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The impact of step count reduction and remobilisation on body composition, blood glucose disposal and muscle protein turnover in healthy, active young and older volunteers

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Thesis Abstract

Physical inactivity is the fourth leading cause of death worldwide. It is a global health problem with evidence that directly attributes physical inactivity to multiple metabolic diseases and mortality. Guidance on the recommended minimum levels of physical activity is available, however knowledge of and uptake in the general population is poor. There has been a shift in modern day society as to what normal daily physical activity should consist of with the increasingly held belief that physical activity is a luxury adjunct to daily life that consists only of MVP during structured classes or within purpose-built establishments. In addition to changing work practices, the vast array of labour-saving devices and the increasing uptake of electronic mobility aids at all ages has resulted in a progressively inactive population. This, combined with the exponential increase in the ageing population will ultimately induce significant growth in the inactivity associated metabolic disease burden with subsequent public health and economic consequences.

Several experimental models of physical inactivity have been developed by researchers to try and interrogate the time course and mechanistic basis through which inactivity exerts

detrimental metabolic and physiological changes. Bed rest and limb immobilisation are the most studied models but there has been increasing interest in step count reduction to try and emulate inactivity induced changes that may be observed secondary to reduced daily ambulatory activity. This novel approach allows participants to remain in their free-living state while partaking in research studies.

This thesis describes a series of longitudinal studies that have employed a step count reduction model and subsequent remobilisation in young and older participants. The principle aims of this thesis were to investigate the impact of a 14-day period of step count reduction and subsequent remobilisation on body composition, glucose regulation and muscle protein turnover in healthy, physically active young and older participants. To achieve our aims, we have utilised proven research techniques alongside novel, oral based tracer methods that have not previously been employed in such context.

The principal findings of this thesis were that a period of 14 days of SR to <1500 steps per day had no impact on leg cumulative MPS, whole-body MPB, or several body-composition measures (with the exception of D3-creatine

derived whole body muscle mass). In older volunteers, 48 hours of SR induced an increase in the AUC of both blood glucose and serum insulin during an OGTT, and for serum insulin in the younger volunteers after 14 days of SR. Following remobilisation, all measures of glucose homeostasis returned to baseline. It is reasonable to conclude that short term reduced ambulatory activity in well-matched otherwise fit and healthy, habitually active young and older humans did not have major, persistent effects on skeletal muscle metabolism as might be predicted from human bed rest and limb immobilisation research.

Declaration

All the experimental data presented in this thesis were generated by me with the assistance of academic and technical staff at the David Greenfield Human Physiology Unit (DGHPU), Queen's Medical Centre, University of Nottingham; the Stable Isotope Mass Spectrometry Facility, Derby Royal Hospital, University of Nottingham; and the Sir Peter Mansfield Imaging Centre, University of Nottingham. Throughout the study, almost all the experimental visits and clinical procedures were performed by me with the assistance of technical staff at the DGHPU.

In **Chapter 3** measurement of serum insulin via radioimmunoassay was performed by Sally Cordon.

In **Chapter 4** muscle myofibrillar protein FSR analyses were performed by Hannah Crossland and Matthew Brook at the Stable Isotope Mass Spectrometry Facility, Derby Royal Hospital, University of Nottingham. Plasma sample analysis to estimate muscle protein breakdown via D_3-3-methylhistidine was performed by Jessica Cegielski, also in **Chapter 5** analysis of urine samples to estimate whole body muscle mass by the D3-creatine dilution method was performed by Jessica

Cegielski and Daniel Wilkinson at the Stable Isotope Mass Spectrometry Facility, Derby Royal Hospital.

All MRI and MRS scanning in **Chapters 3** and **5** were performed by Christopher Bradley and Jan Paul at the Sir Peter Mansfield Imaging Centre. MRI analysis and data acquisition were performed by me, utilising software created by Olivier Mougin. DEXA scanning for **Chapter 3** and **5** were performed by technical staff at the David Greenfield Human Physiology Unit.

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 the experiments, unless otherwise stated, were performed by me.

Signed:

Date:

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First and foremost, I would like to thank my long-suffering wife, Jodie. The path that I have taken since completing my medical degree in 2009 has been long, arduous and difficult. Completion of this thesis has complimented said journey exceptionally and I am very grateful for Jodie's almost universal acceptance of my chosen path and absence at children's bedtime.

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Research really is a team game, and I have felt part of a great time. I am however more than happy to return to the land of anus and rectum for good. Thank you everyone, it has been great.

COVID Impact Statement

The COVID-19 pandemic had a significant impact upon the analysis and thus completion of my PhD studies which required a 6-month extension to the thesis pending period, which was accepted by the University of Nottingham. Due to national lockdown measures and resultant closure of laboratory facilities at the University of Nottingham between March 2020 and September 2020, analytic throughput was halted. Subsequent reopening of facilities was achieved with restricted occupancy alongside prioritisation of COVID related studies, further delaying sample analysis and data generation. In addition, I was required to be working fulltime clinically within the National Health Service with no ability to negotiate further time out of training.

Analysis of the tracer-based methods including muscle protein synthesis via D_2O (**Chapter 4**), muscle protein breakdown via D_3-3- methylhistidine (**Chapter 4**) and whole-body muscle mass via D3-creatine (**Chapter 5**), all required mass spectrometry and was thus significantly delayed due to COVID-19 related closures. The time losses related to sample analysis translated into delays in thesis generation and the requirement for an extension to the thesis pending period.

Thankfully this extension was sufficient for completion of my studies and the generation of novel data that has not previously been published.

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List of Abbreviations

3MH	3-Methylhistidine
3T	3 Tesla
AEE	Active energy expenditure
AFFM	Appendicular fat free mass
APE	Atom percent excess
AUC	Area under the curve
BMI	Body mass index
COV	Coefficient of variation
CSA	Cross sectional area
CT	Computerised tomography
D_2O	Deuterium oxide
D_3-3HM	D_3-3-methylhistidine
DEXA	Dual-energy x-ray absorptiometry
DIT	Dietary induced thermogenesis
DVT	Deep vein thrombosis
EAA	Essential amino acid
EMCL	Extramyocellular lipid
FFA	Free fatty acids
FFM	Fat free mass
FSR	Fractional synthetic rate
GLUT	Glucose transporter
IMCL	Intramyocellular lipid
IMF	Intermyofibrillar
MPB	Muscle protein breakdown
MPS	Muscle protein synthesis
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
MVP	Moderate to vigorous physical activity
NWS	Non water saturated spectra
OGTT	Oral glucose tolerance test
PAL	Physical activity level
REE	Resting energy expenditure
RET	Resistance exercise training
RIA	Radioimmunoassay
SEM	Standard error of the mean
SR	Step count reduction

SS	Subsarcolemmal
TEE	Total energy expenditure
ULLS	Unilateral lower limb suspension
WAS	Water saturated spectra

Chapter 1

General Introduction

1.1 Physical inactivity

Physical inactivity is a global public health endemic with more than 1.4 billion people worldwide not physically active enough to maintain physical health (Guthold et al., 2018). The recommended amount of physical activity according to the four home countries' Chief Medical Officers is at least 150 minutes per week in divided sessions. In 2013 a report published by the Health and Social Care Information Centre identified that only 66% of men and 56% of women in England met the recommended targets, but this was through self-reported data which is known to often over report participants actual level of physical activity (Prince et al., 2008). Prior investigation has traditionally focused on the effects of a lack of participation in regular moderate to vigorous physical activity (MVP). However, increasing evidence suggests that those spending significant parts of their wake time sitting, are at a higher risk of all-cause mortality, with the degree of time spent sitting increasing that risk (de Rezende et al., 2014, Ku et al., 2018). Further work that looks at the mitigating effect of MVP on sedentary behaviour is reporting that even those with >7 hours per week of MVP but also >7 hours per day of television viewing had a higher rate of all-cause and cardiovascular related mortality (Matthews et al., 2012).

There is cumulative evidence to suggest that the implications of progressive physical inactivity are increased risk of metabolic disease in the form of heart disease, vascular disease, insulin resistance, type 2 diabetes, cancer and musculoskeletal problems (Bergouignan et al., 2011).

Human movement is influenced by many factors including personal motivation, health and wellbeing, environmental and social factors. Modern day life has induced a paradigm shift as to what normal daily physical activity should involve with changes in social and physical environments being the biggest driver. The change in working practices, the vast array of labour-saving devices and the increasingly held belief that physical activity is a luxury adjunct to modern day life that consists only of MVP during structured classes or within purpose-built establishments. The current situation is likely to progress, as these beliefs become increasingly engrained into society. This, combined with the western drive of maximising productivity further reduces the indirect periods of physical activity and increases daily sedentary time.

Physical inactivity increases with age (Suryadinata et al., 2020). It is expected that by 2035 the proportion of the

population over the age of 65 years will represent 23% of the United Kingdom population (Office of National Statistics, 2012). It can therefore be expected that the combination of an ageing population with a progressive reduction in daily habitual physical activity will induce significant growth in the inactivity associated metabolic disease burden with subsequent public health and economic consequences. There is a plethora of research into the beneficial effects that physical activity has on metabolic health. Conversely, the investigation into the negative effects of reduced physical activity in the elderly and the underlying mechanistic basis driving the metabolic dysregulation is relatively sparse. Many of the features of skeletal muscle dysmetabolism that are observed in the elderly can be induced in young people through controlled exposure to reduced physical activity, raising the question as to what proportion of these 'age related changes' can be explained by the worsening epidemic of chronic reduced physical activity in both the young and old.

1.2 Models of physical inactivity in human volunteer research

The study of long-term reductions in physical activity are important to try and understand the temporal and mechanistic basis through which alterations in metabolic and physiological processes are induced. However, the true study of individuals over prolonged periods of time is logistically challenging and fraught with potential bias. As a result of these challenges, several models of physical inactivity have been developed to study the effects of physical inactivity over acceptable periods on healthy volunteers that afford the ability to control certain factors. The differing models range in severity and allow the investigation of short and long-term physical inactivity.

1.2.1 Bed rest and dry water immersion

Complete bed rest is the most extensively investigated form of immobilisation. Investigation has taken place in both younger (Ferrando et al., 1996, LeBlanc et al., 1992) and older participants (Kortebein et al., 2007, Kortebein et al., 2008, Drummond et al., 2012) for varying time frames ranging from 3-120 days duration (LeBlanc et al., 1992, Ferrando et al., 1996, Smorawinski et al., 2000, Shur et al., 2022, Kortebein

et al., 2007, Kortebein et al., 2008, Drummond et al., 2012, Suzuki et al., 1994, Hesse et al., 2005). Most bed rest studies have, to a varying degree, induced a reduction in skeletal muscle mass, strength, and whole-body insulin sensitivity, with this effect being demonstrated after just 3 days (Shur et al., 2022). Bed rest is one of the more extreme forms of immobilisation which has afforded extensive insight into the effects of a drastic reduction in daily physical activity, like that of elderly patients that are hospitalised, but allowing the study of the inactivity induced effects without the confounding factors of intercurrent illness. It also allows the control of certain factors such as energy balance and duration, which cannot be predictably controlled within the hospitalised population. The effects of bedrest are rapid in onset with sustained metabolic effects that make it attractive to researchers investigating mechanisms of adaptation in humans.

Dry water immersion is an alternative technique that can induce whole body inactivity. Rather than participants resting in a bed, they are immersed in thermoneutral water within a thin elastic, waterproof fabric. The technique produces an effect of weightlessness, which induces similar physiological

effects to bed rest but often to a more dramatic degree and over a shorter period of time (Shenkman and Kozlovskaya, 2019). Both models of inactivity have generated significant insight into the mechanisms involved in inactivity induced deconditioning which in turn allows the study of ameliorating factors that can be introduced into real world situations. However, due to the severity of the restrictions in physical activity, it is highly unlikely to be representative of the changes that occur secondary to chronic physical inactivity that is being observed throughout society.

1.2.2 Limb immobilisation and suspension

Limb immobilisation, involving both upper and lower limbs, is a common technique used for immobility studies. The benefit of limb immobilisation is that the participant's contralateral limb can serve as an internal control, drastically reducing the number of potential intraparticipant variables. However, there is some debate about the effect of limb immobilisation on the non-immobilised leg with some studies reporting a reduction in overall physical activity (Edwards et al., 2020), increases in skeletal muscle mass (Hespel et al., 2001) and decreases in skeletal muscle mass (Vigelso et al., 2015). In theory, this technique should allow the participant to continue with other

daily activities, therefore it does not induce whole body effects. Several techniques have been employed to induce immobilisation with external braces and casting being common. The use of limb immobilisation in research has more clinical relevance than bed rest due to it being a commonly used technique in the treatment of limb fractures, which are common within all age groups.

Limb suspension in human volunteers is usually performed on the lower limb (Unilateral Lower Limb Suspension, ULLS), traditionally using a harness that flexes and suspends the limb of interest above the ground while providing a shoe with a thick sole for the contra-lateral limb (Berg et al., 1991). Mobilisation is then achieved using under axilla crutches. This technique was found to increase the risk of DVT (Bleeker et al., 2004) and hence was modified by removing the strap and increasing the thickness of the platform shoe and allowing the suspended limb to swing freely (Ploutz-Snyder et al., 1995) with the aid of crutches. As with limb immobilisation it induces limb specific changes due to unloading only the muscles of the suspended limb, rather than the systemic changes that are associated with bed rest, therefore its utilisation and risks differ from that of complete bed rest. ULLS results in a loss of

muscle at a rate of ≈0.44% per day (Hackney and Ploutz-Snyder, 2012) and generally elicits similar performance related adaptations to bedrest of a similar duration (Hackney and Ploutz-Snyder, 2012). Limb suspension does have an inherent weakness associated with compliance that is not present in limb immobilisation but again provides an internal control through the contralateral limb. Although, it remains debated, as with limb immobilisation, how true a control the contralateral limb provides particularly when it comes to highly dynamic changes such as muscle protein synthesis (MPS) and glucose uptake.

1.2.3 Decreased physical activity

Bed rest, dry water immersion and limb immobilisation/suspension techniques provide excellent insight into whole body or limb specific adaptations to reduced activity. However, to replicate the physiological adaptations of reduced daily ambulatory activity, such as that seen increasingly in modern day society, these inactivity models focus on high magnitude of metabolic insult and are in no way representative of a sedentary lifestyle. Decreased physical activity is achieved through step count reduction (SR) models

that allow participants to remain within the free-living state with instructions on how to reduce daily step count or restrictions on the maximal number of daily steps (Breen et al., 2013a, Krogh-Madsen et al., 2010, Bowden Davies et al., 2018, Oikawa et al., 2019). A number of studies have investigated the effects of 1500 daily steps for a set time period with this model being considered the most realistic to the sedentary, free living state (Tudor-Locke and Bassett, 2004). Limitations of such a model include participant adherence and due to the relatively benign level of enforced reduction in activity it may be considered that participants often must have a longer period of restricted mobilisation for physiological or metabolic changes to manifest. Despite the proposed limitations, studies have reported physiological changes after short (7-14 days) periods of reduced step count (<750-1500 daily steps) confirming it as a valid investigative model over such time periods (Breen et al., 2013a, McGlory et al., 2018, Oikawa et al., 2018, Krogh-Madsen et al., 2010, Bowden Davies et al., 2018).

1.3 Drivers of insulin resistance in humans

Insulin resistance describes a state in which despite increasing levels of serum insulin, cellular glucose uptake is impaired. There are several known causes of insulin resistance, along with a few debated causes and theories. Here we will discuss those relevant to this thesis in detail.

1.3.1 Physical inactivity

Whole body and limb specific glucose uptake is impaired following periods of inactivity in both young and older healthy adults. The effects of bed rest on insulin sensitivity have been extensively investigated since the 50's (Lutwak L, 1959). Following this, multiple studies have identified the detrimental effect of bed rest on glucose metabolism: Cree (Cree et al., 2010) investigated the effects of 28 days of bedrest in six healthy, young volunteers and found a reduction in glucose uptake during hyperinsulinaemia (from 9.1 ± 1.3 to 5.2 ± 0.7 mg/kg of leg per minute, $p=0.015$), ten healthy young males were subject to one week of bed rest by Dirks (Dirks et al., 2016) where a 29 ± 5% decrease in whole body insulin sensitivity was induced. Similarly in a study of nine older participants by Coker, ten days of bed rest resulted in a

significant increase in fasting plasma glucose and a 15% decrease in insulin-mediated suppression of glucose production from the liver during hyperinsulinaemic euglycaemic glucose clamp. Furthermore Shur (Shur et al., 2022) reported a 20% reduction in glucose uptake following 4 days of bedrest that was of a similar magnitude to that seen following 56 days of bedrest, suggesting that the effect of inactivity on glucose disposal is fast acting and sustained.

Along with whole-body changes in glucose disposal, limb specific reductions in glucose uptake can be induced following limb immobilisation. These forms of inactivity models restrict motion in the target limb, thus allowing maintenance of everyday activities and restricting the whole-body effects of immobilisation, unlike bed rest. Burns (Burns et al., 2021) noted a sizable decrement in forearm glucose uptake in response to glucose feeding within 24 hours of immobilisation in ten healthy young men, that was sustained and specific to the immobilised limb. Earlier findings by Dirks (Dirks et al., 2020) had established a 50% reduction in forearm glucose uptake in response to a physiologically relevant mixed meal concluding that the lack of muscle contraction was the driving force behind this physiological response. Similar findings

following seven days of knee immobilisation have been reported by Richter (Richter et al., 1989) whom reported local decreased insulin action on thigh glucose uptake in five healthy young men.

Along with bed rest and limb immobilisation studies, there has been a limited number of studies that have interrogated the effects of SR on glucose metabolism. Olsen published a study in 2008 (Olsen et al., 2008) that identified the detrimental effects of a three week period of reduced step count on insulin sensitivity by seeing a significant increase in the area under the curve (AUC) of plasma insulin concentration during an oral glucose tolerance test (OGTT) (757 pmol/L/3h at baseline to 1352 pmol/L/3h) in eight healthy male participants. Krogh Madsen repeated a similar study in 2010 where they recruited ten healthy young males and reduced their step count from ~10,000 to ~1350 steps daily for 14 days, which resulted in a reduced glucose infusion rate of 17% while under a hyperinsulinaemic clamp, indicating reduced whole body insulin sensitivity (Krogh-Madsen et al., 2010). Similarly, Knudsen (Knudsen et al., 2012), whom combined 14 days of SR to <1500 daily steps with overfeeding, found both an increase in the Matsuda index at days three and seven along

with decreased glucose infusion rates at day 14, inferring a reduction in whole body insulin sensitivity in nine healthy young men. It is therefore becoming increasingly apparent that these less severe forms of inactivity induce significant changes in whole body glucose disposal that are likely to become increasingly relevant given the progressive adoption of a sedentary lifestyle.

The mechanisms responsible for the reported changes in glucose disposal induced by bed rest, limb immobilisation and step count reduction are yet to be elucidated. There have been several proposed mechanisms including alterations in GLUT4 mediated glucose transport, central adiposity accumulation, intramyocellular lipid (IMCL) accumulation and mitochondrial dysfunction. Although recent work by Burns (Burns et al., 2021) showed rapid onset insulin resistance, suggesting that is the lack of contraction that is driving the blunting of glucose uptake rather than an adaptation at a cellular or molecular level. In addition, Shur (Shur et al., 2022) maintained energy balance during a 56-day best rest study and found no evidence of IMCL accumulation to account for the 20% reduction in whole body glucose disposal.

1.3.2 Central adiposity

Vague identified that body fat distribution may influence the predisposition to metabolic disease (Vague, 1956) with central obesity being linked with insulin resistance and other metabolic disorders (Bjorntorp, 1988, Carey et al., 1997, Pouliot et al., 1992). The exact mechanisms underlying central adiposity induced insulin resistance are unclear, though hepatic insulin resistance is likely to be a major player (See below). Certainly, short term inactivity does not result in body composition changes or hepatic insulin resistance that are sufficient to drive alterations in whole body insulin sensitivity (Mikines et al., 1991), but chronic inactivity with no concurrent reduction in calorific intake, leads to increases in whole body adipose tissue (Choe et al., 2016) with several mechanisms related to this increased adiposity being suggested as central to insulin resistance. In addition, it has become increasingly evident over recent years that adipose tissue is not merely a storage depot for excess dietary energy: Adipose tissue has several endocrine functions (Coelho et al., 2013) that include secretion of inflammatory mediators including cytokines and adipokines, along with the release of free fatty acids (FFA). These additional functions have been implicated in the pathogenesis of the detrimental effects of obesity including

insulin resistance (Fruhbeck et al., 2001, Lazar, 2005). The "portal hypothesis" describes the situation where an increase in central abdominal fat results in an elevation in the delivery of FFA via the portal vein to the liver. This induces hepatic insulin resistance and liver steatosis (Bjorntorp, 1990). Secondly, it is proposed that excessive production of inflammatory mediators from visceral fat immune cells, that are present in higher numbers, results in a proinflammatory state with negative implications on insulin sensitivity (Curat et al., 2006). However, the evidence to support adipose related inflammation as being a driver of insulin resistance is somewhat inconsistent (Romano et al., 2003) with more work being required.

1.3.3 Intramyocellular lipids

IMCL content has been shown to correlate strongly with insulin resistance (Jacob et al., 1999, Perseghin et al., 1999, Krssak et al., 1999) and it was thought to be important in the development of type 2 diabetes (Michael et al., 2020). However, the discovery of high levels of IMCL in trained athletes despite normal or heightened insulin sensitivity and oxidative capacity (the so called 'athletes paradox') (Goodpaster et al., 2001), have conferred that the

accumulation of IMCL in isolation is not directly causative of insulin resistance. Further research has led to the discovery that certain lipid intermediates, such as ceramides and diacylglycerol, play a more important role than total IMCL in skeletal muscle insulin resistance (Kitessa and Abeywardena, 2016). In addition to this, IMCL can be considered to be made up of subsarcolemmal (SS) and intermyofibrillar (IMF) fractions. Chee (Chee et al., 2016) investigated these two subdivisions and identified a threefold higher volume of lipids in the SS region and similar volume density of IMF lipids in sedentary type 2 diabetic patients compared with non-diabetic BMI matched control subjects and highly trained endurance athletes. Similar findings can be made when comparing young and older, with the older adults having a higher proportion of IMCL content in the SS region (Crane et al., 2010). Despite this recent advancement, the current role of IMCL accumulation in relation to inactivity and alterations in glucose disposal remain poorly understood. In addition, the contribution, if any, that both extramyocellular lipid (EMCL) and the IMCL/EMCL ratio make towards insulin resistance remain unclear.

1.4 Effects of physical inactivity on muscle protein turnover

Muscle mass is maintained through the dynamic balance of MPS and muscle protein breakdown (MPB). The shift between the two opposing states occurs at numerous times per day depending on the presence of stimuli that induce MPS, namely physical activity and/or protein ingestion. For a net change in muscle protein balance, it is required that a prolonged increase in either process occurs. Despite rodent studies suggesting that muscle atrophy occurs because of an increase in MPB (Krawiec et al., 2005), much of the equivalent investigation in humans has suggested that a decline in stimulated MPS is the main driving force behind inactivity muscle atrophy (Ferrando et al., 1996, de Boer et al., 2007).

1.4.1 Muscle protein synthesis

The unloading of healthy muscle results in muscle atrophy through a reduction in exercise induced MPS. Many studies have shown acute and chronic measures of myofibrillar fractional synthetic rate (FSR) are reduced following both bed rest (Ferrando et al., 1996, Symons et al., 2009, Shur et al., 2024) and limb immobilisation (Glover et al., 2008, Wall et al.,

2013, de Boer et al., 2007). Ferrando (Ferrando et al., 1996) reported a reduction in acute post absorptive vastus lateralis FSR by 50% following two weeks of bed rest in six healthy young males. Similar reductions of 52% following 10 days and 57% following 21 days of ULLS were found in 17 young men by de Boer (de Boer et al., 2007). The reduction in MPS is rapid in onset with Brook (Brook et al., 2022) demonstrating a reduced rate of integrated MPS following 4 days of leg immobilisation when compared to the control leg (1.55 ± 0.21% day^{-1} vs 1.29 ± 0.17% day^{-1}, $p < 0.01$)). Similarly, Shur (Shur et al., 2024) reported a 43% reduction in myofibrillar FSR in ten young males after three days of bedrest.

The limited number of SR studies performed have also reported a negative effect on myofibrillar FSR in both young and older participants. Breen (Breen et al., 2013a) studied ten healthy young men and found a reduction of 26% in postprandial acute MPS following 14 days of < 1500 daily steps. Similarly, both Oikawa (Oikawa et al., 2018) and McGlory (McGlory et al., 2018) identified a significant drop in cumulative myofibrillar FSR after a set period of SR. These studies demonstrate that even a short period (14 days) of

reduced daily habitual activity is sufficient to affect the daily rate of MPS, which if prolonged could result in muscle mass loss.

1.4.2 Muscle protein breakdown

In humans during the fasting state there is little evidence that MPB significantly contributes to sarcopaenia (Kim et al., 2020, Phillips and McGlory, 2014). A reduction in MPS appears to be the driver of muscle mass loss both during inactivity and older age (Rennie et al., 2010). This differs from muscle loss in rodents where there is an increase in MPB (Loughna et al., 1986), however skeletal muscle physiology between human and rodent are far from comparable; skeletal muscle comprises a much smaller body percentage of the total body mass in rats as compared to humans (Wolfe, 2017). In addition unlike humans, MPS is not stimulated by amino acids (Waterlow et al., 1978). It must be said however, that the techniques for measuring MPB are limited and less robust than those employed when measuring MPS. These include intravascular monitoring of the appearance of products of proteolysis, quantification of proteolytic gene expression involving one or more of the proteolytic pathways involved in

MPB and more recently D_3-3-methylhistidine (D_3-3MH) dilution technique (Sheffield-Moore et al., 2014).

The number of studies that have directly measured MPB following a period of inactivity are few and the data is far from conclusive. Tesch (Tesch et al., 2008) measured the rate of MPB through vastus lateralis micro dialysis in eight young men after a 72-hour period of unilateral lower limb immobilisation and reported an increase in the rate of MPB. However, Shur (Shur et al., 2024) measured MPB in ten healthy young men and found a decrease in the rate by 30% following 2 days of bed rest when volunteers were maintained in energy balance, while Brook (Brook et al., 2022) did not find any change in the rate of MPB after 4 days of unilateral lower limb immobilisation in nine young men. These contradicting results clearly spell out the need for further controlled studies to try and identified the impact, if any, that MPB has on immobilisation induced muscle atrophy.

1.5 Effects of physical inactivity on body composition

1.5.1 Muscle mass

As discussed above, physical inactivity affects MPS and MPB with the resultant outcome being a change in muscle mass. It is well established through bed rest and limb immobilisation studies that muscle unloading results in muscle atrophy both in young and older healthy volunteers. Muscle atrophy is usually a result of a reduction in the size of individual muscle fibres, rather than a loss of fibre number (Kasper et al., 2002). For example, following three days of bed rest in ten healthy young men, a 2.5% loss of leg lean mass was found by Shur (Shur et al., 2024). Similarly, Jones (Jones et al., 2004) showed a 4.7% loss of quadriceps lean mass in young men following two weeks of full leg immobilisation. In 12 older participants undertaking ten days of bed rest, Kortebein (Kortebein et al., 2007) recorded a 3.2% loss in whole body muscle mass and 6.5% loss of lower limb muscle mass as measured by DEXA. Wall (Wall et al., 2013) studied the effects of one-legged knee immobilisation for 14 days in 12 healthy young men. There were no changes in whole-body muscle

mass but as might be expected they found a 3.1 ± 1.0% reduction in leg lean mass in the immobilised leg with no changes in the control leg. Similarly, whole leg casting for two weeks in 20 healthy men (11 young and nine older) resulted in a loss of quadriceps muscle volume in both young (8.9%) and older (5.2%) (Suetta et al., 2009).

SR models have also reported losses of lean mass, however the data following this form of reduced physical activity is not so conclusive likely due to this being an intervention of less physiological insult. Breen (Breen et al., 2013b) reported a 3.9% reduction in lower limb fat free mass (FFM) following 14 days of <1500 daily steps in ten older adults and Devries (Devries et al., 2015) restricted 30 older men to 14 days of <1500 daily steps and found a 124 ± 61g reduction in leg FFM. Conversely McGlory at al. (McGlory et al., 2018) did not find any change in total body or leg FFM, quantified by DEXA, in either 12 men or ten women following a SR of <1000 daily steps for 14 days. Similarly, Walker (Walker et al., 2024) studied 66 older adults that were restricted to less than 2000 daily steps for two weeks and did not find any change in lean mass utilising DEXA.

1.5.2 Fat mass

A positive energy balance brought about by inactivity or overfeeding results in an increase in fat mass. Dirks (Dirks et al., 2016) showed that ten days of bed rest in ten young male volunteers maintained in energy balance did not result in a measurable change in DEXA-derived whole body adiposity despite a significant drop in DEXA-derived whole body lean mass and a 29 ± 5% decrease in whole body insulin sensitivity via a hyperinsulinaemic-euglycaemic clamp. However, Eggelbusch (Eggelbusch et al., 2024) found that despite energy balance, there was a significant increase in whole body fat mass measured via DEXA in 24 young volunteers after 30 days of bed rest, that was no worse after 60 days. In addition, Reidy (Reidy et al., 2018) found a relative increase in DEXA whole body fat mass in nine older adults following five days of bed rest with energy intake adjusted for zero activity. Therefore, despite energy balance in both young and old groups, there is a tendency for an increase in whole body adiposity following bed rest, even for as short as five days duration.

1.6 Remobilisation from periods of immobilisation in human volunteers

Short periods of immobilisation are common and can occur due to a multitude of reasons. Ill health, hospitalisation or injury can result in either complete bed rest, limb immobilisation or a significant reduction in daily ambulatory activity. As described, these periods of inactivity can be accompanied by alterations in body composition, blood glucose homeostasis and muscle protein balance at a magnitude related to the degree and duration of immobilisation. Knowledge about the anticipated level of and the time course of metabolic recovery during remobilisation is key but at present represents an important knowledge gap. Importantly, it is unclear whether returning to free living habitual activity levels enable recovery or whether structured, regular training sessions are required and whether that is different between young and older adults.

There are a limited number of studies that have investigated recovery following immobilisation, with most data focussed on recovery from bed rest studies. Following 21 days of bed rest in seven healthy young male participants, returning to

habitual activity levels for four days did not restore normal glucose tolerance; between five and 14 days of habitual activity was required to return to baseline parameters (Heer et al., 2014). In a study of five days of bed rest in nine older adults, there was a 39% reduction in insulin sensitivity after bed rest, that returned to baseline following two months of lower limb eccentric exercise rehabilitation (Reidy et al., 2018). However, there was no interval study during the eight-week period of rehabilitation which means a return to normal insulin sensitivity could have occurred at any point during this time. With regards to muscle protein turnover, Shur investigated the effects of three days of bed rest followed by three days of unilateral exercise supplemented leg remobilisation on ten healthy young males. The study reported a 43% reduction in myofibrillar protein FSR and a 2.5% decrease in leg muscle volume that were restored only in the exercised remobilised leg (Shur et al., 2024). Tanner (Tanner et al., 2015) identified that a 4% loss of lower limb lean mass via DEXA following five days of bed rest in nine older adults was restored by an intensive eight-week rehabilitation programme.

With regards to remobilisation following limb immobilisation, Jones (Jones et al., 2004) demonstrated that six weeks of prescribed rehabilitation training in nine young healthy males returned DEXA derived whole body lean mass to baseline (pre-immobilisation) values, with muscle function measures being above this baseline. Although DEXA scans one week after starting exercise training did not demonstrate a return of whole-body lean mass to baseline levels. Furthermore Christensen (Christensen et al., 2008) studied the effects of two weeks of limb immobilisation on 12 healthy young men. They reported a 6% reduction in the volume of triceps surae CSA which returned to baseline after two weeks of habitual daily activity. Finally, there appears to be only a single study that investigates recovery after remobilisation following a SR model of inactivity. McGlory (McGlory et al., 2018) found that two weeks of SR (7362 ± 3294 to 991 ± 97 daily steps) in overweight, prediabetic adults induced impaired glycaemic control and a decline in integrated rates of MPS that was not recovered after two weeks of return to habitual physical activity levels.

The paucity of studies that investigate the effects of remobilisation, either through resumption of habitual physical

activity levels or prescribed rehabilitation programmes, leaves a significant knowledge gap. The heterogeneity of the reported studies regarding duration and type of inactivity, the remobilisation regime and the patient groups studied, means that it is difficult to draw firm conclusions about optimal strategies for post inactivity rehabilitation. Further inactivity studies are required that include measures made after remobilisation, particularly studies of the elderly following SR. Ageing is associated with impaired functionality and increased adiposity, insulin resistance and anabolic resistance (i.e. loss of resilience) therefore if inactivity is additive to these events, then understanding the best strategy for remobilisation will be key to improving age related health span.

1.7 Metabolic and Physiological correlates of ageing

Ageing is associated with a progressive decline in physiological and metabolic health, with reductions in skeletal muscle mass and function resulting in detrimental effects on functional capacity and independence. As suggested by Busse (E. W. Busse, 1969), ageing can be considered to have two facets; primary ageing, the time related decline in physiological and

metabolic processes not related to disease, stress or trauma; and secondary ageing, the resultant effect of environmental and lifestyle factors such as diet, levels of physical activity and smoking. Such factors may be considered reversible or have their impact limited by changing behaviours to reduce exposure. This can be appreciable through cohort studies that compare the average elderly population with master athletes, for example (Wroblewski et al., 2011). This has generated research interest regarding the relative effects that primary and secondary ageing have on both the physiological effect of ageing and the functional aspect of physiological decline. Here I will discuss the physiological and metabolic effects of human ageing most relevant to this thesis.

1.7.1 Impaired glucose disposal with age

Ageing is associated with a decline of skeletal muscle insulin sensitivity and overall muscle oxidative capacity (Lalia et al., 2016, Houmard et al., 1995, Kohrt et al., 1993, Petersen et al., 2003, Shimokata et al., 1991, Short et al., 2003, Short et al., 2005). There has been debate about whether ageing per se is the cause of this metabolic dysfunction or whether reduced physical activity/muscle contraction (Burns et al., 2021), adiposity (Kohrt et al., 1993), mitochondrial

dysfunction (Short et al., 2005, Petersen et al., 2003), inflammation (Shoelson et al., 2006) or changes in the cellular transport mechanisms of glucose (Houmard et al., 1995) are responsible. Clearly there may be multiple causative and or additive factors. There remains a degree of uncertainty about how each of these elements interact, and the time scale or relative contribution of each to the ageing phenotype.

1.7.1.1 Physical activity

It is well established that physical inactivity in older people results in a reduction in whole body glucose disposal (Coker et al., 2014, Fink et al., 1983). Alongside this, one in three adults are classified to be inactive in the United Kingdom (England, 2021), with the current population reported to be around 20% less active than in the 1960s (Statistics, 2018). As people age, they become progressively less active with reports suggesting that as few as 42% of 50–74-year-olds are meeting the recommendations set out by the Chief Medical Officers strength guidelines (Brainard et al., 2020). There are multiple reasons that may contribute to reduced activity in the elderly including retirement, physical inability due to musculoskeletal disease or ill health, social isolation or even the socially

accepted expectation that ageing should be accompanied by less physical exertion.

