

Effect of immobilisation on human forearm fuel metabolism

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Thesis abstract

Worldwide it has been estimated that physical inactivity causes between 6-10% of global deaths, with low physical activity levels directly linked to 25-27% of the incidence of type 2 diabetes, 21-25% of breast and colon cancers, and approximately 30% of ischaemic heart disease. Thus, elucidating the underlying mechanisms between physical inactivity and the associated negative implications for health is imperative to establish and implement effective countermeasures. Currently bed rest and limb immobilisation represent the most popular experimental models used to investigate the functional and metabolic perturbations associated with physical inactivity. Forearm immobilisation was employed in each of the studies presented in this thesis, a model which allows the contralateral, non-immobilised arm to act as a within volunteer control. Furthermore, upper limb immobilisation facilitates the exploration of the reduced muscle use without the confounding effects of decreasing overall levels of physical activity and has a decreased risk of deep vein thrombosis compared to lower limb immobilisation.

The studies included in this thesis aimed to investigate the time course of metabolic and physiological changes in response to forearm immobilisation. Initial pilot work was conducted to determine which post-sampling processing methods are most appropriate to produce accurate and stable measures of whole blood glucose concentration in conjunction with the arterialised venous-venous substrate difference technique. Previous studies have shown intermittent bouts of resistance exercise during forearm immobilisation are not sufficient to protect against the decline in insulin stimulated muscle glucose uptake, a marker of insulin sensitivity, measured 36-48 hrs after the last bout of exercise. Thus, the first study in this thesis aimed to

elucidate the time course of changes in insulin sensitivity over 3 days of immobilisation in 10 healthy, male volunteers and the findings revealed a marked decline (38%) in insulin sensitivity within 24 hrs of immobilisation. This reduction was sustained throughout the immobilisation period, and was not accompanied by parallel decrements in brachial artery blood flow and insulin concentration. The second study included 9 healthy, young males and aimed to investigate the impact of 2 days of immobilisation on forearm lipid clearance, for which no effect was observed. This study also aimed to identify any additional impact of increasing dietary lipid supply on decrements in insulin sensitivity and demonstrated increased dietary lipid supply does not affect the magnitude of decline in insulin sensitivity during forearm immobilisation. Overall, findings from the first two studies indicate impairments driving the rapid decline in insulin sensitivity associated with forearm immobilisation reside within the muscle. To further investigate the role of reduced muscle contraction on muscle glucose uptake during forearm immobilisation, a pilot study was carried out to determine whether regular low-grade electrically-evoked muscle contraction could blunt the immobilisation induced changes in muscle glucose uptake in response to an oral glucose challenge. The findings from this pilot study suggest that frequent muscle contraction, elicited via PES, throughout the immobilisation period is unable to prevent this decline in forearm glucose uptake.

A growing body of work links the accumulation of intramyocellular lipid during muscle disuse with reduced insulin sensitivity, thus the third study aimed to investigate any relationship between decreased insulin sensitivity with increased lipid content of forearm muscle, assessed via proton magnetic resonance spectroscopy (^{1}H-MRS), during16 days of limb immobilisation in 5 healthy male

volunteers. Additionally, changes in forearm muscle cross section area and handgrip strength were also assessed. The major findings from this study support those of the earlier studies demonstrating rapid and marked decrements in muscle insulin sensitivity, which are sustained over 8 days of immobilisation. Forearm muscle cross sectional area and handgrip strength also significantly declined, however, decrements in strength were of a much greater magnitude than muscle cross sectional area. Despite extensive method development, the scanning protocol established was unable to obtain sufficient quality data to evaluate changes in forearm lipid content associated with immobilisation. Thus, due to the small volume and structure of forearm muscles, the lower limb remains the optimal model for obtaining quality spectra using ^{1}H-MRS.

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Declaration

All the work presened in this thesis was the result of preocedures conducted by myself with the following exceptions:

In Chapters 3, 4 and 5 cannulations were performed by Aline Nixon, Dr. Liz Simpson and Dr. Tariq Taylor, and measurement of forearm blood flow was conducted by Aline Nixon and Dr. Jo Mallinson. Sally Cordon performed the blood analyses for studies described in Chapters 3,4 and 5, and Dr. Jo Mallinson performed forearm casting for these studies. The staff of the Sir Peter Mansfield MRI Centre for conducted the MRI and MRS scans for the study included in Chapter 5.

I hereby declare that this thesis has been composed by myself and it is a record of the work performed by myself, exccept where assistance has been acknowledged. No part of this thesis has been submitted in any other applpication for a higher degree and all sources of information have been appropriately referenced.

Aisling Burns

December 2018

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List of presentations arising from this thesis

Impact of forearm immobilisation on metabolic function of skeletal muscle in healthy volunteers. Oral presentation at the School of Life Sciences Annual Symposium, University of Nottingham, UK (July, 2017).

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Novel insight regarding the trajectory and time-course of immobilisation induced impairment of limb glucose uptake in healthy volunteers. Poster presentation at the annual American Physiology Meeting at Experimental Biology, San Diego, USA (April, 2016).

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List of abbreviations

° C	Degrees celcius
Acetyl CoA	Acetyl CoenzymeA
Akt	Protein kinase B
AMPK	5’ adenosine monophosphate-activated kinase
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BMI	Body mass index
BR	Bed rest
Ca^{2+}	Calcium
CaMK	Calmodulin-dependent protein kinase
CD36	Fatty acid translocase
CGI	Continuous glucose infusion
CSA	Cross sectional area
CV	Coefficient of variation
DAG	Diacylglycerol
ECG	Electrocardiogram
EM	Electron microscopy
EMCL	Extramyocellular lipid
FAK	Focal adhesion kinase
FABPpm	Plasma membrane associated fatty acid binding protein
FATP	Fatty acid transport protein
FFA	Free fatty acid
FDP	Flexor digitorium profundus
FGU	Forearm glucose uptake
FOM	Fibre orientation modelling
GLUT	Glucose transporter
H_2O_2	Hydrogen peroxide
H-E clamp	Hyperinsulinemic-euglycemic clamp
H-I clamp	Hyperinsulinemic-isoglycemic clamp
HK II	Hexokinase II
^{1}H MRS	Proton magnetic resonance spectroscopy
HOMA-IR	Homeostatic model of analysis of Insulin Resistance

IMCL	Intramyocellular lipid
IMM	Immobilised
IRS	Insulin receptor substrate
IVGTT	Intravenous glucose tolerance test
LGU	Leg glucose uptake
LL	Lower limb immobilisation
mTOR	Mammalian target of rapamycin
MRI	Magnetic Resonance Imaging
MRS	Magnetic Resonance Spectroscopy
ms	Milliseconds
NCAM	Neural cellular adhesion molecule
NIM	Non-immobilised
OGTT	Oral glucose tolerance test
PDC	Pyruvate dehydrogenase complex
PDH	Pyruvate dehydrogenase
PI3-k	Phosphatidylinositol-3 kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIP_3	Phosphatidylinositol (3,4,5)-triphosphate
PKC	Protein kinase C
QUIKI	Quantitative insulin sensitivity check index
RA	Reduced ambulation
ROI	Region of interest
SD	Standard deviation
S-E clamp	Sequential euglycemic clamp
SEM	Standard error of the mean
SNR	Signal to noise ratio
SPMIC	Sir Peter Mansfield Imaging Centre
TAG	Triacylglycerol
TE	Echo time
TR	Repetition time
UL	Upper limb immobilisation
WBGU	Whole body glucose uptake
WET	Water suppression enhanced through longitudinal relaxation time

Chapter 1: Introduction

Although the study of the specific negative health effects of physical inactivity is relatively recent, early work from Professor Jerry Morris led to the recognition of the negative health implications associated with sedentary lifestyles. Morris and colleagues were amongst the first to perform rigorous, epidemiologic studies comparing disease outcomes associated with varying levels of physical activity by investigating the impact of sitting time on the health of London double decker bus drivers, and compared this group with physically active bus conductors (Morris *et al.*, 1953). The authors reported men with jobs requiring long periods of sitting had a 2-fold increased risk of cardiovascular disease, and these findings were later reproduced in active postmen compared with physically inactive telephonists in the British Civil Service (Morris and Crawford 1958). More recent reports attribute 6% of global deaths to physical inactivity (WHO 2009), with low physical activity levels directly linked to 25-27% of the incidence of type 2 diabetes, 21-25% of breast and colon cancers, and approximately 30% of ischaemic heart disease (WHO 2009). The ever increasing levels of chronic sedentary behaviour have been in part attributed to technological advances resulting in reduced occupation and transport related movement, in combination with increased time spent on leisure activities, such as playing computer games. Dunstan *et al.* reported an association between television viewing time and increased risk of cardiovascular disease and all-cause mortality, with each one hour increment in television viewing time associated with an 11% and 18% increase in risk of all-cause and cardiovascular disease mortality respectively (Dunstan *et al.*, 2010). Overall, it is clear that a greater understanding of the early physiological alterations which occur as a result of increasing levels of physical inactivity are necessary to combat the global health burden associated with sedentary lifestyles.

1.1 Experimental models inducing muscle disuse

Many longitudinal studies have established correlations between sedentary lifestyles and increased risk of cardiovascular disease, obesity and type 2 diabetes, as determined by time spent watching television (Hu *et al.,* 2003, Jakes *et al.,* 2003, Dunstan *et al.,* 2010). To explore the physiological responses to decreased muscle use associated with physical inactivity and sedentary lifestyles, several experimental models have been developed. These experimental models typically either reduce physical activity levels or induce limb or whole body immobilisation. Physical inactivity is a behaviour characterised by low levels of physical activity, whereas immobilisation describes the deprivation of muscle use in which the entire body or a single limb is mechanically unloaded. While immobilisation is indeed on the physical inactivity continuum, it is a more extreme state than physical inactivity and involves distinct experimental designs. To a large extent, the terms physical inactivity and immobilisation are used interchangeably in the literature, and physical inactivity is often used to describe adaptations induced via immobilisation experiments (Hamburg *et al.,* 2007, Bergouignan *et al.,* 2011). While there are numerous studies investigating the effects of immobilisation using animal models, this review of the current literature will focus on those studies using human models.

1.1.1 Space flight

In a microgravity environment such as those experienced during space flight, muscles are not required to perform their normal load bearing functions and this soon results in both functional and metabolic adaptations, which has provided insight into metabolic responses to reduced muscle use. However, findings obtained during

spaceflight are limited due to the difficulty in obtaining such data, high financial costs and the inconsistencies in flight conditions.

1.1.2 'Dry' water immersion

'Dry' water immersion can be used as a model of weightlessness as the volunteer is immersed in thermoneutral water, while wearing an elastic wetsuit. This form of unloading results in the rapid decline in both muscle tone and motor activity level (Grigor'ev *et al.,* 2004), which cannot be replicated even by prolonged bed-rest (Kovalenko EA 1980). Koryak *et al*. reported an 8.2% decrease in muscle mass following 7 days of dry water immersion (Koryak 1998). This model of immobilisation may be favoured as it can produce similar outcomes to those observed using other models at an accelerated rate (Shenkman *et al.,* 1997), however, the necessary equipment and personnel required may be considered to be a limitation.

1.1.3 Bed rest

Bed-rest is a popular models to study the physiological effects of muscle disuse in a controlled environment. This model can be used for short or extended periods of time, requiring volunteers to remain in a horizontal or head down tilt position. While minimal muscle use during bed-rest induces measurable physiological adaptations, this model also facilitates the effect of other factors such as energy balance, diet and pharmacological interventions to be investigated. However, bed rest studies are limited by high financial costs, substantial manpower required and

access to the necessary clinical settings, which are out of the reach of many individual academic investigative teams.

1.1.4 Limb suspension and immobilisation

In 1991, Tesch and colleagues pioneered the mechanical unloading of human muscles by immobilising a lower limb (Tesch *et al.*, 1991), which is now among the most popular models used to explore muscle disuse. Studies using limb suspension and immobilisation involve suspending the limb using a shoulder harness or placing the limb in a cast to restrict movement, whilst still allowing other daily habitual activities as much as possible. This model of immobilisation produces similar physiological adaptations to those observed with bed-rest and the changes in young, healthy volunteers at least are usually confined to the immobilised limb, meaning the localised effects of the intervention may then be compared to the contralateral, non-immobilised limb. A major advantage of limb immobilisation and suspension studies is the less invasive nature for volunteers compared to studies requiring subjects to be suspended in water or confined to bed. Although some methods of limb immobilisation and suspension may require extra precautions to ensure participant compliance, overall this represents a relatively inexpensive method to examine the physiological responses to muscle unloading and disuse. Upper limb immobilisation may be favoured in some study designs as lower limb immobilisation has been indicated to increase risk of deep venous thrombosis, with Bleeker *et al*. reporting an incidence of approximately 2.7 % (Bleeker *et al.*, 2004). Thus, use of lower limb immobilisation requires extra precautionary measures to be undertaken to prevent possible thrombosis. Upper limb immobilisation also permits the study of

reduced muscle contraction without the confounding effects of reducing overall physical activity, which also effects energy balance.

1.1.5 Reduced ambulatory activity

An increasingly popular model of physical inactivity involves decreasing ambulatory activity by reducing the daily step-count or increasing time spent sitting by participating volunteers. Typically investigations transition participants from a high to a low level of ambulatory activity (>10,000 to <2,000steps per day). This method of reducing step count has been employed to induce a decline of 18-37% in whole body insulin sensitivity over 1-3 days, in concert with a decline in cardiovascular fitness and lean leg mass (Krogh-Madsen 2010, Stephens *et al.,* 2011, Knudsen *et al.,* 2012). Although reducing ambulatory activity may be considered by some to be the most applicable model of physical inactivity representing the increasingly sedentary global population, the associated physiological alterations may take longer to reach measurable levels compared to other models of physical inactivity.

1.2 Insulin sensitivity

As reduced insulin sensitivity is an independent risk factor for many chronic conditions such as type 2 diabetes and cardiovascular disease, the development of several tools to assess insulin sensitivity have benefited both clinicians and academic researchers alike. While the majority of methods for measuring insulin sensitivity are generally comparable, some inconsistencies have been reported and should be considered when selecting the most appropriate methods to answer the question at hand.

1.2.1 Direct measures of insulin sensitivity

1.2.1.1 Hyperinsulinemic-euglycemic clamp

The hyperinsulinemic-euglycemic clamp technique was first developed by Andres and DeFronzo in 1979 and is now frequently referred to as the reference standard for measuring insulin sensitivity (DeFronzo *et al.,* 1979). After an overnight fast, a continuous intravenous insulin infusion is used to elevate and sustain an individual's plasma insulin concentration above basal values. This results in a decrease in hepatic glucose production, while glucose disposal in adipose tissue and skeletal muscle is increased. Meanwhile, blood glucose levels are frequently monitored and 20% dextrose is intravenously infused at a variable rate to 'clamp' blood glucose concentrations within the normal range. When near steady state for both glucose and insulin has been achieved, the mean glucose infusion rate (M-value) provides a measure of whole body insulin sensitivity. While this method is conceptually straightforward and widely used, there are some technical issues which complicate the utilisation of this technique. The major drawback of utilising a hyperinsulinemic-euglycemic clamp relate to its labour intensive and time-consuming nature, which is expensive compared to other methods and requires very experienced personnel. Therefore, this technique is not feasible for studies using large populations, or indeed for many smaller, independent academic research groups.

1.2.1.2 Insulin suppression test

Following an overnight fast endogenous insulin and glucagon secretion is suppressed by the intravenous infusion of either somatostatin or an analogue of somatostatin, such as ocreotide, at a rate of 250μg per hour or via an initial bolus of 25μg and

0.5µg per minute thereafter for three hrs. Concurrently, insulin and glucose are also infused into the same antecubital vein, while blood sampling from the contralateral arm facilitates the determination of glucose and insulin concentrations, which are measured every 30 minutes for the initial 2.5 hrs and every 10 minutes for the final 30 minutes. A steady state of plasma insulin and glucose, assumed to be from 150-180 minutes after the start of the insulin suppression test, is achieved by the constant infusions of glucose and insulin. The concentration of insulin in the steady state is usually similar between individuals, therefore, an individual's steady state plasma glucose concentrations are reflective of their level of insulin sensitivity, as glucose concentrations during this period are inversely proportional to insulin sensitivity, i.e. higher in insulin resistant conditions and lower in insulin sensitive individuals. In this way the insulin suppression test uses the steady state plasma glucose level as a direct measure of the capacity of the exogenous insulin infused to increase the disposal of the infused glucose load. Assessment of insulin sensitivity using the insulin suppression test has been shown to be similar to determinations made using the gold standard hyperinsulinemic-euglycemic clamp method in healthy subjects ($r=0.93$) and largely reflects insulin sensitivity of skeletal muscle (Greenfield *et al.*, 1981). Furthermore, the insulin suppression test requires less personnel and is not as technically demanding compared to the hyperinsulinemic-euglycemic clamp method. However, running an insulin suppression test can be expensive and still requires experienced personnel which may limit this methods feasibility for determining insulin sensitivity. Furthermore, hypoglycaemia associated with an insulin suppression test can cause additional adverse side-effects for participants, including nausea, coldness, sweating or even cardia arrhythmias (Binder *et al.*, 2004).

1.2.2 Indirect measures of insulin sensitivity

1.2.2.1 Models using intravenous glucose tolerance tests

In 1979 Bergman, Cobelli and co-workers developed a protocol to indirectly measure insulin sensitivity based on information acquired from an intravenous glucose tolerance test (IVGTT) (Bergman *et al.*, 1979). Following an infusion of an intravenous glucose bolus, blood samples are frequently obtained over three hrs to determine blood glucose and insulin concentrations, which undergo minimal modelling analysis by a computer programme (MINMOD) to generate an insulin sensitivity index (Bergman *et al.*, 1979, Yang *et al.*, 1989). The 'minimal model' describes the mathematical model with the least number of parameters identified as providing a good fit for the data and produces reliable estimates of glucose disappearance during the IVGTT. The physiological nature of this test, along with its relative simplicity are enticing attributes of this method for determining insulin sensitivity. In addition, unlike the hyperinsulinemic-euglycemic clamp method, obtaining steady state conditions or insulin infusions necessitating continuous attention are not required, thus, this method requires less specialised personnel. However, this indirect method of measuring insulin sensitivity requires repeated blood sampling and intravenous glucose infusions, and has also been criticised as oversimplifying the physiology of glucose homeostasis (Muniyappa *et al.*, 2008).

1.2.2.2 Oral glucose challenge/ Mixed meal challenge

An oral glucose or mixed meal challenge is a simple test which can be used to determine the body's capacity for glucose clearance in response to an oral glucose bolus or a meal consisting of carbohydrate, protein and fat. Following an overnight fast, blood samples are drawn prior to and at set intervals after an oral glucose load

or meal ingestion. Both of these tests represent more accurate physiological responses to glucose or meal ingestion compared to methods previously described, and are also less technically demanding to perform than direct methods measuring insulin sensitivity. The oral glucose challenge and mixed meal challenge measure glucose tolerance, from which glucose uptake can be calculated and used in conjunction with surrogate indexes of insulin sensitivity. Thus, an oral glucose/ mixed meal challenge was selected to provide an oral load to investigate changes in metabolite uptake in response to limb immobilisation for the studies presented in this thesis.

1.3 Metabolic adaptations during muscle disuse

1.3.1 Diminished insulin sensitivity

One of the major metabolic adaptations associated with periods of muscle disuse is the reduced sensitivity to insulin action, which is a strong predictor of the future development of type 2 diabetes. In addition to type 2 diabetes, lowered insulin sensitivity is also a key feature in other metabolic disorders, including obesity, endothelial dysfunction, hypertension and dyslipidemia (Jallut *et al.*, 1990, DeFronzo 2009). While the association between reduced insulin sensitivity and impaired metabolic health is well recognised gaps remain in understanding its aetiology, particularly in response to muscle disuse.

While the vast amount of research to date has explored the biological responses to both acute and chronic exercise, the area of "inactivity physiology" is now gaining much attention. Various models of physical inactivity and immobilisation, as

described in Section 1.1, have been employed to investigate the mechanistic pathways leading to reduced insulin sensitivity associated with reduced muscle use, and some of the key publications to date are listed in Table 1.1. Study designs incorporating extreme muscle unloading, such as bed-rest for 24 hrs per day, have also demonstrated significant reductions in insulin-stimulated glucose uptake. Using a hyperinsulinemic-isoglycemic clamp, Sonne *et al*. reported a 34% reduction in whole body insulin sensitivity in 20 healthy males following 10 days of bed-rest (Sonne *et al.,* 2010), while Coker *et al*. reported a 15% reduction in insulin mediated glucose uptake during a multistage insulin infusion in 9 older, overweight individuals, over the same time frame (Coker *et al.,* 2014). Similarly, 7 days of bed-rest induced a 40-47% increase in the insulin response to oral glucose ingestion in healthy young males (Charles A. Stuart 1988, Blanc *et al.,* 2000), while a 67% increase was observed in women by Blanc *et al,* (Blanc *et al.,* 2000). Gender differences in the insulin response to glucose ingestion reported by Blanc *et al*. could be representative of decrements in insulin sensitivity occurring primarily in skeletal muscle in men, whereas resistance to the action of insulin following bed rest occurs at both the hepatic and muscular level in women. Hamburg *et al*. are among the relatively few investigating short-term bed rest on metabolic health, reporting a 67% increase in the insulin response to glucose loading in 20 healthy adults after 5 days of bed rest (Hamburg *et al.,* 2007). Yanagibori *et al*. measured an increase of 17% in men and 75% in women of insulin area under the curve during an oral glucose tolerance test (Yanagibori *et al.,* 1997) and Smorawinski *et al*. reported a 30% increase in glucose area under the curve after 3 days of bed rest in inactive males (Smorawinski *et al.,* 2000). However, not all studies are in agreement as a crossover study showed 48 hrs of bed-rest had no effect on insulin sensitivity, as calculated by

the quantitative insulin sensitivity check index, in healthy adults (Duran-Valdez *et al.,* 2008). In concert with these findings, a recent publication from Dirks and colleagues observed no change in whole body insulin sensitivity after 1 day of bed rest, determined using Homeostatic model of analysis of insulin resistance and the Matsuda Index, which suggests that measurable changes to whole body insulin sensitivity are established over a longer time frame (Dirks *et al.,* 2018).

A number of studies have reduced the habitual ambulation of subjects in an attempt to closely mimic the increasingly sedentary lifestyles now adopted by a large proportion of the global population. After reducing step count from 6203 to 1394 for one week, Olsen *et al*. reported a 53% increase in area under the curve of plasma insulin, measured using an oral glucose tolerance test (Olsen *et al.,* 2008). Both Stephens *et al*. and Dunstan *et al*. also reported harmful effects of uninterrupted sedentary behaviour on insulin sensitivity (Stephens *et al.,* 2011, Dunstan *et al.,* 2012). The first of these randomised crossover studies measured an 18% decrease in insulin-stimulated glucose uptake during continuous glucose infusion after 1 day of acute sitting, when compared to one day with minimal sitting (Stephens *et al.,* 2011). After 5 hrs of uninterrupted sitting Dunstan *et al*. observed a 30% increase in insulin area under the curve following glucose ingestion in a cohort of overweight adults, in comparison to 5 hrs of sitting interrupted with sporadic bouts of walking (Dunstan *et al.,* 2012). Likewise, Nygaard *et al*. investigated the effects of 2 hrs of sitting in 13 middle aged women and showed a 45% increase in the blood glucose response to a standardised meal (Nygaard *et al.,* 2009). The overarching implication of these findings, that reducing physical activity induces harmful metabolic perturbations, is

convincing. Although the problem appears simplistic in nature the time-frame for development, progression and mechanistic cause remain elusive.

A serious limitation of many of the studies using immobilisation, particularly bed rest, is the lack of dietary control, resulting in the possibility that a positive energy balance exaggerated the negative impact of immobilisation on insulin sensitivity. To investigate this, Bergounian and co-workers performed bed rest studies for 2 months in women (Bergouignan *et al.*, 2006) and 3 months in men (Bergouignan *et al.*, 2009), during which time dietary intake was tightly controlled to clamp fat mass. These studies revealed an increase in plasma insulin concentration without any corresponding change in plasma glucose concentration in response to consumption of a standard meal. Therefore, extended immobilisation significantly reduces insulin sensitivity even during energy balance.

Following 7 days of bed rest, Mikines and colleagues observed a 24% decrease in whole body glucose uptake, which is considerably less than the 53% decrease in leg glucose uptake concurrently measured using the sequential euglycemic clamp technique (Mikines *et al.*, 1991). The authors concluded the decrease in whole body insulin sensitivity was accounted for by reduced insulin sensitivity in the inactive muscle. These findings are significant as they demonstrate that immobilisation induced decrements of insulin stimulated glucose uptake occur as a result of impairments residing within the muscle itself, rather than a global effect. Thus, limb immobilisation is a practical alternative to explore alterations in skeletal muscle glucose uptake in response to reduced muscle use and allows the contralateral limb to act as a within-subject control. Following 7 days of lower limb immobilisation

Richter *et al*. reported significant reductions in glucose uptake in the thigh muscle of healthy men, using the two-step euglycemic-hyperinsulinemic clamp technique (Richter *et al.,* 1989), while 7 days of forearm immobilisation led to a 64% decrease in forearm glucose uptake, determined via an oral glucose tolerance test (Skirrow 2014). However, no study to date has used limb immobilisation to investigate the acute effects of muscle disuse on skeletal muscle glucose uptake or to establish the time course of immobilisation induced changes in glucose uptake.

Table 1.1 Summary of key publications linking physical inactivity with reduced insulin sensitivity.

Author	Model of inactivity	Duration (days)	Findings	Methodology
Stephens *et al*, (Stephens *et al.*, 2011)	RA	1	↓18% decrease in insulin action	CGI, *n*=14
Duran-Valdez *et al*.(Duran-Valdez *et al*., 2008)	BR	2	No changes	QUIKI, *n*=5
Lipman *et al*.(Lipman *et al.*, 1972)	BR	3	↓ 45% FGU	OGTT, *n*=23
Smorowanski *et al*.(Smorawinski *et al.*, 2000)	BR	3	↑ 40% insulin AUC, ↑ 30% blood glucose response	OGTT, *n*=11
Knudsen *et al*.(Knudsen *et al.*, 2012)	RA	3	↓37% insulin sensitivity	OGTT, *n*=9
Yanagibori *et al*.(Yanagibori *et al.*, 1994)	BR	3	↑ insulin AUC (17% in 10 men, 75% in 7 women)	OGTT, *n*=14
Hamburg *et al*.(Hamburg *et al.*, 2007)	BR	5	↑ 67% insulin response to glucose load	OGTT, *n*=20
Mikines *et al*.(Mikines *et al.*, 1991)	BR	7	↓ 23.6% WBGU rate; ↓ 52.5% LGU rate	S-E clamp, *n*=6
Blanc *et al*.(Blanc *et al*., 2000)	BR	7	↑ insulin response (47% in 8 men, 67% in 8 women)	CGI, *n*=16
Richter *et al*.(Richter *et al.*, 1989)	LL	7	↓ insulin action on LGU	H-E clamp, *n*=5
Dirks *et al*.(Dirks *et al.*, 2016)	BR	7	↓ 30% glucose disposal	H-E clamp, *n*=10
Stuart *et al*.(Charles A. Stuart 1988)	BR	7	↑ 40% insulin response to glucose load	OGTT clamp, *n*=6
Knudsen *et al*.(Knudsen *et al.*, 2012)	RA	7	↓ 43% insulin sensitivity	OGTT, *n*=9
Skirrow *et al*. (Skirrow 2014)	UL	7	↓ 66% FGU	OGTT, *n*=8
Alibegovic *et al*.(Alibegovic *et al.*, 2009)	BR	9	↓ whole body insulin sensitivity	IVGTT, *n*= 20
Sonne *et al*. (Sonne *et al.*, 2010)	BR	10	↓ 34% whole body insulin sensitivity; ↓75% FGU	H-I clamp, *n*=20
Coker *et al*. (Coker *et al.*, 2014)	BR	10	↓ 15% insulin mediated glucose uptake	CGI, *n*=9
Knudsen *et al*.(Knudsen *et al.*, 2012)	RA	14	↓ 26% insulin sensitivity	OGTT, *n*=9
Krogh-Madson *et al*.(Krogh-Madsen 2010)	RA	14	↓ 17% glucose infusion rate	H-E clamp, *n*=10
Skirrow *et al*. (Skirrow 2014)	UL	21	↓ 54% FGU	OGTT, *n*=8
Cree *et al*.(Cree *et al.*, 2010)	BR	28	↓ 43% LGU	H-E clamp, *n*=6

Abbreviations: RA, reduced ambulation; BR, bed rest; LL, lower limb immobilisation; UL, upper limb immobilisation; FGU, forearm glucose uptake; LGU, leg glucose uptake; WBGU, whole body glucose uptake; AUC, area under the curve; CGI, continuous glucose infusion; QUIKI, quantitative insulin sensitivity check index; OGTT, oral glucose tolerance test.; S-E clamp; sequential euglycemic clamp; H-E clamp, hyperinsulinemic-euglycemic clamp; IVGTT, intravenous glucose tolerance test; H-I clamp, hyperinsulinemic-isoglycemic clamp

1.3.2 Glucose uptake and metabolism in healthy skeletal muscle

1.3.2.1 Glucose transport

Skeletal muscle is well recognised as the primary consumer of glucose both during exercise and after the consumption of a meal (DeFronzo and Tripathy 2009). However, as glucose is a hydrophilic molecule which cannot cross the plasma membrane of myocytes, a family of transporter proteins, known as glucose transporters (GLUT), are employed to carry glucose into muscle via facilitated diffusion. While several isoforms of these transporter proteins are found in various tissues, GLUT-1 and GLUT-4 are the main isoforms present in skeletal muscle. Although the molar ratio of GLUT-1: GLUT-4 in the plasma membrane is 0.6:1.0 (Klip and Marette 1992), there is much more GLUT-4 protein present throughout the whole muscle and therefore, GLUT-4 trafficking has a more significant role to play in skeletal muscle glucose uptake. In the quiescent state, GLUT-4 is primarily sequestered within the membrane of intracellular storage vesicles from where it can be rapidly translocated to the sarcolemma, resulting in its relocation throughout the plasma membrane, thereby amplifying glucose uptake (Marette *et al.*, 1992). The mobilisation of GLUT-4 to the plasma membrane occurs as a result of a multipart intracellular cascade which may be stimulated by a number of stimuli, including pharmacological agents or hypoxia, however, the primary factors inducing the translocation of GLUT-4 are muscular contraction and stimulation by insulin. Intracellular impairments in these signalling pathways resulting in reduced GLUT-4 translocation to the cell membrane have been identified as rate limiting steps in glucose uptake and utilisation by the myocyte.

1.3.2.2 Insulin stimulated glucose uptake

Insulin has a crucial role in glucose and lipid metabolism and its action is initiated by binding to insulin receptors found on the surface of most mammalian cells, particularly adipocytes and myocytes. The insulin receptor is a tetrameric protein composed of two extracellular α- subunits to which insulin binds, causing a conformational change to occur to the intracellular domain of the receptor, composed of two β-subunits. This conformational change of the receptor results in the activation of tyrosine kinase domains attached to each β-subunit (Chang *et al.,* 2004). This activity leads to the auto-phosphorylation of many tyrosine residues in the β-subunit, triggering the activation of docking proteins, known as insulin receptor substrates 1 through 4 (IRS 1-4), marking the initiation of the ”classical insulin cascade”. Phosphorylation of the IRS proteins facilitates interaction with the p85 regulatory subunit of the phosphatidylinositol (PI)-3-kinase, leading to the activation of the catalytic subunit, p110, of this enzyme (Fruman *et al.,* 1998). Once activated PI-3-kinase phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP_2), producing a second messenger, phosphatidylinositol (3,4,5)-triphosphate (PIP_3), which regulates the activity of 3-phosphoinositide-dependent protein kinase (PDK) (Cantley 2002). One of the primary roles of PDPK is the activation of a signalling protein, a serine/threonine kinase known as protein kinase B (PKB or Akt), and protein kinase C, which serves as an activator for several actions of insulin, including GLUT-4 translocation and glycogen synthesis (Foran *et al.,* 1999, Bruss *et al.,* 2005). The action of Akt in linking insulin signalling with glucose trafficking is an integral component in the glucose uptake process of skeletal muscle, which is impaired in type 2

diabetic individuals, therefore contributing to reduced GLUT-4 translocation (Karlsson *et al.,* 2005). Other indirect effects of insulin signalling on glucose uptake include the upregulation of mitogen activated protein kinases (MAPK). The MAPK stimulate transcription factors related to cell proliferation and differentiation, and indirectly alter glucose uptake (Saltiel and Kahn 2001).

1.3.2.3 Intracellular glucose metabolism

Following transport into a muscle cell, glucose either becomes irreversibly phosphorylated or it may be transported back outside the cell by the same glucose transporters. Once phosphorylated, glucose-6-phosphate can either be non-oxidatively metabolised, in glycogen synthesis, or oxidatively by glycolysis. As glucose uptake occurs via facilitated diffusion, it is imperative that the transmembrane glucose gradient is sustained, via the rapid phosphorylation of inbound glucose, to maintain efficient glucose uptake. In the fasting state, glucose utilisation in tissues with an obligatory glucose requirement, such as the brain, is prioritised over utilisation by skeletal muscles. Therefore, glucose utilisation by skeletal muscle in resting conditions is low, however, this significantly increases following meal ingestion. In fact, skeletal muscle has been identified as the most important site for post-prandial glucose disposal, accounting for up 60-85% of glucose clearance (DeFronzo *et al.,* 1981, Jackson *et al.,* 1983).

Although glucose transport is generally considered to be the rate limiting step in glucose utilisation, glucose phosphorylation may become rate-limiting under certain situations, such as during insulin stimulation. Intracellular

phosphorylation of glucose, the final step of glucose uptake by skeletal muscle, adds a charged phosphate group from the hydrolysis of ATP to the glucose molecule, forming glucose-6-phosphate and thereby preventing it from diffusing back across the plasma membrane. This phosphorylation is mediated by a hexokinase isoenzyme, Hexokinase II (HK II), resulting in the formation of glucose-6-phosphate which can then be further metabolised or stored as glycogen. Many elegant investigations have employed isotopic glucose analogues to reveal the impact of disruption to steps distal to glucose transport on glucose utilisation by myocytes (Ferrannini *et al.*, 1985, Kelley *et al.*, 1999). Compromised glucose phosphorylation has been identified as a feature of several insulin resistant states, with some studies suggesting this occurs as a result of lower basal HK II activity, and activity of this enzyme in response to insulin stimulation during a euglycemic-hyperinsulinemic clamp in obese and type 2 diabetic patients compared to lean subjects (Kruszynska *et al.*, 1998, Pendergrass *et al.*, 1998). Similar observations from Vestergaard and colleagues revealed reduced gene expression of HK II in conjunction with reduced enzyme activity in muscle of type 2 diabetic patients (Vestergaard *et al.*, 1995). Inhibition of hexokinase activity has also been associated with an increase in long chain fatty acyl-coenzyme A, suggestive of lipid intermediates interfering with insulin-mediated glucose metabolism by reducing glucose phosphorylation (Thompson and Cooney 2000). Thus, considerable evidence supports the notion that impairments in glucose phosphorylation by HK II may contribute to reduced glucose uptake and utilisation by skeletal muscle, and this may represent a site for disruption to glucose metabolism during muscle disuse.

1.3.3 Potential mechanisms underlying immobilisation induced impairments of muscle glucose uptake

Despite increasingly abundant evidence linking decreased insulin stimulated glucose uptake with muscle disuse, the exact mechanism(s) behind such an effect are unknown. Periods of immobilisation have been associated with decreased lean tissue mass (discussed in Section 1.4.1). Such reductions of metabolically active tissue result in the reduced glucose clearance capacity of the body and, thus, may contribute to decrements in insulin stimulated glucose uptake by skeletal muscle. However, reductions of muscle mass are generally of a much smaller magnitude than reductions in glucose uptake (Mikines *et al.*, 1991), therefore, muscle atrophy cannot fully account for the decrements to insulin sensitivity. Moreover, it has not yet been confirmed if sufficient muscle atrophy could precede decrements in insulin sensitivity, and assessing the interaction between these mechanisms remains challenging until the independent time-course of change for each mechanism has been established.

As discussed above glucose transport into skeletal muscle, mediated by GLUT-4, is the rate limiting step in glucose uptake under normal physiological conditions. A positive association is known to exist between insulin sensitivity and GLUT-4 content in the vastus lateralis muscle of both men and women (Houmard *et al.*, 1995). Several lines of evidence now attribute immobilisation induced impairments in muscle glucose uptake, a marker of insulin sensitivity, to a reduction in the content of some of the key proteins involved in glucose transport (Garvey and Birnbaum 1993, Biensø *et al.*, 2012). Current data indicate that this reduction in glucose uptake may be due to insufficient

expression of GLUT-4, or shortcomings in the recruitment of GLUT-4 to the plasma membrane, even with normal GLUT-4 expression (Garvey *et al.,* 1998). Sedentary individuals have a lower GLUT-4 mRNA and protein content compared to their endurance trained counterparts (Houmard *et al.,* 1991, Seki *et al.*, 2006), while bed-rest ranging from 7-19 days was associated with a 16-26% decrease in skeletal muscle GLUT-4 protein content, which was accompanied by a concurrent decline in insulin sensitivity (Tabata *et al.,* 1999, Biensø *et al.,* 2012). Investigations focusing on skeletal muscle glucose transport in insulin resistant conditions have revealed abnormal subcellular localization of GLUT-4, despite normal GLUT-4 expression (Garvey *et al.,* 1992, Zierath *et al.,* 1996, Garvey *et al.,* 1998). These observations indicate the lower insulin stimulated glucose uptake in human skeletal muscle during periods of muscle disuse may be due to impaired GLUT-4 translocation to the plasma membrane. Although it is not possible to definitively conclude from these findings that GLUT-4 translocation *per se* is directly affected by periods of muscle disuse, it does lend credence to the concept that impaired glucose transport is a major player in the onset of decrements in insulin mediated glucose uptake associated with immobilisation, whether these changes are a cause or consequence of immobilisation remains to be determined.

Other intracellular events which may also be affected by impairments in the glucose transport system in skeletal muscle include glucose phosphorylation and glycogen synthesis. Following 7 days of bed rest, Biensø *et al*. measured a 34% decline in levels of HK II, which suggests decrements in glucose phosphorylation associated with muscle disuse may contribute to decreased

insulin stimulated glucose uptake. Furthermore, in insulin resistant states use of the triple tracer technique has demonstrated glucose phosphorylation is more profoundly reduced in the forearm muscle of lean type 2 diabetes patients compared to insulin-stimulated glucose transport, although glucose transport was also impaired (Bonadonna *et al.*, 1993, Bonadonna *et al.*, 1996). Therefore, although both glucose transport and phosphorylation are impaired in this insulin resistant population, defective glucose phosphorylation may be more obstructive to insulin sensitivity than glucose transport. Findings from DeFronzo *et al*. state that insulin-mediated glucose disposal in normal subjects, determined using the euglycemic clamp technique, occurs mainly through non-oxidative pathways, primarily glycogen synthesis, and accounts for 59% of the total glucose metabolized (DeFronzo *et al.*, 1981). Several publications agree that faults in glycogen synthesis, secondary to a decline in glycogen synthase activity, may be the earliest measurable metabolic defect in type 2 diabetes (Bogardus *et al.*, 1984, DeFronzo 1997). Findings from immobilisation studies support this hypothesis, as Biensø *et al*. propose that decrements in insulin sensitivity following 7 days of bed-rest may also be related to a concurrent decline in insulin-stimulated glycogen synthase activity (Biensø *et al.*, 2012). Thus, as glycogen synthase is the rate-controlling enzyme in glycogen synthesis, a decline in this enzymes activity in response to reduced muscle use may contribute to decreased insulin mediated glucose uptake. However, as mechanistic insight is still lacking, whether these impairments instigate or occur as a consequence of reduced insulin sensitivity has yet to be elucidated.

Muscle from insulin resistant individuals and type 2 diabetes patients have been shown to exhibit reduced Akt phosphorylation (Karlsson *et al.,* 2005, Hojlund *et al.,* 2008) and may be an early impairment in glucose transport and uptake during muscle disuse. Using the hyperinsulinemic-euglycemic clamp technique, Krogh-Madsen *et al*. reported decreased insulin sensitivity following 2 weeks of reduced ambulation, which was accompanied by a decrease in insulin-stimulated Akt phosphorylation (Krogh-Madsen 2010). These observations are in agreement with reports of 20-30% lower Akt protein content and phosphorylation after 7 days of bed rest and 2 days of knee immobilisation (Urso *et al.,* 2006, Biensø *et al.,* 2012). However, this finding is not universal as no changes were observed in the phosphorylation of Akt after 2-21 days of unilateral lower limb immobilisation (de Boer *et al.,* 2007, Abadi *et al.,* 2009). Further investigation is necessary to clarify these inconsistent findings. Nonetheless, the potential reduction in Akt phosphorylation, as a result of reduced muscle disuse, may inhibit GLUT-4 activity and contribute to the decline in muscle glucose uptake. These findings point to a mechanism linking reduced Akt protein content and phosphorylation with immobilisation induced impairment of insulin stimulated glucose uptake which requires additional examination.

As mitochondria are the primary producer of ATP, via oxidative phosphorylation, a reduction in their number and/or function has been conjectured to contribute to the altered fuel metabolism associated with immobilisation. Following 14 days of unilateral limb immobilisation Nielsen *et al*. measured a 19% decrease in mitochondrial fibre volume measured using

electron microscopy (Nielsen *et al.*, 2010). Similarly, after 42 days of bed rest Ferretti *et al*. reported a 17% reduction in the volume density of mitochondria (Ferretti *et al.*, 1997). Gram *et al*. also observed a reduction in mitochondrial respiratory capacity and protein content after 14 days of immobilisation of the quadriceps muscle (Gram *et al.*, 2014). These authors demonstrated that a decrease in citrate synthase activity, a marker of mitochondrial content, accounted for the decreased respiratory capacity rather than changes in mitochondrial function.

1.3.4 Immobilisation induced accumulation of lipid in skeletal muscle

It is now over twenty years since intramyocellular lipid (IMCL) content in the vastus lateralis was first reported by Pan *et al*. to be negatively associated with insulin sensitivity, and to date, the precise mechanisms resulting in this diminution of insulin sensitivity remain to be fully elucidated (Pan *et al.*, 1997). Several studies have since reinforced these findings (Jacob *et al.*, 1999, Krssak *et al.*, 1999, Virkamäki *et al.*, 2001), although the majority of these studies focused on sedentary populations which were overweight, obese, diabetic or offspring of type 2 diabetic parents. Cree *et al*. utilised proton magnetic resonance spectroscopy (^{1}H-MRS) to expose a negative correlation between IMCL content and glucose uptake in healthy, young subjects following 28 days bed rest (Cree *et al.*, 2010). However, investigations of IMCL accumulation over shorter periods of immobilisation have reported opposing findings to those of Cree *et al*.(Wall *et al.*, 2015, Dirks *et al.*, 2016). Dirks *et al*. did not detect any change in IMCL content, determined using Oil Red O staining, after 7 days of bed rest, despite substantial atrophy and

decreased insulin sensitivity in the muscle of healthy subjects (Dirks *et al.*, 2016). Similarly, Wall *et al*. observed no change in IMCL content, measured using biochemical analysis, following 5 days of one-legged knee immobilisation in a healthy cohort (Wall *et al.,* 2015). While these findings may indicate that short-term immobilisation is insufficient to induce measurable changes in IMCL content, it may be reasoned that the analysis of tissue from muscle biopsies, used by both Dirks *et al*. and Wall *et al*., to determine IMCL content is not sensitive enough, or requires a larger sample size, to detect small changes. Thus, further investigations of IMCL accumulation during short-term physical inactivity, measured using methods with greater sensitivity, are warranted to establish the early metabolic responses to reduced muscular contraction.

1.3.4.1 Lipid metabolism in healthy skeletal muscle

The excessive storage of lipid within skeletal muscle has been associated with many detrimental states attributed to modern Western lifestyles, such as obesity, and has been strongly linked to impaired metabolic health (WHO 2009). Excessive IMCL accumulation may occur due to increased rate of fatty acid uptake, decreased fatty oxidation or both. Indeed, extended periods of immobilisation, enforced via bed rest, has been associated with decreased fat oxidation in skeletal muscle, contributing to IMCL accumulation (Bergouignan *et al.*, 2011). Although skeletal muscle has the ability to oxidise fatty acids, there is a relatively narrow range between the triacylglycerol content of skeletal muscle in healthy, lean individuals compared to their obese counterparts, reported as 0.5% versus 3.5% lipid droplet volume density in lean

and obese individuals respectively (Watt and Hoy 2012). This variance in skeletal muscle lipid content represents the relatively small increment in IMCL capable of inducing impairments in metabolic health, which have been underlined by numerous studies reporting strong correlations between IMCL content and decreased insulin sensitivity of skeletal muscle (Jacob *et al.,* 1999, Krssak *et al.,* 1999, Virkamäki *et al.,* 2001).

1.3.4.1.1 Supply and cellular uptake of lipids by skeletal muscle

Free fatty acids, or non-esterified fatty acids, are the most important form of lipid for myocytes, and are derived from either intra- or extra-cellular sources. Intracellular sources of fatty acids are found in triacylglycerol, which is stored in lipid droplets within the cytoplasm (Gorski 1992). Extracellular supplies are derived from triacylglycerol contained within the lipid core of circulating chylomicrons or very low density lipoproteins, which are hydrolysed by endothelial lipoprotein lipase, releasing glycerol and free fatty acids. Extracellular supply of free fatty acids also include fatty acids bound to albumin released by adipocytes (Hodgetts *et al.,* 1991). Liberated free fatty acids can be taken up by myocytes through either passive diffusion or protein mediated diffusion, chiefly facilitated by fatty acid translocase (CD36), fatty acid transport proteins 1 and 4 (FATP-1 and -4), and plasma membrane associated fatty acid binding protein (Glatz *et al.,* 2010). Recent evidence indicates CD36 and FATP-4 may be quantitatively most important as they have been associated with the greatest transport efficiency (Nickerson *et al.,* 2009). Much debate exists around the definite mechanism by which fatty acids are transported inside myocytes and, although the precise pathway has not been

identified, the translocation of CD36 is believed to have an important role to play in this process. Similar to the translocation of GLUT-4, CD36 translocation to the sarcolemma from an intracellular pool is activated by PI-3-kinase following insulin stimulation (Luiken *et al.,* 2002, Chabowski *et al.,* 2005). Moreover, the translocation of many of these fatty acid transporters to the sarcolemma is also stimulated by muscle contraction (<30 minutes) (Jain *et al.,* 2009, Glatz *et al.,* 2010). Interestingly, Bonen *et al*. reported a linear relationship between the intensity of muscle contraction and rate of fatty acid transport, suggesting the metabolic demand of the muscle determines fatty acid uptake (Bonen *et al.,* 2000).

1.3.4.1.2 Fatty acid metabolism in healthy skeletal muscle

Once transported inside the myocyte plasma free fatty acids have a number of possible fates, primarily involving oxidisation or storage. While a small quantity of free fatty acids may be used in phospholipid synthesis for structural use within the cell membrane, the majority of extracellular derived free fatty acids are utilised in triacylglycerol synthesis, with 50-63% of this free fatty acid source reported to be re-incorporated into intracellular triacylglycerol pools (Dyck and Bonen 1998, Sacchetti *et al.,* 2002, Sacchetti *et al.*, 2004). This triacylglycerol, along with cholesterol esters and diacylglycerol, is stored within lipid droplets close to mitochondria and the endoplasmic reticulum, thereby in close proximity to sites of both fatty acid oxidation and triacylglycerol esterification (Stein and Stein 1968, Shaw *et al.,* 2008). Alternatively, free fatty acids may also be oxidised within the mitochondria, generating ATP (Feher 2012). Upon entering the sarcoplasm fatty acids are

coupled with coenzyme A forming fatty acyl-CoA, catalysed by acyl-CoA synthase, which can then be used to synthesise triacylglycerol or it can be bound to carnitine palmitoyl transferase I for transportation into the mitochondria. Binding to carnitine releases free CoA, allowing the acyl-carnitine to pass the mitochondrial membrane. Once inside the mitochondria carnitine palmitoyl transferase II converts the acyl-carnitine complex back to acyl-CoA, before the carnitine molecule diffuses out of the mitochondria. The reformed fatty acyl-CoA complex undergoes β-oxidation, generating ATP to be used in the tricarboxylic acid cycle and reducing equivalents for the electronic transport chain (Watt and Hoy 2012).

1.3.4.2 Measuring intramyocellular lipid content

The accumulation of lipid within skeletal muscle occurs in the form of either spherical droplets within the cytoplasm of myocytes (IMCL) or as tubular structures between muscle fibres (extramyocellular lipids; EMCL) (Khuu *et al.*, 2009). Several publications now support the association between increased IMCL content with decrements in insulin sensitivity, and numerous methods are now available for the quantification of IMCL concentrations. While there has been significant development of non-invasive methodologies for the measurement of IMCL concentration, the more established techniques using invasive biopsy techniques also remain popular and, in some instances, represent a more accessible method for determination of the physiological role of IMCL. Table 1.2 provides a summary of the main advantages and drawbacks of the main techniques currently employed to investigate lipid content of skeletal muscle.

1.3.4.2.1 Analysis of muscle tissue from biopsies

For several decades the biochemical analysis of muscle biopsy samples has been extensively used for quantification of lipid concentration. Following biopsy extraction, visible fat is removed from the muscle sample prior to the sample being freeze dried. Lipids are then isolated by chloroform-methanol extraction, and the lipid concentration is determined from the amount of glycerol and free fatty acids liberated from the hydrolysed triacylglycerols. Despite this being the most widely used method, inherent difficulties in minimising contamination by EMCL reduce the accuracy of biochemical analysis of muscle biopsies in the determination of IMCL content. Findings by Wendling and colleagues illustrate the large variability in data obtained using the needle muscle biopsy technique, by measuring a between biopsy variation of 23.5% in triacylglycerol content, determined from three simultaneous biopsies performed on the same muscle of the same individual (Wendling *et al.,* 1996). Such high variability renders this method unsuitable for detection of small changes in lipid content. Furthermore, the invasive nature of muscle biopsies reduces the feasibility for multiple assessments of IMCL quantification in longitudinal studies. Advantages of obtaining muscle biopsy samples include using electron microscopy (EM), Bodipy-493/503 or oil red O staining for structural and histological analysis (Hoppeler *et al.,* 1973, De Bock *et al.,* 2007). These techniques can yield valuable information regarding the localisation of lipid droplets within the myocyte and their position relative to mitochondria. Importantly, certain methodological issues have been associated with oil red O staining, primarily relating to this particular dye binding to intracellular organelle other than IMCL. Therefore, Bodipy-493/503 has been

identified as the fluorescent dye of choice in IMCL imaging (Prats *et al.*, 2013).

1.3.4.2.2 Use of ^{1}H-MRS in IMCL measurement

A growing number of studies are now using ^{1}H-MRS to investigate associations between accumulation of IMCL with decrements in insulin sensitivity. Early work employing ^{1}H-MRS revealed strong correlations between increased IMCL content of the soleus muscle and lower insulin sensitivity in the offspring of type 2 diabetic parents compared to healthy controls (Perseghin *et al.*, 1999). Jacob *et al*. reported IMCL content to be 84% and 57% higher in the soleus and tibalis anterior muscles respectively, in the insulin resistant first degree relatives of type 2 diabetic patients compared to healthy controls (Jacob *et al.*, 1999). These crucial preliminary findings from studies using ^{1}H-MRS suggested defects in lipid metabolism were associated with lower insulin sensitivity in individuals at increased risk of developing type 2 diabetes. Similar observations have also been reported in healthy individuals. Forouhi *et al*. measured insulin sensitivity, using an insulin tolerance test in a healthy cohort, and reported strong correlations between IMCL content of the soleus muscle and lower insulin sensitivity (r=-0.53, P=0.016) (Forouhi *et al.*, 1999). Additionally, Krssak *et al*. measured insulin sensitivity using a hyperinsulinemic-euglycemic clamp in healthy individuals, in conjunction with quantification of IMCL content of the soleus muscle by ^{1}H-MRS, observing a similar inverse correlation (r=-0.692, P=0.0017) (Krssak *et al.*, 1999). The culmination of findings from studies using ^{1}H-MRS offer credible indications that IMCL accumulation may be

responsible, at least in part, for defects in skeletal muscle glucose uptake. However, all of the existing research has been confined to muscles of the lower limbs, primarily the soleus and tibialis anterior muscles. Extrapolating findings from one muscle group to represent metabolic perturbations at the whole body level would be injudicious, considering the substantial heterogeneity between muscles groups. Although the large volume of the lower limb muscles has led to fast-paced development of this method, the viability of using MRS to assess IMCL content of upper limb muscles, which are of a considerably smaller volume and have varying pennation angles , has not yet been determined. Furthermore, no study to date has interrogated the relationship between IMCL content and glucose uptake in muscles of the upper limb, such a study would provide valuable insight into associations between muscle disuse induced abnormalities of IMCL metabolism contributing to decrements in insulin sensitivity.

Table 1.2. Summary of major advantages and limitations of common techniques used to measure intramyocellular lipid content.

Technique	Advantages	Limitations
Magnetic Resonance Spectroscopy	1. Non-invasive 2. High sensitivity 3. Repeated measurement of large muscle volume	1. Operational costs 2. Limited availability of MRI scanner and spectroscopy acquisition software 3. Highly trained personnel required 4. Problematic in obese populations
Magnetic Resonance Imaging	1. Non-invasive 2. Repeated measurement of large muscle volume	1. Operational costs 2. Limited availability of MRI scanner 3. Highly trained personnel required 4. No differentiation between IMCL and EMCL
Biochemical analysis	1. Relatively inexpensive 2. Widely used allowing comparison between investigations 3. Facilitates further structural and histological analysis	1. Invasive, increased risk and discomfort for research subject 2. Low sensitivity for detection of small changes 3. Less feasible for time course studies 4. Highly trained personnel required
Histochemistry	1. Relatively inexpensive 2. Not biased by extracellular lipid	1. Invasive, increased risk and discomfort for research subject 2. No differentiation between lipid species
Electron microscopy	1. Additional morphological information may be obtained 2. Not biased by extracellular lipid	1. Invasive, increased risk and discomfort 2. Limited to small muscle volume 3. Labour intensive

1.3.4.3 Links between intramyocellular lipid content and insulin sensitivity

For several decades the potential mechanisms linking increased IMCL content with reduced insulin sensitivity, presented in Figure 1.1, have been postulated although the exact relationship remains to be fully defined. Over 50 years ago Randle and colleagues proposed the hypothesis that skeletal muscle insulin resistance associated with obesity occurred as a result of decreased glucose utilisation and increased oxidation of fatty acids (Randle *et al.,* 1963). Randle hypothesised that increased fatty acid oxidation caused an accumulation of citrate and inhibited phosphofructokinase, an essential enzyme in glycolysis, resulting in increased intramyocellular concentration of glucose-6-phosphate and glucose, thereby impacting glucose uptake and utilisation. More than 30 years later this theory was further explored non-invasively using MRS and the findings showed a decrease in intramyocellular concentrations of glucose-6-phosphate and glucose, in association with acute skeletal muscle insulin resistance induced by a lipid infusion (Dresner *et al.,* 1999, Roden *et al.,* 1999). Furthermore, Dresner *et al*. observed substantial reductions in IRS-1 associated PI3K activity during lipid infusion (Dresner *et al.,* 1999). These findings, in conjunction with the elucidation of insulin signalling pathways and identification of mechanisms involved in the translocation of glucose transporter proteins to the sarcolemmal membrane, indicate that fatty acids impair insulin-stimulated glucose uptake by disrupting insulin-stimulated glucose transport into the myocyte, rather than inhibition of glycolytic enzymes as suggested by Randle.

Due to their encapsulation within lipid droplets it is generally accepted that intramuscular triacylglycerol are incapable of interrupting insulin signalling and are therefore, innocuous to insulin sensitivity (Goodpaster *et al.*, 2001). Instead, much evidence points to a mechanistic role of the bioactive intermediates of lipid metabolism in the impairment of insulin signalling in skeletal muscle, with ceramides and diacylglycerol identified as the chief contenders (Itani *et al.*, 2002, Adams *et al.*, 2004, Bruce *et al.*, 2006). Itani *et al*. detailed a fourfold increase in diacylglycerol mass in muscle of healthy volunteers associated with lipid induced insulin resistance, assessed by lipid/heparin infusion during a euglycemic-hyperinsulinemic clamp (Itani *et al.*, 2002), while Amati and co-workers reported greater ceramide content in the insulin resistant muscle of obese sedentary individuals, compared to normal weight, sedentary controls, as measured during a glucose clamp (Amati *et al.*, 2011). Meanwhile, Bruce *et al*. observed reductions of 42% and 15% in total ceramide and diacylglycerol content of muscle respectively, in association improved glucose tolerance assessed during an oral glucose tolerance test, following 8 weeks of moderate intensity endurance training (Bruce *et al.*, 2006).

Proposed mechanisms linking bioactive lipid intermediates with reduced insulin sensitivity centre primarily on impairments in points of phosphorylation of the insulin signalling cascade. These impairments are thought to disrupt the signal initiating GLUT-4 translocation to the sarcolemmal membrane, thereby inhibiting glucose uptake by the myocyte. Specifically, ceramides are reported to contribute to diminished insulin

sensitivity by inhibiting insulin-stimulated Akt/PKB serine phosphorylation and activation (Summers *et al.,* 1998, Schmitz-Peiffer *et al.,* 1999, Bruce *et al.,* 2006). Ceramides are also capable of activating inflammatory molecules such as IkappaB-alpha, which have been implicated in the development of insulin resistance (Itani *et al.,* 2002). However, existing evidences indicates these inflammatory molecules do not have a role to play in the instigation of lipid-induced insulin resistance, instead contributing to the progression of insulin resistance once established (Lee *et al.,* 2011). Whereas, diacylglycerol accumulation has been shown to be associated with activation of PKC isoforms, which phosphorylates IRS-1 at serine 1101, thereby inhibiting tyrosine phosphorylation and preventing insulin-stimulated activation of the Akt pathway (Li *et al.,* 2004).

Interestingly, the muscle content of long chain acyl CoA esters has been proposed as a more accurate predictor of insulin resistance than intramuscular triacylglycerol content due to the reduced risk of contamination from extracellular lipids (Ellis *et al.,* 2000). Previous investigations utilising high fat diets or lipid infusions to increase skeletal muscle long chain fatty acyl CoA content reported a concurrent decline in insulin sensitivity (Chalkley *et al.,* 1998, Ellis *et al.,* 2000), while the pharmacological inhibition of lipolysis, using Acipmox, has been demonstrated to reduce total muscle long chain fatty acyl CoA content in conjunction with significantly increasing whole body glucose clearance (Bajaj *et al.,* 2004). Overall, these data suggest that potential accumulation of long chain fatty acyl CoA during muscle disuse may also contribute to reduced insulin mediated glucose uptake by skeletal muscle.

Although, further work is still necessary to fully understand mechanisms linking long chain fatty Acyl CoA's with reduced insulin sensitivity, findings from Yu *et al*. indicate the activation of PKC by long chain fatty acyl CoA's, resulting in increased IRS-1 serine phosphorylation and decreased IRS-1 tyrosine phosphorylation, may contribute to disruptions in insulin stimulated glucose transport.

Alternative lines of research have proposed the accrual of intermediates of mitochondrial fatty acid oxidation as mediators of decrements in skeletal muscle insulin sensitivity. Ussher *et al*. suggest greater lipid supply results in the increased β-oxidation of fatty acids, which surpass the capacity of the Krebs cycle (Ussher *et al.,* 2010). However, data in humans is currently lacking and requires considerable development to fully elucidate the potential role of the accumulation of by-products of fatty acid oxidation in decreased insulin signalling.

