.01$) and older (23%, $P<0.05$) individuals and to the same extent when compared to the free living state (Figure 4.7). Furthermore, whilst myofibrillar protein FSR in the immobilised limb of young volunteers was also less than in the contralateral non-immobilised limb (15%, $P<0.05$), this was not the case for the older volunteers where no difference between limbs was evident, such that myofibrillar protein FSR was less in both limbs compared with the free-living state (Figure 4.7). This suggests that immobilisation impacts upon chronic muscle protein FSR to the same extent in young and older volunteers, but that older volunteers probably sit down when one limb is immobilised, which is corroborated by physical activity data (Figure 4.3 and 4.4)

The majority of research into myofibrillar protein FSR during muscle disuse in humans has involved in healthy, young volunteers (de Boer et al., 2007;

Glover et al., 2008; Wall et al., 2013), but some studies have documented changes in older people (Kortebein et al., 2007; Tanner et al., 2015) using acute (hours) tracer infusion methodologies. Whilst acute studies have documented MPS to be attenuated from the free living state following several days of limb immobilisation in both young and older volunteers, this approach has failed consider the chronic myofibrillar FSR response to muscle unloading which will be influenced by nutrition (Aagaard et al., 2001) and physical activity (Breen et al., 2013). Mitchell and colleagues quantified chronic myofibrillar protein FSR in middle aged (45-60 years old) men over 14 days of unilateral lower limb immobilisation and reported it to be unaffected from baseline (Mitchell et al., 2018), despite thigh muscle cross-sectional area declining by ~4% when quantified using peripheral quantitative computed tomography. This observation contrasts findings from acute tracer infusion studies involving limb immobilisation in young (de Boer et al., 2007; Glover et al., 2008; Wall et al., 2013) and older participants (Tanner et al., 2015), and with the those of the current study where myofibrillar protein FSR declining over 3 days of unilateral lower limb immobilisation and to the same extent in young and older volunteers. Mitchell and colleagues (Mitchell et al., 2018) measured the average myofibrillar protein FSR response over 14 days of unilateral lower limb immobilisation and therefore any fluctuation in myofibrillar protein FSR over this time, particularly at the onset of immobilisation where the decline in myofibrillar protein FSR is thought to be greatest (de Boer et al., 2007) would have been missed. However, the findings of Mitchell and colleagues are difficult to rationalise as it is difficult understand how muscle protein FSR could be expected to increase (and above that seen in

the basal state) during sustained immobilisation, such as to achieve no overall change in the average protein FSR from the basal state over the 14 days of immobilisation, particularly as the authors reported muscle cross-sectional area declined. The incongruous observations could possibly be explained by recycling labelled amino acid within muscle. Muscle mass is always being turnover i.e. muscle proteins are catabolised into amino acids and re-synthesised into proteins. Therefore, this leads to the possibility of labelled-amino acid re-entering the protein pool. As there is no mechanism to detect how many times a labelled amino acid has been re-incorporated into the protein pool coupled with the extended period of study by Mitchell and colleagues (3 weeks) and the higher doses of D_2O used (150ml daily for 3 days then 100ml for the remainder), this will undoubtedly lead to an underestimated rate of protein synthesis.

By utilising the non-immobilised contralateral limb as a within subject control in tandem with quantification chronic myofibrillar protein FSR a second novel observation has emerged in the present study. Specifically, medial gastrocnemius myofibrillar protein FSR in the older volunteers was no different between the immobilised limb and the contralateral non-immobilised limb, i.e. myofibrillar protein FSR declined in both limbs from the free living state and to the same extent. This was not seen in the young volunteers, where myofibrillar protein FSR different between limbs and the rate in the non-immobilised limb was no different from the free living state. This reduction in myofibrillar protein FSR from the free living state in the non-immobilised limb of the older group is likely to be attributable to total physical activity levels declining more during

immobilisation in this group, i.e. they sat down rather than using the crutches provided. Indeed, in the older volunteers, habitual physical activity levels remained unchanged with immobilisation (Figure 4.4) and during the immobilisation period, average daily physical activity levels were ~22% lower in the older group when compared to the young group (Figure 4.4). Therefore, the less dramatic response observed between limbs in the older group is probably as a result of low habitual physical activity. Indeed, habitually reduced physical inactivity levels is known to impact negatively on muscle protein FSR and mass. For example, two weeks of reduced (~80%) ambulatory activity in young volunteers is sufficient to induce lower limb muscle atrophy when measured using DXA in young participants (Krogh-Madsen et al., 2010) which appears to be underpinned by reductions in postprandial myofibrillar FSR (Breen et al., 2013).

Aging is associated with a decline in skeletal muscle mass and anabolic resistance (i.e. the reduced ability to stimulate MPS in response to protein feeding) observed with aging (Cuthbertson et al., 2005) has been identified as a likely contributing factor for sarcopenia. However, during a period of 3-4 days of free living conditions between young and older volunteers we observed chronic myofibrillar FSR in the medial gastrocnemius to be similar between young (1.61 ± 0.20%d-1) and older (1.61 ± 0.21 %d-1) volunteers. Indeed, two weeks of unilateral lower limb immobilisation has shown to induce anabolic resistance in young humans (Glover et al., 2008) and aging is associated with habitually reduced physical activity (Frisard et al., 2007), such that the reduced sensitivity of

older muscle to a given protein dose as compared with the young may not be related to age *per se* but rather age-related reduced habitually physical activity.

The literature is in unclear as to whether older humans are more susceptible to muscle disuse related atrophy. For example, studies have shown older volunteers to lose less (Suetta et al., 2009) or more (Pisot et al., 2016; Tanner et al., 2015) muscle during muscle unloading when compared to young volunteers. The present data shows 3d of unilateral lower limb immobilisation to induce ~7% medial gastrocnemius muscle volume loss in young volunteers with no change in the older volunteers when measured using ultrasonography (Figure 4.5A). Furthermore, this occurred in the face of the decline in chronic myofibrillar protein FSR being the same between groups. The literature has drawn attenuation to initial muscle mass rather than aging per se being an independent driver of muscle volume decline during muscle unloading (Miles et al., 2005; Suetta et al., 2009). Smaller baseline forearm flexor muscle volume has shown to elicit lower declines in muscle volume during 21 d of elbow muscle arm suspension in young volunteers (Miles et al., 2005). Similarly, lower declines in quadriceps muscle volume measured using MRI were observed in older volunteers when compared to younger volunteers who had a higher baseline muscle volume during 2 weeks unilateral full leg cast immobilisation (Suetta et al., 2009). Therefore, it could be proposed that initial muscle mass can influence the magnitude of muscle mass loss during subsequent immobilisation. In this study, the young volunteers had a greater medial gastrocnemius volume when compared to the older

volunteers (YL: 154.76 ± 44.29 cm^3 vs. OL: 133.15 ± 27.48 cm^3) and the young group lost ~7% muscle volume during 3 days of immobilisation whilst muscle volume in the older group remained unchanged. Furthermore, there was a significant association (R^2= 0.33 P <0.05) between muscle volume in the free-living state and the decline in muscle volume during immobilisation in young, but not older, volunteers, however remained significant when the young and older data was combined (Figure 4.6).

Despite myofibrillar protein FSR declining by 22% from the free living state in immobilised limb of the older normal weight volunteers, medial gastrocnemius muscle volume did not change in the immobilisation limb (unlike in the young). In addition to the relatively lower decline in muscle FSR during immobilisation, the older volunteers were also less physically active and had a comparatively lower medial gastrocnemius muscle volume at baseline when compared to the young volunteers. Collectively, these factors would have likely resulted in less of a drive for muscle volume to decline during immobilisation in the older volunteers. This, together with the likelihood that older volunteers also had greater muscle lipid content and greater muscle fibrous connective tissue, which would not be discernible using ultrasonography, suggests that the ultrasound method employed was not sensitive enough to reflect the impact of a 22% reduction in myofibrillar protein FSR over 3 days of immobilisation.

Muscle pennation angle has been shown to increase during resistance exercise (Aagaard et al., 2001) in young volunteers and decline during unilateral lower limb immobilisation in young volunteers (Narici and

Cerretelli, 1998). Higher muscle pennation angles in pennate muscles make it possible to increase the quantity of contractile components in parallel into a certain volume therefore allowing the muscle to generate greater force. As shown previously (Turton et al., 2016) and in our study, baseline muscle pennation angle was lower in the older group when compared to the young, but this was only significant in the non-immobilised limb at baseline. During immobilisation, muscle pennation angle declined in the older group by ~10% but remained unchanged in the young group. Indeed, it has been shown the elderly are more susceptible to reductions in muscle force activation following 14 days of unilateral lower limb immobilisation, when compared to young volunteers (Suetta et al., 2009) and this could be linked to greater declines in muscle pennation angle during immobilisation. However, why muscle pennation angle declines, yet muscle volume in the older group remains unchanged requires further investigation.

The AKT/mTOR/p70S6K signalling axis by regulating protein translation initiation is thought to be the dominant pathway controlling MPS in response to exercise and feeding (Brook et al., 2015; Cuthbertson et al., 2005). Conversely, it may be assumed the total expression and phosphorylation of these proteins may be attenuated during immobilisation. However, the total expression and phosphorylation status of AKT/mTOR and p70S6K has been demonstrated to remain unchanged following 8 days of bed rest in young participants (Jespersen et al., 2014) and following 10 or 21 of unilateral lower limb suspension model in young participants (de Boer et al., 2007). Similar, we did not observe any

changes in total expression of mTOR and AKT and phosphorylation level of AKT and p70S6K despite observing declines in declines in medial gastrocnemius myofibrillar protein FSR in young and older volunteers during immobilisation. However, the phosphorylation level of 4E-BP1 (Thr37/46) was 52% ($P<0.05$) and 51% ($P<0.01$) less in the young and older volunteers respectfully in the immobilised limb when compared to the non-immobilised limb (Figure 4.7A). Eukaryotic initiation factor 4E (elF4E) binds to mRNA at the 5' end and induces translational initiation. However, the action of elF4E is prevented by 4E-BP1. Phosphorylation of 4E-BP1 prevents the binding of 4E-BP1 to elF4E promoting cap-dependent translation (reviewed by Richter and Sonenberg, 2005). Therefore, the reduction in myofibrillar protein FSR following 3 days of unilateral lower limb immobilisation in both young and older volunteers could be due to reduced phospho-4E-BP1 thereby attenuating cap dependent translational initiation and reducing myofibrillar protein FSR during immobilisation. However, our finding of reduced phospho-4E-BP1 (Thr37/46) goes against previous data from the literature (de Boer et al., 2007; Jespersen et al., 2014). The discordance in our results could be due to the difference in muscle group studied. We studied the medial gastrocnemius muscle which is most susceptible to muscle disuse induced atrophy (Belavy et al., 2017; de Boer et al., 2008) whilst *de Boer et al* and *Jespersen et al* studied the medial vastus lateralis. In addition, our immobilisation duration was 3 days in which the rate of muscle atrophy is at its highest (de Boer et al., 2007) whilst *de Boer et al* immobilisation duration was 10 or 23 days and *Jespersen et al* immobilisation period was 8 days and any acute transient changes in protein expression could have been missed.

In conclusion, this study for the first time demonstrates a reduction in chronic (measured through a sustained period of unloading) in medial gastrocnemius muscle myofibrillar protein FSR of 25% in young and 23% in older volunteers in the immobilised limb after 3 days of unilateral lower limb immobilisation when compared to the non-immobilised limb before immobilisation. In addition, following immobilisation a 15% reduction in medial gastrocnemius muscle myofibrillar protein FSR is observed in the immobilised limb when comparing limbs in young volunteers, however was no different in the older group which could be attributable to reduced average physical activity in the older group. Medial gastrocnemius muscle volume declined by 7% in the young group however remained unchanged in the elderly which may be as a result of a combination of factors such as; habitually reduced physical activity, reduced baseline muscle volume, increased contribution of lipid and fibrous connective tissue contributing to the overall contribution of muscle mass, such that the ultrasound method employed may not have been sensitive enough to reflect the impact of a 22% reduction in myofibrillar protein FSR over 3 days of immobilisation.

Chapter 5

5 The effect of bed-rest and subsequent rehabilitation exercise on muscle volume and targeted muscle gene networks in healthy young and older male volunteers

5.1 Introduction

Musculoskeletal deterioration during hospital admission and during recovery from illness and/or injury is a major clinical challenge in both young and older adults, but especially in the latter. Mandatory periods of bed rest or immobilisation lead to a number of negative metabolic and physiological changes, these include (but are not limited to); skeletal muscle atrophy (de Boer et al., 2008; Ferrando et al., 1996; Kortebein et al., 2007; LeBlanc et al., 1992), muscle weakness (Suetta et al., 2009), reduced muscle glucose uptake (Mikines et al., 1991; Richter et al., 1989), and a blunted protein synthetic response to amino acid feeding (Glover et al., 2008), all of which may be exacerbated in older people and may prolong recovery (Suetta et al., 2009).

Muscle mass is constantly being turned over through the dynamic processes of MPS and MPB. In mature adults, MPS and MPB fluctuate throughout a diurnal cycle but remain in overall balance to maintain muscle mass (Atherton and Smith, 2012). Nevertheless, sustained periods of muscle unloading, in the form of reduced step count (Breen et al., 2013), unilateral limb immobilisation (Jones et al., 2004) and bed-rest (Ferrando et al., 1996; LeBlanc et al., 1992; Symons et al., 2009) lead to

muscle mass loss. This is thought to occur principally through a decline in post-absorptive MPS (de Boer et al., 2007; Ferrando et al., 1996) and a blunting of post-prandial amino acid induced increases in MPS, termed anabolic resistance (Glover et al., 2008). Furthermore, ageing is accompanied by anabolic resistance in response to protein ingestion and diminished anabolic signalling even in the absence of a muscle unloading intervention (Cuthbertson et al., 2005). Therefore, muscle unloading and ageing both impair muscle protein metabolism.

Whether older people are more susceptible to muscle disuse related atrophy currently remains unclear. Fourteen days of unilateral full leg immobilisation has been shown to result in a decline in quadriceps muscle volume of ~9% in young males using MRI compared to ~5% in older males (Suetta et al., 2009), conversely 14 days of bed rest has been reported to result in a quadriceps muscle volume decline of ~6% in young males and ~8% in older males, again measured using MRI (Pisot et al., 2016). Furthermore, there are mixed reports concerning whether the muscle of older individuals is as responsive as younger muscle to resistance exercise, with reports of attenuated (Welle et al., 1996), similar (Ivey et al., 2000) and greater (Kosek and Bamman, 2008) muscle mass recovery in the elderly. In addition, little is known about the molecular pathways regulating these changes in muscle volume during muscle disuse atrophy and subsequent rehabilitation in humans, especially in context to ageing. Indeed, in **Chapter 4**, in keeping with previous human muscle unloading studies (de Boer et al., 2007; Jespersen et al., 2014), I did not observe any impact of immobilisation on the expression of putative

regulators of MPS, MPB and ER stress related pathway with exception to phospho-4E-BP1 (Thr37/46) which was 52% ($P<0.05$) less in the young group and 51% ($P<0.01$) in the older group.

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After a period of immobilisation adaptive changes in skeletal muscle result in deleterious metabolic effects. Seven days of a unilateral limb immobilisation in young males results in a blunting of local muscle glucose uptake following a euglycemic hyperinsulinemic clamp, whilst utilising the contralateral non-immobilised limb as a control (Richter et al., 1989). Similarly, bed rest induces whole body insulin resistance in human, as Mikines and colleagues demonstrated following seven days of bed rest the action of insulin was depressed in the whole body with reduced glucose uptake measured using a euglycemic clamp. It would seem the time course of muscle insulin resistance occurs fairly early as following 3 days of bed-rest whole body muscle glucose uptake following an Oral glucose tolerance test was depressed (Smorawinski et al., 2000). In addition, it has been shown peripheral insulin resistance develops following 2 weeks of reduced physical activity measured using a hyperinsulinemic-euglycemic clamps (Krogh-Madsen et al., 2010). Therefore, taken together the decline in muscle contractile activity drives the attenuation in postprandial muscle glucose uptake. However, the full mechanism by which this occurs has not been elucidated. In addition, aging is also associated with muscle insulin resistance, however whether this due to aging per se, or due to age-related reduced physical activity (Frisard et al., 2007) or age related changes in body composition, namely a reduction in skeletal tissue (lean

body mass) and relative increase in adipose tissue, remains unknown. Indeed, it is well documented that obesity leads to muscle insulin resistance (DeFronzo and Tripathy, 2009). One possible mechanism for the development of muscle insulin resistance during muscle unloading could be due to the increase in IMAT following muscle unloading. Manini and colleagues demonstrated following 4 wk of unilateral lower limb suspension in young subject, IMAT increased by 20% in the calf and 14% in the thigh when quantified using MRI (Manini et al., 2007). Furthermore, Boettcher and colleagues demonstrated increased levels of IMAT quantified using MRI was associated with insulin resistance quantified using a glucose clamp (Boettcher et al., 2009). Therefore, it seems logical that increased IMAT content may play a role in the aetiology of skeletal muscle insulin resistance during muscle unloading. Furthermore, the increase in IMAT will increase intracellular fatty acid metabolites, such as long chain fatty acyl CoAs, long chain acylcarnitines, diacylglycerols and ceramides, which are known to inhibit insulin signalling and pyruvate dehydrogenase flux thereby reducing muscle glucose utilisation.

5.2 **Aims**

Given little is known about the molecular pathways regulating changes in muscle volume and fuel metabolism during immobilisation and subsequent rehabilitation in humans, especially in context to ageing, and furthermore, that immobilisation is known induce alterations in muscle mass and fuel selection in humans (Crossland et al., 2019). The overall aim of this study was to determine the effect of 14 days bed rest and subsequent exercise rehabilitation on quadriceps muscle volume and mRNA expression of genes

thought to regulate muscle architecture and mass, and muscle carbohydrate and lipid metabolism in young and older volunteers. Analysis was performed using RT-PCR microfluidic, low-density array cards focused on 96 targeted gene transcripts, and subsequent bioinformatics analysis (Ingenuity Pathway Analysis). The specific aim being to unravel novel insights into gene responses to muscle disuse in bed-rest and subsequent rehabilitation in the context of aging.

5.3 Methods

The muscle biopsy collection and MRI data generated for use in this study has previously been reported (Biolo et al., 2017; Pisot et al., 2016; Rejc et al., 2018). However, all mRNA and bioinformatics analyses were performed at the University of Nottingham, and is unpublished data and specific to this thesis.

Study volunteers

Twenty-three healthy men were recruited into the study. Seven volunteers aged 18 –30 yr represented a young group (YG), whilst 16 volunteers aged 55– 65 yr were allocated to an older (OG). Each group had similar anthropometric characteristics **Table 5.1.** Prior to recruitment into the study, all participants were informed of the purpose, procedures, and potential risks of the study before providing written consent. All participants underwent a medical health examination which consisted of a routine blood and basic anthropometric parameters **Table 5.1.** Exclusion criteria included: smoking; regular alcohol consumption over >21 units; ferromagnetic implants; history of deep vein thrombosis supported by a

blood D-dimer concentration >500 μg/l; acute or chronic skeletal, neuromuscular, metabolic, and cardiovascular disease conditions; and any history of pulmonary embolism. The study was performed in accordance with the ethical standards of the 1964 Declaration of Helsinki and was approved by the National Ethical Committee of the Slovenian Ministry of Health on April 17, 2012, under the acronym IR-aging 1200.

Study protocol

The study was conducted in the Orthopaedic Hospital of Valdoltra, Slovenia in a controlled medical environment and replicated a scenario similar to that of hospital admissions. Fourteen days of bed rest were conducted in an air-conditioned room with 24 hr surveillance. Subjects performed all daily activities in bed and were allowed to freely communicate, watch television and listen to the radio, read, use computer and to receive visitors. During bed rest, all subjects received passive physiotherapy treatments (i.e. joint mobility and stretching, relief massage in the presence of acute back pain) three times per week to avoid cardiovascular disorders. To prevent thrombosis, a D-dimer test was repeated on Day 7 of bed rest where participants reached elevated values but scored < 500 μg/l −1. Dietary energy requirements were calculated for each subject multiplying resting energy expenditure by 1.2 in the bed rest period, in accordance with ESA guidelines to maintain energy balance (Beals et al., 2018). Meals were given 3 times per day with the macronutrient food content set at 60% carbohydrate, 25% fat and 15% proteins. Energy balance was checked weekly by fat mass assessment using bioimpedance analysis.

Following bed rest, participants underwent a 14 day programme of mixed exercise (resistance and endurance) rehabilitation at the hospital and in a gym near the hospital. Each rehabilitation session took place 3 times per week and comprised of 12-min warm-up, 15–20 min of balance and strength training, and 20–30 min of endurance training. The rehabilitation programme was aimed at stimulating both muscular and cardiorespiratory

adaptation. The exercises chosen to be performed during the rehabilitation programme did not require specific training equipment so that they could be translated to any home and community environment. However, training was supervised by 6 physical trainers who instructed and ensured exercise manoeuvres were performed properly. Specifically, the first 12 min of each training session was devoted to warm-up; subjects performed 6 min of Nordic walking, its speed being determined from a 2-km walking test performed before bed rest, and 6 min of active stretching (10 exercises). Subjects then performed 20 min of strength and balance exercises. This section started with half squats (one set; 10 repetitions; overload: from no overload to a 6 kg ball held with both hands), and continued with circuit training (30 s of exercise followed by 30 s of rest) that consisted of 8 motor tasks including lower body exercise such as a frontal and sagittal plane lunge, a double leg heel raise with elastic resistance and hip extension with elastic resistance. The balance exercises mainly consisted of dynamic standing balance activities (i.e. standing on toes; standing on one leg on balance foams) and functional movements that involved reaching and passing objects. Strength and balance exercises were followed by 30 min of aerobic exercise (e.g. Nordic walking, brisk walking, running). The final 3 min of each session was devoted to cool down (relaxation and breathing exercises). Heart rate was monitored during each training session. All subjects performed all planned training sessions.

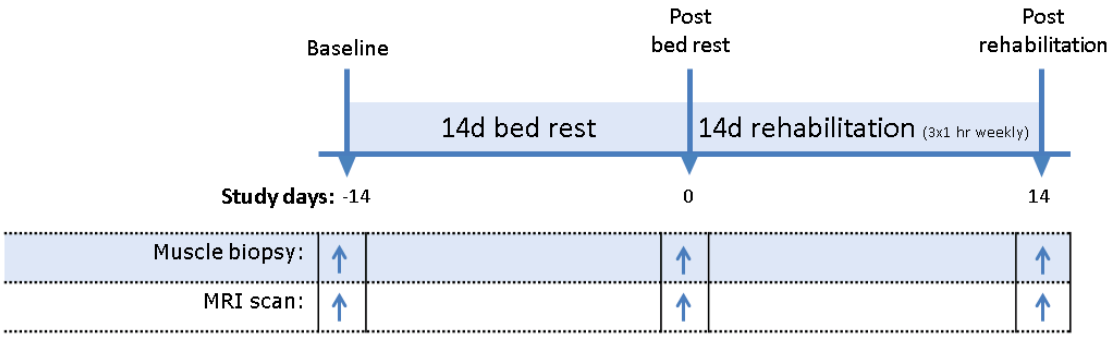

Figure 5.1. A schematic overview of the study protocol

Study procedures

Study procedures were performed at 3 time-points; the start of bed rest, after 14 days of best rest and on day 14 of the rehabilitation programme (see Figure 5.1). Muscle biopsies were performed on the mid-region of the left vastus lateralis muscle. Local anaesthesia (lidocaine (2 ml, 2%) was applied to the skin and surrounding tissue before a small incision was made to the fascia of the muscle allowing the passage of a purpose-built rongeur (Zepf Instruments, Tuttlingen, Germany) to harvest muscle. The muscle sample was frozen in isopentane cooled with liquid nitrogen and stored in a freezer at -80°C until analysis (see below). A portion of muscle was shipped to the University of Nottingham for mRNA analysis

Quadriceps muscle volume of the right leg was determined using turbo spin-echo, T1-weighted, magnetic resonance imaging (MRI) obtained with 1.5 T MRI system (Magnetom Avanto; Siemens Medical Solution, Erlangen, Germany). MRI images were analysed by an expert in MRI imaging using image processing tool OsiriX (version 4.1.2; Pixmeo Sarl). Quadriceps

muscle volume was then calculated by summing a series of evenly spaced truncated cones between each two axial images, a process that included an average of 25 images (range 23–28) and covered the entire length of the quadriceps.

mRNA expression analysis

Total muscle RNA was extracted (as described in detailed in **Chapter 2 section 9**) using the Trizol Method. RNA concentration was quantified spectrophotometrically using a NanoDrop® (NanoDrop® Technologies Inc, Wilmingtion, USA). Following spectrophotometric quantification, cDNA was synthesised as described in earlier in Chapter 2.10 using SuperScriptTM III reverse transcriptase. Following cDNA synthesis, mRNA expression was quantified using an ABI PRISM 7900T sequence detection system and SDS 2.1 software (Applied Biosystems, Foster City, CA, USA). A custom designed low-density RT-PCR microfluidic array card with 96 unique gene targets (Appendix 1.2) was utilised to measure relative mRNA abundance of genes representing multiple pathways involved in regulation of muscle architecture and mass, and carbohydrate, lipid and protein metabolism. Target selection was directed by data from three published studies from our laboratory. The first study reported an Affymetrix based analysis of tissue sampled at rest pre- and 24 hrs-post resistance exercise in healthy volunteers (Pi-Sunyer, 1999). The second, documented mRNA expression levels for 90 genes spanning carbohydrate metabolism, fat metabolism, insulin signalling and proteolysis using low-density microfluidic cards as per the present study (Arentson-Lantz et al., 2016). The third study highlighted mRNA expression changes following a period of immobilisation

and resistance exercise based rehabilitation in young healthy volunteers (Jones et al., 2004).

Data was analysed using SDS RQ manager which automatically determined threshold cycle CT for each gene. Relative mRNA abundance was calculated using the ΔΔCT method with the participant's pre-bed rest sample as the calibrator (post-bed rest and post-rehabilitation) and HMBS as the endogenous control. HMBS exists in two isoforms with the first isoform, and the one used in the present study, being ubiquitously expressed and a reliable endogenous reference for use in real-time PCR (Pietiläinen et al., 2008). The second isoform of HMBS is involved in haem biosynthesis.

Ingenuity Pathway Analysis

Muscle mRNA abundance was analysed using Ingenuity Pathway Analysis (IPA) software (Redwood City, CA, USA). IPA is a web-based commercial software application that enables analysis, integration and understanding of data obtained from gene expression, miRNA and SNP microarrays, as well as metabolomics, proteomics and RNA sequencing experiments. It can also be used for analysis of small-scale experiments that generate gene and chemical lists. Data analysis and interpretation with IPA is built on a comprehensive, manually curated data base (Ingenuity Knowledge Base, https://analysis.ingenuity.com/). Powerful algorithms identify regulators, relationships, mechanisms, functions and pathways relevant to changes observed in an analysed dataset. Analytics go beyond pathway analysis to understand the experimental results within the context of biological systems (https://www.qiagen.com/ie/products/life-science-

research/research-applications/gene-expression-analysis/analysis/ingenuity-pathway-analysis/).