1.7.1.2 Adiposity

The ageing process is associated with progressive declines in physical activity which predisposes to dietary energy oversupply and subsequent increases in adiposity (Dela et al., 1996). Alongside increasing adiposity, ageing is associated with a decline in muscle insulin sensitivity and therefore increasing abdominal and/or visceral adiposity has been suggested as a cause of insulin resistance (Kohrt et al., 1993). Links between excessive adiposity and insulin resistance are longstanding with evidence to suggest that ageing combined with increasing adiposity compounds insulin resistance (Kohrt et al., 1993, Seals et al., 1984, Basu et al., 2003). In addition to whole body adiposity, there are links between the varying adipose storage sites and insulin resistance with the strongest associations existing between subcutaneous fat (Kelley et al., 2000, Smith et al., 2001) and abdominal adiposity (Ross et al., 2002).

There is, however, evidence to suggest that lipid accumulation associated with age is likely secondary to lifestyle factors and

thus the associated insulin resistance is not due to skeletal muscle ageing in isolation. Chee (Chee et al., 2016) studied young and older groups matched for body composition and habitual activity levels finding comparable insulin sensitivity between groups.

1.7.1.3 Mitochondrial function

Mitochondrial dysfunction has been linked to insulin resistance through the comparison of young healthy volunteers and older healthy volunteers. Petersen (Petersen et al., 2003) identified a 40% reduction in the rate of mitochondrial oxidative and phosphorylation activity associated with an increase in fat accumulation in muscle when comparing older against young. However, other studies have shown that when the apparent decrease in mitochondrial function is corrected for the loss of mitochondrial mass seen with age (probably attributable to decreased physical activity) then mitochondrial dysfunction disappears (Broskey et al., 2014). This is also true of Type 2 diabetes; Boushel compared older volunteers with and without Type 2 Diabetes and found mitochondrial function to be normal in the diabetic cohort, concluding that the blunting of respiration could be attributed to lower mitochondrial content (Boushel et al., 2007).

Evidence suggests that an increase in intra-myocellular lipid (IMCL) content could have detrimental effects on mitochondrial function, resulting in the inability of mitochondria to switch from lipid to glucose oxidation during insulin stimulation (Petersen et al., 2015). However, much of the existing research, focussed on the link between insulin resistance and mitochondrial dysfunction, has been contradictory and the mechanistic link between the two is clearly far more complex than first thought (Dela and Helge, 2013).

1.7.1.4 Muscle blood flow deficits

With skeletal muscle being responsible for >80% of post prandial glucose uptake through an insulin mediated process, the maintenance of adequate and dynamic muscle blood flow is essential for this process to remain efficient. Insulin binds to endothelial receptors, which results in enhanced microvascular blood flow through nitric oxide induced vasodilation and subsequent delivery of both glucose and insulin to myocytes (Vincent et al., 2003). Ageing is associated with an alteration in the response to vasodilators (Taddei et al., 1995) along with reduced nitric oxide bioavailability

(Taddei et al., 2001). The effect of such age-related changes is a reduction in insulin mediated microvascular changes and thus skeletal muscle blood flow. Timmerman has shown that insulin mediated microvascular responses to an insulin infusion in one leg was markedly impaired in older when compared to younger participants (Timmerman et al., 2010). Such loss of responsiveness has since been confirmed by Mitchell at al. by quantifying skeletal muscle blood flow in both young and older following an amino acid meal (Mitchell et al., 2013). Following ingestion, they observed an increase in skeletal muscle blood flow using a novel contrast-enhanced ultrasound technique in the young that was completely absent in the older group, concluding that age related declines in postprandial circulation could impact age related declines in muscle glucose disposal.

1.7.1.5 GLUT4 related glucose transport

It has been suggested that age related defects in the transport mechanism for glucose into the skeletal muscle cells may be associated with age related impairments in glucose uptake. A study by Houmard (Houmard et al., 1995) identified an age related decline in both males and females of insulin stimulated GLUT4 protein concentration in skeletal muscle; the

relationship persisting despite adjusting for overall adiposity, regional adiposity, and cardiorespiratory fitness. This age-related decline was also observed in the fast twitch fibres of the vastus lateralis, but not the slow twitch fibres, when comparing older and younger participants in a study by Gaster in 2000 (Gaster et al., 2000). However contrasting findings in studies by Dela (Dela et al., 1994) and Cox (Cox et al., 1999) found no age-related differences for GLUT4 protein abundance in the vastus lateralis, and furthermore Cox identified similar relative increases in vastus lateralis GLUT4 protein concentration, irrespective of age, following a short term exercise programme. Therefore, the current evidence linking muscle GLUT4 protein content with the age-related decline in insulin sensitivity is inconclusive and unable to account for these changes.

1.7.2 Reduced muscle mass and function with age

Sarcopaenia is a condition characterised by loss of muscle mass that is associated with ageing. Ageing is often associated with loss of muscle strength and power, a process known as dynapaenia (Mitchell et al., 2012). These two processes can coexist but the relative contributions can vary; the reported loss of strength can be up to 2-5 times the loss of mass

(Delmonico et al., 2009). These changes ultimately affect functional ability (Wolfson et al., 1995) and independent living (Tinetti and Williams, 1997) as well as putting those affected at risk of comorbidity and death (Brown et al., 2016). However, there is little agreement in the pattern of muscle loss with Kyle reporting a linear loss of muscle mass with age (Kyle et al., 2001) but other studies showing the rate increases after the age of 60 (Melton et al., 2000). Similarly, the rate of reported muscle mass loss varies widely, with Holloszy reporting muscle mass loss at anything between 3-8% per decade after the age of 30 (Holloszy, 2000). In addition to whole body mass loss, muscle groups can be differentially affected by sarcopaenia (Ikezoe, 2020) with the rate of loss in the antigravity, lower limb muscles reported to be twice that of the upper body (Janssen et al., 2000).

Regardless of the reported variation in both pattern and rate of muscle mass loss, there is universal agreement that muscle strength is lost more rapidly than mass. Loss of strength is reported to occur 2-5 times faster than the loss of mass, at a rate of 3-4% per year in men and 2.5-3% in women over the age of 75 (Mitchell et al., 2012). Loss of strength is not only the result of muscle mass loss, in addition neuromuscular

function also declines with age and may well be a causative factor in the age-related loss of muscle strength (Rudolf et al., 2014, Punga and Ruegg, 2012). Muscle function is measurable through several facets including strength (The amount of force that can be generated by a muscle group), power (the ability to generate force rapidly) and fatiguability (exercise induced decrease in the ability to produce force). Assessment of muscle function is dependent on other skeletal component including joints, tendons, and ligaments and neuromuscular dysfunction, therefore the quantification of dynapaenia is far more challenging than the simple measurement of mass. It therefore follows that despite muscle function being more predictive of musculoskeletal performance, quantification and change in muscle mass continues to be more reliably reported within intervention studies.

Net protein balance is maintained through the diurnal variation of MPS and MPB with protein ingestion and exercise being the stimulants to MPS. The average rate of MPS in the fasted state equates to around 1% turnover daily (Cuthbertson et al., 2005) . Oral feeding induces an anabolic response in the form of increased MPS. This occurs primarily in response to essential amino acids (EAAs) from dietary protein, especially

leucine (Smith et al., 1992, Leenders and van Loon, 2011). Basal levels of MPS and MPB are not detectably lower in older people when compared to younger people, however increasing age seems to result in the development of 'anabolic resistance', a term that describes the inability of exercise or feeding to stimulate MPS and inhibit MPB. Cuthbertson demonstrates this through showing a blunted myofibrillar FSR in response to EAAs in the older when compared with the young in a study including 44 healthy men (Cuthbertson et al., 2005). Similarly, Kumar et al. (Kumar et al., 2009) found the MPS response to RET in elderly men to be significantly blunted when compared to their young counterparts. This metabolic derangement is thought to be the driver of the chronic reduction in stimulated MPS that results in net muscle loss over time and contributes to the physiological state of sarcopaenia. To date however, well controlled longitudinal studies are missing to determine the actual relative contribution of anabolic resistance to sarcopaenia. Despite this gap in knowledge, increasing numbers of studies are being conducted that are trying to develop methods to counteract this process through increased protein nutrition (Bauer et al., 2013) and increased habitual activity (Burd et al., 2013).

The phenomenon of anabolic resistance is however not just limited to the elderly, it is also a recognised consequence of inactivity in the young. Glover investigated the rate of post absorptive MPS in 12 participants following 14 days of unilateral knee immobilisation and concluded that the intervention induced a decline in post absorptive MPS, with evidence of resistance to amino acid infusion (Glover et al., 2008). These findings further add to the debate of whether the physiological and metabolic changes seen in the elderly are to a degree related to the declining activity levels that are seen in the majority with advancing age.

1.8 The importance of appropriate experimental controls in ageing studies

Most published inactivity studies report on outcomes from a single defined age range. Whilst the outcomes of these studies offer valuable insight into the effects of the desired model of inactivity, they are often not able provide a true comparative group that can be used to compare against other studies, such as comparing young with old. This is due to the variability within each study such as the different techniques to calculate body composition or the varying analytical techniques to

estimate serum insulin concentration for example. In addition, each study will have inbuilt variability or biases that may or may not be significant. For these reasons, studies that compare young and older groups simultaneously may reduce some of the variability and offer a more true comparison. Unfortunately, several of the studies that have been performed do not have comparative groups matched for habitual physical activity and/or body composition at baseline, meaning any conclusion must be interpreted with caution. In addition, many ageing studies are cross sectional comparisons, rather than longitudinal studies.

Reidy (Reidy et al., 2018) subjected 14 young and nine older participants to five days of bed rest followed by eight weeks of exercise rehabilitation. The groups were matched for lean leg mass (DEXA), quadricep CSA and BMI. However, the older group had a significantly greater relative whole body fat mass and abdominal fat mass, with no measurement of preintervention physical activity levels. Following bed rest there was no difference in quadriceps CSA in either group. After rehabilitation, the young group had no difference from baseline, but the older group had a quadriceps CSA significantly greater than baseline. The older group also had

reductions in whole body lean mass, trunk lean mass and leg lean mass after bed rest that returned to baseline after rehabilitation, whereas these measures did not alter following bed rest in the young. In addition to body composition changes, there was a significant decrease in insulin sensitivity in both young (36%) and the older (39%) following bedrest that returned to baseline following two months of rehabilitation. Despite the results suggesting a clear difference in response between young and older groups, given that there was no measure of habitual pre-study activity levels, the results should be interpreted with caution. This finding is common amongst studies that compare young and older groups.

Young and older participants were compared in a leg immobilisation study by Vigelso in which 17 young and 15 older men had one leg immobilised for two weeks followed by six weeks of bicycle endurance retraining (Vigelso et al., 2015). Immobilisation induced a 485 ± 105g loss of lean mass in the young which returned to baseline following three weeks of rehabilitation. Of interest, there was no lean mass loss in the older participants. The younger participants had a greater baseline leg lean mass and based on pre-study measures of activity via accelerometery the younger participants spent

more time in sedentary activities with no difference between groups in time spent in moderate and vigorous activities. In a similar study by Suetta (Suetta et al., 2009), where 11 young and nine older healthy volunteers were subjected to two weeks of randomised, unilateral whole leg casting which was followed by four weeks of heavy resistance training, there was a decline in DEXA measured quadriceps volume in both young (-8.9%) and old (-5.2%). Following six weeks of retraining the younger group recovered volume to their initial baseline level whereas despite a degree of recovery, the older group did not. At baseline the groups were matched for habitual physical activity however the younger group had an 11% higher quadriceps volume than the older group as well as a lower percentage of total body fat and a lower BMI than the older group, which could explain the greater percentage loss following leg immobilisation.

1.9 Thesis structure and aims

Despite reduced step count models likely being more relevant than bed rest or limb immobilisation for studying physiological adaptations to sedentary lifestyles, I believe this is the first study to compare young and old following SR and subsequent remobilisation. Furthermore, there is a paucity of inactivity

studies that have compared young and older participants that are well matched for habitual physical activity and body composition at baseline. Clearly if the mechanisms behind the real-world adaptations to sedentary behaviour are to be elucidated in a bid to improve human health, then better controlled studies utilising state of art technologies in longitudinal study designs are required.

This thesis reports the outcomes of a single large study that was designed in such a way to include multiple endpoint measures from sequential interventions: SR and remobilisation. The overall aim of this thesis was to investigate the impact of a 14-day period of SR and a subsequent 14-day period of remobilisation on body composition, blood glucose regulation and muscle protein turnover in healthy, physically active young and older participants. In addition, we examined the ability of the D3-Creatine dilution method to estimate whole-body muscle mass in a longitudinal study design. The overarching hypothesis of the Thesis was that a 14-day period of SR would induce metabolic and physiological changes of a similar magnitude in both young and older participants, that would be completely resolved upon resolution of normal

habitual activity. The outcomes were organised into three distinct studies as follows;

Chapter 3: It has previously been shown that a period of SR in both young and older participants result in detrimental effects on glucose regulation. However, the underlying mechanisms have yet to be elucidated with ongoing debate about whether body composition changes are causative or indeed the result of reduced physical activity. This chapter therefore investigated the impact of SR on blood glucose regulation and whole body and regional body composition in young and older volunteers that were as well matched as possible at baseline for habitual physical activity levels and body composition. In addition, the effects of remobilisation on end-point measures were investigated in the same volunteers. We hypothesised that 14 days of SR would induce changes in blood glucose regulation that were independent of body composition changes and reverted to baseline following 14 days of remobilisation to normal activity levels.

Chapter 4: Bed rest and limb immobilisation studies have repeatedly and conclusively reported loss of whole body and limb specific lean mass following a period of intervention.

These changes occur in the presence of blunted MPS responses or a reduction in cumulative MPS, with little data to support an increase in MPB. SR studies to date have reported fewer uniform outcomes for the effect on MPS and at present no study has investigated the effect on MPB following SR. The aims of this study were to assess the impact that a 14-day period of SR and subsequent 14-day period of remobilisation had on MPS, whole-body MPB, and MRI determined thigh muscle volume and CSA in healthy, active younger and older volunteers matched as well as possible for habitual physical activity levels and body composition. Based on prior studies we hypothesised that following 14 days of SR, we would see a reduction in the cumulative rate of MPS in both young and older groups that would revert to baseline following remobilisation. We did not expect to see any change in the rate of MPB following either intervention in both groups.

Chapter 5: Sarcopaenia, the age-related loss of skeletal muscle mass and quality characterised by loss of strength and physical performance, is associated with multiple adverse outcomes. The measure of muscle volume has traditionally been achieved through CT, MRI or more commonly DEXA. The D3-creatine dilution method has emerged as a reliable

measure of whole-body muscle mass utilising an orally ingested stable isotope tracer. As far as I am aware, no study has reported on repeat measures of whole-body muscle mass using this method in a longitudinal study, rather than a single timepoint cross-sectional design. The aim of this study therefore was to compare the performance of the D3-creatine dilution technique with DEXA and MRI derived measures of whole-body and regional muscle volume before and following SR and after a period of remobilisation in healthy, active younger and older volunteers matched as well as possible for habitual physical activity levels and body composition. Based on prior studies that have investigated single time point interventions, we expected to see a good correlation between D3-creatine measures of whole-body muscle mass and other forms of body composition measures during each time point in the study.

Chapter 2

General Methods

This chapter describes the methods that are common to the experimental chapters in this thesis. Details regarding recruitment of volunteers, medical screening, the study protocol, specimen collection and statistical analysis will be given. Where an experiment employed a method unique to that chapter, it is detailed in the relevant Methods section of each chapter.

2.1 Study Design

The general methods and experimental chapters within this thesis describe a single large study, that was designed in such a way as to capture multiple outcome measurements from the same participant as they progressed through the study protocol. Each participant being enrolled in the study for a total of 38 days, requiring 7 comprehensive study visit days in addition to a screening visit and having to adhere to a 14-day period of SR to less than 1500 daily steps.

2.2 Ethical Considerations

The large study design was scrutinised and approved by the University of Nottingham Ethics Committee (Ethics Reference

No: A10022017) and conducted in accordance with the Declaration of Helsinki. Following ethics approval there was one amendment; the age range of the younger participants was altered from 25-40 years to 18-40 years. There was also a 6-month extension to the project requested and granted, due to delays attributable to the COVID-19 pandemic.

2.3 Recruitment of study volunteers

Posters advertising the study and displaying contact details of the investigators were placed around the University of Nottingham campuses and Queens Medical Centre, Nottingham. Advertisements displaying the same information were also be placed on popular advertising (e.g., www.gumtree.com) and social media (e.g., www.facebook.com) websites.

Detailed information and the opportunity to discuss the protocol was then provided to interested parties who were then given time to think about the study before agreeing to take part. For those interested, a screening visit was arranged at the David Greenfield Human Physiology Unit, University of Nottingham, to discuss the study in more detail and ensure suitability for participation.

Participant inclusion/exclusion criteria were;

- Aged 18-40 or 60-75 years
- Non-smokers
- Non obese (BMI>30)
- No history of metabolic disease including diabetes
- No history of cancer
- No history of cardiovascular disease
- No history of activity limiting musculoskeletal disease
- No regular extreme exercise routines or significant pre-participation sedentary behaviors (Baseline physical activity monitored by Actiheart prior to commencing study)
- No use of medications including anti-inflammatories, steroids, antidepressants, or medication that affects glucose handling (Oral hypoglycaemics) and/or cardiovascular status (beta blockers, antihypertensives)
- No metal implants within the body
- Not participating in any other studies concurrently or within the prior three months

All inclusion and exclusion criteria were sent to the volunteer as part of the study information leaflet. It was again confirmed

with each participant during the screening visit that they were eligible to participate within the confines of the above criteria.

2.4 Pre-study medical screening

The first part of the medical screening involved an in-depth explanation of the aims and rationale of the study. This was followed by a step-by-step explanation about what would be expected from the participants at each stage of the study and how each procedure was to be conducted. A medical screening was then performed which involved the completion of a general health questionnaire, a physical examination, measurement of height and weight, a supine, sitting and standing blood pressure, the acquisition of a 12-lead electrocardiogram and blood sampling for full blood count, urea and electrolytes, clotting profile and liver function tests. All blood samples were analysed within the departments of Haematology and Clinical Chemistry, Queens Medical Centre, Nottingham. Finally, baseline leg strength was determined during isometric contraction of the knee extensors using a dynamometer (HUMAC Norm, CSMi Solutions, MA, US). Three measures were obtained, and the highest value used. Using a technique formulated by Harbo (Harbo et al., 2012), peak estimates and standard deviation for maximal strength of the

knee extensors were devised using demographics including gender, height, weight and age. A measure >2 standard deviations below the calculated peak estimate rendered the participant ineligible to participate.

2.5 Subjects, sample size and power calculation

Ten young (age 18-40 years) and ten older (age 60-75 years) healthy volunteers were recruited to partake in this study. All participants provided informed, written consent (Appendix 1) and completed a general health questionnaire (Appendix 1) as part of the medical screening visit (See section 2.4). A *a priori* power calculation was performed based on data from previous studies (de Boer et al., 2007, Glover et al., 2008) for MPS. Assuming a realistic reduction in MPS from 0.040% h^{-1} to 0.036% h^{-1} with σ 0.005% h^{-1} then we would require a total of 10 participants in each age group (α: 0.05 β: 0.8) to detect a significant decline in muscle protein synthesis induced by inactivity. This formed the basis of recruiting 10 participants to each age group and no *a priori* power calculations were performed for other primary or secondary variables that were studied.

2.6 Study protocol

Participants attended the David Greenfield Human Physiology Unit, University of Nottingham, on study visits as shown in the study schematic within each Chapter. Following a medical screening, participants were enrolled to the study if suitable. The primary intervention was to reduce daily step count to less than 1500 steps per day, for a total of 14 consecutive days. Baseline measurements of habitual activity, physiological function and body composition were conducted before and after the 14-day period of SR as described below. Measures were repeated a further 14 days after returning to normal activity, with activity monitoring also being recorded for this period.

2.7 Measure of physical activity

2.7.1 Measurement of physical activity before and following step count reduction using Actiheart monitoring

Participants underwent seven days of habitual physical activity monitoring through the use of the Actiheart monitor (CamNtech, Cambridgeshire, UK) prior to commencing on the

sedentary period of the study. A second 14-day period of analysis took place following completion of the reduced step count. This was compared to the original activity measure to identify whether participants returned immediately to habitual activity levels, or if non return to pre-intervention levels could account for non-recovery of pre-intervention, metabolic function. During assessment periods, participants were requested to carry out their normal daily routines with no regard for the monitoring of activity. The monitor is worn over the chest using an elastic strap, undertaking heart rate and accelerometery recordings at 1-minute intervals throughout. Accelerometery is the measurement of acceleration in three directions which is achievable through its inbuilt 3 axis accelerometer. The Actiheart utilizes a piezoelectric accelerometer and combines this data with that obtained for heart rate to estimate active energy expenditure (AEE). AEE is the modifiable component of total energy expenditure (TEE) derived from all activities, both volitional and nonvolitional. AEE combined with the estimated resting energy expenditure (REE) (calculated using age, sex and weight inputted into Schofield's equation (Schofield, 1985)) and dietary induced thermogenesis (DIT), total energy expenditure (TEE) can be estimated. Physical activity level (PAL) is defined as an

individual's TEE over a 24-hour period divided by the REE. The Actiheart monitor has been reported to be a reliable method to estimate these end-point parameters (Brage et al., 2005, Brage et al., 2004).

During the assessment period the average daily step-count was estimated using the participants' weight, height and the Actiheart generated AEE. These values were entered into an online step-count calculator (Michalowska, 2005) that was based on research by Brooks that allows estimation of step count based on the above parameters (Brooks et al., 2005).

2.7.2 Measurement of step count via pedometer during step count reduction

Participants were requested to reduce their daily step count to less than 1500 steps per day for a 14-day period. Daily step count was recorded using a pedometer (Omron HJ-321) which was worn on the participants waist at all times during waking hours. The pedometer uses a tri-axis sensor which has previously been validated against a number of other step-tracking devices, giving step count errors within 1% (Huang et al., 2016). Participants were requested to manually record their daily step count each night prior to going to bed and to

replace the pedometer upon waking. The recorded step count was checked against the inbuilt memory of the pedometer following its return. During SR, the number of daily steps were used alongside the participants weight and height to calculate the AEE (See section 2.7.1).

2.7.3 Measurement of dietary intake

Participants were requested to complete daily diet diaries for the first seven days of the SR period and the first seven days of remobilisation. The standard diet diary (Appendix 1) requested volunteers to record all oral intake including drinks and record the weight (Either from the packaging or by manually weighing each item or ingredient) and number of each food items that were ingested. The data was then manually uploaded onto Nutritics, an online food data management software (Nutritics, 2019), which reported the average daily macronutrient (i.e. data) and caloric intake. Participants were requested to abstain from alcohol and continue to eat their normal diet throughout the duration of the study.

2.8 Blood sampling

2.8.1 Retrograde cannulation and hand warming for arterialised- venous blood sampling

In order to obtain arterialised-venous blood during the OGTT, a retrograde cannula was placed into a dorsal hand vein and the hand placed into a hand warmer at 55 degrees centigrade to enable regular sampling of arterialised venous blood via a 3-way tap. This technique has been shown to give an accurate representation of arterial blood (Gallen and Macdonald, 1990) and is the standard procedure performed in our laboratory. The hand was warmed for a minimum of 10 mins prior to fasting samples being taken. At all times where an indwelling intravenous catheter was in place, an intravenous drip infusion of 0.9% sodium chloride ensured that the catheter remained patent.

2.8.2 Plasma sampling

Plasma collection was required for measuring D_3-3MH. Plasma sampling did not require for the blood to be arterialised and thus cannulation of the antecubital vein sufficed. A three-way tap was employed, as described for the whole blood sampling,

with a slow infusion of intravenous 0.9% sodium chloride to ensure catheter patency.

2.9 Whole body and regional fat mass and fat free mass quantification using dual-energy x-ray absorptiometry

Dual-energy x-ray absorptiometry (DEXA) took place at the DGHPU. DEXA is a non-invasive, painless investigation that uses X-rays to estimate body composition. Two low dose X-ray beams are passed through the subject being scanned (40 and 70keV). The X-rays pass through different constituents of the body at a different rate due to the differences in elemental composition. The degree of beam attenuation is used to calculate several measures including regional and whole-body fat mass, lean mass or fat free mass (see below) and bone mineral density. The terms lean mass and fat free mass are often used interchangeably when referring to skeletal muscle. However, the terms refer to different DEXA derived measures. Lean mass is the sum of body water, total body protein, carbohydrates, non-fat lipids and soft tissue mineral, whereas, fat-free mass includes bone as well as skeletal muscle, organs,

and connective tissue (Prado and Heymsfield, 2014) Throughout this thesis the measure used was fat free mass. Over the duration of the study, three DEXA scans were obtained per participant at time points as indicated on the study protocol (Chapter 3, Figure 3.1). Participants require no specific preparation to undergo a DEXA and the scan takes around 10 minutes to complete. It is important that female participants are not pregnant prior to scanning due to the scan involving ionising radiation which can be harmful to an unborn foetus. All participants were required to complete a DEXA safety questionnaire prior to being scanned (Lunar Prodigy DEXA, GE Medical Systems, Bedford UK), which was reviewed by a trained operator. They were asked to remove any metal containing item on their person and to remain motionless while laying supine with their arms placed by their side. The acquired data is processed via the operating software (encore; Ge Medical Systems, Chicago, USA) and gives whole body and regional analysis. The radiation dose is very low (~1mSv (millisievert) variable to height and weight). This is the equivalent to the amount of radiation that you would be exposed to if you spent 15 minutes on a transatlantic flight or the amount contained in 10 Brazil nuts.

Chapter 3

The impact of step-count restriction and remobilisation on whole-body glucose regulation and body composition in healthy active young and older participants

3.1 Introduction

There is an abundance of data indicating that physical inactivity in young people, induced through either bed rest, limb immobilisation or reduced ambulatory activity, is detrimental to whole body glucose handling (Burns et al., 2021, Shur et al., 2022, Bowden Davies et al., 2018, Alibegovic et al., 2009, Olsen et al., 2008, Krogh-Madsen et al., 2010, Knudsen et al., 2012). Of particular interest to this work are the findings of the limited number of studies utilising SR or restriction, over a set time-period. Olsen found that a reduction in daily step count from ~10,000 to ~1300 daily steps for 21 days in ten young men resulted in an increase in the AUC for insulin during a 3-hour OGTT from 757 pmol/l/3h (95% confidence interval [CI], 488-1026 pmol/l/3h) at baseline to 1352 pmol/l/3h (95% CI, 1025-1678 pmol/l/3h) at 3 weeks (P=0.004) (Olsen et al., 2008). Similar findings were reported by Krogh-Madsen where a reduction in step count from ~10,000 daily steps to < 1500 daily steps for 14 days resulted in a 17% reduction of the glucose infusion rate under insulin clamp conditions in ten healthy young men (Krogh-Madsen et al., 2010). In addition, 45 young volunteers took part in a SR study by Bowden-Davies where habitual daily step count was reduced from 12,500-13,000 daily steps by a

mean average of 10,285 steps (Bowden Davies et al., 2018). The participants were split into 16 that had a first degree relative with type 2 diabetes and 29 that did not. There was a significant reduction in the Matsuda index following SR in both groups, which was accompanied by a significant increase in glucose and insulin AUC, with no between group differences being identified.

The mechanisms underpinning the reduction in glucose disposal following a period of reduced mobilisation, regardless of the inactivity technique, remain largely unresolved. One theory suggests that given skeletal muscle accounts for about 85% of post-prandial insulin mediated glucose disposal (DeFronzo et al., 1981), a reduction in the total body sink for glucose disposal through muscular atrophy results in a reduced whole body ability for cellular glucose uptake. Given that the studies by Krogh-Madsen and Bowden-Davies reported significant reductions in leg lean mass in the order of 0.5kg and 0.2kg respectively, it is plausible that the loss of metabolically active tissue diminishes the capacity for whole-body glucose uptake. However, a recent upper limb immobilisation study by Burns found a decline in forearm glucose uptake within 24 h which was sustained over several

days of immobilisation (Burns et al., 2021), with no associated loss of muscle mass following this duration of immobilisation. It was therefore suggested that a lack of contraction *per se* results in a reduction in muscle glucose uptake and consequent cellular glucose oxidation and storage. Furthermore Shur (Shur et al., 2022) showed after 4 days bed rest (BR) glucose uptake and storage during a hyperinsulinaemic euglycaemic clamp were diminished, but substrate oxidation was not different compared to pre-BR. However, after 56 days BR, glucose uptake, storage and oxidation were all reduced compared to pre-bed rest. This suggests that impairment of glucose uptake and storage is a rapid adaptation to BR, whereas changes in fuel oxidation take longer to become established. Zierath however argue that cellular uptake is diminished due to inactivity induced alterations in the signalling and/or translocation of GLUT4, an insulin regulated glucose transporter, to the plasma membrane (Zierath et al., 1996). Though, Burns (Burns et al., 2021) debates this as being a consequence of decreased glucose uptake rather than a cause, stating that muscle level changes could at least to some extent reflect an adaptive response to reduced glucose muscle uptake. The data is therefore suggestive that certainly in the early phases of

inactivity, the reduction in glucose uptake is secondary to lack of contractile activity with adaptive changes, such as a reduction in GLUT4 translocation and loss of lean mass, occurring later. Another theory suggests that mitochondrial dysfunction results in alterations in carbohydrate metabolism (Abadi et al., 2009), with mitochondrial down regulation occurring as early as 48 hours following the onset of inactivity. Although a study by Noone (Noone et al., 2023) showed that the reduction in mitochondrial function may in fact be because of a decrease in mitochondrial numbers rather than a decline in individual mitochondrial respiratory capacity. Therefore, the contribution of mitochondrial dysfunction to changes in glucose disposal should be considered carefully given the lack of conclusive data at present.

It has long been recognised that human adiposity, in particular abdominal adiposity, is associated with detrimental health outcomes and insulin resistance (Bogardus et al., 1985, Lebovitz and Banerji, 2005). Reductions in physical activity without a concurrent reduction in dietary energy intake results in energy oversupply and accumulation of adipose tissue in both central and peripheral locations. DEXA measurements of abdominal fat, which include visceral and subcutaneous fat

depots, correlate well with insulin resistance (Paradisi et al., 1999, Carey et al., 1996). In the above mentioned study by Olsen, 14 days of reduced step count induced an increase in the intra-abdominal fat mass by 7% ($p=0.046$) which correlated with an increase in the plasma insulin AUC (from 599 pmol/L/3h [95% CI, 489-709 pmol/L/3h] to 942 pmol/L/3h [95% CI, 443-1440 pmol/L/3h]; $p=0.04$) (Olsen et al., 2008). Bowden-Davies reported similar findings of increased abdominal or central fat measures being associated with alterations in glucose handling, typically with maintenance of normal glucose profiles but hyperinsulinaemia (Bowden Davies et al., 2018). Conversely, a study by Knudsen that enforced 14 days of SR along with overfeeding in nine healthy young men found decrements in insulin sensitivity that proceeded body composition changes (Knudsen et al., 2012). This contrasting data therefore raises the question as to whether body composition changes with prolonged inactivity and positive energy balance are causative of a reduction in insulin sensitivity, or an associated adaptation due to the enforced inactivity. Studies by Shur et al. (Shur et al., 2022) and Egglebusch (Eggelbusch et al., 2024) further support the notion that short term inactivity is the driver of the observed rapid onset insulin resistance rather than this being a function

of energy oversupply. It is suggested that lipid accumulation represents the long-term adaptation to energy oversupply and may or may not contribute to the sustained impaired glucose disposal.

There has been less work on the relative contribution of peripheral lipid deposition in the form of IMCL or EMCL on glucose handling in the muscle. Traditional methods of peripheral lipid quantification have relied on muscle biopsy techniques that are timely, expensive, and invasive. Improved availability and refinement of non-invasive MRS based methodology has resulted in renewed interest in the measurement of these lipid depots and the understanding of their relative contribution to insulin resistance. It is thought that persistent lipid oversupply to skeletal muscle and diminished lipid oxidation through a lack of physical activity results in the accumulation of IMCL (Kitessa and Abeywardena, 2016). Quantification of IMCL either by muscle biopsy (Abadi et al., 2009) or MRS (Krssak et al., 1999) (Perseghin et al., 1999), correlate strongly with measures of insulin resistance and increased IMCL concentrations have been considered important in the development of type 2 diabetes (Dube and Goodpaster, 2006). Again however, data

from Shur (Shur et al., 2022), where energy balance was maintained for the duration of a 56 day bed rest study, suggest that lipid accumulation is in fact a feature of positive energy balance rather than inactivity given that no increase in IMCL was observed after the BR period, but insulin resistance occurred rapidly. However, lipid accumulation did occur in the presence of energy oversupply during the BR period in the study by Eggelbusch (Eggelbusch et al., 2024).

Fewer studies have attempted to identify the contribution that increasing amounts of EMCL may have on skeletal muscle function both in terms of muscle contractility and glucose handling (Goodpaster et al., 2000, Goodpaster et al., 1997, Goodpaster and Wolf, 2004). At present the metabolic entity of EMCL is unknown but it is generally considered to be metabolically inert when compared with IMCL. The ratio of IMCL to EMCL has also garnered interest as a marker of lipid redistribution within the muscle following changes in activity levels. However, along with EMCL, the data regarding this measure is scarce when compared to IMCL in isolation. Continued improvements in the availability and accuracy of the non-invasive MRS technique for quantifying IMCL and EMCL will further shed light on this parameter and may

ultimately prove to be a useful tool in the metabolic profiling of individuals.

The described alterations in whole body glucose handling and body composition with inactivity have historically been attributed to aging in isolation. Aging is associated with a significant reduction in skeletal muscle mass, with cross-sectional studies demonstrating the difference in muscle mass between young and old is in the range of 8% to 45% (Tzankoff and Norris, 1977, Mitchell et al., 2012). In addition there is an increase in both central and peripheral adiposity (Kohrt et al., 1993) which, as already described, is linked with detrimental effects on insulin sensitivity. The trajectory of which these changes occur remains unclear and is further complicated by the declines observed in physical activity that occur with ageing (Milanovic et al., 2013). At present, despite the acknowledgement that aging is associated with reduced physical activity, detrimental changes in body composition and whole-body glucose disposal, there remains a degree of uncertainty about whether these changes are simply a result of aging per se or whether chronic, progressive declines in physical activity and/or increases in positive energy balance are responsible for the 'ageing phenotype'. The paucity of well-

designed longitudinal studies that have compared matched cohorts, controlled for dietary energy intake, and employed an inactivity technique more representative of that seen in free living individuals, ultimately means that we are unable to draw robust conclusions on the relative contributions of these drivers to the ageing phenotype.

As individuals age, there will almost certainly be periods of unplanned, significant reductions in physical activity through injury or ill health. Current research can provide an insight into the potential metabolic and physiological consequences of such periods in both younger and older cohorts. However, despite several studies investigating the effects of short-term step count restrictions, there is little data investigating the metabolic and physiological response of returning to habitual physical activity. Certainly in a study by Shur, exercise supplemented remobilisation in 10 young healthy males after three days of BR restored leg muscle volume and myofibrillar FSR but not leg glucose uptake or glycogen storage, highlighting differences in metabolic recovery following remobilisation (Shur et al., 2024). Furthermore, it is not known if there is a difference between young and older groups' response to remobilisation and if recovery is complete. Neither

is it known whether either group immediately returns to habitual physical activity levels or there is any form of lag following SR. Short periods of inactivity that follow either injury or illness appear to result in a stepwise decline in the elderly population, thus data on recovery is of paramount importance if we are to interrogate the mechanisms behind this demise.