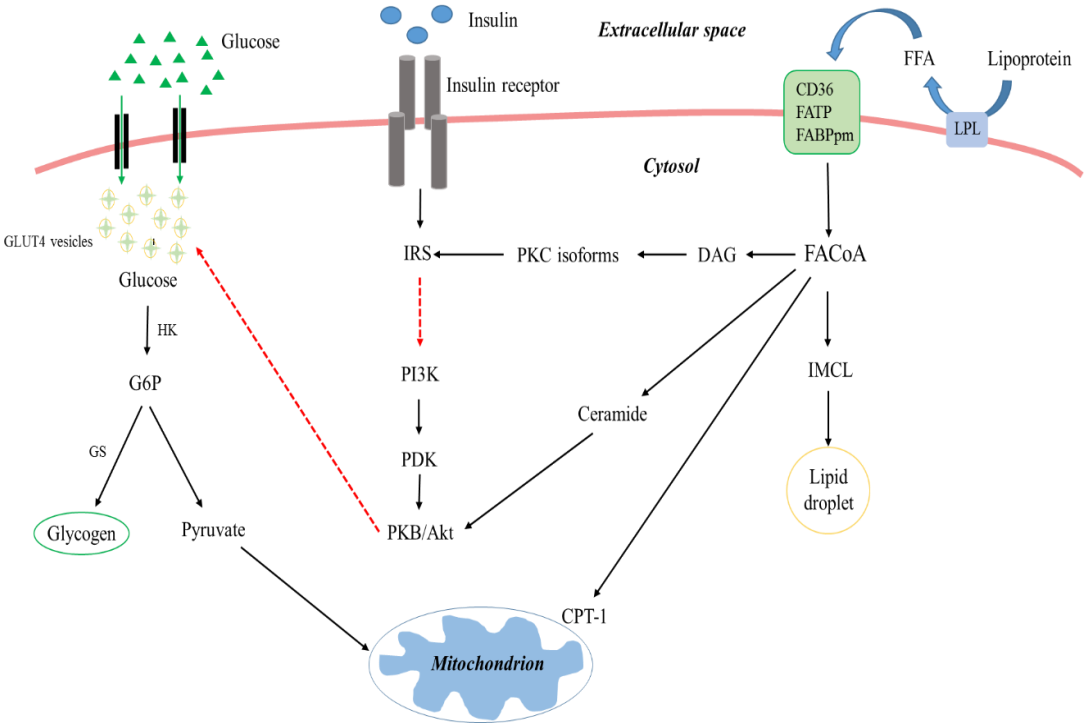


Figure 1.1 Potential mechanisms linking intramyocellular lipid accumulation with inhibited insulin signaling within skeletal muscle. Diacylglycerol (DAG) has been proposed to interfere with insulin sensitivity by activating PKC isoforms which serine phosphorylate IRS-1, thereby reducing tyrosine phosphorylation and preventing further stimulation of the Akt pathway. Ceramides are reported to inhibit insulin-stimulated serine phosphorylation and activation of Akt/PKB. Black arrows represent activating pathways; red dashed arrows represent inhibited processes. Abbreviations: FFA, free fatty acid; CD36, fatty acid translocase; FATP, fatty acid transport proteins; FABPpm, plasma membrane associated fatty acid binding protein; FACoA, fatty acyl CoA; IMCL, intramyocellular lipid; CPT-1, carnitine palmitoltransferase-1; PKC, protein kinase C; IRS, insulin receptor substrate; PI3K, phosphatidylinositol-3 kinase; PDK, phosphoinositide-dependent kinase; PKB (Akt) protein kinase B; GS, glycogen synthase; HK, hexokinase.

1.4 Physiological adaptations during immobilisation

Muscle disuse has been shown to result in a decline in functional capacity of most physiological systems within the body. While chronic sedentary lifestyles have long been associated with increased risk of premature mortality, cardiovascular disease and type 2 diabetes, an ever increasing wealth of evidence now exists revealing the substantial muscle weakness and wasting, decrements in bone mass and cardiovascular deconditioning induced by short-term muscle disuse.

1.4.1 Muscle atrophy

Muscle atrophy is one of the major physiological responses to muscle disuse. Changes in muscle size as a result of deconditioning are generally indicated by alterations in muscle volume, cross sectional area (CSA) or muscle fibre CSA, commonly measured using magnetic resonance imaging (MRI), ultrasound or computerised tomography scans. Numerous studies employing long periods of bed rest, ranging from 35-119 days, have consistently reported considerable declines of 10-14% in leg muscle volume, usually focusing on calf and thigh muscles, assessed using MRI (LeBlanc *et al.*, 1988, LeBlanc *et al.*, 1992, Berg *et al.*, 1997). Shorter periods of muscle disuse, typically 10-28 days, have also been well investigated using bed rest or limb immobilisation with findings from these studies reporting decrements in quadriceps muscle CSA ranging from 5-21% (White *et al.*, 1984, Davies *et al.*, 1987, Veldhuizen *et al.*, 1993, Hespel *et al.*, 2001, Kawakami *et al.*, 2001, Thom *et al.*, 2001, Schulze *et al.*, 2002, Akima *et al.*, 2003, Yasuda *et al.*, 2005, de Boer *et al.*, 2007, Glover *et al.*, 2010). Remarkably, even brief periods of lower limb immobilisation have

induced substantial muscle atrophy, with quadriceps muscle CSA reduced by 3.5-5% after 5-7 days (White *et al.,* 1984, Dirks *et al.,* 2016), while Suetta and colleagues employed unilateral lower limb immobilisation for 4 days to reduce myofibre size by 10% (Suetta *et al.,* 2012). The latter findings are in accordance with multiple studies reporting decrements of approximately 10% in myofibre CSA after three days of dry water immersion, highlighting the promptitude of disuse induced changes in muscle mass (Demangel *et al.,* 2017, Pagano *et al.,* 2017). Although investigations into these acute changes in muscle mass in response to muscle disuse are relatively scarce compared to longer term investigations, existing findings indicate the greatest loss of muscle mass occurs early in response to muscle disuse (1-2 weeks), with an average loss of 0.5-0.6% per day reported (Wall and van Loon 2013).

Alterations in muscle size of the lower limbs in response to deconditioning has been widely investigated due to the greater predisposition of weight-bearing, anti-gravity muscles of the lower limbs to atrophy. Conversely, there is currently a paucity of studies investigating the impact of muscle disuse on muscle mass of forearm muscles. The limited number of studies investigating the impact of upper limb immobilisation on muscle volume have provided conflicting results with some authors observing no change in muscle volume, assessed using MRI, following 21 days of forearm immobilisation (Kitahara *et al.,* 2003, Skirrow 2014). In contrast, MRI and computed tomography have revealed decrements in flexor volume of 7-10% following 21 days of immobilisation (Miles *et al.,* 1994, Seki *et al.,* 2001), while 7-9 days of forearm immobilisation has produced a 4% decrease in both forearm muscle

CSA and volume, evaluated via MRI and dual energy x-ray absorptiometery (Miles *et al.,* 1994, Johnston *et al.,* 2009). The inconsistent findings arising from studies using upper limb immobilisation are likely to stem from the use of different models of immobilisation and various techniques for assessment of muscle volume.

In the literature to date, controversy surrounds the identification of the primary driver of muscle atrophy during muscle disuse as either decreased muscle protein synthesis or increased muscle protein degradation. Previous studies utilising both unilateral lower limb suspension and bed rest for 7-14 days have demonstrated 30-50% reductions in muscle protein synthesis in both the fasting and fed states (Ferrando *et al.,* 1996, Paddon-Jones *et al.,* 2004, Glover *et al.,* 2008, Drummond *et al.,* 2012, Wall and van Loon 2013). No change in muscle protein breakdown was observed by Symons and colleagues in healthy young volunteers following 21 days of bed rest (Symons *et al.,* 2009). However, upregulation of ubiquitin ligases (Muscle RING finger protein-1 and Atrogin-1), which regulate ubiquitin-mediated protein degradation, has been reported within 2-4 days of muscle unloading (Abadi *et al.,* 2009, Gustafsson *et al.,* 2010, Suetta *et al.,* 2012). Interestingly, after 14 days of lower limb immobilisation expression levels of these ubiquitin ligases returned to basal levels, which suggests the ubiquitin proteasome pathway may have a part to play in a transient increase in muscle protein degradation early on during muscle disuse but may not have a significant role in long-term muscle atrophy response (Abadi *et al.,* 2009, Suetta *et al.,* 2012). Furthermore, several investigations indicate that down-regulation of muscle protein synthesis occurs

concurrent to a transient rise in signalling markers of protein degradation in response to both bed rest and limb immobilisation (Gibson *et al.,* 1987, Ferrando *et al.,* 1996, Paddon-Jones *et al.,* 2006, de Boer *et al.,* 2007, Glover *et al.,* 2008). Overall, existing evidence currently points towards reduced muscle protein synthesis as the primary culprit in reducing muscle mass during immobilisation, although increased muscle protein degradation may also contribute to the initial decline observed in the early stages.

1.4.3 Strength changes

A consequence of the reduced muscle volume as a result of muscle disuse is the associated reduction in the force generating capacity of the muscle, resulting in decreased muscle strength. Interestingly, reductions in muscle strength following muscle disuse have been found to be of a much greater magnitude compared to decrements in muscle volume. Bed rest for 10-42 days has been reported to reduce maximal voluntary contraction by 20-42% (MacDougall *et al.,* 1977, Bamman *et al.,* 1998, Kitahara *et al.,* 2003, Paddon-Jones *et al.,* 2004, Miles *et al.,* 2005, Kortebein *et al.,* 2007, Brooks *et al.,* 2008, Lundbye-Jensen and Nielsen 2008, Matsumura *et al.,* 2008, Johnston *et al.,* 2009), while studies using limb immobilisation for 9-45 days has been shown to reduce maximal voluntary contraction by 9-41% (Berg *et al.,* 1991, Dudley *et al.,* 1992, Thom *et al.,* 2001, Labarque *et al.,* 2002). Overall, a daily strength loss of 0.3-4.2% per day has been linked with muscle disuse during immobilisation (Wall and van Loon 2013). Such extensive reductions in muscle strength cannot be accounted for by comparable decrements in muscle volume and a considerable body of evidence now points towards

alterations in the capacity for motor unit recruitment and activation by muscle, a phenomenon preceding the onset of muscle atrophy (Duchateau and Hainaut 1987, Berg *et al.,* 1991, Hortobagyi *et al.,* 2000). Duchateau and Hainout reported a 20% greater reduction in maximal voluntary force compared to electrically evoked maximal force, highlighting a less effective neural drive following 6 weeks of forearm immobilisation (Duchateau and Hainaut 1987). Reports from Hortobágyi *et al*. revealed a 40% decline in electromyogram activity of the vastus lateralis and vastus medialis following 21 days of knee immobilisation, indicating a significant decline in the neural activity required to elicit muscular contraction occurs as a result of muscle disuse (Hortobagyi *et al.,* 2000). Findings from recent studies implicate neural cellular adhesion molecule (NCAM) as having a role to play in the denervation of myofibres during muscle disuse. First, Arentson-Lantz *et al*. reported a significant increase (2%) in the number of myofibres expressing NCAM following 14 days of bed rest (Arentson-Lantz *et al.,* 2016). Demangel and colleagues then added to these findings, measuring a 6-fold increase in the NCAM-positive myofibres after 3 days of dry water immersion (Demangel *et al.,* 2017), which indicates the loss of motor innervation begins very early on during muscle disuse and may account for a significant proportion of strength loss associated with muscle hypoactivity.

1.4.4 Cardiovascular changes

Despite the recognition of the serious health risks associated with common sedentary behaviours, the exact aetiology of the progression of cardiovascular disease remains unclear (Dunstan *et al.,* 2012, Foundation 2017). Various

approaches have been adopted to investigate the effect of physical inactivity and immobilisation on vasculature, each with their own limitations which predominantly relate to difficulties quantifying levels of muscle disuse. While bed rest has been utilised in several studies to provide valuable insight into the impact of complete immobilisation, this approach is accompanied by alterations to plasma volume (Convertino *et al.,* 1998), thereby potentially confounding findings relating to physical inactivity. Limb immobilisation facilitates exploration of the localised effects of immobilisation, allowing comparison to the contralateral, non-immobilised limb. Upper limb immobilisation may be the favoured model due to the increased risk of deep venous thrombosis associated with lower limb immobilisation (Bleeker *et al.*, 2004).

Observations from investigations of blood flow changes in response to muscle disuse are conflicting. Leg blood flow, measured using occlusion plethysmography, has been reported to decrease by 44-49% after 42-120 days bed rest (Louisy *et al.,* 1997, Christ *et al.,* 2001) and 18-42 days of bed rest reduced calf blood flow by 26-38% (Convertino *et al.*, 1989, Louisy *et al.*, 1997, Kamiya *et al.,* 2000), whereas no change in femoral artery blood flow has been observed in response to 12-28 days of lower limb immobilisation (Bleeker *et al.,* 2005, Rakobowchuk *et al.,* 2011). The discrepancy in findings from bed rest studies compared to those using limb immobilisation may be the result of more modest vascular alterations associated with limb immobilisation or longer periods of disuse are necessary to induce quantifiable changes to blood flow. Confounders of bed rest studies, such as microgravity, may also

contribute to the more pronounced blood flow changes which were not observed using other experimental models which did not induce total immobilisation.

Evaluation of the adaptations to artery structure, such as lumen dimension and wall thickness, also provide valuable insight regarding the mechanisms underlying vascular changes as a result of immobilisation. Evidence gathered to date indicates arterial diameter is significantly decreased by immobilisation in a dose and time-dependent manner (Bleeker *et al.*, 2005). Femoral artery diameter was reduced by 13% and 24% following 25 and 60 days of bed rest respectively, assessed using Doppler ultrasound (Bleeker *et al.*, 2005, van Duijnhoven *et al.*, 2010), while the same technique was used to measure decrements of 6% and 13% in arterial diameter following 7 and 28 days lower limb immobilisation (Sugawara *et al.*, 2004, Bleeker *et al.*, 2005). However, Birk *et al*. did not detect any change in brachial artery diameter following 8 days of forearm immobilisation as measured by ultrasonography (Birk *et al.*, 2013). These contrasting findings may be due to insufficient immobilisation of the forearm muscle in the study executed by Birk and colleagues, as only a sling was used to restrict forearm movement, allowing no measure of subject compliance. More robust investigations are necessary to fully comprehend the impact of upper limb immobilisation on arterial diameter. Overall, the available literature to date suggests changes to arterial diameter occur rapidly during immobilisation, and a significant proportion of the decrease in diameter occurs early on. The exact cause of alterations to luminal dimension is unknown, however, this may be related to muscle atrophy occurring as a result

of deconditioning. Furthermore, using sonography, van Duijnhoven and colleagues observed a 20% increase in the arterial wall thickness of both the carotid artery and superficial femoral artery following 60 days of bed rest (van Duijnhoven *et al.,* 2010). Thus, narrowing of the arterial lumen during immobilisation has been postulated to occur as a result of inward remodelling resulting in thickening of the luminal wall, leading to narrowed luminal diameter (Pauletto *et al.,* 1991, Chen *et al.,* 1995).

The inner wall of blood vessels are lined by a single layer of endothelial cells, known as the endothelium, which have a major role to play in vascular tone and blood flow by releasing substances with actions of either vasodilation or vasoconstriction. Vasodilators, particularly nitric oxide, have been highlighted as having a potential role to play in the mechanisms driving vascular changes as a result of muscle disuse. Thus, immobilisation induced impairments of vasodilation by the vascular endothelium, via inhibition of the nitric oxide pathway, represents a potential site for alterations to vascular structure. Much research has explored the impact of immobilisation on endothelial function, typically measured using flow mediated dilation or vascular reactivity, and the results to date have been conflicting. Bed rest for 7, 25 and 52 days, and 28 days of lower limb suspension, was found to augment the flow mediated dilation response of the brachial and femoral arteries (Bonnin *et al.,* 2001, Bleeker *et al.,* 2005, Bleeker *et al.,* 2005), while Hamburg *et al*. observed no change in flow mediated dilation response of the brachial artery after 5 days of bed rest (Hamburg *et al.,* 2007). Methodological discrepancies in flow mediated dilation measurement may

contribute to the variability of these findings. Furthermore, artery lumen size has been inversely correlated with flow mediated dilation response and indicates a strong association between inward remodelling of arteries and increased flow mediated dilation response. Thus, it may not be appropriate to use flow mediated dilation as a measure of endothelial function following inward remodelling of arteries in response to immobilisation. Overall, existing evidence suggests that nitric oxide-dependent endothelial function is preserved during immobilisation (Bonnin *et al.*, 2001, de Groot *et al.*, 2004, Bleeker *et al.*, 2005), indicating vasoconstrictor function may instead be upregulated in the vascular adaptation to deconditioning.

Immobilisation acts a potent stimulus for rapid inward remodelling and increased wall thickness of vessels, by disrupting the balance between the vasoconstrictor and vasodilator pathways. Evidence from the existing literature indicates that structural changes are preceded by functional changes, both of which happen quickly in response to immobilisation, and are likely to occur within days or weeks although the precise time frame is yet unknown. In conclusion, it is clear that immobilisation greatly effects vascular health, resulting in a pro-atherosclerotic environment and clarification of the time period over which these pathological changes occur is imperative to establish and implement effective countermeasures.

1.5 Aims

1. To investigate the time course of changes (if any) in skeletal muscle glucose uptake, brachial artery blood flow and serum insulin concentration prior to, during and following 3 days of forearm immobilisation in healthy, young volunteers.
2. To quantify the time course of change (if any) in postprandial lipid clearance by skeletal muscle during 2 days of forearm immobilisation in healthy, young volunteers.
3. To determine any additive effect of increasing dietary lipid availability on the pattern of change in forearm glucose uptake during forearm immobilisation.
4. To measure changes in forearm muscle cross sectional area and strength before and after 16 days of forearm immobilisation.
5. To establish an effective protocol for quantification of forearm lipid content using 7T proton magnetic resonance spectroscopy, and highlight possible inter-relationships between lipid accumulation in forearm muscle and attenuated forearm glucose uptake over 16 days of immobilisation.

Chapter 2: General methods

2.1 Common Methods

The following sections outline the experimental procedures common to each of the studies in this thesis, including volunteer recruitment, medical screening, laboratory visits and statistical analysis.

2.1.1 Ethical considerations

All of the experimental protocols described in this thesis were approved by the University of Nottingham Medical School Ethics Committee and were conducted in accordance with the principles detailed in the Declaration of Helsinki from the World Medical Association (2013). Informed, written consent was obtained from all volunteers before participation.

2.1.2 Volunteer recruitment and screening

Volunteers were recruited from the staff and student population of the University of Nottingham. After expressing their interest in taking part, volunteers were sent an information sheet to read through before being invited to attend the David Greenfield Human Physiology Unit at the University of Nottingham for a medical screening visit. During this visit the study was verbally explained in detail, including study timeframe, protocol, procedures and participants right to withdraw at any time, before written consent was obtained. Subjects then completed a general health questionnaire before having their body weight and height measured for the calculation of body mass index. Duplicate measurements of both standing and supine blood pressure were recorded (Dinamap Pro 1000, GE Healthcare, Little Chalfont, UK), in addition to a 12-lead electrocardiogram (ECG; Schiller, Altgasse, Switzerland) being conducted. A blood sample (10ml) was taken from an

antecubital vein and screened for full blood cell count, hepatic, renal and clotting functions by the Departments of Haematology and Clinical Chemistry, Queens Medical Centre, Nottingham, UK. Screening data was reviewed and approved by a medic before subjects were recruited onto the study. Exclusion criteria for study participation included any metabolic or endocrine abnormalities, history of varicose veins or deep vein thrombosis, regular medications, clinically significant abnormalities detected by ECG or blood tests, or any contraindications to magnetic resonance imaging scans for the study detailed in Chapter 5 (including metal present in the body or extensive tattoos on the body, especially on the arm).

2.1.3 Forearm immobilisation

Subjects attended the David Greenfield Human Physiology Unit for a fibre-glass cast to be fitted by a trained technician. Fibreglass was chosen as it is lightweight, durable and technicians were familiar with the use of this material. This cast extended from the 2nd phalanges of the fingers, also encasing the thumb, to below the elbow prohibiting any gripping action (Figure 2.1). A stockinet sleeve covered the area to be casted and extended both proximally and distally beyond the end of the cast. Two layers of cast padding were then applied in a spiral manner from distal to proximal ends with overlap of one-half the width of the padding, plus extra padding placed over bony prominences, while the arm was held at a 90° angle at the elbow with the forearm in a neutral position without pronation or supination. Finally, the casting tape was immersed in water and applied in the same fashion as with the cast padding. Before a second layer of casting tape was applied to

finish the cast, the surplus stockinet was folded down. The immobilised arm was supported by a broad-arm sling, which volunteers were repeatedly instructed to wear at all times, except when bathing and sleeping (2.2). A protective cover was provided to volunteers to ensure the cast did not get wet whilst bathing. The non-immobilised arm served as the control limb.



Figure 2.1. Forearm fibreglass cast.



Figure 2.2. Volunteer with fibreglass cast and sling.

2.1.4 Laboratory visit

2.1.4.1 Cannulation

All laboratory visits took place in a temperature controlled room at 25°C and 50% humidity. After voiding their bladder, volunteers were asked to rest semi-supine (elevation of 30°) with their dominant foot warmed in a hot-air warming unit (55°C; University of Nottingham Medical Engineering Unit, Queens Medical Centre, Nottingham), in order to facilitate cannulation. Following the intradermal infiltration of approximately 0.1ml local anaesthetic (1% Lidocaine Hydrochloride, B.Braun, Melsungen, Germany) to minimise discomfort, a 20-gauge sterile cannula (BD Venflon, Becton Dickinson Infusion Therapy, Helsingborg, Sweden) was inserted in a retrograde direction in a dorsal vein of the heated foot. The cannula was kept patent during the sampling period by a slow running saline infusion (0.9%, sodium chloride, Baxter Healthcare, Norwich, UK). The foot was then returned into the hot-air warming unit to allow sampling of arterialised-venous blood from the foot and remained there for the duration of the sampling period, with the temperature regularly monitored and recorded to ensure it remained constant throughout each laboratory visit. Arterial cannulation carries considerable associated risk and discomfort for participants, thus arteriovenous cannulation offers a safer and well-tolerated alternative, for which the difference between whole blood glucose concentrations between arterialised venous and true arterial samples is negligible (Forster *et al.,* 1972). Furthermore, multiple arterial cannulations are not possible thus eliminating this technique from temporal studies. Additionally, Gallen and Macdonald demonstrated minimal effects of using a

hot air warming unit on skin and forearm blood flow in the contralateral heated limb (Gallen and Macdonald 1990).

As it was necessary to sample venous blood draining directly from the forearm musculature an ultrasound scan (Aplio SSA-770A; Toshiba Medical Systems) was performed using a 12-14 MHz transducer probe, which was used to identify a vessel with an adjoining perforating vein draining the deep vein of each forearm. Once this was identified, a retrograde cannula was inserted, as described above, into the antecubital deep vein in each arm, so the tip lay below the level of the bifurcation for blood sampling from the muscle bed. Correct placement was then confirmed using ultrasound. Insertion of each retrograde cannulae within a deep vein requires more practitioner experience and takes substantially longer compared to antegrade cannula insertion in a superficial vein. Furthermore, repeated cannulation of the same deep vein can lead to thrombosed or hardened veins, leading to increased difficulty in achieving successful cannulation. Figure 2.3 provides an illustration of the setup for laboratory visits.

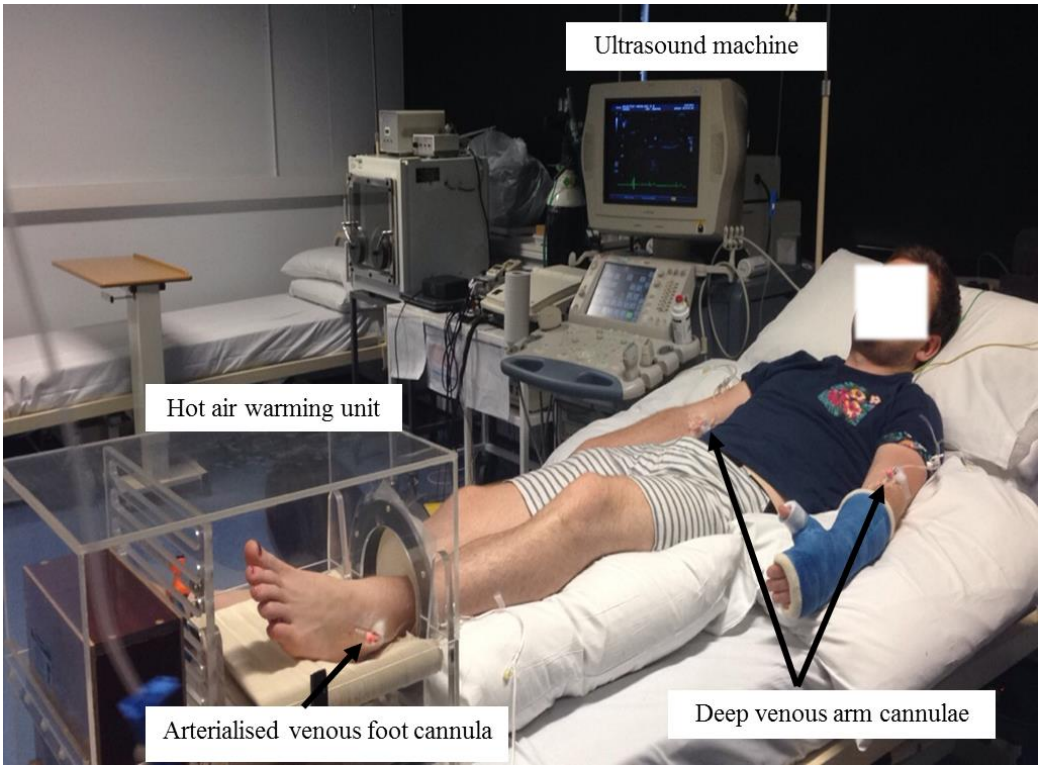


Figure 2.3. Photograph of setup for each laboratory visit demonstrating positioning of cannulae and placement of foot in hot-air warming unit to achieve arterialisation of venous blood.

2.1.4.2 Blood flow measurement

Doppler ultrasound is most frequently used to measure blood velocity in the femoral and brachial arteries. Although there is currently no definitive gold standard for the measurement of blood flow through muscle in human limbs, Doppler ultrasound is commonly used as it is non-invasive and is considered to provide the best measure of absolute blood flow. This technique uses a handheld transducer to emit ultrasonic waves of a set frequency, which are scattered by moving erythrocytes within a blood vessel. The scattered waves return to the transducer at an altered frequency, this difference in frequency is known as the Doppler effect. The shift in frequency is determined by the speed of the moving erythrocytes, from which the mean blood velocity may be calculated. The handheld transducer can also be used to determine diameter of

a blood vessel, which is needed in addition to mean blood velocity for calculation of blood flow.

For the studies in this thesis brachial artery blood flow was measured concurrent to blood sampling by Doppler ultrasound imaging (Aplio SSA-zier70A Diagnostic ultrasound system, Toshiba Medical Systems) equipped with a linear array transducer (12-14MHz), connected to a 3-lead electrocardiogram. To ensure the position of the transducer was constant throughout each experimental visit, its location was marked onto the skin using semi-permanent ink, while subjects lay semi-supine on a hospital bed. The mean blood velocity and luminal diameter of the brachial artery were measured longitudinally at the same anatomic location, 2-5cm above the antecubital fossa in each arm. The diameter of the brachial artery was defined as the distance between the media-adventitia interface of the near wall and the luminal-intima-interface of the opposite wall of the vessel as these landmarks are easily identifiable. Luminal diameter measurements were determined using online video callipers at a perpendicular angle to the axis of the vessel and were measured at the same time point in the cardiac cycle, to overcome changes in vessel diameter during the cardiac cycle. To determine average brachial artery diameter over the cardiac cycle, longitudinal vessel images were stored and later analysed at the peak systolic (mid-T wave) and end diastolic phase (mid-R wave; Figures 2.4 and 2.5). Diameter of the brachial artery was then calculated from the mean of three measurements taken at systole and diastole, with diastole contributing two thirds and systole contributing one third to the mean diameter. The reproducibility of the within-

and between-subject measurements of baseline diameter in this laboratory is 1.7% and 8.5%, respectively. Blood velocity was measured using the pulsed-wave Doppler signal at an insonation angle of 60°. The same measurements were then made on the contralateral, non-immobilised arm at pre- and post-immobilisation visits. Mean blood velocity and brachial artery diameter images were stored and, at a later date, used to determine brachial artery blood flow (see Section 2.4). The same Doppler ultrasound unit and ultrasonographer (intrarater coefficient of variation (CV) = 16%; determined from 60 pairs of repeated baseline measurements) were used to measure blood flow in each limb across all laboratory visits within the same study.

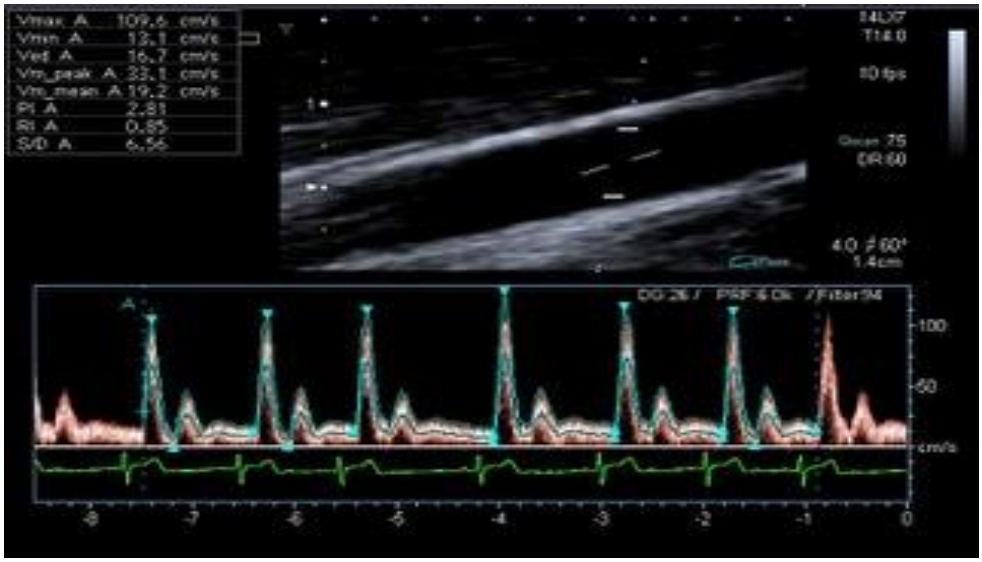


Figure 2.4. Ultrasound image demonstrating 60° insonation angle, images were stored for the subsequent determination of mean blood velocity.

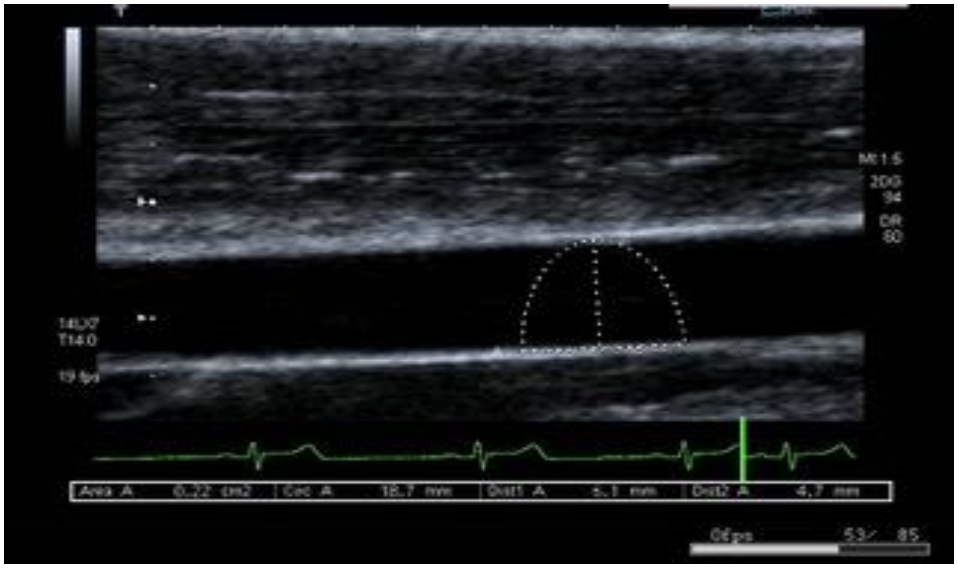


Figure 2.5. Ultrasound image of brachial artery longitudinal section stored for the subsequent determination of vascular diameter. Online video callipers were positioned at a perpendicular angle to the axis of the blood vessel and vessel diameter was determined using in-built computer software.

2.1.4.3 Oral glucose challenge

In Chapters 3 and 5 oral glucose challenges were used to determine forearm glucose uptake. Participants were asked to refrain from caffeine, alcohol and strenuous exercise for 24 hrs before each experimental visit. Participants were also asked to fast overnight, consuming nothing except unflavoured water. On the morning of each laboratory visit two baseline blood samples were taken in duplicate in the fasted state. Subjects consumed a 30% oral glucose solution (75g dextrose dissolved in 250ml water) within 5 minutes. Blood samples were then collected simultaneously from each cannula at 10 min intervals for the initial 2 hrs and every 15 min for the final hour of the 180 min oral glucose challenge. Brachial artery blood flow was measured concurrent to blood sampling, using Doppler ultrasound, at all time-points.

2.1.5 Blood sample collection and analyses

To allow the comparison of metabolite concentration between the arteriovenous and venous samples, blood samples were simultaneously drawn into syringes from a three-way tap attached to each cannula. Syringes containing the first 2ml of sample were discarded to avoid dilution by saline. A further 0.5ml of blood was drawn from each cannula at every time point for blood glucose determination, while an additional 1ml was taken every 20 minutes for the initial 120 mins and every 30 minutes for the final 60 minutes of the oral glucose challenge, for determination of serum insulin concentration. Overall, 360ml and 432ml were collected in total for the oral glucose challenges described in Chapter 3 and 5. For the mixed meal challenge in the study detailed in Chapter 4, 0.5ml of blood was collected every 10 minutes for the first 2 hrs, every 15 minutes for the third hour, and every 60 minutes for the final 4 hrs for measurement of whole blood glucose concentration. Insulin concentration was measured in 1 ml of blood drawn every 20 minutes for the initial 2 hrs, every 30 minutes for the third hour and every 60 minutes for the final hr. Finally, a further 2 ml of blood was collected every 30 minutes for the first 3 hrs and every 60 minutes thereafter to determine triacylglycerol and free fatty acid concentrations. Overall, 490 ml of blood was collected in the study detailed in Chapter 4.

2.1.5.1 Whole blood glucose concentration

As multiple blood samples were to be simultaneously collected in all studies detailed in this thesis, a pilot study was initially conducted to determine which post-sampling processing methods are most appropriate to produce accurate

and stable measures of whole blood glucose concentration in conjunction with the forearm balance model (Appendix 1). This pilot work showed the most profound decline in whole blood glucose concentration occurs within the first three minutes of being drawn, after which time decrements in glucose concentration become less marked. Thus, as multiple samples were drawn at each time point, glucose concentration was measured after 3 minutes to reduce the impact of time taken to analyse, which would introduce variation due to continued glycolysis. Directly after the collection of blood samples, 0.5ml from each sample was added to microtubes (Sarstedt, Nümbrecht, Germany), containing16 IU heparin and 1.0 mg fluoride to inhibit glycolysis, and placed on a roller (Stuart Roller Mixer SRT6, Bibby Scientific, Staffordshire, UK) for 3 minutes. The samples were then analysed for whole-blood glucose concentration using a YSI automated analyser (YSI 2300 STATplus, Yellowsprings Instruments, Yellowspring, Ohio, USA), which was calibrated with 10 mmol/l glucose standards before and during each experimental visit. The YSI analyser has two enzyme specific membranes, targeting either glucose or lactate, each containing three layers (YSI STAT 2300 Laboratory manual 5-4). The outer polycarbonate layer is porous and limits the diffusion of glucose to the inner layer, thereby preventing an enzyme limiting reaction. The middle layer incorporates the immobilised enzyme, glucose oxidase, which catalyses the oxidation of glucose to gluconic acid, producing H_2O_2:

$$\text{D-glucose} + O_2 \xrightarrow{\text{Glucose oxidase}} \text{D-gluconolactone} + H_2O_2$$

The third cellulose acetate layer prevents larger molecules (molecular weight >200) from diffusing through to the platinum electrode. H_2O_2 molecules are sufficiently small enough to diffuse through this membrane and are oxidised at the platinum electrode, liberating electrons as follows:

$$H_2O_2 \xrightarrow[\text{Platinum anode}]{} 2H+ + O_2 + 2e^-$$

The glucose concentration of a given sample is proportional to electrons produced by the oxidation of H_2O_2, measured at the platinum electrode after the injection of the sample into the YSI (Chua and Tan 1978).
Finally, the circuit is concluded at a silver cathode where oxygen is reduced to water:

$$4H + O_2 \longrightarrow 2H_2O + 4e^-$$

The intra-assay CV describes the variation of results measured from one subject and is calculated by dividing the standard deviation of a set of repeated measurements from the same subject by the pooled mean, and multiplying by 100. The intra-assay CV for measurement of whole blood glucose concentration using the YSI is 1.0%. The inter-assay CV is 0.6%, which describes assay to assay consistency, or consistency between YSI machines, and is determined by dividing the overall mean of the measured samples by the pooled standard deviation, and then multiplied by 100, is 0.6% for whole blood glucose determination using the YSI.

2.1.5.2 Serum insulin concentration

Approximately 1ml of each blood sample was added to serum separating tubes (BD vacutainers, Helsingberg, Sweden), which were then gently inverted 5 times and allowed to clot over 30 min before being centrifuged for 10 min at 3,000 rcf. Serum was pipetted and divided into aliquots in screw-top microtubes (Sarstedt, Nümbrecht, Germany) before being stored at -40°C. At a later date, serum insulin concentration was determined using a solid phase radioactive immunoassay (Millipore Insulin, Linco Research, Missouri, USA). This assay uses the sandwich method, where two monoclonal antibodies are aimed at separate antigenic determinants on the surface of the insulin molecule. Insulin in the samples interacts with the anti-insulin antibodies attached to the microtitration well, and also with peroxidase-conjugated anti-insulin antibodies. Following the incubation period the microtiter plate is washed to remove unbound enzyme labelled antibody, and the bound conjugate is then detected by reaction with a chromogenic (3,3',5,5'-tetramethylbenzidine). Hydrochloric acid is added to stop the reaction and the microtiter plate is read spectrophotometrically at a wavelength of 450nm. The concentration of insulin in the original sample is directly proportional to the intensity of colour produced. This assay has an inter-assay co-efficient of variation, of <10.0%.

2.1.5.3 Serum triacylglycerol concentration

For the study described in Chapter 4 a further 1ml of each blood sample was added to serum separating tubes, gently inverted 5 times and allowed to clot for 30 min before being centrifuged for 10 min at 3,000 rcf. Serum was then

aliquoted, using pipettes, into screw-top mictotubes (Sarstedt, Nümbrecht, Germany) before being stored at -80°C. At a later date serum triacylglycerol concentration was determined enzymatically using coupled reactions, in which triacylglycerols are hyrolyzed by lipase to produce glycerol and free fatty acids. The liberated glycerol formed in equation 1 below is phosphorylated by adenosine triphosphate to glycerol-3-phosphate, a reaction catalysed by glycerol kinase (equation 2 below). Glycerol phosphate oxidase then oxidises glycerol-3-phosphate, producing dihydroxyacetone phosphate and hydrogen peroxide (H_20_2; equation 3). The peroxidase catalysed coupling of 4-aminoantipyrine (4-AAP) and sodium N-ethyl-N-(3-sulfopropyl)*m*-anisidine (ESPA) with H_2O_2 then produces a quinoneimine dye (equation 4), which was measured spectrophotometrically on an automated analyser (ABX Pentra 400, Horiba Medical, Montpellier, France) at an absorbance maximum at 540nm. The inter-assay co-efficient of variation for this assay is <3.0%.

(1) Triglyceride $\xrightarrow{\text{Lipoprotein lipase}}$ Fatty acid + Glycerol

(2) Glycerol + ATP $\xrightarrow{\text{Glycerol kinase}}$ Glycerol-3-Phosphate +ADP

(3) Glycerol-3-Phosphate + O_2 $\xrightarrow{\text{Glycerol phosphate oxidase}}$ Dihydroxyacetone phosphate + H_2O_2

(4) H_2O_2 + 4-AAP+ESPA $\xrightarrow{\text{Peroxidase}}$ Quinoneimine dye + $4H_20$

2.1.5.3 Serum glycerol concentration

Although plasma is the optimal sample for determination of free glycerol concentration, free glycerol was measured in the same samples collected to determine serum triacylglycerol concentration (Section 2.1.5.3) for the study

detailed in Chapter 4 to minimise the volume of blood collected from participants. Serum glycerol concentration was measured using a Free Glycerol Determination Kit (Sigma-Aldrich), which measures endogenous glycerol, excluding initial lipase hydrolysis. Glycerol kinase firstly phosphorylates glycerol in a given sample, forming glycerol-3-phosphate (G-3-P) and adenosine-5'-diphosphate (ADP; equation 1). Glycerol phosphate oxidase then oxidises G-3-P producing dihydroxyacetone phosphate and hydrogen peroxide (equation 2). H_2O_2 is then coupled with 4-AAP and ESPA by peroxidase (equation 3), resulting in the production of a quinoneimine dye which displays an absorbance. Free glycerol concentrations are directly proportional to increases in absorbance at 540nm, as determined using automated analyser (ABX Pentra 400, Horiba Medical, Montpellier, France). The inter-assay co-efficient of variation for this assay is <2.5%.

(1) $\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerol kinase}} \text{Glycerol-3-Phosphate} + \text{ADP}$

(2) $\text{Glycerol-3-Phosphate} + O_2 \xrightarrow{\text{Glycerol phosphate oxidase}} \text{Dihydroxyacetone phosphate} + H_2O_2$

(3) $2H_2O_2 + \text{4-AAP+ESPA} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine dye} + 4H_20$

2.1.5.4 Plasma free fatty acid concentration

The final 1ml of whole blood from each sample was added to lithium heparin coated anticoagulant tubes pre-filled with 7.5µl of ethylene glycol tetraacetic acid-gluthathione (BD vacutainers, Helsingberg, Sweden), a preservative which inhibits degradation of free fatty acids during storage. Tubes were then gently inverted 8 times and centrifuged for 10 min at 3,000 rcf. Plasma was then pipetted into tubes containing 5µl tetrahydrolipstatin to prevent *in vitro*

lipolysis (Krebs *et al.*, 2000), before being stored in screw-top microtubes (Sarstedt, Nümbrecht, Germany) at -80°C until analysis for determination of plasma free fatty acid concentration. At a later date these plasma samples were analysed for free fatty acid concentration using a coupled enzymatic reaction (Wako Chemicals GmbH, Neuss, Germany). Firstly Acyl-CoA synthetase catalyzes fatty acylation of Coenzyme A. The acyl Co-A formed from this reaction is then oxidised by acyl CoA oxidase, generating H_2O_2. In the presence of peroxidase H_2O_2 facilitates the oxidative condensation of 4-AAP with 3- methyl-N-ethyl-N-(-hydroxyethyl)-aniline producing 10-acyl-3,7-dihydroxyphenoxazine (ADHP), a purple product which absorbs light at 550nm and was measured with an automated analyser (ABX Pentra 400, Horiba Medical, Montpellier, France). This assay has an inter-assay co-efficient of variation of <3.0%.

(1) $\text{FFA} + \text{ATP} + \text{CoA} \xrightarrow{\text{Acyl CoA synthetase}} \text{Acyl-CoA} + \text{ADP}$

(2) $\text{Acyl-CoA} + O_2 \xrightarrow{\text{Acyl-CoA oxidase}} \text{2,3-trans-Enoyl-CoA} + H_2O_2$

(3) $2H_2O_2 + \text{4-AAP} + \text{3- methyl-N-ethyl-N-(-hydroxyethyl)-aniline} \xrightarrow{\text{Peroxidase}} \text{ADHP} + 4H_20$

2.1.6 Measurement of handgrip strength

Handgrip strength is a measure the maximal voluntary isometric force produced by muscles of the hand and forearm. In Chapter 3 (visits 1 and 4)

handgrip strength was measured using a standard handgrip dynamometer (University of Nottingham Medical Engineering Unit, Queens Medical Centre, Nottingham) while in Chapter 5 (visits 1 and 5), a handgrip dynamometer compatible with a Magnetic Resonance scanner was used (Current Designs, USA). At the end of the oral glucose challenge and before the cannulae were removed in the laboratory visits detailed in Chapter 3, subjects were asked to sit on the edge of the bed with their arms resting on a table, with the elbow by the side of the body and the forearm at right angles. The handgrip was held in an upright position, with the base resting at the first metacarpal (heel of the palm) and the fingers gripped around the handle. When ready, subjects were strongly encouraged to squeeze the dynamometer with maximum voluntary effort three times on each hand, with 2 minutes rest between each contraction to avoid muscle fatigue. For the study described in Chapter 5, handgrip strength measured according to the same testing protocol prior to entering the MRI scanner. Measurement of handgrip strength has a CV of 10%.

2.2 Principles underlying the measurement of arterialised venous-venous substrate difference

In 1887, Chauveau was the first to utilise an "input-output method" to measure glucose uptake of the masseter muscle in horses, by multiplying blood flow by the local arterial-venous difference (Chauveau MA 1887). Considerable development of this method since then has led the arterialised venous-venous difference model to be extensively used for the determination of uptake or release of a metabolite by a tissue, by calculating the difference between arterialised venous and venous concentrations of the metabolite of interest. A

positive difference represents net uptake by the tissue, while a negative difference represents net release of the metabolite by the tissue. This technique is based on the Fick principle, with the hypothesis that the metabolic activity of a tissue equates to the difference between the concentration of a metabolite in the arterial supply to a tissue and the venous supply draining the same tissue, when measured simultaneously (Zierler 1961). If blood flow is concurrently measured the rate of uptake or release of a metabolite by the tissue can be determined. Application of the Fick principle is most fitting during a steady state, when the arterial concentration and tissue metabolism of a substrate are constant, and blood flow is steady. Some fluctuations may be sufficiently small enough to permit the application of the Fick principle, such as changes to blood flow due to the cardiac cycle. Although certain conditions, such as increasing arterial glucose concentrations in response to a glucose load or meal, may complicate the interpretation of information collected whilst glucose concentrations fluctuate, glucose uptake may be estimated by assimilating the arterialised venous-venous differences in between the two steady states of metabolite concentrations. Although insulin sensitivity was not directly measured in the studies comprising this thesis, measurement of forearm glucose uptake, substrate delivery and circulating insulin concentration were used as markers of insulin sensitivity.

While the use of both forearm and leg in limb balance models is now frequently used to investigate muscle metabolism, selection of the forearm as the limb of choice provides several advantages. The majority of forearm tissue mass is composed of skeletal muscle (Barrett and Rattigan 2012), which

thereby lessens the probability of sampling blood draining adipose. Additionally, the vascular anatomy of the femoral vein complicates cannula positioning and makes it difficult to avoid sampling blood draining subcutaneous adipose tissue. Although additional imaging techniques, such as ultrasound guidance, are required for cannulation of veins draining forearm musculature, the vascular anatomy of upper limbs is relatively well understood. Thus, cannulation of antecubital deep veins is comparatively more straightforward and ensures sampling of blood draining forearm muscle.

2.3 Basic Principles of Magnetic Resonance Spectroscopy

Proton MRS records signals from chemicals or metabolites which are then converted to peaks and used to identify and quantify the chemical or metabolite. Only nuclei with non-zero spin are detectable by MRS, therefore ^{1}H, ^{13}C and ^{31}P are among the most commonly used nuclei in this process. As ^{1}H is the most naturally abundant of these nuclei, this produces stronger MR signals. The frequency location of a metabolite or chemical determined by ^{1}H MRS depends on the configuration of protons within the metabolite.

In humans, MRS data are obtained using a MRI scanner composed of several different elements. Inside the MR scanner a magnet produces a magnetic field which causes nuclei within this field to align either with (low energy state) or against (high energy state) the magnetic field. The homogeneity of the magnetic field is one of the major factors contributing to metabolite spectrum line width resolution or peak sharpness. Thus, it is crucial that the strength of the magnetic field is homogenous and a shim system is used to ensure this

remains constant. A radio frequency coil transmits pulses at set timings, duration and power, and can also receive signals. Energy applied by the radio frequency coil causes the magnetisation to flip nuclei to a different orientation, nuclei immediately start to revert to their original orientation and emit small amounts of energy, which is detected by the receiver of the radio frequency coil, and this is known as precession. Fourier transformation is then used to generate MRS spectra from the signals detected by the coil. The area under each peak on the spectrum is proportional to the metabolite concentration.

For several decades magnetic resonance technology has been used in brain imaging for the detection of tumours, strokes, neurodegenerative disorders etc. In recent years, much attention has turned to utilising this methodology to non-invasively quantify IMCL content. This technique is based on the differing resonance frequencies emanating from the different structure of IMCL and EMCL. Lipids may be stored within skeletal muscle in either intra- or extra-myocellular spaces. The EMCL are stored between muscle fibres, assuming long, tubular shapes and may have different sizes or orientations, whereas IMCL are small, spherical fat droplets found near mitochondria within the cyotosol (Ren *et al.,* 2010). These differing characteristics allow IMCL and EMCL to be differentiated using ^{1}H MRS, as the magnetic field within each structure varies depending on its orientation, shape and magnetic susceptibility (Khuu *et al.,* 2009). Thus, these compartments were shown to be separated by approximately 0.2 ppm on the spectrum from human calf muscle almost 20 years ago (Schick *et al.,* 1993, Boesch *et al.,* 2006). Since then the use of ^{1}H MRS to quantify IMCL content has become increasingly common in both physiology and clinical research, with its ability to

distinguish between IMCL and EMCL, its non-invasive nature and high accuracy all major lures for the application of this technique.

The non-invasive nature of ^{1}H-MRS means it is an attractive alternative to the muscle biopsy technique for IMCL quantification, in addition to having a lower coefficient of variation of 6-12% for repeated measurements (Boesch *et al.*, 1997, Szczepaniak *et al.*, 1999). Furthermore, this method allows lipid content of a large area of muscle to be assessed, whereas only a single muscle fibre may be targeted using electron microscopy (EM) or oil red O techniques on muscle biopsy samples. Comparison of the various methods available to quantify IMCL have shown ^{1}H-MRS to correlate strongly with EM analysis of muscle biopsy samples, while biochemical analysis of muscle biopsy samples showed poor correlation with both ^{1}H-MRS and EM analysis (Howald *et al.*, 2002). The major limitations of using ^{1}H-MRS to quantify IMCL primarily relate to overlapping peaks of EMCL and IMCL spectra, and unless this overlap is accounted for concentrations of IMCL may be over-estimated. Thus, specialised line fitting software must be available to accurately quantify each peak, which further adds to the high costs and level of expertise required to employ this method. Nonetheless, the high precision and non-invasive nature of ^{1}H-MRS make this an increasingly popular choice for investigating IMCL content, particularly when answering research questions requiring multiple measurements.

In vivo studies of tissue metabolism and measurements of lipid concentrations are preferential at high field strengths of 7.0T and above, particularly if

detecting spectra from small volumes due to the increased signal-to noise ratio (van der Graaf 2010). While ^{1}H MRS performed at ultra-high field strengths holds many advantages, there are also associated technological limitations which must be considered. Such limitations include increased magnetic field inhomogeneity resulting in spectral line broadening and thereby, inaccurate quantification of metabolite concentrations (Moser 2010). Dominant methylene proton signals are used to measure IMCL content, which originate from the CH_2 chain. However, because EMCL and IMCL spectra tend to overlay each other, both peaks must be fit to a symmetrical line shape such as Gaussian, Lorentzian or Voigt function. While this method is widely used, many investigators report difficulties of successfully fitting spectra obtained from most muscles other than the anterior tibialis (Steidle *et al.,* 2002, Boesch *et al.,* 2006). Unless such limitations are addressed they may offset the benefits of using high field strengths.

2.4 Calculations

The primary variable in the studies included in this thesis was change in area under the curve for forearm glucose uptake from 0 to 72 hrs. Based on previous data from this laboratory observing a 35% decline in forearm glucose uptake after 7 days of forearm immobilisation (Skirrow 2014), as well as findings from Stettler et al. reporting a 20% decline in whole body glucose uptake within 2.5 days of bed rest (Stettler et al. 2005), we expected to see a 20-30% reduction in forearm glucose uptake after 72 hrs of forearm immobilisation. In pilot data we found standard deviation to be 2.4

[mmol/min.180min] in the measurement of forearm glucose uptake between volunteers, which would give an effect size of 0.95. Thus, this effect size should be detectable in 8 subjects with a power of 80% at 5% significance level on a paired t-test basis (Lenth 2006). Therefore, a sample size of n=10 in each study would allow for dropouts.

The arterialised venous-venous substrate difference was calculated as the difference between the arterialised-venous sample (from the heated foot) and the deep venous brachial sample from each arm. Using mean blood velocity and arterial diameter, brachial artery blood flow was calculated in ml/min as brachial artery blood flow= MBV x (πr^2), where MBV =mean blood velocity, measured in cm/second, and *r*= brachial artery radius, measured in cm^2. Using the Fick principle (Zierler 1961) forearm glucose uptake (Chapters 3, 4 and 5), triacylglycerol clearance (Chapter 4), glycerol clearance (Chapter 4) and free fatty acid uptake (Chapter 4) were calculated as: brachial artery blood flow x ([AV] – [V]), where [AV] is the arterialised-venous and [V] is the deep-venous metabolite concentration. These calculations were performed on 180 glucose samples for each subject in Chapter 3, 216 glucose samples for each subject in Chapter 5, and on 210 glucose samples, 120 triacylglycerol, glycerol and free fatty acids respectively for each subject in the study described in Chapter 4. For the studies in this thesis incremental area under the curve analysis was calculated for forearm glucose uptake (Chapters 3, 4 and 5), triacylglycerol clearance (Chapter 4), free fatty acid uptake (Chapter 4), brachial artery blood flow (Chapters 3, 4 and 5), arterialised venous-venous difference (Chapters 3, 4 and 5) and serum insulin (Chapters 3, 4 and 5) and employed curve integration using the trapezium rule.

2.5 Statistical analyses

All statistics, graphs, and calculations presented in this thesis were analysed using GraphPad Prism (version 6.05; GraphPad Software Inc, USA). Data was assessed for normality and treated appropriately using either parametric or non-parametric tests. A two-way analysis of variance (ANOVA) was used to compare all time dependent variables to detect main effects of immobilisation on blood glucose, serum triacylglycerol, glycerol, plasma free fatty acid and brachial artery blood flow. Significant main effects were further investigated using a Sidak post hoc test. One-way repeated measures ANOVA with Dunnett post hoc analysis, students t-test or Friedman test with Dunn's multiple comparison were performed to measure single variables over time. Correlation analysis, presented in Chapter 4, was performed using Pearson product-moment correlation coefficient. Statistical significance was set at $p<0.05$, and all data are presented as means ± standard error of the mean (SEM).

Chapter 3: Immobilisation induced impairment of limb glucose uptake: novel insight regarding the trajectory and time-course in healthy volunteers.

3.1 Introduction

Decreased insulin stimulated glucose uptake by skeletal muscle has frequently been associated with immobilisation in human volunteers (Dolkas and Greenleaf 1977, Charles A. Stuart 1988, Mikines *et al.,* 1991, Smorawinski *et al.,* 2000). Accordingly, interest in the negative health impacts of muscle disuse as a result of our increasingly sedentary lifestyles is becoming a research priority. However, little is known about the temporal relationship or mechanisms linking immobilisation with reduced muscle glucose uptake in humans.

Immobilisation models have frequently been utilised to investigate the negative impact of complete immobility on insulin-mediated glucose uptake at a whole body and limb level. Bed rest has been employed for various durations in human volunteers, most commonly 7-28 days, to induce decrements in whole body and limb glucose uptake (Lipman *et al.,* 1970, Mikines *et al.,* 1988, Mikines *et al.,* 1991, Alibegovic *et al.,* 2009, Biensø *et al.,* 2012), and a number of mechanisms have been suggested to be responsible for these decrements. Following 20 days of bed rest Tabata *et al*. observed a 16% decline in vastus lateralis protein content of glucose transporter 4 (GLUT-4) (Tabata *et al.,* 1999). More recently, Biensø *et al.* also reported lower skeletal muscle protein levels of GLUT 4 and Hexokinase II, in addition to the reduced ability of insulin, delivered using a hyperinsulinemic-euglycemic clamp, to activate glycogen synthase and phosphorylate Akt after 7 days of bed rest (Biensø *et al.,* 2012). These findings suggest that decrements in whole body glucose uptake following bed rest may be a result

of impaired insulin signalling, leading to decreased glucose transport and phosphorylation, along with decreased glycogen synthesis in skeletal muscle. One of the strongest predictors of skeletal muscle insulin resistance is intramyocellular lipid content (Krssak *et al.,* 1999) and yet, little is known about the interaction between muscle fat metabolism and reduced glucose uptake during bed rest. Bed rest for 28 days has been shown to increase intramyocellular triacylglycerol content, measured by magnetic resonance spectroscopy in human subjects, which was associated with a decrease in whole-body glucose uptake during a hyperinslinemc-euglycemic clamp (Cree *et al.,* 2010). A potential explanation for the increased storage of lipids during bed rest is a reduction in fat oxidation (Bergouignan *et al.,* 2011), leading to the incorporation of fatty acids into intracellular lipid stores. Thus, the associated accumulation of lipid intermediates such as diacylglycerols and ceramides, which occur secondary to elevated concentrations of intramyocellular triacylglycerol, are thought to blunt insulin signalling, thereby reducing the insulin activated translocation of GLUT4-containing vesicles to the plasma membrane and reducing muscle glucose uptake.

Impairments in insulin stimulated glucose uptake have also been observed over relatively shorter periods of immobilisation. Following 7 days of bed rest, Mikines *et al.* reported more pronounced decrements in glucose uptake over a wide range of steady-state insulin concentrations under clamp conditions, expressed per kilogram of muscle, in the leg than at the whole body level (Mikines *et al.,* 1991). Similarly, Richter *et al*. reported significant reductions in glucose uptake in the thigh muscle of healthy men, using the

two-step euglycemic-hyperinsulinemic clamp technique, following 7 days of leg immobilisation (Richter *et al.,* 1989). As the leg predominantly consists of muscle, these findings suggest that impairments driving the development of immobilisation induced decrements in muscle glucose uptake reside within the muscle itself and occur as a consequence of muscle inactivity. Thus, limb immobilisation offers a less invasive alternative to bed rest for determining the drivers of immobilisation induced deficits in glucose uptake by skeletal muscle. This model is novel as it permits the contralateral limb to act as a within volunteer control, thereby allowing direct comparisons to be drawn on the effects of the intervention between a non-immobilised and immobilised limb. Additionally, it permits the study of reduced contraction *per se* on intrinsic muscle function, without the confounding effects of reducing overall physical activity, such as a positive energy balance. While these studies have provided substantial insight into the perturbations in glucose uptake associated with lowered muscle use, questions remain regarding the time course of these metabolic disturbances. Two days of bed rest did not induce any measurable change in insulin-mediated glucose disposal (Duran-Valdez *et al.,* 2008), while Lipman and colleagues document the earliest decrease (45%) in muscle glucose uptake following three days of bed rest (Lipman *et al.,* 1970). Previous work from this group has demonstrated a substantial decline in forearm glucose uptake 48 hrs following a bout of resistance exercise, indicating changes in muscle glucose uptake in response to muscle disuse are rapid (Skirrow 2014). However, no investigation to date has explored the time frame of development or progression of these impairments in glucose uptake, therefore the present study aimed to address this. Such a study would be

invaluable in the design of future investigations into the mechanistic basis of immobilisation induced decrements in skeletal muscle glucose uptake.

Periods of immobilisation have been associated with vascular deconditioning resulting in reduced resting and insulin-stimulated blood flow, arterial stiffening and increased arterial tone (Takenaka *et al.,* 1994, Sonne *et al.,* 2010, Nosova *et al.,* 2014). Of particular interest is the potential role of immobilisation induced reductions of blood flow in decreased glucose uptake by skeletal muscle. Such a reduction in blood flow may result in diminished glucose and insulin availability to muscle, despite no change in arterial concentrations, thus leading to lower muscle glucose uptake. Bed-rest for 18-42 days led to a 26-38% decrease in calf blood flow (Convertino *et al.,* 1989, Louisy *et al.,* 1997, Kamiya *et al.,* 2000), whereas 12-28 days of lower limb immobilisation did not result in any changes to femoral artery or leg blood flow (Bleeker *et al.,* 2005, Rakobowchuk *et al.,* 2011), determined via venous occlusion plethysmography. Moreover, insulin has been shown to increase blood flow by increasing microvasculature recruitment, therefore, defects in insulin stimulated microvasculature recruitment can contribute to lowered insulin sensitivity (Lillioja *et al.,* 1987, Baron *et al.,* 1994). Such links between reduced insulin sensitivity and altered microvascular function in response to immobilisation have been proposed by Hamburg and co-workers (Hamburg *et al.,* 2007). The authors demonstrated a reduction in endothelial function, which occurred in parallel to the development of insulin resistance (assessed via Homeostasis model assessment- insulin resistance) following 5 days of bed rest (Hamburg *et al.,* 2007). The findings from these

immobilisation studies raise the possibility that vascular dysfunction and decreased insulin sensitivity during immobilisation may progress through shared mechanistic pathways. Studies exploring the acute vascular changes in response to limb immobilisation would circumvent any confounding factors associated with bed rest studies. Furthermore, immobilisation induced changes in blood flow may have negative implications for overall cardiovascular health (Widlansky *et al.,* 2003), independent of associated impairments in muscle glucose uptake, which warrant further investigation. The existing absence in the literature of investigations into the time-course of immobilisation induced vascular alterations potentially leading to reductions in blood flow thwarts a comprehensive understanding of the implications of short periods of immobilisation on overall cardiovascular and metabolic health.