5.4 **Results**

Table 5.1. Baseline age and anthropometric characteristics for the two groups of healthy, male participants

Subject parameter	**Young group (YG)**	**Older group (OG)**	***P* value**
N	7	16	
Age (years old)	23.1 ± 2.9	59.6 ± 3.4	< 0.001
Height (m)	1.77 ± 0.07	1.73 ± 0.05	0.192
Weight (kg)	74.8 ± 8.8	79.9 ± 12.3	0.336
Body mass index (kg/m^2)	24.0 ± 2.4	26.6 ± 4.4	0.142

Values are means ± SD

Quadriceps Muscle Volume

Fig. 5.2A shows quadriceps muscle volumes at baseline, following 14 days bed rest and following 14d rehabilitation in young and older volunteers. Baseline quadriceps muscle volume was 1988 ± 270 cm^3 in the YG which was significantly greater than that of the OG at the corresponding time-point (1666 ± 234 cm^3, P<0.05). Following 14d of bed rest, quadriceps muscle volume had declined by 6% in the YG to 1867 ± 204 cm^3 (p = 0.001); the average absolute muscle volume decline being 121 ± 37 cm^3

(Fig. 5.2B). Similarly, quadriceps muscle volume declined by 8% during bed rest in the OG to 1525 ± 211 cm^3 (p = 0.05), which represents an average absolute muscle volume decline of 141 + 17 cm^3. Need to sate here whether the absolute and relative decline in muscle volume was different when comparing YG and OG (and give P values). From Fig 5.2 they look to be no different

Subsequent 14 days of exercise rehabilitation training in the YG resulted in an increase in quadriceps muscle volume to 1954 ± 211 cm^3 (P<0.05), which represents a 4% increase from the post-bed rest value, and restored muscle volume in the YG to a value no different from baseline. Similarly, exercise rehabilitation increased quadriceps muscle volume in the OG following bed rest to 1615 ± 188 cm^3 (P<0.001), but this remained significantly less than the baseline volume in this group (P<0.05; probably because of the lower between individual variance in this group compared to the YG). Indeed, despite this age-related difference, the absolute muscle volume gain during exercise rehabilitation in the YG (86 ± 81 cm^3) was no different from that observed in the OG (89 ± 50 cm^3, P>0.05).

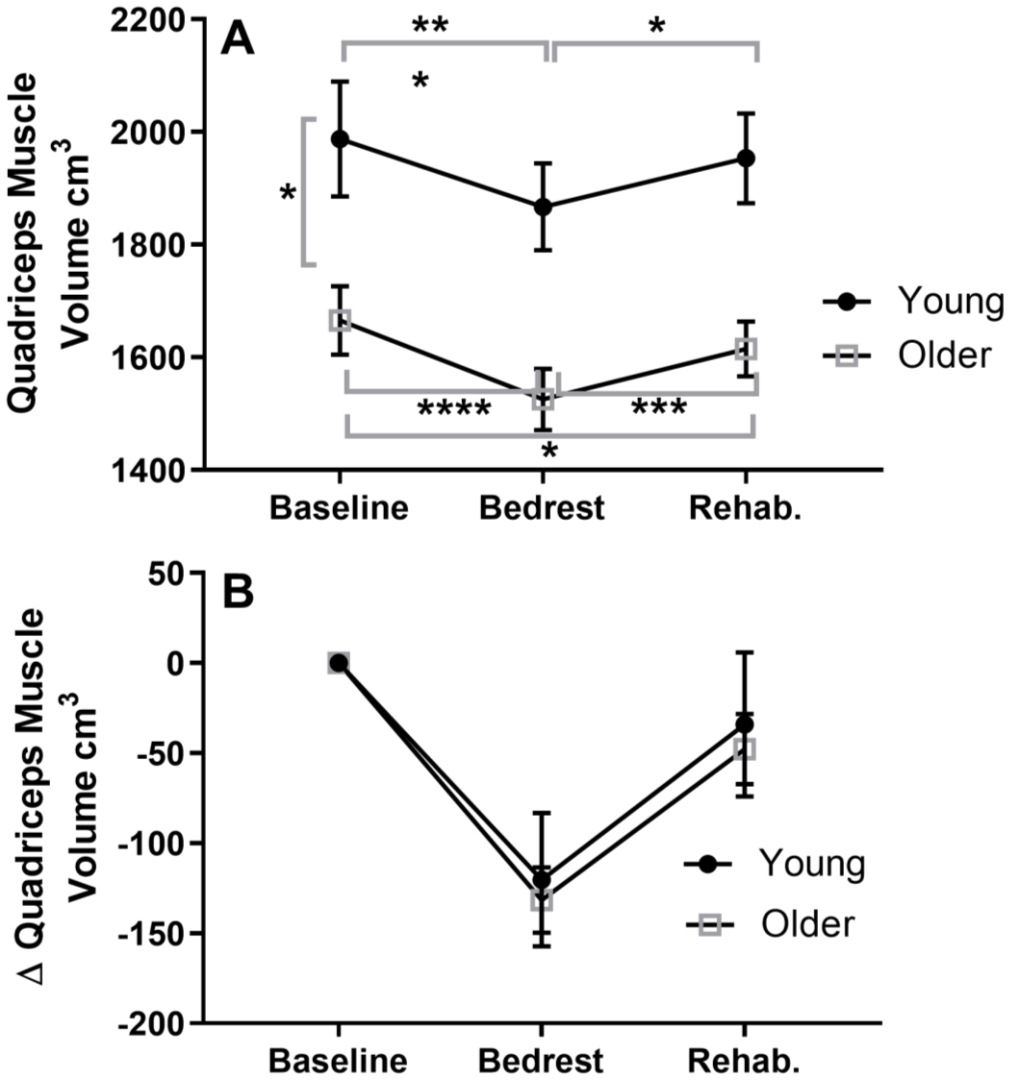

Figure 5.2. *Quadriceps muscle volume measured using 1.5T MRI in young and older participants; during baseline, following 14 days of bed rest and 14 days of mixed (endurance and resistance) rehabilitation. Figure* ***A*** *represents absolute muscle volume whilst* ***B*** *represents the change in muscle volume.*

Fig. 5.3 illustrates the association between baseline quadriceps muscle volume and the decline in quadriceps muscle volume during 14 days of

bed rest in young and older volunteers. The X and Y axis indicates baseline quadriceps muscle volume and 14d bed rest induced quadriceps muscle volume decline, respectively. There was a significant association (R^2 = 0.18, P<0.05), such that the greater muscle volume was at baseline the more muscle volume declined during bed rest, which appeared to be independent of age. No association was observed with between muscle volume following bed rest and muscle volume gain during the 14 days of rehabilitation training.

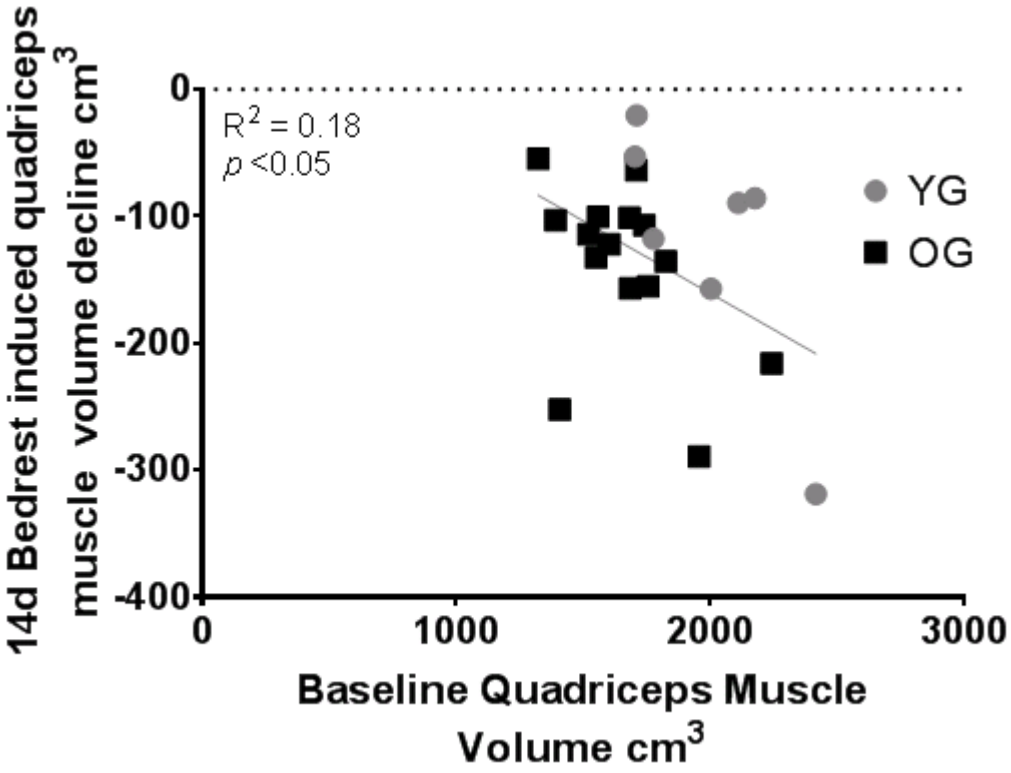

Figure 5.3. *The relationship between baseline quadriceps muscle volume measured using 1.5T MRI in young (YG) and older (OG) participants and muscle volume decline during 14 days bedrest.*

Fig. 5.4 depicts the cellular functions altered during 14d bed rest in young and older volunteers. These cellular functions were predicted by Ingenuity

Pathway Analysis based on the mRNA expression changes recorded over the course of bed rest. The X axis depicts cellular functions, and the Y axis -log of the P value (calculated by IPA using Fisher's exact right-tailed test, set at P <0.05) and the threshold indicates the minimum significance level [scored as −log(p-value) from Fisher's exact test, set here to 1.25]. Cellular carbohydrate metabolism, protein synthesis, lipid metabolism and inflammatory response functions were identified as being markedly altered by bed rest, with the magnitude of response for each function being similar between young and older volunteers.

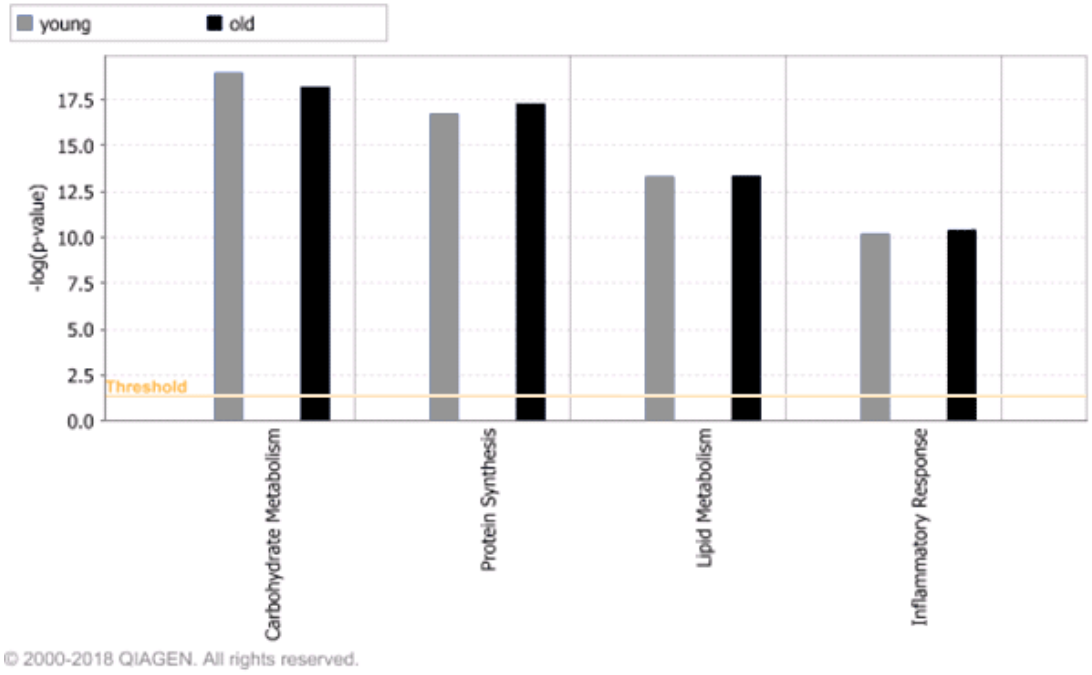

Figure 5.4. *Cellular functions altered from baseline during 14d bed rest in young and older volunteers as predicated by IPA based on mRNA expression changes observed in young and older volunteers*

Fig. 5.5 shows cellular gene functions as predicted by IPA which changed the most during 14d rehabilitation in young and older volunteers based on mRNA expression data generated using the low-density microarray cards. The X and Y axis indicates cellular functions most affected by 14d bed rest and -log of the P value, respectively. The –log of the P value was calculated by Fisher's exact test right-tailed ($P < 0.05$) and the threshold indicates the minimum significance level [scored as −log(p-value) from Fisher's exact test, set here to 1.25]. Cellular development, lipid metabolism, protein synthesis and Cell death and survival were identified as functional pathways which significantly changed during rehabilitation in young and older volunteers.

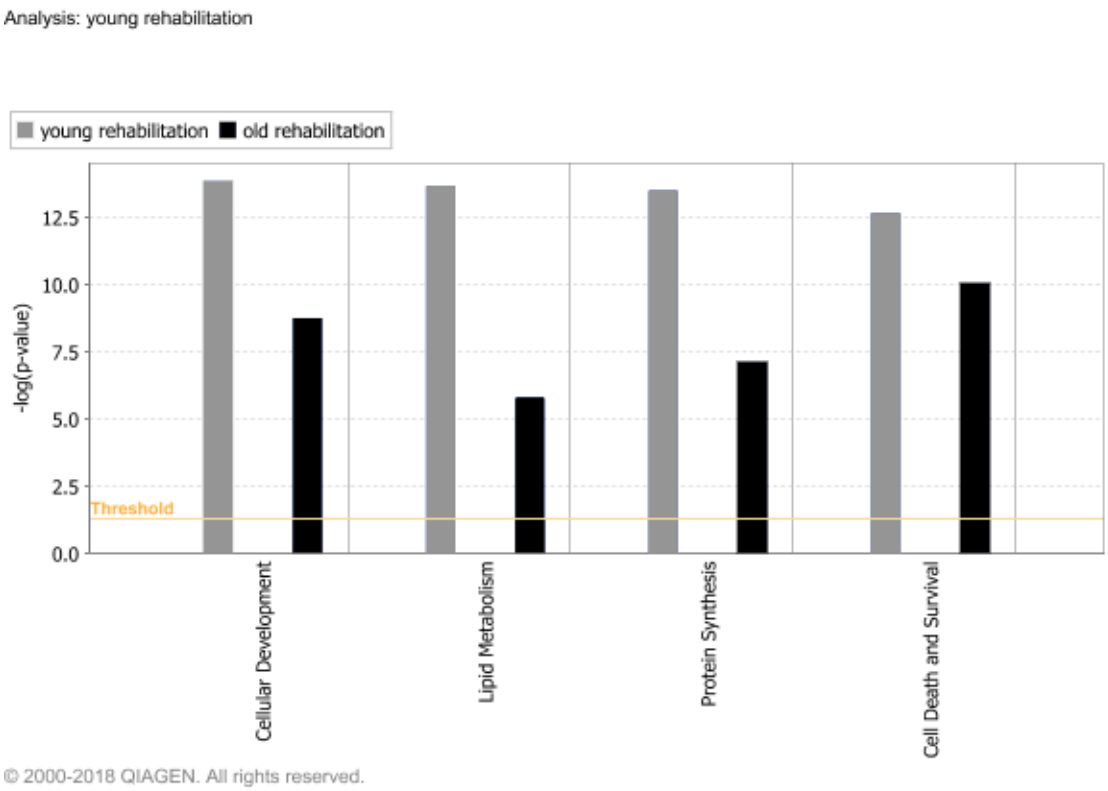

Figure 5.5. *Cellular functions altered from baseline during 14 d exercise rehabilitation in young and older volunteers as predicated by IPA based on mRNA expression changes observed in young and older volunteers.*

Figure 5.6 depicts differentially regulated mRNAs (outer ring) and IPA prediction of changes in muscle carbohydrate metabolism functions based on changes in mRNA expression from baseline following 14 days of bed rest in young and older volunteers. In both the young and older group, HIF1A, HK2, IGF1, IL6, INSR, MTOR, MYOG, PDK4, PFKM, PPAR, PRKAA1, PRKAA2, RPTOR, STAT3, ADIPOQ, FABP2, NFE2L2, OSTN, CPT1A, PPARG, IKBKB, MAPK9, SOCS3, AKT1 and GSK3B increased in abundance during bed rest with exception to LDHA, PKM and PPARGC1A which were

upregulated in the young only and PDK2 and UCP which was upregulated in the older volunteers only. IPA predicted activation in muscle carbohydrate metabolism functions in; glycolysis of cells, uptake of 2-deoxyglucose, glycolysis, uptake of D-glucose and uptake of monosaccharide in both the young and older groups whilst only predicting muscle carbohydrate metabolism functions in; production of lactic acid, quantity of lactic acid and transport of D-glucose in the young group only during bed rest.

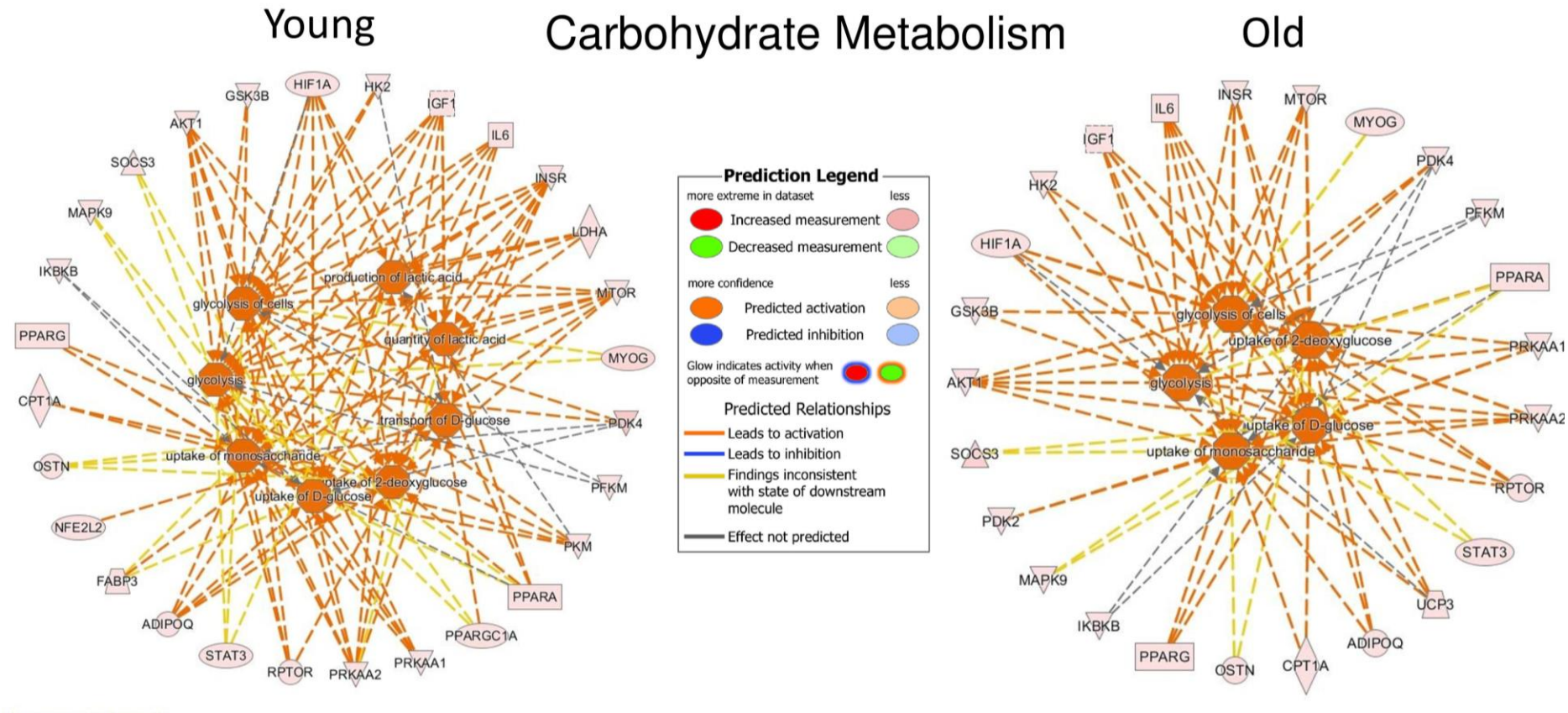

Figure 5.6. Differentially regulated mRNAs (outer ring) and IPA prediction of changes in muscle carbohydrate metabolism based on changes in mRNA expression from baseline following 14 days of bed rest in young and older volunteers.

Abbreviations: HIF1A = Hypoxia-inducible factor 1-alpha, HK2 = Hexokinase 2, IGF 1 = Insulin-like growth factor 1, IL6 = Interleukin 6, INSR = Insulin receptor, LDHA = Lactate Dehydrogenase A, MTOR = Mechanistic Target Of Rapamycin Kinase, MYOG = Myogenin, PDK2 & 4 = Pyruvate Dehydrogenase Kinase 2 and 4, PFKM = Phosphofructokinase (Muscle), PPARA & PPARG = peroxisome proliferator-activated receptor α, and γ, PPARGC1A = PPARG Coactivator 1 Alpha, PRKAA1 & 2 = Protein Kinase AMP-Activated Catalytic Subunit Alpha 1 and 2, RPTOR = Regulatory Associated Protein Of MTOR Complex 1, STAT3 = Signal Transducer And Activator Of Transcription 3, UCP3 = Uncoupling Protein 3, ADIPOQ = Adiponectin, C1Q And Collagen Domain Containing, FABP3 = Fatty Acid Binding Protein 3, NFE2L2 = Nuclear Factor, Erythroid 2 Like 2, OSTN = Osteocrin, CPT1A = Carnitine Palmitoyltransferase 1A, IKBKB = Inhibitor Of Nuclear Factor Kappa B Kinase Subunit Beta, MAPK9 = Mitogen-Activated Protein Kinase 9, SOCS3 = Suppressor Of Cytokine Signaling 3, AKT1 = AKT Serine/Threonine Kinase 1 and GSK3B = Glycogen Synthase Kinase 3 Beta

Figure 5.7 depicts differentially regulated mRNAs (outer ring) and IPA prediction of changes in muscle protein metabolism functions based on changes in mRNA expression from baseline following 14 days of bed rest in young and older volunteers. In both the young and older group, EIF2S1, EIF4B, EIF4EBP1, FBXO32, FST, GSK3B, HSPA8, IGF1, IKBKB, IL6, INSR, MSTN, MTOR, NFKB1, PPARA, PPARG, PRKAA1, PRKAA2, RPS6KB2, SOCS3, SOD2, STAT3, TLR4, TRAF3, TRIM63, TUFM, IL6R, NFE2L2, ADIPOQ, AKT1, ATF4, ATF6, CAPN1, CAPN2 and CASP8 increased in abundance during bed

rest with FOXO1 in the young group and CCL19 in the older group being shown as highly upregulated. IPA predicted activation in muscle protein metabolism functions in; metabolism of protein and quantity of cytokine in both the young and older groups during bed rest.

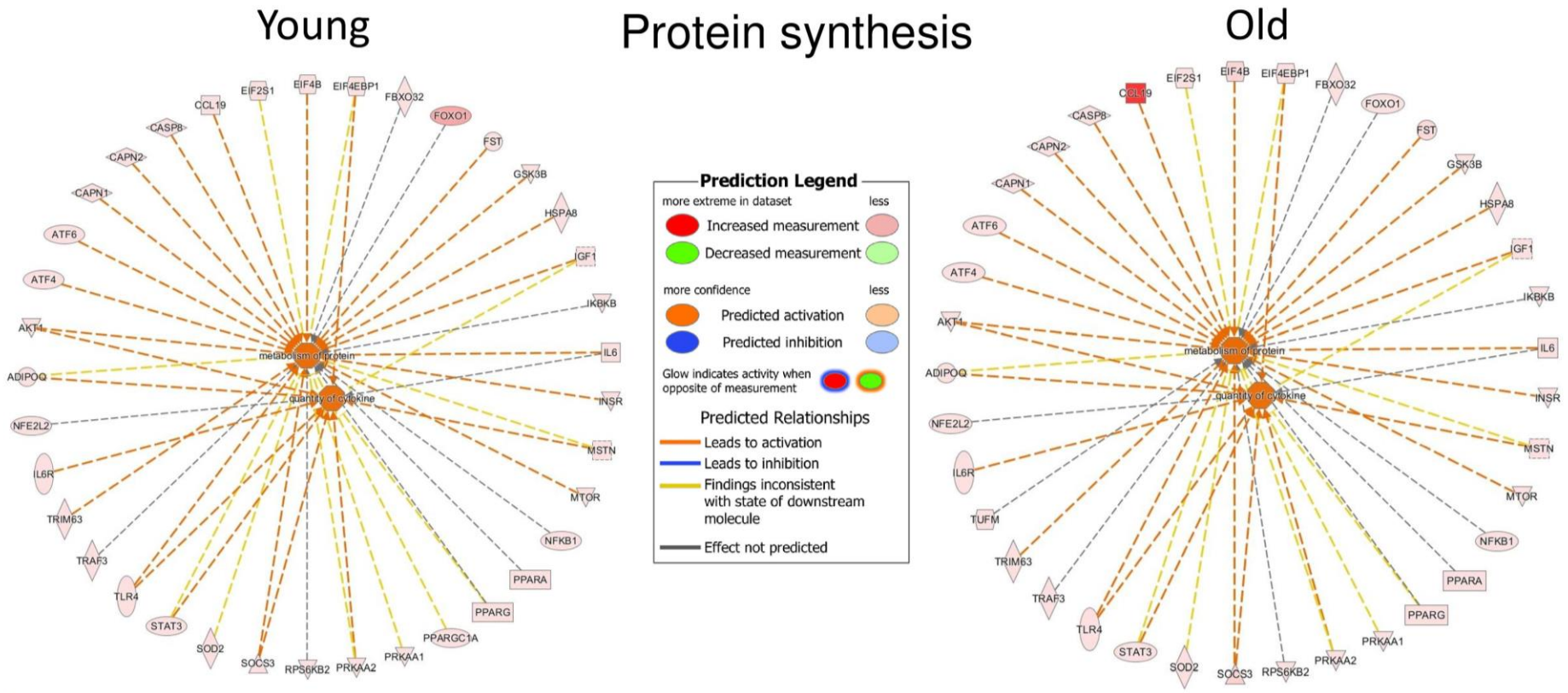

Figure 5.7. Differentially regulated mRNAs (outer ring) and IPA prediction of changes in muscle protein synthesis metabolism based on changes in mRNA expression from baseline following 14 days of bed rest in young and older volunteers.