3.2 Aims

An aim of this study was to determine the impact of 14 days of SR and subsequent remobilisation on whole body glucose disposal in young and older volunteers. In addition, it aimed to investigate whether any changes in glucose disposal observed with reduced physical activity are associated with concurrent changes in whole body or regional tissue composition. Finally, any impact of age on these endpoints was considered.

3.3 Methods

3.3.1 Study protocol

Participants attended the DGHPU, University of Nottingham, on study visits as shown in Figure 3.1. Following a medical screening (See General Methods section 2.4) participants were enrolled to the study if suitable. The inclusion and exclusion criteria are given in the General Methods (Section 2.3). There were 4 study visits in total at set time points; baseline (prior to SR), following two days and then 14 days of SR and finally 14 days post remobilisation. The intervals between study visits were strictly adhered to and participants attended following an overnight fast for each visit. During three of the four visits, vastus lateralis IMCL and EMCL measurements at mid-thigh were acquired using MRS.

MR scans were performed at the Sir Peter Mansfield Imaging Centre, University of Nottingham prior to attending the DGHPU, University of Nottingham, for body composition measurements using DEXA. This was followed by an OGTT. Participants were fed on completion of the study day. There was no MRI/MRS or DEXA scan performed on the 2-day SR study visit (See Figure 3.1).

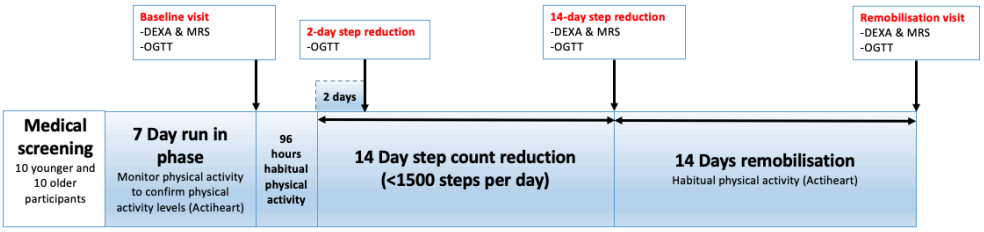


Figure 3.1 - Study Schematic.

3.3.2 Physical activity measurement, step count measurement and step count reduction

Habitual physical activity was measured using an Actiheart monitor (CamNTech, Cambridgeshire, UK) during the 7-day run in phase and again during the 14-day remobilisation as described in the General Methods (Section 2.6.1). The Actiheart monitor provided data for RMR, TEE, DIT and AEE during the run-in phase and during remobilisation. TEE is a sum of RMR, DIT and AEE. AEE for the SR phase were calculated based on the individual step count and the participants height and weight (See General Methods section 2.6.1). Baseline daily step count was not directly recorded but was computed using a calculator and values for each participants height, weight and Actiheart measured AEE (See General Methods section 2.6.1). Step count monitoring was performed during the SR part of the study which is described

in detail in the General Methods (Section 2.6.2). Participants were requested to limit their daily step-count to less than 1500 steps daily for 14 days, with transport being provided for general travel and travelling to and from study visits.

3.3.3 Nutritional intake

Detail regarding the collection of data and the analysis of nutritional intake during the study is given in the General Methods (Section 2.7). Briefly, participants kept self-recorded diet diaries during the first seven days of the SR period and again during the first seven days of remobilisation. Participants were advised to continue to eat their normal diet and not alter their intake during the SR period. Data was uploaded onto Nutritics, an online nutritional database and data management software.

3.3.4 Oral glucose tolerance test

OGTT was performed at all four study visits. For each visit, participants attended following an eight hour overnight fast. Fasting arterialised-venous blood samples were taken (See General methods, section 2.8.1) at time points -10, -5 and 0 min. Following this, an oral bolus of 75g dextrose dissolved in

250mls of water was consumed over a 5-minute period. Further blood samples were taken at 10 min intervals for 80 mins and then 15 min intervals to complete 155 mins of sampling post oral glucose (See Figure 3.2). Two ml of whole blood was drawn and divided into two tubes; 0.5mls for blood glucose analysis which occurred at the bedside (see below) and one and a half ml for serum insulin analysis, which took place later following sample storage at -80 degrees centigrade.

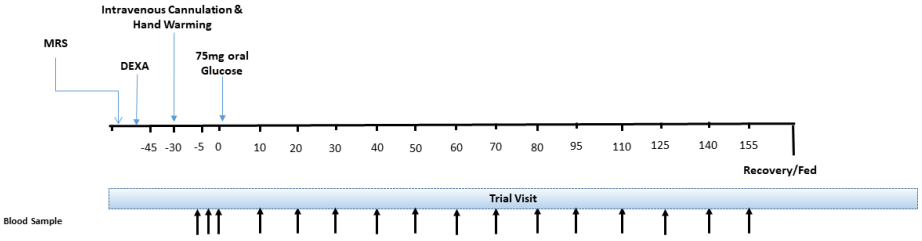


Figure 3.2 – OGTT protocol indicating blood sampling times.

3.3.4.1 Determination of whole blood glucose concentration

Whole blood glucose concentration was measured at the bedside using the YSI 2300 STAT PLUS™ glucose analyser (YSI Life Sciences, Yellow Springs, OH). Following collection, 0.5mls of whole blood was mixed on a roller mixer in a fluoride oxalate tube for a minimum of 3 min prior to

analysis. Prior to commencing each study day, the analyser was calibrated using a 10mmol/l glucose standard.

The YSI 2300 uses steady-state measurement methodology in which immobilised glucose oxidase in a membrane overlying an electrode catalyses the oxidation of glucose into gluconic acid and hydrogen peroxide, the latter being oxidised at the platinum electrode, releasing electrons. The glucose concentration is proportional to the difference between the plateau current generated by the sample and the initial baseline current (YSI STAT 2300 Laboratory manual 5- 4). The coefficient of variation (COV) is 1.2% or less for glucose concentrations of 5.2 – 22.1mmol/l and 5.8% or less for glucose concentrations of 1.6-16.2mmol/l, respectively, identifying it as a reliable, rapid and easy to use method (Chua and Tan, 1978). Studies comparing the use of the YSI with the HemoCue system have shown good correlation and thus reliability of the results (Stork et al., 2005).

3.3.4.2 Determination of serum insulin concentration

One and a half ml of whole blood was collected into a gold topped serum separator tube (SST) which was left to clot for

20 mins prior to centrifugation at 3000 rpm for 10 min. A 1ml aliquot of serum was pipetted off the cell pellet into a micro tube and stored at -80 degrees prior to insulin quantification later in batches using human insulin specific RIA kit (EMD Millipore Corporation, Missouri, USA).

All samples were analysed in duplicate. Firstly, calibration standards were prepared from human insulin of known concentrations. Assay buffer was then added to either the standards or the serum samples followed by hydrated ^{125}I radio-labelled insulin and human insulin antibody. The tubes were vortexed and then incubated for 24h at room temperatures. The endogenous insulin competes with the exogenous radioactive insulin for sites on the insulin specific antibody. Next cold precipitating reagent was added to all tubes which were vortexed, incubated and centrifuged at 2600xg for 40 minutes. Following centrifugation, the supernatant was decanted from the tube which terminates the competitive binding and leaves the bound insulin in a pellet within the tube. The radioactivity of the precipitated pellet was measured using a gamma counter. A standard binding curve was plotted using the standard calibrators and the study sample concentrations were determined using this standard

curve. The mean inter-assay COV was 9.0% and the mean intra-assay COV was 3.3%.

3.3.5 Calculation of the Matsuda index and QUICKI

The Matsuda index and the Quantitative Insulin Sensitivity Check Index (QUICKI) are composite measures that can be derived from the circulating concentrations of glucose and insulin. The Matsuda index was originally proposed by Matsuda and DeFronzo (Matsuda and DeFronzo, 1999) and provides an evaluation of whole body (Skeletal muscle and hepatic) insulin resistance in the insulin-stimulated state using plasma glucose and serum insulin measures taken at 0, 30, 60, 90 and 120 minutes during an OGTT. These values were inserted into an online calculator (https://mmatsuda.diabetes-smc.jp/MIndex.html) which generated individual values for each OGTT. It is generally considered that a Matsuda index of less than 3 indicates insulin resistance. The QUICKI index is an assessment of fasting insulin sensitivity based on fasting plasma

glucose and serum insulin concentration. It is based on the mathematical equation;

$$1/(\log(fasting\ insulin) + \log(fasting\ glucose))$$

Where insulin concentration is expressed in μU/ml and glucose concentration is expressed as mg/dl. A value of greater than 0.45 is considered normal whereas a score of 0.30-0.45 suggests that insulin resistance is likely.

3.3.6 Measurement of absolute whole body, appendicular and abdominal fat masses and fat free masses

DEXA scanning was performed at the DGHPU as described in the General Methods (Section 2.11). Scans were performed at baseline, following 14 days of SR and after 14 days of subsequent remobilisation. The automated software provides whole body and regional measures of fat and fat free masses

3.3.7 Measurement of intra-myocellular and extra-myocellular muscle lipids using Magnetic Resonance Spectroscopy

MRI scans were performed at the Sir Peter Mansfield Imaging Centre at the University of Nottingham. Participants were required to complete a safety questionnaire prior to scanning (Appendix 1) and an explanation of the process was given. A three tesla (3T) MRI scanner (Phillips Ingenia) was utilised to acquire axial images of the dominant thigh at baseline, 14 days post SR and 14 days post remobilisation. Magnetic resonance spectroscopy (MRS) was performed to determine IMCL and EMCL content of the vastus lateralis of the dominant leg. The basis of the method used in this study was that developed by Khuu (Khuu et al., 2009) and Ren (Ren et al., 2010). The method

involves a 15x15x50mm voxel being manually placed such that the direction of the longer voxel dimension lay in the same fibre orientation as the muscle being measured, in our case the vastus

lateralis. Data acquisition by STEAM (STimulated Echo Acquisition Mode) was obtained using a flip angle of 90 degrees and a repetition time of 2s. A total of five different

echo times were acquired (20, 40, 60, 80, 100 and 160ms) and repeated eight times to gain signal to noise ratio (SNR) in the spectra (total acquisition time of 6min 40sec). A single acquisition was also performed without water saturation for quantification purposes (acquisition time of 2 mins). Spectra were exported using the Philips format (SPAR/SDAT), loaded and processed using an in-house MATLAB (Mathworks, MA, USA) based software written by Dr Olivier Mougin, University of Nottingham. Processing involved selecting the non-water saturated spectra (NWS) and water saturated spectra (WS) from the same acquisition. The software was used to adjust the zero-order phase followed by the individual phase per spectra as required. The start and end points of the spectra of interest were isolated and the EMCL frequency was adjusted to fit the first peak of the spectra and the IMCL frequency fitted to the second peak. The software then counted the raw data to give values for total IMCL, total EMCL and the IMCL:EMCL ratio.

Scan analysis was performed by one of two trained operators. Neither operator was blinded to the participant or to which stage of the study the scan was being undertaken. The COV

was not calculated for the MRI or MRS techniques used in this study but would ideally be done for future studies.

3.3.8 Statistics and Calculations

Data for each parameter were tested for normal distribution using the D'Agostino and Pearson normality test within Prism. Individual glucose and insulin concentrations were used to calculate the net incremental AUC over the duration of the OGTT using Prism. Statistical analysis was performed to compare measures in young vs older volunteers at baseline (when fasted) using Student's unpaired t-test. Two-way ANOVA with Tukey's and Sidak's multiple comparisons tests were applied to locate within (time) and between (treatment) group differences. All analyses were performed in Prism 10 (Version 10.1.1, Graphpad). All values in the text, Tables and Figures are reported as mean ± standard error of the mean (SEM), with a $P<0.05$ value considered to be statistically significant.

3.4 Results

3.4.1 Body composition, metabolic characteristics and physical activity levels at baseline

At baseline DEXA derived abdominal fat mass and voxel concentration of EMCL in relation to water were greater in the older vs younger volunteers (p=0.028 and p=0.001, respectively; Table 3.1). No difference was observed when comparing age groups for the other body composition and metabolic characteristics at baseline and both groups were metabolically healthy based on fasting blood glucose and serum insulin concentrations (Table 3.1). Nevertheless, there was a trend for BMI, fasting blood glucose concentration, and AUC for blood glucose during the OGTT to be greater in the older volunteers (P=0.072, P=0.069, P=0.085, respectively; Table 3.1). Blood glucose and insulin concentrations relative to time during the OGTT are shown in Figure 3.3. TEE was higher in the younger group. However, there was no difference in PAL, AEE, RMR or mean daily step-count observed at baseline (Figure 3.4) between the young and older groups, but both groups could therefore be described as active (average step count >10,000 steps per day) (Table 3.1).

	Younger	Older	P value
Age (years)	26.1 ± 3.8	68.9 ± 3.2	<0.0001
Height (cm)	170.5 ± 9.6	164.7 ± 10.3	0.209
Sex	4 male, 6 female	4 male, 6 female	
Weight (kg)	70.0 ± 13.4	72.1 ± 12.6	0.720
BMI (kg/M²)	23.8 ± 2.4	26.5 ± 3.7	0.072
DEXA body fat %	30.6 ± 2.9	35.9 ± 2.9	0.233
DEXA whole body fat mass (kg)	20.7 ± 2.6	25.1 ± 2.6	0.292
DEXA appendicular fat mass (kg)	10.9 ± 1.7	10.4 ± 1.6	0.799
DEXA whole body fat free (kg)	46.0 ± 3.0	44.0 ± 3.0	0.636
DEXA appendicular fat free mass (kg)	21.3 ± 1.6	19.0 ± 1.7	0.328
DEXA abdominal (Android) fat mass (kg)	1.5 ± 1.6	2.4 ± 3.4	0.028*
Habitual physical activity level (PAL)	1.44 ± 0.02	1.45 ± 0.01	0.783
Total energy expenditure (kcal/day)	2059 ± 81	1799 ± 41	0.010*
Resting metabolic rate (kcal/day)	1552 ± 79	1350 ± 56	0.052
Active energy expenditure (Kcal/day)	454 ± 26	412 ± 17	0.195
Baseline daily step-count	12542 ± 1133	11410 ± 896	0.443
Fasting glucose (mmol/L)	4.0 ± 0.1	4.4 ± 0.0	0.069
Fasting insulin (mIU/l)	8.04 ± 0.48	10.71 ± 0.73	0.130
AUC for glucose (mmol/l/155min)	316.3 ± 39.4	418.8 ± 40.3	0.085
AUC for insulin (mIU/l/155min)	315.9 ± 59.5	462.0 ± 69.6	0.127
IMCL concentration	0.470 ± 0.100	0.812 ± 0.155	0.170
EMCL concentration	0.805 ± 0.158	2.84 ± 0.530	0.001*
IMCL/EMCL ratio	0.604 ± 0.105	0.382 ± 0.080	0.397

Table 3.1 Baseline characteristics of the older and younger groups. Values are means ± SEM. * indicates a significant difference between groups ($p<0.05$). BMI = body mass index. IMCL = intramyocellular lipids. EMCL = extramyocellular lipids.

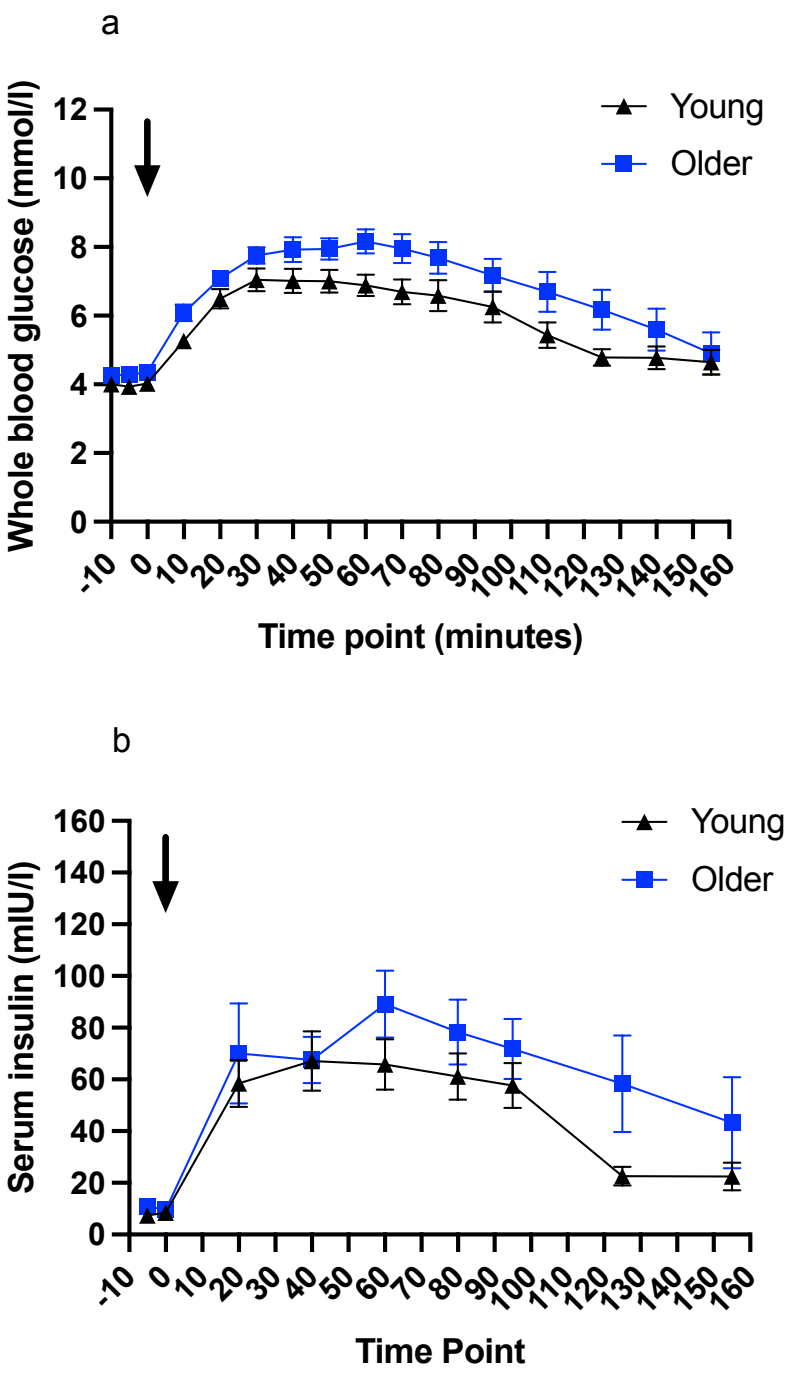


Figure 3.3 a Whole blood glucose concentration (mmol/l) before and during 155m OGTT at baseline. **b** Serum insulin concentration (mIU/l) before and during 155m OGTT at baseline. Values are means ± SEM. The arrow indicates the point of glucose consumption.

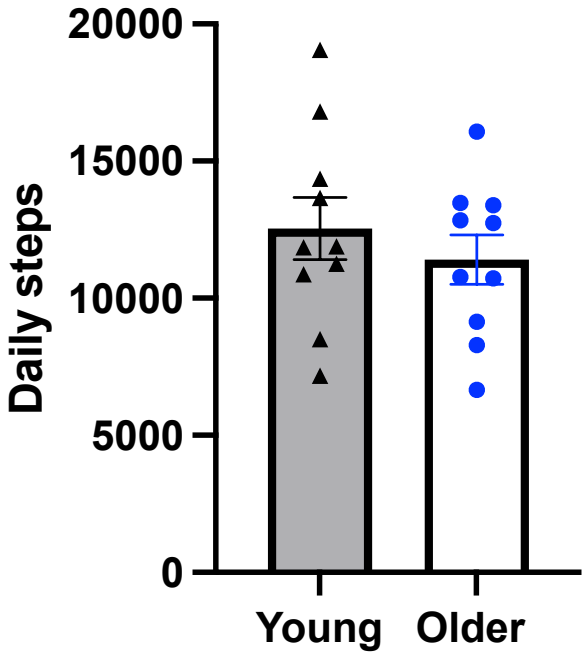


Figure 3.4 Baseline average daily step count in young and older participants. Values are mean ± SEM and individual values.

3.4.2 Impact of step count reduction and remobilisation on body composition, whole body glucose regulation and muscle lipid content

3.4.2.1 Step-count reduction intervention

For a period of 14 days, all participants reduced their average daily step-count by >90%. The young group averaged 1053 (± 94) daily steps, down from a baseline of 12542 (± 1133) (Figure 3.5 a). Similarly, the older group averaged 1095 (± 85) daily steps, down from a baseline measure of 11410 (±

896) (Figure 3.5b). There was no difference between groups in the number of daily steps taken during this period (p=0.983). Following remobilisation, the daily step count returned to baseline levels in both the younger (13724 ± 1196 steps) and older (11683 ± 776) group, with no difference between groups (p=0.659).

3.4.2.2 Energy expenditure, energy and macronutrient intake

As expected, the >90% reduction in daily ambulatory activity translated into a reduction in both TEE and AEE in both groups (Table 3.2). Resting metabolic rate did not change throughout the study due to the lack of change in body weight. The young mean daily TEE dropped from 2059 ± 81 kcal/day to 1752 ± 87 (p=0.004) and the older 1799 ± 41 kcal/day to 1531 ± 64 kcal/day (p=0.001). The drop in TEE was largely a result of the significant drop in AEE in both groups: The young mean daily AEE was reduced from 454 ± 26 Kcal/day to 41 ± 6 Kcal/day (p=0.0001) and similarly the older AEE was reduced from 412 ± 17 Kcal/day to 41 ± 5 Kcal/day (p=0.0001). Remobilisation returned the mean daily AEE to baseline levels in both the young and older groups (481 ± 27 Kcal/day and

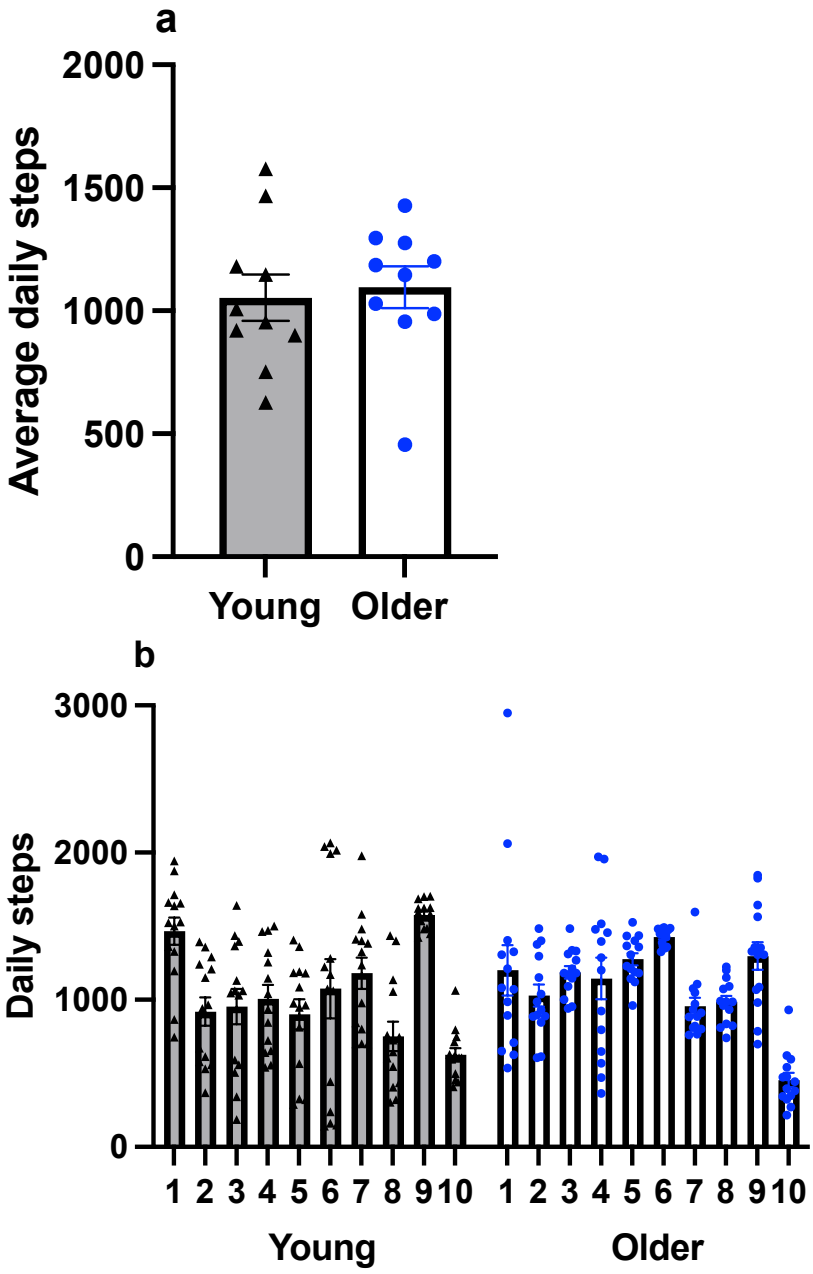


Figure 3.5 a Average daily step count and **b** Individual participant daily step counts during SR in young and older participants. Values are mean ± SEM and individual values.

	Step reduction		Remobilisation	
	Younger	**Older**	**Younger**	**Older**
Total energy expenditure (kcal/day)	1752 ± 87 †	1531 ± 64 *	1943 ± 230	1809 ± 43 *
Active energy expenditure (Kcal/day)	41 ± 6 †	41 ± 5 *	481 ± 27	423 ± 8 *
Calorific intake (Kcal)	1455 ± 77	1456 ± 78	1348 ± 94	1445 ± 73
Carbohydrate (g)	164 ± 9	170 ± 14	153 ± 12	163 ± 14
Protein (g)	65 ± 5	51 ± 4	64 ± 3	58 ± 3
Fat (g)	60 ± 6	59 ± 2	53 ± 6	63 ± 3

Table 3.2 Average daily energy expenditure, calorific intake and macronutrient intake during the first seven days of SR and the first seven days of remobilisation in young and older groups. Values are means ± SEM. † Indicates a significant difference ($p<0.05$) between timepoints in the younger group. * Indicates a significant difference ($p<0.05$) between timepoints in the older group.

423 ± 8 Kcal/day, respectively) which in turn returned the TEE to baseline (1943 ± 230 kcal/day in the young and 1809 ± 43 kcal/day in the older). There was no difference in average daily TEE or AEE between groups during SR or remobilisation.

Diet diary derived daily energy intake was no different within or between groups during SR and remobilisation. During SR the mean daily energy intake was 1456 ± 77 Kcal/day in the

younger group and 1456 ± 78 Kcal/day (p>0.999). During remobilisation the daily calorific intake was 1349 ± 94 Kcal/day in the younger group vs 1445 ± 73 Kcal/day in the older group (p=0.646). There were no differences in macronutrient intake within or between groups throughout the study (Table 3.2).

3.4.2.3 Absolute whole body, appendicular and abdominal fat masses and fat free masses

Absolute whole body, abdominal and appendicular fat and FFM are displayed in Table 3.3 (See Table 3.1 for baseline measures). There was no difference within age group over time or between age groups following 14 days of SR or 14 days of remobilisation.

3.4.2.4 Fasted state whole blood glucose concentration and responses to an oral glucose challenge

Mean fasted state blood glucose concentration for each age group at baseline, two days and 14 days SR and 14 days of remobilisation are shown in Figure 3.6. As outlined above there was a trend for fasted blood glucose to be different between age groups at baseline (Table 3.1), but values were

	14-day step reduction		Remobilisation	
	Younger	Older	Younger	Older
Whole body fat mass (kg)	21.1 ± 2.7	25.1 ± 2.6	21.6 ± 2.6	25.3 ± 3.3
Whole body fat free mass (kg)	45.8 ± 3.0	43.7 ± 3.0	45.5 ± 3.2	43.2 ± 3.2
Appendicular fat mass (kg)	11.1 ± 1.8	10.3 ± 1.7	11.0 ± 1.5	10.7 ± 1.7
Appendicular fat free mass (kg)	21.2 ± 1.5	18.8 ± 1.5	20.9 ± 1.8	19.2 ± 1.7
Abdominal fat mass (kg)	1.6 ± 0.2	2.4 ± 0.4	1.6 ± 0.2	2.5 ± 0.4

Table 3.3 DEXA derived body composition measures following 14-day SR and following 14 days of remobilisation. Values are means ± SEM.

very much in the 'healthy' range in both groups (4.0 mmol/l ± 0.1 vs 4.3 mmol/l ± 0.0). There was a significant increase in fasted state blood glucose concentration from baseline in the young volunteers following two days of SR to 4.3 mmol/l ± 0.1 (p=0.01), which returned to the baseline concentration after 14 days of SR (4.1 mmol/l ± 0.1, p=0.919) and remained no different to baseline after 14 days remobilisation (4.1mmol/l ± 0.1, p=0.858). In the older volunteers, fasted state blood glucose concentration was no different from baseline after two days (4.4mmol/l ± 0.1, p=0.839) or 14

days (4.4mmol/l ± 0.2, p=0.994) of SR, and following 14 days remobilisation (4.1mmol/l ± 0.1, p=0.067). However, fasting blood glucose concentration was significantly greater after two days (4.4mmol/l ± 0.1, p=0.002) and 14 days (4.4mmol/l ± 0.2, p=0.01) of SR when compared to the concentration after remobilisation (4.1mmol/l ± 0.1), but these differences were small such to be of minor physiological significance. Furthermore, there was no difference in mean fasting blood glucose concentration found between age groups at any timepoint through the study.

There was a trend for the AUC of blood glucose concentration during the OGTT to be different between groups at baseline (Table 3.1, p=0.085). Blood glucose AUC in the young group was no different to that recorded at baseline after two days (316.3 ± 39.4 mmol/l/155min vs 325.2 ± 35.6 mmol/l/155min, p=0.998) and 14 days SR (321.6 ± 21.8 mmol/l/155min, p=0.999) and following 14 days remobilisation (314.5 ± 32.1 mmol/l/155min, p=0.999) (Figure 3.7 a). In the older group, the AUC for blood glucose was increased from baseline after two days of SR (418.8 ± 40.3 mmol/l/155min vs 518.7 ± 40.1 mmol/l/155min, p=0.0386; Figure 3.7 a) and then returned to baseline AUC

after 14 days of SR (432.8 ± 34.0 mmol.l.155min, p=0.9648), and remained no different from baseline after 14 days remobilisation (459.5 ± 26.2 mmol/l/155min, p=0.4708). Blood glucose AUC for the OGTT was significantly greater in the older group compared with the younger group following 2 days SR (325.2 ± 35.6 mmol/l/155m vs 518.7 ± 40.1 mmol/l/155m, p=0.0083) and following remobilisation (314.5 ± 32.1 mmol/l/155m vs 459.5 ± 26.2 mmol/l/155m, p=0.0107). These differences in blood glucose AUC between age groups is reflected by the blood glucose concentration vs time curves after two days SR (Figure 3.7 b) and following two weeks remobilisation (Figure 3.7 d).

