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Exploring mechanisms of disuse atrophy and optimal rehabilitation strategies for the restoration of muscle mass, structure & function

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Abstract

Disuse atrophy (DA) occurs during situations of unloading and is characterised by a loss of muscle mass and function. These reductions may be observed as early as 5 days into a period of unloading. While the reduction of muscle size is well studied, the reduction in muscle function is less well characterised. Furthermore, different muscles of the lower leg have been shown to express diverging profiles of muscle size loss as a result of DA. In particular, the medial gastrocnemius (MG) is relatively susceptible to DA while the tibialis anterior (TA) is resistant to even long-term bed rest of over a month. The average length of stay in hospital in the UK was last reported at 4.5 days which is enough time for DA to occur in the quadriceps. In older individuals, loss of muscle mass and function may reduce quality of life to the point of frailty and are less well suited to performing resistance exercise. Hence, alternative therapies to attenuate DA may be needed.

This thesis introduces skeletal muscle and its function as an organ in the human body, along with its composition and how this influences its function. It then discusses the study of DA and the situations in which it occurs, before covering the response of different muscles, the time course and strategies used for rehabilitation. General methods used within this thesis are detailed in Chapter 2. In Chapter 3, results of muscle size, strength, and various aspects of function from the vastus lateralis (VL), the MG and the TA to investigate the difference in response to 15-day unilateral lower limb immobilisation in young adults.

In Chapters 4 and 5, this thesis investigates the neuromuscular adaptation to this intervention in the VL compared to the non-immobilised control, and then the immobilised MG and TA, respectively. These results show an impairment of neural input to the VL and the MG following immobilisation which is not seen in the TA.

Finally, in Chapter 6, peripheral nerve stimulation is shown to potentially recruit from a broader pool of motor units than traditional neuromuscular electrical stimulation and as such may be more favourable for rehabilitation.

Publications

Inns, TB., Hardy, EJO., Bass, JJ., Wilkinson, DJ., Stashuk, DW., Atherton, PJ., Phillips, BE., Piasecki, M. (2022). Motor unit dysregulation following 15 days of unilateral lower limb immobilisation. J. Physiol, 600 (21).

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Presentations

Oral presentations

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Poster presentations

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Awards

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COVID-19 impact statement

To mention the impact the COVID-19 pandemic on this thesis would be appropriate considering the effects on the research studies detailed in the forthcoming chapters. Primarily, as this thesis aims to investigate human participants, this was the most impacted aspect. The University of Nottingham closed in March 2020 and all research was halted. Unfortunately, the immobilisation study had been gaining momentum in recruitment, so this was stifled, and two 15-day participants were unable to take part. During a brief cessation of lockdown in late autumn 2020, we managed to recruit three participants until we had to end human research again until summer 2021. Fortunately, the electrical stimulation study had generated enough data to analyse, write up and publish during this time. Recruitment was arguably more difficult following the pandemic also which slowed the progress of the studies. However, given the difficulties, the body of work below represents the progress achieved despite these circumstances.

Acknowledgements

The following thesis contains investigations carried out over three and three-quarter years at the University of Nottingham as part of the Centre for Metabolism, Ageing and Physiology within the School of Medicine. I am extremely thankful to the Centre for Musculoskeletal Ageing Research and by extension the Medical Research Council and Versus Arthritis for supporting and funding my PhD studies. Having undertaken my undergraduate degree at the University of Nottingham, it is fair to say that my time studying there has taken my fascination with medical science and honed it into a drive to further the knowledge of the neuromuscular system in different situations and particularly into disuse as presented here. Over those years, I have connected with many people who I would like to express my thanks for:

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Last but by no means least, I would like to thank my **Mum.** A permanent rock in the storm of life who has never stopped believing in me reaching this point and beyond. For her love and support every day, sharing the smiles, the tears, and the excitement that this PhD has brought, I dedicate this thesis to her.

Declaration

The data in this thesis was collected and analysed by myself unless otherwise acknowledged below.

In Chapters 3, 4 and 5, ultrasound data of the vastus lateralis, tibialis anterior and medial gastrocnemius were analysed by Mr Edward Hardy.

I declare that this thesis has been constructed by myself and that all the data presented are my own work unless otherwise stated. All published literature within this thesis has been appropriately referenced. None of the data presented in this thesis has been submitted towards the assessment of a higher degree previously.

Thomas Inns

January 2023

List of abbreviations

1RM	1-repetition maximum
4E-BP1	eukaryotic translation initiation factor 4E
AA	amino acid
ACh	acetylcholine
AChR	acetylcholine receptors
ACSA	anatomical cross-sectional area
ADP	adenosine diphosphate
Akt	protein kinase B
ATP	adenosine triphosphate
BIA	bioimpedance analysis
BMI	body mass index
BP	blood pressure
BR	bed rest
Ca^{2+}	calcium ion
CMAP	compound muscle action potential
COP	centre of pressure
COPD	chronic obstructive pulmonary disease
CSA	cross sectional area
CT	computerised tomography
DA	disuse atrophy
DMS	dynamic muscle strength
DQEMG	decomposition-based quantitative electromyography
DXA	dual x-ray absorptiometry
EAA	essential amino acid
E-C	excitation contraction
ECM	extracellular matrix
eIF4F	eukaryotic initiation factor 4F
EMG	electromyography

EWGSOP	The European Working Group on Sarcopenia in Older People
$_{F}L$	muscle fascicle length
FR	firing rate
FS	force steadiness
FSR	fractional synthetic rate
HDBR	-6° head-down tilt bed rest
HMB	β-hydroxy-β-methylbutyrate
H-reflex	Hoffmann's reflex
HS	hindlimb suspension
ICU	intensive care unit
ICU-AW	intensive care unit acquired weakness
iEMG	intramuscular electromyography
IGF1	insulin-like growth factor 1
IL-6	interleukin 6
KE	knee extensor
LG	lateral gastrocnemius
MAFbx	atrogin-1
MG	medial gastrocnemius
MHC	myosin heavy chain
MPB	muscle protein breakdown
MPS	muscle protein synthesis
MRI	magnetic resonance imaging
mRNA	micro ribonucleic acid
MT	muscle thickness
mTOR	mammalian target of rapamycin
MU	motor unit
MuFR1	muscle RING-finger protein-1
MUNE	motor unit number estimate
MUP	motor unit potential

MUPT	motor unit potential trains
MVC	maximal voluntary isometric contraction
MVT	maximal voluntary isometric torque
Na^+/K^+-ATPase	sodium potassium pump
NCAM	neural cell adhesion molecule
NF-MUP	near-fibre motor unit potential
NM	neuromuscular
NMES	neuromuscular electrical stimulation
NMJ	neuromuscular junction
p70S6K	ribosomal protein S6 kinase
$_P$A	muscle fibre pennation angle
PCSA	physiological cross-sectional area
PI3K	phosphoinositide 3-kinase
PIC	persistent inward current
PNS	peripheral nerve stimulation
QF	quadriceps femoris
RET	resistance exercise training
RF	rectus femoris
RNA	ribonucleic acid
RTD	rate of torque development
RVE	resistive vibration exercise
S6K	serum 6 kinase
SCI	spinal cord injury
sMUP	surface motor unit potential
SOL	soleus
SPPBT	short physical performance battery test
SR	step-count reduction
TA	tibialis anterior
TNF-α	tumour necrosis factor alpha

TUG	timed up and go
ULLS	unilateral lower limb suspension
UPP	ubiquitin-proteasome pathway
VAS	visual analogue scale
VL	vastus lateralis
VM	vastus medialis
VO_2 peak	peak oxygen consumption

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Chapter 1 Introduction

1.1 An introduction to skeletal muscle

1.1.1 The importance of skeletal muscle for whole-body health

1.1.1.1 Skeletal muscle structure and movement

A key function of skeletal muscle is movement, achieved via the contraction of muscle cells, or fibres, which collectively form a whole muscle. Muscle fibres are multinucleated cells which are collected in bundles called fascicles (Frontera & Ochala, 2015). These fascicles are in turn collectively grouped into whole muscles, stretching between tendons. The apparatus which muscle fibres use to contract are protein structures referred to as myofilaments.

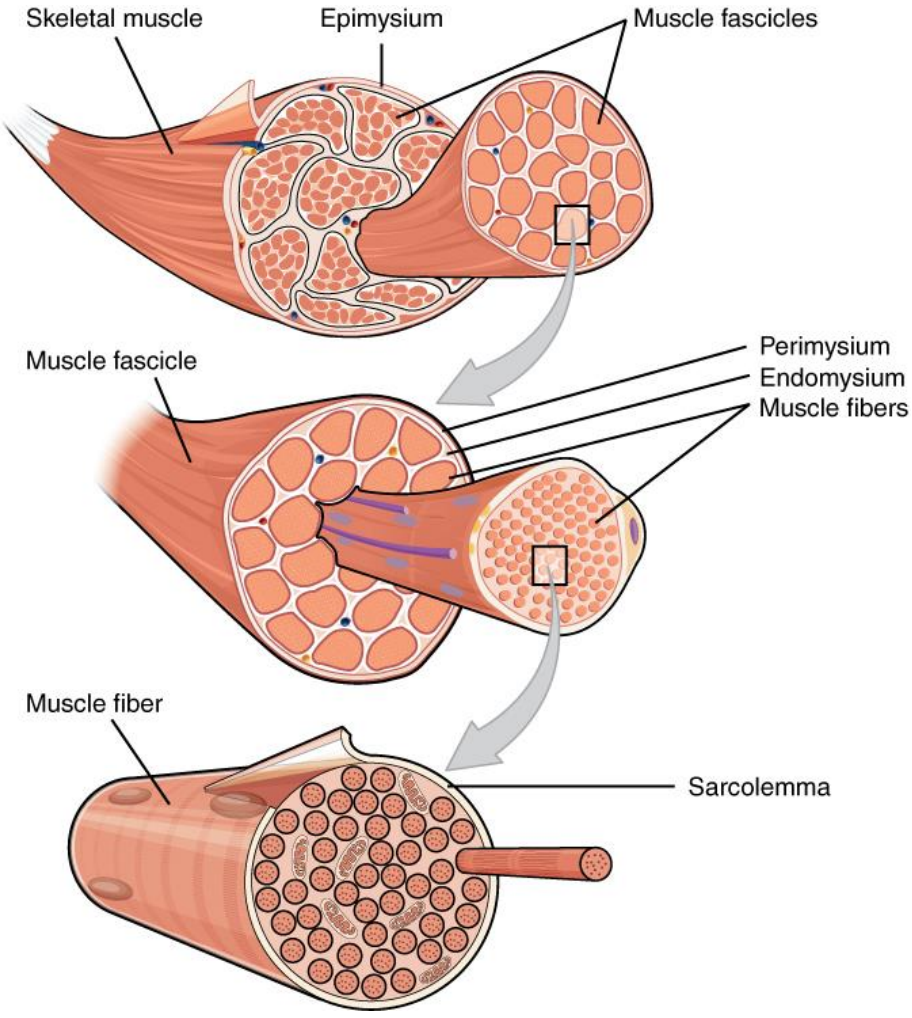


Figure 1.1: *A diagram of muscle structure from whole muscle to muscle fibres.*

There are two key myofilaments that allow the contraction of the individual fibres and collectively, the whole muscle (Szent-Györgyi, 2004). Actin is a thin myofilament which contains multiple binding sites for part of the other key protein, myosin (Rayment *et al.*, 1993). In a resting state, these binding sites remain covered by a third myofilament called tropomyosin, preventing myosin from binding to it. Myosin is a thicker protein, and contains numerous protrusions called myosin heads. The release of calcium ions (Ca^{2+}) from the sarcoplasmic reticulum of myofibres is triggered in response to an action potential from a motor nerve reaching the neuromuscular junction (NMJ). Ca^{2+} binds to troponin C, causing a conformational change in tropomyosin, which cover the binding sites on actin filaments. Tropomyosin moves away from the binding sites so that myosin heads may attach to their binding sites on the actin molecule. This is brought about by the cleaving of a single molecule of adenosine triphosphate (ATP) attached to the myosin head by the myosin ATPase enzyme (Powers *et al.*, 2021). Energy released from this process causes the myosin head to bind to actin. Once the myosin head is attached to the actin filament, the resulting products of adenosine diphosphate (ADP) and inorganic phosphate detach from the myosin head which performs 'power stroke', pulling the actin filament closer to the middle of the sarcomere, or the 'M-line'. A new ATP molecule then attaches to the myosin head which causes it to return to the same alignment that it was in before at the start of the process. Once it is in a neutral position again; the process, referred to as excitation-contraction (E-C) coupling, is repeated until the contraction is complete. The accumulated mechanical action of sarcomere shortening throughout the muscle leads to a muscle contraction (Frontera & Ochala, 2015).

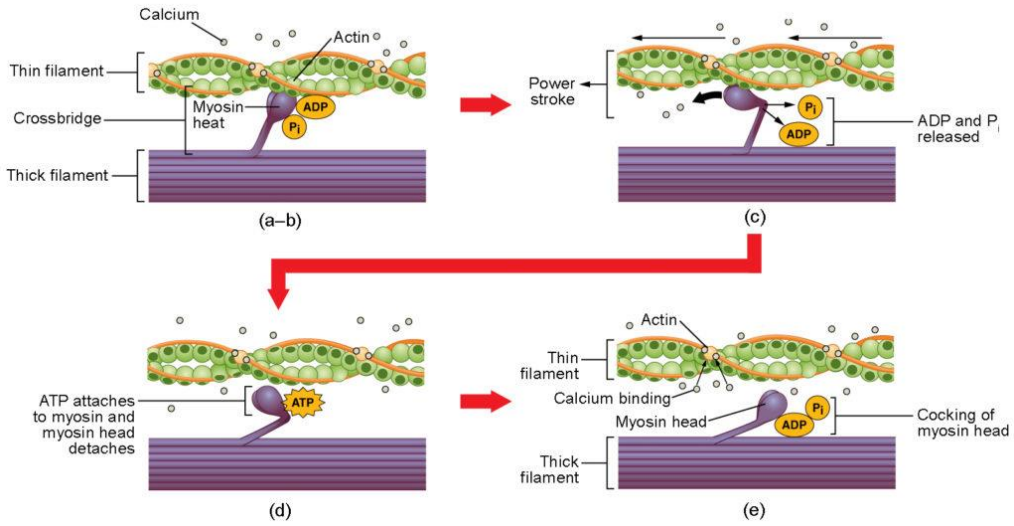


Figure 1.2: *A diagram of the contractile process.*

As a contraction occurs in skeletal muscle, it pulls on the attached tendons which in turn change the angle of the joint which they are responsible for. Moving multiple joints in concert with each other allows the performance of simple tasks such as walking, bending down to pick up an object or climbing a flight of stairs.

Muscle fibre type is determined by the myosin heavy chain (MHC) phenotype(s) which it expresses (Spangenburg & Booth, 2003). These differing fibre types have different properties determining their function, with proportions of each varying between individual muscles. MHC I containing-fibres, also known as type I, rely on oxidative phosphorylation as a source of ATP to produce a contraction as described above. These fibres are slower to contract but in the presence of enough oxygen are extremely resistant to fatigue. MHC IIa containing-fibres, or type IIa fibres, may produce ATP through either anaerobic or aerobic respiration, and are quicker to contract than type I but less resistant to fatigue. The final main type of muscle fibre contains MHC IIx. These fibres have the greatest twitch speed but are highly prone to fatigue, due to their reliance on anaerobic respiration. In addition to these main fibre types, some exist which co-express two or even all of the mentioned MHC isoforms and are labelled as hybrid fibres with features shared between the expressed MHC forms (Medler, 2019).

Table 1.1: *Differences between standard skeletal muscle fibre types.*

	Type I	Type IIa	Type IIx
MHC isoform	MHC I	MHC IIa	MHC IIx
Contraction time	Slow	Fast	Very fast
Oxidative capacity	High	High	Low
Fatigue resistance	High	Moderate	Low
Fibre diameter	Small	Medium	Large
Capillary density	High	Moderate	Low
Mitochondrial density	High	High	Low
Metabolic pathway	Aerobic (Oxidative phosphorylation)	Aerobic and anaerobic	Anaerobic (glycolysis)
Force production	Low	Moderate	High

1.1.1.2 Skeletal muscle and health

In addition to the well-recognised role of skeletal muscle for movement, the role of skeletal muscle on numerous other aspects of whole-body health is largely underappreciated (Wolfe, 2006), with skeletal muscle having a proven role to play in, amongst other things, insulin sensitivity (Jensen *et al.*, 2011). To exemplify this importance, a number of studies have shown a relationship between skeletal muscle mass and mortality (Li *et al.*, 2018) and morbidity (Chen *et al.*, 2013) in both 'healthy' and disease-specific cohorts (Weijs *et al.*, 2014; Srikanthan *et al.*, 2016; Lucidi *et al.*, 2018). Many of the positive roles of skeletal muscle on aspects of whole-body health relate to the ability of skeletal muscle cells to act as a reservoir for amino acids (AAs) for use across the body. Indeed, skeletal muscle is the largest site of AA storage in the body. For example, in times of fasting, AAs are made available by skeletal muscle to replenish plasma AAs taken up by other tissues, such as in the liver for gluconeogenesis (Schutz, 2011), and throughout the immune system for vital

roles to increase resistance to infection and disease (Li *et al.*, 2007). Due to their critical function throughout the human body, AAs stored in skeletal muscle are found primarily forming sarcomeres as described above. Considering skeletal muscle mass maintenance, skeletal muscle proteins exist in a constant cycle of muscle protein synthesis (MPS) and breakdown (MPB), with MPS being stimulated in postprandial states (primarily by essential AA [EAA]), while MPB takes precedence in the post-absorptive state (Burd *et al.*, 2009). In the post-absorptive state AA produced from the breakdown of skeletal muscle protein may be recycled by the myofibre itself or transported by the circulatory system to other organs in the body to construct proteins there (Argilés *et al.*, 2016). The regulation of this dynamic equilibrium and the primary anabolic drivers (i.e., EAA nutrition and contractile activity) are discussed in more detail in section 1.1.3.

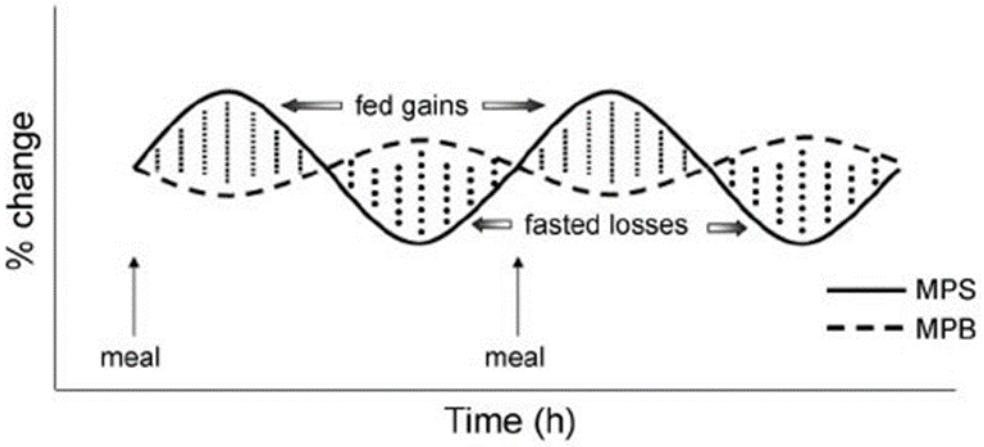


Figure 1.3: *A diagram to represent changes in muscle protein synthesis and breakdown over the course of a day with regular meals. Reproduced from* (Burd *et al.*, 2009)*.*

Reduced muscle mass has been shown to be associated with functional decline in ageing (Janssen, Heymsfield and Ross, 2002, discussed further in sections 1.1.4.1). Furthermore, reduced lean body mass (which should be noted is not purely muscle mass but total mass minus fat mass (Buckinx *et al.*, 2018), and as such may also represent changes in bone mineral density) has been linked to increased prevalence of cardiometabolic diseases in overweight or obese males

(Khazem et al., 2018) and increased length of stay following hospital admission (Pichard et al., 2018).

1.1.2 The neuromuscular system

1.1.2.1 Structure and function of the neuromuscular system

The smallest working part of the neuromuscular system is the motor unit (MU). This was first defined by Sherrington (1925) to consist of 1) an alpha motor neuron and 2) the muscle fibres which it innervates. Alpha motor neurons originate from the motor cortex and exit from the ventral horn of the spinal cord. At each level of the vertebrae, bundles of alpha motor neurons, collectively a motor nerve, which will branch off to supply different muscles. The neuron will terminate as the pre-synaptic end of the (NMJ where it interfaces with the muscle fibre membrane. Action potentials which have propagated down the motor neuron will trigger the release of acetylcholine (ACh) from the pre-synaptic NMJ. Action potential generation is a result of electrical impulses from the brain or directly from sensory efferent pathways overcoming the resting membrane potential of the motor axon and subsequently propagating along the axon to the motor end plate, resulting in the release of ACh molecules. These will travel across the synaptic cleft to the ACh receptor (AChR), a ligand-gated sodium ion channels on the muscle fibre membrane which open in response to ACh binding. The influx of sodium ions into the cell depolarises the membrane, propagating the action potential along the muscle fibre membrane and through the T-tubules within the muscle (Calderón *et al.*, 2014). This leads to a release of Ca^{2+} from the sarcoplasmic reticulum followed by the steps described in section 1.1.1.1 leading to a muscle contraction.

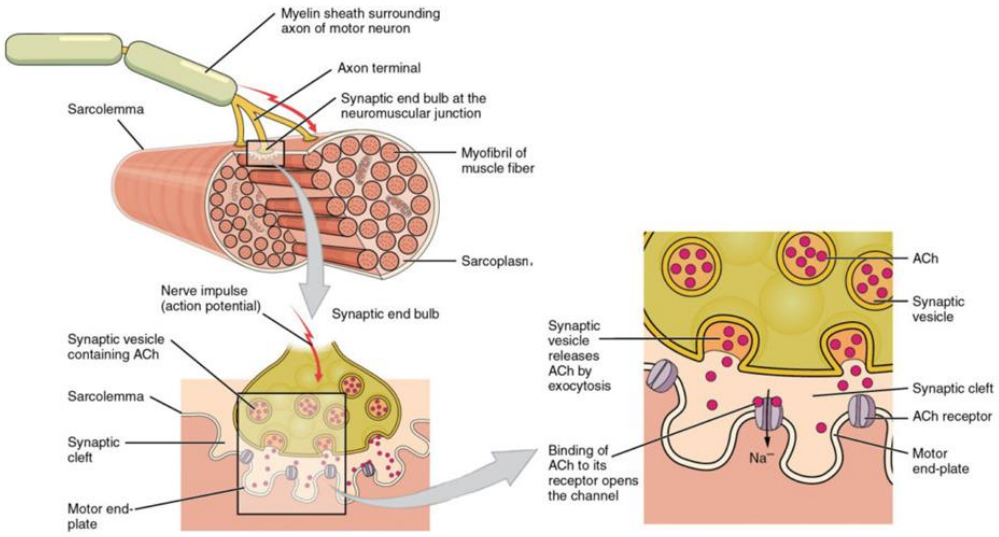


Figure 1.4: *A diagram of synaptic transmission of an action potential at the motor end plate.*

1.1.2.2 Characteristics of motor units

The characteristics of individual motor units within a muscle which relate to neuromuscular function are most commonly investigated using electromyography (EMG). EMG is performed using electrodes to record the electrical activity produced by a target muscle, with many electrode configurations used. In simple terms, the 2 major categories of EMG include surface EMG (sEMG) where electrodes are placed over the surface of the muscle itself, or intramuscular EMG (iEMG) where electrodes are positioned within the muscle itself on the tip of a needle or using a fine wire. Although the specific numbers and patterning of electrodes for both of these EMG methods can vary, the overarching premise is that EMG enables visualisation and distinction of the signals produced by MU's using complex pattern recognition (Stashuk, 1999). Motor unit characteristics can be quantified using high-density surface or intramuscular EMG techniques to provide information relating to the size, stability, and complexity of an individual motor unit within the target muscle. A key characteristic pertaining to neuromuscular remodelling is motor unit number estimate (MUNE), which has been shown to be lower in older individuals (Piasecki *et al.*, 2016*c*), an observation further described in the following section. This is done using a comparison of average surface motor

unit potential (sMUP) size with the compound muscle action potential (CMAP), i.e. the maximal electrically stimulated activity of the muscle (Piasecki *et al.*, 2018*a*). In addition to this, two measurements of NMJ transmission stability can be taken from iEMG recordings; namely jitter and jiggle. The former relates to the variation of intervals between individual instances of the same MUP (Sanders & Stålberg, 1996) while the latter quantifies variation of amplitude between individual instances of the same MUP (Stålberg & Sonoo, 1994). High variability of MUPs can be a sign of unstable neuromuscular transmission and may be a result of reinnervation of denervated muscle fibres as a feature of ageing or disuse (Piasecki *et al.*, 2016*b*). MU firing rate is also a quantifiable characteristic, with variability measurements of this parameter relating to impaired neuromuscular function and assisting diagnosis of neuromuscular disorders (Dorfman *et al.*, 1989) and other diseases with neuromuscular components (Watanabe *et al.*, 2013). Current understanding of MU recruitment is based on Henneman's size principle stating that MUs are recruited in order from smallest to largest as workload increases (Henneman, 1957). This is observable in EMG traces as smaller MUs may be detectable throughout a set-intensity contraction while the larger may be seen to only begin to be activated towards the end of said contraction to allow the muscle to continue generating a consistent force.

1.1.2.3 Neuromuscular plasticity

As is the case for skeletal muscle which demonstrates a high degree of plasticity through its adaptation to internal (i.e., ageing (Piasecki *et al.*, 2016*b*) and disease (Rudolf *et al.*, 2014)) and external (exercise training (Hakkinen *et al.*, 2000)) stimuli, both in terms of structure and function, the neuromuscular system also appears to be highly adaptable.

1.1.2.4 The neuromuscular system and exercise training

There is no doubt that neuromuscular adaptation is closely tied to increase in muscle strength following resistance exercise training (RET). Early gains in

strength during RET have been shown to occur despite no measurable muscle mass gain, i.e. hypertrophy (Moritani & DeVries, 1979). For example, quadriceps cross sectional area (CSA) and fibre areas were not seen to change whereas peak isometric and isokinetic torque increased following 9 sessions of unilateral RET over two weeks (Akima *et al.*, 1999). With the use of electromyography, these neural changes have been quantified to elucidate the mechanisms behind adaptations (Aagaard, 2003). Quadricep muscle EMG amplitude and rate of amplitude rise (measures of myoelectrical activity) were both increased following 15 weeks of RET, along with contractile rate of force development (Aagaard *et al.*, 2002). Following 4 weeks of plantar flexor RET, soleus EMG, rate of torque development, voluntary activation and rate of activation were all improved along with torque increases (Del Balso & Cafarelli, 2007). Another neural factor expressing adaptation following RET is motor unit firing rate, with increases seen as early as 1 week alongside maximal force gains, although these features were shown to be enhanced further in young participants compared to older (Kamen & Knight, 2004). More recently, the use of high-definition electromyography (HDEMG) has facilitated deeper understanding of the neuromuscular adaptation to resistance exercise. For example, greater motor unit discharge rate was recorded from individually tracked motor units following 4-week RET of the ankle dorsiflexors (Del Vecchio *et al.*, 2019). These collective findings highlight clear evidence for the neural component of muscle strength adaptation in response to resistance exercise.

1.1.2.5 The neuromuscular system and advancing age

A number of MU characteristics decline with advancing age, with these changes shown to precede the more obvious signs of muscle size and function losses (Piasecki et al., 2016*c*). These changes include a reduction in MU number coupled with an increase in MU size, potentially suggesting a degree of muscle fibre rescue by sprouting of new axonal branches to re-innervate lost fibres following the death of the original supplying neuron (Hepple & Rice, 2016). Supporting the concept of age-associated declines in neuromuscular function, reduced voluntary torque and voluntary activation of the quadriceps have been

reported in older men and women compared with younger individuals (Mau-Moeller *et al.*, 2013). Similarly, v*astus medialis* (VM), *vastus lateralis* (VL) and *rectus femoris* (RF) muscle activity was also decreased during maximal contractions in the older participants with suggestions that decreased neural drive might be attributed to this decline, due to a reduced input from central stimuli. Considering sarcopenia (the age-associated loss of muscle mass and function (Cruz-Jentoft *et al.*, 2010)), there does appear to be a relationship between neuromuscular degeneration and sarcopenia. When assessing groups of pre-sarcopenic, sarcopenic, and severely sarcopenic participants, severely sarcopenic participants had much lower maximum voluntary contractions (MVC) and rate of torque development of the dorsiflexor muscle group than both sarcopenic and pre-sarcopenic groups (Morat *et al.*, 2016). This differentiation is further characterised by a failure to increase MU size by rescuing denervated muscle fibres by nearby motor axons in sarcopenic men, while non-sarcopenic and pre-sarcopenic men appear to retain this capacity (Piasecki *et al.*, 2018*b*). Collectively, this suggests a strong association between progression of sarcopenia and neuromuscular function degeneration, and more specifically in loss of force generation and power. Again, as seen in skeletal muscle, age-associated losses in neuromuscular function are exacerbated by age-associated diseases. For example, when looking at older individuals with osteoarthritis of the hip, differences between the affected and unaffected legs were seen for quadriceps size and strength as well reduced rates of force development and neuromuscular activity in both VL and VM muscles (Suetta *et al.*, 2007).

1.1.3 Skeletal muscle maintenance

An increase of myofibrillar structural proteins within a particular muscle fibre causes that fibre to grow in size. Subsequently, as this occurs throughout the muscle fibre pool, whole-muscle mass increases (Conceição *et al.*, 2018). Not only do muscle fibres just increase in protein content, but small tears in muscle fibres caused by exercise leads to the release of factors which increases the

rate of myoblast proliferation and differentiation. Myocytes produced from this process incorporate into the damaged muscle fibre to repair the tissue by fusing their membranes together. The addition of cellular material from these differentiated myoblasts further increases the mass of the fibre and therefore the whole muscle. A greater muscle mass implies a greater storage pool of amino acids which can be broken down and used to synthesis other proteins for tissues throughout the body aiding in processes detailed above in section 1.1.1.2.

There are two primary aspects determining this process of muscle growth, or muscle hypertrophy. Namely, nutrition and exercise. The anabolic component of nutrition has been whittled down throughout the past few decades and continues to be developed. The consumption of a mixed meal of macronutrients was found to double the volume of skeletal muscle protein synthesis (MPS) (Rennie *et al.*, 1982). Additional investigations using protein alone confirmed that the protein component of the mixed meal was responsible for increases in MPS (Bennet *et al.*, 1989). Following investigations into muscle protein turnover, it was discovered that a particular group of amino acids were responsible for the majority of muscle protein synthesis and increased anabolism, i.e., essential amino acids (EAAs). Furthermore, intravenous flooding using a labelled amino acid, L-leucine, was found to increase incorporation of infused L-valine into skeletal muscle protein (Smith *et al.*, 1992). This and other work provided a basis for individual EAAs providing significant anabolic stimulus independent of other EAAs. It was then established that leucine in particular provided the greatest anabolic stimulus among EAAs (Churchward-Venne *et al.*, 2012). Beyond this, the possibility of a leucine metabolite, β-hydroxy-β-methylbutyrate (HMB), as the key to the potency of this particular EAA has been suggested, as it has been observed to stimulate MPS to a similar degree to leucine supplementation whilst concurrently reducing MPB (Wilkinson *et al.*, 2013). The ‘muscle full’ effect was a further key discovery in the area of skeletal muscle nutrition. This phenomenon essentially means that once the optimum dose of protein has

been ingested to maximise muscle growth following a resistance training session, the addition of further protein will not increase that growth (Atherton *et al.*, 2010).

The second of these aspects, exercise, is also essential to muscle hypertrophy. MPS has been shown consistently to increase following various formats of RET. A primary focal point of studies in this area has been to determine the effectiveness of varying training intensities and structures to maximise muscle hypertrophy while minimising necessary workload. Following an acute bout of RET at 80% of each participant's 1-repetition maximum (1RM), muscle fractional synthetic rate (FSR) and net balance were increased up to 48 hours following this exercise bout (Phillips *et al.*, 1997). A more recent investigation found MPS response to RET to plateau between 60-90% 1RM after testing with multiple intensities from 20-90%, with a caveat of this study being that responses were somewhat blunted in older participants compared to younger (Kumar *et al.*, 2009). The impact of nutrition and exercise combined was clearly demonstrated in a group of healthy young men when trained to failure (Burd *et al.*, 2011). Specifically, mixed and sarcoplasmic muscle protein synthesis were shown to increase significantly following feeding (15 g whey protein) and exercise combined with feeding, although only when trained to failure at 90% or 30% of maximal strength was myofibrillar protein synthesis significantly greater than following feeding.

1.1.4 Skeletal muscle atrophy

1.1.4.1 Sarcopenia

Sarcopenia was first defined in 1988 by Dr Irwin Rosenberg to describe the change noted in body composition and functional decline with ageing (Rosenberg, 1989). Literally translating from the Greek "poverty of the flesh", this definition began an avalanche of research aimed at better understanding the causes, consequences, and potential mitigation of this phenomenon (Marzetti *et al.*, 2017). In an effort to consolidate research efforts in this field, 'The European Working Group on Sarcopenia in Older People' (EWGSOP) was

formed in 2008 with a stated aim of “discussing the clinical definition and diagnostic criteria of sarcopenia as a syndrome”. The 2008 EWGSOP meeting concluded with a diagnosis of sarcopenia defined as:

Evidence of low muscle mass and one other of the following:

- low muscle strength or
- low physical performance (Cruz-Jentoft *et al.*, 2010).

A subsequent meeting of the EWGSOP in 2018 led to an adjustment of the defining criteria for sarcopenia with a greater focusing more on declines in muscle strength. These new guidelines state that low muscle strength indicates probable sarcopenia, which can be confirmed by having low muscle quantity or quality, with severe sarcopenia considered if low physical performance is also apparent (Cruz-Jentoft *et al.*, 2019).

This new definition has sparked a series of published debates as to its suitability, specificity and ease of use. For example, some have found that when comparing the definitions and their differing cut-off points, prevalence of sarcopenia falls in men but rises in women (Van Ancum *et al.*, 2020), while others have found it to fall in both (Reiss *et al.*, 2019).

However, it was seen by some that using sarcopenia as a term to include age-related decline in muscle mass and strength implied that these two facets of health have a causative relationship, which in fact they do not (Clark and Manini, 2008).

1.1.4.2 Dynapenia

Dynapenia was a term originally coined to describe age-related declines in muscle strength independent of changes in mass (Clark & Manini, 2008). The term was developed as there was concern regarding the lack of research on strength declines compared to declines in muscle mass, especially considering that declines in strength have a greater impact on the functional capacity of older adults (Goodpaster *et al.*, 2006). To further promote a clinical diagnosis of dynapenia, an algorithm was constructed to help characterise individuals

that may be at risk of this condition in a similar way to the diagnosis of sarcopenia (Manini & Clark, 2012).

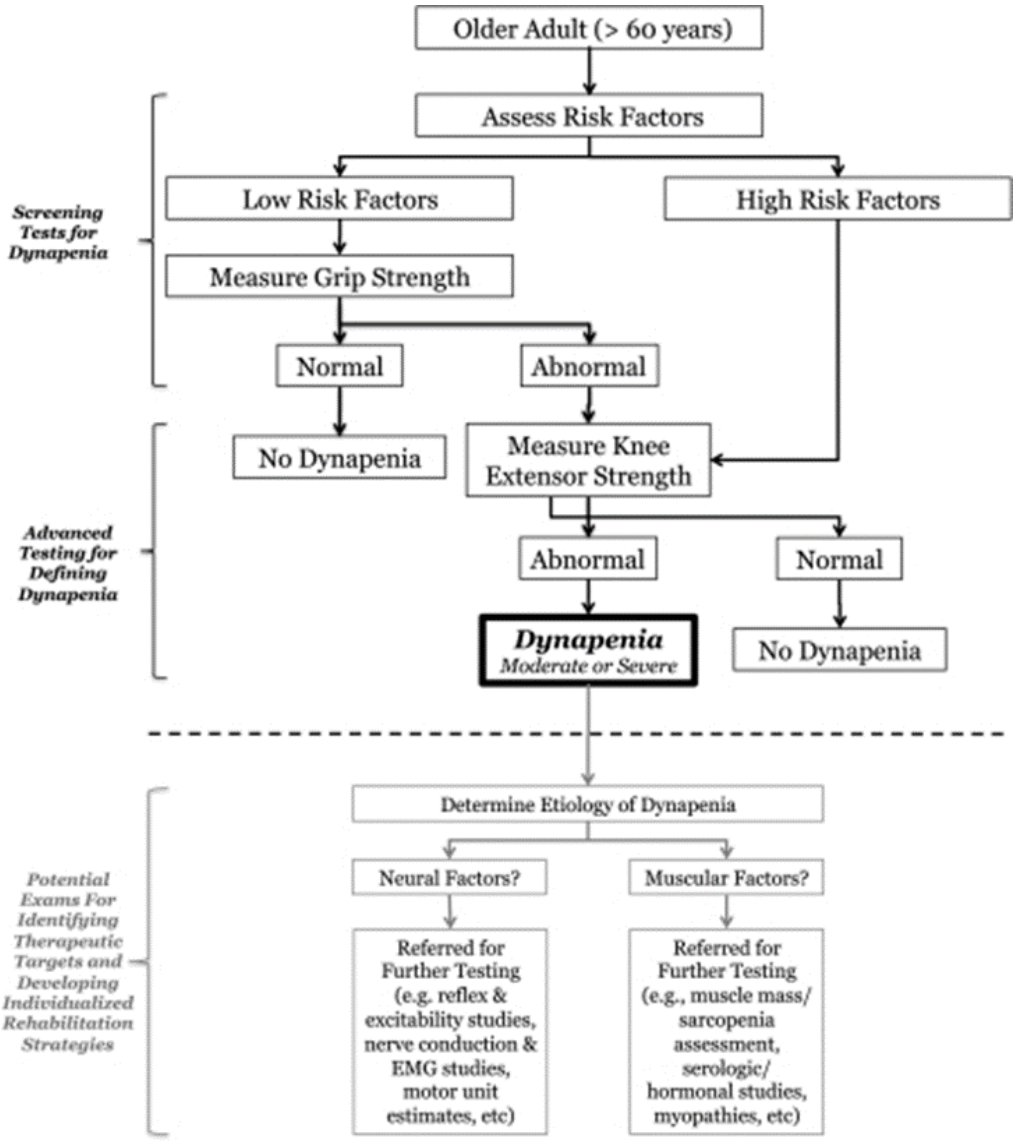


Figure 1.5: *a flow-chart designed to define progression of dynapenia in older adults* (Manini & Clark, 2012).

1.2 Skeletal muscle atrophy

1.2.1 Types of muscle atrophy

1.2.1.1 Sarcopenia

As described in detail above, sarcopenia is a form of skeletal muscle atrophy, specifically describing age-related changes in muscle mass and function.

However, other forms of muscle atrophy are not strictly age related, although exacerbated by age, and are detailed below.

1.2.1.2 Cachexia

Cachexia is a condition commonly seen accompanying chronic diseases such as cancer, chronic obstructive pulmonary disease, and chronic renal failure among others (Morley *et al.*, 2006). A combination of declining nutrition and abnormal metabolism can lead to drastic loss of adipose and muscle tissue, adding the deleterious effects of this to the already damaging impact of the primary disease. When presenting in cancer in particular, it displays a high level of heterogeneity, with a complex relationship between the location of the tumour, it's size and density (Fearon *et al.*, 2012). In patients with COPD and accompanying cachexia, mortality rates were found to be three-fold that of non-cachexic patients (McDonald *et al.*, 2019).

1.2.1.3 Disuse atrophy

Disuse atrophy (DA) refers to the loss of muscle mass caused by a period of unloading. This phenomenon occurs within various real-world scenarios causing a multitude of deleterious effects and negative consequences throughout the whole body (Fitts *et al.*, 2000). The primary focus of this thesis will be to investigate DA, suggest the key gaps in the current literature and establish the need to carry out true time-course studies, assessing both atrophy-resistant and atrophy-susceptible muscles. Perhaps the most highlighted impact of disuse atrophy is the general loss of muscle mass. It has been reported by the vast majority of studies investigating disuse atrophy in different situations and models while investigating different aspects. Collectively, there are data available from as long as 16 to 28 weeks of space flight to as short a time frame as 5-day unilateral lower limb suspension (ULLS) (LeBlanc *et al.*, 2000; Wall *et al.*, 2014). Quadriceps CSA decline of ~3% after just 5 days, developing to ~8% after an additional 9 days in healthy young adults may be less severe than it would be in older adults, in part due to the greater

potential for hypertrophy at a younger age to replenish the losses, and also since younger individuals are likely to have greater muscle mass to begin with. Quadriceps volume after such an extended duration in space of 16 to 28 weeks was only reduced by ~12%, which by comparison with 5-day and 14-day ULLS does not seem to be as much. However, during spaceflight countermeasures will have been in place to reduce atrophy as much as possible whereas there is a much greater level of control in the disuse model of ULLS. The subsequent sections contain a more detailed description of the various situations and models of DA

1.2.2 Situations of disuse atrophy

1.2.2.1 Spaceflight

One of the primary drivers of disuse atrophy research is the loss of muscle mass experienced by individuals sent into space, which coupled with reduction in neural drive lead may lead to catastrophic health issues if countermeasures are not put into place (Narici & De Boer, 2011). To exemplify the magnitude of DA experienced during spaceflight, LeBlanc *et al.* (1995), reported that 8 days in space led to triceps surae volume and quadriceps volume decrease by 6%. Nine to 16 days in space reduced knee extensor (KE) volume by between ~6 to 15% and knee flexor volume by ~9 to 14%, while plantar flexor volume decreased between ~8 to 16% (Akima *et al.*, 2000*a*). KE CSA and gluteal muscle CSA both reduced by 8% following 17 days of spaceflight (Tesch *et al.*, 2005), while another study reported a ~7% quadricep volume decrease after the same length of time, along with ~12% and ~10% reductions in gastrocnemius and soleus volumes, respectively (LeBlanc *et al.*, 2000). Taken together, it is clear that there is a large heterogeneity of DA across individual muscle groups with relatively short-term space flight- although it must be acknowledged that measurement techniques did differ between studies. Also, the variation in physical activity carried out in space, and pre-flight physiological characteristics (i.e., muscle mass) must also be considered as potential confounding factors in such investigations (Narici & De Boer, 2011).

In the case of longer-term spaceflight missions, 180 days of spaceflight reportedly reduced thigh volume by 4 to 7%, while whole calf volume reduced by 10 to 16% (Gopalakrishnan *et al.*, 2010). Furthermore, 115 to 197 days in space was reported to reduced quadriceps volume by ~12%, gastrocnemius volume by ~24% and soleus volume by ~20% (LeBlanc *et al.*, 2000). Again, these data illustrate the significant heterogeneity in the DA of different muscles.

Beyond the heterogeneity of different muscles rates of DA, another interesting finding to have arisen from the spaceflight literature is the lack of linearity between time and rates of DA. For example, quadriceps volume, measured using MRI, reduced by ~6% after 8 days, ~7% after 17 days, but only ~12% after 115 to 197 days (LeBlanc *et al.*, 1995, 2000), suggesting that DA predominates in the early phases of disuse, a finding that reflects the temporal nature of hypertrophy in response to an anabolic stimuli (Atherton *et al.*, 2010). In support of this suggestion, a more recent study investigated changes in the calf muscles following 6 months of spaceflight in 9 crewmembers (Trappe *et al.*, 2009). In this study total calf volume decreased by ~13% (specifically 15% in the soleus and 10% in the gastrocnemius), with similar % declines in function (~14% reduction in plantar flexion MVC at 90°). It must however be noted that these changes were with consistent aerobic (~5 hours per week) and resistance (3 to 6 days per week) exercise training performed during the mission. In addition to the heterogeneity in DA between muscles, this study also illustrated muscle-specific differences in the degree of fibre-type shifting from one to another. Although both the gastrocnemius and soleus favoured a shift from MHC I to MHC I/IIa hybrid and MHC IIa, the gastrocnemius had a decrease of 12% MHC I fibres with significant increases of 4% and 9% for MHC I/IIa and MHC IIa fibres, respectively. Comparatively, the soleus showed a 17% decrease in MHC I fibres, with non-significant increases in the other fibre types individually (~4% each), although a collective increase of 12% in MHC I/IIa, IIa and IIa/IIx fibres was seen. Of note, the increase in MHC IIx fibres, or hybrids containing it, was not observed in the gastrocnemius suggesting this muscle to be more susceptible to atrophy.

1.2.2.2 Intensive Care

Disuse atrophy is also commonly experienced in patients admitted to intensive care units (ICU). For example, in a cohort of 63 critically ill patients studied for a minimum of 10 days ICU treatment, the CSA of the rectus femoris decreased by ~12% from days 1 to 7, with a further decline up to ~18% lost by day 10 (Puthucheary *et al.*, 2013). From this cohort, a sub-group of patients had vastus lateralis muscle biopsies taken on days 1 and 7, which exhibited a muscle fibre CSA decline of ~18% (Puthucheary *et al.*, 2013). This study showed that, similar to that seen with spaceflight, rapid muscle atrophy occurred early on in these patients' ICU treatment. This observation was even more pronounced in those patients who experienced multi-organ failure (in comparison to those with single-organ failure), marking them as a high priority for disuse atrophy attenuation interventions (Puthucheary *et al.*, 2013). In fact early mobilisation may be the key to interrupting DA and the accompanying health issues, since greater length of time immobile has greater impact, while early mobilisation reduces time spent in ICU (Needham, 2008). This is demonstrated further by the finding that following as little as 5 days in critical care, vastus lateralis pennation angle (a measure discussed in section 1.3.1 in more detail), was decreased and to a greater extent following 10 days (Turton *et al.*, 2016). The reduction in force production as a result of this decline contributes to the functional decline seen in ICU patients following reambulation, commonly known as ICU-acquired weakness (ICUAW). Indeed, following discharge from ICU, those who had mobilised during mechanical ventilation had lower scores of ICUAW than those who had not (Hodgson *et al.*, 2015). Furthermore, of those who had passed away within 90 days, their ICUAW at discharge was greater than survivors beyond day 90 (Hodgson *et al.*, 2015).

1.2.2.3 Limb casting

Limb casting is also a healthcare intervention which can induce muscle atrophy. It is less well studied clinically than DA induced by ICU, but nevertheless remains an important situation to target, and is well linked to limb suspension

models of DA as discussed below. One study investigated fracture patients across ~4 weeks of casting to treat 5th metatarsal or fibular fractures whose ankle joints underwent casting. Using magnetic resonance imaging (MRI), thigh muscle CSA decreased from ~88cm^2 to ~77cm^2 and calf muscle CSA decreased from ~53 cm^2 to ~49 cm^2 (Yoshiko *et al.*, 2018). Beyond muscle size, ankle casting following fracture reduced plantar flexion isometric torque and isokinetic peak torque by half after 1 week, compared with the uninjured leg (Shaffer *et al.*, 2000). Compared with uninjured controls, fracture patients expressed large functional decrements, particularly in stair descent time which was ~4-5 times longer. These results highlight the importance of early mobilisation and monitored functional recovery following fractures.

1.2.3 Different models of disuse

1.2.3.1 Bed Rest

In order to study the effects of disuse atrophy in a research setting, a number of models have been used to elicit atrophy in vivo. In order to mimic whole-body disuse, monitored bed rest (BR) in a research laboratory setting provides a highly controlled stimulus with which to study disuse atrophy, offering a model that replicates real-life situations of acute hospitalisation and intensive care or prolonged illness requiring long-term BR. This model has been used for over 20 years, and was the basis of a 2006 review collating information on the impact of this model on multitude of organ systems and mechanisms, including skeletal muscle changes (Pavy-Le Traon *et al.*, 2007). This review demonstrated the need for more integrated investigations into the impact of disuse atrophy throughout the body, with the take-home message about skeletal muscle being the most affected muscles appeared to be the knee and ankle extensors. Providing more detail on the impact of BR on skeletal muscle, it has been shown that 20-days bed rest resulted in 7% decline in the knee extensors and flexors (Funato *et al.*, 1997). However, this study established that maximal static force declines for both muscle groups were more pronounced than the loss of CSA, linking back to the previously discussed declines in neuromuscular function

preceding loss of muscle size. Furthermore, eight weeks of BR led to reductions in quadriceps femoris CSA and maximal voluntary torque of ~14% and ~17% respectively (Mulder *et al.*, 2006). Both of these parameters decreased linearly, with MRIs taken fortnightly for CSA and torque measurements at increasing intervals throughout the 8 weeks. The linear time course of these changes contradict more recent findings, albeit using a different model (ULLS), of an initial rapid phase of atrophy followed by a less pronounced period (Kilroe *et al.*, 2020). However, the timing of the testing in the former study may not have been early enough to detect changes in muscle mass. Other than measures of muscle mass, MPS was also shown to be impaired following 2 weeks of BR in middle aged adults by ~30% (English *et al.*, 2016). Leucine supplementation, however, was shown to rescue two thirds of this decline in MPS, resulting in only ~10% declines after the same period. Furthermore, HMB was shown to protect against losses in lean body mass in older adults following 10 days of bed rest (Deutz *et al.*, 2013). These findings show the necessity of adequate nutritional interventions to be put in place when these models translate into real situations of DA. Further to changes in muscle mass, just 5 days of bed rest were found to induce insulin resistance in healthy volunteers (Hamburg *et al.*, 2007), which highlights the underlying metabolic impact that periods of disuse can impose on healthy individuals, while it is likely that this dysfunction would be more drastic in those with underlying health conditions, particularly obesity and diabetes.

In addition to 'simple' bed-rest models, the addition of a -6° head-down tilt (HDBR) has been shown to mimic the shifts of body fluids that occur with spaceflight, providing an additional level of similarity to this real-life situation (Montgomery, 1993). Studies using this model have shown 20 days of HDBR can reduce physiological CSA (measured at a 90° angle to muscle fibre pennation) by ~8% in the knee extensors, ~11% in the knee flexors and ~13% in the ankle extensors (Akima *et al.*, 2000*b*, 2003). 29-day HDBR led to 10 and 16% declines in quadriceps and triceps surae volume (Alkner & Tesch, 2004*a*), while a 30-day study reported 11 and 10.5% declines in the same muscle groups

(Berry, Berry and Manelfe, 1993). In this 30-day study, plantar flexor maximal torque reduced by ~20% while dorsiflexor torque reduced by ~15% (Portero, 1996). 90 days of HDBR resulted in knee extensor volume reduction of 18% while plantar flexor volume reduced by 29% (Alkner & Tesch, 2004*b*). Although a ~10% difference was seen between volume reduction, force reduction was similar, decreasing by 31 to 60% and 37 to 56% respectively. The large amount of heterogeneity between changes in different muscle groups over varying lengths of HDBR studies adds to the need for a true time course of such changes to be established.

In more recent studies, exposure to different degrees of artificial gravity each day during a 60-day HDBR intervention was studied for the effect on the gluteal muscles (Tran *et al.*, 2021). Alongside a control group with no artificial gravity, either 30 minutes continuous or 6 x 5 minutes of artificial gravity, the latter with rest intervals, were assigned to two groups. However, muscle volumes for the gluteal muscles declined between ~8% to ~11% and no attenuation of artificial gravity was observed. Concurrently, the effect of artificial gravity on standing balance and postural stability of the trunk muscles was investigated (de Martino *et al.*, 2021). In this study, some beneficial effects of artificial gravity were seen in postural control, although balance performance was reduced in all groups detailed above. Some potential may be noted in this method to attenuate atrophy related decrements but further refinement is likely required.