Abbreviations: EIF2S1 = Eukaryotic Translation Initiation Factor 2 Subunit Alpha, EIF4B = Eukaryotic Translation Initiation Factor 4B, EIF4EBP1 = Eukaryotic Translation Initiation Factor 4E Binding Protein 1, FBXO32 = F-Box Protein 32, FOXO1 = Forkhead Box O1, FST = Follistatin, GSK3B = Glycogen Synthase Kinase 3 Beta, IGF 1 = Insulin-like growth factor 1, IKBKB = Inhibitor Of Nuclear Factor Kappa B Kinase Subunit Beta, IL6 = Interleukin 6, INSR = Insulin receptor, MSTN = Myostatin, MTOR = Mechanistic Target Of Rapamycin Kinase, NFKB1 = Nuclear Factor Kappa B Subunit 1, PPARA & PPARG = peroxisome proliferator-activated receptor α, and γ, PRKAA1 & 2 = Protein Kinase AMP-Activated Catalytic Subunit Alpha 1 and 2, RPS6KB2 = Ribosomal Protein S6 Kinase B2, SOCS3 = Suppressor Of Cytokine Signaling 3, SOD2 = Superoxide Dismutase 2, STAT3 = Signal Transducer And Activator Of Transcription 3, TLR4 = Toll Like Receptor 4, TRAF3 = TNF Receptor Associated Factor 3, TRIM3 = Tripartite Motif Containing 3, TUFM = Tu Translation Elongation Factor, Mitochondrial, IL6R = IL6 = Interleukin 6 receptor, NFE2L2 = Nuclear Factor, Erythroid 2 Like 2, ADIPOQ = Adiponectin, C1Q And Collagen Domain Containing, AKT1 = AKT Serine/Threonine Kinase 1, ATF4 & 6 = Activating Transcription Factor 4 & 6, CAPN1 & CAPN2 = Calpain 1 & 2, CASP5 = Caspase 5, CCL19 and C-C Motif Chemokine Ligand 19.

Figure 5.8 depicts differentially regulated mRNAs (outer ring) and IPA prediction of changes in muscle lipid metabolism functions based on changes in mRNA expression from baseline following 14 days of bed rest in young and older volunteers. In both the young and older groups, GPD2, IGF1, INSR, PDK4, PPARA, PPARG, PRKAA1, PRKAA2, CASP8, HSPA8, IL6,

MAPK9, MTOR, NFKB1, SDHA, SOCS3, STAT3, TLR4, HIF1A, NRF1, ADIPOQ, AKT1 and CPT1A increased in abundance during bed rest with exception to FABP3, SMAD2, RPTOR, MSTN, ATF6, PFK and PPARGC1A which were upregulated in the young only and UCP3 and PDK2 which was upregulated in the older volunteers only. IPA predicted activation in muscle lipid metabolism functions in; oxidation of palmitic acid, oxidation of fatty acid, oxidation of fatty acid and fatty acid metabolism in both the young and older groups during bed rest. However, IPA predicted activation in muscle lipid metabolism functions in synthesis of prostaglandin E2 and oxidation of long chain fatty acid in the older group whilst, synthesis of fatty acid, synthesis of lipid and uptake of fatty acid in the young group during bed rest. Furthermore, IPA predicted inhibition if concentration of fatty acid in muscle lipid metabolism functions during bed rest.

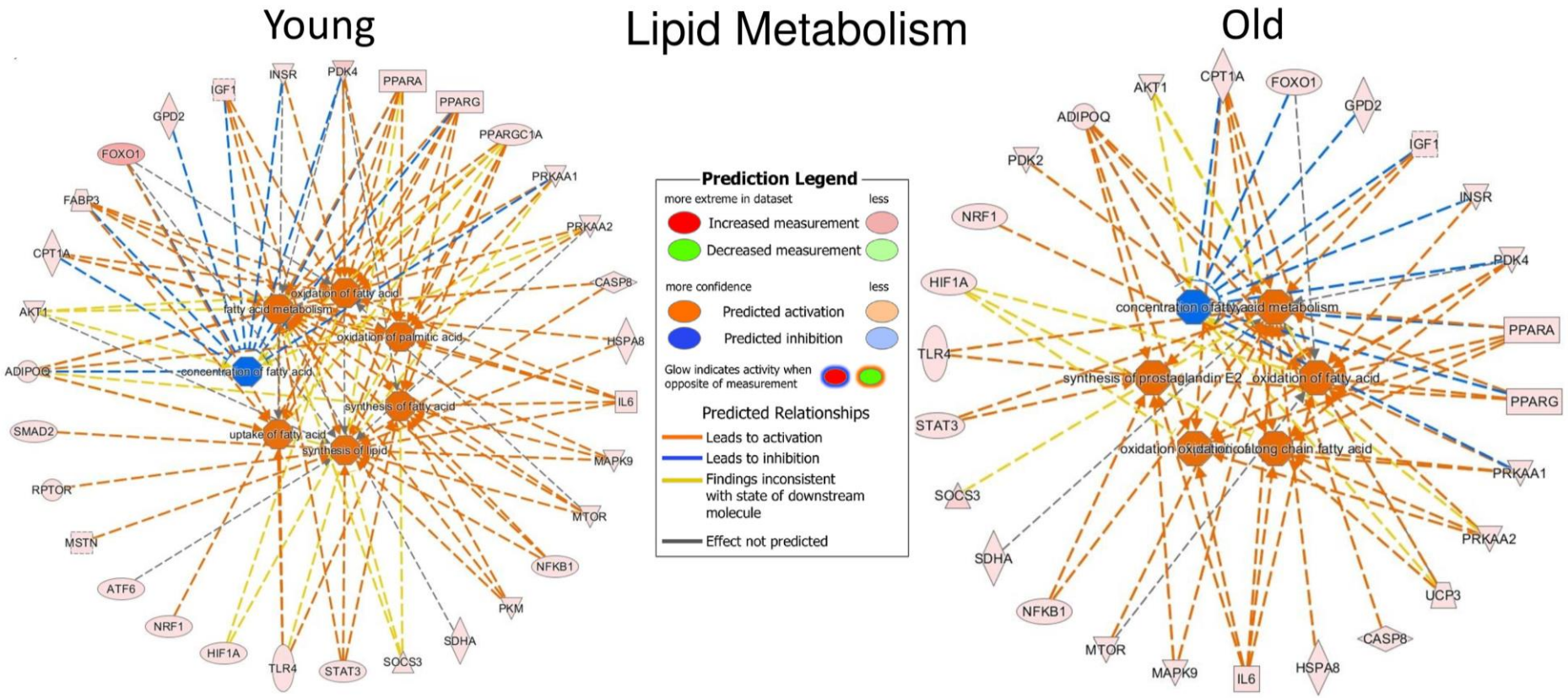

Figure 5.8. Differentially regulated mRNAs (outer ring) and IPA prediction of changes in muscle lipid metabolism based on changes in mRNA expression from baseline following 14 days of bed rest in young and older volunteers.

Abbreviations: CPT1A = Carnitine Palmitoyltransferase 1A, FOXO1 = Forkhead Box O1, GPD2 = Glycerol-3-Phosphate Dehydrogenase 2, IGF 1 = Insulin-like growth factor 1, INSR = Insulin receptor, PDK2 & 4 = Pyruvate Dehydrogenase Kinase 2 and 4, PPARA & PPARG = peroxisome proliferator-activated receptor α, and γ, PRKAA1 & 2 = Protein Kinase AMP-Activated Catalytic Subunit Alpha 1 and 2, UCP3 = Uncoupling Protein 3, CASP8 = Caspase 8, HSPA8 = Heat Shock Protein Family A (Hsp70) Member 8, IL6 = Interleukin 6, MAPK9 = Mitogen-Activated Protein Kinase 9, MTOR = Mechanistic Target Of Rapamycin Kinase, NFKB1 = Nuclear Factor Kappa B Subunit 1, SDHA = Succinate Dehydrogenase Complex Flavoprotein Subunit A, SOCS3 = Suppressor Of Cytokine Signaling 3, STAT3 = Signal Transducer And Activator Of Transcription 3, TLR4 = Toll Like Receptor 4, HIF1A = Hypoxia-inducible factor 1-alpha, NRF1 = Nuclear Respiratory Factor 1, ADIPOQ = Adiponectin, AKT1 = AKT Serine/Threonine Kinase 1, PPARGC1A = PPARG Coactivator 1 Alpha, PKM = Pyruvate Kinase M1/2, ATF6 = Activating Transcription Factor 6, MSTN = Myostatin, RPTOR = Regulatory Associated Protein Of MTOR complex 1, SMAD2 = SMAD Family Member 2 and FABP3 = Fatty Acid Binding Protein 3.

Figure 5.9 depicts differentially regulated mRNAs (outer ring) and IPA prediction of changes in muscle inflammatory response functions based on changes in mRNA expression from baseline following 14 days of bed rest in young and older volunteers. In both the young and older groups; IL, ILR, MTOR NFE2L2, NFKB1, PPARG, RPTOR, SOCS3, STAT3, TLR4, ADIPOQ, ATF4, FBXO32, HIF1A, IGF1, IKBKB, INSR, MAPK9, PPARA, SOD2, TRAF3,

PDK4, SMAD2, AKT1 increased in abundance during bed rest with exception to PPARGC1A and PKM which were upregulated in the young only. IPA predicted inhibition of muscle inflammation body cavity and inflammation of body organ functions during bed rest in both the young and older groups.

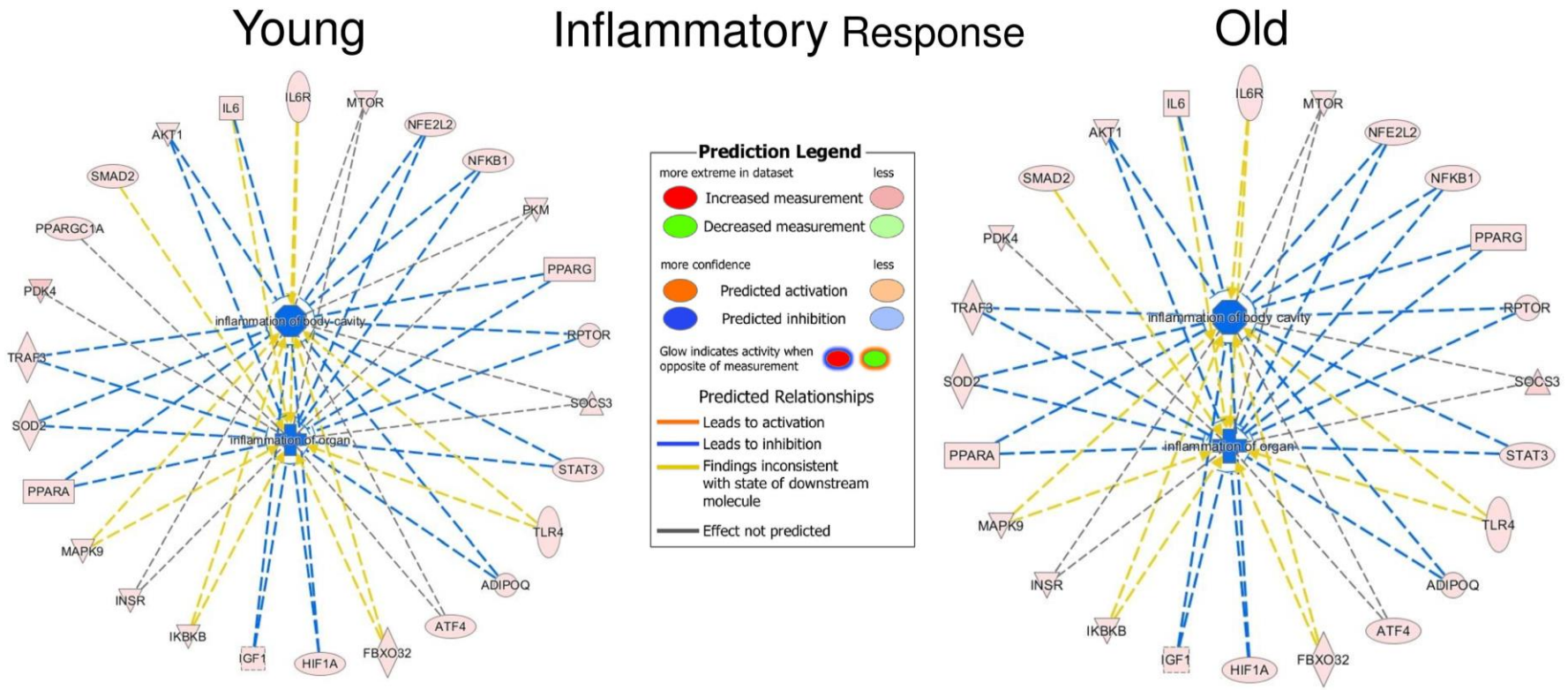

Figure 5.9. Differentially regulated mRNAs (outer ring) and IPA prediction of changes in muscle inflammatory response based on changes in mRNA expression from baseline following 14 days of bed rest in young and older volunteers.

Abbreviations: IL = Interleukin 6, ILR = Interleukin 6 receptor, MTOR = Mechanistic Target Of Rapamycin Kinase, NFE2L2 = Nuclear Factor, Erythroid 2 Like 2, NFKB1 = Nuclear Factor Kappa B Subunit 1, PPARA & PPARG = peroxisome proliferator-activated receptor α, and γ, RPTOR = Regulatory Associated Protein Of MTOR Complex 1, SOCS3 = Suppressor Of Cytokine Signaling 3, STAT3 = Signal Transducer And Activator Of Transcription 3, TLR4 = Toll Like Receptor 4, ADIPOQ = Adiponectin, ATF4 = Activating Transcription Factor 4, FBXO32 = F-Box Protein 32, HIF1A = Hypoxia-inducible factor 1-alpha, IGF1 = Insulin-like growth factor 1, IKBKB = Inhibitor Of Nuclear Factor Kappa B Kinase Subunit Beta, INSR = Insulin receptor, MAPK9 = Mitogen-Activated Protein Kinase 9, SOD2 = Superoxide Dismutase 2, TRAF3 = TNF Receptor Associated Factor 3, PDK4 = Pyruvate Dehydrogenase Kinase 4, SMAD2 = SMAD Family Member 2, AKT1 = AKT Serine/Threonine Kinase 1, PPARGC1A = PPARG Coactivator 1 Alpha and PKM = Pyruvate Kinase M1/2

Figure 5.10 depicts differentially regulated mRNAs (outer ring) and IPA prediction of changes in muscle cellular development based on changes in mRNA expression from baseline following 14 days of exercise rehabilitation in young and older volunteers. In both the young and older groups VEGFA, TLR4, MTOR, IL6, IGF1, ADIPOQ and CCL19 increased in abundance during rehabilitation with exception to ILR, INSR, MSTN, MTOR, OSTN, PPARA, PPARG, PPARGC1A, SMAD2, SOCS3, STAT3, FST, PRKAA1, TRAF3, CASP8, FOXO1, GSK3B and IKBKB which were all upregulated in the young group only with PDK4 being highly upregulated whilst HIF1A was upregulated in the older group only. IPA predicted activation in muscle cellular

development functions in; maturation of cells in both the young and older group whilst IPA predicted activation in lymphopoiesis and differentiation of connective tissue in the young group only.

Table 4.2. A summary of IPA predicted changes in muscle cellular responses based on changes in mRNA expression from baseline following 14 days of bed rest in young and older volunteers

	Young	**Older**
Carbohydrate Metabolism	(+) Glycolysis of cells (+) Glycolysis (+) Uptake of monosaccharide (+) Update of D-Glucose (+) Update of 2-deoxyglucose (+) Transport of D-Glucose (+) Quantity of lactic acid (+) Production of lactic acid	(+) Glycolysis of cells (+) Glycolysis (+) Uptake of monosaccharide (+) Update of D-Glucose (+) Update of 2-deoxyglucose
Protein synthesis	(+) Metabolism of protein (+) Quantity of cytokine	(+) Metabolism of protein (+) Quantity of cytokine
Lipid Metabolism	(+) Fatty acid metabolism (-) Concentration of fatty acid (+) Uptake of fatty acid (+) Synthesis of lipid (+) Synthesis of fatty acid (+) Oxidation of palmitic acid (+) Oxidation of fatty acid	(+) Fatty acid metabolism (-) Concentration of fatty acid (+) Oxidation of fatty acid (+) Synthesis of prostaglandin E2 (+) Oxidation of lipid (+) Oxidation of long chain fatty acid
Inflammatory response	(-) Inflammation of body cavity (-) Inflammation of organ	(-) Inflammation of body cavity (-) Inflammation of organ

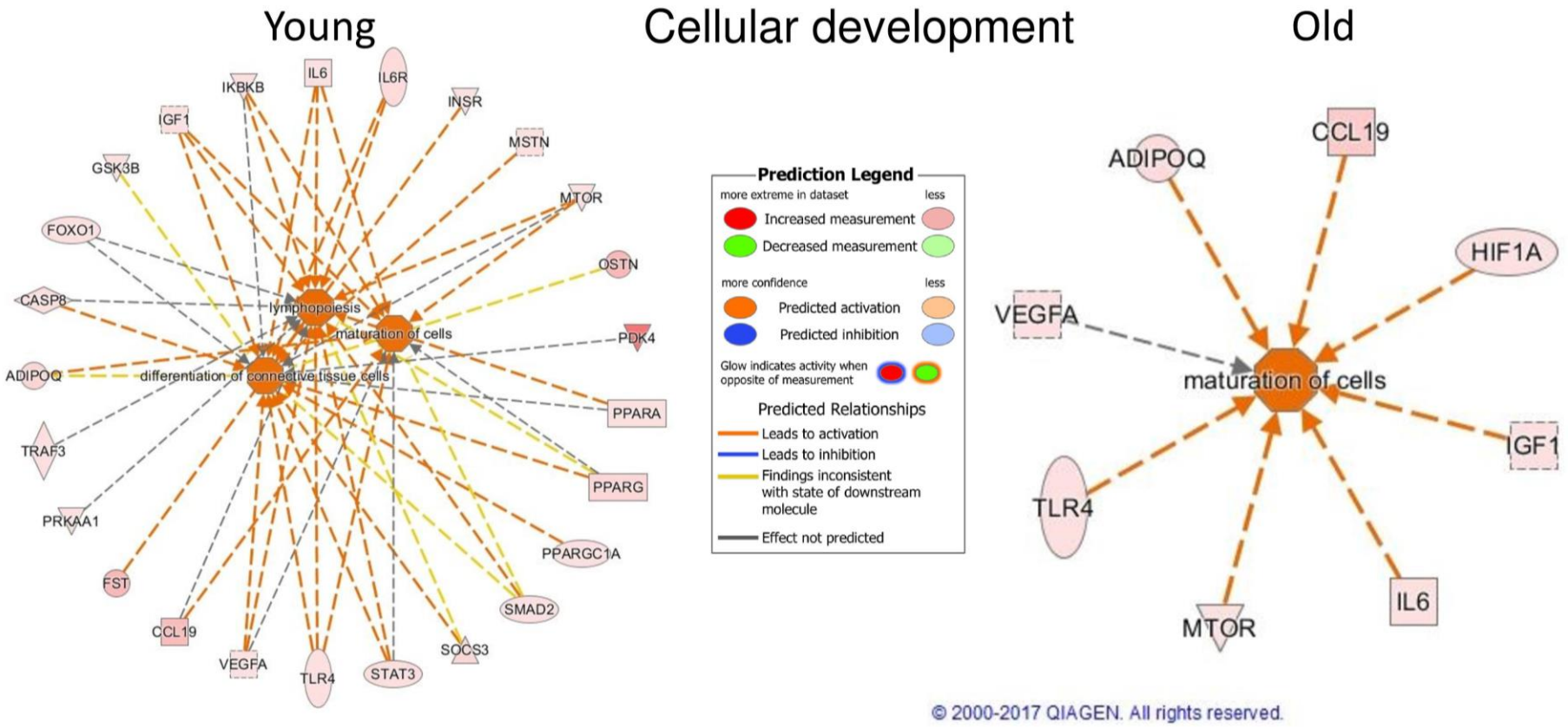

Figure 5.10. Differentially regulated mRNAs (outer ring) and IPA prediction of changes in muscle cellular development based on changes in mRNA expression from baseline following 14 days of exercise rehabilitation in young and older volunteers.

Abbreviations: CCL19 = C-C Motif Chemokine Ligand 19, HIF1A = Hypoxia-inducible factor 1-alpha, IGF 1 = Insulin-like growth factor 1, IL6 = Interleukin 6, MTOR = Mechanistic Target Of Rapamycin Kinase, TLR4 = Toll Like Receptor 4, VEGFA = Vascular Endothelial Growth Factor A, ADIPOQ = Adiponectin, IL6R = Interleukin 6 receptor, INSR = Insulin receptor, MSTN = Myostatin, MTOR = Mechanistic Target Of Rapamycin Kinase, OSTN = Osteocrin, PDK4 = Pyruvate Dehydrogenase Kinase 4, PPARA & PPARG = peroxisome proliferator-activated receptor α, and γ, PPARGC1A = PPARG Coactivator 1 Alpha, SMAD2 = SMAD Family Member 2, SOCS3 = Suppressor Of Cytokine Signaling 3, STAT3 = Signal Transducer And Activator Of Transcription 3, FST = Follistatin, PRKAA1 = Protein Kinase AMP-Activated Catalytic Subunit Alpha 1, TRAF3 = TNF Receptor Associated Factor 3, CASP8 = Caspase 8, FOXO1 = Forkhead Box O1, GSK3B = Glycogen Synthase Kinase 3 Beta, IKBKB = Inhibitor Of Nuclear Factor Kappa B Kinase Subunit Beta

Figure 5.11 depicts differentially regulated mRNAs (outer ring) and IPA prediction of changes in muscle lipid metabolism development based on changes in mRNA expression from baseline following 14 days of exercise rehabilitation in young and older volunteers. In both the young and older group ADIPOQ, CASP8, IGF1, IL6, MTOR, SOCS3, TLR4 and VEGFA increased in abundance during rehabilitation with exception to INSR, PPARA, PPARG, PPARGC1A, PRKAA1, GSK3B, MSTN, RPS6KB2, STAT3, TRAF3, SDHA, CPTA1, FABP3 and FOXO1 which were all upregulated in young only with PDK4 being highly upregulated. IPA predicted activation in lipid metabolism functions in; fatty acid metabolism in both the young and

older group, whilst activation in oxidation of fatty acid, oxidation of palmitic acid, synthesis of fatty acid and uptake of fatty acid was activated in the young only and IPA predicted inhibition in; concentration of fatty acid and concentration of lipid in the young only.

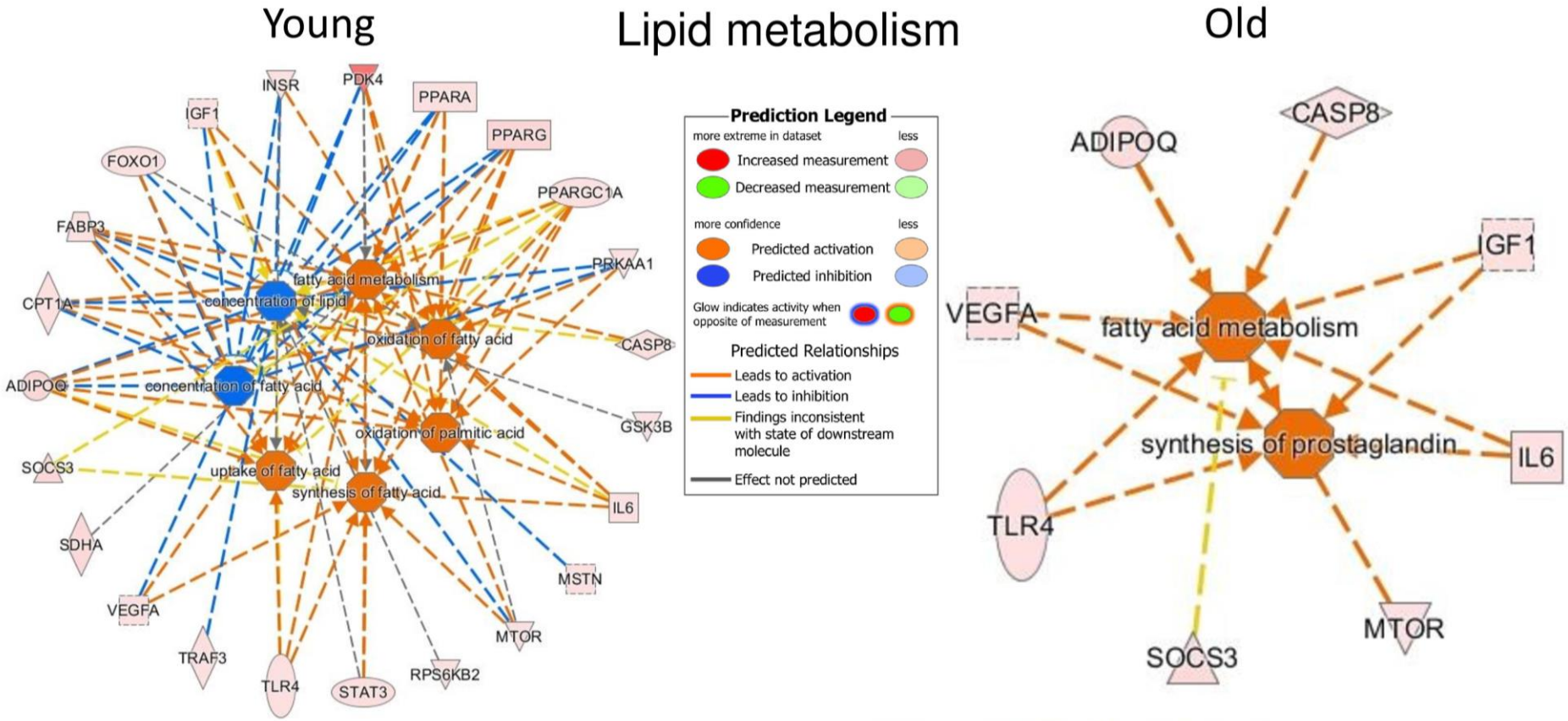

Figure 5.11. Differentially regulated mRNAs (outer ring) and IPA prediction of changes in muscle lipid metabolism based on changes in mRNA expression from baseline following 14 days of exercise rehabilitation in young and older volunteers.

Abbreviations: CASP8 = Caspase 8, IGF 1 = Insulin-like growth factor 1, IL6 = Interleukin 6, MTOR = Mechanistic Target Of Rapamycin Kinase, SOCS3 = Suppressor Of Cytokine Signaling 3, TLR4 = Toll Like Receptor 4, VEGFA = Vascular Endothelial Growth Factor A, ADIPOQ = Adiponectin, PPARA & PPARG = peroxisome proliferator-activated receptor α, and γ, PPARGC1A = PPARG Coactivator 1 Alpha, PRKAA1 = Protein Kinase AMP-Activated Catalytic Subunit Alpha 1, GSK3B = Glycogen Synthase Kinase 3 Beta, MSTN = Myostatin, RPS6KB2 = Ribosomal Protein S6 Kinase B2, STAT3 = Signal Transducer And Activator Of Transcription 3, TRAF3 = TNF Receptor Associated Factor 3, SDHA = Succinate Dehydrogenase Complex Flavoprotein Subunit A, CPT1A = Carnitine Palmitoyltransferase 1A, FABP3 = Fatty Acid Binding Protein 3, FOXO1 = Forkhead Box O1, INSR = Insulin receptor and PDK4 = Pyruvate Dehydrogenase Kinase 4.