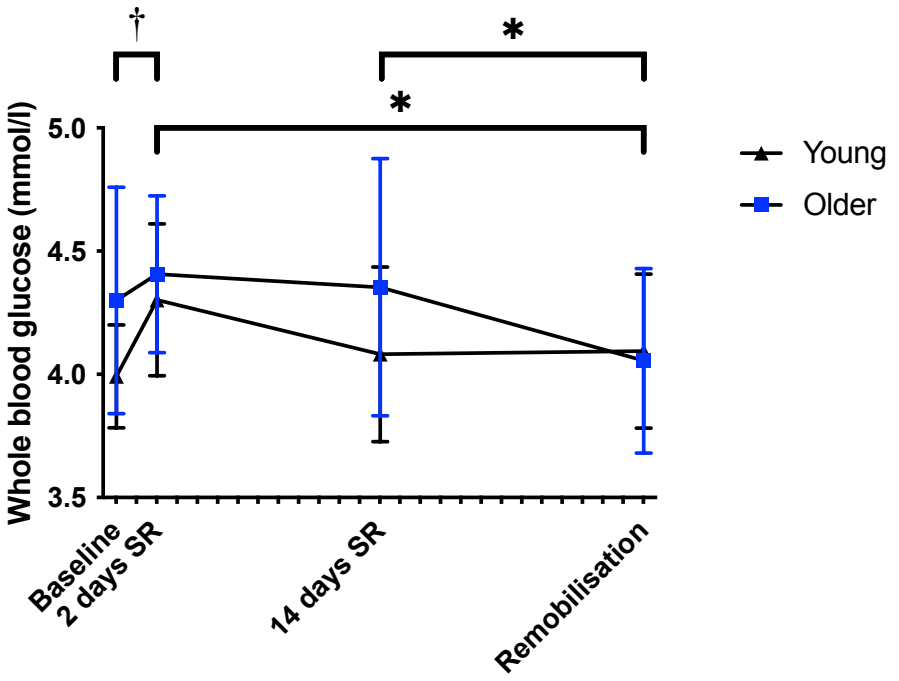
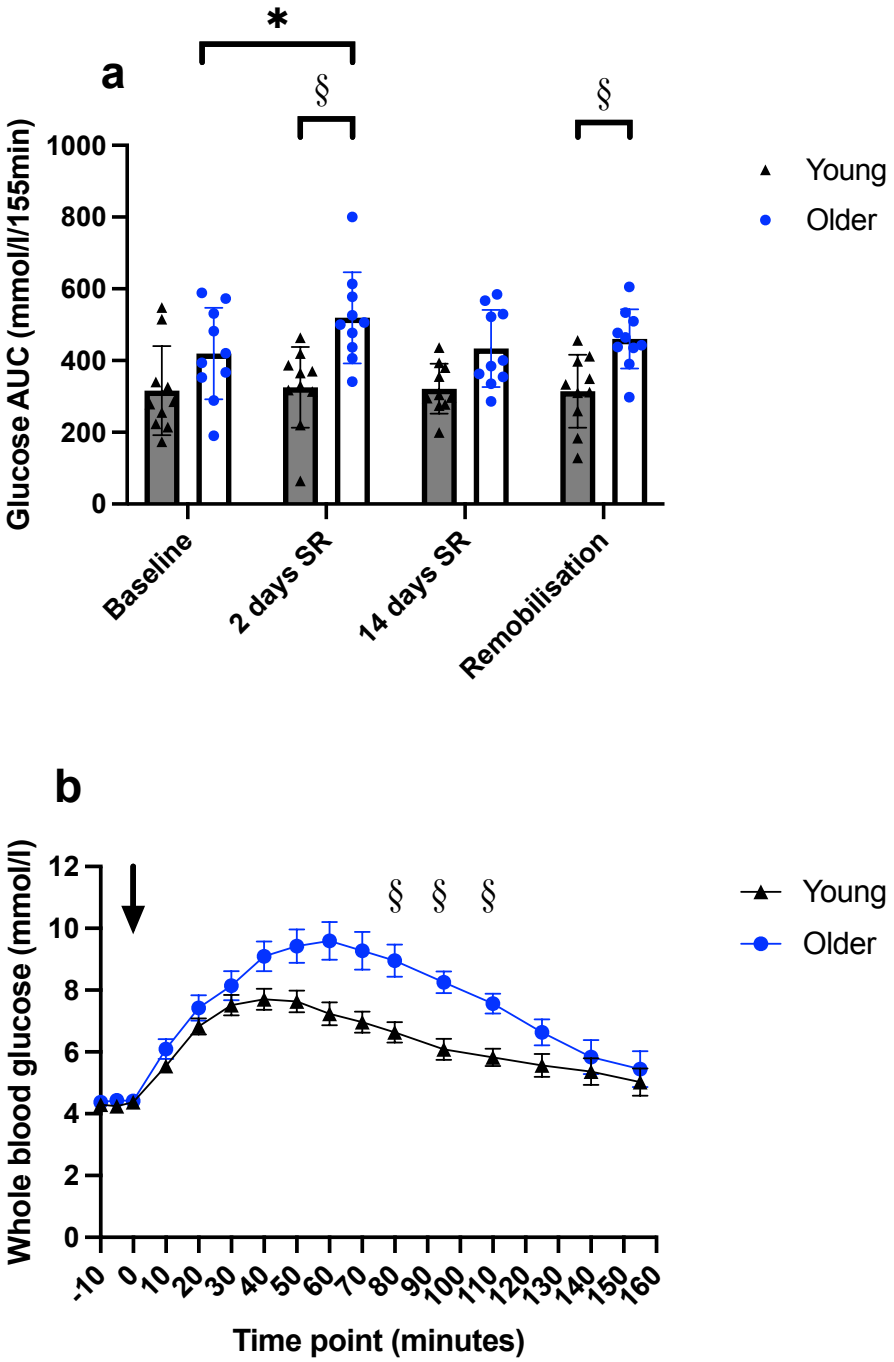


Figure 3.6 Fasting whole blood glucose concentration (mmol/l) in younger and older groups at baseline (Baseline), following two days and 14 days of step count reduction (SR) and after 14 days of remobilisation (Remobilisation). Values are means ± SEM. † indicates a significant difference ($p<0.05$) between timepoints in the younger group. * Indicates a significant difference ($p<0.05$) between timepoints in the older group.


a
*
§
§
Glucose AUC (mmol/l/155min)
1000
800
600
400
200
0
Baseline
2 days SR
14 days SR
Remobilisation
Young
Older
b
§ § §
Whole blood glucose (mmol/l)
12
10
8
6
4
2
0
-10
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
Time point (minutes)
Young
Older

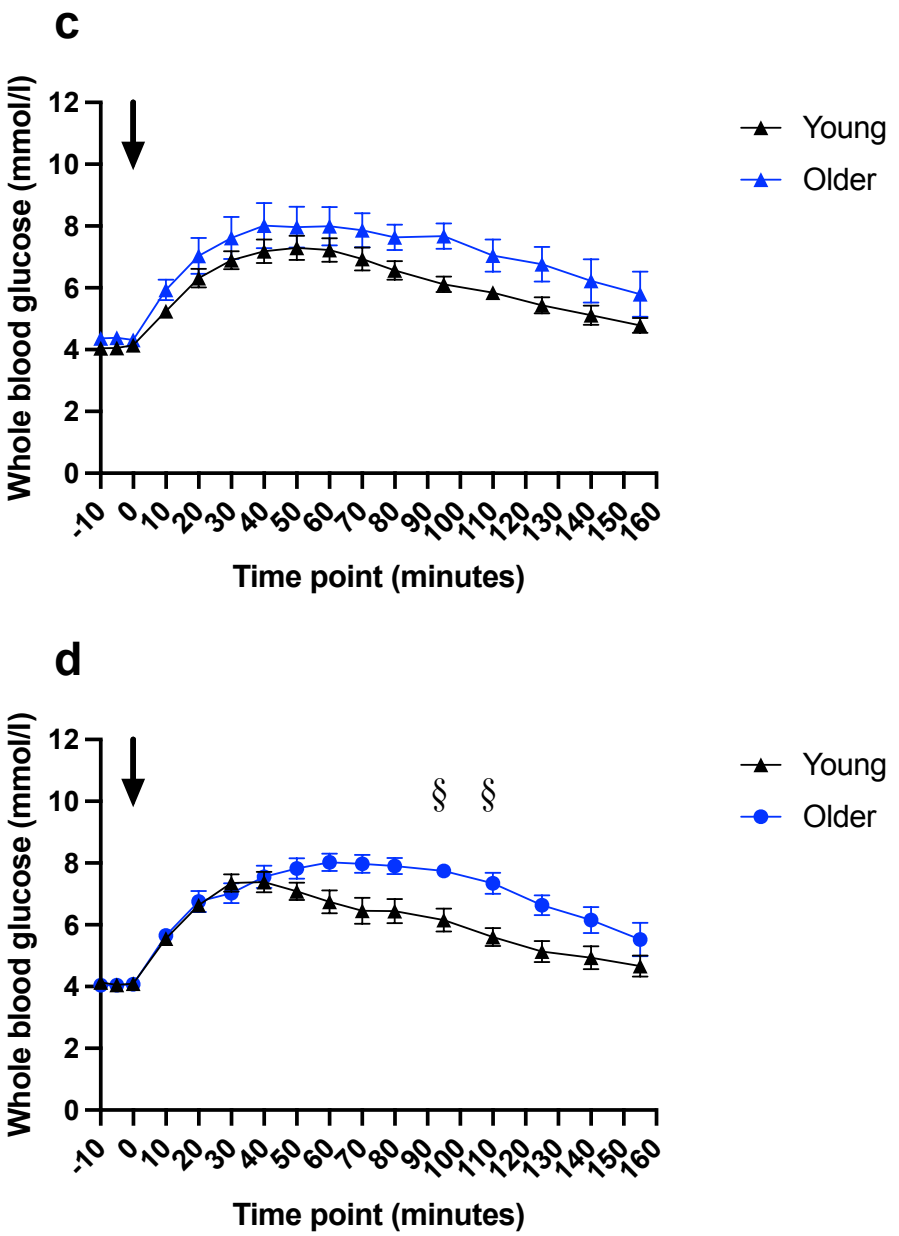


Figure 3.7 a Blood glucose area under the curve during an OGTT (mmol/l/155min) at baseline, after two and 14 days step count reduction (SR) and after 14 days remobilisation (Remobilisation). Fig 3.7 **b, c** and **d** depict whole blood glucose concentration (mmol/l) at individual time points during the OGTT performed two and 14 days SR, and after 14 days remobilisation, respectively. Values are means ± SEM and individual values in **a**. § indicates between group difference ($p<0.05$), * indicates within group differences ($P<0.05$). The arrow indicates the point of glucose consumption.

3.4.2.5 Fasted state serum insulin concentration and responses to an oral glucose challenge

Fasted state serum insulin concentration was no different at baseline between age groups (Table 3.1) and was no different from baseline in the young and older volunteers after two and 14 days of SR and 14 days of remobilisation (Figure 3.8). Accordingly, there were no difference in fasted state serum insulin concentration between age groups at any of these study time-points.

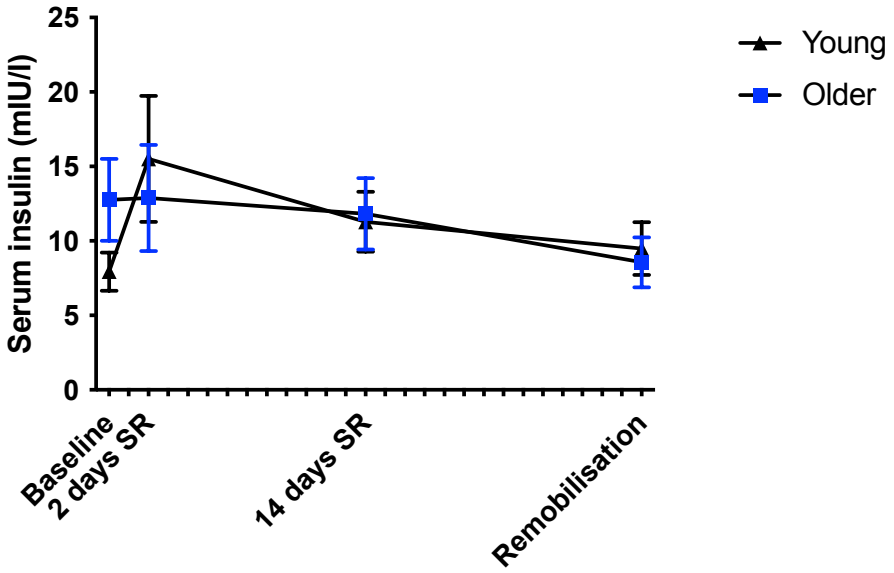


Figure 3.8 Fasting serum insulin concentration (mIU/l) in younger and older age groups at baseline (Baseline), following two and 14 days of step count reduction (SR) and following 14 days of remobilisation (Remobilisation). Values are means ± SEM.

Serum insulin concentration AUC during the OGTT was no different from baseline in the young group after two days of SR (5982 ± 847 mIU/l/155min vs 7077 ± 1296 mIU/l/155min, p=0.498), but was greater than baseline after 14 days of SR (9115 ± 1491 mIU/l/155min, p=0.042). Following remobilisation, serum insulin concentration AUC during the OGTT had returned to that observed at baseline (5918 ± 578 mIU/l/155min, p=0.999) (Figure 3.9). In the older group, two days of SR induced an increase in the AUC for serum insulin from baseline (8431 ±1673 mIU/l/155min vs 12010 ± 1712 mIU/l/155min, p=0.005), but not after 14 days SR (9699 ± 2287 mIU/l/155min) or 14 days remobilisation (8103 ± 1000 mIU/l/155min). Serum insulin concentrations during the OGTT were no different between the younger and the older group at any sampling point during each study visit (Figure 3.10).

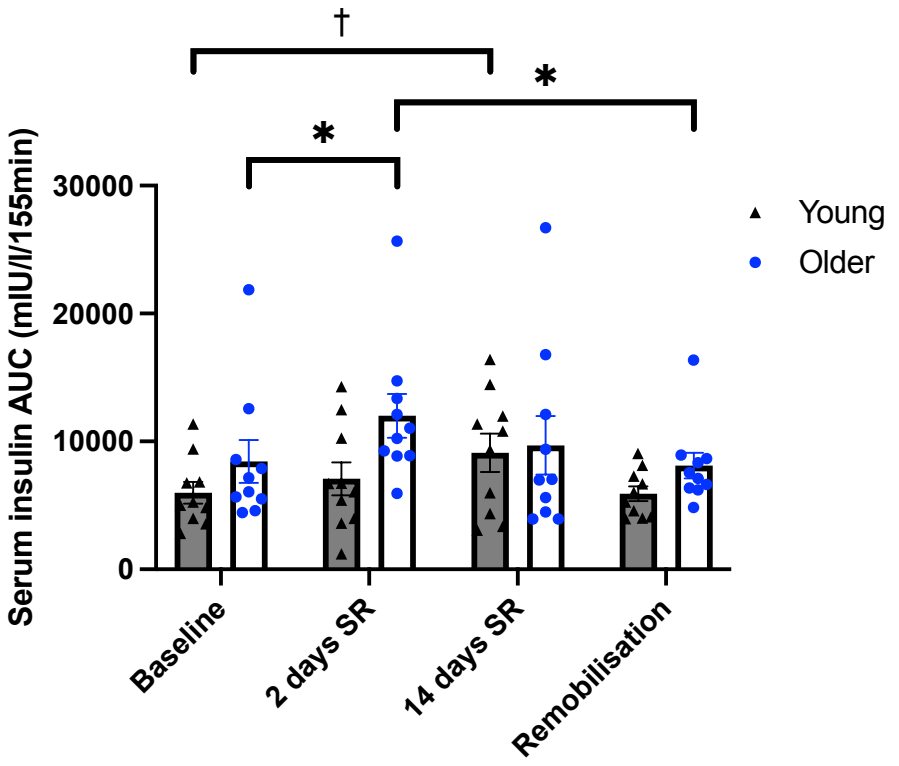
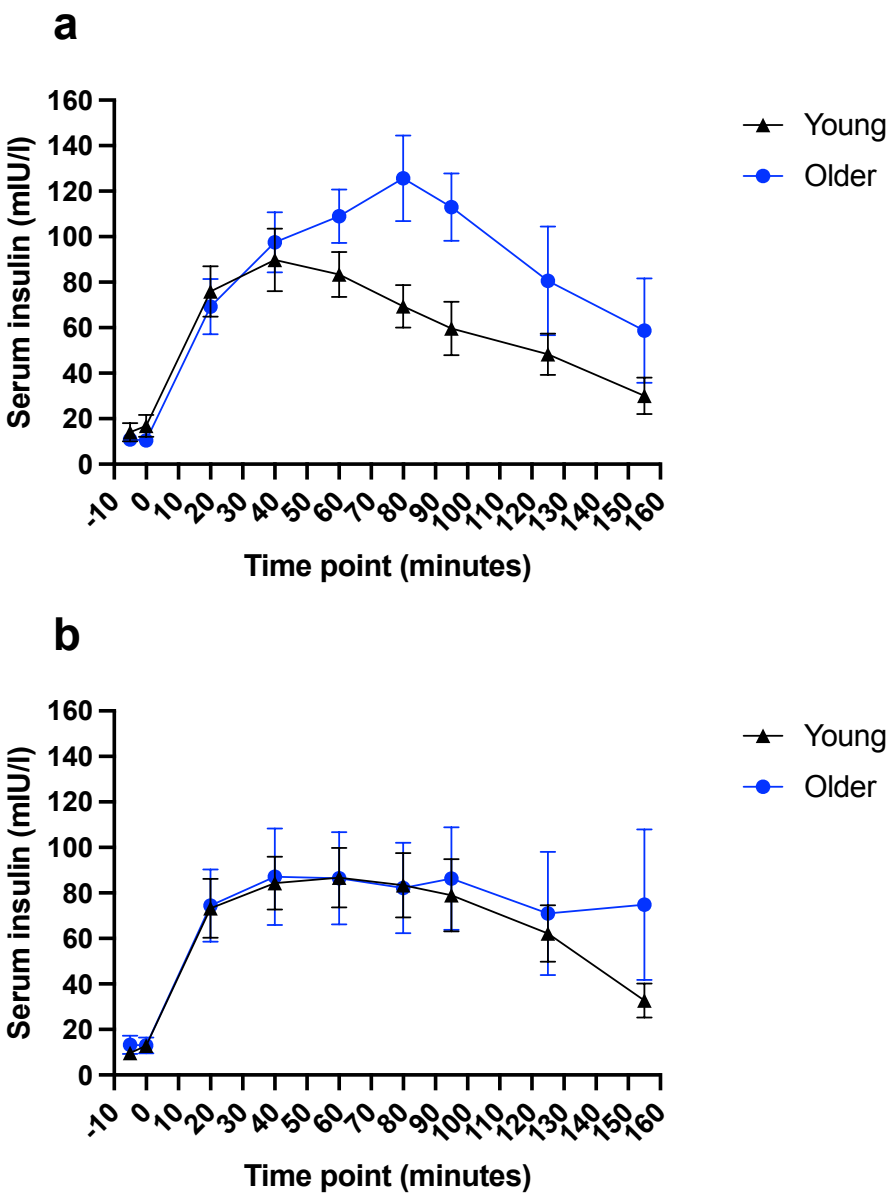


Figure 3.9 Area under the curve for serum insulin concentration during the OGTT (mIU/l/155min) at baseline, after 2 and 14 days of step count reduction (SR) and after 14 days of remobilisation (Remobilisation). Values are mean ± SEM and individual values. † Indicates a significant difference ($p<0.05$) between timepoints in the younger group. * Indicates a significant difference ($p<0.05$) between timepoints in the older group.


a
Serum insulin (mIU/l)
160
140
120
100
80
60
40
20
0
-10
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
Time point (minutes)
Young
Older
b
Serum insulin (mIU/l)
Time point (minutes)
Young
Older

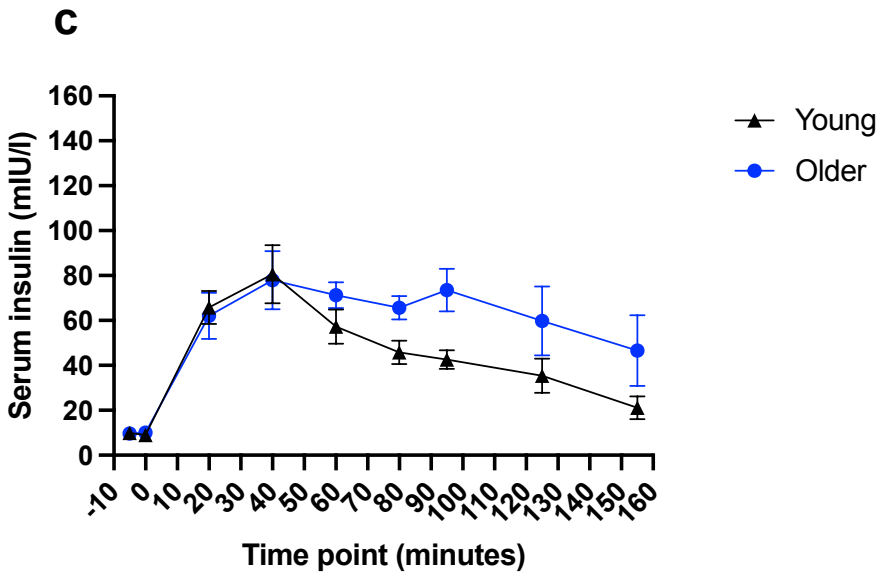


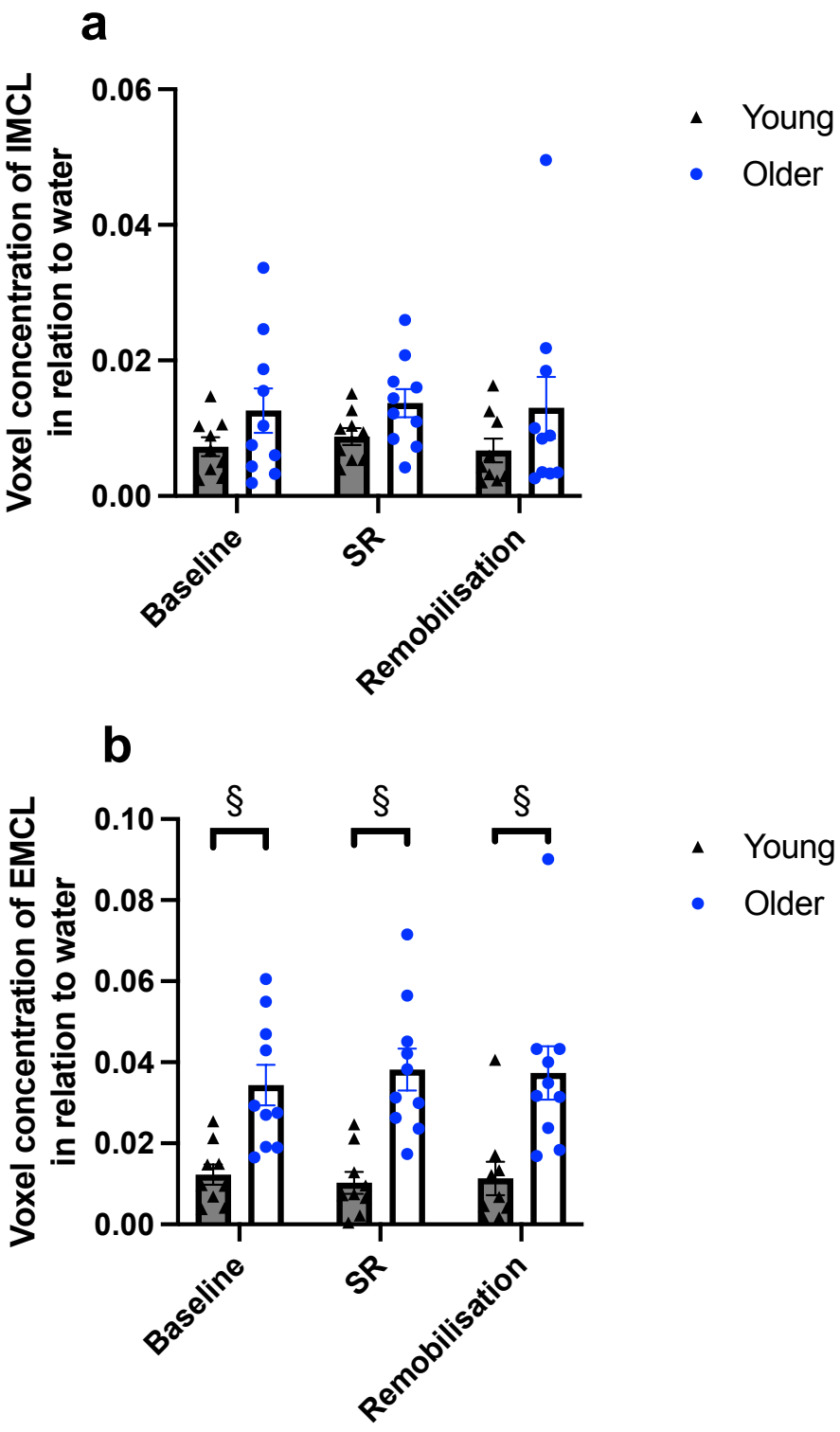
Figure 3.10 a, b and c Serum insulin concentration (mIU/l) at individual time points during the OGTT performed at after two (**a)** and 14 days (**b**) step count reduction and 14 days remobilisation (**c**). Values are mean ± SEM.

3.4.2.6 Muscle IMCL, EMCL and IMCL/EMCL ratio

IMCL (Figure 3.11a) and EMCL (Figure 3.11b) concentrations (in relation to water) were no different from baseline following 14 days of SR and 14 days remobilisation in the young and older volunteers. Furthermore, there was no difference between age groups in IMCL concentration at any study time point. However, as with the baseline measure, EMCL concentration was significantly greater in the older group compared to the young group at both 14 days of SR (3.433 ±

0.831 vs 0.930 ± 0.272, p=0.001) and 14 days of remobilisation (3.842 ± 1.415 vs 0.835 ± 0.345, p=0.013).

The IMCL/EMCL ratio remained unchanged from baseline within the older group following SR (0.382 ± 0.080 vs 0.408 ± 0.047, p=0.961) and remobilisation (0.337 ± 0.059, p=0.876). There was however a significant increase from baseline in the young group after 14 days of SR (0.604 ± 0.105 vs 0.991 ± 0.151, p=0.001). This increase from baseline persisted following remobilisation (0.923 ± 0.173, p=0.007) and was significantly higher in the young group at both SR (0.991 ± 0.151 vs 0.408 ± 0.047, p=0.001) and remobilisation (0.923 ± 0.173 vs 0.337 ± 0.059, p=0.001) when compared with the older group (Figure 3.11c).


a
Voxel concentration of IMCL in relation to water
0.06
0.04
0.02
0.00
Baseline
SR
Remobilisation
Young
Older
b
Voxel concentration of EMCL in relation to water
§
§
§
0.10
0.08
0.06
0.04
0.02
0.00
Baseline
SR
Remobilisation
Young
Older

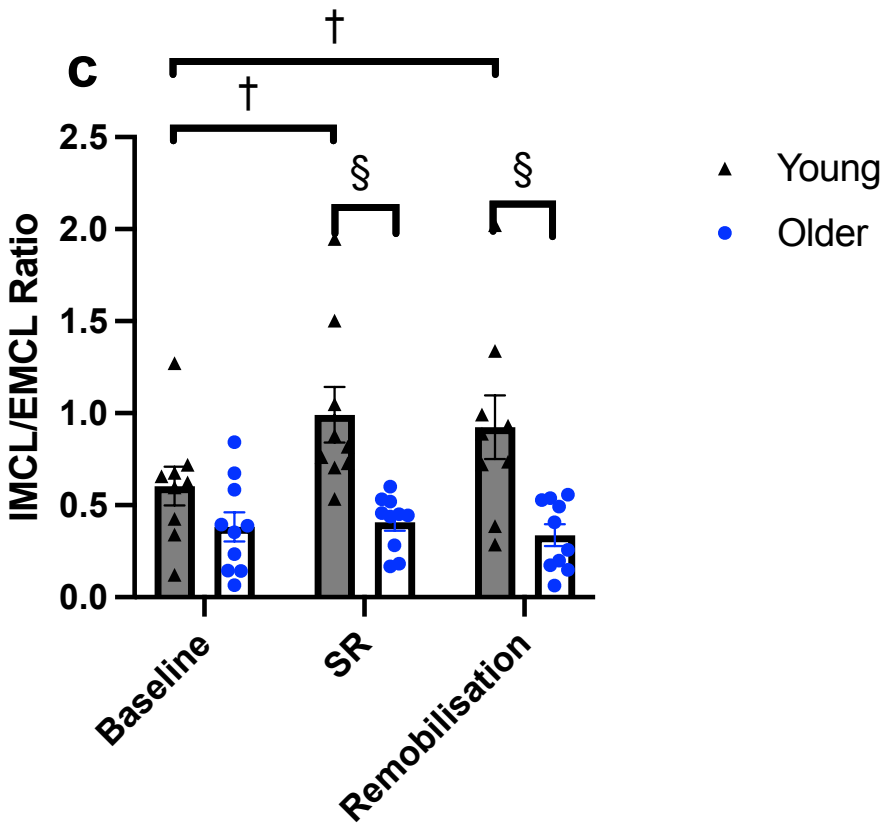


Figure 3.11 a, b and c Voxel concentration of IMCL (**a**), EMCL (**b**) in relation to water and the IMCL/EMCL ratio (**c**) at baseline, after 14 days of step count reduction (SR) and after 14 days remobilisation (Remobilisation). Values are mean ± SEM and individual values. § indicates between group difference ($p<0.05$). † Indicates a significant difference ($p<0.05$) between timepoints in the younger group.

3.4.2.7 Correlation between skeletal muscle IMCL content and composite indices of insulin sensitivity

There was no correlation identified between the voxel concentration of IMCL in relation to water and either the Matsuda index (Figure 3.12a) or the QUICKI (Figure 3.12b)

when all participants, at all timepoints, were grouped together.

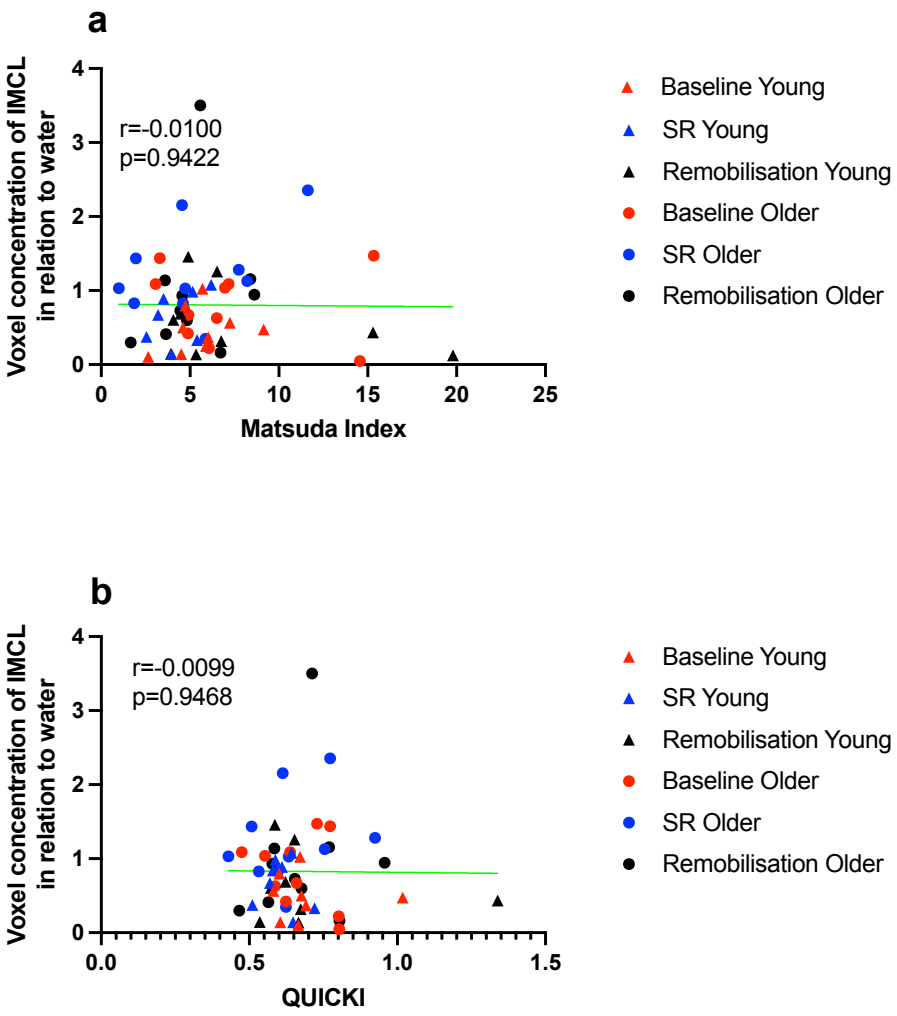


Figure 3.12 a and b Association between the voxel concentration of IMCL in relation to water and (**a**) Matsuda and index and (**b**) QUICKI at all study timepoints in both groups of volunteers combined. Green line reflects Pearson linear correlation, along with the correlation coefficient and statistical p value reflecting the strength of the linear association between the two variables.

3.5 Discussion

The major findings of this study are firstly, in this study of healthy active volunteers, there were no age-related differences at baseline in habitual physical activity levels (step count and AEE), body composition or blood glucose regulation, except for abdominal fat and EMCL content. However, both groups were clearly healthy with good glucoregulation. Secondly, both age groups were able to maintain good glucoregulation following a major SR to less than 1500 steps per day for 14 days, but this was accompanied by a significant increase in the AUC for serum insulin concentration during an OGTT in young participants. Thirdly, this change in the serum insulin response was mirrored by a significant increase from baseline in the IMCL/EMCL ratio, in the absence of an accompanying change in whole body or regional body composition determined by DEXA or any change in the vastus lateralis IMCL concentration. Finally, the older participants displayed an increase in both insulin and glucose AUC after 48 hours of SR. However, following 14 days of SR, both measures had returned to baseline, unlike in the young group. There was no associated change in the IMCL/EMCL ratio at any point in the older group, but their EMCL content was consistently approximately 3-fold greater than young volunteers

throughout the study (whilst IMCL content was not significantly different from the young volunteers).

Step count reduction is a relatively novel technique that has proven to be effective in the study of inactivity (Alibegovic et al., 2009, Krogh-Madsen et al., 2010, Knudsen et al., 2012, Bowden Davies et al., 2018, Shad et al., 2019). The aim of the model is to allow investigation into the metabolic and physiological effects of reduced ambulatory activity, without the profound effects of total body or limb immobilisation. Our data shows that the SR was adhered to by both groups. The induced inactivity is hoped to be more representative of the chronic reduction in daily ambulation that is associated with age (Milanovic et al., 2013) and allow the comparison of these changes between younger and older groups. Here we have presented and compared two groups of participants within defined age ranges that were well balanced at baseline with regards body composition, baseline activity levels and glucose handling. The 'aging phenotype' describes the physiological and metabolic changes that are expected as we age. The mechanistic basis behind these observations is less well described as they simply represent the findings of cross-sectional studies comparing young and old.

Despite multiple previous SR studies validating the technique as an effective way to control physical activity, alongside this present study demonstrating alterations in glucoregulation occurring in association with a reduced step count, it must be considered that step count alone as a measure of physical activity is not absolute. Step count is not affected by intensity of physical activity, nor does it consider time spent standing but not moving, which has significant physiological benefits. In addition to this, it would be quite possible for study participants to avoid overstepping by not always wearing the pedometer, thus ameliorating the effect SR has on end point measures. This is however an unavoidable potential pitfall faced during healthy volunteer research.

Ageing is associated with declines in physical activity and it has been reported that 39% of adults are not meeting the recommended activity level of 150 minutes per week, with 54% of those over the age of 75 undertaking less than 30 minutes of physical activity per week (2017). However, in this current study the baseline measures of both PAL and mean daily step-count were no different between groups (Table 3.1), with both groups being healthy and physically active. It is plausible to consider that the level of physical activity in

individuals that are actively seeking to partake in research studies may well not be representative of that of the population as a whole. Nonetheless, prior studies looking at young and old have not compared matched groups and therefore it is difficult to draw conclusions based on age alone. This study has shown that when matched at baseline, ageing is not associated with poor metabolic heath when those being studied are physically active and healthy.

Aging is associated with a decline in whole body glucose disposal in response to insulin (Fink et al., 1983). There is ongoing debate as to whether this is an effect of age in isolation or whether other age-related changes underpin the pathological metabolic demise. One of the proposed mechanisms is the increase in central and peripheral adiposity (Kohrt et al., 1993). Despite there being no difference in our data at baseline in either body weight or whole-body fat mass between young and older, there was a trend towards a higher BMI ($p=0.072$) and there was a significantly higher mass of DEXA derived abdominal fat in the older group ($p=0.028$) (Table 1). It is well documented that accumulation of visceral fat is strongly correlated with insulin resistance and is considered central in the pathophysiological processes of type

2 diabetes mellitus (Hardy et al., 2012). This form of diabetes is characterised by skeletal muscle and adipose insulin resistance with resultant hyperinsulinaemia that is insufficient to maintain blood glucose concentration below defined thresholds. However, in our data at baseline, despite the significantly higher mass of abdominal fat and EMCL content, there was no difference in either fasted state insulin or insulin AUC during the OGTT between young and older groups (Table 3.1). There was however a trend for both fasting glucose and AUC during OGTT to be higher in the older group, although the relevance of this is not clear given that the absolute values were physiologically very small and the compensatory response to insulin resistance is hyperinsulinaemia in order to maintain euglycaemia (Reaven, 2005). Indeed, both age groups were metabolically healthy based on fasted state and OGTT responses; pointing to 'age related' decline being lifestyle driven rather than age per se.

Along with abdominal adiposity, accumulation of IMCL has been mechanistically linked to skeletal muscle insulin resistance (the lipid overspill hypothesis). IMCL are used as fuel by the muscle. However, excessive accumulation within skeletal muscle has been implicated as a driver of insulin

resistance (Jacob et al., 1999, Perseghin et al., 1999). In this study, of well-matched healthy and active volunteers, baseline measures of IMCL were no different between groups (Table 3.1), which is in contrast to the findings of others (Tsintzas et al., 2017, Crane et al., 2010). However, given the similarity in habitual physical activity, energy intake, body composition and glucoregulation between groups in the present study the similarity in IMCL is perhaps not at all surprising. Nevertheless, it is acknowledged that the notion of simply measuring the total concentration of IMCL is perhaps too simplistic, with research focused on different lipid subtypes and perilipins to try and explain the association between insulin resistance and skeletal muscle lipid droplets (Bosma et al., 2012, Harber et al., 2010).

EMCL was significantly greater in the older vs young group at baseline (Table 3.1). EMCL exist as adipocytes situated in the extracellular space between muscle fibres, rather than as lipid droplets within myocytes as in the case of IMCL (Larson-Meyer et al., 2006). IMCL is distributed evenly within skeletal muscle, whereas EMCL is not, it is found around and between skeletal muscles in concentrated, distinct structures such as the subcutaneous fat or fascial layers (Boesch et al., 2006). The

relationship between IMCL and EMCL is yet to be resolved and it appears that EMCL is a relatively inert entity representing fat storage in a peripheral location (Rico-Sanz et al., 1998, Haus et al., 2011). The quantification of EMCL is not so straight forward as that of IMCL; when placing the voxel during the MRS scan, care is taken to avoid excess marbling within the muscle. Due to the even distribution of IMCL, small variations when placing the voxel would be insignificant, however the effect of EMCL quantification, due to its distribution pattern, could be significant (Boesch et al., 2006). Probably due to these technical difficulties, few studies have focused on the function of and mechanistic role of EMCL and its accumulation, but it is thought to accumulate with age in a manner that is independent to IMCL (Yoshiko et al., 2022), which the findings of the present study corroborate. DEXA derived appendicular fat mass was no different between groups and thus the contribution of EMCL to appendicular fat mass is likely negligible.