1.2.3.2 Unilateral Lower Limb Suspension

Another model of DA, and one of particular relevance to the work presented in this thesis, is unilateral lower limb unloading, or suspension (ULLS); a model first presented in the literature in the early 1990's. This first study of ULLS used a harness to keep the ankle above the ground and prevent weight bearing, with a 5cm platform shoe on the control leg and crutches to facilitate locomotion (Berg *et al.*, 1991). This study was performed with a stated aim to simulate the effects of microgravity experienced by astronauts in space as joint mobility

remained unhindered. A modified version of ULLS was developed shortly after the work of Berg and colleagues was published to allow the leg to be moved more freely. This model involved no harness but a 10 cm platform shoe instead to ensure no accidental weight-bearing on the suspended leg (Hather *et al.*, 1992). The studies using ULLS for the 20 years from the first study in 1991 were collated and reviewed to investigate the multitude of outcomes that had been studied and compare the temporal nature of disuse (Hackney & Ploutz-Snyder, 2012). Data from these studies collectively pointed towards a decrease in muscle size (measured by CSA or volume) of the knee extensors and plantar flexors by approximately 0.4% and 0.36% per day throughout ULLS. This was remarked to be similar to the collective data from similar durations of bed rest, i.e. approximately 0.41% and 0.42% muscle size decline per day in the knee extensors and plantar flexors respectively per day (Narici & De Boer, 2011). Even with this cross-study comparison, the two methods of studying disuse atrophy had not been directly compared. Although it was hypothesised that bed rest and ULLS may show differences in amount of muscle size and strength lost over the same time period of 7 days, a study found that there were no significant differences between quadriceps CSA and KE one-repetition maximum between the methods (Dirks *et al.*, 2016).

1.2.3.3 Step-count Reduction

In addition to the previously discussed models of DA, reduced physical activity is also simulated by step-count reduction (SR) studies. Although this model is less severe in its implementation, the impact of SR is still significant, resulting in reduced skeletal muscle mass and function (Oikawa *et al.*, 2019*b*). Reduction in step count by approximately 76% in older adults for two weeks was shown not only to reduce lean leg mass but also increase insulin resistance while reducing insulin sensitivity (Breen *et al.*, 2013). These findings demonstrate the significant impact this form of DA can have on the ageing population, particularly since periods of reduced activity such as these may often be experienced in order to convalesce from a period of illness or as a result of sarcopenia directly, further exacerbating already present issues. Following a

pilot study of two-week SR combined with energy restriction, again in older individuals, maximal voluntary contraction torque was decreased (Oikawa *et al.*, 2019*a*). Male participants torque was found to have returned to baseline following a single week of normal activity, although this was not the case in the older female group which suggests further strategies should be investigated in this demographic in particular to reduce the impact of these periods of reduced activity.

1.3 Impact of disuse atrophy beyond muscle mass

As illustrated in the sections above, different scenarios and muscle disuse elicit DA. However, disuse has implications for skeletal muscle beyond losses of muscle mass, including alterations in architecture and function.

1.3.1 Impact on muscle architecture

The specific architecture of the muscle has not attracted as much attention as whole muscle mass. This is despite the fact that declines in muscle quality, as measured by muscle fascicle length ($_{F}L$) and pennation angle ($_{P}A$), have been shown to occur with advancing age (Straight *et al.*, 2015). In addition, different aspects of muscle architecture have been shown to be associated with different functional capabilities and the plasticity of these features has also been shown to change following exercise training regimens. For example, $_{F}L$ was increased following 12-week eccentric RET in both heads of the gastrocnemius and the soleus, whereas $_{P}A$ remained similar (Geremia *et al.*, 2019). Increased $_{F}L$ following eccentric RET is theorised to be due to increasing sarcomeres in series within myofibrils, reducing individual sarcomere length and thereby both increasing range of motion and shifting optimal force production towards a longer length muscle (Geremia *et al.*, 2019).

Exemplifying the muscle-specific response of these parameters to a disuse stimuli, a study of ventilated patients in ICU showed that the medial gastrocnemius (MG) did not change in muscle thickness or pennation angle at 5 or 10 days, whereas VL muscle thickness decreased by 11 and 29% at this

time-point, with pennation angle declines of 14 and 29%, respectively (Turton *et al.*, 2016). Following 5 weeks of bed rest however, both VL and MG expressed reductions in fascicle length (~5-6%) and pennation angle (~13-14%) (de Boer *et al.*, 2008). No changes were seen in the TA which suggests atrophy resistance in this muscle following unloading (discussed later in 1.3.4). The contrast in these results could partly be due to duration, since ICU ventilation was relatively short term compared to 5 weeks of bed rest or could be due to patient demographics. De Boer *et al.* (2008) recruited young healthy male participants while Turton *et al.* (2016) did not report the demographics of the patients. They were all above 18 years of age, critically ill without neurological, neuromuscular or muscle wasting diseases among other disease criteria, but other than this we could be seeing results from a wide age range and this makes it difficult to effectively compare the two studies.

ULLS in healthy young males led to significant declines in lateral gastrocnemius (LG) pennation angle after 2 weeks (3%) and both pennation angle and fascicle length after 3 weeks (5% and 4% respectively) (Seynnes *et al.*, 2008). This could suggest that muscle architectural changes are slower to present than changes in mass and neuromuscular function, but there is no further evidence to support that since this appears to be the only study to date which has assessed muscle architecture parameters using ULLS.

1.3.2 Impact on functional capacity

In addition to the changes in muscle architecture, discussed above, situations of disuse also negatively impact muscle function. For example, young, healthy men (~23 years), participating in 7 days of bed rest, exhibited reductions in both leg press 1RM and leg extension 1RM, from ~220kg to ~200kg (Dirks *et al.*, 2016). Although no change in hand grip strength was observed, peak oxygen consumption (VO_2 peak), assessed on a static cycle ergometer, was also significantly reduced by ~6%.

Functional reductions have also been reported in older adults following a period of bed rest. Older males and females completing a 10 day bed rest study

showed a reduced isometric force in the right leg from ~130N to ~120 N (Kortebein *et al.*, 2008). Stair ascent power, calculated from participant's weight and time taken to ascend 10 steps, was measured in 8 participants, showing a reduction following bed rest by ~15%. VO_2 max was also reduced by ~7% in 9 participants. Mean collective score on the short physical performance battery test (SPPBT) remained unchanged following bed rest.

Considering a true clinical implication of DA in relation to muscle function, in older adults sarcopenia is a major risk factor for falls in the elderly, and as such any additional atrophy (e.g., from a period of disuse), inevitably renders an already susceptible cohort to a higher risk of falls and the associated negative consequences (Cuevas-Trisan, 2017). To illustrate this concept, in a study of octogenarians, those diagnosed as sarcopenic based on EWGSOP definitions (at the time) were over three times more likely to experience a fall over a two-year period than those without sarcopenia (Landi *et al.*, 2012). In addition to increased risk of falls and fractures, sarcopenia diagnosis was not reduced following surgical hip fracture repair, although some fluidity was observed, i.e. some previously sarcopenic patients recovered while previously non-sarcopenic patients became sarcopenic (Chiles Shaffer *et al.*, 2020).

1.4 Mechanisms of disuse atrophy

Both cellular turnover and protein turnover are necessary for the regulation of muscle content. These two systems rely on independent signalling pathways in order to produce their effects. Cellular development in skeletal muscle is limited in adults, having a more predominant role in embryonic development (Sandri, 2008). Therefore, the key mechanisms involved in regulating muscle mass in adult humans focus on the turnover of muscle protein as introduced in section 1.1.3. Protein turnover relies on the ribosome, specifically their translational capacity and translational efficiency (McCarthy & Esser, 2010) to produce skeletal muscle proteins from free nucleotides in response to anabolic stimulus and resultant mRNA production. In relation to DA, although the

situations eliciting this physiological effect are varied, a set of transcriptional changes appear to be common (Sandri *et al.*, 2004).

1.4.1 Impaired anabolism

1.4.1.1 Anabolic blunting

Part of the cause of these decrements in muscle mass and strength is the anabolic resistance experienced in ageing, wherein the intake of protein, specifically essential amino acids, no longer produces an adequate response of muscle protein synthesis (Cuthbertson *et al.*, 2005). This resistance expresses itself in the form of reduced phosphorylation of numerous signalling components such as mTOR and p70S6K by comparison to younger participants in addition to reduced MPS as a response to supplementation with protein (Wall *et al.*, 2015). This effect appears to be exacerbated in obese, inactive older individuals, who showed no increases in postprandial myofibrillar protein synthesis following 15 g of milk protein isolate, while lean older and lean younger participants showed some increases (~38% and ~81% respectively) (Smeuninx *et al.*, 2017). Furthermore, the response to essential amino acid intake was blunted in healthy older participants following a 7-day bed rest study, causing a slowing of muscle protein synthesis and no doubt contributing to the loss of lean leg mass observed (Drummond *et al.*, 2012). Anabolic blunting was also evident in young adults following 14 days of ULLS, with immobilisation inducing reduction in post-prandial MPS after 4 hours compared to the non-immobilised leg using a low AA dose (Glover *et al.*, 2008). Interestingly, a higher dose of AAs showed a reduction in MPS of the immobilised leg after both 2 and 4 hours of infusion. Furthermore, evidence suggested that Akt phosphorylation was delayed, suggesting a mechanistic effect of the immobilisation-induced blunting and a potential target for therapeutic countermeasures. In older adults, this effect appears more pronounced, as 14 days of reduced activity (76% less steps) induced a ~26% decline in MPS following a 25g bolus of protein (Breen *et al.*, 2013).

1.4.1.2 Mammalian Target of Rapamycin (mTOR) pathway degradation

IGF1 is upregulated as a response to training, as shown by an increased gene expression following functional overload in mice (McCall *et al.*, 2003). The activity of IGF1 induces the activation of Akt via PI3K. Exercise has been shown to increase activation of Akt1 in vivo within human skeletal muscle (Sakamoto *et al.*, 2004). Downstream of Akt, mTOR (the mammalian target of rapamycin) is subsequently activated. This is a key regulator in muscle protein synthesis, which was shown by blocking it with rapamycin leading to a suppression of muscle growth and hypertrophy in rodents (Bodine *et al.*, 2001). mTOR1, one of the two proteins within the mTOR complex, increases phosphorylation, and therefore activity, of S6K1 and 2 which regulate mRNA translation (Drummond *et al.*, 2012). It also leads to the activation of 4E-BP1, which in turn leads to the upregulation of eIF4F which promotes mRNA translation initiation. 5-day ULLS in young healthy adults reduced postabsorptive and postprandial myofibrillar muscle protein synthesis by ~41% and ~53% respectively compared with the non-immobilised leg (Wall *et al.*, 2014).

1.4.2 Catabolism

1.4.2.1 Ubiquitin-proteasome pathway

One of two primary catabolic mechanisms postulated to be involved in DA is the ubiquitin-proteasome pathway (UPP). The UPP incorporates downstream factors of the IGF1-Akt axis described in the previous section; the Forkhead-box-O proteins, which have been shown to have a role in muscle protein breakdown. This role is the upregulation of MAFbx, or Atrogin-1, and MuRF1, both of which are E3 ubiquitin ligases.

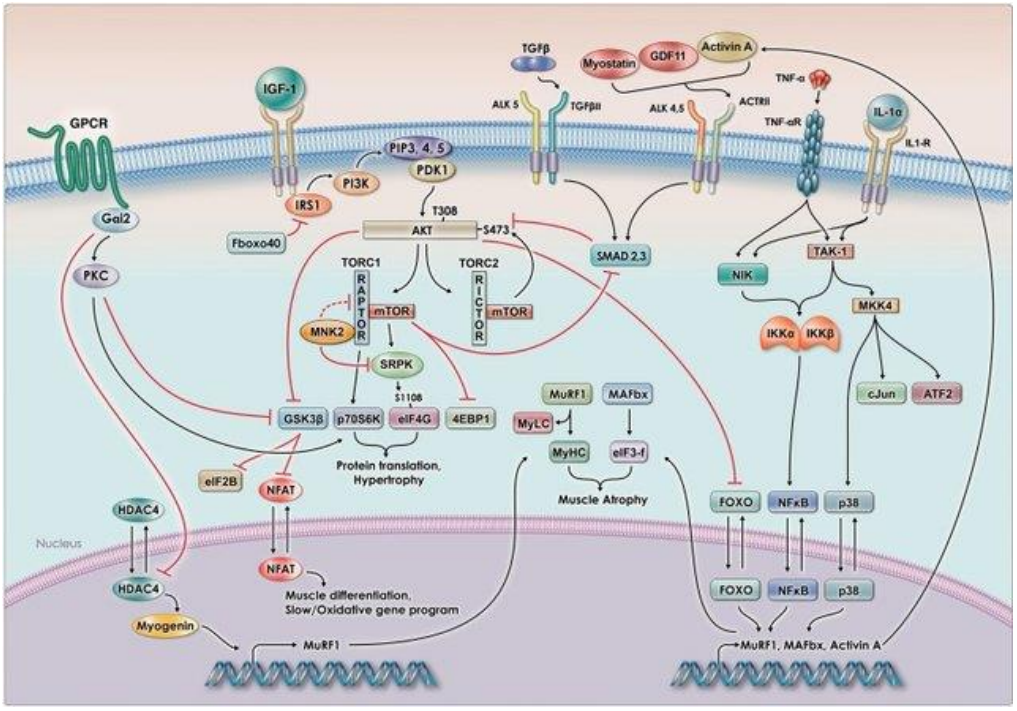


Figure 1.6: *Skeletal muscle protein signalling pathways* (Egerman & Glass, 2014).

The process of ubiquitination begins with the activation of the ubiquitin protein by E1 ligases, followed by a transfer of ubiquitin to the target for breakdown, which an E3 ligase has bound to for marking (Ciechanover, 1994). Depending on the level of ubiquitination, different fates await the marked proteins, such as polyubiquitination leading to protein degradation by the 26S proteasome (Bodine & Baehr, 2014). In the context of disuse atrophy, 8 healthy young men underwent 3 days of ULLS, which resulted in significantly greater levels of MAFbx and MuRF1 mRNA in the vastus lateralis, although not in the soleus, compared to baseline values which did not differ between the muscles (Gustafsson *et al.*, 2010). Protein and phosphorylated Akt 1 did not show a significant difference between either muscle before or after the ULLS period, suggesting that the changes seen in MAFbx and MuRF1 are not completely reliant on the inhibition of Akt activation. Forty-eight hours of ULLS in healthy young male participants did not show an increased expression of MAFbx or MuFR1, which could suggest that this was too short a time for atrophic mechanisms to begin at a significant level in this group of participants (Urso *et al.*, 2006). From 0 to 10 days of ULLS, MuRF1 mRNA expression increased 3-fold

in 5 of a group of 9 healthy young men, although MAFbx mRNA showed no changes (de Boer *et al.*, 2007*b*). The remaining 4 participants were investigated from 10 to 21 days of ULLS, but levels of both MuRF1 and MAFbx mRNA reduced between these time-points. After 2 weeks of immobilisation using ULLS, an increase of MAFbx expression was observed in a group of 9 healthy male participants (18 to 30 years), with a slight but non-significant trend towards increased expression of MuRF1 (Jones *et al.*, 2004). Interestingly, both of these markers were significantly decreased following reloading and immediate participation in a single session of maximal isokinetic KE training. This suggests that although these factors play a role in disuse atrophy, early reloading of the muscle has a marked impact on the ubiquitin-proteasome pathway activity (Jones *et al.*, 2004).

1.4.2.2 Apoptosis

Another mechanism known to play a role in skeletal muscle atrophy is autophagy, the process of programmed cell death (Jiao & Demontis, 2017). This occurs throughout the body to remove old or damaged cells to prevent dysfunction in a particular tissue. Caspase-3 is an important enzyme in apoptosis as it is responsible for cleaving specific proteins in the target cell (Porter and Jänicke, 1999). It is a downstream factor of the apoptosis pathway mediated by cytochrome-C. Mice deficient in this enzyme have shown a decreased atrophic response to hindlimb suspension (HS) (Zhu et al., 2013) and denervation (Plant et al., 2009). Caspase-3 activity was found to increase in young rat models of the soleus during HS and was linked to a loss of muscle fibre nuclei that was not seen in older HS rats (Leeuwenburgh *et al.*, 2005). However, cytochrome-C-independent pathways were seen to be the driver of apoptosis seen in the gastrocnemius of aged rats undergoing HS, while cytochrome-C-linked pathways increased in both young and old gastrocnemius. This suggests a potential shift of preferential apoptosis pathways in ageing muscle although this could be simply a muscle specific difference, the two have yet to be assessed together as far as is known. It was also shown that caspase-3 directly breaks down actin filaments within skeletal

muscle and it was suggested that it may not be the case that increased caspase-3 activity leads to apoptosis as a mechanism of disuse atrophy but the secondary role comes into play in the case of skeletal muscle (Dupont-Versteegden, 2006). Furthermore, a recent review suggested that skeletal muscle fibres do not in fact undergo apoptosis at all (Schwartz, 2019). The explanation of increased activity of apoptotic markers in skeletal muscle cells was suggested to be the degradation of other mono-nucleated cells in the vicinity rather than the breakdown of myonuclei within the fibre itself. This is further evidenced by the presence of anti-apoptosis proteins found within skeletal muscle fibres (Schwartz, 2019). It could be the case that daughter cells of proliferating myoblasts in response to exercise and damage which are not used to increase myonuclear number or repair muscle tissue are the targets of apoptosis which would explain the presence of increased apoptosis-linked activity regardless of the protection of skeletal muscle fibres themselves against this process. There seems to be a lack of literature on these pathways in humans, and as all of the previously mentioned studies have been conducted in rodent models, unlike ubiquitin-proteasome pathways, it is unknown how well this research translates into humans. In terms of the impact of caspase-3 in disuse atrophy, mRNA content was not found to change following 3-day ULLS, which provides little insight into the role it may play in humans (Gustafsson *et al.*, 2010).

1.4.3 Neuromuscular alterations

Disuse atrophy does not only affect the muscle tissue itself, but also the motor nerve as introduced in section 1.1.2. In a recent meta-analysis of 40 studies on the topic of NM decline and disuse, it was reported that, similar to the temporal nature of DA, the majority of neuromuscular decline occurred within the first week of unloading, although it must be noted that only a few studies had investigated such a short timeframe (Campbell *et al.*, 2019). Considering the two main aspects of NM function, central drive and peripheral activation, it has been found upon meta-analysis that central motor drive declines most notably

with upper limb immobilisation, while peripheral activation is decreased to a greater extent with disuse of the lower limb. This disparity is likely attributed to the fact that central neural control is higher in the upper than the lower limbs and as such should be considered when carrying out studies of this nature (Campbell *et al.*, 2019). This suggestion is reinforced by a study investigating central activation of the lower limb during unloading; following 30 days of ULLS, healthy male participants did not show significant declines in central activation of the knee extensors or the plantar flexors but there did seem to be a trend towards decline (Cook, Kanaley and Ploutz-Snyder, 2014). This implies a confirmation of a larger peripheral input to lower limb muscle activation.

Although 7-day immobilisation using ULLS did not induce any significant decline in surface EMG activity, age comparisons showed a greater decline in older participants than younger, suggesting that short-term unloading reduces neuromuscular activity to a greater extent in older adults (Deschenes *et al.*, 2008). However, neuromuscular efficiency was greater in older than younger participants, as measured by dividing maximal isometric torque by average EMG activity. This is likely caused by pre-unloading values of EMG activity being ~50% greater in young than old, and the fact that EMG was not decreased in either age group following immobilisation.

Following 4 weeks of ULLS, soleus CMAP duration was greatly increased, which could be attributed to a number of factors such as slowing in conduction velocity and an increased dispersion in activity between individual motor units (Clark *et al.*, 2006*a*). However, CMAP amplitude was not affected by ULLS, which suggests that although maximal activity remains at a similar level, there could be a reduction in activity of Na^+/K^+-ATPase.

1.5 The time-course of disuse atrophy

Despite suggestions from the available literature that DA (and NM declines) predominate in the early stages of disuse (Wall *et al.*, 2014), few studies have investigated the true time-course of DA. Furthermore, of the numerous studies covering the changes in muscle mass and neuromuscular function with disuse,

a number of different muscles are studied, and diverse outcomes are measured making it difficult to collate even a theoretic time-course of DA. This section does however attempt to synthesise the results of a number of investigations into the effects of disuse over multiple time-points, to elucidate a pattern of changes in muscle mass and structure along with neuromuscular function and the relationships between these parameters.

1.5.1 Changes in muscle mass

While several studies have aimed to investigate the time course of DA, few have been able to achieve good temporal resolution to fully define this time course, since many of the studies have used two individual groups. The reason this aspect of DA is important is that understanding when the atrophy occurs most rapidly will allow timely interventions in those individuals experiencing or at risk of DA. One such study which compared the DA responses of two groups following two different periods of cast immobilisation was published by Wall and colleagues in 2014. This study showed that in young male participants a loss of ~3.5% quadriceps CSA (measured using computerised tomography (CT)) was caused by 5 days in a cast, with losses after 14 days of ~8.5% (Wall *et al.*, 2014). A 0.7% decrease per day over 5 days compared with 0.6% per day over 14 days may suggest that a larger magnitude of effect is seen in the first 5 days compared with the subsequent nine days. However, since these were separate groups of participants and the difference not striking, it may simply be due to individual differences between participants. Furthermore, a single cross-section from a CT scan may not accurately represent the whole muscle volume, although a high correlation coefficient exists between single-slice CSA and muscle volume of the quadriceps (r=0.956, Marcon *et al.*, 2015).

1.5.2 Alterations in neuromuscular function

As with DA, the temporal nature of alterations in NM function with disuse has not yet been well defined. Based on different studies of differing durations, it has been reported that with just one week of forearm immobilisation, maximal

voluntary wrist flexion strength was reduced by 27%, being followed by further reductions in the subsequent two weeks, falling by 31% after two weeks then 41%, after 3 weeks (Clark *et al.*, 2008). Following immobilisation, central activation decreased by 19%. H-reflex, normalised to M-max as a measure of spinal excitability, did not change over the course of immobilisation which could suggest that central properties are resilient to changes caused by disuse. However, previous work by the same group on soleus immobilisation showed an increase in H-reflex after 4 weeks of suspension of the lower limb (Clark *et al.*, 2006*b*). Their explanation of the different findings was the use of different methods of inducing disuse, i.e., immobilisation with a cast compared to limb suspension using a platformed shoe and crutches. Also, there may be intrinsic differences between the muscles studied as to why this difference may be the case, a facet discussed in more detail in the following section.

1.5.3 Alterations in muscle protein metabolism and associated cellular pathways

Alongside larger-scale changes occurring as a result of DA, intracellular changes are also of high importance. One study assessed myofibrillar protein synthesis over 21 days of ULLS, in two subgroups of participants: a) from days 0 and 10, and b) from days 10 and 21 (de Boer *et al.*, 2007*b*). No changes were seen throughout the study in protein level or phosphorylated protein level of components involved in the Akt-p70S6K-mTOR pathway. However, postabsorptive myofibrillar protein synthesis was reduced by half after 10 days and remained maintained at this level following an additional 11 days. This could suggest that other anabolic pathways were reduced to reduce overall synthesis. An increase in catabolic signalling was observed in the *vastus lateralis* following 5 and 14 days of ULLS in a group of 24 healthy young men (Wall *et al.*, 2014). Namely, MAFbx mRNA expression increased by 48% and 40% following 5 and 14 days respectively, while MuRF1 mRNA showed an increased expression by 56% following 5 days but was not different from baseline after 14 days. This could suggest that catabolic changes occur rapidly

following the onset of disuse, but after a slightly longer duration begin to stabilise somewhat to pre-disuse levels. However, this may not be the case when concerning disuse caused by injury or illness as other influences such as the inflammatory pathway may take effect, expressing increased activity of proinflammatory cytokines also associated with muscle atrophy such as TNF-α and IL-6 (Costamagna *et al.*, 2015). Furthermore, since the 5-day and 14-day groups were independent, there may be a greater level of individual difference between participants which may negatively impact the reliability of this suggestion. This appears to be the case in the vast majority of 'time-course' focussed studies which leads to great difficulty when trying to generalise results and accurately predict changes during DA over time.

1.6 Muscle-specific disuse atrophy

1.6.1 Differences in rates of muscle atrophy and structural alterations

As outlined above, lower limb muscle atrophy has been studied using a mixture of models including bed rest and ULLS, with different muscles from the same limb appearing to have divergent atrophy responses to disuse. To exemplify this, following 5 weeks bed rest in 9 healthy middle-aged males, plantar flexor (contains the MG, LG and the soleus (SOL)) area decreased by 12%, while the dorsiflexor (tibialis anterior (TA)) area did not decrease significantly (Leblanc *et al.*, 1988). These findings have been recently supported by further studies where even within a functional muscle group (i.e., the plantar flexors) differences in DA were observed. Volume declines in SOL, MG and LG were 5%, 6% and 5% respectively following 14 days ULLS, with changes of 7%, 10% and 6% respectively following 23 days (Seynnes *et al.*, 2008). Corresponding with the greater decline seen in the MG in this study, and the atrophy susceptibility of the plantar flexors compared to the dorsiflexors in the work of LeBlanc *et al*, patients cast following ankle fractures saw MG CSA declines after 29 days of 23% compared with 19% in the SOL and 17% in the LG (Psatha *et al.*, 2012). Interestingly, in the same study the TA only declined by 11%. A 56-day bed rest study reported similar divergent findings across muscles with MG and TA CSA

losses following 14 days of 9 and 1%, respectively; a pattern that remained at 28 (14 and 1%), 42 (18 and 1%) and 56 (22 and 5%) days (Belavý *et al.*, 2009).

Collating the results discussed from MG CSA, 29 days of casting caused a reduction of ~23% (Psatha *et al.*, 2012), 56 days of bed rest resulted in a decline of ~22% (Belavý *et al.*, 2009) and a study involving 90 days of bed rest saw whole calf muscle CSA decline by ~25% in their control group (Rittweger *et al.*, 2005). Three similar findings from very different durations could suggest that muscle atrophy, at least in the MG or calf muscles, slows significantly after a certain time point. However, this assumes that casting and bed rest cause the same degree of atrophy, and conflicting evidence exists on this topic. Clark (2009) reported that different models of disuse result in differing degrees of muscle wasting, with bed rest inducing greater atrophy. Conversely, Dirks *et al.* (2016) found no significant differences in muscle loss between bed rest and leg cast immobilisation over 7 days.

Following the first Berlin Bed-rest study (Belavý *et al.*, 2009), a similar investigation was carried out to study muscle atrophy changes along the whole length of target muscles. 9 healthy male adults underwent 60 days of head-down-tilt bed rest to investigate muscle specific atrophy using MRI to test the hypothesis that different muscles atrophy to different extents and within a single muscle this decline would not be uniform throughout the muscle length (Miokovic *et al.*, 2012). Scans at the mid-point of the study (day 27 or 28) showed a significantly lower volume of muscle in all anterolateral leg muscles and posteromedial calf muscles, along with the vasti, medial and lateral hamstrings and the adductor magnus. All of these muscles were also significantly smaller compared with baseline at the end-of-study scans (day 55 or 56), with the addition of the adductor longus, sartorius, and rectus femoris muscles. However, given the use of muscle volume as opposed to CSA in this study, it is difficult to compare with the original study and other studies which only used this measurement. Another outcome of this study provides good insight into the variability of CSA changes over the length of the muscle (Miokovic *et al.*, 2012). The data suggested that the majority of muscle atrophy

is not necessarily seen at the level of greatest bulk, which should be considered when studies are focusing solely on CSA as an outcome measure. For example, MG and LG atrophied to a greater extent in their distal region. It is not clear whether any comparisons were done between the mid-point and post-bed rest scans, which could have provided interesting data as to whether rates of atrophy were maintained throughout the study or if they slowed or increased across individual muscles.

It is clear that different muscles respond differently to disuse situations, with stark differences between MG and TA, potentially caused by several factors such as differences in gene expression and therefore muscle protein turnover pathway regulation along with neural adaptations affecting different muscle fibre types.

1.6.2 Differences in neuromuscular function

Following 14 days of ULLS, 8 healthy young male participants showed a reduced plantar flexor MVC, although this reduction did not increase at 23 days but was instead maintained (Seynnes *et al.*, 2008). Normalised EMG, a measure of muscle activation, was decreased in the soleus by 29% after 14 days but recovered to a small extent to a 16% decline after 23 days. LG normalised EMG did not significantly differ at either time point.

1.6.3 Differences in muscle protein metabolism and associated cellular pathways

The majority of invasive studies investigating metabolic and molecular pathways of disuse atrophy appear to be of the vastus lateralis which is not necessarily surprising as it is the muscle of choice for most studies of that nature in many models. However, given the differences in rates of atrophy, structural alterations, and changes in neuromuscular function between different muscle groups, the cellular changes should not be so easily overlooked.

1.7 Interventions targeting disuse atrophy

1.7.1 Rehabilitation

Old and young male cohorts were investigated following 14 days of bed rest and 14 subsequent days of rehabilitation. This was in the form of combined resistance and endurance training, with three sessions per week for a total of six. Quadriceps volume was significantly decreased in both groups, ~8% and ~5% for old and young participants respectively (Pišot *et al.*, 2016). This decline was significantly greater in older males than in younger. Following rehabilitation, there was no reported change in volume from baseline in the young group. In the old group, the text reported a significant difference between baseline and post-rehabilitation values, but this was not displayed on the graphs presented in the paper. The numerical changes were not reported for volume changes in either group following the intervention and as such, the extent of this change cannot be commented upon. Force and power of the quadriceps was also measured by this study, with significant declines only occurring within the old group of ~13% and ~12% respectively (Pišot *et al.*, 2016). Following rehabilitation, only force recovered to values similar to the baseline results, while power remained significantly decreased by ~7%. While a conclusion could be drawn that power declines following unloading are not significantly recovered by concurrent exercise training, the standard deviation of the mean is greater than the mean itself. This suggests a high level of variability in the individual responses to exercise. If the high spread of data in the post-bed rest results is also considered, the force and power may not have declined at all in some individuals. Taking the two together, it could be the case that participants who showed the least decline following bed rest may have recovered by the least amount as well, but this is merely conjectured as individual values are not reported here so we cannot see these changes.

Using the model of ULLS, two weeks of unloading was performed by old and young participants, followed by four weeks of unilateral resistance training on the immobilised leg (Hvid *et al.*, 2010). Baseline maximal isometric knee

extension strength (MVC) was lower in the old group (~2Nm/kg) compared to the young (~3Nm/kg) by 40%, as was dynamic muscle strength (DMS) by 42% in old (~1 Nm/kg) compared to young (~2Nm/kg). Furthermore, maximal isometric strength per muscle volume (mVol) was 27% lower in older participants (~9Nm/cm^2) compared to young (~12Nm/cm^2). Following ULLS, MVC and DMS were decreased significantly in both groups by ~15% and ~25% respectively in old, and ~15% and ~23% respectively in young. Strikingly, these declines are quite similar and contrast somewhat to previously discussed work which found no declines in MVC in young participants following 14 days of unloading via bed rest (Pišot et al., 2016). Both MVC and DMS recovered to baseline values in each group following rehabilitation. However, only the old group saw declines in MVC per mVol following unloading. Regardless of conflicting results in young participants, the results in old participants from Hvid *et al.* (2010) and Pišot *et al.* (2016) could suggest a decreased neural drive during contraction is induced during unloading, a phenomena which has been reported previously in ambulatory participants in this age group (Mau-Moeller *et al.*, 2013).

Older adults lost a significant amount of leg lean muscle mass following 5 days of bed rest while young participants saw no reduction (Tanner *et al.*, 2015). They then participated in 24 sessions of eccentric contractions performed at a high intensity over eight weeks. The decline in mass that the older group saw was recovered to baseline values. Isometric strength was also measured, showing a similar pattern to losses in lean mass between old and young. However, following rehabilitation, this was improved to a significantly greater level than even pre-bed rest values in both groups. It should be noted however that following each training session, participants were provided with 17 g of whey protein as a drink. Therefore, this study reports changes in response to combined exercise and nutritional intervention. This remains an interesting finding but raises the question as to the separate effect of exercise alone or protein provision in each age group. It has already been reported that protein supplementation provided during longer-term bed rest elicits a slightly

protective effect on the transcriptome (Chopard *et al.*, 2009), but it is yet to be investigated whether protein alone can provide benefits following this extended time of unloading, or indeed whether this has an impact in older populations considering the anabolic blunting seen in this age group (Drummond *et al.*, 2012; Wall *et al.*, 2015).

1.7.2 Intra-disuse therapy

Following 20 days of bed rest, a control group saw declines of ~11% in KE force and declines of ~8% in VL CSA, whereas daily resistance exercise protected against these declines (Kawakami *et al.*, 2001). No change was seen in muscle architecture measured by pennation angle following this training, although a significant decline was seen in neural activation (measured using supramaximal evoked twitch during MVC) of the VL in the control (~6%) but again no significant decline was seen in the training group. However, given that the standard deviation of this result was so high, it may have been the case that a control group size of four could well be underpowered to detect such a change. If this is true, then it may also be the same case for the training group which only contained five participants. There are no power calculations reported to support their chosen sample size so these findings may be contentious.

During a longer-term bed rest study, the impact of resistance training with the addition of whole-body vibration on muscle volumes in the leg was investigated in 20 young adults. Following 56 days of bed rest, the control group expressed significant declines of -~13% in vasti volume and ~22% in MG (Belavý *et al.*, 2009). 89 sessions of resistive vibration exercise (RVE) did not prevent a significant decline in MG volume, although at a much lower value than the control group of ~9%. Although VL volume decline trended towards ~3%, the change was not significant, suggesting that this was an effective method in reducing declines of muscle volume during unloading. Whether this was an effect of RET alone or in combination with whole-body vibration however remained to be seen.

To test that question, the previous study was repeated over the same time course and similar participant demographics with the addition of a group performing standard resistance exercise (RET), aiming to find the specific changes caused by the addition of vibration itself. 8 participants were randomised to each group, and similar changes were seen when comparing the control group of this and the previous study. Vasti volume decline was ~15% and MG volume decline was ~25% (Miokovic *et al.*, 2014). This alone suggests a good level of reliability within the protocol and measurement. Comparing the effects of the two interventions, vasti volume showed no significant changes in either group, although trending towards greater decline in the RVE group (~7%) than in the RET group (~1%). For MG, a significant decline in volume was seen in both groups. Although these were similar between RVE (~11%) and RET (~8%) groups, it does not appear that direct comparison was performed between the groups themselves and as such it is unclear as to whether the groups varied significantly. The results concluded that both RET alone and RVE were effective strategies to somewhat or entirely mitigate the deleterious declines in muscle volume caused by this extended period of bed rest.

Another point to note is that the former study involved a much higher load of resistance training, of eight sessions per week (Belavý *et al.*, 2009). In contrast, the latter study involved three sessions per week (Miokovic *et al.*, 2014). Considering their reductions in muscle volume decline were so similar, it suggests that there may be a cut-off point for effective resistance training during this scenario. This raises the question of how little a training load is necessary to see the maximum protective effects. This is an important aspect of resistance exercise training in general, especially in the subject of ageing. The lower the volume of work required would increase the probability that more of the population would be able to carry out such training and therefore maximise the number of people who would see the benefits of reduced DA.

A study of 60 days bed rest in young women investigated the effects of combined resistance and endurance exercise on the skeletal muscle transcriptome during unloading. The control group exhibited a differential

expression pre- to post-bed rest of 472 and 207 genes in SOL and VL respectively, 124 of which were common to both (Chopard *et al.*, 2009). Downregulations were seen in the gene network involved in protein synthesis, RNA damage and repair and also genes involved in cell death, cytoskeleton, and extracellular matrix (ECM) remodelling. Upregulations were seen within the fatty acid metabolism and protein degradation network. Analysis of the exercise group, however, showed a 'correction' of ~90% and ~95% of these transcripts in SOL and VL respectively. An additional group in this study carried out bed rest with a nutritional supplement, with dietary protein content being increased to 1.45 g/kg BW per day, from the 1 g/kg BW provided to the control and exercise groups. This group expressed a moderate 'correction' of ~40% and ~25% of the transcripts in SOL and VL respectively. These data suggests that protein alone may somewhat reduce atrophy-related gene expression during unloading, while exercise alone acts as a highly protective mechanism. An interesting follow-on from this study would be the impact of a combination of these two interventions on the transcriptome, and also a comparison between both male and female along with old and young participants. A further consideration would be the direct functional and structural adaptations that follow from these changes, including a time course effect. It could be the case that these gene expressions are pre-emptive of such changes, and therefore may act as a useful biomarker to measure in similar situations. This way, an intervention could be put in place before direct changes to the skeletal muscle occur.

1.7.3 Prehabilitation

The concept of pre-habilitation has been explored in a number of scenarios, perhaps most prominently before elective surgery as a method to improve patient outcome (Boereboom *et al.*, 2016). This concept in application to DA would derive from the idea to improve fitness or strength as a pre-emptive measure to protect against the decline seen without it, however, it does not seem that this concept has been explored in research. The use of endurance

exercise before a period of unloading has been considered with a focus on the role of heat shock proteins in protecting against DA and by raising their activity before unloading there could be a theoretical reduction of the deleterious effects of this period (Wiggs, 2015). Although this theory may have promise in the future, it is as of yet untested. The question of the temporality of the effect it would have is also an issue, considering certain periods of unloading are prolonged. Furthermore, it would be an unavailable countermeasure if the period of unloading were an unexpected one.

1.7.4 Neuromuscular stimulation

Neuromuscular electrical stimulation (NMES) has been used as an alternative strategy to mitigate the impact of disuse atrophy. It has been shown to implement various positive outcomes when used in disease states, reducing muscle atrophy in chronic kidney disease (Hu *et al.*, 2015) and improving functional mobility in chronic obstructive pulmonary disease (Coquart *et al.*, 2016). Following nine weeks, which included 24 training sessions, of NMES applied the quadriceps in 16 healthy older participants (8 male, 73.1 ± 6.9 years), numerous health-related outcomes improved such as improved maximal isometric torque, increased SPPBT score and reduced timed-up-and-go test time (Kern *et al.*, 2014*a*). Alongside this, mRNA of IGF1 and numerous isoforms was significantly increased, while a down-regulation of MuRF1 expression and a declining trend of MAFbx expression were observed. To investigate the effect of combined NMES and protein intake, a single session of 70 minutes was carried out in 18 healthy older men (~69 years) followed by 20 g of casein (Dirks *et al.*, 2016). Although phosphorylation of mTOR and P70S6K was observed to increase in the stimulated leg, there were no differences between the stimulated and control legs in terms of mixed or myofibrillar muscle protein synthesis rates following stimulation and feeding. NMES has also been shown to restore muscle mass in spinal cord injury patients with paralysed lower limbs, increasing by up to 40% (Bickel *et al.*, 2015). Critical care settings have also made use of NMES protocols to improve measures of

strength and function, preserve muscle mass and restore independence. However, the variation seen in studies of this nature regarding the specific NMES protocol is wide and outcome measures are not standardised (Trethewey *et al.*, 2019). There still remains a need for a well-developed and reliable protocol to be tested and come into practice which would offer the best results for the target population.

1.8 Aims and hypothesis

Based on the current literature, it is clear that studies investigating the impact of DA throughout a wide variety of endpoints are necessary to elucidate the key mechanisms of DA at all levels, from neuromuscular to cellular, and their interactions. Furthermore, it is also necessary to investigate the true time course of these mechanisms to determine when the mechanisms promoting DA begin to increase in activity. This would allow interventional strategies to be put in place at the appropriate time to reduce the impact of DA on the previously discussed systems.

Therefore, the experiments of this thesis will aim to:

a) Investigate muscle size and function impacts and potential neuromuscular mechanisms involved in DA.
b) Determine the time-course of these mechanisms over 15-day unilateral lower limb immobilisation.
c) Investigate novel rehabilitative strategies to counteract the impact of DA.

It was hypothesised that a multifactorial analysis of DA mechanisms will reveal a large neuromuscular impact, and that these will predominantly display their impact in the more atrophy susceptible medial gastrocnemius following 15-day unilateral lower limb immobilisation.

Chapter 2 General methods

2.1 Skeletal muscle size and function measurements

2.1.1 Skeletal muscle ultrasound

2.1.1.1 Cross-sectional area

Skeletal muscle ultrasound CSA scans were taken from the VL, TA and MG (fig. 2.1A, 2.1B and 2.1C). Participants lay supine on a clinical couch with straight legs. For the VL, the mid-belly of the muscle was located by measuring the length from the midline of the patella to the greater trochanter and taking the middle value of that line. Following this, a narrow ultrasound probe (LA523 probe and MyLab™50 scanner, Esaote, Genoa, Italy) was used to find the medial and proximal borders of the muscle where the aponeurosis of the VL intersected with the *vastus intermedius*. Three axial plane images were collected following this line from both legs. For the TA, the muscle length was measured from the midline of the patella to the lateral malleolus of the ankle. 30% of the length was then measured from the midline of the patella to mark the region for scanning. The same narrow probe (LA523, Esaote) was used to collect three axial plane images from this region in both legs. For the MG, the participant was asked to bend their knee to 90° and twist their hip laterally so that the MG was accessible to the operator. The length of the MG was measured from the crease at the back of the knee to the medial malleolus of the ankle. 30% of this length was then measured from the crease of the knee to determine the region for scanning. The narrow probe (LA523, Esaote) was used to collect three axial plane images from this region in both legs. CSA scans of each muscle in both legs were performed before and after the immobilisation period. For each participant, the same operator performed all scans to reduce inter operator bias for within-participant comparisons. The same operator analysed all scans to further reduce inter operator bias.

Quantification of CSA for each muscle was performed using ImageJ (Laboratory of Optical and Communication, University of Wisconsin-Madison, WI, USA).

Images were imported into the software before the scale was set based on the number of pixels relative to the measurement bar on the captured image. Using a polygonal tool, the muscle cross-section was drawn out and area recorded three times for each image then averaged across all images to provide the muscle CSA value used for statistical analysis.

2.1.1.2 Pennation angle and fascicle length

Sagittal ultrasound images of the relevant muscles were captured following the axial plane images as described above. For the VL, the midpoint of the muscle as previously recorded for CSA was used as the starting point to measure for the appropriate scan location. The medial and lateral borders of the muscle belly were marked with a surgical pen and the distance between these points was measured. A wide ultrasound probe (LA923, Esaote) was placed on the intersection of the length and breadth of the muscle. The probe was adjusted in line with the muscle fascicles to provide a clear image of the upper and lower aponeurosis of the VL. Three sagittal images were taken with the probe in the same approximate area to provide a broader view of muscle architecture. For the TA, at 30% of the length of the muscle from the midpoint of the patella as measured for the CSA scan, the medial and lateral borders of the TA were marked with a surgical pen and the distance between these borders was measured. As before, a wide ultrasound probe (LA923, Esaote) was placed on the intersection of the length and breadth of the muscle. The probe was adjusted in line with the muscle fascicles to provide a clear image of the upper and lower aponeurosis of the TA. Three sagittal images were taken with the probe in the same approximate area to provide a broader view of muscle architecture. Similarly for the MG, a measurement between the medial and lateral borders was made to identify the midpoint of the breadth of the muscle. The wide probe was placed here and oriented to provide a clear view of the fascicles along with the upper and lower aponeuroses. Three sagittal images were taken in the same approximate area to provide a broader view of muscle architecture. These images were analysed for $_{P}A$ and $_{F}L$ (fig. 2.1D).

An angle tool was used to measure the $_{P}$A of three fascicles visible in the image. An angle measurement tool was placed along the deep aponeurosis to the origin of the fascicle and then along the fascicle (fig. 2.1D). Three measurements were taken from each sagittal image, providing an average $_{P}$A value based on the nine values recorded across three images. This was done for each muscle at each time point and the values were taken forward for statistical analysis.

The length of individual fascicles was carried out using a line tool drawn from the deep aponeurosis to the superficial aponeurosis (fig. 2.1D). Three fascicles were measured from each sagittal image, providing an average muscle $_{F}$L based on the nine values recorded across the three images taken from each muscle. This average value was taken forward for statistical analysis.

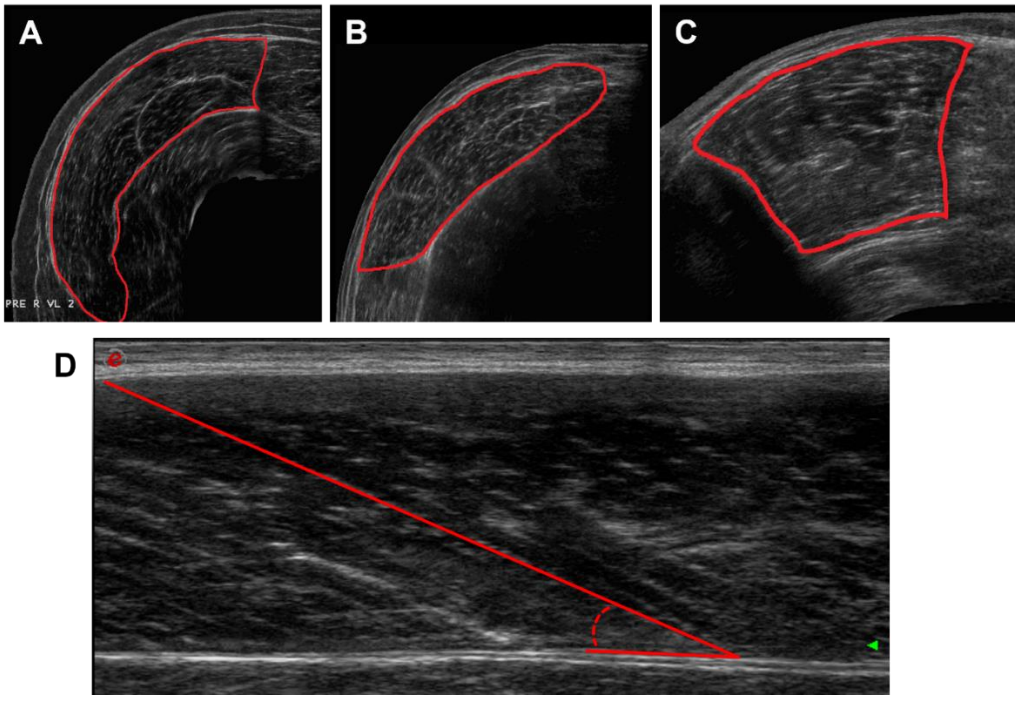


Figure 2.1: *Images taken during muscle ultrasound for cross-sectional area of the vastus lateralis (A), medial gastrocnemius (B) and tibialis anterior (C) and for muscle architecture (D) showing fascicle length measurements from deep to superficial aponeurosis and pennation angle of the fascicle.*

2.1.2 Lower limb power

Overall lower limb power was assessed using a countermovement jump assessment (fig 2.2A). A BTS G-sensor (BTS Bioengineering S.p.A., Milan, Italy) was placed on the participant's waist to sit on the S1 vertebrae secured with a belt. The G-sensor was connected via Bluetooth to G-Studio (version 3.3.22.0, BTS Bioengineering). A countermovement jump was demonstrated before a practice jump was carried out. This jump had the participant stand with feet shoulder width apart, hands on hips, able to bend down up to 90 degrees knee angle before springing up in a smooth motion and landing in an upright position (fig. 2.2A). The participant carried out three jumps with a gap of 30 seconds between each. The jump with the greater maximal power was recorded for analysis along with the respective take-off speed and jump height measurements.

2.1.3 Knee extension power

Unilateral leg extension power was assessed using the Nottingham Power Rig (fig. 2.2B, University of Nottingham; Bassey and Short, 1990). This device allowed the measurement of lower limb power using a leg press plate attached to a flywheel in turn connected to a device which calculated movement power. The seat was adjusted so that the participant's knee joint was at 90° when the foot was resting on the plate. The seat measurement was recorded for the follow-up visit. As the seat did not have a full back rest, this prevented any input from postural changes during the power measurement. The flywheel brake was applied, and the seated participant placed their foot on the plate. Following a countdown from three, the participant was instructed to push as hard as possible as the brake was simultaneously released. A digital display provided a power measurement which was recorded. For each leg, participants carried out two attempts. If these attempts were within 10% of each other, then that concluded this measurement. If the attempts were greater than 10% apart, a final attempt was made. The highest measurement was taken from the two or three attempts.

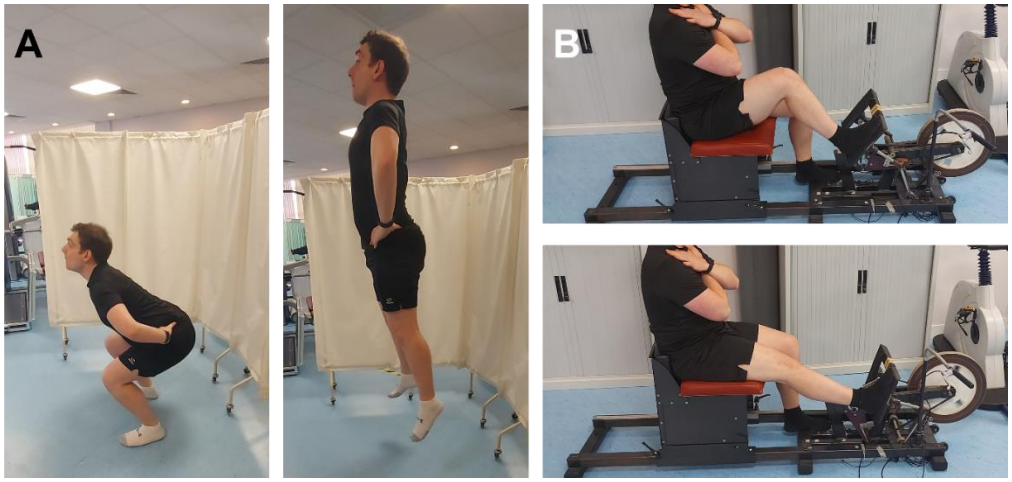


Figure 2.2: *Pictures demonstrating the countermovement jump used to assess bilateral lower limb explosive power (A) and to demonstrate the Nottingham power rig used to assess unilateral leg extension power (B).*

2.1.4 Knee extension strength

Unilateral knee extensor strength was measured using a 1RM test. Participants were seated on the machine with one leg rested behind the extension lever. Five warm-up contractions were carried out at a low weight (fig. 2.3A). Following a 3-minute rest, a single contraction at ~50% of investigator-perceived 1RM was carried out. A modified Borg scale rating perceived exertion from 1 to 10 was presented to the participants who expressed the level of exertion the contraction demanded. With a 3-minute rest between each 1RM attempt, participants carried out one unilateral contraction before assessing their exertion. A 1RM was deemed to have been achieved at a modified Borg scale rating of 10. The same assessment was then carried out on the contralateral leg. The weight value of the true 1RM contraction from each leg was taken forward for analysis.

2.1.5 Handgrip strength

Peak unilateral handgrip strength was measured using a handgrip dynamometer (T.K.K. A5401, Takei Scientific Instruments, Niigata, Japan). Participants were seated at a table with their elbow and forearm of the arm

being tested resting on the table with the wrist in a neutral position holding the dynamometer (fig. 2.3B). The grip width was adjusted to fit the participants' hand. Participants were instructed to squeeze the dynamometer as hard as they could for three seconds before relaxing. With a 30-second rest between attempts, three attempts were recorded from each arm and the peak values were taken forward for analysis. For this measurement, n=10.