Figure 5.12 depicts differentially regulated mRNAs (outer ring) and IPA prediction of changes in muscle protein metabolism based on changes in mRNA expression from baseline following 14 days of exercise rehabilitation in young and older volunteers. In both the young and older group, ADIPOQ, VEGFA, TLR4, SOCS3, PRKAA1, MTOR, IL6, IGF1, FST, CCL19 and CASP8 increased in abundance during rehabilitation with exception to FOXO1, GSK3B, INSR, MSTN, MTOR, PPARA, PPARG, PPARGC1A, RPS6KB2, STAT3, TRAF4, TUFM, ATF6 and CAPN2 which were all upregulated in young whilst ATF4 and EIF4B in the older group only. IPA predicted activation in metabolism of protein in both groups.

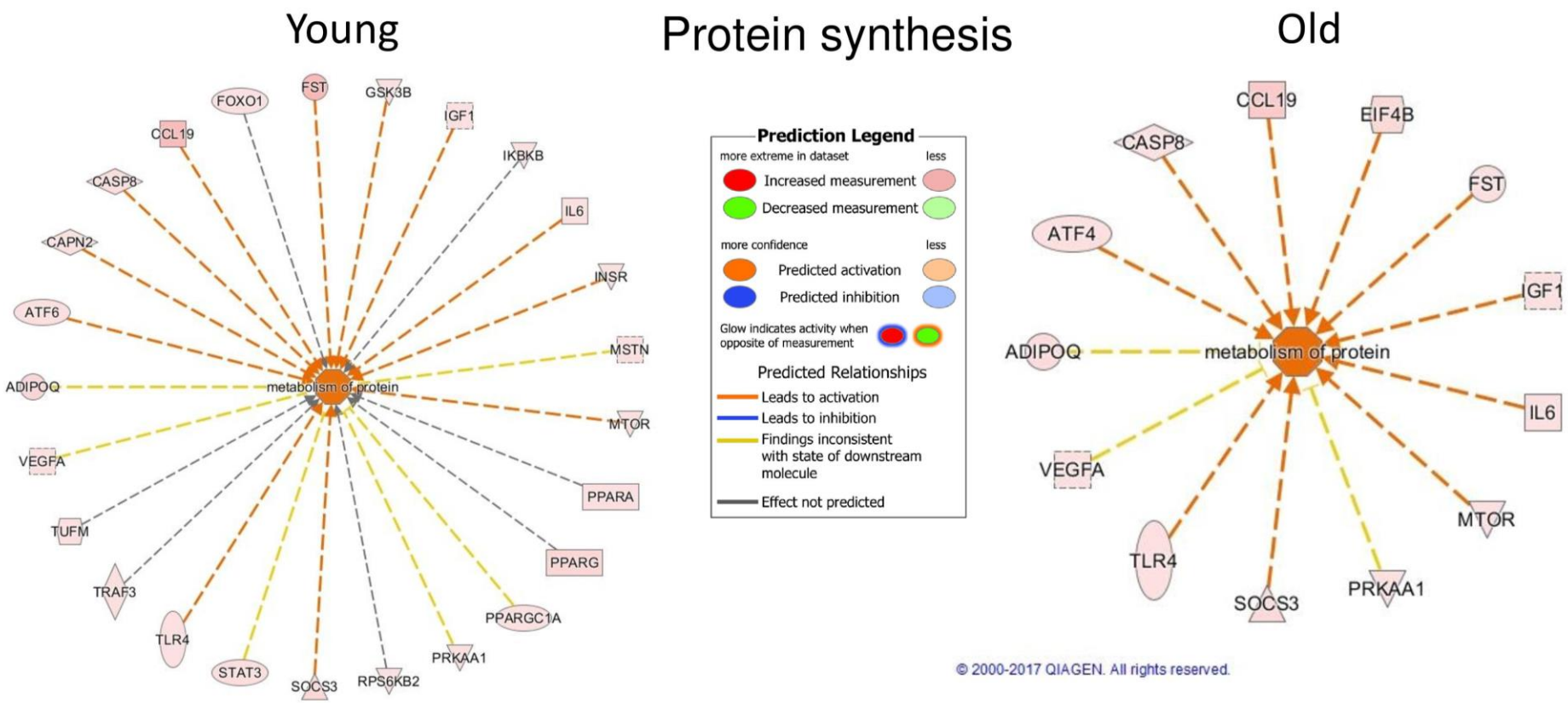

Figure 5.12. Differentially regulated mRNAs (outer ring) and IPA prediction of changes in muscle protein synthesis metabolism based on changes in mRNA expression from baseline following 14 days of exercise rehabilitation in young and older volunteers.

Abbreviations: CCL19 = C-C Motif Chemokine Ligand 19, EIF4B = Eukaryotic Translation Initiation Factor 4B, FST = Follistatin, IGF 1 = Insulin-like growth factor 1, IL6 = Interleukin 6, MTOR = Mechanistic Target Of Rapamycin Kinase, PRKAA1 = Protein Kinase AMP-Activated Catalytic Subunit Alpha 1, SOCS3 = Suppressor Of Cytokine Signaling 3, TLR4 = Toll Like Receptor 4, VEGFA = Vascular Endothelial Growth Factor A, ADIPOQ = Adiponectin, ATF4 & 6 = Activating Transcription Factor 4 & 6c, CASP8 = Caspase 8, GSK3B = Glycogen Synthase Kinase 3 Beta, INSR = Insulin receptor, MSTN = Myostatin, PPARA & PPARG = peroxisome proliferator-activated receptor α, and γ, PPARGC1A = PPARG Coactivator 1 Alpha, RPS6KB2 = Ribosomal Protein S6 Kinase B2, STAT3 = Signal Transducer And Activator Of Transcription 3, TRAF3 = TNF Receptor Associated Factor 3, TUFM = Tu Translation Elongation Factor, Mitochondrial, CAPN1 & CAPN2 = Calpain 2 and FOXO1 = Forkhead Box O1.

Figure 5.13 depicts differentially regulated mRNAs (outer ring) and IPA prediction of changes in muscle cell death and survival based on changes in mRNA expression from baseline following 14 days of exercise rehabilitation in young and older volunteers. In both the young and older groups, ADIPOQ, CASP8, IGF1, IL6, LDHA, MTOR, PRKAA1, SOC3, TLR4 and VEGFA increased in abundance during rehabilitation with exception to ATF6, CAPN2, FOXO1, GSK3B, IKBKB, IL6R, INSR, PPARA, PPARG, RPS6KB2, SMAD2, STAT3, MYH4, PPARGC1 and TTN which were all upregulated in young with PDK4 being highly upregulated whilst HIF1A, HK2 was upregulated in the older group with PSMA1 being highly

upregulated. IPA predicted activation in cell viability in both the young and older group whilst IPA predicted activation in cell survival in the older group only and IPA predicted inhibition of atrophy of myofiber.

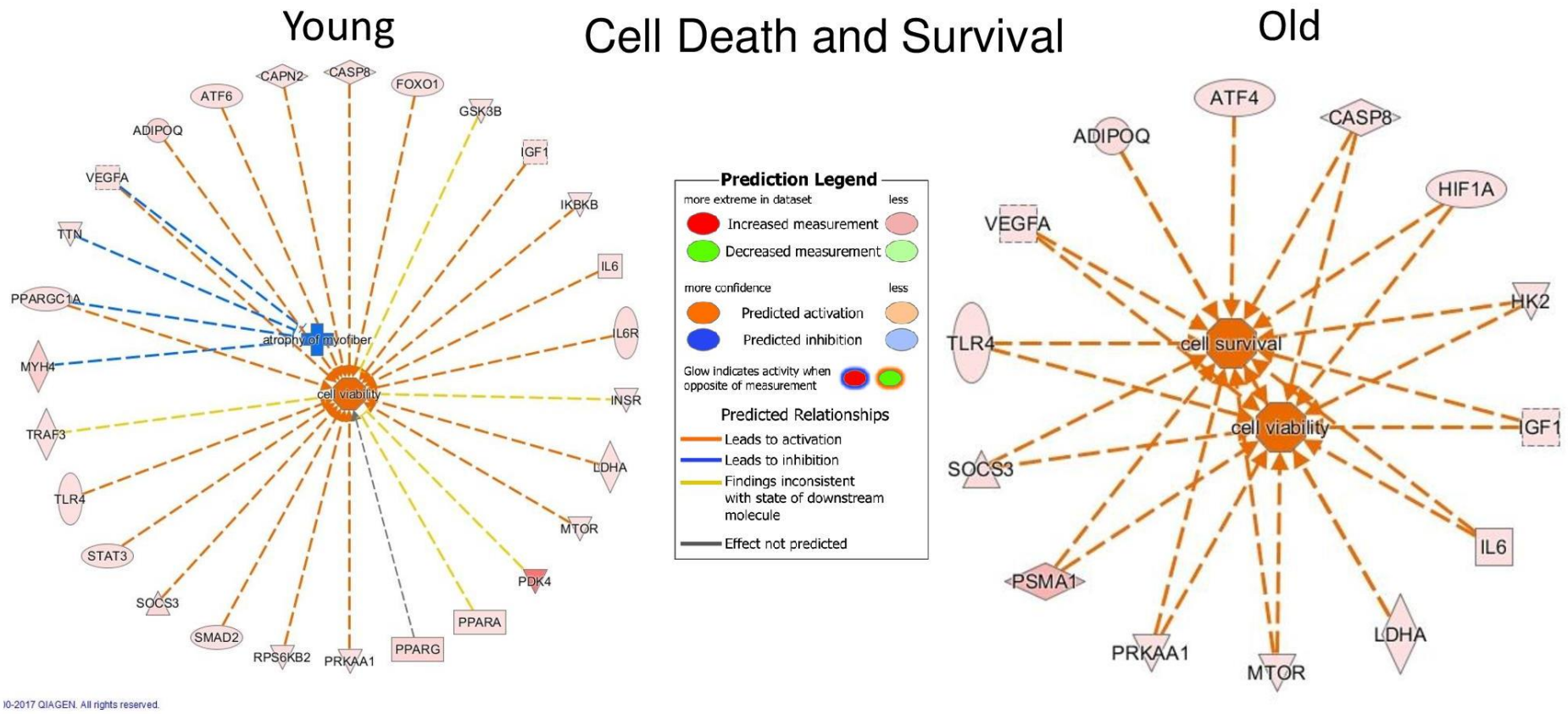

Figure 5.13. Differentially regulated mRNAs (outer ring) and IPA prediction of changes in muscle cell death and survival based on changes in mRNA expression from baseline following 14 days of exercise rehabilitation in young and older volunteers.

Abbreviations**:** ATF4 & 6 = Activating Transcription Factor 4 & 6, CASP8 = Caspase 8, HIF1A = Hypoxia-inducible factor 1-alpha, HK2 = Hexokinase 2, IGF 1 = Insulin-like growth factor 1, IL6 = Interleukin 6, LDHA = Lactate Dehydrogenase A, MTOR = Mechanistic Target Of Rapamycin Kinase, PRKAA1 = Protein Kinase AMP-Activated Catalytic Subunit Alpha 1, PSMA1 = Proteasome Subunit Alpha 1, SOCS3 = Suppressor Of Cytokine Signaling 3, TLR4 = Toll Like Receptor 4, VEGFA = Vascular Endothelial Growth Factor A, ADIPOQ = Adiponectin, FOXO1 = Forkhead Box O1, GSK3B = Glycogen Synthase Kinase 3 Beta, IKBKB = Inhibitor Of Nuclear Factor Kappa B Kinase Subunit Beta, IL6R = Interleukin 6 receptor, INSR = Insulin receptor, PDK4 = Pyruvate Dehydrogenase Kinase 4, PPARA & PPARG = peroxisome proliferator-activated receptor α, and γ, RPS6KB2 = Ribosomal Protein S6 Kinase B2, SMAD2 = SMAD Family Member 2, STAT3 = Signal Transducer And Activator Of Transcription 3, MYH4 = Myosin Heavy Chain 4, PPARGC1A = PPARG Coactivator 1 Alpha, TTN = Titan and CAPN2 = Calpain 2

Table 4.3. A summary of IPA predicted changes in muscle cellular responses based on changes in mRNA expression from baseline following 14 days of exercise rehabilitation in young and older volunteers.

	Young	**Older**
Cellular Development	(+) Lymphopoiesis (+) Differentiation of connective tissue cells (+) Maturation of cells	(+) Maturation of cells
Lipid metabolism	(-) Concentration of lipid (-) Concentration of fatty acid (+) Uptake of fatty acid (+) Synthesis of fatty acid (+) Oxidation of palmitic acid (+) Oxidation of fatty acid (+) Fatty acid metabolism	(+) Fatty acid metabolism (+) Synthesis of prostaglandin
Protein synthesis	(+) Metabolism of protein	(+) Metabolism of protein
Cell death and survival	(-) Atrophy of myofiber (+) Cell viability	(+) Cell viability (+) Cell survival

5.5 Discussion

This present study aimed to unravel novel insights into gene responses regulating muscle architecture and mass, muscle carbohydrate and lipid metabolism to muscle disuse in bed-rest and subsequent rehabilitation in the context of aging. A comprehensive bioinformatics pathway based analysis using IPA was performed to identify the most significant pathways and discover potential novel regulatory molecular networks associated with bed rest and rehabilitation in context to aging. The main findings from this study were; (i) Absolute quadriceps muscle volume decline during bed rest was similar in both the young and older group and absolute quadriceps muscle volume gain during rehabilitation was similar in both the young and older group (Figure 5.2). (ii) Based on total gene card mRNA expression changes during bed rest, IPA predicted carbohydrate metabolism, protein synthesis, lipid metabolism and inflammatory response to be the most altered cellular functions whilst during rehabilitation, IPA predicted cellular development, lipid metabolism, protein synthesis and cell death and survival to be the most altered cellular functions. (iii) Based on mRNA changes relating to carbohydrate metabolism, lipid metabolism, protein synthesis and inflammatory response IPA analysis predicted bed rest initiated similar cellular metabolic events in young and older participants (Figure 5.6, Figure 5.7, Figure 5.8 and Figure 5.9). (iii) IPA analysis predicted muscle gene functions to respond differently with age, with attenuated signalling in cellular development and cell death and survival with aging (Figure 5.10 and Figure 5.13); (iv) IPA predicated pronounced activation of

multiple lipid metabolism related genes in the young during rehabilitation which were not predicted in the elderly (Figure 5.11)

There are differing reports as to whether aging accelerates muscle disuse related atrophy. For example, fourteen days of unilateral full leg immobilisation in a light weight fibre cast resulted in a significant decline in quadriceps muscle volume of ~9% in young males when compared to ~5% in the elderly males when measured using MRI (Suetta et al., 2009). On the other hand, *Pisot et al* demonstrated quadriceps muscle volume following 14 days of bed rest had significantly declined by ~6% in young males and ~8% in older males when measured using MRI (Pisot et al., 2016). Furthermore, following 5 days bed rest, leg lean mass was found to have declined by 4% in older volunteers but was unchanged in young volunteers when measured using DXA (Tanner et al., 2015). Therefore, there appears to be no clear consensus as to whether older people are more or less susceptible to muscle disuse related atrophy than younger people, or indeed whether they are no different. In our study during bed rest, quadriceps muscle volume declined by 6% in the YG and 8% in the OG (Figure 5.2). However, baseline quadriceps muscle volume was significantly lower in the OG when compared to the YG such that relative changes were amplified in the OG. Indeed, absolute muscle volume loss during bed rest was similar between the YG and OG (Figure 4.2B). It has been proposed initial muscle volume, independent of age may be the overriding factor dictating muscle volume decline during disuse related atrophy, rather than age per se (Suetta et al., 2009). Indeed, we observed a significant association between initial muscle

volume and magnitude of volume decline during bed rest, with individuals with a larger muscle volume exhibiting greater muscle volume decline during 14 days bed rest (Figure 4.3).

In keeping with the similarity in absolute muscle volume decline in both YG and OG, based on mRNA changes relating to carbohydrate metabolism, lipid metabolism, protein synthesis and inflammatory response, IPA analysis predicted bed rest initiated similar cellular metabolic events in YG and OG (Figure 5.6, Figure 5.7, Figure 5.8 and Figure 5.9). Therefore, there does not seem to a difference in the regulation of muscle unloading induced atrophy in young and older volunteers as IPA predicted similar cellular metabolic based on mRNA changes related to carbohydrate metabolism, lipid metabolism, protein synthesis and inflammatory response and absolute volume decline irrespective of age was similar between the young and older group.

Some workers argue during muscle unloading, a heighted inflammation response may aid muscle atrophy during unloading. For example, following a 7 day bed rest in older participants, TLR4 protein expression increased by 70% and mRNA gene expression of NF-κB1 and IL-6 increased during bed rest whilst serum concentrations of IL (1β, -2, -4, -5, -6, -7, -8, -10, -12, -13, -17) remained unchanged (Drummond et al., 2013). Furthermore, mRNA upregulation of TNF-alpha, IL-1, and IL-6 has been observed in rodent models of muscle unloading using a stapling technique to immobilise the limb and crushing the sciatic nerve to immobilise the limb (Caron et al., 2009). However, based on mRNA changes from our data, IPA predicted a robust inhibition of muscle

inflammation with bed rest with no difference in the response between YG and OG. The nature and methodologies used to impose hind limb immobilisation in animals is profoundly more severe than experimented on humans such as severing the motor nerve or limb stapling. Thus, the inflammatory response observed in animal's models may not be a reflection of immobilisation but rather the severity of the method used to induce muscle immobilisation. Furthermore, Drummond and colleagues measured a couple mRNA gene responses relating to inflammation in a hand full (n=6) older volunteers only (Drummond et al., 2013) whilst we measured multiple mRNA gene responses related to inflammation during bed rest in both young and older volunteers.

After a period of immobilisation adaptive changes in skeletal muscle result in deleterious metabolic effects. IPA predicated bed rest initiated a similar muscle gene functions related to carbohydrate and lipid metabolism with a pattern of gene functions consistent with muscle deconditioning i.e. upregulation of non-oxidative carbohydrate metabolism functions (glycolysis of cells) and inhibition of concentration of fatty acid in both the young and older volunteers. Indeed, it has been observed following 7 days of bed rest basal carbohydrate oxidation increased by 40% in young males whilst lipid oxidation was reduced by 90% (Blanc et al., 2000). Muscle unloading has shown to depress the action of insulin on muscle glucose uptake (Krogh-Madsen et al., 2010; Mikines et al., 1991; Richter et al., 1989). However, the full mechanism by which this occurs has not been elucidated. From a molecular signalling perspective, seven days of bed-rest has shown to attenuate insulin

stimulated AKT phosphorylation in young humans (Kiilerich et al., 2011). Furthermore, 7 days of bed-rest has shown to reduce GLUT4 protein content, reduce muscle hexokinase activity and decrease glycogen synthase activity (Bienso et al., 2012). However, whether responses to muscle unloading a drivers of muscle insulin resistance or occur as a consequence remain unknown. PDC governs the entry of pyruvate into the mitochondria for oxidation to acetyl-CoA and therefore is the rate-limiting step in carbohydrate oxidation. In both the YG and OG group, PDK4 gene expression was amplified during bed rest (YG: Fold change: 6.580, $P \leq 0.001$; OG: Fold change: 3.681, $P \leq 0.05$). In doing so, increased PDK4 expression may impart be responsible for muscle insulin resistance observed during muscle unloading by blunting muscle carbohydrate oxidation by inhibiting PDC activity (Mikines et al., 1991; Richter et al., 1989).

Following 2 weeks rehabilitation, IPA analysis predicted muscle gene functions to respond differently with age, with attenuated mRNA signalling in pathways related to lipid metabolism, cellular development and cell death and survival with aging (Figure 5.10 - 5.13). IPA analysis predicted genes regulating the transport (FABP3, CPT1A), uptake, catabolism and oxidation (PPAR-α) of lipids were upregulated in the young only. Based on mRNA expression changes from baseline, IPA prediction activation in lipid metabolism functions in; fatty acid metabolism in both the young and older group, whilst activation in oxidation of fatty acid, oxidation of palmitic acid, synthesis of fatty acid and uptake of fatty acid was activated in the young only and IPA

predicted inhibition in; concentration of fatty acid and concentration of lipid in the young only. Indeed, immediately after immobilisation a difference in lipid utilisation during exercise recovery has been demonstrated in context to ageing with young men primarily utilising endogenous fat stores, whist elderly men primarily utilised exogenous fatty acid during moderate intensity exercise (Vigelso et al., 2016).

Following rehabilitation, IPA analysis predicted based on changes in mRNA expression from baseline related to muscle cellular development the activation of maturation of cells in both the young and older group whilst only predicting activation in lymphopoiesis and differentiation of connective tissue in the young group only. Furthermore, following rehabilitation, IPA analysis predicted based on changes in mRNA expression from baseline related to muscle cell death and survival the activation of cell viability in both the young and older group whilst IPA predicted activation in cell survival in the older group only and IPA predicted inhibition of atrophy of myofiber. However, despite a blunting in mRNA gene responses related to muscle cellular development, protein metabolism, muscle cell death and survival and lipid metabolism in the older group, the absolute muscle volume decline assessed using MRI during bed rest was restored in the OG. A possible explanation could be that the OG had reached their full capacity to maximise the cellular functions (i.e. they had returned to baseline), but the YG had the potential to continue having changes in the networks.

Genes related to the regulation of MPB (FoXO1 and Caspase2) were elevated following rehabilitation in the young leading to predicted

pathway activation by IPA which was not present in the OG (Figure 5.10 and Figure 5.13). FoXO1 in the active state resides in the nucleus upregulating gene transcripts responsible for the regulation of glucose oxidation (PDK4), fatty acid oxidation (lipoprotein lipase (LPL) and fatty acid translocase (FAT)) and MPB (MAFbx and MuRF1) (Sanchez et al., 2014). In addition, caspases are a family of cysteine proteases which hold an integral role in protein degradation during apoptosis (Fan et al., 2005). Elevation of MPB following rehabilitation aids in the remodelling of muscle. Furthermore, following rehabilitation, gene expression of structural proteins (MYM4; Myosin Heavy Chain 4 and TTN; Titin) were upregulated in the young but not the elderly. Indeed, functional recovery is delayed in the elderly following skeletal muscle unloading when compared to the young (Sanchez et al., 2014; Tanner et al., 2015) and attenuated muscle remodelling following rehabilitation in the elderly could be linked to delayed functional recovery.

Fourteen days of mixed (resistance and endurance) exercise rehabilitation restored the absolute decline in muscle volume from bed rest in both the YG and OG (Figure 5.2). It is difficult to put these findings into context as the rehabilitation programme wasn't strictly controlled to individual ability. Rather, the aim of the rehabilitation programme was to restore functional capacity. Nevertheless, there is mixed reports concerning whether older muscles are as responsive as younger muscles to resistance exercise, with reports of attenuated (Welle et al., 1996), similar (Ivey et al., 2000) and greater (Kosek and Bamman, 2008) muscle recovery in the elderly. From a molecular

perspective, Kumar and colleagues showed older volunteers to have attenuated myofibrillar protein FSR stimulation in the post-absorptive state following a single repetition maximum between 20%-90% resistance exercise (Kumar et al., 2009). Therefore, with aging the muscle becomes less receptive to resistance exercise induced myofibrillar protein FSR stimulation. Furthermore, ageing is associated with diminished muscle satellite cell activation following 4 weeks of supervised resistive exercise in older volunteers when compared to young volunteers (Suetta et al., 2013). Therefore, aging is associated diminished myofibrillar protein FSR stimulation following resistance exercise and a reduced capability of stimulating myogenesis. However, we did not observe any differential prediction by IPA in protein synthesis gene functions during rehabilitation between ages. Furthermore, two weeks of unilateral lower limb immobilisation has shown to induce anabolic resistance in young humans (Glover et al., 2008) and aging is associated with habitually reduced physical activity (Frisard et al., 2007), such that the reduced sensitivity of older muscle to a resistance exercise may be due to reduced habitually physical activity. Indeed, physical activity has shown to make the muscle more sensitive to amino acids feeding in young males (Burd et al., 2011).

Advances in scientific technology and increases in funding resources now make it possible to profile the relative mRNA gene expression of one to the whole genome within a relatively short space of time. The microfluidic cards allow for the validation of tens or hundreds of microarray hits because they can be customised to include up to 384 of

those hits in one easy-to-use qPCR plate. Therefore, in a relativity short space a large dataset of gene responses can be identified and probed further. However, such an approach does have some shortcomings. Measuring the relative abundance of a gene does not directly correlate with the activation of a specific pathway as genes can be further regulated by transcriptional and posttranscriptional mechanisms. Furthermore, increased expression of a gene transcription does not necessarily mean there will be a rise in protein product. In addition, gene expression measurements were taken at 3 specific time points (baseline, following 14d bed rest and following 14d mixed rehabilitation) therefore any transient changes in gene expression changes could have been missed.

In conclusion, bed rest resulted in a similar decline in absolute quadriceps muscle volume in both the YG and OG. In addition, based on mRNA changes relating to carbohydrate metabolism, lipid metabolism, protein synthesis and inflammatory response IPA analysis predicted bed rest initiated similar cellular metabolic events in YG and OG. However, multiple mRNA genes responses relating to lipid metabolism, cellular development, protein synthesis and cell death and survival were blunted in the OG when compared to the YG following rehabilitation and based on the mRNA gene responses, IPA predicted reduced cellular functions relating to lipid metabolism, cellular development, protein synthesis and cell death and survival. However, despite a blunting in mRNA gene responses related to muscle cellular development, protein metabolism, muscle cell death and survival and lipid metabolism and attenuated IPA

predicted cellular functions in the OG, the absolute muscle volume decline assessed using MRI during bed rest was restored in the OG during rehabilitation. Therefore, it would seem the OG had reached their full capacity to maximise cellular functions (i.e. they had returned to baseline), but the YG had the potential to continue having changes in the networks.

Chapter 6

6 The effect of three-days unilateral lower limb immobilisation on chronic muscle protein synthesis, muscle thickness and volume and the expression of protein targets implicated in muscle mass regulation in young, healthy, normal weight and obese volunteers

6.1 Introduction

Generally speaking, obesity results when habitual energy intake is in excess of energy expenditure over chronic time periods. Obesity is starkly on the rise, as obese adults accounted for 15% of the UK population in 1993 which rose markedly to 26% by 2016 (NHS England, 2018). Obesity is associated with a number of comorbidities including type 2 diabetes, coronary artery disease and certain types of cancer (Pi-Sunyer, 1999). Skeletal muscle is crucial for locomotion and metabolic health as skeletal muscle plays a central role in whole body protein metabolism by serving as the principal reservoir for amino acids to maintain protein synthesis in vital tissues and organs. Furthermore, altered muscle metabolism plays a key role in the formation of multiple chronic disease states such as Diabetes and Sarcopenia, and therefore plays a key role in the prevention of these chronic disease states.

The regulation of muscle mass in adults is dependent on the balance between MPS and MPB and both processes are responsive to external factors i.e. muscle unloading measured using a chronic tracer approach

in young volunteers **(Chapter 3)** and older volunteers **(Chapter 4)** and measured using an acute tracer methodologies (Breen et al., 2013; de Boer et al., 2007; Glover et al., 2008; Wall et al., 2016) , protein supplementation (Cuthbertson et al., 2005; Glover et al., 2008; Smith et al., 1992), resistance exercise (Brook et al., 2015) and infection, trauma and inflammation (Puthucheary et al., 2013). Similar to aging, Murton and colleagues demonstrated the in postabsorptive state MPS rates between older normal weight and older obese humans to be similar, however the stimulatory effect of amino acid infusion on MPS stimulation was blunted in the obese, termed anabolic resistance (Murton et al., 2015). In addition, Beals and colleagues demonstrated the combined stimulatory effect of resistance exercise and protein feeding on myofibrillar protein FSR is blunted in young obese humans when compared to normal weight counterparts (Beals et al., 2018). Therefore, obesity is associated with altered protein metabolism which may accelerate muscle atrophy during muscle unloading.