The ratio between IMCL and EMCL concentrations is often included in literature that is focussing on the changes between IMCL and EMCL concentration following an intervention. It may emphasise changes in the lipid depots that in isolation

are not significant but represent changes in the overall storage pattern. As discussed, the relationship between the two entities appears complex and is unresolved. We did not identify a difference in the IMCL/EMCL ratio at baseline between the groups, which may be expected given that the groups were very well matched and that it is generally thought that both storage locations increase with aging.

To further assess the purported relationship between IMCL and insulin resistance, total IMCL concentration was correlated against well validated composite measures of insulin sensitivity; the Matsuda index and QUICKI. However, we did not observe any association between MRS derived IMCL concentration and either the Matsuda index or QUICKI.

Participants were instructed to reduce their step-count to less than 1500 steps per day for the 14-day period of SR. This was achieved in both groups with the young group averaging 1053 daily steps and the older group 1095 daily steps. The participants were advised to continue with their normal daily nutritional intake and diet diaries were taken during this period. The significant reduction in physical activity resulted in a dramatic change in the participants AEE in the order of

>90%, which translated into a positive energy balance and thus oversupply of dietary energy intake. Excess dietary lipid results in the increase in number and size of adipocytes (Jo et al., 2009), the natural storage site for lipid. Ongoing oversupply results in ectopic storage in sites including the heart, the liver and skeletal muscle. Furthermore, it is becoming increasingly apparent that energy oversupply is more a driver of increased IMCL than inactivity per se, with Shur (Shur et al., 2022) demonstrating that immobilisation combined with maintained energy balance results in glucose dysregulation without IMCL accumulation. However, despite significant reductions in physical activity and no energy balance maintenance during this period, we did not see any increase in IMCL.

Participants returned after two days of SR for an early OGTT. There were a number of interesting observations; fasted state blood glucose concentration in the younger group was greater after two days SR than at baseline (Figure 3.6), but this was physiologically small and accompanied by no difference in fasting serum insulin concentration in either age group. This demonstrates little difference in fasting state responses with two days SR in both age groups. However, OGTT AUC

measurements after 48 hours of SR identified significant increases in both insulin (Figure 3.9) and glucose (Figure 3.7 a) responses in the older group, with no difference found in the young. Despite the short time frame of reduced activity, both groups had a degree of measurable metabolic derangement. No other study has previously utilised step count restriction and examined the effects on glucose disposal following only 48 hours in both young and older groups. Unfortunately, neither DEXA or MRS was performed at this timepoint and therefore drawing conclusions as to the underlying mechanistic basis are not possible. However, it is reasonable to assume that following 48 hours of SR it is unlikely that measurable changes in body composition (either fat or lean mass) would have occurred and thus be instrumental in the above findings. As in the study by Burns (Burns et al., 2021), it could be hypothesised that muscle uptake of glucose is closely matched to utilisation and thus inactivity results in substrate oversupply and reduced muscle glucose uptake.

It might be expected that following 14 days of SR, any changes in glucose disposal found following two days of SR may remain, or indeed be more prominent. As previously

discussed, prior SR studies (Olsen et al., 2008, Krogh-Madsen et al., 2010, Bowden Davies et al., 2018, Breen et al., 2013a) have found alterations in glucose handing in both young and old participants following 14 days of SR. In accordance with the published literature, the young participants had a significant increase in the AUC for insulin following 14 days of SR (Figure 3.9). This response maintained normoglycaemia and measures of fasting glucose and insulin reverted to baseline. However, our findings in the older group were not altogether in keeping with prior studies with both blood glucose (Figure 3.7) and serum insulin (Figure 3.9) OGTT AUC responses recovering back to baseline after being significantly raised following two days of SR. The overall picture is that of increased insulin resistance (i.e. a greater insulinaemic response) in the younger but not the older participants following SR in this well-matched group of volunteers, which contrasts with prior SR studies.

We did not find any change from baseline in either group in whole body fat or fat free mass following either two or 14 day SR (Table 3.3). Our findings do not support those of other studies where 14 days of reduced step-count has induced whole body or regional changes in fat (Olsen et al., 2008,

Krogh-Madsen et al., 2010) and lean mass (Krogh and Lindhard, 1920, Breen et al., 2013a) in both young and older groups, with these changes being linked to impairments in glucose disposal. Our findings demonstrate that detectable changes in conventional measures of body composition are not central to inactivity induced alterations in whole-body glucoregulation over a two-week period of reduced activity. The use of DEXA results in reliable, reproducible data that are less user dependant than manually analysed MRI measures and given the large p values associated with these outcomes; it is unlikely that the inability to detect a difference is related to insufficient sample size.

Along with whole body composition measures, IMCL was also no different from baseline following 14 days of SR in either group (Figure 3.11 a). These results are congruent with those of Bowden-Davis study (Bowden Davies et al., 2018) and also support the current view that IMCL accumulation alone is unlikely to be responsible for short term, inactivity induced insulin resistance (Dirks et al., 2016). EMCL was significantly greater at baseline in the older group and remained so after SR (Figure 3.11 b). There was however no effect of SR on the concentration of EMCL in either group, which is in keeping with

the lack of change of DEXA measured whole body fat masses. The IMCL/EMCL ratio has been considered to be a marker of lipid redistribution within the muscle, however the significance of this, along with the concentration of EMCL, is yet to be established. We identified a significant increase in the young in the IMCL/EMCL ratio following 14 days of SR (Figure 3.11 c), corresponding with the increase in the OGTT AUC response for serum insulin. No other study has examined the changes in EMCL or the IMCL/EMCL ratio following SR and thus the significance of our findings are difficult to interpret. However, it is clear that the association of IMCL, EMCL and the IMCL/EMCL ratio with inactivity, glucose homeostasis and body composition remain poorly understood and further work needs to be done to understand how they relate, if at all.

Remobilisation back to habitual physical activity levels following SR returned almost all the study end-point measurements back to baseline values in both groups. Average daily step counts during remobilisation were no different to baseline in both groups, confirming both groups to be active and well balanced. Diet diary entries showed that dietary energy intake during remobilisation was no different from SR and thus could be considered as habitual dietary

intake. Accordingly, given that SR did not induce any detectable changes in DEXA whole body or regional composition measures, values taken following 14 days of habitual activity were no different to baseline (Table 3.3).

There were a number of between group differences identified following remobilisation. Firstly, the blood glucose AUC during the OGTT was significantly higher in the older group (Figure 3.7 a). This is likely the result of a non-significant increase in the AUC in the older group along with a reduction in the variation, given that the young group maintained normoglycaemia throughout the study. However, it must be maintained that the difference between the two is small and both groups demonstrated a healthy blood glucose response following remobilisation. Secondly, as with at baseline and following 14 days of SR, the EMCL concentration was significantly higher in the older group compared to the young group (Figure 3.11 b). This finding likely reinforces the belief that EMCL storage is not affected by short-term inactivity and has little role in activity induced changes in glucose disposal in this time period. Given that EMCL is stored in adipocytes like central adiposity, it would be unlikely that any intervention that does not result in whole-body changes in adiposity would

induce peripheral changes. Finally, the IMCL/EMCL ratio remained significantly elevated from baseline after remobilisation in the young group (Figure 3.11 c), whereas in the older group there was no change throughout the study. Given that the AUC for insulin in the younger group returned to baseline after remobilisation, this finding further confuses what contribution the ratio between IMCL and EMCL play in glucose homeostasis and physical inactivity.

As discussed, there are several findings in this study that were not in keeping with prior studies utilising similar techniques, interventions and outcome measures. There was no *a priori* power calculation performed for either the primary or secondary outcome measures and as the study was part of a larger study, sample sizes were generated based on changes in MPS following SR (See General Methods Section 2.5). However, it must be noted that most of the prior studies investigating changes in glucose homeostasis and body composition have used similar sample sizes. In addition, in those outcomes where no difference was found, such as the body composition measures and the AUC for insulin and glucose in the older group after 14 days of SR, the large p value makes it likely that there is not a difference. It must

however be considered a weakness of the study that no *a priori* power calculation was performed.

3.6 Conclusion

Aging in isolation has for a long time been attributed to the observed differences in muscle mass, body composition and glucose regulation identified between younger and older groups through cross sectional studies. However, many of these studies have failed to accurately define habitual levels of physical activity and thus match groups at baseline. The data presented here has shown through the use of well-matched healthy, physical active young and older volunteers, that many of the presumed age-related changes could well be representative of differences in habitual activity level rather than aging per se. Despite changes in glucose regulation following SR, both younger and older groups in this study demonstrated a return to baseline after remobilisation which could suggest that living a physically active life infers a degree of protection from short periods of reduced physical activity. Alternatively, it could simply demonstrate that the changes induced by 14 days of SR are clinically insignificant and longer periods of inactivity of such a benign nature are required to demonstrate significant changes that are not rapidly

reversible. The definitive link between IMCL, EMCL and glucose homeostasis continues to be elusive.

Chapter 4

The impact of step count reduction and remobilisation on muscle protein turnover, leg muscle volume and mid-thigh cross-sectional area in healthy, physically active young and older participants

4.1 Introduction

Muscle mass is maintained through the net balance of MPS and MPB. These processes form a dynamic equilibrium that is influenced by anabolic drivers, such as physical activity, nutrition or endogenous hormones (Burd et al., 2009, Kumar et al., 2009). Sustained changes in this balance induces muscle hypertrophy or atrophy. In healthy humans, the current literature suggests that muscle mass change; both positive and negative, is predominantly driven by alterations in the rate of MPS, rather than the rate of MPB (Biolo et al., 1995, Brook et al., 2022).

Muscle contraction induces an increase in post-exercise MPS, and it is well established that resistance exercise training (RET) (Loughna et al., 1986) and aerobic training (Tanner et al., 2015), such as walking, cycling or running, are beneficial for the maintenance of muscle volume and muscle health. Research into the form, duration and intensity of exercise required to induce optimal increases in MPS and resultant hypertrophy, is well founded. Likewise the effects of immobilisation and bed rest on MPS is well documented (Kortebein et al., 2007). There is increasing interest in the study of less severe models of reduced physical activity, such

as SR (Tudor-Locke et al., 2013) on muscle protein balance and mass/volume, particularly since sedentarism is associated with sarcopenia and poor health outcomes with age (Shur et al., 2021) and increased rates of all-cause mortality (Paluch et al., 2022).

Prolonged whole body and limb immobilisation is associated with disproportionate losses of lean mass in the antigravity, lower limb muscle groups when compared with the upper limbs (de Boer et al., 2008, Krogh-Madsen et al., 2010). More benign interventions such as SR has also been found to result in loss of lean mass in these muscle groups. Breen (Breen et al., 2013b) found that 14 days of SR in ten healthy older adults with low levels of habitual physical activity (5962 ± 695 steps per day), to less than 1500 daily steps resulted in a reduction of leg FFM of approximately 3.9%, accompanied by a post prandial reduction in MPS in the region of 26%. A further SR study performed by Krogh-Madsen also found a post intervention reduction in DEXA derived leg FFM from 18.6kg to 18.1kg ($P<0.001$) in ten physically active (10,501 ± 808 steps/day), healthy young men following 14 days of SR to an average of 1344 daily steps (Krogh-Madsen et al., 2010).

Despite several studies investigating the effects of short-term SR, there is little data investigating the effect of SR in healthy, physically active older volunteers (>10,000 steps/day) on muscle protein turnover, or the response of returning to habitual physical activity levels following a period of reduced ambulation. Furthermore, it is not known if there is a difference between healthy, physically active young and older volunteers in response to remobilisation and if recovery is complete. It is known however that older people appear to be at greater risk of 'anabolic resistance'; the term that describes the attenuation of MPS to a given dose of anabolic stimuli (Morton et al., 2018), which may act negatively on protein metabolism during remobilisation. A number of studies have identified an age related blunting of the anabolic response to amino acids (Shad et al., 2016, Volpi et al., 2000) and resistance exercise (Kumar et al., 2009). For example, in the study of Breen (Breen et al., 2013a) involving ten healthy older men with low levels of habitual physical activity, a blunting of postprandial MPS of approximately 26% was found following 14 days of reduced daily steps. The existence of anabolic resistance to protein nutrition and/or the response to physical activity in older people suggests the elderly may be at a greater risk of incomplete or delayed recovery from illness

and injury compared to younger volunteers. This is important given ageing is associated with greater prevalence of periods of inactivity that follow injury or illness and negative muscle centric changes (Hartley et al., 2019). Ultimately this puts older people at risk of loss of independence with both social and financial implications. Greater understanding of muscle responses to sustained SR and subsequent recovery is therefore warranted. Importantly, SR research in older people to date has generally not included a young comparator group or involved groups of volunteers matched for habitual physical activity levels.

Deuterium oxide (D_2O) has been developed as an orally administered stable-isotope tracer that can be used to quantify acute and chronic rates of MPS (Brook et al., 2017). This technique involves the collection of saliva and muscle samples and removes the need for volunteers to remain in a laboratory setting for several hours whilst stable isotope infusions take place. D_3-3MH has emerged as a stable isotope tracer technique to quantify whole-body MPB, which can also be administered orally (Sheffield-Moore et al., 2014). Plasma samples taken at specified time points post D_3-3MH ingestion provide a technique for calculating the rate of MPB that could

potentially help in the detection of those at risk of sarcopaenia or other muscle wasting disorders. D_3-3MH has been used to investigate the impact of immobilisation on whole-body MPB (Shur et al., 2024) but as far as I am aware, no prior study has employed D_3-3MH in the context of SR.

4.2 Aims

The aims of this study were two-fold. Firstly, to assess the impact that a 14-day period of SR and subsequent 14-day period of remobilisation had on muscle protein turnover, in the form of rates of quadriceps MPS and whole-body MPB, in healthy, active young and older volunteers displaying similar habitual physical activity levels. Secondly, to identify whether any changes in these metabolic parameters were reflected by changes in thigh muscle volume or mid-thigh cross-sectional area (CSA) determined using MRI.

4.3 Methods

4.3.1 Subjects and sample size

Ten physically active, young (age 18-40y) and ten physically active older (age 60-75y) healthy volunteers took part in the study. Both groups demonstrated healthy glucoregulation and

were matched at baseline with regards body composition and physical activity levels (See Chapter 3 for physical characteristics). Volunteers were recruited locally and consented following a medical screening and a discussion about what the study involved. The study was approved by the University of Nottingham Medical School Ethics Committee in accordance with the Declaration of Helsinki (Ethics Reference No: A10022017).

4.3.2 Study protocol

As part of a larger study, participants attended study visits at the DGHPU at the University of Nottingham Medical School. Participants were required to make six study visits over a period of 32 days, following a seven day run in period where habitual physical activity levels were recorded using an Actiheart accelerometer (See Chapter 3 for details) (Figure 4.1).

The study was grouped into baseline, SR and remobilisation phases. Participants were required to ingest D_2O, as indicated in the study schematic (Figure 4.1), at three time points in order to maintain a 'steady state' of body water distribution throughout the study (as described below). Similarly,

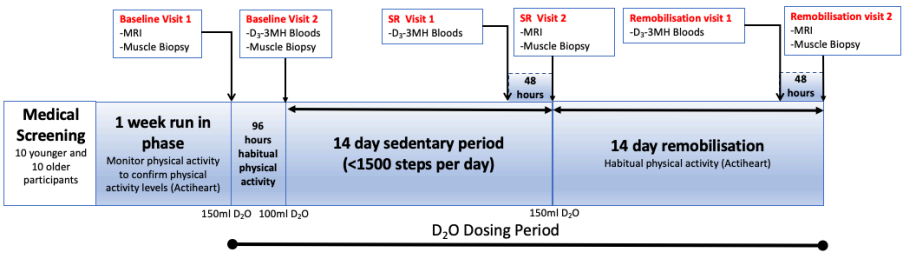


Figure 4.1 – Study Schematic.

participants ingested D_3-3MH on three separate occasions during each study phase (Figure 4.1). Measures of MPS, MPB, thigh muscle volume and mid-thigh muscle CSA were made at baseline prior to SR. Due to logistical study constraints, measures of MPB were repeated after 12 days (rather than 14) of SR and after 12 days of remobilisation, whereas measures of MPS and MRI measures of thigh muscle volume and mid-thigh muscle CSA were made after 14 days of SR and remobilisation (Figure 4.1). On study visits where MRI scanning was required, this was performed at the Sir Peter Mansfield Imaging Centre, University of Nottingham, prior to attending the David Greenfield Human Physiology Unit. Participants attended each study visit following an overnight fast and were fed on completion of the study day.

4.3.3 Muscle Sampling

This study utilised the minimally invasive percutaneous microneedle, or BARD microbiopsy technique, to obtain muscle from the vastus lateralis. This technique is performed using the spring-loaded Bard Magnum reusable core biopsy system (MG1522) with the BARD 12-gauge microneedle (MN1210) as described by Hayot (Hayot et al., 2005). Volunteer consent was obtained prior to biopsy and then a point 10-15cm caudally and 4-5cm laterally to the upper boundary of the patella was marked. The patient was requested to voluntarily contract the quadriceps muscle group to ensure the marked point was within the belly of the vastus lateralis.

The marked biopsy point and the surrounding area was cleaned with betadine solution and 8-10mls of 1% lignocaine was injected into the skin and progressively deeper into the subcutaneous tissues and muscle prior to a 3-4mm skin incision being made. The biopsy needle was passed through the skin incision and into the belly of the muscle with firm, constant pressure being applied to pass the needle through the tough fascial layer. Once confident that the fascia has been traversed and the needle was sitting in the muscle, the spring-

loaded mechanism was activated, and the needle biopsy performed. During each of the biopsies, six passes of the biopsy needle were made to obtain adequate muscle tissue which was snap frozen in liquid nitrogen after dissection from blood and adipose tissue. In between passes of the biopsy needle, firm pressure was applied to the biopsy site by an assistant in an attempt to minimize bleeding and potential haematoma formation.

After the final pass of the microbiopsy needle, pressure was applied for a minimum of two minutes and the skin edges were approximated using steristrips. The leg was then bandaged over the biopsy site and a compression bandage (tubigrip) applied, again to reduce the risk of haematoma formation. Participants were advised to remove the compressive dressings within 24 hours and the steristrips at 5-7 days. An information leaflet regarding care of the biopsy site and contact details if there were any concerns were given prior to the participants leaving the unit.

This biopsy technique was chosen over the commonly used Bergstrom technique due to all samples being obtained in a resting state, which allowed multiple passes to be obtained.

The procedure is also associated with minimal volunteer discomfort (Hayot et al., 2005).

4.3.4 Quantification of muscle protein fractional synthetic rate (FSR)

4.3.4.1 Body water deuterium enrichment and monitoring

Three oral boluses of D_2O (70 atom %; Sigma-Aldrish, Poole, UK) were consumed over the study period to maintain a 'pseudo-steady state' of D_2O within the body water pool. During the first study day a baseline muscle biopsy was taken prior to any D_2O ingestion and then participants were given 150mls of D_2O split into two 75ml doses and taken at least ten minutes apart. The splitting of the dose was to try and reduce the risk of the potential side effect of temporary light headedness and nausea that is reported in the literature (Jones and Leatherdale, 1991). The second bolus of 100mls D_2O was taken on study day five after muscle biopsy and the final 150mls post biopsy in two split doses on day 19.

Deuterium is one of two stable isotopes of hydrogen which contains an extra neutron and is 11% heavier than H_2O. The

human body treats D_2O as normal water, and it is taken up in newly synthesised molecules and thus the rate of MPS can be calculated. Saliva samples were taken to calculate the body water enrichment prior to and post muscle biopsies and at least two hours following ingestion of further doses of D_2O, with body water enrichment values being required to calculate the myofibrillar FSR.

Saliva was collected into a sterile galley pot at least 30 minutes after any oral intake. The sample was pipetted into a sterile plastic tube and cold centrifuged at 16,000 g to separate any debris. A 1ml aliquot was then taken into a further sterile Eppendorf tube and stored at -80 degrees centigrade until analysis.

4.3.4.2 Sample preparation and processing

Previously collected saliva samples were defrosted and 80-90µL of saliva was pipetted into 2ml inverted autosampler vials which were then placed onto a heating block for 4 hours at 90-100°C to purify fractions of the body water. The vials were then quickly cooled by placing on ice for ten minutes and the water distillate transferred to a clean autosampler vial for injection. A high-temperature conversion elemental analyser

(TC/EA: Thermo Finnigan, Thermo Scientific, Hemel Hempstead, UK) connected to an isotope ratio mass spectrometer (Delta V advantage, Thermo Scientific) was employed to measure deuterium labelling in saliva (0.1μL). Each sample was injected a minimum of four times to minimize the effect of carryover between samples. As saliva passed through the TC/EA it was converted into H_2 gas and on passage through the IRMS, the $^2H/^1H$ ratio was determined. Total body water enrichment was then able to be calculated using the following equation;

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

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

4.3.4.3 Isolation and derivatisation of the myofibrillar protein fraction

To assess the protein-bound alanine muscle fraction enrichment, approximately 30mg of muscle was homogenized in ice-cold homogenization buffer to isolate myofibrillar proteins. 10 min rotary mixing was followed by centrifugation at 11 000g for 15 min at 4°C forming a muscle sarcoplasmic supernatant and a myofibrillar/collagen pellet. The supernatant (sarcoplasmic fraction) was then removed, and the pellet was resuspended in 500µL mitochondrial extraction buffer (MEB) and homogenized by Dounce prior to centrifugation at 1000g for 5 min at 4°C. Insoluble collagen was separated following centrifugation from myofibrillar proteins that were solubilized in NaOH (750µL, 0.3M), and subsequently precipitated using 1 M perchloric acid (PCA) then pelleted by centrifugation. Following overnight hydrolysis at 110°C in a 0.1 M HCl and Dowex H+ resin slurry, the amino acids were eluted with 2 M NH4OH and dried down. Dried samples were suspended in 60 µL distilled water, 32µL methanol, and 10µL pyridine and 8µL methyl chloroformate with intermittent vortex. The n-methoxycarbonyl methyl esters of the amino acids were then extracted after adding 50µL chloroform. A molecular sieve was added to remove

water for around 20s before being transferred to clean vials. Newly formed muscle protein was determined through incorporation of deuterium into the protein-bound alanine as detected by gas chromatography–pyrolysis–isotope ratio mass spectrometry (Delta V Advantage, Thermo, Hemel Hempstead, UK).

4.3.4.4 Calculation of muscle protein fractional synthetic rate

MPS was calculated from the deuterium enrichment (APE) in alanine in myofibrillar proteins, using the body water enrichment as the precursor labelling between subsequent biopsies. The FSR was calculated as follows:

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

Where APE_{Ala} is deuterium enrichment of protein-bound alanine, APE_P is mean precursor enrichment of the body water over the period, and *t* is the time between biopsies. APE_p was corrected for the mean number of deuterium moieties incorporated per alanine (3.7), and the dilution from the total number of hydrogens in the derivative, i.e. 11.

4.3.5 Quantification of whole-body muscle protein breakdown using D_3-3-methylhistidine

3-MH is formed by the post-translational methylation of histidine residues in actin and myosin. When these proteins undergo proteolysis (muscle protein breakdown), 3-MH is released into the blood and is not reutilised for protein synthesis. 3-MH is then renally excreted and can be quantified in the either the plasma or the urine. Work by Sheffield-Moore has shown that using a stable isotope tracer of 3MH (D_3-3MH), it is possible to obtain an index of MPB from both urine and plasma samples collected the day after oral tracer consumption (Sheffield-Moore et al., 2014). Further work by Cegielski (Cegielski et al., 2021) have identified this technique to give rates of MPB in elderly human volunteers equivalent to that published in the scientific literature using alternative techniques.

4.3.5.1 Sample collection and storage

MPB was quantified using a non-invasive oral tracer technique (Sheffield-Moore et al., 2014) where participants receive a 10mg dose of D_3-3MH dissolved in 50mls of distilled water 24 hours prior to a lab visit. On the 24-hour mark, a blood sample was drawn from a three-way tap connected to a cannula

placed in the median cubital vein under local anaesthetic. Further blood samples were taken hourly for 6 hours while the patient rested semi-recumbent on a bed within the DGHPU. The collected blood was transferred into lithium/heparin bottles prior to being centrifuged at 3000 rpm for ten minutes, with the plasma being aliquoted into microtubes for storage at -80 degrees prior to analysis.

4.3.5.2 Sample Preparation and Processing

Plasma samples were defrosted and centrifuged at 10,000 rpm for 3 min. 100μl of plasma was de-proteinised using 1ml of MeCN:MeOH and samples were vortex mixed and stored at -20°C for 1 hour. The chilled samples were subsequently centrifuged at 13,000 rpm for 5 min at 4°C. The supernatant was dried down in a Techne heating block with a flow of nitrogen gas at <40°C. The supernatant was resuspended in 100μl ACN:ddH_2O and analysis performed using High-Performance Liquid Chromatography mass spectrometry (HPLC/MS).

An enrichment curve of 0-10% D_3-3MH was prepared alongside the batch of samples to be analysed. Liquid chromatography techniques were used prior to mass

spectrometry to determine the ratio of D_3-3MH and 3MH for each sample. Briefly, 2μl of plasma was injected into the Agilent 1100 series liquid chromatograph, which was connected to the Thermo Scientific Q Exactive Mass spectrometer. A flow rate of 0.4μl /min was set with an initial buffer gradient of 95:5 (Buffer A:B); where buffer A was 10mM ammonium formate (90:10 Acetonitrile: ddH_2O), with 0.1% formic acid and buffer B was 10mM ammonium formate (50:50 Acetonitrile: ddH2O) with 0.1% formic acid. Following a 2.5 min hold, the buffer gradient was increased to 100% buffer B and held for a further 2.5 min prior to returning to a 95:5 gradient where it was held for 13.5 minutes.

The mass spectrometer polarity was set positive, and the resolution was set at 35,000. A full scan method was utilised and the masses for the labelled D_3-3MH and the endogenous 3MH were identified as 173.11131 and 170.09230, respectively. Peak areas were integrated using Xcalibur software after these masses were set up in a selective ion monitoring (SIM) method.

The ratio of D_3-3MH to 3MH was determined for each sample and then corrected relative to the enrichment curve, run with

each batch, and the APE determined. The pre-dose/baseline blood sample was used to correct for background and calculation of the APE. The ratios were log transformed and plotted against time to determine the rate of decay. The gradient of the line connecting these points provides the rate of MPB (k) and was performed at each time point for each participant.

4.3.6 Quantification of thigh muscle volume and mid-thigh muscle cross sectional area using MRI

During the same MR visit described in Chapter 3, a 3T MRI (Phillips Ingenia) was utilised to acquire axial images of the dominant thigh at baseline, 14 days post inactivity and 14 days post remobilisation. A T1-TSE sequence acquisition was performed using a field of view of 200x200x74.7mm with a resolution of 1x1x1mm with a 0.1mm gap between slices. The total acquisition time was three minutes for a total of 68 slices. Whole thigh muscle volume and mid-thigh CSA was calculated from the axial scans though a semi-automated method using an in-house software (developed by Christoph Arthofer, Sir Peter Mansfield Imaging Centre, University of Nottingham) based on 3D Slicer software (Version 4.10.1, www.slicer.org). Briefly DICOM images were

converted from axial scans to the NIFTI format using MRIConvert software (Lewis Centre for Neuroimaging, University of Oregon) and loaded into the 3D Slicer Software where the region of interested was isolated and the muscle segmented. Firstly, the number of iterations and the convergence threshold was adjusted in order to correct the bias threshold. Multiple fiducial points were set within the muscle tissue and parameters applied for segmentation. The segment editor then performed an automated volume and mid-thigh CSA quantification of skeletal muscle. Each scan was performed by one of two trained operators. Neither operator was blinded to the participant or which stage of the study the scan was being undertaken.

4.3.7 Statistics and Calculations

Graphpad Prism 10 (Version 10.1.1) was used to perform statistical analysis on the raw output of the study. Measures of myofibrillar FSR, MPB, thigh muscle volume and mid-thigh CSA made at baseline were compared between young and older groups using an unpaired, two tailed t test. Two-way ANOVA, and Tukey's and Sidak's multiple comparisons tests were applied to locate within (time) and between (treatment) group differences over the course of the study. Statistical

significance within and between groups was accepted at $p<0.05$ in all cases. Values in the text, Tables and Figures represent mean ± SEM.

Due to an unforeseen issue with the frozen storage of some samples, complete datasets were only available for n=5 young and n=8 older. Through statistical methods it was possible to estimate a single value in the younger group to give n=6 and two values in the older group to give n=8 for myofibrillar protein FSR. The one missing data point (from a total of 24) for the rate of myofibrillar protein FSR following remobilisation in the young participants was estimated by plotting individual values for FSR following SR vs FSR following remobilisation. A regression line for these data (r=0.986) was then used to predict the missing data point using the equation of the regression line ((1.9452 x 2.02) – 1.132 = 2.797).

In the older group there were two missing data points (one data point for two participants), out of a total of 32, for rate of myofibrillar protein FSR following remobilisation. Due to a poor correlation when plotting the individual myofibrillar FSR data (r value) these two missing values could not be robustly predicted using the equation of the regression line, as above.

Instead, SPSS (IBM, Version 29.0.1.0) was utilised to generate multiple imputations for the missing data points, based on the individual myofibrillar FSR data for all of the older participants. The multiple imputation data set was then processed using the Bar procedure (Baranzini, 2018) to generate individual values for each missing data point. These predicted data points could then be used to complete the individual participant data sets and the participant included in the analysis performed on Prism, as discussed previously.

Furthermore, complete results for whole-body MPB were limited to n=9 for each group and MRI data were not collected from one young volunteer due to ongoing protocol development in the early part of the study. For transparency, all individual values are presented in figures in the results.

4.4 Results

4.4.1 Measures of physical activity, muscle protein turn over, thigh muscle volume and mid-thigh cross sectional area at baseline

4.4.1.1 Physical activity levels and body composition at baseline

As presented in Chapter 3, there was no difference in the baseline PAL between young and older participants (1.44 ± 0.02 vs 1.45 ± 0.01, p=0.783) or baseline daily step count (12542 ± 1133 steps vs 11410 ± 896 steps, p=0.443). In addition, all body composition measures, other than DEXA abdominal fat mass, were matched at baseline between groups (See Chapter 3).

4.4.1.2 Myofibrillar protein FSR at baseline

Myofibrillar protein FSR at baseline (Figure 4.2) were no different between the young (1.38% ± 0.22% d^{-1}) and the older (1.33 ± 0.07% d^{-1}) volunteers (p=0.840).

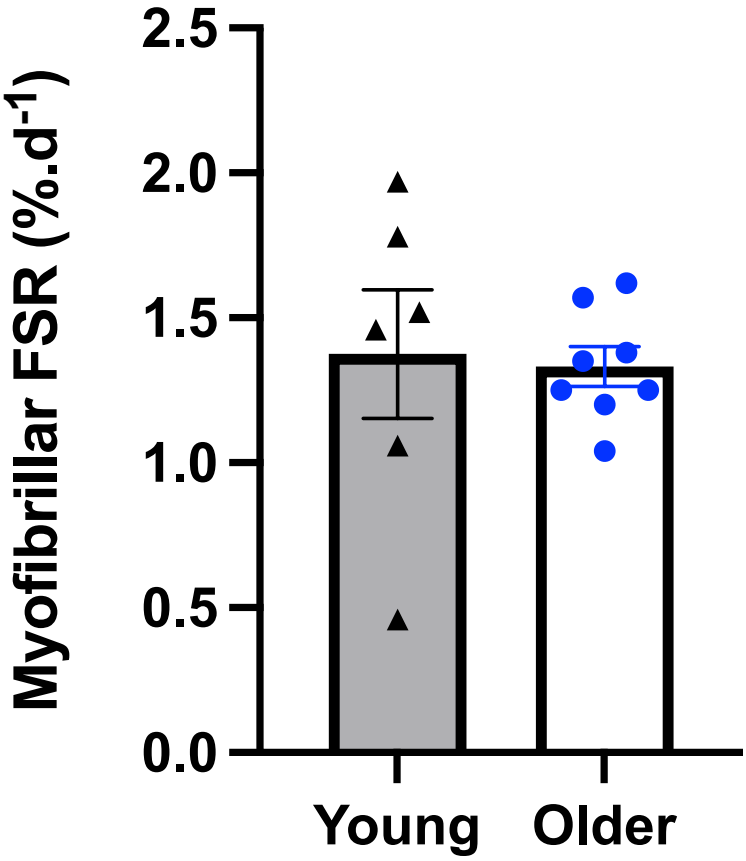


Figure 4.2 – Mean rate of myofibrillar protein fractional synthetic rate (FSR). Values are mean ± SEM and individual values.

4.4.1.3 Determination of rates of muscle protein breakdown by D_3-3-methylhistidine at baseline

There was no measurable difference between the mean rate of whole body MPB in the young (0.045 ± 0.005 h^{-1}) and the older (0.035 ± 0.003 h^{-1}) participants (p=0.136) (Figure 4.3).

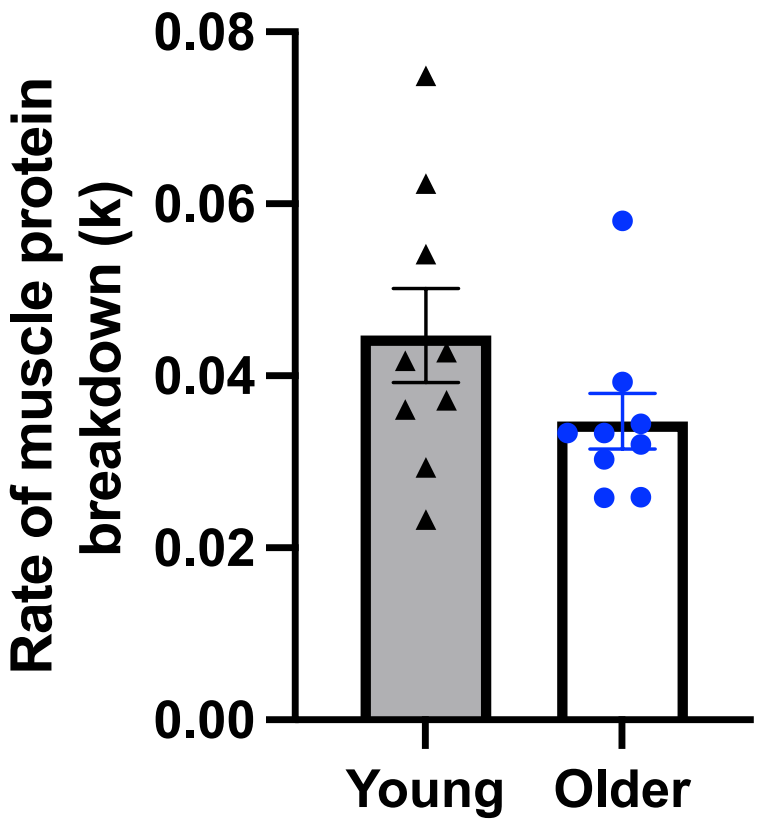


Figure 4.3 – Mean rate of whole-body muscle protein breakdown at baseline. Values are means ± SEM and individual values.