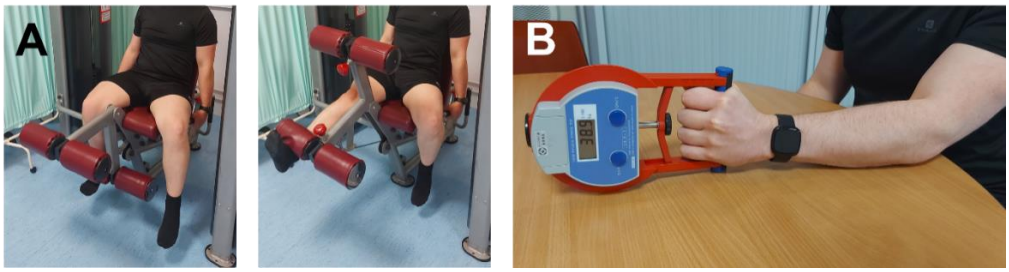


Figure 2.3: *Pictures demonstrating the use of the knee extensor employed to perform one-repetition maximum assessment (A) and to demonstrate handgrip strength assessment (B).*

2.1.6 Gait and balance

General mobility and function was assessed with the TUG (Podsiadlo and Richardson, 1991). Using a BTS G-sensor (BTS S.p.A., Milan, Italy) placed on the L2 vertebrae, participants were seated in a chair without arms. An obstacle was placed 3 metres to the front of the chair. Participants were instructed to stand from the chair without the use of the hands, walk as fast as they were able without running up to and around the obstacle, back to the chair then turn and sit without the use of the hands again. The process was demonstrated to the participant before they carried out two attempts themselves. The quickest time was recorded from G-Studio (BTS S.p.A.) for analysis.

Using a Materialise Footscan 1-metre force plate (Materialise, Leuven, Belgium), balance was assessed for both legs and each leg individually (fig. 2.4A). Participants stood naturally in the centre of the force plate, and this was recorded for 30 s to measure the COP distance moved and ellipse area. For the individual leg balances, participants were instructed to raise their opposite foot

to the rear by at least 5 inches from the floor. They were allowed to use their arms to keep stability if necessary and told to put their foot down if they felt they might fall to avoid injury. They were given a countdown from 5 to start the balance recording and instructed to raise their foot on 2. This prevented balancing longer than necessary. As with both legs, the balances for each individual leg were 30 s and COP distance moved and ellipse area were measured (fig. 2.4B and 2.4C). For these measurements, n=8 as the equipment was acquired midway through the study.

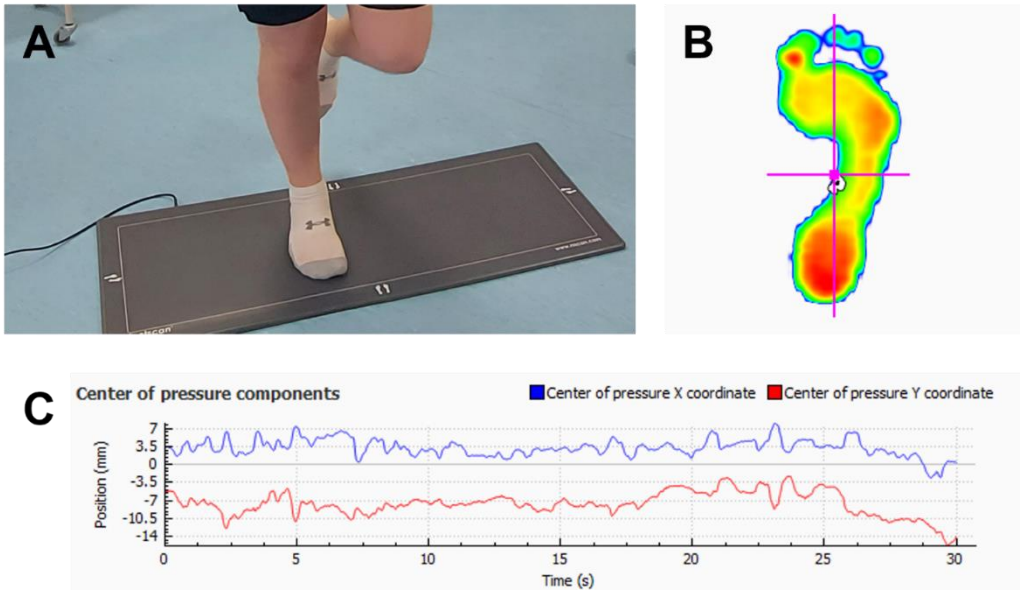


Figure 2.4: *A picture to demonstrate unilateral balance assessment on a force plate (A). A heat map of the foot following 30 s unilateral balance showing the movement of centre of pressure as a white line (B) and a figure plotting the variation in centre of pressure in the X and Y coordinates during the balance assessment (C).*

2.1.7 Maximal voluntary isometric contractions

MVC assessment of the knee extensors, participants were seated in a custom-built isometric dynamometer with their knee joint fixed at 90° while the hip joint angle was approximately 110° (fig. 2.5A). The ankle was secured in place to a plate connected to a force transducer. Three moderate intensity warm-up contractions were carried out with visual feedback of force traces on a screen in front of the participants. Using a waist belt to prevent hip lifting and facilitate

isolation of the knee extensors, participants were verbally encouraged to perform an isometric knee extension at maximal capacity for 3 to 5 seconds. Three attempts were carried out and the highest value was taken as the maximal and used to determine voluntary contraction intensity.

For dorsiflexion MVC, participants were seated in a chair at an appropriate distance from the custom-built ankle isometric dynamometer to set the knee at 90° (fig. 2.5B). The participant's foot was placed on a plate connected to a force transducer with a strap securing the ball of the foot onto the plate. For this contraction, participants were instructed to push the ball of their foot into the plate while keeping their heel on the plate to activate the dorsiflexors. Three moderate intensity warm-up contractions were carried out with visual feedback of force traces on a screen in front of the participants. Following the warm-up, three attempts were carried out with verbal encouragement for 3 to 5 seconds and the highest value was taken as the maximal and used to determine voluntary contraction intensity.

For plantar flexion MVC, participants were seated in the same position as for plantar flexion MVC described above (fig. 2.5B). For this contraction, participants were instructed to dig their heel into the plate while lifting their foot against the strap from the ankle. Three moderate intensity warm-up contractions were carried out with visual feedback of force traces on a screen in front of the participants. Following the warm-up, three attempts were carried out with verbal encouragement for 3 to 5 seconds and the highest value was taken as the maximal and used to determine voluntary contraction intensity. Force signals were measured from their respective force transducers at 100 Hz.

2.1.8 Isometric force control

Force control, measured as force steadiness (FS) was recorded during voluntary isometric contractions carried out following MVC assessment. For each contraction type, participants carried out 2 – 6 voluntary contractions at

intensities of 10%, 25% and 40% of their previously recorded MVC (fig. 2.5C). A target line was visible on screen for participants to follow as closely as they could. Contractions were held for 12 s with a ~20 s rest between them. For analysis purposes, the first two passes of the target line (<1 s) were excluded from calculations to avoid corrective actions when reaching the target line. The coefficient of variation of the force during the recorded contraction was calculated for each contraction and averaged for each contraction intensity for each contraction type.

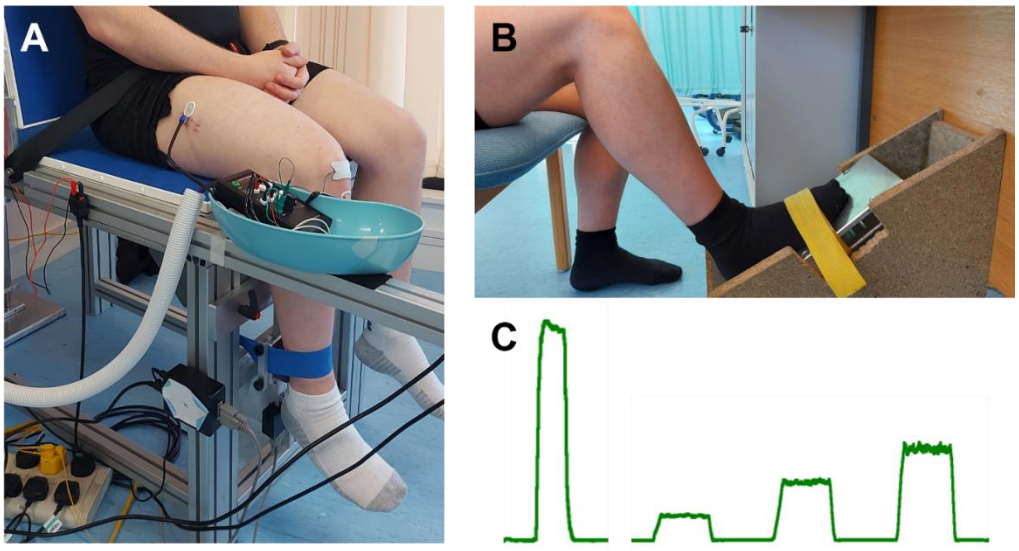


Figure 2.5: *Pictures showing the isometric dynamometer for knee extension maximal voluntary contraction (MVC) and force control (A) and the isometric dynamometer for plantar flexion and dorsiflexion MVC and force control (B). Representative traces of MVC and subsequent force control at 10%, 25% and 40% MVC (C).*

2.2 Electromyography assessments

2.2.1 Vastus lateralis motor point identification

Using low intensity percutaneous electrical stimulation (400 V, pulse width 50 µS, current ~10 mA; delivered via a Digitimer DS7A, Welwyn Garden City, UK), the surface of the VL was explored to find the point producing the greatest

visible twitch with the lowest current, i.e., the motor point (Piasecki *et al.*, 2016*b*).

2.2.2 Surface electromyography

Surface electromyography was arranged in a bipolar configuration. The recording electrode was placed over the identified motor point and the reference electrode over the patellar tendon (disposable self-adhering Ag-AgCl electrodes; 95 mm^2; Ambu Neuroline, Baltorpbakken, Ballerup, Denmark). A ground electrode was placed just above the reference electrode on the patella (Ambu Neuroline Ground). EMG signals were digitised (CED Micro 1401; Cambridge Electronic Design, Cambridge, UK) and Spike2 (version 9.00a, CED) software was used to provide a real-time display of the signal on screen. Peak twitch force was calculated using percutaneous electrical stimulation (Digitimer, UK), and the maximal M-wave was measured with surface electromyography at the motor point. A stimulating pen was placed over the femoral nerve in the inguinal fold and stimulation intensity (400 V, pulse width 50 µS, current typically 70-110 mA) was increased until the M-wave amplitude displayed in Spike2 plateaued. The peak force generated corresponding to the maximal M-wave was recorded.

2.2.3 Intramuscular electromyography

A concentric needle electrode (Ambu Neuroline model 740 25-45/25, Ambu, UK) was inserted at the *vastus lateralis* motor point. The recorded iEMG signals were sampled at 50 kHz and bandpass filtered from 10 Hz to 10 kHz (1902 amplifier, CED). A real-time display was observed using Spike2 (CED) and data were stored offline for analysis. Once the needle was inserted, contractions were carried out at 10% and 25% of the participants MVC following a visual target line. Six contractions were recorded at each intensity, with the needle electrode position altered between each to sample a broader range of MUPs (Jones *et al.*, 2021). From the original position, the needle electrode was slightly

withdrawn, and the bevel rotated 180° at each depth and positioned to maximise signal to noise ratio. These voluntary contractions were held for 12 s with a ~20 s rest in between each contraction.

2.2.4 Electromyography analysis

Decomposition-based quantitative electromyography (DQEMG) was used for all iEMG data analysis. This involved the detection of MUPs and extraction of MUP trains (MUPTs) from individual MUs generated during sustained iEMG signals recorded during voluntary contractions at set intensities. MUPTs were excluded if they contained MUPs from multiple MUs or fewer than 40 MUPs. MUP templates were visually inspected to ensure markers were placed correctly to start, end, positive and negative peaks of the waveforms. MUP area was defined as the integral of the absolute value of MUP values recorded between start and end markers multiplied by the sampling time interval in µV/ms (Piasecki *et al.*, 2021*b*). MUP amplitude was determined as the measurement from the maximal positive and negative peaks of the waveform (Guo *et al.*, 2022). MUP complexity, measured as the number of turns, was defined as the number of significant slope direction changes within the duration of the MUP of a height >20 µV. A near fibre MUP (NF-MUP) was obtained from each MUP by estimating the slopes of each MUP (Piasecki *et al.*, 2021*b*) and NMJ transmission instability, measured as near-NF-MUP jiggle, was determined as the normalised means of median consecutive amplitude differences (Piasecki *et al.*, 2021*b*). MU FR was recorded as the rate of occurrence per second of MUPs within a MUPT in Hz, and MU FR variability was determined as the coefficient of variation of the inter-discharge interval. iEMG derived motor unit characteristics have been assessed as reliable in young and old male participants in the vastus lateralis and tibialis anterior (Piasecki *et al.*, 2018*a*). These methods, although not formerly applied to the study of immobilisation, have been consistently applied to age and sex comparisons of

motor unit and near-fibre motor unit characteristic analysis (Swiecicka *et al.*, 2019; Piasecki *et al.*, 2021*a*, 2021*b*).

2.3 Unilateral limb immobilisation procedure

For the 15 days of immobilisation, a unilateral lower-limb immobilisation model was used. Although limb immobilisation has been reported to impact muscle tone and cellular metabolism in rats (Booth, 1977; Goldspink *et al.*, 1986; Hackney & Ploutz-Snyder, 2012), it was used in favour of ULLS in this study to prevent accidental weight bearing. The knee joint was fixed at 75° flexion using a hinged leg brace (Knee Post op Cool, Össur, Iceland) with the ankle joint fixed using an air-boot (Rebound Air Walker, Össur), ensuring that the immobilised leg was not able to bear any weight. Crutches were provided and adjusted according to the height of the participant, and training on their effective use was provided. The brace and boot remained in place at all times, including sleeping and bathing, with tamper tags attached to each to monitor intervention adherence.

Chapter 3 Determining the effects of 15-days lower limb immobilisation on muscle size, structure, and function in lower limb muscles of young adults

3.1 Abstract

Disuse atrophy, the loss of muscle following muscle inactivity, is well studied in regard to muscle size parameters. Significant knowledge gaps exist in muscle function adaptation to disuse, both in terms of global and specific muscle function, in particular between muscles with diverging atrophy profiles, such as the atrophy susceptible medial gastrocnemius and the atrophy resistant tibialis anterior.

13 male participants were recruited to undergo 15-day unilateral limb immobilisation of the knee and ankle joint, with the contralateral leg acting as an internal control. Before and after the immobilisation period, muscle ultrasound to measure muscle size and architecture was performed along with a battery of functional tests including MVC, bilateral and unilateral power assessments, balance, gait, and strength measurements.

The key findings were a reduction in the muscle cross sectional area of the immobilised VL (−12%, $p<0.001$), corresponding with reductions in knee extensor MVC (−29%, $p<0.001$) and strength (−19%, $p<0.001$). No changes were seen in any measure of size, architecture, or specific function of the control leg aside from dorsiflexion MVC reduction (−16%, $p<0.05$). Reduction was also seen in unilateral power output (-28%, $p<0.001$) and balance performance (31%, $p<0.05$) of the immobilised leg with a reduction in bilateral jump height (−18%, $p<0.01$) and take-off speed (−9%, $p<0.01$). A reduction in gait speed following immobilisation was also present (10%, $p<0.01$). The MG only expressed reductions in measures of muscle size (−13%, $p<0.05$) and MVC in the immobilised leg. The TA went against expectations in also expressing a

change in dorsiflexion MVC (−23%, $p<0.01$), although no alterations to muscle size or architecture as expected. No muscle size or force, measured by MVC, adaptation in the immobilised leg were able to explain the decrements in unilateral power of that same leg or of reduced jump take-off speed.

In agreement with the hypotheses, no decrements were observed in any muscle of the control leg for muscle size and structure, and most function measurements, although unexpectedly control leg dorsiflexion MVC did decrease. The VL expressed the greatest decrements in muscle size following immobilisation. The MG did reduce, and the TA resisted atrophy. In the case of muscle function, reduction was seen in the knee extensors and plantar flexors following immobilisation as expected, while dorsiflexion MVC also reduced. Unilateral leg extension power also reduced, a decrement which reduction in VL size or strength did not significantly contribute to a greater extent than other variables. This may suggest that other factors, such as neuromuscular adaptations, may be present in muscles not expressing reductions in size or function.

3.2 Introduction

3.2.1 Human skeletal muscle

As introduced in Chapter 1 (section 1.1.1), skeletal muscle is an organ with multiple roles, being crucial for mobility and function while also encompassing metabolic and thermal regulation (Ivanenko *et al.*, 2005; Jensen *et al.*, 2011; Schutz, 2011). To focus on the former and primary role of this organ, it enables us to carry out activities such as those important for daily living at the simplest level such as standing from a chair to a more complex motor control task such as making a cup of coffee. These are driven by the action potential propagated along the motor nerve to the connected muscle fibres, which form the motor unit, the most basic functional part of the neuromuscular system responsible for successful muscle contraction (Enoka & Farina, 2021). The following sections will introduce methodologies and previous findings for the measurements of muscle size, structure, and function, both broadly through the lens of ageing and sex differences and more specifically in the context of disuse atrophy where findings are available.

3.2.2 Skeletal muscle size and structure

MRI remains the gold standard technique for the measurement of human skeletal muscle size in healthy individuals and is commonly used by investigations centred around human muscle adaptation to provide measures of muscle volume and CSA (Mijnarends *et al.*, 2013). However, MRI is expensive and time consuming, requiring large facilities and highly trained operators. As an alternative to MRI, skeletal muscle ultrasound has been employed as a cheaper, quicker, and portable method for the measurement of skeletal muscle size and architecture (Naruse *et al.*, 2022). Change in muscle thickness, measured from the superficial to the deep aponeurosis of the VL using transverse ultrasound scans, was found to correlate highly with change in MRI-derived anatomical CSA following a 12-week resistance training protocol (Franchi *et al.*, 2018). This highlights the reliability of skeletal muscle ultrasound

to not only correlate with the gold standard of MRI muscle size measurement at a single time-point as previously reported in young (Reeves *et al.*, 2004*a*) and old (Sipilä & Suominen, 1993) individuals, but also across hypertrophic adaptation to resistance exercise (Franchi *et al.*, 2018). Muscle thickness at mid-thigh, measured from the rectus femoris and vastus intermedius using ultrasound, also correlated highly with muscle volume using MRI in a broad age range of men from 20 to 70 years (Miyatani *et al.*, 2002), emphasising the reliability of this comparison across the lifespan. The same group also saw a correlation between muscle thickness of elbow flexors and extensors, knee extensors and plantar flexors with muscle volume derived from MRI (Miyatani *et al.*, 2004), suggesting this is not solely limited to the muscles of the quadriceps. However, the prediction of muscle volume from just muscle thickness as an independent variable was found to be less reliable than when incorporating limb length into multiple regression equations, an aspect which bears consideration when attempting to estimate muscle volume in this way (Miyatani *et al.*, 2004).

Muscle structure is commonly measured using ultrasound which has been validated as reliable for static measurements (May *et al.*, 2021) and during active contractions (van Hooren *et al.*, 2020). Muscle thickness (MT) is the length between the peripheral and deep aponeurosis of the muscle. Fascicle length ($_{F}L$) is the length of the individual fascicles within the muscle from deep to peripheral aponeurosis, with increases in $_{F}L$ representing addition of sarcomeres in series (Franchi *et al.*, 2014). Pennation angle ($_{P}A$) is the angle at which muscle fascicles extend from the deep aponeurosis as the origin of the fascicle, with greater $_{P}A$ representing an increase in contractile material bundling at the tendon aponeurosis (Kawakami *et al.*, 1993). Knee extensor resistance training was found to induce significant increases in VL $_{F}L$ following 10 days of training, followed by quadriceps CSA increase at 20 days and an increase in $_{P}A$ after 35 days (Seynnes *et al.*, 2007). An early increase to $_{F}L$ ahead of other parameters suggests sarcomeres may be added in series before other adaptive responses occur. This is consistent with findings from a training study

targeting improvements to muscle power using plyometrics: VL volume and $_{F}L$ were increased after two weeks of training, followed by quadriceps volume at four weeks and then $_{P}A$ increase at six weeks (Monti *et al.*, 2020). These studies suggest that, at least following training of the knee extensor muscles, $_{F}L$ precedes $_{P}A$ in architectural adaptation to exercise. Interestingly, resistance training for 14 weeks was found to increase both the $_{F}L$ and $_{P}A$ of older individuals in the VL, with resting $_{F}L$ increasing ~8% at a 10° knee angle throughout the knee extension range of motion to ~10% by 90°, while the opposite effect was observed for $_{P}A$ which increased ~28% at 90° through to ~35% at 10° (Reeves *et al.*, 2004*b*). These results may have implications for the appropriate knee angle measurement of studies investigating these parameters.

There are contrasting findings of the baseline muscle architecture of older adults, however. When comparing young (between 27 and 42 years old) and older (70 to 81) men, older men were found to have a ~10% reduction in $_{F}L$ and a ~13% reduction in $_{P}A$ of the medial gastrocnemius (MG) (Narici *et al.*, 2003). This was corroborated by a further investigation into the broader triceps surae comparing ~25 year old men and ~74 year old men: the older cohort had significantly reduced $_{F}L$ in the MG and also reduced $_{P}A$ in the MG, lateral gastrocnemius and soleus muscles (Morse *et al.*, 2005). Alternatively, in mixed sex groups of young and older individuals, ~24 and ~70 years old respectively, no difference was found between the groups for $_{F}L$ and $_{P}A$ in any of the muscles of the triceps surae (Pinel *et al.*, 2021). However, there may be a sex difference effect confounding these data as this study used a mixed sex group and, when studying female participants exclusively, no differences were observed between younger and older women in $_{F}L$ and $_{P}A$, albeit from the VL (Fitzgerald *et al.*, 2020). As such, older men may be more responsive to resistance training adaptations of muscle architecture.

3.2.3 Skeletal muscle function

While muscle size may be measured using a number of methods, the term 'muscle function' is more nebulous. In this case, function is used in the sense of physical capacity rather than the multitude of functions exhibited in skeletal muscle at a cellular level. Numerous different methods have been employed in various different contexts to measure various aspects of skeletal muscle function. Certain measurements of muscle function provide broader measures of global muscle function such as complex movement cycles such as gait, postural balance which incorporates multiple muscles acting harmoniously and explosive jump performance assessing collective lower limb muscle explosive power. Alternatively, measures may provide a more specific output of an individual muscle or muscle group. 1RM tests measure the strength of a given muscle group carrying out a movement such as the knee extensors. Similarly, MVCs measure the isometric force of a contraction, such as plantar flexion and dorsiflexion.

Some methods used to measure more global muscle function are accompanied by a scale or framework of assessment which will define the status of that individual by their capacity to carry out the given task. For example, 'timed up-and-go' (TUG) time is used to predict risk of falls based on the score, i.e., the time taken to completion and was originally implemented to assess frailty in older people (Podsiadlo & Richardson, 1991). It was developed to standardise the measurement of the 'up-and-go' test which was scored from one to five by an observer based on their perception of likelihood to fall which was liable to inter-operator bias (Podsiadlo & Richardson, 1991). Although reliability to predict future falls has been questioned due to protocol variability and lack of longer-term follow-ups (Beauchet *et al.*, 2011), it has been associated with a history of falls in older (>65) individuals (Shumway-Cook *et al.*, 2000). In a clinical context, it is used in the NHS for the diagnosis of high fall risk in different cohorts with individual cut-off times ranging from 13.5 seconds in community dwelling adults to 32.6 seconds in frail elderly (NHS Digital, n.d.).

Measurement of more specific muscle function can also be carried out in a number of ways. One of the most common is the use of a MVC which provide a value of maximal force exerted by a muscle group or, when normalised to lever length, maximal voluntary torque (MVT). These measurements typically precede neuromuscular assessments which will be discussed in later chapters. However, as a measure of muscle strength they still provide useful insight into muscle function of different cohorts and different muscle groups. For example, comparing young and older women, ~71% reduction in plantar flexion MVT was observed in the older women (Vandervoort & Hayes, 1989). Reductions in plantar flexor MVC has also been observed between young and older men, although not to the same extent, of ~32% (Bemben *et al.*, 1991). Similar gradual decline in plantar flexor MVT across the age span has been presented more recently, however between healthy young, middle aged and older individuals, dorsiflexor MVT was not significantly reduced (Cattagni *et al.*, 2014). In the knee extensors, ~32% reduction of MVC force in older compared to younger men (Piasecki *et al.*, 2016*c*) while in the knee flexors, ~50% MVT reduction was seen from young to older men (Kirk *et al.*, 2018). Although different muscle groups express varying deficiency in MVC or MVT with age, it remains clearly a common factor of skeletal muscle adaptation to age.

Different forms of muscle power measurements are also used, such as jump power, handgrip dynamometry, leg press power and strength, to investigate dynamic movement. These have also been tested against measures of physical performance, including gait speed, walk time and repeated sit-to-stand time. Jump power was reported to have a greater association with quicker gait speed compared to other measures of muscle power (Winger *et al.*, 2021). In contrast, all other measures of muscle power were better associated with chair stand speed than jump power. These findings may suggest, at least in older individuals, that selected methods of assessing muscle power are more appropriate when investigating different measures of physical performance and also that physical performance may benefit from a varied training regimen to target multiple aspects of performance. Power relative to lean leg mass is

also seen to reduce throughout the lifespan from ~40 years old when compared to a young adult group (Alcazar *et al.*, 2020). The initial decline in relative power was driven by a reduction in absolute muscle power, while after the ages of ~65 and ~75 in men and women respectively, relative lean mass reduction added to the continued decline of absolute power with a more even responsibility. Handgrip strength, measured by a handgrip dynamometer, has been a consistently used method to assess sarcopenia due to the strong association of low handgrip strength and multiple unfavourable outcomes which severely reduce quality of life (Cruz-Jentoft *et al.*, 2019). It was also found that asymmetry between bilateral handgrip strength values predict an increased risk of falls and subsequent fractures in older men (McGrath *et al.*, 2021). Conversely, this was not the case with asymmetry between bilateral leg extension power measurements as no association between these events was observed. This adds to the evidence that different muscle power and strength measurements may not individually provide the entire picture of physical performance and risk of adverse events, particularly in older age.

Balance performance is one such measure which provides a measure of multiple muscle function and also may relate to adverse events experienced in older age. While peak torque of plantar flexor MVT was not significantly correlated with single leg standing balance, there were significant negative associations observed with centre of pressure (COP) displacement and normalised rate of torque development (RTD) in a mixed-sex group of older adults (Ema *et al.*, 2016). However, when sex was separated, significant correlations were observed only in men between COP displacement and normalised RTD, which suggests that the mixed-sex findings were primarily driven by men and plantar flexor RTD may be less relevant to the balance performance of older women. The alternative may be found in the hip extensors, which was measured alongside dynamic balance performance in young and older women. A reduced balance performance, which was reflected by higher scores on the overall stability index which in turn relates to a greater risk of falls, was observed in older women compared to the younger

counterparts (Palmer *et al.*, 2017). Alongside this, older women expressed a reduced absolute peak torque and absolute RTD measured from 0 to 50 ms and 200 ms. Negative correlations were observed between overall stability index and early RTD in older women only (Palmer *et al.*, 2017), which may suggest that reduced balance performance and the associated greater risk in falls may be caused by early torque development of the hip extensors rather than later torque development or peak torque. In terms of relation to adverse events, plantar and dorsiflexor MVT and COP displacement over a 30 second static balance task were compared across four groups: young, middle-aged and healthy older adults along with older adults who had experienced a fall six months prior to the study (Cattagni *et al.*, 2014). Across the groups, the normalised total plantar and dorsiflexor MVT and normalised total centre of pressure displacement expressed a strong negative correlation, suggesting that overall weakness in the lower limb postural muscles presents a greater risk of falls in older age. However, this study did not report the sex of the participants and as mentioned previously, plantar flexor MVT and RTD was not associated with balance performance in older women (Ema *et al.*, 2016). With this in mind, it is difficult to interpret whether any sex differences may have impacted these findings.

3.2.4 Skeletal muscle size and function relationship

Muscle size and muscle strength are intrinsically linked (Young *et al.*, 1984, 1985). Strong correlations were found between MVT of the knee extensors and quadriceps femoris (QF) muscle volume, moderate correlations between MVT and both QF anatomical CSA (ACSA) and physiological CSA (PCSA) while MVT correlated weakly with QF muscle thickness (Balshaw *et al.*, 2021). These same methods were used to measure triceps surae size, along with dual x-ray absorptiometry (DXA)-measured lower leg lean mass and circumference, which were correlated against plantar flexor MVC (Bamman *et al.*, 2000). In contrast to the findings from the VL, ACSA and PCSA correlated more strongly with MVC than muscle volume, with other methods even less so. The negative impact of

ageing on the relationship between muscle size and muscle strength has also been documented. Studying cohorts of middle-aged (55 to 64), 'young-old' (65 to 74) and 'old-old' (75+), an age-related deterioration in muscle strength per unit of muscle size, in this case lean muscle mass, was observed (Reed *et al.*, 1991). Although arm strength remained moderately correlated with mid-arm muscle area in those >65 years, this contrasted with a weak correlation of knee extensor strength with mid-thigh area, suggesting a differing trajectory of muscle groups in older age. More recently in older women (~78 years old), thigh muscle volume measured by MRI was found to be associated with a multitude of functional outcomes (Lindemann *et al.*, 2016). Sit-to-stand performance power had the strongest association followed by moderate associations with leg extension power, while weak correlations were seen with handgrip strength, fast and preferred gait speed. In this case, volume may be seen as a useful determinant of sit-to-stand and leg extension power. However, care should be taken when interpreting these results as hand-held dynamometers, as used in this study, may not be entirely reliable for the measurement of knee extension power (Chamorro *et al.*, 2017) while handgrip dynamometers may also lack reliability in predicting overall muscle strength in healthy young and old individuals (Yeung *et al.*, 2018). Further supporting a deterioration in the relationship of muscle size and function in ageing; while healthy older men and male masters athletes did not have altered TA CSA compared with young participants, both older groups expressed reduced dorsiflexion MVC (Piasecki *et al.*, 2016*a*). This suggests other factors are responsible for age related decline in muscle function which is not attenuated by lifelong exercise.

In terms of gait performance, across young and old age groups neither leg muscle strength of the plantar flexors, knee and hip extensors, nor leg lean tissue mass were found to predict gait speed at either preferred or maximal speed (Muehlbauer *et al.*, 2017). Although limited by the use of bioimpedance analysis (BIA) to measure lean mass rather than the gold standard DXA, part of these findings correspond with previously mentioned results that jump power

provided a better predictor of gait performance than other measures (Winger *et al.*, 2021). Furthermore, while significant, only weak correlations with muscle volume, comparative with muscle mass, were seen with both fast and preferred gait speed (Lindemann *et al.*, 2016), suggesting that not all measures of muscle size and function share close or predictive relationships.

Considering the influence of training on the muscle size and function relationship, comparisons between long-term trained individuals and untrained individuals unsurprisingly revealed greater muscle size and strength in those consistently performing resistance exercise for at least three years (Maden-Wilkinson *et al.*, 2020). Further analysis of the contributions of muscle function, size and architecture parameters revealed that the greater MVT of trained individuals was primarily driven by the increased size rather than other variables, such as specific tension. Skeletal muscle mass of the arm and torso estimated from segmental bioimpedance analysis has also been found to correlate with shoulder strength derived from five different shoulder movements measured using the Nottingham Mecmesin myometer (Alizadehkhaiyat *et al.*, 2014). These findings, in young adults, were also independent of sex differences which may suggest that a closer relationship between muscle size and muscle strength measurements exists in younger individuals contrasting to the previously mentioned more diverging relationship expressed in older individuals.

3.2.5 Impact of disuse on skeletal muscle

To contrast with the research centred on understanding the growth of skeletal muscle in terms of both size and function, the loss or atrophy of skeletal muscle during unloading provides a different picture. Skeletal muscle DA has been discussed in the previous chapter and briefly can be described as muscle loss as a result of unloading. DA occurs in numerous situations such as spaceflight, limb casting for orthopaedic treatment and intubation, with various models designed for its study for research purposes including ULLS, bed rest and step count reduction (Rudrappa *et al.*, 2016). Each model has its own strengths and

limitations, such as the much more resource dependant bed rest studies which may provide a closer representation of acute hospitalisation scenarios. Conversely ULLS allows a less restrictive and resource dependent approach but is argued to not precisely reflect all cellular changes observed in bed rest and space flight (Widrick *et al.*, 2002). However equivalent reductions in muscle mass and strength were observed in age-matched groups which underwent seven days of bed rest or ULLS (Dirks *et al.*, 2016*a*).

3.2.5.1 Size and structure

To summarise, muscle size and structure changes in response to disuse (previously introduced in Chapter 1 sections 1.2.3 and 1.3.1 respectively), results from bed rest and ULLS studies will be considered here, with a focus on the muscles of the quadriceps. Quadriceps femoris CSA was reduced by ~3% following seven-day bed rest (Dirks *et al.*, 2016*c*), while ~5% and ~10% reductions in knee extensor CSA were observed after 14 and 23 days of ULLS respectively (de Boer *et al.*, 2007*a*). Following 20 days of HDBR, knee extensor physiological CSA was reduced by ~8% (Akima *et al.*, 2000*b*) while 29-day HDBR reduced QF volume by ~10% (Alkner & Tesch, 2004*a*). Further, after 8 week bed rest, QF CSA reduced by ~14% (Mulder *et al.*, 2006) and lastly following 90-day HDBR, knee extensor volume reduced by ~18% (Alkner & Tesch, 2004*b*). Although various methods and muscle combinations were used in these studies, in muscles of the quadriceps there appears to be a steep initial reduction of size which eventually stabilises and plateaus. In terms of structural adaptations, ~3% reduction in VL_{PA} was seen after 14-day ULLS, which declined further to ~8% after 23 days and was accompanied by ~8% reduction in $_{FL}$ (de Boer *et al.*, 2007*a*). In contrast, 5-week bed rest caused only a ~6% reduction in $_{FL}$, although a greater reduction of ~14% in $_{PA}$ (de Boer *et al.*, 2008). A similar reduction in VL_{PA} after merely 5 days of ventilated intubation in intensive care, which almost doubled after 10 days to ~29%, highlights the severe additional consequences of the conditions which require such treatment (Turton *et al.*, 2016).

3.2.5.2 Function

Considering muscle function (covered in Chapter 1 section 1.3.2) of the quadriceps, ~9% reduction in both leg press and knee extension one-repetition maximum (1RM) was observed after 7-day bed rest (Dirks *et al.*, 2016*c*). Knee extensor MVT reduced progressively by ~15% and ~21% following 14- and 23-day ULLS (de Boer *et al.*, 2007*a*) while after 8-week bed rest, this was only reduced by ~17% (Mulder *et al.*, 2006). These collective findings further suggest that a similar pattern to muscle size loss is mirrored by function in the muscles of the quadriceps. However, this may not be the case for muscle groups which express diverging responses to situations of disuse atrophy. Although further methodologies for the investigation of muscle function have been introduced, a significant knowledge gap exists in measurements of global function in the context of disuse atrophy. While the relevance of specific muscle or muscle group measures such as 1RM, MVT and MVC cannot be understated for determining the specific impact of periods of disuse atrophy, the impact on postural balance and gait remains unclear. These more global measures of function are also of greater relevance to older populations in which age-related decrements may be further exacerbated by short-term periods of disuse.

3.2.6 Diverging atrophy profiles in disuse

The majority of studies into DA have focused on the VL muscle of the quadriceps. A wealth of data exists on numerous aspects of human muscle physiology in this muscle due to the functional relevance, ease of testing and, particularly relevant to DA studies, a moderate susceptibility to atrophy. However it has been known for some time that different muscles express diverging atrophy profiles, as after 5-week bed rest, plantar flexors reduced in area by 12% with no change in the dorsiflexors (Leblanc *et al.*, 1988). Further to this, muscles from the same muscle group have also been shown to express diverging degrees of DA. Reductions of 23%, 19% and 17% were observed in the MG, soleus and LG respectively (Psatha *et al.*, 2012). However, it should be noted that this study was conducted in patients with ankle fracture rather than

healthy participants. The association with fractures and muscle atrophy has been reported previously, mainly in the context of ageing and sarcopenia where muscle atrophy is often seen to both precede fractures and become exacerbated following treatment (Kramer *et al.*, 2017; Wong *et al.*, 2019). Furthermore, localised inflammation following injury may limit muscle regenerative capacity and exacerbate muscle atrophy (Howard *et al.*, 2020). This study had a mixed sex group of participants with a broad age range between 22 and 73 years old, a limitation mentioned by the author, which should be considered when interpreting these results (Psatha *et al.*, 2012). However, the cohort was screened for general health, aside from the ankle fracture, before taking part in the study which likely excluded any individuals with sarcopenia which may have altered the results due to the known associations. Nevertheless, diverging atrophy profiles of muscles in response to disuse have also been reported in healthy individuals with no potentially confounding impacts of age or health status.

3.2.6.1 Size and structure

To focus on diverging changes in muscle size, as mentioned in Chapter 1 (section 1.6.1), much of these findings come from the use of bed rest models. To summarise previously mentioned examples with this method, 5 weeks induced a reduction of ~12% plantar flexor muscle group area while dorsiflexors were unaffected (Leblanc *et al.*, 1988). This corresponds with findings following 20-day HDBR, in which the plantar flexor PCSA was reduced by ~13% with no change in the TA (Akima *et al.*, 2000*a*). This was also accompanied by ~12% PCSA reduction in the knee flexors compared to the previously mentioned ~8% reduction in the knee extensors. More recently, after 56-day bed rest, MG CSA was reduced by 22% with the TA only reducing by 5% (Belavý *et al.*, 2009*a*). From this evidence it appears that the TA eventually experiences a small degree of atrophy between five and eight weeks of disuse, while the plantar flexors atrophy much earlier and continue to do so. Differential atrophy has also been observed within the same functional muscle group. As described in the aforementioned section, soleus, MG and LG volume

reduced following 23-day ULLS (Seynnes *et al.*, 2008). In terms of structural adaptations, less evidence is present. The VL and MG have been shown to express similar changes following 5-week bed rest, with respective $_{F}L$ reductions of ~6% and ~5% and $_{P}A$ reductions of ~14% in both (de Boer *et al.*, 2008). This contrasts with changes observed in knee extensor and plantar flexor volume changes, (Alkner & Tesch, 2004*a*) and after 90-day HDBR (Alkner & Tesch, 2004*b*). This suggests that architectural changes do not necessarily follow changes in total muscle volume. However, it should be noted that the latter two studies incorporated the collective muscle groups rather than the individual VL and MG muscles, and adaptations in the muscles without architectural data may be responsible for the varying changes in volume. While muscle size of the lower limb muscles is well characterised following periods of disuse, structural adaptations are less well studied. This knowledge gap may prevent the optimal rehabilitation strategy from being enacted following disuse, as previously discussed evidence may suggest greater decrement in $_{P}A$ may be attenuated by concentric exercise while reduction in $_{F}L$ may be attenuated by eccentric-focused exercise.

3.2.6.2 Function

Although diverging profiles of muscle size reduction are well established, whether this holds true for muscle function is less clear. Following 10-day bed rest in older individuals, knee extensor MVC reduced by ~11% while knee flexor MVC reduced by ~14%, although it was not stated whether these slightly dissimilar findings were significantly different from each other (Kortebein *et al.*, 2008). A reduction in plantar flexion MVT of 10% was reported following 14 days of ULLS which then persisted to day 23 has been reported previously (Seynnes *et al.*, 2008). However, while dorsiflexion MVC was stated to be recorded in this study, the values were not reported. This presents a significant knowledge gap in the context of the application for which disuse atrophy is studied. If there is a diverging response to disuse in muscle *function* alongside the established response in size across muscles, then this may inform the

priority of targeted rehabilitation to those particular muscles or muscle groups which might express a greater reduction in muscle function.

3.2.7 Aims and hypothesis

The primary aim of this chapter is to investigate muscle size and structure and function adaptation to disuse atrophy using unilateral limb immobilisation across muscles with previously established diverging profiles of muscle size loss. A secondary aim is to address the knowledge gap of diverging profiles of disuse across a broad spectrum of muscle function outcome measures.

Based on previous research, the following hypotheses were made:

- Muscle size of the immobilised limb will decline to the greatest extent in the medial gastrocnemius, followed by the vastus lateralis while the tibialis anterior will resist changes.
- No adaptations in the control limb will be observed in muscle size or unilateral function assessments.
- Unilateral function of the immobilised limb will be decreased and, where specific muscles are tested, plantar flexion will have greater decrements than knee extension while dorsiflexion will remain unaffected.
- Bilateral measures of function will be decreased and driven by the immobilised limb.

3.3 Methods

3.3.1 Study overview

3.3.1.1 Participant recruitment and screening

This study was approved by the University of Nottingham Faculty of Medicine and Health Sciences Research Ethics Committee (103-1809). Participants were recruited locally through advertisement posters placed around the University, student cohort emails and online social groups and forums. The Participant Information Sheet (appendix 1) was provided to interested individuals providing a more detailed explanation of the study protocol. 13 participants were recruited and screened for the study and more detailed participant information is found in the results section. For screening visits, participants were invited to attend the Royal Derby Hospital Medical School building. Following the taking of informed consent, participant height, weight and blood pressure was measured along with an electrocardiogram. A routine blood test was also carried out and sent to the pathology department to ensure participants were eligible based on the criteria below.

3.3.1.2 Exclusion criteria

To ensure the recruited participants were eligible for the study, they were excluded if evidence of the following was apparent:

- A BMI <18 or >35 kg/m^2
- Active cardiovascular disease:
    - uncontrolled hypertension (BP > 160/100), angina, heart failure (class III/IV), arrhythmia, right to left cardiac shunt, recent cardiac event
- Cerebrovascular disease:
    - previous stroke, aneurysm (large vessel or intracranial), epilepsy
- Respiratory disease including:
    - pulmonary hypertension, COPD
- Metabolic disease:
    - hyper and hypo parathyroidism, untreated hyper and hypothyroidism, Cushing's disease, type 1 or 2 diabetes

- Active inflammatory bowel or renal disease
- Active malignancy
- Recent steroid treatment (within 6 months) or hormone replacement therapy
- Clotting dysfunction
- Musculoskeletal or neurological disorders
- Inability to use crutches, including stair negotiation.

3.3.2 Immobilisation strategy

15 days was chosen as the length of immobilisation for this study as prior studies of disuse atrophy have used similar durations which allows directly comparable results. Furthermore, 15 days was also used more practically to allow a time course of disuse adaptation in the comparison with a 5-day immobilisation study following this. Before and after the immobilisation period, participants underwent a battery of functional assessments and muscle ultrasound scans (fig. 3.1A). The immobilisation strategy is detailed in Chapter 2 section 2.3. Briefly, for the 15 days of immobilisation, a modified ULLS model was used (fig. 3.1B). The knee joint was fixed at 75 degrees flexion and the ankle joint was fixed using an air-boot. Crutches were provided and adjusted according to the height of the participant.

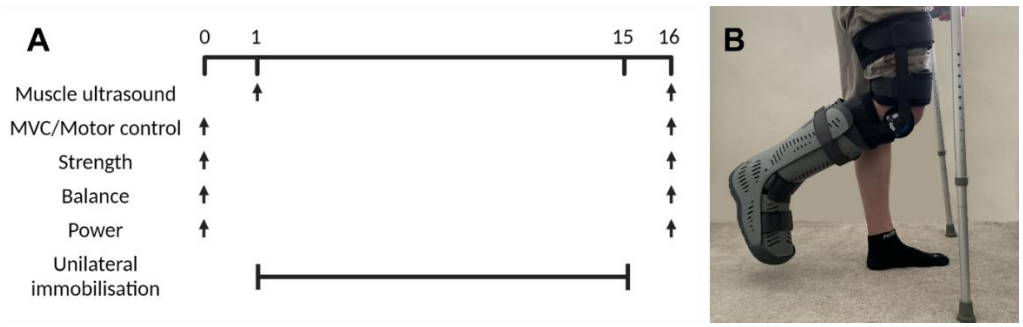


Figure 3.1: *A schematic of the time frame of the study including pre and post immobilisation muscle size and function assessments (A) and a picture showing the immobilisation strategy used (B).*

3.3.3 Assessment of muscle size

Skeletal muscle ultrasound CSA scans were taken from the VL, TA and MG. These methods are detailed in Chapter 2 section 2.1.1.1. Briefly, three axial plane images were collected following this line from both legs in each of these . Scans of each muscle in both legs were performed before and after the immobilisation period

Quantification of CSA for each muscle was performed using ImageJ (Laboratory of Optical and Communication, University of Wisconsin-Madison, WI, USA). The muscle cross-section was drawn out and area recorded three times for each image then averaged across all images to provide the muscle CSA value used for statistical analysis. For the VL, n=11, for the TA, n=10 and for the MG, n=12.

3.3.4 Assessment of muscle structure

3.3.4.1 Sagittal ultrasound scans

Sagittal ultrasound images of the relevant muscles were captured following the axial plane images as described above. These methods are detailed in Chapter 2 section 2.1.1.2. Briefly, three sagittal images were taken with the probe in the same approximate area for each muscle to provide a broader view of muscle architecture. These images were analysed for $_{P}A$ and $_{F}L$ as detailed in Chapter 2 section 2.1.1 For these measurements, n=12 in each muscle.

3.3.5 Assessment of muscle function

3.3.5.1 Lower limb power

Overall lower limb power was assessed using a countermovement jump assessment using a BTS G-sensor (BTS Bioengineering S.p.A., Milan, Italy). These methods are detailed in Chapter 2 section 2.1.2. Briefly, a countermovement jump was demonstrated before a practice jump was carried out. The participant carried out three jumps with a gap of 30 seconds between each. The jump with the greater maximal power was recorded for analysis

along with the respective take-off speed and jump height measurements. For this measurement, n=7 as the equipment was acquired midway through the study.

Unilateral leg extension power was assessed using the Nottingham Power Rig (Bassey and Short, 1990). These methods are detailed in Chapter 2 section 2.1.3. Briefly, with the participant seated on the Rig, they were instructed to push as hard as possible with one leg onto the force plate as the brake was simultaneously released. A digital display provided a power measurement which was recorded. For each leg, participants carried out two attempts. The highest measurement was taken from the two or three attempts. For this measurement, n=11.

3.3.5.2 Timed up and go

General mobility and function was assessed with the TUG (Podsiadlo and Richardson, 1991). These methods are detailed in Chapter 2 section 2.1.6. Briefly, the participant stood from a seated position, walked 3 metres, turned around an obstacle before returning to the chair and sitting down. The quickest time of three attempts was recorded for analysis. For this measurement, n=10.

3.3.5.3 Unilateral knee extensor strength

Unilateral knee extensor strength was measured using a 1RM test. These methods are detailed in Chapter 2 section 2.1.4. Briefly, participants were seated on the machine with one leg rested behind the extension lever. With a 3-minute rest between each 1RM attempt, participants carried out one unilateral contraction until a true 1RM was achieved. The same assessment was then carried out on the contralateral leg. The weight value of the 1RM contraction from each leg was taken forward for analysis. For this measurement, n=12.

3.3.5.4 Handgrip strength

Peak unilateral handgrip strength was measured using a handgrip dynamometer (T.K.K. A5401, Takei Scientific Instruments, Niigata, Japan). This method is detailed in Chapter 2 section 2.1.5. Briefly, participants were instructed to squeeze the dynamometer as hard as they could for three seconds before relaxing. With a 30-second rest between attempts, three attempts were recorded from each arm and the peak values were taken forward for analysis. For this measurement, n=10.

3.3.5.5 Balance

Using a Materialise Footscan 1-metre force plate (Materialise, Leuven, Belgium), balance was assessed for both legs and each leg individually. These methods are detailed in Chapter 2 section 2.1.6. Briefly, participants stood naturally in the centre of the force plate, and this was recorded for 30 s to measure the COP distance moved and ellipse area. This was carried out in three conditions: both legs standing, left leg, and right leg.. For these measurements, n=8 as the equipment was acquired midway through the study.

3.3.5.6 Maximal voluntary contraction

The following methods are detailed in Chapter 2 section 2.1.7. Briefly, for knee extension MVC, participants were seated in a custom-built isometric dynamometer with their knee joint fixed at 90° while the hip joint angle was approximately 110° Participants were verbally encouraged to perform an isometric knee extension at maximal capacity. Three attempts were carried out and the highest value was taken as the maximal and used to determine voluntary contraction intensity. For this measurement, n=11 due to equipment failure at follow-up.

For dorsiflexion MVC, participants were seated in a chair at an appropriate distance from the custom-built ankle isometric dynamometer. Following a warm-up, three attempts were carried out and the highest value was taken as

the maximal and used to determine voluntary contraction intensity. For this measurement, n=12 due to equipment failure at follow-up.

For plantar flexion MVC, three attempts were carried out and the highest value was taken as the maximal and used to determine voluntary contraction intensity. For this measurement, n=11 due to equipment failure at follow-up and muscle stiffness in one participant at follow-up.

3.3.5.7 Force control

Force control, measured as force steadiness (FS) was recorded during voluntary isometric contractions carried out following MVC assessment. These methods are detailed in Chapter 2 section 2.1.8. Briefly, for each contraction type, participants carried out 2 – 6 voluntary contractions at intensities of 10%, 25% and 40% of their previously recorded MVC Contractions were held for 12 s with a ~20 s rest between them. For VL FS, n=12 at 10% and 25% and n=11 at 40%. For TA FS at 10%, 25% and 40%, n=11. For MG FS, n=11 at 10% and 25% and n=10 at 40% due to participant muscle stiffness at follow-up.

3.3.6 Statistical analysis

Statistical analysis of ultrasound features (CSA, $_{F}$L and $_{P}$A), MVC, power, single leg balance, TUG and FS were performed using GraphPad Prism version 9.4.1 (GraphPad Software, CA, USA) using repeated measures two-way analysis of variance (ANOVA) with Šidak's post-hoc analysis in the event of a significant interaction. Two within-subject factors were included; leg (immobilised and control) and time (pre and post), and leg x time interactions were included in all analyses. For the variables related to jump performance and both leg balance, Student's paired T-tests were used. To compare between percentage change in muscle size (CSA) and muscle strength (MVC) measurements in the immobilised leg pre to post, one-way repeated measures ANOVA was used. Contributory analysis: to further investigate potential contributions of changes in muscle strength and size to changes in broader function parameters, additional analyses were performed using R (Version 4.2.0, (https://cran.r-

project.org/) implemented using R studio. These analyses were carried out with n=6 including participants with complete data sets. Following normality testing using the Shapiro-Wilks test for normality, correlative analysis was carried out with Pearson's product moment correlation coefficient and visualised using corrplot (https://cran.r-project.org/web/packages/corrplot) to highlight the strength of relationships between variables investigated. Cluster analysis and principal component analysis for variables were performed using the ClustOfVar (https://cran.r-project.org/web/packages/ClustOfVar) and factoextra (https://cran.r-project.org/web/packages/factoextra) packages respectively, to determine which variables strongly clustered with and related to the independent variable of interest. Once clusters were identified, multivariate linear regression was performed to determine whether any variables within the cluster significantly impacted the change in the independent variable of interest. Significance was assumed if $p<0.05$.

3.4 Results

3.4.1 Participant information

Thirteen male participants took part in this study. Characteristics are shown in table 3.1.