Erskine and colleagues demonstrated obese young and older individuals display higher medial gastrocnemius muscle volume when compared to normal weight counterparts, measured using ultrasonography (Erskine et al., 2017). Furthermore, Murton and colleagues demonstrated increased lean mass in older obese individuals in the trunk (18% higher), android (26% higher), and gynoidal (14% higher) assed using a DXA scan (Murton et al., 2015). Therefore, despite the acute MPS response to amino acid infusion being blunted in the obese (Murton et al., 2015), obesity is associated with higher muscle mass when compared to aged

matched counterparts (Erskine et al., 2017; Murton et al., 2015). A possible explain as to why obese individuals have increased muscle mass may be as of result of the increased bodyweight which acts as a positive training stimulus on weight bearing muscles, increasing muscle mass (Bosco et al., 1986). Therefore, during muscle unloading, obese individuals may experience higher muscle volume declines during immobilisation when compared to normal weight counter parts. Indeed, in **Chapter 4** and **Chapter 5** we have observed a positive correlation between the absolute decline in muscle volume and baseline muscle volume during unloading (Figure 4.6 and Figure 5.3). However, no previous human muscle unloading study has compared muscle volume decline during muscle unloading between young normal weight and young obese participants.

6.2 Aims and Hypothesis

The aim of this study to provide novel insight of the impact of unilateral, below knee, limb immobilisation on chronic myofibrillar FSR (%/day) in the medial gastrocnemius muscle of healthy, young normal weight and young obese volunteers over a 3 day period, when compared to both the free living pre-immobilised state and the contralateral non-immobilised limb of the same individual. These FSR measurements were made in conjunction with the quantification of medial gastrocnemius muscle volume and protein expression levels of the putative regulators of anabolic, catabolic and ER stress related pathways. It was hypothesised that immobilisation would reduce chronic myofibrillar FSR in the immobilised limb compared to the basal state and contralateral limb in

both the young normal weight and young obese groups, with the effect more pronounced in the young obese due to the increase bodyweight acting as a daily training stimulus on muscle mass, and this would relate to immobilisation induced decrements in medial gastrocnemius muscle volume in both the young normal weight and young obese groups. Additionally, we hoped to provide novel insight of how changes (if any) in the putative regulators of muscle mass aligned with these muscle protein metabolic and architectural measurements in context to obesity.

6.3 **Methods**

Participants

Following informed consent, 15 healthy, normal-weight, young and 9 healthy, obese, young volunteers (see **Table 6.1** for anthropometric parameters) were recruited to participate in a 3 day unilateral lower limb immobilisation study (see Chapter 2 section 18 for detailed protocol). For the sake of clarity, the healthy, normal-weight, young data presented in Chapter 3 are used as a control comparator group in this chapter.

6.4 **Results**

Table. 6.1 Baseline anthropometric characteristics of participants with body composition assessed using a DXA scan (n.s = not significant; ** signifies *p* value < 0.01, *** signifies *p* value < 0.001).

Variables	Mean + SD	Mean + SD	*P* value

Group	Young normal Weight	Young obese	-
Age	23 ± 4	33 ± 5	-
***n* (Male:Female)**	15 (7M:8F)	9 (5M:4F)	-
Height (m)	1.77 ± 0.11	1.74 ± 0.09	*n.s.*
Mass (kg)	72.10 ± 3.3	96.76 ± 5.2	**
BMI (kg·m-2)	22 ± 2	32 ± 3	***
Total body fat (g)	18004 ± 1986	37641 ± 2625	***
Total body fat (%)	26.2 ± 2.5	41.40 ± 3.0	**
Total body lean (g)	50783 ± 2951	54222 ± 4602	*n.s.*
Legs fat (g)	7304 ± 850	12285 ± 1365	**
Legs lean tissue (g)	17460 ± 1110	18054 ± 1572	*n.s.*

Physical activity levels

Fig. 6.3 shows the daily PAL score during periods of free-living and unilateral lower limb immobilisation in healthy young normal weight and young obese volunteers. The X axis indicates study day. There was no difference in the daily PAL score between days during the free-living period within or between groups. Similarly, during unilateral lower limb immobilisation there was no difference in the PAL score between days within or between groups. Daily physical activity tended to be less when comparing the free living state with the immobilised state between groups, however this was only significant on day 2 of immobilisation

compared to day 2 of the free living state in both the young normal weight and young obese group (P<0.05). Furthermore, obesity did not have a differential effect on daily physical activity when comparing to the young lean.

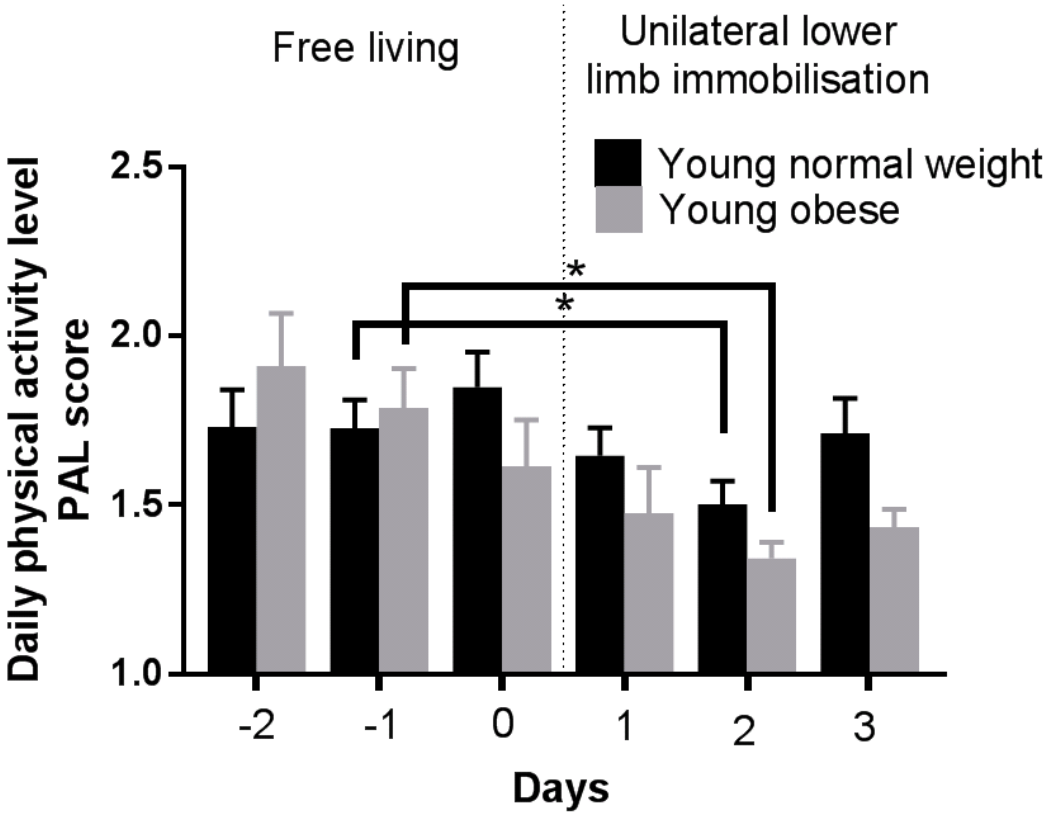

Figure 6.3. *Daily physical activity level (PAL) score measurements recorded using an ActiHeart during 3 days of free living and 3 days of unilateral lower limb immobilisation in young normal weight and young obese volunteers. * signifies P< 0·05 and ** signifies P<0·001.*

Fig. 6.4 shows the average daily PAL during periods of free living and 3 days of unilateral lower limb immobilisation in young normal weight and young obese volunteers. There was no difference between groups in the free living state. Average daily PAL score declined from the free-living state in both groups during immobilisation (normal weight group 8% ($p < 0.05$) decline, obese group 19% ($p < 0.01$) decline). There was no significant difference in average daily PAL between groups during immobilisation, but there was a trend for average PAL in the obese group to be less than in the normal weight group ($P=0.055$).

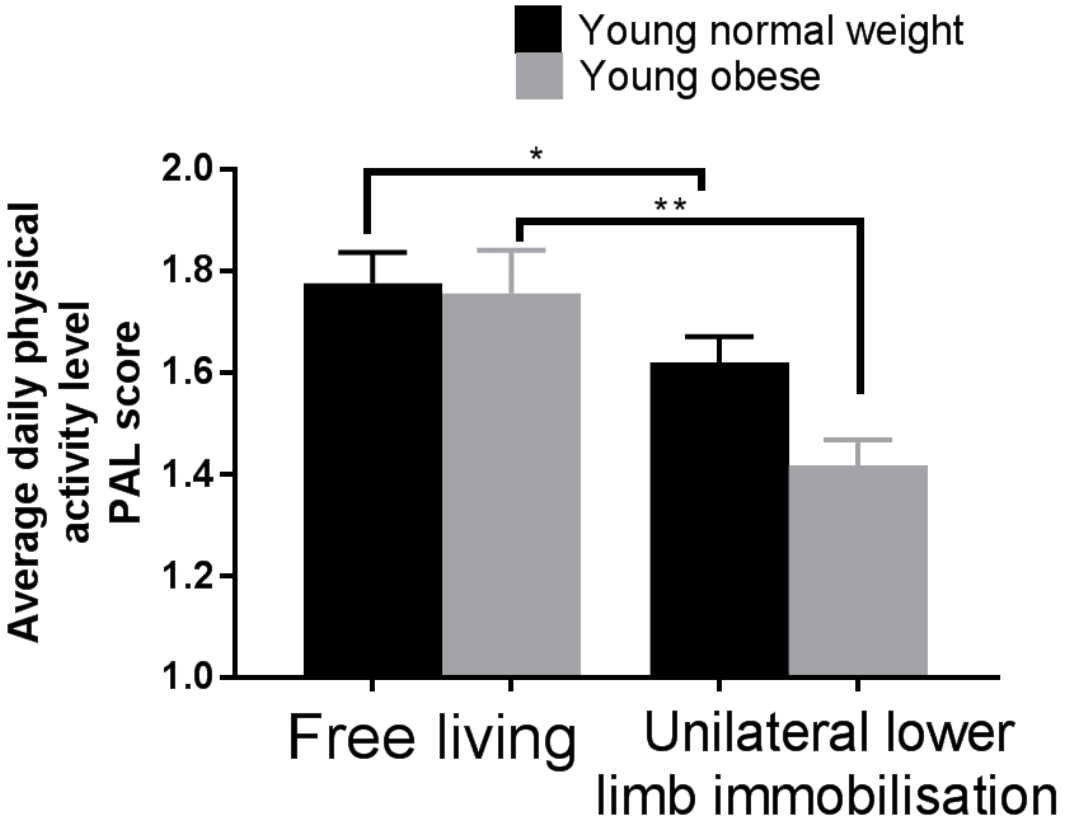

Figure 6.4. *Average daily physical activity level (PAL) score recorded using an ActiHeart during free living and 3 days of unilateral lower limb immobilisation in young normal weight and young obese volunteers. * signifies P< 0·05 and ** signifies P<0·001.*

Fig. 6.5A shows medial gastrocnemius muscle volume (cm^3) before and immediately following unilateral lower limb immobilisation and at the corresponding time points in the non-immobilised limb in young lean and young obese participants. Medial gastrocnemius muscle volume was significantly lower at baseline in both limbs in the young normal weight group when compared to the young obese group (P<0.05). Muscle volume in the non-immobilised limb did not change during immobilisation of the contralateral limb in both the young normal weight and young

obese group. However, muscle volume in the immobilised limb declined by ~7% (mean absolute decline: -10.48 ± 4.79 cm^3; $P<0.05$) from the pre-immobilised state in the young lean group, and this decline in volume was discernible in 11 of the 15 volunteers. Furthermore, muscle volume in the immobilised limb declined by ~15% (mean absolute decline: -31.33 ± 9.74 cm^3; $P<0.01$) from the pre-immobilised state in the young obese group, and this decline in volume was discernible in all 9 of the volunteers.

Fig. 6.5B shows medial gastrocnemius muscle thickness (cm) before and immediately following unilateral lower limb immobilisation and at the corresponding time points in the non-immobilised limb in young normal weight and young obese participants. Medial gastrocnemius muscle thickness was significantly lower at baseline in both limbs in the young normal weight group when compared to the young obese group ($P<0.05$). Muscle thickness of the non-immobilised was unchanged during immobilisation of the contralateral limb in both the young lean and young obese groups. Similarly, muscle thickness of the immobilised limb did not change during immobilisation in the young lean group. However, muscle thickness of the immobilised limb declined by 4% ($P<0.05$) during immobilisation in the young obese group.

Fig. 6.5C shows medial gastrocnemius muscle fascicle length (cm) before and immediately following unilateral lower limb immobilisation and at the corresponding time points in the non-immobilised limb in young normal weight and young obese participants. Muscle fascial length was no different in both limbs, between groups at baseline. Muscle fascicle

length of the immobilised and contralateral non-immobilised limb was unchanged during immobilisation in both groups.

Fig. 6.5D shows medial gastrocnemius muscle pennation angle (°) before and immediately following unilateral lower limb immobilisation and at the corresponding time points in the non-immobilised limb in young normal weight and young obese volunteers. Muscle pennation angle was significantly lower in the young normal weight group when compared to the young obese group at baseline in the non-immobilised limb. Muscle pennation angle of the non-immobilised was unchanged during immobilisation of the contralateral limb in both groups. However, whilst muscle pennation angle of the immobilised limb did not change during immobilisation in the young normal weight group, muscle pennation angle declined in the young obese group by (6%) in the immobilised limb ($P<0.05$).

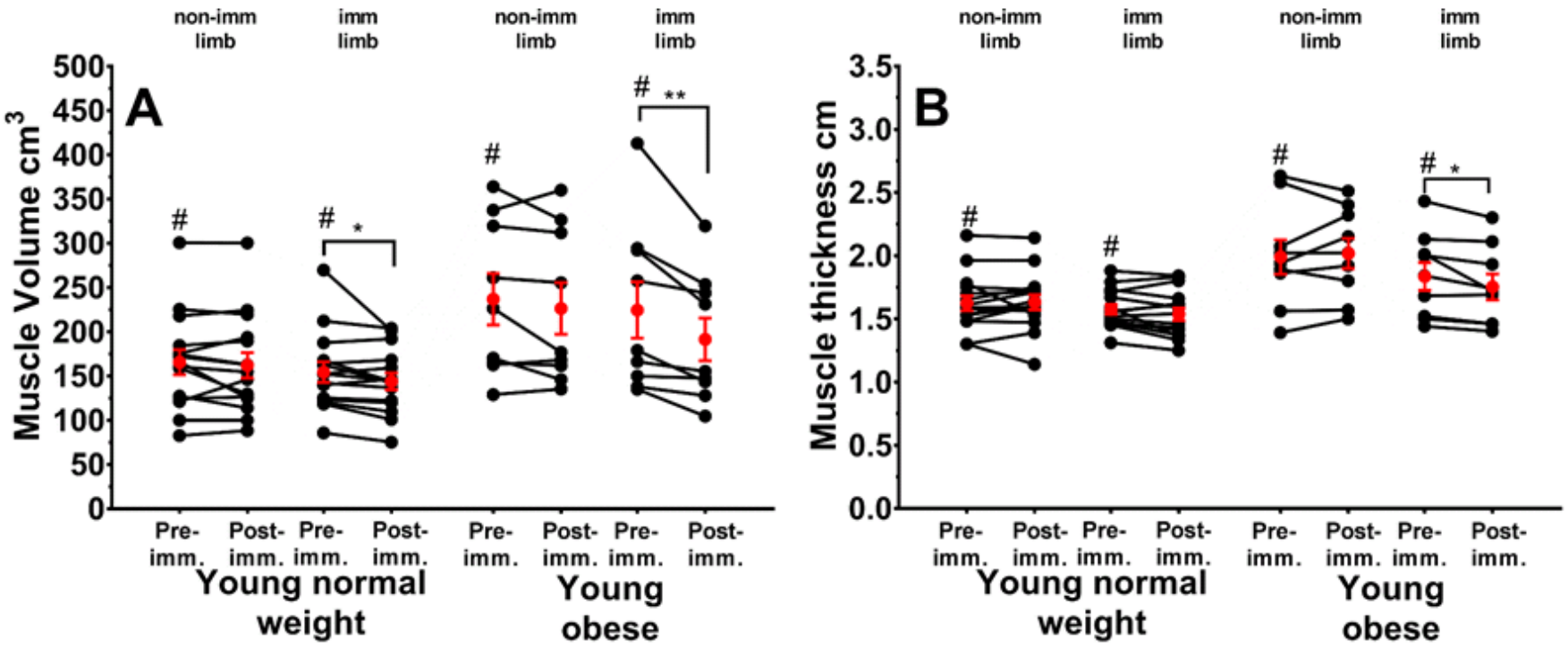

Figure 6.5. *Medial gastrocnemius muscle volume (A) and muscle thickness (B) in young normal weight and young obese volunteers measured using ultrasonography in the non-immobilised (non-imm.) limb and the contralateral immobilised (imm.) limb before 3 days of unilateral lower limb immobilisation (pre-imm.) and following three days of unilateral lower limb immobilisation (post-imm.) . Significant difference within limb over time * (P<0.05), ** (P<0.01), # signifies significant difference between groups at corresponding time-point (P<0.05).*

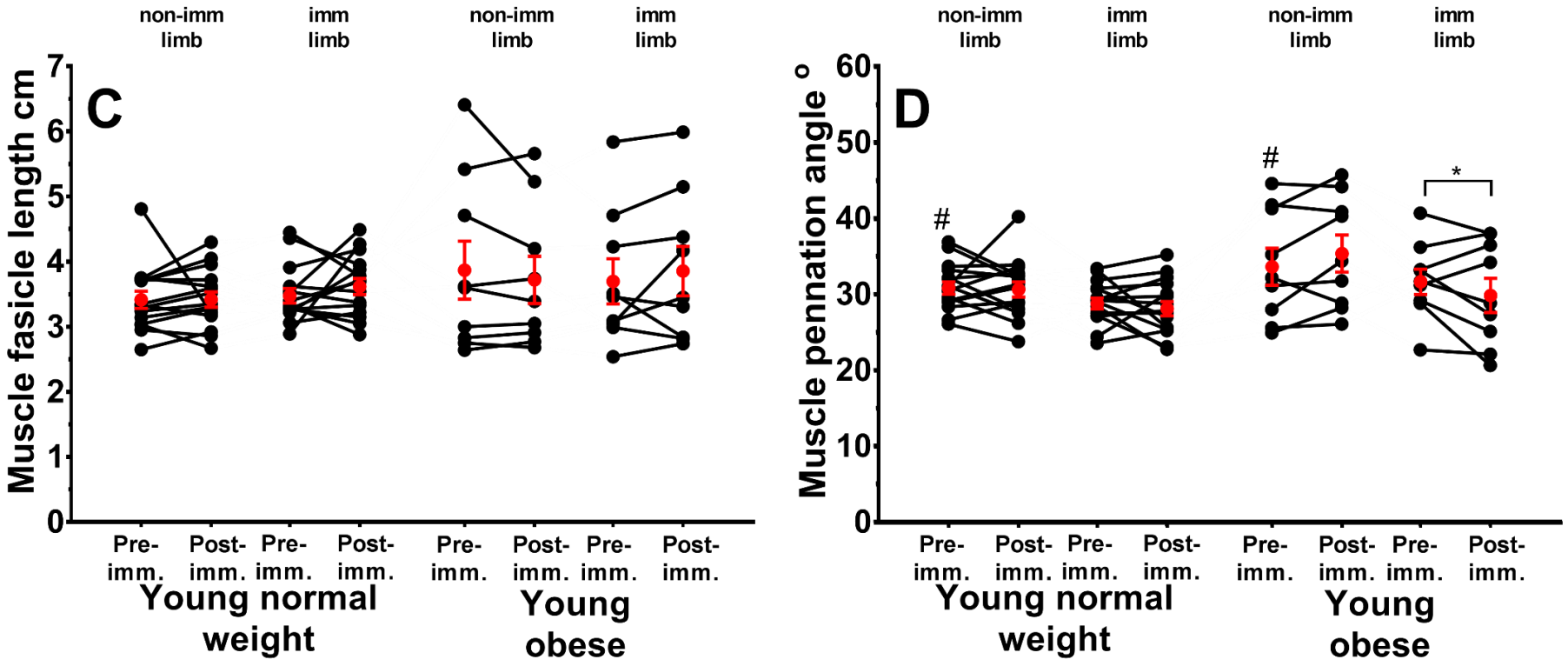

*Figure 6.5. Medial gastrocnemius muscle fascicle length (C) and muscle pennation angle (D) in young normal weight and young obese volunteers measured using ultrasonography in the non-immobilised (non-imm.) limb and the contralateral immobilised (imm.) limb before 3 days of unilateral lower limb immobilisation (pre-imm.) and following three days of unilateral lower limb immobilisation (post-imm.) . Significant difference within limb over time * (P<0.05), # signifies significant difference between groups at corresponding*

Fig. 6.6 shows the correlation between baseline medial gastrocnemius muscle volume (x-axis) and the absolute change in medial gastrocnemius muscle volume during 3d unilateral lower limb immobilisation (y-axis) in young normal weight and young obese volunteers. There was a significant correlation (R^2= 0.71, P<0.01) between baseline muscle volume and the absolute decline in muscle volume during immobilisation in the obese group, which was also evident in the normal weight group but weaker (R^2= 0.33, P<0.05). Furthermore, when data from both groups was combined, a robust significant relationship was maintained (R^2= 0.64, P<0.001).

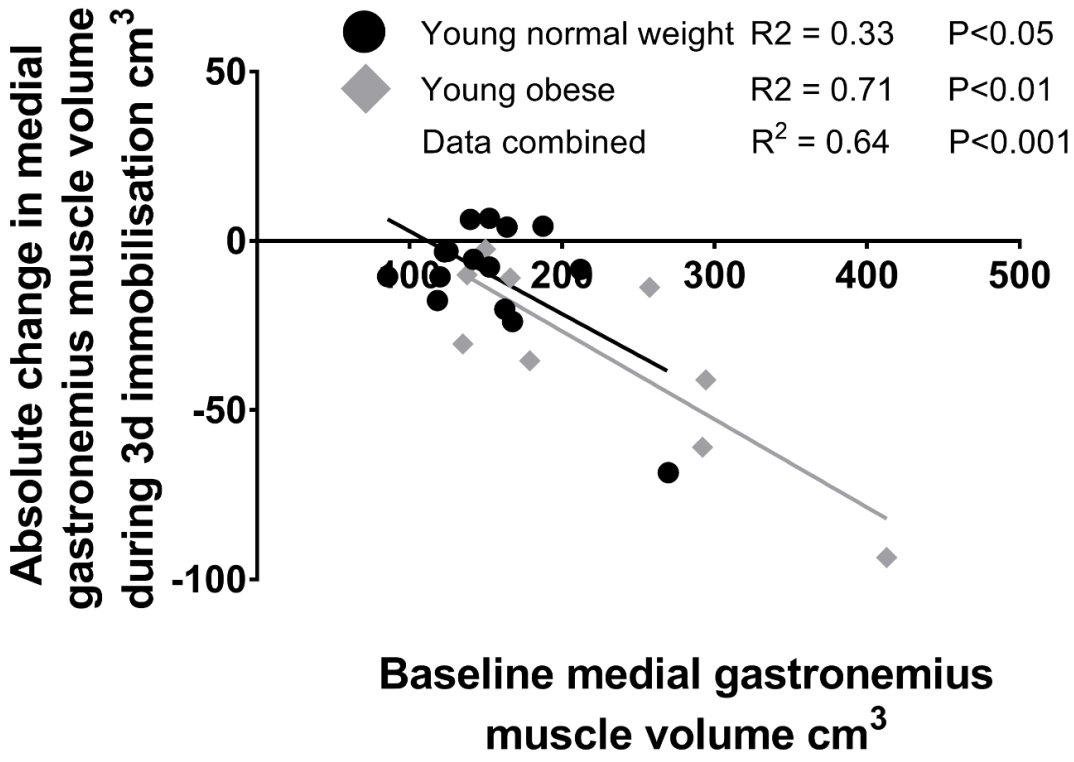

Figure 6.6. *The correlation between baseline medial gastrocnemius muscle volume and the absolute change in muscle volume during 3d unilateral lower limb immobilisation in young normal weight (black) and young obese (grey) volunteers.*

Fig. 6.7 shows the correlation between the absolute change in medial gastrocnemius muscle volume during muscle unloading (x-axis) and the absolute change in average physical activity level from the free living state to the immobilised state (y-axis) in young normal weight and young obese volunteers. There was no correlation between the absolute change in muscle volume during immobilisation and the absolute change in average physical activity level from the free living state to the immobilised state in young normal weight and young obese volunteers and with the data combined.

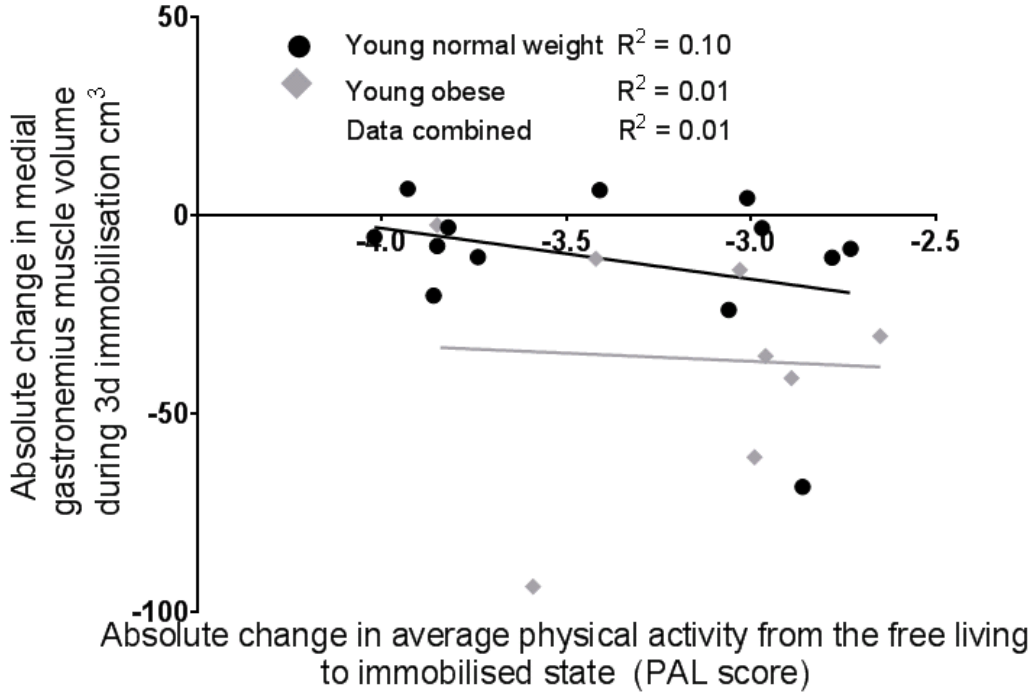

Figure 6.7. *The correlation between absolute change in medial gastrocnemius muscle volume during 3 days unilateral lower limb immobilisation and the absolute change in average physical activity from the free living state to the immobilised state in young normal weight (black) and young obese (grey) volunteers.*

Chronic Myofibrillar FSR

Fig. 6.8 shows chronic myofibrillar protein FSR in the medial gastrocnemius muscle during free living conditions and following immobilisation in the immobilised and contralateral non-immobilised limb in young normal weight and young obese volunteers. Data values are individual (red spots) and mean ± SEM. Chronic myofibrillar protein FSR in the medial gastrocnemius muscle during free living conditions was similar when comparing young normal weight and young obese volunteers, 1.62 ± 0.21%.d^{-1} vs. 1.52 ±

0.34%.d^{-1}, respectively. Following 3 days of unilateral lower limb immobilisation in the young normal weight group, myofibrillar protein FSR in the non-immobilised limb (1.44 ± 0.30%.d^{-1}) was no different from the free living state (1.62 ± 0.21%.d^{-1}, Figure 5.7). However, in the immobilised limb myofibrillar protein FSR (1.22 ± 0.37%.d^{-1}) was 25% less than in the free living state ($p<0.01$) and 15% less than in the contra-lateral non-immobilised limb ($p<0.05$; Figure 5.7). Furthermore, myofibrillar FSR in the immobilised limb less in 13 of the 15 volunteers when compared to the free living state, and less in 11 of the 15 volunteers when compared to the contralateral non-immobilised limb. In the young obese group following 3 days of unilateral lower limb immobilisation, myofibrillar protein FSR remained unchanged in both limbs.