Muscle disuse has been shown to considerably reduce muscle strength, with the largest decrements reported to manifest in the early stages of disuse (1-2 weeks) after which the rate of decline significantly slows down (de Boer *et al.,* 2007, Homma *et al.,* 2009). Studies exploring the impact of muscle disuse on the strength of upper limb muscles are much less abundant than those focusing on lower limb muscles. To date, three publications have reported a 15-30% decline in muscle strength following 7-9 days of immobilisation of the wrist and upper arm (Miles *et al.,* 1994, Lundbye-Jensen and Nielsen 2008, Johnston *et al.,* 2009). Overall, muscle disuse is associated with a loss of muscle strength in the range of 0.3-4.2% per day (Wall and van Loon 2013), although no study to date has investigated changes in upper limb strength

within the initial 7 days of immobilisation. Such investigations are important as upper limbs are crucial for many simple tasks of daily living, and decrements in strength may significantly affect quality of life.

3.2 Aims and Hypothesis

There has been no investigation in the published literature to date, into the timeframe of the development and progression of impairments in limb glucose uptake in response to reduced muscular activity. Therefore, this study was designed to ascertain the magnitude and time-course of impairment in forearm glucose uptake during three days of immobilisation, and to compare this to a contralateral, non-immobilised arm in healthy male volunteers. Additionally, this study also aimed to determine the effects of three days of forearm immobilisation on brachial artery blood flow and handgrip strength. The a priori hypothesis was that forearm glucose uptake will decrease over the immobilisation period, without any significant changes to blood flow or handgrip strength.

3.3 Research design and methods

3.3.1 Participants and sample size

Ten healthy male volunteers (mean±SD; age 24.9±1.9 years, weight 71.9±8.2 kg, body mass index 22.6±2.2 kg/m^2) participated in the present study.

3.3.2 Experimental protocol

After a medical screening visit and providing informed written consent (Section 2.1.2) participants attended the laboratory on four occasions (Figure 3.1) to complete the experimental protocol as described in Section 2.1.4. Briefly, participants arrived at the laboratory for a pre-immobilisation visit (0 hrs), having fasted overnight, and rested in a semi-supine position on a hospital bed. A retrograde cannula was inserted into a dorsal foot vein under local anaesthesia, and the foot was then placed into a hot-air warming unit in order to arterialise venous drainage of the foot (Gallen and Macdonald 1990). An antecubital deep vein in each arm, identified using an ultrasound scan (Aplio SSA-770A, Toshiba Medical Systems) was then cannulated to allow sampling of venous blood from a deep vein draining the forearm tissue (predominantly skeletal muscle; (Barrett and Rattigan 2012)). Baseline blood samples were taken in duplicate, while brachial artery blood flow was simultaneously measured in each arm, prior to participants ingesting a 75g glucose drink to begin the 180 minute oral glucose challenge (detailed in Section 2.1.4.3). Blood samples from each cannula were collected simultaneously every 10 minutes for the first 2 hrs and every 15 minutes for the third hr. Brachial artery blood flow in each arm was measured concurrent to blood sampling (Figure 3.2).

Within 7 days of the pre-immobilisation visit participants were asked to attend the David Greenfield Human Physiology Unit to have a fibreglass cast fitted to their non-dominant (IMM) arm, allowing the dominant, non-immobilised (NIM) arm to act as a within volunteer control (Section 2.1.3). Participants

returned to the laboratory 24, 48 and 72 hrs after the cast being fitted to repeat the post-immobilisation visits. The 24 and 48 hour post-immobilisation visit were similar to the pre-immobilisation visit, except that only the dorsal foot vein and antecubital deep vein in the immobilised arm were cannulated following immobilisation. Previous work by our group has shown that forearm glucose uptake is unlikely to change in the non-immobilised limb over several days (Skirrow 2014) and so the non-immobilised arm was cannulated at only the pre- and post-immobilisation time-points to reduce the amount of blood and number of time-consuming cannulations for the participants. The 72 hour post-immobilisation laboratory visit was identical to the pre-immobilisation visit and the cast was removed at the end of the oral glucose challenge. Following the oral glucose challenge at the 0 and 72 hour visit subject's handgrip strength was tested using a handgrip dynamometer. Participants completed three bouts of maximal handgrip contractions on each hand with 2 minute rests between each bout (Section 2.1.6). Calculations and statistical analyses were performed as described in Sections 2.4 and 2.5 respectively.

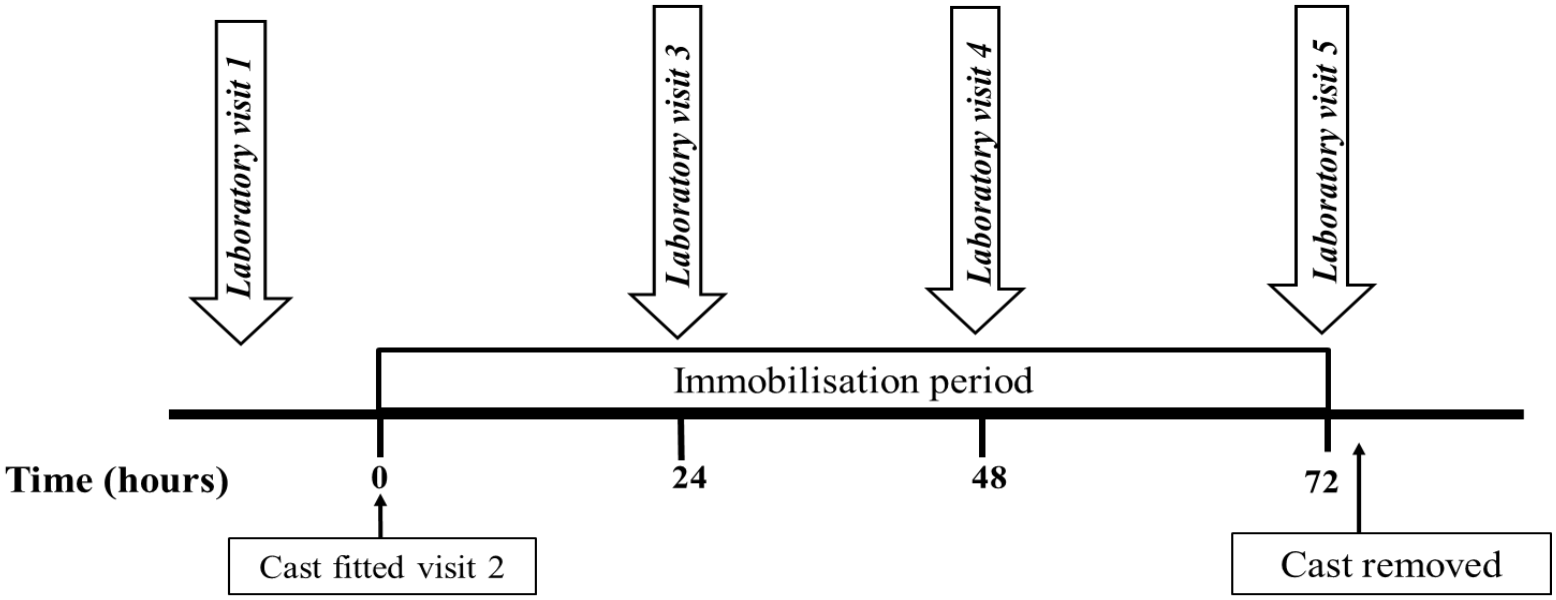


Figure 3.1 Timeline of study protocol.

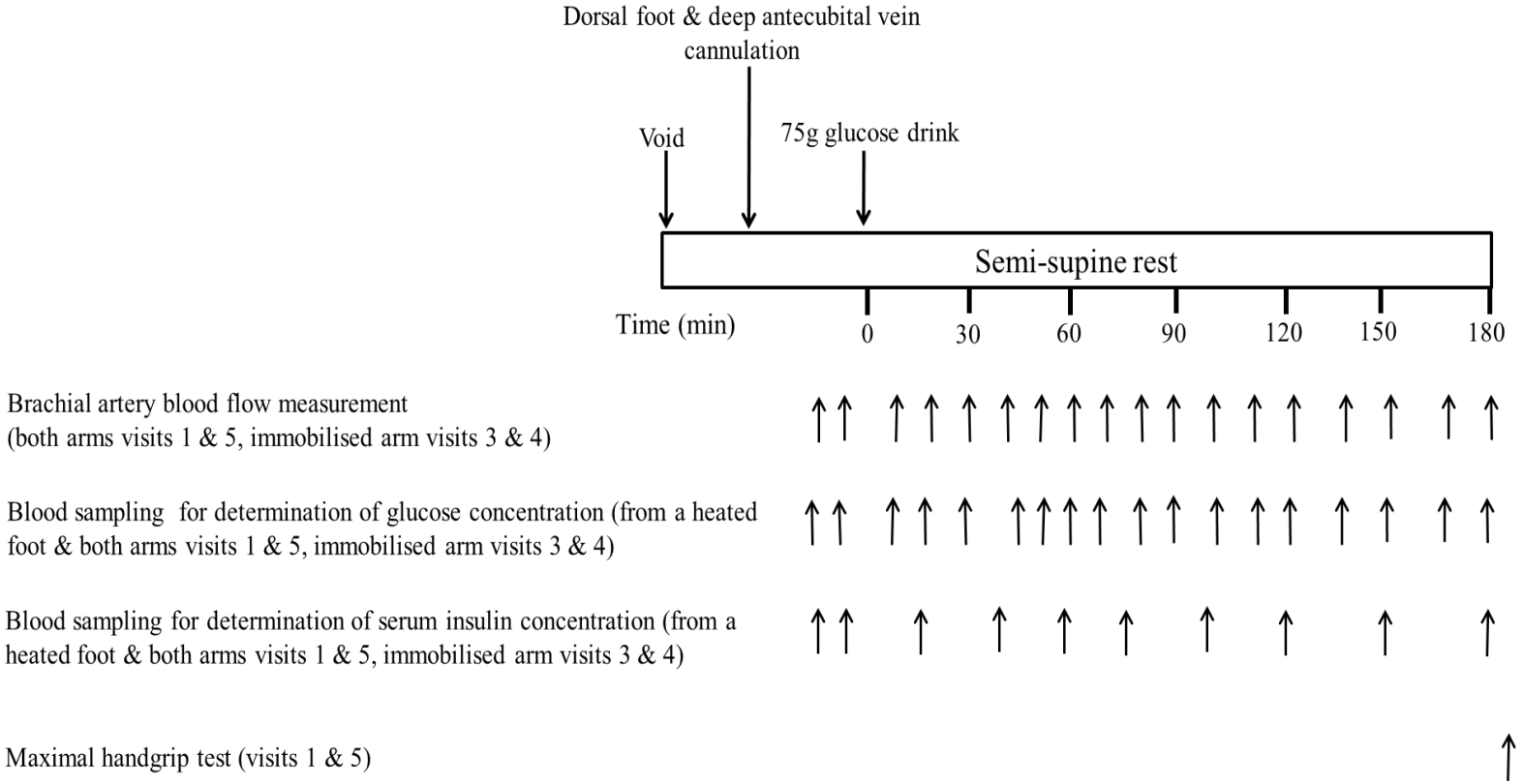


Figure 3.2 Schematic diagram of protocol for laboratory visits.

3.4 Results

Supplementary data, from which the figures in Sections 3.4.3, 3.44, 3.45 and 3.46 are derived from, is included in Appendix 2.

3.4.1 Characteristics of study participants

Ten participants were recruited to the current study. All participants were within normal BMI range of 18.5-25 kg/m^2. Participant characteristics are shown in Table 3.1.

Table 3.1 Characteristics of study participants

Parameters	**Mean ± SD**
Age	24.9±1.9
Body mass (kg)	71.9±8.2
BMI (kg/m^2)	22.6±2.2
Fasting glucose (mmol/L)	4.36±0.39
Fasting insulin (mIU/L)	6.91±2.95

3.4.2 Brachial artery diameter

Basal brachial artery diameter, displayed in Figure 3.3, was measured prior to each oral glucose challenge and was comparable between limbs prior to immobilisation (4.2±0.1 and 4.3±0.1 mm in the NIM and IMM limbs respectively), and did not change from pre-intervention values in the NIM arm following 72 hrs of immobilisation (4.2±0.15 mm; P=0.4). In the IMM limb, there was no significant change from baseline values at 24 hrs (4.2±0.1 mm; P=0.7), however, following 48 and 72 hrs of immobilisation, brachial artery diameter in the IMM limb was less (4.1±0.1 mm and 4.05±0.1 mm; P<0.05,

respectively) compared to pre-immobilisation measurements. This represents a reduction in brachial artery diameter from pre-immobilisation values in the IMM limb of 3.5% and 4.2% at 48 and 72 hrs of forearm immobilisation, respectively.

3.4.3 Brachial artery mean blood velocity

The area under the curve of mean blood velocity for the total 180 min oral glucose challenge is shown in Figure 3.4 and was similar between arms prior to immobilisation (2013±175.8 and 1976±218.7 [cm/s]·180min in the NIM and IMM limbs respectively; P=0.9). After 72 hrs of immobilisation, mean blood velocity during the oral glucose challenge did not change from baseline in the NIM limb (2054±205.9 [cm/s]·180min; P=0.88). In the IMM limb, mean blood velocity during the oral glucose challenge increased from baseline at 24 hrs (2478±204.5 [cm/s]·180min; P<0.01) but showed no change at 48 (2221±161.5 [cm/s]·180min; P=0.21) or 72 hrs (2195±199.9 [cm/s]·180min; P=0.27), such that mean blood velocity in response to glucose ingestion was not different between arms following 72 hrs of immobilisation (P=0.26).

3.4.4 Brachial artery blood flow

According to the area under the curve for brachial artery blood flow during the total 180 min oral glucose challenge, shown in Figure 3.5, the brachial artery blood flow response to glucose ingestion was not different between the NIM and IMM limbs at baseline (17.3±1.7 and 17.8±2.1 [l/min]·180min for NIM and IMM arms respectively; P=0.86) and did not significantly change in the

NIM limb over the immobilisation period (18.1±2.0 [l/min]·180min; P=0.59). However, brachial artery blood flow in the IMM limb was greater at 24 hrs compared to pre-immobilisation (21.6±2.0 [l/min]·180min; P<0.05), but was no different from baseline at 48 hrs (19.0±1.6 [l/min]·180min; P=0.7) or 72 hrs (18.1±2.0 [l/min]·180min; P=0.97). Brachial artery blood flow was similar in both the NIM and IMM arms after 72 hrs of immobilisation (P=0.99).

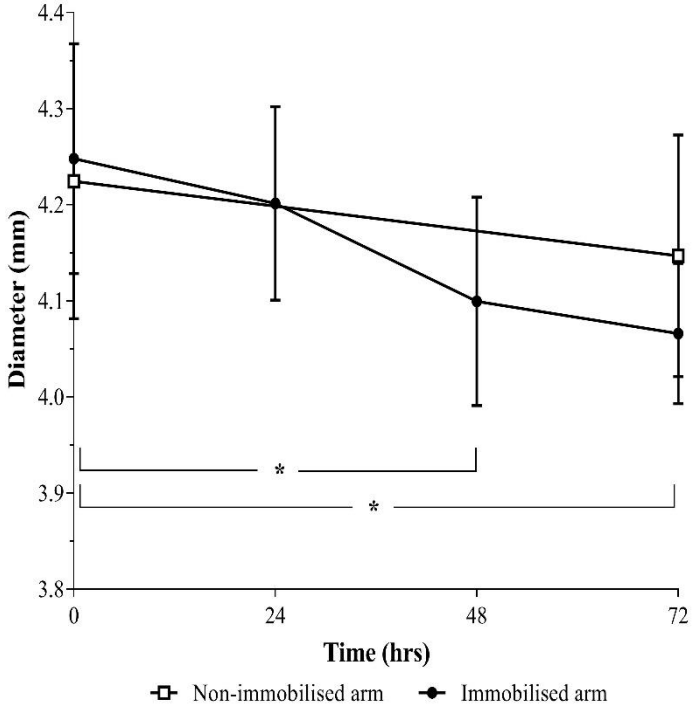


Figure 3.3 Basal brachial artery diameter measured in a non-immobilised and immobilised forearm before (0), and at 24, 48 and 72 hrs of forearm immobilisation. Values are mean ±SEM (*n*=10). *P<0.05 significantly different to corresponding baseline value.

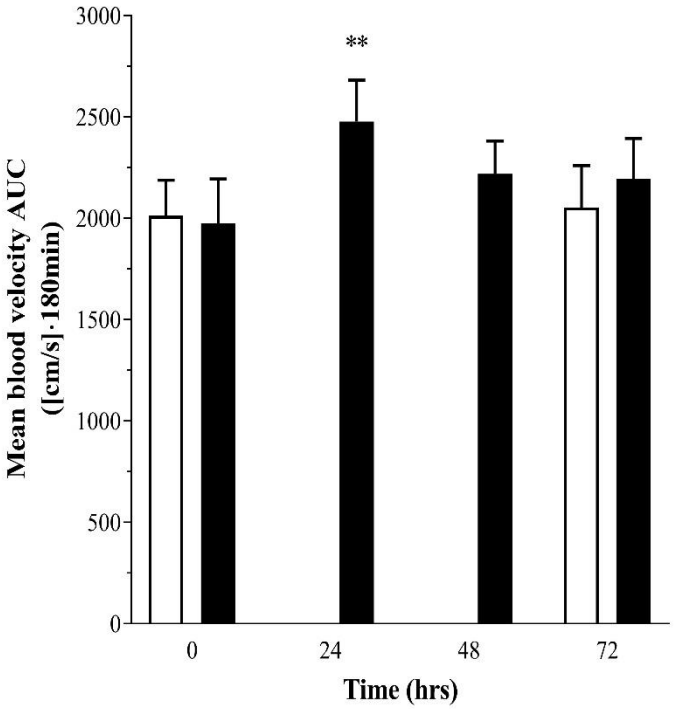


Figure 3.4 Area under the curve (AUC) of mean blood velocity for the total 180 min oral glucose challenge is shown in a non-immobilised (white) and immobilised (black) forearm before (0), during (24, 48) and after 72 hrs of forearm immobilisation. Values are mean ±SEM (*n*=10). **P<0.01 significantly different to corresponding baseline value.

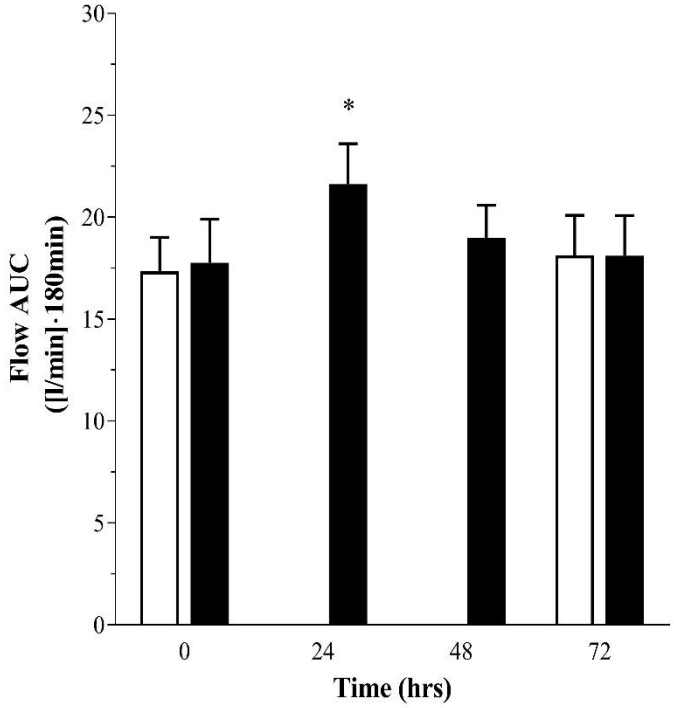


Figure 3.5 Area under the curve (AUC) of brachial artery blood flow for the total 180 min oral glucose challenge was determined for a non-immobilised (white) and immobilised (black) forearm before (0), during (24, 48) and after 72 hrs of forearm immobilisation. Values are mean ±SEM (*n*=10). *P<0.05 significantly different to corresponding baseline value.

3.4.5 Serum insulin

The total area under the curve for arterialised-venous serum insulin concentration during each 180min oral glucose challenge is shown in Figure 3.6. Serum insulin concentration did not change from pre-immobilisation values (6733±862 [mIU/l]·180min) at 24 (8326±1145 [mIU/l]·180min; P=0.18), 48 (8963±1317 [mIU/l]·180min; P=0.14) and 72 hrs (88241±1104 [mIU/l]·180min; P=0.16) of forearm immobilisation.

3.4.6 Arterialised venous-venous glucose difference

Figure 3.7 presents the total area under the curve for arterialised venous-venous glucose difference over the 180 min oral glucose challenge, which was similar between limbs pre-immobilisation (97.39±17.59 and 90.59±19.68 [mmol/l]·180min in the NIM and IMM limb respectively; P=0.94). Following 72 hrs of immobilisation, the arterialised venous-venous glucose difference was greater in NIM limb when compared to baseline (157.2±30.48 [mmol/l]·180min; P<0.05). In IMM arm, the arterialised venous-venous glucose difference was reduced from baseline at 24 (45.84±7.76 [mmol/l]·180min; P<0.05), 48 (37.4±4.71 [mmol/l]·180min; P<0.05) and 72 hrs (51.91±8.09 [mmol/l]·180min; P<0.05). The arterialised venous-venous glucose difference was significantly different between NIM and IMM limbs following 72 hrs of immobilisation (P<0.001).

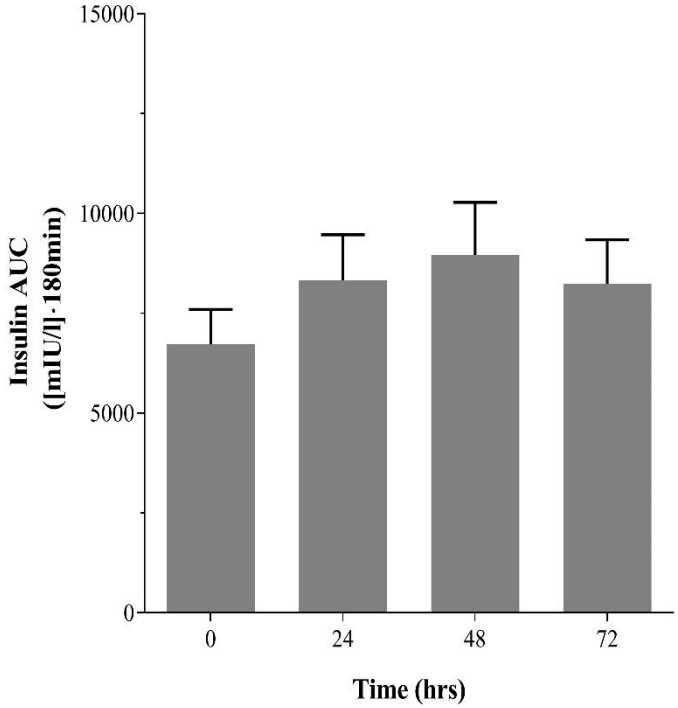


Figure 3.6 Area under the curve (AUC) of serum insulin concentration for each 180 min oral glucose challenge was determined from the arterialised-venous blood samples. Values are mean ±SEM (*n*=10).

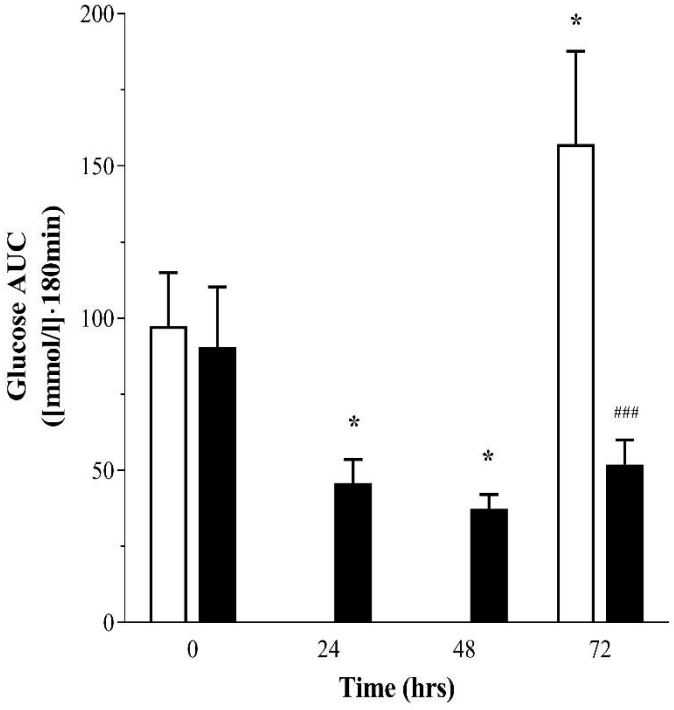


Figure 3.7 Area under the curve (AUC) of arterialised venous – venous glucose difference for the total 180 min oral glucose challenge was determined for a non-immobilised (white) and immobilised (black) forearm before (0), during (24, 48) and after 72 hrs of forearm immobilisation. Values are mean ±SEM (*n*=10). *P<0.05 significantly different from corresponding baseline value. ### P<0.001 significantly different between arms.

3.4.7 Forearm glucose uptake

Based on the total area under the curve for the 180 min oral glucose challenge (Figure 3.8), forearm glucose uptake was similar between arms prior to immobilisation (9.5±2.2 and 8.7±1.8 [mmol/min]·180min for the NIM and IMM limb respectively; P=0.58), and did not change following 72 hrs in the NIM limb (12.7±3.0 [mmol/min]·180min; P=0.16). However, forearm glucose uptake was less than at baseline in the IMM limb at 24 (38%; 5.4±1.1 [mmol/min]·180min; P<0.05), 48 (57%; 3.8±0.5 [mmol/min]·180min; P=0.01) and 72 hrs (46%; 4.7±0.7 [mmol/min]·180min; P=0.06) of immobilisation. Following 72hrs of immobilisation forearm glucose uptake was less in the IMM arm when compared to the NIM arm (P<0.01).

3.4.8 Hand grip strength

Handgrip strength, presented in Figure 3.9, did not change from pre-immobilisation values at the end of the intervention period in the NIM or the IMM arm (P=0.38 and P=0.16 for NIM and IMM arms respectively).

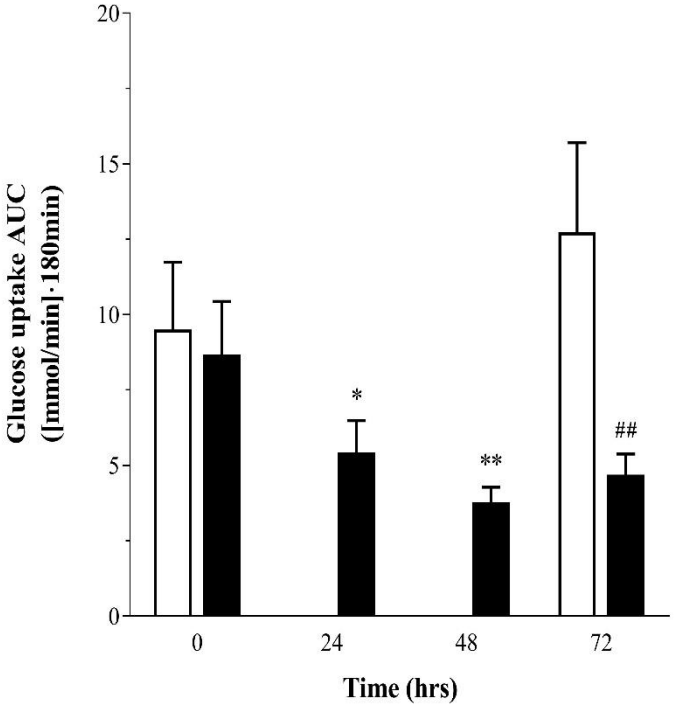


Figure 3.8 Area under the curve (AUC) of forearm glucose uptake for the total 180 min oral glucose challenge was determined for a non-immobilised (white) and immobilised (black) forearm before (0), during (24, 48) and after 72 hrs of forearm immobilisation. Values are mean ±SEM (*n*=10). *P<0.05 and **P<0.01 significantly different to corresponding baseline value. ## P<0.01 significantly different between arms.

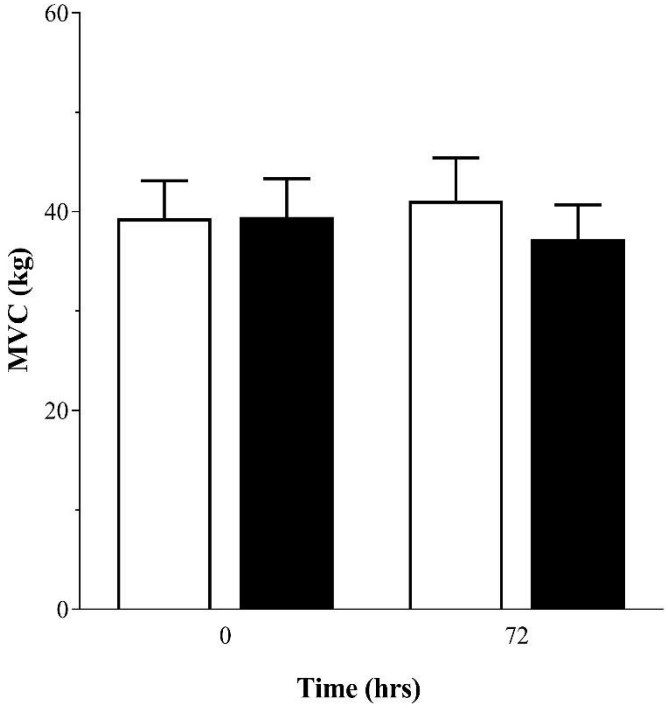


Figure 3.9 Handgrip strength in a non-immobilised (white) and immobilised (black) arm prior to immobilisation (0) and 72 hrs after immobilisation. Values are mean ±SEM (*n*=10).

3.5 Discussion

The study presented in the current Chapter examined the effect of 24, 48 and 72 hrs of forearm immobilisation on forearm glucose uptake and brachial artery blood flow following an oral glucose challenge, and to compare this to a contralateral, non-immobilised arm in healthy, young male volunteers. The present study aimed to provide novel insight regarding the time-course of development of immobilisation induced declines in forearm glucose uptake. The principal finding was that forearm glucose uptake following an oral glucose challenge was blunted within 24 hrs of immobilisation, which occurred without a concurrent decline in brachial artery blood flow or arterialised-venous serum insulin concentration. This study also aimed to investigate the impact of three days of forearm immobilisation on handgrip strength, for which there was no effect observed. These novel data demonstrate that the development of resistance to insulin action on glucose uptake during immobilisation of the human forearm occurs rapidly, is specific to the immobilised limb and appears to be muscle centric.

3.5.1 Effect of short-term immobilisation on forearm glucose uptake

The central finding of the current study shows that forearm glucose uptake substantially diminishes by 38% within 24 hrs of immobilisation, suggesting that the action of insulin on glucose uptake declines expeditiously when muscular contraction is reduced. Numerous studies utilising an extreme stimulus of muscle disuse, such as sustained bed rest, have repeatedly reported significant reductions in whole body glucose uptake, with the earliest detectable declines using this model reported to occur within 3-7 days (Stuart

et al., 1988, Mikines *et al.*, 1991, Yanagibori *et al.*, 1994, Smorawinski *et al.*, 2000, Hamburg *et al.*, 2007). Lipman and colleagues were among the first to suggest an impairment in forearm glucose uptake occurs within 14 days of bed-rest, before going on to report forearm glucose uptake, determined using a glucose infusion, to be almost 50% lower following 3 days of bed-rest compared to forearm glucose uptake measured prior to bed rest (Lipman *et al.*, 1970, Lipman *et al.*, 1972). However, immobilisation induced declines in glucose uptake have not previously been systematically investigated over an intervention period to the same degree as that of the current study. Hence, this study is the first to document markedly lower glucose uptake as a result of forearm immobilisation within just 24 hrs, which is much more rapid than previously recognised. Furthermore, the progression of immobilisation-induced decrements in glucose uptake has not been formerly explored and the present study demonstrated that forearm glucose uptake continued to fall from baseline by 57% at 48 hrs and this decrement remains evident at 72 hrs (46% lower compared to baseline values), despite no decrements in serum insulin concentration or brachial artery blood flow, which corroborates well with reports described above from Lipman *et al*. (Lipman *et al.*, 1970). As the majority of glucose uptake occurs in skeletal muscle (DeFronzo and Tripathy 2009), it may be assumed that this blunting effect reflects the muscle specific inhibition of glucose uptake. Furthermore, absence of blunted forearm glucose uptake in the NIM limb highlights the localised effects of immobilisation and support the proposition that decrements in muscle glucose uptake are a primary metabolic response to reduced muscular contractions.

3.5.1.2 Potential mechanisms linking immobilisation with decreased forearm glucose uptake

Overall, it remains improbable that an immobilisation induced change in forearm glucose uptake is due to insufficient delivery of glucose to the muscle. Instead any reductions in glucose uptake are likely to be as a result of defects occurring at other sites of muscle glucose uptake, such as the transport of glucose into the cell by GLUT-4 or phosphorylation of glucose within the myocyte (Wasserman *et al.,* 2011). Further evidence indicating a muscle specific inhibition of glucose uptake following bed rest indicate major defects in muscle glycogen storage rates, which may be a consequence of lower muscle GLUT-4 content and activity (Mikines *et al.,* 1991). Additional reports also align well with these findings of reduced GLUT-4 content, in addition to lower insulin-stimulated muscular glycogen synthase activity, Akt signalling and protein level of Hexokinase II following 7 days of bed rest (Biensø *et al.,* 2012). These findings do indeed point towards both decreased non-oxidative glucose metabolism and glucose transport/phosphorylation as potential factors driving the observed decrements in glucose uptake in skeletal muscle. Increased intramyocellular lipid content has also been implicated in the inhibition of skeletal muscle glucose uptake associated with immobilisation, with the accumulation of lipid intermediates suggested to interfere with insulin signalling (Cree *et al.,* 2010, Sonne *et al.,* 2010). However, the precise mechanisms which instigate these effects of immobilisation, and if such intracellular defects could be established within 24 hrs, have yet to be experimentally determined.

3.5.2 Effect of short-term immobilisation on brachial artery diameter

Baseline brachial artery diameter was shown to be 3.5 % narrower following 48 hrs of immobilisation when compared with pre-immobilisation values. While this finding fits in well with the current literature which indicates that muscle disuse is a potent stimulus for inward remodelling of conduit artery diameter, however, whether such inward remodelling could occur within 48hrs remains to be determined. Previous studies have reported a 13% and 24% decrease in femoral artery diameter following 25 and 60 days of bed rest respectively (Bleeker *et al.,* 2005, van Duijnhoven *et al.,* 2010). Furthermore, findings from studies using 7 days of lower limb casting or 28 days of leg suspension describe decrements of 6% and 12% in femoral artery diameter respectively (Bleeker *et al.,* 2005, van Duijnhoven *et al.,* 2010). Contrary to these findings, Birk *et al*. observed no change in baseline diameter of the brachial artery following 8 days of forearm immobilisation, measured using ultrasound (Birk *et al.,* 2013). However, as only a sling was used to immobilise the forearm in this particular study, it is possible that mobility of the forearm was not sufficiently restricted to induce measurable changes in brachial artery diameter. However, no study to date observed a measurable effect of immobilisation on artery diameter over the brief time frame as demonstrated in the present study.

It has been suggested that the narrowing of conduit arteries in response to reduced muscle use may be due to inward remodelling as a result of reduced periods of high peak shear stress or smooth muscle hypertrophy stimulated by increased sympathetic nerve activity, thereby causing in increased artery wall

thickness (Pauletto *et al.*, 1991, Chen *et al.*, 1995). Reports from the second Berlin bed rest study support this hypothesis by demonstrating an increase in femoral artery wall thickness following 60 days of bed rest (van Duijnhoven *et al.*, 2010). However, Palombo *et al.* did not observe any change artery wall thickness of the femoral artery despite the lumen diameter being reduced following 35 days of bed-rest (Palombo *et al.*, 2015). The discrepancy between these findings may be due to the shorter duration of bed rest utilised by Palombo and colleagues. Although the precise mechanisms driving changes in arterial diameter remain unknown, the current literature indicates a "dose-dependent" relationship between change in diameter and duration of inactivity, a hypothesis further supported by findings from the current study.

3.5.3 Effect of short-term immobilisation on brachial artery blood flow

Brachial artery blood flow was shown to be increased from pre-immobilisation values in response to glucose ingestion in the present study, which is contrast to previous studies reporting no change in blood flow following immobilisation (Bleeker *et al.*, 2005, Rakobowchuk *et al.*, 2011). There are a credible number of reports in the published literature which have demonstrated that an increase in insulin can in turn lead to an increase in vasodilation and blood flow (Pisters *et al.*, 1991, Baron 1994, Dela *et al.*, 1995, Serne *et al.*, 2002, Vincent *et al.*, 2004). However, in the majority of findings an insulin induced increase in vasodilation generally occurred in response to supraphysiological insulin levels or following intravenous insulin administration over 4-6 hrs, which is much longer than typical postprandial hyperinsulinemia (Yki-Jarvinen and Utriainen 1998). As serum insulin

concentration did not change significantly during the immobilisation period, it is unlikely that insulin augmented blood flow in the present study. Another possible explanation for the observed increase in brachial artery blood flow and velocity at 24 hrs of immobilisation may be related to the cast itself causing an increase in skin temperature, thereby resulting in vasodilation and an increased blood flow response (Charkoudian 2003). However, an increase in vasodilation as a result of increased skin temperature is not supported by a parallel increase in brachial artery diameter, which suggests other mechanisms are driving the increased blood flow and velocity. Brachial artery blood flow is determined by the resistance of the brachial artery to the flow of blood, and by the pressure pushing blood through the vessel. Previous studies have shown blood pressure to increase during bed rest (Hamburg *et al.,* 2007, Nosova *et al.,* 2014), and, although blood pressure was not measured during laboratory visits, this may be a factor for increased blood flow observed at 24 hrs of immobilisation in the present study. Furthermore, small arterioles represent the main site for vascular resistance, thus reduced vascular resistance in arterioles due to endothelium-dependent vasodilation could also have contributed to an initial increase in blood flow, which may have then become impaired as the period of immobilisation continued (Hamburg *et al.,* 2007). Although the increase in blood flow and blood velocity observed following 24 hrs of forearm immobilisation in the present study was unexpected, it is not surprising this occurrence did not supersede the reduction in forearm glucose uptake. However, it is difficult to fully account for the increased blood flow at 24 hrs without concurrent increases in brachial artery diameter based on the parameters measured in the current study. Future work should investigate

changes in response to forearm immobilisation at the arteriolar level using techniques such as plethysmography.

It is possible that bulk brachial artery blood flow does not accurately reflect microvascular blood flow and therefore, microvascular changes during immobilisation may have contributed to the observed decline in forearm glucose uptake. Although nutritive or microvascular blood flow were not assessed in the present study, our group has previously demonstrated that increasing blood flow, via pharmacologically induced vasodilation, did not prevent an immobilisation induced deficit in forearm glucose uptake (Skirrow 2014). Furthermore, although the study performed by Skirrow *et al*. did not directly measure microvascular blood flow or muscle perfusion (Skirrow 2014), blood flow was increased using glycerl trinitrate, a potent vasodilator previously shown to augment microvascular blood flow in patients undergoing gastric tube reconstruction, measured by laser Doppler flowmetry (Buise *et al.*, 2005). Capillary perfusion has also been increased using glycerl trinitrate in acute heart failure patients, assessed via Sidestream Dark Field imaging (den Uil *et al.*, 2009). These reports demonstrate the capacity of glycerl trinitrate to increase capillary perfusion and microvascular flow, suggesting the decline in forearm glucose uptake observed in the present chapter is driven by factors unrelated to delivery of substrates to the myocyte.

3.5.4 Effect of short-term immobilisation on muscle strength

Current literature suggest the greatest decrements in muscle strength of upper limbs occurs within 1-2 weeks of muscle disuse, with a 15-30% decrease

reported to occur within 7-9 days of upper limb immobilisation (Miles *et al.*, 1994, Lundbye-Jensen and Nielsen 2008, Johnston *et al.*, 2009). The present study is the first to investigate changes in muscle strength during a period of immobilisation of <7 days, and demonstrated that three days of muscle disuse did not significantly impact muscle strength. Therefore, it is apparent that measurable changes in forearm muscle strength in response to limb immobilisation are likely to occur between 3-7 days of disuse. Future investigations should focus on this time frame of muscle disuse to further elucidate the time-course of alterations to muscle strength, and to identify the mechanisms underlying these decrements, which will undoubtedly help in maintaining quality of life during periods of low physical activity.

3.5.5 Conclusions

The present study is one of few which have utilised upper limb immobilisation and included a within subject control, and is the only study to date investigating the localised effects forearm immobilisation on skeletal muscle glucose uptake over 72 hrs. Our findings indicate that immobilisation induces a rapid decline in insulin mediated glucose uptake by skeletal muscle within 24 hrs, and that this decline is sustained for 72 hrs. Additionally, this impairment in forearm glucose uptake occurs independently of a parallel decrease in bulk blood flow or insulin response. Such findings of dramatic reductions in glucose uptake, within just 1 day of reduced muscular contractile activity, strongly support the plausibility of a cause-and-effect relationship between muscle disuse and its immediate, negative implications on our metabolic health.

Chapter 4: Effects of immobilisation on forearm postprandial lipid clearance and glucose uptake during 2 days of forearm immobilisation.

4.1 Introduction

Several lines of evidence now promote the supposition linking immobilisation with impairments in lipid trafficking, accumulation of intramuscular lipid and diminished insulin-stimulated glucose uptake by skeletal muscle (Bergouignan *et al.,* 2006, Cree *et al.,* 2010, Bergouignan *et al.,* 2011). Although the deleterious effects of lipids on insulin action have been well documented over many years (Thiebaud *et al.,* 1982, Boden *et al.,* 1994, Roden *et al.,* 1996, Boden 1997), there remains an absence of data linking the time course of changes in insulin-stimulated glucose uptake during immobilisation with temporal changes (if any) in postprandial lipid clearance. Such information would be valuable in helping to elucidate potential functional relationships between alterations in glucose and lipid clearance in response to periods of muscle disuse during immobilisation. The study presented in Chapter 3 revealed an expeditious decline in feeding induced forearm glucose uptake in response to forearm immobilisation. The current study intended to build on this insight by providing a comprehensive assessment on the impact of limb immobilisation on forearm lipid clearance following an oral high fat mixed meal challenge, and to relate any observed changes in lipid clearance to concurrent decrements in forearm glucose uptake.

A steadily growing body of research has highlighted the positive effect of breaks in sedentary time on postprandial lipid clearance. A single bout of low to moderate intensity physical activity, such as standing or walking, prior to a high fat meal has been reported to lower postprandial blood lipid concentrations (Aldred *et al.,* 1994, Tsetsonis *et al.,* 1997, Zhang *et al.,* 1998,

Gill and Hardman 2003). Recently, following 2 days of prolonged sitting (14.3 hrs/day) Kim *et al*. reported the incremental area under the curve for triacylglycerol after a high fat tolerance test to be 26% higher compared to interrupted sitting or active walking in healthy, lean subjects in response to the same lipid challenge (Kim *et al.*, 2016). This delayed removal of postprandial chylomicrons and triacylglycerol-rich lipoproteins is known to impair endothelial function by elevating free fatty acid concentration in the bloodstream, creating an atherogenic environment within vasculature and promoting increased deposition of lipoprotein remnants on the arterial wall (Zilversmit 1995). Thus, impairments in the efficacy of the clearance of postprandial triacylglycerol-rich lipoproteins is a potential driver in the development of atherosclerosis (Simons *et al.,* 1987, Sharrett *et al.,* 1995, Cohn 1998, Lopez-Miranda *et al.,* 2006). Findings from Kim *et al*. indicate that short periods of reduced physical activity lead to deleterious modifications in postprandial lipid clearing activity and defining the precise timeframe of these pathological changes should be a research priority.

Over 20 years ago findings from Jeppesen and colleagues showed resistance to insulin-mediated glucose uptake, determined from the steady state plasma glucose concentration during an insulin suppression test, was an independent predictor of the magnitude of postprandial lipemia, characterised by increased triacylglycerol-rich lipoproteins following a meal (Jeppesen *et al.,* 1995). Since then several publications using healthy cohorts have utilised lipid infusions to raise plasma fatty acid concentrations leading to the development of insulin resistance within 3.5-5 hrs, assessed using glucose infusion or

hyperinsulinemic-euglycemic clamp (Boden *et al.,* 1991, Boden *et al.,* 1994, Dresner *et al.,* 1999). Noteworthy findings from Boden *et al*. addressed the initial effect of intravenously administered high fat loading on insulin sensitivity and demonstrated reduced insulin-mediated glucose disposal during euglycemic hyperinsulinemia, following 3.5 hrs of a lipid /heparin infusion in healthy, lean individuals (Boden *et al.,* 1991). These findings are in contrast to several studies which previously failed to detect any inhibition of glucose disposal during a lipid infusion lasting less than 3 hrs (Bevilacqua *et al.*, 1987, Wolfe *et al.,* 1988, Bonadonna *et al.,* 1989). Boden and colleagues conclude that a sufficient timeframe is necessary to measure lipid induced inhibition of insulin signalling, which may be due to the obstruction of glycogen synthesis by lipids, for which the underlying mechanism remains to be identified. To date, no study has explored the additive effect of increased dietary lipid availability during muscle disuse on skeletal muscle glucose uptake. Such an investigation would provide invaluable insight considering the high proportion of the global population consuming high fat diets during chronic physical inactivity.

Although many lines of evidence point to an association between increased blood lipid availability and decreased insulin stimulated whole body glucose uptake, there has been little progress in elucidating the mechanistic basis of this relationship. A number of possible candidates have been identified. For example, hydrolysis of circulating triacylglycerols by lipoprotein lipase located in the capillary endothelium will liberate glycerol and free fatty acids. Glycerol can either be taken up by skeletal muscle or transported to the liver,

where it can be used in gluconeogenesis or lipid synthesis (Coppack *et al.*, 1999), while the free fatty acids are transported into muscle via fatty acid transporters, such as plasma membrane associated fatty acid binding proteins. From here fatty acids can undergo mitochondrial β-oxidation forming acetyl-CoA, which is used to sustain mitochondrial ATP generation (Jeukendrup 2002). Accumulation of mitochondrial acetyl-CoA will inhibit pyruvate dehydrogenase complex activity (via activation of pyruvate dehydrogenase kinase which phosphorylates pyruvate dehydrogenase) and flux by end-product inhibition, thereby limiting the rate of mitochondrial pyruvate utilisation. Alternatively, fatty acids may be esterified and stored within muscle as intramyocellular lipids (IMCL) or lipid intermediates such as diacylglycerols (DAG) and ceramides (Jeukendrup 2002). Accumulation of these lipid intermediates has been postulated by many to blunt insulin signalling and thereby blunt muscle glucose uptake (Itani *et al.*, 2002, Summers 2006, Timmers *et al.*, 2008, Schmitz-Peiffer 2010, Ussher *et al.*, 2010). Firstly, DAG, a bioactive lipid species, is capable of activating novel protein kinase C (PKC) (Schmitz-Peiffer 2000) which can inhibit the insulin signalling cascade through the serine phosphorylation of insulin receptor substrate-1 (IRS-1) ultimately resulting in impaired translocation of glucose transporters to the cell membrane (Bosma *et al.*, 2012). Moreover, fatty acid induced activation of PKC has also been proposed to promote pro-inflammatory pathways, such as Nuclear-factor kappa-B, further negatively impacting insulin action (Itani *et al.*, 2002). Secondly, ceramides disrupt insulin signalling downstream of IRS-1, by stimulating the dephosphorylation of protein kinase B (Akt) via protein phosphatase 2A, and interrupting the

translocation of Akt to the plasma membrane, resulting in decreased translocation of GLUT-4 to the plasma membrane and thus, resulting in reduced glucose uptake (Reali *et al.,* 2017).

With sedentarism rapidly becoming more prevalent, investigating the impact of increasing dietary fat on impairments to feeding induced glucose uptake during reduced muscle use would provide novel insight applicable to a large proportion of the global population. Skeletal muscle accounts for approximately 70-80% of exogenous glucose uptake and approximately 90% of the energy requirements of this tissue at rest are catered for by fatty acid oxidation (DeFronzo and Tripathy 2009). Thus, forearm immobilisation is valuable model to examine the role of muscle disuse in the development of pathological changes of skeletal muscle lipid metabolism, which is likely to lead to disturbances in whole body homeostasis. As reported in the previous chapter, glucose uptake by skeletal muscle rapidly declines during immobilisation, but whether the time-course of this decline corresponds with changes in postprandial lipid metabolism remains unknown. Considering both decrements in myocellular glucose uptake and postprandial lipid clearance are associated with low levels of physical activity, unravelling the interplay between these conditions could greatly contribute to our understanding.

4.2 Aims and hypothesis

The novel research detailed in Chapter 3 revealed a marked decline (38 %) in feeding induced forearm glucose uptake after 24hrs of immobilisation, which was sustained for 72 hrs. The present study aimed to add to these findings by, firstly, quantifying the time-course of change (if any) in postprandial forearm lipid clearance in response to forearm immobilisation, over the same timeframe investigated in Chapter 3, in healthy, young volunteers. Secondly, to determine any impact of increasing dietary lipid availability on the pattern of change in immobilisation induced forearm glucose uptake over the course of 48 hrs of immobilisation compared to that seen in Chapter 3. It is hypothesised that forearm lipid clearance will decrease in response to forearm immobilisation and the high fat meal, in addition to immobilisation will further reduce forearm glucose uptake. This is the first study to evaluate if any relationship exists between muscle glucose uptake and lipid clearance during limb immobilisation in humans.

4.3 Research design and methods

4.3.1 Participants and sample size

Overall, 10 subjects participated in the present study. However, due to problems associated with repeated deep-venous cannulation, 3 participants were withdrawn from the study following the laboratory visit at 48 hrs and a fourth subject was withdrawn at the 24 hr time-point. As 3 consecutive days of cannulation of the antecubital deep vein in the IMM arm proved to be problematic, resulting in a 40% drop-out rate at the 48 hr time-point, analysis

was performed on all participants who completed the laboratory visits at baseline (0), and at 24 and 48 hrs following immobilisation. Findings presented in Chapter 3 demonstrate how the vast majority (57 %) of the decline in forearm glucose uptake occurs within 48 hrs of immobilisation, thus it was deemed acceptable to perform data analysis on participants completing the 48 hr laboratory visit in the current study. Furthermore, as also detailed in Chapter 3, no change in forearm glucose uptake in the NIM limb was previously observed in 10 participants following 72 hrs of immobilisation. Therefore, it was determined that measurements of glucose uptake in the control arm of 6 participants, at baseline and 72 hrs, would be sufficient to verify that any responses to the intervention were localised to the IMM arm. Thus, 9 healthy male volunteers (mean±SD; age 22.4±4.3 yrs, weight 71.4±9.4kg, BMI 22.6±2.3kg/m^2) were included in the analysis for the present study. A subset of 6 participants completed a laboratory visit 72 hrs post-immobilisation to detect any changes in glucose uptake in the NIM arm. All participants completed a medical screening and provided informed written consent, as described in the General Methods chapter (Section 2.1.2).

4.3.2 Mixed meal challenge

A high fat, mixed meal challenge involves the consumption of an oral test meal containing carbohydrate, fat and protein by fasting individuals, and was employed for the measurement of postprandial blood glucose, serum insulin, serum triacylglycerol, plasma free fatty acid and serum glycerol responses in the current study. A high fat mixed meal challenge was chosen as this method has been shown to correlate well with model-based estimates of insulin

sensitivity from oral glucose tolerance tests (Maki *et al.*, 2009, Brodovicz *et al.*, 2011), and would evoke a sufficient insulin response for postprandial triacylglycerol processing.

Participants were requested to complete a 3 day diet diary prior to commencing the study to determine total energy consumed per day, as well as proportions of fats, carbohydrates and proteins consumed, ensuring excessive dietary fat consumption was avoided (*Nutritics-Professional Nutritional Analysis Software*, Swords, Co. Dublin, Ireland, 2016). Participants were also asked to refrain from strenuous physical activity and alcohol for 48 hrs prior to laboratory visits, as these factors are known to effect the postprandial triacylglycerol response (Lairon *et al.*, 2007). A low-fat ready meal (65% carbohydrate, 20% fat, 15% protein) was provided to participants for consumption between 8pm-9pm the evening before each laboratory visit. Participants were then asked to fast overnight, consuming nothing except unflavoured water.

The mixed meal challenge commenced with the collection of baseline blood samples on the morning of the laboratory visit, participants then consumed a high fat mixed meal (16.7 kcal, 1.21g fat, 1.11g carbohydrate and 0.27g protein per kg body weight, respectively) within 15 minutes. Participants were also provided with water (2ml per kg body weight) to rinse their mouth following the test meal. The mixed meal challenge is described in detail in Section 4.3.3.

4.3.3 Experimental protocol

Figures 4.1 and 4.2 present schematic illustrations of the timeline and experimental protocol for study visits. Following a medical screening and providing informed consent (detailed in Section 2.1.2), participants who satisfied the inclusion criteria were invited back to the laboratory to complete a baseline mixed meal challenge (laboratory visit 1). Within 7 days of this baseline visit, participants had a fibreglass forearm cast fitted to their non-dominant arm (laboratory visit 2, Section 2.1.3) before completing subsequent mixed meal challenge at 24 (laboratory visit 3) and 48 (laboratory visit 4) hrs following immobilisation. As discussed above (Section 4.3.1) complications with repeated cannulation of the antecubital deep vein in the IMM arm on consecutive days resulted in a subset of 6 volunteers completing a fourth experimental visit, 72 hrs after cast fitting (laboratory visit 5).

On the morning of each laboratory visit participants arrived at the laboratory, having fasted for 12 hrs, and rested semi-supine on a hospital bed. Cannulation then took place to facilitate blood sampling throughout the mixed meal challenge, and is detailed in Section 2.1.4.1. In brief, following intradermal infiltration with local anaesthetic, a dorsal foot vein was cannulated in the retrograde direction before the foot was placed in a hot-air warming unit to achieve arterialisation of the venous drainage in the foot. An ultrasound scan was used to identify a blood vessel draining the forearm muscles in the IMM arm, which was then cannulated, under local anaesthetic, in the retrograde direction. This process was then repeated on the NIM arm for the baseline visit. Only the dorsal foot vein and antecubital deep vein in

the IMM arm were cannulated in the study visits performed at the 24 and 48 hr time-points. At the 72 hr visit the dorsal foot vein and antecubital deep veins in both the IMM and NIM arm were cannulated for the subset of volunteers completing this additional laboratory visit.

Following cannulation, participants rested for 30 minutes before basal measurements of brachial artery blood flow were made in duplicate, while blood samples were simultaneously drawn from each cannula. Participants then consumed a body weight adjusted high fat mixed meal within 15 min (outlined in Section 4.3.2), after which blood samples were collected from each cannula every 10 minutes for the first 2 hrs, every 15 minutes for the third hour and every 60 minutes for the final 4 hrs. Concurrent to blood sampling, brachial artery blood flow was also measured. Subsequently, the arterialised-venous to venous substrate difference was determined using the arterialised-venous sample from the foot and the deep venous sample from the antecubital deep vein in each arm. Lipid clearance and forearm glucose uptake during the mixed meal challenge were calculated by multiplying the arterialised- venous to venous difference of glucose, triacylglycerol, free fatty acids and glycerol by brachial artery blood flow (described in Section 2.4). At the end of the final laboratory visit, participants had their arm cast removed. Calculations and statistical analyses were performed as described in Sections 2.4 and 2.5 respectively.

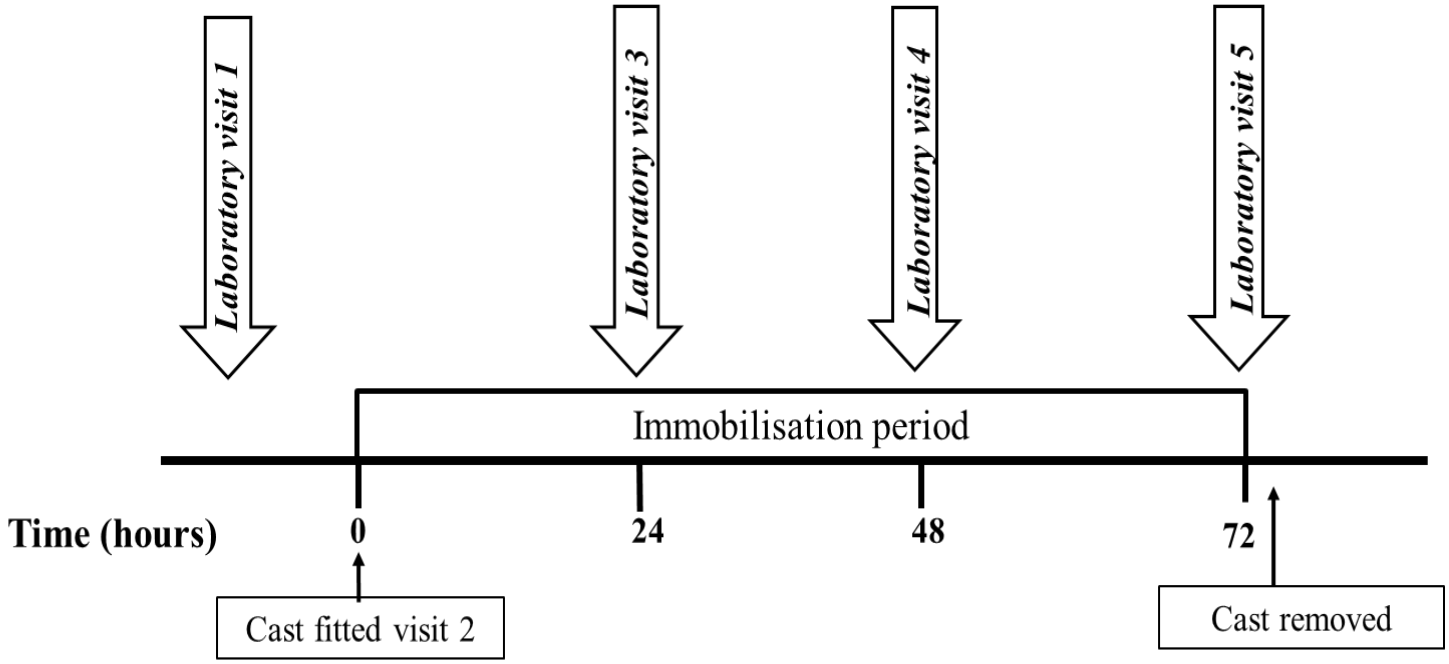


Figure 4.1 Timeline of study protocol.