4.4.1.4 Mid-thigh muscle cross sectional area and thigh muscle volume at baseline

Figure 4.4 shows that dominant limb mid-thigh CSA was no different at baseline between the young and older (140.4 ± 11.7 cm^2 vs 116.1 ± 8.8 cm^2, p=0.112). Dominant limb thigh muscle volume was also no different at baseline between the

young and older participants (5211.6 ± 437.2 cm^3 vs 4310.1 ± 425.2 cm^3, p=0.158) (Figure 4.5).

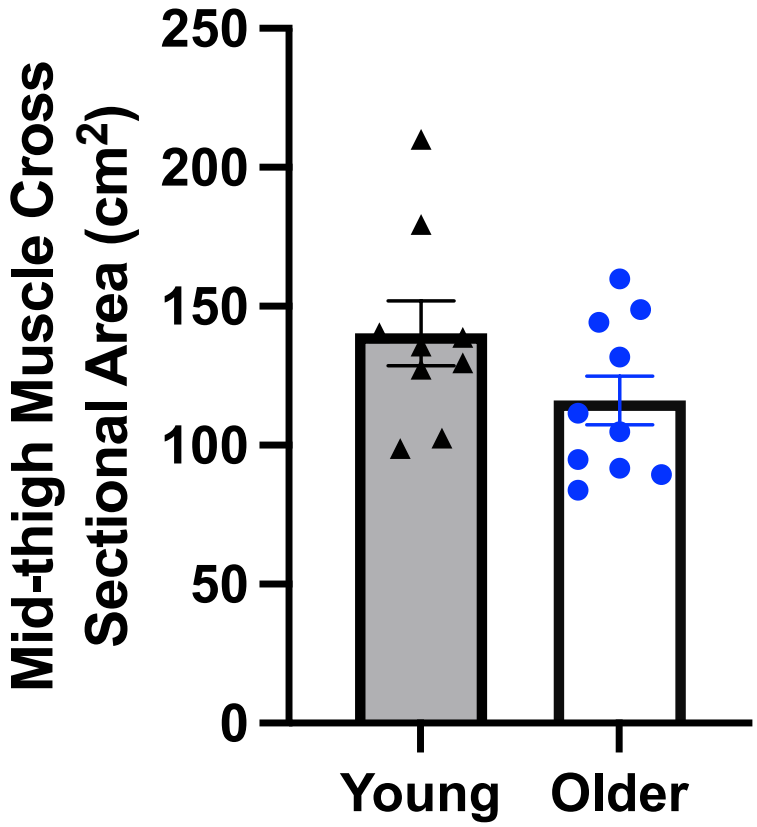


Figure 4.4 – Dominant mid-thigh cross-sectional area at baseline. Values are means ± SEM and individual values.

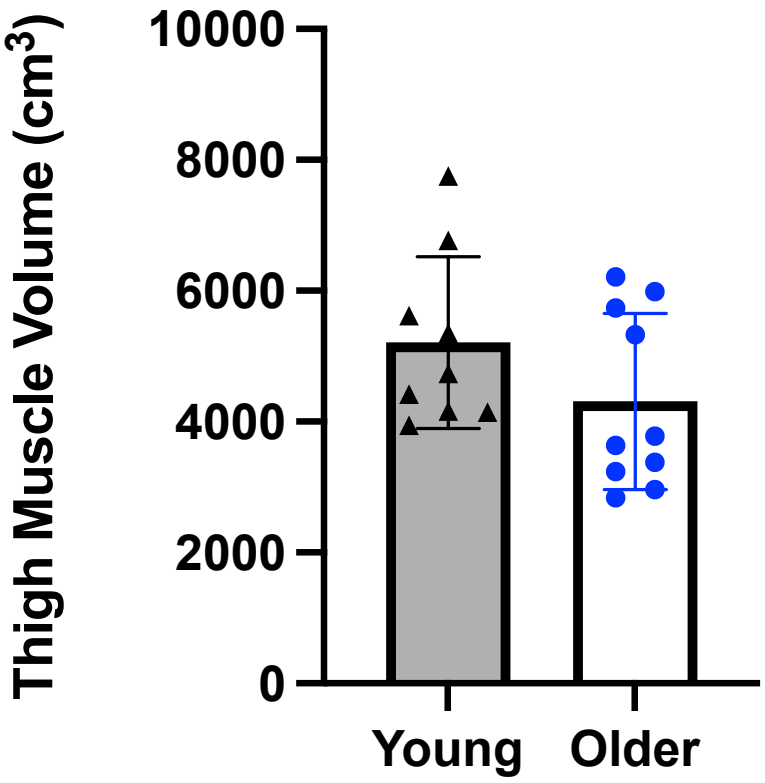


Figure 4.5 – Dominant thigh muscle volume at baseline. Values are means ± SEM and individual values.

4.4.2 Impact of step count reduction and remobilisation on physical activity level, muscle protein turn over, thigh muscle volume and mid-thigh cross sectional area

4.4.2.1 Step count reduction

Daily SR data is presented in detail in Chapter 3. Briefly, the young participants reduced daily step count by 92% on

average from 12542 ± 1133 steps to 1053 ± 94 daily steps. The older participants reduced daily step count on average by 90% from 11410 ± 896 steps to 1095 ± 85 steps per day. Following remobilisation, both groups returned to baseline with the younger group averaging 13724 ± 1196 steps and the older group 11683 ± 776 group, with no difference between groups (p=0.659).

4.4.2.2 Myofibrillar protein FSR

Rates of myofibrillar protein FSR were unchanged from baseline in both the young (1.38 ± 0.22% d^{-1} vs 1.31 ± 0.18% d^{-1}, p=0.905) and older (1.33 ± 0.07% d^{-1} vs 1.20 ± 0.08% d^{-1}, p=0.367) groups after 14 days SR (Figure 4.6). Similarly there were no differences from baseline in rates of FSR following 14 days of habitual physical activity in either young (1.42 ± 0.35% d^{-1}, p=0.980) or older (1.47 ± 0.% d^{-1}, p=0.71).

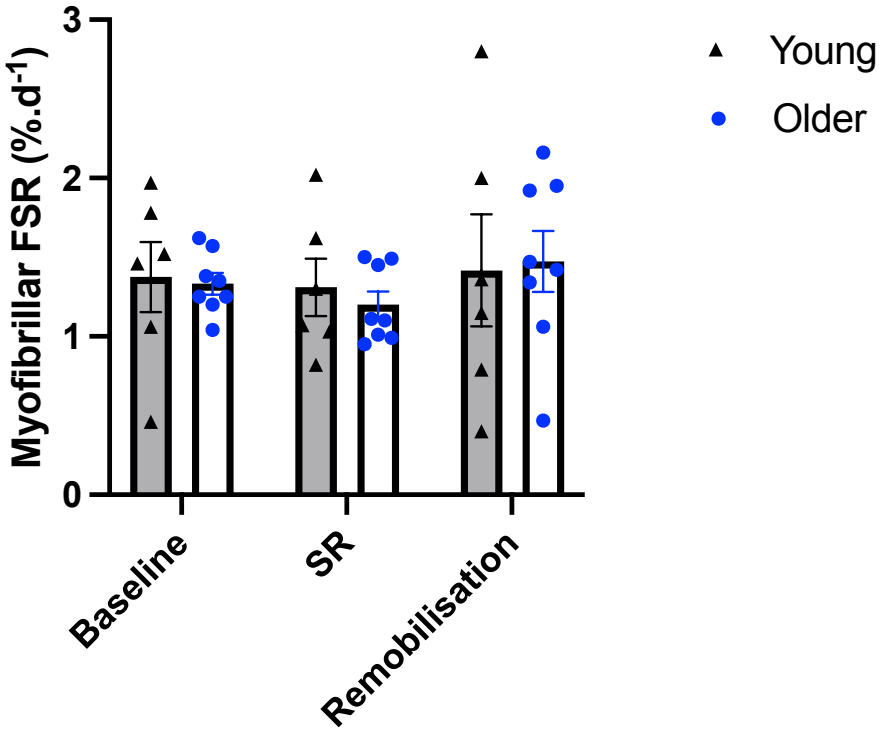


Figure 4.6 – Mean rate of myofibrillar protein FSR at baseline (Baseline), over 14 days of step count reduction (SR) and over 14 days of returning to habitual activity levels (Remobilisation). Values are means ± SEM and individual values.

4.4.2.3 Determination of rates of whole-body muscle protein breakdown by D_3-3-methylhistidine

The rate of whole-body MPB (k) following SR and remobilisation are shown in Figure 4.7. There was no difference from baseline in the young volunteers following SR (0.045 ± 0.005 h^{-1} vs 0.048 ± 0.006 h^{-1}, p=0.853) or following remobilisation (0.043 ± 0.007 h^{-1}, p=0.987). Similarly, there

was no difference from baseline in the rate of MPB in the older group after SR (0.035 ± 0.010 h^{-1} vs 0.043 0.004 h^{-1}, p=0.244) or following remobilisation (0.038 ± 0.003 h^{-1}, p=0.707). Two-way ANOVA did not identify between group differences at any of the three time points during the study.

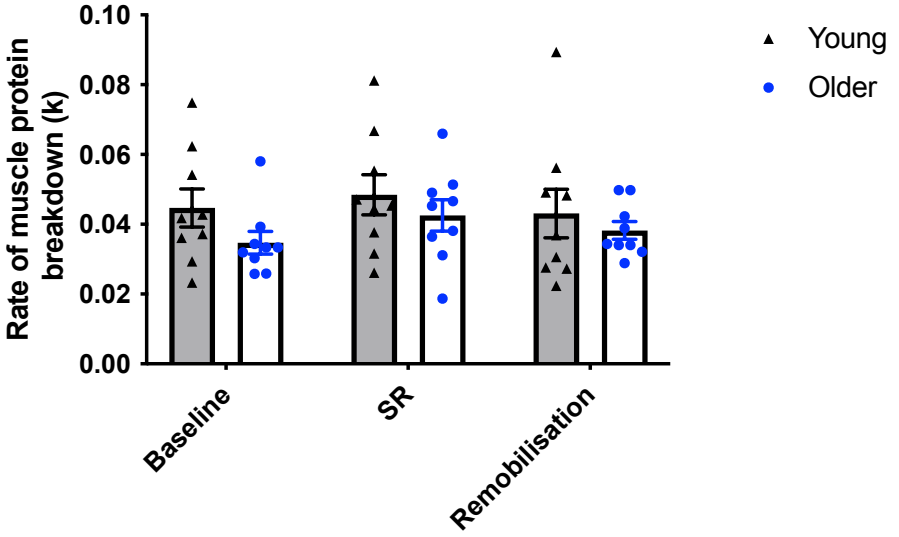


Figure 4.7 – Mean rate of whole-body muscle protein breakdown at baseline (Baseline), after 14 days of step count reduction (SR) and following 14 days of returning to habitual activity levels (Remobilisation). Values are means ± SEM and individual values.

4.4.2.4 Dominant mid-thigh muscle cross sectional area and thigh muscle volume

Mid-thigh CSA and thigh muscle volume after SR and remobilisation are shown in Figure 4.8 and 4.9, respectively. No difference from baseline in mid-thigh CSA was seen within

the young participants following SR (140.4 ± 11.7cm^2 vs 137.8 ± 12.2cm^2, p=0.79) or after remobilisation (142.5 ± 10.4cm^2, p=0.86). Similarly, muscle CSA in the older participants was unchanged from baseline after SR (116.1 ± 8.8cm^2 vs 111.3 ± 8.9cm^2, p=0.26) and remobilisation (110.0 ± 9.4cm^2, p=0.11). There was no significant difference in mid-thigh CSA when comparing young and older volunteers following SR or after remobilisation. Thigh muscle volume was unchanged from baseline across all timepoints in the young (5211.6 ± 437cm^3 vs 5270.7 ± 429.3cm^3 (p=0.78) vs 5265.9 ± 462.4cm^3, p=0.81). Similarly, SR did not affect thigh muscle volume in the older group (4310.1 ± 425.1cm^3 vs 4334.3 ± 464.0cm^3 (p=0.95) vs 4300.1 ± 452.1cm^3, p=0.99). As with muscle CSA, there were no between group differences across at each study time-point.

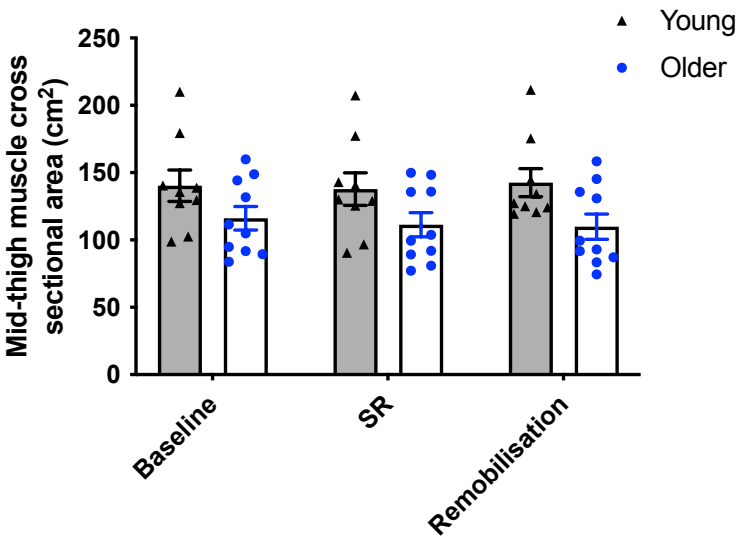


Figure 4.8 Mid-thigh muscle cross sectional area (cm^2) at baseline (Baseline), following 14 day of step count reduction (SR) and after 14 days of remobilisation (Remobilisation). Values are means ± SEM and individual values.

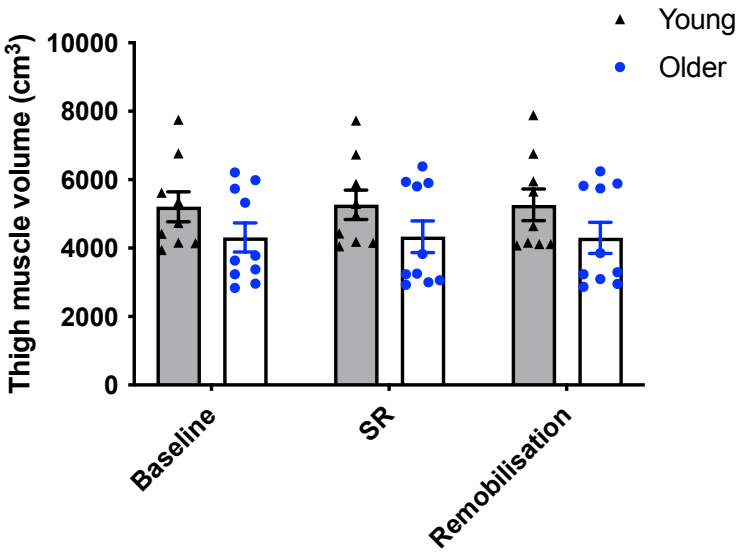


Figure 4.9 Thigh muscle volume (cm^3) at baseline (Baseline), following 14 day of step count reduction (SR) and after 14 days of remobilisation (Remobilisation). Values are means ± SEM and individual values.

4.5 Discussion

The aim of this study was to assess the impact that a 14-day period of SR and a subsequent 14-day period of remobilisation had on muscle protein turnover and thigh muscle cross-sectional area and volume in healthy, physically active young (18-40 years) and older (60-75 years) volunteers displaying similar body composition and habitual physical activity levels at baseline. A major finding was that a daily SR of >90% for 14 days did not result in a measurable change in MPS or MPB from baseline in young or older volunteers. In keeping with this, neither mid-thigh muscle CSA nor thigh muscle volume were reduced following SR. Furthermore, a 14-day period of remobilisation that increased daily step count back to baseline in both young (13724 ± 1196 steps) and older (11683 ± 776) volunteers had no measurable impact on muscle end-point measures. These findings add further to the contrasting outcomes of other studies that have applied SR interventions, which will be discussed below.

Despite the notion that changes in net protein balance in healthy volunteers are predominantly a result of alterations in the rate of myofibrillar protein FSR, and thus the theoretical assumption that muscle loss associated with ageing (i.e.

sarcopaenia) may be a result of a blunting of MPS to anabolic stimuli, the present study found no difference in MPS at baseline when comparing young and older volunteers (1.38% ± 0.222% d^{-1} vs 1.33% ± 0.068% d^{-1}). Similar baseline rates of cumulative myofibrillar protein FSR in young volunteers have been reported by Wilkinson (1.45% ± 0.10% d^{-1}) (Wilkinson et al., 2014) and by Brook (1.35 ± 0.08% d^{-1}) in young males (Brook et al., 2015). However, in the study by Oikawa (Oikawa et al., 2018) that included 31 older participants, the baseline rate was significantly greater at 1.5-1.7% d^{-1}. Similarly, McGlory (McGlory et al., 2018) reported higher baseline rates in ten older women (1.55% d^{-1}), but not 12 older men (1.43% d^{-1}). The participants in both studies had a markedly lower baseline daily step count and a higher mean whole body FFM compared with the older volunteers in the present study. Indeed, the participants in the study of McGlory were overweight and prediabetic, which might explain the differences from the present study. Both studies adopted the D_2O methodology. However, there were slight differences in the oral dosing schedule with the Oikawa study employing a daily dosing regimen of three x 60mls per day during the five days prior to the first muscle biopsy. Each of the above studies aimed to achieve ~ 1% body water enrichment. Nevertheless,

it is difficult to easily rationalise the greater (not lower) rate of cumulative MPS at baseline in the studies of Oikawa and McGlory when compared with the present study. Reassuringly, the rate of MPS at baseline in the younger and older participants in the present study is in keeping with rates reported in other studies using D_2O methodology (Brook et al., 2015, Wilkinson et al., 2014). In the study by Breen (Breen et al., 2013a) where the pre-intervention step count was again significantly lower than the present experiment, the rate of MPS was calculated using an acute tracer based method, thus comparisons of baseline rates between studies may not be directly comparable.

Muscle protein turnover is a dynamic process that can be affected by numerous factors. The net balance of this process in humans results in maintenance of muscle mass, with changes in MPS, rather than MPB, thought to be the main driver of atrophy and hypertrophy of muscle tissue in healthy individuals (Brook et al., 2022, Glover et al., 2008). Ageing is associated with declines in physical activity levels and an increase in sedentary behaviour (Milanovic et al., 2013) which may play a part in the age-related loss of muscle mass (sarcopaenia). This study employed SR as a model of inactivity

that has previously been used in both young and older participants for varying periods of time. We induced a reduction in daily steps > 90% from baseline in both groups for a period of 14 days (See Chapter 3, Figure 3.5). Breen's study reduced step count by approximately 75% for 14 days (Breen et al., 2013a), Olsen by 77% for 7 days (Olsen et al., 2008), Krogh-Madsen by 87% for 14 days (Krogh-Madsen et al., 2010), Oikawa by 84% and 90% for 14 days (Oikawa et al., 2018) and finally McGlory by 70% for 7 days (McGlory et al., 2018). Each of these studies identified acute changes in body composition and/or acute myofibrillar protein FSR (i.e. measured over three to four hours before and after SR) or cumulative FSR using D_2O. However, despite a greater relative reduction in daily step count in the present study the physiological responses observed did not corroborate these earlier findings.

Over 14 days of SR, we observed no change from baseline in the average daily rate of myofibrillar protein FSR in either the young or the older participants. In studies by Oikawa (Oikawa et al., 2018) and McGlory (McGlory et al., 2018), there was a significant drop in cumulative myofibrillar FSR to rates between 1.3 and 1.4% d^{-1}, which were similar to the rates

found (both at baseline and after SR) in this study. Breen found a 26% reduction in post prandial rates of acute MPS following 14 days of SR in ten healthy older adults compared with baseline (Breen et al., 2013a), with no change in the rate of post absorptive MPS in the same volunteers. This may have some bearing on the present study if energy intake was reduced during SR, but this was not the case (see Chapter 3), nor was there a change in body composition which corroborates that the volunteers were not in negative energy balance during SR (see Chapter 3).

The reduction in post prandial MPS following SR in the Breen study (Breen et al., 2013a) could be explained by the process known as 'anabolic resistance'. This has been purported as the underlying process responsible for sarcopaenia, with intermittent episodes of reduced activity in the elderly culminating in mass loss. The term describes the reduced stimulation of MPS to anabolic stimuli such as exercise and/or protein nutrition and is demonstrated more readily in the acute measurement of MPS with both postabsorptive and postprandial measures. This would be represented by a global fall in cumulative FSR but likely to a lesser extent given that post absorptive rates of MPS were not changed following the

period of SR. The differing methods of FSR measurement make it difficult to draw direct comparisons between the studies, in addition the baseline characteristics identify a significant difference in preintervention average daily step-count along with a difference in body composition measures meaning the groups are not matched. Leading on from this, it would be appropriate to consider the potential impact of low numbers on the FSR responses, i.e. a lack of impact of SR on FSR. I think it would be reasonable to conclude that this may influence the interpretation of the young data, where variation can be seen to be quite large, particularly given that the power calculation determined that a minimum number of ten participants was required and due to sample loss we only had six complete data sets. It is however unlikely to explain the lack of response in the older volunteers, where n=8 and the inter individual variance is low. Furthermore, if FSR was indeed lower in the young and has been masked by high variation and low numbers, it might have been expected to be accompanied by a loss in muscle CSA and volume, which was not observed.

Given that there was no change from baseline in all end-point measures in both younger and older participants in the study,

it comes as no surprise that there was no difference in responses from baseline following remobilisation. The physical activity data (Chapter 3) indicated that both groups returned back to pre-study levels of activity and daily steps immediately, implying no lasting mobility effects from 14-day SR, which is in keeping with the data on remobilisation from other SR studies (McGlory et al., 2018, Oikawa et al., 2018, Bowden Davies et al., 2018). It is interesting to note that following SR and remobilisation in the Oikawa study, despite MPS rates being significantly lower than baseline, there was an increase in whole body FFM and leg fat mass following remobilisation. Similarly, McGlory found no change in body composition measures, despite there being a significant drop in the rate of MPS, which did not recover after remobilisation, after SR.

Historically, the technical challenges associated with measuring MPB have limited the inclusion of this parameter to a small number of inactivity studies (Fujita et al., 2009, Jahn et al., 1999). However, D_3-3MH has re-emerged as a promising, non-invasive oral tracer-based technique to measure acute rates of whole-body MPB over time. In 1954 endogenous 3MH was identified in the urine (Tallan et al.,

1954) and later found to be released upon the breakdown of the striated muscle proteins actin and myosin (Long et al., 1975), thus considered to potentially provide a reliable measure of muscle protein turnover in humans (Long et al., 1975). Despite initial optimism over the technique, reservations existed over the potential contribution of 3-MH from other organs such as the gastrointestinal tract, given that 3-MH is also present in gut actin (Long et al., 1988). These apprehensions along with further concerns about the relative contribution of exogenous sources of 3-MH, such as from meat ingestion, have largely been allayed through the study by Sheffield-Moore that utilised the isotopically labelled methyl-D_3-3MH (Sheffield-Moore et al., 2014). In addition, the increased sensitivity of mass spectrometry technology has dramatically improved this analytical technique and has helped to unlock the potential of D_3-3MH as a non-invasive oral tracer-based technique (Li et al., 2021).

We did not identify a difference at baseline in whole-body MPB between the young (0.045 ± 0.005 h^{-1}) or the older (0.035 ± 0.003h^{-1}) volunteers (Figure 4.3). Published research has consistently reported no age-related difference to the rate of MPB at rest (Volpi et al., 2001, Volpi et al., 1999). In the

present study, there was also no change in the rate of MPB with SR or remobilisation in either group of volunteers (Figure 4.7). As far as we are aware, this is the first study to have applied the D_3-3MH technique to quantify MPB following SR, thus there is an absence of directly comparable data. However, a limited number of studies have addressed the impact of immobilisation on MPB; Shur (Shur et al., 2024) identified a 30% decline in the rate of MPB after two days of bed rest in ten healthy young men, and the authors suggested this may be explained by the volunteers being maintained in energy balance during bed rest. Brook (Brook et al., 2022) however reported MPB was unchanged from baseline following four days of unilateral leg immobilisation in nine healthy young men (Brook et al., 2022). In addition to this, Tesch (Tesch et al., 2008) found an increase in the rate of MPB after 72 hours of unilateral leg immobilisation in eight young men after noting a 44% increase in interstitial 3MH within the vastus lateralis on skeletal muscle microdialysis.

In line with whole body measures for lean mass (Chapter 3), this study did not identify difference between age groups in dominant thigh muscle volume or dominant mid-thigh muscle CSA at baseline (Figure 4.8 and 4.9). The results for mid-thigh

CSA in the present study are similar to that in the current literature for both the young (Roth et al., 2001) and the older group (Mathur et al., 2008). The measures of thigh muscle volume sit within the middle of a wide range of published MRI based estimates for both old and young: In the SOMMA study by Cawthon (Cawthon et al., 2023), 827 participants aged > 70 years were grouped by sex. Their estimates of 11.2 ± 1.6L for older men and 7.5 ± 1.1L for older woman dwarfed our measures of 4310 ± 452cm^3 for the older group, which we did not differentiate by sex. The participants in this study were not matched for physical activity levels or body composition and were eligible provided they had a BMI<40 kg/m^2 and were able to complete a 400m walk. Similarly, in a study performed in the UK by Linge (Linge et al., 2018) 6,021 participants taken from the UK biobank had body composition profiles generated which involved whole body MRI scanning. Both this and the SOMMA study outsourced MRI analysis to AMRA medical (AMRA Medical AB, Linkoping, Sweden), with thigh muscle volumes being similar with males averaging 12.39L and females 8.23L. At the other extreme was a study by Yoshiko (Yoshiko et al., 2017) that estimated young adults (21 ± 0.4 years) thigh volume as 2397.9 ± 712.1 cm^3 and older adults (70.7 ± 3.8 years) as 1616.8 ± 469.0 cm^3. In addition,

Taniguchi estimated a volume of 2450 ± 344cm^3 in 17 young men, taking the volume of the quadriceps and the hamstrings, but not the adductor muscles, as the volume for the thigh (Taniguchi et al., 2021). It is likely the variability between studies is due to variation in technique of MRI analysis given the large difference of baseline measures. Certainly, the results in chapter 5 demonstrates that the methods employed to determine body composition in the present study were robust (Figure 5.3 and Figure 5.6).

As with the whole-body composition changes presented in Chapter 3, this study did not identify any difference from baseline in thigh muscle volume or CSA in either group following 14 days of SR, which may have been predicted based on the lack of change in either cumulative MPS or MPB. In line with our findings, McGlory found no change in leg or whole body FFM post SR (McGlory et al., 2018), despite reporting a significant reduction in cumulative MPS after SR, which persisted following remobilisation. In addition, Oikawa (Oikawa et al., 2019) found no change in either measure following SR despite a significant reduction in cumulative MPS. Furthermore, they reported that remobilisation accompanied with whey protein supplementation did not recover baseline

rates of MPS but did induce an increase in both leg FFM and whole body FFM. This is in contrast to the findings of Breen (Breen et al., 2013a) and Devries (Devries et al., 2015) where a small but significant reduction in leg FFM accompanied a reduction in post prandial MPS. However, each of the above referenced studies employed DEXA for body composition measures as opposed to our utilisation of MRI for thigh volume and CSA. Despite DEXA being a well utilised and more readily available method of calculating body composition, MRI (along with CT) have been shown to be near perfectly correlated with cadaveric values for both muscle groups (Engstrom et al., 1991) and CSA (Mitsiopoulos et al., 1998). As far as we are aware, no prior study has utilised MRI measures of these parameters following a reduced step count intervention.

Remobilisation back to baseline levels of physical activity in the present study was accompanied by no change in the rate of MPS or MPB, or body composition. As discussed, this result might well have been expected and again is in line with the other body composition measures discussed in Chapter 3. As alluded to above, other step count restriction studies that have examined the effects of remobilisation on MPS and body composition have found differing results (Oikawa et al., 2019,

McGlory et al., 2018, Bowden Davies et al., 2018). Suetta (Suetta et al., 2009) (See below) showed that that remobilisation in the young, returned body composition measures back to baseline levels, similarly as already discussed, Bowden Davis (Bowden Davies et al., 2018) showed body composition changes in young habitually active participants returned to baseline after remobilisation. However, in the older participants in the above study of Suetta (Suetta et al., 2009), following a two week period of unilateral whole leg casting and subsequent four week supervised retraining programme, the authors reported a diminished capacity to restore muscle size and architecture following retraining. The younger participants returned to baseline despite displaying a greater loss of quadriceps muscle volume (8.9% loss vs 5.25% loss). Although unlike our study, the groups were not matched at baseline with regards to physical activity levels or baseline muscle volume with the older group having an 11% smaller quadriceps volume when compared to the young group.

4.6 Conclusion

This study did not find any effect in muscle protein turnover (With regards to MPS and MPB) or thigh muscle volume and

mid-thigh muscle CSA in either young or older groups following SR. Additionally, returning to habitual physical activity also had no effect on these measured parameters. Given the short time period of SR utilised during this study, it remains to be found whether longer periods of reduced ambulatory activity are in part responsible for the detrimental physiological and metabolic changes that accompanying ageing. It could be considered that the small number of complete data sets for MPS in the young due to loss of samples may have impacted the findings in this group.

Chapter 5

Evaluation of whole-body muscle mass using the D3-creatine dilution method in a repeated measures longitudinal study design in active healthy, young and older volunteers

5.1 Introduction

Skeletal muscle forms the largest single organ in the human body (Koopman, 2011). Along with its functions in locomotion, posture and breathing it is a highly metabolic tissue involved in several processes including glucose homeostasis, fuel storage and cytokine excretion. The loss of volume and quality of skeletal muscle secondary to reduced physical activity or, the age-related loss known as sarcopaenia, is likely to result in reduced physical ability but also perhaps metabolic dysfunction. The measurement of whole body or regional skeletal muscle mass could be considered as a useful adjunct to profiling the metabolic health of an individual. Certainly, most proposed definitions of sarcopaenia include the measure of skeletal muscle mass. The currently available techniques to measure body composition are magnetic resonance imaging (MRI), computerised tomography (CT), DEXA and bioelectrical impedance analysis (BIA). MRI and CT measure muscle volume and are considered the gold standard due to their accuracy, however routine use within the general population is expensive and impractical. DEXA estimates fat mass, FFM and bone density. BIA can be used to estimate FFM, total body water, total body fat percentage plus intracellular and

extracellular water. The assessment of muscle volume in the non-research setting is generally limited to DEXA and BIA.

DEXA is a widely used tool for the assessment of body composition both for research and clinical purposes. It is relatively cheap when compared with MRI and CT and less time consuming both in terms of scanning time and analysis due to automated software that provides values for bone density, whole body and regional fat and fat free masses. However, DEXA does not directly measure muscle mass, rather it measures FFM which includes skin, connective tissue, bone marrow and viscera along with skeletal muscle (Clarys et al., 2010). This can result in an overestimation of muscle mass, the effects of which are more pronounced in older or obese individuals (Buckinx et al., 2018). AFFM is the sum of the lean soft tissue found in the limbs. Much of the appendicular lean soft tissue is skeletal muscle, as opposed to other non-fat tissues. In addition, a large proportion of the whole-body skeletal muscle compartment is found in the limbs so despite not providing a value for whole body skeletal muscle mass, it is likely a more accurate surrogate than whole body measures utilising DEXA (Buckinx et al., 2018). Certainly, DEXA measured AFFM is highly correlated with both

MRI ($r=0.88$, $p<0.001$) and CT ($r=0.77$-0.95, $p<0.0001$) measured skeletal muscle volume (Bredella et al., 2010, Bilsborough et al., 2014, Carver et al., 2013, Knapp et al., 2015, Toombs et al., 2012).

In addition to imaging techniques, the D3-creatine dilution method has emerged as an apparently reliable measure of whole-body muscle mass. Creatine is an organic compound that was discovered in meat extracts by the chemist Michel Eugene Chevreul in 1832 (Chevreul, 1835). Myers and Fine confirmed the presence of creatine in skeletal muscle and other tissues in 1913 (Myers and Fine, 1913) and by 1960, with a greater understanding of creatine biology, Meador had developed a technique for estimating total body skeletal muscle mass by directly measuring the creatine pool size with a radiolabelled isotope (creatine-1-^{14}C) (Meador et al., 1968). However, by the mid to late 70's, developments in MRI, CT and DEXA had all but made the creatine method redundant as a technique for measuring skeletal muscle mass, particularly given that at this stage the tracer used was radioactive, and it was still required to have a muscle biopsy in addition to the facilities required to manage radiolabelled compounds. In 2012, facilitated by advances in high sensitivity mass

spectrometry, Stimpson (Stimpson et al., 2012) further developed the method in rats using the non-radioactive isotope D3-creatine, which was later used in humans by Clarke (Clark et al., 2014). This method directly measures whole body skeletal muscle mass through a non-invasive, oral tracer-based technique, which negates the need for muscle biopsies.

Ninety eight percent of total body creatine is found within skeletal muscle where it is turned over at a constant rate of about 2g/day in an irreversible, non-enzymatic reaction in healthy individuals from creatine into creatinine (Casey and Greenhaff, 2000). This reliable metabolic process forms the basis of the technique; as with endogenous creatine, ingested low dose oral creatine is absorbed and transported into muscle by a specific muscle creatine transport protein, where it is irreversibly and non-enzymatically converted into creatinine before being excreted in the urine. Any surplus creatine not retained by skeletal muscle, is excreted by the kidneys as creatine. Through the provision of a stable-isotope labelled form, such as D3-creatine, the enrichment of D3-creatinine in the urine over time following ingestion can be used to determine the total body creatine pool which is then divided

by 4.3 (4.3g/kg represents the concentration of creatine per kg of wet muscle based on historical studies (Baldwin et al., 1952, Barnes et al., 1957, Fitch and Sinton, 1964)) to calculate an estimate for whole body muscle mass.

The validity of the D3-creatine dilution method rests on several key assumptions about creatine similar to those that were required for the 24 hour urinary creatinine excretion method of skeletal muscle mass estimation (Heymsfield et al., 1983):

- **The oral bolus of D3-creatine is fully absorbed with no faecal or urinary losses**

Although not studied in humans, Stimpson (Stimpson et al., 2012) compared oral vs intravenous dosing and found that faecal loses of isotopically labelled creatine do not alter the accuracy of the pool size measurement. However tracer can be lost into the urine, which can result in an overestimation of the creatine pool size that is proportional to the fraction that is spilled. The degree of spillage appears to vary dependent on sex (Clark et al., 2018) with an average of 1% in males and 3% in females. This has led to the development of dose

spillage correction equations to try and minimise this effect (Clark et al., 2018).

- **D3-creatine is exclusively taken up by skeletal muscle**

Creatine can be distributed across several organs which includes the brain, the heart, the lungs, the liver and the intestine. It is estimated that the extra skeletal muscle creatine uptake can vary between 2-10% (Picou et al., 1976, Wallimann et al., 2017, Farshidfar et al., 2017, Stimpson et al., 2012, Borsook and Dubnoff, 1947, Hunter, 1922) meaning that the skeletal muscle creatine pool size could potentially sit within 90-98% of whole-body creatine.