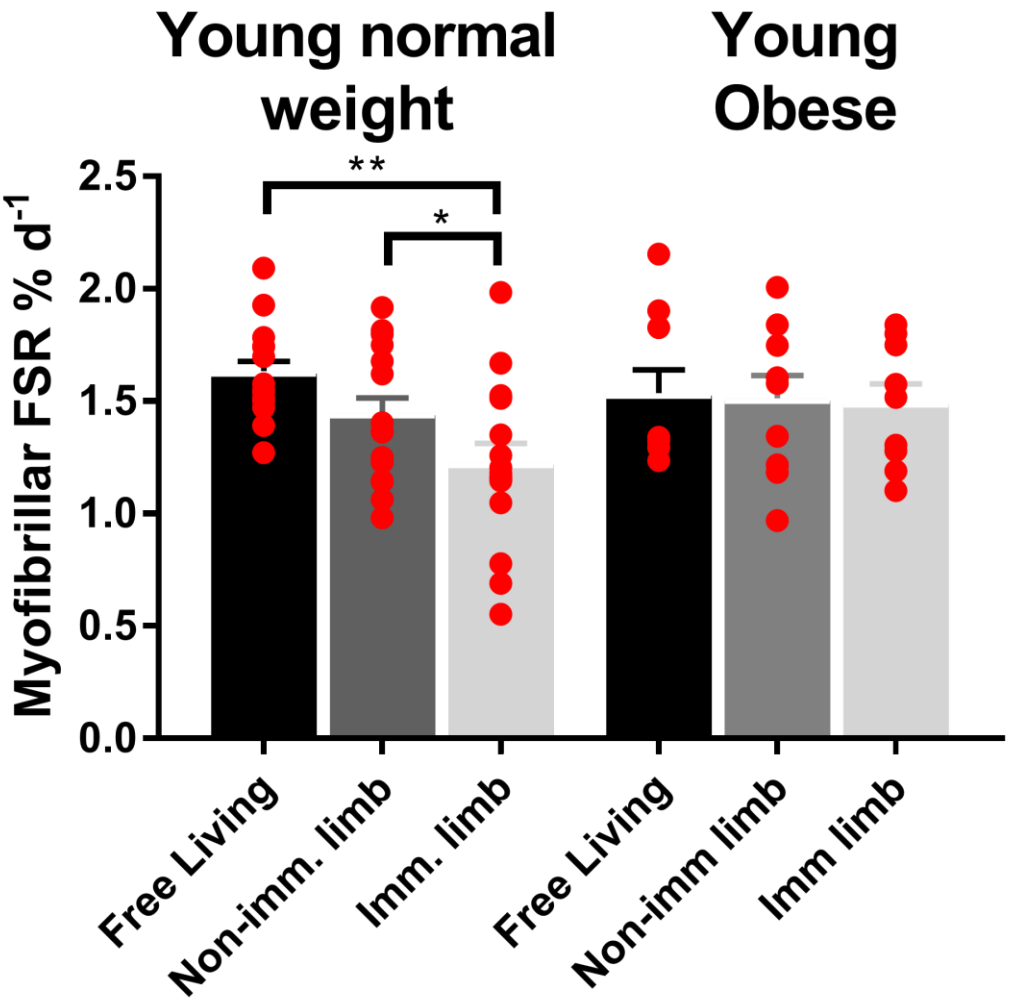

Figure 6.8. *Chronic myofibrillar protein FSR in the medial gastrocnemius muscle during free living conditions, following three days unilateral lower limb immobilisation (Imm.) and over the same time period in the non-immobilised limb (Non-imm.) contralateral immobilised limb in young, healthy volunteers. Values are mean + SEM. * (P<0.05) and ** (P<0.01) signify significant difference between conditions.*

Muscle protein expression

Fig. 6.9 shows muscle protein expression levels of targeted anabolic signalling proteins (A) and proteins thought to regulate MPB (B). No differences were observed when comparing target protein expression levels between limbs following 3 days of unilateral lower immobilisation in the young normal weight and young obese (Figure 6.9 A and B), with exception to phospho-4E-BP1 (Thr37/46) expression which was less in the immobilised limb in young normal weight volunteers when compared to the contralateral non-immobilised limb (51%, $p<0.05$).

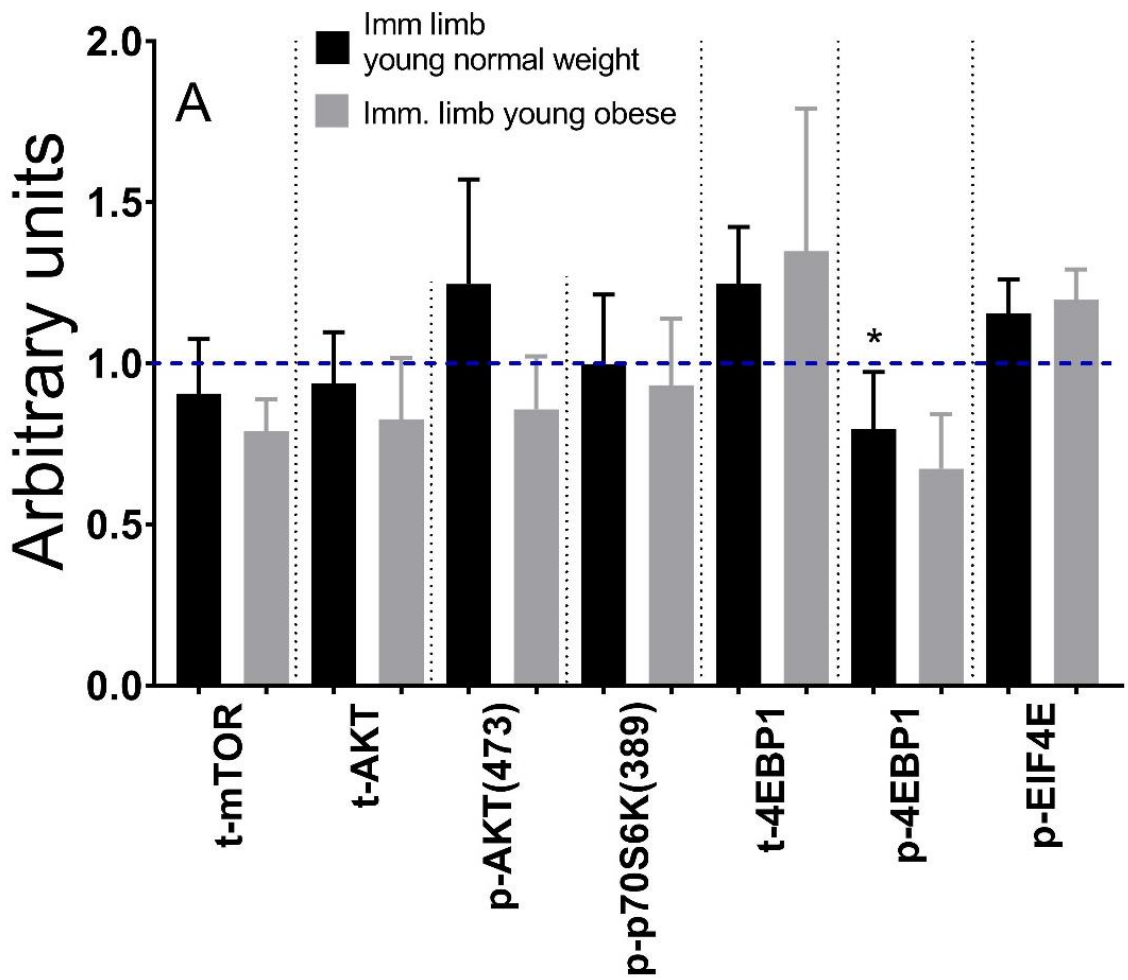

Figure 6.9A. *Medial gastrocnemius muscle protein expression in the immobilised limb presented as a fraction from the contralateral non-immobilised limb (blue line) following 3 days of unilateral lower limb immobilisation in young normal weight and young obese volunteers.* Values represent mean + SEM. * signifies significant difference between limbs ($p < 0.05$). AKT = Protein kinase B, mTOR = mammalian target of rapamycin, 4EBP1 = Eukaryotic translation initiation factor 4E-binding protein 1, P70S6K = Ribosomal protein S6 kinase beta-1, EIF4E = Eukaryotic translation initiation factor 4E.

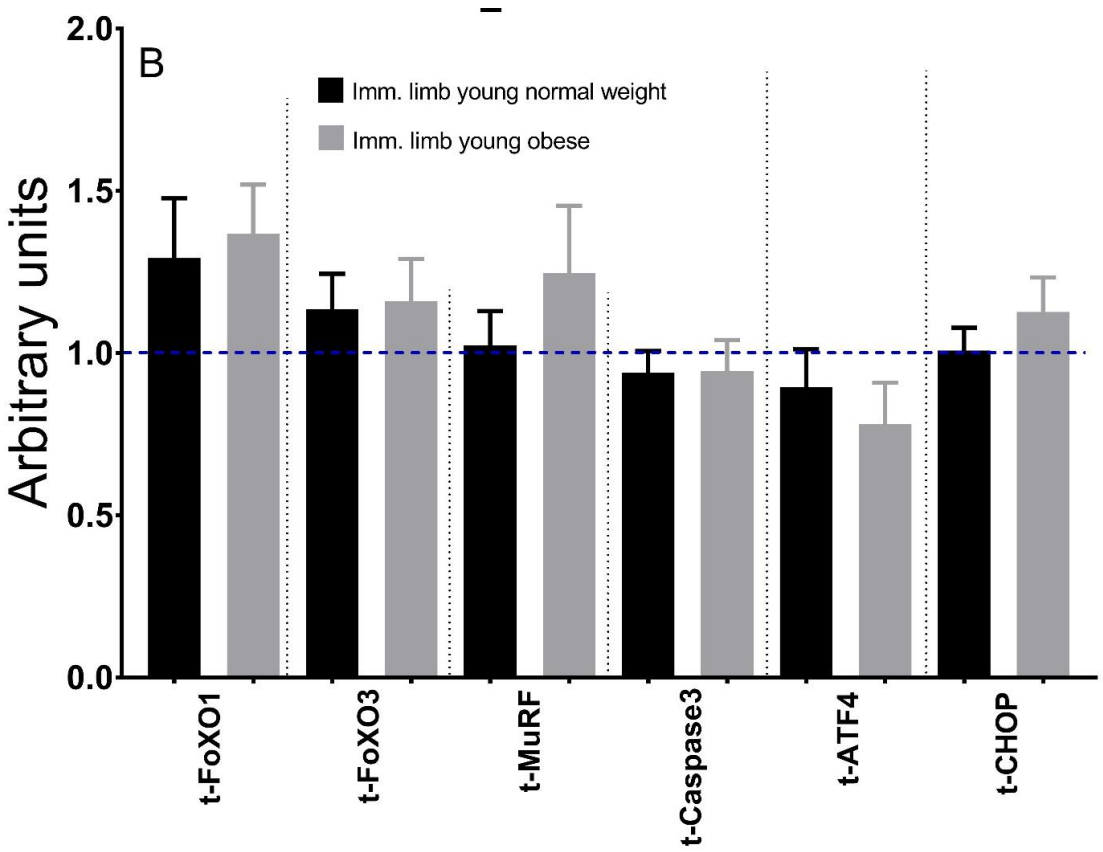

Figure 6.9B. *Medial gastrocnemius muscle protein expression in the immobilised limb presented as a fraction from the contralateral non-immobilised limb (blue line) following 3 days of unilateral lower limb immobilisation in young normal weight and young obese volunteers.* Values represent mean + SEM. FoXO1 = Forkhead box protein O1, FoXO3 = Forkhead box protein O3, CHOP = C/EBP homologous protein and ATF4 = Activating transcription factor 4.

6.5 Discussion

The aim of this study was to determine for the first time the impact of obesity in young volunteers on the free living pre-immobilised myofibrillar FSR in the medial gastrocnemius muscle and the impact of 3 days unilateral below knee limb immobilisation on chronic myofibrillar FSR in the immobilised limb and the contralateral non-immobilised limb. Medial gastrocnemius muscle myofibrillar FSR during free living conditions was similar in the young normal weight group (1.61 ± 0.20%d^{-1}) and in the young obese group (1.52 ± 0.34 %d^{-1}). However, chronic myofibrillar protein FSR over 3 days of unilateral lower limb immobilisation was significantly attenuated by 25% ($P<0.01$) in the young normal weight group in the immobilised limb when compared to the free living values (Figure 6.8) which remained unchanged in the young obese group. In addition, following 3 days of unilateral lower limb immobilisation in the young normal weight group myofibrillar protein FSR was 15% ($P<0.05$) less than in the contra-lateral non-immobilised limb when comparing limbs which remained unchanged in the young obese group.

No study has comparatively documented changes in myofibrillar protein FSR during muscle unloading in young normal weight and young obese volunteers. Here we demonstrate for the first time using D_2O tracer, following 3 days of unilateral lower limb immobilisation myofibrillar protein FSR remained unchanged in both limbs in young obese volunteers. This is rather surprising, given previous muscle unloading studies utilising an acute tracer methodology have unequivocally demonstrated an attenuation in myofibrillar protein FSR during muscle disuse induced atrophy in young

normal weight volunteers (de Boer et al., 2007; Glover et al., 2008; Wall et al., 2013). However, to date, no study has documented the impact of muscle unloading in an obese cohort and there doesn't seem to a major methodical limitation in studying such a population. Using the D_2O tracer, the body water pool was enriched to a similar concentration in both the young normal weight and young obese groups (0.25% ± 0.01 vs 0.24% ± 0.02 respectfully) and any additional lipid in the obese muscle biopsy would have been removed during the analytical process. However, one study has documented chronic myofibrillar protein FSR in response to immobilisation, which was reported for overweight middle aged (45-60 years old) men (Mitchell et al., 2018). Fourteen days of unilateral lower limb immobilisation induced no change in myofibrillar protein FSR from the basal to the immobilised state. However, this was despite mid-thigh muscle cross-sectional area declining significantly by ~4% when quantified using peripheral quantitative computed tomography (Mitchell et al., 2018). Similarly, medial gastrocnemius muscle volume in the present study declined significantly by ~15% in the young obese group over 3 days unilateral lower limb immobilisation despite myofibrillar protein FSR remaining unchanged. One possible explanation could be, obesity is associated with systemic low-grade chronic inflammation (Murton et al., 2015) and as a result of the inflammation, MPB is accelerated during muscle unloading which contributes to the decline in muscle volume during unloading. Indeed, trauma induced inflammation is known to upregulate leg protein breakdown in critically ill patients (Puthucheary et al., 2013) in line with pathway signalling data depicting the suppression of AKT signalling and increased muscle-specific E3-ligase (MAFbx and MuRF1) and 20S proteasome

mRNA and protein expression levels in critically ill patients when compared to healthy to controls (Constantin et al., 2011). However, we did not observe any changes in muscle protein expression of putative markers of MPS nor MPB pathway related proteins between the immobilised and non-immobilised limb following immobilisation in the young obese group (Figure 6.9). Furthermore, if increased systemic inflammation is giving rise to MPB in obese individuals, then it is difficult to explain why the obese individuals have increased baseline muscle mass when compared to normal weight counter parts. Further explanation could be provided in the light of obesity being associated with elevated basal FFAs, therefore increased lipid availability which Stephens and colleagues demonstrated suppresses post-prandial myofibrillar protein FSR in young lean individuals. Therefore, in the obese the ability to stimulate myofibrillar protein FSR is blunted in response to feeding (Murton et al., 2015; Smeuninx et al., 2017) and exercise (Beals et al., 2018). Conversely, we show in young obese the ability to attenuate myofibrillar protein FSR is response to unloading is not apparent. Collectively, this suggest obesity may be associated with protein metabolism inflexibility i.e. the inability to stimulate/attenuate myofibrillar protein FSR in response to external factors.

Medial gastrocnemius muscle volume measured using ultrasonography declined by 7% in the young normal weight group but declined to a higher degree of 15% in the young obese group following 3 days of unilateral lower limb immobilisation Figure 6.6A. Obesity is associated with a greater medial gastrocnemius muscle volume in the free living state when compared to normal weight counterparts, measured using ultrasonography (Erskine et

al., 2017). Erskine and colleagues demonstrated the medial gastrocnemius muscle in young obese individuals to be 45% greater when compared to young normal weight individuals. Similarly, we observed the obese group had on average 45% greater medial gastrocnemius muscle volume (Figure 5.6) and greater muscle thickness than age matched normal weight volunteers. In chapter 4 we observed baseline medial gastrocnemius muscle to correlate with the absolute decline in muscle volume during 3 days of unilateral lower limb immobilisation (Figure 4.6) across ages. Following on from this finding, we observed obesity was associated with a greater medial gastrocnemius muscle volume compared to the normal weight volunteers (45%, Figure 6.6). Furthermore, there was a robust correlation (R^2= 0.71, P<0.01) between baseline muscle volume and the absolute decline in muscle volume during 3 days of unilateral lower limb immobilisation in the young obese group (Figure 6.7). Therefore, it seems individuals with a larger muscle volume would experience greater muscle volume loss during immobilisation when compared to individuals with a lower initial muscle mass. The increased medial gastrocnemius muscle volume observed in obesity is probably as a result of the greater body weight in the obese group, which acts as a positive training stimulus during acts of daily living (i.e. standing up and walking) on weight bearing muscles, increasing muscle mass (Bosco et al., 1986). We did not observe any correlation between absolute change in medial gastrocnemius muscle volume during 3 days unilateral lower limb immobilisation and the absolute change in average physical activity from the free living state to the immobilised state in young normal weight and young obese volunteers, or with the data combined. In addition, muscle volume and thickness in the non-immobilised limb did not

change during immobilisation of the contralateral limb in both the young normal weight and young obese group. Therefore, this suggest, during muscle unloading in the obese with a greater body weight, the greater positive training stimulus during acts of daily living is withdraw such that this has a greater detraining effect thus leading to greater muscle volume decline during muscle unloading.

In conclusion, this study is the first to comparatively document changes in myofibrillar protein FSR in young normal weight and young obese volunteers during free living conditions and 3 days of immobilisation. Chronic medial gastrocnemius muscle myofibrillar protein FSR declined by 25% in the young normal weight group but did not change in the young obese group during immobilisation when compared to free living conditions. Obesity is associated with a reduced stimulation of MPS to a given dose of amino acid infusion (Murton et al., 2015; Smeuninx et al., 2017) and exercise (Beals et al., 2018) whilst we demonstrate the ability to attenuate myofibrillar protein FSR is response to unloading is not apparent therefore suggesting obesity may be associated with protein metabolism inflexibility i.e. the inability to stimulate/attenuate myofibrillar protein FSR in response to external factors. Obesity was associated with a higher baseline medial gastrocnemius muscle volume perhaps as a result of the increased bodyweight acting as a positive training stimulus. The young obese group lost more than twice the relative (7% vs. 15%) and three times the absolute (-10.48 ± 4.79 cm^3 vs. -31.33 ± 9.74 cm^3) medial gastrocnemius muscle volume during muscle unloading. We observed a negative correlation between higher medial gastrocnemius

muscle volume and a higher absolute decline in muscle volume during immobilisation within groups and with the data combined.

Chapter 7

7 Overall discussion

The overall aim of this thesis was to provide novel insight into the impact of 3 days unilateral lower limb immobilisation on muscle architecture (volume, thickness, fascicle length and pennation angle), myofibrillar protein FSR and the putative regulators of MPS, MPB and ER-stress related pathways during 3 days unilateral lower limb immobilisation in young, normal weight, volunteers compared to the free living state, whilst also quantifying physical activity levels (Chapter 3). Furthermore, the thesis aimed to determine whether aging (Chapter 4) and obesity (Chapter 6) further impacted on muscle architecture and protein metabolism and physical activity responses to unilateral lower limb immobilisation. Finally, as we observed limited changes in muscle protein expression in response to short-term immobilisation (Chapter 3 and 4) we explored the long term (14 days) impact of bed rest and subsequent 14 days rehabilitation on quadriceps muscle volume and mRNA expression of 96 gene targets known to regulate muscle mass and structure, and muscle carbohydrate and lipid metabolism in young and older volunteers utilising a RT-PCR microfluidic, low-density array cards approach and IPA bioinformatics analysis muscle protein expression to unravel novel insights into gene responses to muscle disuse in bed-rest and subsequent rehabilitation in the context of aging.

7.1 Deuterium derived muscle myofibrillar protein FSR and ultrasonography determined muscle volume

In adults, muscle mass is regulated through the dynamic process of MPS and MPB, which fluctuate throughout a diurnal cycle, but remain in overall

balance to maintain muscle mass (Atherton and Smith, 2012). In **Chapter 3**, utilising D_2O tracer methodology (Wilkinson et al., 2014) which allows for myofibrillar protein FSR quantifications over chronic periods (days and weeks as opposed to hours), it was found that over 3 days of unilateral lower limb immobilisation, medial gastrocnemius muscle myofibrillar protein FSR was attenuated by 25% compared to free living conditions ($P<0.01$; Figure 3.6). In keeping with this, myofibrillar protein FSR attenuation in the immobilised limb was accompanied by a 7% decline in medial gastrocnemius muscle volume determined using ultrasonography. These observations are in accordance with data demonstrating acute myofibrillar protein FSR when measured acutely before and at the end of immobilisation is attenuated in young participants (de Boer et al., 2007; Glover et al., 2008). Glover and colleagues demonstrated a reduction of 27% in the post-absorptive MPS rate following 14 days unilateral lower limb immobilisation whilst de Boer and colleagues demonstrated a 51% reduction in post-absorptive myofibrillar protein FSR in young humans. In addition, utilising the contralateral limb as a within subject control in this thesis, it was found that myofibrillar protein FSR was less in the immobilised limb compared with the immobilised limb (15%, $P<0.05$), which was no different from the free living state.

In **Chapter 4,** 3 days of unilateral lower limb immobilisation attenuated myofibrillar protein FSR from the free living state in older normal weight people (15%, $P<0.05$), which was less than the decline seen in normal weight young volunteers during immobilisation. Furthermore, unlike young normal weight volunteers, there was no difference in myofibrillar protein FSR when comparing between limbs in the older normal weight volunteers, which

is likely to be at least partly attributable to average physical activity levels remaining unchanged from the free living state in the older normal weight volunteers during immobilisation (Figure 4.4). This supports the stance that at least some of the decline in muscle mass attributable to ageing is likely being driven by an age-related reduction in habitual physical activity levels.

Despite myofibrillar protein FSR declining by 15% from the free living state in immobilised limb of the older normal weight volunteers, medial gastrocnemius muscle volume did not change in the immobilisation limb (unlike in the young). In addition to the relatively lower decline in muscle FSR during immobilisation, the older volunteers were also less physically active and had a comparatively lower medial gastrocnemius muscle volume at baseline when compared to the young volunteers. Collectively, these factors would have likely resulted in less of a drive for muscle volume to decline during immobilisation in the older volunteers. This, together with the likelihood that older volunteers also had greater muscle lipid content and greater muscle fibrous connective tissue, which would not be discernible using ultrasonography, suggests that the ultrasound method employed was not sensitive enough to reflect the impact of a 15% reduction in myofibrillar protein FSR over 3 days of immobilisation. Indeed, in chapter 5 the absolute decline in muscle volume was similar between young and older volunteers when quantified using MRI, albeit over a long period of muscle unloading.

Building on from this, **Chapter 6** demonstrated 3 days of unilateral lower limb immobilisation did not change myofibrillar protein FSR in young obese volunteers when compared to the free living state or the non-immobilised limb. This is a rather surprising finding, given previous muscle unloading

studies utilising an acute tracer methodology have unequivocally demonstrated an attenuation in myofibrillar protein FSR during muscle disuse induced atrophy (de Boer et al., 2007; Glover et al., 2008; Wall et al., 2013). However, to date, no study has documented the impact of muscle unloading in an obese cohort and there doesn't seem to a methodical limitation in studying an obese population. Using the D_2O tracer, the body water pool was enriched to a similar concentration in both the young normal weight and young obese groups (0.25% ± 0.01 vs 0.24% ± 0.02 respectfully) and any additional lipid in the obese muscle biopsy would have been removed during the analytical process. However, one study has documented chronic myofibrillar protein FSR in response to immobilisation, which was reported for overweight middle aged (45-60 years old) men (Mitchell et al., 2018). In agreement, 14 days of unilateral lower limb immobilisation induced no change in myofibrillar protein FSR from the basal to the immobilised state. However, this was despite mid-thigh muscle cross-sectional area declining significantly by ~4% when quantified using peripheral quantitative computed tomography. Similarly, medial gastrocnemius muscle volume significantly declined by ~15% in the young obese group during 3 days unilateral lower limb immobilisation despite myofibrillar protein FSR remaining unchanged. One possible explanation could be, obesity is associated with systemic low-grade chronic inflammation (Murton et al., 2015) and as a result of the inflammation, MPB is accelerated during muscle unloading which contributes to the decline in muscle volume during unloading. Indeed, trauma induced inflammation is known to upregulate leg protein breakdown in critically ill patients (Puthucheary et al., 2013) in line with pathway signalling data depicting the suppression of AKT

signalling and increased muscle-specific E3-ligase (MAFbx and MuRF1) and 20S proteasome mRNA and protein expression levels in critically ill patients when compared to healthy to controls (Constantin et al., 2011). However, we did not observe any changes in muscle protein expression of putative markers of MPS nor MPB pathway related proteins between the immobilised and non-immobilised limb following immobilisation in the young obese group (Figure 6.9). Furthermore, if increased systemic inflammation is giving rise to MPB in obese individuals, then it is difficult to explain why the obese individuals have increased baseline muscle mass when compared to normal weight counter parts. Further explanation could be provided in the light of obesity is associated with elevated basal FFAs, therefore increased lipid availability which Stephens and colleagues demonstrated suppresses post-prandial myofibrillar protein FSR in young lean individuals. Therefore, in the obese the ability to stimulate myofibrillar protein FSR is blunted in response to feeding (Murton et al., 2015; Smeuninx et al., 2017) and exercise (Beals et al., 2018). Conversely, we show in young obese the ability to attenuate myofibrillar protein FSR is response to unloading is not apparent. Collectively, this suggest obesity may be associated with protein metabolism inflexibility i.e. the inability to stimulate/attenuate myofibrillar protein FSR in response to external factors.