Table 3.1: *Descriptive characteristics of participants showing mean and standard deviation (SD)*

N = 13	Mean (SD)
Age (years)	22.7 (3.2)
Height (cm)	180.5 (7.2)
Weight (kg)	78.1 (9.6)
BMI (kg/m^2)	24.0 (2.5)

3.4.2 Changes in muscle size and structure with unilateral immobilisation

3.4.2.1 Muscle cross-sectional area

A significant time x leg interaction was observed in VL CSA ($p<0.01$, fig. 3.2A), with a decrease in the immobilised leg (−12%, $p<0.001$) and no change in the control leg ($p = 0.700$) after 15-day immobilisation. No significant interaction was observed in TA CSA ($p = 0.477$, fig. 3.2B). A significant interaction was observed in MG CSA ($p<0.05$, fig. 3.2C), with a reduction in the immobilised leg ($p<0.05$) and no change was seen in the control leg ($p = 0.060$).

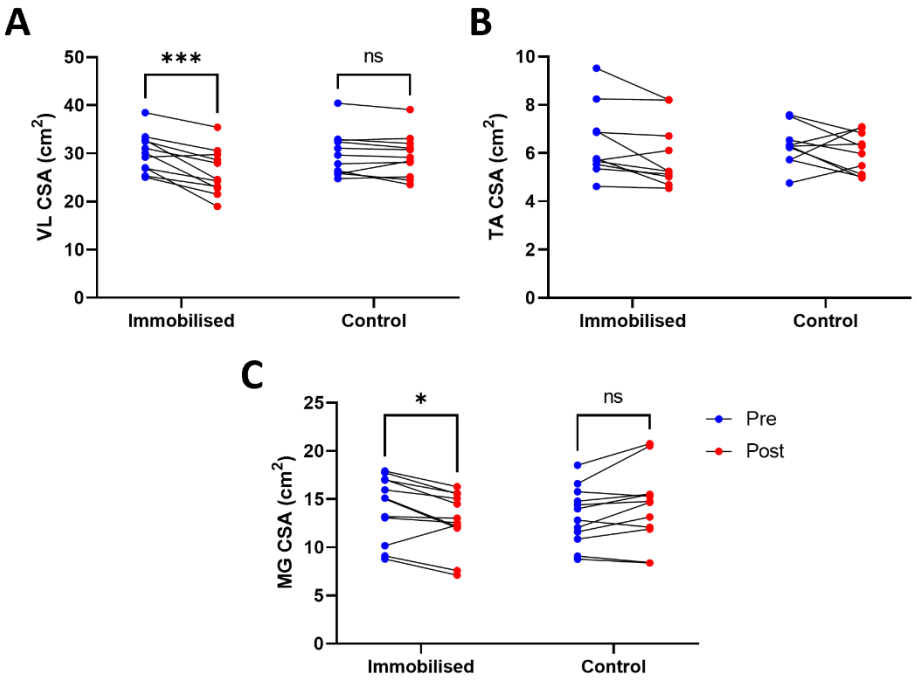


Figure 3.3: *Muscle cross-sectional area in cm² measured by ultrasound before and after 15-day unilateral limb immobilisation in the immobilised and control limbs. Measurements taken from the vastus lateralis (A, n = 11), tibialis anterior (B, n = 10) and medial gastrocnemius (C, n = 12). * = p<0.05, *** = p<0.001.*

3.4.2.2 Pennation angle

For ₚA, no significant interaction was observed in the VL (p = 0.736, fig. 3.3A).

Similarly for TA ₚA, no significant interaction was observed (p = 0.200, fig 3.3B).

Also, for MG ₚA, no significant interaction was observed (p = 0.052, fig. 3.3C).

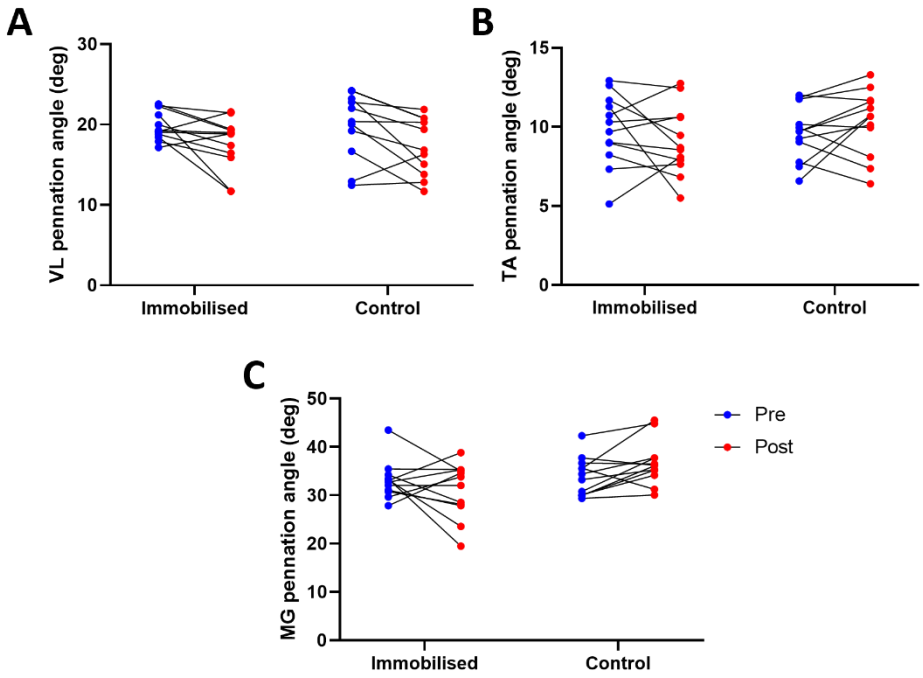


Figure 3.3: *Muscle fascicle pennation angle in degrees measured by ultrasound before and after 15-day unilateral limb immobilisation in the immobilised and control limbs. Measurements taken from the vastus lateralis (A), tibialis anterior (B) and medial gastrocnemius (C, all n = 12).*

3.4.2.3 Fascicle length

For muscle $_{F}L$, no significant interaction was observed in the VL ($p = 0.080$, fig. 3.4A). For TA $_{F}L$, no significant interaction was observed ($p = 0.521$, fig. 3.4B). Additionally, no significant interaction was observed in MG $_{F}L$ ($p = 0.157$, fig. 3.4C).

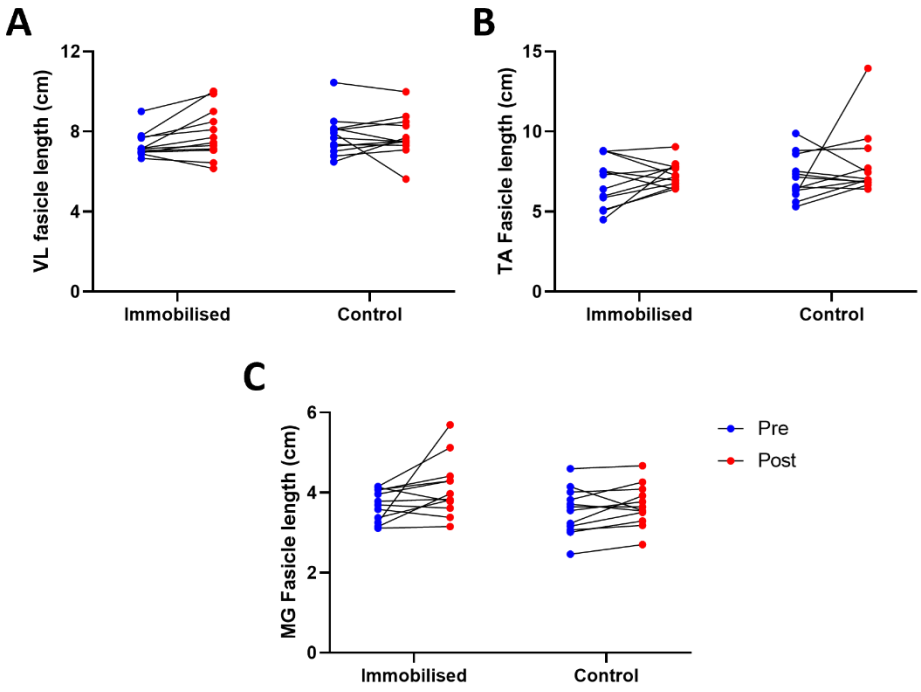


Figure 3.4: *Muscle fascicle length in cm measured by ultrasound before and after 15-day unilateral limb immobilisation in the immobilised and control limbs. Measurements taken from the vastus lateralis (A), tibialis anterior (B) and medial gastrocnemius (C, all n = 12).*

3.4.3 Changes in muscle function with unilateral immobilisation

3.4.3.1 Lower limb power

For overall lower limb power, there was no significant change in jump maximum concentric power (p = 0.058, fig 3.5A). However, there was a significant reduction in both jump height (−18%, p<0.01, fig. 3.5B) and jump take-off speed (−9%, p<0.01, fig. 3.5C).

For unilateral leg extension power, a significant interaction was observed (p<0.05, fig. 3.6A). A significant reduction was seen in the immobilised leg (-28%, p<0.001) while no significant change was seen in the control leg (p=0.621).

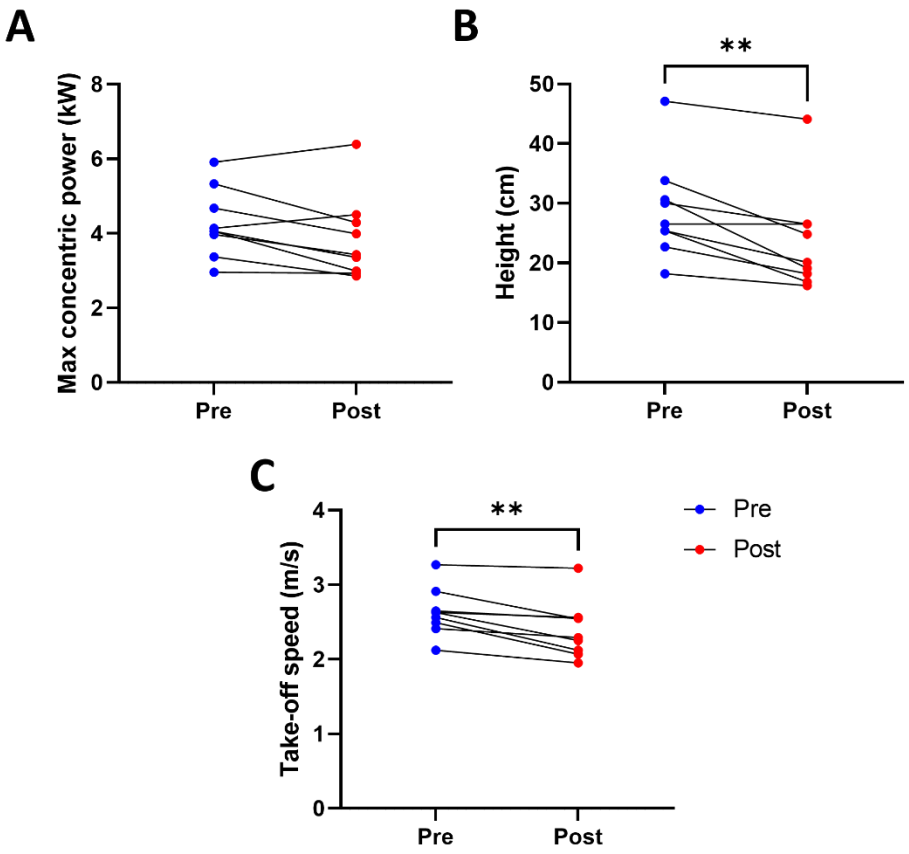


Figure 3.5: *Measurements taken from countermovement jumps before and after 15-day immobilisation. Maximum concentric power (A) measured in kW, maximum jump height (B) measured in cm and peak take-off speed (C) measured in m/s. n = 7.*

3.4.3.2 Timed up and go

For TUG, there was a significant increase in time taken to complete the task following immobilisation (10%, $p<0.01$, fig. 3.6B).

3.4.3.3 Unilateral knee extensor strength

For knee extensor 1RM, there was a significant leg x time interaction ($p<0.001$, fig. 3.6C). A reduction was observed in the immobilised leg (−19%, $p<0.001$), but no change was seen in the control ($p = 0.800$).

3.4.3.4 Handgrip strength

For unilateral handgrip strength there was no significant arm x time interaction (p = 0.356, fig. 3.6D).

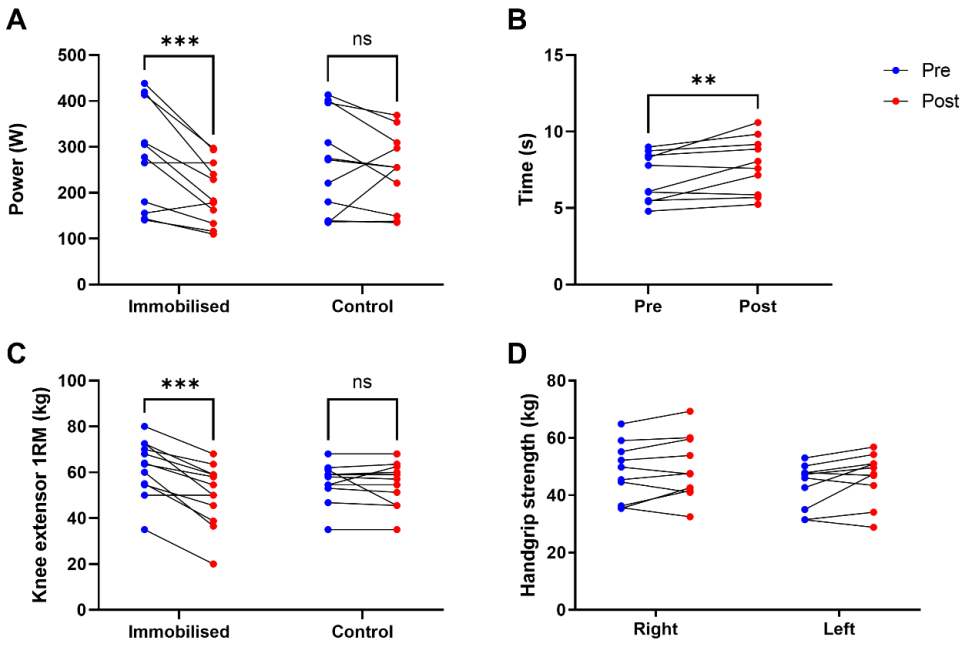


Figure 3.6: *Measurements of muscle function taken before and after 15-day unilateral limb immobilisation. Unilateral leg extension power in the immobilised and control leg (A, n = 11), timed up-and-go time (B, n = 10), knee extensor 1-repetition maximum (C, n = 12) and handgrip strength (D, n = 12).*

3.4.3.5 Balance

For distance moved during balance assessments, there was no significant interaction (p = 0.129, fig. 3.7A). However, a significant main effect of leg was observed which was reflected in a significantly greater distance moved in the immobilised leg following immobilisation (31%, p<0.05). For ellipse area during balance assessments, there was also no significant interaction observed (p = 0.925, fig. 3.7B).

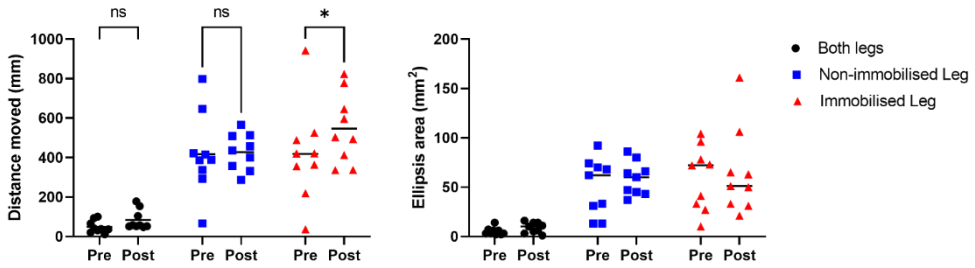


Figure 3.7: *Measurements taken from both leg and individual leg balance before and after 15-day unilateral immobilisation. Centre of pressure distance moved (A) measured in mm and ellipsis area (B) of the centre of pressure measured in mm². n = 9.*

3.4.3.6 Maximal voluntary contraction

For knee extensor MVC, there was a significant interaction between leg and time factors ($p<0.01$, fig. 3.8A). A significant reduction was observed in the immobilised limb only (−29%, $p<0.001$) with no change observed in the control ($p = 0.560$). For dorsiflexion MVC, no significant interaction was observed following immobilisation ($p = 0.393$, fig. 3.8B) However, a significant main effect of time was observed ($p<0.01$) which was reflected in a reduction in the immobilised leg (−23%, $p<0.01$) and a reduction in the control leg (−16%, $p<0.05$). Similarly for plantar flexion MVC, although no significant interaction was observed ($p = 0.259$, fig. 3.8C), there was a significant main effect of time ($p<0.05$). This was reflected in a significant decrease in the immobilised leg only (−13%, $p<0.05$).

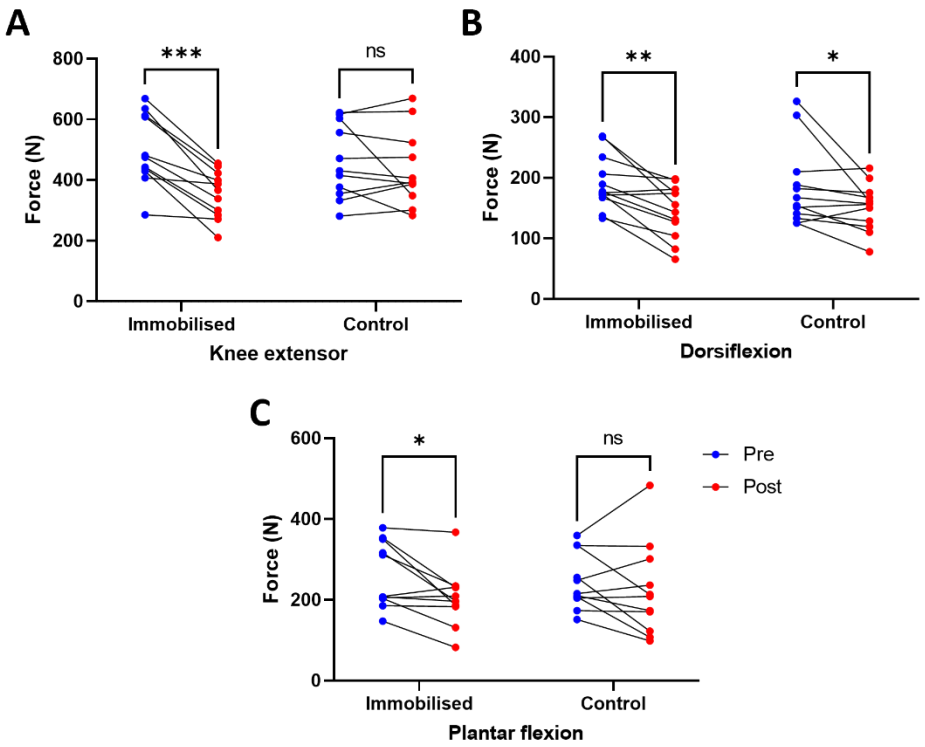


Figure 3.8: *Measurements of maximal voluntary isometric contraction measured in newtons taken from knee extensor (A, n = 11), dorsiflexion (B, n = 12) and plantar flexion (C, n = 11) contractions before and after 15-day unilateral limb immobilisation in the immobilised and control limbs.*

3.4.3.7 Force control

For VL FS, there was no significant interaction at 10% (p = 0.173), 25% (p = 0.228) or 40% (p = 0.382, all fig. 3.9A). For TA FS, there was no significant interaction at 10% (p = 0.419), 25% (p = 0.351) or 40% (p = 0.076, all fig. 3.9B). For MG FS, there was no significant interaction at 10% (p = 0.367), 25% (p = 0.355) or 40% (p = 0.163, all fig. 3.9C).

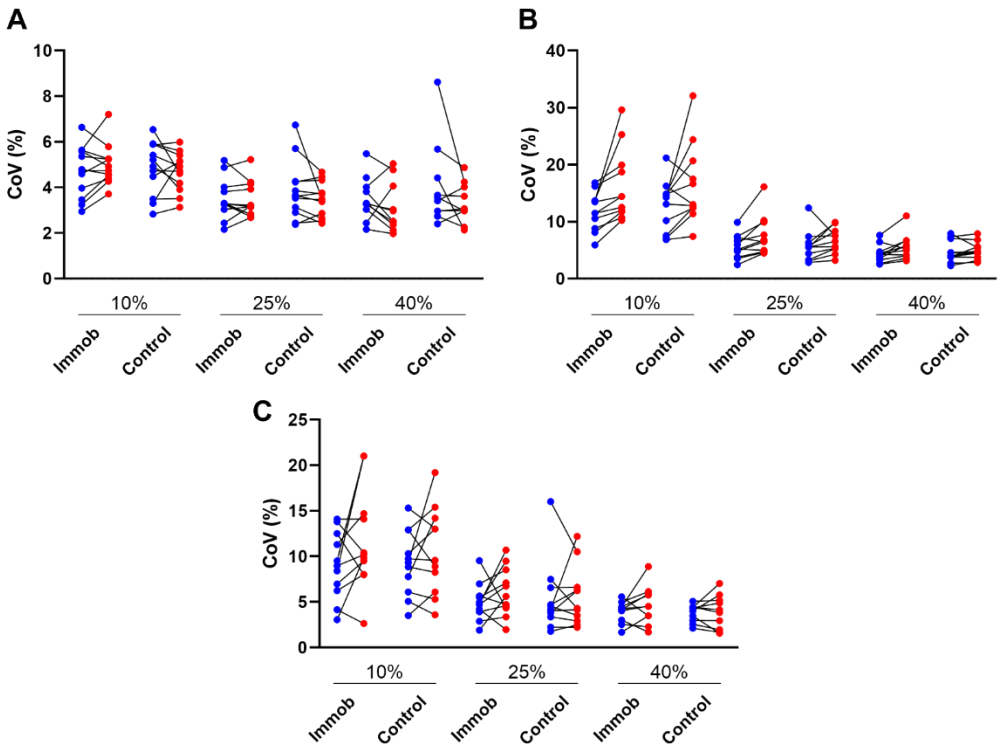


Figure 3.9: *Measurements of force steadiness reported as the coefficient of variation of deviation from a target line during contractions set at 10%, 25% and 40% of maximal voluntary contraction force before and after 15-day unilateral limb immobilisation in the immobilised (immob) and control limbs. Contractions recorded from knee extension (A), dorsiflexion (B) and plantar flexion (C). At 10%, 25% and 40%; knee extension n = 12, 12 and 11, dorsiflexion n = 11 for all, plantar flexion n = 11, 11, and 10 respectively.*

3.4.4 Immobilised muscle comparison

When comparing percentage change in CSA across VL, TA and MG in the immobilised limb before and after 15-day unilateral limb immobilisation, no significant main effect was seen (n = 9, p = 0.095, fig. 3.10A). When comparing percentage change in MVC across knee extension, dorsiflexion and plantar flexion, there was no significant effect (n = 11, p = 0.546, fig. 3.10B).

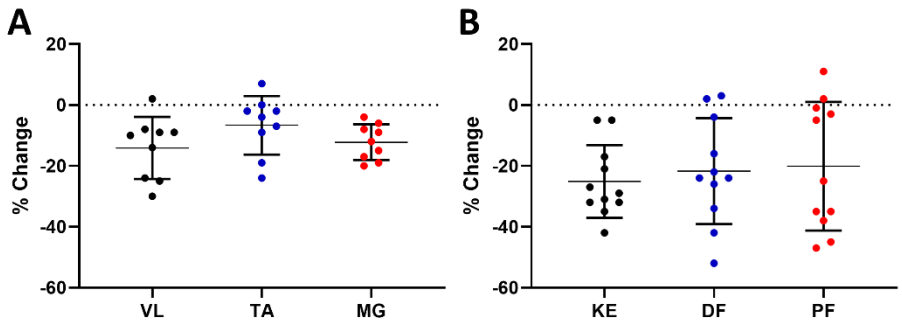


Figure 3.10: *A: Analysis of percentage change in muscle cross-sectional area of the vastus lateralis (VL), tibialis anterior (TA) and medial gastrocnemius (MG) before and after 15-day unilateral limb immobilisation in the immobilised limb (n = 9). B: Analysis of percentage change in maximal voluntary isometric contraction of the knee extensors (KE), dorsiflexors (DF) and plantar flexors (PF) before and after 15-day unilateral limb immobilisation in the immobilised limb (n = 11). Group means are presented with error bars showing standard deviation with individual values overlaid.*

3.4.5 Contributory analysis

Correlative analysis was performed to investigate the contribution of changes in muscle size using CSA or muscle strength using MVC of individual muscles of the immobilised limb to parameters of muscle function. Correlations were performed on the mean absolute change of VL, TA and MG CSA and MVC of the immobilised limb with mean absolute change in jump take-off speed and also, independently, with mean absolute change in immobilised leg extensor power measured on the Nottingham power rig. A strong positive correlation was observed between TA MVC and jump take-off speed (r = 0.83, y = 0.0042x – 0.1338, fig. 3.11A) and a moderate negative correlation between MG CSA and take-off speed (r = −0.55, y = -0.0887x - 0.4655, fig. 2.16A). Muscle power correlated strongly with MG MVC (r = 0.73, y = 0.6558x - 69.373, fig. 3.11B) and moderately with TA CSA (r = 0.67, y = 47.7x - 129.08, fig. 2.16B).

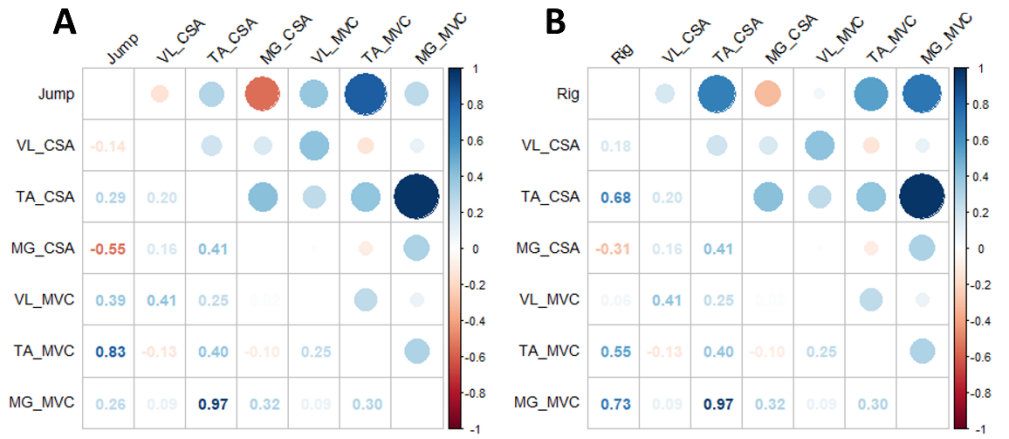


Figure 3.11: *Correlation plots between jump take-off speed (A) and unilateral power (B) with VL, TA, and MG CSA and MVC. Blue colouring indicates positive correlation, red indicates negative correlation. n = 6. Jump; jump take-off speed, VL; vastus lateralis, TA; tibialis anterior, MG; medial gastrocnemius, CSA; cross-sectional area, MVC; maximal voluntary contraction, Rig; unilateral leg extensor power measured by Nottingham power rig.*

In order to reflect relationships between these parameters, variables were clustered using the hierarchical clustering algorithm provided in ClustOfVar (fig. 3.12A and B) and visualised further using principal component analysis score plots (PCA, fig. 3.12C and D). For jump take-off speed, the key cluster of interest included jump take-off speed, TA MVC, VL CSA and VL MVC. Using multivariate linear regression, the contribution of size and strength parameters were tested against jump take-off speed to determine whether any of these parameters significantly contributed to the change observed. No significant relationships were observed between jump take-off speed and TA MVC ($p = 0.199$), VL CSA ($p = 0.744$) and VL MVC ($p = 0.597$). For power, the key cluster of interest contained rig power, TA CSA, MG MVC and TA MVC. Following the same multivariate linear regression analysis with these clustered variables, no significant relationships were observed between power and TA CSA ($p = 0.501$), MG MVC ($p = 0.368$) and TA MVC ($p = 0.349$).

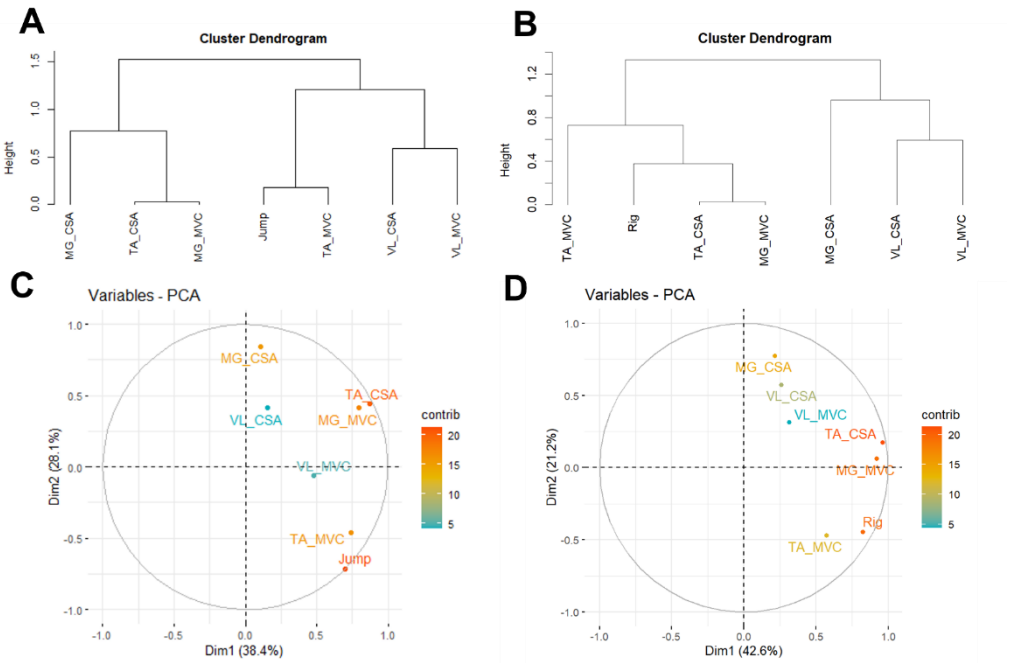


Figure 3.12: *Cluster dendrograms and principal component analysis score plots to visualise clustering between jump take-off speed (A and C) and unilateral power (B and D) independently with VL, TA, and MG CSA and MVC. Jump; jump take-off speed, VL; vastus lateralis, TA; tibialis anterior, MG; medial gastrocnemius, CSA; cross-sectional area, MVC; maximal voluntary contraction, Rig; unilateral leg extensor power measured by Nottingham power rig.*

3.5 Discussion

The key findings of these measurements were a reduction in CSA of the VL and the MG and no change in the TA in the immobilised leg which was in line with the primary hypothesis. Additionally, no changes in CSA were observed in the control leg, which also agrees with the hypothesis of this chapter. No changes were seen in muscle architecture across any of the muscles studied in either the immobilised or the control limb. In terms of functional adaptations to disuse, although maximum jump power was not altered, jump height and take-off speed were reduced. There was also a significantly greater time taken to complete the timed up-and-go test. For unilateral power, a reduction was seen in the immobilised leg with no change in the control which agrees with the hypothesis that unilateral measures of function would decrease in the immobilised leg. No significant alterations to handgrip strength were observed in either arm. In line with expectations, unilateral balance of the immobilised limb showed a reduction in some aspects of balance performance compared to performance in both legs and the control leg. MVC force in the knee extensors of the immobilised leg was reduced, with reductions in the immobilised and control TA, contrasting the hypothesis, while the immobilised MG also declined. The VL and MG MVC of the control leg remained unchanged as expected. No alterations in force control variation were observed in any contraction type at any intensity. When comparing the magnitude of change in CSA and MVC of the muscles studied in the immobilised leg, none of the muscles expressed a lesser or greater degree of change compared to each other. When investigating the contribution of CSA and MVC changes to jump take-off speed reduction and unilateral muscle power reduction in the immobilised leg, none of the variables which clustered together were found to provide a significantly greater contribution to the changes in jump speed and leg extension power over the other variables within the cluster.

3.5.1 Muscle size and structure

Adaptations of muscle size reflected by CSA observed by this study generally agree with previous findings from disuse interventions. However, they were of a greater magnitude: the present findings in the VL were a 12% reduction after 15 days, compared to ~5% reduction seen after 14-day ULLS (de Boer *et al.*, 2007*a*) which only advanced to ~10% after 23 days. Even after 8-week bed rest, quadriceps CSA was reduced by just ~14% (Mulder *et al.*, 2006), although this does incorporate the entire quadriceps rather than just the VL which may express a smaller degree of atrophy. The individual vasti have not been studied separately but the rectus femoris has been reported to resist volume reduction after 29-day bed rest before reducing by 9% after 89 days, whereas the vasti collectively decreased by 10% by day 29 and 19% by day 89 (Alkner & Tesch, 2004*b*). Additionally, rectus femoris was not found to atrophy after 56-day bed rest while the vasti progressively reduced in volume to ~16% by day 56 (Belavý *et al.*, 2009*a*). While covering a much longer duration, these findings suggest disparity exists among the quadriceps and may explain the greater loss seen here in the VL compared to the quadriceps overall, although with a lack of conclusive evidence the definitive cause remains unclear. The lack of change in the TA is in agreement with bed rest studies which also show no reduction (Leblanc *et al.*, 1988; Akima *et al.*, 2000*b*). The collective anterior tibial muscles were seen to lose ~5% volume following 56-day bed rest (Belavý *et al.*, 2009*a*), however it is unclear whether the TA atrophied equally with the EDL and EHL, a similar issue as previously discussed with the VL and the broader vasti and quadriceps. Continuing in agreement with the hypothesis, MG CSA was significantly reduced in the immobilised leg. Similar to the findings in the VL, the reduction in MG size is in agreement with previous literature but also exceeds it at a comparative duration. Following 14-day ULLS, MG volume was reduced by 6% (Seynnes *et al.*, 2008) while the present findings saw a reduction of 10% after 15-day immobilisation. It may be considered that, although ultrasound measurements of plantar flexor size correlate well with MRI-measured volume (Miyatani *et al.*, 2004), CSA reduction at the mid-belly of the

muscle may not fully represent muscle loss across the length of the muscle and regional changes may account for the lesser decrement in muscle volume after a similar length intervention. Atrophy across the MG has been shown to be non-uniform following 56-day HDBR, with the majority of change occurring between 30% and 95% of muscle length (Miokovic *et al.*, 2012), with the scanned region of the present study sitting within that range at 70%. Hence a lack of atrophy at the proximal end of the muscle may account for the differing volume and CSA reductions.

Changes in muscle structural parameters were not seen in any of the muscles investigated by this study. Although this was hypothesised to take place in the VL and the MG, the TA remained unaltered in agreement with the hypothesis. Some of these findings from the VL are in agreement with previous studies. Following 14-day ULLS, no changes were seen in $_{F}L$, although a ~8% reduction was seen after 23 days (de Boer *et al.*, 2007*a*). Contrasting with the present findings however, after 14 days a ~3% reduction was observed in $_{P}A$, with ~8% reduction at day 23 which was not reflected by the findings of the present study. Even after 5-week bed rest, VL $_{F}L$ was only reduced by ~6% with $_{P}A$ reducing by ~14% (de Boer *et al.*, 2008) which may suggest a longer duration of disuse is required before small, while significant, changes are seen in muscle architecture. In this same study, MG $_{F}L$ was reduced by ~5% and $_{P}A$ similar to VL with ~14% reduction also (de Boer *et al.*, 2008). This also suggests that muscle architecture may resist adaptation to shorter-term disuse even in conjunction with reductions in muscle size overall. It has recently been discussed that $_{p}A$ may not hold any functional significance in the study of human skeletal muscle (Lieber, 2022). While the evidence presented towards a 'modern view' of this characteristic may challenge the current thought of the relationship of $_{p}A$ and force generation, it remains an adaptive feature of muscle atrophy as described above which may still warrant investigation.

3.5.2 Muscle function

3.5.2.1 Power

Although maximum concentric power was not reduced in the jump performance of participants following immobilisation, the other measurements taken from jump performance of height and jump take-off speed were both reduced. While as yet unstudied in the context of disuse, jump power is known to reduce as a factor of age, albeit in masters athletes which may not represent the broader older population (Michaelis *et al.*, 2008; Piasecki *et al.*, 2021*a*). In fact, due to the high levels of activity, this removes disuse from the equation to suggest that a maintained high activity level in older age does not preserve jump performance, even in power-event focused masters athletes (Michaelis *et al.*, 2008). Fatigue has been found to influence jump performance, with countermovement jump peak force and peak velocity reducing following a fatiguing protocol (Cooper *et al.*, 2020). Prior to the jump assessment in the present study, participants had performed MVC and force control tasks for VL, MG and TA in both legs, along with balance and gait performance assessments, which could collectively have caused a fatigue-related reduction in jump performance. However, the assessments were performed in the same order on pre- and post-immobilisation testing visits which should account for any effect and participants were able to take short breaks between each assessment while the subsequent test was prepared.

In terms of unilateral power, in line with the hypothesis there was a significant reduction in the immobilised leg which was not mirrored by the control leg. Leg extension power has been found to decline in both men and women during healthy ageing from the 6th decade by comparison to individuals in the 3rd decade with decrements present even before significant declines in lean tissue mass (Suetta *et al.*, 2019). Asymmetry knee extensor power has also been associated with functional decrements in community dwelling older women. Although the present study consisted of young male participants, a similar asymmetry in power was observed following immobilisation: the immobilised

limb had a 19% lower power while ~15% (Portegijs *et al.*, 2005) and ~11% (Straight *et al.*, 2016) have been reported in older women. Those with a greater asymmetry in power also expressed a reduced balance performance and a lower walking velocity than those with less asymmetry in power (Portegijs *et al.*, 2005). Similarly, significant negative associations between power asymmetry percentage and six-minute walk distance, 8-foot up-and-go time and 30-second chair stand performance (Straight *et al.*, 2016). While the demographics are not directly comparable, it remains interesting to note that decrements from short term disuse are somewhat similar to those seen in ageing individuals. The asymmetry between muscle power following short-term immobilisation may hold negative implications for older individuals undergoing periods of reduced activity or even bed rest as prior asymmetry may be exacerbated resulting in more extreme functional decrements.

3.5.2.2 Balance and gait

There was a significant increase in the distance moved during balance following immobilisation in the immobilised leg. However, there were no changes observed in ellipse area of the immobilised leg or in the balance performance of both legs and the control leg. For both legs and the control leg, this was the case for both the distance travelled by the centre of pressure, which reflects postural sway, and for centre of pressure ellipse area, which represents postural sway variation. While the lack of change in the control leg agrees with the hypothesis of this investigation, the lack of change in both leg balance does not. However, the partial reduction in balance performance of the immobilised leg does agree with the hypothesis in the case of distance moved. Since there do not appear to be any data on balance performance in young males following short-term immobilisation, it is difficult to draw any comparisons as to whether this was expected or not. Balance performance has been shown to decline with age and associated with increased risk of falls (Cattagni *et al.*, 2014), but crucially this was alongside reduced strength of the plantar flexor and dorsiflexor muscles which was not observed in this study. As balance performance in older men appears to be more associated with plantar flexor

RTD (Ema *et al.*, 2016), investigating changes in this parameter may be worthwhile in future studies of short-term immobilisation in young individuals, perhaps explaining the absence of balance performance decrement in both leg balance and of the postural sway variation in the immobilised leg.

Gait performance assessed by the TUG did show a significantly greater time taken to completion, further suggesting some systemic effects result from unilateral immobilisation. Again, there does not appear to be any comparative data in other studies of disuse atrophy, but in the same theme as muscle power there is a greater time taken observed in older adults compared to younger (Montgomery *et al.*, 2020). Interestingly, normalised jump power was found to be a key contributor of TUG performance in older males, accounting for 19% of the variance observed across this cohort. Although the present study did not find a difference in jump power, the reduction in unilateral limb power of the immobilised leg may contribute to the reduction in TUG performance observed here.

3.5.2.3 Strength and force control

Unilateral knee extensor strength of the immobilised leg was reduced as was expected following the intervention. There was no change in the control leg which fulfils the hypothesised finding of unilateral decrements solely being expressed in the immobilised leg. There was no interaction present for handgrip strength and therefore no change was seen in this parameter following the immobilisation period in either arm. Although changes in the immobilised lower limb were hypothesised, it is not entirely unexpected that handgrip strength changes were not seen in the present study. The arms of the participants were both freely able to move, while also remaining vital to the locomotion of the participant on crutches throughout the study. Although handgrip is widely associated with sarcopenia and the contributing and resulting decrements and greater risk factors (Cruz-Jentoft *et al.*, 2019), this is likely not relatable to the young cohort involved in the present study. While some parameters present a similar phenotype, albeit less extreme, as observed

in ageing, the participants involved were inherently far removed from the broader ageing phenotype as healthy young male adults. Therefore, comparisons are not able to be drawn between these differing demographic groups in the case of handgrip strength.

As expected, and in line with both the hypothesis of immobilised-limb only decrements, along with matching reduced unilateral knee extensor power and 1RM strength reductions, knee extensor MVC was significantly reduced in the immobilised leg. This parameter is also in line with previous findings of reduced MVC/MVT with disuse. However, while decrements of ~15% MVT after 14-day ULLS which reached ~21% after 23 days (de Boer *et al.*, 2007*a*), the present findings of ~29% reduction almost double the magnitude of change in a similar time frame. This pattern reflects the findings of VL CSA with a greater magnitude of reduction also observed in that parameter compared to a similar time frame. When considering the dorsiflexor and plantar flexor MVC findings, the hypothesis that the function of the MG by comparison to the TA in the immobilised leg would express greater decrements was not found to be true. Decrements were found in both muscles of the immobilised leg and the TA of the control following the 15-day intervention. The reduction in plantar flexion MVC agrees with findings following 14-day and 23-day ULLS, in which plantar flexion MVC was reduced by ~10% at day 14 with no further decrement by day 23 (Seynnes *et al.*, 2008). Furthermore, a ~17% reduction in plantar flexion MVC was observed following 21-day ULLS (Schulze *et al.*, 2002). It was expected that the plantar flexor MVC was reduced, and alongside the reductions in MG muscle size, in agreement with the hypothesis. In contrast, the reductions in the TA are difficult to explain, particularly in the control leg and especially in the absence of muscle size reduction of the TA, although there may be further neuromuscular determinants which warrant investigation.

There were also no changes in force control across the muscle groups tested and at any of the contraction intensities used in this study. As there does not appear to be comparable data following disuse of any kind or duration, it is difficult to compare with a relevant group. Specific training of force control

using sinusoidal contractions in the knee extensors has been shown to improve sinusoidal and plateau force control in young individuals (Ely *et al.*, 2022). However, as this was not accompanied by any changes in strength, rather that neuromuscular adaptations were concluded to be responsible, it may be the case that force control is not a parameter governed by muscle size and/or muscle strength. This could explain why force control remained preserved in young males following immobilisation in the present study while also experiencing decrements to muscle size and strength of the knee extensors.

Reductions were observed in jump take-off speed, a measure of overall lower limb explosive contraction velocity, and also in unilateral leg extensor power of the immobilised leg. However, no significant contributions were ascertained with changes to CSA and MVC in the studied muscles/muscle groups following multivariate linear regression analysis of clustered variables. Interestingly, jump take-off speed clustered with VL CSA and MVC, along with TA MVC which did not express a significant change pre to post when compared to the control leg. Although none of these variables presented a significantly greater relationship to jump take-off speed, it does suggest that reduction in both VL CSA and knee extensor MVC may impair jump performance to some extent. However further investigation into this relationship would be required to draw any conclusions. In terms of leg extensor power, variables in the cluster of interest, i.e., TA CSA and MVC along with MG MVC were not altered significantly when compared to their respective controls. In contrast to the potential relationship of the former variable of interest, it would not seem appropriate to suggest that these parameters would be more highly related to leg extensor power change.

3.6 Conclusion

In conclusion, this study accomplished the aim to investigate muscle size, structure and function adaptation to disuse atrophy using unilateral limb immobilisation across muscles known to express diverging profiles of muscle size loss. However, contrary to the stated hypotheses, the medial

gastrocnemius did not express the greatest degree of reduction in muscle size, structure, and function as expected. Instead, the vastus lateralis of the immobilised limb was the only muscle to express decrements to muscle size, in cross-sectional area, and muscle strength, in knee extensor maximal voluntary contraction and one-repetition maximum. The medial gastrocnemius did still express decrements as expected in size and strength parameters, while although the tibialis anterior did not express muscle size reduction, it went against expectations in expressing reductions in muscle strength. The hypothesis that the control limb would not express decrements to any parameters solely studying muscles of this limb was found to be correct in both the vastus lateralis and the medial gastrocnemius, although the reductions in dorsiflexion strength of the control leg refute this. Unilateral reductions in muscle function of the immobilised limb were found to reduce in both leg extension power and one measure of unilateral balance performance. Some reduction in bilateral muscle function was seen in gait speed and jump performance, however bilateral balance was not affected by immobilisation. Investigations into the contributions of variables of muscle size and strength to bilateral and unilateral function decrements did not provide answers as to which variables may be responsible for these changes. This provides a further question as to alternate adaptations which may drive reductions in bilateral and unilateral function, rather than simply reductions in muscle size and specific function. Further investigations into neuromuscular parameters may uncover changes which may better explain these findings.

Chapter 4 Determining motor unit dysregulation of the vastus lateralis following 15 days of unilateral lower limb immobilisation

4.1 Abstract

Disuse atrophy, caused by situations of unloading such as limb immobilisation, causes a rapid yet diverging reduction in skeletal muscle function when compared to muscle mass. While mechanistic insight into the loss of mass is well studied, deterioration of muscle function with a focus towards the neural input to muscle remains underexplored. This study aimed to determine the role of motor unit adaptation in disuse-induced neuromuscular deficits.

Ten young, healthy male volunteers underwent 15 days of unilateral lower limb immobilisation with intramuscular electromyography (iEMG) bilaterally recorded from the vastus lateralis (VL) during knee extensor contractions normalised to maximal voluntary contraction (MVC), pre and post disuse. Muscle cross-sectional area (CSA) was determined by ultrasound. Individual MUs were sampled and analysed for changes in motor unit (MU) discharge and MU potential (MUP) characteristics.

VL CSA was reduced by approximately 15% which was exceeded by a two-fold decrease of 31% in muscle strength in the immobilised limb, with no change in either parameter in the non-immobilised limb. Parameters of MUP size were reduced by 11 to 24% with immobilisation, while NMJ transmission instability remained unchanged, and MU firing rate decreased by 8 to 11% at several contraction levels.

All adaptations were observed in the immobilised limb only. These findings highlight impaired neural input following immobilisation reflected by suppressed MU firing rate which may underpin the disproportionate reductions of strength relative to muscle size.

4.2 Introduction

4.2.1 Disuse atrophy

Disuse atrophy is the loss of skeletal muscle mass associated with decreased external loading or complete immobilisation. It commonly occurs following joint trauma, nerve injury, or prescribed bed rest (Bodine, 2013), progressing rapidly with reductions of strength occurring after just 5 days of immobilisation (Wall *et al.*, 2014). As such, it has been widely applied as an experimental model to investigate underpinning mechanisms in scenarios of space flight, prolonged bed rest, spinal cord injury, and ageing (Castro *et al.*, 2000; Narici & De Boer, 2011; Puthucheary *et al.*, 2013). The trajectory of decline is not linear; five days of unilateral lower limb suspension lead to ~3 % reduction in quadricep (CSA (Wall *et al.*, 2014) and eight weeks of bed rest reduced quadriceps CSA by ~14% (Mulder *et al.*, 2006).

4.2.2 Muscle mass and size loss

Although these aspects are discussed in more detail in Chapter 1 (sections 1.2.3 and 1.3.1) and Chapter 3 (section 3.2.5.1) they will be briefly recounted to add to the context of this chapter. The loss of muscle strength with disuse is commonly reported to exceed the loss of muscle size. Following 8 weeks of bedrest, quadriceps CSA declined by 14% compared to a 17% decline in strength (Mulder *et al.*, 2006), while only 10 days of bed rest was enough to elicit a ~6% reduction in CSA and a ~14% reduction in knee extensor MVC (Monti *et al.*, 2021). Furthermore, meta-analysis of bed rest studies with a combined 118 participants across various durations of disuse found no relationship between length of bed rest and reductions in muscle size, whereas muscle power reduction and bed rest duration were strongly related (Di Girolamo *et al.*, 2021). Numerous data provide mechanistic insight to muscle *atrophy*, including decreased MPS, increased MPB, mitochondrial dysfunction, insulin resistance, and histochemical markers of fibre denervation (Phillips *et al.*, 2009; Rudrappa *et al.*, 2016; Monti *et al.*, 2021), yet mechanistic insight into loss of muscle *function* is less clear.

4.2.3 Motor units in disuse atrophy

4.2.3.1 Surface electromyography use in disuse

Further determinants of muscle function other than just muscle mass may provide the answer. Thus the focus turns to the motor unit (MU), the basic functional unit of the neuromuscular system, identified as the alpha motor neuron and the muscle fibres it innervates (Sherrington, 1925). However, while a wealth of previous data describes the changes over varying lengths of unloading in different models (Wall *et al.*, 2013), in-depth research into the impact of disuse atrophy on the neuromuscular system is highly limited. A recent systematic review consolidated previous work to date (Campbell *et al.*, 2019). Of the 40 studies included, 20 immobilised the knee, preventing use of one of the most functionally relevant muscle groups, i.e., the quadriceps. Furthermore, just 20 studies carried out voluntary neuromuscular assessment using EMG, although all using surface EMG. Maximum torque was found to reduce by 13% after 10 days of ULLS in young healthy male participants, but EMG activity at maximum torque remained unchanged (Berg & Tesch, 1996). EMG activity at submaximal contractions, fixed at 100 Nm, was increased after unloading however, suggesting that greater neural input was required to reach the same torque values. Furthermore, torque in the loaded leg remained unchanged after the ULLS period and comparable to pre-ULLS values in the unloaded leg. Torque values were returned to baseline levels following 4 days of ambulation which implies that at least in young, healthy males there is a good degree of plasticity following short-term ULLS (Berg & Tesch, 1996). 14 days of ULLS elicited a reduction in peak torque at different isokinetic contraction velocities at an average of 17.2% in a mixed group of 6 male and 4 female participants. (Deschenes *et al.*, 2002). Average integrated EMG, the area under the curve of a rectified surface EMG signal, recorded during the MVC was reduced following unloading in the vastus lateralis and vastus medialis by ~16%. Neuromuscular efficiency, presented as torque relative to integrated EMG, was reported to be unchanged which may contrast the previous study that neural input relative to torque output is preserved in ULLS

(Deschenes *et al.*, 2002). Later, 23-day ULLS was performed in 9 young participants with 8 ambulatory controls (de Boer *et al.*, 2007*a*). Knee extensor CSA was reduced by 5.2% after 14 days and 10% after 2 days, and KE torque reduced by 14.8% after 14 days and 21% after 23 days, an example of the disparity seen between muscle size reduction and a greater muscle function reduction. Central activation, measured by imposing a supramaximal doublet stimulation at the plateau phase of an MVC to derive the percentage of voluntary and involuntary activation ratio, was not found to change following unloading nor was the maximal stimulated m-wave peak that represents the maximum potential activity of the total muscle (de Boer *et al.*, 2007*a*). This may suggest that the reductions in torque are not driven by a reduction in the voluntary activation of the muscle, potentially pointing towards more peripheral than central adaptations. This is corroborated by a study which investigated 24 days of ULLS in 6 male participants, where voluntary activation was also unchanged post-unloading while KE MVC was similarly reduced by 21% (Horstman *et al.*, 2012). Understanding the impact of unloading on an older population is also important to the preservation of muscle function in a population at greater risk of such declines (Cruz-Jentoft *et al.*, 2019). Seven days of ULLS was carried out by 10 young and 10 old male participants, although no change in EMG activity was seen in either group following unloading (Deschenes *et al.*, 2008). Although it should be noted that activity was lower in the older group than the younger group at baseline and post-unloading, however the older group saw a greater neuromuscular efficiency than the young group post-unloading. This was suggested to be caused by a preferential atrophy of fast twitch fibres in ageing which produce more EMG activity and therefore the ratio of force relative to EMG activity is inflated by this preferential atrophy or an inability to recruit these motor units (Deschenes *et al.*, 2008). Individual muscle fibres have been studied to understand potential mechanisms behind these changes before and after 4 days of knee brace immobilisation, muscle biopsies were taken from young and old men (Hvid *et al.*, 2013). Before the intervention, MHC I fibres had a lower force than MHC IIa in both young and old, with MHC 2a force also being lower in old than

in young. Following the intervention, single fibre specific force decreased in MHC I in both young and old fibres, while MHC IIa force only decreased in young. The reduction in MHC I force was the same between young and old (Hvid *et al.*, 2013). Based on force alone, these findings suggest that age does not affect MHC I fibre force decline following immobilisation, while interestingly older muscle exhibited a greater preservation of MHC IIa force than young. Calcium sensitivity was also measured in these tissues, with a lower sensitivity in MHC IIa fibres than MHC I in both young and old, while sensitivity decreased in MHC IIa fibres post-immobilisation in young but increased in MHC I fibres in old (Hvid *et al.*, 2013). This suggests that immobilisation exerts differing effects on young and old muscle tissue in terms of contractile function, although the mechanisms are yet to be determined. Collectively investigations into neuromuscular function following disuse atrophy as a result of unloading or immobilisation to date suggest that central, voluntary activation of the muscle may not be impaired and rather peripheral mechanisms may be responsible for the disparity in the greater loss of muscle function than muscle size following short-duration atrophy. While the breadth of measured parameters remains helpful, they reveal little of the adaptation of MU characteristics, particularly at the NMJ.