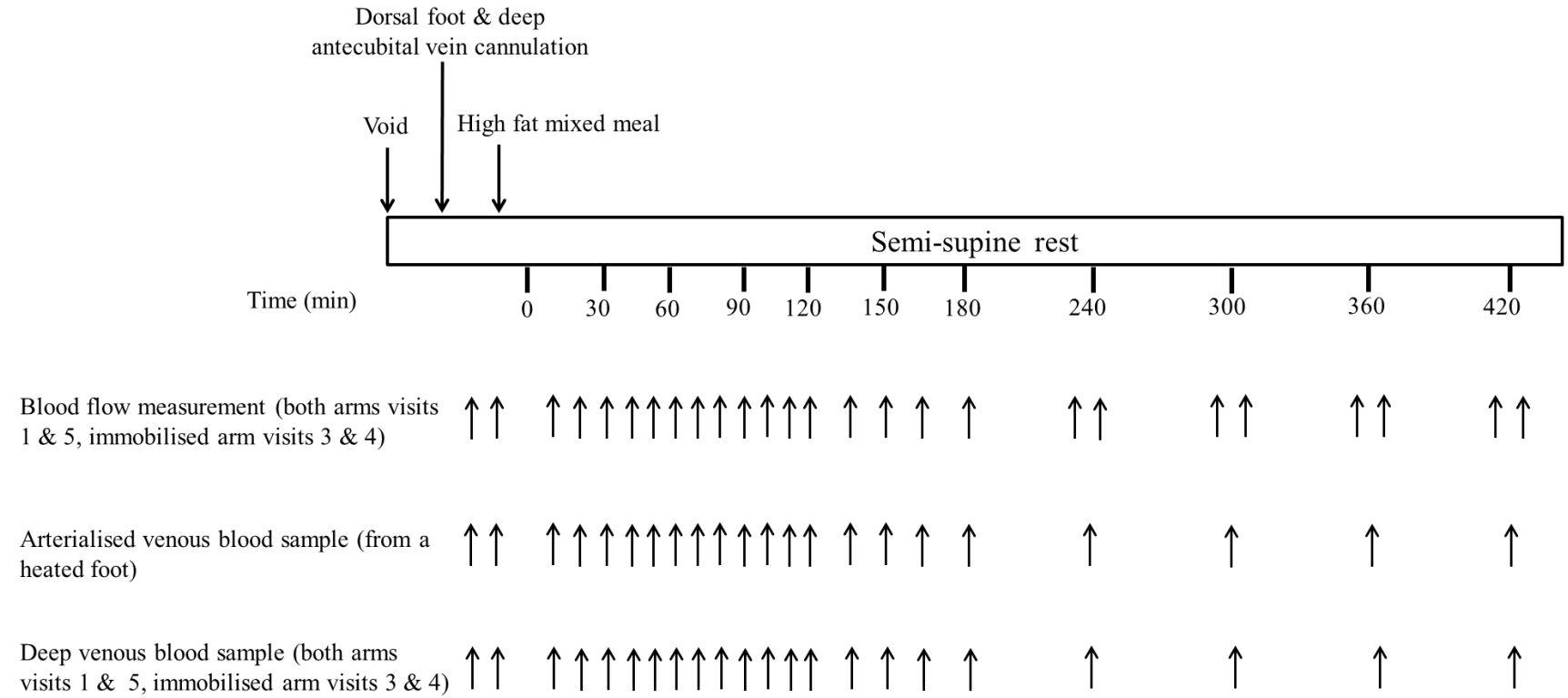


Figure 4.2 Schematic diagram of protocol for laboratory visits..

4.4 Results

The main purpose of this study was to determine the impact of immobilisation on postprandial forearm triacylglycerol, free fatty acid and free glycerol clearance, and to interrogate any impact of increased dietary lipid availability on the pattern of change in forearm glucose uptake over the course of immobilisation. These data are therefore presented in the proceeding section. However, supplementary data from which the following figures are derived are included in Appendix 3.

4.4.1 Characteristics of study participants

Nine participants were initially recruited, however**,** 3 participants withdrew after the 48 hr experimental visit due to issues with repeated cannulation of the antecubital deep vein in the immobilised arm on consecutive days (Section 4.3.1). All participants were within normal BMI range of 18.5-25 kg/m^2. Participant characteristics are shown in Table 4.1.

Table 4.1 Characteristics of study participants

Parameters	**Mean ± SD**
Age	22.4±4.3
Body mass (kg)	71.4±9.4
BMI (kg/m^2)	22.6±2.3
Fasting glucose (mmol/L)	4.24±0.36
Fasting insulin (mIU/L)	9.96±4.06
Fasting triacylglycerol	0.74±0.22
Fasting free fatty acid	0.46±0.14
Fasting glycerol	0.027±0.054

4.4.2 Plasma free fatty acid

4.4.2.1 Fasting plasma free fatty acid concentration

Figure 4.3 A and B presents the fasting concentrations of free fatty acid in arterialised venous blood sampled from a heated foot and deep-venous blood sampled from the IMM arm at baseline (0), 24 and 48 hrs of immobilisation, while Figure 4.3 C presents the free fatty acid concentration of deep venous blood sampled from the NIM arm at baseline and at 72 hrs. When compared to baseline (0.47±0.05 mmol/l), the fasting arterialised venous free fatty acid concentration was lower at 24 and 48 hrs of immobilisation (0.32±0.06 mmol/l, $P<0.05$ and 0.22±0.04 mmol/l, $P<0.01$ respectively). Likewise, fasting deep venous free fatty acid concentration in the IMM arm was less than baseline (0.4±0.06 mmol/l) at 24 and 48 hrs of immobilisation (0.25±0.04 mmol/l, $P<0.05$ and 0.17±0.03 mmol/l, $P<0.01$ respectively). Similarly fasting venous free fatty acid concentration sampled in the NIM arm was also lower than baseline (0.44±0.04 mmol/l) at 72 hrs of immobilisation (0.21±0.03 mmol/l, $P<0.01$).

4.4.2.2 Forearm free fatty acid uptake

Forearm free fatty acid uptake for each limb, represented as total area under the curve over the 420 min mixed meal challenge, is displayed in Figures 4.4 A and B, respectively. At baseline, free fatty acid uptake was similar between limbs (3.25±0.87 and 2.04±0.45 [mmol/min]·420min in the NIM and IMM arms respectively; $P=0.26$). In the NIM arm, free fatty acid uptake did not significantly change from baseline in the subgroup of 6 volunteers studied following 72 hrs of immobilisation (2.34±0.63 [mmol/min]·420min; $P=0.23$).

In the IMM arm, free fatty acid uptake following 24 (2.04±0.39 [mmol/min]·420min, P=0.99) and 48 (2.14±0.33 [mmol/min]·420min, P=0.94) hrs of immobilisation was not different from the baseline rate.

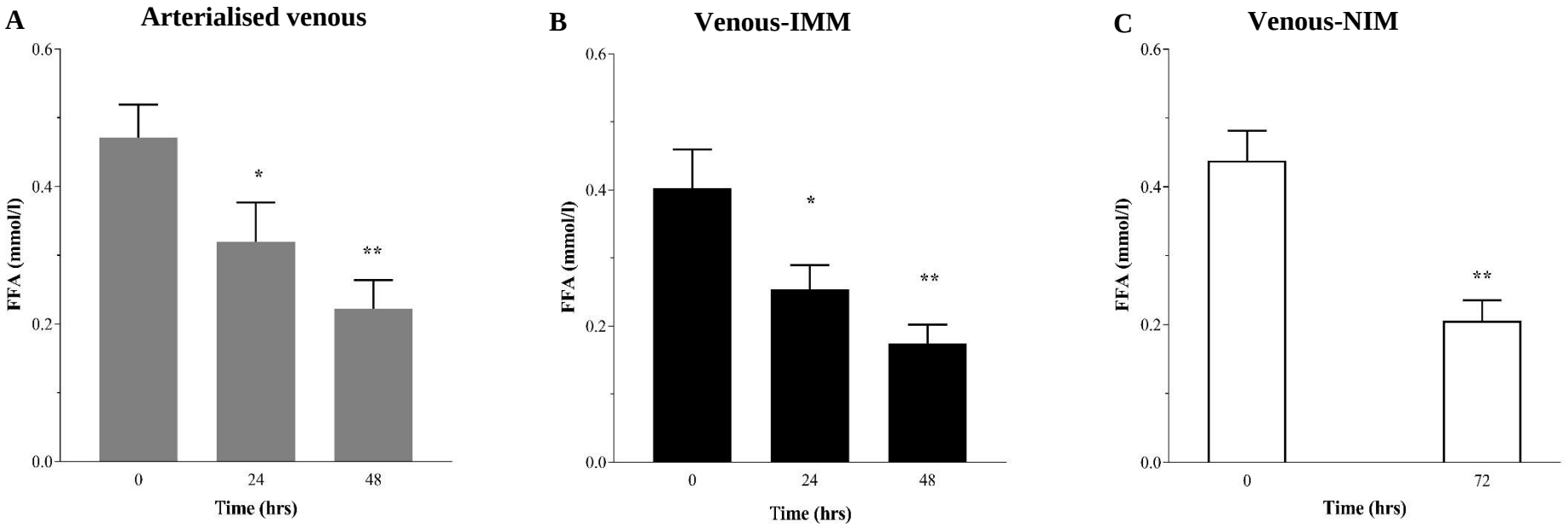


Figure 4.3 Free fatty acid (FFA) concentration in arterialised venous blood samples from a heated foot (Arterialised venous; **A**) and venous blood samples from an immobilised (Venous-IMM; **B**) and non-immobilised (Venous-NIM; **C**) arm following an overnight fast, before (0), and at 24, 48 and 72 hrs of forearm immobilisation. Values are mean ±SEM (*n*=9 in Arterialised venous and Venous-IMM respectively, and *n*=6 in Venous-NIM). *P<0.05 and ** P<0.01 significantly different from corresponding baseline value.

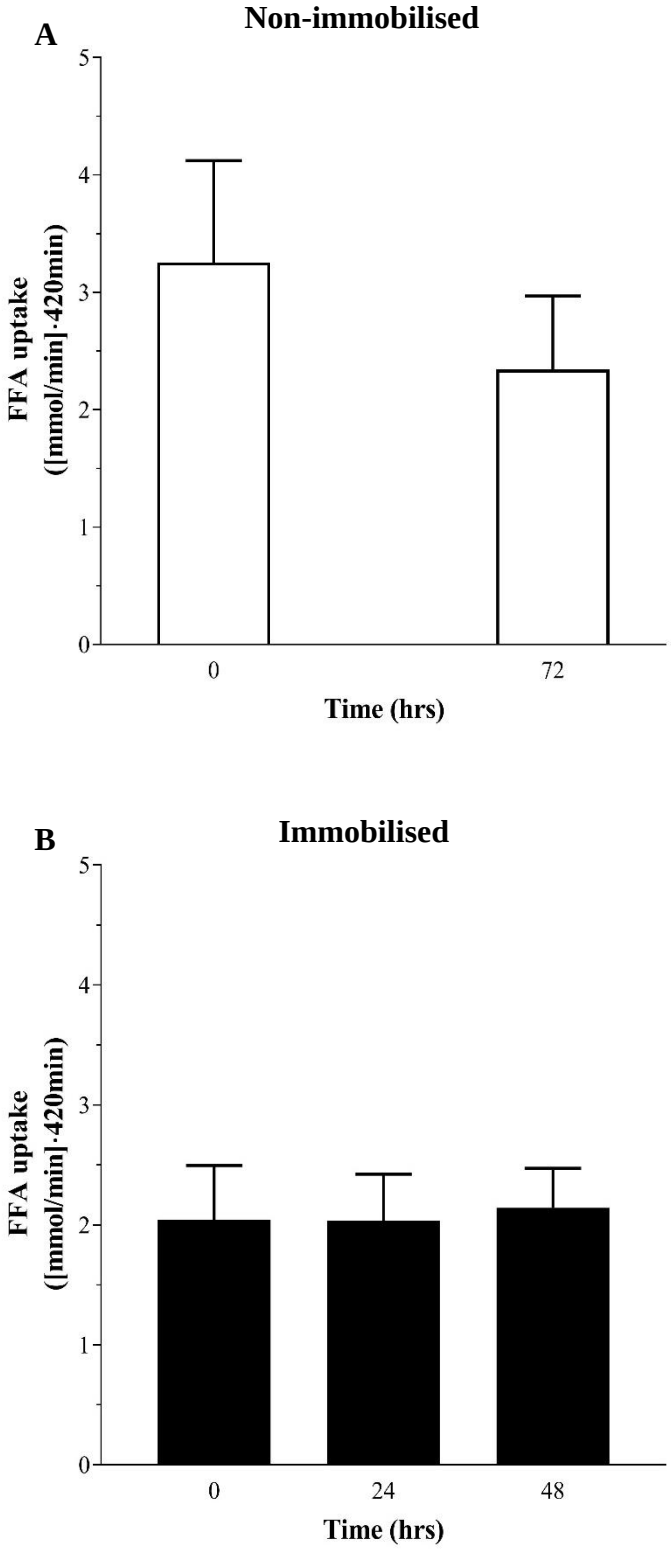


Figure 4.4 Area under the curve of free fatty acid (FFA) uptake for the total 420 min high fat mixed meal challenge was determined for a non-immobilised **(A)** and immobilised **(B)** forearm before (0), during (24, 48) and after 72 hrs of immobilisation. Values are mean ±SEM (*n*=6 and *n*=9 for the non-immobilised and immobilised arms, respectively).

4.4.3 Serum triacylglycerol

4.4.3.1 Fasting serum triacylglycerol concentration

Figure 4.5 A and B depicts the fasting concentrations of triacylglycerol in arterialised-venous blood sampled from a heated foot and deep-venous blood sampled from the IMM arm at baseline (0), 24 and 48 hrs, while Figure 4.5 C presents the fasting triacylglycerol concentration of deep venous blood sampled from the NIM arm at baseline and at 72 hrs of immobilisation. The fasting arterialised-venous triacylglycerol concentration was similar to baseline (0.72±0.07 mmol/l) at 24 hrs (0.7±0.06 mmol/l, P =0.97) of immobilisation, and was less than baseline following 48 hrs of immobilisation (0.58±0.04 mmol/l, P<0.05). Fasting venous triacylglycerol concentrations from the IMM arm did not change from baseline (0.68±0.07 mmol/l) at 24 and 48 hrs of immobilisation (0.67±0.06 mmol/l, P=0.93 and 0.58±0.04 mmol/l, P=0.14; respectively). In the NIM arm, fasting triacylglycerol concentration tended to be lower than baseline (0.68±0.07 mmol/l) at 72 hrs (0.59±0.05 mmol/l; P=0.07).

4.4.3.2 Forearm triacylglycerol clearance

Figure 4.6 A and B present the total area under the curve for forearm triacylglycerol clearance during each 420 min mixed meal challenge performed at baseline (0) and following 72 hrs in the NIM arm and at 0, 24 and 48 hrs of immobilisation in the IMM arm. At baseline, triacylglycerol clearance was similar between arms (3.9±0.7 and 2.8±0.5 [mmol/min]·420min for the NIM and IMM arm, respectively; P=0.33) and did not change from baseline in the NIM arm in the subset of 6 volunteers studied 72 hrs following

immobilisation (3.6±0.8 [mmol/min]·420min; P=0.75). In the IMM arm, forearm triacylglycerol clearance was no different from baseline following 24 (3.1±0.9 mmol/min/420min; P=0.85) and 48 hrs (4.7±1.3 [mmol/min]·420min; P=0.17) of immobilisation.

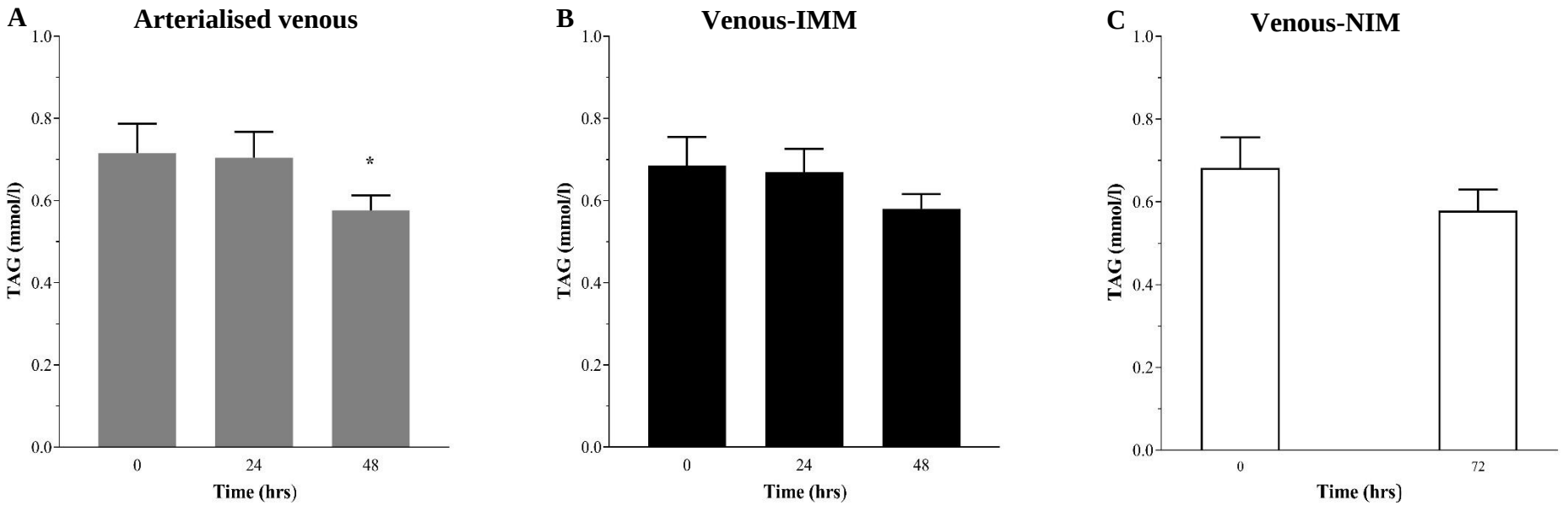


Figure 4.5 Triacylglycerol (TAG) concentration in **(A)** arterialised venous blood samples from a heated foot and venous blood samples from **(B)** an immobilised and **(C)** non-immobilised arm following an overnight fast, before (0), and at 24, 48 and 72 hrs of forearm immobilisation. Values are mean ±SEM (*n*=9 in Arterialised venous and Venous-IMM respectively, and *n*=6 in Venous-NIM). *P<0.05 and ** P<0.01 significantly different from corresponding baseline value.

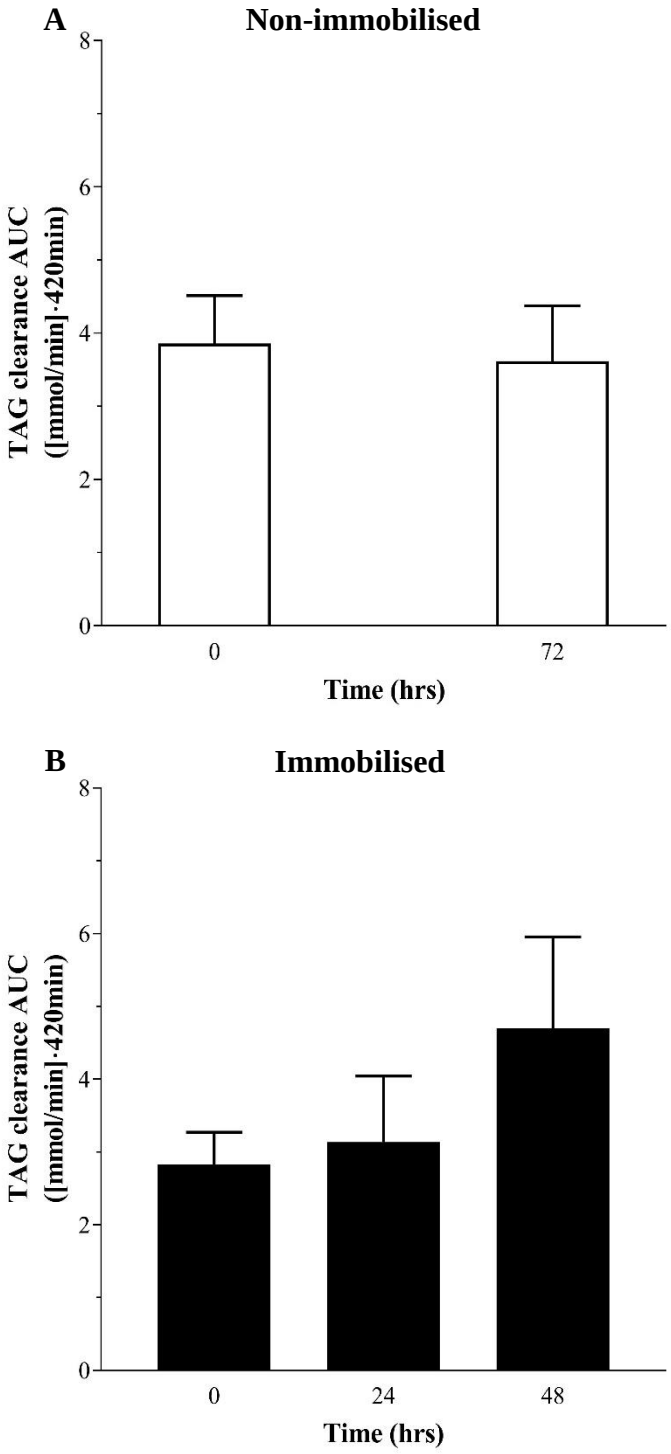


Figure 4.6 Area under the curve (AUC) of triacylglycerol (TAG) clearance for the total 420 min high fat mixed meal challenge was determined for a non-immobilised **(A)** and immobilised **(B)** forearm before (0), during (24, 48) and after 72 hrs of immobilisation. Values are mean ±SEM (*n*=6 and *n*=9 in NIM and IMM respectively).

4.4.4 Serum free glycerol

4.4.4.1 Fasting serum free glycerol concentration

Figure 4.7 A and B depicts the fasting concentrations of free glycerol in arterialised venous blood sampled from a heated foot and deep-venous blood sampled from the IMM arm at baseline (0), 24 and 48 hrs, while Figure 4.7 C presents the free glycerol concentration of deep venous blood sampled from the NIM arm at baseline and at 72 hrs of immobilisation. The fasting arterialised venous free glycerol concentration was similar to baseline (10.0±1.67μmol/l) after 24 (8.44±2.8 μmol/l, P =0.76), and 48 (7.0±1.77 μmol/l, P=0.24) hrs of immobilisation. Fasting venous serum free glycerol concentration in the IMM arm did not significantly change from baseline (8.0±2.66 μmol/l) after 24 (4.5±1.25 μmol/l, P=0.15) and 48 (4.83±1.29 μmol/l, P=0.48) hrs of immobilisation. In the NIM arm, fasting free glycerol concentration at baseline (7.58±2.25 μmol/l) did not significantly change than after 72 hrs of immobilisation (4.83±1.71 μmol/l, P=0.23).

4.4.4.2 Forearm free glycerol clearance

The total area under the curve for free glycerol clearance during each 420 min mixed meal challenge at 0 and 72 hrs of immobilisation in the NIM arm, and at 0, 24 and 48 hrs of immobilisation in the IMM arm is presented in Figures 4.8 A and B. Free glycerol clearance was similar between arms at baseline (104.9±34.27 and 119.5±23.27 [μmol/min]·420min in the NIM and IMM arms respectively, P=0.8), and did not change from baseline in the NIM arm at 72 hrs (271.9±132.4 [μmol/min]·420min; P=0.33). In the IMM arm, free glycerol clearance was similar to baseline values following 24 hrs of

immobilisation (111.3±27.22 [μmol/min]·420min, P=0.92), however free glycerol clearance increased from baseline at 48 hrs of immobilisation (215.1±45.5 [μmol/min]·420min, P<0.05).

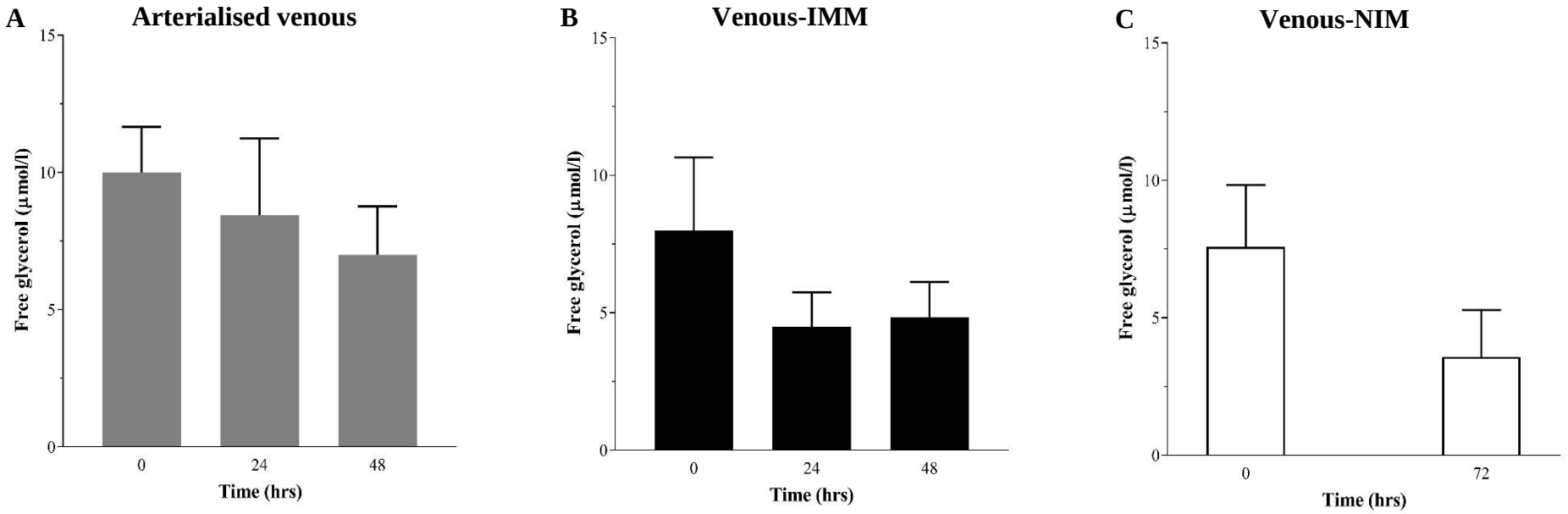


Figure 4.7 Free fatty acid glycerol concentration in **(A)** arterialised venous blood samples from a heated foot and venous blood samples from **(B)** an immobilised and **(C)** non-immobilised arm following an overnight fast before (0), and at 24, 48 and 72 hrs of immobilisation. Values are mean ±SEM (*n*=9 in Arterialised venous and Venous-IMM respectively, and *n*=6 in Venous-NIM).

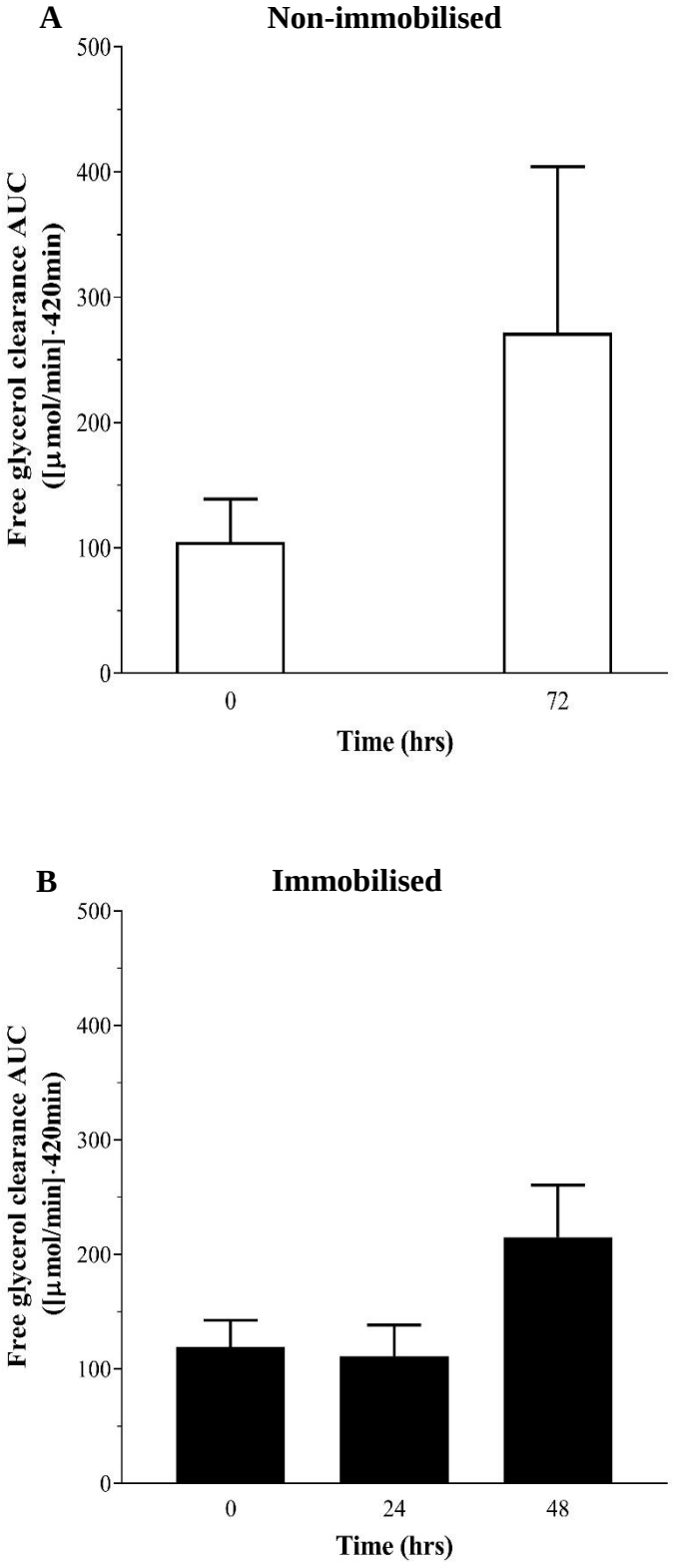


Figure 4.8 Area under the curve (AUC) of free glycerol clearance for the total 420 min high fat mixed meal challenge was determined for a non-immobilised **(A)** and immobilised **(B)** forearm before (0), during (24, 48) and after 72 hrs of immobilisation. Values are mean ±SEM (*n*=6 and *n*=9 in NIM and IMM respectively). *P<0.05 significantly different from corresponding baseline value

4.4.5 Forearm glucose uptake

The total area under the curve of forearm glucose uptake during the 420 min high fat mixed meal challenge in the NIM and IMM limbs is displayed in Figure 4.9 A and B, respectively. Prior to immobilisation, forearm glucose uptake was similar between limbs (21.6±4.9 and 14.9±3.3 [mmol/min]·420min in the NIM and IMM limbs, respectively; P=0.27) and forearm glucose uptake had not changed from baseline after 72 hrs in the NIM limb (24.3±5.2 [mmol/min]·420min; P=0.65) in the subset of participants studied at this time point. Conversely, forearm glucose uptake was less than baseline after 24 hrs (45.8%; 8.1±1.8 [mmol/min]·420min; P<0.01) and 48 hrs (51%; 7.3±1.5 [mmol/min]·420min; P<0.05) of immobilisation in the IMM limb.

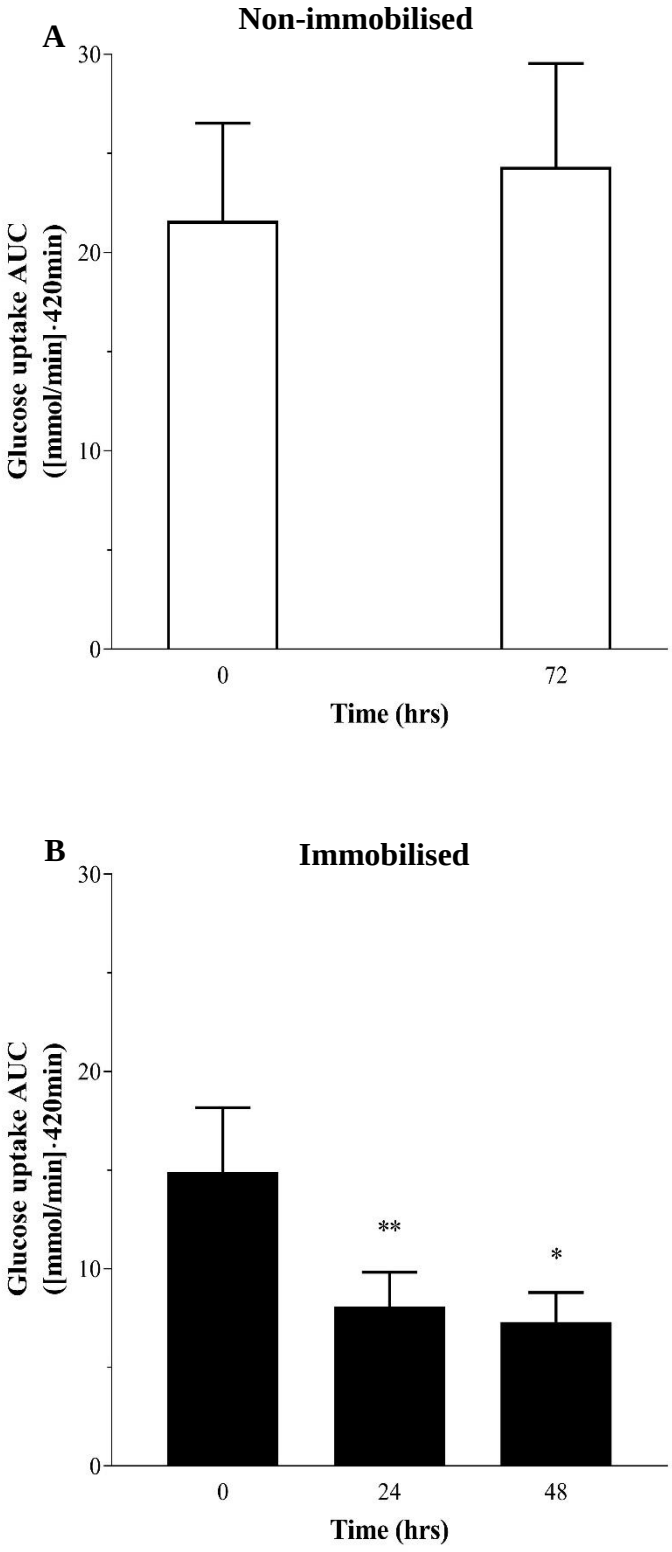


Figure 4.9 Area under the curve (AUC) of forearm glucose uptake for the total 420 min high fat mixed meal challenge was determined for a non-immobilised **(A)** and immobilised **(B)** forearm before (0), during (24, 48) and after 72 hrs of immobilisation. Values are mean ±SEM (*n*=6 and *n*=9 for the non-immobilised and immobilised limbs, respectively). *P<0.05 and **P<0.01 significantly different to corresponding baseline value.

4.4.6 Association analysis

No association was found between forearm glucose uptake and triacylglycerol clearance at baseline (R^2=0.00002; P=0.99), 24 (R^2=0.08; P=0.47) or 48 (R^2=0.02; P=0.75) hrs of forearm immobilisation. There was no association detected between forearm glucose uptake and free fatty acid clearance at baseline (R^2=0.03; P=0.68), 24 (R^2=0.2; P=0.23) or 48 (R^2=0.06; P=0.52) hrs of forearm immobilisation. Similarly, no association was found between forearm glucose uptake and glycerol clearance at baseline (R^2=0.07; P=0.5), 24 (R^2=0.03; P=0.66) or 48 (R^2=0.38; P=0.76) hrs of forearm immobilisation.

4.5 Discussion

The current study was conducted to build on the findings described in Chapter 3 by exploring the temporal changes (if any) in postprandial lipid clearance over 48 hrs of forearm immobilisation. This study also intended to investigate any additional impact of increasing dietary lipid availability on the magnitude of decline in forearm glucose uptake in response to 48 hrs of immobilisation in comparison to that observed in Chapter 3. Arterialised venous balance measurements and brachial artery blood flow measurements were used to determine glucose uptake and lipid clearance across the forearm. The current study confirms earlier findings which revealed that attenuated glucose uptake occurs expeditiously in response to forearm immobilisation. Furthermore, this swift immobilisation induced decrease in glucose uptake was not accompanied by concurrent changes in lipid clearance. The present findings therefore strongly suggest that the marked attenuation of glucose uptake during 48 hrs of forearm immobilisation is not additionally influenced by increased lipid availability, and support the supposition that this immediate decline in skeletal muscle glucose uptake is driven primarily by impairments of mechanisms related to contraction-induced glucose uptake.

4.5.1 Effect of short-term immobilisation on post-prandial lipid clearance

Delayed clearance of postprandial triacylglycerol leaves the arterial wall exposed to a greater magnitude of triacylglycerol-rich lipoproteins, particularly pro-artherogenic low-density lipoproteins, for a longer duration of time leading to endothelium dysfunction. The literature to date is lacking investigations into the time-course of changes in postprandial lipid clearance

during short-term muscle disuse, and such investigations would yield invaluable information regarding the susceptibility of postprandial lipid metabolism to pro-atherogenic changes due to immobilisation. Findings from the current study indicate that postprandial lipid metabolism does not significantly change in response to a high fat mixed meal within 48 hrs of limb immobilisation, and a longer time frame may be necessary to elicit measurable modifications to the postprandial lipid response as a result of the deleterious effects of muscle disuse. In contrast to the present findings, following 2 days of sitting (14.3 hrs/day) Kim and co-workers demonstrated that postprandial lipid response was 26% greater when compared with 2 days of active walking/standing (Kim *et al.*, 2016). These conflicting outcomes may be due to the different study designs employing upper limb immobilisation versus reduced ambulatory activity. It is possible that the upper limb immobilisation design employed in the current study was not sufficient to induce measurable changes in postprandial triacylglycerol response to a high fat meal in 9 volunteers within 48 hrs. The prolonged sitting model employed by Kim *et al*. reduced participants step count to <1700 steps per day, resulting in substantially lower activity levels compared to the walking group (approximately 17,000 steps per day). Moreover, for two days prior to starting the intervention of either prolonged sitting or walking, all participants were instructed to "maintain a low level of physical activity" (6,500-7,500 steps per day), which may have also contributed to the increased postprandial lipid response observed in the sitting group compared with the active walking group. Overall, these findings indicate that 48 hrs of decreased muscular

contraction, using forearm immobilisation, is not a sufficient timeframe to induce significant deleterious changes in postprandial lipid metabolism.

4.5.2 Effect of increased lipid supply on forearm glucose uptake during immobilisation

Several lines of evidence demonstrate substantial declines in skeletal muscle glucose uptake associated with decreased physical activity or immobilisation, further underlining a major consequence of the current trend for snowballing levels of sedentarism. In addition to increasing global levels of physical inactivity, the proportion of fat in modern diets is also increasing. Numerous publications have shown a link between decreased whole body and muscle insulin sensitivity with increased lipid availability (Schrezenmeir *et al.,* 1993, Dresner *et al.,* 1999, Griffin *et al.,* 1999), few however, have studied the impact of increased lipid supply on insulin-stimulated glucose uptake during immobilisation, which the present study aimed to address. Boden *et al*. previously reported a 55% decrease in rates of glucose disappearance 2-4 hrs following the intravenous infusion of lipid and heparin during euglycemic hyperinsulinemia in healthy men (Boden *et al.,* 1991), indicating that increased intravascular lipid availability hinders glucose uptake. The current findings indicate the diminution in skeletal muscle glucose uptake as a result of 48 hrs of immobilisation, was of a similar magnitude to that previously reported in Chapter 3 ($P>0.05$ at all time-points) and that increasing dietary lipid availability had no additional impact on the observed decrement in forearm glucose uptake. This may be due to the impact of forearm

immobilisation on muscle glucose uptake being so profound no additional effect of increasing dietary lipid could be measured, or indeed the metabolic demands of the immobilised muscle may have decreased so that substrate uptake was negligible. Previous reports suggesting a causative link between increased lipid supply and compromised insulin action highlight impaired insulin transduction in skeletal muscle, primarily through inhibition of IRS-1 tyrosine phosphorylation, IRS-1 associated PI3-Kinase activity and Akt phosphorylation and activity, as the most significant mechanisms which underlie this relationship (Itani *et al.*, 2002, Reali *et al.*, 2017). However, much of this research has been conducted in obese, Type 2 diabetic or lipid-infused participants making it difficult to determine if dietary lipid induced impairments in insulin signalling could occur in healthy individuals within 48 hrs of muscle disuse.

The lack of association between decrements in muscle glucose uptake and lipid availability during forearm immobilisation observed in the current study is in agreement with some (Weintraub *et al.*, 1987, Byrne *et al.*, 1997) but not all previous reports (Jeppesen *et al.*, 1995). As triacylglycerol clearance increased in 6 out of the 9 participants included in the current study, it may be the case that the relatively small sample size accounts for failure to establish an association between decreased glucose uptake and increased lipid availability during 48 hrs of forearm immobilisation. However, Byrne *et al.* failed to detect any association between impaired insulin sensitivity, evaluated using the Homeostasis model assessment of insulin resistance, and changes in postprandial triacylglycerol response in a cohort of 57 subjects (Byrne *et al.*,

1997), while Weintraub and colleagues reported no link between postprandial triacylglycerol concentration and insulin response in 15 individuals (Weintraub *et al.*, 1987). Furthermore, associations between triacylglycerol clearance and glucose uptake, as well as FFA uptake and forearm glucose uptake, as reported in the present study were very weak at each time point. Overall, the lack of association between decreased skeletal muscle glucose uptake and increased dietary lipid supply observed in the present study indicates that using dietary sources of lipid to increase postprandial lipid concentration within physiological levels, is insufficient to induce deleterious changes in myocellular glucose uptake over the course of 48 hrs of forearm immobilisation. Early speculation by Randle and colleagues suggested that increased lipid availability leading to substrate competition, resulted in inhibition of glucose metabolism (Randle *et al.*, 1963). However, findings from the current chapter complement more recent studies indicating that increased lipid supply is not directly responsible for the almost immediate impairments of insulin-mediated glucose uptake. Boden *et al*. using proton nuclear magnetic resonance spectroscopy in conjunction with a euglycemic-hyperinsulinemic clamp and lipid plus heparin infusion, demonstrated that elevated plasma free fatty acids must be first reincorporated into lipid stores within the myocyte before resistance to insulin action develops within skeletal muscle (Boden *et al.*, 2001). The authors also propose that a minimal threshold of intramuscular lipid must be reached before decrements to insulin action transpire, which may occur over a prolonged period if circulating lipids remain within the physiological range achieved by dietary ingestion as in the current study, instead of the elevation of lipids achieved via the combination

of lipid and heparin infusions. Thus, further investigation is necessary to determine the precise time frame over which dietary lipid over supply inhibits muscle glucose uptake during immobilisation.

4.5.3 Effect of short-term immobilisation on circulating lipid concentration

The decrease in fasting free fatty acid concentrations in the present study is similar to observations from previous investigations. Blanc *et al.* reported reduced fasting plasma fatty acid concentrations in young adults following 7 days of bed rest and concluded this was due to increased hepatic lipogenesis, evaluated using measurements of lipid oxidation (Blanc *et al.*, 2000). Based on measurements carried out in the current study it is not possible to definitively conclude whether the decline in fasting free fatty acid concentrations are indeed due to increased hepatic lipogenesis. However, due to the study design, participants rested in bed for approximately 9 hrs during each consecutive laboratory visit, therefore it may be possible that hepatic lipogenesis was increased as a result.

Immobilisation is typically associated with increased circulating triacylglycerol levels, as demonstrated by Hamburg *et al*., reporting an increase of approximately 35% in fasting triacylglycerol concentrations following 5 days bed-rest (Hamburg *et al.*, 2007). Such reports are contradictory to findings in the present study which show reduced fasting arterialised venous triacylglycerol concentrations following 48 hrs of immobilisation, which may be due to increased muscle lipoprotein lipase activity. Although high fats diets for 4-6 weeks have been reported to increase

hepatic lipase activity by 8% (Campos *et al.*, 1995) and muscle lipoprotein lipase activity by 20-80% after lipid ingestion (Kiens *et al.,* 1987, Campos *et al.,* 1995), it is unlikely that such diet induced increases in lipase activities are possible within 48 hrs. Kiens *et al*. reported a close correlation between insulin mediated changes in glucose uptake by thigh muscle with reduced muscle lipoprotein lipase activity (Kiens *et al.,* 1987). The mechanism linking plasma insulin with lower muscle lipoprotein lipase activity may be indirect, occurring as a result of increased glucose metabolism within the myocyte. Thus, decreased glucose metabolism as a result of diminished muscle glucose uptake during muscle disuse may stimulate lipoprotein lipase activity which increases the hydrolysis of triacylglycerol, releasing glycerol and free fatty acids. The liberated glycerol and free fatty acids may then be taken up by skeletal muscle, before being reincorporated into lipid droplets within the myocyte, or transported to the liver to be used in gluconeogenesis or lipid synthesis (Coppack *et al.,* 1999). However, as the decrease in skeletal muscle glucose uptake was localised to the immobilised limb, augmented muscle lipoprotein lipase activity due to decreased glucose metabolism is unlikely to account for the reduced fasting triacylglycerol and free fatty acid concentrations measured in the current study. Utilising direct methods to measure lipids, such as isotopic labelling and muscle biopsies for determination of lipoprotein lipase activity would provide more insight in future studies.

4.5.4 Conclusions

From the results presented in this chapter it is improbable that the diminished glucose uptake, observed within 24 hrs of immobilisation, occurs as a result of lipid-mediated impairments in insulin signal transduction. However, the possibility that forearm glucose uptake was inhibited so profoundly over 48 hrs of immobilisation that increasing dietary lipid supply could have no additional effect remains. The intervention period in the present study was relatively short, thus, over a longer time-frame these mechanisms may have a more significant role to play in impairments of skeletal muscle glucose uptake during periods of muscle disuse. However, consideration should be given to the practicalities of such an intervention utilising repeated high fat mixed meal challenges, such as feasibility of repeated cannulations of the same blood vessel, participant compliance and recruitment. Nonetheless, a primary objective of this study was to determine if increasing dietary lipid supply amplified the magnitude of decrements in muscle glucose uptake during 48 hrs of forearm immobilisation, for which no additive effect was observed. Future research questions arising from this study include identification of the mechanisms leading to reduced fasting triacylglycerol and free fatty acid concentrations within 48 hrs of immobilisation. Additionally, investigation into the time frame of limb immobilisation necessary to induce lipid accumulation within skeletal muscle, and its impact on muscle glucose uptake, would provide new evidence for the discussion and continued exploration of associations between lipid supply, accumulation and insulin action.

Chapter 5: Effects of limb immobilisation on muscle glucose uptake and lipid content in healthy volunteers.

5.1 Introduction

Links between muscle disuse and reduced metabolic health are now well established, and the findings presented in Chapter 3 clearly demonstrate the substantial detrimental effects of immobilisation on decreased insulin stimulated muscle glucose uptake, reflecting reduced insulin sensitivity and impaired metabolic health. Several mechanisms may be responsible for this decrement in muscle glucose uptake in response to forearm immobilisation, including muscle atrophy and IMCL accumulation. Better understanding of the aetiology of these undesirable metabolic perturbations caused by immobilisation is important, and may also help understanding of chronic disease where decreased sensitivity of skeletal muscle to insulin action is prevalent, such as type 2 diabetes.

As discussed in Section 1.3.4.2 the accrual of lipid within myocytes may occur either as spherical droplets within the cytoplasm, referred to as IMCL, or as EMCL, characterised by tube like strands between muscle fibres (Khuu *et al.*, 2009). This accumulation of lipid within or between muscle is often a consequence of sedentary lifestyles and numerous publications link periods of muscle disuse with reduced lipid oxidation, resulting in increased lipid accumulation (Ritz *et al.*, 1998, Bergouignan *et al.*, 2006). After 4 weeks of unilateral lower limb suspension Mannini and colleagues used MRI to demonstrate an 8% reduction in leg muscle volume in concurrence with a 20% and 15% increase in EMCL within the regions of the thigh and calf muscles respectively (Manini *et al.*, 2007). Moreover, Brooks *et al*. used computed tomography to measure a decrease of 11% in mid-thigh muscle cross sectional

area, while mid-thigh IMCL increased by 14% following 4 weeks of bed rest (Brooks *et al.,* 2008). These studies indicate an accumulation of lipid occurred both within and surrounding atrophied muscles as a result of muscle disuse. There are an increasing number of studies highlighting the negative metabolic health implications of IMCL accumulation. In particular, a strong correlation between increased IMCL content and both reduced whole body and skeletal muscle insulin sensitivity has been repeatedly demonstrated (Pan *et al.,* 1997, Jacob *et al.,* 1999, Krssak *et al.,* 1999, Virkamäki *et al.,* 2001), whereas the relationship between EMCL and insulin sensitivity remains unclear and comparatively under investigated (Brechtel *et al.,* 1999, Sinha *et al.,* 2002).

It was not until the early 1990's that IMCL was recognised as having a role to play in the reduced insulin sensitivity of skeletal muscle (Falholt *et al.,* 1988, Pan *et al.,* 1995, Phillips *et al.,* 1996). Traditionally, biochemical extraction methods were used to measure IMCL content in muscle biopsy samples in the investigation of links between IMCL concentration and reduced skeletal muscle insulin sensitivity (Pan *et al.,* 1997). However, potential contamination by EMCL reduces the sensitivity of this method for identifying increased IMCL as the instigator of decrements in insulin sensitivity of skeletal muscle. Considerable advancements in proton Magnetic Resonance Spectroscopy (^{1}H MRS) have helped to further define a relationship between increasing IMCL accumulation and decreased insulin sensitivity by eliminating the confounding influence of EMCL, thereby identifying IMCL as the likely culprit of decreased insulin sensitivity. The majority of IMCL is

stored as inert triacylglycerols, however, the proposed association between IMCL and decreased insulin sensitivity relates to the lipid intermediates of triacylglycerol metabolism. These lipid intermediates, such as ceramides and diacylglycerol, are thought to interfere with insulin signalling, thereby reducing insulin stimulated glucose uptake by the myocyte. Specifically, diacylglycerol activates novel protein kinase C isoforms, leading to the serine phosphorylation of insulin receptor substrate-1 (Samuel and Shulman 2012). Insulin action may also be disrupted by ceramides, through inhibition of protein kinase B (PKB/Akt) activity, a signal transduction intermediate which is crucial for glucose transport into the myocyte. The actions of ceramides on Akt are two-fold: *firstly*, ceramides stimulate dephosphorylation of Akt via protein phosphatase 2A. *Secondly*, ceramides block the translocation of Akt to the plasma membrane, resulting in reduced translocation of GLUT 4 to the plasma membrane, thereby impairing glucose uptake by the cell (Stratford *et al.*, 2004, Reali *et al.*, 2017). Furthermore, increased lipid availability leads to increased acetyl CoenzymeA (CoA): CoA and citrate concentration. Increased acetyl CoA activates pyruvate dehydrogenase kinase, which then inhibits pyruvate dehydrogenase via its phosphorylation, thereby limiting the entry of pyruvate into the oxidative metabolism pathway. Increased citrate levels inhibits phosphofructokinase and lowers rates of glycolysis, this in turn results in an increased concentration of glucose-6 phosphate, which could inhibit hexokinase activity and thereby decrease glucose uptake (Randle *et al.*, 1963, Jeukendrup 2002). Numerous studies have also utilised insulin and lipid infusions to demonstrate the effect of increased circulating and intramuscular lipid availability on the activation of the pyruvate dehydrogenase complex, a

mitochondrial enzyme with an important role in insulin-stimulated glucose oxidation, which subsequently impairs both carbohydrate oxidation and insulin stimulated glucose uptake (Boden *et al.,* 1991, Kelley *et al.,* 1993, Griffin *et al.*, 1999, Boden *et al.*, 2001, Itani *et al.,* 2002, Belfort *et al.*, 2005). Additionally, reduced glycolytic flux associated with lipid induced impairments in insulin stimulated glucose uptake may also suppress pyruvate dehydrogenase complex activity (Holness and Sugden 2003).

Jacob and colleagues were among the first to use ^{1}H MRS to identify an association between decreased skeletal muscle glucose uptake with increased IMCL content in the soleus and tibialis anterior muscles of first degree relatives of type 2 diabetic patients (Jacob *et al.,* 1999). Following 28 days of bed rest, a negative association between glucose uptake, during a hyperinsulinemic-euglycemic clamp, and IMCL content, assessed using MRS, was observed by Cree *et al.* (Cree *et al.*, 2010). In contrast, Stettler *et al*. reported no association between insulin-mediated glucose uptake and IMCL content, also measured by hyperinsulinemic-euglycemic clamp and MRS, however these divergent findings may be due to the relatively brief bed rest period of 2 days employed by Stettler and colleagues (Stettler *et al.*, 2005). Considering Bergounian *et al*. measured a significant increase of 2.7% in IMCL within the gastrocnemius muscle following a much more extended period of 2 months of bed rest, determined using 1.5T MRI (Bergouignan *et al.*, 2009), it is possible that skeletal muscle insulin sensitivity, and thereby muscle glucose uptake, may indeed be inhibited by IMCL accumulation. However, the exact timeframe over which such measurable alterations occur

remains unknown. Moreover, not all reports are in agreement with the hypothesis that IMCL accumulation has a role to play in attenuated insulin sensitivity of skeletal muscle. Following one week of bed rest Dirks *et al*. employed a hyperinsulinemic-euglycemic clamp to measure a decline of almost 30% in whole body insulin sensitivity without any concurrent changes in IMCL content of biopsy samples taken from the vastus lateralis muscle, determined using Oil Red O staining (Dirks *et al.*, 2016). Although the authors did report a change in some diacylglycerol species following bed rest, these were not the species which have been specifically linked to insulin resistance. Likewise, Wall *et al*. did not observe any change in IMCL content of the vastus lateralis muscle, measured using biochemical analysis., after 5 days of leg immobilisation (Wall *et al.*, 2015). This absence of quantifiable changes in IMCL content within 5-7 days of muscle disuse indicates the decreased whole body insulin sensitivity observed over this period of bed rest is unrelated to lipid accumulation within skeletal muscle. Thus, the question still remains regarding the period of muscle disuse necessary to induce measurable changes in IMCL in conjunction with decrements in whole body and skeletal muscle insulin sensitivity.

Consideration must be given to the most appropriate method used to quantify IMCL. The use of muscle biopsy samples for histochemical quantification of IMCL, as used by Dirks *et al*. is susceptible to contamination by intermuscular adipose (Dirks *et al.*, 2016). Furthermore, air drying of muscle cryosections is not recommended, although still commonly performed prior to histochemical analysis, due to reports of reductions in both lipid droplet size and density by

37% and 66% respectively, further reducing the accuracy of this method for determination of IMCL content (Prats *et al.,* 2013). Additionally, the Oil Red O stain used by these authors has been shown to bind not only to the neutral lipids within lipid droplets, but also to other intracellular organelle producing variable results (Prats *et al.,* 2013). Moreover, the use of repeated muscle biopsies to quantify the time course of changes in IMCL is not feasible. Instead, the use of ^{1}H MRS represents an ideal non-invasive method to investigate alterations in IMCL using a longitudinal study design. Recent studies using MRS to quantify IMCL have focused on lower limb muscles due to their large volume (Li *et al.,* 2008, Cree *et al.,* 2010) meaning considerable development work is needed to establish a viable protocol to achieve IMCL quantification of forearm muscles. Although time-consuming and labour intensive, such developments would provide novel insight into the relationship between temporal changes in insulin sensitivity of skeletal muscle and both IMCL and EMCL accumulation during immobilisation, thereby expanding on existing knowledge regarding the aetiology of immobilisation induced decrements in skeletal muscle insulin sensitivity.

It is well established that prolonged periods of muscle disuse over weeks or months, primarily induced through extended bed rest interventions, lead to considerable alterations in body composition through reduced bone and muscle mass, and increased fat mass (LeBlanc *et al.,* 1992, Alkner and Tesch 2004, Miokovic *et al.,* 2012). The impact of shorter periods of immobilisation, typically ranging from 7-28 days, on body composition has also received attention with the recognition that short periods of muscle disuse

may diminish the capacity of skeletal muscle to lower circulating glucose levels (Cree *et al.,* 2010, Dirks *et al.,* 2016), and importantly, may have a cumulative harmful effect, thereby impacting negatively on long term metabolic health. Various models of muscle disuse have been employed to study changes in body composition, using changes in muscle volume or cross sectional area as indices of alterations to muscle mass. Seven days of bed rest resulted in a 3% decrease in quadriceps cross sectional area assessed using MRI (Ferrando *et al.,* 1996), whilst, Manini *et al*. reported a decrease in volume of 7.4% and 7.9% in mid-thigh and mid-calf muscles, respectively, following 4 weeks of unilateral lower limb suspension using 1.5T MRI (Manini *et al.,* 2007). Typically, 10-14 days lower limb immobilisation results in a decline of quadriceps cross sectional area ranging from 5-12% (Hespel *et al.,* 2001, Thom *et al.,* 2001, Yasuda *et al.,* 2005, de Boer *et al.,* 2007, Glover *et al.,* 2010), however, a limited number of studies have investigated changes in muscle mass in response to shorter periods of muscle disuse. Three days of lower limb immobilisation decreased cross sectional area of type 1 muscle fibres by 14% (Lindboe and Platou 1984) and dry water immersion over the same time frame reduced cross sectional area of type 1 fibres by 11% (Pagano *et al.,* 2017). Similar reports from Demangel *et al*. support these findings, by demonstrating a 10% reduction in type 1 muscle fibres as a result of 3 days of dry water immersion (Demangel *et al.,* 2017).

Reductions in muscle size following prolonged disuse (>10 days) have been attributed in the main to decreased muscle protein synthesis rather than increased muscle protein breakdown (Ferrando *et al.,* 1996, Kortebein *et al.,*

2007, Symons *et al.*, 2009). However, increased rates of muscle protein breakdown during short-term muscle disuse (<10 days) have been suggested to combine with a simultaneous decline in muscle protein synthesis rates, thereby also contributing to the rapid muscle atrophy observed early in muscle disuse (Abadi *et al.,* 2009, Gustafsson *et al.,* 2010, Suetta *et al.,* 2012). Studies utilising short-term upper limb immobilisation to investigate alterations to muscle size are less abundant and the results are also more variable. Using both MRI and peripheral quantitative computed tomography, Skirrow *et al*. showed no change in muscle cross sectional area or volume following 21 days of forearm immobilisation (Skirrow 2014), whereas, over the same period of upper limb immobilisation, Miles *et al*. reported a 7% decrease in flexor muscle volume, also assessed via MRI (Miles *et al.,* 2005). The same authors also reported a 4% decrease in forearm cross sectional area after 9 days immobilisation of the wrist (Miles *et al.,* 1994). These inconsistent findings relating to changes in muscle size derived from studies utilising upper limb immobilisation are likely to stem from differences in immobilisation methods (e.g. cast versus sling only) and the degree of immobilisation achieved. Furthermore, variations in methods of assessment of muscle size are also likely to influence these findings. Although MRI is considered the gold standard for determination of muscle size, it is possible that minor yet significant alterations in upper limb muscle volume are not detectable by some MRI scanners. Smeulders *et al*. investigated the feasibility of 3T MRI for quantification of changes in forearm muscle volume and concluded this was a reliable method for detecting changes exceeding 7% (Smeulders *et al.,* 2010). Investigations using ultra-high strength 7.0T MRI to

investigate changes in muscle size in response to upper limb immobilisation are absent in the current literature. Such investigations unravelling the time course of decrements in muscle size with immobilisation would provide novel insight, allowing comparison between disuse-induced alterations in the size of upper versus lower limb muscles.

5.2 Aims and hypothesis

As described in Chapter 3 forearm glucose uptake rapidly declines within 24 hrs of immobilisation, however, the time course of any further progression beyond 72 hrs has yet to be characterised. Therefore, the primary objective of this study was to expand on these findings by establishing the time-course of changes in forearm glucose uptake, in response to feeding over 16 days of arm immobilisation in healthy, young volunteers. This study also aimed to investigate the mechanisms driving this reduction in muscle glucose uptake by using ^{1}H-MRS to identify any relationship existing between alterations in muscle glucose uptake with concurrent changes IMCL/EMCL content over the immobilisation period. The primary hypothesis was that forearm glucose uptake would continue to decrease over the extended immobilisation period and this period of muscle disuse would be associated with intramuscular lipid accumulation. In addition, this study intended to explore any association between decrements in forearm muscle cross sectional area with reduced forearm strength as a result of immobilisation, determined via MRI.

5.3 Research design and methods

5.3.1 Participants and sample size

Five healthy male volunteers (mean±SD; age 20.7±1.7 years, weight 69.2±5.9kg, body mass index 21.5±1.8kg/m^2) completed the protocol for the current study. A routine medical screening was completed and informed written consent was obtained (as detailed in General Methods, Section 2.1.2) from each participant before commencing the study. In total 9 participants

were initially enrolled into the present study. However, two participants decided to discontinue the study due to nausea and vomiting in response to entering the MRI scanner, a third subject decided to discontinue due to discomfort around a permanent dental retainer following MRI scans and a fourth subject decided to withdraw due to the inconvenience of wearing the forearm cast. Data was assessed for normality and treated appropriately using either parametric or non-parametric tests. Forearm glucose uptake data was analysed using either Wilcoxon test or Friedman test with Dunn's multiple comparisons, while forearm muscle cross sectional area and handgrip strength data were analysed using either a 1-way ANOVA or paired t test, with Dunnetts or Holm Sidak multiple comparison tests, and 2-way ANOVA with Sidak multiple comparison test.

5.4.2 Experimental protocol

5.4.2.1 Forearm glucose uptake measurements

Participants fulfilling inclusion criteria for the study and having completed a medical screening (Section 2.2) were invited back to the David Greenfield Human Physiology Unit on 5 occasions to determine forearm glucose uptake. These measurements of forearm glucose uptake followed the protocol described in Section 2.1.4.3 and were conducted at baseline (Day 0) and following 1, 3, 8 and 15 days of forearm immobilisation (Figure 5.1). An additional arm of this study, coordinated by staff at the MRI Centre, aimed to quantify changes in brain sensorimotor cortex architecture and activation during immobilisation. Thus, the timeframe for the current study was based

on an earlier publication from Langer *et al.* reporting a decrease in cortical thickness after 16 days upper limb immobilisation (Langer *et al.,* 2012).