Rather surprisingly in the context of the FSR responses, medial gastrocnemius muscle volume declined by ~15% during 3 days of unilateral lower limb immobilisation in the young obese volunteers. These individuals had on average 45% greater medial gastrocnemius muscle volume (Figure 6.6) and x% greater muscle thickness than their age matched normal

weight counterparts. Furthermore, a significant association was observed between baseline medial gastrocnemius muscle volume and the absolute decline in muscle volume during immobilisation across all age groups and BMI ranges of volunteers from Chapters 3, 4, 6 (Figure 7.1. In keeping with this, a significant association between baseline quadriceps muscle volume (measured using MRI) and the absolute decline in muscle volume during 14 days of bedrest in Chapter 5 was also observed (Figure 5.3). Overall therefore it would seem baseline muscle volume is influencing the absolute decline in muscle volume during unloading i.e. during muscle unloading individuals with a higher medial gastrocnemius muscle volume have greater muscle loading on a daily basis such that during muscle unloading, this has a greater detraining effect thus leading to greater muscle volume decline during muscle unloading.

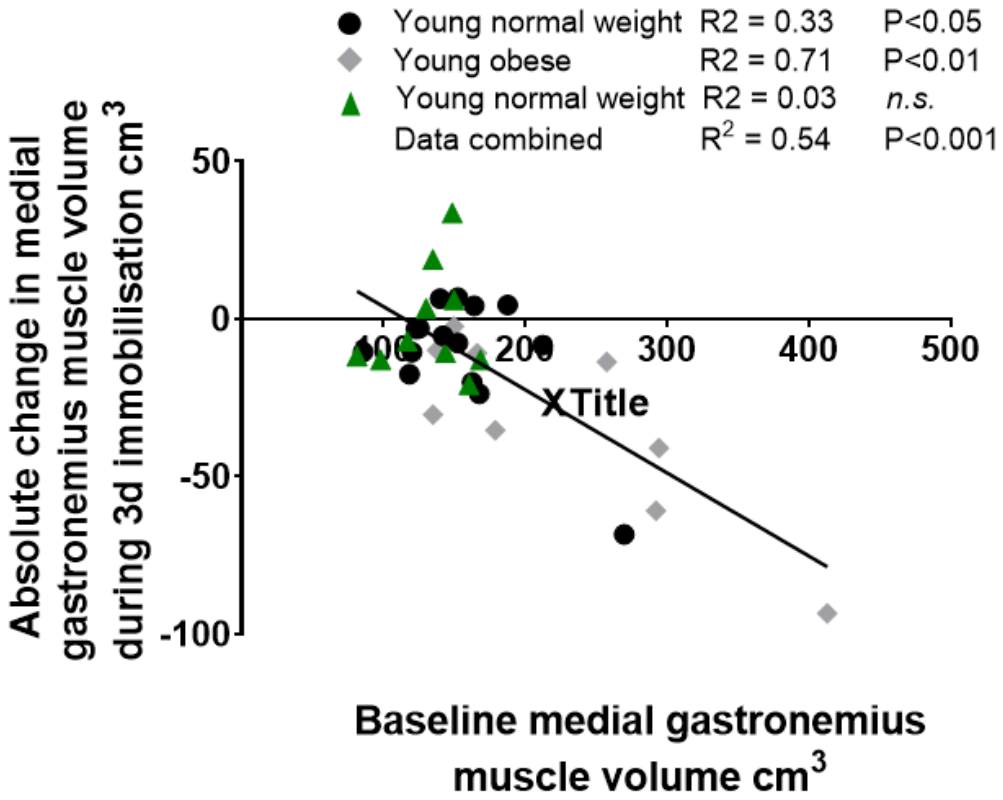

Figure 7.1. *Association between baseline medial gastrocnemius muscle volume and the absolute change in muscle volume during 3d unilateral lower limb immobilisation in young normal weight (black circle) and young obese (grey diamond) volunteers, older normal weight (green triangle) and 6 older obese volunteers (blue square).*

7.2 **Muscle protein expression and gene signalling during muscle unloading and rehabilitation**

In keeping with previous human muscle unloading studies (de Boer et al., 2007; Jespersen et al., 2014), in Chapter 3, 4 and 6 we did not observe any impact of immobilisation on the expression of putative regulators of MPS, MPB and ER stress related pathway with exception to phospho-4E-BP1

(Thr37/46) which was 52% ($P<0.05$) less in the young normal weight group and 51% ($P<0.01$) less in the older normal weight group in the immobilised limb when compared to the non-immobilised limb (Figure 4.7A). Eukaryotic initiation factor 4E (eIF4E) binds to mRNA at the 5' end and induces translational initiation. However, the action of eIF4E is prevented by 4E-BP1. Phosphorylation of 4E-BP1 prevents the binding of 4E-BP1 to eIF4E promoting cap-dependent translation (reviewed by Richter and Sonenberg, 2005). Therefore, the reduction in myofibrillar protein FSR following 3 days of unilateral lower limb immobilisation could be due to reduced phospho-4E-BP1 thereby attenuating cap dependent translational initiation and reducing myofibrillar protein FSR during immobilisation.

A reduction in myofibrillar protein FSR during muscle unloading could be modulated through the mechanotransduction sensing protein FAK which resides on the extracellular matrix of the muscle and converts mechanical stimulus (muscle contraction) into electrochemical activity (Kosek and Bamman, 2008). Indeed FAK phosphorylation expression has been reported to be attenuated during immobilisation in humans by 30% following 14 days of unilateral lower limb suspension (de Boer et al., 2007). Likewise, a 6-fold increase in neural cellular adhesion molecule (NCAM) positive muscle fibres has been demonstrated following 3 days of bed rest in healthy young male volunteers (Pi-Sunyer, 1999) and a 2-fold increase in NCAM positive muscle fibres following 14 days of bed rest in middle aged men (Arentson-Lantz et al., 2016) suggesting an early muscle denervation process which would presumably involve changes in both muscle myofibrillar protein FSR and MPB, which warrants further investigation.

Chapter 5 involved utilisation of RT-PCR microfluidic, low-density array cards focused on 96 targeted gene transcripts, and subsequent bioinformatics analysis using IPA. Bed rest resulted in a similar decline in absolute quadriceps muscle volume in both the young and older volunteers. In addition, based on mRNA changes relating to carbohydrate metabolism, lipid metabolism, protein synthesis and inflammatory response IPA analysis predicted bed rest initiated a similar cellular metabolic event in young and older volunteers. However, multiple mRNA genes responses relating to lipid metabolism, cellular development, protein synthesis and cell death and survival were blunted in the OG when compared to the YG following rehabilitation and based on the mRNA gene responses, IPA predicted reduced cellular functions relating to lipid metabolism, cellular development, protein synthesis and cell death and survival. However, despite a blunting in mRNA gene responses related to muscle cellular development, protein metabolism, muscle cell death and survival and lipid metabolism and attenuated IPA predicted cellular functions in the OG, the absolute muscle volume decline assessed using MRI during bed rest was restored in the OG during rehabilitation. Therefore, it would seem the older had reached their full capacity to maximise cellular functions (i.e. they had returned to baseline), but the young had the potential to continue having changes in the networks.

7.1 The impact of unilateral lower limb immobilisation on physical activity levels.

Muscle metabolic and physiological deterioration during hospital admission and recovery from illness and/or injury are major clinical challenges in both young and older adults, but especially in the latter. Primary aging is the inevitable decline of cellular structure and biological function, independent of disease or harmful lifestyle or environmental factors (Holloszy, 2000). Secondary aging, is defined as additional environmental factors (life-style [including diet and physical activity] and non-communicable diseases) which may accelerate the deterioration in muscle metabolic health. Due to the difficulty in delineating the impact of primary aging from drivers of secondary ageing on muscle mass and metabolic health status in humans, this has led to the uncertainty in the literature with regards to the primary determinants of muscle atrophy during muscle unloading.

The research studies in the thesis are the first to our knowledge to quantify physical activity levels prior to and during unilateral limb immobilisation across groups differing in age and body composition. Summarising the findings from Chapters 3, 4 and 6, average daily habitual physical activity levels in the free living state (albeit over 3-4 days) were similar between young normal weight and young obese individuals, but lower in normal weight older people when compared with either group (~20%, $P<0.001$; Figure 7.2). Aging is known to associated with reduced habitual physical activity levels (Frisard et al., 2007) which will no doubt have deleterious effect on muscle metabolic health (Breen et al., 2013; Tanner et al., 2015).

Importantly, during periods of immobilisation in this thesis, average daily habitual physical activity was found to be 23% lower in the normal weight older people compared with young normal weight people. It would seem therefore that the older normal weight group had relatively low levels of habitually physical activity in the free living state, which remained unchanged during unilateral immobilisation, whilst the young volunteers reduced their habitual physical activity levels during unilateral limb immobilisation. This has an important public health message; that older people are habitually inactive and unilateral limb immobilisation does not reduce PAL further. However, young people (whether normal or over weight) are at the greatest risk of reduced physical activity levels during lower limb immobilisation.

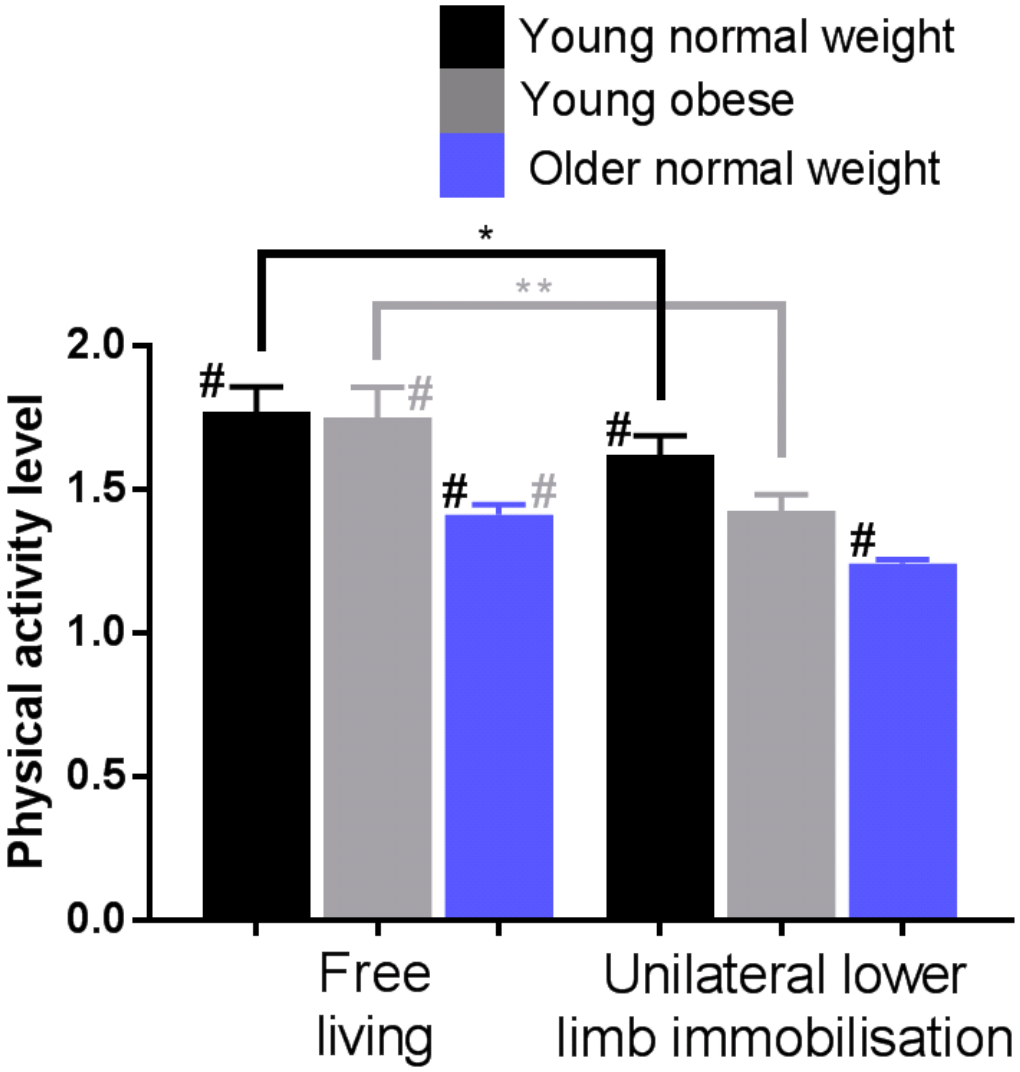

Figure 7.2. *Average daily physical activity level (PAL) score recorded using an ActiHeart during 3 or 4 days of free living and 3 days of unilateral lower limb immobilisation in healthy, normal-weight (black), young obese (grey) and older normal weight (blue) volunteers. * signifies P< 0·05 and ** signifies P<0·001. # signifies P< 0·001 between same time points across groups.*

Strengths, limitations and future perspective

This thesis is the first to describe a series of novel experiments. No previous study has comparatively documented changes in myofibrillar protein FSR

during muscle unloading in context to aging (Chapter 4) or obesity (Chapter 6). In addition, the application of D_2O tracer, as used in thesis provides a distinct methodological advantages as researchers are able to quantify accumulative changes in muscle myofibrillar protein FSR over days as opposed to hours in a clinical setting thereby providing a more accurate reflection of the dynamic process of muscle myofibrillar protein synthesis in a 'free-living' environment and during immobilisation.

A good number of volunteers enrolled to participate in ambitious clinical research studies with protocols involving multiple biopsies. Chapters 3, 4, and 6 utilised the unilateral lower limb immobilisation approach which allows for the contralateral non-immobilised limb to act as a within subject control thereby providing novel insight into the regulation between the immobilisation and contralateral non immobilised limb during unilateral lower limb immobilisation but also being able to reflect these changes to the baseline muscle biopsy. We also measured habitual physical activity levels using an ActiHeart throughout the study intervention as physical activity level is known impact on myofibrillar protein FSR however research studies often overlook this aspect.

As with all clinical research studies there are some short comings. It would have definitely been beneficial to have performed MRI scans as opposed to ultrasonography to assess medical gastrocnemius muscle volume during immobilisation due to the increased sensitivity of the method. We would have also liked to recruited more participants to an older obese group (n=6) and due to the low numbers, the data was not published in this thesis.

In designing this study, I selected to study the medial gastrocnemius muscle for muscle disused induced atrophy as the medial gastrocnemius muscle has shown to be the most susceptible to disuse induced atrophy (Belavy et al., 2017; de Boer et al., 2008). Furthermore, the medial gastrocnemius muscle has been studied previously during muscle induced atrophy by utilising a below knee cast (Nedergard et al., 2012). Therefore, although the point is valid in that the medial gastrocnemius muscle has its origins attached to the femur, a below knee cast is still able to result in atrophy of the muscle. Furthermore, performing soleus biopsies is methodically difficult due to the small size of the muscle and its anatomical location. In addition, the soleus muscle in humans is composed predominantly of slow-twitch muscle fibres which are resistant to atrophy. Therefore, studying the medial gastrocnemius muscle was most appropriate.

As the primary focus of research was intended to be performed on the medial gastrocnemius muscle for which muscle metabolism and physiological parameters were measured during muscle unloading. No muscle function tests were performed on the medial gastrocnemius muscle during this study as making isolated functional measurements would have been very difficult. In addition, we were concerned that making functional measurements during plantar flexion would not be representative of muscle level measurements given the ankle had been fixed in a rigid position for 3 days. The analytical approach which was performed for the comparing protein expression data between limb post-immobilisation does have some drawbacks. By utilising the contralateral non-immobilised limb as a within subject control it is assumed that the control limb is performing the same

workload as during free living condition. However, with having the one leg immobilised, the contralateral non-immobilised leg is being overworked. This may lead to exaggerated differences in protein expression between limbs as one limb is being trained whilst the other is being de-trained. As we did not observe major differences between limbs following immobilisation it can only be assumed the contribution of the extra workload and positive training stimulus on the non-immobilised limb during immobilisation was minimal. In an ideal world, if the experimental analysis were to be performed again, comparisons from the immobilised limb should have been drawn to the non-immobilised post immobilisation and during free living condition (baseline biopsy). Furthermore, during the western blotting process gel loading was quantified using Coomassie staining as a within loading gel control. However, sadly we did not utilise a between gel loading control which is a limitation. To circumvent this limitation, I compared the difference in protein expression in the immobilised limb relative to the contralateral non-immobilised limb, and all these measurements were made on the same gel. Therefore, comparison of pre-vs post immobilisation responses between volunteer groups won't be influenced by protein loading between gels which negated the use of utilising an internal gel loading control to make comparison between gels. In Chapter 4 we determined the impact of aging on short-term muscle unloading. We therefore studied older volunteers. In order to do this, we chose to use the World Health Organisation definition of an older person in a developed country of 65 years of age and older. We recruited individuals who were healthy and free from metabolic disease, cardiovascular disease, overt muscle wasting, cerebrovascular disease, respiratory disease, inflammatory bowel disease, renal disease and/or

clotting dysfunction was present. In addition, participants were non-smokers and did not consume more than >21 units of alcohol per week, as smoking reduces basal myofibrillar protein FSR (Petersen et al., 2007) and alcohol consumption suppresses muscle anabolic responses (Parr et al., 2014). Therefore, in having a strict recruitment criteria and with older volunteers demonstrating to have sufficient upper body strength to use crutches, there may have been some selection bias. Then again, this could also be seen as a study strength from the perspective of recruitment and study power.

In the immobilisation studies I used a coin-toss in selecting which limb was to be immobilised. This method of selecting which limb is to be immobilised is different from previous muscle unloading studies which immobilise the dominant limb only and are more likely to see a difference in muscle metabolic and physiological measurements during unloading between limbs. To balance this out, I used a coin-toss method to select which limb was to be immobilised. Indeed, the use of a coin-toss can be influenced by the coin "tosser" (Clark and Westerberg, 2009). However, for the purpose of this study, this has little importance. In this study, we immobilised the dominant leg on 19 occasions and the non-dominant leg on 15 occasions. I thought this approach is more clinically relevant.

The work presented in Chapter 6 is thought-provoking and of great interest. Obesity is associated with greater baseline muscle volume. However, during immobilisation, the young obese group lost the greatest volume of muscle despite myofibrillar muscle protein FSR remaining unknown. No doubt this finding warrants further investigation to understand the potential drivers of muscle atrophy in the young obese group.

Over the past few years, our understanding of the cellular and molecular mechanisms involved in human muscle disuse induced atrophy has significantly increased. However, gaps in our understanding still remains with numerous unanswered questions. The importance of protein turnover in driving muscle disuse induced atrophy has been widely recognised. When the rate of muscle protein synthesis becomes slower than the rate of muscle protein breakdown, muscle atrophy occurs. Is it well established from a number of human clinical experiments, suppressed muscle protein synthesis seems to be the major driver of muscle disuse induced atrophy rather than increased muscle protein breakdown. However, with the emergence of D2O tracer which allows measurements of myofibrillar protein FSR over a sustained period (1 day to 2 weeks), in free living conditions, future studies should seek to unravel the temporal changes in muscle protein metabolism in response to unloading. For example, how quickly does muscle unloading lead to an attenuation in muscle protein synthesis. Is 1-day sufficient to attenuate to muscle protein synthesis? Following on from this, we know muscle unloading leads to a decline in muscle protein synthesis which is greater at the onset of muscle unloading and slowly plateaus off, but what is the daily decline in the rate of muscle protein synthesis during unloading?

On the other hand, we currently lack effective therapeutical (with exception to resistance exercise training) and pharmaceutical countermeasures to attenuate muscle disuse induced atrophy in humans. The major obstacle is our lack of understanding regarding the cellular and molecular mechanisms involved in human muscle disuse induced atrophy, which is far more complicated than initially envisaged. Rodent models of muscle disuse

induced atrophy provide a good foundation for knowledge base, however the work does not always translate into human models of muscle atrophy. Therefore, before work can fully begin on counteracting the loss of muscle atrophy during muscle unloading through means of pharmaceutical treatment, our understanding of the cellular and molecular mechanisms drivers of muscle atrophy needs to increase to unravel potential pharmaceutical treatment.

Physical inactivity and sedentary behaviour is the fourth leading cause of global mortality (Lee et al., 2012) and a major driver of ill health in today's society. Therefore, understanding the musculoskeletal deterioration associated with physical inactivity is a major public health problem and embodies a vital area for scientific investigation. This thesis has provided novel insight regarding muscle metabolic (muscle protein metabolism and protein expression), muscle physiological (volume, thickness, fascicle length and pennation angle) and physical activity level changes during muscle unloading in humans and accessed the impact of aging and obesity on these parameters. In addition, this thesis has provided novel insight regarding mRNA gene expression changes during bed rest in young and older volunteers and during rehabilitation and has offered insight of metabolic functions most affected by these changes.

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9 Appendix

9.1 **Appendix 1.1 –** General health questionnaire

Three day leg cast study health questionnaire

Participant Contact Details

Today's date: …….

Please record below the participant's contact details

Title: ……

First name: ……..... **Surname**: ……..

Date of Birth:……..

Address: **Telephone:**

House number: …….. Home: ……..

Street name: …… Work: ……..

Town/City: …… Mobile: ……..

Postcode: …….. Preferred method of contact:

Email: ……..... ……

Please provide details of someone who can be contacted by the research team in case of an emergency

---

Title: ……

First name: ……..... **Surname** :……

Address: **Telephone:**

House number: …….. Home: ……..

Street name: …… Work: ……..

Town/City: …… Mobile: ……..

Postcode: …….. Preferred method of contact:

Email: ……..... ……

GP Details

Doctor/Surgery name: ……..

Address: ……..

……

Telephone: ……

GENERAL HEALTH QUESTIONAIRE

In general would you say your health is; Please tick one response

a. **……** *Much better than a year ago*

b. **……** *Somewhat better than a year ago*

c. **……** *About the same*

d. **……** *Somewhat worse than a year ago*

e. **……** *Much worse than a year age*

2. The following questions are concerned with any medical conditions you might have.

Please tick one box on each line

Condition	No	Yes	Don't Know
Diabetes			
Epilepsy			
Depression			
Hayfever			
Asthma			
Eczema			
High blood pressure			
Heart problems			
Breathing problems *			
Indigestion / heartburn			
Irritable bowel			
Any other medical condition not listed above (please detail below)			

*** Other than Asthma**

Please detail any other medical condition/s:

……

……

……

If you answered '*yes*' to any of the above, when did you first start having the problem and what medication (if any) do you use?

……

……

……

……

……

……

3. Do you take any regular medication? Yes…….. No……..

If yes please provide further information: (drug, dose, frequency, duration)

……

……

4. Do you have a tendency to faint in certain situations?

Yes…….. No…….. Don't Know……..

eg. on standing, in a warm room or after fasting

5. On average, how many cigarettes do you smoke a day? ……..……..
Did you ever smoke in the past? ……
If so, how when did you quit? ……

6. Have you had any previous injuries* which affected any of your lower limbs?

* An injury which limited your walking ability for more than 2 days in the last year.

Yes…….. No……..

If yes please provide further information:

……

……

……

……

……

……

7. Do you drink alcohol:

Yes…….. No……..

If yes please provide further information: (units per average week)

……

……

DIET AND HEALTH

The following questions relate to your diet and your health

Please tick next to your answer.

1. Are you on a medically prescribed diet? eg diabetic, gluten-free etc

Yes…….. No…….. Don't Know……..

If '*yes*', what diet are you following?

……

……

2. Do you suffer from any food allergies? Eg nuts, wheat, milk

Yes…….. No…….. Don't Know……..

If '*yes*', which foods are you allergic to?

……

……

3. Do you regularly (at least once a week) take **vitamin** and / or **mineral** supplements

Yes…….. No…….. Don't Know……..

If '*yes*', which supplements do you take?

……

……

4. Do you regularly (at least once a week) take any other supplements for your health? Eg. Creatine, Carnitine, Echinacea, St John's Wort, etc

Yes…….. No…….. Don't Know……..

If '*yes*', which supplements do you take?

……

……

Work and leisure activities

This section asks about the type of work you do if you work and followed by the duration and intensity of any work and leisure activities. **Physical Activity Record** (Please think of a **usual day** during the last 3-months)

Please tell us the type and amount of physical activity involved in your work.

		Please mark one box only
A.	I am not in employment (e.g. retired, retired for health reasons, unemployed, full-time carer etc.)	
B.	I spend most of my time at work sitting (such as in an office)	
C.	I spend most of my time at work standing or walking. However, my work does not require much intense physical effort (e.g. shop assistant, hairdresser, security guard, childminder, etc.)	
D.	My work involves definite physical effort including handling of heavy objects and use of tools (e.g. plumber,	

	electrician, carpenter, cleaner, hospital nurse, gardener, postal delivery workers etc.)	
E.	My work involves vigorous physical activity including handling of very heavy objects (e.g. scaffolder, construction worker, refuse collector, etc.)	

Clinical Physical Activity Questionnaire (CPAQ) (Please think of a **usual week** during the last 3-months)

If you do not work or are not at college, please go straight to Part B (leave Part A blank).

When completing the questionnaire, please consider:

- **MODERATE** activity as any activity that gets you mildly sweaty and out of breath, (e.g. brisk walking, carrying loads, digging, climbing ladders)
- **VIGOROUS** activity as any activity that involves hard physical effort and makes you breathe much harder than normal (e.g. heavy lifting, aerobics, or fast bicycling)

Please tick here if you DO NOT complete ANY physical activity as outlined below in the normal week ☐

Part A: AT WORK/COLLEGE		MODERATE	VIGOROUS
1. How much time do you spend doing physical activity **at work/college** every week? The activity much be done for at least **10** minutes at a time. Please include time spent travelling to/from work or college (if **moderate or vigorous** in nature).	Monday Tuesday Wednesday Thursday Friday Saturday Sunday	Minutes ……. Minutes ……. Minutes ……. Minutes ……. Minutes ……. Minutes ……. Minutes …….	Minutes ……. Minutes ……. Minutes ……. Minutes ……. Minutes ……. Minutes ……. Minutes …….
Part B: AT HOME/LEISURE TIME			

2. How much time do you spend doing **sport/exercise when not at work or college?** The activity much be done for at least 10 minutes at a time. Please include time spent travelling to/from events (if **moderate or vigorous** in nature).	Monday Tuesday Wednesday Thursday Friday Saturday Sunday	Minutes ……. Minutes ……. Minutes ……. Minutes ……. Minutes ……. Minutes ……. Minutes …….	Minutes ……. Minutes ……. Minutes ……. Minutes ……. Minutes ……. Minutes ……. Minutes …….