4.2.3.2 Neuromuscular junction in disuse

The successful activation of muscle relies upon coordinated input to the motoneuron pool and synaptic transmission across the NMJ. A recent review highlighted the current knowledge concerning the breakdown of the NMJ observed during the ageing process (Gonzalez-Freire *et al.*, 2014). However much of this work has been carried out in mouse models since the NMJ is notoriously difficult to image and study in humans, especially during active contractions to understand the cellular dynamics and how they may be altered by different conditions. Numerous factors have been proposed for the dysregulation of activity at the NMJ, such as motor endplate remodelling (Kurokawa *et al.*, 1999), i.e., the size of the nerve terminal and number of folds in the post-synaptic section are reduced which decreases the efficiency of ACh

and therefore Ca^{2+} ion transport, ultimately disrupting action potential transmission. In the context of disuse atrophy, there has been very limited human research into the NMJ and associated neuromuscular maladaptation. One study took Biopsies from the VL following 10 days of bed rest and revealed structural disruption at the NMJ (Monti *et al.*, 2021) highly suggestive of contributing to decreased activation and function. Serum c-terminal agrin fragment (CAF) concentration was increased following bed rest. This has been identified as a biomarker for the breakdown of the NMJ structure, as the native, motoneuron produced agrin exists in balance with neurotrypsin to ensure the correct structure and function of the NMJ, but when neurotrypsin excessively cleaves native agrin, the NMJ becomes dysfunctional and CAF is detectable in serum (Drey *et al.*, 2013). As discussed by Monti *et al.* (2021) however, it is not clear as to whether these changes at the NMJ are responsible for the disparity in strength and size loss in human muscle during disuse or if they are a consequence. Furthermore, these findings do not provide information regarding the direct function of the NMJ during active contractions.

4.2.4 Aims and hypothesis

The purpose of the study was to quantify neuromuscular decrements and individual MU features in the immobilised and non-immobilised limbs by utilising iEMG to sample individual MUs from the VL pre and post 15-days immobilisation. We hypothesised that:

- Coinciding with a reduction in muscle mass and strength, corresponding from that observed in Chapter 3, NMJ transmission instability and firing rate variability would increase in the immobilised leg.
- Alongside this, there would be no change to the non-immobilised, i.e., control, leg.
- A reduction in MUP area and amplitude would be present in contractions performed relative to the predicted reduced MVC post-immobilisation, but that these would be the same in contractions relative to baseline MVC providing MVC did decline.

4.3 Methods

4.3.1 Ethical approval

This study was approved by the local University Research Ethics Committee (ethics code: 103-1809) and conformed with the Declaration of Helsinki. It was also registered online at clinicaltrials.gov (NCT04199923). Participants aged 18 to 40 were recruited locally from the community via advertisement posters in print and on research group social media pages.

4.3.2 Participants

Ten healthy, young male participants were recruited to take part in the study. These participants are a subset of those who took part in the experiments carried out in Chapters 3 and 5. Characteristics are provided in Table 4.1. After providing written informed consent to participate in the study, potential participants were screened for eligibility against pre-determined exclusion criteria. These are described in detail in Chapter 2 (section 2.3.1.2) but briefly; active cardiovascular, cerebrovascular, respiratory, renal, or metabolic disease, active malignancy, musculoskeletal or neurological disorders. Once eligibility was confirmed, participants were invited to the laboratory for baseline testing, as described below (fig. 4.1A).

4.3.3 Initial testing visit

4.3.3.1 Muscle ultrasound

VL cross sectional area scans (CSA) were taken at the mid-belly of the muscle (fig. 4.1D). These methods are detailed in Chapter 2.1.1.1. Briefly, three axial plane images were collected following this line from both legs and subsequently analysed using ImageJ (Laboratory of Optical and Communication, University of Wisconsin-Madison, WI, USA) to quantify CSA (Scott *et al.*, 2017). Three measurements were made from each image which were averaged, providing three mean values per participant that were subsequently averaged for each leg and timepoint for analysis. Due to equipment malfunction at follow-up, n=9 for US.

4.3.3.2 Nottingham Power Rig

Explosive unilateral lower limb power was assessed using the Nottingham Power Rig (University of Nottingham; (Bassey & Short, 1990)). These methods are detailed in Chapter 2 section 2.1.2. Briefly, the power exerted from the highest of three attempts recorded. Due to equipment malfunction at follow-up, n=9 for unilateral lower limb power assessment.

4.3.3.3 Maximal voluntary isometric contraction

Knee extensor MVC force was measured from both legs. Thes methods are detailed in Chapter 2 section 2.1.7. Briefly, three MVC attempts were carried out and the highest value was taken as the maximal and used to determine voluntary contraction intensity (fig. 4.1B).

4.3.3.4 Vastus lateralis motor point identification

These methods are detailed in Chapter 2 section 2.2.1. Briefly, the surface of the VL was explored to find the point producing the greatest visible twitch with the lowest current (Piasecki *et al.*, 2016*b*).

4.3.3.5 Force and surface electromyography recording

Surface electromyography was arranged in a bipolar configuration. These methods are detailed in Chapter 2 section 2.2.2. Briefly, the recording electrode was placed over the identified motor point and the reference electrode over the patellar tendon and a ground electrode was placed just above the reference electrode on the patella.Peak twitch force was calculated using percutaneous electrical stimulation. The peak force generated corresponding to the maximal M-wave was recorded (fig. 4.1C). As three participants were unable to tolerate femoral nerve stimulation at follow-up visits, n=7 for this assessment. Force steadiness (FS) was assessed during the sustained voluntary contractions and these methods are detailed in Chapter 2 section 2.1.8.

4.3.3.6 Intramuscular electromyography procedure

These methods are detailed in Chapter 2 section 2.2.3. Briefly, a concentric needle electrode was inserted at the VL motor point. Contractions were carried out at 10% and 25% of the participants MVC following a visual target line (fig. 4.1B). These voluntary contractions were held for 12 s with a ~20 s rest in between each contraction.

4.3.3.7 EMG analysis

These methods are detailed in Chapter 2 section 2.2.4. Briefly, decomposition-based quantitative electromyography (DQEMG) was used for all iEMG data analysis. This involved the detection of MUPs and extraction of MUP trains (MUPTs) recorded during voluntary contractions MUP area was defined as the integral of the absolute value of MUP values. MUP amplitude was determined as the measurement from the maximal positive and negative peaks of the waveform (Guo *et al.*, 2022). MUP complexity, measured as the number of turns, was defined as the number of significant slope direction changes within the duration of the MUP of a height >20 µV (fig. 4.1G). A near fibre MUP (NF-MUP) was obtained from each MUP by estimating the slopes of each MUP (Piasecki *et al.*, 2021*b*) and NMJ transmission instability, measured as near-NF-MUP jiggle, was determined as the normalised means of median consecutive amplitude differences (Piasecki *et al.*, 2021*b*)(fig. 4.1F). MU FR was recorded as the rate of occurrence per second of MUPs within a MUPT in Hz, and MU FR variability was determined as the coefficient of variation of the inter-discharge interval.

4.3.4 Immobilisation procedure

The immobilisation strategy is detailed in Chapter 2 section 2.3. Briefly, for the 15 days of immobilisation, a modified ULLS model was used. The knee joint was fixed at 75 degrees flexion and the ankle joint was fixed using an air-boot. Crutches were provided and adjusted according to the height of the participant.

4.3.5 Follow-up testing visit

Following 15 days of immobilisation, participants were invited back to the laboratory for post-immobilisation testing (fig. 4.1A). All procedures carried out in the baseline testing visit were repeated in both legs with iEMG performed at the same motor point as the initial visit. In addition to measuring FS and iEMG at 25% of post-immobilisation MVC (i.e., relative force, referred to as follow-up force), iEMG was also performed at 25% of pre-immobilisation MVC (i.e., absolute force, referred to as baseline force) to compare FS and MU characteristics normalised to pre and post disuse-induced strength loss.

4.3.6 Statistical analysis

Statistical analysis of CSA, MVC, twitch force, power, and FS was performed using GraphPad Prism version 9.1.0 (GraphPad Software, CA, USA) using repeated measures 2-way analysis of variance with Šidak's post-hoc analysis in the event of a significant interaction. Multi-level mixed effect linear regression models were used to analyse MU parameters, in StataSE (v16.0, StataCorp LLC, TX, USA). For these models the first level was single motor unit; single motor units were clustered according to each participant to form the second level, which was defined as the participant level and reflects the total n. Two within-subject factors were included; leg (immobilised and control) and time (pre and post), and leg x time interactions were included in all models. Additional exploratory analyses were performed to investigate relationships between iEMG variables and key physiological outcome variables – muscle strength (MVC) and size (CSA) using R (Version 4.2.0, (https://cran.r-project.org/) implemented using R studio. Firstly, correlative analysis was assessed with Pearson's product moment correlation coefficient and visualised using corrplot (https://cran.r-project.org/web/packages/corrplot) to determine any strong relationships between variables. Cluster analysis and principal component analysis for variables were performed using the ClustOfVar (https://cran.r-project.org/web/packages/ClustOfVar) and factoextra (https://cran.r-project.org/web/packages/factoextra) packages respectively, to determine which variables strongly clustered and related to others. The variables used for

these analyses included those measured from 25% pre immobilisation and 25% follow-up in the immobilised limb only, namely MVC, CSA, MUP area, MUP amplitude, MUP complexity, NMJ transmission instability, MU FR and MU FR variability. Finally, using a subset of these best clustering variables, namely MVC, CSA, FR, FR variability and NMJ transmission instability, multivariate linear regression was performed to determine which clustered variables best predict changes in MVC and CSA. Significance was assumed if $p<0.05$.

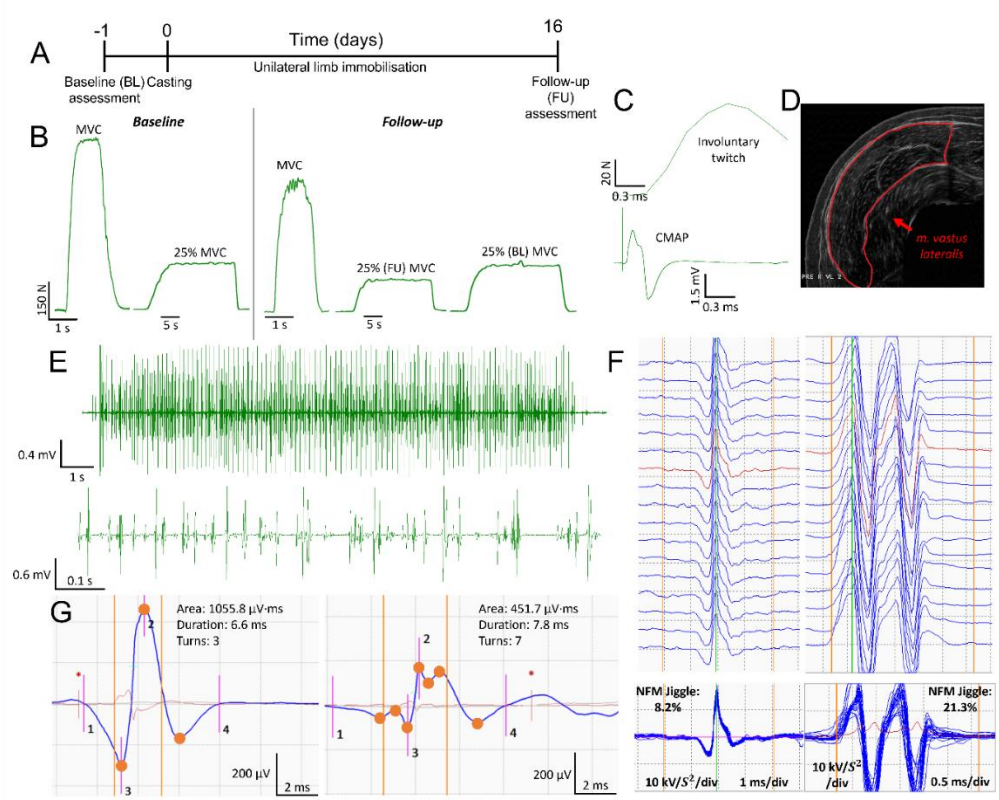


Figure 4.1: (A) *Schematic of the study timeframe.* ***(B)*** *Knee extensor force traces (left to right) from a maximal voluntary isometric contraction (MVC), 25% of MVC contraction pre 15-day unilateral limb immobilisation, 25% contraction post-immobilisation relative to follow-up MVC, and 25% contraction post-immobilisation relative to baseline MVC.* ***(C)*** *Peak twitch force recording showing force trace (upper) and corresponding surface electromyography EMG recorded M-wave (lower) from a maximal stimulation of the femoral nerve.* ***(D)*** *An example ultrasound scan of the m. vastus lateralis used to calculate cross-sectional area.* ***(E)*** *Example intramuscular EMG (iEMG) trace recorded during a 12 second voluntary isometric contraction (upper) and magnified (lower) with visible motor unit potentials (MUP).* ***(F)*** *Example raster plots (upper) and corresponding shimmer plots (lower) from near-fibre MUPs (NF-MUP) before (left) and after (right) immobilisation recorded during active contractions.* ***(G)*** *Example MUPs recorded before (left) and after (right) immobilisation. Turns are*

marked with an orange circle. N; newtons, s; seconds, ms; milliseconds, V; volts, µV; microvolts, kV/s^2; kilovolts per second squared, µV·ms; microvolts per millisecond.

4.4 Results

4.4.1 Participant Characteristics

Nine male participants took part in this study. Characteristics can be seen in table 4.1.

Table 4.1: *Descriptive characteristics of participants showing mean and standard deviation (SD)*

N = 10	Mean (SD)
Age (years)	23.7 (3.4)
Height (cm)	181.6 (6.7)
Weight (kg)	79.4 (9.7)
BMI (kg/m^2)	24.0 (2.1)

4.4.2 Muscle Size and Function

There was a significant leg x time interaction in VL CSA ($p<0.001$), which decreased in the immobilised leg after 15 days (−15%, $p<0.001$) and remained unchanged in the control leg ($p=0.761$, fig. 4.2A). Similarly, there was a significant leg x time interaction of knee extensor MVC ($p=0.005$), which decreased in the immobilised leg (−31%, $p<0.001$) while no change was seen in the control leg ($p=0.498$, fig. 3.2B). Peak twitch force showed no significant leg x time interaction ($p=0.549$, fig. 4.2C). Unilateral lower limb power output presented a significant leg x time interaction ($p=0.017$), which reduced significantly in the immobilised limb (−26%, $p=0.003$) while remaining unchanged in the control leg ($p=0.939$, fig. 3.2D). FS at 10% MVC presented a significant leg x time interaction ($p=0.020$, fig. 4.2E), with non-significant changes in the immobilised limb (−14%, $p=0.077$) and the control ($p=0.278$). No interaction was present in FS at 25% MVC ($p=0.255$, fig. 4.2E), nor was an interaction present in FS at 25% baseline MVC ($p=0.349$).

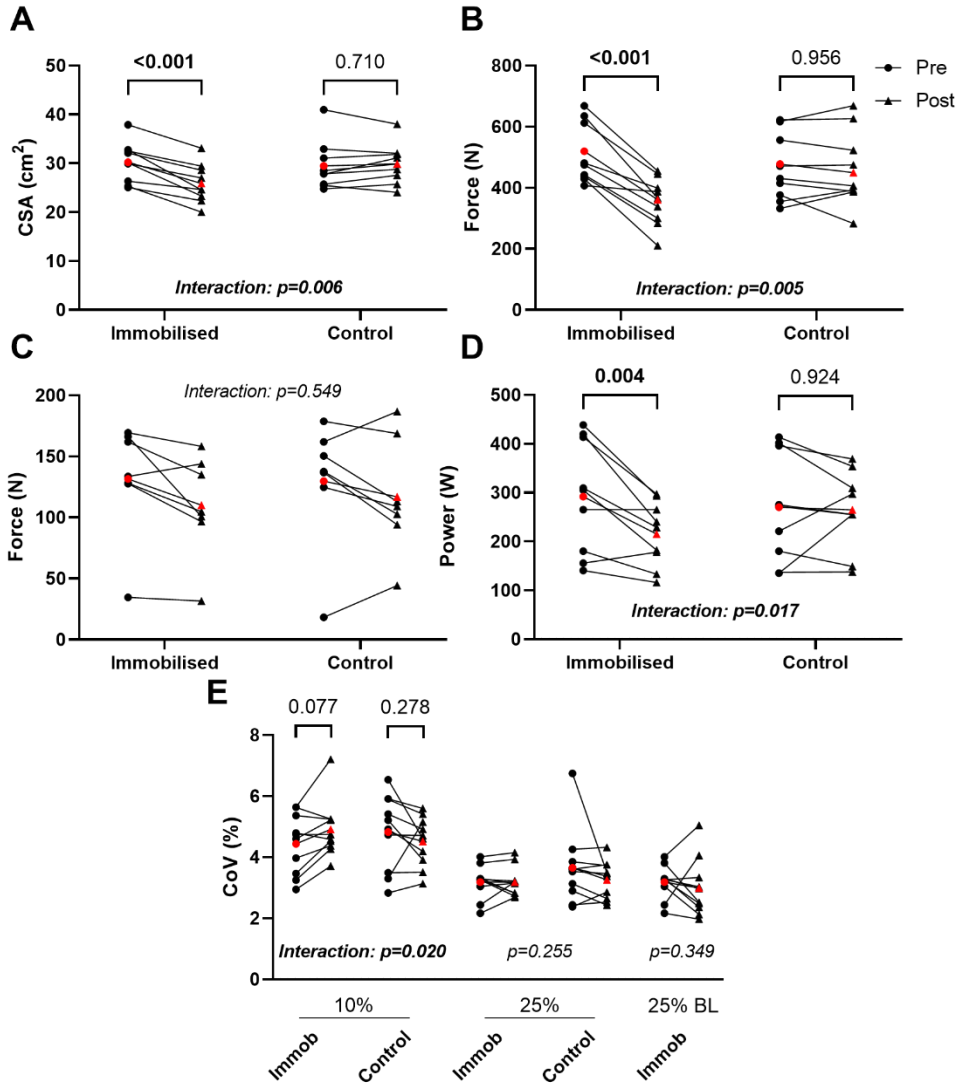


Figure 4.2: *All figures show data before and after 15-day unilateral lower limb suspension in the immobilised and control legs.* ***A:*** *Vastus lateralis cross-sectional area (cm²) measured by ultrasound (n=9).* ***B:*** *Knee extensor maximal voluntary isometric contraction force (N).* ***C:*** *Peak twitch force (N, n=7) measured from maximal involuntary contraction elicited by femoral nerve stimulation.* ***D:*** *Unilateral lower limb power measured using the Nottingham Power Rig.* ***E****: Knee extensor force steadiness measured during contractions at 10 and 25% relative to both follow-up and baseline maximal voluntary isometric contraction. Coefficient of variation shown as average deviation from target line (individual mean values shown pre to post in immobilised and control legs at each contraction intensity). For all parameters, analysis performed via repeated measures 2-way analysis of variance with Šidak's post-hoc analysis.*

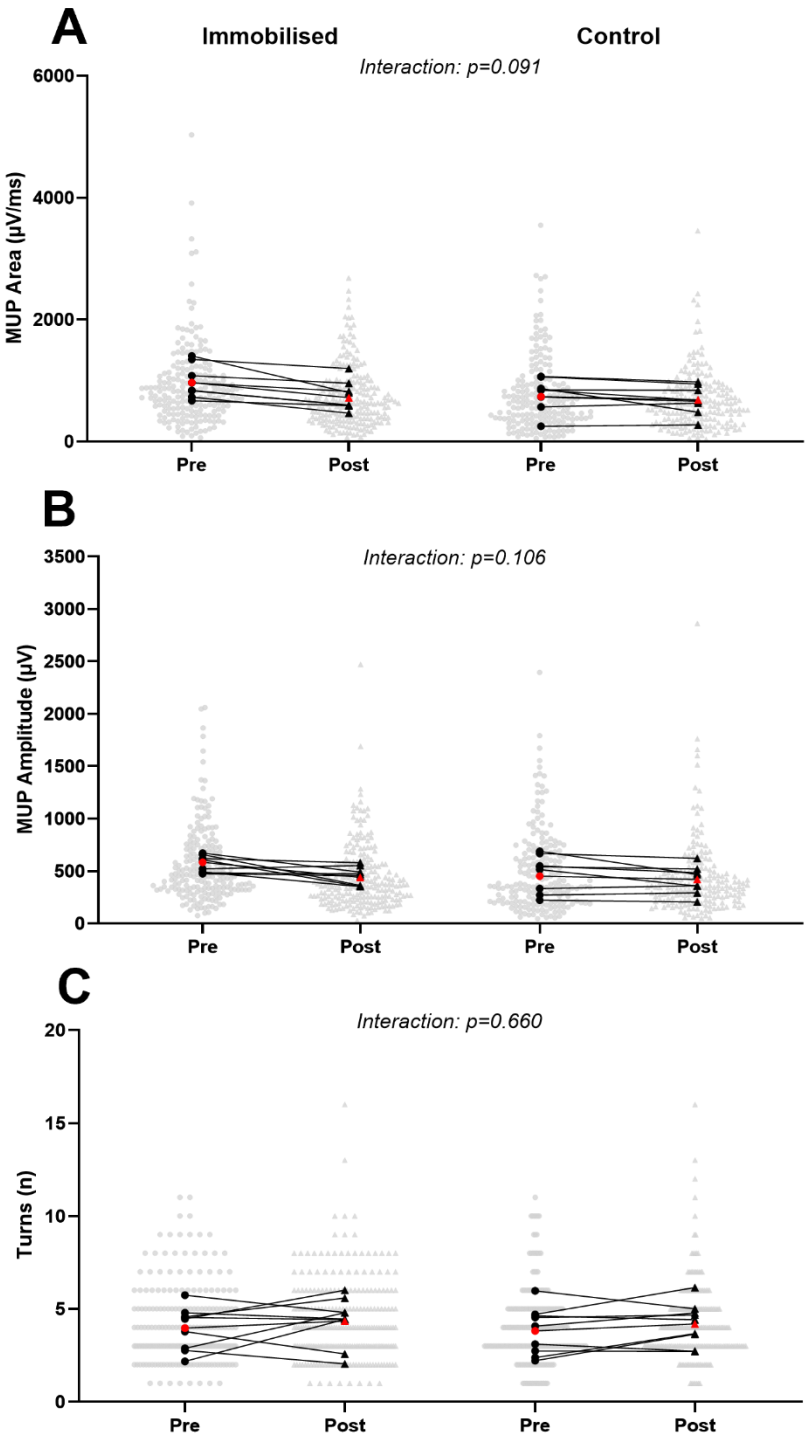
4.4.3 Neuromuscular characteristics

MUP area and amplitude at 10% MVC presented no significant leg x time interaction (p=0.091 and p=0.106, respectively, fig. 4.3A, 4.3B, 4.6A and 4.6B). MUP complexity, defined as the number of turns, presented no significant leg x time interaction at 10% MVC (p=0.660, fig. 4.3C and 4.6C). At 10% MVC, NMJ transmission instability showed no leg x time interaction (p=0.191, fig. 4.3D and 4.6D). MU FR presented a significant leg x time interaction at 10% MVC (p=0.022, fig. 4.3E and 4.6E), with a reduction in the immobilised leg (p<0.001) but no change in the control (p=0.848). Firing rate variability presented no leg x time interaction at 10% (p=0.070, fig. 4.3F and 4.6F).

MUP area at 25% follow-up MVC presented a significant leg x time interaction (p=0.028, fig. 4.4A and 4.6A), reflected by a reduction in the immobilised leg (p<0.001) but no change in the control (p=0.717). Similarly, MUP amplitude presented a significant leg x time interaction (p=0.046, fig. 4.4B and 4.6B), with a reduction in the immobilised leg (p=0.007) but no change in the control (p=0.881). MUP complexity showed a significant leg x time interaction (p=0.007, fig. 4.4C and 4.6C), with an increase in the immobilised leg (p=0.009), but no change in the control (p=0.600). For NMJ transmission instability, as assessed by NF-MUP Jiggle, at 25% follow-up MVC, no leg x time interaction was observed (p=0.966, fig. 4.4D and 4.6D). For FR at 25% follow-up MVC a significant leg x time interaction was also observed (p<0.001, fig. 4.4E and 4.6E), again showing a reduction in the immobilised leg (p<0.001) but no change in the control (p=0.563). Firing rate variability at 25% follow-up MVC presented no significant leg x time interaction (p=0.082, fig. 4.4F and 4.6F).

At 25% baseline MVC, a significant leg x time interaction was seen in MUP area (p<0.001, fig. 4.5A and 4.6A), with a reduction in the immobilised leg (p<0.001) not reflected in the control leg (p=0.891). Similarly, MUP amplitude also presented a significant leg x time interaction (p=0.008, fig. 4.5B and 4.6B) with a significant reduction in the immobilised leg (p=0.021) but no change in the control leg (p=0.634). MUP complexity did not present a significant interaction (p=0.086, fig. 4.5C and 4.6C), nor did NMJ transmission instability (p=0.856, fig.

4.5D and 4.6D). There was a significant interaction for firing rate ($p<0.001$, fig. 4.5E and 4.6E), which decreased in the immobilised leg ($p<0.001$) with no change in the control ($p=0.482$). At 25% baseline MVC FR variability showed a significant interaction ($p<0.001$, fig. 4.5F and 4.6F), which increased in the immobilised ($p<0.001$) but not the control ($p=0.951$) leg.


A
Immobilised
Control
Interaction: p=0.091
MUP Area (µV/ms)
6000
4000
2000
0
Pre
Post
Pre
Post
B
Interaction: p=0.106
MUP Amplitude (µV)
3500
3000
2500
2000
1500
1000
500
0
Pre
Post
Pre
Post
C
Interaction: p=0.660
Turns (n)
20
15
10
5
0
Pre
Post
Pre
Post

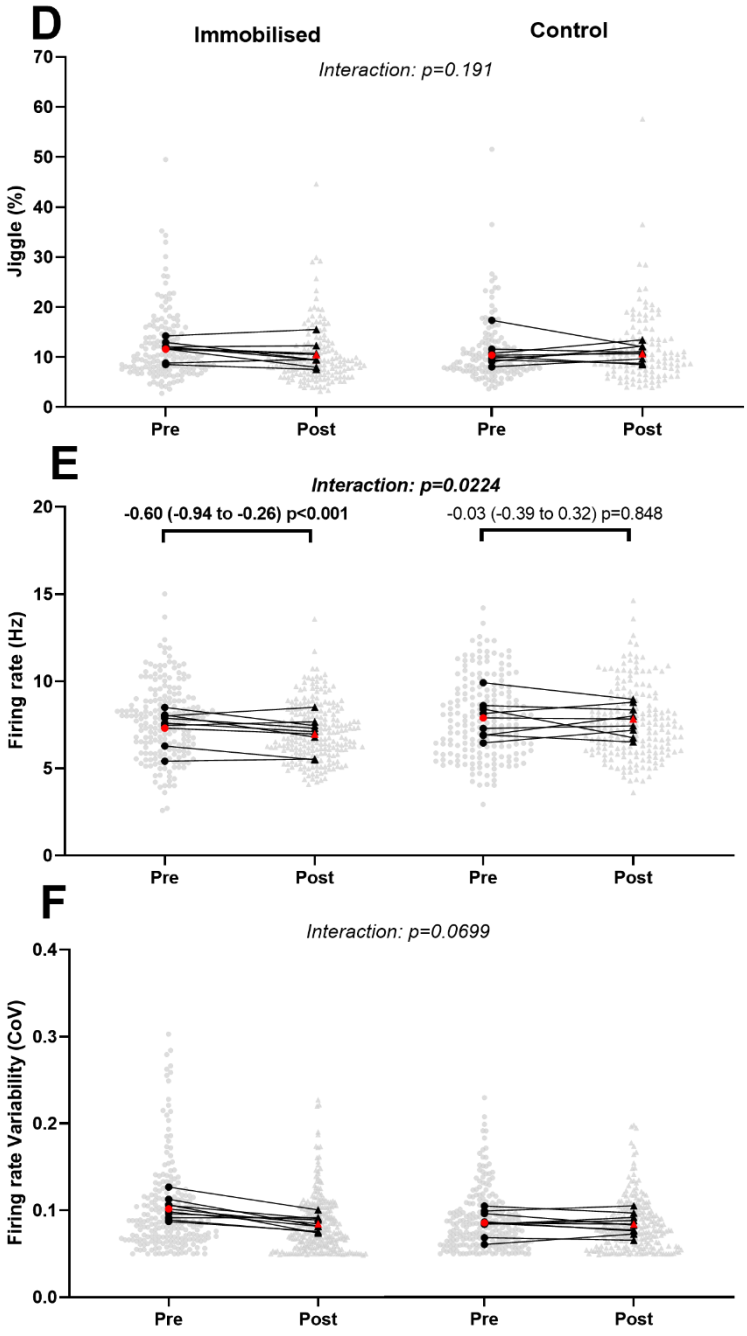
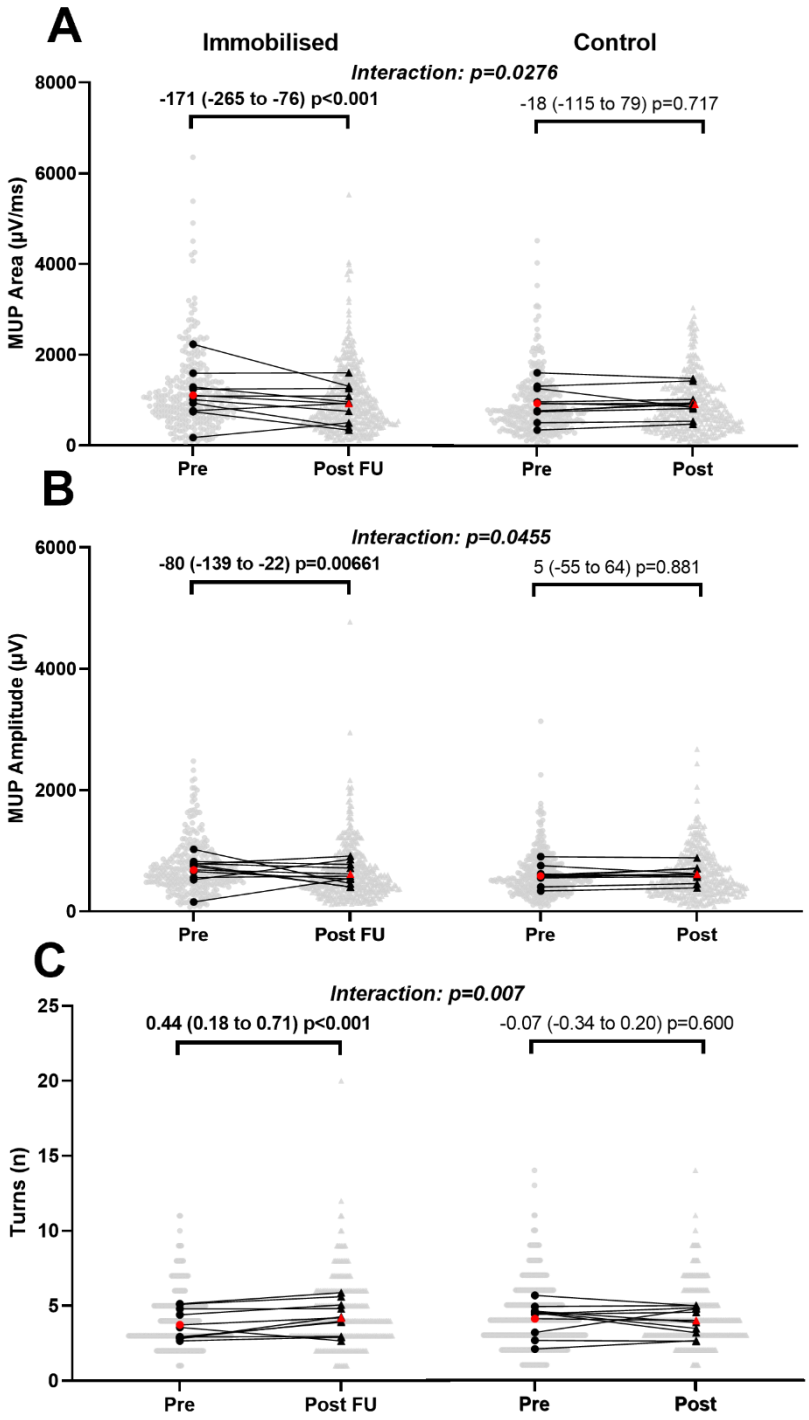


Figure 4.3: *Motor unit potential (MUP) and discharge characteristics of motor units sampled during contractions performed at 10% maximal voluntary force before and after 15-day unilateral leg immobilisation in both immobilised and control legs. Data show individual mean values and pooled MUs with comparison bars showing β coefficient and 95% CI from multi-level mixed effects models. Group mean of individual means is shown in red. N = 9. µV/ms; microvolts per millisecond, µV; microvolts, n; number, Hz; hertz, CoV; coefficient of variation.*


A
Immobilised
Control
Interaction: p=0.0276
-171 (-265 to -76) p<0.001
-18 (-115 to 79) p=0.717
MUP Area (µV/ms)
8000
6000
4000
2000
0
Pre
Post FU
Pre
Post
B
Interaction: p=0.0455
-80 (-139 to -22) p=0.00661
5 (-55 to 64) p=0.881
MUP Amplitude (µV)
6000
4000
2000
0
Pre
Post FU
Pre
Post
C
Interaction: p=0.007
0.44 (0.18 to 0.71) p<0.001
-0.07 (-0.34 to 0.20) p=0.600
Turns (n)
25
20
15
10
5
0
Pre
Post FU
Pre
Post

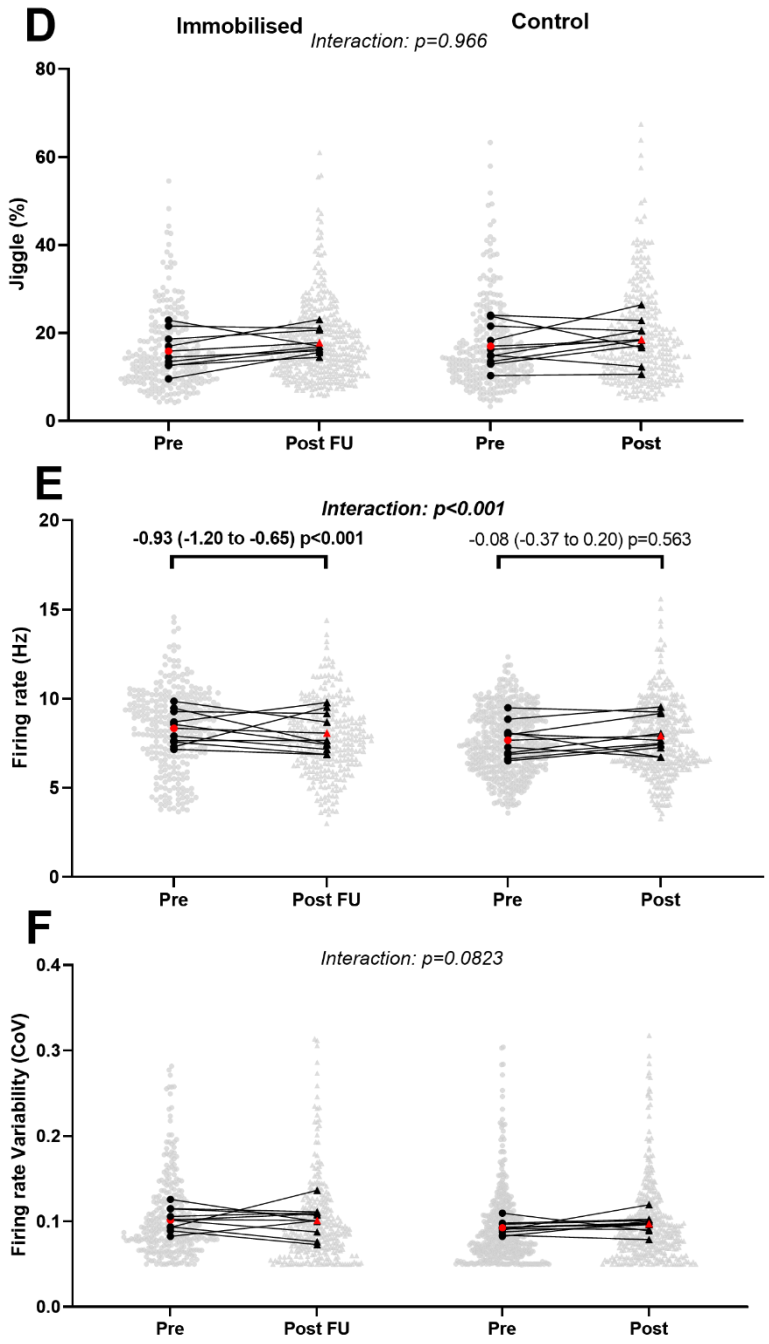
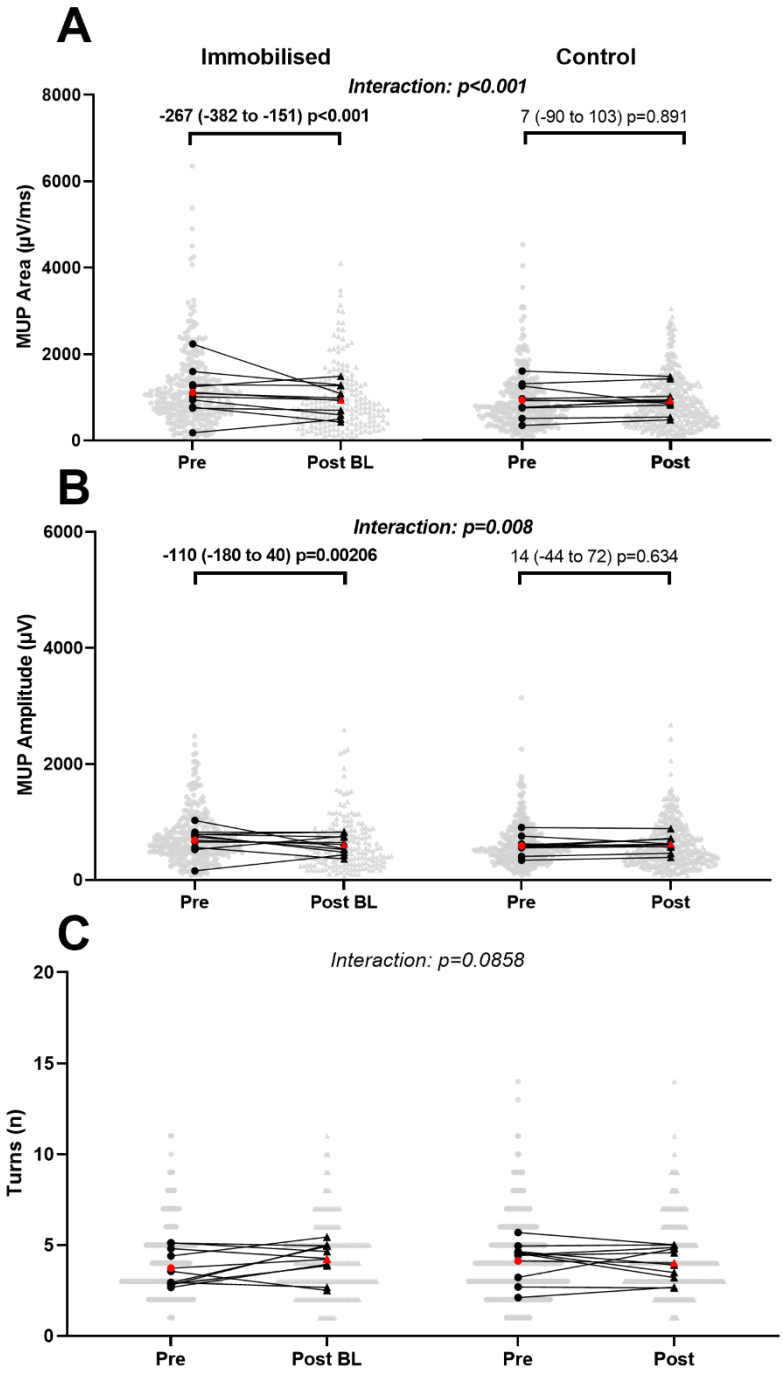


Figure 4.4: *Mean values of MUP and MU discharge characteristics sampled during contractions performed at 25% maximal voluntary force before and after, relative to follow-up (FU) MVC, 15-day unilateral leg immobilisation in both immobilised and control legs. Values in the immobilised leg post-immobilisation are relative to follow-up MVC. Data show mean values and pooled MUs with comparison bars showing β coefficient and 95% CI from multi-level mixed effects models. Group mean of individual means is shown in red. µV/ms; microvolts per millisecond, µV; microvolts, n; number, Hz; hertz, CoV; coefficient of variation.*


A
Immobilised
Control
Interaction: p<0.001
-267 (-382 to -151) p<0.001
7 (-90 to 103) p=0.891
MUP Area (µV/ms)
8000
6000
4000
2000
0
Pre
Post BL
Pre
Post
B
Interaction: p=0.008
-110 (-180 to 40) p=0.00206
14 (-44 to 72) p=0.634
MUP Amplitude (µV)
6000
4000
2000
0
Pre
Post BL
Pre
Post
C
Interaction: p=0.0858
Turns (n)
20
15
10
5
0
Pre
Post BL
Pre
Post

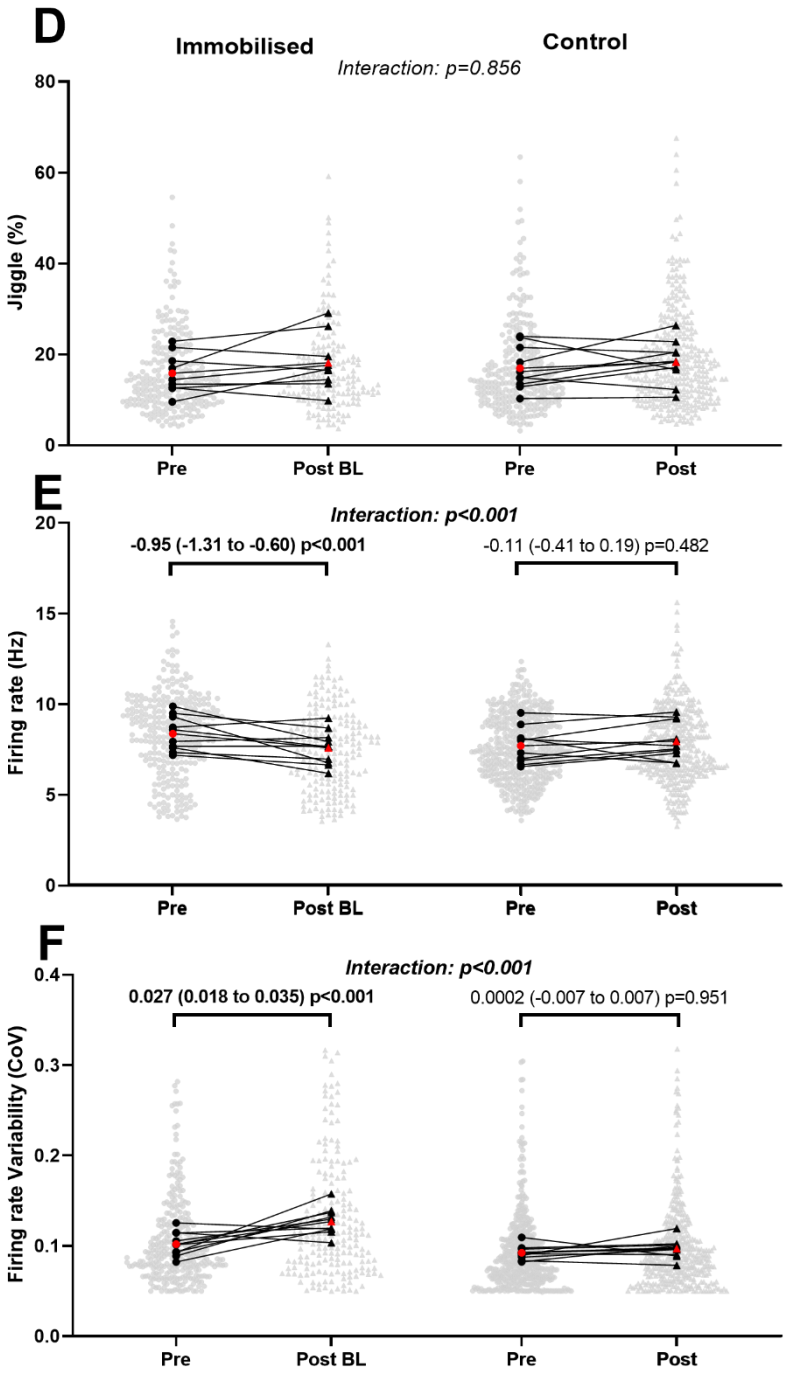
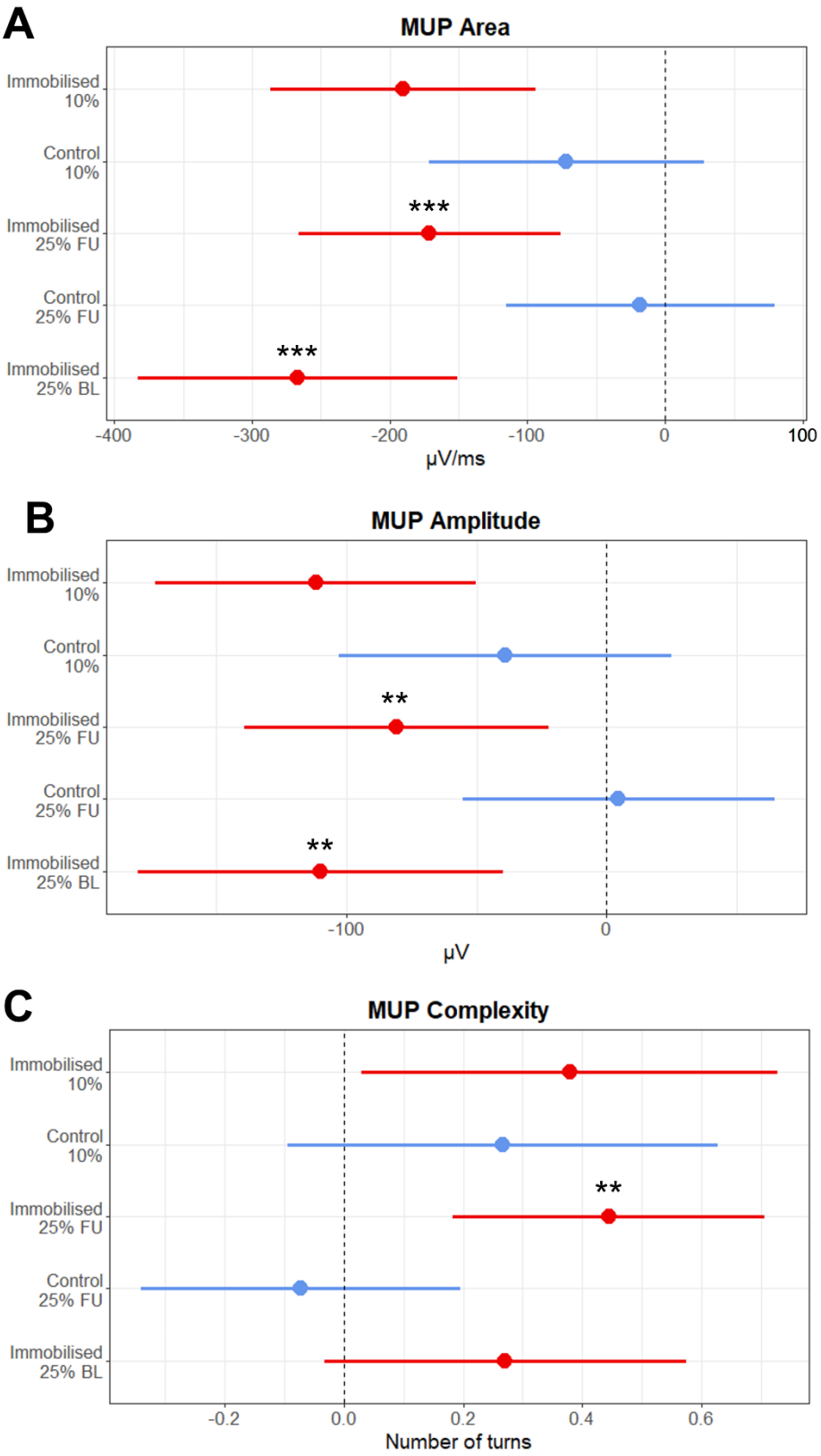


Figure 4.5: *Mean values of MUP parameters and MU discharge characteristics of motor units sampled during contractions performed at 25% maximal voluntary force before and after, relative to baseline (BL) MVC, 15-day unilateral leg immobilisation in both immobilised and control legs. Values in the immobilised leg post-immobilisation are relative to follow-up MVC. Data show mean values and pooled MUs with comparison bars showing β coefficient and 95% CI from multi-level mixed effects models. Group mean of individual means is shown in red. µV/ms; microvolts per millisecond, µV; microvolts, n; number, Hz; hertz, CoV; coefficient of variation.*


A
MUP Area
Immobilised 10%
Control 10%
Immobilised 25% FU
Control 25% FU
Immobilised 25% BL
***
***
-400
-300
-200
-100
0
100
μV/ms
B
MUP Amplitude
Immobilised 10%
Control 10%
Immobilised 25% FU
Control 25% FU
Immobilised 25% BL
**
**
-100
0
μV
C
MUP Complexity
Immobilised 10%
Control 10%
Immobilised 25% FU
Control 25% FU
Immobilised 25% BL
**
-0.2
0.0
0.2
0.4
0.6
Number of turns

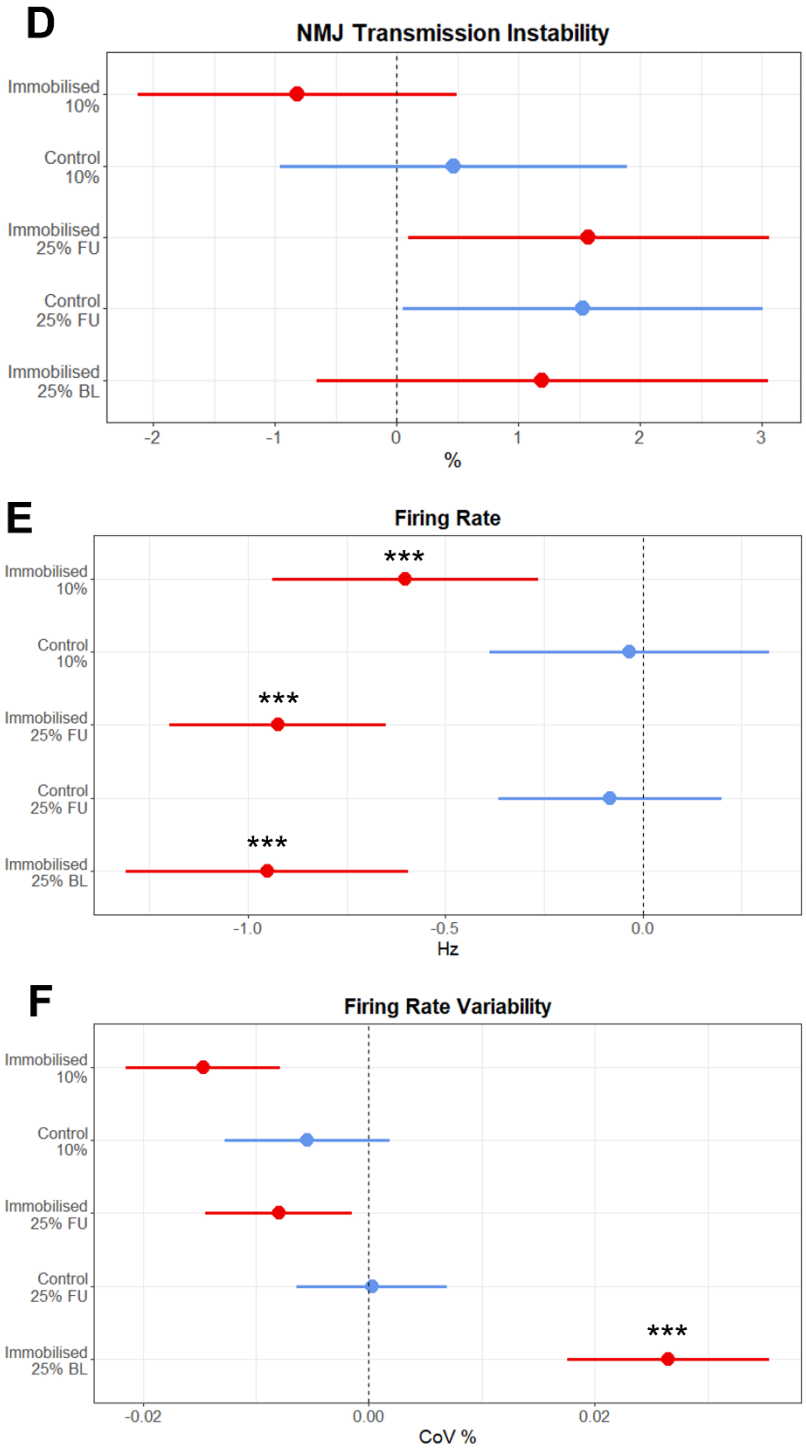


Figure 4.6: *Forest plots summarising model outputs from motor unit characteristics measured from the vastus lateralis during 10% MVC, and 25% normalised to follow-up (FU) and baseline (BL) MVC. Plots display beta coefficient and 95% confidence intervals from multi-level mixed linear regression models. **=p<0.01, ***=p<0.001. µV/ms; microvolts per millisecond, µV; microvolts, Hz; hertz, CoV; coefficient of variation.*

4.4.4 Exploratory analysis

In exploratory analyses, to investigate potential relationships between neuromuscular parameters and muscle strength and size, correlation analysis was performed on the mean difference between MU characteristics assessed at 25% MVC at baseline, and 25% of FU MVC (fig. 4.7). Strong correlations were observed between MUP area and amplitude (r^2=0.92, p=0.0003) and between NMJ transmission instability and MVC (r^2=0.89, p=0.0082). To more accurately reflect relationships between these variables, clustering of these variables was performed using the hierarchical clustering algorithm provided in ClustOfVar and further visualised using principal component analysis score plots (PCA, fig. 4.8). The key cluster of interest consisted of MVC, CSA, NMJ transmission instability (jiggle), FR and FR variability. To test whether the neuromuscular parameters in this cluster had any influence on the changes observed in muscle strength and size, the values were first normalised to the same dynamic range before multivariate linear regression was performed. There were no significant relationships for either MVC or CSA with NMJ transmission instability (p=0.191 and p=0.625 respectively), FR variability (p=0.759 and p=0.495) and FR (p=0.350 and p=0.907).