- **Labelled intramuscular D3-creatine is non-enzymatically converted to D3-creatinine**

Endogenous creatine is synthesised in the liver and kidneys before being transported into skeletal muscle where it is converted irreversibly into creatinine through a non-enzyme dependent process. Labelled creatine will undergo the same process, the rate limiting step is transport into skeletal muscle as discussed previously

- **Skeletal muscle creatine concentrations are consistent at 4.3g/kg**

The creatine dilution method uses the assumption that there is approximately 4.3g of creatine per kg of wet muscle, based on work by Kriesberg (Kreisberg et al., 1970). There are several factors that can affect the skeletal muscle creatine concentration which include the individual muscle (the concentration is higher in type 2 fibres than type 1 fibres hence muscles with a higher number of type 2 fibres, such as the vastus lateralis, will have a higher concentration of creatine than the soleus, which is composed of more type 1 fibres), age (Forsberg et al., 1991), diet (Bleiler and Schedl, 1962) and level of physical activity (Morris-Paterson et al., 2020). The result of these factors means that the creatine concentration in skeletal muscle can vary to anything between 3-5g/kg of wet muscle.

- **Labelled D3-creatinine is entirely excreted by the kidneys in the urine**

It is well established that endogenous creatinine is not stored in the body and undergoes a predictable excretion in the urine via the kidneys (Wyss and Kaddurah-Daouk, 2000). The same is true of labelled creatine; after it undergoes conversion into

creatinine within skeletal muscle it diffuses out of the muscle and is renally excreted into the urine in its entirety.

In the face of these apparent potential pitfalls in the technique, the D3-creatine dilution method continues to emerge as a useful, non-invasive tracer based technique that has been validated against whole body MRI in both healthy men and women over a wide age range (Clark et al., 2014). Furthermore, this method is increasingly being explored as a reliable technique to measure muscle mass in both research (Balachandran et al., 2023, Cawthon et al., 2019) and clinical settings (Cheng et al., 2023, Cawthon et al., 2022, Banack et al., 2024). Despite this technique showing promise as an isolated measure of whole-body muscle mass, there are limited data available regarding its reliability in longitudinal study designs, in particularly its ability to estimate muscle mass before and after a study intervention. A study by Balachandran (Balachandran et al., 2023) assessed the ability of the D3-creatine dilution method to detect change in muscle mass in response to resistance exercise training in older volunteers. The study included 21 volunteers with a mean age of 82.1 years that performed a full body strength training programme 3 days per week for a total of 15 weeks, with

participants being assessed at baseline and then again at 16 weeks. Following the 15 weeks of strength training there were increases in DEXA estimated appendicular lean mass and D3-creatine determined muscle mass. However, the training induced change in D3-creatine and DEXA estimates of muscle mass were poorly correlated, with the change in D3-creatine muscle mass being approximately double the increase observed for DEXA AFFM, with the authors concluding that D3-creatine is more sensitive to longitudinal changes than DEXA lean mass measures.

As far as I am aware, there are no published studies that have examined the utility of the D3-creatine dilution method to detect change in muscle mass following a period of reduced physical activity, such a bed rest or SR. Further research is therefore required before this technique can be considered a suitable means in which to non-invasively determine the magnitude of change in whole-body skeletal muscle mass following a period of altered physical activity or any intervention or condition purported to alter muscle mass.

5.2 Aims and hypotheses

The aim of this study was firstly to evaluate the D3-creatine dilution technique to estimate whole body muscle mass in healthy and habitually active young and older volunteers at baseline, following 12 days of SR and 12 days of remobilisation. Secondly, to assess the magnitude of association of individual estimates of whole-body muscle mass using the D3-creatine dilution method with individual measures of DEXA AFFM, MRI thigh muscle volume and MRI mid-thigh cross sectional area (CSA) in the same volunteers across multiple time-points in the same study.

5.3 Methods

5.3.1 Subjects and sample size

Ten young (age 18-40y) and ten older (age 60-75y) healthy volunteers took part in the study (See Chapter 3 for physical characteristics). They were recruited locally and consented following a medical screening and a discussion about what the study involved. The inclusion and exclusion criteria are given in the General Methods (Section 2.3). The study was approved by the University of Nottingham Medical School Ethics

Committee in accordance with the Declaration of Helsinki (Ethics Reference No: A10022017).

5.3.2 Study protocol

Participants attended for study visits at the DGHPU as indicated in Figure 5.1. Recruited participants made 6 study visits over a 32-day period following medical screening (See General Methods section 2.4) and a seven-day run in phase during which habitual physical activity was recorded with an Actiheart accelerometer, as described in the General Methods (Section 2.6.1). Study visits were grouped into baseline, SR (after a 12-day period of daily SR to <1500 steps) or remobilisation where participants returned to normal habitual physical activity levels. Measurements were taken as per the protocol (Figure 5.1) and participants were required to ingest D3-creatine 72 hours prior to the stated study visit. Due to the restraints of being part of a larger study, measures of whole-body muscle mass using D3-creatine were made following 12 days of both SR and remobilisation, whereas DEXA and MRI measures of body composition and thigh muscle volume respectively, were made follow 14 days of SR and remobilisation. On study visits where MRI scanning was required, this was performed at the Sir Peter Mansfield

Imaging Centre, University of Nottingham, prior to attending the DGHPU for DEXA scanning. Following ingestion of the D3-creatine, complete 24-hour urine collections were performed at home, along with a single 'spot' sample at 48 hours and then 72 hours post tracer ingestion (indicated by D3-creatine urine on the protocol). Participants attended each study visit following an overnight fast and were fed on completion of the study day.

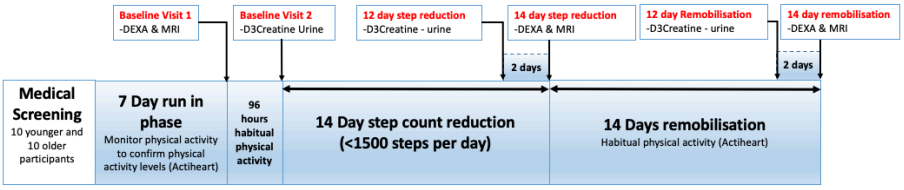


Figure 5.1 – Study schematic.

5.3.3 Habitual physical activity and step count measurement

Habitual physical activity was measured using an Actiheart monitor (CamNTech, Cambridgeshire, UK) during the seven-day run in phase and again during the 14-day remobilisation as described in the General Methods (Section 2.6.1). Step count monitoring was performed during the SR part of the

study using a clip-on pedometer, which is described in detail in the General Methods (Section 2.6.2).

5.3.4 Whole body muscle mass quantification using the D3-Creatine dilution method

5.3.4.1 Tracer ingestion, sample collection and storage

Whole-body muscle mass quantification was performed using the D3-creatine dilution technique based on the work by Clark (Clark et al., 2018). Participants received a 30mg oral dose of D3-creatine dissolved in 50mls of distilled water along with detailed instructions on when to ingest the tracer. They were then requested to collect all urine produced over the 24 hours following ingestion. Further "spot" samples were collected at 48 and 72 hours post ingestion. Each sample volume was recorded and 1ml aliquots were taken and stored at -80°C until analysis. Prior to each ingestion of tracer, a single urine sample was requested.

5.3.4.2 Sample preparation and processing

Urine was thawed at room temperature and then vortex mixed. 250μl of ice cooled acetonitrile was added to each of the samples. An internal standard was produced using 50ml of urine with 10μl of ^{13}C-creatine along with 250μl of acetonitrile. The samples and the standard were vortex mixed for 10 minutes and left to incubate for 30 minutes on ice. Samples were then centrifuged at 17,000 rpm for 20 min and the supernatant then filtered through a 0.2μm filter before being transferred into vials for analysis. Standard curves using ^{12}C- and ^{13}C-creatine were prepared (0.25μg - 5μg) for the determination of creatine concentration and a D_3-creatine enrichment curve of 0-0.1% was prepared and run alongside each batch. 2μl of prepared sample was injected into the Agilent 1100 series liquid chromatograph that was connected to the Themo Scientific Q Exactive Mass spectrometer. A 60:40 buffer mix (100% acetonitrile LC-MS grade:Ammonium acetate pH 5.8) was added and the flow set to 0.2μl/min. The mass spectrometer polarity was set to positive with a resolution of 70,000. Using the full scan method the masses of D_3-Creatine, ^{13}C-creatine and ^{12}C-creatine were identified as 135.09549, 133.08006 and 132.07666 respectively. The same method was employed to identify ^{12}C-creatinine and D_3-

creatinine where the mass was identified as 114.00638 and 117.08515, respectively. The SIM method utilised the identified masses as inclusion masses for creatine and creatinine and Xcalibur software was used to integrate peak areas for the calculation of isotope ratios and then concentration and enrichment. The creatine pool size for each sample can be calculated which is then divided by 4.3 (4.3 g/kg represents the concentration of creatine per kg of wet muscle) to calculate an estimate for whole body muscle mass. The following equation is used to calculate creatine pool size:

$$Creatine\ pool\ size = \frac{\left(\frac{MW\ Unlabelled\ Cr}{MW\ Labelled\ Cr}\right) x\ \left(Amount\ of\ D3{-}Cr\ dosed\ (g) {-} Amount\ of\ D3{-}Cr\ excreted\ (g)\right)}{(mean\ steady\ state{-}state\ D3{-}creatinine\ enrichment\ ratio)}$$

Where MW unlabelled Cr is the molecular weight of creatine (131.13 g/mol), and MW labelled Cr is the molecular weight of D_3-creatine (133.09 g/mol).

5.3.5 Whole body and appendicular fat free mass quantification using dual-energy X-ray absorptiometry

DEXA was employed to calculate whole body and AFFM as described in the General Methods (Section 2.11). These

measures were taken at baseline, following 14 days of SR and following a further 14 days of remobilisation.

5.3.6 Measurement of dominant thigh muscle volume and mid-thigh cross sectional area using magnetic resonance imaging

Thigh muscle volume and mid-thigh cross sectional area measurements were made of the dominant thigh at baseline, following 14 days of SR and following a further 14 days of remobilisation using 3T MRI as described in the General Methods (Section 2.12).

5.3.7 Statistical analysis

All data sets were presented as mean ± SEM, unless otherwise stated. Two-way ANOVA with Tukey's and Sidak's multiple comparisons tests were applied to locate within (time) and between (treatment) group differences. Pearson correlation coefficients and simple linear regression models were generated to compare the two measurement techniques. A Bland-Altman plot was created to compare D3-Creatine derived whole body muscle mass with DEXA derived AFFM and

assess for systematic bias. All analyses were performed in Prism 10 (Version 10.1.1, Graphpad).

5.4 Results

Results for physical activity levels, DEXA AFFM, MRI calculated mid-thigh CSA and thigh muscle volume are presented in Chapters 3 and 4 but are briefly presented in this section to provide context and allow direct comparison with the D_3-Creatine dilution method.

5.4.1 Physical activity levels

As presented in Chapter 3, there was no difference in the baseline PAL between younger and older participants (1.44 ± 0.02 vs 1.45 ± 0.01, p=0.783) or baseline daily step-count (12542 ± 1133 steps vs 11410 ± 896 steps, p=0.443). In addition, all body composition measures, other than DEXA abdominal fat mass, were matched at baseline between groups (See Chapter 3). Daily SR data is presented in detail in Chapter 3. Briefly, the young participants reduced daily step count by 92% on average from 12542 ± 1133 steps to 1053 ± 94 daily steps. The older participants reduced daily step count on average by 90% from 11410 ± 896 steps to 1095 ±

85 steps per day. Following remobilisation, both groups returned to baseline with the young group averaging 13724 ± 1196 steps and the older group 11683 ± 776 group, with no difference between groups (p=0.659).

5.4.2 Appendicular fat free mass, mid-thigh cross sectional area and thigh muscle volume

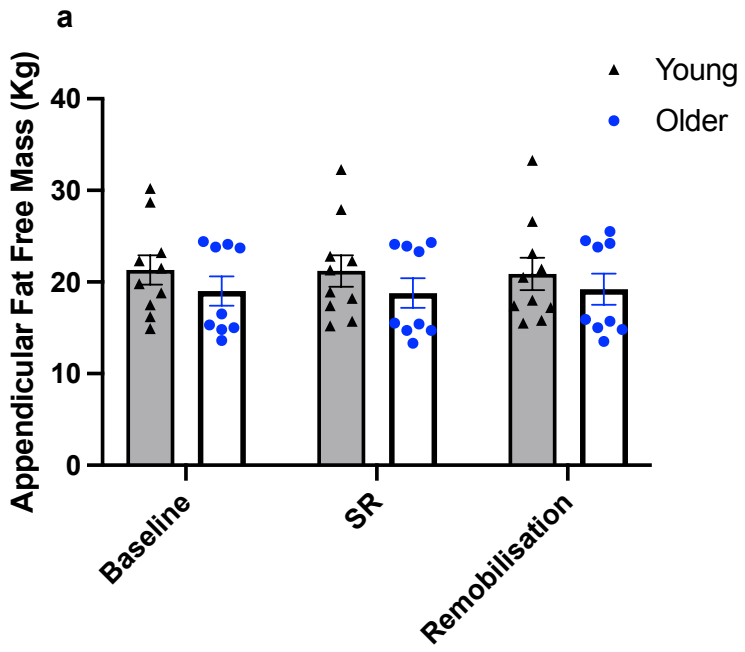
Figure 5.2 a depicts baseline, post SR and post remobilisation measures of DEXA AFFM. Detailed results of DEXA AFFM are given in Chapter 3, Section 3.4.1 and 3.4.2.2. Detailed results of mid-thigh CSA (Figure 5.2 b.) and thigh muscle volume (Figure 5.2 c.) are given in Chapter 4 (Section 4.4.1.4 and 4.4.2.4) but are given here for reference.

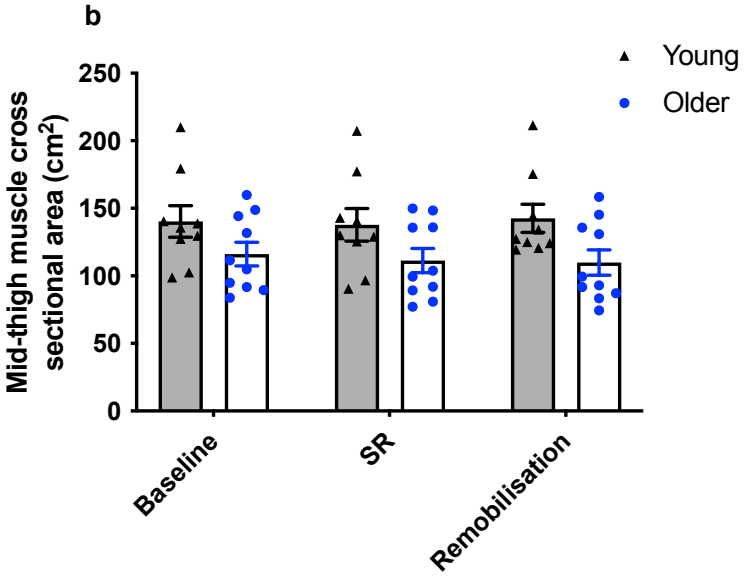
5.4.3 D3-creatine whole body muscle mass quantification

At baseline the D3-creatine derived measure of whole-body muscle mass was significantly greater in the young vs older volunteers (25.17 ± 2.15 kg vs 18.72 ± 1.29 kg, p=0.019) (Figure 5.2 d). Following 12 days of SR there was a significant decrease in the D3-creatine measured whole body muscle mass in the young group (25.17 ± 2.15 kg vs 22.10 ±

1.95 kg, p=0.034) but no equivalent change was observed in the older group (18.72 ± 1.29 kg vs 22.1 ± 1.96, p=0.45). There was no between group difference after SR. Remobilisation resulted in a return to baseline in D3-creatine whole body muscle mass in the young group, a significant increase when compared to 12-day SR (22.10 ± 1.95 kg vs 27.99 ± 2.27 kg, p=0.006). In the older group, there was no change following remobilisation after 12 days of SR. There was however a significant difference between groups in D3-creatine muscle mass

following remobilisation (27.99 ± 2.27 kg vs 19.29 ± 1.41 kg, p=0.0012).


a
Young
Older
Appendicular Fat Free Mass (Kg)
40
30
20
10
0
Baseline
SR
Remobilisation


b
Young
Older
Mid-thigh muscle cross sectional area (cm2)
250
200
150
100
50
0
Baseline
SR
Remobilisation

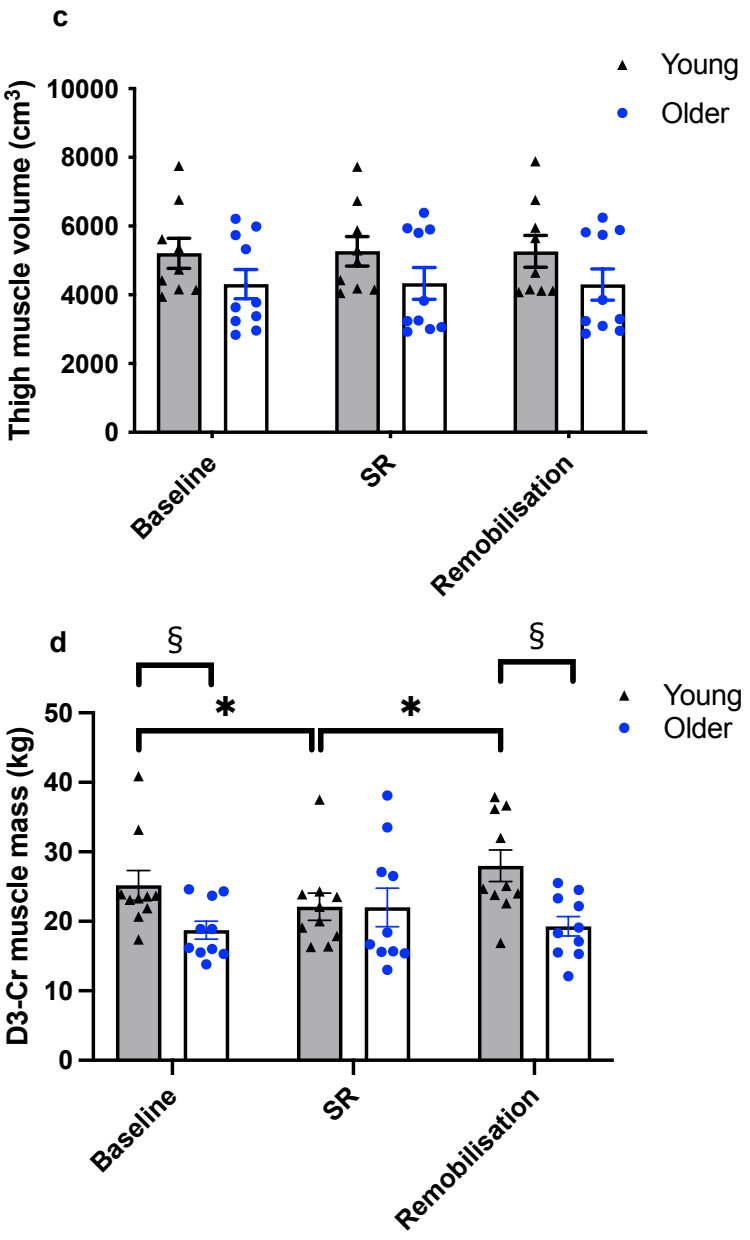


Figure 5.2 a DEXA appendicular fat free mass, **b MRI** mid-thigh muscle cross sectional area, **c** MRI thigh muscle volume and **d** D3-creatine derived whole body muscle mass at baseline, following 14 days of step count reduction and 14 days of subsequent remobilisation in young and older. Values are means ± SEM and individual values. § indicates between group difference ($p<0.05$), * indicates within group differences ($P<0.05$).

5.4.4 Correlation and Bland-Altman analysis between muscle mass measured by D3-creatine and DEXA derived appendicular fat free mass

D3-creatine derived muscle mass was positively correlated with DEXA AFFM for all time points in the young (Figure 5.3 a; $r=0.8299$, $p<0.0001$). A similar association was observed in the older group ($r=0.8414$, $p<0.0001$) (Figure 5.3 b). When all times points for D3-creatine derived muscle mass and DEXA derived AFFM from both age groups were plotted a positive association remained evident $r=0.8242$, $p<0.0001$ (Figure 5.3 c.).

The Bland-Altman analysis revealed a systematic bias (figure 5.4) when comparing D3-creatine derived whole body muscle mass and DEXA measured AFFM as shown through an increase in the difference between the two measures as the AFFM increases.

5.4.5 Correlation between muscle mass measured by D3-creatine and MRI thigh muscle volume

D3-creatine estimated muscle mass was strongly positively associated with MRI derived thigh muscle volume for all time

points in the young ($r=0.8521$, $p<0.0001$) (Figure 5.5 a.). This positive association was also evident in the older volunteers ($r=0.7762$, $p<0.0001$) (Figure 5.5 b). Figure 5.5 c shows that there was a strong correlation when all time points for both young and old were combined ($r=0.8231$, $P<0.0001$).

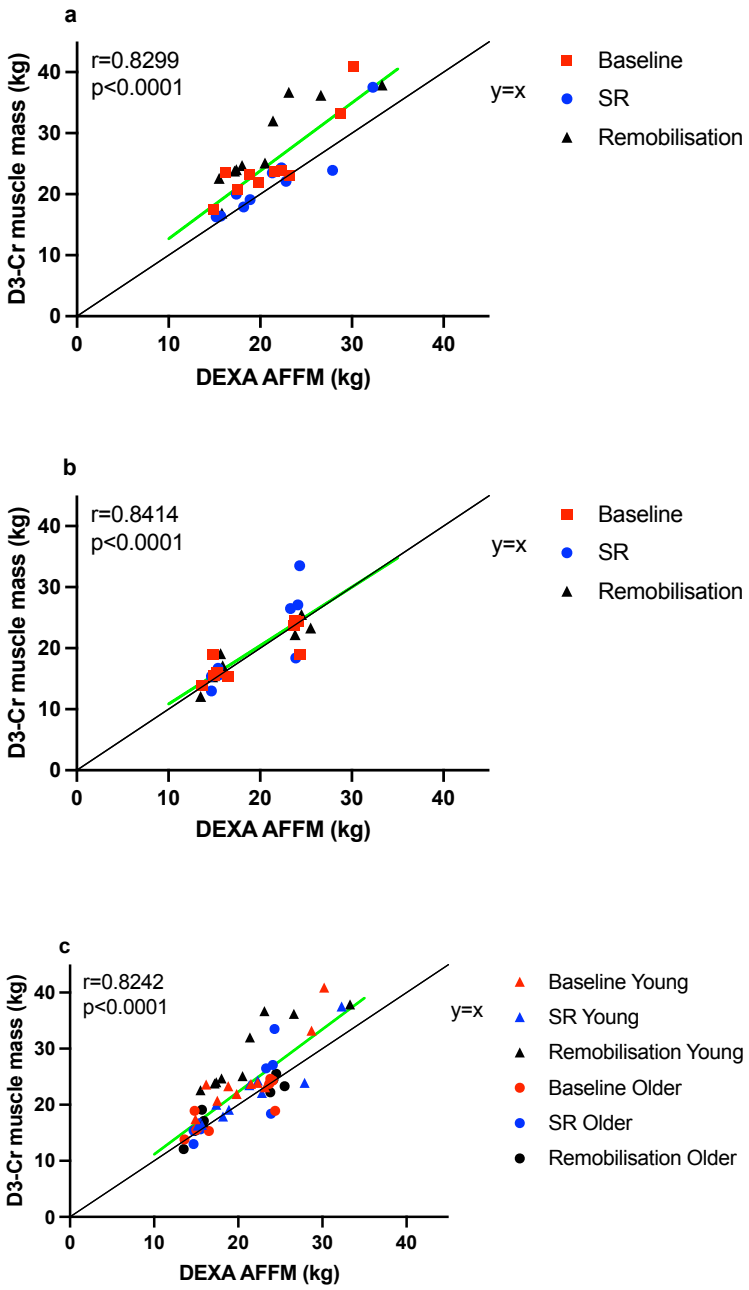


Figure 5.3 Association between DEXA derived appendicular FFM vs D3-creatine derived whole body muscle mass at all study time points in young volunteers (**a**), in older volunteers (**b**), and in both groups of volunteers combined (**c**). Black line reflects the line of identity (y=x). Green line reflects Pearson linear correlation, along with the correlation coefficient and statistical p value reflecting the strength of the linear association between the two variables

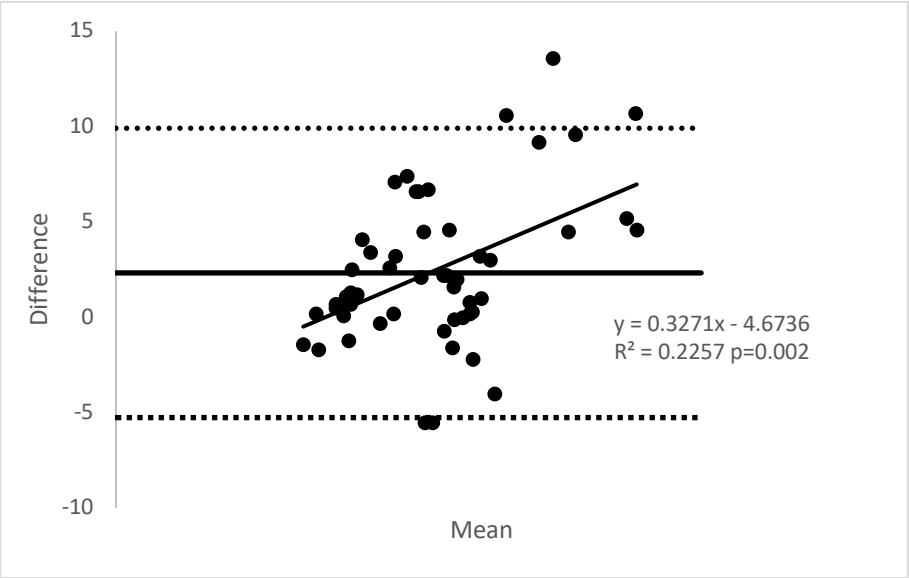


Figure 5.4 Bland-Altman plot comparing D3-creatine derived whole body muscle mass (Kg) and DEXA derived AFFM (Kg) at all time points for both younger and older volunteers. The dotted lines represent the upper and lower 95% limits of agreement and the horizontal line solid line represents the mean difference. The oblique line represents Pearsons linear correlation

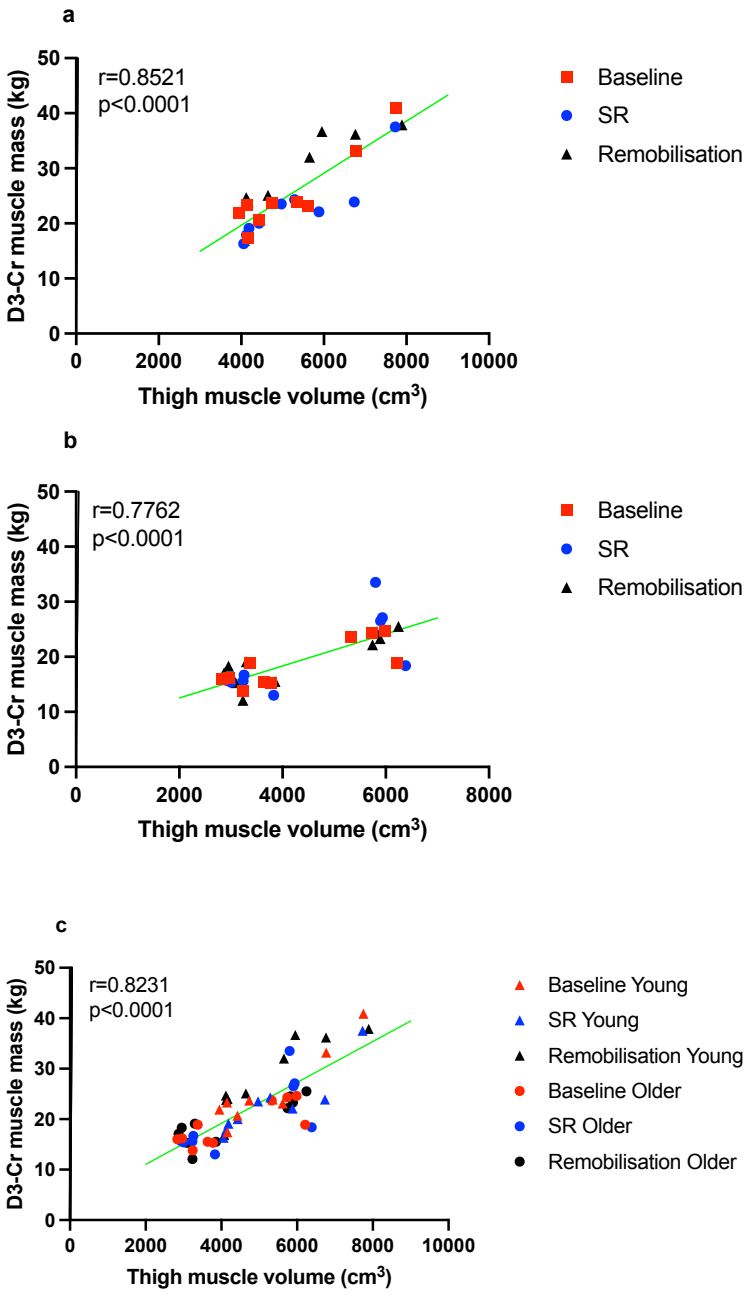


Figure 5.5 Association between MRI thigh muscle volume vs D3-creatine derived whole body muscle mass at all study time points in young volunteers (**a**), older volunteers (**b**), and in both groups of volunteers combined (**c**)**.** Green line reflects Pearson linear correlation, along with the correlation coefficient and statistical p value reflecting the strength of the linear association between the two variables.

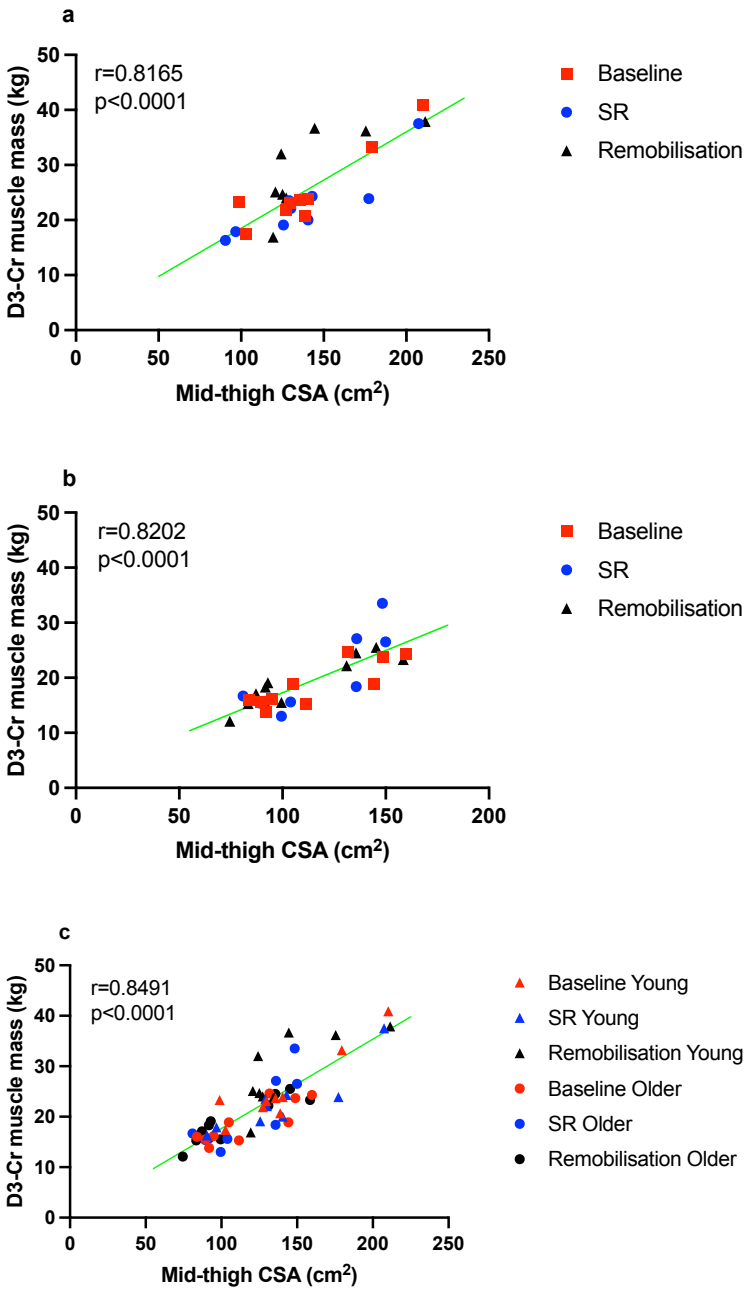


Figure 5.6 Association between MRI mid-thigh cross sectional area vs D3-creatine derived whole body muscle mass at all study time points in young volunteers (**a**), older volunteers (**b**), and in both groups of volunteers combined (**c**)**.** Green line reflects Pearson linear correlation, along with the correlation coefficient and statistical p value reflecting the strength of the linear association between the two variables.

5.4.2.4 Correlation between muscle mass measured by D3-creatine and MRI mid-thigh cross sectional area

There was a strong linear association between D3-creatine derived muscle mass and MRI derived mid-thigh CSA in both the young ($r=0.8165$, $p<0.0001$) and the older (0.8202, $p<0.0001$) volunteers (Figure 5.6 a and b). It therefore follows that this strong association was maintained when all timepoints in both age groups were combined ($r=0.8491$, $p<0.0001$) (Figure 5.6 c).

5.5 Discussion

This study aimed to evaluate the ability of the D3-creatine dilution technique to estimate whole body muscle mass in healthy and habitually active young and older volunteers at baseline, following 12 days of SR and 12 days of remobilisation. As far as I am aware, there have been no prior studies that have used the D3-creatine dilution method to estimate whole body skeletal muscle mass at several timepoints during a longitudinal study design while employing a SR model of inactivity. Given that neither DEXA (the most widely used estimate of body composition measurement) or

MRI (the gold standard measure of muscle volume) identified a difference in their respective skeletal muscle quantification measures following SR or remobilisation, either within or between groups, it could be assumed that the D3-creatine dilution method would generate similar results. However, the D3-creatine method identified a significant difference in whole body muscle mass between the young and old both at baseline and after remobilisation. It also showed a significant reduction in whole body muscle mass in the young group following SR (Figure 5.2 d), with a return to baseline following remobilisation. There were no within group changes in the older group at any time during the study.

Given that there were no within or between group differences in AFFM or muscle volume using either DEXA or MRI, these results need to be interpreted with some caution. To validate the technique, associations between D3-creatine measures with DEXA AFFM, MRI thigh muscle volume and MRI mid-thigh CSA were assessed in the young volunteers, the older volunteers and then in both age groups combined using linear regression analysis along with a Bland-Altman analysis between D3-creatine measures and DEXA AFFM. Positive associations between DEXA AFFM and D3-creatine were observed in both young and older volunteers. Numerous

previous studies have compared single time point D3-creatine derived estimates of muscle mass with DEXA, with differing observations (Clark et al., 2014, Buehring et al., 2018, Cawthon et al., 2019, Orwoll et al., 2020, Zhu et al., 2021, Cegielski et al., 2021, Balachandran et al., 2023). As previously stated, DEXA does not directly measure muscle mass, rather FFM, of which muscle along with other soft tissue, such as viscera and connective tissue, are included. This can result in an overestimate of skeletal muscle mass. AFFM is considered to give a better estimate for muscle mass given that 75% of body skeletal muscle volume is located in the limbs and the majority of the limb fat free tissue is skeletal muscle (Kim et al., 2002). Of the studies that have previously compared the D3-creatine dilution method with DEXA, a number found positive correlations with whole body FFM while others found positive correlations with AFFM (Table 5.1). Despite several positive correlations, none of the studies found correlations as strong as our results for AFFM vs D3-creatine whole body muscle mass.