Have 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; having an invasive procedure (eg blood sample >50ml) or being exposed to ionising radiation?

If yes please provide further information:

……

……

……

……

……

……

……

……

……

……

			-						

MUSCLE HEALTH QUESTIONNAIRE

Only answer these questions if you are **65 and older**

The following questions relate to muscle health. Please answer each question as accurately as possible. If you would like any question explained or if you would like help, please speak with the researcher.

1. Do you suffer from muscle discomfort such as aches / pains or tiredness?

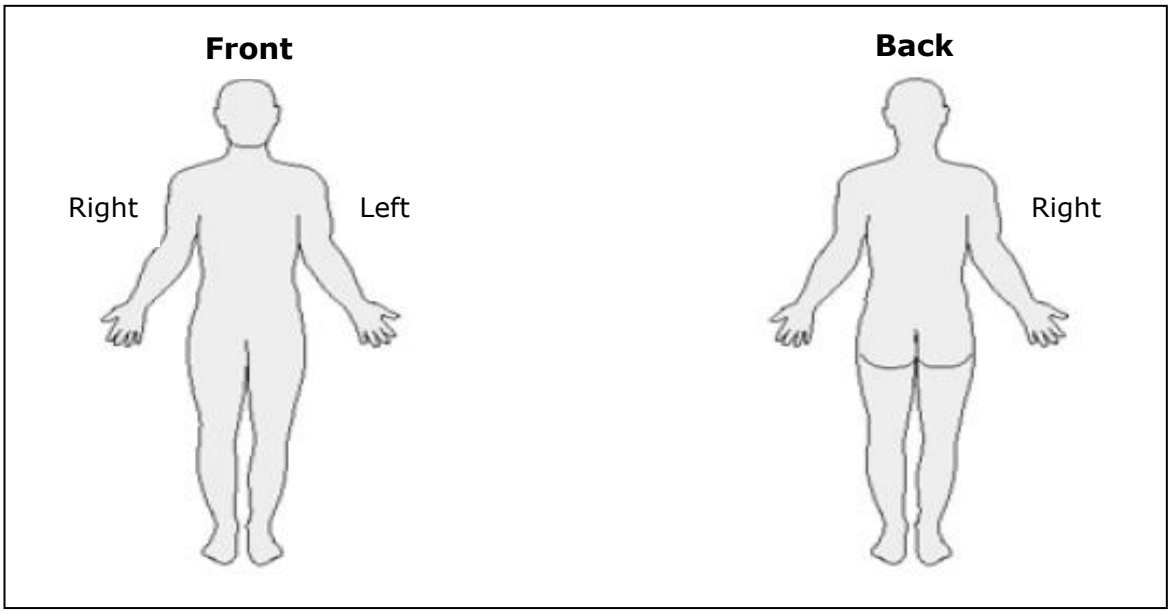
Yes…….. No……..

If no, please go to Page 14.

2. Is the discomfort:

All over the body …….. **OR** In a certain area of the body ……..

If the discomfort is in a certain area of the body, please indicate on the diagram below where it is.



3. Please tick which box best describes your discomfort.

	On most days	At it's worst	At it's best
No discomfort			
Mild			
Distressing			
Horrible			
Excruciating			

4. Look at the words below which can be used to describe muscle discomfort. If any of these describe your muscle discomfort, please indicate its intensity by placing a tick in the appropriate column.

	None	Mild	Moderate	Severe
Weakness				
Loss of strength				
Throbbing				
Shooting				
Stabbing				
Cramping				
Gnawing				
Stiffness				
Tender				
Numb				
Aching				
Tiring/exhausting				
Unbearable				

5. On a scale of 1 to 10, please circle where your discomfort is **usually**.

No discomfort **1** **2** **3** **4** **5** **6** **7** **8** **9** **10** **Worst possible**

6. How often do you feel this discomfort?

Rarely **Occasiona** **Often** **Frequentl** **All the**

7. Approximately, how long have you had this discomfort?

☐ ☐ ☐ ☐ ☐

1-2 weeks **1-2 month** **Over 6 months** **1 year** **Over a year**

8. Is your muscle discomfort due to a previous injury/accident? ☐ **Yes** ☐ **No**

.....

.....

.....

9. What kinds of things make the discomfort better (e.g. heat, pain killers, rest)?

.....

.....

.....

.....

10. What kinds of things make the discomfort worse (e.g. walking, standing, lifting)?

.....

.....

.....

.....

.....

11. If you experience muscle discomfort, has it got worse since taking the statin?

☐ **Yes** ☐ **No**

Thank you for completing the questionnaire.

HEIGHT AND WEIGHT

Height		cm	
Weight		Kg	
BMI (researcher can calculate if required)		BMI	
BP Standing		mm/hg	Heart Rate: …….. BPM
BP Sitting		mm/hg	Heart Rate: …….. BPM
BP Supine		mm/hg	Heart Rate: …….. BPM
Cybex Strength	1)	nm	
	2)	nm	
	3)	nm	
Dominant side:			

Research Associate completing this form:

Signature: . . . . . Designation . . . . . .

Date: | | | . | | | . | 2 | 0 | 1 | |

9.2 Appendix 1.2 – Gene card

Detector	Comments
18S-Hs99999901_s1	Eukaryotic 18S rRNA
EIF4EBP1-Hs00607050_m1	eukaryotic translation initiation factor 4E binding protein 1
PRKAA1-Hs01562315_m1	protein kinase, AMP-activated, alpha 1 catalytic subunit
ACTA1-Hs00559403_m1	actin, alpha 1, skeletal muscle
ADIPOQ-Hs00605917_m1	adiponectin, C1Q and collagen domain containing
AKT1-Hs00178289_m1	v-akt murine thymoma viral oncogene homolog 1
ATF6-Hs00232586_m1	activating transcription factor 6
ATG12-Hs00740818_m1	autophagy related 12
ATG9A-Hs01036946_m1	autophagy related 9A
ATP5B-Hs00969569_m1	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide
ATP5I-Hs00273015_m1	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit E
BECN1-Hs00186838_m1	beclin 1, autophagy related
CAPN1-Hs00559804_m1	calpain 1, (mu/I) large subunit
CAPN2-Hs00965097_m1	calpain 2, (m/II) large subunit
CASP8-Hs01018151_m1	caspase 8, apoptosis-related cysteine peptidase
CCL19-Hs00171149_m1	chemokine (C-C motif) ligand 19
COL1A1-Hs00164004_m1	collagen, type I, alpha 1
COL2A1-Hs00264051_m1	collagen, type II, alpha 1
COX5A-Hs00362067_m1	cytochrome c oxidase subunit Va
COX5B-Hs00426950_g1	cytochrome c oxidase subunit Vb
CPT1A-Hs00912671_m1	carnitine palmitoyltransferase 1A (liver)
CPT1B;CHKB-CPT1B-Hs00189258_m1	carnitine palmitoyltransferase 1B (muscle),CHKB-CPT1B readthrough (NMD candidate)
CTSL-Hs00964650_m1	cathepsin L
EIF4B-Hs04368067_g1	eukaryotic translation initiation factor 4B
FABP3-Hs00997360_m1	fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor)
FBXO32-Hs01041408_m1	F-box protein 32
FOXO1-Hs01054576_m1	forkhead box O1
FOXO3B;FOXO3-Hs00921424_m1	forkhead box O3B pseudogene,forkhead box O3
FST-Hs00246256_m1	follistatin
GSK3B-Hs01047719_m1	glycogen synthase kinase 3 beta
HIF1A-Hs00153153_m1	hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)
HMBS-Hs00609297_m1	hydroxymethylbilane synthase
HSPA8-Hs00941880_m1	heat shock 70kDa protein 8
IGF1-Hs01547656_m1	insulin-like growth factor 1 (somatomedin C)
IKBKB-Hs00233287_m1	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta
IL6-Hs00985639_m1	interleukin 6 (interferon, beta 2)
IL6R-Hs01075666_m1	interleukin 6 receptor
INSR-Hs00961560_m1	insulin receptor
MAPK9-Hs00177102_m1	mitogen-activated protein kinase 9

MAP1LC3A-Hs00738808_m1	microtubule-associated protein 1 light chain 3 alpha
LDHA-Hs01378790_g1	lactate dehydrogenase A
MCUR1-Hs00254417_m1	mitochondrial calcium uniporter regulator 1
MNF1-Hs00276889_m1	mitochondrial nucleoid factor 1
MPC2-Hs00209889_m1	mitochondrial pyruvate carrier 2
MSTN-Hs00976237_m1	myostatin
MTFR1-Hs00206110_m1	mitochondrial fission regulator 1
MTOR-Hs00234522_m1	mechanistic target of rapamycin (serine/threonine kinase)
MYH1-Hs00428600_m1	myosin, heavy chain 1, skeletal muscle, adult
MYH3-Hs01074230_m1	myosin, heavy chain 3, skeletal muscle, embryonic
MYH4-Hs00757977_m1	myosin, heavy chain 4, skeletal muscle
MYL1-Hs00984899_m1	myosin, light chain 1, alkali; skeletal, fast
MYL6-Hs00819642_m1	myosin, light chain 6, alkali, smooth muscle and non-muscle
MYLPF-Hs00203261_m1	myosin light chain, phosphorylatable, fast skeletal muscle
MYO5C-Hs00218921_m1	myosin VC
MYOG-Hs01072232_m1	myogenin (myogenic factor 4)
NFKB1-Hs00765730_m1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
NRF1-Hs01031046_m1	nuclear respiratory factor 1
NFE2L2-Hs00232352_m1	nuclear factor, erythroid 2-like 2
OSTN-Hs00898258_m1	osteocrin
RPS6KB2-Hs00177689_m1	ribosomal protein S6 kinase, 70kDa, polypeptide 2
PDK2-Hs00176865_m1	pyruvate dehydrogenase kinase, isozyme 2
PDK4-Hs01037712_m1	pyruvate dehydrogenase kinase, isozyme 4
PFKM-Hs00175997_m1	phosphofructokinase, muscle
PKM-Hs00761782_s1	pyruvate kinase, muscle
POLRMT-Hs04187596_g1	polymerase (RNA) mitochondrial (DNA directed)
PPARA-Hs00947536_m1	peroxisome proliferator-activated receptor alpha
PPARGC1A-Hs01016719_m1	peroxisome proliferator-activated receptor gamma, coactivator 1 alpha
PPARG-Hs00234592_m1	peroxisome proliferator-activated receptor gamma
PRKAA2-Hs00178903_m1	protein kinase, AMP-activated, alpha 2 catalytic subunit
PSMA1-Hs01027360_g1	proteasome (prosome, macropain) subunit, alpha type, 1
PSMC1-Hs02386942_g1	proteasome (prosome, macropain) 26S subunit, ATPase, 1
PYGM-Hs00989942_m1	phosphorylase, glycogen, muscle
RICTOR-Hs00380903_m1	RPTOR independent companion of MTOR, complex 2
RPTOR-Hs00375332_m1	regulatory associated protein of MTOR, complex 1
SDHA-Hs00417200_m1	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)
SMAD2-Hs00183425_m1	SMAD family member 2
SOCS3-Hs01000485_g1	suppressor of cytokine signaling 3
SOD2-Hs00167309_m1	superoxide dismutase 2, mitochondrial
STAT1-Hs01013996_m1	signal transducer and activator of transcription 1, 91kDa
STAT3-Hs01047580_m1	signal transducer and activator of transcription 3 (acute-phase response factor)
TFAM-Hs01073349_g1	transcription factor A, mitochondrial
TFB2M-Hs00915025_m1	transcription factor B2, mitochondrial
TGFB1-Hs00998133_m1	transforming growth factor, beta 1
TLR4-Hs00152939_m1	toll-like receptor 4
TRAF3-Hs00936781_m1	TNF receptor-associated factor 3

TRIM63-Hs00822397_m1	tripartite motif containing 63, E3 ubiquitin protein ligase
TTN-Hs00399225_m1	titin
TUFM-Hs00607042_gH	Tu translation elongation factor, mitochondrial
UCP1-Hs00222453_m1	uncoupling protein 1 (mitochondrial, proton carrier)
UCP3-Hs01106052_m1	uncoupling protein 3 (mitochondrial, proton carrier)
VEGFA-Hs00900055_m1	vascular endothelial growth factor A
ZNF711-Hs00254359_m1	zinc finger protein 711
EIF2S1-Hs00187953_m1	eukaryotic translation initiation factor 2, subunit 1 alpha, 35kDa
ATF4-Hs00909569_g1	activating transcription factor 4
HK2-Hs00606086_m1	hexokinase 2
GPD2-Hs01090711_m1	glycerol-3-phosphate dehydrogenase 2 (mitochondrial)

9.3 Appendix 1.3 – Oral glucose tolerance test data

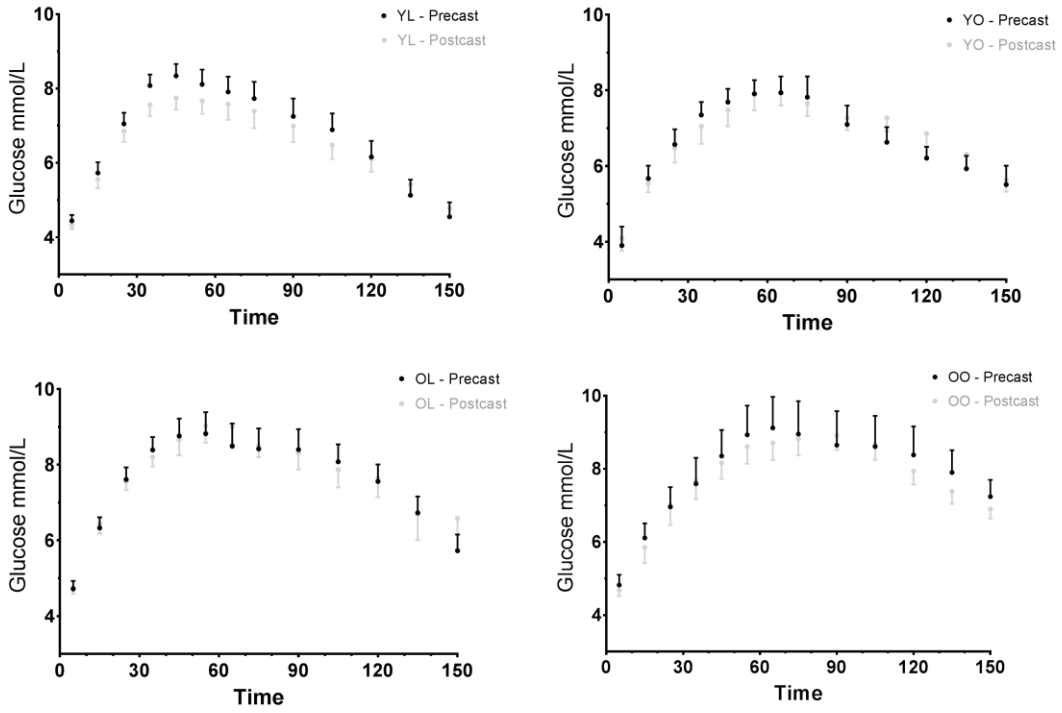


Figure 8.1. Whole body blood glucose values measured during an oral glucose tolerance test in young lean (YL), young obese (YO), older lean (OL) and older obese (OO) before immobilisation (precast) and immediately following 3 days unilateral lower limb immobilisation (post-cast). Values are mean ± SEM

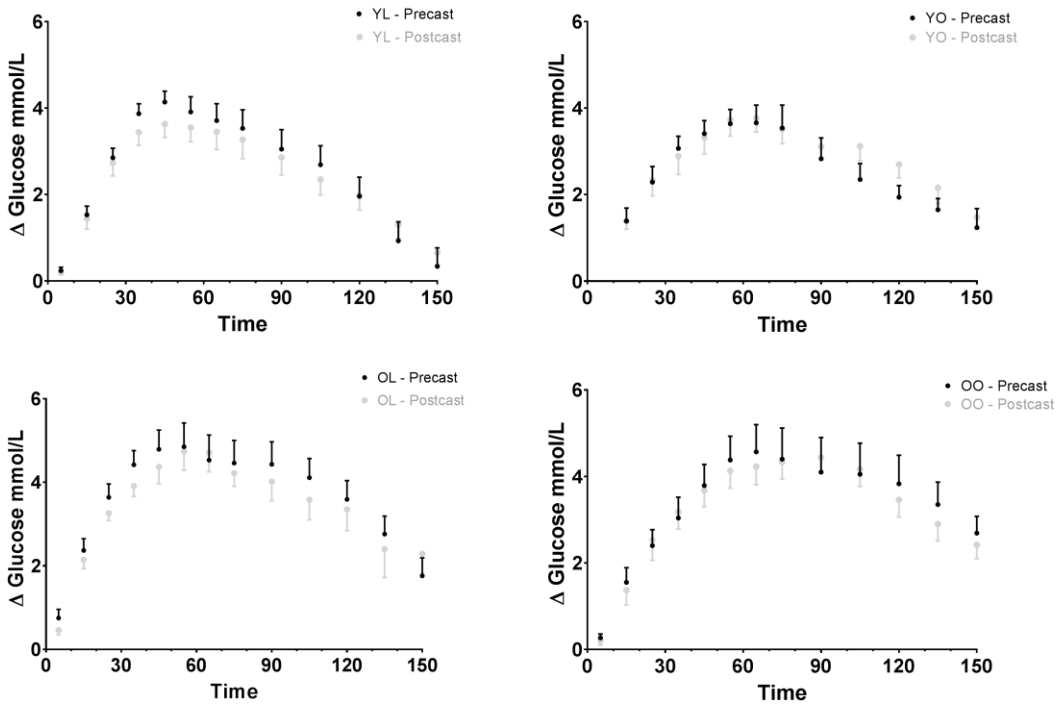


Figure 8.2. Whole body Δ blood glucose values measured during an oral glucose tolerance test in young lean (YL), young obese (YO), older lean (OL) and older obese (OO) before immobilisation (precast) and immediately following 3 days unilateral lower limb immobilisation (post-cast). Values are mean ± SEM

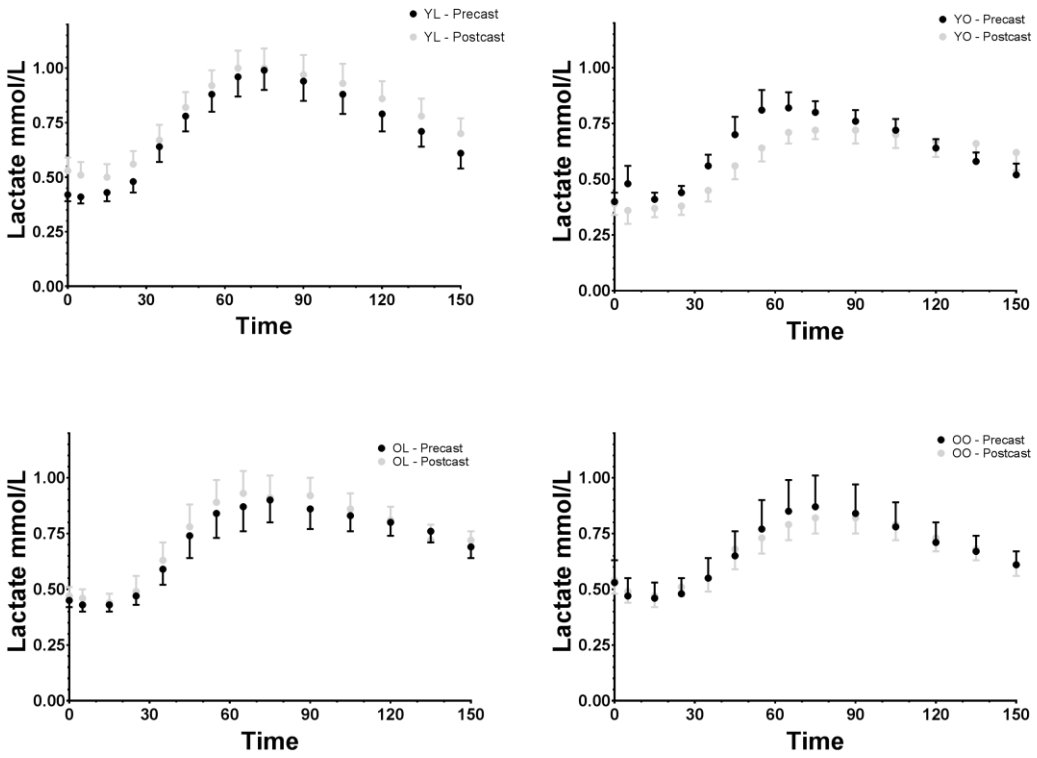


Figure 8.3. Whole body blood lactate values measured during an oral glucose tolerance test in young lean (YL), young obese (YO), older lean (OL) and older obese (OO) before immobilisation (precast) and immediately following 3 days unilateral lower limb immobilisation (post-cast). Values are mean ± SEM

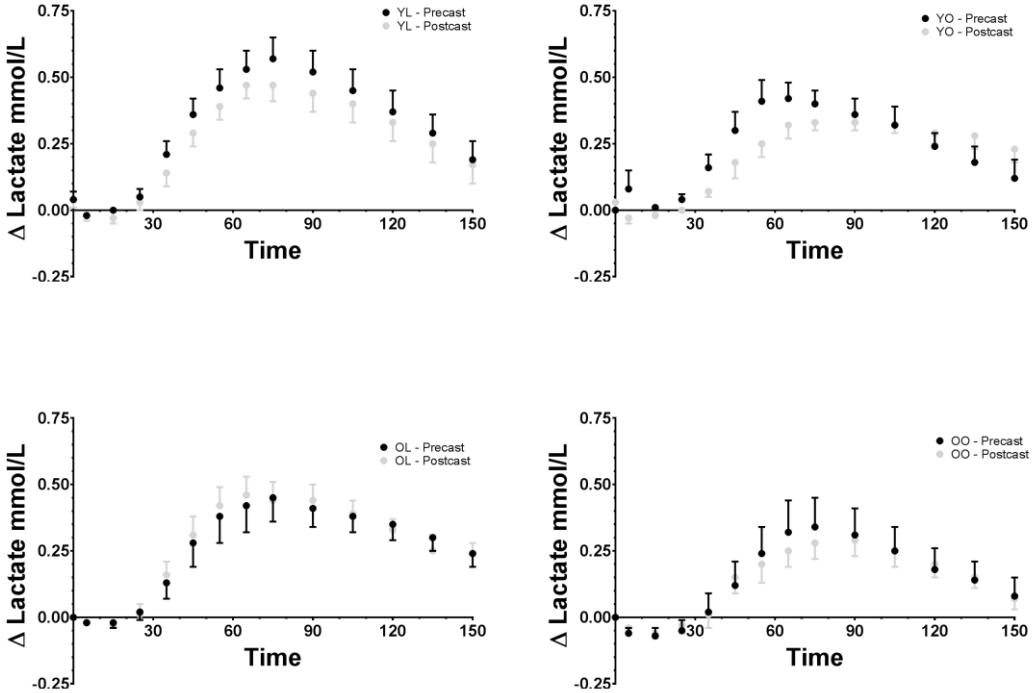
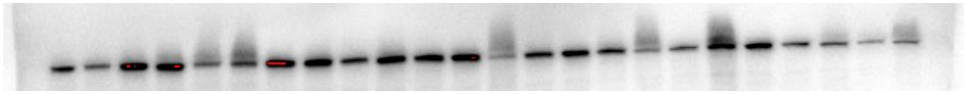


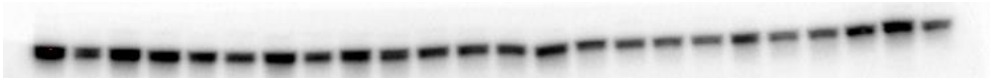
Figure 8.4. Whole body Δ blood lactate values measured during an oral glucose tolerance test in young lean (YL), young obese (YO), older lean (OL) and older obese (OO) before immobilisation (precast) and immediately following 3 days unilateral lower limb immobilisation (post-cast). Values are mean ± SEM

Appendix 1.3: Example Western Blots

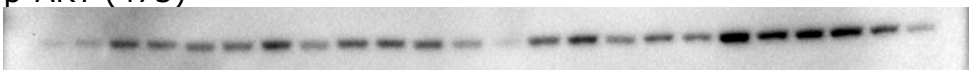
t-mTOR



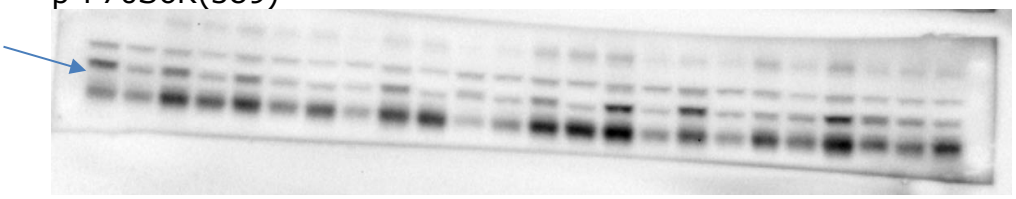
t-AKT



p-AKT (473)

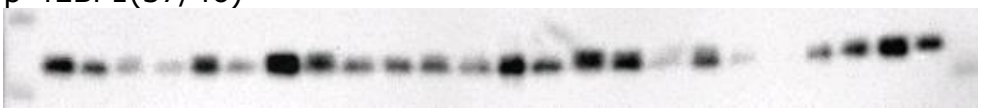


p-P70S6K(389)



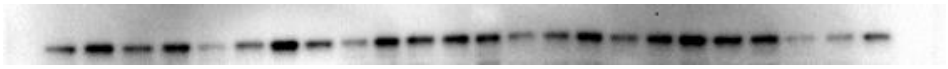
t-4EBP1

p-4EBP1(37/46)

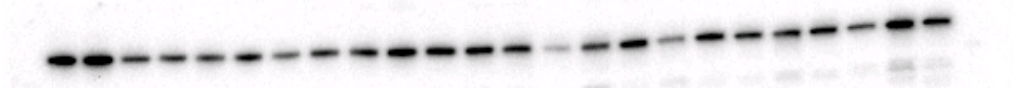


p-EIF4E (209)

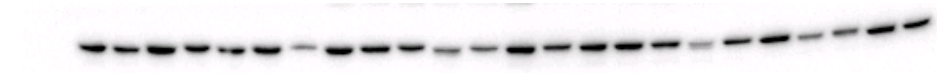
tFOXO1



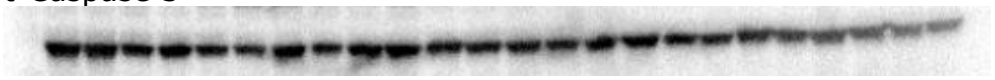
tFOXO3



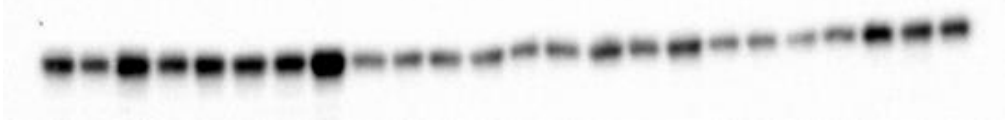
t-MuRF



t-Caspase 3



t-ATF4



tCHOP