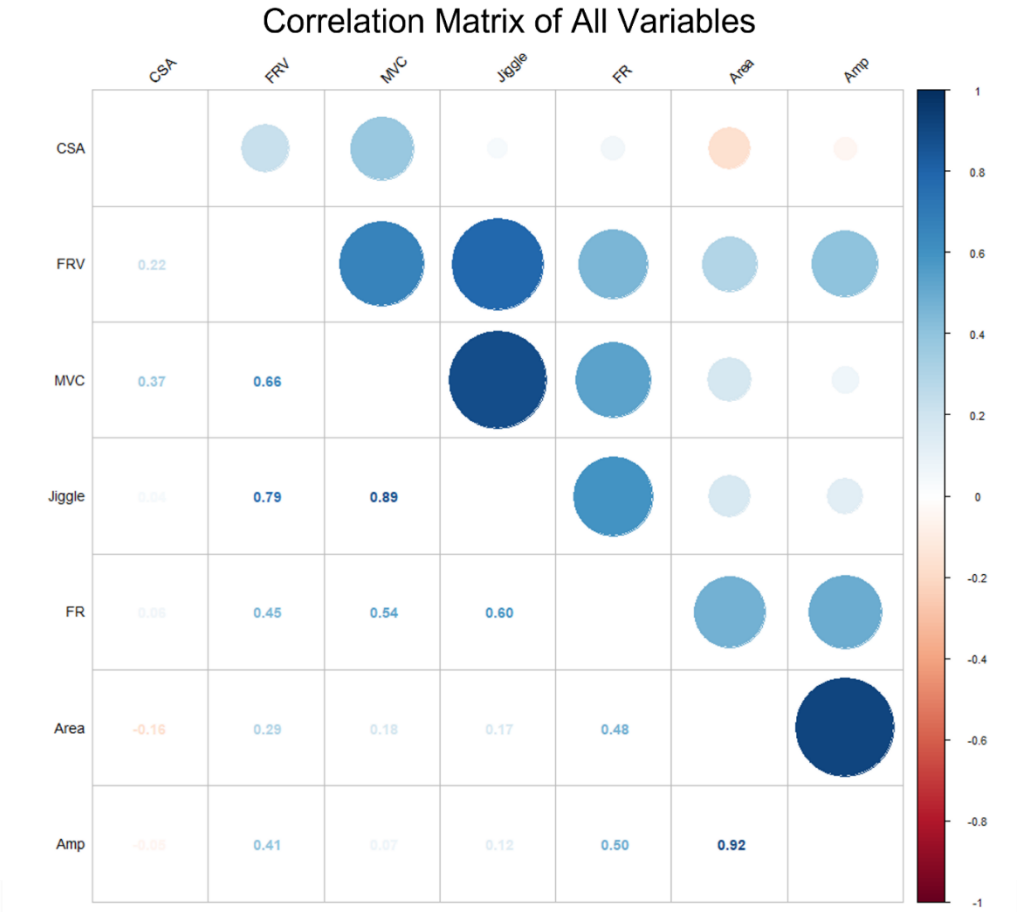


Figure 4.7: *Correlation matrix of relationships between eight variables measured in this study. CSA; cross-sectional area, FRV; firing rate variability, MVC; maximal voluntary contraction, FR; firing rate, Amp; amplitude.*

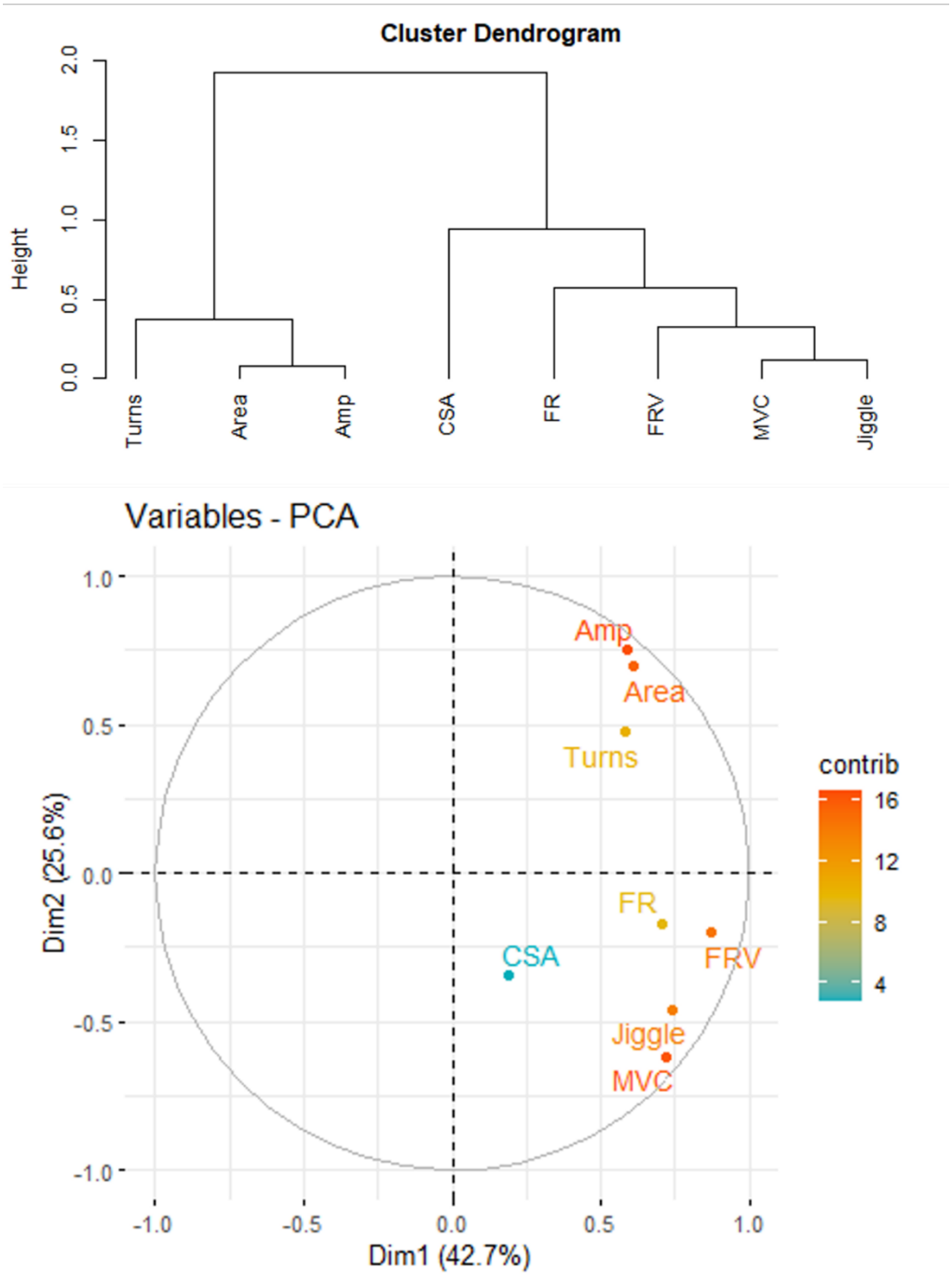


Figure 4.8: *Cluster tree plot (upper) and accompanying principal component analysis plot (lower) to illustrate relationships between variables within the first two dimensions of variance between variables. Amp; amplitude, FRV; firing rate variability, MVC; maximal voluntary contraction, CSA; cross-sectional area, FR; firing rate, Dim; dimension.*

4.5 Discussion

This study has characterised the adaptations of individual VL MUs following 15 days unilateral lower-limb immobilisation, in immobilised and non-immobilised limbs. Our findings reveal a number of adaptations that are unique to the immobilised limb only, with the non-immobilised limb largely unaffected. Muscle of the immobilised leg became smaller and weaker, and individual MUPs became smaller and more complex. Motor units of the immobilised leg also had reduced FR. Notably, the majority of these decrements were still apparent when force levels were normalised to that achieved prior to immobilisation, highlighting impaired neural input to muscle as a prominent contributor to the observed reduction in muscle function.

4.5.1 Strength and size

The observed decline in muscle strength (−31%) in the immobilised limb exceeded the decline in muscle CSA (−15%), and this discordant finding supports several other studies employing a ULLS model. For example, 14-day unilateral knee immobilisation resulted in a ~5% decrease in quadriceps CSA and a ~25% decline in isometric strength (Glover *et al.*, 2008). Additionally, following 14 days of limb cast immobilisation, an ~8.5% decline in quadriceps CSA was observed alongside a ~23% decline in muscle strength (Wall *et al.*, 2014). Unilateral power output was reduced in the immobilised limb only. Bed rest studies have collectively shown a reduction in muscle power of ~3% per day, although this reduction was seen to stabilise between day 5 and day 14, suggesting a sharp initial reduction (Di Girolamo *et al.*, 2021) which may have also occurred in the present study. Furthermore, the control limb remained unaffected in terms of functional loss and clearly evidences the non-immobilised limb is spared from these decremental adaptations. Peak twitch force was unaltered when assessed with an involuntary contraction elicited from a single pulse applied over the femoral nerve. However, given muscle force is highly dependent on the rate of MU firing, this method may lack sensitivity when assessing involuntary force loss from a single electrical pulse.

Similarly, although a leg x time interaction in force steadiness was apparent at lower contraction levels, immobilisation did not notably influence force steadiness and indicates a preservation of basic motor control following disuse.

4.5.2 Motor unit potential size

The size of a MUP (area and amplitude) is reflective of the depolarisation of all fibres within a single MU, within the detection area of an indwelling electrode and is proportional to the size and number of fibres contributing to it. Area and amplitude were significantly decreased post-intervention at 25% follow-up and 25% baseline MVC contraction levels in the immobilised leg only. While greater MUP size is commonly observed in aged muscle reflecting MU remodelling and reinnervation of denervated muscle fibres (Jones *et al.*, 2022), smaller MUPs differentiated sarcopenic from non-sarcopenic older men in this muscle group (Piasecki *et al.*, 2018*b*). The reduction observed here may indicate partial denervation of MU fibres as a result of disuse, or it may reflect extensive fibre atrophy across MU fibres sampled at these contraction levels. MUP size reduction has also been observed in dystrophinopathies (Zalewska *et al.*, 2013), where amplitude in the biceps brachii was significantly below reference values in patients with Duchene and Becker muscular dystrophies (Zalewska *et al.*, 2004). All markers of MUP size increase with increasing contraction level, reflecting the recruitment of additional, larger MUs (Guo *et al.*, 2022), and the decreases observed in the immobilised leg were apparent at force levels normalised to maximum pre and post disuse-induced strength loss. Therefore, the reduction of force post-immobilisation cannot explain the decline in MUP size and more likely reflects a reduction in muscle fibre size and/or partial denervation/reinnervation of fibres.

4.5.3 Motor unit complexity

Similar to MUP size, MUP complexity, defined as the number of MUP turns, was significantly greater in the immobilised leg only following the intervention, although not at all contraction levels, which is indicative of a greater temporal electrophysiological dispersion between individual fibres of the same MU

(Stålberg & Sonoo, 1994; Piasecki *et al.*, 2021*b*) as a result of an increased difference in conduction times along axonal branches and/or MU fibres. Increased MUP complexity has been observed in various myopathies which are suggestive of increased fibre diameter variability and also in neuropathies, expressing 75% greater turns than the control cohort, which is thought to be a product of the reinnervation and longer conduction times along axonal sprouts (Stewart *et al.*, 1989). This has been reinforced specifically in myopathic conditions, as data from primarily the biceps brachii along with recordings of the vastus lateralis and gastrocnemius showed an 82% increase in polyphasic MUPs (Uncini *et al.*, 1990). While much less severe, the changes observed here following immobilisation may suggest that some reinnervation has occurred, or alternatively that selective reduction in muscle fibre diameter has taken place, affecting MU fibres unequally resulting in more variable timing muscle fibre action potential propagation. Although needle insertions around the muscle motor point help to minimise the effects of variable muscle fibre conduction times and enable greater focus on axonal branch conduction variability, the specific motor endplate location remains an unknown *in vivo* and these effects cannot be completely excluded.

4.5.4 NMJ transmission instability

Greater NMJ transmission instability, as quantified by NF-MUP jiggle, has been reported in patients with chronic inflammatory demyelinating polyneuropathy (Gilmore *et al.*, 2017), diabetic neuropathy (Allen *et al.*, 2015), and healthy ageing (Hourigan *et al.*, 2015; Piasecki *et al.*, 2016*c*, 2021*a*; Power *et al.*, 2016), corresponding with larger MUPs indicative of MU remodelling (Jones *et al.*, 2022). Here we found no statistically significant leg x time interaction in NF-MUP jiggle, at any contraction levels assessed and as such, does not support increased transmission instability at the NMJ. Recent histological findings demonstrate a greater proportion of NCAM positive fibres following 10 days bed rest which was interpreted as increased NMJ disruption (Monti *et al.*, 2021). This method has previously been used in humans to identify denervated fibres following 3-day dry immersion (Demangel *et al.*, 2017) and 14-day bed

rest (Arentson-Lantz *et al.*, 2016). Although the current electrophysiological findings do not support this, it may reflect differences of severity in bed rest compared to unilateral limb immobilisation. Furthermore, it is also possible disuse-induced denervation is more apparent in later recruited MUs, beyond the range of the *in vivo* methods applied here.

4.5.5 Motor unit firing rate

MU FR was reduced following immobilisation and loss of strength and may initially be explained by the lower absolute forces produced. However, this suppression of FR was also apparent at contractions normalised to baseline strength and clearly highlights it as a contributor to functional decrements. Ionotropic synaptic inputs and neuromodulation control the excitability of motoneurons, the latter of which is largely mediated by the amplitude of persistent inward currents (PICs) which act to amplify synaptic input and are proportional to the level of localised monoamine release (Heckman *et al.*, 2008). Although difficult to quantify *in vivo*, it is possible that monoamine levels decrease in response to reduced activity, yet it is unclear how this would influence the immobilised limb only. PIC amplitudes are also sensitive to inhibition (Hyngstrom *et al.*, 2007; Mesquita *et al.*, 2022), and recent RNAseq data highlight increased ligand-receptor interactions between muscle and dorsal root ganglion neurons following disuse, suggestive of an increased nociceptor sensitivity and susceptibility to pain (McFarland *et al.*, 2022). As such, it is possible increased inhibition occurred in the immobilised limb only and suppressed PIC amplitudes and MU FR, similar to that believed to explain decreased FR following knee joint trauma (Nuccio *et al.*, 2021). No change was observed in FR variability at 10% or 25% follow-up MVC. However, when forces were normalised to MVC recorded pre-immobilisation (i.e., a greater absolute force), FR reduced to a similar extent yet with an increase of FR variability. The variability of MU FR can be reduced with training (Vila-Chã & Falla, 2016) and the current findings highlight opposing effects with muscle disuse.

4.5.6 Alternative factors

Several alternative factors may also partly explain the disparity in strength and size adaptation following disuse, such as impaired calcium handling (Monti *et al.*, 2021) suggesting a reduced efficiency of cross-bridge cycling resulting in reduced force output. Additionally, suppression of muscle protein synthesis (MPS), specifically myofibrillar proteins such as actin and myosin, is well reported as the driving mechanism of reduced muscle size during disuse (Glover *et al.*, 2008; Nunes *et al.*, 2022). A net negative protein balance may result in a disproportionate loss of muscle fibre contractile protein, contributing to reduced function. Although the mTOR pathway, a key driver of MPS, does not appear to be downregulated following disuse (Glover *et al.*, 2008), others have suggested a reduction in mitochondrial protein turnover may be related to these declines in MPS during short-term immobilisation (Abadi *et al.*, 2009) and therefore contribute to reduced muscle function. Furthermore, reduced specific tension may partly explain this disparity (Berg *et al.*, 1997).

Additional analysis into factors potentially related to the reduction in force and changes in neuromuscular parameters found a clustering of NMJ transmission instability, MU FR and FR variability with MVC and CSA. Following multivariate simple linear regression, no significant relationships were observed between either muscle size or strength with those neuromuscular parameters. This suggests that, in these data, no single variable fully explained the decline in muscle strength and size.

4.5.7 Future Work

Since the average length of hospital stay in the United Kingdom as of 2018/19 was 4.5 days (Ewbank *et al.*, 2020), future work in disuse should focus on the impact of such a short time frame on the neural input to muscle. However, with the increasing volume of adults >65 years old requiring short-term hospital admission (NHS Digital, 2017), this age group is also a priority for future study. Understanding these changes will provide a mechanistic basis on which to

optimise rehabilitation protocols to counteract reduced muscle function. Although there are clear neural adaptations present in the VL following disuse, it remains unclear as to the impact of immobilisation in other muscle groups and whether this corresponds to known diverging atrophy profiles. As such, neural adaptations in different muscles may provide a broader picture of the response to disuse and corresponding patterns of adaptation.

4.5.8 Limitations

The contraction levels at which motor units were sampled in the current study are of the low to mid-level and reveal nothing of adaptation to later recruited MUs. Knee extensor movements are not uniquely controlled by the VL, and although it may be a useful proxy for total quadriceps, we cannot rule out greater decrements in other muscles of this group. The current data are available in males only, and although we have highlighted similar differences across contractions in young males and females (Guo *et al.*, 2022), there are sex-based differences in MU FR at normalised contraction levels which may respond differently to this intervention.

4.5.9 Conclusion

These results support previous findings that unilateral short-term immobilisation of just 15 days leads to a decline in muscle strength unmatched by that in muscle size. Following on from the findings of Chapter 3 in the vastus lateralis of declining muscle size and function, they provide insight into potential causes for this unmatched decrement. Importantly, the current data highlight adaptations to neural input to muscle, evidenced by suppressed MU FR at contraction intensities relative to both the reduced maximal force following immobilisation and relative to baseline maximal force.

Chapter 5 Determining the effects of 15-days lower limb immobilisation on neuromuscular function in divergent lower limb muscles of young adults

5.1 Abstract

Skeletal muscles vary widely in size, structure, and function throughout the human body. The TA is the primary contributor to ankle dorsiflexion, an important contraction which lifts the foot during the gait cycle. The MG is forms part of the triceps surae along with the lateral gastrocnemius and the soleus which collectively perform ankle plantar flexion. The MG is an anti-gravity muscle and as such helps to maintain posture while standing. Previous research has found that these two muscles express diverging responses to situations of disuse. The TA appears to be relatively atrophy resistant, and conversely, the MG appears to be largely atrophy susceptible. Although the adaptation of muscle size in the TA and MG following disuse is well characterised, the adaptation of measures of neuromuscular function, specifically at a motor unit level, are less well understood. The question remains as to whether motor unit level adaptation profiles match those of the respective muscle size change, i.e., their resistance and susceptibility.

Eight male participants were recruited to take part in a 15-day unilateral limb immobilisation of the knee and ankle joint. Before and after immobilisation, muscle ultrasound was carried out to measure muscle size in the immobilised TA and MG, while MVCs of dorsiflexion and plantar flexion were also measured. Alongside this, balance assessments were carried out while standing on both legs then separately on the leg chosen for immobilisation. Lastly, iEMG was performed in the TA and MG independently to record motor unit potentials measured during 25% MVC voluntary contractions. These recordings were analysed to investigate adaptations to MU characteristics as a result of immobilisation.

Following the immobilisation period, reduction in muscle size was only observed in the MG (−11%, $p<0.001$), while MVC of both dorsiflexion (−22%,

p<0.05) and plantar flexion (−23%, p<0.01)was reduced. Aside from an increase in the centre of pressure distance travelled during both leg balance, no other parameters of balance were significantly different. Both muscles expressed reduction in MUP size (TA; −41%, p<0.001, MG; (−18%, p<0.05), although no alterations to complexity or neuromuscular junction transmission instability were observed. The key finding between these muscles was a suppression of firing rate in the MG (−10%, p<0.05) along with a greater firing rate variability (+23%, p<0.01) which was not matched in the TA. No single variables were found to solely explain reductions in MVC of either muscle.

These findings of a greater neural dysregulation of the MG compared to the TA following immobilisation may suggest that the TA is preserved from these changes to an extent. Although both muscles reduced in strength, the MG may provide a more crucial target for rehabilitation following disuse situations due to the greater degree of maladaptation alongside muscle size and strength reductions.

5.2 Introduction

5.2.1 Diverging skeletal muscles

A large heterogeneity exists between human skeletal muscles. Multiple factors may be used to divide skeletal muscles into different groups including fibre type composition, and function, i.e., anti-gravity. Crossovers exist between these groups however, making muscle classification somewhat complex. For example, the MG is an anti-gravity, or postural, muscle primarily comprising of type I muscle fibres (Bass *et al.*, 2021). On the other hand, the TA does not have a postural function while also composed of primarily type I. As formerly discussed in Chapter 3 (section 3.2.5), atrophy resistance and atrophy susceptibility may also be used to characterise different muscles. With this in mind, the following chapter will focus on the comparison of the TA, an atrophy resistant muscle, and the MG, an atrophy susceptible muscle.

The TA is the primary muscle involved in ankle dorsiflexion a contraction largely performed as part of the gait cycle (Ruiz Muñoz *et al.*, 2015). Dorsiflexion range of motion is important for maintaining ankle stability as rehabilitative mobilisation of the joint in patients with chronic ankle instability were found to improve outcome measures such as postural control and instability (Cruz-Díaz *et al.*, 2015). To further evidence the importance of this muscle in the gait cycle, weight-bearing ankle dorsiflexion range of motion was found to be the most representative factor in the performance of balance and dynamic tasks by an older cohort (Hernández-Guillén *et al.*, 2021). Conversely, a delay in the contraction of the TA during the swing phase of the gait cycle was found to suggest a greater tendency towards falls in older individuals (Kemoun *et al.*, 2002).

The MG, along with the lateral head and the soleus, carries out ankle plantar flexion (LaPrade *et al.*, 2007; Contreras-Hernandez *et al.*, 2022). As a postural muscle, it has a role in supporting the body while standing. In determining the proportional contribution of the stiffness of the ankle joint to postural stability while standing, it was deemed insufficient (Loram & Lakie, 2002). The

hypothesis that the triceps surae were also involved in the support of standing posture was confirmed in the discovery that these muscles are activated during electromyographic investigation during quiet standing (Loram *et al.*, 2005). These findings suggested an almost constant activity of the triceps surae during standing in a predictive fashion to maintain standing balance.

Early work using intramuscular stimulation techniques characterised the MU organisation of the MG. This established the composition of slow and fast-fatigable MUs of type I and type 2b muscle fibres respectively and the probability of fast-fatigue resistant units being comprised of type 2a fibres (Garnett *et al.*, 1979). During postural standing using intramuscular and surface EMG, it was found that MU distribution is localised throughout the longitudinal axis of the muscle which suggests a potential for regional sub-groups of muscle contraction to take place (Vieira *et al.*, 2011). However, a more recent study has found contrasting evidence. Using multi-wire intramuscular electrodes, seven longitudinal sites were recorded during plantar flexion contractions and knee extensions independently with the same protocol followed using five medial sites (Héroux *et al.*, 2015). Findings from this study suggested that there were no specific regional distributions present in the human MG during isometric contractions, as identified MUs were spanned longitudinally from between one to all seven sites of recording and similarly across horizontal recordings identified territories ranging between one to five sites. The latter of these studies did suggest that the former findings may have been a result of methodological issues combined with inter-individual differences (Héroux *et al.*, 2015). However, it should be noted that the former recorded data during quiet standing, the primary functional task of this muscle group (Vieira *et al.*, 2011), whereas isometric ramp contractions in a prone position were carried out in the latter (Héroux *et al.*, 2015). As such, and as noted by the later study's authors, while findings suggest that the MG is more uniformly activated rather than regionally, the absence of localised MU territories should be interpreted cautiously.

5.2.2 Muscle strength

As muscles with different structure and role, the TA and MG also express differing muscle function. In young healthy individuals, 14 male and 4 female, plantar flexion isometric peak torque was ~124 Nm at 90° knee angle and ~150 Nm at 80° knee angle (Trappe *et al.*, 2001). Both the soleus and lateral gastrocnemius expressed greater proportions of type I muscle fibres, although ~23% greater in the soleus than the lateral gastrocnemius. The torque values are similar to those measured before a bed rest intervention in healthy young male participants. One group averaged ~140 Nm while the other ~127 Nm tested at 90° knee angle (Akima *et al.*, 2003). In contrast, dorsiflexion measured in a cross-sectional group of 15 male and 16 female participants reported a maximum isometric torque of ~50 Nm (Ruiz Muñoz *et al.*, 2015). These findings suggest a greater strength in the plantar flexors of healthy young individuals than in dorsiflexion, although literature is somewhat limited. Dorsiflexion force has been shown to reduce with age, although this is attenuated to an extent by high levels of activity seen in master athletes. In a comparison between mixed sex groups of young and old participants, maximal dorsiflexion torque was ~35 Nm in the young and ~28 Nm in the old (Cogliati *et al.*, 2020). When including highly active older individuals, young men had an average dorsiflexion MVC of ~370 N, significantly greater than the ~245 N of old and ~290 N of master athletes (Piasecki *et al.*, 2016*a*). Although these findings show activity in older age may maintain dorsiflexion strength better, there is still a clear age related decline in master athletes of varied endurance and power disciplines (Piasecki *et al.*, 2021*a*). There does not appear to be similar investigations of plantar flexion strength and as such any disparity between those muscle groups remains seemingly unexplored.

5.2.3 Neuromuscular characteristics

Since the MG has an important role in the maintenance of balance during quiet standing (Loram *et al.*, 2005), this has encouraged investigation into recruitment patterns of MUs of the MG in this state. Intramuscular EMG across

three positions during quiet standing, alongside measurement of centre of pressure displacement from postural swaying, was recorded to determine features of MG recruitment (Vieira *et al.*, 2012). Results suggested that not discharge rate, but rather increased recruitment of smaller MUs was responsible for stabilisation during quiet standing. A similar investigation was carried out in which iEMG was performed on the MG in both legs while anterior load was added infrequently using a weighted pulley attached to a belt worn around the participants waist(Pollock *et al.*, 2014). The results suggested that, similar to simple quiet standing, additional MU recruitment was used to maintain a steady state of balance rather than increased discharge rate (Pollock *et al.*, 2014). However, it should also be noted that in the acute response to the addition of incremental load, there was a transient increase in discharge rate of the formerly recruited MUs, a mechanism which may be in place to maintain stability before additional MUs are recruited. A further observation was that the MG was the primary muscle responsible for reacting to increased perturbations compared with EMG activity from the soleus, biceps femoris, lumbar erector spinae, tibialis anterior and rectus femoris (Pollock *et al.*, 2014). Concerning the adaptation of balance performance in response to ageing, dynamic perturbations were applied to young and old individuals during standing balance (Piirainen *et al.*, 2013). Older participants expressed a greater displacement of centre of pressure during slow perturbations and peak displacement occurred earlier than young. This was thought to be a result of the age-related loss of faster MUs and MU remodelling, features which are known to lead to larger, more complex and harder to control MUs consequently impairing function (Piasecki *et al.*, 2016*b*).

The MU characteristics of the TA have also been investigated albeit without the context of a posture-related function. Instead, studies have focused on the impact of ageing on the TA. As this muscle expresses a reduction in strength in advancing age, this may lead to functional issues such as impaired gait and foot drop, a significant contributor to falls (Bland *et al.*, 2011). Age group comparisons have found that, alongside the previously discussed changes in

strength, there is a suppression of firing rate in the TA from old to very old men (McNeil *et al.*, 2005). This was found alongside the typical increases in MUP size in old and very old men compared to the younger cohort which is an expected feature of age-related MU remodelling. Although the suppression of firing rate may suggest a further level of neural dysregulation in the transmission and/or propagation of action potentials to and throughout the muscle. Voluntary activation capacity between young and older men was found to be similar, suppressed firing rate was also observed at similar relative torque contractions (Connelly *et al.*, 1999). This may compound the suggestion of neural dysregulation to MU characteristics as a feature of older age. These patterns of dysregulation are also not wholly attenuated by lifelong exercise. Both older recreationally active males and male master athletes with median weekly training times of ~6 hours from 30 years to present (averaging 69 years at the time of the study) expressed suppressed TA firing rate compared to young males (Piasecki *et al.*, 2016*a*). Also accompanied by greater MUP area and amplitude than young males, these findings suggest that master athletes are not spared from age-related MU remodelling. In fact, measurement of MUP features may provide a useful determinant between sarcopenic and non-sarcopenic individuals. As the former studies have shown an increase in TA MUP size accompanies the healthy ageing process, the lack of capacity to successfully reinnervate lost muscle fibres and thus express the observed larger MUPs was found to be a feature of sarcopenia in an older male cohort (Piasecki *et al.*, 2018*b*).

5.2.4 Response to disuse

As covered in detail in previous sections of this thesis (Chapter 1 sections 1.2.3 and 1.3.1 and Chapter 3 section 3.2.6), there is a clear divergence in the response of the TA and MG to disuse atrophy. Suffice it to say that multiple findings in the TA show a very slight or complete lack of muscle size reduction following extended periods of disuse contrasting with significantly greater loss in the MG (Akima *et al.*, 2000*b*; Belavý *et al.*, 2009*a*; Miokovic *et al.*, 2012).

Furthermore, results from Chapter 3 of this thesis also show a similar trend in the reduction of MG CSA and lack of alterations in TA CSA following 15-day unilateral lower limb immobilisation (Chapter 3 section 3.4.2). The impact of disuse on function in lower limb muscles with diverging atrophy profiles remains less clear. Recent findings showed no difference in the balance performance of young individuals following two weeks of unilateral lower limb immobilisation (Elam *et al.*, 2022). Concerning muscle strength, although former studies have shown a reduction in plantar flexion MVC following disuse in young (Seynnes *et al.*, 2008) and older (Kortebein *et al.*, 2008) participants, a lack of findings from the TA in literature is present. However, the findings reported in Chapter 3 (section 3.4.4.6) show significant reductions in both plantar flexion and dorsiflexion MVC, an unexpected finding considering the lack of size change in the TA. This may suggest further adaptation beyond muscle size is responsible for these reductions in MVC. Patients with ankle osteoarthritis expressed a reduction in both plantar flexion and dorsiflexion strength compared with the contralateral healthy leg and the healthy control group (Valderrabano *et al.*, 2006). This was accompanied by a significantly lower mean EMG frequency and intensity in both the TA and MG compared to the contralateral leg measured during maximal voluntary contractions. However, the confounding factor of osteoarthritis, accompanying symptoms such as pain and inflammation and extent of muscle atrophy as a result may not compare directly with situations of disuse in healthy individuals. As shown in Chapter 4, neural dysregulation may explain the greater reduction in muscle strength than muscle size seen in the vastus lateralis. This may also be the case in the TA and MG. A significant knowledge gap appears to be present in the neuromuscular investigation, specifically at a MU level, of muscles which express diverging atrophy profiles.

5.2.5 Aims and hypotheses

The primary aim of this study was to address the knowledge gap present in the alteration of neuromuscular characteristics between two muscles formerly

identified as atrophy resistant, i.e., the tibialis anterior, and atrophy susceptible, i.e., the medial gastrocnemius.

Based on previous research, the following hypotheses were made:

- Muscle size of the medial gastrocnemius would express a reduction following immobilisation while the tibialis anterior would remain unaffected.
- The medial gastrocnemius would express a greater degree of motor unit level maladaptation in response to short-term disuse than the tibialis anterior.
- As a result of neural dysregulation of the medial gastrocnemius, aspects of postural balance performance would worsen following immobilisation.

5.3 Methods

5.3.1 Study overview

This study was approved by the University of Nottingham Faculty of Medicine and Health Sciences Research Ethics Committee (103-1809) and conformed with the Declaration of Helsinki. Participants were recruited locally through advertisement posters placed around the University, student cohort emails and online social groups and forums. While a total of 13 participants were recruited and screened for the overall study also described across Chapters 3 and 4, a subset of 8 will be used for this chapter due to issues with data quality. A more detailed participant information is found in the results section. For screening visits, participants were invited to attend the Royal Derby Hospital Medical School building. Following the taking of informed consent, participant height, weight and blood pressure was measured along with an electrocardiogram. A routine blood test was also carried out and sent to the pathology department to ensure participants were eligible based on the exclusion criteria. These are described in detail in Chapter 2 (section 2.3.1.2) but in a brief summary; active cardiovascular, cerebrovascular, respiratory, renal, or metabolic disease, active malignancy, musculoskeletal or neurological disorders.

5.3.2 Immobilisation strategy

The immobilisation strategy is detailed in Chapter 2 section 2.3. Briefly, for the 15 days of immobilisation, a modified ULLS model was used. The knee joint was fixed at 75 degrees flexion and the ankle joint was fixed using an air-boot. Crutches were provided and adjusted according to the height of the participant.

5.3.3 Assessment of muscle size

These methods are detailed in Chapter 2 section 2.1.1.1. In brief, skeletal muscle ultrasound CSA scans were taken from the TA and the MG while participants lay supine on a clinical couch An average of three values from each

scan, totalling nine images per muscle per participant, was used for statistical analysis.

5.3.4 Balance assessment

These methods are detailed in Chapter 2 section 2.1.6. Briefly, assessment of both leg and individual leg balance was performed using a Materialise Footscan 1-metre force plate (Materialise, Leuven, Belgium). Balance performance was recorded for 30 seconds in which features of the COP were measured. For these measurements, n=6 as the equipment was acquired midway through the study.

5.3.5 Maximum voluntary contraction

The following methods are detailed in Chapter 2 section 2.1.7. Briefly, for dorsiflexion MVC, participants were seated in a chair at an appropriate distance from the custom-built ankle isometric dynamometer. Following a warm-up, three attempts were carried out and the highest value was taken as the maximal and used to determine voluntary contraction intensity. For plantar flexion MVC, three attempts were carried out and the highest value was taken as the maximal and used to determine voluntary contraction intensity.

5.3.6 Force control

Force control, measured as FS, was recorded during voluntary isometric contractions carried out following MVC assessment. These methods are detailed in Chapter 2 section 2.1.8. Briefly, for each contraction type, participants carried out 2 – 6 voluntary contractions at intensities of 10%, 25% and 40% of their previously recorded MVC Contractions were held for 12 s with a ~20 s rest between them. These contraction intensities were chosen to give a range of low to mid-level contractions which represent activities of daily living. 10% represents walking on a flat surface while 25% represents standing from a chair or climbing stairs (Tikkanen *et al.*, 2013).

5.3.7 Surface electromyography recording

The motor point for the TA and MG were identified using a cathode probe (Compex Motor Point Pen Electrode, Digitimer Ltd, Welwyn Garden City, UK) and an anode electrode (BioTENS 2" round cloth electrode, Nissha Medical Technologies, NY, USA) placed on the medial knee joint cleft for the TA and on the head of the *lateral gastrocnemius* for the MG. For the TA, the motor point was generally located ~20% of the muscle length from the muscle origin on the lateral surface of the tibia. The recording surface electrode (disposable self-adhering Ag-AgCl electrodes; 95 mm^2; Ambu Neuroline, Baltorpbakken, Ballerup, Denmark) was placed here, and the reference electrode was also placed on the patellar tendon with the ground electrode (Ambu Neuroline Ground) on the patella. For the MG, the motor point was generally located centrally in the mid-belly of the muscle. The recording electrode was placed over the motor point and a reference electrode on the patellar tendon. A ground electrode was placed on the patella.

5.3.8 Intramuscular electromyography

For the both the TA and MG, a concentric needle electrode (Ambu Neuroline model 740 25-45/25) was inserted into the muscle adjacent to the respective surface electrode (fig. 5.1A and fig. 5.1B). These methods are detailed in Chapter 2 section 2.3.3. Briefly, four contractions were recorded at each intensity, with the needle electrode position altered between each to sample a broader range of MUPs (Jones *et al.*, 2021). These voluntary contractions were held for 12 s with a >10 s rest in between each contraction.

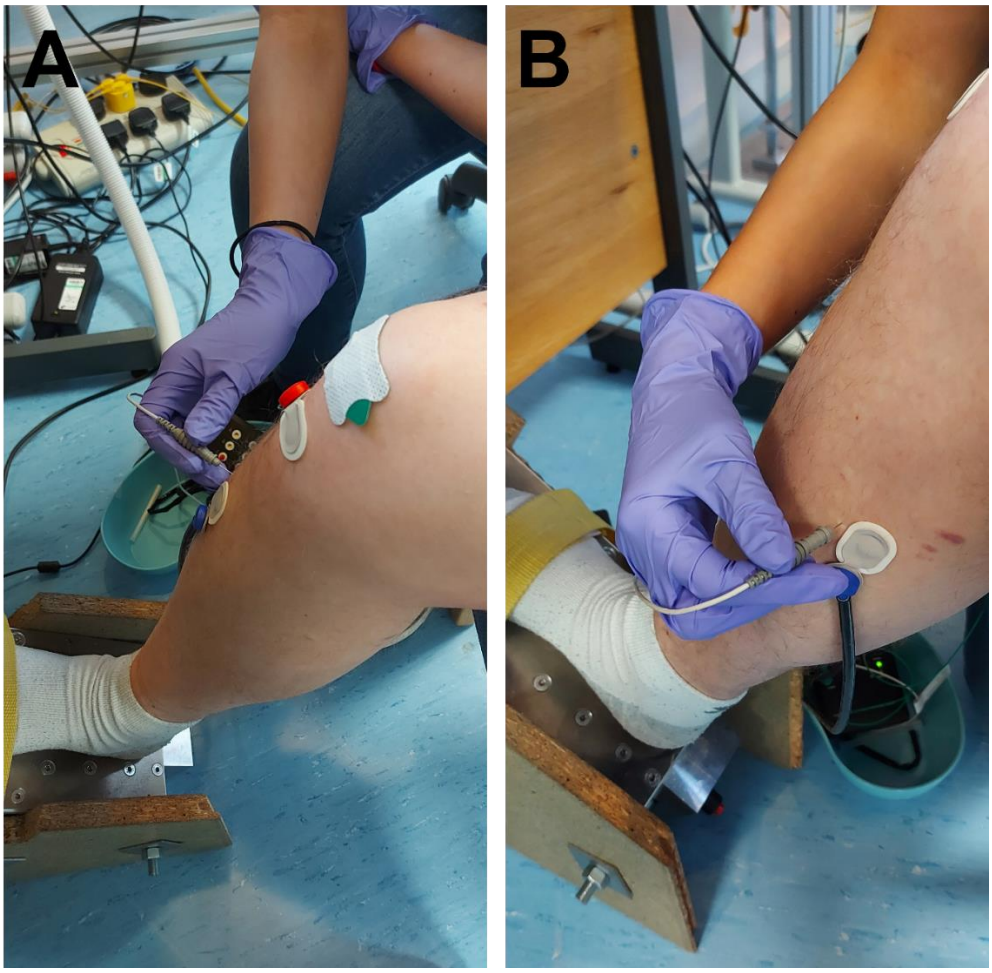


Figure 5.1: *Intramuscular electromyography and force transducer positioning for the tibialis anterior and dorsiflexion (A) and for the medial gastrocnemius and plantar flexion (B).*

5.3.9 EMG Analysis

These methods are detailed in Chapter 2 section 2.4.4. Briefly, identified MUPs and their corresponding MUPTs were analysed for electrophysiological characteristics including MUP area, amplitude, and complexity, MU FR and FR variability and NF-MUP jiggle representing NMJ transmission instability.

5.3.10 Statistical analysis

Statistical analysis of CSA, MVC, and FS was performed using GraphPad Prism version 9.1.0 (GraphPad Software, CA, USA) using repeated measures 2-way analysis of variance with Šidak's post-hoc analysis in the event of a significant main effects. To assess balance parameters, two-tailed Student's t tests were

performed where data were normally distributed or using Wilcoxon matched-pairs signed rank test if not normally distributed. Multi-level mixed effect linear regression models were used to analyse MU parameters, in StataSE (v16.0, StataCorp LLC, TX, USA). For these models the first level was single motor unit; single MUs were clustered according to each participant to form the second level, which was defined as the participant level and reflects the total n. Two within-subject factors were included; muscle (tibialis anterior and medial gastrocnemius) and time (pre and post), and muscle x time interactions were included in all models. Further analyses were performed to investigate relationships between MU characteristics, muscle size and muscle strength. These analyses were performed using R (Version 4.2.0, (https://cran.r-project.org/) implemented using R studio. Firstly, correlative analysis was assessed with Pearson's product moment correlation coefficient and visualised using corrplot (https://cran.r-project.org/web/packages/corrplot) to determine any strong relationships between variables. Cluster analysis and principal component analysis for variables were performed using the ClustOfVar (https://cran.r-project.org/web/packages/ClustOfVar) and factoextra (https://cran.r-project.org/web/packages/factoextra) packages respectively, to determine which variables strongly clustered and related to others. A subset of the variables clustered with the respective MVC force were analysed with multivariate linear regression to determine whether clustered variables were significantly able to predict changes in MVC. These analyses were carried out independently for dorsiflexion force, tibialis anterior size and MU parameters in the first instance and also for plantar flexion force, medial gastrocnemius size and MU parameters. For all tests, significance was assumed if $p<0.05$.

5.4 Results

5.4.1 Participant characteristics

For this subgroup of the study, eight male participants were used. Characteristics are shown in table 5.1.

Table 5.1: *Descriptive characteristics of participants showing mean and standard deviation (SD)*

N = 8	Mean (SD)
Age (years)	23.8 (3.6)
Height (cm)	181.6 (6.7)
Weight (kg)	79.8 (9.3)
BMI (kg/m^2)	24.1 (2.1)

5.4.2 Muscle size

A significant muscle x time interaction was observed between TA and MG CSA ($p<0.01$, fig. 5.2). Following 15-day immobilisation, a reduction was observed in the MG (−11%, $p<0.001$) with no change in the TA ($p = 0.841$).

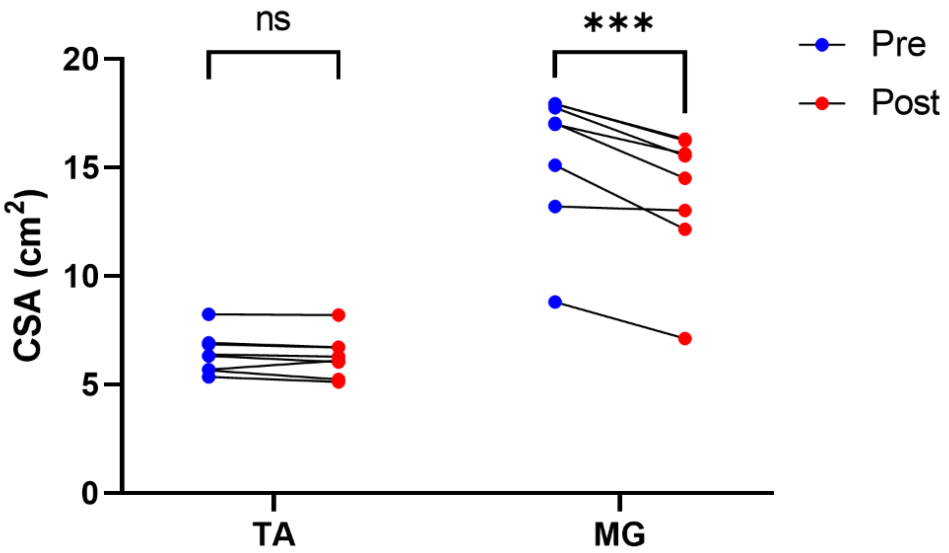


Figure 5.2: *Cross-sectional area (CSA) differences in tibialis anterior and medial gastrocnemius of the immobilised leg following 15-day unilateral limb immobilisation. *** = p<0.001.*

5.4.3 Muscle function

No significant interaction was observed between dorsiflexion and plantarflexion MVC in the immobilised limb following immobilisation ($p = 0.428$, fig. 5.3A), however there was a significant main effect of time ($p<0.01$). This was reflected by a significant reduction in both dorsiflexion (−22%, $p<0.05$) and plantar flexion (−23%, $p<0.01$). No interaction was seen between dorsiflexion and plantarflexion force control following immobilisation ($p = 0.627$, fig. 5.3B).

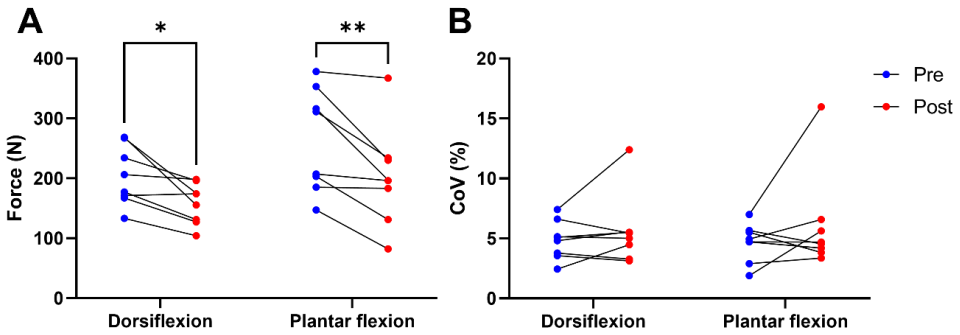


Figure 5.3: A: *Dorsiflexion and plantar flexion maximal voluntary isometric contraction (MVC) force measured in the immobilised leg before and after 15-day unilateral limb immobilisation.* ***B:*** *Dorsiflexion and plantar flexion force steadiness measured during contractions at 25% MVC before and after immobilisation in the immobilised limb. CoV shown as average deviation from target line. N; newtons, CoV; coefficient of variation. * = p<0.05, ** = p<0.01.*

For individual leg balance, there was no significant change pre to post in COP distance moved ($p = 0.281$, fig. 5.4A), ellipse area ($p = 0.398$, fig. 5.4B), average COP velocity ($p = 0.716$, fig. 5.4C) or peak COP velocity ($p = 0.392$, fig. 5.4D). For both leg balance there was a significant increase in COP distance moved following the immobilisation period ($p<0.05$, fig. 5.4E). There was no significant change in COP ellipse area ($p = 0.052$, fig. 5.4F), average COP velocity ($p = 0.181$, fig. 5.4G) or peak COP velocity ($p = 0.233$ fig. 5.4H). For balance measurements, $n = 6$.