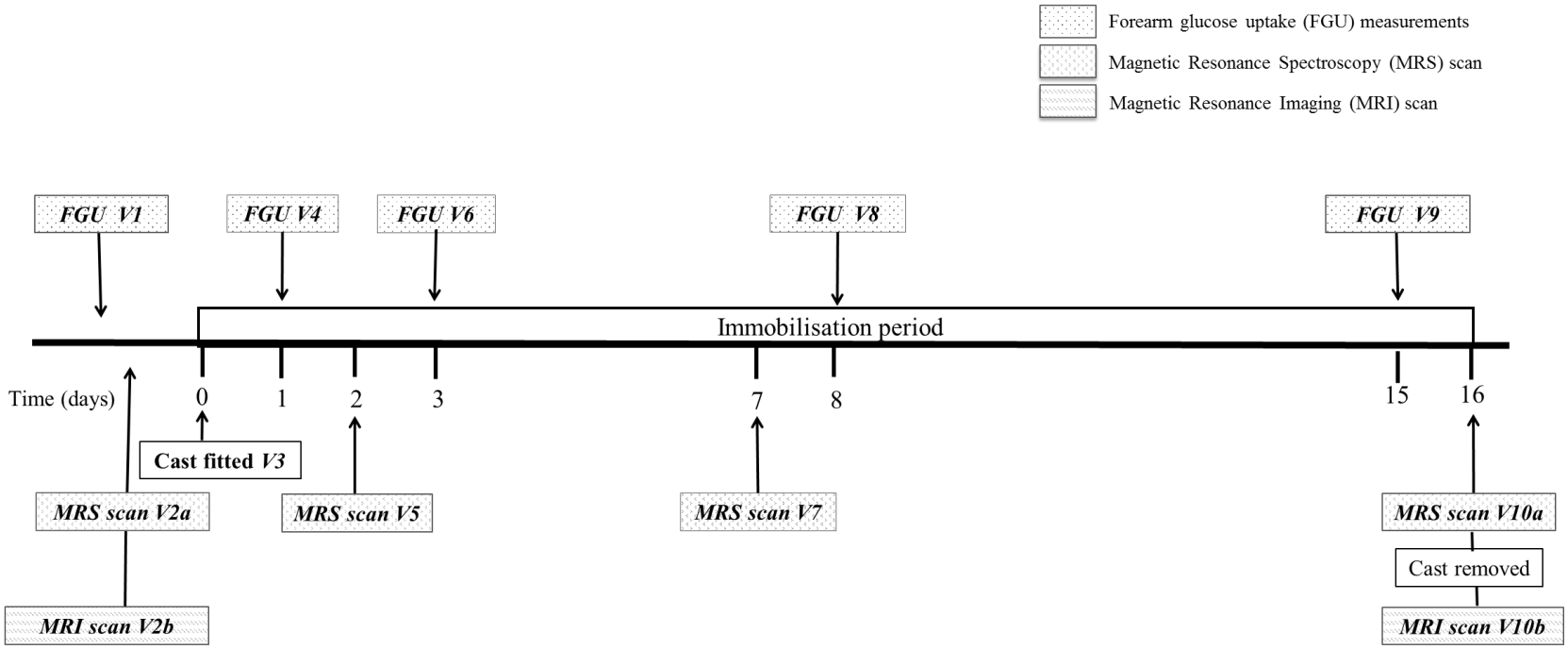


Figure 5.1 Summary of study timeline.

5.4.2.2 Scanning protocols

Within three days of the first laboratory visit measuring forearm glucose uptake, participants attended the Sir Peter Mansfield Magnetic Resonance Imaging Centre, having fasted overnight, for ^{1}H MRS and MRI scans on a 7.0 Tesla Achieva Magnetic Resonance Imaging whole body scanner (Philips Medical Systems, Cleveland, Ohio) for the determination of forearm lipid content and forearm cross sectional area. Participants completed a standard MRI safety questionnaire (Appendix 7) and were provided with ear plugs and defenders prior to entering the scanner. At the beginning of the baseline visit, each participants forearm was measured from the styloid process of the ulna to the crease of the antecubital fossa for positioning of an anatomical marker to be used for consistent voxel positioning during each scan. The anatomical marker was placed approximately 30% of forearm length away from the antecubital crease (Figure 5.2). This anatomical marker was then positioned in the same location for subsequent scans. Participants were also asked to wear a dummy cast during baseline scans, and on the control arm during scans throughout the immobilisation period, to ensure consistent conditions for all scans.

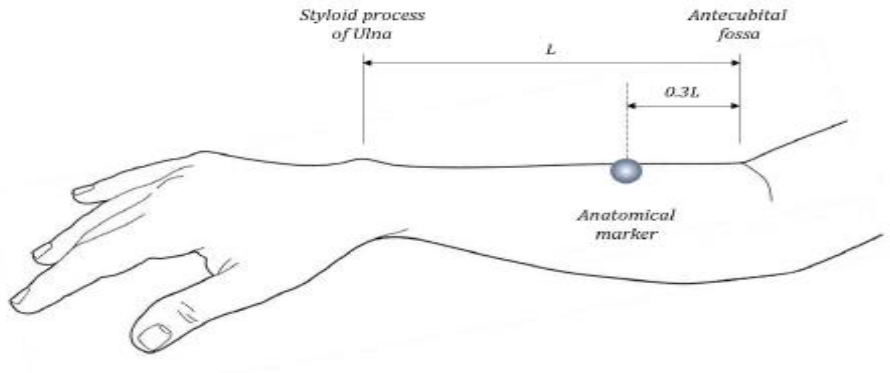


Figure 5.2 Illustration demonstrating the location of the anatomical marker for consistent voxel positioning during each scan throughout the immobilisation period.

Participants entered the scanner head-first, in the prone position with the arm to be scanned outstretched and secured using Velcro straps within a head volume transmit coil containing a pair of 16-channel surface receive coils (MR Coils, Netherlands), which were positioned around the anatomical marker. Sagittal images were initially acquired for positioning of the volume of interest.

Within 7 days of the baseline laboratory visits measuring forearm glucose uptake and MRS/MRI scans, participants attended the David Greenfield Human Physiology Unit to have a forearm cast fitted by trained technicians (see Section 2.1.3). Participants then returned to the Sir Peter Mansfield Magnetic Resonance Imagine Centre 2, 7 and 16 days after the forearm cast was fitted to repeat the MRS scans for determination of forearm lipid content and MRI scans to measure forearm cross sectional area.

5.4.2.2.1 Forearm lipid determination

For the ^{1}H MRS scans, a 10 x 10 x 50mm^{3} voxel was manually aligned within the flexor digitorum profundus (FDP) muscle, avoiding contamination by intermuscular adipose tissue. After shimming (calibration of the magnet) a head volume transmit coil (Nova Medical Onc., Wilmington, Massachusetts) and two 16 channel surface receive coils (MR Coils, Netherlands) were used to collect spectra. The area of resonances collected from protons of water (4.8 ppm) and methylene groups of fatty acid chains of the IMCL (1.4ppm) and EMCL (1.6ppm) were processed using fibre orientation modelling (FOM), described in Section 5.6.5.2.

The outcome of method development steps (outlined in Section 5.6.5) was an optimised protocol for the acquisition of spectra using ^{1}H MRS in the quantification of forearm lipid. Scan parameters were as follows: varying TE= 20 (x2), 50, 80, 150ms; TR 2000ms; water suppression by WET; 148 averages; spectral bandwidth = 4kHz; 1024 samples; total scan time= 12 minutes. Spectra were collected in duplicate at 20ms to increase signal to noise ratio (SNR).

5.4.2.2.2 Forearm muscle cross sectional area

In addition to MRS scans, a structural forearm MRI scan at a field strength of 7.0T, using an in-phase and out-of-phase gradient echo sequence was also performed on each forearm to determine muscle cross sectional area, with the imaging volume centred on the anatomical marker. Scan parameters were as follows: field of view= 122 x 130mm^2; in-plane spatial resolution 0.9 x 0.9 mm^2; slice thickness 1mm; 80 continuous slices; TE = 5ms; TR = 20ms; NSA = 1; acquisition time = 2.8 minutes.

After all cross sectional images were acquired, regions of interest were then identified using the free online package "NeuRoi", available from www.nottingham.ac.uk/research/groups/clinicalneurology/neuroi.aspx . The anatomical landmark was used as a reference point to ensure repeatability of voxel placement and, thus, consistent slice analysis in each visit, with the areas of the radius and ulna bones excluded from analysis. Figure 5.3 provides an example of the ROI analysis from one subject.

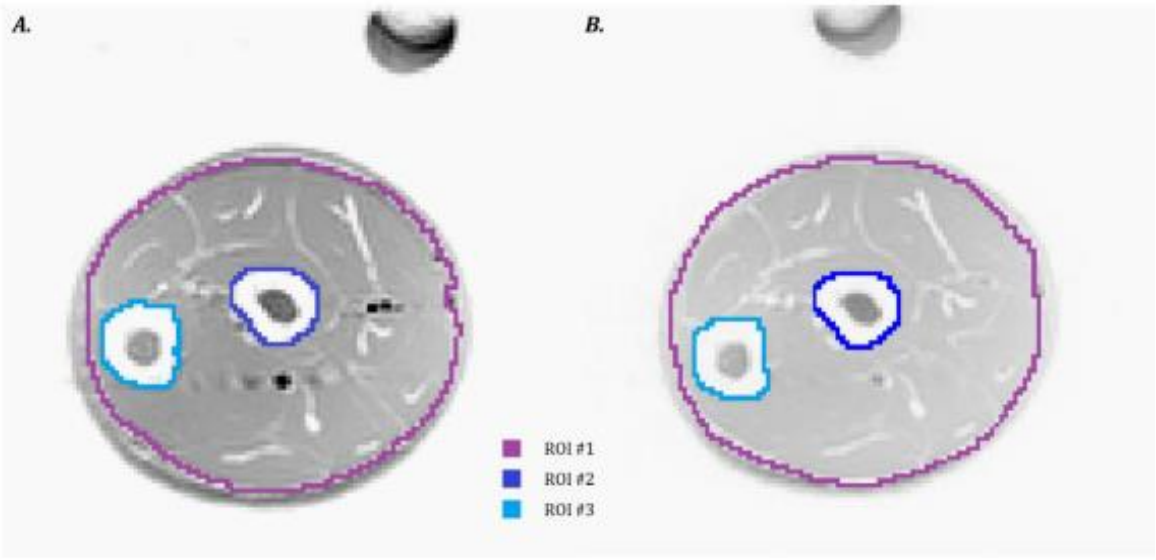


Figure 5.3 Example of region of interest (ROI) determination using NeuRoi software in Visit 1 **(A)** compared with Visit 3 **(B)**. Cross sectional area was determined by subtracting ROI #2 and ROI #3 from ROI #1.

5.4.2.2.3 Hand-grip strength

Hand-grip strength was assessed prior to (Day 0) and post-immobilisation (Day 16). A 500 Newton MR compatible handgrip dynamometer (Current Designs, USA; Figure 5.4) was used, with each participant being asked to perform three bouts of maximum voluntary contraction using each hand, with a 2 minute rest period between each bout.



Figure 5.4 Image of handgrip dynamometer used for determination of muscle strength (images sourced from http://www.curdes.com/mainforp/responsedevices).

5.5 Results

The primary objective of this study was determination of the effects of immobilisation on forearm glucose uptake, forearm muscle lipid content, muscle cross sectional area and handgrip strength, and these data are detailed below. As no impact of forearm immobilisation on brachial artery blood flow or serum insulin concentration was observed, these data are included in Appendix 4.

5.5.1 Characteristics of study participants

Nine participants were initially recruited, however**,** 4 participants withdrew for numerous reasons (Section 5.3.1). All participants were within normal BMI range of 18.5-25 kg/m^2. Participant characteristics are shown in Table 5.1.

Table 5.1 Characteristics of study participants

Parameters	**Mean ± SD**
Age	20.7±1.7
Body mass (kg)	69.2±5.9
BMI (kg/m^2)	21.5±1.8
Fasting glucose (mmol/L)	4.38±0.34
Fasting insulin (mIU/L)	6.44±2.39

5.5.2 Brachial artery diameter

Basal brachial artery diameter measurements were made prior to commencing the oral glucose challenge at days 0, 1, 3, 8 and 15 of forearm immobilisation in the IMM limb and at days 0 and 15 in the NIM limb (Figure 5.5). There was no difference in basal brachial artery diameter between arms at pre-

immobilisation (Day 0; 4.1±0.1 and 4.1±0.1 mm for NIM and IMM arms respectively; P=0.95) and was similar to baseline in the NIM limb at the end of the intervention period (4.2±0.2 mm; P=0.62). Although basal brachial artery diameter did not change in the IMM limb following 1 (4.1±0.1 mm; P=0.46), 3 (4.0±0.05 mm; P=0.29) or 15 (4.0±0.1 mm; P=0.4) days of immobilisation, at day 8 of forearm immobilisation basal brachial artery diameter was significantly less compared to baseline (3.9±0.1 mm; P<0.05). At the end of the immobilisation period basal brachial artery diameter was not different between arms (P=0.25).

5.5.3 Forearm glucose uptake

The area under the curve for forearm glucose uptake over the course of each 180 min oral glucose challenge is shown in Figure 5.6 and, prior to immobilisation, was comparable between arms (16.2±2.6 and 16.7±3.5 [mmol/min]·180min in the NIM and IMM arms respectively; P>0.99). There was no significant difference from baseline in the NIM arm at the end of the immobilisation period (21.5±2.6 [mmol/min]·180min; P=0.14). In the IMM arm, forearm glucose uptake was not significantly different from pre-immobilisation values at day 1 (9.8±2.9 [mmol/min]·180min; P>0.99) or 15 (7.7±1.4 [mmol/min]·180min; P>0.99) following immobilisation. However, forearm glucose uptake was significantly reduced from baseline in the IMM limb at day 3 (5.6±1.7 [mmol/min]·180min; P<0.05) and day 8 (5.2±1.7 [mmol/min]·180min; P<0.05) of forearm immobilisation. This represents a 41.3%, 66.9%, 69.2% and 54% decrease in forearm glucose uptake within the IMM limb from baseline (Day 0) at 1, 3, 8 and 15 days of immobilisation

respectively. After 15 days of immobilisation forearm glucose uptake tended to be lower in the IMM arm compared to the NIM arm (P=0.06).

5.5.4 Forearm muscle lipid content

Forearm lipid content was measured in 5 participants using ^{1}H MRS, however, spectra from 4 participants was of insufficient quality to undergo further analysis (the reasons for this will be covered in detail in Section 5.6.4). Thus, Figure 5.7 displays longitudinal MRS data of EMCL concentration (A) and IMCL concentration (B) within the FDP muscle of a NIM and IMM forearm of 1 subject before (day 0) and after 2, 7 and 16 days of immobilisation. Prior to immobilisation both EMCL and IMCL concentrations of the immobilised arm were approximately 4 times greater than corresponding values measured in the non-immobilised arm. In the NIM limb EMCL and IMCL concentration of the FDP muscle slightly increased from baseline at 2 days, after which both measures declined at day 7 and had returned close to the baseline values by day 16. Within the IMM limb, the EMCL concentration in the FDP muscle appeared to decrease below baseline at day 2 and remained lower than baseline at day 7 and day 16. In the case of IMCL concentration, values appeared to be lower than baseline at day 2 of immobilisation but increased thereafter.

5.5.5 Forearm muscle cross sectional area

As shown in Figure 5.8, forearm muscle cross sectional area was comparable between arms before immobilisation (37.4±0.9 and 36.5±1.7 cm^2 for the NIM

and IMM arms respectively; P=0.18). In the NIM limb, muscle cross sectional area showed no change from baseline following 2 (37.7 ±1.0 cm^2; P=0.75), 7 (37.9±1.1 cm^2; P=0.77) or 16 (38.1±1.1 cm^2; P=0.37) days of forearm immobilisation. In the IMM limb, muscle cross sectional area was similar to pre-immobilisation values at day 2 (36.2±1.5 cm^2; P=0.38) of immobilisation, however, muscle cross sectional area was less than baseline at day 7 (-2.9%, 35.5±1.5 cm^2; P<0.05) and 16 (-3.6%, 35.2±1.6 cm^2; P<0.05). After 16 days of immobilisation forearm muscle cross sectional area was significantly less in the IMM arm compared to the NIM arm (P<0.01).

5.5.6 Handgrip strength

Handgrip strength was measured in both limbs prior to (Day 0) and following 16 days of immobilisation and is presented in Figure 5.9. Handgrip strength was similar between arms at baseline (47.44±2.47 and 46.73±3.39 kg in the NIM and IMM limbs respectively; P= 0.59) and was not different from baseline values in the NIM limb at the end of the immobilisation period (48.07±2.3 kg; P=0.73). In the IMM limb, handgrip strength was less than baseline following 16 days of immobilisation (-22.6±2.8%; 36.16±3.22 kg; P<0.0001), such that strength in the IMM arm was less than NIM arm at day 16 (P=0.02).

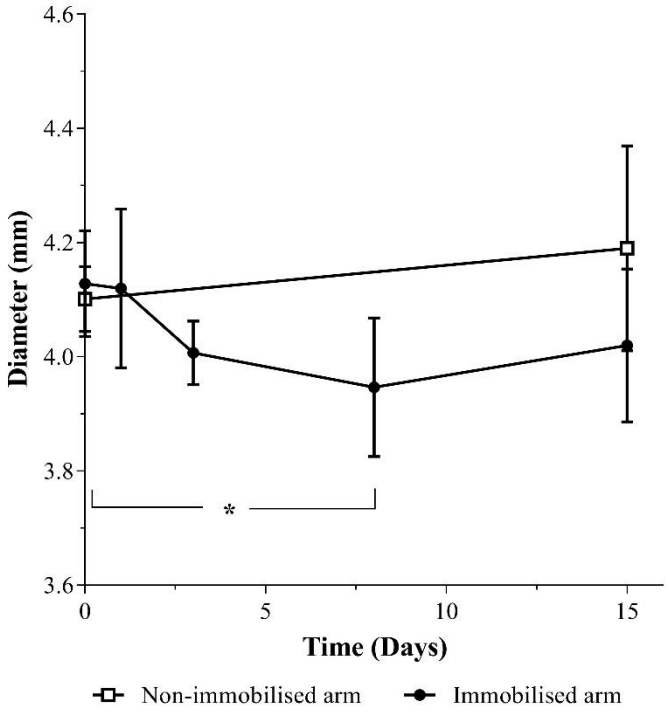


Figure 5.5 Basal brachial artery diameter measured prior to glucose ingestion in a non-immobilised forearm (open squares) before (0) and 15 days following forearm immobilisation, and in an immobilised forearm (closed circles) before (0), and at 1, 3, 8 and 15 days of immobilisation. Values are mean ±SEM (*n*=5). *P<0.05 significantly different to corresponding baseline value.

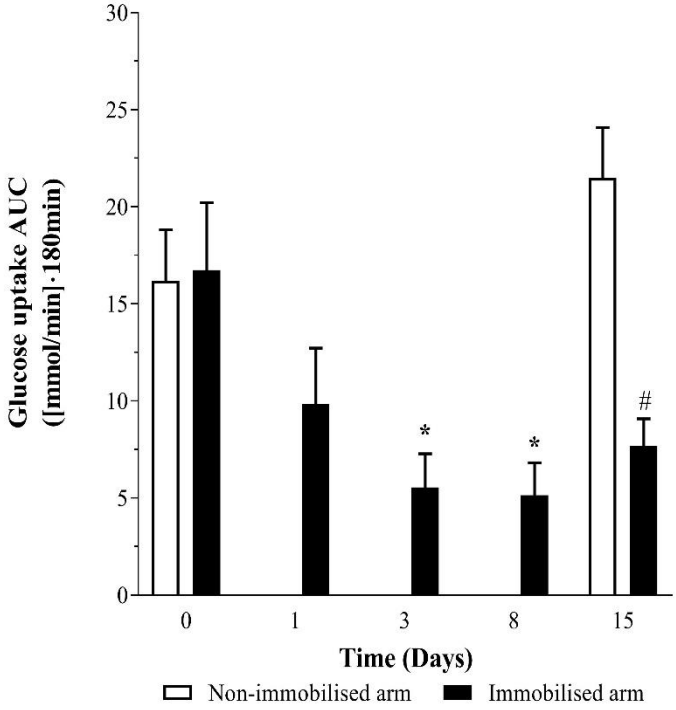


Figure 5.6 Area under the curve (AUC) for forearm glucose uptake over the course of each 180 min oral glucose challenge in non-immobilised (white) and immobilised (black) limbs before (day 0), during (days 1, 3 and 8) and after 15 days of immobilisation. Values are mean ±SEM (*n=5*). *P<0.05 significantly different to corresponding baseline value. # P<0.05 significantly different between arms.

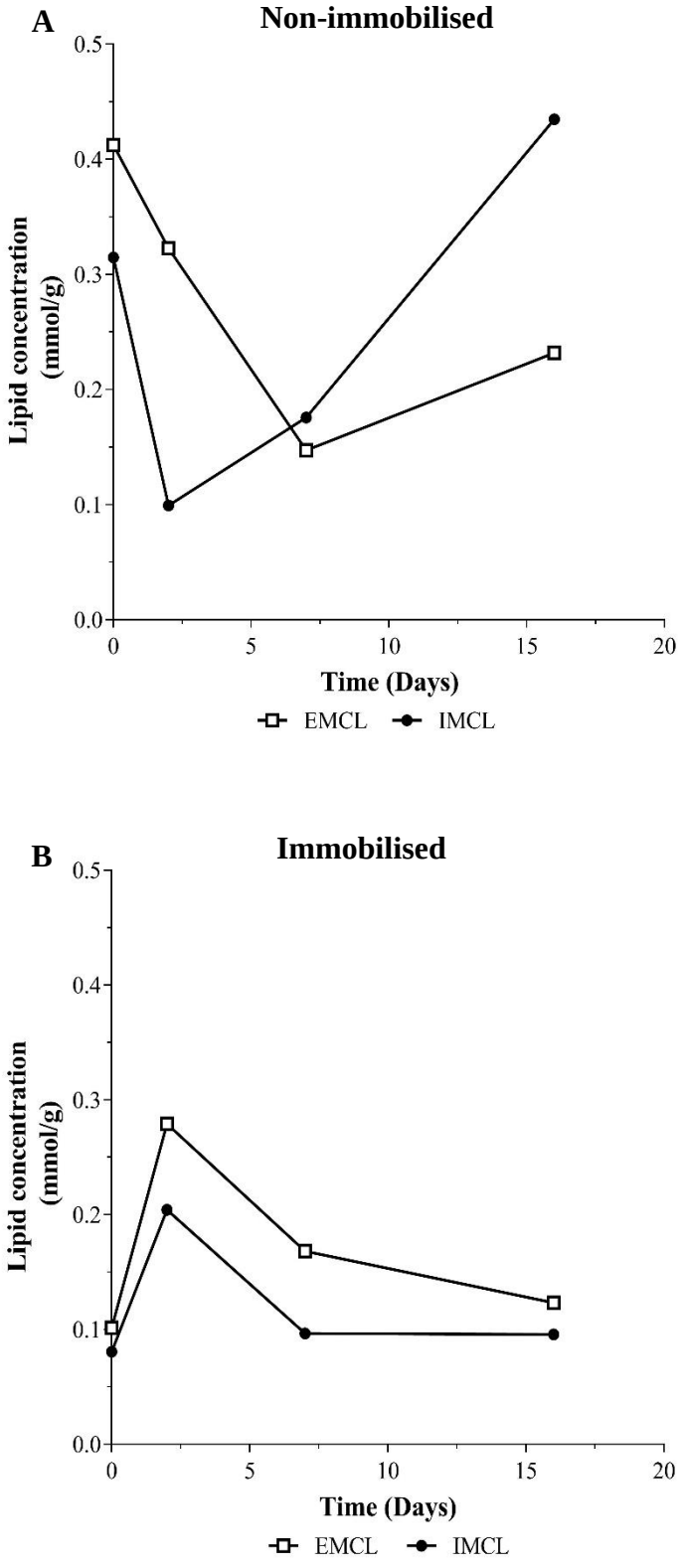


Figure 5.7 Extramyocellular lipid **(**EMCL; open squares) and intramyocellular lipid (IMCL; closed circles) concentration of non-immobilised arm **(A)** and immobilised arm **(B)** measured within the flexor digitorium profundus muscle, using magnetic resonance spectroscopy, prior to (0), during (2 and 7) and following 16 days of forearm immobilisation (n=1).

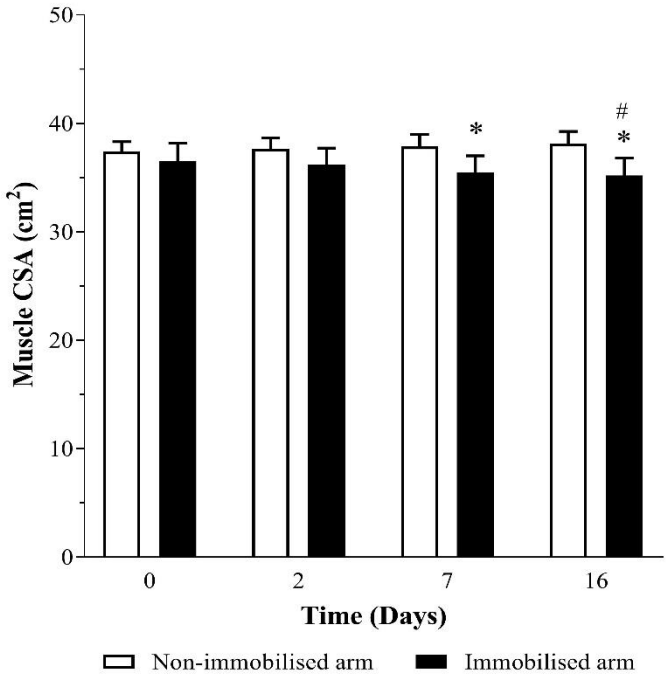


Figure 5.8 Forearm muscle cross sectional area (CSA) measured using 7T magnetic resonance imaging in a non-immobilised (white) and immobilised (black) arm before (day 0), during (days 2 and 7) and at 16 days of arm immobilisation. Values are mean ±SEM (*n*=5). *P<0.05 significantly different to corresponding baseline value. # P<0.05 significantly different between arms at 16 days.

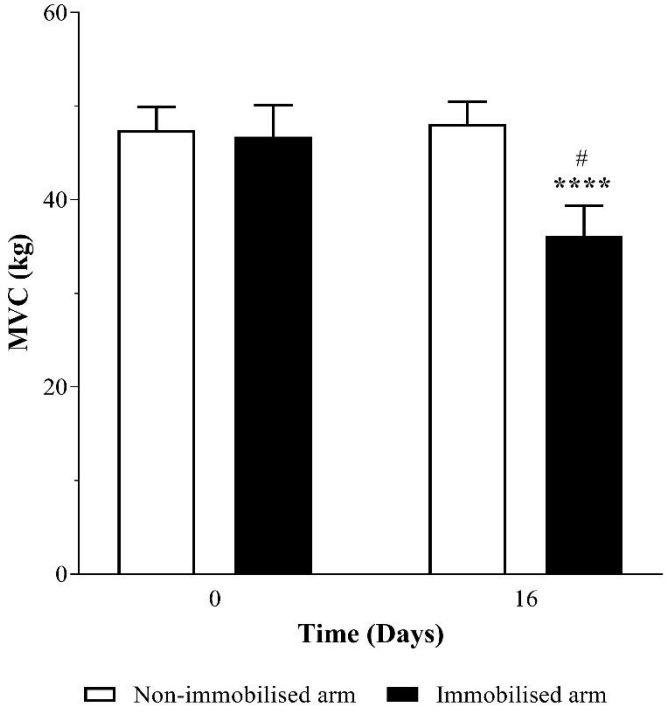


Figure 5.9 Handgrip strength in a non-immobilised (white) and immobilised (black) arm before (0) and at 16 days of forearm immobilisation. Values are mean ±SEM (*n*=5). ****P<0.0001 significantly different to corresponding baseline value. # P<0.05 significantly different between arms at 16 days.

5.6 Discussion

The present study supports the outcomes detailed in Chapter 3, which revealed a rapid inhibition of glucose uptake by skeletal muscle induced by forearm immobilisation, without any concurrent changes in brachial artery blood flow or serum insulin concentration. The current work builds on these findings, indicating that this decline is sustained for 8 days of forearm immobilisation in healthy, young participants. In addition the present study also aimed to investigate the mechanisms accounting for this reduction in insulin stimulated muscle glucose uptake by quantifying any concurrent alterations in IMCL content, using ^{1}H MRS, as well as determining any changes to forearm muscle cross sectional area during the immobilisation period, assessed via MRI scanning. Forearm immobilisation reduced muscle cross sectional area in the IMM limb by 2.9% within 7 days and this continued to decline to 3.6% after 16 days, without any change in the NIM limb. Likewise, a substantial loss of 23% muscle strength was measured in the IMM limb at the end of the immobilisation period, while no change was observed in the NIM limb. Interestingly, the magnitude of decline of cross sectional area was considerably less than the decrease in muscle strength. Furthermore, the decline in glucose uptake occurs prior to the observed decrements in both forearm muscle cross sectional area, which indicates initial immobilisation induced declines in forearm glucose uptake are unlikely to occur as a direct consequence of muscle atrophy. While extensive measures were undertaken to optimise scanning protocols for quantification of forearm lipid content using ^{1}H MRS (discussed in Section 5.6.4), this study illustrates the inherent

difficulties of using muscles of the forearm, rather than those of lower limb, in conjunction with this technique.

5.*6.1 Effects of immobilisation on forearm glucose uptake*

Findings from the current study support the hypothesis that decrements in muscle size occur swiftly in response to muscle disuse. Such a reduction of skeletal muscle presents a credible means of lowering the body's capacity for glucose clearance, and was indeed accompanied a 70% decline in forearm muscle glucose uptake. Dirks *et al*. reported a 3.2% decrease in cross sectional area of the quadriceps during 1 week of bed-rest, while concurrently observing a 30% reduction in glucose disposal, assessed by a hyperinsulinemic-euglycemic clamp (Dirks *et al.,* 2016). Earlier bed rest studies also demonstrate that although changes in muscle size during muscle disuse may indeed contribute to reduced glucose clearance, significant declines in glucose disposal occur before any measurable changes in muscle size manifest (Berg *et al.,* 1997). The addition of findings from the present study to the existing literature, indicates immobilisation induced muscle atrophy may indeed contribute to lowered glucose clearance capacity of skeletal muscle, however, the decrement in glucose clearance is of a much greater magnitude, therefore other mechanisms must be primarily responsible. After 7 days of bed rest, Biensø *et al*. reported reductions in the content/activity of key proteins involved in glucose transport, phosphorylation and storage, including glycogen synthase and glucose-transporter-4 (Biensø *et al.,* 2012), while intramuscular lipid deposition in response to bed rest has also been associated reduced muscular and whole body insulin sensitivity (Cree *et*

al., 2010). However, it remains to be established precisely which mechanisms are accountable for the rapid decrements in glucose uptake during forearm immobilisation, but clearly it is restricted to the immobilised muscle so is likely to be muscle centric. The rapid development of decrements in muscle glucose uptake indicate intracellular perturbations in response to reduced muscle contraction are potentially responsible and requires further investigation.

5.6.2 Effects of immobilisation on forearm muscle cross sectional area

Extended periods of muscle disuse, predominantly achieved using bed rest, have been extensively shown to cause changes in body composition through substantial muscle loss and/or increased fat mass (Deitrick 1948, LeBlanc *et al.*, 1988, LeBlanc *et al.*, 1992). Using unilateral lower limb suspension, quadriceps cross sectional area was reported to decrease by 5% following 7 days (White *et al.*, 1984); 5-10% after 14 days (Yasuda *et al.*, 2005, de Boer *et al.*, 2007, Glover *et al.*, 2010) and 10% following 23 days (de Boer *et al.*, 2007). Earlier work by this group showed no change in forearm muscle cross sectional area, assessed using MRI, following 3 weeks of immobilisation (Skirrow 2014), which is in agreement with some (Kitahara *et al.*, 2003) but not all previous reports. Miles *et al*. reported a decrease of 4.1 % in muscle cross sectional area following 9 days of forearm immobilisation, measured by computed tomography (Miles *et al.*, 1994). These contrasting findings may be due to differences in the degree of immobilisation achieved or varying techniques for assessment of muscle cross sectional area in respective studies. In view of the general consensus that weight bearing muscles of the lower

limbs are considered to have greater susceptibility to muscle atrophy compared to muscles of the upper limbs, it is logical to conclude that upper limb immobilisation is less likely to induce measurable changes in muscle size or cross sectional area. However, as discussed earlier, Smeulders *et al*. highlighted the low sensitivity of MRI scanners with field strengths below 3.0T for the quantification of changes in muscle volume of less than 7% (Smeulders *et al.*, 2010). Hence, as Skirrow *et al*. measured muscle cross sectional area using 1.5T MRI, re-evaluation of these findings using a more sensitive, ultra-high strength 7.0 T MRI was considered pertinent. The current findings, revealing significant decrements in muscle cross sectional area within 7 days of forearm immobilisation, demonstrate 7T MRI is a highly sensitive method for the detection of changes in forearm muscle cross sectional area, which maybe be <5%. This non-invasive method will be invaluable in determining the time-course of changes in muscle mass as a result of muscle disuse.

Following 4 days of lower limb immobilisation Suetta *et al*. reported a 10% decrease in muscle fibre cross sectional area, measured in vastus lateralis muscle biopsy samples taken from young males (Suetta *et al.*, 2012). This demonstrates a rapid remodelling of skeletal muscle in response to disuse, however, there is an absence of studies determining the time-frame of changes in muscle size during short-term disuse. Thus, the current study aimed to explore the time-course of alterations in muscle cross sectional area during 16 days of forearm immobilisation In the present study, cross sectional area of the immobilised forearm significantly declined by 2.9% following 7 days of

immobilisation and continued to decline so that forearm muscle cross sectional area was 3.6% lower than baseline values after 16 days. Similar to these findings, Dirks *et al*. reported a decline of 3.5% in cross sectional area of the quadriceps, measured using computed tomography, following 5 days of unilateral lower limb suspension (Dirks *et al.*, 2016). A decline of 0.9% in forearm muscle cross sectional area was also measured at 2 days of immobilisation in the present study. Though non-significant, these findings are in keeping with reports of a 0.5% reduction in cross sectional area per day over 23 days of unilateral lower limb suspension from de Boer *et al*. (de Boer *et al.*, 2007). This magnitude of reduction in muscle cross sectional area was maintained only until day 7 of immobilisation, after which the rate of muscle atrophy slowed for the remainder of the immobilisation period. Remarkably, 17 weeks of bed rest resulted in a decline in muscle mass of approximately 0.1% per day (LeBlanc *et al.*, 1992). The amalgamation of these findings suggest the greatest loss in muscle mass occurs quickly (within 1 week), followed by a deceleration of the rate of muscle atrophy as the period of muscle disuse extends, pointing towards the possibility that mechanisms driving the early muscle loss may differ from those responsible for muscle atrophy during later periods of disuse. Indeed, disuse induced decrements in muscle volume represent a potential reduction in the body's ability to lower circulating blood glucose concentrations and thereby contribute to impairments in metabolic health. Thus, additional investigations are necessary to further our understanding of the contribution of immobilisation-induced alterations in body composition to diminished metabolic health.

The precise mechanisms capable of producing a rapid reduction in muscle mass have not yet been identified, although some likely candidates have been proposed. Phosphorylation of Akt was reportedly reduced by 10-25% within 2-4 days of immobilisation in healthy subjects, suggesting that reductions in Akt phosphorylation may contribute to the decreased muscle mass associated early periods muscle disuse (Urso *et al.,* 2006, Suetta *et al.,* 2012). However, not all investigations support these findings. Several studies have reported reduced muscle protein synthesis in response to longer periods of muscle disuse, induced by 14 days of knee immobilisation or 23 days of bed rest, without any concurrent decrease in Akt content or signalling (de Boer *et al.,* 2007, Glover *et al.,* 2010). Moreover, Glover *et al*. demonstrated an immobilisation induced inhibition of increased muscle protein synthesis following amino acid infusion, when compared to the contralateral non-immobilised leg, while no difference in the phosphorylation state of Akt was observed between limbs (Glover *et al.,* 2008). These findings suggest other mechanisms may be implicated in the decline in muscle protein synthesis during muscle disuse. Focal adhesion kinase (FAK), a regulator in the assembly of costamere proteins with an important role in regulation of muscle size, has also been identified as having a potential role to play in immobilisation induced muscle atrophy and its expression has been shown to be load dependent (Glover *et al.,* 2008). Unilateral lower limb immobilisation for 3 days reduced FAK phosphorylation by 20% in the vastus lateralis, while de Boer and colleagues also report a 30% decrease in the vastus lateralis following 10 days of bed rest (de Boer *et al.*, 2007, Fluck *et al.,* 2014). While these reports indicate that decreased phosphorylation of Akt, and/or FAK may

have a significant impact on the catabolic loss of muscle occurring in response to muscle disuse, further investigations are necessary fully elucidate the precise mechanisms driving the decrease in muscle mass associated with immobilisation.

5.6.3 Effects of immobilisation on forearm muscle strength

Reductions in muscle size, as a result of periods of muscle disuse, represent a decrease in the force generating capacity of the muscle, thus leading to a decrease in muscle strength. Such reductions in muscle strength are reported to range between 0.3%-4.2% per day (Wall and van Loon 2013). Studies investigating changes in muscle strength following upper limb immobilisation vary in the reduction of muscle strength reported, ranging from 22-24% after 7 days (Lundbye-Jensen and Nielsen 2008, Johnston *et al.*, 2009), 15-30% after 9 days (Miles *et al.*, 1994), 15-20% after 21 days (Miles *et al.*, 2005, Matsumura *et al.*, 2008, Farthing *et al.*, 2009) and 27% after 35 days (Skirrow 2014). The wide range of decrements in strength reported in these studies are likely to be derived from the model of immobilisation used (e.g. finger, wrist or entire forearm immobilisation). The present study showed a decrease of 23% in handgrip strength of the immobilised arm following 16 days of immobilisation, almost 6-fold greater than the corresponding loss in muscle cross sectional area. In concert with the present findings, the literature similarly indicates that decrements in muscle strength following periods of disuse are of a much greater magnitude compared to reductions in muscle size (Wall and van Loon 2013). This has been suggested to be the result of alterations in motor unit activation and recruitment, resulting in reduced

ability of the nervous system to fully activate muscle. Duchateau and Hainout reported changes in neural drive following 6 weeks of forearm immobilisation, as evidenced by a 55% reduction in maximal voluntary force, compared to a 35% reduction in electrically evoked maximal force (Duchateau and Hainaut 1987). Structural changes in grey matter cortical thickness have also been proposed by Langer *et al*. as a potential cause of the disproportional loss in muscle strength compared to size following periods of muscle disuse (Langer *et al.*, 2012). Following 16 days of upper limb immobilisation, surface based morphometry analysis performed using MRI, showed a decrease in thickness of the areas governing sensorimotor control of upper limbs, which indicates the sensorimotor cortex has the capacity to be rapidly remodelled in response to muscle disuse. Although these proposed mechanisms present credible explanations for the greater loss of muscle strength compared to muscle size, the exact mechanisms remain to be elucidated. Further investigations are merited to identify novel targets that may reduce decrements in muscle strength during periods of muscle disuse.

5.6.4 Effects of immobilisation on forearm muscle lipid content

As reported in both previous chapters, forearm immobilisation rapidly induces a pronounced decrement in glucose uptake, and this reduction was not associated with alterations in lipid clearance by skeletal muscle. Hence, the major questions arising from these findings relate to changes in forearm glucose uptake over an extended period of immobilisation, and if any changes over this extended period are associated with the accumulation of IMCL. Following 3 and 8 days of forearm immobilisation forearm glucose uptake

was reduced by almost 70%. Unfortunately, this study is underpowered due to difficulties with participant retention encountered as result of issues associated with the side effects of entering the bore of the MRI scanner. Of the 5 participants that completed the protocol, ^{1}H MRS spectra from just one participant was suitable for further analysis. Spectra from this participant suggested that both IMCL and EMCL concentrations of the FDP muscles in both the NIM and IMM limbs are labile and may be an early indication that measurements made during different time points of muscle disuse are incomparable. However, further research is first necessary to determine if these changes in lipid concentrations are of any physiological significance.

5.6.5 Methodological considerations for Magnetic Resonance Spectroscopy acquisition

To date, the majority of publications investigating muscle lipid content have focused on lower limb muscles. Thus, considerable work was put into method development to produce scanning protocols capable of quantifying IMCL concentration of forearm muscles for the use in the current study and is detailed below.

5.6.5.1 Voxel size and positioning

The SNR refers to the ratio between an amplitude of resonance from a metabolite of interest and the amplitude of random noise, and thereby describes the performance of an MRI system. While many of the variables determining SNR are unchangeable, such as coil size, voxel size represents one of the few adjustable variables. Since SNR is directly proportional to voxel size, increasing voxel size also increases SNR. While this presents a

simple method to increase SNR in large lower limb muscles, the smaller muscles typical of the forearm prove more difficult in accommodating larger voxel sizes. To assist in overcoming this difficulty, the largest muscle of the forearm, the FDP muscle (Figure 5.10), was selected in the present study design to allow voxel placement of the largest possible size. The elongated profile of this muscle allowed the voxel length to be extended to lie parallel with the muscle fibres, thereby increasing the volume from which signal is collected whilst still remaining within the muscle itself and avoiding contamination by EMCL.

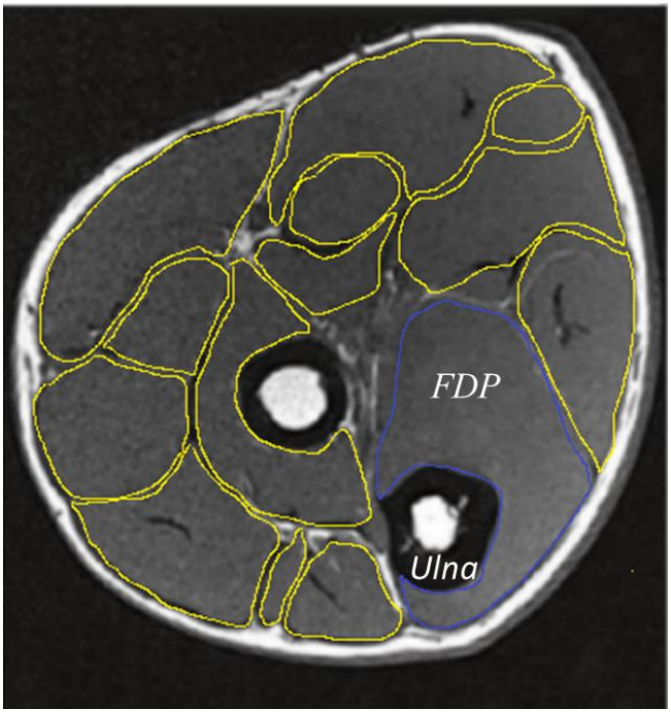


Figure 5.10. Cross sectional image of human forearm muscle groups with the flexor digitorium profundus (FDP) outlined in blue.

To identify the optimal voxel size within the FDP for determination of IMCL content, one subject was scanned using three different voxel volumes: 10 x 10 x 10 mm^3, 10 x 10 x 25 mm^3 and 10 x 10 x5 0mm^3. Scan parameters were as follows: repetition time = 2000 ms, spectral bandwidth of 4 kHz, 4096 samples and 32 averages. As shown in Figure 5.11, the voxel with the largest

volume produced the highest SNR between the peaks, while also avoiding any obvious line broadening or contamination by extramyocellular compartments. Thus, a voxel sixe of 10x10x50mm^3 was used for measurement of metabolite concentration in the present study.

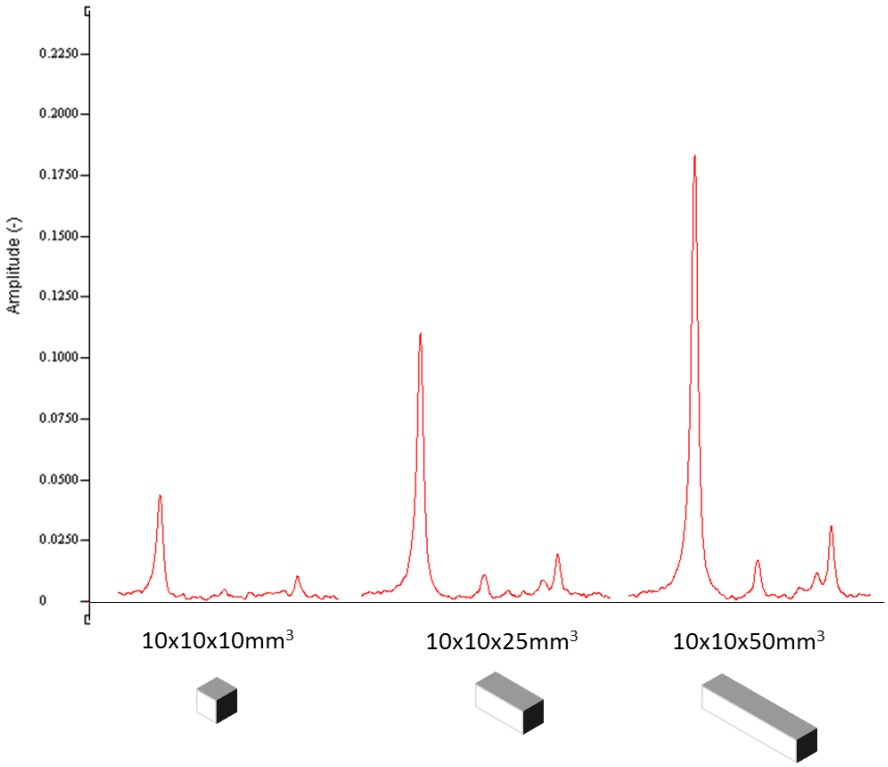


Figure 5.11 Spectra showing increased signal produced by increasing voxel volume for quantification of lipid within the flexor digitorum profundus muscle of a human forearm.

5.6.5.2 Orientation

Although the characteristic shapes of EMCL and IMCL aid in distinguishing spectra originating from each separate compartment, the orientation of these shapes in relation to the main magnetic field (B_0) can impact on the resolution of the frequency of a signal, referred to as chemical shift. Due to the spherical shape of IMCL droplets their orientation to the magnetic field has a negligible effect on the chemical shift and resulting spectra. However, the chemical shift produced by EMCL is orientation dependent, thus, as the angle of EMCL strands increases relative to the direction of B_o, the chemical shift of the EMCL signal increases accordingly (Khuu *et al.*, 2009). As the EMCL signal and linewidth broadens it begins to overlap with the IMCL signal resulting in inferior resolution between the lipid peaks. Moreover, since muscle fibres of forearm musculature are generally not parallel to B_0, this can further increase the angle of EMCL to the main magnetic field. Thus, preliminary investigations were conducted to explore the extent of the impact of lipid orientation within forearm muscle on collected spectra, using the same parameters as described above in Section 5.6.4.1.

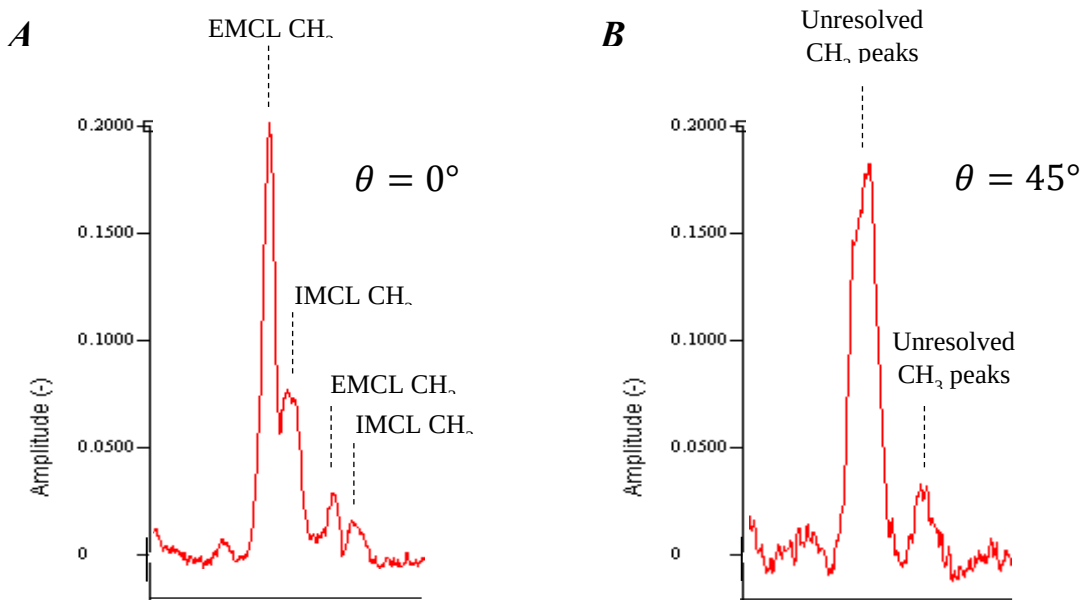


Figure 5.12 Lipid spectra obtained from forearm muscle fibres positioned in parallel **(A)** and at a 45° angle **(B)** to the main magnetic field.

As shown in Figure 5.12, increasing the angle of muscle fibres to the magnetic field produced indistinguishable lipid peaks, which underlines the importance of maintaining a forearm position parallel with B_0. Consequently, it was considered essential to produce custom-made equipment, suitable for use within the MRI scanner, to keep the forearm musculature in parallel with B_0 for the duration of the scanning period. This simple instrument consisted of a wooden base, specifically designed to fit inside the transmit coil for the 7.0 T Philips MRI scanner, within which the participants forearm was held in place using a pair of Velcro straps (Figure 5.13).

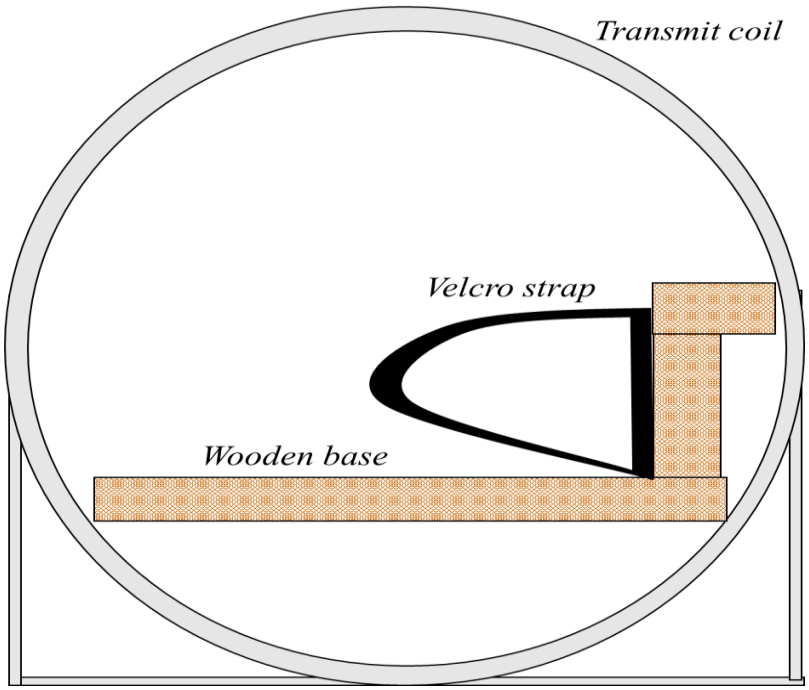


Figure 5.13 Schematic representation of the instrument designed to maintain participants forearm in a stationary position, in parallel to the magnetic field for the duration of scanning.

For the quantification of IMCL and EMCL content the spectra obtained must first be fitted with symmetrical line shapes, such as Gaussian and Voigt functions. While this procedure is relatively straightforward for the anterior tibialis, in which muscle fibres run parallel to each other and thus, EMCL strands are also in parallel to the main magnetic field, this becomes a much more complex issue in muscle groups in which fibres are not uniformly aligned. As this is the case with the FDP muscle, the line shape of EMCL peaks are likely to be asymmetrical leaving conventional line fitting methods inappropriate for use in the present study. To overcome such a problem, seminal work by Khuu and colleagues established FOM, a new method for spectral analysis which accounts for lipid orientation and dispersion (Khuu *et al.*, 2009). Building on the work of these authors, Olivier Mougin (SPMIC, Nottingham, UK) developed a FOM based program for robust line fitting of

spectra originating from FDP muscle specifically for the present study and, thus, this is the first study to apply FOM analysis in the interrogation of forearm muscle lipid content.

5.6.5.3 Optimisation of scan parameters

5.6.5.3.1 Echo time

The acquisition parameters of a scan affect the characteristics of a spectrum, and so, it is essential that these parameters are diligently planned. Two of the major parameters requiring careful planning are the echo time (TE) and repetition times (TR) used during scanning. The TE of the scan is typically measured in milliseconds (ms) and describes the time between the application of the radiofrequency excitation pulse and the peak signal measured. After excitation, each metabolite has a characteristic transverse relaxation time (T_2) from which it can be identified and quantified. Although the acquisition of a non-water suppressed spectrum may be required to act as an internal standard for metabolite quantification, the abundant water signal is generally suppressed to allow concentrations of other metabolites to be both detected and quantified. Water suppression is commonly achieved through pre-saturation of the water signal by applying a water suppression signal at the beginning of the excitation pulse sequence. However, a major drawback of this method is the potential for these signals to also encroach upon lipid spectra, thereby resulting in the misrepresentation of lipid concentrations. Choosing long TE offers an alternative to using water-suppression methods, by allowing the water signal to decay before acquiring spectra from lipid signals which still remain due to their longer relaxation times. Longer TE

reduces the number of detectable peaks and simplify the spectra for lipid quantification, and the lipid signal may also be partially reduced, thus multiple averages must be taken to overcome this decrease in fat signal. One publication by Ren *et al*. investigating the outcome of short (20ms) and long (280ms) TE on the determination of lipid concentration within the soleus muscle showed that the longer TE produced clearly distinguished EMCL and IMCL peaks (Ren *et al.,* 2010). Whether such a long TE as that employed by Ren *et al*. is appropriate for lipid quantification within forearm muscles is unknown, therefore, preliminary trials were conducted to identify the optimal TE time to be used in the current study. One subject was scanned using the same parameters as detailed by Ren and colleagues (TE of 20ms and 280 ms, using a stimulated echo acquisition mode (STEAM) sequence and collecting 128 averages). At the low TE, a water suppression enhanced through longitudinal relaxation time (T_1) effects (WET) method was utilised to saturate the water signal. These preliminary scans revealed that no fat signal could be detected at the long TE and so the experiment was repeated using a TE of 100ms. The complete deterioration of fat signal at this length TE is likely a result of the smaller voxel size necessary when studying musculature of upper limbs. The voxel size used by Ren and colleagues in the soleus muscle was 3-5 times greater than could be used in the present study, thus, the smaller volume restricts the SNR. Analysis of these revised longer TE revealed well separated peaks from CH_2 and CH_3 resonances of both EMCL and IMCL (Figure 5.14). However, the greater decay of the lipid signal reduced SNR (0.17AU) so that it was four fold less than the SNR of the short TE signal (0.7AU) as a consequence.

A limitation of using longer TE to overcome dominant water signals is the associated signal decay from both metabolites of interest and internal physiological standards necessary for determination of lipid concentrations (Blüml 2013). This unpredictable decay may lead to discrepancies in peaks representing the reference metabolite and peaks of other metabolites of interest. To account for this decay, multiple TE time of varying lengths were employed throughout the acquisition times. Furthermore, signal relaxation correction was also employed to account for the difference in loss of signal between water and lipid, by using varying TE's to determine T_2 relaxation times of both water and lipid peaks.

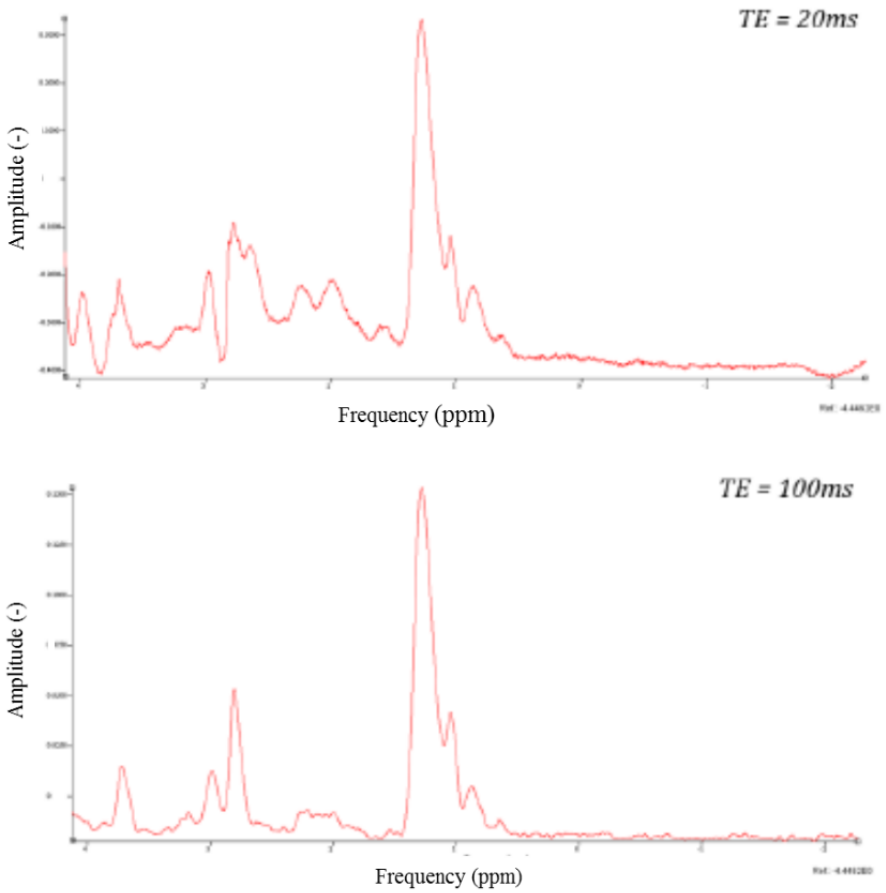


Figure 5.14 Presentation of spectra obtained from short (20ms) and long (100ms) echo times (TE).

5.6.5.3.2 Repetition time

The second changeable scan parameter which can influence the acquisition of high quality spectra is TR, which refers to the time between each radiofrequency pulse. Each metabolite has its own unique T_1 and must be allowed to return to equilibrium after each excitation pulse for accurate determination of absolute metabolite concentrations. Long TR are typically used to ensure each metabolite has had sufficient time to return to equilibrium, therefore negating the need for prior knowledge of each individual metabolites T_1 (Blüml 2013). However, extended TR can reduce SNR and result in an increase in overall scan acquisition times, particularly when averaging over multiple excitations. Determination of the minimum TR for the reference metabolite is important as lipid concentration could be over-estimated if the signal of the internal standard was reduced due to insufficient relaxation time. Thus, an experiment was conducted in one volunteer to identify a TR time which optimises both SNR and scanning time. As the T_1 of water, the physiological internal standard, is longer than the T_1 of lipids, water signal recovery was examined in a 10 x 10 x 50mm^3 voxel positioned in the FDP muscle, using a STEAM sequence with multiple TR. As shown in Figure 5.15 the water signal was adequately recovered after 2000ms.

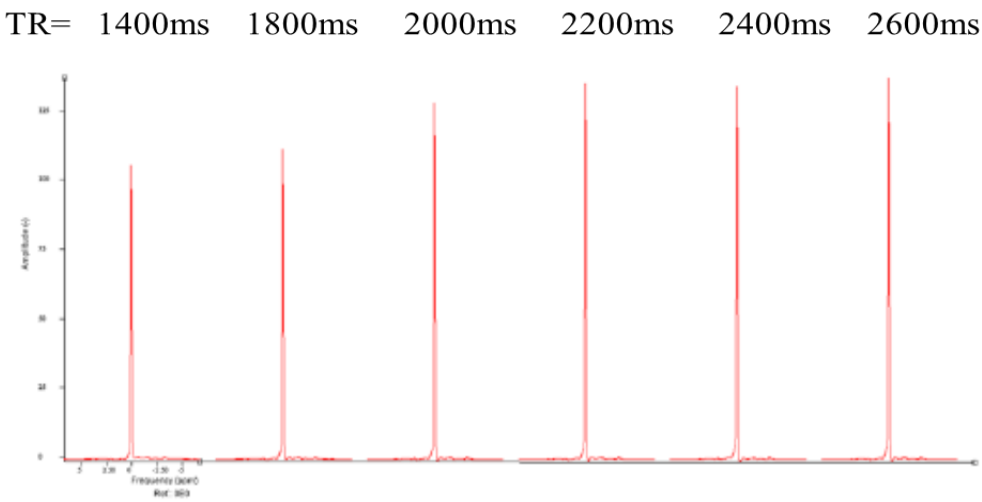


Figure 5.15 Multiple repetition times (TR) assessing water signal recovery.