Study	DEXA measurement	Correlation vs D3-creatine muscle mass
(Clark et al., 2014)	Whole body FFM	r=0.754, p<0.0001
(Balachandran et al., 2023)	AFFM	r=0.79, p<0.001
(Cawthon et al., 2019)	Whole body FFM AFFM	r=0.66, p<0.001 r=0.68, p<0.001
(Buehring et al., 2018)	Whole body FFM	r=0.60 ^
(Zhu et al., 2021)	Whole body FFM AFFM	r=0.50 ^ r=0.50 ^
(Cegielski et al., 2021)	AFFM	r=0.69, p=0.027

Table 5.1 Studies correlating DEXA whole body fat free mass (FFM), appendicular fat free mass (AFFM) or both. ^indicates p value not given.

In addition, some of the studies utilised corrective formulas using height, weight or BMI to obtain stronger positive correlations (Cawthon et al., 2019, Zhu et al., 2021, Zanker

et al., 2022). Our results show that D3-creatine estimates whole body muscle mass to be greater than AFFM in the young, whereas in the older group the estimates appear very similar. This may be at least partly explained by the scatter of the data in the older group is much more restricted compared to the young. However, when both groups are combined the greater muscle mass estimation with the D3-creatine method remains evident and is very likely explained by AFFM not being representative of total body muscle volume, unlike D3 creatine. This is further supported by the observation that the difference between methods as per the Bland-Altman analysis appears to become greater, the larger the AFFM (Fig. 5.4).

MRI is regarded as the gold standard to quantify muscle volume, both for whole body and limb specific volume measurements (Tothill and Stewart, 2002, Nakatani et al., 2016). MRI scanning is costly and can be time consuming when considering both scanning time and manual analysis of each scan. These problems are becoming less important with the advent of higher power MRI scanners and machine learning volume assessment methods. Nevertheless, participants still need to be ambulant enough to mobilise onto the scanner table and lay flat for the duration of the scan. The

present results revealed very strong positive associations between the D3-creatine dilution estimate of muscle mass and MRI estimates of thigh muscle volume (Figure 5.4) and mid-thigh CSA (Figure 5.5) in both the young and older participants. Prior studies that have compared D3-creatine with MRI have also found good associations between the two measures; Clarke correlated MRI total muscle mass with D3-creatine finding a strong correlation ($r=0.868$, $p<0.0001$) (Clark et al., 2018), whereas Sagayama also found a strong correlation between D3-creatine and MRI whole body muscle mass with $r=0.840$, $p<0.001$ (Sagayama et al., 2023). Thus, in both young and older participants, D3-creatine dilution estimate of muscle mass associates with the gold standard method for quantifying skeletal muscle for both a single timepoint measure and during a longitudinal study design, such as in this study, where three separate estimates of muscle mass were made.

Less work has been done on the ability of D3-creatine to measure muscle mass following an intervention, either in the form of increased physical activity to induce a positive change in muscle mass or reduced physical activity, as in this study. A potential strength of the D3-creatine derived muscle mass

estimation as a tool for detecting small changes in muscle mass is that it is inexpensive and non-invasive and may be able to detect small but significant changes in populations over time. To date, there are few published studies that have aimed to quantify longitudinal change in muscle mass with D3-creatine. Balachandran (Balachandran et al., 2023) compared D3-creatine derived estimates of muscle mass with DEXA derived measures of appendicular and whole-body FFM before and after a 15-week period of high intensity strength training in 21 older volunteers. They found increases in both the D3-creatine and DEXA derived measures after intervention, however despite strong baseline associations between D3-creatine and DEXA AFFM (r=0.79; 95% CI: 0.53, 0.92), following the 15-week period of training there was no significant association (r=0.19; 95% CI: -0.35-0.64). Duchowny (Duchowny et al., 2020) studied 40 male participants as part of the Osteoporotic Fractures in Men Study (Cawthon et al., 2016) whereby repeated measures, including DEXA and D3-creatine derived muscle mass estimations were made 1.6 years apart. At baseline they report the DEXA total body lean mass to be 55.22kg, with AFFM 23.3kg and the D3-creatine estimated whole body muscle mass 24.64kg. Over the duration of the study there was a loss of 0.31kg in DEXA

total body lean mass, 0.08kg of DEXA AFFM and 1.42kg D3-creatine muscle mass. The study correlated the percentage change from baseline of the DEXA measures with D3-creatine and found moderate positive correlations between DEXA total body lean mass ($r=0.50$, $p<0.001$) and DEXA AFFM ($r=0.58$, $p<0.001$). The study concluding that The D3-creatine method may be a feasible tool to measure declines in muscle mass over time. Both studies identified a degree of potential in the technique as a non-invasive measure of skeletal muscle mass following an intervention but concluded that further work needed to be performed. Certainly, the present study found significantly stronger correlations between D3-creatine and DEXA AFFM despite including both genders and young and older participants rather than a single age range as per the above studies.

The D3-creatine dilution method was the only quantification technique that identified any difference between groups with regards to lean mass and/or volume in the present study. It is however important to emphasise that each technique measure different entities. DEXA measures FFM, both whole body and appendicular, which includes tissue other than muscle (connective tissue, viscera, intramuscular fat), whereas MRI

in this context is measuring only dominant thigh muscle volume or mid-thigh CSA, rather than a direct measure for whole body muscle mass. Following changes in activity, muscle groups can differentially adapt to the varying demands placed upon them. Therefore, quantifying muscle volume of a single region (i.e., the thigh) following a change in activity and comparing it to a whole-body measure may not provide corresponding results. Similarly with DEXA whole body FFM, which includes a higher proportion of non-muscle tissue, whole body muscle mass is not actually being quantified and thus there is potential for discrepancies due to changes in other non-muscle tissue following a change in activity levels. Irrespective of the differences between the tests, it would seem unlikely that the 12.1% decline in whole body muscle mass with SR in the young volunteers using the D3-creatine dilution method, which was restored upon remobilisation, was a true finding given that it was not accompanied by a parallel decline and subsequent recovery in cumulative MPS over the course of the study (See Chapter 4).

5.6 Conclusion

Estimation of whole-body muscle mass in a longitudinal study design using the D3-creatine dilution method in the present

study associated well with other established forms of body composition measurement. This consistency extended to both young and older participants which is in keeping with prior research, but perhaps none that have considered as many timepoints, which is a positive indictment of the method. The apparent over-estimation compared to AFFM using DEXA is very likely to be a result of the latter estimation not including the whole of the body muscle compartment. Use of the D3-creatine dilution technique did however reveal a 12.1% decline in whole body muscle mass after SR in the younger participants, that was restored by remobilisation but was not mirrored by changes in DEXA derived AFFM or MRI derived muscle volume or CSA measures. Furthermore, there was no associated change in myofibrillar MPS to support such a change in muscle mass with SR and remobilisation. The number of studies employing the D3-creatine dilution method are increasing to try and refine and establish the technique as a viable alternative to DEXA for a single measure of whole-body muscle mass. However, when comparing D3-creatine whole body muscle mass with DEXA AFFM via Bland-Altman analysis, there is evidence of systematic bias which needs to be considered when utilising the technique. Further inclusion in studies where there is a change in muscle mass over time

would enhance the robustness of the method. In addition to its potential ability to provide a non-invasive muscle mass measure, the association between mass measure and functional status could prove to be a very useful adjunct in population-based surveys.

Chapter 6 - General Discussion

An increasing proportion of the world's population fail to undertake the minimum recommended level of physical activity with associated detrimental health effects (Organization, 2022). As previously discussed, ageing is associated with a progressive decline in the level of daily physical activity due to a varied number of reasons. In addition to this, the elderly population is growing, in the last 40 years the number of people in England aged 65 and over has increased by over 3.5 million (a 52% increase) (Better, 2023). The combination of an ageing population that is increasingly sedentary paves the way for a socioeconomic disaster given the clear evidence linking sedentary behaviour with poor health outcomes (Biswas et al., 2015, Stamatakis et al., 2019).

The importance of investigating the effects of inactivity on human health is not only because of the well-known link with metabolic disease, but it is becoming increasingly apparent that there are similarities in the metabolic and physiological changes that occur in both the young and older sedentary person. In essence, it appears you can metabolically age a young person just by making them inactive. The principle aims of this thesis were to investigate the impact of a 14-day

period of SR and subsequent remobilisation on body composition, glucose regulation and muscle protein turnover in healthy, physically active young and older participants. The project was composed of a single large study through which volunteers were exposed to two interventions (SR and remobilisation). The data was then segregated based on investigation type to create three experimental chapters which had a degree of overlap in parts. The benefits of this form of study design are that multiple rounds of participant recruitment are not required, participants become accustomed to the laboratory techniques and measures being taken and given that the SR intervention is quite intrusive, multiple measures were possible following just a single 14-day period of SR. The limitations to this study design are organising the logistics of multiple measures being required at the same time. For example, we were unable to perform OGTT and plasma sampling for MPB (D3-MH) during the same study visit which resulted in measures for MPB being taken on day 12 rather than 14 of SR. In addition, it can get somewhat confusing for participants to understand the instructions regarding when and how to take oral tracers and extra care had to be taken to ensure these were taken at the correct times given that the participants were taking them at home at a set time prior to a

study visit. Logistically it is easier to perform studies with a single intervention and a single key aim. However, for studies such as this where participants are recruited for a significant period this was not possible.

Much of the published research involving older volunteers has not quantified baseline physical activity levels adequately and similarly studies that have compared both young and older groups have failed to match groups based on habitual physical activity levels. Thus, at present there is a significant knowledge gap in the comparison of well-matched young and older volunteers. Despite there not being a deliberate effort to recruit participants that were well matched at baseline for physical activity levels, glucoregulation and body composition measures, fortuitously this was achieved. This was seen as a significant benefit given that no prior published inactivity study comparing young and old has achieved this. Our study populations allowed a true comparison of several metabolic and physiological endpoint measures in well matched, healthy, physically active young and older groups. In addition, the study did not include a control group, which given that the participants were physiologically healthy and well matched at baseline, was not considered a weakness. The overarching aim

of the project was to compare the differential effects of the described interventions to baseline measures (within group) as well as a direct comparison between the young and older groups, rather than intervention versus control. In addition, recruiting a control group would have required both young and older controls, increasing the total number of participants significantly which would have been logistically impossible due to the time restraints.

Both groups underwent a 14-day period of SR and subsequent remobilisation. Here I will summarise the results of the main outcome measures, the limitations of each study and make suggestions for further research related to these outcomes.

Glucoregulation and body composition have been well studied in bedrest (Mikines et al., 1991, Shur et al., 2022, Dirks et al., 2016) and limb immobilisation (Abadi et al., 2009, Berg et al., 1991, Burns et al., 2021) experimental scenarios. More recently there has been a growing interest in the effects of increasing sedentary behaviour or SR on these parameters (Walker et al., 2024, Krogh-Madsen et al., 2010, Knudsen et al., 2012), in a bid to try and emulate the progressive decline in habitual physical activity levels observed in the general

population. We found in Chapter 3 that SR induced alterations in glucose regulation in response to an OGTT in both younger and older participants, with the younger group maintaining good glucoregulation at the expense of hyperinsulinemia following 14 days of SR. In the older participants, we found the AUC of both serum insulin and blood glucose during the OGTT was increased after just 48 hours of SR, which resolved after 14 days of SR. Importantly, following remobilisation both groups displayed normal blood glucose responses to OGTT. The changes in blood glucose regulation occurred without concurrent changes in body composition and under conditions where physical activity levels were well controlled and monitored in all volunteers, further supporting the belief that these changes in glucoregulation are an early manifestation of reduced physical activity, whereas body composition changes occur later and are not causative of the acute changes in glucose dysmetabolism.

The main weakness of the data in Chapter 3 relates to the lack of priori power calculation. The study design meant that participants were recruited to a single large study that was reported as individual studies based on outcome measures and the number of participants recruited to each group (Young

and older) was a result of the power calculation based on changes in MPS following a period of SR (i.e. 10 participants in each group). It must however be said that in the outcome measures were no difference was found, the p values are high, and it could therefore be considered that there is unlikely a difference, and this finding is not because of a lack of data. In addition, similar prior studies have used participant groups of similar number and found differences. Clearly further studies investigating the effects of SR on glucose homeostasis should perform outcome specific power calculations.

The finding of a return to baseline of all glucoregulatory measures after remobilisation infers that there was no significant, long-lasting effect of short-term SR. This poses the question of the significance of the observed changes and whether, particularly in the elderly where there was an increase in the AUC after 48 hours but not 14 days, further SR would detrimentally effect glucoregulation. In addition, despite demonstrating an effect of SR on glucoregulatory parameters, it remains impossible to confirm this as being a significant causative factor in the association between increasing age and reduced insulin sensitivity. Furthermore, based on these results it cannot be determined what effect

further SR in the young group would have had on glucoregulation and despite the logistical issues that we encountered we would encourage further studies that utilise a longer period of SR, particularly in the young participants.

Body composition measured by DEXA is a well-established technique with reliable, well refined results being obtained over multiple timepoints. We have no reason to question the results obtained in this study following SR and would challenge the notion that body composition changes are acutely responsible for alterations in glucose homeostasis. We believe that a benign intervention over a relatively short period is unlikely to result in significant, physiology altering changes in body composition despite other studies suggesting this to be the case. Clearly long-term or more significant forms of inactivity has been proven to induce muscle atrophy or result in accumulation of adipose tissue which may in part be responsible for changes in glucoregulation but through a different mechanism to those seen in mild, short-term inactivity.

The role of skeletal muscle IMCL accumulation and insulin resistance is not well defined. Early studies had suggested that

IMCL accumulation had a role in regulating whole body insulin resistance (Krssak et al., 1999), but further studies in tandem with the introduction of quantification of IMCL via 1H-MRS techniques, have shown the relationship between IMCL and whole body insulin resistance to be more complex (Gemmink et al., 2017, Kitessa and Abeywardena, 2016). In Chapter 3, 14 days of SR had no effect on vastus lateralis IMCL content despite changes in glucoregulation in both groups, further supporting the current understanding that the relationship is not of a linear nature. The contribution of EMCL and the IMCL/EMCL ratio to whole body insulin resistance remains unclear. Despite an increase in the IMCL/EMCL ratio in the young group following 14 days SR, which was mirrored by an increase in the AUC for insulin, the mechanistic relationship between the two remains elusive. Indeed, while remobilisation resulted in a return to baseline for insulin AUC during an OGTT, the IMCL/EMCL ratio remained elevated. A similar situation exists for the role of EMCL. Throughout the study EMCL content was significantly greater in the older group, despite there being no significant difference in the DEXA measured leg fat mass between groups. There was no change associated with SR or remobilisation thus the search for any specific

function of EMCL continues, other than it simply being a site of lipid deposition and storage.

1H-MRS has significantly improved the detection of IMCL and EMCL in human volunteers and is opening the door to novel research. However, it must be acknowledged that both the acquisition of and the analysis of the spectra require significant manual processing which has the potential to increase the COV and thus decrease the accuracy of the results. This is also true of muscle lipid determination using wet lab based biochemical and imaging analyses, but further work on the development of an automated processes for 1H-MRS IMCL and EMCL quantification would be welcome as it will likely improve methodological reproducibility. The technique employed in this study is novel and unfortunately no COV was calculated. As discussed previously, the technique involved two different MRI scanner operators who were not blinded to the stage of the study the participants were in, and it is likely that a learning curve of scan acquisition could have influenced the results obtained and further refinement of the processes involved are required. It is however clear that the link between inactivity, glucose homeostasis and IMCL, EMCL and the IMCL/EMCL ratio are far more complex than originally thought

and the findings identified in this study are likely irrelevant from a clinical viewpoint.

Muscle mass is maintained by the net balance of MPS and MPB. MPS is stimulated by anabolic drivers including exercise and EAA ingestion. In healthy humans, changes in muscle mass are the result of prolonged alterations in the rate of MPS, with MPB playing a minor role (Brook et al., 2022). Inactivity results in a blunting of the exercise stimulus of MPS with resultant reductions in skeletal muscle mass over time. Bed rest and limb immobilisation have shown a reduction in skeletal muscle mass with associated reductions in myofibrillar FSR following a relatively short period of time (Shur et al., 2024, Brook et al., 2022). In addition, similar findings have been reported in a limited number of studies following 14 days of SR (Breen et al., 2013b). However as described in Chapter 4, despite both young and older volunteers adhering to the SR protocol and achieving a >90% reduction in daily step count for 14 days, we did not demonstrate any decline in cumulative myofibrillar FSR in either young or older volunteers over 14 days SR. Furthermore, neither did we find a change in whole body MPB after 14 days of SR, which is in keeping with literature reporting no change in leg MPB following 4 days of

limb immobilisation (Brook et al., 2022). These findings do not really come as a surprise given the lack of evidence in the literature demonstrating MPB as having any significant effect on inactivity induced human muscle turnover and despite no pre-study power calculation for this outcome measure, there is no reason to doubt the results. As far as I am aware, this is the first study that has utilised the D_3-3MH technique to quantify rates of skeletal muscle breakdown following a period of SR. As previously discussed, in support of the lack of decline in MPS after 14 days of SR, there were no changes from baseline in DEXA whole body FFM or AFFM, and neither MRI mid-thigh CSA nor thigh muscle volume. Interestingly in Chapter 5 however, estimation of whole-body skeletal muscle mass using the D3-creatine dilution method showed whole body muscle mass was reduced by 12.1% following 14 days SR and returned to baseline on remobilisation (See later).

Unlike the findings within this Thesis, there are published SR studies that have reported a reduction in cumulative MPS following a 14-day period of SR (McGlory et al., 2018, Oikawa et al., 2019). However, the baseline rates of MPS in both studies were significantly higher than our findings. In addition, in both studies following SR the cumulative daily rate was

more in keeping with our baseline results and failed to return to baseline following remobilisation. Furthermore, the body composition changes that were reported in these studies were not what might be expected based on the changes in rate of FSR. Certainly, the findings reported in Chapter 4 suggest that a short period of reduced daily ambulatory activity is not as detrimental on cumulative MPS muscle protein turnover as previously suggested, and this is supported by the lack of body composition changes. It is clear that further work is needed to clarify the true effects on MPS following SR and as discussed within Chapter 4, the *a priori* power calculation indicated that 10 participants were required within each group and unfortunately due to sample loss this was not achieved. It must be considered that this could have affected the results obtained and despite our findings being consistent with the lack of body composition changes, it cannot be said with certainty that no difference exists in myofibrillar FSR following 14 days of SR.

The measurement of body composition is traditionally performed through the utilisation of imaging techniques such as MRI, CT and DEXA as well as BIA. The cost and logistical implications of each imaging technique limit the widespread

application to large cohorts, both in the patient clinic and research setting. With the development of the D3-creatine dilution method (Clark et al., 2018), excitement has been garnered in the potential of this tracer method to measure whole-body muscle mass in cross-sectional study designs (Banack et al., 2022, Cawthon et al., 2019, McCarthy et al., 2022). Chapter 5 assessed the utility of the D3-Cr dilution method to quantify change in whole-body muscle mass in a longitudinal study design, i.e. at baseline, after 12 days of SR and following 12 days of remobilisation, in both young and older cohorts. Associations between D3-creatine derived muscle mass and DEXA measures of appendicular fat free mass (AFFM), MRI derived mid-thigh CSA and thigh muscle volume were also assessed.

D3-Cr estimated muscle mass was significantly lower following SR than at baseline in young volunteers, and returned to baseline with remobilisation, which was not evident with DEXA derived AFFM or MRI measures of thigh muscle volume and CSA. No change in body composition with any of the techniques employed was observed in the older volunteers, identifying a clear disconnect between the established measures of body composition and D3-Cr estimated whole

body muscle mass. However, in keeping with previous reports, strong associations were observed between D3-creatine derived whole body muscle mass and DEXA AFFM ($r=0.8242$, $p<0.0001$), MRI mid-thigh CSA ($r=0.8491$, $p<0.0001$) and thigh muscle volume ($r=0.8231$, $p<0.0001$).

It must also be acknowledged that the loss of whole-body muscle mass as measured by D3-Cr was not associated with a change in skeletal muscle FSR, which is not in keeping with prior studies that have utilised other body composition measures (i.e DEXA); loss of muscle mass is associated with a reduction in skeletal muscle FSR. It is therefore difficult to interpret these collective body composition measurements, particularly given no change in MPS, MPB or fat mass (DEXA) was identified with SR and remobilisation. It must be acknowledged however that D3-creatine dilution measures whole-body muscle mass, as opposed to DEXA and MRI that measure regional and whole-body muscle volumes, not mass. The strong associations between D3-creatine derived whole-body muscle mass and DEXA derived AFFM, MRI measures of thigh volume and CSA, provide some support for the D3-Cr dilution technique, but there was evidence of systematic bias identified by the Bland Altman indicating that further

refinement is required and at present it does not have an established body of evidence to fully support the findings that have been reported in this study. Clearly further studies are required to establish the technique as a robust alternative to more invasive measures, but these results show promise.

The public health implications of a progressively inactive, ageing population, as discussed throughout this thesis, are well founded and significant. There is no doubt that a sedentary lifestyle increases the risk of metabolic disease, cancer and all-cause mortality. What is yet to be established is the degree of inactivity and/or time over which inactivity induced detrimental effects occur. Here we have reported the effects of a very short period of inactivity, when in all probability it is the effects of many years of reduced physical activity that culminates in the development of significant disease processes. However, despite the relatively mild technique of inactivity that was enforced over a short period of time, other studies have identified changes in body composition, glucoregulation and muscle FSR. A major difference between these prior studies and the current study is the recorded baseline physical activity levels of participants. These findings could suggest that those who are physically

active at baseline (young and older) may have a degree of protection against a short period of physical inactivity, as opposed to the step wise decline that is often observed in habitually inactive older people that suffer intermittent short periods of immobility.

There are several questions raised from the findings of this study that could direct future research. Emphasis must be placed on whether habitually active people have a degree of protection to short term inactivity and future studies that aim to recruit habitually active older people and compare them to habitually inactive older people following a period of inactivity would help answer this question further. A study of this nature could further direct research into the level of habitual activity that confers a level of protection and help to further guide healthy ageing.

Future physical activity research should focus greater attention to ensuring volunteers are well-matched at baseline, as based on the findings of this thesis, this is likely to be a confounding factor in the interpretation of study outcomes. Finally, future research efforts should be devoted to undertaking a direct comparison of step count reduction vs

bed-rest vs immobilisation models on metabolic and body composition end-point measurements.

In summary, this thesis has reported that in well-matched, healthy, physically active young and older participants, a period of 14 days of SR to less than 1500 steps per day had no impact on leg cumulative MPS, whole-body MPB, or several body-composition measures (with the exception of D3-creatine derived whole body muscle mass). In older volunteers, 48 hours of SR induced an increase in the AUC of both blood glucose and serum insulin during an OGTT, and for insulin in the younger volunteers after 14 days of SR. Following remobilisation, all measures of glucose homeostasis returned to baseline. As far as I am aware this thesis describes the first longitudinal study to utilise the D3-creatine dilution method to quantify whole-body muscle mass at multiple times, and is the first study to employ the D_3-3MH technique to estimate MPB following SR. It is reasonable to conclude that short term reduced ambulatory activity in well-matched, otherwise fit and healthy, habitually active young and older humans did not have major, persistent effects on skeletal muscle metabolism as might be predicted from human bed rest and limb immobilisation research.

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Appendix 1

Physical Inactivity Study Health Questionnaire

Participant Contact Details

Today's date: ……..

Title: ……
First name: …… **Surname**: ……
Date of Birth:……

Address:
House number: ……
Street name: ……
Town/City: ……
Postcode: ……
Email: ……

Telephone:
Home: ……
Work: ……
Mobile: ……
Preferred method of contact:
……

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

---

Title: ……
First name: …… **Surname** :……

Address:
House number: ……
Street name: ……
Town/City: ……
Postcode: ……
Email: ……

Telephone:
Home: ……
Work: ……
Mobile: ……
Preferred method of contact:
……

GP Details
Doctor/Surgery name: ……
Address: ……
……
Telephone: ……

GENERAL HEALTH QUESTIONAIRE

1. 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 must 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 ever 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 **60 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?

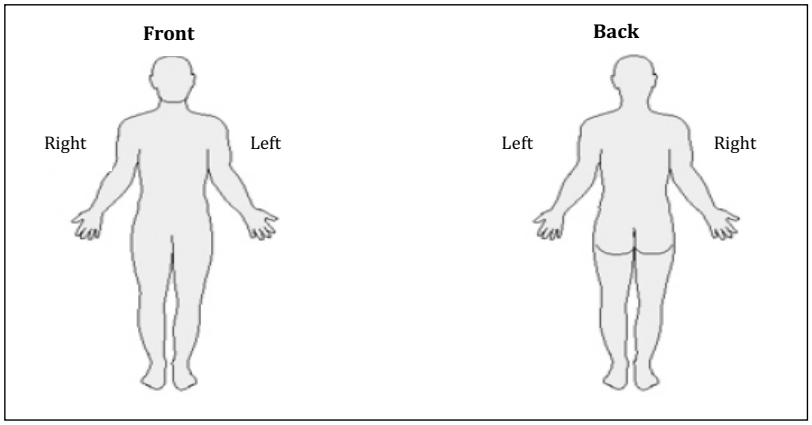
Yes…….. No……..

If no, the questionnaire is now complete.

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 or pain **1** **2** **3** **4** **5** **6** **7** **8** **9** **10** **Worst possible discomfort**

6. How often do you feel this discomfort?

☐ **Rarely** ☐ **Occasionally** ☐ **Often** ☐ **Frequently** ☐ **All the time**

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

☐ **1-2 weeks** ☐ **1-2 months** ☐ **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.

Screening Checklist:

Consent	
ECG	
Blood Pressure	
Bloods	
Height/Weight	
Strength Test	
Actiheart	

HEIGHT AND WEIGHT

Height		cm	
Weight		Kg	
BMI		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 | |

University of Nottingham
UK | CHINA | MALAYSIA

School of Life Sciences

Human Tissue Act:
Healthy Volunteer Consent Form (HVCF)

Premises covered	Nottingham Medical School	This is version	1.1
Document Created	06/09/2018	Document issued in	
Review Period		To be reviewed in	

Project Title: Are the metabolic effects of physical inactivity more pronounced in younger than older healthy volunteers?
Name of Investigators: Mr Lee Creedon, Prof. Paul Greenhaff, Prof Phillip Atherton, Mr Jonathon Lund
REC REF: A10022017

Please read this form and sign it once the investigator above-named, or their designated representative, has explained fully the aims and procedures of the study to you.

Please Initial Box

- I voluntarily agree to take part in this study. ☐
- I confirm that I have been given a full explanation of the study and that I have read and understand the information sheet (version 1.3) for the above study and have had the opportunity to ask questions. ☐
- I understand that I am free to withdraw from the study at any time, without having to give a reason for withdrawing. Should I withdraw then the information collected so far cannot be erased and that this information may still be used in the project analysis. ☐
- I agree to comply with the reasonable instructions of the research team and will notify them immediately of any unexpected unusual symptoms or deterioration of health. ☐
- I give permission to collect, store, analyse and publish information obtained from my participation in this study. I understand my personal details will be kept confidential. ☐
- I understand that data collected in the study may be looked at by responsible individuals from the University of Nottingham and regulatory authorities where it is relevant to my taking part in this study. ☐
- I understand that information about me recorded during the study will be kept in a secure database. If data is transferred to others it will be made anonymous. Data will be kept for 7 years or after all results of this study have been published. After this time, it will be disposed of in line with University policy. ☐
- I understand that the tests or procedures are carried out for research only and not for clinical diagnostic purposes. However, if the study investigator should feel it necessary to inform my GP of my participation in the study, or of an adverse event or abnormal test result, I understand I am giving my consent to do so. ☐
- I confirm that I have disclosed relevant medical information before the study. ☐

RECIPES AND COMMENT

Amount	Ingredient	Comments

The University of Nottingham
UNITED KINGDOM · CHINA · MALAYSIA

Inactivity Study

DIET DIARY

Please complete your diary on 7 consecutive days
(including 5 weekdays and 2 weekend days)

Day 1 …….. Day 2 ……..

Day 3 …….. Day 4 ……..

Day 5 …….. Day 6 ……..

Day 7 ……..

Code:

INSTRUCTIONS FOR FILLING IN YOUR FOOD DIARY

- Please keep a food diary for the 7-day period shown on the front cover. Please include **seven consecutive days including five week days plus two weekend days**. The record should include as much detail as possible. Please start a new page each day (ensure the day and date is recorded at the top of the page).

- Fill in the food diary each time you consume a drink, a meal or a snack. Please note the time, and describe everything you eat or drink. Use a further page if you need more space. (Please remember to record the day and date at the top of the new page).

- When you record a food or drink, please give a thorough description and include any Brand names. You cannot give us too much detail! For example:-
  - Was the bread white/wholemeal/granary
  - Did you have a baguette/cob/pitta/slice?
  - Did you use butter or margarine, was the margarine made with olive/sunflower oil?
  - Was the product a diet product- e.g. sugar-free lemonade?
  - Was anything added- e.g. sugar to tea, butter to toast, tomato ketchup or mayonnaise etc?
  - Do you use full fat/semi-skimmed/skimmed milk?
  - How did you cook your food – eg. boiled/fried/baked/raw?
  - How was the food processed – eg. fresh/tinned/frozen?
  - Was the product Heinz/Tesco's own etc.?
  -
- The amounts you have should be described as fully as possible using household measures
  - Use kitchen equipment – a measuring jug, a mug (of know volume), a teaspoon (see below) and a tablespoon

DIET DIARY

Day 7	Food/Drink Consumed	Amount /portion size As much description as possible
Meal 1		
Meal 2		
Meal 3		
Meal 4		
Snacks		

Please circle:

Was the bread white/wholemeal/granary thick/med/thin slice

Did you use butter or margarine

Please put brand of product where possible

DIET DIARY

Day 6	Food/Drink Consumed	Amount /portion size As much description as possible
Meal 1		
Meal 2		
Meal 3		
Meal 4		
Snacks		

Please circle:

Was the bread white/wholemeal/granary thick/med/thin slice

Did you use butter or margarine

Please put brand of product where possible

- Look on cans and packets to see whether the weight/volume is given and describe how much of the packet you had (eg. Half a 400g can of Heinz tomato soup)
- Record the number of sausages/fish fingers/bacon rashers etc. that you ate
- Describe the size of a piece of food – eg. egg sized potato, small matchbox sized piece of cheese
- Please record each item of food separately. If you drink white tea with sugar, please record the type and amount of tea, milk and sugar separately.
- If you had a packed lunch, each part must be recorded in the diary.
- It is important that you complete the 'leftovers' section with anything you did not actually eat. Please give us as much detail as possible
- For homemade recipes, the raw ingredients should be recorded please record all ingredients and then write down how much you have eaten e.g ¼ etc

Examples of Portion sizes

3

DIET DIARY

Day 5	Food/Drink Consumed	Amount /portion size As much description as possible
Meal 1		
Meal 2		
Meal 3		
Meal 4		
Snacks		

Please circle:

Was the bread white/wholemeal/granary thick/med/thin slice

Did you use butter or margarine

Do you use full fat/semi-skimmed/skimmed milk?

Please put brand of product where possible

8

DIET DIARY

Day 4	Food/Drink Consumed	Amount /portion size As much description as possible
Meal 1		
Meal 2		
Meal 3		
Meal 4		
Snacks		

Please circle:

Was the bread white/wholemeal/granary thick/med/thin slice

Did you use butter or margarine

Do you use full fat/semi-skimmed/skimmed milk?

7

DIET DIARY

Day 1	Food/Drink Consumed	Amount /portion size As much description as possible
Meal 1		
Meal 2		
Meal 3		
Meal 4		
Snacks		

Please circle:

Was the bread white/wholemeal/granary thick/med/thin slice

Did you use butter or margarine

Do you use full fat/semi-skimmed/skimmed milk?

4

DIET DIARY

Day 2	Food/Drink Consumed	Amount /portion size As much description as possible
Meal 1		
Meal 2		
Meal 3		
Meal 4		
Snacks		

Please circle:

Was the bread white/wholemeal/granary thick/med/thin slice

Did you use butter or margarine

Do you use full fat/semi-skimmed/skimmed milk?

Please put brand of product where possible

DIET DIARY

Day 3	Food/Drink Consumed	Amount /portion size As much description as possible
Meal 1		
Meal 2		
Meal 3		
Meal 4		
Snacks		

Please circle:

Was the bread white/wholemeal/granary thick/med/thin slice

Did you use butter or margarine

Do you use full fat/semi-skimmed/skimmed milk?

Please put brand of product where possible

The University of Nottingham

Sir Peter Mansfield Imaging Centre

MR Volunteer Safety Screening Questionnaire:

NAME	Date of Scan	Date of Birth
ADDRESS	Volunteer Number	
	Ethics Code	
Phone number	Weight	Height if applicable

MR scanning uses strong magnetic fields. For your own safety and the safety of others it is **very important** that you do not go into the magnet halls with any metal in or on your body or clothing. Please answer the following questions carefully and ask if anything is not clear. All information is held in the strictest confidence.

1. Do you have any implants in your body? e.g. replacement joints, drug pumps Y/N
2. Do you have aneurysm clips (clips put around blood vessels during surgery)? Y/N
3. Do you have a pacemaker or artificial heart valve? *(These stop working near MR Scanners)* Y/N
4. Have you ever had any surgery? Please give brief details over. Y/N
   (We do not need to know about uncomplicated caesarean delivery, vasectomy or termination of pregnancy)
5. Do you have any foreign bodies in your body (e.g. shrapnel)? Y/N
6. Have you ever worked in a machine tool shop without eye protection? Y/N
7. Do you wear a hearing aid or cochlear implant? Y/N
8. Could you be pregnant? (Pregnancy tests are available in the female toilets) Y/N
9. Have you ever suffered from tinnitus? Y/N
10. Do you wear dentures, a dental plate or a brace? Y/N
11. Are you susceptible to claustrophobia? Y/N
12. Do you suffer from blackouts, epilepsy or fits? Y/N
13. Do you have any tattoos? (If yes, you may be asked to read and sign another form) Y/N
14. Do you have any body piercing jewellery that cannot be removed? Y/N
15. Do you have any skin patches (trans-dermal patches)? Y/N
16. Do you have a coil in place (IUD) for contraception? Do you know what type? Y/N
17. Do you have any condition that may affect your ability to control your temperature ?
    (e.g. Do you have a fever, cardiovascular disease, hypertension, diabetes or cerebrovascular disease?) Y/N
18. Will you remove all metal including coins, body-piercing jewellery, false-teeth, hearing aids etc. before entering the magnet hall? *(lockers available by the changing rooms)* Y/N
19. Is there anything else you think we should know? Y/N

I have read and understood all the questions **Signature:**	**Date:**
Verified by: **Scanner Operator/MR Assistant Signature :**	**Date**