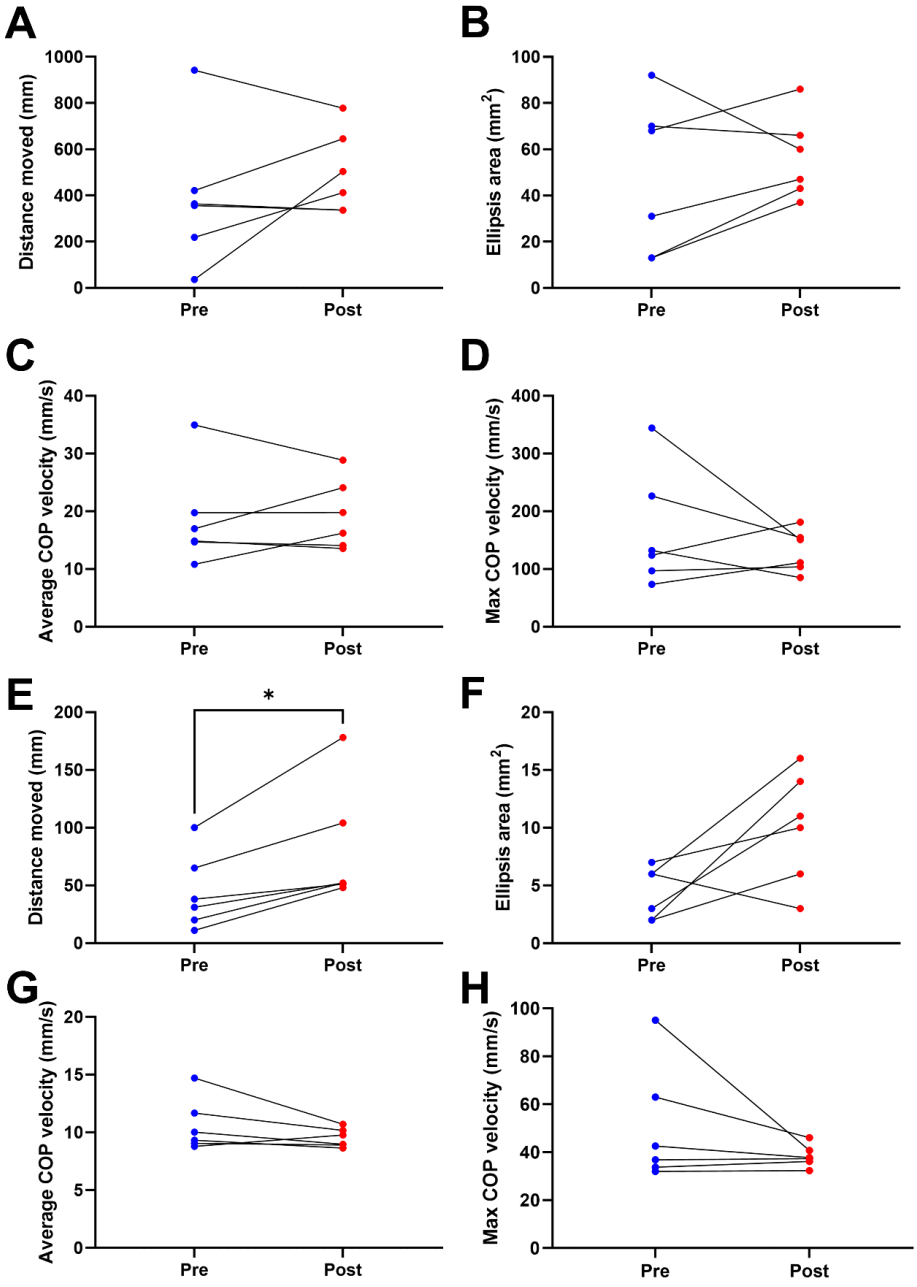
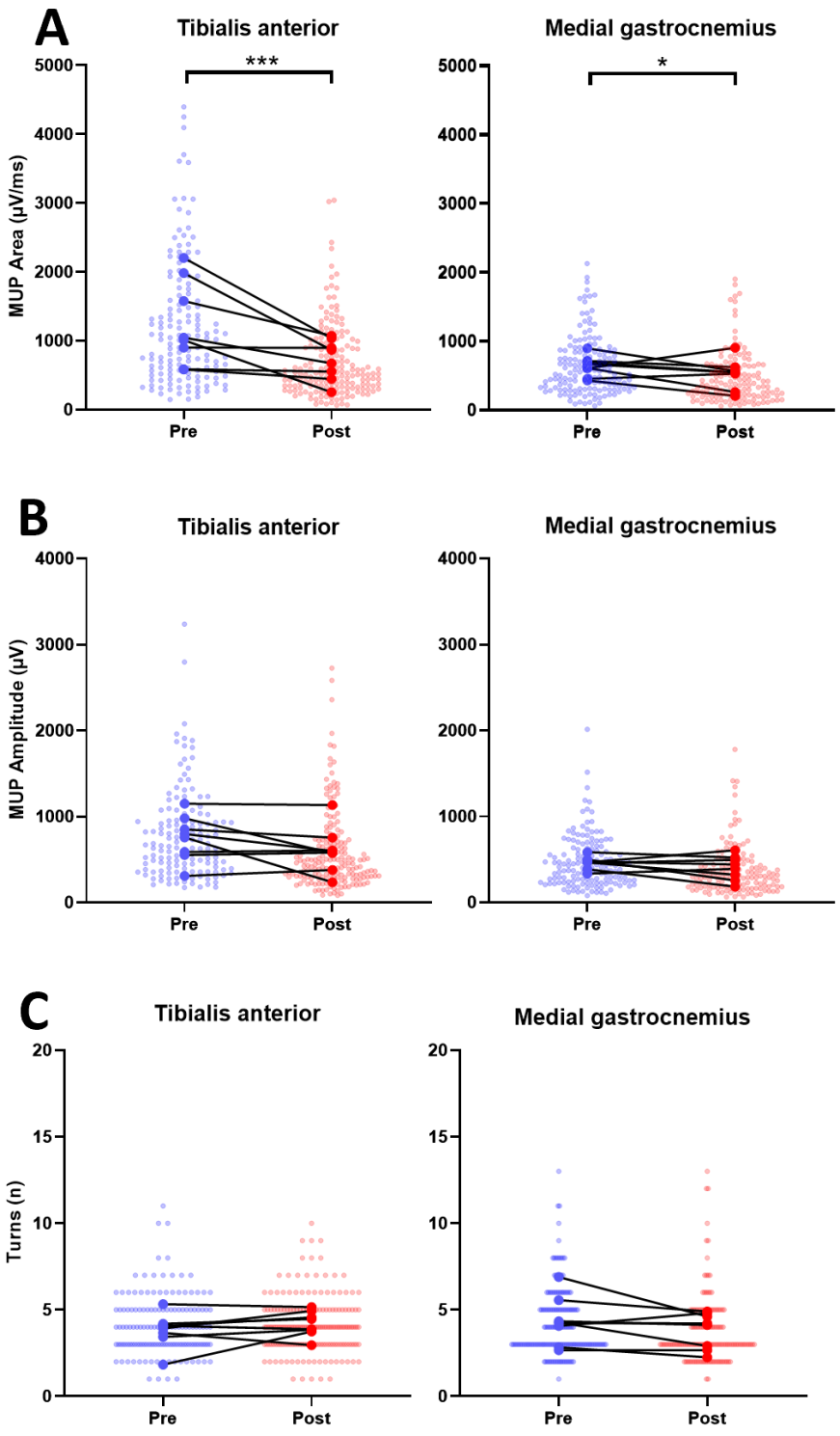


Figure 5.4: A to D; *Single leg balance of the immobilised leg before and after immobilisation.* ***E to H;*** *both leg balance before and after immobilisation. n=6 for all measurements. * = p<0.05. COP; centre of pressure.*

5.4.4 Neuromuscular function

MUP area presented a significant muscle x time interaction (p<0.001, fig. 5.5A), explained by the greater reduction in TA (−41%, p<0.001) when compared to the MG (−18%, p<0.05). There was no significant interaction present for MUP amplitude (p = 0.167, fig. 5.5B). Although a significant interaction was observed

for MUP turns ($p<0.05$, fig. 5.5C), there were no changes observed in either TA ($p = 0.271$) or MG ($p = 0.075$). NMJ transmission instability did not present a significant interaction ($p = 0.655$, fig. 5.5D). For MU firing rate, there was a significant muscle x time interaction ($p<0.05$, fig. 5.5E). A significant reduction was observed in the MG (−10%, $p<0.05$) whereas no change was seen in the TA ($p = 0.444$). Similarly, an interaction was present in firing rate variability ($p<0.05$, fig. 5.5F). A significant increase was present in the MG (23%, $p<0.01$) with no change observed in the TA ($p = 0.582$).


A
Tibialis anterior
Medial gastrocnemius
***
*
MUP Area (μV/ms)
5000
4000
3000
2000
1000
0
Pre
Post
B
Tibialis anterior
Medial gastrocnemius
MUP Amplitude (μV)
4000
3000
2000
1000
0
Pre
Post
C
Tibialis anterior
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Turns (n)
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Pre
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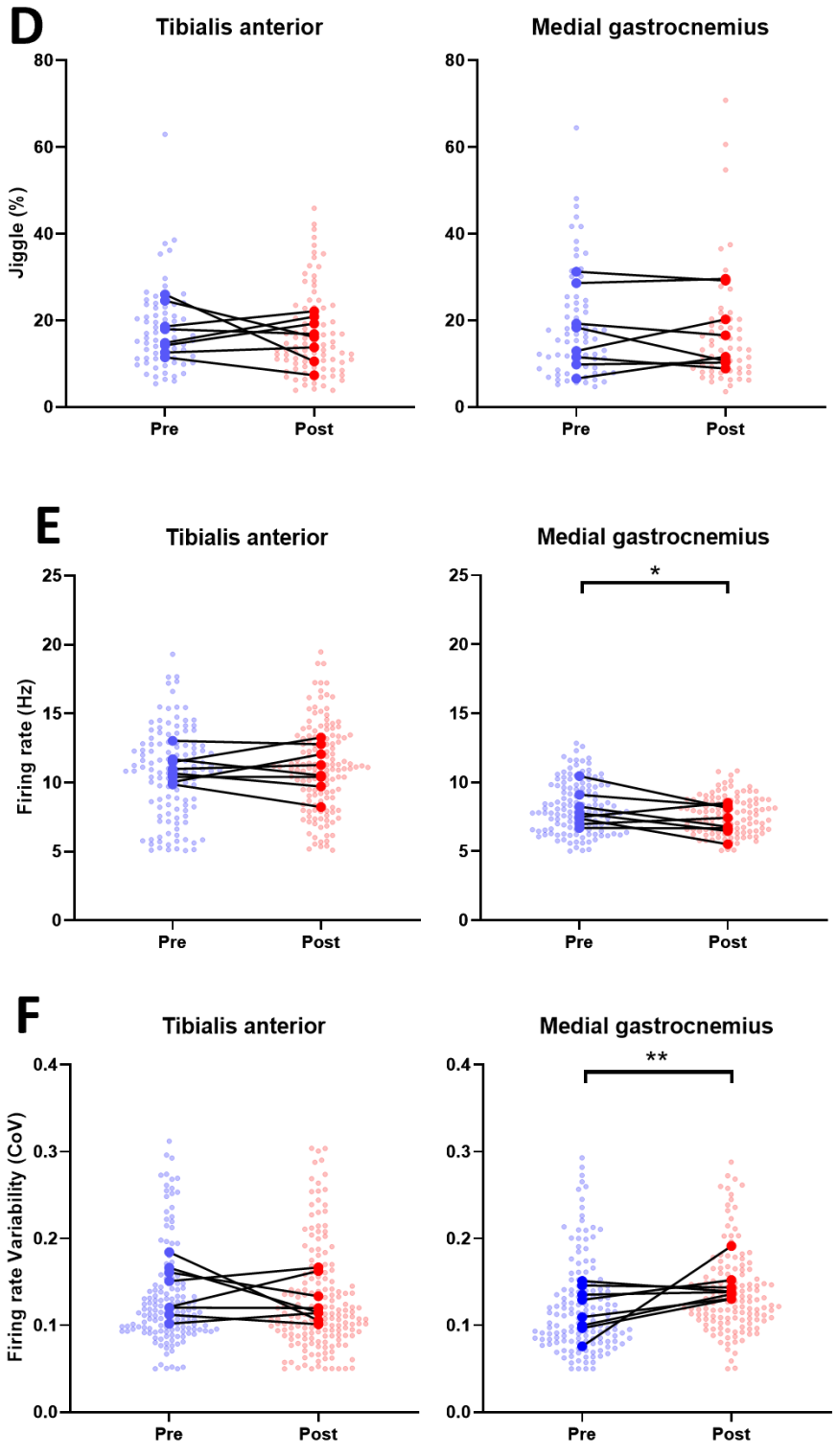

Figure 5.5: *Motor unit potential (MUP) and discharge characteristics sampled from contractions performed at 25% maximum voluntary force from the tibialis anterior and medial gastrocnemius of the immobilised limb following 15-day unilateral limb immobilisation. µV/ms; microvolts per millisecond, µV; microvolts, n; number, Hz; hertz, CoV; coefficient of variation. * = p<0.05, ** = p<0.01, *** = p<0.001.*

5.4.5 Exploratory analysis

In order to investigate potential contributors to reductions in muscle size, strength and MU characteristics, correlative analysis was performed on the mean differences for these data in both the TA and dorsiflexion (fig. 5.6A) and the MG and plantar flexion (fig. 5.6B) respectively. For the tibialis anterior, strong negative correlations were observed between turns and MU firing rate variability (r^2 = −0.80) and between turns and neuromuscular junction transmission instability (r^2 = −0.72). For the MG, strong positive correlations were observed between MUP area and MUP amplitude (r^2 = 0.91) and between MUP area and firing rate variability (r^2 = 0.70). These variables were then clustered to more accurately represent potential relationships using the hierarchical clustering algorithm provided in ClustOfVar. Additional visualisation of these clusters was performed using principal component analysis score plots (PCA, fig. 5.7 and fig. 5.8). For the tibialis anterior, the key cluster of interest consisted of MVC, CSA, NMJ transmission instability, MU firing rate, MUP area and MUP amplitude. For the MG, the key cluster of interest included MVC, MU firing rate variability, MUP area and MUP amplitude. To test whether the parameters in these clusters had any influence on the changes observed in muscle strength, the values were first normalised to the same dynamic range before multivariate linear regression was performed. For the tibialis anterior, there were no significant relationships observed between MVC and MUP area (p = 0.460), MUP amplitude (p = 0.419), MU firing rate (p = 0.285), CSA (p = 0.402) and NMJ transmission instability (p = 0.621). For the MG, there were no significant relationships observed between MVC and MUP area (p = 0.485), MUP amplitude (p = 0.278) and MU firing rate variability (p = 0.979).

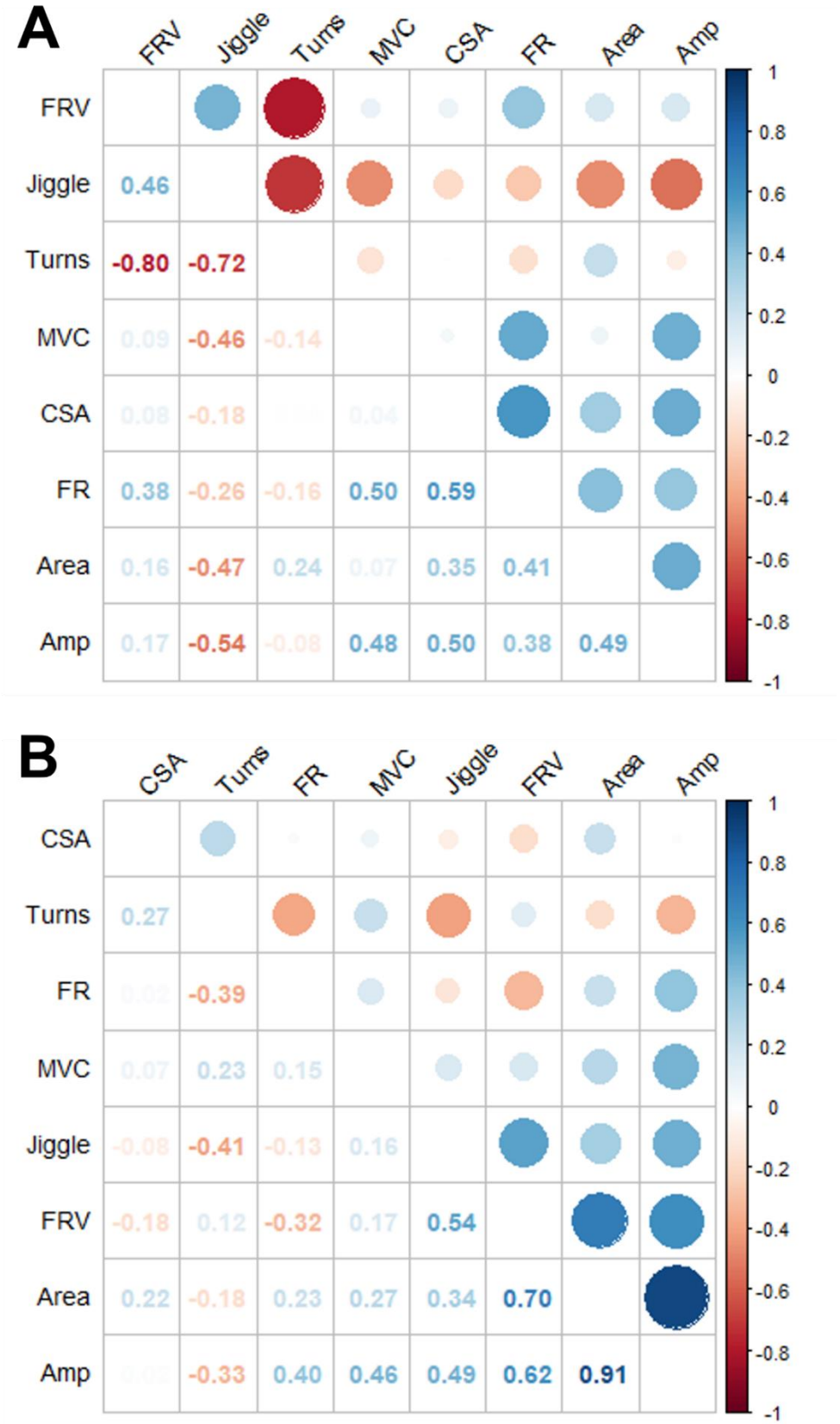

Figure 5.6: *Correlation matrix of relationships between variables measured from the tibialis anterior (A) and medial gastrocnemius (B). CSA; cross-sectional area, FRV; firing rate variability, MVC; maximal voluntary contraction, FR; firing rate, Amp; amplitude.*

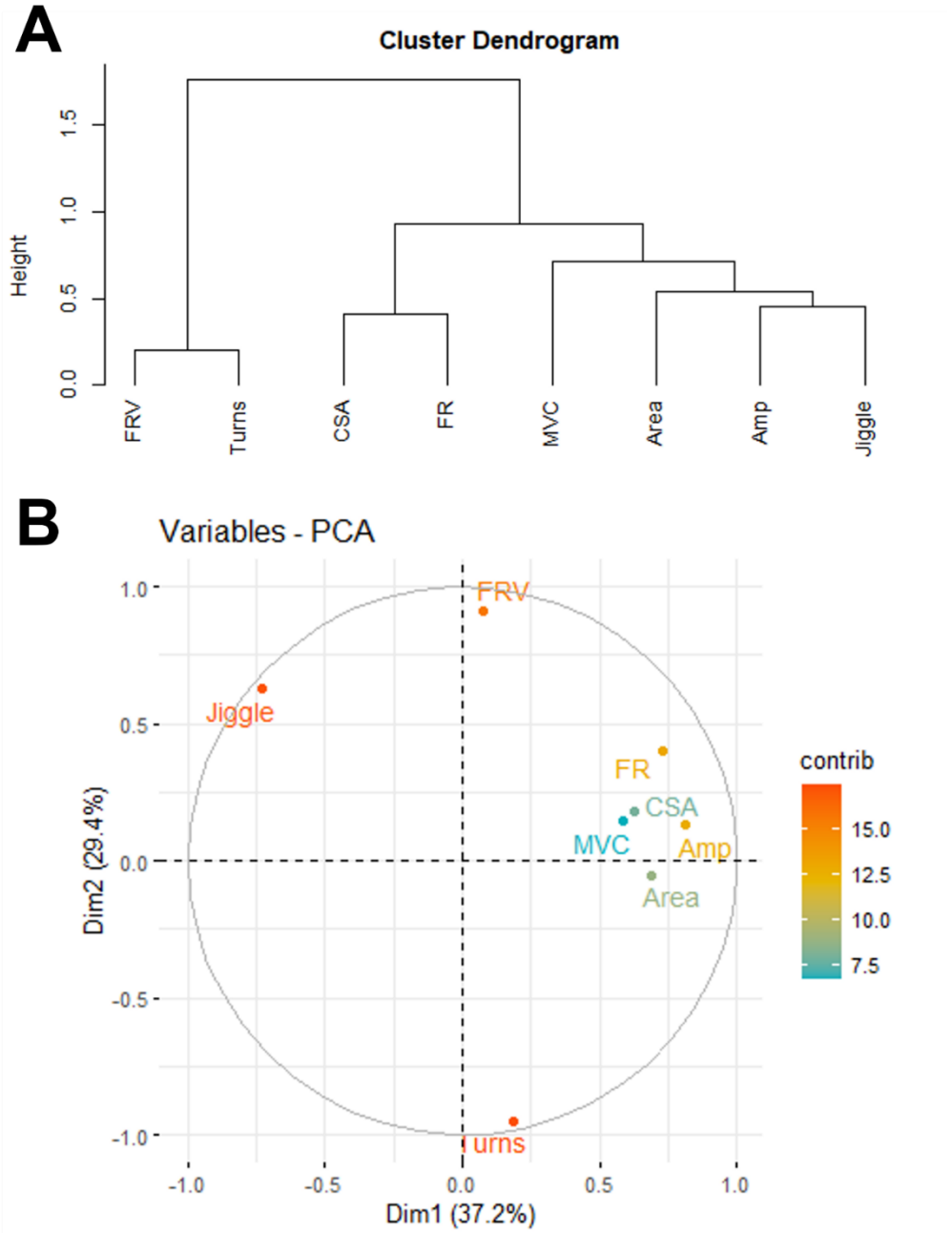

Figure 5.7: *Variables from the tibialis anterior presented in a cluster tree plot (A) and accompanying principal component analysis plot (B) to illustrate relationships between variables within the first two dimensions of variance between variables. Amp; amplitude, FRV; firing rate variability, MVC; maximal voluntary contraction, CSA; cross-sectional area, FR; firing rate, Dim; dimension.*

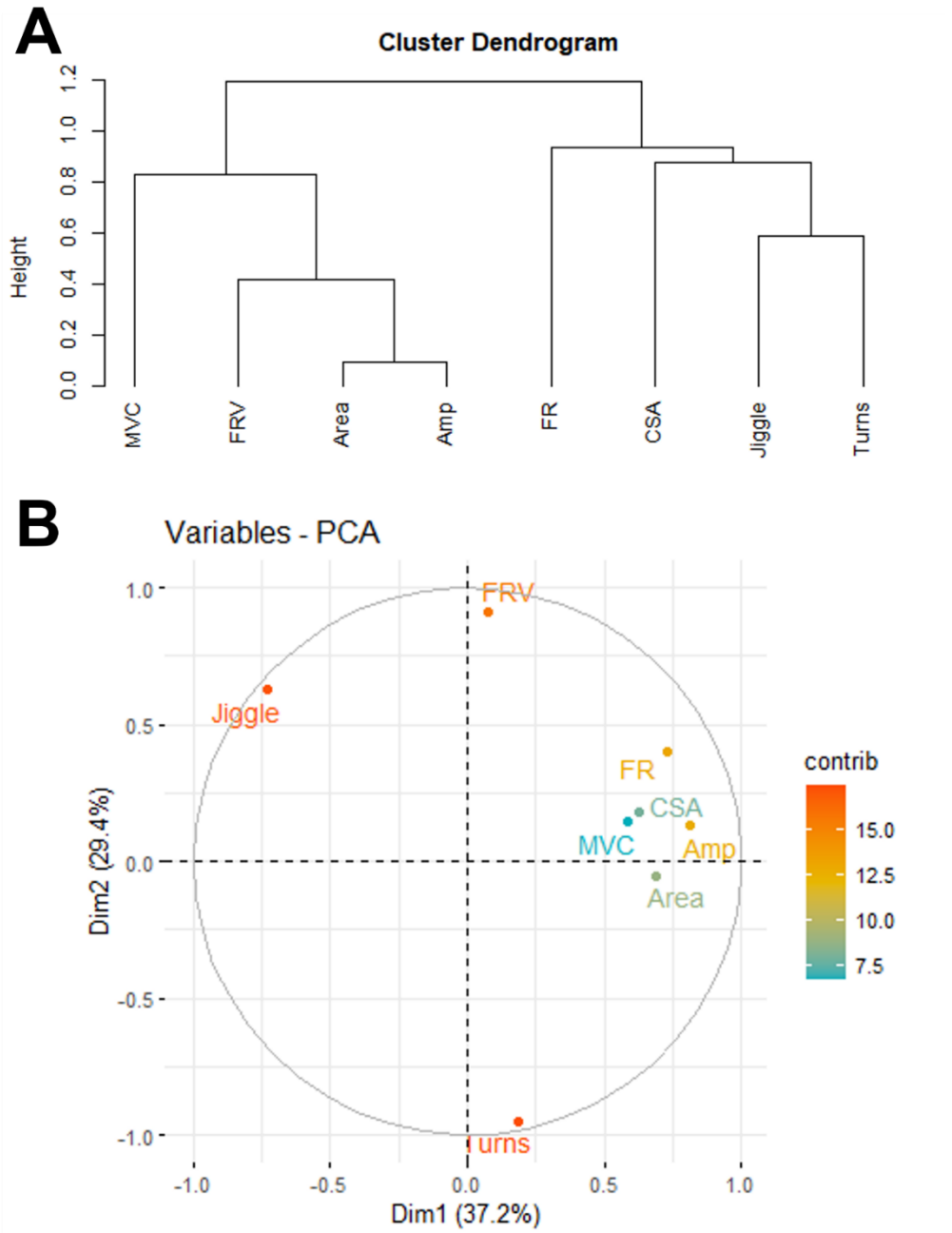

Figure 5.8: *Variables from the medial gastrocnemius presented in a cluster tree plot (A) and accompanying principal component analysis plot (B) to illustrate relationships between variables within the first two dimensions of variance between variables. Amp; amplitude, FRV; firing rate variability, MVC; maximal voluntary contraction, CSA; cross-sectional area, FR; firing rate, Dim; dimension.*

5.5 Discussion

The key findings of this study were that a reduction in muscle size was only observed in the immobilised MG following a 15-day period of immobilisation while no change was seen in the tibialis anterior. However, there were significant reductions to muscle strength in both muscles as an effect of time following immobilisation. For the most part this was not reflected by changes in postural balance of either single leg balance on the immobilised limb or both leg balance, although there was a greater centre of pressure distance travelled in both leg balance. In terms of MU level adaptations, these were more pronounced in the MG with reductions in MUP size, suppression of MU firing rate and a greater firing rate variability. In contrast, the TA only expressed reductions in MUP size. Investigating any potential contributions of these parameters to the observed reductions in muscle strength did not uncover any single significant relationships which may explain this change.

5.5.1 Muscle size and function

In agreement with our hypothesis, a reduction in muscle size was only observed in the MG of the immobilised leg while the TA did not express any size changes. This is also in line with findings from long-term bed rest in which the MG expressed the greatest atrophy susceptibility in terms of size change while the anterior tibial muscles were not significantly smaller after 20-day bed rest (Akima *et al.*, 2000*b*) or until after 56 days (Belavý *et al.*, 2009*a*). In agreement with findings from Chapter 3, a significant reduction in the muscle strength, i.e., MVC, of the plantar flexors was observed following immobilisation. The finding that dorsiflexion MVC was significantly reduced in this subset of participants also agrees with findings from Chapter 3. While there does not appear to be any comparative literature on dorsiflexion MVC changes following disuse, the present plantar flexion MVC reduction is in alignment with prior studies reporting similar reductions during disuse (Kortebein *et al.*, 2008; Seynnes *et al.*, 2008). However, the former study was carried out in older participants and as such may not be an appropriate comparison.

Although strength reduction was seen in both muscles studied from the immobilised limb, this was not reflected in alterations to balance performance while standing solely on the immobilised leg. Findings of COP distance moved, ellipse area, average and peak COP displacement velocity remained unchanged following immobilisation. When assessing balance performance during both leg balance, there was a significantly greater COP distance travelled but this was not accompanied by any changes in the further balance parameters mentioned prior. While the majority of these findings correspond to recent results of from a study utilising two-week unilateral lower limb immobilisation in which no balance characteristics worsened significantly in young people (Elam *et al.*, 2022), the present study contrasts with this in the both-leg balance performance. However, the former study measured balance for 15 seconds while the present study used a duration of 30 seconds which may explain the discrepancy as greater assessment time may introduce greater variation. There were significant decrements in the balance performance of the older cohort however (Elam *et al.*, 2022), which may be a result of age-related MU dysregulation, providing an avenue for future study.

5.5.2 Motor unit characteristics

Comparing MU characteristics between TA and MG, MUP area expressed a significant interaction which was reflected in reductions in both muscles. Although a similar measure of MUP size, MUP amplitude did not express any changes in either muscle following the immobilisation period. This may be a result of a reduction in MUP duration which may explain a reduction in area with no corresponding alteration in MUP amplitude. MUP size is a representation of the depolarisation of all fibres within an MU detected by the intramuscular electrode. This in turn represents the size and number of the fibres contributing to the MUP and as such greater MUP size is seen in ageing where MU remodelling has taken place resulting in more muscle fibres per MU (Jones *et al.*, 2022). However, the reduction present here in both muscles may suggest these muscles are undergoing a partial degree of MU remodelling in

which lost fibres have not yet been reinnervated. This finding follows a similar trend to the findings from the vastus lateralis in Chapter 4, further reinforcing the suggestion of partial MU remodelling in immobilisation. Alternatively, selective muscle fibre atrophy within an MU, reducing the size of the MUP, although this would have implications for MUP complexity, which did not significantly increase in either muscle. An increase in MUP complexity, measured here by number of turns, reflects a greater temporal electrophysiological dispersion, caused by variation in conduction time between axonal branches and/or muscle fibres within the same MU (Stålberg & Sonoo, 1994; Piasecki *et al.*, 2021*b*). Polyphasic, more complex MUPs have been observed from the gastrocnemius in myopathies, although of the 995 MUPs recorded, only two of the 43 muscles measured were gastrocnemii which somewhat confounds comparison here (Uncini *et al.*, 1990). These findings also differ from those in Chapter 4 in which the vastus lateralis expressed greater MUP complexity following immobilisation whereas no alterations were seen in the TA or MG.

There were no changes observed in neuromuscular junction (NMJ) transmission instability, measured by near-fibre MUP jiggle, in either TA or MG following immobilisation. Increases NMJ transmission instability has been reported during the healthy ageing process in the TA and correlates significantly with parameters showing greater MUP size (Hourigan *et al.*, 2015). The older cohort expressed a significantly greater NMJ transmission instability than the younger cohort with ~36% and ~27% respectively, which are both greater than the ~17% and ~18% values expressed before and after immobilisation respectively. However, the contractions used to obtain the recorded near-fibre MUPs differed greatly, as the former study performed submaximal contractions until a specific discharge rate was achieved for 30 seconds (Hourigan *et al.*, 2015) while the present study used 25% MVC contractions held for 10 seconds. These methodological differences may explain the disparity in findings between two young cohorts and it is difficult to estimate the level of relative force used in the study by Hourigan et al. The lack

of change in the TA and MG agrees with the findings from the vastus lateralis in both Chapter 4 and previous findings following bed rest (Monti *et al.*, 2021), which may suggest NMJ transmission is preserved in muscles of the lower leg following immobilisation.

Regarding the discharge properties of the MUs measured, no change was seen in the TA whereas a suppression of firing rate was observed in the MG within the immobilised leg. Firing rate is mediated by ionotropic synaptic input and neuromodulation via PICs. These provide amplification to the synaptic input of the motor neuron and are proportional to monoamine release in localised areas (Heckman *et al.*, 2008). Reduction in PIC efficiency may be caused by reciprocal inhibition from the antagonist muscle group during voluntary contractions (Mesquita *et al.*, 2022). This may suggest that immobilisation might cause a degree of dysregulation in the capacity of PICs to amplify neural input to a voluntarily contracting muscle or a greater level of inhibition of the PICs may be causing a similar effect. Concerning the lack of change in TA firing rate following immobilisation, although no comparative findings following disuse are available, suppression of TA firing rate is observed in older populations (Connelly *et al.*, 1999; McNeil *et al.*, 2005; Piasecki *et al.*, 2016*a*). The present findings suggest that the TA remains resistant to suppression of firing rate and as such does not display tendencies towards an ageing phenotype in this parameter. Firing rate variability was found to increase in the MG with no change in the TA. Again the results from the MG follow the pattern from the vastus lateralis reported in Chapter 4 in which it was suggested that an opposing effect may be seen in disuse than the reduction of variability with training (Vila-Chã & Falla, 2016; Inns *et al.*, 2022). Furthermore, the TA again proved resilient to adaptations, fulfilling the hypothesis that MU level characteristics would become dysregulated in the MG to a greater extent than the TA.

To investigate whether any specific features of neural dysregulation were significantly more responsible than others for the decrements seen in the MVC of both muscles, characteristics recorded from each muscle were

independently clustered. Once multivariate simple linear regression was carried out on these clusters of interest, no significant relationships were observed with the MVC change in each muscle and the muscle size or MU characteristics recorded in this study. This may suggest that a range of contributing factors are responsible for the decrements observed rather than just one single parameter.

5.5.3 Conclusion

The results of the present study have achieved the aim of investigating adaptations to disuse in muscles expressing diverging atrophy profiles. Specifically in the case of the medial gastrocnemius which, alongside decrements to muscle size and strength, displayed significant neural dysregulation in motor unit discharge properties. This was a similar pattern of maladaptation caused by disuse as observed in the vastus lateralis in Chapter 4. In contrast, while tibialis anterior strength was decreased, no size adaptation was observed and a lesser degree of neural dysregulation was found, suggesting some degree of resilience matching the atrophy resistant profile it exhibits.

Chapter 6 Investigating nerve versus muscle stimulation as potential countermeasures in disuse atrophy

6.1 Abstract

Neuromuscular electrical stimulation (NMES) is increasingly viewed as a central tenet to minimise muscle loss during periods of disuse/illness - typically applied directly over a muscle belly. Peripheral nerve stimulation (PNS) is afforded less attention, despite providing a more global contractile stimulus to muscles. We investigated NMES versus PNS in relation to performance fatigability and peripheral contributions to voluntary force capacity.

Two fatigue protocols were assessed separately: 1) over-quadriceps NMES and, 2) peripheral [femoral] nerve stimulation (PNS). Before and after each session, a maximal voluntary contraction (MVC) was performed to assess force loss. Knee-extensor force was measured throughout to assess contractile function in response to submaximal electrical stimulation, and m-wave features quantified myoelectrical activity.

NMES and PNS induced similar voluntary (MVC, NMES: −12 ± 9%, PNS: −10 ± 8%, both $p<0.001$) and stimulated (NMES: −45 ± 12%, PNS −27 ± 27%, both $p<0.001$) force reductions. Although distinct between protocols, myoelectrical indicators of muscle recruitment (m-wave area and amplitude) and nerve conduction time did not change throughout either protocol. Myoelectrical propagation speed, represented as m-wave duration, and the delay before muscle relaxation began both progressively increased during NMES only ($p<0.05$ and $p<0.001$ respectively).

NMES myoelectrical changes suggested performance fatigability, indicating activation of superficial fibres only, which was not observed with PNS. This suggests PNS recruits a wider pool of muscle fibres and motor units and is a favourable alternative for rehabilitation. Future work should focus on implementing PNS interventions in clinically relevant scenarios such as immobilisation, care homes and critical illness.

6.2 Introduction

6.2.1 Skeletal muscle and its importance in daily life

Skeletal muscle is a crucial organ, not just for movement, but for a host of other reasons. It acts on the majority of organs throughout the body to increase functions such as vascularisation and bone repair leading to reduced disease risk in many ways (reviewed in Giudice and Taylor, 2017). It plays a particularly key role in glucose metabolism, with greater muscle mass leading to reduced risk of diabetes in later life (reviewed in Wolfe, 2006). These influences are further enhanced by exercise, providing wide-spread beneficial effects with examples including reduced fat mass and improved cognitive function (reviewed in Hoffmann and Weigert, 2017).

Evidence of muscle loss as a pervasive function of ageing has been present for a number of decades. Generally beginning at middle age, this loss of muscle mass progressively increases in magnitude (Grimby & Saltin, 1983). Since the phenomena was discovered, it has been defined as 'sarcopenia' and has become an increasingly important research focus over the last few decades (Rosenberg, 1997). In the older population, this condition is associated with reduced capacity to perform functional tasks, physical disability and reduced strength (Hughes *et al.*, 2001; Janssen *et al.*, 2002). If sarcopenia is not addressed using dietary or exercise interventions, functional status can rapidly degrade and lead to reduced rates of recovery from illness and hospitalisation coupled with an increased rate of all-cause mortality (García-Hermoso *et al.*, 2018).

6.2.2 Rehabilitation and neuromuscular electrical stimulation

Rehabilitation is an important part of the recovery process from illness and hospitalisation due to the impact this has on the neuromuscular system in reducing strength and muscle mass, especially in the older population (Graf, 2006). This is often seen in cases of joint replacement surgeries where returning the muscles around the particular joints is of vital importance for remobilising these individuals, especially as muscle loss in this area most likely

begins to decrease before hospitalisation and surgery occurs due to chronic pain (Alnahdi *et al.*, 2012). Rehabilitative exercises prescribed by physiotherapists following total hip replacement across five randomised trials, including a mixture of resistance and aerobic exercises, have been shown to increase strength, in particular of the knee extensors, and improve gait speed in this population using various functional exercises (Coulter *et al.*, 2013). It has also been suggested that in order to maximise the potential for improvement, resistance exercises should be used as these provide greater neuromuscular activation than many conventional exercises (Andersen *et al.*, 2006). Furthermore, evidence also points towards a focus on the first month of rehabilitation as the most important for functional recovery, at least in adults following hip replacement surgery (Judd *et al.*, 2014).

There are scenarios where traditional rehabilitation exercises may not be performed effectively if at all. For example, intensive care unit patients, being intubated on ventilators and sedated, are unable to voluntarily contract their muscles (Trethewey *et al.*, 2019). Spinal cord injury (SCI) patients may not be able to move whole limbs due to the impairment to their nervous system (Bickel *et al.*, 2015). Neuromuscular electrical stimulation (NMES) has been used in order to produce involuntary contractions, following the principle that the contraction of muscle using a training modality will still have beneficial effects, even involuntarily. The Dudley protocol is used to deliver NMES in a way to mirror resistance training in patients with paralysed muscle due to SCI and was delivered to the studied patients bi-weekly for between 3 to 6 months (Bickel *et al.*, 2015). The stimulation procedure was designed to produce full isotonic knee extensions, with current turned up to the target intensity for this to be achieved then turned down again. Patients perform 10 knee extensions per set up to 4 sets as capacity allows then following this external load may be added to maintain resistance. Following 3 months using this protocol, SCI patients showed reported increases of 39 ± 27% quadriceps volume in both legs by MRI in one study and another reporting 45 ± 25% and 43 ± 23% increases after 3 months in right and left legs, respectively. This second set of participants

continued for a further 3 months, after which they showed increases of 80 ± 59% and 75 ± 41% of right and left again respectively. They do state however that although this is a useful advancement, the beneficial effects have yet to be studied (Bickel *et al.*, 2015). It is also likely that this treatment would need to be continued indefinitely to maintain the hypertrophic gains and thus sustain any beneficial effect, such as metabolic, that is produced from this. The review of NMES in critically ill patients reflects a wider body of work, however the protocols used are highly heterogeneous (Trethewey *et al.*, 2019). Furthermore, investigated aspects were also variable so it may be difficult to compare the improvements seen between different configurations of stimulation. Inconsistencies between studies included different time courses of treatment, muscle measurement techniques and modalities of NMES. .For the NMES studies identified, functional outcomes in 4 of the 7 were measured using the MRC muscle scale. This determines discrete ability or lack thereof to produce a contraction, with 0 being no contraction at all to 5 being normal power. However, even using the same scale the results were not all consistent, although 3 of the 4 showed a greater preservation of strength which suggests a more positive outcome than otherwise (Trethewey *et al.*, 2019).

The potential benefits of NMES have been reported to extend beyond those immobile or with limited mobility. Protocols have been shown to attenuate muscle decline in older adults following 9 weeks of training (Kern *et al.*, 2014). A group of 44 older care home residents underwent 6 weeks of NMES thrice-weekly and improvements in a number of functional measurements such as timed up and go, Berg balance scale score and 6-minute walk test (Acaröz Candan *et al.*, 2019). Alongside strength improvements, the aerobic capacity of healthy, sedentary adults was also improved using NMES applied to a number of muscles in the lower limb (Banerjee *et al.*, 2005).

Regardless of the proven benefits that NMES offers, there still remains a difficulty in comparing sets of studies that employ vastly different methodologies. There are multiple variables to consider when designing stimulation protocols, such as pulse width, frequency, and dose of stimulation

'repetitions', and very few are the same. Furthermore, all of the studies previously mentioned, and the majority of the literature apply stimulation solely to the muscle belly of the target. As effective as it may seem, depending on the muscle structure there is a high possibility that only the more superficial muscle fibres are being activated in this way. It could be postulated that the motor nerve of the particular target muscle would be a more favourable target for stimulation, yet little evidence exists in the current literature to evidence this claim which will be discussed in more detail later.

6.2.3 Fatigue

The aspect of fatigue is widely studied in relation to skeletal muscle. It is one of the key limiting factors of exercise capacity and has thus been defined as the inability for muscle to maintain its maximum force generation (Boerio *et al.*, 2005). Although fatigue may not be critical for muscle adaptation, it remains a useful stimulus for it (Folland *et al.*, 2002). It has historically been separated into two factors: central, relating to neural drive and peripheral, relating to the motor unit (Davis, 1995). Facets of central fatigue include lack of neural drive or motivation to perform a contraction which has been linked to a number of neurotransmitters and their relative levels within the central nervous system such as serotonin, dopamine and noradrenaline (Wan *et al.*, 2017).

Peripheral fatigue may be caused by a multitude of different factors and often a combination of them rather than just one in particular. These can include depletion of muscle glycogen stores, desensitisation of calcium ion channels in the neuromuscular junction and muscle fibres leading to inefficient excitation-contraction coupling, and an accumulation of unbuffered hydrogen ions which reduce the pH of the muscle fibre (Kirkendall, 1990). The latter point being suggested to have negative effects on membrane excitability, sarcoplasmic reticulum calcium ion uptake and numerous enzymes' ability to function including ATPases (Kirkendall, 1990). However, this assertion was more recently disputed as further work has been produced which do not substantiate the claims that pH changes do more than indirectly influence fatigue in

humans. This work suggested that lower pH in muscle fibres had only a minimal effect on cross-bridge mechanics (Stackhouse *et al.*, 2001), although the entire mechanisms are still not fully understood. It is also highly dependent on the muscle fibres which are being used. Considering Henneman's size principle, motor units consisting of the motor nerve and constituent muscle fibres are recruited in order of size (Henneman, 1957) described in more detail in Chapter 1 (section 1.1.1.1).

More recently, fatigue has been reclassified as perceived fatigability and performance fatigability (Enoka & Duchateau, 2016). The former relating to body homeostasis and psychological state while the latter refers to changes in contractile function and muscle activation. Electrical simulation protocols target performance fatigability and rely less on perceived fatigability, allowing the elicited activity to extend further than perceived fatigue. This is of particular use in situations where activity is reduced due to high perceived fatigability or to the extreme where ambulation is not possible such as intensive care units (Dirks *et al.*, 2015).

Fatigue has been shown to be sex dependent in that young females are more resistant to fatigue than young males. In the knee extensors, this was seen to be the case using low-level voluntary contractions (Akagi *et al.*, 2019) and using electrically evoked involuntary contractions (Wüst *et al.*, 2008). It is also suggested that this phenomena lessens as contraction intensity increases (Hicks *et al.*, 2001). The results seen with voluntary and involuntary contractions suggest that the majority of this contribution is a result of differences in peripheral fatigue between males and females (Wüst *et al.*, 2008; Akagi *et al.*, 2019). As the work of Wüst *et al.* (2008) effectively eliminated aspects of central fatigue by directly stimulating the muscle, this provides a useful method for investigating peripheral fatigue in an isolated manner. This was done using a similar stimulation protocol, along with sustained voluntary contractions, in order to compare between age groups along with sex (Mcphee *et al.*, 2014). Their results found that older participants were significantly more fatigue resistant when tested using voluntary contractions sustained at 50%

MVC. However, similar levels of fatigue were seen with intermittent isometric involuntary contractions of 30 Hz, 1 second on/1 second off for two minutes at approximately 25% MVC. Women were also found to fatigue slower than men in voluntary contractions and less than men with involuntary contractions.

In an attempt to investigate the effects of NMES configuration parameters on muscle fatigue, four different protocols were tested on the knee extensors (Gorgey *et al.*, 2009). Voluntary fatigue, measured in this case by peak torque, was not seen to be affected by the duration of the pulses or the amplitude of the current, i.e., stimulation intensity. Their key finding is that frequency of stimulation was seen to reduce fatigue almost two-fold. Between protocols of 100 Hz and 25 Hz, voluntary torque was reduced by 76 ± 10% and 39 ± 19% respectively. Aspects of peripheral and central fatigue were investigated by applying a session of NMES to the triceps surae muscles (Boerio *et al.*, 2005). Thirty contractions at 6.25 second on/20 seconds off were delivered using 75 Hz pulses at maximum tolerated intensity. Their results suggested a contribution of both aspects of fatigue to reduction in overall torque of the plantar flexors, evidenced by reduced maximal m-wave (peripheral) and central activation ratio. However, the following section discusses potential problems with the measurement of peripheral fatigue in this way.

6.2.4 The m-wave

The m-wave is a result of collective motor unit potentials being recorded simultaneously and providing an accumulated signal in the form of a single curve to represent the electrophysiological activity of the muscle. It is also referred to as a compound motor action potential, or CMAP. In neurophysiological research this is usually recorded by applying a single pulse to the supplying nerve in order to evoke a single instance of activity, commonly delivered supramaximally to elicit a maximal CMAP, reflecting total muscle activation. The properties of this wave have been shown to change during the activation of motor units using transcutaneous electrical stimulation at varying stimulation intensities in the biceps brachii (Farina *et al.*, 2004). An increase in

duration of the m-wave was suggested to occur as intensity was increased from 40% of current required for a maximal m-wave to be produced in 20% increments up to 100% with each as a 15 second contraction. Another suggestion from their results was that stimulation recruited motor units from superficial to deep layers of muscle and high to low conduction velocities which has implications for the recruitment pattern of NMES, a topic discussed in more detail below.

Historically, the m-wave has been used in the measurement of peripheral fatigue This is generally done using the peak-to-peak amplitude: from the initial negative peak of depolarisation to the positive peak of hyperpolarisation. There are difficulties with this since other evidence suggests that the positive peak, or 2nd phase, of the m-wave is highly susceptible to alteration based on external factors such as muscle fascicle pennation angle and tendon length (Rodriguez-Falces & Place, 2018). Using recording sites along the length of the biceps brachii, the features of m-waves were analysed to show progressive changes as the m-wave propagated from the point of stimulation along the muscle fibres to the tendon. Aside from showing that the positive peak of the m-wave is susceptible to changes, it also highlights the importance of recording location when investigating changes in m-waves. Placing recording electrodes away from innervation zones and close to tendons should be avoided. At low contraction intensities up to 40% MVC, muscle architecture parameters can change dramatically in isometric contractions and therefore the shortening of muscle fibres reduces the propagation time of the m-wave and causes changes in the positive peak that cannot be accounted for by the electrophysiological activity of the muscle itself. They go on to suggest that m-wave duration may be more essential than the features of area and amplitude, since this factor influences the latter two (Rodriguez-Falces & Place, 2018). Further, it is suggested that since amplitude and duration may be altered in opposing directions during fatiguing voluntary contractions, this leads us to question the validity of area as a measurement of fatigue. The m-wave should be separated into constituent parts for analysis: positive and negative peaks, with the

positive peak being the focal point. It should also be noted that changes in the m-wave reflect changes in the sarcolemmal excitability of the muscle fibres which are being recorded from using electromyography. There are other fatigue-related parameters which are quantifiable such as Ca^{2+} reuptake and sensitivity, force capacity, blood flow and cellular metabolism which are able to present a wider picture of the impact of fatigue (Enoka & Duchateau, 2016), the former being the primary cause of acute decrements in contractile function and this should be considered at least as a limiting factor when studying muscle fatigue (Cheng *et al.*, 2018).

6.2.5 Muscle versus nerve stimulation

While the majority of studies investigating NMES have applied it to the muscle belly of the target muscles, a select few have applied stimulation to the motor nerve directly. Using protocols of wide pulse-width (1 ms), high frequency (100 Hz) stimulations, they aimed to investigate central and peripheral contributions to stimulation applied over both muscle and nerve. The premise of this being that recruitment of muscle fibres through central pathways in a seemingly normal recruitment pattern, i.e., small to large but perhaps more pertinent: fatigue resistant first and therefore more sustainable. Firstly, the triceps surae and wrist flexors, along with the tibial and medial nerves respectively, were studied (Baldwin *et al.*, 2006). This was done using a stepwise frequency of 20-100-20 Hz for 2-2-3 seconds and repeated 5 times at low intensity (2% MVC) and moderate intensity (4% MVC). In the triceps surae their findings were that significantly greater torque was produced during 100 Hz and following 20 Hz stimulation when applied over the muscle compared to the nerve at both intensities. These findings were not reflected in the wrist flexors, as no difference was seen between muscle and nerve stimulation although extra contraction torque was significantly greater at each intensity in each individual stimulation method.

Using a similar design, another study was carried out in the triceps surae. This time, alongside a stepwise frequency slightly modified to 3-2-3 seconds,

included a continuous 20 Hz stimulation for 8 s (Bergquist *et al.*, 2011). The continuous stimulation test was performed to act as a control within the recommended range of frequency for lower limb NMES stated in a review by Sheffler and Chae (2007). This investigation again used two different intensities of stimulation: one at approximately 10% MVC and the other between 20 to 40% MVC. These intensities were chosen as between 10 and 40% is estimated to be the torque of plantar flexion necessary for walking. In 10 participants at ~10% torque, collectively in both nerve and muscle stimulation, the 100 Hz section caused torque to increase significantly during the 2nd 20 Hz section. Similar to their previous work, m-wave amplitude was increased using the stepwise pattern only in muscle stimulation. However, no significant main effect was seen between m-wave pre to post in muscle stimulation pre to post in nerve simulation when the 1st and 2nd 30 Hz sections were compared. Instead, significant location and time-point interactions were found. They do state that the m-wave and h-reflex amplitudes were greater pre to post in muscle and nerve stimulation respectively, but it is unclear how their statistics support this.