5.6.6 Conclusion

Despite the extensive effort put into the development of spectroscopy techniques specifically designed for this study, the intrinsic complications associated with scanning an upper limb ultimately hindered the acquisition of quality spectra. *Firstly*, typical muscle volumes of forearm muscle are considerably less than muscle volumes of the lower limb, for which the majority of existing work investigating lipid concentrations has concentrated on. Thus, the size of a voxel which can be placed within a forearm muscle is substantially restricted, resulting in diminished SNR and an increased risk of including EMCL within the voxel from which signal is being acquired, leading to potential line broadening of the spectra. *Secondly*, the requirement to place participants in a head-first prone position for the duration of scanning compromises their comfort, which leads to an increased likelihood of motion artefacts, resulting in further line broadening. Furthermore, common side effects of ultra-high field strengths include vertigo, an effect well recognised in MRI studies, which did indeed provoke an intolerable response, including nausea, vomiting and dizziness, in 2 out of 9 participants enrolled in the current study. Physicists have identified the Lorentz effect as the cause of this phenomenon, occurring as a result of the magnetic field pushing on the current of charged particles within fluid of the inner ear, thus effecting an individual's sense of motion (Roberts *et al.,* 2011). Although the likelihood of this response is completely unpredictable, it has been suggested that the subject positioning used in the present study, entering the bore of the scanner head first, is more likely to elicit an intolerable response to the magnetic field (Mian 2013, Glover 2015). Overall, the outcomes of the present study indicate

further development of the forearm model is needed for optimal voxel positioning within muscle fibres for uniform orientation. In addition, the high dropout rate of approximately 45% in the present study strongly emphasises the need for further work to determine the ideal subject positioning to minimise the side-effects associated with ultra-high field strengths. In summary, it is clear that until extensive development work takes place to improve the use of forearm musculature in ^{1}H MRS quantification of IMCL, muscles of the lower limb remain as the optimal model in terms of quality spectra acquisition.

In conclusion, 16 days of forearm immobilisation has been shown to result in considerable decrements in forearm glucose uptake, muscle cross sectional area and muscle strength. Although the time course of intramuscular lipid accumulation is still unclear, the current findings strongly support the need for further longitudinal studies exploring the relationship between lipid accumulation within myocytes with decreased glucose uptake, and underlines the necessity for further development of MRS scanning protocols in the interrogation of IMCL content.

Chapter 6: General discussion

6.1 Overview

The negative health implications of immobilisation induced muscle disuse are now well recognised (Bergouignan *et al.*, 2011). Thus, it is surprising that a number of unresolved questions remain pertaining to the time course of development of metabolic impairments during immobilisation associated with negative health outcomes, and the resolution of these impairments if remobilisation occurs. Diminished insulin stimulated glucose uptake by skeletal muscle is one of the early metabolic responses to immobilisation and yet the precise time frame, magnitude and causative factors driving such impairments are not yet fully characterised. Furthermore, whether these negative responses to immobilisation are heightened by factors known to impair insulin stimulated glucose uptake, such as increased dietary fat intake, are unknown. Therefore, the studies presented in this thesis endeavoured to fill gaps in the existing literature, primarily aiming to establish the time course of development of changes in forearm glucose uptake in response to short-term muscle disuse using immobilisation in healthy individuals (Chapter 3), and whether increasing dietary lipid availability (Chapter 4) modulated the impact of immobilisation on forearm glucose uptake. Additionally, this thesis aimed to elucidate whether quantification of IMCL accumulation using MRS (Chapter 5) could explain the deficits in insulin mediated glucose uptake during short-term forearm immobilisation identified in Chapter 3, as well exploring the impact of forearm immobilisation on muscle size, using MRI, and handgrip strength.

With increasing knowledge of the metabolic impairments associated with immobilisation establishing a time course of these metabolic responses is

imperative before the development and implementation of effective countermeasures can be achieved. This gap in the existing literature was first explored in the study detailed in Chapter 3, where 3 days of forearm immobilisation was used to determine the impact of muscle disuse on insulin-stimulated glucose uptake. Forearm immobilisation resulted in substantial decrements in forearm glucose uptake within 24 hrs (38%), and this decline was sustained for 72 hrs, without concurrent reductions in blood flow or insulin concentration. The subsequent study (Chapter 4) investigated the effect of increasing dietary lipid availability on the previously observed decrements in forearm glucose uptake during forearm immobilisation, and the results showed no additional deleterious effect on glucose uptake. It is worth considering these findings may indeed indicate the immobilisation induced decrement in muscle glucose uptake observed was so profound that no further quantifiable impact of increasing dietary lipid was possible. This study also aimed to establish the time course of alterations in lipid clearance by skeletal muscle in response to short-term immobilisation and the results demonstrate no significant change in skeletal muscle lipid clearance during 48 hrs of immobilisation. While these findings may indicate forearm immobilisation is not a sufficient stimulus to invoke changes in lipid clearance by skeletal muscle within 48 hrs, it may also be the case the study is not sufficiently powered to detect changes in lipid clearance over 48 hrs of immobilisation (discussed in section 6.5). Nonetheless, results from these two studies clearly show immobilisation induced decrements in forearm glucose uptake occur rapidly, are localised to the immobilised limb and the driving mechanisms instigating such impairments are likely to reside within the muscle as there

was no change in substrate delivery or circulating insulin concentration observed. Accumulation of IMCL is one such muscle centric mechanism identified as having a potential role in the disruption of insulin signalling, resulting in reduced glucose uptake by myocytes in response to muscle disuse. Thus, the third study in this thesis (Chapter 5) aimed to, firstly, develop a protocol capable of quantifying forearm IMCL content using ultra-high strength 7T proton MRS that could be employed in longitudinal study designs. This study also aimed to investigate any relationship between changes in muscle IMCL content over the course of 16 days of forearm immobilisation with concurrent changes in forearm glucose uptake. However, due to inherent complexities associated with the architecture of forearm muscle and necessary positioning of participants within the relatively small bore of the 7T scanner, the scanning protocol established failed to successfully quantify forearm IMCL content in a sufficient number of volunteers to allow robust changes in IMCL content to be resolved. Furthermore, this study demonstrated that decrements in glucose uptake, as a result of muscle disuse, occur prior to measurable reductions in muscle volume, and therefore, the underlying mechanisms are unlikely to be attributed to muscle atrophy *per se*. The primary findings from these studies are discussed in detail within each experimental chapter, and therefore the remainder of this chapter will provide an overall perspective of the body of work, identify limitations of the studies and discuss potential future directions for research aimed at investigating negative metabolic health outcomes associated with muscle disuse.

6.2 Metabolic adaptations during forearm immobilisation

6.2.1 Forearm glucose uptake

The studies presented in this thesis consistently demonstrate short-term forearm immobilisation rapidly induces substantial decrements in forearm glucose uptake, in the absence of comparable reductions to circulating insulin concentrations. Furthermore, each study confirmed this reduction in glucose uptake was localised to the immobilised limb, without any corresponding decrements in glucose uptake in the non-immobilised contralateral control limb. While an abundance of evidence exists linking immobilisation with decreased insulin sensitivity, no such study to date has investigated the acute impact of just 24 hrs of forearm immobilisation on skeletal muscle glucose uptake. Thus, findings from the studies described in Chapters 3 and 5 are novel as an oral glucose challenge was used in each to measure a 40% decline in forearm glucose uptake after 24 hrs of forearm immobilisation. These findings demonstrate a prompt reduction in the insulin stimulated glucose uptake by skeletal muscle, a phenomenon for which there has been no evidence reported in any published studies to date. At the global level, Stephens *et al*. reported an 18% reduction in whole body insulin action, measured during a continuous glucose infusion, following one day of prolonged sitting (Stephens *et al.*, 2011). These observations support the hypothesis that diminished insulin sensitivity is an early metabolic response to reduced muscle use. However, not all observations are in agreement with those presented in this thesis or from Stephens and colleagues. A recent publication from Dirks and colleagues reported no change in whole body insulin sensitivity, assessed using indices of insulin sensitivity (HOMA-IR and

Matsuda index), following 1 day of bed rest (Dirks *et al.*, 2018). The discrepancy between these findings may be due to limb immobilisation acting as a more potent instigator of complete muscle disuse, whereas, although bed rest induces significant muscle unloading, this model of physical inactivity has been assigned a physical activity level of 1.45 due to fidgeting movements, which is above the level of total inactivity (Bergouignan *et al.*, 2011). Moreover, the use of venous blood (instead of arterial or arterialised-venous blood) in the determination of insulin sensitivity by Dirks *et al*. may also contribute to the contradictory findings. Recently, Edinburgh *et al.* highlighted the variance in estimation of insulin sensitivity based on the use of venous versus arterialised venous blood, with venous blood associated with higher estimates of insulin sensitivity (Edinburgh *et al.*, 2017). Overall, these studies support the supposition that impairments driving immobilisation induced reductions in muscle glucose uptake occur primarily within skeletal muscle, thus the underlying mechanisms must reside within skeletal muscle and this should be the focus of future investigations.

To date there has been no temporal investigation into changes in insulin mediated skeletal muscle glucose uptake as a result of the reduced muscle use associated with limb immobilisation. Therefore, the studies presented in this thesis are the first to document substantial reductions in skeletal muscle glucose uptake within 24 hrs of limb immobilisation, which continues to decline after 48 hrs (Chapter 3) and is sustained thereafter, with forearm glucose uptake measured to be 54% lower than baseline after 15 days of immobilisation (Chapter 5). These findings are in line with earlier work from

this laboratory, which measured a 66% and 54% decrease in forearm glucose uptake following 7 and 21 days of forearm immobilisation respectively (Skirrow 2014). Previous bed rest and lower limb immobilisation studies have also demonstrated lowered insulin sensitivity both at the whole body (Mikines *et al.,* 1991, Hamburg *et al.,* 2007, Alibegovic *et al.,* 2009, Sonne *et al.,* 2010, Coker *et al.,* 2014) and muscle level (Richter *et al.,* 1989, Cree *et al.,* 2010, Sonne *et al.,* 2010) after 5-28 days. While these studies have added greatly to existing knowledge of the negative health impact of muscle disuse during immobilisation, unravelling the precise time course and underlying mechanisms of such negative metabolic adaptations will also further contribute to establishing effective interventions to prevent or delay the harmful consequences of muscle disuse.

Building on findings detailed in Chapter 3, the study described in Chapter 4 was designed to investigate any additional negative implications of increasing dietary lipid availability on insulin mediated forearm glucose uptake during short term immobilisation, using a high fat mixed meal challenge. Findings from Chapter 4 show decrements in forearm glucose uptake following 24 and 48 hrs of immobilisation to be of the same magnitude as those observed in Chapter 3 ($P=0.7$ and $P=0.5$ at 24 and 48 hrs respectively; displayed in Figure 6.1). Thus, increasing dietary lipid availability had no additive negative effect on immobilisation induced reductions in forearm glucose uptake. This may indicate the impact of muscle disuse via forearm immobilisation alone is so profound, no further impact of increasing dietary lipid was measurable or

immobilisation reduced the metabolic demands of the muscle so that tissue uptake was negligible.

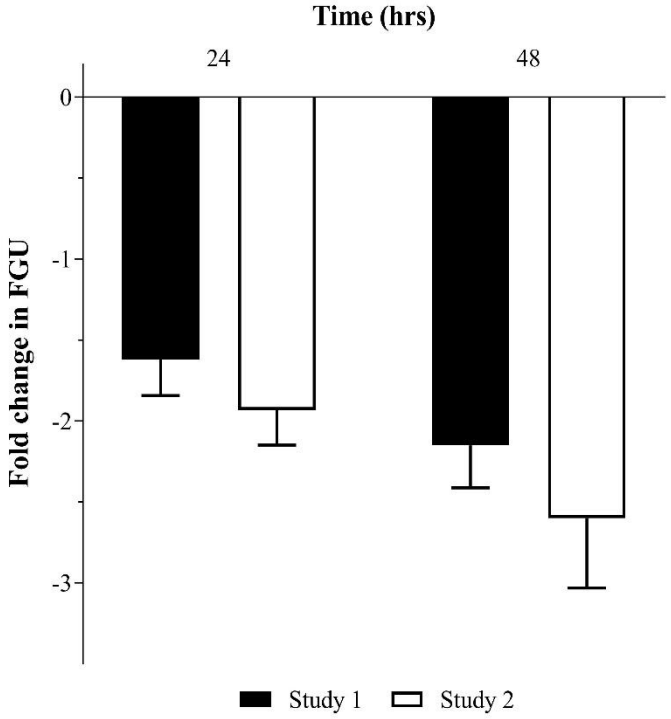


Figure 6.1 Fold changes in forearm glucose uptake (FGU) measured prior to (0), and at 24 and 48 hrs following forearm immobilisation, determined using 180 min oral glucose challenge detailed in Chapter 3 (Study 1; black) and 420 min high fat mixed meal challenge detailed in Chapter 4 (Study 2; white). Values are mean ±SEM, *n*=10 and *n*=9 in Study 1 and Study 2 respectively.

6.2.1.1 Potential mechanisms linking immobilisation with reduced muscle glucose uptake

Findings presented in this thesis demonstrate a rapid (within 24 hrs) decline in forearm glucose uptake in response to oral glucose ingestion, which cannot be accounted for by insufficient delivery of glucose to the muscle. Instead, alterations at other sites of muscle glucose uptake are likely to account for the decrements observed, such as glucose transport or phosphorylation of glucose within the myocyte (Wasserman *et al.,* 2011). Reports from bed rest studies

provide further evidence supporting a muscle specific inhibition of glucose uptake by revealing major defects in muscle glycogen synthesis rates, which may be a consequence of lower muscle GLUT-4 protein content and activity and/or lower glycogen synthase activation and activity (Mikines *et al.,* 1991, Tabata *et al.,* 1999, Biensø *et al.,* 2012), while 7 days of bed rest has also been associated with a reduction in protein content of Hexokinase II (Ringholm *et al.,* 2011, Biensø *et al.,* 2012). Defective insulin signalling via the IRS-1/Akt pathway may also contribute to decrements in muscle glucose uptake associated with immobilisation. Kiilerich *et al*. reported reduced insulin stimulated Akt phosphorylation in humans following 7 days of bed rest (Kiilerich *et al.,* 2011), while animal studies have revealed decreased Akt activity and IRS-1 protein expression after 7 days of hind-limb immobilisation (Hirose *et al.,* 2000). Further research is necessary to fully elucidate possible defects in insulin signalling occurring alongside muscle disuse, in addition to the timeframe over which such defects manifest.

Numerous exercise studies have identified mechanisms by which chronic muscular contraction increases glucose uptake by stimulating GLUT-4 translocation, while also reducing intracellular glycogen levels (Goodyear and Kahn 1998, Philp *et al.,* 2012). Richter *et al*. were among the first to provide evidence of a regulatory role of glycogen in the GLUT-4 content of the cell membrane (Richter *et al.,* 1984). These authors findings revealed muscle glucose uptake, during 1 hour of bicycling, to be considerably lower in muscles loaded with glycogen compared to muscles in the glycogen depleted state (Richter *et al.,* 2001), which further suggests that high muscle glycogen

concentration inhibits both GLUT-4 translocation and glucose transport. Although the precise means by which intracellular glycogen exerts an inhibitory effect on GLUT-4 action is unknown, a potential physical attachment between the glycogen particles and the GLUT-4 containing vesicles has been postulated (Richter *et al.,* 2001). In this scenario GLUT-4 translocation would be initially inhibited by high muscle glycogen levels, and depletion of muscle glycogen concentrations through muscular contraction would release GLUT-4, facilitating an increase in glucose uptake by the myocyte. Previously, muscle glycogen concentration has been shown to increase by approximately 20 % after 7-9 days of bed rest (Kiilerich *et al.,* 2011, Ringholm *et al.,* 2011). Thus, the sustained absence of muscular contraction induced by forearm immobilisation may prevent sufficient depletion of glycogen, thereby inhibiting GLUT-4 release prior to translocation to the plasma membrane for facilitation of glucose uptake. Again, however, whether this mechanism could explain the marked reduction in muscle glucose uptake measured within 24 hrs remains to be clarified.

Previous investigations have shown lipid oxidation to be reduced during immobilisation (Ritz *et al.,* 1998, Bergouignan *et al.,* 2006), which is likely to contribute to IMCL accumulation during periods of muscle disuse (Manini *et al.,* 2007, Cree *et al.,* 2010). This increased IMCL content has also been implicated in the inhibition of skeletal muscle glucose uptake associated with immobilisation, with the accumulation of lipid intermediates, such as ceramides and diacylglycerol, suggested to interfere with insulin signalling (Blanc *et al.,* 2000, Itani *et al.,* 2002, Cree *et al.,* 2010, Sonne *et al.,* 2010).

However, the timeframe for accumulation of such lipid intermediates is unknown. Further investigation is needed to elucidate whether or not increased IMCL content, and associated lipid intermediates, could account for the substantial decrease in forearm glucose uptake observed within 24 hrs of forearm immobilisation in the study detailed in Chapter 3.

6.2.2 Skeletal muscle lipid clearance

The effect of forearm immobilisation on skeletal muscle lipid clearance was explored in Chapter 4, and revealed no significant changes over 48 hrs. Although large scale epidemiological studies have identified exaggerated and prolonged post-prandial lipid concentrations as an independent risk factor for the development of cardiovascular disease (Bansal *et al.*, 2007, Nordestgaard *et al.*, 2007), very few reports to date have investigated the impact of immobilisation on postprandial lipid clearance. Recently, Kim *et al.* reported a 30% increase in postprandial lipid concentration following 2 days of prolonged sitting (14.3 hrs/day) in energy balance compared to an active walking condition (Kim *et al.*, 2016). However, Kim *et al*. instructed participants to maintain low levels of physical activity for 2 days prior to starting the intervention which may have an additional impact on postprandial lipid clearance. These contrasting findings suggest 48 hrs of forearm immobilisation does not allow measureable changes in skeletal muscle lipid metabolism to manifest and a longer time period may be required to detect deleterious alterations using this model of muscle disuse.

6.3 Physiological adaptations during forearm immobilisation

6.3.1 Forearm muscle strength

Numerous studies demonstrate the marked negative impact of periods of immobilisation on muscle strength (Veldhuizen *et al.,* 1993, Thom *et al.,* 2001, Paddon-Jones *et al.,* 2004, Kortebein *et al.,* 2007) and this was a point of interest for the studies detailed in Chapters 3 and 5. Three days of forearm immobilisation led to a non-significant reduction of 6% in handgrip strength, reported in Chapter 3, however, 16 days of forearm immobilisation was shown to elicit a substantial decrease of 23% in handgrip strength from baseline in Chapter 5. While several authors have investigated the impact of upper limb immobilisation over 21-42 days, reporting an average reduction of 15-41% in muscle strength (MacDougall *et al.,* 1977, MacDougall *et al.,* 1980, Miles *et al.,* 1994, Seki *et al.,* 2001, Kitahara *et al.,* 2003, Matsumura *et al.,* 2008), Johnston *et al*. were the first to report a 22% decrease in muscle strength following 7 days of upper limb immobilisation (Johnston *et al.,* 2009). Findings from the studies presented in this thesis, showing no change in muscle strength after 3 days of forearm immobilisation, add to this existing knowledge by demonstrating the greatest decline in forearm muscle strength during muscle disuse is likely to occur expeditiously between days 3 to 7 of upper limb immobilisation, with the majority of the literature indicating that decrements in muscle strength decelerate during further periods of muscle disuse (Seki *et al.,* 2001, Kitahara *et al.,* 2003, Paddon-Jones *et al.,* 2004, Brooks *et al.,* 2008). Future studies investigating the mechanisms instigating reduced muscle strength should focus on the period of 3-7 days of muscle disuse.

Furthermore, as reported in Chapter 5, this decrease in muscle strength was 6-fold greater than the corresponding decrease in muscle mass, hence, muscle atrophy alone cannot fully account for decrements in strength. Instead, reduced motor activation of muscle during periods of muscle disuse has been identified as a potential factor leading to reduced muscle strength (Duchateau and Hainaut 1987, Berg *et al.,* 1991, Hortobagyi *et al.,* 2000). Additionally, structural changes in brain grey matter during immobilisation have been highlighted as potential factors contributing to decreased central drive producing decrements in muscle strength (Langer *et al.,* 2012). Therefore, it is possible that the loss of muscle strength measured in the studies of the present thesis is accounted for by the combination of reduced muscle mass, lower motor unit activation and brain grey matter structural changes.

6.3.2 Forearm muscle cross sectional area

In Chapter 5, forearm muscle cross sectional area, measured using 7T MRI, was shown to decrease by 2.9% after 7 days of immobilisation, and continued to decrease, such that forearm cross sectional area was 3.6% lower than pre-intervention values following 16 days of immobilisation. These findings are in keeping with reports from Smeulders *et al*. proposing investigations employing MRI at field strengths above 3.0T have sufficient sensitivity to detect changes in muscle volume of <5% (Smeulders *et al.,* 2010). A novel aspect of the study detailed in Chapter 5 is the use of the contralateral non-immobilised limb as a within volunteer control, which very few publications have concurrently studied to date. Forearm muscle cross sectional area did not

change in the control limb following the 16 day immobilisation period, further highlighting the negative implications of short-term muscle disuse.

Numerous studies have highlighted muscle atrophy as one of the major physiological adaptations to muscle disuse, with weight-bearing, antigravity muscles of the lower limb repeatedly shown to waste considerably in response to bed-rest and lower limb immobilisation (White *et al.,* 1984, Davies *et al.,* 1987, LeBlanc *et al.,* 1988, LeBlanc *et al.,* 1992, Ferrando *et al.,* 1996, Thom *et al.,* 2001, de Boer *et al.,* 2007, Glover *et al.,* 2010) Much fewer studies report on upper limb atrophy in response to muscle disuse, and those which have delivered variable outcomes. A number of studies to date report no change in muscle volume or cross sectional area following 3-5 weeks of upper limb immobilisation, and, in general, these investigations resolutely conclude a longer period of upper limb immobilisation is necessary to induce muscle atrophy at a measurable level (Kitahara *et al.,* 2003, Homma *et al.,* 2009, Skirrow 2014). While the majority of these findings were indeed surmised using MRI, the gold standard measurement of muscle volume and cross sectional area, the conclusions do not hold true in every investigation. Miles *et al*. demonstrated a 7% reduction in flexor muscle volume after 21 days of forearm immobilisation and, in an earlier publication, the same authors also reported a 4% decrease in forearm muscle cross sectional area after 9 days of immobilisation of the wrist (Miles *et al.,* 1994, Miles *et al.,* 2005). Similarly, Johnston *et al*. also reported a 4% decrease in muscle mass following 7 days of upper arm immobilisation (Johnston *et al.,* 2009). These inconsistent findings may be due to different techniques used to measure muscle atrophy or

as a result of varying degrees of muscle disuse achieved by different methods of immobilisation (cast vs sling only). Decrements in muscle mass due to disuse are reported to be primarily driven by reduced muscle protein synthesis, with a transient rise in signalling markers of protein degradation during the initial stages of muscle disuse (Gibson *et al.*, 1987, Ferrando *et al.*, 1996, Paddon-Jones *et al.*, 2006, de Boer *et al.*, 2007, Glover *et al.*, 2008).

The vast majority of the existing literature indicates muscle disuse must be drastic, such as unloading of the weight bearing, anti-gravity muscles of the lower limbs, to elicit physiologically significant muscle wasting which is likely to impact daily tasks and mobility. The study detailed in Chapter 5, along with other publications using upper limb immobilisation (Miles *et al.*, 1994, Johnston *et al.*, 2009), illustrate decrements in volume of non-weight bearing muscles to be of a similar magnitude to reductions in size of lower limb muscles during immobilisation or bed rest (Ferrando *et al.*, 1995, Hespel *et al.*, 2001, Thom *et al.*, 2001, Yasuda *et al.*, 2005, de Boer *et al.*, 2007, Kortebein *et al.*, 2007). Overall, data presented in Chapter 5 strongly supports the concept that reduced muscle use results in atrophy of both weight bearing and non-weight bearing muscle groups, albeit to a varying extent between each muscle, and even within each muscle. Although muscles of the upper limbs are not weight bearing, they are intrinsic in the basic yet fundamental tasks of daily living, and thus, it is essential to recognise the potentially debilitating impact of any atrophy of these muscle groups.

6.3.3 Brachial artery diameter

The findings from the studies comprising this thesis support the theory that reduced physical activity leads to a reduction in arterial diameter, in the resting state, in a dose-dependent manner. Basal brachial artery diameter was shown to decrease by 4.2% during 3 days of immobilisation in the study described in Chapter 3. Similarly, in Chapter 5, basal brachial artery diameter in the immobilised arm was narrower after 8 days of immobilisation when compared to pre-immobilisation values. The absence of significant reductions in brachial artery diameter at other time points in the study detailed in Chapter 5 may be due to the data being underpowered, as only measurements from 5 subjects were included in this analysis, whereas measurements from 10 subjects were analysed for the study detailed in Chapter 3. In Chapter 4 no change in basal brachial artery diameter was identified over the 48 hour period of immobilisation despite 9 subjects being included in this analysis, which may indicate a minimum of 3 days of immobilisation is necessary to induce measurable changes. Furthermore, manual analysis of brachial artery diameter measurement is subject to significant inter-observer variation and observer error. An average basal brachial artery diameter of 4.25±0.12 and 4.13±0.09 mm, as measured pre-immobilisation (Day 0), in Chapters 3 and 5 respectively, compared with an average basal brachial artery diameter of 3.88±0.25 mm measured prior to immobilisation in Chapter 4. Therefore, the variation in the detection of changes in brachial artery diameter in response to forearm immobilisation may stem from a different scanner operator determining brachial artery diameter for the study detailed in Chapter 4,

whereas the same scanner operator performed brachial artery diameter measurements in the studies of Chapter 3 and Chapter 5.

The amalgamation of findings from Chapter 3 and Chapter 5 demonstrate a considerable reduction of 3.5%, 4.2% and 4.4% in basal brachial artery diameter after 2, 3 and 8 days of forearm immobilisation respectively. Moreover, in both Chapter 3 and Chapter 5, basal brachial artery diameter was similar to baseline values at the end of the intervention period, further highlighting the negative impact of muscle disuse. These findings indicate brachial artery diameter rapidly decreases in response to reduced muscle use, with the majority of this decline occurring early on (within 2-3 days) and this reduction is sustained thereafter. In contrast to the outcomes presented in this thesis, previous studies investigating the impact of forearm immobilisation on brachial artery diameter reported no change (Birk *et al.*, 2013). However, as discussed earlier in Chapter 3, these discrepancies are likely to arise from different models of forearm immobilisation used and the varying degree of muscle disuse achieved. In a recent publication Doppler ultrasound was used to measure a 6.5% decrease in brachial artery diameter following 5 days of bed rest, while femoral artery diameter was reduced by 3% in the same study (Credeur *et al.*, 2018). Additionally, the impact of 7 and 28 days lower limb immobilisation has been reported to induce reductions of 6-12 % in femoral artery diameter (Bleeker *et al.*, 2005, van Duijnhoven *et al.*, 2010), which are in line with the current observations. Extended periods of immobilisation further suggest a dose-dependent relationship between periods of immobilisation with alterations to arterial diameters following 25 and 60 days

of bed rest shown to decrease femoral artery diameter by 13% and 24 % respectively (Bleeker *et al.,* 2005, van Duijnhoven *et al.,* 2010). Overall, findings presented in this thesis align well with the current literature supporting a dose-dependent relationship between the extent and duration of immobilisation with the change in arterial diameter. Such changes have been proposed to occur as the result of inward remodelling due to smooth muscle hypertrophy which contributes to arterial wall thickening, an integral part of the atherosclerotic process (Pauletto *et al.,* 1991, Chen *et al.,* 1995).

6.3.4 Associations between muscle atrophy and reduced basal brachial artery diameter

Skeletal muscle atrophy during immobilisation may be associated with decreased basal arterial diameter. Previous reports by Olive *et al*. have proposed links between vascular atrophy and skeletal muscle atrophy after differences between the diameter of the femoral artery in spinal cord injury patients with considerable muscle atrophy, compared to those of healthy controls, were abolished by expressing the cross sectional area of the femoral artery per unit muscle mass (Olive *et al.,* 2003). Additionally, de Groot *et al*. report simultaneous reductions of 25% in femoral artery diameter and 22% decline in muscle volume within the first 3 weeks of bed rest in spinal cord injury patients, which may be indicative of a functional link between muscle atrophy and changes in vascular dimensions during deconditioning (de Groot *et al.,* 2006). Chapter 5 demonstrates a similar rate of change between basal brachial artery diameter and cross sectional area of forearm muscle, represented by a 2.9% decline in muscle cross sectional area after 7 days of

forearm immobilisation, in conjunction with a 4.4% decrease in basal brachial artery diameter after 8 days of immobilisation. In contrast, no change in either brachial artery diameter or forearm muscle cross sectional area was observed in the NIM arm, highlighting the localised effects of immobilisation. Furthermore, 16 days of forearm immobilisation resulted in a 3.5% reduction in muscle cross sectional area, while there was a non-significant 3% decline in brachial artery diameter measured in the IMM limb after 15 days of immobilisation, this lack of statistical change may be due to the study being under-powered. Animal models of congestive heart failure have demonstrated that apoptosis of endothelial cells occurs prior to apoptosis of skeletal muscle fibres, suggesting vascular atrophy precedes muscle atrophy (Vescovo *et al.*, 1998). However, the mechanisms of vascular and muscular adaptions during deconditioning, and any relationships between the two, remains largely unexplored in humans. Overall, the similar time course for changes in muscle cross sectional area and basal brachial artery diameter indicate a similar pattern, or existence of a common pathway, between muscle atrophy and vascular remodelling which warrants further investigation.

6.5 Limitations

A major limitation of two of the studies presented in this thesis relate to the relatively small sample size of the studies detailed in Chapter 4 and Chapter 5. Difficulties with repeated cannulation of the same deep vein on 3 consecutive days, as required for the study described in Chapter 4, resulted in a high proportion (approximately 40%) of participants not completing the high fat mixed meal challenge at the 72 hour time point. The lack of statistically significant alterations in postprandial triacylglycerol clearance may also be due to high inter-individual variability, as an increase in postprandial triacylglycerol clearance was detected in 6 subjects, while 2 subjects produced a decreased postprandial triacylglycerol clearance and 1 subject showed no change from baseline values. Similarly, problems associated with entering the 7T ultra-high field strength MRI scanner, including nausea, vomiting and pain around a fixed dental retainer, caused several participants to withdraw from the study included in Chapter 5. Additionally, to avoid confounding issues regarding scheduling laboratory visits around the same phase of the menstrual cycle only young men participated in each of the studies included in this thesis. Due to gender differences in muscle metabolism and muscle morphology, such as fibre type composition and capillarisation, as well as hormonal differences, it is difficult to determine if the current observations would be observed at the same magnitude in skeletal muscle of females.

6.6 Conclusions and future directions

To conclude, the studies included in this thesis have established the rapid onset of changes in muscle metabolism and function in response to forearm

immobilisation. These investigations demonstrate that forearm glucose uptake declines markedly within 24 hrs of forearm immobilisation and this reduction is sustained thereafter over 8 days of immobilisation. Furthermore, the studies comprising this thesis have shown that immobilisation induced alterations to blood flow, serum insulin concentration, muscle cross sectional area or increased dietary lipid availability cannot account for these major decrements in muscle glucose uptake. Instead, impairments residing within the muscle itself, likely to be related to the sustained removal of muscular contraction, are the chief candidates believed to drive these muscle disuse induced impairments in muscle metabolism, such as impaired Akt phosphorylation, decreased muscle hexokinase activity and reduced GLUT-4 protein content (Tabata *et al.*, 1999, Biensø *et al.*, 2012, Mortensen *et al.*, 2014). Despite extensive efforts, the development of a scanning protocol capable of measuring forearm muscle lipid content using ultra-high filed strength 7T ^{1}H-MRS was ultimately hindered by intrinsic complexities associated with the forearm architecture (long, thin muscles) and the narrow bore of the magnet. Until further development of these protocols is undertaken, the work presented in this thesis underscores the lower limb as the ideal model for quantification of lipid accumulation during immobilisation.

Although the work presented in this thesis adds to exiting knowledge regarding temporal changes in muscle metabolism during periods of disuse, several new questions and lines of investigation arise from this research.

1. Investigation of the temporal changes in insulin-stimulated glucose uptake over 24 hrs of immobilisation.

2. Identification of the exact mechanistic pathways underlying decrements in muscle glucose uptake are necessary. Future investigations should aim to elucidate whether the molecular pathways driving the initial decline in glucose uptake during acute immobilisation are the same as those sustaining the decrease in glucose uptake over extended periods of muscle disuse. Subsequent limb immobilisation studies involving muscle biopsies would facilitate this.
3. Exploration of the minimum amount of muscular contraction necessary to deter decrements in muscle glucose uptake.
4. Identification and exploration of the interplay between motor and brain factors contributing to reduced muscle strength during muscle disuse.
5. While forearm immobilisation has proven to be an excellent model to investigate early metabolic dysfunction in response to muscle disuse, using this model in combination with ^{1}H-MRS to determine temporal changes in intramuscular lipid content has proven problematic. Further development of scanning protocols in the upper limbs is necessary to optimise this technique.

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Appendices

Appendix 1: A pilot study to compare the impact of processing methods on whole blood glucose concentrations.

1. Introduction

For many years the decrease in glucose concentration following blood sample collection has been extensively investigated . Glycolysis within erythrocytes has been identified as the mechanism underlying the declining glucose concentration, which can be amplified under certain conditions such as increased ambient temperature and high white blood cell counts (Bruns and Knowler 2009). The degradation of glucose in whole blood occurs at a rate of 5-7% of the samples glucose content per hour, or approximately 0.67 mmol/l decline after 2 hrs at room temperature at a starting concentration of 5.5 mmol/l (Chan *et al.,* 1989). Much of the research into this decline in glucose concentration has been focussed over a period of 1.5- 4 hrs, which is the maximum time between sample collection and analysis recommended in the clinical setting (Sacks *et al.,* 2011). However, it has not yet been identified if this decline in whole blood glucose concentration is linear or occurs exponentially over this time period. This is particularly relevant for studies utilising the arterialised venous-venous substrate difference technique, which measures substrate concentrations in blood samples collected simultaneously from multiple ports, thereby precluding immediate measurement of substrate concentration. As the highest level of accuracy achievable is needed to measure small arterialised venous-venous differences it is particularly important in these studies that measurement error is quantified and, if possible, minimised.

2. Aims

The purpose of the present study was to determine which post-sampling processing methods are most appropriate to produce accurate and stable measures of whole blood glucose concentration.

3. Methods

3.1 Subjects and sample size

Blood samples were collected from 6 healthy volunteers, consisting of laboratory staff and students, with an age range of 25-35 years, including 5 males and 1 female.

3.2 Experimental protocol

Approximately 7ml of blood was collected from each volunteer and within 20 seconds blood glucose concentration was measured using a YSI automated analyser (YSI 2300 STATplus, Yellowsprings Instruments, Yellowspring, Ohio, USA). Prior to sampling the YSI automated analyser was calibrated using glucose standards of known concentration. The remaining blood sample was then aliquoted equally into four tubes: two yellow top tubes (HEP) containing 16 IU heparin and 1.0mg fluoride (Sarstedt, Nümbrecht, Germany) and two grey top tubes (SFPO) containing 5mg sodium fluoride and 4 mg potassium oxalate (Becton-Dickinson, Franklin Lakes, NJ). Following the addition of whole blood, one of each yellow (HEP-ice) and grey (SFPO-ice) tube was immediately placed in an ice slurry while the remaining yellow (HEP-roll) and grey (SFPO-roll) top tubes were placed on a tube roller (Stuart Roller Mixer SRT6, Bibby Scientific, Staffordshire, UK), to inhibit blood clot

formation and ensure adequate mixing with tube additives. Glucose concentrations were then measured at 1 minute intervals in whole blood samples in the HEP-roll tube. Blood glucose concentrations were measured in each of the other tubes 10 minutes following sample collection. A one-way ANOVA was used to detect differences between conditions after 10 minutes and the percentage decline from 0 mins in each condition.

4. Results

Figure 1 shows the comparison of blood glucose concentrations from each of the processing methods described above. The most profound decline in glucose concentration in yellow top tubes placed on the tube roller occurs within the first three minutes (4.0%), after which time decrements in glucose concentration become less marked. Figure 2 displays the percentage decline in glucose concentration in each respective tube 10 minutes after the initial blood sample was obtained. There was no difference between any of the processing methods relating to the decrease in glucose concentration after 10 minutes ($P>0.05$ for each condition).

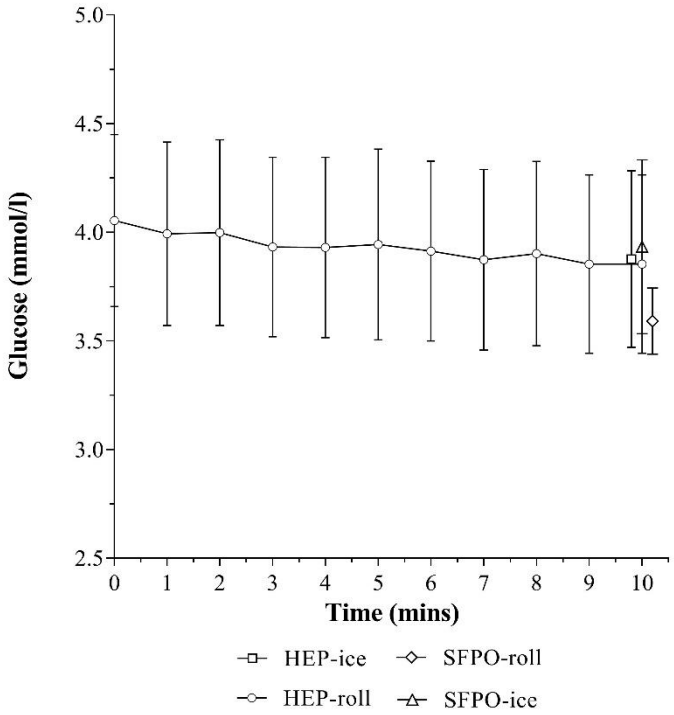


Figure 1. Whole blood glucose concentrations for each processing method. Following sample collection glucose concentration in whole blood was immediately measured using an automated analyser. The collected whole blood sample was then aliquoted into 4 tubes: two microtubes containing fluoride and heparin, one of these tubes was then placed on a sample roller (HEP-roll; circles) and the other in an ice slurry (HEP-ice; squares). The remainder of the blood sample was divided between two vacutainers containing sodium fluoride and potassium oxalate, with one of these tubes then placed on a tube roller (SFPO-roll; diamonds) and the second tube then placed in an ice slurry (SFPO-ice; triangles). Values are mean ±SEM (*n*=6).

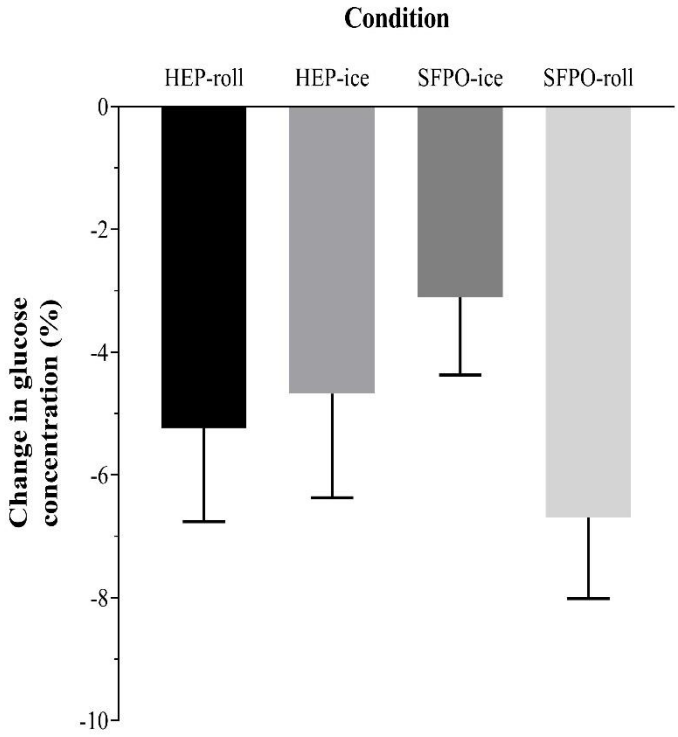


Figure 2. Decline in glucose concentration (%) for each processing method. Following immediate glucose determination using an automated analyser, the whole blood sample was aliquoted into 4 tubes: two microtubes containing fluoride and heparin, one of these tubes was then placed on a sample roller (HEP roll) and the other in an ice slurry (HEP-ice). The remainder of the blood sample was divided between two vacutainers containing sodium fluoride and potassium oxalate, with one of these tubes then placed on a tube roller (SFPO-roll) and the second tube then placed in an ice slurry (SFPO-ice). Glucose concentration in each tube was measured again after 10 minutes. Values are mean ±SEM (*n*=6).

5. Discussion

The present study was conducted to determine the optimal method of whole blood sample processing to ensure accurate measurement of blood glucose concentrations. The blood tubes included in the present study contained fluoride, which inhibits glycolysis, and an anticoagulant, either heparin or potassium oxalate, preventing the formation of blood clots which distort determination of glucose concentration (H. Major 2018). Indeed, placing blood samples in an ice slurry has also been shown to promptly and completely stop glucose metabolism (Mikesh and Bruns 2008, Gambino *et al.*, 2009). The current data demonstrates each of the processing methods investigated are equivocal when glucose concentration is measured 10 minutes after the initial measurement.

The key discovery from this pilot study highlights the importance of the timing of glucose determination in whole blood samples. Findings from the present study demonstrate a rapid decline in glucose concentration within the first three minutes (4.0%), after which time glucose degradation stabilises. Previous work by Skirrow *et al*. determined arterialised venous-venous glucose difference to peak at 1.2 mmol/l prior to immobilisation, which then declined to peak at 0.5 mmol/l after 7 days of immobilisation (Skirrow 2014). If, for example, the glucose concentration of a venous blood sample was immediately measured to be 4.2 mmol/l and 3 minutes later the glucose concentration of an arterialised-venous blood sample, collected simultaneously to the venous blood sample, declined by 4% and now measured 4.03 mmol/l. This difference of 0.17 mmol/l in glucose concentration represents 24% of the

arterialised venous-venous glucose difference measured by Skirrow and colleagues, which may hinder the ability to accurately detect changes in muscle substrate uptake in response to an intervention, such as limb immobilisation. Therefore, if glucose concentration is to be measured in multiple samples collected simultaneously, it is imperative such measurements are made when glucose degradation has stabilised (after 3 minutes) to minimise inter-sample variance.

Appendix 2: Supplementary figures from Chapter 3

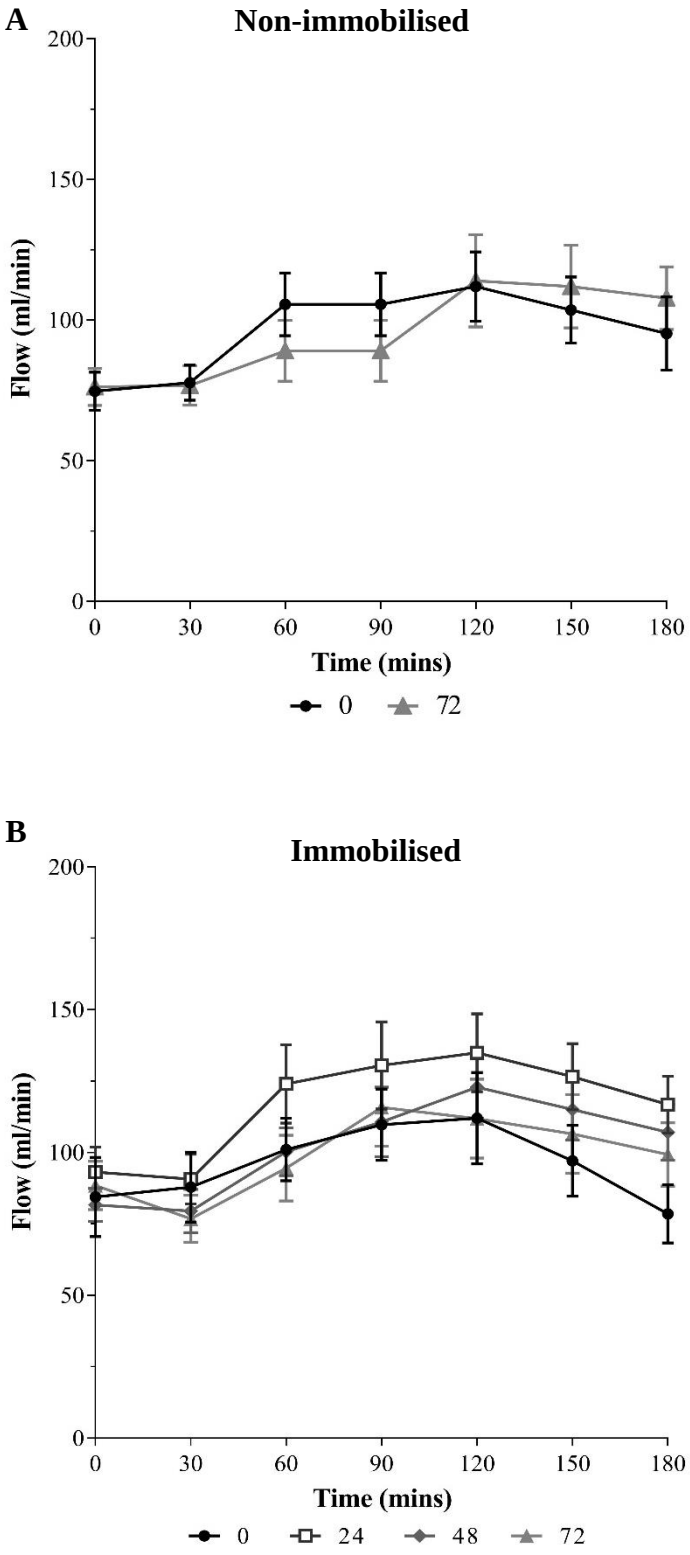


Figure 1 A and B Brachial artery blood flow following the ingestion of 75 g of glucose (t=0 min) in a non-immobilised **(A)** and immobilised **(B)** forearm before, and at 24, 48 and 72 hrs of immobilisation, values are averaged over 30 min periods. Values are mean ±SEM (n=10).

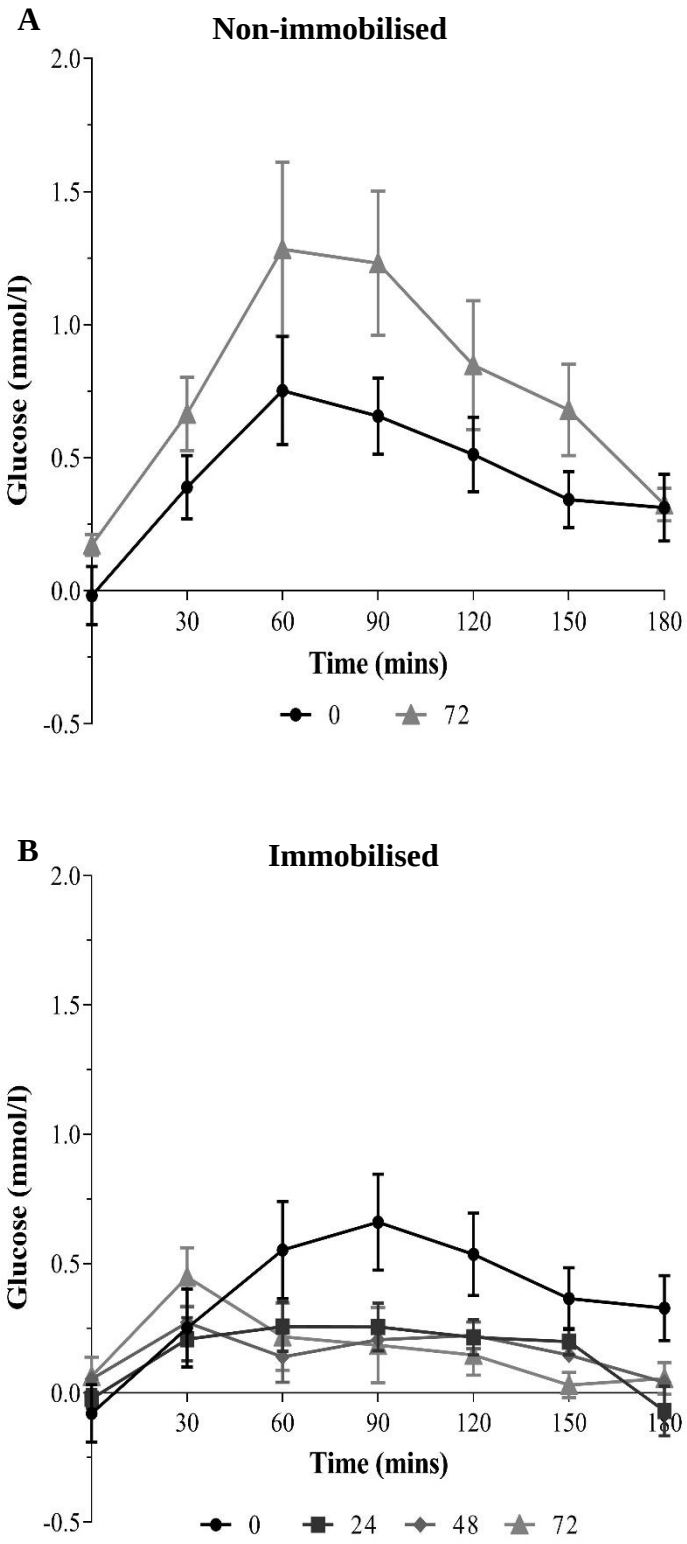


Figure 2.1 A and B Arterialised venous – venous glucose difference following the ingestion of 75 g of glucose (t=0 min) in a non-immobilised **(A)** and immobilised **(B)** forearm before (0), and at 24, 48 and 72 hrs of immobilisation, values are averaged over 30 min periods. Values are mean ±SEM (*n*=10).

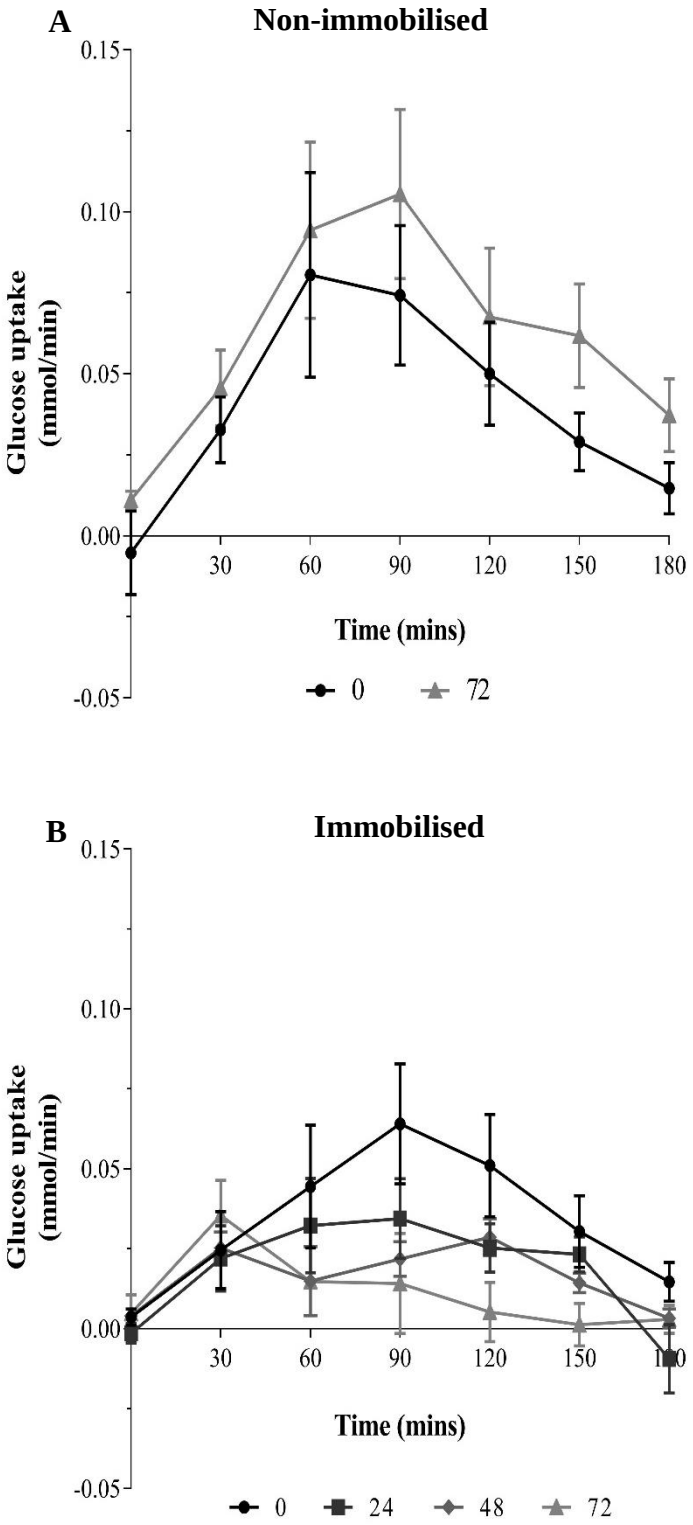


Figure 2.2 A and B Forearm glucose uptake following the ingestion of 75 g of glucose (t=0 min) in a non-immobilised **(A)** and immobilised **(B)** forearm before (0), and at 24, 48 and 72 hrs of immobilisation, values are averaged over 30 min periods. Values are mean ±SEM (*n*=10).

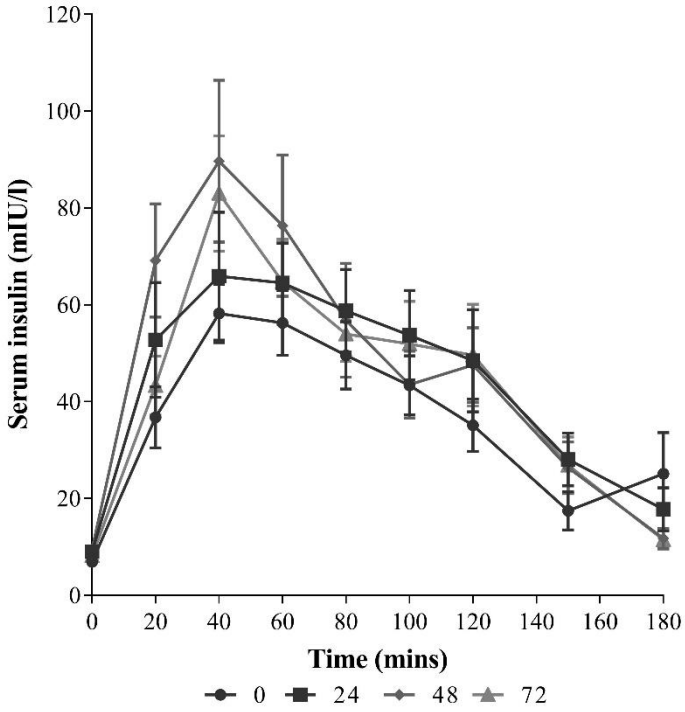


Figure 3 Arterialised venous (from the heated foot) serum insulin concentration following the ingestion of 75 g of glucose (t=0 min) before (0; circles), and at 24 (squares), 48 (diamonds) and 72 (triangles) hrs after immobilisation. Values are mean ±SEM (*n*=10).

Appendix 3: Supplementary figures from Chapter 4

1. Brachial artery blood flow

Measurements of brachial artery blood flow were performed during each mixed meal challenge at baseline (0) and 72 hrs in the NIM arm, and at baseline, 24, 48 and 72 hrs in the IMM arm, and is presented in Figure 1.1 A and B. In Figure 1.2 A and B the total area under the curve for brachial artery blood flow is shown for the 420 min mixed meal challenge. Following ingestion of a mixed meal, brachial artery blood flow was similar between arms prior to immobilisation (74.5±12.9 and 64.7±9.5 [l/min]·420min in NIM and IMM limbs respectively; P=0.95). After 72 hrs, brachial artery blood flow in the NIM arm tended to decrease from baseline (63.8±9.5[l/min]·420min; P=0.06) in the subset of volunteers studied at this time point. Similarly, brachial artery blood flow in the IMM arm also tended to decline from baseline at 24 (59.5±9.3[l/min]·420min; P=0.065) and 48 hrs (58.9±7.5[l/min]·420min; P=0.07) of immobilisation.

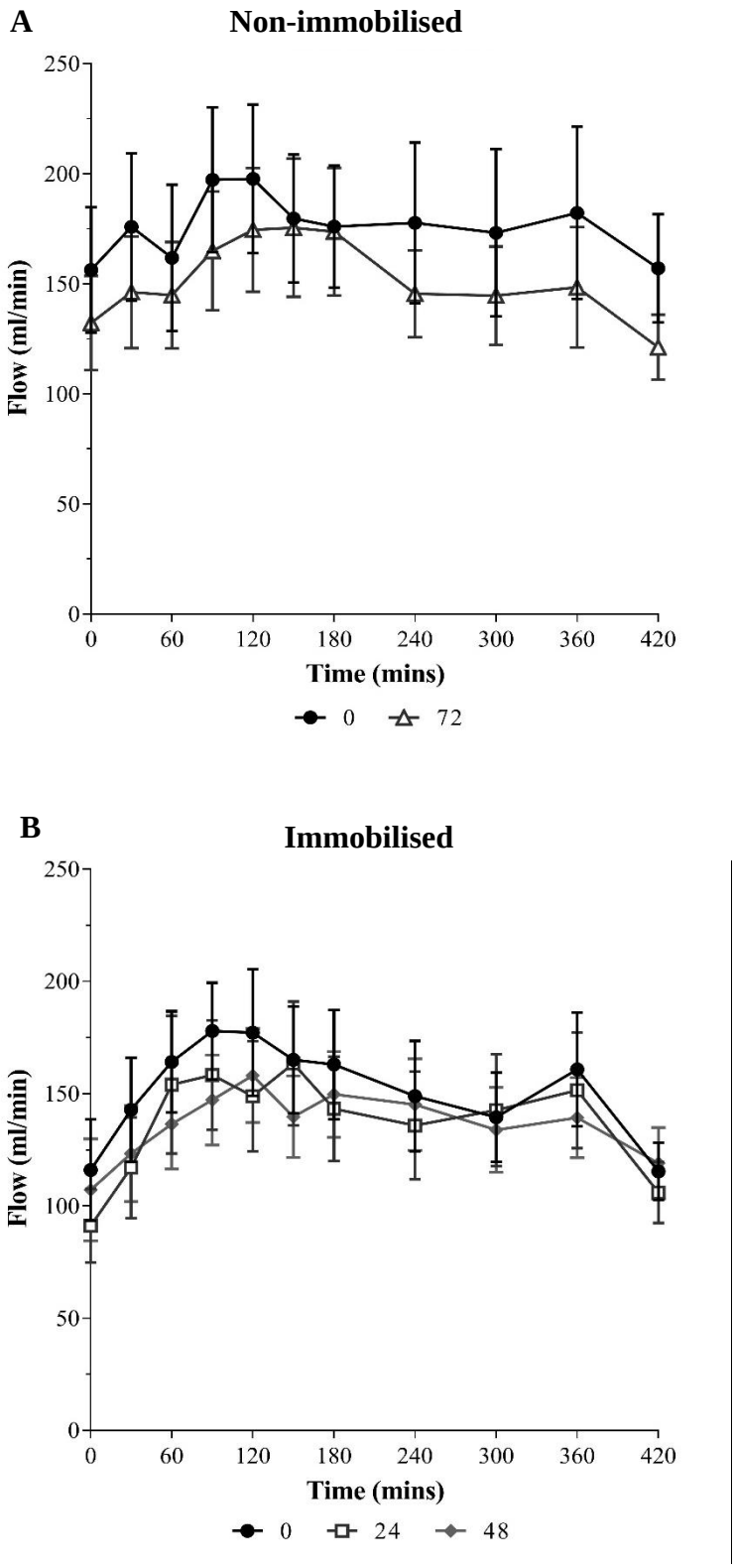


Figure 1.1 Brachial artery blood flow following the ingestion of a mixed meal (t=0 min) in a non-immobilised **(A)** and immobilised **(B)** forearm before (0; circles), and at 24 (squares), 48 (diamonds) and 72 (triangles) hrs of immobilisation, values are averaged over 30 mins. (*n*=6 and *n*=9 in non-immobilised and immobilised arms respectively).

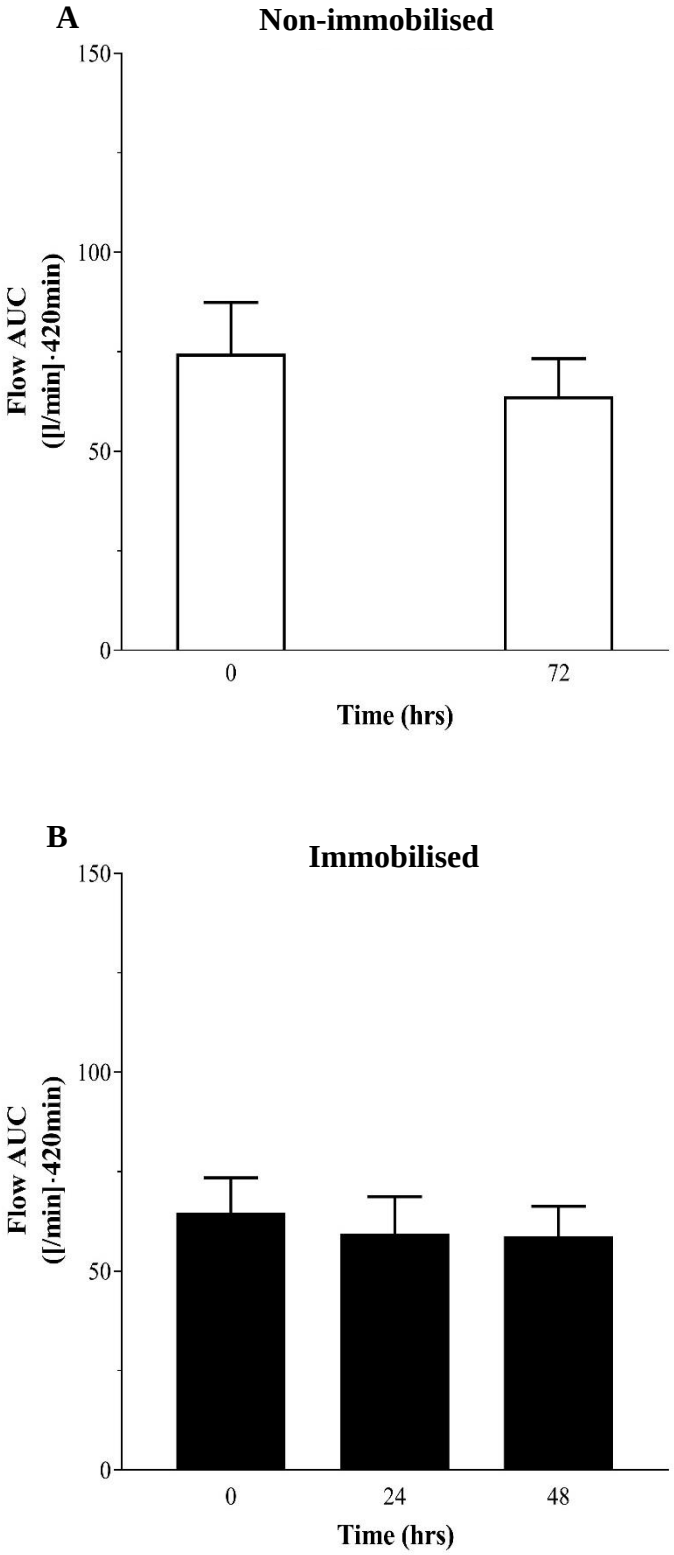


Figure 1.2 Area under the curve (AUC) of brachial artery blood flow for the total 420 min high fat mixed meal challenge was determined for a non-immobilised **(A)** and immobilised **(B)** forearm before (0), during (24, 48) and after 72 hrs of immobilisation. Values are mean ±SEM (n=6 and n=9 for non-immobilised and immobilised arms respectively).

2. Free fatty acids

2.1 Plasma free fatty acid concentrations

Figure 2.1 presents the plasma free fatty acid concentration of arterialised-venous blood sampled from a heated foot (A), venous blood sampled from a deep vein in both the immobilised (B) and non-immobilised (C) arms in response to ingestion of a high-fat mixed meal prior to (0), and during (24, 48 hrs) and after 72 hrs of forearm immobilisation.

2.2 Free fatty acid arteriovenous-venous difference

The arterialised venous-venous difference for free fatty acid concentrations in both the NIM and IMM limbs during each 420 min mixed meal challenge is shown in Figure 2.2 (A and B). Figures 2.3 A and B presents the total area under the curve of venous-venous free fatty acid arterialised difference in the NIM and IMM limbs for each 420 min mixed meal challenge. The arterialised venous-venous free fatty acid difference was comparable between the NIM and IMM arm at baseline (20.9±5.35 and 14.9±3.1 [mmol/l] ·420min; P=0.24 respectively). No change in arterialised venous-venous free fatty acid difference in NIM arm was detected in the group of subjects measured following 72 hrs (14.3±2.7 [mmol/l] ·420min; P=0.29). Similarly, no alteration in arterialised venous-venous free fatty acid difference was detected in the IMM arm at 24 and 48 hrs of immobilisation (16.4±2.9 and 16.1±1.7 [mmol/l] ·420min; P=0.81 and P=0.88 at 24 and 48 hrs respectively).

2.3 Free fatty acid uptake

Figure 2.4 A and B presents free fatty acid uptake in response to ingestion of a mixed meal in the NIM arm at baseline (0) and 72 hrs, and in the IMM arm at baseline and at 24 and 48 hrs of immobilisation.

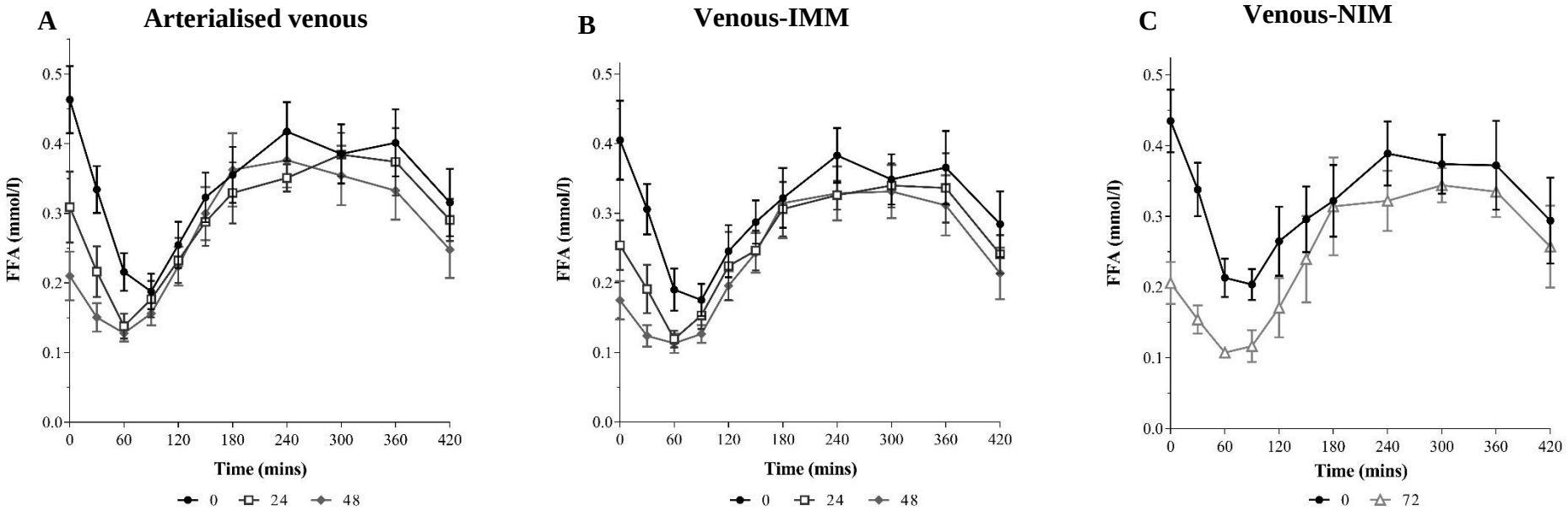


Figure 2.1 Free fatty acid concentration in arterialised venous blood samples from a heated foot (Arterialised venous; **A**) and venous blood samples from an immobilised (Venous-IMM; **B**) and non-immobilised (Venous-NIM; **C**) arm in response to high-fat meal ingestion, before (0), and at 24, 48 and 72 hrs of forearm immobilisation. Values are mean ±SEM (*n*=9 in Arterialised venous and Venous-IMM respectively, and *n*=6 in Venous-NIM).

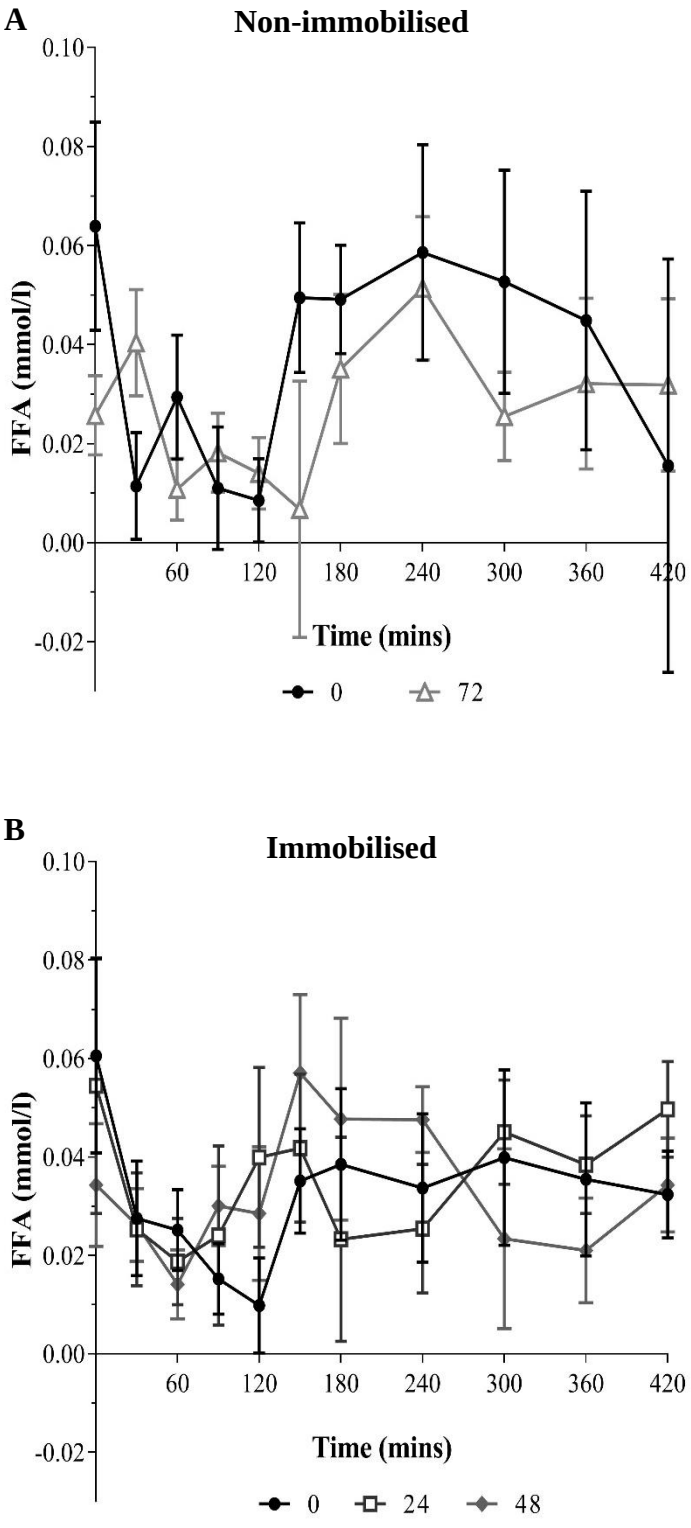


Figure 2.2 Arteriovenous – venous free fatty acid (FFA) difference following the ingestion of a mixed meal (t=0 min) in a non-immobilised **(A)** and immobilised **(B)** forearm before (0; circles), and at 24 (squares), 48 (triangles) and 72 (diamonds) hrs of immobilisation. Values are mean ±SEM (*n*=6 and *n*=9 in the non-immobilised and immobilised arms respectively).

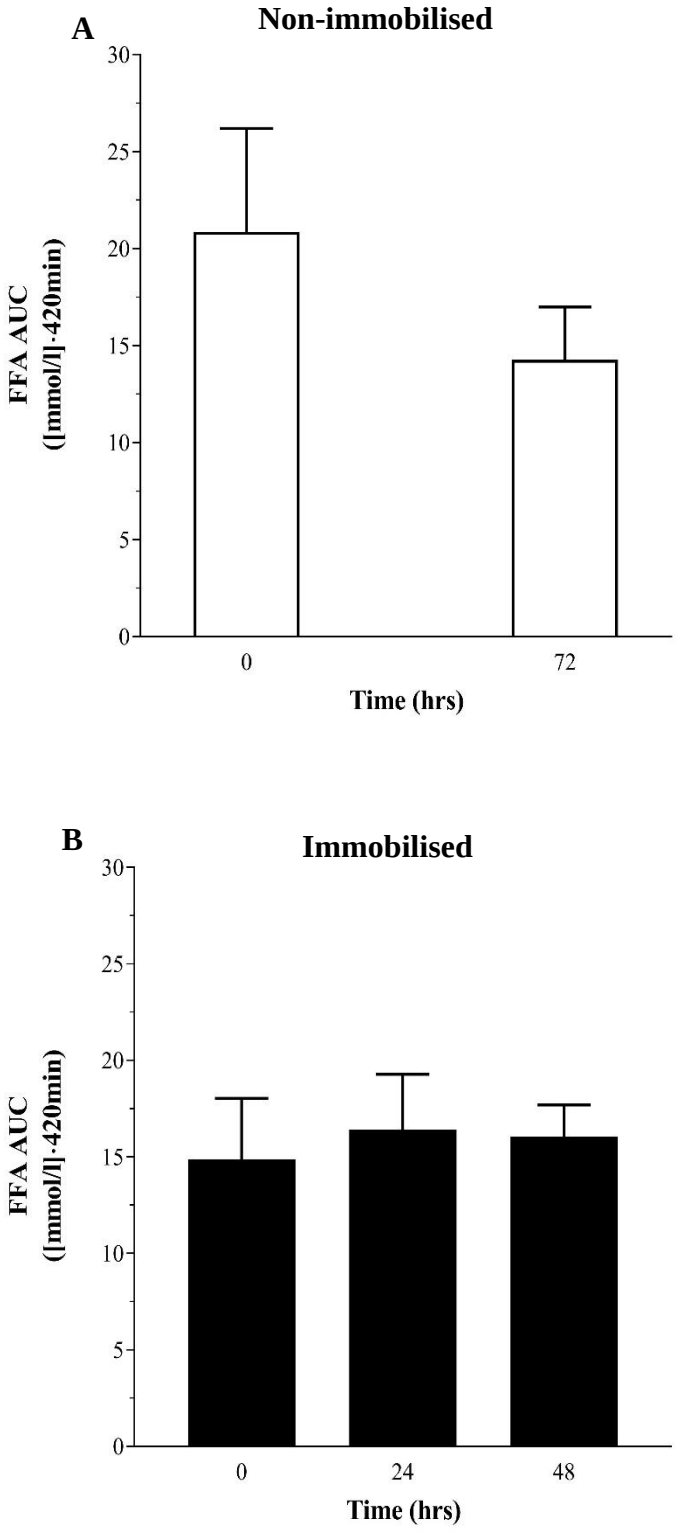


Figure 2.3 Area under the curve (AUC) of arteriovenous-venous free fatty acid (FFA) difference for the total 420 min high fat mixed meal challenge was determined for a non-immobilised **(A)** and immobilised **(B)** forearm before (0), during (24, 48) and after 72 hrs of immobilisation. Values are mean ±SEM (*n*=6 and *n*=9 in the non-immobilised and immobilised arms respectively).

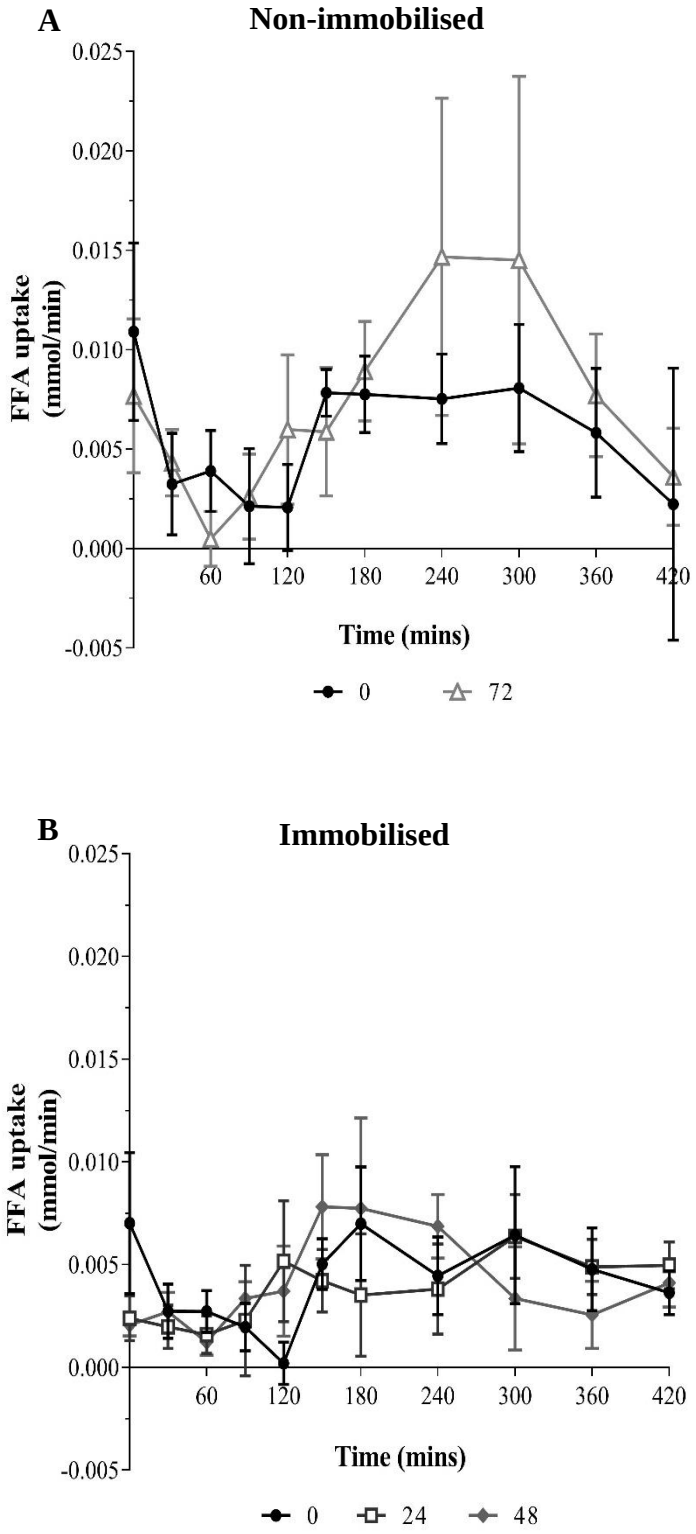


Figure 2.4 Free fatty acid (FFA) uptake following the ingestion of a mixed meal (t=0 min) in a non-immobilised **(A)** and immobilised **(B)** forearm before (0; circles), and at 24 (squares), 48 (triangles) and 72 (diamonds) hrs of immobilisation. Values are mean ±SEM (*n*=6 and *n*=9 in the non-immobilised and immobilised arms respectively).

3. Triacylglycerol

3.1 Serum triacylglycerol concentrations

Figure 3.1 displays the serum triacylglycerol concentration of arterialised-venous blood sampled from a heated foot (A), venous blood sampled from a deep vein in both the immobilised (B) and non-immobilised (C) arms in response to ingestion of a high-fat mixed meal prior to (0), and during (24, 48 hrs) and after 72 hrs of forearm immobilisation.

3.2 Triacylglycerol arteriovenous-venous difference

Arterialised venous-venous difference for triacylglycerol in both the NIM and IMM limbs during each 420 min mixed meal challenge is shown in Figure 3.2 (A and B). The total area under the curve for triacylglycerol arterialised venous-venous difference during each 420 min mixed meal challenge in the NIM and IMM arms is displayed in Figure 3.3 A and B. There was no difference in triacylglycerol arterialised venous-venous difference at baseline between the NIM (25.1±3.8 [mmol/l] ·420min) and IMM arms (19.8±2.3 [mmol/l] ·420min; P=0.09). The triacylglycerol arterialised venous-venous difference did not change from baseline in the NIM arm at 72 hrs (21.9±2.7 [mmol/l] ·420min; P=0.65) or in the IMM arm at 24 and 48 hrs (21.2±4.3 and 34.2±7.4 [mmol/l] ·420min; P=0.73 and P=0.15 respectively).

3.3 Triacylglycerol clearance

Triacylglycerol clearance in response to ingestion of a mixed meal in the NIM arm at baseline (0) and 72 hrs, and in the IMM arm at baseline and at 24 and 48 hrs of immobilisation is shown in Figure 3.4 A and B.

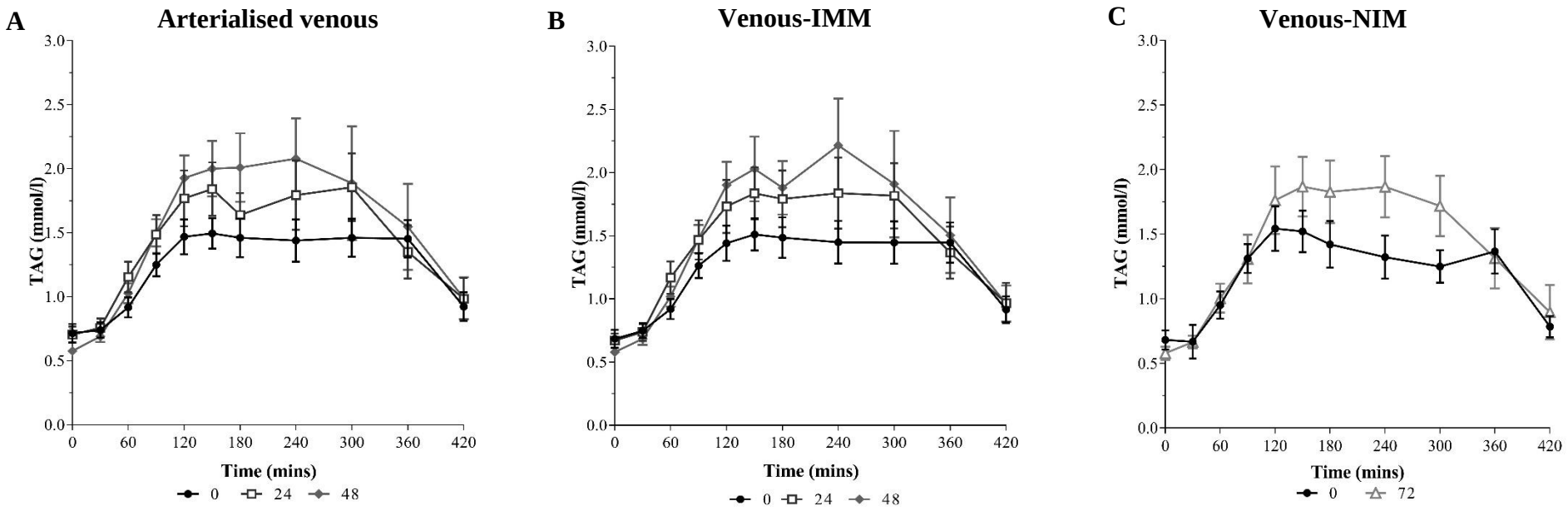


Figure 3.1 Triacylglycerol concentration in arterialised venous blood samples from a heated foot (Arterialised venous; **A**) and venous blood samples from an immobilised (Venous-IMM; **B**) and non-immobilised (Venous-NIM; **C**) arm in response to high-fat meal ingestion, before (0), and at 24, 48 and 72 hrs of forearm immobilisation. Values are mean ±SEM (*n*=9 in Arterialised venous and Venous-IMM respectively, and *n*=6 in Venous-NIM).

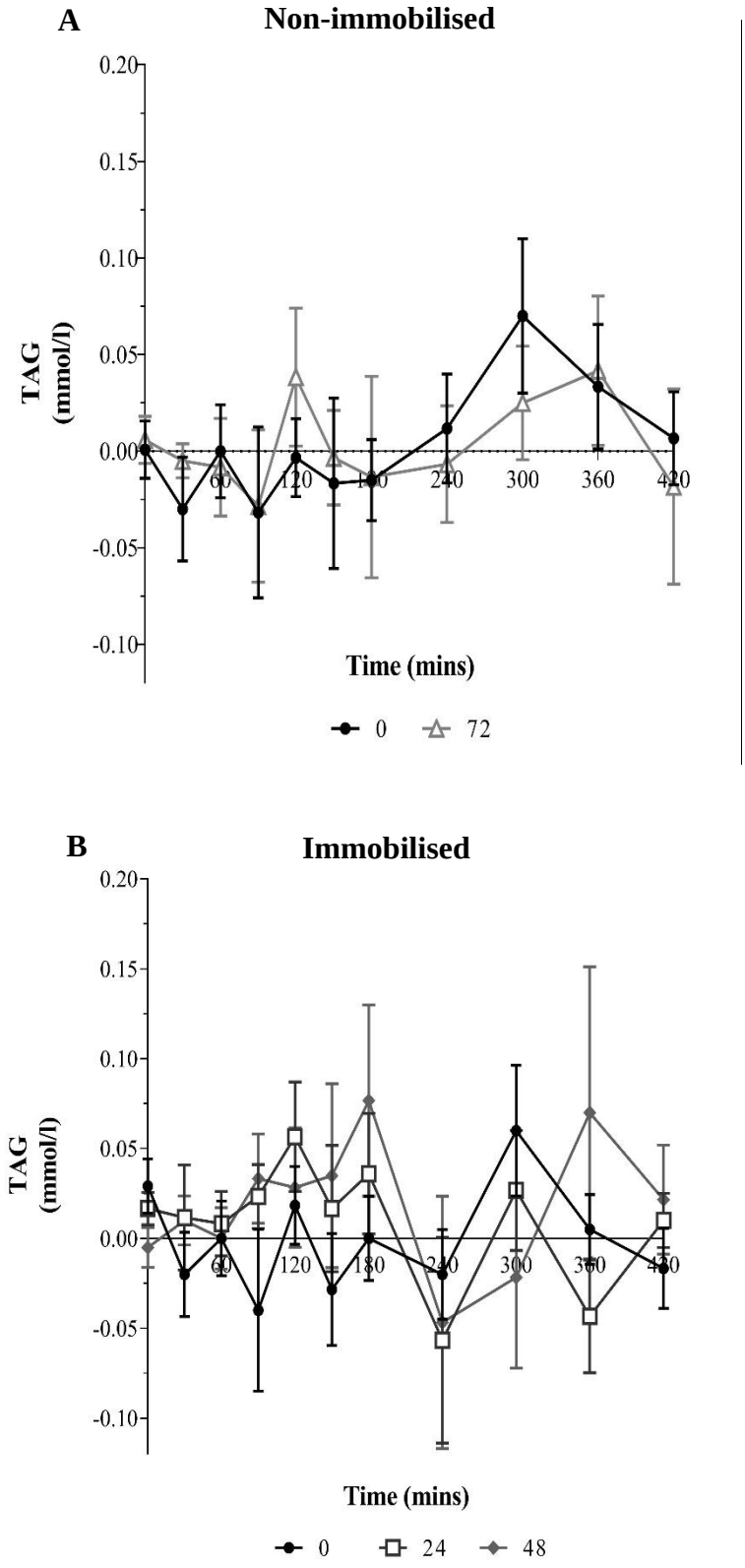


Figure 3.2 Triacylglycerol (TAG) arterialised venous – venous difference following the ingestion of a mixed meal (t=0 min) in a non-immobilised **(A)** and immobilised **(B)** forearm before (0; circles), and at 24 (squares), 48 (triangles) and 72 (diamonds) hrs of immobilisation. Values are mean ±SEM (*n*=6 and *n*=9 in the non-immobilised and immobilised arms respectively).

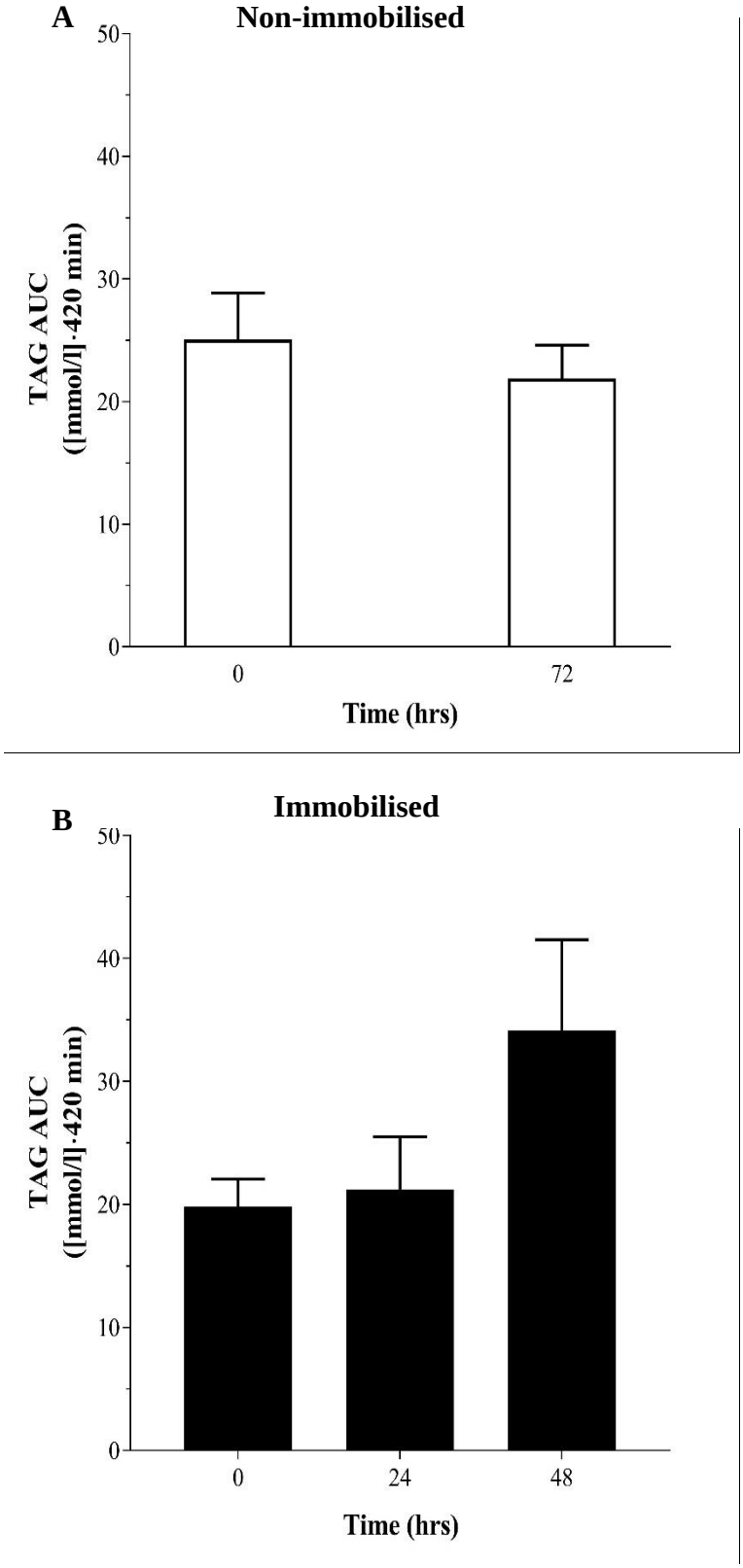


Figure 3.3 Area under the curve (AUC) of triacylglycerol (TAG) arteriovenous-venous difference for the total 420 min high fat mixed meal challenge was determined for a non-immobilised **(A)** and immobilised **(B)** forearm before (0), during (24, 48) and after 72 hrs of immobilisation. Values are mean ±SEM (*n*=6 and *n*=9 in NIM and IMM respectively).

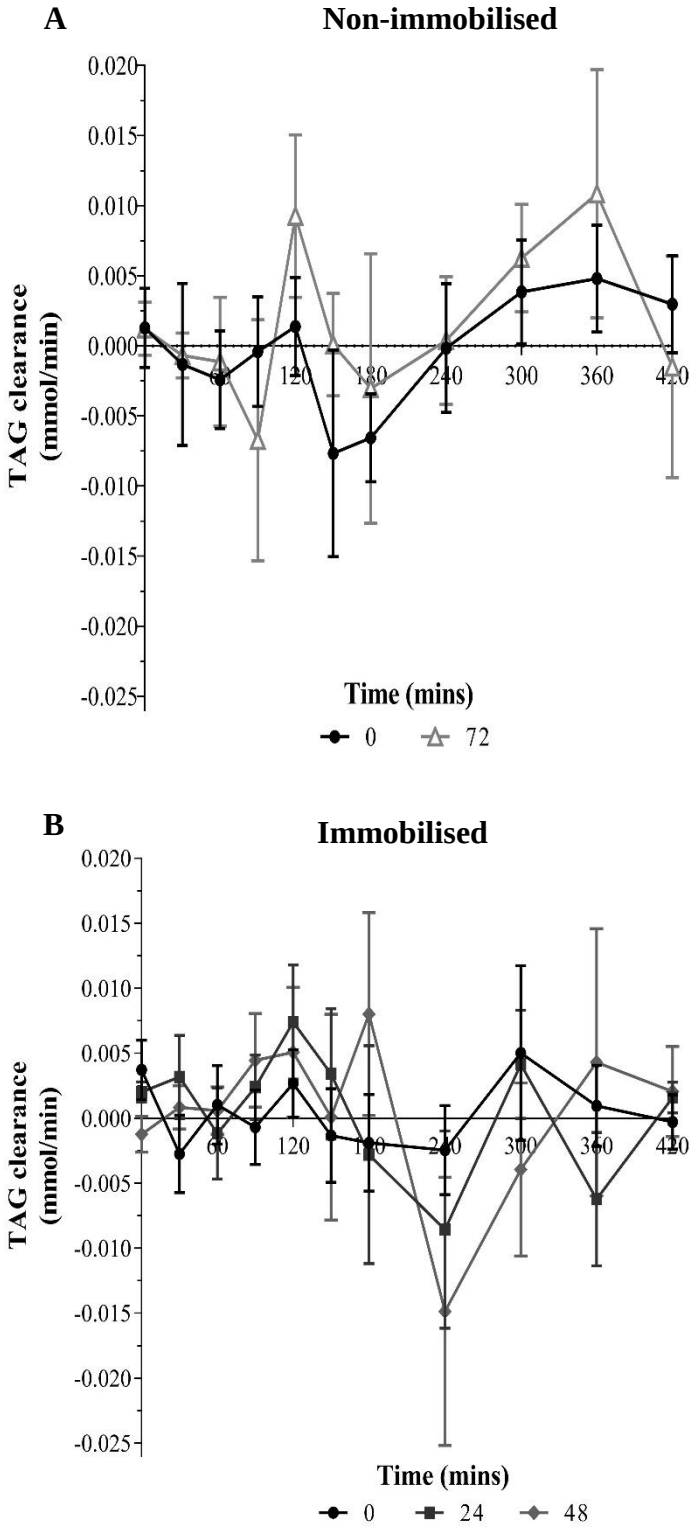


Figure 3.4 Triacylglycerol (TAG) clearance following the ingestion of a mixed meal (t=0 min) in a non-immobilised **(A)** and immobilised **(B)** forearm before (0; circles), and at 24 (squares), 48 (triangles) and 72 (diamonds) hrs of immobilisation. Values are mean ±SEM (*n*=6 and *n*=9 in the non-immobilised and immobilised arms respectively).

4. Free glycerol

4.1 Serum free glycerol concentrations

Figure 4.1 depicts the serum free glycerol concentration of arterialised-venous blood sampled from a heated foot (A), venous blood sampled from a deep vein in both the immobilised (B) and non-immobilised (C) arms in response to ingestion of a high-fat mixed meal prior to (0), and during (24, 48 hrs) and after 72 hrs of forearm immobilisation.

4.2 Free glycerol arterialised venous-venous difference

The arterialised venous-venous difference for free glycerol in both the NIM and IMM limbs during each 420 min mixed meal challenge is shown in Figure 4.2 (A and B). The total area under the curve for free glycerol arterialised venous-venous difference during each 420 min mixed meal challenge at pre- and post-immobilisation (0 and 72 hrs) in the NIM arm and at 0, 24 and 48 hrs in the IMM arm are shown in Figures 4.3 A and B respectively. The free glycerol arterialised venous-venous difference was similar between arms prior to immobilisation (639.0±175.5 and 836.7±139.8 [mmol/l] ·420min in the NIM and IMM arms respectively; P=0.77), and showed no significant change after 72 hrs in the NIM arm (1772.0±817.3 [mmol/l] ·420min; P=0.28). Similarly, the free glycerol arterialised venous-venous difference in the IMM arm did not change from baseline after 24 (9818±230.5 [mmol/l] ·420min; P=0.67) or 48 hrs (1368.0 ±261.0 [mmol/l] ·420min; P=0.13) of immobilisation.

4.3 Free glycerol clearance

Figure 4.4 A and B depicts free glycerol clearance in response to mixed meal ingestion in the NIM arm at baseline (0) and 72 hrs, and in the IMM arm at baseline and at 24 and 48 hrs of immobilisation.

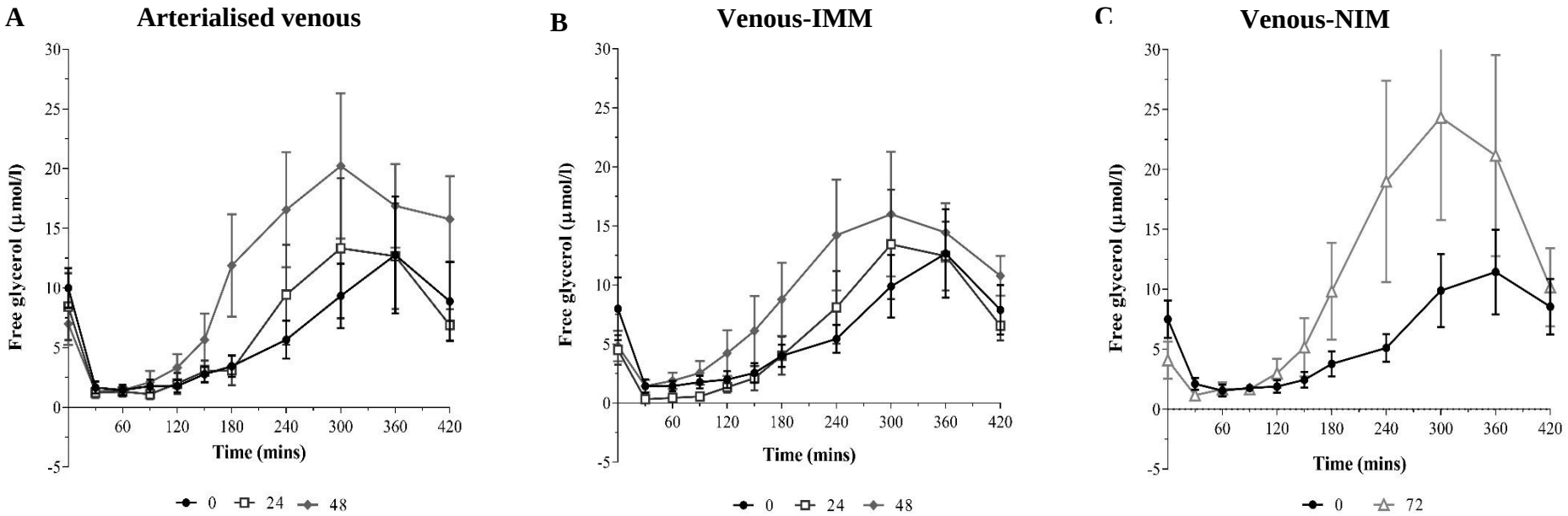


Figure 4.1 Free glycerol concentration in arterialised venous blood samples from a heated foot (Arterialised venous; **A**) and venous blood samples from an immobilised (Venous-IMM; **B**) and non-immobilised (Venous-NIM; **C**) arm in response to high-fat meal ingestion, before (0), and at 24, 48 and 72 hrs of forearm immobilisation. Values are mean ±SEM (*n*=9 in Arterialised venous and Venous-IMM respectively, and *n*=6 in Venous-NIM).

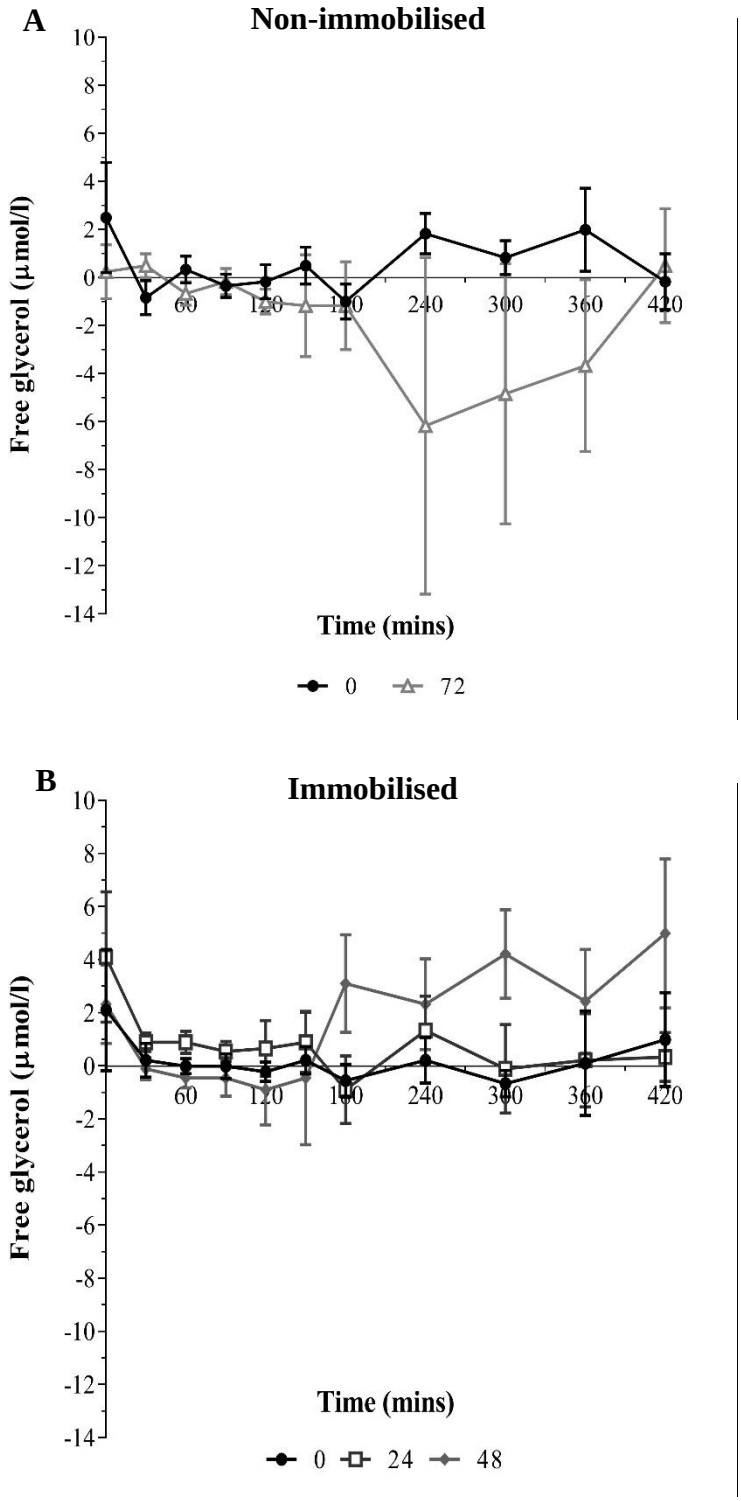


Figure 4.2 Free glycerol arteriovenous – venous difference following the ingestion of a mixed meal (t=0 min) in a non-immobilised **(A)** and immobilised **(B)** forearm before (0; circles), and at 24 (squares), 48 (triangles) and 72 (diamonds) hrs of immobilisation. Values are mean ±SEM (*n*=6 and *n*=9 in the non-immobilised and immobilised arms respectively)

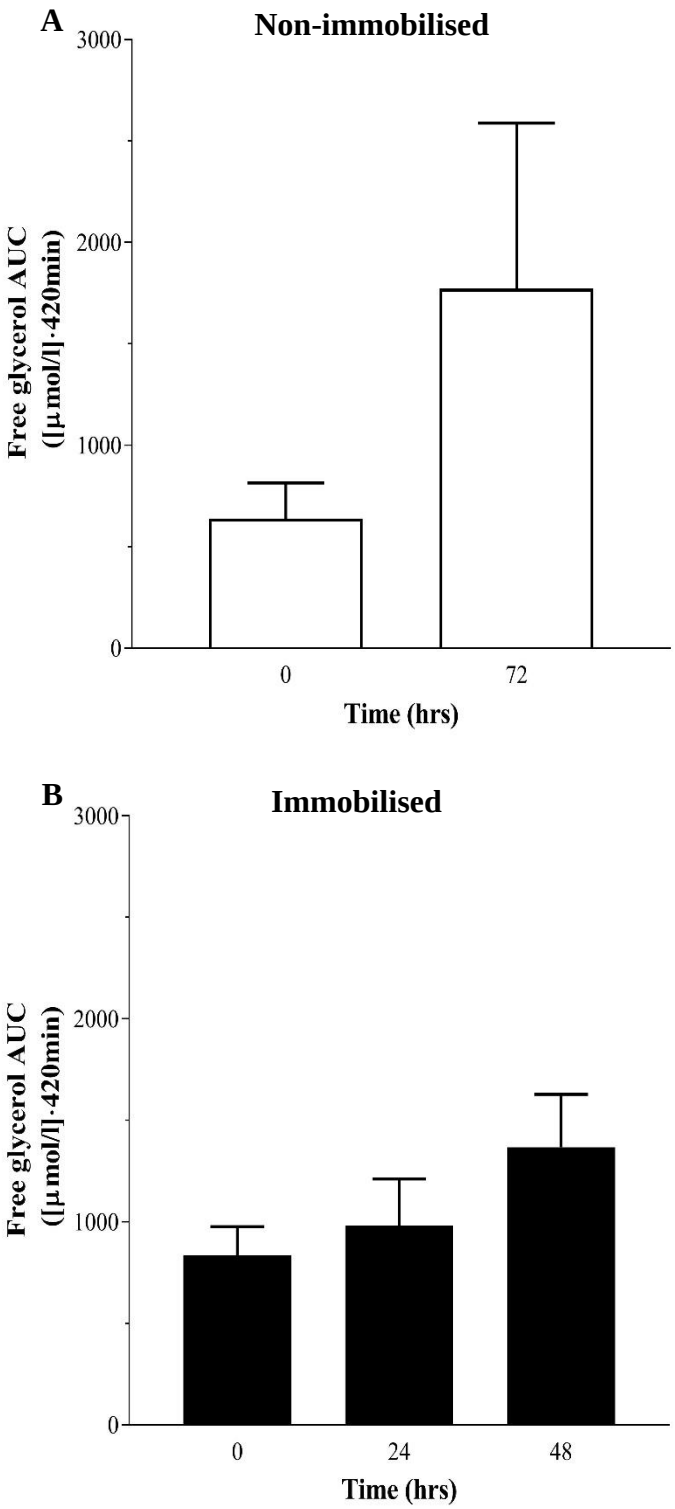


Figure 4.3 Area under the curve (AUC) of free glycerol arteriovenous-venous difference for the total 420 min high fat mixed meal challenge was determined for a non-immobilised **(A)** and immobilised **(B)** forearm before (0), during (24, 48) and after 72 hrs of immobilisation. Values are mean ±SEM (*n*=6 and *n*=9 in NIM and IMM respectively).

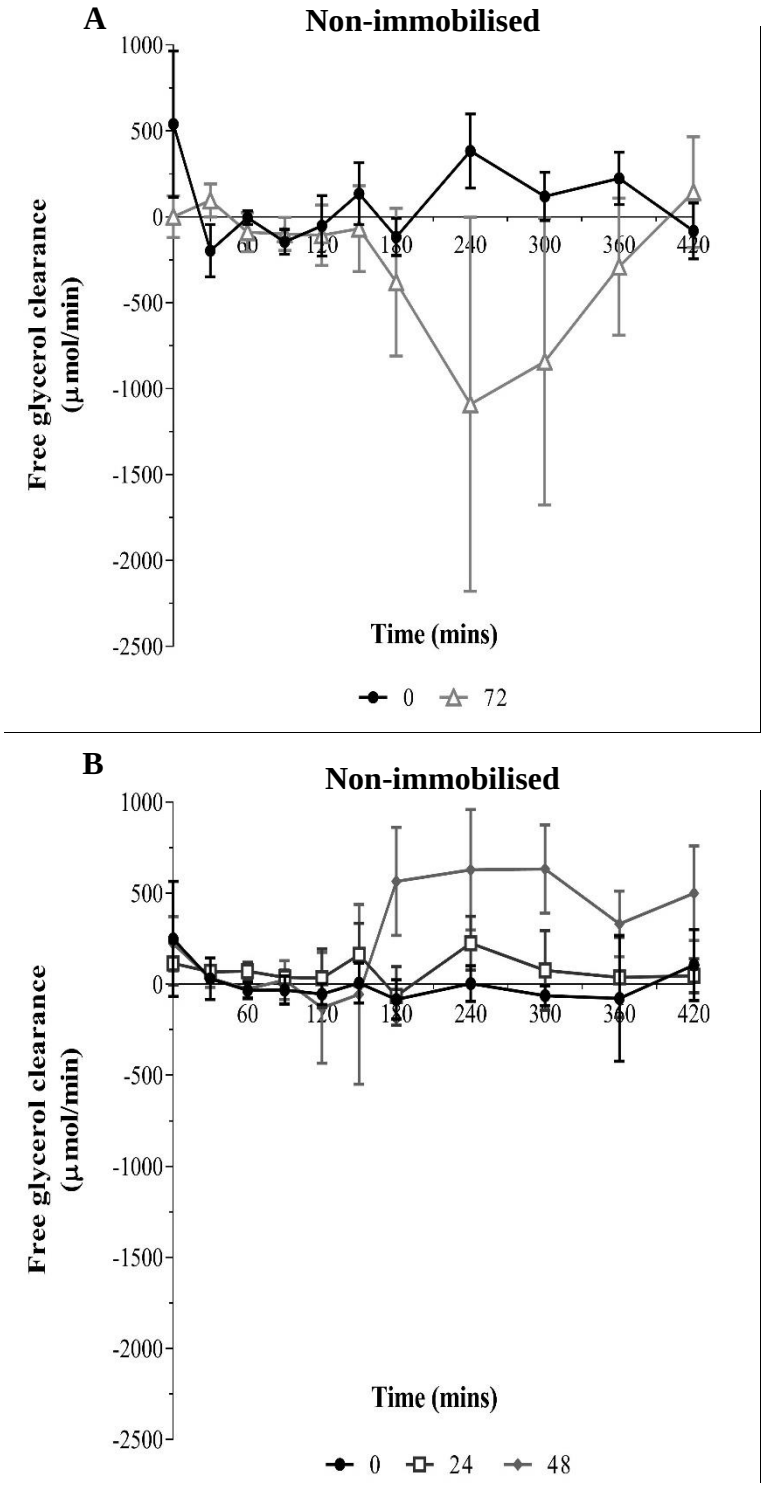


Figure 4.4 Free glycerol clearance following the ingestion of a mixed meal (t=0 min) in a non-immobilised **(A)** and immobilised **(B)** forearm before (0; circles), and at 24 (squares), 48 (triangles) and 72 (diamonds) hrs of immobilisation. Values are mean ±SEM (*n*=6 and *n*=9 in the non-immobilised and immobilised arms respectively).

5. Glucose

5.1 Whole blood glucose concentrations

Figure 5.1 presents the glucose concentration of arterialised-venous blood sampled from a heated foot (A), venous blood sampled from a deep vein in both the immobilised (B) and non-immobilised (C) arms in response to ingestion of a high-fat mixed meal prior to (0), and during (24, 48 hrs) and after 72 hrs of forearm immobilisation.

5.2 Arterialised venous-venous glucose difference

The arterialised venous-venous glucose difference in both the NIM and IMM limbs is shown in Figure 5.2 (A and B). Figure 5.3 A and B depict the total area under the curve of arterialised venous-venous glucose difference for the 420 min mixed meal challenge in both the NIM and IMM limbs respectively. The glucose arterialised venous-venous difference between arms was not different between arms at baseline (123.2±23.2 and 107.8±25 [mmol/l] ·420min for the NIM and IMM limbs respectively; P=0.67). In the NIM arm there was no change from baseline in arterialised venous-venous glucose difference (107.7±23.3 [mmol/l] ·420min; P=0.24) after 72 hrs, as measured in 6 subjects. In the IMM arm, arterialised venous-venous glucose difference decreased from baseline at 24 (63.2±10.8 [mmol/l] ·420min; $P<0.05$) and 48 hrs (54.3±8.6 [mmol/l] ·420min; $P<0.05$) of-immobilisation.

5.3 Forearm glucose uptake

Figure 5.4 A and B presents forearm glucose uptake in response to ingestion of a high-fat meal, measured in both a non-immobilised and immobilised arm prior to (0) and after 24, 48 and 72 hrs of forearm immobilisation.

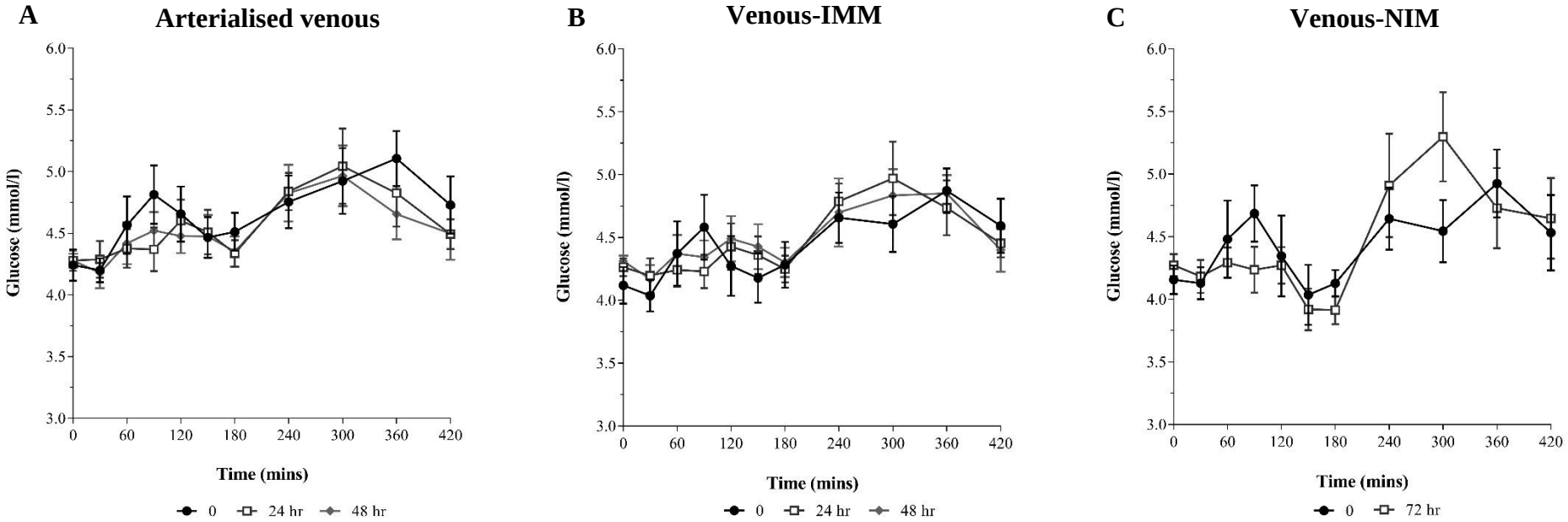


Figure 5.1 Whole blood glucose concentration in arterialised venous blood samples from a heated foot (Arterialised venous; **A**) and venous blood samples from an immobilised (Venous-IMM; **B**) and non-immobilised (Venous-NIM; **C**) arm in response to high-fat meal ingestion, before (0), and at 24, 48 and 72 hrs of forearm immobilisation. Values are mean ±SEM (*n*=9 in Arterialised venous and Venous-IMM respectively, and *n*=6 in Venous-NIM).

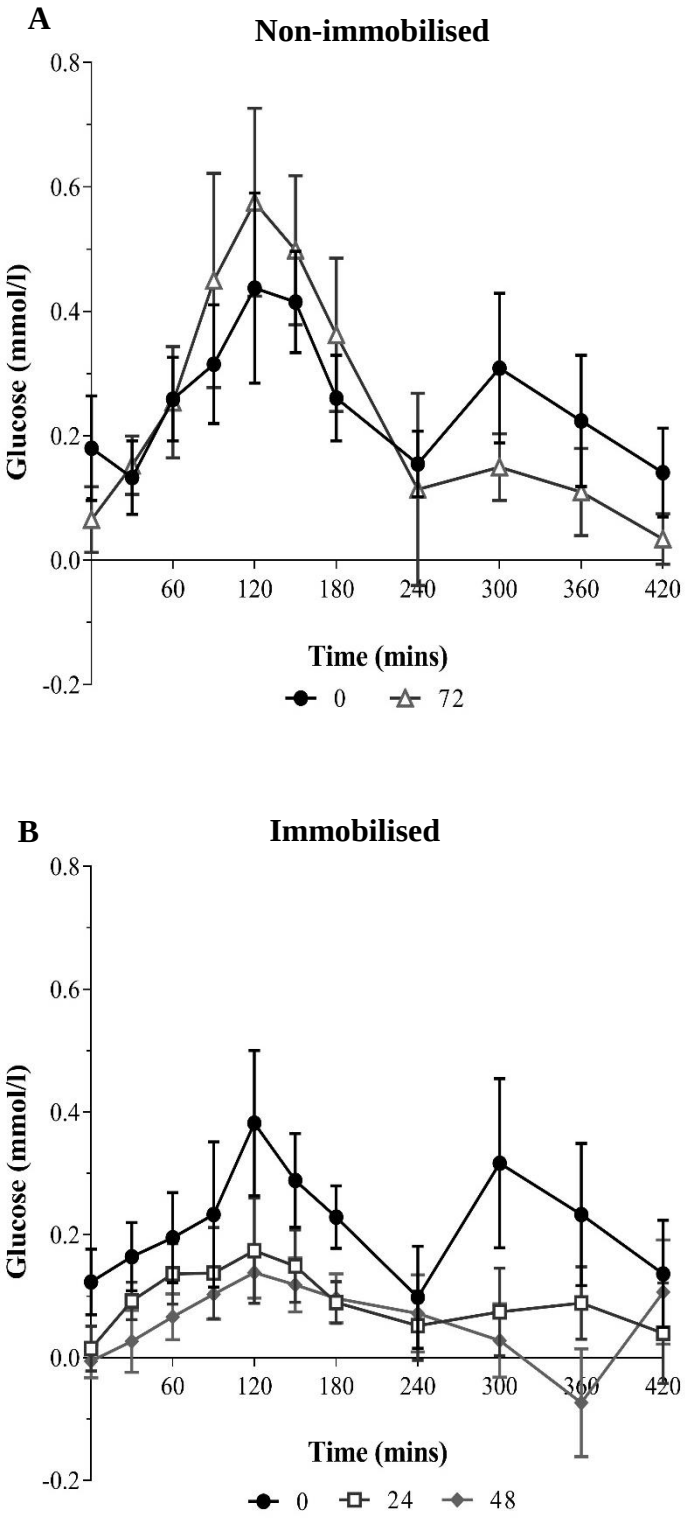


Figure 5.2 A and B Arterialised venous – venous glucose difference following the ingestion of high fat mixed meal (t=0 min) in a non-immobilised **(A)** and immobilised **(B)** forearm before (0; circles), and at 24 (squares), 48 (diamonds) and 72 (triangles) hrs of immobilisation, values are averaged over 30 min periods. Values are mean ±SEM (*n*=6 and n=9 in non-immobilised and immobilised arms respectively).