Their subsequent study transferred the same techniques into the quadriceps femoris (Bergquist *et al.*, 2012). A further altered stepwise pattern of 15-100-15 Hz for 3-2-3 seconds was used. 15 Hz was used as opposed to 20 Hz from their previous work due to pilot studies suggesting that 15 Hz was the maximum frequency allowing asynchronous activity to be accurately quantified. This experiment also used a control stimulation of continuous 25 Hz stimulation for 8 seconds. It is worth noting also that they were unable to record any EMG from the 25 Hz stimulation or any of the 100 Hz sections from any of their work due to interference of stimulus artefacts. Intensities used in their experiments were 10, 20 and 30% of MVC, with 11, 8 and 1 participant(s) producing data sets for each intensity, respectively. Investigating torque, no changes were seen in any intensity or using each modality in the two configurations pre to post in contrast to their previous work in triceps surae. However, m-wave amplitude changes were shown to be increased only in

muscle stimulation and nerve stimulation respectively, adding further weight to their previous findings. Results were not reported as to whether pre to post m-wave differences were seen but from their graphical representation they do not seem to be.

Collectively, their main finding in this regard is that sensory axons are activated by nerve stimulation due to their proximity and clustering at the point of stimulation whereas in muscle stimulation they are much more dispersed and less likely to be stimulated. However, we cannot be certain, just based on this evidence, that this factor causes an afferent feedback loop to produce force and therefore recruit following Henneman's size principle to resist fatigue to a greater extent in contrast to the proposed recruitment strategy of muscle stimulation reversing the recruitment pattern. It is in fact more likely that muscle stimulation is non-selective in the recruitment of motor units, "spatially fixed and temporally synchronous" (Bickel *et al.*, 2011). A further issue with these amplitude data is that the peak-to-peak amplitude of the m-wave were measured. As discussed previously, this may not provide accurate data purely relating to the propagation of activity occurring within the muscle fibres, instead relating to the termination of the action potential as well (Rodriguez-Falces & Place, 2017, 2018). Furthermore, as these studies were only investigating the effects of stimulation on electrophysiological characteristics over an 8 second period in each instance, we cannot draw inferences from this work as to how the m-wave may change in response to a protocol designed to induce fatigue.

6.2.6 Aims and hypotheses

To our knowledge, no studies have compared NMES and PNS protocols and the impact they have on performance fatigability by considering myoelectrical and mechanical aspects of voluntary and involuntary force decrement. Due to the potential use of electrical stimulation techniques in situations of disuse atrophy, optimisation of this intervention is necessary before any implication into mainstream practice. Therefore, the purpose of the present study was to

investigate the impact of identical fatiguing protocols elicited via stimulation of the femoral nerve or muscle belly, on vastus lateralis (VL) myoelectrical, and quadriceps mechanical markers of fatigue. We hypothesised that PNS would induce a greater level of performance fatigue than NMES, which would be reflected by greater force decrements and larger differences in m-wave characteristics.

6.3 Methods

6.3.1 Ethical approval

This study was approved by the local University Research Ethics Committee (ethics code 523-2002) as to be conforming with the standards of the Declaration of Helsinki. We recruited 8 male and 8 female participants between the ages of 18 and 40 locally from the University of Nottingham using advertisement posters. Upon receipt of written informed consent to participate in the study, participants were screened for eligibility. All included participants fulfilled recruitment criteria of being healthy, recreationally active, and of normal weight or overweight i.e., with BMI within the range of 18 and 30. Once eligibility was confirmed, participants were invited to the research laboratory for two visits separated by an average of 7 days to ensure muscle function was not impaired from the previous session. For all assessments, the dominant leg was used in each participant aside from one male who had an injury to their dominant leg. Participants were requested to refrain from vigorous exercise 3 days prior to each visit.

6.3.2 Muscle ultrasound cross-sectional area

An ultrasound of the VL (n=13) was carried out for characterisation purposes. This took place on the first study visit. These methods are detailed in Chapter 2 section 2.1.1.1. Briefly, CSA measurements were taken from the mid-belly of the VL and were analysed by ImageJ to quantify CSA.

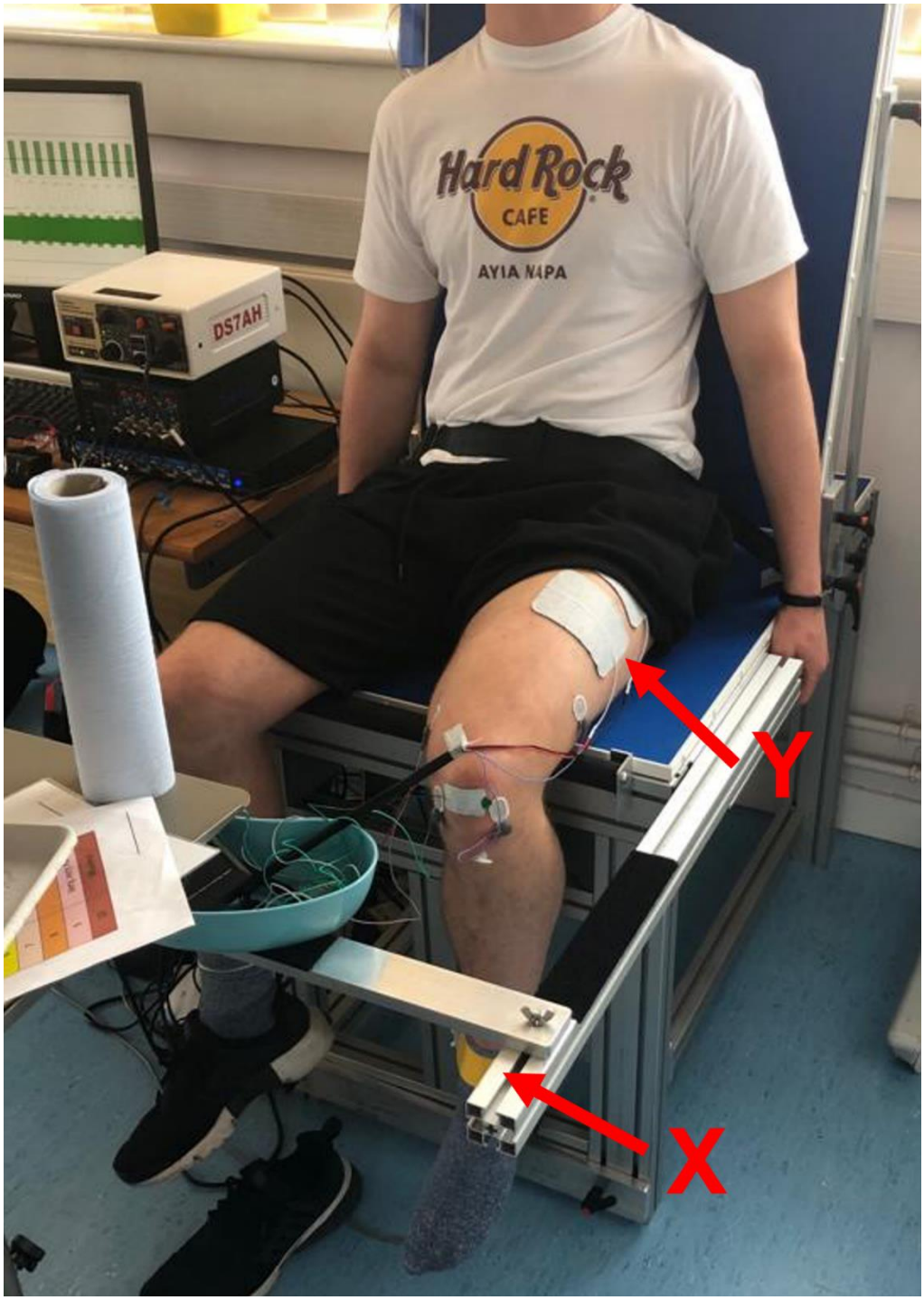

Figure 6.1: *A picture showing the custom dynamometer 'Derby Chair' with the participant's knee joint fixed at 90 degrees with an ankle strap (X) to perform maximal voluntary isometric contractions against a force transducer. Also shown are the stimulation electrodes in position for NMES (Y).*

6.3.3 Maximal voluntary contraction

These methods are detailed in Chapter 2 section 2.1.7. Briefly, following a standardised warm-up, verbal encouragement was given while the participant performed an isometric knee extensor MVC for 3 to 5 s. Three attempts were made with a 30 s minimum rest between attempts.

6.3.4 Vastus lateralis motor point identification

These methods are detailed in Chapter 2 section 2.2.1. Briefly, the motor point of the VL was identified using low intensity percutaneous electrical stimulations and marked with a surgical pen.

6.3.5 Surface electromyography and force recording

A recording electrode (E1) was placed over the motor point and a reference electrode (E2) was placed over the patellar tendon (disposable self-adhering Ag-AgCl electrodes; 95 mm^2; Ambu Neuroline, Baltorpbakken, Ballerup, Denmark) in a bipolar configuration. A ground electrode (E0) was placed just above this on the patella (Ambu Neuroline Ground). This configuration was used to reduce the signal cancellation error observed when using nearby bipolar electrode configurations, since this has been shown to reduce the m-wave signal from test muscles during contractions, providing unreliable data (Tucker & Türker, 2005). Sampling of surface EMG signals was performed at 10 kHz then further bandpass filtered at 5 Hz to 5 kHz (1902 amplifier, Cambridge Electronics Design Ltd, Cambridge, UK). Force transducer signals were sampled at 100 Hz. EMG signals were digitized (CED Micro 1401; Cambridge Electronic Design, Cambridge, UK) and Spike2 (version 9.09a; Cambridge Electronic Design) was used to display the signal in real-time on screen.

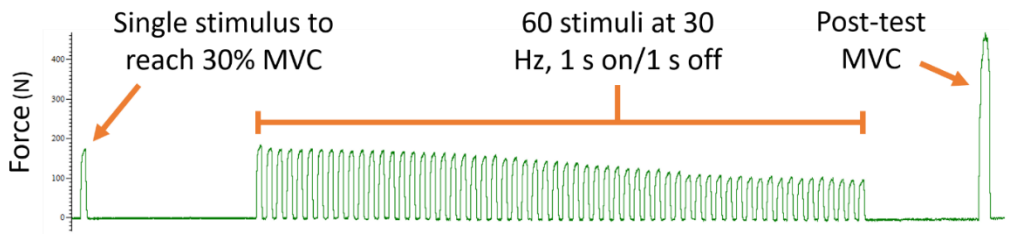

Figure 6.2: *A schematic of the electrically stimulated fatigue protocol, consisting of a single stimulus to ensure 30% of maximal voluntary contraction was achieved by the initial impulse, followed by sixty stimuli at 30 Hz, with 1s between each stimulation. Following the stimulations, a second maximal voluntary contraction was performed to test voluntary fatigue.*

6.3.6 Electrically stimulated fatigue protocol

All participants received two different stimulation modalities in the same format as an involuntary fatigue test. Neuromuscular electrical stimulation was delivered over the femoral nerve (PNS) and the VL muscle belly (NMES) on each of the separate visits. The order in which these were received was randomised. To perform PNS, large stimulating electrodes (ValuTrode cloth electrodes, 8x13 cm; Axelgaard Manufacturing Company, CA, USA) were placed in the right inguinal fold (anode) and on the lower back above the right gluteal muscles (cathode). For NMES, the electrodes were placed over the right quadricep, 1 cm apart above (anode) and below (cathode) the mid-belly of the VL as previously measured (fig. 6.1). The stimulation used was based on previously published literature (Wüst *et al.*, 2008; Mcphee *et al.*, 2014). Regarding the size of the electrodes, previous literature has used smaller circular electrodes to stimulate the femoral nerve, albeit for a shorter duration such as to elicit a compound motor action potential (Lanza *et al.*, 2019). During development of this protocol a smaller electrode was tested as the anode to deliver the stimulation, but it was found to be uncomfortable or even painful. A 30 Hz pulse was applied at 400 V, 25 µS pulse width with a current to elicit an involuntary contraction of 30% MVC. Once the appropriate current had been determined, 60 pulses were delivered 1 s in length with 1 s intervals between each pulse (fig. 6.2). Participants were encouraged to relax as much as possible between pulses to remove any voluntary activation. Following the 2-minute test, an MVC was performed within 15 s to measure voluntary fatigue.

Discomfort was measured following each test using an arbitrary Visual Analogue Scale (VAS) from 0 to 10 with 0 being no discomfort and 10 being maximum possible discomfort.

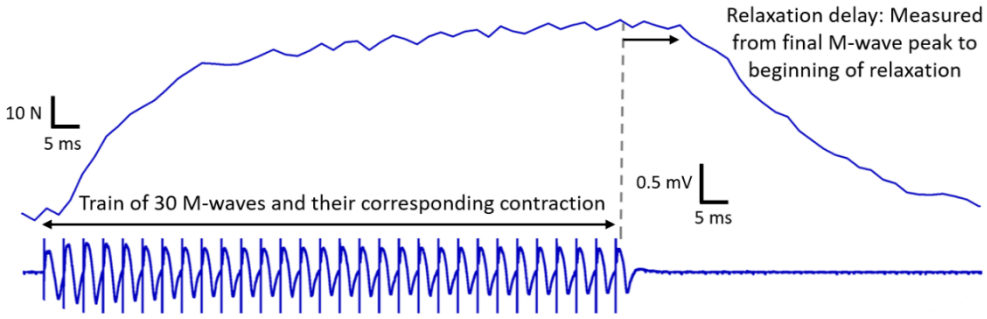

Figure 6.3: *A schematic of a single stimulated contraction and the corresponding m-waves recorded from the vastus lateralis. Including a representation of the measurement of relaxation delay: from the peak of the final m-wave to the turning point at which force begins to decline.*

6.3.7 Neuromuscular parameters

Voluntary and involuntary force were recorded via a force transducer and raw data was extracted using Spike2 version 9.09a (CED). Force traces consisted of a pre-MVC, 60 involuntary contractions and a post-MVC (fig. 6.2). Relaxation delay was measured from the peak of the final m-wave in a train to the last turning point in the force trace before it began to decline (fig. 6.3). M-wave parameters were measured from the final 3 m-waves in each train from the 1st, 15th, 30th, 45th and 60th contractions. These were the negative peak area, duration, and amplitude along with conduction time (fig. 6.4).

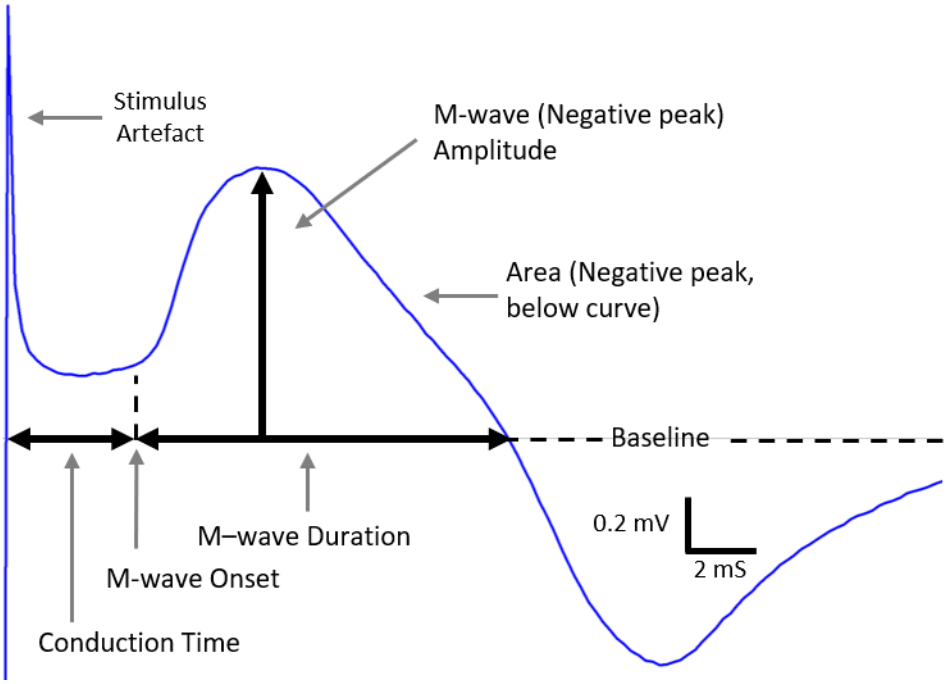

Figure 6.4: *A diagram of a single m-wave to represent the measurements taken from it to generate the myoelectrical data for this study. Specifically, the m-wave negative peak area, amplitude, and duration along with nerve conduction time.*

6.3.8 Statistical analysis

Data was analysed using GraphPad Prism version 8.4.1 (GraphPad Software, CA, USA). N=16 unless otherwise stated. Paired t-tests were used to assess stimulation current and VAS. All other variables were investigated using repeated measures 2-way analysis of variance and post-hoc testing was done using Šidak's multiple comparisons. Data are expressed as mean ± standard deviation. Significant differences were considered to be $p<0.05$.

6.4 Results

6.4.1 Participant characteristics

Sixteen participants (8 male) completed the study. Participant characteristics are displayed in Table 6.1.

Table 6.1. *Descriptive characteristics of participants showing mean and standard deviations (SD).*

N=16 (8 male)	Mean (SD)
Age (years)	27.06 (4.88)
Height (cm)	172.28 (11.05)
Weight (kg)	70.47 (17.83)
BMI (kg/m^2)	23.26 (3.84)
Vastus lateralis cross sectional area (cm^2)	23.18 (7.95)

6.4.2 Stimulation intensity and discomfort

Stimulation current (mA) required to elicit an involuntary contraction of 30% MVC was greater in NMES than PNS (132.4 ± 55 vs. 90.1 ± 25 mA, p<0.001; Figure 6.5A). Correspondingly, participants reported greater discomfort during NMES than PNS (5.3 ± 1.8 vs. 3.3 ± 1.6, p<0.001; Figure 6.5B).

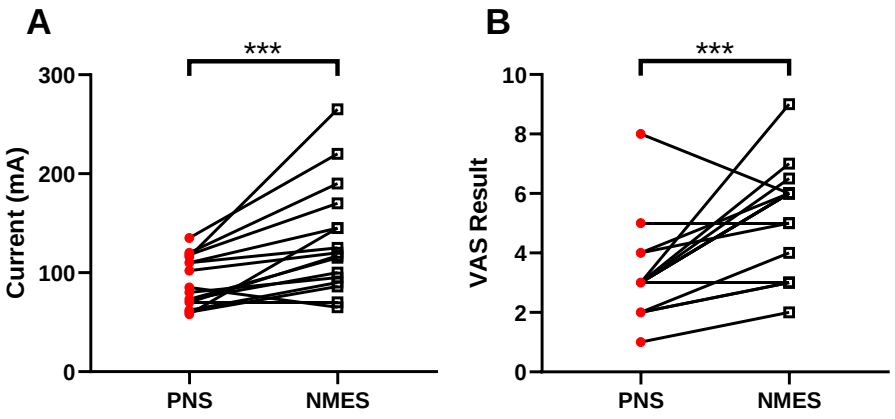

Figure 6.5. *Stimulation intensity (A) and visual analogue scale (VAS) of discomfort (scale 1 to 10) (B) for nerve (PNS) and muscle stimulation (NMES). Analysis via paired Students t-test. *** = p<0.001.*

6.4.3 Performance fatigue

MVC decreased following both PNS (459.9 ± 184.7 vs. 411.2 ± 166.9 N (−10 ± 8%), p<0.001) and NMES (474.7 ± 188.1 vs. 412.5 ± 153.2 N (−12 ± 9%), p<0.001), with no significant interaction (−8.057, 95% CI 32.22 − 78.65, p=0.23, Figure 6.6A). Similarly, involuntary force also decreased from the start to the end of the test in both PNS (136.0 ± 60.5 vs. 90.6 ± 38.9 N (−27 ± 27 %, p<0.001) and NMES (133.7 ± 48.1 vs. 72.5 ± 26.9 N (−45 ± 12%), p<0.001), with no significant interaction (53.28, 95% CI 35.9 – 70.7, p=0.16, Figure 6.6B).

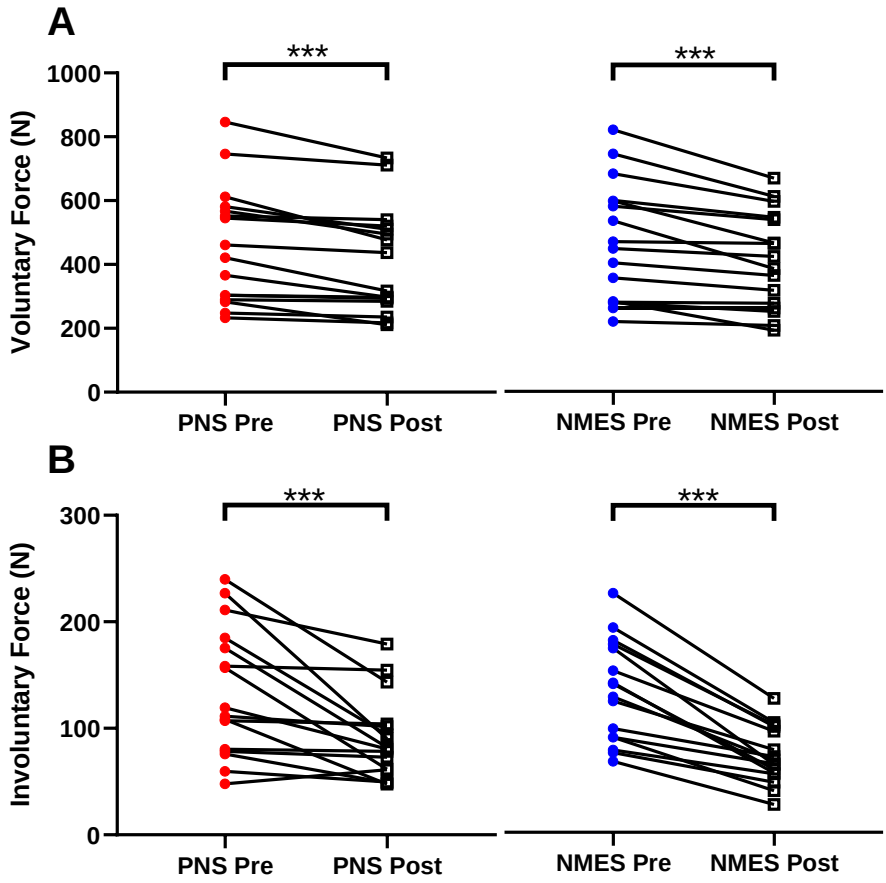

Figure 6.6. *Voluntary (A) and involuntary (B) force before and after nerve (PNS) and muscle stimulation (NMES). Analysis via repeated measures 2-way analysis of variance with Šidak's post-hoc analysis. *** = p<0.001.*

6.4.4 Relaxation delay

There was a significant interaction for both time and stimulation modality on relaxation delay (RD) ($p<0.001$, fig. 6.7). Šídák's post-hoc analysis demonstrated that NMES increased RD throughout the stimulation, with RD significantly longer than with PNS at contraction 30 ($p<0.01$), 45 and 60 ($p<0.001$). This increase in RD was progressive throughout NMES, with RD at each contraction being greater than the first to an increasing degree (between contractions 1 and 15, $p<0.01$; between contractions 1 and 30, 1 and 45 and 1 and 60, all $p<0.001$).

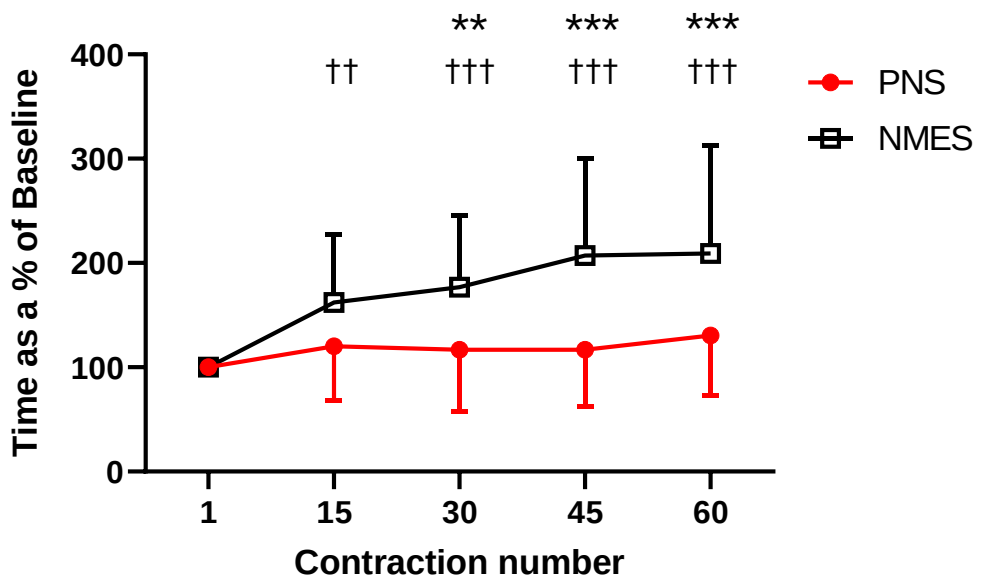

Figure 6.7. *Relaxation delay with nerve (PNS (●)) and muscle stimulation (NMES (□)). Analysis via repeated measures 2-way analysis of variance with Šídák's post-hoc analysis. ** = p<0.01, *** = p<0.001 between stimulation modality; †† = p<0.01, ††† = p<0.001 vs. contraction 1 for NMES only.*

6.4.5 M-wave characteristics

M-wave characteristics are each reported as the average value of the last 3 recorded m-waves (from 30 in each pulse) at contractions 1, 15, 30, 45 and 60. Data are shown as a percentage of baseline with contraction 1 values set at 100%. Raw data are presented in figure 6.9.

For m-wave area, there was a significant interaction effect between time and condition (−12.3, 95% CI −24.5 − −0.11, $p<0.05$). When analysed separately, m-wave area was greater for NMES than PNS at each contraction (all $p<0.001$, Figure 6.8A).

M-wave amplitude analysis revealed a significant effect of condition with a moderate effect size (partial $\eta 2 = 0.13$, $p<0.05$) and no significant interaction between conditions (−1.99, 95% CI −3.8 − −0.14, $p=0.71$). Following post-hoc analysis, m-wave amplitude was greater for NMES than PNS overall ($p<0.05$, fig. 6.7B); this was apparent for each of the five contraction times (all $p<0.001$, fig. 6.8B).

M-wave duration analysis showed a significant interaction effect (2.07, 95% CI 1.02 – 3.12, $p<0.01$). When analysed separately, m-wave duration was lower with NMES than PNS at contractions 1, 15 (both $p<0.001$), 30 ($p<0.05$) 60 ($p<0.01$). A progressive increase in m-wave duration seen with NMES only (from contraction 1 to 30 ($p<0.05$), 1 to 45 ($p<0.001$) and 1 to 60 ($p<0.001$)) (fig. 6.8C).

As expected, based on stimulation sites, there was a large main effect of condition for nerve conduction time (partial $\eta 2 = 0.32$, $p<0.001$), with nerve conduction time lower with NMES than PNS for all contractions ($p<0.001$) (fig. 6.8D). No significant interaction was present (1.94, 95% CI 1.08 – 2.80, $p=0.9$).

As this was a mixed-sex sample, secondary analyses using 3-way ANOVAs (factors: time, condition, sex) were performed to investigate the influence of sex. No 3-way interaction was revealed for MVC ($p=0.17$) and involuntary force ($p=0.38$) decline, or relaxation delay ($p=0.18$). Similarly, there was no 3-way interaction for m-wave amplitude ($p=0.07$) and duration ($p=0.68$) along with nerve conduction time ($p=0.75$). However, there was a significant 3-way interaction for m-wave area ($p<0.05$).

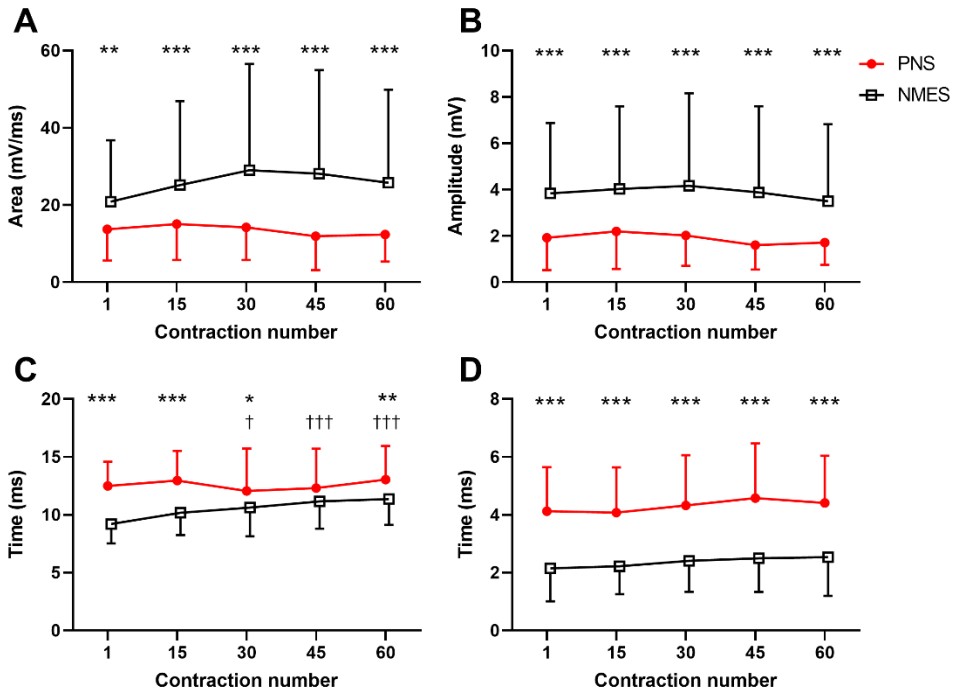

Figure 6.8. *M-wave characteristics of motor nerve (PNS (●)) and muscle stimulation (NMES (□)). A) Negative peak area, B) Negative peak amplitude, C) Negative peak duration and D) Conduction time from stimulation to m-wave onset. Analysis via repeated measures 2-way analysis of variance with Šidak's post-hoc analysis. * = p<0.05, ** = p<0.01, *** = p<0.001 between stimulation modality; † = p<0.05, ††† = p<0.001 vs. contraction 1 for NMES only.*

	PNS		NMES	
Voluntary force (N)	Mean	SD	Mean	SD
Pre	459.53	187.68	473.62	153.37
Post	411.25	166.92	412.40	153.37
Involuntary force (N)				
Pre	135.97	60.54	133.66	48.07
Post	90.61	38.87	72.45	26.88

M-wave area (mV/ms)	PNS		NMES	
Contraction	Mean	SD	Mean	SD
1	13.72	8.10	20.85	15.91
15	15.05	9.26	25.18	21.73
30	14.22	8.46	29.02	27.55
45	11.95	8.79	28.10	26.86
60	12.39	7.00	25.79	24.10

M-wave amplitude (mV)	PNS		NMES	
Contraction	Mean	SD	Mean	SD
1	1.92	1.39	3.84	3.04
15	2.19	1.63	4.03	3.57
30	2.02	1.32	4.17	3.99
45	1.60	1.06	3.88	3.72
60	1.72	0.96	3.50	3.33

M-wave duration (ms)	PNS		NMES	
Contraction	Mean	SD	Mean	SD
1	12.50	2.10	9.21	1.68
15	12.96	2.56	10.17	1.92
30	12.05	3.67	10.60	2.48
45	12.30	3.41	11.16	2.35
60	13.05	2.89	11.35	2.22

Conduction time (ms)	PNS		NMES	
Contraction	Mean	SD	Mean	SD
1	4.12	1.52	2.15	1.14
15	4.08	1.56	2.22	0.96
30	4.32	1.74	2.41	1.07
45	4.58	1.89	2.50	1.17
60	4.41	1.63	2.53	1.34

Relaxation delay (ms)	PNS		NMES	
Contraction	Mean	SD	Mean	SD
1	43.21	10.95	43.21	10.95
15	65.00	12.99	65.00	12.99
30	50.72	24.74	71.18	17.31
45	50.06	18.21	83.24	27.89
60	54.69	18.32	82.82	25.14

Figure 6.9. *Raw data from measured myoelectrical and force parameters.*

6.5 Discussion

These data demonstrate that fatiguing electrical stimulation protocols applied via the motor nerve (PNS) or muscle (NMES) result in similar voluntary and involuntary force decrements. However, PNS was more tolerable and required a reduced stimulation intensity compared to NMES. Myoelectrical activity, as assessed by the m-wave, showed no progressive change across fatiguing contractions with PNS. However, m-wave duration and relaxation delay progressively increased throughout the protocol with NMES, with little change across other variables.

6.5.1 Discomfort and intensity

As expected, a higher current was required to elicit the same level of involuntary force with NMES than PNS. It is likely that this is the cause of the lower discomfort reported following PNS. As the same type of electrodes were used in each modality, any effect of electrode size on discomfort was eliminated, as smaller electrodes produce a greater current density at the same current compared to larger electrodes, thus heightening discomfort (Herzig *et al.*, 2015). These data suggest that PNS is better tolerated than NMES, potentially increasing the favourability of PNS for use in therapeutic or rehabilitative treatment.

6.5.2 Voluntary and involuntary force

This study has shown that PNS and NMES induce similar levels of both voluntary and involuntary fatigue following a 2-minute fatigue protocol, with the degree of fatigue similar to that reported in previous studies which have used the same protocol in young adults, applied over the muscle only. One study reporting ~38% and ~30% declines in men and women respectively (Wüst *et al.*, 2008), while the later reporting ~45% declines in both men and women (Mcphee *et al.*, 2014). Although we report no statistical difference in the involuntary force reductions elicited by PNS and NMES, the two methods clearly did induce differing effects on contractile force (−45 N and −62 N respectively). NMES progressively decreased contractile force across the stimulation protocol,

whereas PNS was more variable with some increases in force mid protocol (Table 2). This somewhat erratic pattern of force with PNS is indicative of variable MU recruitment, occurring in a non-selective and non-physiological manner (Bickel *et al.*, 2011) Furthermore, this is consistent with the findings of Okuma *et al.* (2013) that peroneal nerve stimulation recruited equally from deep and superficial MUs. This is also in line with the recruitment pattern curves produced by Rodrigues-Falces *et al.* (2013) suggesting the random recruitment of MUs using NMES with depth of activity increased by stimulation intensity, while PNS targets a tightly packed bundle of axons producing a more uniform activation pattern.

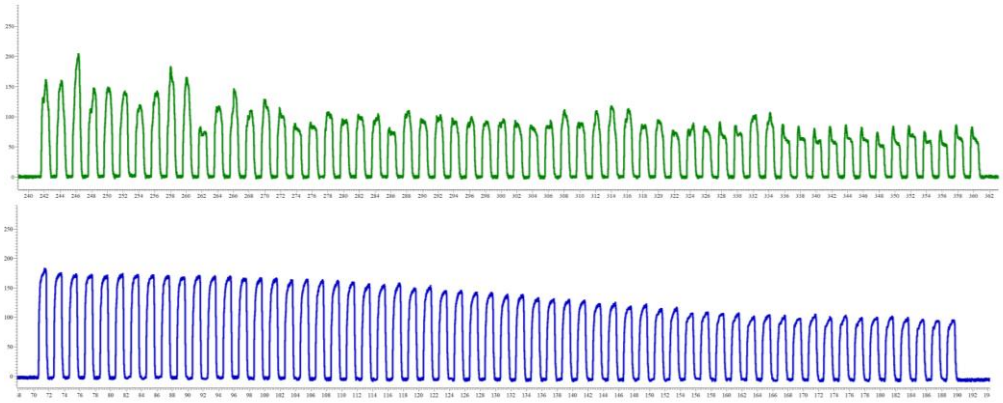

Figure 6.10: *A representative trace from a male participant of corresponding PNS (upper) and NMES (lower) stimulated force traces of sixty contractions throughout 2 minutes highlighting the force variability between the stimulation protocols.*

6.5.3 Relaxation delay

Slowing of relaxation was first shown to occur in fatigued single fibres of mouse muscles following a lack of available Ca^{2+} ions, thought to be caused by sarcoplasmic reticulum calcium pump (SERCA) impairment (Westerblad & Allen, 1993). However, when this principle was later investigated in fatigued human muscle cells, although SERCA was shown to be impaired, this did not lead to a slower relaxation of involuntary contractions (Booth *et al.*, 1997). Most, if not all studies investigating the relaxation of human skeletal muscle have focused on the time taken for the muscle to relax, measured from the

beginning of force reduction to force returning to baseline, rather than on the time delay before reduction of force takes place, with the latter of these measures better representing an impairment of muscle fibre relaxation. Indeed, to our knowledge, this is the first study to show a delay of relaxation (temporal difference of m-wave and force) in fatigued muscle following NMES applied to the muscle belly. This is suggestive of NMES recruiting from a select group of muscle fibres which are being activated and subsequently fatiguing, with no potentiation from muscle fibres which do not receive the stimulus. Furthermore, the lack of this observation in PNS further supports the suggestion that this stimulation modality recruits from a wider pool of muscle fibres than NMES.

6.5.4 M-wave characteristics

The key finding from assessing m-wave parameters was the progressively increasing m-wave duration observed in NMES which was absent in PNS. This finding is in agreement with previous studies which have applied sustained stimulation to the muscle belly (Farina *et al.*, 2004). The lack of change in m-wave duration with PNS again supports the theory that PNS is stimulating a wider pool of muscle fibres. The increased m-wave duration with NMES could be caused by a localised fatigue of a select number of superficial muscle fibres and a dysregulation of excitation-contraction coupling, in particular reduced Na^+-K^+ATPase activity (McKenna *et al.*, 2008). This reduction combined with repeated stimulation causes an accumulation of extracellular potassium ions and reduces the efficiency of membrane repolarisation (Macintosh *et al.*, 2012). With a delay in repolarisation, m-wave duration would be extended until the excess extracellular K^+ ions are pumped back across the membrane, which may be prevented with higher stimulation frequency, such as that applied here.

The m-wave has been commonly used as a marker for peripheral fatigue (Farina *et al.*, 2004). Using NMES to remove any aspects of central fatigue and isolate peripheral aspects, this study shows that the majority of m-wave characteristics do not change as fatigue is induced, either with PNS or NMES.

The m-wave duration change seen with NMES may be relevant to this but can only be applied to this form of NMES and is most likely caused by fatigue in superficial muscle fibres which it stimulates and not of the entire muscle itself. Therefore, based on the findings of this study, it seems that the m-wave may not be a useful biomarker to investigate the effects of fatigue. In relation to this suggestion, comparison of our data with previous studies highlights a key issue: In the present study m-wave amplitude was measured as the negative peak only, based on the idea that the positive peak is heavily influenced by several external factors as the action potential is terminated (Rodriguez-Falces & Place, 2018), however this is not the approach reported in previous studies (Clark *et al.*, 2006*b*; Vitry *et al.*, 2019). M-wave characteristics may have altered as a result of NMJ fatigue also. The concept of this possibility has been postulated historically (Stephens & Taylor, 1972). More recently, this has been investigated by NMES of the common peroneal nerve and decreases in axonal excitability from three different frequencies of stimulation was the best predictor of torque decline (Luu *et al.*, 2021). However, as this has not been studied in PNS to my knowledge, it remains unclear whether differing stimulation location would influence levels of axonal excitability enough to be an explanation for differences in fatigue and m-wave characteristic alteration between NMES and PNS.

The evidence presented here supports the previously suggested theory that NMES recruits muscle fibres in a non-selective manner (Bickel *et al.*, 2011). In the context that the majority of studies apply NMES over the muscle surface directly, it remains to be seen whether the same principle applies to stimulation applied over the nerve. However, irrespective of recruitment pattern, results from the present study suggest that the pool of muscle fibres available for recruitment using PNS is greater and potentially encapsulates a larger volume of muscle, rather than the superficial area targeted by NMES. With PNS, the lack of change in conduction time indicates that nerve function is not affected throughout the fatiguing protocol.

6.5.5 Limitations and future work

The data herein demonstrate clear differences between electrical stimulation protocols with regards to myoelectrical measures of muscle and neuromuscular performance. Furthermore, in must be acknowledged that these data are from healthy, young participants and it is not clear if the same outcomes, including levels of tolerability, would be observed in older participants, in whom such interventions would be more applicable. There may be further limitations to these findings in the potential input from anatomically close-by nerves and muscles to the site of PNS. As other nerves innervating other muscles of the thigh may have also been stimulated alongside the quadriceps, these may have provided some confounding force input. To determine whether this was the case, surface EMG may be performed on these muscles, such as the sartorius, during PNS to investigate potential input although this may prove difficult in deeper muscles such as the hip adductors. Given the evidence that chronic NMES applied directly over the muscle improves muscle function (Acaröz Candan *et al.*, 2019) and attenuates muscle atrophy (Kern *et al.*, 2014*b*), while seemingly only activating a superficial area of muscle fibres, PNS over a similar time course may provide similar benefits, potentially with better acceptability. A longer-term protocol would require optimisation based on the responses of participants, and could provide further mechanistic insight, such as local and non-local muscle molecular and neural adaptations. Following this, a translation into a clinical setting would be recommended to measure responses in a population which may find a greater benefit from it, as previously shown (Dirks *et al.*, 2015).

6.6 Conclusion

This investigation found that the NMES modality used does not have an impact on the level of whole muscle force reduction. However, myoelectrical characteristics were found to change in response to NMES only, specifically m-wave duration and relaxation delay. We suggest that this difference provides strong evidence of a larger pool of muscle fibres being recruited when

stimulating the motor nerve. Furthermore, PNS requires a lower intensity of stimulation to produce the same force and is more comfortable as a result of this. Collectively, these results suggest that PNS may be a more effective tool for rehabilitation than NMES. Future long-term interventions, particularly in clinically relevant populations are warranted. In the broader context of this thesis, as short-term immobilisation has shown to reduce muscle mass and function, the use of PNS to attenuate these changes would be advised, however this should be investigated further to define the appropriate dose of treatment and how effective it can be in different muscle groups.

Chapter 7 Discussion

7.1 Overview of aims

The health, size and function of skeletal muscle is of crucial importance in day-to-day life, as discussed throughout this thesis. Numerous situations exist in which these aspects are compromised with varying degrees of severity. Disuse atrophy (DA) is commonly observed following periods in which skeletal muscle remains unused and may be localised to a specific limb or even muscle, or present globally in severe cases such as intubation. Research into DA has provided insight primarily into the adaptation of muscle size, and large advances in this field came from striving to understand the effects of spaceflight on skeletal muscle and novel ways to counteract the DA observed in low gravity (Narici & De Boer, 2011). However, numerous knowledge gaps still remain in this advancing area of research. Adaptation of muscle function to situations inducing DA is less well understood. It is known that a disparity in the loss of function and size are expressed, at least in some muscles, as a result of DA, although the cause of this is unclear (Mulder *et al.*, 2006; Glover *et al.*, 2008). Further to this, while a number of works have shown that different muscles express diverging response to disuse (Belavý *et al.*, 2009*a*; Miokovic *et al.*, 2012), the mechanisms driving atrophy susceptibility in certain muscles such as the medial gastrocnemius (MG) and the atrophy resistance of muscles including the tibialis anterior (TA) have yet to be disentangled. With these aspects in mind, this thesis set out to achieve the following aims: firstly, to investigate aspects of muscle size and function parameters in functionally relevant, yet diverging in their known atrophy profile, muscle groups of the lower limb. Secondly, to uncover potential causes of disparity in the muscle size and strength decline of these muscles, with a particular focus on neuromuscular parameters measured using intramuscular electromyography. Thirdly, to carry out work which may further potential avenues of research into optimal rehabilitation strategies which may be used to attenuate the impact of DA.

7.2 Developing methods to study diverging atrophy profiles

In order to study DA, a model was implemented which would allow individuals to remain mobile using crutches while preventing any contraction of the immobilised leg. This also allowed the use of the mobile leg as an internal control to compare findings from the immobilised leg with. This model was shown to be effective at both inducing DA over a short, 15-day period in the immobilised leg measured by MRI muscle volume analysis while maintaining muscle size of the control leg despite all locomotion performed by it (Bass *et al.*, 2021). In terms of the methods of primary interest to this thesis, the use of electromyography (EMG) was essential to achieving the aim of uncovering motor unit level adaptation to DA. A prior systematic review had collated findings from studies of DA which had incorporated some degree of neuromuscular function assessment into the investigation (Campbell *et al.*, 2019). Of particular note, just 20 of the 40 studies included used EMG during voluntary contractions to provide measures of muscle activation, while others tested evoked capacity such as twitch force and central motor drive. However, all EMG used in these studies was recorded from surface electrodes, providing useful yet superficial measurements of broad motor unit activity. Intramuscular EMG (iEMG) has been used to investigate age-related adaptations across different muscle groups in the past (Piasecki *et al.*, 2016*c*, 2016*a*) along with comparisons between disease and healthy control cohorts (Berger *et al.*, 2011). However, before the conception of this thesis, it was not apparent that any studies had incorporated iEMG to investigate motor unit level adaptations to DA to our knowledge. With this in mind, the use of custom-built force dynamometers for knee extension, dorsiflexion and plantar flexion were employed alongside iEMG measurements to provide data on motor unit characteristics in multiple muscle groups across both immobilised and control legs before and after immobilisation. In addition to this, the knee extension apparatus were used alongside surface electromyography and nerve & muscle stimulation techniques to achieve the aim of development towards potential rehabilitation strategies which may attenuate DA.

7.3 Summary of key findings

There were a number of key findings throughout the thesis, and these will be summarised in order of chapter. Firstly, the primary findings from the investigations of muscle size and function following DA were that the vastus lateralis (VL) and the MG demonstrated significant reductions in size whereas the TA remained resistant following 15-day immobilisation. Crucially there were also no size changes in the control leg. Knee extension and plantar flexion strength reduced, although the former to a greater degree than the latter to the contrary of the hypothesis based on their prior size-related atrophy profiles. In further contrast to the hypotheses, dorsiflexion strength was not only reduced in the immobilised leg but also in the control leg. Other aspects of muscle function were also reduced, including, unilaterally, knee extension power and one-repetition maximum knee extension strength along with jump performance and both-leg balance decrements. Secondly, in terms of the adaptation of the immobilised vastus lateralis compared with the control, the reduction in muscle strength outweighed that of muscle size. Neuromuscular analyses found significant impairments to motor unit level characteristics using iEMG. In the immobilised VL only, motor unit potential (MUP) size was reduced and a suppression of motor unit firing rate was observed alongside a greater degree of firing rate variability, suggesting unilateral impairments to neural input as a result of immobilisation. Thirdly, comparing the immobilised TA and immobilised MG to investigate whether neuromuscular changes in muscles which express diverging muscle size adaptation in DA; only the MG decreased in size while both plantar flexion and dorsiflexion strength declined. However, while MUP size was seen to decrease in both muscles, suppression of motor unit firing rate and an increased firing rate variability was only found in the MG. Lastly, neuromuscular electrical stimulation (NMES) of the VL was compared with peripheral nerve stimulation (PNS) over the femoral nerve to assess potential features which may be favourable for rehabilitation from DA or attenuation during situations of disuse. Findings from this study included a progressive increase of myoelectrical propagation speed and a greater delay

preceding muscle relaxation observed only in NMES. This suggests a greater degree of performance fatigability not seen in PNS which may suggest PNS recruits from a wider pool of motor units than NMES, making it a potentially more favourable method of rehabilitation.

7.4 Further thoughts and speculations

Reflection of this thesis following its completion raises a number of interesting questions which should be mentioned. Importantly, are the findings documented here indeed real? Broadly I believe we can take these findings seriously due to the use of well validated and widely used methodologies. However, there are some specifics which can be mentioned. The majority of the key findings from Chapters 4 and 5 concerning the neuromuscular dysregulation as a result of disuse atrophy were recorded using iEMG during low-intensity contractions. It is important to note that at higher-intensity contractions, we may observe a different profile of neuromuscular function and also a different response to disuse atrophy in higher threshold motor units. Contractions of the intensity necessary to record this information are difficult to record with iEMG due to the potential pain from the needle electrode along with increased noise artefacts from the greater movement and even shaking of the muscle at very high intensities. It may be possible to use HDEMG to record from such contractions, but the capacity to record near-fibre motor units remains highly unlikely until methodologies develop to enable it.

Variation between the immobilisation studies presented here and others using similar methods to induce disuse atrophy such as bed rest and ULLS is worth discussing briefly. As our method of unilateral limb immobilisation prevents the movement of two joints in the leg, the knee and the ankle, ULLS allows the free movement of these while removing any potential weight bearing. The capacity to carry out unloaded contractions may mimic the weightlessness of spaceflight while perhaps being less similar to immobilisation following fracture, ALC surgery or other limb fixing reasons compared to the model used here. Comparing either of these methods to bed rest also proves difficult as the

systemic effects of total unloading are not present in ULLS, or the model used here. When putting these results into a real-world context, it is important to recognise that while the relevant model to the real-world situation may be the most useful reflection of the potential adaptation, the collective understanding of different mechanisms from various models still remains useful. With that in mind, I believe no one model for disuse atrophy is more important and all add value to our understanding of unloading-related adaptations.

Based on the findings presented in the chapters of this thesis, I believe that valuable aspects of measurement include muscle size, which would be elevated by muscle volume measurement or segmental ultrasound; muscle strength, although if investigating older adults or other disorders and disabilities then functional tasks focused on activities of daily living should be used; neuromuscular function, with particular attention to motor unit potential characteristics at differing contraction intensities and incorporation of HDEMG and potentially active EMG measurements.

While these studies highlight findings particularly centred on muscle size and neuromuscular function, the influence of muscle cellular and metabolic features were not addressed. Whether this was addressed or not, it may be important for future studies to control for diet or at least use diet diaries to have some data which might explain individual variation which may occur beyond intrinsic individual differences. Furthermore, this study recruited only male participants and it is crucial for future studies to include females if only to highlight that males and females experience similar degrees of muscle atrophy or if sex differences do exist then how must these be addressed clinically when females experience situations of disuse atrophy.

7.5 Final conclusions and future work

Overall, this thesis achieved its aims of investigating adaptation to disuse atrophy in multiple muscle groups across parameters of muscle size, aspects of muscle function and characteristics of motor units. It also reported potential features differing between NMES and PNS during fatiguing contractions which

may favour the later for development of future rehabilitation techniques. The findings from these studies suggest that the model used for immobilisation to study DA provides a useful feature of an internal control limb which generally does not express similar declines to the immobilised leg or compensatory increases. Furthermore, the use of iEMG across different limbs and muscle groups proved a valuable and sensitive tool to detect motor unit level adaptations to short-term immobilisation. It would be of interest to see whether similar changes to motor unit characteristics would also be observed during a shorter duration of DA, as early declines in muscle size and function have been observed within 5 days (Wall *et al.*, 2014). Additionally, the use of other EMG techniques, namely high-density EMG, may provide an alternative angle of study in which individual MUP trains may be tracked across the muscle following disuse to confirm the present iEMG findings and investigate features of motor unit recruitment and derecruitment. The application of this model in an ageing population would also be of interest, as the age-related decline of motor unit parameters may be further impaired by the additional stressors of DA. The understanding of how ageing and DA may collectively impact the neuromuscular system may provide crucial targets for therapeutic interventions to attenuate these declines. With that in mind, the further investigation of PNS as a potential strategy to rehabilitate post-DA or even attenuate decrements during periods of DA may be worthwhile as recruiting a greater number of muscle fibres and motor units may provide more benefit than just those located superficially. In closing, it is clear from these findings that short-term lower limb immobilisation induces a significant impairment of neural input to muscle contractions of functionally relevant muscle groups. This effect varies in severity across muscles with differing atrophy responses, and as such may point towards the knee extensors and plantar flexors as more important targets when developing rehabilitative strategies. It is also crucial that these strategies not only restore muscle size but also muscle function and by extension the neural input to muscle.

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