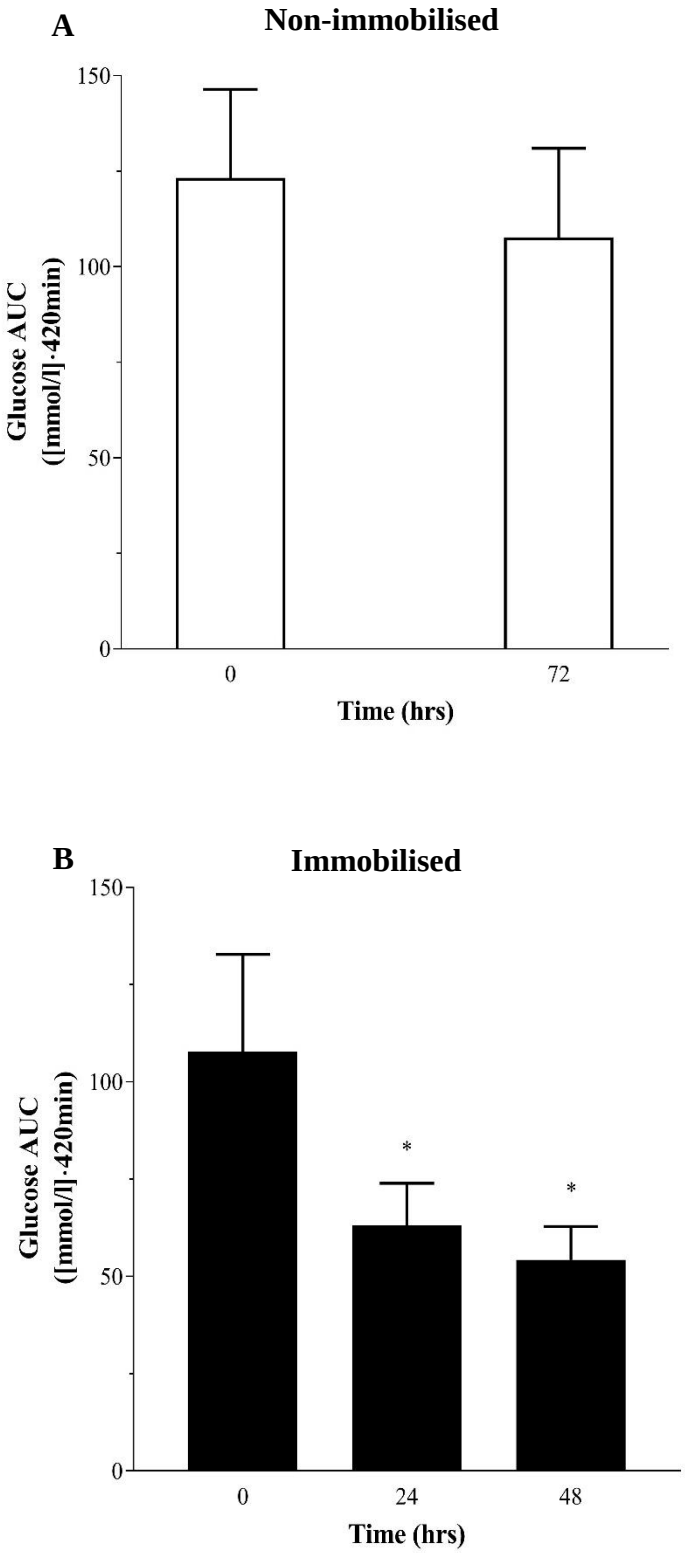


Figure 5.3 Area under the curve (AUC) of arterialised venous-venous glucose difference for the total 420 min high fat mixed meal challenge was determined for a non-immobilised **(A)** and immobilised **(B)** forearm before (0), during (24, 48) and after 72 hrs of immobilisation. Values are mean ±SEM (*n*=6 and *n*=9 in the non-immobilised and immobilised arms respectively). *P<0.05 significantly different from corresponding baseline value.

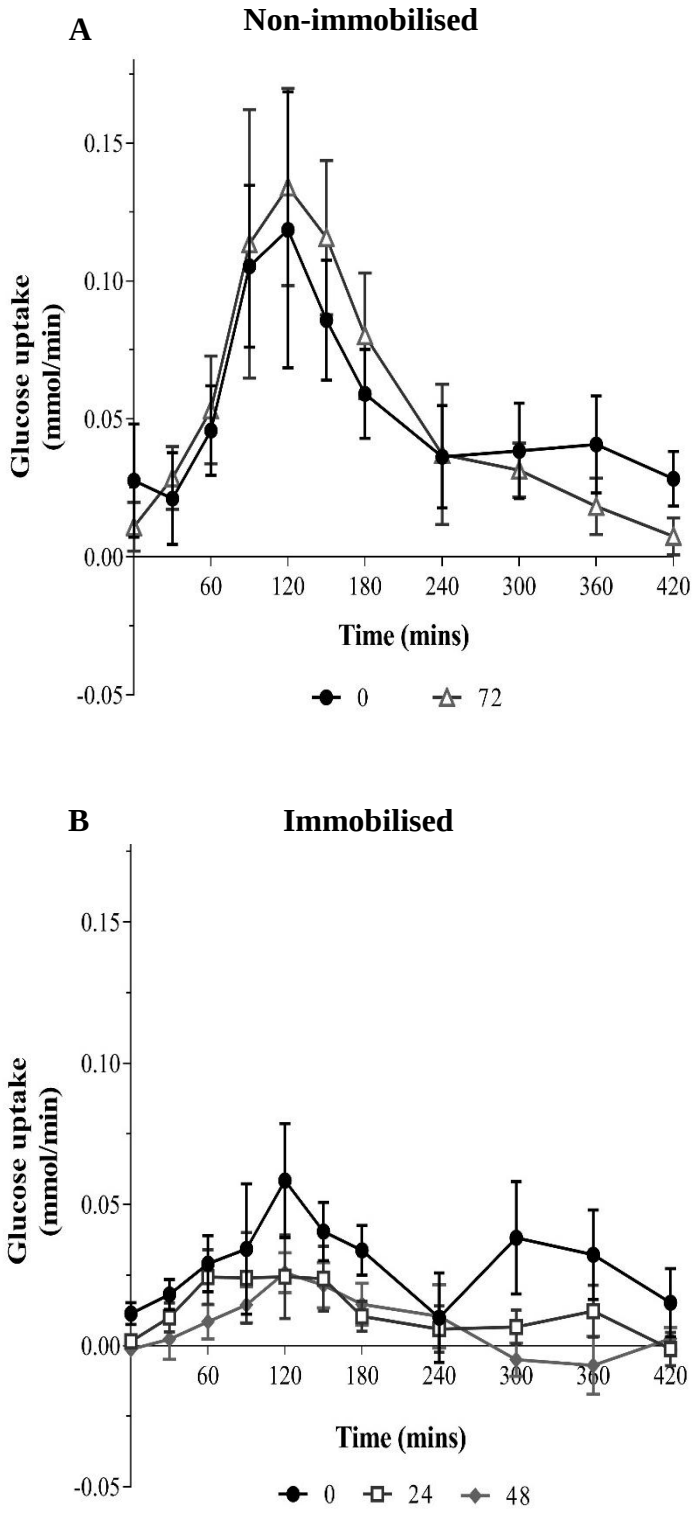


Figure 5.4 Forearm glucose uptake following the ingestion of a mixed meal (t=0 min) in a non-immobilised **(A)** and immobilised **(B)** forearm before (0; circles), and at 24 (squares), 48 (diamonds) and 72 (triangles) hrs of immobilisation, values averaged over 30 min (*n*=6 and *n*=9 in non-immobilised and immobilised arms respectively).

6. Serum insulin

Serum insulin concentration measured in arterialised venous blood at baseline (0) 24 and 48 hrs of immobilisation is presented in Figure 6.1. The total area under the curve for arterialised-venous serum insulin concentration, collected from a heated foot, for the 420 min mixed meal challenge is presented in Figure 6.2. Serum insulin concentration was not different from baseline (10295±953 [mIU/l]·420min) following 24 (10595±951 [mIU/l]·420min; P=0.94) or 48 hrs (10653±797 [mIU/l]·420min; P=0.91) of immobilisation.

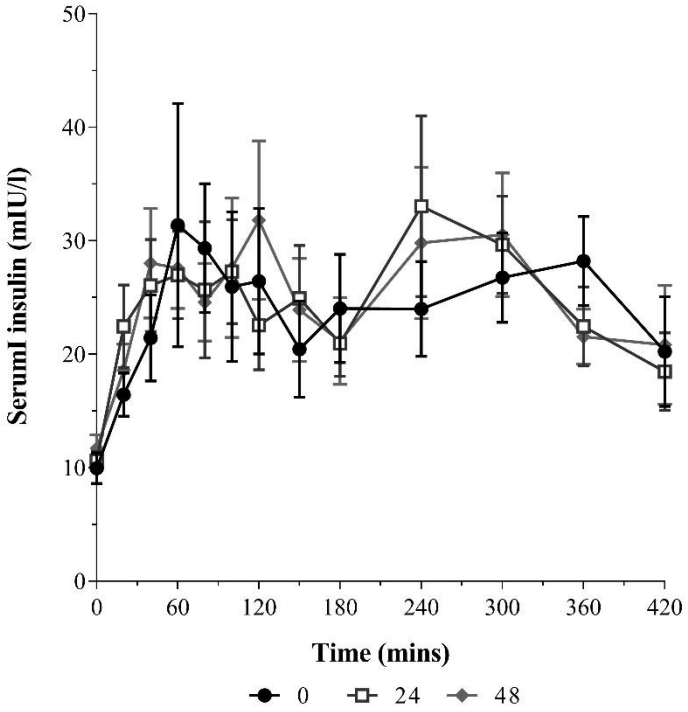


Figure 6.1 Arterialised venous (from the heated foot) serum insulin concentration following the ingestion of a mixed meal (t=0 min) before (0; circles), and at 24 (squares) and 48 (diamonds) hrs after immobilisation. Values are mean ±SEM (*n*=9).

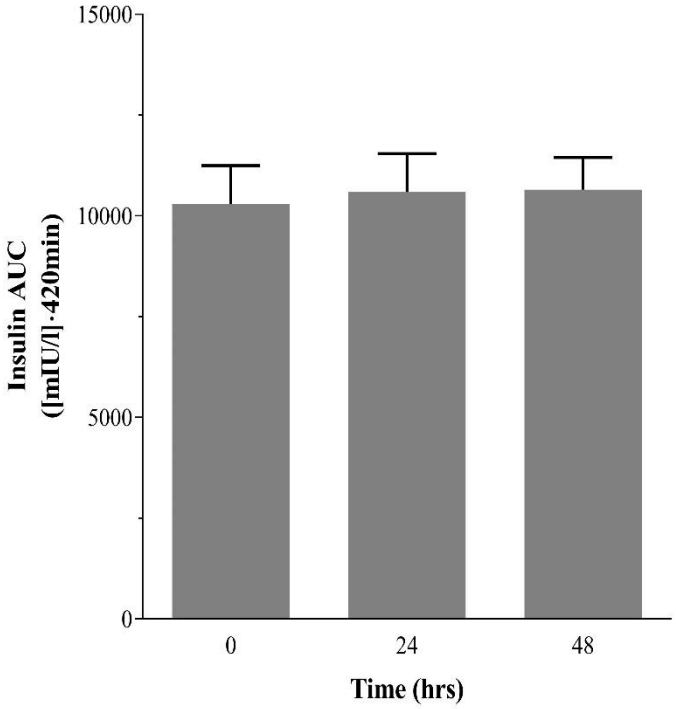


Figure 6.2 Area under the curve (AUC) of arterialised venous serum insulin concentration for the total 420 min high fat mixed meal challenge, collected from a heated foot. Values are mean ±SEM (*n*=9).

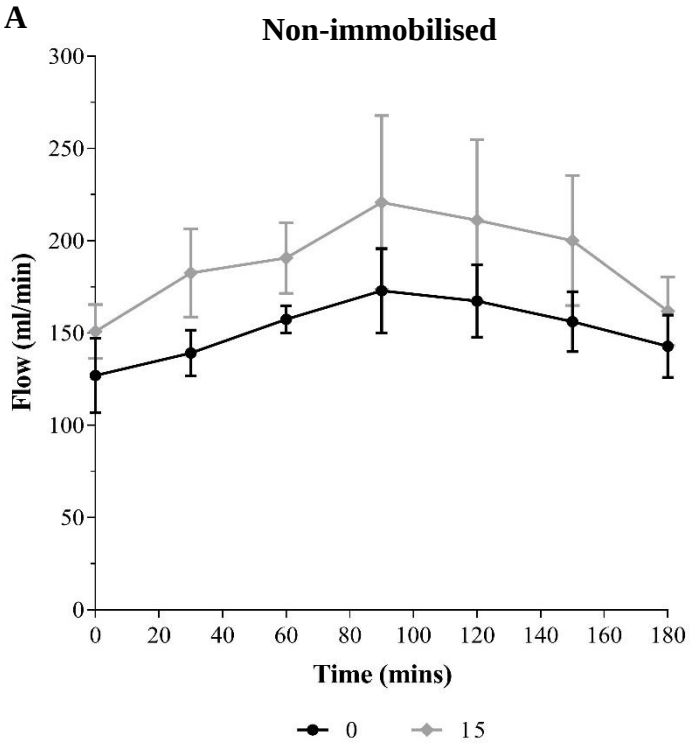
Appendix 4: Supplementary figures from Chapter 5

1. Brachial artery blood flow

Brachial artery blood flow averaged over consecutive 30 min periods during each 180 min oral glucose challenge is shown in Figure 1.1 A for the non-immobilised limb and Figure 1.1 B for the immobilised limb. The total area under the curve for brachial artery blood flow over the whole 180 min is displayed in Figure 1.2. Total area under the curve for brachial artery blood flow was no different between arms at prior to immobilisation (28.1±2.5 and 28.8±3.7 [l/min]·180min in the NIM and IMM arms respectively; P=0.84) and showed no statistically significant change from pre-immobilisation values in the NIM limb following 15 days of immobilisation (35.1±5.4 [l/min]·180min; P=0.24). There was no difference in brachial artery blood flow from baseline in the IMM limb at day 1 (30.2 [l/min]·180min; P=0.99), 3 (25.2±2.6 [l/min]·180min; P=0.76), 8 (28.2±2.5 l/min/180min; P=0.99) and 15 (33±4.7 [l/min]·180min; P=0.85). At the end of the immobilisation period brachial artery blood flow was not different between arms (P=0.35).

2. Arterialised-venous serum insulin concentration

Figure 2.1 displays arterialised-venous serum insulin concentration during each 180 min oral glucose challenge prior to immobilisation (0), and on days 1, 3, 8 and 15 of immobilisation. The area under the curve for arterialised-venous serum insulin concentration over the course of each 180 min oral glucose challenge is presented in Figure 2.2. There was no difference in arterialised-venous serum insulin concentration from baseline at any point over the course of the study.



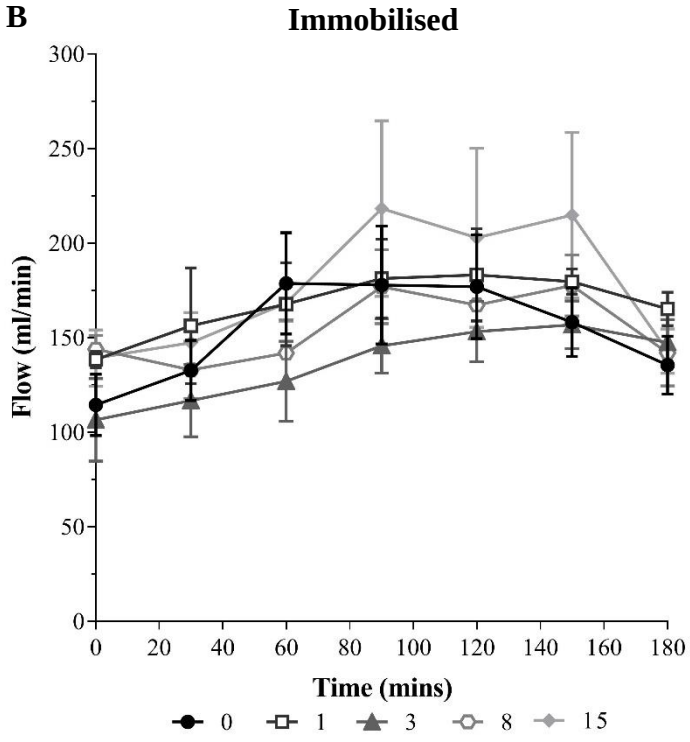


Figure 1.1 A and B Brachial artery blood flow following the ingestion of 75 g of glucose (t=0 min) is shown in a non-immobilised **(A)** and immobilised **(B)** forearm before (Day 0; circles), and at 1 (hexagons), 3 (diamonds), 8 (triangles) and 15 (squares) days of immobilisation, values are averaged over 30 min periods. Brachial artery blood flow determined before (0), during (1, 3, 8) and after 15 days of immobilisation. Values are mean ±SEM (*n*=5).

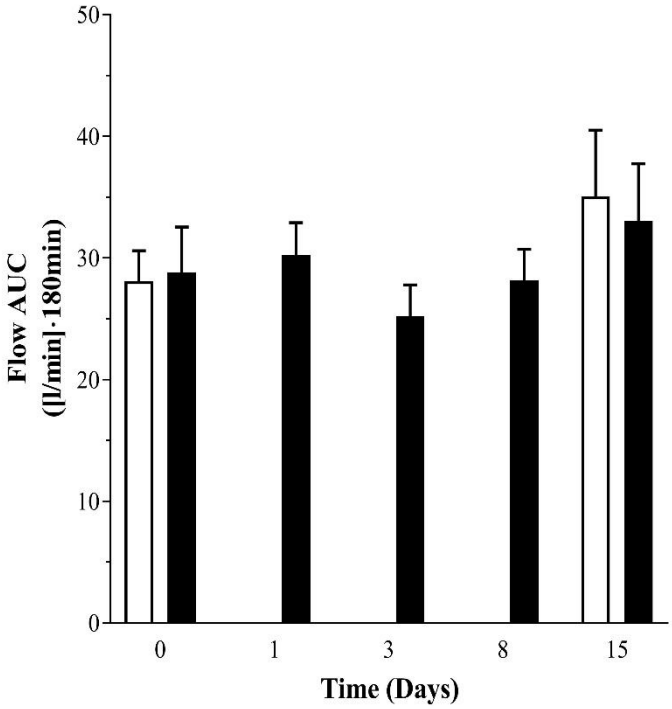


Figure 1.2 Area under the curve (AUC) for brachial artery blood flow over the course of 180 min of an oral glucose challenge in non-immobilised (white) and immobilised (black) limbs determined before (day 0), during (days 1, 3, 8) and after 15 days of immobilisation. Values are mean ±SEM (*n*=5).

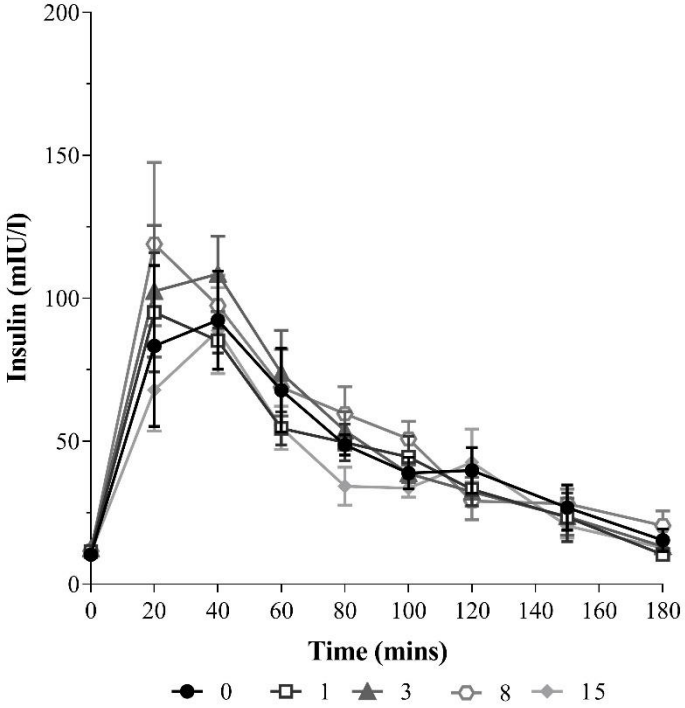


Figure 2.1 Arterialised-venous serum insulin concentration (from the heated foot) following the ingestion of 75 g of glucose (t=0 min) before (0; circles), and at 1 (squares), 3 (triangles), 8 (hexagons) and 15 (diamonds) days after immobilisation. Values are mean ±SEM (*n*=5).

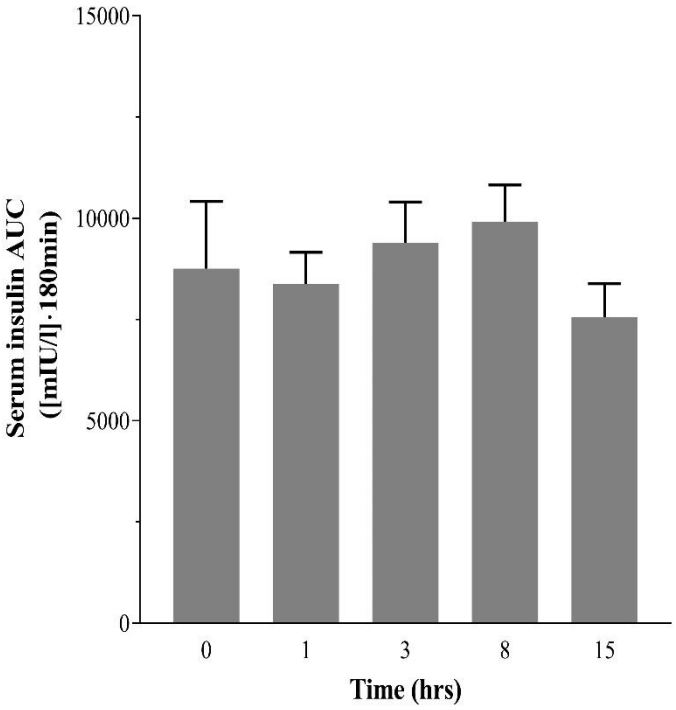


Figure 2.2 Area under the curve (AUC) for arterialised-venous serum insulin concentration over the course of each 180 min oral glucose challenge. Values are mean ±SEM (*n*=5).

3 Arteriovenous-venous glucose difference

Figures 3.1 A and B display the arteriovenous-venous glucose difference following glucose ingestion, with values averages over 30 min periods. As shown in Figure 3.2, the area under the curve for arteriovenous-venous glucose difference over the course of each 180 min oral glucose challenge was similar between arms before immobilisation (108.61 ±26.61 and 117.68±27.52 [mmol/l]·180min for the NIM and IMM limbs respectively; P=0.9) and was no different from pre-immobilisation value in the NIM arm after 15 days of immobilisation (127.85±24.33 [mmol/l]·180min; P=0.56). Similarly, arteriovenous-venous difference was unchanged in the IMM arm at days 1 (66.49±14.3 [mmol/l]·180min; P=0.3), 3 (38.48±7.02 [mmol/l]·180min; P=0.12), 8 (31.87±10.21 [mmol/l]·180min; P=0.12) and 15 (39.11±7.55 [mmol/l]·180min; P=0.14) of forearm immobilisation. However, arteriovenous-venous glucose difference was significantly different between arms at the end of the immobilisation period (P<0.01).

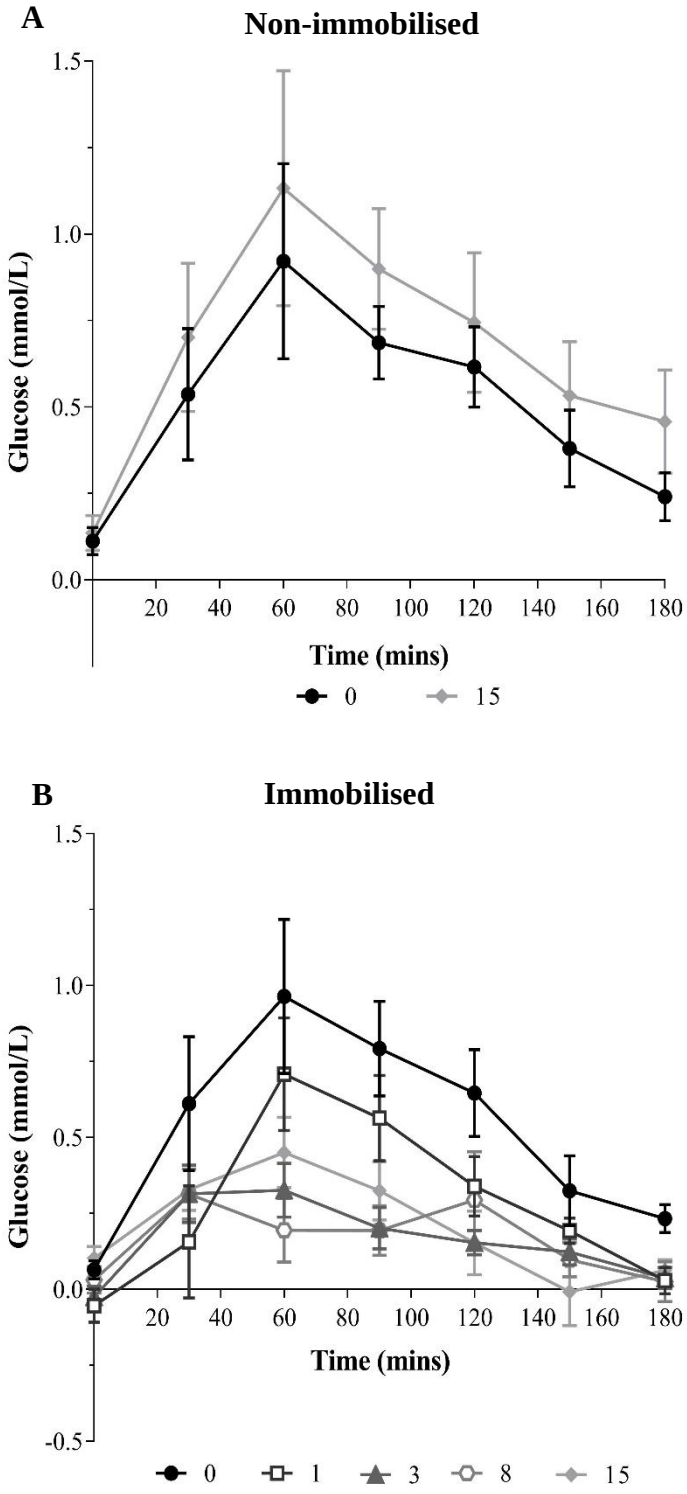


Figure 3.1 A and B Arterialised-venous glucose difference following the ingestion of 75 g of glucose (t=0 min) in a non-immobilised (A) and immobilised (B) forearm before (Day 0; circles), and at 1 (hexagons), 3 (diamonds), 8 (triangles) and 15 (squares) days of immobilisation, values are averaged over 30 min periods. Values are mean ±SEM (*n*=5).

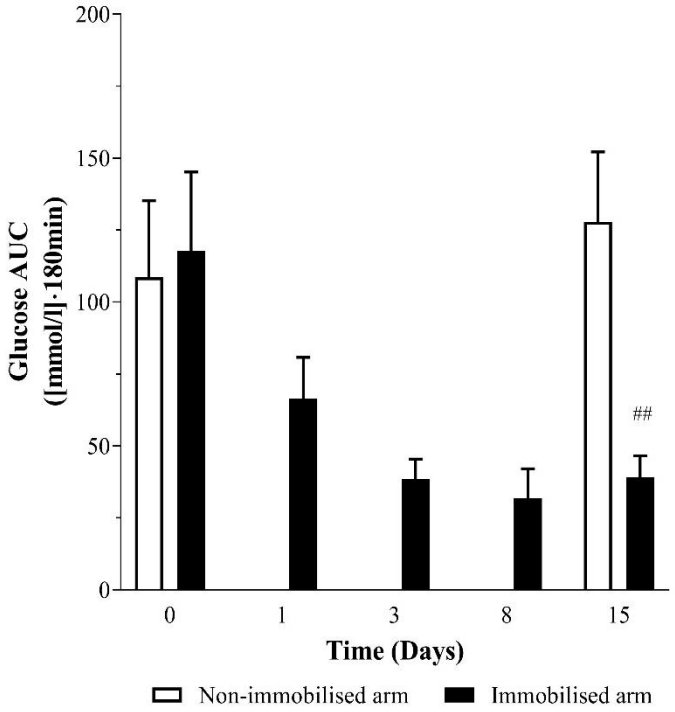


Figure 3.2 Area under the curve (AUC) for arterialised-venous glucose difference over the course of 180 min of an oral glucose challenge in non-immobilised (white) and immobilised (black) limbs before (day 0), during (day 1, 3 and 8) and after 15 days of immobilisation. Values are mean ±SEM (*n=5*). ## P<0.01 significantly different between arms.

Appendix 5: A pilot study to determine the efficacy of percutaneous electrical stimulation to maintain muscle glucose uptake during forearm immobilisation.

1. Introduction

Previous work by this group (Skirrow 2014) and others (Mikines *et al.,* 1991) have shown that immobilisation induces acute reductions in muscle glucose uptake. Furthermore, findings reported in Chapter 3 have shown that this effect occurs within 24 hrs of limb immobilisation, and is profound (38% decline from pre-immobilisation values). Given that the control arm (non-immobilised) was protected from these changes, it seems likely that the reduction in muscle contraction per se is driving the inhibition of forearm glucose uptake. Although the cellular mechanisms responsible for these changes are unclear, previous attempts to restore forearm glucose uptake with glyceryl trinitrate (to enhance blood flow) or to prevent the decline in forearm glucose uptake with intense bouts of intermittent resistance exercise, have been unsuccessful (Skirrow 2014). The latter supports the contention that immobilisation is a rapid and profound driver of decrements of insulin sensitivity.

As demonstrated by Skirrow *et al*. one bout of 150 forearm contractions does not protect against the development of immobilisation-induced reduction in muscle glucose uptake that is seen 48 hrs later (Skirrow 2014). Thus, the question remains regarding the efficacy of frequent bouts of muscular contraction to inhibit such decrements in muscle glucose uptake over this 48 hr time frame. The use of percutaneous electrical stimulation (PES) offers a feasible intervention to allow low-grade muscle contraction to be delivered at prescribed regular intervals during limb immobilisation. A single bout of low frequency electrical stimulation, characterised as ranging from 3-20 Hz, has been shown to enhance whole body glucose uptake in the absence of limb movement in healthy volunteers (Hamada *et al.,* 2003, Hamada

et al., 2004), while there was a 0.8% improvement in haemoglobin A1c of Type 2 Diabetic patients using PES for 1 hour per day over 8 weeks (Crowe and Caulfield 2012). To date, however, the use of low-grade PES has not been investigated as an intervention to preserve muscle glucose uptake in healthy volunteers during limb immobilisation.

2. Aims

The aim of this pilot study was to determine whether regular low-grade electrically-evoked muscle contraction could blunt the immobilisation induced changes in muscle glucose uptake in response to an oral glucose challenge.

3. Methods

3.1 Participants

Two healthy male volunteers (age 22.5±4.6 years, weight 65.7±0.42, body mass index 20.9±0.3 kg/m^2) participated in the pilot study.

3.2 Study design

After a routine health screening and the provision of informed written consent as described in Section 2.1.2, volunteers attended the laboratory on 3 separate occasions (pre-immobilisation and again after 24 and 48 hrs of immobilisation; protocol depicted in Figure 1) for determination of forearm glucose uptake. Prior to each laboratory visit participants fasted over-night and abstained from alcohol, caffeine and strenuous exercise for the previous 24 hrs. On the morning of each laboratory visit participants attended a thermoneutral laboratory (room temperature 25°C) at 08.00hr.

Upon arrival at the laboratory participants were asked to void their bladder and then rested quietly on a hospital bed. During this rest period in visits 3 and 4, participants underwent one 30 min bout of PES on the forearm muscle of their non-dominant, immobilised arm (see below). Following the same procedure described in Section 2.1.4.1, a retrograde cannula was inserted into a dorsal vein in the dominant foot to facilitate the collection of arterialised-venous blood samples, while a deep vein in each arm was also cannulated for sampling of venous blood. The foot was then placed in a hot-air warming unit (55°C) for 30 min before blood sampling began. On each occasion, an oral glucose challenge, detailed in Section 2.1.4.3, was performed to determine changes in forearm glucose uptake (Section 2.1.4.3; Fig. 2).

3.3 Blood sample collection and analysis

Previous studies detailed in this thesis demonstrated a significant change in arterialised venous-venous glucose difference, a marker for forearm glucose uptake, over respective immobilisation periods. Therefore, this measure was used in the present pilot study as a marker for immobilisation induced changes in forearm glucose uptake. Two baseline blood samples were obtained from arterialised-venous and both deep venous cannulae before beginning the 180 min oral glucose challenge. Blood samples were then collected simultaneously from each cannula at 10 min intervals for the initial 2 hrs and every 15 min for the final hour of the 180 min oral glucose challenge (Figure 2). Directly after the collection whole blood samples were analysed for glucose concentration as described in Section 2.1.5.1.

Figure 1. Schematic showing the protocol of pilot study.

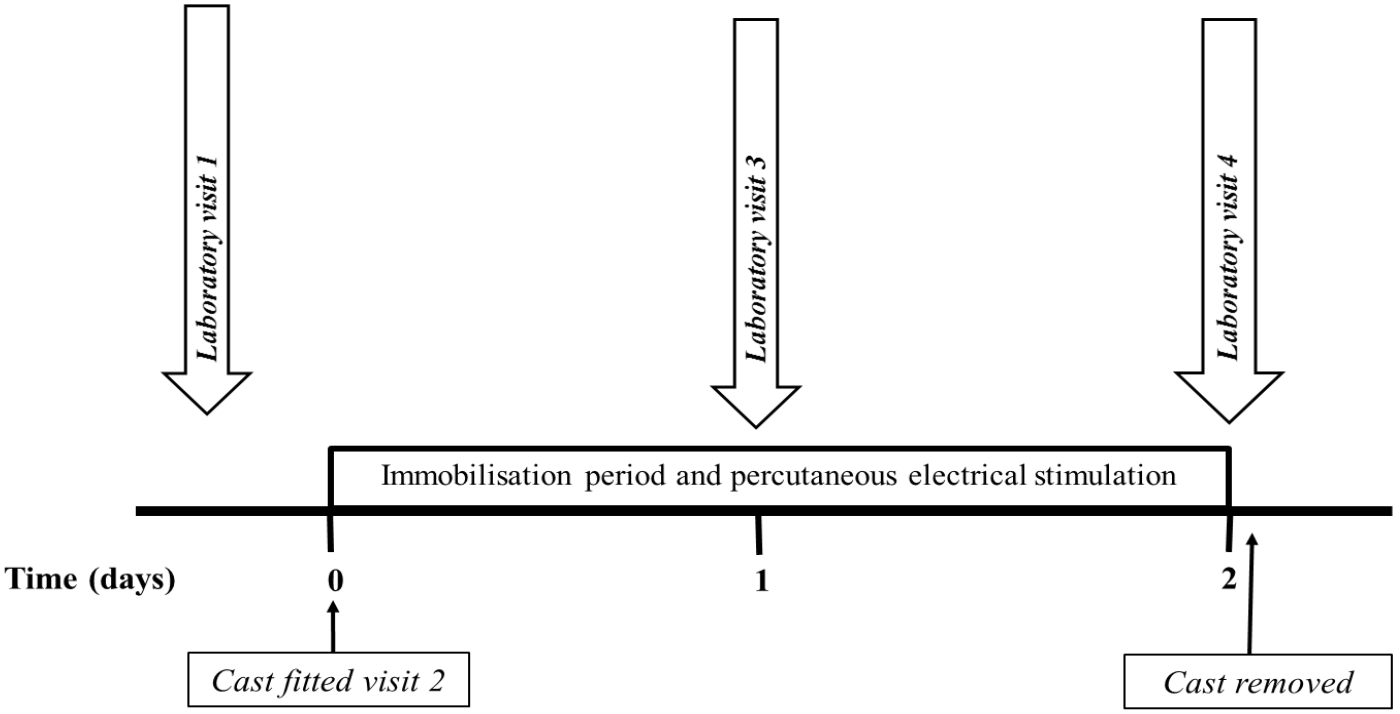
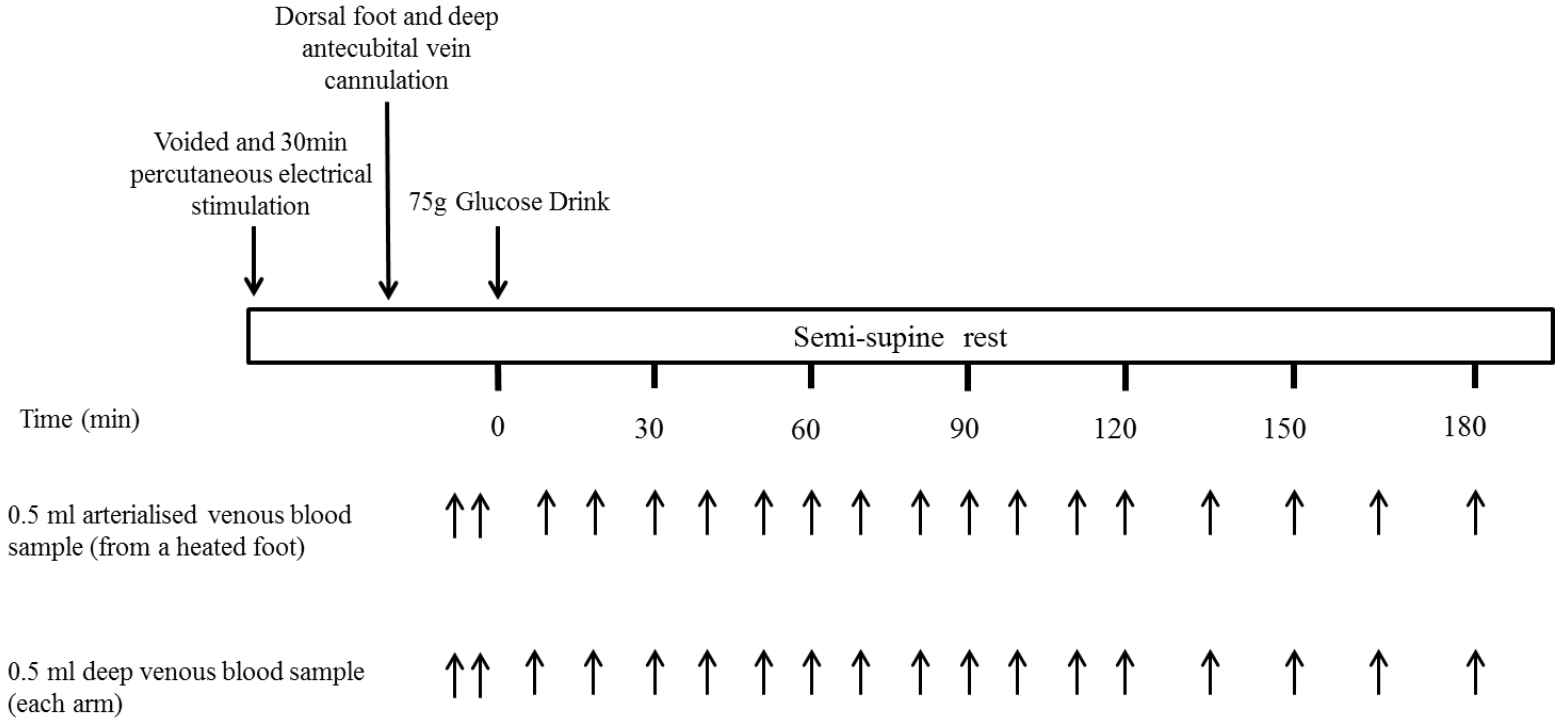


Figure 2. Schematic representation of laboratory visit.



3.4 Percutaneous electrical stimulation

Within 7 days of the pre-immobilisation visit (Laboratory visit 1), participants returned to the laboratory to have pairs of electrodes placed over the major motor points innervating the forearm muscles of their non-dominant arm (Figure 3). These electrodes were moistened by a water based gel and held in position by a medical dressing (Tegaderm™) to ensure continued good electrode contact. Participants were then instructed on how to use a hand-held, battery operated, stimulation device (Electronic Pulse Massager HV-F05, Omron Healthcare Europe B.V., Netherlands) to deliver low frequency (3 Hz) electrical stimulation producing low level contraction of the forearm muscles for 30 min every 2 hrs from 8am-10pm each day. The stimulation intensity of 3 Hz was chosen as this was sufficient to elicit visible muscular contraction of the forearm muscles without causing discomfort to the participant.

3.5 Forearm immobilisation

Once electrodes were fixed in place a below elbow fibreglass cast was fitted (as detailed in Section 2.1.3) over the electrodes by an experienced technician. The immobilised (IMM) arm was supported by a broad-arm sling, which volunteers were repeatedly instructed to wear at all times, except when bathing and sleeping. The dominant arm served as the non-immobilised (NIM) control arm.

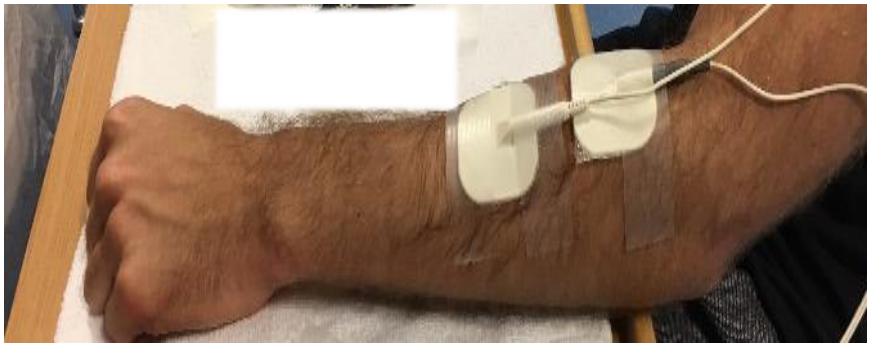


Figure 3. Electrode placement over major motor points of the forearm prior to cast fitting.

4. Results

4.1 Arterialised venous-venous blood glucose difference

The arterialised venous-venous blood glucose difference in the NIM and IMM arms in response to glucose ingestion is shown in Figure 4. The arterialised venous-venous glucose difference increased in response to the oral glucose challenge in all visits. Prior to immobilisation arterialised venous-venous glucose difference peaked in the NIM arm at 1.59±0.62 mmol/l and at 1.64±0.56 mmol/l in the IMM arm (Figure 4 A). Following 24 hrs of immobilisation arterialised venous-venous glucose difference peaked at 2.07±1.04 mmol/l in the NIM and at 1.36±0.63 mmol/l in the IMM arm (Figure 4 B). After 48 hrs of immobilisation arterialised venous-venous glucose difference peaked at 2.17±1.62 mmol/l in the NIM arm and at 1.14±0.87 mmol/l in the IMM arm (Figure 4 C).

Figure 5 compares the total area under the curve for the arterialised venous-venous glucose difference, during an 180 min oral glucose challenge, measured over 48 hrs of immobilisation in the current pilot study (PES) with that of the study detailed in Chapter 3, which did not employ PES during immobilisation (non-PES). Following 24 hrs of immobilisation the area under the curve for arterialised venous-venous

glucose difference declined by 31.3 and 56.2% from baseline in the non-PES and PES conditions respectively. After 48 hrs the area under the curve for arterialised venous-venous glucose difference was 68.8 and 61.8% lower than pre-immobilisation values in the PES and non-PES conditions respectively.

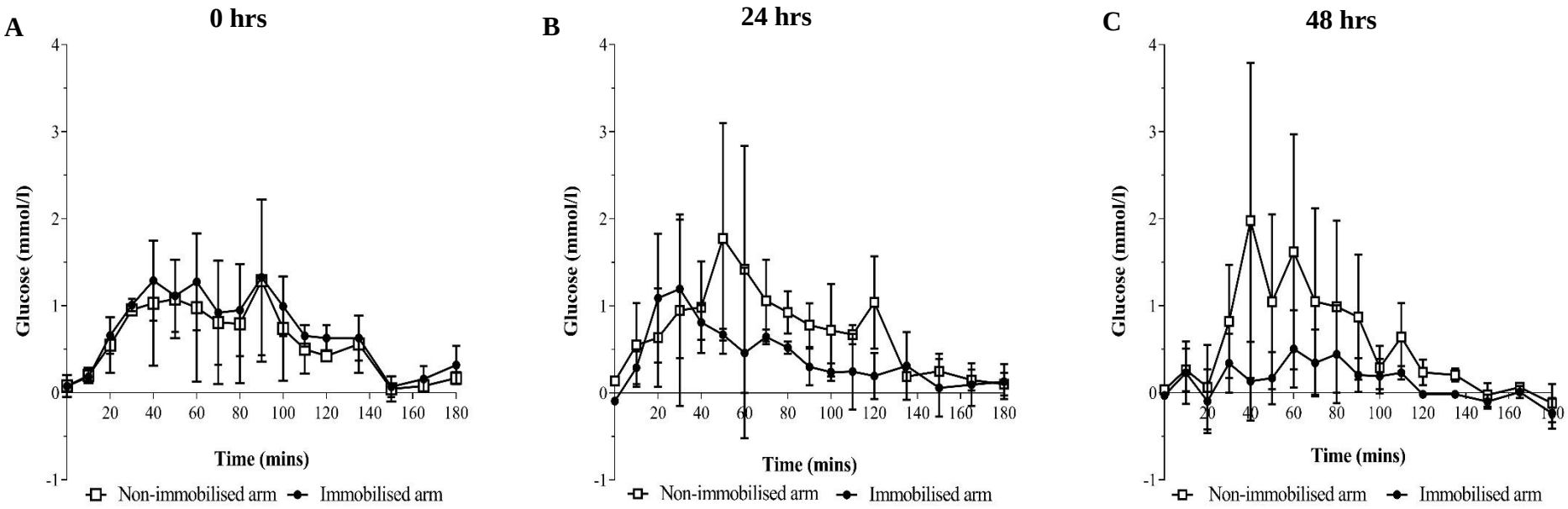


Figure 4 Arterialised venous–venous glucose difference following the ingestion of 75 g of glucose (t=0 min) in a non-immobilised (open squares) and immobilised (black circles) forearm before (0 hrs; **A**), during (24 hrs; **B**) and 48 hrs (**C**) after immobilisation. Values are mean ±SEM (*n*=2).

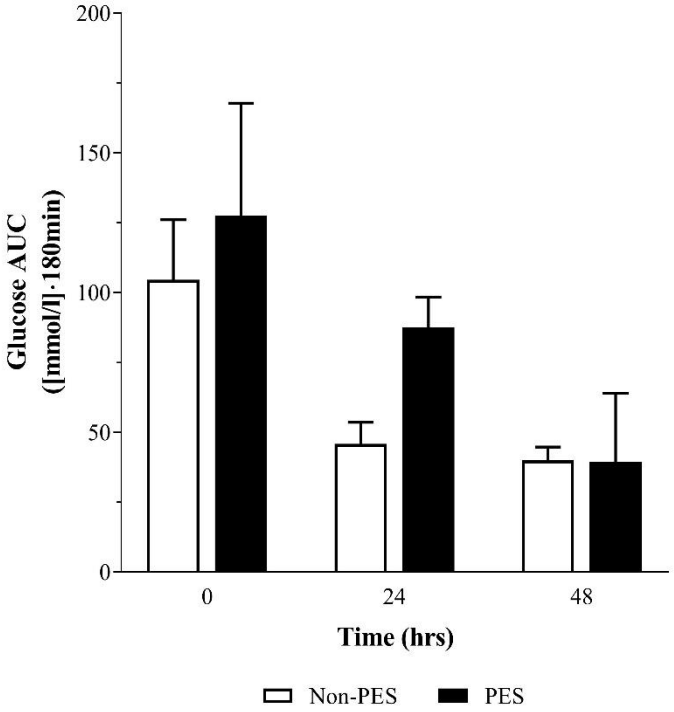


Figure 5 Area under the curve for arterialised venous–venous glucose difference measured prior to (0), and at 24 and 48 hrs following forearm immobilisation, determined using 180 min oral glucose challenge detailed in Chapter 3 (Non-PES; white) and in the present study (PES; black). Values are mean ±SEM, *n*=10 and *n*=2 in Non-PES and PES respectively.

5 Discussion

Decreased muscle glucose uptake is apparent within the first 24 hrs of limb immobilisation. These pilot data suggest that frequent muscle contraction, elicited via PES, throughout the immobilisation period is unable to prevent this decline in forearm glucose uptake. Although PES has been used for several decades to provoke muscle contractions via an electrical impulse, primarily for the purpose of muscle strengthening or restoration of neuromuscular function following stroke or spinal cord injury (Lake 1992), the associated metabolism of this exercise modality is not fully understood. A more comprehensive understanding of this method may lead to greater consistency in its implementation as an alternative to voluntary exercise.

In Chapter 3 forearm glucose uptake markedly decreased after just 24 hrs of immobilisation. As this decline in glucose uptake occurred in the absence of changes to brachial artery blood flow or insulin concentrations, these findings point towards the absence of muscle contractile activity as the potential driver of the observed inhibition of muscle glucose uptake. Furthermore, an acute exercise bout has been shown to increase skeletal muscle insulin sensitivity by augmenting capacity for glucose transport, uptake and glycogen synthesis by increasing GLUT4, Hexokinase-II and TBC1D4 protein content, as well as glycogen synthase activity (Biensø *et al.,* 2012). The present pilot study aimed to investigate whether or not contraction induced glucose uptake could be preserved during forearm immobilisation by replacing voluntary contraction with PES. As depicted in Figure 5, inhibition of forearm glucose uptake was of a similar magnitude in the PES condition in comparison to the

non-PES condition, following 48 hrs of immobilisation. Interestingly, after 24 hrs of immobilisation the decline in forearm glucose uptake was approximately 25% greater in the non-PES state compared to the PES state. While this may indicate some preservation of forearm glucose uptake by contraction induced pathways associated with PES in the early stages of immobilisation, further investigations with larger sample sizes are necessary to explore this further.

While the precise mechanisms driving the immobilisation associated decline in muscle glucose uptake remains unknown, a number of potential candidates have been proposed, such as insufficient depletion of muscle glycogen concentrations due to the lack of muscular contraction (Garetto *et al.,* 1984, Richter *et al.,* 1984). However, many of these proposed mechanisms are simply the opposite of mechanisms known to increase muscle glucose uptake, which have been derived from exercise studies. A growing body of evidence suggests the molecular mechanisms underlying beneficial effects of exercise are not simply the converse of pathways regulated by muscle disuse (Thijssen *et al.,* 2010, Booth *et al.,* 2017). Reports from Thijssen *et al*. describe the cardioprotective effects of exercise occurring as the result of vasodilation by nitric oxide pathway, whereas vasoconstrictor pathways are activated during bed rest (Thijssen *et al.,* 2011). These findings point toward different regulatory pathways for cardiovascular adaptations to exercise and deconditioning, supporting the view that the metabolic pathways of immobilisation are not simply the reverse of exercise-regulated pathways. Findings from the present pilot study support the supposition suggesting the

mechanisms underlying the decline in muscle glucose uptake in response to reduced muscle contraction are not simple the reversal of pathways driving the increase in glucose uptake associated with increased muscular contraction. However, further investigation is necessary to elucidate if the mechanisms through which exercise/contraction improve insulin-stimulated glucose uptake are the same or different mechanisms as those through which immobilisation impairs muscle glucose uptake.

Frequent muscular contraction, elicited via PES, during 48 hrs of forearm immobilisation was not sufficient to preserve muscle glucose uptake in 2 participants. While the possibility of the present study being underpowered due to the small sample size is undeniable, the findings are in agreement with earlier findings of a substantial decline in muscle glucose uptake within 48 hrs of immobilisation (Chapter 3). Much of the published data using electrical stimulation of the musculature to elicit improvements in insulin sensitivity have used devices capable of delivering complex pulses in unusual patterns, most of which have been specifically designed for their purpose (Hamada *et al.*, 2003, Hamada *et al.,* 2004, Crowe and Caulfield 2012). The handheld device used in the present study is available over the counter and may not have the capacity to produce muscular contractions sufficient to induce a high enough metabolic demand capable of preserving muscle glucose uptake. Therefore, further studies are warranted to elucidate the precise frequency and duration of electric pulses needed to mimic the metabolic profile of forearm musculature during voluntary contraction.

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Appendix 6: Human study screening forms

Metabolic Physiology Group,
School of Life Sciences,
University of Nottingham Medical School,
Queen's Medical Centre Nottingham

Effects of immobilisation on skeletal muscle lipid, insulin resistance and brain function in healthy volunteers.

Name of Investigators*:*

A Burns	PhD student
PL Greenhaff	Professor of Muscle Physiology
PA Gowland	Professor of Physics (Magnetic Resonance)
IA Macdonald	Professor of Metabolic Physiology
S Francis	Associate Professor and Reader of Physics (Magnetic Resonance)
FB Stephens	Lecturer
A Hale	PhD student

HEALTHY VOLUNTEER'S QUESTIONNAIRE

Please remember, all information will be treated in the strictest of confidence.

Personal Information

Volunteer Number _____ Date _____/_____/_____

1. Name
   _____
   a. Contact Address
      _____
      _____
2. Contact Telephone Number
   _____
3. Email Address
   _____
4. Date of Birth _____
5. Age _____

General Health

1. In general would you say your health is;

   Please tick one response

   - □ Excellent
   - □ Very Good
   - □ Good
   - □ Fair
   - □ Poor

2. Compared to one year ago, how would you rate your health in general now?

   Please tick one response

   - □ Much better than a year ago
   - □ Somewhat better than a year ago
   - □ About the same
   - □ Somewhat worse than a year ago
   - □ Much worse than a year ago

3. The following questions are concerned with any medical conditions you might have.
   Do you suffer from any of the following

   Please tick one box on each line

	No	Yes	Don't know
Diabetes			
Epilepsy			
Depression			
Hayfever			
Asthma			
Eczema			
High blood pressure			
Heart problems			
Breathing problems*			
Indigestion/heartburn			
Irritable Bowel			
Nervous disorders			
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?

_____

_____

4. Have you had any operations in the last year? Yes/No

   If 'yes' please detail

   _____

   _____

5. Do you have a tendency to faint in certain situations?
   E.g. on standing in a warm room, after fasting

   - ☐ Yes
   - ☐ No
   - ☐ Don't know

6. Smoking On average how many cigerettes do you smoke a day?
   _____

7. Alcohol On average, how many of the following alcohol containing drinks do you consume in a week?

   - ☐ None
   - ☐ 125ml (Pub measure) glass red/white wine
   - ☐ Bottles of wine (750ml): in addition to any wine intake recorded above)
   - ☐ Single 'shot' (35ml) of spirits
   - ☐ Bottle (330ml) of lager
   - ☐ Pint of lager (5%)
   - ☐ Half pint of lager (5%)
   - ☐ Pint of bitter
   - ☐ Half pint of bitter
   - ☐ 'Alco-pops'
   - ☐ Other

8. Do you have any allergies?

   _____

   _____

Physiologic Measurements

1. Height: _____cm
2. Weight:_____kg
3. BMI:_____kg/m^2
4. BP lying:_____
5. BP standing:_____
6. HR lying:_____
7. HR standing:_____
8. Blood glucose:_____

Recruited?

- ☐ Yes
- ☐ No

School of Life Sciences,
University of Nottingham Medical School,
Queen's Medical Centre Nottingham

Effects of immobilisation on skeletal muscle lipid, insulin resistance and brain function in healthy volunteers.

Name of Investigators*:*

A Burns	PhD student
PL Greenhaff	Professor of Muscle Physiology
PA Gowland	Professor of Physics (Magnetic Resonance)
IA Macdonald	Professor of Metabolic Physiology
S Francis	Associate Professor and Reader of Physics (Magnetic Resonance)
FB Stephens	Lecturer
A Hale	PhD student

HEALTHY VOLUNTEER'S QUESTIONNAIRE

Please remember, all information will be treated in the strictest of confidence.

Personal Information

Volunteer Number _____ Date _____/_____/_____

Name _____

Contact Address _____

_____

_____

_____

Contact Telephone Number _____

Email Address _____

Date of Birth _____

Age _____

General Health

In general would you say your health is;

Please tick one response

Excellent
Very Good
Good
Fair
Poor

Compared to one year ago, how would you rate your health in general now?

Please tick one response

Much better than a year ago
Somewhat better than a year ago
About the same
Somewhat worse than a year ago
Much worse than a year ago

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

Do you suffer from any of the following

Please tick one box on each line

	No	Yes	Don't know
Diabetes			
Epilepsy			
Depression			
Hayfever			
Asthma			
Eczema			
High blood pressure			
Heart problems			
Breathing problems*			
Indigestion/heartburn			
Irritable Bowel			
Nervous disorders			
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?

_____

_____

Have you had any operations in the last year? Yes/No

If 'yes' please detail

_____

_____

Do you have a tendency to faint in certain situations?
E.g. on standing in a warm room, after fasting

Yes
No
Don't know

Smoking On average how many cigerettes do you smoke a day?

_____

Alcohol drinks do On average, how many of the following alcohol containing you consume in a week?

- None
- 125ml (Pub measure) glass red/white wine
- Bottles of wine (750ml): in addition to any wine intake recorded above)
- Single 'shot' (35ml) of spirits
- Bottle (330ml) of lager
- Pint of lager (5%)
- Half pint of lager (5%)
- Pint of bitter
- Half pint of bitter
- 'Alco-pops'
- Other

Do you have any al rgies? le

_____

_____

Physiologic Measurements

1. Height: _____cm
2. Weight:_____kg
3. BMI:_____kg/m^2
4. BP lying:_____
5. BP standing:_____
6. HR lying:_____
7. HR standing:_____
8. Blood glucose:_____

Recruited?

- Yes
- No

MRI CONSENT FORM
(Final version 1.0: 03/03/2015)

Title of Study: *Effects of immobilisation on skeletal muscle lipid, insulin resistance and brain function in healthy volunteers.*

REC ref: A12022015 SoLS MRI PhD

Name of Researcher: Aisling Burns

Name of Participant:

Please initial box

1. I voluntarily agree to take part in the above study. ☐

2. I confirm that I have read and understand the information sheet version number 1 dated 05/03/2015 for the above study and have had the opportunity to ask questions. ☐

3. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, and without my legal rights being affected. ☐

4. I understand that relevant sections of data collected in the study may be looked at by authorised individuals from the University of Nottingham, the research group and regulatory authorities where it is relevant to my taking part in this study. I give permission for these individuals to have access to these records and to collect, store, analyse and publish information obtained from my participation in this study. I understand that my personal details will be kept confidential. ☐

5. I agree to comply with the reasonable instructions of the supervising investigator and will notify him immediately of any unexpected unusual symptoms or deterioration of health. ☐

6. I authorise the investigators to disclose the results of my participation in the study but not my name. ☐

7. 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 after the results of this study have been published. After this time they will be destroyed securely. ☐

8. I understand that I can ask for further instructions or explanation at any time. ☐

9. I understand that I am free to withdraw from the study at any time, without having to give a reason for withdrawing. ☐

10. I confirm that I have disclosed relevant medical information before the study. ☐

11. I shall receive an inconvenience allowance of £300. If I withdraw from the study for medical reasons not associated with the study a payment will be made to me proportional to the length of the period of participation, but if I withdraw for any other reason, the payment to be made, if any, shall be at the discretion of the supervising investigator. ☐

12. **Optional: Consent for storage and possible use in future research** I agree that the samples I give and the data gathered about me can be stored by Prof. Paul Greenhaff's research team at the University of Nottingham for possible use in future studies (approved by an Ethics Committee). I understand that some of these studies may be carried out by researchers other than current team of Prof. Paul Greenhaff, who ran the first study, including researchers working for commercial companies. Any samples or data used will be anonymised, and I will not be identified in any way. ☐

13. I have not 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 exposure to ionising radiation. ☐

Abnormal Findings in Scans

14. I understand that the Imaging and Radiological Sciences MR Suite is not a clinical diagnostic facility and so does not routinely inspect images for abnormalities. I understand that my MR scans will NOT routinely be reviewed by a radiologist (or any other medically qualified person) to look for any signs of disease, and it is unlikely that any abnormalities that may be present will be detected. ☐

15. On the other hand I understand that if one of the investigators should happen to notice something on my scan which they think is abnormal then they will show my scans to a radiologist who will contact my GP to organise any further action required. ☐

16. I agree to my GP being informed of any medically relevant information gained from the study. ☐

GP Name:_____

Surgery Address:_____

_____

Telephone number: _____

_____ _____ _____
Name of Participant Date Signature

Information below to be completed by one the named investigators:

I confirm that I have fully explained the purpose of the study and what is involved to:

……

I have given the above named a copy of this form together with the information sheet.

_____ _____ _____
Name of Investigator Date Signature

_____ _____
Ethics Code Study Volunteer Number

2 copies: 1 for participant, 1 for the project notes.